Bioengineering: An Interface with Biology and Medicine

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Lecture – 36
Techniques to Study Protein & Proteome-III

Welcome to MOOC-NPTEL course on Bioengineering, an Interface with Biology and Medicine. In the last week, we have discussed about some of the basic properties of amino acids and proteins. Then we discussed about few tools and techniques which could be utilized to study proteins and at a much complex level of proteins at the proteome level. We talked about technology like SDS page, 2D electrophoresis.

Then we moved on to advanced form difference gel electrophoresis and then we talked about protein purification strategies such as chromatography based methods, gel filtration chromatography, ion exchange chromatography and affinity chromatography. From gel based protein work as well as from the chromatography based work, you are utilizing some property to separate the proteins or to purify the proteins.

But you cannot confirm that this is the right protein that you have identified until unless you do a mass spectrometry based analysis. So mass spectrometry has become very prominent and started paying a very strong role in the entire field of protein biochemistry and proteomics. And today, we are mainly going to talk about mass spectrometry, the basics of it as well as mass spectrometry based proteomics.

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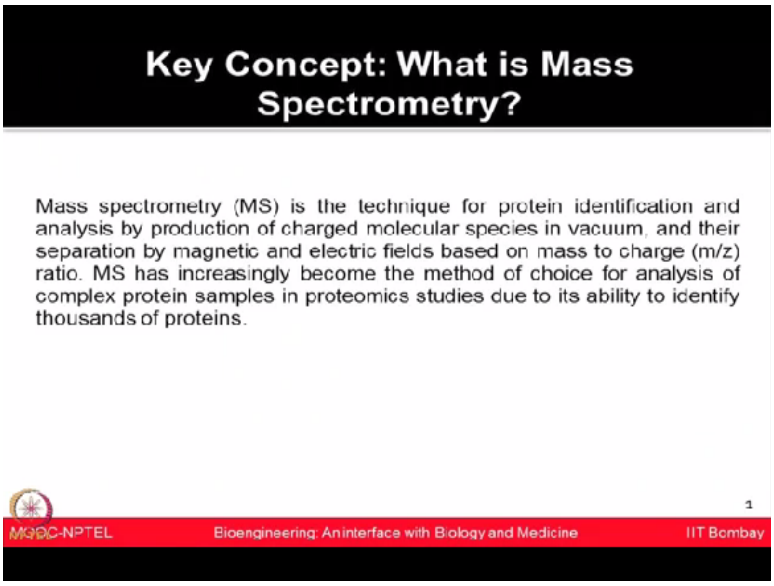

So what is mass spectrometry? It is a technique for protein identification and analysis by producing the charge molecular species in vacuum and then their separation by magnetic and electric field based on the mass to charge ratio. This technology has become increasingly in use in the proteomics field and now has become a method of chaise for analyzing the complex protein samples for proteomics studies.

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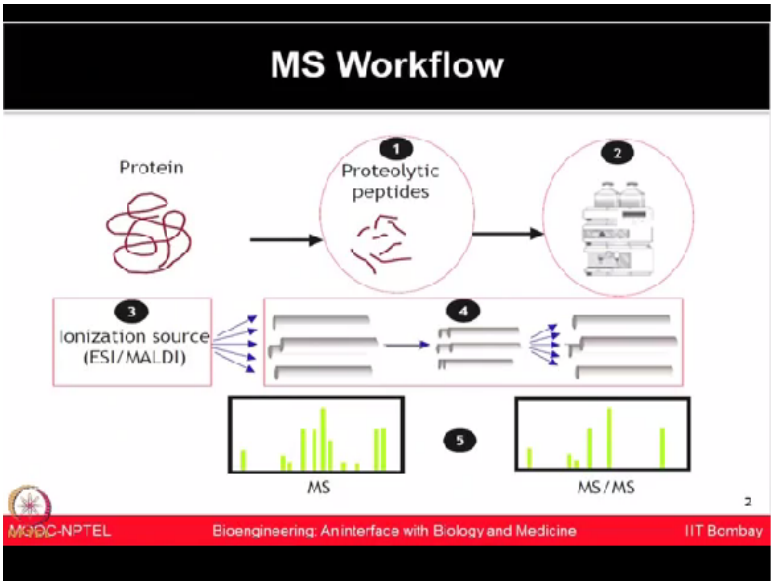

In the mass spectrometry based proteomics workflow, let us say first thing we are starting that we have a protein of interest which we want to analyze and that protein either you have purified it or you have excised a spot from the 2D gel or SDS page band and now you want to know what is the protein identification. So in this workflow, initially let us say you have the intact protein

which you want to digest using enzymes like trypsin, chymotrypsin, lysine, etc.

Then you are generating the peptides, the proteolytic peptides. Now these peptides can be further separated based on the liquid chromatography based methods and then now you want to, you have the proteins in solution form, which you want to ionize and you want to now move inside the mass spectrometers.

So first thing is, you want to use ionization source to ionize these molecules. Then you want to separate them in the mass to charge ratio using different type of properties of mass analyzers. And then in the process, either you can use only 1 mass analyzer or you can use 2 mass analyzers. Accordingly, you can do either MS or MS/MS-based analysis. So let us start with the first component of the mass spectrometer which is ionization source.

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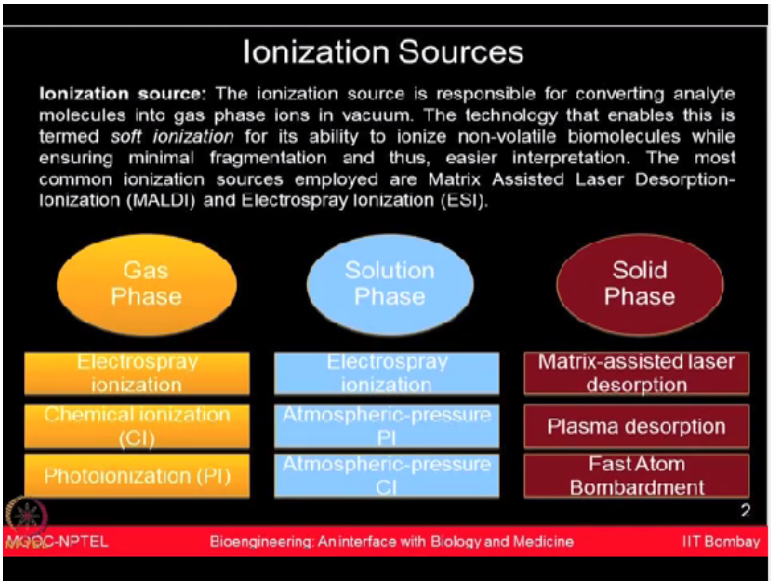

The soft ionization is known for its ability to ionize non-volatile biomolecules while ensuring the minimal fragmentation and therefore, one could easily interpret the data. The most commonly used ionization methods include electrospray ionization or ESI or matrix assisted laser desorption ionization method which is MALDI. Let us first talk about MALDI or matrix assisted laser desorption ionization method.

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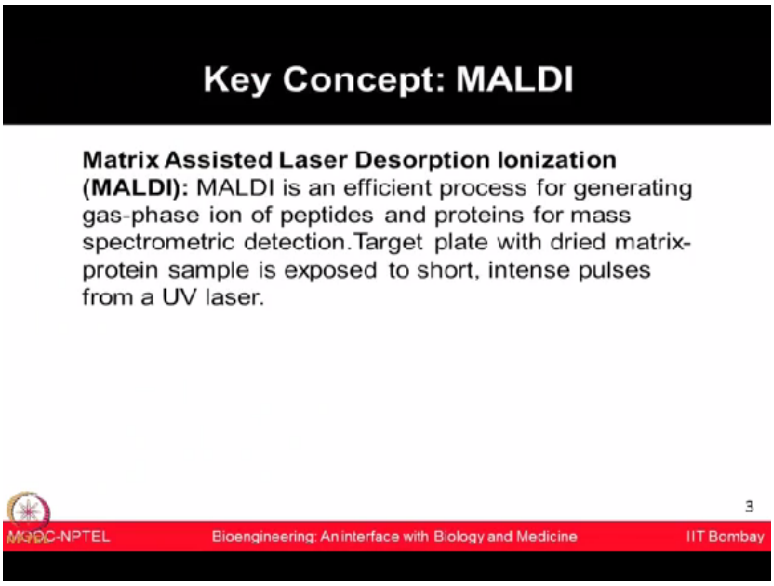

It is one of the efficient process for generating gas phase ions of peptides and proteins for the mass spectrometry detection. In this, there is a target plate which has a dry matrix and the protein sample which is exposed to a short, intense pulse of a laser light.

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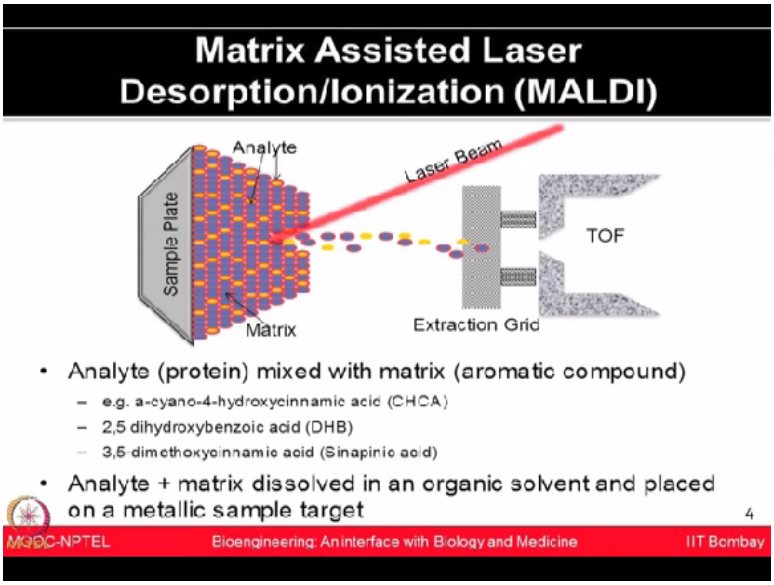

And that is going to generate these ionized forms of these peptides which could be further analyzed in the mass analyzers. So here in this image, it is shown that analyte or the protein of interest is mixed with the matrix which could be some aromatic compound, it could be different type of matrix could be used like alpha-cyano-4-hydroxycinnamic acid or it can be 2,5 dihydroxybenzoic acid or DHB. It could be Sinapinic acid.

So there are different type of matrix which can be used to crystallize these kind of protein and now analyte and the matrix is dissolved in organic solvents which is now crystallized and put on the metallic sample target plate on which now you are doing the laser bombardment. And these ions now based on the mass to charge ratio could be further separated and Time of Flight tube.

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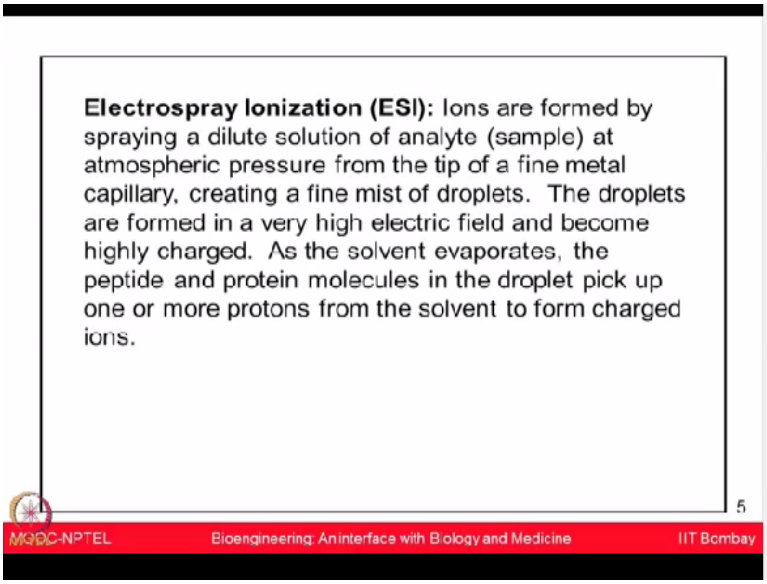

Let us now talk about another ionization method which is electrospray ionization or ESI. In ESI, the ions are formed by spraying a dilute solution of analyte at the atmospheric pressure from the tip of a fine metal capillary and that creates a fine mist of droplets. These droplets, they are formed in very high electric field and becomes highly charged.

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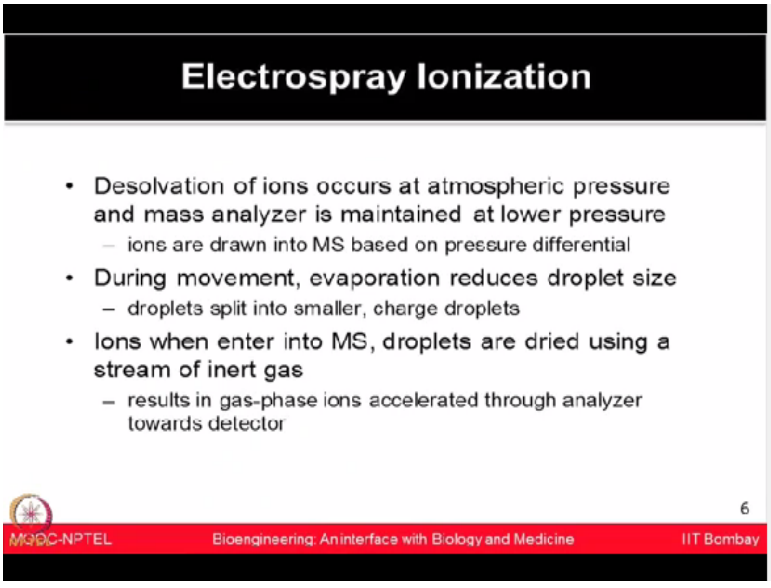

So in electrospray ionization, the desolvation of ions occur at atmospheric pressure and the mass analyzer is maintained at the lower pressure. So by using kind of differential pressure, you are trying to move all these ions inside the mass analyzers. And during the movement, the evaporation happens which reduces the droplet size and now these becomes the charge droplets. These ions when they are entering inside the mass analyzer, it results into the gas phase ions accelerated through the analyzers towards the detector.

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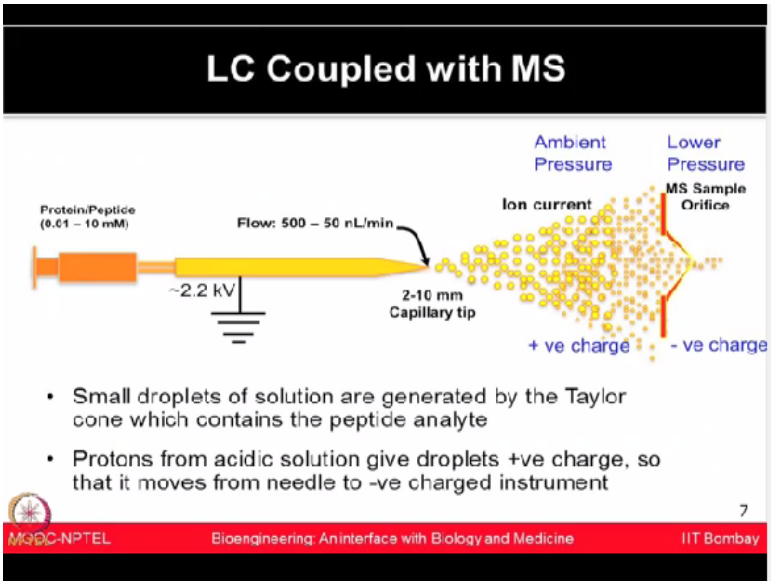

So this is small droplets from a solution forms are generated that is known as a Taylor cone which contains the peptide analytes and now the protons from the acidic solution, they provide droplets a positive charge so that now it can move to the negatively charged instrument and both by using the differential pressure and the charge, you are trying to move all these ions which are coming from the peptides towards the mass analyzer.

And therefore, now you can do the further separation using the mass analyzers. There are different type of mass analyzers available. It can be Time of Flight or TOF tube. It can be ion trap, quadrupole, ion cyclotron resonance, orbitrap, magnetic sector. There are multiple options available for the mass analyzers. Usually people want to utilize different properties of mass analyzers and therefore, they use in tandem.

So that is known as the hybrid MS technology where you can use, you know, 2 different mass

analyzers and utilize both of their property. For example, Q-TOF or quadrupole. Time of Flight is one of the hybrid configuration. Or you can use even tribrid technology like, you know, for example an orbitrap. We have quadrupole, we have orbitrap and ion trap, that is a tribrid technology. So let us look at, you know, some of the basic properties of these mass analyzers, especially the popular ones like Time of Flight or TOF mass analyzers.

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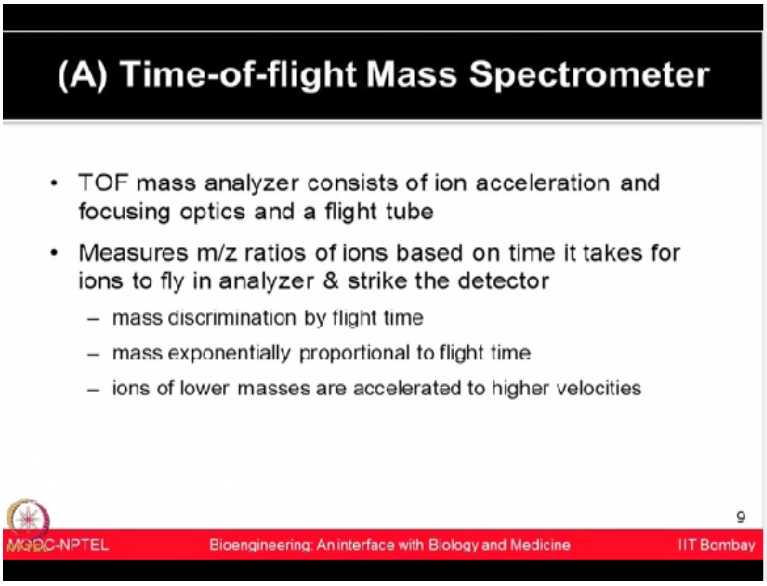

The TOF mass analyzers, it consists of the ion acceleration and the focusing optics inside the flight tube. It measures the mass to charge ratio of ions based on how much time it takes for the ions to fly inside the analyzer and how much time it takes to strike to the detector. So you are using mass discrimination based on the flight time and the mass is exponentially proportional to the flight time. So ions of lower masses are accelerated to the higher velocities here.

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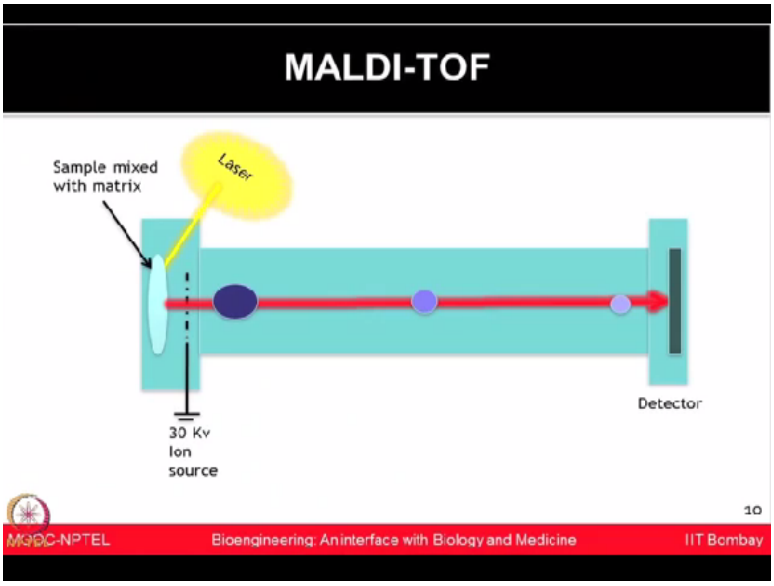

You can see this in this diagram that, you know, laser is bombarded. Now these ions are based on the mass to charge ratio, they are flying inside the Time of Flight tube and reaching to the detector.

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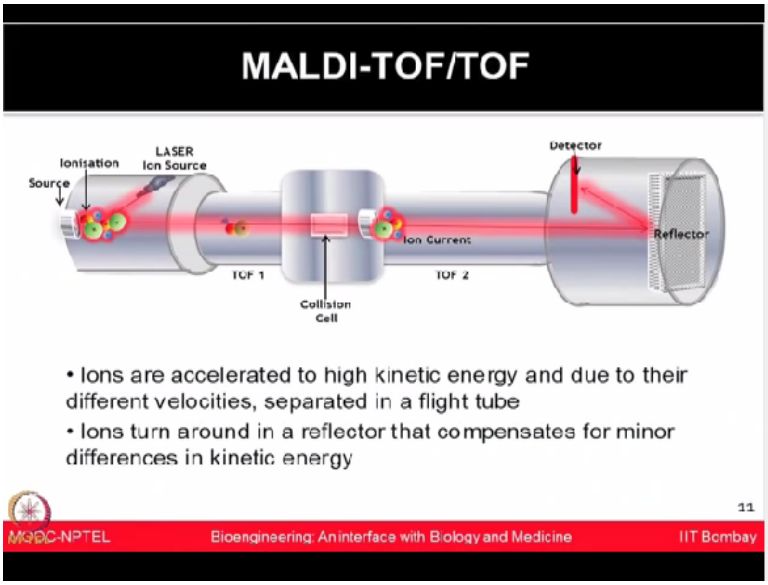

Now if you have 2 different type of Time of Flight tubes, that is the hybrid configuration. You can make MALDI-TOF/TOF. So in this case after the first mass analyzer which is TOF1, there is a collision cell which will select the precursor fragment them further and then generate the spectrum from those which could be utilized to obtain the peptide sequence information.

So in this case, you are using 2 TOF tubes and then you are utilizing their properties to further

separate each one of these ions and their fragmentation pattern is obtained and this could be utilized to obtain the basic peptide sequences. Another popular configuration is quadrupole based mass spec.

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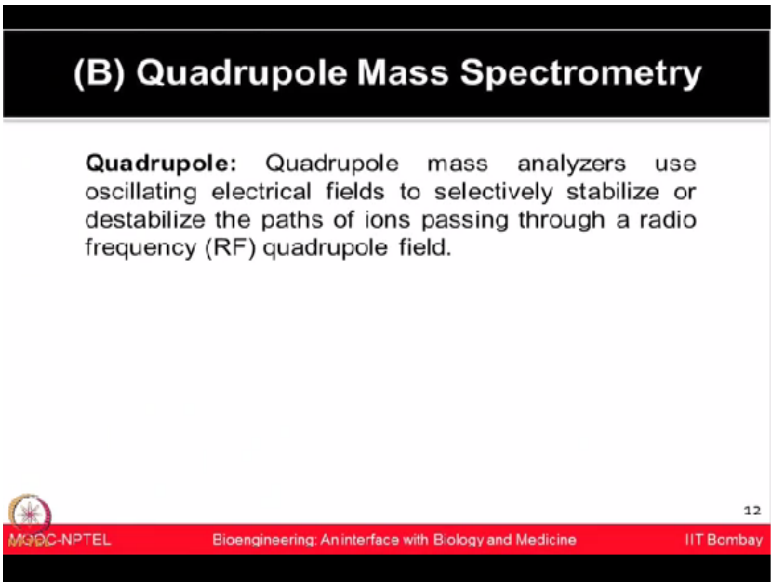

So this mass analyzers, they use oscillating electric fields to selectively stabilize or destabilize the path of ions which is moving inside these quadrupoles and then they are passing through the radio frequency of these quadrupole field. So this is one of the, again, one of the popular configuration.

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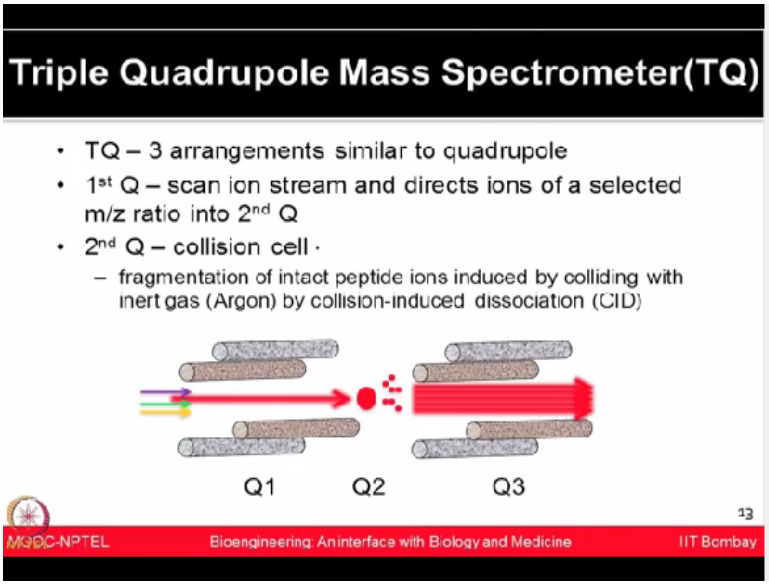

And there are certain mass spec like triple quadrupole. They are based on these principles where

in the first quadrupole, you are scanning the ion stream and directing the ions of a selected mass to charge ratio. That mass to charge ratio, that selected ions is now selected and that is going to get further fragmented in the collision cell where you are using some collision induced dissociation based on some inert gases like Argon.

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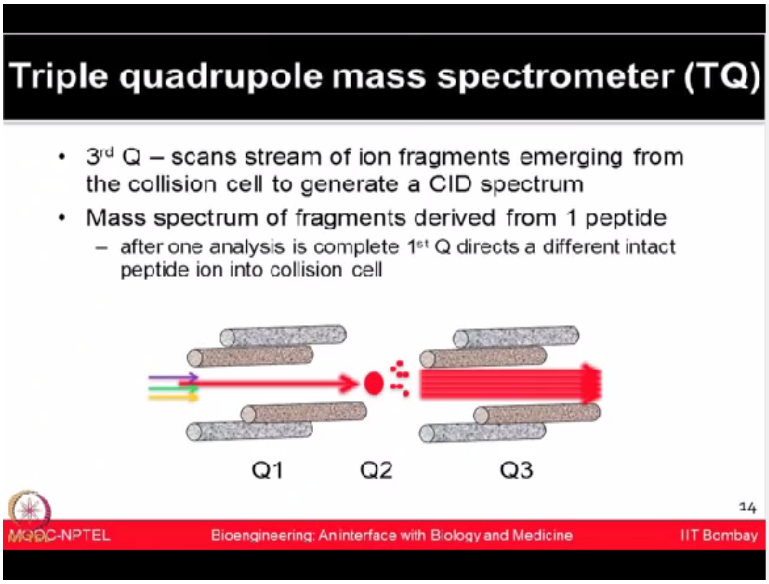

And then in the third quadrupole, you are scanning the stream of these ion fragments which is emerging from the collision cell which generates the CID spectrum. So therefore, by utilizing these properties of mass analyzers, different type of mass spectrometry configurations have come forward. Now I have shown you this mass spec which is Q-TOF or quadrupole Time of Flight.

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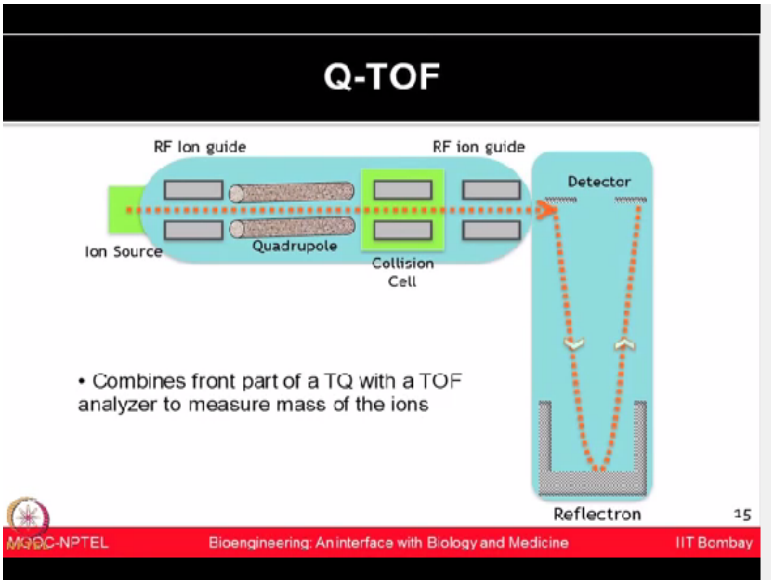

It utilizes 2 different mass analyzers based on, the first one is a quadrupole property and second one is the time of flight. So not only you are resolving them in the electric field but also separating them based on the Time of Flight tube, looking at the mass to charge ratio, how much time they take to separate them.

And then further you have a reflectron tube which also increases the path length in the Time of Flight tube. So by utilizing many of these properties, your intention is to separate, resolve these ions much more accurately and much more high throughput manner to provide the much deeper coverage of the proteins. A new technology which has come forward is orbitrap technology.

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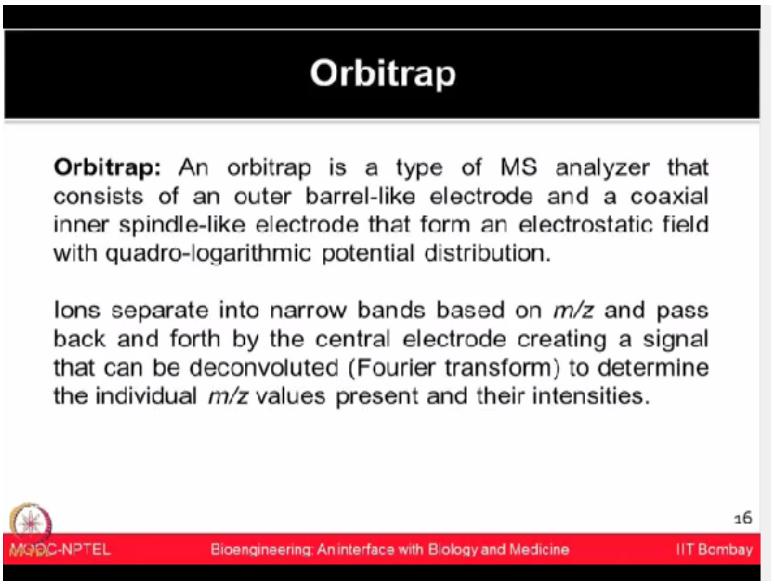

Orbitrap is type of mass analyzers that consists of an outer barrel-like electrodes and a coaxial inner spindle-like electrode that form an electrostatic field with quadro-logarithmic potential distribution. These ions then separate into narrow bands based on the mass to charge ratio and then they pass back and forth by the central electrode which creates a signal that can be deconvoluted to determine the individual mass to charge values which could be present and their intensities can be monitored.

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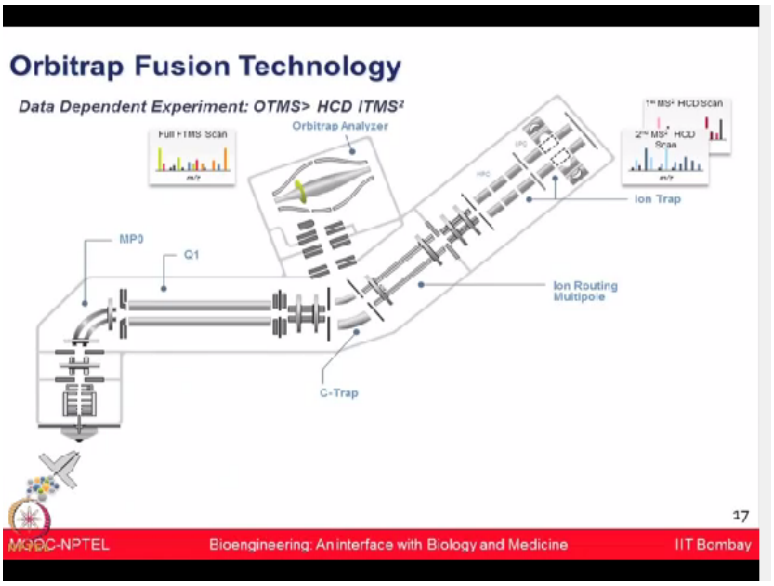

So in this manner, one of the latest technology which has come forward is orbitrap fusion technology where we have quadrupoles, ion trap and orbitraps and both ion trap and orbitrap have their own detectors as well. So now, you know, this kind of tribrid technology not only provides the highest resolution but also provides, you know, multiple capability to do different type of fragmentation, different ways of analysis and it opens up many type of applications by using this kind of technology.

Now the way we talked about, you know, the DIGE technology, difference gel electrophoresis in context of gel based proteomics, similarly when intention is to not only identify a protein but also to quantitate them well, then technologies like iTRAQ which is Isobaric tag for relative and absolute quantification have come forward as a very strong platform to do the quantitative proteomics.

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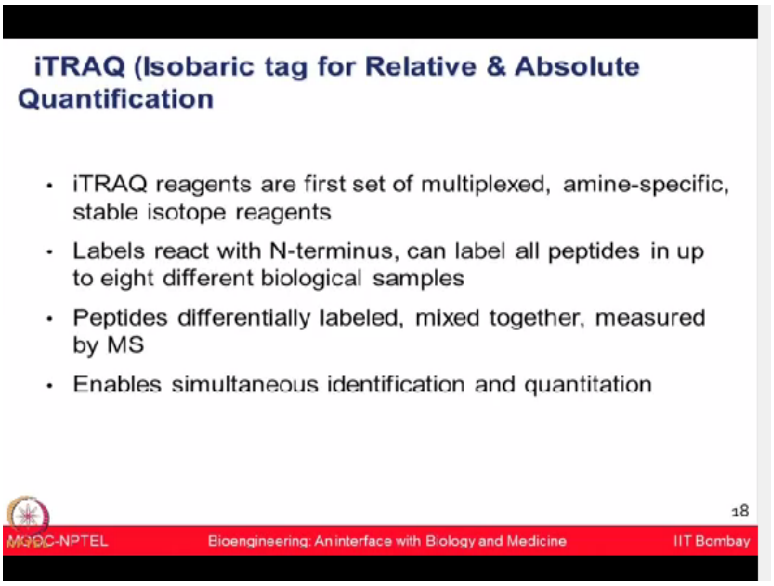

The iTRAQ reagent, these are the multiplexed reagents which are mainly amine-specific stable isotopic reagents. They can label the proteins by reacting on their N-terminal sequence and then you can actually use 4 or 8 plex iTRAQ or you can use even 4 to 8 comparisons, different biological conditions to be compared by labelling with these type of iTRAQ reagents. So now after labelling them, then these peptides could be, you know, mixed together and now you can analyze them in the mass spec simultaneously for their identification and quantification.

A similar technology like iTRAQ has also come forward which is known as TMT or tandem mass tags. Even they use the same property. In both the cases, you are using some isobaric tags where intention is to not to add any extra mass on the protein for 4 of the conditions or 8 of the conditions to be compared because you do not want to change any, you know, extra mass from your side, otherwise the differential changes are not real.

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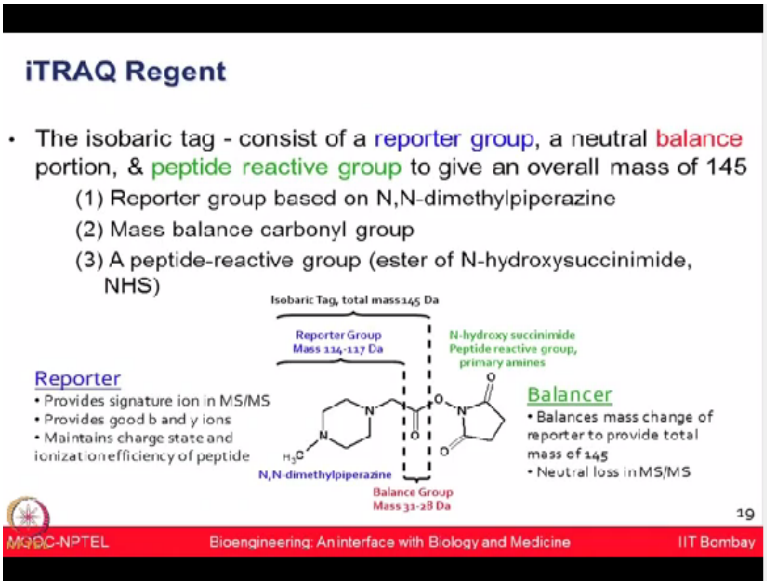

So if you look at, you know, one of the iTRAQ 4 plex reaction, we have shown you here the labelling strategy in which it is a condition A, B, C and D are compared. The iTRAQ reagent is having this reporter ions of 114, 115, 116 and 117.

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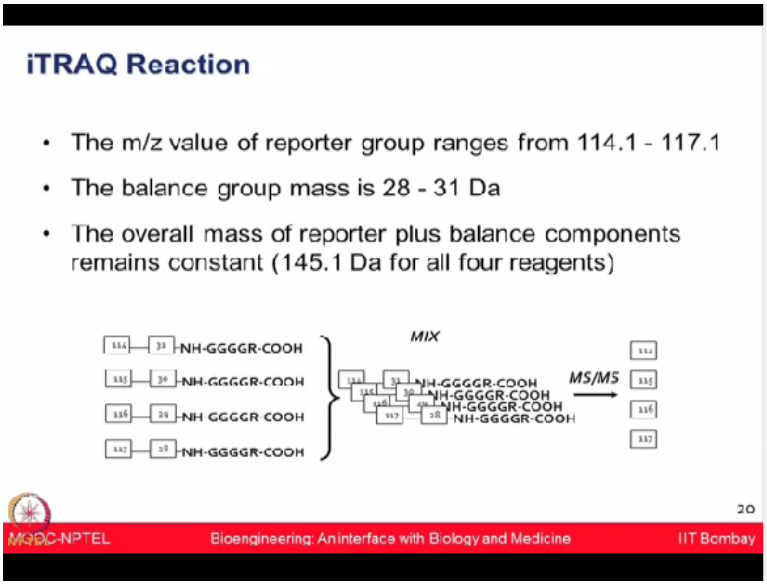

And to balance them there is a balancer reagent which is 31, 30, 29 and 28. Now together they are always going to add mass 145 which is the isobaric for all the 4 conditions. After labelling them, now you can mix these peptides from 4 different conditions. Now you can do further analysis in the mass spec, do MS and MS/MS analysis and these reporter ions will be seen at the MS/MS level.

And now you can not only identify what is this protein but also quantify what happens to this protein when you compare across 4 conditions. So this whole field of doing quantitative proteomics using mass spectrometry is highly relevant for lot of biological applications but it is not in the scope of this particular lecture and for this course. So I am not talking in too much detail about it but I just wanted to give you the glimpse and some idea that in which way scientist are now using mass spectrometry technology for doing various quantitative based analysis.

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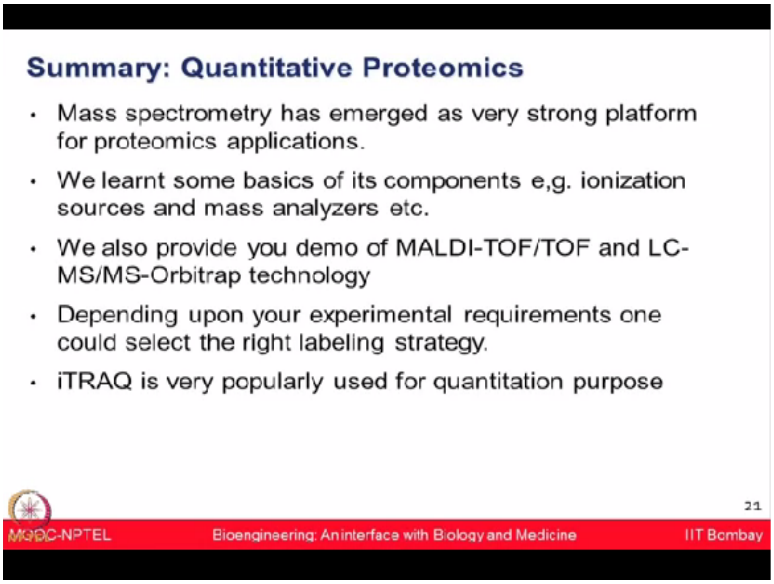

So in today's lecture and conclusions, we mainly talked about mass spectrometry platform which has emerged as a very strong technology for doing the proteomics applications. We have tried to understand some of the basic components of a mass spectrometers, especially the ionization sources and the mass analyzers. We have also provided you some demonstration for the MALDI-TOF/TOF technology and LC-MS/MS-orbitrap based technology.

And I tried to convey you that like DIGE technology, you can also do labelling in the mass spectrometry based methods, one powerful platform is iTRAQ or TMT based labelling strategies which could be utilized to do the quantitative proteomics using mass spectrometry. So some of these are just the kind of basic overview of these technologies to give you an idea and a flavour that in which way now the protein scientists, they are utilizing different type of technologies and platforms to study the proteins more accurately and much more high throughput manner.

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So let us start our first demonstration on MALDI-TOF/TOF. (Video Starts 15:38 - Video Ends 21:44)

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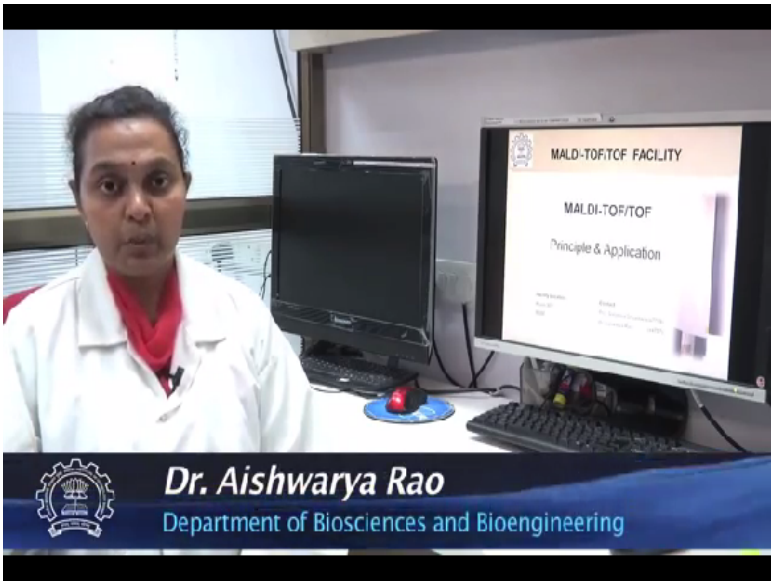

Hello. I am Dr. Aishwarya Rao incharge of the MALDI facility in professor Sanjeeva Srivastava's lab. MALDI is one such instrument which is used as a mass spectrometer. The instrument that you can see behind me is a MALDI-TOF/TOF which is called the autoflex speed and it is made by Bruker. The ionization source in this instrument is a smart beam laser which is used to ionize the samples.

The mass analyzer in this particular instrument is called the Time of Flight. It separates the ions on the basis of the time the ions take to traverse a vacuum tube and therefore, the name Time of Flight. MALDI is an acronym that stands for matrix assisted laser desorption ionization. It is very important to understand the term matrix. The matrix is actually a chemical compound which has to be mixed with the sample.

It is very important to understand what the matrix is. These are chemical compounds which are capable of absorbing the energy from the laser beam and transferring them to the sample so that the sample can get ionized. The matrices are chosen based on the type of sample to be analyzed. They help the sample to be ionized with minimum fragmentation. This helps the analysis of very fragile samples like DNA, proteins, sugars, polymers, etc.

The samples are mixed with the matrix and loaded on to a plate. The plate that is used here is a ground steel plate. It has a number of wells demarcated on it and the samples are to be loaded

inside these wells. There are different ways in which the sample and the matrix can be loaded on to the plate. One of the common methods is called the direct droplet method. In the direct droplet method, matrix is first mixed with the sample in tube and the resulting mixture is then loaded on to the plate.

So I would be now demonstrating this technique. The matrix is taken into a tube after which the sample is taken in equal proportion into this tube and it is mixed externally. The mixing has to be made proper. The resulting mixture is then loaded on to the plate. While loading the mixture on the plate, care has to be taken that the tip should not touch the plate. This drop is then allowed to dry during which the sample and the matrix co-crystallizes.

The laser beam would then hit these crystals and the matrix would absorb all the energy from the laser and transfer the required amount of energy to the sample to help it get ionized. Once the spot has dried completely, this plate has to be inserted inside the instrument. The sample has not to be introduced inside the instrument on top of this platform. So the plate is placed on the platform taking care that the north side of the plate is towards the instrument.

Once the plate is placed inside the instrument, with the help of a software called flexControl, the instrument is now operated. The instrument will first have to be calibrated using certain calibrants which are standard calibrants that are provided by the company itself which would be placed in when any one of these wells. When we calibrate the instrument, we can then use it for analyzing the samples.

In this particular case, the sample has been loaded in C5. We can see this sample in the picture that is showing up over here. This is the image of the region where the sample has been loaded. The crosshair can be adjusted to any region and the laser beam can be fired to that exact point. Once that happens, the matrix will absorb all the energy from the laser and transfer it to the sample so that the sample is converted to the ions.

These ions can then be detected either in the linear or the reflectron mode and this kind of a graph will then be formed. This spectrum is a typical pattern of a high molecular weight

compound that was used. This is the typical mass spectrum that is obtained for high molecular weight samples. The spectrum obtained from the matrix looks like this. So what you see to the right is actually all the peaks that have got high intensity in this particular spectrum. So in this way MALDI is a very versatile technique that can be used to study a number of small compounds, intact proteins and also to establish the identity of the proteins.

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Let us start Orbitrap MS demonstration session now. (Video Starts 21:50 - Video Ends 27:12)

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Hello everyone. I am Saicharan, a doctoral fellow in Dr. Sanjeeva Srivastava's lab. I would now take you to the basics of mass spectrometry and how you can use this instrument for different

proteomics experiments. The mass spectrometer basically consists of 3 components, the ionization source, the analyzer and the detector. However, for proteomics experiments to be carried out, there is a need of an upstream component which can separate complex mixtures of proteins.

For this, we use chromatography based techniques and the most common hyphenation of mass spectrometry is with liquid chromatography. So for proteomics based experiments, the most widely used mass spectrometric technique is LC-MS which stands for liquid chromatography hyphenated with mass spectrometry. I would now introduce you briefly to the different components which make up and LC-MS setup.

So coming to the basic of a mass spectrometry based experiment for proteomics, we have the sample of interest which is digested using a protease. The most commonly used protease usually is trypsin, okay. And we now take a sample which has already been digested to give rise to number of peptides. So say if my sample consists of thousands of proteins, the addition of a protease and digesting the proteins with a protease would result in smaller fragments of these proteins giving rise to a very complex mixture which consists of smaller fragments of proteins, that is peptides.

So these peptides are now in the solution form. For LC based techniques, you need to have the compound in the solution form. So we now look into a nano-LC system. This is the EASY nano-LC 1200 setup. So this is the assembly where the samples are placed. As you can see, there is a 48 well slot available here to place these vials. So these are vials which contain the sample that is to be analyzed.

So inside the unit is a needle which picks up the sample from the designated slot and injects it into the, on to the column. There are 2 separate bottles, one for washing the needle after it has picked the sample and one to discard the waste. On top of the unit, are 2 slots for the solvents that are used. So solvent A contains 0.1% formic acid and solvent B contains 0.1% formic acid in 80% ACN.

So the ratio of these solvents determines when the peptides elute out form the column. So what we now see is a column, a hydrophobic C18 column which is used for separating the samples of interest. This is a 50 cm column which indicates the length of the column and what you see here is the end where the sample gets sprayed in to the mass spectrum. So the nano-LC setup is connected to the mass spectrometer through these silicon tubes.

As you can see, here is the 50 cm column which I just showed you a while back, okay. So the sample enters into the mass spectrometer through these columns. What you see here is a guard column which acts as a preventive device to make sure that the pressure does not exceed a threshold value. If the pressure exceeds for any reason, it can potentially damage the column. So the guard column prevents any damage to the column because of high pressure.

So once the sample enters on to the column, the gradient of the solvents from the nano-LC setup is responsible for continuous elution of the peptides based on the hydrophobicity. You can now understand that the more the hydrophilic a peptide, the earlier it is going to come out of the column. As the time progresses during a run, the hydrophobicity inside also increases because there is an increase in the amount of solvent B.

So the more hydrophobic peptides get eluted later on during the LC/MS run. So what you see here is the camera view of the needle from the column and the formation of a spray and thus the peptides entering inside the mass spectrometer. So it is at this stage that the ionization of the peptides takes place for the mass spectrometer to separate the ions and later on detect them.

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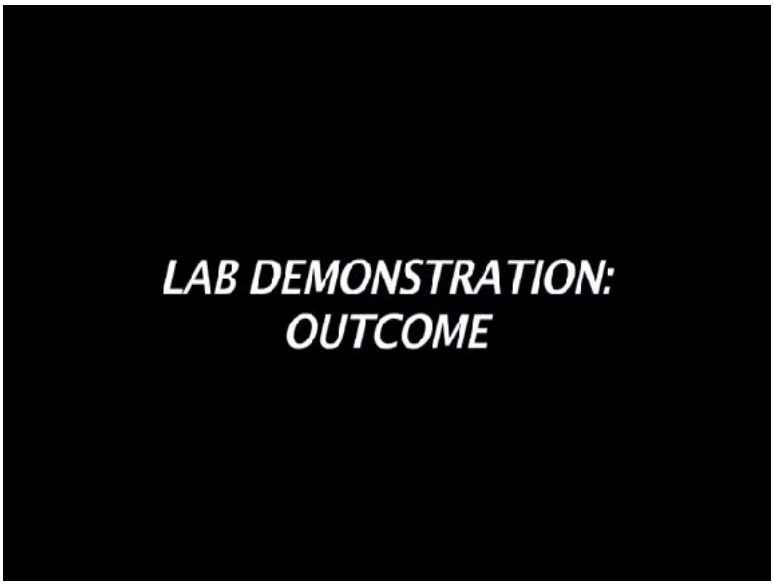

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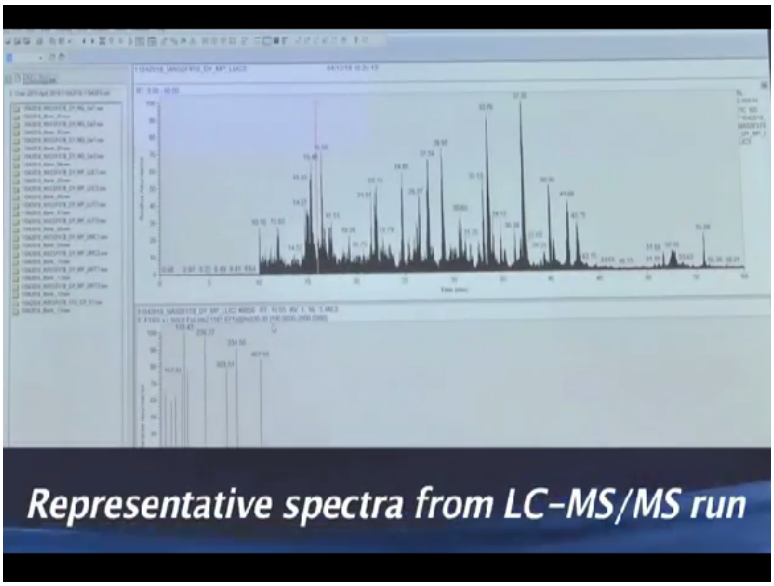

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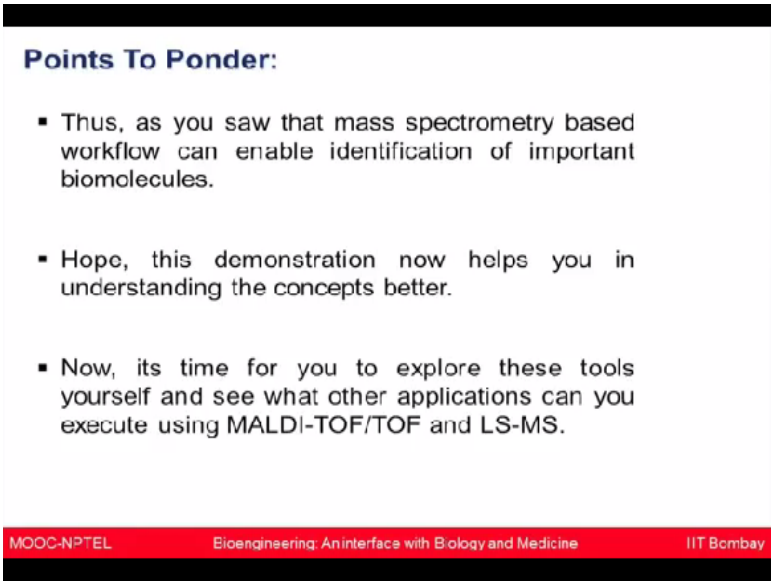

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