Ma5: Dynamic Processes in lipid membranes

1. Overview of the topic and goals of the experiments

Fluorescence is a highly sensitive method for detection of tiny amounts of molecules (nanomolar). In biophysics, intrinsic fluorophores (e.g. tryptophane, tyrosine) can be used or fluorescent reporter molecules can be used as probes.

The range of applications spans:

- Calculation of pH and polarity of microenvironment by analyzing the shape of the emission spectra;
- Calculation of microviscosity and conformational space by measuring fluorescence anisotropy;
- Calculation of distance to acceptor molecule by determination of energy transfer rate.

The goal of this experiment is:

- Getting to know steady-state and time-resolved fluorescence methods;
- Study the influence of environment on form, position and excitation- and emission spectra of the fluorophore;
- Analyze the dynamics of a lipid membrane at different temperatures by means of steady state- as well as time-resolved fluorescence measurements. The membranes microviscosity, order, van't Hoff enthalpy and cooperativity of the lipids phase transition can be deduced;
- Computer-assisted analysis of steady state fluorescence anisotropy as well as time-resolved fluorescence anisotropy data.

2. Theoretical background

An anisotropic distribution of emitting transition dipoles is created by excitation of an isotropic sample with a nanosecond pulse of linearly polarized light (photoselection). The emitted fluorescence is highly polarized. Relaxation of the polarized emission takes place due to rotational diffusion. This leads to depolarization of fluorescence. Depending on the equilibrium state of the sample, the final anisotropy reaches zero or not (in the case of restricted rotational diffusion) for "long" measurement times. The decay times and final anisotropies yield information about fluorophor 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).

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.
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, check the following literature excerpts (see appendix). It will be sufficient to be able to explain the catchwords with a little sketch or equation/formula:

- Transition dipole moment, $\pi - \pi^*$ 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.

3. Important information about hardware and measurements

The used hardware is a current research setup and as such prone to erroneous use!

3.1. Fluorescence lifetime setup

- Switch off PM-voltage before opening the lid of the measurement chamber!
- PM-voltage no higher than 1000 V.
- Ma5.3 -

- Pressurize flashlamp with 500 mbar H₂, set lamp voltage no higher than 7 kV.

3.2. Steady state fluorescence setup

- 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).
- Set RANGE controls at least to 3 µA. If OVERLOAD indicator lights up, press RESET button (red) and decrease PM Voltage.
- To record spectra, use the “MESS” software implemented on the PC. Set start values for wavelength manually, then switch to “remote”. Instrument indicators mustn’t go off scale! Also see that I(PM₁) < I(PM₂). Don’t forget to write down all needed parameters (PM settings, Temperature). Time constant, slit widths, and scan speed have to be adjusted optimally.
- For temperature measurements in 4.3. the thermocouple is directly inserted into the cuvette until the final temperature is reached. Remove thermocouple before measurement.
- For anisotropy measurements only one arm (one photometer) is used (L-setup). Set all slid widths to “half”. Use (0) = Test of MESS software. Set excitation polarizer to horizontal. Adjust intensities by varying slit width, PM voltage and RANGE. Then switch to vertical excitation polarization and write down the values (Iᵥ, three digits). Average ~ 10 values. Repeat measurement for other emission polarization to obtain Iᵥ. Calculate the anisotropy R using

\[ R = \frac{Iᵥ - I_H}{Iᵥ + 2I_H} \]  

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- In 4.2. first measure the vesicle sample (DPH in DMPC) and then measure DPH in H₂O changing the RANGE only!

4. Measurements

4.1. Draw a sketch of both measurement setups

- Steady state fluorescence
- Time resolved fluorescence

4.2. Fluorescence as a function of solvent polarity

Take two Eppendorf tubes and fill them with

a) 1 ml vesicle solution
b) 1 ml buffer solution
a) + 8 µl 1 mM DPH in THF (Diphenylhexatriene in Tetrahydrofurane) (A)
b) + 8 µl 1 mM DPH in THF (Diphenylhexatriene in Tetrahydrofurane) (B)

Measurements are in 4 x 10 mm quartz cuvettes.

First, take an absorption spectrum of sample A (λₑₓ = 270 – 420 nm, λₑₘ = 450 nm).
Then take emission spectra of both A and B (λ_ex = 360 nm, λ_em = 380 - 530 nm).

Important: Write down all parameters of PM controls! Keep samples A and B for further measurements.

4.3. Steady state fluorescence depolarization

Use the vesicle sample from 4.2. (A; DPH in DMPC in phosphate buffer). Take measurements at temperatures T = 10°C, 15°C, 18°C, 21°C, 22°C, 22.5°C, 23°C, 23.5°C, 24°C, 24.5°C, 25°C, 26°C, 27°C, 30°C, 35°C. Set the excitation polarization to horizontal, set voltage to defined value, and then switch to vertical excitation polarization. Now read the value for I_V and I_H respectively. Calculate the anisotropy R as in equation (1).

Hint: Close apertures for excitation and emission half way (Why?).

4.4. Time resolved fluorescence depolarization of DPH in DMPC vesicles.

Take the vesicle sample (A) from 4.2. and 4.3. and measure the fluorescence (S(t)) and anisotropy (R(t)) for temperatures T = 10°C, 23°C, 35°C. Measurements are taken using the right emission arm with vertical excitation polarization. To calibrate the setup, record the count rates for both emission polarizations for one minute each with the excitation polarizer set horizontally. The G-factor is calculated as G = N_{par}/N_{per}. Additionally a calibration of the time channels by changing the time delay is required.

Important: Switch off PM Voltage prior to opening the lid of the measurement chamber!

5. Tasks and data analysis

5.1. How would you prepare 100 ml of 1 mM DPH solution in THF (M_W(DPH) = 232.3 g/mol)

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

5.3. Estimate the ratio of quantum yields

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

(2)

From the structured spectra (DPH in Lipid), determine the corresponding oscillation frequencies (in cm^{-1}) of the ground state and the excited states. Calculate the force constant k and the zero point oscillation x_0 from the spectra using

$$x_0^2 = \frac{\hbar}{m \omega} \quad \text{and} \quad k = m \omega^2$$

(3)
with \( m \approx m_c \). The zero level difference \( D_x \) of the potentials depends harmonically on the difference in energy

\[
E_{\text{max}} - E_{\text{iso}} + h \omega = k \Delta x^2 / 2
\]  

(4)

5.4. Plot the anisotropy \( R(T) \) over the temperature \( T \) and the conversion yield

\[
\Theta = \frac{M_2}{(M_1 + M_2)}
\]  

(5)

for the phase transition of the lipid vesicle.

The function \( \Theta(T) \) can be interpreted by an equilibrium reaction \( M_1 \leftrightarrow M_2 \) with equilibrium constant

\[
K = \frac{M_2}{M_1} = k_0 e^{-\frac{\Delta H}{RT}}.
\]  

(6)

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

\[
\frac{d\Theta}{dT} \bigg|_{T_m} = \frac{\Delta H}{4RT^2}.
\]  

(7)

A very high van’t Hoff enthalpy results compared to the calorimetric measurement \( H_{\text{th}} = 30 \text{ kJ/mol} \). Explain why that is.

5.5. Compare the values for \( \langle R \rangle \) calculated by the computer software with the values determined in 4.3. Calculate the microviscosity in \( [\text{Pa} \cdot \text{s}] \) using the rotational correlation times \( \Phi_{\text{rot}} \). Calculate the cone angles for the simple wobbling-in-cone model using the ratio \( R_0/R_\infty \) for three temperatures.

5.6. Discuss the temperature dependence of lifetime \( \tau \), rotational correlation time \( \Phi_{\text{rot}} \), \( R_\infty \) and \( \eta \) (see reference [4]).

6. References

The references can be found in the lab course folder in the “FP collection” of the library (FB-Bibliothek).


