LABORATORY OF ELEMENTARY BIOPHYSICS

Experimental exercises for III year of the First cycle studies

Field: „Applications of physics in biology and medicine”

Specialization: „Molecular Biophysics”

Quenching of SPQ fluorescence by chloride – steady state and time-resolved fluorescence (PB18d)

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Projekt *Fizyka wobec wyzwań XXI wieku* współfinansowany ze środków Unii Europejskiej w ramach Europejskiego Funduszu Społecznego

I. Introduction

The goal of this experiment is to determine the mechanism of 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) fluorescence quenching by chloride. You will perform steady-state and time-resolve fluorescence measurements.

I.1. Collisional and static fluorescence quenching

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching. These include excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation (static quenching), and collisional (dynamic) quenching.

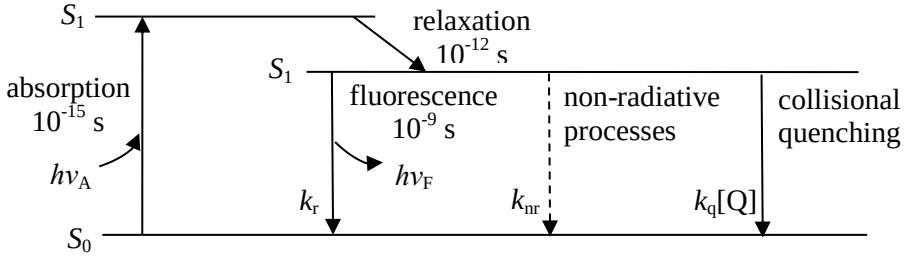

Figure 1. Jabłonski diagram showing the processes of absorption, fluorescence, solvent relaxation and collisional quenching. For simplicity only the first singlet excited state S_1 is indicated and neither vibrational nor triplet levels are shown. k_r and k_{nr} are the rate constants for fluorescence emission and non-radiative processes, respectively (for convenience all possible non-radiative decay paths are characterized by the single rate constant k_{nr}) and k_q is the bimolecular quenching constant.

In case of **collisional quenching** the excited-state fluorophore (A*) is returned to the ground state without photon emission during a diffusive encounter with the quencher (Q):

$$A^* + Q \xrightarrow{k_q} A + Q + heat$$

where k_q is the bimolecular quenching constant, proportional to the diffusion-controlled bimolecular rate constant k_0. The efficiency of quenching depends on quencher concentration [Q] and the rate of fluorohore-quencher diffusion (described by the k_0 rate constant).

According to the Jablonski diagram shown in Figure 1, the ratio of quantum yields, and hence the ratio of fluorescence intensities in the absence and presence of quencher is given by:

$$\frac{F_0}{F} = \frac{\frac{k_r}{k_r + k_{nr}}}{\frac{k_r}{k_r + k_{nr} + k_q[Q]}} = \frac{k_r + k_{nr} + k_q[Q]}{k_r + k_{nr}} = 1 + \frac{k_q}{k_r + k_{nr}}[Q] \qquad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, and k_r and k_{nr} are the rate constants for fluorescence emission and non-radiative processes, respectively.

Bearing in mind that the fluorescence lifetime is the inverse of the sum of all rate constants leading to depopulation of the excited state, equation (1) can be rearranged to:

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \qquad (2)$$

where $\tau_0 = (k_r + k_{nr})^{-1}$ and $\tau = (k_r + k_{nr} + k_q[Q])^{-1}$ are the lifetimes in the absence and presence of quencher, and $K_{SV} = k_q\tau_0$ is the Stern-Volmer quenching constant.

The expression (2) is called **the Stern-Volmer equation**. It follows a plot of F_0/F and τ_0/τ is linear with an intercept of 1 and a slope equal to K_{SV}. The K_{SV} constant indicates the sensitivity of the fluorophore to a quencher.

Static quenching results from the formation of a nonfluorescent ground-state complex between the fluorophore and quencher:

$$A + Q \overset{K}{\leftrightarrow} AQ \overset{h\nu}{\rightarrow} AQ^* \rightarrow AQ + ciepło$$

where $K = \frac{[AQ]}{[A][Q]}$ is the equilibrium constant for the formation of the „dark" complex, AQ.

From definition of the association constant K and from the fact that fluorescence intensity F in the presence of quencher is proportional to the fraction of the total fluorophores that are not complexed ([A] = $[A_0]$-[AQ]) one can obtained the dependence of the fluorescence intensity upon quencher concentration:

$$\frac{F_0}{F} = 1 + K[Q] \qquad (3)$$

Note that the dependence of F_0/F on [Q] is linear, which is identical to that observed for dynamic quenching (Eq. 2), except that the quenching constant is now the association constant. Hence, fluorescence quenching data obtained by intensity measurements alone cannot determine the quenching mechanism. The best method to distinguish static and dynamic quenching is the measurement of fluorescence lifetimes. Equation (2) follows that $\frac{F_0}{F} = \frac{\tau_0}{\tau}$ for collisinal quanching. In contrast, in case of static quenching, the fluorophore-quencher interaction takes place in the ground-state, hence it does not influence the value of life time. Therefore, for static quenching $\frac{\tau_0}{\tau} = 1$

A wide variety of small molecules or ions can act as collisional quenchers of fluorescence. These substances include chloride (Cl^-), iodide (I^-), oxygen, and acrylamide.

The accessibility of fluorophores to such quenchers can be used to determine the location of probes on macromolecules.

I.2. SPQ – fluorescent indicator for chloride

SPQ (6-methoxy-N-[3-sulfopropyl]-quinoline) is the fluorescence probe sensitive to quenching by chloride (probably by photoinduced electron transfer). Therefore, it is used to determine the intracellular chloride concentration.

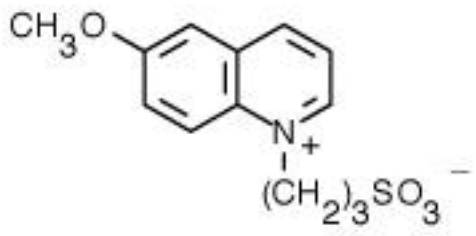

Figure 2. The structure of 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ)

During the exercise you will investigate the effect of KCl concentration (source of chloride) on the SPQ fluorescence intensity and lifetimes. On the basis of the measurements you will determine the mechanism of quenching.

II. The requirements for entrance test

1. The phenomenon of absorption and emission of electromagnetic radiation
2. Fluorescence, fluorescence emission spectrum, fluorescence decay, fluorescence quantum yield and lifetime, analysis of a fluorescence decay data
3. Collisional and static fluorescence quenching, the Stern-Volmer equation, the quenching constant

III. Experimental part

During the exercise you will measure the fluorescence emission spectra and fluorescence lifetimes of SPQ in water in the absence and presence of different KCl concentrations. Steady-state measurements you will perform using Cary-Eclipse fluorimeter (Agilent). The fluorescence decays you will record using time-resolved fluorescence lifetime spectrometer FT300 (PicoQuant) and time-correlated single photon counting method.

You will be provided with:

– SPQ in water

– deionized water

– 100 mM KCl in water

– Ludox scattering solution

– a pair of absorption quartz cuvettes of 1 cm optical path length

– fluorescence quartz cuvette of 1 cm optical path length

1. Prepare SPQ aqueous solutions with different KCl concentrations: 0, 10, 20, 30 i 40 mM.

2. Register absorption spectrum of based SPQ solution in the range of 250-450 nm using a spectrophotometer. What is the maximum position of the absorption spectrum? Determine SPQ concentration (the extinction coefficient of SPQ at 344 nm is equal $\varepsilon(344) = 3700\ M^{-1}cm^{-1}$).

3. Register the fluorescence emission spectra in the range of 360-620 nm for each SPQ solution prepared at point 1. On the basis of the measured absorption spectrum, select appropriate excitation wavelength. Where have you observed the maxima of emission spectra? Read the value of fluorescence intensity at the maximum for each SPQ sample.

4. Measure the fluorescence decays for each sample. On the basis of the measured absorption and emission spectra, select appropriate excitation sources and observation wavelength. Register also the instrument response function (IRF) using Ludox scattering solution. What excitation source and emission wavelength should be selected for IRF measurements?

5. Do an analysis of the acquired decay data using FluoFit software to determine lifetimes for each sample. Perform IRF reconvolution with exponential decay model.

IV. Final report

Final report should include all elements typical for the experiment description (abstract, introduction, description of the experimental system, results and discussion).

The results should be shown in tables and figures. The fluorescence decay data should be analysed using FluoFit program.

The following issues should be taken into account:

1. Presentation of the plot with the F_0/F and τ_0/τ dependence on KCl concentration
2. Determination of the mechanism of SPQ fluorescence quenching by chloride

3. Calculation of the Stern-Volmer quenching constant K_{SV} and the bimolecular quenching constant k_q
4. Comparison of the k_q value with the diffusion-controlled bimolecular rate constant k_0 in water ($k_0 = 10^{10}$ $M^{-1}s^{-1}$)

V. Bibliography

1. „Principles of Fluorescence Spectroscopy" Joseph R. Lakowicz
2. „Fluorescence decay kinetics measurements of typical fluorescence dyes", A. Modrak-Wójcik – instruction for exercise PB16 (Laboratory of Elementary Biophysics)