HANDOUT

LECTURE-21

MATRIX ASSISTED LASER DESORPTION/IONIZATION-TIME OF FLIGHT

Slide 1:

In this lecture, matrix assisted laser desorption/ionization-Time of flight or MALDI-TOF will be discussed. This technique provides a high-throughput platform for several applications including molecular weight determination, protein identification as well as post-translational modification studies.

Slide 2:

This lecture will cover the basics of MALDI-TOF, preparation of samples for MALDI-TOF or TOF/TOF analysis which will include in-gel digestion, zip-tip sample clean-up and matrix and sample plating and then MALDI instrumentation will be discussed.

Slide 3:

Basic principles of MALDI-TOF: MALDI is an efficient process for generating gas phase ions of peptides and proteins for mass spectrometric detection. It is one of the most widely used ionization techniques currently applicable in the proteomics area. This ionization method was independently developed by two scientists; Koichi Tanaka and Hillenkamp. Tanaka received a Nobel prize for his contribution to soft ionization techniques such as MALDI. So let's go through some of the basic concepts involved in MALDI-TOF. We can split that in two parts, one is MALDI which is an ionization source and another is TOF which is a mass analyzer.

Slide 4:

MALDI: The analyte or proteins of interest are mixed with the matrix which is usually an aromatic compound. There are various types of matrices available which we talk about in detail when we come to the sample preparation and matrix selection. But for your reference, you can use 2-5-dihydroxy benzoic acid, sinipinic acid etc. Once you have selected a matrix for the experiment then the analyte and matrix are dissolved in an organic solvent and placed on a metallic target. As you can see in the slide, the first left section shows you how to place the analyte and matrix together on the sample plate. Now, once you have placed the matrix and the analyte on the target plate you can place that in the vacuum chamber and apply high voltage. These crystals are targeted with short laser beams, which then sublimate to convert the solid phase analyte into gas phase ions. Once generated, these ions accelerate away from the target into the mass

analyzer through the TOF tube and reach the detector. This process is shown in the right hand side of the slide.

Slide 5:

There are various advantages and disadvantages of using MALDI as an ionization source.

Advantages:

- The sample preparation is very easy.
- MALDI demonstrates high tolerance to salts as compared to electrospray ionizers (ESI).
- It produces singly charged species, as most analytes accept a single photon. The single charge current results in some molecules having large mass-to-charge values. Therefore MALDI is typically integrated with the TOF mass analyzers which can provide the m/z range for the large ions as well.

Disadvantages:

- There is a strong dependence on good sample preparation for this analysis, so the ample preparation methods heavily influence the spectrum generated from these experiments.

Slide 6:

TOF mass analyzers

- These consist of an ion accelerator, focusing optics and a flight tube. As shown in the slide you have a source where the sample ionization occurs due to the laser beam bombardment. Then the ions move in the time-of-flight tube and reach towards the detector. Common additions include a reflector and an ion mirror, which can increase the path length. This time-of-flight tube measures the mass-to-charge ratio of ions based on the time it takes for the ions to travel across the analyzer and strike the detector. So the mass is exponentially proportional to the time-of-flight. So ions of the lower masses are accelerated to higher velocities. Time-of-flight tubes often out- perform scanning mass analyzers in sensitivity and scanning speed.

Slide 7:

The time-of-flight of a charged ion can be calculated by the equation shown in this slide. The flight time is directly proportional to the square root of mass of the ion. In this equation t represents time-of-flight, m is mass of the ion, q is charge on ion, V_0 is accelerating potential and L is the length of the flight tube.

Slide 8:

In time-of-flight tubes, the ions are accelerated to high kinetic energy and due to the different velocities, they are separated in a flight tube. As I mentioned earlier by adding a reflectron or a reflector, the ions can turn around in the reflector that can compensate for minor differences in the kinetic energy. If you take an example where you have 3 ions as shown in this slide as dark blue, light blue and red, you will expect the small red ion will show the first peak followed by the light blue ion and then the dark blue ion.

Slide 9:

After discussing some of the basic concepts of MALDI and TOF, an overview of entire MALDI-TOF experiment is shown by the following animation.Animation 1

Fundamentals of MALDI-TOF MS

Part 1 – Fundamentals of MALDI-TOF MS

The time-of-flight analyzer resolves ions produced by the ionization source on the basis of their mass-to-charge ratio. The TOF tube can be operated in the linear mode or the reflectron mode depending on the sample to be detected. In case of small molecules, this mode usually provides sufficient resolution. The generated ions are accelerated towards the detector with the lighter ions travelling through the TOF tube faster than the heavier ions. The flight time of the ions is correlated with the m/z ratio.

The TOF analyzer can also be operated in the reflectron mode, which is more commonly used for proteomics studies. A reflectron, which acts as an ion mirror, is incorporated at one end of the TOF tube. This helps in extending the path length and in turn the flight time of the ion without having to increase the actual size of the instrument. This helps to even out any kinetic energy differences between ions having the same mass and thereby improves the resolution.

The time of flight of a charged ion can be calculated by means of the equation shown. The flight time is directly proportional to the square root of mass of the ion.

Part 2 – Sample preparation and spotting

The protein sample must be prepared suitably before it can be analyzed by MS. The purified protein of interest is excised from the gel on which it has been electrophoresed and dissolved in a suitable buffer. Trypsin is then added to this in order to carry out digestion of the protein. This enzyme cleaves the protein at the C-terminal of the arginine & lysine residues, unless there is a proline present immediately after. The protein is thus digested into smaller fragments of manageable size.

Once the protein sample has been digested, all the salt, buffers and any detergents must be removed from this sample. This can be efficiently done with the help of filters (e.g. ZipTip). It offers several advantages such as quick purification, sample enrichment and ensuring there is no contamination. However, it can purify only a limited volume of the sample and also adsorbs some amount of the protein sample thereby leading to losses.

The purified protein sample is then mixed with an aromatic matrix compound like α-cyano-4-hydroxycinnamic acid, sinapinic acid etc, in the presence of an organic solvent. After through mixing, the solution containing the organic matrix with the embedded analyte is then spotted on to a metallic MALDI sample plate. MALDI thus gives you an opportunity to analyze large number of samples in a high-throughput fashion.

Part 3: Ionization and detection

The target plate containing the spotted matrix and analyte 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.

The gas phase ions generated are accelerated and travel through the flight tube at different rates. The lighter ions move rapidly and reach the detector first while the heavier ions migrate slowly. The ions are resolved and detected on the basis of their m/z ratios and a mass spectrum is generated. Parameters such as geometric design, power supply quality, calibration method, sample morphology, ion beam velocity etc. all affect the accuracy of mass detection.

Slide 10:

After looking at the animation, now let's talk about preparation of the sample. The first part isthe in-gel digestion of the protein samples. So the mass spectrometric identification of target protein greatly depends upon the efficacy of in-gel digestion that generates a mixture of peptides from the proteins through proteolytic digestion.

Slide 11:

This slide gives you an overview of the process. The spot of interest on the 2DE gel can be excised and subjected to in-gel digestion followed by mass spectrometric analysis. In-gel digestion is a multi-step procedure which includes spot selection, spot excision, removal of the stain, reduction, alkylation, proteolytic cleavage as well as peptides extraction. A good in-gel digestion is critical to successful generation of a spectrum through mass spectrometry. While the above overview begins with 2-DE, but the same subsequent methodology can be applied following gel-free proteomic techniques. If you want to analyze your sample obtained from gel-free proteomics approaches, it is often a good idea to separate that complex protein mixture on the SDS-PAGE gel, excise the bands and then extract the proteins from that, perform the in-gel digestion so that you can simplify the proteome and increase the overall proteome coverage.

Slide 12:

This slide provides various recipes for performing in-gel digestion. The first step is destaining of the spots or band, if you have stained the gels with coomassie brilliant blue or some other non-MS-compatible stains. So the stain removal is essential prior to MS analysis. The excised pieces should be washed with bicarbonate buffer and acetonitrile for the removal of the stains. Acetonitrile reduces the hydrophobic interactions between the protein and the stain while the ionic solution decreases the ionic interactions between negatively charged coomassie brilliant blue dye and the positively charged proteins. The destaining step is followed by the dehydration step done by the addition of acetonitrile. After the incubation is done, then you are ready for the reduction step. After the stain removal, the next steps include the reduction as well as alkylation of protein residues so that we can denature the protein into its primary structure.

Slide 13:

Following reduction, the reformation of disulphide bonds may occur. To prevent that, iodoacetamide, an alkylating agent, is used. Next, dehydration using the recipe shown in the slide is performed by adding acetonitrile and then you are ready to perform digestion which is usually done by adding trysin. Prior to the MS identification, proteins are digested to generate peptides. There are various enzymes to perform this step but trypsin is most widely used proteolytic enzyme used. It breaks the peptide bonds at the carboxy terminals of basic amino acids such as argenine and lysine. Once the digestion is done over-night, then one needs to perform peptide extraction from the digested proteins. The peptides generated through proteolytic cleavage can be extracted by

using a solution including formic acid or triflouroacetic acid (TFA) in 50% acetonitrile solution.

Slide 14:

Coming back to the importance of reduction and alkylation of the proteins, we mentioned that we need to add DTT and IAA in various steps during the in-gel digestion. DTT is important to break the disulphide bonds while IAA adds the iodoacetamide group to sulphydyrl group and prevents disulphide bond formation.

Slide 15:

You want to generate the peptides with the molecular weight with in the mass range of mass spectrometer. It is always desirable to simplify the process for even superior analytical instruments such a mass spectrometer so that you can increase the efficiency of the process. The enzymatic digestion is performed with various enzymes, but typically with trypsin which cleaves at the c-terminal of lysine or arginine residues but an exception occurs if the lysine or argenine is followed by a proline residue. In such cases, one can use a modified trypsin which is a serine indopeptidase. However it cleaves the proline-lysine or proline-arginine bonds at a much slower rate.

Slide 16:

So the in-gel digestion of the proteins isolated by the gel electrophoresis remains a core area in mass spectrometry or in any proteomics applications. So in the following video a broad coverage e of in-gel digestion is provided. However the recipe is very flexible and it varies from lab to lab to meet the specific requirements of particular proteomic experiments. The in-gel digestion procedure is compatible with the down-stream MS analysis whether you want to continue with MALDI-TOF or LS-MS-based proteomic analysis.

Video 1
ZipTip

- In-gel digested protein samples are processed further using ZipTip pipette tips containing C18 or C4 media for enrichment of peptides prior to MS analysis.
- The ZipTip pipette tip is a 10 µL pipette tip with a bed of chromatography media fixed at its end. It is used for concentrating and purifying peptides as well as removing salts, detergents and interfering agents.
- Attach the ZipTip pipette tip on the top of a suitable micropipette.

- Condition the ZipTip with 10 µL of acetonitrile (ACN). Perform the step three times. Wash the tip thrice with 0.1% trifluoroacetic acid (TFA).

Slide 17:

Once you have done the in-gel digestion, you can use a ZipTip digest for the MS analysis. But often it is recommended that you add one more step which is sample clean up. You do not want your columns or instruments to get clogged due to salts or other interfering residues present in the mixture. So it is recommended that one should use the clean-up step in between. So the in-gel digested protein can be cleaned up by processing further using ZipTip pipette tips which contain C_{18} or C_4 media for the enrichment of peptides. Salts and interfering agents are washed and finally the samples can be eluted in a very small volume of solvent.

Slide 18:

The ZipTip is a device for removal of salts as well as other interfering components from the protein sample. This step should be performed prior to injecting the sample for the mass spectrometric analysis. The ZipTips can be incorporated into high-throughput devices or multi-channel pipettes for the high-throughput applications. Let me show you this video for the sample clean-up by using ZipTips.

Video 2
Sample clean-up

- In-gel digested protein samples are processed further using ZipTip pipette tips containing C18 or C4 media for enrichment of peptides prior to MS analysis.
- The ZipTip pipette tip is a 10 µL pipette tip with a bed of chromatography media fixed at its end. It is used for concentrating and purifying peptides as well as removing salts, detergents and interfering agents.
- Attach the ZipTip pipette tip on the top of a suitable micropipette.
- Condition the ZipTip with 10 µL of acetonitrile (ACN). Perform the step for three times. Wash the tip thrice with 0.1% trifluoroacetic acid (TFA).
- Load the sample onto the ZipTip by pipetting 5-10 µL samples up and down 10-15 times and discard the liquid.
- Samples are passed through activated ZipTips where they are captured in particular bed of chromatography media.

- Wash the C_{18}/C_4 tip thrice with 10 µL of 0.1% TFA to remove salts.
- Salts and interfering agents, detergents are washed and finally samples are eluted in a very small volume of solvent.
- Elute the sample from the ZipTip with 10 µL 50-70% ACN in 0.1% TFA.
- Keep the processed samples in cryo box and store at -20^{o}C.
- In-gel digested samples processed using ZipTips will be subjected to MS analysis.

Slide 19:

Next, the various steps involved before you can start the MALDI experiment are shown. You need to select and prepare the matrix. You have already done the sample purification. Now sample needs to be deposited on the MALDI plate. Either you can mix it with matrix or you can do this separately. There are combinations one can try and then once both sample and matrix are deposited on the MALDI target plate then you are ready for drying and the plate can be used in MALDI-TOF instrument for further analysis.

Slide 20:

Matrix selection: The important step in MALDI-TOF analysis is the selection of an appropriate matrix for the sample. The matrix selection mostly depends on the molecular weight of the target to be analyzed and often the type of application which you intend to do by using this type of instrument. These matrices are low molecular weight organic compounds with low vapour pressure and a volatile nature. Most of the matrices are acidic in nature so it can easily donate aproton and ionize the analyte for the analysis. However there are few basic matrices which are available. In the slide I am giving you an overview of few matrices and some of their properties. One choice is a-cyano-4-hydroycinnamic acid (a-cyano) which can be used when you have peptides less than 5000 Da or lipids and nucleic acidsOne can also use sinapinic acid if peptides and proteins are larger than 5000 Da and it can also be sometimes used for the lipids.

Slide 21:

Then you have options such as 2-5-dihydroxybenzoic acid (DHB). Small molecules and peptides which are not ionized by other molecules can be analyzed by this matrix. Trihydroxyacetophenone (THAP) is used for small nucleotides and also used for phosphorylation and specialized applications. Then we have picolinic acid which is generally used for nucleic acids.These matrices absorb energy from the laser source

and converts both matrix and analyte into the gaseous phase. The matrix can also ionize the analyte molecule by transfer of energy which comes from the laser bombardment.

Slide 22:

Once you have selected the matrix, it can be prepared by mixing it in a suitable solvent and vortexing it for few minutes so that it can dissolve properly. Now you are ready with both analyte as well as the matrix which we have selected for your application. There are many ways of deposition of sample and matrix onto the MALDI plate. Mostly sample and matrix are mixed in an eppendorf tube and then mixture is directly deposited by using a micropipette onto the MALDI plate. But one can also try various combinations. In one approach the sample is directly deposited on to the MALDI plate followed by the matrices deposited above it and then it is properly mixed before drying process can happen. Another way of doing it is to apply that with the sandwich-based method, in which a small amount of matrix is deposited on the plate and then you add the protein sample and again the matrix is spotted on top of it so that you have enough matrix below and above of the analyte. So one can try different combinations of deposition of the matrix and the analyte. After deposition, you are ready to dry the target plate.

Slide 23:

After spotting is done and the MALDI plate is dried for 30 min, then the instrument can be turned on and MS analysis can be performed. Now there are various types of configurations of these instruments available as well as there are various commercial software which help to operate the hardware. It is not possible to go into individual details but the following are generic steps of MALDI-TOF instrumentation.

Video 3
MALDI is performed in two steps. In the first step, the compound for the analysis should be dissolved in a solvent containing small organic molecules, known as matrix. This mixture is dried before analysis and liquid solvent used in the preparation of the solution is removed, which results into analyte-doped matrix crystals deposition.

So in this video, by depicting the matrix preparation as well as instrumentation I'll try to give you the overview of MALDI-TOF instrumentation.

- Spot the mixture on the MALDI plate. The uniformity of deposition of these mixtures on the MALDI plate ensures the good spectra quality later on.

- Once the deposition of mixture on the MALDI plate is complete, the samples are allowed to dry for 30 min.
- After that the instrument is switched on and the MS analysis is performed. Click on the software and open the acquisition window and then click on “open door”.
- Insert MALDI target plate face-up with the cut-off corner to the front.
- Close the door using the software. The door of the insertion chamber is now closed.
- You can view the plate on the screen and then select the spot which you want to analyze. So click on the yellow target on the acquisition window and select “go to the location”.
- You can now do the laser bombarding and peptide spectrum is generated. Shown here is one standard protein, bovine serum albumin. So you have to look at various locations where you can get best spectra from that spot and then you can freeze it.
- Same process can be performed for different spots and different regions. Shown here is a spectra for Pepmix.

Video 4

In-gel digestion of proteins for MS analysis

- Often one- or two-dimensional gel electrophoresis is performed for separation of complex mixtures of proteins prior to mass spectrometric analysis. In-gel proteolytic digestion of separated proteins is done to cleave the protein of interest present within the polyacrylamide matrix.
- The gel-based techniques increase the dynamic range of analysis since they involve sequential separation of proteins based on molecular weight (lower to higher molecular weight).
- Mass spectrometric identification of the target protein greatly depends on the efficacy of the in-gel digestion process, that generates mixture of peptides from the target protein through proteolytic digestion. In-gel digestion is a multi-step

procedure, which include spot selection, spot excision, stain removal, reduction, alkylation, proteolytic cleavage and finally extraction of the peptides.

- Rinse the entire gel with water for few hours with intermittent changing of water.
- Keep a glass plate inside a laminar hood and clean the surface carefully.
- Excise protein spots with a clean sterile scalpel and place gel slice into a 1.5 mL Eppendorf tube.
- Place the excised spot on a clean glass plate.
- Cut the slices into cubes (1 x 1 mm) while avoiding too small pieces as they can clog pipette tips.
- Keep the small gel pieces in a sterile microcentrifuge tube.
- Add 50-100µL of stain removal solution (for large gel slices, add enough liquid to cover it completely).
- Rotate on a shaker for 30 minutes at room temperature for removal of the stain from the gel pieces. Change the solution after every 10 mins.
- Remove the solution. The CBB-stained gel pieces become colourless.
- Add 50-100µL dehydration solution and mix at room temperature, until gel pieces become white and stick together. Change the solution after every 10 mins.
- Spin gel pieces down at ~1000g for 30 sec and remove all liquid.
- Add 30–50 µL of the Reduction solution to completely cover gel pieces.
- Incubate 30 minutes at 56^{o}C.
- Treatment of the protein residues with DTT breaks the disulfide bonds.
- Chill down the tubes to room temperature; add 50µL of dehydration solution, mix properly and incubate for 10 min and remove all liquid.
- Add 30–50 µL (more for a larger gel slices) of the alkylation solution and incubate for 20 minutes at room temperature in the dark,
- IAA prevents the reformation of disulfide bonds. It an alkylating agent. It adds acetoamide group to the sulfhydral group and prevents the disulfide bond formation.
- Add 50µL of dehydration solution, mix properly and incubate for 10 minutes and remove all liquid.

- Air-dry the gel pieces.
- Add 25 µL Trypsin solution [~400ng] to the dry gel pieces and keep on ice for 30 min for absorption of enzyme by the gel pieces.
- Add 25µL ammonium bicarbonate buffer [In which Trypsin is prepared] and incubate at 37^{o}C for overnight (12-16 hours).
- Prior to MS identification, proteins are digested to generate peptides. Several proteolytic enzymes are available. Chymotrypsin, trypsin, pepsin are some of the enzymes used for proteolysis. CNBr is one of the chemical agents that cleaves peptide bond at Met residue. Trypsin is most widely used proteolytic enzyme used for protein digestion prior to MS analysis. It breaks the peptide bond at carboxyl terminal of basic amino acids such as arginine and lysine.
- The next morning, stop the reaction by keeping the reaction mixtures in ice.
- After overnight incubation, peptides generated through proteolytic digestion are extracted using extraction buffer containing 0.1% FA/TFA in 50% ACN solution.
- Extracted samples are stored in aliquots.
- Efficient extraction process is essential to ensure the release of peptides from gel-matrix to solution, which is further subjected to mass spectrometric analysis.

Slide 24:

Now, it should be clear how to perform MALDI-TOF experiment. One more mass analyzer can be added to provide a configuration of MALDI-TOF-TOF. MALDI can be coupled to the tandem- time of flight in combination with another time of flight or with hybrid quadrupole-time of flight analyzer which are separated by collision cells. For proteomic applications, it is recommended to use TOF-TOF or Q-TOF. The peptide ions are accelerated through the first time of flight tube as you can see in the slide and then they are disassociated by introducing an inert gas into the collision cell. This process allows collision induced disassociation spectra from MALDI produced from the precursor ions. These hybrid configurations are more sensitive than triple-quad and single TOF. So the combination of TOF-TOF allows the protein identification through peptide mass fingerprinting and high-throughput analysis of proteins or proteome is possible with hybrid-TOF analyzers.

Slide 25:

So in summary, this lecture talked about basics of MALDI-TOF. We have also discussed the sample preparation, various steps involved including in-gel digestion, matrix selection and ZipTipping. After that we talked briefly about MALDI-TOF instrumentation and then we talked about various types of configurations which can be used to increase the overall sensitivity and various applications for proteomics. We will continue our discussion on MS in the next lecture where we talk about liquid chromatography-based methods.