Diblock copolymers enhance folding of a mechanosensitive membrane protein during cell-free expression

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The expression and integration of membrane proteins into vesicle membranes is a critical step in the design of cell-mimetic biosensors, bioreactors, and artificial cells. While membrane proteins have been integrated into a variety of nonnatural membranes, the effects of the chemical and physical properties of these vesicle membranes on protein behavior remain largely unknown. Nonnatural amphiphiles, such as diblock copolymers, provide an interface that can be synthetically controlled to better investigate this relationship. Here, we focus on the initial step in a membrane protein’s life cycle: expression and folding. We observe improvements in both the folding and overall production of a model mechanosensitive channel protein, the mechanosensitive channel of large conductance, during cell-free reactions when vesicles containing diblock copolymers are present. By systematically tuning the membrane composition of vesicles through incorporation of a poly(ethylene oxide)-b-poly(butadiene) diblock copolymer, we show that membrane protein folding and production can be improved over that observed in traditional lipid vesicles. We then reproduce this effect with an alternate membrane-eliciting molecule, C12E2. Our results suggest that global membrane physical properties, specifically available membrane surface area and the membrane area expansion modulus, significantly influence the folding and yield of a membrane protein. Furthermore, our results set the stage for explorations into how nonnatural membrane amphiphiles can be used to both study and enhance the production of biological membrane proteins.

Significance

Membrane protein folding is a critical step that underlies proper cellular function as well as the design of technologies like vesicle-based biosensors and artificial cells. Membrane composition is known to play a role in membrane protein folding; however, the specific mechanical properties of membranes that govern protein folding remain unclear. Using a highly elastic nonnatural amphiphile, we highlight the importance of a membrane mechanical property, membrane elasticity, on the spontaneous insertion and unfolding of a model \(\alpha\)-helical membrane protein. Through this study, we gain a deeper understanding of cellular membrane protein folding and offer a potential approach to improve the production of membrane proteins through optimizing the mechanical properties of synthetic scaffolds present in cell-free reactions.

Author contributions:
M.L.J., M.A.B., and N.P.K. designed research; M.L.J. and M.A.B. performed research; M.L.J., M.A.B., and N.P.K. analyzed data; and M.L.J., M.A.B., and N.P.K. wrote the paper.

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such as lipid vesicles (31–33), polymer scaffolds, polymersomes (15, 16, 34–36), and nanodiscs (37, 38). While these studies suggest that the presence of membranes is important for preventing protein aggregation, an open question remains as to how membrane properties affect protein folding.

Using a cell-free expression system and synthetic vesicles, we show that the production of a mechanosensitive channel protein, the mechanosensitive channel of large conductance (MscL), is affected by physical features of membranes present in the reaction mixture, including membrane concentration and membrane area expansion modulus. We use diblock copolymers to tune the chemical and mechanical properties of synthetic vesicle membranes present in the reaction mixture and show that protein folding is dependent on a balance of steric repulsive interactions at the membrane surface and available membrane surface area. Our study points to the unexplored role of available membrane surface area and membrane area expansion moduli in membrane protein folding.

**Results**

**Lipid Vesicles Increase Production of a Membrane Protein.** We first investigated the folding of MscL in a standard 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) lipid vesicle membrane. While vesicles have been shown to enhance the yield of membrane proteins in these reactions (39), the effect of vesicle concentration on this process has not been widely explored. To investigate this relationship, we used an in vitro transcription–translation system (PURExpress) with a construct expressing an MscL green fluorescent protein fusion (MscLGFP) with varying concentrations of DOPC vesicles (Fig. 1A and B). Because monomeric enhanced green fluorescent protein (eGFP) does not readily dimerize (40) and because its folding and fluorescence is dependent on the proper folding of the N-terminal fusion partner (41), this fusion protein is a useful marker for MscL folding during production. MscL is known to cotranslationally insert into lipid membranes without a chaperone, making it a useful model protein for membrane protein folding studies conducted in cell-free systems (33).

We observed that MscLGFP fluorescence increased in the presence of increasing DOPC vesicle concentration, leading to a sevenfold increase in GFP fluorescence at the maximum vesicle concentration with respect to protein production in the absence of vesicles (Fig. 1C and SI Appendix, Fig. S1A). The upper limit of vesicles used in our studies (100 mM) was constrained by the inability to form vesicles at high concentrations. We expect, however, that if MscLGFP folding is dependent on available membrane surface area, protein folding should eventually plateau when available membrane surface area exceeds the surface area occupied by expressed proteins. In contrast to MscL, the folding of soluble GFP decreased when expressed in solutions containing increasing vesicle concentrations (Fig. 1C). These results suggest that lipid vesicles improve the folding of MscLGFP through direct interactions with the protein. When we added vesicles postexpression, we were unable to rescue misfolded proteins (SI Appendix, Fig. S1B), indicating that membranes act as chaperones to facilitate cotranslational folding of MscL. Protein fluorescence was observed during cell-free reactions correlated monotonically with Western blot densitometry analysis of the relative protein produced, indicating that lipid vesicles improve not only the functional folding of MscL, but also the overall production (Fig. 1D). Finally, we assessed the functional integration of MscLGFP by assessing protein activity. Using a calcein dye release assay, we observed the activity of MscLGFP G22C, a chemically activatable mutant of MscL, after integration into vesicle membranes (SI Appendix, Fig. S2). Our results confirm that MscLGFP is functionally integrated into vesicle membranes present during cell-free protein synthesis.

**Diblock Copolymers Enhance MscLGFP Folding During Cell-Free Protein Synthesis.** Next, we used the poly(ethylene oxide)-b-poly (butadiene) (PEO-b-PBD) diblock copolymer to gain insight into how physical features of membranes affect membrane-assisted protein folding. Of the various diblock copolymers that have been studied, PEO-b-PBD is one of the best characterized within vesicle membranes. PEO-b-PBD has been particularly useful in providing insight into how chemical properties of individual amphiphiles affect the macroscopic properties of assembled membranes and how membrane physical properties affect biological processes of interest (13, 42, 43).

A unique feature of diblock copolymers in comparison with phospholipids is that chemical and physical features of the polymer can be systematically changed. For the PEO-b-PBD polymer, the molecular weight (MW) of the hydrophilic PEO chain and the hydrophobic PBD chain can be modified, resulting in a controlled shift in the hydrophilicity of membrane surface groups and membrane hydrophobic thickness, respectively, while maintaining the overall chemical identity of the polymer. While polymersomes have been successfully used as an insertion platform in the cell-free synthesis of membrane proteins (16), a systematic study of how diblock copolymers influence membrane protein folding has yet to be conducted.

We investigated the effect of diblock copolymer content in vesicles on MscLGFP production by preparing DOPC vesicles with increasing fractions of PEO-b-b-PBD (MW = 1.8 kDa, hereafter referred to as PEO-b-PBD 1.8 kDa) in the membrane, and monitoring GFP fluorescence (Fig. 2A). The total amphiphile concentration was kept constant in these studies (10 mM), and we varied the ratio of lipid:polymer. The number of amphiphiles per vesicle is expected to be similar across vesicle blends as DOPC lipids and PEO-b-PBD 1.8-kDa polymer chains are expected to have similar
Increasing Membrane Elasticity Improves Membrane Protein Folding.

To confirm the role of the membrane area expansion modulus on the cotranslational folding of MscL, we sought to change the area expansion modulus using alternate molecules. Specifically, we used membrane additives that would decrease and increase the \( K_a \) of DOPC vesicles by incorporating a detergent, \( C_{12}E_8 \), and cholesterol, respectively. The detergent \( C_{12}E_8 \) has been previously proposed to increase bilayer elasticity in lipid vesicles as a function of \( C_{12}E_8 \) content (48). We confirmed that \( C_{12}E_8 \) significantly reduced the area expansion modulus of DOPC vesicles, while maintaining membrane stability with respect to pure DOPC vesicles (SI Appendix, Figs. S7 and S8). We then prepared DOPC vesicles with increasing amounts of \( C_{12}E_8 \) and decreasing amounts of DOPC while maintaining an overall constant amphiphile concentration and observed a commensurate increase in MscLGFP fluorescence when vesicles contained up to 25 mol\% detergent (Fig. 3). Typical methods for protein reconstitution use detergents to structurally alter lipid vesicles (e.g., forming micelles) or destabilize vesicles to a degree that results in membrane permeabilization (49). Our approach differs from these studies in that the concentrations of detergent that resulted in the most efficient protein folding (25 mol\%), did not lead to significant permeabilization of vesicles (SI Appendix, Fig. S8), indicating membrane cohesion was maintained. To confirm MscLGFP fluorescence was due to interactions with the membrane and not free detergent micelles, we evaluated MscLGFP folding in the presence of \( C_{12}E_8 \) alone, in which \( C_{12}E_8 \) was added in comparable concentrations to that used in the studies with vesicles (SI Appendix, Fig. S9). We observed that detergent alone reduces protein folding relative to samples containing pure DOPC vesicles. In addition, we observed through microscopy that detergent added at 50 mol\% or higher disrupted vesicle formation, helping to explain why MscLGFP fluorescence decreased in these samples.

We then observed how MscL folding was affected when membrane stiffness was increased. Cholesterol is known to increase the area expansion modulus of lipid membranes (50),

![Diagram](image)

**Fig. 3.** Increasing membrane elasticity with a membrane additive improves MscLGFP folding. MscLGFP fluorescence after cell-free reactions conducted with DOPC vesicles containing varying fractions of \( C_{12}E_8 \). MscLGFP fluorescence was normalized to reactions performed in water. **P \( \leq 0.001 \) (values were generated by ANOVA using the Dunnett test for multiple comparisons to the DOPC vesicle sample containing 0 mol\% \( C_{12}E_8 \); ns = 3; error bars represent SEM.)

We then observed how MscL folding was affected when membrane stiffness was increased. Cholesterol is known to increase the area expansion modulus of lipid membranes (50),
For example, incorporation of 50% cholesterol into stearoyloleoylphosphatidylcholine (SOPC) vesicles increased the K_a from ~193 to 781 dyne/cm (50). When we incorporated cholesterol into DOPC vesicles, we observed a decrease in MscLGFP folding relative to pure DOPC vesicles (SI Appendix, Fig. S10). Our observations with C_12E_8 and cholesterol indicate that using membrane additives that decrease the membrane area expansion modulus is a potential route to enhance MscL folding during cell-free reactions.

Poly(Ethylene Glycol) Headgroups Prevent MscLGFP Folding and Insertion into Membranes. Finally, we explored what feature of PEO-b-PBD was inhibiting MscL folding when the polymer was present at higher concentrations (>25%) in vesicles. Diblock copolymers contain a hydrophilic PEO chain and PEO-modified amphiphiles can generate an effective steric barrier to prevent protein interaction with a membrane interface (51). To isolate the effect of PEO headgroups, otherwise known as poly(ethylene glycol) (PEG), in our cell-free expression system, we used a 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine (DSPE) lipid with PEG modifications of three sizes: 550 Da, 1,000 Da, and 2,000 Da. We performed cell-free reactions expressing MscLGFP in the presence of a constant amphiphile concentration (10 mM) of DOPC vesicles containing 10 mol% DSPE-PEG, and monitored MscLGFP fluorescence. MscLGFP fluorescence decreased with increasing PEG chain length (Fig. 4A). Our results are consistent with the expected steric behavior of PEG-modified amphiphiles, or the enhanced surface tension PEG chains can add to bilayers (52), which can prevent protein interaction with membranes.

We then confirmed the inhibitive role of PEG chains on membrane protein folding using a series of diblock copolymers. PEO-b-PBD amphiphiles of different molecular weights have similar area expansion moduli and chemical composition (43) but differing sizes of PEG and PBD groups. We performed cell-free reactions at a constant vesicle concentration (10 mM) with DOPC and 25 mol% PEO-b-PBD, but varied the molecular weight of the copolymer between 1.05 kDa (PEO_24-b-PBD_24), 1.8 kDa (PEO_36-b-PBD_36), and 3.5 kDa (PEO_56-b-PBD_56) (Fig. 4B). As the MW of the diblock copolymer increased, protein folding decreased. Though changes in membrane thickness could also contribute to this decrease, diblock copolymers like those used in our study are expected to be more flexible and therefore more able to change conformation to accommodate membrane proteins, even with membrane thickness mismatches (53). An additional property of diblock copolymers that could inhibit cotranslational protein folding is interdigitation of polymer segments in the hydrophobic core of the membrane. We would expect polymer interdigitation to increase with increasing polymer content in lipid vesicles and subsequently reduce protein insertion through increases in membrane bending rigidity. While diblock copolymers commonly interdigitate across assembled monolayers, smaller molecular weight diblock copolymers such as PEO-b-PBD 1.8 kDa are not expected to significantly interdigitate (54). Taken together, these results suggest steric interactions between MscL and diblock copolymer PEO groups are responsible for the reduced efficiency of MscL folding.

Discussion

Here, we have shown that the titer of a properly folded α-helical membrane protein can be increased by altering membrane concentration and composition in a cell-free reaction. While previous studies have shown that it is possible to cotranslationally fold membrane proteins into diblock copolymer membranes using cell-free expression techniques (34–36), we show that these synthetic membrane amphiphiles can enhance the cotranslational folding and synthesis of a membrane protein, which to the best of our knowledge, has not been previously shown.

There are two major steps in membrane protein assembly: insertion into the membrane and folding. Bilayer membranes have a variety of mechanical properties that could influence either step, including bending rigidity, spontaneous curvature elasticity, hydrophobic thickness, area expansion moduli, and membrane fluidity/viscosity. While it is difficult to vary any one of these parameters in isolation, diblock copolymers impart unique combinations of mechanical changes to membranes that are difficult to achieve with biological lipids alone, allowing us to examine the effect of these properties on folding in a different way. For example, the diblock copolymers we used are expected to have either an equivalent or higher bending rigidity and membrane thickness (42) and less fluid/more viscous membranes (SI Appendix, Fig. S11) (55) compared with lipid vesicles. These parameters would be expected to decrease membrane protein folding efficiency, which is inconsistent with our observations (21, 22, 56). Additionally, unlike detergents used to solubilize membranes to allow protein reconstitution, diblock copolymers also do not appear to affect the stability of vesicles in our study (SI Appendix, Fig. S8). One of the remaining features of diblock copolymers that
appear to be most relevant to protein folding efficiency in the current study are the chemical and structural features of the polymer chains. We are unable to exclude the role of membrane curvature or chemical interactions between MscL and PEO-b-PBD in enhancing protein folding at this time. However, our observation that small amounts of the polymer (10%) lead to significant increases in protein folding and the reproducibility of this effect with C12E8 points to an important role of the global mechanical features of the hybrid polymer/lipid membrane in MscL folding. Our study suggests that membrane elasticity, specifically the area expansion modulus, which has not been widely considered in membrane protein folding studies, may be important.

The area expansion modulus is a membrane property that is commonly interconnected to other membrane physical properties, one of them being interfacial tension. Amphiphiles that integrate into vesicle membranes (diblock copolymers and C12E8) can reduce interfacial tension significantly (57), which we can measure through the area expansion modulus (Kc ~ 4γ). Reduced interfacial tension has been shown to lead to effects that should affect MscL folding: increased surface area per amphiphile and decreased energetic costs of membrane deformation. First, with reduced interfacial tension, the average surface area per amphiphile increases (57), leading to an increase in total membrane surface area available for membrane protein folding. This remains even when total amphiphile density (amphiphiles/volume or amphiphiles/surface) is maintained.

Our results with DOPC vesicles (Fig. 1) show the importance of total membrane surface area, at a constant chemical composition and with constant membrane mechanical properties, on enhancing protein folding and yield. Similarly, the diblock copolymers used in our study are expected to provide increases in membrane surface area through native size and increased accommodation of strain. While the area per chain of PEO-b-PBD 1.8 kDa (expected to be ~0.76 nm2) (44) is similar to the area per lipid of DOPC (0.72 nm2) (58) in a unstretched/relaxed membrane, we experimentally observed through FRET studies that polymers increase the average distance between lipids when blended into lipid vesicles (SI Appendix, Fig. S3D). Polymers could therefore increase available membrane surface area because they occupy more area per chain than lipids, by reducing interfacial tension of the membrane, which would increase the area per lipid chain, and by increasing the total strain that vesicles can experience.

A consequence of reduced interfacial tension is that the energetic cost of bilayer compression and expansion will be reduced relative to the scenario where no elasticizing amphiphile is present. The energy required to create a vacancy in a bilayer membrane is proportional to the Kc (59). In studies with gramicidin, a channel whose function is regulated by elastic properties of the bilayer, Lundbaek et al. (60) showed that amphiphiles which increased membrane elasticity (here described as a combination of area expansion modulus, fluidity, and curvature) and reduced the free energy of membrane deformation shifted the proportion of proteins in a dimerized, functional conformation. These results indicated that global changes in membrane elastic properties that lower the energy of membrane deformation can increase the conformational freedom of a protein in the bilayer, thereby increasing the probability of membrane protein assembly. We suggest that diblock copolymers decrease the area expansion modulus and increase available surface area in membranes with respect to the lipid used in this study, enabling more available membrane area as well as reduced energy costs to deform membranes (Fig. 5). These effects should enhance protein folding by providing more surface area and more conformational freedom for protein segments.

The extent to which our observations with MscL can be generalized to other membrane proteins is an important question. Toward answering this, we explored how the incorporation of PEO-b-PBD 1.8-kDa polymers affected the folding of another model α-helical membrane protein, channelrhodopsin (ChR2). We observed that the folding of ChR2 increased monotonically with the increasing fraction of diblock copolymer, and a maximum level of folding was observed in the 100% polymer vesicles, in contrast to MscL, which exhibited maximum folding in 25% polymer vesicles (SI Appendix, Fig. S12). This may be due to differences in structure between MscL and ChR2. For example ChR2 has seven transmembrane domains while MscL has two; this difference in structure could lead to differences in membrane affinity and folding thermodynamics. Overall, it appears that polymeric membranes could facilitate improved folding of different types of α-helical membrane proteins and merits further investigation.

Our findings have interesting implications for both biological systems and technological applications. Toward the former, our study contributes to mechanistic studies of cotranslational folding. Studies using cell-free systems have become an important tool to investigate this process and to date have revealed that several membrane proteins can be produced without translocase insertion machinery so long as vesicles are present (29, 33). A recently proposed model posits that transmembrane helices spontaneously insert into membranes, driven by the transfer of free energy between aqueous and lipid phases (61). Our study supports this thermodynamic model by showing that increasing the amount of membrane area increases both the rate of protein folding and the total amount of MscL expressed (Fig. 1D).

Finally, our results have potential use in technological applications to increase the yield of membrane protein production in cell-free reactions. An important issue in membrane protein synthesis is improving the yield of protein or expanding the environments in which proteins are expressed (1). Cell-free expression methods have overcome a variety of issues associated with heterologous membrane protein expression and purification, yet membrane protein yield remains an area to be optimized (62). An emerging approach to address this problem is to change the reaction environment by using nonnatural polymers as scaffolds or chaperones (13, 15, 16). Our use of nonnatural amphiphiles that change membrane properties present in cell-free reactions should provide a complementary approach to protein design strategies to enhance the expression of membrane proteins that behave similarly to MscL.

Materials and Methods

Materials.

Chemicals. The 18:1 (α9) DOPC and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-550] (ammonium salt) (DSPE-PEG550) were obtained from Avanti. PEO-b-PBD12, PEO-b-PBD24, and PEO-b-PBD36 were obtained from Polymer Source. Octaethylen glycol monododecyl ether (C12E8), P85, sucrose, and Sepharose 4B were obtained from Sigma-Aldrich. Plasmids. The EcMscL gene (pADet-His6-MscL) was a gift from Allen Liu (Addgene plasmid no. 83373, University of Michigan, Ann Arbor, MI) (63); the pET119b backbone was a gift from Douglas Rees (Addgene plasmid no. 79028, California Institute of Technology, Pasadena, CA) (64), and mEGFP-P8AD was a gift from Michael Davidson (Addgene plasmid no. 54622, Florida State University, Tallahassee, FL).

Lipid and Polymer Vesicle Preparation. Vesicles were prepared through thin-film hydration as described by Kamat et al. (57). Briefly, DOPC lipids were dissolved in chlororform and mixed with PEO-b-PBD, polymers of varying molecular weights, DSPE-PEG, or C12E8 in chlororform at varying molar percentages relative to DOPC before addition to glass vials. Chlorform was evaporated under water and incubated at 60 °C overnight. The films were rehydrated in a stream of nitrogen to create a uniform film, and films were placed under vacuum for 4 h. For studies with MscL expression, films were rehydrated in water and incubated at 60 °C overnight. The films were vortexed and vesicles were extruded to 100 nm by seven passes through a polycarbonate membrane using a minietruder set (Avanti). For studies with hybrid vesicles and membrane additives, the total amphiphile concentration per sample (moles amphiphile/solution volume) was kept constant and the effective concentration of amphiphiles after vesicle assembly, typically 10 mM, is reported in the text.

PURExpress Reactions. Protein expression was performed with the PURExpress In Vitro Protein Synthesis kit (E6800, NEB) according to the manufacturer’s protocol. Final reaction volumes were 30 μL and contained PURExpress components, 200 ng plasmid (EcMscLGFP), and desired vesicle content (10 mM final unless otherwise stated). The reactions were monitored in a
Molecular Devices Spectra Max I3 plate reader at 37 °C. GFP fluorescence was measured at excitation 480 nm, and emission at 507 nm for 3.5 h.

Quantitative Western Blotting. Using the WesternBreeze cheluminescent kit for mouse antibodies (Thermo Fisher Scientific), 12% SDS/PAGE gels were treated according to kit protocol with anti-GFP (Abcam). Blots were imaged using an Azure Biosystems c280 imager. Densitometry quantification was performed in ImageJ (65), measuring band density and comparing relative levels within the same blot; n = 3 averaged between blots.

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Elasticity Measurements. Area expansion moduli were measured using micropipette aspiration techniques similar to those described by Kamat et al. (57). Further details are provided in SI Appendix.

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