Spectroscopic Techniques for Pharmaceutical and Biopharmaceutical Industries
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Lecture 22 - UV-Vis & Fluorescence Spectroscopy

Hello friends! Welcome to the lecture. In the last lecture I have discussed about applications of UV Visible Spectroscopy. I will continue with that and then I will go into Fluorescence Spectroscopy. In this lecture I will discuss about what is the principle of fluorescence spectroscopy.

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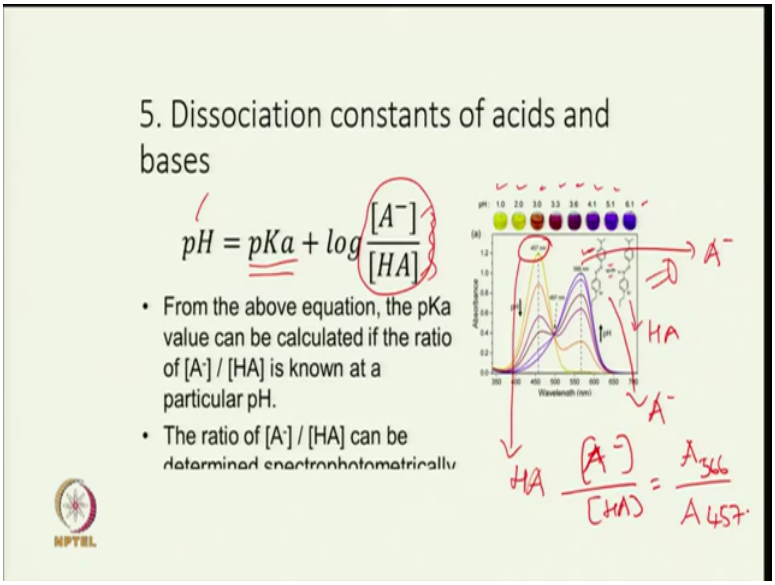

So in the last lecture I started discussing about how to get dissociation constants of acids and bases. So as you remember that the equation of Henderson–Hasselbalch which is pH is equal to p K a plus log A minus by HA. So this is basically salt by acid ratio.

So if somehow we can determine the salt and acid ratio at a particular given pH I can know what is the p K a value at that pH and that is what we try to do using the UV visible spectroscopy and then we determine the salt-acid ratio to get p K a value. So here is one example, where one dye is given whose HA form is this and A minus form this. Now, what you do is, you take the acid at different pH conditions, so from 1 pH to pH 6.1 and since this is an equilibria, so it changes from one form to another. So your acidic form will be at pH 1 or salt form A minus form will be at pH 6.1.

Now, as pH increases you can see this peak is going down and this peak is going up. So peak at 457 going down and peak at 566 going up. So what does that mean is 457 nanometer

corresponds to HA peak. Whereas 566 nanometer correspond to A minus peak and just by measuring the absorbance at 457 nanometer and 566 nanometer, I can get the ratio between A minus to HA. So A minus concentration by HA is equal to absorbance at 566 nanometer divided by absorbance at 457 nanometer. So just by doing that we can calculate the ratio between salt and acid and based on that we can calculate what is the p K a value at particular pH.

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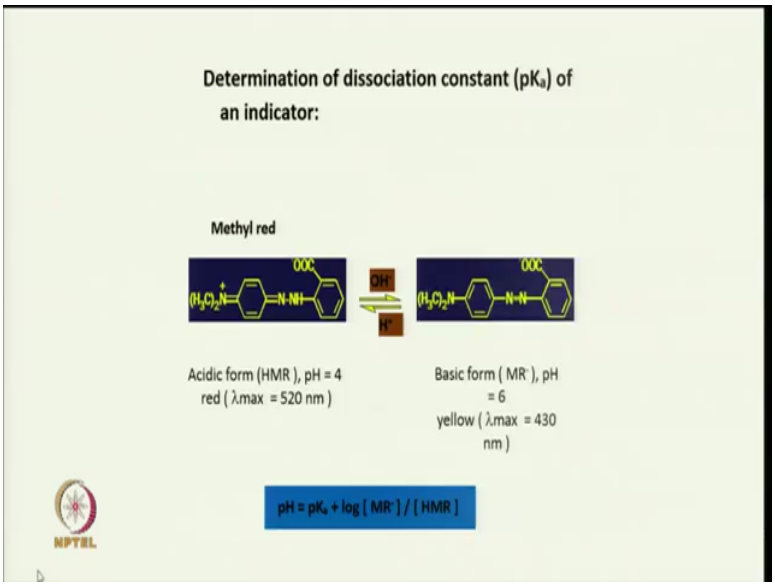

For example, we can calculate p K a value for methyl red at some given pH. So methyl red has this structure which is basically your acidic form and you can say as HMR, give this a name of HMR and this is your MR minus 1 H plus which is here as dissociated. So this is MR minus form. This happens at pH6. So suppose I want to calculate the ratio of MR minus and HMR between 4 and 6. What we can do is you need to measure absorbance at 430 nanometer ad 520 nanometer. So MR minus absorbs at 430 nanometer and HMR absorbs at 520 nanometer. So just by taking the ratio of this, we can get the ratio of salt and acid and that can help you in the determination of K a value at a particular pH.

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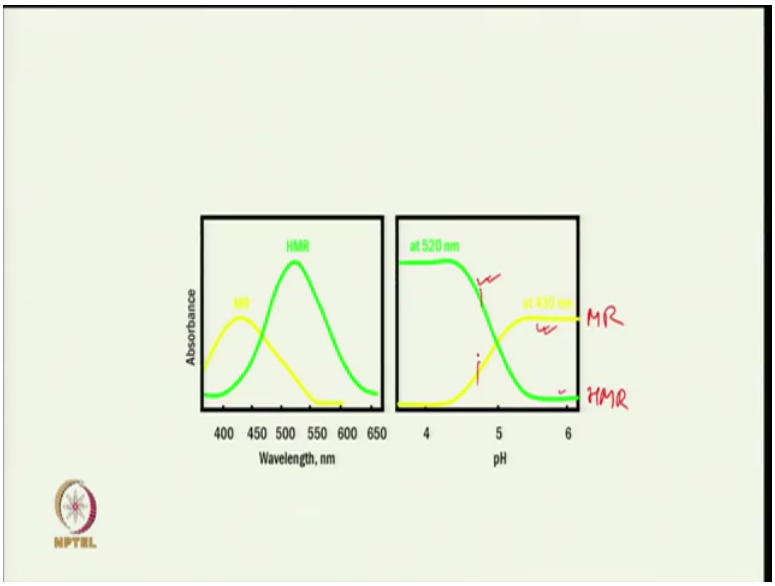

So this is your absorbance of the two species as a function of wavelength. So this is your peak for HMR and this is peak for your MR. and if I plot the absorbance, how absorbance at 520 nanometer decreases with increase in pH, this is your plot and as this will start decreasing, this starts increasing which is the peak due to MR. So this is a peak due to MR and this is a peak due to HMR. So at 4 HMR is present and at 6 MR is present. In between, we can get the acid-salt ratio and by measuring acid-salt ratio or by calculating acid-salt ratio I can now p K a value at a particular pH.

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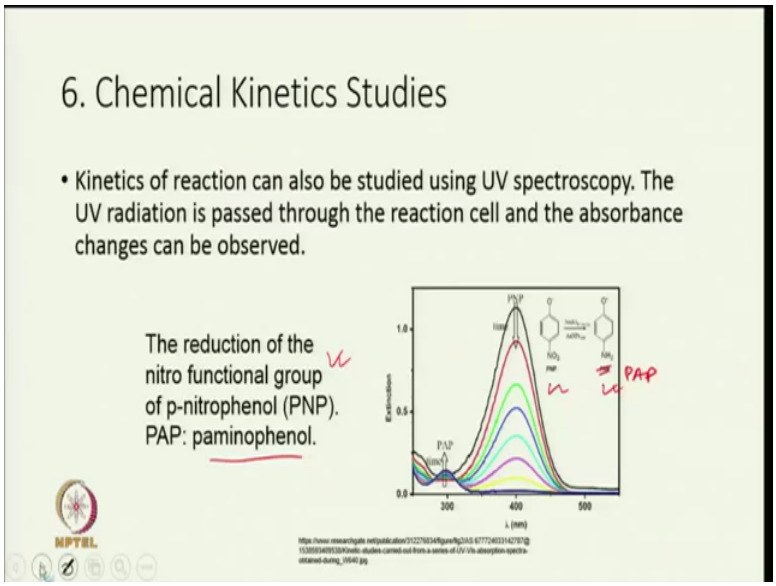

UV visible spectroscopy can also be used to know the chemical kinetic parameters. Kinetics of any reaction can be studied using UV visible spectroscopy. The UV radiation is passed through reaction cell and absorbance can be observed as a function of time.

For example, this is the reduction of nitro functional group of paranitrophenol to paraaminophenol. So this is paranitrophenol (PNP) and this is your paraaminophenol, so this is PAP. So PNP to PAP. So now you can see that PNP decreases with increase in time whereas PAP increases with time, it means PNP is getting converted into PAP. And you can look at minus D PNP by DT or you can also look at D PAP by DT to know the rate of reaction. And kinetics is quite often used to know the mechanism of the reaction and so measuring the rate of reaction is quite important when we are dealing with the mechanism of the reaction and UV visible spectroscopy can be used to know the kinetics or can be used to observe the kinetics of a reaction.

Here, I am not going to discuss in detail how to get the kinetic parameters using UV visible spectroscopy. But I am going to discuss, once I complete the fluorescence spectroscopy what I will do is I will go and show you how your UV visible spectroscopy or fluorescence can be used to determine different kinetic parameters. In fact, I will also go and show you how to obtain different binding parameter using spectroscopic signal. For example, absorbance of fluorescence intensity. You can calculate the Delta G of a reaction or the equilibrium constant, you can calculate Delta H, you can calculate Delta S and you can also know whether a reaction is a first order, is your reaction is a second order and once you know that you can also propose a mechanism, you can also calculate the activation parameter.

This all can be done using a spectroscopic signal. Here in UV visible spectroscopy a spectroscopic signal is absorbance whereas in fluorescence intensity spectroscopic signal can be fluorescence intensity.

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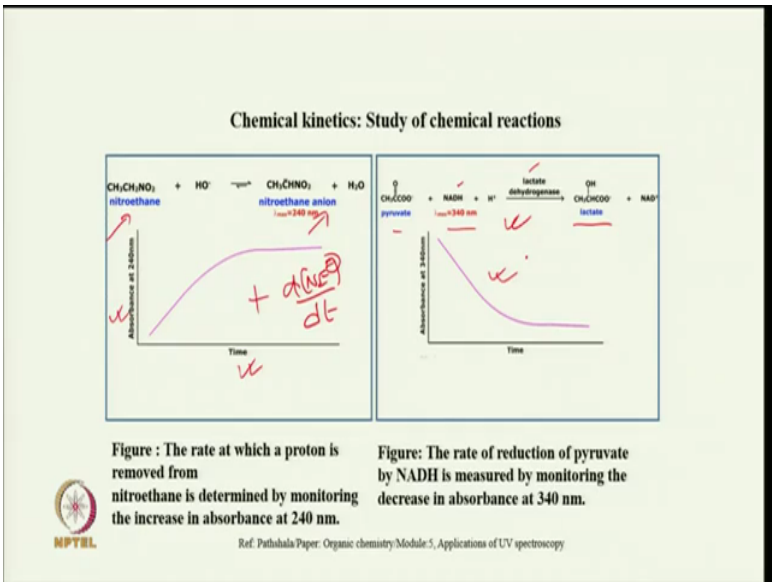

So here is one of the example where UV visible spectroscopy can be used to study the chemical reaction. In this reaction nitroethane is converted into nitroethane anion which absorbs as 240 nanometer and you can look at the absorbance at 240 nanometer with respect to time to look at the progress of the reaction. And from this, we can always calculate the your nitroethane by dt, d nitroethane by dt. ethane anion, this is nitroethane anion by dt, okay. Sorry, this should be plus since product is increasing.

So here, in this case what we looked at is the kinetics of product formation. We can also look at the kinetics of reactant use. How a reactant is utilized in a particular reaction? For example, in this case pyruvate plus NADH gives you lactate. In presence of lactate dehydrogenase, you can look at the absorbance at 340 nanometer which is due to NADH which is a reactant. And as expected, the absorbance will decrease with time. But here, the absorbance signal of reactant can be used to look at the kinetics of the reaction.

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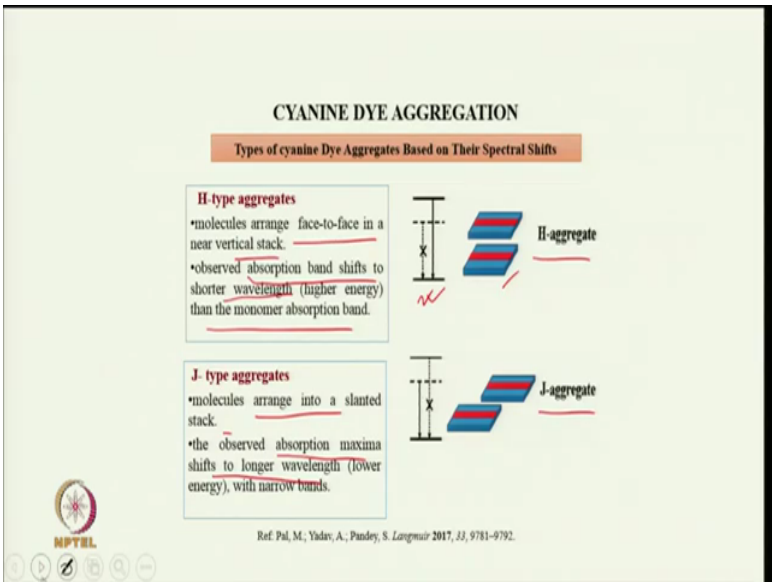

So UV visible can be used to discuss cyanine dye aggregation, different kind of aggregation. The different kind of aggregate has different spectral shift. For example, there are two different kind of aggregates; cyanine dye. One is H-type aggregate where molecules arrange face-to-face in a near vertical stack. So observed absorption band shift to shorter wavelength then the monomer absorption band and this is your typical diagram and this is your H-aggregate.

J-type aggregate, the molecules arrange in slanted stack. It has a slanted arrangement and that arrangement is called J-aggregate and the difference between these two can be done using UV-Visible Spectroscopy because here absorbance maxima shifts to longer wavelength with narrow band. So, H-aggregate has shorter of wavelength then the monomer whereas J-aggregate has longer wavelength than monomer.

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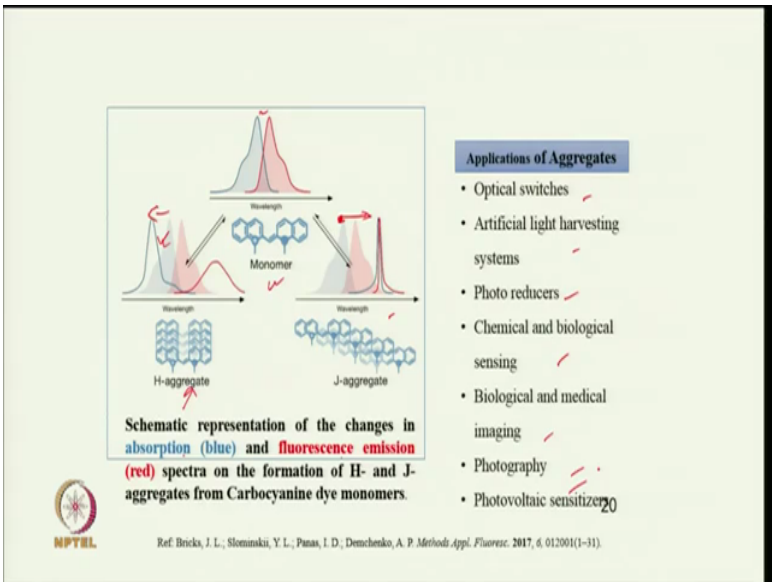

And here is your the spectra. This is for monomer, this blue is absorption. This red one is fluorescence emission. So absorption band if you look it is here and if you see at this position, it is going to sort a wavelength and if you look at this is the absorbance and this goes to higher wavelength in case of J-aggregates. These J-aggregates and H-aggregates are quite useful in optical switches. They are used in artificial light harvesting system. They are used in photo reducers. They are used in chemical and biological sensing. They have applications in biological and medical imaging. They have application in photography and they can be used as photovoltaic sensitizer.

So these aggregates have several different applications and UV visible spectroscopy can be used to know whether a particulate aggregate form is of H-type or J-type.

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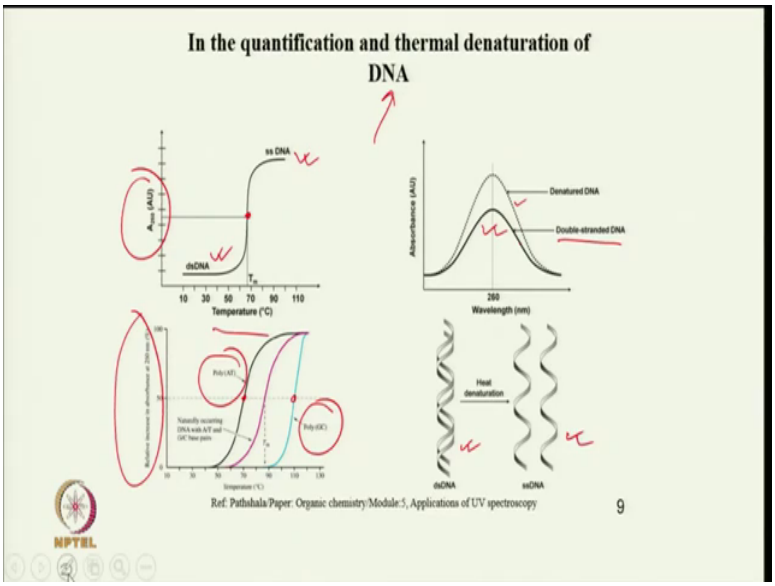

Not only that UV visible spectroscopy is quite of often used in biochemistry for quantifying and to study thermal denaturation. So this is for DNA molecule and you see this is double stranded DNA going to single stranded DNA with the temperature and the denaturation temperature can be calculated by looking at absorbance at 260 nanometer. I have already told that 260 nanometer corresponds to your DNA Lambda max and you can see that at higher temperature absorbance increases when double stranded DNA goes to single stranded DNA. And just by looking at the 260 maximum by 2 you can know what is the TM value.

This is the typical diagram of double stranded DNA. This is typical diagram of denatured DNA. Although Lambda max does not change but there is change in absorbance. So double stranded DNA has lower absorbance compared to denatured DNA. Again, you see the AT base pair and GC pair is going to have a different kind of TM. And if you look at this, that can also be looked through the absorbance at 260 nanometer and now, you can see this is the TM for Poly-AT and this is the TM for Poly-GC and you can see that Poly-GC is more stable compared to Poly-AT. Poly-AT, it is around 70 degree Celsius. But Poly-GC it is around 110 degree Celsius and heat denaturation can be thought of like this from double stranded to single stranded.

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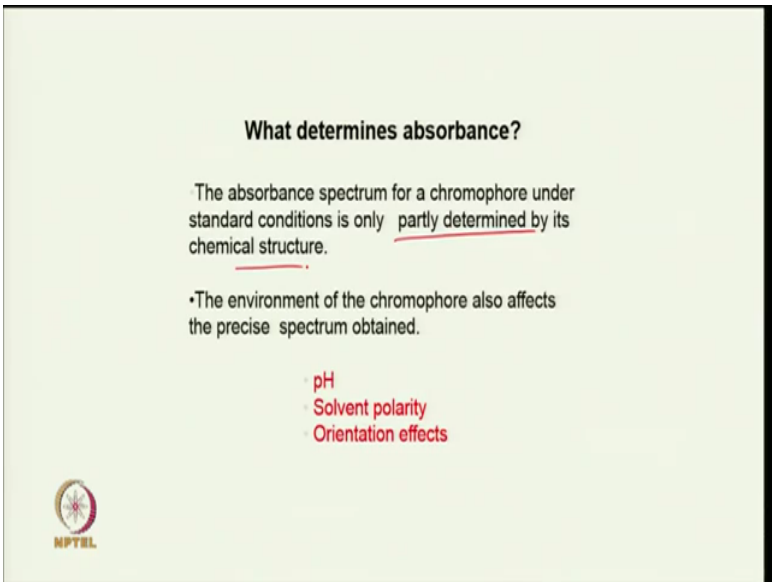

Now absorbance is not only dependent on a particular functional group. It is only partly determined by its chemical structure. The environment of chromophore also affect precise spectrum obtained. For example, pH can affect the spectrum. The solvent polarity can affect the spectrum and orientation can affect the system. For example, I showed you one case.

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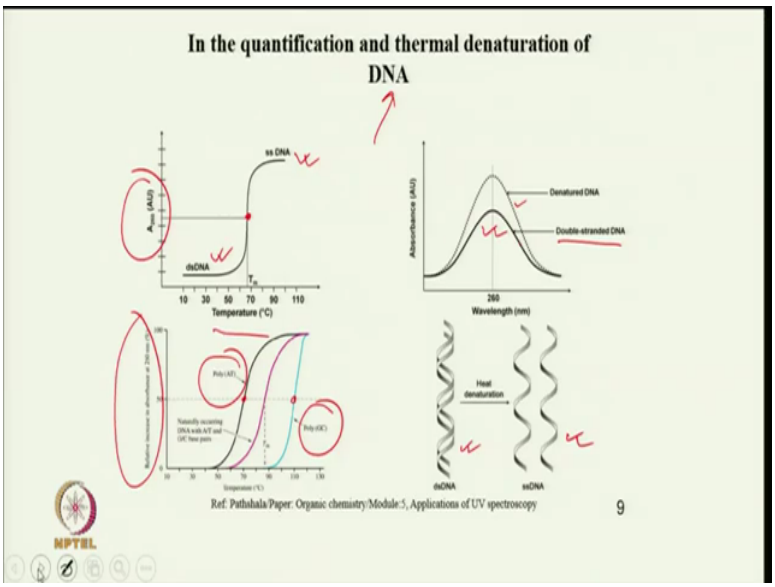

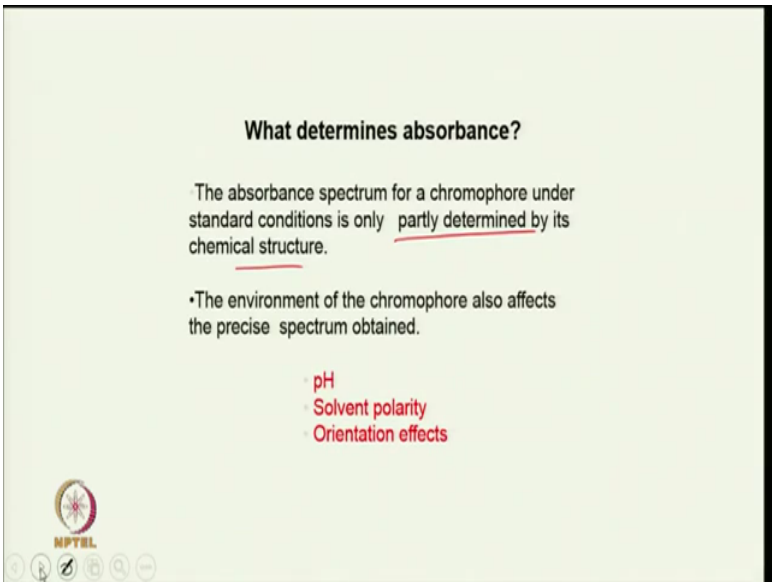

That here your single stranded DNA and double stranded DNA has different spectrum profile, different spectrum profile, that is because of change in the environment. And that can be used to look at the environment associated with particular chromophore.

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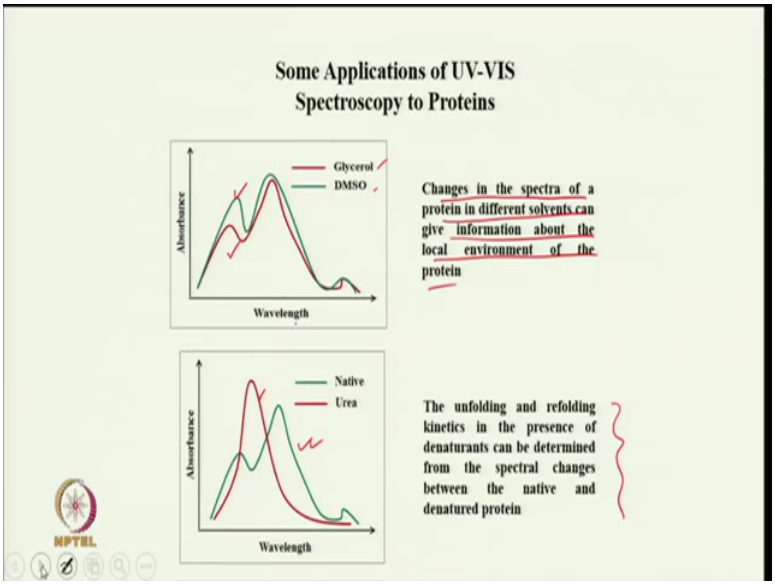

For example, changes in a spectra of protein in different solvent can give information about the local environment of the protein. You see this is absorbance versus wavelength, same protein, same concentration. If you look at the spectra profile in presence of two different solvent, the spectra profile is quite different. This is in the presence of DMSO. This is presence of Glycerol. And just by looking at the spectra you can tell what is the changes going on around the chromophore in that particular solvent.

The unfolding, refolding kinetics in the presence of the denaturants can also be determined from the spectral changes between native and denatured protein. For example, this is a native protein. The spectra is quite different than spectra of that protein in urea. And so the spectra can tell you about the different confirmations of the protein present.

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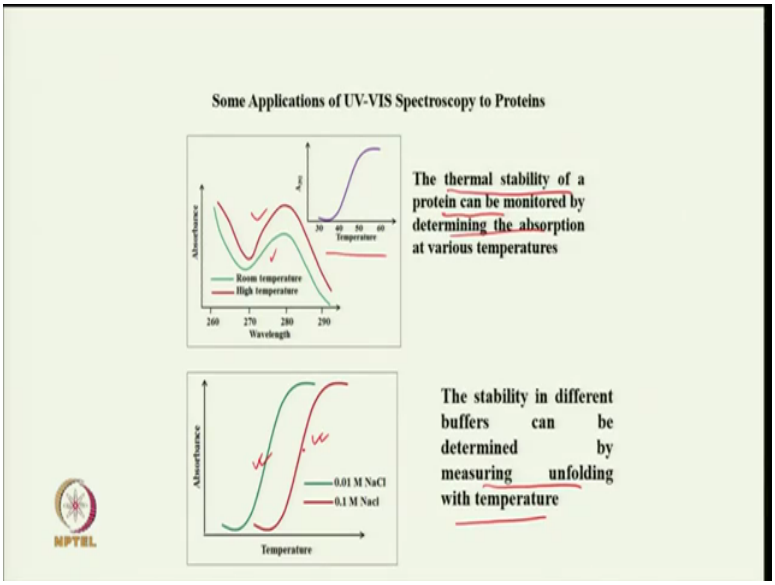

Other application is just like DNA, we can also monitor the thermal stability of protein by monitoring the absorption at various temperature. Absorption at 292 nanometer with temperature can give you the information about the TM value. And you can look that room temperature and high temperature spectra of a given protein is quite different.

We can also look at stability in different buffers while measuring unfolding with the temperature. So this is the curve obtained for, this is the stability curve which is basically absorbance versus temperature curve for a particular protein at 0.1 molar NaCl whereas this is at 0.01 molar NaCl. So with increasing concentration, with the increasing concentration what is happening is TM is shifting to higher value, it tells you that the introduction of salt which is NaCl in this case is increasing stability of that protein.

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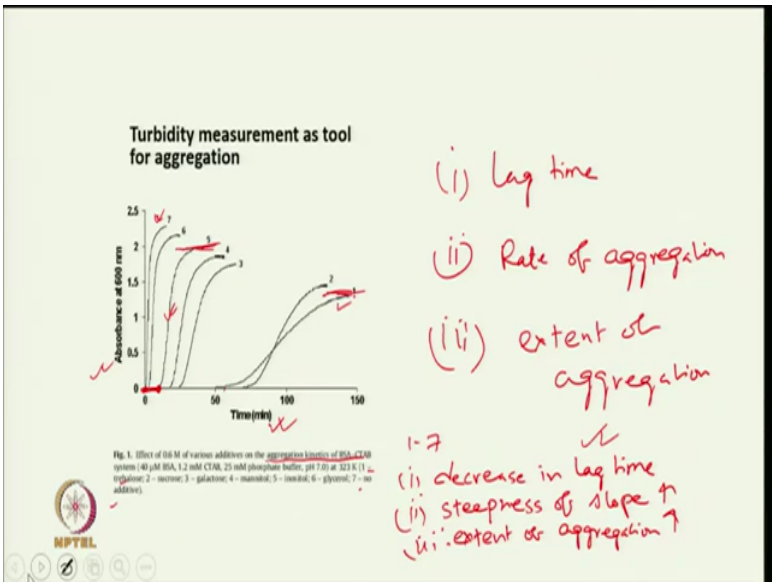

So not only we can know the thermal stability of a particular protein at a given pH or given solvent condition by looking at the absorbance as a function of temperature. We can also tell about the aggregation or effect of a particular solvent on the aggregation kinetics. So aggregation kinetics can also be measured using absorbance as 600 nanometer.

As I discussed earlier, here what we are looking at the aggregation phenomena or a scattering phenomena which is due to formation of aggregate, it is not the absorbance. So we generally measure absorbance as 600 nanometer, it is just to avoid looking at the absorbance which generally happens around 280 nanometer because of (())(20:17). So we are trying ourselves to be in a region which is quite far from the absorbance. Quite far from the 280 nanometer where our (())(20:30) absorbs. So when we look at absorbance as 600 nanometer, we are looking at the increase in absorbance due to a scatter and that is basically because of increase in aggregates, increase in the aggregates. So by looking at the absorbance at 600 nanometer versus time what we are looking at is the aggregation kinetics.

And in aggregation, three things basically we look at, first is your lag time, the second is rate of aggregation, and third thing is your extent of aggregation. And all three things can be qualitatively obtained from the measurement of aggregation kinetics using UV visible spectrophotometer.

So what we are looking at absorbance as 600 nanometer. So basically the distance here where the increase in absorbance is very minimal is known as lag time. Whereas, if you look at this

slope, that will give you idea about extent of aggregation and here this plateau can give you the idea about extent of aggregation. And if you look from this place, what you can see is that from 1 to 7, you see there is first increase in lag time. If you go from 7 to 1 or you go from 1 to 7, let us go from 1 to 7, what we are seeing is first decrease in lag time. Second is steepness of slope increases and the third thing is extent of aggregation which is given by this plateau increases. So extent of aggregation increases.

And if you look at the first is in presence of trehalose. So this is in presence of trehalose where 7 is in presence of no additives. This is no additives, okay. So if you go from 7 to 1 then what you see, increase in lag time, steepness of slope decreases, extent of aggregation decreases. So the first which corresponds to trehalose is able to increase the lag time and able to decrease the rate of aggregation and able to also decrease extent of aggregation. So if you are looking at inhibitor for aggregation, trehalose will be the best for the aggregation of, this is basically for BSA CTAB system**.** So if you are looking for inhibitor of BSA CTAB system and trehalose is going to be the best inhibitor.

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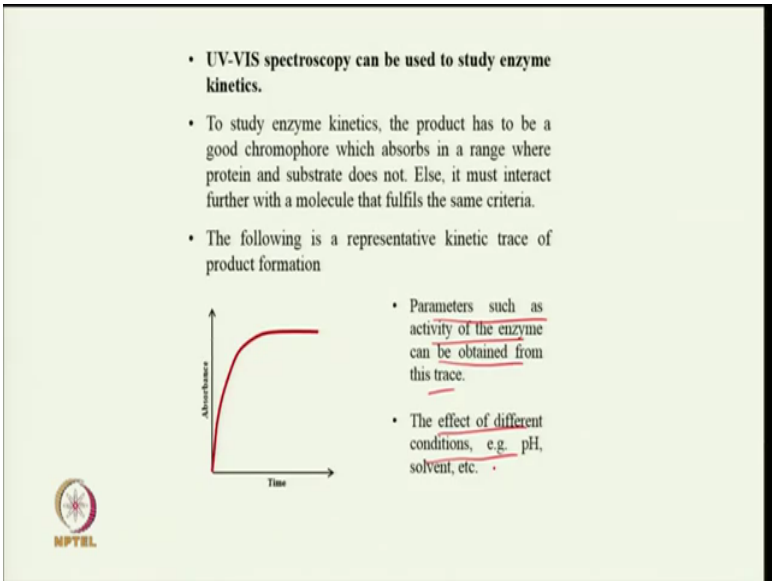

UV visible spectroscopy can also be used to study enzyme kinetics. For studying enzyme kinetics, you need to have a good chromophore which absorbs in a range where protein and substrate does not. So this is the representative kinetic trace of product formation and parameters such as activity of enzyme can be obtained from this trace and we can also look at the different, effect of different conditions on rate of product formation. For example, pH effect on rate of product formation or solvent effect.

So these are the kind of things we can do. Here, I am not telling you in detail but one lecture I am going to devote where I am going to look at how to get different thermodynamic and kinetic parameters using spectroscopic signal.

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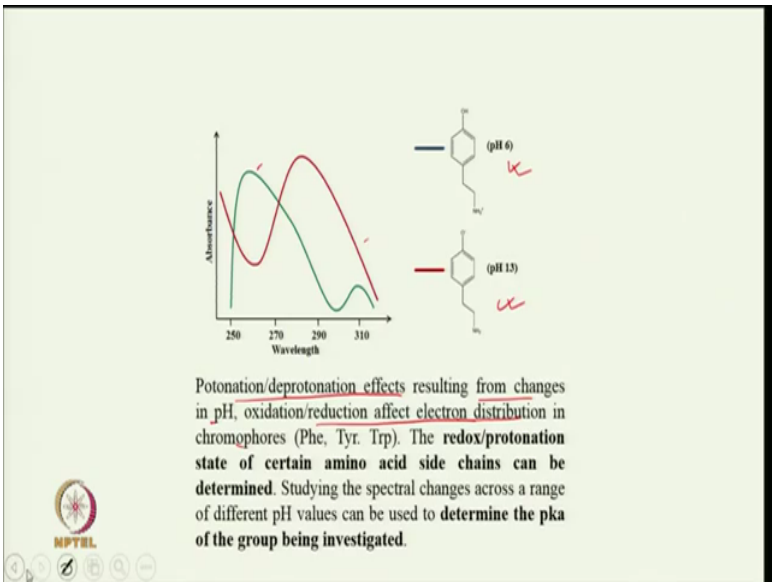

So now look at this the potonation, deprotonation effect resulting from change in pH. This oxidation, reduction affect your electron distribution in chromophore and here you see this is your pH 6, this at pH 6, this is at pH 13, and both have different kind of spectra. So at pH 13 you have this spectra, at pH 6 you have this spectra. So I have already told this can be used to get the p K a value.

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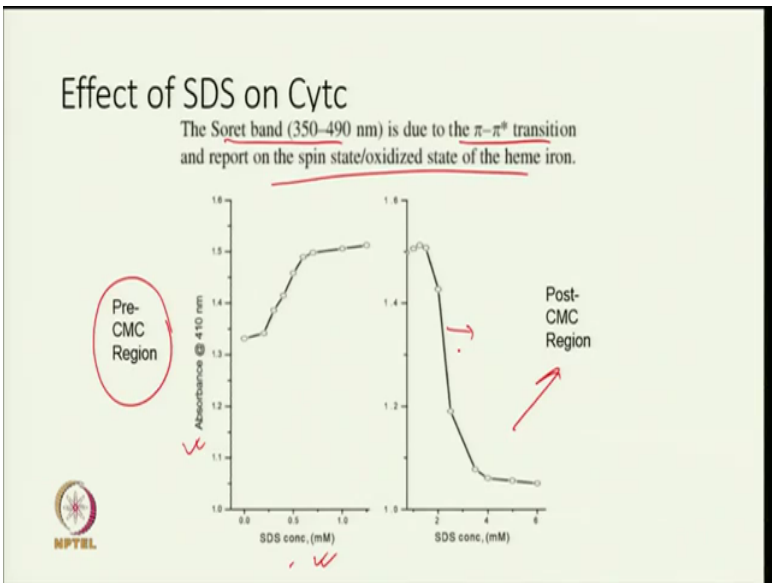

Now, I will tell you one effect of ligand on a protein. So effect of ligand on protein can also be studied. You can see how the structure of a particular protein changes when you add a particular additive or ligand. Cytochrome C has a heme group and it has well-known solid band which is between 350 nanometer to 490 nanometer and that is due to pi to pi star transition and the change in the Lambda max can happen due to spin state, oxidized state of heme group.

So here I am showing you effect of SDS which is sodium dodecyl sulfate, a surfactant on cytochrome C. so we can look at absorbance as 410 nanometer with SDS concentration and this is in in pre-CMC Region of SDS and you can see that absorbance is increasing with increase in SDS concentration. But in post post-CMC Region, the effect is quite different and what you can see is with the increase in SDS concentration there is a decrease in the absorbance.

So you can tell that within one region there is a different kind of confirmation chain and in the post-CMC region there is a different kind of structural chain. And then you can use different kind of spectroscopy to get more idea about what kind of structural changes happening in the protein.

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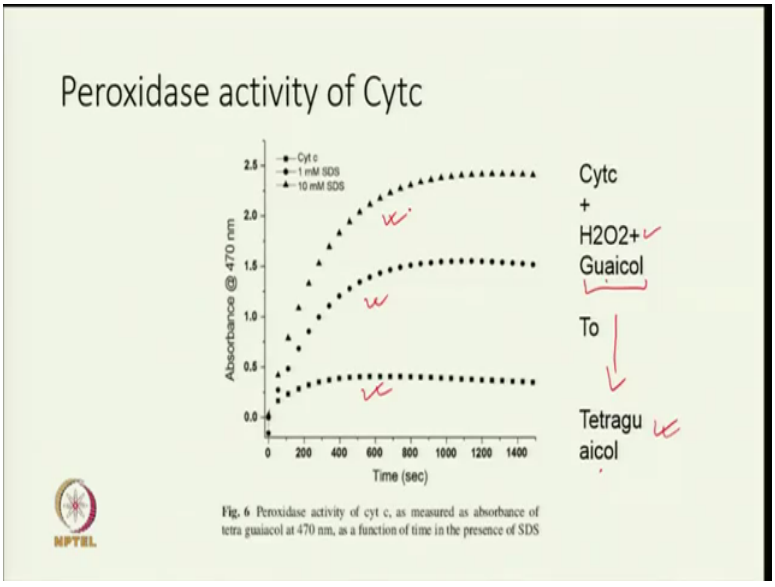

I will discuss in one of my lecture in detail how we can use different kind combination of a spectroscopy to know about what kind of structure our protein gets on interaction with ligand. Here I am showing you peroxidase activity of Cytochrome C. Again, this kind of activity can be looked at by looking at absorbance. What we do is we add H2O2 and Guaicol and what we

get a Tetraguaicol which has absorbance as 470 nanometer. So if cytochrome C is active or cytochrome C has peroxidase activity then it can convert this compound into this one which absorbs at 470 nanometer.

Now, you can see that cytochrome C is here, cytochrome C gives you this kind of curve, so absorbance at 470 nanometer is a smaller compared to when I put 1 millimolar SDS and 10 millimolar SDS. So just by looking at this kind of curve you can tell what is happening to peroxidase activity of cytochrome C when SDS is added. So this kind of study can also be done through UV visible spectroscopy.

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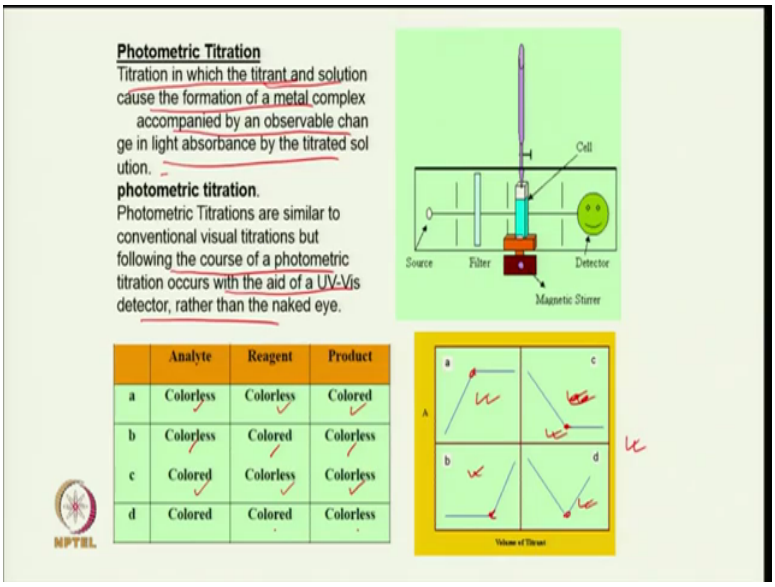

We can also study the titration, okay. And we can know the protein ligand ratio using UV visible spectroscopy. Okay. This is a titration in which titrant and solution causes the formation of metal complex accompanied by an observable change in light absorbance by titrated solution. They are similar to conventional visual titration, only thing is here we are following the course with add of UV visible detector, rather than naked eye. So we can have different kind of color in a light reagent and product and based on that we will get different kind of your titration curves.

For example, if you have colorless in light, reagent is colorless and product is colored in that case, we will get this kind of titration profile. If an analyte is colorless, reagent is colored and product is colorless, then we will get this kind of titration profile. When analyte is colored is colored, reagent is colorless and product is colorless, in that case certainly there will decrease

in the, so you will get this kind of titration profile. This is colored, and this is colorless, then you can get this kind of profile.

So different kind of titration profile will be obtained depending on the type of analyte reagent and product, a color of analyte, reagent and product. But you can get the end point. You can get the end point from all this titration profile. So here is the end point, here is the end point, here is the end point and here is the end point. And that can tell you about the stoichiometric ratio of analyte and a reagent which results into a product.

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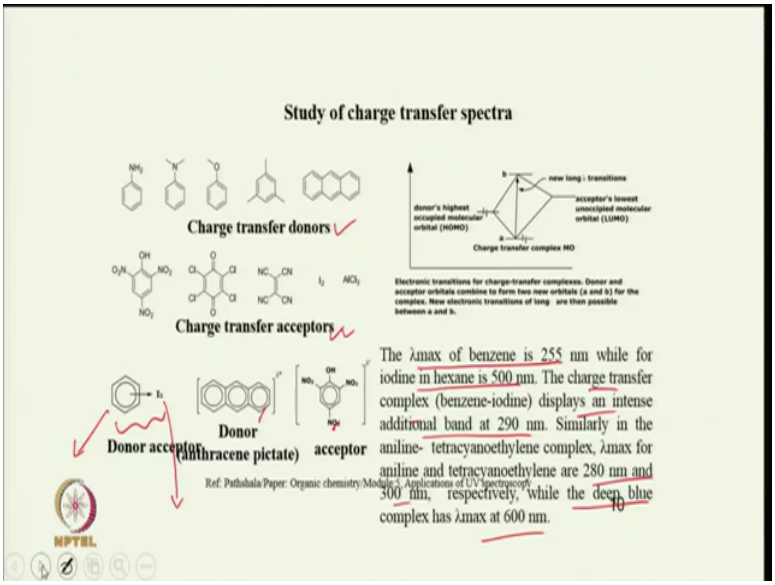

We can study charge transfer spectra. So these are the charge transfer donor. These are the charge transfer acceptor and we can look at the complexes. For example, Lambda max of benzene is 255 nanometer while iodine in hexane is 500 nanometer. The charge transfer complex displays an intense additional band at 290 nanometer. So this benzene is at 255 nanometer, this absorbs at 500 nanometer. But this complex absorbs at 280 nanometer and similarly in aniline and tetracyanoethylene complex Lambda max of aniline and tetracyanoethylene is 300 nanometer whereas complex as Lambda max around 600 nanometer. Here you see anthracene pictate. So this is donor, this is acceptor and they have Lambda max and we can study a charge transfer spectra while looking at absorbance at different nanometer.

So this is all about UV visible spectroscopy. There are hundreds of applications of UV visible spectroscopy. Few of them I have showed you and a lot more I will be showing in next set of lectures. But the idea is same either you can do qualitative analysis or quantitative analysis.

Quantitative analysis using your UV visible spectroscopy. For qualitative analysis Lambda max is important. For quantitative analysis absorbance at Lambda max is important. What does that means is epsilon at Lambda max is important and where Lambert's law can be used to get the quantity using UV visible spectroscopy.

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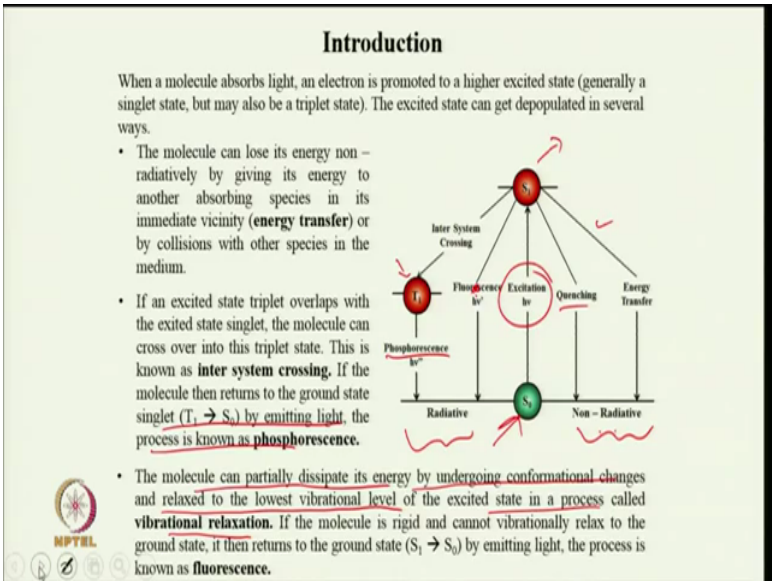

The next thing is fluorescence spectroscopy and this is based on emission spectrum, emission spectra or emission phenomena. So when a molecule absorbs light, an electron is promoted to higher excited state, the excited state can get depopulated in several different way and that is what is shown here. So this is your ground state, it goes to this one on absorption of energy and then there can be different things happening, some can be non-radiative and some can radiative. Non-radiative means now light is getting produce, only energy will be lost as a heat whereas in radiative process energy is lost as a light.

So first thing quenching can happen in the excited state. Quenching is your non-radiative process, energy transfer can take place which is non-radiative whereas two different kind of radiative process can happen, it will directly go to ground state, in that case that is known as fluorescence. Whereas, if it does undergo intersystem crossing to a triplet state and then emission takes place, that process is known as phosphorescence.

So there are two radiative processes, one is fluorescence and another is phosphorescence. Fluorescence happens from singlet excited state to singlet ground state whereas phosphorescence happens from T 1 to S naught. So this is T 1 to S naught by emitting light, then this process is called phosphorescence. And in the fluorescence what happens is first

molecule dissipates its energy by undergoing conformational changes and relaxed to the lowest vibrational level of excited state in the process called vibrational relaxation. And that is basically non-radiative process. And after that from this place, so this is suppose this is the lowest vibrational level of the excited state, so first it comes to lowest vibrational levels of excited state and then radiative process happens and the radiative process is called fluorescence.

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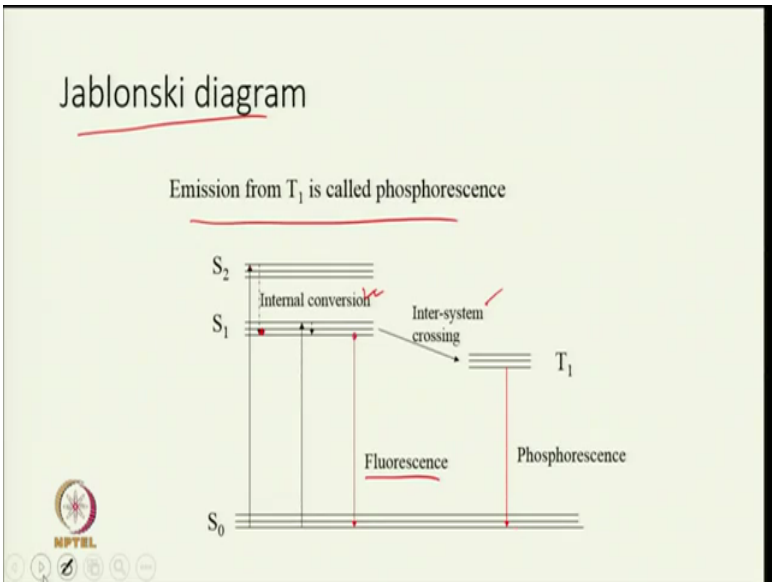

This is another way to represent same phenomena. This diagram is basically called Jablonski diagram. Here we have already told the emission from T 1 is called phosphorescence. So what is happening that if the molecule absorbs light it goes from S naught state to S 2 and then internal conversion takes place such that it comes back to lowest vibrational state and then the fluorescence will happen from the lowest vibrational state. So the excited molecules come back to ground state by emitting light and that process is called fluorescence. So coming from the upper vibrational level to lowest vibrational level of the excited stage is known as internal conversion. Whereas going from singlet state to artipulate state is known as inter-system crossing and coming from triplet state to ground state is known as your phosphorescence.

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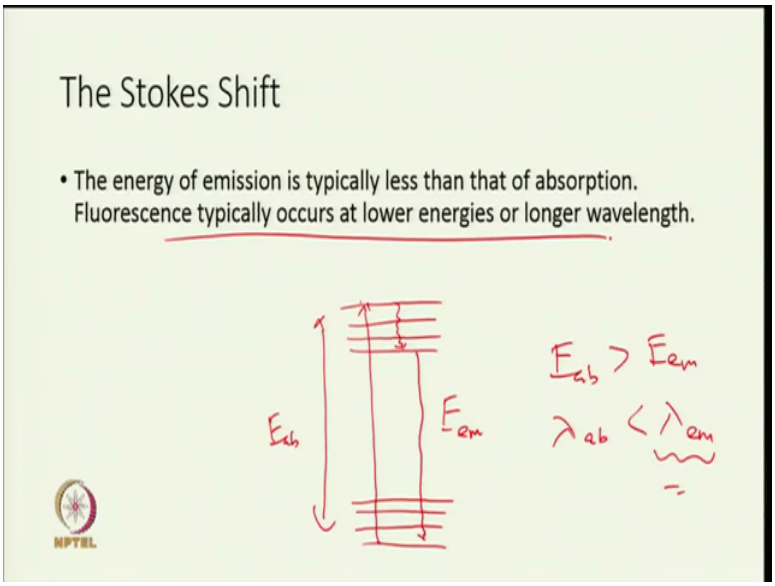

Now, there are some important facts about the fluorescence. First is known as stokes shift. What does it tells you that energy of emission is typically less than that of absorption and so fluorescence typically occurs at lower energy or longer wavelength compared to absorbance. And this is quite clear because if you remember, these are the excited state vibrational levels, this is ground state vibrational levels. So what happens that first your molecule goes from the ground state to some of the vibrational levels. Some of the vibrational level depending on how much energy you have supplied. But what happens that after that it comes back to the lowest vibrational level of the excited state and from there fluorescence takes place.

So energy of absorption is always higher and energy of emission. And so, energy of absorption is always higher than energy of emission and so Lambda max for absorption is always lower than Lambda for emission. And so Lambda emission is always high. So that is what it is written that fluorescence typically occurs at lower energies or longer wavelength.

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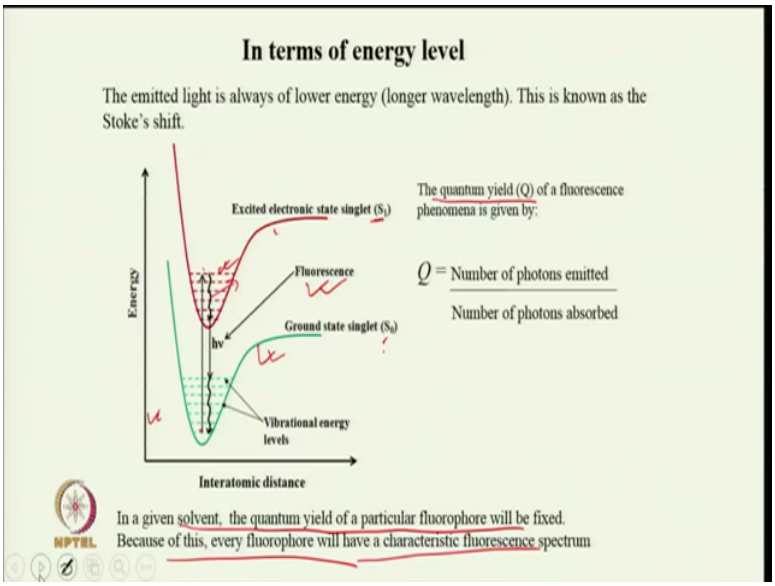

Same thing we are trying to show through electronic picture. The emitted light is always of lower energy or longer wavelength and this process is basically known as stoke shift and same thing is shown here energy versus interatomic distance, this is your potential energy diagram. These are the two electronic states, this is S0 and S1. These are the different vibrational level of S0. These are the different vibrational level of S1.

So on absorption of light, the excited molecule goes from lowest vibrational level to some of the vibrational level of S1. And then what happens that there is non-radiative transition from higher vibrational level of S1 to lowest vibrational level of S1 and that is your non-radiative. But from the lowest vibrational level to ground state, it is the radiative transition, the radiative transition and that process is called as fluorescence. That process is known as fluorescence. Okay.

There is important parameter what is known as quantum yield and that is given by number of photons emitted by number of photons absorbed. So if there is no loss here then number of photons and number of emitted and number of photons absorbed will be equal but there are losses at this place and so quantum yield is going to be less than 1. In a given solvent, quantum yield of particular fluorophore will be fixed, because of this every fluorophore will have a characteristic fluorescence spectrum.

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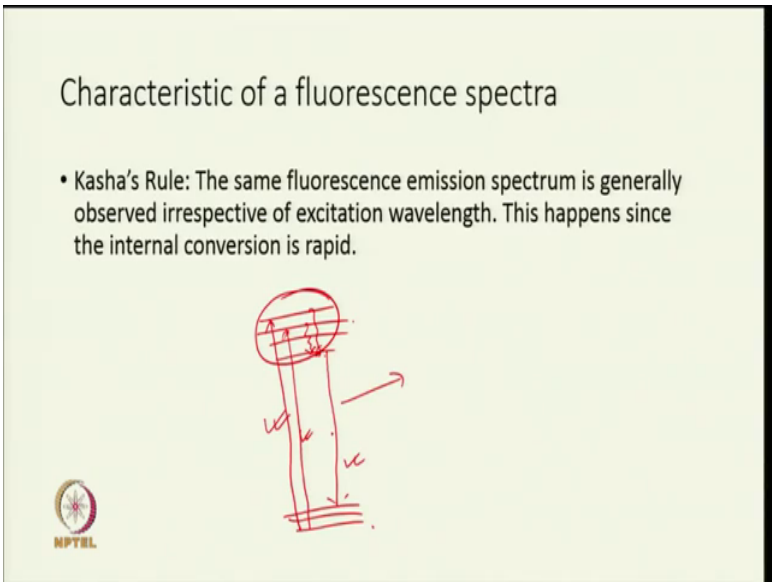

The second characteristic of fluorescence spectra is known as Kasha rule. This tells you that same fluorescence emission spectrum is generally observed irrespective of excitation wavelength. This happens since internal conversion is rapid. So internal conversion is rapid. So same fluorescence emission spectrum is generally going to be obtained.

So basically what does this means is if this is your excited state, this is ground state then if there is absorption, this process is very fast compared to this process. And so a fluorescence emission spectrum is generally same irrespective of excitation wavelength. Because this process whether it goes to at this vibrational level, it has to come back to this level, it has to come to ground level and this process is rapid. So this process internal conversion is not going to affect the fluorescence emission spectrum. Whether excitation wavelength is smaller or larger, emission spectrum is going to be same.

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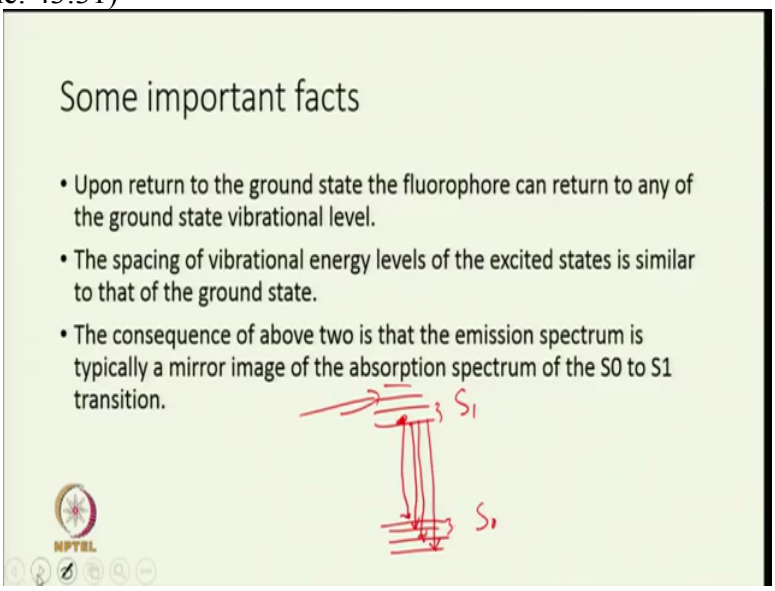

Some other important facts about fluorescence, upon return to the ground state the fluorophore can return to any of the ground state vibrational levels. The spacing of vibrational levels of the excited state is similar to that of the ground state. And consequence of above two is emission spectrum is typically a mirror image of absorption spectrum of the S naught to S1 transition. This is very important, these two facts are very important and that leads to this observation.

So fluorophore can return to any of the ground state vibrational levels. So suppose these are here. So from this state it can come to here, it can come to this place, it can come to this vibrational level, it can come to this vibrational level. So the fluorophore can return to any of the ground state vibrational level. And then spacing between these two levels are same, okay. There is a gap between these two energy levels and S1 or S naught is going to be same. And so what happens is emission spectrum is going to be mirror image of absorption spectrum. We will explain again in the next slide.

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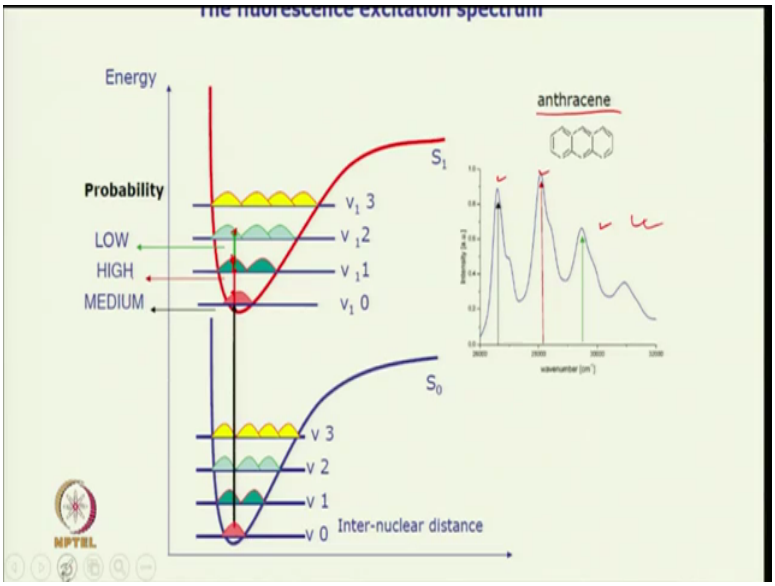

So look at this is the fluorescence excitation spectrum, what is happening? So if a fluorophore absorb light then it can go to any of the vibrational level. And you see here, since the electronic transition is quite rapid compared to a movement in nucleus and so, this transition can be shown a vertical line and if you are sure it has a vertical line, you can see that it is reaching here, it is reaching at this place, it is reaching at this place and so intensity will be like this.

So this is going to be highest because you see this is reaching at this place. And this is going to be lower than this one, this is going to be medium, depending kind of a wave function where it is ending up. So this is your the spectra, electronic spectra of anthracene. Electronic spectra of fluorescence excitation spectrum of anthracene.

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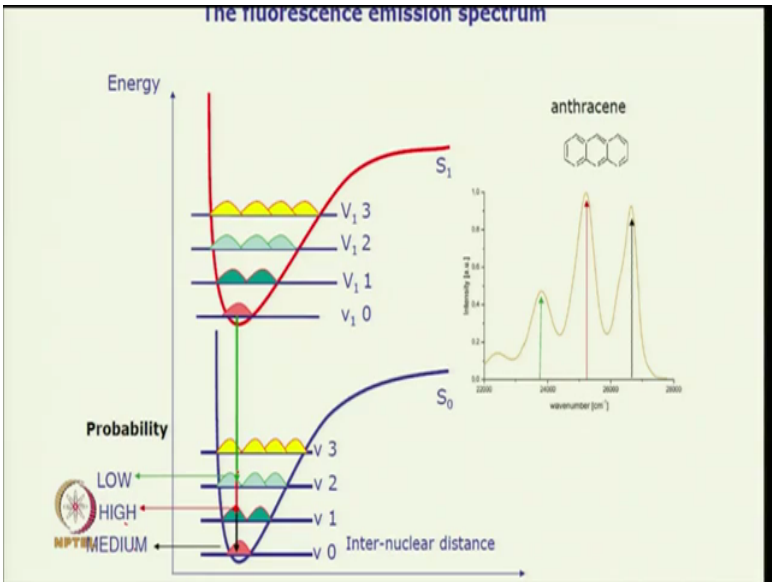

Now, look at the emission. Emission will take place from this place to this place. Again, it has to come to this and since spacing is quite similar again, coming from this to this place this will have highest intensity whereas this one will have medium and this one is a lower. So if you look at, it is going to be mirror image, so anthracene emission spectrum is going to be mirror image of excitation spectrum.

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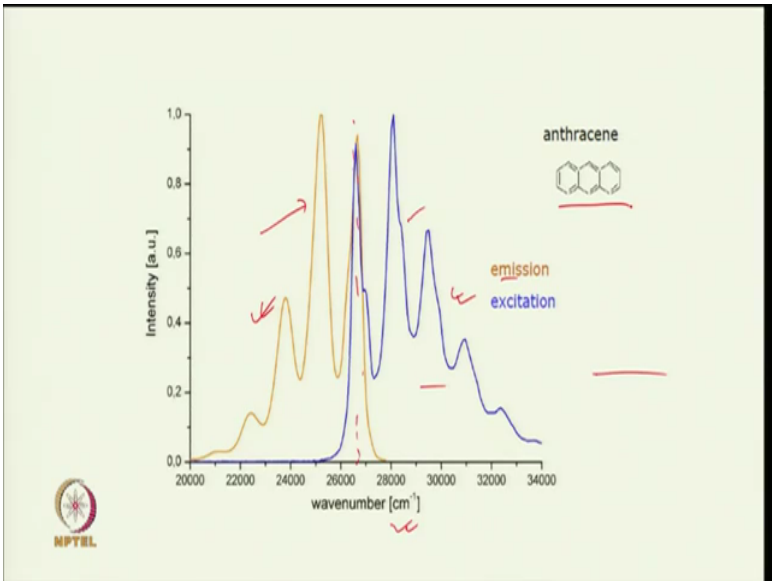

So I will show you it here. So this is your emission spectrum and this is excitation spectrum, this is the wave number. So wave number for emission is going to be lower and wave number

of excitation is going to be higher. But if you look at this, you see, if you put here mirror then this excitation spectrum is a mirror image of emission spectrum, this is for anthracene.

And this is because of two important facts that from the lowest energy level of the excited state the chromophore can come to any of the vibrational level of the ground state and second thing is the gap between the vibrational level and S0 state is same as the gap between the vibrational levels of S1 state.

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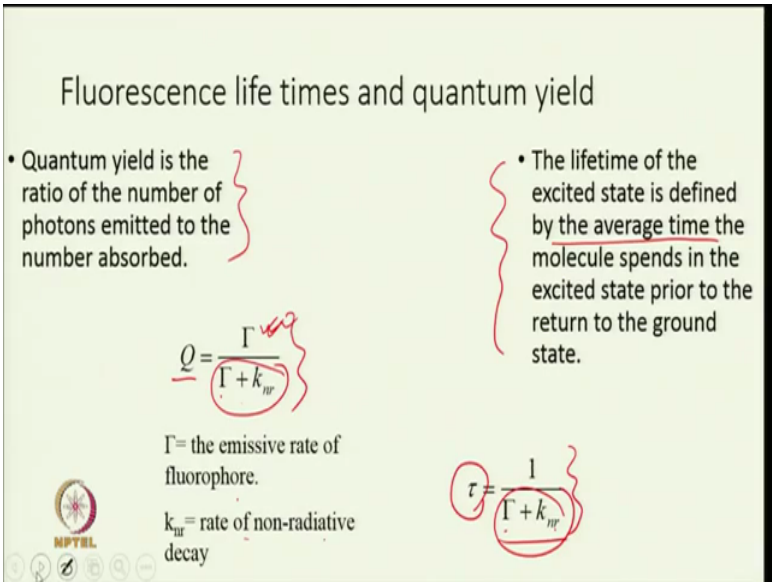

There are two other important parameters, one is called fluorescence lifetime and other is called quantum yield. Quantum yield I have already discussed. But let us again talk about quantum yield. Quantum yield is the ratio of the number of photons emitted to the number of absorbed and this Q will be given by the emission rate of fluorophore divided by emission rate of fluorophore plus rate of rate of non-radiative decay.

So you can think of that when fluorophore absorbs certain number of photons during absorption process some of these photons are lost due to non-radiative process and some of the photons are lost due to the radiative process. So the sum of this emissive rate of fluorophore and the rate of non-radiative decay will be proportional to number of photon absorbed. Whereas this is the emissive rate of fluorophore and that will be the proportional to number of photons emitted. So Q is basically the ratio between the rate of emission divided by total rate of emission. Total rate basically consist of rate of emission and rate of non-radiative decay.

Then there is a lifetime. Lifetime of excited state is defined by the average time. The molecule spends in excited state prior to the return to the ground state and so, this is lifetime will be inversely proportional to the rate of emissive decay, or emissive rate of fluorophore plus rate of non-radiative decay. So inverse of this rate of decay is known as lifetime. And this is the basically average time the molecule spends in excited state prior to the return to the ground state.

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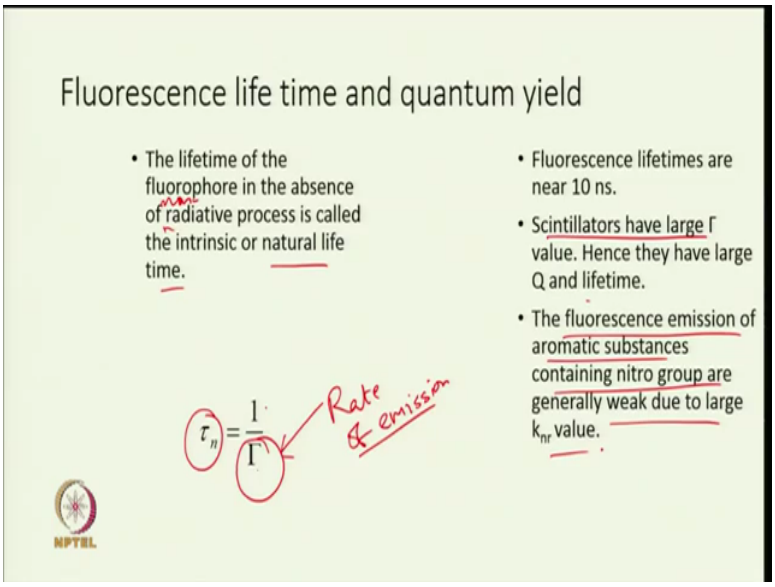

Prior to the return to the ground state. There are few more important terms which you need to know in the fluorescence. One is your natural lifetime. So natural lifetime is the lifetime of fluorophore in absence of your non-radiative process and that is given by Tau n is 1 by Tau. Tau n is 1 by Tau is a rate of this lifetime is 1 by cap Tau and that is basically your rate of emission. So this is your rate of you can say radiative emission.

Fluorescence lifetimes are near 10 nanosecond. This is well-known fact and I will tell you what is the importance of that. Scintillators have large Tau value and so they have large Q and lifetime. The fluorescence emission of aromatic substances containing nitro groups are generally due to large k nr value.

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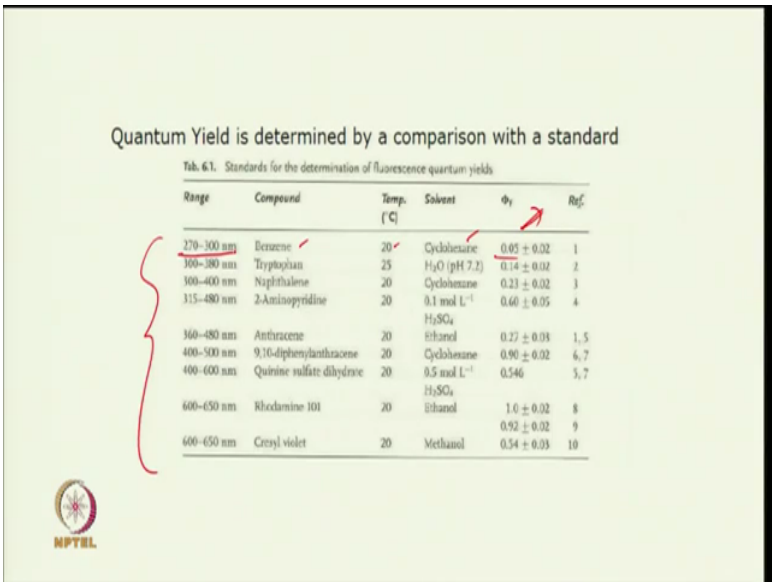

Quantum yield is generally determined by compare with a standard and these are the standards given and their quantum yield at a particular condition. For example benzene at 20 degree Celsius in cyclohexane solvent as 0.05 if you obtain spectrum in 270 to 300 nanometer.

So depending on the chromophore you are using, you can use different standard compounds whose quantum yield is known and based on their quantum yield and the fluorescence intensity obtained for a particular chromophore, you can obtain the quantum yield.

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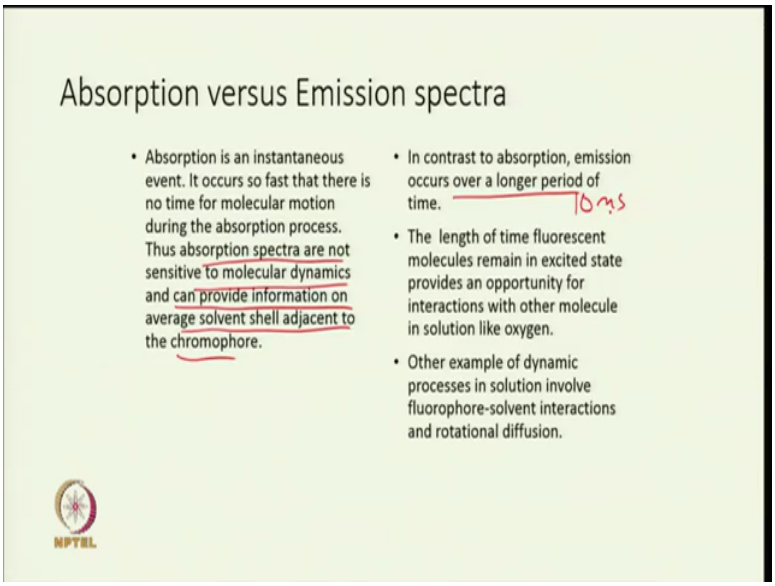

Now, let us understand what is the importance of the lifetime, what is the importance of lifetime. The absorption is an instantaneous event. What does it mean is lifetime of absorption process is very small, it is in femtosecond to picosecond time square. It occurs so fast that there is no time for molecular motion during the absorption process. And thus absorption spectra are not sensitive to molecular dynamics and cannot provide information on the effect of the different effect of process, effect of certain change in condition on the processes which are happening in time scale greater than femtosecond or picosecond.

So absorption spectra are not sensitive to molecular dynamics and can provide information every solvent shell adjacent to the chromophore. So it can only provide average property. In contrast to absorption, emission occurs over longer period of time. And if you remember lifetime I told you, is around 10 nanosecond. So what does that mean is that the length of time fluorescent molecule remains in excited state is around 10 nanosecond and so this provides an opportunity for interaction with other molecule in solution like oxygen.

And so fluorescence can be used to study fluorophore solvent interaction. But UV visible spectra cannot be used. Since the time scale in which fluorescence solvent interaction takes place is in nanosecond, is in nanosecond whereas absorption is absorption lifetime happens in femtosecond and picoseconds region, so you cannot use absorption to study the fluorophore solvent interaction.

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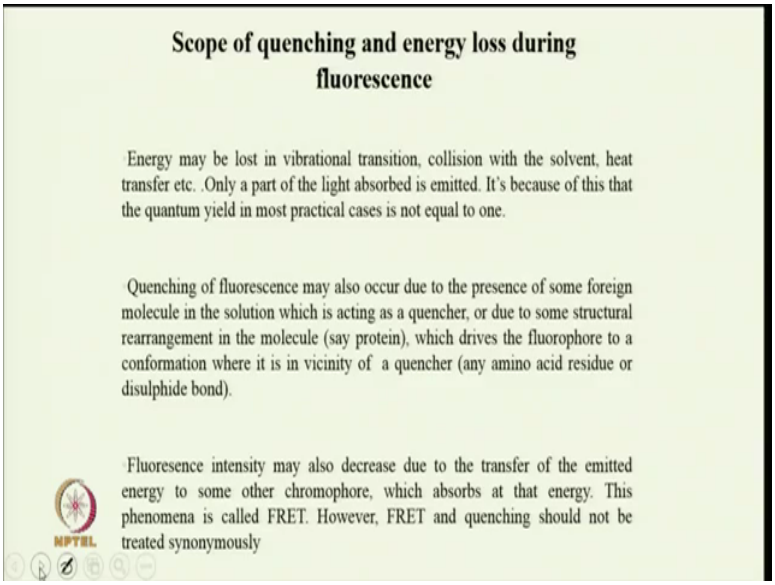

So then we need to talk about quenching phenomena. But since now time is up so I am going to stop here. In the next class, I will discuss about quenching and FRET, these are the two

very important processes in fluorescence and which is quite often used to study different kind of systems. So thank you very much for listening. See you in the next lecture. Bye.