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Trends in Biochemical Sciences

Review

Membrane protein synthesis: no cells required

Zachary A. Manzer,[1,2] Ekaterina Selivanovitch,[1,2] Alexis R. Ostwalt,[1] and Susan Daniel[1,*]

Despite advances in membrane protein (MP) structural biology and a growing interest in their applications, these proteins remain challenging to study. Progress has been hindered by the complex nature of MPs and innovative methods will be required to circumvent technical hurdles. Cell-free protein synthesis (CFPS) is a burgeoning technique for synthesizing MPs directly into a membrane environment using reconstituted components of the cellular transcription and translation machinery *in vitro*. We provide an overview of CFPS and how this technique can be applied to the synthesis and study of MPs. We highlight numerous strategies including synthesis methods and folding environments, each with advantages and limitations, to provide a survey of how CFPS techniques can advance the study of MPs.

Highlights

Membrane proteins are the frontier of fundamental biochemistry and drug discovery and require innovations to break down the technical barriers that exist for their broader study.

Cell-free protein synthesis has the potential to revolutionize the study of membrane proteins by enabling higher protein yields of complex targets.

Supplementing cell-free synthesis with membrane environments can produce structurally intact membrane proteins for a variety of applications.

Both soluble and solid supported membrane mimics serve as excellent environments for cell-free synthesized membrane proteins and can be used readily in this format to answer questions in biophysics and biotechnology.

Traditional approaches to, and limitations of, transmembrane protein isolation

Biological membranes are complex and dynamic environments with embedded macromolecules that carry out various functions. Two classifications of such macromolecules include **monotopic membrane proteins (MMPs)** (see Glossary) and **transmembrane proteins (TMPs)**, both of which associate with the lipid leaflets of the membrane (Figure 1). The last several decades have been devoted to identifying the structure and function of these complex molecules [1]. MMPs bind to the interior or exterior leaflet and perform various tasks, such as anchoring the cytoskeleton to the membrane, mediating cell–cell interactions, and facilitating signaling pathways [2]. TMPs span the entire lipid bilayer, bridging the extracellular and cytosolic environments. These proteins can function to transport molecules along a concentration gradient (e.g., Ca^{2+} channels [3], glucose transporters [4]), actively transport molecules to move species across the membrane (e.g., Na^{+}/K^{+} pump [5], proton pumps [6]), or participate in signal transduction (e.g., integrins, G-protein coupled receptors) [7]. Although computational studies have provided some insight into these proteins [8–10], understanding MP structure, function, and energetics lags behind that of soluble proteins due to limitations associated with the purification and isolation of MPs in their native conformations [11,12].

The structure and functions of MPs are highly dependent on their interactions with the bilayer constituents and removing them often has detrimental effects on MP activity [13]. Removing the protein domains that are embedded within the hydrophobic core of the membrane exposes the hydrophobic amino acid chains to water; a thermodynamically unfavorable process that often results in protein unfolding [14]. In eukaryotic cells, MPs are synthesized by ribosomes located on the surface of the endoplasmic reticulum (ER) and are typically inserted into an ER lipid membrane during synthesis (co-translationally) where they can continue to fold correctly in an amphiphilic environment [15]. To isolate full-length MPs synthesized within a cell, the lipid bilayer must first be disrupted without deleterious effects to the protein, then the MP is reconstituted into a membrane environment using amphiphiles, such as detergents. This process is typically not straightforward, as no universal detergents can reconstitute every MP. Detergent reconstitution involves finding a delicate balance between the solubilization efficiency of the detergent and protein stability; both of which are dictated by the physicochemical properties of the surfactant and protein [e.g., **critical**

[1]R.F. School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY 14853, USA
[2]These authors contributed equally.

*Correspondence:
sd386@cornell.edu (S. Daniel).

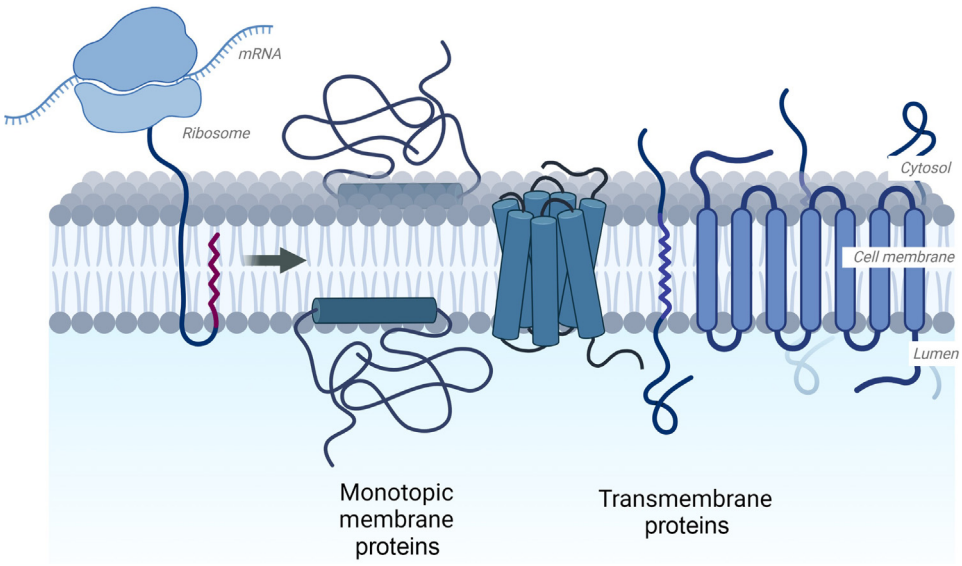

Figure 1. Most membrane proteins (MPs) are synthesized co-translationally and interface with membranes in various ways. MPs are typically synthesized directly into the lipid membrane to enable their proper folding. Various chaperones (not shown) and a signal peptide sequence in the protein itself (purple) direct this process. Monotopic membrane proteins (MMPs) and transmembrane proteins (TMPs) display different structural motifs and physical characteristics. Monotopic proteins associate to a single leaflet on either side of the lipid membrane. These can be transiently associated (peripheral) or permanently attached (integral). TMPs pass through the membrane and include various types of channels, receptors, and enzymes.

micelle concentration (CMC) of the detergent, pH stability, and water solubility] [16]. Finding this balance is time consuming and, even if solubilization is achieved, the micellar environment still differs from that of a biomembrane, which can lead to protein instability. Other methods developed to study MPs include exchanging detergents for styrene–maleic acid (SMA) lipid particles or fusing the MPs to an amphipathic protein [17,18]. Despite these advancements, each method presents different challenges, and expressing and scaling up MP production using cell-based expression systems remains laborious. In this Review, we highlight the potential of using cell-free expression systems to provide insights into MPs. We provide a brief overview of the development of cell-free synthesis and highlight several approaches that can be used to support the cell-free synthesis of MPs in a variety of formats and applications. These include both soluble and surface-based methods for structural, functional, and biotechnology applications that showcase this technique and what still needs to happen to push the boundaries of cell-free production of MPs.

Cell-free protein synthesis: an emerging addition to traditional membrane protein synthesis methods

Given the challenges associated with cell culture-based MP synthesis, purification and isolation, alternative methods are needed to advance the field, particularly for large, multi-pass TMPs. **Cell-free protein synthesis (CFPS)** techniques use the transcription and translation machinery found in cells to express proteins of interest without the need for living cells to execute the synthesis itself. CFPS offers shorter protein synthesis timelines and circumvents the complications that arise using traditional methods, such as the need for intensive sterile environments, cytotoxic protein expression, cellular growth constraints, or successful genetic engineering [19,20]. These *in vitro* synthesis reactions, at minimum, require the translation and transcription machinery, an

Glossary

Atomic force microscopy (AFM): topographic technique used to measure surface roughness by monitoring the interaction between a cantilever probe tip and the surface of a substrate.
Cell-free protein synthesis (CFPS): technique where cellular transcription and translation machinery is taken out of cells and recombined with necessary cofactors *in vitro* to synthesize proteins from added genetic material.
Critical micelle concentration (CMC): the concentration above which amphiphilic molecules (i.e., lipids and surfactants) will begin to aggregate and form micelle structures.
Cryogenic electron microscopy (Cryo-EM): advanced microscopy technique using cryogenic temperatures to flash-freeze protein samples prior to imaging.
Fluorescence microscopy: form of optical microscopy that uses a high-intensity light source to excite a molecule and then monitors the emission of a specific wavelength of light.
G-protein coupled receptor (GPCR): large, diverse class of transmembrane receptors that detect external stimuli by binding to a G protein and triggering downstream signaling cascades.
Lipid vesicles/liposomes: spherical assembly of lipid molecules that forms an enclosed structure separating the interior solution from the exterior.
Membrane protein (MP): a protein consisting of large hydrophobic regions that is associated with or attached to a lipid bilayer.
Monotopic membrane proteins (MMPs): membrane proteins that associate with one leaflet of the lipid membrane.
Nanodisc: an assembly of lipids surrounded by small molecules, proteins, or other materials that forms a bounded bilayer structure.
Nuclear magnetic resonance (NMR): spectroscopic technique that measures local magnetic fields to determine structural information of proteins.
Supported lipid bilayer (SLB): 2D lipid bilayer supported by a solid interface providing greater stability than other model membrane systems.
Surface plasmon resonance (SPR): spectroscopic technique that monitors molecular interactions.

energy source, and the genetic material encoding the protein of interest (Figure 2) [21]. Because of the modular and customizable nature of these systems, the reactions can be modified to optimize yield and protein activity. Supplementing the reactions can improve energy regeneration [22], disulfide bond formation [23], or provide a hydrophobic environment to support MP folding [24]. A recent example of this continued development is the addition of glycosylation machinery to *Escherichia coli* cell lysate synthesis reaction mixture [25–27]. In this area, both N- and O-linked glycans are built in either one-pot reactions or in microfluidics to achieve glycosylation in a cell-free environment.

Transmembrane proteins (TMPs): membrane proteins that span across both leaflets of the lipid membrane.
X-ray crystallography: technique where proteins are prepared into solid crystals and then exposed to x-rays to determine diffraction patterns and calculate the underlying structure.

Cell-lysate-based protein synthesis has been used since the mid 1950s to study biological processes [28]. *E. coli* has been the most common lysate source although reaction lysates can be produced from eukaryotic sources including mammalian cells [29], plants [30], and yeast [31]. Initially, technical constraints limited the use of CFPS; however, in the early 2000s the development of recombinant, or purified, cell-free protein systems (such as the PURE system) [32] and optimized cell extracts made them more accessible to researchers and demonstrated the true potential of this approach. Lysate-based systems reached impressive total protein yields well above a gram per liter. One recent commercial system for CFPS, TXTL, exemplifies the progress made since then for soluble proteins [33]. Factors that contributed to the continual increase in protein yield include ATP and amino acid regeneration [22,34,35], new energy sources [36], improved lysate preparation [37,38], extensions to the reaction lifetime [39,40], and initial reaction rates [41]. Here, we only provide a cursory summary of the robust history of CFPS and direct interested readers to more extensive reviews on the development of cell-free systems [42,43]. When synthesizing a specific protein, the choice of recombinant versus lysate-derived systems and each step in the development of a cell-free system requires careful consideration with regards to protein size, structural motifs, and post-translational modifications.

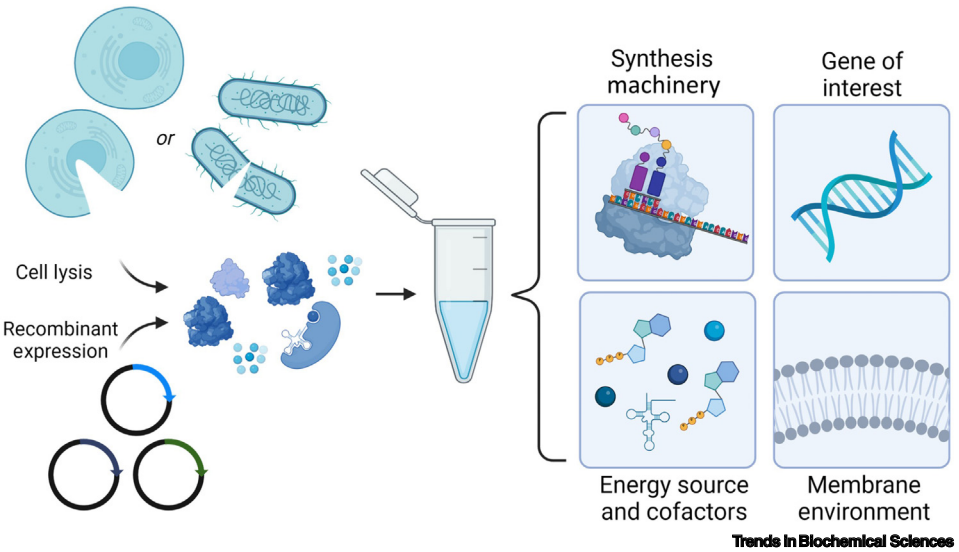

Figure 2. Cell-free synthesis provides modular control of protein expression. There are four main components of the *in vitro* reaction: (i) a source of translation and transcription machinery, which can be obtained from a cell lysate directly or generated by recombinantly expressing and purifying the components (as depicted on the left); (ii) an energy source and energy regeneration system to prolong the reaction; (iii) a gene of interest, typically in the form of a plasmid, but linear DNA fragments can also be used; and (iv) additional synthetic components, such as membranes vesicles, that are optional but can enhance the production and stability of a protein of interest.

Assisted CFPS methods for MPs

The integration of cell-free systems with MP synthesis has the potential to recapitulate native biological functions and serve as a platform to study a broad range of proteins (Figure 3, Key figure). Regardless of the CFPS system selected, a stabilizing and solubilizing environment is needed when expressing MPs to gain insights into their native mechanistic, biophysical, and structural properties. Such an environment can be provided via the addition of different detergents or micelles, **nanodiscs**, **lipid vesicles (liposomes)**, or **supported lipid bilayers** during CFPS (Figure 4) [44,45]. Consideration for each strategy should be given, as they have specific advantages and limitations.

Adding detergents to CFPS reactions during or after protein synthesis is a common approach used to solubilize MPs [46]. In early studies, when CFPS methods resulted in precipitate formation or partially unfolding of MPs, detergents were supplemented to solubilize aggregated or misfolded proteins [47,48]. The alternative is to directly supplement CFPS mixtures with mild detergents that can render a MP more soluble during the reaction (e.g. digitonin and Brij35) [47,49–52]. With a small amount of detergent present during synthesis, the hydrophobic protein

Key figure

Cell-free membrane protein (MP) synthesis advanced biotechnology

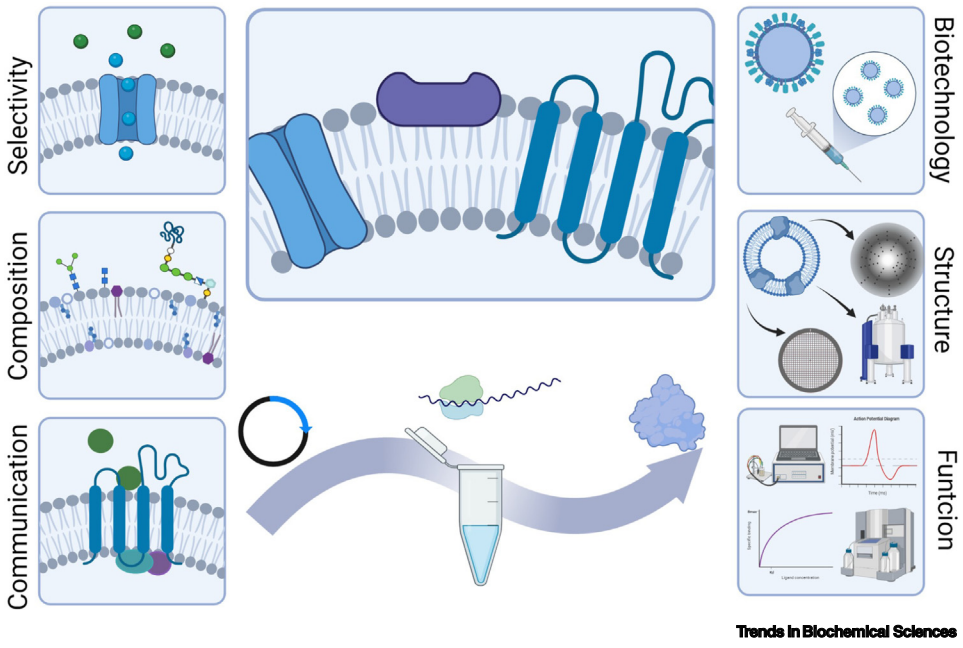

Figure 3. There are several important properties of MPs that make them compelling candidates to study. This includes their exquisite selectivity in transport across the membrane, reliance on membrane composition for proper folding and function, and the ability to transmit information in a complex environment (communication). Each of these properties needs to be maintained to accurately understand the fundamental mechanisms of a protein's function. Cell-free protein synthesis provides the ability to recapitulate this function *in vitro* in a single test tube reaction. As shown in the center, genetic material is provided and then used to directly synthesize proteins using the isolated cellular machinery. By providing a membrane environment during cell-free synthesis, this opens the door for widespread use of membrane proteins in biotechnology applications, elucidating complex structure, and understanding how to modulate their function.

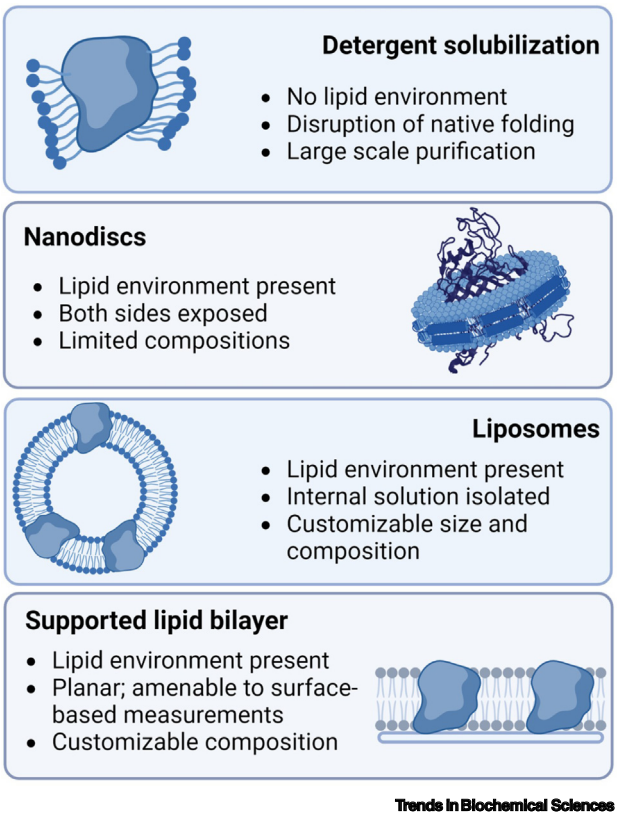

Figure 4. Different cell-free synthesis approaches to consider for optimal membrane protein synthesis and folding. Detergent solubilization allows for large scale refolding and purification but can disrupt native protein folding. Nanodiscs are a lipid bilayer bound by a protein or small molecule scaffold. These discs provide a native-like membrane environment and access to both sides of the membrane but offer a limited range of lipid compositions. Liposomes are spherical membrane structures with an internal, sealed cavity that offers more customization (e.g., liposome size and composition). Supported membranes provide the benefits of a native lipid environment and are compatible with surface sensitive measurements and assays for functional and single molecule studies.

domains are stabilized by the acyl chain domains, forming proteomicelles. Not all detergents will be suitable for a particular protein and stronger detergents can disrupt the transcriptional and translational machinery [47,51]. The detergent of choice should be carefully considered prior to supplementing the CF reaction. A second consideration is that proteomicelles, which are structurally considered closed monolayers, do not replicate the bilayer structure of a lipid membrane, therefore MPs associated with them may not retain their native shapes and activity.

Another method for the CF synthesis of MPs involves supplementing the reaction with a variety of lipid-based nanostructures, such as nanodiscs (Figure 4). In these approaches the lipids of choice are assembled into bilayers that mimic the cell membrane, followed by MPs insertion during translation. Nanodiscs have discoid morphologies and are organized with the support of membrane scaffold proteins or apolipoproteins [53]. These disks have been added directly to the CF synthesis reaction and facilitated the production of functional MPs [54,55], provided they have the proper lipid composition [56,57]. It has also been shown that an apolipoprotein, required for nanodisc formation, along with a desired MP can be coexpressed, resulting in the simultaneous formation of nanodiscs and co-translational insertion MPs into the lipid bilayer [58–60]. In addition to the compatibility of nanodiscs with CFPS reagents, they can be used as protein carriers, on which, following CFPS into the disk, MPs can be delivered elsewhere without reconstitution [59,61]. Such an example is found in Patriarchi *et al.*, where nanodiscs delivered a CF-synthesized functional **G-protein-coupled receptor** to a plasma membrane of a living cell [59]. The discoid structure of these nanoparticles allows for interrogation of the cytosolic and extracellular domains of TMPs, introducing both a benefit and a limitation of using nanodiscs for orientation-dependent studies.

For instance, such is the case when studying transporters, where the direction-dependent passage of ions or other molecules cannot be easily probed. Tools are currently being developed to address these issues [62,63], and it is likely that they will soon be integrated with CFPS.

An alternative approach to improving the synthesis and stability of MPs in CF reactions is to add synthetic or cell-derived membrane liposomes [64]. Like nanodiscs, liposomes contain a lipid bilayer but have a spherical morphology with an interior cavity separated from the exterior environment. Libraries of proteins have been co-translationally inserted into synthetic lipid vesicles and their structures and functions have been evaluated in accordance with various parameters such as lipid environment [65], membrane curvature [66], and temperature [24,64,67,68]. Certain proteins require specific lipids or accessory proteins that are present in the native membrane environment that would be difficult to mimic synthetically [69], although there are emerging reports of heterologous chaperone-assisted cell-free synthesis [70]. Additionally, given the customizable nature, synthetic components like diblock copolymers can also be added to study the effect of mechanical properties on protein folding [71]. While specific essential lipids and proteins can be added during CF synthesis, it would be cumbersome to identify the lipids (without an available crystal structure) and proteins (without fully understanding their roles). To circumvent this, liposomes directly from biological membranes can be acquired [72]. For instance, vesicles harvested from the ER, termed microsomes, can successfully incorporate functional MPs [73,74]. Even though a wide variety of different vesicles are suitable for CFPS, because the hydrophobic bilayer domain needs to match the membrane spanning region of the MP, this approach often requires some level of optimization. Overall, there are a variety of choices and membrane-like additives available for the CF expression of functional MPs that offer different advantages and trade-offs. In recent years, scientists and engineers have used cell-free approaches to produce proteins for structure–function studies, including viral and bacterial MPs [75–79], and to design new technologies for sensing, catalysis, and drug discovery applications [68,80,81].

Membrane-assisted cell-free synthesis can overcome hurdles in structural studies of MPs

Even though solving the structures of MPs is critical for learning about fundamental biological processes and are advantageous for identifying drug targets, their complexity has long been a barrier for studying their structural properties. These difficulties with MP structural studies are often presented by the large hydrophobic domains that require the supporting structure of a membrane bilayer. Additionally, milligram amounts of purified protein are required for certain techniques, which means the protein must be overexpressed, isolated, and purified from its native lipid environment [82]. Currently, the main techniques used in protein structure determination are **cryogenic electron microscopy (cryo-EM)**, **NMR**, and **X-ray crystallography**.

Common NMR techniques can be used for small (≤30 kDa) proteins, due to the spectral crowding of signals from larger proteins; they also need to be isotope-labeled, which is not trivial for most eukaryotic proteins in cell-based systems [83]. Most MPs are too large for NMR structural determination studies and are also frequently too small (≤100 kDa) for cryo-EM studies, which affects the ability to acquire a high resolution structure [84,85]. For crystallographic methods to be successful, milligram amounts of pure, stable, and functional protein are required. The customizable and open-system nature of CFPS makes this method particularly advantageous for structural biology [86–90]. Along with the ability to overexpress proteins without the concern for cytotoxicity, purification is simpler, optimization of protein production is independent of cell viability, pH, redox potential, or temperature, and additives that can help co-translational folding can be readily included [91,92]. It should be mentioned that yields for MP CFPS can still be suboptimal, though have seen much improvement in recent years.

When coupling CFPS with structural biology studies, an *E.coli*-based cell-free system is the most popular route for soluble proteins and may emerge as the standard for MPs. There have been great efforts in the improvement and development of CFPS methods for the preparation and study of MP structures, especially by crystallographic methods [86,93]. There have already been some reports of MP crystallization utilizing CFPS [88,94], such as a 3.7Å resolution crystal structure of the *E. coli* membrane transporter EmrE [94], but overall few resolved MP structures at high atomic resolutions [62,95]. This is partly due to the challenges mentioned previously, but also the obstacle of post-translational modifications (PTMs), which are often critical for MP structure, function, and stability. PTMs are notoriously difficult to address with most recombinant systems, especially CFPS [21,96]. A lack of PTMs can lead to reduced stability and functionality, but a more conformationally homogenous sample can be beneficial when carrying out structure determination techniques that require uniform protein samples. Although coupling CFPS with structural determination techniques remains challenging, this method can serve as a complementary approach to traditional cell-based techniques for comparison purposes. For example, the crystal structure of the bacterial membrane kinase DgkA, produced by CFPS methods, was solved to a similar resolution and structure compared with the same protein generated using a cell-based approach, with the CFPS optimization approach being much less extensive [95].

Another benefit of using CFPS for protein structure studies is that additives such as cofactors, ligands, and substrates can be included into the system to help improve protein stability during crystallization. For instance, small molecule binding agents were chosen to enhance stability and improve crystal quality, which resulted in high quality crystals of the human claudin-4 (T4L-claudin-4) protein when bound to an enterotoxin (*Clostridium perfringens*-CPE) (Figure 5A,B) [86,97].

Different types of micelles, nanodiscs, liposomes, detergents, and chaperones can mimic the native lipid environment found in the cell and promote MP solubility and functionality [62]. Cell free expression of the chemokine GPCRs, CX3CR1 and CCR5, in the presence of nanodiscs, micelles, or giant unilamellar vesicles (GUVs) resulted in enhanced conformational stability, particularly with the nanodisc-incorporated method (Figure 5C,D) [98]. In the case of both proteins, the solubility and yield were substantially higher in the presence of nanodiscs (ND+) than in their absence (ND^-) (Figure 5C), with Figure 5D showing that CX3CR1–GFP (C-terminal tag) successfully localized to the GUV membrane. Similarly, the MP bacteriorhodopsin displayed increased stability for NMR methods when it was cell-free expressed with detergent micelles, amphipols, and nanodiscs [99]. Improving membrane mimetics for CFPS applications will enable the use of techniques such as cryo-EM or NMR for the determination of MP structures. Overall, continued investigation and optimization of these methods is needed for CFPS to become a go-to technique for MP structure studies. As CFPS systems and structural determination techniques continue to merge and evolve, MP studies will undoubtedly advance.

Solid supported membranes for the study of MPs

Using supported membranes in CFPS reactions provides alternative methods to study MPs not possible with model membranes suspended in solution. These membrane platforms create 2D bilayers whose geometry is compatible with surface-based techniques such as **atomic-force microscopy (AFM)**, **fluorescence microscopy**, and **surface plasmon resonance (SPR)**, among many others [100]. These surfaces have compatibility with surface-based measurements and have the ability to coat large surface areas to functionalize a surface to present molecules for applications ranging from biosensors to cell culture platforms. Throughout the development of supported lipid bilayers (SLBs), the integration of properly folded, functional TMPs into these biomimetic platforms has long been a goal of the field (Figure 4). By performing the synthesis reaction in the presence of an SLB, TMPs can be directly integrated into planar membranes as highlighted

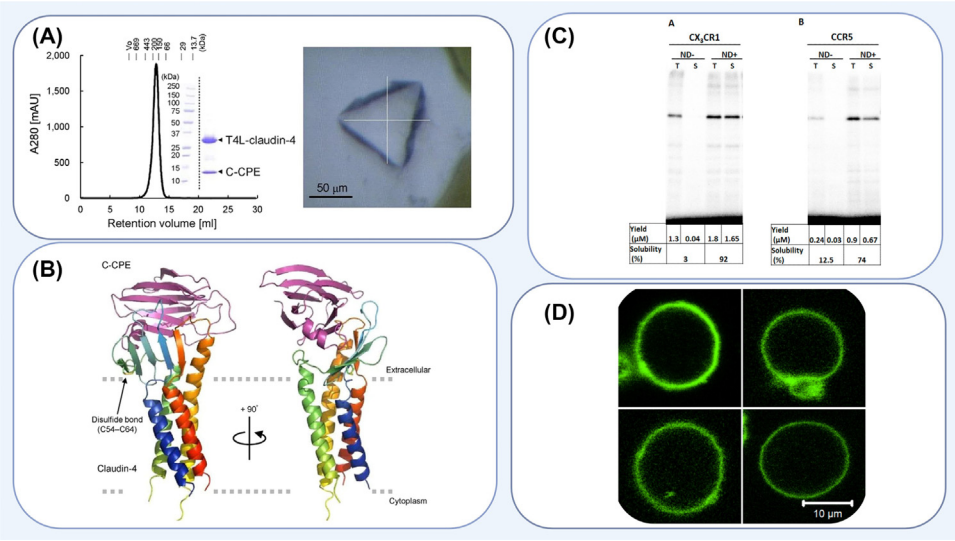

Figure 5. Cell-free synthesis can be used to interrogate the structure of MPs. (A) Human-claudin-4 was fused with T4 phage lysozyme to promote crystal formation and synthesized using cell-free protein synthesis (CFPS) [86]. This protein was eluted in a single step and then used to form crystals and achieve a 4 Å resolution structure. (B) Using this technique, a 3.5 Å resolution structure of the claudin-4–C–CPE complex was resolved [97]. (C) In another study, a nanodisc-based system was used to support the expression of chemokine receptors. An SDS-PAGE image of the cell-free expression and study of CX3CR1 and CCR5 with (ND^+) and without (ND^-) nanodiscs, showing total protein synthesized (T) versus soluble protein recovered (S) and their respective yields [98]. This shows the improvement when using a membrane environment in conjunction with CFPS. (D) Giant unilamellar liposomes were also used to show the localization of GFP-tagged CX3CR1 to the giant unilamellar vesicle membrane taken from different fields of vision, proving that the protein is correctly associating with membranes. The above figures have been reproduced from their original source under the Creative Commons 4.0 License. Credit is given to the authors listed under the respective citations.

by a few examples shown in Figure 6. In one study, CFPS was used to study the interaction of bacterial proteins FtsZ and FtsA with SLBs [101]. Using fluorescence microscopy, the authors characterized various membrane structural phenotypes on both supported membranes and vesicles by using cell-free coexpression to mimic the process involved in *E. coli* cell division (Figure 6A). By using the combination of cell-free synthesis and model membranes, the authors were able to dissect the effect of each component. Additional analytical techniques such as quartz crystal microbalance, can also be used with SLBs to monitor SLB formation and insertion of the MP of interest, as has been done with α-hemolysin in real time during its cell-free expression [102].

An early demonstration of the direct synthesis of a TMP into an SLB was done with a GPCR using fluorescence imaging [103]. GPCRs are seven-pass TM receptors that control many physiological functions such as olfaction, vision, and taste, and represent important therapeutic targets. Synthesis of functional GPCRs into biosensors would be a major advancement in GPCR biotechnology. In one GPCR study, an SLB was first tethered to a gold interface with peptide spacers [104]. Using this platform, a variety of lipids were used to characterize the binding of an antagonist to the model GPCR protein CXCR4. This platform was then adapted to a microarray support, showing the potential of this cell-free method to be scaled-up for use in high throughput screening applications [105]. The direct cell-free synthesis of TMPs into SLBs has been coupled with more sophisticated measurement techniques, like neutron reflectivity to determine protein structures. This approach has been used to probe MP structure of the hepatitis C virus protein p7

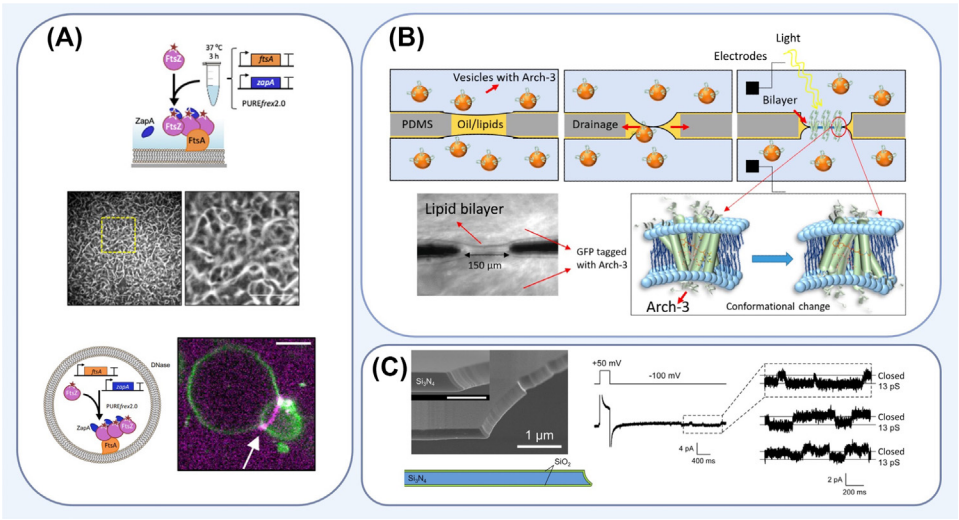

Figure 6. Model membranes can be coupled with cell-free protein synthesis for the study of membrane proteins. (A) FtsZ was added to the surface of a supported bilayer along with the cell-free expression of ZapA and FtsA, components of the bacterial division process [101]. Fluorescence images with tagged FtsZ show the formation of protein bundles on the membrane surface. A liposome model was also used to study the effect of this process, and the subsequent re-creation of membrane-tethered bundles is seen in purple, as indicated by the arrow. (B) A schematic is shown for a device using microfluidic chambers that was created to form a lipid membrane across an aperture using an oil–water interface with liposomes embedded with the cell-free expressed plant photoreceptor Arch-3 [108]. The top sequence shows the formation of the functionalized membrane and the bottom depicts the details of the aperture and a cartoon showing the integral membrane protein. This system was electrically sealed and was used to monitor electrical current changes through the membrane upon light excitation. (C) More advanced microfabrication techniques were used to create a tapered aperture with better mechanical properties as shown in the image and schematic [110]. Using this device, an important cardiac ion channel (hERG) was cell-free-expressed into liposomes to form a planar membrane. The conductance of single channel hERG-containing membranes was monitored as shown in the image. The above figures have been reproduced from their original source under the Creative Commons 4.0 License. Credit is given to the authors listed under the respective citations.

[106]. Researchers have also synthesized the chimeric potassium channel KcsA-Kv1.3 into a membrane supported SPR surface [107].

Beyond simple supports, more complicated surfaces can be used to provide sensing capabilities to CF membrane systems. In a recent study, a microfluidic device was fabricated to create an aperture opening in which a lipid membrane could be supported across a micron-scale channel. The authors used commercial *E. coli* lysate to synthesize the plant photoreceptor archaerhodopsin-3 into this membrane to carry out single protein electrophysiological studies (Figure 6B) [108]. This allowed the authors to create an electrically sealed membrane that was able to detect the function of cell-free expressed proteins. In subsequent work, more advanced nano- and microtaper apertures were fabricated with silicon nitride supports [109]. These served to provide increased stability to the lipid membrane while enabling the researchers to test the effect of astemizole on single hERG channels synthesized using a wheat-germ lysate. This technique was further refined to better characterize the fabrication process and produce more robust hERG sensors (Figure 6C) [110].

A critical step in SLB fabrication is the successful integration of the biological membrane with the underlying support. In early work, this supporting layer was comprised of bacterial S-layer proteins, which self-assemble into a lattice and provide a protective meshwork [111]. This coating

is an example of a proteinaceous supporting layer that biosensing platforms can contain; S-layer SLBs have since been used for the CFPS of a human voltage gated ion channel, VDAC [112]. Our research group also recently demonstrated that diblock copolymers can be used to create a cushioning support while tuning the biophysical properties of the membrane itself [113]. These types of systems can also be coupled with conducting polymer supports for electrical monitoring [114,115]. Supported membranes provide access to a broad range of applications and capabilities that, with CFPS, can advance the study and implementation of MPs.

Cell free MP synthesis: a tool for biopharmaceutical applications

There is a growing interest in using CFPS to manufacture biopharmaceutical remedies, such as antibodies and therapeutic enzymes [116,117]. In recent years, biotherapeutic MPs, such as vaccines, have been generated due to the development of membrane mimics. Traditionally, conjugate vaccines incorporate a weak and strong antigen, where the strong antigen behaves as a carrier to enhance the immune response to weak ones. Stark *et al.* designed a conjugate vaccine in which CF-expressed MPs served as immunostimulatory protein carriers, to which nanodiscs were supplemented to improve solubility [118]. This CF-based conjugate vaccine design can overcome some of the limitations associated with vaccine manufacturing, including slow and costly production times. Similarly, CFPS has also been used to produce proteoliposome-based vaccine particles against *Pseudomonas aeruginosa* – one of the leading causes of pneumococcal infections in hospitals [119]. A bacterial outer MP was co-translationally expressed in the presence of liposomes using CFPS and the authors showed that its epitope assembled into an active state outside of its native bacterial environment. It is especially encouraging that in both of the aforementioned studies, the designed vaccines showed immunogenically promising results in murine models. Although few studies to date have showcased the potential of CFPS MPs as therapeutic targets, the accessibility and robustness of the CF method will likely expand the use of MPs for therapeutic development in the coming years.

Concluding remarks

MPs represent a significant scientific frontier, rife with opportunities for discovery at a basic molecular level up to complex physiological systems. Developing new techniques to interrogate the complex structures and functions of MPs in their native environments will be required to make progress in this area. CFPS is well suited to be used for studies of MPs, but several open questions will need to be addressed (see Outstanding questions). These include increasing overall yields from CFPS, achieving proper folding, extending reaction systems to complex protein classes, and studying molecular targets that prove too difficult to adequately study today. The biggest challenge will be in increasing protein yields and maintaining complex structures as these are fundamental to unlocking these future studies. Soluble protein expression has seen dramatic improvements in this realm, but there is still much to learn about expanding membrane protein synthesis yields. When these critical needs can be met, we expect the cell-free expression of MPs to become more routine and adopted more widely. Beyond accelerating the discovery of MP structures using techniques such as cryogenic electron microscopy, more efficient and cost-effective MP production will usher in a new era in therapeutic development. With the continuing development in the field of CFPS, this paradigm shift is only a matter of time.

Outstanding questions

Can CFPS of membrane proteins be improved to reach high enough yields to enable structural characterization studies?

Can we develop cell-free systems capable of supporting complex folding and post-translational modifications in a controlled manner?

Does the cell-free synthesis approach extend to all membrane protein classes and thus open new ways to study such proteins?

Can CFPS be used to study TMPs present in organelle membranes, opening a means to study them in *in vitro* platforms and use them for drug screening, structure determination, etc.?

Acknowledgments

The project described was supported by T32GM138826 from the National Institute of General Medical Sciences (ZAM). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health. ZAM was supported by a School of Chemical and Biomolecular Engineering Fleming Fellowship, Cornell Commercialization Fellowship, and National I-Corps Teams (Grant 2229505). E.S. was supported by Cornell University. This project was supported by National Science Foundation Grant

1935370. A.O. was supported by a National Science Foundation Graduate Research Fellowship. We are grateful to J. Carten for providing valuable feedback on the manuscript. Schematic figures were created with BioRender.com.

Author contributions

Z.A.M and E.S. co-authored the manuscript with contributions from A.O.. S.D. is the corresponding author and provided key input. All authors approve of the final version.

Declaration of interests

The authors declare no conflicts of interest.

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