Chapter 4: Standardization of methods to incorporate GPI analogues into plasma membrane of live cells

4.1 Introduction

In literature very few methods are reported for incorporating lipids into the plasma membrane of live cells. Amongst these, a common method of lipid transfer involves the use of lipid vesicles. In this case, vesicles containing the lipid of interest are incubated with the cell membrane at low temperature to transfer them to the plasma membrane (Mayor et al., 1993). However in addition to the desired lipid, other lipids on the vesicles may also get incorporated into the cell membrane and therefore reduces the specificity of the purpose. Another frequently used method of lipid incorporation is based on lipid binding proteins, namely bovine serum albumin (BSA). This protein contains lipid pockets to which lipids can bind (number depending on the length of lipid chain length) and when incubated with the cell membrane can exchange it with the lipids in the membrane (Martin and Pagano, 1994). More recently Mirkka Koivusalu et al have utilized gamma-cyclodextrin (γ-CD) to incorporate sphingomylein of different chain length into cell membranes in vivo (Koivusalo et al., 2007).

The GPI analogues, with variations in the glycan head group, acyl chain hydrophobicity or polarity of the attached fluorophore has brought about difficulty in utilizing a single method for its incorporation into live cell membranes. Therefore different lipid incorporation methods were optimized for incorporating various GPI analogues into live cell membrane. During this process, a novel method of lipid incorporation using commercially available lipofectamine reagent was identified in our laboratory.
4.2. Methods of lipid incorporation

4.2.1 BSA method: Bovine serum albumin (BSA) contains hydrophobic fatty acid binding pockets to stably incorporate lipid molecules (Mukherjee et al., 1999). BSA-lipid complex formation is dependent on the acyl chain length of lipid molecules. Longer the chain length, greater is the ability to complex with BSA and lower is the ability to transfer the lipid molecules into the membrane. For example, the ratio of BSA:lipid for lipids with short acyl chain length (C8:0 to C12:0) is 1:2 whereas the ratio for long acyl chain length lipid (C14:0 to C18:0) (Spector et al., 1969; Eggeling et al., 2009) is 2:1. In my study, BSA method was identified as an appropriate vehicle to efficiently transfer GPI analogues carrying short acyl chains (Figure 4.3A) into the plasma membrane of live cells.

4.2.2 Gamma-cyclodextrin (γ-CD) method: γ-CD is a cyclic oligosaccharide made up of eight α-D-glucopyranoside units. The glucose units are linked to each other by means of 1-4 linkages such that it maintains hydrophobic within and hydrophilic outside the cyclic ring. This feature makes it amenable to carry hydrophobic lipids and was previously reported as an efficient molecule to transfer lipids especially with long acyl chains (>C16:0) into live cell membranes (Koivusalo et al., 2007) (Figure 4.3B).

4.2.3 Lipofectamine method: Lipofectamine is a commercially available transfection reagent, a mixture of polycationic lipid DOSPA (2,3-dioleoyloxy-N[2(sperminecarboxamido)ethyl]-N,Ndimethyl-1-propanaminium trifluoroacetate) and neutral co-lipid DOPE (dioleoyl phosphatidylethanolamine) that has been shown to assist nucleic acid molecules to traverse through the plasma membrane. A low concentration (8 µg/ml or less) of lipofectamine required to efficiently transfer GPI analogues into live cell
plasma membrane and at the same time do not perturb the native membrane organization of endogenous GPI-APs (Figure 4.3C) was optimized.

4.3 Methods to get rid of lipid sticking on coverslips:

Lipids can bind to traces of paraffin wax (used to seal the glass coverslip to the petri dish) or cell debris (dead cells) on the glass coverslip leading to non-specific sticking. Fluorescence signals from non-specific sticking of fluorescent GPI analogues or lipids to organic residues on glass coverslips can lead to a serious error in the anisotropy calculated and consequently lead to incorrect predictions of the result. Thus it is very important to prevent non-specific sticking and reduce the background signals as low as possible. For this purpose, different methods were utilized and are detailed below.

4.3.1 Cleaning coverslips with chemicals:

4.3.1.1 Piranha Solution: A 3:1 mixture of concentrated sulfuric acid and hydrogen peroxide forms a strong oxidizing agent and helps to remove organic residues from the glass cover slip. This solution is prepared fresh every time. Cover slips were incubated in piranha solution for 30 minutes followed by thorough cleaning with double distilled water and finally PBS buffer to neutralize the pH.

4.3.1.2 Hellmanex\textsuperscript{III} is a strong alkaline, commercially available reagent that can be used to clean the surface of glass cover slips and cuvettes. Basically, the cover slips were incubated with 1% Hellmenax\textsuperscript{III} (in ion free or double distilled water) at room temperature for 45 minutes, washed thoroughly with copious amounts of double distilled water and then finally with PBS buffer.
The cover slips treated with above mentioned methods were air dried, exposed to UV and then plated with cells for anisotropy measurements.

4.3.2 Replating method: Certain lipids namely those tagged to Bodipy\textsuperscript{TMR} dye were found to non-specifically stick to glass coverslips. Cleaning the cover slips with chemicals may not minimize non-specific sticking. In this case, the cells post lipid incorporation was detached from the coverslip by incubating in 5 mM EDTA (in M1 without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}). These cells were re-adhered on to fresh cover slips that were coated with fibronectin (aids in re-adhering faster) (Figure 4.1.A).

Coating glass coverslips with fibronectin: Briefly, the coverslips were incubated with 10 \( \mu \text{g/mL} \) of fibronectin at 37\( ^\circ \text{C} \) for 1 hour or 4\( ^\circ \text{C} \) overnight. Fibronectin was gently removed and washed once with PBS before plating cells.

EDTA treatment for detaching cells: The cells incorporated with fluorescent GPI analogues or lipids were incubated with 5 mM EDTA solution (in Ca\textsuperscript{2+} and Mg\textsuperscript{2+} free M1 or PBS, pH 7.2-7.4) for 5-10 minutes at 37\( ^\circ \text{C} \). During this period, the cells rounded up, detached and started floating in the buffer. These cells were gently transferred into 1.5 mL eppendorf tubes and centrifuged at 82 g for 10 minutes at 4\( ^\circ \text{C} \). The supernatant was carefully removed and the cell pellet was re-suspended in HF-12 media and seeded on to fibronectin-coated dishes followed by incubation at 37\( ^\circ \text{C} \) for 30 minutes. During this incubation time, the cells settle down on the glass coverslip and re-adhere completely which could then be used for anisotropy measurements (Figure 4.1.B). During 37\( ^\circ \text{C} \), the fluorescent lipids incorporated into plasma membrane of cells underwent endocytosis thereby marking the various endocytic compartments. Co-incubating the cells with known
endocytic markers (transferrin, Tf for clathrin and dextran for GPI-AP enriched early endosomes) allowed marking of the endocytic compartments, which were then excluded for anisotropy measurements. Therefore anisotropy of GPI analogues or lipids were calculated by marking 10-13 µm² regions on the cell membrane that were devoid of endocytic pool.

In certain cases, a combination of the above mentioned methods (4.3.1 and 4.3.2) were helpful in overcoming the problem of non-specific sticking (Figure 4.2).

4.4 Protocols for incorporating GPI analogues into plasma membrane of live cells.

In each method mentioned below, prior to lipid incorporation, the cells were incubated in HF-12 media for 15 minutes at 37°C to get rid of serum components (capable of capturing lipids) (Saha et al., 2015).

4.4.1 BSA-GPI complexes: This complex is prepared by vigorous mixing or probe sonication (3 x 2 sec) of 20 µM fatty acid free BSA in Hams F12 media with desired lipid molecules such that the final BSA-lipid complex ratio is at 1:x (x > 1 for short lipid tails C8-C12 and x < 1 for long lipid tails). The complexes are then incubated on cells for 30 minutes on ice and at 37°C for short and long acyl chain length containing GPI analogues respectively (Figure 4.3 A). The same concentration of BSA however did not have an effect on the native membrane organization of endogenous GPI-APs. IA2.2F cells with or without 200 µM unloaded BSA, incubated with PLBFL and the anisotropy was measured (Figure 4.4 B).
4.4.2 γ-CD-GPI complexes: γ-CD was dissolved in HF-12 media at milli Molar concentration and mixed with lipids at micro Molar concentrations such that the final γ-CD:lipid molar ratio is 1000:1. This mixture was then probe sonicated for 3 x 2 sec and this step was repeated thrice. The γ-CD-lipid complexes formed were incubated with cells at 37°C for 30 minutes (Figure 4.3 B). The native organization of GPI-APs remains unperturbed when treated with 10 mM γ-CD suggesting that (Figure 4.4 A) it has no effect on the native organization of GPI-APs. For this assay, I incubated the cells with 10 mM γ-CD at 37°C for 30 minutes and then subjected to anisotropy measurements.

4.4.2.1 Comparison of the efficiency of BSA and γ-CD to incorporate lipids with long acyl chains.

Assuming that the mechanism of lipid incorporation for both GPI analogues and phosphatidylethanolamine (PE) is dependent only on its acyl chain length, commercially available Bodipy<sup>FL</sup> tagged dihexadecanoyl phosphatidylethanolamine (Bodipy<sup>FL</sup>-DHPE, invitrogen) was utilized to assess the efficiency of different lipid incorporation methods. Briefly, the same concentration of Bodipy<sup>FL</sup>-DHPE (50 µM) was complexed with BSA (200 µM) or γ-CD (10 mM). These complexes were then incubated with cells for 30 minutes at 37°C, washed and fluorescence intensity images acquired. The concentration of DHPE incorporated by γ-CD method was found to be higher than that by BSA method. The images with different camera exposure settings (higher exposure for BSA incorporated DHPE as compared to γ-CD incorporated DHPE) were utilized to obtain images at the similar intensity regime (Figure 4.5), suggesting that the γ-CD is a better vehicle than BSA for incorporating long acyl chain containing lipids into the cell membrane.
4.4.3 Methods to prepare lipofectamine-GPI complexes and its incorporation into cell membrane: 20 µL of 18 µg/mL of lipofectamine reagent (diluted in HF-12 medium) was added to GPI lipids in a glass vial and incubated at room temperature for 30 minutes. The solution is then made up to 100 µL to give lipofectamine-lipid complexes. This mixture is further diluted to desired lipid concentrations prior to use. The cells were incubated with lipofectamine-GPI complexes at 10°C for 10-30 minutes, dependent on the acyl chain length (lower and higher incubation times for short and long acyl chains respectively) (Figure 4.3 C).

γ-CD was utilized to efficiently incorporate BodipyTMR tagged GPI analogues whereas lipofectamine was utilized for NBD tagged GPI analogues (Figure 4.3).

4.4.3.1 Effect of Lipofectamine on the membrane organization of folate receptor (a GPI-AP)

Since lipofectamine contains a mixture of cationic and neutral lipids, it was important to check if lipofectamine has an effect on the clustering properties of the cell membrane. Since any change in the membrane property is reflected in the nanoclustering of GPI-AP, post lipofectamine treatment the anisotropy of folate receptor was measured. The anisotropy of folate receptor (PLB\textsuperscript{TMR} bound) was found to be similar (Figure 4.6) in case of lipofectamine treated and untreated cells suggesting that the concentration of (18 µg/mL) lipofectamine used to incorporate lipids does not affect the native membrane organization of GPI-APs. Alternatively the diffusion coefficients of PLB\textsuperscript{FL} bound folate receptor (IA2.2F cells) (Figure 4.7) pre-incubated with or without 18 µg/mL lipofectamine was also measured. It was observed that the diffusion coefficient of folate receptor remains
unchanged in both the conditions, suggesting that the properties of the cell membrane remains unperturbed when treated with lipofectamine reagent. Additionally it was also confirmed that the endocytic uptake and fate of GPI-APs are unperturbed on pretreatment with 18 µg/mL lipofectamine (Figure 4.8).

Plasma membrane incorporated GPI analogues were sensitive to cleavage by exogenously added PI-PLC (phosphatidylinositol specific phospholipase C). PI-PLC enzyme is known to cleave GPI at the phosphodiester bond. The loss of fluorescence (for head group tagged GPI analogues) and trafficking of cleaved DAG to intracellular compartments (for lipid acyl chain tagged GPI analogues) is a direct measure of PI-PLC cleavage (Figure 4.9), thus confirming that it is retained at the outer leaflet of the plasma membrane.

4.5 Conclusions

In this chapter I have

1. Identified γ-CD as an appropriate vehicle to efficiently incorporate GPI analogues into plasma membrane of live cells in comparison to BSA for lipids containing long acyl chains.

2. Commercially available Lipofectamine reagent was identified as a novel method for lipid incorporation into live cell membrane.

3. Confirmed that the concentration of BSA, γ-CD or lipofectamine used for incorporating GPI analogues into plasma membrane of CHO cells, does not affect the plasma membrane composition or fluidity, as measured by the anisotropy, endocytic fate and diffusion characteristics of the folate receptor (GPI-AP).
4. GPI analogues incorporated using BSA, γ-CD or lipofectamine methods retained them in the outer leaflet of plasma membrane.

5. Standardized methods to minimise non-specific sticking of Bodipy tagged GPI analogues on to the glass coverslips.
**Figure 4.1**

**A**

- **i** lipid:BSA or γ-CD complexes at 37°C for 30 minutes
- **ii** 5mM EDTA in PBS at 37°C for 10 minutes
- **iii** cells replated on fibronectin costed dishes for 30 minutes at 37°C
- **iv**

**B**

- PLB<sup>TMR</sup>
- FITC dextran
- A647- transferrin
- Colour combine

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**Replating Method. A)** Schematic representation of replating method depicted at the level of single cell i) cell (pink) grown on coverslip dishes for 24 hours are well adhered. Post incubation with HF-12 to remove serum components, it is incubated with lipid:BSA or γ-CD complexes for 30 minutes at 37°C ii) the cells (magenta) are now incorporated with lipid molecules and then prepared for de-adhering by incubation with 5mM EDTA 10 minutes at 37°C. iii) Due to this, the cells round up and float in the buffer when they are collected, centrifuged at 1000 rpm for 10 minutes, pellet suspended in HF-12 and incubated on fibronectin (blue) coated dishes for re-adhering iv) depicts lipid incorporated cell readhered onto fibronectin coated coverslip. **B)** TIRF images of IA2.2F cells labeled with PLB<sup>TMR</sup> and re-plated on to fibronectin coated coverslip dishes.
To differentiate the internalized pool of folate or GPI lipids (red) from that on the plasma membrane, the cells are incubated with endocytic markers such as FITC dextran (green) and Alexa-643 Transferrin (magenta) during the process of replating. The color combine clearly differentiate between the membrane from the endocytic pool. The anisotropy analysis is obtained from 10 x 10 pixel boxes (depicted in the colour combine) that are devoid of internal endocytic pool.
Flow chart of the complete protocol for FR-GPI labeling/GPI analogue incorporation: The entire processes of FR-GPI labeling or lipid incorporation in combination with the different cover slip cleaning methods are depicted in a flow chart.
Figure 4.3:

A  BSA method       B  γ-CD method       C  Lipofectamine method

Figure 4.3: Images of cells incorporated with lipids using different methods:

Bodipy<sup>TMR</sup> labeled DHPE lipids were incorporated into IA2.2F cell by A) BSA method or B) γ–CD method and GlcNPI-NBD lipid by C) lipofectamine method.
The concentration γ-CD or BSA used to incorporate lipids does not affect the native clustering properties of folate receptor: IA2.2F cells were either treated with or without BSA/ Lipofectamine or subsequently labeled with PLB\textsuperscript{FL} on ice. These cells were washed and anisotropy was measured using TIRF microscopy. The graph A) represents anisotropy of PLB\textsuperscript{FL} with (blue diamonds) or without γ-CD incubation (green diamonds) B) represents anisotropy of PLB\textsuperscript{FL} with (red diamonds) or without BSA incubation (green diamonds) C) Gray scale images of cell labeled with PLB\textsuperscript{FL} obtained for above measurements.
Figure 4.5:

Comparisons of γ-CD method and BSA method for lipid incorporation. Bodipy^{FL}-DHPE (50 µM) was incorporated into IA2.2F cells either using γ-CD (red diamonds) or BSA (cyan diamonds) and anisotropy measured in each case, using the TIRF microscope. The lipid incorporation for γ-CD is evidently higher than BSA method and hence in the former case the images were acquired at lower (20 ms exposure for γ-CD lipid incorporated cells) camera exposure settings compared to the latter (100 ms exposure for BSA lipid incorporated cells).
**Figure 4.6:**

**Lipofectamine treatment does not affect the membrane organization of Folate receptor.** Gray scale images and graphs representing anisotropy of IA2.2F cells treated with and without lipofectamine (18 μg/mL) at 10°C for 30 minutes followed by labeling with PLB\textsuperscript{TMR}. The cells were also subjected to cholesterol depletion by saponin. The results indicate no change in average value of anisotropy for PLB\textsuperscript{TMR} bound folate receptor when treated with or untreated with lipofectamine. On cholesterol depletion, the anisotropy values increase to the same extent in both the treated and untreated case.
**Figure 4.7:**

A  
PLB^FL (control)  
PLB^FL (lipofectamine)

B

<table>
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<tr>
<th>N</th>
<th>Sample</th>
<th>Diffusion time scales (ms)</th>
<th>Diffusion coefficient (μm^2 s^-1)</th>
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<td>5</td>
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<td>273.5814 + 24.89</td>
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<tr>
<td>5</td>
<td>GFP</td>
<td>0.088 + 0</td>
<td>85.55 + 0</td>
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<td>PLB^FL</td>
<td>3.12 + 0.45</td>
<td>2.47 + 0.33</td>
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<td>4</td>
<td>PLB^FL+ lipofectamine</td>
<td>3.18 + 0.18</td>
<td>2.42 + 0.39</td>
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</table>

**FCS measurements on lipofectamine treated IA2.2F cells.** Cells were treated with or without lipofectamine prior to labeling with PLB^FL and then subjected to
Figure 4.8:

A

<table>
<thead>
<tr>
<th></th>
<th>Dextran</th>
<th>Anti-Transferrin</th>
<th>Transferrin</th>
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<tr>
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Fluid uptake and Transferin uptake graphs.

B

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<tr>
<th>PLB&lt;sup&gt;FL&lt;/sup&gt;</th>
<th>TMR Dextran</th>
<th>A647-Transferrin</th>
<th>Color combine</th>
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<td><img src="treated.png" alt="Image" /></td>
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Lipofectamine at 18ug/mL does not affect the endocytic trafficking of folate receptor. IA2.2F cells were pulsed with TMR-Dextran and Alexa-488 Transferrin for 2 minutes at 37°C. These cells were cooled on ice, then stripped the surface bound Transferrin using ascorbate buffer and marked the surface Transferrin receptor (TfR) using Anti-TfR antibody (OKT9) followed by Alexa-647 labeled mouse secondary antibody. The cells were then fixed and subjected to whole cell measurements (20X). A) Gray scale images of cells with internalized TMR Dextran, Alexa-488 Transferrin and Alexa-647 labeled secondary antibody. These images are processed and analysed quantitatively to obtain the graphs below. In order to check if lipofectamine induces any alteration in the general trafficking route of folate receptor, IA2.2F cells were incubated with PLB<sup>FL</sup>, Alexa-643 Transferrin and TMR-dextran for 1 hr at 37°C washed and immediately imaged at 60X. B) In untreated case, PLB<sup>FL</sup> endosomes co-localize with Transferrin in the recycling endosomes located at the perinuclear region whereas the TMR-Dextran localize to the late endosomes, as reported earlier<sup>59</sup>. The fate of PLB<sup>FL</sup> remains the same in case of cells that were subjected to lipofectamine treatment.
GPI analogues incorporated by BSA, γ-CD and lipofectamine method were susceptible to PI-PLC cleavage. IA2.2F cells were either labeled with PLB\textsuperscript{TMR} or incorporated with GPI analogues Bodipy\textsuperscript{TMR}-GlcNPI\textsubscript{c16:0c16:0} by BSA or γ-CD method and then re-plated followed by imaging or incubated with PI-PLC on ice and then imaged at high resolution. Alternatively GlcNPI-NBD were incorporated into cells by lipofectamine method and then imaged with or without PI-PLC treatment. PLB-TMR and Bodipy\textsuperscript{TMR}-GlcNPI\textsubscript{c16:0c16:0} (BSA method) were imaged in TIRF mode while as Bodipy\textsuperscript{TMR}-GlcNPI\textsubscript{c16:0c16:0} (γ-CD method) and GlcNPI-NBD (lipofectamine method) were imaged using the Andor spinning disc modality.