Ma 5: Dynamic Processes in lipid membranes

1. Introduction

Fluorescence is a powerful tool for investigating the structure and dynamics of systems at a molecular or supramolecular level. Biological membranes, proteins, nucleic acids and living cells are well-known systems in which local parameters such as polarity, fluidity, order and molecular mobility is possible to be investigated by means of fluorescent molecules acting as probes. The latter can be intrinsic or externally applied. The high sensitivity and specificity of the response allows for an accurate readout of the response of the microenvironment. Another factor is the ability of probes to provide information on dynamics of fast phenomena and the structural parameters of the system under study.

The aim of this experiment is to give an overview of molecular fluorescence, allowing to understand the fundamental phenomena, which is a prerequisite for its practical use in analyzing for example biological membrane properties, such as fluidity, order parameters, lipid–protein interactions, translational diffusion, site accessibility, structural changes, lateral organization, dynamics and many more.

In this experiment we focus on using steady-state and time-resolved fluorescence methods to

- Characterize the fluorophore probe by identifying the excitation- and emission spectra of the fluorophore, the environmental interaction;
- Analyze the dynamics of a lipid membrane at different temperatures by means of steady state- as well as time-resolved fluorescence measurements by measuring the fluorescence anisotropy. The membranes microviscosity, order, van't Hoff enthalpy and cooperativity of the lipids phase transition can be deduced.

Theoretical background

Light is an electromagnetic wave consisting of an electric field E and a magnetic field B perpendicular both to each other and to the direction of propagation, and oscillating in phase. Most chromophores absorb light along a preferred direction, depending on the electronic state. In contrast, the emission transition moment is the same whatever the excited state reached by the molecule upon excitation, because of internal conversion towards the first singlet state. Thus, when a population of fluorophores is illuminated by a linearly polarized incident light, those whose transition moments are oriented in a direction close to that of the electric vector of the incident beam are preferentially excited. This is called photoselection. Because the distribution of excited fluorophores is anisotropic, the emitted fluorescence is also anisotropic. Any change in direction of the transition moment during the lifetime of the excited state will cause this anisotropy to decrease, i.e. will induce a partial (or total) depolarization of fluorescence. The decay times and final anisotropies yield information about fluorophore order and steric restrictions.

In the experiment, the fluorophore DPH (Diphenylhexatriene, see Fig. 1) is used as a reporter probe which gets inserted into the hydrophobic core of the DMPC vesicle membrane (see Fig. 1). Biological membranes allow life as we know it to exist. They form cells and enable separation of an organism, setting not only a barrier for substances, but also controlling by means of their selective permeability which may pass. Gradients of ions across them, enable living organisms to generate energy. Biological membranes consist of a double sheet (known as a bilayer) of lipid molecules, called phospholipid bilayer.

DPH molecules in aqueous solution are quenched by the polar water molecules so that its fluorescence is only ~ 1/200 of DPH fluorescence in a hydrophobic environment. Hence, fluorescence of free DPH in solution can be neglected.

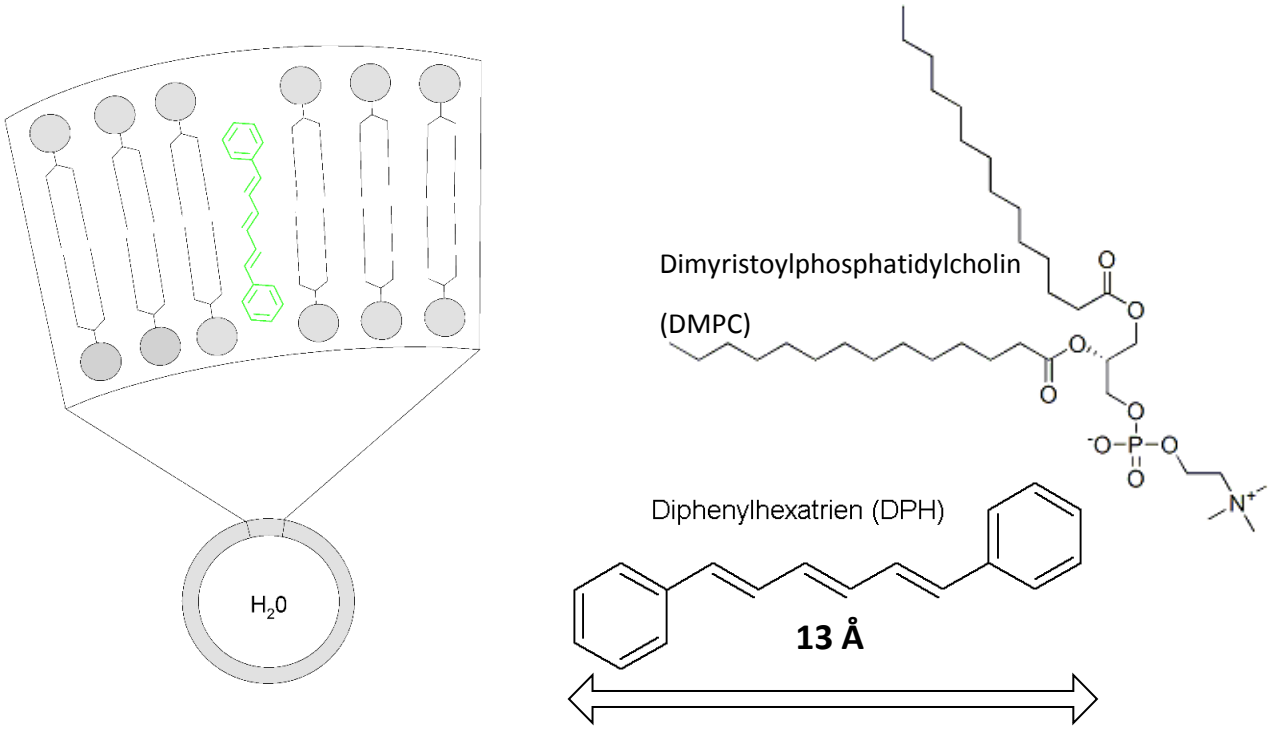


Fig 1.: Vesicle (DMPC molecules) in solution with DPH molecule inserted. Chemical structure of DMPC and DPH.

At ~ 23°C the DMPC molecules in the vesicle membrane undergo a phase transition from an ordered state towards a disordered state. The ordered state corresponds to a gel like phase, whereas the disordered state reflects a liquid crystal like phase. The fluorescence probe DPH within the DMPC vesicle membrane reports on the conformational freedom of the DMPC molecules by exhibiting a faster anisotropy decay in the liquid crystal phase.

In preparation for the experiment, you should be familiar with the following terms:

Transition dipole moment, □ -□□* transition, Born-Oppenheimer approximation, Franck-Condon principle, absorption, emission, fluorescence, non-radiative transitions, quenching, fluorescence lifetime, quantum yield, rotational diffusion, anisotropy, stokes shift, rate equations. Experimental setup for polarized fluorescence measurements, distribution of transition dipole moments after photoselection, diffusional equation, Time correlated single photon counting (TCSPC).

2. Measurements/ Tasks in the lab

2.1 Setup

Draw a sketch of both measurement setups (Steady state **and** Time resolved fluorescence). The sketches and the description must be in such a way, that an experimentalist could recreate the setups and the experiments!

2.2. Sample preparation

Measurements are in 4 x 10 mm quartz cuvettes: Take two cuvettes and fill them with
A) 1 ml vesicle solution
B) 1 ml buffer solution
add than to:
A) + 8 µl 1 mM DPH in THF (Diphenylhexatriene in Tetrahydrofurane)
B) + 8 µl 1 mM DPH in THF (Diphenylhexatriene in Tetrahydrofurane)
Creating the two samples investigated further on.

2.3. Fluorescence excitation and emission spectra

What are the main features/property information you will gain by these spectra?

1) First, take an excitation spectrum of sample **A** (λ_{ex} = 270 – 420 nm, λ_{em} = 450 nm).
2) Then take emission spectra of both **A** and **B** (λ_{ex} = 360 nm, λ_{em} = 380 - 530 nm).
   First measure the vesicle sample A (decide and set all parameters) and then measure B changing the RANGE only!

Choose the scan steps (nm) and the integration time of each step.

Important:

- Choose the settings for each spectra to have a good S/N ratio and no overload of the detector: Check beforehand especially the settings of the PMT!

- Write down all parameters of the PMT controller and your chosen settings in the program (not all of this settings will be written in the data file)!

2.4. Steady state fluorescence depolarization

Use the vesicle sample (**A**; DPH in DMPC in phosphate buffer). (Why only this one is needed?)
Choose an appropriate excitation and emission wavelength by looking at the previous recorded spectra.
Measure zero voltage (closed shutter) and G-factor beforehand ($G = {}^{I_{HV}}/_{I_{HH}}$). Why?
Take measurements at following temperatures:
T = 10°C, 12°C, 16°C, 18°C, 20°C, 21°C, 22°C, 22.5°C, 23°C, 23.5°C, 24°C, 24.5°C, 25°C, 26°C, 27°C, 29°C, 31°C, 33°C, 35°C.
Record both intensities (I_{VV} and I_{VH}) and plot anisotropy for each temperature.

2.5 Time resolved fluorescence depolarization of DPH in DMPC vesicles.
Switch setup to time resolved setup. Turn on laser (key based) and check in the program (B&H software) the snychornisation signal between Time correlated single photon counting (TCSPC) card and laser. When opening the measuring chamber make sure the PMT is NOT overloaded, by **switching off PM Voltage prior to opening the lid** carefully (check also that the ADC signal in the Program stays low). Choose appropriate measurement time (S/N ratio).

1) Measure milk powder (scattering sample) in solution (cuvette at the setup). What information is gained by this?
2) Take the vesicle sample (A) and measure the fluorescence S(t) and anisotropy R(t) for temperatures T = 10°C, 23°C, 35°C. Install the long pass filter 410nm provided in the excitation path. Why?

3. Additional information for the setups/experimental procedure:
The used hardware is a current research setup and as such prone to erroneous use! Please act thought- and careful.

Steady state fluorescence setup
To turn on the fluorescence excitation lamp (Xe-Lamp) turn on the power switch, power the ventilator on with the dip-switch (EIN) behind the lamping housing, enable the water cooling, wait a few minutes and press the red button to light up the lamp.
Set high voltage adjust controls no higher than 650 (corresponds to 1300 V PM Voltage). Set reference PM (channel 2) to max. 450 (corresponds to 900 V PM Voltage). (**preset by the tutor**)

Set RANGE controls of PMT1 at least to 3 μA (sensitivity). Adjust for better signal to noise ratio, while experimenting (keep record of the chosen setting). If OVERLOAD indicator lights up, decrease PM Voltage and press RESET button (red).
Use the LabView-based software implemented on the PC. Set starts values for wavelength manually at both monochromators, and then switch one monochromator to "remote", which is desired to change over the experiment. Instrument indicators mustn't go off scale! Also see that $I(PM_1) < I(PM_2)$.
For temperature measurements, a thermocouple is inserted next to the cuvette. Wait for the desired temperature to be stable, since it is most important to be stable for the **whole measurement time at a given temperature**!
Don't forget to write down all needed parameters (PM settings, G-factor). Integration time constant, slit widths, and scan steps have to be adjusted optimally.

For steady anisotropy measurements also the setup is used. Use the LabView-based software (second register card). Measure the zero voltage (measure with closed emission/excitation shutter). Insert the detected intensity as zero voltage in the program. Each intensity must be corrected by the zero voltage! Measure the G-factor ($G = I_{HV}/I_{HH}$).

Therefore set excitation polarizer to horizontal (pulled out) and the emission polarizer to vertical (pushed in) and measure I_{HV}. Repeat measurement for other emission polarization to obtain I_{HH}. Then switch to vertical excitation polarization (pushed in) for further measurements. When desired temperature is achieved measure first with vertical emission polarization and afterwards with horizontal polarization. Calculate the anisotropy R using

$$R = \frac{I_{VV} - (G * I_{VH})}{I_{VV} + 2 * I_{VH}}$$

Time resolved fluorescence setup

- A ps pulsed laser in the UV (370nm, 20 kHz pulses) is used. This laser is almost not visible for the eye and students should take extra care! A mechanical shutter stops the laser beam while opening the measurement chamber, but still precaution is advised while opening the chamber. Ask and remember for safety advise. Check for closed shutter while operating inside chamber.
- Switch off PM-voltage before opening the lid of the measurement chamber!
- Use the Becker&Hickel hardware/software implemented on the left PC.
- Laser is horizontal polarised, but detector is optimized for vertical polarization (measure signal with installed λ/2 plate. What is this plate changing?)
- Take care of lever for horizontal/vertical emission polarization (horizontal → lever pushed in; inverse to steady state levers)

4. Tasks and data analysis presented in the protocol

For task 2.1:
Draw a sketch of the measurement setups (steady state and time resolved) and explain the function and principle of measurement (max. one page)

For task 2.2:
How would you prepare 100 ml of 1 mM DPH solution in THF (M_W(DPH) = 232.3 g/mol)? Include formula for calculation.

For task 2.3:
Estimate the ratio of quantum yields

$$\frac{\Phi(k_r, k_{nr}, DPH_{H_2O})}{\Phi(k_r, k_{nr}, DPH_{Lipid})}$$

From the structured spectra (DPH in Lipid), determine the corresponding oscillation frequencies ω (in Hz) of the ground state and the excited states.

$$E_n - E_0 = n\hbar\omega$$

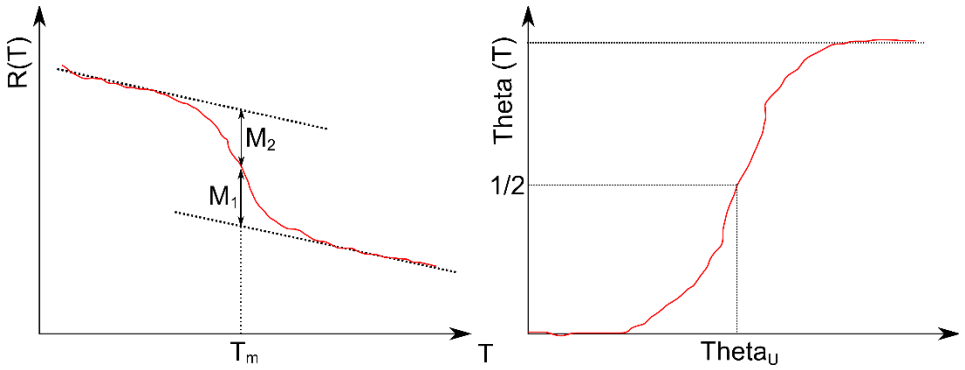
E_n being the energy of a vibrational state (S_0^n) and E_0 the corresponding electronic ground state (S_0^0).

For tasks 2.4:
Plot the anisotropy R(T) over the temperature T and the conversion yield

$$\Theta = \frac{M_2}{(M_1 + M_2)}$$

for the phase transition of the lipid vesicle.
The function Θ(T) can be interpreted by an equilibrium reaction $M_1 \leftrightarrow M_2$ with equilibrium constant

$$k = \frac{M_2}{M_2} = k_0 e^{-\frac{\delta H}{RT}}.$$



From the slope d Θ/dT, calculate the van't Hoff enthalpy. From equations (5) and (6) follows

$$\left.\frac{d\Theta}{dT}\right|_{(T_m)} = \frac{\delta H}{4RT^2}$$

A very high van't Hoff enthalpy results compared to the calorimetric measurement H_{th}= 30 kJ/mol. Explain why the cooperative effect is most likely the reason for this.

For task 2.5:

Calculate the time dependent anisotropy curves.

Compare the values for <R> calculated with the values determined in 2.3.

Calculate the microviscosity in [Pa * s] using the rotational correlation times Φ_{rot}.

Calculate the cone angles for the simple wobbling-in-cone model using the ratio R_0/R_∞ for three temperatures.

Discuss the temperature dependence of lifetime τ, rotational correlation time Φ_{rot}, R_∞ and η(see reference [3]).

References

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