Fluorescence-based approach for bio-membrane studies

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Introduction

In this thesis the isomerization process of the Merocyanine 540 fluorophore is studied. The presence of that process, when the dye is attached to the lipid membrane, was not reported before, and thus become a main interest of this work. For the further investigation: the reaction of the isomerization dynamics to the different properties of the membrane was studied. The influence of such membrane’s parameters as the size, polarity and fluidity were checked.

It was revealed that the isomerization of the MC 540 is present inside the lipid membranes, and its dynamics is changed with the membrane’s polarity and viscosity. So the MC 540 could be in use as a probe for the investigation of the membrane’s properties.

The isomerization process was studied with the help of the fluorescence correlation spectroscopy (FCS) which is a powerful technique for the single molecule studies.
Chapter 1. Fluorescence and fluorophores

1.1 Historical basis and general descriptions

The phenomena of fluorescence was first observed and reported in 1564 by the Spanish scientist Nicolas Monardes. From the end of XIX century, substances that have fluorescent properties become in a widespread use for different research applications. For example, the first applied use of fluorescence was documented in 1877, when addition of the fluorescein into the water of Danube River proved that it has an underground connection to the Rhine [Lakowicz 2000]. For the medical issues, fluorescence was first used in 1950s for the development of antimalaria drugs. Nowadays, it is hardly possible to imagine any medical or biological research, which takes place on the level of the cell without fluorophores, especially when imaging processes are concerned.

Significant contribution to the development of fluorescence theory was done by the Polish scientist of Ukrainian origin Professor Alexander Jablonski. Main processes that give rise to the phenomenon of fluorescence easily can be described by his diagram. In Figure 1.1 a typical Jablonski diagram is shown [Jablonski 1935]. There both fluorescence and phosphorescence are presented and their principles are intuitively transparent.
The diagram is organized in such a way that in a vertical direction the electronic states are arranged in concordance with their energy, while horizontally – in concordance with their spin multiplicity. The presence of five basic energy states are shown: $S_0$ – ground state, $S_1$ – first excited state, $S_2$ – simplified representation of all higher excited states, $T_1$ – first triplet state and $T_2$ – simplified representation of all higher triplet states. By the thin lines the internal sub-states of each of the main states are presented. Two important energy transfer mechanisms – intersystem crossing and internal conversion are
represented on the diagram by the abbreviations ISC and IC. This diagram will be used in what follows to describe the processes that occur in fluorophores after excitation.

There are three main events that are involved in a process of photoluminescence. All of them can be explained from the quantum mechanical point of view. Since, in photoluminescent substances, the difference between the ground energetic state and the first excited energetic state is large enough to make impossible the intersystem crossing (from lower to higher states) only due to thermal energy – light should be used to provoke an excitation. The process of excitation is fast and occurs on femtosecond timescale. Within such small time interval, the electron, due to its small mass (in comparison to the mass of nuclei), can not provoke any displacement of the nuclei, so the electronic transitions occur only in vertical direction (Frank-Cordon principle, Figure 1.2). The Frank-Cordon principle is equally applied to the tractions from higher to lower energetic states as well.

![Figure 1.2 The Frank-Cordon principle. $E_0$ and $E_1$ represent the ground and the first excited energy levels. $\nu^*$ and $\nu'$ represent the internal vibrational levels of the $E_0$ and $E_1$, respectively. [Adopted from http://wikipedia.org]](http://wikipedia.org)
After absorption of the energy of the light photon by the atom, transitions between the lower and higher electronic states become possible. As it is seen from Figure 1.2 – each electronic state has some number of the vibrational levels, thus, in a dependence on what amount of energy is carried by incoming photon \((E = h\nu)\), electrons can move: a) – to higher vibrational levels of the ground state, b) – cross the gap and move to some vibrational level of the higher electrical state, or c) – if energy is large enough, the electron can be kicked out from the atom. If the electron moves to the higher vibrational levels of the ground state it will relax to the lowest level \(\nu''_0\) and all accumulated energy will be spent on heat.

So, if the energy, carried by the photon, was enough for the electron to cross interstate energy gap, then it moves to some vibrational level of higher energy state. There, due to the internal conversion, it relaxes to the lowest vibrational level of the current excited state. Emission of the new photon is possible only from the lowest vibrational level \(\nu'_0\) (Kasha rule - named after Michael Kasha, American physicist of Ukrainian origin). That process is called internal conversion and occurs within picoseconds time range. After the electron has occupied level \(\nu'_0\) three general scenarios become possible. The simplest one is when the electron returns to the ground state without photon emission. For the other two scenarios, such factor as a spin direction should be taken into account.

Initially at the lowest electronic state \((S_0)\) fluorescent molecule is in the singlet state, which means that the orbital is occupied by the two electrons which have opposite spin directions. The electron spin projection number for the fluorescent molecule is \(m_s = \pm \frac{1}{2}\), so the multiplicity, which is \(2nm_s + 1\) (where \(n\) – is the number of singly occupied electrons) equals 1, since all electrons are paired. Such arrangement is required due to the Pauli Exclusion
Principle. Excitation also occurs to the singlet states. If the spin of the excited electron flips to the opposite direction, then the spin conversion to a triplet state occurs ($T_1$ or $T_2$ in Figure 1.1). Emission from the triplet state is called phosphorescence, while emission from the first excited singlet state $S_1$ is called fluorescence. The photon, emitted from the triplet state, will have a longer wavelength, due to the lower energy difference between the states $T_1$ and $S_0$, compared to the difference between $S_1$ and $S_0$ (Stokes shift\(^1\)).

Another important characteristic of the phosphorescence is the lifetime. Fluorescence lifetime is the time that the electron spends at excited singlet states, before it returns to the ground state $S_0$. For the process of fluorescence (transition $S_1 \rightarrow S_0$) the lifetime is in the range of $10^{-10} - 10^{-7}$ sec, for phosphorescence (transition $T_1 \rightarrow S_0$) $- 10^{-6} - 1$ sec. Such difference arises due to the fact that transition $T_1 \rightarrow S_0$ is not an “easy to go” path for the electron. Such path is “forbidden” by quantum mechanics, but since this transition is dissipative from the energetic point of view it can be possible. If the molecule was excited to a higher electronic state ($T_n, n > 1$), then the opposite transition from triplet to singlet states also becomes possible (reverse intersystem crossing, for example: $T_1 \rightarrow T_2 \rightarrow S_1$) [Widengren and Seidel 2000]. It should be mentioned, that the probability that a photon will be emitted from the triplet state is much lower then it is for the singlet state ($\Delta E_{T_1 \rightarrow S_0} < \Delta E_{S_1 \rightarrow S_0}$), irradiative

\(^1\)The Stokes shift, named after the Sir George Gabriel Stokes who was the first person who noticed that photoluminescense occurs at the longer wavelengths. From the energetic point of view such statement is clear. Since the fluorescent molecule can be considered as a closed system, then the emitted photon will have a lower energy then the excited had, because due to Kasha rule, excitation can not occur directly from the vibrational state within the electronic state to which the electron was excited and some energy must be spend on internal convection processes, so the resulting photon energy can be presented as $E_{\text{em. photon}} = E_{\text{exc. photon}} - E_{\text{internal processes}}$. Also relaxation can occur to a higher vibrational level of the ground state, which also decreases the energy of the emitted photon. The process is marked on the figure 1.3.
transition for $T_i \rightarrow S_0$ is more possible than for $S_i \rightarrow S_0$), so the dyes that go to the triplet state are much less bright.

It might be expected that since electronic transitions occur between discrete energy levels, the absorption and emission spectra should be like a series of sharp lines, and it would be like that if the fluorophores were just simple separate atoms. However, since organic fluorophores consist of several tenths of atoms, the amount of different vibrational levels is very high (vibrational levels could be related to: electronic transitions, molecular skeletal vibrations or flexures, collision with solvent molecules, etc). Population of those levels is determined by a Boltzmann distribution.

Due to mentioned reasons absorption spectrum is continuous all over the absorption band, as well as emission spectrum, which generally looks like a mirrored image of the absorption spectrum, but is shifted to the lower energies.
due to Stokes shift. Schematic representations of absorption and emissions spectra are presented in Figure 1.3 and Figure 1.4.

In addition to fluorescence, phosphorescence and internal conversion, there are several other possible de-excitation pathways that are not described in detail here; for example, processes such as: conformational change, electron transfer, proton transfer, energy transfer, excimer formation, exciplex formation and photochemical transformation.
1.2 Fluorophores

First of all, it is good to mention the difference between the ordinary dyes and the fluorophores: fluorophores work in accordance to the principles that were described in a previous chapter, while dyes just absorb photons with appropriate wavelengths and reflect the non-absorbed part of the visible spectrum. That difference arises from the fact that fluorophores have the conjugated carbon bounds in their structure.

Generally, in fluorescence spectroscopy and imaging the visible region (380-750nm) of the electromagnetic spectrum is in use. Since fluorophores are typically organic molecules, they can have double chemical bounds consisting from covalent bonds in their structure. Organic compounds without such kind of bonds absorb and emit wavelengths which are out of the visible region (160nm). If the molecule has only one carbon-carbon double or triple bound then it is still out of the region of interest (with one carbon-carbon double bound the absorption is around 170nm). In order to be suitable for fluorescent applications, or in other words, in order to shift absorption/emission spectrums to the visible region, molecule should consists of the sequence of carbon-carbon double bounds separated by a single bond (-C=C-C=C-), and exactly such bounds which are separated in such a way are called conjugated.
Many fluorescent measurements could not be possible without fluorescent probes (for example when intrinsic fluorophores, such as tryptophan, phenylalanine and tyrosine, are not presented in a specimen structure). Some fluorescent probes are sensitive to the surrounding medium, and by investigation of the changes in such fluorophore’s emission it is possible to make conclusions about physicochemical and biochemical properties of the studied sample. Generally probes are sensitive to such parameters as: pH, polarity, viscosity temperature, electric potential, pressure, etc.
Chapter 2. Lipid membranes

Lipids are amphipathic molecules which have polar, hydrophobic part ("tale") and a non polar, hydrophilic part ("head"). Due to such structure, lipids preferentially group themselves into the energetically most favorable configuration – lipid bilayers, with the "heads" directed towards the water and "tales" against it. Thus lipids are held together by entirely non-covalent forces. The formation of chemical bounds between the individual molecules is typically not involved. This allows lipids to be unstuck and flow inside the membrane. In a Figure 2.1 a typical lipid conformation is shown.

Any cell in the live organisms is surrounded by a lipid bilayer membrane that forms a barrier around the cell which is impermeable to most water-soluble molecules and particularly impermeable to ions; keeping all cell components (ions, proteins, other molecules) at the correct places, preventing them to diffuse out of the cell.

Figure 2.1, a) typical lipid conformations and d) the structure of the DOPC lipids which were widely use in this study [adopted from http://wikipedia.org and http://www.avantilipids.com]
Chapter 3. Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy was developed in early 1970s [Magde 1972]. Due to the low technical level of the required equipment at that time FCS did not get widespread use. With the development of light sources, detectors and filters, combined with confocal arrangement, which was proposed in 1990s by Rudolf Rigler and co-workers [Rigler, Widengren 1990], FCS became a useful and powerful single molecule method to study processes such as: diffusion, binding, charge transfer, singlet-triplet transition dynamics, isomerization, antibunching etc.

A general scheme of a confocal FCS setup is presented in Figure 3.1. Typically it consists of a confocal microscope and photodetectors of high sensitivity and temporal resolution. In order to get different fill levels of the back aperture of an objective, the laser beam is expanded by two lenses placed in a telescope arrangement. After that, the beam is reflected by a dichroic mirror and focused on the sample by an objective with a high numerical aperture. Fluorescence is collected by the same objective and directed through a confocal pinhole and emission filters to the detectors. The detection volume size is defined by the projected confocal pinhole size in the object plane and the excitation volume. The excitation volume depends on the fill level of the back aperture of the objective by the laser beam. If the size of laser beam matches the size of the aperture then the beam will be focused to the diffraction limited spot.
and the detection volume will have close to Gaussian properties [Hess and Webb 2002].

Figure 3.1, Typical FCS setup with all named components. Inset – example of a correlation curve with the main parameters: triplet fraction $\langle T \rangle$, triplet relaxation time $\tau_T$, number of molecules $\langle N \rangle$, diffusion time $\tau_D$. [Adopted from Andriy Chmyrov Doctoral thesis 2010]

FCS is based on the analysis of the correlation function

$$G(\tau) = 1 + \frac{1}{\pi^{3/2} \omega_x \omega_z \omega_C} \left( 1 + \frac{4D\tau}{\omega_x^2} \right)^{-1} \left( 1 + \frac{4D\tau}{\omega_z^2} \right)^{-1/2}$$

(1)

where:

$\langle C \rangle$ – in Poisson statistics is equal to $\langle C(\vec{r},t) \rangle$, where $C(\vec{r},t)$ is the concentration of the molecules at position $\vec{r}$ at time $t$,

$\omega_x$, $\omega_z$ – the lateral and axial radii of the detection volume,
\[ D = \frac{k_B T}{6\pi \eta R_b} \]  — the diffusion coefficient of a molecule, in which \( k_B \) - Boltzmann constant, \( T \) - absolute temperature, \( \eta \) - viscosity of the solvent, \( R_b \) - hydrodynamic radius.

\( \tau \) – time parameter

One of the crucial parameters in FCS measurements is the detection (effective) volume, which is defined as:

\[ V_{\text{eff}} = \frac{W_1^2}{W_2}, \quad W_n = \frac{1}{E^n(0)} \int E^n(\hat{r}) d^3\hat{r} \]  \hspace{1cm} (2)

where \( E(\hat{r}) \) is the molecular detection efficiency function, which is proportional to the excitation intensity \( I_{\text{exc}}(\hat{r}) \) and the collection efficiency function of the confocal microscope setup \( CEF(\hat{r}) \), which is the probability of detecting a photon as a function of the position of the emitter [Rigler at al. 1993]. Thus \( E(\hat{r}) \) is calculated as:

\[ E(\hat{r}) = I_{\text{exc}}(\hat{r}) CEF(\hat{r}) \]  \hspace{1cm} (3)

For the confocal detection, the molecular detection efficiency (MDE) function can be approximated to be a 3-dimensional Gaussian function:

\[ E(\hat{r}) = E(0) \exp \left( -\frac{2x^2}{\omega_x^2} - \frac{2y^2}{\omega_y^2} - \frac{2z^2}{\omega_z^2} \right) \]  \hspace{1cm} (4)

By applying those definitions, equation (3) can be rewritten as:

\[ V_{\text{eff}} = \pi^2 \omega_x^2 \omega_y \omega_z \]  \hspace{1cm} (5)

It is logically clear that a number of molecules in the detection volume is equal to the concentration of the molecules in a unit volume multiplied by the volume, thus \( N = V_{\text{eff}} \langle C \rangle \). The characteristic diffusion time is defined as
\[ \tau_D = \frac{\omega_y^2}{4D}. \] The axial and spatial radii of the detection volume can be substituted by their ratio as \( \omega = \frac{\omega_y}{\omega_x} \). Thus, the correlation function can be rewritten as:

\[
G(\tau) = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + \frac{\tau}{\omega^2 \tau_D} \right)^{-\frac{1}{2}} + 1 = \frac{1}{N} G_D(\tau) + 1 \tag{6}
\]

Such correlation function represents only the diffusion process and does not take into account other processes (triplet state, isomerization, rotation, etc) which occur in a majority of fluorophores and have an influence on a correlation curve. To be able to resolve all ongoing processes in fluorophores, the correlation function should be modified.
3.2 Fluorescence correlation spectroscopy as a technique to study the Isomerization in the Cyanine dyes

Cyanine dyes were developed for use in photographic emulsion but later became in a widespread use for lasers (mode lockers), media (CD-R DVD-R dicks) and fluorescence microscopy. They are characterized by short fluorescence lifetimes, high fluorescence quantum yield, reasonable photostability and high extinction coefficients. Cyanine dyes cover the span from green to far-red wavelengths of the electromagnetic spectrum. The ability to be used in the far-red region made them popular for use in biomedical applications due to low autofluorescence and low damage to cells.

![Chemical structures and reaction scheme](image_url)

Figure 3.2. Chemo-physical scheme (A) and the electronic states model (B) for the isomerization process. [Adopted from Widengren at al 2000]
The presence of several conjugated carbon bonds is a specific structural feature of Cyanine dyes. Because of that, they have trans- cis- isomerization as an additional de-excitation mechanism [McCartin 1965]. Terms “trans” and “cis” comes from the Latin and mean “on the other side” and “on the same side” respectively. The double chemical bounds, due to rigidity, allow 180° degree twist around their axis (but do not allow free rotation). Such twist changes the structure of the molecule and makes its new isomer with different properties. When a molecule is at its “cis” state it is claimed to be non fluorescent. In reality the cis-isomer still emits photons but the relative quantum yield of the cis- form is much lower then that of the trans- form. [Aramendia 1998].

As it was noticed in the previous chapter, the correlation function must be modified in order to represent the specific processes, which take place in the particular dye. Merocyanine 540 is a fluorophore from the Cyanine dye family for which the isomerization is claimed to be a primary phenomenon. After the excitation of the cyanine from the all-trans form $^1N_0$ to its first excited state $^1N_1$ the major de-excitation processes are [Chibisov 1977]:

\[
^1N_1 \xrightarrow{k_f} ^0N_0 + h\nu \quad \text{(fluorescence)}
\]

\[
^1N_1 \xrightarrow{k_{isc}} ^3N \quad \text{(intersystem crossing to the lowest triplet state)}
\]

\[
^1N_1 \xrightarrow{k_{ic}} ^0N \quad \text{(internal convection)}
\]

\[
^1N_1 \xrightarrow{k_{ic}} ^0P \quad \text{(photo-induced trans-cis isomerization)}
\]

The scheme, presented in Figure 3.2, represents the photo-physical behavior of the cyanine dyes. After the molecule gets excited and moves from the state $^1N_0$ to the state $^1N_1$ (singlet states, assumed to be all-trans) it goes to the partially twisted, intermediate state $^1Perp$. Since molecule reaches $^1Perp$ it
rapidly (picosecond or even femtosecond) relaxes either to the singlet ground trans-state $^1_0N$ or to the ground cis-state $^1_0P$. Neither transitions between ground and excited singlet states, nor $^1\text{Perp}$ deactivation will be resolved by a correlator, since they take place at the pico- or nanoseconds time scale which is lower than a resolution of the PC-based correlator, which is 12.5 ns.

The cis-state of cyanine dyes is assumed to be non fluorescent at room temperature, and to be deactivated through internal conversion. The quantum yield of the triplet formation from $^1_1N$ is very low and is generally neglected, but it can be resolved and taken into account for measurements at high excitation intensities [Widengren at al 2000]. The thermal deactivation coefficient from $^1_0P$ to the $^1_0N$ state ($k_{PN}$) is relatively small and can generally be neglected, but it can play an important role in studies under very low excitation intensities. With all those assumptions, the model of Figure 3.2 can be simplified as it presented in Figure 3.3.

![Figure 3.3, Simplified photophysical model for cyanine dyes.](Adopted from Widengren at al, PCCP, 2000)

It contains a fluorescent trans form $^1\text{N}$ ($^1_0N$ and $^1_{1N}$), assumed to be non-fluorescent cis-form $P$ ($^1_0P$ and $^1_1P$) and non fluorescent triplet state of the trans form $^3\text{N}$. The effective transition coefficients from $^1\text{N}$ and $P$ will be the same as those for the excited states of $^1\text{N}$ and $P$, differences will only arise as a scaling factor that correspond to the fractions of the singlet state dyes in the $^1\text{N}$ and $P$ forms that are in their excited states [Widengren at al 2000].

$$k'_{\text{ISC}} = k_{\text{ISC}} \frac{k_{N10}}{k_{N10} + k_{N01}} = \frac{\sigma_N I_{\text{exc}}}{\sigma_N I_{\text{exc}} + k_{N10}} \quad (7)$$
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\[ k'_{\text{ISO}} = k_{\text{ISO}} \frac{k_{N10}}{k_{N10} + k_{N01}} = \frac{\sigma_N I_{\text{exc}}}{\sigma_N I_{\text{exc}} + k_{N10}} k_{\text{ISO}} \]  

\[ k'_{\text{BISO}} = k_{\text{BISO}} \frac{k_{P10}}{k_{P10} + k_{P01}} = \frac{\sigma_p I_{\text{exc}}}{\sigma_p I_{\text{exc}} + k_{P10}} k_{\text{BISO}} = \{k_{P10} \gg \sigma_p I_{\text{exc}}\} = \sigma_{\text{BISO}} I_{\text{exc}} \]  

where \( \sigma_N \) is the excitation cross section of the trans form, and \( \sigma_{\text{BISO}} = \frac{\sigma_p k_{\text{BISO}}}{k_{P10}} \) is the effective cross section for the back-isomerization of the cis state, \( \sigma_p \) denotes the excitation cross section of the cis form.

The detected fluorescence rate is given by

\[ F(t) = \int CEF(\vec{r}) c(\vec{r}, t) k_{N10} q \Phi_f \, \rho \, N(\vec{r}, t) \, dV \]  

(10)

All terms from this equation were explained in previous expressions.

As it was noticed before, the general expression for the correlation function represents only the translational diffusion process, and it should be modified in accordance to the particular dye properties. As many other photo-induced transient states observed by FCS, isomerization occurs at a faster time scale than the translational diffusion. The fluctuation in the fluorescence generated by the isomerization is denoted by \( \delta F_{\text{fast}} \) and can be added to the correlation function separately from the diffusion part \( \delta F_D \). The modified correlation function can be written as:

\[ G(\tau) = \frac{\langle F(t) F(t + \tau) \rangle}{\langle F \rangle^2} = \left[ \frac{\langle F \rangle + \delta F_D(t) + \delta F_{\text{fast}}(t) \rangle}{\langle F \rangle^2} - \frac{\langle F \rangle + \delta F_D(t + \tau) + \delta F_{\text{fast}}(t + \tau) \rangle}{\langle F \rangle^2} \right] = \frac{\langle \delta F_D(t) \delta F_D(t + \tau) \rangle + \langle \delta F_{\text{fast}}(t) \delta F_{\text{fast}}(t + \tau) \rangle}{\langle F \rangle^2} + 1 \]  

(11)

\( G_{\text{fast}} \) - corresponds to the part of the correlation function originating from the photo physically generated fluctuation in fluorescence [Widengren and Schwille 2000]. To find the expression for the \( G_{\text{fast}} \), the following system of the coupled
first order linear differential equations (which are set up from the kinetic scheme) should be solved:

\[
\begin{bmatrix}
\frac{d}{dt}^{1}N(t) \\
\frac{d}{dt}^{3}N(t) \\
P(t)
\end{bmatrix} = 
\begin{bmatrix}
-(k'_{\text{ISC}} + k'_{\text{ISO}}) & k'_{T} & k'_{\text{BISO}} \\
k'_{\text{ISC}} & -k'_{T} & 0 \\
k'_{\text{ISO}} & 0 & -k'_{\text{BISO}}
\end{bmatrix}
\begin{bmatrix}
^{1}N(t) \\
^{3}N(t) \\
P(t)
\end{bmatrix}
\tag{12}
\]

here \(k_{T}\) is the triplet state deactivation rate to the ground singlet state. The probability of a photon emission (detection) at a time \(\tau\) is expressed by the correlation function. For the fluorophore at a fixed position and at constant excitation this probability will be proportional to \(^1N(\tau)\), that is obtained from the solution of the equation (13) by applying such boundary conditions (which reflect the fact that initially a fluorophore resides in the \(^1N\) state):

\[
\begin{bmatrix}
^{1}N(0) \\
^{3}N(0) \\
P(0)
\end{bmatrix} = 
\begin{bmatrix}
1 \\
0 \\
0
\end{bmatrix}
\tag{13}
\]

with such boundary conditions, the solution of the (Eq.12) will be:

\[
\begin{bmatrix}
^{1}N(\tau) \\
^{3}N(\tau) \\
P(\tau)
\end{bmatrix} = 
\sum_{i=1}^{3}
\begin{bmatrix}
A_{i}\nu_{1}(1)e^{\lambda_{i}\tau} \\
A_{i}\nu_{2}(2)e^{\lambda_{i}\tau} \\
A_{i}\nu_{3}(3)e^{\lambda_{i}\tau}
\end{bmatrix}
\tag{14}
\]

where \(A_{i}\) is a scaling factor to the \(i\) th eigenvectors, \(\nu_{i} = (\nu_{1}, \nu_{2}, \nu_{3})\) are the eigenvectors and \(\lambda_{i}\) is the \(i\) th eigenvalue for the matrix above and given by:

\[
\lambda_{i} = 0
\]

\[
\lambda_{2,3} = -\left\{ \frac{1}{2}(k'_{\text{ISC}} + k'_{T} + k'_{\text{ISO}} + k'_{\text{BISO}}) \pm \left[ \frac{1}{4}(k'_{\text{ISC}} + k'_{T} + k'_{\text{ISO}} + k'_{\text{BISO}})^{2} - k'_{\text{ISO}} k'_{T} - k'_{\text{ISC}} k'_{\text{BISO}} - k'_{T} k'_{\text{BISO}} \right]^{\frac{3}{2}} \right\}
\]

where the first eigenvalue \(\lambda_{1}\), is equal to zero. The first component of the eigenvector \(A_{i}\nu_{1}(1)\) represents a steady state concentration of \(^1N\), denoted by \(^1N\). The multiplicative factors of the exponential terms of \(^1N\) are given by:

\[
A_{1}\nu_{1}(1) = k_{T} \frac{k'_{\text{BISO}}}{\alpha} = ^{1}N
\tag{15}
\]

\[
A_{2}\nu_{2}(1) = (\beta + \gamma) \frac{k_{T} \gamma + \delta}{4\alpha \gamma}
\tag{16}
\]
\[ A_3 N_3(t) = (\beta - \gamma) \frac{k_T \gamma - \delta}{4 \alpha \gamma} \]  

where:

\[ \alpha = k'_{ISO} k_T + k'_{ISC} k_{BISO} + k_T k_{BISO} \]  

\[ \beta = k'_{ISC} + k'_{ISO} + k_T - k_{BISO} \]  

\[ \gamma = \left( k'_{ISC} + k'_{ISO} \right)^2 + (k'_{BISO} - k_T)^2 + 2(k'_{ISO} - k'_{ISC})(k_{BISO} - k_T)^2 \]  

\[ \delta = k_T (k'_{ISO} + k'_{BISO} - k_T - k'_{ISC}) + 2k'_{ISC} k'_{BISO} \]

For the photophysically generated fluctuations in fluorescence, the normalized correlation function can be expressed as (assuming stationary excitation intensity):

\[
G_{\text{fast}}(t) = \frac{\langle \delta F_{\text{fast}}(t) \delta F_{\text{fast}}(t + \tau) \rangle}{\langle F \rangle^2} = \frac{k_{N_{10}} q \Phi_{j_1}^N k_{N_{10}} q \Phi_{j_1}^N (N(t) - \frac{1}{\tau})}{(k_{N_{10}} q \Phi_{j_1}^N)^2}
\]

here all term are defined in the previous expressions. The steady-state \( \langle \frac{1}{\tau} N \rangle \) as well as the time dependent \( \langle \frac{1}{\tau} N(t) \rangle \) population of the excited singlet state of the trans-form have a fixed relationship to \( \frac{1}{\tau} N \) and \( \frac{1}{\tau} N(t) \), respectively, given by \( \frac{k_{N_{01}}}{k_{N_{01}} + k_{N_{10}}} \). Thus \( G_{\text{fast}} \) can be written as [Widengren and Schwille 2000]:

\[
G_{\text{fast}} = \frac{\langle \frac{1}{\tau} N(t) - \frac{1}{\tau} N \rangle}{\langle \frac{1}{\tau} N \rangle^2} = \frac{\langle \frac{1}{\tau} N(t) - \frac{1}{\tau} N \rangle}{\langle \frac{1}{\tau} N \rangle} = \frac{A_3 N_3(t) e^{-\lambda \tau}}{A_3 N_3(1)}
\]

And finally the full correlation function (using the fact that \( \frac{1}{\lambda_2}, \frac{1}{\lambda_3} \ll \tau_D \approx \frac{\sigma_0^2}{4D} \)) can be rewritten as:
\[
G(\tau) = \frac{1}{N} \left( \frac{1}{1 + 4D \frac{\tau}{\omega_1^2}} \right) \left( \frac{1}{1 + 4D \frac{\tau}{\omega_2^2}} \right)^{\frac{1}{2}} [1 + G_{\text{fast}}(\tau)] + 1 = \\
= \frac{1}{N^2} \left( \frac{1}{1 + 4D \frac{\tau}{\omega_1^2}} \right) \left( \frac{1}{1 + 4D \frac{\tau}{\omega_2^2}} \right)^{\frac{1}{2}} \cdot N(\tau) + 1 = \\
= \frac{1}{N \cdot A \nu_1(l)} \left( \frac{1}{1 + 4D \frac{\tau}{\omega_1^2}} \right) \left( \frac{1}{1 + 4D \frac{\tau}{\omega_2^2}} \right)^{\frac{1}{2}} \left[ A_1 \nu_1(l) + A_2 \nu_2(l)e^{i\omega\tau} + A_3 \nu_3(l)e^{i\omega\tau} \right] + 1
\]
Chapter 4. Materials and methods.

4.1 Materials.

Merocyanine 540 (MC540) fluorophore was obtained from Sigma-Aldrich and the stock solution was prepared by dissolving the fluorophore in Ethanol to the concentration of 2mM. Two types of lipids with different polarity were used during the experiments: DOPC (neutrally charged) and DOPG (negatively charged). Lipids and cholesterol were ordered from the Avanti Polar Lipids as dissolved in Chloroform (to concentration 10mg/mL) and as powder.

For the investigation of the changes in the absorption and emission spectrums, as well as changes in brightness of the MC540, the fluorophore was dissolved to suitable concentrations in solvents of different polarity: Methanol, Ethanol, Butanol and Propanol.

0.2mM solution of the Potassium chloride (KCl) with pH equal to 7.4 was used as a buffer.
4.2 Sample preparation and labeling.

According to the protocol for preparation and labeling of liposomes, the appropriate amount of dye (exact amounts will be mentioned later, for each measurement) was added to the 2.5ml of lipids dissolved in chloroform. Resulting solution was left to evaporate under a low flow of nitrogen for 1.5 hour. After that, 2ml of buffer was added and the solution was shaked on a high speed at a vortex for 2 hours (until sediment gets completely dissolved). Resulting solution was subjected to 5 cycles of freezing in liquid nitrogen (1min), heating to 30° C (4min) and mixing (20 sec). Then, by using extruder (Avanti Polar Lipids mini extruder) liposomes were shaped to the appropriate size (from 30 nm to 300 nm depending on experimental needs). To ensure that all the liposomes get the same size, the shaping procedure was repeated 25 times. After that, by using PD10 column (GE Life Science) the free dye was removed from the prepared sample.

4.3 FCS setup.

In this study, fluorescent samples are excited by Melles Griot 643-RYB-A02 krypton-argon laser. From all of the wavelengths which such laser can emit, the appropriate wavelength was separated by using the Z568/10 (Chroma Technology Corp.) excitation filter. After reflection from the dichroic beam splitter (FF576/661-Di01-18-D, Semrock Inc.), the laser light was focused on the sample by the cover glass corrected, water-immersion objective (Zeiss, 63x/1.2 Plan-Neofluar, 160 mm tube length.). Fluorescence was collected by the same objective and focused to the two avalanche photodiodes (SPCM-AQR-14, Perkin-Elmer Optoelectronics) in a beam splitting arrangement. Two detectors were used in order do eliminate all influence of the inherent noise,
detector’s dead times and afterpulsing effects. In order to discriminate the fluorescence from the Raman scattering (from solvent’s molecules) and the excitation laser light, a band-pass filter HQ640/115M (Chroma Technology Corp.) was inserted in front of the each detector. In order to spatially discriminate the fluorescence – a 50 nm pinhole was placed at the image plane. The signal from the two avalanche photodiodes was analyzed by a PC-based correlator (ALV-5000, ALV Gmbh). The laser power was controlled by a laser power controller (BEOC LPC) and measured before objective by a laser power meter PM-100 (Thor Labs). Deoxygenation procedure was done by flushing nitrogen over the sample in an air-tight chamber for 1 hour. Measurements were done under a low continuous flow of nitrogen over the sample.
Chapter 5. Results and discussion

5.1 Free dye studies

MC 540 is a polar dye, so it should react on the solvent polarity. All main photo physical properties of the dye can vary significantly for different solvents. In order to select a proper excitation wavelength, as a first step of this work, the absorption and emission spectrums of MC 540 were measured in different solvents. The study of the excitation and emission spectra of MC540, as well as its brightness, was done by using a FluoroMAX -3 (Horiba Jobin Yvon Inc.) spectrofluorometer.

Figure 5.1, Excitation and emission spectra of the MC 540 dissolved in solvents of different polarity. Excitation peaks were observed at: 544nm for methanol, 559 for ethanol, 564 for butanol and 563 for propanol. Emission peaks were observed at: 567nm for methanol, 579 for ethanol, 582 for butanol and 583 for propanol.
The excitation and emission spectra were recorded from the dye dissolved in Ethanol, Methanol, Butanol and Propanol (in the order of increasing polarity). The resulting spectrums are shown in Figure 5.1. It can be clearly noticed that with increase of polarity, the maximums shift to the direction of longer wavelengths.

Especially big difference is observed between low-polar Methanol and Ethanol, which contributes to the statement of the high dye’s sensitivity of the dye to solvent properties. Solutions of the same concentration were used at the brightness comparison. In Figure 5.2 un-normalized emission spectrums are shown. MC540 dissolved in Ethanol becomes brighter by approximately 45% than when dissolved in Methanol, and almost 100% brighter when dissolved in Butanol. In FCS experiments the difference in count per molecule between the signal from the MC 540 dissolved in Ethanol and Butanol is 36% (more in Butanol), which is roughly the same difference that was observed during the spectral measurements. As a result of this study, the excitation wavelength of 568 nm was chosen as suitable for the further experiments.
During the FCS studies the fraction of isomerization stays roughly the same for the dye dissolved in ethanol, propanol, and butanol.

Curves were fitted to the following one exponential model:

\[
G(\tau) = \frac{1}{N \left(1 + \frac{\tau}{\tau_D}\right) \sqrt{1 + \frac{\tau}{\tau_D} S^2 \left(1 - T_1 T_1 e^{-\tau/\tau_D}\right)}} + \text{offset} \tag{25}
\]

where \( N \) is the average number of molecules inside the detection volume, \( T_1 \) is the isomerization fraction, \( \tau_i \) is the isomerization relaxation time, \( \tau_D \) is the diffusion time, \( S \) is the ratio between \( \omega_z, \omega_{xy} \) (Eqs. 1, 2) and \( \text{offset} \) comes mainly from uncorrelated fluorescence signal for \( \tau \gg \tau_D \).

![FCS curves for Merocyanine 540 dissolved in ethanol and excited with different intensities.](image)

Figure 5.3, FCS curves for Merocyanine 540 dissolved in ethanol and excited with different intensities. The fraction of isomerization remains constant independent on excitation, but the isomerization relaxation time shifts to lower values (from \( \tau_i = 0.24 \text{ \mu s} \) at 2 kW/cm\(^2\) to \( \tau_i = 0.065 \text{ \mu s} \) at 23,01 kW/cm\(^2\)). As a consequence, for high excitation intensities isomerization becomes hardly resolvable. Insert: residuals.
FCS data for methanol is not presented, due to a low quantum yield of the dye in methanol and the fact that 568nm is not a good excitation wavelength for such solution. In Figure 5.3 the power series recorded from MC 540 dissolved in ethanol are presented, and typical behavior of isomerization is seen. At the insert the residuals of the fitting are presented. The fraction of isomerization (a) and inverse of its relaxation time (b), versus the excitation intensities, for the different solvents are presented in Figure 5.4. The fraction of the isomerization ($T_{i}$) stays in a range of 50% – 60% independent of solvent. The relaxation time ($1/T_{\tau}$) also shows a solvent independent behavior.

Figure 5.4, The fraction of the isomerization (a) and the inverse of the isomerization relaxation time (b) for the merocyanine 540 dissolved in different solvents. The $T_{i}$ remains constant and stays in a range of 55% – 50% independently on the solvent. The $1/T_{\tau}$ shows a linear behavior and does not significantly change its values in the different solvents.
5.2 Determination of appropriate amounts of the dye in the FCS vesicle studies.

To be able to track the differences in the isomerization process of MC 540 dye due to the different membrane’s properties, first the proper amount of dye, that should be added to the lipids at the step of liposome labeling, must be investigated. To be able to properly record the isomerization dynamics of the MC 540 by FCS, labeling should not exceed one dye per liposome. In the other case – when there is more than one dye per vesicle, the correlation curve cannot properly resolve the ongoing intersystem processes. Moreover, since it is impossible to control the dye distribution per vesicle, they will not be homogeneously distributed – some liposomes will have much more fluorophores attached to their surfaces, so the signal from the vesicles will get highly disturbed and that will ruin all measurements. The difference between the correlation curves is shown on Figure 5.3, where two correlation curves which correspond to the different amount of the dye per vesicle, is presented.

Figure 5.3, Correlation curves for the different amount of MC 540 per liposomes of 100 nm radius. Blue line $0.2 \cdot 10^{-4}$ L of 2mM concentrated MC 540, red line – $0.2 \cdot 10^{-5}$ L of 2mM concentrated MC 540.
The investigation is performed in an experimental way. Theoretical idea is to track the “counts per molecule” parameter and decrease the amount of dye until the signal reaches the lowest level and becomes insensitive to further dye reduction. Experimental results are presented in Figure 5.4, and the plateau that corresponds to the presence of one fluorophore (or less) per liposome is clearly seen.

For the further experiments in order to safely neglect the probable volume errors, \(0.2 \cdot 10^{-8} \text{L}\) of 2mM concentrated MC 540 was added to the lipids at the labeling procedure. That guarantees the presence of on average less then one fluorophore per liposome.

![Graph showing counts per vesicle versus volume of added dye](image-url)

Figure 5.4. “Counts per molecule” versus the different amount of MC 540 added to 100nm large liposome during the labeling procedure.
5.3 Isomerization in lipid membranes.

The presence of the isomerization process of the MC 540 in the lipid membranes was not noticed before and thus, it should be justified. Isomerization is a process that occurs due to the chemical structure of the molecule, so its fraction at the correlation curve should be independent on excitation intensity and remain constant. The isomerization relaxation time should quickly decrease with the increase of the excitation intensity. Series of measurements were recorded and results are presented in Figure 5.5. Since the measurements are done on vesicles which are 100 nm large, it should be noticed that the rotation process could not be neglected. It was stated before [Ehrenberg and Rigler 1974], that the relaxation time of the rotation process is independent on excitation intensity and can be assumed to be in a range of 100 nanoseconds [James M. Tsay at al 2006]. At correlation curves three bulbs are clearly resolved: the first one is in the range of milliseconds – represents the process of translational diffusion, the second one is in the range of microseconds – could represent trans-cis isomerization process, and the third one is in the region of tens - hundreds of nanoseconds is assumed to represent the rotational process.

In Figure 5.6 the isomerization fraction and its relaxation time are presented. As it was expected, the fraction of isomerization does not differ much and can be assumed to be constant. The isomerization relaxation time shifts to a faster time scale with the increase of the excitation intensity, so its inversion is a straight line as it is presented in Figure 5.6
Figure 5.5, Correlation curves recorded from 100 nm large liposomes which were labeled by the MC 540. Relaxation time of the isomerization shifts to the lower values with an increase in the excitation intensity. Clear trend which corresponds to the behavior of isomerization process is seen.

The two-exponential additive model was used for the fitting:

\[
G(\tau) = \frac{1}{N \left(1 + \frac{\tau}{\tau_D}\right) \left(1 + \frac{\tau}{\tau_D}\right) S^2} \left(\frac{1 - T_1 - T_2 + T_1 e^{\frac{-\tau}{T_1}} + T_2 e^{\frac{-\tau}{T_2}}}{1 - T_1 - T_2}\right) + \text{offset}
\]

where: \(N\) is the number of molecules inside the detection volume, \(\tau_D\) is the diffusion time, \(\tau_{I_1}\) - relaxation time of the isomerization process, \(\tau_{I_2}\) - rotation time, \(T_1\) - fraction of the isomerization, \(T_2\) - fraction of the rotation.
To investigate the presence of the isomerization and its behavior inside the lipid membranes, the two different approaches were used, as described in parts 5.4 and 5.5.

5.4 Isomerization in a free dye and inside the membranes

According to the previous chapter, the isomerization process is assumed to be present not only in a free dye but also when the dye is attached to a lipid membrane. To investigate the difference in dye’s behavior when free in solution and when bound to lipid vesicles, the appropriate correlation curves were recoded and are presented in Figure 5.7.
From Figure 5.7 the change in the diffusion time, that indicates the difference in the size of the detected molecules, is clearly seen: for the free MC 540 $\tau_D \approx 0.05 \mu$s, for the MC 540 in liposomes – $\tau_D \approx 5.5 \mu$s. At the curve that corresponds to the liposomes, the two bulbs are distinguishable: the first one corresponds to the isomerization process while the second one to the rotation of the liposome. The difference in the isomerization fraction and its relaxation time for those measurements are presented in Figure 5.8.
As it is presented in Figure 5.8 a) the fraction of isomerization ($T_i$) for the MC 540 dye dissolved in ethanol stays in a range of 55% - 50%. When the MC 540 dye is attached to the lipid membrane, the fraction of the isomerization is dropping to the range of 40%-30%. That could be explained by the fact that when the dye is attached to the membrane by its two “anchors”, it needs an extra force to get unstuck and make a twist, so the probability of such event gets lower. From Figure 5.8 b), where the inverse of the isomerization relaxation times are presented, it could be concluded that the isomerization occurs much slower when the dye is attached to the liposomes,
then when it is free. The difference in the $\tau$ parameter for the free and for the attached dye is more than one order of magnitude. The same theory as for the fraction of isomerization could be applied to explain this difference.

5.5 Deoxygenation

In the previous chapter the presence of the triplet state in the range of 6%-3% was assumed, but not resolved due to complication with the fitting of such small amplitude process in the FCS curves. In order to check the presence of the assumed by the model triplet state $^3N$, the sample was studied under deoxygenated conditions. Also this study could be used to prove by another approach that the observed process is the isomerization and not the triplet state.

Oxygen is a well known triplet state quencher, and under deoxygenated conditions the triplet state fraction should rise up dramatically. Labeled by the MC 540, 100 nm size liposomes made from DOPC lipids were placed into a hermetical chamber and oxygen was replaced by a continuous flow of nitrogen over the sample. A set of normalized correlation curves is presented in Figure 5.9.
Figure 5.9, Correlation curves recorded from 100 nm large liposomes made from DOPC lipids under the deoxygenated condition for the different excitation intensity. Insert: total signal trace.

As it was previously seen from the oxygen saturated measurements, the isomerization fraction stays constant and shows the same behavior to the excitation intensity – shifts to a faster time scales with the increase in the excitation intensity. The rise of one extra bulb (that corresponds to the fraction of the triplet state) between the diffusion and isomerization parts is clearly seen. In Figure 5.10, the comparison between the oxygen saturated and deoxygenated data is presented. Curves were recorded from 100nm large liposomes made from DOPC lipids under 14.78 kW/cm² excitation intensity.
Figure 5.10, Correlation curves recorded from 100 large liposomes made from DOPC lipids under 14.78 kW/cm² excitation intensity. Insert a) the fraction of the isomerization for the deoxygenated and oxygen saturated conditions. Insert b) the fraction of the triplet state for the deoxygenated conditions.

The curves which are presented in Figure 5.10 were fitted by the following tree exponential model:

\[ G(\tau) = \frac{1}{N\left(1 + \frac{\tau}{\tau_D}\right)^2 \left(1 + \frac{\tau}{\tau_D}\right)^2} \left(1 + \frac{\tau}{\tau_D}S^2\right) \left(1 + \frac{\tau}{\tau_D}S\right) \left(1 - T_1 - T_2 - T_3 + T_1 e^{\tau/\tau_D} + T_2 e^{\tau/\tau_D} + T_3 e^{\tau/\tau_D}\right) + \text{offset} \ (27) \]

where the terms \( T_3 \) and \( \tau_D \) corresponds to the fraction of the triplet and its relaxation time respectively. From the curve recorded under the air saturated conditions the triplet traction \( T_3 = 3\% \) was obtained, while from the curve recoded under the deoxygenated conditions the triplet fraction rises up to 20\%. The isomerization fraction (insert a) in Figure 5.10, rises up not significantly and taking into account the fitting and experimental inaccuracies it could be assumed to stay the same for the both studied conditions. In the insert b) the
triplet fraction for the deoxygenated conditions is presented. Observed behavior, when it first rises up and then falls down with the increase of excitation intensity could be explained by the strong photobeaching of the sample, which occurs even at relatively low excitation intensities due to the long diffusion time of the vesicles though the detection volume.

### 5.6 Study of the different sizes of the vesicles

In this study the influence on the liposome’s size to the isomerization process of the MC 540 was investigated. Previously it was reported [Williamsom at al. 1983] that the brightness of the MC 540 is depended on the vesicle size, that it is brighter when it is attached to the smaller vesicles. One extra task in this experiment was the investigation of the reasons behind the fluorophore’s brightness decrease, and one of such reasons could be a change in the isomerization dynamics, which was studied. In Figure 5.11 the fractions of the isomerization ($T_i$) and the inverses of the isomerization relaxation times ($1/\tau_i$) are presented for the different sizes of the liposomes. Measurements performed on the liposomes made from DOPC lipids.
It could be seen that the fractions of the isomerization as well as the isomerization relaxation times do not change for the different sizes of the liposomes. Thus, the isomerization process could not be the reason of the observed changes in the fluorophore’s brightness.
5.7 Membrane’s polarity

Since the MC 540 is a polar dye, it should be sensitive to the membrane’s polarity. To study the influence of a membrane’s polarity on the isomerization dynamics, the samples were prepared from two different kinds of lipids: DOPC, which is neutrally charged and DOPG which is negatively charged. Results are presented in Figure 5.12.

From the obtained data it could be noticed that with the membrane polarity the dynamics of the isomerization process changes. For the liposomes made from the DOPG lipids the fraction of isomerization is not significantly but less than it is for the liposomes made from the DOPC lipids. The
isomerization relaxation time is faster for the DOPC liposomes then it is for the DOPG. All that, indicates, that the change in the dynamics of the isomerization process could be used as an extra parameter for the investigation of the membrane’s properties.

5.8 Membrane’s fluidity

As well as the changes in the membrane’s charge, the changes in membrane’s fluidity should have an influence to the photophysical properties of the MC 540. To investigate that, cholesterol was added to the lipids at the liposome preparation stage. Two combinations of DOPC and Cholesterol were used: 50% of DOPC + 50% of Cholesterol and 75% of DOPC + 25% of Cholesterol. Obtained data was compared to the previously measured isomerization dynamics for the MC 540 attached to the 100nm large liposomes made from DOPC lipids only. Results are presented in Figure 5.13. As it could be seen from the obtained data, the isomerization dynamics change with the changes membrane fluidity. The fraction of isomerization ($T_i$) decrease from the range of 35%-30%, as it is for the liposomes made from DOPC lipids only, to the range of 25%-20% and 30-25% for the liposomes made from the combinations of 50% of DOPC + 50% of Cholesterol and 75% of DOPC + 25% of Cholesterol respectively. So the trend of the changing of the fraction of isomerization due to the changes in membrane’s fluidity could be clearly resolved. From the inverse of the isomerization relaxation time ($1/\tau_i$) it could be concluded, that the isomerization occurs faster with an increase in membrane’s fluidity, and clear trend is observed.
Figure 5.13, the fraction of the isomerization (a) and the inverse of the isomerization relaxation time (b) for the 100 nm large liposomes made from the different combinations of the DOPC lipids and Cholesterol.
Chapter 6. Conclusions

It this works the dynamics of the isomerization process of the Merocyanine 540 dye was studied. During the free dye studies the influence of the solvent’s polarity and viscosity to the shifts in the absorption and emission spectrums as well as to the brightness of the dye was investigated. It was proven that for the free dye the dynamics of the isomerization process is independent on the solvents properties. The isomerization fraction stays constant at the range of 50%-55% and the inverse of the isomerization relaxation time shows the linear behavior.

The occurrence of the isomerization of the Merocyanine 540 dye inside the lipid membranes was not reported before and thus become a main subject of this study. Firstly the isomerization was observed in the 100 nm large liposomes made from DOPC (neutrally changed) lipids. The isomerization fraction decrease to 35%-30% and its relaxation time become significantly (orders of magnitude) shorter in comparison to the free dye studies.

In the model of the isomerization process in a free dye, the presence of the triplet state was assumed. To check that, the isomerization dynamics was studied under the deoxygenated conditions (MC 540 was attached to the 100 nm liposomes, made from DOPC lipids). A significant rise of the triplet state, by up to 20%, was observed while the isomerization fraction and its relaxation time stayed unaltered.
As a next step, it was investigated of how such parameters of liposomes as size, the membrane’s polarity and viscosity influence the isomerization dynamics. It was revealed that the size of liposomes has no influence on the isomerization dynamics, thus, the previously observed change in the fluorophore’s brightness, due to the different size of the vesicles, has nothing to do with the isomerization process. For the membrane’s polarity studies, liposomes were prepared from two different kinds of lipids: DOPC (neutrally charged) and DOPG (negatively charged). A change in the fraction as well as in the relaxation time of the isomerization process was observed. Membrane’s fluidity also shows an influence on the isomerization dynamics. To change the fluidity of the membranes, the different fractions of the cholesterol were added to the lipid’s solution at the stage of the liposome’s preparation.

In summary, the isomerization process is revealed to occur in the Merocyanine 540 fluorophore, attached to the lipid membrane. Tracking the changes in the isomerization dynamics of MC 540 could be used as an extra parameter to investigate the membrane’s polarity, and fluidity.
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References