

Basics of Fluorescence Spectroscopy
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Lecture – 23

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Lecture 23: Content

- ❑ Fluorescence Quenching (continued)
- ❑ Combined static and dynamic quenching
- ❑ Apparent static quenching

Welcome to the lecture number 23. Till last lecture we are discussing about this fluorescence quenching. And what we have seen is two type of quenching.

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Fluorescence Quenching

Dynamic quenching

$$\frac{I_0}{I} = 1 + k_D [Q]$$

$$\frac{\tau_0}{\tau} = 1 + k_D [Q]$$

Static Quenching

$$\frac{I_0}{I} = 1 + k_S [Q]$$

$\frac{\tau_0}{\tau}$ is always 1

$k_D (C_0)$ Lifetime of the fluorophore without any quencher.

slope = k_D

slope = k_S

$F \xrightarrow{k_{f2}} F^*$

$F + Q \xrightarrow{k_S} F-Q$

F^* : Concentration of fluorophore in the excited state will decrease

Fluorescence intensity will decrease

In the first type of quenching I said that this is a dynamic quenching, and in this type of quenching the equation what we got its look something like this: I_0/I is equal to $1 + K_D$ into concentration of the quencher. And then another type of quenching what I got is discussed is static quenching, and in this case the equation what I got is I_0/I is equal to $1 + K_S$ concentration of quencher. And I was discussing about that the nature of this two plot I_0/I and I_0 for this dynamic quenching and I_0/I for this static quenching they are exactly same.

However, in static quenching if you plot the τ_0/τ you will not get any dependence, this is always 1. But in this case τ_0/τ will also give you a linear dependence with the concentration of the quencher add it. Here this K_D is I mentioned k_q into τ_0 where this τ_0 is the lifetime of the fluorophore without any quencher.

So, what is the prescription? That I have two measure both I_0/I and τ_0/τ if both of them are showing a linear dependency of having some slope; that means, having some value of K_D then this is a dynamic quenching: I_0/I or τ_0/τ . How will going to measure this τ_0 ? This is by the lifetime measurement either TCSPC or up conversion method. So, then I will get a straight line with slope is equal to K_D and intercept is equal to 1. So, this is for both τ_0/τ and I_0/I both for this as well as for this.

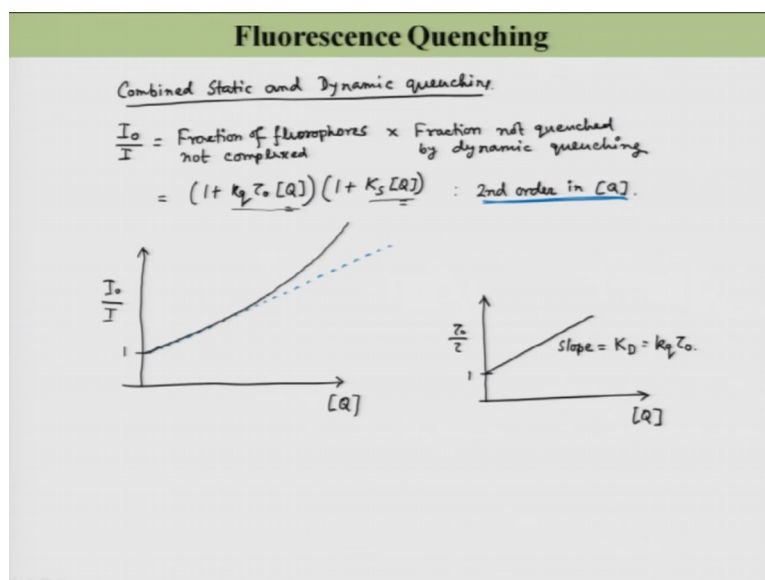
However, in case of static quenching if I plot this I_0/I I will get like this slope will be equal to K_S here this is equal to 1, but for τ_0/τ what you will get the value is constant 1, even you change the concentration. And that what we have discussed, this is because of the ground state complex formation or kind of complex formation of between the fluorophore and the quencher. And because of these complex formations the system will reduce the concentration of fluorophore which can be excited to the excited state.

So, if I have this F right this is in the ground state with λ_{nu} excitation this will go to the excited state. Now, if F will make a complex with Q forming FQ which you cannot excite and this is K_S . So, eventually the concentration of fluorophore in the excited state; this is concentration of fluorophore in the excited state will also decrease and because of this the fluorescence intensity will decrease. Yes, I will increase the concentration of quencher. As I will increase the concentration of quencher more and more F molecule will form the complex with Q. So, the availability of F for the

excitation will be less; that means, the number of fluorophores in the excited state will decrease as the fluorescence intensity will decrease. This is what we have discussed.

Now, we will go to look at different phenomena, here in this case we will go to see what happens if both the static and dynamic quenching are present.

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So, here we will go to see a combined static and dynamic quenching. So, here the change of the fluorescence intensity that is I_0 by I , I can be written as the product of two terms: the first term is coming from the dynamic quenching and the second part is coming from the static quenching. It means that the fluorophores are the same, the similar types of fluorophores are present in the solution and the quencher is also the same, but some of the fluorophores that means the fraction of the fluorophores are not complexed in the ground state by the quencher. And the rest of the fluorophores which are taking part in the quenching, those are not quenched by the dynamic quenching.

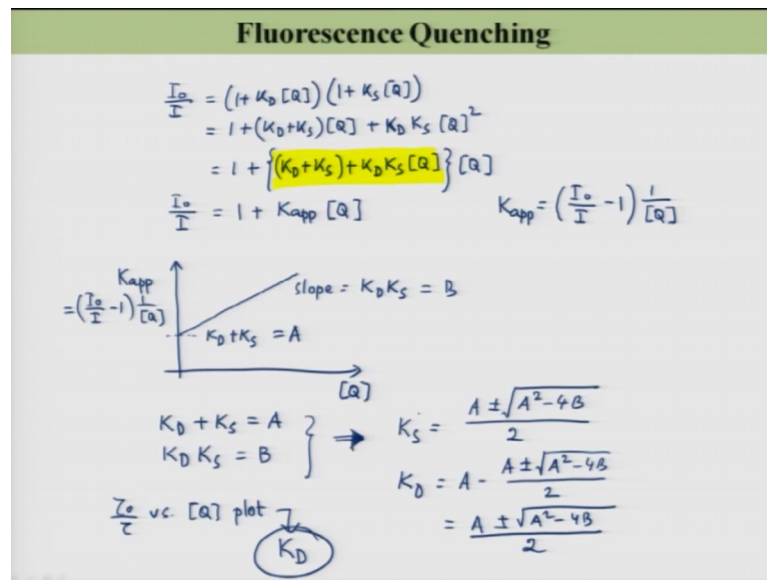
Let me write then it will be clear. So, the first fraction is the fraction of fluorophores that are not complexed multiplied by the fraction of fluorophores that is not quenched by dynamic quenching. So, I can write this the first part obviously I know what is that, this is $1 + k_d \tau_0 [Q]$ into the concentration of the quencher and multiplied by the second term this is nothing but $1 + K_s [Q]$ this is my equilibrium constant for the complex formation. And I cannot write this K_s as $k_q \tau_0$ because this is a ground state completely different thing; you remember this- into Q .

So, if I just try to simplify it I can do that, but it is not necessary later on we will do that. But here you can see this is like here is my Q and here is my Q , so it must be a second order in Q . So, I can write a comment here this is second order in the concentration of the quencher. Now if you plot these I_0 by I versus concentration of Q obviously this is no longer a straight line. Obviously, it will start from 1 as you can see here, when Q equal to 0 so this whole part is 0, this whole part is 0, then is 1 into 1 is equal to 1; so it will start from 1 there is an no doubt. But for lower quencher concentration it looks straight line, but as you increase the quencher concentration you will see the curve is like this upward nature.

Originally, if it is only static quenching or only dynamic quenching then you know that the curve is a straight line where the slope value is equal to either K_S or K_D , depending on whether it is static quenching or it is dynamic quenching. So, in this case what you can see this curve is going upward because of the second order in Q , the Q square term will come over here. However, if you plot τ_0 by τ versus q obviously you will not going to see such kind of upward curvature, because for the τ_0 of τ there is no contribution from the static quenching; only dynamic quenching is operation or is visible both the quenching is operational, but only dynamic quenching is visible for the lifetime measurement.

So, in this case this is purely will be a straight line intercept equal to 1 and here the slope will be equal to K_D is equal to k_q into τ_0 . Let us do something so that we can get this.

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Let us write this as I_0 by I equal to $1 + K_D$ to Q to $1 + K_S$ into Q . So, as I wrote let us write it as $1 + K_D + K_S$ into Q plus $K_D K_S Q$ square, so this is equal to $1 + K_D + K_S + K_D K_S$ into Q that whole thing is multiplied by Q . And let me write it as $1 + K_{app}$ into Q . So, you see I got this equation: I_0 by I equal to $1 + K_{app}$ into Q . And here the K_{app} is, I can write this K_{app} is equal to I_0 by I minus 1 into 1 over concentration of the quencher.

Now see if I plot K_{app} versus 1 over K_{app} versus Q . What is K_{app} ? K_{app} is I_0 by I minus 1 into 1 over Q . So, if I plot K_{app} versus Q what I will get, I will get simply a straight line because here you see K_{app} versus Q if I plot I will simply get a straight line and the intercept will be here you see here is my K_{app} let me highlight it this part. So, if I plot K_{app} versus Q how I will going to calculate K_{app} ? I will going to calculate K_{app} by the measured value of I_0 and I .

So, in case of K_{app} versus Q I will get such kind of equation where the intercept will be $K_D + K_S$ and the slope will be equal to $K_D K_S$. Now the K_{app} will be calculated from the measured value of I when there is no quencher that I is equal to I_0 and in presence of quencher those values are called the I , this nothing but the fluorescence intensity, clear.

Now, if I get this value K_D plus K_S and $K_D K_S$ as A and B: let us say write here K_D plus K_S that is equal to A and $K_D K_S$ because this is this will gives you some value; the slope this equal to B let us say and this equal to A right some value will come from the graph $K_D K_S$ equal to B. And then we can just do some simple rearrangement and we will get K_S is equal to $A \pm \sqrt{A^2 - 4B}$ divided by 2. And K_D will also get as $A \mp \sqrt{A^2 - 4B}$ divided by 2; here $A \mp K_S \mp \sqrt{A^2 - 4B}$ divide by 2 which is equal to $A \pm \sqrt{A^2 - 4B}$ by 2. So, in case one if the plus is K_S then the minus is k_b and vice versa.

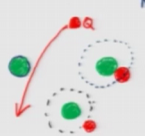
One of them will be K_S one of them will be K_D , but from the lifetime dependence from the τ_0 by τ versus Q plot we already know what is the value of K_D , so this is already know. So, I will get we will calculate the value of K_S . So, this is not so difficult to find out the value of K_D as well as K_S , when there is a combined quenching that combined means combine static and dynamic quenching. But, some time what people have found is that the value of K_S obtained by such kind of procedure comes out to be too small too tiny value.

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Fluorescence Quenching

$$K_S = \frac{[F-Q]}{[F][Q]}$$

When the value of K_S is too small
 \downarrow
 Not Suitable for complex formation.



Assume the existence of a volume (V) within which the probability of immediate quenching is unity.

$$V \approx \frac{4}{3} \pi (r_F + r_Q)^3$$

Mean number of quencher in the volume " V " = λ

The probability of finding " n " quencher molecule in the given volume

$$P(n) = \frac{\lambda^n}{n!} e^{-\lambda} \quad : \text{Poisson distribution.}$$

And if there is if this equilibrium constant is because of the complex formation; so this value is this case is $F Q$ by F into Q - if this is a complex formation and the complex stable equilibrium should be shifted to the right side; that means, in this case the value of

K_S should be higher, if the value of K_S is too small that means the complex is not that stable; which is not so convincing to the people.

So, what I was telling that when the value of K_S is too small. So, probably such situation is not suitable for complex formation, so obviously people have started thinking then the that what is the source of such kind of small value of K_S . So, what we have seen here that the value of K_S is really small and this is not suitable for the complex formation. And to explain this phenomena that very small value of K_S people have try to describe the situation in a different way, let us see that.

Let us have a fluorophore sitting over here right and my quencher let me draw my fluorophores like this way let us say this is my fluorophore over sitting over here another fluorophore is sitting over here and then quencher will come and interact, let me draw the three fluorophores over here. And let me draw the quenchers with this red one. So, let us say this is my quencher just I will write Q. So, the Q will come and interact then it will undergo this dynamic quenching.

However, for a moderate concentration of the quencher is situation may arise in such that the quencher molecule is present very near to the fluorophore molecule. This possibility is there always there is such kind of possibility. So, let me draw such possibility here. So, here is my quencher, and let us say in this case that quencher molecule is sitting just next to the fluorophore. And I can imagine is situation where if the quencher is present within the volume I showed in this diagram right, so this is the volume made up with half of the radius of the fluorophore and half of the radius. So, radius of the fluorophore plus radius of the quencher with that I make a volume and I consider that if the quencher is present within this volume the probability of quenching is unity.

That means, whatever the fluorescence is there by this fluorophore that will be completely quenched by this quencher molecule; if the situation is like this, if the situation is like this then it is not, if the situation is like this then it is not. So, here that particular volume is like this. So, that let me write in words.

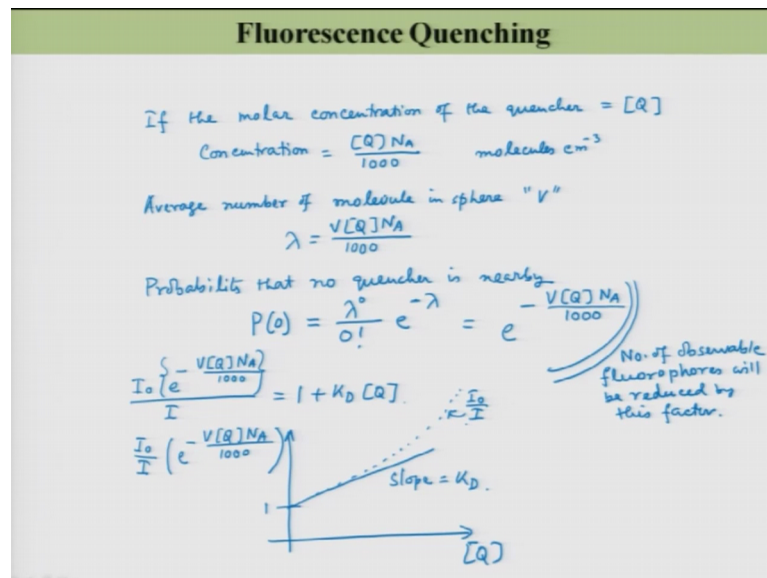
Let us assume the existence of a volume and that volume I termed as V within which the probability of immediate quenching is unity. So, if the radius of the fluorophore is I write as r_F if the radius of the quencher I write r_q . So, that particular volume v is equal to 4

by $3 \pi r^2 F + r^3 q$ it is about this kind of volume. So, if the fluorophore is present within it then the probability of quenching is unity; that is what I assume.

And let me also tell that the mean number of quencher molecule within this volume A is λ . So, I will define here that mean number of; obviously, I can write later on this λ in terms of the concentration that will do just after few seconds. Number of quencher in the volume V let us say λ denoted as a λ .

Now, I can write the probability of finding just a number n quencher molecule in the given volume; given volume means that V , in the given volume can be written as in terms of Poisson distribution. So, I can write this as P_n is equal to λ^n to the power n divided by factorial $n e^{-\lambda}$; this is coming from the Poisson distribution.

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Let us proceed. So, if the molar concentration of the quencher that is equal to Q then I can write this concentration in terms of molecules per C ; this will be Q into Avogadro number divided by 1000. That means, the average number of molecule in this sphere of volume V that λ is equal to $V Q N A$ divided by 1000.

Now, if I want to calculate the probability that is no quencher is nearby. So now, you see this probability that no quencher is nearby that same as according to the Poisson distribution P_0 which is equal to λ^0 by factorial λ to $e^{-\lambda}$ to the

power minus lambda. So, I can write this as $e^{-\lambda \tau} N_A$ divided by 1000. It means that number of observable fluorophore will be reduced by this factor, because this is P_0 no quencher is nearby.

No quencher is nearby that means you will get the fluorescence from that. When the quencher is nearby those quencher will immediately quench the all the fluorescence of those fluorophores. So, I want this, this quantity right $e^{-\lambda \tau} N_A$ by 1000 that must be multiplied with I_0 , then whatever that I_0 I will get that is that realise I_0 . Then when I will add the quencher, quencher, quencher then the decrees of the fluorescence intensity should follow my dynamic quenching, otherwise it will look like is a combine static and dynamic quenching.

So, what I wanted to say is. Now, if I multiply with I_0 , multiplied by $e^{-\lambda \tau}$ into Avogadro number by thousand divided by I_0 this should follow this equation. So, the I_0 is modified with this quantity, I_0 is modified with this quantity that is the number of observable fluorophore, because some other as immediate in their immediate vicinity they have the quencher which immediately quench all the fluorescence so those I should not take care. So, those I should not take into account for my calculation.

So, in this case is simple now, I_0 by I into $e^{-\lambda \tau} N_A$ will be $1 + K_D Q$. So, what I should plot? I should plot this one versus Q ; then what I will get, then I will get a straight line and the intercept will be equal to 1. And the slope will be equal to K_D .

See that although instead of this if I have plot the I_0 by I , I would get this type of line deviating from the linearity and I would think that this is because of the combined static and dynamic quenching, but it is not because it is just because of the apparent static quenching. So, let us finish here, and we will continue our discussion on the next class.

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Lecture 23: Summary

- ❑ For the combined static and dynamic quenching, I_0/I vs $[Q]$ will have an upward curvature due to the $[Q]^2$ term. However, a plot of τ_0/τ vs $[Q]$ would give a straight line with positive slope.
- ❑ It is possible to determine K_D and K_S experimentally by measuring I_0/I in different quencher concentration. In this case, we need to plot K_{app} vs $[Q]$ where $K_{app} = (I_0/I - 1) \cdot (1/[Q])$
- ❑ Apparent static quenching may mislead us about the combined static and dynamic quenching process.

Thank you very much.