Basics of Fluorescence Spectroscopy

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Lecture – 25

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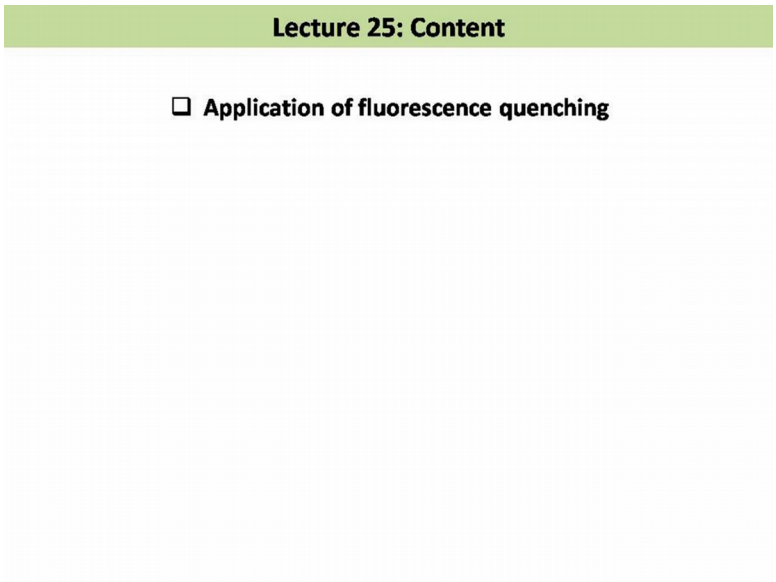

So, welcome to the lecture number 25. So, let us continue our discussion on fluorescence quenching that we were doing for last few lectures and we have already get some idea about the types of quenching, we got the idea of static quenching, dynamic quenching, combine static and dynamic quenching then we have seen the quenching sphere of action. So, in that case the apparent static quenching term is coming in where we are getting a smaller value of k s which is because of the immediate presence of quencher next to the fluorophore. And then we have also seen the quenching resolved emission spectra and now today I wanted to show you some application of this fluorescence quenching method.

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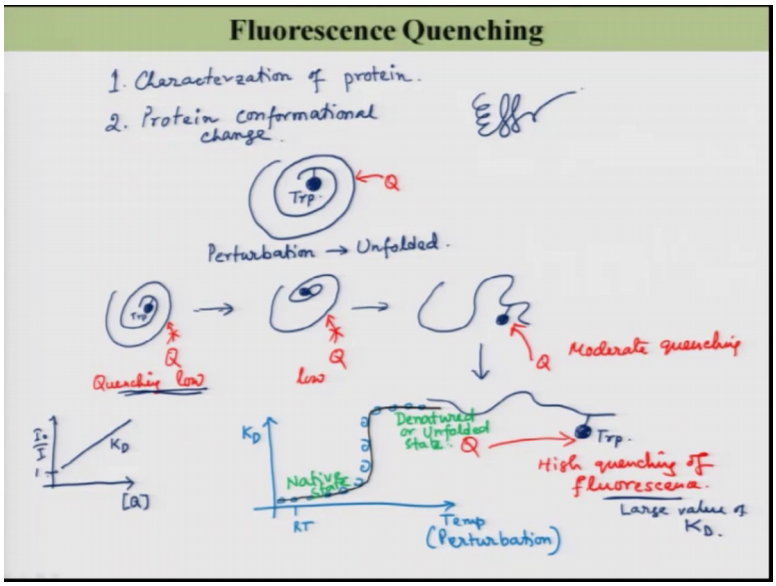

So, let us start with one by one. So, there is several things we can do with this fluorescence quenching method like the characterisation of protein let us say in this case fluorescence quenching method is being used for example, let me take a protein right I can just draw my protein like this way as usual and there was a debate between 2 diffractions one diffraction is said that I have a 2 tryptophan residue as I told you this tryptophan residues are kind of amino acid which are fluorescent in nature right. So, one diffraction is said that I have 2 tryptophan residue one is present at the surface and another is also present at the surface of this protein and the second diffraction is come in and he told no my tryptophan results tells me that both the sorry one of them are present at the surface and another is buried right.

Now, what to do right, but using a fluorescence quenching you can resolve this and suppose you are doing this quenching experiment for the tryptophan both are present at the surface the quenching will be much more. Now if the tryptophan one of them is present in the buried location and another is exposed to the surface then you will going to see a lesser amount of quenching because that tryptophan which is present in the buried location will not contribute to the fluorescence quenching right I mean that quencher will not be able to reach that buried tryptophan and it will not able to quench the tryptophan emission. So, the fluorescence intensity will not decrease that way that it would be for the case when both the tryptophan will be present at the surface right.

Number 2; so, you can also do this protein conformational change you can also study by fluorescence quenching method for example, suppose you have a protein let us say this is you protein and you have this tryptophan residing over here, now if you add a quencher molecule from outside that quencher molecule will not be able to reach this tryptophan residue 100 percent. So, the fluorescence intensity of this tryptophan which is the intensity fluorescence of this protein will not going to be affected by addition of the quencher in the solution now consider that you have done something on this protein. So, that this protein chain right the secondary structure of the protein is getting distorted. So, that is what is referred as the unfolding of the protein or the change in the conformation of this project right. So, you do something you put some perturbation. So, that the protein is unfolded.

Now, as the protein is unfolded right the structure of the protein will change suppose you have this structure and this structure as it is unfolding the structure is getting changed like this way. So, that tryptophan which was originally here on the buried location this is my TRP, it is now over here still again in the buried location now it is open somewhat and now it is fully open this is my tryptophan residue. So, depending on the condition depending on the secondary structure of this protein the accessibility of this quencher will be different. So, in this case the quencher cannot interact with this tryptophan here also it cannot interact with this tryptophan, but here somewhat it can interact with this tryptophan and here the interaction with the tryptophan is quite easy.

Now, it tells me that if I do this quenching experiment here the quenching will be low here also low here moderate quenching, but here I will get high quenching of fluorescence. Now consider that as your giving this perturbation this perturbation could be anything this perturbation could be the raise in the temperature this perturbation could be the adding of some foreign agents like guanidine in hydro chloride urea these are the well known chemical denature and which actually unfold some protein. So, this perturbation could be anything and this perturbation also could be the lower in the p H. So, if you change the p H of the medium from neutral p H 7.4 to 2 or 1 then also this is also access the perturbation. So, the protein is getting denatures or unfolded.

In this case right if the, now, if I plot the quenching right I can plot the K D right because this is typical and dynamic quenching. So, if the K D value is more right you remember here. So, when I plot I 0 by I then versus Q I get a straight line with intercept equal to 1

and my slope is K D right. So, K D more means the quenching is more. So, in this case I will get for this case where the quenching is low I will get a low value of K D then as I will go in this direction then the K D value will larger and larger, so large value of K D.

So, now, if I plot the observed K D value as a function of this perturbation right if it is the temperature then what I can simply do I can simply plot the measured K D value as a function of temperature. So, here temperature is my perturbation it could be other thing also right. So, then what I will going to see the value of K D, K D value was really small right over here initial temperature or the temperature is lower. So, let us say here is my room temperature and as I increase the temperature. So, it may suddenly increase like this and then remains same like such kind of feature right the feature is it is almost similar here then suddenly increase and then remains same right such kind of feature by looking at such kind of feature you can comment that this unfolding of these protein when you raise the temperature is a 2 step process, right.

Here is your native state in this part is your native state and this part is your denatured or unfolded state. So, you see by simply doing this quenching experiment right of such kind of molecule we can comment on half of the structure of the protein is changing by increasing the temperature suppose you have done this exactly same experiment, but not temperature as your perturbation, but the change p H as your perturbation.

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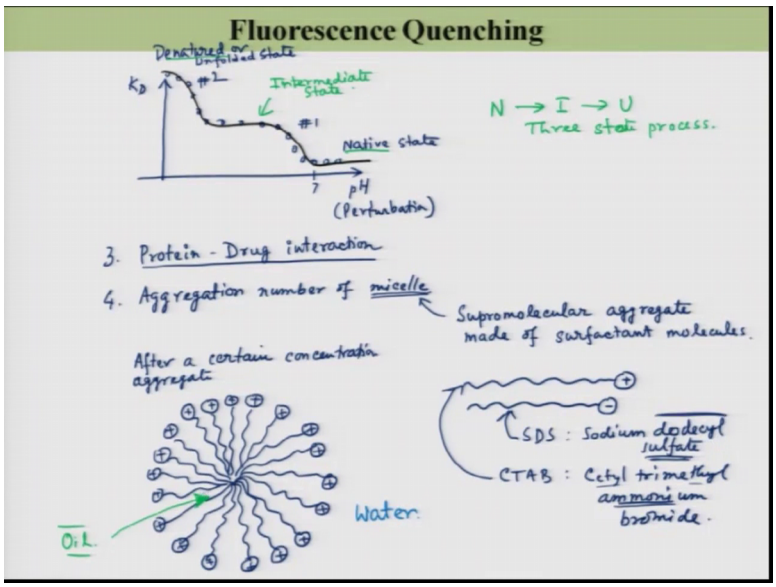

So, in this case let us assume that here you have used p H as your perturbation. So, and you have measured this K D value at different different p H and your plotting it this versus p H and what you have seen is that such kind of feature at neutral p H let us say here is my 7 at neutral p H the value of K D what you have observed from your experiment is really low value right and as and little bit of higher p H 7.4; 7.6, 7.8 this value is like this as you increase the p H probably you have seen such kind of data and the trend is like this it clearly tells you that when your perturbation is this p H here is your perturbation then the change of K D is 2 step here is a transition number 1 and here is a transition number 2. If I assign these you can do so [FL] assignment, if obviously, the physiological p H is my native state I assign this as native state and if this is my denatured or unfolded state I can clearly tell that I have some state which is not denatured, not denatured, nor native right intermediate state.

So, I would have the signature of intermediate state in this case right. So, in this case the denaturation is not a 2 step process this is a 3 step process. So, I can write the native state of the protein is first changing to some several intermediate state and then this is converting to the unfolded state. So, this is a three state process.

We can also study the protein drug interaction by this method number four which I will discuss in elaborately is you can also use this fluorescence quenching for the determination of aggregation number of micelle, so, for the aggregation number of micelle. So, let me first tell you that; what is micelle? So, micelle is a supra molecular aggregate. So, this supra molecular aggregates made of surfactant molecules what is surfactant molecules we have a long alive tic chain with a charge head group either positive or it could looks something like this negative. So, this is charge head group like the famous name of this surfactant is SDS right sodium dodecyl sulphate. So, you see this sulphate this is negatively charge head group and dodecyl this is alive tic chain right similarly CTAB acetyl trimetheyl ammonium bromide. So, here is my positive head group and here is the long chain right. So, this is a positively charged surfactant head group this is the negatively charged and so on.

So, if you keep on adding this surfactant in water let us say in water then after a certain concentration this surfactants will form supra molecular aggregate right then after a certain concentration this surfactants right will forming supra molecular aggregate that looks something like this. For this surfactant all the head groups of the surfactant will

come outside and these hydro carbon chain of the surfactant will come together to form a kind of spherical structure is a kind of spherical structure as I have drawn over here like these are all the positively charge. So, this positively charge is exposed to the water and this hydro carbon part I am making a core like of thing. So, basically this part is like oil. So, this inside part is like oil and the outside is water and the outside is water. So, this is like a I have a droplet of oils, but these are very tiny very tiny right in size is nanometre size droplet oil droplet in water and because of these actually the surfactants like our detergents actually helps to wash our clothes right that is the different story; let us proceed.

This surfactant molecules form such kind of supra molecular aggregate only after a certain concentration right.

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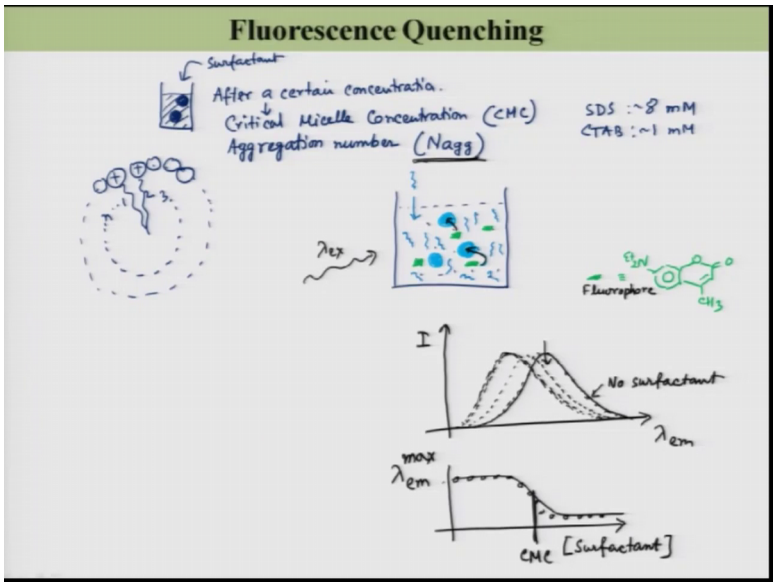

If you may if you add this surfactant let us say this is your water and your keep on adding this surfactant just for a very small concentration it will not form this micelle only after a certain concentration after a certain concentration those kind of micelles will form like this these balls like micelles will form and that concentration is known as critical micelle concentration and generally denoted written as CMC. So, for this different different surfactant has a different values of CMC for this as I said this SDS the CMC of SDS is 8 milli molar. So, after it cross the 8 milli molar concentration in water then only such kind of micelles will form.

On the other hand for the CTAB it is about one mill molar. So, after one mill molar this CTAB will be form such kind of micelles right now as I have drawn this figure like it consists of several surfactants molecules is number 1 2 3 dot, dot, dot, dot, dot, dot, dot, dot n number of surfactant molecule actually makes this micelle the question is how I will going to determine these CMC value and the aggregation number. So, for this micelle formation from the surfactant I encounter 2 thing one is that critical micelle concentration and after it the critical micelle concentration n number of surfactant will molecule this micelle and that is called the aggregation number of this micelle and this called aggregation number and denoted as Nagg. Now my question is that how will going to experimentally determine this CMC and this N aggregation.

So, there are several method to determine a for the determination of the same CMC for example, surface tension the surface tension of the air water interface right will keep on increase as you as add the surfactant, but after the CMC that will remains same the if you measure the molar conductance will be almost similar right before CMC, but once the surfactant will form the micelle the molar conductance will decrease because of the bulky nature of this micelle.

However as we are doing the basic course on the fluorescence spectroscopy we will try to tell you a method based on the fluorescence how to determine this CMC and the n aggregation for the measurement of this CMC right you see the situation I have solution let us say this is water and let us take one fluorophore inside this water right some concentration let us say these are my fluorophore these are my fluorophore and this structure of this fluorophore is this is my fluorophore and this looks something this something like this looks something like this. So, you see this is not a polar molecule, but it is a low solubility in a water.

So, the solubility of this molecule is higher in non polar medium than in the polar medium now you are keep on adding this surfactant molecule let us say these are your surfactant molecule. So, you keep on adding. So, surfactant molecules are they are as you increase the concentration the concentration of this surfactant molecule is keep on increasing and there is not much change in the local environment of this fluorophore and in this case if I now plot the florescence spectra of this molecule how will get you just excite with a suitable light lambda excitation and then measure the fluorescence intensity as a function of wavelength then you will get the fluorescence spectra.

So, I will just plot intensity versus lambda emission. So, you will get the fluorescence spectra of this molecule let us say the fluorescence spectra looks something like this you are not changing the number of molecules in this medium. So, it remains same because you are not adding more number of this fluorophore you are not adding more number of fluorophore with time. So, the number is remains same what you adding is a surfactant right. So, the fluorescence intensity will not change that way right.

Now, as you are adding the surfactant you keep on measure the fluorescence spectra of this molecule. So, when there is no surfactant let us say it looks like this no surfactant and as you are adding surfactant you are measuring initially there is no change it will remain like this remain like this remain like this, but once there is a formation of those micelles when it will be once the concentration of the surfactants will cross or will reach the CMC value. So, then there will be formation of supra molecular aggregates of this surfactant molecule and automatically as these fluorophores likes to stay in oil. So, then this fluorophore will go inside it will go inside this micelle because inside the micelle as I said oil light outside is water. So, it will preferred to go inside the micelle right. So, it will go inside.

Once it will go inside it will not going to see that may much of water in inside it as that inside the micelle there is no water it is like a oil hydrocarbon. So, when you will going to excite this fluorophore in the excited state we will not going to be stabilised in the same way that it was in case of water right when it was present in the water then the stabilisation of the excitation state was quite large because of the high polarity of the water, but inside this micelle micelle environment the polar the polarities low. So, it will not going to stabilise. So, what will going to see you will see a sudden change of emission maxima why because the emission maxima tells me that how much it is solo rated if it is solo rated more it is same as leapat mathaga right when you increase this is the case of excited state dipole moment is more than the ground state dipole moment. So, here you see there is a change and when all the fluorophores will be inside then there will be not much change in the shape of the emission spectra.

So, if I know plot in a different way the concentration of surfactant versus lambda emission maximum then for the low value of the surfactant right these lambda was high value right lower energy as you seen here. So, you will get such kind of thing and then it will, once there is formation of micelles this value will decrease and then it will be again

constant. So, from here what you can get the midpoint is nothing, but the value of this CMC we can use this fluorescence quenching now to get this aggregation number or this micelle. Now let us see how we can use this fluorescence quenching to find out the aggregation number right like we have found the CMC value by fluorescence method, but in this case we will use the fluorescence quenching method to find the aggregation number of the micelle we will find the n aggregate let me define all the terms step by step and then it will very easy for you to understand.

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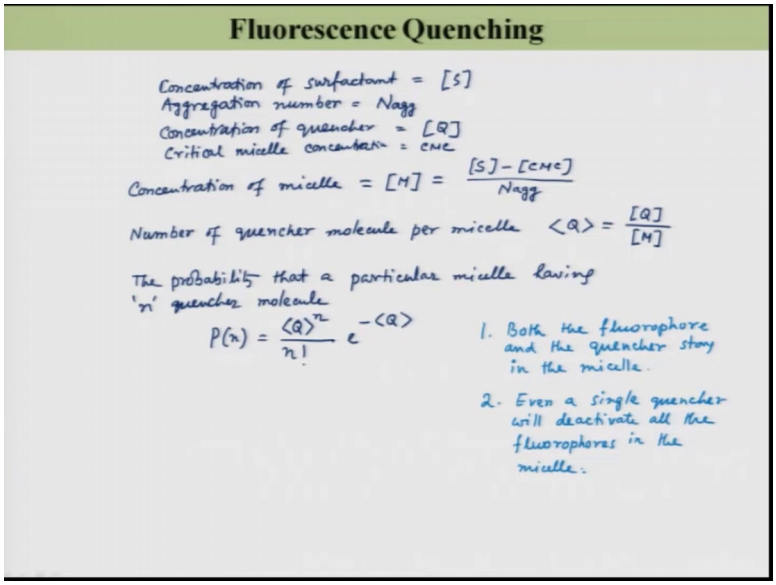

So, here first term I will going to define is let us say the concentration of surfactant let me use this symbol s to denote this aggregation number let us say N a g g as I said earlier concentration of quencher lets denote s Q is usual that critical micelle concentration that is my CMC. Now I can write from here the concentration of micelle or I can write here concentration of micelles this will be equal to lets represent this as m this is equal to concentration of surfactant minus that CMC value because only after CMC this surfactant will form the micelle and divided by aggregation number. That means, n a g g right and I can now also try to define this quantity that number of quencher molecule for micelle right represent by this average Q this is nothing, but the concentration of Q concentration of the quencher divided by the concentration of the micelle and that will going to be the average it is not necessarily that you all this micelle will have that exact number of quencher, but that is my average right.

So, I will just simply write here concentration of Q divided by this m right and I know how to write this probability. So, let me write that the probability that a particular micelle having that is the same way earlier I have done right having N quencher molecule that using my poison distribution I can write P n this is my average Q right to the power n factorial n e to the power minus average Q right.

Now, I will take 2 assumptions which are; obviously, valid assumptions right let us take this assumptions with this other colour here the first one I will take as the fluorophores and quenchers are such that the can be present in the micelle it is not that the fluorophore is completely hydro it is a charge and micelle surface is positively charged fluorophore is also positively charge. So, fluorophore will never come to the micelle I will not take such kind of situation I will take a neutral molecule neutral fluorophore neutral quencher. So, that it has a hydro part hydro fabric interaction is favour favourable in that case those all these fluorophores and quenchers will go and sit the micelle. So, that depends on me I can choose the type of fluorophore and quencher which will go and sit on the micelle will not remain in the solution right.

So, the first assumption is valid assumption I will write here both the fluorophore and the quencher stay in the micelle, and the second one is that even I have a single quencher present in the micelle that is good enough to quench the fluorescence completely of the fluorophore which is present in the micelle clear.

So, even a single quencher will deactivate all the fluorophores in the micelle in this case I will not create such kind of situation at all I will use the fluorophore concentration is low. So, that each micelle probably we will have on and average less than one it means that some micelle will have fluorophores some are not right, but I will use a quencher more. That means, the micelle which are having fluorophore that must contain a quencher I will create the condition like that way right and there is a very little chance that a micelle is having more than one fluorophore, but only one quencher that condition I will not let it come, right. So, in this case both will be satisfied.

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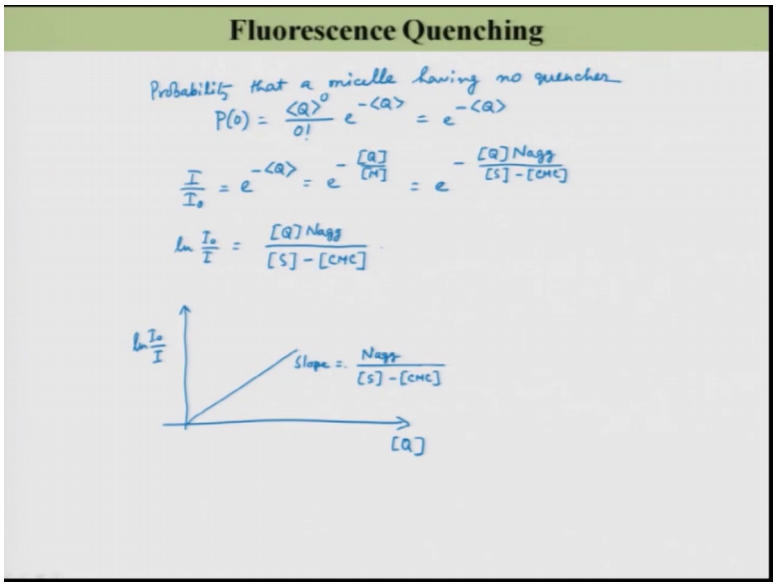

If so, then let us see what will happen. So, then I can write the probability that a micelle having no quencher that is simply p 0 which is Q that average Q to the power 0 by factorial 0 e to the power minus this average Q which is nothing, but e to the power minus this average Q right.

So, here this means right I can write I by I 0 is equal to e to the power minus Q right this means this. So, this is equal to e to the power minus Q by m which is equal to e to the power minus Q n aggregation because I know this means that right s minus that CMC value whatever it is. So, if I take l n. So, l n I 0 by I is equal to Q n aggregation divided by s minus CMC right now I can make a plot easily. So, the plot I will do like this way I will plot l n this measured quantity right I 0 and I they are measured quantity I 0 is the fluorescence intensity without quencher and I is with quencher. So, I can take the l n of that and I plot the l n I 0 by I versus q. So, then this should be a straight line passing through the origin. So, then this should be a straight line passing through the origin and the slope would be N a g g divided by concentration of the surfactant minus the CMC value.

I know this concentration of the surfactant right for a particular experiment and the slope is known CMC you already have determined from other experiment. So, you can simply get the n aggregation value. So, see that using fluorescence quenching easily we can determined the n aggregation value. So, we will finish here.

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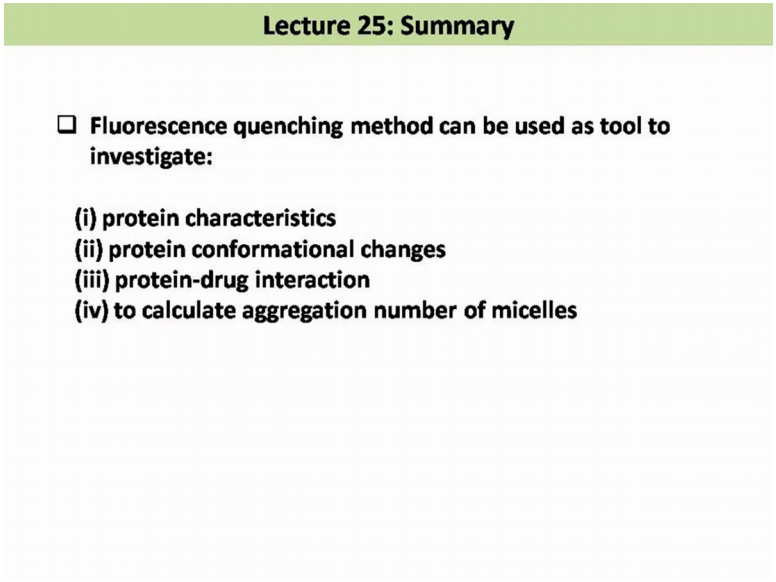

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