LECTURE-22

Liquid chromatography-Mass spectrometry (LC-MS/MS)

TRANSCRIPT

Welcome to the proteomics course. In this module we are discussing about mass spectrometry techniques. In the previous lectures we talked about basic concepts of mass spectrometry, then we discussed about MALDI-TOF-MS and today we will talk about liquid chromatography-mass spectrometry (LC-MS/MS). MS is based on production ions which are subsequently separated according to their m/z ratio. The resulting mass spectrum provides a plot of relative abundance of generated as a function of m/z. MS provides most versatile platform and comprehensive analytical technique for a proteomics scientist for a wide variety of applications.

So in today's lecture I will give you overview of mass spectrometry workflow. We will then talk about individual components in detail such as liquid chromatography, ionization sources, mass analyzers and tandem mass spectrometry.

As you can see in the slide, in the MS experiment workflow there are multiple steps involved. I have numbered those from 1 to 5. Protein samples are digested using trypsin and peptide mixtures are fractionated using liquid chromatography or LC. These fractions are subjected to electrical potential which results into a spray formation. In ESI or electrospray ionization it leads to desolvation and ionization of peptides. Mass to charge ratio is measured in mass analyzer and specific ions are randomly selected in the collision cell. Then based on the collision-disassociation the resulting fragment ions are further analyzed in another mass analyzer. The MS precursor ion intensity can be used for peptide quantification and MS/MS ion fragmentation can be used for sequence information and protein identification. So in this workflow all the steps are equally important, starting from the first step; the tryptic digestion, second; pre-fractionation strategies from liquid chromatography, then ionization sources, mass analyzer and then spectrum generation MS or MS/MS.

Now we discuss the first step; in-gel digestion is a multi-step procedure which includes spot selection from the gels if you have used 2-DE or if you have used gel-free techniques you can still run the samples on the gel, excise the bands. After that you would have to remove the stain, reduce, alkylate and perform the proteolytic cleavage and finally peptides can be extracted. This slide gives you an overview of various steps involved in in-gel digestion. I have discussed this in more detail in the previous lecture while discussing about MALDI-TOF-MS.

But just to refresh you the concept I will discuss this in the following animation of in-gel digestion. Animation - In-gel digestion - Electrophoretic separation of a protein mixture results in distinct protein bands. These proteins can be used for analytical purposes by carrying out in-gel digestion. The entire gel is fragmented into small pieces with each piece being dissolved in a suitable buffer.

The protein solution is treated with a reducing agent like dithiothreitol (DTT), which cleaves the disulphide bonds in the protein. This is followed by treatment with iodoacetamide (IAA), which alkylates the sulfhydryl groups and thereby prevents reformation of the disulphide bonds.

Following cleavage of the disulphide bonds, the protein is treated with a proteolytic enzyme, the most commonly used enzyme is trypsin. This cleaves the protein at specific residues and generates smaller peptide fragments. This tryptic digest is used for further purification and analysis.

After doing the in-gel digestion, let's move on to separation technology-liquid chromatography (LC). Chromatography in general is a physical separation method in which components for separation are selectively distributed in two immiscible phases, a mobile phase flowing through the stationary phase. Depending upon the mobile phase the technique is termed as either liquid chromatography or gas chromatography.

So what is liquid chromatography? The peptide mixtures can be fractionated in-line with instrument prior to the introduction into mass spectrometer. That is one of the

advantages for doing the proteomic applications. So LC can separate mixtures or components on the basis of difference in the affinity for stationary and mobile phase. LC is also useful for removing the undesirable impurities. It also increases sensitivity of the samples as well as the robustness of the MS measurement. LC along with further enrichment can help concentrating the diluted samples. It also helps in detection of low-level proteins and further it can separate peptide mixtures.

There are various types of chromatographic methods one can use for different applications. In proteomics most commonly used method for peptide fractionation is reverse phase liquid chromatography or RPLC. RPLC separates peptides based upon the hydrophobic binding of interaction between the peptides or proteins in the mobile phase and immobilized hydrophobic ligands in the stationary phase. By utilizing this hydrophobicity one can separate the peptides. However, if your proteome mixture or the peptides are complex then one can further use another type of chromatographic method such as strong cationic exchange as well as different type of multidimensional separation can be employed.

In this slide I have shown the configuration for RP-HPLC. As you can see the two components in the mobile phase, A and B linked with the HPLC pump. A buffer can provide between the range of 0.1% formic acid to 5% of acetonitrile and in B 0.1% formic acid to 80% of acetonitrile. So first of all you equilibrate the system in buffer A and then load the peptides and wash those. Then run the gradient of increasing mobile phase B. Now wash the reverse phase with buffer B and then re-equilibrate in buffer A. In this way the peptides can be separated and prior to MS analysis it can be desalted so that there is no interference of salt.

Reverse phase most commonly employed with the electrospray ionization because of its compatibility of reverse phase acidic aqueous and polar mobile with ESI. As I mentioned, in proteomics one can use ESI and LC in-line. So directly the samples can be pre-fractionated and further analyzed using MS. The in-line RP-HPLC is very useful because it can do desalting of peptides prior to ionization in ESI. There is no need for

doing separate off-line desalting and pre-fractionation. It can focus peptides from dilute samples into narrow chromatographic bands and it also enhances the sensitivity.

Let us now talk about another separation technique which also commonly employed in proteomics which is strong cation exchange (SCX). In SCX resin the silica based cation exchange used in the stationary phase. The sulfonic acid cation-based exchange ligands are used. These ligands are covently bound to the polymer coated silica. Now as I have shown in the slide there two important phenomena here; one is retention and another is elution. Retention is based on the electrostatic attraction between the negatively charged sulfonic acid and positively charged peptides. The elution can be performed by an exchange of peptides for cation of mobile phase additive, the ammonium ions. This is a reaction to the high concentration of cations.

HPLC can be microcapillary, nano-LC or different type of chip-based chromatography separation. For proteomics various types of microcapillary, nano-LC or different types of chip-based technologies are currently used. In the microcapillary HPLC, the flow rate, which is less than 1 µL per minute, is more sensitive than the standard RP-HPLC, which is around 50 µL per minute. The microcapillary-HPLC is required for the analysis of low femtomole amount of the peptides. One can prepare the microcapillary-HPLC by using fused silica capillaries and then pack that with the reverse phase packing material.

For pre-fractionation multidimensional separations are used. There are different types of principles which are involved for separating these peptides. One can use size exclusion chromatography (SEC), ion exchange chromatography (IEX), capillary electrophoresis (CE), Reversed-phase (RP) and affinity chromatography. SEC separates the peptides based on the molecular weight or size. IEX and CE separate based on the charge. RP separates based on the hydrophobicity. Affinity separates peptides based on the biological interactions.

Multidimensional approaches can be coupled with the MS. So one can use various types of liquid chromatography methods in tandem and do the multidimensional separation. Different types of approaches have been tried. For example SEC followed

by RP, but it has resulted in the poor resolution of peptides in SEC. So this not so widely used. Other approaches include RP followed by CE or SEC followed by CE. Due to the limited loading capacity and the low loading volume of the CE, it is not very popular. The affinity chromatography separation such as IMAC and Avidin followed by RP are commonly used and SCX followed by RP is most commonly used. So this slide gives you an overview of various types of methods. Liquid chromatography separation which can be employed prior to the injection into the ionization source.

As I mention SCX as well as RP chromatography together can be employed for various proteomics applications which have been demonstrated in the multidimensional protein identification technology or MudPIT. In this technique SCX separates the peptides based on charge and provides low resolution fractionation in the beginning and then the RP (C-18) column separates peptides based on hydrophobicity and provides high resolution gradient.

Let me describe some of the concepts involved in liquid chromatography in following animation. I will also discuss MudPIT and some of the chip-based approaches which are integrated for proteomic application with the liquid chromatography. So let's discuss these concepts in the following animation.

Animation - 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 the 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.

Multidimensional Protein Identification Technology (MudPIT) is a widely adopted strategy that carries out two consecutive protein separations based on different principles as shown in the figure below. Shown on either side is a protein with different properties. Drag & drop the protein that will interact with the SCX & RP regions of the column respectively.

MudPIT is a non-gel technique to separate and identify individual components of complex proteins and peptide mixtures of a proteome. It has been shown that MudPIT has potential to be used as a substitute of traditional two-dimensional gel electrophoresis since it separates peptides in 2D liquid chromatography. MudPit technique allows greater separation of peptides, which can directly be interfaced with

MS ionization source. It also avoids band broadening, which is one of the drawbacks of many chromatographic methods.

Agilent's HPLC-Chip technology

We have discussed different liquid chromatography systems. In a traditional nanoflow LC/MS system several fittings and connections are required, which is one of the major limitation. Agilent's HPLC-Chip technology is a microfluidic device which carrys out nanoflow high performance liquid chromatography and reduces limitations of several fittings and connections. Microfluidic devices contain circuits of tiny closed channels and wells, which are etched onto a glass or plastic microchip. Different forces such as pressure or electrokinetic can push small volume of fluids in a defined manner.

This technology integrates functional components onto a reusable, biocompatible chip, which integrates sample enrichment and analytical nanocolumns, nanospray emitter, fittings and connection capillaries on a reusable biocompatible polymer chip. It reduces the possibility of leaks and dead volumes, no clogging of spray needle and improves sensitivity, and reliability during analysis.

Another important component of this technology is the HPLC-Chip/MS interface. A chip is inserted into the interface, which mounts on a mass spectrometer. The design configuration ensures that the electrospray tip is in the optimal position for mass analysis when the chip is inserted.

Compared to the conventional nanospray techniques, this technology achieves maximum sensitivity with minimal sample sizes by integrating sample preparation, separation, and electrospray tip on a single chip.

So far we have talked about trypsonization or doing the peptide cleavage, then we talked about pre-fractionation strategies, liquid chromatography. Now these samples are ready to be injected into the ionization sources.

In the previous lecture when we talked about the basic concepts and overview of the procedures involved I gave you list of ionization techniques available. We also

discussed that soft ionization techniques are also required for the proteomic applications. So the soft ionization techniques such as ESI and MALDI were introduced in the late 80's and now they have overcome the problem of hard ionization and now these are widely adopted for the proteomic applications, so we will focus more on ESI and MALDI. Since we discussed MALDI in detail in the last lecture, I will focus on ESI in today's lecture.

ESI requires the sample of interest to be in solution and that's why I mentioned that we can use in-line separation along with liquid chromatography. To ionize the sample, high voltage is applied to high conductively coated needle. To results into the sample becoming charged either negative or positive. The positive ions are primarily used for the analysis of proteins. The distinguishing feature of ESI includes its ability to produce multiple charged ions. The number of charges that can be accepted by a particular molecule depends on its basicity and size.

Here you can get an overview of the process involved in the ESI. The small droplets of solution are generated by the tailored cone which contains the peptide analyte. Protons from the acidic solution provide the droplets with positive charge so that they can move from the needle to the negatively charged instrument.

In ESI the desolvation of ions occur at atmospheric pressure and the mass analyzer is maintained at a lower pressure so that the ions can be drawn in to the MS because of difference in the pressure. During movement the evaporation reduces the sizes of the droplets and then it spreads into the small charged droplets. When the ions enter the mass spectrometer, droplets are dried using the vacuum of the inert gas which results into gas phase ion acceleration through analyzer towards the detector.

You can see the process much clearly here in this slide. The top panel is showing the tailored cone generation, the center panel shows the production of multiple charged ions which is usually coupled to MS via real-time separation.

After discussing three important components now let's move on to the 4th part, the mass analyzer. There are different types of mass analyzers currently available. But for proteomics there are two configurations which are most commonly used, the quadrupole-time of flight or Q-TOF-based configurations and hybrid linear ion orbitrap instruments. The TOF configurations separate peptides in time as they reach the detector, so the time of flight is measured. Whereas the orbitrap mass analyzers measure the frequency of peptide ions which are oscillating in the ion trap. Different types of resolution and sensitivity can be obtained from each of these configurations.

In the previous lecture I gave you an overview of different types of mass analyzers. Each of those has their own unique properties in mass range, analysis speed, resolution, sensitivity, ion transmission and dynamic range. The time of flight analyzers use time flight, IonTrap, Orbitrap and ion cyclotron resonance. Ion cyclotron resonance separates ions based on their mass to charge resonance frequency. Whereas, quadrupole uses oscillating electrical field for selective stabilization of ions. This just gives you an overview of various types of mass analyzers and briefly we discussed about their principle.

Mass analyzers can be categorized broadly into scanning MS, ion-beam MS and trapping MS. Scanning MS is commonly used with TOF which is further coupled with MALDI ionization source. The ion-beam MS is commonly used with quadrupole, whereas, trapping MS with the iontraps, orbitrap and FT-ICR. All these can be coupled with ESI.

Let's discuss in detail about some of the important mass analyzers. Let's talk about TOF first, which is one of the simplest mass analyzers currently used in combination with MALDI. The TOF has emerged as one of the mainstream techniques for the analysis of the biomolecules and it is widely used for various applications.

In TOF ions are accelerated to high kinetic energy and due to their velocities they are separated in a flight tube. One can also use the reflectron mirror so that ions can turn

around into a reflector and it compensate for minor differences in kinetic energy and provide long separation.

Another commonly used mass analyzer is Quadrupole or Q. The Q instruments are one of the most widely used mass analyzers in proteomics. It consists of four matched parallel metal rods and mass separation is accomplished by the stable vibratory motion of ions in a high-frequency oscillating electric field that is created by applying direct current and radio frequency potentials to these electrodes. So as we talked quadrupole is a set of 4 parallel metallic rods where opposite pairs are electrically connected. There are different modes one can use for this analysis such as 1. Radiofrequency (RF) mode which allows ions of any m/z ratio to pass though, 2. Scanning mode where the ions of selected mass and charge can be allowed by the detector. Potential difference can be applied and the instrument can be used as a mass filter, 3. The neutral loss scan and precursor ion scanning mode used for the phosphorylation to distinguish the phosphorylated and nonphosphorylated peptides.

The triple quard arrangement of quadrupoles is widely used in proteomics. In triple quad, Q1 casts ionic streams. It directs ions of a selected m/z ratio into the second quadrupole Q2 which is a collision cell. As you can see in the slide the collision cell operates in the radio frequency mode. The fragmentation of intact peptide ions can be induced by colliding with inert gases and then selected ions are further moved into the Q3. Q3 scans the streams of ions fragments which emerge from the collision cell to generate a collision induced diassociation spectrum. The mass spectrum of fragments derived from one peptide after one analysis is complete. Then Q1 directs different intact peptide into the collision cell. So in this sequential manner it can process various peptides.

Now let's talk about another important mass analyzer, ion trap. The ion trap traps ions using electric fields and it measures by selectively ejecting them to a detector. It consisits of a chamber which is surrounded by a ring electrode and two end-cap electrodes, as you can see in the slide here. The voltage applied to the ring electrode

determines which ions remain inside the trap. So ions above a threshold of m/z ratio remain inside the trap and the others are ejected through a small hole. Theoretically ion trap can provide MS analysis and it can also provide a mass filter.

Another important mass analyzer is fourier transform ion cyclotron resonance or FT-ICR. Due to its high resolution and MS/MS capabilities application of FT-ICR MS in combination with ESI has been employed for large biomolecules and now it's also used in proteomics.

An FT-ICR MS can be considered as ion trap system where ions are trapped in the magnetic field. It uses cyclotron motion or cyclotron frequency to resolve the ions. Although it is operationally complex it provides highest resolution, mass accuracy and sensitivity. It also provides the capability of multiple tandem experiments and MS/MS of very large molecules is possible by using FT-ICR.

So we have discussed all the important components of LC-MS. Now one can apply these configurations in tandem. One can select different types of mass analyzers and use it based on their applications. Now we will look at some of the popular Hybrid-MS and MS/MS configurations.

MALDI TOF-TOF is one of the widely used tandem-MS configuration. In this the TOF-TOF or two time of flight tubes as well hybrid quadrupole-time of flight analyzers can be used. We have discussed the MALDI TOF-TOF system in detail in the previous lecture. So I'll move on to some other configurations which is Q-TOF.

The Q-TOF combines front part with quadrupole or it can be triple quard along with the TOF analyzers to measure the mass of ion. I will describe some of the important concepts involved in ionization, mass analyzers and tandem-MS in the following animation.

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 like Matrix Assisted Laser Desorption-Ionization and Electrospray Ionization,

which ensure that the non-volatile protein sample is ionized without completely fragmenting it. In MALDI, the analyte of interest is mixed with an aromatic matrix and bombarded with short pulses of laser. The laser energy is transferred to the analyte molecules which undergo rapid sublimation into gas phase ions. 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. These ions are then accelerated towards the mass analyzer depending upon their mass and charge.

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 analyzer 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.

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.

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 moves towards the detector and the rest are filtered out.

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 behavior of ions in an oscillating electrical field.

MALDI-TOF-TOF-MS-This 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 TOF 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 finally, there are so many mass spectrometers currently available commercially. Now depending on individual's application, one can select different type of configuration. Based on an excellent review from Yates et al. I provided the performance comparison of MS instruments in this slide. Here you can see the linear ion traps (LIT or LTQ) have resolution of 2000, mass accuracy of 100 ppm, sensitivity femtomole and the scan rate is very fast. TQ or triple quadrupole with resolution of 2000, mass accuracy 100 ppm, sensitivity in attomole and scan rate is moderate. The LTQ-orbitraps can provide high resolution of 100,000, mass accuracy 2ppm, sensitivity in femtomole and moderate scan rate. LTQ-FT-ICR can provide very high resolution of 500,000, mass accuracy less

than 2 ppm, sensitivity femtomole and slow scan rate. Q-TOF provides resolution of 10,000, mass accuracy 2-5 ppm, sensitivity attomole and scan rate is moderate to fast.

I hope in today's lecture you learnt various concepts together, how one can use MS epxeriments, we have discussed individual components of in-gel digestion, liquid chromatography, ionization source, mass analyzers and tandem mass spectrometry. And finally we compared various configurations commercially available so that we can get an idea what type of resolution, sensitivity, scanning speed, etc. they can provide. We will continue our discussion on MS and data analysis in the following lecture. Thank you!