LECTURE-21

Matrix assisted laser desorption/ionization-Time of flight

TRANSCRIPT

Welcome to the proteomics course. Today we will talk about matrix assisted laser desorption/ionization-Time of flight or MALDI-TOF. In the previous lecture we talked about basics of mass spectrometry, various combinations of mass analyzers and ionization sources. Now it's time for us to combine those and start discussing these in more detail. So today let's focus on MALDI-TOF, which is one of the widely used technique in proteomics. This provides a high-throughput platform for several applications including molecular weight determination, protein identification as well as post-translational modification studies.

So in today's lecture we will talk about basics of MALDI-TOF, preparation of sample 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 we will talk about MALDI instrumentation.

Let's start with basics of MALDI-TOF. MALDI is an efficient process for generating gas phase ions of peptides and proteins for mass spectrometric detection. MALDI is one of the most widely used ionization technique 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 technique 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 ionization source and another is TOF which is a mass analyzer.

Let's first talk about MALDI. So analyte or proteins of interest are mixed with 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 analyte and matrix can be dissolved in an organic solvent after that it can be 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 put that in the vacuum chamber and apply high voltage. These crystals are targeted with short laser beams, as you can see in the slide, then rapid sublimation can convert analyte into gas phase ions. These ions once generated, they can accelerate away from the target to the mass analyzer through TOF and they can reach the detector. This process is shown in the right hand side of the slide.

There are various advantages and disadvantages of using MALDI as an ionization source. The sample preparation is very easy. MALDI provides high tolerance to salts as compared to electrospray ionizers (ESI). MALDI produces single charged species. Most analytes can accept the single photon. It produces mainly single charged ions since most ions can accept single protons. 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. These are various merits of using MALDI. Obviously, it has to be collected with the TOF. There are various demerits of using this system. There is strong dependence on good sample preparation for this analysis. So sample preparation methods heavily influence the spectrum generated from these experiments.

The TOF mass analyzers consist of ion accelerator and 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. Often we can add the reflector, 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 fly in 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.

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.

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.

After discussing some of the basic concepts of MALDI and TOF, let me give you and overview of entire MALDI-TOF experiment by showing you the following animation.

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.

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 its 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 limited volume of the sample and also adsorbs some amount of the protein sample thereby leading to losses.

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

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.

After looking at the animation, now let's talk about preparation of the sample. We will discuss this in more detail. The first part will be the 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.

This slide gives you an overview. In the last module we discussed about 2-DE. It shows that if you have a spot of interest you can excise the spot from the 2-DE gel and subject that spot 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 going to ensure the success of the spectrum generated from the mass spectrometry. Although this overview shows the process to start with the 2-DE but the same can be applied for gel-free proteomic techniques. If you want to analysis your sample even 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 so that you can increase the overall proteome coverage. So similar protocol can be modified and can be used for various type of applications in the proteomics.

This slide gives you various recipes for performing in-gel digestion. The first step is destaining of the spots or band because you have stained the gels with coomassie brilliant blue or some other stains. That's why you would have to remove these 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. You can see the recipe in the slide and we will talk more about performing the experiments when I show you the animation on how to perform these steps. Acetonitrile reduces the hydrophobic interacts 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. Once the destaining step is done we need to perform the dehydration step which can be 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.

Continuing on the same theme of in-gel digestion and various steps required in performing such experiments let's now look at the next step which is alkylation. In alkylation you need to add iodoacetamide. The reformation of disulphide bonds may occur. To prevent that iodoacetamide, an alkylating agent, is used. Then you would have to do dehydration using the recipe shown in the slide. You can add 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 for the protein digestion. It breaks the peptide bonds at the carboxy terminals of basic amino acids such as Argenine and Lysine. Once the digestion is done which is usually the over-night step then one needs to do the peptides extraction of the digested proteins. So the peptides generated through the proteolytic cleavage can be extracted by using recipe including formic acid or triflouroacetic acid (TFA) in 50% acetonitrile solution.

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 for the treatment of proteins to break the disulphide bonds which we have talked in the sample preparation when we discussed earlier. So IAA adds the iodoacetamide group to sulphydryl group and prevents disulphide bond formation. So these steps are very important in in-gel digestion process.

Coming to the specificity of trypsin. First of all I'll discuss why we need to do the proteolytic digestion. You want to generate the peptides with the molecular weight with

in the mass range of mass spectrometer. So you always want to simplify the process for even superior analytical instrument 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 exception can occur with proline. If proline is in then that breakage will not happen. Then one can use a modified trypsin which is serine indopeptidase. However it cleaves at proline-lysine or proline-arginine bonds at much slower rate. You can see the cleavage process and the specificity in the slide where it shows that if you have lysine or arginine residues it can break the bonds. But when there is a proline resudue then it can not cleave.

So the in-gel digestion of the proteins isolated by the gel electrophoresis remains a core area in mass spectrometry or in any of the proteomics applications. So in the following video which we are going to watch is going to provide broad knowledge on in-gel digestion. 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: ZipTip

- In-gel digested protein samples can be further processed using ZipTip pipette tips containing C_{18} or C_4 media for enrichment of peptides prior to MS analysis.
- 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).

After watching this video you are very clear about the various steps are performed in these experiments. Once you have done the in-gel digestion you can use this 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.

So the ZipTip is a very device for removal of salts as well as other interfering components from the protein sample and perform this before 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: Sample clean-up

- 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.

Now you know how to perform the cleaning step by using ZipTips. Now you have the sample ready and you have selected the matrix. So now let me show you the various steps involved before you can start the MALDI experiment. 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.

Let's first talk about matrix selection. The important step in MALDI-TOF analysis is the selection of 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 volatile nature. Most of the matrices are acidic in nature so it can easily excite the photon 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. But there are many more properties which are not mentioned here. But just to give you certain main features of few matrices commonly used for various applications. One is a-cyano-4-hydroycinnamic acid (a-cyano) when you have peptides less than 5000 Da or lipids and nucleic acids one can use this matrix. One can also use sinapinic acid if peptides and proteins have more than 5000 Da and it can also be sometimes used for the lipids.

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 acid. So these are few representative matrices. As you can see there are many options available for selecting the matrix depending upon the molecular weight and the type of applications. Regardless of this, these matrices absorb energy from the laser source and converts both matrix and analyte into the gaseous phase. Matrix can also analyze analyte molecule for providing energy which comes from the laser bombardment.

Once you have selected the matrix, matrix 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 which you want to analyze as well as the matrix which we have selected for your application. Now one needs to think how to deposit sample on the MALDI target plate. So 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. Other 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 placing the matrix and the analyte. And then once you have placed all of this sample of interest on MALDI plate then you are ready to dry the target plate.

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's not possible to go into individual detail but I am going to show you the generic steps in the following video of MALDI-TOF instrumentation.

Video - MALDI is performed in two steps. In 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 deposit.

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, the samples are allowed to dry for 30 min.
- After that the instrument is switched on and the MS analysis is performed. While steps are happening you need to ensure that the instrument is on.
- 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. We are showing here 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. Now we have shown here a spectra for Pepmix.

Video - In-gel digestion of proteins for MS analysis

- Often one- or two-dimensional gel electrophoresis is applied for separation of complex mixtures of proteins prior to mass spectrometric analysis. In-gel proteolytic digestion of separated proteins is performed 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 micro centrifuge tube.
- Add 50-100µL of stain removal solution (for large gel slices take 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. CBB stained gel pieces become colourless.
- Add 50-100µL dehydration solution and rotate 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.
- 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.

So now you are clear with how to perform MALDI-TOF experiment. Now let's add one more mass analyzer so now we have a configuration of MALDI-TOF-TOF. So MALDI can be couple 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.

In summary today we talked about basics of MALDI-TOF. We have also discussed the sample preparation, various steps involved including in-gel digestion, matrix selection, 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 lecture where we talk about liquid chromatography-based methods. Thank you!