Video Article

Measuring Peptide Translocation into Large Unilamellar Vesicles

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Abstract

There is an active interest in peptides that readily cross cell membranes without the assistance of cell membrane receptors[1]. Many of these are referred to as cell-penetrating peptides, which are frequently noted for their potential as drug delivery vectors[1-3]. Moreover, there is increasing interest in antimicrobial peptides that operate via non-membrane lytic mechanisms[4,5], particularly those that cross bacterial membranes without causing cell lysis and kill cells by interfering with intracellular processes[6,7]. In fact, authors have increasingly pointed out the relationship between cell-penetrating and antimicrobial peptides[1,8]. A firm understanding of the process of membrane translocation and the relationship between peptide structure and its ability to translocate requires effective, reproducible assays for translocation. Several groups have proposed methods to measure translocation into large unilamellar lipid vesicles (LUVs)[9-13]. LUVs serve as useful models for bacterial and eukaryotic cell membranes and are frequently used in peptide fluorescent studies[14,15]. Here, we describe our application of the method first developed by Matsuzaki and co-workers to consider antimicrobial peptides, such as magainin and buforin II[16,17]. In addition to providing our protocol for this method, we also present a straightforward approach to data analysis that quantifies translocation ability using this assay. The advantages of this translocation assay compared to others are that it has the potential to provide information about the rate of membrane translocation and does not require the addition of a fluorescent label, which can alter peptide properties[18], to tryptophan-containing peptides. Briefly, translocation ability into lipid vesicles is measured as a function of the Foster Resonance Energy Transfer (FRET) between native tryptophan residues and dansyl phosphatidylethanolamine when proteins are associated with the external LUV membrane (Figure 1). Cell-penetrating peptides are cleaved as they encounter uninhibited trypsin encapsulated with the LUVs, leading to disassociation from the LUV membrane and a drop in FRET signal. The drop in FRET signal observed for a translocating peptide is significantly greater than that observed for the same peptide when the LUVs contain both trypsin and trypsin inhibitor, or when a peptide that does not spontaneously cross lipid membranes is exposed to trypsin-containing LUVs. This change in fluorescence provides a direct quantification of peptide translocation over time.

Video Link

The video component of this article can be found at https://www.jove.com/video/3571/

Protocol

1. Preparation of Large Unilamellar Lipid Vesicles (LUVs)

1. Prepare LUVs to serve as cell membrane mimics for the assay[19].
2. Mix phosphatidylcholine (POPC, 760.10 g/mol), phosphatidylglycerol (POPG. 770.99 g/mol), 5-dimethylaminonaphthalene-1-sulfonyl phosphatidylethanolamine (DNS-POPE, 994.350 g/mol) (Avanti Polar Lipids) dissolved in chloroform in a 50:45:5 ratio. As every assay requires the separate preparation of both experimental and control LUVs, two lipid cake vials should be prepared. 0.376 mg of total lipid (0.188 mg POPC, 0.169 mg POPG, and 0.019 mg DNS-POPE) is usually sufficient to make enough vesicles for a single translocation trial.
3. Evaporate off chloroform using an N_2 gas stream.
4. Dry the lipid cake 9-14 hours in a vacuum desiccator.
5. Prepare a stock 10 mM HEPES buffer solution (10 mM HEPES, 238.3 g/mol, 45 mM NaCl, 58.44 g/mol, 1 mM EDTA, 372.24 g/mol pH 7.4). HEPES buffer is stored at 4°C.
6. Prepare a stock solution of 0.4 mM porcine trypsin (23.3 kg/mol) in 10 mM HEPES buffer prepared above. Trypsin is stored at -20°C and, in our practice, usually does not undergo more than 2 freeze-thaw cycles before use.
7. Prepare a stock solution of 4.0 mM Bowman-Birk trypsin inhibitor (8 kg/mol) in 10 mM HEPES buffer solution and store at -20°C. In our use, this stock is replenished monthly and usually does not undergo more than 3 freeze/thaw cycles before it is made again.
8. Reconstitute experimental multilamellar vesicles (MLVs) in a 0.2 mM trypsin solution by adding an equal volume of 0.4 mM trypsin stock and 10 mM HEPES buffer solution to the dried lipid cake. For a single sample of 0.376 mg total lipid, a mixture of 150 µL of trypsin solution and 150 µL of HEPES buffer is sufficient.
9. Reconstitute control MLVs in a solution with 0.2 mM trypsin and 2.0 mM Bowman-Birk trypsin inhibitor by adding an equal volume of 0.4 mM trypsin stock and 4.0 mM trypsin inhibitor to the dried lipid cakes. For a single sample of 0.376 mg total lipid, a mixture of 150 µL of trypsin solution and 150 µL of trypsin inhibitor is sufficient.
10. Transfer MLV solutions from glass vials to 1.7 ml microcentrifuge tube.

11. Subject MLV solutions to 5 freeze/thaw cycles in liquid nitrogen.
12. Prepare lipid extruder (Avanti Polar Lipids) by placing two filter supports moistened in HEPES buffer on each Teflon extruder piece. Place a nuclepore track etched membrane with 0.1 µm pores (Whatman, United Kingdom) in between the two Teflon extruder pieces, and place the extruder pieces inside the metal extruder canister. Place the donut spacer over the two extruder pieces before tightening the metal top of the extruder canister and placing it in the holder.
13. Fill the void volume within the extruder with 500 µL 10 mM HEPES buffer using a 250 µL glass syringe to prevent loss of vesicle sample.
14. Load each MLV sample into the extruder and push the sample through the membrane at least 21 times to ensure uniform unilamellar vesicle size. LUVs are obtained from the MLV solution following extrusion.
15. Store LUVs at 4°C and use within 48 hours.

2. Quantifying LUV Concentration

1. LUV concentration was quantified by determining the total phosphorus content in the sample[20].
2. Prepare 8.9 N H_2SO_4 in nanopure water and store at room temperature.
3. Prepare a 2.5% w/v ammonium molybdate VI (588.04 g/mol) solution in nanopure water. Prepare a 10% w/v ascorbic acid (176.1 g/mol) solution in nanopure water. Protect the ascorbic acid solution from light exposure by covering the tube with foil. Store both solutions at 4 °C for up to 2 months.
4. Prepare 0.65 mM phosphate buffer solution (monobasic anhydrous sodium phosphate, 120 g/mol).
5. Prepare six standard tubes. Standards should contain 0.0 µL, 50 µL (0.0325 µmoles), 100 µL (0.065 µmoles), 175 µL (0.114 µmoles), 250 µL (0.163 µmoles), or 350 µL (0.228 µmoles) of phosphorus solution.
6. Prepare LUV sample tubes in triplicate. Each sample tube should contain approximately 0.1 µmoles of LUVs. Experimental and control LUV concentrations must be measured separately.
7. To correct for any dried phosphorus salts in the Bowman-Birk inhibitor powder, a control LUV blank must be prepared. The blank should contain the same volume of the 0.2 mM trypsin/2.0 mM Bowman-Birk inhibitor solution as was placed in the control LUV sample tube.
8. Place 450 µL of 8.9 N H_2SO_4 into each of the sample, standard, and blank tubes.
9. Heat samples, standards, and blanks for 25 minutes at 175-210 °C in an oven.
10. Remove all tubes and allow them to cool. Add 150 µL of 30% H_2O_2 to each of the tubes.
11. Heat samples, standards, and blanks for 30 minutes at 175-210 °C.
12. Remove the samples, standards, and blanks from heat. If any of the solutions are not yet colorless, add 50 µL H_2O_2 to all the tubes and heat for an additional 15 minutes at 175-210 °C.
13. Add 3.9 mL nanopure H_2O, 0.5 mL ammonium molybdate solution and 0.5 mL ascorbic acid solution to each tube.
14. Heat all tubes for 5-7 minutes in a boiling water bath.
15. Measure the absorbance of each standard and sample at 820 nm using an Agilent 8453 UV-Visible spectrophotometer. The tube containing 0.0 mmoles phosphorus should be used as the blank for all standards and experimental LUV samples. The tube containing the 0.2 mM trypsin/2.0 mM Bowman-Birk inhibitor solution should be used as the blank for the control LUV sample.
16. A linear absorbance vs. phosphorus content standard curve can be produced and used to calculate the total phosphorus content and LUV concentration of the samples.

3. Preparing Peptide Solutions

1. Dissolve peptide in nanopure H_2O. Peptides used in this assay as described must contain one tryptophan residue.
2. Measure the absorbance of the peptide solution at 282 nm in triplicate. Calculate peptide concentration using the molar extinction coefficient for tryptophan of 5700 $M^{-1}cm^{-1}$.
3. Dilute peptide in nanopure water to a final concentration of 30 µM. Store at -20°C

4. Translocation Quantification

1. Prepare experimental and control samples in 1.7 mL microcentrifuge tubes. Add enough LUVs to each solution for a final concentration of 250 µM. Add enough Bowman-Birk inhibitor solution so that inhibitor is a final concentration 10X greater than the trypsin concentration. Add enough 10 mM HEPES buffer to bring the final solution volume to 450 µL.
2. Place 50 µL peptide solution into a Starna micro quartz fluorometer cell. The ratio between LUVs and peptide is high enough to ensure that virtually all intact peptides are in close enough proximity to a dansyl group to give a FRET signal even if none have translocated into the vesicle. This is consistent with the similar FRET observed in control conditions for peptides that do and do not translocate into vesicles.
3. Add the experimental or control sample to the peptide solution in the cuvette. This should bring the final peptide concentration to 3 µM.
4. Begin a 25 minute fluorescent kinetics experiment *immediately* upon addition of experimental or control LUV solution, taking a fluorescence reading at least once per second. Set the excitation wavelength at 280 nm, the emission wavelength at 525 nm, and the temperature to 25 °C. Using a Cary Eclipse Fluorescence Spectrophotometer, we set the PMT sensitivity to high.

5. Generating a Quantitative Translocation Ratio

1. Define initial fluorescence (F_o) as the fluorescence reading after fifteen seconds of data collection. In our instrument setup, we have found that the first fifteen seconds of fluorescence data collected are unreliable due to mixing, closing the sample chamber, and other perturbations to the system upon sample addition. Divide each subsequent reading by F_o to obtain relative fluorescence (F/F_0) at each time point.
2. Average all relative fluorescence readings from the last minute of the experiment to obtain a final average relative fluorescence $\left[\frac{F}{F_0}\right]_{avg}$.

3. Divide the $\left[\frac{F}{F_0}\right]_{avg}$ for control samples by the $\left[\frac{F}{F_0}\right]_{avg}$ for experimental samples to obtain a corrected final fluorescence value.

6. Representative Results

Figure 2 shows the results of this assay for a representative peptide that showed robust translocation. The signal in this experiment (black trace) shows a marked drop in FRET signal over time. However, it is important to control for the potential loss of FRET signal due to incomplete trypsin inhibition or other factors unrelated to translocation ability. To this end, we always also measure the FRET signal between peptide and LUVs containing both trypsin and Bowman-Birk trypsin inhibitor (gray trace, Figure 2). In our hands, it has been important to perform this control for every peptide every time an experiment is run. This allows us to clearly correct for any changes in signal due to any differences between lipid vesicle preparations or instrument noise, which can be significant for the relatively weak fluorescent signals typically observed at these concentrations.

The importance of the control experiment using LUVs containing trypsin inhibitor is highlighted by the data shown in Figure 3. In this case, the peptide signal decreased a similar amount to that in Figure 2 in the experimental sample (black trace). However, the control sample shows a more rapid decrease for this peptide, so its net translocation is lower.

Section 5 of our protocol describes a straightforward method for quantifying translocation from this experiment. Higher translocation ratios are indicative of peptides that translocate efficiently; the translocation ratio for the cell penetrating peptide shown in Figure 2 is 1.16, while weakly translocating peptides have translocation ratios closer to 1. In our experience, the standard error for three independent experiments performed with different vesicle preparations is in the range of 0.01 to 0.06.

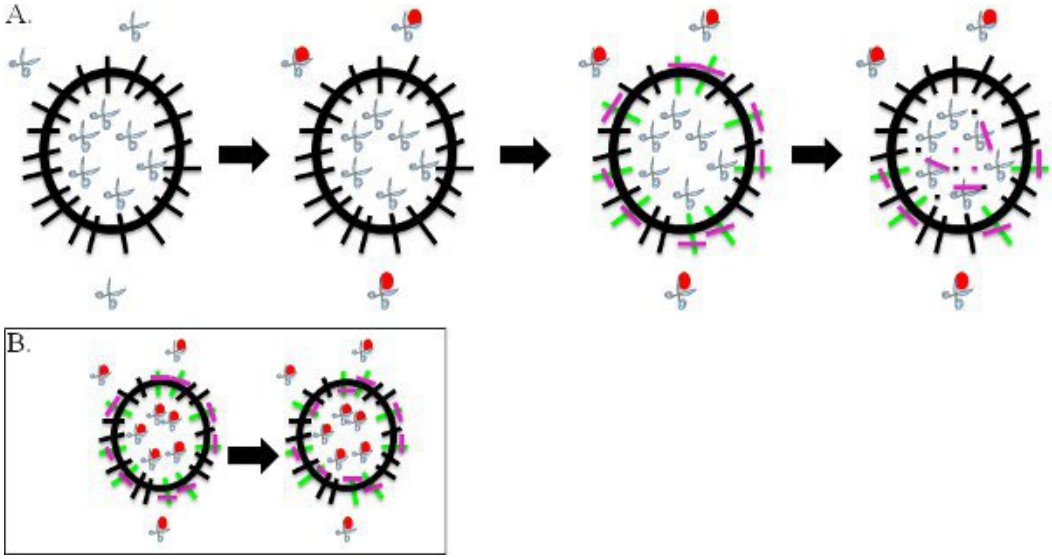

Figure 1. Schematic of translocation assay. Experimental LUV samples (**A**) are doped with fluorescent dansyl POPE (black bars) and contain encapsulated trypsin (scissors). Trypsin inhibitor (red circle) is used to inhibit trypsin outside the LUVs. The LUVs are exposed to peptide (purple). Peptide association with LUV membranes yields a FRET signal (green) that decreases as translocting peptides encounter uninhibited internal trypsin. Both trypsin and trypsin inhibitor are encapsulated in control LUV samples (**B**) to measure decreases in FRET signal unrelated to translocation.

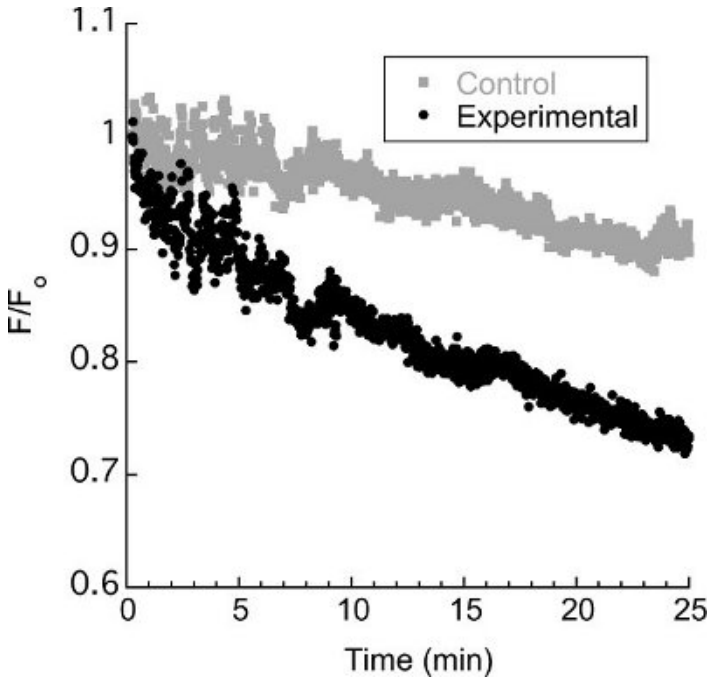

Figure 2. Representative data for a cell-penetrating peptide. A representative translocating antimicrobial pepide shows a significant drop in FRET signal compared to its control.

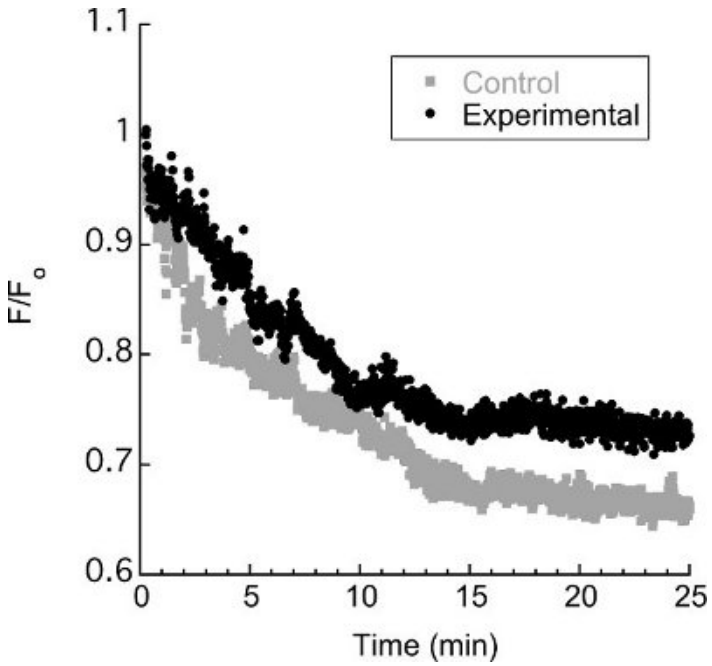

Figure 3. Representative data highlighting the importance of the control. Incomplete inhibition of tryptic digestion of a non-translocating peptide leads to a drop in FRET signal over time in both experimental and control LUV solutions. Because the difference between the control and experimental traces is relatively small, this mutant is characterized as a weakly translocating peptide.

Discussion

The protocol presented here can be used to assess the relative change in the concentration of peptides inside and outside of lipid vesicles. These changes are related to translocation ability. This protocol can be used to identify cell-penetrating peptides with potential as drug delivery vectors. As interest in cell-penetrating peptides grows, it will be interesting to see how methods that directly measure the translocation event are developed and used in a quantitative manner.

In our lab, we have found that the consistency of the assay can be improved by carefully monitoring a few particular aspects of the experiment. First, quantification of both experimental and control vesicles improves the consistency of the results obtained using this protocol. This assay also depends on the ability to detect an accurate initial fluorescence prior to the beginning of protein translocation and digestion. Therefore, it

is essential for the fluorescence signal collection to begin as soon as the protein or peptide is exposed to the LUV solution. This assay is also sensitive to the particular trypsin inhibitor used; to date, we have obtained the most consistent results with Bowman-Birk trypsin inhibitor (Sigma T-9777). Importantly, the degree of degradation observed in control scenarios seems to vary significantly between peptides (as highlighted in Figures 2 and 3) and in some cases between different vesicle preparations for the same peptide. This further emphasizes the need to run a control with each experimental replication. As an additional control, one could also measure the FRET response for peptide exposed to a vesicle sample containing neither trypsin nor trypsin inhibitor. However, this control does not provide any additional information that is necessary in order to evaluate data for peptide translocation.

One concern is that membrane-lytic peptides could permeabilize the membrane enough to allow trypsin or trypsin inhibitor to travel across the membrane. The peptides used for examples in this paper have been shown to cause little membrane permeabilization. Nonetheless, many membrane-lytic peptides also are amenable to this and similar translocation assays[9,16,21,22]. This is likely because the volume is much greater outside than inside the vesicles. Thus, any trypsin that leaks out of the vesicle can be inhibited by excess trypsin inhibitor, preventing any cleavage of peptide outside the vesicle. Thus, a positive result from this assay likely denotes a peptide that translocates while causing relatively minimal membrane disruption, preventing inhibitor from leaking into the vesicles. Regardless, a thorough characterization of peptide properties should include an assessment of membrane permeabilization.

This assay is amenable to adaptation for a wider variety of experimental circumstances. Given that translocation is measured as a function of FRET signal between protein and membrane, as described this assay is best-suited to proteins and peptides that spontaneously associate with anionic LUVs, contain one tryptophan residue and have trypsin cut sites near the tryptophan residue. The proximity of the tryptophan to a cut site ensures that the fragment containing tryptophan has a negligible membrane affinity, and thus a negligible FRET signal. However, one could also use peptides containing tyrosine or other chemically conjugated fluorescent moities along with vesicles containing an appropriate FRET acceptor. Similarly, trypsin could be replaced with another protease that targets alternative cut sites in a peptide of interest. However, altering the enzyme and inhibitor may require additional optimization since some commercially available trypsins and trypsin inhibitors led to aggregation or instability of vesicle samples. By altering the lipid composition of the LUVs, this assay may also be used to determine the role that lipid charge or structure plays in determining peptide translocation. Additionally, this assay could also be readily adapted to provide high-throughput measurements of translocation in an approach similar to that of Wimley and co-workers[9].

Disclosures

No conflicts of interest declared.

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