SEMESTER III

17BTU304B BIO- ANALYTICAL TOOL 3H - 3C

Total hours/week: L:3 T:0 P:0 Marks: Internal: 40 External: 60 Total: 100

Scope: This course deals with the study of bio-analytical tools and their applications.

Objective: This paper will enable the students to learn in detail about microscopy, colorimetry, chromatography, electrophoresis, blotting techniques and bio sensors.

UNIT I

Microscopy: Simple microscopy, phase contrast microscopy, florescence and electron microscopy (TEM and SEM), pH meter, absorption and emission spectroscopy

UNIT II

Colorimetry: Principle and law of absorption fluorimetry, colorimetry, spectrophotometry (visible, UV, infrared), centrifugation, cell fractionation techniques, isolation of sub-cellular organelles and particles.

UNIT III

Chromatography : Introduction to the principle of chromatography. Paper chromatography, thin layer chromatography, column chromatography: silica and gel filtration, affinity and ion exchange chromatography, gas chromatography, HPLC.

UNIT IV

Electrophoresis: Introduction to electrophoresis. Starch-gel, polyacrylamide gel (native and SDS-PAGE), agarose-gel electrophoresis,

UNIT V

Applications: Pulse field gel electrophoresis, immuno- electrophoresis, isoelectric focusing, Western blotting. Introduction to Biosensors and Nanotechnology and their applications**.**

References

1. Karp, G. (2013). *Cell and Molecular Biology: Concepts and Experiments* (7th ed.). Hoboken, US: John Wiley & Sons. Inc.
2. Cooper, G.M., & Hausman, R.E. (2013). *The Cell: A Molecular Approach* (6th ed.). Washington, USA: ASMPress & Sunderland, D.C., Sinauer Associates.
3. De Robertis, E.D.P., & De Robertis, E.M.F. (2006). *Cell and Molecular Biology* (8th ed.). Lippincott Williams and Wilkins, Philadelphia.
4. Becker, W.M., Kleinsmith, L.J., Hardin. J., & Bertoni, G. P. (2009). *The World of the Cell* (7th ed.). San Francisco: Pearson Benjamin Cummings Publishing.

KARPAGAM ACADEMY OF HIGHER EDUCATION

DEPARTMENT OF BIOTECHNOLOGY

II B.Sc., BIOTECHNOLOGY – SEMESTER 3

LECTURE PLAN – BIO-ANALYTICAL TOOL 17BTU304B

S.No	Lecture Duration (hr)	Topic to be covered	Support materials
		UNIT I	
1	1	Microscope - Introduction	T1 Pg 26-29
2	1	Simple Microscope and its history behind	T2 Pg 37-41
3	1	Phase contrast microscopy	T1 Pg 31-33
4	1	Fluorescence microscopy	T2 Pg 47-48
5	1	Electron Microscopy	T1 Pg 43-44
6	1	Ph Meter & Absorption spectroscopy	T1 Pg 57-64
7	1	Emission spectroscopy	T1 Pg 200-201
8	1	Revision	
		UNIT II	
9	1	Principle and Law of absorption fluorimeter	T1 Pg 137-139
10	1	Colorimeter and Spectrophotometry (UV, Visible, Infrared)	T1 Pg 82-84
11	1	Centrifugation	T1 Pg 114-116
12	1	Different, Types of centrifuge &its uses	T2 Pg 91-117
13	1	Cell fractionation techniques	T1 Pg 82-84
14	1	Difference between colorimeter & spectrophotometer	T2 Pg 355-356
15	1	Isolation of subcellular organelle and particles	T2 Pg 356-357
16	1	Revision	
		UNIT III	
	1	Introduction to Chromatography	T1 Pg 139-140
17	1	Paper Chromatography	T1 Pg 141
18	1	Thin Chromatography	T2 Pg 129
19	1	Column Chromatography	T1 Pg 147-148
20	1	Silica & gel filtration	T1 Pg 150-151

21	1	Affinity &Ion Exchange Chromatography	T1 Pg 148-150
22	1	Gas chromatography	T1 Pg 158-159
23	1	HPLC & its uses	T1 Pg 159-161
24	1	Revision	
29			
25	1	Introduction to electrophoresis	T1 Pg 167-171
26	1	Starch gel electrophoresis	T1 Pg 167-171
27	1	PAGE	T1 Pg 172-174
28	1	SDS-PAGE	T1 Pg 172-174
29	1	AGE	T1 Pg 178-179
30	1	Revision	
UNIT V			
31	1	PFGE	T1 Pg 179-180
32	1	Immunoelectrophoresis	T1 Pg 183-187
33	1	Isoelectric focusing	T1 Pg 174-177
34	1	Western blotting	T4 Pg 78-80
35	1	Introduction to Biosensors and nanotechnology, its application	T3 Pg 63-78
36	1	Revision	T3 Pg 139-151

Reference Books

1. T1. Sabari Ghosal., A.K Srinivastav (2010), Fundamentals of Bioanalytical techniques & Instrumentation, PHI learning private limited, New Delhi.
2. T2: Abhilasha, Shourie and Shilpa (2005), Bioanalytical Technology, TERI, New Delhi.
3. T3: Mascini. M (2009), Aptamers in Bioanalysis, John Wiley& Sons, New Jersy.
4. T4: Sadasivam.S and Manikam. A (2005), Biochemical Methods, New Age International Limited, New Delhi.

UNIT-I

SYLLABUS

Microscopy: Simple microscopy, Phase contrast microscopy, fluorescence and electron microscopy (TEM and SEM), pH meter, absorption and emission spectroscopy.

Simple Microscope

A microscope is an optical instrument which is used to see highly magnified images of tiny objects such as bacteria, cells , viruses and protozoans etc. because these tiny objects cannot be seen by naked eyes. In this chapter we shall study two types of microscopes:

1. Simple microscope
2. Compound microscope

Simple Microscope

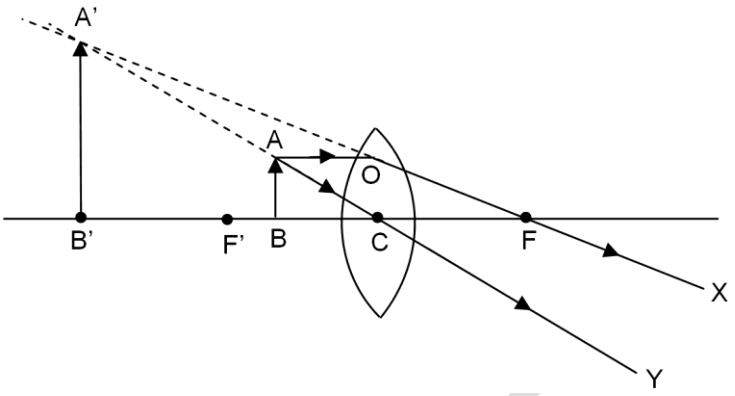
A simple microscope is also called magnifying glass. It is actually a convex lens of small focal length, which is used for seeing the magnified images of small objects.

Principle of Simple Microscope

A simple microscope works on the principle that when a tiny object is placed within its focus, a virtual, erect and magnified image of the object is formed at the least distance of distinct vision from the eye held close to the lens.

Working of Simple Microscope

The ray diagram to show the working of simple microscope is shown in figure. A small object AB which is to be magnified is placed between the principal focus F' and optical centre C of the convex lens. Now, a ray of light AO parallel to principal axis which is coming from the point A of the object passes through the focus F along the straight line OX after getting refracted by the convex lens. A second ray of light AC coming from the point A of the object passes through the optical centre C of the convex lens along the straight line CY. As is clear from the figure that the two rays i.e. OX and CY are diverging rays so these rays can intersect each other only at point A' when produced backward. Now, on drawing A'B' perpendicular from point A' to the principal axis, we get the image A'B' of the object which is virtual, erect and magnified.



Uses of Simple Microscope: Following are the important uses of simple microscope:

1. The simple microscope is commonly used by watch makers to see the magnified view of small parts of a watch.
2. It is also used by the jewelers to see the magnified view of the fine parts of jewellery.
3. Simple microscope is used to see the enlarged image of letters of a book, textures of fibers or threads of a cloth.
4. Simple microscope is used to see the magnified view of different particles of different types of soils.
5. It is used by palmists to see enlarged view of the lines of our hand.
6. Simple microscope is used by skin specialists to find out various diseases of skin.
7. It is also used to see the details of stamp and engravings.

Magnification of Simple Microscope

The magnifying power of a simple microscope is given by: $m = 1 + \frac{D}{f}$

Where, D = least distance of distinct vision, F = focal length of the convex lens

It should be noted that the focal length of the convex lens should be small because smaller the focal length of the lens, greater will be its magnifying power. Also the maximum magnification of a simple microscope is about 10, which means that the object will appear 10 times larger by using the simple microscope of maximum magnification.

Phase-contrast microscopy

It 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 travel 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, phase changes often carry important information.

Phase-contrast microscopy is particularly important in biology. It reveals many cellular structures that are not visible with a simpler bright-field microscope, as exemplified in the figure. These structures were made visible to earlier microscopists by staining, but this required additional preparation and thus killing the cells. The phase-contrast microscope made it possible for biologists to study living cells and how they proliferate through cell division. After its invention in the early 1930s,phase-contrast microscopy proved to be such an advancement in microscopy that its inventor Frits Zernike was awarded the Nobel Prize in Physics in 1953.

Working principle

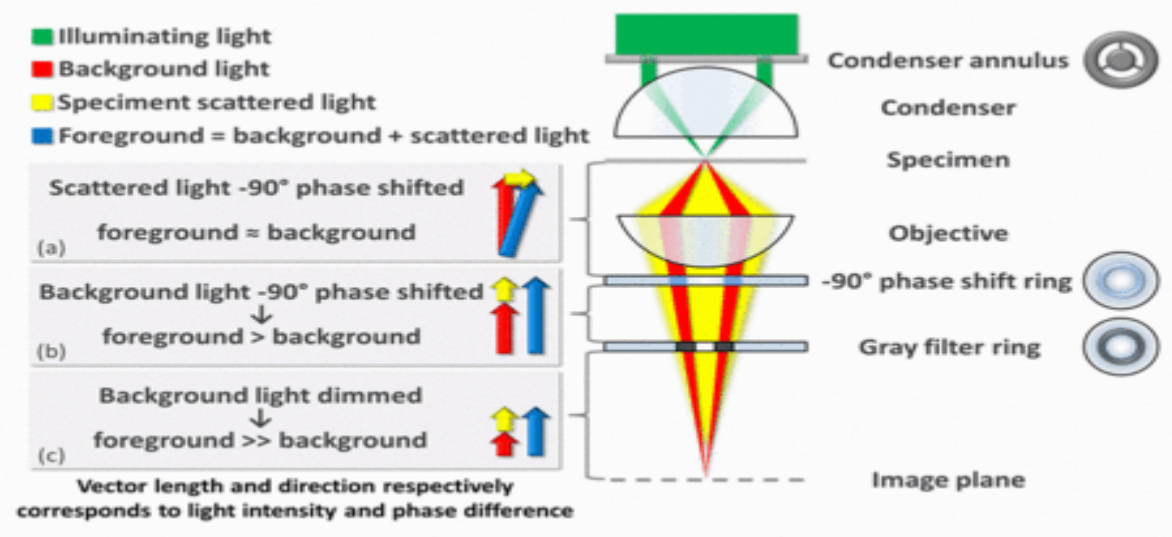
Dark field and phase contrast microscopies operating principle

The basic principle to making phase changes visible in phase-contrast microscopy is to separate the illuminating (background) light from the specimen-scattered light (which makes 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 forms the background light (red). When observing an unstained biological specimen, the scattered light is weak and typically phase-shifted by −90° (due to both the typical thickness of specimens and the refractive index difference

between biological tissue and the surrounding medium) relative to the background light. This leads to the foreground (blue vector) and background (red vector) having nearly the same intensity, resulting in low image contrast.

In a phase-contrast microscope, image contrast is increased in two ways: by generating constructive interference between scattered and background light rays in regions of the field of view that contain the specimen, and by reducing the amount of background light that reaches the image plane. First, the background light is phase-shifted by −90° by passing it through a phase-shift ring, which eliminates the phase difference between the background and the scattered light rays.



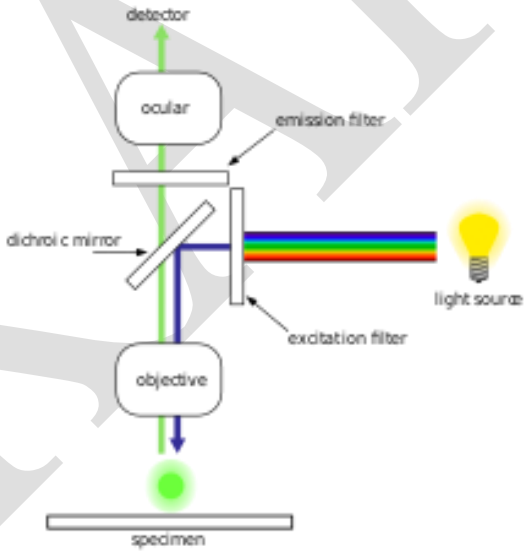
When the light is then focused on the image plane (where a camera or eyepiece is placed), this phase shift causes background and scattered light rays originating from regions of the field of view that contain the sample (i.e., the foreground) to constructively interfere, resulting in an increase in the brightness of these areas compared to regions that do not contain the sample. Finally, the background is dimmed ~70-90% by a gray filter ring—this method maximizes the amount of scattered light generated by the illumination (i.e., background) light, while minimizing the amount of illumination light that reaches the image plane. Some of the scattered light (which illuminates the entire surface of the filter) will be phase-shifted and dimmed by the rings, but to a much lesser extent than the background light (which only illuminates the phase-shift and gray filter rings).

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. The scattered light will then be subtracted from the background light to form an image with a darker foreground and a lighter background, as shown in the first figure.

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.

In the year 2014, the Nobel Prize in Chemistry was awarded to Eric Betzig, William Moerner and Stefan Hell for "the development of super-resolved fluorescence microscopy," which brings "optical microscopy into the nanodimension".

Epifluorescence 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 illuminates the

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 greater resolution will need objective lens with higher 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. The dichroic beam splitter acts as a wavelength specific filter, transmitting fluoresced light through to the eyepiece or detector, but reflecting any remaining excitation light back towards the source.

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, super continuum 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. By placing two microlens arrays into the illumination path of a widefield epifluorescence microscope, highly uniform illumination with a coefficient of variation of 1-2% can be achieved.

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 labeling 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.

Immunofluorescence

Immunofluorescence is a technique which uses the highly specific binding of an antibody to its antigen in order to label specific proteins or other molecules within the cell. A sample is treated with a primary antibody specific for the molecule of interest. A fluorophore can be directly conjugated to the primary antibody. Alternatively a secondary antibody, conjugated to a fluorophore, which binds specifically to the first antibody can be used. For example, a primary antibody raised in a mouse which recognises tubulin combined with a secondary anti-mouse antibody derivatised with a fluorophore could be used to label microtubules in a cell.

Fluorescent proteins

The modern understanding of genetics and the techniques available for modifying DNA allow scientists to genetically modify proteins to also carry a fluorescent protein reporter. In biological samples this allows a scientist to directly make a protein of interest fluorescent. The protein location can then be directly tracked, including in live cells.

Limitation

Fluorophores lose their ability to fluoresce as they are illuminated in a process

called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence. Photobleaching can severely limit the time over which a sample can be observed by fluorescent microscopy. Several techniques exist to reduce photobleaching such as the use of more robust fluorophores, by minimizing illumination, or by using photoprotective scavenger chemicals.

Fluorescence microscopy with fluorescent reporter proteins has enabled analysis of live cells by fluorescence microscopy, however cells are susceptible to phototoxicity, particularly with short wavelength light. Furthermore, fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.

Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been labeled for fluorescence. For example, observing a tissue sample prepared with a fluorescent DNA stain by fluorescent microscopy only reveals the organization ofthe DNA within the cells and reveals nothing else about the cell morphologies

Electron microscopes have emerged as a powerful tool for the characterization of a wide range of materials. Their versatility and extremely high spatial resolution render them a very valuable tool for many applications. The two main types of electron microscopes are the Transmission Electron Microscope (TEM) and the Scanning Electron Microscope (SEM). In this blog we briefly describe their similarities and differences.

Working Principle of Scanning Electron Microscopes and Transmission Electron Microscopes

For both techniques, electrons are used in order to acquire images of samples. Their main components are the same;

- An electron source;
- A series of electromagnetic and electrostatic lenses to control the shape and trajectory of the electron beam;
- Electron apertures.

All of these components live inside a chamber which is under high vacuum.Now over to the differences. SEMs use a specific set of coils to scan the beam in a raster-like pattern and collect the scattered electrons (read more about the different type of electrons detected in a SEM).

The transmission electron microscopy (TEM) principle, as the name suggests, is to use the transmitted electrons; the electrons which are passing through the sample before they are collected. As a result, TEM offers invaluable information on the inner structure of the sample, such as crystal structure, morphology and stress state information, while SEM provides information on the sample's surface and its composition.

Moreover, one of the most pronounced differences between the two methods is the optimal spatial resolution that they can achieve; SEM resolution is limited to ~0.5 nm, while with the recent development in aberration-corrected TEMs, images with spatial resolution of even less than 50 pm have been reported.

Advantages:

This all depends on what type of analysis you want to perform. For example, if you want to get information on the surface of your sample, like roughness or contamination detection, then you should choose a SEM. On the other hand, if you would like to know what the crystal structure of your sample is, or if you want to look for possible structural defects or impurities, then using a TEM is the only way to do so.

SEMs provide a 3D image of the surface of the sample whereas TEM images are 2D projections of the sample, which in some cases makes the interpretation of the results more difficult for the operator.

Due to the requirement for transmitted electrons, TEM samples must be very thin, generally below 150 nm, and in cases that high-resolution imaging is required, even below 30 nm, whereas for SEM imaging there is no such specific requirement.

This reveals one more major difference between the two techniques; sample preparation. SEM samples require little or no effort for sample preparation and can be directly imaged by mounting them on an aluminum stub.

In contrast, TEM sample preparation is a quite complex and tedious procedure that only trained and experienced users can follow successfully. The samples need to be very thin, as flat as possible, and the preparation technique should not induce any artefacts (such as precipitates or amorphization) to the sample. Many methods have been developed, including electropolishing, mechanical polishing and focused ion beam milling. Dedicated grids and holders are used to mount the TEM samples.

SEM vs TEM: differences in operation

The two EM systems also differ in the way they are operated. SEMs usually use acceleration voltages up to 30 kV, while TEM users can set it in the range of 60 – 300kV.

The magnifications that TEMs offer are also much higher compared to SEMs: TEM users can magnify their samples by more than 50 million times, while for the SEM this is limited up to 1-2 million times.

However, the maximum Field of View (FOV) that SEMs can achieve is far larger than TEMs, which users can only use to image a very small part of their sample. Similarly, the depth of field of SEM systems are much higher than in TEM systems.

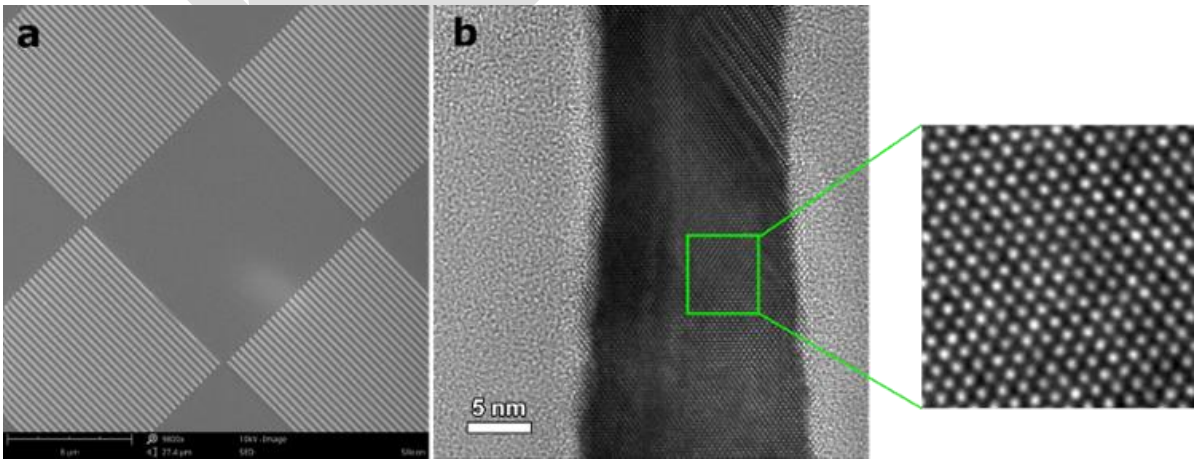


Figure 1: Electron microscopy images of silicon. a) SEM image with SED offers information on the morphology of the surface, while b) TEM image reveals structural information about the inner sample.

In addition, the way images are created are different in the two systems. In SEMs, samples are positioned at the bottom of the electron column and the scattered electrons (back-scattered or secondary) are captured by electron detectors. Photomultipliers are then used to convert this signal into a voltage signal, which is amplified and gives rise to the image on a PC screen.

In a TEM microscope, the sample is located in the middle of the column. The transmitted electrons pass through it, and through a series of lenses below the sample (intermediate and projector lenses). An image is directly shown on a fluorescent screen or via a charge-coupled device (CCD) camera, onto a PC screen.

	SEM	**TEM**
Type of electrons	Scattered, scanning electrons	Transmitted electrons
High tension	~1 – 30 kV	~60 – 300 kV
Specimen thickness	Any	Typically <150 nm
Type of info	3D image of surface	2D projection image of inner structure
Max. magnification	Up to ~1 – 2 million times	More than 50 million times
Max. FOV	Large	Limited

Optimal spatial resolution	~0.5 nm	< 50 pm
Image formation	Electrons are captured and counted by detectors, image on PC screen	Direct imaging on fluorescent screen or PC screen with CCD
Operation	Little or no sample preparation, easy to use	Laborious sample preparation, trained users required

Table I: Summary of the main differences between a SEM and a TEM

Generally, TEMs are more complex to operate. TEM users require intensive training before being able to operate them. Special procedures need to be performed before every use, with several steps included that ensure that the electron beam is perfectly aligned.

A **pH Meter** is a scientific instrument that measures the hydrogen-ion activity in water-based solutions, indicating its acidity or alkalinity expressed as pH. The pH meter measures the difference in electrical potential between a pH electrode and a reference electrode, and so the pH meter is sometimes referred to as a "potentiometric pH meter". The difference in electrical potential relates to the acidity or pH of the solution. The pH meter is used in many applications ranging from laboratory experimentation to quality control.

Principle of operation

Potentiometric pH meters measure the voltage between two electrodes and display the result converted into the corresponding pH value. They comprise a simple electronic amplifier and a pair of electrodes, or alternatively a combination electrode, and some form of display calibrated in pH units. It usually has a glass electrode and a reference electrode, or a combination electrode. The electrodes, or probes, are inserted into the solution to be tested.

The design of the electrodes is the key part: These are rod-like structures usually made of glass, with a bulb containing the sensor at the bottom. The glass electrode for measuring the pH has a glass bulb specifically designed to be selective to hydrogen-ion concentration. On immersion

in the solution to be tested, hydrogen-ions in the test solution exchange for other positively charged ions on the glass bulb, creating an electrochemical potential across the bulb. The electronic amplifier detects the difference in electrical potential between the two electrodes generated in the measurement and converts the potential difference to pH units. The magnitude of the electrochemical potential across the glass bulb is linearly related to the pH according to the Nernst Equation.

The reference electrode is insensitive to the pH of the solution being composed of a metallic conductor which connects to the display. This conductor is immersed in an electrolyte solution, typically potassium chloride, which comes into contact with the test solution through a porous ceramic membrane. The display consists of a voltmeter which displays voltage in units of pH.

On immersion of the glass electrode and the reference electrode in the test solution, an electrical circuit is completed in which there is a potential difference created and detected by the voltmeter. The circuit can be thought of as going from the conductive element of the reference electrode to the surrounding potassium chloride solution, through the ceramic membrane to the test solution, the hydrogen-ion selective glass of the glass electrode, to the solution inside the glass electrode, to the silver of the glass electrode, and finally the voltmeter of the display device.[10] The voltage varies from test solution to test solution depending on the potential difference created by the difference in hydrogen ion concentrations on each side of the glass membrane between the test solution and the solution inside the glass electrode. All other potential differences in the circuit do not vary with pH and are corrected for by means of the calibration.

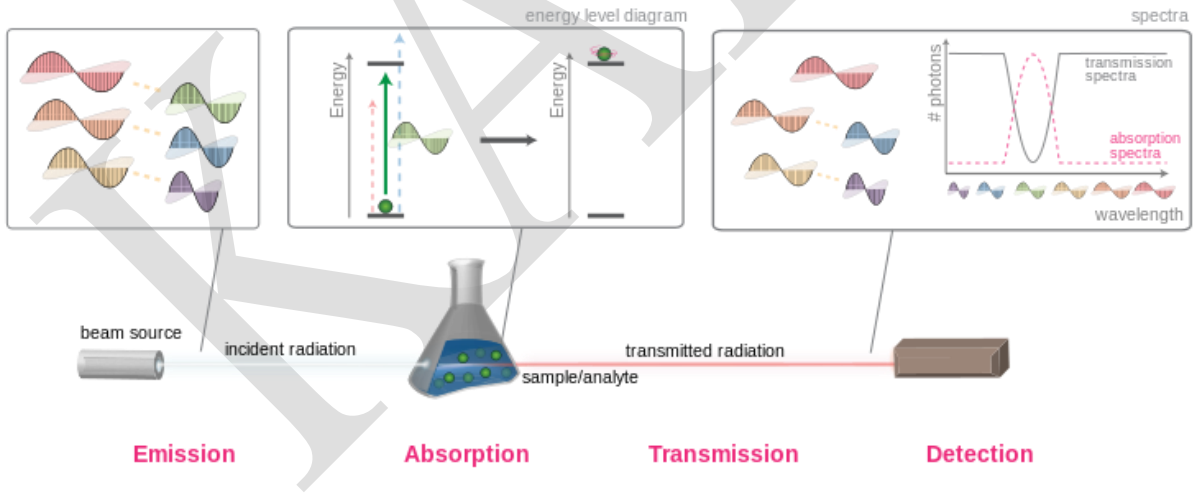
For simplicity, many pH meters use a combination probe, constructed with the glass electrode and the reference electrode contained within a single probe. A detailed description of combination electrodes is given in the article on glass electrodes.

The pH meter is calibrated with solutions of known pH, typically before each use, to ensure accuracy of measurement. To measure the pH of a solution, the electrodes are used as probes, which are dipped into the test solutions and held there sufficiently long for the hydrogen-ions in the test solution to equilibrate with the ions on the surface of the bulb on the glass electrode. This equilibration provides a stable pH measurement.

Absorption spectroscopy refers to spectroscopic techniques that measure the absorption of radiation, as a function of frequency or wavelength, due to its interaction with a sample. The sample absorbs energy, i.e., photons, from the radiating field. The intensity of the absorption varies as a function of frequency, and this variation is the absorption spectrum. Absorption spectroscopy is performed across the electromagnetic spectrum.

Absorption spectroscopy is employed as an analytical chemistry tool to determine the presence of a particular substance in a sample and, in many cases, to quantify the amount of the substance present. Infrared and ultraviolet-visible spectroscopy are particularly common in analytical applications. Absorption spectroscopy is also employed in studies of molecular and atomic physics, astronomical spectroscopy and remote sensing.

There are a wide range of experimental approaches for measuring absorption spectra. The most common arrangement is to direct a generated beam of radiation at a sample and detect the intensity of the radiation that passes through it. The transmitted energy can be used to calculate the absorption. The source, sample arrangement and detection technique vary significantly depending on the frequency range and the purpose of the experiment.



A material's absorption spectrum is the fraction of incident radiation absorbed by the material over a range of frequencies. The absorption spectrum is primarily determined[1][2][3] by the atomic and molecular composition of the material. Radiation is more likely to be absorbed at frequencies that match the energy difference between two quantum mechanical states of the

molecules. The absorption that occurs due to a transition between two states is referred to as an absorption line and a spectrum is typically composed of many lines.

The frequencies where absorption lines occur, as well as their relative intensities, primarily depend on the electronic and molecular structure of the sample. The frequencies will also depend on the interactions between molecules in the sample, the crystal structure in solids, and on several environmental factors (e.g., temperature, pressure, electromagnetic field). The lines will also have a width and shape that are primarily determined by the spectral density or the density of states of the system.

Theory

Absorption lines are typically classified by the nature of the quantum mechanical change induced in the molecule or atom. Rotational lines, for instance, occur when the rotational state of a molecule is changed. Rotational lines are typically found in the microwave spectral region. Vibrational lines correspond to changes in the vibrational state of the molecule and are typically found in the infrared region. Electronic lines correspond to a change in the electronic state of an atom or molecule and are typically found in the visible and ultraviolet region. X-ray absorptions are associated with the excitation of inner shell electrons in atoms. These changes can also be combined (e.g. rotation-vibration transitions), leading to new absorption lines at the combined energy of the two changes.

The energy associated with the quantum mechanical change primarily determines the frequency of the absorption line but the frequency can be shifted by several types of interactions. Electric and magnetic fields can cause a shift. Interactions with neighboring molecules can cause shifts. For instance, absorption lines of the gas phase molecule can shift significantly when that molecule is in a liquid or solid phase and interacting more strongly with neighboring molecules.

The width and shape of absorption lines are determined by the instrument used for the observation, the material absorbing the radiation and the physical environment of that material. It is common for lines to have the shape of a Gaussian or Lorentzian distribution. It is also common for a line to be described solely by its intensity and width instead of the entire shape being characterized.

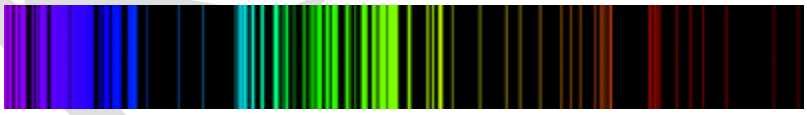
The integrated intensity—obtained by integrating the area under the absorption line—is proportional to the amount of the absorbing substance present. The intensity is also related to the temperature of the substance and the quantum mechanical interaction between the radiation and the absorber. This interaction is quantified by the transition moment and depends on the particular lower state the transition starts from, and the upper state it is connected to.

The width of absorption lines may be determined by the spectrometer used to record it. A spectrometer has an inherent limit on how narrow a line it can resolve and so the observed width may be at this limit. If the width is larger than the resolution limit, then it is primarily determined by the environment of the absorber. A liquid or solid absorber, in which neighboring molecules strongly interact with one another, tends to have broader absorption lines than a gas. Increasing the temperature or pressure of the absorbing material will also tend to increase the line width. It is also common for several neighboring transitions to be close enough to one another that their lines overlap and the resulting overall line is therefore broader yet.

Relation to transmission spectrum

Absorption and transmission spectra represent equivalent information and one can be calculated from the other through a mathematical transformation. A transmission spectrum will have its maximum intensities at wavelengths where the absorption is weakest because more light is transmitted through the sample. An absorption spectrum will have its maximum intensities at wavelengths where the absorption is strongest.

Relation to emission spectrum

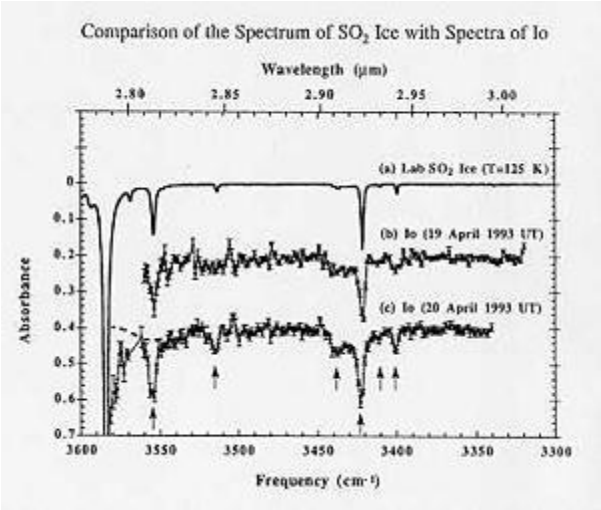


Emission spectrum of iron

Emission is a process by which a substance releases energy in the form of electromagnetic radiation. Emission can occur at any frequency at which absorption can occur, and this allows the absorption lines to be determined from an emission spectrum. The emission spectrum will typically have a quite different intensity pattern from the absorption spectrum, though, so the two are not equivalent. The absorption spectrum can be calculated from the emission spectrum using

appropriate theoretical models and additional information about the quantum mechanical states of the substance.

Applications



The infrared absorption spectrum of NASA laboratory sulfur dioxide ice is compared with the infrared absorption spectra of ices on Jupiter's moon, **Io** credit NASA, Bernard Schmitt, and UKIRT.

Absorption spectroscopy is useful in chemical analysis because of its specificity and its quantitative nature. The specificity of absorption spectra allows compounds to be distinguished from one another in a mixture, making absorption spectroscopy useful in wide variety of applications. For instance, Infrared gas analyzers can be used to identify the presence of pollutants in the air, distinguishing the pollutant from nitrogen, oxygen, water and other expected constituents.

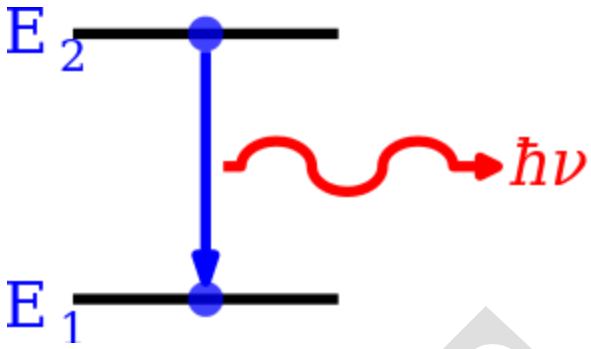
The specificity also allows unknown samples to be identified by comparing a measured spectrum with a library of reference spectra. In many cases, it is possible to determine qualitative information about a sample even if it is not in a library. Infrared spectra, for instance, have characteristics absorption bands that indicate if carbon-hydrogen or carbon-oxygen bonds are present.

An absorption spectrum can be quantitatively related to the amount of material present using the Beer-Lambert law. Determining the absolute concentration of a compound requires knowledge of the compound's absorption coefficient. The absorption coefficient for some compounds is available from reference sources, and it can also be determined by measuring the spectrum of a calibration standard with a known concentration of the target.

Emission spectroscopy

Light consists of electromagnetic radiation of different wavelengths. Therefore, when the elements or their compounds are heated either on a flame or by an electric arc they emit energy in the form of light. Analysis of this light, with the help of a spectroscope gives us a discontinuous spectrum. A spectroscope or a spectrometer is an instrument which is used for separating the components of light, which have different wavelengths. The spectrum appears in a series of lines called the line spectrum. This line spectrum is called an atomic spectrum when it originates from an atom in elemental form. Each element has a different atomic spectrum. The production of line spectra by the atoms of an element indicate that an atom can radiate only a certain amount of energy. This leads to the conclusion that bound electrons cannot have just any amount of energy but only a certain amount of energy.

The emission spectrum can be used to determine the composition of a material, since it is different for each element of the periodic table. One example is astronomical spectroscopy: identifying the composition of stars by analysing the received light. The emission spectrum characteristics of some elements are plainly visible to the naked eye when these elements are heated. For example, when platinum wire is dipped into a strontium nitrate solution and then inserted into a flame, the strontium atoms emit a red color. Similarly, when copper is inserted into a flame, the flame becomes green. These definite characteristics allow elements to be identified by their atomic emission spectrum. Not all emitted lights are perceptible to the naked eye, as the spectrum also includes ultraviolet rays and infrared lighting. An emission is formed when an excited gas is viewed directly through a spectroscope.



Schematic diagram of spontaneous emission

Emission spectroscopy is a spectroscopic technique which examines the wavelengths of photons emitted by atoms or molecules during their transition from an excited state to a lower energy state. Each element emits a characteristic set of discrete wavelengths according to its electronic structure, and by observing these wavelengths the elemental composition of the sample can be determined. Emission spectroscopy developed in the late 19th century and efforts in theoretical explanation of atomic emission spectra eventually led to quantum mechanics.

There are many ways in which atoms can be brought to an excited state. Interaction with electromagnetic radiation is used in fluorescence spectroscopy, protons or other heavier particles in Particle-Induced X-ray Emission and electrons or X-ray photons in Energy-dispersive X-ray spectroscopy or X-ray fluorescence. The simplest method is to heat the sample to a high temperature, after which the excitations are produced by collisions between the sample atoms. This method is used in flame emission spectroscopy, and it was also the method used by Anders Jonas Ångström when he discovered the phenomenon of discrete emission lines in the 1850s.[1]

Although the emission lines are caused by a transition between quantized energy states and may at first look very sharp, they do have a finite width, i.e. they are composed of more than one wavelength of light. This spectral line broadening has many different causes.

Emission spectroscopy is often referred to as **optical emission spectroscopy** because of the light nature of what is being emitted.

History

Emission lines from hot gases were first discovered by Ångström, and the technique was further developed by David Alter, Gustav Kirchhoff and Robert Bunsen.

Experimental technique in flame emission spectroscopy

The solution containing the relevant substance to be analysed is drawn into the burner and dispersed into the flame as a fine spray. The solvent evaporates first, leaving finely divided solid particles which move to the hottest region of the flame where gaseous atoms and ions are produced. Here electrons are excited as described above. It is common for a monochromator to be used to allow for easy detection.

On a simple level, flame emission spectroscopy can be observed using just a flame and samples of metal salts. This method of qualitative analysis is called a flame test. For example, sodium salts placed in the flame will glow yellow from sodium ions, while strontium (used in road flares) ions color it red. Copper wire will create a blue colored flame, however in the presence of chloride gives green (molecular contribution by CuCl).

Emission coefficient is a coefficient in the power output per unit time of an electromagnetic source, a calculated value in physics. The emission coefficient of a gas varies with the wavelength of the light. It has units of $ms^{-3}sr^{-1}$.[2] It is also used as a measure of environmental emissions (by mass) per MWh of electricity generated, see: Emission factor.

Scattering of light

In Thomson scattering a charged particle emits radiation under incident light. The particle may be an ordinary atomic electron, so emission coefficients have practical applications.

If $\boldsymbol{X}\, dV\, d\Omega\, d\lambda$ is the energy scattered by a volume element dV into solid angle $d\Omega$ between wavelengths λ and $\lambda+d\lambda$ per unit time then the Emission coefficient is $\boldsymbol{X}$.

The values of $\boldsymbol{X}$ in Thomson scattering can be predicted from incident flux, the density of the charged particles and their Thomson differential cross section (area/solid angle).

Spontaneous emission

A warm body emitting photons has a monochromatic emission coefficient relating to its temperature and total power radiation. This is sometimes called the second Einstein coefficient, and can be deduced from quantum mechanical theory.

Possible Questions

Short questions

1. What is Simple microscope?
2. What are the parts of the microscope? Explain.
3. What is Magnification?
4. What are the uses of microscopy?
5. Write short note on Phase contrast microscopy?
6. Define fluorescence microscope.
7. What is electron microscope?
8. What is absorption and emission?
9. What is a spectroscopy?
10. What is a immunofluorescence?

Essay type questions

1. Explain in detail the principle and working of simple microscope. Add notes on the magnification.
2. Give in detail about the phase contrast microscope and dark field microscope with neat diagram.
3. Explain in detail about the immunofluorescent microscopy and its uses.
4. Explain in detail about the principle and working of electron microscope.
5. Write in detail about the difference between the SEM and TEM.
6. Discuss the difference between the absorption and emission spectroscopy.

UNIT-II

SYLLABUS

Colorimetry: Principle and law of absorption fluorimetry, colorimetry, spectrophotometry (Visible, UV, infrared), Centrifugation, cell fractionation techniques, isolation of sub-cellular organelles and particles.

Fluorescence spectroscopy (also known as **fluorometry** or **spectrofluorometry**) is a type of electromagnetic spectroscopy that 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 there are various vibrational states.[1]

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.

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.

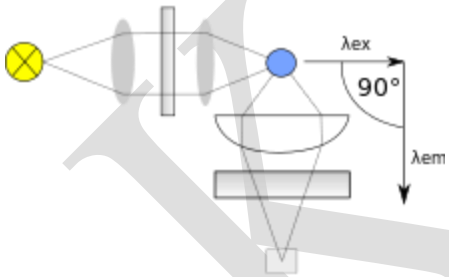
In a typical fluorescence (emission) measurement, the excitation wavelength is fixed and the detection wavelength varies, while in a fluorescence excitation measurement the detection

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 gratingmonochromators 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 is 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,[3]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 through 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.

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.

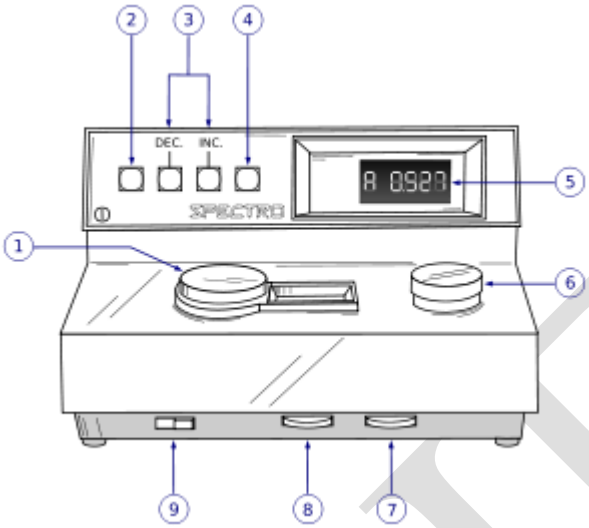
Applications

Fluorescence spectroscopy is used in, among others, biochemical, medical, and chemical research fields for analyzing organic compounds. There has also been a report of its use in differentiating malignant skin tumors from benign.

Atomic Fluorescence Spectroscopy (AFS) techniques are useful in other kinds of analysis/measurement of a compound present in air or water, or other media, such as CVAFS which is used for heavy metals detection, such as mercury. Fluorescence can also be used to redirect photons, see fluorescent solar collector. Additionally, Fluorescence spectroscopy can be adapted to the microscopic level using microfluorimetry. In analytical chemistry, fluorescence detectors are used with HPLC.

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 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.

Construction



(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

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.

In addition, there may be:

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

There are many commercialized colorimeters as well as open source versions with construction documentation for education and for research.

Filters

Changeable optics filters are used in the colorimeter to select the wavelength 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.

Cuvettes

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.

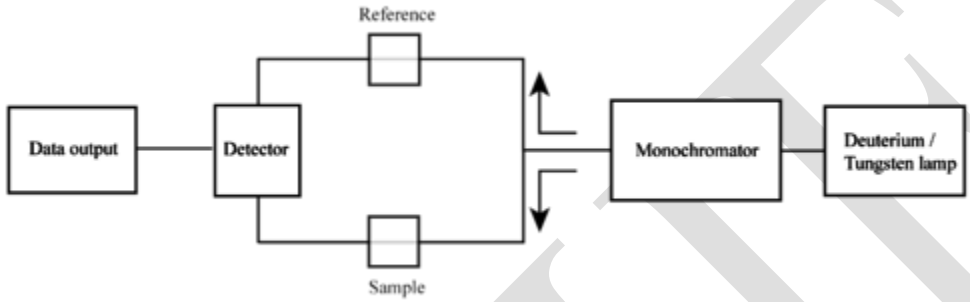
In addition, the output may be sent to a chart recorder, data logger, or computer.

Ultraviolet-visible spectrophotometer

The UV-visible spectrophotometer can also be configured to measure reflectance. In this case, the spectrophotometer measures the intensity of light reflected from a sample and compares it to the intensity of light reflected from a reference material (such as a white tile). The ratio is called the *reflectance*, and is usually expressed as a percentage (%R).

The basic parts of a spectrophotometer are a light source, a holder for the sample, a diffraction grating in a monochromator or a prism to separate the different wavelengths of light, and a detector. The radiation source is often a Tungsten filament (300-2500 nm), a deuterium arc lamp, which is continuous over the ultraviolet region (190-400 nm), Xenon arc lamp, which is continuous from 160-2,000 nm; or more recently, light emitting diodes (LED) for the visible wavelengths. The detector is typically a photomultiplier tube, a photodiode, a photodiode array or a charge-coupled device (CCD). Single photodiode detectors and photomultiplier tubes are used

with scanning monochromators, which filter the light so that only light of a single wavelength reaches the detector at one time. The scanning monochromator moves the diffraction grating to "step-through" each wavelength so that its intensity may be measured as a function of wavelength. Fixed monochromators are used with CCDs and photodiode arrays. As both of these devices consist of many detectors grouped into one or two dimensional arrays, they are able to collect light of different wavelengths on different pixels or groups of pixels simultaneously.



Simplified schematic of a double beam UV- visible spectrophotometer.

A spectrophotometer can be either *single beam* or *double beam*. In a single beam instrument all of the light passes through the sample cell.

It measured by removing the sample. This was the earliest design and is still in common use in both teaching and industrial labs.

In a double-beam instrument, the light is split into two beams before it reaches the sample. One beam is used as the reference; the other beam passes through the sample. The reference beam intensity is taken as 100% Transmission (or 0 Absorbance), and the measurement displayed is the ratio of the two beam intensities. Some double-beam instruments have two detectors (photodiodes), and the sample and reference beam are measured at the same time. In other instruments, the two beams pass through a beam chopper, which blocks one beam at a time. The detector alternates between measuring the sample beam and the reference beam in synchronism with the chopper. There may also be one or more dark intervals in the chopper cycle. In this case, the measured beam intensities may be corrected by subtracting the intensity measured in the dark interval before the ratio is taken.

Samples for UV/Vis spectrophotometry are most often liquids, although the absorbance of gases and even of solids can also be measured. Samples are typically placed in a transparent cell, known as a cuvette. Cuvettes are typically rectangular in shape, commonly with an internal width of 1 cm. (This width becomes the path length, in the Beer-Lambert law.) Test tubes can also be used as cuvettes in some instruments. The type of sample container used must allow radiation to pass over the spectral region of interest. The most widely applicable cuvettes are made of high quality fused silica or quartz glass because these are transparent throughout the UV, visible and near infrared regions. Glass and plastic cuvettes are also common, although glass and most plastics absorb in the UV, which limits their usefulness to visible wavelengths.

Specialized instruments have also been made. These include attaching spectrophotometers to telescopes to measure the spectra of astronomical features. UV-visible microspectrophotometers consist of a UV-visible microscope integrated with a UV-visible spectrophotometer.

A complete spectrum of the absorption at all wavelengths of interest can often be produced directly by a more sophisticated spectrophotometer. In simpler instruments the absorption is determined one wavelength at a time and then compiled into a spectrum by the operator. By removing the concentration dependence, the extinction coefficient (ε) can be determined as a function of wavelength.

A **centrifuge** is a piece of equipment that puts an object in rotation around a fixed axis (spins it in a circle), applying a potentially strong force perpendicular to the axis of spin (outward). The centrifuge works using the sedimentation principle, where the centripetal acceleration causes denser substances and particles to move outward in the radial direction. At the same time, objects that are less dense are displaced and move to the center. In a laboratory centrifuge that uses sample tubes, the radial acceleration causes denser particles to settle to the bottom of the tube, while low-density substances rise to the top.

There are 3 types of centrifuge designed for different applications. Industrial scale centrifuges are commonly used in manufacturing and waste processing to sediment suspended solids, or to separate immiscible liquids. An example is the cream separator found in dairies. Very

high speed centrifuges and ultracentrifuges able to provide very high accelerations can separate fine particles down to the nano-scale, and molecules of different masses.

Large centrifuges are used to simulate high gravity or acceleration environments (for example, high-G training for test pilots). Medium-sized centrifuges are used in washing machines and at some swimming pools to wring water out of fabrics. Gas centrifuges are used for isotope separation, such as to enrich nuclear fuel for fissile isotopes.

Types

A centrifuge machine can be described as a machine with a rapidly rotating container that applies centrifugal force to its contents. There are multiple types of centrifuge, which can be classified by intended use or by rotor design:

Types by rotor design:

- Fixed-angle centrifuges are designed to hold the sample containers at a constant angle relative to the central axis.

Analytical ultracentrifuge

In an analytical ultracentrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption and/or interference optical refractive index sensitive system. This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field. With modern instrumentation, these observations are electronically digitized and stored for further mathematical analysis. Two kinds of experiments are commonly performed on these instruments: sedimentation velocity experiments and sedimentation equilibrium experiments.

Preparative ultracentrifuge

Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments. Most rotors are designed to hold tubes that contain the samples. *Swinging bucket rotors* allow the tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially accelerates.

Fixed angle rotors are made of a single block of material and hold the tubes in cavities bored at a predetermined angle. *Zonal rotors* are designed to contain a large volume of sample in a single central cavity rather than in tubes. Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high speed.

Preparative rotors are used in biology for pelleting of fine particulate fractions, such as cellular organelles (mitochondria, microsomes, ribosomes) and viruses. They can also be used for gradient separations, in which the tubes are filled from top to bottom with an increasing concentration of a dense substance in solution. Sucrose gradients are typically used for separation of cellular organelles. Gradients of caesium salts are used for separation of nucleic acids. After the sample has spun at high speed for sufficient time to produce the separation, the rotor is allowed to come to a smooth stop and the gradient is gently pumped out of each tube to isolate the separated components.

- Swinging head (or swinging bucket) centrifuges, in contrast to fixed-angle centrifuges, have a hinge where the sample containers are attached to the central rotor. This allows all of the samples to swing outwards as the centrifuge is spun.
- Continuous tubular centrifuges do not have individual sample vessels and are used for high volume applications.

Centrifugation of subcellular organelles

Once the cell-free homogenate has been prepared the next step is the use of differential centrifugation to separate out the various organelles on the basis of their size and density.

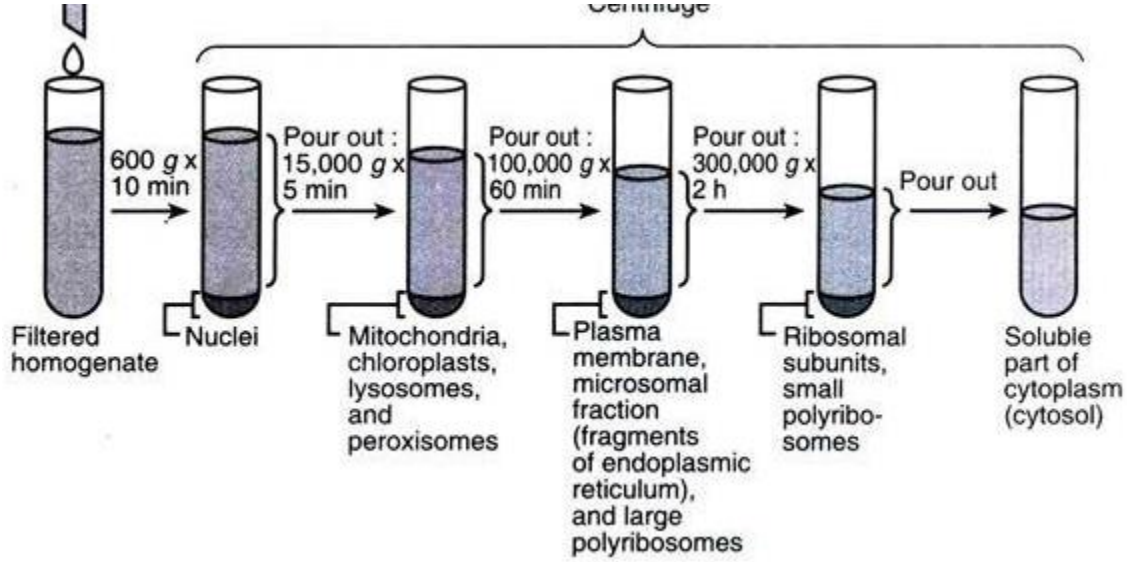
Table 1. Size and Density of Some Sub-cellular Organelles from Liver

Cell organelles	Size mm	Density (g/cm^3) (in sucrose medium)
Nuclei	3 - 12	>1.30
Mitochondria	0.5 - 2.0	1.17 - 1.21
Lysosomes	0.2 - 0.4	1.20 - 1.22
Peroxisomes	0.2 - 0.5	1.23
Endoplasmic reticulum vesicles (microsomes)	0.05 - 0.30	1.15 (smooth ER) 1.22 (rough ER)
Golgi stacks	~ 1.0	1.10 - 1.13
Golgi vesicles	~0.05	
Plasma membrane sheets	20	1.15 - 1.19
Plasma membrane vesicles	0.05	< 1.17

Organelles sedimented by centrifugation at increasing speeds

[1] 1,000 x g (10 min)	Unbroken cells, nuclei, plasma membrane sheets, heavy mitochondria plus smaller, trapped particles.
[2] 3,000 x g (10 min)	Heavy mitochondria, plasma membrane fragments plus smaller, trapped particles.
[3] 10,000 x g (20 min)	Mitochondria, lysosomes, peroxisome, some Golgi membranes and rough endoplasmic reticulum.
[4] 100,000 x g (60 min)	Membrane vesicles derived from smooth and rough endoplasmic reticulum, Golgi vesicles and plasma membrane vesicles.
[5] 100,000 x g Supernatant	Cytoplasmic components plus any soluble organelle

The material to be separated is divided centrifugally into number of fractions by increasing the applied centrifugal field. The centrifugal field at each step is chosen so that particular type of material sediments. Any type of particle originally present in homogenate may be found in pellet or the supernatant or both fractions, depending upon the time and speed of centrifugation and size and density of particles. At the end of each stage the pellet and supernatant are separated and pellet washed several times by re-suspension and re-centrifugation in homogenation medium.



Initially all particles of homogenate are homogenously distributed throughout the centrifuge tube. During centrifugation particles move down the centrifuge tubes at their respective sedimentation rates and start to form a pellet on the bottom of centrifuge tube. Ideally centrifugation is continued enough to pellet all the largest class of particles, the resulting supernatant then being centrifuged at a higher speed to separate medium-sized particles and so on.

Possible Questions

Short questions

1. What is fluorimeter?
2. What is colorimeter?
3. What are filters?
4. What is wavelength and absorption?
5. Write short note spectroscopy?
6. Define the wavelength ranges of colorimeter.
7. What is visible range?
8. What is centrifugation?
9. What is sedimentation rate?
10. What is centrifugal force?
11. What is density gradient centrifuge?

Essay type questions

1. Explain the principle and instrumentation of colorimeter in detail.
2. What is the principle and instrumentation of centrifuge in detail?
3. What are the different types of centrifuge? Add notes on the density gradient centrifugation.
4. What is the methodology of cell fractionation?
5. Describe the importance of absorption fluorimeter.

UNIT-III

SYLLABUS

Chromatography: Introduction to the principle of chromatography, Paper chromatography, thin layer chromatography, column chromatography, silica and gel filtration, affinity and ion exchange chromatography, Gas chromatography, HPLC.

Chromatography is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the *mobile phase,* which carries it through a structure holding another material called the *stationary phase.* The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for later use, and is thus a form of purification. Analytical chromatography is done normally with smaller amounts of material and is for establishing the presence or measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.

Chromatography was first employed in Russia by the Italian-born scientist Mikhail Tsvet in 1900.He continued to work with chromatography in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll, carotenes, and xanthophylls.

- A *chromatograph* is equipment that enables a sophisticated separation, e.g. gas chromatographic or liquid chromatographic separation.
- *Chromatography* is a physical method of separation that distributes components to separate between two phases, one stationary (stationary phase), the other (the mobile phase) moving in a definite direction.
- The *eluate* is the mobile phase leaving the column. This is also called effluent.
- The *eluent* is the solvent that carries the analyte.
- The *eluite* is the analyte, the eluted solute.
- An *eluotropic series* is a list of solvents ranked according to their eluting power.
- An *immobilized phase* is a stationary phase that is immobilized on the support particles, or on the inner wall of the column tubing.
- The *mobile phase* is the phase that moves in a definite direction. It may be a liquid (LC and Capillary Electrochromatography (CEC)), a gas (GC), or a supercritical fluid (supercritical-

fluid chromatography, SFC). The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. In the case of HPLC the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or a polar solvent such as methanol in reverse phase chromatography and the sample being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.

- *Preparative chromatography* is used to purify sufficient quantities of a substance for further use, rather than analysis.
- The *retention time* is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions. See also: Kovats' retention index
- The *sample* is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as waste.
- The *solute* refers to the sample components in partition chromatography.
- The *solvent* refers to any substance capable of solubilizing another substance, and especially the liquid mobile phase in liquid chromatography.
- The *stationary phase* is the substance fixed in place for the chromatography procedure. Examples include the silica layer in thin layer chromatography
- The *detector* refers to the instrument used for qualitative and quantitative detection of analytes after separation.

Chromatography is based on the concept of partition coefficient. Any solute partitions between two immiscible solvents. When we make one solvent immobile (by adsorption on a solid support matrix) and another mobile it results in most common applications of chromatography. If the matrix support, or stationary phase, is polar (e.g. paper, silica etc.) it is forward phase chromatography, and if it is non-polar (C-18) it is reverse phase.

Paper chromatography is an analytical method used to separate colored chemicals or substances. It is primarily used as a teaching tool, having been replaced by other chromatography methods, such as thin-layer chromatography. A paper chromatography variant, two-dimensional chromatography involves using two solvents and rotating the paper 90° in between. This is useful for separating complex mixtures of compounds having similar polarity, for example, amino acids. The setup has three components. The mobile phase is a solution that travels up the stationary phase, due to capillary action. The mobile phase is generally mixture of polar organic solvent with water, while the stationary phase is water. Paper is used to support stationary phase (water). Difference between TLC and paper chromatography is that stationary phase in TLC is a layer of adsorbent (usually silica gel, or aluminium oxide), and stationary phase in paper chromatography is water.

Types

Descending

Development of the chromatogram is done by allowing the solvent to travel down the paper. Here, mobile phase is placed in solvent holder at the top. The spot is kept at the top and solvent flows down the paper from above.

Ascending

Here the solvent travels up the chromatographic paper. Both descending and ascending paper chromatography are used for the separation of organic and inorganic substances. The sample and solvent move upward.

Ascending-descending

This is the hybrid of both of the above techniques. The upper part of ascending chromatography can be folded over a rod in order to allow the paper to become descending after crossing the rod.

Radial

This is also called circular chromatography. A circular filter paper is taken and the sample is deposited at the center of the paper. After drying the spot, the filter paper is tied horizontally on a petri dish containing solvent, so that the wick of the paper is dipped in the solvent. The solvent rises through the wick and the components are separated into concentric circles.

Two-dimensional

In this technique a square or rectangular paper is used. Here the sample is applied to one of the corners and development is performed at a right angle to the direction of the first run.

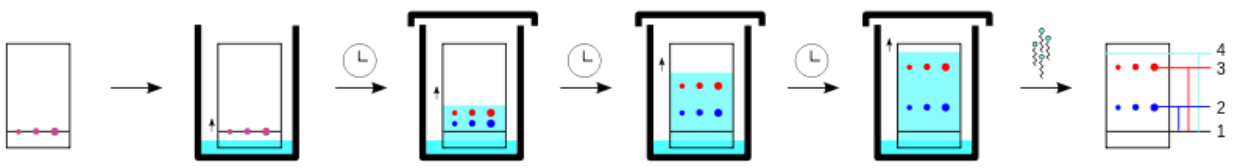
Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures.Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase.

Plate preparation

TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate (gypsum) and water. This mixture is spread as a thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. The resultant plate is dried and *activated* by heating in an oven for thirty minutes at 110 °C. The thickness of the absorbent layer is typically around 0.1 – 0.25 mm for analytical purposes and around 0.5 – 2.0 mm for preparative TLC.

Technique

The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products. Plates can be labeled before or after the chromatography process using a pencil or other implement that will not interfere or react with the process.



To run a thin layer chromatography plate, the following procedure is carried out.

- Using a capillary, a small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. The solvent is allowed to completely evaporate off to prevent it from interfering with sample's interactions with the mobile phase in the next step. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a vacuum chamber. This step is often repeated to ensure there is enough analyte at the starting spot on the plate to obtain a visible result. Different samples can be placed in a row of spots the same distance from the bottom edge, each of which will move in its own adjacent lane from its own starting point.
- A small amount of an appropriate solvent (eluent) is poured into a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. A strip of filter paper (aka "wick") is put into the chamber so that its bottom touches the solvent and the paper lies on the chamber wall and reaches almost to the top of the container. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber. (Failure to saturate the chamber will result in poor separation and non-reproducible results).
- The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the eluent in the chamber, and the lid is closed. The solvent moves up the plate by capillary action, meets the sample mixture and carries it up the plate (elutes the sample). The plate should be removed from the chamber before the solvent front reaches the top of the stationary phase (continuation of the elution will give a misleading result) and dried.
- Without delay, the *solvent front*, the furthest extent of solvent up the plate, is marked.
- The plate is visualized. As some plates are pre-coated with a phosphor such as zinc sulfide, allowing many compounds to be visualized by using ultraviolet light; dark spots appear where the compounds block the UV light from striking the plate. Alternatively, plates can be sprayed or immersed in chemicals after elution. Various visualising agents react with the spots to produce visible results.

    - **Column chromatography** in chemistry is a chromatography method used to isolate a single chemical compound from a mixture. Chromatography is able to separate substances based on differential adsorption of compounds to the adsorbent; compounds move through the column at different rates, allowing them to be separated into fractions. The technique

is widely applicable, as many different adsorbents (normal phase, reversed phase, or otherwise) can be used with a wide range of solvents. The technique can be used on scales from micrograms up to kilograms. The main advantage of column chromatography is the relatively low cost and disposability of the stationary phase used in the process. The latter prevents cross-contamination and stationary phase degradation due to recycling. Column chromatography can be done using gravity to move the solvent, or using compressed gas to push the solvent through the column.

- A thin-layer chromatograph can show how a mixture of compounds will behave when purified by column chromatography. The separation is first optimised using thin-layer chromatography before performing column chromatography.

Column preparation

A column is prepared by packing a solid absorbent into a cylindrical glass or plastic tube. The size will depend on the amount of compound being isolated. The base of the tube contains a filter, either a cotton or glass wool plug, or glass frit to hold the solid phase in place. A solvent reservoir may be attached at the top of the column.

Two methods are generally used to prepare a column: the dry method and the wet method. For the dry method, the column is first filled with dry stationary phase powder, followed by the addition of mobile phase, which is flushed through the column until it is completely wet, and from this point is never allowed to run dry.[1] For the wet method, a slurry is prepared of the eluent with the stationary phase powder and then carefully poured into the column. The top of the silica should be flat, and the top of the silica can be protected by a layer of sand. Eluent is slowly passed through the column to advance the organic material.

The individual components are retained by the stationary phase differently and separate from each other while they are running at different speeds through the column with the eluent. At the end of the column they elute one at a time. During the entire chromatography process the eluent is collected in a series of fractions. Fractions can be collected automatically by means of fraction collectors. The productivity of chromatography can be increased by running several columns at a time. In this case multi stream collectors are used. The composition of the eluent flow can be monitored and each fraction is analyzed for dissolved compounds, e.g. by analytical

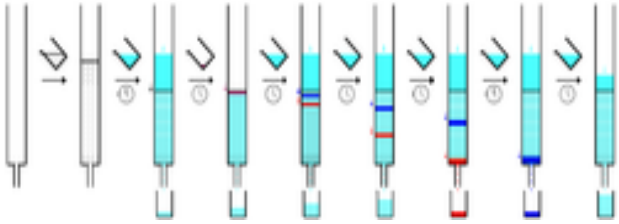
chromatography, UV absorption spectra, or fluorescence. Colored compounds (or fluorescent compounds with the aid of a UV lamp) can be seen through the glass wall as moving bands.

Stationary phase

The *stationary phase* or *adsorbent* in column chromatography is a solid. The most common stationary phase for column chromatography is **silica gel**, the next most common being **alumina**. **Cellulose** powder has often been used in the past.

A wide range of stationary phases are available in order to perform ion exchange chromatography, reversed-phase chromatography (RP), affinity chromatography or expanded bed adsorption (EBA). The stationary phases are usually finely ground powders or gels and/or are microporous for an increased surface, though in EBA a fluidized bed is used. There is an important ratio between the stationary phase weight and the dry weight of the analyte mixture that can be applied onto the column. For silica column chromatography, this ratio lies within 20:1 to 100:1, depending on how close to each other the analyte components are being eluted.

Mobile phase (eluent)



Column chromatography proceeds by a series of steps.

The *mobile phase* or *eluent* is a solvent or a mixture of solvents used to move the compounds through the column. It is chosen so that the retention factor value of the compound of interest is roughly around 0.2 - 0.3 in order to minimize the time and the amount of eluent to run the chromatography. The eluent has also been chosen so that the different compounds can be separated effectively. The eluent is optimized in small scale pretests, often using thin layer chromatography (TLC) with the same stationary phase.

There is an optimum flow rate for each particular separation. A faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation. However, the maximum flow rate is limited because a finite time is required for the analyte to equilibrate between the stationary phase and mobile phase, see Van Deemter's equation. A simple laboratory column runs by gravity flow. The flow rate of such a column can be increased by extending the fresh eluent filled column above the top of the stationary phase or decreased by the tap controls. Faster flow rates can be achieved by using a pump or by using compressed gas (e.g. air, nitrogen, or argon) to push the solvent through the column (flash column chromatography).

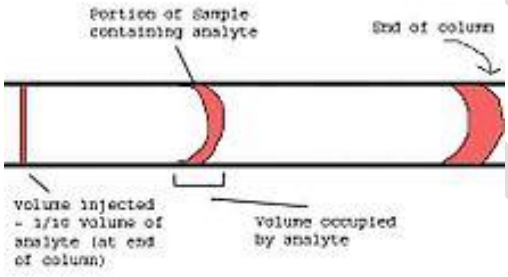
The particle size of the stationary phase is generally finer in flash column chromatography than in gravity column chromatography. For example, one of the most widely used silica gel grades in the former technique is mesh 230 – 400 (40 – 63 μm), while the latter technique typically requires mesh 70 – 230 (63 – 200 μm) silica gel.

A spreadsheet that assists in the successful development of flash columns has been developed. The spreadsheet estimates the retention volume and band volume of analytes, the fraction numbers expected to contain each analyte, and the resolution between adjacent peaks. This information allows users to select optimal parameters for preparative-scale separations before the flash column itself is attempted.

Gas chromatography (**GC**) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can also be determined). In

some situations, GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture.

In gas chromatography, the *mobile phase* (or "moving phase") is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. Helium remains the most commonly used carrier gas in about 90% of instruments although hydrogen is preferred for improved separations. The *stationary phase* is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column (a homage to



the fractionating column used in distillation). The instrument used to perform gas chromatography is called a *gas chromatograph* (or "aerograph", "gas separator").

The gaseous compounds being analyzed interact with the walls of the column, which is coated with a stationary phase. This causes each compound to elute at a different time, known as the *retention time* of the compound. The comparison of retention times is what gives GC its analytical usefulness.

Gas chromatography is in principle similar to column chromatography (as well as other forms of chromatography, such as HPLC, TLC), but has several notable differences. First, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase, whereas in column chromatography the stationary phase is a solid and the mobile phase is a liquid. (Hence the full name of the procedure is "Gas–liquid chromatography", referring to the mobile and stationary phases, respectively.) Second, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas

column chromatography (typically) has no such temperature control. Finally, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas.

Gas chromatography is also similar to fractional distillation, since both processes separate the components of a mixture primarily based on boiling point (or vapor pressure) differences. However, fractional distillation is typically used to separate components of a mixture on a large scale, whereas GC can be used on a much smaller scale (i.e. microscale).

Gas chromatography is also sometimes known as **vapor-phase chromatography** (VPC), or **gas–liquid partition chromatography**(GLPC). These alternative names, as well as their respective abbreviations, are frequently used in scientific literature. Strictly speaking, GLPC is the most correct terminology, and is thus preferred by many authors.

Sample injection

The real chromatographic analysis starts with the introduction of the sample onto the column. The development of capillary gas chromatography resulted in many practical problems with the injection technique. The technique of on-column injection, often used with packed columns, is usually not possible with capillary columns. The injection system in the capillary gas chromatograph should fulfil the following two requirements:

1. The amount injected should not overload the column.
2. The width of the injected plug should be small compared to the spreading due to the chromatographic process. Failure to comply with this requirement will reduce the separation capability of the column. As a general rule, the volume injected, V_{inj}, and the volume of the detector cell, V_{det}, should be about 1/10 of the volume occupied by the portion of sample containing the molecules of interest (analytes) when they exit the column.

Some general requirements which a good injection technique should fulfill are:

- It should be possible to obtain the column's optimum separation efficiency.
- It should allow accurate and reproducible injections of small amounts of representative samples.

- It should induce no change in sample composition. It should not exhibit discrimination based on differences in boiling point, polarity, concentration or thermal/catalytic stability.
- It should be applicable for trace analysis as well as for undiluted samples.

However, there are a number of problems inherent in the use of syringes for injection:

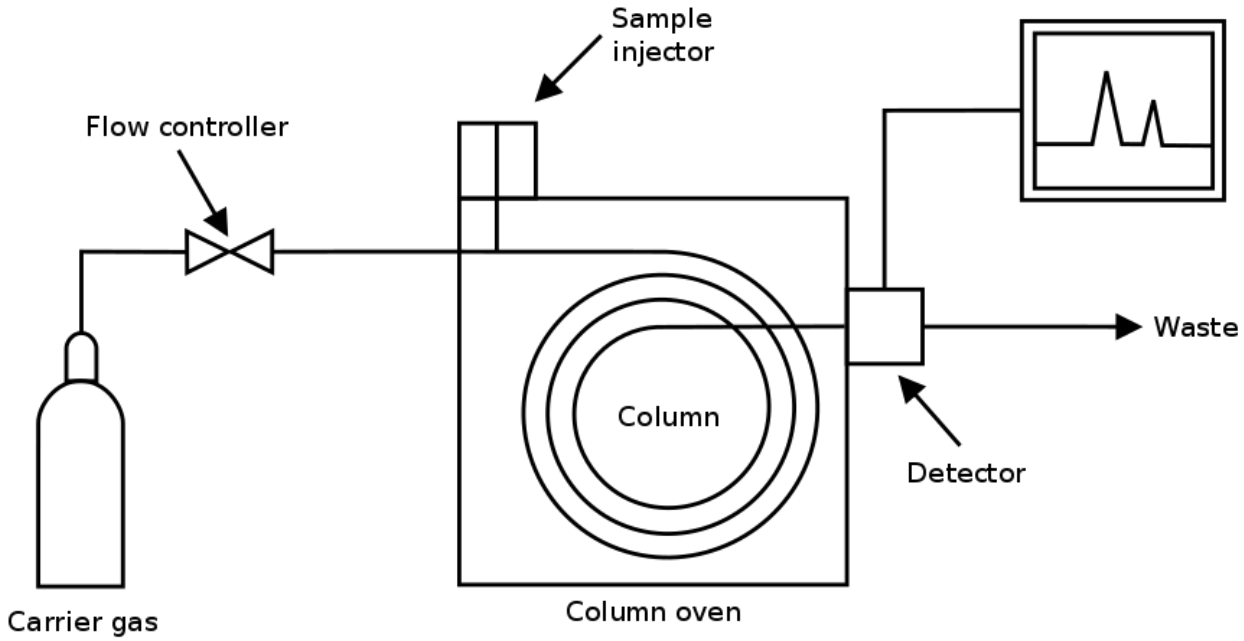
- Even the best syringes claim an accuracy of only 3%, and in unskilled hands, errors are much larger
- The needle may cut small pieces of rubber from the septum as it injects sample through it. These can block the needle and prevent the syringe filling the next time it is used. It may not be obvious that this has happened.
- A fraction of the sample may get trapped in the rubber, to be released during subsequent injections. This can give rise to ghost peaks in the chromatogram.
- There may be selective loss of the more volatile components of the sample by evaporation from the tip of the needle.

Column selection

The choice of column depends on the sample and the active measured. The main chemical attribute regarded when choosing a column is the polarity of the mixture, but functional groups can play a large part in column selection. The polarity of the sample must closely match the polarity of the column stationary phase to increase resolution and separation while reducing run time. The separation and run time also depends on the film thickness (of the stationary phase), the column diameter and the column length.

Qualitative analysis

Generally chromatographic data is presented as a graph of detector response (y-axis) against retention time (x-axis), which is called a chromatogram. This provides a spectrum of peaks for a sample representing the analytes present in a sample eluting from the column at different times. Retention time can be used to identify analytes if the method conditions are constant. Also, the pattern of peaks will be constant for a sample under constant conditions and can identify complex mixtures of analytes. However, in most modern applications, the GC is connected to a mass spectrometer or similar detector that is capable of identifying the analytes represented by the peaks.



Quantitative analysis

The area under a peak is proportional to the amount of analyte present in the chromatogram. By calculating the area of the peak using the mathematical function of integration, the concentration of an analyte in the original sample can be determined. Concentration can be calculated using a calibration curve created by finding the response for a series of concentrations of analyte, or by determining the relative response factor of an analyte. The relative response factor is the expected ratio of an analyte to an internal standard (or external standard) and is calculated by finding the response of a known amount of analyte and a constant amount of internal standard (a chemical added to the sample at a constant concentration, with a distinct retention time to the analyte).

In most modern GC-MS systems, computer software is used to draw and integrate peaks, and match MS spectra to library spectra.

Applications

In general, substances that vaporize below 300 °C (and therefore are stable up to that temperature) can be measured quantitatively. The samples are also required to be salt-free; they should not contain ions. Very minute amounts of a substance can be measured, but it is often

required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

Various temperature programs can be used to make the readings more meaningful; for example to differentiate between substances that behave similarly during the GC process.

Professionals working with GC analyze the content of a chemical product, for example in assuring the quality of products in the chemical industry; or measuring toxic substances in soil, air or water. GC is very accurate if used properly and can measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.

In practicals, students sometimes get acquainted to the GC by studying the contents of Lavender oil or measuring the ethylene that is secreted by *Nicotiana benthamiana* plants after artificially injuring their leaves. These GC analyse hydrocarbons (C2-C40+). In a typical experiment, a packed column is used to separate the light gases, which are then detected with a TCD.

High-performance liquid chromatography (**HPLC**; formerly referred to as **high-pressure liquid chromatography**), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column.

Operation

The sample mixture to be separated and analyzed is introduced, in a discrete small volume (typically microliters), into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are a function of specific physical interactions with the adsorbent (also called stationary phase). The velocity of each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the mobile phase. The time at which a specific analyte elutes (emerges

from the column) is called its retention time. The retention time measured under particular conditions is an identifying characteristic of a given analyte.

Many different types of columns are available, filled with adsorbents varying in particle size, and in the nature of their surface ("surface chemistry"). The use of smaller particle size packing materials requires the use of higher operational pressure ("backpressure") and typically improves chromatographic resolution (*i.e.* the degree of separation between consecutive analytes emerging from the column). Sorbent particles may be hydrophobic or polar in nature.

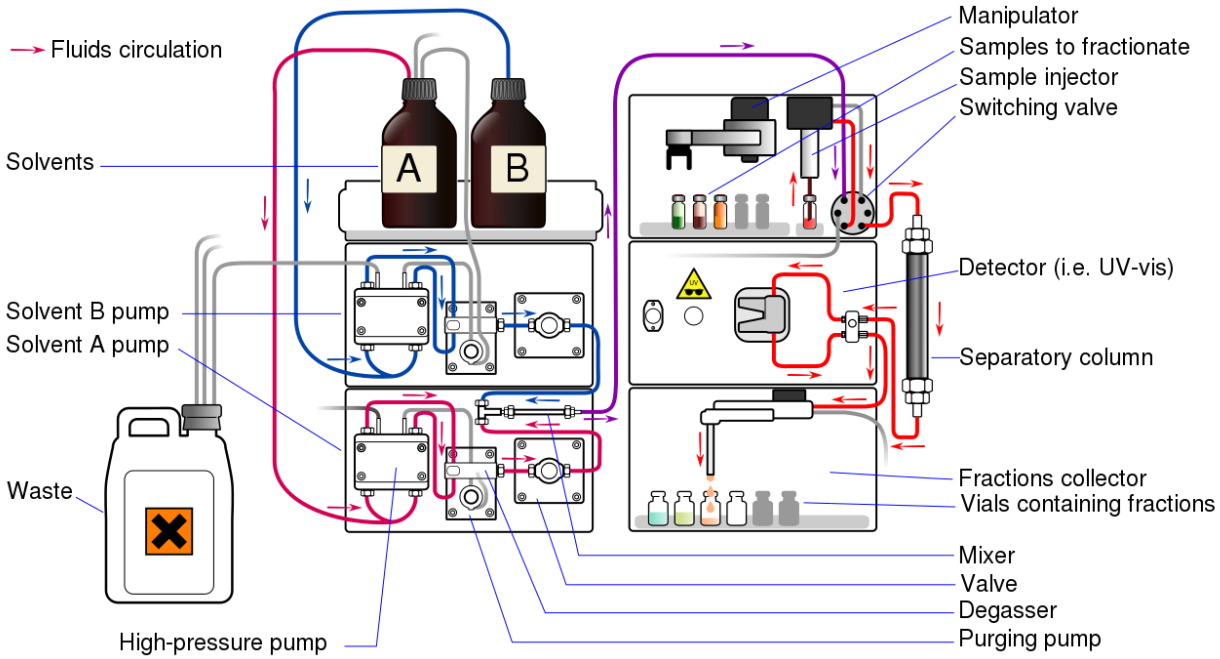
Common mobile phases used include any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol). Some HPLC techniques use water-free mobile phases (see Normal-phase chromatography below). The aqueous component of the mobile phase may contain acids (such as formic, phosphoric or trifluoroacetic acid) or salts to assist in the separation of the sample components. The composition of the mobile phase may be kept constant ("isocratic elution mode") or varied ("gradient elution mode") during the chromatographic analysis. Isocratic elution is typically effective in the separation of sample components that are very different in their affinity for the stationary phase.

In gradient elution the composition of the mobile phase is varied typically from low to high eluting strength. The eluting strength of the mobile phase is reflected by analyte retention times with high eluting strength producing fast elution (=short retention times). A typical gradient profile in reversed phase chromatography might start at 5% acetonitrile (in water or aqueous buffer) and progress linearly to 95% acetonitrile over 5–25 minutes. Periods of constant mobile phase composition may be part of any gradient profile. For example, the mobile phase composition may be kept constant at 5% acetonitrile for 1–3 min, followed by a linear change up to 95% acetonitrile.

The chosen composition of the mobile phase (also called eluent) depends on the intensity of interactions between various sample components ("analytes") and stationary phase (*e.g.* hydrophobic interactions in reversed-phase HPLC). Depending on their affinity for the stationary and mobile phases analytes partition between the two during the separation process taking place in the column. This partitioning process is similar to that which occurs during a liquid–liquid extraction but is continuous, not step-wise. In this example, using a water/acetonitrile gradient, more hydrophobic components will elute (come off the column) late,

once the mobile phase gets more concentrated in acetonitrile (*i.e.* in a mobile phase of higher eluting strength).

The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depends on the nature of the column and sample components. Often a series of trial runs is performed with the sample in order to find the HPLC method which gives adequate separation

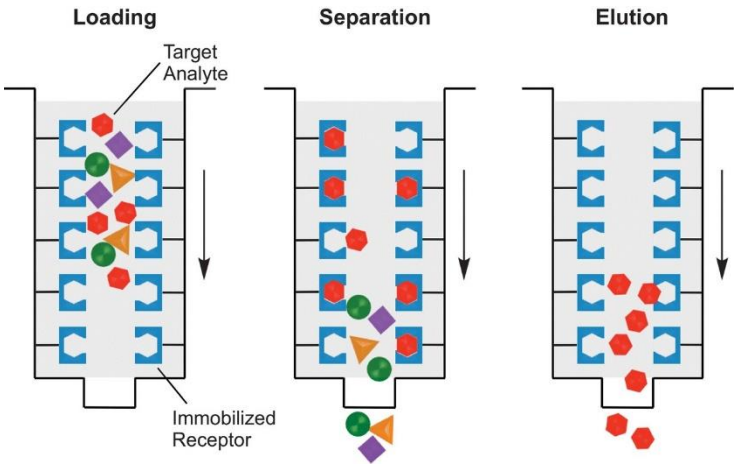


Affinity chromatography is a type of chromatographic laboratory technique used for purifying biological molecules within a mixture by exploiting molecular properties. Biological macromolecules, such as enzymes and other proteins, interact with other molecules with high specificity through several different types of bonds and interaction. Such interactions including hydrogen bonding, ionic interaction, disulfide bridges, hydrophobic interaction, and more. The high selectivity of affinity chromatography is caused by allowing the desired molecule to interact with the stationary phase and be bound within the column in order to be separated from the undesired material which will not interact and elute first. The molecules no longer needed are first washed away with a buffer while the desired proteins are let go in the presence of the eluting solvent (of higher salt concentration). This process creates a competitive interaction between the

desired protein and the immobilized stationary molecules, which eventually lets the now highly purified proteins be released.

Uses

Affinity chromatography can be used to purify and concentrate a substance from a mixture into a buffering solution, reduce the amount of unwanted substances in a mixture, identify the biological compounds binding to a particular substance, purify and concentrate an enzyme solution. The molecule of interest can be immobilized through covalent bonds. This occurs through an insoluble matrix such as chromatographic medium like cellulose or polyacrylamide. When the medium is bound to the protein of interest it becomes immobilized.



Principle

In summary, affinity chromatography exploits the differences in interactions' strengths between the different biomolecules within a mobile phase, and the stationary phase. The stationary phase is first loaded into a column with mobile phase containing a variety of biomolecules from DNA to proteins (depending on the purification experiment). Then, the two phases are allowed time to bind. A wash buffer is then poured through a column containing both bound phases. The wash buffer removes non-target biomolecules by disrupting their weaker interactions with the stationary phase. Target biomolecules have a much higher affinity for the stationary phase, and remain bound to the stationary phase, not being washed away by wash buffer. An elution buffer is then poured through the column containing the remaining target biomolecules. The elution buffer disrupts interactions between the bound target biomolecules with the stationary to a much greater extent

than the wash buffer, effectively removing the target biomolecules. This purified solution contains elution buffer and target biomolecules, and is called elution.

The stationary phase is typically a gel matrix, often of agarose; a linear sugar molecule derived from algae. To prevent steric interference or overlap during the binding process of the target molecule to the ligand, an inhibitor containing a hydrocarbon chain is first attached to the agarose bead (solid support). This inhibitor with a hydrocarbon chain is commonly known as the spacer between the agarose bead and the target molecule.

Usually, the starting point is a crude, heterogeneous group of molecules in a whole cell extract, such as a cell lysate, growth medium or blood serum. The molecule of interest will have a well known and defined property, and can be exploited during the affinity purification process. The process itself can be thought of as an entrapment, with the target molecule becoming trapped on a solid or stationary phase or medium. The other molecules in the mobile phase will not become trapped as they do not possess this property.

The stationary phase can then be removed from the mixture, washed and the target molecule released from the entrapment in a process known as dialysis. The desired molecules are eluted with specific substances after washing the non-interacting molecules away. Thus, this results in a highly purified material. Highly specific elution of the desired macromolecule from the stationary phase is usually effected by adding to the eluting buffer a gradient of the same kind on the macromolecule and displaces it.[6] Possibly the most common use of affinity chromatography is for the purification of recombinant proteins. Affinity chromatography is an excellent choice for the first step in purifying a protein or nucleic acid from a crude mixture.

If the molecular weight, hydrophobicity, charge, etc. of a protein is unknown, affinity chromatography can still apply to this situation. An example of this situation is when trying to find an enzyme with a particular activity, where it can be possible to build an affinity column with an attached ligand that is similar or identical to the substrate of choice. The way that the desired enzyme would be eluted would be from the mixture based on the strong interaction of enzyme and the immobilized substrate analog, which would be done selectively through the affinity column. Then, the elution of the enzyme with the appropriate substrate can be done

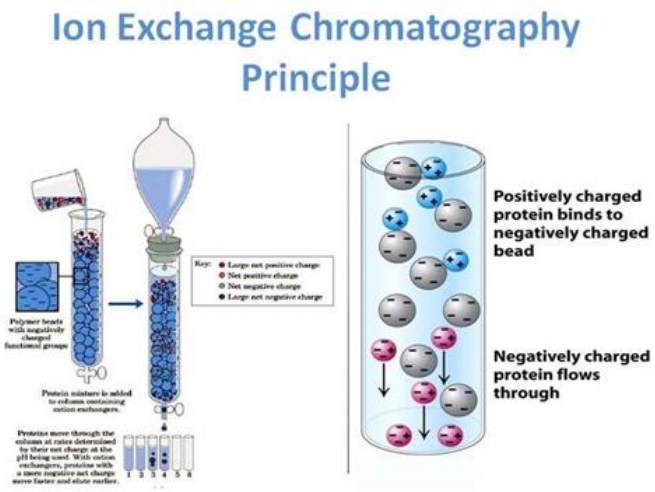
Ion chromatography (or **ion-exchange chromatography**) is a chromatography process that separates ions and polar molecules based on their affinity to the ion exchanger. It works on almost any kind of charged molecule—including large proteins, small nucleotides, and amino acids. However, ion chromatography must be done in conditions that are one unit away from the isoelectric point of a protein. The two types of ion chromatography are anion-exchange and cation-exchange. Cation-exchange chromatography is used when the molecule of interest is positively charged.

The molecule is positively charged because the pH for chromatography is less than the pI. In this type of chromatography, the stationary phase is negatively charged and positively charged molecules are loaded to be attracted to it. Anion-exchange chromatography is when the stationary phase is positively charged and negatively charged molecules (meaning that pH for chromatography is greater than the pI) are loaded to be attracted to it.

It is often used in protein purification, water analysis, and quality control. The water-soluble and charged molecules such as proteins, amino acids, and peptides bind to moieties which are oppositely charged by forming ionic bonds to the insoluble stationary phase. The equilibrated stationary phase consists of an ionizable functional group where the targeted molecules of a mixture to be separated and quantified can bind while passing through the column—a cationic stationary phase is used to separate anions and an anionic stationary phase is used to separate cations. Cation exchange chromatography is used when the desired molecules to separate are cations and anion exchange chromatography is used to separate anions.The bound molecules then can be eluted and collected using an eluant which contains anions and cations by running higher concentration of ions through the column or changing pH of the column. One of the primary advantages for the use of ion chromatography is only one interaction involved during the separation as opposed to other separation techniques; therefore, ion chromatography may have higher matrix tolerance.

Another advantage of ion exchange, is the predictably of elution patterns (based on the presence of the ionizable group). For example, when cation exchange chromatography is used, cations will elute out last. Meanwhile, the negative charged molecules will elute out first. However, there are also disadvantages involved when performing ion-exchange chromatography, such as constant

evolution with the technique which leads to the inconsistency from column to column. A major limitation to this purification technique is that it is limited to ionizable group



Procedure

Before ion-exchange chromatography can be initiated, it must be equilibrated. The stationary phase must be equilibrated to certain requirements that depend on the experiment that you are working with. Once equilibrated, the charged ions in the stationary phase will be attached to its opposite charged exchangeable ions. Exchangeable ions such as Cl- or Na+. Next, a buffer should be chosen in which the desired protein can bind to. After equilibration, the column needs to be washed. The washing phase will help elute out all impurities that does not bind to the matrix while the protein of interest remains bounded.

This sample buffer needs to have the same pH as the buffer used for equilibration to help bind the desired proteins. Uncharged proteins will be eluted out of the column at a similar speed of the buffer flowing through the column. Once the sample has been loaded onto to the column and the column has been washed with the buffer to elute out all non-desired proteins, elution is carried out to elute the desired proteins that are bound to the matrix. Bound proteins are eluted out by utilizing a gradient of linearly increasing salt concentration. With increasing ionic strength of the buffer, the salt ions will compete with the desired proteins in order to bind to charged groups on the surface of the medium. This will cause desired proteins to be eluted out of the column. Proteins that have a low net charge will be eluted out first as the salt concentration increases

causing the ionic strength to increase. Proteins with high net charge will need a higher ionic strength for them to be eluted out of the column.

It is possible to perform ion exchange chromatography in bulk, on thin layers of medium such as glass or plastic plates coated with a layer of the desired stationary phase, or in chromatography columns. Thin layer chromatography or column chromatography share similarities in that they both act within the same governing principles; there is constant and frequent exchange of molecules as the mobile phase travels along the stationary phase. It is not imperative to add the sample in minute volumes as the predetermined conditions for the exchange column have been chosen so that there will be strong interaction between the mobile and stationary phases.

Furthermore, the mechanism of the elution process will cause a compartmentalization of the differing molecules based on their respective chemical characteristics. This phenomenon is due to an increase in salt concentrations at or near the top of the column, thereby displacing the molecules at that position, while molecules bound lower are released at a later point when the higher salt concentration reaches that area. These principles are the reasons that ion exchange chromatography is an excellent candidate for initial chromatography steps in a complex purification procedure as it can quickly yield small volumes of target molecules regardless of a greater starting volume.

A simple device can be used to create a salt gradient. Elution buffer is consistently being drawn from the chamber into the mixing chamber, thereby altering its buffer concentration. Generally, the buffer placed into the chamber is usually of high initial concentration, whereas the buffer placed into the stirred chamber is usually of low concentration. As the high concentration buffer from the left chamber is mixed and drawn into the column, the buffer concentration of the stirred column gradually increase. Altering the shapes of the stirred chamber, as well as of the limit buffer, allows for the production of concave, linear, or convex gradients of counterion.

A multitude of different mediums are used for the stationary phase. Among the most common immobilized charged groups used are trimethylaminoethyl (TAM), triethylaminoethyl (TEAE), diethyl-2-hydroxypropylaminoethyl (QAE), aminoethyl (AE), diethylaminoethyl

(DEAE), sulpho (S), sulphomethyl (SM), sulphopropyl (SP), carboxy (C), and carboxymethyl (CM).

Successful packing of the column is an important aspect of ion chromatography. Stability and efficiency of a final column depends on packing methods, solvent used, and factors that affect mechanical properties of the column. In contrast to early inefficient dry- packing methods, wet slurry packing, in which particles that are suspended in an appropriate solvent are delivered into a column under pressure, shows significant improvement. Three different approaches can be employed in performing wet slurry packing: the balanced density method (solvent's density is about that of porous silica particles), the high viscosity method (a solvent of high viscosity is used), and the low viscosity slurry method (performed with low viscosity solvents).

Polystyrene is used as a medium for ion- exchange. It is made from the polymerization of styrene with the use of divinylbenzene and benzoyl peroxide. Such exchangers form hydrophobic interactions with proteins which can be irreversible. Due to this property, polystyrene ion exchangers are not suitable for protein separation. They are used on the other hand for the separation of small molecules in amino acid separation and removal of salt from water. Polystyrene ion exchangers with large pores can be used for the separation of protein but must be coated with a hydrophillic substance.

Cellulose based medium can be used for the separation of large molecules as they contain large pores. Protein binding in this medium is high and has low hydrophobic character. DEAE is an anion exchange matrix that is produced from a positive side group of diethylaminoethyl bound to cellulose or Sephadex.

Agarose gel based medium contain large pores as well but their substitution ability is lower in comparison to dextrans. The ability of the medium to swell in liquid is based on the cross-linking of these substances, the pH and the ion concentrations of the buffers used.

Possible Questions

Short questions

1. What is Chromatography?
2. What is absorbant used in different chromatography?
3. What is the solvents used in chromatography?
4. What is retention factor?
5. Write short on column chromatography?
6. What are the molecules separated in the chromatography.
7. What is the history of discovery of chromatography?

Essay type questions

1. Explain the working of chromatography in detail.
2. What is column chromatography? Write its types in detail.
3. What are the different types of Stationary phase? Add notes on the mobile phases.
4. What is the methodology of HPLC?
5. Describe the importance of GC Chrmatography.

UNIT-IV

SYLLABUS

Electrophoresis: Introduction to Electrophoresis, Starch gel, Polyacrylamide, gel (native and SDS- PAGE), Agarose gel electrophoresis.

Electrophoresis (from the Greek meaning "to bear electrons") is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field. Electrophoresis of positively charged particles (cations) is sometimes called **cataphoresis**, while electrophoresis of negatively charged particles (anions) is sometimes called **anaphoresis**.

The electrokinetic phenomenon of electrophoresis was observed for the first time in 1807 by Russian professors Peter Ivanovich Strakhov and Ferdinand Frederic Reuss at Moscow State University),who noticed that the application of a constant electric field caused clay particles dispersed in water to migrate. It is ultimately caused by the presence of a charged interface between the particle surface and the surrounding fluid. It is the basis for analytical techniques used in chemistry for separating molecules by size, charge, or binding affinity.

Electrophoresis is used in laboratories to separate macromolecules based on size. The technique applies a negative charge so proteins move towards a positive charge. Electrophoresis is used for both DNA and RNA analysis.

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. It is used in clinical chemistry to separate proteins by charge and/or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge.

Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose or other substances. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving.[2] Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins. Gel electrophoresis can also be used for separation of nanoparticles.

Gel electrophoresis uses a gel as an anticonvective medium and/or sieving medium during electrophoresis, the movement of a charged particle in an electrical field. Gels suppress

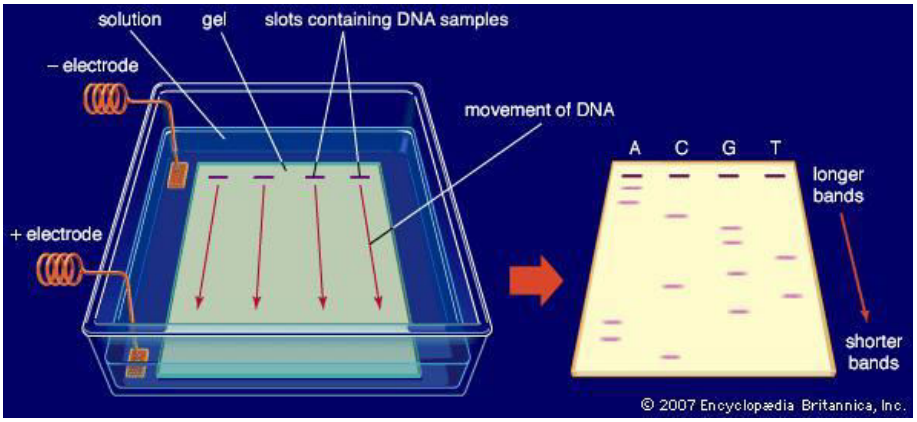
the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules; gels can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied.[3] DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via polymerase chain reaction (PCR), but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterization.

Types of gel

The types of gel most typically used are agarose and polyacrylamide gels. Each type of gel is well-suited to different types and sizes of analyte. Polyacrylamide gels are usually used for proteins, and have very high resolving power for small fragments of DNA (5-500 bp). Agarose gels on the other hand have lower resolving power for DNA but have greater range of separation, and are therefore used for DNA fragments of usually 50-20,000 bp in size, but resolution of over 6 Mb is possible with pulsed field gel electrophoresis (PFGE).[6] Polyacrylamide gels are run in a vertical configuration while agarose gels are typically run horizontally in a submarine mode. They also differ in their casting methodology, as agarose sets thermally, while polyacrylamide forms in a chemical polymerization reaction.

Agarose gel electrophoresis

Agarose gels are made from the natural polysaccharide polymers extracted from seaweed. Agarose gels are easily cast and handled compared to other matrices, because the gel setting is a physical rather than chemical change. Samples are also easily recovered. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.



Agarose gels do not have a uniform pore size, but are optimal for electrophoresis of proteins that are larger than 200 kDa. Agarose gel electrophoresis can also be used for the separation of DNA fragments ranging from 50 base pair to several megabases (millions of bases), the largest of which require specialized apparatus. The distance between DNA bands of different lengths is influenced by the percent agarose in the gel, with higher percentages requiring longer run times, sometimes days. Instead high percentage agarose gels should be run with a pulsed field electrophoresis (PFE), or field inversion electrophoresis.

"Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10kb DNA fragments) and 2% (good resolution for small 0.2–1kb fragments) agarose dissolved in electrophoresis buffer. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly. 1% gels are common for many applications.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore size provided by the polyacrylamide gel. Pore size is controlled by modulating the concentrations of acrylamide and bis-acrylamide powder used in creating a gel. Care must be used when creating this type of gel, as acrylamide is a potent neurotoxin in its liquid and powdered forms.

Traditional DNA sequencing techniques such as Maxam-Gilbert or Sanger methods used polyacrylamide gels to separate DNA fragments differing by a single base-pair in length so the

sequence could be read. Most modern DNA separation methods now use agarose gels, except for particularly small DNA fragments. It is currently most often used in the field of immunology and protein analysis, often used to separate different proteins or isoforms of the same protein into separate bands. These can be transferred onto a nitrocellulose or PVDF membrane to be probed with antibodies and corresponding markers, such as in a western blot.

Typically resolving gels are made in 6%, 8%, 10%, 12% or 15%. Stacking gel (5%) is poured on top of the resolving gel and a gel comb (which forms the wells and defines the lanes where proteins, sample buffer and ladders will be placed) is inserted. The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample. The smaller the known weight, the higher the percentage that should be used. Changes on the buffer system of the gel can help to further resolve proteins of very small sizes.

Starch

Partially hydrolysed potato starch makes for another non-toxic medium for protein electrophoresis. The gels are slightly more opaque than acrylamide or agarose. Non-denatured proteins can be separated according to charge and size. They are visualised using Napthal Black or Amido Black staining. Typical starch gel concentrations are 5% to 10%

Native

Native gels are run in non-denaturing conditions, so that the analyte's natural structure is maintained. This allows the physical size of the folded or assembled complex to affect the mobility, allowing for analysis of all four levels of the biomolecular structure. For biological samples, detergents are used only to the extent that they are necessary to lyse lipid membranes in the cell. Complexes remain—for the most part—associated and folded as they would be in the cell. One downside, however, is that complexes may not separate cleanly or predictably, as it is difficult to predict how the molecule's shape and size will affect its mobility. Addressing and solving this problem is a major aim of quantitative native PAGE.

Unlike denaturing methods, native gel electrophoresis does not use a charged denaturing agent. The molecules being separated (usually proteins or nucleic acids) therefore differ not only in molecular mass and intrinsic charge, but also the cross-sectional area, and thus experience different electrophoretic forces dependent on the shape of the overall structure. For proteins,

since they remain in the native state they may be visualised not only by general protein staining reagents but also by specific enzyme-linked staining.

A specific experiment example of an application of native gel electrophoresis is to check for enzymatic activity to verify the presence of the enzyme in the sample during protein purification. For example, for the protein alkaline phosphatase, the staining solution is a mixture of 4-chloro-2-2methylbenzenediazonium salt with 3-phospho-2-naphthoic acid-2'-4'-dimethyl aniline in Tris buffer. This stain is commercially sold as kit for staining gels. If the protein is present, the mechanism of the reaction takes place in the following order: it starts with the de-phosphorylation of 3-phospho-2-naphthoic acid-2'-4'-dimethyl aniline by alkaline phosphatase (water is needed for the reaction). The phosphate group is released and replaced by an alcohol group from water. The electrophile 4- chloro-2-2 methylbenzenediazonium (Fast Red TR Diazonium salt) displaces the alcohol group forming the final product Red Azo dye. As its name implies, this is the final visible-red product of the reaction. In undergraduate academic experimentation of protein purification, the gel is usually ran next to commercial purified samples in order to visualize the results and make confusions of whether or not purification was successful.[18]

Native gel electrophoresis is typically used in proteomics and metallomics. However, native PAGE is also used to scan genes (DNA) for unknown mutations as in Single-strand conformation polymorphism

Applications

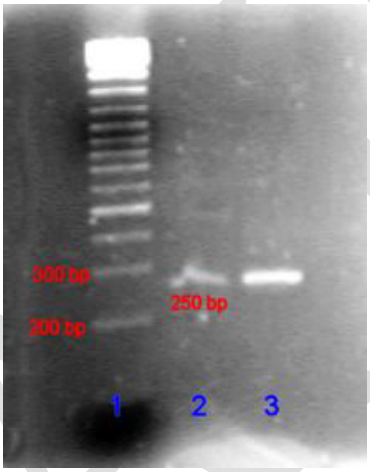
- Estimation of the size of DNA molecules following restriction enzyme digestion, e.g. in restriction mapping of cloned DNA.
- Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting
- Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.

Gel electrophoresis is used in forensics, molecular biology, genetics, microbiology and biochemistry. The results can be analyzed quantitatively by visualizing the gel with UV light and a gel imaging device. The image is recorded with a

computer operated camera, and the intensity of the band or spot of interest is measured and compared against standard or markers loaded on the same gel. The measurement and analysis are mostly done with specialized software.

Depending on the type of analysis being performed, other techniques are often implemented in conjunction with the results of gel electrophoresis, providing a wide range of field-specific applications.

Gel electrophoresis of nucleic acids



An agarose gel of a PCR product compared to a DNA ladder.

In the case of nucleic acids, the direction of migration, from negative to positive electrodes, is due to the naturally occurring negative charge carried by their sugar-phosphate backbone.

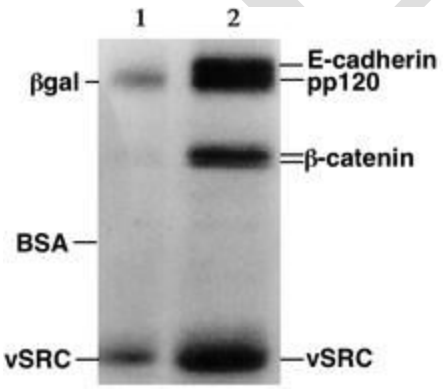
Double-stranded DNA fragments naturally behave as long rods, so their migration through the gel is relative to their size or, for cyclic fragments, their radius of gyration. Circular DNA such as plasmids, however, may show multiple bands, the speed of migration may depend on whether it is relaxed or supercoiled. Single-stranded DNA or RNA tend to fold up into molecules with complex shapes and migrate through the gel in a complicated manner based on their tertiary structure. Therefore, agents that disrupt the hydrogen bonds, such as sodium

hydroxide or formamide, are used to denature the nucleic acids and cause them to behave as long rods again.

Gel electrophoresis of large DNA or RNA is usually done by agarose gel electrophoresis. See the "Chain termination method" page for an example of a polyacrylamide DNA sequencing gel. Characterization through ligand interaction of nucleic acids or fragments may be performed by mobility shift affinity electrophoresis.

Electrophoresis of RNA samples can be used to check for genomic DNA contamination and also for RNA degradation. RNA from eukaryotic organisms shows distinct bands of 28s and 18s rRNA, the 28s band being approximately twice as intense as the 18s band. Degraded RNA has less sharply defined bands, has a smeared appearance, and intensity ratio is less than 2:1.

Gel electrophoresis of proteins



SDS-PAGE autoradiography – The indicated proteins are present in different concentrations in the two samples.

Proteins, unlike nucleic acids, can have varying charges and complex shapes, therefore they may not migrate into the polyacrylamide gel at similar rates, or at all, when placing a negative to positive EMF on the sample. Proteins therefore, are usually denatured in the presence of a detergent such as sodium dodecyl sulfate (SDS) that coats the proteins with a negative charge.[3] Generally, the amount of SDS bound is relative to the size of the protein (usually 1.4g SDS per gram of protein), so that the resulting denatured proteins have an overall negative charge, and all the proteins have a similar charge-to-mass ratio. Since denatured proteins act like

long rods instead of having a complex tertiary shape, the rate at which the resulting SDS coated proteins migrate in the gel is relative only to its size and not its charge or shape.

Proteins are usually analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), by native gel electrophoresis, by preparative gel electrophoresis (QPNC-PAGE), or by 2-D electrophoresis.

Characterization through ligand interaction may be performed by electroblotting or by affinity electrophoresis in agarose or by capillary electrophoresis as for estimation of binding constants and determination of structural features like glycan content through lectin binding.

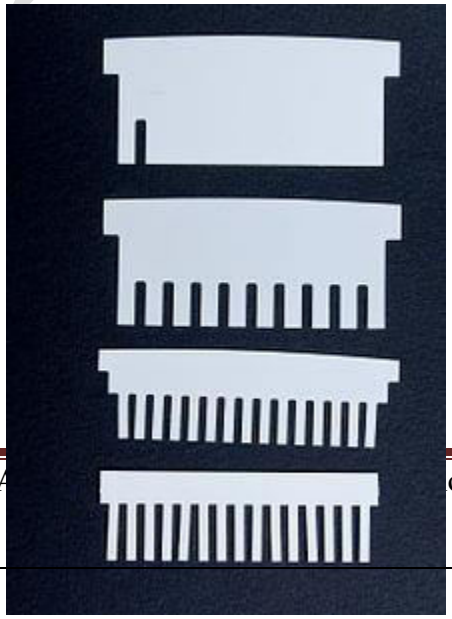
SDS-PAGE (**sodium dodecyl sulfate–polyacrylamide gel electrophoresis**) is a variant of polyacrylamide gel electrophoresis, an analytical method in biochemistry for the separation of charged molecules in mixtures by their molecular masses in an electric field. It uses sodium dodecyl sulfate (SDS) molecules to help identify and isolate protein molecules.

This discontinuous electrophoretic system developed by Ulrich K. Laemmli allows a good separation of proteins with molecular masses between 5 and 250 KDa.[1] The publication describing it is the most frequently cited paper by a single author, and the second most cited overall.

Procedure

The SDS-PAGE method is composed of gel preparation, sample preparation, electrophoresis, protein staining or western blotting and analysis of the generated banding pattern.

Gel production



Sample combs with different numbers of pockets, each prong leaves a pocket in the gel when pulled out

Polymerised separating and stacking gel before removing the sample comb (white) between the spacers (black), in the stacking gel are small amounts of bromophenol blue for improved visibility, the separating gel is unstained

When using different buffers in the gel (discontinuous gel electrophoresis), the gels are made up to one day prior to electrophoresis, so that the diffusion does not lead to a mixing of the buffers. The gel is produced by radical polymerisation in a mold consisting of two sealed glass plates with spacers between the glass plates. In a typical mini-gel setting, the spacers have a thickness of 0.75 mm or 1.5 mm, which determines the loading capacity of the gel. For pouring the gel solution, the plates are usually clamped in a stand which temporarily seals the otherwise open underside of the glass plates with the two spacers. For the gel solution, acrylamide is mixed as gel-former (usually 4% V/V in the stacking gel and 10-12 % in the separating gel), methylenebisacrylamide as a cross-linker, stacking or separating gel buffer, water and SDS. By adding the catalyst TEMED and the radical initiator ammonium persulfate (APS) the polymerisation is started. The solution is then poured between the glass plates without creating bubbles. Depending on the amount of catalyst and radical starter and depending on the temperature, the polymerisation lasts between a quarter of an hour and several hours. The lower gel (separating gel) is poured first and covered with a few drops of a barely water-soluble alcohol (usually buffer-saturated butanol or isopropanol), which eliminates bubbles from the meniscus and protects the gel solution of the radical scavenger oxygen.

After the polymerisation of the separating gel, the alcohol is discarded and the residual alcohol is removed with filter paper. After addition of APS and TEMED to the stacking gel solution, it is poured on top of the solid separation gel. Afterwards, a suitable sample comb is inserted between the glass plates without creating bubbles. The sample comb is carefully pulled out after polymerisation, leaving pockets for the sample application. For later use of proteins for protein sequencing, the gels are often prepared the day before electrophoresis to reduce reactions of unpolymerised acrylamide with cysteines in proteins.

By using a gradient mixer, gradient gels with a gradient of acrylamide (usually from 4 to 12%) can be cast, which have a larger separation range of the molecular masses.[13] Commercial gel systems (so-called *pre-cast gels*) usually use the buffer substance Bis-tris methane with a pH value between 6.4 and 7.2 both in the stacking gel and in the separating gel.[14][15] These gels are delivered cast and ready-to-use. Since they use only one buffer (continuous gel electrophoresis) and have a nearly neutral pH, they can be stored for several weeks. The more neutral pH slows the hydrolysis and thus the decomposition of the polyacrylamide. Furthermore, there are fewer acrylamide-modified cysteines in the proteins.[14] Due to the constant pH in collecting and separating gel there is no stacking effect. Proteins in BisTris gels can not be stained with ruthenium complexes. This gel system has a comparatively large separation range, which can be varied by using MESor MOPS in the running buffer.

Sample preparation

During sample preparation, the sample buffer, and thus SDS, is added in excess to the proteins, and the sample is then heated to 95 °C for five minutes to disrupt secondary and tertiary structures by disrupting hydrogen bonds and stretching the molecules. Optionally, disulfide bridges can be cleaved by reduction. For this purpose, reducing thiols such as β-mercaptoethanol (β-ME, 5% by volume), dithiothreitol (DTT, 10 millimolar) or dithioerythritol (DTE, 10 millimolar) are added to the sample buffer. After cooling to room temperature, each sample is pipetted into its own pocket in the gel, which was previously immersed in electrophoresis buffer in the electrophoresis apparatus.

In addition to the samples, a molecular-weight size marker is usually loaded onto the gel. This consists of proteins of known sizes and thereby allows the estimation (with an error of ± 10%) of the sizes of the proteins in the actual samples, which migrate in parallel in different tracks of the gel. The size marker is often pipetted into the first or last pocket of a gel.

Electrophoresis

Electrophoresis chamber after a few minutes of electrophoresis. In the first pocket a size marker was applied with bromophenol blue, in the other pockets, the samples were added bromocresol green

For separation, the denatured samples are loaded onto a gel of polyacrylamide, which is placed in an electrophoresis buffer with suitable electrolytes. Thereafter, a voltage (usually around 100 V, 10-20 V per cm gel length) is applied, which causes a migration of negatively charged molecules through the gel in the direction of the anode (plus pole). The gel acts like a sieve. Small proteins migrate relatively easily through the mesh of the gel, while larger proteins are more likely to be retained and thereby migrate more slowly through the gel. The electrophoresis lasts between three quarters of an hour and several hours depending on the voltage and length of gel used.

The fastest-migrating proteins (with a molecular weight of less than 5 KDa) form the buffer front together with the anionic components of the electrophoresis buffer, which also migrate through the gel. The area of the buffer front is made visible by adding the comparatively small, anionic

dye bromophenol blue to the sample buffer. Due to the relatively small molecule size of bromophenol blue, it migrates faster than proteins. By optical control of the migrating colored band, the electrophoresis can be stopped before the dye and also the samples have completely migrated through the gel and leave it.

The most commonly used method is the discontinuous SDS-PAGE. In this method, the proteins migrate first into a collecting gel with neutral pH, in which they are concentrated and then they migrate into a separating gel with basic pH, in which the actual separation takes place. Stacking and separating gels differ by different pore size (4-6 % T and 10-20 % T), ionic strength and pH values (pH 6.8 or pH 8.8). The electrolyte most frequently used is an SDS-containing Tris-glycine-chloride buffer system. At neutral pH, glycine predominantly forms the zwitterionic form, at high pH the glycines lose positive charges and become predominantly anionic. In the collection gel, the smaller, negatively charged chloride ions migrate in front of the proteins (as leading ions) and the slightly larger, negatively and partially positively charged glycinate ions migrate behind the proteins (as initial trailing ions), whereas in the comparatively basic separating gel both ions migrate in front of the proteins.

The pH gradient between the stacking and separation gel buffers leads to a stacking effect at the border of the stacking gel to the separation gel, since the glycinate partially loses its slowing positive charges as the pH increases and then, as the former trailing ion, overtakes the proteins and becomes a leading ion, which causes the bands of the different proteins (visible after a staining) to become narrower and sharper - the stacking effect. For the separation of smaller proteins and peptides, the TRIS-Tricine buffer system of Schägger and von Jagow is used due to the higher spread of the proteins in the range of 0.5 to 50 KDa.

Gel staining

Coomassie-stained 10% Tris/Tricine gel. In the left lane, a molecular weight size marker was used to estimate the size (from top to bottom: 66, 45, 35, 24, 18 and 9 kDa). In the remaining lanes purified yeast proteins were separated.

At the end of the electrophoretic separation, all proteins are sorted by size and can then be analyzed by other methods, e. g. protein staining such as Coomassie staining (easy to use, most commonly used), silver staining (highest sensitivity), stains allstaining, Amido black

10B staining, Fast green FCF staining, fluorescent stains such as epicocconone stain and SYPRO orange stain, and immunological detection such as the Western Blot. The fluorescent dyes have a comparatively higher linearity between protein quantity and color intensity of about three orders of magnitude above the detection limit, i.e. the amount of protein can be estimated by color intensity. When using the fluorescent protein dye trichloroethanol, a subsequent protein staining is omitted if it was added to the gel solution and the gel was irradiated with UV light after electrophoresis.

Analysis

Protein staining in the gel creates a documentable banding pattern of the various proteins. Glycoproteins have differential levels of glycosylations and adsorb SDS more unevenly at the glycosylations, resulting in broader and blurred bands.Membrane proteins, because of their transmembrane domain, are often composed of the more hydrophobic amino acids, have lower solubility in aqueous solutions, tend to bind lipids, and tend to precipitate in aqueous solutions due to hydrophobic effects when sufficient amounts of detergent are not present. This precipitation manifests itself for membrane proteins in a SDS-PAGE in "tailing" above the band of the transmembrane protein. In this case, more SDS can be used (by using more or more concentrated sample buffer) and the amount of protein in the sample application can be reduced. An overloading of the gel with a soluble protein creates a semicircular band of this protein (e. g. in the marker lane of the image at 66 KDa), allowing other proteins with similar molecular weights to be covered. A low contrast (as in the marker lane of the image) between bands within a lane indicates either the presence of many proteins (low purity) or, if using purified proteins and a low contrast occurs only below one band, it indicates a proteolytic degradation of the protein, which first causes degradation bands, and after further degradation produces a homogeneous color ("smear") below a band. The documentation of the banding pattern is usually done by photographing or scanning. For a subsequent recovery of the molecules in individual bands, a gel extraction can be performed.

Possible Questions

Short questions

1. What is Electrophoresis?
2. What is anode and cathode?
3. What is the buffer used in electrophoresis?
4. What is stacking gel and separating gel?
5. Write short on PAGE?
6. What are the Molecules separated by SDS PAGE.
7. What is the history of discovery of Electrophoresis?

Essay type questions

1. Explain the working of electrophoresis in detail.
2. What is PAGE? Write its types in detail.
3. What are the different types of Electrophoresis? Add notes on SDS-PAGE.
4. What is the methodology of agarose gel electrophoresis?
5. Describe the importance of electrophoresis.

UNIT-V

SYLLABUS

Application: Pulse field electrophoresis, immune-electrophoresis, isoelectric focusing, Western blotting. Introduction to Biosensors and Nanotechnology and their applications.

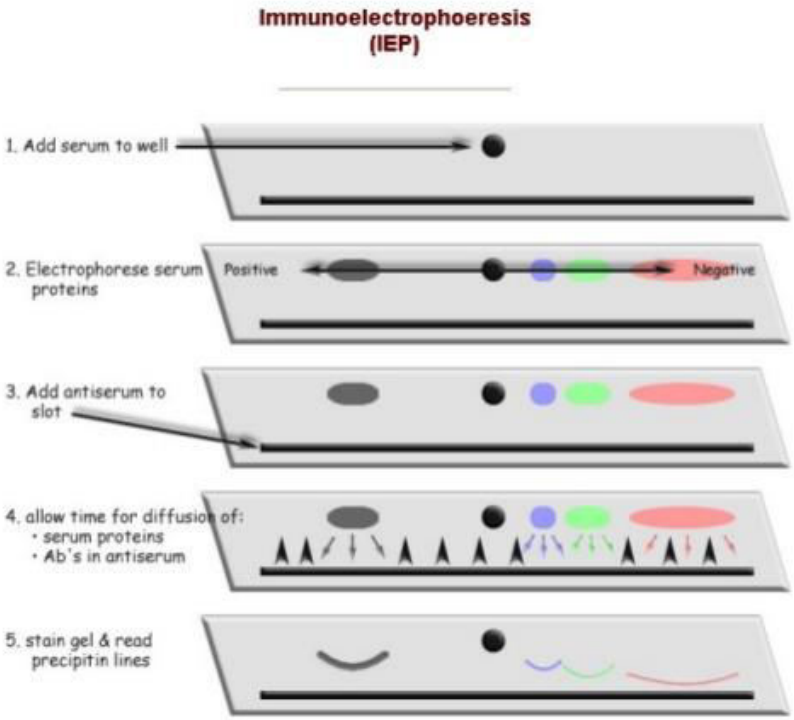
Immunoelectrophoresis is a general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies. All variants of immunoelectrophoresis require immunoglobulins, also known as antibodies, reacting with the proteins to be separated or characterized. The methods were developed and used extensively during the second half of the 20th century. In somewhat chronological order: Immunoelectrophoretic analysis (one-dimensional immunoelectrophoresis *ad modum* Grabar), crossed immunoelectrophoresis (two-dimensional quantitative immunoelectrophoresis *ad modum* Clarke and Freeman or *ad modum* Laurell), rocket-immunoelectrophoresis (one-dimensional quantitative immunoelectrophoresis *ad modum* Laurell), fused rocket immunoelectrophoresis *ad modum* Svendsen and Harboe, affinity immunoelectrophoresis *ad modum* Bøg-Hansen.

Agarose as 1% gel slabs of about 1 mm thickness buffered at high pH (around 8.6) is traditionally preferred for the electrophoresis as well as the reaction with antibodies. The agarose was chosen as the gel matrix because it has large pores allowing free passage and separation of proteins, but provides an anchor for the immunoprecipitates of protein and specific antibodies. The high pH was chosen because antibodies are practically immobile at high pH. An electrophoresis equipment with a horizontal cooling plate was normally recommended for the electrophoresis.

Immunoprecipitates may be seen in the wet agarose gel, but are stained with protein stains like Coomassie Brilliant Blue in the dried gel. In contrast to SDS-gel electrophoresis, the electrophoresis in agarose allows native conditions, preserving the native structure and activities of the proteins under investigation, therefore immunoelectrophoresis allows characterization of enzyme activities and ligand binding etc. in addition to electrophoretic separation.

Rocket immunoelectrophoresis is one-dimensional quantitative immunoelectrophoresis. The method has been used for quantitation of human serum proteins before automated methods became available.

Fused rocket immunoelectrophoresis is a modification of one-dimensional quantitative immunoelectrophorsis used for detailed measurement of proteins in fractions from protein separation experiments.



Affinity immunoelectrophoresis is based on changes in the electrophoretic pattern of proteins through specific interaction or complex formation with other macromolecules or ligands. Affinity immunoelectrophoresis has been used for estimation of binding constants, as for instance with lectins or for characterization of proteins with specific features like glycan content or ligand binding. Some variants of affinity immunoelectrophoresis are similar to affinity chromatography by use of immobilized ligands.

Pulsed field gel electrophoresis is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying to a gel matrix an electric field that periodically changes direction.

Historical background

Standard gel electrophoresis techniques for separation of DNA molecules provided huge advantages for molecular biology research. However, it was unable to separate very large molecules of DNA effectively. DNA molecules larger than 15–20 kb migrating through a gel will essentially move together in a size-independent manner. At Columbia University in 1984, David C. Schwartz and Charles Cantor developed a variation on the standard protocol by introducing an alternating voltage gradient to improve the resolution of larger molecules. This technique became known as pulsed-field gel electrophoresis (PFGE). The development of PFGE expanded the range of resolution for DNA fragments by as much as two orders of magnitude.

Procedure

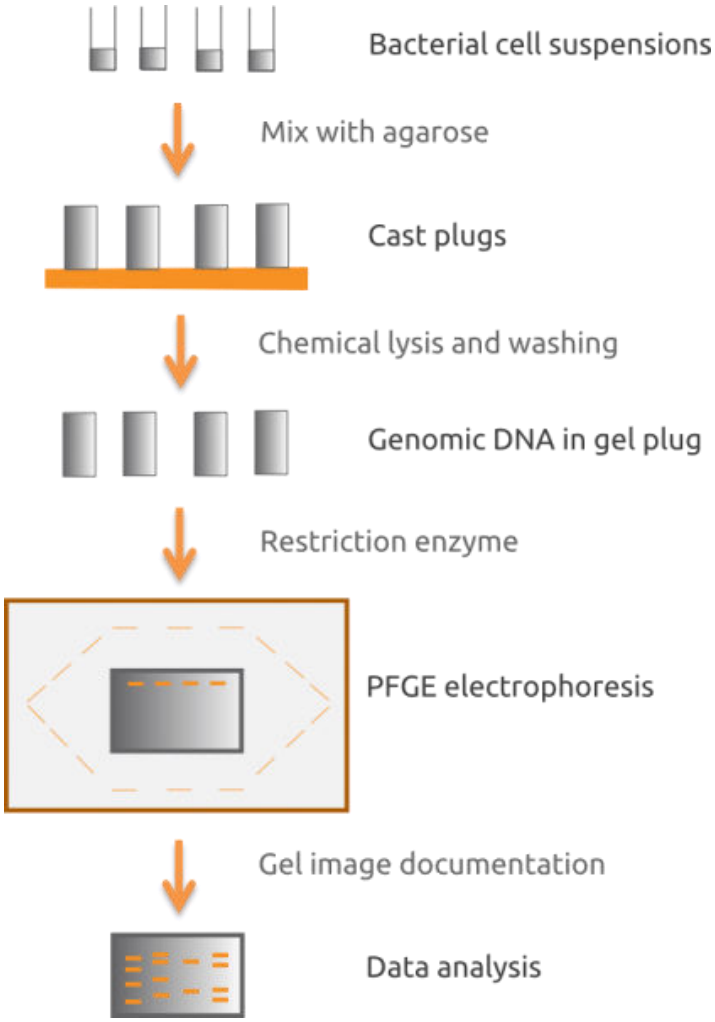
The procedure for this technique is relatively similar to performing a standard gel electrophoresis except that instead of constantly running the voltage in one direction, the voltage is periodically switched among three directions; one that runs through the central axis of the gel and two that run at an angle of 60 degrees either side. The pulse times are equal for each direction resulting in a net forward migration of the DNA. For extremely large molecules (up to around 2 Mb), switching-interval ramps can be used that increases the pulse time for each direction over the course of a number of hours—take, for instance, increasing the pulse linearly from 10 seconds at 0 hours to 60 seconds at 18 hours.

This procedure takes longer than normal gel electrophoresis due to the size of the fragments being resolved and the fact that the DNA does not move in a straight line through the gel.

Applications

PFGE may be used for genotyping or genetic fingerprinting. It is commonly considered a gold standard in epidemiological studies of pathogenic organisms. Subtyping has made it easier

to discriminate among strains of Listeria monocytogenes and thus to link environmental or food isolates with clinical infections.



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*, which transforms one signal into another one, works in a physicochemical way: optical, piezoelectric, electrochemical, electrochemiluminescence etc., resulting from the interaction of the analyte

with the biological element, to easily measure and quantify. The biosensor reader device with the associated electronics or signal processors that are primarily responsible for the display of the results in a user-friendly way. This sometimes accounts for the most expensive part of the sensor device, however it is possible to generate a user friendly display that includes transducer and sensitive element (holographic sensor). The readers are usually custom-designed and manufactured to suit the different working principles of biosensors.

Isoelectric focusing (IEF), also known as electrofocusing, is a technique for separating different molecules by differences in their isoelectric point (pI). It is a type of zone electrophoresis, usually performed on proteins in a gel, that takes advantage of the fact that overall charge on the molecule of interest is a function of the pH of its surroundings.

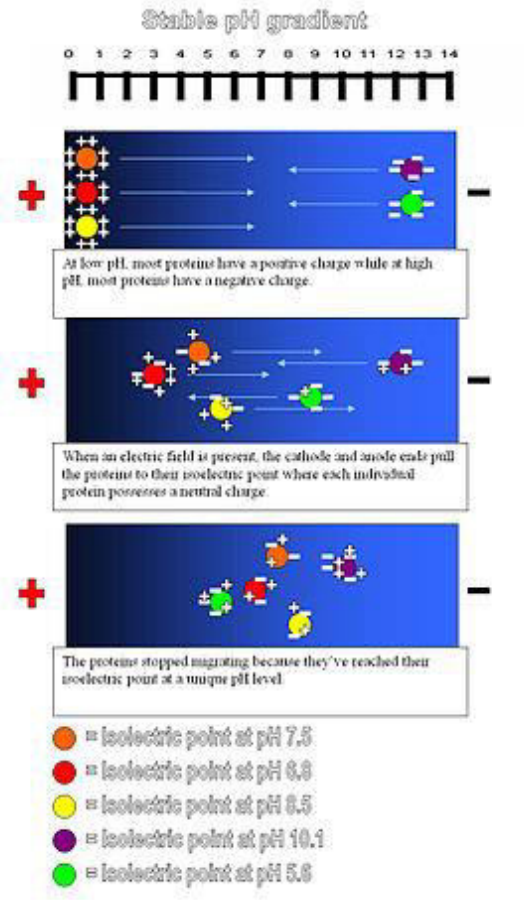
Procedure

IEF involves adding an ampholyte solution into immobilized pH gradient (IPG) gels. IPGs are the acrylamide gel matrix co-polymerized with the pH gradient, which result in completely stable gradients except the most alkaline (>12) pH values. The immobilized pH gradient is obtained by the continuous change in the ratio of *immobilines*. An immobiline is a weak acid or base defined by its pK value.

A protein that is in a pH region below its isoelectric point (pI) will be positively charged and so will migrate towards the cathode (negatively charged electrode). As it migrates through a gradient of increasing pH, however, the protein's overall charge will decrease until the protein reaches the pH region that corresponds to its pI. At this point it has no net charge and so migration ceases (as there is no electrical attraction towards either electrode). As a result, the proteins become focused into sharp stationary bands with each protein positioned at a point in the pH gradient corresponding to its pI. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands.

Molecules to be focused are distributed over a medium that has a pH gradient (usually created by aliphatic ampholytes). An electric current is passed through the medium, creating a "positive" anode and "negative" cathode end. Negatively charged molecules migrate through the pH gradient in the medium toward the "positive" end while positively charged molecules move

toward the "negative" end. As a particle moves towards the pole opposite of its charge it moves through the changing pH gradient until it reaches a point in which the pH of that molecules isoelectric point is reached.



At this point the molecule no longer has a net electric charge (due to the protonation or deprotonation of the associated functional groups) and as such will not proceed any further within the gel. The gradient is established before adding the particles of interest by first subjecting a solution of small molecules such as polyampholytes with varying pI values to electrophoresis.

The method is applied particularly often in the study of proteins, which separate based on their relative content of acidic and basic residues, whose value is represented by the pI. Proteins are introduced into an Immobilized pH gradient gel composed of polyacrylamide, starch, or agarose where a pH gradient has been established. Gels with large pores are usually used in

this process to eliminate any "sieving" effects, or artifacts in the pI caused by differing migration rates for proteins of differing sizes. Isoelectric focusing can resolve proteins that differ in pI value by as little as 0.01. Isoelectric focusing is the first step in two-dimensional gel electrophoresis, in which proteins are first separated by their pI and then further separated by molecular weight through SDS-PAGE.

Biosensor system

A biosensor typically consists of a bio-recognition site, biotransducer component, and electronic system which includes 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 of bioreceptor interactions involving: antibody/antigen, enzymes/ligands, nucleic acids/DNA, cellular structures/cells, or biomimetic materials.

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. In another approach, small protein scaffolds with favorable biophysical properties have been engineered to generate artificial families of Antigen Binding Proteins (AgBP), capable of specific binding to different target proteins while retaining the favorable properties of the parent molecule. The elements of the family that specifically bind to a given target antigen, are often selected in vitro by display techniques: phage display, ribosome display, yeast display or mRNA display. The artificial binding proteins are much smaller than antibodies (usually less than 100 amino-acid residues), have a strong stability, lack disulfide bonds and can be expressed in high yield in reducing cellular environments like the bacterial cytoplasm, contrary to antibodies and their derivatives. They are thus especially suitable to create biosensors.

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 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.

Biosensors can be classified by their biotransducer type. The most common types of biotransducers used in biosensors are:

- electrochemical biosensors
- optical biosensors
- electronic biosensors
- piezoelectric biosensors
- gravimetric biosensors
- pyroelectric biosensors

Nanotechnology ("nanotech") is manipulation of matter on an atomic, molecular, and supramolecular scale. The earliest, widespread description of nanotechnology referred to the particular technological goal of precisely manipulating atoms and molecules for fabrication of macroscale products, also now referred to as molecular nanotechnology. A more generalized description of nanotechnology was subsequently established by the National Nanotechnology Initiative, which defines nanotechnology as the manipulation of matter with at least one dimension sized from 1 to 100 nanometers.

This definition reflects the fact that quantum mechanical effects are important at this quantum-realm scale, and so the definition shifted from a particular technological goal to a research category inclusive of all types of research and technologies that deal with the special properties of matter which occur below the given size threshold. It is therefore common to see the plural form "nanotechnologies" as well as "nanoscale technologies" to refer to the broad range of research and applications whose common trait is size. Because of the variety of potential applications (including industrial and military), governments have invested billions of dollars in nanotechnology research. Through 2012, the USA has invested $3.7 billion using its National Nanotechnology Initiative, the European Union has invested $1.2 billion, and Japan has invested $750 million.

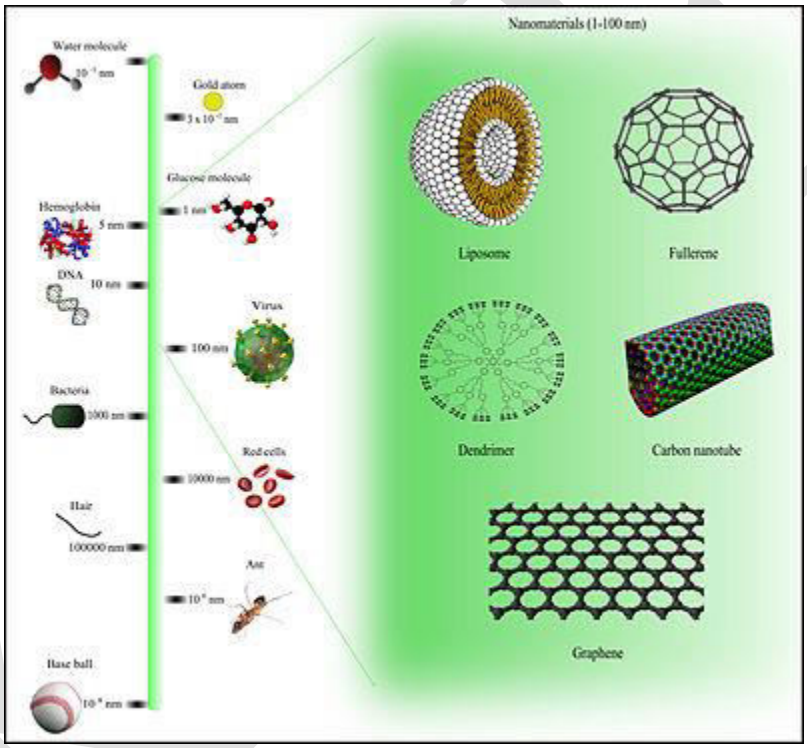
Nanotechnology as defined by size is naturally very broad, including fields of science as diverse as surface science, organic chemistry, molecular biology, semiconductor physics, energy storage, microfabrication molecular engineering, etc. The associated research and applications are equally diverse, ranging from extensions of conventional device physics to completely new approaches based upon molecular self-assembly, from developing new materials with dimensions on the nanoscale to direct control of matter on the atomic scale.

Scientists currently debate the future implications of nanotechnology. Nanotechnology may be able to create many new materials and devices with a vast range of applications, such as in nanomedicine, nanoelectronics, biomaterials energy production, and consumer products. On the other hand, nanotechnology raises many of the same issues as any new technology, including concerns about the toxicity and environmental impact of nanomaterials and their potential effects on global economics, as well as speculation about various doomsday scenarios. These concerns

have led to a debate among advocacy groups and governments on whether special regulation of nanotechnology is warranted.

History of nanotechnology

The concepts that seeded nanotechnology were first discussed in 1959 by renowned physicist Richard Feynman in his talk There's Plenty of Room at the Bottom, in which he described the possibility of synthesis via direct manipulation of atoms. The term "nano-technology" was first used by Norio Taniguchi in 1974, though it was not widely known.



Thus, emergence of nanotechnology as a field in the 1980s occurred through convergence of Drexler's theoretical and public work, which developed and popularized a conceptual framework for nanotechnology, and high-visibility experimental advances that drew additional wide-scale attention to the prospects of atomic control of matter. In the 1980s, two major breakthroughs sparked the growth of nanotechnology in modern era.

First, the invention of the scanning tunneling microscope in 1981 which provided unprecedented visualization of individual atoms and bonds, and was successfully used to manipulate individual

atoms in 1989. The microscope's developers Gerd Binnig and Heinrich Rohrer at IBM Zurich Research Laboratory received a Nobel Prize in Physics in 1986.

Governments moved to promote and fund research into nanotechnology, such as in the U.S. with the National Nanotechnology Initiative, which formalized a size-based definition of nanotechnology and established funding for research on the nanoscale, and in Europe via the European Framework Programmes for Research and Technological Development.

By the mid-2000s new and serious scientific attention began to flourish. Projects emerged to produce nanotechnology roadmaps which center on atomically precise manipulation of matter and discuss existing and projected capabilities, goals, and applications.

Fundamental concepts

Nanotechnology is the engineering of functional systems at the molecular scale. This covers both current work and concepts that are more advanced. In its original sense, nanotechnology refers to the projected ability to construct items from the bottom up, using techniques and tools being developed today to make complete, high performance products.

One nanometer (nm) is one billionth, or 10^{-9}, of a meter. By comparison, typical carbon-carbon bond lengths, or the spacing between these atoms in a molecule, are in the range 0.12–0.15 nm, and a DNA double-helix has a diameter around 2 nm. On the other hand, the smallest cellular life-forms, the bacteria of the genus Mycoplasma, are around 200 nm in length.

By convention, nanotechnology is taken as the scale range 1 to 100 nm following the definition used by the National Nanotechnology Initiative in the US. The lower limit is set by the size of atoms (hydrogen has the smallest atoms, which are approximately a quarter of a nm kinetic diameter) since nanotechnology must build its devices from atoms and molecules. The upper limit is more or less arbitrary but is around the size below which phenomena not observed in larger structures start to become apparent and can be made use of in the nano device. These new phenomena make nanotechnology distinct from devices which are merely miniaturised versions of an equivalent macroscopic device; such devices are on a larger scale and come under the description of microtechnology.

To put that scale in another context, the comparative size of a nanometer to a meter is the same as that of a marble to the size of the earth. Or another way of putting it: a nanometer is the amount an average man's beard grows in the time it takes him to raise the razor to his face.

Two main approaches are used in nanotechnology. In the "bottom-up" approach, materials and devices are built from molecular components which assemble themselves chemically by principles of molecular recognition In the "top-down" approach, nano-objects are constructed from larger entities without atomic-level control. Areas of physics such as nanoelectronics, nanomechanics, nanophotonics and nanoionics have evolved during the last few decades to provide a basic scientific foundation of nanotechnology.

Larger to smaller: a materials perspective

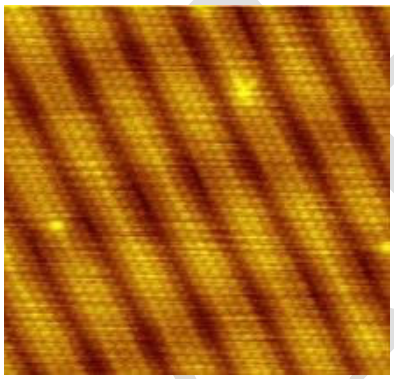


Image of reconstruction on a clean Gold(100) surface, as visualized using scanning tunneling microscopy. The positions of the individual atoms composing the surface are visible.

Several phenomena become pronounced as the size of the system decreases. These include statistical mechanical effects, as well as quantum mechanical effects, for example the "quantum size effect" where the electronic properties of solids are altered with great reductions in particle size. This effect does not come into play by going from macro to micro dimensions. However, quantum effects can become significant when the nanometer size range is reached, typically at distances of 100 nanometers or less, the so-called quantum realm. Additionally, a number of physical (mechanical, electrical, optical, etc.) properties change when compared to macroscopic systems. One example is the increase in surface area to volume ratio altering mechanical, thermal and catalytic properties of materials. Diffusion and reactions at nanoscale,

nanostructures materials and nanodevices with fast ion transport are generally referred to nanoionics. *Mechanical* properties of nanosystems are of interest in the nanomechanics research. The catalytic activity of nanomaterials also opens potential risks in their interaction with biomaterials.

Materials reduced to the nanoscale can show different properties compared to what they exhibit on a macroscale, enabling unique applications. For instance, opaque substances can become transparent (copper); stable materials can turn combustible (aluminium); insoluble materials may become soluble (gold). A material such as gold, which is chemically inert at normal scales, can serve as a potent chemical catalyst at nanoscales. Much of the fascination with nanotechnology stems from these quantum and surface phenomena that matter exhibits at the nanoscale.

Simple to complex: a molecular perspective

Modern synthetic chemistry has reached the point where it is possible to prepare small molecules to almost any structure. These methods are used today to manufacture a wide variety of useful chemicals such as pharmaceuticals or commercial polymers. This ability raises the question of extending this kind of control to the next-larger level, seeking methods to assemble these single molecules into supramolecular assemblies consisting of many molecules arranged in a well defined manner.

These approaches utilize the concepts of molecular self-assembly and/or supramolecular chemistry to automatically arrange themselves into some useful conformation through a bottom-up approach. The concept of molecular recognition is especially important: molecules can be designed so that a specific configuration or arrangement is favored due to non-covalent intermolecular forces. The Watson–Crick base pairing rules are a direct result of this, as is the specificity of an enzyme being targeted to a single substrate, or the specific folding of the protein itself. Thus, two or more components can be designed to be complementary and mutually attractive so that they make a more complex and useful whole.

Such bottom-up approaches should be capable of producing devices in parallel and be much cheaper than top-down methods, but could potentially be overwhelmed as the size and complexity of the desired assembly increases. Most useful structures require complex and

thermodynamically unlikely arrangements of atoms. Nevertheless, there are many examples of self-assembly based on molecular recognition in biology, most notably Watson–Crick basepairing and enzyme-substrate interactions. The challenge for nanotechnology is whether these principles can be used to engineer new constructs in addition to natural ones.

Molecular nanotechnology: a long-term view

Molecular nanotechnology, sometimes called molecular manufacturing, describes engineered nanosystems (nanoscale machines) operating on the molecular scale. Molecular nanotechnology is especially associated with the molecular assembler, a machine that can produce a desired structure or device atom-by-atom using the principles of mechanosynthesis. Manufacturing in the context of productive nanosystems is not related to, and should be clearly distinguished from, the conventional technologies used to manufacture nanomaterials such as carbon nanotubes and nanoparticles.

When the term "nanotechnology" was independently coined and popularized by Eric Drexler (who at the time was unaware of an earlier usage by Norio Taniguchi) it referred to a future manufacturing technology based on molecular machine systems. The premise was that molecular scale biological analogies of traditional machine components demonstrated molecular machines were possible: by the countless examples found in biology, it is known that sophisticated, stochastically optimised biological machines can be produced.

It is hoped that developments in nanotechnology will make possible their construction by some other means, perhaps using biomimetic principles. However, Drexler and other researchers have proposed that advanced nanotechnology, although perhaps initially implemented by biomimetic means, ultimately could be based on mechanical engineering principles, namely, a manufacturing technology based on the mechanical functionality of these components (such as gears, bearings, motors, and structural members) that would enable programmable, positional assembly to atomic specification. The physics and engineering performance of exemplar designs were analyzed in Drexler's book *Nanosystems*.

Nanomaterials

The nanomaterials field includes subfields which develop or study materials having unique properties arising from their nanoscale dimensions.

- Interface and colloid science has given rise to many materials which may be useful in nanotechnology, such as carbon nanotubes and other fullerenes, and various nanoparticles and nanorods. Nanomaterials with fast ion transport are related also to nanoionics and nanoelectronics.
- Nanoscale materials can also be used for bulk applications; most present commercial applications of nanotechnology are of this flavor.
- Progress has been made in using these materials for medical applications; see Nanomedicine.
- Nanoscale materials such as nanopillars are sometimes used in solar cells which combats the cost of traditional silicon solar cells.
- Development of applications incorporating semiconductor nanoparticles to be used in the next generation of products, such as display technology, lighting, solar cells and biological imaging; see quantum dots.
- Recent application of nanomaterials include a range of biomedical applications, such as tissue engineering, drug delivery, and biosensors.

Bottom-up approaches

These seek to arrange smaller components into more complex assemblies.

- DNA nanotechnology utilizes the specificity of Watson–Crick basepairing to construct well-defined structures out of DNA and other nucleic acids.
- Approaches from the field of "classical" chemical synthesis (Inorganic and organic synthesis) also aim at designing molecules with well-defined shape
- More generally, molecular self-assembly seeks to use concepts of supramolecular chemistry, and molecular recognition in particular, to cause single-molecule components to automatically arrange themselves into some useful conformation.
- Atomic force microscope tips can be used as a nanoscale "write head" to deposit a chemical upon a surface in a desired pattern in a process called dip pen nanolithography. This technique fits into the larger subfield of nanolithography.
- Molecular Beam Epitaxy allows for bottom up assemblies of materials, most notably semiconductor materials commonly used in chip and computing applications, stacks, gating, and nanowire lasers.

Top-down approaches

These seek to create smaller devices by using larger ones to direct their assembly.

- Many technologies that descended from conventional solid-state silicon methods for fabricating microprocessors are now capable of creating features smaller than 100 nm, falling under the definition of nanotechnology. Giant magnetoresistance-based hard drives already on the market fit this description as do atomic layer deposition (ALD) techniques. Peter Grünberg and Albert Fert received the Nobel Prize in Physics in 2007 for their discovery of Giant magnetoresistance and contributions to the field of spintronics.
- Solid-state techniques can also be used to create devices known as nanoelectromechanical systems or NEMS, which are related to microelectromechanical systems or MEMS.
- Focused ion beams can directly remove material, or even deposit material when suitable precursor gasses are applied at the same time. For example, this technique is used routinely to create sub-100 nm sections of material for analysis in Transmission electron microscopy.
- Atomic force microscope tips can be used as a nanoscale "write head" to deposit a resist, which is then followed by an etching process to remove material in a top-down method.

Functional approaches

These seek to develop components of a desired functionality without regard to how they might be assembled.

- Magnetic assembly for the synthesis of anisotropic superparamagnetic materials such as recently presented magnetic nano chains.
- Molecular scale electronics seeks to develop molecules with useful electronic properties. These could then be used as single-molecule components in a nanoelectronic device. For an example see rotaxane.
- Synthetic chemical methods can also be used to create synthetic molecular motors, such as in a so-called nanocar.

Biomimetic approaches

- Bionics or biomimicry seeks to apply biological methods and systems found in nature, to the study and design of engineering systems and modern technology. Biomineralization is one example of the systems studied.
- Bionanotechnology is the use of biomolecules for applications in nanotechnology, including use of viruses and lipid assemblies. Nanocellulose is a potential bulk-scale application.

Possible Questions

Short questions

1. What is Immunoelectrophoresis?
2. What is PFGE?
3. What is IEF?
4. What is Biosensor system?
5. Write short on Bioreceptors?
6. What are Enzymatic interactions?
7. What is Affinity binding receptor?
8. What is Nucleic acid interaction?
9. What are biosensors type?
10. What is Nanotechnology?

Essay type questions

1. Explain the working of Immunoelectrophoresis in detail.
2. What is PFGE? Write its mechanism in detail.
3. What are the different types of Biosensor? Add notes on Biotransducers.
4. What is the methodology of Nanomaterial synthesis?
5. Describe the importance of Nanotechnology in medicine.