Quantitative Methods in Chemistry
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Lecture-38
Analytical Separations-Electrophoresis, Capillary Electrophoresis, Isoelectric Focusing

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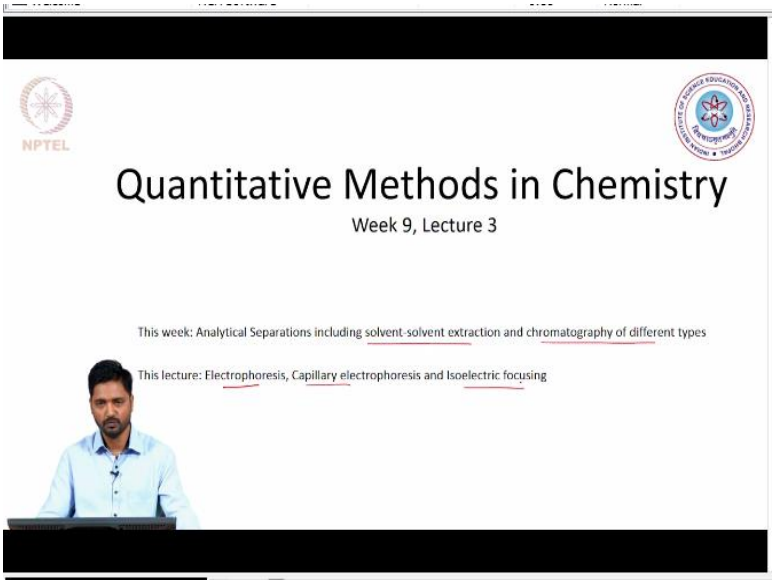

So welcome back to lecture 3 of week 9 for this course quantitative methods in chemistry. This week our efforts have focused on a wide variety of analytical separations that we carry out as chemists. And we have already explored in the previous 2 lectures what are the principles and applications of solvent-solvent extraction, and what are the different types of chromatographic techniques that are available to us for purifying samples and isolating analytically pure compounds.

Now, we also got introduced to the concept of size exclusion chromatography, that is often utilized for separating macromolecules or polymers based on their molecular weight. However, one can also extend it to separating proteins of interest by the same logic. Now, this lecture is specifically focused on techniques that are more utilized in biological context for achieving separations.

So, we will be talking about electrophoresis and its capillary version and also we will be discussing isoelectric focusing in the current lecture. So, let us get started with the concept of electrophoresis.

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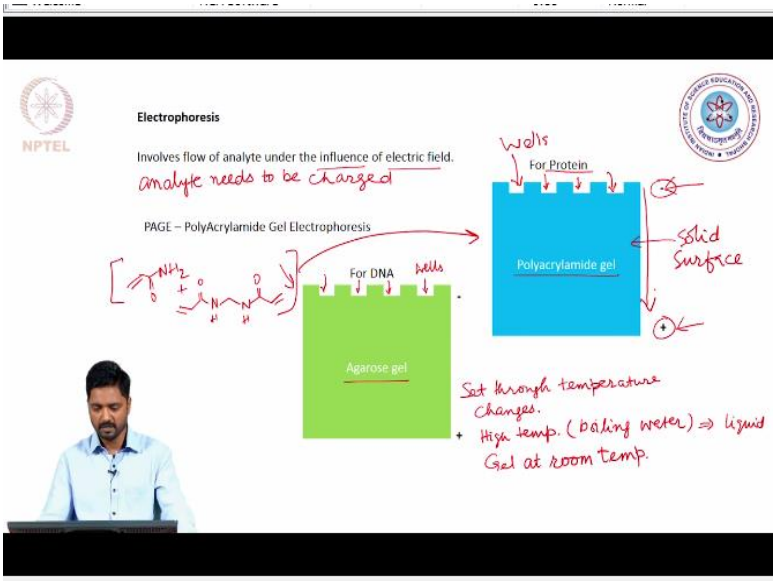

So, electrophoresis is nothing but when we apply an electric field, for example, here we have applied that electric field and we move over analyte of interest under the influence of this electric field. Now, the analyte of course, has to be charged and the material that is used as the solid surface has to be electrically conductive or should have a porous architecture through which ions can migrate and analyte can also to move through.

So, what we are essentially talking about is a gel like architecture, which retains the mechanical integrity while providing porous architecture within it for the movement of ions and the analyte. So, the most commonly utilized gel frameworks for purifying proteins for example, are the polyacrylamide gels and in biological context and in biological labs polyacrylamide gels are routine affair.

Similarly, when we are talking about separating nucleic acids based on their molecular weight, or the number of base pairs present, we use a agarose gel for this purpose and agarose gel is basically polysaccharide gel which can be set through temperature changes. So, for example, at high temperatures in boiling water agarose will give rise to viscous liquid. So, in high temperature that is boiling water.

Agarose is a liquid. But as the solution cools, we get a gel room temperature. So, it is very straightforward to cast this gel. That means we can make the gel fluid through use of high temperature and pour the gel into the corresponding dye or cassette and allow it to cool down

to form the gel which now becomes a rigid network and is also inherently polar. The polyacrylamide gel is made through a chemical reaction instead.

And is not thermal responsive. So here what we do is we simply take acrylamide as the polymerizing agent and we also add the BIS acrylamide as the cross linker and we undertake a chemical polymerization of this mixture in appropriate amounts to obtain a polyacrylamide gel of different pore sizes. Now, we apply a electric field by attaching electrodes to **to** the gel and these structures here, which are known as the wells are where we insert our sample, whether it is a protein mixture or DNA fragments.

So, these are again wells for inserting the sample into the gel, now, when the protein or the DNA fragment moves through this gel, it experiences 2 very different forces here one obviously, is a force that moves the protein from the negative layer electrode to the positive electrode. However, as this protein moves through this gel network, it also experiences are dragged. So, we will get introduced to these concepts in subsequent slides.

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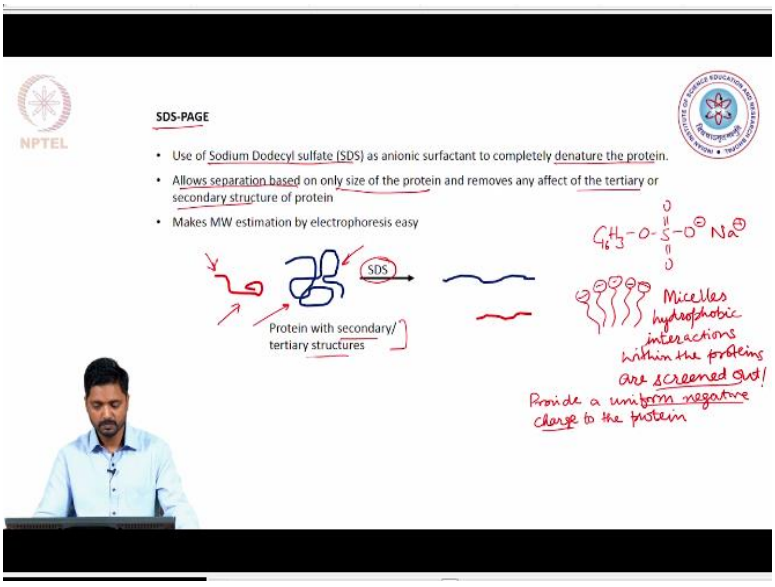

But let us see a different protocol which is often used when purifying proteins based on the molecular weight and that is known as the SDS page. So, PAGE page is an acronym that is used for poly acrylamide gel electrophoresis and SDS page is a refined version or a different version of the same polyacrylamide gel electrophoresis where we utilize sodium dodecyl sulfate as the anionic surfactant to completely denature the protein.

Now, why this is required is because if we have say 2 proteins, which have a certain charge along with the charge they will also have secondary or the tertiary structures and when they move through the electrophoresis, this red colored protein, which is having an extended conformation will seems like having higher molecular weight compared to more globular blue color protein despite the fact that the opposite is true.

So, to screen out any secondary or tertiary structure effects, we utilize the sodium dodecyl sulfate plus moreover, SDS also incorporates a uniform negative charge to the protein. Now, this protocol hence allows separation based only on the size of the protein and removes any effect of the tertiary or secondary structure that the protein may have. So, what we have essentially done is by use of the sodium dodecyl sulfate as the surfactant, we utilize this as surfactant that can form micelles in aqueous medium.

And these micelles screen out all the hydrophobic interactions. So, the key effect of these an ionic micelles is that they screen out all the hydrophobic interactions that were originally existing and giving rise to the tertiary and secondary structures and they also provide a uniform negative charge to the protein and facilitates is its movement from the electrophoresis chamber.

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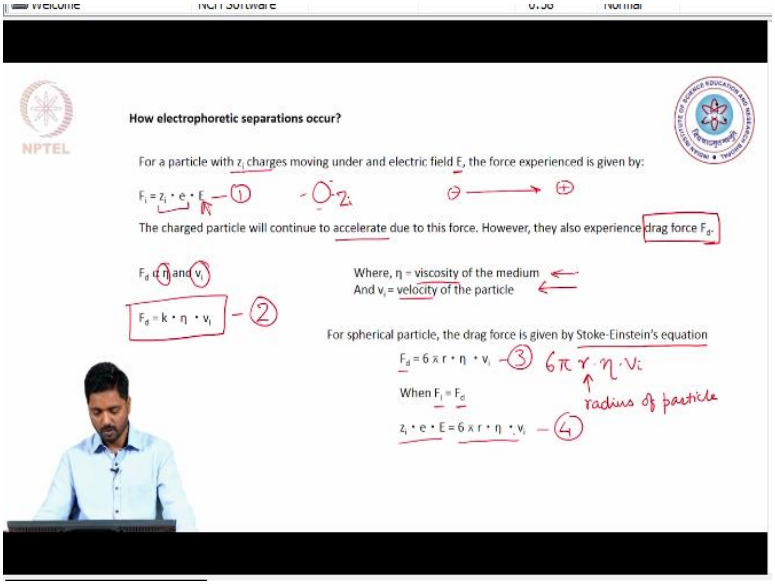

Now, the principle by which the electrophoresis separation occur is provided here. So, if we have a spherical particle for example, that has different charges on it, and, these charges are defined as Z i charges and this particle is moving under, an electric field which is given as E,

the force experienced by this particle is given by this equation 1 here where the electric field generated force is the total charge on the particle, where E is the electronic charge.

And capital E is the strength of the electric field. So, this force accelerates the particle towards the cathode and the particle moves from the negative electrode to the positive electrode under the influence of this force. However, when the particle is undergoing this acceleration, due to the electric force, it also experiences a drag force, which is defined by F d. And this drag forces proportional to the viscosity of the medium as well as the velocity of the particle.

So, both these factors viscosity and velocity play a part in dictating what is the drag force that is being experienced by the particles that is undergoing acceleration. Now, this drag force can then be finally written as, what is given in equation 2 and that is the drag force is nothing but some constant into the viscosity of the medium into the velocity of the particle. Now, in the context of spherical particle, this drag force is given by the Stokes Einstein's equation.

And that is nothing but the drag force is defined as 6 pi r, r is the radius of the particle. So, this essentially dictates the dimensions of the particle into the eta, which is the viscosity of the medium and V i which is the velocity of the particle. Now, it may happen that the force due to the electric field becomes equal to the drag force. And in those conditions we can write equation 4 where the Z i e E term becomes equal to the 6 pi r eta V i term coming from the drag force.

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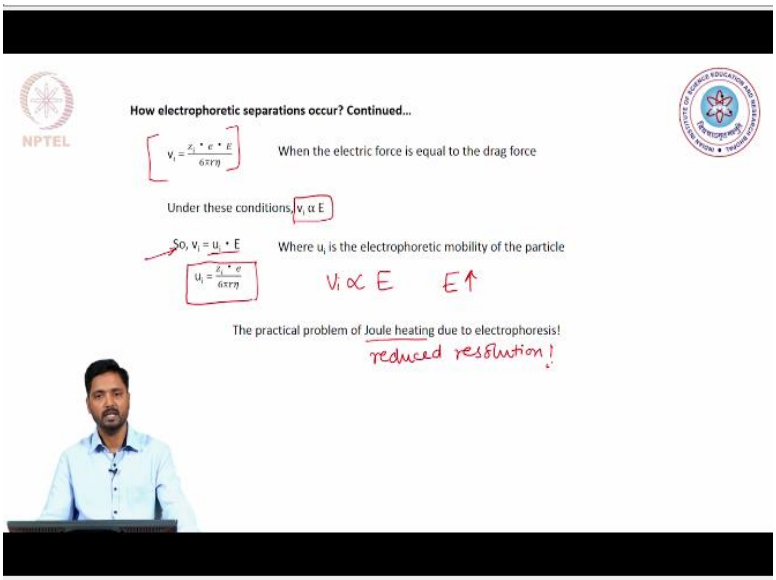

So, when we solve this further what we get is that V i is **is** the total charge on the particle into the electric field divided by 6 pi r eta. Now, under these conditions, it has been found, that the velocity of the particle is proportional to the electric field that is being applied. And this can be further written by this equation here, that is the velocity of the particle is nothing but, electrophoretic mobility into the electric field that is present in the medium.

And the electrophoretic mobility hence is given by this particular equation which takes into consideration the charge on the particle, the size of the particle and the viscosity of the medium. Now, since V i is proportional to E, what we would want is that the E value or the electric field that is being applied should be really high. So, that effective separations and resolutions can be achieved with high velocity of the particle movement under the electric field.

Now, this brings us to an important problem that is often encountered during electrophoresis and that is known as the joule heating. That means, when a current is passed through a medium which can offer resistance and we know that water is not so good conductor of current and that is why we will have to add salt into it but still if the electric field is very high then this results in generation of heat during the electrophoresis.

And this heat is what can often cause either the protein to denature or will result in reduced resolution. So, we would want to minimize the joule heating during electrophoresis and it was figured out that if we use some sort of high surface area material, then it should be possible to undertake electrophoresis with high resolution by applying high electric field.

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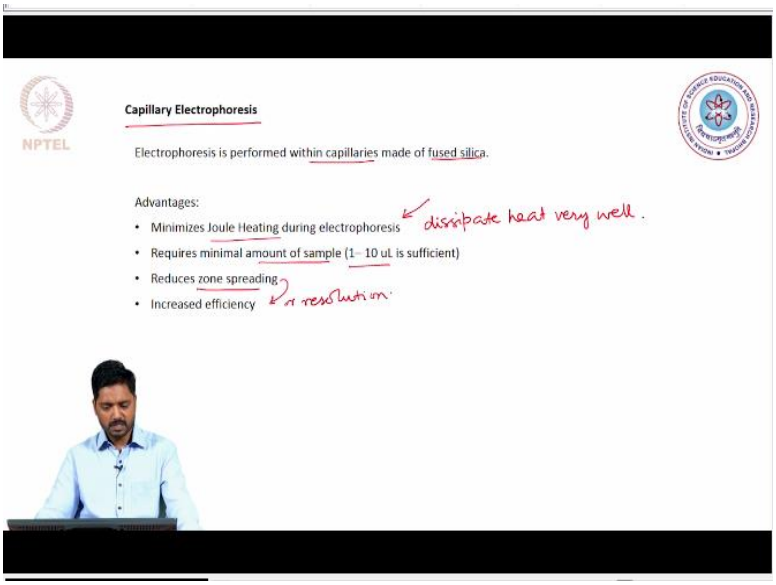

So, this brought out the idea of capillary electrophoresis where electrophoresis is performed within thin capillaries, which are made of fused silica. Now, why few silica is used, because it is having good mechanical strength when thin capillaries are drawn from it. Now, as mentioned already, that capillary electrophoresis offers certain very key advantages.

The first obviously is that it minimizes the joule heating during electrophoresis because of its capillary nature, it can dissipate heat much better and allows application of high electric fields to achieve sufficient resolutions. Another key advantage of capillary electrophoresis is that it requires very less amount of sample. And often times 1 to 10 microliter of the sample is good enough to achieve the relevant separation.

So, this is one of the very profound techniques for achieving separation of small amounts of biological samples, which includes proteins and nucleic acids. Now, of course, since the heating is being avoided, the zone in which our material is present will not spread as much and hence a reduced zone spreading and as a result, increased efficiency or resolution is achieved through the capillary electrophoresis.

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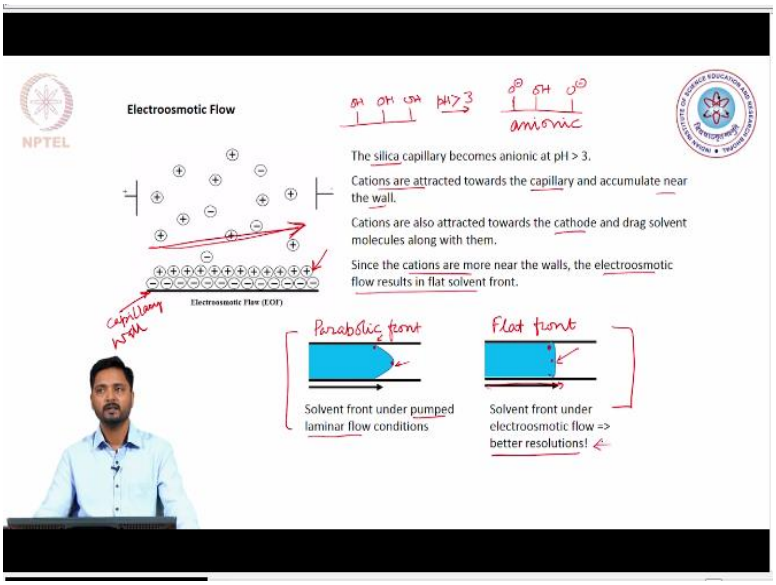

Let us look at how the solvent flow happens under the electroosmotic conditions. Now, I have already told you that the capillary are made up of fused silica and these silica as discussed in the previous classes have hydroxyl residues present on their surface and as the pH becomes greater than 3 these hydroxy residues undergo partial ionization and this makes the silica surface anionic under the typical conditions in which electrophoresis is undertaken.

Now, when that happens and that is demonstrated here by showing the capillary wall which now has an anionic charge and as a result the cations present in the electrolyte are attracted towards this negatively charged surface and they start accumulating close to the surface or the wall of the capillary. So, cations are attracted towards the capillary and like accumulate near the wall.

But under the electric potential that has been applied, the net flow of the cations is towards the cathode. And when they are moving towards the cathode in the direction that is demonstrated here, they also drag with them the solvent or the water molecules. And since, more cations are present near the walls, the electro osmotic flow also results in a flat solvent front. So, here I have contrasted the differences between the flow that happens in pump conditions and laminar flow conditions.

The solvent obtains a parabolic front. However, under electroosmotic flow, the solvent has a more flat front and this is very advantages when we are undertaking electrophoresis or using the electroosmotic flow for separation purposes, because, now the solvent does not contribute

to the spread of the band and it results in better resolutions. So, let me explain this concept a little further.

Since we are having a very flat front it does not matter whether our particle is here or here or here, in all the 3 cases, the particle is at a very similar position within the capillary at a particular instant of time. This is in clear contrast with the flow that happens under the pump conditions. For example, in our typical liquid chromatography separations, where a particle near the surface is lagging behind a particle, which is more at the center of the flow.

The central particle is significantly ahead of the particle which is near the wall of the capillary and this results in the solvent contributing to the spread of the band in case of pumped laminar flow conditions and in the electroosmotic flow conditions this does not happen and results in better resolutions during electrophoresis.

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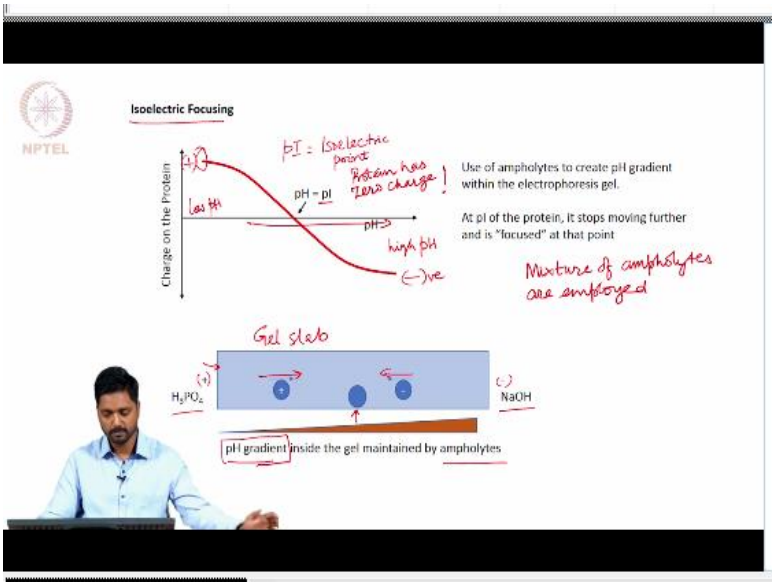

The last topic of this lecture will deal with what is known as isoelectric focusing as the term indicates, here, we are trying to focus our charged particle at a particular location within the gel. And this is based on the concept of the change in the charge of the protein or the particle as the pH of the medium is altered. So, at acidic pH or low pH different residues of the protein can be protonated.

And hence, the protein will have a positive charge on the clean are extreme at very high pH the residues present in the protein would have all ionized and given our net negative charge to the protein. Of course, we would be passing through point where the pH is equal to the point

of zero charge or the isoelectric point. And this is the point at which the protein has zero charge.

Now, any particle if it attains a zero charge, it will have no particular inclination to move under an electric field. And this is the concept that is exploited in isoelectric focusing. So, the way this is done in isoelectric focusing is by achieving a pH gradient inside the electrophoresis gel by use of certain molecules, which are known as ampholytes. It is not a single molecule it is very important to understand.

But mixture of ampholytes are employed to create a pH gradient within the gel. Now, consider the case where you have the positive electrode on the left and the negative electrode on the right. And you create a pH gradient by using phosphoric acid near the positive electrode and sodium hydroxide near the negative electrode and you also maintain a pH gradient inside this gel, gel slap, by use of this mixture of ampholytes.

So, as our protein is added from the left side, it is having our experiencing a low pH region and is positively charged. So, it will start migrating towards the negatively charged electrode. And as it moves through this point, it may reach a point where the pH becomes equal to the isoelectric point of the protein and the protein is no longer charged. In that case, the protein molecule will start accumulating at this point.

And similarly, if we have the protein present as the in the in it is anionic form, it will have a net flow towards the positive electrode and will ultimately reach again the P i region or the isoelectric point region at a particular pH and will no move no further. So, this is the idea that is applied in isoelectric focusing to focus the charged particle into a pH regime, where it is no longer charged.

Now, I will quickly give you some example of the structures of them for ampholytes that are used to create the pH gradient. So that you can appreciate how chemistry is utilized in solving biological problems. So, let us go to the board.

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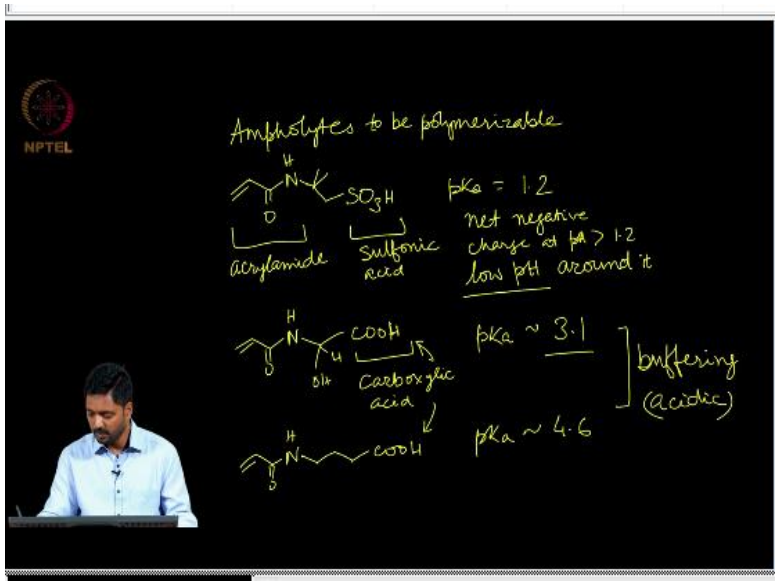

Now, these ampholytes also needs to be polymerizable and that is very nicely incorporated in the chemical structure of ampholytes. So, we usually have acrylamide and this part is what is known as the acrylamide part. But we can create molecules which will have a charge along with the presence of the polymerizable residue. So for example, we can attach sulfonic acid residue here on our molecule, which has the acrylamide part present.

And this is the sulfonic acid part and the sulfonic acid gives a pKa value of 1.2. So at all pH values greater than 1.2 this molecule will have a net negative charge at pH greater than 1.2, but because of its acidic nature, it will a create low pH around it. Similarly, we can use a variety of carboxylic acid molecules. For example simple structure is this molecule where the carboxylic acid part is now you utilised as the ionisable residue.

And this results in a pKa value of about 3.1. So, when we use sulfonic acid, we achieve a very low pH in the region where it is present with carboxylic acid the pH is still low, but somewhat higher than the sulfonic acid. Similarly, we can space out the carboxylic acid to further increase the pKa value to 4.6, also note that these molecules are weak acids. So, they will also create a buffering region around them.

But these regions will still be acidic in nature. Now, to create systems which will give neutral or basic pH, we utilize amines and morpholines.

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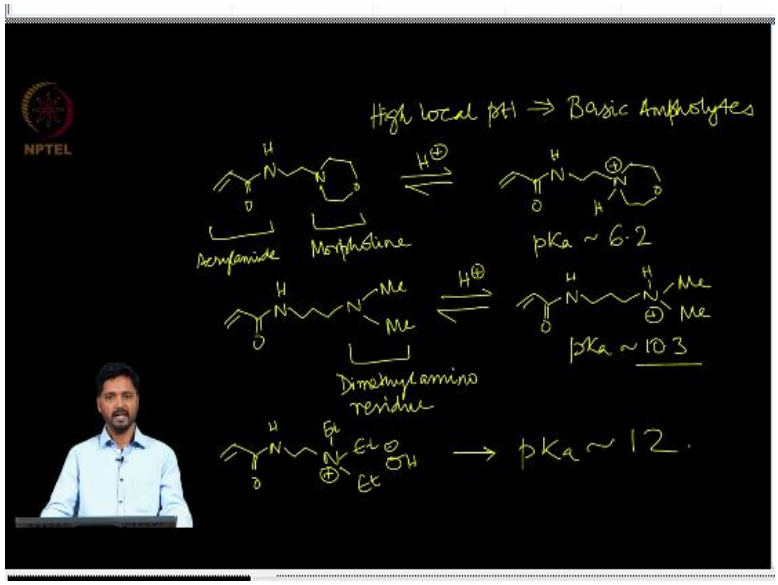

Now to create high local pH we use what are known as the basic ampholytes. And now I will give you some examples of what these basic ampholytes are. So, again they will be based on the acrylamide framework. However, here we will utilize or exploit either morpholine residue as it is shown here, this part is the morpholine part and this is our acrylamide part. Now, this morpholine is a basic molecule.

So, it can be protonated under the appropriate conditions to give rise to positively charged species. And this protonation can as well occur from the water of the medium and as a result, this morpholine derivative will give rise to a slightly higher local pH of 6.2 or around that. So, in the same way, we can use a molecule which has the dimethyl amino residue, now when we use this dimethyl amino residue we again create high local pH because of its basic nature.

And formation of this protonated structure in presence of water. So, this results in a pKa value of about 10.3. So when we use this dimethyl amino acrylamide system our gel will have a local pH of about 10 or so. Finally, we can use ammonium hydroxide salt. So we can use triethylammonium salt. And this molecule can have hydroxyl anion as the counter anion and this will have a pKa value of about 12.

So, we can use these different basic ampholytes in combination with the acidic ampholytes to create a wide range of local pH regimes within our gel, and that will help us in achieving the isoelectric focusing where the protein of interest will attain a net neutral charge and will no longer migrate under the electric field. And this will allow us to carefully focus the protein of interest in a narrow region within the gel.

So, I hope this lecture has given you some basic ideas of how capillary electrophoresis and isoelectric focusing is applied in biological context. This brings us to the end of this week's lecture. In the subsequent weeks, we will be focusing on how we analyze chromatograms and identify what are the resolution of the peaks, what are the retention factor, selectivity factor.

And what is the theoretical basis through which separations happen inside a chromatographic column and for that the plate theory and the rate theory will be investigated. And finally, as time permits, we will also understand the basic principle of how different detectors in the chromatography work. So, thank you all for your attention.