Basics of Fluorescence Spectroscopy
Prof. Pratik Sen
Department of Chemistry
Indian Institute of Technology, Kanpur

Lecture – 24

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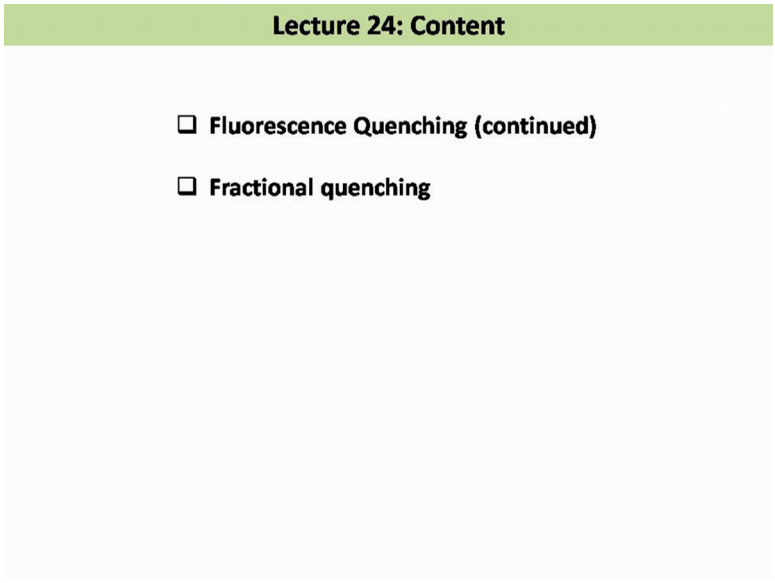

Welcome to the lecture number 24. The last few lectures what we were discussing is the fluorescence quenching. And we have seen basically few things, let me list it out.

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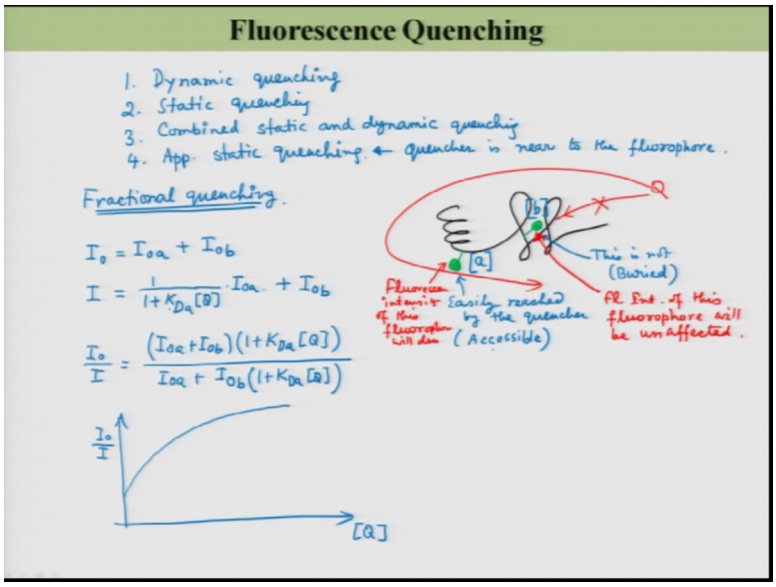

Dynamic quenching, I also have seen static quenching, I have seen combined static and dynamic quenching. I also have seen that apparent static quenching, this is because of the quencher is near to the fluorophore. And now what I will try to tell you is another type of quenching which is for the fractional quenching. Let us say I have two such kind of fluorophore in a molecule and the proteins are one of the typical example for such kind of fractional quenching.

So, let us say I have this protein over here, I have just drawn my protein like this way looks good. I have one fluorophore which is present here and one fluorophore which is present over here. What I can comment about the position of this two fluorophore? This fluorophore is easily reached by the quencher, but this is not this is present in buried location. So, this is accessible and this is not accessible. If so, now if I add quencher; so quencher will come and interact with this fluorophore, but quencher cannot come and interact with this fluorophore. So, the fluorescence intensity due to these fluorophore will decrease; this fluorophore will decrease fluorescence intensity of this fluorophore will decrease. But fluorescence intensity of this fluorophore will be unaffected right; fluorescence intensity of these fluorophore will be unaffected, clear.

Now, let me write this as site a, because this is accessible so this is site a; and let us me write this as my site b because this is in the buried. Without addition of quencher, when there is no quencher then the total fluorescence intensity I 0 can be written as I 0 for a position of the a and plus I 0 of b I can simply write that one. Now as I argued that when you will add the quencher the quencher will quench the fluorescence of a, but it will not going to quench the fluorescence of b. That I argued like that way.

So, the fluorescence intensity in presence of quencher which is typically denoted by I, I can write this I as 1 over 1 plus K D; this transformer quenching constant for this so let me write K Da into Q it initial intensity I 0a right remember I 0 by I equal to 1 plus K Da into Q so from here I have written plus I 0b, because the I 0b fluorescence unaffected because quencher cannot reach to that site b site two quench the fluorescence of this fluorophore which is present here which is marked as b. So, I 0b will be I 0b even after you will add the quencher molecule, clear.

So, what I can do? I can take the ratio of I 0 by I as usual: I 0 by I is equal to I 0a plus I 0b into 1 plus K Da into Q divided by I 0a plus I 0b into multiply it by 1 plus K Da into

Q. Now what I intended to do? I intend to plot I 0 by I versus Q. Obviously, this is a complicated right think. And if you plot this I 0 by I versus Q, let me plot it somewhere here. So, this is my I 0 by I versus Q what you will see that the curve will look something like this, the curve will look something like this. It means that this is not a straight line, obviously it is not.

So, in this case my question is how I will going to calculate what is the value of K Da.

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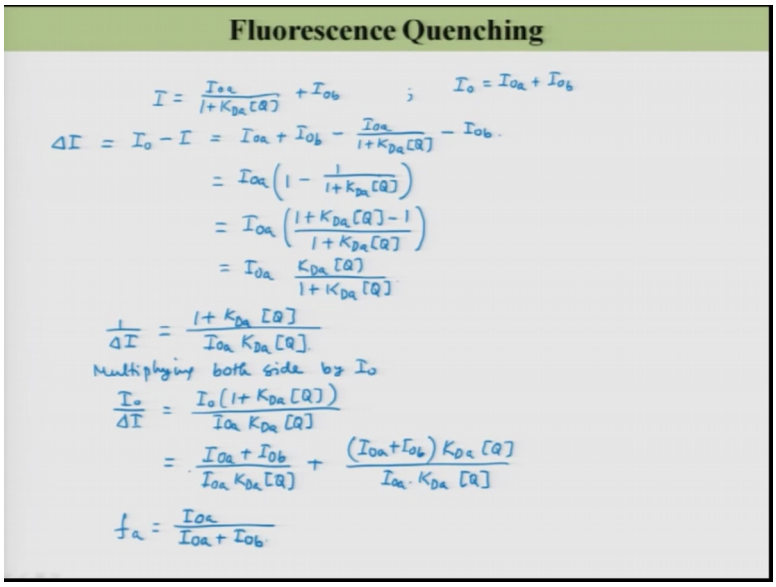

Let us move forward and see how can we do that. Let us write this I equal to I 0a by 1 plus K Da into Q plus I 0b that is what I was writing, and let me write this quantity I 0 minus I like. So, what is I 0? Write here I 0 equal to I 0a plus I 0b. So, I 0 minus I, I can write it as I 0a plus I 0b and then minus I minus I 0a divided by 1 plus K Da, I do not have any K Db because that fluorophore present in that b site, buried it site is not accessible to the quencher- into Q minus I 0 b.

So, this is equal to I 0a to 1 minus 1 over 1 plus K Da into Q. This is equal to I 0a to 1 plus K Da into Q minus 1 divided by 1 plus K Da into Q. This is equal to I 0a K Da into Q divided by 1 plus K Da into Q. So, this one I write as delta I, I can also write this as a delta I. So, 1 over delta I is equal to 1 plus K Da into Q divided by I 0a K Da into Q.

Now, I multiply both side by I 0; I will get I 0 by delta I is equal to I 0 1 plus K Da into Q divided by I 0a K Da into Q. So, if you just simplify this and proceed what you will

get is I 0a plus after rearranging I 0b divided by I 0a K Da into Q plus I 0a plus I 0b K Da into Q divided by I 0a K Da into Q.

Let us assume that f a this fraction is equal to I 0a divided by I 0a plus I 0b, then this equation will change too.

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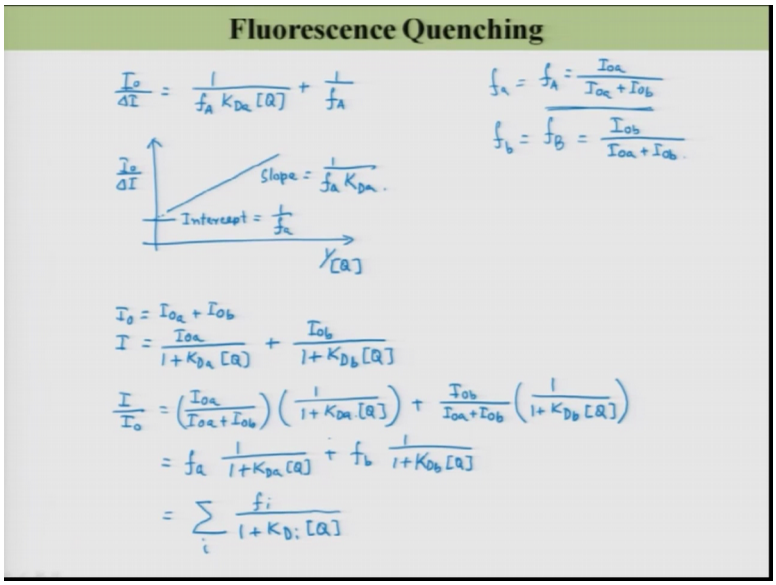

So I 0 by delta I, this equation will be equal to 1 over f a K Da Q plus 1 over f A. Remember that f A is equal to I 0a divided by I 0a plus I 0b.

Now, you see we got this equation, now if I plot instead of plotting I 0 by I now I am plotting I 0 by delta I as a function of 1 over Q. If I plot I will get a straight line right where the slope will be equal to 1 over f a K Da and the intercept will be equal to 1 over f a. So, like this I will be able to calculate what is the value of K Da.

Now the question is that as I said that in this particular case this position is completely buried position, it is not accessible at all. Now if both are partially accessible. So, in this case my equation will be anyway. So, I 0 is equal to I 0a plus I 0 b, but in this case I will be equal to I 0a divided by 1 plus K Da into Q plus I 0b divided by 1 plus K Db, it is for the site b so I have written K Db.

In this case what I am telling in this case both the sides are accessible, but not at same extent. So, the stun harmer quenching constant may be defined for the different side although the same molecule. Here my condition is the quencher is the same molecule it

is also interacting with the site a, it is also interacting with the site b, the fluorophore which is present in site a and site b they are also be same, but their accessibility is little bit different. And that is why all the differences are coming. The first case what I showed is that the one is fully accessible another is 0 accessibility, there is completely inaccessible to that quencher, but here I said that is somewhat accessible. So, then this will be equal to Q.

Now, if I just write this ratio I by I 0 what will happen, I will simply get you can just see later on this will be equal to I 0a plus I 0b into 1 over 1 plus K Da into Q plus I 0b divided by I 0a plus I 0b to 1 over 1 plus K Db that K Db will come over here into Q. So, this I will write as f a, that is that is what I have defined like this way here you see f a so f b also this will be small a, anyway it does not matter- f a f b same thing. So, I 0b divided by I 0a plus I 0b is defined like this way.

So, this is f a to 1 by 1 plus K Da to Q plus f b to 1 by 1 plus K Db into Q. So, for having more than two sites, so I will just simply write the sum is f i divided by 1 plus K Di into Q. So, this will going to be little complicated. And one of the limiting thing is little easier, but let us proceed with this. Let me show you the emission spectra of that what I showed you earlier that protein having two fluorophore, this is the same diagram let me draw over here.

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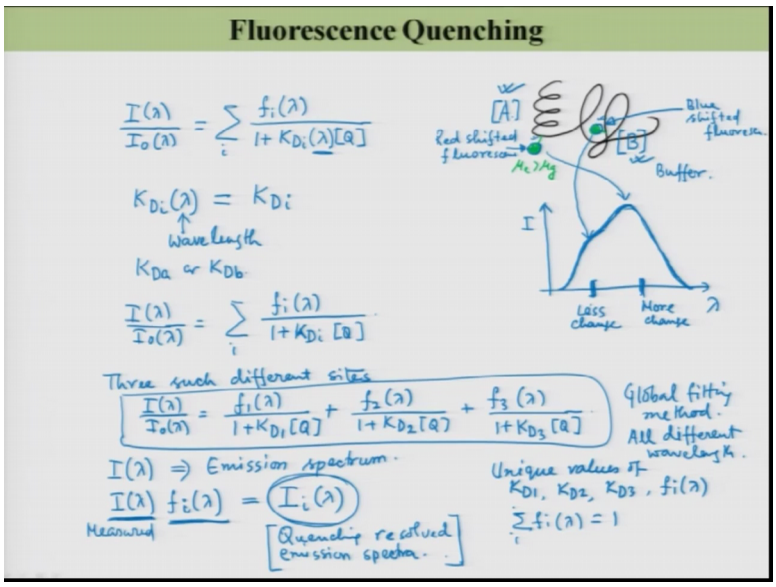

That is what I, that one right the same thing. Now I have 1 over here, as I said I have 1 over here and this protein having these two fluorophore present in two different locations and consider that for this fluorophore mu e is greater than mu g. And this whole protein is present inside water buffer; so outside there is a buffer.

So, this fluorophore is accessible to buffer more compare to this fluorophore (Refer Time: 19:43) moment is more than grossly double moment you will remember the solver to provision of a fluorescence. So, the fluorescence of this one will be red shifted compared to this one let me write that: red shifted fluorescence, this will be blue shifted fluorescence. So, if you take these fluorescence spectra of this what you will going to see is fluorescence spectra emission spectra right intensity versus wavelength.

See this something over here, probably you will going to see such kind of thing. Looks like there is something over here, because one is little red shifted other is little blue shifted. So, in this case this part of fluorescence is given by this fluorophore and this part of the fluorescence because this is a red shift higher longer wavelength lower energy. This part of the fluorescence is given by this fluorophore.

As I said that one of them is more accessible compare to the other. That means, these fluorescence intensity that these I by I 0 that is what I just have calculated this is a function of wavelength. If I calculate at this wave length then this I by I 0 value, and if I calculated this wave length then the I by I 0 value will be different suddenly it will be. Because, when I will calculate the I by I 0 for this wavelength then there will be lot of change in the fluorescence intensity. When I will calculate the I by I 0 at this wavelength then there will be less change in the fluorescence, so here is the less change, is a more change in the fluorescence intensity.

So, these are the function of lambda. So, this function of lambda then that summation what I have written this f i that also has to be function of lambda, because at this particular wavelength the contribution of this one and this one is something different than the at this wavelength because the emission spectrum shifted for the location A and location B. You remember here is this is my location A and this is my location B. So, emission spectrum is shifted. If the emission spectra is exactly same for the location A and location B then I want to have such kind of wavelength dependency. And this f i is also the wavelength independent.

But in this case as there is a change so I have this f i is a function it depends on the wavelength. So, then this divided by 1 plus K Di lambda into Q. So, I can write this K Di lambda. This K Di lambda whether it should be depends on the wavelength or not; this is my question whether this K Di, I means for the I-th one it could be A, it can be B, if you have multiple then C, D and so on and so forth. So, whether for a particular site either its K Da or K Db does it depends on the wave length. Obviously no, so I should write this only K Di. This K Di lambda right it does not depend on the wavelength because it is a stung harmer quenching constant, so will not going to depends on the wavelength. It is the interaction between the fluorophore and the quencher. So, the where that molecule will emit I mean it does not matter for it.

So ultimately what will going to have: this I lambda by I 0 lambda is equal to sum over I f i lambda by 1 plus K Di into Q. Like if I have three such different sites then I can simply write I lambda by I 0 lambda equal to f 1 that is for my first site divided by 1 plus K D1 to Q plus f 2 1 plus K D2 to Q plus f 3 1 plus K D3 into Q. Now if I lambda denotes my excitation; sorry emission spectra right emission spectrum, this I lambda has the component of three different species in this case you see: this is for the f 1, f 2 and f 3. So, this I lambda if I multiply it by f i lambda that will going to give me intensity for the I-th site at this particular lambda I will simply get that one.

So obviously, this I lambda is a measured quantity. I will get this one, this is my I lambda; it has the component of B and it has component of A. So, I measured it together. And now this f i is that fractional fluorescence intensity for this I-th component, and I will get this I I lambda. So, if I analyse let us say for example: if I analyse these thing in a global fitting method then what you will get, you will get the global fitting method means that same equation will be applied for all different wavelengths- note it down. These contribution f 1, f 2, f 3 they are wavelength dependent; I by I 0 wavelength dependent, but K D2 or K D1 or K D3 they are not wavelength dependent.

So, for each wavelength if you plot I by I 0 and then just feet this one you will get the unique value of K D1, K D2, K D3. So, you will get unique values of K D1, K D2, K D3 and also all the f i lambdas at each wavelength; f I lambda means at each wavelength. And what I know another information is f i sum of our I f i is equal to 1 that I know. That means, what you will going to get is all these a f i lambda right you will get all these f i lambda, and this is your measured spectrum. So, if you multiply this measured one with

all these f i lambda you will get the spectrum for the I-th species. And that is what we called this as quenching resolved emission spectra.

So let us stop here, and we will continue on the next day.

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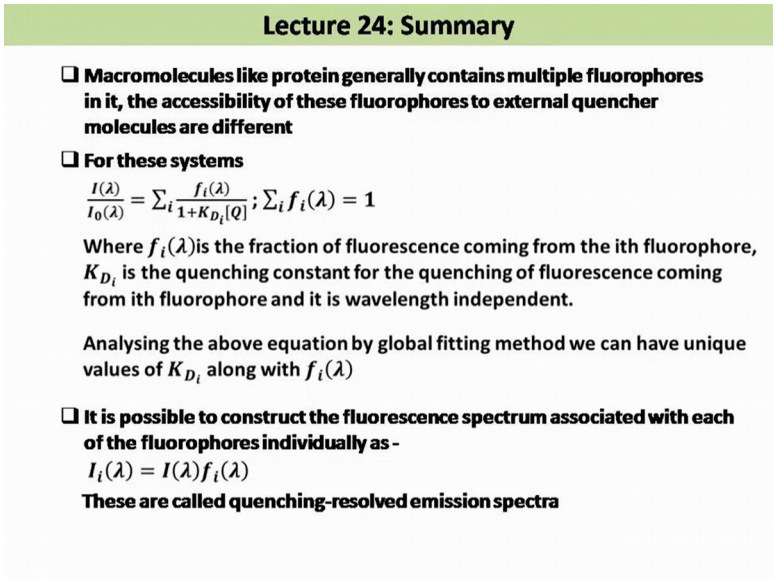

Thank you very much.