

Dye Release Experiments with Dextran Loaded Vesicles

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[Abstract] Dye release experiments are a widely used method to assess the interactions between membrane-active molecules and lipid membranes. Of particular interest is the ability to assess the degree of the lipid bilayer perturbation by simultaneously encapsulating dye of different sizes, such as dextrans grafted with a chromophore. In this assay, dextran linked to rhodamine or fluorescein are both encapsulated in lipid vesicles to allow quantifying the leakage of each dextran individually from a single sample. For instance, the size evaluation of the lipid pore formed by an antimicrobial peptide has been recently achieved using this protocol (Sani *et al.*, 2013).

Materials and Reagents

1. Rhodamine-dextran (RD) of different molecular weights [for instance 40 kDa RD molecular weight (RD-40)]
2. Fluorescein-dextran (FD) of different molecular weights [for instance 4.4 kDa FD (FD-4)]
3. Tris-HCl
4. NaCl
5. MQ-water
6. Triton-X100
7. Peptide of interest *e.g.* Maculatin 1.1 (Sani *et al.*, 2013)
8. Lipids of interest *e.g.* 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol (TOCL) (Sani *et al.*, 2013)

Equipment

1. Polycarbonate membrane filters (200 nm diameter)
2. Extruder (for instance Avanti Mini-extruder, Alabaster)
3. Bench centrifuge

4. Spectrofluorimeter and quartz cuvette

Procedure

A. Stock preparation

1. Prepare a Tris:HCl + X NaCl buffer (buffer A) and a Tris:HCl + (X-Dextran concentration) NaCl buffer (buffer B) at desired pH, with X being the NaCl concentration.
2. Prepare a peptide stock solution (The concentration will depend on the peptide solubility and the required lipid to peptide molar ratio under investigation.) in buffer A. Glass vials are recommended.
3. Prepare a stock solution of equimolar RD and FD in buffer B.
4. Co-solubilize lipids in chloroform/methanol (3:1 v/v), remove the organic solvents under vacuum using a rotary evaporator. Rehydrate the lipids in Milli-Q water and lyophilize.
5. Resuspend the require mass of fluffy lipid powder with the dextran stock solution. A 15 mM lipid stock solution usually allows practical dilution to reach required lipid to peptide molar ratio, but will be dependent on the peptide stock concentration. Perform 3-5 freeze-thaw cycles to homogenise the liposome dispersion, usually without a time delay between cycles, by dipping the solution in liquid nitrogen and then melting above the lipid fluid phase transition for ~ 5 min.
6. Extrude 10 times through an Extruder using 0.2 mm pore size polycarbonate filters to produce large unilamellar vesicles (LUV) of 200 nm diameter. Extrusion must be performed above the gel-to-fluid lipid phase transition to avoid vesicle aggregation or demixing of heterogeneous lipid compositions, if used.
7. Remove un-encapsulated dye from LUV dispersions by centrifuging three times at 22,000 x g for 30 min at 25 °C. After each centrifugation, remove the supernatant and replace with an equal volume of fresh buffer B solution and mixed gently.
8. Determine the lipid concentration of washed LUV dispersions in triplicate using the phosphorus assay of Sani *et al.* (2013).

B. Sample preparation

1. Equilibrate the LUV dispersion and peptide solution at the desired temperature.
2. Depending on fluorimeter performance, prepare an adequate dextran-encapsulated solution, ~ 200 μ M gives a high signal in our experience.
3. Add the require amount of peptide to obtain the lipid to peptide molar ratio (L/P) of interest. For reproducibility and statistical analysis, it is best to prepare the sample in triplicate.

4. Produce negative control by adding buffer B instead of the peptide solution. A non-membrane active peptide or protein, such as bovine serum albumin, can also be used.
5. Produce positive control by adding 0.5% Triton X-100 (Tx).
6. Incubate the samples for 30 min at the desired temperature.
7. Centrifuge the samples at 22,000 x g for 30 min.
8. Recover the supernatant, with care to leave the pellet unperturbed. Usually, 500 µl sample are prepared and 300 µl recovered for analysis.
9. Transfer the supernatant to a quartz cuvette.

C. Fluorescence measurement

1. Record the fluorescence emission of RD (then FD) using an excitation wavelength set at 550 nm (then 480 nm). Fluorescence emission is recorded from 560 to 650 nm using adequate integration time and PMT voltage. Triplicate the measurements.
2. The amount of fluorescence is determined by integrating the area under the emission curve. The % release of each sample is calculated by using the following equation:

$$\%_{Dye\ Release} = 100 \times (I - I_0) / (I_{max} - I_0)$$
where, I is the fluorescence intensity of peptide treated vesicle supernatant, I₀ obtained from the peptide-free sample (negative control) supernatant, and I_{max} from the Tx treated supernatant (positive control).

Acknowledgments

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References

1. Anderson, R. L. and Davis, S. (1982). [An organic phosphorus assay which avoids the use of hazardous perchloric acid](#). *Clinica Chimica Acta* 121(1): 111-116.
2. Sani, M. A., Whitwell, T. C., Gehman, J. D., Robins-Browne, R. M., Pantarat, N., Attard, T. J., Reynolds, E. C., O'Brien-Simpson, N. M. and Separovic, F. (2013). [Maculatin 1.1 disrupts *Staphylococcus aureus* lipid membranes via a pore mechanism](#). *Antimicrob Agents Chemother* 57(8): 3593-3600.