CHAPTER ELEVEN

Biophysical approaches to study actinoporin-lipid interactions

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Abstract

Protein-lipid interactions are crucial events from a biochemical point of view, like the interaction of proteins with the cell plasma membrane, and their study is of great importance. Actinoporins are a very powerful tool to study this kind of interactions, since they are soluble proteins in an aqueous environment, capable of inserting into membranes when they have the adequate composition. In fact, actinoporins have been used to study protein-lipid interactions for many years now. Sometimes it is not possible to use real biological membranes in the experiments, so model membranes need to be used. This article aims to give a thorough description of many of the techniques used to study actinoporin-lipid interactions, using both biological and model membranes: Hemolysis, release of vesicles content, surface plasmon resonance, isothermal titration calorimetry, fluorescence-based measurements, etc. Some of these techniques measure

the actinoporins activity and some measure their binding properties. The combination of all the techniques described can offer valuable information about the thermodynamics and the kinetics of the actinoporin-lipid interaction.

1. Introduction

Proteins can be classified based on many different principles: Folding patterns, structural domains, sequence motifs, function, shape, or enzymatic activity, to name a few. One of the most general categorizations, based on a rather broad criterion, sorts proteins according to whether they are soluble or reside at the membrane. While this method can comprise most proteins, there are some that elude this categorization. Those are the proteins that can change their partition between aqueous environments and membrane phases depending on environmental features, which would need a category of their own.

A family of proteins that can be included in that separate group are pore-forming toxins (PFTs) (Dal Peraro & van der Goot, 2016). These toxins remain stably folded in solution. That conformation is also maintained when they encounter a membrane that lacks the receptor required. However, if the specific molecule recognized by the toxin is accessible in that membrane, binding occurs, triggering a conformational change that leads, ultimately, to oligomerization and the formation of pores, driving the death of the targeted cell by osmotic shock.

Some of the best known PFTs are actinoporins. These are small, basic, single-polypeptide proteins, produced by sea anemones which specifically recognize sphingomyelin (SM) (Alegre-Cebollada, Oñaderra, Gavilanes, & Martínez-del-Pozo, 2007; Anderluh & Maček, 2002; Maček, 1992). There are four actinoporins that have been studied in detail: Sticholysins I and II (StnI and StnII) produced by *Stichodactyla helianthus*, equinatoxin II (EqtII) produced by *Actinia equina*, and fragaceatoxin C (FraC) produced by *Actinia fragacea*. The three-dimensional soluble structures of these four proteins have been solved by crystallographic and/or nuclear magnetic resonance methods (Athanasiadis, Anderluh, Maček, & Turk, 2001; García-Linares et al., 2013; Hinds, Zhang, Anderluh, Hansen, & Norton, 2002; Mancheño, Martín-Benito, Martínez-Ripoll, Gavilanes, & Hermoso, 2003; Mechaly et al., 2011; Mechaly, Bellomio, Morante, González-Mañas, & Guerin, 2009). Over the years, the pore complex of FraC could

be isolated, and its structure resolved using X-ray crystallography (Tanaka, Caaveiro, Morante, González-Mañas, & Tsumoto, 2015).

These structures have provided great insight for those of us investigating actinoporin behavior. However, they only represent the initial and final states of the process. And even if, in principle, most of the information regarding the process of pore formation is contained in their structure, it cannot be inferred completely directly and solely from the structures, since other aspects, especially lipids, play an essential role in this process. That is to say, the action mechanism of these proteins cannot be understood without studying their membrane interactions.

Therefore, understanding the behavior of these toxins demands a thorough characterization of their interaction with the membranes. This is accomplished with the combination of a great variety of methods, of which we take advantage of their respective strengths. The use of this assortment of techniques is essential, since each one reveals only a few aspects of the interaction. It is only when put together that the results obtained really become meaningful.

The first step to characterize actinoporin-lipid interactions is, obviously, having availability of such protein, purified to homogeneity. This process shall not be covered in this chapter. Nevertheless, the reader is encouraged to consult the following references (Alegre-Cebollada et al., 2007; Anderluh, Pungerear, Štrukelj, Maček, & Gubenšek, 1996; Bellomio et al., 2009). Next, the membranes that the toxin of interest interacts with need to be prepared. This is covered in the section regarding vesicle preparation if model membranes are to be used. This process, quite straight forward, is a common place for most of the subsequent techniques, sometimes requiring some experiment-specific adjustments. In case the activity of actinoporins is to be tested with biological membranes, erythrocytes are usually chosen, due to their easy availability. Preparation of the erythrocytes is covered in the section regarding hemolysis assays.

As for the assays, they can be classified in two categories: those that report on the activity of the toxins, and those that do not. It should be noted that, when it comes to actinoporins, and PFTs in general, the term activity refers to their capacity to form pores and induce cell/vesicle damage. No enzymatic activity is carried out by these toxins, i.e., pore formation entails no change whatsoever on the covalent bonds in neither of the intervening parts and the toxins are nor recovered after pore formation to damage other suitable target membranes.

The usual activity experiments performed with actinoporins are the hemolysis and the aqueous contents release assays. Other procedures reported in this article give information about the actinoporins binding capability to membranes. The information provided by all other most commonly performed approaches does not directly inform about the activity. In some cases, though, the results can correlate since the parameters reported do affect the activity.

There are two techniques that can be used to obtain information regarding the overall process of pore formation. On the one hand, surface plasmon resonance (SPR) can be used, in principle, to measure the affinity of a toxin for a given membrane composition. Nevertheless, this is often not possible with actinoporins since their binding to membranes is largely irreversible. Still, SPR can be used to compare different membrane compositions in a qualitative manner. If the mass of vesicles attached to the chip is registered, one can also estimate how many lipids per bound protein are for each combination. On the other hand, isothermal titration calorimetry (ITC) can provide a complete thermodynamic characterization of the interaction between a selected actinoporin and the chosen membrane composition, including the affinity constant, and other thermodynamic data such as the enthalpy of the interaction.

Finally, a series of fluorescence experiments can be used to gather information regarding details of the interaction between the toxins and specific lipid species. The intrinsic fluorescence of the proteins, and the use of specific labels and fluorescent lipid analogs can provide great insight regarding specific interactions, protein oligomerization, and the effects toxin binding can have on the distribution of a given lipid in the membrane.

2. Hemolysis

It is common to use erythrocytes as a substrate to measure actinoporin's activity. Red blood cells can be easily obtained from a wide variety of animal sources and are stably stored at 4 °C. Each species shows a specific lipid composition. However, they share common features that make them suitable to study actinoporins' activity: The presence of sphingomyelin and a high cholesterol content (around 26%) (Nelson, 1967). One should be careful, nevertheless, since some animal red blood cells have a remarkably low SM content. Eventually, they can be used as controls as well. Actinoporins form pores in the cell membrane of erythrocytes, leading to cell burst by osmotic shock. It is therefore possible to measure actinoporins'

activity by following the turbidity decrease of a red blood cell suspension upon interaction with actinoporin solutions.

2.1 Materials and equipment

- Buffer: 10 mM Tris–HCl, pH 7.4, 145 mM NaCl
- 0.1% (*w*/*v*) Na_2CO_3
- Defibrinated/heparinized blood
- Multichannel pipettor
- 96-multiwell plate
- Centrifuge suitable for standard 15 mL tube centrifugation
- 96-multiwell plate reader equipped with 620–655 nm filter

2.2 Step-by-step method details

2.2.1 Preparation of erythrocytes suspensions

Timing: 1 h

This step is intended to remove plasma proteins. Actinoporins could avidly bind to lipoproteins.

1. 10 mL of blood are centrifuged at 700 × *g* for 20 min at 25 °C
2. Supernatant is carefully discarded with a Pasteur pipette, avoiding disturbing the sediment. Then, the sediment is gently resuspended in Tris–HCl, NaCl buffer by inverting the tube. Cells are washed by centrifugation, again, at 700 × *g* for 10 min
3. Repeat step 2 at least four more times or until the supernatant is clear
4. To ensure that the suspension's turbidity is within the sensitivity range of the 96-multiwell plate reader, it is necessary to test erythrocyte dilutions between 1/10 to 1/100 dilution range, in identical conditions as the ones that will be used in the assay: 100 μL of erythrocytes suspension dilution mixed with 100 μL of buffer (that will be substituted by protein solution in the assay) have to display an optical density value at 620–655 nm lower than 1.0
5. Washed erythrocytes can be used for hemolysis assays within 24 h, although it is recommendable to check suspension dilution every 8 h

Note: While resuspending sediment or diluting erythrocytes it is not recommended to shake or hit the tube, since erythrocytes would burst due to mechanical stress. It is possible that, when blood is stored in the fridge for a long time, erythrocytes need more washes since they become more fragile because of aging.

2.2.2 Hemolysis assay

Timing: 50 min/plate

1. Twofold serial dilutions are prepared in 96-multiwell plates. This format allows for assaying two proteins with triplicates or three proteins in duplicates simultaneously, which is especially useful when comparing different wild-type or modified versions of the protein. The maximum concentration of protein employed will depend on the specific toxin used. A good orientation is the already determined HC_{50} value for well-known actinoporins (García-Linares et al., 2016). The final volume on each well should be 100 μL
2. Every plate must include a control for 0% hemolysis, obtained with 100 μL of buffer that will allow to monitor spontaneous hemolysis during the kinetic assay and a control for 100% hemolysis, obtained with 100 μL of 0.1% (*w*/*v*) Na_2CO_3
3. Upon addition of 100 μL of erythrocytes dilution to each well, the $OD_{620–655}$ is registered every 20 s for 20 min

Note: avoid shaking steps when measuring.

2.2.3 Hemolysis assay to test synergy

Actinoporins appear as multigene families in the sea anemone genomes. Typically, a single sea anemone species has a battery of independent genes encoding highly similar actinoporins, each displaying different pore-forming activity (García-Linares et al., 2016). It has been previously described that some of these actinoporins can act in synergy when mixed, displaying higher activity than expected if both isoforms would act independently. To test synergy between two actinoporin isoforms using a hemolysis assay, it is necessary to test the relative concentration of each isoform separately and in combination within the same plate. For example, when testing a final concentration of 10 nM, at a 20%–80% proportion, it is necessary to assay 2 and 8 nM of the isoforms representing the 20% and 80% fractions of the combined sample, respectively. Such strategy allows to compare experimental kinetic values with the theoretical activity obtained by the simple addition of the activity values displayed by the relative concentrations of the isolated proteins.

2.3 Expected outcomes

For each concentration assayed, it will be possible to build a kinetic curve by representing the percentage of hemolysis as a function of time (Fig. 1A).

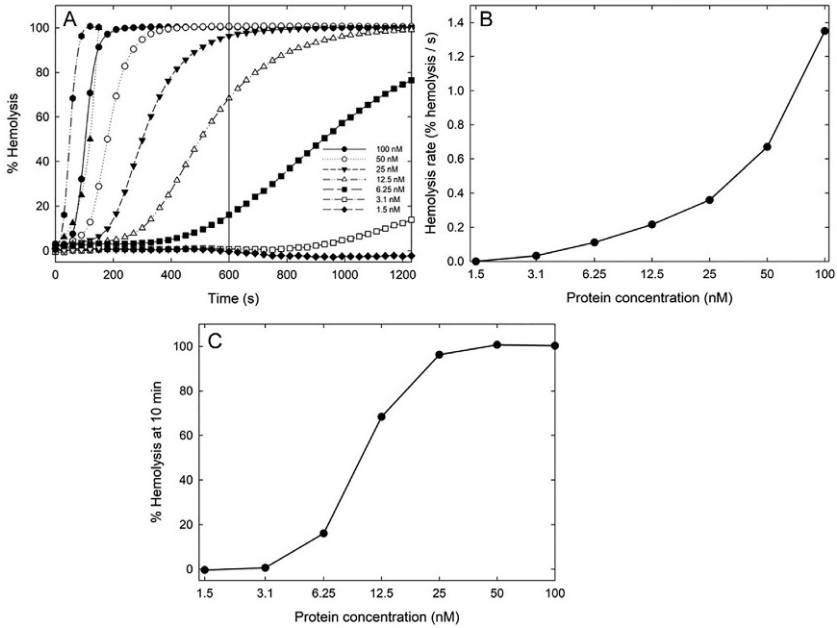

Fig. 1 *Hemolysis*. (A) Curves represent hemolytic activity exerted by a range of concentration of the actinoporin StnI. (B) and (C) are dose-response curves built from graph A data. (B) represents the maximum hemolytic rate values (expressed as percentage of hemolysis/s) vs the logarithm of total protein concentration. (C) represents the % of hemolysis reached after 10 min of StnI addition (vertical line in (A) indicates this time).

From each %hemolysis/time curve, the initial hemolytic rate is calculated as the slope of the linear increment range of the graph (Fig. 1B). In addition, it is also possible to determine the maximum hemolysis obtained for each protein concentration after 10 min (Fig. 1C). Both approximations allow for the construction of dose-response curves, enabling for different subsequent interpretations.

2.4 Quantification and analysis

Initial hemolytic rate curves allow for the evaluation of the activity in terms of binding and pore formation steps, while maximum hemolysis values are related to the number of pores that can be formed and are therefore related to the stoichiometry.

Every experiment should be tested at least by duplicate on each plate and it should be repeated three times with different blood batches.

2.5 Advantages

Hemolysis assays are simple, cheap, accessible, and versatile experiments that allow for a fast interpretation of actinoporins' activity.

2.6 Limitations

Unlike model vesicles, that have a well-defined composition, erythrocytes are complex membranes that, while better mimicking physiological activity of actinoporins, yield results which are also more difficult to interpret.

3. Preparation of vesicles

The preparation of vesicles is a common procedure for many of the experiments discussed in this article and for protein-lipid interactions studies in general. This section will cover the main aspects of the vesicles preparation process to obtain a population of mainly large unilamellar vesicles of a certain diameter.

3.1 Materials and equipment

- Lipids
- Methanol or chloroform:methanol 2:1 (*v*/v) solution
- System for drying lipids under nitrogen flow (it is ideal to also have a separate system that can generate vacuum to ensure complete removal of the organic solvent)
- Graduated glass syringes
- Disposable glass tubes
- Vesicle hydration buffer: Tris–HCl, NaCl
- Water bath
- Vortexer
- Mini extruder
- Polycarbonate filters (Whatman - Millipore), typically with diameters between 100 and 200 nm

3.2 Step-by-step method details

3.2.1 Preparation of lipid films

Timing: 30 min to 2 h

The commercial presentation of lipids is usually a solid powder, although it is possible to purchase them already dissolved in organic solvents such us

methanol or mixtures containing chloroform. It is *extremely important* to consider that most plastic consumables in the laboratory can be affected by organic solvents. Thus, to prepare lipid films it is necessary to work with glass graduated pipettes and disposable glass tubes. Methanol or chloroform: methanol (2:1) solvents are used to dissolve pure lipids at a known concentration. Lipids can be then mixed to obtain the desired lipid mixture for the assay, in the wished molar ratio. Typical mixtures used when assaying actinoporins are PC:SM:Chol (1:1:1), PC:SM:Chol (80:20:10), and PC:SM (80:20), from 0.5 to 10 mM.

The lipid solutions are dried out under a stream of nitrogen. Ideally, vacuum is applied to the dried films for 2–3 h to ensure the evaporation of all the organic solvent. Any solvent left in the film might affect the formation and behavior of vesicles or damage the extrusion filter. Once the solvent is completely evaporated, aliquots might be gassed with nitrogen or argon to avoid oxidation of certain lipids, like polyunsaturated fatty acids, which are especially prone to oxidation. Dried lipid films aliquots can be stored for long term at −20 °C until use.

3.2.2 Preparation of large unilamellar vesicles (LUVs) from lipid films

1. Lipid films are hydrated with the amount of hydration buffer necessary to achieve the desired final lipid concentration. This will be the buffer that will define the inner content of the vesicles
2. The mixture is vortexed intensely. The lipid films, in contact with an aqueous solution, will form multilamellar vesicles (MLVs). The suspension is then hydrated at a temperature above the melting temperature of the lipid mixture for 30 min to 1 h. It is recommendable to vortex every 10 min to ensure the homogeneity of the suspension
3. The MLVs preparation is intensely vortexed one more time, and then the suspension is passed through a 100 or 200 nm polypropylene filter with a mini extruder device 15 times, to ensure a homogeneous population of LUVs. An odd number of passes are used to retain any large contaminant on one side of the extruder

3.3 Expected outcomes

After extrusion, you will end up with a vesicle preparation in which the majority of the particles will be large unilamellar vesicles of the specific diameter you use to extrude them.

3.4 Quantification and analysis

Take aliquots of your LUV preparation after it has been extruded to determine the actual concentration of lipids by means of phosphorus quantification methods like (Bartlett, 1959) or (Rouser, Fkeischer, & Yamamoto, 1970). This calculation will not be possible if the buffer used was phosphate based, such as PBS.

3.5 Advantages

This technique allows to prepare lipid vesicles of the desire composition and obtain a large population of the specific size we need.

3.6 Limitations

A small proportion of the vesicles may still have a different diameter or be multilamellar. Homogeneity of the LUVs can be checked by means of laser scattering measurements, for example.

4. Release of aqueous contents from model lipid vesicles

Actinoporins bind to SM-containing membranes forming stable pores that are selectively permeable to some cations. According to electrophysiology studies and leakage of PEGs of different molecular mass, the hydrodynamic size of the pore ranges between 1 and 2 nm (Casallanovo et al., 2006; Tejuca, Serra, Ferreras, Lanio, & Menestrina, 1996).

In comparison with hemolysis assays, leakage of soluble contents from model vesicles allows controlling lipid composition while also giving insight of the primary steps of pore formation. Release of aqueous contents from model lipid vesicles is a standard procedure that has been used to evaluate pore-formation efficiency of actinoporins. However, regardless of the probe of choice, actinoporins action does not empty vesicles completely, at least with the probes so far employed. Calcein and carboxifluorescein have been for decades the standard ones in the field (Alm, García-Linares, Gavilanes, Martínez-del-Pozo, & Slotte, 2015; Carretero et al., 2018; García-Linares et al., 2015, 2016; Maula et al., 2013; Palacios-Ortega et al., 2019; Rivera-de-Torre et al., 2016; Ros et al., 2019). However, recent studies remarked that both fluorescent probes were indeed negatively charged

and too big to leak through the pore (Palacios-Ortega, Rivera-de-Torre, Gavilanes, Slotte, & Martínez-del-Pozo, 2020). This same study revealed that positively charge fluorescent probes such as rhodamine 6G, though still too big, seem to be a better choice for performing this type of experiments. Irrespective of the probe chosen, once the final pores are stably formed on the membrane, the release through the pore seems impossible, since they are too small for the probes. It is for this reason that leakage of fluorescent probes, which are generally larger than the pore of actinoporins, report transient permeation of the membrane as consequence of the assembly into the final oligomeric pore structures, rather than the passage of solutes through the stably assembled pores. Leakage assays demonstrate then that, besides the proper pore, the first stages of pore-formation would inflict serious damages to living cells as well, thus being an important feature to study about the activity of actinoporins.

4.1 Materials and equipment

- Material and chemicals for LUVs preparation (see Section 3)
- Vesicle hydration buffer: Typically, 10 mM Tris–HCl, pH 7.4, 140 mM NaCl
- Actinoporins (sticholysins)
- Fluorescent probe, such as calcein or rhodamine 6G
- Sephacryl S200HR (Sigma-Merck)
- Triton X-100 10% (*v*/v)
- $C_{12}E_8$ (octaethylene glycol monododecyl ether) 10% (v/v)
- Liquid nitrogen
- Empty Poly-Prep chromatography columns (BioRad)
- Fluorimeter
- Fluorescence quartz cuvette

4.2 Step-by-step method details

4.2.1 Calcein

If calcein is dissolved at high enough concentrations, it becomes self-quenched. Hence, its fluorescence emission is negligible when encapsulated in liposomes at such concentrations. Release of calcein from LUVs leads to a dramatic increase in fluorescence emission due to the dilution of the probe in the total volume of the spectrophotometric cell. Fluorescence emission is proportional to the fraction of dye leaked from the model vesicles.

Preparation of calcein loaded vesicles

Timing: 2 h

- 250–500 nmol lipid films are hydrated with 200 μL of 100 mM calcein dissolved in Tris–HCl, NaCl hydration buffer
- After hydration, and before extrusion, the MLVs are subjected to 10 freezing/thawing cycles with liquid nitrogen to improve encapsulation efficiency. Thawing is done at the same temperature as hydration
- Once extruded, LUVs are separated from non-entrapped calcein by gel filtration on Sephacryl S200HR. Briefly, plastic columns are filled with the resin and equilibrated with the vesicle hydration buffer. Then, the 200 μL LUVs suspension is applied in the column, 1 mL of hydration buffer is added, and vesicles are eluted by centrifugation in that unique 1 mL elution fraction
- Calcein-loaded vesicles are used for permeabilization studies within 8 h from preparation

Calcein leakage assay

Timing: 10 min/assay

- Lipid concentration during the assay should be about 2.5 μM
- Emission is recorded at 550 nm as a function of time, using excitation at 480 nm. Temperature must be constantly controlled. Typically, experiments are performed at a fixed temperature between 23 °C and 25 °C, depending of the lipid composition of the vesicles used
- To ensure that non-spontaneous leakage does not take place, fluorescence emission should be recorded for a few minutes before toxin addition. A steady signal should be obtained for stable vesicles
- Protein is then added in a volume less than 2% of the total volume of the cuvette to avoid dilution effects. Different concentrations (typically ranging from 1 to 100 nM) should be tested to build dose-response curves in terms of calcein leakage rates
- Once the signal has reached a steady level after the addition of the toxin and has maintained it for some time, 10 μL of 10% (*v*/v) Triton X-100 is added to the cuvette to induce LUVs disintegration. The fluorescence value obtained after total release of the probe makes possible to calculate the fraction of calcein released by action of the toxin. The following expression is used to that end:

$$\%\ \text{leakage} = \frac{(F_{obs}(t) - F_0)}{(F_{max} - F_0)} \times 100$$

where F_{obs} (t) is the emission at a given time, F_0 is the basal emission prior to protein addition, and F_{max} is the maximum emission value upon lysis of the vesicles by the addition of the detergent.

4.2.2 Rhodamine 6G (R6G)

R6G, in contrast with calcein, is a cationic probe. Therefore, it is a more suitable molecule to test leakage upon actinoporins' action, since they form cation-selective pores (Varanda & Finkelstein, 1980). R6G release rates are faster and much more sensitive than any other tested probe, including calcein. Therefore, charge is a critical factor to measure permeation, even considering that the probes do not leak through the actual final stable pores. Consequently, given that neither calcein nor R6G would have the right size to cross the pore lumen, the final leakage value reaches a plateau below 100%. Nevertheless, the faster release of R6G suggests that it can escape from vesicles easier than calcein through transient pores because of its positive charge (Palacios-Ortega et al., 2020).

Preparation of R6G loaded vesicles

Timing: 2 h

- R6G is dissolved in distilled water to saturation and then a five times concentrated version of the hydration buffer is added to the R6G solution. Insoluble fraction of the probe is discarded by centrifugation. The approximately concentration of R6G in the hydration buffer should be 5 mM
- R6G loaded vesicles are then prepared as detailed for calcein loaded vesicles

R6G leakage assay

Timing: 10 min/assay

- Lipid concentration during the assay should be around 2.5 μM as in calcein assays
- Emission is followed at 555 nm as a function of time upon excitation at 525 nm. Temperature must be constantly controlled between 23 °C and 25 °C
- As in calcein assays it is necessary to control spontaneous leakage before adding different protein concentrations
- Once the signal is steady after the addition of the lytic protein, 10 μL of 10% (*v*/v) $C_{12}E_8$ is added to the cuvette to induce the disintegration of LUVs. Triton X-100 should not be used for R6G assays since it quenches the probe. Percentage of released probe is calculated as described above

4.3 Expected outcomes

For each concentration assayed, it will be possible to build a kinetic curve by representing the percentage of leakage as a function of time (Fig. 2A). From each %leakage/time curve, the initial lytic rate is calculated as the slope of the linear increment range of the graph (Fig. 2B). Experimental data can be fitted in a model for peptide-induced membrane perturbation defined by (Andersson, Danielsson, Gräslund, & Mäler, 2007):

$$\frac{F_{obs}(t)}{F_{max}} = 1 - \exp\left(J_2\left(e^{-t \cdot v_{relax}} - 1\right)\right)$$

The presented model describes release curves resulting from transient perturbations affecting the membrane, where F_{obs} (t) is the fluorescence registered at a certain time and F_{max} is the maximum fluorescence registered in

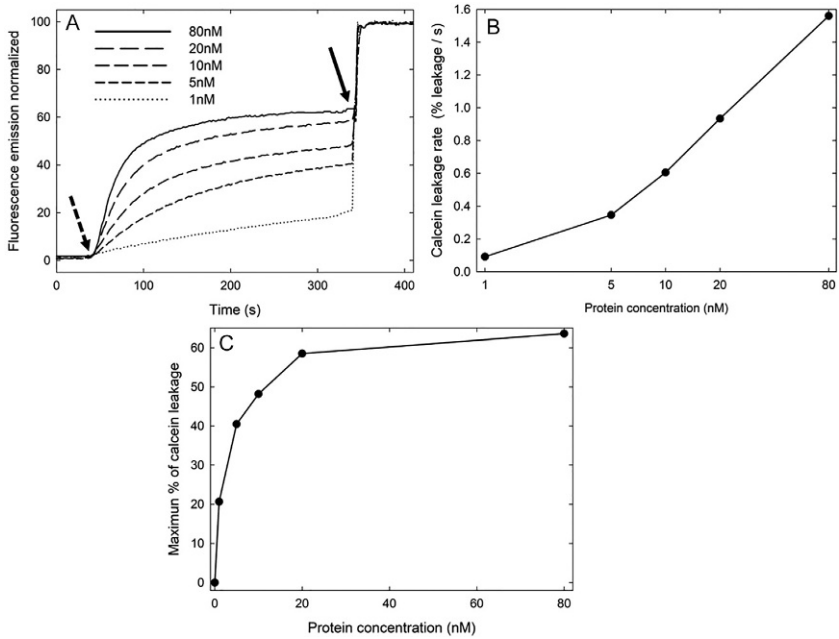

Fig. 2 *Calcein leakage*. (A) Curves represent calcein release activity exerted by a range of concentration of the actinoporin StnI from DOPC:SM:Chol (1:1:1) model vesicles. Dashed arrow indicates the addition of the lytic protein and solid arrow indicates de addition of detergent to lysate vesicles. (B) and (C) are dose-response curves built from graph A data. (B) represents the maximum calcein release rate values (expressed as percentage of leakage/s) vs the logarithm of total protein concentration. (C) represents the maximum steady percentage of leakage reached before adding detergent to obtain 100% release control.

the system after addition of detergent. Once the system is stable and there are no more perturbations, the leak ceases. The model has two parameters that defines the behavior of the system. J_2 is proportional to the magnitude of the perturbations and if the model fits the experimental data, it can be calculated as:

$$J_2 = -\ln(1 - L_{max})$$

where L_{max} is the value of the final, maximum release induced by the toxin. The term v_{relax} is related to the duration of the perturbations. The product $J_2 \cdot v_{relax}$ is equal to the slope of the release curves at $t = 0$. In addition, it is also possible to determine the maximum release rate obtained for each protein concentration after as the steady value obtained before the addition of the detergent (Fig. 2C). Both approximations allow for the construction of dose-response curves that lead to different interpretations.

4.4 Quantification and analysis

Due to their particular features, actinoporins are not able to induce 100% leakage from vesicles. The size of the actinoporin pore does not enable the pass of the probe through the lumen, as explained. Therefore, it is usually considered that final release values are not highly representative of the process of pore formation. Instead, it is a common practice to calculate dose-response curves in terms of leakage rate to evaluate the permeabilization activity of these proteins.

Every experiment should be tested at least by duplicate with different LUVs batches.

4.5 Advantages

Release of soluble contents from vesicles is an easy way to test actinoporins' activity in terms of permeabilization due to membrane disruption/perturbations prior to the formation of the final stable pores (once the pores are formed, the probe is too large to escape through them and leakage reaches a plateau). The development and interpretation of the results are usually simple, and the use of model vesicles allows to select the specific composition desired to test the activity.

4.6 Limitations

Release of the soluble probes presented does not account for leakage through the stable pores.

5. Surface plasmon resonance (SPR)

Surface plasmon resonance is a highly sensitive and label-free method used to study surface changes and it is a well-established method utilized to measure binding affinity and kinetics. Lipid vesicle layers are spread on a sensor surface and actinoporins interaction on lipids is determined by measuring the change of the angle of reflection of an incident beam due to the adsorption of actinoporins to the vesicle layer.

5.1 Materials and equipment

- Material and chemicals for LUVs preparation (see Section 3)
- Buffer: 10 mM Tris–HCl, pH 7.4, 140 mM NaCl
- Binding agent: Actinoporins (sticholysins) or membrane binding agent (MBA) of interest
- Detergents: 3-[(3-Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS)
- Bovine serum albumin (BSA)
- NaOH
- Isopropanol
- Bath sonicator
- SPR instrument (BioNavis)
- Sensor gold chip coated with a carboxymethylated dextran layer treated with N-hydroxysuccinimide and N-ethyl-N′-(dimethylaminopropyl)-carbodiimide (Löfås & Johnsson, 1990)

5.2 Step-by-step method details

Timing: 50 min; LUVs preparation, 1 h

1. Filter and degas by sonication (using an ultrasonic bath) the Tris–HCl, NaCl buffer
2. Prepare:
   50 mM NaOH in Tris–HCl, NaCl buffer
   50 mM NaOH:isopropanol (2,3)
   0.1 mg/mL BSA in Tris–HCl, NaCl buffer
   10 mM CHAPS in filtered and degassed water
   4 μM actinoporin in Tris–HCl, NaCl buffer
   0.5 mM LUVs of the desired composition (see Section 3)

3. Degas the protein preparation by centrifugation (12,000 g, 5 min, 25 °C).
4. Place the carboxymethyl dextran coated sensor chip in the instrument
5. Clean the sensor chip surface using the detergent CHAPS (2 × 2 min injection).
6. Apply the lipid vesicles (0.5 mM) to the dextran surface for 12 min
7. Unbound vesicles were removed using 50 mM NaOH (3 min injection), in order to make the experiment only with stably bound vesicles
8. Use bovine serum albumin (BSA) as a blocking molecule to prevent any non-specific binding to the substrate (3 min injection).
9. Apply actinoporins (4 μM) for 10 min on the lipid surface
10. Inject buffer for 5 min to study toxin dissociation
11. Regenerate the sensor surface using CHAPS. For vesicles containing cholesterol, 50 mM NaOH:isopropanol (2,3) gives better results than CHAPS

Notes

1. Temperature = 23 °C.
2. Flow = 10 μL/min.
3. To finish every injection of the different reactants, 200 μL of buffer are injected.
4. For a more detailed explanation of the protocol see (Cooper, Try, Carroll, Ellar, & Williams, 1998) and (Del Vecchio & Stahelin, 2016).

5.3 Expected outcomes

The instrument returns bond mass in ng/cm^2 which can be represented versus time to obtain a sensorgram that shows the progress of the interaction (Fig. 3).

5.4 Quantification and analysis

The relative molecular ratios can be calculated if the ng/cm^2 are recorded for both the lipids and the MBA, provided the molecular mases are known.

5.5 Advantages

SPR can be utilized to measure the binding of molecules in real-time without the use of labels.

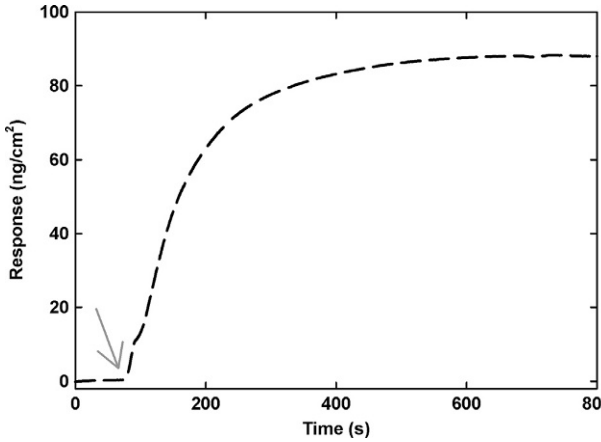

Fig. 3 *Surface plasmon resonance*. Typical sensorgram showing the interaction of the actinoporin FraC with supported DOPC:SM (80:20) model vesicles. Protein was added at time indicated by arrow, and the signal was recorded as a function of time.

5.6 Limitations

Mass transport limitations can occur if the actinoporin transfer to the vesicles on the sensor surface is not adequate, in which case the actinoporin concentration near the surface will be different from the bulk concentration. Increasing the flow rate is an easy way to limit mass transport effects.

6. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry is a highly suitable technique for studying the energetics of interactions between biological molecules (Velázquez-Campoy, Ohtaka, Nezami, Muzammil, & Freire, 2004). This technique has been widely used to study actinoporins' affinity for membranes of different compositions, by injecting aliquots of the lipid suspension into a protein solution continuously stirred. The heat released in each injection can be calculated from the raw data by integration of the peaks after subtraction of the baseline. Experimental data can be fitted to obtain the thermodynamic parameters of the reaction.

6.1 Materials and equipment

- Material and chemicals for LUVs preparation (see Section 3)
- Buffer: 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA
- Binding agent: Actinoporins (sticholysins) or MBA of interest

- Dialysis system
- Bath sonicator
- VP-ITC calorimeter (Malvern MicroCal)
- ThermoVac (Malvern MicroCal)
- Origin software (OriginLab) provided by MicroCal

6.2 Step-by-step method details

Timing: 3.5 h; MBA dialysis, 2–3 h; LUVs preparation, 1 h

1. Prepare the actinoporin at the proper concentration (see note 1) in Tris–HCl, NaCl buffer
2. Dialyze the actinoporin against 500 mL of buffer for 2–3 h at room temperature (20–25 °C) and keep the outer dialysis medium
3. Prepare LUVs of the desired composition (see Section 3) using the actinoporin dialysis media (see note 2) and let them stabilize to experimental temperature. The concentration of vesicles depends on the concentration of MBA and the stoichiometry of the reaction (see note 3).
4. Filter the dialysis media and degas by bath sonication. Degas both the membrane-binding agent and LUVs under constant stirring using a vacuum system
5. Fill the calorimeter cell using the proper Hamilton syringe. It is crucial not to introduce bubbles in the cell
6. Fill the injection syringe with the vesicle preparation following the calorimeter manufacturer instructions
7. Run the adequate protocol for your experiment. Some parameters (total number of injections, volume of injection, cell temperature, reference power, stirring speed, etc.) should be adjusted to better register the binding reaction depending on the characteristics of the reaction (stoichiometry, exothermic/endothermic reaction, etc.)
8. Injection of vesicles against buffer in the cell under the same experimental conditions must be done to eliminate dilution heat contribution

6.3 Expected outcomes

ITC experiments give a thermogram showing the characteristic sequence of peaks corresponding to each ligand injection into the sample cell (Fig. 4A). A binding isotherm is obtained by integrating each injection peak as a function of lipid/protein molar ratio in the calorimetric cell (Fig. 4B).

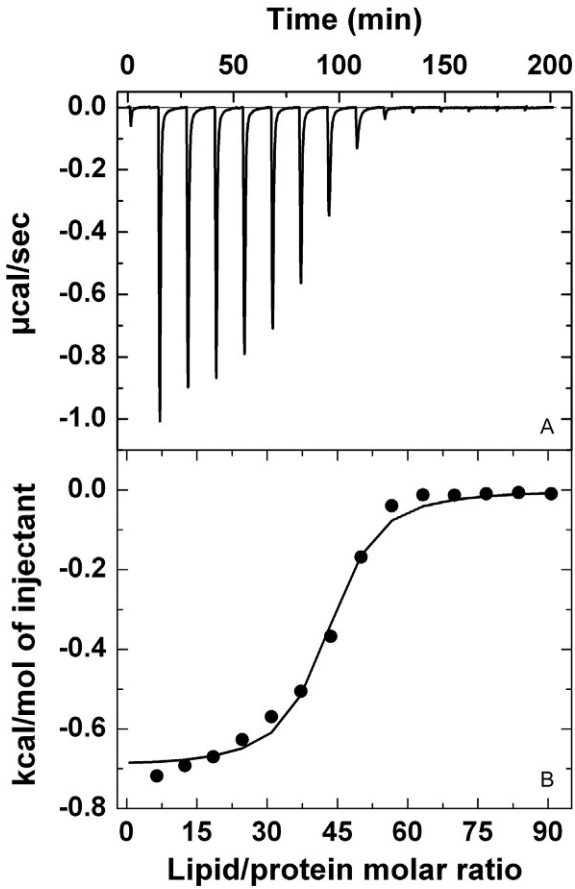

Fig. 4 *Isothermal titration calorimetry*. Panel (A) shows the characteristic heat peaks of an exothermic reaction, in this case the interaction of StnI with DOPC:SM (80:20) model vesicles. Panel (B) represents the binding isotherm obtained by integration of the peaks as a function of lipid/protein molar ratio.

Notes

1. The concentration of the MBA should be adjusted so the c value ($c = K_b \times N \times [MBA]$) is between 10 and 1000 (preferably 500). In our case, we used actinoporins at 1–10 μM, depending on the value of the binding constant (K_b) and the stoichiometry of the reaction (N) (García-Linares et al., 2016). There are alternate ways of analyzing thermodynamics of membrane interactions for cases that cannot be described by stochiometric binding but are consistent with phase partitioning instead (White, Wimley, Ladokhin, & Hristova, 1998).

2. It is imperative that the buffer matches exactly between the actinoporin and vesicles preparations to avoid heat contributions from buffer mixing and dilution.
3. In our case, lipid vesicles concentration was between 1 and 10 mM.
4. The volume of samples depends on the specific model of the calorimeter.
5. For extraordinarily high binding constants (i.e., nano-picomolar range) in combination with high heat release, a good experimental approximation to obtain a curve that can be fitted in the correct c interval (note 1) is reducing the concentration of cell and titration components and increasing the number of injections while reducing their volume (Rivera-de-Torre et al., 2016).

6.4 Quantification and analysis

(Alegre-Cebollada, Cunietti, Herrero-Galán, Gavilanes, & Martínez-del-Pozo, 2008):

1. Use the Origin software to open the raw data
2. Follow the software instructions to calculate the heat released in each injection from the raw data by integration of the peaks after subtraction of the baseline
3. The binding isotherms are adjusted to a model in which a molecule of protein binds the membrane involving N lipid molecules
4. Obtain K_b, N, and ΔH values from the Origin integration
5. Obtain ΔG and ΔS values according to the expression: $\Delta G = -R \cdot T \cdot \ln(K_b/(N \cdot \nu_L)) = \Delta H - T \cdot \Delta S$ (where ν_L is the specific molar volume of lipids in L/mol).

6.5 Advantages

ITC gives a basic thermodynamic characterization of the actinoporin-lipid interaction in a single experiment (including association, stoichiometry, and binding enthalpy) without the need of labeling or immobilizing the reactants.

6.6 Limitations

ITC requires large amounts of samples and only one ligand can be tested at a time. ITC methodology requires heat is either consumed or generated by the reaction. The lower the changes in enthalpic component of binding, the lower is the signal, and the less reliable the measurements becomes.

7. Evaluating the effect of actinoporin binding on SM-clustering

The effect that actinoporin binding has on SM organization in the membrane can be studied using pyrene-labeled SM analogs. Pyrene moieties can form excited-state dimers, known as excimers, if two of them are in very close range of each other during the lifetime of their excited state (Lakowicz, 2006). Pyrene emission, which regularly displays three peaks, becomes unstructured and red-shifted when excimers are formed (Lakowicz, 2006).

Therefore, pyrene-labeled lipids can be used to detect lipid clustering and declustering by means of measuring the relative pyrene monomer (M) and excimer (E) emission, i.e., the E/M ratio (Hresko, Sugár, Barenholz, & Thompson, 1986, 1987; Jones & Lentz, 1986). This has been used, for example, to detect formation of domains. In our case, we have used it to monitor the effect of actinoporin membrane binding on the distribution of SM in the membrane, using SM analogs labeled in their acyl chain. In practice, this technique can be used to follow the effect of any MBA on any lipid that can be labeled with pyrene.

7.1 Materials and equipment

- Material and chemicals for LUVs preparation (see Section 3)
- Buffer: 10 mM Tris–HCl, pH 7.4, 140 mM NaCl
- Binding agent: Actinoporins (sticholysins) or MBA of interest
- Probes: Pyrene-labeled lipids
- Steady-state fluorimeter, preferably one that can operate in T-format (to measure simultaneously emission of the monomer at 392 nm and the excimer at 480 nm)
- Quartz cuvette

7.2 Step-by-step method details

Timing: 10–15 min/assay (depending on the kinetics of the process); LUVs preparation, 1 h

1. Prepare LUVs of the desired composition (see Section 3) and let them stabilize to experimental temperature (see note 1).
2. Set the excitation wavelength at 345 nm. Monomer and excimer emission are recorded at 392 and 480 nm respectively (see general note 1).
3. Record E/M emission for the vesicles alone (see notes 2–4).

4. Record E/M emission in the presence of the MBA (see general note 2). If the kinetics of the process are of interest, the MBA can be added to the measurement started on 3

7.3 Expected outcomes

Results allow to build dose-dependent curves to quantify the effect of a given MBA on the distribution of SM, with the possibility of also comparing different membrane systems.

7.4 Advantages

This is a straightforward system to evaluate effect of membrane binding on SM-clusters.

7.5 Limitations

Requires pyrene-labeled SM, which is not always easy to acquire, and might require self-manufacturing. The labeled compound may also behave slightly different from endogenous SM.

Notes

1. In our case, we used POPC:PSM:pyrSM:Chol in ratios 5:3:2:0 and 5:3:2:1, because we also wanted to see the effect of Chol presence (Palacios-Ortega et al., 2019). Depending on which MBA is tested, and the pyrene-labeled lipid, the ratio might need to be tuned for best sensitivity.
2. Vesicle concentration would need to be adjusted depending on total pyrene concentration, instrument sensitivity and, later, desired protein/lipid ratio. In our case, we used 1.0 μM lipids, with the protein lipid ratio being below 0.2 (maximum value assayed).
3. We recorded the E/M ratio in a time dependent manner, measuring emission at 392 and 480 nm, because initially we were interested in the kinetics of the process. Nevertheless, as long as all measurements are consistent and comparable, the ratio can also be calculated from the emission spectra, as long as the sample has reached equilibrium when they are recorded. Either way, light scattering due to vesicles needs to be evaluated and corrected for if required.
4. If the software of the fluorimeter allows for it, the E/M ratio can be calculated on the go. If else, E and M signals can be acquired independently and processed later.

8. Measuring the effect of actinoporin binding on lipid distribution

Actinoporin binding can affect lipid distribution on the membrane. In order to test that effect, we used a lipid-mimicking fluorescent probe in combination with a phase-selective quencher which is also embedded within the membrane as a lipid component. When the quencher is in contact with the fluorescent probe, it prevents the probe from emitting. In practice, we can consider that collisional quenching only takes place upon molecular contact (Lakowicz, 2006).

Thus, by measuring the relative quantum yield of the probe, we can assess its exposure to the quencher. Since the quencher is confined to one phase, this method allows to determine if the selected MBA affects the phase partitioning of the fluorescent probe. In our case, we used it to determine that StnII binding to the membrane alters the distribution of cholesterol in the membrane, using a fluorescent analog of cholesterol, cholestatrienol (CTL) (Palacios-Ortega et al., 2019).

8.1 Materials and equipment

- Material and chemicals for LUVs preparation (see Section 3)
- Buffer: 10 mM Tris–HCl, pH 7.4, 140 mM NaCl
- Binding agent: Actinoporins (sticholysins) or MBA of interest
- Probes: Fluorescent lipid analogs, such as CTL
- Phase selective quencher: 7-SLPC (1-palmitoyl-2-(7-doxyl)-stearoyl-phosphatidylcholine) or equivalent
- Steady-state fluorimeter
- Quartz cuvette

8.2 Step-by-step method details

Timing: 10–20 min/sample; LUVs preparation, 1 h

1. Prepare two sets of LUVs of the desired composition (see Section 3), differing only in the quencher. In one of them, the quencher should be absent and replaced by a regular lipid with similar physicochemical properties (see note 1).
2. Let the prepared vesicles stabilize to experimental temperature
3. Set the excitation and emission wavelengths according to the spectral properties of the probe of choice. For CTL, we used 310 and 400 nm for excitation and emission, respectively (see general note 1).

4. Starting with, say, the F_0 sample, record probe emission in absence of the MBA
5. Add the MBA and record probe emission until equilibrium is reached
6. Repeat points 4 and 5 for the F sample. If the kinetics of the process is of interest, add the MBA at the same time point as for the previous sample

8.3 Expected outcomes

Results (Fig. 5) allow to conclude if an MBA affects the micro-environment surrounding a given lipid species (in our case, CTL).

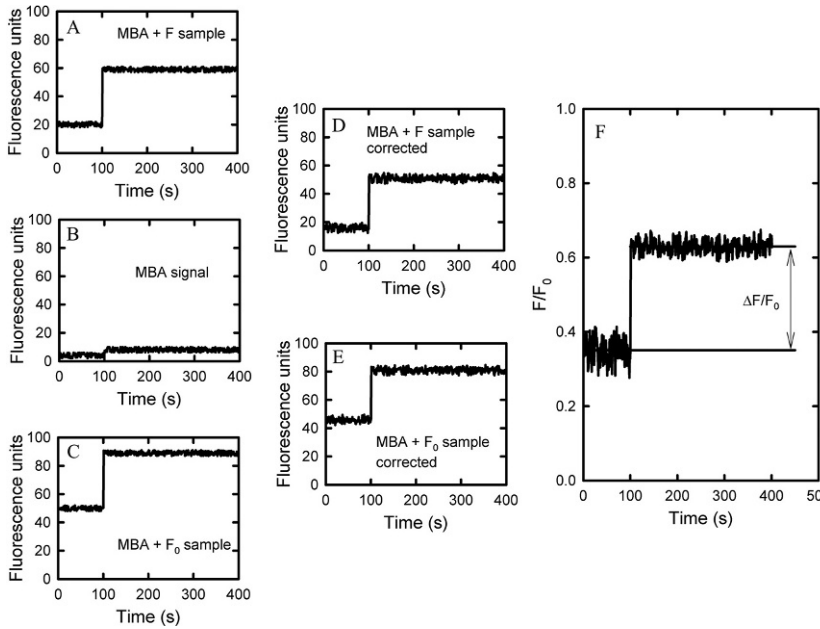

Fig. 5 *Lipid distribution*. Panels (A) to (C) represent the experimental results expected when adding a MBA to a quencher-containing sample (F sample; panel (A)), directly to plain buffer (panel (B)), and to a quencher-free sample (panel (C)). Data in panels (A) and (C) need to be corrected for MBA contribution by subtraction of data in (B), as shown in panels (D) (for data in (A)) and (E) (for data in (C)). Finally, the F/F_0 ratio can be calculated by division of data in (D) over (E), yielding the result shown in (F). In the example, the F/F_0 ratio before time 100 s corresponds to the quenching of the probe in absence of the MBA. Addition of the MBA, in the example, increases the value of the F/F_0 ratio (i.e., $\Delta F/F_0 > 0$). Since the probe is less exposed to the quencher than it was in absence of the MBA, it can be concluded that MBA binding hinders the probe-quencher interaction.

8.4 Quantification and analysis

1. If the MBA interferes with the signal of the probe, the final signal should be corrected for MBA effects (note 2).
2. Obtain the F/F_0 ratio, using the time point of MBA addition as reference to align the traces
3. If the F/F_0 ratio does not change due to MBA addition, it can be concluded that the MBA does not change the distribution of the probe
4. If the F/F_0 is affected by MBA addition, the $\Delta F/F_0$ can be calculated. If $\Delta F/F_0 > 0$, the MBA can be said to reduce the exposure of the probe to the quencher. If $\Delta F/F_0 < 0$, the MBA can be said to increase probe-quencher interactions. Elucidation of the mechanisms causing the changes in probe exposure to quencher requires further experiments

Notes

1. Since the intensity of the probe's emission is the parameter of interest, the concentration of the probe in the membrane has to be such that it does not quench itself. CTL, for example, has to be kept below 5 mol % (Schroeder, Nemecz, Gratton, Barenholz, & Thompson, 1988). The samples with and without quencher will be referred to as F and F_0, respectively.
2. For example, if the MBA is a protein, like sticholysins, and the probe used is CTL, excitation at 310 nm does excite Trp residues (though not much), which also emit at 400 nm. In that case, Trp emission has to be measured and subtracted from the final signal to obtain the contribution of CTL.

8.5 Advantages

The method is very precise in elucidating if a given MBA promotes or hinders the displacement of the probed lipid to the quencher-rich phase.

8.6 Limitations

Further experiments are required to gain mechanistical insight in the process.

9. Membrane binding of proteins based on Trp emission

Membrane binding of proteins can be observed monitoring Trp fluorescence. In order to do that, the protein of interest needs to have exposed Trp residues that change their exposure to the solvent upon membrane

binding, either by going into the membrane, or by being shielded from the solvent by a neighboring monomer if the protein oligomerizes.

If the protein has multiple Trp residues, and Trp mutants are available, this approach also enables to find out which Trp residues are in close contact with the membrane or involved in protein-protein interactions as a consequence of oligomerization upon pore-formation. Furthermore, if a three-wdimensional structure of the soluble protein structure is available, this approach enables to estimate the orientation of the membrane-bound form of the protein relative to the bilayer based on the changes in the emission of each Trp residue. This is only possible, however, if the structural changes that occur concomitant to membrane binding do not significantly alter the soluble fold or the protein, or at least the regions that include the Trp residues. This latter scenario is the one observed for actinoporins.

9.1 Materials and equipment

- Material and chemicals for LUVs preparation (see Section 3)
- Buffer: 10 mM Tris–HCl, pH 7.4, 140 mM NaCl
- Binding agent: Actinoporins (sticholysins), or MBA of interest
- Steady-state fluorimeter
- Quartz cuvette

9.2 Step-by-step method details

Timing: 3–5 min/spectrum, i.e., 1 h/titration; LUVs preparation, 1 h

1. Prepare a set of LUVs of the desired composition (see Section 3) and let them stabilize to experimental temperature
2. Since emission of Trp is to be measured, excitation can be set to 295 nm to avoid contributions from Tyr and Phe. However, to determine the thermodynamics of binding via titration, the relevant observation is the net change in the spectroscopic signal, and for that, selective excitation of Trp residues is not required. In that case, excitation at 280 nm can be used. For more detailed suggestions see (Ladokhin, Jayasinghe, & White, 2000).
3. Record the emission spectra of the soluble protein from 305 to 475 nm
4. Titrate the sample with vesicles until saturation is reached. Record the emission spectra after each addition of vesicles
5. If Trp mutants are available, repeat steps 3 and 4 using those

9.3 Expected outcomes

Results (Fig. 6) allow to determine the lipid-protein ratio required to achieve complete binding of the available MBA in solution.

9.4 Quantification and analysis

1. The relative quantum yield of the protein after each addition of the vesicles can be calculated by integrating the emission spectra and comparing the result to that of the protein in solution
2. The calculated relative quantum yields can be plotted either as a function of lipid/protein molar ratio (for stoichiometric binding), or as a function of lipid concentration (for phase partitioning, as described in (Ladokhin et al., 2000)).

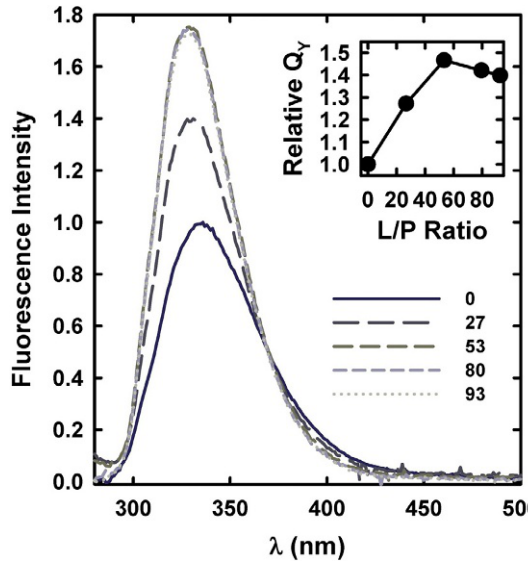

Fig. 6 *Trp emission*. Fluorescence emission of StnII under 260 nm excitation at increasing lipid/protein (L/P) molar ratios. Notice how the emission is greatly increased as the lipid amount is increased in the sample, while the position of the emission maximum is blue shifted. Insert: the emission of all spectra in the graph was quantified by numerical integration and normalized by the value obtained for the spectra obtained in absence of lipids. Plot of the quantum yield as a function of the L/P ratio in the sample clarifies that saturation is reached at ~55 L/P molar ratio. Increased resolution can be achieved by using smaller increments when adding lipids to the sample

3. If Trp mutants have been used, the relative quantum yields (calculated to each protein's emission in absence of lipids) can be used to see which Trp residues interact with the membrane. The details depend on the mutants that are available

9.5 Advantages

The only requirement is that the MBA has Trp residues, or that one can be introduced via mutagenesis.

9.6 Limitations

At least one of the Trp residues needs to change its environment as a consequence of binding, otherwise no change in signal is observed, unless specialized quencher-enhanced titration protocols are used (Posokhov, Gottlieb, & Ladokhin, 2007).

General notes:

1. In all fluorescence experiments it is good practice to first record an emission scan to assess the emission spectra of the sample. If this is the case, it should be remembered to perform all regular corrections, such as subtraction of the background, including the Raman peak of water
2. Make sure, before performing the experiment, that the spectral properties of the MBA do not interfere with those of the probe. If that is the case, the interference should be estimated, and the signals corrected accordingly

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