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Research Paper

Production of membrane proteins without cells or detergents

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The production of membrane proteins in cellular systems is besieged by several problems due to their hydrophobic nature which often causes misfolding, protein aggregation and cytotoxicity, resulting in poor yields of stable proteins. Cell-free expression has emerged as one of the most versatile alternatives for circumventing these obstacles by producing membrane proteins directly into designed hydrophobic environments. Efficient optimisation of expression and solubilisation conditions using a variety of detergents, membrane mimetics and lipids has yielded structurally and functionally intact membrane proteins, with yields several fold above the levels possible from cell-based systems. Here we review recently developed techniques available to produce functional membrane proteins, and discuss amphipols, nanodisc and styrene maleic acid lipid particle (SMALP) technologies that can be exploited alongside cell-free expression of membrane proteins.

Introduction

Membrane proteins represent challenging targets for structural biology and drug discovery due to their hydrophobicity and reliance on a lipid bilayer environment for stability. They play key roles in diverse cellular processes including signal transduction, cell division, growth and differentiation, often as multimers or complex assemblies. Not only are they functionally critical, constituting a third of all gene products in living organisms, but membrane proteins also account for roughly half of all pharmaceutical targets [1], making their production vitally important.

Most membrane proteins are expressed in low copies on cell surfaces and hence endogenous forms are insufficient for structure/function studies. Overexpression of recombinant membrane proteins often results in cytotoxicity, misfolding and aggregation. Owing to their hydrophobic surface, conventional purification of membrane proteins involves extraction with detergents, which are often destabilizing. The structural and functional integrities of membrane proteins depend on associated lipids which are lost upon detergent extraction. Hence the entire process of expression, solubilisation and purification of membrane proteins using conventional methods is technically demanding and risk prone.

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In recent years, cell-free expression technologies have been developed to surmount the technical hurdles involved in membrane protein expression primarily by the elimination of cellular toxicity seen in cell-based systems. The absence of an enclosed membrane system is also advantageous for the systematic screening of detergents or membrane mimetics, the optimisation of reaction conditions, as well as for testing the effects of adding protease inhibitors, ligands, cofactors and lipids with higher throughput. The general *in vitro* protein transcription, translation and optimisation methods are the subjects of recent in-depth reviews [2–4]. Here we have focussed on the latest strategies available for the stable production of membrane proteins in different hydrophobic environments using cell-free expression and nanoparticle solubilisation systems.

Cell-free production of membrane proteins in the presence of detergents

The two most popular cell-free expression systems for membrane proteins are based on *Escherichia coli* and wheat germ extracts. Whilst the *E. coli* method involves a coupled transcription/translation system where protein synthesis can be initiated from a plasmid DNA or PCR product [5], the wheat germ system uses decoupled translation and requires mRNA to initiate protein

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synthesis [6]. In both schemes, during extract preparation the cell-free systems are stripped of the membranes that could provide a lipidic environment to receive membrane proteins. Consequently conventional cell-free expression of a membrane protein results in insoluble precipitates that must be solubilised using detergents [7]. Successes have been largely restricted to β-stranded outer membrane proteins rather than those with α-helical folds [8], consistent with higher stabilities of β-barrel folds. Some of the detergents used to efficiently solubilise membrane protein precipitates are lysopalmitoyl phosphatidylglycerol (LPPG) and lysomyristoyl phosphatidylglycerol, dodecylphosphocholine (DPC) and sodium dodecyl sulphate (SDS). Despite the recent progress in regenerating functional multidrug transporter protein EmrE from precipitates following cell-free expression [8], the solubilisation of membrane proteins from precipitates is generally disfavoured because the majority are functionally compromised [7].

Direct expression of folded membrane proteins can be enabled by the addition of detergents to cell-free reaction chambers. The presence of detergent micelles in proximity to the translation apparatus aids the direct solubilisation of the synthesised membrane protein. Moreover, the elimination of the rate-limiting steps of protein transport through endoplasmic reticulum, Golgi apparatus and translocation to membranes, as found in living cells, results in improved protein yields. The choice of detergent influences the solubilisation efficiency and therefore needs to be carefully evaluated for individual membrane proteins. Such optimisations can be readily accomplished due to the open nature of the cell-free systems. Generally detergents with high critical micellar concentration (CMC), such as *n*-octyl-β-D-glucopyranoside (β-OG) or CHAPS are not suitable for cell-free expression because they inhibit the transcription/translation machinery. The most successful class of detergents is long chain polyoxyethylene-ethers such as Brij derivatives and steroid glycosides such as digitonin [7]. Some proteins are stabilized by specific cellular lipids [9] which can be combined with the detergents to help maintain their structural integrity. Functional membrane proteins such as mechanosensitive channel protein MscL [10], small multidrug transporter EmrE [8], light-harvesting protein of LH1 [11], nucleoside transporter Tsx [7] and β_2-adrenergic receptor [12], all from *E. coli* cell-free expression, have been successfully expressed in preparative scales. Interestingly, Park *et al.* recently reported the use of hemifluorinated surfactants in the production of the bacterial mechanosensitive channel, MscL, in *E. coli* cell-free systems [13]. Being naturally lipophobic, these hemifluorinated surfactants find little use in extracting membrane proteins from natural membranes, but could be advantageous for cell-free expression of proteins that fail to retain their native fold in the presence of detergents. A further advantage of these surfactants lies in their ability to directly deliver the membrane proteins to preformed lipid bilayers or plasma membranes of living cells.

The wheat germ system is particularly compatible with detergents because it is a translation only system and hence does not suffer from detergent damage to the transcriptional machinery. Efficient production of an olfactory GPCR, olfactory receptor h-OR17-4, was reported with detergents digitonin and Brij-58 [14]. Successful production of the β-barrel PagP and α-helical DsbB *E. coli* membrane proteins was achieved using the detergents Brij-35, octaethylene glycol monododecyl ether ($C_{12}E_8$) and CHAPS (Table 1), whilst other detergents including DPC, dihexanoylphosphatidylcholine (DHPC) and LPPG were found to inhibit translation (S. Rajesh and M. Overduin, unpublished results). The major disadvantage in the use of detergents for membrane protein solubilisation stems from the differences between the protein's state in micellar and membrane environments. The absence of bound lipids and diminished lateral pressure can compromise the stability of membrane proteins in micelles and can render some functional states inaccessible [15]. This has prompted efforts to produce membrane proteins within more native membrane-mimicking environments to facilitate more accurate analysis of their biophysical properties and structures.

TABLE 1

Detergents for the production of soluble membrane proteins in *E. coli* and wheat germ cell-free systems

Abbr	Long name	Soluble membrane protein expression[a]		Reference
		E. coli	Wheat germ	
Detergents				
Brij35	Polyoxyethylene-(23)-lauryl-ether	++	+++	[7,12][b]
Brij 58	Polyoxyethylene-(20)-cetyl-ether	++	–	[7][b]
Brij-78	Polyoxyethylene-(20)-stearyl-ether	++	n.d.[c]	[7]
Brij-98	Polyoxyethylene-(20)-oleyl-ether	++	n.d.	[7]
C12E8	Octaethylene glycol monododecyl ether	n.d	++/–	[b]
Digitonin	Digitonin	++	++	[7,12][b]
DDM	*n*-Dodecyl-β-D-maltoside	+/–	+/–	[7,12][b]
Triton X-100	PEG P-1,1,3,3-tetra-methyl-butylphenyl ether	++	n.d.	[7,12,10]
diC8PC	1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine	++	n.d.	[7]
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propansulfonate	–	++/–	[7][b]
β-OG	n-Octyl-β-D-glucopyranoside	–	–	[7][b]
LPPG	1-Palmitoyl-2-hydroxy-*sn*-glycerol-3-(phosphor-*rac*-(1-glycerol))	+/–	+/–	[7][b]
Lipids				
DPC	Dodecylphosphocholine	–	–	[7][b]
DHPC	1,2-Diheptanoyl-*sn*-glycero-3-phosphocholine	+	–	[7][b]

[a] Expression was classified into five groups, +++ highly soluble; ++ good solubility; ++/– good solubility with some precipitation; +/– soluble but yield is reduced; – inhibits translation.
[b] Rajesh and Overduin (unpublished results).
[c] n.d.: not determined.

Cell-free production of membrane protein into liposomes and vesicles

Several strategies have been exploited to express membrane proteins from cell-free reactions into a lipid bilayer mimicking environment. In the first approach, precipitated membrane protein is first solubilised using detergents, as discussed above, before reconstitution in liposomes made from lipids found in membrane, for example phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylserine or cholesterol. The detergent solubilised membrane protein is reconstituted into liposomes by freeze–thaw [16–18] through the formation of proteoliposomes or by the removal of detergents in the presence of liposomes using adsorbent beads [19]. In the second approach, direct cell-free synthesis in the presence of unilamellar liposomes composed of soya bean lipids, 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC), DMPC or microsomal fractions obtained from lysed cells have been successfully used to incorporate membrane proteins [20–22]. This technique is advantageous for assaying the activity of proteins, because the membrane proteins are in an inside out orientation within the lipid vesicles. Because cell-free systems can tolerate high concentrations of lipids and liposomes, there is scope for optimisation to improve membrane protein yields. However, the heterogeneity of unilamellar vesicles (sizes vary between 30 nm and 200 nm) and presence of multilamellar vesicles can render this method unsuitable for structural biology studies. *In vitro* translocation of membrane protein into liposomes can be problematic due to the absence of suitable translocation machinery. In a recent development, integral membrane proteins were successfully expressed in a cell-free system supplemented with *E. coli* inner membrane inverted vesicles and proteins (SecA/B and SRP/SR) that aid translocation/integration [23,24]. The major drawbacks in this approach are the lack of commercial availability of inverted vesicles and the heterogeneity in vesicle preparation, which limit utility for structural studies.

Nanolipoproteins and cell-free expression of membrane protein

Nanodiscs were developed by Sligar and co-workers and consist of a bilayer composed of 130–160 lipid molecules and surrounded by an amphipathic helical lipoprotein (recently reviewed in Ref. [25]). These monodisperse nanodiscs vary in size between 10 and 20 nm, depending on the type of scaffold protein used, whilst the thickness is equal to that of a bilayer. The molecular mass of empty nanodisc is ~150 kDa and can be tuned to contain a single membrane protein per disc; however, they can be constrained in accommodating membrane proteins beyond a certain size [26]. The obvious advantages of nanodiscs over liposomes reside in their similarity to the normal lipid bilayer, simple method of preparation and accessibility of both surfaces of the lipid bilayer [27]. Membrane protein functions that are dependent on association with cellular lipids, cofactors or additives for activity could be addressed in nanodiscs with ease. The first attempts at cell-free production of membrane proteins in nanodiscs involved the addition of preformed or empty nanodiscs to the reaction mixture to provide a hydrophobic support for the membrane protein [28]. The resulting product was a mixture of nanodisc membrane protein complex, empty nanodiscs and precipitated protein from which soluble membrane protein in nanodiscs could be separated using conventional affinity and size exclusion chromatography techniques. Nanodiscs were found to be very effective at increasing the solubility of a wide variety of cell-free produced membrane proteins that were also correctly folded, a key indicator of functional activity of membrane proteins. The nanodiscs did not inhibit the translation machinery in cell-free extracts from *E. coli*, wheat germ and rabbit reticulocytes, and solubilised expressed EmrE protein [28]. As seen in the case of the liposome method, the major drawback with nanodiscs is the absence of translocon machinery for the correct insertion and folding of the membrane proteins into the lipid environment. However by adding components from the *E. coli* Sec YEG complex into nanodiscs it might be possible to overcome the translocation problem and boost the success of this technology [29].

By exploiting the ability of apolipoprotein to sequester lipid bilayer patches in solution, Cappuccio *et al.* [30] recently reported the successful co-expression of the scaffold protein with bacteriorhodopsin in the presence of phospholipids and cofactors to produce functional bacteriorhodopsin–nanodisc complexes in a single reaction. The lack of requirement for a preformed nanodisc is a clear advantage of this method, although a source of unilamellar liposomes is still a necessity. Separation of membrane protein–nanodiscs from the large amount of reaction products such as empty nanodiscs, liposomes, scaffold proteins and precipitated proteins is still a difficult task. The yield of nanodisc-solubilised membrane protein can be low when compared to methods involving use of preformed nanodiscs, because the translation machinery is necessarily used to also express the scaffold protein.

Amphipols in membrane protein solubilisation

Recently, novel solubilisation techniques based on using alternative surfactant technologies [31–35] have emerged that could be exploited for cell-free expression. Amphipathic polymers are promising alternatives to detergents in view of their design flexibility, stability and versatility. For example, amphipols are water-soluble, linear short-chain copolymers comprise strongly hydrophilic backbones presenting hydrophobic chains for engaging lipids and membrane proteins. Their periodicity can promote hypercoiling under defined conditions and when in contact with membrane proteins, can coil around their hydrophobic transmembrane regions. They can enhance protein stability, and can be used to fold membrane proteins or retain their native forms.

Several polymer systems have been developed for the study of membrane proteins, with four showing considerable promise: A8-35 [35], PC-amphipol [36], PMAL [37] and styrene maleic acid (SMA) [38]. The A8-35 polymer is the most studied one and solubilises and maintains the activity of numerous membrane proteins [35,39] as well as helping refold G-protein-coupled receptors (GPCR) from inclusion bodies [40]. The polymers increase protein stability [41], presumably by providing lateral pressure whilst being of sufficiently small and homogeneous to permit structural studies by NMR [42]. However, the carboxylate groups of A8-35 limit its use to a pH threshold of ~6.5, as below this point the polymer becomes insoluble. To combat this attempts have been made to make polymers more soluble, either by making them zwitterionic or by converting the carboxylate group to other more soluble groups. The first of these is PMAL (Anatrace) which is a

fully zwitterionic polymer that preserves the activity of diacyl glycerol kinase at neutral pH [37]. Furthermore the solubilisation is reversible as the polymers are able to deliver proteins back into micelles and vesicles [43]. The second polymer to be more pH versatile is PC–amphipol and is based on replacing the carboxylate groups with phosphorylcholine [36]. Unlike carboxylated polymers PC–amphipol remains soluble in aqueous media under conditions of low pH, high salt concentrations, or in the presence of divalent ions.

Although A8-35, PMAL and PC–amphipol show significant promise as an improvement over detergent based solubilisation of the proteins from their native membrane environment, they still require the presence of detergent before the addition of the polymer. A solution being developed for this limitation is styrene maleic acid lipid particles (SMALPs) [38]. This carboxylated amphipathic polymer is composed of alternating styrene and maleic acid groups and solubilises transmembrane proteins in active forms directly from membranes without the need for detergents at any stage. The SMA can be added to a membrane and forms discoidal structures approximately 11 nm in diameter that encloses both the protein and a layer of lipid molecules which are maintained throughout purification. Furthermore, like nanodiscs, the protein's stability is retained as the lipid environment and lateral pressure are maintained. Their advantages are the absence of any interfering scaffold protein components or signals, as well as low cost of production and biocompatibility, making SMALP system advantageous for biophysical analyses and scaling up production.

Perspectives

Large-scale production of membrane proteins using cell-free expression is still a nascent technology and remains a challenging area especially for structural biology projects; recent successes include the X-ray crystal structure of multidrug transporter EmrE [44] and NMR structures of three histidine kinase receptors [45]. The different hydrophobic environments highlighted above offer interesting alternatives to overcome the pitfalls in the X-ray or NMR structural analyses of cell-free synthesised membrane protein in detergents systems. The exploitation of amphipathic polymers for cell-free synthesis of membrane protein is the subject of interest in laboratories across the world. A major technology to benefit from the use of amphipathic polymers for cell-free synthesis could be the design of novel nanodevices with membrane proteins for various applications including improved drug delivery.

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