HANDOUT

LECTURE-20

FUNDAMENTALS OF MASS SPECTROMETRY

Slide 1:
This lecture will cover the fundamentals of mass spectrometry.

Slide 2:
This lecture's outline is as follows:

- Fundamentals of mass spectrometry that covers the basic concepts.
- A discussion of the individual components such as ionization source, mass analyzer and others followed by a discussion on tandem mass spectrometry.

Slide 3:
The gel-based proteomics technique discussed in the previous module resolved several hundred proteins. However the scale at which the proteome has to be studied requires a much higher analytical capability. Mass spectrometry has that ability to provide a platform for comprehensive coverage of the proteome. MS has become an important analytical tool in proteomics and also biology in general during the last decade. Various applications have emerged using MS-based platform. It offers high throughput, sensitive and specific analysis for many applications.

Slide 4:
Mass spectrometry is an analytical technique to measure the molecular mass of individual compounds and atoms accurately by converting them into charged ions. By definition this is a technique for the production of charged molecular species in vacuum, followed by their separation in magnetic and electric fields based on mass to charge ratio. You can see the MS spectrum shown in the slide m/z on x-axis and the abundance (intensity) on y-axis.

Slide 5:
So what are the unique features of mass spectrometry?

- Due to its unique ability to accurately measure molecular mass and provide fragment ions of the analyte, mass spectrometry offers molecular specificity.

- It provides ultra-high detection sensitivity. In theory MS can detect even a single molecule and its sensitivity at atomole and femtomole range has also been demonstrated.
- It provides a versatile platform to determine the structure of compounds and it is applicable to all elements and samples whether they are volatile, non-volatile, polar, non-polar or solids, liquids or gases. Analysis of complex samples such as the entire proteome is thus very much possible by MS.

Slide 6:
The main steps involved in a MS operation are as follows:

- The first step is ionization, ie to convert analyte molecules or atoms into the gas phase ionic species. It removes or adds electrons or protons.
- The second step is separation and mass analysis of molecular ions and charged fragments on the basis of mass to charge ratio.
- The final step is the detection and generation of mass spectrum.

Slide 7:
Now let's discuss about general properties of MS. The sensitivity, resolution and accuracy vary among various mass spectrometers. The sensitivity drops off as the mass increases. And as I mentioned the sensitivity for protein detection can go as low as the atomolar or femtomolar range. Ion sources generate positive, negative and neutral ions. The neutrals cannot be focused or accelerated by the ion optics. So one can analyze either positive or negative ions. The positive ions have an adduct which is typically a proton and the sensitivity for negative ions is generally lower.

Slide 8:
The MS data is presented as mass to charge ratio which is mass of an ion (m) divided by the number of charges (z) it carries. So the total charge on the ion is represented by q=ze, where e is the charge on the electron. So how can m/z of any peptide be calculated? As mentioned, multiple charged states (ie +1, +2, +3) are possible. So if you need to calculate m/z you need to add m+h or m+2h or m+3h as shown and then divide by 2 or 3 depending on the number of charges carried.

Slide 9:
The choices and details of ionization sources will be provided at a later point. But in this context, it should be mentioned that there are multiple charge states generated by in the

electrospray ionization (ESI) technique. So in many charge states in proteins there many possible proton acceptors in equilibrium with the solution. Multiple charged states are quite useful because they form ions which are in the mass range of mass analyzers such as TOF, quadrupoles, ion poles etc.

Slide 10:
To cover some of the basic terminology and basic concepts involved in the mass spectrometry, average and monoisotopic masses of amino acids should be discussed. As shown in the table here there are amino acids with their 3 letter codes, single letter code, average and the monoisotopic masses. You can use this for reference later on which can be used in the data analysis and calculations. So what is monoisotopic mass of a molecule? It is the sum of masses for most abundant isotope of each element. The average mass of a molecule is the average of isotopic masses of each element weighted for the isotopic abundance.

Slide 11:
By employing MS, 2 different types of approaches can be used: top down and bottom up. The Top down approach involves separating and analyzing intact proteins without any previous proteolytic digestion. To provide a brief explanation of the steps involved in the top down approach, the proteins are first introduced into a mass spectrometer directly, without performing in-gel digestion or proteolytic cleavage. Proteins are then broken into fragments inside the mass spectrometer. The fragments are analyzed by either MS or MS-MS and then the proteins can be identified using databases. So the advantage of top down approach is that it retains information on protein isoforms, sequence and modifications. The disadvantage is that sensitivity and the proteome coverage is very limited.

Slide 12:
Moving on to the bottom up approach, this involves the separation and analysis of peptides following proteolytic digestion of a sample protein, usually using trypsin. Peptides generated are separated by using liquid chromatography and introduced inside the mass spectrometer. The peptides are fragmented inside the MS and amino acid composition in the peptide sequence can be identified by running the obtained spectra through the MASCOT database. The advantage of this approach is that it yields higher sensitivity and proteome coverage. The disadvantage of this method is that the information about protein isoforms is lost and it is unable to distinguish very close homologues.

Slide 13:
The different parts of mass spectrometer are next discussed.

Slide 14:

The major components of the mass spectrometer include the sample inlet, the ionization source, the mass analyzer, the detectorm signal processing components and the data output. Let's look at each of these components in more detail.

- The sample inlet transfers the samples into the ionization source. It is important to maintain the integrity of the sample molecules during transition from the atmospheric pressure to the vacuum of ionization source.
- The ionization source converts neutral sample molecules into gas phase ions.
- The mass analyzer separates and analyses mass of the ionic species. Various types of mass analyzers available which will be discuss in greater detail later.
- The Detector amplifies and measures the ion current of the mass-resolved ions
- The electronics control the operation of various units. The data system records various processes and stores and displays the data output. In addition to the 3 major components the ionization source, mass analyzer and detector, various accessory components exist that are integral to the process.

Slide 15:

The various processes include:

- Sample introduction: This can be coupled with HPLC or CHIP-based technologies for liquid chromatography based separations.
- Sample ionization, which can be accomplished by various methods depending on the ionization source used.
- Sample transfer to the high vacuum region, wherein the ion mass to charge filtering is performed by mass analyzers.
- Ion detection by using detectors.
- Data acquisition and analysis.

Slide 16:

The above is an overview of the workflow involved in performing MS-based experiments. First of all, one can pre-fractionate the sample by using liquid chromatography or or by alternate methods. After pre-fractionation, then in-gel digestion or proteolytic digestion can be performed by using various enzymes such as

trypsin and then the sample is injected into the ionization source. The 2 most common methods of ionization include ESI or MALDI(discussed later). Then these ions can be further resolved using mass analyzers and then data can be analyzed in MS or MS-MS mode.

Slide 17:
This slide provides an overview of the in-gel digestion process. Protein digestion (using trypsin or other proteolytic enzymes) can be done regardless of whether gel-based gel-free proteomics have been performed. In gel based proteomics experiments, the proteins are first resolved on the gel, followed by excision of either the spots or bands of interest, which are then subjected to in-gel digestion or in-solution digestion wherein proteins are cleaved into small peptides. These peptides are then ionized and analyzed by the mass analyzer component of the Mass spectrometer.

Slide 18:
The success of a MS experiment lies in efficiency of converting a neutral compound to a gaseous phase ionic species. Various types of options to do this are currently available. The type of ionization source selected can be tailored to a specific application. The choice of the ionization method is dictated largely by the nature of sample being investigated. With gas phase samples, electron ionization, chemical ionization and photo ionization are the commonly used methods of ionization. With solution phase samples, electrospray, atmospheric pressure PI and atmospheric pressure CI are commonly used methods. With samples in solid phase, matrix-assisted laser desorption ionization (MALDI), plasma desorption and fast atom bombardment are commonly used.

Slide 19:
The traditional ionization sources used for analysis of small molecules relied on chemical or electrical ionization methods. But these processes are too energy rich to ionize intact large biomolecules and lead to the unpredictable decomposition. So for proteomic applications, there was a need to develop soft ionization methods for mass spectrometry. These are non-selective fragmentation methods.

Slide 20:
The main function of an ion source is to convert sample molecules or atoms into the gas phase ionic species. It should possess following desirable characteristics.

- It should have high ionization efficiency.
- A Stable ion beam.

- A low energy spread in secondary ion beam, with minimum background ion current.
- Minimal cross-contamination so the risk of cross-contamination of successive samples is low.

Slide 21:
This animation shows two of the most commonly used soft ionization methods; MALDI and ESI.

Animation 1

Fundamentals of mass spectrometry:
Mass spectrometry is a 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 ratio. MS has increasingly become the method of choice for the analysis of complex protein samples in proteomics studies due to its ability to identify thousands of proteins. A mass spectrometer is an instrument that produces charged molecular species in vacuum and then separates them electric and magnetic fields and measures the mass to charge ratio and the relative abundance of the ions thus produced. It is being increasingly used for detection and analysis of proteins from complex samples. The various components which are involved in the mass spectrometry experiments are shown here, starting from the sample inlet, the ionization source, the mass analyzer, detector and then data analysis and data processing.

Sample inlet: This is the first point of contact where the sample is introduced into the mass spectrometer either as liquid nano-droplets or within a solid matrix.

Ionization source: The ionization source is responsible for converting the analyte molecules into gas phase ions in vacuum. The ions generated by the ionization source are thenintegrated with the mass analyzers. The technology used is called soft ionization for its ability to ionize non-volatile biomolecules while ensuring minimum fragmentation and thus, easier interpretation. The commonly used ionization source include MALDI and ESI.

Mass analyzers: The mass analyzers resolve the ions produced by the ionization source on the basis of their mass to charge ratio. There are various types of mass analyzers available including time of flight, quadrupole, ion trap etc.

Detector: The ion detector determines the mass of ions that are resolved by the mass analyzer and generates data, which is then subjected to further analysis. The electron multiplier is the most commonly used detection technique.

The next section studies the function of each of these components in greater detail.

Ionization sources

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 ensure 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 alpha-cyano-4-hydroxycinnamic acid or sinapinic acid. 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.

In **ESI**, the sample is present in the liquid state 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.

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

Slide 22:
Continuing with the overview of MS methodology, after discussing liquid chromatography-based pre fractionation, in-gel digestion, different types of ionization sources, mass analyzers are now examined. A mass analyzer plays two very important functions. First of all it disperses all the ions based on their mass to charge ratio.

Secondly, it focuses all the mass-resolved ions at a single focal point. So therefore all the transmission of all ions which enter the mass spectrometer can be maximized

Slide 23:
Several different types of mass analyzers, capable of the above mentioned functions, are currently available. Some of the popular mass analyzer configurations are shown in this slide which include time of flight (TOF), ion trap, quardupole, ion cyclotron resonance, orbitrap and magnetic sector.

Slide 24:
What are the desirable features of mass analyzers? Their performance can be evaluated on the basis of various desirable characteristics.

- Mass range, which is the maximum allowable m/z ratio which is amenable to analysis. If you have higher value that will be useful for analysis of high mass compounds.
- Resolution or the ability to separate two neighbouring mass ions,
- Adaptability, the possibility of outfitting the mass analyzer with other ionization sources as well as other devices such as chromatography systems and multi-channel array detectors.

Slide 25:
- Efficiency, which is the transmission multiplied by the duty cycle which is defined by the fraction of ions of interest formed in a single ionization event.
- Mass accuracy,which is determined by how closethe measured mass is from the actual mass. It is expressed in ppm units.
- Linear dynamic range, the range over which the ion signal is linear with analyte signal.
- Speed, ie how many spectra can be acquired per unit time.

Slide 26:
Within the list of desirable features of a mass analyzer, high sensitivity is very important. Sensitivity is determined by the minimum concentration of compound that the instrument can detect with a particular signal to noise ratio. So the detection sensitivity is the smallest amount of analyte that can be detected at a certain defined confidence

level. Mass stability is another important feature: how reproducible is the measured mass?

Slide 27:
Another property previous mentioned is mass resolution, which is the ability of mass spectrometer to resolve different molecular species with similar but distinct masses. Mass resolution is the dimensionless ratio of m/z value of a peak divided by its width at half maximum intensity. It can range from 1000 to 100,000 in different mass spectrometers. If a resolution of 1000 is obtained, it means that the instrument can resolve 2 peptides that differ by 1 unit at a mass of 1000.

Slide 28:
Obviously high resolution is desirable because it can help to perform accurate mass measurement, it can resolve isotopically labelled species when the percentage incorporation of the label is to be determined. It can resolve an isotope cluster when the charge state of a high mass compound is to be determined. It can enhance the accuracy of quantitation, enabling the identity of mass selected precursors ions in MS-MS to be unambiguously established.

All of these factors need to be taken into account while selecting a mass analyzer for any given application

Slide 29:
This slide shows you full width, half maximum or FWHM. Mathematically mass resolution is the inverse of the resolving power R_f shown as $R=m/W_{1/2}$, where m is the average of mass and $W_{1/2}$ is difference in the accurate mass of the two neighbouring ions.

Slide 30:
Now let's talk about mass accuracy, which is a measure of how close a mass measurement is to its true theoretical or exact value. It is expressed in parts per million or ppm. Absolute volume is mass dependent. The equation for ppm is shown here which is theoretical mass – experimental mass divided by theoretical mass multiplied by 10^6. So mass accuracy affects the number of peptides in a database with similar masses and so lower the ppm values, fewer the possible matches in the database. Now currently there are many mass analyzers which can measure both in MS and MS-MS mode less than 1 ppm mass accuracy.

Slide 31:
The following animation discusses a few available mass analyzers.

Animation 2
It resolves the ions produced by the ionization source on the basis of their mass to charge ratio. Various characteristics such as resolving power, accuracy, mass range and speed determine the efficiency of these mass analyzers. Let us discuss few most commonly used mass analyzers for the proteomics applications.

Currently various types of mass analyzers are available including TOF, ion traps, quadrupole, ion cyclotron resonance, orbitrap and magnetic sector.

The TOF analyzer accelerates the charged ions generated by the ion source MALDI along the long tube known as the Flight Tube or TOF. Ions are accelerated at different velocities depending on their mass to charge ratios. Ions of lower masses are accelerated to high velocities and reach the detector first. The time of flight under such circumstances is inversely proportional to square root of molecular mass of the ion. The TOF analyzer has several applications in proteomics.

Moving on to the ion trap mass analyzer, this, makes use of a combination of electric and magnetic fields and captures the ion in a region of vacuum system or tube and traps it using the electric field and then measures the mass by selectively ejecting them to a detector.

The quadrupole mass analyzers use oscillating electric fields to selectively stabilize or destabilize the paths of ion passing through a radio frequency (RF) quadrupole field.

The quadrupole mass analyzer can be operated in either radio frequency or scanning mode. In the RF mode, ions of all m/z are allowed to pass through which are then detected by a detector. In the scanning mode the quadrupole analyzer selects ions of specific m/z value as set by the user. A range can also set in which case only those specific ions which satisfy the criteria will move towards the detector and the rest will be filtered out.

The ionization source and the mass analyzer can be combined in different ways to give various configurations for the mass spectrometer. So the most commonly used MS configurations are MALDI with TOF, ESI with ion trap, ESI with Q-TOF and MALDI with ion trap.

Slide 32:
After the mass analyzer, the next important component is detector. A detector provides information on the ion fluxes or abundance of the ions after their exit from the mass analyzer. It converts the beam of ions into an electrical signal that can be amplified, stored and displayed by the data system. There are two major categories of detectors;

the focal point detector and focal plane detector. The focal point detector detects an ion 1 m/z at a time and is used with the scanning mass analyzers. The focal plane detector monitors all ions constantly upon their arrival along the plane and are used with the m/z special dispersion mass analyzers.

Slide 33:

Moving on to Tandem mass spectrometry or tandem MS, this involves two mass spectrometry systems. The first MS performs the mass selection of a desired target ion from a stream of ions produced in the ionization source. As you can see in the slide the precursor ion 251 is selected. This mass selected ion undergoes fragmentation or a chemical reaction and then the second MS system performs mass analysis of the product ions that are formed in the intermediate step.

Slide 34:

There are various types of fragmentation methods currently available.A lst is provided above: ;collision induced disassociation (CID), infra-red multi photon disassociation (IRMPD), electron capture disassociation (ECD), electron transfer disassociation (ETD), electron impact (EI) which is used for small molecules, and chemical ionization (CI) also used for the small molecules. For MALDI especially, post-source decay (PSD), in-source decay (ISD) and CID are commonly used fragmentation methods. The most commonly used means of ion activation and disassociation in organic and bio-organic materials is CID.

Slide 35:

The above slide provides a comparison of MS versus MS/MS analysis. In MS/MS, a peptide is fragmented and masses of the fragment ions are recorded in a spectrum. The tandem MS or MS/MS uses 2 stages of mass analysis as mentioned earlier; the first stage wherein selection of an ion for its subsequent fragmentation occurs and the second stage during which this ion is fragmented by using different types of fragmentation methods such as CID or ECD. And then the sequence information is obtained by *de novo* analysis of the spectrum.

Slide 36:

For some of the commonly used tandem MS configurations, see the following animation.

Animation 3

A combination of various mass analyzers in tandem gives rise to tandem MS.

The triple **quadrupole** consists of two sets of parallel metallic rods interspersed by a collision cell. The first quadrupole scans the ions coming from the ionization source and allows only ions of a particular m/z ratio to pass through. These ions enter the collision cell, which is the second qudrupole, where they are fragmented by collision against an inert gas like argon. The smaller fragments then enter the third quadrupole which scans all the ions in the radio frequency mode to generate a spectrum based on the varying behaviour of ions in an oscillating electrical field.

Very commonly used tandem MS configurations are now discussed.

MALDI-TOF-TOF is another common tandem MS configuration in which the ions are first resolved on the basis of their time of flight in the first TOF analyzer. The selected ions enter the collision cell where they are further fragmented. The fragmented ions are accelerated and further resolved on the basis of their m/z values in the second flight tube, after which they are detected.

ESI-Q-TOF is a commonly used tandem MS configuration that first selects ions in the radio frequency mode. The selected peptide is then fragmented in the collision cell and the resulting ions are accelerated and resolved on the basis of their time of flight.

Slide 37:

In summary, this lecture we have discussed the fundamental concepts involved in the mass spectrometry. Different types of ionization sources, mass analyzers, detectors, and different types of terminology involved in evaluating the performance of these instruments have been covered. So what qualities should an ideal mass spectrometer have? It should possess wide mass range, high sensitivity, high resolution, high mass accuracy, true MS/MS and MRM capabilities, wide dynamic range, multiple charge separation capability, rapid or low polarity switching capability & complementary ionizations, and adaptability—the ability to combine, say, different types of sources with ESI and CI should be available. It should be possible to perform targeted analysis for a wide range of applications, such as post-translational modifications, label-free quantifications or MRM type of assays. In other words, the ideal mass spectrometer should be able to perform a range of applications and that's only possible if it has very high specifications. This lecture has hopefully conveyed fundamental concepts involved in mass spectrometry. The subsequent lectures will cover the topics discussed today, the ionization sources, mass analyzers and different types of mass spectrometry configurations and its applications in much greater detail.