Spectroscopic Techniques for Pharmaceutical and Biopharmaceutical Industries
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Lecture 24
Application of Fluorescence Spectroscopy

Hello students, welcome back to the lecture on Fluorescence Spectroscopy. So, during the last lecture I started with what is the principle of fluorescence spectroscopy we discussed about quantum belt life time and quenching I will start from wherever I left and I will discuss the applications of fluorescence spectroscopy in this lecture.

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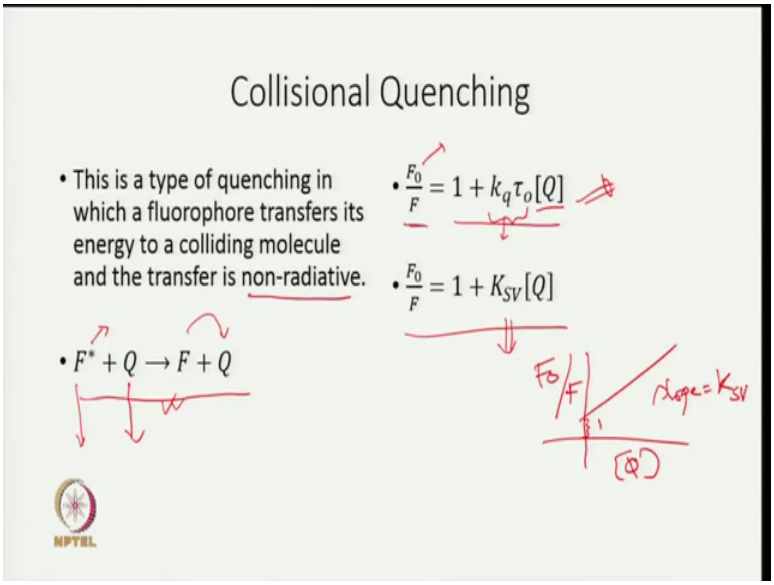

So, in the last lecture I was discussing about two different kind of quenching one is collisional quenching and another is static quenching. Collisional quenching is also known as dynamic quenching. This is the type of quenching in which a fluorophore transfers its energy to a colliding molecule and the transfer is non-radiative. So, this point is very important that the transfer is non-radiative and the whole process can be shown by this equation where F is fluorophore, Q is your quencher, stars tells you that the particular compound is in excited state so F star means fluorophore is in excited state.

So, quencher reacts with fluorophore in its excited state and there is a transfer of energy from fluorophore to quencher. I discuss the kinetic equation which holds true in presence of and when we solve it we got this equation which is known as Stern-Volmer equation. So, F naught by F is

equal to 1 plus k q tau naught multiplied by concentration of Q. So, F naught is fluorescence intensity in absence of quencher, F is fluorescence intensity in presence of quencher, k q is rate constant for this process, tau naught is life time and this term gives you a concentration of quencher and this is well known Stern-Volmer equation.

F naught by F can also be written as 1 plus K SV into concentration of Q where Q is a quencher. K SV is basically equal to k q into tau naught and it is known as a Stern-Volmer constant and if I plot F naught by F, F naught by F versus Q, I will get a straight line the intercept will be equal to 1 and the slope will give you value of K SV and that is how we can calculate a Stern-Volmer constant.

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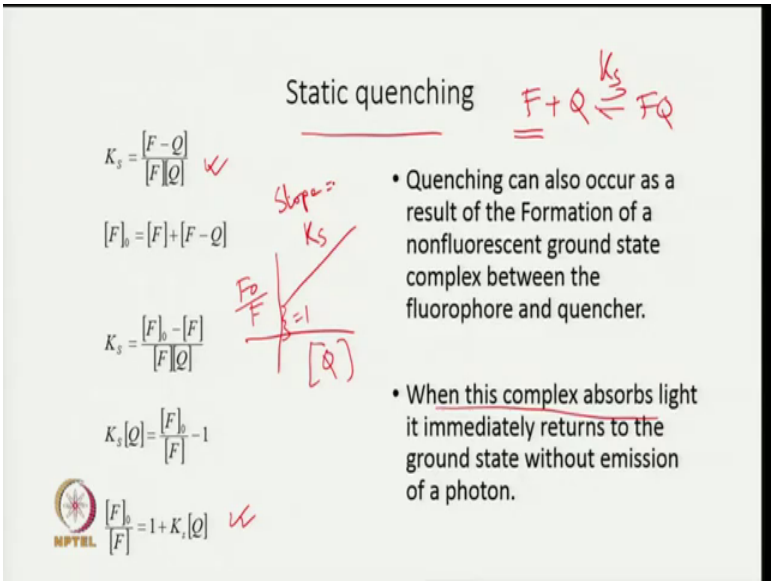

I also discuss about the second type of quenching which is known as static quenching. In this quenching takes place because of formation of non-fluorescent ground state complex between fluorophore and quencher. So, here quencher is associating with fluorescent spaces in it is ground state so please keep that thing in mind. So, when this complex absorbs light it immediately returns to ground state without emission of a photon.

So, this complex does not contribute to fluorescence intensity. And the process is shown as F plus Q giving you FQ and now you can see that I am not writing F star it means that quencher is associating with fluorescent spaces in it is ground state and if I take a equilibrium constant as K S. I can write this equation and I showed you last time that, that will give you this equation

where F naught by F is equal to 1 plus KS or K a Association constant K is your static quenching constant you can say multiplied by the concentration of Q.

So, again if I plot F naught by F versus Q I will get a straight line where intercept will be equal to 1 and the slope will give you the value of K S slope will be equal to value of K S. So, both K SV, K S can be calculated using this plot F naught by F versus the concentration of Q plot. So, either we are going to get a K SV value or K S value. Now, one of the very important thing is to know how to differentiate between how to differentiate between K SV and K S Stern-Volmer constant and this is static quenching constant.

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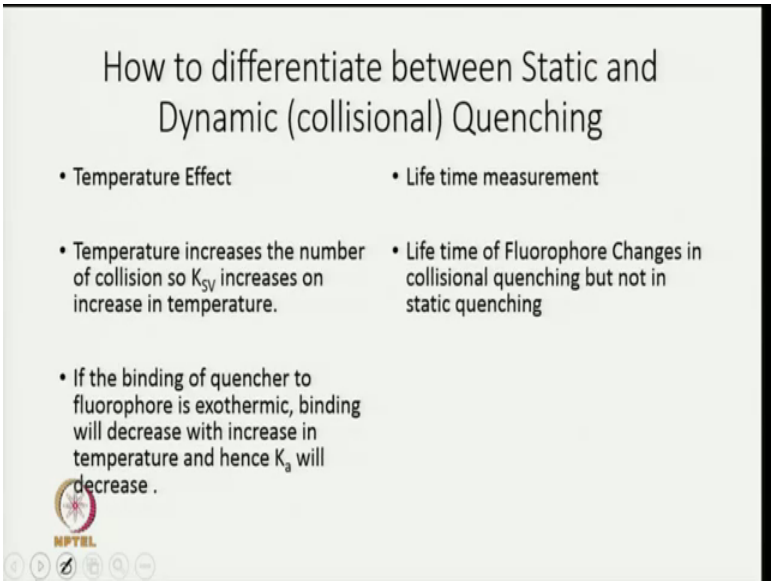

And the way we can differentiate is, we can look at the effect of temperature and the second thing which we can do is measure the life time in presence of quencher. Let us look at effect of temperature, what we expect that if I increase the temperature it will increase the number of collision and since K SV is dependent on number of collision so K SV will increase on increase in temperature.

In the second case, I mean in static quenching case. If the binding of quencher to fluorophore is exothermic what it means is that if I increase the temperature binding will decrease and hence K a decrease. So, if I increase the temperature and then do a plot between F naught by F versus Q and what we see is that slope is increasing then it is probably because of collisional quenching whereas if it is decreasing then it is sure surely because of a static quenching.

So, if slope is decreasing with temperature then you are sure that it is due to a static quenching. Second way to differentiate between static and dynamic quenching is by life time measurement and I will talk about life time measurement in next lectures but one thing you need to keep in mind that life time of fluorophore will change in collisional quenching.

So, the life time of fluorophore without quencher if life time of fluorophore without quencher is different than in presence of quencher then you are sure that it is a case of collisional quenching, if it is equal then quenching must be due to static quenching. A quencher must be associative with fluorophore to give you a non-fluorescent spaces, to give a non-fluorescent spaces in that case life time is not going to change.

It is quite obvious because in case of collisional quenching Q interacts with the excited state and so it affects life time whereas in case of a static quenching Q interacts with the ground state and so there is no there is no way it can affect a life time.

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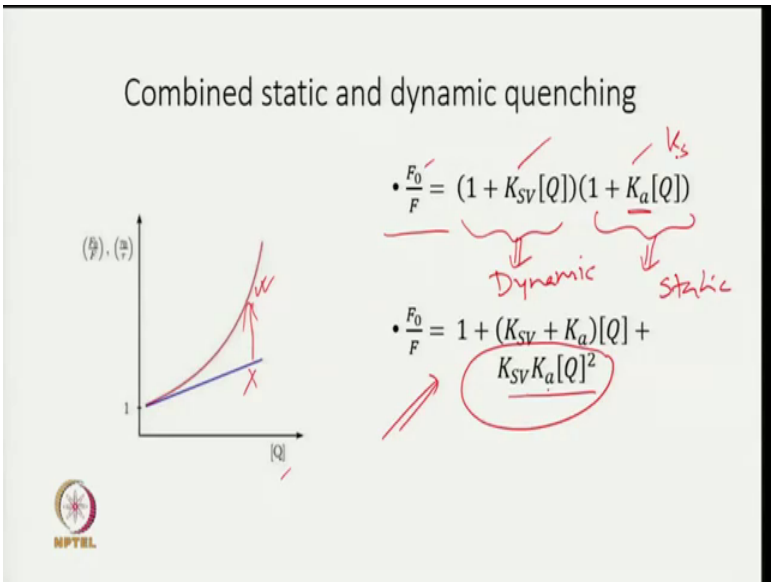

You can have a case where both static and dynamic quenching takes place. In that case, F naught by F versus Q plot will not going to be linear it will have upward curvature and when you see this a quad curvature in Stern–Volmer plot. It means you are dealing with a case where both kind of quenching is present and you can see why there is a quad curvature. So, when you have a case where both static and dynamic quenching is taking place in that case F naught by F will be multiple of these two terms one due to dynamic quenching and another due to static quenching.

So, this is for a static and this is for dynamic and if you expand this you will get this equation. So, F not by F is equal to 1 plus KSV plus K a multiplied by concentration of quencher plus K SV into K a multiplied by concentration of quench square and because of this square term you will get this deviation, you get this deviation. Now, can we calculate from this what is the value of K SV and K a? So, this is K SV is nothing but K S.

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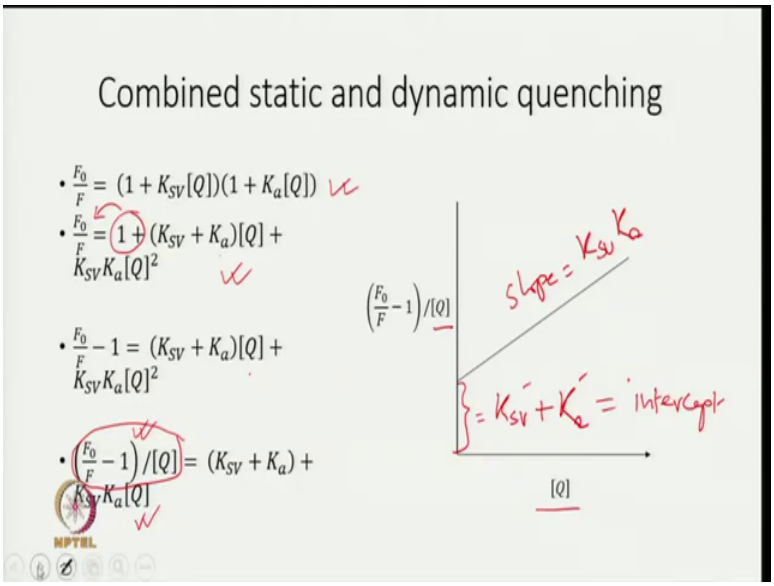

So, now we are going to look at whether we can get the value of K SV and K a by a different method. This is the equation for F naught by F, if the quenching is through both static and dynamic quenching and if we expand then we will get three terms and if I take 1 to this side what I will get is F naught by F minus 1 is equal to KSV plus K a into Q plus K SV into K a multiplied by Q square and if I divide the whole equation by Q what I am going to get is this is equal to K SV plus K a plus K SV into K a multiplied by the concentration of Q this is now a equation of a straight line.

If I plot this quantity versus Q, this quantity versus Q. So, F naught by F minus 1 divided by Q versus Q plot is going to be a straight line with intercept is equal to K SV plus K a and slope is equal to K SV into K a this is equal to intercept and this is equal to slope. So, you have a two unknowns K SV and K a and you have two equations and so you can get the value of K SV and K a

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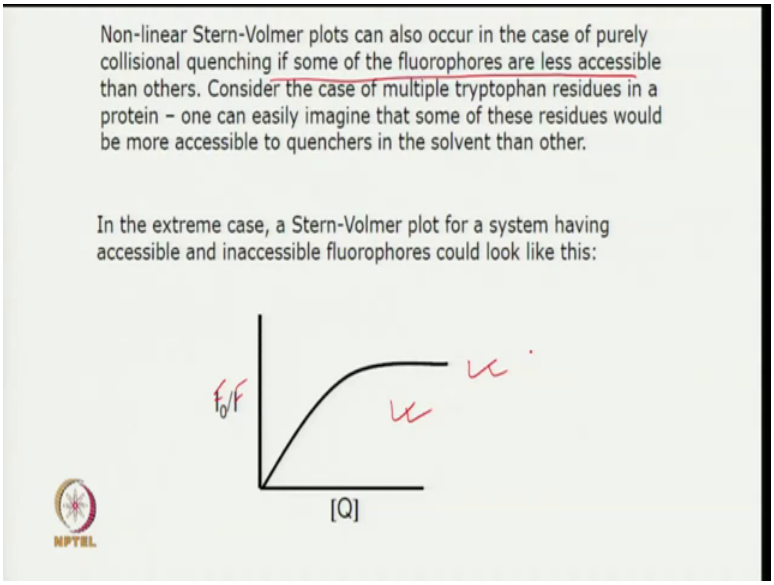

Sometimes you can also get this kind of curve when you plot F naught by F versus concentration of Q and this can happen if some of the fluorophores are less accessible than others. For example, if you take a protein molecule it is going to have a multiple tryptophan residues and at certain times it is possible that some of the residues are more accessible comparison to other residue to quenchers and in that case your plot will look like this. So, this is about your quenching.

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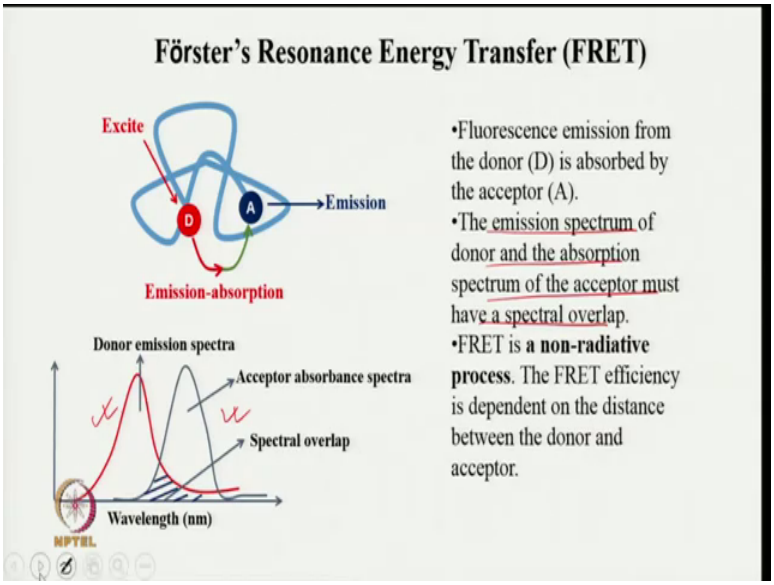

Now, I am going to talk about another phenomena which is quite often used in different applications which is known as FRET Forster Resonance Energy Transfer. The emission spectrum of donor and absorption spectrum of the acceptor must have a spectral overlap. So, this phenomena takes place when emission spectrum of donor and absorption spectrum acceptor must have as a spectral over.

So, basically here there are two species one is known as donor another is called acceptor and the criteria for FRET is that emission spectrum of donor and absorption spectrum of acceptor must have spectral overlap and only then this phenomena can take place, only then this phenomena can take place. The another important point about FRET is, it is a non-radiative process and the FRET efficiency is dependent on the distance between donor and acceptor and you can see this is donor emission spectra and this is acceptor absorbance spectra if there is a spectral overlap then only FRET can take place.

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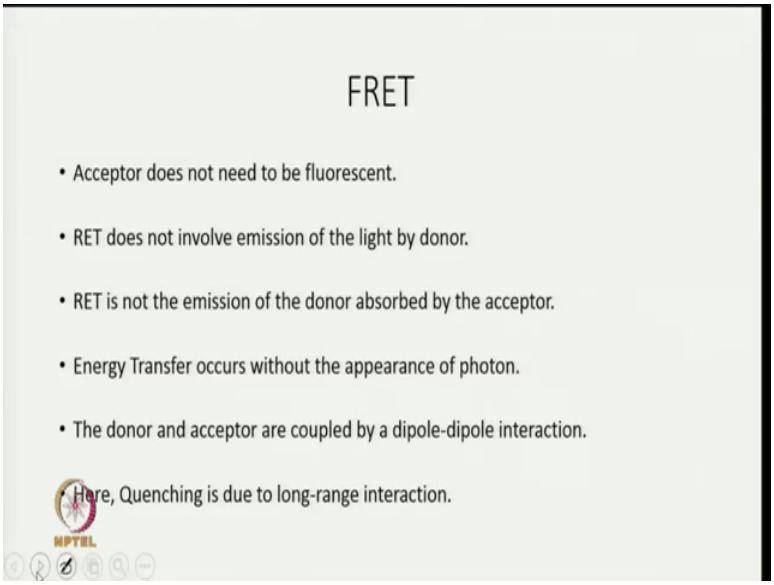

There some of the very important point of about FRET is acceptor does not need to be fluorescent, donor need to be fluorescent but acceptor does not need to be frozen it can be fluorescent it can be non-fluorescent. FRET does not involve emission of the light by donor so please keep this thing mind that it does not involve emission of the light by donor. The third point is, rate is not the emission of donor absorbed by the acceptor.

So, it is not that donor is emitting and that energy is absorbed by acceptor. Energy transfers occurs without the appearance of Photon. So, energy transfer between donor and acceptor takes place without the appearance of a photon and that is what we mean by it is FRET is a non-radiative process. The energy transfer takes place because donor and acceptor are coupled by dipole-dipole interaction and here quenching is due to long range interactions.

So, what happens that quenching of donor take place and that is because of long range interaction with acceptor. So, that is a difference between quenching as such and your quenching due to FRET, quenching due to FRET and FRET there is long range interaction whereas simple quenching is because of short range interaction.

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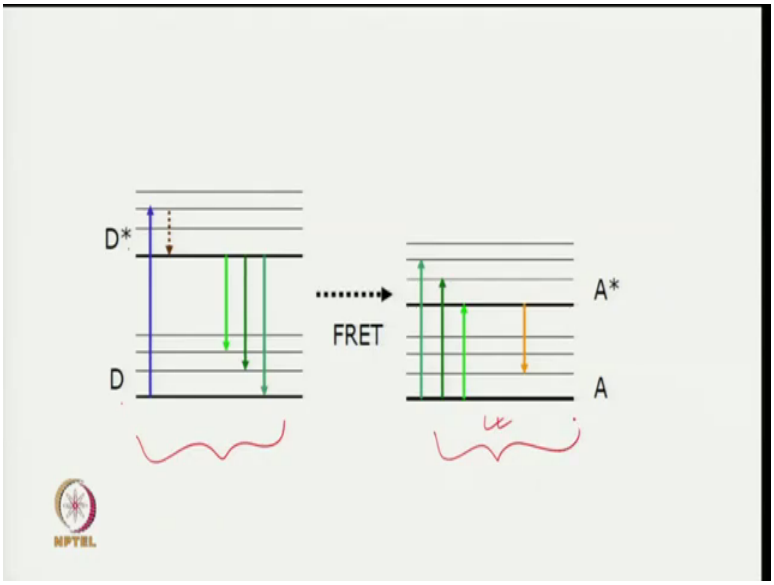

So, what happens that D goes to D star and from there, there is non-radiative transfer of energy to the acceptor and due to that it undergoes emission, it undergoes emission. So, first the energy which is transferred to acceptor that he used to excite the acceptor molecule to the excited state and when the excited molecules comes back to ground state there is emission. So, there is a decrease in emission of donor molecule and there is increase in emission of acceptor molecule.

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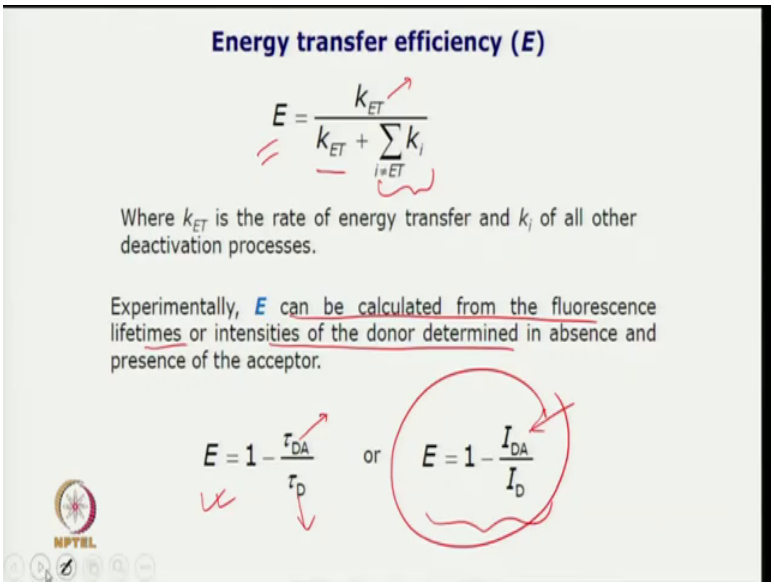

So, since there is energy transfer from donor to acceptor we define a term called energy transfer efficiency how much this energy transfer is efficient? And that is given by where K energy transfer is the rate constant of energy transfer divided by rate constant of energy transfer plus other kind of rate constant for other kind of energy transfer other kind of energy transfer. It can be calculated from the fluorescence lifetime or intensity of the donor determined in absence and presence of acceptor and this is the formula for E.

So, E is one minus I DA divided by I D this is intensity of donor in presence of acceptor and they say intensity of donor. So, this is your energy transfer efficiency and we can also write this transfer efficiency in terms of life time by using the formula 1 minus life time of donor in presence of acceptor divided by life time of donor in absence of acceptor.

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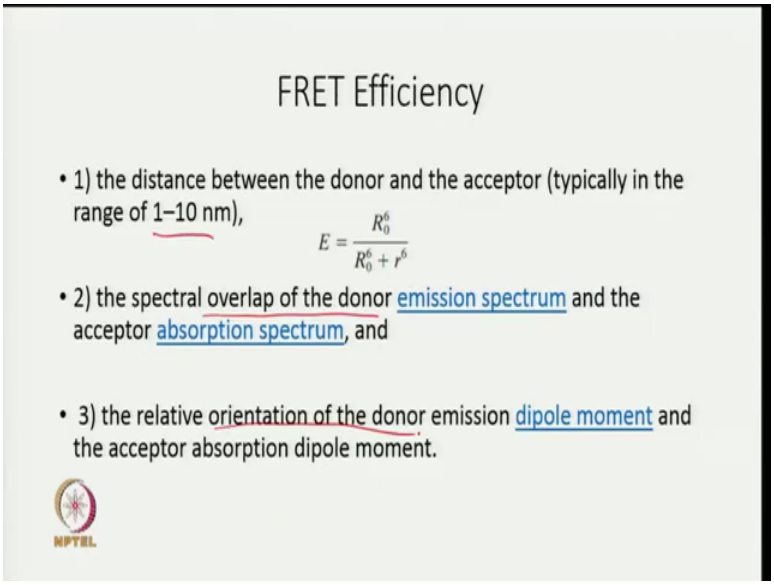

One of the very important aspect of this FRET is that we can know the distance between donor and acceptor if the distance is in the range of 1 to 10 nanometer. So, if there is a donor, if there is acceptor nearby and I can even know the distance between donor and acceptor and this has a huge implication. The second thing, on which rate efficiency depends is a spectral overlap of donor emission spectrum and acceptor absorption spectrum.

And the last one is a relative orientation of donor emission dipole moment and acceptor absorption dipole moment. Since we have already talked that FRET is due to dipole interaction

between acceptor and donor molecule and so relative orientation of the two dipole moments matter.

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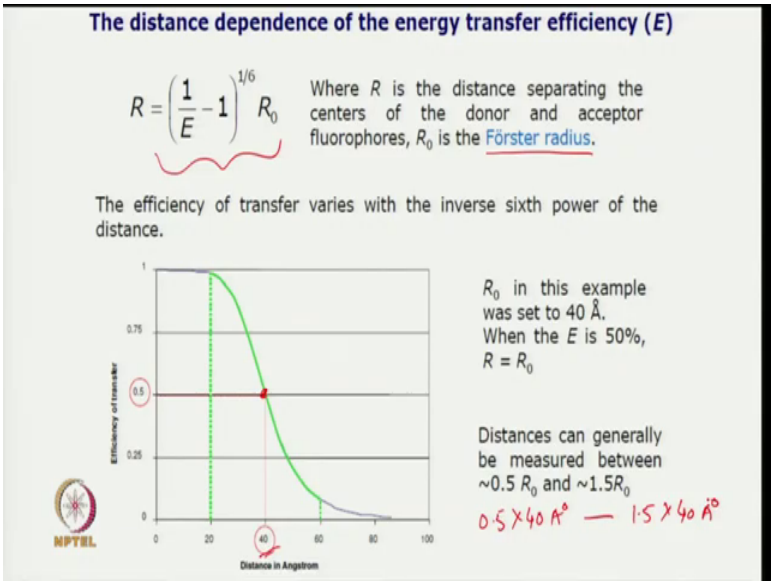

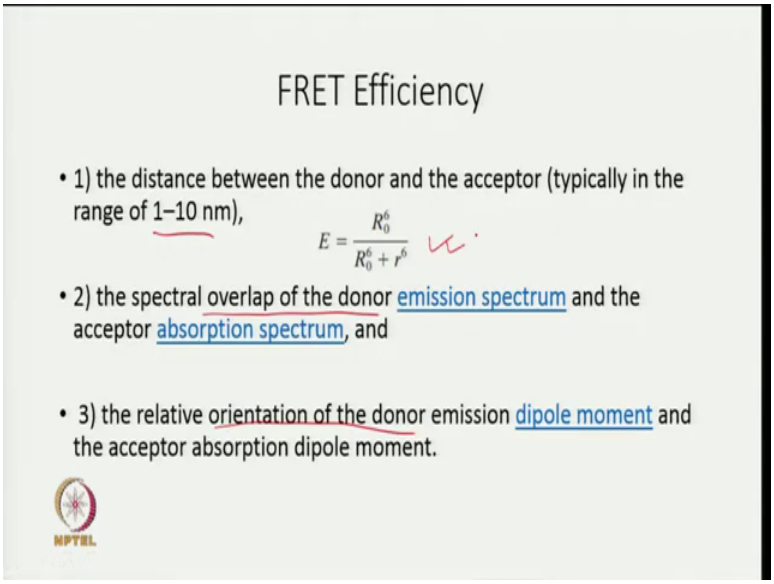

So, R is given in terms of R naught and E by this equation and this is basically derived from E is equal to R naught power 6 divided by R naught power 6 plus R 6. So, first thing is what is R naught? R naught is called Forster radius and the way we define this R naught is R naught is the distance between donor and acceptor when energy transfer is 50 percent, energy transfer efficiency is 0.5 and you can put value here and so E will be your half here.

So, this is 2 minus 1 power 1 by 6 into R naught which is 1 power 1 by 6 R naught which is basically R naught. So, R is equal to R naught when E is equal to 0.5, and so Forster radius is the distance between center of the donor and acceptor of fluorophore when E is 0.5. In this case, you can look at that E is equal to 0.5 when the distance between the center of donor and acceptor is 40 angstrom what this means is that Forster radius in this case is equal to 40 angstrom and you can measure the distance between 0.5 R naught and 1.5 are naught. So, in this case you can calculate 0.5 into 40 angstrom to 1.5 into 40angstrom. So, if donor and acceptor is between these two distance we can see the quenching effect because of FRET.

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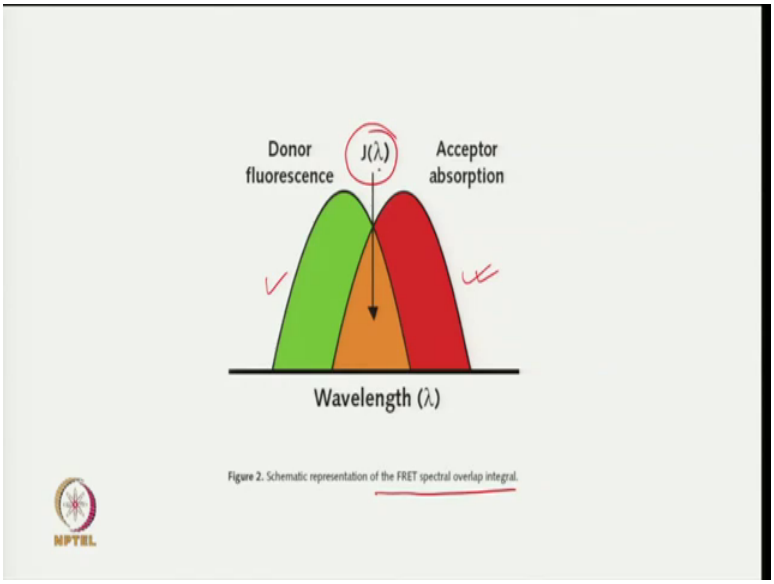

The second thing on which your transfer efficiency depends is FRET spectral overlap integral which is basically JJ. So, this is donor fluorescence, this is acceptor absorbance. So, the energy transfer will depend on J lambda.

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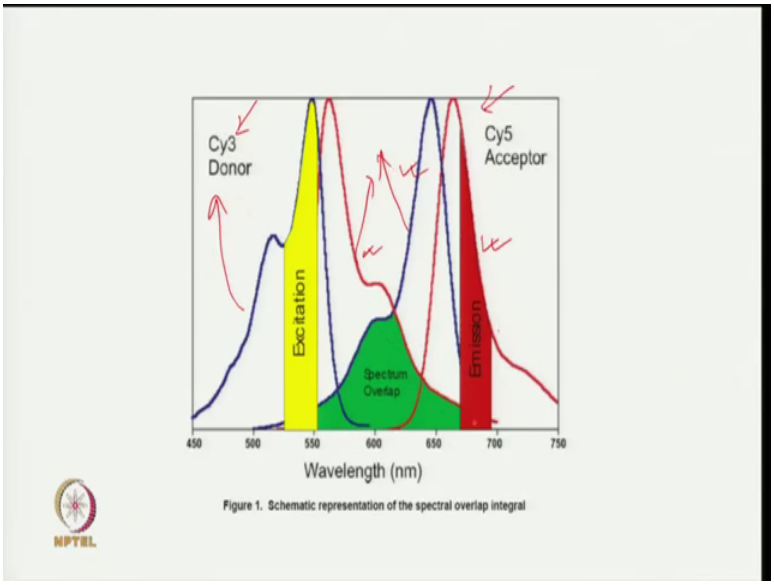

Now, here is one of the example this is Cy3 donor molecule and this is Cy5 acceptor molecule, this is your excitation, this is your emission spectra and this is your absorption spectra for Cy5, this is emission spectra for acceptor. So, blue one is absorption spectra or excitation spectra of Cy3 donor, this is emission spectra of Cy3 donor, this is absorption spectra of Cy5 acceptor and this is emission spectra of Cy5 acceptor and this is your overlap integral.

So, you can see this is emission of donor and this is absorption of acceptor and this gives you a spectral overlap and energy transfer efficiency will depend on a spectral overlay. If a Spector overlap is small then energy transfer will be small.

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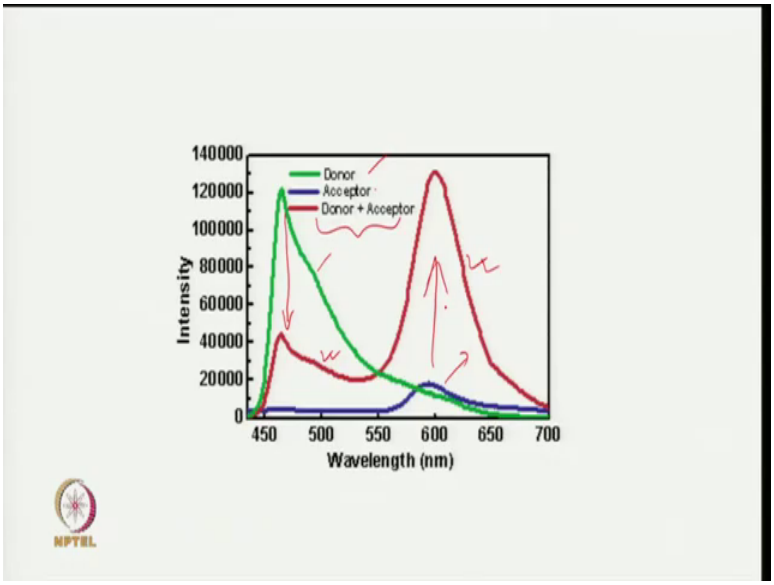

Here is the fluorescence intensity of donor when there is no acceptor, this is the fluorescence intensity of acceptor and there is no donor and when donor and acceptor are present together then you can see the emission spectra of donor and acceptor. What you see that there is a quenching of there is a quenching of donor emission and there is increase in acceptor emission if acceptor is a fluorophore but acceptor may not be a fluorophore in that case you can simply see the quenching.

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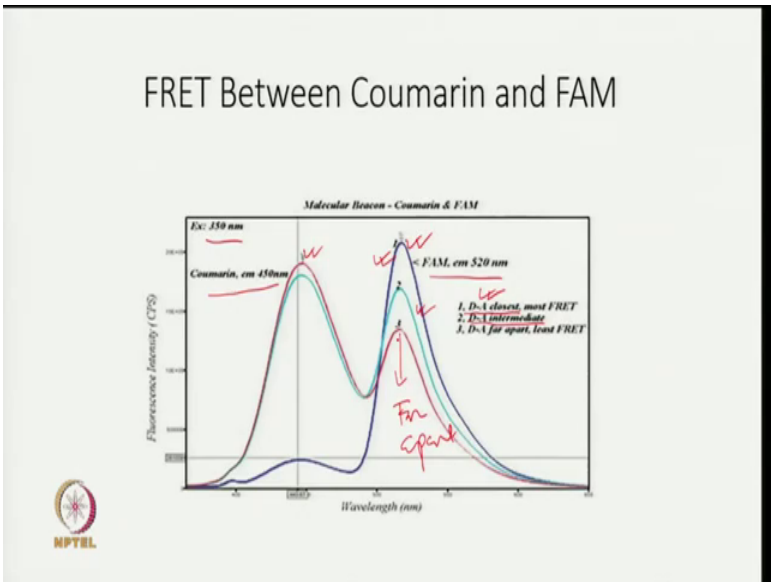

This is one of the example where FRET is shown between coumarin and FAM. So, suppose I excite coumarin at 350 nano meter, coumarin will give a emission peak at 450 nanometer. So, there are 3 emission spectra you can see for FAM. One is when D A is closest, so this is when D A is closest. The second when D A is intermediate and this is when D A are far apart, D stands for donor and A stands for acceptor.

So, when D and A are closest and you can see that there is a maximum FRET, when D and A are far apart then FRET a least. So, the increase in fluorescence intensity of acceptor well depend on the difference or depend upon the distance between coumarin and FAM. So, just by looking at the transfer efficiency we can tell about the distance between coumarin and FAM. If energy transfer is high then the distance between the donor and acceptor is going to be small.

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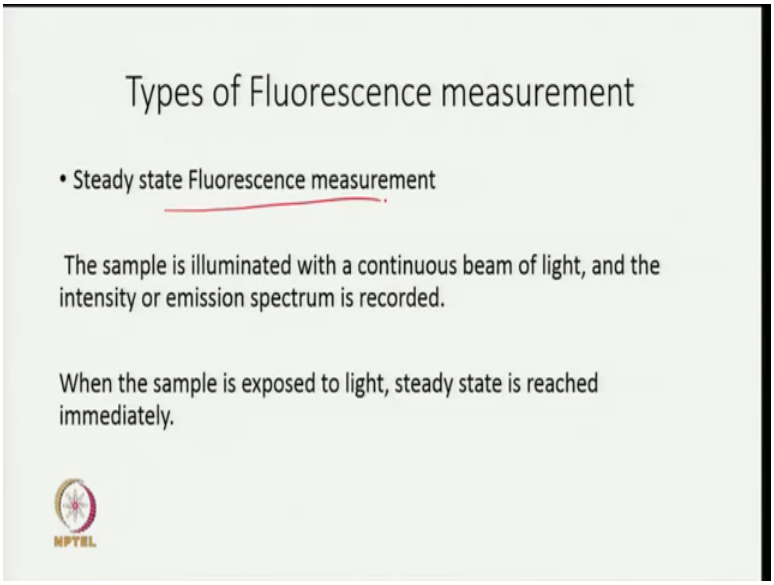

So, till now I talked about basics of fluorescence spectroscopy. Now, let us go to the types of fluorescence measurement and then we will discuss about the applications of fluorescence spectroscopy. So, there are two types of fluorescence measurement. One is a steady state fluorescence measurement in which sample is illuminated with a continuous beam of light and intensity or emission spectrum is recorded here when sample exposed to light steady state is reached immediately.

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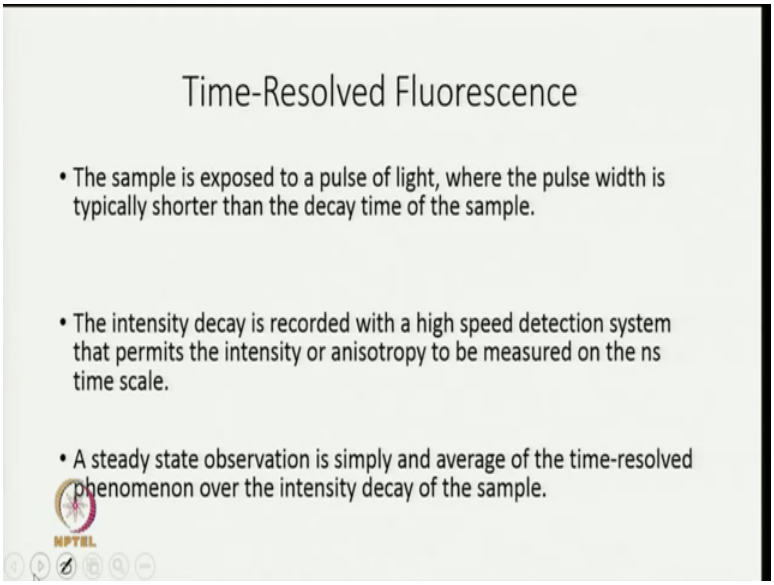

Whereas in the second kind of measurement which is known as time resolved fluorescence measurement. Sample is exposed to a pulse of light not the continuous light. Here the pulse width is typically shorter than the decay time of the sample and then what we do is we record intensity decay with a high speed detection system that permits the intensity or anisotropy to be measured on the nanosecond time scale.

Steady state observation is simply the average of time resolved phenomena over the intensity decay of the sample. So, these are the two different kind of measurement. I will start with the application of steady state fluorescence measurement and then I will go to time resolved fluorescence there I will discuss how time resolved fluorescence are measured and then what is the application.

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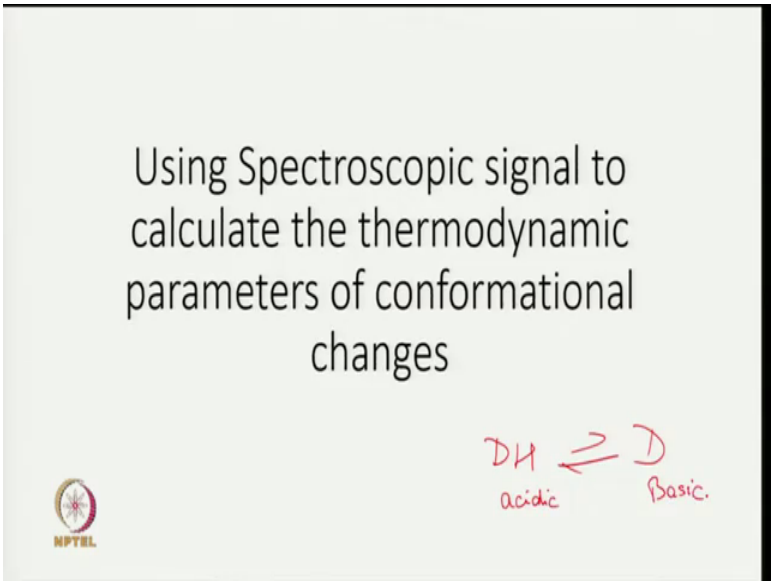

So, let us first go to look at the applications of the steady state fluorescence measurement. One of the very important application is to look at thermodynamic parameters of conformational changes. So, suppose that a molecule exists as one conformation and in the presence of suppose acid and it goes to another confirmation in presence of suppose acid and it goes to another conformation in presence of the base. So, we can look at the transition between suppose a protonated form n acidic solution to un protonated form in basic solution.

So, we can look at the thermodynamic parameters what is the equilibrium constant. For example, if we have a suppose D with H and it is going to D this is an acidic condition, this is in basic condition we can know the equilibrium constant we can know the equilibrium constant and that is quite useful in various cases, in various applications. So, I am going to tell you how to know the equilibrium constant when there is conformational changes.

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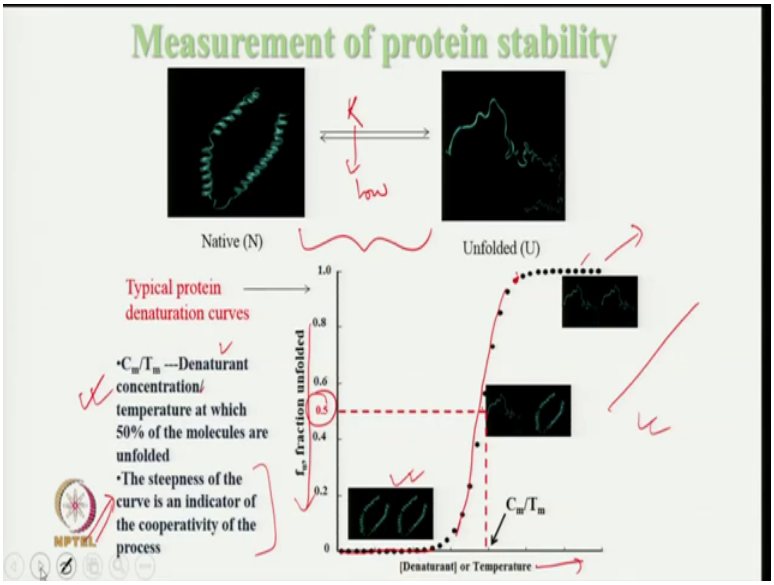

I will start with the example of a protein since protein stability is quite important in biopharmaceutical industry and we study protein stability to know how the protein is the stable and how we can change the protein stability, how we can increase the protein stability by changing the environment. So, one of the important parameter for protein is it is a stability. So, first look at what we mean by protein stability.

Protein stability is basically the conformational change of protein from native to unfolded if the equilibrium constant of this is high it means protein is unstable, if this K is low it means equilibrium is more towards native form and protein is more stable. The typical stability study is what we know as protein denaturation studies. So, what we do? We take protein solution we denature it somehow either by using pH or by increasing the temperature or by adding denaturing.

And what we do is we look at these two parameters C m, T m and the steepness. C m is basically your denaturant concentration and T m is temperature. So, C m is denaturant concentration at which 50 percent of molecules are unfolded whereas T m is temperature at which 50 percent of molecules are unfolded and your steepness of curve is an indicator of cooperativity of the process.

These are two very important parameters, I will explain with this profile this is your denaturation profile of a protein. So, what we are plotting in this is we are plotting fraction of unfolded form versus denaturant concentration or temperature. So, if denaturant concentration low or temperature is low it means that protein is in native form and when it is high than protein is unfolded form and so fraction of unfolded form will increase with the increase in concentration of denaturant or increase in temperature.

So, what you will see is that initially there is no increase in fraction of unfolded form but at a particular concentration of denaturant or temperature there is a sharp increase in fraction of unfolded form and at about this temperature almost all native protein has gone to unfolded protein. So, you can see unfolded protein here, folded protein here in between there is a mixture of folded and unfolded form what is C m? I already told you that this is a concentration at which fraction of unfolded form is 0.5 or is T m is the temperature at which the fraction of unfolded form is 0.5.

Now, this stiffness is also very important because if it is very steep it means it is a cooperative process and basically this is this only involves a native estate and unfolded state to state process. If it is like slope is like this not very steep then there is a chances of intermediates being present between native and unfolded process and then the process is not called cooperative. So, to calculate the stability of the protein we need to know what is the delta G value for this process.

So, if Delta G is negative means this process is spontaneous and it means protein is unstable. If delta G is positive it means this conformational change is not a spontaneous and it means that native state is more stable. So, one of the criteria is your C m or T m if C m and T m is large it means protein is more stable but more important parameter is delta G because delta G tells you about whether a process is spontaneous or not.

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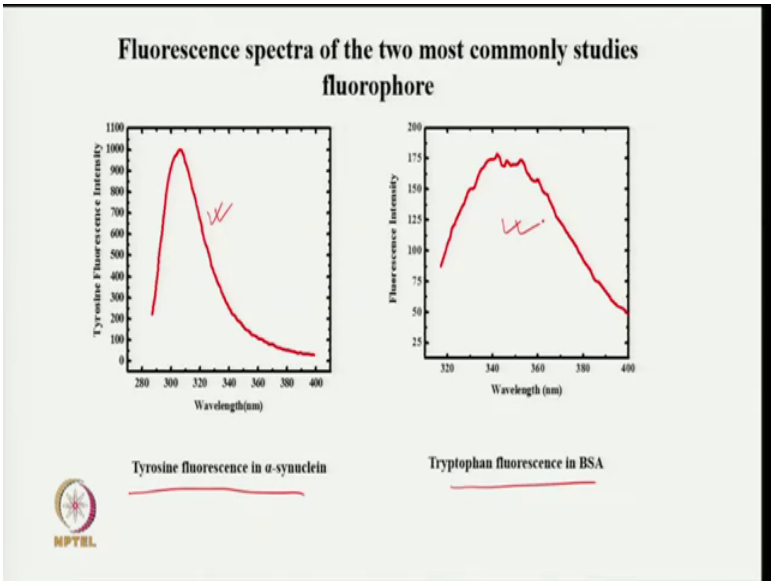

So, if I want to calculate the a delta G value or we want to know T m value or C m value we need to look at the fluorophore which is a part of protein and there are two very important fluorophore one is a tyrosine and another is tryptophan and here I can show you the tyrosine fluorescence in alpha synuclein. So, when protein does not have tryptophan then you look at tyrosine otherwise you look at tryptophan fluorescence because tryptophan has higher quantum melt.

So, alpha synuclein does not have tryptophan and so we generally look at tyrosine fluorescence in this case and this is your tyrosine fluorescence. Here is the tryptophan fluorescence for BSA, BSA is bovine serum albumin which has tryptophan residues and so we look at tryptophan.

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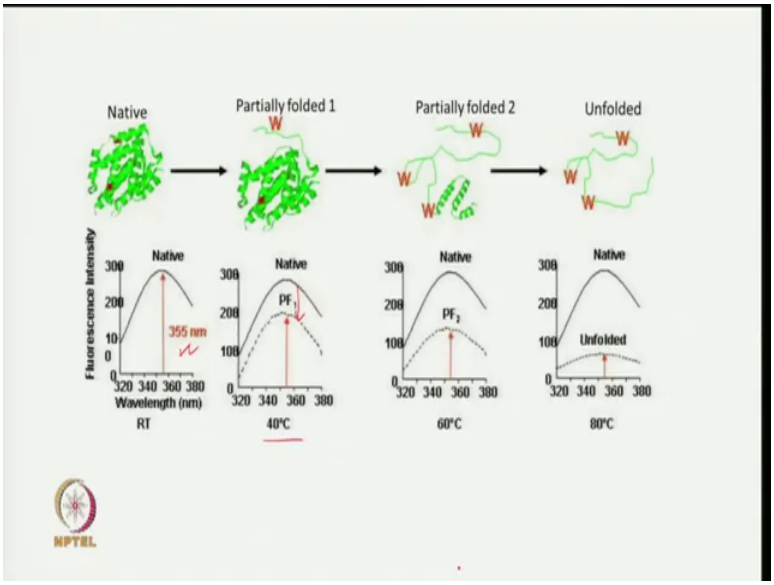

So, let us understand first what happens to fluorescence intensity of this fluorophores when you go from native state to unfolded form. So, this is native state and you can see that the fluorescence intensity is maximum at 355 nano meter at room temperature. Now, suppose I increase the temperature what will happen? That for example, at 40 degree Celsius the intensity of fluorescence decreases and you can see it here.

If it goes to this partially folded state where the tryptophan is less exposed comparatively to unfolded state and it is more exposed comparatively to partially fold at one state if further decrease and in unfolded form you see there is a large decrease of fluorescence intensity when protein goes from native state to unfolded state either due to addition of denaturant or on increase in temperature then I expect fluorescence intensity to decrease.

This decreases is because the fluorophore is going from nonpolar environment to polar environment and I have already talked about what is the effect of solvent on the fluorescence intensity of a fluorophore. So, the decrease is expected since tryptophan is going from more hydrophobic environment to polar environment.

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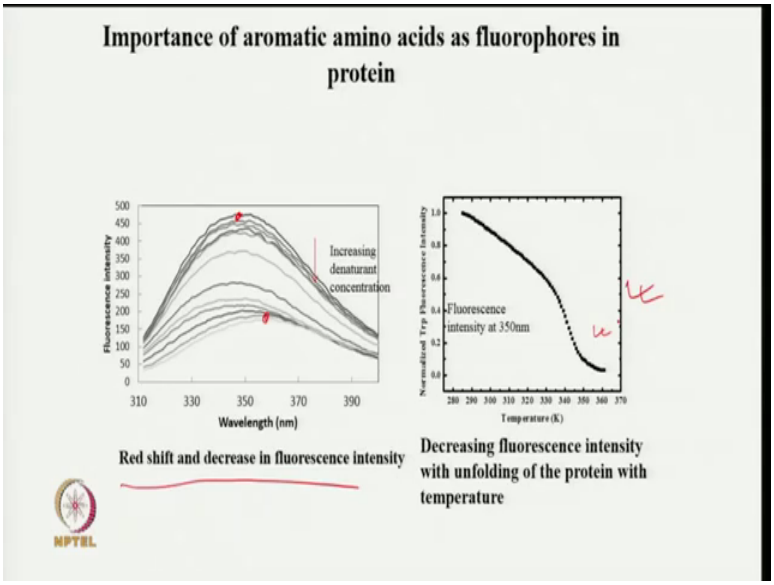

So, there is a red shift and decrease in fluorescence intensity when I add a denaturant R when I unfold a protein. So, you can see lambda max is here when you have a high amount of denaturant whereas lambda max is here so there is a red shift and again when you look at fluorescence intensity there is decrease in fluorescent intensity, this is the fluorescence intensity decrease when temperature is increased. So, when we increase the temperature protein is going to unfolded form and so we expect decrease in fluorescence intensity and that basically tells you about unfolding of protein.

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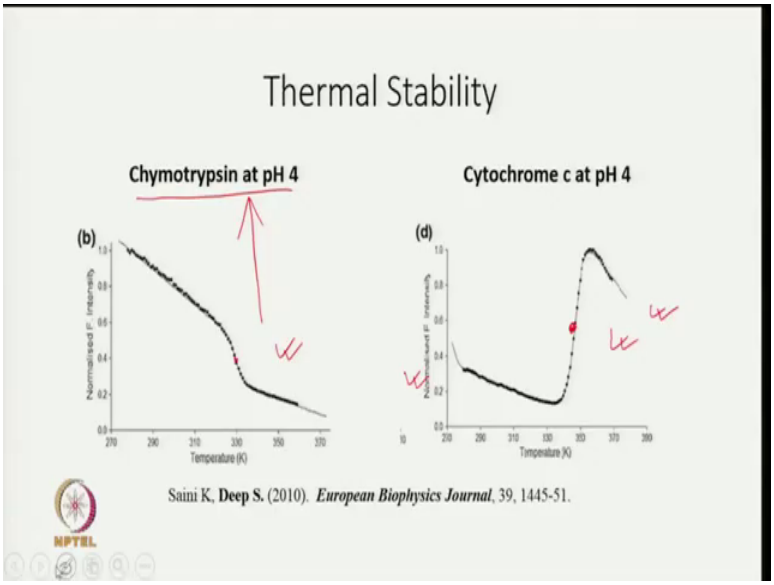

So, these are two different proteins and their thermal stability profile. The first profile is of chymotrypsin at pH 4, if I increase the temperature this is the way your denature is some profile will look like and your T m is somewhere at this point and so you can calculate the T m of chymotrypsin at pH 4. This is for cytochrome C at pH 4 and here T m is around this point this temperature and so I can also know what is the T m for cytochrome C at pH 4.

There is a 1 difference between chymotrypsin and cytochrome C. Here what is happening is tryptophan intensity is decreasing when temperature is increased and so you are looking at decrease of intensity but in this case cytochrome C what we are looking at is not tryptophan fluorescence but we are looking at basically fluorescence due to haem and what happens in this case that when protein unfolds your haem comes into contact with water and comes in contact with water and it is now no longer quince by residue inside the protein.

And so what we see is against the normal trend but it is quite obvious because here we are not looking at tryptophan fluorescence we are looking at the fluorescence due to haem and when haem is inside cytochrome C it is fluorescence intensity is quenched when it comes out as fluorescence intensity increases.

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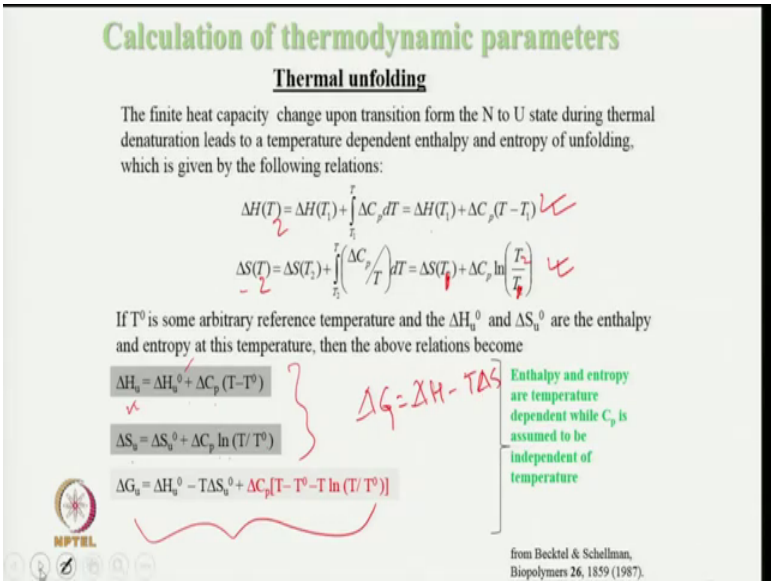

So, once we have thermal profile we can go and calculate delta G. so, suppose I have taken thermal unfolding profile and now I want to calculate delta G what we can do? This is the delta H dependence on temperature this is well known Kirchhoff equation which tells you about delta H dependent on temperature, this is steady state delta S dependence on temperature.

So, how delta S will change with the temperature? So, delta S T, T 2 you can say, this is delta S T 1 this is delta S T 2. So, Delta S T 2 is equal to Delta S T 1 plus delta C P T minus T 1 and here delta S T 2 so you can do like this T 2 will be equal to delta S T 1 plus Delta C P ln T 2 by T 1. So, if I try to write delta H of unfolded form so what I will get it? Delta H of unfolded form and delta H of unfolded form at some temperature, some temperature which I said reference temperature plus delta C P T minus reference temperature delta S U is equal to delta S U naught plus delta C P log T by T naught. So, these are the two equations and from that I can get the delta G value, delta G value since delta G is equal to delta H minus T delta S.

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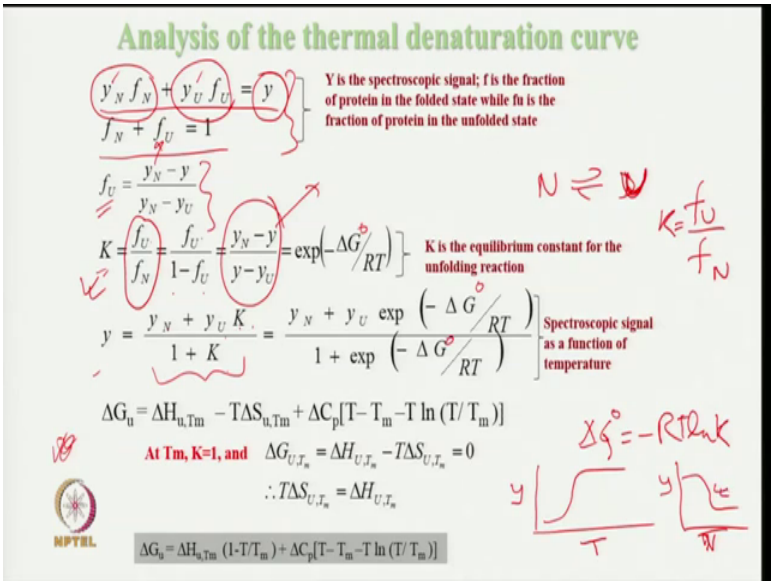

Now the next question is how to get the delta G value. So, if I want to know the thermodynamic parameter from thermal denaturation profile the way we can go is first we need to calculate K of the into D process and K equilibrium constant of this process can be determined by if N to U. Let us write into you K can be determined by taking fraction of unfolded form by fraction of native form and so these fractions can be calculated from fluorescence intensity.

Now, think of if there are only two species the fluorescence intensity which I am representing here by Y can come from either native form or unfolded form and this gives you contribution of native form to fluorescence intensity overall fluorescence intensity and this gives you the contribution of unfolded form to overall intensity, what is y N? y N is fluorescence intensity when f N is equal to 1 while y U is the fluorescence intensity of unfolded form when f U is equal to 1.

So, y N is fluorescence intensity of native form when f N is 1 and y U is fluorescence intensity of unfolded form when f U which is fraction of unfolded form is 1. So, if fraction of native form is 1 it means f U will be 0 so y is simply y N into f N which is equal to 1. So, y is simply y N when fraction of unfolded form is 1 in that case y will be equal to y U. So, overall intensity is due to intensity from native form and intensity from unfolded form.

The second equation is quite obvious. So, fraction of native form plus fraction of unfolded form is equal to 1. Now, using these two i can calculate fraction of unfolded form and when we calculate what we are going to get is f U is equal to y N minus y divided by y N minus y U and if I take this K, K is f U by f U so we can simply write f U divided by 1 minus f U because f N is equal to 1 minus f U and so this is the formula for K.

So, now equilibrium constant is related to fluorescence intensity. So, equilibrium constant is related to fluorescence intensity measured at different time of denaturation and we know that K is equal to exponential minus delta G naught by RT since delta g naught is equal to minus RT ln k. So, from fluorescence intensity I know K and since I know K I also know delta G naught if I use this equation I can express y in terms of K y N and y U.

So, y is equal to y N plus y U into K divided by 1 plus K we know K is equal to exponential minus delta G naught by RT so we can put it here and delta G U remember what is the denaturation profile? What we are doing is we are looking at y versus T and we are getting this kind of curve or we are getting y versus T we are getting this kind of curve. And so if I fit this then I can get the value of delta G.

In the next lecture, I will discuss about how these equation comes and how we can calculate the stability of protein or we can calculate the k value for our conformational change, we can calculate delta G value of a conformational change, we can calculate delta H of a conformational change, delta S of conformational change and then I can talk about the other kind of equilibria.

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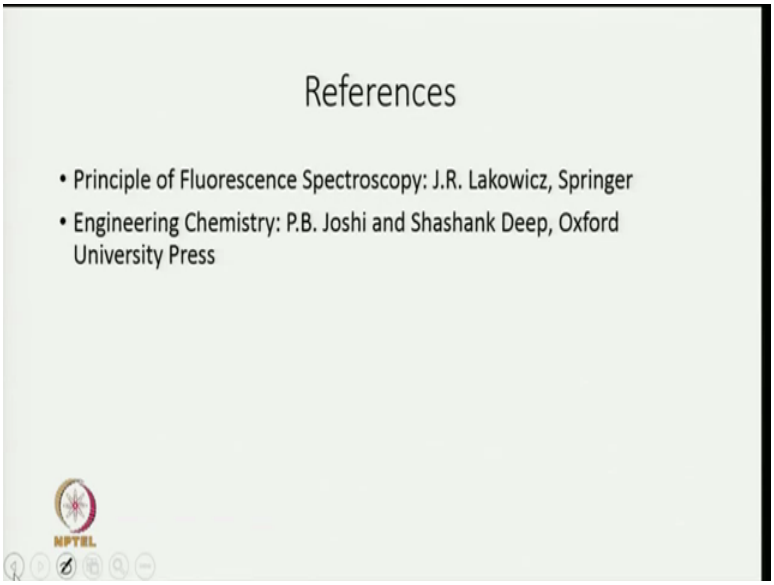

So, I would like to stop here, thank you for listening and I will like to acknowledge these two books please go and read this principle of Fluorescence Spectroscopy by Lakowicz this is a very good book on fluorescence, it has discussed in detail about each and every aspect of fluorescence. It is from the springer and if you want in short what is a fluorescence? Then you can look at the book authored by me, thank you very much.