Universität Leipzig

Biophysics Internship

Cation-induced membrane fusion detected by fluorescence spectroscopy (MF)

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Cation-induced membrane fusion detected by fluorescence spectroscopy

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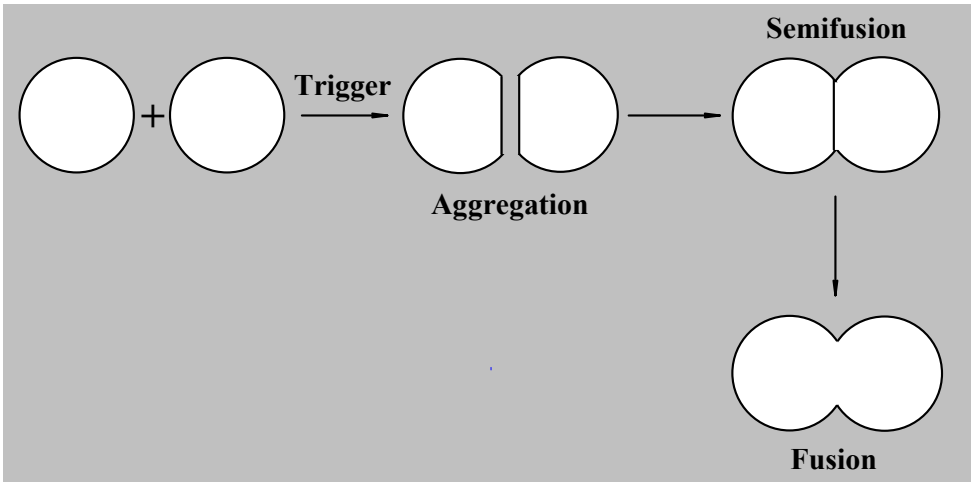
1 Introduction

Membrane fusion occurs in various intra- and intercellular processes, such as, exocytosis, endocytosis, membrane genesis, and fertilization. The molecular mechanism of cellular membrane fusion is not yet fully clear. However, it was established by studies on model systems as phospholipid vesicles that all the diverse biological membrane fusion events have definite physical principles in common. Cation-induced aggregation and fusion experiments were important to elucidate the role of surface forces and membrane destabilization. Differences in the charge state of the participating lipids are expected to influence the cation-induced fusion, especially, via electrostatic forces. Other factors related to membrane fusion (membrane stability and hydrophobicity of the vesicle surface) are specifically influenced by the lipid species.

1.1 Fusion of liposomes



Trigger

Cations (Ca^{2+}, Mg^{2+}, La^{3+}, …), Calcium binding proteins, viral proteins, peptides, polymers (PEG, …)

1.2 Fusion reaction

A	**Aggregation:** nonspecific interactions (vdW attraction, reduced electrostatic repulsion) → **interbilayer forces**
B	**Direct molecular contact:** reduced hydration repulsion → **interbilayer forces**
C	**Local perturbation of lipid bilayer:** hydrophobic interactions → merging of outer monolayers → **intrabilayer forces**
D	**Coalescence of vesicle interiors:** mixing of inner-monolayer lipids → **intrabilayer forces**

2 Methods

Preparation of liposomes: Small unilamellar vesicles (SUV; $d < 100$ nm) are prepared by ultrasonification (BRANSON Sonifier) of aqueous lipid suspensions. Large unilamellar vesicles (LUV; $d = 100$, 200 or 400 nm) are prepared by extrusion of aqueous lipid suspensions through polycarbonate membranes (LIPEX Biomembranes Extruder).

Fluorescence spectroscopy: The most important method for the measurement of membrane fusion is to incorporate fluorescent probes into the lipid bilayer or into the aqueous interior of vesicles and to follow changes of the distribution of these probes by spectroscopic approaches. Different assays are used to investigate single steps of membrane fusion separately (aggregation, lipid mixing, leakage, internal content mixing). Additionally, fluorescence spectroscopy allows to investigate membrane fluidity, membrane hydrophobicity or adsorption of charged or uncharged molecules to membranes by fluorescence resonance energy transfer. All measurements will be done using a PERKIN ELMER fluorimeter LS 50B with temperature controlled sample changer and magnetic stirrer.

Characterization of membrane surfaces: The electrostatic surface potential of phospholipid vesicles is calculated from their electrophoretic mobility (EPM). EPM is measured by cell electrophoresis using photon correlation spectroscopy (ZETASIZER 4, Malvern Instruments).

Particle size measurements: Using photon correlation spectroscopy (ZETASIZER 4, Malvern Instruments), the mean particle diameter of liposomes before and after cation-induced fusion can be detected.

2.1 Particle sizing with photon correlation spectroscopy

In a classical static light scattering experiment, the intensity of light scattered by particles is taken as a measurement for the size of the particles. For particles smaller then the wavelength of the applied light ($R<\lambda/10$), one can assume Rayleigh scattering and write

$$I = I_0 16\pi^4 R^6 \frac{n^2-1}{\left(n^2+2\right)^2}\frac{1}{r^2\lambda^4}$$

λ - wavelength of incident light, I_0 – intensity of incident light, n – ratio of refraction index of medium (n_0) and scattering substance (n_1), R - radius of scattering particles, r – distance from the detector.

With Rayleigh's number R_q, it follows:

$$R_q = 16\pi^4 R^6 \frac{n^2-1}{\left(n^2+2\right)^2}\frac{1}{r^2\lambda^4}$$

$$\frac{I}{I_0} = \frac{R_q}{r^2}$$

For increasing particle radii, an angle dependence of the intensity of the scattered light occurs originating from interferences of light that is scattered by different parts of the particle. When θ is the angle between incident and scattered light beam, the wave number vector K can be written as follows and the scattered intensity can be represented by a series:

$$K = \frac{4\pi n_0}{\lambda}\sin\frac{\theta}{2}$$

$$I = I_0 P(\theta)$$

$$P(\theta) = 1 - \frac{1}{3}\left(R_G K\right)^2 + \ldots$$

R_G is the radius of gyration and depends on the size and on the geometry of the scattering particles. For spheres, it holds

$$R_G = \frac{3}{5}R^2$$

The radius of gyration can be obtained by measurements for varying angles. However, the estimation of particle sizes by static light scattering has some serious limitations.

The **photon correlation spectroscopy (PCS)**[1] was developed in the 1970ies. In principle, it measures fluctuations of the scattered intensity that are caused by the movement of the scattering particles. One obtains the diffusion coefficient and can calculate the particle size from it.

The scattering particles in a suspension follow the Brownian motion. The mean square displacement can be calculated according to the Einstein-Smoluchowski equation:

$$\left\langle x^2 \right\rangle = 6Dt$$

The diffusion is described by Einstein's equation in combination with Stokes' law, where D is the diffusion coefficient, η the viscosity of the medium, and R_h is the hydrodynamic radius of the particles:

$$D = \frac{kT}{6\pi\eta R_h}$$

The fluctuations of the particle position due to the Brownian motion are leading to fluctuations of the intensity spectrum of the scattered light. A displacement x during the measurement time is creating the same intensity shift as the interference of scattered light from two particles with the distance x would create in the static case.

All measurements are carried out with a Zetasizer 4 (Malvern Instruments). Here, the beam of a 5-mW-Helium-Neon-laser is passing through the sample chamber. The scattered light is detected by a photo multiplier. A computer-based analysis of the detected signals is used to obtain the auto-correlation function of the intensity of scattered light:

$$G_2(t, t+\tau) = \left\langle I(t) \cdot I(t+\tau) \right\rangle$$

This auto-correlation function of second order ($I \sim E^2$) provides information how the light intensity at time (t+τ) is correlated with the intensity at the time point t. It is possible to obtain a characteristic time t_c for the particle motion from the auto correlation function. This time is affected by the diffusion coefficient:

$$t_C = \frac{1}{DK^2}$$

Therefore, D can be calculated from the measured auto correlation function. from t_c. In consequence, the hydrodynamic radius R_h can be obtained. In general, for monodisperse samples, this hydrodynamic radius is equal to the mean particle radius within reasonable margins. For polydisperse samples with a broad size distribution, the auto correlation function can be related to the size distribution function.

[1] Several names are in use for the method: dynamic light scattering, intensity correlation spectroscopy, quasi-elastic light scattering (QELS).

2.2 Zetapotential estimation by electrophoresis

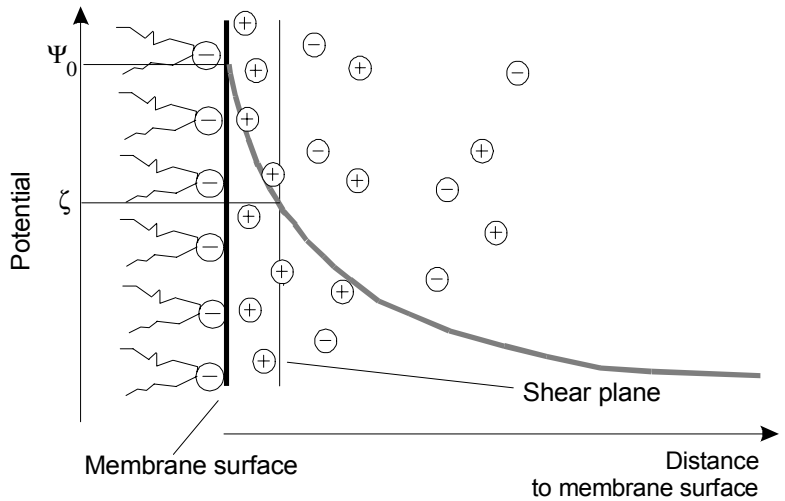
In general, cellular membranes are negatively charged. Therefore, an electrostatic surface potential exists. To bring two membranes together or to fuse them, it is necessary to overcome the resulting repulsive force. The electrostatic surface potential can be measured by electrophoresis. In our lab, we use the Zetasizer 4 for the electrophoretic characterization of liposome surfaces.

Electrophoresis is the movement of suspended charged particles (e.g. solid or colloidal particles, cells, drops or vesicles) in an external electric field. According to their charge, the particles are exposed to a force leading to a velocity *v*. The ratio of the velocity and the strength of the electric field is called electrophoretic mobility *b*:

$$b = \frac{v}{E} = \frac{s}{t \cdot E}$$

The particle charge can be obtained from the electrophoretic mobility. For the simplest case of a point charge, the mobility is proportional to the charge.

The situation is more complex for liposomes in an electrolyte. Fixed charges are present at the liposome surface while counterions are present in the surrounding medium. Due to the attractive potential near the surface, the concentration of counterions near the surface is increased with respect to the bulk. With increasing distance from the surface, it decreases and reaches the bulk ion concentration at long distances. Concomitantly, the concentration of coions is decreased near the surface and increasing with increasing distance. The cloud of counterions and coions near a charged surface can be described as diffuse electric double-layer.



Diffuse electric double-layer and zeta potential

In an external electric field, a charged particle moves together with a cloud of absorbed ions. All other not absorbed ions in the electrolyte are moving according to the sign of their charge. In consequence, a separation of ions moving with the particle (absorption layer) and ions not moving with the particle (diffusion layer) occurs. The separation layer is called shear plane. The thickness of the absorption layer is approx. 20 nm for negatively charged phospholipid vesicles. The electrostatic potential in the shear plane is called zeta potential ζ. The zeta potential is connected with the electrophoretic mobility *b* by the Helmholtz-Smoluchowski equation:

$$\zeta = \frac{\eta}{\varepsilon_0 \varepsilon_r} b$$

Here, η is the viscosity of the medium. ε_0 and ε_r are the dielectric numbers of the vacuum and of the medium, respectively.

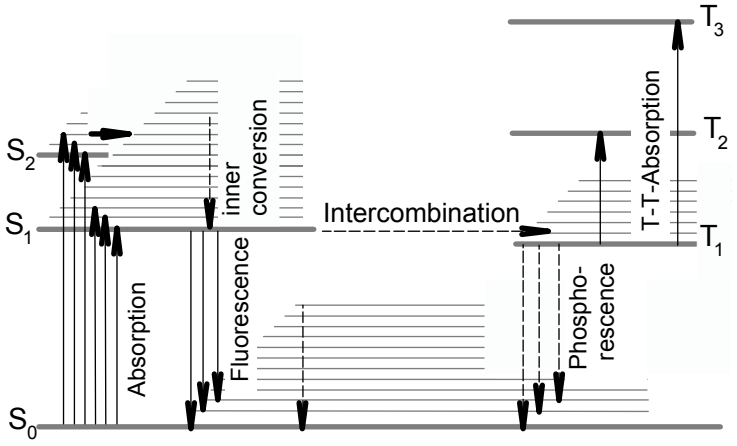
Using the Gouy-Chapman theory, one can obtain the electrostatic potential near the liposome surface from zeta potential measurements, and describe the reduction of repulsive electrostatic forces between membranes in the presence of fusiogenic trigger substances.

2.3 Fluorescence spectroscopy

Fluorescence spectroscopy is a very sensitive spectroscopic method with numerous applications for studies with model membranes.

2.3.1 Physical principles

Fluorescence is the result of a three-stage process that occurs in certain molecules (generally polyaromatic hydrocarbons or heterocycles) called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to localize within a specific region of a biological specimen or to respond to a specific stimulus. The process responsible for the fluorescence of fluorescent probes and other fluorophores is illustrated by the simple electronic-state diagram (Jablonski diagram)



Jablonski diagram of a fluorophore

1. Excitation: A photon of energy ν_{EX} is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S_1' or S_2'). This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

2. Excited-State Lifetime: The excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption return to the ground state (S_0) by fluorescence emission. Other processes such as collisional quenching, fluorescence resonance energy transfer (FRET) and intersystem crossing

(intercombination) may also depopulate S_1. The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted to the number of photons absorbed, is a measure of the relative extent to which these processes occur.

$$\Phi = \frac{\text{Number of emitted photons}}{\text{Number of absorbed photons}} \leq 1 .$$

3. Fluorescence Emission: A photon of energy ν_{EM} is emitted, returning the fluorophore to its ground state S_0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon ν_{EX}. The difference in energy or wavelength represented by ($\nu_{EX} - \nu_{EM}$) is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. In contrast, absorption spectrophotometry requires measurement of transmitted light relative to high incident light levels at the same wavelength.

Fluorescence Spectra: The entire fluorescence process is cyclical. Unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as photobleaching), the same fluorophore can be repeatedly excited and detected. The fact that a single fluorophore can generate many thousands of detectable photons is fundamental to the high sensitivity of fluorescence detection techniques. For polyatomic molecules in solution, the discrete electronic transitions are replaced by rather broad energy spectra called the fluorescence excitation spectrum and fluorescence emission spectrum, respectively. The bandwidths of these spectra are parameters of particular importance for applications in which two or more different fluorophores are simultaneously detected. With few exceptions, the fluorescence excitation spectrum of a single fluorophore species in dilute solution is identical to its absorption spectrum. Under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy during the excited-state lifetime. The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength.

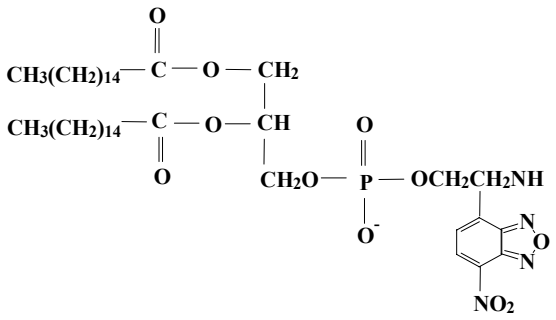
The properties of absorption and emission spectra can be summarized as follows:

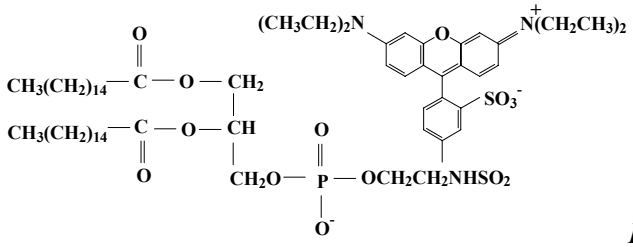
- Every fluorophore has two typical spectra: absorption spectrum and emission spectrum.
- The emission spectrum is independent on the absorption wavelength, since fluorescence is always emitted from the lowest vibrational level of the S_1 state.
- The emission spectrum is shifted to higher wavelengths with respect to the absorption spectrum.
- When the vibrational structure of S_0 and S_1 is equivalent, the absorption and the emission spectrum is identical but reversed left to right.

2.3.2 Fluorescence assay for lipid mixing

Phospholipid mixing can be investigated by the NBD-PE / Rh-PE-assay (Struck, Hoekstra & Pagano, 1981). The method is based on the fluorescence energy transfer (FRET) from 7-nitrobenz-2-oxa-1,3-diazol (NBD) acting as donor to Rhodamine acting as acceptor.

NBD-PE





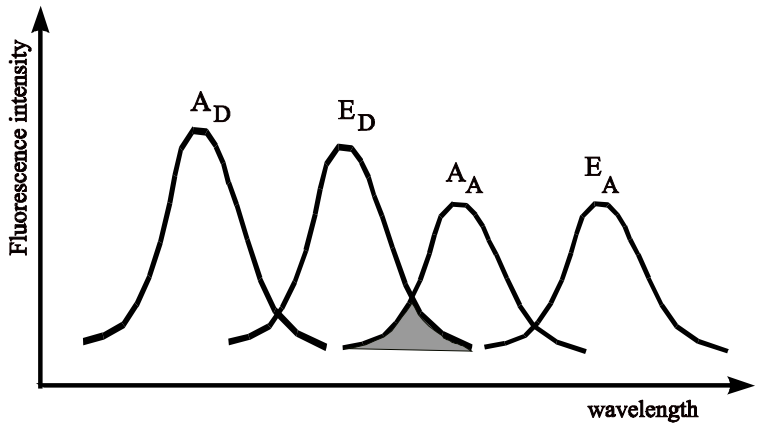
Rhodamine-PE

Both fluorophores are present in chemically modified lipids (phosphatidylethanolamine, PE) and incorporated into vesicle membranes by mixing the organic solutions of NBD-PE (1 mol%), Rh-PE (1 mol%) and the matrix lipids, and preparing fluorophore-labeled vesicles from this mixture. A second vesicle population is prepared from the same lipids but in the absence of fluorophore-labeled lipids. For the measurements, 1 part of a stock of labeled vesicles and 1 part of a stock of unlabeled vesicles are combined in buffer solution representing a 1:1 ratio of labeled and unlabeled vesicles.

Name	*Molecular weight*	*Supplier*	*Excitation wavelength*	*Emission wavelength*
NBD-PE	880 g/mol	Molecular Probes	460 nm	520 nm
Rh-PE	1259 g/mol	Molecular Probes	520 nm	588 nm

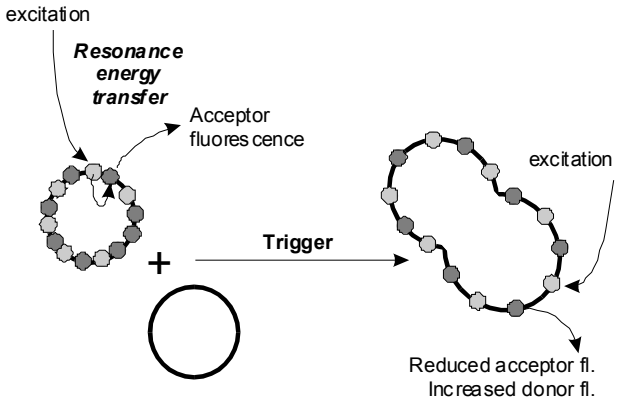
Data for NBD-PE and Rh-PE

The donor molecule NBD has the absorption maximum at 460 nm. The emission maximum of NBD is at 520 nm. At this wavelength, Rhodamine has the absorption maximum. Therefore, the excitation energy of the donor can be transferred to the acceptor, leading to an excitation of Rhodamine and, in consequence, an emission of Rhodamine at 588 nm.



Schematic representation of spectra of donor and acceptor for FRET: A_D – absorption of donor; E_D – emission of donor; A_A – absorption of acceptor; E_A – emission of acceptor

The fluorescence measurements are carried out by exciting the NBD at 460 nm and recording the fluorescence emission of both NBD and Rh in emission spectra ranging from 500 to 600 nm. When phospholipid mixing between fusing labeled and unlabeled vesicles occurs, the average distance between NBD- and Rh-labeled molecules increases on the membrane due to the lateral diffusion of lipids over the fused membrane. In this case, the acceptor emission intensity at 588 nm decreases whereas the donor emission intensity at 520 nm increases, since the FRET efficiency strongly depends on the distance between donors and acceptors (Förster mechanism).



Principle of NBD/Rh lipid mixing assay

The extent of phospholipid mixing M is calculated from

$$M = (q - q_0)/(q_{\text{Triton}} - q_0) \cdot 100\%$$

where $q = I_{\text{NBD}} / I_{\text{Rh}}$ is the ratio of the intensity of NBD at 520 nm and the intensity of Rhodamine at 588 nm. The values for q_0 are measured in the absence of the fusion trigger. The values for q_{Triton} are measured to define the 100% value of phospholipid mixing by solubilization of the vesicles in 0.2% (v/v) Triton X-100. By using the ratio q instead of the NBD-intensity, no intensity correction for light scattering or for quenching of the NBD-fluorescence by Triton X-100 is necessary.

3 Experimental instructions

1. Vesicle preparation

Unlabeled vesicles

- Fill 1 mg **phosphatidylserine (PS)** (chloroform solution) with (cleaned!) Hamilton syringe into a 50-ml-round bottom flask
- Rotational evaporator: Remove organic solvent by rotation under very low pressure for 15 – 20 min
- Suspend dry lipid by adding 1 ml buffer solution (10 mM HEPES, 100 mM NaCl, pH 7.4) → Lipid concentration in suspension: 1 mg/ml
- Vortex for 10 min
- Fill suspension into plastic vials
- Prepare SUV by ultrasonic treatment (Branson Tip Sonifier: temperature bath, clean Tipp, power supply, temperature control, approx. 10 min to obtain clear suspension)
- Repeat whole procedure for **phosphatidylinositol (PI)**

Fluorescently-labeled vesicles for NBD/Rh lipid mixing assay

- Mix chloroform solutions of 98 mol% phospholipid (PS or PI) + 1 mol% NBD-PE + 1 mol% Rh-PE . Before, calculate necessary volumes to obtain the same total molar lipid amount as with the unlabeled vesicles. Use appropriate Hamilton syringe to fill the respective chloroform solutions into a 50-ml-round bottom flask.
- Follow same procedure as with the unlabeled vesicles to obtain SUV.

2. Fusion experiment

- Four experiments / systems:

$$\left.\begin{matrix}\text{PS - SUV}\\ \text{PI - SUV}\end{matrix}\right\} + \left\{\begin{matrix}Ca^{2+}\\ La^{3+}\end{matrix}\right.$$

- Fluorescence spectrometer LS 50-B (Perkin-Elmer), Temperature control at 37°C
- Quartz cuvettes with stirrer (Handle with care! Fragile and expensive!)

For each of the four systems:

- Mix in fluorescence cuvette: 1 part unlabeled + 1 part labeled vesicles (20 µl + 20 µl + 2500 µl buffer solution → calculate molar lipid concentration
- Record fluorescence spectrum: experimental conditions (excitation wavelength and emission wavelength) according to assay principle
- Titration of Ca^{2+} or La^{3+}: Calculate amounts of ionic solutions with existing BASIC program (note logarithmic gradation!). Add to cuvette and vortex gently. Record spectrum for each titration step
- After titration: lysis of vesicles by addition of 1 vol% Triton-X100

3. Data analysis

- Read from every spectrum: I(520 nm) und I(588 nm)
- Calculate $q = \frac{I(520\,\mathrm{nm})}{I(588\,\mathrm{nm})}$ for initial spectrum (q_0), spectrum with Triton-X-100 (q_{Triton})and for every cation concentration (q_c)
- Fusion extent $M = \frac{q_c - q_0}{q_{\mathrm{Triton}} - q_0} \cdot 100\%$
- Create Graph M=M(c_{Ion}) for each of the four systems; logarithmic scaling for c_{Ion} axis

4. Protocol

1. Aim
2. Method
3. System
4. Preparation
5. Measurements
   - Data table
   - Observations
   - Print-out of spectra
   - Graphical presentation of results
6. Discussion

4 Literature

Phospholipids and Liposomes:

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