HANDOUT

LECTURE-22

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS/MS)

Slide 1:

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

Slide 2:

So in today's lecture I will provide an 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.

Slide 3:

As you can see in the slide, in the MS experiment workflow there are multiple steps involved.

1. Protein samples are digested using trypsin.
2. Peptide mixtures are fractionated using liquid chromatography or LC.
3. These fractions are subjected to an electrical potential by a technique called electrospray ionization or ESI. ESI leads to desolvation and ionization of peptides.
4. Mass to charge ratio is measured in mass analyzer and specific ions are randomly fragmented in the collision cell. Then based on the collision-disassociation the resulting fragment ions are further analyzed in another mass analyzer.
5. 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; prefractionation strategies from liquid chromatography, then ionization sources, mass analyzer and then spectrum generation MS or MS/MS.

Slide 4:

Now we discuss the first step; in-gel digestion is a multi-step procedure which includes spot selection from the gels. After that you would have to remove the stain, reduce and alkylate the protein and then perform proteolytic cleavage and finally peptides can be extracted.

Slide 5:

This slide provides an overview of various steps involved in in-gel digestion. I have discussed this in greater detail in the previous lecture while discussing about MALDI-TOF-MS.

Slide 6:

Butt to refresh the concept, I will discuss this in the following animation of in-gel digestion.

Animation 1

1. 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.

Slide 7:

After the in-gel digestion step, let's move on to separation technique of -liquid chromatography (LC). Chromatography in general is a physical separation method in which components for separation are selectively distributed amongst 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.

Slide 8:

What does Liquid chromatography achieve?

- The peptide mixtures can be fractionated in-line with the instrument prior to the introduction into mass spectrometer. So LC can separate mixtures or components on the basis of difference in their affinity for the stationary and mobile phase.
- LC is also useful for removing undesirable impurities.
- It increases the sensitivity of sample detection. LC, in concert with MS, can help concentrate the diluted samples.
- It helps in detection of low-level proteins and further it can separate peptide mixtures.

Slide 9:

There are various types of chromatographic methods one can use for different applications. In proteomics, the most commonly used method for peptide fractionation is reverse phase liquid chromatography or RPLC. RPLC separates peptides based upon the hydrophobic binding or interaction between the peptides or proteins in the mobile phase and immobilized hydrophobic ligands in the stationary phase. 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 types of multidimensional separation methods.

Slide 10:

In this slide is shown the configuration for RP-HPLC. As you can see, the two components in the mobile phase, A and B are linked with the HPLC pump. The 'A' buffer consists of 0.1% formic acid to 5% of the hydrophobic solvent acetonitrile while the 'B' buffer consists of 0.1% formic acid and 80% of acetonitrile.

- First, equilibrate the system in the less hydrophobic buffer, buffer A and then load the peptides and wash those.
- Then run the gradient of increasing hydrophobicity with Buffer 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 to remove interfering salts.

Slide 11:

- Reverse phase is most commonly employed with the electrospray ionization- the reasons for this are twofold: ESI requires the analyte to be in the liquid phase, and moreover, In RP, samples are eluted in an organic solvent (ACN), which is compatible with ESI.
- In proteomics, one can use ESI and LC in-line. The samples can be directly pre-fractionated and further analyzed using MS.
- The in-line RP-HPLC is very useful because it can desalt the peptides prior to ionization by ESI.
- It can focus peptides from dilute samples into narrow chromatographic bands and it also enhances the sensitivity.

Slide 12:

Let us now talk about another separation technique which is also commonly employed in proteomics, strong cation exchange (SCX). In the SCX resin, a silica based cation exchange is used in the stationary phase. Sulfonic acid cation-based exchange ligands are covently bound to the polymer coated silica. There are 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 cations from the mobile phase additive, the ammonium ions. This is a reaction to the high concentration of cations.

Slide 13:

HPLC can be a microcapillary, nano-LC or a 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 amounts of the peptides. One can prepare the microcapillary-HPLC by using fused silica capillaries and then pack that with the reverse phase packing material.

Slide 14:

For pre-fractionation multidimensional separations are used. There are different types of principles 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 biological interactions.

Slide 15:

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 was followed by RP, but it has resulted in the poor resolution of peptides in SEC. So this was 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-based approaches 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 of liquid chromatography separation which can be employed prior to the injection into the ionization source.

Slide 16:

As I mentioned, 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 a high resolution gradient.

Slide 17:

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

Animation 2

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, the mobile phase flows into the column through the pump. The column consists of a stationary matrix that preferentially binds certain analytes. An Outlet from the column enters the flow cell where detection can occur.

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.

MudPIT

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. 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 the 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 limitations. Agilent's HPLC-Chip technology is a microfluidic device which carries 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 force 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.

Slide 18:

So far we have talked about trypsinization 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.

Slide 19:

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 are widely adopted for the proteomic applications. Since we discussed MALDI in detail in the last lecture, I will focus on ESI in today's lecture.

Slide 20:

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, which results in the sample becoming either positively or negatively charged either . 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.

Slide 21:

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.

Slide 22:

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.

Slide 23:

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

Slide 24:

After discussing three important components, now let's move on to the 4^{th} 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.

Slide 25:

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 flight time while the IonTrap, Orbitrap and ion cyclotron use resonance. Ion cyclotron resonance separates ions based on their mass to charge resonance frequency,whereas the quadrupole uses an oscillating electrical field for selective stabilization of ions.

Slide 26:

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 the 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.

Slide 27:

Let's discuss in detail 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.

Slide 28:

In TOF, ions are accelerated to possess a high kinetic energy and due to their velocities 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.

Slide 29:

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. Under defined DC and RF potentials ions of specific m/z value can pass through the quadrupole rods. The mass

spectrum is obtained by changing the DC and RF potentials while keeping their ratios constant.

Slide 30:

The 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, which is used to detect phosphorylated peptides.

Slide 31:

The triple quad arrangement of quadrupoles is widely used in proteomics. In this 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. 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 disassociation spectrum. The mass spectrum of fragments derived from a peptid is generated after one analysis is complete. Then Q1 directs another intact peptide into the collision cell. So in this sequential manner,multiple peptides are processed.

Slide 32:

Now let's talk about another important mass analyzer, ion trap.

Slide 33:

The ion trap traps ions using electric fields and selectively ejects them to a detector. It consists 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 produce MS analysis and it can also act as a mass filter.

Slide 34:

Another important mass analyzer is fourier transform ion cyclotron resonance or FT-ICR. Due to its high resolution and MS/MS capabilities, FT-ICR MS in combination with ESI can be used to study large biomolecules in proteomics applications.

Slide 35:

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.

Slide 36:

We have discussed all the important components of LC-MS. These configurations can be applied in tandem, wherein different mass analyzers available can be selected based on the application.

Now we will look at some of the popular Hybrid-MS and MS/MS configurations.

Slide 37:

MALDI TOF-TOF is one of the widely used tandem-MS configurations. 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 the other configuration which is Q-TOF.

Slide 38:

The Q-TOF combines the frontal portion, a triple quad with a TOF analyzer to measure the mass of ion.

Slide 39:

I will describe some of the important concepts involved in ionization, mass analyzers and tandem-MS in the following animation.

Tandem mass spectrometry

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.

Slide 40:

There are many mass spectrometers currently available commercially. Now depending on the individual application, one can select different types of configurations. Based on an excellent review from Yates et al. I provided the performance comparison of MS instruments in this slide.

- The linear ion traps (LIT or LTQ) have resolution of 2000, mass accuracy of 100 ppm, sensitivity in the femtomolar range with a a fast scan rate.
- TQ or triple quadrupole have a resolution of 2000, mass accuracy 100 ppm, sensitivity in attomolar range with a moderate and scan rate is moderate.
- The LTQ-orbitraps can provide a high resolution of 100,000, mass accuracy 2ppm, sensitivity in femtomole and a moderate scan rate.
- LTQ-FT-ICR can provide very high resolution of 500,000, mass accuracy less than 2 ppm, sensitivity in the fentomolar range and slow scan rate.
- Q-TOF provides resolution of 10,000, mass accuracy 2-5 ppm, sensitivity in the attomolar range and with a moderate to fast scan rate

Slide 41:

I hope in today's lecture helped integrate various concepts, of the MS workflow with the individual steps 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.