Protocol
Preparation and purification of calcein-filled, large unilamellar vesicles and fluorescence lifetime-based leakage

Leaky membrane fusion: an ambivalent effect induced by antimicrobial polycations

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1 The method

We'd all be grumpy people if we frequently walked into lamp-posts—no matter if the lamps are on or not. It is perhaps understandable then, that an excited molecule of calcein simply loses its exciation when it collides with another calcein molecule. This property of collisional self-quenching of calcein allows us to apply time-resolved fluorescence to study membrane permeabilization *via* the leakage of calcein-loaded vesicles (CLVs).

In dilute solutions ($\sim$5 µM), calcein has a characteristic fluorescence lifetime (τ) of $\sim$4 ns. In concentrated solutions of $\sim$70 mM, it has a fluorescence lifetime of $\sim$0.4 ns. Addition of membrane-active molecules or application of membrane-perturbing conditions to vesicles with high concentrations of entrapped calcein can cause dye efflux, *i.e.*, leakage. A leakage assay therefore allows us to study the mechanism by which a membrane-active substance or membrane-perturbing condition leads to membrane permeabilization.

Fluorescence lifetimes may be measured with the technique of time-correlated single photon counting (TCSPC). The fluorescent dye is excited with a light source of high pulse frequency (at 467 nm for calcein); the detector bins the arrival times of photons at the emission wavelength of the dye (515 nm for calcein). For a single dye population, the binned data follow the form of an exponential decay.

In the case of calcein-loaded vesicles, it is not experimentally possible to remove all of the dye from bulk solution. Therefore, a CLV solution would have two calcein populations—entrapped at $\tau_{\mathrm{E}} \approx 0.4$ ns (majority) and small amounts of free, diluted dye with $\tau_{\mathrm{F}} \approx 4$ ns. The measured fluorescence decay would be a sum of exponential components with these two lifetimes.

Let us consider two extreme cases: if the membrane perturbation occurs such that only a small amount of calcein leaks at a time, the entrapped calcein concentration decreases and, in turn, the average fluorescence lifetime of entrapped calcein would increase gradually as the dye is gradually diluted by several perturbations. This scenario was termed graded leakage. In the other extreme, the perturbation is such that all of the calcein has the chance to equilibrate with the surrounding at once. This scenario is termed all-or-none leakage. Such a classification is only generally descriptive and there are other possibilities to classify leakage behavior. Some options are described in Section 3.3.2.

The method of fluorescence lifetime-based calcein leakage assay is first described in [1].

2 Materials

All materials are examples for which the protocol worked with us and are probably replaceable.

2.1 Reagents

- Lipid (PC, PS, PG, PE, CL, cholesterol, yeast polar lipid extract, or E. coli polar lipid extract)
- Chloroform ($> 99\%$ HPLC grade)
- Membrane-active substance of interest
- Dimethylsulfoxide (DMSO)

- Buffering agent of choice (e.g. Tris, HEPES; ⚠ do not use phosphate)
- NaCl or KCl
- NaOH/KOH, HCl
- Ethylenediaminetetraacetic acid (EDTA, free acid form)
- Calcein
- Colloidal silica (LUDOX AM, 30 wt.% suspension in water, e.g. Sigma Aldrich,)

- Dry ice or liquid nitrogen
- PD-10 desalting columns, Sephadex G-25 (GE Healthcare Life Sciences)

For the ashing + molybdate assay:

- Phosphate Colorimetric Assay Kit (e.g. Biovision)
- $Mg(NO_3)_2$
- 95% ethanol
- 0.5 N HCl

For the Bartlett assay:

- KH_2PO_4
- 10 N H_2SO_4
- 30% w/w H_2O_2, *pro analysis* grade
- Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$)
- Fiske-Subbarow reducer (Sigma)

COMMENT: We could in principle add a protocol to use ^{31}P NMR for phosphate analysis.

2.2 Labware and equipment

- Positive-displacement pipette or glass syringe (Hamilton Company Gastight, cemented needle)
- Micropipettes and pipette tips

- Amber glass vials (∼4 mL capacity) with Teflon-lined caps
- Centrifuge tubes
- 1.5 mL Eppendorf tubes
- Disposable polystyrene fluorescence cuvettes (1 cm path length, unfrosted sides)

- Water filtration system (Millipore)
- Hot/stir plate
- pH meter and calibration standards
- Osmometer
- Retort stand and clamp
- Chamber/oven with vacuum pump
- Vortex mixer
- Barrel extruder (e.g. Northern Lipids) with nitrogen source or mini-extruder (e.g. Avanti polar lipids);
  drain discs
- Polycarbonate membrane filters with appropriate pore size (80 nm or 100 nm work best for 100 nm LUVs) (Whatman™ Nuclepore™ track-etched)
- Rotatory shaker

- Particle sizer (e.g., ZetaSizer from Malvern Instruments, NanoBrook from Brookhaven Instruments)
- Fluorescence lifetime fluorometer with thermostated sample chamber (e.g., Horiba Fluorolog, PicoQuant FluoTime)
- MS Excel and Origin for data analysis
- Any instrumentation needed to apply the membrane-perturbing conditions under investigation

For the ashing + molybdate assay:

- 5 mL glass test tubes and rack
- Test tube tongs
- Hot plate
- Aluminum foil or marbles
- Propane torch
- 96-well plate or UV/Vis cuvettes
- Microplate reader or UV/Vis spectrophotometer

For the Bartlett assay:

- 10–15 mL glass test tubes and rack
- Oven (160 °C)
- Heating block (95 °C)
- Marbles
- UV/Vis cuvettes

- UV/Vis spectrophotometer

2.3 Reagent setup

Solution of the membrane-active substance of interest
If the substance has low solubility in buffer but is soluble in water: first prepare a stock solution in water, then dilute with buffer when preparing leakage samples.
If the substance has low aqueous solubility, consider supplementing the external buffer with up to 5% v/v DMSO. During sample preparation, ensure that all leakage samples contain the same total amount of DMSO. DMSO may change partitioning. Beware of problems wit solubility that may occur after the complete sample solution has been prepared.

Calcein buffer
Calcein, 70 mM
EDTA, 0.5 mM
Buffering agent, 10–25 mM
Adjust to the desired pH ($6.5 <$ pH < 9) with NaOH/KOH

Prepare this buffer under red light or under low light conditions. The optimal batch size is 25 mL.
Steps:

1. Weigh out all buffer components into a 50 mL beaker, wrap the sides of the beaker in Al foil for light protection
2. Add 17–20 mL water. Immerse pH electrode completely (avoid stirbar)
3. Add 1 M NaOH/KOH drop-wise, let pH stabilize between additions (calcein does not completely dissolve until enough base has been added to reach pH 6–7)
4. Take care to avoid going above the desired pH (the addition of HCl renders the buffer unusable due to mismatched osmolarity)
5. Wait for the pH reading to stabilize completely before topping the total buffer volume to 25 mL in a volumetric flask; minimize calcein loss
6. Store buffer in amber vials at 4 °C for up to 6 months

External buffer
Buffering agent, same as the calcein buffer
HCl/NaOH as needed
EDTA, 0.5 mM
NaCl/KCl, isosmotic to the calcein buffer (determine with osmometer). If there is a slight mismatch in osmolarity, the outside buffer should have the slightly higher value to avoid vesicle bursting because of water influx.

Recipes of isosmotic calcein/external buffers

- 10 mM Tris, 70 mM calcein, 0.5 mM EDTA, pH 7.4
  10 mM Tris, 110 mM NaCl, 0.5 mM EDTA, pH 7.4

- 20 mM HEPES, 70 mM calcein, 0.8 mM EDTA, pH 8.0
  20 mM HEPES, 120 mM NaCl, 0.8 mM EDTA, pH 8.0
- 25 mM MOPS, 70 mM calcein, pH 6.5 to pH 7.5
  25 mM MOPS, 130 mM NaCl, pH 6.5 to pH 7.5

Required solutions for the ashing + molybdate assay

30% w/v $Mg(NO_3)_2$ in ethanol

Phosphate standard (0.1 mM, prepared from the commercial kit)

Required solutions for the Bartlett assay

1 mM KH_2PO_4 in water (13.61 mg $+100$ mL water)

0.22% w/v Ammonium heptamolybdate tetrahydrate in water (220 mg $+100$ mL water)

14.8% w/v Fiske-Subbarow reducer in water (740 mg $+5$ mL water)

3 Procedure

3.1 Preparation of calcein-loaded vesicles (CLVs)

3.1.1 How much lipid to use?

m_{L}	total mass of lipid required for a leakage series
c_{L}	lipid concentration of each sample in a leakage series (typically 30 µM, and/or additionally 100 µM, 200 µM, or 400 µM)
V	solution volume of each sample (typically 1.5 mL to 2 mL for the spectroscopy)
MW_{A}	weight-average molecular weight of lipid by membrane composition
N	number of samples in each series, including blank
f_c	factor of reduction in lipid concentration in the desalting column ($f_c \approx 3$ for synthetic lipids, $f_c > 5$ for polar lipid extracts)
f_{e}	contingency/experience multiplier, $f_e \approx 1.3$–2

$$m_{\mathrm{L}} = c_{\mathrm{L}} \cdot V \cdot MW_{\mathrm{A}} \cdot N \cdot f_c \cdot f_{\mathrm{L}}$$

If working with binary or ternary lipid compositions, determine the mass of each lipid required from the total m_{L}.

For multiple leakage series with different lipid concentrations to determine the partition coefficient Section 3.2.4, sum all calculated m_{L} values.

3.1.2 Liposome preparation

Multilamellar vesicles (MLVs)

1. Label and weigh an empty amber vial (⚠ do not use labeling tape)

2. If lipid is dissolved in chloroform, determine the required volume; add the solution to the vial with a positive-displacement pipette or a glass syringe
   If using powdered lipid, weigh out the required mass and add chloroform to dissolve (at $\sim$25 mg mL^{-1})
   Note: Let lipid reach room temperature before handling
3. Evaporate chloroform under a gentle stream of nitrogen
4. Place vial under vacuum overnight to remove residual chloroform
5. Weigh vial and calculate the mass of the resulting lipid film
6. If working with binary or ternary lipid compositions, carry out steps 2–5 for each lipid to achieve the target composition. Adding one compound after the other. ⚠ Ensure that the lipid film was dissolved completely before evaporating the chloroform.
7. Store the lipid film at $-20\,^{\circ}$C (with Teflon-lined cap + parafilm) or continue as below

8. Hydrate lipid with the calcein buffer:
   Determine the volume of calcein buffer required to obtain 30–40 mM total lipid, then add that volume to the lipid film; vortex or gently shake solution until lipid film disappears and no chunks are visible ⚠ Avoid extensive foam formation.
9. Complete 5–8 freeze-thaw cycles:
   Freeze solution in dry ice/liquid nitrogen, thaw completely in water bath (25–60 °C). Each freeze or thaw step takes approximately 3 mins. Then vortex solution, repeat
   ⚠ Avoid drastic temperature changes to prevent vial breakage
10. Store the MLVs at $-20\,^{\circ}$C or continue as below

Large unilamellar vesicles (LUVs)

1. Extrude the MLVs with a mini-extruder ($<$1 mL) for 31 (or a larger, uneven number of) passes or a barrel extruder ($>$1 mL) for 15 passes through a polycarbonate membrane filter (80 nm pore size to obtain 100 nm LUVs) (following the manufacturer's instructions).
2. Verify liposome size (e.g. diameter $<$ 120 nm) and uniformity (polydispersity index < 0.1)
3. Store the LUVs at a temperature above or below lipid phase transitions, e.g. at room temperature for not more than one week, depending on the stability of the lipid composition or continue as below.
   For some types of lipids, it might be beneficial to allow the vesicles to anneal after production over night. There is not much experience on that issue yet. Often, vesicles containing fluid PO lipids do not require annealing.

3.1.3 Exchange external buffer

The capacity of the PD-10 desalting column is 2 mL. Use more than one column if the volume of LUVs is >2 mL. For optimal results, load 1–2 mL per column.

1. mount a PD-10 desalting column on a post, let drain
2. Equilibrate the column with 5 column volumes of external buffer ($\sim 25\,\mathrm{mL}$)
3. Prepare a large number of small Eppendorf tubes for collecting the fractions in a stand (at least 15 to 25).
4. Add the LUV solution all at once, wait for the solution to seep into the gel
5. Elute the column, collect fractions (1–2 drops per Eppendorf tube). If you are less experienced, collect even very early fractions. The relevant fractions are colored only very faintly .
6. For each yellow/orange fraction, remove $< 5\,\mu\mathrm{L}$ and dilute to $1\,\mathrm{mL}$ or $2\,\mathrm{mL}$ with external buffer in a cuvette; measure each fraction in the fluorescence lifetime fluorometer, retain fraction if $B_\mathrm{E}/B_\mathrm{F} > 9$ (see Section 3.3.1)
   Note: Lightly coloured fractions may have low lipid concentration
7. Verify CLV size (diameter $< 120\,\mathrm{nm}$) and uniformity (polydispersity index < 0.1)
8. Store the CLVs at a temperature above or below lipid phase transitions similar to the temperature during preparation and intended measurement conditions for not more than one week (e.g. POPC, POPG), or proceed immediately, depending on the stability of the lipid composition. Caution is needed especially for lipids prone to chemical degradation, such as oxidation of unsaturated acyl chains, lipids that favor non-zero curvature, compositions prone to demixing, natural extracts.

CLV storage times:
Lipids that are in the fluid phase at room temperature—up to one week
E. coli polar lipid extract—up to two days
Yeast polar lipid extract—up to one day

3.1.4 Determine lipid concentration

There are various options to determine the lipid concentration. Here, we describe an ashing + molybdate assay (which involves a commercial kit) and the Bartlett assay.

The ashing + molybdate assay
This assay is adapted from Ames (1966) [2] and makes use of a commercial assay kit. The kit contains ammonium molybdate and malachite green and can be > 7 times more sensitive than the Fiske-Subbarow procedure [2]. When in doubt, follow the manufacturer's directions.

1. Make five dilutions of the CLV stock:
   1/5 (5 µL +20 µL external buffer),
   1/10 (5 µL +45 µL external buffer),
   1/20 (5 µL +95 µL external buffer),
   1/30 (5 µL +145 µL external buffer), and
   1/50 (5 µL +245 µL external buffer)
2. Prepare the $0.1\,\mathrm{mM}$ phosphate standard according to kit instructions

3. Label 22 5 mL glass test tubes; add the dilutions and the 0.1 mM phosphate standard to the bottom of each test tube as per Table 1 below

Table 1: Volumes of diluted CLV stock solutions to assay

Tube #	Dilution	Volume
1	1/5	2 µL
2	1/5	2 µL
3	1/5	2 µL
4	1/10	2 µL
5	1/10	2 µL
6	1/10	5 µL
7	1/10	5 µL
8	1/20	10 µL
9	1/20	10 µL
10	1/20	10 µL
11	1/30	10 µL
12	1/30	10 µL
13	1/30	10 µL
14	1/50	15 µL
15	1/50	15 µL
16	1/50	15 µL
17	–	–
18	0.1 mM phosphate	10 µL
19	0.1 mM phosphate	20 µL
20	0.1 mM phosphate	30 µL
21	0.1 mM phosphate	40 µL
22	0.1 mM phosphate	50 µL

4. Add 30 µL of the % $Mg(NO_3)_2$ solution (in 95% ethanol) to each test tube; lightly shake to mix
5. Ash the mixtures (ensure the reactions are complete); let cool
6. Add 300 µL of 0.5 N HCl to each test tube; shake until the white ashing product is dissolved
7. Cap each tube with Al foil or marbles and boil for 15 min in a water bath; let cool
8. Add 200 µL of the solution from each test tube to a 96-well plate in order
9. Add 30 µL of the assay reagent to each well and mix (be consistent, e.g. pipette 3 times up and down)
10. After 30 min, scan the plate with a UV-Vis plate reader at 650 nm
11. Construct the calibration curve; calculate the lipid concentration of the CLV solution

The Bartlett assay

This method is adapted from Grant R. Bartlett (1959) [3] and is based on the colourimetric determination of inorganic phosphate.

1. Weigh 0 (blank), 50, 100, 150, 200, 250, 300, and 350 µL of the phosphate standard solution (1 mM KH_2PO_4) into glass test tubes for the calibration curve
2. Weigh some CLV stock solution into several test tubes—aim for ∼150–200 nmol phos-

pholipid (weigh 20 µL per test tube if the expected phospholipid concentration is ∼10 mM)
3. Add 500 µL of 10 N H_2SO_4 to each test tube; vortex thoroughly
4. Bake the test tubes for 3 h at 160 °C
5. Add 200 µL of the 30% w/w H_2O_2 solution to each test tube
6. Bake the test tubes for 1.5 h at 160 °C
7. The samples should be clear and colourless; if not, repeat steps 5 and 6
8. Preheat the heating block to 95 °C; fill wells with water for better transfer of heat
9. Prepare the fresh 14.8% w/v Fiske-Subbarow reducer solution
10. Add 4.5 mL of the 0.22% w/v ammonium heptamolybdate tetrahydrate solution and 200 µL of the Fiske-Subbarow reducer solution to each test tube; vortex thoroughly
11. Cover the test tubes with marbles and incubate in the heating block for 10 min at 95 °C
12. Cool the test tubes to room temperature; vortex thoroughly
13. Transfer the solutions to cuvettes and measure the absorbances at 830 nm
14. Construct the calibration curve; use the initial masses to calculate the lipid concentration of the CLV stock solution

3.2 Leakage assay

Generally, perform all steps involving CLVs or calcein under red light or low light conditions. For screening, incubate measrue at least for 1 hour in order not to miss slow permeabilization behavior. The incubation time starts with the addition of the CLVs. Incubate in the dark on a rotatory shaker and measure with the fluorescence lifetime fluorometer. Thermostating the sample chamber and rotary shaker is always recommended. Measurements are possible at room temperature for lipid mixtures that are in the fluid phase at room temperature. For all other conditions, the sample chamber and shaker should be thermostated at a temperature well below or above lipid phase transitions.

3.2.1 Instrument setup

The excitation wavelength is 467 nm; emission is measured at 515 nm.

The instrument response function (IRF), or prompt, is obtained at an emission wavelength of 467 nm with a diluted solution of colloidal silica (LUDOX AM). Ensure a narrow FWHM (full width at half maximum) before proceeding. Other instrument settings (e.g., filter bandwidth) should be consistent between the prompt and the measured samples.

3.2.2 Screening

If the concentration range at which the test substance is membrane active or the intensity of the intensity of the membrane-perturbing condition are known, screening is likely unnecessary.

Otherwise, screen for the active concentration range by preparing a series of samples with variable concentrations of the membrane-active substance that span several orders of magnitude—e.g., 0.1 µM, 1 µM, 10 µM, 100 µM, and 1 mM—at fixed lipid concentration (e.g., 30 µM).

Once the range of active concentration or membrane-perturbing condition has been determined, carry out the leakage assay as below.

3.2.3 A single leakage series

For a well-defined dose-response curve, the concentrations of the membrane-active substance or intensity of the leakage inducing condition of interest should yield various leakage values (well-distributed) between 0% and 100% total leakage.

Prepare samples directly in the cuvettes according to the pipetting scheme and in the order : solution of the membrane active-substance, DMSO if adding, external buffer. These can be prepared. Incubation starts with the successive addition of CLVs according to timing indicated in the excel scheme. Samples are incubated in the dark on a rotatory shaker and measured after $10\,\mathrm{min}$, $30\,\mathrm{min}$, 1 h, 2 h, (3 h,) 5 h, and 24 h with the fluorescence lifetime fluorometer. Only measuring for incubation times less than one hour might miss slow permeabilization behavior.

3.2.4 Determining the partition coefficient (K) and general leakage curve independent of the lipid concentration

Multiple leakage series at different lipid concentrations may be performed to determine the mole-ratio partition coefficient, K, and general leakage curve independent of the lipid concentration through an equi-activity analysis [1]:

1. Obtain full dose-response curves at four different lipid concentrations (e.g., 30 µM, 100 µM, 200 µM, and 400 µM)
   Note: For higher lipid concentrations and for a single incubation time only (e.g. 1h), you can reduce sample volumes during incubation to conserve lipid—e.g., for a leakage series with 100 µM lipid, prepare 600 µL of each sample, incubate, then top up to 2 mL final volume with buffer immediately prior to measurement. We recommend at least one sample prepared in the usual way for comparison.
2. Plot Leakage vs. Concentration of membrane-active substance
3. For the leakage value of 10%, obtain the corresponding concentration of the membrane-active substance required to achieve this for each leakage series/lipid concentration (interpolate as necessary)
4. Plot the concentrations from above vs. their lipid concentrations (4 data points)

5. Perform linear regression as governed by

$$c_{\mathrm{p}}^{10\%} = c_{\mathrm{p}}^{\mathrm{aq},10\%} + R_{\mathrm{e}}^{10\%} c_{\mathrm{L}} \ ,$$

   where $c_{\mathrm{p}}^{10\%}$ is the concentration of the membrane-active substance at 10% leakage, $c_{\mathrm{p}}^{\mathrm{aq},10\%}$ is the concentration of the membrane-active substance in aqueous solution, R_{e} is the molar ratio between the membrane-active substance that is membrane bound and the lipid, and c_{L} is the lipid concentration
6. Perform linear regression as above (steps 2-4) for 20%, 30%, 40%, 50%, and 60% leakage, or other leakage values that result in minimal uncertainty when interpolating in step 2
7. Plot the resulting R_{e} vs. $c_{\mathrm{p}}^{\mathrm{aq}}$ values; the molar ratio partition coefficient is the derivative (if the trend is linear, intercept is 0)

3.3 Data analysis and interpretation

3.3.1 Calculating leakage

Entrapped calcein (∼70 mM) has a lifetime τ_{E} of ∼0.4 ns; leaked/free/diluted calcein has a lifetime τ_{F} of ∼4 ns. The fluorescence decay trace is saved with the IRF. The data is modeled with a sum of exponential terms, each in the form $Be^{-t/\tau}$:

$$I(t) = A + B_{\mathrm{E}} e^{-t/\tau_{\mathrm{E}}} + B_{\mathrm{F}} e^{-t/\tau_{\mathrm{F}}} + \dots \ ,$$

where A is a constant. $I(t)$ is then convoluted with the IRF and fitted to the decay trace by the software provided with the instrument. Usually, a biexponential fit of the decay is used.

Caution is needed if additional terms with intermediate lifetimes are to be fitted as they are rarely well-defined and might change between samples in a series or over time.

Leakage (L) or percent of vesicle leakage is defined as exchanged content relative to the maximum exchange possible in the entire sample by

$$L_{total} = \frac{B_{\mathrm{F}} - B_{\mathrm{F0}}}{B_{\mathrm{F}} - B_{\mathrm{F0}} + Q_{\mathrm{stat}} B_{\mathrm{E}}} \ ,$$

where B_{F0} is the B_{F} of the reference sample without any membrane-active substance, and $Q_{\mathrm{stat}} \approx 1.2$ is a static quenching factor that accounts for, among other effects, some statically quenched calcein dimers while entrapped at high concentrations in the CLV.

It's important to remember that leakage values are not significant beyond the decimal point of their percentage value. In addition, the significance of the digit before the decimal is debatable.

Due to difficulties in distinguishing two similar exponential terms, exact values of $L_{total} \geq$ 70% are less reliable, too.

Note: Check that $B_{\mathrm{F}} - B_{\mathrm{F0}} + Q_{\mathrm{stat}} B_{\mathrm{E}}$ is consistent between samples—Q_{stat} may need to be adjusted to achieve this. Further problems arise when samples aggregate, cause light scattering, or sediment. Stirring is not always improving this situation and leakage might be overestimated or underestimated.

We can provide templates and example scripts for data handling.

3.3.2 Mechanisms of leakage

There is a huge number of proposed leakage mechanisms and a distinction on the basis of leakage data alone is not possible. Mechanisms may be defined by structural features such as oligomeric pores, toroidal pores, defects, or membrane solubilization. Additionally, leakage mechanisms might be distinguished more phenomenologically by different aspects of leakage behavior. For example, vesicle leakage experiments with a range of incubation times yield the time course of occurrence or formation of leakage events (fast and transient or stochastically re-occuring over time). Determining the lifetime of entrapped calcein relates to the concentration of entrapped calcein and, in turn, to the strength of leakage events, *i.e.*, pores that can exchange the entire dye entrapped in one vesicle at once (all-or-none) or a mechanism that requires many leakage events to occur in each vesicle for substantial leakage (graded). Most of this information can be obtained with a single leakage series, plotting leakage not only as a function of concentration, but also as a function of lifetime of entrapped calcein and over longer incubations times (Figure 1).

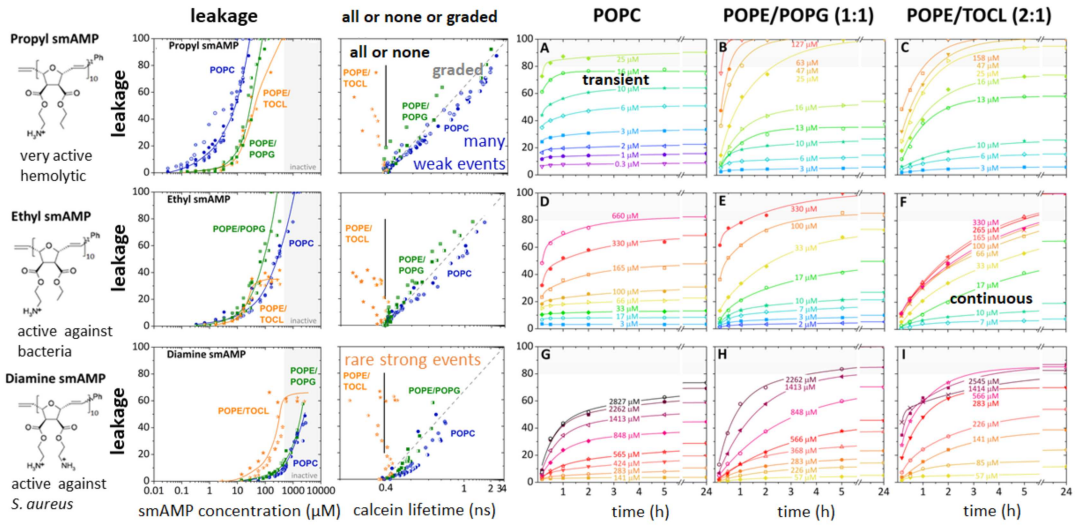

Figure 1: Structure of three synthetic mimics of antimicrobial peptides (smAMPs), leakage as a function of smAMP concentration, leakage mechanisms (strong all-or-none leakage events on the vertical line, weak gradual leakage on the diagonal), and long leakage kinetics (panels A-I). All y-axes align and show leakage (%). Leakage induced in POPC vesicles (blue), POPG/POPE 1:1 (green), and POPE/ TOCL (orange). Leakage kinetics (A-I) as a function of smAMP concentration are color-coded from low concentrations to high concentrations (from purple via green to dark red). [4]

Transient leakage

Transient leakage typically occurs fast (within 10 minutes) and does not increase much more after 1 hour of incubation. Currently, there are two processes associated with this signature: Asymmetric packing stress or leakage related to membrane fusion [5]. Asymmetric packing stress is supposed to result from a material imbalance after insertion of material into the outer membrane leaflet only. Upon cracking in of material, leakage may occur and afterwards, the imbalance is relieved so that the membrane anneals and leakage stops. This mechanism seems to be induced by relatively hydrophobic molecules and might have reduced selectivity for different lipid head groups compositions. An example for transient leakage that occurs independent of fusion, *i.e.*, presumably by asymmetric packing stress is the polymer MM:CO [6].

Another process that occurs in a similar time span (before 10 min and up to 1 hour of incubation) is vesicle fusion that might be linked to leakage, *i.e.*, leaky fusion. Leaky fusion was demonstrated for example for cell-penetrating TAT-peptides [7, 8].

Continuous leakage

Leakage that proceeds over several hours must be caused by events that reoccur stochastically over time. To date, it can only be speculated about the molecular processes leading to this behavior. We consider rare events or nucleation. For example, one might imagine that a certain number of bound molecules needs to combine in a certain orientation/ conformation to form an oligomeric pore that disassembles and reassembles again. An example for continuous leakage is ethyl smAMP acting on POPE/TOCL (Figure 1 F) [4].

All-or-none leakage

Classically, all-or-none leakage is observed when leakage increases with permeabilizer concentration, but τ_{E} remains unchanged at $\sim$0.4 ns. This corresponds to a scenario where the CLVs in solution are either intact or have lost their calcein gradient completely. Examples of all-or-none leakage are the peptide magainin added to 1:1 POPC/POPG liposomes [9], the polycations poly-NM and poly-MM with YPLE [10], CHAPS with POPC [11] or various smAMPs with POPE/TOCL (middle row Figure 1) [4].

Graded leakage

Classically, graded leakage is observed when τ_{E} increases with permeabilizer concentration along with efflux. This corresponds to a scenario where all of the CLVs in solution lose a fraction of their calcein gradient. Examples of graded leakage are the polycation MM:CO or the detergent octyl glucoside with YPLE [10], various synthetic AMPs with POPE/POPG and POPC (middle row Figure 1) [4], or the polycation MM:CO with POPE/POPG and POPC [6].

The different mechanisms can apparently occur in mixed forms. Note that there is no assignment of all-or-none or graded to transient or continuous leakage, respectively.

3.3.3 Interpreting leakage behavior

The observed leakage mechanism is dependent on how a membrane-active substance interacts with the membrane. Many mechanisms have been proposed to explain the observed leakage behavior or propose molecular structures. A single membrane-active substance may show different leakage mechanisms with different membrane compositions (providing evidence for the membrane-selectivity observed in some peptides). In addition, leakage mechanisms may change over incubation time or concentration ranges and may not follow the textbook examples.

When interpreting leakage data, consider the following questions:

1. How are the hydrophobic and hydrophilic groups/moieties distributed in your membrane-active molecule?
2. Does the the molecule change its conformation upon binding to the membrane? What timescale does this need?
3. Does the buffer or the ionic strength influence the conformation of your molecule?
   Do the molecule's interactions with the membrane change with these and other solution factors?
4. Does the molecule form oligomers in solution? Are the oligomers in equilibrium with monomers?
   Does the molecule aggregate in solution? What are the kinetics of the process?
5. If your molecule requires leakage assays at higher temperatures, what are the caveats?
   What is the phase-behavior of the lipid composition?
6. Does your membrane-active molecule interact with charged lipids? E.g., repel, attract, cluster, shield, cause de-mixing?
   How would this interaction change with membrane composition? E.g., with more/less/lack of positive/negative/zwitterionic lipids?
7. Are hydrogen bonds formed/interrupted with membrane binding?
8. What is the partition coefficient of your molecule?
   How would the partition coefficient change with membrane composition?
9. Does your molecule interact with or sequester membrane sterols?
10. What is the time scale of your molecule's interaction with the membrane?
    Should the incubation time of the leakage samples be adjusted?
11. Does your molecule cause the liposomes to adhere/flocculate/aggregate/fuse?
12. Does your molecule adsorb at the membrane surface or does it insert into the membrane?
    Or does it do both sequentially, on a time scale that can be probed with the leakage assay?
13. Does your molecule interact with both membrane leaflets or does it interact with one leaflet at a time? E.g., is it a transmembrane peptide or a surfactant that undergoes fast flip-flop in the membrane?
14. If your molecule interacts with one membrane leaflet at a time, does it translocate from

one leaflet to the other rapidly (i.e. with low energy barrier)?
How does it translocate?

15. Does your molecule form oligomers in the membrane?
Are the oligomers associated with membrane thinning?
Would the oligomers be coupled in the two leaflets?
Would a stable pore be possible?
16. Does your molecule cause curvature stress within the membrane?
What sort of packing factor does it have?

4 Quality Assurance and Troubleshooting

4.1 The Alex test

The Alex Test gauges the reliability of the fluorescence decay data, the robustness of the data analysis, and to a certain extent, the quality of the IRF. To perform this test, the fluorometer must be able to pause and resume data acquisition on command.

Two samples with the same lipid concentration are required: a control sample containing only CLVs (0% leakage) and a sample "killed" by Triton X-100 or $C_{12}EO_8$ (100% leakage).

If the control sample is in the sample chamber for 50% of the total duration of data acquisition and the "killed" sample is in the sample chamber for the remaining 50%, the resulting decay trace should yield 50% leakage. The same principle applies for all other leakage values. Acquire data in increments of 10% leakage and compare the expected leakage values to the measured values. Deviations of up to 10% may be observed beyond 60% leakage. Below 60%, deviations of more than 2–3% warrant further investigation; possible causes are the instrument setup, poor IRF, and poor sample quality.

4.2 Vesicle adhesion/flocculation/aggregation/fusion

If the membrane-active substance of interest causes CLVs to flocculate or aggregate, fuse and sediment or float, the experiment cannot yield accurate leakage values. Flocculation/aggregation may only be visible to the eye 1–12 h after sample preparation. Measured decay traces yield decreasing values of $B_{\mathrm{F}} - B_{\mathrm{F}0} + Q_{\mathrm{stat}} B_{\mathrm{E}}$ with increasing concentrations of the membrane-active substance.

The CLVS may be supplemented with 1-4 mol% PEG-lipids as part of the lipid film (e.g. DSPE-PEG_{2000} Avanti polar lipids), which should prevent flocculation/aggregation/fusion [12]. It has to be ensured, that the binding of the permeabilizer to the lipid membrane is not altered by the PEG-chains and that the PEG-lipids do not solubilize the membrane into bicells. The resulting steric hindrance at the surface of the CLVs may shift the leakage profile

to higher concentrations of the membrane-active substance. Caution should be exercised when interpreting the data. Also, vesicle fusion is altered by the addition of PEGylated lipids.

4.3 Higher temperatures

For some subjects, it may be necessary to perform the leakage assay at above 30 °C. As the assay relies on the collisional self-quenching of calcein, one should be aware that increased temperatures lead to increased diffusion and hence shorter fluorescence lifetimes. As the diluted (leaked) calcein molecules outside the liposomes collide relatively infrequently, higher temperatures have little effect on the corresponding lifetime (τ_0) of 4 ns. However, the lifetime of the concentrated calcein inside the liposomes may become shorter than 0.4 ns. Additional effects include increases in solvent interactions at higher temperatures and changes in buffer pH.

If enough lipid is available, consider loading the liposomes with different concentrations of the calcein buffer and checking the resulting fluorescence lifetimes against the Stern-Volmer equation

$$\tau = \frac{\tau_0}{(1 + K_\mathrm{D} \cdot c_\mathrm{calcein})} \ ,$$

where τ is the fluorescence lifetime measured at a particular calcein concentration c_calcein, τ_0 is the fluorescence lifetime at infinite dilution, and K_D is the Stern-Volmer constant of the sample. τ_0 and K_D are obtained by fitting τ vs. c_calcein.

5 Selected examples

Lipopeptides
Surfactins [9, 13]
Fengycins [9, 13, 14]
Iturins [13]
Serenade mix [13]
Pseudodesmin A [15]

Detergents
$C_{12}EO_8$ [1, 16]
Octyl-glucoside [9, 10, 16]
CHAPS [9]
Digitonin [17]
n-dodecyl b-D-maltoside [16]

Cationic polymers
Nylon-3 polymers: poly-NM, polyMM, poly-MM:CO [6, 10, 18]

antimicrobial polymers: propyl, ethyl, diamine smAMP [4]

Synthetic peptides
Magainin 2 [9]
Multifunctional, pH-triggered C8 [19]

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