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Reconstitution of membrane budding with unilamellar vesicles

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Abstract

Enveloped virus particles select their lipid-protein components and egress by budding from the host cell membranes. The matrix protein of many enveloped viruses has been proposed as a crucial element for viral budding; however, molecular mechanisms behind membrane budding by the matrix protein are yet to be unraveled. Here we describe a set of *in vitro* functional reconstitution assays that allows quantitative evaluation of both, membrane binding and creation of membrane curvature by the matrix protein isolated from Newcastle Disease Virus. Individual budding events orchestrated by the matrix protein can be resolved in real time. The assays may be applied for direct reconstitution of the on-membrane action of cellular proteins involved in membrane curvature induction upon binding *in vivo*.

Introduction

Cellular life and death depends critically on the membrane curvature creation by cellular proteins. Since the works by Palade (Palade, 1975; Palade, 1953), the mechanisms of cellular trafficking and membrane deformation have been the target of extensive studies and many protein candidates for curvature induction have been proposed. However, it is not easy to extract the exact molecular mechanism behind the cellular curvature creation from *in vivo* observations, as cellular membranes are very crowded environments. The bottom-up approach, where isolated membrane systems are used to reconstitute cellular events with a minimum set of proteins, simplifies greatly this task. Recent reconstitution studies have proved this approach be a handful tool for our better understanding of curvature creation in cells. One good example of the usefulness of these kinds of studies is the reconstitution of budding of enveloped viruses on GUVs (Shnyrova et al., 2007; Solon et al., 2005).

Despite the generally accepted notion that the host cell participates in viral budding, final virus particles include only small traces of cellular proteins (Briggs et al., 2003). This suggests that enveloped viruses ultimately rely on their own protein machinery for vesicle egression. Budding of most retroviruses (except Foamy Virus) and many paramyxoviruses has been proposed to rely critically on the viral matrix protein, although other viral proteins are often enlisted as required components of a minimal budding machinery (reviewed in (Welsch et al., 2007)). Matrix (M) protein is the major component of most enveloped viruses, where it forms a tightly packed shell beneath the lipid envelope of the virus, thus resembling the caveolin coat of the endocytic vesicles, though with the opposite topology (Welsch et al., 2007). For many paramyxoviruses, including the Newcastle Disease Virus (NDV), expression of M protein in the absence of other viral proteins in a cell leads to formation of Virus-Like Particles (VLPs) (Jayakar et al., 2004; Pantua et al., 2006;

Takimoto and Portner, 2004). These results indirectly suggested that NDV M protein possesses the energy and functionality necessary to create membrane shape of desired geometry. However, the mechanism of negative membrane curvature induction still remained unresolved and the question remained open whether M alone, with just a lipid bilayer, is the minimum requirement for this process of membrane remodeling.

Here we describe an *in vitro* assay that allows to evaluate, to monitor in real time, and to quantify the ability of a membrane bound protein (the NDV matrix protein) to induce negative curvature upon its interaction with the membrane of giant liposomes. This assay offers unique insights into the molecular mechanisms driving this type of membrane remodeling *in vivo*.

M protein purification

An *in vitro* reconstitution of membrane activity of a protein requires highly purified protein in its native conformation, as misfolded proteins or sample contaminants can bind to the membrane and alter its behavior. These requirements often preclude and limit the use of recombinant membrane-active proteins from bacteria, as they often fold incorrectly in the absence of other components of their native environment and tend to aggregate in physiological conditions. Instead, we purify the M protein directly from its native environment: the NDV. This method yields high amount of sufficiently purified protein, however, we would note here that the ultimate test is the comparison of the membrane behavior of purified and recombinant proteins.

Newcastle Disease Virus *LaSota* strain (Charles Rivers Laboratories Inc., MA) is grown in the allantoic cavity of eleven-day old pathogen-free chick embryos (CBT Farm, MD). Upon infection, embryos are allowed to grow for 48 hours at 37°C and 55% humidity. The allantoic fluid is then collected and stored at -4°C. Virus is purified the next day as described previously (Garcia-Sastre et al., 1989).

Scheid and Choppin's purification protocol is used as the reference for M protein purification from NDV (Scheid and Choppin, 1973). To separate the lipid anchored proteins from the viral nucleocapsid, the virus pellet is resuspended in 10 mL of 1 M KCl, 10 mM Tris-HCl and 5 mM CaCl₂ at pH 7.4 (*buffer A*) with 2% of detergent Triton X-100 and then incubated 30 minutes at room temperature with constant agitation. After incubation, the sample is centrifuged at 200,000 g during 2 hours at 4°C. The supernatant containing M, HN and F proteins, as well as viral lipids, is recovered and dialyzed against 2 L of *buffer A*, lacking KCl (5 changes during 36 hours). The low ionic strength of the dialysis buffer induces M protein precipitation and separation from the rest of the viral membrane proteins. The contents of the dialysis bag are centrifuged at 9,000 g for 30 minutes at 4°C. M protein pellet is then dissolved in 1 mL of *buffer B*, which contains 1 M KCl, 20 mM Hepes, and 0.2 EDTA, pH 7.4. Final protein concentration is determined using the BCA protein assay kit (Pierce, IL) and is adjusted to lower than 75 µM in order to avoid protein precipitation during storage.

Various viral matrix proteins are known for their ability to self-assemble into ordered structures in low salt conditions. The speed and efficiency of such aggregations are usually determined by the ionic strength and protein concentration (McCreedy et al., 1990). This sensitivity has been routinely used to separate matrix protein from the rest of the viral proteins: M protein appears mostly as a monomer at high ionic strength, but aggregates slowly and irreversibly at physiological ionic strength (Sagrera et al., 1998). Accordingly, the use of high ionic strength (>1M) solution during purification is critical for disassembly of the interior of NDV. However, experimental observations indicate that M protein interaction with NP protein is not totally disrupted during the purification procedure based

only on salt concentration changes, and that the aggregation of M protein may be in part due to the formation of this M-NP complexes. Thus, addition of calcium in milimolar concentration during M protein purification allows for a better separation of M protein from the NP complex, presumably through disruption of some electrostatic interactions, therefore leading to highly purified M protein.

The protein purity is estimated from the reducing SDS-PAGE by analysis of the areas under the intensity peaks correspondent to protein bands, as was described in a previously developed procedure (Coorssen et al., 2002).

Evaluation of the membrane activity of M protein through interaction with intermediate-sized unilamellar liposomes

Giant unilamellar vesicles (GUVs) provide an excellent tool for studying the membrane activity of a particular protein, as they allows for real time observation of shape transformations by bright-field or fluorescence microscopy. However, experiments on GUVs may end up frustrating if the binding of the protein to a particular lipid composition is not previously analyzed by quantitative methods. In this aspect, large (200-500 nm in diameter) and intermediate-sized (100-200 nm in diameter) unilamellar liposomes (LUVs and IUVs, respectively) are suitable for the initial check-up of the protein-membrane interactions.

Remarks about IUV formation and characterization

There are many methods for IUV preparation described elsewhere (a great reference source is (New, 1990b)). The choice of a particular method greatly depends on the desired lipid composition of the vesicles and the buffer used. For the compositions and conditions described below, a combination of gentle hydration method followed by extrusion through appropriate polycarbonate membrane gives reproducible results. However, we encourage the reader to explore a variety of methods for IUV preparation in order to find an appropriate one or a combination of several that suits the particular experimental case.

The success in the IUV preparation relies on achieving both the desirable size and the unilamellarity of the vesicles. The first parameter is crucial in terms of the curvature as well as the lateral membrane tension of the vesicles. For example, for many proteins their membrane binding depends on membrane curvature (e.g. (Peter et al., 2004; Vaccaro et al., 1993)). Thus, if the vesicle population exhibits high size variability, results of the binding assays may be truly confusing. Hence, the size distribution of the IUV sample is to be measured routinely, e.g. by dynamic light-scattering.

The lamellarity of the vesicles is extremely important when quantitative results are obtained from a binding assay. Even if the phospholipid concentration is determined for a particular IUV sample, the amount of the lipid accessible to the proteins, i.e. lipids in the outer monolayer of a vesicle, may be up to orders of magnitude lower than for a unilamellar vesicle. Multilamellarity also affects the amount of encapsulated material into the vesicle and the effective membrane rigidity. This may lead to potential artifacts in experiments, e.g. in measurements of the protein-induced leakage of encapsulated material by fluorescence spectroscopy. Thus, the routine of checking the unilamellarity of the IUVs should be adopted as well, e.g. by TNBS assay (New, 1990a). A direct measurement of the IUVs lamellarity is offered by the electron microscopy imaging of ammonium molybdate negatively stained samples, as described previously (Bugelski P.J., 1990). The use of uranyl acetate (UA) stain with this kind of samples is highly discouraged, as UA interferes with lipid membranes, especially with the charged ones (Caffrey et al., 1987; Parsegian et al., 1981).

IUV preparation

For the M protein experiments, IUVs are prepared by a combination of the gentle hydration method (Reeves and Dowben, 1969) with the extrusion procedure (Mayer et al., 1986). IUV are formed from mixtures of the lipid stocks in chloroform of dioleoyl phosphatidylcholine (DOPC), palmitoyl oleoyl phosphatidylcholine (POPC), dioleoyl phosphatidylethanolamine (DOPE), dioleoyl phosphatidylglycerol (DOPG) and cholesterol (chol), doped with 0.2 molar percent of lipid fluorescence marker Lissamine Rhodamine B DOPE (Rh-DOPE). All lipids are from Avanti Polar Lipids, AL. IUVs used for the binding assay with M protein are prepared from the following lipid mixtures (in molar ratios): DOPC:DOPE:Chol:Rh-PE 60:29.8:10:0.2 (PE-chol neutral mixture); POPC:Chol:Rh-DOPE 69.8:30:0.2 (chol neutral mixture); POPC:Rh-PE 99.8:0.2 (neutral mixture); POPC:DOPG:Rh-PE 84.8:15:0.2 (charged mixture). The total lipid concentration in the final IUV sample for these mixtures is 1 g/L. For the assay of the M protein induced IUV leakage, the PE-chol neutral mixture is employed, with total lipid concentration of 0.5 g/L in the IUV sample.

Each lipid mixture (initially in chloroform) is added to a round bottom of 5 or 10 mL flask, suitable for a rotary-evaporator system (such as VV Micro Evaporator (Heidolph Brinkmann LLC, IL) or equivalent). Chloroform and methanol (9:1 vol/vol) are added to the flask until a final volume of ~ 0.5 mL. The flask is rapidly transferred to the rotary evaporator, where the evaporation of the organic solvents is carried out with gentle warming of the flask (30°C or above the phase transition temperature(s) of the lipids in the mixture) and an intermediate flask rotation speed, in order to obtain a thin and uniform lipid film. Once the visible traces of the organic solvent have disappeared, the evaporation continues for another 15-30 minutes. Then, the flask is transferred to a high pressure lyophilizer at room temperature for 1 hour to ensure the removal of residual solvents.

Then the desired aqueous buffer is added to the flask and the lipid film is left to hydrate overnight at 37°C. Special care is taken to avoid shaking the flask during the hydration process. In the case of the IUV preparation for the binding assay, the aqueous buffer is 100 mM KCl, 20 mM Hepes and 0.2 mM EDTA, pH 7.4 (*buffer C*). For leakage measurements, an aqueous fluorescent dye should be entrapped inside the IUVs. Thus, *buffer D* (12.5 mM ANTS, 45 mM DPX, 20 mM KCl, 0.2 mM EDTA, 20 mM HEPES, pH 7.4) contains 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) quenched with *p*-xylenebis(piridinium bromide) (DPX) (Molecular Probes, Invitrogen Corp., CA). The quenching efficiency is proportional to the concentration of ANTS/DPX. The dyes are loaded to IUV in high concentration ensuring effective quenching of ANTS fluorescence. The leakage measurements are based on the increase of the ANTS fluorescence upon dilution. A small (< 1 nm) hole in the IUV membrane is sufficient to let ANTS/DPX release to the bulk solution, thus causing the dilution. Encapsulation of dyes different in size allows estimating the size of such holes. For example, 70 kDa fluorescein isothiocyanate conjugated dextran, FITC-dextran (Sigma-Aldrich Corp, MO), contained in the buffer E (75 mg/mL 70 kDa FITC-dextran dissolved in *buffer C*), requires a much larger (~ 5 nm) hole in the membrane to escape. Iso-osmolarity of *Buffer D*, *buffer E* and *buffer C* is controlled using 5500 vapor pressure osmometer (Wescor, UT) or equivalent.

The lipid cloud formed after hydration (mainly containing MLVs and LUVs) is dispersed in the buffer by gentle vortexing of the flask. Using a glass Pasteur pipette, the vesicle dispersion is loaded into an extruder (Lipex Biomembrane Inc., Canada) preassembled with a drain disc and one or two (usually 0.1 micron, if not indicated otherwise) polycarbonate filter (GE Osmonic Labstore, MN). The samples are extruded 15 times. Non-encapsulated ANTS/DPX is separated from IUVs using pre-packed Sephadex PD-10 columns (GE Healthcare Biosciences Corp, NJ). Non-trapped 70 kDa FITC-dextran is removed using a column packed with Superose 6 prep grade (GE Healthcare Biosciences Corp, NJ) at a 1

mL/min flow rate. In both cases, *buffer C* is used as the elution buffer. A crude estimation of the amount of lipids in the preparation is made by measuring the fluorescence signal from Rh-DOPE. For that, 96-well plate apt for fluorescence measurements with a plate reader instrument (Synergy 4 microplate reader (Biotek, VT) or similar) is used. 10 μ L aliquots from the IUV suspension before and after extrusion are diluted by 100 μ L with *buffer C*. The Rhodamine (Rh) fluorescence is measured at 550 nm excitation and 590 nm emission wavelengths. The fluorescence of each sample is measured three times and the arithmetic mean of the fluorescence signals is used to estimate the amount of lipid lost during extrusion process by the following formula:

$$\text{lipid loss} \sim \frac{F_{\text{before}} - F_{\text{after}}}{F_{\text{before}}}, \quad \text{Eq. (1)}$$

where F_{before} stands for the mean Rh fluorescence signal before extrusion and F_{after} , for mean Rh fluorescence signal after the extrusion for each sample prepared. The total lipid concentration of the IUV preparation is then estimated by applying the following formula:

$$C_{\text{IUV}} \sim C_i * (1 - \text{lipid loss}), \quad \text{Eq. (2)}$$

where C_{IUV} stands for the total lipid concentration in the IUV preparation and C_i stands for the total lipid concentration of the initial lipid mixture.

We note that this method provides a rough estimation of the amount of total lipid in the final IUV preparation and cannot discriminate between lipid species in non-homogeneous lipid mixtures. Alternatively the phosphate analysis (Bartlett, 1959; Duzgunes, 2003) or liquid chromatography combined with mass spectrometry (Byrdwell, 2005) should be employed if the amount of a particular lipid in the sample is a critical parameter.

The size distribution and the lamellarity level of the IUV samples are routinely controlled before using them in M protein experiments. Only IUV preparations that show unimodal size distributions with mean diameters of 120 ± 30 nm by dynamic light scattering measurements with a dynamic light scattering instrument (N4 Plus Submicron Particle Sizer (Beckman Coulter Inc., FL) or equivalent) are used in the experiments. Furthermore, the unilamellarity of these samples is also confirmed periodically by negative-staining EM as described by (Bugelski P.J., 1990).

M protein binding to IUVs: the composition dependence analysis

Stable binding of M protein to PE-chol neutral, chol neutral, neutral and charged mixtures is assayed by ficoll gradient flotation or sedimentation methods (Fraley et al., 1980). 50 % w/v ficoll solution is prepared by dissolving 25 g of Ficoll PM400 (GE Healthcare Life Sciences, NJ) in *buffer C* (the amount of this stock increases or decreases proportionally to the amount of samples in the binding assay). This concentration is near the solubility limit of ficoll, so the solution preparation requires slight heating and extensively mixing. Solution containing smaller amount of ficoll are prepared by simple dissolving of the appropriate amount of Ficoll PM400 in *buffer C*.

In simple two-step flotation experiments, 111 μ g of M protein (2.8 nmol) is incubated with IUVs at 1:200 molar ration of matrix protein to lipid during 5 min at room temperature. In the control experiments, the same amount of the protein without IUVs and IUVs without the protein are diluted to the same volume as the protein/lipid mixtures and similarly incubated in *buffer C*. After incubation, each sample is mixed with 50% (w/v) ficoll so that the ficoll is diluted to 20% (w/v). The final volume of this mixture should not exceed 1.2 mL. Then, two step ficoll gradients are prepared in a 13 $\times$ 51 mm (4 mL total volume) polycarbonate

centrifuge tube (Beckman Coulter Inc., CA). The 20% (w/v) ficoll solution mixed with the protein-IUV samples is carefully transferred to the bottom of the centrifuge tubes. Next, the 10% ficoll solution is layered on top (the volume of this layer is usually greater than 2.5 mL). The last gradient step is composed of 200 μ L of the *buffer C*. The gradients are then centrifuged using a swinging bucket rotor SW 55Ti (Beckmann Coulter Inc., CA) at 4°C and 150,000xg for 30 minutes. IUV, seen as the Rh labeled band settle at the boundary between buffer C and 10% ficoll layers. Similar Rh-labeled band is seen in IUV-M protein mixture. These bands can be better observed and documented by illuminating the centrifuge tubes from the top with a light source and acquiring a photograph (Fig. 1).

Multi-step gradients are required to resolve whether several sub-populations of IUVs are formed upon the protein adsorption. For example, a six step gradient is prepared by layering, from the bottom to the top of the centrifuge tube, 500 μ L layers of 20, 15, 12, 9, 6 and 3% ficoll. The samples are very carefully deposited on top of the last layer. The gradients are centrifuged at 150,000xg in a SW55Ti rotor (Beckmann Coulter Inc., CA) at 4°C for 90 min. The location of the protein-IUV band/s depends on the amount of protein and membrane deformation upon its binding to the IUVs. Again, the bands can be observed and documented by illuminating the centrifuge tubes from the top with a light source. These images may be very illustrative, as they give qualitative macroscopic description of the behavior of each sample as compared to the IUV control (Fig.1).

Independently of the method used, the gradients are fractionated into aliquots (smaller than the volume of a gradient layer, e.g. 100 μ L) by using a peristaltic pump connected through tygon type tubing to 1.5 mm capillary borosilicate glass (#PG10150-4, WPI Inc., FL) carefully brought to the bottom of the centrifuge tube. A fraction collector (Model 2110 (BioRad Labs., CA) or equivalent) is attached to the output of the pump. After being collected, the 10 μ L of each fraction is diluted to 100 μ L with *buffer C* in a 96-well plate suitable for fluorescence measurements (Fig. 1). The Rhodamine fluorescence, indicating the amount of lipid in each fraction, is then measured at 550 nm excitation length and 590 nm emission wavelength in a plate reader instrument (Synergy 4 microplate reader (Biotek, VT) or similar). This allows one to construct a plot of fluorescence intensity of each fraction versus the fraction number by using a suitable software (Origin 7.0, OriginLab Corp., USA or similar) (Fig. 1). The peaks in this plot show the distribution of IUV between fractions.

The fractions corresponding to the peaks of the distribution of Rh fluorescence (“Rh-pick fractions”) are selected to explore for the M protein contents by the reduced SDS-PAGE analysis. The same fractions from the control samples are also selected and analyzed by SDS-PAGE. It is recommendable to load the maximum sample volume possible into the gel (the 10-well Pierce Precise Protein Gels (Pierce, IL) are a good option to consider, as they allow to load as much as 50 μ L of a sample per line). As it is very difficult to guess the percentage of protein bound to the IUV in each particular case, it is recommendable the use of sensitive stains, such as SyproRuby Red Gel Stain (Molecular Probes, Invitrogen Corp., CA) or similar in the initial binding experiments. Once the binding has been characterized, less sensitive stains, such as SimplyBlue SafeStain (Invitrogen Corp., CA) or equivalent may be used.

Equal volumes of the selected fractions are subjected to the reducing treatment and then loaded into the gel together with a line marker. The gel should also include a calibration line with a known amount of protein loaded. Stained gel images are acquired and analyzed using a gel imaging (FluorChem FC (Alpha Innotech Corp., CA) or equivalent). M protein exhibits stronger binding to chol containing liposomes, with preference for the PE-chol neutral composition, likely because of less dense lipid packing in such mixtures related high spontaneous curvature of PE (Fuller and Rand, 2001). M protein shows lower affinity for the

charged compositions. No protein signal is detected for in the equivalent protein control fraction (Shnyrova et al., 2007).

The areas under the intensity peaks of the protein bands are further analyzed using an appropriate software (Origin 7.0 (OriginLab Corp., MA), or similar). This analysis allows establishing the percentage of the protein bound to each IUV type. First the amounts of protein in each line of the gel are found by normalizing to the area of the protein intensity peak in the calibration line:

$$g_L = \frac{A_L}{A_{CL}} * g_{CL}, \quad \text{Eq. (3)}$$

where g_L are the grams of protein loaded in the sample line, A_L is the area under the intensity peak of the protein band, A_{CL} is the area under the intensity peak of the protein band in the calibration line and g_{CL} are the grams of protein loaded into the calibration line. The amount of protein calculated this way is per volume of sample loaded into the gel. Thus, the amount of protein for the fraction with the maximum Rh signal will be:

$$g_{fmax} = \frac{V_f}{V_L} * g_L, \quad \text{Eq. (4)}$$

where g_{fmax} stands for the grams of protein in the studied fraction, V_f is the total volume of the fraction (100 μ L), V_L is the volume of the fraction loaded into the gel line and g_L are the grams of protein loaded in the gel line (see Eq. (3)).

However, this is not enough to estimate the amount of protein bound to IUVs, as the IUV-protein band usually extends for more than one fraction collected from the centrifuge tube (plot in Fig. 1). To correct for the smearing of IUV over the gradient, the plot of Rh fluorescence versus the fraction collected (see above) is used. It is assumed that the distribution of M protein follows the distribution of IUV in the fractions, i.e. the fractions which signals contribute to the Rh fluorescence peak on the plot. Each point of the fluorescence represents a homogeneous fraction of IUV with bound M proteins. The maximum point of the peak corresponds to the fraction analyzed by SDS-PAGE and which protein amount is a known parameter (g_{fmax}). To calculate the amount of protein in the entire band (i.e. for all the fractions that forms the fluorescence peak), first, the plot showing the distribution of Rh fluorescence is baseline corrected leading to elimination of the fractions with no detectable peaks of Rh fluorescence (plot in Fig. 1). Next, the fluorescence values for each fraction of the fluorescence peak are normalized by dividing for the fluorescence value of the peak maximum. By doing so, the amount of protein bound to the single population of IUVs is calculated as follows:

$$g_B = g_{fmax} * \sum_{n=1}^n \frac{F_{fn}}{F_{fmax}}, \quad \text{Eq. (5)}$$

where g_B denotes the grams of protein bound to the IUVs, g_{fmax} is calculated using Eq. (4), F_{fn} is the fluorescence value minus the baseline for the fraction n of the fluorescence peak and F_{fmax} is the fluorescence value minus the baseline for the fraction with the maximum fluorescence intensity of the peak. If peaks of the Rh fluorescence corresponding to different populations of IUV (or gradient bands) overlap, simple deconvolution analysis is needed to estimate the amount of M protein in each IUV population.

Finally, the percent of the total protein bound to a single population of IUVs is estimated by:

$$\% \text{ bound protein} = \frac{g_B}{g_i} * 100, \quad \text{Eq. (6)}$$

where g_B is calculated from Eq. (4) and g_i denotes the grams of protein initially in the binding assay (111 μg in the case considered).

The fractions can be further analyzed for IUV morphological deformations caused by adsorbed proteins via EM imaging. Cryo-EM techniques are favorable as IUV deformations caused by the adhesion and fluttering to the supporting film of the grids used for negative-staining EM are avoided altogether.

M protein interaction with IUVs: Leakage of aqueous dyes

The IUV stability upon M protein adsorption is explored using IUVs loaded with aqueous fluorescent markers, either small (ANTS/DPX) or large (70 kDa FITC-dextran). Leakage of these aqueous dyes from IUVs upon the M protein additions is detected at ambient temperature and under constant stirring by spectrofluorimetric measurements using a spectrofluorometer (Aminco-Bowman SLM-2 (Spectronic Instruments, Inc.) or equivalent). In a cell suitable for fluorescence measurements and containing a stirrer bar IUV sample is diluted with *buffer C*. Protein is added in an amount such as the protein/lipid molar ratio increases 0.01 units after each protein addition. The increase in integral fluorescence due to the release of the dyes is detected. The normalized fluorescence intensity (F_N) is recalculated from the integral IUV fluorescence intensity (F) as:

$$F_N = \frac{F - F_i}{F_T - F_i}, \quad \text{Eq. (7)}$$

where F_i corresponds to the fluorescence intensity before the protein addition and F_T to the fluorescence intensity after complete disruption of IUV (infinite dilution of the fluorophores) by detergent (0.1% of Triton X-100). The excitation/emission wavelengths used are 380/520 nm for ANTS/DPX detection and 550/590 nm for Rh-DOPE detection. The experiments are carried out in triplicates. Data analysis is performed with Origin 7.0 software or similar.

Starting at ~ 0.01 protein/lipid ratio, release of both markers is observed with slower kinetics for the larger marker (Shnyrova et al., 2007). The possibility that the observed effects are caused by some artifact present in the protein solution (such as traces of detergent triton that is used during the protein purification) is ruled out by α -chymotrypsin proteolytic (α -chymotrypsin from bovine pancreas (Sigma-Aldrich Corp, MO)) treatment of a 5 μM protein solution by a 5 minute incubation at room temperature with in 1:5 molar ratio. The digestion of M protein upon this treatment is confirmed by an SDS-PAGE (Shnyrova et al., 2007). After this treatment, the M protein digested sample is added to the IUVs in the same conditions as the M protein without digestion. The efficiency of content release from IUV after protein addition is greatly impaired (Shnyrova et al., 2007).

Release efficiencies are comparable for both high and low molecular weight markers for the same amount of the protein added, thus most probably indicating vesicle bursting, rather than membrane pore formation. Also, expected changes in IUV curvature induced by M protein adsorption increases membrane tension, thus increasing the possibilities for membrane bursting, versus small pore formation. Thus, “massive” leakage with no size selectivity, indicating bursting, can also be used as a rough preliminary criterion for the curvature activity of a protein.

Reconstitution of M protein-driven membrane budding on giant unilamellar vesicles (GUVs)

GUV preparation

Giant unilamellar vesicles are prepared using a slight modification of the electroformation protocol (Angelova and Dimitrov, 1988). The design of a custom made electroformation chamber used is depicted in Fig. 2A. The chamber consists of a polytetrafluoroethylene (PTFE) body and two 0.8 mm platinum (Pt) electrodes (Goodfellow Corp., PA) inserted into the PTFE block in such a way that on one side both electrodes are parallel to each other (this is the place for the GUVs to form), while on the other side the electrodes go in opposite directions. This is the place where the connector cables to the voltage generator are attached.

Lipid mixtures used for GUV preparation are the following (in molar ratios): DOPC:DOPE:Chol:Rh-DOPE (48:43:4:5); POPC:Chol:Rh-DOPE (90:5:5); POPC:Rh-PE (95:5); POPC:DOPG:Rh-PE (80:15:5), similar to those used in IUV experiments. In some cases, the difference in composition between IUV and GUV is dictated by the electroformation protocol where each particular lipid mixture, upon drying, has to form a film covering platinum electrodes. When lipid mixture includes charged species, like DOPG, the mixture is dissolved in chloroform:methanol:ethyl ether (4:1:5 volume ratio), to a final concentration of 0.1 g/L of total lipid. In all other cases, the mixtures are dissolved in chloroform:methanol (9:1 volume ratio), to a final concentration of 0.1 g/L.

A custom made application device is used for application of the lipid mixture to the Pt electrodes (Fig. 2A). This device consists of a 0.56 mm ID $\times$ 1.07 mm OD PTFE microbore tubing (Cole Palmer, IL) of ~ 2 cm length inserted into flexible tubing of 1.02 mm ID (such as Masterflex Tygon tubing, Saint-Gobain Corp., PA) with 1 cm length that is hermetically closed on the opposite side to the PTFE tubing with clogged by a small screw at the end. This device offers the advantage of being disposable after use (alternatively, a 10 μ L Hamilton syringe (Hamilton company, NV) can be used for lipid application, but care should be taken to clean the syringe thoroughly after each use with appropriate lipid solvent combination, such as ethanol, methanol, chloroform. Sonication is also a good option for syringe cleaning). Before using the lipid solution, the device is washed with pure chloroform by repetitive cycles of squeeze/release of the flexible tubing part, while a small part of the PTFE tubing is immersed into the chloroform solvent. After washing, the PTFE end of the device is immersed into the lipid solution. By squeezing-releasing of the flexible part of the device a small amount of the solution is withdrawn. Two small drops of it are deposited on each one of the two platinum electrodes of the electroformation chamber by gently squeezing the flexible part of the application device. After the application, the electrodes are dried under vacuum for at least 1 hour or overnight. Then, the electroformation chamber is assembled on a plastic disposable dish with a 0.17 mm thick cover glass bottom (Delta TPG Dish (Biopatch, PA) or similar; Fig. 2B) and the dish is mounted on a stage of an inverted microscope (Axiovert 200 (Carl Zeiss, Inc., Germany) with a fluorescence excitation source, a filter cube combination suitable for Rhodamine observation and a 40x/0.65 and a 100x/1.25 objectives (ACHROPLAN; Carl Zeiss, Inc., Germany) or equivalent microscope configuration). The electrodes are connected to a function generator (GFG-3015 model function generator (Instek America Corp., CA) or equivalent) pre-set to apply a 10 Hz and 0.2 V sinewave. Right after the initial voltage application, a buffer containing 20 mM Hepes and approximately 200 mM sucrose, pH 7.4 is gently added to the dish in such a way that it is slightly above the electrodes. This buffer should be scrupulously equilibrated osmotically with *buffer C* by using the procedure described above. During the following 15 minutes, the sinewave amplitude is gradually increased from 0.2 to 1.0 V. Electroformation times varied from 15 minutes to 1 hour for different lipid compositions of the GUV.

After GUVs become visible on the electrode by bright-field or fluorescence microscopy observation with a 40x objective, the voltage is lowered to 0.2V and then turned off completely. If the GUVs are to be detached from the electrodes, the frequency is lowered to 0.1 Hz before lowering of the voltage. Then the GUVs are collected with 200 μ L pipette and stored in a microcentrifuge tube until usage. If the GUVs are to be left on the electrodes, the plastic dish is perfused with *buffer C* for ~ 15 minutes by using an appropriate microscope perfusion system (such as the micro perfusion system (Bioscience Tools, CA) or equivalent). Visualization of GUVs in the attached configuration is performed directly on the inverted microscope stage immediately after perfusion of the chamber with *buffer C*.

Interaction of matrix protein with GUVs

Visualization of GUVs in the attached to the electrode configuration (Fig. 3A) is performed directly on the inverted microscope stage immediately after perfusion of the chamber with *buffer C*. The biggest advantage of this configuration is the relative immobilization of the GUVs on the electrodes, which facilitates greatly the application of the protein in the proximity of the GUV, as well as allows to easily patching the GUV membrane. However, the fluorescence background from the electrode and other GUVs in the vicinity sometimes interferes with the imaging of a single GUV. Also, the proximity of the electrodes to the bottom of the dish becomes a critical factor when high magnification objectives (such as 100x or bigger) are used. These objectives have small working distances, thus there is a requirements for the electrodes to be close to the bottom of the dish, something not easily achievable with 0.8 mm thick electrodes.

In order to observe individual vesicles at high magnifications, the detached GUVs are routinely used. In this case, a 0.17 mm-thin glass 35 mm dish (Delta TPG Dish (Biotech, PA) or similar), is pretreated with 1 g/L BSA for 1 min and thoroughly washed with the *buffer C* in order to avoid GUV spreading and bursting upon contacting the glass bottom of the dish. The dish is set up on the microscope stage and 1 mL of *buffer C* is added. 100-200 μ L of GUV suspension collected after electroformation is added to the 1 mL of *buffer C* in the dish. As GUVs are formed in the sucrose buffer, osmotically equilibrated with *buffer C*, after 5-10 minutes all the GUVs are settled in the glass coverslip of the bottom of the dish due to a difference in density between the two buffers (Fig. 3B). In this case, GUVs are easily visualized by high resolution fluorescence microscopy, however, the fact that GUVs are only slightly attached to the bottom, makes the experiments with protein delivery more involved.

For both configurations, prior to the protein addition the intrinsic behavior of each vesicle is recorded for a period of time (1-3min). The recording is made using a digital camera (iXon (Andor, CT) or similar) attached to the camera port of the microscope. The camera is controlled through a PC with MicroManager open source microscope software (University of California, San Francisco, CA) or equivalent. The unilamellarity of the GUV may be established by fluorescence analysis of the GUV contour, as was described previously (Akashi et al., 1996).

Upon establishing the stability (and unilamellarity) of the GUV, the M protein applied through a pipette brought to the close proximity of the GUV (Fig. 3B). The pipettes for M protein application are obtained by pulling a 1.5 mm capillary borosilicate glass (#PG10150-4, WPI Inc., FL) with a micropipette puller (Flaming Brown Micropipette puller, Model P80/PC (Sutter Instruments Co., CA)). The pipette is backfilled with M protein solution using a 1 mL syringe equipped with MicroFil™ (WPI Inc., FL). Three motorized actuators (12.5 mm Travel Open-loop DC CMA Actuator (Newport Corp., CA)) attached to a crossed-roller bearing linear stage, 0.5 inch, with XYZ travel (461-XYZ-M, Newport Corp., CA) are used to control the pipette position. The pipette filled with M

protein solution is inserted into a microelectrode holder with pressure port (WPI Inc., FL) attached to the linear stage (Fig. 2B). A 1 mL syringe is attached to one port in a three way luer valve (WPI Inc., FL). Another port of the valve is connected through long PTFE tubing to the pressure port of the microelectrode holder. This allows controlling the pressure difference applied to the pipette (i.e., for protein application or liposome patch isolation). The luer valve should be closed (i.e. turned toward the unoccupied port) during the pipette search in the objective focus area.

The delivery pipette containing concentrated solution of the protein (4 μ M) is carefully brought to the proximity of the studied GUV, the pipette motion being observed by bright field microscopy. Once the pipette is located in the proximity of the vesicle, it is directed toward the GUV and the luer lock in the pressure loop is opened. A pulse of positive hydrostatic pressure is applied to the pipette by gently pressing the pressure syringe. Immediately, a small flow of protein can be seen exiting the pipette. Prior to the protein application, the microscope light pass is switched to the fluorescence mode and the morphological changes of the GUV membrane are monitored and documented by acquiring time lapse series. The protein concentration in the GUV vicinity increases transiently. Shortly after protein application, invagination of the GUV membrane is detected. After each protein application the apparent size of the GUV decreases and the steady-state fluorescence in the GUV projection area increased (Shnyrova et al., 2007). Internalized membrane vesicles are clearly detected, thus justifying the fluorescence changes.

Special care should be taken about the pressure applied to the delivery pipette when GUVs slightly attached to the dish bottom are used. If the pressure is too strong, the GUV can be detached from the bottom of the dish and flow away. In this case, the pressure valve of the pressure syringe should be closed, the pipette should be lift immediately and a new GUV, far enough from the initial observation point, should be found. Several experiments can be performed in the same dish. However, the pipette should be exchanged after each one of them to avoid protein dilution effects.

GUVs of different compositions are used in order to establish the preferences for M protein induced budding. Surprisingly, and against the prediction made upon the binding preferences of M protein detected with IUVs, vesicle formation is more effective when cholesterol or charged lipids are present in the GUV composition. Thus, the budding activity of M protein is not directly proportional to the efficiency of its membrane binding (Shnyrova et al., 2007). Another important observation is formation of transient, fluid-like membrane domains upon application of M proteins to GUV. The appearance of these domains indicates that M proteins self-organize into domains which then bud away from the membrane (Shnyrova et al., 2007). Overall, these experiments directly demonstrate that M protein implements the genetically encoded information required to create the closed spherical membrane particles through its interactions with lipid bilayer;

The validity of these observations should be routinely checked by doing buffers and negative protein controls (i.e., by applying the M protein buffer containing no proteins or a reference protein, such as albumin, in similar concentration as M protein). Those are very important controls, as osmotic pressure, as well as bulk protein binding, can greatly affects the behavior of the GUVs. For example, differences in osmosis pressure between the exterior and the interior of the GUV can cause spontaneous budding and formation of vesicles in the GUV lumen without the need of a protein, as in the case described by (Guedeau-Boudeville, 2000). Thus, iso-osmolarity of every solution mixed with GUVs should be particularly monitored.

When experiments are performed on electrode-attached GUVs, the procedure is almost the same as with detached GUVs. However, the manipulations are eased by the fact that GUV is immobilized. Patch-clamp type experiment, hard on the slightly attached GUV, can be performed using single GUVs on the electrode (Fig. 3A). For these experiments, the luer pressure valve on the pressure syringe is opened before the pipette approaches a GUV closely. After touching GUV, a very small negative pressure is applied. The GUV membrane is sucked in and a stable contact, seen as a small membrane patch inside the pipette, forms. Protein adsorption on this isolated patch inside the pipette starts instantaneously, and the morphological changes of the patch membrane, as well as of the whole liposome are observed and documented by time lapse series as described before. The membrane rearrangements quickly lead to the budding directed toward the interior of the GUV. Round vesicles of different diameters become visible near the patch and inside the GUV. The membrane outside the patched area provided a lipid reservoir to support this small vesicle formation. Thus the GUV shrinks while more vesicles are produced. Finally the GUV membrane detaches from the pipette and multiple vesicles are seen moving inside the GUV (Shnyrova et al., 2007).

The patch-clamp experiments emphasize the localized character of the M protein action. It is an important observation, as GUVs are notorious for their extreme sensitivity to the global changes in GUV membrane, such as introduction of area asymmetry (difference in area) between monolayers of GUV membrane. These experiments also indicate the power of M protein assembly: constitutive formation of membrane vesicles lead to building up the lateral membrane tension powerful enough to induce the GUV detachment from the pipette. Analyzing these observations provide valuable mechanistic insights into curvature-driven activity of M protein (Shnyrova et al. 2007).

Conclusions

The assays described in this chapter represent a relatively easy approach for studying the activity of cellular protein involved in controlling membrane curvature. The approach is composed of two parts. The initial analysis is based upon quantification of membrane binding and leakage for membranes of different lipid compositions. Although less spectacular than the GUV based experiments, these assays offers a significant amount of information about the mechanism of protein-lipid interaction. They can also provide ultra-structural information upon EM analysis of membrane shape transformations by bound proteins. Nevertheless, these measurements are lacking the dynamic aspects of the budding process. Microscopic observations of GUV's membrane provide the versatile tool to visualize the dynamics of membrane curvature creation. These observations reveal dynamic, transient intermediates of the budding process, such as membrane domains, which would not be resolved by structural analysis. However these observations are limited by the optical resolution of the microscope itself. In order to resolve smaller structures (i.e. less than 200 nm), as well as to confirm fission of the vesicle neck, more sensitive techniques should be used in combination with the methods described here (such as measurements of membrane area and permeability by electrical admittance measurements on planar bilayer lipid membrane. See (Shnyrova et al., 2007)). Ultimately, the combination of ultrastructural, electrophysiological and microscopy techniques form an experimental environment critical for obtaining real mechanistic insights into complex processes of membrane morphogenesis.

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Fig.1. Steps of the assay for the protein binding to IUUV by sedimentation (or flotation)

Left panel shows the photographs of the control (only IUUV) and experimental sample (IUUV plus protein sample) bands after centrifugation in ficoll gradient. The samples are then fractionated and each fraction is analyzed by measuring the Rh fluorescence at 550/590 excitation/emission wavelengths using a microplate reader. The plot on the right represents the fluorescence intensity of the small aliquots from each fraction versus the fraction number. For the case shown, the IUUV in the control (black squares) and in the assayed (grey circles) samples distribute predominantly in 2 similar populations (two fluorescence peaks). SDS-PAGE analysis of the fraction corresponding to the maximum of the smaller peak shows no significant presence of protein. Thus, the quantification of the protein binding is performed only for the fractions in the peak marked with a grey frame. The plot is normalized by subtracting the baseline (black dashed line) and dividing by the maximum value of the fluorescence peak (f_{max}). The normalized scale is shown on the right side of the plot. The detailed analysis of the protein binding is further described in the main text.

Fig. 2. Electroformation of GUV

(A) Schematic representation of the lipid film application device and the electroformation chamber with Pt electrodes. (B) Schematic representation of the setup for observation of the GUV formation and for further manipulations of the electrode-bound GUVs.

Fig. 3. Main configurations used in GUV experiments

(A) GUV remains bound to the electrode and the M protein is applied by patching the GUV membrane with a patch-pipette filled with the protein solution of choice. (B) A GUV detached from the electrode is situated on the glass bottom of the dish (pretreated with BSA) and the M protein is applied to the GUV through the patch-pipette brought in proximity to the GUV. Pulses of positive hydrostatic pressure cause protein efflux.