University of Leipzig

Biophysics Practical Course

Preparation of liposomes and analysis of cation-induced membrane fusion detected by fluorescence resonance energy transfer

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1 Introduction

Cell membranes consist of a fluid double layer composed of phospholipids and cholesterol. Furthermore, proteins and a multiplicity of different other molecules are embedded in these membranes (Fig. 1). Due to the high complexity of biological systems, phospholipid vesicles are used as model systems for the analysis of highly diverse processes occurring at cell membranes.

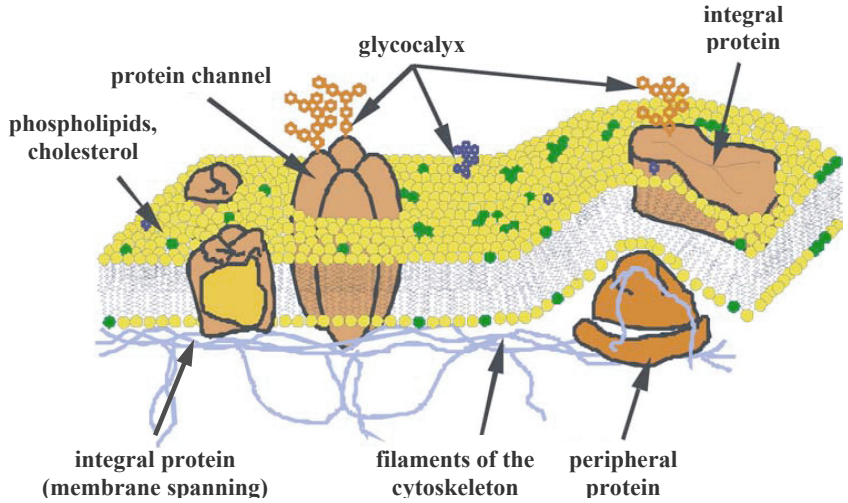


Fig. 1: The fluid mosaic model developed by Singer and Nicholson characterizes cell membranes as fluid double layers consisting of phospholipids and cholesterol which embed intrinsic membrane proteins. Additionally, peripheral proteins bind to the membrane surface.

A phospholipid molecule is composed of a polar head group, the glycerol backbone and also the two nonpolar, hydrophobic acyl chains (Fig. 2). For the preparation of liposomes the negatively charged phosphatidylglycerol (PG) is used, which was extracted from natural cell membranes (hen's egg) and, therefore, exhibits a mixture of different acyl chains.

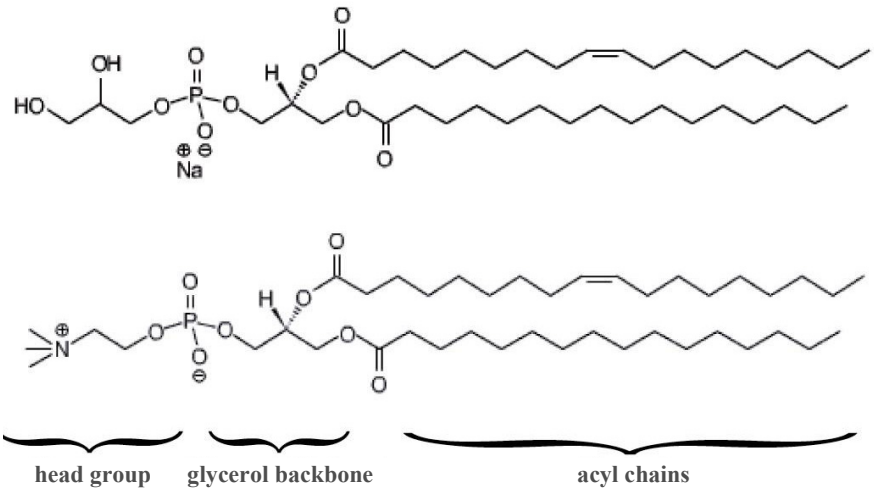


Fig. 2: Chemical structure of phosphatidylglycerol (upper) and phosphatidylcholine (lower). The length of the acyl chains and the number of double bonds may vary.

Based on their amphiphilic character (polar head group and nonpolar acyl chains), phospholipids in aqueous solutions arrange spontaneously into different superstructures to minimize the free enthalpy due to the dissolving of the hydrophobic acyl chains in water (hydrophobic effect). The formation of the superstructures is dependent on the interaction between the lipids (electrostatic, van der Waals and hydrophobic interactions and, further, hydrogen bonds), their shape (dimension of the head group in relation to the acyl chains), the water content of the membrane, and also the temperature. The transfer of the phospholipids, used in the practical course, into an aqueous solution causes a spontaneous assembly of multilamellar vesicles (MLV), which can be converted into single-layer vesicles (liposomes) of different size by varying methods (Fig. 3). The multilamellar vesicles, which were formed in the aqueous solution, are composed of multiple lipid double layers which are ordered onionskin like. Therefore, MLVs can achieve huge diameters of up to 10 µm.

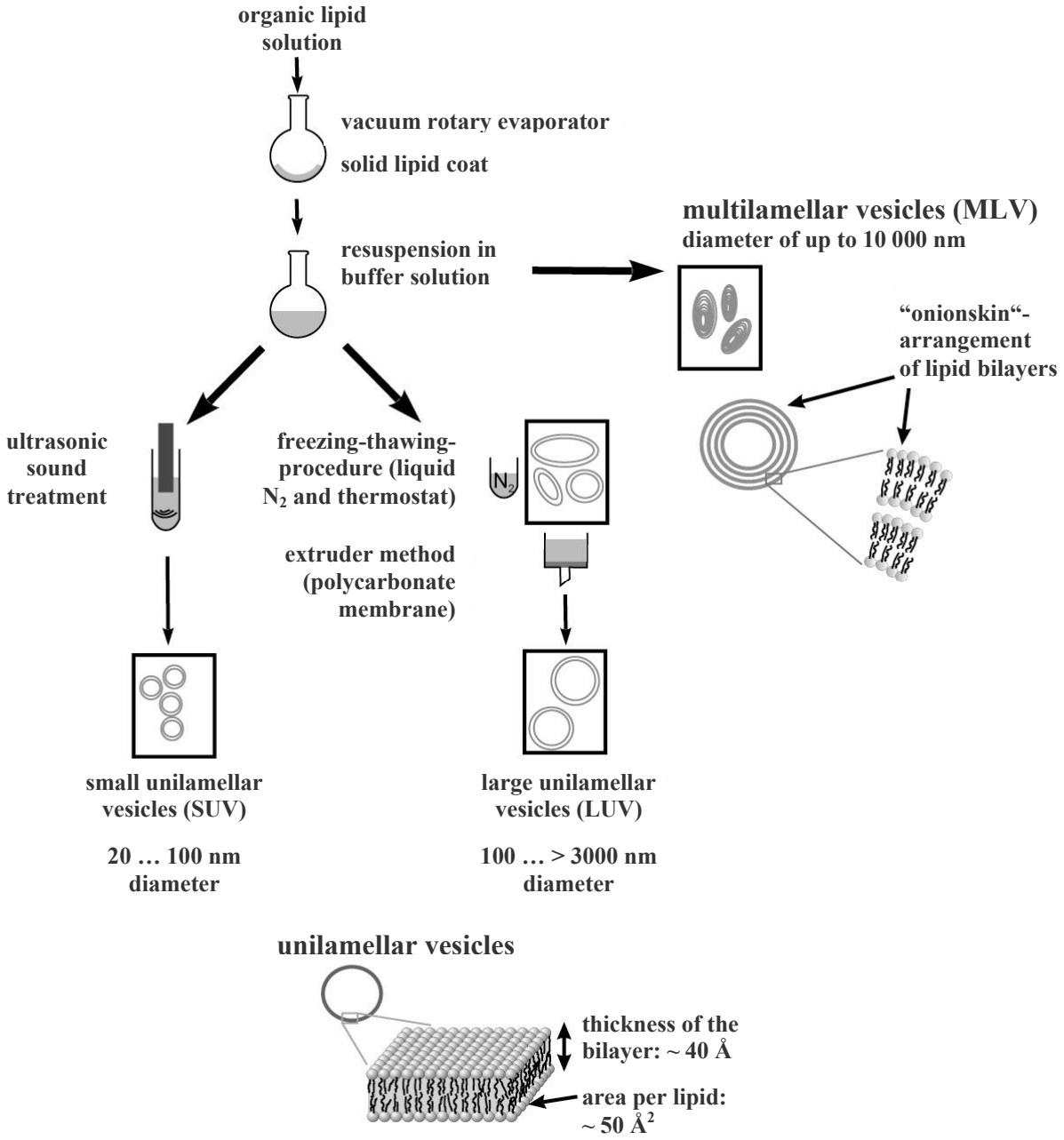


Fig. 3: Lipid vesicle preparation protocol and classification of lipid vesicles.

The MLVs collapse, when they are treated with ultrasonic sound, and small unilamellar vesicles (SUV) with diameters of 20 to 100 nm are formed. Another method for the preparation of liposomes is the so-called freezing-thawing-procedure. Repeated freezing in liquid nitrogen and subsequent thawing of the vesicle suspension generates a suspension of single-layer liposomes of varying size. If this suspension is extruded several times with high pressure through a polycarbonate membrane with pores of a defined dimension (extruder method), liposomes of homogeneous size (typically 100 nm) are produced.
Liposomes are simple models mimicking cell membranes. Depending on the preparation, they can enclose proteins and similar molecules and are used as transport vehicles for pharmacological agents.

2 Basic principles

2.1 Membrane fusion

Membrane fusions are part of essential cell biological processes, like exocytosis, endocytosis, the intracellular vesicle transport, fertilization, and also viral and bacterial infection of cells. However, the molecular mechanism of membrane fusions is not fully understood. In the multistage model for phospholipid vesicle fusions (Fig. 4) different physical interactions between the vesicles and the trigger molecules play a role. The practical course analyzes the mixing of vesicle membranes during fusion under influence of Ca^{2+} ions via fluorescence spectroscopy.

A	aggregation	unspecific interactions (van der Waals attraction, reduced electrostatic repulsion)
B	direct molecular contact of surfaces	reduced hydrostatic repulsion
C	local disruption of the phospholipid bilayer	among other things, hydrophobic interactions
D	mixing of liposomes • mixing of outer layer lipids possibly without real fusion (semifusion) • mixing of liposome contents (total „real“ fusion)	

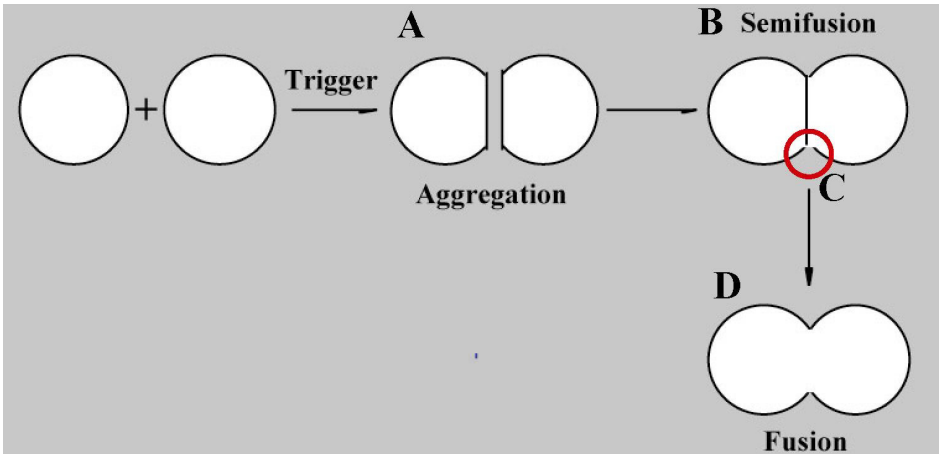
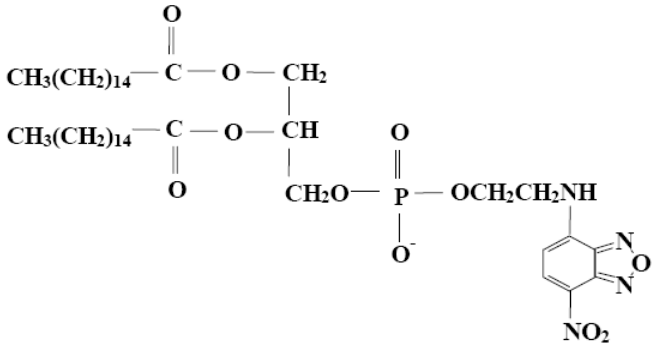


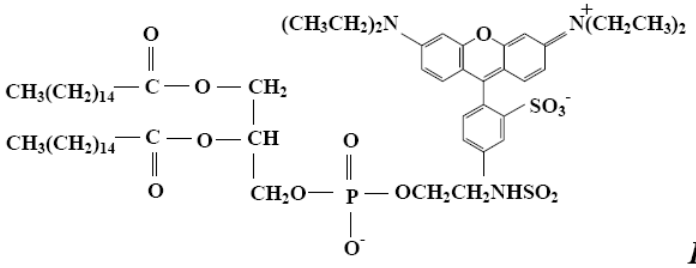
Fig. 4: Membrane fusion scheme. Cations (Ca^{2+}, Mg^{2+}, La^{3+}, …), calcium binding proteins, viral proteins, peptides and polymers can be considered as trigger molecules for vesicle fusion.

2.2 Fluorescence spectroscopy

The basis of fluorescence spectroscopy is the interaction of electromagnetic radiation with matter. If a molecule absorbs electromagnetic radiation with frequencies of the ultraviolet or visible range transitions between the electron energy levels of the molecule are stimulated. The absorbed radiation lifts an electron from an energetically lower to an energetically higher orbital, which leads to an excited higher energy state of the molecule.
Subsequent to the absorption, different deactivation processes take place, which result the release of the absorbed energy. This energy release can either be radiationless or under emission of radiation. Thus, a molecule possesses an absorption and emission spectrum. Due to the lower quantum energy, the emission spectrum is shifted to higher wavelengths compared to the absorption spectrum.
Fluorescence is mostly observed for aromatic or heterocyclic molecules. In particular, molecules with two or more condensed rings are of considerable importance in this context. Such molecules are also termed fluorophores or fluorescent dyes.
In the practical course membrane fusion will be detected with the help of fluorescent probes, which will be embedded into the model membranes during liposome preparation. The mixing of the membrane lipids, which occurs in the course of a membrane fusion or of a strong aggregation of liposomes, will be detected with help of the NBD-Rhodamine mixing assay. For that purpose, the fluorophores are bound to the head group of the phospho-lipids (Fig. 5).

NBD-PE





Rhodamine-PE

Fig. 5: Chemical structure of Rhodamine-PE and NBD-PE.

The donor molecule NBD (7-Nitrobenz-2-oxa-1,3-diazole) is a fluorophore whose absorption maximum is located at a wavelength of 460 nm. The emission maximum of NBD at 520 nm is located in the absorption maximum of Rhodamine (Rho). So, the excitation energy of the donor molecule can be transferred radiationless to the acceptor molecule Rhodamine on condition that the distance between the molecules is sufficient small (Förster mechanism). Thereby,

the excited Rhodamine emits radiation with an emission maximum at a wavelength of 588 nm (Fig. 6).

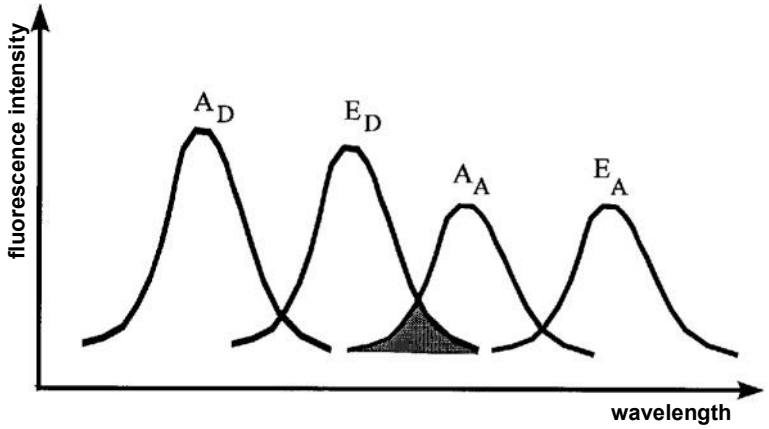


Fig. 6: Absorption and emission spectra of a donor acceptor pair. A indicates the absorption spectrum and E the emission spectrum. The indices identify donor (D) und acceptor (A).

When mixing of membrane lipids during the fusion of liposomes with and without fluorophores occurs, the fluorophores are diluted via lateral diffusion over the larger surface. The distance between donor and acceptor becomes larger and, thus, the transfer efficiency decreases. Therefore, the acceptor fluorescence also decreases while the donor fluorescence increases (Fig. 7).

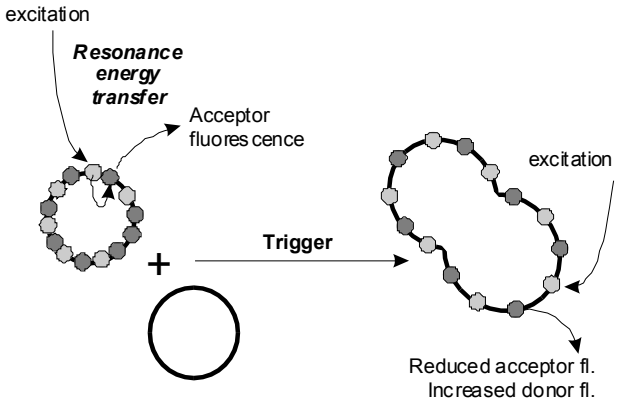


Fig. 7: Principle of the NBD-Rhodamine mixing assay.

The calculation of the mixing yield M is carried out by the following equation:

$$M = \frac{\left(\frac{I_{520}^{Ca}}{I_{588}^{Ca}} - \frac{I_{520}^{0}}{I_{588}^{0}}\right)}{\left(\frac{I_{520}^{Triton}}{I_{588}^{Triton}} - \frac{I_{520}^{0}}{I_{588}^{0}}\right)} \cdot 100\% \,.$$

The calculation comprises both the donor (I_{520}) and acceptor fluorescence intensity (I_{588}). In this regard, the intensities in absence of calcium ions are indexed “0“. The fluorescence intensities after lysis of the liposomes via 1 vol% Triton X-100 are indexed “Triton“. After addition of Triton, the state of the system is characterized by a high dilution of the fluorescence probes and is assumed as the 100% value of lipid mixing.

3 Schedule

3.1 Liposome preparation

Material:
Egg PG M_w = 771 g/mol
Rho-PE M_w = 1276 g/mol
NBD-PE M_w = 898 g/mol

- Prepare 0.5 L buffer solution containing 10 mM HEPES, 100 mM NaCl, pH 7.4. Calculate required amounts **before the practical course begins**. (M_{HEPES} = 238.3 g/mol, M_{NaCl} = 58.44 g/mol)
- At first, prepare multilamellar vesicles (MLV) composed of PG with and without fluorescent probes.
- The phospholipids are provided as powder. Weigh the needed amount of lipids to achieve 3 mL of vesicle suspension with a concentration 1 mg/mL. For the MLVs without fluorophores dissolve the lipids directly in buffer.
- For the vesicles with fluorophores dissolve the lipids in chloroform (Work under the extractor hood!). The amount and concentration of the lipids shall be identical to phospholipids without fluorophores with the exception of 1 mol% Rhodamine-PE and 1 mol% NBD-PE (Fig. 5). Add the needed amounts of Rho-PE and NBD-PE. Evaporate the chloroform with help of the rotary evaporator. Detach the produced lipid coating with buffer from the glass flasks. Calculate required amounts **before the practical course begins**.
  ($Conc_{Rho\text{-}PE}$ = 1 mg/mL, $Conc_{NBD\text{-}PE}$ = 1 mg/mL)
- Incubate the lipid suspensions for 30 minutes at 37°C in a shaking bath.
- Partition the MLV suspensions (2 mL for LUV, 1 mL for SUV) and prepare small unilamellar vesicles (SUV) by the ultrasonic sound procedure and accordingly large unilamellar vesicles (LUV) by the extruder method.
- **Preparation of SUV:** Ultrasonic sound treatment (Use EAR PROTECTION! Refrigeration of the samples by sludge. Clean cone end. Cone end in the centre of the tubes without contact to tube edge.) for 15 min - cycle 0.5 - output up to 100% (Raise slowly beginning from the minimum to prevent fluid ejection).
- **Preparation of LUV (diameter of 100 nm):** 10 freezing-thawing cycles with liquid nitrogen. Lipex Biomembranes Extruder: clean extruder, insert 2 polycarbonate membranes, nitrogen pressure 20-25 bar, prior to sample extrusion rinse two times with buffer, 5 extrusion cycles.

3.2 Detection of membrane fusion via FRET

- Measure the mixing of lipid membranes for different systems under influence of Ca^{2+} ions, i.e., LUV/LUV*, LUV/SUV*, SUV/LUV*, SUV/SUV* (*=labeled vesicles)!
- Mix labeled and unlabeled vesicles at a ration of 1:1 (20 µL each) with 2 mL of buffer in the cuvette (What is the molar lipid concentration in the cuvette?).
- The measurement of the spectra is carried out at the Fluoromax 2 Spectrometer (excitation at 460 nm, data acquisition of spectra between 500 and 630 nm, increments 1 nm, integration time 0.2 s, temperature 37°C).
- Measure the fluorescence spectra for Ca^{2+} concentrations of 0/0.2/0.5/1/2/5/8/10/20 and 50 mM in the cuvette. (The quantities of Ca^{2+} solutions to be added will be calculated with a special program (stock solutions: 10/100/1000 mM Ca^{2+}).)

- Measure the fluorescence spectrum after addition of 1 vol% Triton X-100 solution (20% stock solution) as reference value of total lipid mixing.
- Analyze the intensities at 520 and 588 nm for initial spectra (I^0), Triton spectra (I^{Triton}) and every spectrum for different ion concentrations (I^{Ca}). Calculate the mixing yield M according to the above equation and plot M as a function of the Ca^{2+} concentration (semi-logarithmical).

Protocol

1. Goal
2. Methods
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5. Measurement
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Comparative graphical presentation of results
6. Discussion