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Membrane Augmented Cell-Free Systems: A New Frontier in Biotechnology

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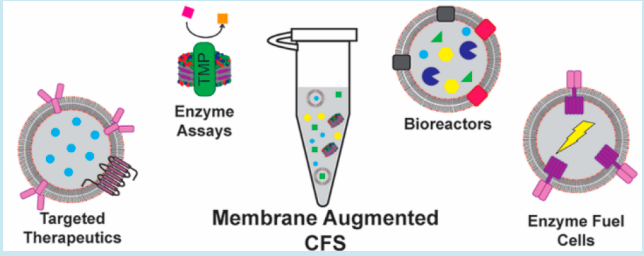
ABSTRACT: Membrane proteins are present in a wide array of cellular processes from primary and secondary metabolite synthesis to electron transport and single carbon metabolism. A key barrier to applying membrane proteins industrially is their difficult functional production. Beyond expression, folding, and membrane insertion, membrane protein activity is influenced by the physicochemical properties of the associated membrane, making it difficult to achieve optimal membrane protein performance outside the endogenous host. In this review, we highlight recent work on production of membrane proteins in membrane augmented cell-free systems (CFSs) and applications thereof. CFSs lack membranes and can thus be augmented with user-specified, tunable, mimetic membranes to generate customized environments for production of functional membrane proteins of interest. Membrane augmented CFSs would enable the synthesis of more complex plant secondary metabolites, the growth and division of synthetic cells for drug delivery and cell therapeutic applications, as well as enable green energy applications including methane capture and artificial photosynthesis.



KEYWORDS: *cell-free systems, membrane proteins, synthetic cells, natural products, liposomes*

The functional, heterologous expression of membrane proteins is one of the missing puzzle pieces in establishing industrially relevant biological processes ranging from the production of medicinal compounds to the capture of methane (CH_4) to the bioremediation of heavy metal pollutants (Figure 1A). Plant-based medicinal compounds are synthesized *via* multienzyme cascades composed of several transmembrane cytochromes P450 (CYPs) that decorate the compounds' scaffolds.[1,2] Particulate methane monooxygenase (MMO) oxidizes CH_4 to methanol,[3−5] which enters C1 assimilation pathways in natural and synthetic methanotrophs,[3] potentially able to convert the ∼650 million tons of CO_2 equivalents produced in the U.S.[6] into high density fuels to power trucks and airplanes. Heavy metals, such as uranium from nuclear waste, can be bioremediated using MtrCAB, which facilitates the transfer of electrons from the organism to the heavy metal.[7] Toward therapeutic applications, the robust functional heterologous expression of surface receptors would support the development of drug delivery vehicles and cell therapies. For instance, G protein-coupled receptors (GPCRs) are the target of more than 30% of FDA approved drugs. Routine heterologous expression of GPCRs would facilitate the development of high-throughput screening platforms for the discovery of new drugs or the study of signaling cascades in the absence of endogenous GPCRs.[8] Access to a wider array of functional receptors would also expand cell therapies beyond detection of cell surface antigens on cancer cells to the detection of soluble, small molecule ligands around the tumor to improve targeting and reduce on-target off-tumor toxicity, *i.e.*, targeting a non-tumor tissue expressing the same antigen.[9] For example, by using GPCRs, which mediate most cellular responses to small molecules.[10] Finally, transmembrane proteins are pivotal in primary metabolism, determining the biosynthetic performance of the production host. For instance, a network of transmembrane proteins synthesizes the phospholipids needed to build the cell's membranes. The oxidative phosphorylation pathway used to produce ATP in plants, bacteria, and humans is also composed of transmembrane proteins.

Application of membrane proteins is hindered by their difficult production outside their endogenous host, with successful applications often requiring engineering of the transmembrane domain. For example, the microbial synthesis

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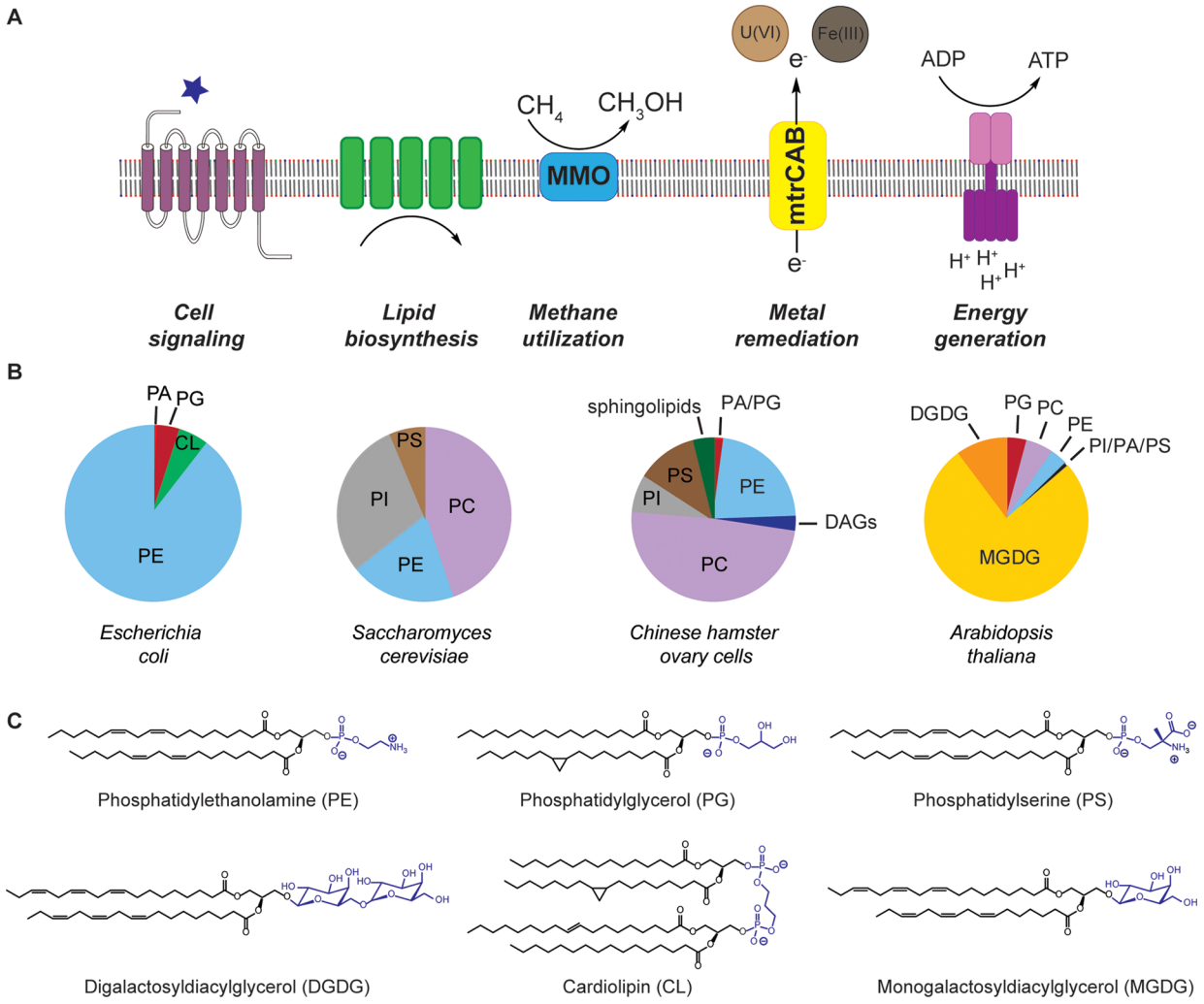

Figure 1. Biological roles of membrane proteins and cell membranes. (A) Cellular processes that occur at the cell membrane include cell signaling, lipid biosynthesis, methane utilization, metal remediation, and energy generation. (B) Membrane compositions in different organisms. PG: Phosphatidylglycerol, PA: Phosphatidic acid, PE: Phosphatidylethanolamine, CL: Cardolipin, PC: Phosphatidylcholine, PI: Phosphatidylinositol, PS: Phosphatidylserine, DAG: Diacylglycerol, MGDG: Monogalactosyldiacylglycerol, DGDG: Digalactosyldiacylglycerol. (C) Sample phospholipid structures. Phospholipid heads in blue.

of plant natural products is limited to hosts amenable to plant transmembrane CYP production, such as *Saccharomyces cerevisiae*, in spite of other hosts, such as *Escherichia coli*, having achieved higher precursor yields.[11] The development of protein chimeras and truncation of plant CYPs to generate soluble variants has been successful;[12] however, only one or two CYPs are usually engineered at a time, far from the five to ten CYPs required for many plant biosynthetic pathways.

Beyond transmembrane protein production, the physicochemical properties of the membrane, including composition, fluidity, curvature, and molecular crowding, influence the production and activity of membrane proteins.[13,14] For example, expression of *Catharanthus roseus* geraniol 10-hydroxylase in *S. cerevisiae* has a 8.3-fold lower activity than the same protein synthesized in a plant membrane, likely due to decreased enzyme stability and suboptimal reductase pairing.[15] In addition, a molecular dynamics simulation of a human CYP, CYP3A4, showed that lipid composition and electrostatics impact membrane incorporation and membrane protein orientation.[16] Indeed, the membrane compositions of mammalian, microbial, and plant cells vary vastly from one another[17−20] (Figure 1B).

As nonliving systems devoid of membranes, cell-free systems (CFSs) could be augmented with tailor-made membranes to fulfill specific membrane protein requirements and applications, thus functionally producing membrane-bound proteins that are challenging to synthesize using cells. Briefly, CFSs are composed of a cell lysate or purified cell machinery (PURE) supplemented with the nucleotides, energy sources, amino acids, cofactors, and salts necessary for transcription and translation.[21] PURE systems are often preferred for more complex protein synthesis due to reduced background and optimized conditions. For example, bacteriorhodopsin, ATP synthase, and enzymes in the lipid biosynthesis pathway have all been synthesized in PURE systems.[22] On the other hand, preparation of PURE reaction mix is low throughput and expensive. Thus, for potential scale-up applications cell lysate-based systems are required. Among cell-lysate-based CFSs, the *E. coli* based CFSs are the most commonly used platform with applications to the production of therapeutics, genetic circuit

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engineering, construction of synthetic cells, chemical biosynthesis, and protein production.[23]

In membrane augmented CFSs, the user has complete control over composition, fluidity, and crowding of membranes, in addition to curvature and vesicle size, in the case of encapsulated CFSs. Lipids of different structure, length, saturation, and charge, a number of them commercially available, could be used to optimize the membrane composition (Figure 1C). Further, encapsulated CFSs could have different lipid compositions depending on the location of the bilayer leaflet. A membrane augmented CFS would open the doors to important bioindustrial applications, such as the development of hybrid chemical-biological processes, the use of organic solvents for *in situ* product extractions, and more efficient downstream processes for product separation. Developing genetic control systems to control both the lipid and protein composition of membrane augmented CFSs will be pivotal to achieve the high levels of membrane enzyme activity to enable these applications. For example in the bioremediation space, expression of MtrCAB in a membrane augmented CFS would enable circumvention of cell toxicity issues to facilitate applications at high contaminant concentrations.

In this review, we highlight recent advances in membrane-based CFSs and their application in the heterologous production of membrane proteins. Further, we examine the potential for genetic control systems, such as those implemented with CRISPR-based transcriptional regulation, to improve the cell-free synthesis of membrane proteins for chemical production and for the study of protein−membrane interactions. Although we are not yet at the level of on-demand membrane augmented CFS generation, the potential advantages of such systems in terms of enabling new chemistry and improving chemical bioproduction processes make it a new frontier in biotechnology.

1. PRODUCTION OF MEMBRANE PROTEINS IN CFSS

1.1. Membrane Augmented CFSs. Membrane protein production in CFSs is often limited by self-aggregation,[13] and addition of oil droplets to a PURE CFS has enabled the production of single-span transmembrane proteins, such as FasL and TRAIL used as anticancer therapeutics.[24] Oil droplets, however, are limited to the display of surface receptors, and cannot recapitulate the physicochemical properties of native lipids that support membrane protein activity.[13] Phospholipid-like additives, such as nanodiscs and liposomes, recapitulate membrane composition better (Figure 2).[25] Nanodiscs are phospholipids stabilized by membrane scaffold proteins,[26] which form a planar bilayer akin to a lipid raft. Both sides of a nanodisc are indistinguishable from one another and open to interact with the CFS environment.[13] Nanodiscs are commercially available and can be directly added to CFSs. Nanodiscs, however, may not work to produce every transmembrane protein,[13] and their composition cannot be easily changed.[25] The cost of nanodiscs (~$1/μL of CFS reaction volume), currently limits this technology to mostly research applications. Liposomes are spherical phospholipid vesicles[13] that provide a tunable environment in terms of shape, size, and composition to mimic both prokaryotic (d = 0.1−5.0 μm) and eukaryotic (d = 10−100 μm) cells. The availability of a wide array of phospholipids allows the generation of liposomes with tunable composition and size. Given liposomes' large surface area, they are the preferred scaffold for coexpression of multiple membrane proteins. Liposomes can be added to a CFS reaction or the CFS can be encapsulated within the liposomes, albeit the yield of liposomes loaded with CFS is typically small (~100 liposomes per μL of reaction mix), limiting their application. Using commercial phospholipids, liposomes have a cost of < $0.01/μL of CFS reaction, enabling high-end biotechnology applications.[27] Finally, inverted vesicles formed during the CFS cell lysis procedure have been used to produce complex membrane proteins, including the oxidative phosphorylation pathway[28] and transmembrane oligosaccharyltransferases for protein glycosylation.[29] As used, inverted vesicles require production and insertion of membrane proteins at the cellular stage, which brings the usual challenges with heterologous production of membrane proteins. The cell used for protein production controls the membrane composition and vesicle size, making it difficult to explore the extent to which these variables have an effect on enzyme activity or control these variables for the desired application.

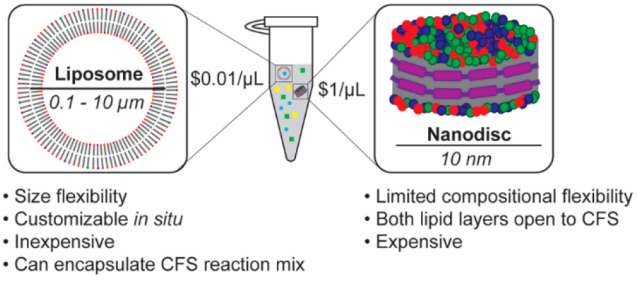

Figure 2. Membrane augmented cell-free systems. Mimetic membranes, in the form of liposomes and nanodiscs, are added directly to CFSs to aid in membrane protein production.

In situ phospholipid synthesis by CFSs would reduce the cost of membrane augmented CFSs, opening the doors to bioindustrial applications. *In situ* phospholipid synthesis has been achieved by feeding glycerol-3-phosphate and acyl-CoA. Because the lipid biosynthesis enzymes are themselves membrane-bound proteins, the CFS must be inoculated with preformed liposomes.[30] To enable feeding of fatty acids as a substrate, FadD was expressed in the CFS leading to phospholipid production and observable vesicle growth.[31] Nevertheless, low fatty acid solubility and its detrimental impact on protein stability limited this work, making the case for using glucose as the starting material in the future. Of note, as the fatty acids are located on the outside of the liposome, the phospholipids are only incorporated to the outer leaflet of the bilayer, limiting the synthesis of asymmetric membranes and potentially disturbing the incorporation of transmembrane proteins.

1.2. Chemical Composition of Membrane Augmented CFSs. Membrane proteins often rely on the phospholipids around them for activity. For example, the reaction rate, substrate affinity, and reductase coupling efficiency of the human cardiomycete epoxygenase responsible for oxidation of fatty acids and xenobiotics (CYP2J2) was shown to be impacted by the concentrations of phosphatidylcholine (PC) and phosphatidylserine (PS).[32] In another example, the bacterial structural protein MreB was polymerized when expressed in a liposome composed of PC and phosphatidylethanolamine-polyethylene glycol (PE−PEG) due to PEG's effect on membrane crowding, changing the

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Table 1. Applications of Membrane Proteins in Cell-Free Systems[a,b,c,d]

Protein Type Key
Structural/Cell Maintenance
Transporter/Porin
Energy Production
Receptor
Secondary Metabolism

Protein	Description	Application	Membrane type	Membrane composition	Protein Synthesis	CFS Method	Citation
MreB	Cell shape-determining protein	Synthetic cells	Liposome	Egg PC PE-PEG	Cell-Free	Cell lysate	[33]
				E. coli total lipid extract	Purified enzymes	N/A	[77]
HyaA	Hydrogensase-1 small chain	Protein characterization	Liposome	DOPC DGS-NTA DMPE-RhoB	Cell-Free	PURE	[78]
OmpA	Outer membrane protein A						
YfbF	Glycosyl transferase	Protocol development	Liposome	DOPC	Cell-Free	PURE	[79]
CyoE	Protoheme IX farnesyltransferase						
FtsZ	Cell division protein	Synthetic cells	Liposome	POPC POPG DOPE-RhoB	Cell-Free	PURE	[80]
FtsA							
ZipA							
GPAT	Lipid biosynthesis	Synthetic cells	Liposome	DOPC DOPE DOPG cardiolipin	Cell-Free	PURE	[30, 36]
LPAAT							
CdsA							
PgsA							
PgpABC					Purified enzymes	N/A	[31]
PssA							
Psd							
CYP2J2	Cytochrome P450	Protein characterization	Nanodisc	POPC POPS Cholesterol	Purified enzymes	N/A	[32]
CYP5A1	Cytochrome P450	Protein characterization	Nanodisc	POPC POPS POPE	Purified enzymes	N/A	[81]
CYP2B4	Cytochrome P450	Protein characterization	Nanodisc	DMPC POPC POPS	Purified enzymes	N/A	[82]
Opi3	Methyltransferase	Protein characterization	Nanodisc	DMPG DOPG DOPMME	Cell-Free	Cell lysate	[37]
OST	Oligosaccharyltransferases	Protein characterization	Nanodisc	POPC	Cell-Free	Cell lysate	[46]
		Protocol development	Inverted vesicle	N/A	Cell-Free	Cell lysate	[29]
SecYEG	Translocon	Protocol development	Liposome	Soybean lipid extract	Cell-Free	PURE	[42]
YidC	Insertase						
LepB	Signal peptidase						
MscL	Large-conductance mechanosensitive channel	Synthetic cells	Liposome	Egg PC	Cell-Free	Cell lysate	[83]
		Protein characterization	Liposome	DOPC + PEG diblock copolymer or detergent	Cell-Free	PURE	[39]
ErmE	Multidrug transporter	Protein characterization	Liposome	POPC	Cell-Free	PURE	[38]
		Protocol development	Liposome	POPC	Cell-Free	PURE	[84]
LacY	Sugar transporter	Protein characterization	Liposome	DMPC DOPC DOPE DOPG	Cell-Free	PURE	[40]
XylE							
α-hemolysin	Pore forming protein	Protein characterization	Liposome	POPC Cholesterol	Cell-Free	PURE	[85]
		Synthetic cells	Liposome	POPC Cholesterol	Cell-Free	Cell lysate	[60]
PFO	Pore protein perfringolysin O	Synthetic cells	Liposome	POPC Cholesterol	Cell-Free	Cell lysate	[59]

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Table 1. continued

Protein	Description	Application	Membrane type	Membrane composition	Protein Synthesis	CFS Method	Citation
ATP synthase	ATP synthesizing protein complex	Synthetic cells	Liposome	DOPC DOPE DOPG	Cell-Free	Cell lysate	[43]
				POPC	Purified enzymes	N/A	[68]
					Cell-Free	PURE	
				POPC POPE POPG Cholesterol	Purified enzymes	N/A	[67]
		Protocol development	Inverted vesicle	N/A	*In vivo*[1]	N/A	[28]
Bacterio-rhodopsin	Photoconverter	Synthetic cells	Liposome	POPC	Purified enzymes	N/A	[68]
					Cell-Free	PURE	
				POPC POPE POPG Cholesterol	Purified enzymes	N/A	[67]
Photo-system II	Photoconverter	Synthetic cells	Liposome	POPC POPE POPG Cholesterol	Purified enzymes	N/A	[67]
PsbS	Photosystem II subunit S	Synthetic cells	Liposome	Asolectin	Cell-Free	Cell lysate[2]	[86]
MtrCAB	Electron transfer pathway	Protein characterization	Liposome	PC	Purified enzymes	N/A	[87]
CX3CR1	Chemokine G-protein coupled receptors	Protein characterization	Liposome	POPC PE-PEG	Cell Free	PURE	[47]
CCR5			Nanodisc	POPC POPS Cholesterol			
CYP725A	Cytochrome P450	Protein characterization	Nanodisc	POPC	Purified enzymes	N/A	[88]

[a]DGS-NTA = Dioleoylglycero-[(*N*-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt). DMPC = Dimyristoylphosphatidylcholine. DMPE-RhoB = Dimyristoylphosphatidylethanolamine-rhodamine B. DMPG = Dimyristoylphosphatidylglycerol. DOPC = Dioleoylphosphatidylcholine. DOPE = Dioleoylphosphatidylethanolamine. DOPE-RhoB = Dioleoylphosphatidylethanolamine-rhodamine B. DOPG = Dioleoylphosphatidylglycerol. DOPMME = Dioleoylphosphatidylmonomethylethanolamine. PC = Phosphatidylcholine. PE−PEG = Phosphatidylethanolamine-polyethylene glycol. POPC = Palmitoyl-oleoyl-phosphatidylcholine. POPE = Palmitoyl-oleoyl-phosphatidylethanolamine. POPG = Palmitoyl-oleoyl-phosphatidylglycerol. POPS = Palmitoyl-oleoyl-phosphatidylserine. [b]In the protein synthesis column: "Cell-Free" indicates that the enzyme was synthesize in the cell-free system. "Purified enzymes" indicates that the protein was synthesized *in vivo*, purified, and reconstituted in CFS. [c(1)] ATP synthase produced *in vivo* and recovered in CFS after cell lysis. [d(2)] Cell lysate reaction mix contains added, purified T7 polymerase.

shape of the liposome from spherical to rod-like.[33] Therefore, membrane protein activity can be altered not only by protein engineering, but also by engineering the membranes in which they are embedded.

Changing the chemical structure of the phospholipid heads or tails causes changes in the membrane's physical properties such as fluidity, thickness, and charge.[34] *In situ* changes in phospholipid composition have been achieved by using different acyltransferases to convert phosphatidic acid (PA) to either phosphatidylglycerol (PG) or PE.[30] Importantly, the PE:PG ratio was maintained to the *E. coli* membrane composition, 75% PE,[35] both genetically, by placing PE and PG production under control of different promoters and dosing the respective polymerases, and enzymatically, using PssA, which associates with PG rich membranes and catalyzes the synthesis of PE.[36] Finally, conversion of phosphatidylmonomethylethanolamine (PMME) into PC, the most prevalent phospholipid in eukaryotes, was achieved by expressing the methyltransferase Opi3 in nanodiscs.[37]

1.3. Physical Properties of Membrane Augmented CFSs. Liposome vesicle diameter, shape, curvature, and number of lamella can be optimized to create a native-like membrane environment. Optimizing the physical properties of the mimetic membrane is key to protein folding, membrane incorporation, and activity. Using the multidrug transporter ErmE as a model, it was shown that surface area-to-volume ratio in smaller vesicles improved membrane insertion. Additionally the ratio of incorporated ErmE to total synthesized ErmE was a function of liposome size, not of DNA concentration.[38] It remains to be seen if this correlation holds true for other membrane proteins, or in membranes composed of more than one type of lipid. Optimal membrane size may depend on other factors such as membrane fluidity and rigidity and may change depending on the transmembrane protein source organism. Another important membrane physical parameter is elasticity. Using diblock copolymers to increase the membrane elasticity, it was shown that decreasing the membrane area expansion modulus improved membrane folding of the mechanosensitive channel of large conductance (MscL) using the PURE system.[39] However, differing results for the model membrane protein channel rhodopsin (ChR2) indicated that this conclusion may not be generalizable to all membrane proteins and the number of transmembrane regions may influence the optimal membrane elasticity. Importantly, in

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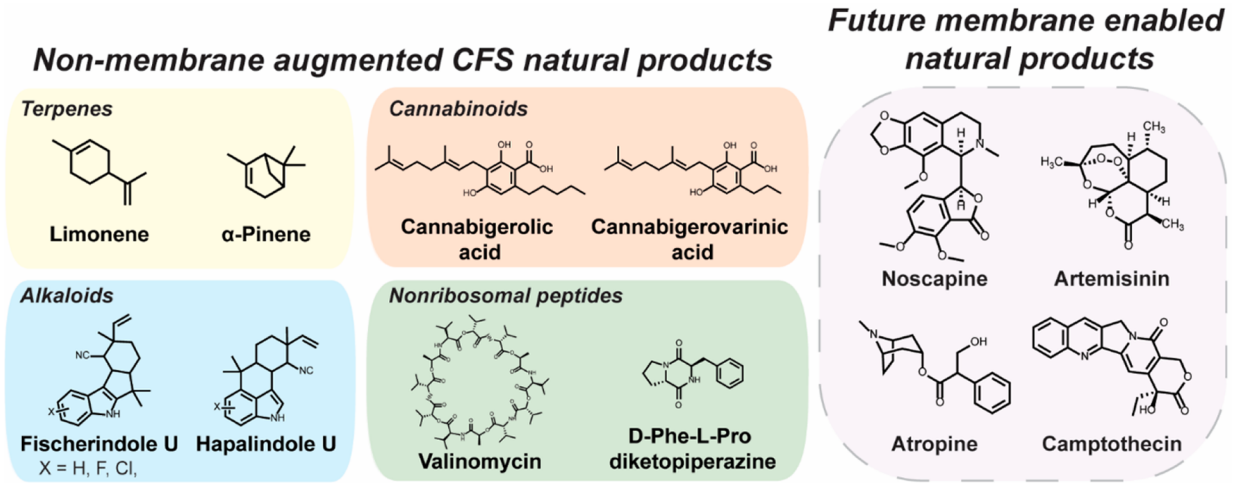

Figure 3. Production of natural products using cell-free systems. Compounds produced to date do not require membrane proteins or membrane augmented CFSs. In the dashed box, natural products that depend on the production of transmembrane proteins that could be synthesized by using a membrane augmented CFS.

addition to polymers, biosynthetic compounds, such as cholesterol, can tune the elasticity of membranes.

1.4. Membrane Protein Insertion into Membrane Augmented CFSs. Chaperones facilitate the insertion of membrane proteins in cells; however, solely membrane composition can affect the efficiency and directionality of this process. In a PURE-based CFS lacking chaperones, the 12-transmembrane proteins LacY and XylE were expressed and spontaneously incorporated into the liposome membrane. The membrane protein incorporation was lipid dependent with 2.6- and 1.5-fold increases in LacY and XylE incorporation, respectively, between the worst (100% PC) and best (100% PG) performing membrane compositions.[40] Importantly, lipid composition played a role in establishing the correct directionality for membrane insertion. While the 100% PG membrane had the highest protein incorporation, the lack of PE resulted in LacY being incorporated in an inverted membrane orientation. This result supported previous work that identified PE as a nonproteinaceous chaperone of LacY.[41] Nonspontaneous membrane protein insertion can be facilitated in a PURE system by the secYEG translocon, which successfully integrated YidC and LepB into an exogenous liposome.[42] Spontaneous cotranslational integration of the multisubunit secYEG[42] and ATP synthase[43] complexes into liposomes in CFSs suggests that many multisubunit membrane proteins will be functional in CFSs without need for additional reaction components.

In encapsulated CFSs, orientation is critical for protein function, and membrane proteins often have to asymmetrically localize in the bilayer leaflet. Using SNAP-tag modified fluorescent proteins and liposomes composed of benzylguanine-modified phospholipids, membrane asymmetry was achieved by encapsulating a CFS expressing mCherry-SNAP within the modified liposome, and suspending the encapsulated CFS in a second CFS expressing GFP-SNAP. Fluorescence microscopy confirmed mCherry was localized to the inner membrane of the liposome while GFP localized to the outer membrane.[44] Controlling protein localization is key in applications that benefit from separating intermediates in metabolic pathways. For example, the production of CMP-*N*-acetylneuraminic acid was improved 2-fold by encapsulating the first pathway enzyme, *N*-acyl-D-glucosamine-2-epimerase, within a polymersome and attaching the rest of the pathway on the outside of the polymersome, thus reducing inhibition of *N*-acyl-D-glucosamine-2-epimerase by a late pathway intermediate.[45]

2. APPLICATIONS

Expanding the use of CFSs to include membrane proteins offers a wide range of applications from single enzyme assays to use of the membrane proteins as part of longer enzyme pathways. Table 1 offers an overview of current literature in which membrane proteins are applied in a CFS using nanodiscs, liposomes, or inverted vesicles, highlighting both *in vitro* synthesized and reconstituted proteins from multiple protein classes.

2.1. Enzyme Assays. Transmembrane protein production in CFSs enables their study in the absence of endogenous metabolic pathways or metabolites that may confound the results. For example, oligosaccharyltransferase homologs have been synthesized in cell lysate based CFSs using nanodiscs and used to rapidly identify acceptor proteins,[46] bypassing competition from an endogenous glycosylation system. Furthermore, the chemokine GPCRs CX_3CR1 and CCR5 have been produced in CFSs using nanodiscs in a PURE system for structural (electron microscopy) and functional (surface plasmon resonance) studies.[47] As membrane augmented CFSs become more widely available, we expect other membrane protein classes to make use of this technology.

2.2. Energy Production. CFSs hold incredible promise for the production of fuels and chemicals due to their high productivity when compared to microbes, up to 815 mg/L/h in the case of mevalonate.[48] Cell-free production of butanol and hydrogen could be improved by extending the respective pathways using particulate MMO for the assimilation of CH_4 to enable use of a C1 feedstock.[49] Such a system would also take advantage of the improved separation and reduced effects of toxicity provided by using CFSs. It is worth noting that CFS production platforms are currently not cost competitive with microbial ones for biofuel production and have not been scaled to the volume or run as long as microbial cell factories.[50] The generation of electricity in microbial fuel cells would also benefit from improvements in membrane protein expression in

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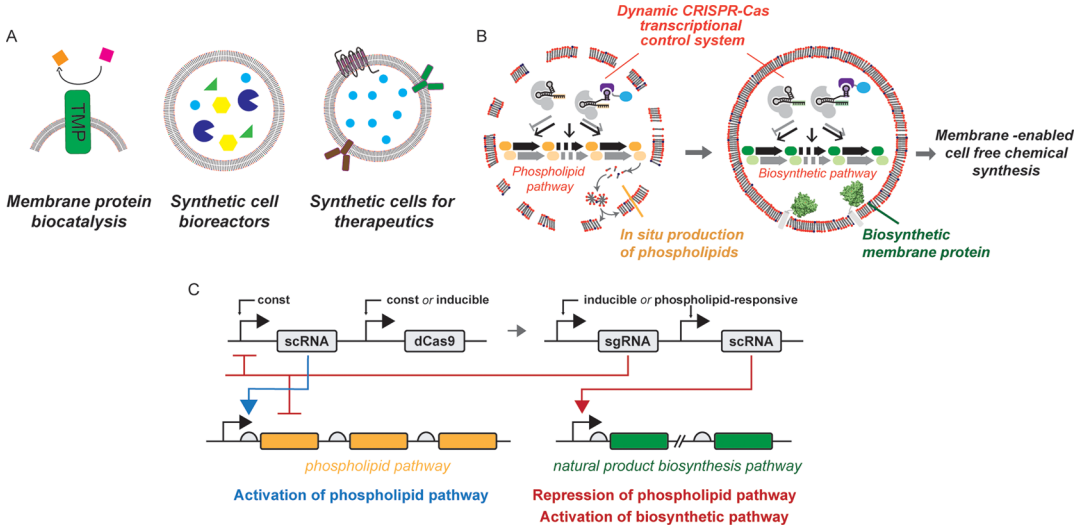

Figure 4. Applications of membrane augmented cell-free systems. (A) Potential bioindustrial applications of membrane proteins in membrane augmented cell-free systems. (B) Dynamic CRISPR-Cas control system can be implemented to stagger phospholipid and natural product biosynthesis, allowing for *in situ* liposome self-assembly prior to membrane protein production and chemical synthesis. (C) Sample two-stage control strategy for staggered phospholipid and natural product biosynthesis. First, constitutive (const) or induced expression of a modified guide RNA that enables the recruitment of a transcriptional activator (scaffold RNA, or scRNA) activates expression of the phospholipid pathway. Second, a user-supplied inducer or sufficient phospholipid concentration triggers expression of the natural product biosynthesis pathway (through targeted scRNA) as well as repression of the phospholipid pathway using a sgRNA coupled with catalytically inactive Cas9 (dCas9). Suppression of lipid biosynthesis during stage two helps conserve precious CFS resources.

CFSs by helping to overcome barriers in feedstock toxicity and limits on electric current production due to organism viability.[51] To this end, enzymatic fuel cells using purified enzymes can produce electricity from hydrogen, methanol, formate, pyruvate, and sugars without the need for membrane proteins.[52] However, these systems often have a lifetime of hours to days. Incorporation of these enzymes into a CFS would allow for enzyme and cofactor regeneration, extending the lifetime of the fuel cell. Furthermore, use of membrane augmented CFS could further improve enzyme stability and expand the range of usable fuels to include CH_4.

2.3. Chemical Biosynthesis. Despite the now large array of chemicals synthesized using CFSs,[50] none of them relies on a transmembrane protein for biosynthesis (Figure 3). Focusing on plant natural products, CFSs have been used to produce monoterpenes[53] and cannabinoids,[54] but improved expression of membrane proteins would increase the structural diversity of reachable products. Specifically, expression of transmembrane plant CYPs, such as those found in monoterpene indole alkaloid (MIA) and benzylisoquinoline alkaloid (BIA) pathways,[1] would allow CFS production of the anticancer drug Taxol, which requires 19 enzymatic steps after geranylgeranyl diphosphate with eight of those steps catalyzed by transmembrane CYPs.[55] Furthermore, synthesis of the MIA intermediate strictosidine requires four CYPs,[56] while the synthesis of the BIA intermediate noscapine requires five.[57] Today, *S. cerevisiae* is used to produce plant CYPs; however, a long doubling time (3 h) limits the rapid prototyping of CYPs and reductase partners. Highlighting the challenges associated with natural product production in *S. cerevisiae*, recent production of the tropane alkaloid scopolamine required N-terminal engineering of the membrane-bound littorine synthase from *Atropa belladonna* for functional expression in *S. cerevisiae*.[58] Finally, the membrane compositions of yeast and plants are very different (Figure 1B), likely impacting transmembrane protein functionality. Membrane augmented CFSs offer a potential upgrade on *S. cerevisiae* in terms of productivity, reduced effects of product toxicity, and flexibility in membrane composition.

2.4. Synthetic Cells. Synthetic cells, particles that mimic biological cell but have different characteristics, functions, or parts, can be generated by encapsulating CFSs in liposomes. Membrane proteins play pivotal roles in the development of synthetic cells: from growth and division *via* phospholipid biosynthesis and Z-ring proteins, to cell-to-cell communication mediated by cell surface receptors for the development of cell therapies, to the generation of NADH and ATP to extend their chemical bioproduction time.

In the context of cell-to-cell communication, a synthetic cell constitutively expressing a brain-derived neurotrophic factor was activated by addition of homoserine lactone, leading to production of the pore forming perfringolysin O, secretion of the neurotrophic factor, and differentiation in cocultured stem cells.[59] Similarly, the pore forming protein α-hemolysin has been leveraged to control uptake or secretion of doxycycline and isopropyl-β-D-thiogalactoside (IPTG), which activated downstream luciferase expression as a proof of concept reporter gene.[60]

In the context of chemical bioproduction, synthetic cells act as miniature bioreactors that, unlike cells, do not use the carbon for cell growth or maintenance, and essentially route all carbon for chemical production (Figure 4A). Thus, bioreactor synthetic cells act as immobilized enzyme catalysts, potentially enjoying easy reuse and separation, while allowing the enzyme inside the reactor to work within the simulated synthetic cell environment with limited loss of activity. Liposome volume and stability is particularly important for bioreactor synthetic cells. Decreased synthetic cell volume results in higher local substrate concentration, speeding up the reaction of low substrate affinity enzymes.[61] Nevertheless, high local product concentrations may limit yields through increased enzyme product inhibition. The stability of the liposome will determine

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the lifetime of the reactions that take place inside it, making it crucial to understand how each of these physical properties affects liposome stability, which is known to be affected by liposome structure[62] and synthesis method.[63]

Membrane proteins often require prosthetic groups for activity. For instance, CYPs need heme, and membrane-bound glucose dehydrogenases used for NAD(P)H regeneration use pyrroloquinoline quinone (PQQ). Prosthetic groups need to be incorporated into proteins as they fold and are inserted in the membrane to achieve proper enzyme function. Often, CFSs lack the biosynthetic pathways to synthesize prosthetic groups and these compounds need to be exogenously added to the reaction. For scale-up applications, however, enriched CFSs from organisms where the prosthetic group biosynthetic pathways are expressed and/or upregulated will be needed for the functional expression of membrane proteins. Some inroads toward this goal have been made. For example, PQQ was synthesized by *Gluconobacter oxydans*-based CFS carrying the machinery to convert fed pqqA precursor to PQQ.[64] In another example, heme was biosynthesized in *E. coli* CFS *via* addition of purified 5-aminolevulinic acid synthase, and heme was successfully incorporated into P450 BM3.[65] Of note, prosthetic group biosynthesis should be carefully regulated to avoid CFS poisoning or unnecessarily diverting carbon flux from the desired product. Additionally, chaperones could be introduced to help in the incorporation of prosthetic groups, such as ferrochelatases for heme loading.[66]

The final challenge in bioreactor synthetic cells is the need for reducing power (NADH) and energy (ATP) regeneration to drive reactions for extended periods of time to reduce process cost. Cofactor regeneration in CFSs has been achieved using glyceraldehyde-3-phosphate dehydrogenase and applied to monoterpene production.[53] Production of ATP in CFSs was achieved early on using oxidative phosphorylation.[28] More recently, efforts have moved to produce ATP from light using purified ATP synthase, photosystem II, and proteorhodopsin reconstituted in liposomes,[67] or using bacteriorhodopsin to generate the proton gradient necessary to produce ATP upon light induction.[68] Importantly, all ATP production mechanisms need membrane proteins to generate an electron gradient, which requires proteins to have the correct membrane orientation. This can be partially controlled by limiting spontaneous membrane integration through modulating cholesterol and diacylglycerol concentration.[69] However, the effect of these compounds is phospholipid dependent and requires continued study on more complex lipid mixtures.[70]

3. CONTROL STRATEGIES FOR THE GENERATION OF MEMBRANE AUGMENTED CFSS

3.1. Genetic Control of Membrane Properties. CFSs have a limited amount of resources for the formation of enzymes and metabolites as well as transcription and translation machinery. For synthetic cells to become industrially relevant, the cost-effective synthesis of both phospholipids for membrane formation and membrane-bound actuating biomolecule(s) is needed. The actuating biomolecule(s) can be single enzymes for biocatalysis applications, multienzyme pathways for chemical bioproduction, or receptors for synthetic therapeutic applications. To enable these applications, a control system that can dynamically program gene expression of multiple units is needed. The control system should (1) have low overhead resource consumption, (2) be capable of turning genes both on and off to generate sequential phases of gene expression programs, (3) be tunable to precisely regulate gene expression dynamics, and (4) be scalable to allow for construction of increasingly complex gene regulatory systems.[71,72] Such a control system would enable synthesis of phospholipid-producing enzymes early in a reaction to form membranes, followed by a shift in production to membrane proteins to produce the chemicals, both with programmable stoichiometry and timing (Figure 4B,C). Control systems in liposomes will need to go beyond controlling the enzyme ratios,[36] and dynamically control on/off gene expression. This capability would allow for controlled changes in membrane properties through the course of the reaction without outside intervention.

The control system should not only regulate lipid synthesis, but also balance the expression of the membrane bound actuating biomolecule to prevent aggregation while maximizing efficiency and rate of membrane protein insertion. Most of the current understanding on membrane protein insertion relies on expression rates to prevent saturation of membrane insertion machinery.[14] In the case of liposomes, hydrophobic interactions among membrane proteins lead to self-aggregation and interactions between membrane proteins and the lipid membrane play an outsized role in protein insertion, posing additional challenges to the control system. Implementation of a gene control system capable of delivering distinct gene expression profiles would develop a better understanding of how liposomes change over time, how liposomes adsorb proteins from the CFS, and how the density of proteins already in a liposome affects how much membrane protein uptake occurs.

3.2. CRISPRa/i: A Control System for the Expression of Membrane Proteins in Membrane-Augmented CFSs. While elementary gene regulation has been implemented in CFSs,[73] efforts toward developing multigene control systems to provide precise regulation over gene expression, membrane protein insertion, and function remain at an early stage. The CRISPR-Cas system provides a powerful suite of tools for multigene transcriptional control.[71] Briefly, catalytically inactive Cas9 protein can be directed to specific DNA sequences by guide RNAs that recognize target sequences based on predictable Watson−Crick base pairing to activate (CRISPRa) or repress (CRISPRi) gene expression. Although the rules governing CRISPRa from bacterial promoters are complex,[74] a growing set of validated CRISPRa components enables the rapid construction of increasingly complex gene regulatory systems.[71] Multigene CRISPR circuits have been engineered through the regulated expression of up to seven distinct sgRNAs in the same system,[75] and CRISPRi has been shown to operate efficiently in CFSs.[76] Thus, by combining new capabilities for CRISPRa with existing tools for CRISPRi in CFSs, it should be possible to engineer multigene programs for membrane augmented CFSs operating through the regulated expression of guide RNAs. Multiguide RNA CRISPRa/i circuits could then be used to program distinct gene expression modes to enhance functional membrane protein expression. Consequently, CRISPRa/i circuits could provide an efficient mechanism for implementing dynamic multigene control, while preserving valuable CFS metabolites and cofactors.

A toolbox of pulse-generating CRISPRa/i circuits could be used to both investigate and optimize how membrane protein expression dynamics impact membrane insertion and function. Here, the network topology would specify the timing of gene

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expression pulses or regulatory functions. Further, incorporation of input-responsive pulses of gene expression or regulation into CRISPRa/i networks would extend tunable control to semicontinuous reactions and at any specific time within a membrane CFS reaction. Ultimately, it may be possible to engineer CRISPRa/i programs as process controls for membrane augmented CFS bioindustrial applications that regulate protein expression, minimize the waste of valuable precursors and energy molecules and prevent the accumulation of destabilizing intermediates.[72]

4. FUTURE DIRECTIONS

Membrane proteins play a pivotal role in bioenergy, biomedical, and bioindustrial applications, and our ability to harness their potential hinges on their functional production outside their endogenous host. Although to some extent heterologous membrane proteins can be engineered for optimal functional heterologous production, an alternate and now more and more feasible strategy is to engineer CFSs with tailor-made augmented membranes to ensure optimal transmembrane protein activity. Although we are far from on-demand membrane augmented CFS generation, the potential advantages of such systems in terms of enabling new chemistry and improved chemical bioproduction processes make this a worthwhile endeavor. The realization that we have not only protein engineering, but also membrane engineering in our toolkit when tackling transmembrane protein challenges should help us accelerate some of these applications.

The biggest step forward in this field will be moving from using membrane augmented CFSs for protein characterization and analysis to larger scale application for chemical bioproduction, bioremediation, or synthetic cells. This will require effective scale up of membrane protein synthesis and controlled expression of phospholipid biosynthesis enzymes and biosynthetic pathway enzymes. Due to cost and tunability, liposomes appear to be the better option for scaled up membrane protein synthesis in CFSs. This said, work toward spontaneous assembly of liposomes *in situ* and without need for organic solvents would help lower process cost for liposome production. Furthermore, study and improvement on membrane protein−liposome stability will be necessary for widespread application. Metrics such as total turnover number and half-life may be useful to help quantify scalability of these systems.

Successful implementation of membrane protein synthesis in CFSs will open the door for enzymatic production of toxic chemicals in nonliving systems as well as nonliving biosensors and bioremediation tools that can be applied environmentally without risk of biological contamination, or loss of function due to environmental toxicity. Finally, more sophisticated synthetic cells aided by membrane proteins hold great promise in therapeutic applications for targeted drug therapies as well as communicating with cellular environments to make expression decisions based on external stimuli. If aided by CRISPRa/i, these decisions and logic gates can be made significantly more complex to respond to combinations of signals and give the synthetic cell temporal, on/off control over gene expression.

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Notes

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ABBREVIATIONS

CFS, cell-free system; CYP, cytochrome P450.

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