LECTURE-20

Fundamentals of Mass Spectrometry

TRANSCRIPT

Welcome to the proteomics course. In today's lecture we will talk about fundamentals of mass spectrometry. So let's switch gears from previous module on gel-based proteomics to mass spectrometry and its applications.

So today we will talk about fundamentals of mass spectrometry. I will describe you the role of MS and some of the basic concepts involved in mass spectrometry. We will look at the individual components such as ionization source, mass analyzer as well other components. Then we will talk about tandem mass spectrometry.

So in the previous module we talked about gel-based proteomics. The gel-based proteomics technique resolved several hundred proteins. However the scale, at which we want to study the proteome requires much more analytical instrument capability. And mass spectrometry has the ability to provide that platform for comprehensive coverage of proteome. MS has become an important analytical tool in biology in general and in proteomics during the last decade. Now various applications have emerged using MS-based platform. It offers high throughput and sensitive analysis for many proteomics applications. Let's look at some of the basic concepts of MS.

So first of all what is mass spectrometry? It is an analytical technique to measure the molecular mass of individual compounds and atoms accurately by converting them into the charged ions. By definition this is a technique for the production of charged molecular species in vacuum and their separation by 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.

So what are the unique features of mass spectrometry?

Due to its unique ability to accurately measure molecular mass and provide fragment ions of 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 has also been demonstrated. It provides versatile platform to determine the structure of compounds and it is applicable to all the elements and samples whether it is volatile, non-volatile, polar, non-polar as well as solid, liquid or gases. Analysis of complex samples such as proteome is very much possible by MS.

What is the basic principle of mass spectrometry? So the first step is ionization. To convert analyte molecules or atoms into the gas phase ionic species. It removes or add electrons or protons. The second step is separation and mass analysis of molecular ions and charge fragments on the basis of m/z ratio. The final step is the detection and generation of mass spectrum. These are the main steps involved in the mass spectrometry operation.

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 as low as atomolar or femtomolar range. Ion sources generate positive, negative and neutral ions. The neutrals can not be focused or accelerated by the ion optics. So one can analyse 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.

So the mass spectrometer measures m/z. The MS data is presented as m/z ratio, i.e. mass of an ion divided by the number of charges it carries. So the total charge on ion is represented by q=ze, where e is the charge on electron. So how one can calculate the m/z of any peptide? As I mentioned here you can have multiple charge states; +1, +2, +3 or mutiple charge states 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 charge state it carries.

I will talk in much detail about how various types of ionization sources work that will be in the later part of the lecture. But in this context I would like to mention that there are multiple charge states present in the electrospray ionization (ESI). So in many charge states in proteins there many possible proton acceptors in equilibrium with the solution. So the multiple charge states are quiet useful because the y form ions which are in the mass range of mass analyzers such as TOF, quadrupoles, ion poles etc.

Now during the initial part we are trying to cover some of the basic terminology and basic concepts involved in the mass spectrometry. So let's talk about average and monoisotopic masses of amino acids. As shown in the table here there are amino acids, 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 protein? It is sum of masses for most abundant isotope of each element. The average mass of an element is the average of isotopic masses of each element weighted for the isotopic abundance. I hope you are able to distinguish the average and monoisotopic masses of amino acids.

By employing MS, different types of approaches people use in proteomics; top down and bottom up. So what is top down approach? Top down is an analytical approach of separating and analysing intact proteins without any previous proteolytic digestion. So let me give you a brief explanation of the procedure, steps involved in top down approach. The proteins are first introduced into MS directly because we are not doing the in-gel digestion or proteolytic cleavage. Proteins are broken into fragments inside the MS. The fragments are analysed by either MS or MS-MS and then 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.

So now let's discuss the bottom up approach. This is analytical approach of separating and analysing peptides following proteolytic digestion of a sample. So let me tell you about the various steps involved in doing the bottom up approach. So first of all proteins

are digested by using trypsin and other proteolytic enzymes. Peptides are separated by using liquid chromatography and introduced inside the mass spectrometer. The peptides are fragmented inside the MS and proteins can be identified by using data bases. The advantage of this approach is that one can obtain more sensitivity and higher proteome coverage. The disadvantage includes the information about the protein isoforms is lost and also it is unable to distinguish very close homologues. So both top down and bottom up approaches have their own advantages and disadvantages.

Now let's talk about different parts of mass spectrometer. So the major components include the sample inlet, ion source, mass analyzer, detector, signal processing components and data output. Let's look at each of these components in more detail. Sample inlet transfers the samples into the ionization source. The ionization source converts neutral sample molecules into gas phase ions. Mass analyzer separates and analyzes mass of the ionic species. Various types of mass analyzers available which we will discuss in more detail during the subsequent part of the lecture. One need to maintain the vacuum condition, very low pressure is maintained in the mass spectrometer. Detector measures and amplifies the ion current of mass resolved ions and then we need the electronics to control the operation of various units. The data system records processes, stores and helps to display the data output. Although there are 3 major components involved, ionization source, mass analyzer and detector, there are some accessory components which are important for doing the mass spectrometry based experiments.

So various components included sample introduction which can be couple with HPLC or CHIP-based technologies for doing the liquid chromatography based separations, sample ionization; there are various types of ionization sources currently available, sample transfer to the high vacuum region, so the ion mass to charge filtering can be performed by mass analyzers, ion detection by using detectors and then data acquisition and analysis by using data system. All these are integral part of MS components.

This slide shows you an overview of workflow involved in performing MS-based experiments. First of all you can pre-fractionate your sample by using liquid chromatography or one can also try different methods of doing the pre-fractionation and then applying that for doing the further MS analysis. After pre-fractionation, then in-gel digestion or proteolytic digestion can be performed by using various enzymes such as trypsin and then the sample can be injected into the ionization source whether it can be ESI or MALDI most commonly used for proteomics as well as other types of ionization sources. And then these ions can be further resolved into mass analyzers. There are various types of mass analyzers and then data can be analysed in MS or MS-MS mode.

This slide gives you an overview of in-gel digestion process. We will talk in more detail about each of these steps as they go along in the next lectures dealing more specifically about MS techniques. But today I am giving you sort of an overview of various types of concepts and various steps involved in performing MS experiments. In-gel digestion, regardless of you have done gel-based proteomics or you want to perform the gel-free proteomics you can resolve the proteins on the gels, simplify the proteome, excise either the spots or bands of your interest and then subject those to in-gel digestion or in-solution digestion so that the proteins can be cleaved into small peptides. These peptides can be further analysed by using ionization source and mass analyzers.

So what are different types of ionization sources involved in the MS analysis. So the success of MS experiment lies in efficiency of converting a neutral compound to a gas phase ionic species. Various types of options are currently available. You can select what type of ionization source you want for a specific application. So the choice of ionization is dictated largely by the nature of sample with which one wants to investigate. With the gas phase, electron ionization, chemical ionization and photo ionization are the commonly used ionization sources. With solution phase, electrospray, atmospheric pressure PI and atmospheric pressure CI commonly used ionization sources. With solid phase, matrix-assisted laser desorption ionization (MALDI), plasma desorption and fast atom bombardment are commonly used.

The traditional ionization sources used for the small molecule chemical application relied on the chemical or electrical ionization. But these processes are too energy rich to ionize intact large biomolecules and they lead to the unpredictable decomposition. So for proteomic application, there was a need for the soft ionization methods in mass spectrometry. These are non-selective fragmentation. The hard ionization is very difficult to predict. Therefore it led to the need for soft ionization methods in proteomics.

What are the properties of ionization sources? The main function of an ion source is to convert sample molecules or atoms into the gas phase ionic species.

In this animation I will show you two of the most commonly used soft ionization methods; MALDI and ESI. 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. MS 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 ratio and the relative abundance of the ions thus produced. It is being increasingly used for detection and analysis of proteins from the complex samples. the various components which are involved in the mass spectrometry experiments are shown here. Starting from the sample inlet, the ionization source mass analyzer, detector and then data analysis and data processing.

Let us first define these terms so that our understanding for each component becomes more clear when we talk about the advanced concepts.

Sample inlet: This is the first point of contact where the sample is introduced with in the MS either as liquid nano-droplets or the mixture of matrix.

Ionization source: The ionization source is responsible for converting the analyte molecules into gas phase ions in vacuum. Ionization source enables the ionization

which can be further integrated with the mass analyzers. The technology that enables is called soft ionization for its ability to ionize non-volatile biomolecules while ensuring minimum fragmentation and this easier interpretation. The commonly used ionization source include MALDI and ESI.

Mass analyzers: The mass analyzers resolves 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 analyzed. The electron multiplier is the most commonly used detection technique.

Now let us look at the function of each of these components in more detail.

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 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 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.

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 these techniques were awarded the Nobel Prize in 2002.

So we are talking about an MS experiment and I am trying to give you an overview of various steps involved. First of all we looked at liquid chromatography-based pre-fractionation, in-gel digestion, different types of ionization sources and now let's move on to the mass analyzers. A mass analyzer plays two most 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 ions which enter in mass spectrometer can be maximized their transmission.

Several different types of mass analyzers are currently available that use properties which we discussed. 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.

What are the desirable features of mass analyzers? The performance of mass analyzers can be evaluated on the basis of various desirable characteristics. Mass range; which is 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 ability to separate two neighboring mass ions, so how well the mass of interest can be separated from the other species that describes mass resolution. Adaptability; the possibility of out-fitting the mass analyzer with certain ionization techniques as well as

other devices such as chromatography systems and multi-channel array detectors also desirable feature of the mass analyzer.

Efficiency; 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; how far is the measured mass 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; how many spectra can be acquired per unit time. All these are desirable features of mass analysers.

Again in the desirable features one needs to look at the sensitivity. 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; how reproducible is the measured mass. So while you are comparing various types of mass analyzers one needs to look at all of these parameters and then only one can decide for what application they can select for which type of mass analyzers.

While we discussed various properties which are desirable for the mass analyzers, let's talk about mass resolution and mass accuracy in little more detail. So mass resolution 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 have a resolution of 1000 it means that the instrument can resolve 2 peptides that differ by 1 unit at a mass of 1000.

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

This slide shows you full width, half maximum or FWHM. So mathematically mass resolution is inverse of resolving power (rf) 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.

Now let's talk about mass accuracy. 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 the 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. So let me show you few available mass analyzers in the following animation.

Animation - 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.

Now let's discuss the mass analyzer, ion trap. An ion trap makes use of a combination of electric and magnetic fields and captures ion in a region of vacuum system or tube. Ion trap traps the ion using the electric field and 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 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. The range can also entered in which case only those specific ions which satisfy the criteria will move towards the detector and the rest can 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.

Now let's talk about tandem mass spectrometry or tandem MS. Tandem MS 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.

There are various types of fragmentation methods currently available. I have given a list of methods on this slide; 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. So most commonly means of ion activation and disassociation in organic and bio-organic materials is CID.

This slide gives you 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 I told earlier; the first stage which can do selection of an ion for its subsequent fragmentation and in the second stage this ion is fragmented by using different types of fragmentation methods such as CID or ECD. And then the sequence information can also be obtained via de novo analysis of the spectrum.

For some of the commonly used tandem MS configuration let me show you this animation. 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 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.

Now let's talk about some of the very commonly used tandem MS. 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. So in summary today we have about some of the fundamental concepts involved in the mass spectrometry. We talked about different types of ionization sources, mass analyzers, detectors, and different types of terminology involved in evaluating the performance of

these instruments. So what does an ideal MS should be like? 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, one can also use the modular where different types of sources can be combined such as ESI and CI. Targeted analysis can be performed for post-translational modifications, label-free quantifications or MRM type of assays. So from your ideal mass spectrometer you would like to have range of applications and that's only possible if it has very high specifications. So from today's lecture I hope you are able to understand some of the fundamental concepts involved in mass spectrometry, very briefly we touched upon ionization sources and mass analyzers and then we talked about tandem MS configurations. In the subsequent lectures we will talk in more detail about some of these ionization sources, mass analyzers and different types of mass spectrometry configurations and its applications. Thank you!