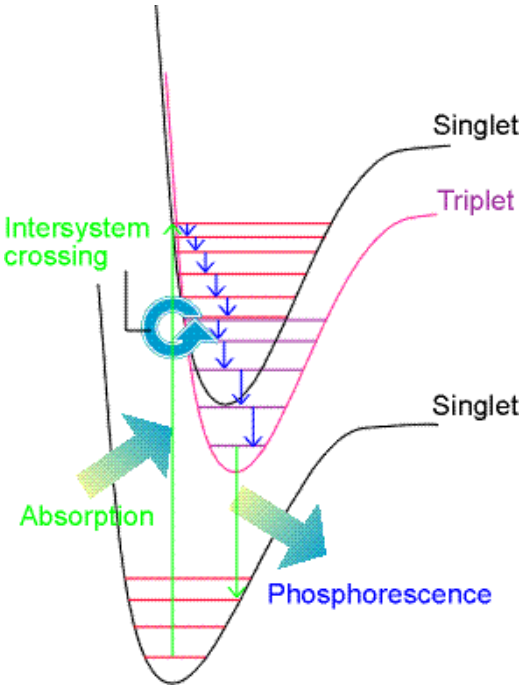
CH454 Physical Chemistry Lab: Luminescence Quenching Experiment

Introduction

This laboratory experiment will provide you with an introduction to the study the quantum yield and kinetics of luminescence quenching. Luminescence encompasses emission by both the singlet and triplet states, fluorescence and phosphorescence, respectively. Your study will use uranine as the sample. Uranine is a water soluble variant of fluorescein. Fluorescein and its derivatives has been widely used in protein labeling studies and microscopy. Fluorescein is a fluorophore and has an observed lifetime of less than 10 nanoseconds. In this laboratory you will determine the lifetime and relative quantum yield of uranine in the presence of a quencher.

3.1 Photophysical processes and quenching

Fluorescence vs. Phosphorescence. Excited states can be deactivated either by be **radiative** or **nonradiative** processes. Spin allowed radiative transitions are usually short-lived ($< 10^{-8}$ s) and give rise to luminescence, which is known as ***fluorescence***. Spin forbidden radiative transitions can also be observed as a long-lived luminescence, which is called ***phosphorescence***. Since singlet-triplet transitions are spin-forbidden, triplet excited states can be form only by means of a process known as **intersystem crossing**. The intersystem designation refers to the fact that the molecular system crosses from the singlet to the triplet state manifold. Although phosphorescence is often difficult to observe in many organic molecules, it is readily observed in $Ru(bipy)_3^{2+}$. Fluorescein, on the other hand normally radiates from the singlet state, but can undergo quenching via formation of a triplet state in the presence of a heavy atom, such as iodine in triiodide.



In order to understand the observations in this experiment you will want to review first order and second order kinetics, fluorescence and phosphorescence, and various quenching mechanisms. These include energy transfer, charge transfer and intersystem crossing mechanisms among others. Experiments involving $Ru(bpy)_3^{2+}$ are based on energy transfer quenching. Since $Ru(bpy)_3^{2+}$ rapidly forms a triplet state, quenching can be combined with a second process of triplet-triplet annihilation in the acceptor to make a higher energy singlet state. Fluorescein, on the other hand, radiates from a singlet state and is quenched by intersystem crossing to form a triplet state.

Quenching of uranine

Uranine is the disodium salt of fluorescein, which is one of the historically most used fluorescent dye molecules. Uranine is readily water soluble, but the pH must be greater than 8.0 in order for it to maintain the appropriate charge state. Fluorescein is an aromatic molecule that has a very high fluorescence quantum yield. Fluorescein can be synthesized in a functionalized form with an isocyanate group that is reactive towards primary amines, appropriate for labeling proteins. Fluorescein has some disadvantages such as pH-sensitivity and tendency to photobleach. Fluorescein undergoes a protonation with a pKa of 6.8. Below pH ~ 7 it has low absorption consequently it is a poor fluorophore. However, above pH 8 it has a high absorption cross section and it is an excellent fluorophore. The photobleaching has been addressed by replacement of the hydrogen atoms with fluorine atoms in the derivative Oregon Green.

3.2 **Kinetics of luminescence and quenching.**

In the following discussion, we will refer to the luminescence as fluorescence. The discussion applied equally well to phosphorescence. Without a quencher, a fluorophore decays radiatively with an observed rate constant of $k_{obs} = k_r + k_{nr}$, where the radiative rate constant is k_r and the non-radiative rate constant is k_{nr}. If we assume that the laser pulse produces a population N_o of excited molecules, ^{1}F at time $t = 0$, the population can be shown to decay exponentially (first order kinetics), resulting in the following time dependence of the emission intensity I (photons/sec):

$$I = k_r[\,^1F] = k_r N_0 \exp\{-t/\tau_0\} \tag{4}$$

where

$$\tau_0 = (k_r + k_{nr})^{-1} \tag{5}$$

The quenched emission decay will proceed with an accelerated speed. In other words, its lifetime will be shorter:

$$I = k_r N_0 \exp\{-t/\tau\} \tag{6}$$

Where

$$\tau = (k_r + k_{nr} + k_q[Q])^{-1} \tag{7}$$

Thus, the rate constant for the quenching reaction, k_q, can be obtained by analyzing the luminescence data. The quenching rate constant, k_q, is determined from the so-called **Stern-Volmer** relation:

$$\frac{\tau_0}{\tau} = 1 + \tau_0 k_q[Q] \tag{8}$$

which can be obtained from the rearrangement of Equations 5 and 7. The quantum yield of luminescence is proportional to the luminescence intensity, so that the Stern-Volmer relation may also be written as:

$$\frac{I_0}{I} = 1 + \tau_0 k_q [Q] \tag{9}$$

where I_o and τ_o are the luminescence intensity and the luminescence lifetime of $^3Ru(bpy)_3^{2+}$ in the absence of quencher.

The equations above suggests that k_q may be obtained from an experiment where the luminescence lifetime or the luminescence intensity of $Ru(bpy)_3^{2+}$ is measured at different quencher concentrations. A plot of τ_o/τ (or I_o/I) versus [Q] is expected to be linear, with a slope:

$$K_{sv} = \tau_0 k_q \tag{10}$$

where K_{sv} is the Stern-Volmer rate constant.

Stern-Volmer quenching is derived with the assumption of dynamic quenching, which means that the quenching is free to diffuse in solution and that it interacts with the luminescent molecule by a second-order process. However, one must always be aware of the possibility that two molecules have formed a complex and therefore that the quenching is static, rather than dynamic. In order to be sure that a process is due to dynamic quenching it is necessary to determine both the yield and the lifetime.

Diffusion limited reactions. In a dynamic process the quenching rate constant k_q is a bimolecular rate constant (has units $M^{-1}s^{-1}$) and describes a process, in which the reactants first have to encounter and then react. Such reactions are often referred to as diffusion-assisted reactions. It is convenient to separate a reaction into two steps. First, there is a diffusion controlled formation of intermediate complex (A*...Q) or transition state, with k_{diff}, and second the transition state can either react with the (first order) rate constant k_p or dissociate without reacting with the rate constant k_{-diff} (also first order) :

$$A^*+Q \quad \underset{k_{-diff}}{\overset{k_{diff}}{<=>}} \quad (A^*...Q) \xrightarrow{k_p} \text{Products} \tag{11}$$

Then the overall rate constant k' can be written as:

$$k' = \frac{k_{diff} k_p}{k_{diff} + k_p} \tag{12}$$

which in the limit of fast reaction rate $k_p >> k_{-diff}$ simplifies to:

$$k' = k_{diff} \quad (13)$$

when the diffusion becomes a limiting step. This case would correspond to the diffusion controlled (or diffusion limited) regime, when no matter how fast k_p is, the observed k' would be defined by how fast molecules encounter due to diffusion. The value of k_{diff} obviously depends on the diffusion coefficients of both reactants:

$$D = D_A + D_Q \quad (14)$$

According to the rate equation:

$$k_{diff} = 4\pi N_a D R_q \quad (15)$$

where N_a is the Avogadro's number 6.022 x 10^{23} mol^{-1}, and R_q is the separation at which the quenching takes place, also called the quenching radius. For neutral molecules, the quenching radius equals the sum of molecular radii:

$$R_q = R_A + R_Q \quad (16)$$

but the situation can differ dramatically if both species are charged. The Coulombic interaction leads to the following condition:

$R_q > R_A + R_Q$- for oppositely charged ions (17)
$R_q < R_A + R_Q$- for ions of the same charge (18)

Mechanisms of quenching. A variety of inorganic and organic species may act as quenchers. Determining k_q does not give any insight into the mechanism of quenching. For a given system, k_q may reflect a combination of energy and electron transfer processes. Energy transfer could occur in either the singlet or the triplet manifold. By detailed study of the system and application of control experiments one can determine whether the mechanism involves energy transfer or electron transfer. Triplet energy transfer can be established in part using the sensitivity of the process to O_2. Since O_2 has a triplet ground state it is an effective triplet quencher (see below). When applied to simple systems, these criteria help determine the predominant mechanism of quenching.

***Energy transfer**:*	$^*Ru(bpy)_3^{2+} + Q \rightarrow Ru(bpy)_3^{2+} + Q^*$	(19)
*Reductive **electron transfer**:*	$^*Ru(bpy)_3^{2+} + Q \rightarrow Ru(bpy)_3^{+} + Q^{+}$	(20)
*Oxidative **electron transfer**:*	$^*Ru(bpy)_3^{2+} + Q \rightarrow Ru(bpy)_3^{3+} + Q^{-}$	(21)
Intersystem crossing:	^{1}Fluorescein + Q $\rightarrow$ ^{3}Fluorescein + Q	(22)

3.3 Experimental Protocols: Quenching of uranine by iodide

Fluorescence quenching of uranine can involve either intersystem crossing using the heavy atom effect or energy transfer, using an acceptor fluorophore such as rhodamine 6G. One of the advantages of studying fluorescence is that there is no competing quenching by O_2 as observed in phosphorescence. Phosphorescent samples must be degassed to exclude O_2. We do not need to concern ourselves with degassing. However, given the much shorter lifetimes we will need to use a time-resolved experiment that has nanosecond time resolution. For this we will use time-correlated single photon counting (TCSPC). The Horiba instrument in the lab is both a fluorometer and TCSPC instrument. Please use appropriate care when turning on the instrument. The order is very important.

Protocol

To conduct this experiment you will need the following reagents:

A. 1 M stock sodium borate buffer at pH 8.5. This is diluted to 0.1 M in the sample.
B. Stock solution of uranine such that the maximum **absorbance** in the sample will be 0.1.
C. 0.5 M KI Solution in 0.1 M borate buffer.

See Appendix 2 for more information on the borate buffer system used in this experiment.

Weigh out approximately 14 milligrams of uranine and place it in a 10 milliliter volumetric flask. Add pH 8.5 0.1 M borate buffer to the mark. The uranine solution can be conveniently diluted first by a factor of 4 by dispensing 2.5 mL of this first stock solution into a 10 mL volumetric flask and diluting to the mark. The absorbance at 494 nm of this experimental stock solution will hopefully be slightly less than 1.0. This is appropriate as a stock solution of uranine because you will want the final concentration to have an absorbance of less than 0.1. For fluorescence measurements the volume in the cuvette needs to be 2.5 mL. You can vary the KI concentration By adding 250 mL of the uranine stock solutions to mixtures of the KI and buffer stock you will make samples containing various concentrations of KI and a constant concentration of uranine. The KI can range from 0.01 to 0.5 M. The molar absorptivity of uranine is ε_{494} = 92,300 $M^{-1}cm^{-1}$ and the molar mass is 378 g/mol.

Overview: Measure the time-resolved (TCSPC) and steady state fluorescence spectra for uranine and uranine in the presence of at least five concentrations of the quencher KI ranging from 0.05 to 0.5 M. A constant concentration of uranine should be used in all samples and the absorbance should be less than 0.1 at 494 nm. Check to make sure you are not saturating the fluorometer! You should see a significant difference between the pure uranine and 0.5 M KI solution of uranine.

Instrumental procedures

1. First use the switch on the side of the main fluorometer to turn on the instrument. NOTE: This switch turns on the Xe arc lamp, which has a damaging voltage pulse.

This should always be turned on before any other instrumentation including the computer.

2. Turn on the DataHub and NanoLED last. First turn on the FluoroMax and then the computer. Make sure the lamp is on (on the left-hand side of the Fluoromax). Finally, turn on the DataJHub and NanoLED.
3. Turn on the computer. Use DataStation software.
4. Set the detector bias voltage to 950 V using the software. Open the shutter. The bandwidth should be set to 1 nm. Peak Preset: 10000 counts.
5. For the Prompt collection (i.e. the standard sample used to obtain the instrument response function) you will use a monochromator setting of 509 nm, which is equal to the laser frequency. The scatterer used to obtain instrument function is a 0.01% solution of LUDOX. (Ludox AS40 colloidal silica(Sigma-Aldrich order code 42, 084-0 is used in purified water). We have used DI water.
6. The Decay function is the sample of interest. Note that you must save the decay together with the "prompt", which contains the instrument function. Once you have saved it you may delete the decay, but keep the same instrument function for repeated use on a series of samples, e.g. with different quencher concentrations.

Before commencing a decay measurement, collect absorbance data and steady-state fluorescence data for the sample to be measured, which will aid in determining the best wavelengths for excitation and emission. Verifying that the absorbance at your chosen excitation wavelength is approximately 0.1 (certainly no greater than 0.2). This is important to ensure that you do not encounter inner filter effects during your measurement.

3.4 Experimental and analytical methods

3.4.1 Obtaining kinetic data using time correlated single photon counting (TCSPC)

TCSPC is used to measure fast kinetics of fluorescence or other emission processes. Direct measurement of processes that have lifetimes less than 10 ns is difficult since most circuits have an RC time constant that is at least several ns. This time constant limits the rise time of the circuit. We would like the rise time of the electronics to be rapid relative to the kinetics. The "trick" of counting single photons permits us to measure rapid kinetics down to 1 ns or even less with high quality equipment.

The method uses a laser that has low power and high repetition rate. In our instrument, the laser is a 1 MHz laser with a pulse that is approximately 1 ns at 509 nm. The laser light may be scattered in which case there is no delay between the incident light and the light on the detector. Actually, to be precise there is a delay equal to the time it takes for the light to reach the detector from the sample. Let's estimate this as 0.1 m. Then given the speed of light of 3.8 x 10^8 m/s that delay is

$$\tau_{delay} = \frac{0.1\ m}{3.0\ x\ 10^8\ m/s} = 3.33\ x\ 10^{-9}\ s$$

But, this 3.33 ns delay is a constant for any sample. If a molecule in the cuvette has a fluorescence emission then there is an additional delay possible as the photon promotes the molecule to the excited state and then an photon at a different wavelength is emitted some nanoseconds later. The idea behind TCSPC is to measure the individual delays of the emitted photons in electronic bins that count how many photons are emitted with a given delay. More photons will be emitted in the 1 ns bin than the 2 ns bin etc. So by collecting thousands of photons we build up a histogram of the delay times. That histogram should have the form of a kinetic trace. In theory it is the same as what we would measure with a photodiode if it were possible to build a circuit with a response time that is fast enough. But, there is one caveat. The laser itself has a pulse shape on the nanosecond time scale. There are lasers that can generate very short pulses, but these are more expensive than our laser. Therefore, we must measure the shape of the pulse of the 509 nm laser and then "deconvolve" that pulse from our kinetic signal. For this reason we perform an experiment with a sample that scatters light (LUDOX) as a means of measuring the laser pulse. This is called the "prompt". Then we measure the kinetic trace of the fluorescence. Both of these are stored together in one data file.

3.4.2 Fitting data based on the instrument response function convolution integral

You may import your data into the fitting program provided by Horiba for data analysis. The data set consists of the instrument response function (prompt) and the fluorescence data (decay). If we assume that the instrument response function has the form $f(t)$ then the fitting of a single exponential $g(t) = e^{-kt}$ involves non-linear fitting of the convolution integral

$$S(t) = \int_0^{\tau} f(t)g(\tau - t)dt \tag{19}$$

Where $S(t)$ is the signal in the data (decay) and $f(t)$ is the prompt. The deconvolution integral becomes

$$S(t) = \int_0^{\tau} f(t)e^{-k(\tau - t)}dt \tag{20}$$

This type of fitting is non-trivial. It has been described in article by Tang and Norris.[10] Fortunately, the software provided by Horiba carries out this fit. Note that for first order kinetics it is true that

$$k_{obs} = \frac{1}{\tau_{obs}} \tag{21}$$

where τ_{obs} is the observed lifetime. Remember that in your experiment the observed lifetime in the absence of quench is given by

$$\frac{1}{\tau_{obs}} = \frac{1}{\tau_f} + \frac{1}{\tau_{nr}} \tag{22}$$

But we usually write the experiment in terms of the rate constants when quenching is included. Note that $k_Q$is a pseudo-first-order rate constant since quenching is actually a second order process.

$$k_{obs} = k_f + k_{nr} + k_Q[Q] \tag{23}$$

It also properly estimates the noise based on the base line. You should understand that the x-axis of the data is not time, but bin number. Normally, your time window is 200 ns. There are 4096 bins in that time. Thus, there are approximately 20 bins per ns. The program takes all of this into account and provides you with the rate constant, baseline and error estimate for the non-linear fit.

3.5 Report

You will acquire three kinds of data in this laboratory experiment, 1. UV-vis absorption spectra on the Cary UV-vis spectrophotometer, 2. Fluorescence spectra from a fluorometer and 3. Time-resolved emission kinetics. You will use the Stern-Volmer relation to make a linear plot of the relative integrated fluorescence yield as a function of [Q], the quenching concentration. From a straight-line fit of these data (using linear least squares) you will obtain a slope. However, to further analyze that slope you need an independent measure of the observed fluorescence/phosphorescence decay time from the time-resolved setup. If the quenching is dynamic quenching, then you should also see a change in the lifetime as you increase the quencher concentration. Thus, the time-resolved experiment is a check on the assumptions of the Stern-Volmer derivation (dynamic quenching).

Record the fitted values of the rate constant for first-order kinetics from the deconvolution program available on the Horiba TCSPC computer. Non-linear least squares fitting of a deconvolution integral is an advanced topic so we will use commercial software for this part. Assuming you can obtain a single exponential fit, please record the rate constant in your lab notebook to 3 significant figures. Also record the standard error from the fitting program. These measurements should be conducted in triplicate so that the error you will report is based on the standard error obtained from the average of the three measurements. The time constant of the fit is the observed lifetime, τ_{obs}. Report those time constants as a function of the quencher concentration.

Obtain the fluorescence spectrum of the same samples and record the relative fluorescence quantum yield. The relative quantum yield is obtained by comparing the area under the fluorescence spectrum over conservative limits that include most of the emission. You can obtain the error in Excel, Igor or any other plotting and fitting program that allows you to integrate data. Stern-Volmer quenching only requires a relative quantum yield. You will need the absolute quantum yield of uranine to obtain all of the requested parameters. You are free to look up the literature value for the absolute fluorescence quantum yield of uranine (see references).

From these two measurements you should be able to obtain k_f, k_{nr} and k_Q (with appropriate errors). Describe how you obtained these fundamental rate constants by showing the plots of raw data, explaining the fitting procedures, relating the fits to various observed rate constants and relative quantum yields. Integrate each fluorescence signal in a software package such as Excel,

Origin or Igor. Fit each decay to an exponential model in a software package. Examine the residuals to ensure that the fit to a single exponential is adequate. Use the integrated values obtained from the experiment to determine the Stern-Volmer rate constant (Eqn. 9). You will then need to use the measured lifetime, τ_{obs}, to determine the quenching rate constant (Eqn. 10).

To determine the errors in the rate constants you will need to use the values you can measure, which are the relative quantum yield and τ_{obs}. Therefore, you will obtain estimates of fundamental rate constants using the measured value of $\sigma(SV) = \sigma(\Phi_0/\Phi)$ and $\sigma(\tau_{obs})$. Use propagation of error for the Stern-Volmer equation and solve for the error in the quenching rate constant, $\sigma(k_Q)$ (NOTE: once you have looked up Φ_0). For k_f and k_{nr} you may use the expression for the quantum and assume that you know Φ_0 and you measure τ_{obs}. Again use propagation of error, but for the unquenched fluorescence process to obtain $\sigma(k_f)$ and $\sigma(k_{nr})$.

Points for discussion

In your discussion, please mention the possible quenching mechanisms (please consult the literature and provide references) and potential sources of error. Compare these to literature values and provide an analysis of the quenching. Can you differentiate between static and dynamic quenching?

Look in the chemical literature and find diffusion coefficients for the molecules studied here or similar molecules. Using an estimate of the diffusion coefficient calculate the quenching radius. Discuss the assumptions that go into this calculation (as discussed above).

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