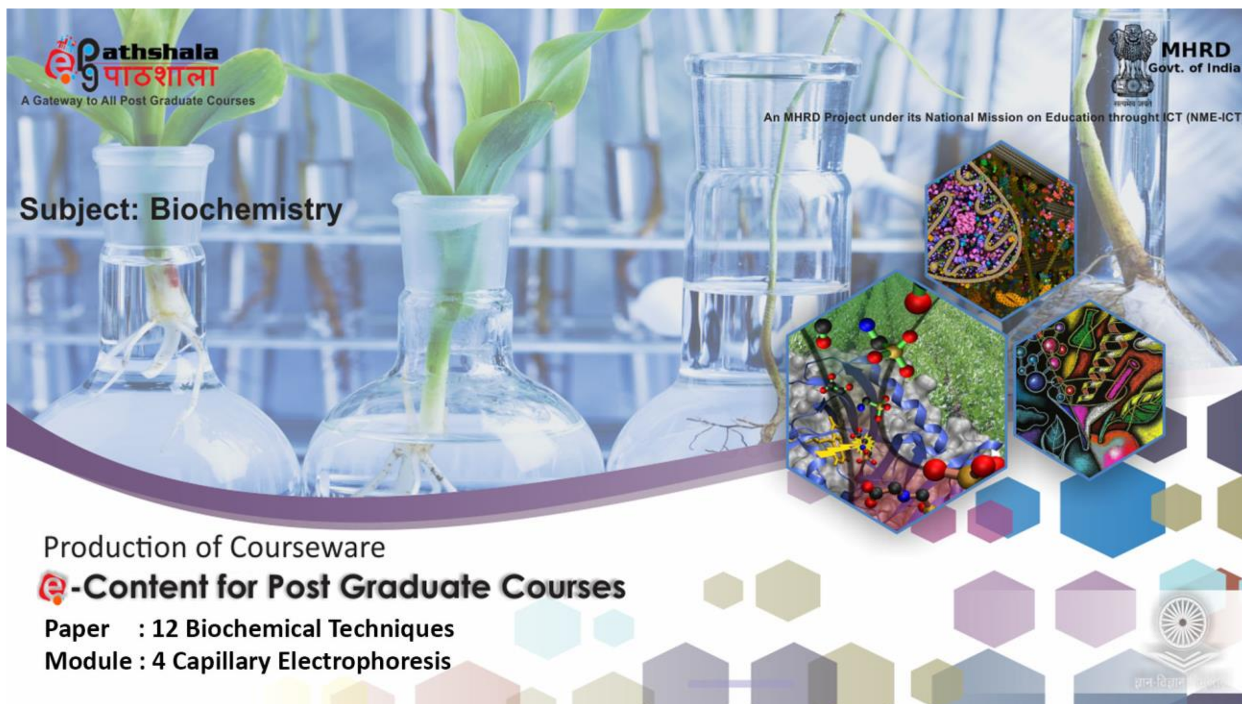

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Description of Module	
Subject Name	Biochemistry
Paper Name	12 Biochemical Techniques
Module Name/Title	04 Capillary Electrophoresis

1. Objectives

1.1 To understand principle of Capillary Electrophoresis.

1.2 How is Capillary Electrophoresis performed?

1.3 What are applications of Capillary Electrophoresis?

Capillary Electrophoresis (CE)

This technique is known for its high performance and also has been mentioned as **high performance capillary electrophoresis** or **free solution capillary electrophoresis**, **capillary zone electrophoresis** or **capillary electrophoresis**. In this technique, the reagents used are in microliter scale, sample detected are in nanoscale and the time taken is in minutes. Techniques such as PAGE, SDS-PAGE, isoelectric focusing and pulsed field gel electrophoresis can be carried out in capillary systems.

This technique is very useful for the separation and analysis of wide variety of compounds such as amino acids, peptides, proteins, oligonucleotides, DNA fragments, nucleic acids, metal ions, drugs.

During the performance of different methods of capillary electrophoresis, the analyte is separated on the basis of its migration in the applied electric field. The main advantage of using CE over the conventional methods using the slab gels is to overcome the heating effects generated during the electrophoresis. In electrophoresis, high voltage generates heat which has deleterious impact on the resolution as well as on the structural integrity of biopolymer being separated. This problem can be resolved by performing the electrophoresis in thin capillaries having small internal volumes and large surface to volume ratio. Therefore, heat generated in such capillaries during electrophoresis can be dissipated efficiently.

Theoretical Plates: The number of theoretical plates are calculated by following equation

$N = \mu V/2D$

where,

μ = electrophoretic mobility
V = voltage applied
D = Diffusion coefficient of the sample in the electrophoresis medium
Number of theoretical plates is dependent on electrophoretic mobility, applied voltage and diffusion coefficient. With increase in electrophoretic mobility or voltage,, number of theoretical plates will increase while with increase in diffusion coefficient coefficient, number of theoretical plates will decrease.

Retention Time

Time (t) taken for sample to pass through capillary is presented by following equation.

$t = L^2/ \mu V$

Where L is length of tube, μ is electrophoretic mobility and V is applied voltage.

From these above two equations, it is clear that column length does not influence theoretical plates and hence resolution. However, it does influence time required to pass the sample through capillary tube. High voltage will result in increase of number of theoretical plates and hence resolution. Essentially it is to be also understood that higher voltage also results in more generation of heat and ultimately it results in increase in diffusion coefficient. In electrophoresis, increase in voltage will result in increase in current and later will result in more generation of heat. Generated heat must be dissipated fast for minimizing its effect on diffusion. In capillary electrophoresis, internal volume is very low ie in microlitres while surface area is very large. Thus, generated heat is very quickly dissipated. This is great advantage seen in capillary electrophoresis. Because of quick dissipation of heat in capillary electrophoresis, no support such as agarose or acrylamide is required to minimize diffusion. Thus in capillary electrophoresis, molecules move freely in solution.

Length of capillary tube does not influence number of theoretical plates and thus longer capillary tubes are not advised. However, it is to be noted that shorter capillary tubes will result in decreased resistance and thus increase in heat. In shorter capillary, surface area available for heat dissipation will also decrease and can result in increase in diffusion coefficient. Also, there will be physical limit to reduce the size of capillary tube. Capillary length is kept usually in the range of 10 to 100 cm. internal diameter of tube is between 10 and 100 µm while external diameter is usually 300 µm.

INSTRUMENTATION

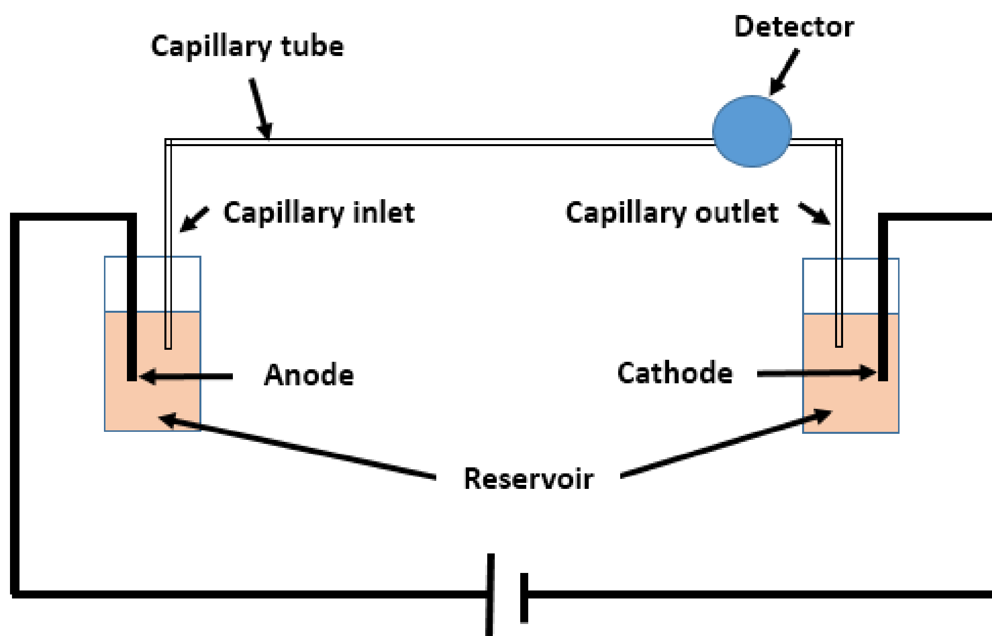
Instrumentation of capillary electrophoresis is essentially consists of two reservoirs, capillary tube, detector, integrator and power supply. Therefore, instrumentation is very simple in design. These two reservoirs are called anode reservoir or cathode reservoirs. In anode reservoir, anode electrode is placed whereas in cathode reservoir, cathode electrode is placed. Capillary ends are placed in these reservoirs. End of capillary placed in anode reservoir is called anodic end while other end of capillary placed in cathode reservoir is called cathodic end. Electrodes are connected to power supply. Detector is connected towards cathodic end of capillary. Power supply should be able to provide voltage in the range of 10 to 100 kV.

The sample injection is very critical in CE where 5-30 μm^3 sample solution (1-10 nL) is introduced from the anode end of the capillary by one of the two methods: pressure injection or by high voltage injection.

Pressure injection: Capillary is removed from the buffer reservoir present at the anodic end and carefully placed in the sample solution which is present in air tight or sealed container. Another tube provides pressure to the sample solution which forces the sample to enter the capillary from one end. Once the sample is introduced into the capillary, it is returned back into the buffer reservoir. And then, voltage is applied to start the electrophoresis.

High Voltage injection: Firstly, when the voltage is not applied, the anodic buffer reservoir is replaced by the sample solution reservoir. Once the sample reservoir is present in the position of anodic buffer reservoir, then high voltage is applied which introduces the sample into the capillary tube. Again by turning off the voltage, the sample solution reservoir is removed, anodic buffer reservoir is placed at its original position and the voltage is switched on to start the electrophoresis

In CE, the high voltage power supply which are commonly used should be capable of supplying up to 50 kV direct current. The high voltage applied across anodic and cathodic end of the capillary tube moves the sample components at the different rates along the length of the capillary tube.



Electroendosmosis or electroosmotic flow is of much significance in capillary electrophoresis. Capillaries made up of glass contains negatively charged silica on the surface. These negative charges attract the positive charges present in the buffer and forms a layer on the inner surface of the capillary. This positively charged layer further attracts the hydration layer of water and forms a double layer. When electric field is applied the positive charges along with layer of water moves towards the cathode. The volume of the capillary is very small and the major fraction of volume is occupied by the hydration layer of the water, therefore, it effects the separation. Electroendosmotic flow is affected by the pH of the buffer used. Electroendosmotic flow is approximately ten times faster at low pH buffers than at high pH buffers.

In addition to electroendosmotic flow, the samples also move according to the electrophoretic flow. Electrophoretic flow is the movement of positive ions towards cathode and movement of negative ions towards the anode. Combining the effects of both the electroendosmotic flow and electrophoretic flow, the positively charged ion will move towards the cathode, where the effect of flow is additive. Uncharged sample will move towards the cathode because of the electroendosmotic flow. Negatively charged ions will move towards the anode because of the electrophoretic flow but also experience the flow towards the cathode as consequence of electroendosmotic flow and therefore the net movement depends upon the difference between the electrophoretic and electroendosmotic flow.

In another version where electroendosmotic flow is undesirable and not required, the inner surface of the capillaries is coated a polymer (polyimide or teflon) which blocks the interaction of positively charged components with the negatively charged silanol groups. In such capillaries, when electrophoresis is conducted, the positively charged ions will move towards cathode, negatively charged ions will move towards the anode and the neutral ions will remain immobile. Since, detection is possible at only one end at a time therefore, only few components are detected and these components are selected depending upon the practical utility. CE is modified depending upon the version.

A typical run time in CE is 10 to 30 minutes. As the separated components reaches the cathodic end of the capillary tube, detection system at this end is installed for the detection and analysis of the sample components. Transmitted signals are then recorded by the recorder. Detection is possible via electrochemical detection, mass spectroscopy which allows two dimensional detection of sample components, laser induced fluorescence detection in UV-visible range in case of peptides, proteins and nucleic acids. Many commercial CE systems use UV or UV-visible absorption detection system. The fluorescence detection system can also be applied when the separated analyte is a fluor and if not then extrinsic fluor is attached to the analyte. This method of detection increases the sensitivity and selectivity of the separated components. Laser induced fluorescence detection in CE detects the analytes at the level of as low as 10^{-18} to 10^{-21}M. The detection system which detects the analyte while moving towards the cathodic end gives results with good resolution.

Thermostatting or heat dissipation mechanism is very critical for the CE. There should be minimum heat gradient between the interior and exterior capillary wall. This depends on the heat transfer rate between the outermost surface of the exterior wall and its surrounding medium. The temperature gradient between the interior and exterior capillary wall is 125°C/mm when air is the surrounding medium and this temperature is decreased to 51°C/mm with the forced air cooling setup is installed, Further, with the arrangement of temperature controlled aluminium plates , the temperature gradient decreased to 7°C/mm.

Sample	**CE mode**	**Detection**
Catecholeamines	Free solution	Electrochemical
Metals	Free solution	UV
Propanolol	Free solution	UV
Amino acids	Free solution	UV
Peptides	Free solution	UV
Insulin	SDS-PAGE	UV
Serum proteins	Discontinuous free solution	UV
DNA	Free solution	Fluorescence
DNA (mutation detection)	Pulsed field in ultradilute sieving solutions	UV
DNA (restriction fragments)	Agarose	Fluorescence
Oligonucleotides	Free solution	UV
Virus particles	Free solution	UV
Bacteria	Free solution	UV

Advantage of capillary electrophoresis over the slab gels

1. The main advantage of using capillary systems is that high voltage (5-50kV) can be applied without effecting the resolution and structural integrity of the samples as the set up for these systems is very effective in heat dissipation.
2. Requires less samples volumes and therefore increases the sensitivity (can detect as little as 10^{-15} M).
3. Exhibits high resolution. It can also separate the chiral mixtures. The separation efficiency of components in CE is greater than the HPLC.
4. It separates the complex mixtures within minutes. This technique is known for its rapidity.

Limitations of Capillary electrophoresis

1. In free solution, where electroendosmotic flow is advantageous, it can also be a shortcoming as the positively charged proteins or peptides or any other sample component can be adsorbed on

the inner surface of the capillary wall. This can lead to the tailing or the complete loss of component during detection.

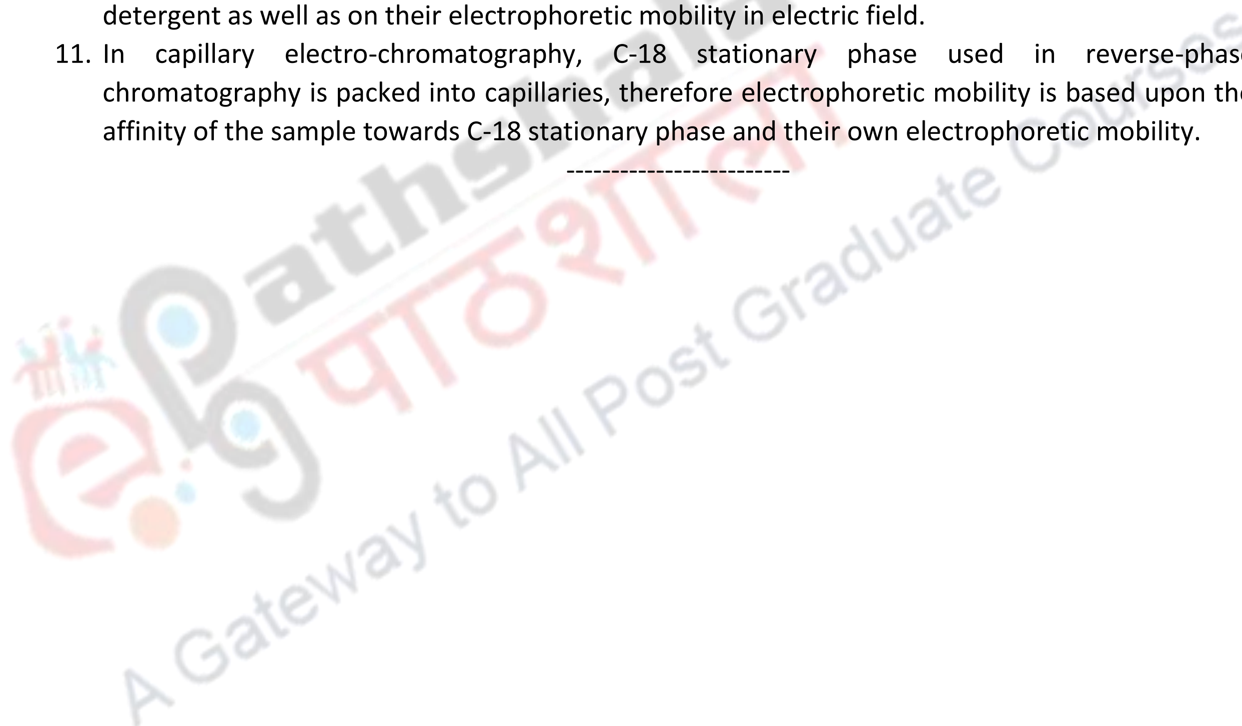
2. Small volume of the sample used in the capillary electrophoresis. If the sample is diluted, even the highly sensitive detectors cannot detect the sample.
3. High voltages used in CE may cause lethal damage if necessary heat dissipation methods are not applied.

APPLICATIONS

1. Point mutations which are responsible for the disease can be identified using CE.
2. CE is used for the quantitative analysis of DNA and RNA.
3. It can be applied for the purity detection of the synthetic oligonucleotides
4. Wide range of metals, drugs, small molecules and other compounds are being detected in the biological samples.
5. It helps in the separation of chiral mixtures. The separation is normally carried out using cyclodextrins as chiral selectors.
6. Isotachophoresis has wide application in CE. Similarly, as in SDS-PAGE, the sample ion is stacked between the leading and the terminating ion. Under the constant current leading ion has the high electrophoretic mobility than the sample ion, and sample ion has the high electrophoretic mobility than the terminating ion. **(Electrophoretic mobility**: Leading ion > sample ion > terminating ion). Therefore, when the electric field is applied, the ion with low electrophoretic mobility experiences high field strength and the ion with high electrophoretic mobility experiences low field strength (**Field Strength:** Leading ion < sample ion < terminating ion) and all the ions move with the same velocity causing the sample ion to stack between leading ion and the terminating ion, also known as zone sharpening effect. This effect relies on the principle that the ions having the higher electrophoretic mobility will diffuse in the zone where these ions experiences low field strength and vice versa. Isotachophoresis is conducted in quartz and teflon cuvettes at voltages up to 30 kV.
7. Capillary Isoelectric Focusing: The buffer used in this type of capillary electrophoresis have pH gradient is which provided by commercially available carrier ampholytes. The pH gradient of different range (narrow or broad) can be setup with these ampholytes having their isoelectric points in this range. These ampholytes are very small, therefore in an applied electric field they move very quickly and pH gradient is formed. The capillary in which electrophoresis will be conducted is filled with ampholytes and analytes which are to be separated. When the voltage is applied all the ampholytes starts to migrate on the basis of their isoelectric point and establishes the pH gradient. As already discussed that being very small, these ampholyte molecules moves faster than the analyte molecule. Each analyte molecule will move according to their isoelectric point. For the identification of analytes chemical markers are available with known isoelectric

points and can be identified when placed in the electropherogram. These markers can be can be added along with the markers.

8. Ligand affinity of protein or DNA can be applied with electrophoresis. Depending upon the electrophoretic mobility, the uncomplexed ligand and uncomplexed protein can be separated from ligand-protein complex as all the three species have different electrophoretic mobility.
9. Separation by capillary electrophoresis is in line with HPLC system, which facilitates 2-D analysis of sample. In can be performed by the directing the eluted samples from HPLC to the CE system where it can be analysed rapidly.
10. Micellar electrokinetic capillary electrophoresis: In this type of capillary electrophoresis, separation can be performed by including SDS in the system. Here, SDS when suspended in an appropriate buffer and above certain concentration it form micelles and act as a stationary phase, therefore, the separation occurs on the basis of the interaction of the sample with the detergent as well as on their electrophoretic mobility in electric field.
11. In capillary electro-chromatography, C-18 stationary phase used in reverse-phase chromatography is packed into capillaries, therefore electrophoretic mobility is based upon the affinity of the sample towards C-18 stationary phase and their own electrophoretic mobility.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