

Lipophilic Anchors that Embed Bioconjugates in Bilayer Membranes: A Review

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ABSTRACT

A wide range of biomaterials and engineered cell surfaces are comprised of bioconjugates embedded in liposome membranes, surface-immobilized bilayers, or the plasma membranes of living cells. This review article summarizes the various ways that Nature anchors integral and peripheral proteins in a cell membrane and describes the strategies devised by chemical biologists to label a membrane protein in living cells. Also discussed are modern synthetic and semisynthetic methods to produce lipidated proteins. Subsequent sections describe methods to anchor a three-component synthetic construct that is comprised of a lipophilic membrane anchor, hydrophilic linker, and exposed functional component. The surface exposed payload can be a fluorophore, aptamer, oligonucleotide, polypeptide, peptide nucleic acid, polysaccharide, branched dendrimer, or linear polymer. Hydrocarbon chains are commonly used as the membrane anchor, and a general experimental trend is a two chain lipid anchor has higher membrane affinity than a cholesteryl or single chain lipid anchor. Amphiphilic fluorescent dyes are effective molecular probes for cell membrane imaging and a zwitterionic linker between the fluorophore and the lipid anchor promotes high persistence in the plasma membrane of living cells. A relatively new advance is the development of switchable membrane anchors as molecular tools for fundamental studies or as technology platforms for applied biomaterials.

INTRODUCTION

Many types of biomaterials and engineered cell surfaces are comprised of bioconjugates embedded in bilayer membranes. Various preclinical research fields and next-generation therapies require surface-decorated cells, functionalized liposomes, and surface-immobilized bilayers for applications like targeted drug delivery, cell-based therapy, cell transplantation, vaccine development, membrane fusion, biosensing, bioimaging, and single molecule tracking.¹²³⁴ Similarly, cell microscopists often need to label cell plasma membranes with fluorophores for static and dynamic cell imaging. In all scenarios, it is crucial to ensure that the bioconjugate remains anchored in the membrane for the entire period of the experiment. This challenging task depends heavily on the structure of the bilayer and the type of functional payload that is attached to the lipophilic anchor.

The purpose of this review article is to concisely summarize the different ways that researchers can anchor a bioconjugate or multicomponent molecular construct in a cell plasma membrane or synthetic bilayer. The article focuses on the different biosynthetic, semi-synthetic, and synthetic strategies that are used to prepare an anchored construct, and we summarize the overarching concepts that underlie the molecular designs with citations directing the interested reader to the specialized synthetic and technical publications. Topics that are beyond the scope of this article include molecular and nanoparticle designs that promote membrane permeation,⁵ amphiphilic nanoparticles that alter membrane curvature,⁶ detailed biosynthetic pathways that produce membrane-anchored proteins,⁷ and molecular probes for the study of membrane diffusion and lipidomics.⁸

In terms of presentation format, the article starts by briefly reviewing the various ways that Nature anchors proteins in a cell membrane, and then describes the strategies that chemical biologists use to fluorescently label a membrane protein in living cells. The latter sections describe bioconjugation methods that anchor synthetic conjugates in liposome membranes or cell plasma membranes and includes a section on switchable anchors. Membrane anchor stability trends and several helpful design rules are also summarized.

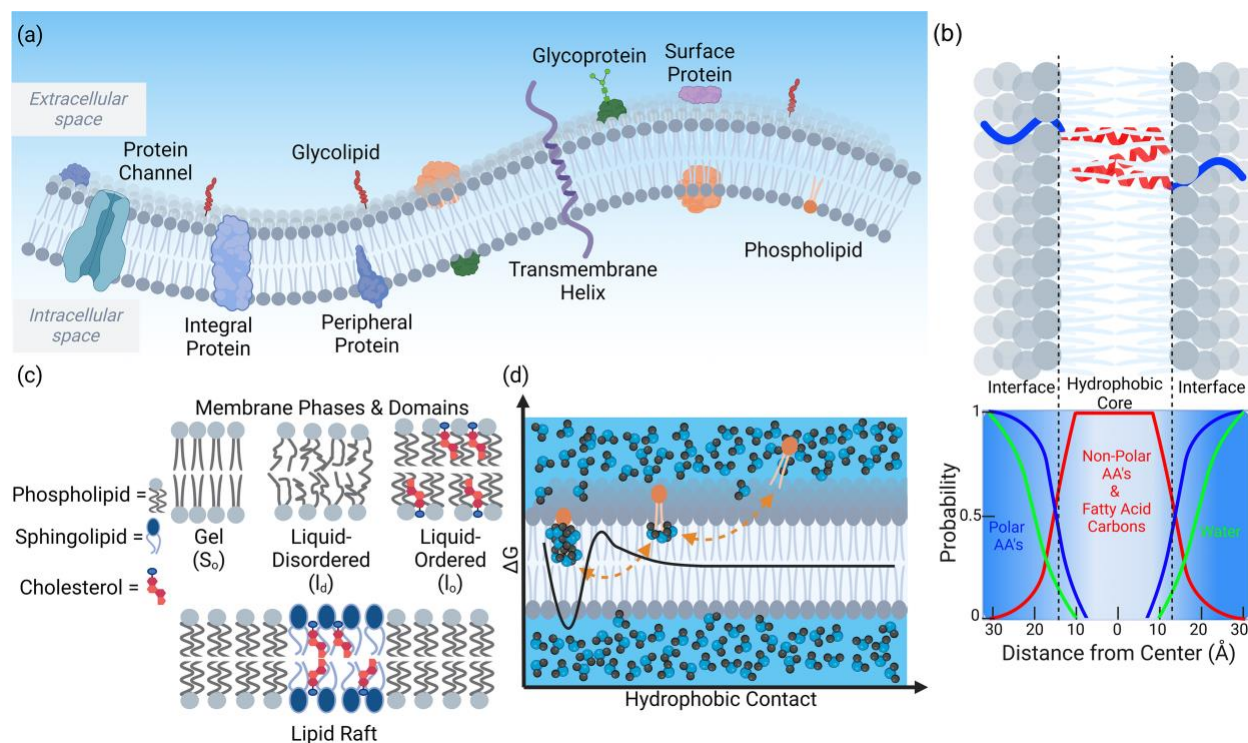
GENERAL SUMMARY OF CELL MEMBRANE STRUCTURE

Before describing the different biochemical anchors that occur in Nature, we provide a concise background summary of mammalian cell membrane structure. Illustrated in Scheme 1a is a generic picture of a simplified mammalian cell plasma membrane showing a polar lipid bilayer decorated with peripheral and integral proteins. Polar lipids are defined as amphiphilic molecules that have a polar head group and one or two lipophilic tails. The polar lipids in eukaryotic membranes have broad structural diversity, and the most common classes are glycerophospholipids, sphingolipids, glycolipids, cholesterol, and fatty acids.⁹

A bilayer membrane is a fluctuating ensemble with an average hydrophobic core that is ~ 30 Å thick (Scheme 1b), and a ~ 15 Å wide polar interfacial region on either side.¹⁰ Molecular dynamics simulations indicate very little water residing in the hydrophobic core. Similarly, amino acids with polar side chains strongly favor the interfacial regions over the hydrophobic core. A homogeneous bilayer composed of one type of polar lipid can exist in a gel phase at a relatively low temperature or a liquid phase at temperatures above the phase temperature (Scheme 1c). Additives within the membrane such as cholesterol can promote lateral phase separation to produce localized spatial domains that are rich in cholesterol (liquid-ordered) and domains that are cholesterol deficient (liquid-disordered). All biological membranes are in a fluid phase, although there is evidence for dynamic, nanoscale domains called lipid rafts that are comprised of cholesterol and other polar lipids, along with membrane proteins, in an ill-defined ensemble with a variable organizational lifetime.⁹

Insertion of a polar lipid into a bilayer membrane is not a barrier-less process. Molecular dynamics simulations have recently calculated the energy profile for the reaction coordinate (Scheme 1d) and found that the low energy path for polar lipid entry (or exit) from a bilayer involves transient formation (or disruption) of a local hydrophobic environment around the polar lipid.¹¹ The rates of passive lipid exchange in model bilayer membranes are on the order of hours, and much slower than the lipid exchange rates seen in biology where specific lipid exchange proteins catalyze lipid transfer. Lipid exchange proteins efficiently extract lipids from donor membranes by lowering the free energy activation barrier for breakage of hydrophobic contact. But they cannot bind the lipids too strongly as they must ultimately release them into acceptor membranes.

A membrane composed of longer phospholipids has a wider hydrophobic core than a membrane composed of shorter phospholipids, and stability of an embedded lipophilic anchor is greatest when it matches the membrane's hydrophobic core. A typical cell membrane is composed of multiple molecular components (glycerophospholipids, sphingolipids, glycolipids, cholesterol, and integral proteins) and the bilayer will assemble in a way that minimizes free energy. One of the contributing energetic factors is the degree of hydrophobic mismatch in the bilayer, and it can be lowered by lateral phase separation to produce nanoscale two-dimensional clusters of neighboring polar lipids with similar amphiphilic dimensions.¹² Consequently, lateral phase separation provides a mechanism to modulate local bilayer thickness, and a “long” lipophilic membrane anchor can recruit a surrounding cluster of long polar lipids to create a localized region of the membrane that is thicker than the average.



Scheme 1: (a) Schematic picture of a simplified mammalian cell membrane. (b) The hydrophobic core within a bilayer membrane is ~ 30 Å thick, and the interfacial regions on either side are ~ 15 Å wide. (c) Different membrane phases and domains. (d) Reaction coordinate for lipid entry (or exit) from a bilayer membrane due to the creation (or disruption) of a locally hydrophobic

environment around the incoming (or outgoing) lipid. Adapted with permission from, Rogers, J. R.; Geissler, P. L. *J. Phys. Chem. B*, 2020, 124, 5884–5898. Copyright 2020, American Chemical Society.

SUMMARY OF BIOCHEMICAL MEMBRANE ANCHORS

Nature uses appended lipid chains to anchor proteins to membranes. Shown in Scheme 2 are examples of different lipid anchors that are linked to proteins, that is, the 16-carbon fatty acid palmitoyl (Scheme 2a), glycosylphosphatidylinositol (GPI, Scheme 2b), or an oligoprenyl chain with 15 or 20 carbons (Scheme 2c). The various biosynthetic pathways that produce these lipid anchors are known, as well as the enzyme-catalyzed protein/lipid conjugation reactions.^{7 13} The high practical value of lipidated proteins has motivated protein chemists to develop synthetic and semisynthetic methods for protein/lipid conjugation.^{14 15} The synthetic methods include various ligation reactions such as maleimide-cysteine addition, native chemical ligation, diselenide-selenoester ligation, serine/threonine ligation, and α -keto-hydroxylamine ligation; whereas the semisynthetic methods include expressed protein ligation and sortase-mediated ligation.

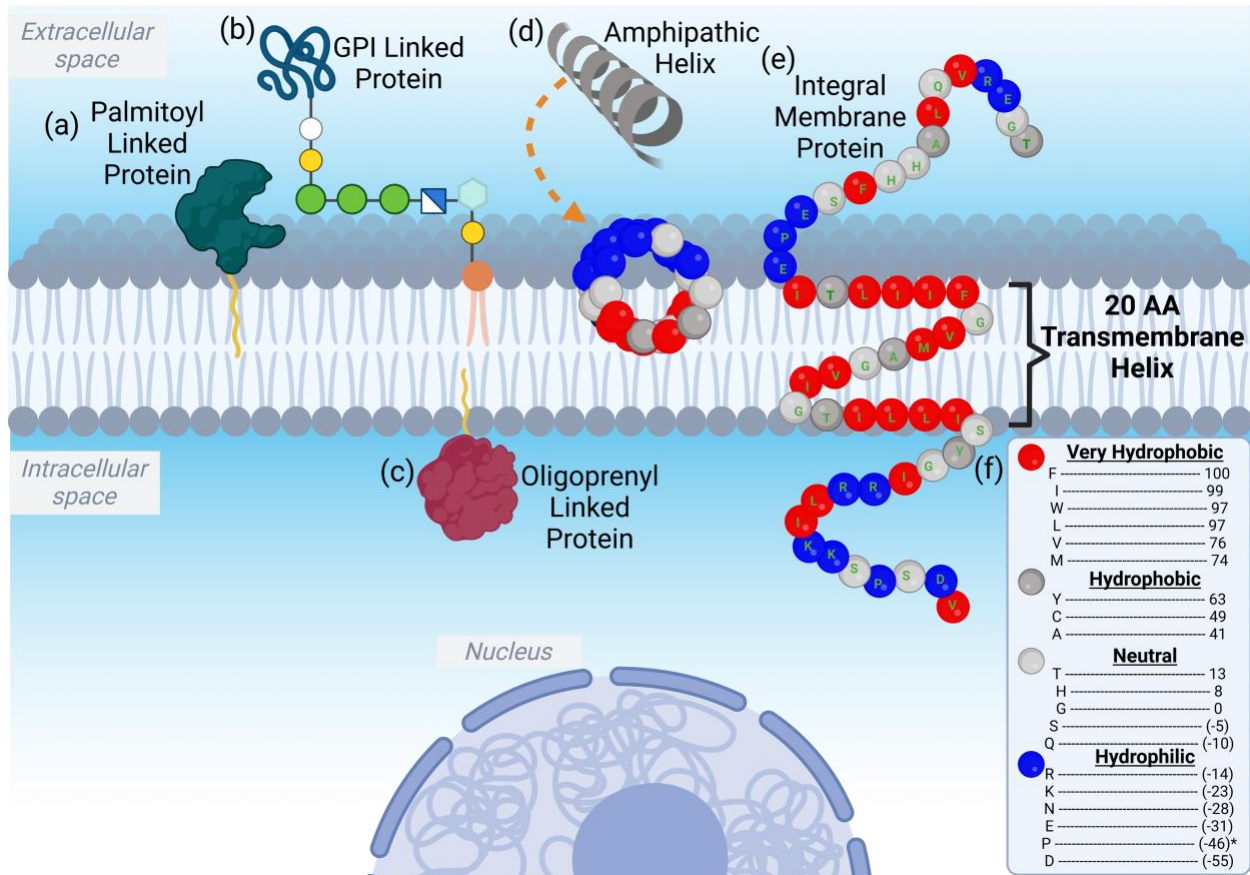
In the absence of lipid anchors, peripheral proteins can still embed into a bilayer membrane if a section of a protein's secondary structure contains enough non-polar amino acids. Tyrosine and tryptophan are commonly observed at the membrane interface because the aromatic side chains provide hydrophobicity, and they can interact directly with the head groups of the membrane polar lipids via hydrogen bonding or cation- π interactions.¹⁶ A specific type of protein secondary structure with membrane affinity is the amphipathic helix, whose key structural feature is a segregated arrangement of amino acid side chains such that one face of the helix is non-polar and the opposite face is polar. The facial amphiphilicity makes it thermodynamically favorable for the helix to embed its non-polar face sideways into a membrane interface with the polar face exposed to the aqueous exterior (Scheme 2d).¹⁷ Amphipathic helices have diverse biological functions based on their capacity to deform membrane packing and alter surface curvature, and they are often involved in dynamic cell membrane processes such as sensing, remodeling, or fusion.¹⁸ The sideways, partial penetration limits the maximum

membrane affinity that can be achieved by a single amphipathic helix, but the membrane localization can be enhanced by helix oligomerization.

In general, an amphipathic helix is not an optimal protein secondary structure if the functional goal is to produce a long-lived, high-affinity membrane anchor. A better membrane protein anchor is a transmembrane helix with high hydrophobicity and sufficient length to span the membrane. As a simple rule of thumb, a polypeptide helix, comprised of ~20 hydrophobic amino acids, is long enough to span the ~30 Å hydrophobic core of a membrane bilayer (Scheme 2e). Listed in Scheme 2f is the hydrophobicity index for the side chains of the naturally occurring amino acids which are classified into four groups: very hydrophobic (red), hydrophobic (dark gray), neutral (light gray), and hydrophilic (blue). Many integral membrane proteins have multiple transmembrane helices, and one of the central tasks of computational protein structure prediction algorithms is identification of the transmembrane helices. Recent computational approaches have exploited deep-learning methodology as a way to reduce the number of parameters while maintaining high prediction accuracy.¹⁹ The dimensions of a transmembrane helix affects its spatial location within a cell plasma membrane. A transmembrane helix with a small lipid accessible surface area (typically rich in Ala/Gly) commonly partitions into tightly packed raft-like domains, whereas, a transmembrane helix with high lipid accessible surface area (typically rich in Leu/Phe) is usually excluded from these domains.²⁰ Association with other membrane components also depends on the hydrophobic length of the transmembrane helix, with a preference for neighboring transmembrane proteins and lipids of similar length to reduce the energetic cost of hydrophobic mismatch.²¹

Some plasma membrane proteins have a relatively short lifespan, on the order of hours to days, while others persist in the plasma membrane for weeks or even months. Fluorescence microscopy studies have found that a labeled membrane protein can be visualized in a plasma membrane for several days at least; in part because it can recycle back, in bioactive form, to the plasma membrane after internalization by endocytosis.^{22 23} The extended duration in the plasma membrane makes fluorescently labeled membrane proteins excellent probes for microscopic studies of cell membrane structure and dynamics, or in vivo longitudinal studies of cell migration. In addition to fluorescent labels, bioactive molecules such as polysaccharides or enzymes can be

attached to membrane proteins for long-term display on cell surfaces, thus creating a new way to manipulate cell physiology and produce new classes of chemotherapy.^{22 23}



Scheme 2: Examples of the different biochemical membrane anchors. (a) Palmitoyl linked protein, (b) Glycosylphosphatidylinositol (GPI) linked protein. (c) Oligoprenyl linked protein. (d) Facially amphipathic helix, (e) Integral protein with transmembrane helix. (f) Hydrophobicity index for amino acid side chains, * = value at pH 2.

BIOSYNTHETIC OR SEMISYNTHETIC MEMBRANE ANCHORS

One way to fluorescently label a membrane protein in a living cell is to genetically express a fused protein construct that connects a fluorescent protein to the C- or N- terminus of the membrane protein. The biosynthetic technology is straightforward for labs that are experienced in protein expression and there are publications that describe optimized methods. For example,

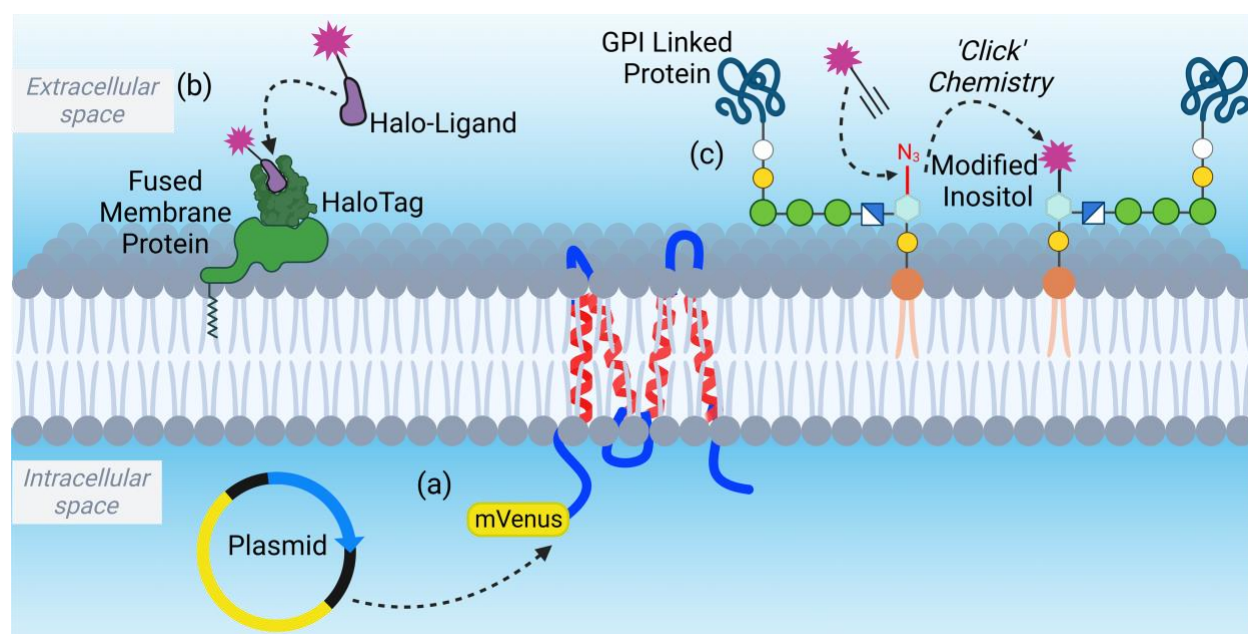
it has been demonstrated that the expression and yield in HEK293 cells of membrane protein, DHHC20 protein acyltransferase, tagged with yellow mVenus are 2 - 5 fold better over mEGFP (Scheme 3a).²⁴ However, there are sometimes challenges with specific fused protein constructs caused by low protein expression levels or improper protein folding, and there are also performance drawbacks due to the relatively large size of the fluorescent protein along with limited fluorescence brightness, photostability, and wavelength range. This has led to nongenetic methods for labeling membrane proteins with synthetic fluorophores that exhibit enhanced brightness, stability, and long wavelength fluorescence that facilitate in vivo imaging.²⁵ These protein labeling methods exploit selective bioconjugation concepts such as proximity-enhanced chemistry and photoaffinity labeling.²⁶

The selectivity and efficiency of the protein labeling step can be greatly improved by using two-step Tag protein protocols. In short, cells are genetically programmed to biosynthesize a membrane protein that is fused with a reactive Tag protein, and then a functional molecular label is selectively attached to the Tag protein. A range of conceptually related Tag-probe labeling methods have been developed over the years and a recent review article described seventeen separate protocols that enable live-cell imaging of membrane proteins.²⁷ The representative example shown in Scheme 3b is based on HaloTag® technology. Genetically modified cells are induced to express a membrane surface protein that is fused to a halotag protein, and subsequent treatment of the cells with a functional molecule (fluorophore or bioactive sequence) that has a halotag reactive motif leads to covalent attachment.²⁸

An alternative two-step example, shown in Scheme 3c, utilizes metabolic engineering to introduce a bioorthogonal reactive site on a glycosylphosphatidylinositol (GPI) anchor within the cell plasma membrane. This is achieved by treating the living cells with an azide-modified phosphatidylinositol, which is recognized as a biosynthetic precursor and integrates into the cell biosynthesis/trafficking pathways to produce an azido-labeled GPI-anchored protein. Subsequent treatment of the cells with an alkyne-bearing functional molecule leads to a selective click reaction and cell surface labeling.²⁹

In each of these cell engineering cases, the anchor component is biosynthesized inside the living cells and trafficked to the cell plasma membrane. A variation of this two-step paradigm

adds a synthetic lipid anchor molecule to cell culture and allows incubation time for anchor insertion into the cell plasma membrane from the exterior surface. After the reactive lipophilic anchor is embedded in the cell membrane, the functional payload component is added to the cell culture where it covalently attaches to the anchor molecule via a selective bioorthogonal reaction. Early examples of this two-step method employed synthetic lipophilic anchor molecules with carbonyl groups that can undergo subsequent condensation reactions with amine nucleophiles,³⁰ whereas later work has expanded the scope of conjugation reactions to include cycloaddition and related click reactions.^{31 32}



Scheme 3: (a) Biosynthetic fused protein construct that covalently connects the fluorescent protein, mVenus, to the C- or N-terminus of a membrane protein. Panels b and c illustrate representative examples of two-step semisynthetic protocols for labeling membrane-bound proteins in living cells. (b) Cell expression of a membrane protein fused with the HaloTag® protein enables covalent attachment of a functional molecule (fluorophore or bioactive sequence). (c) Cells metabolize an azide-modified phosphatidylinositol precursor and produce an azido-labeled

glycosylphosphatidylinositol (GPI)-anchored protein which is covalently labeled with an alkyne-bearing functional molecule.

SYNTHETIC LIPID MEMBRANE ANCHORS

The simplest way to functionalize the exterior surface of a bilayer membrane is to simply treat the membrane (living cell, liposome, or immobilized bilayer) with a pre-synthesized three-component molecular construct that is comprised of a lipophilic membrane anchor, flexible hydrophilic linker, and exposed functional component such as a stealth polymer, reporter molecule, or bioactive sequence (Scheme 4). The method is employed extensively to functionalize the surface of liposomes with a bioactive sequence such as peptide,³³ immunogenic protein,³⁴ antibody,³⁵ aptamer,³⁶ oligonucleotide,^{37 38} or polysaccharide.³⁹

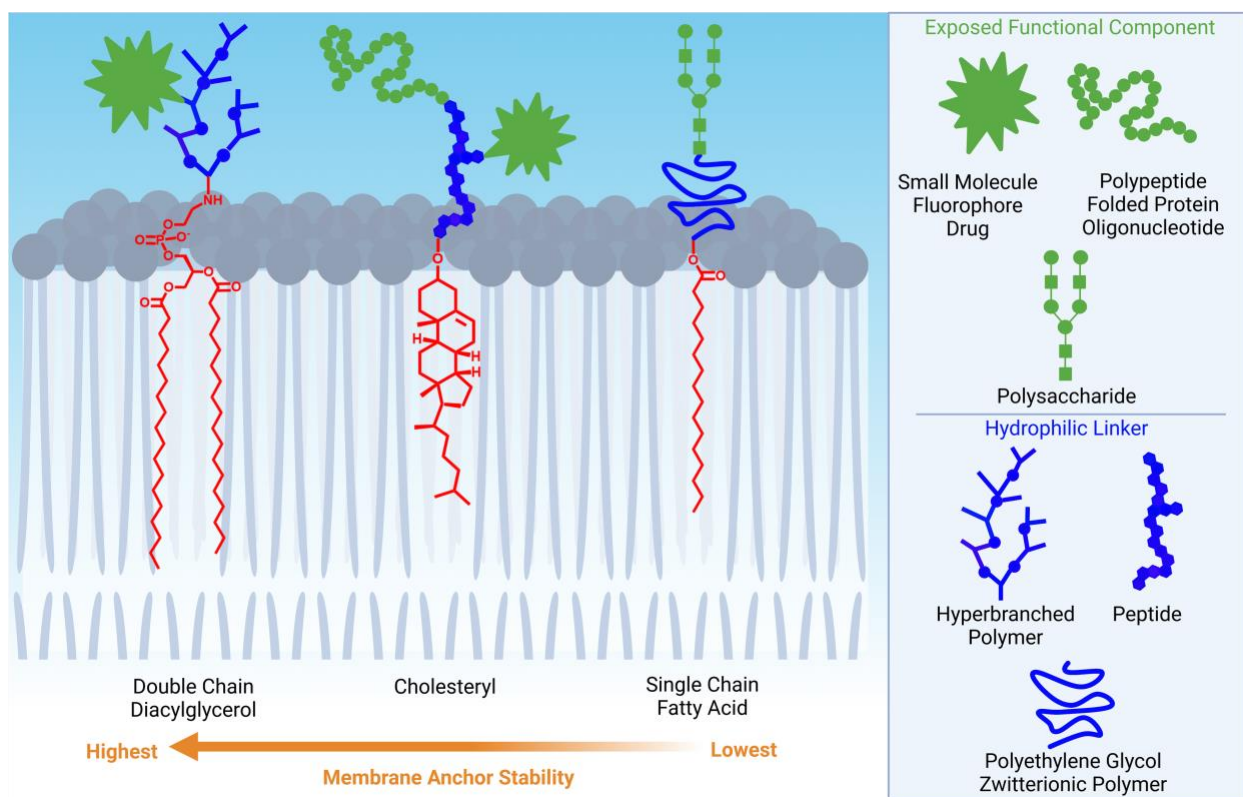
Ideally, the high lipophilicity of the membrane anchor will thermodynamically drive insertion into the hydrophobic core of the membrane. The thermodynamics for polar lipid insertion into a synthetic membrane can be measured by isothermal calorimetry or fluorescence spectroscopy, although the experiment is technically demanding due to self-aggregation and non-ideal behavior of the polar lipid.^{40 41 42} The partitioning of a single chain polar lipid (fatty acid or lysolipid) into a phospholipid bilayer has been determined to be favored enthalpically and entropically; thus, the partition coefficient increases with temperature and can be as high as 10^5 . A longer non-polar acid chain has higher membrane affinity than a shorter chain, and lengthening a saturated non-polar chain by two carbons produces an order of magnitude increase in the partitioning coefficient.^{41 42}

In practice, there are several thermodynamic and kinetic factors that slow the membrane insertion process and lower insertion efficiency. The most problematic factor is the propensity of an amphiphilic three-component construct to immediately self-aggregate as micelles upon dosing. Depending on stability, the micellar aggregates might slow or even prevent the transfer of the construct to the target membrane. Furthermore, the micellar aggregates may even extract polar lipids from the target membrane and destabilize its structure.^{43 44} It is possible to circumvent these technical problems by using dosing protocols that add the three-component construct slowly to ensure a consistently low concentration. If membrane insertion is still

unsuccessful, then it may be necessary to synthetically alter the amphiphilic structure of the three-component construct so that it does not immediately self-aggregate upon dosing. For example, the structure of the lipophilic membrane anchor can be altered in a way that disfavors self-aggregation but does not diminish membrane affinity. This type of logic is apparent in work reported by Fan and coworkers who used cell-based biosynthesis to fabricate the three-component construct as a single polypeptide. Moreover, the amino acid sequence of the lipophilic membrane anchor component was based on a cell-penetrating peptide and contained a small number of polar residues that attenuated self-aggregation of the construct after dosing but the anchor component was sufficiently lipophilic for membrane insertion with high affinity.⁴⁵

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Synthetically, the simplest choice of a membrane anchor component is one of the readily available hydrocarbon anchors within naturally occurring polar lipids and shown in Scheme 4 are the three most used lipid anchors; double chain diacylglycerol, cholesteryl, and single chain fatty acid. Relatively few studies have attempted to systematically compare the membrane affinities of these three different lipophilic anchors, and most of this work has indirectly assessed relative anchor stability by measuring the loss of fluorescently labeled anchor molecules from liposomes.^{47 48} The general experimental trend is that a two chain anchor has higher membrane affinity than a cholesteryl or one chain anchor (Scheme 4).^{32 47 48 49 50 51 52} This matches the order of log P values for 1-octanol/water partitioning, for example: dipalmitoylphosphatidylcholine (DPPC) (log P = 11.5)⁵³ > cholesterol (log P = 8.6)⁴² > palmitic acid (log P = 7.2).



Scheme 4: Relative membrane stability of three-component molecular constructs that have double chain diacylglycerol, cholesteryl, or single chain fatty acid as the lipophilic Membrane Anchor. Also listed are examples of the Hydrophilic Linker, and Exposed Functional Component.

Bioconjugation with cholesterol is synthetically straightforward and cholesteryl derivatives are convenient and common choices as lipophilic anchor. However, a less appreciated point with cholesterol is its variable interaction with other polar lipids in a target membrane and its capacity to form cholesterol-rich domains. For example, measurements of cholesterol partitioning into liposome membranes revealed a dependence on phospholipid headgroup in the order of sphingomyelin (SM) > phosphatidylserine (PS) > phosphatidylcholine (PC) > phosphatidylethanolamine (PE), and among the common acyl chains, the order of cholesterol partitioning was 18:0 (stearic acid) ~ 18:1n-9 (oleic acid) PC > di18:2n-6 (linoleic acid) PC > 16:0 (palmitic acid).⁵⁴ This information can be used to customize target liposome membrane composition for high affinity anchoring of bioconjugates that have a cholesteryl anchor

component. Another synthetic idea is to replace cholesteryl as the anchor component with a more lipophilic multi-ring hydrocarbon such as tocopheryl ($\log P = 10.4$).⁵⁵

The magnitude of the membrane partition constant also varies with the structure of the other two components in the amphiphilic construct, namely the hydrophilic linker and the exposed functional payload. Thermodynamically, a short hydrophilic linker does not have much effect on the partitioning of a lipophilic anchor into a charge-balanced bilayer membrane. However, the plasma membrane and glycocalyx of a living cell are rich in anionic biomolecules and a polycationic linker can enhance electrostatic attraction for the cell surface and decrease the rate of construct internalization by endocytosis. A fascinating but underappreciated molecule is cholesterylamine which has been used as a cell plasma membrane anchor.⁵⁶ Various derivatives of cholesterylamine have been shown to cycle between the cell surface and early endosomes leading to high persistence on the cell surface.⁵⁷ A final point concerning cell surface stability is the susceptibility of the linker bonds to chemical cleavage. In this regard, it is known that amide and carbamate bonds are more stable than ester bonds which are susceptible to hydrolytic cleavage by serum esterases.⁵⁸

Decorating a liposome surface with linear polyethylene glycol (PEG) is a classic way to introduce stealth liposome behavior; that is, resistance to undesired liposome association with proteins or other membranes, and attenuation of liposome uptake by the reticuloendothelial system. The large body of work on stealth liposomes includes in vitro and in vivo studies that have measured the blood lifetime of different stealth liposome compositions and the residence time of PEGylated lipids within a liposome membrane. PEGylated lipids with longer PEG chains have increased hydrophilicity and larger steric size, and the two factors combine to decrease liposome membrane affinity and increase the rate of intermembrane transfer.⁵⁹ PEG₂₀₀₀ is a commonly used chain length for creation of stealth liposomes and a systematic comparison of glycerophospholipid anchors found that intervesicle transfer of saturated diacyl conjugates decreases exponentially with increasing chain length.⁵⁹ In vivo studies revealed a more subtle structural difference in the residence time of PEGylated lipids within liposomes composed of distearoylphosphatidylcholine (DSPC) and cholesterol (DSPC/cholesterol/lipid-PEG₂₀₀, 50 : 45 : 5, molar percentage), namely, DSPE is considerably more effective than 1-palmitoyl-2-oleoyl-*sn*-

glycero-3-phosphoethanolamine (POPE) as a membrane anchor.⁵⁸ Indeed, DSPE-PEG₂₀₀₀ is a very common choice of PEGylated lipid for the creation of stealth liposomes.⁶⁰ Typically, stealth liposomes are generated by hydrating a lipid film that contains the constituent polar lipids mixed with the lipid-PEG₂₀₀₀ construct and the resulting unilamellar vesicles have a symmetrical distribution of lipid-PEG₂₀₀ across the bilayer. An interesting structural strategy to improve retention of the embedded PEGylated lipid was reported by Uhrich and coworkers who developed PEGylated bolaamphiphiles.⁶¹ The basis of the PEG-bola design is a rigid, non-polar anchor that is sufficiently long to span the entire ~ 30 Å hydrophobic core of the bilayer membrane and project the two appended PEG chains into the opposing aqueous phases. Liposomes stabilized by PEG-bolas exhibited similar storage and biological stability compared to conventional PEGylated lipid stabilized liposomes, but significantly improved retention of the PEG-lipid conjugates upon dilution. Finally, it is worth noting that there are alternatives to PEG as the hydrophilic polymer within stealth liposomes and examples include promising membrane-anchored conjugates comprised of lipid connected to zwitterionic poly(carboxybetaine).³⁷

Beyond liposomes, PEGylated lipids have been embedded in other classes of self-assembled nanoparticles. Most notably, there has been extensive research on solid lipid nanoparticles (LPNs) for delivery of small interfering RNA (siRNA) to silence pathological genes in hepatocytes. It is known that the in vivo potency of LNP-siRNA systems depends strongly on the ionizable amino-lipid within the LNP.⁶² In addition, the co-constituent PEG-lipid provides a protective PEG coating that extends in vivo circulation time but inhibits cell uptake and thus reduces potency. This has led to the use of PEG-lipids with short (C14) acyl chains that can rapidly exchange out of the LNP following administration. However, lipid dissociation is strongly dependent on LNP size, and small LNP with diameter ≤ 30 nm are considerably less potent than their larger counterparts due to dissociation of the protective PEG-lipid and subsequent loss of the amino-lipid from the LNP. Small LPNs stabilized by PEG-lipids with longer (C18) acyl chains exhibit reduced amino-lipid dissociation rates, however, the systems are relatively impotent due to the continued presence of the PEG coating.⁶² Thus, there is a complex interplay between

particle size, amino-lipid content, and the type of PEG-lipid that determines the in vivo potencies of NP-siRNA systems.

Polymeric constructs with multiple lipophilic anchors (such as multiple copies of cholesterol or 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, DPPE) appended to a hydrophilic synthetic or biopolymer have been found to rapidly partition into liposomes or cell plasma membranes.^{39 63 64} With cells, the hydrophilic polymeric strongly maintains the construct at the exterior surface and there is no substantial cellular internalization by endocytosis after incubation periods of many hours. In separate work, charged, hydrophilic polymers have been coated on the surface of cells or liposomes by physisorption methods that exploit favorable electrostatics.⁴ The technical simplicity of this electrostatic coating approach is attractive but the polymer coating covers the entire membrane surface and greatly modifies the surface properties which makes the method unsuitable for many applications that intend to exploit cell phenotypic behavior.

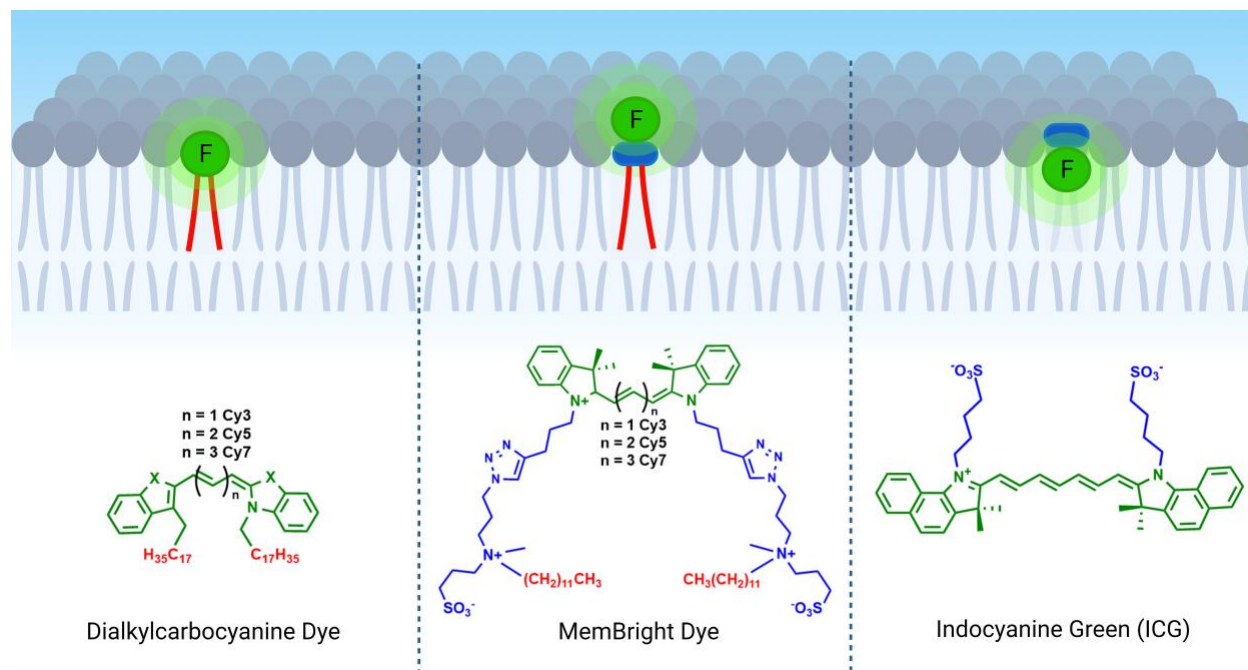
FLUORESCENT DYES FOR MEMBRANES

Small-molecule fluorescent probes are used often to stain cell plasma membranes for imaging, tracking, and sensing experiments and the broad topic has recently been summarized in excellent review articles.^{63 65 66} The focus of this present article is on the structural features that promote membrane anchoring of the dye conjugates.

Uncharged lipophilic dyes with highly positive log P values will partition into the hydrophobic core of cell plasma membranes, and subsequently distribute to the intracellular membranes. Longer residence time in the plasma membrane is achieved using dyes with amphiphilic structures, and several common cyanine dyes have partial amphiphilic character due to the cationic polymethine fluorochrome. Classic examples are the commercially available fluorescent dialkylcarbocyanines (known as DiI or DiO dyes) whose cationic charge and appended hydrocarbon chains combine to produce high affinity for cell plasma membranes (Scheme 5).⁶⁷ However, dialkylcarbocyanine dyes are water-insoluble and extremely hydrophobic (log P = 12.2),⁶⁸ and sometimes there is high background signal due to residual crystals in the sample.⁶⁷ Ongoing research efforts have produced amphiphilic fluorescent dyes that exhibit partial water

solubility and high affinity for a cell plasma membrane. The use of zwitterionic linker(s) between the dye and the lipophilic membrane anchor has been quite effective and a notable example is the MemBright family of fluorescent membrane probes produced by Klymchenko and coworkers. (Scheme 5).⁶⁹ These plasma membrane probes are well-suited for long-term live-cell imaging, with a bright plasma membrane signal persisting for six or more hours after cell treatment.⁷⁰ Upon dosing, these amphiphilic probes form micelles in solution and the close proximity of the dyes within the micelles leads to self-quenching of dye fluorescence. Subsequent transfer of the probes from the micelles into a target cell membrane leads to turn-on fluorescence and thus enables no-wash fluorescence imaging.⁷¹ More recent work has expanded the molecular design to include sensing dyes that accumulate in cell plasma membranes and report changes in membrane properties such as voltage.⁷²

In addition to membrane labels for cell microscopy, fluorescent dye-labeled liposomes are often used for in vivo fluorescence imaging and therapeutics. However, recent publications have noted the propensity of many commercial dye-labeled phospholipids to transfer from liposomes during experiments that involve plasma or living subjects.^{73 74} Moreover, there is a higher amount of fluorophore transfer from liposomes composed of unsaturated phospholipids compared to analogous liposomes composed of saturated phospholipids.⁷³ A similar dye transfer problem can occur with the well-known near-infrared cyanine dye, Indocyanine Green (ICG) whose amphiphilic structure is comprised of a cationic heptamethine cyanine fluorophore with two appended sulfonates (Scheme 5) and has a log P value of 2.05.⁶⁸ ICG is clinically approved for various in vivo imaging and diagnostics, and there are many reports of imaging and light-mediated drug release using liposomes containing ICG. However, anionic ICG has high affinity for blood proteins, such as albumin, and a recent study demonstrated that ICG can readily transfer from liposomes to albumin, which weakens the light-absorbing capacity of the liposomes.⁶⁸ The same study showed that ICG affinity for the liposomes is enhanced by including α -tocopherol as a liposome membrane additive that stabilizes the ICG within the liposome membrane. The experimental caution that emerges from this collection of studies is to treat each dye-labeled liposome system as a unique nanoscale formulation that likely requires detailed characterization of its structure, stability and colloidal properties.⁷⁵



Scheme 5: Common fluorescent dyes used to stain liposomes or cell plasma membranes.

SWITCHABLE MEMBRANE ANCHORS

A relatively new technical advance is the development of membrane anchors with switchable affinity. The field of synthetic biology is developing “membrane switches” to control complex behavior in synthetic cells such as signaling and polarity induction. One example is the *Escherichia coli* MinCDE system which couples ATP-dependent switching of protein dimerization to membrane targeting. Reversible phosphorylation/dephosphorylation of suitably designed heterodimeric coiled-coil peptides was shown to modulate processes such as membrane tethering of a cargo peptide to an anchor protein and oligomeric switching of membrane-binding state.⁷⁶ Another synthetic biology example exploits protein prenylation as a biochemical reaction that triggers membrane association, with membrane dissociation induced by the addition of guanine nucleotide Rho GDP dissociation inhibitor which sequesters the prenyl anchor.⁷⁷

The inherent spatiotemporal control of light-switchable membrane affinity is very attractive for fundamental research and development of functional biomaterials. A conjugate comprised of a spiropyran photoswitch connected to a tetrapeptide (GLFD) was shown to self-

assemble under physiological conditions and form ~3.5 nm thick, foil-like peptide bilayers that exhibited a strong affinity for bilayer membranes.⁷⁸ Photoisomerization of the photoswitch triggered rearrangements within the foils and a substantial change in membrane-binding properties. Moreover, the nanofoils could efficiently induce liposome fusion as judged by lipid mixing assays. A separate light-switchable conjugate was prepared by coupling a 24-residue hydrophobic peptide to an azobenzene photoswitch.⁷⁹ The hydrophobic peptide adopted a helical structure and associated with bilayer membranes in a transmembrane or interface orientation with azobenzene photoisomerization significantly altering the population distribution. The construct has promise as a light-switchable molecular probe to study the dynamic process of helical peptide insertion into membranes. It has performance advantages compared to pH-switchable transmembrane helical membrane peptides such as pHLP or cell penetrating peptides.^{80 81}

Finally, a conceptually new way to reversibly anchor molecular payload in a PEG-coated membrane was reported based on the affinity of polycyclic aromatic hydrocarbons, such as pyrenyl, for a layer of PEG chains.⁸² Upon mixing, pyrenyl conjugates with appended dyes and functional groups were rapidly embedded in the PEG corona surrounding nanoscale polymersomes. In follow up work, an adaptive surface was created by appending a pH-sensitive group to the pyrenyl anchor which allowed reversible insertion into the PEG layer.⁸³ This novel approach to switchable anchoring suggests a path towards new classes of smart nanomedicine and nanoreactors.

CONCLUSIONS

Many biomedical applications require bioconjugates to be embedded in liposomes, surface-immobilized bilayers, or the plasma membranes of living cells. Naturally occurring membrane proteins often persist for extended periods of time in plasma cell membranes; therefore, they are good choices for chemical or biosynthetic conversion into fluorescent probes or scaffolds for the presentation of bioactive sequences on cell surfaces. There are also efficient modern synthetic and semisynthetic methods for protein/lipid conjugation to produce lipidated proteins for membrane anchoring. The simplest way to rapidly functionalize a bilayer membrane

is to treat the membrane (living cell, liposome, or immobilized bilayer) with a pre-synthesized three-component molecular construct that is comprised of a lipophilic membrane anchor, hydrophilic linker, and an exposed functional component such as stealth polymer, reporter molecule, or bioactive sequence. Lipid hydrocarbon anchors are commonly used, and generally a two chain lipid anchor has higher membrane affinity than a cholesteryl or single chain lipid anchor. In addition, a longer non-polar acid chain has higher membrane affinity than a shorter chain. Amphiphilic fluorescent molecular probes are often used to stain cell membranes for imaging, tracking, or sensing experiments. Fluorescent probes with a zwitterionic linker between the dye and the lipid anchor exhibit high persistence in cell plasma membranes and enable high-performance microscopic imaging. Listed in Table 1 are general attributes of the different lipophilic membrane anchor strategies. However, it is important to realize that as the structural complexity of the three-component molecular construct increases, there is increased need to fully characterize the entire membrane-embedded assembly to ensure that it exhibits the anticipated dynamic self-assembled structure, high stability, and interfacial properties. Ongoing improvements in computer simulations of membrane dynamics will be very helpful.⁸⁴

Table 1. Attributes of Different Membrane Anchor Strategies

Anchor Strategy	Requires Protein Engineering?	Permits Synthetic Reporters?	High Persistence in Cell Membranes?	Membrane Persistence Sensitive to Linker Structure?
Biosynthetic	Yes	No	Yes	No
Semisynthetic	Sometimes	Yes	Yes	Sometimes
Synthetic	No	Yes	Sometimes	Yes

ASSOCIATED CONTENT

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The authors declare no competing financial interest.

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REFERENCES

- (1) Luchini, A.; Vitiello, G. Mimicking the Mammalian Plasma Membrane: An Overview of Lipid Membrane Models for Biophysical Studies. *Biomimetics* **2021**, *6* (1), 1–18. DOI: 10.3390/biomimetics6010003.
- (2) Xia, Y.; Xu, C.; Zhang, X.; Ning, P.; Wang, Z.; Tian, J.; Chen, X. Liposome-Based Probes for Molecular Imaging: From Basic Research to the Bedside. *Nanoscale* **2019**, *11* (13), 5822–5838. DOI: 10.1039/C9NR00207C.
- (3) Khan, A. A.; Allemailem, K. S.; Almatroodi, S. A.; Almatroudi, A.; Rahmani, A. H. Recent Strategies towards the Surface Modification of Liposomes: An Innovative Approach for

- Different Clinical Applications. *3 Biotech* **2020**, *10* (4), 1–15. DOI: 10.1007/s13205-020-2144-3.
- (4) Park, J.; Andrade, B.; Seo, Y.; Kim, M. J.; Zimmerman, S. C.; Kong, H. Engineering the Surface of Therapeutic “Living” Cells. *Chem. Rev.* **2018**, *118* (4), 1664–1690. DOI: 10.1021/acs.chemrev.7b00157.
 - (5) Anwar, S.; Mir, F.; Yokota, T. Enhancing the Effectiveness of Oligonucleotide Therapeutics Using Cell-Penetrating Peptide Conjugation, Chemical Modification, and Carrier-Based Delivery Strategies. *Pharmaceutics* **2023**, *15* (4), 1130. DOI: 10.3390/pharmaceutics15041130.
 - (6) Lavagna, E.; Güven, Z. P.; Bochicchio, D.; Olgiati, F.; Stellacci, F.; Rossi, G. Amphiphilic Nanoparticles Generate Curvature in Lipid Membranes and Shape Liposome-Liposome Interfaces. *Nanoscale* **2021**, *13* (40), 16879–16884. DOI: 10.1039/d1nr05067b.
 - (7) Kinoshita, T. Biosynthesis and Biology of Mammalian GPI-Anchored Proteins. *Open Biol.* **2020**, *10* (3), 190290. DOI: 10.1098/rsob.190290.
 - (8) Schultz, C. Chemical Tools for Lipid Cell Biology. *Accounts Chem. Res.* **2022**, *56* (10), 1168–1177. DOI: 10.1021/acs.accounts.2c00851.
 - (9) Harayama, T.; Riezman, H. Understanding the Diversity of Membrane Lipid Composition. *Nat. Rev. Mol. Cell Biol.* **2018**, *19* (5), 281–296. DOI: 10.1038/nrm.2017.138.
 - (10) Tristram-Nagle, S.; Nagle, J. F. Lipid Bilayers: Thermodynamics, Structure, Fluctuations, and Interactions. *Chem. Phys. Lipids* **2004**, *127* (1), 3–14. DOI: 10.1016/j.chemphyslip.2003.09.002.
 - (11) Rogers, J. R.; Geissler, P. L. Breakage of Hydrophobic Contacts Limits the Rate of Passive Lipid Exchange between Membranes. *J. Phys. Chem. B* **2020**, *124* (28), 5884–5898. DOI: 10.1021/acs.jpcb.0c04139.
 - (12) Kaiser, H. J.; Orłowski, A.; Róg, T.; Nyholm, T. K. M.; Chai, W.; Feizi, T.; Lingwood, D.; Vattulainen, I.; Simons, K. Lateral Sorting in Model Membranes by Cholesterol-Mediated Hydrophobic Matching. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (40), 16628–16633. DOI: 10.1073/pnas.1103742108.
 - (13) Suazo, K. F.; Park, K.; Distefano, M. D. A Not-So-Ancient Grease History: Click Chemistry

- and Protein Lipid Modifications. *Chem. Rev.* **2021**, *121* (12), 7178–7248. DOI: 10.1021/acs.chemrev.0c01108.
- (14) Hanna, C. C.; Kriegesmann, J.; Dowman, L. J.; Becker, C. F. W.; Payne, R. J. Chemical Synthesis and Semisynthesis of Lipidated Proteins. *Angew. Chem. Int. Ed.* **2022**, *61* (15). DOI: 10.1002/anie.202111266.
- (15) Schäfer, B.; Orbán, E.; Borics, A.; Huszár, K.; Nyeste, A.; Welker, E.; Tömböly, C. Preparation of Semisynthetic Lipoproteins with Fluorescent Cholesterol Anchor and Their Introduction to the Cell Membrane with Minimal Disruption of the Membrane. *Bioconjugate Chem.* **2013**, *24* (10), 1684–1697. DOI: 10.1021/bc4002135.
- (16) Hanshaw, R. G.; Stahelin, R. V.; Smith, B. D. Noncovalent Keystone Interactions Controlling Biomembrane Structure. *Chem. Eur. J.* **2008**, *14* (6), 1690–1697. DOI: 10.1002/chem.200701589.
- (17) Giménez-Andrés, M.; Čopič, A.; Antonny, B. The Many Faces of Amphipathic Helices. *Biomolecules* **2018**, *8* (3), 1–14. DOI: 10.3390/biom8030045.
- (18) Zhukovsky, M. A.; Filograna, A.; Luini, A.; Corda, D.; Valente, C. Protein Amphipathic Helix Insertion: A Mechanism to Induce Membrane Fission. *Front. Cell Dev. Biol.* **2019**, *7* (December), 1–29. DOI: 10.3389/fcell.2019.00291.
- (19) Liu, Z.; Gong, Y.; Bao, Y.; Guo, Y.; Wang, H.; Lin, G. N. TMPSS: A Deep Learning-Based Predictor for Secondary Structure and Topology Structure Prediction of Alpha-Helical Transmembrane Proteins. *Front. Bioeng. Biotechnol.* **2021**, *8* (January), 1–12. DOI: 10.3389/fbioe.2020.629937.
- (20) Lorent, J. H.; Levental, K. R.; Ganesan, L.; Rivera-Longworth, G.; Sezgin, E.; Doktorova, M.; Lyman, E.; Levental, I. Plasma Membranes Are Asymmetric in Lipid Unsaturation, Packing and Protein Shape. *Nat. Chem. Biol.* **2020**, *16* (6), 644–652. DOI: 10.1038/s41589-020-0529-6.
- (21) Levental, I.; Lyman, E. Regulation of Membrane Protein Structure and Function by Their Lipid Nano-Environment. *Nat. Rev. Mol. Cell Biol.* **2023**, *24* (2), 107–122. DOI: 10.1038/s41580-022-00524-4.
- (22) Roy, S.; Brasino, M.; Beirne, J. M.; Harguindey, A.; Chapnick, D. A.; Liu, X.; Cha, J. N.;

- Goodwin, A. P. Enzymes Photo-Cross-Linked to Live Cell Receptors Retain Activity and EGFR Inhibition after Both Internalization and Recycling. *Bioconjugate Chem.* **2020**, *31* (1), 104–112. DOI: 10.1021/acs.bioconjchem.9b00781.
- (23) Capicciotti, C. J.; Zong, C.; Sheikh, M. O.; Sun, T.; Wells, L.; Boons, G. J. Cell-Surface Glyco-Engineering by Exogenous Enzymatic Transfer Using a Bifunctional CMP-Neu5Ac Derivative. *J. Am. Chem. Soc.* **2017**, *139* (38), 13342–13348. DOI: 10.1021/jacs.7b05358.
- (24) Rana, M. S.; Wang, X.; Banerjee, A. An Improved Strategy for Fluorescent Tagging of Membrane Proteins for Overexpression and Purification in Mammalian Cells. *Biochemistry* **2018**, *57* (49), 6741–6751. DOI: 10.1021/acs.biochem.8b01070.
- (25) Roy, S.; Cha, J. N.; Goodwin, A. P. Nongenetic Bioconjugation Strategies for Modifying Cell Membranes and Membrane Proteins: A Review. *Bioconjugate Chem.* **2020**, *31* (11), 2465–2475. DOI: 10.1021/acs.bioconjchem.0c00529.
- (26) Ayele, T. M.; Knutson, S. D.; Heemstra, J. M. Covalent Live-Cell Labeling of Proteins Using a Photoreactive Fluorogen. *Methods Enzymol.* **2020**, *639* (April), 355–377. DOI: 10.1016/bs.mie.2020.04.019.
- (27) Yano, Y.; Matsuzaki, K. Tag-Probe Labeling Methods for Live-Cell Imaging of Membrane Proteins. *Biochim. Biophys. Acta - Biomembr.* **2009**, *1788* (10), 2124–2131. DOI: 10.1016/j.bbamem.2009.07.017.
- (28) Berki, T.; Bakunts, A.; Duret, D.; Fabre, L.; Ladavière, C.; Orsi, A.; Charreyre, M. T.; Raimondi, A.; Van Anken, E.; Favier, A. Advanced Fluorescent Polymer Probes for the Site-Specific Labeling of Proteins in Live Cells Using the HaloTag Technology. *ACS Omega* **2019**, *4* (7), 12841–12847. DOI: 10.1021/acsomega.9b01643.
- (29) Kundu, S.; Jaiswal, M.; Craig, K. C.; Guo, J.; Guo, Z. Labeling Cell Surface Glycosylphosphatidylinositol-Anchored Proteins through Metabolic Engineering Using an Azide-Modified Phosphatidylinositol. *Biochem. Biophys. Res. Commun.* **2023**, *645* (February), 103–109. DOI: 10.1016/j.bbrc.2023.01.029.
- (30) Elahipanah, S.; O'Brien, P. J.; Rogozhnikov, D.; Yousaf, M. N. General Dialdehyde Click Chemistry for Amine Bioconjugation. *Bioconjugate Chem.* **2017**, *28* (5), 1422–1433. DOI: 10.1021/acs.bioconjchem.7b00106.

- (31) Takayama, Y.; Kusamori, K.; Nishikawa, M. Click Chemistry as a Tool for Cell Engineering and Drug Delivery. *Molecules* **2019**, *24* (1), 172. DOI: 10.3390/molecules24010172.
- (32) Vabbilisetty, P.; Sun, X. L. Liposome Surface Functionalization Based on Different Anchoring Lipids via Staudinger Ligation. *Org. Biomol. Chem.* **2014**, *12* (8), 1237–1244. DOI: 10.1039/c3ob41721b.
- (33) Lu, R. M.; Chen, M. S.; Chang, D. K.; Chiu, C. Y.; Lin, W. C.; Yan, S. L.; Wang, Y. P.; Kuo, Y. S.; Yeh, C. Y.; Lo, A.; et al. Targeted Drug Delivery Systems Mediated by a Novel Peptide in Breast Cancer Therapy and Imaging. *PLoS One* **2013**, *8* (6), e66128. DOI: 10.1371/journal.pone.0066128.
- (34) Hartwell, B. L.; Melo, M. B.; Xiao, P.; Lemnios, A. A.; Li, N.; Chang, J. Y. H.; Yu, J.; Gebre, M. S.; Chang, A.; Maiorino, L.; et al. Intranasal Vaccination with Lipid-Conjugated Immunogens Promotes Antigen Transmucosal Uptake to Drive Mucosal and Systemic Immunity. *Sci. Transl. Med.* **2022**, *14* (654), 1413. DOI: 10.1126/scitranslmed.abn1413.
- (35) Nellis, D. F.; Ekstrom, D. L.; Kirpotin, D. B.; Zhu, J.; Andersson, R.; Broadt, T. L.; Ouellette, T. F.; Perkins, S. C.; Roach, J. M.; Drummond, D. C.; et al. Preclinical Manufacture of an Anti-HER2 ScFv-PEG-DSPE, Liposome-Inserting Conjugate. 1. Gram-Scale Production and Purification. *Biotechnol. Progr.* **2005**, *21* (1), 205–220. DOI: 10.1021/bp049840y.
- (36) Nsairat, H.; Mahmoud, I. S.; Odeh, F.; Abuarqoub, D.; Al-Azzawi, H.; Zaza, R.; Qadri, M. I.; Ismail, S.; Al Bawab, A.; Awidi, A.; et al. Grafting of Anti-Nucleolin Aptamer into Preformed and Remotely Loaded Liposomes through Aptamer-Cholesterol Post-Insertion. *RSC Adv.* **2020**, *10* (59), 36219–36229. DOI: 10.1039/d0ra07325c.
- (37) Cao, Z.; Zhang, L.; Jiang, S. Superhydrophilic Zwitterionic Polymers Stabilize Liposomes. *Langmuir* **2012**, *28* (31), 11625–11632. DOI: 10.1021/la302433a.
- (38) Rubio-Sanchez, R.; Fabrini, G.; Cicuta, P.; Di Michele, L. Amphiphilic DNA Nanostructures for Bottom-up Synthetic Biology. *Chem. Commun.* **2021**, 57 (95), 12725–12740. DOI: 10.1039/d1cc04311k.
- (39) Cosco, D.; Tsapis, N.; Nascimento, T. L.; Fresta, M.; Chapron, D.; Taverna, M.; Arpicco, S.; Fattal, E. Polysaccharide-Coated Liposomes by Post-Insertion of a Hyaluronan-Lipid Conjugate. *Colloid Surfaces B* **2017**, *158* (October), 119–126. DOI:

- 10.1016/j.colsurfb.2017.06.029.
- (40) Moreno, M. J.; Bastos, M.; Velazquez-Campoy, A. Partition of Amphiphilic Molecules to Lipid Bilayers by Isothermal Titration Calorimetry. *Anal. Biochem.* **2010**, *399* (1), 44–47. DOI: 10.1016/j.ab.2009.11.015.
- (41) Høyrup, P.; Davidsen, J.; Jørgensen, K. Lipid Membrane Partitioning of Lysolipids and Fatty Acids: Effects of Membrane Phase Structure and Detergent Chain Length. *J. Phys. Chem. B* **2001**, *105* (13), 2649–2657. DOI: 10.1021/jp003631o.
- (42) Cardoso, R. M. S.; Filipe, H. A. L.; Gomes, F.; Moreira, N. D.; Vaz, W. L. C.; Moreno, M. J. Chain Length Effect on the Binding of Amphiphiles to Serum Albumin and to POPC Bilayers. *J. Phys. Chem. B* **2010**, *114* (49), 16337–16346. DOI: 10.1021/jp105163k.
- (43) Paolino, D.; Accolla, M. L.; Cilurzo, F.; Cristiano, M. C.; Cosco, D.; Castelli, F.; Sarpietro, M. G.; Fresta, M.; Celia, C. Interaction between PEG Lipid and DSPE/DSPC Phospholipids: An Insight of PEGylation Degree and Kinetics of de-PEGylation. *Colloid Surfaces B* **2017**, *155* (July), 266–275. DOI: 10.1016/j.colsurfb.2017.04.018.
- (44) Johnsson, M.; Edwards, K. Interactions between Nonionic Surfactants and Sterically Stabilized Phosphatidyl Choline Liposomes. *Langmuir* **2000**, *16* (23), 8632–8642. DOI: 10.1021/la000190r.
- (45) Fan, X.; Xu, H.; Song, J.; Jin, Y.; Wink, M.; Wu, G. Using a Membrane-Penetrating-Peptide to Anchor Ligands in the Liposome Membrane Facilitates Targeted Drug Delivery. *Bioconjugate Chem.* **2020**, *31* (1), 113–122. DOI: 10.1021/acs.bioconjchem.9b00798.
- (46) Fan, X.; Xu, H.; Zhao, F.; Song, J.; Jin, Y.; Zhang, C.; Wu, G. Lipid-Mimicking Peptide Decorates Erythrocyte Membrane for Active Delivery to Engrafted MDA-MB-231 Breast Tumour. *Eur. J. Pharm. Biopharm.* **2020**, *152* (July), 72–84. DOI: 10.1016/j.ejpb.2020.04.024.
- (47) Tokunaga, T.; Kuwahata, K.; Sando, S. Systematic Exploration of Lipophilic Tags That Allow Efficient Anchoring of Aptamers to Live Cell Surfaces. *Chem. Lett.* **2013**, *42* (2), 127–129. DOI: 10.1246/cl.2013.127.
- (48) Woods, E. C.; Yee, N. A.; Shen, J.; Bertozzi, C. R. Glycocalyx Engineering with a Recycling Glycopolymer That Increases Cell Survival in Vivo. *Angew. Chem. Int. Ed.* **2015**, *54* (52),

- 15782–15788. DOI: 10.1002/anie.201508783.
- (49) Fritz, T.; Voigt, M.; Worm, M.; Negwer, I.; Müller, S. S.; Kettenbach, K.; Ross, T. L.; Roesch, F.; Koynov, K.; Frey, H.; et al. Orthogonal Click Conjugation to the Liposomal Surface Reveals the Stability of the Lipid Anchorage as Crucial for Targeting. *Chem. Eur. J.* **2016**, *22* (33), 11578–11582. DOI: 10.1002/chem.201602758.
- (50) Versluis, F.; Voskuhl, J.; Van Kolck, B.; Zope, H.; Bremmer, M.; Albregtse, T.; Kros, A. In Situ Modification of Plain Liposomes with Lipidated Coiled Coil Forming Peptides Induces Membrane Fusion. *J. Am. Chem. Soc.* **2013**, *135* (21), 8057–8062. DOI: 10.1021/ja4031227.
- (51) Gleue, L.; Schupp, J.; Zimmer, N.; Becker, E.; Frey, H.; Tuettenberg, A.; Helm, M. Stability of Alkyl Chain-Mediated Lipid Anchoring in Liposomal Membranes. *Cells* **2020**, *9* (10), 1–13. DOI: 10.3390/cells9102213.
- (52) Beales, P. A.; Vanderlick, T. K. Application of Nucleic Acid-Lipid Conjugates for the Programmable Organisation of Liposomal Modules. *Adv. Colloid Interface Sci.* **2014**, *207* (1), 290–305. DOI: 10.1016/j.cis.2013.12.009.
- (53) Souza, L. M. P.; Souza, F. R.; Reynaud, F.; Pimentel, A. S. Tuning the Hydrophobicity of a Coarse Grained Model of 1,2-Dipalmitoyl-sn-Glycero-3-Phosphatidylcholine Using the Experimental Octanol-Water Partition Coefficient. *J. Mol. Liq.* **2020**, *319* (December), 114132. <https://doi.org/10.1016/j.molliq.2020.114132>.
- (54) Niu, S. L.; Litman, B. J. Determination of Membrane Cholesterol Partition Coefficient Using a Lipid Vesicle-Cyclodextrin Binary System: Effect of Phospholipid Acyl Chain Unsaturation and Headgroup Composition. *Biophys. J.* **2002**, *83* (6), 3408–3415. DOI: 10.1016/S0006-3495(02)75340-X.
- (55) You, M.; Lyu, Y.; Han, D.; Qiu, L.; Liu, Q.; Chen, T.; Sam Wu, C.; Peng, L.; Zhang, L.; Bao, G.; et al. DNA Probes for Monitoring Dynamic and Transient Molecular Encounters on Live Cell Membranes. *Nat. Nanotechnol.* **2017**, *12* (5), 453–459. DOI: 10.1038/nnano.2017.23.
- (56) Boonyarattanakalin, S.; Martin, S. E.; Dykstra, S. A.; Peterson, B. R. Synthetic Mimics of Small Mammalian Cell Surface Receptors. *J. Am. Chem. Soc.* **2004**, *126* (50), 16379–

16386. DOI: 10.1021/ja046663o.
- (57) Woods, E. C.; Yee, N. A.; Shen, J.; Bertozzi, C. R. Glycocalyx Engineering with a Recycling Glycopolymer That Increases Cell Survival in Vivo. *Angew. Chem. Int. Ed.* **2015**, *54* (52), 15782–15788. DOI: 10.1002/anie.201508783.
 - (58) Parr, M. J.; Ansell, S. M.; Choi, L. S.; Cullis, P. R. Factors Influencing the Retention and Chemical Stability of Poly(Ethylene Glycol)-Lipid Conjugates Incorporated into Large Unilamellar Vesicles. *BBA Biomembranes* **1994**, *1195* (1), 21–30. DOI: 10.1016/0005-2736(94)90004-3.
 - (59) Silvius, J. R.; Zuckermann, M. J. Interbilayer Transfer of Phospholipid-Anchored Macromolecules via Monomer Diffusion. *Biochemistry* **1993**, *32* (12), 3153–3161. DOI: 10.1021/bi00063a030.
 - (60) Che, J.; Okeke, C. I.; Hu, Z. B.; Xu, J. DSPE-PEG: A Distinctive Component in Drug Delivery System. *Curr. Phar. Des.* **2015**, *21* (12), 1598–1605. DOI: 10.2174/1381612821666150115144003.
 - (61) Zhang, Y.; Mintzer, E.; Uhrich, K. E. Synthesis and Characterization of PEGylated Bolaamphiphiles with Enhanced Retention in Liposomes. *J. Colloid Interface Sci.* **2016**, *482* (November), 19–26. DOI: 10.1016/j.jcis.2016.07.013.
 - (62) Chen, S.; Tam, Y. Y. C.; Lin, P. J. C.; Sung, M. M. H.; Tam, Y. K.; Cullis, P. R. Influence of Particle Size on the in Vivo Potency of Lipid Nanoparticle Formulations of siRNA. *J. Control. Release* **2016**, *235* (August), 236–244. DOI: 10.1016/j.jconrel.2016.05.059.
 - (63) Wang, H. Y.; Jia, H. R.; Lu, X.; Chen, B.; Zhou, G.; He, N.; Chen, Z.; Wu, F. G. Imaging Plasma Membranes without Cellular Internalization: Multisite Membrane Anchoring Reagents Based on Glycol Chitosan Derivatives. *J. Mater. Chem. B* **2015**, *3* (30), 6165–6173. DOI: 10.1039/c5tb00930h.
 - (64) Kim, J. C.; Tae, G. The Modulation of Biodistribution of Stem Cells by Anchoring Lipid-Conjugated Heparin on the Cell Surface. *J. Control. Release* **2015**, *217* (November), 128–137. DOI: 10.1016/j.jconrel.2015.08.053.
 - (65) Klymchenko, A. S. Fluorescent Probes for Lipid Membranes: From the Cell Surface to Organelles. *Accounts Chem. Res.* **2023**, *56* (1), 1–12. DOI: 10.1021/acs.accounts.2c00586.

- (66) Liu, C.; Gao, X.; Yuan, J.; Zhang, R. Advances in the Development of Fluorescence Probes for Cell Plasma Membrane Imaging. *TrAC Trends Anal. Chem.* **2020**, *133* (December), 116092. DOI: 10.1016/j.trac.2020.116092.
- (67) Cheng, C.; Trzcinski, O.; Doering, L. C. Fluorescent Labeling of Dendritic Spines in Cell Cultures with the Carbocyanine Dye "Dil." *Front. Neuroanat.* **2014**, *8* (May), 1–8. DOI: 10.3389/fnana.2014.00030.
- (68) Gamage, R. S.; Smith, B. D. Spontaneous Transfer of Indocyanine Green from Liposomes to Albumin Is Inhibited by the Antioxidant α -Tocopherol. *Langmuir* **2022**, *38* (39), 11950–11961. DOI: 10.1021/acs.langmuir.2c01715.
- (69) Collot, M.; Ashokkumar, P.; Anton, H.; Boutant, E.; Faklaris, O.; Galli, T.; Mély, Y.; Danglot, L.; Klymchenko, A. S. MemBright: A Family of Fluorescent Membrane Probes for Advanced Cellular Imaging and Neuroscience. *Cell Chem. Biol.* **2019**, *26* (4), 600–614.. DOI: 10.1016/j.chembiol.2019.01.009.
- (70) Collot, M.; Boutant, E.; Lehmann, M.; Klymchenko, A. S. BODIPY with Tuned Amphiphilicity as a Fluorogenic Plasma Membrane Probe. *Bioconjugate Chem.* **2019**, *30* (1), 192–199. DOI: 10.1021/acs.bioconjchem.8b00828.
- (71) Karpenko, I. A.; Collot, M.; Richert, L.; Valencia, C.; Villa, P.; Mély, Y.; Hibert, M.; Bonnet, D.; Klymchenko, A. S. Fluorogenic Squaraine Dimers with Polarity-Sensitive Folding as Bright Far-Red Probes for Background-Free Bioimaging. *J. Am. Chem. Soc.* **2015**, *137* (1), 405–412. DOI: 10.1021/ja5111267.
- (72) Klymchenko, A. S. Solvatochromic and Fluorogenic Dyes as Environment-Sensitive Probes: Design and Biological Applications. *Accounts Chem. Res.* **2017**, *50* (2), 366–375. DOI: 10.1021/acs.accounts.6b00517.
- (73) Münter, R.; Kristensen, K.; Pedersbæk, D.; Larsen, J. B.; Simonsen, J. B.; Andresen, T. L. Dissociation of Fluorescently Labeled Lipids from Liposomes in Biological Environments Challenges the Interpretation of Uptake Studies. *Nanoscale* **2018**, *10* (48), 22720–22724. DOI: 10.1039/c8nr07755j.
- (74) Wang, G.; Zannikou, M.; Lofchy, L.; Li, Y.; Gaikwad, H.; Balyasnikova, I. V.; Simberg, D. Liposomal Extravasation and Accumulation in Tumors as Studied by Fluorescence

- Microscopy and Imaging Depend on the Fluorescent Label. *ACS Nano* **2021**, *15* (7), 11880–11890. DOI: 10.1021/acsnano.1c02982.
- (75) Cauzzo, J.; Nystad, M.; Holsæter, A. M.; Basnet, P.; Škalko-Basnet, N. Following the Fate of Dye-Containing Liposomes in Vitro. *Int. J. Mol. Sci.* **2020**, *21* (14), 1–17. DOI: 10.3390/ijms21144847.
- (76) Harrington, L.; Fletcher, J. M.; Heermann, T.; Woolfson, D. N.; Schwille, P. De Novo Design of a Reversible Phosphorylation-Dependent Switch for Membrane Targeting. *Nat. Commun.* **2021**, *12* (1), 1–11. DOI: 10.1038/s41467-021-21622-5.
- (77) Kai, L.; Sonal, N.; Heermann, T.; Schwille, P. Reconstitution of a Reversible Membrane Switch via Prenylation by One-Pot Cell-Free Expression. *ACS Synth. Biol.* **2023**, *12* (1), 108–119. DOI: 10.1021/acssynbio.2c00406.
- (78) Udyavara Nagaraj, V.; Juhász, T.; Quemé-Peña, M.; Szigyártó, I. C.; Bogdán, D.; Wacha, A.; Mihály, J.; Románszki, L.; Varga, Z.; Andréasson, J.; et al. Stimuli-Responsive Membrane Anchor Peptide Nanofoils for Tunable Membrane Association and Lipid Bilayer Fusion. *ACS Appl. Mater. Inter.* **2022**, *14* (50), 55320–55331. DOI: 10.1021/acсами.2c11946.
- (79) Gutiérrez-Salazar, M.; Santamaría-Aranda, E.; Schaar, L.; Salgado, J.; Sampedro, D.; Lorenz-Fonfria, V. A. A Photoswitchable Helical Peptide with Light-Controllable Interface/Transmembrane Topology in Lipidic Membranes. *iScience* **2021**, *24* (7), 102771. <https://doi.org/10.1016/j.isci.2021.102771>.
- (80) Wu, H.; Zheng, L.; Ling, N.; Zheng, L.; Du, Y.; Zhang, Q.; Liu, Y.; Tan, W.; Qiu, L. Chemically Synthetic Membrane Receptors Establish Cells with Artificial Sense-and-Respond Signaling Pathways. *J. Am. Chem. Soc.* **2023**, *145* (4), 2315–2321. DOI: 10.1021/jacs.2c10903.
- (81) Reissmann, S.; Filatova, M. P. New Generation of Cell-Penetrating Peptides: Functionality and Potential Clinical Application. *J. Pept. Sci.* **2021**, *27* (5), 1–14. DOI: 10.1002/psc.3300.
- (82) Zhang, S.; Li, W.; Luan, J.; Srivastava, A.; Carnevale, V.; Klein, M. L.; Sun, J.; Wang, D.; Teora, S. P.; Rijpkema, S. J.; et al. Adaptive Insertion of a Hydrophobic Anchor into a Poly(Ethylene Glycol) Host for Programmable Surface Functionalization. *Nat. Chem.* **2023**, *15* (2), 240–247. DOI: 10.1038/s41557-022-01090-0.

- (83) Zhang, S.; Srivastava, A.; Li, W.; Rijpkema, S. J.; Carnevale, V.; Klein, M. L.; Wilson, D. A. Molecular Engineering of PH-Responsive Anchoring Systems onto Poly(Ethylene Glycol) Corona. *J. Am. Chem. Soc.* **2023**, *145* (19), 10458–10462. DOI: 10.1021/jacs.3c00986.
- (84) Róg, T.; Giryh, M.; Bunker, A. Mechanistic Understanding from Molecular Dynamics in Pharmaceutical Research 2: Lipid Membrane in Drug Design. *Pharmaceuticals* **2021**, *14* (10), 14101062. DOI: 10.3390/ph14101062.

Graphic for TOC

