Video Article

Using Scaffold Liposomes to Reconstitute Lipid-proximal Protein-protein Interactions *In Vitro*

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Abstract

Studies of integral membrane proteins *in vitro* are frequently complicated by the presence of a hydrophobic transmembrane domain. Further complicating these studies, reincorporation of detergent-solubilized membrane proteins into liposomes is a stochastic process where protein topology is impossible to enforce. This paper offers an alternative method to these challenging techniques that utilizes a liposome-based scaffold. Protein solubility is enhanced by deletion of the transmembrane domain, and these amino acids are replaced with a tethering moiety, such as a His-tag. This tether interacts with an anchoring group (Ni^{2+} coordinated by nitrilotriacetic acid ($NTA(Ni^{2+})$) for His-tagged proteins), which enforces a uniform protein topology at the surface of the liposome. An example is presented wherein the interaction between Dynamin-related protein 1 (Drp1) with an integral membrane protein, Mitochondrial Fission Factor (Mff), was investigated using this scaffold liposome method. In this work, we have demonstrated the ability of Mff to efficiently recruit soluble Drp1 to the surface of liposomes, which stimulated its GTPase activity. Moreover, Drp1 was able to tubulate the Mff-decorated lipid template in the presence of specific lipids. This example demonstrates the effectiveness of scaffold liposomes using structural and functional assays and highlights the role of Mff in regulating Drp1 activity.

Video Link

The video component of this article can be found at https://www.jove.com/video/54971/

Introduction

Studying membrane-proximal protein-protein interactions is a challenging endeavor due to difficulty in recapitulating the native environment of the integral membrane proteins involved[1]. This is due to the necessity of detergent solubilization and the inconsistent orientation of proteins in proteoliposomes. In order to avoid these issues, we have employed a strategy whereby soluble domains of integral membrane proteins are expressed as His-tag fusion proteins, and these soluble fragments are anchored to scaffold liposomes via interactions with $NTA(Ni^{2+})$ headgroups at the lipid surface. Using these scaffolds, lipid-proximal protein interactions can be investigated over a range of lipid and protein compositions.

We have effectively applied this method to investigate the critical protein-protein interactions that govern assembly of the mitochondrial fission complex and examine lipid interactions that modulate this process[2]. During mitochondrial fission, a conserved membrane remodeling protein, called Dynamin-related protein 1 (Drp1)[3], is recruited to the surface of the Outer Mitochondrial Membrane (OMM) in response to cellular signals that regulate energy homeostasis, apoptotic signaling, and several other integral mitochondrial processes. This large, cytosolic GTPase is recruited to the surface of mitochondria through interactions with integral OMM proteins[4-8]. The role of one such protein, Mitochondrial Fission Factor (Mff), has been difficult to elucidate due to an apparent weak interaction with Drp1 *in vitro*. Nevertheless, genetic studies have clearly demonstrated that Mff is essential for successful mitochondrial fission[7,8]. The method described in this manuscript was able to overcome previous shortcomings by introducing simultaneous lipid interactions that promote Drp1-Mff interactions. Overall, this novel assay revealed fundamental interactions guiding assembly of the mitochondrial fission complex and provided a new stage for ongoing structural and functional studies of this essential molecular machine.

To date, examination of interactions between Drp1 and Mff have been complicated by the inherent flexibility of Mff[9], the heterogeneity of Drp1 polymers[2,10], and the difficulty in purifying and reconstituting full-length Mff with an intact transmembrane domain[11]. We addressed these challenges by using $NTA(Ni^{2+})$ scaffold liposomes to reconstitute His-tagged Mff lacking its transmembrane domain($MffΔTM\text{-}His_6$). This strategy was advantageous because MffΔTM was extremely soluble when over-expressed in E. *coli*, and this isolated protein was easily reconstituted on scaffold liposomes. When tethered to these lipid templates, Mff assumed an identical, outward facing orientation on the surface of the membrane. In addition to these advantages, mitochondrial lipids, such as cardiolipin, were added to stabilize Mff folding and association with the

membrane[11]. Cardiolipin also interacts with the variable domain of Drp1[2,12] which may stabilize this disordered region and facilitate assembly of the fission machinery.

This robust method is widely applicable for future studies that seek to evaluate membrane-proximal protein interactions. Through the use of additional tethering/affinity interactions, the sophistication of these membrane reconstitution studies can be enhanced to mimic additional complexity found at the surface of membranes within cells. At the same time, lipid compositions can be modified to more accurately mimic the native environments of these macromolecular complexes. In summary, this method provides a means to examine the relative contributions of proteins and lipids in shaping membrane morphologies to during critical cellular processes.

Protocol

1. Scaffold Liposome Preparation

NOTE: Ideally, initial experiments should use a relatively simple and featureless scaffold (comprised of DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine or PC) and DGS-NTA(Ni^{2+}) (1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl](nickel salt)). Building off of these experiments, lipid charge, flexibility, and curvature can be introduced as individual factors with the potential to alter membrane-proximal interactions. These changes can be achieved by adding defined amounts of specific lipid constituents, including phosphatidylserine or cardiolipin (CL), phosphatidylethanolamine (DOPE or PE), or galactosyl(β) ceramide.

1. Combine lipids dissolved in chloroform in a clean glass test tube. Evaporate the solvent with dry nitrogen gas while rotating the tube to form a thin lipid film. Remove residual solvent with a centrifugal evaporator for 1 h at 37 °C.
   NOTE: Various liposome formulations are used in the protocols described below: scaffold liposomes (3.3 mol% DGS-NTA(Ni^{2+}) / 96.7 mol% DOPC), scaffold liposomes with cardiolipin (3.3 mol% DGS-NTA(Ni^{2+}) / 10 mol% cardiolipin / 86.7 mol% DOPC), flexible scaffold liposomes with cardiolipin (3.3 mol% DGS-NTA(Ni^{2+}) / 10 mol% cardiolipin / 35 mol% DOPE / 51.7 mol% DOPC), and enriched scaffold liposomes (10 mol% DGS-NTA(Ni^{2+}) / 15 mol% cardiolipin / 35 mol% DOPE / 40 mol% DOPC).
2. Add Buffer A (25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 150 mM KCl, pH adjusted to 7.5 with KOH) preheated to 37 °C such that the final lipid concentration is 1 - 2 mM. Incubate 30 min at 37 °C with occasional vortexing to fully resuspend the lipid mixture (**Figure 1a**).
3. Transfer to a plastic test tube, place the tube in liquid nitrogen until completely frozen (roughly 30 s), and place in a 37 °C water bath until fully thawed (roughly 1 - 2 min). Repeat for a total of 4 freeze-thaw cycles (**Figure 1b**).
4. Prepare a lipid extruder by soaking 4 filter supports and a polycarbonate filter in buffer and assembling the extruder according to manufacturer instructions. Extrude the lipid solution through the filter 21 times. Use gentle, constant pressure to ensure a homogenous size distribution (**Figure 1c**).
   NOTE: For all experiments described in this protocol, a 1.0 µm polycarbonate filter was used for extrusion. Drp1 interaction with anionic lipids can be observed with a variety of liposome diameters ranging from 50 nm to 400 nm[12] or larger[13]. Hence, the filter size of 1 µm was chosen to be ideal for both GTPase activity and for electron microscopy. If other liposome diameters are desired, preparation of giant unilamellar vesicles[14,15] (GUVs) or small unilamellar vesicles[16] (SUVs) can be used. Dynamic light scattering can be used to assess liposome size heterogeneity[13].
5. Store extruded liposomes at 4 °C and discard after 3 - 5 d.

2. Use of Scaffold Liposomes for Protein Binding Analysis

1. Sample Preparation
   1. Incubate His-tagged MffΔTM (5 µM final) with scaffold liposomes (40 mol% PC / 35 mol% PE / 15 mol% CL / 10 mol% DGS-NTA(Ni^{2+}); 50 µM final) for at least 15 min at RT in Buffer A + BME (25 mM HEPES, 150 mM KCl, 10 mM β-mercaptoethanol (BME), pH adjusted to 7.5 with KOH). For an Mff-free control, incubate liposomes with a his-tagged control protein (such as GFP) to bind and shield exposed NTA(Ni^{2+}).
      NOTE: MffΔTM was expressed and purified as described in a previous study[2]. GFP was purified in a similar manner, but the ion-exchange step was omitted. BME was required for these experiments because Drp1 is sensitive to oxidation, which can alter its activity and assembly properties.
   2. Add Drp1 (2 µM final) and incubate for 1 h at RT.
      NOTE: Drp1 was expressed and purified as described in a previous study[2]. After incubation with Drp1, the effect of nucleotide binding on membrane deformation can be investigated by incubating one additional hour with 2 mM $MgCl_2$ and either 1 mM GTP, 1 mM GMP-PCP, or Buffer A + BME.

2. Negative Stain Transmission Electron Microscopy (EM) Analysis
   1. Transfer 5 µL of sample to a sheet of laboratory film, and lay a carbon-coated Cu/Rh grid on the sample. Incubate the grid 1 min on the sample, blot away excess liquid on filter paper, and transfer to a drop of 2% uranyl acetate. Incubate 1 min, blot excess stain on filter paper, and transfer to a grid box. Store under vacuum O/N to ensure full desiccation.
   2. Image samples using a transmission electron microscope at 18,500 - 30,000X magnification to observe ultrastructural changes in protein and liposome morphologies[17].
      Note: Ultrastructural changes can be quantified using image analysis software, such as ImageJ[13] (http://imagej.nih.gov/ij/). Protein decoration can be measured when compared with naked lipid templates. Additionally, the diameters of tubular segments can be measured from the outermost portion of the assemblies[13]. A more detailed analysis can be performed using cryo-electron microscopy[17]. This method can be used to image native protein-lipid assemblies in solvent without the use of heavy metal stains

that coat the sample. In this way, detailed structural features not apparent in negative stain, including changes in the underlying lipid morphology, can be examined and quantified.

3. Use of Scaffold Liposomes for Enzymatic Assay

Note: A colorimetric GTPase assay[18] was used to measure phosphate liberation via GTP hydrolysis. Alternative GTPase assays are available[19] and can be implemented as needed.

1. Incubate His-tagged MffΔTM (Mff), Fis1ΔTM(Fis1), or GFP (5 μM final for all) with scaffold liposomes (150 μM final) for 15 min at RT in Buffer A + BME (volume = 30 μL). Add Drp1 (500 nM final) and incubate an additional 15 min at RT (volume = 80 μL).
   NOTE: Fis1 was purified in a similar manner to Mff[2], but the ion-exchange chromatography step was omitted. The purpose of His-tagged GFP is to shield the NTA(Ni^{2+}) headgroups and prevent nonspecific charge interactions with other proteins. If no effect is observed in the absence of GFP, then this control may not be required. Alternative blocking proteins (of comparable size to the protein of interest) can be used as well, but GFP allows for direct visualization of the interactions with scaffold liposomes.
2. Transfer tubes to a thermocycler set to 37 °C, and initiate reactions by addition of GTP and $MgCl_2$ (1 mM and 2 mM final, respectively; volume = 120 μL).
3. At desired time points (*i.e.* T = 5, 10, 20, 40, 60 min), transfer 20 μL of reaction to wells of a microtiter plate containing 5 μL of 0.5 M EDTA to chelate Mg^{2+} and stop the reaction.
4. Prepare a set of phosphate standards by diluting KH_2PO_4 in Buffer A + BME to calibrate results. A useful set of standards is 100, 80, 60, 40, 20, 10, 5, and 0 μM. Add 20 μL of each to wells containing 5 μL of 0.5 M EDTA.
5. Add 150 μL of Malachite green reagent (1 mM malachite green carbinol, 10 mM ammonium molybdate tetrahydrate, and 1 N HCl) to each well, and read OD_{650} 5 min after addition.
   NOTE: GTP is acid labile, and will hydrolyze in the presence of malachite green reagent. Ensure that the time between adding malachite reagent and reading is constant to ensure reproducible results.
6. Generate a standard curve by plotting OD_{650} of the standards as a function of phosphate concentration. Use linear regression to determine the relationship between OD_{650} and phosphate concentration in a sample.
7. Using the linear regression, convert the OD_{650} of the protein reaction samples to μM phosphate. Determine the rate of phosphate generation for each reaction mixture by plotting phosphate concentration as a function of time, and convert to k_{cat} by dividing the rate by the Drp1 concentration (0.5 μM).
   NOTE: Only the initial linear rate should be used to determine the rate of phosphate generation, and a minimum of 3 data points must be used. If the rate of reaction is sufficiently rapid that the first three data points are not linear (*i.e.* the r^2 of the linear fit is less than 0.9) a significantly shorter time course with at least 3 time points should be performed.

Representative Results

While the interaction between Drp1 and Mff has been demonstrated to be important for mitochondrial fission, this interaction has been difficult to recapitulate *in vitro*. Our goal was to better emulate the cellular environment wherein Drp1 and Mff interact. To this end, liposomes containing limiting concentrations of NTA(Ni^{2+}) headgroups were prepared by rehydrating a lipid film as described above. The lipid solution initially consists of unilamellar and multilamellar vesicles of heterogeneous diameters as evidenced by the opacity of the solution (**Figure 1a**). This opacity is reduced by freeze-thawing (**Figure 1b**), which reduces the prevalence of multilamellar vesicles. The liposome diameters are further homogenized by extrusion through a polycarbonate filter, which results in a clear solution (**Figure 1c**).

In previous studies, we found that Drp1 was able to assemble on Mff-decorated scaffold liposomes, and membrane tubulation was observed when flexible membranes were employed[2]. Building on these findings, we utilized a new template composed of PC, PE, Ni, and CL (called Enriched Scaffold Liposomes or ESL) to promote ordered assembly of a polymeric Drp1-Mff complex capable of inducing membrane deformation. Specifically, increased NTA(Ni^{2+}) and cardiolipin lipids were utilized (10 mol% and 15 mol% respectively) for this application. Then, GFP or Mff was tethered to ESL templates in the presence and absence of Drp1 (**Figure 2**), and the ability of Drp1 to remodel membranes was qualitatively assessed. In the absence of Drp1, neither Mff nor GFP resulted in membrane deformation (**Figure 3a, b**), and similarly in the case of GFP-decorated ESL, only featureless liposomes were observed (**Figure 3c**). However, when Drp1 was added to Mff-decorated ESL templates, remodeling of the liposomes was evident (**Figure 3d**).

While macromolecular complex formation clearly demonstrates an interaction between Drp1 and Mff, this qualitative analysis alone is incapable of determining the functional effects of such an interaction. Therefore, we utilized a malachite green phosphate generation assay[18] to assess alterations in the catalytic activity of Drp1 in response to interaction with Mff. As described previously[2], we initially utilized a simple scaffold liposome (SL; 3.3 mol% DGS-NTA(Ni^{2+}), 96.7 mol% DOPC) to investigate the effect of Mff alone on Drp1 structure and function. Nonspecific interaction of Drp1 with NTA(Ni^{2+}) has previously been described[20], so SL was initially designed to contain low concentrations of NTA(Ni^{2+}) to avoid nonspecific activity stimulation of Drp1. With the larger amounts of NTA(Ni^{2+}) in ESL, the use of His-tagged GFP as a control was found to be critical to shield the Ni^{2+} and prevent non-specific Drp1 interactions. After decoration of SL liposomes by Mff or GFP (as illustrated in **Figure 2**), the extent of self-assembly can be assessed by measuring the GTPase activity of Drp1. In the absence of liposomes, Drp1 has a relatively low basal GTPase activity, which is slightly enhanced by addition of SL. Decoration of these scaffold liposomes with Mff enhanced GTPase activity (**Figure 4a**, 1.8 fold). Conversely, when the exposed NTA(Ni^{2+}) headgroups were blocked with His-tagged GFP, this augmented GTPase activity was ablated. We also tested the role of Fis1, an OMM protein that has been suggested to have a role in mitochondrial fission[21,22], though this has been challenged in recent studies[7,23]. Tethering of Fis1 lacking its transmembrane domain to SL also failed to elicit a stimulation of Drp1's GTPase activity (**Figure 4a**).

We then utilized a slightly more complex lipid scaffold containing a small amount of cardiolipin (SL/CL: SL with 10 mol% cardiolipin replacing DOPC) to determine the role of this mitochondrial lipid in the interaction of Drp1 and Mff. This moderate concentration of cardiolipin was specifically chosen to limit the stimulation of Drp1 by cardiolipin as described previously[10]. Similar to SL, addition of SL/CL to Drp1 resulted in a slight stimulation of GTPase activity that was reversed by tethering His-tagged Fis1 or GFP to the liposomes. A synergy between Mff and cardiolipin was observed as the GTPase activity of Drp1 was stimulated 2.6 fold when it was incubated with Mff-decorated SL/CL (**Figure 4b**).

Membrane fluidity and the ability of Drp1 to remodel lipid bilayers have been proposed to enhance its GTPase activity. Therefore, we sought to examine the effect of membrane fluidity/flexibility using a flexible scaffold liposome. This was achieved by replacing 35 mol% of DOPC in SL/CL with DOPE (SL/PE/CL), which has previously been shown to allow for Drp1-mediated membrane remodeling[10]. Addition of undecorated SL/PE/CL scaffold liposomes to Drp1 slightly enhances Drp1 GTPase activity, and decoration of these liposomes with GFP eliminates this effect. When SL/PE/CL templates were decorated with Mff, Drp1 activity was enhanced (**Figure 4c**, 2.4 fold). As we have previously shown, the ability of Drp1 to remodel liposomes into lipid tubules was enhanced by the addition of PE to the scaffold liposomes. Interestingly, this improved tubulation leading to the formation of a helical Drp1 polymer did not result in any greater stimulation when compared to liposomes that Drp1 was unable to remodel[2].

Using these adaptable lipid templates, Mff and Drp1 were found to interact in a more native environment *in vitro*. This technique has enabled us to control the relative abundance of Drp1, Mff (through NTA(Ni^{2+}) concentration), and specific lipids (cardiolipin and PE specifically) that appeared to regulate the assembly of this macromolecular complex. As we have demonstrated, this method can be utilized to visualize the membrane remodeling of Mff-recruited Drp1 by electron microscopy, and to determine the effects of Drp1 assembly on its catalytic activity using GTPase activity assay.

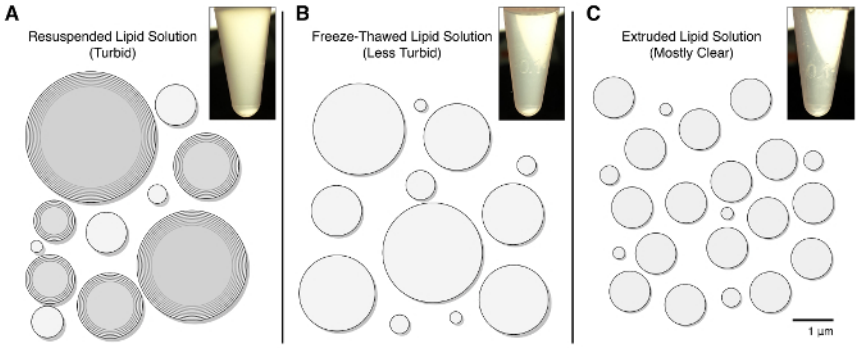

Figure 1: Lipid Preparation Schematic. (**a**) Upon resuspension, liposomes of diverse sizes form and consist of unilamellar and multilamellar vesicles, which results in an opaque solution (inset). (**b**) Freeze-thawing the solution results in a more unilamellar population of liposomes, which are still heterogeneous in diameter. Freeze-thawing clarifies the solution (inset). (**c**) Extrusion of the lipid solution homogenizes the liposome diameter (1.0 µm in this example), and results in a clear solution (inset). Please click here to view a larger version of this figure.

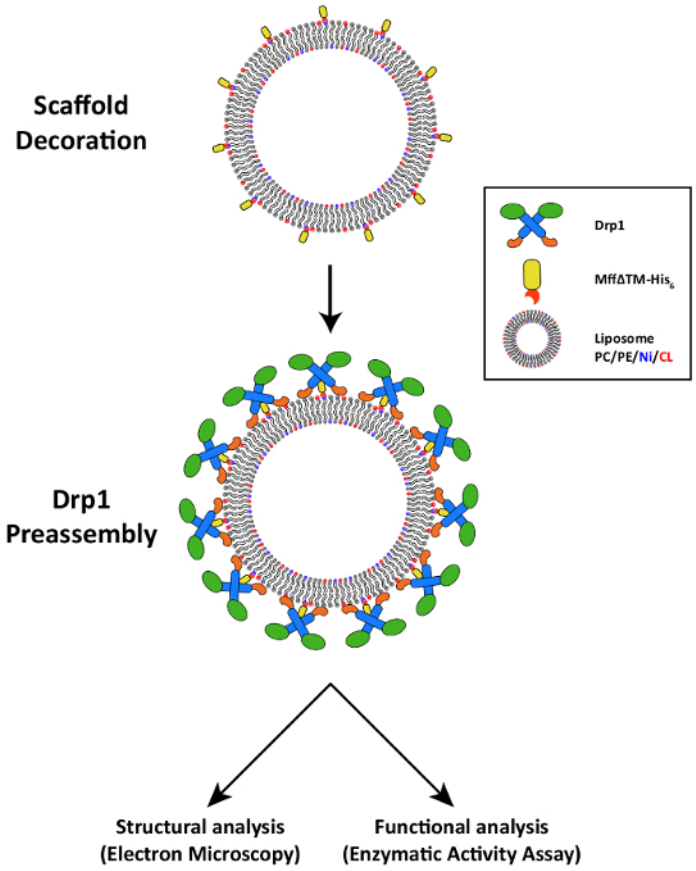

Figure 2: Methods to Assess Protein assembly. A schematic depicting partner protein assembly on scaffold liposomes is presented. His-tagged partner proteins or GFP are incubated with scaffold liposomes, and then Drp1 is incubated with decorated or undecorated liposomes. These Drp1-preassembled liposomes can then be analyzed by structural methods (electron microscopy) and functional assays (GTPase assay). Please click here to view a larger version of this figure.

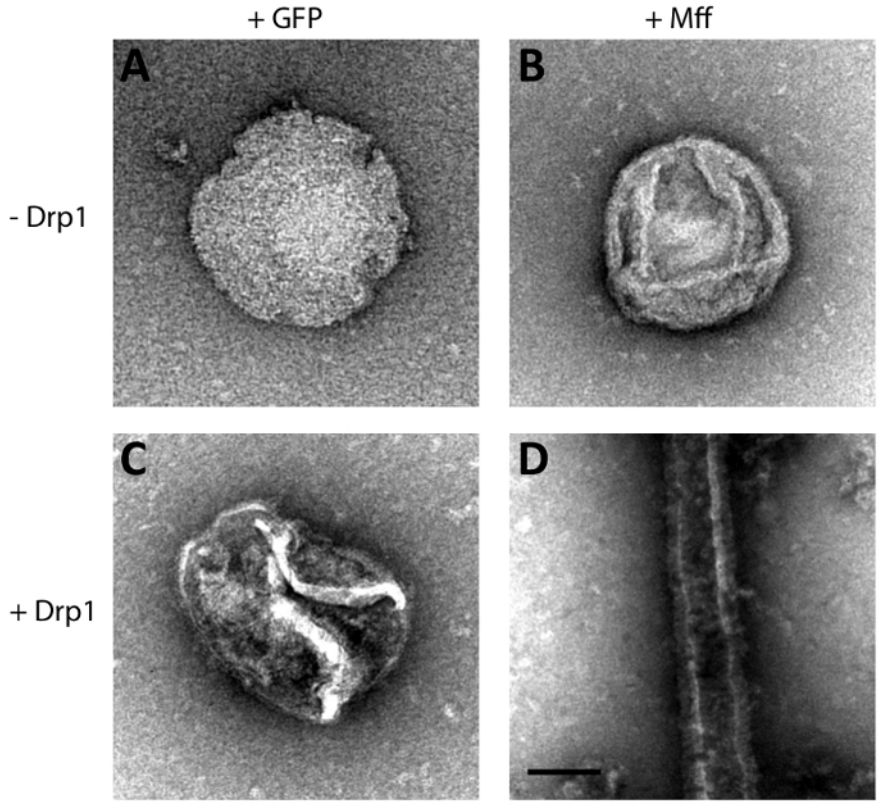

Figure 3: Structural Assessment of Drp1 Recruitment. Negative stain transmission micrographs of GFP or Mff decorated liposomes alone (**A, B**, respectively) or incubated with Drp1 (**C, D**, respectively). Scale bar = 100 nm. Please click here to view a larger version of this figure.

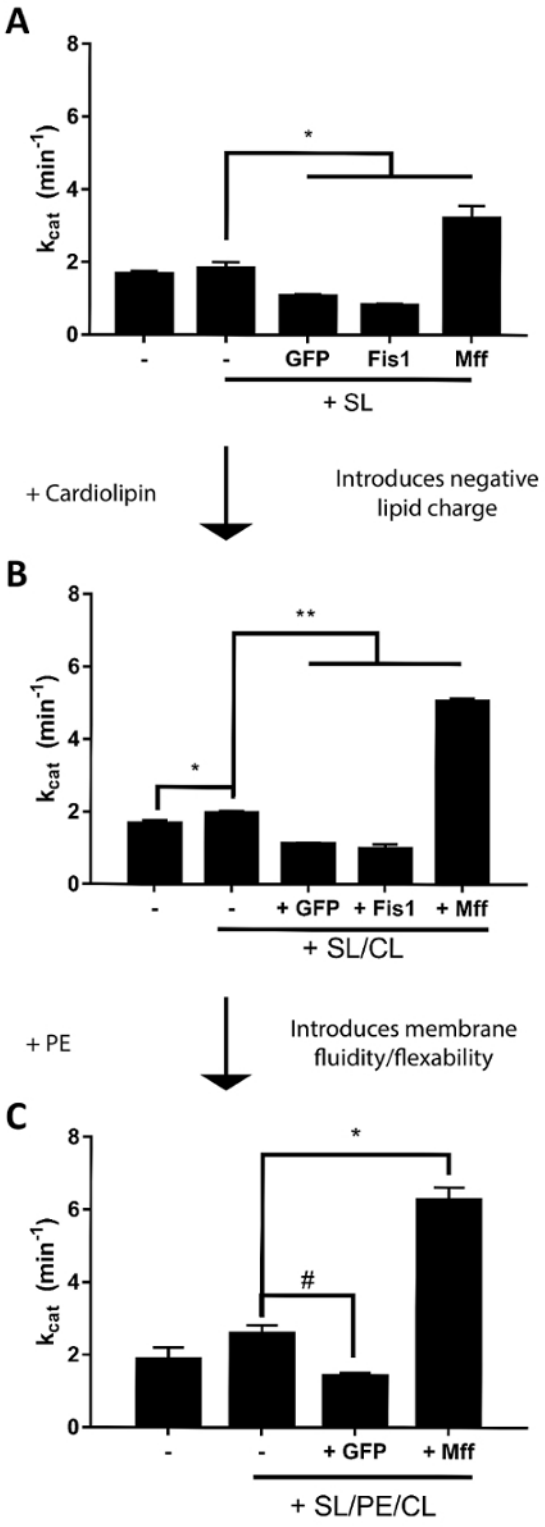

Figure 4: Scaffold Liposome Enzymatic Assay. (**A - C**) The generation of phosphate over time was measured (inset), and the k_{cat} was determined. This method was applied to SL-tethered proteins (**A**), SL/CL-tethered proteins (**B**), or SL/PE/CL-tethered proteins (**C**). #: $p < 0.05$, *: $p < 0.0001$, **: $p < 0.000001$ as determined by unpaired student's T-test. All error bars represent standard deviation from 3 independent samples. Please click here to view a larger version of this figure.

Discussion

This protocol offers a method for investigating protein-protein interactions involving integral membrane proteins. By utilizing a modular liposome scaffold, investigators are capable of assessing the activity of one or more proteins in a lipid-proximal environment. Previous studies have demonstrated a similar method for receptor enzymes of the plasma membrane[24-26]. We have expanded this method to incorporate lipid cofactors and explore interactions between proteins that make up the mechanoenzymatic core of the mitochondrial fission machinery.

For the model system presented above, we found that Mff-decorated SL enhanced Drp1 self-assembly. Moreover, we now show that, Mff-decorated ESL templates were efficiently remodeled by wild-type Drp1 to form extended tubular structures. We also assessed the roles of various mitochondrial lipids, including the negatively charged cardiolipin and the conical lipid PE. Cardiolipin synergizes with Mff to further enhance Drp1 self-assembly, while the membrane flexibility and fluidity imparted by PE enhances membrane tubulation but does not further augment Mff-induced stimulation.

To assess ultrastructural changes in membrane morphologies, EM analyses were required. Drp1 GTPase activity was elevated through clustering and assembly of filamentous polymers that did not reshape the liposomes to any great extent[2]. However, membrane deformation was observed when the more mitochondria-like SL/PE/CL template was used. Interestingly, the enzyme activity was not enhanced. Therefore, the EM studies were essential in identifying key differences that would otherwise be missed using the functional assay.

While this technique is powerful for exploring the function and interaction of soluble proteins and soluble protein domains, these lipid scaffolds cannot account for the role of transmembrane domains. This is an important consideration because the transmembrane domain can effect dynamic protein processes such as self-assembly[27] and lateral diffusion[28-30] in lipid bilayers. If these factors are critical for evaluating protein interactions at the membrane surface, then traditional lipid reconstitution experiments with detergent would be favored. Alternative tethers may also be explored to control the recruitment and mobility of the membrane anchored proteins.

In addition to using His-tagged proteins with NTA(Ni^{2+}) lipid anchors, other tethers such as biotin-conjugated[31] or reactive group-conjugated lipids can be utilized. These covalent modifications would more stably trap proteins at the lipid surface, but mobility and exchange of these factors would likely be diminished. As such, the tether should be carefully considered in the context of the protein complexes being studied. When considering the use of this method, the mode of tethering proteins to lipid templates have the potential to influence certain assays. For instance, the His-tag tethering to NTA(Ni^{2+}) method may be more appropriate for *in situ* assays rather than separation experiments, especially in the case of transient protein-protein interactions. This is clearly demonstrated in **Figure 3** by the discrepancy between the *in situ* negative stain electron microscopy and the sedimentation assay.

In the future, a combination of two or more of these anchor lipids with distinct target headgroups could be implemented to allow for recruitment of multiple proteins to a scaffold template without competition for a single lipid tether. Moreover, the relative abundance of each component can be managed by altering the lipid composition. Additional lipid cofactors, such as phosphoinositides, cardiolipin, and phosphatidylserine, can easily be introduced in these templates to assess the isolated impact of a variety of factors.

Overall, these lipid scaffolds represent a novel platform for investigating complex protein interactions near lipid membranes. These templates are easily generated and are simple to tailor to a range of diverse applications, including enzymatic assays, electron microscopy, or fluorescence imaging. In addition, the lipid composition can be formulated to resemble an organelle or membrane microdomain of interest to better recapitulate protein function at these specific regions. Using these techniques, biochemists can probe the complex interactions of membrane bound and membrane associated proteins with their partners and their environment.

Disclosures

The authors have nothing to disclose.

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