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A Biosynthetic Membrane-Anchor/Protein System Based on a Genetically Encoded "Aldehyde Tag"

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INTRODUCTION

The biochemical basis of cellular function involves the intricate interplay of signaling molecules, receptors, and lipid (fatty-acid) membranes. The ubiquity of membranes, from the outer membrane that protects a eukaryotic cell to the different membranes that surround each intracellular constituent, accentuates their importance in cellular life. Proteins, associated with biological membranes, carry out the majority of the biological functions of these fluid lipid bilayers and are responsible for regulating the flow of chemical information in and out of the cell. Membrane fluidity adds versatility to the cell by allowing its membrane surface components to easily redistribute, to readily recognize cellular signals, and to participate in all kinds of life-sustaining biological reactions. Because of the specificity in evolved biochemical recognition, mimicking these functionalities in artificial membrane systems for their application as biosensors, biochemical reactors, drug-screening devices and hybrid-cell communication systems is desirable [1].

Anchoring a protein to a lipid membrane is generally achieved by modifying the protein of interest with a lipid molecule (i.e., lipidating) that can anchor into a biomimetic lipid membrane. Efforts to capture the natural elegance and efficiency of in vivo post-translational modifications and coordination of these modified proteins in artificial membrane systems have been limited by both the types of lipids that can be attached to the protein and by the location along the protein at which lipidation can be successfully performed. These limitations can potentially be addressed by using a recently developed “aldehyde tag”
technology, i.e., the site-specific integration of the non-standard amino acid formylglycine which bears a reactive aldehyde side chain for further chemical modification [2]. This aldehyde functional group potentially opens also a versatile avenue for synthetic protein lipidation by facilitating the site-specific covalent attachment of lipids with aminooxy-modified head groups. The main goal of this study is to develop a proof of principle demonstrating the successful site-specific insertion of an aldehyde side chain to an enhanced green fluorescence protein (EGFP) for subsequent lipidation at the aldehyde site and incorporation into a fluid supported lipid bilayer. An EGFP was aldehyde-tagged at the carboxyl-terminus and covalently bonded to an aminooxy-modified phospholipid (specifically a phosphatidylethanolamine). The successful incorporation of the resulting aldehyde-tagged EGFP lipid anchor constructs into a solid-supported lipid bilayer was verified by fluorescence microscopy. Fluorescence recovery after photo-bleaching (FRAP) was used to confirm the preservation of the fluidity of both the EGFPs and lipids in the bilayer.

BACKGROUND

1. The Biological Importance of Lipid Bilayers

The prevalence of lipid membranes in partitioning cellular function, from the homeostatic processes across the bilayers that separate intracellular organelles to the more complex signaling mechanisms that drive organ systems, confirms their importance in mediating cellular life. Cells are dynamic; they communicate, react, and constantly change their physical and chemical characteristics to ensure
proper cell function, survival, and proliferation [3]. The chemical recognition of both intra- and extracellular signals is one of the most important membrane-bound biochemical processes. In many of these processes, receptors must aggregate around a bound antigen in order for the subsequent steps in signal transduction to occur. For example, through a process called receptor-mediated endocytosis, specific molecules are recognized by outer-membrane proteins to coordinate a small section of a membrane to pinch off and encase the molecules, thereby facilitating their transport between organelles and cells. Therefore, a large number of life-sustaining biochemical events are coordinated on and/or by the body’s cellular membranes [3]. In addition, the lipid membrane protects and holds much of the cellular machinery required for cellular communication and cell maintenance [4].

Lipids are amphipathic molecules with a hydrophilic (water-loving) head group bonded to long hydrophobic (water-fearing) fatty acid chains [Figure 1]. In aqueous solution this amphipathic nature allows lipids to self-assemble into thermodynamically stable ellipsoidal, closed lipid structures called liposomes. The wall of such liposomes is a lipid bilayer that contains at least two sheets.
of lipids, one on top of the other, with the hydrophilic heads facing out, and the hydrophobic tails facing in [Figure 2]. An important consequence of this dynamically stable configuration is its properties as a barrier. The membrane houses the cell’s machinery in an aqueous environment, while protecting its inner constituents by prohibiting the trans-membrane movement of most molecules and ions. Furthermore, the bilayer structure permits lipids and other molecules located within each plane of the bilayer to move and diffuse laterally across the plane of the membrane. This mobility within the membrane leaflets permits cellular mobility and the necessary dynamic rearrangement of the membrane-associated proteins and receptors that mediate cellular adhesion and the communication that is integral to cellular life and applications in biotechnology [3].

2. Biomimetic Membrane Interfaces and Their Important Properties

Biological membranes serve as barriers that separate and safeguard neighboring cells and the intracellular organelles of a cell. Their stability as barriers allows them to partition cellular space and function by limiting the
movement of molecules across the membrane, while allowing certain processes to take place in different areas of the living cell. As a result, cell membranes not only efficiently divide up cellular life, but also must closely regulate signaling pathways, chemical gradients, and chemical trafficking among separated cellular environments [1]. Given these properties, the reasons why much of the cell’s important chemical activity occurs at the membrane is clear.

From the membranes that encapsulate individual organelles to the one membrane protecting the entire cell, membranes in eukaryotic cells vary dramatically in regard to lipid composition, organization, and the cellular processes that take place on the surface. However regardless of a membrane’s chemical identity, all biological membranes must be dynamic, in the rearrangement of membrane constitutes in the plane of the membrane, in order to effectively partake in the cell’s conformational, signaling, trafficking, and recognition events [1]. Because of the dynamic spatial requirements for proper membrane function, the development of biomimetic membrane systems has progressed to help elucidate the biochemical and biophysical phenomena that take place at the membrane interface. In addition, many simplified model membrane systems have been created to preserve the fundamental bilayer structure and lipid dynamics, while enabling analysis of the roles and organization of individual membrane components [1]. Model membrane systems allow for the study of biomembrane processes at the cellular level, providing information about membrane properties, ligand-receptor interactions, cellular signaling events, and
immune responses [4, 7]. For example, supported lipid bilayers (SLBs) allow for the easy study of membrane surface assembly, organization, and interactions [4].

Most important, because biomimetic lipid membranes may be tailored with the same lipids that make up the eukaryotic cell, they are therefore naturally biocompatible with living cells. Lipid-based biomembrane devices provide biocompatible surfaces that work in the human body and its cells. This is exemplified in the current developments of membrane-based technologies such as biosensors, biochemical reactors, drug-screening devices, clinical imaging nanostructures, drug/gene delivery systems and hybrid-cell communication systems [1,8,9]. Biosensors, such as on-chip immunoassays, which measure the kinetics and thermodynamics of antibody binding to membrane antigens, serve as an example of the powerful sensor applications of biomimetic lipid surfaces [4]. In medical applications, liposomal anticancer drugs have proven to be a successful alternative treatment for tumors [10]. Hydrophilic head groups of the inner membrane leaflet enclose the hydrophilic drug (like attracts like) to form a liposome around the drug [4]. Then, the drug is delivered to the tumor, using antibodies or ligands that are mounted on the liposome’s outer surface, and are specific for antigens on the tumor cell surfaces [4]. Finally, the drug-containing liposome fuses with the tumor’s cell membrane and enters the cell by receptor-mediated endocytosis to deposit the drug.

Most, if not all, of these applications require specific proteins that functionalize the bilayer, allowing the membrane to take part in biochemical interactions. Similarly, in the body, proteins mediate the exchange of chemical
signals between the cellular organelles and the cell’s external environment through channel proteins, receptor proteins, and antibodies. Because of the high level of specificity of biochemical processes, biomimetic lipid interfaces must be developed using proteins that can dynamically reorganize within the bilayer [3]. When interfacing with a live eukaryotic cell, for example, biomimetic membrane-bound proteins should be able to follow the movement of their binding partners on the cell surface; e.g., the dimerization, or general oligomerization, of protein receptors that is often essential to create the proper signal. There are many chemical identification processes that require protein receptors to aggregate and localize at the site of the chemical signal in order to ensure that the signal gets transported across the membrane [11].

There is one more feature of a biological membrane that is difficult to replicate in biomimetic membrane models: the ability to phase separate (i.e., to alter its lipid organization), depending on its lipid composition. Phase separation in a bilayer, depends on the amount of the different types of lipids present, which may include glycopospholipids with saturated and unsaturated fatty acid chains, cholesterol, and sphingomyelin. During a separation event, different membrane components take on different configurations [Figure 3]. Coupling this membrane property with protein anchoring will further allow biomimetic protein-lipid systems to actively rearrange themselves, leading to the formation of large-scale dynamic structures of proteins that depend on what types of lipids the proteins are attached to on the biomimetic membrane [8]. For these reasons, it is imperative
that surface membrane proteins be anchored onto the bilayer in a fashion that preserves their overall function and motility.

![Figure 3: A schematic overview of phase separation. By raising and lowering the temperature to be above or below the transition temperature, $T_m$, the lipids will redistribute across the membrane (i.e., phase separate) according to their chemical identity. In the above image, the bilayer contains two types of lipids: one labeled red and the other black.](image)

3. Solid-Supported Lipid Bilayer Systems

Solid-supported lipid bilayers (SLBs) are stable membrane models that support a variety of surface analysis techniques for studying membrane surface function and assembly. There are a limited variety of surfaces that can support a high quality membrane (i.e., one with little to no defects with high lipid mobility) because substrates need to be hydrophilic, smooth, and clean [11]. SLBs have been deposited on silicon oxide, borosilicate glass, mica, and silicon substrates [11]. Most important, the optical clarity of glass and silicon substrates allows for the easy access of the SLB’s structure with various microscopy techniques.

Through a process called vesicle fusion, small unilamellar vesicles (SUVs) fuse into an enlarged vesicle before they rupture into a planar fluid bilayer on the substrate [Figure 4] [12]. With the help of intermolecular forces, the smaller vesicles fuse with each other until a critical size is reached, at which point the
internal tension of the vesicle’s curvature, reaches a point where the continued fusion of SUVs, causes the large vesicle to rupture into two leaflets of lipid membrane that settle onto the solid substrate [Figure 4 and 5] [12]. Intermolecular forces – such as electrostatic forces, van der Waals interactions, and hydration forces between the lower leaflet of the supported lipid bilayer and the substrate enable the membrane to remain stable [4]. The fluid-supported bilayer rests on a 10-20Å layer of water, which is trapped between the bilayer and the glass substrate [Figure 5] [11]. This layer of water serves to keep the lipids in both sheets of the membrane fluid [4]. Once the membrane is formed, it must be constantly kept in aqueous solution as the hydrophobic and hydrophilic interactions and intermolecular forces stabilize the lipid bilayer structures. If the sample dries, the bilayer will fall apart. Generally, buffer solutions at a pH and with ionic conditions that mirror the in vivo environment of the eukaryotic cell (pH ~7.5 with ~120mM of NaCl) are appropriate for ensuing deposition of proteins onto the SLB surface. Besides the widely used vesicle fusion techniques, SLBs have also been deposited onto substrates one lipid monolayer (i.e., lipid leaflet) at a time onto substrates using the Langmuir-Blodgett and Langmuir-Schaefer techniques [11].
4. Anchoring Proteins onto Supported Lipid Bilayers

Attaching proteins to a fluid lipid membrane may be achieved by protein lipidation to (or acyl chain modification of) lipid anchors and by the addition of hydrophobic protein anchors that are made of selected amino acids [13]. The latter method requires a hydrophobic environment for the stable production of proteinaceous membrane anchors, and therefore, it is difficult to carry out and maintain in vitro [13]. Current lipidation methods for integrating proteins into fluid artificial biomembranes employ either a glycosylphosphatidylinositol (GPI) anchor, a His-tag + nickel linkage, or a biotin + streptavidin binding. Each of these techniques has drawbacks that limit control over where and how a protein is positioned on the membrane, what type of lipids the protein may attach to, and at what positions the attachment can take place.

Figure 5: Diagram of a solid-supported lipid bilayer. Of note is that the water molecules surround the bilayer from above and below, thus supporting the lipids’ membrane configuration. The solid-supported lipid bilayer maintains its fluidity by resting on a 10-20Å layer of water, which is trapped between the bilayer and the glass substrate [11].
Naturally occurring GPI anchors are actively used in eukaryotic cells, covalently attaching modified proteins to the extracellular side of the cell membrane [14]. The GPI lipidation method has positioning limitations that allow GPI anchors to attach only at the protein’s carboxyl terminus [Figure 6] [14].

Thus, the GPI anchor motif severely restricts the possible orientations of a protein incorporated on the membrane surface. Furthermore, the chemical identities of the two resulting lipid anchors are fixed by a post-translational modification mechanism in the eukaryotic cell’s Golgi apparatus, thereby limiting the types of supported lipid bilayer compositions that may be constructed. An in vitro synthesis of the GPI anchors would permit the choice of acyl chain modification, but the chemical production of GPI is complicated [16].

The His-tag method involves an inserted polyhistidine sequence (6-12 Histidine residues) at the protein’s C or N terminus, which is used to bind to nickel (Ni) atoms present on lipid head groups at the membrane surface [Figure
The His-tag procedure involves a carboxyl or amino terminus bound polyhistidine chain that binds nickel atoms on the surface of a membrane [17]. Currently, only one phospholipid with a nickelated head group is commercially available, once again limiting the variety of lipid membrane compositions. The histidine + nickel interaction is also reversible and thus the amount of bound protein is highly sensitive to changes in pH, temperature, and time [17]. Finally, histidine + nickel binding is ill-defined, due to the His-tags possibly interacting with up to two nickel head groups and the reversible nature of this His + Ni interaction on the membrane surface [Figure 7].

The biotin-streptavidin method utilizes proteins and lipids that are biotinylated to bind with streptavidin, a molecule that acts as a “glue” that links up to four biotinylated constructs [Figure 8]. However, biotinylation of particular amino acid side-groups may occur at multiple regions along the protein, resulting in a non-site specific binding with streptavidin and consequently, non-specific lipid attachment [18]. Furthermore, streptavidin at elevated surface concentrations
forms large-scale aggregates that impede the lateral motion of the lipid-bound proteins and prohibits the deposition of large amounts of protein [18]. Thus, biotin-streptavidin binding is difficult to predict, measure, and control. Also, the commercial availability of biotinylated lipid anchors is limited.

In summary, both the His + nickel and biotin + streptavidin approaches have been proved to successfully attach proteins onto the membrane surface. However, in addition to the limitations of, and the problems regarding, the choice of commercially available lipid anchors and the possible sites of protein lipidation (i.e., the use of only the C and N terminus in His tag modification and the random biotinylation of proteins), both methods are faced with problems of dynamic consistency in protein attachment, competitive binding for single substrates, and the specificity of modified lipids for binding. These constraints prohibit the
exploitation of all the dynamic physicochemical properties of biomimetic membranes. Thus, there is a need to develop a new method that allows the freedom to choose from a large variety of lipid anchors and to attach them at virtually any location along a protein.

5. The Aldehyde Tag

To meet this need and to overcome the limitations of current methodologies described above, the aldehyde tag offers a new approach to protein attachment that permits proteins to bind at well-defined positions with one of many different lipid anchor motifs. It was recently found that the site-specific insertion of a six-amino-acid consensus sequence (LCTPSR) into a protein’s amino acid chain is sufficient to target the cysteine within the hexapeptide for a post-translational modification by a formylglycine-generating enzyme (FGE) [19]. This consensus sequence, also known as the “sulfatase sequence,” along with FGEs, occurs naturally in eukaryotic organisms and plays a vital role in activating the catalytic function of sulfatases, which are enzymes that hydrolyze sulfate esters in the cell [20].

While there are a variety of protein modifications, few offer the site-specificity that is guaranteed by this consensus motif. In addition, the consensus sequence’s relatively small size minimizes the structural effects that would destabilize the protein’s structure. As seen in Figure 8, the FGE recognizes and acts specifically on this consensus motif and oxidizes the cysteine into a formylglycine, which provides a reactive aldehyde group for chemoselective reactions with aldehyde-specific reagents that include: aminooxy- (−ONH₂) and
hydrazide-modified molecules [20]. The aldehyde side chain binds covalently with these reagents to produce very stable linkages. Of great benefit is the fact that the consensus sequence may be inserted into any site along a polypeptide chain, allowing for site-specific protein modification with high chemical accuracy, such as the covalent attachment of aminooxy-modified lipids to the aldehyde tag [Figure 9]. As a result, the aldehyde tag provides the site-specificity and precise chemical control that is necessary to produce post-translational

**Figure 8:** The consensus sequence LCTPSR is inserted anywhere along a protein’s amino acid chain. A FGE will read only this consensus sequence and translate the cysteine to a formylglycine, thus site-specifically introducing a reactive aldehyde side chain.

**Figure 9:** A diagram of the aldehyde tagging procedure. The conversion of the cysteine (C) to a formylglycine (FGly) generates a reactive aldehyde side chain for subsequent chemical coupling to an aminooxy-modified partner (e.g., the chemical label).
modifications on any type of protein and anywhere along its peptide chain.

6. The Enhanced Green Fluorescence Protein (EGFP)

A naturally occurring protein that is found in a variety of aquatic life, Green Fluorescent Protein (GFP), emits a green light when excited by blue light and has become a well-established fluorescence marker in biological cell studies [21]. The discovery of GFP and its derivatives such as the more photo-stable Enhanced Green Fluorescent Protein (EGFP) has revolutionized molecular imaging with its easy application and diverse uses [21]. A potent advantage of this protein is the ability of the EGFP gene to be easily integrated into virtually any part of a protein’s DNA sequence and to be translated into a functional fluorophore, an excitable light-emitting structure, within its final protein structure.

EGFP allows for the quantitative measurement of successful protein-lipid anchor integration. Since EGFP detection is straightforward using fluorescence microscopy, it is the perfect protein for testing the aldehyde tag-based lipidation of proteins. By measuring the green fluorescence, a quantitative measurement of our GFP-lipid anchor’s abundance across the supported lipid membrane can be achieved.

7. Fluorescence Microscopy

Fluorophores are essential to fluorescence microscopy and have been widely used in microbiology to tag and identify, with high accuracy, cells and their intracellular constituents. By using multiple fluorophores of different colors, various structures of the cell may be simultaneously imaged [22]. Fluorophores are molecules that emit light at defined wavelengths after they have been excited
with excitation light at shorter wavelengths [22]. Currently, there is a wide variety of synthetic and naturally occurring fluorophores available, each with their specific chemical target and tagging procedure [22].

A fluorescent microscope simultaneously irradiates excitation photons and collects the photons emitted by the fluorophores. This is done with the help of special filters that can separate the excitation and emission wavelengths of the fluorophores and project the emitted light onto the eyes or detector [Figure 10a].

![Fluorescence microscopy diagram](image)

**Figure 10:** (a) Separation of excitation and emission light with special band filters. Notice how photons hit the specimen and radiate spherically in all directions. The filters collect and separate only a portion of these spherical light waves [23]. (b) Filter cube containing the excitation filter, the fluorescence emission filter, and the dichromatic mirror [22].

First, photons at different wavelengths pass through an excitation filter, which picks out the desired wavelengths of light to excite the fluorophores. Next, the selected excitation photons reflect off the surface of a dichromatic (also called a dichroic) mirror and travel to the specimen [Figure 10b and 11]. The excited fluorophores on the specimen will emit light through an objective lens, then hit the dichroic mirror, and pass an emission filter (at the top of a filter cube) that only allows the emission photons to pass through and project an image of only the fluorophores on the specimen [Figure 11] [22].
8. **Fluorescence Recovery After Photo-Bleaching (FRAP)**

Photo-bleaching results in the irreversible break down of excited fluorophores when they react with oxygen right before they emit light. Fluorescence recovery after photo-bleaching (FRAP) exploits this phenomenon to examine the diffusion of tagged biological molecules on the specimen [22]. In this process a region of the specimen is exposed to excitation light, which subsequently permanently bleaches (i.e. deactivates) the fluorophores. Bleached fluorophores have their chemical structure permanently disrupted, so that they may no longer undergo the conformational changes that are necessary for their fluorescence activity. By observing the rates and patterns in fluorescence recovery in the bleached region over a period of time, the motion of the tagged molecules may be determined [22].
MATERIALS AND METHODS

1. Preparation of EGFPs with the Aldehyde Tag

   The aldehyde tag’s consensus sequence was fused to the carboxyl terminus of an enhanced green fluorescence protein (EGFP). All of the EGFPs were expressed in and extracted from an *E. Coli* system. In addition, to improve the yield of aldehyde production after EGFP translation, the consensus sequence-EGFP fusion construct was co-expressed with the DNA sequence for a formylglycine-generating enzyme (FGE). FGEs specifically target the six-amino-acid sequence to enzymatically convert the cysteine in the consensus sequence into a formylglycine (FGly), leading to the site-specific introduction of an aldehyde side chain for further chemical modification [Figure 12]. Next, the EGFP’s aldehyde group was reacted with commercially available phospholipid anchor motifs that were previously modified to bear an aminooxy reactive head group [For details see Appendix A].

2. Aminooxy-Modification of Commercially Available Phospholipids

   Commercially available phosphatidylethanolamine (PE) phospholipids were aminooxy-modified to bear the (-ONH$_2$) required to covalently react with the modified-EGFP’s aldehyde side chain [Figure 12 and Appendix A]. PE phospholipids with different acyl chains (or fatty acid chains) are available at Avanti Polar Lipids. The coupling of the aminooxy-group to the lipid required an initial reaction with a Boc-protected aminooxy-reagent, followed by boc-deprotection to form the aminooxy-modified lipid product.
Overview of Aldehyde-Tagged EGFP + Aminooxy-Lipid Anchor Preparation

Figure 12:
A schematic of the aldehyde-tagging and lipidation process:

(a) The consensus sequence (LCTPxR) was inserted at the EGFP’s C-terminus. Co-expression with FGE on the same plasmid yields the site-specific introduction of the aldehyde side chain for reaction with aldehyde-specific binding partners (e.g., aminooxy-modified lipids).

(b) Chemical reaction between an aldehyde tagged-EGFP and aminooxy- (ONH$_2$)-lipid (phosphatidylserine) forms a stable covalently bonded protein-lipid anchor. Different phosphatidylethanolamine (PE) phospholipids of varying structure and acyl-chain (tail) compositions may be lipidated on an aldehyde-tagged protein.
Boc groups are often used in chemical reactions to protect amine groups from reacting until they are needed. Therefore, first, Boc -2- (aminooxy) acetic acid was added to a 3-neck flask. The flask was evacuated and filled with argon. Next, dry methylenechloride (CH$_2$Cl$_2$) and carbonyldiamidazole (CDI) were added to the flask. The solution was left to stir for 30 minutes to allow the activation of the carboxylic acid group. Next, the PE phospholipid was added to the reaction and allowed to stir overnight at room temperature, ~25°C to facilitate coupling. Afterwards, the reaction intermediates were washed with 1M hydrochloric acid (HCl) and brine (salt water). The organic layer was then dried over magnesium sulfate (MgSO$_4$), and filtered, and the remaining solvent was evaporated to yield the boc-protected lipid [Appendix A]. Next, the modified lipids were dissolved in 1:1 CH$_2$Cl$_2$: TFA (Trifluoroacetic acid) and allowed to stir for 2 hours. To remove the boc- group, TFA and CH$_2$Cl$_2$ were evaporated to generate the deprotected aminooxy-modified lipid for the subsequent reaction with an aldehyde-tagged EGFP. Due to the presence of residual TFA at the end of the reaction, 4M HCl in dioxane will be substituted in future coupling reactions.

3. Preparation of Small Unilamellar Vesicles (SUVs) for Supported Lipid Bilayer (SLB) Formation

Small unilamellar vesicles (SUVs) consisting of two molar percent Marina Blue® 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (a lipid with a blue fluorophore attached to its head group) and 98 molar percent 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were preformed. Marina Blue® DHPE was purchased from Invitrogen, Carlsbad, CA. All other lipids were obtained from
Avanti Polar Lipids, Alabaster, AL. A 50mL round-bottom flask was first washed with piranha etch (a mixture of sulfuric acid and hydrochloric acid). The flask was rinsed with distilled H$_2$O and dried in an oven before use. Next, the determined volumes of lipids were pipetted into a cleaned 50mL round-bottom flask containing 1mL of chloroform. The contents were mixed manually before the chloroform was evaporated with a Büchi® RE-111 Rotovapor. Next, 1mL of distilled H$_2$O was added to the dried lipid mix to initiate the formation of vesicles of varying sizes. These vesicles were allowed to hydrate overnight. The vesicles were extruded using a LIPEX™ extruder (from Northern Lipids Inc., Burnaby, BC, Canada) to produce a solution of uniformly sized 100nm or smaller vesicles. These small vesicles are normally stable for a week and are necessary for vesicle fusion to occur during bilayer deposition. Once extruded, the resulting solution of vesicles (~1mL of solution of ~100nm lipid vesicles in H$_2$O) was ready for deposition on glass coverslips (solid substrate). Fisherbrand® 25CIR-1 25mm round glass coverslips were purchased from Fisher Scientific, Pittsburg, PA.

4. Deposition of SLBs

To construct a high-quality fluid bilayer, the glass substrate must be vigorously cleaned to minimize the number of defects in the bilayer. Glass coverslips were first sonicated and degassed in a beaker of isopropanol, rinsed in double ionized water, and dried under nitrogen gas. Next, the glass was treated with piranha etch, a solution of three parts 98% sulfuric acid with one part 33% hydrochloric acid for a 10-minute interval. Piranha acid dissolves organic molecules and leaves the glass negatively charged. Finally, the coverslips were
thoroughly rinsed with double distilled H$_2$O and dried under nitrogen right before bilayer deposition. SUVs were allowed to fuse together and form a bilayer on the glass surface. The deposition procedure took two forms (both via vesicle fusion):

**Method 1**

A 3:1 volume mixture of buffer solution (containing 150mM NaCl and 20mM Tris salt at pH ~7.4) to 100nm lipid-vesicle solution was prepared. SLBs were kept in salt buffer at approximately pH 7.4, to mimic the *in vivo* conditions of a eukaryotic cell. All standard salts and chemicals were purchased from Sigma-Aldrich®, USA. Forty microliters of this lipid mixture was pipetted onto a plastic petri dish. Next, a cleaned glass coverslip was placed over the droplet of vesicles to expedite the vesicle fusion process and initiate bilayer formation. The droplet of vesicles was smeared onto the glass substrate, resulting in the deposition of two layers of lipids across the coverslip. As shown in Figure 5, to preserve the bilayer structure, a layer of water lies between one side of the bilayer and the substrate, while another layer of water is located in between the other side of the bilayer and the petri dish. The dish is next submerged in the same ~7.4 pH salt buffer solution, where the coverslip holding the SLB is mounted on a watertight coverslip holder that allows easy access with an inverted-epifluorescence microscope.

**Method 2**

In this method, a coverslip is first mounted on the microscope coverslip holder. Next, a 30-40µL drop of the desired lipid vesicles is placed on a clean coverslip and allowed to incubate for 10-15 minutes. Then, the coverslip is
submerged with buffer solution at ~7.4 pH. A SLB should form at the spot where the drop was initially placed.

5. Incubation of Aldehyde-Tagged EGFP Lipid Anchors on a SLB

Aldehyde-tagged EGFP lipid anchors were incorporated onto a SLB with three techniques. The first technique involved passive integration, where 100µL to 200µL of EGFP-lipids were added directly onto a previously formed SLB composed of 2 mol percent MB DHPE and 98 mol percent DOPC. The EGFP-lipid anchors were allowed to incubate and passively integrate into the SLB, for 12 to 24 hours at a time.

The second approach involved the use of detergents to help keep a larger quantity of aldehyde-tagged EGFP lipid anchors in solution (i.e., water soluble and away from the large aggregates). Detergents, like lipids, are amphipathic molecules that contain a single hydrophobic acyl chain. They form micelles around molecules and are used to gather the EGFP-lipid anchors, thereby driving the formation of EGFP-lipid containing micelles while reducing the production of the larger lipid aggregates. Micelles are similar to liposomes where amphipathic molecules containing only one hydrophobic chain (as oppose to the two fatty acid chains found in lipids), aggregate in aqueous solution with their hydrophobic tails protected at the micelle center [Figure 2]. The EGFP-containing micelles were directly pipetted onto a SLB and allowed to incubate for 12 to 24 hours.

The third method required the deposition of proteoliposomes that resulted from reacting the aldehyde-tagged EGFP onto SUVs that contained the
aminooxy-lipid. A vesicle solution containing 1 molar percent of the aminoxy-modified lipids, along with 1 mol percent MB-DHPE, and 98 mol percent DOPC, was prepared in the same fashion as described above. These aminoxy-containing liposomes were extruded to form SUVs and allowed to react with the aldehyde-tagged EGFP. On average, three aldehyde-tagged EGFPs were added for one aminoxy-modified lipid on the SUVs. The aldehyde side chains of the EGFPs covalently bonded to the free aminoxy-groups on the preformed vesicles, resulting in a solution of vesicles that contain the modified or tagged EGFP. These vesicles were next separated by size exclusion chromatography to separate the successfully coupled EGFP-proteoliposomes from the unbound or unmodified EGFPs (i.e., non-aldehyde tagged) in solution. To improve the yield of EGFP-proteoliposomes, SUVs containing 3 molar percent of the aminoxy-modified lipids, along with 2 mol percent MB-DHPE, and 95 mol percent DOPC were made to react with, on average, thirty aldehyde-tagged EGFPs per aminoxy-modified lipid. Given the presence of non aldehyde-tagged EGFP (that has no binding partners) in solution, the higher EGFP concentration was used to ensure ample amounts of aldehyde-tagged EGFPs were coupled to the liposomes.

6. Size Exclusion Column Chromatography

A column was prepared with Sephacryl™ beads made from a polymer of allyl dextran and N, N’-methylene bisacrylamide. A 133mm glass column was set up to contain 1500µL of Sephacryl™ CL2B-300 beads in suspension. Beads had pores of various sizes. Large molecules can easily pass around the beads and elute out of the column, while smaller molecules must travel a longer path, through the
bead’s pores, before coming out of the column. A sample of the EGFP-proteoliposomes was run through the column. Sephacryl™ beads were chosen with a 50µm pore size, which hindered the journey of the smaller unbound or unmodified EGFPs as significantly larger EGFP-liposomes were quickly filtered. During the separation, fractions were collected after each drop, and at the end each fraction was profiled for its contents by probing each fraction for EGFP fluorescence with laser light. A clean separation should clearly indicate where the

![Intensity Profile of Size Exclusion Fractions](image)

**Figure 13**: An intensity plot of the fractions obtained from a size exclusion column. Each fraction was exposed to excitation photons and probed for the presence of EGFP and MB DHPE. Fractions containing the successfully coupled aldehyde-tagged EGFP liposomes displayed both blue and green fluorescence.

EGFP-proteoliposomes and free EGFPs started and finished traveling through the column [Figure 13]. Fractions verified to contain EGFP-proteoliposomes were deposited via vesicle fusion and imaged with fluorescence microscopy.
7. FRAP Analysis of Supported Lipid Bilayers

The quantitative determination of integration efficiencies as a function of physical parameters (mostly the solution concentration of the EGFP-anchor construct) calls for fluorescence microscopy and fluorescence recovery after photo-bleaching (FRAP). Supported lipid bilayers (SLBs) were imaged with either a Nikon 60X water or oil immersion objectives. FRAP analysis images were taken with an epifluorescence microscope and investigated using NIS Elements Advance Research imaging software provided by Nikon, Japan. In SLBs, FRAP is used to study the changes in intensity by exploiting the self-rearranging ability of the lipid membranes to provide evidence that the macromolecules in the bilayer are functional and mobile. A Photometrics® (Tucson, AZ) Cascade II 1024 EMCCD camera connected to the microscope was used to take time lapse photographs of the FRAP recovery. A strong pulse of light was used to deactivate (“bleach”) fluorophores (i.e., the MB DHPE and EGFP-

![Figure 14: A FRAP sequence of a bilayer containing His-tagged EGFP. The bleached (black) region gradually recovers its intensity as mobile lipids carrying functional EGFP move into the space. The amount of time it takes for strong recovery depends on the protein-lipid anchor and its membrane environment.](image)
lipid anchors) on a SLB. The laser light was kept on at specific wavelengths that excited the fluorophores. With the laser turned off, active fluorophores attached to neighboring lipids diffused into the region and effectively brightening the space with a slightly darker shade of the original fluorescence color [Figure 14] [25]. Therefore, FRAP directly measured the percentage of fluorescence recovery and the speed and time it took for the fluorophores to re-occupy the *darkened* region [25].
RESULTS AND DISCUSSION

1. Successful Aminooxy-Modification of Phospholipids

Nuclear magnetic resonance (NMR) was used to study the magnetic resonance within the aminooxy-modified phospholipids in order to verify its chemical structure. A Bruker 300MHz NMR was used to obtain $^1$H NMR spectra (CDCl3) to verify the successful aminooxy-modification of lipids. The initial spectrum in appendix B showed the initial reactants as outlined in the materials and methods section. On the second NMR spectrum, the presence of methyl (CH$_3$) protons on the boc-protecting group at 1.6ppm confirmed product formation [Appendix C]. On the last spectrum, the aminooxy-modified lipid product is clearly deprotected, as indicated by the loss of the signal at 1.6ppm. The rest of the spectra in appendix D were consistent with the proposed structure identified in the reaction scheme [Appendix A].

2. Successful Attachment of EGFP to Aminooxy- Lipid

In the production of the first batch of aldehyde-tagged EGFP lipid anchors a ~10:1 volume ratio of aminooxy-lipids to aldehyde-tagged EGFPs was reacted with the aldehyde-tagged EGFP to ensure the limited amounts of EGFP would all be conjugated to an aminooxy- lipid. However, the excess lipids that did not get conjugated (to the EGFP) formed large lipid structures, as verified by fluorescence microscopy [Figure 15]. As a result, these proteins were unable to integrate into the membrane. However, aggregation of the usually very water-soluble EGFP is a clear indication that the protein lipidation reaction per se was
successful. EGFP is not a hydrophobic protein and normally would not seek lipids to interact and form aggregates with. Therefore, the formation of these lipid “clumps” verified the successful coupling of the aldehyde-tagged EGFPs to the aminooxy-modified lipids. The integration of individual EGFP-lipid constructs would have produced a uniform field of green fluorescence, because mobile EGFP-lipid molecules would distribute evenly in the membrane.

**Figure 15**: The initial reaction of aldehyde-tagged EGFP with an excess amount of aminooxy-lipids resulted in large amounts of “clumping,” where uncoupled aminooxy-lipids would form larger lipid structures around the modified EGFP-lipid anchor constructs. Aggregation supported the successful reaction of the aldehyde-tagged EGFP to the aminooxy-lipid, since EGFP is not hydrophobic in nature and would therefore not collect around lipids. As a result, the aldehyde-tagged EGFPs must have successfully reacted with the aminooxy-lipids and acquired some of the lipids’ hydrophobic nature.

3. **Fluorescence Analysis of SLB from Proteoliposomes Deposition**

The results of the protein integration to a supported lipid bilayer (SLB) were obtained by examining the fluidity of the protein and the lipid environment. To that end, membrane integrity, as well as protein and lipid motility, were investigated using fluorescence recovery after photo-bleaching (FRAP). After the aldehyde-tagged EGFP reacted with the aminooxy-lipid containing small unilamellar vesicles (SUVs), a bilayer was made from these proteoliposomes. The final proteoliposomes contained about 1 mol percent MB DHPE, 98 mol
percent DOPC, and 1 mol percent of aminooxy-modified lipid anchors with aldehyde-tagged EGFP bound to some fraction of them. The blue fluorescent MB-DHPE was used to check the integrity of the bilayer without having to compete with EGFP’s green fluorescence. A perfect and intact bilayer displays a uniform field of blue before and after GFP integration. FRAP analysis on the blue channel displayed an intact bilayer, with few visible defects, that recovered well [Figure 16]. In figure 16, FRAP analysis images were taken within 5 to 10 seconds apart. Inspection on the green channel showed noticeable recovery over an extended period of time (~2 to 5 hours) [Figure 17]. This slow recovery may be due to the presence of unbound and unmodified EGFP in solution. Free EGFP was found to have likely bonded to the glass substrate and remained immobile, hindering the lateral motion of its neighboring lipids and thereby increasing the time it takes for lipids carrying functional EGFP to travel into the bleached zone. Furthermore, the dark silhouette that remained in the last FRAP image in Figure 17, was likely due to permanently bound EGFP on the glass, that had been bleached and then essentially occupied the space where mobile lipids carrying aldehyde-tagged EGFP were trying to enter. A comparison of the normalized intensity profiles of
the first and last image in the EGFP FRAP sequence indicated an expected increase in the intensity of the bleached region from the first frame to the last [Figure 15]. The obvious recovery in green intensity demonstrated the presence of mobile EGFP-lipids and the overall successful incorporation of the aldehyde-tagged EGFP aminooxy-lipid anchors into the SLB.

![Image of FRAP sequence](image)

**Figure 17**: A FRAP sequence of aldehyde tagged EGFP + Aminooxy-modified Lipid anchors on a SLB. The intensity plot tracks the increasing change in intensity from the first time capture to the last frame (over a period of 5 hours) as mobile EGFP-carrying lipids diffuse into the bleached region.

4. **Size Exclusion Purification of Proteoliposomes**

The noticeable presence of unbound or unmodified (i.e., non-aldehyde-tagged) EGFP influenced the decision to add a purification step after the aldehyde-tagged EGFPs were reacted with the aminooxy-containing liposomes. Separation of the EGFP-proteoliposomes was successful and produced a good
range of fractions that contained proteoliposomes. From Figure 13, the aldehyde-tagged EGFP proteoliposomes were likely found in fractions 5 through 11 where intensity measurements revealed sufficient amounts of blue and green signal. Fractions 12 through 19 displayed only high amounts of green signal giving evidence that only unbound aldehyde-tagged or unmodified EGFPs were present in these fractions [Figure 13]. However, deposition of the proteoliposomes in fractions 5 through 11 produced SLBs with too little green fluorescence for a FRAP analysis. For the next reaction, a higher concentration of aminooxy-lipids will be added to the initial vesicle production, in addition to adding more aldehyde-tagged EGFP to the reaction.


In the development of biomimetic lipid surfaces, the aldehyde tag method offers unprecedented freedom to site-specifically modify proteins at any location along its peptide structure, thereby controlling the site where the protein will couple with a lipid anchor. As mentioned above, current protein attachment strategies such as the addition of a GPI lipid anchor may be added only at a protein’s C terminus and the His-tag may be applied only at both the C and N terminus. As a result, these two techniques only allow a lipid anchor to bind at one or two sites on the protein, respectively. In the biotin-streptavidin method, biotinylation is non-site specific resulting in the binding of biotin on various places on a protein. Therefore, the biotin-streptavidin method remains difficult to predict and control how the modified protein will attach on the lipid bilayer.
The His-tag and biotin-streptavidin methods are also hampered by the lack of diverse, and commercially available, lipid anchor motifs. In the aldehyde tag method, the aminooxy-modification may be performed on any commercially available phosphatidylethanolamine (PE) to generate diverse lipid anchors. PE phospholipids with varying acyl chains are commercially available from many vendors such as Avanti Polar Lipids (Alabaster, AL). Therefore, an aldehyde-tagged protein may bind to a variety of different phospholipid anchors with different fatty acid chains. This versatility promotes the development of diverse bilayers with unique lipid compositions for applications such as phase separation studies.

The His-tag and biotin-streptavidin methods also require modified proteins (i.e., His-tagged or biotinylated proteins) to compete for single substrates (e.g., nickel or streptavidin) on the membrane surface. If multiple modified-proteins were deposited on to a bilayer, they would compete for the same binding partners on the bilayer. Therefore, competitive binding on the membrane surface prohibits the production of biomimetic membranes with pre-determined concentrations for each type of modified protein. On the other hand, in the aldehyde tag method, because aldehyde-tagged proteins are reacted on small unilamellar vesicles (SUVs) containing aminooxy- lipids prior to SLB formation, multiple aldehyde-tagged proteins may be reacted to different solutions of aminooxy- containing SUVs generating SUVs with the same lipid composition but varying aldehyde-tagged proteins. Next these assorted SUVs may be combined (at specific volume ratios) through vesicle fusion, and deposit into a supported lipid bilayer. Table 1
below summarizes the advantages of the different protein attachment strategies that are currently used to attach proteins on biomimetic membranes:

**Table 1: A summary of the advantages and disadvantages of current membrane protein attachment strategies**

<table>
<thead>
<tr>
<th>Protein Attachment Strategy</th>
<th>C Terminus Modification</th>
<th>N Terminus Modification</th>
<th>Site-specific Modification Along the Protein</th>
<th>Diversity in Lipid Anchor Motifs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPI</td>
<td>✓</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>His Tag + Nickel</td>
<td>✓</td>
<td>✓</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Biotin + Streptavidin</td>
<td>✓</td>
<td>✓</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Aldehyde Tag</td>
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</tr>
</tbody>
</table>
CONCLUSION AND FUTURE WORK

Biological membranes not only provide a protective barrier for cells and their intracellular compartments, but also host all the machinery for cellular communication and transport across the bilayer that allows cellular function to proceed. Due to the high level of specificity in cellular signaling and trafficking, the correct arrangement and orientation of receptor proteins is integral to accurately managing chemical recognition. The aldehyde tag based method of covalent lipidation developed in this study permits protein modifications with an unrivaled freedom and accuracy that stretches beyond the biofunctionalization of biomimetic membranes. By varying the site of lipidation to include the aldehyde tag on the amino (N-) terminus or along the middle body of the EGFP, aldehyde tag expression at different areas of the EGFP, along with their effects on protein lipidation and membrane integration, will be explored in future work. In addition, by using a variety of anchoring lipid motifs, the dependence of the protein integration on the chemical structure of the lipid will be studied. By varying the chemical structure of the lipid anchors to reflect those with known phase separation (lipid arrangement) behavior, the ability of proteins (lipidated using the aldehyde-tag strategy) to partake in more complex membrane dynamics will be evaluated. Depending on the specific lipid motif, if the membrane system undergoes a phase transition induced by temperature change, the EGFP should redistribute in the membrane in expected patterns consistent with its lipid composition. Extending the aldehyde tag to lipidate antibodies would be a step towards defining sensory and drug delivery applications. Once the integration
technique is refined, different proteins may be aldehyde-tagged and lipidated for membrane integration and provide steps towards advancing biological, medical and biotechnological applications.
REFERENCES


APPENDICES

Appendix A: Reaction Schematics for the Aminooxy-Modification of a Lipid and Subsequent Conjugation to a Aldehyde-Tagged Protein
Appendix B: $^1$H NMR Spectra of the Starting Materials in the Aminooxy-Modification Reaction
Appendix C: $^1$H NMR Spectra of the Protected Lipids in the Aminooxy-Modification Reaction
Appendix D: $^1$H NMR Spectra of the De-protected Lipids in the Aminooxy-Modification Reaction
CAPSTONE SUMMARY

A distinguishing feature of cellular life is the ability for animal cells to communicate, exchange, and process biochemical information. A variety of important chemical recognition events transpire on the cell membrane, which protects the cell’s inner environment. Proteins, such as antibodies and receptor proteins, attached to the membrane surface play a key role in the essential chemical recognition and information exchange within a cell or with the exterior environment outside the cell. Because of the high level of specificity in biochemical recognition, artificial membranes that interface with living cells must be developed in a way that allows proteins to freely reorganize themselves on the membrane surface. Therefore, efforts in maximizing the effectiveness of cell surface functionality in synthetic or artificial membrane systems is important in the advancement of biomedicine, biotechnology, and basic science.

Human and animal cells contain a variety of lipid membrane-enclosed organelles – most importantly a nucleus that carries the organism’s genetic information. The entire exterior is protected by a cell membrane, composed of phospholipids, which are amphipathic molecules containing a hydrophilic (water-loving) head region and two hydrophobic (water-fearing) fatty acid chains. In aqueous solution, the lipids form closed three-dimensional structure, called liposomes or vesicles, where all the hydrophilic heads facing out and the hydrophobic tails facing in. This configuration enforces a “like attracts like” phenomenon that keeps all the hydrophilic heads and hydrophobic tails facing their preferred environments. The wall of a liposome is therefore a continuous
bilayer where two leaflets of lipids situate on top of each other in the configuration mentioned above. At the surface, a cell is essentially a liposome with specialized organelles and membranes carrying out its life-sustaining processes. Therefore, many simplified model membrane systems or biomimetic membranes have been synthesized to study certain cell surface interactions and properties by mimicking sections or aspects of the cell membrane.

Much like living cell membranes, biomimetic membrane systems often require specific proteins to be attached, via e.g., a lipid anchor on its surface, in order to carry out a specialized function. Lipids and proteins must be able to freely diffuse and rearrange across the surface of the bilayer. In many cases of cellular signaling, the coming together of multiple protein receptors is required for the proper signal to be created. However, current protein attachment strategies have been limited by both the site where a protein may be modified to attach to a lipid anchor and the types of commercially available lipids that may be coupled to a protein. For example, the histidine + nickel interaction has been exploited in the production of biomimetic lipid surfaces. A series of histidine amino acid residues are linked to either the C or N terminus (i.e., the two poles of a protein’s structure) and allowed to bind to nickel atoms attached on lipid anchors on a bilayer. However, since the His-tag may only be added to the two ends of a protein it does not permit proteins to be anchored to a lipid from its side or some location along the body of the protein. Furthermore, only one lipid anchor with a nickel atom on its head group is commercially available, thereby severely limiting the production of diverse membrane compositions.
With the recently developed aldehyde tag modification, a protein may be modified at virtually any location along its amino acid chain (the pre-protein structure) to bear a reactive aldehyde group that may bind to an aldehyde-specific binding partner including one of many modified lipid anchors. Lipid anchors modified to have an aminooxy- group may covalently bind with an aldehyde-tagged protein for subsequent incorporation into a biomimetic bilayer.

In this study, the aldehyde tag technology was incorporated into an enhanced fluorescence protein (EGFP) and later coupled to an aminooxy-modified lipid anchor for deposition onto a supported lipid bilayer. A supported lipid bilayer is a biomimetic membrane system that allows the easy study of membrane surface assembly and structure with microscopy techniques. By using EGFP, which fluoresces green when exposed to the right, blue excitation light, the successful incorporation of the aldehyde-tagged EGFP lipid anchors will produce bilayers that can be imaged and examined for fluidity with a fluorescence microscope.

A special six-amino-acid consensus sequence is first inserted into the C terminus EGFP’s amino acid chain. Next a formylglycine-generating enzyme (FGE) reads this sequence and converts the cysteine amino acid (that is in the sequence), into a formylglycine, which contains the aldehyde group. This consensus sequence offers the site-specific introduction of an aldehyde group, i.e., wherever the consensus sequence is inserted, the protein will be translated into its final structure with the aldehyde group at that location. Next commercially available phosphatidylethanolamine (PE) lipid anchors with varying fatty acid
chains may be aminooxy- modified and coupled with the tagged EGFP. This permits the freedom to generate assorted biomimetic membranes with unique lipid compositions for various biological studies and applications. In the experiment, aldehyde-tagged EGFPs were coupled/reacted on to small lipid vesicles containing aminooxy- lipids. The resulting solution of EGFP proteoliposomes were allowed to fuse, through a process called vesicle fusion, and flatten into a bilayer on glass substrate.

Once a bilayer was formed, fluorescence recovery after photo-bleaching (FRAP) was used to analyze the mobility of the EGFP lipid anchors. In a FRAP study, a strong pulse of light is used to deactivate (“bleach”) the EGFP leaving a dark region of space, while over time, active EGFP attached to neighboring lipids will begin to diffuse into the region and effectively fill it with a darker shade of the original fluorescence color. FRAP studies of the bilayers revealed successful recovery supported the EGFP-lipid anchor integration. However, the presence of extra unbound or free non-aldehyde tagged EGFP, during the coupling reaction, may have interfered with the bilayer deposition process. These excess EGFP would permanently bind to the glass, bleach, and remain immobile, as neighboring lipids carrying functional EGFP must diffuse around them. This issue will be addressed with additional filtering to separate out the successfully reacted EGFP proteoliposomes before bilayer deposition.

The successful FRAP analyses proved that the aldehyde tag may be used to couple proteins to lipid anchors while remaining functional and mobile when attached to a supported lipid bilayer. For the development of biomimetic
membranes, effectively any protein may be aldehyde tagged from any position to attach to a lipid anchor on a bilayer. In future work, by introducing the aldehyde tag at different sites along the EGFP, the effects of location on the successful aldehyde tag modification may be examined. Furthermore, by tagging a different protein, such as an antibody, sensory applications may be further explored. Therefore, the aldehyde tag method provides a versatile and powerful way to modify proteins not only for attachment to lipid anchors but also for many other biological studies.