LECTURE-10

High Performance Liquid Chromatography Mass Spectrometry

HANDOUT

PREAMBLE

Mass Spectrometry is the most versatile technique in the emerging field of proteomics. MS is technique for production of charged molecular species, and their separation by magnetic and electric fields based on mass to charge ratio Unlike genomics, where the availability of whole genome sequence, microarray chips etc. has revolutionized the growth of the field; proteomics appears to be a challenging field when it comes to identifying proteins, post-translational modification or differential expression of proteins subject to different conditions. Mass spectrometry has emerged as an excellent tool capable of performing several applications. Mass spectrometry has emerged from an analytical technique to identify molecules based on the mass, to an advanced form, capable of identifying complex molecules like proteins, their features like post translational modification and sequence. Various advancements in soft ionization techniques and combinations of mass analyzers are used to achieve these objectives. The inclusion of HPLC with Mass spectrometry has increased the resolution and sensitivity of the technique, whereby; peptides are separated and identified by the mass spectrometer.

OUTLINE OF LECTURE

I. Principle of Mass Spectrometry

II. Components of Liquid Chromatography Mass Spectrometry

(a) Chromatographic unit

(b) Ionization Source

(c) Mass Analyzer

(d) Mass Detector

III Tandem Mass Spectrometry

IV. Applications and challenges in clinical proteomics

V. Conclusions

BOX-1: TERMINOLOGY

1. MS = Mass Spectrometry - Mass spectrometry (MS) is the technique for protein identification and analysis by production of charged molecular species in vacuum, and their separation by magnetic and electric fields based on mass to charge (m/z) ratio.

2. RP = Reverse phase chromatography - A technique used to separate analytes based on their hydrophobicity.

3. SXC = Strong Cation Exchanger - A technique used to separate analytes based on their positive charges.

4. MALDI = Matrix Assisted Laser Desorption Ionization (MALDI): is an efficient process for generating gas-phase ion of peptides and proteins for mass spectrometric detection. Target plate with dried matrix-protein sample is exposed to short, intense pulses from a UV laser.

5. ESI = Electrospray Ionization: Ions are formed by spraying a dilute solution of analyte (sample) at atmospheric pressure from the tip of a fine metal capillary, creating a fine mist of droplets. The droplets are formed in a very high electric field and become highly charged. As the solvent evaporates, the peptide and protein molecules in the droplet pick up one or more protons from the solvent to form charged ions.

6. TOF = Time of flight - This is a mass analyzer in which the flight time of the ion from the source to the detector is correlated to the m/z of the ion. An ion trap makes use of a combination of electric and magnetic fields that captures ions in a region of a vacuum system or tube. It traps ions using electrical fields and measures the mass by selectively ejecting them to a detector. This analyzer typically has lower resolution.

7. Quadrupole: Quadrupole mass analyzers use oscillating electrical fields to selectively stabilize or destabilize the paths of ions passing through a radio frequency (RF) quadrupole field.

1. PRINCIPLE OF MASS SPECTROMETRY

The first step in mass spectrometry involves generating charged particles in the gaseous form, which can be accelerated using a strong electric field and then analyzed by the detector.

$$M + e^- \longrightarrow M^{\bullet +} + 2e^-$$

In case of proteomic analysis, the proteins are digested with a suitable protease (usually trypsin) and the peptide fragments so obtained are ionized to generate charged particles. These charged particles are accelerated in vacuum under the presence of an external electric field. They are further fragmented (in tandem MS/MS) before finally being detected by the detector. The data is obtained in the form of relative abundance vs m/z ratio. Relative abundance refers to the abundance of that particular ion in the sample. Using the m/z ratio, an idea about the fragmentation pattern and hence the empirical formula of the molecule or peptide sequence of proteins can be established.

Illustration: Fundamentals of Mass Spectrometry

Mass spectrometer is an instrument that produces charged molecular species in vacuum, separates them by means of electric and magnetic fields and measures the mass-to-charge ratios and relative abundances of the ions thus produced. It is being increasingly used for detection and analysis of proteins from complex samples.

2. COMPONENTS OF LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

A Mass Spectrometer has three basic components – ionization source, mass analyzer and detector (Fig. 1). Mass spectrometer is also coupled with HPLC system to facilitate easy separation of peptides, prior to their identification. These columns utilize the inherent properties of the peptides to separate them in solution, thereby increasing the resolution of the mass spectrometer (Fig. 2).

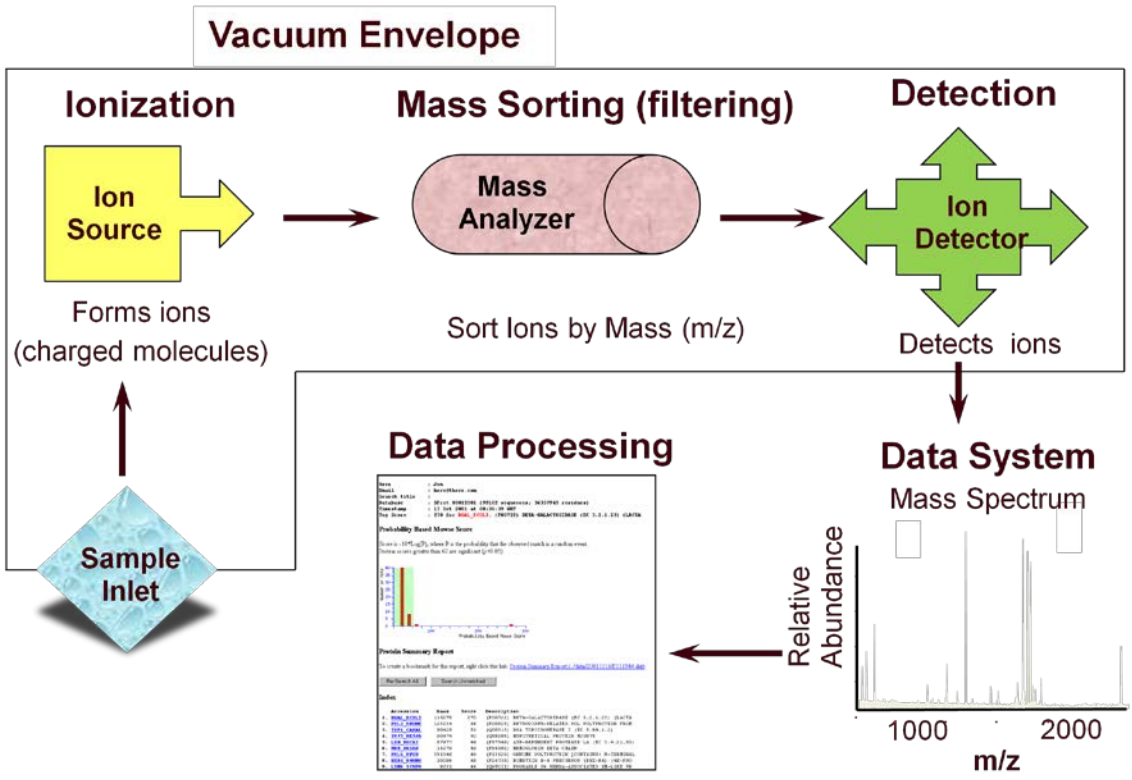

Fig.1. The basic components of mass spectrometer.

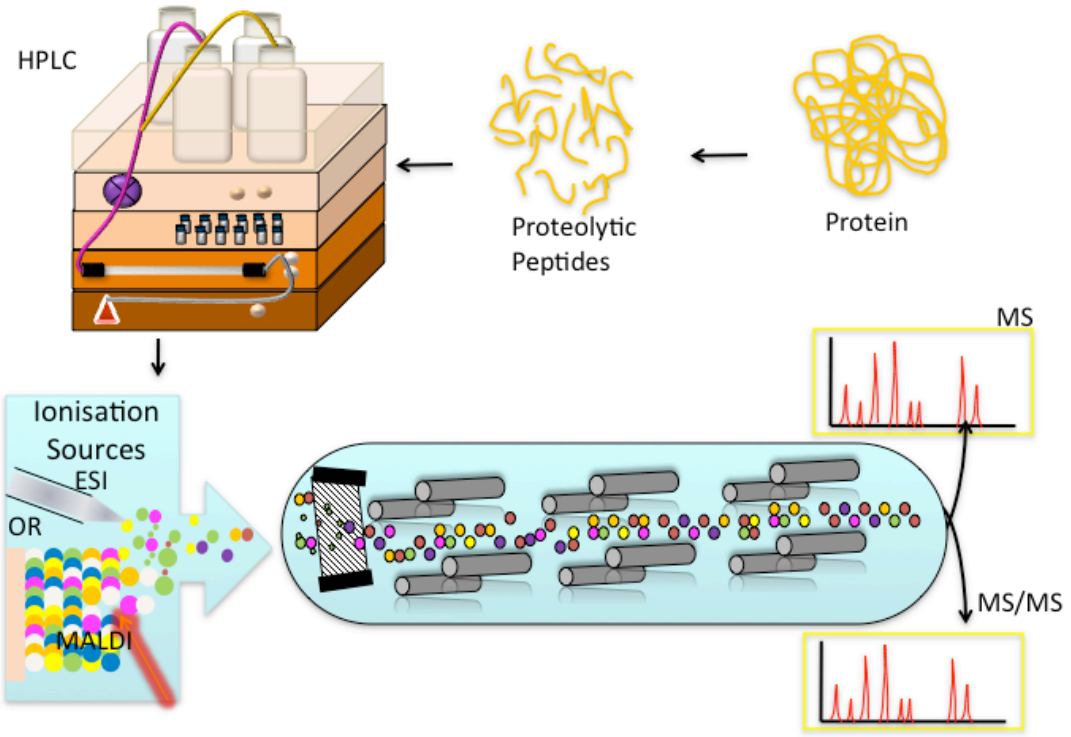

Fig. 2. Schematic of a proteomic experiments. Protein is digested using trypsin and pre-fractionated using HPLC. MS device makes use of a combination of ion source and one or two mass analyzers.

2.1. CHROMATOGRAPHIC UNIT

Protein mass spectrometry is a versatile tool for protein identification and quantification; however, it is subjected to various limitations. Since the detection is solely based on m/z ratio, it might so happen that two different peptide fragments are generated, having same m/z ratio. Assigning their true identity becomes a problem in this case. Also sometimes the highly abundant peptides overshadow the signals of low abundant peptides, which are clinically relevant from the point of view of biomarker discovery.

Therefore, fractionation of complex mixtures of peptides into smaller fractions, which can be analyzed accurately and with high sensitivity, is required. The separation methodology using various types of HPLC techniques like RPLC (Reverse phase liquid chromatography), SXC (Strong Cation Exchanger), Affinity chromatography and Hydrophilic Interaction Liquid Chromatography (HILIC) is effective pre-fractionation strategy. RPLC and SXC are more commonly used to separate peptides.

RPLC: The columns for RPLC are highly hydrophobic C-18 columns, which strongly interact with the hydrophobic patches of the peptides. The major advantage of RPLC is that the mobile phase used to elute peptides is compatible with ESI. With varying degrees of hydrophobicity, the peptides elute at different time intervals and detected by MS (Fig. 3).

SCX: The columns for SCX consist of strong cation exchangers like aliphatic sulfonic acid groups that are negatively charged in aqueous solution. Tryptic peptides containing positive charge are specifically exchanged using these columns. Using suitable solvents of high ionic strengths strong ionic interactions are broken. Ideally, working with only SCX yields very poor resolution of peptides. However, combining SCX with RPC yields better resolution, as two different properties of the peptides are being utilized in fractionating the peptides.

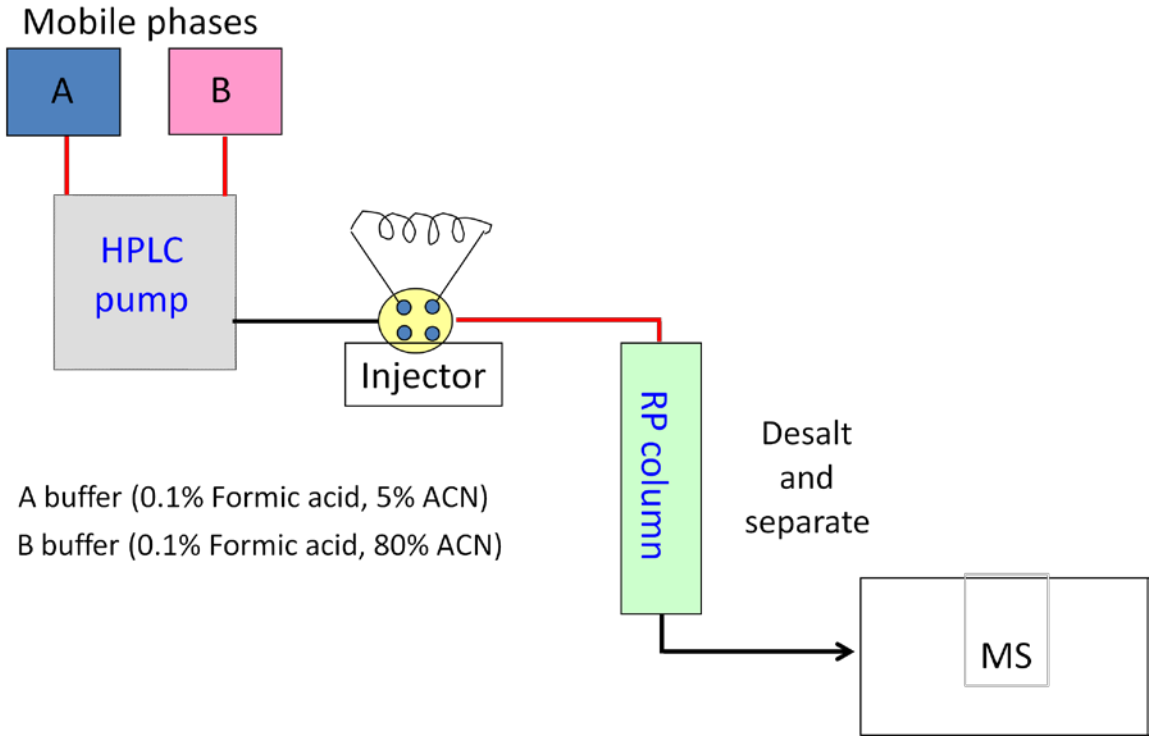

Fig 3. Scheme for RP-HPLC chromatography

Illustration: Liquid chromatography

A typical liquid chromatography setup consists of the solvent bottles, degassifier, dual or quaternary pump, sample injector, column and detector. Different solvents can be placed in the solvent bottles depending upon the purification requirement. These solvents are mixed in the desired ratio and pumped into the column during elution after removal of any trapped air inside it by means of the de-gassifier.

The sample injector system may be automatic or manual. The automatic sampler uses a syringe to inject the sample placed in a vial directly into the column. Once the sample is injected, mobile phase flows into the column through the pump. The column consists of a stationary matrix that preferentially binds certain analytes. Outlet from the column enters the flow cell where it is detected.

Various stationary phase matrices are available that separate the components of the mixture based on different principles. One of the commonly used matrices, the strong cation exchanger, separates charged peptides based on their electrostatic interactions with negatively charged sulphonic acid groups on the resin surface. Reverse phase chromatography is another commonly used tool, which uses a hydrophobic matrix consisting of long aliphatic carbon chains. These retain analytes on the basis of their hydrophobic interactions and can be eluted by changing the polarity of the solvent.

Nano-liquid chromatography, which makes use of C-18 capillary columns, has gained popularity for proteomic studies due to their ability to achieve fine separation.
The separated components pass from the column outlet into the flow cell present in the detector. The most commonly used detector for protein analysis is UV detector, which analyzes the protein absorbance at 280 nm and plots a graph of retention time against intensity. Each peak corresponds to a particular analyte in the sample mixture.

2.2.

IONIZATION SOURCE

An ionization source ionizes the peptides so that they acquire a charge and hence can be accelerated in vacuum under the influence of an external electric field. The ionization source must be effective for all type of molecules (polar, non polar, non volatile etc.) and must be capable of ionizing the analyte without much degradation. Various ionization sources are described in Fig. 4. Two most commonly used ionization sources, MALDI and ESI will be discussed.

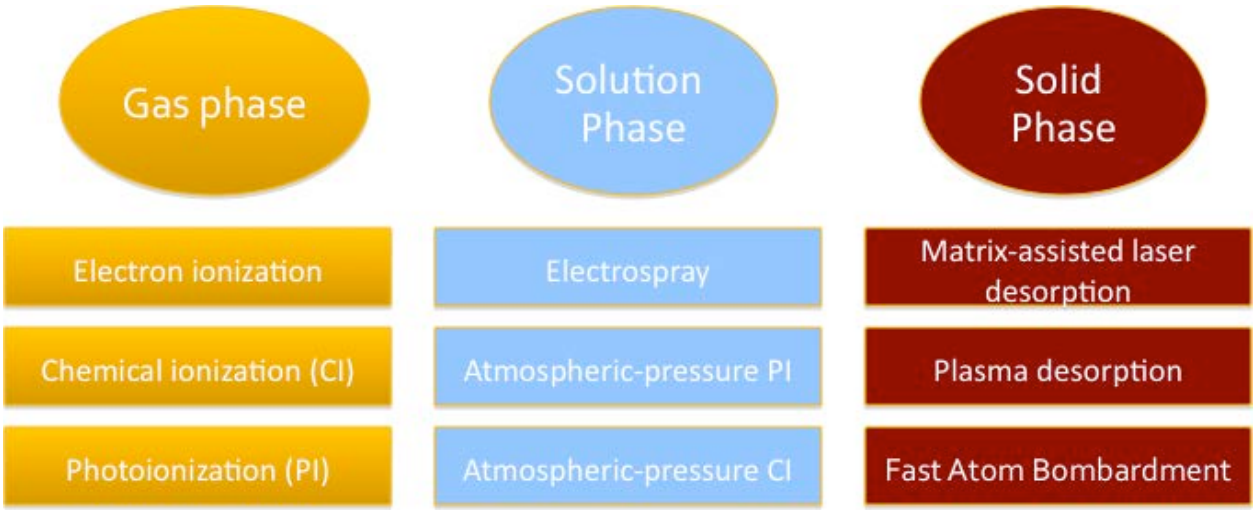

Fig 4. Different ionization sources: gas phase, solution phase & solid phase.

MALDI:

The trypsin digested peptides are mixed with matrices (e.g. Cyanohydroxy cinnamic acid or cinapinic acid) and plated on MALDI plate. Short pulses of laser are inflicted upon the MALDI plate. The matrices absorb the energy from the laser and transmit it to

the peptide fragments, which get ionized. Ideally singly charged ions are produced using MALDI ionization and these ions are accelerated through vacuum (Fig. 5).

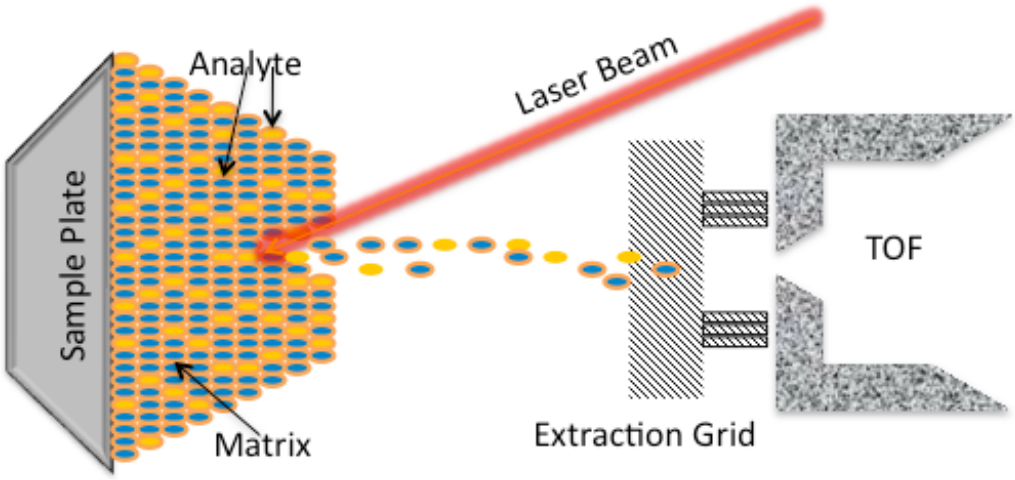

Fig. 5. A schematic diagram of ionization using MALDI

Illustration: Ionization techniques - MALDI

The ionization source is responsible for converting analyte molecules into gas phase ions in vacuum. This has been made possible by the development of soft ionization techniques, which ensures that the non-volatile protein sample is ionized without completely fragmenting it. The most commonly used ionization sources are Matrix Assisted Laser Desorption-Ionization (MALDI) and Electrospray Ionization (ESI). In MALDI, the analyte of interest is mixed with an aromatic matrix compound like a-cyano-4-hydroxycinnamic acid, sinapinic acid etc. This is then dissolved in an organic solvent and placed on a metallic sample plate. The evaporation of solvent leaves the analyte embedded in the matrix. The target plate is placed in a vacuum chamber with high voltage and short laser pulses are applied. The laser energy gets absorbed by the matrix and is transferred to the analyte molecules, which undergo rapid

sublimation resulting in gas phase ions. These ions then accelerate towards the mass analyzer based on their mass-to-charge ratio.

ESI:

The need for softer ionization technique, to preserve peptide integrity, led to the emergence of Electrospray ionization (ESI). Trypsin digested peptides are allowed to pass through a very small capillary, the tips of which are maintained at very high voltage. As the peptides emerge out of the capillary in the form of fine droplets, the droplets get ionized (Fig. 6). All type of charges are formed in ESI but it is the positively charged ions that are accelerated.

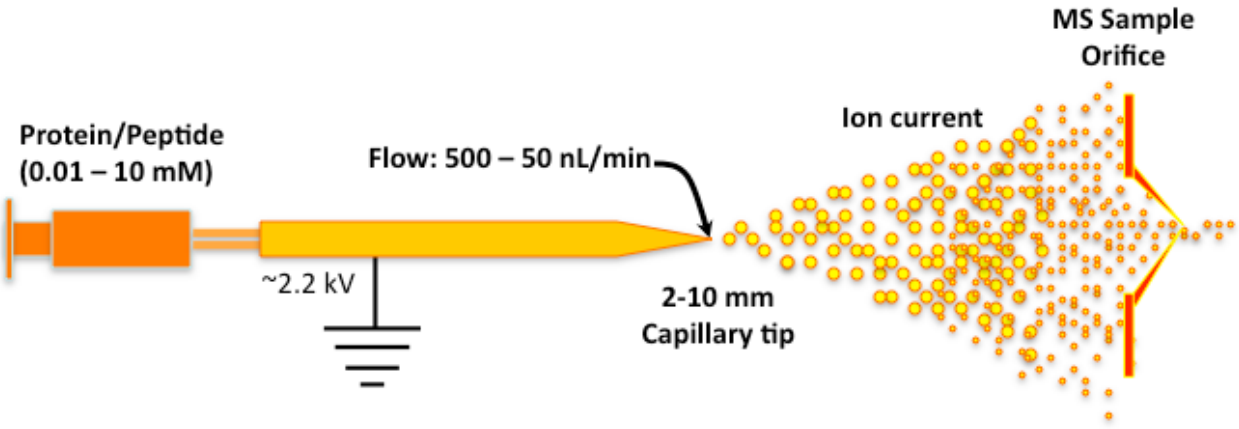

Fig 6. A schematic diagram of ionization using ESI.

Illustration: Ionization techniques - ESI

In ESI, the sample is present in the liquid form and ions are created by spraying a dilute solution of the analyte at atmospheric pressure from the tip of a fine metal capillary, creating a mist of droplets. The droplets are formed in a very high electric field and become highly charged. As the solvent evaporates, the peptide and protein molecules in the droplet pick-up one or more protons from the solvent to form

charged ions. These ions are then accelerated towards the mass analyzer depending upon their mass and charge.

Illustration: Comparison of MALDI and ESI

MALDI and ESI both have their pros and cons and can be used for the analysis of different types of protein samples. Development of both of these techniques was awarded the Nobel Prize in 2002.

Other ionization sources include APPI (Atmospheric Pressure Photo-ionization) and APCI (Atmospheric Pressure Chemical Ionization), where photons generated from specialized UV lamps ionize the peptides in case of APPI and inert gases like charged nitrogen gas transfer their ionic charge to the analyte in case of APCI.

2.3. MASS ANALYZER

The mass analyzer represents the component of the mass spectrometer where the ionized peptides are separated according to their m/z ratio. Various types of mass analyzers are available (Fig. 7), some of which are listed below.

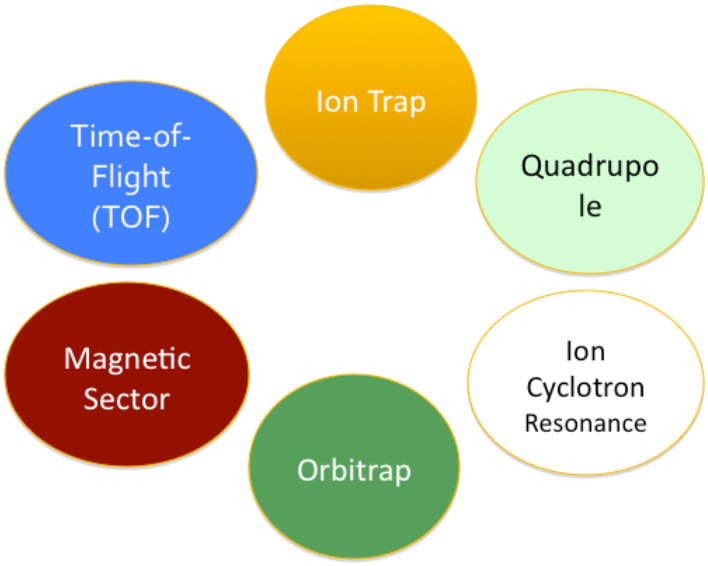

Fig 7. Different types of mass analyzers used for proteomics applications.

TOF: Time of Flight involves accelerating the charged peptides through a long flight tube, maintained at vacuum. The different sized peptides by virtue of their difference in kinetic energy move at different rates and reach to the detector. Usually TOF is in association with MALDI and hence, singly charged peptides are accelerated through TOF. Thus heavier peptides take longer time to reach the detector than smaller peptides (Fig. 8). The time required by a peptide to move across the entire flight tube is given by:

$$t = \left(\frac{m}{2qV_0} \right)^{1/2} L$$

Where

t = time-of-flight (s)
m = mass of the ion (kg)
q = charge on ion (C)
V_0 = accelerating potential (V)
L = length of flight tube (m)

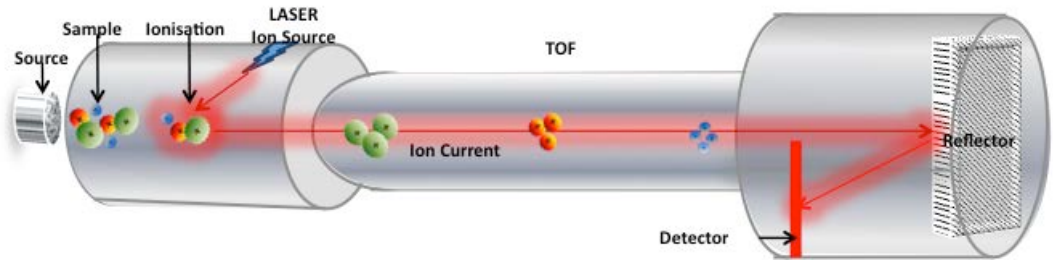

Fig 8. A time of flight (TOF) mass analyzer and its working principle. Ions are accelerated at different velocities depending on their m/z ratios. Ions of lower masses are accelerated to higher velocities and reach the detector first.

Illustration: Time of Flight (TOF)

The mass analyzer resolves the ions produced by the ionization source on the basis of their mass-to-charge ratios. Various characteristics such as resolving power, accuracy, mass range and speed determine the efficiency of these analyzers. Commonly used mass analyzers include Time of Flight (TOF), Quadrupole (Q) and ion trap.

The time of flight analyzer accelerates charged ions generated by the ionization source along a long tube known as the flight tube. Ions are accelerated at different velocities depending on their mass to charge ratios. Ions of lower masses are accelerated to higher velocities and reach the detector first. The TOF analyzer is most commonly used with MALDI ionization source since MALDI tends to produce singly

charge peptide ions. The time of flight under such circumstances is inversely proportional to square root of molecular mass of the ion.

QUADRAPOLE / TRIPLE QUADRAPOLE (TQ):

A Quadrapole mass analyzer consists of a parallel set of four metallic rods, which are maintained at different potential difference, hence allowing particular ions to pass through them. A triple quadrapole consist of an additional collision cell in between two quadrapoles (Fig. 9). The first quadrapole allows a particular set of ions to move into the collision cell, where that particular ion is fragmented into several ion pieces, all of which are then moved into the third quadrapole and hence the detector.

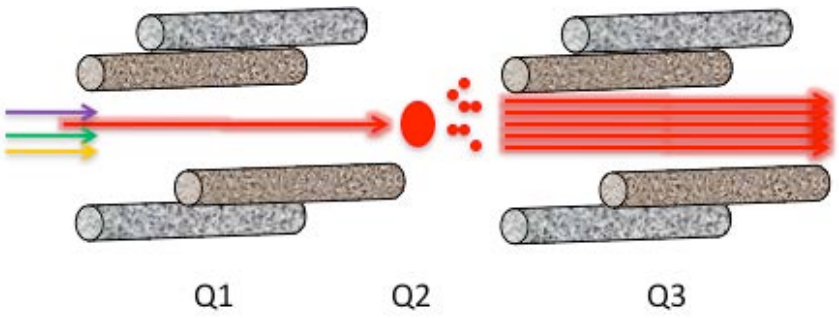

Fig 9. A typical scheme of Triple quadrapole mass analyzer.

Illustration: Quadrupole mass analyzer

Quadrupole mass analyzers use oscillating electrical fields to selectively stabilize or destabilize the paths of ions passing through a radio frequency (RF) quadrupole field. The quadrupole mass analyzer can be operated in either the radio frequency or scanning mode. In the RF mode, ions of all m/z are allowed to pass through which are then detected by the detector.

In the scanning mode, the quadrupole analyzer selects ions of a specific m/z value as set by the user. A range can also be entered in which case only those specific ions satisfying the criteria will move towards the detector and the rest are filtered out.

ION TRAP:

An ion trap mass analyzer consists of two sets of electrodes, the cap electrodes and the ring electrodes maintained at two different voltages. The ions that have a threshold m/z value more than the corresponding m/z value set by the voltage remain trapped in the ring electrode while other ions are allowed to pass into the cap electrode, where they are further fragmented and analyzed (Fig. 10).

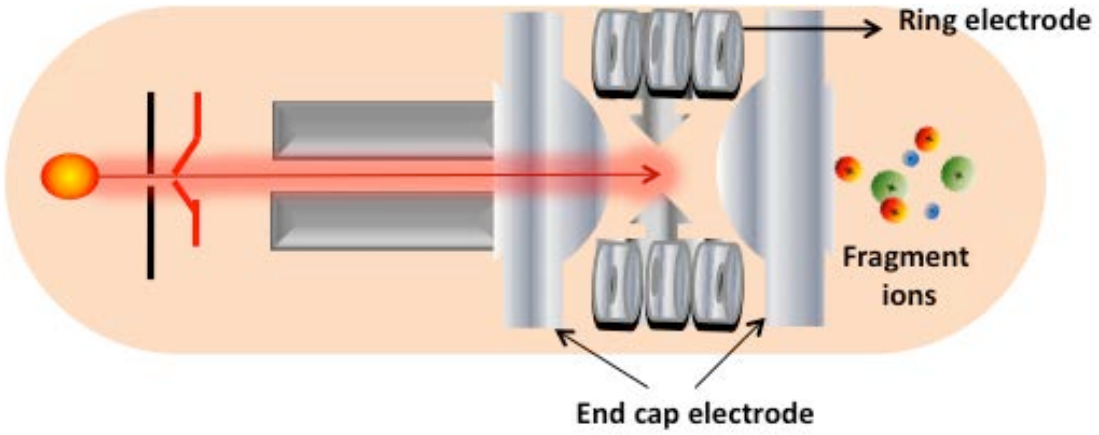

Fig 10. A typical scheme of Ion Trap mass analyzer.

Illustration: Ion-trap mass analyzer

An ion trap makes use of a combination of electric and magnetic fields and captures ions in a region of a vacuum system or tube. It traps ions using electrical fields and measures the mass by selectively ejecting them to a detector.

FOURIER TRANSFORM ION CYCLOTRON RESONANCE (FT-ICR)

Ion cyclotron resonance is the most advanced form of mass analyzer where the ions of a particular m/z ratio orbit around a pole subjected to a uniform magnetic field. This gives the highest resolution; however, it is extremely complicate to handle.

2.4. MASS DETECTOR

The mass detector is the final component of the mass spectrometer. It records the ion flux or the abundance of ions reaching the surface and hence the m/z ratio. Typically, a detector is an electron multiplier or an ion-to-photon converter (photo multiplier). This is because the amount ion reaching to the detector is extremely small to be detected and hence multiplication is required.

3. TANDEM MASS SPECTROMETRY

One of the most important applications of mass spectrometry is in protein sequencing. The Edman degradation method of protein sequencing is limited to a stretch of approximately 40 amino acids and also with the availability of a free N-terminal. Mass spectrometry based amino acid sequencing is hence a better option when it comes to protein identification.

Tandem MS refers to another round of peptide fragmentation, analysis and detection after the first round. Technically, it is represented as MS^N, where N takes up an integer value.

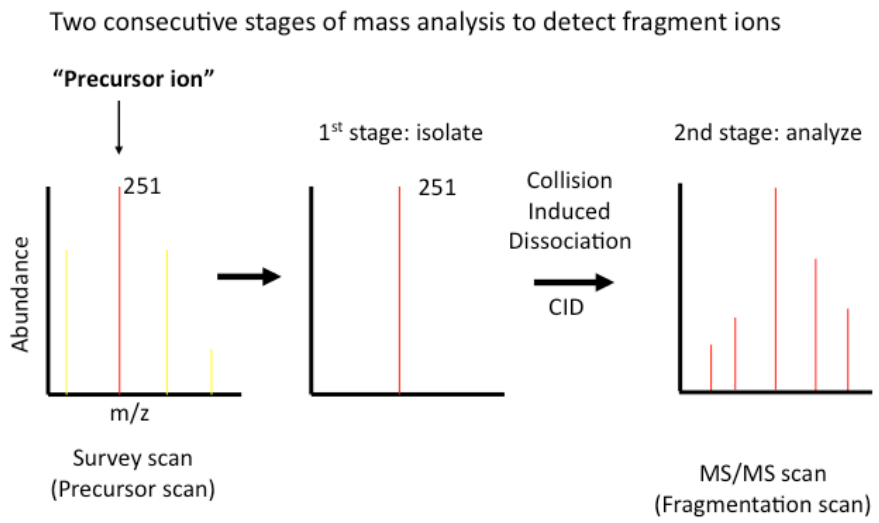

Fig 11. Tandem MS/MS - One of the peaks obtained is chosen and that particular peptide is allowed to move into the next mass analyzer where it is further fragmented and detected. Ideally, the different daughter peptides so obtained differ by one or more amino acid and hence from there, the sequence of the peptide can be determined.

4. APPLICATIONS AND CHALLENGES IN CLINICAL PROTEOMICS

All disorders have some signature molecules for diagnosis, and clinical proteomics aim to identify and characterize proteins involved in those disorders. For example, haptogloblin is one of the biomarkers identified in glioblastoma (Grade IV). One of the most challenging aspects of this approach is the abundance of these biomarkers in the samples. The human serum accounts for almost 70% albumin and 25% immunoglobulin G (IgG). The low molecular weight proteins account for less than 2% of human serum. Amidst the cloud of albumin and IgG, all these biomarkers are hidden. Two Dimensional Electrophoresis can not resolve all of these proteins; neither can the most sophisticated MS. The low molecular weight proteins are either hidden under the intense signal of the most abundant ions or are so low in concentration that the sensitivity limit of the MS cannot detect it. MS is also very sensitive to levels of contaminations like salt and other biomolecules, which interfere with the fragmentation pattern of the peptides.

These challenges are mitigated by the use of depletion columns, which specifically remove the high abundant proteins and thereby enrich the samples for low-abundant proteins, which could be potential biomarkers. Over the period of years, advancement in MS has increased the detection of analytes. Also the sequential elution of peptides using HPLC columns enhances the sensitivity of the MS. Nonetheless, the technology is still developing and with every passing year, it is turning into a more robust platform for use in advanced clinical diagnosis.

5. CONCLUSION

The success of MS in the area of proteomics has been phenomenal. Many biomarkers have been discovered using MS, which no other proteomic technique could identify. However, like other techniques, MS is also subject to certain limitations, some of which are discussed in this lecture. The main issues with MS are its sensitivity and resolution. With the development of sophisticated technology the sensitivity and resolution power of the instrument is increasing, which is extremely advantageous for clinical proteomics applications. The pace of proteomics research has been phenomenal due to the advancement of strong mass spectrometry platform. It can be said that MS can survive without proteomics but the converse is not true.

6. REFERENCES

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