SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

COURSE MATERIAL

Subject Name: ANALYTICAL TECHNIQUES IN BIOTECHNOLOGY Code: SBB1201

UNIT I – V

SBT1207	ANALYTICAL TECHNIQUES IN BIOTECHNOLOGY	L	T	P	Credits	Total Marks
		3	0	0	3	100

COURSE OBJECTIVE

- To give an insight into the working principles of optical methods, radioisotopes, spectroscopy and separation methods. This will enable the students to carry out the research work innovatively

UNIT 1 BASIC INSTRUMENTATION **9 Hrs.**

Cell disruption techniques, Basics of Microscope and its types - Bright field Microscope, Dark field Microscope, Phase contrast Microscope, Fluorescent Microscope, Electron Microscope (TEM, SEM, Tunnelling EM) & Confocal Microscope, Microtechnique, pH meter.

UNIT 2 BASIC SPECTROSCOPY **9 Hrs.**

Principles and Working of colorimetry, Spectroscopy : Basic principles, nature of electromagnetic radiation, Beer-Lambert laws- UV- Visible Spectrophotometry, Fluorescence Spectrophotometry, Atomic Absorption Spectrophotometry, FTIR, Raman Spectroscopy, Mass Spectrometry, Nuclear Magnetic Resonance (NMR) -Electron Spin Resonance(ESR).

UNIT 3 SEPARATION AND PURIFICATION TECHNIQUES **9 Hrs.**

Centrifugation - Principles & types - Differential, Rate zonal and Isopycnic centrifugation. Electrophoresis of nucleic acids - Agarose , PAGE and Pulse field Electrophoresis. Electrophoresis of proteins - SDS-PAGE, IEF and 2D PAGE. Protein purification methods, Chromatography - Principles, methodology and applications of chromatography: paper, Thin layer, column (gel filtration, ion exchange, affinity), GC and HPLC. Basics of flow cytometry

UNIT 4 RADIO ISOTOPE TECHNIQUES **9 Hrs.**

Radioactive isotopes - storage, safety, handling and radioactive waste management. Liquid Scintillation counter - α-counter and β-counter. X- ray Diffraction, Crystallography, Autoradiography. Magnetic Resonance Imaging (MRI) and CT scan.

UNIT 5 MOLECULAR TECHNIQUES **9 Hrs.**

Quantification of proteins, DNA and RNA. Blotting techniques - Southern, Northern and Western blotting. Gene transfer and transfection methods. PCR and its types. Biosensors and types Biosensors

Max. 45 Hours.

TEXT / REFERENCE BOOKS

1. David T. Plummer, An introduction to Practical Biochemistry, Tata McGraw Hill Edition, 1988
2. Keith Wilson and John Walker, Practical Biochemistry - Principles and techniques, Cambridge University Press, U.K; 5th Edition, 2003.
3. Rapley and Walker, Molecular Biomethods Handbook, Humana Press, Totowa, NewYork, 2003.
4. Biophysical chemistry : Principles and Techniques - Upadhayay and Nath - Himalaya publishing house , 2nd Review Edition, 2009.

END SEMESTER EXAMINATION QUESTION PAPER PATTERN

Max Marks : 80 **Exam Duration : 3 Hrs.**

PART A : 10 questions of 2 marks each - No choice **20 Marks**

PART B : 2 questions from each unit of internal choice; each carrying 12 marks **60 Marks**

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UNIT I – V

UNIT I

Cell Disruption / Cell Lysis:

Cell Disruption is the method or process for disrupting or lysing the cell inorder to release the contents out of the cell.

Purpose

Cell disruption is done to release the cell contents. Cell Lysis or Disruption is done for isolating Nucleic acids (DNA / Plasmids), Proteins (Intracellular Proteins), Organelles, etc.

Methods

Cell-disruption / Cell Lysis methods include physical methods, Chemical methods and Enzymatic Methods.

Physical Methods

Manual Grinding:

Tissue is frozen and then crushed using a mortar and pestle. Minimum amount of bufer can be used while grinding. Because of the tensile strength of the cellulose and other polysaccharides constituting the cell wall, this method was the fastest and most efficient way to access plant proteins and DNA. Even for DNA isolation from liver tissue this method can be used.

Mortar & Pestle Used for manual grinding

Sonication:

Sonication is a physical method of cell disruption, in this method a sonicator probe is immersed into a solution containing cell suspension to disrupt, applying high frequency sound waves causes the cell to Lyse. Usually the solution will be kept in ice bath because lot of heat will be generated during the process.

A sonicator Device for cell Disruption

Liquid Homogenization:

Liquid-based homogenization is the most widely used cell-disruption technique for small volumes . Cells are lysed by forcing the cell or tissue suspension through a narrow space, thereby shearing the cell membranes. Three different types of homogenizers are in common use. A Dounce homogenizer, Potter-Elvehjem homogenizer and French Press. A French press consists of a piston that is used to apply high pressure to a sample volume of 40 to 250 ml, forcing it through a tiny hole in the press. Only two passes are required for efficient lysis due to the high pressures used with this process. The equipment is expensive,but the French press is often the method of choice for breaking bacterial cells mechanically.

Freeze Thaw Method:

The freeze/thaw method is commonly used to lyse bacterial and mammalian cells. The technique involves freezing a cell suspension in a dry ice/ethanol bath or freezer and then thawing the material at room temperature or 37°C. This method of lysis causes cells to swell and ultimately break as ice crystals form during the freezing process and then contract during thawing. Multiple cycles are necessary for efficient lysis, and the process can be quite lengthy. However, freeze/thaw has been shown to effectively release recombinant proteins located in the cytoplasm of bacteria and is recommended for the lysis of mammalian cells in some protocols.

Chemical Methods

Chemical agents can be used for lysing cell membranes, chemical agents acts on the cell wall of bacteria causing it to rupture the cells and release the product out. Urea and guanidium thiocyanate are widely used for cell lysis. Detergents are used in cell lysis buffers they help to solubilize membrane proteins and lipids there by causing the cell to lyse and release its contents outside.

Sodium Dodecyl Sulfate (SDS) CHAPS

Enzymatic Methods:

Lysozyme, Chitinase, Pectinase

Enzymatic methods are easy and fast but cost for these methods are also high. Lysozymes are used for Bacterial cell lysis and Chitinase for Yeast cell lysis.Pectinases are used for the lysis of plant cell walls for isolating protoplast and other applications.

Lysis Buffer Composition used in Alkaline Lysis Method for Plasmid Extraction

(Tris pH 8.0, EDTA- Ethylene diamine tetra acetic acid and Glucose) RNase

Lysozyme- optional 50mM glucose

25mM Tris-Cl (pH 8.0) 10mM EDTA (pH 8.0)

Applications of Cell-disruption

1. Isolating intracellular Proteins.
2. Downstream Processing.
3. Isolating intracellular organelles.
4. Nucleic acid isolation.

Disadvantages

1. Whole cell contents are released out which makes it difficult to separate out product of interest from the mixture.

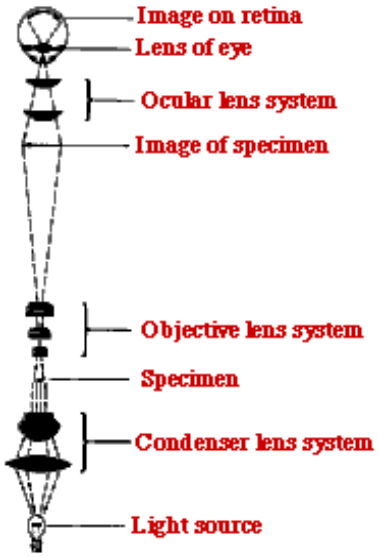
2. Cell lysis increases viscosity of the solution making it difficult to process in the further steps.

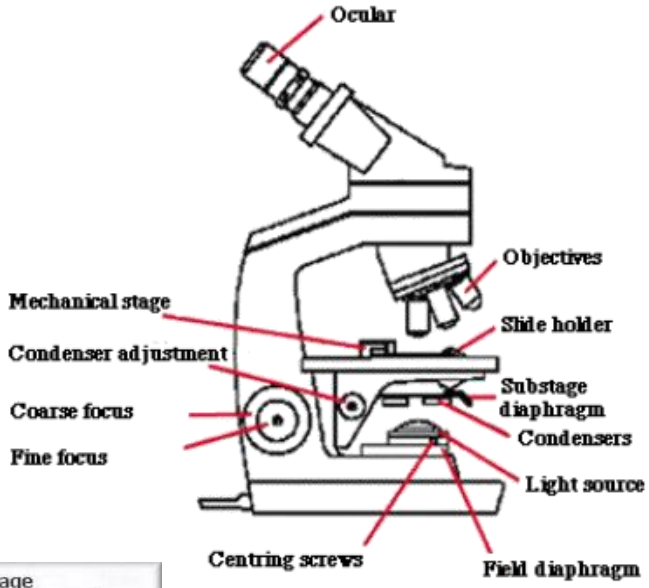
3. Product released into harsh environment causing the product to lose stability or activity.
4. Might cause filtration membranes to foul during the filtration process.
5. Enzymatic cell-disruption in large scale can be expensive.

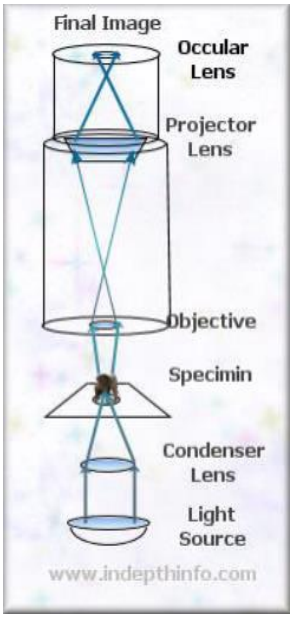
Bright field microscopy

Bright field Microscopy is the simplest of all the optical micrscopy illumination techniques. Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light and contrast in the sample is caused by absorance of some of the transmitted light in dense areas of the sample. Bright field microscopy is the simplest of a range of techniques used for illumination of samples in light microscopes and its simplicity makes it a popular technique. The typical appearance of a bright field microscopy image is a dark sample on a bright background, hence the name.

Light path of bright field microscope







Light path

The light path of a bright field microscope is extremely simple, no additional components are required beyond the normal light microscope setup. The light path therefore consists of:

light source

1. a **transillumination light** source, commonly a halogen lamp in the microscope stand; A halogen lamp, also known as a tungsten halogen lamp or quartz iodine lamp, is an incandescent lamp that has a small amount of a halogen such as iodine or bromine added. The combination of the halogen gas and the tungsten filament produces a halogen cycle chemical reaction which redeposits evaporated tungsten back onto the filament, increasing its life and maintaining the clarity of the envelope

2. a **condenser lens** which focuses light from the light source onto the sample. A condenser is one of the main components of the optical system of many transmitted light compound microscopes. A condenser is a lens that serves to concentrate light from the illumination source that is in turn focused through the object and magnified by the objective lens.

3. **objective lens** : In an optical instrument, the objective is the optical element that gathers light from the object being observed and focuses the light rays to produce a real image. Objectives can be single lenses or mirrors, or combinations of several optical elements. Microscope objectives are characterized by two parameters: magnification and numerical aperture. The typically ranges are 4× , 10x , 40x and 100×. 4. oculars to view the sample image.

4. An **eyepiece**, or **ocular lens**, is a type of lens that is attached to a variety of optical devices such as microscopes. It is so named because it is usually the lens that is closest to the eye when someone looks through the device. The objective lens or mirror collects light and brings it to focus creating an image. The eyepiece is placed near the focal point of the objective to magnify this image. The amount of magnification depends on the focal length of the eyepiece.

Magnification is the process of enlarging something only in appearance, not in physical size. Typically magnification is related to scaling up visuals or images to be able to see more detail, increasing resolution.

Resolving power is the ability of an imaging device to separate (i.e., to see as distinct) points of an object that are located at a small angular distance..

In optics, the **numerical aperture** (NA) of an optical system is a dimensionless number that characterizes the range of angles over which the system can accept or emit light. In most areas

of optics, and especially in microscopy, the numerical aperture of an optical system such as an objective lens is defined by

$NA = n \sin \theta$

where n is the index of refraction of the medium in which the lens is working (1.0 for air, 1.33 for pure water, and up to 1.56 for oils; see also list of refractive indices), and θ is the half-angle of the maximum cone of light that can enter or exit the lens. In general, this is the angle of the real marginal ray in the system

Working Performance

Bright field microscopy typically has low contrast with most biological samples as few absorb light to a great extent. Staining is often required to increase contrast, which prevents use on live cells in many situations. Bright field illumination is useful for samples which have an intrinsic colour, for example chloroplasts in plant cells.

Light is first emitted by the light **source** and is directed by the **condenser lens** on to the specimen, which might be a loose object, a prepared plate or almost anything. A microscope can even be applied to small parts of larger objects, though with a bit more difficulty. (The light does not absolutely need to originate below the specimen.)

The light from the specimen then passes through the objective lens. This lens is often selected from among three or four and is the main determinant for the level of magnification. It bends the light rays and in the case of this example sends them to a **projector lens**, which reverses their direction so that when the image reaches the eye it will not appear "upside-down". Not all microscopes have a projector lens, so the viewer may be seeing a reverse image. In these cases, when the slide is moved, it will appear to be moving in the opposite direction to the viewer.

The light rays then travel to the oracular lens or "eye piece". This is often a 10X magnification lens, meaning it magnifies the magnified image an additional ten times. The image is then projected into the eye. It is very seldom that a specimen is in focus the moment it is placed beneath a microscope. This means that some adjustment will have to be made. Unlike in telescopes, the focal length between lenses remains constant when adjusting the focus. The lens apparatus is brought closer to or further from the object. The focus adjustment is often along the neck of the tube containing the lenses, but it might just as well move the slide up and down. The best way to make this adjustment is to make a course adjustment

so that it is too close to the object and then back off with the fine adjustment2. This helps to ensure that the specimen is not inadvertently smashed by the lens.

Advantages

The name "brightfield" is derived from the fact that the specimen is dark and contrasted by the surrounding bright viewing field. Simple light microscopes are sometimes referred to as bright field microscopes.

Brightfield microscopy is very simple to use with fewer adjustments needed to be made to view specimens.

Some specimens can be viewed without staining and the optics used in the brightfield technique don't alter the color of the specimen.

It is adaptable with new technology and optional pieces of equipment can be implemented with brightfield illumination to give versatility in the tasks it can perform.

Disadvantages

Certain disadvantages are inherent in any optical imaging technique.

- By using an aperture diaphragm for contrast, past a certain point, greater contrast adds distortion. However, employing an iris diaphragm will help compensate for this problem.
- Brightfield microscopy can't be used to observe living specimens of bacteria, although when using fixed specimens, bacteria have an optimum viewing magnification of 1000x.
- Brightfield microscopy has very low contrast and most cells absolutely have to be stained to be seen; staining may introduce extraneous details into the specimen that should not be present.
- Also, the user will need to be knowledgeable in proper staining techniques.
- Lastly, this method requires a strong light source for high magnification applications and intense lighting can produce heat that will damage specimens or kill living microorganisms.

Phase contrast microscopy

Phase contrast microscopy is an optical microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. Phase shifts themselves are invisible, but become visible when shown as brightness variations.

When light waves travels through a medium other than vacuum, interaction with the medium causes the wave amplitude and phase to change in a manner dependent on properties of the

medium. Changes in amplitude (brightness) arise from the scattering and absorption of light, which is often wavelength dependent and may give rise to colors. Photographic equipment and the human eye are only sensitive to amplitude variations. Without special arrangements, phase changes are therefore invisible. Yet, often these changes in phase carry important information.

History and Background Information

Frits Zernike, a Dutch physicist and mathematician, built the first phase contrast microscope in 1938.

It took some time before the scientific community recognized the potential of Zernike's discovery; he won the Nobel Prize in 1953 and the German-based company Zeiss began manufacturing his phase contrast microscope during World War II.

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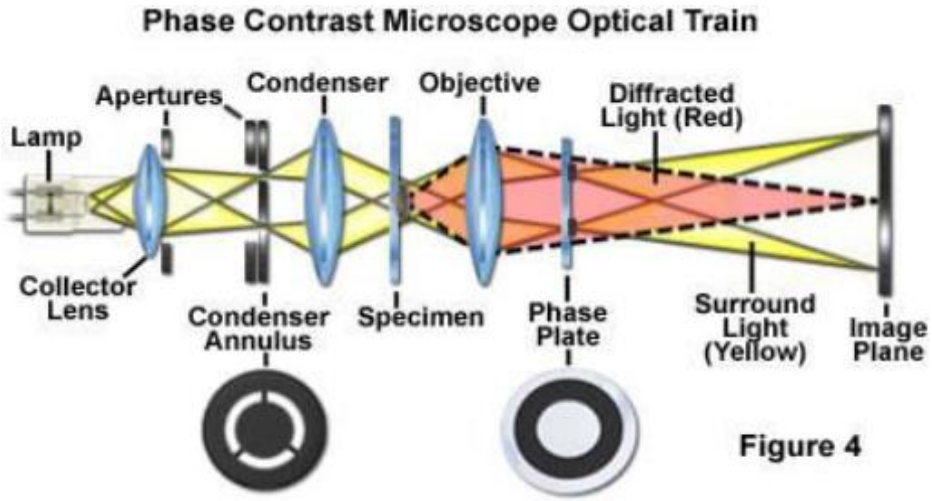
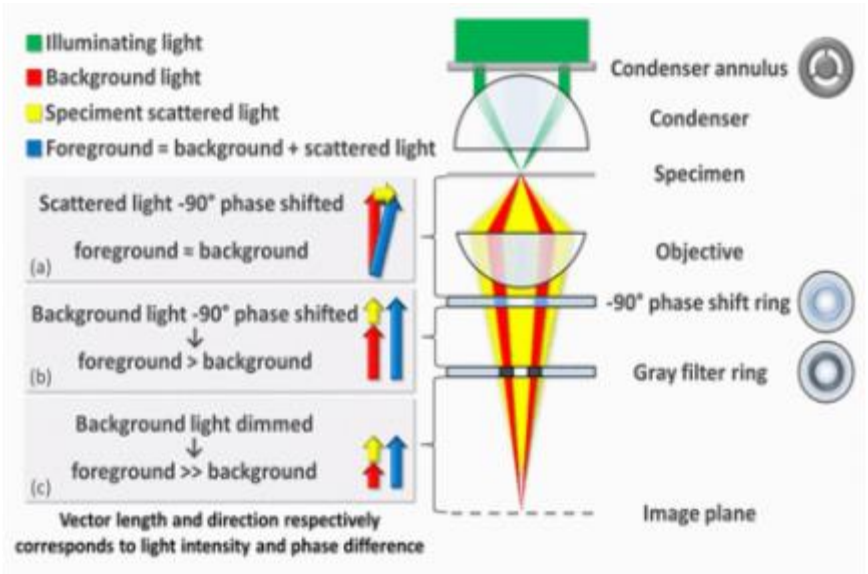


Figure 4



Working Principle

The basic principle to make phase changes visible in phase contrast microscopy is to separate the illuminating background light from the specimen scattered light, which make up the foreground details, and to manipulate these differently.

The ring shaped illuminating light (green) that passes the condenser annulus is focused on the specimen by the condenser. Some of the illuminating light is scattered by the specimen (yellow). The remaining light is unaffected by the specimen and form the background light (red). When observing unstained biological specimen, the scattered light is weak and typically phase shifted by -90° — relative to the background light. This leads to that the foreground (blue vector) and the background (red vector) nearly have the same intensity, resulting in a low image contrast (a). In a phase contrast microscope, the image contrast is improved in two steps. The background light is phase shifted -90° by passing it through a phase shift ring. This eliminates the phase difference between the background and the scattered light, leading to an increased intensity difference between foreground and background (b). To further increase contrast, the background is dimmed by a gray filter ring (c). Some of the scattered light will be phase shifted and dimmed by the rings. However, the background light is affected to a much greater extent, which creates the phase contrast effect.

The above describes negative phase contrast. In its positive form, the background light is instead phase shifted by +90°. The background light will thus be 180° out of phase relative to the scattered light. This results in that the scattered light will be subtracted from the background light in (b) to form an image where the foreground is darker than the background.

Applications in Microscopy

The possible applications of Zernike's phase contrast microscope in microscopy are evident in the fields of molecular and cellular biology, microbiology and medical research.

Specimens that can be observed and studied include live microorganisms such as protozoa, erythrocytes, bacteria, molds and sperm, thin tissue slices, lithographic patterns, fibers, glass fragments and sub-cellular particles such as nuclei and organelles.

Advantages

The advantages of the phase contrast microscope include:

- The capacity to observe living cells and, as such, the ability to examine cells in a natural state
- Observing a living organism in its natural state and/or environment can provide far more information than specimens that need to be killed, fixed or stain to view under a microscope
- High-contrast, high-resolution images
- Ideal for studying and interpreting thin specimens
- Ability to combine with other means of observation, such as fluorescence
- Modern phase contrast microscopes, with CCD or CMOS computer devices, can capture photo and/or video images

In addition, advances to the phase contrast microscope, especially those that incorporate technology, enable a scientist to hone in on minute internal structures of a particle and can even detect a mere small number of protein molecules.

Disadvantages

Disadvantages and limitations of phase contrast:

- Annuli or rings limit the aperture to some extent, which decreases resolution
- This method of observation is not ideal for thick organisms or particles
- Thick specimens can appear distorted
- Images may appear grey or green, if white or green lights are used, respectively, resulting in poor photomicrography
- Shade-off and halo effect, referred to a phase artifacts
- Shade-off occurs with larger particles, results in a steady reduction of contrast moving from the center of the object toward its edges
- Halo effect, where images are often surrounded by bright areas, which obscure details along the perimeter of the specimen

Modern advances and techniques provide solutions to some of these confines, such as the halo effect.

Apodized phase contrast utilizes amplitude filters that contain neutral density films to

minimize the halo effect. Essentially, this is attempting to reverse the definition achieved through phase contrast annuli, but the halo effect can never be eliminated completely.

The pros that phase contrast has brought to the field of microscopy far exceed its limitations. This is easily seen with the myriad of advances in the fields of cellular and microbiology as well as in medical and veterinary sciences.

Conclusion

The **phase contrast microscope** opened up an entire world of microscopy, providing incredible definition and clarity of particles never seen before.

Dark field microscopy

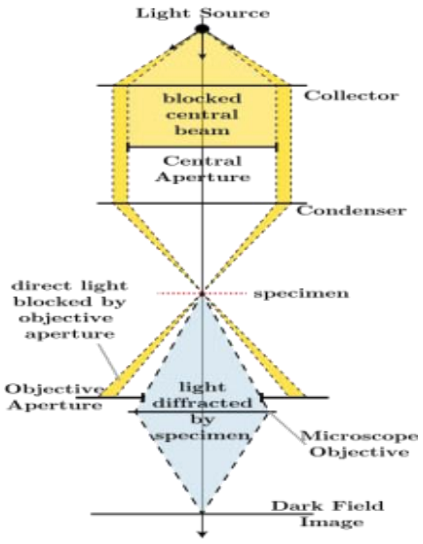
Dark field microscopy (dark ground microscopy) describes microscopy methods, in both light and electron microscopy, which exclude the unscattered beam from the image. As a result, the field around the specimen (i.e. where there is no specimen to scatter the beam) is generally dark.

Light microscopy applications

In optical microscopy, darkfield describes an illumination technique used to enhance the contrast in unstained samples. It works by illuminating the sample with light that will not be collected by the objective lens, and thus will not form part of the image. This produces the classic appearance of a dark, almost black, background with bright objects on it.

The light's path

The steps are illustrated in the figure where an upright microscope is used.



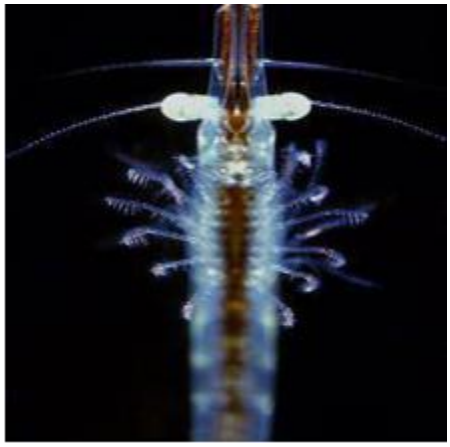


Diagram illustrating the light path through a dark field microscope.

1. Light enters the microscope for illumination of the sample.

2. A specially sized disc, the patch stop (see figure) blocks some light from the light source, leaving an outer ring of illumination. A wide phase annulus can also be reasonably substituted at low magnification.

3. The condenser lens focuses the light towards the sample.

4. The light enters the sample. Most is directly transmitted, while some is scattered from the sample.

5. The scattered light enters the objective lens, while the directly transmitted light simply misses the lens and is not collected due to a direct illumination block (see figure).

6. Only the scattered light goes on to produce the image, while the directly transmitted light is omitted.

Dark field microscopy produces an image with a dark background.

Advantages and disadvantages

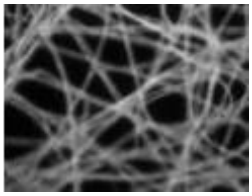
Dark field microscopy is a very simple yet effective technique and well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual water borne single-celled organisms. Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.

The main limitation of dark field microscopy is the low light levels seen in the final image.

This means the sample must be very strongly illuminated, which can cause damage to the sample. Dark field microscopy techniques are almost entirely free of artifacts, due to the nature of the process. However the interpretation of dark field images must be done with great care as common dark features of bright field microscopy images may be invisible, and vice versa.

While the dark field image may first appear to be a negative of the bright field image, different effects are visible in each. In bright field microscopy, features are visible where either a shadow is cast on the surface by the incident light, or a part of the surface is less reflective, possibly by the presence of pits or scratches. Raised features that are too smooth to cast shadows will not appear in bright field images, but the light that reflects off the sides of the feature will be visible in the dark field images.

Comparison of transillumination techniques used to generate contrast in a sample of tissue paper. 1.559 μm/pixel (when viewed at full resolution).



Dark field illumination, sample contrast comes from light scattered by the sample.

Bright field illumination, sample contrast comes from absorbance of light in the sample.

Fluorescence microscope

An upright fluorescence microscope (Olympus BX61) with the fluorescent filter cube turret above the objective lenses, coupled with a digital camera.

A **fluorescence microscope** is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances. The "fluorescence microscope" refers to any microscope that uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image.

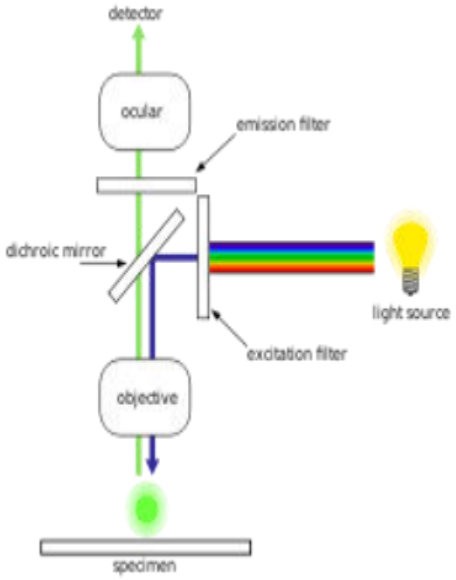
Principle

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp are common; more advanced forms are high-power LEDs and lasers), the excitation filter, the dichroic mirror (or dichroic beamsplitter), and the emission filter (see figure below). The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images.

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective). These microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflectio fluorescence microscope (TIRF).

Fluorescence microscopy

Schematic of a fluorescence microscope.

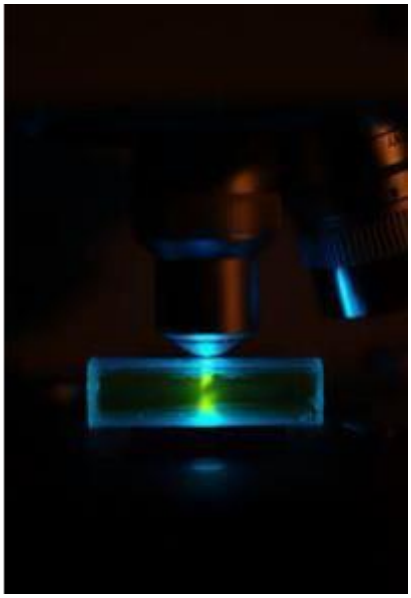


The majority of fluorescence microscopes, especially those used in the life sciences, are of the epifluorescence design shown in the diagram. Light of the excitation wavelength is focused onthe specimen through the objective lens. The fluorescence emitted by the specimen is focused to the detector by the same objective that is used for the excitation which for greatest sensitivitywill have a very high numerical aperture. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light and the epifluorescence method therefore gives a high signal to noise ratio. An additional barrier filter between the objective and the detector can filter out the remaining excitation light from fluorescent light.

Light sources

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide. Four main types of light source are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, supercontinuum sources, and high-power LEDs. Lasers are most widely used for more complex fluorescence microscopy techniques like confocal microscopy and total internal reflection fluorescence microscopy while xenon lamps, and mercury lamps, and LEDs with a dichroic

excitation filter are commonly used for widefield epifluorescence microscopes.



Sample preparation

A sample of herring sperm stained with SYBR green in a cuvette illuminated by blue light in an epifluorescence microscope. The SYBR green in the sample binds to the herring sperm DNA and, once bound, fluoresces giving off green light when illuminated by blue light.

In order for a sample to be suitable for fluorescence microscopy it must be fluorescent. There are several methods of creating a fluorescent sample; the main techniques are labelling with fluorescent stains or, in the case of biological samples, expression of a fluorescent protein. Alternatively the intrinsic fluorescence of a sample (i.e., autofluorescence) can be used.[1] In the life sciences fluorescence microscopy is a powerful tool which allows the specific and sensitive staining of a specimen in order to detect the distribution of proteins or other molecules of interest. As a result there is a diverse range of techniques for fluorescent staining of biological samples

Biological fluorescent stains

Many fluorescent stains have been designed for a range of biological molecules. Some of these are small molecules which are intrinsically fluorescent and bind a biological molecule of interest.

Major examples of these are nucleic acid stains like DAPI and Hoechst (excited by UV wavelength light) and DRAQ5 and DRAQ7 (optimally excited by red light) which all bind the

minor groove of DNA, thus labelling the nuclei of cells. Others are drugs or toxins which bind specific cellular structures and have been derivatised with a fluorescent reporter. A major example of this class of fluorescent stain is phalloidin which is used to stain actin fibres in mammalian cells.

There are many fluorescent molecules called fluorophores or fluorochromes such as fluorescein, Alexa Fluors or DyLight 488, which can be chemically linked to a different molecule which binds the target of interest within the sample.

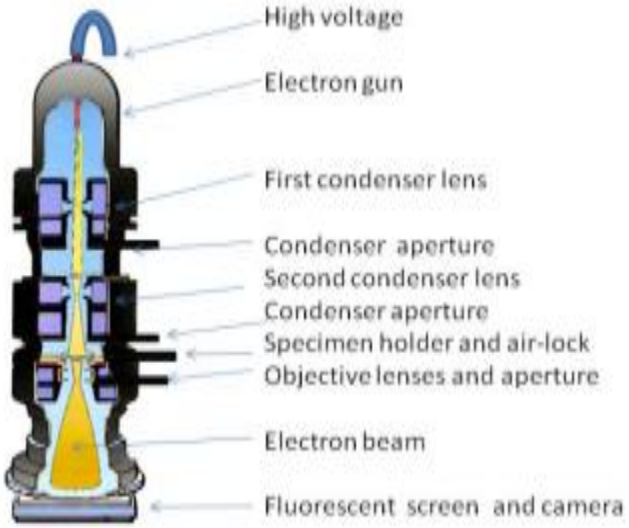
Electron microscope

An electron microscope is a microscope that uses a beam of accelerated electrons as a source of illumination. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of smaller objects. A transmission electron microscope can achieve better than 50 pm resolution[1] and magnifications of up to about 10,000,000x whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x. Transmission electron microscopes use electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image. These electron optical lenses are analogous to the glass lenses of an optical light microscope. Electron microscopes are used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, electron microscopes are often used for quality control and failure analysis. Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the image.

Types

Transmission electron microscope (TEM) The original form of electron microscope, the transmission electron microscope (TEM) uses a high voltage electron beam to illuminate the specimen and create an image. The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part

transparent to electrons and in part scatters them out of the beam. When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in this information (the "image") may be viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or scintillator material such as zinc sulfide. Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a digital camera. The image detected by the digital camera may be displayed on a monitor or computer.



Transmission Electron Microscope

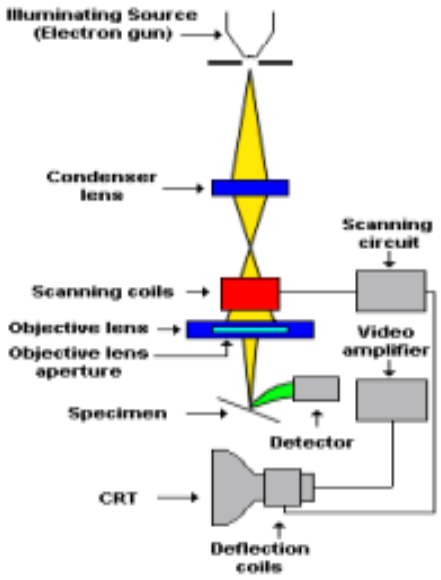
The resolution of TEMs is limited primarily by spherical aberration, but a new generation of aberration correctors have been able to partially overcome spherical aberration to increase resolution. Hardware correction of spherical aberration for the high-resolution transmission electron microscopy (HRTEM) has allowed the production of images with resolution below 0.5 angstrom (50 picometres) and magnifications above 50 million times. The ability to determine the positions of atoms within materials has made the HRTEM an important tool for nano-technologies research and development.

Transmission electron microscopes are often used in electron diffraction mode. The advantages of electron diffraction over X-ray crystallography are that the specimen need not be a single

crystal or even a polycrystalline powder, and also that the Fourier transform reconstruction of the object's magnified structure occurs physically and thus avoids the need for solving the phase problem faced by the X-ray crystallographers after obtaining their X-ray diffraction patterns of a single crystal or polycrystalline powder.

The major disadvantage of the transmission electron microscope is the need for extremely thin sections of the specimens, typically about 100 nanometers. Biological specimens are typically required to be chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning. Sections of biological specimens, organic polymers and similar materials may require special treatment with heavy atom labels in order to achieve the required image contrast.

Scanning electron microscope (SEM)



The SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). When the electron beam interacts with the specimen, it loses energy by a variety of mechanisms. The lost energy is converted into alternative forms such as heat, emission of low-energy secondary electrons and high-energy backscattered electrons, light emission (cathodoluminescence) or X-ray emission, all of which provide signals carrying information

about the properties of the specimen surface, such as its topography and composition. The image displayed by an SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated. In the SEM image of an ant shown below and to the right, the image was constructed from signals produced by a secondary electron detector, the normal or conventional imaging mode in most SEMs.

Generally, the image resolution of an SEM is at least an order of magnitude poorer than that of a TEM. However, because the SEM image relies on surface processes rather than transmission, it is able to image bulk samples up to many centimeters in size and (depending on instrument design and settings) has a great depth of field, and so can produce images that are good representations of the three-dimensional shape of the sample. Another advantage of SEM is its variety called environmental scanning electron microscope (ESEM) can produce images of sufficient quality and resolution with the samples being wet or contained in low vacuum or gas. This greatly facilitates imaging biological samples that are unstable in the high vacuum of conventional electron microscopes.

Color

In their most common configurations, electron microscopes produce images with a single brightness value per pixel, with the results usually rendered in grayscale. However, often these images are then colorized through the use of feature-detection software, or simply by hand editing using a graphics editor. This may be done to clarify structure or for aesthetic effect and generally does not add new information about the specimen.

Sample preparation

Materials to be viewed under an electron microscope may require processing to produce a suitable sample. The technique required varies depending on the specimen and the analysis required:

 Chemical fixation – for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.

 Negative stain – suspensions containing nanoparticles or fine biological material (such as viruses and bacteria) are briefly mixed with a dilute solution of an electron-opaque solution such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid. This mixture is applied to a suitably coated EM grid, blotted, then allowed to dry. Viewing of this preparation in the TEM should be carried out without

delay for best results. The method is important in microbiology for fast but crude morphological identification, but can also be used as the basis for high resolution 3D reconstruction using EM tomography methodology when carbon films are used for support. Negative staining is also used for observation of nanoparticles.

 Cryofixation – freezing a specimen so rapidly, in liquid ethane, and maintained at liquid nitrogen or even liquid helium temperatures, so that the water forms vitreous (noncrystalline) ice. This preserves the specimen in a snapshot of its solution state. An entire field called cryo-electron microscopy has branched from this technique. With the development of cryo-electron microscopy of vitreous sections (CEMOVIS), it is now possible to observe samples from virtually any biological specimen close to its native state.[citation needed]

 Dehydration – or replacement of water with organic solvents such as ethanol or acetone, followed by critical point drying or infiltration with embedding resins. Also freeze drying.

 Embedding, biological specimens – after dehydration, tissue for observation in the transmission electron microscope is embedded so it can be sectioned ready for viewing. To do this the tissue is passed through a 'transition solvent' such as propylene oxide (epoxypropane) or acetone and then infiltrated with an epoxy resin such as Araldite, Epon, or Durcupan; tissues may also be embedded directly in water-miscible acrylic resin. After the resin has been polymerized (hardened) the sample is thin sectioned (ultrathin sections) and stained – it is then ready for viewing.

 Embedding, materials – after embedding in resin, the specimen is usually ground and polished to a mirror-like finish using ultra-fine abrasives. The polishing process must be performed carefully to minimize scratches and other polishing artifacts that reduce image quality.

 Metal shadowing – Metal (e.g. platinum) is evaporated from an overhead electrode and applied to the surface of a biological sample at an angle. The surface topography results in variations in the thickness of the metal that are seen as variations in brightness and contrast in the electron microscope image.

 Replication – A surface shadowed with metal (e.g. platinum, or a mixture of carbon and platinum) at an angle is coated with pure carbon evaporated from carbon electrodes at right angles to the surface. This is followed by removal of the specimen material (e.g. in an acid bath, using enzymes or by mechanical separation) to produce a surface replica that records the surface ultrastructure and can be examined using transmission electron microscopy.

 Sectioning – produces thin slices of specimen, semitransparent to electrons. These can be cut on an ultramicrotome with a diamond knife to produce ultra-thin sections about 60– 90 nm thick. Disposable glass knives are also used because they can be made in the lab and are much cheaper.

 Staining – uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects). In biology, specimens can be stained "en bloc" before embedding and also later after sectioning. Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.

 Freeze-fracture or 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 (cryofixation), then fractured by breaking or by using a microtome while maintained at liquid nitrogen temperature. The cold fractured surface (sometimes "etched" by increasing the temperature to about −100 °C for several minutes to let some ice sublime) is then shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator. A second coat of carbon, evaporated perpendicular to the average surface plane is often performed to improve stability of the replica coating. The specimen is returned to room temperature and pressure, then the extremely fragile "pre-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 still-floating replica is thoroughly washed free from residual chemicals, carefully fished up on fine grids, dried then viewed in the TEM.

 Ion beam milling – thins samples until they are transparent to electrons by firing ions (typically argon) at the surface from an angle and sputtering material from the surface. A subclass of this is focused ion beam milling, where gallium ions are used to produce an electron transparent membrane in a specific region of the sample, for example through a device within a microprocessor. Ion beam milling may also be used for cross-section polishing prior to SEM analysis of materials that are difficult to prepare using mechanical polishing.

 Conductive coating – an ultrathin coating of electrically conducting material, deposited either by high vacuum evaporation or by low vacuum sputter coating of the sample. This is done to

prevent the accumulation of static electric fields at the specimen due to the electron irradiation required during imaging. The coating materials include gold, gold/palladium, platinum, tungsten, graphite, etc.

 Earthing – to avoid electrical charge accumulation on a conductive coated sample, it is usually electrically connected to the metal sample holder. Often an electrically conductive adhesive is used for this purpose.

Disadvantages

Electron microscopes are expensive to build and maintain, but the capital and running costs of confocal light microscope systems now overlaps with those of basic electron microscopes. Microscopes designed to achieve high resolutions must be housed in stable buildings (sometimes underground) with special services such as magnetic field cancelling systems.

The samples largely have to be viewed in vacuum, as the molecules that make up air would scatter the electrons. An exception is the environmental scanning electron microscope, which allows hydrated samples to be viewed in a low-pressure (up to 20 Torr or 2.7 kPa) and/or wet environment.

Scanning electron microscopes operating in conventional high-vacuum mode usually image conductive specimens; therefore non-conductive materials require conductive coating (gold/palladium alloy, carbon, osmium, etc.). The low-voltage mode of modern microscopes makes possible the observation of non-conductive specimens without coating. Non-conductive materials can be imaged also by a variable pressure (or environmental) scanning electron microscope.

Small, stable specimens such as carbon nanotubes, diatom frustules and small mineral crystals (asbestos fibres, for example) require no special treatment before being examined in the electron microscope. Samples of hydrated materials, including almost all biological specimens have to be prepared in various ways to stabilize them, reduce their thickness (ultrathin sectioning) and increase their electron optical contrast (staining).

Applications

Semiconductor and data storage

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UNIT I – V

 Circuit edit Defect analysis Failure analysis

Biology and life sciences

 Cryobiology Cryo-electron microscopy Diagnostic electron microscopy

 Drug research (e.g. antibiotics) Electron tomography

 Particle analysis Particle detection Protein localization

 Structural biology Tissue imaging Toxicology

 Virology (e.g. viral load monitoring)

Materials research

 Device testing and characterization Dynamic materials experiments

 Electron beam-induced deposition Materials qualification

 Medical research Nanometrology Nanoprototyping

Industry

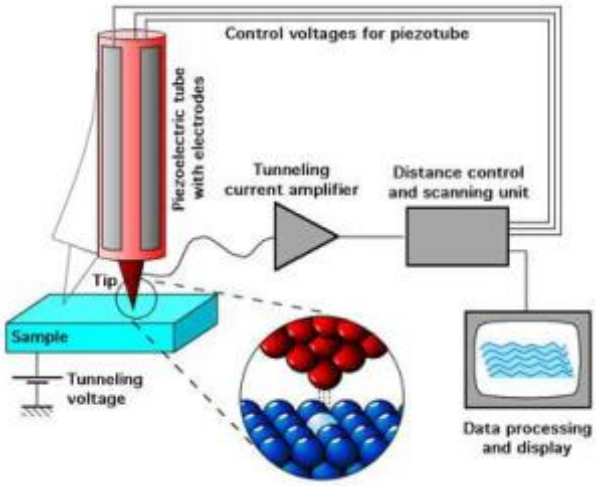
 Chemical/Petrochemical Direct beam-writing fabrication Food science

 Forensics Fractography Micro-characterization

 Mining (mineral liberation analysis) Pharmaceutical QC

Instrumentation of scanning tunneling microscope

Schematic view of an STM



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UNIT I – V

A **scanning tunneling microscope** (**STM**) is an instrument for imaging surfaces at the A scanning tunneling microscope (STM) is an instrument for imaging surfaces at the atomic level. Its development in 1981 earned its inventors, Gerd Binnig and Heinrich Rohrer (at IBM Zürich), the Nobel Prize in Physics in 1986. For a STM, good resolution is considered to be 0.1 nm lateral resolution and 0.01 nm (10 pm) depth resolution. With this resolution, individual atoms within materials are routinely imaged and manipulated. The STM can be used not only in ultra-high vacuum but also in air, water, and various other liquid or gas ambients, and at temperatures ranging from near zero kelvin to over 1000°C.

The components of an STM include scanning tip, piezoelectric controlled height and x,y scanner, coarse sample-to-tip control, vibration isolation system, and computer.

The resolution of an image is limited by the radius of curvature of the scanning tip of the STM. Additionally, image artifacts can occur if the tip has two tips at the end rather than a single atom; this leads to "double-tip imaging," a situation in which both tips contribute to the tunneling. Therefore, it has been essential to develop processes for consistently obtaining sharp, usable tips. Recently, carbon nanotubes have been used in this instance. The tip is often made of tungsten or platinum-iridium, though gold is also used. Tungsten tips are usually made by electrochemical etching, and platinum-iridium tips by mechanical shearing

Due to the extreme sensitivity of tunnel current to height, proper vibration insulation or an extremely rigid STM body is imperative for obtaining usable results. In the first STM by Binnig and Rohrer, magnetic levitation was used to keep the STM free from vibrations; now mechanical spring or gas spring systems are often used Additionally, mechanisms for reducing eddy currents are sometimes implemented.

Maintaining the tip position with respect to the sample, scanning the sample and acquiring the data is computer controlled. The computer may also be used for enhancing the image with the help of image processing as well as performing quantitative measurements.

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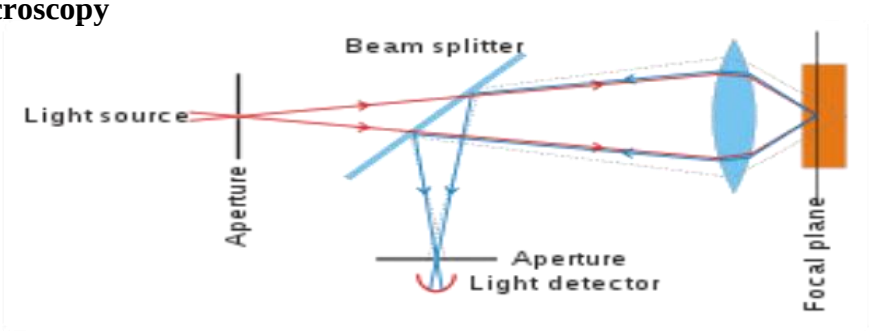
UNIT I – V

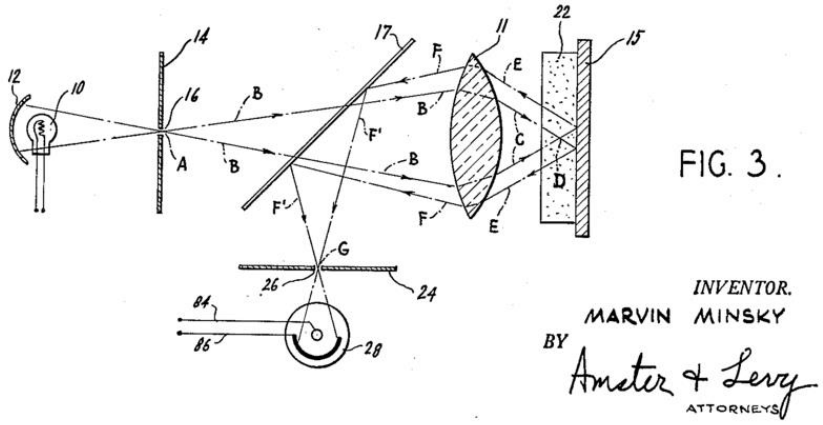
Confocal microscopy



Principle of confocal microscopy

Confocal microscopy is an optical imaging technique used to increase optical resolution and contrast of a micrograph by using point illumination and a spatial pinhole to eliminate out-offocus light in specimens that are thicker than the focal plane. It enables the reconstruction of three-dimensional structures from the obtained images. This technique has gained popularity in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.

Basic concept



Confocal point sensor principle from Minsky's patent

The principle of confocal imaging was patented in 1957 by Marvin Minsky and aims to overcome some limitations of traditional wide-field fluorescence microscopes. In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded evenly in light from a light source. All parts of the specimen in the optical path are excited at the same time and the resulting

fluorescence is detected by the microscope's photodetector or camera including a large unfocused background part. In contrast, a confocal microscope uses point illumination (see Point Spread Function) and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal - the name "confocal" stems from this configuration. As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution, particularly in the sample depth direction, is much better than that of wide-field microscopes. However, as much of the light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity – so long exposures are often required.

As only one point in the sample is illuminated at a time, 2D or 3D imaging requires scanning over a regular raster (i.e., a rectangular pattern of parallel scanning lines) in the specimen. The achievable thickness of the focal plane is defined mostly by the wavelength of the used light divided by the numerical aperture of the objective lens, but also by the optical properties of the specimen. The thin optical sectioning possible makes these types of microscopes particularly good at 3D imaging and surface profiling of samples.

Techniques used for horizontal scanning

Three types of confocal microscopes are commercially available:

 Confocal laser scanning microscopes use multiple mirrors (typically 2 or 3 scanning linearly along the x and the y axis) to scan the laser across the sample and "descan" the image across a fixed pinhole and detector.

 Spinning-disk (Nipkow disk) confocal microscopes use a series of moving pinholes on a disc to scan spot of light.

 Programmable Array Microscopes (PAM) use an electronically controlled spatial light modulator (SLM) that produces a set of moving pinholes. The SLM is a device containing an array of pixels with some property (opacity, reflectivity or optical rotation) of the individual pixels that can be adjusted electronically. The SLM contains microelectromechanical mirrors or liquid crystal components. The image is usually acquired by a CCD camera.

Each of these classes of confocal microscope have particular advantages and disadvantages. Most systems are either optimized for recording speed (i.e. video capture) or high spatial resolution. Confocal laser scanning microscopes can have a programmable sampling density and very high

resolutions while Nipkow and PAM use a fixed sampling density defined by the camera's resolution. Imaging frame rates are typically slower for single point laser scanning systems than spinning-disk or PAM systems. Commercial spinning-disk confocal microscopes achieve frame rates of over 50 per second – a desirable feature for dynamic observations such as live cell imaging. In practice, Nipkow and PAM allow multiple pinholes scanning the same area in parallel as long as the pinholes are sufficiently far apart. Cutting-edge development of confocal laser scanning microscopy now allows better than standard video rate (60 frames/second) imaging by using multiple microelectromechanical systems-based scanning mirrors.

Confocal X-ray fluorescence imaging is a newer technique that allows control over depth, in addition to horizontal and vertical aiming, for example, when analyzing buried layers in a painting.

Variants and enhancements

Improving axial resolution

The point spread function of the pinhole is an ellipsoid, several times as long as it is wide. This limits the axial resolution of the microscope. One technique of overcoming this is 4π microscopy where incident and or emitted light are allowed to interfere from both above and below the sample to reduce the volume of the ellipsoid. An alternative technique is confocal theta microscopy. In this technique the cone of illuminating light and detected light are at an angle to each other (best results when they are perpendicular). The intersection of the two PSFs gives a much smaller effective sample volume. From this evolved the single plane illumination microscope.

Super resolution

There are confocal variants that achieve resolution below the diffraction limit such as STED microscopy.

Low-temperature operability

To image samples at low temperature, two main approaches have been used, both based on the laser scanning confocal microscopy architecture. One approach is to use a continuous flow cryostat: only the sample is at low temperature and it is optically addressed through a transparent window. Another possible approach is to have part of the optics (especially the microscope objective) in a cryogenic storage dewar. This second approach, although more cumbersome,

guarantees better mechanical stability and avoids the losses due to the window.

pH meters

Who invented the pH meter?

Who do we have to thank for this clever stuff? First, Nobel-Prize winning German chemist Fritz Haber (1868–1934) and his student Zygmunt Klemensiewicz (1886–1963) developed the glass electrode idea in 1909. The modern, electronic pH meter was invented about a quarter century later, around 1934/5, when American chemist Arnold Beckman (1900–2004) figured out how to hook up a glass electrode to an amplifier and voltmeter to make a much more sensitive instrument

A typical pH meter has two basic components: the meter itself, which can be a moving-coil meter (one with a pointer that moves against a scale) or a digital meter (one with a numeric display), and either one or two probes that you insert into the solution you're testing. To make electricity flow through something, you have to create a complete electrical circuit; so, to make electricity flow through the test solution, you have to put two electrodes (electrical terminals) into it. If your pH meter has two probes (like the one in the photo at the top of this article), each one is a separate electrode; if you have only one probe, both of the two electrodes are built inside it for simplicity and convenience.

The electrodes aren't like normal electrodes (simple pieces of metal wire); each one is a mini chemical set in its own right. The electrode that does the most important job, which is called the glass electrode, has a silver-based electrical wire suspended in a solution of potassium chloride, contained inside a thin bulb (or membrane) made from a special glass containing metal salts (typically compounds of sodium and calcium). The other electrode is called the reference electrode and has a potassium chloride wire suspended in a solution of potassium chloride.

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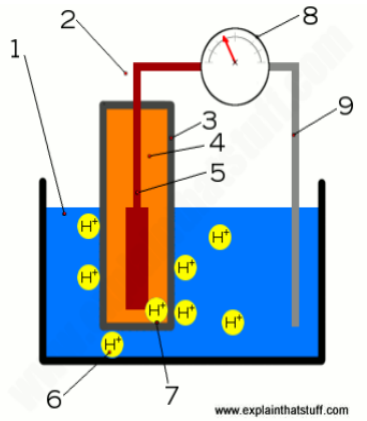
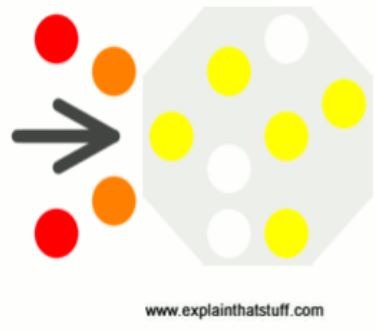


Artwork: Key parts of a pH meter: (1) Solution being tested; (2) Glass electrode, consisting of (3) a thin layer of silica glass containing metal salts, inside which there is a potassium chloride solution (4) and an internal electrode (5) made from silver/silver chloride. (6) Hydrogen ions formed in the test solution interact with the outer surface of the glass. (7) Hydrogen ions formed in the potassium chloride solution interact with the inside surface of the glass. (8) The meter measures the difference in voltage between the two sides of the glass and converts this "potential difference" into a pH reading. (9) Reference electrode acts as a baseline or reference for the measurement—or you can think of it as simply completing the circuit.

The potassium chloride inside the glass electrode (shown here colored orange) is a neutral solution with a pH of 7, so it contains a certain amount of hydrogen ions (H+). Suppose the unknown solution you're testing (blue) is much more acidic, so it contains a lot more hydrogen ions. What the glass electrode does is to measure the difference in pH between the orange solution and the blue solution by measuring the difference in the voltages their hydrogen ions produce. Since we know the pH of the orange solution, we can figure out the pH of the blue solution.



How does it all work? When you dip the two electrodes into the blue test solution, some of the hydrogen ions move toward the outer surface of the glass electrode and replace some of the metal ions inside it, while some of the metal ions move from the glass electrode into the blue solution. This ion-swapping process is called ion exchange, and it's the key to how a glass electrode works. Ion-swapping also takes place on the inside surface of the glass electrode from the orange solution. The two solutions on either side of the glass have different acidity, so a different amount of ion-swapping takes place on the two sides of the glass. This creates a different degree of hydrogen-ion activity on the two surfaces of the glass, which means a different amount of electrical charge builds up on them. This charge difference means a tiny voltage (sometimes called a potential difference, typically a few tens or hundreds of millivolts) appears between the two sides of the glass, which produces a difference in voltage between the silver electrode (5) and the reference electrode (8) that shows up as a measurement on the meter.

Although the meter is measuring voltage, what the pointer on the scale (or digital display) actually shows us is a pH measurement. The bigger the difference in voltage between the orange (inside) and blue (outside) solutions, the bigger the difference in hydrogen ion activity between. If there is more hydrogen ion activity in the blue solution, it's more acidic than the orange solution and the meter shows this as a lower pH; in the same way, if there's less hydrogen ion activity in the blue solution, the meter shows this as a higher pH (more alkaline).

Making accurate pH measurements

For pH meters to be accurate, they have to be properly calibrated (the meter is accurately translating voltage measurements into pH measurements), so they usually need testing and adjusting before you start to use them. You calibrate a pH meter by dipping it into buffers (test solutions of known pH) and adjust the meter accordingly. Another important consideration is that pH measurements made this way depend on temperature. Some meters have built-in thermometers and automatically correct their own pH measurements as the temperature changes; those are best if fluctuations in temperature are likely to occur while you're making a number of different measurements. Alternatively, you can correct the pH measurement yourself, or allow for it by calibrating your instrument and making pH measurements at broadly the same temperature.

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UNIT I – V

Microtechnique

I collection of material

In general plant material should be cut into pieces not exceeding 1 cm in diameter to allow the fixative to penetrate the tissues. Use a clean sharp razor blade for this purpose and be careful not to damage material by squeezing. Make a sketch of the plant before taking specimens and indicate where each piece came from. Place this sketch in your notebook.

II Fixation

Making Up Osmium Tetraoxide Solutions

Making Up Osmium Tetraoxide Solutions

First and foremost - osmium*vapors are extremely toxic and can permanently damage eyes, respiratory tract and skin. Always use in fume hood and wear acid resistant gloves and lab coat. If accident occurs seek medical attention immediately.

For 2% stock OsO4 solution:

1. Dry an acid-cleaned 50 cc reagent bottle in the oven. Stopper and cool to room temperature.
2. Take a ½ gm vial of OsO4 *, and taking care not to warm it in your hand, wash the label off in cold water. Wash vial in acetone, and let air dry.
3. Measure out 25 ml distilled water in a graduated cylinder, and keep it handy.
4. Knock loose crystals of OsO4 to one side of the vial. Place vial on glassine paper, and score lightly with glass saw. Score must be continuous and complete. Do not separate halves.

CAUTION: After acetone washing, the glass is very brittle and will break very easily.

5. Drop vial into bottle and replace stopper. If vial did not break when dropped, give the bottle a sharp rap with the side of your hand. (If still didn't break, start again, or smash vial with thick glass rod.)*
6. Add water, and restopper. Swirl bottle twice only (don't shake) and leave at room temperature overnight. Solution will be clear by morning. Cover bottle with aluminum foil to protect from light.
7. Left-over osmium tetraoxide solutions may be stored frozen in tightly sealed containers

a) Types of fixatives

There are three common ways of classifying fixation: acidic fixation vs. basic, coagulant vs. nonT coagulant, and additive vs. non-additive.

Acidic fixation preserves chromosomes, nucleoli, and the spindle apparatus, while dissolving nucleoplasm and mitochondria. Conversely, basic fixation preserves nucleoplasm, mitochondria and dissolves chromatin and the spindle apparatus.

Coagulant fixatives (such as ethanol, methanol, picric acid, murcuric chloride, acetone, and chromium trioxide) cause cytoplasm to become opaque and congeal into a netlike structure. Nonc coagulant fixatives do not disrupt the cytoplasm ut transform it into a transparent gel. Examples of fixatives of this kind are glutaraldehyde, formaldehyde, acrolein, potassium dichromate, acetic acid and osmium tetraoxide. Non-coagulant fixatives very often stabilize tissues to such an extent that they are protected from disruption by the use of subsequent coagulant reagents during post-fixation or dehydration.

Additive fixatives cross-link with proteins in cells, strengthening cell structure and insuring tissue preservation. Examples of additive fixatives are the aldehydes.

As can be said thus far, there is no one substance that will successfully fix all components of a cell or tissue equally. It is necessary to combine fixatives in such a way so as to minimize the shrinking or swelling action of the various ingredients and to stabilize the various cell structures. Any fixation schedule should be regarded as a starting point from which the best

method of fixation for one's specimen will be developed.

b) Factors Affecting Fixation

1. Temperature

Autolysis, the self-digestion of a cell through the action of hydrolytic enzymes, increases with temperature. Therefore fixation at low temperatures (0°-4°C) is recommended. For some tissues, a second change of fixative to wash out any remaining hydrolytic enzymes is beneficial.

2. pH

Cells in nature are normally buffered and have a certain pH. To minimize cellular disruption the use of buffered fixatives equaling the pH of the tissue is recommended. In general a pH of 7.0 -

7.4 for most tissues is optimum.

3. Osmolarity (concentration of salts and solutes withing the cell)

The osmolarity of the buffer used with the fixative ideally should also match the osmolarity of the specimen. For most specimens an osmolarity between 0.025 - 0.1M buffer is sufficient. Salt water plants (sea grasses) will have higher osmolarities.

4. Length of fixation

The length of fixation is dependent upon the type of fixative used (and thus the rate of penetration) and the size of the specimen. For small specimens (<1 cm in diameter) less time will be needed. Larger specimens (>1 cm) will require longer times and a fast penetrating fixative such as acrolein should be used. Depending then on the type of fixative and the size of the specimen, fixation length can be anywhere from 4 hrs - 24 hrs. (Since some cellular components such as mitochondria and certain proteins may be leached out during fixation and dehydration , times should be kept to a minimum. Again this will depend upon the objectives of the investigator, i.e. the level of resolution desired). Very long specimens, organs, with a thick dense cuticle and tissues of low water content such as seeds and seeweeds.

c) Fixative Formulas

1. FAA - Formalin- Acetic Acid -Alcohol

1. FAA - Formalin- Acetic Acid -Alcohol

50% (or 70%) Ethanol 90 ml

Glacial Acetic Acid 5 ml

Formaldehyde (38%) 5 ml

This is by far the most common fixative used in the botanical studies. It is a stable fluid and material may be stored in it for years. It is ideally suited to large impervious objects such as woody twigs, stems, roots and tough herbaceous stems. More tender material however, may be damaged from the shrinkage caused by the alcohol concentration. This can be remedied by varying the acetic acid concentration from 2 -6% by volume (acetic acid has a swelling action on protoplasm).

Fixation time is approximately 18-24 hrs for normal material and several days for woody material.

This fixative is not recommended for plastic embedding.

Wash in 50% ethanol before proceeding to paraffin.

III. Dehydration and Infiltration and embedding.

In order to section tissues, they must first be supported by an embedding medium to avoid disruption and/or compression. Ideally the embedding medium should match the tissue type in strength and hardness. If the embedding medium is too soft for the material, the tissue will not be supported and sections will be torn or shredded. If the medium is too hard for the tissue, sections will be brittle and will shatter. To infiltrate the tissues with supporting embedding medium, tissues must be free of all water (since usually embedding medium is not miscible with water). This is accomplished by using dehydrating solutions such as ethanol and TBA. If the dehydration solution is not a solvent of the embedding medium, the specimens are infiltrated with it. After the tissue specimen has been properly oriented in a mold, the embedding medium is then hardened (either by cooling for the paraffin method or by heating for the plastic method) and the tissue is then ready to be sectioned.

A. Paraffin Method

1. Fixed material is dehydrated gradually in a graded tertiary butyl alcohol (TBA) series. The material is first brought to 50-70% Ethanol and then transferred to TBA1 proceeding to anhydrous TBA in 5 steps. The material is then transferred to TBA1 proceeding to anhydrous TBA in 5 steps. The material is then transferred first to pure TBA at 37 °C and then to pure TBA at 61 °C. It is then changed into equal portions of paraffin wax and pure TBA at 61 °C. Two changes of pure wax follow and then the material may be embedded.

3. Embedding For Paraffin Method

It is important to have an embedding bench set up near the paraffin oven. Have alcohol lamps on hand, matches, a bucket of crushed ice, a petri dish, and tools such as needles, tweezers, molds, mold holders and a pencil.

The most convenient molds to use for paraffin embedding are metal boats available from Fisher Scientific Co. the mold is first sprayed with mold release compound in a fume hood. The plastic mold holder is labelled with a pencil as to subject, orientation and date if necessary. The metal boat mold is then heated over the alcohol flame and molten wax is poured into it. The mold is

briefly placed on top of a petri dish with crushed ice to form a thin layer of hardened wax on the bottom. The specimen is quickly* transferred to the mold with hot tweezers and orientated** with hot needles or tweezers. The plastic mold holder is quickly placed on top of the metal boat and more molten wax is added. A hot curved needle is quickly moved around underneath the rim of the plastic holder to dislodge any trapped air bubbles. The completed assembly is carefully placed on crushed ice in an ice bucket and allowed to cool. The plastic holder may be topped off with molten wax as it cools. When the block has completely cooled, remove metal boat and store block with attached plastic holder in refrigerator until ready to section.

*It is important to work quickly while transferring specimens or wax from oven because wax hardens quickly. Always remember to put wax container back into oven immediately and close oven door between transfers.

**Specimens may be placed in pith prior to dehydration and oriented under a dissecting microscope. The piece of pith is then made asymmetrical and can be easily oriented during embedding.

4. Sectioning (Microtoming) for Paraffin Method

Trim cooled tissueprep block with a sharp razor blade. Make a trapezoid locating the specimen in the center. Take care that the upper and lower edges of the trapezoid are indeed parallel, otherwise the ribbons produced will be curved. Make the angle of the cuts wide so that the cutting face will have a large sturdy base to support it.

Insert plastic holder containing cooled block into microtome clamp. Insert sharp knife* into holder, adjust knife angle and lock into place. Orient the longer edge of the trapezoid parallel to the knife edge. Adjust block so that cutting face is parallel to knife edge. Carefully bring knife close to paraffin block, do not let knife edge touch block surface and leave enough of a gap between knife edge and block so that the block will not touch the knife on the downstroke of the block. If the block does touch the knife and a thick section is cut off, move to another part of the knife (because the knife edge is ruined) and reposition knife and block as before. Lock knife into place. Adjust section thickness (usually 6-10 µ). Slowly and steadily begin to turn wheel of microtome. Do not turn wheel fast in an attempt to make sections appear faster. To do so will damage the gears of the microtome.

When sections begin to appear, gently pick up the end of the ribbon with a camel hair brush and hold it up, away from the knife side.

When a ribbon** of a sufficient length has been cut, lock microtome and reomve ribbon with camel hair brushes, be careful not to touch knife edge, (never use metal needle when removing ribbon from knife as it is very easy to badly damage the knife edge by touching needle to it) and place on clean piece of cardboard (in a draft free area) until enough ribbons have accumulated to make a slide.

*CAUTION! Knife edge is extremely sharp!

**If ribbon fails to form or if any other sectioning difficulties are encountered consult page entitled “Sectioning Difficulties”.

To mount the ribbons take a clean glass slide, moisten surface with breath and wipe dry with Kimwipe. Carefully label extreme left part of slide and number with a diamond pencil. Do not let fingers or hand touch slide where sections will be placed. Mix 2 drops Haupt’s adhesive and 6 drops 4% Formalin solution on slide. Cut ribbons to desired length and arrange on top of adhesive mixture with a needle.

Place slide on a warming table adjusted to not more than 1° below melting point of the wax used (i.e. 60 °C for 61 °C tissuemat). This gives maximum stretching of sections to counteract the compression incurring during sectioning.

As soon as sections change luster (i.e become transparent) and flatten out, remove from warming table. Do not let sections melt (i.e. if the temperature of the warming table is greater than 61 °C) because cellular disruption will occur. Make sure that the sections have indeed changed luster and expanded because insufficiently heated ribbons do not adhere well and may come off or buckle during subsequent processing.

Drain excess adhesive off slide and blot dry with moistened bibulous paper.

Accumulate slides in glass carrier and dry in 37 °C oven overnight. If Sakai’s toluidine blue method is to be used, dry at room temperature in a dust free place or in an oven only 1 hr or less.

Mounting

Remove slide from XYLENE IV with tweezers and place on blotting paper. (Make sure section side is facing up!) Drain excess xylene. Put a drop of mounting medium (Permount) on sections

and gently lower coverslip with tweezers. When coverslip is on, remove any air bubbles that may have become trapped by pressing down with tweezers and wooden handle of dissecting needle. Gently turn covered slide over and blot and excess permount. Place covered slide on warming table (53 EC) and carefully put weights on top and let harden overnight. Extreme care should be taken when handling newly covered slides as it is easy for coverslip to dislodge and damage tissue.

Never in haste examine freshly covered slides with microscope, as it is possible to get a drop of "wet" permount on the objective. Once on, it is difficult to remove and may damage the lens.

Staining

For the majority of stains (except Sakai's Toluidine Blue), slides need to be deparaffinized prior to staining. To do this, a series of glass dishes is set up along a bench and the slide carrier is passed through them according to the following schedule:

Aqueous Safranin and Delafield's Hematoxylin Method

1. Transfer the sections to the stain from either water or 50% alcohol. The length of the time in stain from 15 to 30 minuted depending on the material. Try a trial slide for 10 minutes.
2. Wash in running tap water a few minutes to remove completely all excess stain. The washing should be as thorough as possible to avoid the formation of troublesome precipitates.
3. Treat briefly (5-10 secs) with acidulated water (2-3 drops of hydrochloric acid in 100 ml water) until the sections turn to a pale pinkish purple color. Care must be taken not to extract too much stain; then transfer quickly to water and wash in slowly running tap water for 20 minutes at least until the sections acquire a rich purple color.
4. Counter stain directly in 0.01% aqueous safranin 0 for 10-15 minutes. (Note: prepare a 2% stock solution by shaking up 2 g of safranin 0 in 100 ml water. A 0.01% solution is made by diluting 1 ml stock with 200 ml water).
5. remove excess stain be rinsing in water then pass sections in 15%, 30%, 50%, 70% alcohol leaving in each strength for 30-60 secs. Destain further in 95% and 100% alcohol until sections appear purple wit only a slight tinge of red. Rinse in 100% alcohol and then in xylene/ethanol, xylene III, IV.

Results:

Purple- Nuclei

cellulose walls

middle lamellae

chromosomes

mitochondria

Slightly red- Cytoplasm

Safranin and Fast Green FCF

Bring sections to 70% ethanol.

1. Stain in Johansen's Safranin overnight. The next day, wash out excess stain in water.
2. Destain briefly in 50% ethanol
3. Stain very quickly for 2 to 5 seconds in Fast Green, then,
4. Differentiate for 10 to 15 seconds (more or less) to remove excess fast green. Dip the slides up and down in the differentiating solution 10 to 15 times. When differentiating solution is used 4 to

5 times and becomes dark, replace.

5. Xylene-clove oil (I) for 5 minutes.
6. Xylene-clove oil (II) for 5 minutes.
7. Xylene (III) for 5 minutes*
8. Xylene (IV) for 5 minutes**
9. Mount.

** replace Xylene (III) with Xylene (IV) and fresh xylene regularly, as done with xylene (I) and (II).

Preparation of staining solutions:

A. Johansen's Safranin:

Dissolve 5 g Safranin in 250 ml methyl cellosolve; add 125 ml 95% ethanol then dissolve 5 g sodium acetate in 125 ml water and 10 ml 40% formaldehyde and add to dye solution.

B. Fast Green Solution:

Saturate a mixture of equal parts of methyl cellosolve and clove oil with fast green FCF. i.e.

mix 30 ml Methyl cellosolve plus 30 ml clove oil, and saturate with fast green.

Filter and add 175 ml 95% ethanol

+ 175 ml TBA

+ 60 ml 1% glacial acetic acid

Differentiating solution for Fast Green:

Differentiate with 95% ethanol and TBA (1:1) plus 0.5% acetic acid (0.5 ml acetic acid per 100 ml; e.g. 150 ml 95% ethanol (1:1)

150 ml TBA

1.5 ml acetic acid

Results obtained:

Red Green

Nuclei Cellulose walls

Nucleoli Cytoplasm

Chromosomes

Lignified and

cutinized cell walls

Colorimeter

A **colorimeter** is a device used in colorimetry. In scientific fields the word generally refers to the device that measures the absorbance of particular wavelengths of light by a specific solution. This device is most 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:

- 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

Filters

Changeable optics filters are used in the colorimeter to select the wavelength of light which the solute absorbs the most, in order to maximize accuracy. The usual wavelength range is from 400 to 700 nanometers (nm). If it is necessary to operate in the ultraviolet range (below 400 nm) then some modifications to the colorimeter are needed. In modern colorimeters the filament lamp and filters may be replaced by several light-emitting diodes of different colors.

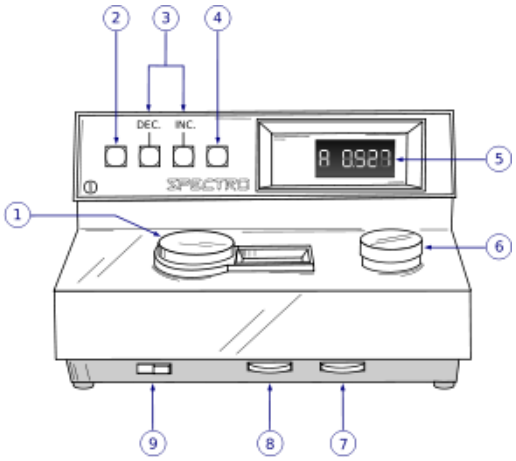
Cuvette

In a manual colorimeter the cuvettes are inserted and removed by hand. An automated colorimeter (as used in an AutoAnalyzer) is fitted with a **flowcell** through which solution flows continuously.

Output

The output from a colorimeter may be displayed by an analogue or digital meter and may be shown as transmittance (a linear scale from 0-100%) or as absorbance (a logarithmic scale from zero to infinity). The useful range of the absorbance scale is from 0-2 but it is desirable to keep

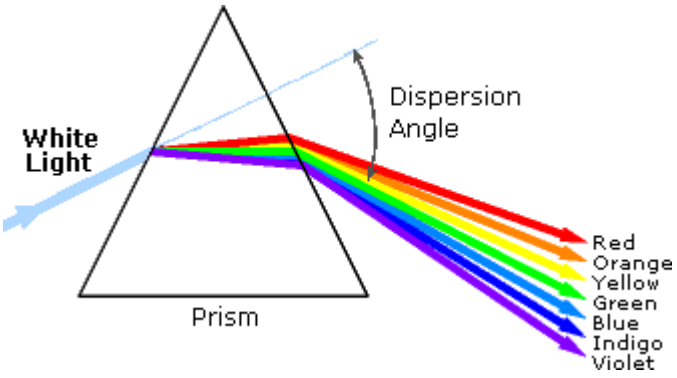
within the range 0-1 because, above 1, the results become unreliable due to scattering of light.



(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

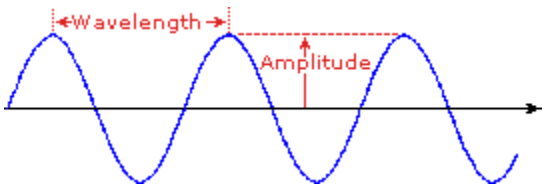
Visible and Ultraviolet Spectroscopy

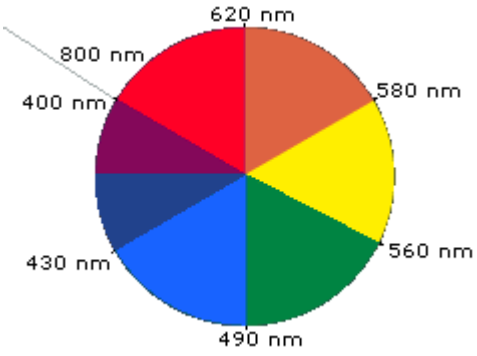
1. Background



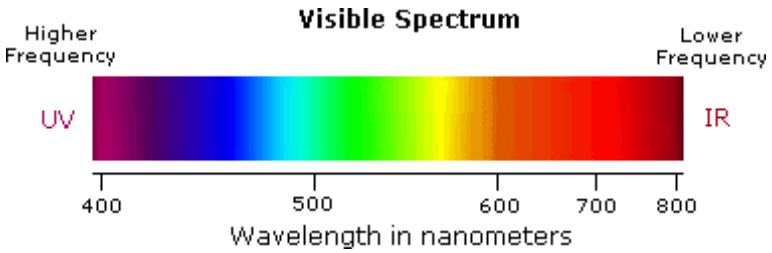
An obvious difference between certain compounds is their color. Thus, quinone is yellow; chlorophyll is green; the 2,4- dinitrophenylhydrazone derivatives of aldehydes and ketones range in color from bright yellow to deep red, depending on double bond conjugation; and aspirin is colorless. In this respect the human eye is functioning as a spectrometer analyzing the light reflected from the surface of a solid or passing through a liquid. Although we see sunlight

(or white light) as uniform or homogeneous in color, it is actually composed of a broad range of radiation wavelengths in the ultraviolet (UV), visible and infrared (IR) portions of the spectrum. As shown on the right, the component colors of the visible portion can be separated by passing sunlight through a prism, which acts to bend the light in differing degrees according to wavelength. Electromagnetic radiation such as visible light is commonly treated as a wave phenomenon, characterized by a wavelength or frequency. **Wavelength** is defined on the left below, as the distance between adjacent peaks (or troughs), and may be designated in meters, centimeters or nanometers (10^{-9} meters). **Frequency** is the number of wave cycles that travel past a fixed point per unit of time, and is usually given in cycles per second, or hertz (Hz). Visible wavelengths cover a range from approximately 400 to 800 nm. The longest visible wavelength is red and the shortest is violet. Other common colors of the spectrum, in order of decreasing wavelength, may be remembered by the mnemonic: **ROY G BIV**. The wavelengths of what we perceive as particular colors in the visible portion of the spectrum are displayed and listed below. In horizontal diagrams, such as the one on the bottom left, wavelength will increase on moving from left to right.





Violet: 400 - 420 nm
Indigo: 420 - 440 nm
Blue: 440 - 490 nm
Green: 490 - 570 nm
Yellow: 570 - 585 nm
Orange: 585 - 620 nm
Red: 620 - 780 nm



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

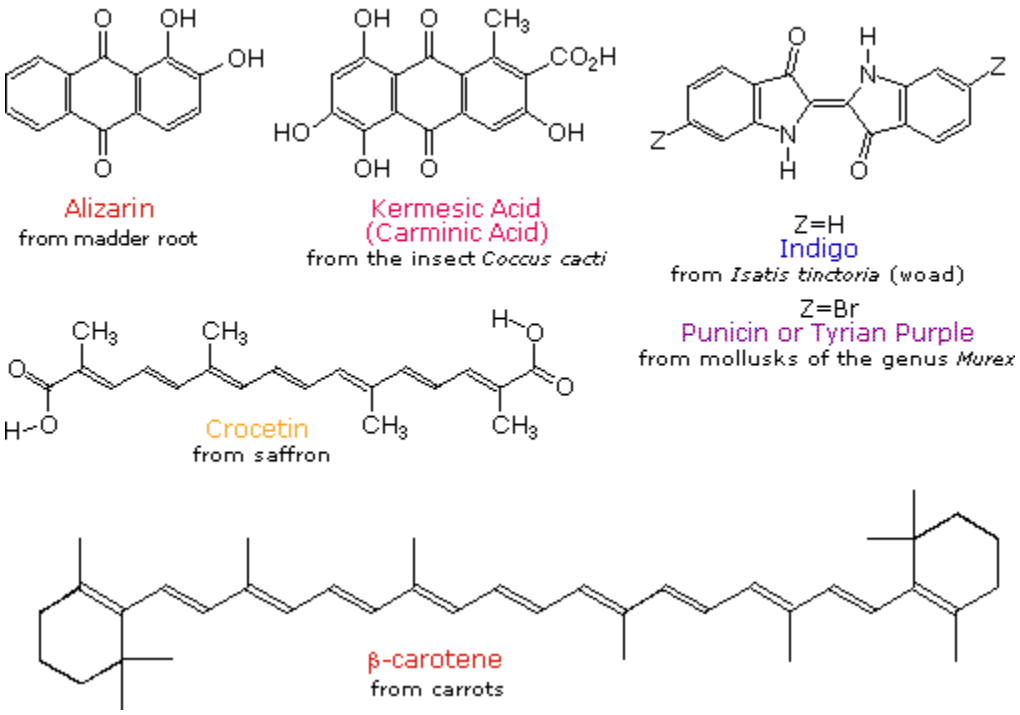
COURSE MATERIAL

Subject Name: ANALYTICAL TECHNIQUES IN BIOTECHNOLOGY Code: SBB1201

UNIT I – V

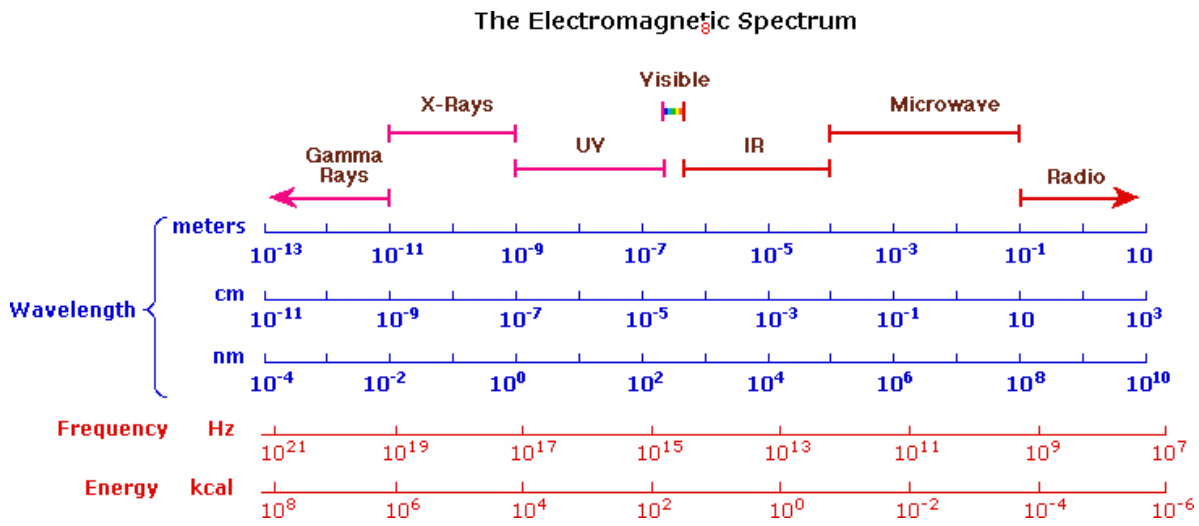
When white light passes through or is reflected by a colored substance, a characteristic portion of the mixed wavelengths is absorbed. The remaining light will then assume the complementary color to the wavelength(s) absorbed. This relationship is demonstrated by the color wheel shown on the right. Here, complementary colors are diametrically opposite each other. Thus, absorption of 420-430 nm light renders a substance yellow, and absorption of 500-520 nm light makes it red. Green is unique in that it can be created by absoption close to 400 nm as well as absorption near 800 nm. Early humans valued colored pigments, and used them for decorative purposes. Many of these were inorganic minerals, but several important organic dyes were also known. These included the crimson pigment, kermesic acid, the blue dye, indigo, and the yellow saffron pigment, crocetin. A rare dibromo- indigo derivative, punicin, was used to color the robes of the royal and wealthy. The deep orange hydrocarbon carotene is widely distributed in plants, but is not sufficiently stable to be used as permanent pigment, other than for food coloring. A common feature of all these colored compounds, displayed below, is a system of **extensively conjugated pi-electrons**.

Some Natural Organic Pigments



2. The Electromagnetic Spectrum

The visible spectrum constitutes but a small part of the total radiation spectrum. Most of the radiation that surrounds us cannot be seen, but can be detected by dedicated sensing instruments. This **electromagnetic spectrum** ranges from very short wavelengths (including gamma and x-rays) to very long wavelengths (including microwaves and broadcast radio waves). The following chart displays many of the important regions of this spectrum, and demonstrates the inverse relationship between wavelength and frequency (shown in the top equation below the chart).



The energy associated with a given segment of the spectrum is proportional to its frequency. The bottom equation describes this relationship, which provides the energy carried by a photon of a given wavelength of radiation.

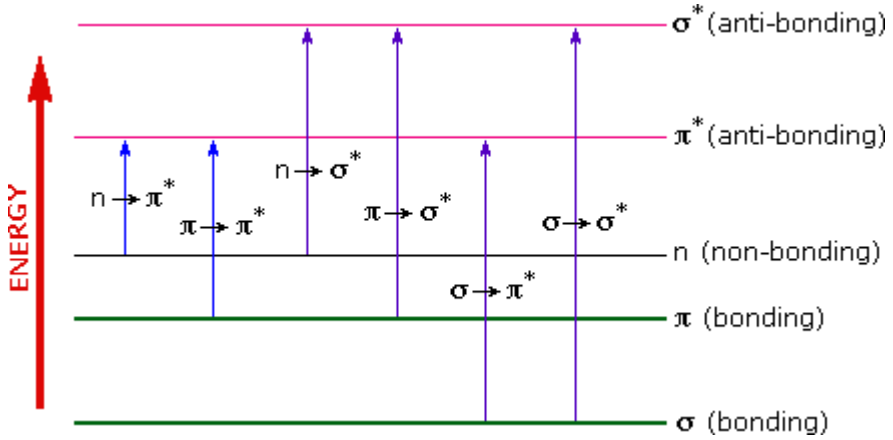
$\upsilon = c/\lambda$ υ=frequency, λ=wavelength, c=velocity of light ($c = 3 \cdot 10^{10}$ cm/sec)
$\Delta E = h\upsilon$ E=energy, υ=frequency, h=Planck's constant ($h = 6.6 \cdot 10^{-27}$ erg sec)

To obtain specific frequency, wavelength and energy values use this calculator.

3. UV-Visible Absorption Spectra

To understand why some compounds are colored and others are not, and to determine the relationship of conjugation to color, we must make accurate measurements of light absorption at different wavelengths in and near the visible part of the spectrum. Commercial optical spectrometers enable such experiments to be conducted with ease, and usually survey both the near ultraviolet and visible portions of the spectrum.

For a description of a UV-Visible spectrometer Click Here.



The visible region of the spectrum comprises photon energies of 36 to 72 kcal/mole, and the near ultraviolet region, out to 200 nm, extends this energy range to 143 kcal/mole. Ultraviolet radiation having wavelengths less than 200 nm is difficult to handle, and is seldom used as a routine tool for structural analysis.

The energies noted above are sufficient to promote or excite a molecular electron to a higher energy orbital. Consequently, absorption spectroscopy carried out in this region is sometimes called "electronic spectroscopy". A diagram showing the various kinds of electronic excitation that may occur in organic molecules is shown on the left. Of the six transitions outlined, only the two lowest energy ones (left-most, colored blue) are achieved by the energies available in the 200 to 800 nm spectrum. As a rule, energetically favored electron promotion will be from the **highest occupied molecular orbital (HOMO)** to the **lowest unoccupied molecular orbital (LUMO)**, and the resulting species is called an **excited state**. For a review of molecular orbitals click here. When sample molecules are exposed to light having an energy that matches

a possible electronic transition within the molecule, some of the light energy will be absorbed as the electron is promoted to a higher energy orbital. An optical spectrometer records the wavelengths at which absorption occurs, together with the degree of absorption at each wavelength. The resulting spectrum is presented as a graph of absorbance (A) versus wavelength, as in the isoprene spectrum shown below. Since isoprene is colorless, it does not absorb in the visible part of the spectrum and this region is not displayed on the graph. **Absorbance** usually ranges from 0 (no absorption) to 2 (99% absorption), and is precisely defined in context with spectrometer operation.

Because the absorbance of a sample will be proportional to the number of absorbing molecules in the spectrometer light beam (e.g. their molar concentration in the sample tube), it is necessary to correct the absorbance value for this and other operational factors if the spectra of different compounds are to be compared in a meaningful way. The corrected absorption value is called "molar absorptivity", and is particularly useful when comparing the spectra of different compounds and determining the relative strength of light absorbing functions (chromophores). **Molar absorptivity** (ε) is defined as:

$$\text{Molar Absorptivity, } \varepsilon = A / c\, l$$

(where *A*= absorbance, **c** = sample concentration in moles/liter & **l** = length of light path through the sample in cm.)

If the isoprene spectrum on the right was obtained from a dilute hexane solution ($c = 4 * 10^{-5}$ moles per liter) in a 1 cm sample cuvette, a simple calculation using the above formula indicates a molar absorptivity of 20,000 at the maximum absorption wavelength. Indeed the entire vertical absorbance scale may be changed to a molar absorptivity scale once this information about the sample is in hand.

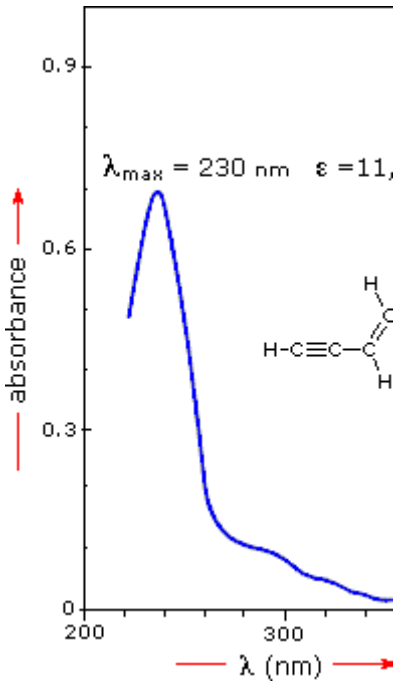
The only molecular moieties likely to absorb light in the 200 to 800 nm region are pi-electron functions and hetero atoms having non-bonding valence-shell electron pairs. Such light absorbing groups are referred to as **chromophores**. A list of some simple chromophores and their light absorption characteristics is provided on the left above. The oxygen non- bonding electrons in alcohols and ethers do not give rise to absorption above 160 nm. Consequently, pure alcohol and ether solvents may be used for spectroscopic studies. The

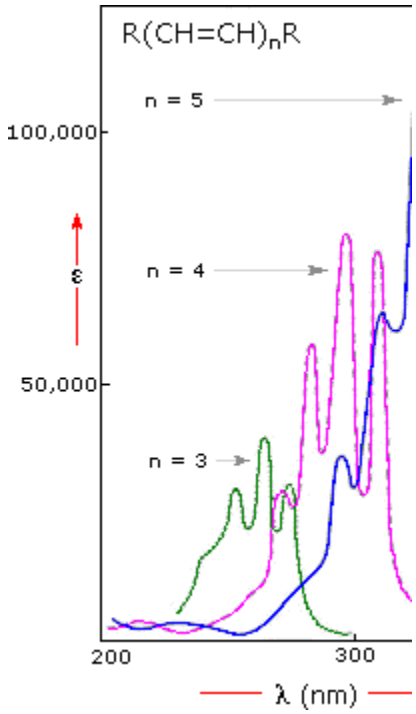
presence of chromophores in a molecule is best documented by UV-Visible spectroscopy, but the failure of most instruments to provide absorption data for wavelengths below 200 nm makes the detection of isolated chromophores problematic. Fortunately, conjugation generally moves the absorption maxima to longer wavelengths, as in the case of isoprene, so conjugation becomes the major structural feature identified by thistechnique.

Molar absorptivities may be very large for strongly absorbing chromophores (>10,000) and very small if absorption is weak (10 to 100). The magnitude ofε reflects both the size of the chromophore and the probability that light of a given wavelength will be absorbed when it strikes the chromophore.

4. The Importance of Conjugation

A comparison of the absorption spectrum of 1-pentene, λ_{max} = 178 nm, with that of isoprene (above) clearly demonstrates the importance of chromophore conjugation. Further evidence of this effect is shown below. The spectrum on the left illustrates that conjugation of double and triple bonds also shifts the absorption maximum to longer wavelengths. From the polyene spectra displayed in the center diagram, it is clear that each additional double bond in the conjugated pi-electron system shifts the absorption maximum about 30 nm in the same direction. Also, the molar absorptivity (ε) roughly doubles with each new conjugated double bond. Spectroscopists use the terms defined in the table on the right when describing shifts in absorption. Thus, extending conjugation generally results in bathochromic and hyperchromic shifts in absorption. The appearance of several absorption peaks or shoulders for a given chromophore is common for highly conjugated systems, and is often solvent dependent. This fine structure reflects not only the different conformations such systems may assume, but also electronic transitions between the different vibrational energy levels possible for each electronic state. Vibrational fine structure of this kind is most pronounced in vapor phase spectra, and is increasingly broadened and obscured in solution as the solvent is changed from hexane to methanol.





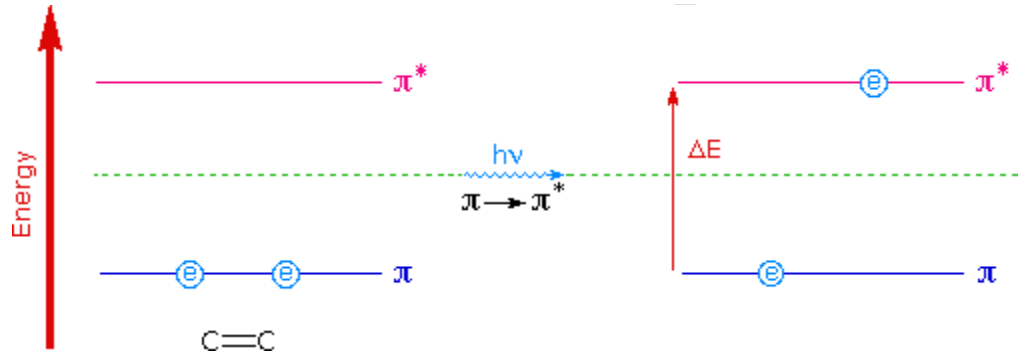
Terminology for Absorption Shifts

Nature of Shift	Descriptive Term
To Longer Wavelength	Bathochromic
To Shorter Wavelength	Hypsochromic
To Greater Absorbance	Hyperchromic
To Lower Absorbance	Hypochromic

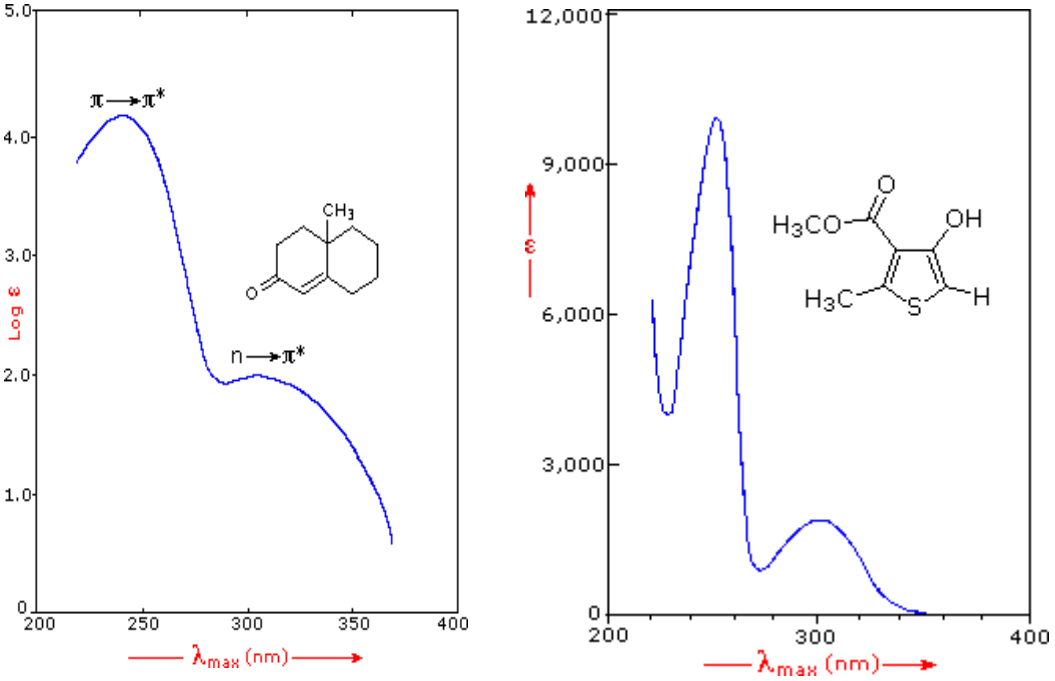
– To understand why conjugation should cause bathochromic shifts in the absorption maxima of chromophores, we need to look at the relative energy levels of the pi-orbitals. When two double bonds are conjugated, the four p-atomic orbitals combine to generate four pi-molecular orbitals (two are bonding and two are antibonding). In a similar manner, the three double bonds of a conjugated triene create six pi-molecular orbitals, half bonding and half antibonding. The energetically most favorable $\pi > \pi^*$ excitation occurs from the highest energy bonding pi-orbital (**HOMO**) to the lowest energy antibonding pi-orbital (**LUMO**). The following diagram illustrates this excitation for an isolated double bond (only two pi-orbitals) and, on clicking the

diagram, for a conjugated diene and triene. In each case the HOMO is colored blue and the LUMO is colored magenta. Increased conjugation brings the HOMO and LUMO orbitals closer together. The energy (ΔE) required to effect the electron promotion is therefore less, and the wavelength that provides this energy is increased correspondingly (remember **λ = h • c/ΔE**).

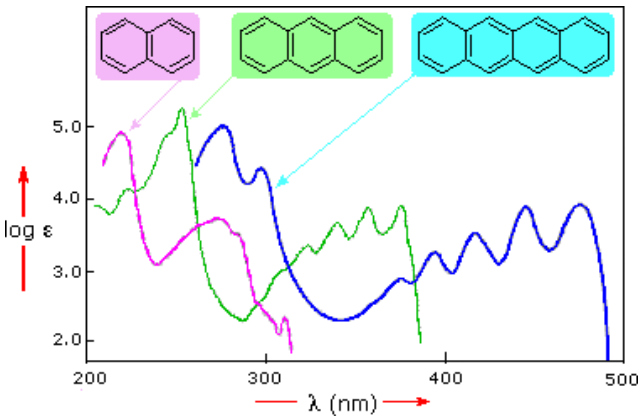
Examples of π > π* Excitation

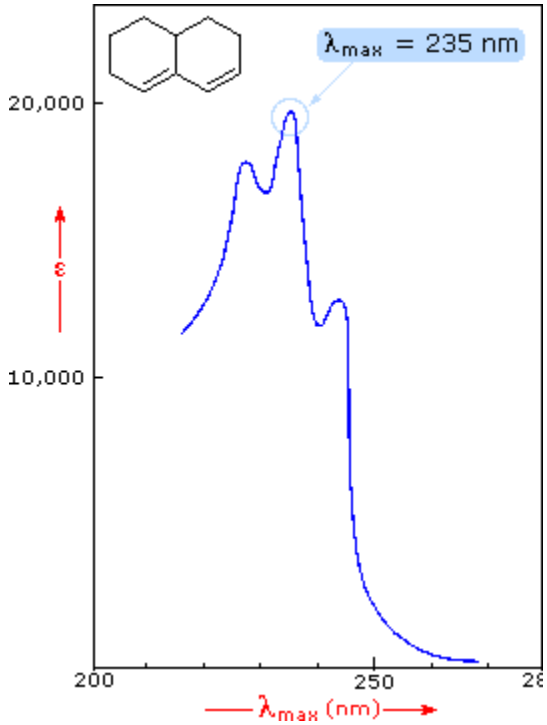


Many other kinds of conjugated pi-electron systems act as chromophores and absorb light in the 200 to 800 nm region. These include unsaturated aldehydes and ketones and aromatic ring compounds. A few examples are displayed below. The spectrum of the unsaturated ketone (on the left) illustrates the advantage of a logarithmic display of molar absorptivity. The π > π* absorption located at 242 nm is very strong, with an ε = 18,000. The weak n > π* absorption near 300 nm has an ε = 100.



Benzene exhibits very strong light absorption near 180 nm (ε > 65,000) , weaker absorption at 200 nm (ε = 8,000) and a group of much weaker bands at 254 nm (ε = 240). Only the last group of absorptions are completely displayed because of the 200 nm cut-off characteristic of most spectrophotometers. The added conjugation in naphthalene, anthracene and tetracene causes bathochromic shifts of these absorption bands, as displayed in the chart on the left below. All the absorptions do not shift by the same amount, so for anthracene (green shaded box) and tetracene (blue shaded box) the weak absorption is obscured by stronger bands that have experienced a greater red shift. As might be expected from their spectra, naphthalene and anthracene are colorless, but tetracene is orange.





The spectrum of the bicyclic diene (above right) shows some vibrational fine structure, but in general is similar in appearance to that of isoprene, shown above. Closer inspection discloses that the absorption maximum of the more highly substituted diene has moved to a longer wavelength by about 15 nm. This "substituent effect" is general for dienes and trienes, and is even more pronounced for enone chromophores.

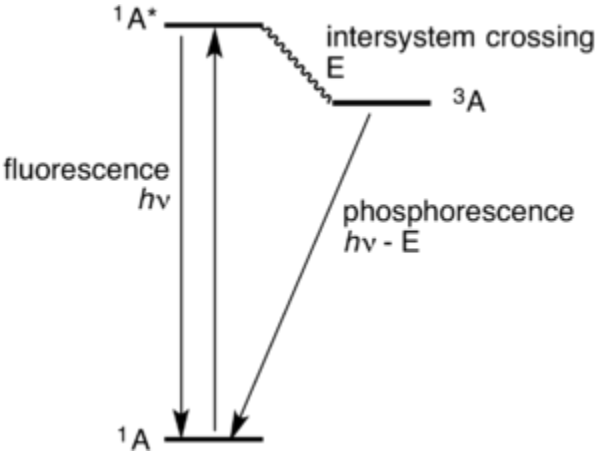
Fluorescence spectroscopy

Fluorescence spectroscopy (also known as fluorometry or spectrofluorometry) is a type of electromagnetic spectroscopy which analyzes fluorescence from a sample. It involves using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light; typically, but not necessarily,visible light. A complementary technique is absorption spectroscopy. In the special case of single molecule fluorescence spectroscopy, intensity fluctuations from the emitted light are measured from either single fluorophores, or pairs of fluorophores.

Devices that measure fluorescence are called fluorometers

Molecules have various states referred to as energy levels. Fluorescence spectroscopy is primarily concerned with electronic and vibrational states. Generally, the species being examined has a ground electronic state (a low energy state) of interest, and an excited electronic state of higher energy. Within each of these electronic states are various vibrational states.

In fluorescence , the species is first excited, by absorbing a photon, from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state of the excited electronic state. This process is often visualized with a Jablonski diagram.



A Jablonski diagram showing the excitation of molecule A to its singlet excited state ($^1A^*$)

followed by intersystem crossing to the triplet state (^3A) that relaxes to the ground state by phosphorescence.

The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process. As molecules may drop down into any of several vibrational levels in the ground state, the emitted photons will have different energies, and thus frequencies. Therefore, by analysing the different frequencies of light emitted in fluorescent spectroscopy, along with their relative intensities, the structure of the different vibrational levels can be determined.

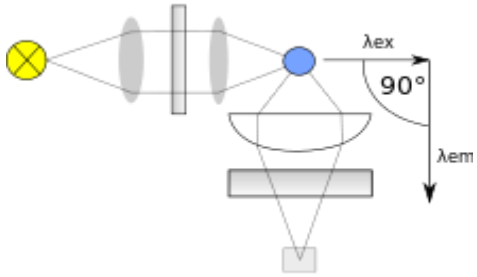
For atomic species, the process is similar; however, since atomic species do not have vibrational energy levels, the emitted photons are often at the same wavelength as the incident radiation. This process of re-emitting the absorbed photon is "resonance fluorescence" and while it is characteristic of atomic fluorescence, is seen in molecular fluorescence as well.

In a typical fluorescence (emission) measurement, the excitation wavelength is fixed and the detection wavelength varies, while in a fluorescence excitation measurement the detection wavelength is fixed and the excitation wavelength is varied across a region of interest. An **emission map** is measured by recording the emission spectra resulting from a range of excitation wavelengths and combining them all together. This is a three dimensional surface data set: emission intensity as a function of excitation and emission wavelengths, and is typically depicted as a contour map.

Instrumentation

Two general types of instruments exist: filter fluorometers that use filters to isolate the incident light and fluorescent light and spectrofluorometers that use a diffraction grating monochromators to isolate the incident light and fluorescent light. Both types use the following scheme: the light from an excitation source passes through a filter or monochromator, and strikes the sample. A proportion of the incident light is absorbed by the sample, and some of the molecules in the sample fluoresce. The fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second filter or monochromator and reaches a detector, which is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light

reaching the detector.



A simplistic design of the components of a fluorimeter

Various light sources may be used as excitation sources, including lasers, LED, and lamps; xenon arcs and mercury-vapor lamps in particular. A laser only emits light of high irradiance at a very narrow wavelength interval, typically under 0.01 nm, which makes an excitation monochromator or filter unnecessary. The disadvantage of this method is that the wavelength of a laser cannot be changed by much. A mercury vapor lamp is a line lamp, meaning it emits light near peak wavelengths. By contrast, a xenon arc has a continuous emission spectrum with nearly constant intensity in the range from 300- 800 nm and a sufficient irradiance for measurements down to just above 200 nm.

Filters and/or monochromators may be used in fluorimeters. A monochromator transmits light of an adjustable wavelength with an adjustable tolerance. The most common type of monochromator utilizes a diffraction grating, that is, collimated light illuminates a grating and exits with a different angle depending on the wavelength. The monochromator can then be adjusted to select which wavelengths to transmit. For allowing anisotropy measurements the addition of two polarization filters are necessary: One after the excitation monochromator or filter, and one before the emission monochromator or filter.

As mentioned before, the fluorescence is most often measured at a 90° angle relative to the excitation light. This geometry is used instead of placing the sensor at the line of the excitation light at a 180° angle in order to avoid interference of the transmitted excitation light. No monochromator is perfect and it will transmit some stray light, that is, light with other

wavelengths than the targeted. An ideal monochromator would only transmit light in the specified range and have a high wavelength- independent transmission. When measuring at a 90° angle, only the light scattered by the sample causes stray light. This results in a better signal-to-noise ratio, and lowers the detection limit by approximately a factor 10000, when compared to the 180° geometry. Furthermore, the fluorescence can also be measured from the front, which is often done for turbid or opaque samples .

The detector can either be single-channeled or multichanneled. The single-channeled detector can only detect the intensity of one wavelength at a time, while the multichanneled detects the intensity of all wavelengths simultaneously, making the emission monochromator or filter unnecessary. The different types of detectors have both advantages and disadvantages.

The most versatile fluorimeters with dual monochromators and a continuous excitation light source can record both an excitation spectrum and a fluorescence spectrum. When measuring fluorescence spectra, the wavelength of the excitation light is kept constant, preferably at a wavelength of high absorption, and the emission monochromator scans the spectrum. For measuring excitation spectra, the wavelength passing though the emission filter or monochromator is kept constant and the excitation monochromator is scanning. The excitation spectrum generally is identical to the absorption spectrum as the fluorescence intensity is proportional to the absorption.

Analysis of data

At low concentrations the fluorescence intensity will generally be proportional to the concentration of the fluorophore.

Unlike in UV/visible spectroscopy, ‘standard’, device independent spectra are not easily attained. Several factors influence and distort the spectra, and corrections are necessary to attain ‘true’, i.e. machine-independent, spectra. The different types of distortions will here be classified as being either instrument- or sample-related. Firstly, the distortion arising from the instrument is discussed. As a start, the light source intensity and wavelength characteristics varies over time during each experiment and between each experiment. Furthermore, no lamp has a constant intensity at all wavelengths. To correct this, a beam splitter can be applied after the excitation monochromator or filter to direct a portion of the light to a reference detector.

Additionally, the transmission efficiency of monochromators and filters must be taken into account. These may also change over time. The transmission efficiency of the monochromator also varies depending on wavelength. This is the reason that an optional reference detector should be placed after the excitation monochromator or filter. The percentage of the fluorescence picked up by the detector is also dependent upon the system. Furthermore, the detector quantum efficiency, that is, the percentage of photons detected, varies between different detectors, with wavelength and with time, as the detector inevitably deteriorates.

Two other topics that must be considered include the optics used to direct the radiation and the means of holding or containing the sample material (called a cuvette or cell). For most UV, visible, and NIR measurements the use of precision quartz cuvettes is necessary. In both cases, it is important to select materials that have relatively little absorption in the wavelength range of interest. Quartz is ideal because it transmits from 200 nm-2500 nm; higher grade quartz can even transmit up to 3500 nm, whereas the absorption properties of other materials can mask the fluorescence from the sample.

Correction of all these instrumental factors for getting a 'standard' spectrum is a tedious process, which is only applied in practice when it is strictly necessary. This is the case when measuring the quantum yield or when finding the wavelength with the highest emission intensity for instance.

As mentioned earlier, distortions arise from the sample as well. Therefore some aspects of the sample must be taken into account too. Firstly, photodecomposition may decrease the intensity of fluorescence over time. Scattering of light must also be taken into account. The most significant types of scattering in this context are Rayleigh and Raman scattering. Light scattered by Rayleigh scattering has the same wavelength as the incident light, whereas in Raman scattering the scattered light changes wavelength usually to longer wavelengths. Raman scattering is the result of a virtual electronic state induced by the excitation light. From this virtual state, the molecules may relax back to a vibrational level other than the vibrational ground state. In fluorescence spectra, it is always seen at a constant wavenumber difference relative to the excitation wavenumber e.g. the peak appears at a wavenumber 3600 cm^{-1} lower than the excitation light in water.

Other aspects to consider are the inner filter effects. These include reabsorption. Reabsorption happens because another molecule or part of a macromolecule absorbs at the wavelengths at which the fluorophore emits radiation. If this is the case, some or all of the photons emitted by the fluorophore may be absorbed again. Another inner filter effect occurs because of high concentrations of absorbing molecules, including the fluorophore. The result is that the intensity of the excitation light is not constant throughout the solution. Resultingly, only a small percentage of the excitation light reaches the fluorophores that are visible for the detection system. The inner filter effects change the spectrum and intensity of the emitted light and they must therefore be considered when analysing the emission spectrum of fluorescent light

Atomic Absorption Spectroscopy (AAS)

Atomic absorption spectroscopy (**AAS**) is a spectroanalytical procedure for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state.

In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 different elements in solution or directly in solid samples used in pharmacology, biophysics and toxicology research.

Principles

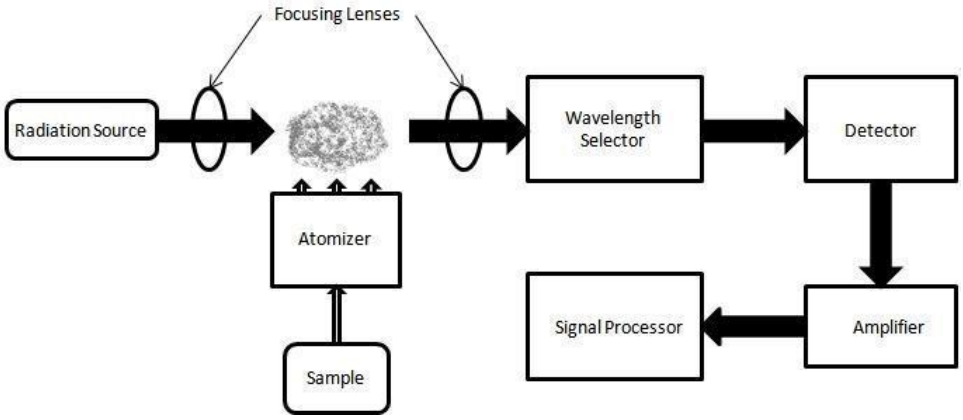
The technique makes use of absorption spectrometry to assess the concentration of an analyte in a sample. It requires standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer-Lambert Law.

In short, the electrons of the atoms in the atomizer can be promoted to higher orbitals (excited state) for a short period of time (nanoseconds) by absorbing a defined quantity of energy (radiation of a given wavelength). This amount of energy, i.e., wavelength, is specific to a particular electron transition in a particular element. In general, each wavelength corresponds to only one element, and the width of an absorption line is only of the order of a few picometers (pm), which gives the technique its elemental selectivity. The radiation flux without a sample and with a sample in the atomizer is measured using a detector, and the ratio between the two

values (the absorbance) is converted to analyte concentration or mass using the Beer-Lambert Law.

Instrumentation

In order to analyze a sample for its atomic constituents, it has to be atomized. The atomizers most commonly used nowadays are flames and electrothermal (graphite tube) atomizers. The atoms should then be irradiated by optical radiation, and the radiation source could be an element-specific line radiation source or a continuum radiation source. The radiation then passes through amonochromator in order to separate the element-specific radiation from any other radiation emitted by the radiation source, which is finally measured by a detector.



Atomizers

The atomizers most commonly used nowadays are (spectroscopic) flames and electrothermal (graphite tube) atomizers. Other atomizers, such as glow-discharge atomization, hydride atomization, or cold- vapor atomization might be used for special purposes.

Flame atomizers

The oldest and most commonly used atomizers in AAS are flames, principally the air-acetylene (compressed high-purity oxygen + C_2H_2) flame with a temperature of about 2300 °C and the nitrous dioxide system (N_2O)-acetylene flame with a temperature of about 2700 °C. The latter flame, in addition, offers a more reducing environment, being ideally suited for analytes with high affinity to oxygen.

Liquid or dissolved samples are typically used with flame atomizers. The sample solution is aspirated by a pneumatic analytical nebulizer, transformed into an aerosol, which is introduced

into a spray chamber, where it is mixed with the flame gases and conditioned in a way that only the finest aerosol droplets (< 10 μm) enter the flame. This conditioning process is responsible that only about 5% of the aspirated sample solution reaches the flame, but it also guarantees a relatively high freedom from interference.

On top of the spray chamber is a burner head that produces a flame that is laterally long (usually 5– 10 cm) and only a few mm deep. The radiation beam passes through this flame at its longest axis, and the flame gas flow-rates may be adjusted to produce the highest concentration of free atoms. The burner height may also be adjusted, so that the radiation beam passes through the zone of highest atom cloud density in the flame, resulting in the highest sensitivity.

The processes in a flame include the stages of desolvation (drying) in which the solvent is evaporated and the dry sample nano-particles remain, vaporization (transfer to the gaseous phase) in which the solid particles are converted into gaseous molecule, atomization in which the molecules are dissociated into free atoms, and ionization where (depending on the ionization potential of the analyte atoms and the energy available in a particular flame) atoms may be in part converted to gaseous ions.

Each of these stages includes the risk of interference in case the degree of phase transfer is different for the analyte in the calibration standard and in the sample. Ionization is generally undesirable, as it reduces the number of atoms that are available for measurement, i.e., the sensitivity.

In flame AAS a steady-state signal is generated during the time period when the sample is aspirated. This technique is typically used for determinations in the mg L^{-1} range, and may be extended down to a few μg L^{-1} for some elements.

Electrothermal atomizers

Although a wide variety of graphite tube designs have been used over the years, the dimensions nowadays are typically 20–25 mm in length and 5–6 mm inner diameter. With this technique liquid/dissolved, solid and gaseous samples may be analyzed directly. A measured volume (typically 10–50 μL) or a weighed mass (typically around 1 mg) of a solid sample are introduced into the graphite tube and subject to a temperature program. This typically consists of stages,

such as drying – the solvent is evaporated; pyrolysis – the majority of the matrix constituents are removed; atomization – the analyte element is released to the gaseous phase; and cleaning – eventual residues in the graphite tube are removed at high temperature.

The graphite tubes are heated via their ohmic resistance using a low-voltage high-current power supply; the temperature in the individual stages can be controlled very closely, and temperature ramps between the individual stages facilitate separation of sample components.

Radiation sources

We have to distinguish between line source AAS (LS AAS) and continuum source AAS (CS AAS). In classical LS AAS, as it has been proposed by Alan Walsh, the high spectral resolution required for AAS measurements is provided by the radiation source itself that emits the spectrum of the analyte in the form of lines that are narrower than the absorption lines. Continuum sources, such as deuterium lamps, are only used for background correction purposes. The advantage of this technique is that only a medium-resolution monochromator is necessary for measuring AAS; however, it has the disadvantage that usually a separate lamp is required for each element that has to be determined. In CS AAS, in contrast, a single lamp, emitting a continuum spectrum over the entire spectral range of interest is used for all elements.

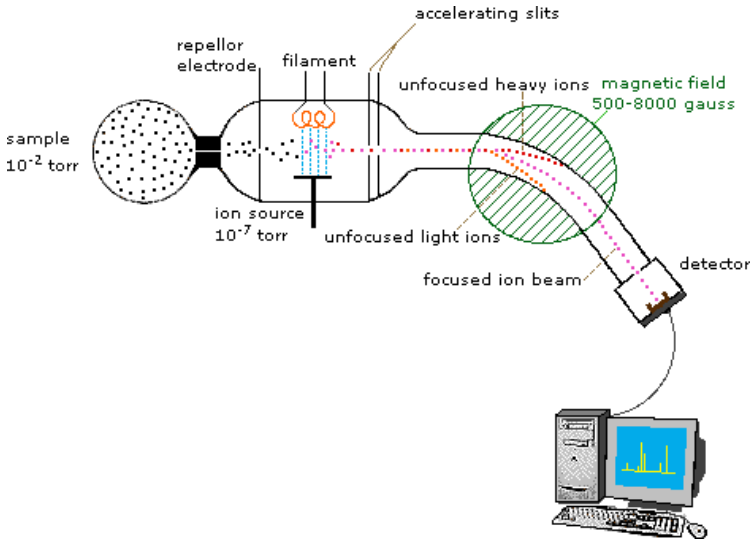
Mass Spectrometry

1. The Mass Spectrometer In order to measure the characteristics of individual molecules, a mass spectrometer converts them to ions so that they can be moved about and manipulated by external electric and magnetic fields. The three essential functions of a mass spectrometer, and the associated components, are:

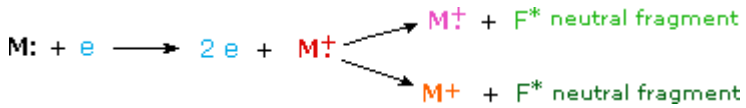
i. **The Ion Source** A small sample is ionized, usually to cations by loss of an electron.
ii. **The Mass Analyze** The ions are sorted and separated according to their mass and charge.
iii. **The Detector** The separated ions are then measured, and the results displayed on a chart.

Because ions are very reactive and short-lived, their formation and manipulation must be

conducted in a vacuum. Atmospheric pressure is around 760 torr (mm of mercury). The pressure under which ions may be handled is roughly 10-5 to 10-8 torr (less than a billionth of an atmosphere). Each of the three tasks listed above may be accomplished in different ways. In one common procedure, ionization is effected by a high energy beam of electrons, and ion separation is achieved by accelerating and focusing the ions in a beam, which is then bent by an external magnetic field. The ions are then detected electronically and the resulting information is stored and analyzed in a computer. A mass spectrometer operating in this fashion is outlined in the following diagram. The heart of the spectrometer is the ion source. Here molecules of the sample (black dots) are bombarded by electrons (light blue lines) issuing from a heated filament. This is called an EI (electron-impact) source. Gases and volatile liquid samples are allowed to leak into the ion source from a reservoir (as shown). Non- volatile solids and liquids may be introduced directly. Cations formed by the electron bombardment (red dots) are pushed away by a charged repeller plate (anions are attracted to it), and accelerated toward other electrodes, having slits through which the ions pass as a beam. Some of these ions fragment into smaller cations and neutral fragments. A perpendicular magnetic field deflects the ion beam in an arc whose radius is inversely proportional to the mass of each ion. Lighter ions are deflected more than heavier ions. By varying the strength of the magnetic field, ions of different mass can be focused progressively on a detector fixed at the end of a curved tube (also under a high vacuum).



When a high energy electron collides with a molecule it often ionizes it by knocking away one of the molecular electrons (either bonding or non-bonding). This leaves behind a **molecular ion** (colored red in the following diagram). Residual energy from the collision may cause the molecular ion to fragment into neutral pieces (colored green) and smaller **fragment ions** (colored pink and orange). The molecular ion is a radical cation, but the fragment ions may either be radical cations (pink) or carbocations (orange), depending on the nature of the neutral fragment. An animated display of this ionization process will appear if you click on the ion source of the mass spectrometer diagram.

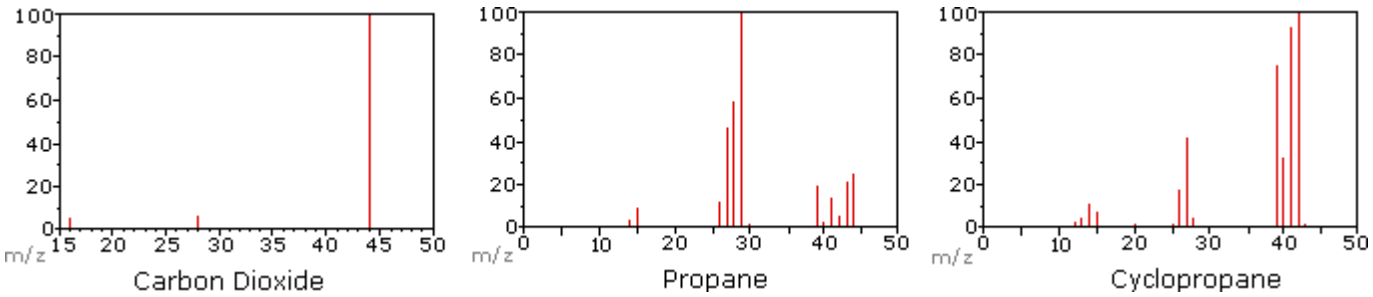


2. The Nature of Mass Spectra

A mass spectrum will usually be presented as a vertical bar graph, in which each bar represents an ion having a specific mass-to-charge ratio (m/z) and the length of the bar indicates the relative abundance of the ion. The most intense ion is assigned an abundance of 100, and it is referred to as the **base peak**. Most of the ions formed in a mass spectrometer have a single charge, so the m/z value is equivalent to mass itself. Modern mass spectrometers easily distinguish (resolve) ions differing by only a single atomic mass unit (amu), and thus provide completely accurate values for the molecular mass of a compound. The highest-mass ion in a spectrum is normally considered to be the molecular ion, and lower-mass ions are fragments from the molecular ion, assuming the sample is a single pure compound.

The following diagram displays the mass spectra of three simple gaseous compounds, carbon dioxide, propane and cyclopropane. The molecules of these compounds are similar in size, CO_2 and C_3H_8 both have a nominal mass of 44 amu, and C_3H_6 has a mass of 42 amu. The molecular ion is the strongest ion in the spectra of CO_2 and C_3H_6, and it is moderately strong in propane. The unit mass resolution is readily apparent in these spectra (note the separation of ions having

m/z=39, 40, 41 and 42 in the cyclopropane spectrum). Even though these compounds are very similar in size, it is a simple matter to identify them from their individual mass spectra. By clicking on each spectrum in turn, a partial fragmentation analysis and peak assignment will be displayed. Even with simple compounds like these, it should be noted that it is rarely possible to explain the origin of all the fragment ions in a spectrum. Also, the structure of most fragment ions is seldom known with certainty.



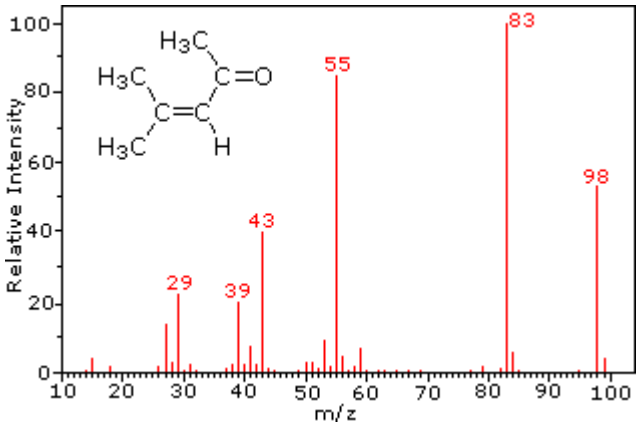
Since a molecule of carbon dioxide is composed of only three atoms, its mass spectrum is very simple. The molecular ion is also the base peak, and the only fragment ions are CO (m/z=28) and O (m/z=16). The molecular ion of propane also has m/z=44, but it is not the most abundant ion in the spectrum. Cleavage of a carbon-carbon bond gives methyl and ethyl fragments, one of which is a carbocation and the other a radical. Both distributions are observed, but the larger ethyl cation (m/z=29) is the most abundant, possibly because its size affords greater charge dispersal. A similar bond cleavage in cyclopropane does not give two fragments, so the molecular ion is stronger than in propane, and is in fact responsible for the the base peak. Loss of a hydrogen atom, either before or after ring opening, produces the stable allyl cation (m/z=41). The third strongest ion in the spectrum has m/z=39 (C3H3). Its structure is uncertain, but two possibilities are shown in the diagram. The small m/z=39 ion in propane and the absence of a m/z=29 ion in cyclopropane are particularly significant in distinguishing these hydrocarbons.

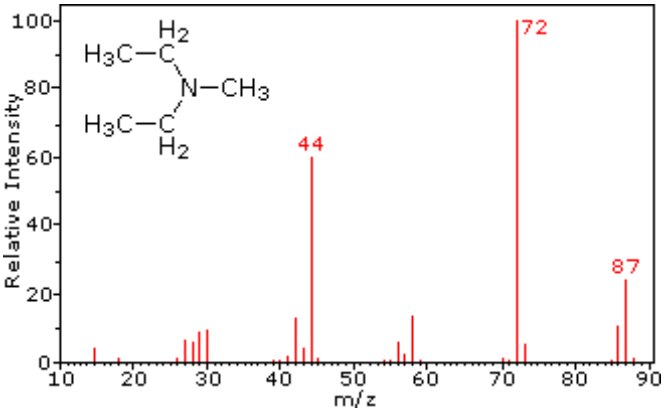
Most stable organic compounds have an even number of total electrons, reflecting the fact that electrons occupy atomic and molecular orbitals in pairs. When a single electron is removed from a molecule to give an ion, the total electron count becomes an odd number, and we refer to such

ions as **radical cations**. The molecular ion in a mass spectrum is always a radical cation, but the fragment ions may either be even-electron cations or odd-electron radical cations, depending on the neutral fragment lost. The simplest and most common fragmentations are bond cleavages producing a neutral radical (odd number of electrons) and a cation having an even number of electrons. A less common fragmentation, in which an even-electron neutral fragment is lost, produces an odd-electron radical cation fragment ion. Fragment ions themselves may fragment further. As a rule, odd-electron ions may fragment either to odd or even-electron ions, but even-electron ions fragment only to other even- electron ions. The masses of molecular and fragment ions also reflect the electron count, depending on the number of nitrogen atoms in the species.

Ions with no nitrogen or an even # N atoms	odd-electron ions even-number mass	even-electron ions odd-number mass
Ions having an odd # N atoms	odd-electron ions odd-number mass	even-electron ions even-number mass

This distinction is illustrated nicely by the following two examples. The unsaturated ketone, 4-methyl- 3-pentene-2-one, on the left has no nitrogen so the mass of the molecular ion (m/z = 98) is an even number. Most of the fragment ions have odd-numbered masses, and therefore are even-electron cations. Diethylmethylamine, on the other hand, has one nitrogen and its molecular mass (m/z = 87) is an odd number. A majority of the fragment ions have even-numbered masses (ions at m/z = 30, 42, 56 & 58 are not labeled), and are even-electron nitrogen cations. The weak even -electron ions at m/z=15 and 29 are due to methyl and ethyl cations (no nitrogen atoms). The fragmentations leading to the chief fragment ions will be displayed by clicking on the appropriate spectrum. Repeated clicks will cycle the display.



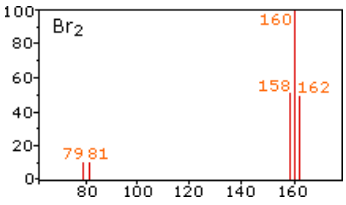


4-methyl-3-pentene-2-one **N,N-diethylmethylamine**

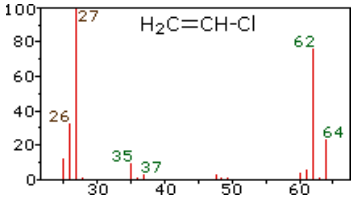
When non-bonded electron pairs are present in a molecule (e.g. on N or O), fragmentation pathways may sometimes be explained by assuming the missing electron is partially localized on that atom. A few such mechanisms are shown above. Bond cleavage generates a radical and a cation, and both fragments often share these roles, albeit unequally.

3. Isotopes

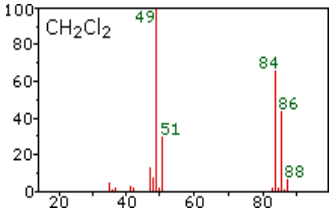
Since a mass spectrometer separates and detects ions of slightly different masses, it easily distinguishes different isotopes of a given element. This is manifested most dramatically for compounds containing bromine and chlorine, as illustrated by the following examples. Since molecules of bromine have only two atoms, the spectrum on the left will come as a surprise if a single atomic mass of 80 amu is assumed for Br. The five peaks in this spectrum demonstrate clearly that natural bromine consists of a nearly 50:50 mixture of isotopes having atomic masses of 79 and 81 amu respectively. Thus, the bromine molecule may be composed of two ^{79}Br atoms (mass 158 amu), two ^{81}Br atoms (mass 162 amu) or the more probable combination of ^{79}Br-^{81}Br (mass 160 amu). Fragmentation of Br2 to a bromine cation then gives rise to equal sized ion peaks at 79 and 81 amu.



Bromine



vinyl methylene chloride



chloride

The center and right hand spectra show that chlorine is also composed of two isotopes, the more abundant having a mass of 35 amu, and the minor isotope a mass 37 amu. The precise isotopic composition of chlorine and bromine is:

Chlorine: 75.77% ^{35}Cl and 24.23% ^{37}Cl

Bromine: 50.50% ^{79}Br and 49.50% ^{81}Br

The presence of chlorine or bromine in a molecule or ion is easily detected by noticing the intensity ratios of ions differing by 2 amu. In the case of methylene chloride, the molecular ion consists of three peaks at m/z=84, 86 & 88 amu, and their diminishing intensities may be calculated from the natural abundances given above. Loss of a chlorine atom gives two isotopic fragment ions at m/z=49 & 51amu, clearly incorporating a single chlorine atom. Fluorine and iodine, by contrast, are monoisotopic, having masses of 19 amu and 127 amu respectively. It should be noted that the presence of halogen atoms in a molecule or fragment ion does not change the odd-even mass rules given above.

To make use of a calculator that predicts the isotope clusters for different combinations of chlorine, bromine and other elements Click Here. This application was developed at Colby College.

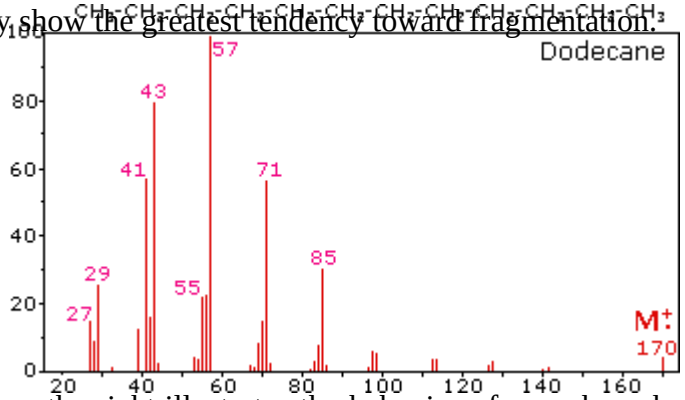
Two other common elements having useful isotope signatures are carbon, ^{13}C is 1.1% natural abundance, and sulfur, ^{33}S and ^{34}S are 0.76% and 4.22% natural abundance respectively. For example, the small m/z=99 amu peak in the spectrum of 4-methyl-3-pentene-2-one (above) is due to the presence of a single ^{13}C atom in the molecular ion. Although less important in this respect, ^{15}N and ^{18}O also make small contributions to higher mass satellites of molecular ions

incorporating these elements.

The calculator on the right may be used to calculate the isotope contributions to ion abundances 1 and 2 amu greater than the molecular ion (M). Simply enter an appropriate subscript number to the right of each symbol, leaving those elements not present blank, and press the "Calculate" button. The numbers displayed in the M+1 and M+2 boxes are relative to M being set at 100%.

4. Fragmentation Patterns

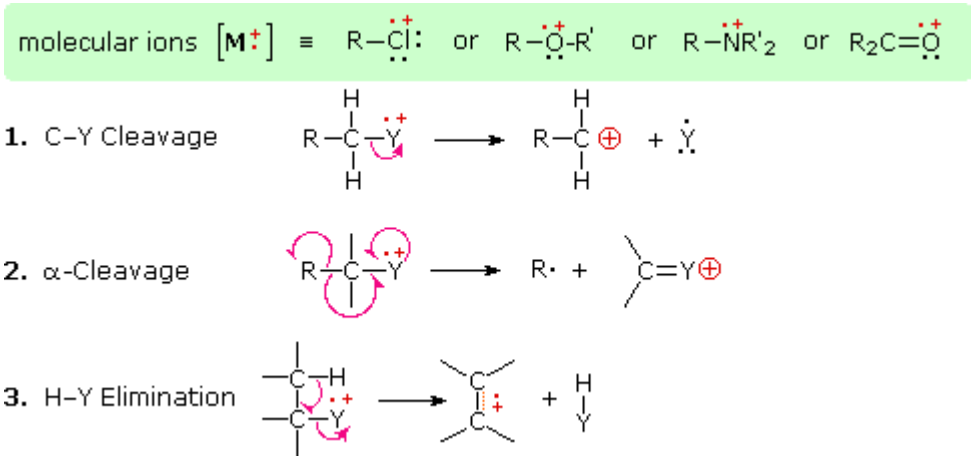
The fragmentation of molecular ions into an assortment of fragment ions is a mixed blessing. The nature of the fragments often provides a clue to the molecular structure, but if the molecular ion has a lifetime of less than a few microseconds it will not survive long enough to be observed. Without a molecular ion peak as a reference, the difficulty of interpreting a mass spectrum increases markedly. Fortunately, most organic compounds give mass spectra that include a molecular ion, and those that do not often respond successfully to the use of milder ionization conditions. Among simple organic compounds, the most stable molecular ions are those from aromatic rings, other conjugated pi-electron systems and cycloalkanes. Alcohols, ethers and highly branched alkanes generally show the greatest tendency toward fragmentation.



The mass spectrum of dodecane on the right illustrates the behavior of an unbranched alkane. Since there are no heteroatoms in this molecule, there are no non-bonding valence shell electrons. Consequently, the radical cation character of the molecular ion (m/z = 170) is delocalized over all the covalent bonds. Fragmentation of C-C bonds occurs because they are usually weaker than C-H bonds, and this produces a mixture of alkyl radicals and alkyl carbocations. The positive charge commonly resides on the smaller fragment, so we see a homologous series of

hexyl (m/z = 85), pentyl (m/z = 71), butyl (m/z = 57), propyl (m/z = 43), ethyl (m/z = 29) and methyl (m/z = 15) cations. These are accompanied by a set of corresponding alkenyl carbocations (e.g. m/z = 55, 41 &27) formed by loss of 2 H. All of the significant fragment ions in this spectrum are even-electron ions. In most alkane spectra the propyl and butyl ions are the most abundant.

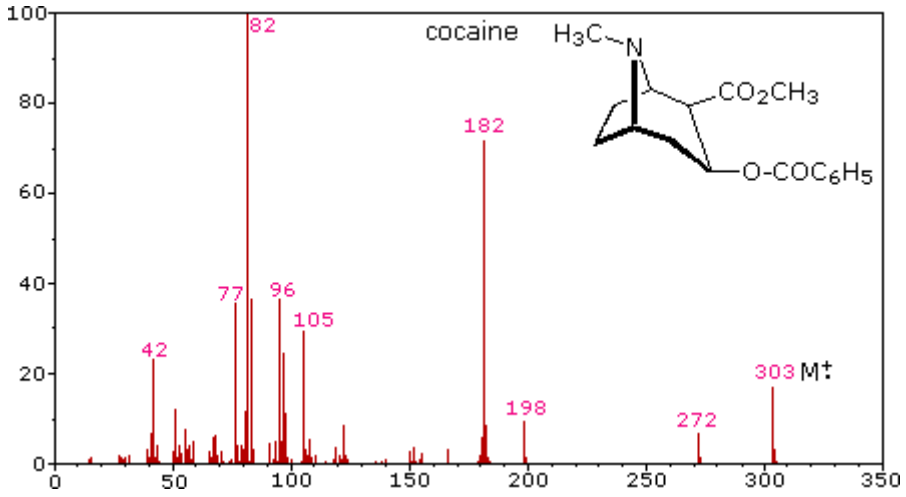
The presence of a functional group, particularly one having a heteroatom Y with non-bonding valence electrons (Y = N, O, S, X etc.), can dramatically alter the fragmentation pattern of a compound. This influence is thought to occur because of a "localization" of the radical cation component of the molecular ion on the heteroatom. After all, it is easier to remove (ionize) a non-bonding electron than one that is part of a covalent bond. By localizing the reactive moiety, certain fragmentation processes will be favored. These are summarized in the following diagram, where the green shaded box at the top displays examples of such "localized" molecular ions. The first two fragmentation paths lead to even- electron ions, and the elimination (path #3) gives an odd-electron ion. Note the use of different curved arrows to show single electron shifts compared with electron pair shifts.



The charge distributions shown above are common, but for each cleavage process the charge may sometimes be carried by the other (neutral) species, and both fragment ions are observed. Of the three cleavage reactions described here, the alpha-cleavage is generally favored for nitrogen, oxygen and sulfur compounds. Indeed, in the previously displayed spectra of 4-methyl-3-

pentene- 2-one and N,N-diethylmethylamine the major fragment ions come from alpha-cleavages. Further examples of functional group influence on fragmentation are provided by a selection of compounds that may be examined by clicking the left button below. Useful tables of common fragment ions and neutral species may be viewed by clicking the right button.

The complexity of fragmentation patterns has led to mass spectra being used as "fingerprints" for identifying compounds. Environmental pollutants, pesticide residues on food, and controlled substance identification are but a few examples of this application. Extremely small samples of an unknown substance (a microgram or less) are sufficient for such analysis. The following mass spectrum of cocaine demonstrates how a forensic laboratory might determine the nature of an unknown street drug. Even though extensive fragmentation has occurred, many of the more abundant ions (identified by magenta numbers) can be rationalized by the three mechanisms shown above. Plausible assignments may be seen by clicking on the spectrum, and it should be noted that all are even-electron ions. The m/z = 42 ion might be any or all of the following: C_3H_6, C_2H_2O or C_2H_4N. A precise assignment could be made from a high-resolution m/z value (next section).



Odd-electron fragment ions are often formed by characteristic rearrangements in which stable neutral fragments are lost. Mechanisms for some of these rearrangements have been identified

by following the course of isotopically labeled molecular ions. A few examples of these rearrangement mechanisms may be seen by clicking the following button.

5. High Resolution Mass Spectrometry

In assigning mass values to atoms and molecules, we have assumed integral values for isotopic masses. However, accurate measurements show that this is not strictly true. Because the strong nuclear forces that bind the components of an atomic nucleus together vary, the actual mass of a given isotope deviates from its nominal integer by a small but characteristic amount (remember $E = mc^2$). Thus, relative to ^{12}C at 12.0000, the isotopic mass of ^{16}O is 15.9949 amu (not 16) and ^{14}N is 14.0031 amu (not 14).

By designing mass spectrometers that can determine m/z values accurately to four decimal places, it is possible to distinguish different formulas having the same nominal mass. The table on the right illustrates this important feature, and a double-focusing high-resolution mass spectrometer easily distinguishes ions having these compositions. Mass spectrometry therefore not only provides a specific molecular mass value, but it may also establish the molecular formula of an unknown compound.

Tables of precise mass values for any molecule or ion are available in libraries; however, the mass calculator provided below serves the same purpose. Since a given nominal mass may correspond to several molecular formulas, lists of such possibilities are especially useful when evaluating the spectrum of an unknown compound. Composition tables are available for this purpose, and a particularly useful program for calculating all possible combinations of H, C, N & O that give a specific nominal mass has been written by Jef Rozenski

How a mass spectrometer works The basic principle

If something is moving and you subject it to a sideways force, instead of moving in a straight line, it will move in a curve - deflected out of its original path by the sideways force.

Suppose you had a cannonball travelling past you and you wanted to deflect it as it went by you. All you've got is a jet of water from a hose-pipe that you can squirt at it. Frankly, its not going to make a lot of difference! Because the cannonball is so heavy, it will hardly be deflected at all

from its original course.

But suppose instead, you tried to deflect a table tennis ball travelling at the same speed as the cannonball using the same jet of water. Because this ball is so light, you will get a huge deflection. The amount of deflection you will get for a given sideways force depends on the mass of the ball. If you knew the speed of the ball and the size of the force, you could calculate the mass of the ball if you knew what sort of curved path it was deflected through. The less the deflection, the heavier the ball.

Note: I'm not suggesting that you personally would have to do the calculation, although the maths isn't actually very difficult - certainly no more than A'level standard!

You can apply exactly the same principle to atomic sized particles.

An outline of what happens in a mass spectrometer

Atoms and molecules can be deflected by magnetic fields - provided the atom or molecule is first turned into an ion. Electrically charged particles are affected by a magnetic field although electrically neutral ones aren't.

The sequence is :

Stage 1: Ionisation

The atom or molecule is ionised by knocking one or more electrons off to give a positive ion. This is true even for things which you would normally expect to form negative ions (chlorine, for example) or never form ions at all (argon, for example). Most mass spectrometers work with positive ions.

Note: *All* mass spectrometers that you will come across if you are doing a course for 16 - 18 year olds work with positive ions. Even if a few atoms in a sample of chlorine, for example, captured an electron instead of losing one, the negative ions formed wouldn't get all the way through the

ordinary mass spectrometer. But it has been pointed out to me that there is work being done on negative ion mass spectrometers, although they use a different ionisation technique.

Stage 2: Acceleration

The ions are accelerated so that they all have the same kinetic energy.

Stage 3: Deflection

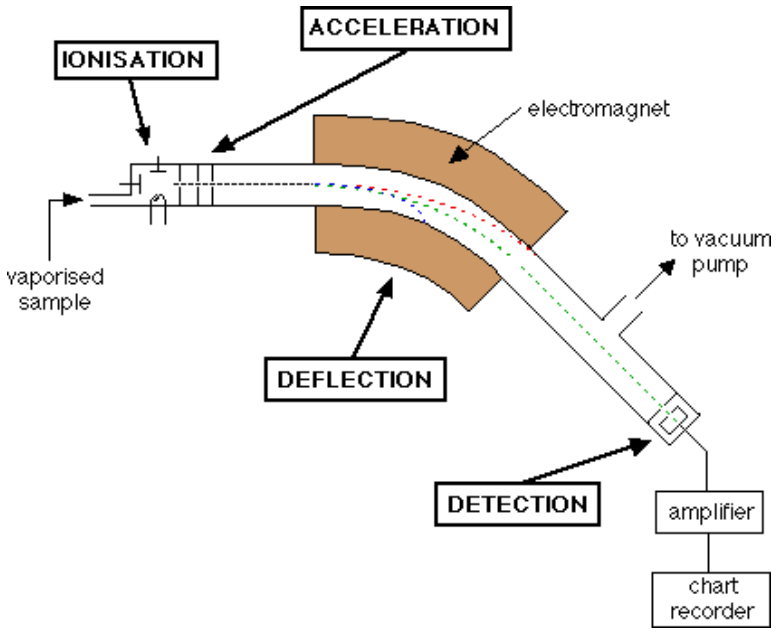
The ions are then deflected by a magnetic field according to their masses. The lighter they are, the more they are deflected.

The amount of deflection also depends on the number of positive charges on the ion - in other words, on how many electrons were knocked off in the first stage. The more the ion is charged, the more it gets deflected.

Stage 4: Detection

The beam of ions passing through the machine is detected electrically.

A full diagram of a mass spectrometer

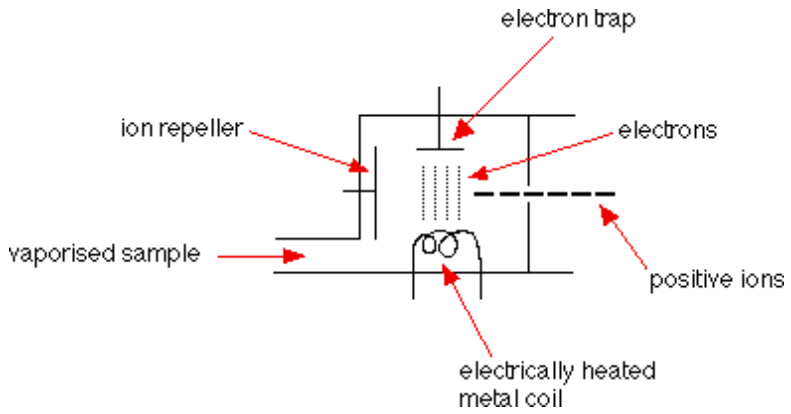


Understanding what's going on

The need for a vacuum

It's important that the ions produced in the ionisation chamber have a free run through the machine without hitting air molecules.

Ionisation



The vaporised sample passes into the ionisation chamber. The electrically heated metal coil gives off electrons which are attracted to the electron trap which is a positively charged plate.

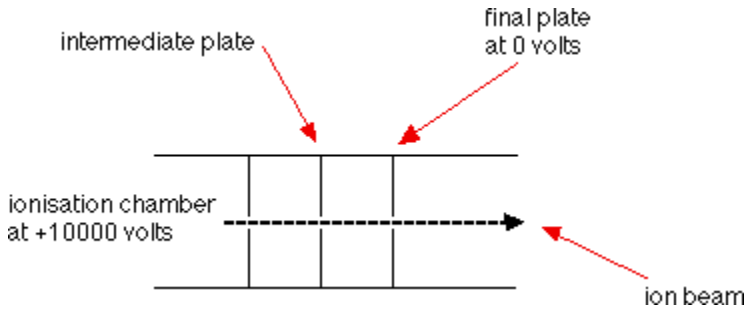
The particles in the sample (atoms or molecules) are therefore bombarded with a stream of electrons, and some of the collisions are energetic enough to knock one or more electrons out of the sample particles to make positive ions.

Most of the positive ions formed will carry a charge of +1 because it is much more difficult to remove further electrons from an already positive ion.

These positive ions are persuaded out into the rest of the machine by the ion repeller which is another metal plate carrying a slight positive charge.

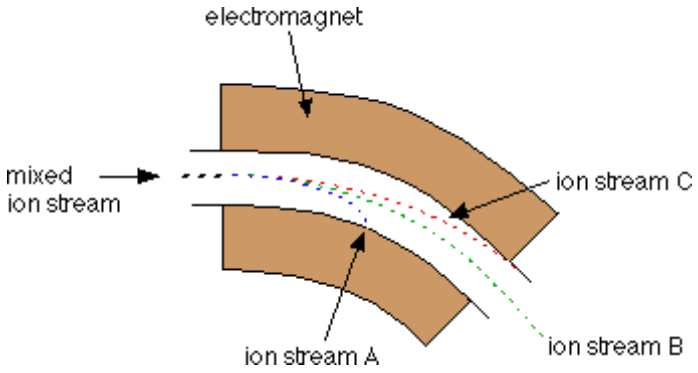
Note: As you will see in a moment, the whole ionisation chamber is held at a positive voltage of about 10,000 volts. Where we are talking about the two plates having positive charges, these charges are in addition to that 10,000 volts.

Acceleration



The positive ions are repelled away from the very positive ionisation chamber and pass through three slits, the final one of which is at 0 volts. The middle slit carries some intermediate voltage. All the ions are accelerated into a finely focused beam.

Deflection



Different ions are deflected by the magnetic field by different amounts. The amount of deflection depends on:

- the mass of the ion. Lighter ions are deflected more than heavier ones.
- the charge on the ion. Ions with 2 (or more) positive charges are deflected more than ones with only 1 positive charge.

These two factors are combined into the ***mass/charge ratio.***Mass/charge ratio is given the symbol m/z (or sometimes m/e).

For example, if an ion had a mass of 28 and a charge of 1+, its mass/charge ratio would be 28. An ion with a mass of 56 and a charge of 2+ would also have a mass/charge ratio of 28.

In the last diagram, ion stream A is most deflected - it will contain ions with the smallest mass/charge ratio. Ion stream C is the least deflected - it contains ions with the greatest mass/charge ratio.

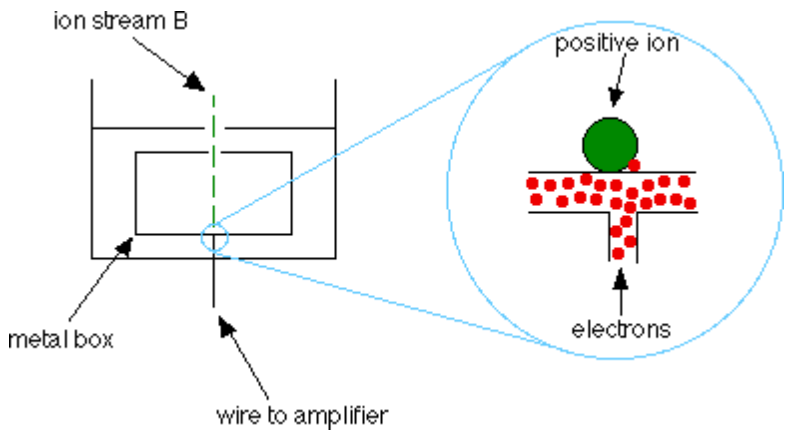
It makes it simpler to talk about this if we assume that the charge on all the ions is 1+. Most of the ions passing through the mass spectrometer will have a charge of 1+, so that the mass/charge ratio will be the same as the mass of the ion.

Note: You must be aware of the possibility of 2+ (etc) ions, but the vast majority of A'level questions will give you mass spectra which only involve 1+ ions. Unless there is some hint in the question, you can reasonably assume that the ions you are talking about will have a charge of 1+.

Assuming 1+ ions, stream A has the lightest ions, stream B the next lightest and stream C the heaviest. Lighter ions are going to be more deflected than heavy ones.

Detection

Only ion stream B makes it right through the machine to the ion detector. The other ions collide with the walls where they will pick up electrons and be neutralised. Eventually, they get removed from the mass spectrometer by the vacuum pump.



When an ion hits the metal box, its charge is neutralised by an electron jumping from the metal

on to the ion (right hand diagram). That leaves a space amongst the electrons in the metal, and the electrons in the wire shuffle along to fill it.

A flow of electrons in the wire is detected as an electric current which can be amplified and recorded. The more ions arriving, the greater the current.

Detecting the other ions

How might the other ions be detected - those in streams A and C which have been lost in the machine?

Remember that stream A was most deflected - it has the smallest value of m/z (the lightest ions if the charge is 1+). To bring them on to the detector, you would need to deflect them less - by using a smaller magnetic field (a smaller sideways force).

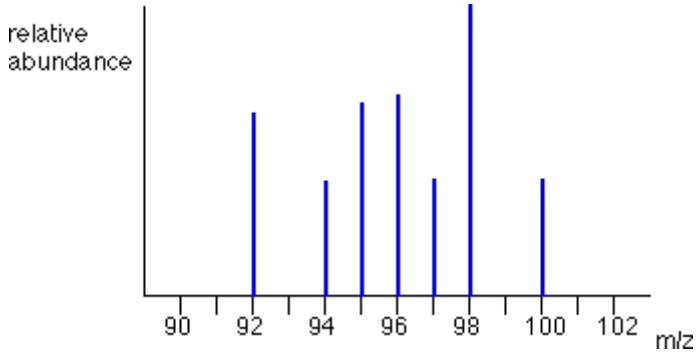
To bring those with a larger m/z value (the heavier ions if the charge is +1) on to the detector you would have to deflect them more by using a larger magnetic field.

If you vary the magnetic field, you can bring each ion stream in turn on to the detector to produce a current which is proportional to the number of ions arriving. The mass of each ion being detected is related to the size of the magnetic field used to bring it on to the detector. The machine can be calibrated to record current (which is a measure of the number of ions) against m/z directly. The mass is measured on the ^{12}C scale.

Note: The ^{12}C scale is a scale on which the ^{12}C isotope weighs exactly 12 units.

What the mass spectrometer output looks like

The output from the chart recorder is usually simplified into a "stick diagram". This shows the relative current produced by ions of varying mass/charge ratio. The stick diagram for molybdenum looks lilke this:



You may find diagrams in which the vertical axis is labelled as either "relative abundance" or "relative intensity". Whichever is used, it means the same thing. The vertical scale is related to the current received by the chart recorder - and so to the number of ions arriving at the detector: the greater the current, the more abundant the ion.

As you will see from the diagram, the commonest ion has a mass/charge ratio of 98. Other ions have mass/charge ratios of 92, 94, 95, 96, 97 and 100.

That means that molybdenum consists of 7 different isotopes. Assuming that the ions all have a charge of 1+, that means that the masses of the 7 isotopes on the carbon-12 scale are 92, 94, 95, 96, 97, 98 and 100.

Nuclear Magnetic Resonance Spectroscopy

Background

Over the past fifty years nuclear magnetic resonance spectroscopy, commonly referred to as nmr, has become the preeminent technique for determining the structure of organic compounds. Of all

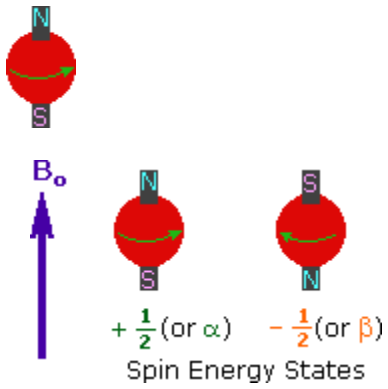
the spectroscopic methods, it is the only one for which a complete analysis and interpretation of the entire spectrum is normally expected. Although larger amounts of sample are needed than for mass spectroscopy, nmr is non-destructive, and with modern instruments good data may be obtained from samples weighing less than a milligram. **To be successful in using nmr as an analytical tool, it is necessary to understand the physical principles on which the methods are based**.

The nuclei of many elemental isotopes have a characteristic spin (**I**). Some nuclei have integral spins (e.g. I = 1, 2, 3 ....), some have fractional spins (e.g. I = 1/2, 3/2, 5/2 .....), and a few have no spin, I = 0

(e.g.^{12}C, ^{16}O, ^{32}S, ...). Isotopes of particular interest and use to organic chemists are ^{1}H, ^{13}C, ^{19}F

and ^{31}P, all of which have I = 1/2. Since the analysis of this spin state is fairly straightforward, our discussion of nmr will be limited to these and other I = 1/2 nuclei.

The following features lead to the nmr phenomenon:

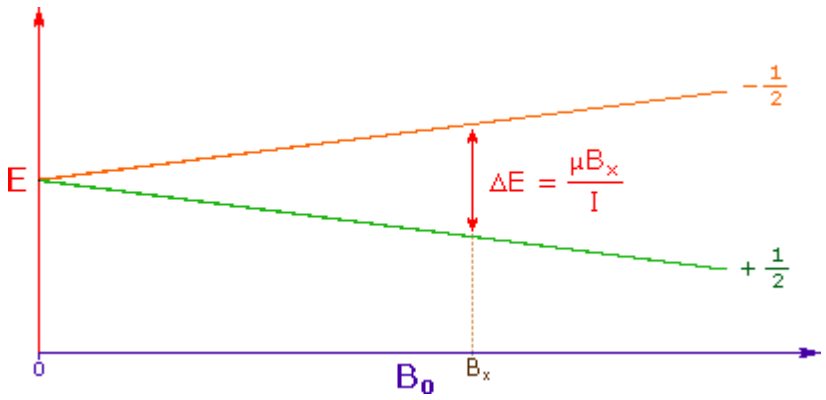


1. A spinning charge generates a magnetic field, as shown by the animation on the right. The resulting spin-magnet has a magnetic moment (**μ**) proportional to the spin.

2. In the presence of an external magnetic field (**B0**), two spin states exist, **+1/2** and-**1/2**.

The magnetic moment of the lower energy +1/2 state is aligned with the external field, but that of the higher energy -1/2 spin state is opposed to the external field. Note that the arrow representing the external field points North.

3. The difference in energy between the two spin states is dependent on the external magnetic field strength, and is always very small. The following diagram illustrates that the two spin states have the same energy when the external field is zero, but diverge as the field increases. At a field equal to B_X a formula for the energy difference is given (remember I = 1/2 and μ is the magnetic moment of the nucleus in the field).



Strong magnetic fields are necessary for nmr spectroscopy. The international unit for magnetic flux is the tesla (**T**). The earth's magnetic field is not constant, but is approximately 10^{-4} T at ground level. Modern nmr spectrometers use powerful magnets having fields of 1 to 20 T. Even with these high fields, the energy difference between the two spin states is less than 0.1 cal/mole. To put this in perspective, recall that infrared transitions involve 1 to 10 kcal/mole and electronic transitions are nearly 100 time greater. For nmr purposes, this small energy difference (ΔE) is usually given as a frequency in units of MHz (10^6 Hz), ranging from 20 to 900 Mz, depending on the magnetic field strength and the specific nucleus being studied. Irradiation of a sample with radio frequency (rf) energy corresponding exactly to the spin state

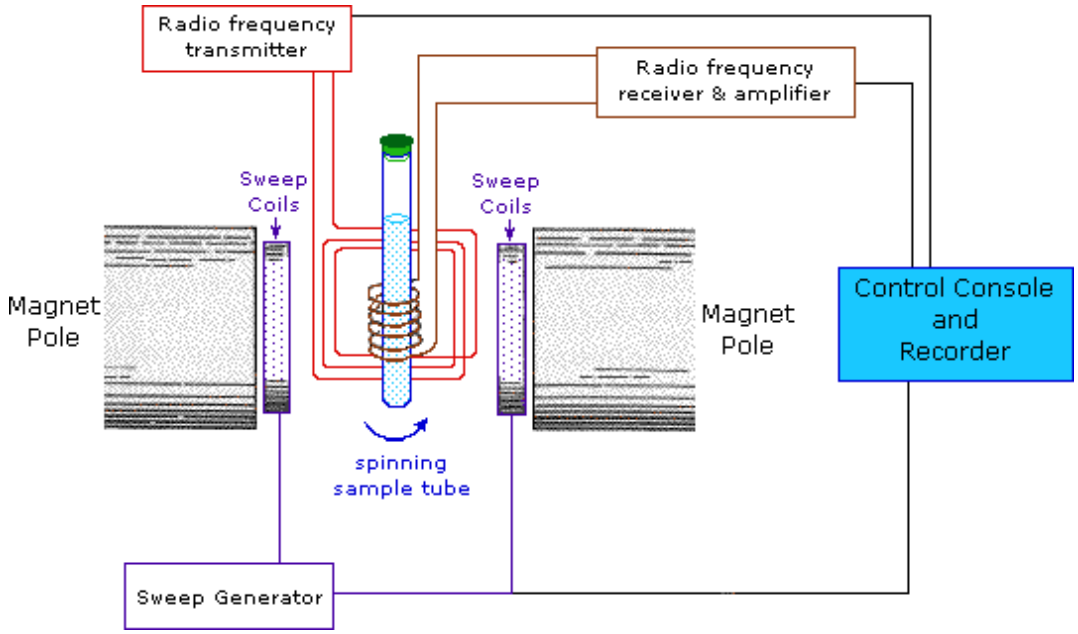
separation of a specific set of nuclei will cause excitation of those nuclei in the +1/2 state to the higher -1/2 spin state. Note that this electromagnetic radiation falls in the radio and television broadcast spectrum. Nmr spectroscopy is therefore the energetically mildest probe used to examine the structure of molecules. The nucleus of a hydrogen atom (the proton) has a magnetic moment μ = 2.7927, and has been studied more than any other nucleus. The previous diagram may be changed to display energy differences for the proton spin states (as frequencies) by mouse clicking anywhere within it.

4. For spin 1/2 nuclei the energy difference between the two spin states at a given magnetic field strength will be proportional to their magnetic moments. For the four common nuclei noted above, the magnetic moments are: ^{1}H μ = 2.7927, ^{19}F μ = 2.6273, ^{31}P μ = 1.1305 & ^{13}C μ = 0.7022. These moments are in nuclear magnetons, which are $5.05078 \cdot 10^{-27}$ JT^{-1}. The following diagram gives the approximate frequencies that correspond to the spin state energy separations for each of these nuclei in an

external magnetic field of 2.35 T. The formula in the colored box shows the direct correlation of frequency (energy difference) with magnetic moment (h = Planck's constant = $6.626069 \cdot 10^{-34}$ Js).

2. ProtonNMR Spectroscopy This important and well-established application of nuclear magnetic resonance will serve to illustrate some of the novel aspects of this method. To begin with, the nmr spectrometer must be tuned to a specific nucleus, in this case the proton. The actual procedure for obtaining the spectrum varies, but the simplest is referred to as the **continuous wave** (CW) method. A typical CW-spectrometer is shown in the following diagram. A solution of the sample in a uniform 5 mm glass tube is oriented between the poles of a powerful magnet, and is spun to average any magnetic field variations, as well as tube imperfections. Radio

frequency radiation of appropriate energy is broadcast into the sample from an antenna coil (colored red). A receiver coil surrounds the sample tube, and emission of absorbed rf energy is monitored by dedicated electronic devices and a computer. An nmr spectrum is acquired by varying or sweeping the magnetic field over a small range while observing the rf signal from the sample. An equally effective technique is to vary the frequency of the rf radiation while holding the external field constant.



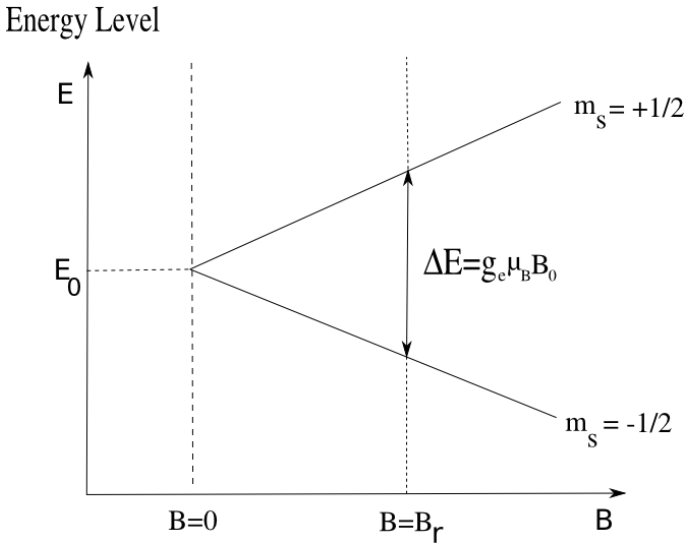
Electron Spin Resonance Spectroscopy

Theory

When a molecule or compound with an unpaired electron is placed in a strong magnetic field, the spin of the unpaired electron can align in two different ways creating two spin states, ms = ± ½. The alignment can either be along the direction (parelle) to the magnetic filed which corresponds to the lower energy state ms = - ½ or opposite (antiparallel) to the direction of the applied magnetic field ms =

+ ½ . The two allignments have different energies and this difference in energy lifts the degeneracy of the electron spin states. The energy difference is given by:

$\Delta E = E+ - E- = hv = gm\beta B$



h = Planck's Constant (6.626 x 10-34 J s-1),
v = the frequency of radiation,
ß = Bohr magneton (9.274 x 10-24 J T-1),
B = strength of the magnetic field inTesla,
g = the g-factor (***more explanation on g factor)***

During the experiment the values of h, v, and ß does not change and g value decrease as B increases. The g-factor is a unitless measurement of the intrinsic magnetic moment of the electron, and its value for a free electron is 2.0023. The concept of g can be roughly equated to that of chemical shift in NMR. EPR spectrum is the absorption of microwave frequency radiation ploted against the magnetic field intensity

3.1. Working principles of EPR:

In an EPR experiment the field of the spectrometer magnet is swept linearly to excite some of the electrons in the lower energy level to the upper energy level while the sample is exposed to fixed

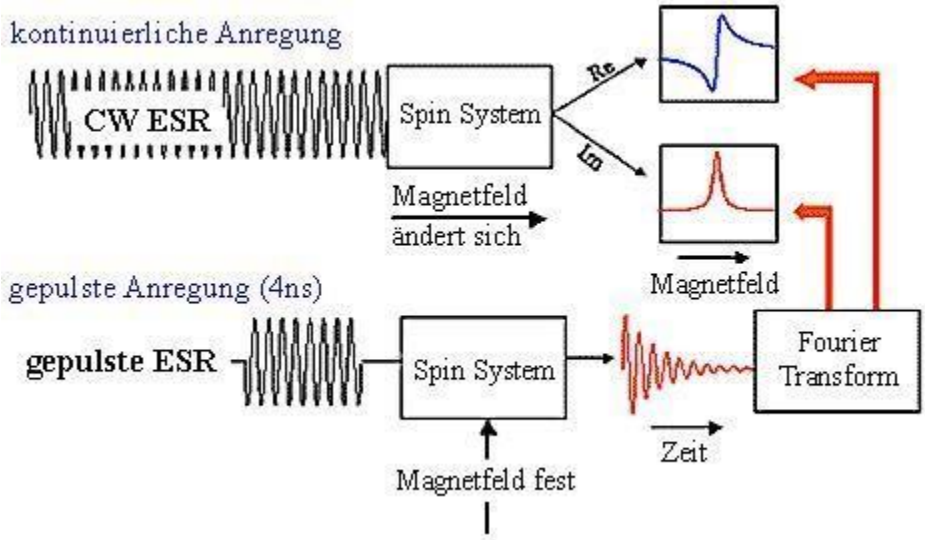
microwave irradiation. The free or the upaired electrons have a small magnetic field and orient themselves parallel to the larger field produced by the spectrometer's magnet. At a particular magnetic field strength the microwave irradiation will cause some of the free electrons to "flip" and orient against the spectrometer's magnetic field. This separation between the lower and the higher energy level is exactly matched by our microwave frequency. The condition where the magnetic field and the microwave frequency are "just right" to produce an EPR resonance (or absorption) is known as the resonance condition is detected by the spectrometer., EPR spectroscopy can be carried out by either

1. varying the magnetic field and holding the frequency constant or
2. varying the frequency and holding the magnetic field constant (as is the case for NMR spectroscopy). Typically in a comericial spectrometer works by varying the magnetic field and holding the frequency constant. EPR spectrometers working at frequencies ranging from several hundred MHz to several hundred Ghz are in use. 1-2 GHz (L-band) and 2-4 GHz (S-band), 8-10 GHZ (X-Band), 35 Ghz (Q- band) and 95 GHz (W-band). The most commonly used EPR spectrometer is in the range of 9-10 GHz (X-band).

3.2 Methods to record EPR spectra:

Continous wave method: the sample is irradiated continuously with microwave radiation of fixed frequency while the magnetic field is slowly swept and the microwave absorption is measured for each field position

Pulse EPR: short pulse of high microwave radiation are sent to the sample and the response in the absence radiation are recorded.



Measuring a CW-EPR spectroscopy can be influenced by various parameters, both instrumental and experimental. The significant aspect of the measurement is to get a high resolved EPR spectrum from a low concentration sample of interest which is dependend on the the sensivity and the resolution of the spectrometer. Microwave bridge and Resonator governs the sensitivity of the signal. Magnet, Magnet field controller, Magnet power supply and signal channel control the resolution of the spectrum obtained.

3.3 Application of EPR:

Only direct method to detect the presence of free radicals and to identify the paramagnetic species
Provides information on :

1. molecular structure near the unpaired electron.
2. EPR spectra lineshape gives insight to dynamic processes molecular motions or fluidity.
3. probes the structure of “active sites” in metalloproteins.
4. dose measurements for sterilization of medical goods and foods,
5. detection of irradiated foods, and the dating of early human artifacts.

Spectrofluorometer

A **spectrofluorometer** is an instrument which takes advantage of fluorescent properties of some compounds in order to provide information regarding their concentration and chemical environment in a sample. A certain excitation wavelength is selected, and the emission is

observed either at a single wavelength, or a scan is performed to record the intensity versus wavelength, also called an emission spectra. See Fluorescence spectroscopy

Operation

Generally, spectrofluorometers use high intensity light sources to bombard a sample with as many photons as possible. This allows for the maximum number of molecules to be in an excited state at any one point in time. The light is either passed through a filter, selecting a fixed wavelength, or a monochromator, which allows a wavelength of interest to be selected for use as the exciting light. The emission is collected at 90 degrees to the exciting light. The emission is also either passed through a filter or a monochromator before being detected by a photomultiplier tube, photodiode, or charge- coupled device detector. The signal can either be processed as digital or analog output.

Systems vary greatly and a number of considerations affect the choice. The first is the signal-to-noise ratio. There are many ways to look at the signal to noise of a given system but the accepted standard is by using water Raman. Sensitivity or detection limit is another specification to be considered, that is how little light can be measured. The standard would be fluorescein in NaOH, typical values for a high end instrument are in the femtomolar range.

UNIT III

Centrifugation

Centrifugation is a process which involves the application of the centripetal force for the sedimentation of heterogeneous mixtures with a centrifuge, and is used in industrial and laboratory settings. This process is used to separate two immiscible substances, but also to analyze the hydrodynamic properties of macromolecules. More-dense components of the mixture migrate away from the axis of the centrifuge, while less-dense components of the mixture migrate towards the axis. Chemists and biologists may increase the effective gravitational force on a test tube so as to more rapidly and completely cause the precipitate (pellet) to gather on the bottom of the tube. The remaining solution(supernatant) may be discarded with a pipette

There is a correlation between the size and density of a particle and the rate that the particle separates from a heterogeneous mixture, when the only force applied is that of gravity. The larger the size and the larger the density of the particles, the faster they separate from the mixture. By applying a larger effective gravitational force to the mixture, like a centrifuge does, the separation of the particles is accelerated. This is ideal in industrial and lab settings because particles that would naturally separate over a long period of time can be separated in much less time. The rate of centrifugation is specified by the angular velocity usually expressed as revolutions per minute (RPM), or acceleration expressed as g. The conversion factor between RPM and g depends on the radius of the centrifuge rotor. The particles' settling velocity in centrifugation is a function of their size and shape, centrifugal acceleration, the volume fraction of solids present, the density difference between the particle and the liquid, and the viscosity.

The most common application is the separation of solid from highly concentrated suspensions, which is used in the treatment of sewage sludges for dewatering where less consistent sediment is produced. In the chemical and food industries, special centrifuges can process a continuous stream of particle-laden liquid. Centrifugation is the most common method used for uranium enrichment, relying on the slight mass difference between atoms of U238 and U235 in uranium hexafluoride gas.

Centrifugation in biological research

Microcentrifuges

Microcentrifuges are used to process small volumes of biological molecules, cells, or nuclei. Microcentrifuge tubes generally hold 0.5 - 2.0 mL of liquid, and are spun at maximum angular speeds of 12,000–13,000 rpm. Microcentrifuges are small enough to fit on a table-top and have rotors that can quickly change speeds. They may or may not have a refrigeration function.

High-speed centrifuges

High-speed or superspeed centrifuges can handle larger sample volumes, from a few tens of millilitres to several litres. Additionally, larger centrifuges can also reach higher angular velocities (around 30,000 rpm). The rotors may come with different adapters to hold various sizes of test tubes, bottles, or microtiter plates.

Fractionation process

General method of fractionation: Cell sample is stored in a suspension which is:

1. Buffered - neutral pH, preventing damage to the structure of proteins including enzymes (which could affect ionic bonds)
2. Isotonic (of equal water potential) - this prevents water gain or loss by the organelles
3. Cool - reducing the overall activity of enzyme released later in the procedure

- Cells are homogenised in a blender and filtered to remove debris
- The homogenised sample is placed in an ultracentrifuge and spun in low speed - nuclei settle out, forming a pellet
- The supernatant (suspension containing remaining organelles) is spun at a higher speed - chloroplasts settle out
- The supernatant is spun at a higher speed still - mitochondria and lysosomes settle out
- The supernatant is spun at an even higher speed - ribosomes, membranes settle out. The ribosomes, membranes and Golgi complexes can be separated by another technique called density gradient centrifugation.

Ultracentrifugation

Ultracentrifugation makes use of high centrifugal force for studying properties of biological particles. Compared to microcentrifuges or high-speed centrifuges, ultracentrifuges can isolate much smaller particles, including ribosomes, proteins, and viruses. Ultracentrifuges can also be used in the study of membrane fractionation. This occurs because ultracentrifuges can reach maximum angular velocities in excess of 70,000 rpm. Additionally, while microcentrifuges and supercentrifuges separate particles in batches (limited volumes of samples must be handled manually in test tubes or bottles), ultracentrifuges can separate molecules in batch or continuous flow systems. In addition to purification, analytical ultracentrifugation (AUC) can be used for determination of the properties of macromolecules such as shape, mass, composition, and conformation. Samples are centrifuged with a high-density solution such as sucrose, caesium chloride, or iodixanol. The high-density solution may be at a uniform concentration throughout the test tube ("cushion") or a varying concentration ("gradient"). Molecular properties can be modeled through sedimentation velocity analysis or sedimentation equilibrium analysis. During the run, the particle or molecules will migrate through the test tube at different speeds depending on their physical properties and the properties of the solution, and eventually form a pellet at the bottom of the tube, or bands at various heights.

Differential Centrifugation

Differential Centrifugation is a type of centrifugation in which one selectively spins down components of a mixture by a series of increasing centrifugation forces. This method is commonly used to separate organelles and membranes found in cells. Organelles generally differ from each other in density in size, making the use of differential centrifugation, and centrifugation in general, possible. The organelles can then be identified by testing for indicators that are unique to the specific organelles.

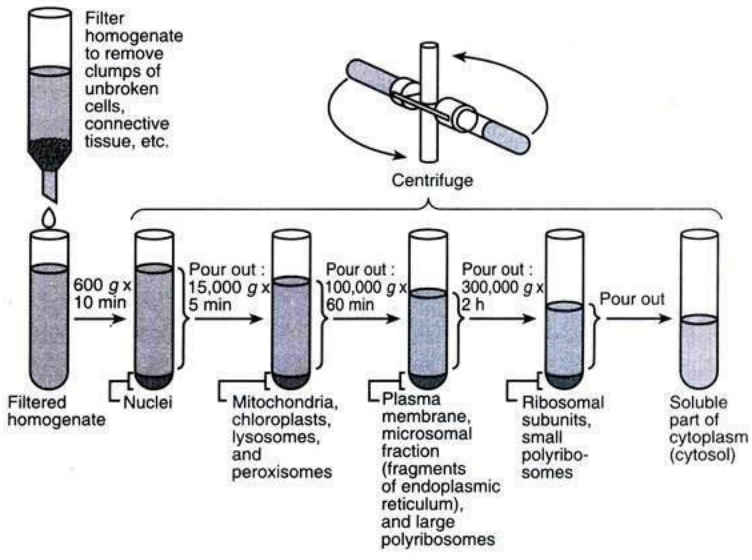


Fig. 5.13: Isolation of different cell organelles by differential centrifugation

Density gradient centrifugation

Density gradient centrifugation Is considered one of the more efficient methods of separating suspended particles. Density gradient centrifugation can be used both as a separation technique and as a method of measuring the densities of particles or molecules in a mixture. A tube, after being centrifuged by this method, has particles in order of density based on height. The object or particle of interest will reside in the position within the tube corresponding to its density.Linderstorm-Lang, in 1937, discovered that density gradient tubes could be used for density measurements. He discovered this when working with potato yellow-**dwarf virus.[6] This method was also used in Meselson and Stahl's famous** experiment in which they proved that DNA replication is semi-conservative by using different isotopes of nitrogen. They used density gradient centrifugation to determine which isotope or isotopes of nitrogen were present in the DNA after cycles of replication.Nevertheless, some non-ideal sedimentations are still possible when using this method. The first potential issue is the unwanted aggregation of particles, but this can occur in any centrifugation. The second possibility occurs when droplets of solution that contain particles sediment. This is more likely to occur when working with a solution that has a layer of suspension floating on a dense liquid, which in fact have little to no density gradient.

(a) Rate Zonal Technique: Particle separation by the rate zonal technique is based upon differences in the size, shape and density of particles, the density and viscosity of the medium and the applied centrifugal field. Subcellular organelles, which have different densities but are similar in size, do not separate efficiently using this method, but sepa¬ration of proteins of similar densities and differing only 3 folds in relative molecular mass can be achieved easily. The technique involves carefully layering a sample solution on top of preformed liquid density gradient, the highest density of which does not exceed that of densest particle to be separated. The function of gradient is primarily to stabilize the liquid column in the tube against the movements resulting from conventional currents and secondarily to produce a gradient that helps to improve the resolution of gradient. The sample is then centrifuged until the desired degree of separation is achieved. Since the technique is time dependent, centrifugation must be terminated before any of the separated zone pellets at he bottom of tube. The technique is employed for the separation of enzymes, RNA-DNA hybrids, ribosomal subunit, subcellular organelle, etc.

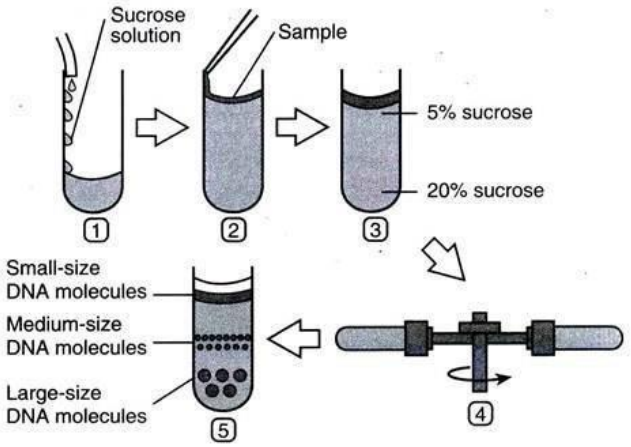


Fig. 5.14: Principle of rate zonal centrifugation technique

(b) Isopycnic Centrifugation Technique: Isopycnic centrifugation depends solely upon the buyout density and not on its shape, size and time, the size of the particle affecting only the rate at which it reaches its isopycnic position in the gradient. The technique is used to separate particles of similar size but of different density. Hence soluble proteins which have very similar densities cannot be usually separated by this method, where as sub cellular organelles can be effectively separated.

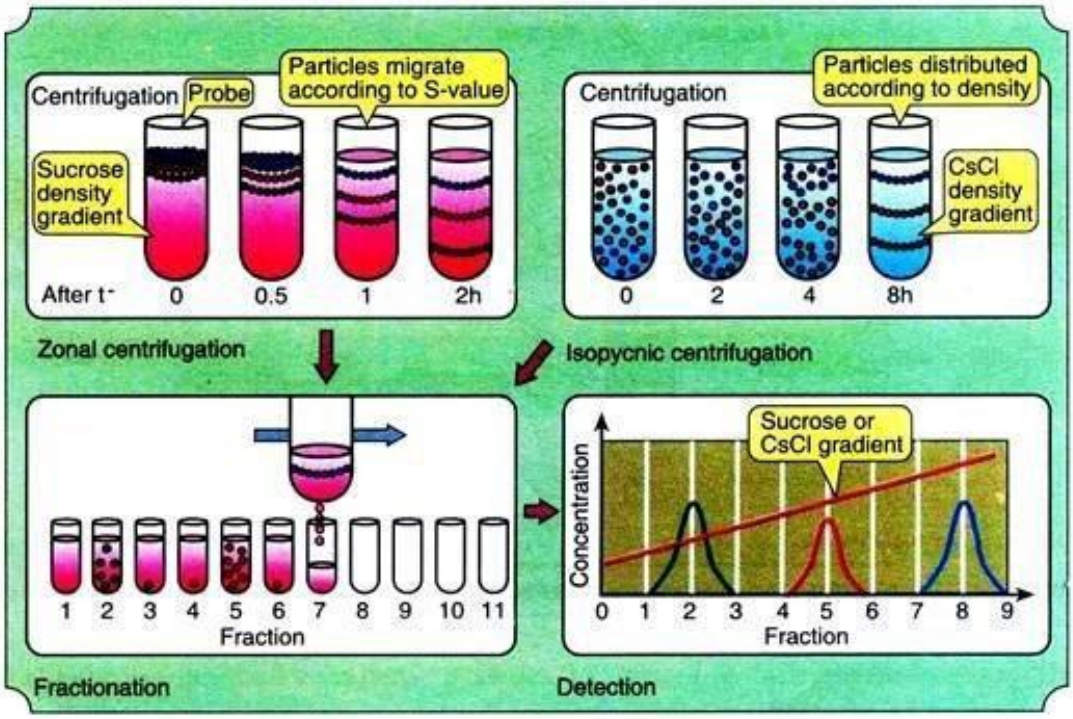


Fig. 5.15: Density gradient centrifugation

The methods are a combination of sedimentation and flotation and involve layering the sample on top of a density gradient that spans the whole range of the particle densities that are to be separated. The maximum density of the gradient, therefore, must always exceed the density of the densest particle. During centrifugation, sedimentation of the particle occurs until the buoyant density of the particle and density of the gradient are equal. At this point of isodensity no further sedimentation occurs, irrespective of how long centrifugation continues, because the particles are floating on the cushion of material that has density greater than their own. Isopycnic centrifugation, in contrast to the rate zonal technique, is an equilibrium method, the particle banding to form zones each at their own characteristic buoyant density. In case when not all components in a mixture of particle are required, a gradient range can be selected in which unwanted materials will be sediment at the bottom of the tube and whole of the particles of interest will float at their respective isopynic positions. Such a technique involves a combination of both the rate zonal and isopynic approaches.

Table 5.3: Densities of various important biological molecules

Material	Density (g/cm^3)
Microbial cells	1.05–1.15
Mammalian cells	1.04–1.10
Organelles	1.10–1.60
Proteins	1.30
DNA	1.70
RNA	2.00

Electrophoresis of DNA

Agarose gel electrophoresis is the most effective way of separating DNA fragments of varying sizes ranging from 100 bp to 25 kb. Agarose is isolated from the seaweed genera Gelidium and Gracilaria, and consists of repeated agarobiose (L- and D-galactose) subunits. During gelation, agarose polymers associate non-covalently and form a network of bundles whose pore sizes determine a gel's molecular sieving properties. The use of agarose gel electrophoresis revolutionized the separation of DNA. Prior to the adoption of agarose gels, DNA was primarily separated using sucrose density gradient centrifugation, which only provided an approximation of size. To separate DNA using agarose gel electrophoresis, the DNA is loaded into pre-cast wells in the gel and a current applied. The phosphate backbone of the DNA (and RNA) molecule is negatively charged, therefore when placed in an electric field, DNA fragments will migrate to the positively charged anode. Because DNA has a uniform mass/charge ratio, DNA molecules are separated by size within an agarose gel in a pattern such that the distance traveled is inversely proportional to the log of its molecular weight. The leading model for DNA movement through an agarose gel is "biased reptation", whereby the leading edge moves forward and pulls the rest of the molecule along. The rate of migration of a DNA molecule through a gel is determined by the following: 1) size of DNA molecule; 2) agarose concentration; 3) DNA conformation;
4) voltage applied, 5) presence of ethidium bromide, 6) type of agarose and 7) electrophoresis buffer. After separation, the DNA molecules can be visualized under UV light after staining with an appropriate dye. By following this protocol, one can: Understand the mechanism by which DNA fragments are separated within a gel matrix Understand how conformation of the DNA molecule will determine its mobility through a gel matrix Identify an agarose solution of appropriate concentration for their needs Prepare an agarose gel for electrophoresis of DNA samples Set up the gel electrophoresis apparatus and power supply Select an appropriate voltage for the separation of DNA fragments Understand the mechanism by which ethidium bromide allows for the visualization of DNA bands Determine the sizes of separated DNA fragments.

1. Preparation of the Gel

1. Weigh out the appropriate mass of agarose into an Erlenmeyer flask. Agarose gels are prepared using a w/v percentage solution. The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2%. The volume of the buffer should not be greater than 1/3 of the capacity of the flask.
2. Add running buffer to the agarose-containing flask. Swirl to mix. The most common gel running buffers are TAE (40 mM Tris-acetate, 1 mM EDTA) and TBE (45 mM Tris-borate, 1 mM EDTA).
3. Melt the agarose/buffer mixture. This is most commonly done by heating in a microwave, but can also be done over a Bunsen flame. At 30 s intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved.
4. Add ethidium bromide (EtBr) to a concentration of 0.5 μg/ml. Alternatively, the gel may also be stained after electrophoresis in running buffer containing 0.5 μg/ml EtBr for 15-30 min, followed by destaining in running buffer for an equal length of time. Note: EtBr is a suspected carcinogen and must be properly disposed of per institution regulations. Gloves should always be worn when handling gels containing EtBr. Alternative dyes for the staining of DNA are available; however EtBr remains the most popular one due to its sensitivity and cost.
5. Allow the agarose to cool either on the benchtop or by incubation in a 65 °C water bath. Failure to do so will warp the gel tray.
6. Place the gel tray into the casting apparatus. Alternatively, one may also tape the open edges of a gel tray to create a mold. Place an appropriate comb into the gel mold to create the wells.
7. Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box. Alternatively, the gel can also be wrapped in plastic wrap and stored at 4 °C until use.

2. Setting up of Gel Apparatus and Separation of DNA Fragments

1. Add loading dye to the DNA samples to be separated. Gel loading dye is typically made at 6X concentration (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol). Loading dye

helps to track how far your DNA sample has traveled, and also allows the sample to sink into the gel.

2. Program the power supply to desired voltage (1-5V/cm between electrodes).

3. Add enough running buffer to cover the surface of the gel. It is important to use the same running buffer as the one used to prepare the gel.

4. Attach the leads of the gel box to the power supply. Turn on the power supply and verify that both gel box and power supply are working.

5. Remove the lid. Slowly and carefully load the DNA sample(s) into the gel . An appropriate DNA size marker should always be loaded along with experimental samples.

6. Replace the lid to the gel box. The cathode (black leads) should be closer the wells than the anode (red leads). Double check that the electrodes are plugged into the correct slots in the power supply.

7. Turn on the power. Run the gel until the dye has migrated to an appropriate distance.

3. Observing Separated DNA fragments

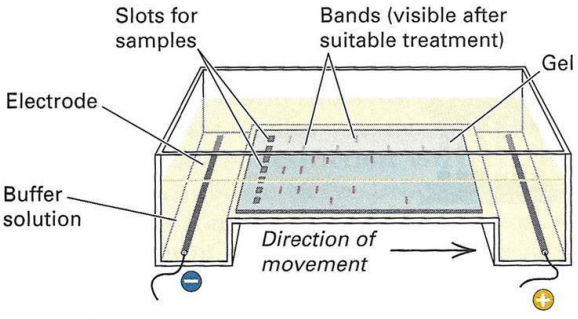
1. When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.

2. Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.

3. Remove the gel from the gel tray and expose the gel to UV light. This is most commonly done using a gel documentation system. DNA bands should show up as orange fluorescent bands. Take a picture of the gel.

4. Properly dispose of the gel and running buffer per institution regulations.

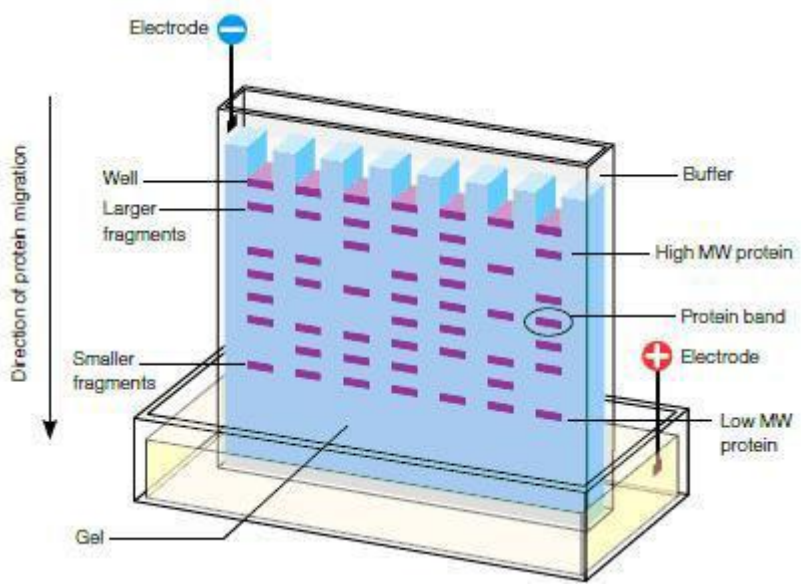
Agarose gel electrophoresis of DNA



Polyacrylamide Gel Electrophoresis (PAGE)

Acrylamide gels serve as a size-selective sieve during separation. As proteins move through a gel in response to an electric field, the smaller molecules travel more rapidly than larger proteins (see figure below).

In most PAGE applications, the gel is mounted between two buffer chambers, and the only electrical path is through the gel. Usually, the gel has a vertical orientation, and the gel is cast with a comb that generates wells in which the samples are applied (see below). Applying an electrical field across the buffer chambers forces the migration of protein into and through the gel.



Schematic of electrophoretic protein separation in a polyacrylamide gel.

Two types of buffer systems can be used:

- **Continuous buffer systems —** use the same buffer (at constant pH) in the gel, sample, and electrode reservoirs. Samples are loaded into wells, and proteins that are closer to the gel enter first. This provides a uniform separation matrix, but yields fuzzy and unresolved protein bands. Continuous systems are rarely used for protein electrophoresis but commonly used for nucleic acid analysis

 Discontinuous buffer systems — use a gel separated into two sections (a large pore stacking gel on top of a small pore resolving gel, see figure below) and different buffers in the gels and electrode solutions. Proteins migrate quickly through the large pore stacking gel and then are slowed as they enter the small pore resolving gel. The proteins stack on top of one another to form a tight band, which helps improve resolution. Discontinuous buffer systems provide higher resolution than continuous systems, and varying the buffers used in the sample, gel, and electrode chambers creates a variety of discontinuous buffer systems that can be used for a variety of applications

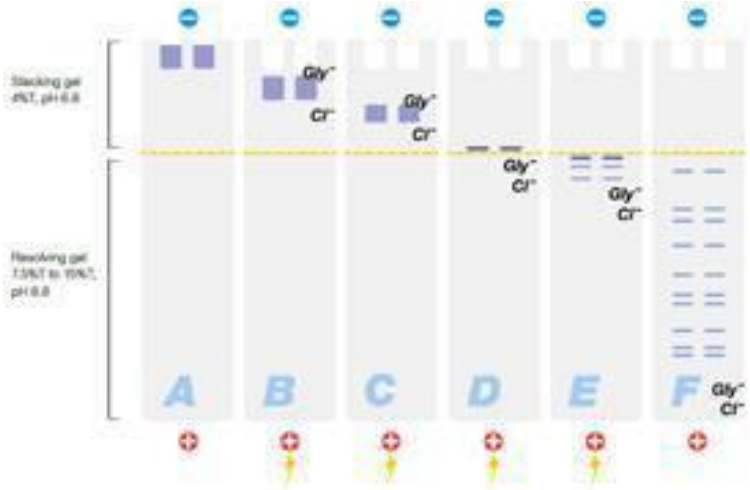


Figure: Migration of proteins and buffer ions in a denaturing discontinuous PAGE system.

A: Denatured sample proteins are loaded into the wells

B: Voltage is applied and the samples move into the gel. The chloride ions already present in the gel (leading ions) run faster than the SDS-proteins and form an ion front. The glycinate ions (trailing ions) flow in from the running buffer and form a front behind the proteins

C: A voltage gradient is created between chloride and glycinate ions, which sandwich the proteins in between

D: The proteins are stacked between the chloride and glycinate ion fronts. At the interface between the stacking and resolving gels, the % of acrylamide increases and the pore size decreases. Movement of the proteins into the resolving gel is met with increased resistance

E: The smaller pore size resolving gel begins to separate the proteins based on molecular weight only, since the charge-to-mass ratio is equal in all the proteins of the sample

F: The individual proteins are separated into band patterns ordered according to molecular weight

Discontinuous Native PAGE

The original discontinuous gel system was developed by Ornstein and Davis (1964) for the separation of serum proteins in a manner that preserved native protein conformation, subunit interactions, and biological activity. Proteins are prepared in nonreducing, nondenaturing sample buffer, and electrophoresis is performed in the absence of denaturing and reducing agents. The native charge is preserved and proteins can migrate towards either electrode, but yields unpredictable separation patterns that are not suitable for molecular weight determination. Nevertheless, native PAGE does allow for separation of proteins in their active state and can resolve proteins of the same molecular weight.

SDS-PAGE

To overcome the limitations of native PAGE systems, Laemmli (1970) incorporated the detergent sodium dodecyl sulfate (SDS) into a discontinuous denaturing buffer system, creating what has become the most popular form of protein electrophoresis, SDS-PAGE.

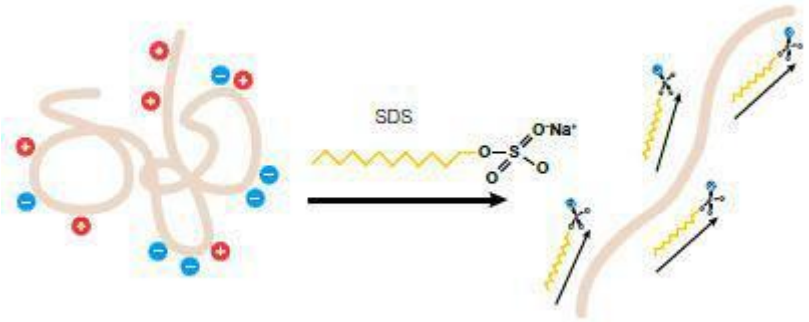
When proteins are separated in the presence of SDS and denaturing agents, they become fully denatured and dissociate from each other (see figure below). In addition, SDS binds noncovalently to proteins in a manner that imparts:

- An overall negative charge on the proteins. Since SDS is negatively charged, it masks the intrinsic charge of the protein it binds
- A similar charge-to-mass ratio for all proteins in a mixture, since SDS binds at a consistent rate of 1.4 g SDS per 1g protein SDS (a stoichiometry of about one SDS molecule per two amino acids)
- A long, rod-like conformation on the proteins instead of a complex tertiary shape

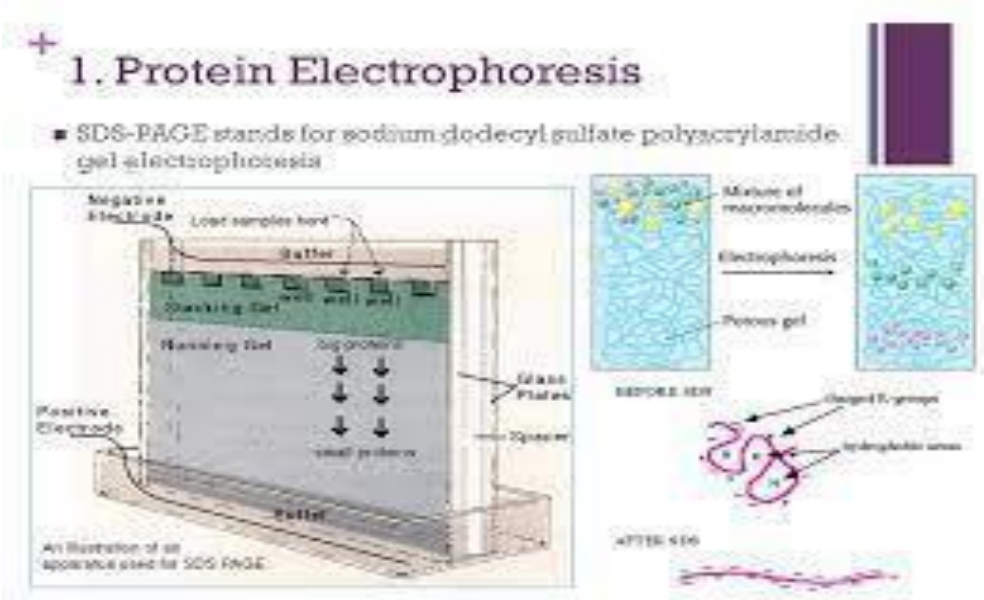
As a result, the rate at which an SDS-coated protein migrates in a gel depends primarily on its size, enabling molecular weight determination.

The original Laemmli system incorporated SDS in the gels and buffers, but SDS is not required in the gel. SDS in the sample buffer is sufficient to saturate proteins, and the SDS in the cathode buffer maintains the SDS saturation during electrophoresis.

A range of gel and buffer combinations can be used for native and SDS-PAGE, each with its own advantages.



Effect of SDS on the conformation and charge of a protein.



Isoelectric focusing

In the course of isoelectric focusing, the conditions are set in a way that proteins will be separated exclusively based on their isoelectric point (Figure 1). The two termini and many side chains of proteins contain dissociable groups (weak acids or bases). The dissociation state of these groups is a function of the pH of the environment. Isoelectric focusing is based on the pH-dependent dissociation of these groups. Due to this pH-dependent phenomenon, the net electric charge of a

protein molecule will be a function of the pH of the medium. If, in a given protein, the number of acidic residues (Asp, Glu) exceeds that of the basic ones (Arg, Lys, His), the protein will have a net negative charge at neutral pH. The isoelectric point (pI) of the protein—i.e. the pH at which the net charge of the protein is zero—will be in the acidic pH range. Such proteins are often denoted as acidic proteins. If the number of basic residues exceeds that of the acidic ones, the protein will be positively charged at neutral pH, and its pI value will be in the basic pH range. These proteins are often called basic proteins.

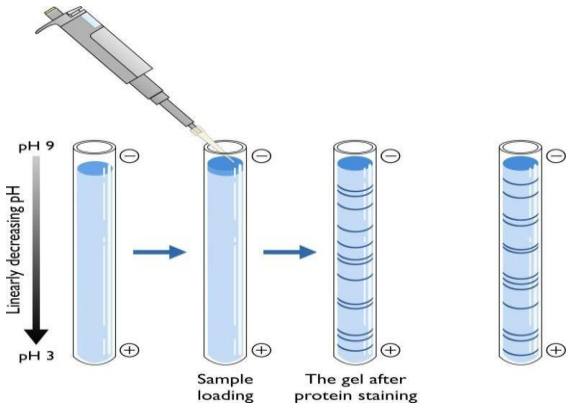


Figure 1. Isoelectric focusing. In the course of isoelectric focusing, a pH gradient is created in the gel (usually made of polyacrylamide, less frequently agarose). Upon electrophoresis, various proteins will accumulate in different narrow regions of the gel where the pH equals their individual pI value. At this pH, the number of positive charges equals that of the negative charges on the protein—the net charge will thus be zero. Consequently, no resultant electric force is exerted on the protein.

Isoelectric focusing is an efficient high-resolution method because the pI values of various proteins are spread across a broad range. If the pH is lower than the pI of the protein, the protein will be positively charged and will move towards the cathode during electrophoresis. If the pH is higher than the pH of the protein, the protein will be negatively charged and will migrate towards the anode. If the pH equals the pI value, the net charge of the protein will be zero and the protein will not migrate in the gel any further.

In the course of isoelectric focusing, proteins are placed in a gel representing a special medium in which the pH gradually decreases by going from the negative cathode towards the positive anode. As the protein migrates, it encounters a gradually changing pH and its net charge will also

change accordingly. If it has a net negative charge and therefore moves towards the cathode, it will encounter a gradually decreasing pH, i.e. a more and more acidic environment. Consequently, the protein will take on more and more protons—up to a level where its net charge will be zero. This state is reached when the protein reaches a location where the pH equals its pI value. At this point, the protein will stop moving because no electric force will be exerted on it. If it spontaneously diffused further towards the anode, it would take on more protons, would become positively charged and would turn back to migrate towards the cathode. Following the same line of thinking, if a positively charged protein moves towards the cathode, it will encounter increasing pH and lose more and more protons. It will migrate to the place where the pH equals its pI value and will thus stop. If it diffused further towards the cathode, it would become negatively charged and would turn back towards the anode. As one can see, by performing electrophoresis in a medium in which **the pH decreases from the cathode towards the anode, each protein will "find its place"** according to its pI value and will become sharply focused at that location. In addition, it does not matter where exactly the proteins were introduced in the medium between the cathode and the anode.

A decisive component of this method is the usually linear pH gradient created inside the gel. There are two methods to create such a gradient. One of them applies carrier ampholytes (ampholyte is an acronym from the words amphoteric and electrolyte). Ampholytes or zwitterions are molecules that contain both weakly acidic and weakly basic groups. Just like in the case of proteins, the net charge of ampholytes is a function of the pH. In the course of isoelectric focusing, a mixture of various ampholytes is used such that the pI of the various **ampholyte components will cover a range in which the pI values of the "neighbouring"** ampholytes differ only slightly. This ampholyte mixture is soaked in the gel and an appropriate electric field is generated by a power supply. This leads to a process analogous to the one already explained for proteins. Each ampholyte will migrate to the location where its net charge becomes zero. As soon as this steady-state is achieved, ampholytes will function as buffers and keep the pH of their immediate environment constant. This establishes the pH gradient in which the proteins can be separated.

The other, more sophisticated method applies special ampholytes that can be covalently polymerised into the polyacrylamide gel. The appropriate ampholyte gradient is created before

the gel is polymerised. This way, the gradient will be covalently fixed in the gel, providing an immobilised pH gradient. The appropriate pH range provided by the ampholyte mixture should be selected based on the pI values of the proteins to be separated.

Regardless of how the pH gradient was created, once the proteins reach the location in the gel where the pH equals their pI, they finally stop moving and the system reaches a steady-state.

One of the potential technical difficulties encountered during isoelectric focusing originates from the fact that the solubility of proteins is lowest at their pI value (see Chapter 5). This can lead to the precipitation of some proteins in the gel. To prevent this unwanted process, urea is most often applied in the gel as an additive. Urea denatures proteins and keeps denatured proteins in solution. As the pI value of proteins is largely independent of their conformational state, this modification does not compromise the method. The solubility of membrane proteins can be further promoted by the addition of non-ionic detergents.

Isoelectric focusing is aimed at separating proteins based exclusively on their pI value—thus, independently of their size. Therefore, the molecular sieving property of the gel in this method should be avoided. The only function of the gel is to prevent free convectional flows in the medium. Accordingly, for isoelectric focusing, polyacrylamide gels are made at very low acrylamide concentrations, and sometimes even agarose gels are applied when very large pores are needed. Isoelectric focusing is usually performed in a horizontally-mounted electrophoresis apparatus and by applying intense cooling.

Two-dimensional (2D) electrophoresis

The various separation methods are all aimed at separating complex systems to individual components. Separation is always based on at least one physicochemical property that shows

diversity among the components. The general problem encountered in the case of complex mixtures is that not all components differ significantly from all other components when only one property is considered. Accordingly, separation based on a single property rarely results in single-component fractions. Some components will be efficiently separated from all others, while some other components will remain in the mixture.

The remaining mixtures can be further fractionated by another separation technique that relies on a different physicochemical property. The most effective separation can be achieved if the combined consecutive separation steps rely on absolutely independent physicochemical properties. A good example of this is the very high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) that combines two already discussed electrophoresis methods, isoelectric focusing and SDS-PAGE (Figure 2).

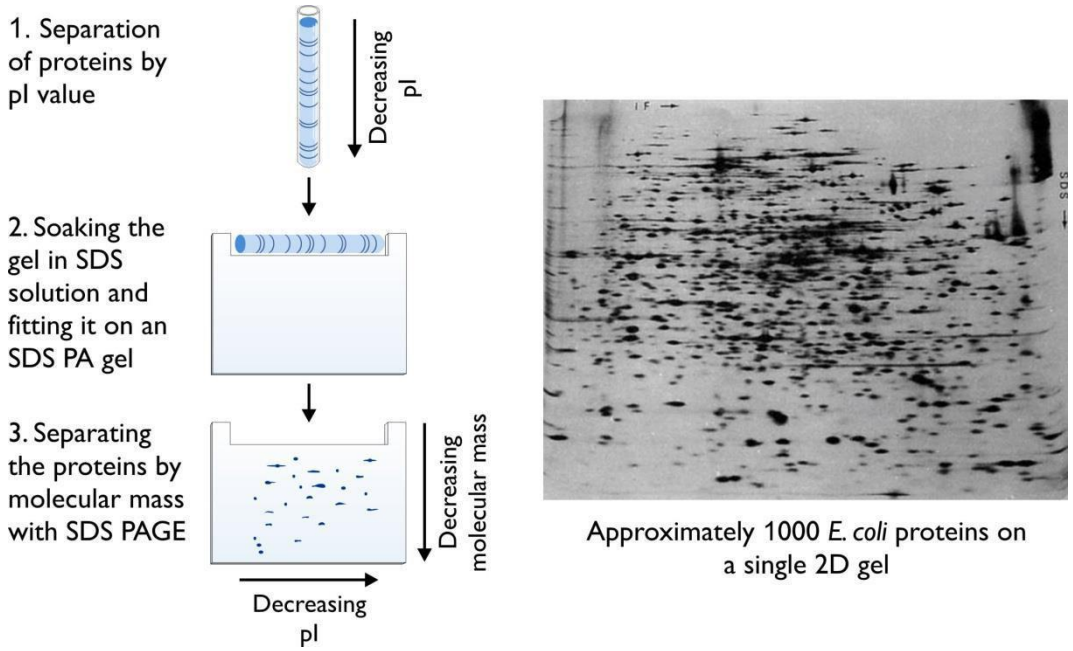


Figure 2. Two-dimensional (2D) electrophoresis. 2D electrophoresis is the combination of isoelectric focusing and SDS-PAGE. Proteins are first separated based on their pI values and then based on their molecular mass. As these properties are completely independent, the combination of the two separation methods provides much higher resolution than either of the two methods alone.

As the first step of 2D gel electrophoresis, isoelectric focusing is performed to separate proteins based on their pI values. Only a single sample is loaded on a gel strip in this step. The sample is separated in one dimension both in a primary and in a figurative sense. In a primary sense because the components are separated along a single line, and in a figurative sense as the separation is based on a single well-defined property, the pI value.

After the first separation step has been completed in the first dimension, the gel strip is **soaked in an SDS solution and is fitted tightly to one side of a "classical" SDS** polyacrylamide gel.

The second separation step is traditional SDS-PAGE, which separates proteins based on their molecular mass. This second step represents a second dimension in both a primary and a figurative sense. The second separation is performed in a second dimension in a direction rectangular to that of the first separation, and the property utilised in the second step (molecular mass) is completely independent of the one utilised in the first step (pI).

If, after the first step, some gel regions contain different proteins that coincidentally have identical pI values, these proteins will be separated from each other in the second step if their molecular mass is different. Note that every aspect discussed for SDS-PAGE also applies to the second separation step of 2D-PAGE. Van der Waals interactions that might have held protein subunits together in the course of isoelectric focusing will break and individual subunits will become separated. If disulfide bridges need to be opened up, some kind of reducing agent needs to be added. Accordingly, in the second separation step, single polypeptide chains will migrate in the gel. If isoelectric focusing collects a multimeric protein at a certain gel location, the second electrophoresis step will dissect it into individual chains. If the multimer contains subunits of different sizes, these subunits will be separated from each other in the second separation step.

DNA purification

DNA Filtration Column

Filter column purification can be used to purify DNA from mammalian cell cultures, bacteria, and yeast, as well as plant and animal tissue. By adjusting the pH and salt of the solution, DNA of interest can be separated from cellular debris or other unwanted contaminants by binding the DNA to a silica membrane situated at the bottom of a filter column. This is a popular method for genomic and plasmid DNA purification. Vacuum and centrifugation protocols are available. After the DNA is bound to the membrane, it is subjected to a low and a high stringency wash to remove contaminants such as RNA, proteins and lipids. The purified DNA is eluted off the membrane with an elution buffer into a collection tube. The volume of the elution buffer used can be varied depending on the final concentration of plasmid or DNA desired. Recovered DNA is suitable for use with PCR and Southern blot analysis.

Traditionally, cesium chloride gradients were used to purify plasmid DNA away from genomic DNA. While this method provides highly pure DNA, it is time consuming and requires removal

of ethidium bromide and cesium chloride from the recovered plasmid. Plasmid purification kits provide a faster and more efficient means for the purification and concentration of DNA > 200 base pairs. DNA is adsorbed onto the silica-based membrane, and RNA, protein, and other cellular components are washed away. The purified DNA is then eluted using elution buffer and is recovered in a form immediately available for fluorescent sequencing, cell transfection, electroporation, and enzymatic restriction and modification.

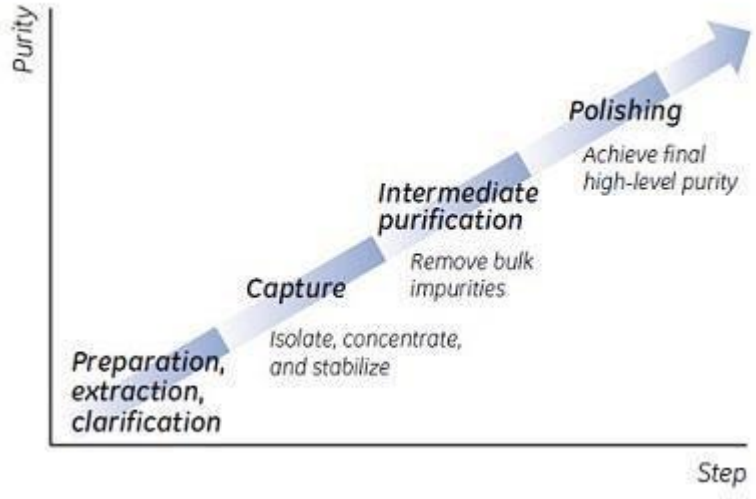
Ion Exchange Resin

As an alternative to filter column purification or phenol/chloroform extraction, a buffered ion exchange resin can be used for the removal of PCR contaminates from blood, cultured cells, and bacteria. Chelex resin that removes PCR-inhibitors from a sample prior to PCR amplification. A single cell lysis step by boiling in the presence of the matrix is sufficient. This is possible because the matrix efficiently absorbs cell lysis products that interfere with the PCR amplification process. The matrix can then be pelleted out by centrifugation and the supernatant can be used for downstream PCR amplification.

Phenol/Chloroform Extraction and Ethanol Precipitation

A traditional DNA purification method that can be used to obtain highly pure DNA is phenol/chloroform extraction followed by ethanol precipitation. This method is intended for the extraction of DNA from animal and plant tissues, cultured mammalian cells, bacteria and yeast cells in under one hour. The aqueous nucleic acid sample is combined with an equal volume of a phenol:chloroform mixture. Phenol dissociates proteins bound to DNA while chloroform denatures proteins and lipids. Three distinct phases will form: the aqueous phase, the interphase, and the organic phase. Of these, the aqueous phase contains the DNA, whereas the proteins and lipids remain in the other two phases. The aqueous phase can then be treated with ethanol to precipitate the DNA. The precipitated DNA can then be pelleted by centrifugation and dissolved in a buffer of choice for use in downstream reactions. DNA purified using the phenol/chloroform extraction and ethanol precipitation method is typically more pure than DNA recovered from filter column purification. The recovered DNA is suitable for use in PCR and Southern blot analysis.

Before starting purification, it is important to define your requirements and objectives. These may include defining the required purity, activity, and quantity of the target, keeping steps and handling to a minimum, and removing critical impurities (e.g., proteases). Whether protein purification is performed at microgram and milligram quantities for research or at kilogram to ton quantities for industry, there are proven strategies that can help you apply a systematic approach to purification.



CIPP Purification Strategy. The capture phase reduces the amount of unwanted proteins and concentrates the target protein. Intermediate purification removes most contaminants that are closely related to the target protein. Polishing removes specified contaminants and unwanted forms of the target protein that may have been formed during isolation and purification.

The overall purification power of CIPP depends on exploiting a combination of techniques with independent selectivities. Together, the techniques give a cost efficient and robust production of the target substance. When planning a CIPP strategy, it is important to take into consideration the scale of the purification process and the desired yield and use of the target protein.

Each purification step in multi-stage purification strategy must have a clearly assigned purpose. Increasing the number of purification steps will often decrease the overall protein recovery, increase purification time, and can be detrimental to activity. For most laboratory-scale work, a two- or three-step purification protocol will be sufficient, whereas difficult purifications may require several additional steps.

A number of protein precipitation methods are frequently used today. Protein precipitation with a neutral salt, such as ammonium sulfate, is useful for purification and enrichment of a target protein from an extract without loss of activity.

At industrial scale, Cohn's cold ethanol fractionation/precipitation methods from the 1940s are still used to produce human serum albumin, immunoglobulins, and other blood-based products. However, chromatography methods, in combination with ultrafiltration, have started to replace Cohn fractionation since they offer higher purity and yield, and are easier to automate. The goals of preparative protein purification are as diverse as isolating enzymes for biochemical characterization or structure determination, process-level protein purification of industrial enzymes, and purification of therapeutic protein molecules for clinical applications.

Extraction

- Depending on the source, the protein has to be brought into solution by breaking the tissue or cells containing it.
- There are several methods to achieve this:
- Repeated freezing and thawing,
- sonication,
- homogenization by high pressure,
- filtration, or permeabilization by organic solvents.

Precipitation and differential solubilization

- In bulk protein purification, a common first step to isolate proteins is precipitation with ammonium sulfate **$(NH_4)_2SO_4$.**
- This is performed by adding increasing amounts of ammonium sulfate and collecting the different fractions of precipitate protein. Ammonium sulphate can be removed by dialysis.

- The hydrophobic groups on the proteins gets exposed to the atmosphere and it attracts other protein hydrophobic groups and gets aggregated. Protein precipitated will be large enough to be visible.

- One advantage of this method is that it can be performed inexpensively with very large volumes.

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

COURSE MATERIAL

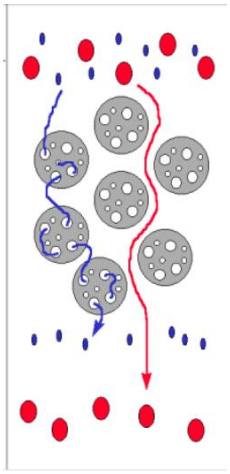
Subject Name: ANALYTICAL TECHNIQUES IN BIOTECHNOLOGY Code: SBB1201

UNIT I – V

CHROMATOGRAPHY METHODS

Gel filtration chromatography

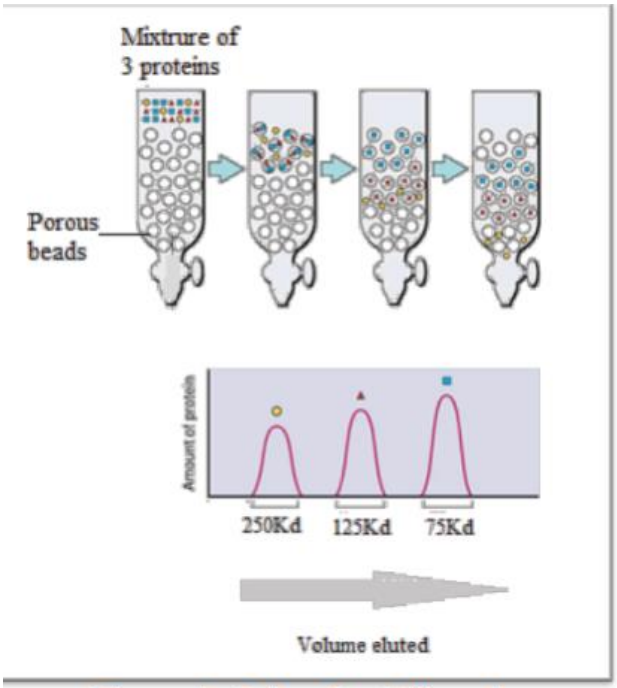
Principle



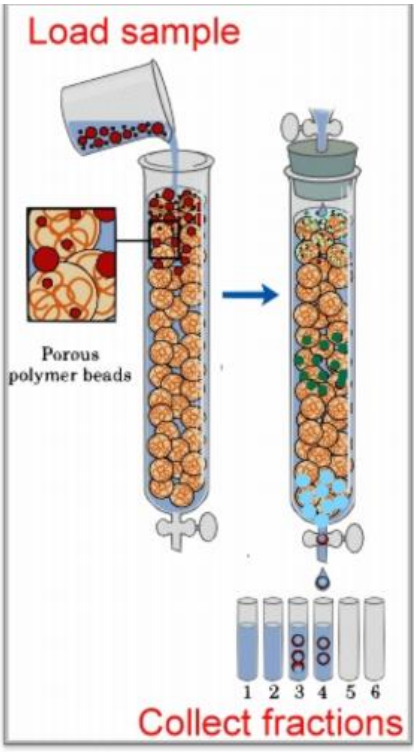
-Gel filtration chromatography separates proteins according to their size. And the molecules are "filtered" through the porous beads.

-The gel filtration matrix [stationary phase] contains pores which permit the buffer, small and medium sized molecules to pass through them.

-Large molecules, can't get through any pores in the beads and move more rapidly through the column, emerging (eluting) sooner.[elute from the column before the smaller proteins]. - Medium-sized molecules, can enter the larger size pores in the matrix, and so they reach the end of the column later -Small molecules, can enter through all pores of the beads and they have the largest volume to pass through before emerging from the column last.à elute last.



The principle of gel filtration chromatography



Phases Stationary phase [beads]….info.:

-The gels used as molecular sieves consist of cross-liked polymers that are generally inert, do not bind or react with the material being analyzed, and are uncharged.

Mobile phase:

- Is the liquid fills the space inside the beads and between the bead particles and this liquid occupies most of the bed volume.

Stationary phase :

Gel Filtration Resins, The gels currently in use are of three types: dextarn, agarose, and polyacrylamide. 1- Dextran [e.g. Sephadex]: -is a polysaccharide composed of glucose residues. -prepared with various degrees of cross-linking to control pores size. -supplied in the form of dry beads that swell when water is added.- -It is mainly used for separation of small peptides and globular proteins with small to average molecular mass

Notes –

The larger the beads, the more rapid the flow rate and the poorer the resolution. [because as the flow rate increases, the time available for the molecules to equilibrate between the mobile phase and the pore space in the stationary phase decreases.] When it is used??? The larger beads are for very large preparation in which resolution is less important than time. -Super fine, is used if maximum resolution is required and the time is less important.

Gel beads come in various sizes: large, medium, fine, and superfine. • All consist of semi-permeable, porous gels of cross linked polymers with a range of pore sizes. •The degree of cross linking is controlled to yield a series of gels having different pore sizes.

Advantages of gel filtration:

It is the best method for separation of molecules differing in molecular weight because:

1. It doesn’t depend on temperature, pH, ionic strength and buffer composition, so, separation can be carried out under any conditions.

2. There is less zonal spreading than in other techniques.

3. The elution volume is related to the molecular weight.

4. Important method in protein purification.

5.This separation method is unique in fractionating without requiring protein binding, thus significantly reducing the risk of protein loss.

The most common application of gel filtration in biochemistry are:

-Molecular weight determination.

-Fractionation of macromolecules.

-purification.

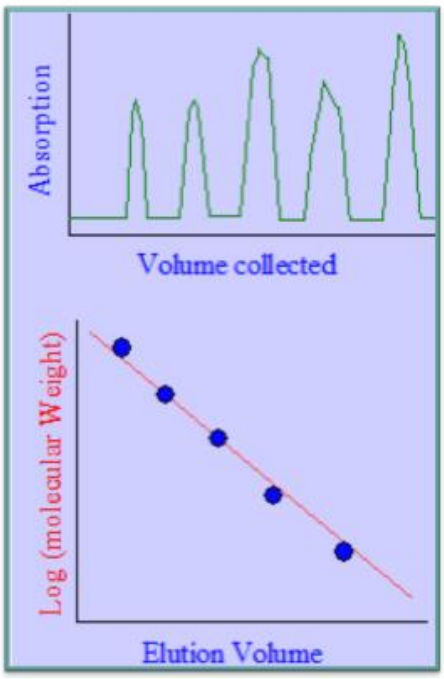
To estimate the molecular weight for a protein

1. Several proteins with known molecular weights are run on the column and their elution volumes determined.

2. The elution volumes are then plotted against the log molecular weight of the corresponding proteins.

3. A straight line is obtained for the separation range of the gel being used.

4. The elution volume of a protein of unknown molecular weight is then found, [it can be compared to the calibration curve and the molecular weight determined.



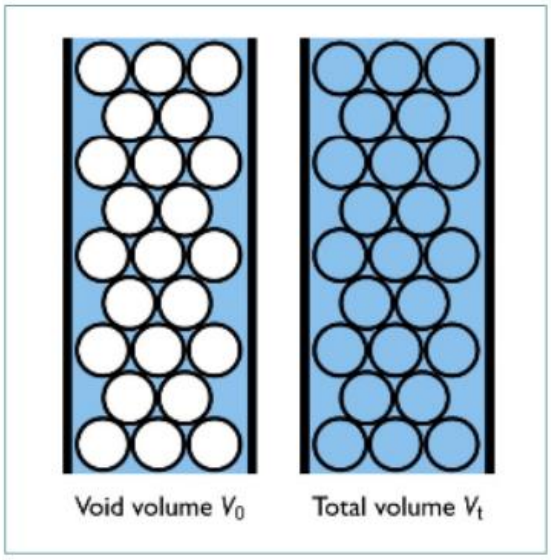
For high molecular weight less elution volume is needed, and for small molecular weight the large elution volume is needed to elute the sample.

For every column, three volumes should be distinguished:

1. The void volume, Vo: - The volume of the mobile phase in the space between the beads . [which is the volume external to the beads]. -Can be determined by the elution of High MW molecules which can not enter any pores .

2. The total volume, Vt (bed volume): -The total volume of material in the column (both solid and liquid). [can be calculated from the dimension of the column.]

3. The elution volume, Ve, of molecules: - the amount of liquid(mobile phase) that must be added to produce a peak of a particular solute in the effluent. Or the volume required for completely eluting the solute from column.



-Loading the sample : small volume of sample is placed on the stationary phase and allowed to enter the column.

-Packing the column: Preparation of the gel and loading it in the column.

Cautions must be taken It is important that the gel should be:

-Homogenous.

-Free from bubbles.

-Free from cracks.

-Free from spaces between the walls.

- And it should be covered by the liquid "mobile phase" all the time.

Ion-exchange chromatography (IEC)

Introduction

Ion-exchange chromatography (IEC) is part of ion chromatography which is an important analytical technique for the separation and determination of ionic compounds, together with ion-partition/interaction and ion-exclusion chromatography. Ion chromatography separation is based on ionic (or electrostatic) interactions between ionic and polar analytes, ions present in the eluent and ionic functional groups fixed to the chromatographic support. Two distinct mechanisms as follows; ion exchange due to competitive ionic binding (attraction) and ion exclusion due to repulsion between similarly charged analyte ions and the ions fixed on the chromatographic support, play a role in the separation in ion chromatography. Ion exchange has been the predominant form of ion chromatography to date. This chromatography is one of the most important adsorption techniques used in the separation of peptides, proteins, nucleic acids and related biopolymers which are charged molecules in different molecular sizes and molecular nature. The separation is based on the formation of ionic bonds between the charged groups of biomolecules and an ion-exchange gel/support carrying the opposite charge. Biomolecules display different degrees of interaction with charged chromatography media due to their varying charge properties.

The earliest report of ion-exchange chromatography date back to 1850, Thompson studied the adsorption of ammonium ions to soils. Spedding and Powell published a series of papers describing practical methods for preparative separation of the rare earths by displacement ion-exchange chromatography in 1947. Beginning in the 1950s, Kraus and Nelson reported numerous analytical methods which are used for metal ions based on separation of their chloride, fluoride, nitrate or sulfate complexes by anion chromatography. In order to separate proteins an ion exchange chromatographic method was reported by Peterson and Sober in 1956. In modern form

ion exchange chromatography was introduced by Small, Stevens and Bauman in 1975. Gjerde et al. published a method for anion chromatography in 1979 and this was followed by a similar method for cation chromatography in 1980. Ion-exchange chromatography has been used for many years to separate various ionic compounds; cations and anions and still continues to be used. The popularity of ion exchange chromatography has been increased in recent years because this technique allows analysis of wide range of molecules in pharmaceutical, biotechnology, environmental, agricultural and other industries.

1.1. Ion exchange mechanism

Ion-exchange chromatography which is designed specifically for the separation of differently charged or ionizable compounds comprises from mobile and stationary phases similar to other forms of column based liquid chromatography techniques. Mobil phases consist an aqueous buffer system into which the mixture to be resolved. The stationary phase usually made from inert organic matrix chemically derivative with ionizable functional groups (fixed ions) which carry displaceable oppositely charged ion. Ions which exist in a state of equilibrium between the mobile phase and stationary phases giving rise to two possible formats, anion and cation exchange are referred to as counter ion (Figure 1) . Exchangeable matrix counter ions may include protons (H+), hydroxide groups (OH-), single charged mono atomic ions (Na+, K+, Cl-), double charged mono atomic ions (Ca2+, Mg2+), and polyatomic inorganic ions (SO42-, PO43-) as well as organic bases (NR2H+) and acids (COO-). Cations are separated on cation-exchange resin column and anions on an anion exchange resin column. Separation based on the binding of analytes to positively or negatively charged groups which are fixed on a stationary phase and which are in equilibrium with free counter ions in the mobile phase according to differences in their net surface charge (Figure 1).

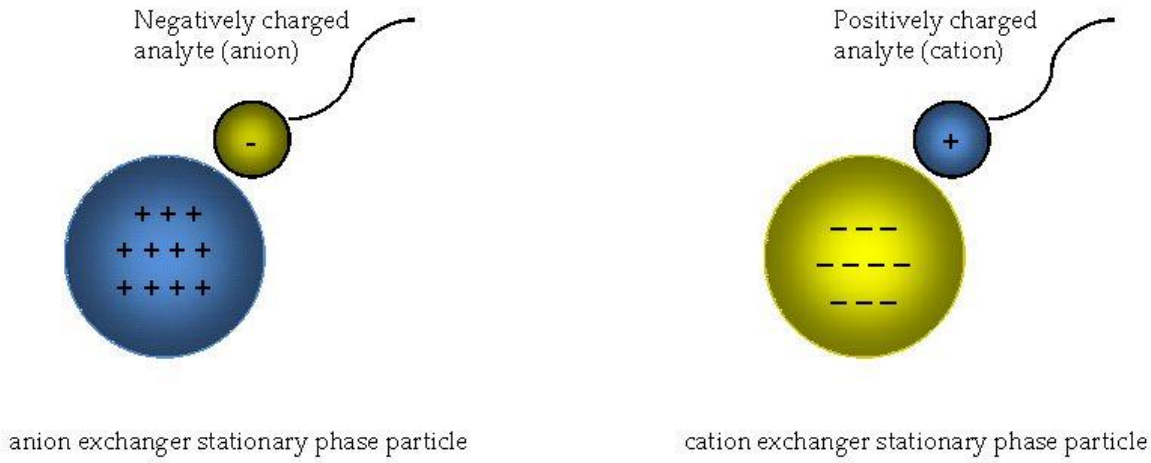


Figure 1.

Types of ion exchangers

Ion exchange chromatography involves separation of ionic and polar analytes using chromatographic supports derivatized with ionic functional groups that have charges opposite that of the analyte ions. The analyte ions and similarly charged ions of the eluent compete to bind to the oppositely charged ionic functional group on the surface of the stationary phase. Assuming that the exchanging ions (analytes and ions in the mobile phase) are cations, the competition can be explained using the following equation;

S-X-C+ + M+↔ S-X-M++ C+S-X-C+ + M+↔ S-X-M++ C+

In this process the cation M+ of the eluent replaced with the analyte cation C+ bound to the anion X- which is fixed on the surface of the chromatographic support (S).

In anion exchange chromatography, the exchanging ions are anions and the equation is represented as follow;

S-X+A- + B-↔ S-X+B-+ A-S-X+A- + B-↔ S-X+B-+ A-

The anion B- of the eluent replaced with the analyte cation A- bound to the positively charged ion X+ on the surface of the stationary phase. The adsorption of the analyte to the stationary phase and desorption by the eluent ions is repeated during their journey in the column, resulting in the separation due to ion-exchange.

Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography support according to differences in their overall charge, charge density and surface charge distribution. Net surface charge of all molecules with ionizable groups is highly pH dependent. Therefore pH of the mobile phase should be selected according to the net charge on a protein of interest within a mixture is opposite to that of matrix functional group, that it will displace the functional group counter ion and bind the matrix. On the other hand oppositely charged proteins will not be retained. Adsorbed protein analytes can be eluted by changing the mobile phase pH which effect the net charge of adsorbed protein, so its matrix binding capacity. Moreover increasing the concentration of a similarly charged species within the mobile phase can be resulted in elution of bound proteins. During ion exchange chromatography for example in anion exchange as illustrated in Figure 2, negatively charged protein analytes can be competitively displaced by the addition of negatively charged ions. The affinity of interaction between the salt ions and the functional groups will eventually exceed that the interaction exists between the protein charges and the functional groups, resulting in protein displacement and elution by increasing gradually the salt concentration in the mobile phase.

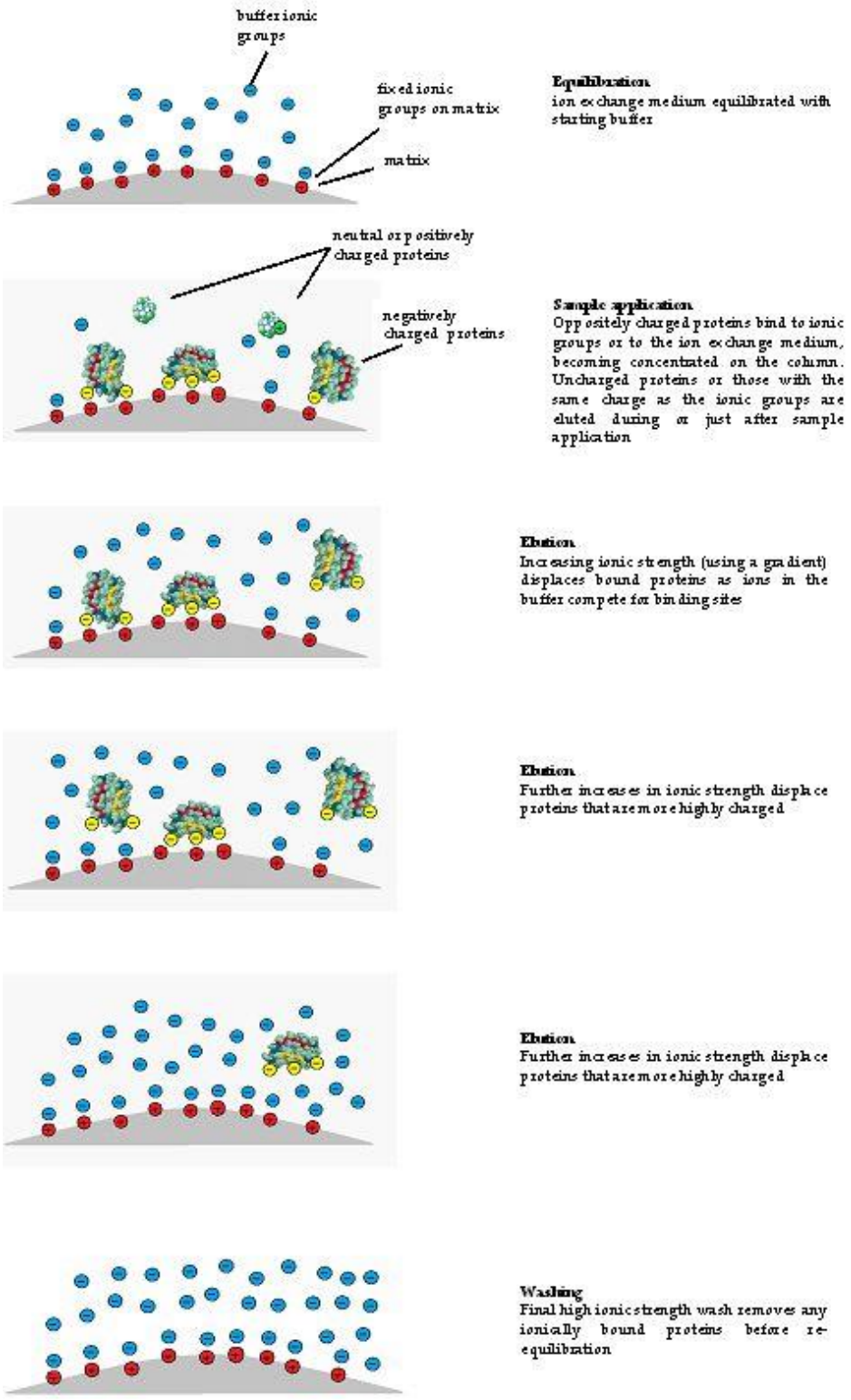


Figure 2. Separation steps in anion exchange chromatography (GE Healthcare)

Complex mixtures of anions or cations can usually be separated and quantitative amounts of each ion measured in a relatively short time by ion exchange chromatography. In classical ion-exchange chromatography separations have been performed in the open-column mode. Column which is loosely packed with stationary phase as small particles made of 1-2 cm diameter glass. The mobile phase or eluent contains the competing ion and is passed continuously into the column and percolates through it under gravity. Sample mixture is applied to the top of the column and allowed to pass into the bed of ion- exchange material. Eluent flow is then resumed and fractions of eluent are collected at regular intervals from the column outlet. Open column ion-exchange chromatography is very slow due to low eluent flow-rates. Increasing flow rate may result in deteriorated separation efficiency (Figure 3). In modern ion-exchange chromatography the usage of high efficiency ion exchange materials combined with flow-through detection have overcome of these challenges. Separations are performed on the column which is filled with ion-exchanger as particles in uniform size. The particles of ion-exchange material are generally very much smaller than those used for classical open column ion-exchange chromatography. However ion-exchange resins used in modern chromatography have lower capacity than older resins. The eluent must be pumped through the column due to the small particle size of stationary phase. The sample mixture is applied into eluent by the injection port. Finally the separated ions are detected with a flow-through detection instrument.

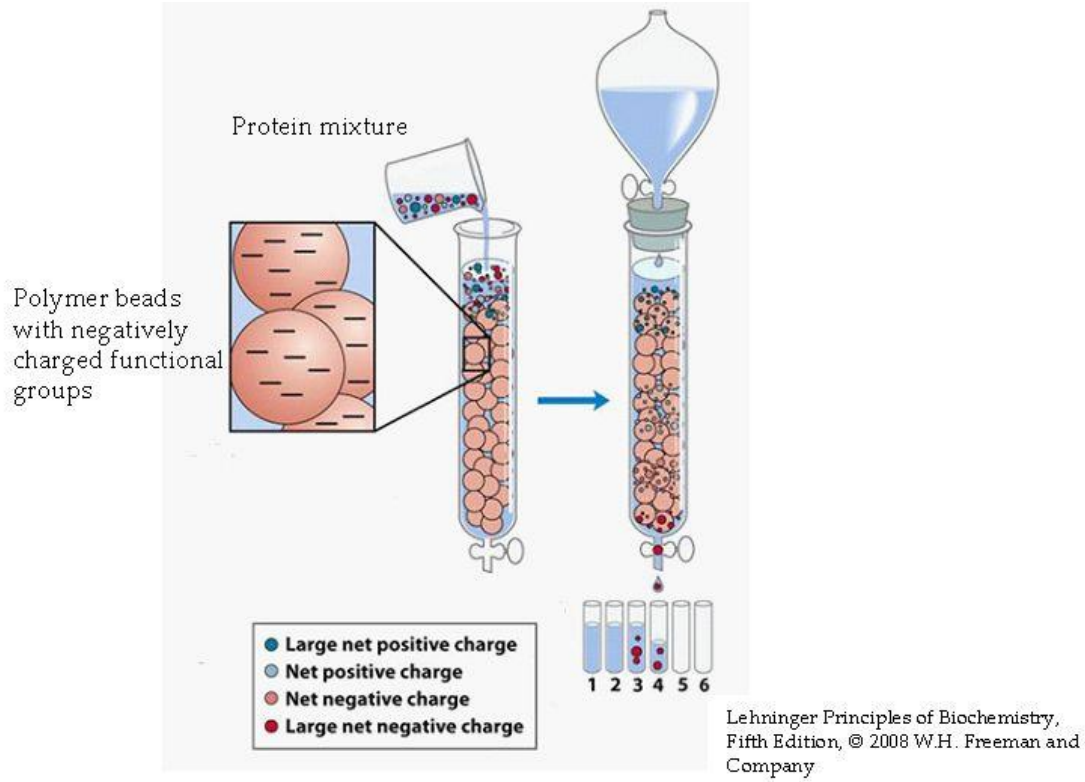


Figure 3.

Ion exchange chromatography technique

This technique has been used for the analyses of anions and cations, including metal ions, mono- and oligosaccharides, alditols and other polyhydroxy compounds, aminoglycosides (antibiotics), amino acids and peptides, organic acids, amines, alcohols, phenols, thiols, nucleotides and nucleosides and other polar molecules. It has been successfully applied to the analysis of raw materials, bulk active ingredients, counter ions, impurities, and degradation products, excipients, diluents and at different stages of the production process as well as for the analysis of production equipment cleaning solutions, waste streams, container compatibility and other applications. Wide applicability including high performance and high-throughput application formats, average cost, powerful resolving ability, large sample handling capacity and ease of scale-up as well as automation allow the ion exchange chromatography has become one of the most important and extensively used of all liquid chromatographic technique.

Although the extensive use of ion exchange chromatography the mechanism of the separation has not completely been elucidated. A considerable effort has been made to describe the ion exchange process theoretically. One of the important disadvantages of this technique is that this method provides no direct information on events occurring at the surface of the stationary phase, because the ion-exchange equilibrium is always determined by the balance between the solute interaction and the eluent interaction with the active sites of resin. Ion exchange is similar to sorption, since in both cases a solid takes up dissolved sample. The most important difference between them is in stoichiometric nature of ion exchange. Each ion removed from the solution is replaced by an equivalent amount of another ion of the same charge, while a solute is usually taken-up non-stoichiometrically without being replaced in sorption . Stoichiometric displacement based on the mass action law and describes the retention of a solute ion as an exchange process with the counter ion bound to the surface. According to this model, the retention of a protein under isocratic, linear conditions is related to counter ion concentration and can be represented by equilibrium as follow;

$\log k = -(Zp/Zs) \log Cm + \log(\varphi Q) \log k = -(Zp/Zs) \log Cm + \log(\varphi Q)$

k is the retention factor and Cm is the concentration of the counter ion in the mobile phase. Zp/Zs (= Z) is the ratio of the characteristic charge of the protein to the value of the counter ion and presents a statistical average of the electrostatic interactions of the protein with the stationary phase as it migrates through the column. The behavior of ion exchange chromatographic system can be explained by stoichiometric models. However, the mechanism of the ion exchange separation is more complex and stiochiometric consideration is inapplicable to long-range mechanisms, such as electrostatic interactions due to the distribution of ions in solution is also influenced by the electrostatic potential. Other interactions between solute-solute, solute-solvent and solvent-solvent also contribute to retention and selectivity in ion exchange. For example ion-dipole and dispersion interaction, should be included as important mechanisms. Additionally entropic contribution originating from solvent, such as water, structures around ion exchange sites should also be regarded as important. In addition to these the primary separation mechanism is the electrostatic interaction between ion-exchange sites and counter ions in ion exchange chromatography.

An important feature differentiating the ion exchange resins from other types of gels is the presence of functional groups. The groups are attached to the matrix. The ion exchange process between the ions in the solution takes place on these functional groups. The exchange of ions between the ion exchange resin and the solution is governed by two principles:

The process is reversible, only rare exceptions are known

The exchange reactions take place on the basis of equivalency in accordance with the principle of electro neutrality. The number of milimoles of an ion sorbed by an exchange should correspond to the number of milimoles of an equally charged ion that has been released from the ion exchange.

Equilibrium is established for each sample component between the eluent and stationary phases when a sample is introduced into the ion-exchange chromatography. The distribution of component (A) between the two phases is expressed by the distribution coefficient, “DA”.

DA= [A]r/ [A]mDA= [A]r/ [A]m

The value of DA is dependent on the size of the population of molecules of component A in the stationary and eluent phases. As the equilibrium is dynamic, there is a continual, rapid interchange of molecules of component A between the two phases. The fraction of time, fm, that an average molecule of A spends in the mobile phase is given by:

fm = Amount of A in the mobile phase / Total amount of A

fm = [A]m Vm / [A]m Vm + [A]r w

= 1/ 1+ DA (w / Vm)

k' = DA (W / Vm)

fm = 1 / 1 + k’

w: Weight of the stationary phase

Vm: Volume of the mobile phase

The mechanism of the anion and cation exchange are very similar. When analytes enter to the ion exchange column, firstly they bind to the oppositely charged ionic sites on the stationary phase through the Coulombic attraction. In accordance with Coulomb's law, the interactions between ions in the solute and oppositely charged ligands on the matrix in ion-exchange chromatography are due to the electrostatic forces. Coulomb's law is given by the equation as follow;

$f = q1q2/\varepsilon r2 f = q1q2/\varepsilon r2$

f: Interaction electrostatic force

q1q2: The charge on ions

: Dielectric constant of the medium

r: The distance between charges.

If the charges on both ions are same (both are positive or negative) the force is repulsive, if they are different (one positive and the other negative) the force is attractive. When the ion charge of the species increase (Divalent ion should interact more strongly than a monovalent ion) and when the dielectric constant decrease (Two oppositely charged molecules increased more strongly in an organic solvent than in water), the interactions increase. On the other hand the distance between the charges increases the interactions decrease. Additionally, other interactions, especially, van der Waals forces participate to the Coulombic forces.

Ion exchange chromatography, which is also known as adsorption chromatography, is a useful and popular method due to its;

high capacity,

high resolving power,

mild separation conditions,

versatility and widespeared applicability,

tendency to concentrate the sample

relatively low cost.

General components of an ion-exchange chromatography are presented as below (Figure 4).

A high pressure pump with pressure and flow indicator, to deliver the eluent

An injector for introducing the sample into the eluent stream and onto the column

A column, to separate the sample mixture into the individual components

An oven, optional

A detector, to measure the analyte peaks as eluent from the column

A data system for collecting and organizing the chromatograms and data

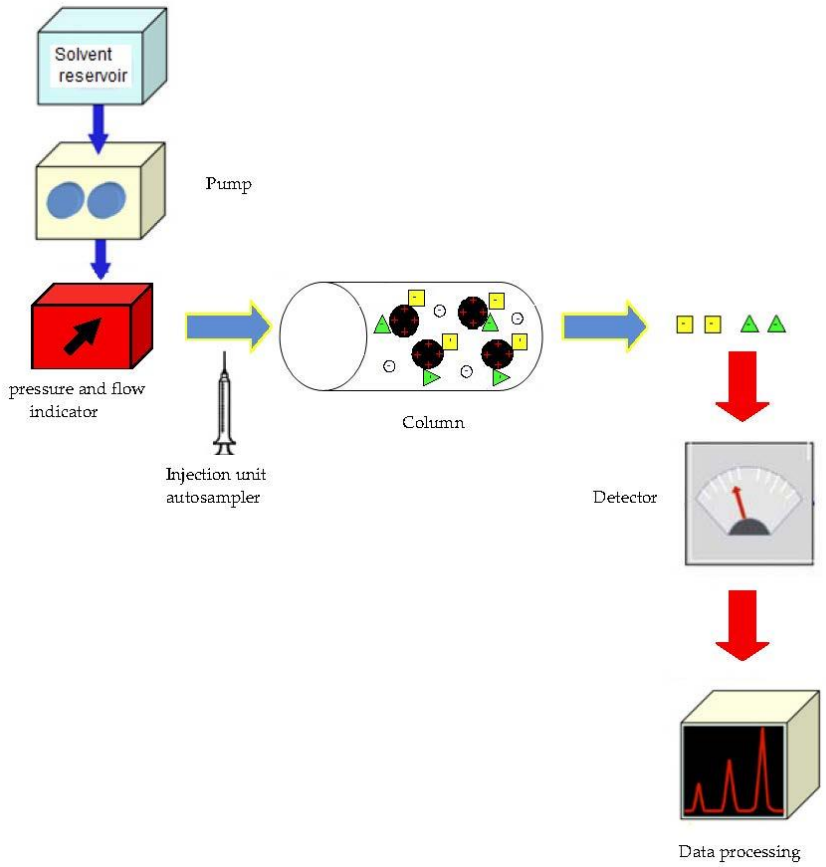


Figure 4. Ion-exchange Chromatography System

In ion-exchange chromatography, adsorption and desorption processes are determined by the properties of the three interacting entities;

The stationary phase,

The constituents of the mobile phase

The solute.

Stationary phase

Selection of a suitable ion-exchange matrix probably is the most important in ion exchange protocol and is based on various factors such as; ion exchanger charge/strength, linear flow rate/sample volume and sample properties. In ion-exchange chromatography, numerous stationary phases are available from different manufacturers, which vary significantly in a number of chemical and physical properties. Stationary phases comprised of two structural elements; the charged groups which are involved in the exchange process and the matrix on which the charged groups are fixed. Ion exchangers are characterized both by the nature of the ionic species comprising the fixed ion and by the nature of the insoluble ion-exchange matrix itself.

Ion exchangers are called cation exchangers if they have negatively charged functional groups and possess exchangeable cations. Anion exchangers carry anions because of the positive charge of their fixed groups. The charged groups determine the specificity and strength of protein binding by their polarity and density while the matrix determines the physical and chemical stability and the flow characteristics of the stationary phase and may be responsible for unspecific binding effects.

General structure (fibrous or beaded form), particle size and variation, pore structures and dimensions, surface chemistry (hydrophilic or hydrophobic), swelling characteristics of matrix are important factors which effect chromatographic resolution. Porosity of ion exchange beads can be categorized as non-porous, microporous and macroporous. (Figure 5 and Figure 6). High porosity offers a large surface area covered by charged groups and so provides a high binding capacity. However when compared with beaded matrix fibrous ion exchangers based on cellulose

exhibit lower chromatographic resolution. On the other hand high porosity is an advantage when separating large molecules and prefractionation. Non-porous matrices are preferable for high resolution separations when diffusion effects must be avoided. Micropores increase the binding capacity but cause to a band broadening. Another disadvantage of microporous beads is that protein can bind to the surface of the beads near to the pores, so penetration of proteins into the pores can prevent or slow down. These problems are overcome by using macroporous particles with pore diameters of about 600-800 nm which are introduced recently. These kinds of particles behave differently compared to microporous materials with respect to microflow characteristics the new term perfusion chromatography has been created.

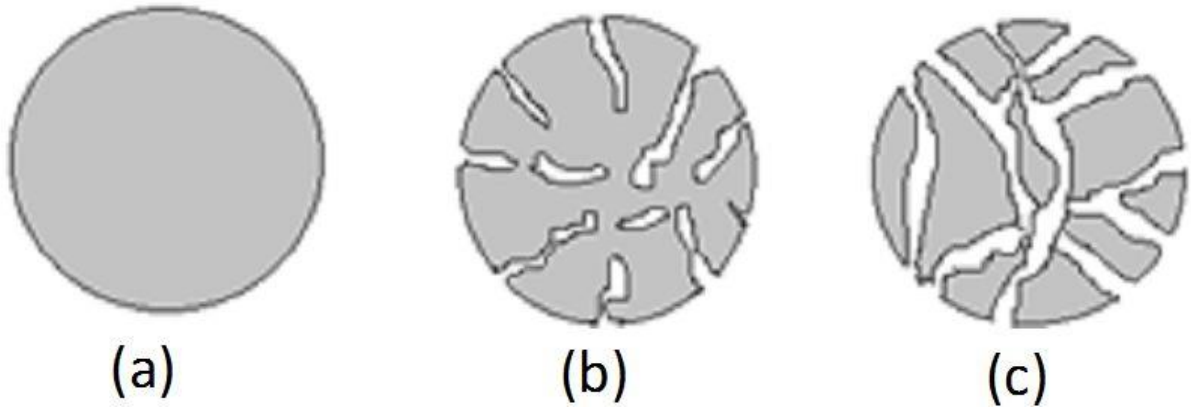


Figure 5. Schematic presentation of different matrix types (a) non-porous beads (b) microporous beads (c) macroporous beads

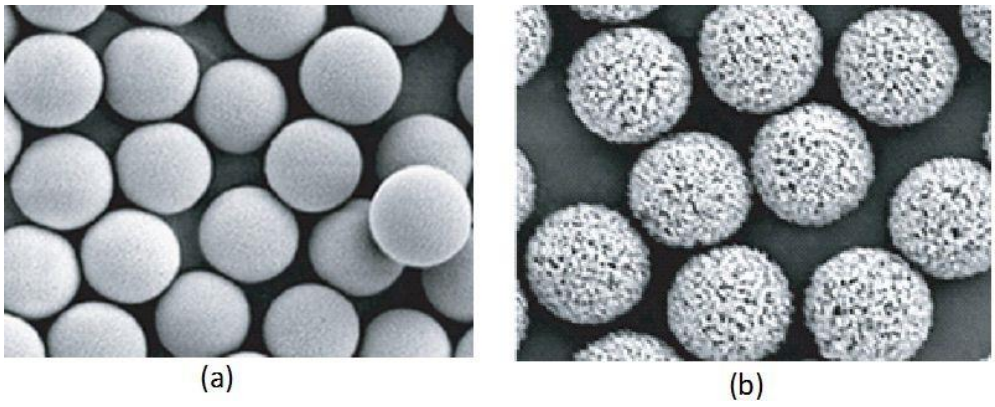


Figure 6. (a) Non-porous beads (b) Porous beads

Furthermore a new matrix type which has been recently introduced is based on a completely new principle and exhibits improved chromatographic features when compared with conventional ion exchangers. This matrix which is known as continuous bed does not consist of ion exchange beads or fibers. The matrix is synthesized in the column by polymerization and established from continuous porous support consisting of a nodule chains (Figure 7). The advantages of that matrix are mainly due to the more homogeneous mobile phase flow and short diffusion distances for the proteins. This is explained by the non-beaded form and the unique pore structure of the support.

Size, size distribution and porosity of the matrix particles are the main factors which affect the flow characteristics and chromatographic resolution. Small particles improved chromatographic resolution. Stationary phases with particle of uniform size are superior to heterogenous materials with respect to resolution and attainable flow rates. The pore size of ion exchange bead directly effect the binding capacity for a particular protein dependent on the molecular weight of the protein because it determines the access of proteins to the interior of the beads. Binding of large proteins can be restricted to the bead surface only so that the total binding capacity of the ion exchanger is not exploited Pore diameter of 30 nm is optimal for proteins up to a molecular weight of about 200.000 Da.

Figure 7. Continuous bed matrix

In order to minimize non-specific interactions with sample components inert matrix should be used. High physical stability provides that the volume of the packed medium remains constant despite extreme changes in salt concentration or pH for improving reproducibility and avoiding the need to repack columns. High physical stability and uniformity of particle size facilitate high flow rates, particularly during cleaning or re-equilibration steps, to improve throughput and productivity. There are pH and pressure limits for each stationary phases. For example pH values higher than 8 should not used in silica based materials which are not coated with organic materials. Matrix stability also should be considered when the chemicals such as organic solvents or oxidizing agents should be required to use or when they are chosen for column cleaning.

Matrices which are obtained by polymerization of polystyrene with varying amounts of divinylbenzene are known as the original matrices for ion exchange chromatography. However these matrices have very hydrophobic surface and proteins are irreversibly damaged due to strong binding. Ion exchangers which are based on cellulose with hydrophilic backbones are more suitable matrices for protein separations. Other ion exchange matrices with hydrophilic properties are based on agarose or dextran.

Several matrix types and their important properties can be listed as follow;

Matrix materials;

Cellulose; Hydrophilic surface, enhanced stability by cross-linking, inexpensive

Dextran; Considerable swelling as a function of ionic milieu, improved materials by cross-linking)

Agarose; Swelling is almost independent of ionic strength and pH, high binding capacity obtained by production of highly porous particles

Polyacrylamide; Swelling behavior similar to dextran

Acrylate-copolymer; High pH stability

Polystyrene-divinilybenzene; Hydrophobic surface, low binding capacity for proteins

Coated polystyrene-divinilybenzene; Hydrophilic surface

Silica; Unstable at pH > 8, rigid particles

Coated Silica; Hydrophilic surface

In addition to electrostatic interactions between stationary phase and proteins, some further mechanisms such as hydrophobic interactions, hydrogen bonding may contribute to protein binding. Hydrophobic interactions especially occur with synthetic resin ion exchangers such as which are produced by copolymerization of styrene and divinylbenzene. These materials are not usually used for separation of proteins. However new ion exchange materials that consist of styrene-divinylbenzene copolymer beads coated with hydrophilic ion exchanger film were introduced. According to the retention behavior of some proteins, it is considered that coating of the beads so efficient that unspecific binding due to hydrophobic interactions cannot be observed. Silica particles have also been coated with hydrophilic matrix. Acrylic acid polymers are also used for the protein separation in ion exchange chromatography. These polymers are especially suitable for purification of basic proteins.

The functional groups substituted onto a chromatographic matrix determine the charge of an ion exchange medium; positively-charged anion exchanger or a negatively-charged cation exchanger. Both exchangers can be further classified as strong and weak type as shown in Table 1. The terms weak and strong are not related to the binding strength of a protein to the ion exchanger but describe the degree of its ionization as a function of pH. Strong ion exchangers are completely ionized over a wide pH range, while weak ion exchangers are only partially ionized a narrow pH range. Therefore with strong ion exchangers proteins can adsorb to several exchanger sites. For this reason strong ion exchangers are generally used for initial development and optimization of purification protocols. On the other hand weak ion exchangers are more flexible in terms of selectivity and are a more general option for the separation of proteins that retain their functionality over the pH 6-9 range as well as for unstable proteins that may require mild elution conditions. Alkylated amino groups for anion exchangers and carboxy, sulfo as well as phosphato groups for cation exchangers are the most common functional groups used on ion exchange chromatography supports. Sulfonic acid exchangers are known as strong acid type cation exchangers. Quaternary amine functional groups are the strong base exchangers whereas less substituted amines known as weak base exchangers. Number and kind of the substituents are

determined the basicity of amino-groups. Immobilized tertiary and quaternary amines proved to be useful for ion exchange chromatography. Immobilized diethylaminoethyl and carboxymethyl groups are the most widely used ion exchangers.

The ion exchange capacity of an ion-exchanger is determined by the number of functional groups per unit weight of the resin. The total ionic capacity is the number of charged functional groups per ml medium, a fixed parameter of each medium and can be given as mval/ml for small ions. Density and accessibility of these charged groups both on the surface and within the pores define the total binding capacity. Ionic medium and the presence of other proteins if a particular protein is considered also affect the binding capacity. However, under defined conditions, the amount of the certain protein which is bound to ion exchanger is more suitable parameter for determining and comparing the capacity of ion exchange chromatography. Albumin for anion exchangers and hemoglobin for cation exchangers is usually used for this purpose. Determination of the binding capacity before the experiment is generally recommended because the capacity for a particular protein depends on its size and also on the sample composition. The binding capacity of a column can be increased for proteins which are retained on the column at high salt concentrations. The salt concentration is adjusted to a suitable concentration in which the protein of interest tightly bound to the ion exchanger while others which have lower affinity pass through the column without occupying binding sites.

Exchange Type	Ion exchange group	Buffer counter ions	pH range	Commercial samples
Strong cation	Sulfonic acid (SP)	Na+, H+, Li+	4-13	Capto®S
				SP Sepharose®
				SP Sephadex®
				TSKgel SP_5PW
Weak cation	Carboxylic acid	Na+, H+, Li+	6-10	CM Cellulose
				CM Sepharose®
				CM Sephadex®
				CM Sepharose® CL6B

Exchange Type	Ion exchange group	Buffer counter ions	pH range	Commercial samples
				TSKgel CM-5PW
Strong anion	Quaternary amine (Q)	Cl-, HCOO3 -, CH3COO-, SO4 2-	2-12	Q Sepharose®
				Capto®Q
				Dowex®1X2
				Amberlite® / Amberjet®
				QAE Sephadex®
Weak anion	Primary amine Secondary amine Tertiary amine (DEAE)	Cl-, HCOO3 -, CH3COO-, SO4 2-	2-9	DEAE-Sepharose®
				Capto® DEAE
				DEAE Cellulose

Table 1.

Weak and Strong type anion and cation exchangers

Mobile phase (Eluent)

In ion exchange chromatography generally eluents which consist of an aqueous solution of a suitable salt or mixtures of salts with a small percentage of an organic solvent are used in which most of the ionic compounds are dissolved better than in others in. Therefore the application of various samples is much easier. Sodium chloride is probably the most widely used and mild eluent for protein separation due to has no important effect on protein structure. However NaCl is not always the best eluent for protein separation. Retention times, peak widths of eluted protein, so chromatographic resolution are affected by the nature of anions and cations used. These effects can be observed more clearly with anion exchangers as compared to cation exchangers. The salt mixture can itself be a buffer or a separate buffer can be added to the eluent if required. The competing ion which has the function of eluting sample components through the column within reasonable time is the essential component of eluting sample. Nature and concentration of the competing ions and pH of the eluent are the most important properties affecting the elution characteristics of solute ions.

The eluent pH has considerable effects on the functional group which exist on the ion exchange matrix and also on the forms of both eluent and solute ions. The selectivity coefficient existing between the competing ion and a particular solute ion will determine the degree of that which competing ion can displace the solute ion from the stationary phase. As different competing ions will have different selectivity coefficients, it follows that the nature of competing ion will be an important factor in determining whether solute ions will be eluted readily. The concentration of competing ion exerts a significant effect by influencing the position of the equilibrium point for ion-exchange equilibrium. The higher concentration of the competing ion in the eluent is more effectively displace solute ions from the stationary phase, therefore solute is eluted more rapidly from the column. Additionally elution of the solute is influenced by the eluent flow-rate and the temperature. Faster flow rates cause to lower elution volumes because the solute ions have less opportunity to interact with the fixed ions. Temperature has relatively less impact, which can be change according to ion exchange material type. Enhancement of the temperature increases the rate of diffusion within the ion-exchange matrix, generally leading to increased interaction with the fixed ions and therefore larger elution volumes. At higher temperatures chromatographic efficiency is usually improved.

Eluent degassing is important due to trap in the check valve causing the prime loose of pump. Loss of prime results in erratic eluent flow or no flow at all. Sometimes only one pump head will lose its prime and the pressure will fluctuate in rhythm with the pump stroke. Another reason for removing dissolved air from the eluent is because air can get result in changes in the effective concentration of the eluent. Carbon dioxide from air dissolved in water forms of carbonic acid. Carbonic acid can change the effective concentration of a basic eluent including solutions of sodium hydroxide, bicarbonate and carbonate. Usually degassed water is used to prepare eluents and efforts should be made to keep exposure of eluent to air to a minimum after preparation. Modern inline degassers are becoming quite popular.

For separation the eluent is pumped through the system until equilibrium is reached, as evidenced by a stable baseline. The time required for equilibrium may vary from a couple of minutes to an hour or longer, depending on the type of resin and eluent used. Before the sample injection to the column should be equilibrated with eluent to cover all the exchange sites on the stationary phase

with the same counter ion. When the column is equilibrated with a solution of competing ion, counter ions associated with the fixed ions being completely replaced with competing ions. In this condition the competing ions become the new counter ions at the ion exchange sites and the column is in the form of that particular ion.

Isocratic elution or gradient elution can be applied for elution procedure. A single buffer is used throughout the entire separation in isocratic elution. Sample components are loosely adsorbed to the column matrix. As each protein will have different distribution coefficient separation will achieved by its relative speeds of migration over the column. Therefore in order to obtain optimum resolution of sample components, a small sample volume and long exchanger column are necessary. This technique is time consuming and the desired protein invariably elutes in a large volume. However no gradient-forming apparatus is required and the column regeneration is needless. Alteration in the eluent composition is needed to achieve desorption of desired protein completely. To promote desired protein desorption continuous or stepwise variations in the ionic strength and/or pH of the eluent are provided with gradient elution. Continuous gradients generally give better resolution than stepwise gradients.

Additives which are protective agents found in the mobile phase are generally used for maintain structure and function of the proteins to be purified. This is achieved by stimulating an adequate microenvironment protection against oxidation or against enzymatic attacks. Any additives used in ion exchange chromatography, should be checked for their charge properties at the working pH in order to avoid undesired effects due to adsorption and desorption processes during chromatography. It is recommended to include in the elution buffer those additives in a suitable concentration which have been used for stabilization and solubilization of the sample. Otherwise precipitation may occur on the column during elution]. For example; zwitterionic additives such as betaine can prevent precipitation and can be used at high concentrations without interfering with the gradient elution. Detergents are generally useful for solubilization of proteins with low aqueous solubility. Anionic, cationic, zwitterionic and non-ionic (neutral) detergents can be used during ion exchange chromatography. Guanidine hydrochloride or urea, known as denaturing agents can be used for initial solubilization of a sample and during separation. However, they

should use if there is a requirement. Guanidine is a charged molecule and therefore can participate to the ion exchange process in the same way as NaCl during separation process.

Commonly used eluent additives which have been successfully used in ion exchange chromatography can be given as follow;

EDTA; Ethylenediamine tetraacetic acid

Polyols; Glycerol, glucose, and saccharose

Detergents;

Urea and guanidinium chloride

Lipids

Organic solvents

Zwitterions

Sulfhydryl reagents

Ligands

Protease inhibitors

Buffer

In ion exchange chromatography, pH value is an important parameter for separation and can be controlled and adjusted carefully by means of buffer substances. In order to prevent variation in matrix and protein net charge, maintenance of a constant mobile phase pH during separation is essential to avoid pH changing which can occur when both protein and exchanger ions are released into the mobile phase. By means of buffer substances pH value can be controlled and adjusted. Concentration of H+ and the buffering component influence the protein binding to the stationary phase, chromatographic resolution and structural as well as functional integrity of the protein to be separated. Thus a suitable pH range, in which the stability of sample is guaranteed, has to be identified. Keeping of the sample function is related with the preservation of its three dimensional structure as well as with its biological activity. A number of buffers are suitable for

ion-exchange chromatography. A number of important factors influences the selection of mobile phase including buffer charge, buffer strength and buffer pH. Properties of good buffers are high buffering capacity at the working pH, high solubility, high purity and low cost. The buffer salt should also provide a high buffering capacity without contributing much to the conductivity and should not interact with the ion exchanger functional groups as well as with media. The buffering component should not interact with the ion exchanger because otherwise local pH shifts can occur during the exchange process which may interfere the elution. Interactions with stationary phase as well as with additives of the mobile phase and with subsequent procedures may be occur with buffer component and selected pH range. Precipitation of the mobile phase components can be observed for example when phosphate buffer and several di- and trivalent metal ions such as Mg^{+2} and Ca^{+2} are mixed or when anionic detergents (i.e. cholate) are used under acidic conditions or in the presence of multivalent metal ions. Precipitation of metal oxides and hydroxides can occur under alkaline conditions. Buffer components may also affect enzymatic assays used for screening and analysis of chromatography fractions. The concentration of buffer salts usually ranges from 10 to 50 mM. Commonly used buffers are presented in Table 2 and Table 3 for cation and anion exchange chromatography.

Generally, applications of ıon exchange chromatography are performed under slightly acidic or alkali conditions, pH range 6.0-8.5 but there are also more acidic and more alkali buffers. Additionally the buffering component should not act as an eluting ion by binding to the ion exchanger. Anionic buffer component such as phosphate or MOPS in cation exchange chromatography and cationic buffers such as ethanolamine, Tris and Tricine in anion exchange chromatography are recommended. Besides interactions of buffer component with stationary phase, there are also possible interactions with additives of the mobile phase. To achieve sufficient buffer capacity the pKa of the buffer component should be as close to the desired pH value as possible difference no more than ± 0.5 pH units. However there are examples of successful separations at which the buffering capacity is very low. It has to be considered that the pKa is a temperature dependent value. Performing on ion exchange separation with the same elution buffer at room temperature or in the cold room can have a remarkable effect on the buffer

capacity. For optimal binding of a sample ion to an ion-exchanger the ionic strength and thus also the buffer concentrations has to be low in sample and equilibration buffers.

Substance	pKa	Working pH
Citric acid	3.1	2.6-3.6
Lactic acid	3.8	3.4-4.3
Acetic acid	4.74	4.3-5.2
2-(N-morpholino)ethanesulfonic acid	6.1	5.6-6.6
N-(2-acetamido)-2-iminodiacetic acid	6.6	6.1-7.1
3-(N-morpholino)propanesulfonic acid	7.2	6.7-7.7
Phosphate	7.2	6.8-7.6
N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)	7.5	7.0-8.0
N,N-bis(2-hydroxyethyl)glycine	8.3	7.6-9.0

Table 2. Commonly used buffers for cation-exchange chromatography

Substance	pKa	Working pH
N-Methyl-piperazine	4.75	4.25-5.25
Piperazine	5.68	5.2-6.2
Bis-Tris	6.5	6.0-7.0
Bis-Tris propane	6.8	6.3-7.3
Triethanolamine	7.8	7.25-8.25
Tris	8.1	7.6-8.6
N-Methyl-diethanolamine	8.5	8.0-9.0
Diethanolamine	8.9	8.4-9.4
Ethanolamine	9.5	9.0-10.0
1,3-Diaminopropane	10.5	10.0-11.0

Table 3.Commonly used buffers for anion-exchange chromatography

Detection

Conductivity detector is the most common and useful detector in ion exchange chromatography. However UV and other detectors can also be useful. Conductivity detection gives excellent sensitivity when the conductance of the eluted solute ion is measured in an eluent of low background conductance. Therefore when conductivity detection is used dilute eluents should be preferred and in order for such eluents, to act as effective competing ions, the ion exchange capacity of the column should be low.

Although recorders and integrators are used in some older systems, generally in modern ion exchange chromatography results are stored in computer. Retention time and peak areas are the most useful information. Retention times are used to confirm the identity of the unknown peak by comparison with a standard. In order to calculate analyte concentration peak areas are compared with the standards which is in known concentration.

Direct detection of anions is possible, providing a detector is available that responds to some property of the sample ions. For example anions that absorb in the UV spectral region can be detected spectrophotometrically. In this case, an eluent anion is selected that does not absorb UV. The eluent used in anion chromatography contains an eluent anion, E-. Anions with little or no absorbance in the UV spectral region can be detected spectrophotometrically by choosing a strongly absorbing eluent anion. An anion with benzene ring would be suitable. Usually Na+ or H+ will be the cation associated with E-. The eluent anion must be compatible with the detection method used. For conductivity the detection E should have either a significantly lower conductivity than the sample ions or be capable of being converted to a non-ionic form by a chemical suppression system. When a spectrophotometric detection is employed, E will often be chosen for its ability to absorb strongly in the UV or visible spectral region. The concentration of E- in the eluent will depend on the properties of the ion exchanger used and on the types of anions to be separated.

Affinity chromatography

Introduction

Affinity chromatography which is known as a liquid chromatographic technique for separation and analysis of biomolecules based on their biological functions or individual structures has become increasingly important and useful separation method in pharmaceutical science, biochemistry, biotechnology and environmental science in recent years. This technique is especially known as the most specific and effective technique for protein purification. Separation of the biomolecules is based on highly specific biological interactions between two molecules, such as enzyme and substrate. These interactions, which are typically reversible, are used for purification by placing one of the interacting molecules, referred to as affinity ligand, onto a solid matrix to create a stationary phase while the target molecule is in the mobile phase [3]. Any component can be used as a ligand to purify its respective binding partner. Some typical biological interactions, frequently used in affinity chromatography, can be given as;

Enzyme. ↔↔ substrate analogue, inhibitor, cofactor.

Antibody ↔↔. antigen, virus, cell.

Lectin ↔↔. polysaccharide, glycoprotein, cell surface receptor, cell.

Nucleic acid ↔↔. complementary base sequence, histones, nucleic acid polymerase, nucleic acid binding protein.

Hormone, vitamin ↔↔. receptor, carrier protein.

Glutathione ↔↔. glutathione-S-transferase or GST fusion proteins.

Metal ions ↔↔. Poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surface.

In case a ligand is immobilized on a polymeric carrier, usually by covalent coupling, and filled in a column, it is possible to separate the substances which have affinity to the ligand and the other substances. As the solution containing the biologically active substance applied to the column, the compounds which have no affinity to the insoluble ligand will pass through the

column and the biologically active compound will be captured on the column, in favorable conditions. The sorbed compounds can then easily be dissociated by changing the external conditions, such as ionic strength, pH, solvent, temperature etc. or alternatively by using dissociating agents. As a result, it is possible to isolate and purify the analyte or make quantitative analysis with a suitable, immobilized ligand by means of molecular recognition.

Macromolecules such as proteins, polysaccharides, nucleic acids differ only in their physico-chemical properties within the individual groups and their isolation on the basis of these differences is therefore difficult and time consuming. Considerable decreases may occur during their isolation procedure due to denaturation, cleavage, enzymatic hydrolysis, etc. The ability to bind other molecules reversibly is one of the most important properties of these molecules. The formation of specific and reversible complexes of biological macromolecules can serve as basis of their separation, purification and analysis by the affinity chromatography.

Affinity chromatography is one of the oldest forms of liquid chromatography method. The first use of the idea of affinity chromatography may be considered as the isolation of α-amylase by using an insoluble substrate, starch, in 1910 by Starkenstein. Similar studies with starch and amylase were carried out in the 1920s through 1940s by other investigators. In another study polygalacturonase was used as a support and ligand for the adsorption of alginic acid, the purification of pepsin through the use of edestin, a crystalline protein and the isolation of porcine elastase with powdered elastin were also performed. Afterwards Willstatter et al. enriched lipase by selective adsorption onto powdered stearic acid. The majority of the previous studies related purification of the enzymes. However the selective purification of antibodies with biological ligands was also being conducted. In 1920, it was reported that antibodies can recognize and bind substances with a specific structure, "antigens". This principle is firstly used in order to isolate rabbit anti-bovine serum albumin antibodies on a specific immunoadsorbent column consisting bovine serum albumin coupled to diazotized p-aminobenzyl-cellulose. According to this approach, antibodies were isolated using urease and exhibited that these antibodies were proteins.

Separation procedure in affinity chromatography can be simply illustrated as shown in Figure 1. A sample containing the compound of interest is applied to the affinity column in the presence of mobile phase which was prepared in suitable pH, ionic strength and solvent composition for

solute-ligand binding. This solvent which is referred as the application buffer presents the weak mobile phase of an affinity chromatography. While the sample is passing through the column compounds which are complementary to the affinity ligand will bind. However other solutes in the sample will tend to be washed off or eluted from the column as nonretained compounds. After all nonretained components are washed off the column, binding solute or together with ligand as solute-ligand complex are eluted by applying a solvent. This solvent which is referred as elution buffer represents the strong mobile phase for the column. Later all the interested solutes are eluted from the column, then application buffer is applied and the column is allowed to regenerate prior to the next sample application.

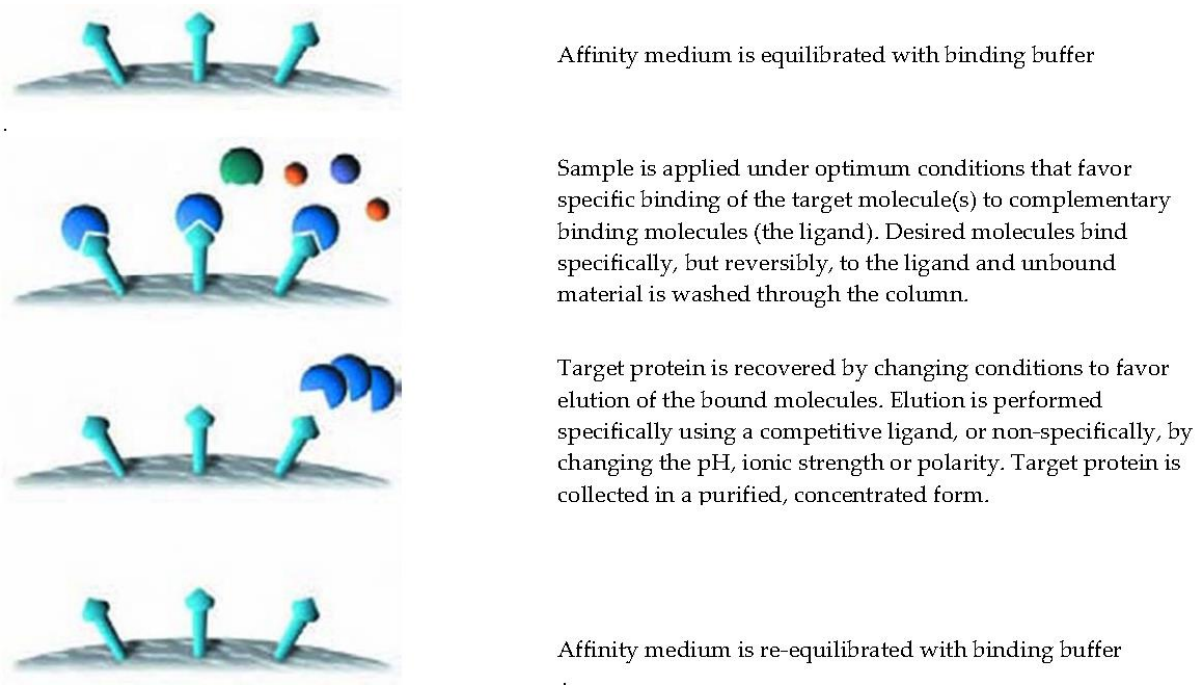


Figure 1. Separation procedure in affinity chromatography

The conditions in which the sample is applied to the column are chosen considering the conditions which the interaction between analyte and ligand is strong, mostly resembling the natural conditions of the analyte and ligand. The content apart from the analyte passes through the column without or with weak binding to the ligand while the analyte is retarded. After the analyte is obtained generally by using an elution buffer, the column is regenerated by washing

with the application buffer in order to prepare the column for the next injection. In the Figure 2, a typical scheme of an affinity chromatography application is shown.

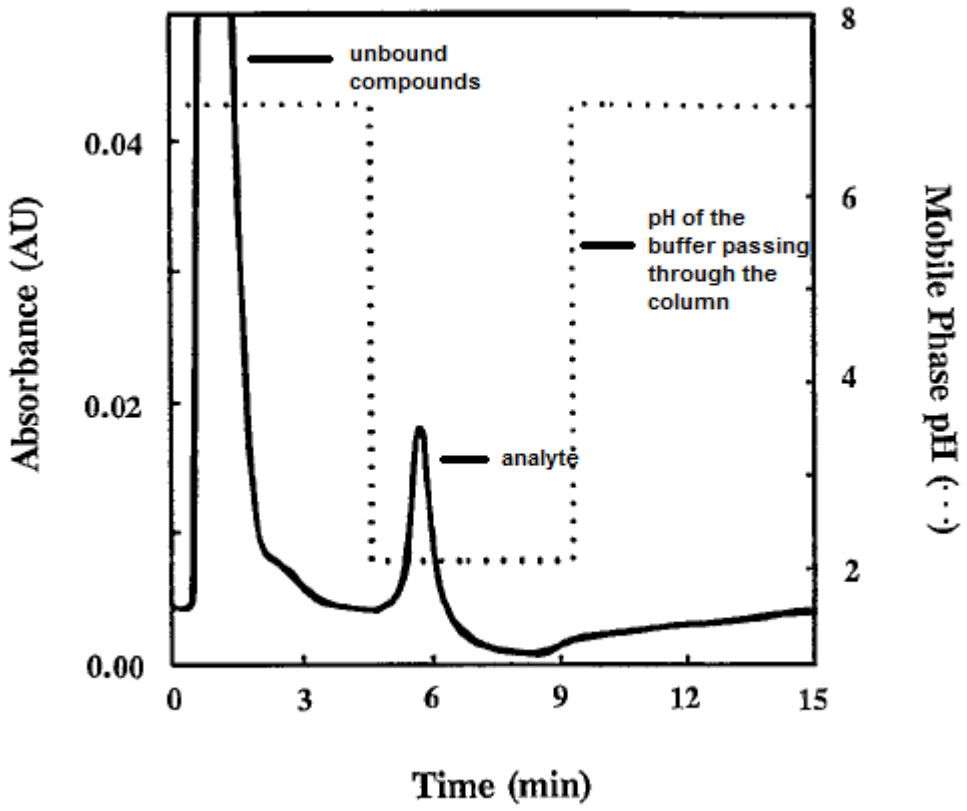


Figure 2. An example of a typical scheme of an affinity chromatography application.

As it is defined above; this technique is based on the interactions between specific bioactive substances, so the ligands are supposed to be originally biological substances, nevertheless columns with nonbiological ligands are also available and the same term “affinity chromatography” is used for the techniques performed by using these ligands. In order to distinguish the techniques according to the origin of the ligand, affinity chromatography with biological ligands may be termed as “bioaffinity chromatography” or “biospesific adsorbtion”. The wide application potential of affinity chromatography leaded to the development of derived techniques some of which are listed below.

Immunoaffinity chromatography

High performance affinity chromatography

Affinity density perturbation

Library-derived affinity ligands

Lectin affinity chromatography

Affinity partitioning

Dye-ligand affinity chromatography

Affinity electrophoresis

Affinity capillay electrophoresis

Centrifuged affinity chromatography

Filter affinity transfer chromatography

Affinity precipitation

Avidin-biotin immobilized system

Affinity tails chromatography

Affinity repulsion chromatography

Perfusion affinity chromatography

Theophilic chromatography

Weak affinity chromatography

Receptor affinity chromatography

Membrane-based affinity chromatography

Molecular imprinting affinity

Metal-chelate affinity chromatography

Covalent affinity chromatography

Hydrophobic chromatography

Affinity chromatography utilizes specific and irreversible biological interactions between a ligand covalently coupled to a support material and its complementary target. The solid support and ligand covalently attached on it, selectively adsorbs the complementary substance from the sample. The unbound part of the sample is removed and the purified substance can easily be

recovered. Selectivity of the ligand, recovery process, throughput, reproducibility, stability and economical criteria are some of the factors that influence the success of affinity chromatography process. Successful affinity purification requires a certain degree of knowledge and understanding of the nature of interactions between the target molecule and the ligand to help determine the selection of an appropriate affinity ligand and purification procedure. Therefore prior to start the process, materials and specifications listed below need to be selected:

Support material

Activation method

Ligand

Immobilization method

Conditions for adsorption and desorption

Support material

For successful separation in affinity chromatography, the important parameter is that solute of interest should be bound firmly and specifically while leaving all other molecules. This requires that the support within the column contain an affinity ligand that is capable of forming a suitably strong complex with the solute of interest. The other important property is that the, support material must be biologically and chemically inert to avoid the unspecific bindings which requires that the support has a chemical character that is very similar to that of the medium in which it is operating. Since almost all affinity separations occur in aqueous solutions, the support should thus be as hydrophilic as possible. As a rule, the mobile phase used in affinity separations has a low ionic strength. The support should therefore contain as few charges as possible to prevent ionic interactions. Many supports are available which have desired properties or they gain such characteristics by hydrophilic coating. Generally solid materials are used as support material though some soluble macromolecular materials are sometimes preferred for two-phase aqueous affinity partition processes. Uniformity in particle size and ease of the activation process are also required for support material that is used in affinity chromatography applications. For the affinity chromatography at low pressure, nonrigid gels with large particle size are generally

used as support materials while materials with smalla, rigid particles or synthetic polymers which are stable under high pressure and flow rates are used in high performance affinity applications.

There are many commercially available support materials for affinity chromatography can be divided into three groups as; natural (agarose, dextrose, cellulose); synthetic (acrylamide, polystyrene, polymethylacrylate) and inorganic (silica, glass) materials. The most popular support material is agarose. Agarose was used in the first modern application of affinity chromatography and still the most commonly preferred one. Agarose consists of alternatively linked 1,3 bound β-D- galactopyranose and 1,4 bound 3,6-anhydro-α-L-galactopyranose, as shown in Figure 3. Agarose gels are stable to eluants with high concentrations of salt, urea, guanidine hydrochloride, detergents or water-miscible organic solvents but its stability is less beyond pH 4-9. To increase the thermal and chemical stability, cross-linked agarose is prepared. Cross-linked agarose is commercially available (Sepharose) and it can be used with many solvents, over pH 3-14 and at high temperatures up to 70°C. However, strong acids, oxidizers as well as some rare enzymes may be harmful to agarose due to their damaging effects. On the other hand mild acid hydrolysis increases the quantity of sterically available galactose residues and turns agarose into an excellent sorbent for galactose binding proteins. Due to its large beads and macroporous, accessible pore structures, agarose is well designed for use with large molecules. High capacity, presence of functional groups, good chemical stability especially at high pH, low non-spesific binding and good reproducibility are the advantage of agarose. Some properties of agarose such as solubility in hot water and non-aqueous solutions, sensitivity to microbial degredation and lack of rigidity restrict the usage under low or medium pressure. Furthermore agarose must not be frozen or air dried. It is sold under several trade names, including Sepharose Fast Flow or Affi-Gel.

Ligand

Ligands are the molecules that bind reversibly to a specific molecule or group of molecules, enabling purification by affinity chromatography. These molecules which play a major role in the specificity and stability of the system are essential for affinity chromatography. The selected ligand must be capable of selectively and reversibly binding to the substance to be isolated and have some groups which are available for modifications in order to be attached to the support

material. It is very important to ensure that the modifications do not reduce the specific binding affinity of the ligands. There are general ligands such as dyes, amino acids, Protein A and G, lectin, coenzyme, methal chelates as well as specific ligands such as enzymes and substrates, antibodies and antigens.

Affinity ligands are classified as synthetic and biological. Biological ligands are obtained from natural sources such as RNA and DNA fragments, nucleotides, coenzymes, vitamins, lectins, antibodies, binding or receptor proteins, or in vitro from biological and genetic packages, employing display techniques including oligonucleotides, peptides, protein domains and proteins. Synthetic affinity ligands are generated either by de novo synthesis or modification of existing molecular structures (triaznyl nucleotide-mimetics, purine and pyrimidine derivatives, non-natural peptides, triazinyl dyes, other triazine-based ligands, oligosaccharide and boronic acid analogues). These can be generated by rational design or selected from ligand libraries. Synthetic ligands are generated using three methods;

The rational method features the functional approach and structural template approach.

The combinatorial method relies on the selection of ligands from a library of synthetic ligands synthesized randomly.

The combined method employs both methods the ligand is selected from an intentionally prepared library based on a rationally designed ligand.

Many parameters have to be taken into account in order to select appropriate ligand. Table 1 exhibits the advantages and disadvantages of synthetic and biological ligands. Selectivity and affinity are the main advantages of biological ligands. Such ligands can be generated by in vitro evolution approaches and selecting from large combinatorial ligand libraries based on biological/genetic packages. Protein ligands display special advantages for example; higher affinities, higher proteolytic stability, preservation of their biological activity when produced by fusion to a different protein or domain. However these ligands can be expensive and unstable to the sterilization and cleanin conditions used in manufacturing process of biologics because of their biological origin, chemical nature and production methods. There is high contamination risk

of the end-product with potentially dangerous leaches, in addition to possible contaminants originated from the biological source.

	Synthetic ligands	Biological ligands
Capacity	High	Low to medium
Cost	Low to medium	Medium to high
Selectivity	Medium to high	Very high
Stability	High	Low to medium
Toxicity	Medium	Low

Table 1.

Comparison of biological and synthetic ligands [20]

Despite the advantages of the affinity chromatography technique, its use is limited due to high cost of affinity ligands and their biological and chemical instability. The development of methods for production of stable synthetic ligands has enabled “utilization of these materials in large scale. For the design of synthetic ligands, information about structure of the target protein and a potential binding site are required, thus a structure-based design can be achieved, in case correct prediction of the ligand’s comformation and the binding affinity of the designed ligand. Function-based design can be applied when the structure of the target is not known. Substantially, selection and design of ligands may be performed by using a template which is a part of a natural protein-ligand couple, modelling a molecule which complements the binding sites of the target or directly resembling the natural interactions.

High selectivity of the biological ligands is a benefit; however these ligands have some handicaps, such as their low binding capacity, cost-efficacy issues, some problems in scale-up and purification process. Hence, synthetic ligands may offer a solution for these issues and enable to provide selectivity, efficacy and inexpensiveness in a body. Biomimetic textile dyes which are developed in 1970s are the most known synthetic ligands. The use of these dyes in biopharmaceutical field is limited due to some issues such as selectivity, purity and toxicity.

These complications have led to new researches and developments about biomimetic dyes and new ligand design techniques.

The selection of the ligand may be done according to the specific binding site of a target, but this manner of selection may fail owing to the fact that immobilization process may change binding affinity. It is known that the affinity of the target to the ligand is dependent on the features of the target as well as support material, activation and coupling chemistry. Some other techniques other than using free ligand solution in order to predict the conditions of three-dimensional matrix. On the purpose of ligand selection, a great number of alternatives may be tested for binding the target or work with more accurate options by employing ligand design techniques. Therefore the idea to combine chemistry with computational tools has accelerated the developments on this field. Along the development of affinity chromatography techniques, different laboratories are established with the purpose of collection of several ligands for affinity chromatography.

Protein-structure-based design of the ligands depends on the correct prediction of the structure of the target protein and the binding site. Apart from this, protein-function-based design is applicable in case the conformation of the target protein is not known. This method is based on the integration of some known properties of the ligand such as an essential molecular structure, a functional group or a derivative of some parts of the structure. The design of a ligand requires several steps to fulfil:

Determination of the binding site or possible biological interactions to use as a template for the modelling,

Initial design of the ligand using this template,

Preparation of a ligand library and chromatographic evaluation,

Selection of the ligand of interest,

Optimisation and chromatographic evaluotion of the adsorbent following the immobilization.

Beyond these design methods, some combinatorial approaches have been developed on the purpose of ligand selection. Synthetic peptide libraries which include all sequences for a length of a protein structure are one of these approaches. By means of these libraries, in vitro prediction

of the action of the library mixture as it passes through the surface where the protein of concern is immobilized is possible.

Immobilization

The immobilized ligand is an essential factor that determines the success of an affinity chromatographic method. The method which is used for affinity ligand immobilization is important because actual or apparent activity of the final column can be affected. Decrease in ligand activity which result in multisite attachment, inappropriate orientation or steric hindrance can be observed if the correct procedure is not pursued. Several methods are available to couple a ligand to a pre-activated matrix. The correct selection of coupling method depends on the ligand characteristics.

Before ligands are coupled matrix is activated. Among several methods used for activation, the cyanogen bromide activation is the most frequently preferred. Activation using this method produces a highly reactive cyanate ester. The ligands are attached to the support via primary aromatic or aliphatic amino groups. High toxicity of cyanogen bromide is the disadvantage of this method. Subsequent coupling of ligands to the activated matrix results in an isourea linkage. Despite the popularity of this method, the isourea linkage of the ligands causes several problems during the purification procedure, including nonspecific binding due to charge and leakage of the ligand because of instability of the isourea bond. N-hydroxysuccinimide (NHS) esters have also been used for immobilizing ligands. The preparation of active esters requires a matrix that contains carboxylic groups. Such matrices can be easily obtained from agarose by activation of the hydroxyl groups with different reagents, including cyanogen bromide, activated carbonates, etc. and successive reaction with ω-amino acids of different sizes depending on the length of the spacer arm required. The NHS ester is then prepared by mixing the carboxylic matrix with dicyclohexylcarbodiimide and NHS. Due to the stability problem a different method based on N,N,N′,N′-Tetramethyl (succinimido) uronium tetrafluoroborate can be also used. The covalent attachment of ligands to such activated carriers provides the production of stable amide bonds. Another method for activating polysaccharides is the use of N′N-disuccinimidyl carbonate (DSC), which forms highly reactive carbonate derivatives with polymers containing hydroxyl groups. These derivatives react with nucleophiles under mild, physiological conditions

(pH 7.4), and the procedure results in a stable carbamate linkage of the ligand coupled to the carrier. The immobilization of different ligands (e.g., enzymes, enzyme inhibitors, antigens and antibodies) on activated carbonate carriers has been achieved, together with excellent maintenance of biological activity of the proteins. Pre-activated commercial matrices are also available (Table 2) to avoid many steps and problems of chemical activation process. A wide range of coupling chemistries, involving primary amines, sulfhydryls, aldehydes, hydroxyls and carboxylic acids are available for covalently attaching ligands to the matrices. The use of commercially available, pre-activated media is recommended to save time and avoid the use of the potentially hazardous reagents that are required in some cases.

Product name	Functional group specificity
UltraLink lodoacetyl resin	-SH
CarboLink Coupling resin	-CHO, C=O
ProfinityTM Epoxide resin	-NH2, -OH, -SH
Affi-Gel 10 and 15	-NH2
Pierce CDI-activated resin	-NH2
Epoxy-activated SepharoseTM 6B	-NH2, -OH, -SH
CNBr-activated Sepharose 4 Fast Flow	-NH2
EAH SepharoseTM 4B	-COOH, -CHO
Thiopropyl SepharoseTM 6B	-SH
Tresyl chloride-activated agarose	-NH2, -SH

Table 2.

Activated commercially available resins of affinity chromatography

After the activation of the support material, it is ready for the immobilization process of the ligand. In case the ligand is a small molecule, steric hindrance will occur between the immobilized support and the compound of interest (Figure 10). This may reduce or totally block specific binding of the substance. Use of the supports having a spacer arm attached or attachment of a spacer molecule to the support before immobilization of the ligand generally solves this

problem. Spacer arm keeps ligand at a suitable distance from the surface of the support (Figure 9), thus the substance of interest will not be prevented to attach to the immobilized ligand. It is possible to bind spacer arms directly to the support prior to the imobilization of the ligand. Then a secondary reaction provides the attachment of ligand to the spacer. The substance of interest doesn't be able to bind the ligand unless the spacer arm is long enough, but it is also possible to shorten the spacer arm in salt buffer.

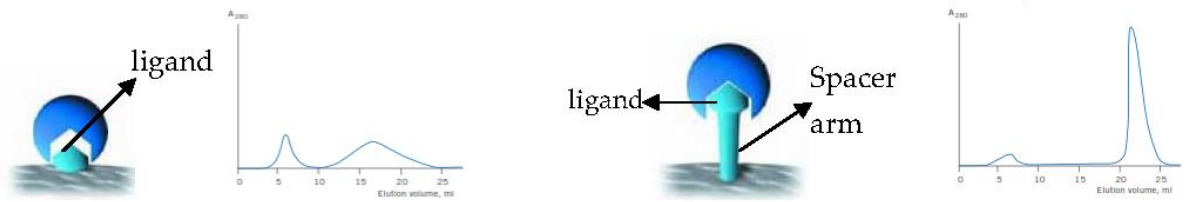


Figure 10. Spacer arm, keeping ligand at a suitable distance from the surface of the support

Properties of an ideal spacer arm are listed below:

It should be long enough (at least 3 atoms) to keep the substance at an appropriate distance.

It should be inactive not to cause a non-specific binding.

It should have bifunctional group for the reaction with both support and the sample.

Compounds which have diamine groups such as hexanediamine, propanediamine and ethylenediamine are the most preferred spacer arms used in affinity cromatography. Some other examples of spacer arms are shown in Table 3.

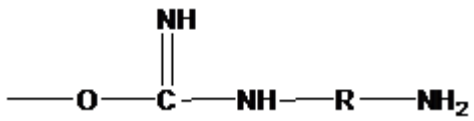


Table 3.

Some examples for spacer arms and their structures

The following step is the immobilization of ligands on the activated matrix by isourea bonds. Immobilization through isourea linkage has some disadvantages including nonspesific binding

of the ligand because of the instability of the bonds. Another method for immobilization is to use active esters such as N-hydroxy-succimide (NHS) esters. The carboxyl groups required for preparation of active esters can be prepared by activation of hydroxyl groups of agarose. The ligands attach to this type of matrix via amide bonds. It is also possible to activate polysacharides by formation of highly reactive carbonate derivatives. In this case the polymer which contains hydroxyl groups is activated by the use of N'N-disuccinimidyl carbonate (DSC). The resultant carbonate derivatives create stable carbamate bonds with nucleophiles under mild, physiological conditions. Immobilization methods can be categorized as follow (Figure 11).

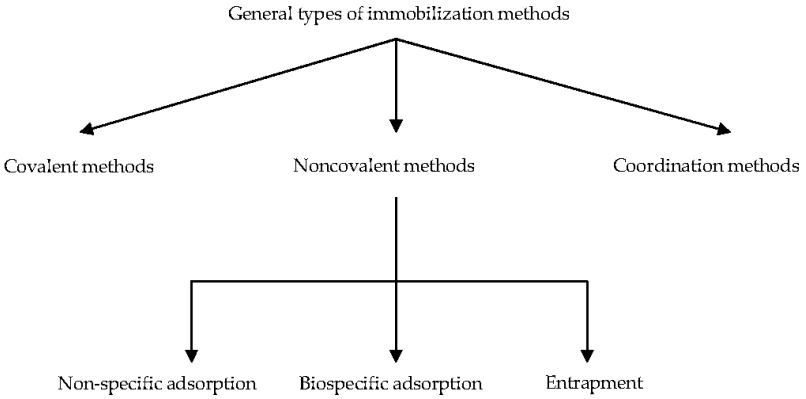


Figure 11. Immobilization methods used in affinity chromatography

Noncovalent immobilization technique

The simple adsorption of ligand to surface, binding to a secondary ligand, or ligand immobilization through a coordination complex can be classified as this type of immobilization. This technique can be subdivided as follow;

Nonspecific Adsorption; It is based on the attachment of ligand to support that has not been specifically functionalized for covalent attachment. Adsorption of the ligand to a support depends on the chemical characteristics of both the ligand and support. Columbic interactions, hydrogen bonding, and hydrophobic interactions involve in this type of immobilization.

Biospecific Adsorption; In this type of noncovalent immobilization method the ligand of interest bind to a secondary ligand attached to the support. Although a variety of secondary ligands can

be used for this purpose, two of the most common are avidin and streptavidin for the adsorption of biotin-containing compounds and protein A or protein G for the adsorption of antibodies.

Coordination Complexes; A coordination complex can be used to prepare an immobilized ligand in some cases. This is used to place metal ions into columns for immobilized metal-ion affinity chromatography (IMAC) which is based on the formation of a complex between a metal ion and electron donor groups.

Covalent immobilization methods

Covalent immobilization is the most popular method in affinity chromatography. In this method, it is necessary to activate the ligand and/or the support first. Activation of the ligand can be conducted when it is desired to couple this ligand through a specific region. An example is the creation of aldehydes in the carbohydrate regions of an antibody for its attachment to a support that contains amines or hydrazide groups. The use of an activated support is more common for ligand immobilization but tends to be less specific in nature. Examples include the immobilization of proteins through their amine groups to supports activated with N-hydroxysuccinimide or carbonyldiimidazole. The support used for covalent immobilization must meet several requirements. First, sufficient number of groups for activation and ligand attachment should be. Hydroxyl groups on the support are employed in most covalent coupling methods. Depending on how its surface is activated, a support can be used to immobilize ligands through their amine, sulfhydryl, hydroxyl, or carbonyl groups, among others.

Amine-Reactive Methods; Amine groups is often used for the immobilization of proteins and peptides. Specific methods are cyanogen bromide method, reductive amination, N-hydroxysuccinimide technique, and carbonyldiimidazole method.

Cyanogen Bromide Method ; The cyanogen bromide (CNBr) method was the first technique used on a large scale for immobilizing amine-containing ligands and involves the derivatization of hydroxyl groups on the surface of a support to form an active cyanate ester or an imidocarbonate group. Both of these active groups can couple ligands through primary amines, but the cyanate ester is more reactive than the imidocarbonate. The CNBr method utilizes relatively mild conditions for ligand attachment, making it suitable for many sensitive biomolecules. But one

problem with this approach is that the isourea linkages obtained by the reaction of CNBr with the support are positively charged at a neutral pH. This means that these groups can act as anion exchangers and nonspecific binding can be occur. Other problems with this method include the toxicity of CNBr, requiring the use of adequate safety precautions during the activation process, and the leakage of ligands that can result from CNBr-activated supports (Figure 12).

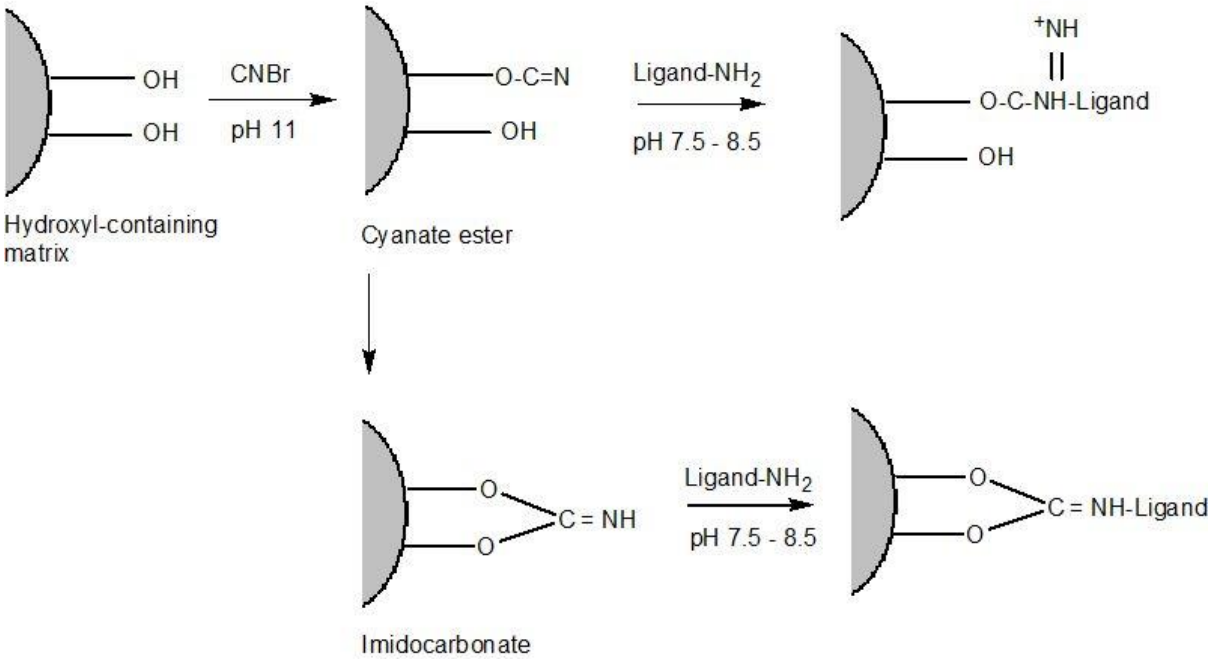


Figure 12. Cyanogen bromide immobilization method pathway

Activation methods which are used in affinity chromatography can be summarized as follow:

Amine groups :

Cyanogen bromide (CNBr) method

Schiff base (reductive amination) method

N-hydroxysuccinimide (NHS) method

Carbonyldiimidazole (CDI) method

Cyanuric chloride method

Azalactone method (for Emphaze supports)

Divinylsulfone (DVS)

Epoxy (bisoxirane) method

Ethyl dimethylaminopropyl carbodiimide (EDC)

method

Tresyl chloride/tosyl chloride method

Sulfhydryl groups:

Azalactone method (for Emphaze supports)

Divinylsulfone method

Epoxy (bisoxirane) method

Iodoacetyl/bromoacetyl method

Maleimide method

Pyridyl disulfide method

TNB-thiol method

Tresyl chloride/tosyl chloride method

Hydroxyl groups:

Cyanuric chloride method

Divinylsulfone method

Epoxy (bisoxirane) method

Aldehyde groups

Hydrazide method

Carboxyl groups

Ethyl dimethylaminopropyl carbodiimide (EDC) method.

Elution

Elution is one of the critical step for successful separation. Sample application in affinity chromatography is performed usually by injection or application in the presence of mobile phase which is prepared in appropriate pH, ionic strength and solvent composition for solute-ligand binding. This solvent is referred as application buffer. In the presence of application buffer, compounds which are complementary to the affinity ligand will bind while the other solutes in the sample will tend to pass through the column as nonretained compounds. After all nonretained

components are washed off the column, the retained solute or together with ligand as solute-ligand complex can be eluted by applying a solvent. This solvent which is referred as elution buffer represents the strong mobile phase for the column. Later all the interest solutes are eluted from the column, regeneration is performed by elution with application buffer and the column is allowed to regenerate prior to the next sample application. Step gradient elution or in other word on/off elution method is the most common method employed for affinity chromatography. Figure 15 shows the typical separation in affinity using step gradient elution.

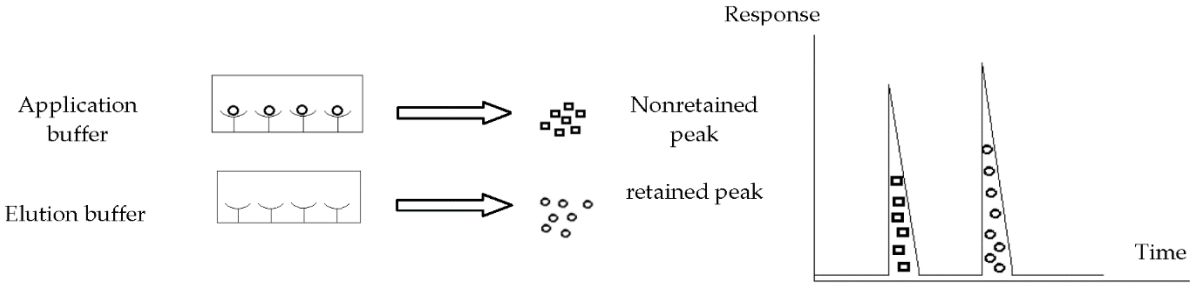


Figure 15.

Typical separation in affinity using step gradient elution.

Step elution mode is employed if the ligands have high affinity for the target molecule. It is also possible to use isocratic elution in affinity chromatography. This elution mode generally selected if the target molecule and ligand have weak interaction. This approach is known as Weak Affinity Chromatography or Dynamic Affinity Chromatography.

In affinity chromatography there are many factors such as strength of solute-ligand interaction, the amount of immobilized ligand present and the kinetics of solute-ligand association and dissociation which have important influences on retention and elution of the compound.

Obtaining stable biomolecules in high yield and purity is aimed for elution process. Elution is achieved by reducing the association constant of the ligand-solute interaction. Biospecific or non-specific elution can be utilized. Biospecific elution is based on solute displacement from the column by addition of molecule that acts as a competing agent. Two different types of biospecific elution can be applied for elution. In first method, normal role elution, molecule competes with the ligand for binding the desired solute. In second type of biospecific elution, reversed role

elution, molecule competes with desired solute for binding the ligand. The main advantage of biospecific elution is that a target can be gently removed from the column. However this elution is slow and generally results in broad solute peaks. Additionally competing agent needs to be removed from the eluted solvent therefore usage is limited. Another disadvantage especially in analytical application is need to use a competing agent that does not produce a large background signal under the conditions used for analyte detection. Non-specific elution is performed by changing solvent conditions like pH, ionic strength and polarity. High concentration of chaotropic salts (NaCl, MgCl2 or LiCl), denaturating agents and detergents (guanidine hydrochloride, sodium dodecyl sulfate and urea) can be used. Organic solvents can be used especially for the elution of low molecular weight compounds. Alteration in structure of the solute or ligand which leads to a lower association constant and lower solute retention is provided by nonspecific elution. Non-specific elution is faster than specific elution but there is a risk for denaturation of solute. The conditions which are applied for the elution may be too hard for column. If this is not considered it may result in long column regeneration times or irreversible loss of ligand activity.

For biospecific elution solvent is selected according to the type of target and ligand. The solvent usually has a pH and ionic composition similar to the application buffer but contains a competing agent. Reversed role elution is generally preferred when the target is a small compound while the normal elution is often used for isolation of macromolecules. Readily available in an inexpensive form and be soluble in the elution buffer are desired properties for competing agent in reverse role elution. In reversed-role elution it must be possible to remove the competing agent from the target when the affinity column is used for purification.

HPLC

- high pressure to drive the solutes through the column faster.
- diffusion is limited and the resolution is improved.
- The most common form is "reversed phase" hplc, where the column material is hydrophobic. The proteins are eluted by a gradient of increasing amounts of an organic solvent, such as acetonitrile. The proteins elute according to their hydrophobicity. After purification by HPLC

the protein is in a solution that only contains volatile compounds, and can easily be lyophilized.

SDS - Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE), describes a technique widely used in biochemistry, forensics, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. Mobility is a function of the length, conformation and charge of the molecule.As with all forms of gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure, or a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. For nucleic acids, urea is the most commonly used denaturant. For proteins, sodium dodecyl sulfate (SDS) is an anionic detergent applied to protein samples to linearize proteins and to impart a negative charge to linearized proteins. This procedure is called **SDS-PAGE**. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Proteins that have a greater hydrophobic content, for instance many membrane proteins, and those that interact with surfactants in their native environment, are intrinsically harder to treat accurately using this method, due to the greater variability in the ratio of bound SDS.

Procedure

Sample preparation

Samples may be any material containing proteins or nucleic acids. These may be biologically derived, for example from prokaryotic or eukaryotic cells, tissues, viruses, environmental samples, or purified proteins. In the case of solid tissues or cells, these are often first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), by sonicator or by using cycling of high pressure, and a combination of biochemical and mechanical techniques – including various types of filtration and centrifugation – may be used to separate different cell compartments and organelles prior to electrophoresis. Synthetic

biomolecules such as oligonucleotides may also be used as analytes. Reduction of a typical disulfide bond by DTT via two sequential thiol-disulfide exchange reactions.

The sample to analyze is optionally mixed with a chemical denaturant if so desired, usually SDS for proteins or urea for nucleic acids. SDS is an anionic detergent that denatures secondary and non–disulfide–linked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass. Urea breaks the hydrogen bonds between the base pairs of the nucleic acid, causing the constituent strands to separate. Heating the samples to at least 60 °C further promotes denaturation.

In addition to SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent, such as dithiothreitol (DTT) or 2-mercaptoethanol (beta-mercaptoethanol/BME), which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). This is known as reducing SDS-PAGE.

A tracking dye may be added to the solution. This typically has a higher electrophoretic mobility than the analytes to allow the experimenter to track the progress of the solution through the gel during the electrophoretic run.

Preparing acrylamide gels

The gels typically consist of acrylamide, bisacrylamide, the optional denaturant (SDS or urea), and a buffer with an adjusted pH. The solution may be degassed under a vacuum to prevent the formation of air bubbles during polymerization. Alternatively, butanol may be added to the resolving gel (for proteins) after it is poured, as butanol removes bubbles and makes the surface smooth. [6] A source of free radicals and a stabilizer, such as ammonium persulfate and TEMED are added to initiate polymerization.[7] The polymerization reaction creates a gel because of the added bisacrylamide, which can form cross-links between two acrylamide molecules. The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. The acrylamide concentration of the gel can also be varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving very high molecular weight molecules, while much higher percentages are needed to resolve smaller proteins.

Gels are usually polymerized between two glass plates in a gel caster, with a comb inserted at the top to create the sample wells. After the gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

Electrophoresis

Various buffer systems are used in PAGE depending on the nature of the sample and the experimental objective. The buffers used at the anode and cathode may be the same or different. An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative electrode (which is the cathode being that this is an electrolytic rather than galvanic cell) and towards the positive electrode (the anode). Depending on their size, each biomolecule moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty. The gel is run usually for a few hours, though this depends on the voltage applied across the gel; migration occurs more quickly at higher voltages, but these results are typically less accurate than at those at lower voltages. After the set amount of time, the biomolecules have migrated different distances based on their size. Smaller biomolecules travel farther down the gel, while larger ones remain closer to the point of origin. Biomolecules may therefore be separated roughly according to size, which depends mainly on molecular weight under denaturing conditions, but also depends on higher-order conformation under native conditions. However, certain glycoproteins behave anomalously on SDS gels.

Further processing

Two SDS-PAGE-gels after a completed run following electrophoresis, the gel may be stained (for proteins, most commonly with Coomassie Brilliant Blue R-250; for nucleic acids, ethidium bromide; or for either, silver stain), allowing visualization of the separated proteins, or processed further (e.g. Western blot). After staining, different species biomolecules appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight in a separate lane in the gel to calibrate the gel and determine the approximate molecular mass of unknown biomolecules by comparing the distance traveled relative to the marker.

For proteins, SDS-PAGE is usually the first choice as an assay of purity due to its reliability and ease. The presence of SDS and the denaturing step make proteins separate, approximately based on size, but aberrant migration of some proteins may occur. Different proteins may also stain differently, which interferes with quantification by staining. PAGE may also be used as a preparative technique for the purification of proteins. For example, **q**uantitative **p**reparative **n**ative **c**ontinuous **p**oly**a**crylamide **g**el **e**lectrophoresis (QPNC-PAGE) is a method for separating native metalloproteins in complex biological matrices.

Chemical ingredients and their roles

Polyacrylamide gel (PAG) had been known as a potential embedding medium for sectioning tissues as early as 1964, and two independent groups employed PAG in electrophoresis in 1959, It possesses several electrophoretically desirable features that make it a versatile medium. It is a synthetic, thermo-stable, transparent, strong, chemically relatively inert gel, and can be prepared with a wide range of average pore sizes. The pore size of a gel is determined by two factors, the total amount of acrylamide present (%T) (T = Total concentration of acrylamide and bisacrylamide monomer) and the amount of cross-linker (%C) (C = bisacrylamide concentration). Pore size decreases with increasing %T; with cross-linking, 5%C gives the smallest pore size. Any increase or decrease in %C from 5% increases the pore size, as pore size with respect to %C is a parabolic function with vertex as 5%C. This appears to be because of non-homogeneous bundling of polymer strands within the gel. This gel material can also withstand high voltage gradients, is amenable to various staining and destaining procedures, and can be digested to extract separated fractions or dried for autoradiography and permanent recording.

Components

- **Chemical buffer** Stabilizes the pH value to the desired value within the gel itself and in the electrophoresis buffer. The choice of buffer also affects the electrophoretic mobility of the buffer counterions and thereby the resolution of the gel. The buffer should also be unreactive and not modify or react with most proteins. Different buffers may be used as cathode and anode buffers, respectively, depending on the application. Multiple pH values may be used within a single gel,

for example in DISC electrophoresis. Common buffers in PAGE include Tris, Bis-Tris, or imidazole.

- **Counterion** balance the intrinsic charge of the buffer ion and also affect the electric field strength during electrophoresis. Highly charged and mobile ions are often avoided in SDS-PAGE cathode buffers, but may be included in the gel itself, where it migrates ahead of the protein. In applications such as DISC SDS-PAGE the pH values within the gel may vary to change the average charge of the counterions during the run to improve resolution. Popular counterions are glycine and tricine. Glycine has been used as the source of trailing ion or slow ion because its pKa is 9.69 and mobility of glycinate are such that the effective mobility can be set at a value below that of the slowest known proteins of net negative charge in the pH range. The minimum pH of this range is approximately 8.0.
- **Acrylamide (C_3H_5NO; mW: 71.08).** When dissolved in water, slow, spontaneous autopolymerization of acrylamide takes place, joining molecules together by head on tail fashion to form long single-chain polymers. The presence of a free radical-generating system greatly accelerates polymerization. This kind of reaction is known as Vinyl addition polymerisation. A solution of these polymer chains becomes viscous but does not form a gel, because the chains simply slide over one another. Gel formation requires linking various chains together. Acrylamide is a neurotoxin. It is also essential to store acrylamide in a cool dark and dry place to reduce autopolymerisation and hydrolysis.
- **Bisacrylamide (*N*,*N*′-Methylenebisacrylamide) ($C_7H_{10}N_2O_2$; mW: 154.17).** Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels. Chemically it can be thought of as two acrylamide molecules coupled head to head at their non-reactive ends. Bisacrylamide can crosslink two polyacrylamide chains to one another, thereby resulting in a gel.

- **Sodium Dodecyl Sulfate (SDS) ($C_{12}H_{25}NaO_4S$; mW: 288.38).** (only used in denaturing protein gels) SDS is a strong detergent agent used to denature native proteins to unfolded, individual polypeptides. When a protein mixture is heated to 100 °C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g SDS/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment

become rod-like structures possessing a uniform charge density, that is same net negative charge per unit weight. The electrophoretic mobilities of these proteins is a linear function of the logarithms of their molecular weights.

Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass-charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

- **Urea ($CO(NH_2)_2$; mW: 60.06).** Urea is a chaotropic agent that increases the entropy of the system by interfering with intramolecular interactions mediated by non-covalent forces such as hydrogen bonds and van der Waals forces. Macromolecular structure is dependent on the net effect of these forces, therefore it follows that an increase in chaotropic solutes denatures macromolecules,
- **Ammonium persulfate (APS) ($N_2H_8S_2O_8$; mW: 228.2)**. APS is a source of free radicals and is often used as an initiator for gel formation. An alternative source of free radicals is riboflavin, which generated free radicals in a photochemical reaction.
- **TEMED (*N*, *N*, *N′*, *N′*-tetramethylethylenediamine)** ($C_6H_{16}N_2$; mW: 116.21). TEMED stabilizes free radicals and improves polymerization. The rate of polymerisation and the properties of the resulting gel depend on the concentrations of free radicals. Increasing the amount of free radicals results in a decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity. Decreasing the amount shows the reverse effect. The lowest catalytic concentrations that allow polymerisation in a reasonable period of time should be used. APS and TEMED are typically used at approximately equimolar concentrations in the range of 1 to 10 mM.

Chemicals for processing and visualization

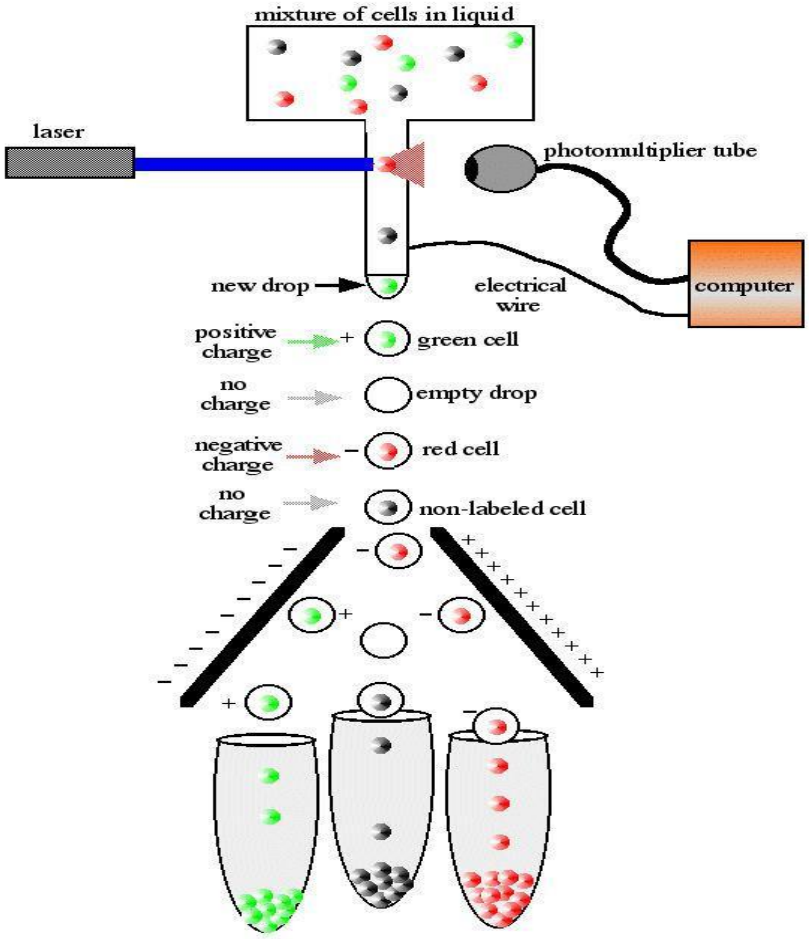
The following chemicals and procedures are used for processing of the gel and the protein samples visualized in it:

- **Tracking dye**. As proteins and nucleic acids are mostly colorless, their progress through the gel during electrophoresis cannot be easily followed. Anionic dyes of a known electrophoretic

mobility are therefore usually included in the PAGE sample buffer. A very common tracking dye is Bromophenol blue (BPB, 3',3'',5',5'' tetrabromophenolsulfonphthalein). This dye is coloured at alkali and neutral pH and is a small negatively charged molecule that moves towards the anode. Being a highly mobile molecule it moves ahead of most proteins. As it reaches the anodic end of the electrophoresis medium electrophoresis is stopped. It can weakly bind to some proteins and impart a blue colour. Other common tracking dyes are xylene cyanol, which has lower mobility, and Orange G, which has a higher mobility.

- **Loading aids**. Most PAGE systems are loaded from the top into wells within the gel. To ensure that the sample sinks to the bottom of the gel, sample buffer is supplemented with additives that increase the density of the sample. These additives should be non-ionic and non-reactive towards proteins to avoid interfering with electrophoresis. Common additives are glycerol and sucrose.
- **Coomassie Brilliant Blue R-250 (CBB)($C_{45}H_{44}N_3NaO_7S_2$; mW: 825.97)**. CBB is the most popular protein stain. It is an anionic dye, which non-specifically binds to proteins. The structure of CBB is predominantly non-polar, and it is usually used in methanolic solution acidified with acetic acid. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated into the gel can be removed by destaining with the same solution without the dye. The proteins are detected as blue bands on a clear background. As SDS is also anionic, it may interfere with staining process. Therefore, large volume of staining solution is recommended, at least ten times the volume of the gel.
- **Ethidium bromide (EtBr)** is the traditionally most popular nucleic acid stain.
- **Silver staining**. Silver staining is used when more sensitive method for detection is needed, as classical Coomassie Brilliant Blue staining can usually detect a 50 ng protein band, Silver staining increases the sensitivity typically 50 times.
- **Western Blotting** is a process by which proteins separated in the acrylamide gel are electrophoretically transferred to a stable, manipulable membrane such as a nitrocellulose, nylon, or PVDF membrane. It is then possible to apply immunochemical techniques to visualise the transferred proteins, as well as accurately identify relative increases or decreases of the protein of interest. For more, see Western Blot.

Flow Cytometry



- What is flow cytometry?

– The analysis of single particles, often cells, within a heterogeneous suspension

- Whole blood, Cell cultures, Separated tissue, Isolated nuclei, Bacteria/yeast/parasites, Algae & plankton
- Signal from individual particles is collected for analysis as they pass through a laser in a stream of fluid.
- Data displayed as events on histograms/dot plots
- Vital that cells pass through the laser bean in single suspension
- Cells injected into a flowing stream of saline solution (sheath fluid)
- Hydrodynamic focusing
- Compresses cell stream to approx 1 cell diameter

- Allows single cells to be interrogated by the laser
- Optimal 'imaging' of cells is achieved with a 'low' flow rate and high concentration of sample

Applications of flow cytometry in research

- Multicolour analysis
- Cell cycle
- Cell proliferation
- Apoptosis and Cell Viability
- Cell Sorting
- Multiplex analysis
- Multicolour analysis
- Immunophenotyping
- Cells surface antigen detection (e.g. receptors, adhesion molecules)
- Intracellular staining
- Assessing infection/transfection levels
- Antibodies/ dyes/ Quantum dots

Cell sorting – Based on charges separation of particles

- Allows rare populations to be isolated from heterogenous populations (cell culture, blood samples, etc)
- Can isolate sub cellular particles (e.g. endosomes, nucleus, chromosomes)
- Allows transfection experiments to be enriched and single cell clones to be isolated
- Can produce purity >95%
- Flow cytometry is a powerful method for rapidly quantitating cellular fluorescence
- A number of functional assays such as cell cycle and apoptosis can be determined by flow and can be used as a method for assessing e.g. the effects of drugs on cell function, or the expression of mutant proteins
- Finally, cells and sub-cellular particles can be sorted from heterogeneous samples to yield near homogeneous populations for subsequent culturing or analysis.

UNIT IV

Radioactive isotopes – storage, safety, handling and radioactive waste management

Introduction

When the number of protons and neutrons does not meet certain proportions, the nucleus will be unstable and will try to reach a steady-state by emitting particles (alpha or beta, see below), sometimes followed by emission of radiation energy. This transition and the associated emission of radiation are called disintegration and radioactive radiation, respectively. The unstable isotopes are called radioactive isotopes

When used in accordance with instructions, radioactive materials can be used safely in the environment. The general public is restricted from unnecessary radiation exposure during hydroprobe use, storage, and transportation by virtue of the operating procedures, locked storage, transportation limitations, and legal restrictions imposed by State and Federal regulations. Operator protection is obtained through training, good gauge design, and following radiological safe work practices (i.e., time, distance, and shielding).

Types of Radiation

Various elements, both naturally occurring (Radium) and reactor produced (Cesium and Americium) are unstable and are slowly decaying to a more stable state. The act of decay produces emissions of energy upon disintegration of the atoms. These emissions are either electromagnetic radiation (gamma rays) or are actual particles (alpha, beta). Other emissions are produced from various radioactive materials; however, we are concerned with only the alpha and gamma radiations and resultant neutrons for purposes of the nuclear soil gauging.

These emissions are detected by appropriate detectors (Geiger Mueller tubes) for gamma rays and (Boron Tri-fluoride or Helium tubes) for neutron measurements. The resultant signals are displayed electronically as an index of soil density and moisture.

All sources are supplied in a sealed stainless steel capsule, doubly encapsulated, and further welded into a stainless steel source rod or located permanently in the gauge housing (see Attachments 1 and 2).

Sources are manufactured by a number of manufacturers' specifications that have been approved by the State of California, Department of Health Services, Radiologic Health Branch.

Sources should never be removed from their mountings and no attempt should ever be made to repair them yourself. Only the manufacturer should perform these source manipulations.

Soil Source Gauges

The most common soil gauge sources are:

1. Cesium-137 for gamma emission
2. Americium-241/Beryllium for neutron emission
3. Radium-226/Beryllium for combined gamma and neutron emissions

Gamma Radiation

Gamma radiation is high energy electromagnetic energy capable of penetrating several inches of most material. It is useful for the total mass measurement of heavy materials and is used to determine the total density of soil.

Gamma radiation is emitted in several energy levels by a sealed Radium source or in a single energy level by a Cesium source. The Cesium level is 0.66 million electron volts (MeV) and requires less shielding that the multi-level output of the Radium source. In addition, the fixed spectrum emission is superior for soil density determination purposes.
Gamma sources are relatively easy to shield with dense material like lead, depleted uranium, tungsten, etc.

Neutron Radiation

Neutron radiation consists of small, non-charged particles emitted from the source at an average energy level of 5 MeV. This is known as fast neutron emission. Neutron detectors see only slow, or thermal neutrons; therefore, the fast neutrons must slow down or they will be ignored by the detectors. Neutrons slow down by colliding with other objects (especially light elements like hydrogen) much like a rifle bullet ricocheting from rock to rock.
A simple analogy is that of a golf ball colliding with a bowling ball. The golf ball would rebound with little loss of energy. However, two golf balls colliding would produce a strong loss of energy in each of them, or a transfer of energy from one to the other.
This is what happens when a fast neutron hits a hydrogen atom. The neutron is markedly slowed down. After a few collisions with hydrogen atoms, a fast neutron is reduced to the slow or thermal energy that the moisture detectors in the soil gauge can detect.
Neutron emission occurs when an alpha particle emitter (Americium, Plutonium, or Radium) is

mixed with Beryllium powder in a tightly compressed pellet. The alpha particles strike the Beryllium atoms to produce fast neutrons of an average energy of 5 MeV. The suffix Be is attached to an alpha source name to identify the type of neutron source (RaBe, AmBe, PuBe). Neutron sources are more difficult to shield. Use of hydrogenous moderators may provide shielding but reduces the measuring capacity of the gauge. It is impossible to moderate the neutrons with heavy plastic shielding and still expect the ground moisture to then moderate more neutrons for measurement. Neutron shielding is further complicated in that the thermal neutrons are captured by the moderating material with a resultant emission of gamma radiation of fairly high energy.

Units of Radiation Measurement

Activity (Unit: Curie)

The Curie (Ci) is defined as the activity of that quantity of radioactive material in which the number of disintegrations per second is 3.7E10 (a number nearly the same as the number of disintegrations per second from 1 gram of radium).
Since a Curie is a large amount of radioactivity sub-units of a Curie, a millicurie (mCi, 1E-3 Curie) or microcurie (μCi, 1E-6 Curie), are commonly used to express the amount of activity.

Exposure (Unit: Roentgen)

The Roentgen (R) is defined as 2.58E-4 coulomb/kg air. This unit is special in that it is defined only for X or gamma radiation in air.

Absorbed Dose (Unit: rad)

The rad is the special unit of absorbed energy. It is defined as that amount of ionizing radiation that deposits 100 ergs/gram of material. For most applications, it can be assumed that 1 Roentgen = 1 rad.

Dose Equivalent (Unit: rem)

The rem is the unit of dose equivalent. The dose equivalent accounts for the difference in biological effectiveness of different types of radiation. It is the product of the absorbed dose (rad) times the quality factor (QF) of the radiation. The QF for x, gamma, and beta radiation is 1, for alpha radiation 20, and varies with energy from 2-11 for neutrons.

Radiation Quantities and Units

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Radioactivity: The rate of disintegration per unit time is called activity, which is measured in curies and based on the following standard:

1 curie (Ci) = 3.7×10^{10} dps (disintegrations per seconds)

1 millicurie (mCi) = 3.7×10^{7} dps = 1×10^{-3} Ci

1 microcurie (μCi) = 3.7×10^{4} dps = 10^{-6} Ci

1 nanocurie (nCi) = 3.7×10^{1} dps = 10^{-9} Ci

1 picocurie (pCi) = 3.7×10^{-2} dps = 1×10^{-12} Ci

dps = cps/efficiency of detector for specific radionuclide

Radiation Exposure: The measurement of radiation exposure in air as ionizations per unit mass of air due to X or gamma radiation.

1 Roentgen (R) = 2.58×10^{-4} Coulomb/kg air

1 milliroentgen = 2.58×10^{-7} Coulomb/kg air = 1×10^{-3} R

Absorbed Dose: The measurement of radiation absorbed dose (rad) represents the amount of energy deposited per unit mass of absorbing material.

1 rad = 100 ergs/gm = 1×10^{-2} Joule/kg

1 millirad (mrad) = 0.1 ergs/gm= 1×10^{-5} Joule/kg = 1×10^{-3} rad

Dose Equivalent: The measure of biological effect of radiation requires a third unit called a quality factor (QF) which takes into account the different degrees of biological damage produced by equal doses of different types of radiation.

1 rem (Roentgen equivalent man) is the product of the amount of energy absorbed (rad) times the efficiency of radiation in producing damage (i.e., QF)

1 rem = 1 rad x QF

For X and gamma radiations and most beta, the QF = 1. Therefore, 1 rem = 1 rad

Alpha radiation has a QF of 20

QF for neutrons is 2-11

S.I. System: There is a new system of radiation units, the S.I. system, which is widely used in Europe and gradually being adopted in the United States. The current and new units are shown below with their conversion factor.

CURRENT UNIT	S.I. UNITS	CONVERSION
Curie (Ci)	Becquerel (Bq)	1 Bq = 1 dps
rad	Gray (Gy)	1 Gy = 100 rad
rem	Sievert (Sv)	1 Sv = 100 rem

NUCLEAR WASTE

•Composed of Radionuclides

• Low, Medium and High level wastes

• High level wastes produced in nuclear reactors consists of oFission products(short-half lives) oActinides(long-half lives)

CLASSIFICATIONS

• Nuclear waste is segregated into following classifications

- Low level waste which is not dangerous but sometimes requires shielding during handling.
- Intermediate waste typically is chemical sludge and other products from reactors.
- High level waste consists of fissionable elements from reactor core.
- Transuranic waste is any waste with transuranic alpha emitting radionuclides whose half-life is longer than 20 years.

LOW LEVEL WASTE (LLW)

• Contains VERY LOW concentration of radioactivity.

• Waste which does not require shielding during normal handling and transportation.

• 90% volume of waste.

INTERMEDIATE LEVEL WASTE (ILW)

• Intermediate level waste contains higher radioactivity levels than low level wastes.

• Waste which requires shielding but needs little or no provision for heat dissipation during its handling and transportation.

• Intermediate level waste typically is chemical sludge, resins etc.

• 7% volume of the waste.

HIGH LEVEL WASTE (HLW)

• High level waste has a large amount of radioactive activity and is thermally hot.

• 3% volume of waste.

• 95% of radioactivity.

• Current levels of HLW are increasing at about 12,000 metric tons per year.

• Most HLW consists of Pu-238, 239, 240, 241, 242, Np-237, U-236.

BASIC STEPS AND ACTIVITIES IN RADIOACTIVE WASTE MANAGEMENT

• Waste generation occurs during the operational period. It can be in the form of solid, liquid or gaseous waste.

• Pretreatment is the initial step that occurs just after generation. It consists of collection, segregation, chemical adjustment and decontamination.

• Treatment involves changing the characteristics of the waste. Basic treatment concepts are volume reduction, radionuclide removal and change of composition.

• Conditioning involves those operations that transform radioactive waste into a form suitable for handling, transportation, storage and handling.

• Storage facilities maybe co-located with a nuclear power plant or licensed disposal facility.

• Retrieval involves the recovery of waste packages from storages either for inspection purpose or further storage in a disposal facility.

• Disposal consists of the authorized emplacement of packages of radioactive waste in a disposal facility.

DISPOSAL OF LOW LEVEL WASTE

NEAR SURFACE DISPOSAL:

• Disposal in a facility consisting of engineered channels or vaults constructed on the ground surface or up to few tens of meters below ground level.

• These type of wastes loses most or all of its radioactivity within 300 years.

• It includes contaminated equipment from the operation of nuclear power plant like clothing, rags, tools and equipment.

• Very short-lived low-level radioactive waste such as that from hospitals, universities and industry generally contains only small amounts of radioactive materials with short half-lives. This means that radioactivity decays away in hours or days.

DISPOSAL OF INTERMEDIATE LEVEL WASTE

• Depending on its characteristics, intermediate level radioactive wastes can be disposed in facilities of different types.

• Disposal could be by emplacement in a facility constructed in caves, vaults or silos at least few hundred meters below the ground level.

• Intermediate level radioactive wastes require long-term management.

• These are much bulkier materials and are first sealed in steel drums and are encased in concrete trenches.

DISPOSAL OF HIGH LEVEL WASTE

GEOLOGICAL DISPOSAL:

• A deep geological repository is a nuclear waste repository excavated deep within a stable geologic environment.

• Most long-lived radioactive wastes are stored here.

• The volume of these HLW can be reduced by Nuclear reprocessing. This reprocessing does not eliminate the need for a repository, but reduces the volume.

• These repositories are at least a few hundred meters below the ground level.

DEEP BOREHOLES

• Deep borehole disposal is the concept of disposing high-level radioactive waste from nuclear reactors in extremely deep boreholes instead of in more traditional deep geological repositories that are excavated like mines.

• Similar concept to geological repositories.

• Kilometers deep rather then hundreds of meters.

• Provide further insulation from ground water.

LAUNCH INTO SPACE

• Near infinite storage space.

- Completely removes waste from biosphere.
- High technical risks and problem of space vehicle failure.
- High energy cost of space launch.
- The current cost to launch an object into orbit around the earth is about $20,000 per kilogram.

Safety measures, safety and handling of radioactive isotopes

These guidelines should be strictly observed.

The radioactive substances used should comply with the following **characteristics**:

radio toxicity must be as low as possible short-living isotopes are preferred to long-living ones the amounts used must be kept to a minimum

• **Never work alone** in a radioactive lab, especially not outside normal working hours. Always make sure to have someone nearby in case of emergency.

• Take all precautions to prevent radioactive **contamination**:

always separate radioactive activities from non-radioactive activities as far as possible, limit the area where radioactive substances are used and mark the area, e.g. by using containers with absorbent paper

Apply a radiation symbol to any containers and items that have come into contact with radioactive substances.

Never bring documents such as notes into the radioactive zone

• When handling radioactive materials, always wear the **appropriate protective clothing**:

lab coat. If there is a risk of serious contamination, wear disposable clothing

Store your lab coat away from your regular clothes

Always wear gloves when handling radioactive substances. Regularly check the radiation level of these gloves. Never touch anything with potentially contaminated gloves; use paper tissues instead wear shoe covers in rooms where the floor may be contaminated keep personal items such as handbags, etc., outside the lab

• Use appropriate **radiation shields**. Return the stock solution to storage immediately after removing the amount needed.

• To avoid internal contamination, **strict hygiene** is essential when handling radioactive materials

• Eating, smoking, drinking, and applying cosmetics are prohibited in all labs.

• Never pipette by mouth. Use pipetting devices instead.

• Wash your hands thoroughly when you leave the lab.

• Regularly **check** the radiation level of your working area and all objects used, or at least at the end of each working day. Replace contaminated absorption paper. Decontaminate contaminated objects.

• Dispose of all radioactive waste in the appropriate containers. Limit the amount of waste to a bare minimum. Separate short-living and long-living radioactive waste.

In case of an **incident** involving radioactive materials (e.g. spills), always remain in the room unless injured.

Radioactive waste management

All waste containing radioactive material is considered radioactive waste. Even if it contains biological and/or chemical waste. As such even chemical and or biological waste containing radioactive material has to be treated as radioactive waste and disposed of accordingly. Waste disposal containers are available in the radioactive room and only these containers are to be used. Never use other containers. Each container must bear a numbered disposal label (completed by the HSE Department) identifying relevant hazards. The container should also be checked for

external contamination. The waste is collected once a week after a Radioactive Waste Collection Request Form was sent to the Department.

Waste class and isotopes	Physical state and specifications		Container
every container should contain only one isotope			
Disposable waste T1/2 > 180 days (54Mn, 57Co, 60Co, 65Zn, 99Tc, 134Cs, 137Cs, 152Eu,...) except 3H and 14C	Solid flammable	black plastic box 25L	
	Solid non-flammable	plastic bucket 30L	
3H - 14C *3H and 14C should be segregated from other disposable waste* *Always indicate the presence of 3H and 14C on the label*	Liquid scintillation bottles	blue plastic box 25L	
	Liquid aqueous and organic	white plastic canister 10L or 20L	

			
	Animal carcasses	plastic bag 50L	
Decay able waste T1/2 < 180 days (32P, 33P, 35S, 45Ca, 51Cr, 75Se, 125I, 131I, ...)	solid	yellow plastic box 50L	
	Liquid aqueous and organic	white plastic canister 10L or 20L	 
	Animal carcasses	plastic bag 50L	
Special waste	Special waste Natural radioactive waste	The container and the cost will be specified by the HSE Department	

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Crystallography

Crystallography is the science of the arrangement of atoms in solids. The word "crystallography" derives from the Greek words *crystallon* = cold drop / frozen drop, with its meaning extending to all solids with some degree of transparency, and *grapho* = write.

Before the development of X-ray diffraction crystallography (see below), the study of crystals was based on their geometry. This involves measuring the angles of crystal faces relative to theoretical reference axes (crystallographic axes), and establishing the symmetry of the crystal in question. The former is carried out using a goniometer. The position in 3D space of each crystal face is plotted on a stereographic net, e.g. Wulff net or Lambert net. In fact, the pole to each face is plotted on the net. Each point is labelled with its Miller index. The final plot allows the symmetry of the crystal to be established.

Crystallographic methods now depend on the analysis of the diffraction patterns of a sample targeted by a beam of some type. Although X-rays are most commonly used, the beam is not always electromagnetic radiation. For some purposes electrons or neutrons are used. This is facilitated by the wave properties of the particles.

Crystallographers often explicitly state the type of illumination used when referring to a method, as with the terms *X-ray diffraction*, *neutron diffraction* and *electron diffraction*.

These three types of radiation interact with the specimen in different ways. X-rays interact with the spatial distribution of the valence electrons, while electrons are charged particles and therefore feel the total charge distribution of both the atomic nuclei and the surrounding electrons. Neutrons are scattered by the atomic nuclei through the strong nuclear forces, but in addition, the magnetic moment of neutrons is non-zero. They are therefore also scattered by magnetic fields. When neutrons are scattered from hydrogen-containing materials, they produce diffraction patterns with high noise levels. However, the material can sometimes be treated to substitute deuterium for hydrogen. Because of these different forms of interaction, the three types of radiation are suitable for different crystallographic studies.

Technique

Some materials studied using crystallography, proteins for example, do not occur naturally as crystals. Typically, such molecules are placed in solution and allowed to crystallize over days, weeks, or months through vapor diffusion. A drop of solution containing the molecule, buffer, and precipitants is sealed in a container with a reservoir containing a hygroscopic solution. Water in the drop diffuses to the reservoir, slowly increasing the concentration and allowing a crystal to form. If the concentration were to rise more quickly, the molecule would simply precipitate out of solution, resulting in disorderly granules rather than an orderly and hence usable crystal.

Once a crystal is obtained, data can be collected using a beam of radiation. Although many universities that engage in crystallographic research have their own X-ray producing equipment, synchrotrons are often used as X-ray sources, because of the purer and more complete patterns such sources can generate. Synchrotron sources also have a much higher intensity of X-ray beams, so data collection takes a fraction of the time normally necessary at weaker sources. Complementary neutron crystallography techniques are used to enhance hydrogen atoms positions. Such techniques are available in Neutron facilities.

Producing an image from a diffraction pattern requires sophisticated mathematics and often an iterative process of modelling and refinement. In this process, the mathematically predicted diffraction patterns of an hypothesized or "model" structure are compared to the actual pattern generated by the crystalline sample. Ideally, researchers make several initial guesses, which through refinement all converge on the same answer. Models are refined until their predicted patterns match to as great a degree as can be achieved without radical revision of the model. This is a painstaking process, made much easier today by computers.

The mathematical methods for the analysis of diffraction data only apply to *patterns*, which in turn result only when waves diffract from orderly arrays. Hence crystallography applies for the most part only to crystals, or to molecules which can be coaxed to crystallize for the sake of measurement. In spite of this, a certain amount of molecular information can be deduced from the patterns that are generated by fibers and powders, which while not as perfect as a solid crystal, may exhibit a degree of order. This level of order can be sufficient to deduce the structure of simple molecules, or to determine the coarse features of more

complicated molecules. For example, the double-helical structure of DNA was deduced from an X-ray diffraction pattern that had been generated by a fibrous sample.

Biology

X-ray crystallography is the primary method for determining the molecular conformations of biological macromolecules, particularly protein and nucleic acids such as DNA and RNA. In fact, the double-helical structure of DNA was deduced from crystallographic data. The first crystal structure of a macromolecule was solved in 1958.[2] A three-dimensional model of the myoglobin molecule obtained by X-ray analysis.[3] The Protein Data Bank (PDB) is a freely accessible repository for the structures of proteins and other biological macromolecules. Computer programs like RasMol or Pymol can be used to visualize biological molecular structures. Neutron crystallography is often used to help refine structures obtained by x-ray methods or to solve a specific bond; the methods are often viewed as complementary, as x-rays are sensitive to electron positions and scatter most strongly off heavy atoms, while neutrons are sensitive to nucleus positions and scatter strongly off many light isotopes, including hydrogen and deuterium. Electron crystallography has been used to determine some protein structures, most notably membrane proteins and viral capsids.

X-ray Diffraction

X-ray diffraction (XRD) is a versatile, non-destructive technique that reveals detailed information about the chemical composition and crystallographic structure of natural and manufactured materials.

Crystal lattice

A crystal lattice is a regular three-dimensional distribution (cubic, rhombic, etc.) of atoms in space. These are arranged so that they form a series of parallel planes separated from one another by a distance *d*, which varies according to the nature of the material. For any crystal, planes exist in a number of different orientations - each with its own specific *d*-spacing.

Constructive interference

When a monochromatic X-ray beam with wavelength *lambda* is projected onto a crystalline material at an angle *theta*, diffraction occurs only when the distance traveled by the rays reflected from successive planes differs by a complete number *n* of wavelengths.

Bragg's Law

By varying the angle *theta*, the Bragg's Law conditions are satisfied by different *d*-spacings in polycrystalline materials. Plotting the angular positions and intensities of the resultant diffracted peaks of radiation produces a pattern, which is characteristic of the sample. Where a mixture of different phases is present, the resultant diffractogram is formed by addition of the individual patterns.

Based on the principle of X-ray diffraction, a wealth of structural, physical and chemical information about the material investigated can be obtained. A host of application techniques for various material classes is available, each revealing its own specific details of the sample studied.

Why XRD?

• Measure the average spacings between layers or rows of atoms
• Determine the orientation of a single crystal or grain
• Find the crystal structure of an unknown material
• Measure the size, shape and internal stress of small crystalline regions

English physicists Sir W.H. Bragg and his son Sir W.L. Bragg developed a relationship in 1913 to explain why the cleavage faces of crystals appear to reflect X-ray beams at certain angles of incidence (theta). The variable *d* is the distance between atomic layers in a crystal, and the variable lambda is the wavelength of the incident X-ray beam; n is an integer. This observation is an example of X-ray wave interference (Roentgenstrahlinterferenzen), commonly known as X-ray diffraction (XRD), and was direct evidence for the periodic atomic structure of crystals postulated for several centuries.

Principle

$n \lambda = 2d\sin\theta$

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Bragg's Law

Although Bragg's law was used to explain the interference pattern of X-rays scattered by crystals, diffraction has been developed to study the structure of all states of matter with any beam, e.g., ions, electrons, neutrons, and protons, with a wavelength similar to the distance between the atomic or molecular structures of interest.

Bragg's Law

The Braggs were awarded the Nobel Prize in physics in 1915 for their work in determining crystal structures beginning with NaCl, ZnS and diamond.

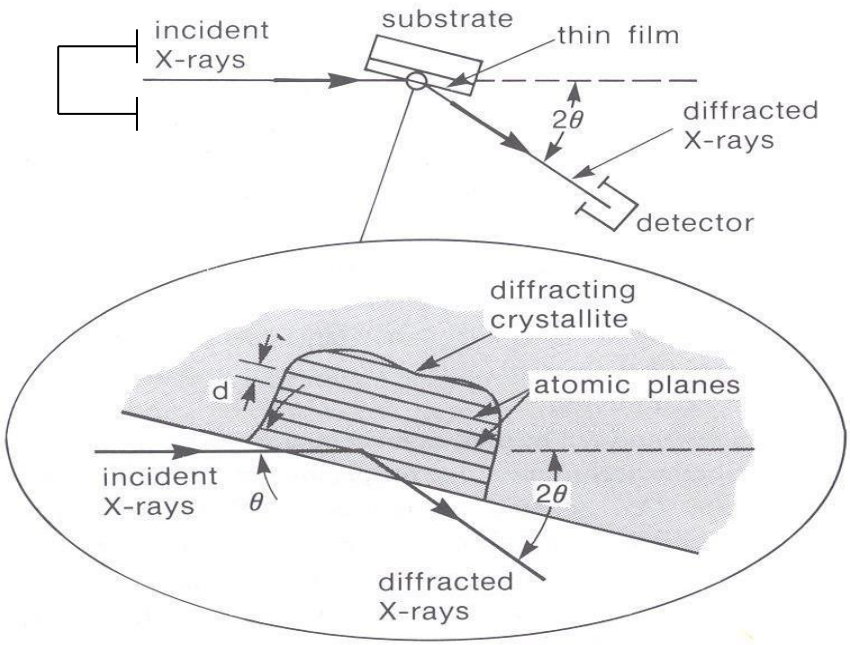
Basic Features of Typical XRD Experiment : Parts

1) Production

2) Diffraction

3) Detection

4) Interpretation



Applications of XRD

• XRD is a nondestructive technique

• To identify crystalline phases and orientation

• To determine structural properties: Lattice parameters (10-4Å), strain, grain size, expitaxy, phase composition, preferred orientation (Laue) order-disorder transformation, thermal expansion

• To measure thickness of thin films and multi-layers*

• To determine atomic arrangement

• Detection limits: ~3% in a two phase mixture; can be ~0.1% with synchrotron radiation Spatial resolution: normally none

Autoradiography

Autoradiography is any technique used to produce an image of the 2D distribution of a radioactive substance. -The first autoradiography was obtained accidently around 1867 when a blackening was produced on emulsions of silver chloride and iodide by uranium salts. -Such studies and the work of the Curie-Curies in 1898 demonstrated autoradiography before, and contributed directly to, the discovery of radioactivity. -The development of autoradiography as a biological technique really started to happen after World war II with the development of photographic emulsions and then stripping film made of silver halide.

General Methods for Autoradiography

Two General Types of Experiments:

1) *In-vivo* autoradiography - receptors are labeled in intact living tissue by systemic administration of the radioligand (like in PET). Tissue is removed, processed, and visualized.

2) *In-vitro* autoradiography - slide-mounted tissue sections are incubated with radioligand so that receptors are labeled under very controlled conditions

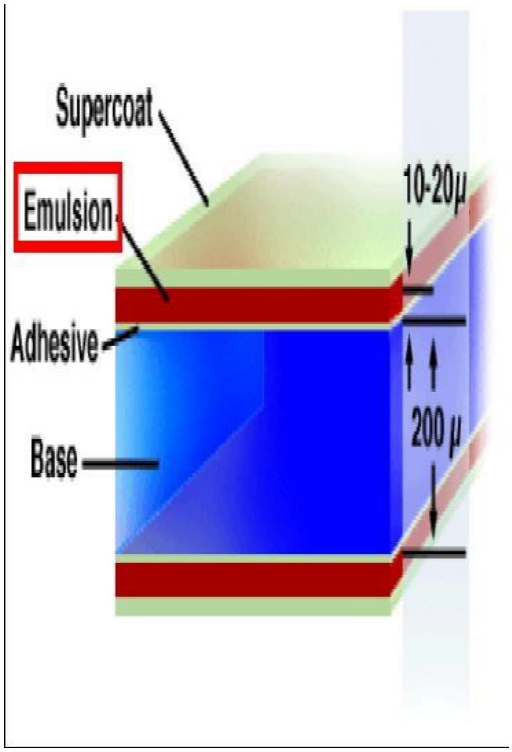
Autoradiographic film is typically composed of 4 layers.

1- An upper protective coat.

2- A layer of gelatin that contains silver halide (AgBr, AgCl, or AgI) crystals. (The type and proportions of the different silver halides determining the speed of the film.)

3- The film base, usually made from a flexible polymer.

4- An anti-halation backing to prevent light from reflecting back onto the emulsion.



This technique was essential to understand

►Oxidative respiration

►Photosynthesis

►The control of protein synthesis by nucleic acids.

►The timing of events throughout the cell cycle.

►the fate of populations of cells.

►Comparison of experimental treatments on events such as above.

PULSE CHASE

►Pulse chase is a technique used to sharpen the resolution in timing in many of the experiments.

►In this method a cellular process occurring over time by successively exposing the cells to a labelled compound (pulse) and then to the same compound in an unlabelled form (chase).

SOME MAJOR ADVANTAGES

►Highly specific detection tool.

►Unlike tissue bath preparations, pharmacologically characterize and localize receptors in tissues.

►Enables characterisation of receptors in different tissues in different animals and brain regions.

APPLICATIONS

►Auto-radiography is used to determine receptor distribution and localization while studying neuro-degenerative disorders.

►Application of auto-radiography in electrophoretic transfer of proteins from polyacrylamide gels to nitro-cellulose sheets during blotting.

►To study cytogenesis of the fore-brain.

►Applications in radio-pharmaceutical research.

►Applications in radioimmunoelectroosmophoresis to study viruses.

►In imaging and analysing rock porosity.

►As a tool for genetic studies.

►For comparison of complex mixtures of proteins.

►Applications in microbial ecology.

►Determining gross absorption and utilization of foliar applied nutrients etc.

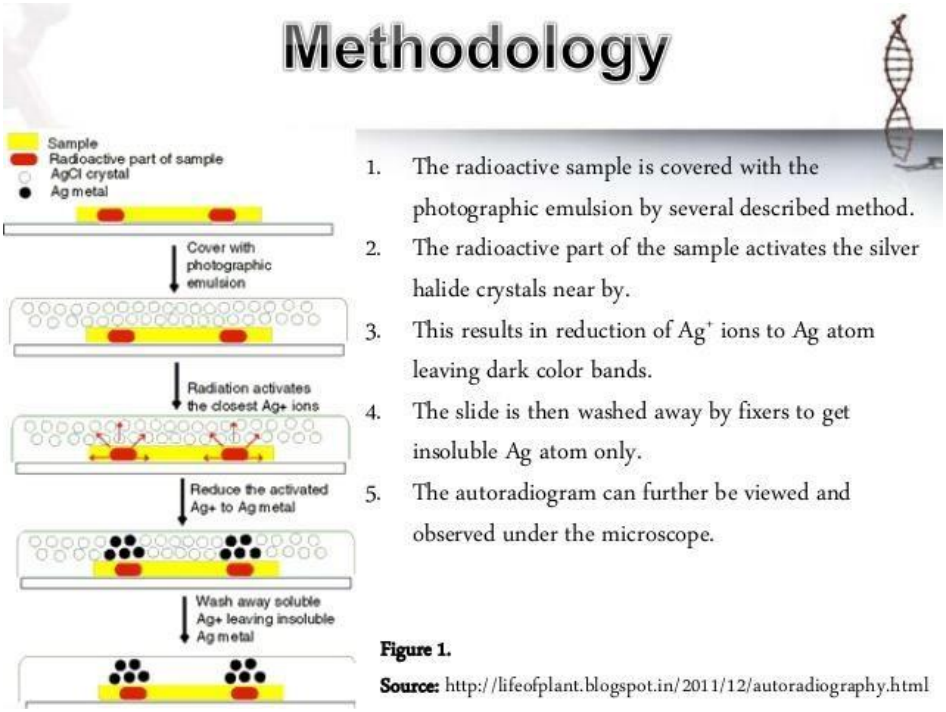


Figure 1.

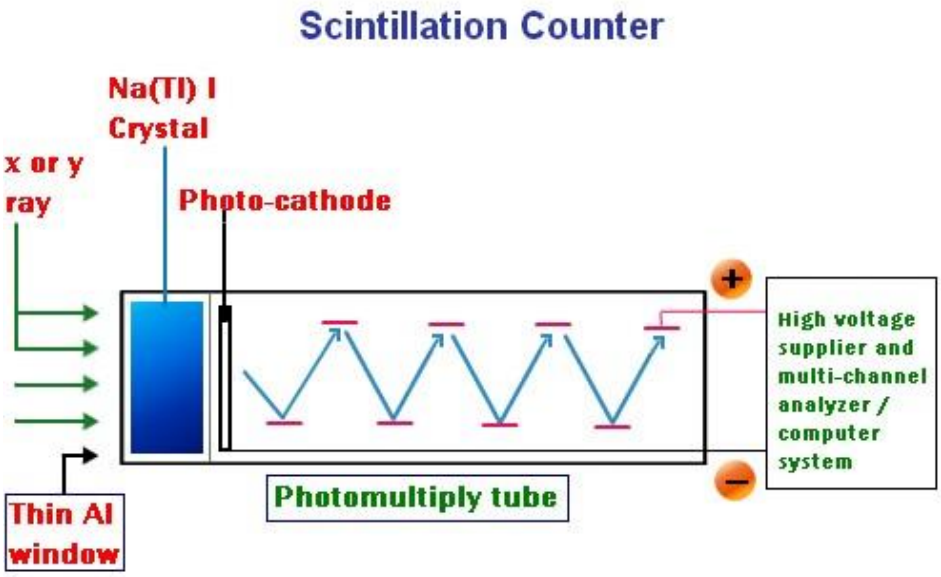
Source: http://lifeofplant.blogspot.in/2011/12/autoradiography.html

Scintillation Counter

The **Scintillation Counter** is a device which measures the ionizing radiation. It consists of sensor, which is also known as **Scintillator**, which fluoresces when it comes in contact of ionizing radiations. This light is then sensed by a photomultiplier tube (PMT). This PMT is attached to sophisticated electronic circuitry which counts and measures the radiation by amplifying the signals received from the PMT.

Liquid scintillation counting (LSC) is the standard laboratory method to quantify the radioactivity of low energy radioisotopes, mostly beta-emitting and alpha-emitting isotopes. The sensitive LSC detection method requires specific cocktails to absorb the energy into detectable light pulses.

The below shown picture is contains main components of the Scintillation Counter.



The one end of the scintillation counter has scintillation phosphor, like thalium activated sodium iodide crystal. This crystal is used as an sensor which senses the radiation. This sensed radiation is then detected by the PMT (Photomultiply tube), which produces the proportional pulses of electric current. This current is then measured by the multi channel analyzer.

The scintillation phosphors are majorly of three types:

1. **Inorganic Crystals :** The inorganic crystals have high atomic number and density. Gamma rays are detected by these types of the crystal very efficiently. In these crystals the pulses get decayed in approx 1 micro seconds. The common examples of inorganic crystals are sodium iodide, zinc sulphide, lithium iodide, etc.
2. **Organic Crystals :** These types of crystals are generally employed in the scintillation counter which is used for the detection of the beta rays. They exhibit the pulse decay time of 10 nano seconds. The common examples of organic crystals are stilbene, anthracene, etc.
3. **Plastic Phosphors :** These types of crystals are used in the fast neutron detectors. They have very high hydrogen content and hence are the ideal fast neutron detectors. The pulse decay in these types of crystal is 1 or 2 nano seconds.

Liquid Scintillation Counter

The **Liquid Scintillation Counters** are majorly used in Life Sciences for measuring the beta emissions from the nuclides. The liquid scintillation counter uses a mixture of solvent and fluors, the mixture is often referred to as cocktail. The sample is added to the cocktail. The sample when emits a beta particle, the energy is transferred to the solvent. The solvent, in turn, transfer this energy to the fluors. The fluors receiving the energy get excited to the unstable state. It releases the extra energy and come down to the stable state. This energy is detected and beta radiations are measured. Few additives are added to these cocktails so as to shift the wavelength of emitted light in a region where it can be easy to detect.

The liquid scintillation counter has two PMTs for better and efficient beta particles detection. There are certain factors which interferes with the beta particles detections, these are:

- **Optical Cross Talk :** This occurs when certain events in one tube induces pulse in the other PMT tube. This factor is overcome by using sophisticated circuitry which can differentiate the events occurred due to sample or due to non sample.

- **Line Transmission Noise :** A faulty line may create a false detection and hence the experiment fails. To overcome such factor the electric line should be free from the noises by using good filters and equalisers.
- **Static Induced Noise :** The static charges are produced all around the PMT due to the friction of the vials containing the sample. For overcoming this factor the vials must be properly grounded.
- **Natural Radioactivity or Cosmic Radiation :** The natural radioactivity is the main cause for the spurious count of the particles. The natural radioactive emission interferes with the count by triggering the false electric pulses which could be counted. To overcome this factor the experimental setup should be properly shielded from the active and/or passive shielding materials.
- **Radio Frequency Interference :** These are common interferes. The source of RF could be any motor or light or switch. To overcome the interference caused by them is to use a RF circuitry which detects these types of Interference and removed them from triggering false pulse count in the counter.
- Apart from these factors there are several other factors which can affect the counting of the scintillation counter. In short, the liquid scintillation device is very sensitive and hence utmost care should be taken to avoid the false triggering of the pulses in the counter.

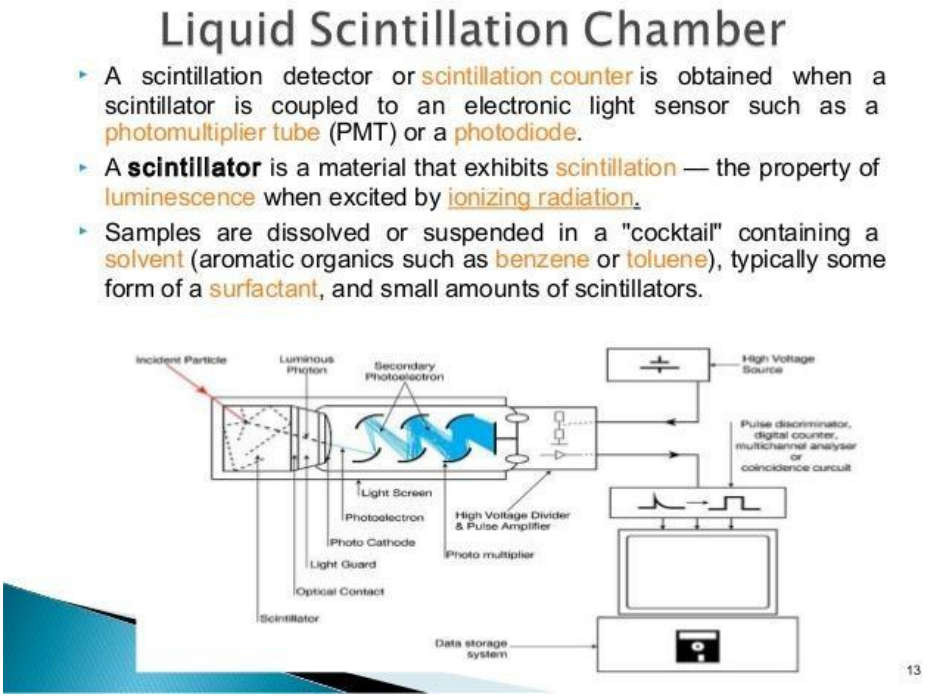
Scintillation Counting

The **Scintillation Counting** is based on count the pulses produced by the fluorescence of the scintillation material. The scintillation counter is composed of scintillator and the photo detector devices. The scintillators fluoresce when they come in contact of the high energy ionizing particle like beta or gamma rays. The electric pulses are produced by the scintillator which are proportional to the amount of the fluoresce.

Applications of the Scintillation Counter

The scintillation counters are used in several places to detect the radiations. Some of the examples are,

1. As a Portable survey meters.
2. Used in medical imaging.
3. Used as border security devices to detect any radiation from the enemy side.
4. Used as a monitoring device in the nuclear plant to monitor and trace the leakage of the nuclear radiation from the plant.
5. Used to detect the radon in the various day to day used materials especially water. The radon is radioactive, odourless, colourless noble gas and can get mixed easily with any substance.
6. In the industry to detect alpha or beta or gamma radiations. The industrial counter are made up of different types of fluoresce. One counter may contain two different fluoresce, like one for alpha particle detection and another rfor beta particle detection.
7. They are used as a spectrometers also, since they contains PMT which converts a high energy photon into several photon with lesser energy. These pulses could then be sorted with respect to their height and the total energy per height is calculated to approximate the energy spectrum of the incident radaitons.



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

COURSE MATERIAL

Subject Name: ANALYTICAL TECHNIQUES IN BIOTECHNOLOGY Code: SBB1201

UNIT I – V

MRI scan

The MRI scan uses magnetic and radio waves, meaning that there is no exposure to X-rays or any other damaging forms of radiation. How is an MRI scan performed?

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What is an MRI scan?

MRI (magnetic resonance imaging) is a fairly new technique that has been used since the beginning of the 1980s.

The MRI scanner uses magnetic and radio waves to create pictures of tissues, organs and other structures within the body, which can then be viewed on a computer.

This means that, unlike some other modes of medical imaging, there is no exposure to X-rays or any other damaging forms of radiation.

The pictures produced by an MRI scan, when compared to other imaging modalities, are much more detailed and therefore are of higher diagnostic quality when compared to more frequently used X-ray scanners for example.

How does an MRI scanner work?

The patient lies inside a large, cylinder-shaped magnet.

Radio waves 10,000 to 30,000 times stronger than the magnetic field of the earth are then sent through the body. This strong magnetic field causes the alignment of particles, called protons which are found naturally within the body, mostly in hydrogen atoms.

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UNIT I – V

Hydrogen, together with oxygen and carbon, make up 99 per cent of the average human body, and therefore almost all the protons found within the body are affected by the strong magnetic field.

This allows detailed MRI images to be created Once the magnetic field is switched off, the protons begin to lose their alignment, and go back to the position they were in before the magnetic field was applied.

As they move back into their original positions, they send out radio waves of their own. The scanner picks up these signals and a computer turns them into a picture. These pictures are based on the location and strength of the incoming signals.

Different protons send out different signals, depending on which tissue the proton can be found in. For example, a proton found in bone will emit a very different radio wave signal when compared to a proton found in blood.

It is these signal differences which allow a picture to be created, and allow different tissues or structures to be distinguished from one another.

What does an MRI scan show?

Using an MRI scanner, it is possible to make pictures of almost all the tissue in the body.

The tissue that has the least hydrogen atoms (such as bones) turns out dark, while the tissue that has many hydrogen atoms (such as fatty tissue) looks much brighter.

By changing the timing of the radiowave pulses it is possible to gain information about the different types of tissues that are present.

An MRI of the brain and spinal cord can be done to look at a multitude of different abnormalities, as it can provide clear pictures of these structures even though they are surrounded by bone tissue.

Changes within the tissues of the brain, whether subtle or gross, can help with a diagnosis and so determine treatment.

For example, an MRI of the brain can be done to look for the changes associated with bleeding or when the brain has been starved of oxygen after a stroke.

It can be used to investigate a traumatic brain injury, and also help diagnose developmental abnormalities.

It can be used to characterise tumours (benign or malignant abnormal growths) and check their progression, for example if they have changed in size and whether there has been spread to nearby tissues.

Within the heart, an MRI scan can give very detailed imaging of the thickness and size of the chamber walls. Damage occurring to the heart tissue after a heart attack or in association with valvular disease can also be assessed.

Other structures, such as the large blood vessels within the surrounding tissue can also be examined, for example to check for the build up of plaques, which can predispose the patient to having a heart attack in the future.

Congenital cardiovascular conditions can more accurately be assessed with MRI, along with changes to the heart which have occurred as a consequence of these conditions.

MRI is the imaging modality of choice for assessing joint problems. This is because joints are typically made up of a number of very different tissues, for example muscle, bone and ligaments, and MRI is very effective at giving clear images of these individual tissues. The method can also sometimes be used to image other parts of your body such as the liver, kidneys, spleen and breasts.

How does an MRI scan differ from a CT scan?

There's no ionizing radiation (X-rays) involved in producing an MRI scan. MRI scans are generally more detailed, too.

This is because they are more capable of illustrating all soft tissues and higher density tissues, such as bone.

For this reason an MRI is preferred over a CT scan for imaging structures such as joints, the brain, and the spinal cord, where clearer and more anatomically detailed images are required. CT scans should therefore not be used if fine detail of soft tissues is needed.

The difference between normal and abnormal tissue is often clearer on the MRI scan than on the CT scan, because the anatomical differences between these types of tissues is seen more clearly.

It is also possible to differentiate cystic lesions from malignant tumours, to identify areas of infection and inflammation and for assessment of join t tendon tears.

MRI scans are also more costly than CT scans, and take longer to complete. The length of the MRI scan depends on the region of the body being imaged. MRI scans may be indicated in those patients who are not able to have a CT scan.

For example patients with surgical clips, metallic fragments, cardiac pacemarkers, and for female patients who are pregnant.

It is also possible to produce images in any plane without having to move the patient when using an MRI scan.

What happens before an MRI scan and how should I prepare? How is an MRI scan performed?

The scan is usually done as an outpatient procedure, which means that the patient can go home after the test. During the scan it is important to lie completely still. For this reason it might be necessary to give a child an anaesthetic before they are tested.

Since you are exposed to a powerful magnetic field during the MRI scan, it is important not to wear jewellery or any other metal objects.

An MRI scan is not suitable for the patient if they have electrical appliances, such an a ear implant, implantable cardioverter defibrillator or pacemaker, or have any metal in their body such as surgical clips.

But orthopaedic metal ware, such as artificial hips or bone screws, are not normally a problem.

Is an MRI scan dangerous?

There are no known dangers or side effects connected to an MRI scan. The test is not painful; you cannot feel it. Since radiation is not used, the procedure can be repeated without problems. There is a small theoretical risk to the foetus in the first 12 weeks of pregnancy, and therefore scans are not performed on pregnant women during this time.

Because patients have to lie inside a large cylinder while the scans are being made some people get claustrophobic during the test. Patients who are afraid this might happen should talk to the doctor beforehand, who may give them some medication to help them relax.

The machine also makes a banging noise while it is working, which might be unpleasant.

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UNIT I – V

CT scan

A CT (computerised tomography) scanner is a special kind of X-ray machine.

What is a CT scanner?

A CT (computerised tomography) scanner is a special kind of X-ray machine. Instead of sending out a single X-ray through your body as with ordinary X-rays,

several beams are sent simultaneously from different angles.

This allows more detailed images from within the body to be constructed, and these images are then interpreted by a doctor.

CT scanners may also be referred to as CAT scans (computerised axial tomography). Unlike an MRI scanner, where you are placed within a tunnel, a CT scanner consists of a doughnut shaped machine and therefore you should not feel claustrophobic.

How does a CT scanner work?

The X-rays from the beams are detected after they have passed through the body and their strength is measured.

Beams that have passed through less dense tissue such as the lungs will be stronger, whereas beams that have passed through denser tissue such as bone will be weaker.

A computer can use this information to work out the relative density of the tissues examined. Each set of measurements made by the scanner is, in effect, a cross-section through the body.

The computer processes the results, displaying them as a two-dimensional picture shown on a monitor. The technique of CT scanning was developed by the British inventor Sir Godfrey Hounsfield, who was awarded the Nobel Prize for his work.

What are CT scans used for?

CT scans are far more detailed than ordinary X-rays.

The information from the two-dimensional computer images can be reconstructed to produce three-dimensional images by some modern CT scanners.

The CT scanner was originally designed to take pictures of the brain. Now it is much more advanced and is used for taking pictures of virtually any part of the body.

They can be used to produce virtual images that show what a surgeon would see during an operation. They are used for a variety of different reasons, mainly because CT imaging is one of the best and fastest tools for examining the chest, abdomen and pelvis, and because it can provide cross-sectional views and highly detailed images.

Some of the most common uses of CT imaging include detecting different types of cancer (for example in the lung, bowel, liver and kidney), examining patients with severe injuries and finding the cause for sudden rapid onset symptoms (such as breathlessness, abdominal pain).

CT is also used for the detection, diagnosis and treatment of a number of vascular disease, which may ultimately lead to stroke, kidney failure or blood clots in the lungs.

This also includes abdominal aortic anueryms (AAAs), which is where the large main artery running downwards in the abdomen becomes enlarged and therefore may be prone to spontaneously rupture.

CT is also used to diagnosing and analysing many spinal problems and injuries which may occur to the hands, feet and other skeletal structures.

This is because CT is good, not just for looking at soft tissue structures, but also in providing detailed images of even very small bones.

CT scans also allows doctors to inspect the inside of the body without having to operate or perform unpleasant examinations.

It allows surgeons to plan surgery prior to starting a procedure, and allows assessment of the results after a procedure has been performed.

CT scanning has also proven invaluable in pinpointing exactly where a tumour is and planning treatment with radiotherapy.

The scanner is particularly good at testing for bleeding in the brain, for aneurysms (when the wall of an artery swells up), brain tumours and brain damage. It can also find tumours and abscesses throughout the body and is used to assess types of lung disease.

In addition, the CT scanner is used to look at internal injuries such as a torn kidney, spleen or liver; or bony injury, particularly in the spine. CT scanning can also be used to guide biopsies and therapeutic pain relieving procedures.

How is a CT scan prepared for?

Patients should always be asked about any recent illnesses or medical conditions they may have, and whether there is a history of heart disease, asthma, diabetes, kidney disease or thyroid problems.

Any of these may increase the risk of the patient having an adverse reaction during the scan procedure.

Prior to starting the procedure, the patient will usually be given a gown to hospital gown to wear. It is important that metal objects, such as jewellery, eyeglasses, dentures and hairpins, are removed before starting the procedure, since these will affect the quality of the images.

The patient may also be asked to remove hearing aids and dental work, and women will be asked to remove bras containing metal underwire. Where possible, piercings should also be removed.

If the patient is receiving an abdomen scan, for example, they will be asked not to eat for six hours before the test. They will be given a drink containing gastrografin, an aniseed flavoured X-ray dye, 45 minutes before the procedure.

This makes the intestines easier to see on the pictures. Sometimes a liquid X-ray dye is injected into the veins during the test. This also makes it easier to see the organs, blood vessels or, for example, a tumour.

The injection might be a little uncomfortable, and some people also experience a feeling of warmth in their arm. It's important the patient inform their doctor prior to the administration of a contrast agent, if they have had a known allergy to contrast material, or 'dye'.

How is a CT scan carried out?

The scan is a non-invasive, painless medical procedure. It usually takes approximately 10 to 30 minutes to perform, depending on the part of the body that is being scanned, the number of pictures taken and the different angles required.

The scanner itself looks like a large doughnut, with a bed passing through it.

During the scan the patient lies on a bed, with the body part under examination placed in the round tunnel or opening of the scanner.

If contrast material is to be used it will either be swallowed, injected via an intravenous line (IV) or administered by enema.

How contrast is given depends on the type of examination to be carried out. The patient will usually be asked to hold their breath during the scan. This is because any kind of motion, such as breathing or body movement, can lead to degradation of the resulting image and so make it harder to analyse and interpret.

The bed then moves slowly backwards and forwards to allow the scanner to take pictures of the body, although it does not touch the patient.

During the scan, only the patient will be in the exam room during the time the scan is carried out, unless there are special circumstances, such as a parent needing to stay in the room with their child, in which case they are required to wear a lead apron to minimise the radiation exposure.

During the scan, the technician will communicate with the patient via a speaker in the scan room, and they will be able to hear and see the patient at all times.

Does a CT scan hurt?

The examination does not hurt but some people find it uncomfortable to lie within the scanner itself.

Although the scanner is not tunnel shaped like an MRI scanner, people who suffer from severe claustrophobia sometimes have problems with CT scans. Let the doctors and radiographers know if this might be a problem.

Other people get slightly nervous because of the whirring noise the machine makes while working.

If the patient feels this might be the case, then this should be discussed prior to the procedure being carried out, since the patient may be unable to lie still during the scan therefore giving pictures of such poor quality they could not be interpreted anyway.

Is a CT scan dangerous?

Far more X-rays are involved in a CT scan than in ordinary X-rays, so doctors do not recommend CT scans without a good medical reason.

However, the amount of radiation a patient is exposed to is small, and therefore are unlikely to cause any long term harm.

The risk is greatest to those who are pregnant, as radiation exposure can cause harm to the fetus, and therefore CT scans are contraindicated in pregnant women, unless the benefits of performing the scan far outweigh the risks.

Risks are also greater in children, when compared to adults, and therefore a CT is only recommended if a child has a serious condition that puts them at greater risk.

Some patients may experience side-effects due to allergic reactions to the liquid dye injected into the veins.

In very rare cases, this dye has been known to damage already weakened kidneys.

It is important to let the X-ray doctors or technicians know if you have any allergies, asthma or kidney trouble, prior to having the X-ray dye injected.

How is a CT scan read?

A CT scan can give the doctor a much clearer picture of the inside of the body than an ordinary X-ray. For example, different types of tissue such as bone, muscle and fatty tissue are easy to see on a CT scan.

When looking at the abdomen, the scan shows various organs such as the pancreas, spleen and liver.

Any pathology or abnormality with these organs can be identified, and the doctor looking after the patient will decide on the best way to follow up any findings made on the CT scan. When it is necessary to look at the brain, the areas containing liquid – the ventricles – are also clearly defined.

Very small shadows on the lungs can also be detected using CT and there are now studies looking into using it as a screening test for lung cancer.

How will I find out the results of my scan?

The results are analysed and interpreted by a radiologist, which is a doctor who is trained to supervise and interpret radiological procedures and images.

The radiologist will write a report based on the scan results, and this will be sent either to your GP or to the doctor who originally referred you for the scan.

They will then be in contact with you to discuss the results. In some cases, it may be necessary to have a follow-up examination, for example if there was a finding on the CT san which needs further investigation before a diagnosis can be made, or if the finding warrants a more specialised imaging technique.

Follow-up scans may also be necessary if an abnormality needs monitoring over a certain amount of time, to see if it is stable, or if treatment is being given whether the abnormality is responding to treatment.

In all cases, the referring doctor will discuss why they feel it is necessary to have a follow- up examination.

UNIT V

Nucleic acid Quantification

In molecular biology, **quantitation of nucleic acids** is commonly performed to determine the average concentrations of DNA or RNA present in a mixture, as well as their purity. Reactions that use nucleic acids often require particular amounts and purity for optimum performance. There are several methods to establish the concentration of a solution of nucleic acids, including spectrophotometric quantification and UV fluorescence in presence of a DNA dye.

Spectrophotometric analysis

Nucleic acids absorb ultraviolet light in a specific pattern. In a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm, and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample.

Using the Beer-Lambert Law it is possible to relate the amount of light absorbed to the concentration of the absorbing molecule. At a wavelength of 260 nm, the average extinction coefficient for double-stranded DNA is 0.020 $(\mu g/ml)^{-1}$ cm^{-1}, for single-stranded DNA it is $(\mu g/ml)^{-1}$ cm^{-1}, for single-stranded RNA it is 0.025 $(\mu g/ml)^{-1}$ cm^{-1} and for short single-stranded oligonucleotides it is dependent on the length and base composition. Thus, an Absorbance (A) of 1 corresponds to a concentration of 50 μg/ml for double-stranded DNA. This method of calculation is valid for up to an A of at least 2. A more accurate extinction coefficient may be needed for oligonucleotides; these can be predicted using the nearest-neighbor model.

Sample purity

It is common for nucleic acid samples to be contaminated with other molecules (i.e. proteins, organic compounds, other). The ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) is used

to assess the purity of nucleic acids. For pure DNA, $A_{260/280}$ is widely considered ~1.8 but has been argued to translate - due to numeric errors in the original Warburg paper - into a mix of 60% protein and 40% DNA.[3] The ratio for pure RNA $A_{260/280}$ is ~2.

Protein contamination and the 260:280 ratio

The ratio of absorbance at 260 nm vs 280 nm is commonly used to assess DNA contamination of protein solutions, since proteins (in particular, the aromatic amino acids) absorb light at 280 nm. The reverse, however, is not true — it takes a relatively large amount of protein contamination to significantly affect the 260:280 ratio in a nucleic acid solution. 260:280 ratio has high sensitivity for nucleic acid contamination in protein:

% protein	% nucleic acid	260:280 ratio
100	0	0.57
95	5	1.06
90	10	1.32
70	30	1.73

260:230 ratio lacks sensitivity for protein contamination in nucleic acids (table shown for RNA, 100% DNA is approximately 1.8):

% nucleic acid	% protein	260:230 ratio
100	0	2.00
95	5	1.99
90	10	1.98
70	30	1.94

This difference is due to the much higher extinction coefficient nucleic acids have at 260 nm and 280 nm, compared to that of proteins. Because of this, even for relatively high concentrations of protein, the protein contributes relatively little to the 260 and 280 absorbance.

While the protein contamination cannot be reliably assessed with a 260:280 ratio, this also means that it contributes little error to DNA quantity estimation.

Other common contaminants

- Contamination by phenol, which is commonly used in nucleic acid purification, can significantly throw off quantification estimates. Phenol absorbs with a peak at 270 nm and a $A_{260/280}$ of 1.2. Nucleic acid preparations uncontaminated by phenol should have a $A_{260/280}$ of around 2.[1] Contamination by phenol can significantly contribute to overestimation of DNA concentration.
- Absorption at 230 nm can be caused by contamination by phenolate ion, thiocyanates, and other organic compounds. For a pure RNA sample, the $A_{230:260:280}$ should be around 1:2:1, and for a pure DNA sample, the $A_{230:260:280}$ should be around 1:1.8:1.
- Absorption at 330 nm and higher indicates particulates contaminating the solution, causing scattering of light in the visible range. The value in a pure nucleic acid sample should be zero

Negative values could result if an incorrect solution was used as blank. Alternatively, these values could arise due to fluorescence of a dye in the solution.

Quantification using fluorescent dyes

An alternative way to assess DNA concentration is to measure the fluorescence intensity of dyes that bind to nucleic acids and selectively fluoresce when bound (e.g. Ethidium bromide). This method is useful for cases where concentration is too low to accurately assess with spectrophotometry and in cases where contaminants absorbing at 260 nm make accurate quantitation by that method impossible.

There are two main ways to approach this. "Spotting" involves placing a sample directly onto an agarose gel or plastic wrap. The fluorescent dye is either present in the agarose gel, or is added in appropriate concentrations to the samples on the plastic film. A set of samples with known concentrations are spotted alongside the sample. The concentration of the unknown sample is then estimated by comparison with the fluorescence of these known concentrations. Alternatively, one may run the sample through an agarose or polyacrylamide gel, alongside some samples of known concentration. As with the spot test, concentration is estimated through comparison of fluorescent intensity with the known samples.

If the sample volumes are large enough to use microplates or cuvettes, the dye-loaded samples can also be quantified with a fluorescence photometer. Minimum sample volume starts at 0.3 μl.

Blotting Techniques

Southern blotting

A Southern blot is a method used for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. The method is named after its inventor, the British biologist Edwin Southern.

The procedure

High molecular weight DNA strands are cut into smaller fragments by restriction endonucleases. The DNA fragments are separated by size by agarose gel electrophoresis and then transferred to a nitrocellulose membrane which is placed on the top of the gel (Figure-1). In Sourthen blotting, before transfer, DNA is usually denatured with alkali for denaturation of the double stranded DNA. The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for later hybridization to the probe, and destroys any residual RNA that may still be present in the DNA. After transfer of the DNA fragments to the nitrocellulose membrane

which is done by capillary action or may be by electrotransfer, vacuum transfer or centrifugation, the membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours to permanently attach the transferred DNA to the membrane. The membrane is then exposed to a hybridization probe(a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined). The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. After hybridization, excess probe is washed from the membrane, and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of color on the membrane if a chromogenic detection method is used.

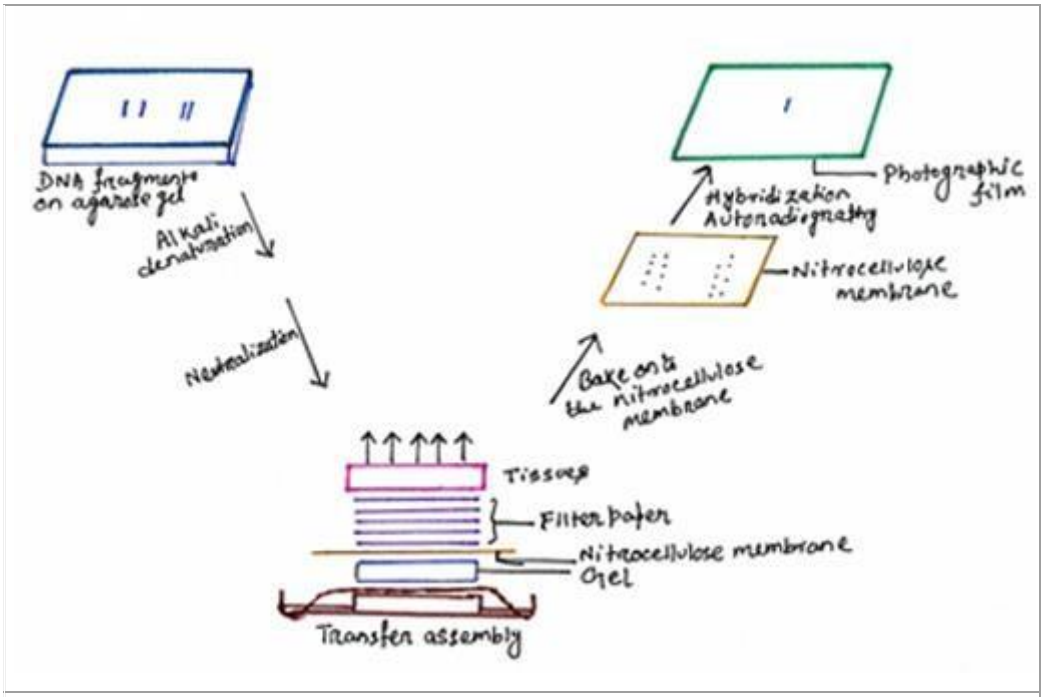

Figure 1: Southern blotting

The hybridization and washing conditions are critical. If the probe and target are 100% identical in sequence, then a high stringency hybridization can be carried out. The stringency is determined by the hybridization temperature and the salt concentration in the hybridization buffer. For probes that don't match the target completely, the stringency must be reduced to a

level that allows imperfect hybrids to form. If the stringency of the hybridization is too low, then the probe may bind to too many sequences to be useful. Formamide can be included in the hybridization buffer to reduce the actual hybridization temperature by about 25°C, from the usual 68°C to the more convenient 43°C.

Applications

Southern hybridization can also be used to locate the exact position of a cloned gene within a recombinant DNA molecule. This is important as often the cloned DNA fragment is relatively large (40 kb for a cosmid vector) whereas the gene of interest, contained somewhere in the cloned fragment, may be less than 1kb in size. Southern blots of cloned genomic DNA fragments can be probed with cDNA molecules to find which parts of the genomic clone correspond to the cDNA fragment. If the Southern blot contains genomic DNA fragments from the whole genome, the probe will give information about the size of the fragment the gene is on the genome and how many copies of the gene are present in the genome.

Northern blotting

Northern blotting, the name was extrapolated from Southern blotting. The northern blot is a technique used to study gene expression by detection of RNA (or isolated mRNA) in a sample.

The Procedure

The nucleic acid molecules (RNA samples) are separated by agarose gel electrophoresis and then transferred to a nitrocellulose membrane but for RNA in Northern blotting, alkali denaturation is not necessary and would in any case hydrolyze the molecules. A nylon membrane with a positive charge is the most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them. The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction, thus preventing RNA degradation by high temperatures. Once the RNA has been transferred to the membrane, it is immobilized through covalent linkage to the membrane

by UV light or heat (Figure-2). After a probe has been labeled, it is hybridized to the RNA on the membrane. Experimental conditions that can affect the efficiency and specificity of hybridization include ionic strength, viscosity, duplex length, mismatched base pairs, and base composition. The membrane is washed to ensure that the probe has bound specifically and to avoid background signals from arising. The hybrid signals are then detected by X-ray film.

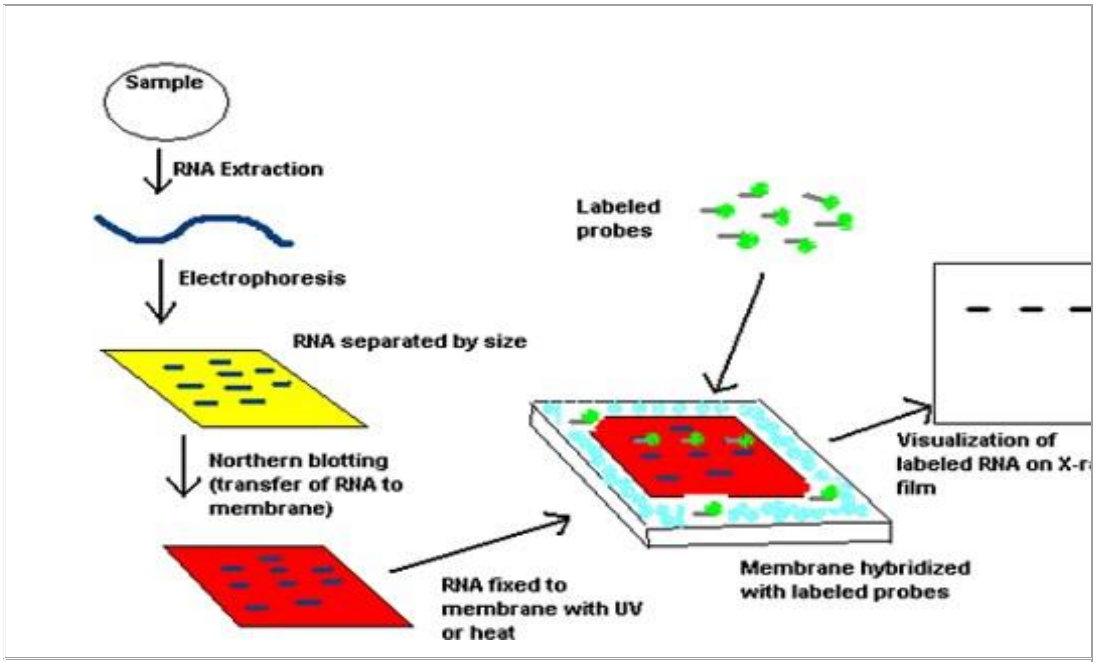

Figure 2: Outline of the general procedure for RNA detection by northern blotting

Applications

Northern blots give information about the size of the mRNA and any precursors, and can be useful to determine whether a cDNA clone used as a probe is full-length or whether it is one of a family of related transcripts. Northern blots can help to identify whether a genomic clone has regions that are transcribed and, if the RNA on the blot is made from different tissues, where these transcripts are made. With northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions.

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INSTITUTE OF SCIENCE AND TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

COURSE MATERIAL

Subject Name: ANALYTICAL TECHNIQUES IN BIOTECHNOLOGY Code: SBB1201

UNIT I – V

Western blotting

Identification of a specific protein in a complex mixture of proteins can be done by a technique known as western blotting. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin, et al. in 1979 and is now a routine technique for protein analysis. The procedure for Western blotting is given below.

Gel electrophoresis

In Western blotting, first a protein mixture is separated by electrophoresis on an SDS-polyacrylamide gel (SDS-PAGE), a slab gel infused with sodeum dodecyl sulphate (SDS), a dissociating agent (Figure-3). Proteins are commonly separated using polyacrylamide gel electrophoresis (PAGE) to characterize individual proteins in a complex sample or to examine multiple proteins within a single sample. When combined with Western blotting, PAGE is a powerful analytical tool providing information on the mass, charge, purity or presence of a protein. Several forms of PAGE exist and can provide different types of information about the protein(s). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilodaltons, kDa). The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots. Samples are loaded into wells in the gel. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different electrophoretic mobilities) separate into bands within each lane.

Transfer of proteins to a membrane

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). There are a variety of methods that have been used for this process, including diffusion transfer, capillary transfer, heat-accelerated convectional transfer, vacuum blotting transfer and electroelution. In capillary transfer, the membrane is placed on top of the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it.But this method of transfer is very time consuming. The transfer method that is most commonly used for proteins is electroelution or electrophoretic transfer because of its speed and transfer efficiency. Electrophoretic transfer of proteins involves placing a protein-containing polyacrylamide gel in direct contact with a piece of nitrocellulose or other suitable, protein-binding support and "sandwiching" this between two electrodes submerged in a conducting solution. When an electric field is applied, the proteins move out of the polyacrylamide gel and onto the surface of the membrane, where the proteins become tightly attached (Figure-3). The result is a membrane with a copy of the protein pattern that was originally in the polyacrylamide gel. The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie Brilliant Blue or Ponceau S dyes.

Blocking non-specific sites

After the transfer of the proteins from the gel, the remaining surface of the membrane is blocked to prevent non-specific binding of the detection antibodies during subsequent steps. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk in Tris-Buffered Saline (TBS), with a minute percentage of detergent such as Tween 20 or Triton X-100. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the western blot, leading to clearer results, and eliminates false positives.

Detection

During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme; when exposed to an appropriate substrate this enzyme drives a colourimetric reaction and produces a colour.

Incubation with the primary antibody

Western blotting is typically performed by probing the blocked membrane with a primary antibody that recognizes a specific protein or epitope on a group of proteins (i.e., SH2 domain or phosphorylated tyrosine). The choice of a primary antibody for a Western blot will depend on the antigen to be detected and what antibodies are available to that antigen.

After blocking, a dilute solution of primary antibody is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. If incubating in blocking buffer overnight, it is imperative to incubate at 4°C or contamination will incur and thus destruction of the protein (especially phospho groups). Agitation of the antibody is recommended to enable adequate homogenous covering of the membrane and prevent uneven binding.

Incubation with secondary antibody

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. A wide variety of labeled secondary detection reagents can be used for Western blot detection. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

Methods of detection

Enzymatic labels are most commonly used for Western blotting and, although they require extra steps, can be extremely sensitive when optimized with an appropriate substrate. Alkaline

phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensively as labels for protein detection. The high activity rate, good stability, low cost and wide availability of substrates make HRP the enzyme of choice for most applications. After binding of the enzyme-antibody conjugate, addition of a chromogenic substrate (Figure-3) that produces a highly coloured and insoluble product causes the appearance of a coloured band at the site of the target antigen. The site of the protein of interest can be determined with much higher sensitivity if a chemiluminescent compound along with suitable enhancing agents is used to produce light at the antigenic site.

The second method method of secondary antibody detection utilizes a near-infrared (NIR) fluorophore-linked antibody. Light produced from the excitation of a fluorescent dye is static, making fluorescent detection a more precise and accurate measure of the difference in signal produced by labeled antibodies bound to proteins on a western blot. Proteins can be accurately quantified because the signal generated by the different amounts of proteins on the membranes is measured in a static state, as compared to chemiluminescence, in which light is measured in a dynamic state. The use of fluorophore-conjugated antibodies in immunoassays requires fewer steps because there is no substrate development step in the assay. This method requires special equipment in order to detect and document the fluorescent signal due to the need for an excitation light source.

Another alternative is to use a radioactive label rather than an enzyme coupled to the secondary antibody. If the protein of interest was bound by a radioactive antibody, its position on the blot can be determined by exposing the membrane to a shit of x-ray film, a procedure called autoradiography.

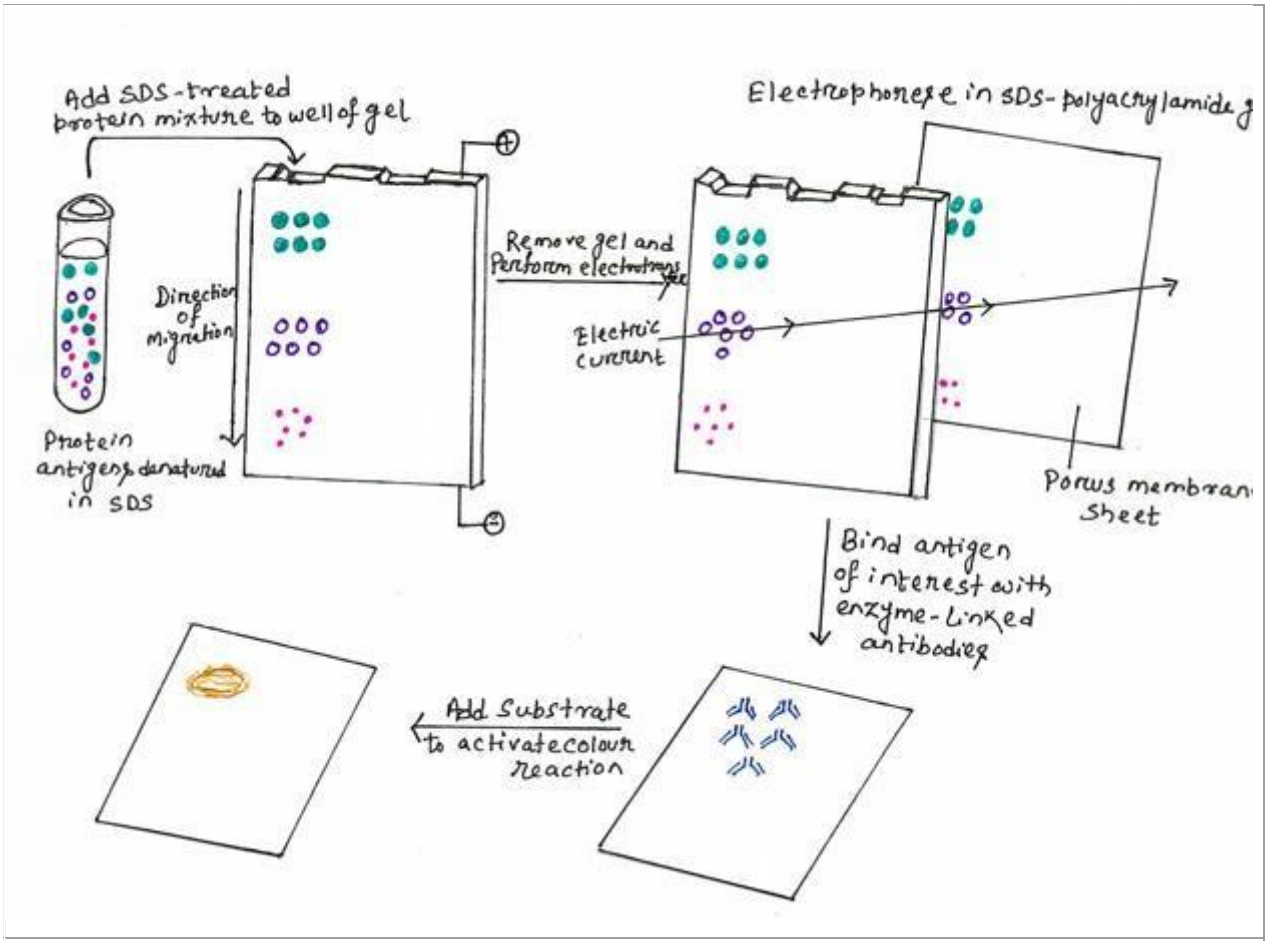

Figure 3: Method of Western blotting

Applications

- Western blotting can be used to identify a specific antibody in a mixture. In this case, known antigens of well-defined molecular weight are separated by SDS-PAGE and blotting onto nitrocellulose. The separated bands of known antigens are then probed with the sample suspected of containing antibody specific for one or more of these antigens. Reaction of an antibody with a band is detected by using either radiolabeled or enzyme linked secondary antibody that is specific for the species of the antibodies in the test sample. The most widely used application of this procedure is in confirmatory testing for HIV, where Western blotting is used to determine whether the patient has antibodies that react with one or more viral

proteins.

- A western blot is also used as the definitive test for mad cow disease.
- Western blot can also be used as a confirmatory test for Hepatitis B infection.

Methods of Gene Transfer

The two methods are: (1) Vector-Mediated Gene Transfer and (2) Direct or Vector-less DNA Transfer.

Gene Transfer Methods:

The gene transfer techniques in plant genetic transformation are broadly grouped into two categories:

I. Vector-mediated gene transfer

II. Direct or vector less DNA transfer

The salient features of the commonly used gene (DNA) transfer methods are given in Table 49.1.

TABLE 49.1 Gene transfer (DNA delivery) methods in plants

Method	*Salient features*
I. Vector–mediated gene transfer	
Agrobacterium (Ti plasmid)-mediated gene transfer	Very efficient, but limited to a selected group of plants
Plant viral vectors	Ineffective method, hence not widely used
II. Direct or vectorless DNA transfer	
(A) Physical methods	
Electroporation	Mostly confined to protoplasts that can be regenerated to viable plants. Many cereal crops developed.
Microprojectile (particle bombardment)	Very successful method used for a wide range of plants/ tissues. Risk of gene rearrangement high.
Microinjection	Limited use since only one cell can be microinjected at a time. Technical personnel should be highly skilled.
Liposome fusion	Confined to protoplasts that can be regenerated into viable whole plants.
Silicon carbide fibres	Requires regenerable cell suspensions. The fibres, however, require careful handling.
(B) Chemical methods	
Polyethylene glycol (PEG)-mediated	Confined to protoplasts. Regeneration of fertile plants is frequently problematical.
Diethylaminoethyl (DEAE) dextran-mediated	Does not result in stable transformants.

Method # I. Vector-Mediated Gene Transfer:

Vector-mediated gene transfer is carried out either by Agrobacterium-mediated transformation or by use of plant viruses as vectors.

Agrobacterium-Mediated Gene Transfer:

Agrobacterium tumefaciens is a soil-borne, Gram-negative bacterium. It is rod shaped and motile, and belongs to the bacterial family of Rhizobiaceae. A. tumefaciens is a phytopathogen, and is treated as the nature's most effective plant genetic engineer.

Some workers consider this bacterium as the natural expert of inter-kingdom gene transfer. In fact, the major credit for the development of plant transformation techniques goes to the natural unique capability of A. tumefaciens. Thus, this bacterium is the most beloved by plant biotechnologists.

There are mainly two species of Agrobacterium:

i. A. tumefaciens that induces crown gall disease.

ii. A. rhizogenes that induces hairy root disease.

Crown Gall Disease and Ti Plasmid:

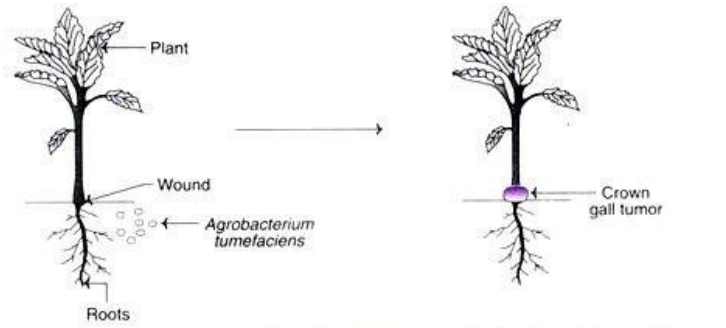

Fig. 49.1 : Formation of a crown gall tumor in a plant infected with Agrobacterium tumefaciens.

Almost 100 years ago (1907), Smith and Townsend postulated that a bacterium was the causative agent of crown gall tumors, although its importance was recognized much later. As A. tumefaciens infects wounded or damaged plant tissues, in induces the formation of a plant tumor called crown gall (Fig. 49.1). The entry of the bacterium into the plant tissues is facilitated by the release of certain phenolic compounds (acetosyringone,

hydroxyacetosyringone) by the wounded sites.

Crown gall formation occurs when the bacterium releases its Ti plasmid (tumor- inducing plasmid) into the plant cell cytoplasm. A fragment (segment) of Ti plasmid, referred to as T-DNA, is actually transferred from the bacterium into the host where it gets integrated into the plant cell chromosome (i.e. host genome). Thus, crown gall disease is a naturally evolved genetic engineering process.

The T-DNA carries genes that code for proteins involved in the biosynthesis of growth hormones (auxin and cytokinin) and novel plant metabolites namely opines — amino acid derivatives and agropines — sugar derivatives (Fig. 49.2).

The growth hormones cause plant cells to proliferate and form the gall while opines and agropines are utilized by A. tumefaciens as sources of carbon and energy. As such, opines and agropines are not normally part of the plant metabolism (neither produced nor metabolised). Thus, A. tumefaciens genetically transforms plant cells and creates a biosynthetic machinery to produce nutrients for its own use.

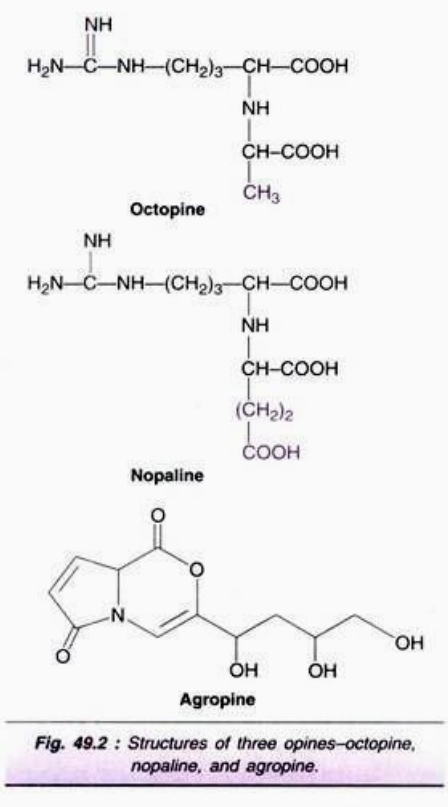

Fig. 49.2 : Structures of three opines–octopine, nopaline, and agropine.

As the bacteria multiply and continue infection, grown gall develops which is a visible mass of the accumulated bacteria and plant material. Crown gall formation is the consequence of the transfer, integration and expression of genes of T-DNA (or Ti plasmid) of A. tumefaciens in the infected plant.

The genetic transformation leads to the formation of crown gall tumors, which interfere with the normal growth of the plant. Several dicotyledonous plants (dicots) are affected by crown gall disease e.g. grapes, roses, stone-fruit trees.

Organization of Ti plasmid:

The Ti plasmids (approximate size 200 kb each) exist as independent replicating circular DNA molecules within the Agrobacterium cells. The T-DNA (transferred DNA) is variable in length in the range of 12 to 24 kb, which depends on the bacterial strain from which Ti plasmids come. Nopaline strains of Ti plasmid have one T-DNA with length of 20 kb while octopine strains have two T-DNA regions referred to as T_L and T_R that are respectively 14 kb and 7 kb in length.

A diagrammatic representation of a Ti plasmid is depicted in Fig. 49.3. The Ti plasmid has three important regions.

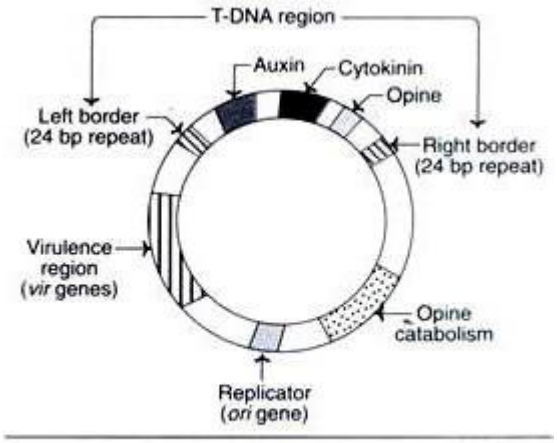

Fig. 49.3 : A dagrammatic representation of a Ti plasmid.

1. T-DNA region:

This region has the genes for the biosynthesis of auxin (aux), cytokinin (cyt) and opine (ocs), and is flanked by left and right borders. These three genes-aux, cyto and ocs are referred to as oncogenes, as they are the determinants of the tumor phenotype.

T-DNA borders — A set of 24 kb sequences present on either side (right and left) of T-DNA are also transferred to the plant cells. It is now clearly established that the right border is more critical for T-DNA transfer and tumori-genesis.

2. Virulence region:

The genes responsible for the transfer of T-DNA into the host plant are located outside T-DNA and the region is referred to as vir or virulence region. Vir region codes for proteins involved in T-DNA transfer. At least nine vir-gene operons have been identified. These include vir A, vir G, vir B1, vir C1, vir D1, D2 and D4, and vir E1, and E2.

3. Opine catabolism region:

This region codes for proteins involved in the uptake and metabolisms of opines. Besides the above three, there is ori region that is responsible for the origin of DNA replication which permits the Ti plasmid to be stably maintained in A. tumefaciens.

T-DNA transfer and integration:

The process of T-DNA transfer and it integration into the host plant genome is depicted in Fig. 49.4, and is briefly described.

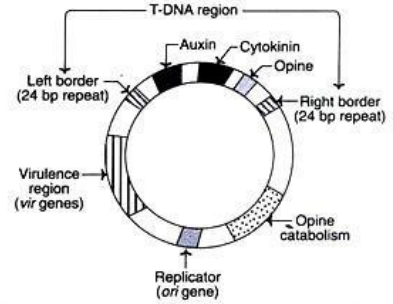

Fig. 49.3 : A dagrammatic representation of a Ti plasmid.

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INSTITUTE OF SCIENCE AND TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

COURSE MATERIAL

Subject Name: ANALYTICAL TECHNIQUES IN BIOTECHNOLOGY Code: SBB1201

UNIT I – V

1. Signal induction to Agrobacterium:

The wounded plant cells release certain chemicals- phenolic compounds and sugars which are recognized as signals by Agrobacterium. The signals induced result in a sequence of biochemical events in Agrobacterium that ultimately helps in the transfer of T-DNA of T-plasmid.

2. Attachment of Agrobacterium to plant cells:

The Agrobacterium attaches to plant cells through polysaccharides, particularly cellulose fibres produced by the bacterium. Several chromosomal virulence (chv) genes responsible for the attachment of bacterial cells to plant cells have been identified.

3. Production of virulence proteins:

As the signal induction occurs in the Agrobacterium cells attached to plant cells, a series of events take place that result in the production of virulence proteins. To start with, signal induction by phenolics stimulates vir A which in turn activates (by phosphorylation) vir C. This induces expression of virulence genes of Ti plasmid to produce the corresponding virulence proteins (D1, D2, E2, B etc.). Certain sugars (e.g. glucose, galactose, xylose) that induce virulence genes have been identified.

4. Production of T-DNA strand:

The right and left borders of T-DNA are recognized by vir D1/vir D2 proteins. These proteins are involved in the production single-stranded T-DNA (ss DNA), its protection and export to plant cells. The ss T-DNA gets attached to vir D2.

5. Transfer of T-DNA out of Agrobacterium:

The ss T-DNA — vir D2 complex in association with vir G is exported from the bacterial cell. Vir B products form the transport apparatus.

6. Transfer of T-DNA into plant cells and integration:

The T-DNA-vir D2 complex crosses the plant plasma membrane. In the plant cells, T-DNA gets covered with vir E2. This covering protects the T-DNA from degradation by nucleases; vir D2 and vir E2 interact with a variety of plant proteins which influences T-DNA transport and integration.

The T-DNA-vir D2-vir E2 — plant protein complex enters the nucleus through nuclear pore complex. Within the nucleus, the T-DNA gets integrated into the plant chromosome through a process referred to illegitimate recombination. This is different from the homologous recombination, as it does not depend on the sequence similarity.

Hairy Root Disease of A. Rhizogenes — R1 Plasmids:

Agrobacterium rhizogenes can also infect plants. But this results in hairy roots and not crown galls as is the case with A. tumefaciens. The plasmids, of A. rhizogenes have been isolated and characterized. These plasmids, referred to as Ri plasmids, (Ri stands for Root inducing) are of different types. Some of the Ri plasmid strains possess genes that are homologous to Ti plasmid e.g. auxin biosynthetic genes.

Instead of virulence genes, Ri plasmids contain a series of open reading frames on the T-DNA. The products of these genes are involved in the metabolism of plant growth regulators which gets sensitized to auxin and leads to root formation.

Vectors of A. rhizogenes:

As it is done with A tumefaciens, vectors can be constructed by using A. rhizogenes. These vectors are alternate strategies for gene transfer. However, employment of A. rhizogene-based vectors for plant transformation is not common since more efficient systems of A. tumefaciens have been developed.

Importance of hairy roots:

Hairy roots can be cultured in vitro, and thus are important in plant biotechnology. Hairy root

systems are useful for the production of secondary metabolites, particularly pharmaceutical proteins.

Ti Plasmid-Derived Vector Systems:

The ability of Ti plasmid of Agrobacterium to genetically transform plants has been described. It is possible to insert a desired DNA sequence (gene) into the T-DNA region (of Ti plasmid), and then use A. tumefaciens to deliver this gene(s) into the genome of plant cell.

In this process, Ti plasmids serve as natural vectors. However, there are several limitations to use Ti plasmids directly as cloning vectors:

i. Ti plasmids are large in size (200-800 kb). Smaller vectors are preferred for recombinant experiments. For this reason, large segments of DNA of Ti plasmid, not essential for cloning, must be removed.

ii. Absence of unique restriction enzyme sites on Ti plasmids.

iii. The phytohormones (auxin, cytokinin) produced prevent the plant cells being regenerated into plants. Therefore auxin and cytokinin genes must be removed.

iv. Opine production in transformed plant cells lowers the plant yield. Therefore opine synthesizing genes which are of no use to plants should be removed.

v. Ti plasmids cannot replicate in E. coli. This limits their utility as E. coli is widely used in recombinant experiments. An alternate arrangement is to add an origin of replication to Ti plasmid that allows the plasmid to replicate in E. coli.

Considering the above limitations, Ti plasmid- based vectors with suitable modifications have been constructed.

These vectors are mainly composed of the following components:

1. The right border sequence of T-DNA which is absolutely required for T-DNA integration into plant cell DNA.
2. A multiple cloning site (poly-linker DNA) that promotes the insertion of cloned gene into the region between T-DNA borders.
3. An origin of DNA replication that allows the plasmids to multiply in E. coli.
4. A selectable marker gene (e.g. neomycin phosphotransferase) for appropriate selection of

the transformed cells.

Two types of Ti plasmid-derived vectors are used for genetic transformation of plants— co-integrate vectors and binary vectors.

Co-integrate vector:

In the co-integrate vector system, the disarmed and modified Ti plasmid combines with an intermediate cloning vector to produce a recombinant Ti plasmid (Fig. 49.5).

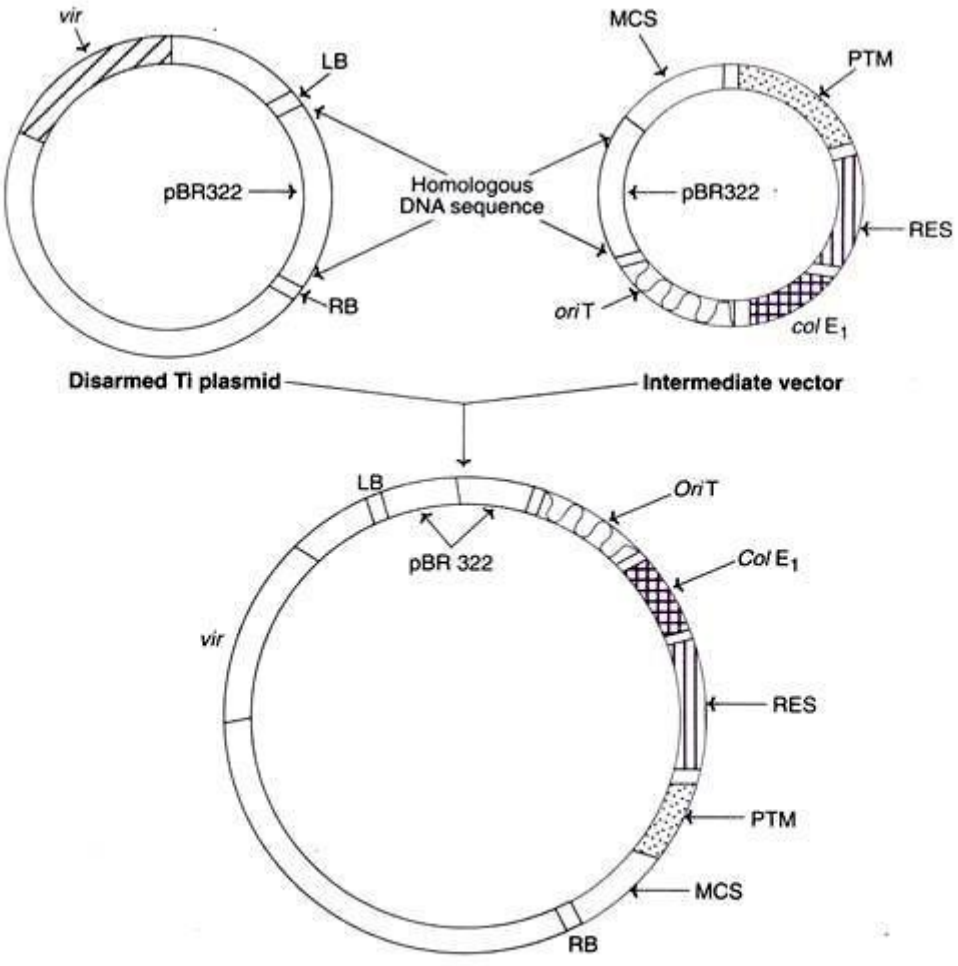


***Fig. 49.5** : Cointegrate vector system (vir-Ti plasmid virulence region; pBR322-Bacterial plasmid 322; LB-Left border; RB-Right border; MCS-Multiple cloning site; PTM-Plant transformation marker; RES-Bacterial resistance marker; col E_1-Origin of a replication from col E_1 plasmid; ori T-Origin of transfer site for conjugative plasmid mobilization).*

Production of disarmed Ti plasmid:

The T-DNA genes for hormone biosynthesis are removed (disarmed). In place of the deleted DNA, a bacterial plasmid (pBR322) DNA sequence is incorporated. This disarmed plasmid, also referred to as receptor plasmid, has the basic structure of T-DNA (right and left borders, virulence genes etc.) necessary to transfer the plant cells.

Construction of intermediate vector:

The intermediate vector is constructed with the following components:

i. A pBR322 sequence DNA homologous to that found in the receptor Ti plasmid.

ii. A plant transformation marker (PTM) e.g. a gene coding for neomycin phosphotransferase II (npt II). This gene confers resistance to kanamycin in the plant cells and thus permits their isolation.

iii. A bacterial resistance marker e.g. a gene coding for spectinomycin resistance. This gene confers spectinomycin resistance to recipient bacterial cells and thus permits their selective isolation.

iv. A multiple cloning site (MCS) where foreign genes can be inserted.

v. A Co/E1 origin of replication which allows the replication of plasmid in E. coli but not in Agrobacterium.

vi. An oriT sequence with basis of mobilization (bom) site for the transfer of intermediate vector from E. coli to Agrobacterium.

Production and use of co-integrate vectors:

The desired foreign gene (target-gene) is first cloned in the multiple cloning site of the intermediate vector. The cloning process is carried out in E. coli, the bacterium where the cloning is most efficient. The intermediate vector is mated with Agrobacterium so that the foreign gene is mobilised into the latter.

The transformed Agrobacterium cells with receptor Ti plasmid and intermediate vector are selectively isolated when grown on a minimal medium containing spectinomycin. The selection

process becomes easy since E. coli does not grow on a minimal medium in which Agrobacterium grows.

Within the Agrobacterium cells, intermediate plasmid gets integrated into the receptor Ti plasmid to produce co-integrate plasmid. This plasmid containing plant transformation marker (e.g. npt II) gene and cloned target gene between T-DNA borders is transferred to plant cells. The transformed plant cells can be selected on a medium containing kanamycin when the plant and Agrobacterium cells are incubated together.

Advantages of co-integrate vector:

i. Target genes can be easily cloned
ii. The plasmid is relatively small with a number of restriction sites.
iii. Intermediate plasmid is conveniently cloned in E. coli and transferred to Agrobacterium.

Binary vector:

The binary vector system consists of an Agrobacterium strain along with a disarmed Ti plasmid called vir helper plasmid (the entire T-DNA region including borders deleted while vir gene is retained). It may be noted that both of them are not physically linked (or integrated). A binary vector with T-DNA can replicate in E. coli and Agrobacterium.

A diagrammatic representation of a typical binary vector system is depicted in Fig. 49.6. The binary vector has the following components.

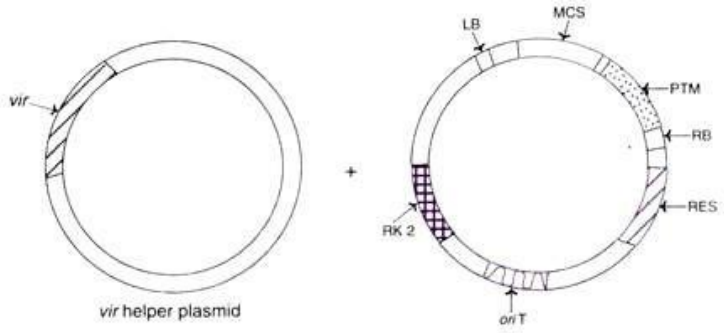


Fig. 49.6 : Binary vector system (vir-Ti plasmid virulence region; LB-Left border; RB-Right border; MCS-Multiple cloning site; PTM-Plant transformation marker; RES-Bacterial resistance marker; oriT-Origin of transfer site for conjugative plasmid mobilization; RK_2-Origin of replication from plasmid).

1. Left and right borders that delimit the T-DNA region.
2. A plant transformation marker (PTM) e.g. npt II that confers kanamycin resistance in plant transformed cells.
3. A multiple cloning site (MCS) for introducing target/foreign genes.
4. A bacterial resistance marker e.g. tetracycline resistance gene for selecting binary vector colonies in E. coli and Agrobacterium.
5. oriT sequence for conjugal mobilization of the binary vector from E. coli to Agrobacterium.
6. A broad host-range origin of replication such as RK2 that allows the replication of binary vector in Agrobacterium.

Production and use of binary vector:

The target (foreign) gene of interest is inserted into the multiple cloning site of the binary vector. In this way, the- target gene is placed between the right and left border repeats and cloned in E. coli. By a mating process, the binary vector is mobilised from E. coli to Agrobacterium. Now, the virulence gene proteins of T-DNA facilitate the transfer of T-DNA of the vector into plant cells.

Advantages of binary vectors:

i. The binary vector system involves only the transfer of a binary plasmid to Agrobacterium without any integration. This is in contrast to co-integrate vector system wherein the intermediate vector is transferred and integrated with disarmed Ti plasmid.
ii. Due to convenience, binary vectors are more frequently used than co-integrate vectors.

Plant Transformation Technique Using Agrobacterium:

Agrobacterium-mediated technique is the most widely used for the transformation of plants and generation of transgenic plants. The important requirements for gene transfer in higher plants through Agrobacterium mediation are listed.

i. The explants of the plant must produce phenolic compounds (e.g. autosyringone) for activation of virulence genes.

ii. Transformed cells/tissues should be capable to regenerate into whole plants.

In general, most of the Agrobacterium-mediated plant transformations have the following basic protocol (Fig. 49.7)

1. Development of Agrobacterium carrying the co-integrate or binary vector with the desired gene.
2. Identification of a suitable explant e.g. cells, protoplasts, tissues, calluses, organs.
3. Co-culture of explants with Agrobacterium.
4. Killing of Agrobacterium with a suitable antibiotic without harming the plant tissue.
5. Selection of transformed plant cells.
6. Regeneration of whole plants.

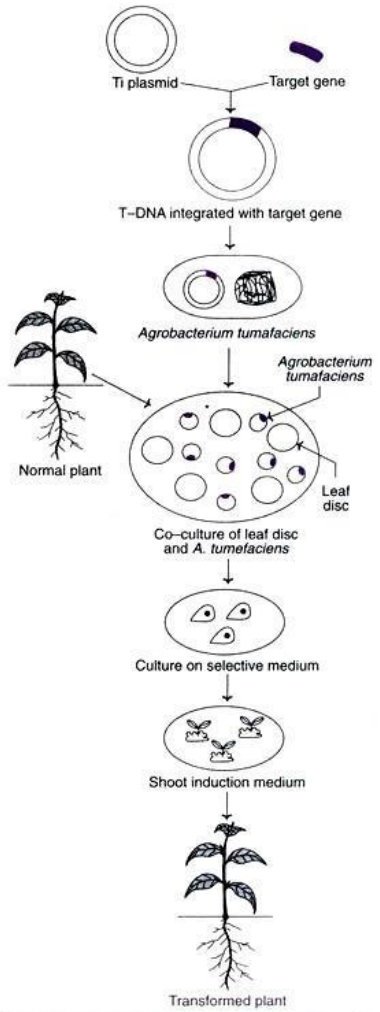


Fig. 49.7 : Transformation technique using Agrobacterium-mediated gene transfer.

Advantages of Agrobacterium- mediated transformation:

i. This is a natural method of gene transfer.

ii. Agrobacterium can conveniently infect any explant (cells/tissues/organs).

iii. Even large fragments of DNA can be efficiently transferred.

iv. Stability of transferred DNA is reasonably good.

v. Transformed plants can be regenerated effectively.

Limitations of Agrobacterium- mediated transformation:

i. There is a limitation of host plants for Agrobacterium, since many crop plants (monocotyledons e.g. cereals) are not infected by it. In recent years, virulent strains of Agrobacterium that can infect a wide range of plants have been developed.

ii. The cells that regenerate more efficiently are often difficult to transform, e.g. embryonic cells lie in deep layers which are not easy targets for Agrobacterium.

Virus-Mediated Gene Transfer (Plant Viruses as Vectors):

Plant viruses are considered as efficient gene transfer agents as they can infect the intact plants and amplify the transferred genes through viral genome replication. Viruses are natural vectors for genetic engineering. They can introduce the desirable gene(s) into almost all the plant cells since the viral infections are mostly systemic.

Plant viruses are non-integrative vectors:

The plant viruses do not integrate into the host genome in contrast to the vectors based on T-DNA of A. tumefaciens which are integrative. The viral genomes are suitably modified by introducing desired foreign genes. These recombinant viruses are transferred, multiplied and expressed in plant cells. They spread systemically within the host plant where the new genetic material is expressed.

Criteria for a plant virus vector:

An ideal plant virus for its effective use in gene transfer is expected to posses the following characteristics:

i. The virus must be capable of spreading from cell to cell through plasmodesmata.

ii. The viral genome should be able to replicate in the absence of viral coat protein and spread from cell to cell. This is desirable since the insertion of foreign DNA will make the viral genome too big to be packed.

iii. The recombinant viral genome must elicit little or no disease symptoms in the infected plants.

iv. The virus should have a broad host range.

v. The virus with DNA genome is preferred since the genetic manipulations involve plant DNA. The three groups of viruses — caulimoviruses, Gemini viruses and RNA viruses that are used

as vectors for gene transfer in plants are briefly described.

Caulimoviruses as Vectors:

The caulimoviruses contain circular double- stranded DNA, and are spherical in shape. Caulimoviruses are widely distributed and are responsible for a number of economically important diseases in various crops. The caulimovirus group has around 15 viruses and among these cauliflower mosaic virus (CaMV) is the most important for gene transfer. The other caulimoviruses include carnation etched virus, dahlia mosaic virus, mirabilis mosaic virus and strawberry vein banding virus.

Cauliflower mosaic virus (CaMV):

CaMV infects many plants (e.g. members of Cruciferae, Datura) and can be easily transmitted, even mechanically. Another attractive feature of CaMV is that the infection is systemic, and large quantities of viruses are found in infected cells.

A diagrammatic view of the CaMV genetic map is depicted in Fig. 49.8. The genome of CaMV consists of a 8 kb (8024 bp) relaxed but tightly packed circular DNA with six major and two minor coding regions. The genes II and VII are not essential for viral infection.

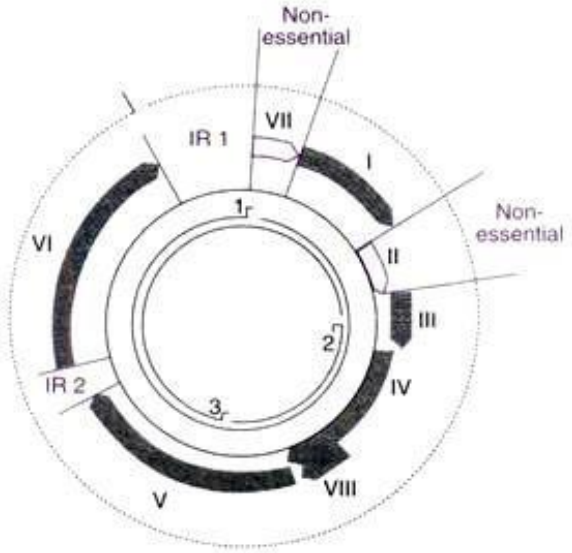


Fig. 49.8 : A diagrammatic representation of the genetic map of cauliflower mosaic virus genome (I...VIII represent coding regions; IR 1and IR 2 are intergeneric regions; The outside dotted circle represents 30S transcript; The two circular lines at the centre indicate viral DNA strands).

Use of CaMV in gene transfer:

For appropriate transmission of CaMV, the foreign DNA must be encapsulated in viral protein. Further, the newly inserted foreign DNA must not interfere with the native assembly of the virus. CaMV genome does not contain any non-coding regions wherein foreign DNA can be inserted. It is fortunate that two genes namely gene II and gene VII have no essential functions for the virus. It is therefore possible to replace one of them and insert the desired foreign gene. Gene II of CaMV has been successfully replaced with a bacterial gene encoding dihydrofolate reductase that provides resistance to methotrexate. When the chimeric CaMV was transmitted to turnip plants, they were systemically infected and the plants developed resistance to methotrexate.

Limitations of CaMV as a vector:

i. CaMV vector has a limited capacity for insertion of foreign genes.

ii. Infective capacity of CaMV is lost if more than a few hundred nucleotides are introduced.

iii. Helper viruses cannot be used since the foreign DNA gets expelled and wild-type viruses are produced.

Gemini Viruses as Vectors:

The Gemini viruses are so named because they have geminate (Gemini literally means heavenly twins) morphological particles i.e. twin and paired capsid structures. These viruses are characterized by possessing one or two single-stranded circular DNAs (ss DNA). On replications, ss DNA forms an intermediate double-stranded DNA.

The Gemini viruses can infect a wide range of crop plants (monocotyledons and dicotyledons) which attract plant biotechnologists to employ these viruses for gene transfer. Curly top virus (CTV) and maize streak virus (MSV) and bean golden mosaic virus (BGMV) are among the important Gemini viruses.

It has been observed that a large number of replicative forms of a Gemini virus genome accumulate inside the nuclei of infected cells. The single-stranded genomic DNA replicates in the nucleus to form a double-stranded intermediate.

Gemini virus vectors can be used to deliver, amplify and express foreign genes in several plants/

explants (protoplasts, cultured cells). However, the serious drawback in employing Gemini viruses as vectors is that it is very difficult to introduce purified viral DNA into the plants. An alternate arrangement is to take the help of Agrobacterium and carry out gene transfer.

RNA Plant Viruses as Vectors:

There are mainly two type's single-stranded RNA viruses:

1. **Mono-partite viruses:**

These viruses are usually large and contain undivided genomes for all the genetic information e.g. tobacco mosaic virus (TMV).

2. **Multipartite viruses:**

The genome in these viruses is divided into small RNAs which may be in the same particle or different particles, e.g. brome mosaic virus (BMV). HMV contains four RNAs divided between three particles. Plant RNA viruses, in general, are characterized by high level of gene expression, good efficiency to infect cells and spread to different tissues. But the major limitation to use them as vectors is the difficulty of joining RNA molecules in vitro.

Use of cDNA for gene transfer:

Complementary DNA (cDNA) copies of RNA viruses are prepared in vitro. The cDNA so generated can be used as a vector for gene transfer in plants. This approach is tedious and cumbersome. However, some success has been reported. A gene sequence encoding chloramphenicol resistance (enzyme- chloramphenicol acetyltransferase) has been inserted into brome mosaic virus genome. This gene expression, however, has been confined to protoplasts.

Limitations of Viral Vectors in Gene Transfer:

The ultimate objective of gene transfer is to transmit the desired genes to subsequent generations. With virus vectors, this is not possible unless the virus is seed-transmitted.

However, in case of vegetatively propagated plants, transmission of desired traits can be done e.g. potatoes. Even in these plants, there is always a risk for the transferred gene to be lost anytime. For the reasons referred above, plant biotechnologists prefer to insert the desired genes of interest into a plant chromosome.

Method # II. Direct or Vector-less DNA Transfer:

The term direct or vector less transfer of DNA is used when the foreign DNA is directly introduced into the plant genome. Direct DNA transfer methods rely on the delivery of naked DNA into the plant cells. This is in contrast to the Agrobacterium or vector-mediated DNA transfer which may be regarded as indirect methods. Majority of the direct DNA transfer methods are simple and effective. And in fact, several transgenic plants have been developed by this approach.

Limitations of direct DNA transfer:

The major disadvantage of direct gene transfer is that the frequency of transgene rearrangements is high. This results in higher transgene copy number, and high frequencies of gene silencing.

Types of direct DNA transfer:

The direct DNA transfer can be broadly divided into three categories.

1. Physical gene transfer methods—electro- portion, particle bombardment, microinjection, liposome fusion, silicon carbide fibres.
2. Chemical gene transfer methods—Polyethylene glycol (PEG)-mediated, diethyl amino ethyl (DEAE) dextran-mediated, calcium phosphate precipitation.
3. DNA imbibition by cells/tissues/organs.

The salient features of the different methods for direct DNA transfer are given in Table 49.1 .

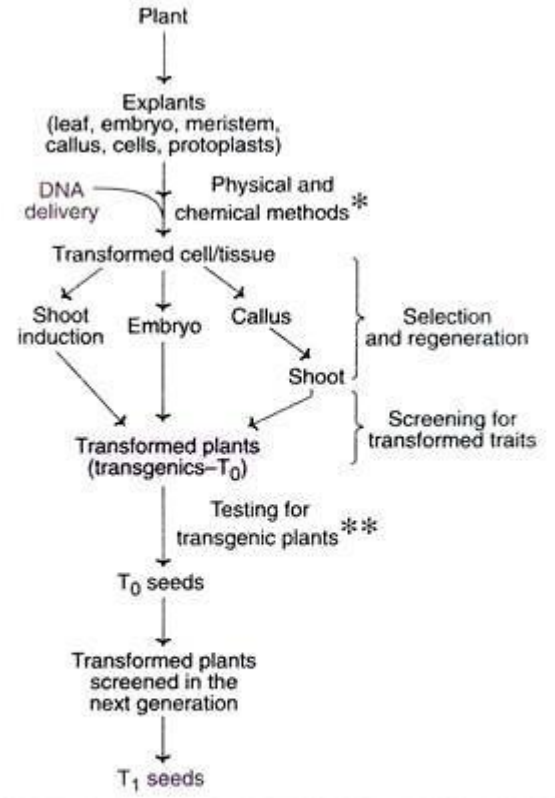


Fig. 49.9 : *An overview of the protocol for the production of transgenic plants using direct DNA delivery methods (*Electroporation, microinjection, macroinjection, bombardment, etc.; **Polymerase chain reaction, Southern hybridization)*

(A) Physical Gene Transfer Methods:

An overview of the general scheme for the production of transgenic plants by employing physical transfer methods is depicted in Fig. 49.9. Some details of the different techniques are described.

1. Electroporation:

Electroporation basically involves the use of high field strength electrical impulses to reversibly permeabilize the cell membranes for the uptake of DNA. This technique can be used for the delivery of DNA into intact plant cells and protoplasts.

The plant material is incubated in a buffer solution containing the desired foreign/target DNA, and subjected to high voltage electrical impulses. This results in the formation of pores in the plasma membrane through which DNA enters and gets integrated into the host cell genome.

In the early years, only protoplasts were used for gene transfer by electroporation. Now a days,

intact cells, callus cultures and immature embryos can be used with suitable pre- and post-electroporation treatments. Electroporation has been successfully used for the production of transgenic plants of many cereals e.g. rice, wheat, maize.

Advantages of electroporation:

i. This technique is simple, convenient and rapid, besides being cost-effective.

ii. The transformed cells are at the same physiological state after electroporation.

iii. Efficiency of transformation can be improved by optimising the electrical field strength, and addition of spermidine.

Limitations of electroporation:

i. Under normal conditions, the amount of DNA delivered into plant cells is very low.

ii. Efficiency of electroporation is highly variable depending on the plant material and the treatment conditions.

iii. Regeneration of plants is not very easy, particularly when protoplasts are used.

2. Particle Bombardment (Biolistics):

Particle (or micro projectile) bombardment is the most effective method for gene transfer, and creation of transgenic plants. This method is versatile due to the fact that it can be successfully used for the DNA transfer in mammalian cells and microorganisms.

The micro projectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Biolistics is a combination of biological and ballistics. There are other names for this technique- particle gun, gene gun, bio blaster. A diagrammatic representation of micro projectile bombardment system for the transfer of genes in plants is depicted in Fig. 49.10, and briefly described below.

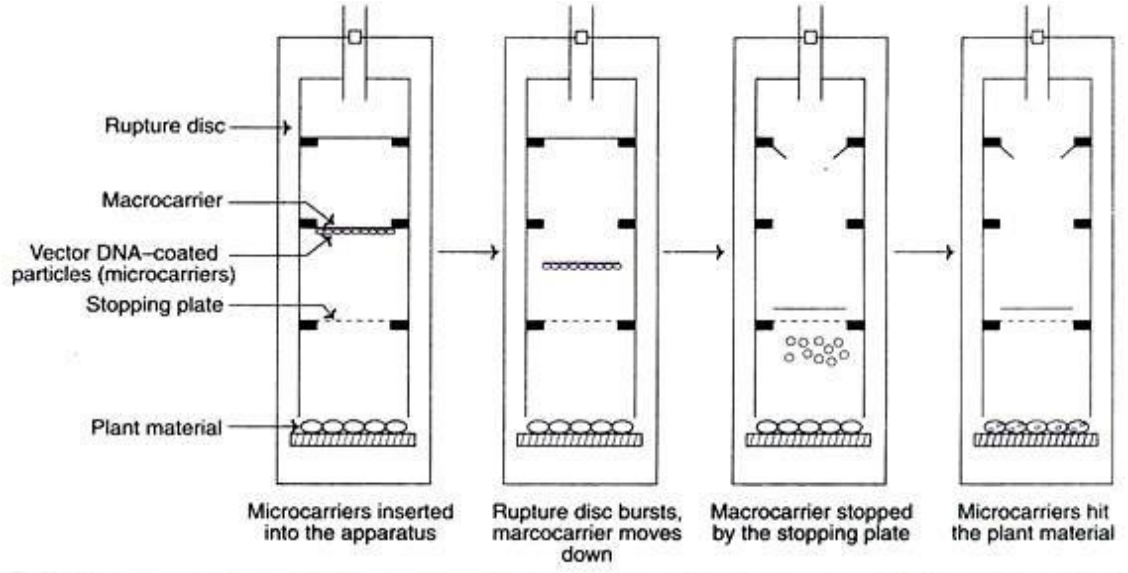


Fig. 49.10 : A diagrammatic representation of particle bombardment (biolistics) system for gene transfer in plants.

Micro carriers (micro projectiles), the tungsten or gold particles coated with DNA, are carried by macro carriers (macro projectiles). These macro-carriers are inserted into the apparatus and pushed downward by rupturing the disc.

The stopping plate does not permit the movement of macro carrier while the micro carriers (with DNA) are propelled at a high speed into the plant material. Here the DNA segments are released which enter the plant cells and integrate with the genome.

Plant material used in bombardment:

Two types of plant tissue are commonly used for particle bombardment:

1. Primary explants which can be subjected to bombardment that are subsequently induced to become embryo genic and regenerate.
2. Proliferating embryonic tissues that can be bombarded in cultures and then allowed to

proliferate and regenerate.

In order to protect plant tissues from being damaged by bombardment, cultures are maintained on high osmoticum media or subjected to limited plasmolysis.

Transgene integration in bombardment:

It is believed (based on the gene transfer in rice by biolistics) that the gene transfer in particle bombardment is a two stage process.

1. In the pre-integration phase, the vector DNA molecules are spliced together. This results in fragments carrying multiple gene copies.
2. Integrative phase is characterized by the insertion of gene copies into the host plant genome.

The integrative phase facilitates further transgene integration which may occur at the same point or a point close to it. The net result is that particle bombardment is frequently associated with high copy number at a single locus. This type of single locus may be beneficial for regeneration of plants.

The success of bombardment:

The particle bombardment technique was first introduced in 1987. It has been successfully used for the transformation of many cereals, e.g. rice, wheat, maize. In fact, the first commercial genetically modified (CM) crops such as maize containing Bt-toxin gene were developed by this approach.

A selected list of the transgenic plants (developed by biolistics) along with the sources of the plant materials used is given in Table 49.2.

TABLE 49.2 A selected list of transgenic plants (along with cell sources) developed by microprojectile bombardment

Plant	*Cell source(s)*
Rice	Embryonic callus, immature zygotic embryos
Wheat	Immature zygotic embryos
Sorghum	Immature zygotic embryos
Corn	Embryonic cell suspension, immature zygotic embryos
Barley	Cell suspension, immature zygotic embryos
Banana	Embryonic cell suspension
Sweet potato	Callus cells
Cotton	Zygotic embryos
Grape	Embryonic callus
Peas	Zygotic embryos
Peanut	Embryonic callus
Tobacco	Pollen
Alfalfa	Embryonic callus

Factors affecting bombardment:

Several attempts are made to study the various factors, and optimize the system of particle bombardment for its most efficient use. Some of the important parameters are described.

Nature of micro particles:

Inert metals such as tungsten, gold and platinum are used as micro particles to carry DNA. These particles with relatively higher mass will have a better chance to move fast when bombarded and penetrate the tissues.

Nature of tissues/cells:

The target cells that are capable of undergoing division are suitable for transformation. Some more details on the choice of plant material used in bombardment are already given.

Amount of DNA:

The transformation may be low when too little DNA is used. On the other hand, too much DNA may result is high copy number and rearrangement of transgenes. Therefore, the quantity of DNA used should be balanced. Recently, some workers have started using the chemical aminosiloxane to coat the micro particles with low quantities of DNA adequate enough to achieve high efficiency of transformation.

Environmental parameters:

Many environmental variables are known to influence particle bombardment. These factors (temperature, humidity, photoperiod etc.) influence the physiology of the plant material, and consequently the gene transfer. It is also observed that some explants, after bombardment may require special regimes of light, humidity, temperature etc.

he technology of particle bombardment has been improved in recent years, particularly with regard to the use of equipment. A commercially produced particle bombardment apparatus namely PDS-1000/HC is widely used these days.

Advantages of particle bombardment:

i. Gene transfer can be efficiently done in organized tissues.
ii. Different species of plants can be used to develop transgenic plants.

Limitations of particle bombardment:

i. The major complication is the production of high transgene copy number. This may result in instability of transgene expression due to gene silencing.
ii. The target tissue may often get damaged due to lack of control of bombardment velocity.
iii. Sometimes, undesirable chimeric plants may be regenerated.

3. Microinjection:

Microinjection is a direct physical method involving the mechanical insertion of the desirable

DNA into a target cell. The target cell may be the one identified from intact cells, protoplasts, callus, embryos, meristems etc. Microinjection is used for the transfer of cellular organelles and for the manipulation of chromosomes.

The technique of microinjection involves the transfer of the gene through a micropipette (0.5-10.0 pm tip) into the cytoplasm/nucleus of a plant cell or protoplast. While the gene transfer is done, the recipient cells are kept immobilized in agarose embedding, and held by a suction holding pipette (Fig. 49.11).

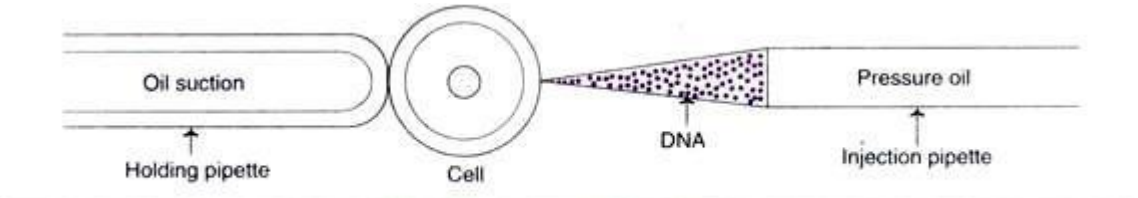


Fig. 49.11 : Microinjection of DNA by holding pipette method.

As the process of microinjection is complete, the transformed cell is cultured and grown to develop into a transgenic plant. In fact, transgenic tobacco and Brassica napus have been developed by this approach. The major limitations of microinjection are that it is slow, expensive, and has to be performed by trained and skilled personnel.

4. Liposome-Mediated Transformation:

Liposomes are artificially created lipid vesicles containing a phospholipid membrane. They are successfully used in mammalian cells for the delivery of proteins, drugs etc. Liposomes carrying genes can be employed to fuse with protoplasts and transfer the genes.

The efficiency of transformation increases when the process is carried out in conjunction with polyethylene glycol (PEG). Liposome-mediated transformation involves adhesion of liposomes to the protoplast surface, its fusion at the site of attachment and release of plasmids inside the cell (Fig. 49.12).

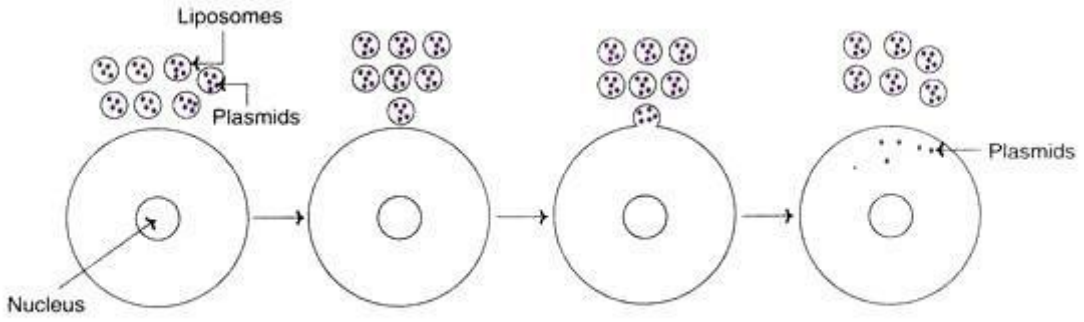


Fig. 49.12 : A diagrammatic representation of fusion of plasmid–filled liposomes with protoplasts.

Advantages of liposome fusion:

i. Being present in an encapsulated form of liposomes, DNA is protected from environmental insults and damage.

ii. DNA is stable and can be stored for some time in liposomes prior to transfer.

iii. Applicable to a wide range of plant cells.

iv. There is good reproducibility in the technique.

Limitations of liposome fusion:

The major problem with liposome-mediated transformation is the difficulty associated with the regeneration of plants from transformed protoplasts.

5. Silicon Carbide Fibre-Mediated Transformation:

The silicon carbide fibres (SCF) are about 0.3-0.6 pm in diameter and 10-100 pm in length. These fibres are capable of penetrating the cell wall and plasma membrane, and thus can deliver DNA into the cells. The DNA coated silicon carbide fibres are vortexed with ‘plant material (suspension culture, calluses). During the mixing, DNA adhering to the fibres enters the cells and gets stably integrated with the host genome. The silicon carbide fibres with the trade name Whiskers are available in the market.

Advantages of SCF-mediated transformation:

i. Direct delivery of DNA into intact walled cells. This avoids the protoplast isolation.
ii. Procedure is simple and does not involve costly equipment.

Disadvantages of SCF-mediated transformation:

i. Silicon carbide fibres are carcinogenic and therefore have to be carefully handled.
ii. The embryonic plant cells are hard and compact and are resistant to SCF penetration.

In recent years, some improvements have been made in SCF-mediated transformation. This has helped in the transformation of rice, wheat, maize and barley by using this technique.

(B) Chemical Gene Transfer Methods:

1. Polyethylene glycol (PEG)-mediated transfer:

Polyethylene glycol (PEG), in the presence of divalent cations (using Ca^{2+}), destabilizes the plasma membrane of protoplasts and renders it permeable to naked DNA. In this way, the DNA enters nucleus of the protoplasts and gets integrated with the genome.

The procedure involves the isolation of protoplasts and their suspension, addition of plasmid DNA, followed by a slow addition of 40% PEG-4000 (w/v) dissolved in mannitol and calcium nitrate solution. As this mixture is incubated, protoplasts get transformed.

Advantages of PEG-mediated transformation:

i. A large number of protoplasts can be simultaneously transformed.
ii. This technique can be successfully used for a wide range of plant species.

Limitations of PEG-mediated transformation:

i. The DNA is susceptible for degradation and rearrangement.
ii. Random integration of foreign DNA into genome may result in undesirable traits.
iii. Regeneration of plants from transformed protoplasts is a difficult task.

2. DEAE Dextran-Mediated transfer:

The desirable DNA can be complexed with a high molecular weight polymer diethyl amino ethyl (DEAE) dextran and transferred. The major limitation of this approach is that it does not yield stable trans-formants.

Calcium Phosphate Co- Precipitation-Mediated Transfer:

The DNA is allowed to mix with calcium chloride solution and isotonic phosphate buffer to form DNA-calcium phosphate precipitate. When the actively dividing cells in culture are exposed to this precipitate for several hours, the cells get transformed. The success of this method is dependent on the high concentration of DNA and the protection of the complex precipitate. Addition of dimethyl sulfoxide (DMSO) increases the efficiency of transformation.

Dna Imbibition By Cells/Tissues:

Some workers have seriously tried to transform cells by incubating cell suspensions, tissues, embryos and even seeds with DNA. The belief is that the DNA gets imbibed, and the cells get transformed. DNA imbibition approach has met with little or no success.

Polymerase Chain Reaction (PCR)- Principle, Procedure, Types, Applications and Animation

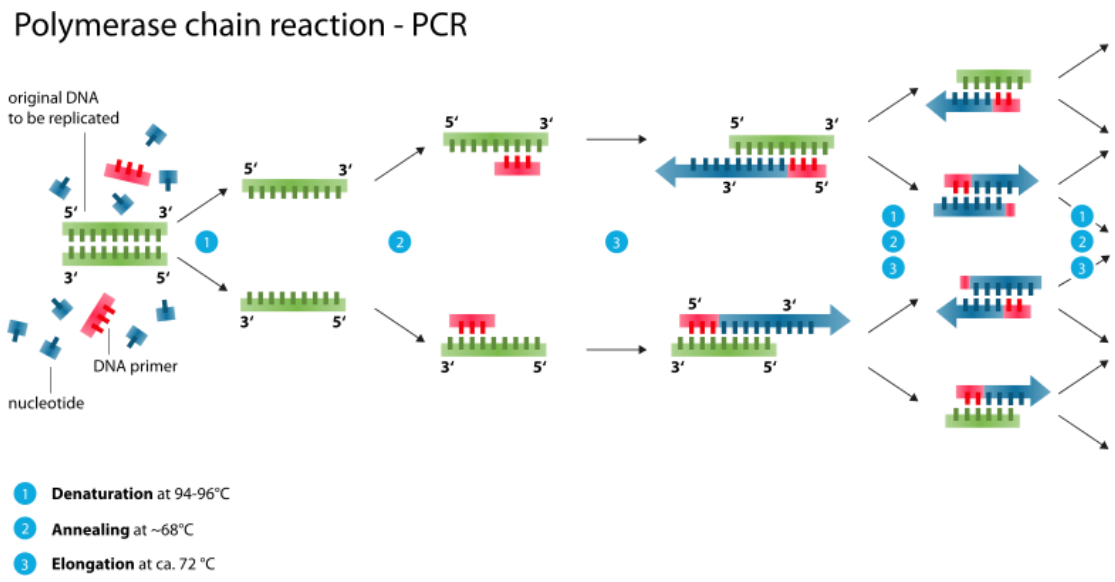
Polymerase Chain Reaction (PCR) is a powerful method for amplifying particular segments of DNA, distinct from cloning and propagation within the host cell. This procedure is carried out entirely biochemically, that is, in vitro. PCR was invented by Kary Mullis in 1983. He shared the Nobel Prize in chemistry with Michael Smith in 1993.

Principle of PCR

PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer

template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Procedure/Steps of PCR



Polymerase Chain Reaction (PCR) By Enzoklop (Own work) via Wikimedia Commons

1. Denaturation

The DNA template is heated to 94° C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

2. Annealing

The mixture is cooled to anywhere from 50-70° C. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

3. Extension

The reaction is then heated to 72° C, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner,

using the target DNA as a template.

With one cycle, a single segment of double-stranded DNA template is amplified into two separate pieces of double-stranded DNA. These two pieces are then available for amplification in the next cycle. As the cycles are repeated, more and more copies are generated and the number of copies of the template is increased exponentially.

Types of PCR

1. Real-time PCR
2. Quantitative real time PCR (Q-RT PCR)
3. Reverse Transcriptase PCR (RT-PCR)
4. Multiplex PCR
5. Nested PCR
6. Long-range PCR
7. Single-cell PCR
8. Fast-cycling PCR
9. Methylation-specific PCR (MSP)
10. Hot start PCR
11. High-fidelity PCR
12. In situ PCR
13. Variable Number of Tandem Repeats (VNTR) PCR
14. Asymmetric PCR
15. Repetitive sequence-based PCR
16. Overlap extension PCR
17. Assemble PCR
18. Intersequence-specific PCR(ISSR)
19. Ligation-mediated PCR
20. Methylation –specifin PCR
21. Miniprimer PCR
22. Solid phase PCR

23. Touch down PCR, etc

Applications of PCR

1. PCR is used in analyzing clinical specimens for the presence of infectious agents, including HIV, hepatitis, malaria, anthrax, etc.

2. PCR can provide information on a patient's prognosis, and predict response or resistance to therapy. Many cancers are characterized by small mutations in certain genes, and this is what PCR is employed to identify.

3. PCR is used in the analysis of mutations that occur in many genetic diseases (e.g. cystic fibrosis, sickle cell anaemia, phenylketonuria, muscular dystrophy).

4. PCR is also used in forensics laboratories and is especially useful because only a tiny amount of original DNA is required, for example, sufficient DNA can be obtained from a droplet of blood or a single hair.

5. PCR is an essential technique in cloning procedure which allows generation of large amounts of pure DNA from tiny amount of template strand and further study of a particular gene.

6. The Human Genome Project (HGP) for determining the sequence of the 3 billion base pairs in the human genome, relied heavily on PCR.

7. PCR has been used to identify and to explore relationships among species in the field of evolutionary biology. In anthropology, it is also used to understand the ancient human migration patterns. In archaeology, it has been used to spot the ancient human race. PCR commonly used by Paleontologists to amplify DNA from extinct species or cryopreserved fossils of millions years and thus can be further studied to elucidate

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DEPARTMENT OF BIOTECHNOLOGY

COURSE MATERIAL

Subject Name: ANALYTICAL TECHNIQUES IN BIOTECHNOLOGY Code: SBB1201

UNIT I – V

Biosensor

A **biosensor** is an analytical device, used for the detection of an analyte, that combines a biological component with a physicochemical detector. The *sensitive biological element* (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.) is a biologically derived material or biomimetic component that interacts (binds or recognizes) with the analyte under study. The biologically sensitive elements can also be created by biological engineering. The *transducer* or the *detector element* (works in a physicochemical way; optical, piezoelectric, electrochemical, etc.) transforms the signal resulting from the interaction of the analyte with the biological element into another signal (i.e., transduces) that can be more easily measured and quantified.

Biosensor system

A biosensor typically consists of a bio-recognition component, biotransducer component, and electronic system which include a signal amplifier, processor, and display. Transducers and electronics can be combined, e.g., in CMOS-based microsensor systems. The recognition component, often called a bioreceptor, uses biomolecules from organisms or receptors modeled after biological systems to interact with the analyte of interest. This interaction is measured by the biotransducer which outputs a measurable signal proportional to the presence of the target analyte in the sample. The general aim of the design of a biosensor is to enable quick, convenient testing at the point of concern or care where the sample was procured.

Bioreceptors

In a biosensor, the bioreceptor is designed to interact with the specific analyte of interest to produce an effect measurable by the transducer. High selectivity for the analyte among a matrix of other chemical or biological components is a key requirement of the bioreceptor. While the type of biomolecule used can vary widely, biosensors can be classified according to common types bioreceptor interactions involving: anitbody/antigen, enzymes/ligands, nucleic acids/DNA, cellular structures/cells, or biomimetic materials.

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DEPARTMENT OF BIOTECHNOLOGY

COURSE MATERIAL

Subject Name: ANALYTICAL TECHNIQUES IN BIOTECHNOLOGY Code: SBB1201

UNIT I – V

Antibody/antigen interactions

An immunosensor utilizes the very specific binding affinity of antibodies for a specific compound or antigen. The specific nature of the antibody-antigen interaction is analogous to a lock and key fit in that the antigen will only bind to the antibody if it has the correct conformation. Binding events result in a physicochemical change that in combination with a tracer, such as a fluorescent molecules, enzymes, or radioisotopes, can generate a signal.

There are limitations with using antibodies in sensors:

1.The antibody binding capacity is strongly dependent on assay conditions (e.g. pH and temperature) and

2. The antibody-antigen interaction is generally irreversible. However, it has been shown that binding can be disrupted by chaotropic reagents, organic solvents, or even ultrasonic radiation.

Artificial binding proteins

The use of antibodies as the bio-recognition component of biosensors has several drawbacks. They have high molecular weights and limited stability, contain essential disulfide bonds and are expensive to produce. In one approach to overcome these limitations, recombinant binding fragments (Fab, Fv or scFv) or domains (VH, VHH) of antibodies have been engineered.

Enzymatic interactions

The specific binding capabilities and catalytic activity of enzymes make them popular bioreceptors. Analyte recognition is enabled through several possible mechanisms: 1) the enzyme converting the analyte into a product that is sensor-detectable, 2) detecting enzyme inhibition or activation by the analyte, or 3) monitoring modification of enzyme properties resulting from interaction with the analyte. The main reasons for the common use of enzymes in biosensors are: 1) ability to catalyze a large number of reactions; 2) potential to detect a group of analytes (substrates, products, inhibitors, and modulators of the catalytic activity); and 3) suitability with several different transduction methods for detecting the analyte. Notably, since enzymes are not consumed in reactions, the biosensor can easily be used continuously. The catalytic activity of enzymes also allows lower limits of detection compared to common binding techniques. However, the sensor's lifetime is limited by the stability of the enzyme.

Affinity binding receptors

Antibodies have a high binding constant in excess of 10^8 L/mol, which stands for a nearly irreversible association once the antigen-antibody couple has formed. For certain analyte molecules like glucose affinity binding proteins exist that bind their ligand with a high specificity like an antibody, but with a much smaller binding constant on the order of 10^2 to 10^4 L/mol. The association between analyte and receptor then is of reversible nature and next to the couple between both also their free molecules occur in a measurable concentration. In case of glucose, for instance, concanavalin A may function as affinity receptor exhibiting a binding constant of 4x10^2 L/mol. The use of affinity binding receptors for purposes of biosensing has been proposed by Schultz and Sims in 1979 and was subsequently configured into a fluorescent assay for measuring glucose in the relevant physiological range between 4.4 and 6.1 mmol/L. The sensor principle has the advantage that it does not consume the analyte in a chemical reaction as is occurs in enzymatic assays.

Nucleic acid interactions

Biosensors that employ nucleic acid interactions can be referred to as genosensors. The recognition process is based on the principle of complementary base pairing, adenine:thymine and cytosine:guanine in DNA. If the target nucleic acid sequence is known, complementary sequences can be synthesized, labeled, and then immobilized on the sensor. The hybridization probes can then base pair with the target sequences, generating an optical signal. The favored transduction principle employed in this type of sensor has been optical detection.

Epigenetics

It has been proposed that properly optimized integrated optical resonators can be exploited for detecting epigenetic modifications (e.g. DNA methylation, histone post-translational modifications) in body fluids from patients affected by cancer or other diseases.

Organelles

Organelles form separate compartments inside cells and usually perform function

independently. Different kinds of organelles have various metabolic pathways and contain enzymes to fulfill its function. Commonly used organelles include lysosome, chloroplast and mitochondria.

Cells

Cells are often used in bioreceptors because they are sensitive to surrounding environment and they can respond to all kinds of stimulants. Cells tend to attach to the surface so they can be easily immobilized. Compared to organelles they remain active for longer period and the reproducibility makes them reusable. They are commonly used to detect global parameter like stress condition, toxicity and organic derivatives. They can also be used to monitor the treatment effect of drugs. One application is to use cells to determine herbicides which are main aquatic contaminant. Microalgae are entrapped on a quartz microfiber and the chlorophyll fluorescence modified by herbicides is collected at the tip of an optical fiber bundle and transmitted to a fluorimeter. The algae are continuously cultured to get optimized measurement. Results show that detection limit of certain herbicide can reach sub-ppb concentration level. Some cells can also be used to monitor the microbial corrosion. Pseudomonas sp. is isolated form corroded material surface and immobilized on acetylcellulose membrane. The respiration activity is determined by measuring oxygen consumption. There is linear relationship between the current generated and the concentration of sulfuric acid. The response time is related to the loading of cells and surrounding environments and can be controlled to no more than 5min.

Tissue

Tissues are used for biosensor for the abundance of enzymes existed. Advantages of tissues as biosensors include the following: 1)easier to immobilize compared to cells and organelles 2)the higher activity and stability from maintain enzymes in natural environment 3)the availability and low price 4)the avoidance of tedious work of extraction, centrifuge and purification of enzymes 5)necessary cofactors for enzyme to function exists 6)the diversity providing a wide range of choice concerning different objectives. There also exists some disadvantages of tissues like the lack of specificity due to the interference of other enzymes and longer response time due to transport barrier.

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Biotransducer

Biosensors can be classified by their biotransducer type. The most common types of biotransducers used in biosensors are 1) electrochemical biosensors, 2) optical biosensors, 3)electronic biosensors, 4)piezoelectric biosensors, 5) gravimetric biosensors, 6) pyroelectric biosensors.

Classification of Biosensors based on type of biotransducer

Electrochemical

Electrochemical biosensors are normally based on enzymatic catalysis of a reaction that produces or consumes electrons (such enzymes are rightly called redox enzymes). The sensor substrate usually contains three electrodes; a reference electrode, a working electrode and a counter electrode. The target analyte is involved in the reaction that takes place on the active electrode surface, and the reaction may cause either electron transfer across the double layer (producing a current) or can contribute to the double layer potential (producing a voltage). We can either measure the current (rate of flow of electrons is now proportional to the analyte concentration) at a fixed potential or the potential can be measured at zero current (this gives a logarithmic response). Note that potential of the working or active electrode is space charge sensitive and this is often used. Further, the label-free and direct electrical detection of small peptides and proteins is possible by their intrinsic charges using biofunctionalized ion-sensitive field-effect transistors.

Another example, the potentiometric biosensor, (potential produced at zero current) gives a logarithmic response with a high dynamic range. Such biosensors are often made by screen printing the electrode patterns on a plastic substrate, coated with a conducting polymer and then some protein (enzyme or antibody) is attached. They have only two electrodes and are extremely sensitive and robust. They enable the detection of analytes at levels previously only achievable by HPLC and LC/MS and without rigorous sample preparation. All biosensors usually involve minimal sample preparation as the biological sensing component is highly selective for the analyte concerned. The signal is produced by electrochemical and physical

changes in the conducting polymer layer due to changes occurring at the surface of the sensor. Such changes can be attributed to ionic strength, pH, hydration and redox reactions, the latter due to the enzyme label turning over a substrate. Field effect transistors, in which the gate region has been modified with an enzyme or antibody, can also detect very low concentrations of various analytes as the binding of the analyte to the gate region of the FET cause a change in the drain- source current.

Ion channel switch

The use of ion channels has been shown to offer highly sensitive detection of target biological molecules. By embedding the ion channels in supported or tethered bilayer membranes (t-BLM) attached to a gold electrode, an electrical circuit is created. Capture molecules such as antibodies can be bound to the ion channel so that the binding of the target molecule controls the ion flow through the channel. This results in a measurable change in the electrical conduction which is proportional to the concentration of the target.

An ion channel switch (ICS) biosensor can be created using gramicidin, a dimeric peptide channel, in a tethered bilayer membrane. One peptide of gramicidin, with attached antibody, is mobile and one is fixed. Breaking the dimer stops the ionic current through the membrane. The magnitude of the change in electrical signal is greatly increased by separating the membrane from the metal surface using a hydrophilic spacer.

Quantitative detection of an extensive class of target species, including proteins, bacteria, drug and toxins has been demonstrated using different membrane and capture configurations.

Reagentless fluorescent biosensor

A reagentless biosensor can monitor a target analyte in a complex biological mixture without additional reagent. Therefore, it can function continuously if immobilized on a solid support. A fluorescent biosensor reacts to the interaction with its target analyte by a change of its fluorescence properties. A Reagentless Fluorescent biosensor (RF biosensor) can be obtained by integrating a biological receptor, which is directed against the target analyte, and a solvatochromic fluorophore, whose emission properties are sensitive to the nature of its local

environment, in a single macromolecule. The fluorophore transduces the recognition event into a measurable optical signal. The use of extrinsic fluorophores, whose emission properties differ widely from those of the intrinsic fluorophores of proteins, tryptophan and tyrosine, enables one to immediately detect and quantify the analyte in complex biological mixtures. The integration of the fluorophore must be done in a site where it is sensitive to the binding of the analyte without perturbing the affinity of the receptor.

Antibodies and artificial families of Antigen Binding Proteins (AgBP) are well suited to provide the recognition module of RF biosensors since they can be directed against any antigen (see the paragraph on bioreceptors). A general approach to integrate a solvatochromic fluorophore in an AgBP when the atomic structure of the complex with its antigen is known, and thus transform it into a RF biosensor, has been described. A residue of the AgBP is identified in the neighborhood of the antigen in their complex. This residue is changed into a cysteine by site-directed mutagenesis. The fluorophore is chemically coupled to the mutant cysteine. When the design is successful, the coupled fluorophore does not prevent the binding of the antigen, this binding shields the fluorophore from the solvent, and it can be detected by a change of fluorescence. This strategy is also valid for antibody fragments.

However, in the absence of specific structural data, other strategies must be applied. Antibodies and artificial families of AgBPs are constituted by a set of hypervariable (or randomized) residue positions, located in a unique sub-region of the protein, and supported by a constant polypeptide scaffold. The residues that form the binding site for a given antigen, are selected among the hypervariable residues. It is possible to transform any AgBP of these families into a RF biosensor, specific of the target antigen, simply by coupling a solvatochromic fluorophore to one of the hypervariable residues that have little or no importance for the interaction with the antigen, after changing this residue into cysteine by mutagenesis. More specifically, the strategy consists

in individually changing the residues of the hypervariable positions into cysteine at the genetic level, in chemically coupling a solvatochromic fluorophore with the mutant cysteine, and then in keeping the resulting conjugates that have the highest sensitivity (a parameter that involves both affinity and variation of fluorescence signal). This approach is also valid for families of antibody fragments.

A posteriori studies have shown that the best reagentless fluorescent biosensors are obtained when the fluorophore does not make non-covalent interactions with the surface of the bioreceptor, which would increase the background signal, and when it interacts with a binding pocket at the surface of the target antigen. The RF biosensors that are obtained by the above methods, can function and detect target analytes inside living cells.

Others

Piezoelectric sensors utilise crystals which undergo an elastic deformation when an electrical potential is applied to them. An alternating potential (A.C.) produces a standing wave in the crystal at a characteristic frequency. This frequency is highly dependent on the elastic properties of the crystal, such that if a crystal is coated with a biological recognition element the binding of a (large) target analyte to a receptor will produce a change in the resonance frequency, which gives a binding signal. In a mode that uses surface acoustic waves (SAW), the sensitivity is greatly increased. This is a specialised application of the Quartz crystal microbalance as a biosensor. Thermometric and magnetic based biosensors are rare.

Placement of biosensors

The appropriate placement of biosensors depends on their field of application, which may roughly be divided into biotechnology, agriculture, food technology and biomedicine.

In biotechnology, analysis of the chemical composition of cultivation broth can be conducted in- line, on-line, at-line and off-line. As outlined by the US Food and Drug Administration (FDA) the sample is not removed from the process stream for in-line sensors, while it is diverted from the manufacturing process for on-line measurements. For at-line sensors the sample may be removed and analyzed in close proximity to the process stream. An example of the latter is the monitoring of lactose in a dairy processing plant. Off-line biosensors compare to

bioanalytical techniques that are not operating in the field, but in the laboratory. These techniques are mainly used in agriculture, food technology and biomedicine.

Applications

There are many potential applications of biosensors of various types. The main requirements for a biosensor approach to be valuable in terms of research and commercial applications are the identification of a target molecule, availability of a suitable biological recognition element, and the potential for disposable portable detection systems to be preferred to sensitive laboratory- based techniques in some situations. Some examples are glucose monitoring in diabetes patients, other medical health related targets, environmental applications e.g. the detection of pesticides and river water contaminants such as heavy metal ions, remote sensing of airborne bacteria e.g. in counter-bioterrorist activities, remote sensing of water quality in coastal waters by describing online different aspects of clam ethology (biological rhythms, growth rates, spawning or death records) in groups of abandoned bivalves around the world, detection of pathogens, determining levels of toxic substances before and after bioremediation, detection and determining of organophosphate, routine analytical measurement of folic acid, biotin, vitamin B12 and pantothenic acid as an alternative to microbiological assay, determination of drug residues in food, such as antibiotics and growth promoters, particularly meat and honey, drug discovery and evaluation of biological activity of new compounds, protein engineering in biosensors, and detection of toxic metabolites such as mycotoxins.

A common example of a commercial biosensor is the blood glucose biosensor, which uses the enzyme glucose oxidase to break blood glucose down. In doing so it first oxidizes glucose and uses two electrons to reduce the FAD (a component of the enzyme) to FADH2. This in turn is oxidized by the electrode in a number of steps. The resulting current is a measure of the concentration of glucose. In this case, the electrode is the transducer and the enzyme is the biologically active component.

A canary in a cage, as used by miners to warn of gas, could be considered a biosensor. Many of today's biosensor applications are similar, in that they use organisms which respond to toxic substances at a much lower concentrations than humans can detect to warn of their presence. Such devices can be used in environmental monitoring, trace gas detection and in water

treatment facilities.

Many optical biosensors are based on the phenomenon of surface plasmon resonance (SPR) techniques. This utilises a property of and other materials; specifically that a thin layer of gold on a high refractive index glass surface can absorb laser light, producing electron waves (surface plasmons) on the gold surface. This occurs only at a specific angle and wavelength of incident light and is highly dependent on the surface of the gold, such that binding of a target analyte to a receptor on the gold surface produces a measurable signal.

Surface plasmon resonance sensors operate using a sensor chip consisting of a plastic cassette supporting a glass plate, one side of which is coated with a microscopic layer of gold. This side contacts the optical detection apparatus of the instrument. The opposite side is then contacted with a microfluidic flow system. The contact with the flow system creates channels across which reagents can be passed in solution. This side of the glass sensor chip can be modified in a number of ways, to allow easy attachment of molecules of interest. Normally it is coated in carboxymethyl dextran or similar compound.

The refractive index at the flow side of the chip surface has a direct influence on the behavior of the light reflected off the gold side. Binding to the flow side of the chip has an effect on the refractive index and in this way biological interactions can be measured to a high degree of sensitivity with some sort of energy. The refractive index of the medium near the surface changes when biomolecules attach to the surface, and the SPR angle varies as a function of this change.

Light of a fixed wavelength is reflected off the gold side of the chip at the angle of total internal reflection, and detected inside the instrument. The angle of incident light is varied in order to match the evanescent wave propagation rate with the propagation rate of the surface plasmon plaritons. This induces the evanescent wave to penetrate through the glass plate and some distance into the liquid flowing over the surface.

Other optical biosensors are mainly based on changes in absorbance or fluorescence of an appropriate indicator compound and do not need a total internal reflection geometry. For example, a fully operational prototype device detecting casein in milk has been fabricated. The device is based on detecting changes in absorption of a gold layer. A widely used research tool, the micro-array, can also be considered a biosensor.

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Biological biosensors often incorporate a genetically modified form of a native protein or enzyme. The protein is configured to detect a specific analyte and the ensuing signal is read by a detection instrument such as a fluorometer or luminometer. An example of a recently developed biosensor is one for detecting cytosolic concentration of the analyte cAMP (cyclic adenosine monophosphate), a second messenger involved in cellular signaling triggered by ligands interacting with receptors on the cell membrane. Similar systems have been created to study cellular responses to native ligands or xenobiotics (toxins or small molecule inhibitors). Such "assays" are commonly used in drug discovery development by pharmaceutical and biotechnology companies. Most cAMP assays in current use require lysis of the cells prior to measurement of cAMP. A live-cell biosensor for cAMP can be used in non-lysed cells with the additional advantage of multiple reads to study the kinetics of receptor response.

Nanobiosensors use an immobilized bioreceptor probe that is selective for target analyte molecules. Nanomaterials are exquisitely sensitive chemical and biological sensors. Nanoscale materials demonstrate unique properties. Their large surface area to volume ratio can achieve rapid and low cost reactions, using a variety of designs.

Other evanescent wave biosensors have been commercialised using waveguides where the propagation constant through the waveguide is changed by the absorption of molecules to the waveguide surface. One such example, dual polarisation interferometry uses a buried waveguide as a reference against which the change in propagation constant is measured. Other configurations such as the Mach–Zehnder have reference arms lithographically defined on a substrate. Higher levels of integration can be achieved using resonator geometries where the resonant frequency of a ring resonator changes when molecules are absorbed.

Recently, arrays of many different detector molecules have been applied in so called electronic nose devices, where the pattern of response from the detectors is used to fingerprint a substance. In the Wasp Hound odor-detector, the mechanical element is a video camera and the biological element is five parasitic wasps who have been conditioned to swarm in response to the presence of a specific chemical. Current commercial electronic noses, however, do not use biological elements.

Glucose monitoring

Blood glucose monitoring

Commercially available gluocose monitors rely on amperometric sensing of glucose by means of glucose oxidase, which oxidises glucose producing hydrogen peroxide which is detected by the electrode. To overcome the limitation of amperometric sensors, a flurry of research is present into novel sensing methods, such as fluorescent glucose biosensors.

Interferometric reflectance imaging sensor

The interferometric reflectance imaging sensor (IRIS) is based on the principles of optical interference and consists of a silicon-silicon oxide substrate, standard optics, and low-powered coherent LEDs. When light is illuminated through a low magnification objective onto the layered silicon-silicon oxide substrate, an interferometric signature is produced. As biomass, which has a similar index of refraction as silicon oxide, accumulates on the substrate surface, a change in the interferometric signature occurs and the change can be correlated to a quantifiable mass. *Daaboul et al.* used IRIS to yield a label-free sensitivity of approximately 19 ng/mL, *Ahn et al.* improved the sensitivity of IRIS through a mass tagging technique.

Food analysis

There are several applications of biosensors in food analysis. In the food industry, optics coated with antibodies are commonly used to detect pathogens and food toxins. Commonly, the light system in these biosensors is fluorescence, since this type of optical measurement can greatly amplify the signal.

A range of immuno- and ligand-binding assays for the detection and measurement of small molecules such as water-soluble vitamins and chemical contaminants (drug residues) such as sulfonamides and Beta-agonists have been developed for use on SPR based sensor systems, often adapted from existing ELISA or other immunological assay. These are in widespread use across the food industry.

DNA biosensors

In the future, DNA will find use as a versatile material from which scientists can craft

biosensors. DNA biosensors can theoretically be used for medical diagnostics, forensic science, agriculture, or even environmental clean-up efforts. No external monitoring is needed for DNA-based sensing devises. This is a significant advantage. DNA biosensors are complicated mini-machines—consisting of sensing elements, micro lasers, and a signal generator. At the heart of DNA biosensor function is the fact that two strands of DNA stick to each other by virtue of chemical attractive forces. On such a sensor, only an exact fit—that is, two strands that match up at every nucleotide position—gives rise to a fluorescent signal (a glow) that is then transmitted to a signal generator.

Microbial biosensors

Using biological engineering researchers have created many microbial biosensors. An example is the arsenic biosensor. To detect arsenic they use the Ars operon. Using bacteria, researchers can detect pollutants in samples.

Ozone biosensors

Because ozone filters out harmful ultraviolet radiation, the discovery of holes in the ozone layer of the earth's atmosphere has raised concern about how much ultraviolet light reaches the earth's surface. Of particular concern are the questions of how deeply into sea water ultraviolet radiation penetrates and how it affects marine organisms, especially plankton (floating microorganisms) and viruses that attack plankton. Plankton form the base of the marine food chains and are believed to affect our planet's temperature and weather by uptake of CO2 for photosynthesis.

Metastatic cancer cell biosensors

Metastasis is the spread of cancer from one part of the body to another via either the circulatory system or lymphatic system. Unlike radiology imaging tests (mammograms), which send forms of energy (x-rays, magnetic fields, etc.) through the body to only take interior pictures, biosensors have the potential to directly test the malignant power of the tumor. The combination of a biological and detector element allows for a small sample requirement, a compact design, rapid signals, rapid detection, high selectivity and high sensitivity for the analyte being studied.

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Compared to the usual radiology imaging tests biosensors have the advantage of not only finding out how far the cancer has spread and checking if treatment is effective, but also are cheaper, more efficient (in time, cost and productivity) ways to assess metastaticity in early stages of cancer.

Biological engineering researchers have created oncological biosensors for breast cancer. Breast cancer is the leading common cancer among women worldwide. An example would be a transferrin- quartz crystal microbalance (QCM). As a biosensor, quartz crystal microbalances produce oscillations in the frequency of the crystal's standing wave from an alternating potential to detect nano-gram mass changes. These biosensors are specifically designed to interact and have high selectivity for receptors on cell (cancerous and normal) surfaces. Ideally this provides a quantitative detection of cells with this receptor per surface area instead of a qualitative picture detection given by mammograms.