Chapter 2
Membrane Protein Quality Control in Cell-Free Expression Systems: Tools, Strategies and Case Studies

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2.1 Introduction

Cell-free (CF) expression has emerged in the last decade as an efficient and fast approach for the production of membrane proteins (MPs) of diverse topologies and origin. Its unique design as an open accessible reaction helps to eliminate several central bottlenecks known from conventional cell-based MP expression systems. In general, problems with cell physiology, expression regulation and cell culture are reduced. On the other hand, the high diversity of CF reaction conditions requests increased time investments in controlling MP quality, fine-tuning of reaction conditions and designing sample evaluation strategies. Poor MP sample quality can be the result if this important requirement is overseen.

CF reactions can basically be operated in two flavors, the single compartment batch configuration and the two-compartment continuous exchange (CECF) configuration (Kigawa and Yokoyama 1991; Spirin et al. 1988). The batch configuration is the method of choice in throughput applications using microplate devices and analytical scale reactions (Kai et al. 2013; Savage et al. 2007; Schwarz et al. 2010). Batch reaction times are limited to few hours with consequently lower yields of protein, although a number of modifications are possible in order to considerably improve efficiencies. Higher protein yields are typically obtained with the CECF configuration containing a reaction mixture (RM) compartment containing all the high molecular weight compounds such as ribosomes, DNA template and enzymes, and a feeding mixture (FM) compartment with a certain amount of precursors such as amino acids and nucleotides. Protocols for batch and CECF configurations are highly variable and among others, expression efficiencies depend on (1) precursor

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concentrations, (2) energy regeneration systems, (3) RM–FM volume ratios and (4) the implementation of repeated FM exchanges.

An array of new applications, modifications, and strategies for the CF production of MP samples has been developed within the last decade. In particular, the tools for the modulation of MP quality already during translation by CF reaction condition tuning have been widely expanded. We therefore provide a current view on options and perspectives for successful MP production and we summarize diverse strategies based on CF expression technologies.

2.2 Selecting the Background: Different Extract Sources

The origin of the CF extract is the first selection to be made by approaching MP expression. In particular, within the last decade, a considerable number of new extract sources covering eukaryotic as well as prokaryotic origins have been introduced (Table 2.1). Major selection criteria before starting a CF expression approach are usually (1) the required amount of synthesized recombinant protein, (2) to provide the most favorable background for promoting protein folding, (3) to increase the likeability of posttranslational modifications, (4) general handling issues, system availability and costs.

Expression efficiencies and other characteristics of the various systems still differ significantly and best compromises have to be found. While few micrograms of recombinant protein can usually be obtained in any system, the production of preparative scale levels approaching milligram yields out of 1 ml of RM is currently only routinely possible with extracts of *Escherichia coli* or wheat germs. Frequent limiting factors for protein production efficiency in cell extracts are high concentrations of endogenous degrading enzymes, poor synchronization of ribosome activity during cell growth, or stability problems of essential enzymes. It should be noted that extracts of cells showing even high expression activities *in vivo* such as yeasts might not be very efficient in CF expression. However, protocols in particular for the efficient CECF configuration are continuously being optimized and further potential for improved protein synthesis might exist. Most systems have now been adjusted as coupled transcription/translation systems including the efficient T7 promoter for protein production and accepting plasmid or linear DNA templates (Table 2.1). The addition of translation factors or considering specific template modifications might further be necessary depending on the selected system.

A critical issue is the availability of the different CF extracts. Most systems are available as standardized commercial kits, but quality optimization and specific applications often require the set up of individual expression reactions. The preparation protocols for the various cell extracts differ significantly with sometimes even high variations in extract batch quality (Table 2.1). For eukaryotic cell extract preparations, species possible to grow in defined cell cultures might be preferred or commercial sources might be considered. The relatively fast and efficient preparation protocol is a major advantage of using *E. coli* extracts. In addition, it is best

Table 2.1 CF extract sources

CF extract source	PTM[a]	cT7[b]	CECF[c]	Yield[d] (mg/ml)	MP[e]	Kit[f]	Protocol[g]
Eukaryotes							
Human HeLa S3 cells CHO	+	+	+	0.05–0.2 ≤0.01	NA	+	Mikami et al. (2006, 2008); Witherell et al. (2001)
Rabbit reticulocytes	+	+	−	≤0.01	+	+	Arduengo et al. (2007); Craig et al. (1992)
Wheat germ	+	+	+	≤2 (CECF) 0.1–0.4 (B)	+	+	Endo and Sawasaki (2005); Madin et al. (2000); Sawasaki et al. (2007); Takai et al. (2010)
Spodoptera frugiperda 9, 21	+	+	−	≤0.1	+	+	Ezure et al. (2007, 2010); Katzen and Kudlicki (2006); Stech et al. (2012)
Saccharomyces cerevisiae	−	−	−	0.05–0.07	NA	−	Wang (2006); Wang et al. (2008)
Leishmania tarantolae	−	+	−	≤0.2	NA	+	Kovtun et al. (2011)
Prokaryotes							
E. coli	−	+	+	≤5 (CECF) 0.5–1 (B)	+	+	Katzen et al. (2005); Schwarz et al. (2008)
Pseudomonas fluorescens (reaction at 8–30 °C)	−	−	−	≤0.1	NA	−	Nakashima and Tamura (2004)
E. coli–PURE (purified translation machinery)	−	+	+	≤0.5	+	+	Shimizu et al. (2001)
Thermus thermophilus (reaction at 37–65 °C)	−	−	+	≤0.06	NA	−	Uzawa et al. (2002); Zhou et al. (2012)
Thermococcus kodakaraensis (reaction at 40–80 °C)	−	−	−	≤0.007	NA	−	Endoh et al. (2008)

[a] Posttranslational modifications other than disulfide bridge formation reported
[b] Coupled transcription/translation protocol with T7 promoter described
[c] Protocols for the more efficient CECF configuration established
[d] Approximate range of protein yield in 1 ml of RM, *B* batch configuration, *CECF* continuous exchange configuration
[e] Expression of MPs reported; *NA* data not yet available
[f] Commercial reaction kits available
[g] Representative recent protocols for extract preparation

characterized and a large variety of compounds useful for reaction modifications is available. The vast majority of current data on MP production have thus been obtained with *E. coli* extracts.

Depending on the intended applications, the proper formation of posttranslational modifications can be a key issue for protein sample quality evaluation. Disulfide bridge formation may be triggered independently from extract origins by modulating the reducing conditions, e.g., by adding redox systems into the reaction, by supporting disulfide bridge formation with chaperones or by chemical pretreatment of extracts (Goerke and Swartz 2008, Kim and Swartz 2004, Yin and Swartz 2004). More complex modifications, such as glycosylation, lipidation, or phosphorylation, are so far only described from systems with eukaryotic extracts such as rabbit reticulocytes, insect cells, or wheat germ and at analytical scales (Table 2.1). Many modifications require supplements such as canine pancreas microsomes into the CF reaction. If modifying enzymes are provided, posttranslational modifications such as N-glycosylation appear to be possible even in extracts of *E. coli* (Guarino and DeLisa 2012). However, it might stay challenging to combine quality and homogeneity of posttranslational modifications with high-level expression purposes.

2.3 Basic Protocol Development: Improving CF Expression Efficiency

Complexity of MP production in CF systems is mainly reduced to the basic transcription/translation process. Coordination of pathways for trafficking or translocation as well as suppressing toxic effects are usually less relevant issues. Protein expression in most CF systems is controlled by the phage T7-RNA polymerase, and the corresponding regulatory promoter and terminator elements in addition to system specific enhancers have to be provided. However, other promoters could work as well. With *E. coli* extracts, derivatives of standard P*tac* promoters recognized by the endogenous *E. coli* RNA polymerase could give even relatively high expression levels (Shin and Noireaux 2010). DNA template constructs can be generated by overlap polymerase chain reaction (PCR) strategies and added as linear DNA fragment into the CF reaction (Ahn et al. 2005; Yabuki et al. 2007). Alternatively, plasmid DNA templates based on standard vectors such as, e.g., the pET or pIVEx series can be provided. DNA templates appear to be quite stable in CF reactions and final concentrations in between 2 and 10 ng/µl RM are already saturating (Haberstock et al. 2012).

Initial problems with low expression efficiency are mainly associated with the translation process. Adjusting the proper Mg^{2+} ion optimum is mandatory for each new target and suboptimal conditions can have severe impacts on protein production (Rath et al. 2011; Schwarz et al. 2007). Abundance of rare codons could further reduce protein expression and induce mis-incorporation of amino acids or even the premature termination of translation. Low protein yields are even more frequently caused by the formation of unfavorable secondary structures of the mRNA

involving the 5-prime end containing the translational initiation site. Modulating the nucleotide sequence of the 5-prime coding sequence can therefore be very efficient in order to improve expression (Ahn et al. 2007; Kralicek et al. 2011). A fast approach is the tag variation screen by analyzing the effects of a small number of short sequence-optimized expression tags (Haberstock et al. 2012). The tag variation constructs are generated by overlap PCR and the resulting products can directly be used as DNA templates in CF expression screens. The construction of large fusion proteins in order to improve expression is therefore usually not necessary. Expression monitoring can initially be performed via immunodetection by using C-terminal purification tags such as a poly$(His)_{10}$-tag as antigen. In an ideal template design, the coding sequence is therefore modified with a C-terminal purification/detection tag, and, if necessary, with a short N-terminal expression tag (Fig. 2.1). If translation can be addressed properly with the above mentioned procedures, the protein production in CF systems is usually very efficient. In expression screens comprising MP targets of diverse sizes, topologies, and functions, high success rates could be achieved (Schwarz et al. 2010; Savage et al. 2007; Langlais et al. 2007).

Expression monitoring by taking advantage of C-terminally attached derivatives of green fluorescent protein (GFP) could be useful for CF expression protocol development and fast protein quantification (Kai et al. 2013; Müller-Lucks et al. 2012; Nozawa et al. 2011; Roos et al. 2012). For MP expression, it must be considered that the folding of wild-type or red-shifted variants of GFP is hampered in the presence of most detergents (Roos et al. 2012). More resistant is the superfolder GFP derivative most likely due to its higher tolerance for chemical denaturants and its faster folding kinetics (Roos et al. 2012; Pedelacq et al. 2006). However, the folding of superfolder GFP might not correlate with the productive folding of the N-terminal target protein as it is speculated for other GFP derivatives (Pedelacq et al. 2006). Superfolder GFP might therefore only be considered as general expression monitor while fusions with other GFP derivatives may in addition also give some preliminary evidence of the target protein folding and quality.

2.4 Folded Precipitates: P-CF Expression

Depending on the strategy and choice of supplemented additives, several basic expression modes are possible for the CF production of MPs (Fig. 2.1). The selection of the expression mode may depend on the intended application of the MP sample, but it can also have drastic consequences on the resulting MP quality (Junge et al. 2010; Lyukmanova et al. 2012). An overview on the implementation of the different CF expression modes is given in Tables 2.2–2.4 and representative case studies published during the last decade are listed.

In absence of any provided hydrophobic environment, the freshly translated MPs instantly precipitate in the RM. Successful expression in this precipitate forming (P-CF) production mode can thus even be monitored by increased turbidity of the RM during incubation. Folded structures of such P-CF-generated MP precipitates

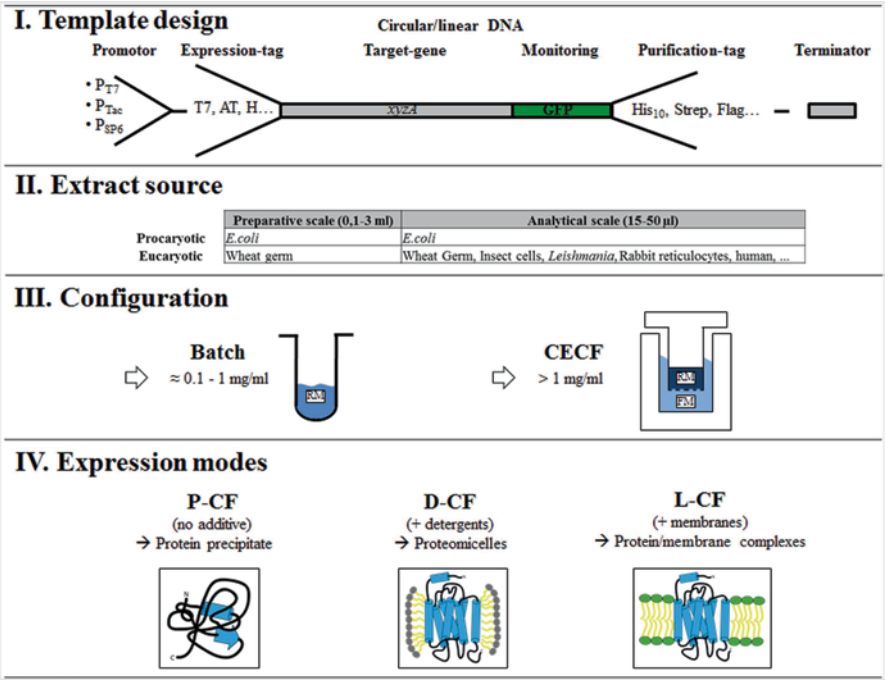

Fig. 2.1 Basic steps for the design of CF expression reactions

can be detected by solid state nuclear magnetic resonance (NMR) and resolubilized precipitates show significant structural overlaps with corresponding MP samples obtained after conventional *in vivo* production (Maslennikov et al. 2010). P-CF-expressed MPs can simply be harvested by centrifugation. The MP pellet is usually contaminated with a number of co-precipitated proteins from the extract. Washing with buffer containing mild detergents such as Brij derivatives can help to selectively reduce such contaminations. The MPs are then solubilized in buffer containing specific detergents. Best results are usually obtained with 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LMPG), 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LPPG), or sodium dodecyl sulfate (SDS; Table 2.2; Klammt et al. 2004; Klammt et al. 2012; Rath et al. 2011). Milder detergents such as n-dodecyl-phosphocholine (DPC) or n-dodecyl-b-D-maltoside (DDM), detergent cocktails or mixtures of detergents and lipids could further be useful depending on the MP target (Ma et al 2011).

Critical parameters for the resulting MP quality can be (1) detergent concentration and volume of the solubilization buffer, (2) temperature of solubilization, and (3) the subsequent exchange of the primary and usually relatively harsh solubilization detergent against secondary and considerably milder detergents, e.g., upon MP immobilization during affinity chromatography. Stabilization and high recovery of ligand binding active GPCRs could be obtained by this strategy (Junge et al. 2010;

Table 2.2 Case studies of P-CF-expressed MPs

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] yield[e]	Solubilization[f] (%)	Reference
Bs-MraY	36 (10)	Enzyme/+	iE [3]	Triton X-100 [2], DPC [2], DHPC [2], DDM [2], LS [0.8], LMPG [0.75], SDS [2]	Ma et al. (2011)
mAqp4-M23	30 (6)	Channel/+	iE [3]	Fos12 [1], DHPC [2], Fos16 [2], LMPG [2], LPPG [1]	Kai et al. (2010)
hETB, hNPY2/5 hMTNR1A/B hSS1/2, hV1BR hHRH1, hV2R rCRF	39–51	GPCR/-	iE [3]	e.g. LMPG [2]	Schneider et al. (2010)
hPS1-CTF	16 (3)	Protease/-	iE [3]	SDS [1–2]	Sobhanifar et al. (2010)
hCRFR1 mCRFR2β	47 (7) 49 (7)	GPCR/+	iE [3]	LMPG [2]/Nvoy	Klammt et al. (2011)
KvAP-VSD *A. pernix*	16.5 (4)	Channel/+	iE [2]	SDS [1]/DPC [0.2]	Lyukmanova et al. (2012)
hErbB3 (639–670)	5 (1)	Receptor/+	iE [3]	DPC	Mineev et al. (2011)
hErbB3 (632–675)	6 (1)	Receptor/+	iE [3]	SDS [1]	Khabibullina et al. (2010)
Ec-ArcB (1–115) Ec-QseC (1–185) Ec-KdpD (397–502)	11–21 (2–4)	Sensor/+	iE [3]	LMPG [5]	Maslennikov et al. (2010)
6 hMPs	~11 (2–3)	Unknown	iE [3]	LMPG	Klammt et al. (2012)
hLAPTM4A hCX32 hGLUT4 hVDAC1	28 (4) 32 (4) 55 (12) 36 (19)	Transporter Gap junction/+ Transporter Channel/+	iE [3]	Fos14	Nguyen et al. (2010)
F_1F_o-ATP synthase *C. thermarum*	542 (26)	Enzyme/+	iE [1]	DDM [2]	Matthies et al. (2011)
PorA/H *C. glutamicum*	5 (7)	Channel/+	iE [3]	LDAO [1], LPPG [1], LMPG [1], LMPC [1], Triton X-100 [1], DHPC [1], DPC [1], SDS [1]	Rath et al. (2011)
134 Ec-MPs	≤112 (≤15)	–	iE [3]	SDS [0.25], LPPG [0.25], LMPC [0.25], DPC [0.1]	Schwarz et al. (2010)

Table 2.2 (continued)

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] yield[e]	Solubilization[f] (%)	Reference
hETA/B	49/50 (7)	GPCR/+	iE [3]	LPPG [1], LMPC [1], SDS [1], Fos16 [1], Fos12 [1]	Junge et al. (2010)
Ec-EmrE	11 (4)	Transporter/+	iE [3]	DDM [2], DPC [1], LMPG	Klammt et al. (2004)
Ec-TehA	36 (10)				
Ec-SugE	11 (4)				
rOCT1/2	55 (12)	Transporter/+	iE [2]	LMPG [1]	Keller et al. (2008, 2011)
rOAT-1	60 (12)				
MPs of diverse origin	Diverse	Diverse	iE [3]	e.g. LMPG, DPC	Schwarz et al. (2007)
Proteorhodopsin	27 (7)	H^+-Pump/+	cE [2]	e.g. LMPG [0.01], LPPG [0.025]	Gourdon et al. (2008)
Bacteriorhodopsin *E. sibiricum*	28/(7)	H^+-Pump/+	iE	SDS, refolding	Shenkarev et al. (2013)
hH1R	56 (7)	GPCR/+	cE [1]	DDM [2]	Sansuk et al. (2008)
~120 MPs	10–30 (1–9)	Diverse	iE	nOG, DDM	Savage et al. (2007)
LH1-α-apoprotein *R. rubrum*	5 (1)	Light harvesting/+	cE [2]	Triton-X100 [0.5–2]	Shimada et al. (2004)
>100 hMPs	8–134	Diverse	cE/cWG	–	Langlais et al. (2007)

DDM n-dodecyl-β-D-maltoside, *DHPC* 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine, *DPC*=Fos12 n-dodecylphosphocholine, *Fos14* n-tetradecylphosphocholine, *Fos16* n-hexadecylphosphocholine, *LDAO* lauryldimethylamine oxide, *LMPC* 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac(1-choline)], *LMPG* 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)], *LPPG* 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)], *LS* n-lauroyl sarcosine, *nOG* n-octyl-β-D-glucopyranoside, *Nvoy* NV10 polymer, *SDS* sodium dodecylsulfate

[a] Approximate size, if documented, the origin of proteins is given in italics; *h* human; *m* murine; *r* rat; *Ec E. coli*, *Bs Bacillus subtilis*

[b] *TMH* Proposed number of transmembrane helices

[c] +: Quality analyzed by structural evaluation or functionality

[d] *iE* individual *E. coli* extract, *cE* commercial *E. coli* extract; *cWG* commercial wheat germ extract

[e] Approximate yields per one ml RM if documented in the corresponding references. 1: ≤0.1 mg/ml; 2: 0.1–1 mg/ml; 3: >1 mg/ml

[f] Detergents and concentrations used for posttranslational solubilization. Exchange into secondary detergents are indicated by slash. Concentrations are given if documented

Klammt et al. 2011). Solubilization of P-CF pellets is fast and usually complete after gentle shaking for approximately 1 h. It should be noted that pellets of CF-expressed soluble proteins cannot usually be solubilized by that procedure as they are much more unstructured. Consequently, MPs having excessive soluble domains could therefore resist solubilization out of P-CF pellets.

Selecting the P-CF expression mode is the fastest approach and usually routinely employed for the first level of MP expression protocol development in order to tune protein production up to the desired yields (Junge et al. 2011). Even complex MPs such as 12 transmembrane segment containing eukaryotic ion transporters or the 10 transmembrane segment containing MraY translocase have been functionally synthesized in the P-CF mode (Keller et al. 2008; Ma et al. 2011). The P-CF mode is furthermore excellent for screening MP libraries (Langlais et al. 2007; Savage et al. 2007; Schwarz et al. 2010) and for the production of MP samples for structural analysis by NMR (Klammt et al. 2004; Maslennikov et al. 2010; Rath et al. 2011; Sobhanifar et al. 2010).

2.5 Production of Proteomicelles: D-CF Expression

CF expression systems can tolerate a considerable number of supplied hydrophobic compounds, while certain variations in between the different extract sources exist. The CF expression in the presence of detergents above their critical micellar concentration (CMC) can result into the co-translational solubilization of the expressed MPs and into the instant formation of proteomicelles (Fig. 2.1).

Extensive evaluation of detergent tolerance has been performed with *E. coli* extracts (Blesneac et al. 2012; Gourdon et al. 2008; Klammt et al. 2005; Lyukmanova et al. 2012) as well as with wheat germ extract systems (Beebe et al. 2011; Genji et al. 2010; Kaiser et al. 2008; Periasamy et al. 2013). As primary compounds of choice, long-chain polyoxyethylene-alkyl-ethers such as Brij35, Brij58, Brij78 or Brij98, and the steroid-derivative digitonin have been determined (Table 2.3). These detergents have been successfully used for the solubilization of different G protein-coupled receptors (GPCRs) as well as of prokaryotic MPs (Table 2.3). Commonly employed detergents for the extraction of MPs out of native membranes such as DPC, the alkyl-glucoside n-dodecyl-β-D-maltoside (DDM), or n-octyl-β-D-glucopyranoside (β-OG) are too harsh or only tolerated at lower concentrations. However, the tolerance can sometimes be increased if critical detergents are provided as mixed micelles together with other detergents, e.g., CHAPS together with Fos-choline derivatives (Genji et al. 2010). It might generally be advantageous to combine the provided detergent micelles with some small amounts of lipids in case the translated MPs require interaction with some lipids for stabilization (Arslan Yildiz et al. 2013; Müller-Lücks et al. 2013; Nozawa et al. 2007). For the expression of mitochondrial carrier proteins, the addition of some cardiolipin together with fluorinated surfactants or Brij35 detergent had significant beneficial effects, whereas cardiolipin had negative effects in combination with the detergent Brij58

Table 2.3 Case studies of D-CF-expressed MPs

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] Yield[e]	Detergent[f]	Reference
Pores and Channels					
Aqp3	32 (6)	Porin/+	iE [3]	Brij98 + Ec polar lipids	Müller-Lucks et al. (2013)
Cx32	32 (4)	Channel/+	cE [2]	Brij35	Nguyen et al. (2010)
VDAC	36 (1 + 13ß)				
hVDAC1	35 (13ß)	Channel/+	cE [1]	DDM, Fos12	Deniaud et al. (2010)
mAqp4	30 (6)	Porin/+	iE [3]	Brij35, Digitonin	Kai et al. (2010)
Ec-MscL	15 (2)	Channel/+	cE [3]	Triton X-100	Berrier et al. (2004); Abdine et al. (2010)
OEP24	24 (12ß)	Channel/+	cE [2]	DDM	Liguori et al. (2010)
PorA/H *C. glutamicum*	5	Channel/+	iE [2]	Brij72	Rath et al. (2011)
hERG	25 (6)	Channel/+	iE [2]	Brij78 + soybean PC	Arslan Yildiz et al. (2013)
Transporters and pumps					
UCP1	30–35 (6)	Carrier/+	cE [2]	Brij35/58, DDM, digitonin, fluorinated surfactants + cardiolipin	Blesneac et al. (2012)
Ec-EmrE	12 (4)	Transporter/+	cE [3]	DDM	Elbaz et al. (2004)
AtPPT1, OpPPT1/2/3	30 (8)	Transporter/+	cE	Brij35 + Asolectin	Nozawa et al. (2007)
Bacteriorhodopsin	28 (7)	H^+-Pump/+	cE [2]	NaPol	Bazzacco et al. (2012)
Bacteriorhodopsin	28 (7)	H^+-Pump/+	WG	Chaps + Fos12, Fos14	Genji et al. (2010)
Bacteriorhodopsin	28 (7)	H^+-Pump/+	WG	Chaps, Fos12	Beebe et al. (2011)
Ec-Tsx	34 (12ß)	Transporter/–	iE [3]	Brij78	Klammt et al. (2005)
Receptors					
Dopamine D2	50 (7)	GPCR/+	iE WG	–	Basu et al. (2013)
hTAAR-T4L	45 (7)	GPCR/+	iE [2]	Brij35	Wang et al. (2013)
hETA, hETB	~45 (7)	GPCR/+	iE [3]	Brij35/78	Junge et al. (2010)
Olfactory Receptors, hFPR3, hVN1R1, hVN1R5	~30 (7)	GPCR/+	cE [2]	Brij35, peptide surfactants	Corin et al. (2011)
Cytokinin Receptor CRE1/AHK4	37 (2)	Receptor/+	iE [3]	Brij58/78	Wulfetange et al. (2011)
CpxA	50 (2)	Receptor/+	cE [3]	Brij35	Miot and Betton (2011)

Table 2.3 (continued)

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] Yield[e]	Detergent[f]	Reference
OR17-4	36 (7)	GPCR/+	WG, cE [2]	Digitonin	Kaiser et al. (2008)
hMTNR1B, hNPY4R, rCRF, hV2R	~40 (7)	GPCR/−	iE [2]	Brij58/78	Klammt et al. (2007)
hCHRM2, hβ2AR, hNTR	~60 (7)	GPCR/+	iE [2]	Brij35, digitonin	Ishihara et al. (2005)
ARII	18 (7)	Receptor/+	cE [1]	Digitonin + PC	Wada et al. (2011)
hOR17-210, mOR103-15, hFPR3, hTAAR5	~40 (7)	GPCR/+	iE [2]	Peptide surfactants	Wang et al. (2011)
hCRF1, CRF2ß	(7)	GPCR/+	iE	Nvoy	Klammt et al. (2011)
Enzymes					
Bs-MraY	36 (10)	Translo-case/+	iE [3]	Brij35	Ma et al. (2011)
Bcl-2	25 (1)	Anti-apoptotic protein/+	iE [2]	Brij58	Pedersen et al. (2011)
ATP synthase	542	Multisub-unit com-plex/+	iE [1]	Brij58	Matthies et al. (2011)
CrdS	70 (7)	Enzyme/+	WG [1]	Brij58, peptide surfactants	Periasamy et al. (2013)
Bs-DesK	~40 (4–5)	Histidine kinase/+	cE [3]	Brij58, digitonin, Triton X-100	Martin et al. (2009)
Diverse					
>100 Ec-MPs	(<15)	Diverse	iE [3]	Brij35/58/78/98	Schwarz et al. (2010)
TM-ErbB3	5 (1)	Receptor/+	iE [3]	Brij35/58/78/98	Lyukmanova et al. (2012)
VSD-KvaP	(4)	Channel		Triton X-100, DDM	
Bacteriorhodop-sin	28 (7)	H^+-Pump/+			

Brij35 polyoxyethylene-(23)-lauryl-ether, *Brij58* polyoxyethylene-(20)-cetyl-ether, *Brij72* Polyoxyethylene-(2)-stearyl-ether, *Brij78* polyoxyethylene-(20)-stearyl-ether, *Brij98* polyoxyethylene-(20)-oleyl-ether, *CHAPS* 3-[(3-Cholamidopropyl) dimethylammonio]-1-propansulfonat, *DDM* n-dodecyl-β-D-maltoside, *Fos12* n-dodecylphosphocholine, *Fos14* n-tetradecylphosphocholine, *NaPol* Nonionic amphipols, *Nvoy* NV10 polymer, *PC* L-α-phosphatidylcholine

[a] If documented, the origin of proteins is given in italics; *h* human, *m* murine, *r* rat, *Ec E. coli*, *Bs Bacillus subtilis*

[b] TMH: Number of transmembrane helices or β-sheets (β)

[c] +: Quality analyzed by structural evaluation or functionality

[d] *iE* individual *E. coli* extracts, *cE* commercial *E. coli* extracts, *WG* wheat germ extracts

[e] Approximate yields per 1 ml RM if documented in the corresponding references. 1: ≤0.1 mg/ml; 2: 0.1–1 mg/ml; 3: >1 mg/ml

[f] Main detergents used for co-translational solubilization. Concentrations are given if documented

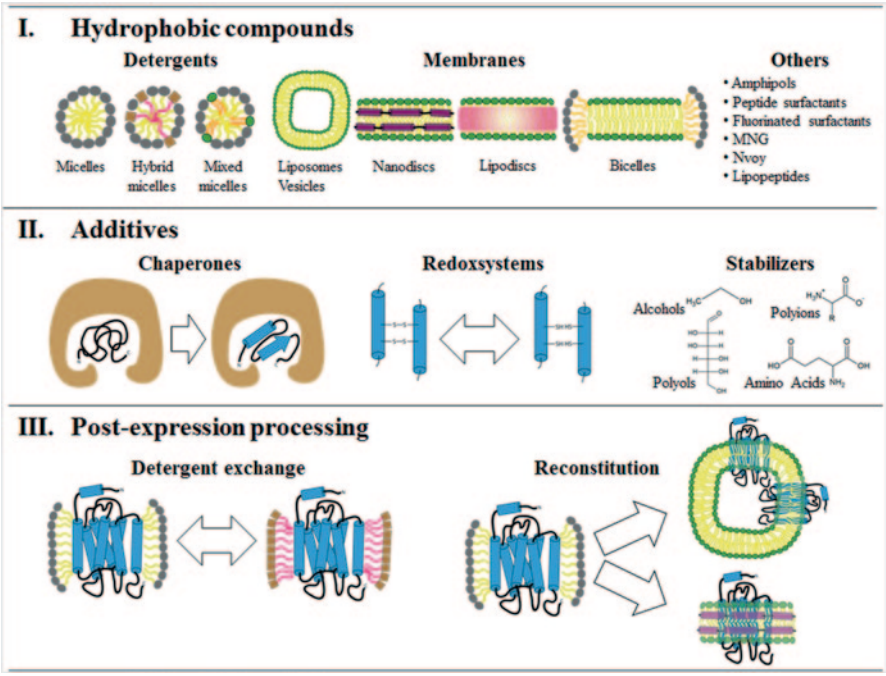

Fig. 2.2 Supplements for the co-translational and posttranslational modification of MP sample quality

(Blesneac et al. 2012). The nature or composition of the selected detergent or detergent mixture can certainly affect the efficiency of solubilization as well as the MP quality in view of folding and stability. Systematic screens for detergent type and concentration are therefore necessary in order to determine optimal conditions for each individually expressed MP (Ishihara et al. 2005; Klammt et al. 2005; Liguori et al. 2008; Martin et al. 2009; Rath et al. 2011).

The open nature of CF reactions has initiated searches for further and new hydrophobic compounds with improved properties that could substitute classical detergents in co-translational MP solubilization (Fig. 2.2). Fluorinated surfactants as well as phospholipid-like surfactants have been considered as mild hydrophobic supplements in D-CF reactions (Park et al. 2011; Blesneac et al. 2012). Amphipols and in particular the charged A8-35 derivative are not well tolerated but might be interesting as solubilizing agents for P-CF-generated MP precipitates. The polyfructose-based uncharged polymer NV10 was claimed to be beneficial in supporting the solubilization of several class B GPCRs (Klammt et al. 2011). Similar positive effects on GPCR solubilization as described for Brij detergents have been observed with peptide surfactants as D-CF supplements (Wang et al. 2011; Corin et al. 2011; Table 2.3). However, curdlan synthase was inactive if D-CF expressed in the presence of Brij58, but active if the detergent was replaced by peptide surfactants (Periasamy et al. 2013). Other compounds such as the recently described

maltose-neopentyl glycol amphiphiles might be considered in future as well (Chae et al. 2010). Although the general availability of several compounds is still somehow limited, it is evident that the variety for designing artificial hydrophobic environments in D-CF reactions is rapidly increasing.

2.6 Designing Protein/Membrane Complexes: L-CF Expression

CF extracts are almost devoid of membranes, although some residual small vesicles originating from the cell membranes might be present after S30 preparation. More complete removal of membrane fragments can be achieved by S100 (centrifugation at 100,000 × g) extract preparation (Berrier et al. 2011). Instead of complex cell membranes, vesicles containing selected lipid compositions can be prepared *in vitro* and provided into the CF reactions. Lipids are, in contrast to detergents, much better tolerated by CF systems and mostly high final concentrations are possible (Kalmbach et al. 2007; Hovijitra et al. 2009; Roos et al. 2013; Umakoshi et al. 2009). This L-CF (lipid membrane-based) expression mode can thus facilitate the co-translational association of the expressed MPs with supplied bilayers provided as liposomes, as bicelles in combination with specific detergents or as planar membrane discs (Fig. 2.2). The L-CF mode is an excellent approach in order to evaluate lipid effects on the quality and activity of MPs (Table 2.4). Subsequent characterization of L-CF-generated MP samples can furthermore be performed in the natural context of membranes. The co-translational insertion might direct the synthesized MPs in a unidirectional inside-out orientation into the supplied membranes. This could be shown with connexins as example and thus more uniform samples can be generated if compared with conventional posttranslational reconstitution approaches (Moritani et al. 2010).

It should be realized that by selecting the L-CF expression mode, translocation problems of the expressed MP can become an issue again. For many MPs, complex translocation machineries are essential for their proper membrane insertion *in vivo* (Shao and Hedge 2011). The knowledge of translocation mechanisms of MPs in artificial L-CF systems is still at the very beginning. However, it is already evident that the dependency of MP insertion on translocation systems might not be as strict as *in vivo*. The membrane insertions of the CF-expressed channel MscL and of the MtlA permease were independent of the YidC insertase and of the SecYEG complex, respectively (Berrier et al. 2011; Nishiyama et al. 2006). Characteristics that could facilitate membrane insertion might be that (1) the provided membranes are empty, (2) the membrane concentration in CF reaction can be high, and (3) lipid compositions of the supplied membranes can specifically be modulated, e.g., increase of anionic lipids can improve MP insertion efficiencies (Roos et al. 2012). It is generally advisable to perform a lipid screening with individual MPs in order to determine the appropriate composition of supplied membranes in view of lipid charge, length, and flexibility. If translocation systems appear to be mandatory,

Table 2.4 Case studies of L-CF-expressed MPs

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] yield[e]	Lipid[f]	Reference
Liposomes/Vesicles					
VDAC	31 (19ß)	Channel/+	cE	Thylakoid vesicles	Liguori et al. (2008)
Ec-AqpZ	24 (6)	Channel/+	iE [2]	DOPC, POPG/E	Hovijitra et al. (2009)
OEP24	24 (7ß)	Channel/+	cE [2]	DOPC/PE/DMPA/cholesterol, Soybean asolectin	Liguori et al. (2010)
P. sativum					
Ec-MscL	15 (2)	Channel/+	cE	Soybean asolectin	Berrier et al. (2011)
Ec-OmpA	37 (8ß)	Pore	PURE [1]	*E. coli* INV	Kuruma et al. (2005)
PulD	68	Pore/+	cE [1]	Soybean asolectin	Guilvout et al. (2008)
K. oxytoca					
α-Hemolysin	33	Pore/+	iE	POPC	Chalmeau et al. (2011)
S. aureus					
Connexin-43	43 (4)	Pore/+	PURE [1]	DOPC/G	Moritani et al. (2010)
Ec-MtlA	70 (6)	Permease	PURE [1]	*E. coli* INV	Kuruma et al. (2005)
MtlA,	637 (6)	Permease	iE [2]	*E. coli* IMV	Wuu and Swartz (2008)
Ec-TetA	41 (12)	Transporter/+			
Ec-MtlA	70 (6)	Permease	iE	PL + DAG	Nishiyama et al. (2006)
BR	262 (7)	H^+-Pump/+	iE [1]	DOPC	Kalmbach et al. (2007)
Ec-FtsQ	31	Enzyme	PURE [1]	*E. coli* INV	Kuruma et al. (2005)
Δgp91	63 (6)	Enzyme/+	cE	MS: Spinach thylacoids	Marques et al. (2007)
hSCD1	40 (4)	Enzyme/+	cWG [2]	MS: Soybean tissue	Goren and Fox (2008)
TbSLS1-4	~30 (6)	Enzyme/+	cWG [2]	Soybean lecithin	Sevova et al. (2010)
T. brucei					
Ec-MraY	40 (10)	Enzyme/+	iE [3]	Soybean PC, PL	Ma et al. (2011)
Bs-MraY					
Cyt B5	17 (1)	Enzyme	cWG	DOPC/egg PC	Nomura et al. (2008)
F_O subunit of ATP synthase	26	Enzyme	cE	Soybean PC	Kuruma et al. (2010)
3 L-Pf3	5 (1)	Coat protein	cE	*E. coli* INV + DAG	Kawashima et al. (2008)

Table 2.4 (continued)

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] yield[e]	Lipid[f]	Reference
hERG,	24 (6)	Channel/+	cE [1]	PC/DMPE	Yildiz et al. (2012)
Ec-Cyt-bo3 ubiquinol oxid.	35	Enzyme/+			
CrdS	75 (7)	Enzyme/+	iWG [2]	DOPG/E, *E. coli* lipids	Periasamy et al. (2013)
Agrobacterium					
Proteorhodopsin	27 (7)	H^+-Pump/+	iE [2]	DMPC/PA	Mörs et al. (2013)
Bacteriorhodopsin	28 (7)	H^+-Pump/+	cE [2]	DMPC	Cappuccio et al. (2008)
Bs-DesK	40 (4–5)	Histidine kinase/+	cE [3]	DOPC	Martin et al. (2009)
40 mammalian MPs	32–130 (1–14)	Diverse/+	cWG [1]	Soybean asolectin	Nozawa et al. (2011)
Connexin-43	43 (4)	Pore/+	RRL	Egg PC	Kaneda et al. (2009)
Lipid+Detergent					
AtPPT1	~30	Transporter/+	cWG	Soybean asolectin + Brij35	Nozawa et al. (2007)
Bacteriorhodopsin	28 (7)	H^+-Pump/+	iE [2]	Egg PC + CHAPS/Cholesterol/ Digitonin	Shimono et al. (2009)
ATP synthase (a + c subunits)	23 + 11	Complex/+	cE [1]	DHPC, DMPC	Uhlemann et al. (2012)
Nanodiscs/lipoprotein complexes					
Ec-EmrE	12 (4)	Transporter/+	cE [2]	apoA1 (DMPC)	Katzen et al. (2008)
Bacteriorhodopsin	28 (7)	H^+-Pump/+	cWG		
32 diverse MPs			RRL		
hTM-ErbB3	5 (1)	Receptor	iE [3]	MSP1 (DMPC/G/POPC, DOPG)	Lyukmanova et al. (2012)
VSD-KvaP	28 (7)	Channel			
Bacteriorhodopsin		H^+-Pump/+			
E. sibiricum					
hß2-AR-T4L	63 (7)	GPCR/+	cE	apoA1 (DMPC)	Yang et al. (2011)
hETB, hETA	–	GPCR/+	iE [2]	MSP1E3 (DMPC/G)	Proverbio et al. (2013)
npSRII (*N.pharaonis*)	26 (6)	Receptor	iE	apoA1 (DMPC)	Isaksson et al. (2012)
pR AND4	32 (7)	H^+-Pump/+			

Table 2.4 (continued)

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] yield[e]	Lipid[f]	Reference
Ec-MraY	40 (10)	Enzyme/+	iE [3]	MSP1E3 (DMPC/G, POPC/G, DOPC/G/E, TL, PL)	Roos et al. 2012
Proteorhodopsin	27 (7)	H^+-Pump/+			
Proteorhodopsin	27 (7)	H^+-Pump/+	iE [2]	MSP1 (DMPC, POPC)	Mörs et al. (2013)
Bacteriorhodopsin *H. halobium*	28 (7)	H^+-Pump/+	cE	–	Cappuccio et al. (2009)
ADRB2, DRD1, NK1R	(7)	GPCR/+	cE	Δ49A1 (DMPC)	Gao et al. (2012)

MS microsomes, *IMV* inner membrane vesicles, *OMV* outer membrane vesicles, *INV* inverted vesicles, *Brij35* polyoxyethylene-(23)-lauryl-ether, *CHAPS* 3-[(3-Cholamidopropyl) dimethylammonio]-1-propansulfonat, *DAG* diacylglycerol, *DHPC* 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine, *DMPA* 1,2-ditetradecanoyl-sn-glycero-3-phosphate, *DMPC* 1,2-dimyristoyl-sn-glycero-3-phosphocholine, *DMPE* 1,2-ditetradecanoyl-sn-glycero-3-phosphoethanolamine, *DMPG* 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol), *DOPA* 1,2-dioleoyl-sn-glycero-3-phosphate, *DOPC* 1,2-dioleoyl-sn-glycero-3-phosphocholine, *DOPE* 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, *DOPG* 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol), *PC* L-α-phosphatidylcholine, *PE* L-α-phosphatidylethanolamine, *PL* polar lipid extract, *POPC* 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, *POPE* 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, *POPG* 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol), *TL* total lipid extract

[a] If documented, the origin of proteins is given in italics; *h* human, *m* murine, *r* rat, *Ec E. coli, Bs Bacillus subtilis*

[b] Size and number of transmembrane helices or ß-sheets (ß), if documented

[c] +: Quality analyzed by structural evaluation or functionality

[d] *iE* individual *E. coli* extracts, *cE* commercial *E. coli* extracts, *iWG* individual wheat germ extracts, *cWG* commercial wheat germ extracts, *PURE* protein synthesis using recombinant elements, *RRL* rabbit reticulocyte extract

[e] Approximate yields per 1 ml RM if documented in the corresponding references. 1:≤0.1 mg/ml; 2: 0.1–1 mg/ml; 3:>1 mg/ml

[f] Lipids or lipid mixtures used for co-translational reconstitution

vesicles isolated out of complex cell membranes and containing natural translocation machineries could be considered as supplements as well (Stech et al. 2012).

L-CF expression in the presence of nanodiscs appears to be in particular promising in order to obtain soluble and functionally active MP/membrane complexes (Cappuccio et al. 2009; Lyukmanova et al. 2012; Proverbio et al. 2013; Roos et al. 2012). Specific advantages might be that nanodisc membranes are accessible from both sides and it could be speculated that inhomogeneities of the membrane/membrane scaffold protein interface may provide additional entry sides for MP integration. MP/nanodisc complexes are highly soluble and can be used for a variety of applications such as surface plasmon resonance (SPR) measurements (Proverbio et al. 2013). Nanodiscs provide furthermore a membrane compartment that is stable in size and topology. In contrast, liposomes supplied to CF reactions show excessive fusion and increase in size, resulting into their almost quantitative precipitation during the reaction (Barrier et al. 2011; Roos et al. 2013). An interesting modification by using the CECF configuration is the initial supply of a lipid/detergent mixture in the RM, whereas the FM is devoid of any detergent. Freshly translated MPs can therefore first physically associate with the provided lipomicelles and become increasingly trapped into membranes that slowly formed by the continuously decreased detergent concentration in the RM. This method was successfully applied for bacteriorhodopsin with combinations of steroid detergents and phosphatidylcholine lipids (Shimono et al. 2009). In a similar approach, the plant solute transporter AtPPT1 was functionally reconstituted by using a mixture of Brij35 and soybean asolectin lipids (Nozawa et al. 2007).

Besides hydrophobic environments, a variety of further additives could be beneficial for the production of high quality MP samples (Fig. 2.2). Chemical chaperones such as sugars, alcohols, or polyions are tolerated by CF systems and synergies of several compounds could be determined in correlated concentration screens (Kai et al. 2013). Such additives might be in particular beneficial for supporting the folding of larger soluble domains of MPs.

2.7 Handling the Toolbox of CF Expression: Strategies for Protein Quality Optimization

During the last decade when CF expression as a new platform for MP production was emerging, the three basic modes P-CF, D-CF, and L-CF have been employed in more or less comparable frequencies. The variety of supplements useful for MP quality optimization is rapidly expanding and defining specific conditions for the production of sufficient MP quantities that are homogenous, functionally folded, and stable is generally the key issue in CF expression protocols. The systematic screening of (1) expression modes, (2) type and concentration of hydrophobic compounds, (3) additives such as stabilizer or chaperones and (4) post-expression processing procedures generate an array of MP samples that have to be analyzed (Fig. 2.2). A strategic plan for MP quality control is therefore crucial and the first evaluation

of solubility, sample homogeneity, protein integrity, stability, or specific activity should be implemented as soon as possible, at best already in the crude reactions. The above mentioned GFP fusion approach can, e.g., provide already a first short list of compounds and compound combinations giving an efficient solubilization of the MP. Enzymatic reactions or binding of labeled ligands might be considered as well in specific cases (Gourdon et al. 2008; Kalmbach et al. 2007). Solubility and integrity can be assayed with immunoblotting by taking advantage of terminal tags. Rare occurring MP fragmentation generated by premature translational termination or proteolytic degradation could be addressed by using synthetic genes and by screening protease inhibitors. In the case of some GPCRs, ligand-binding assays by SPR measurement can be performed with crude RMs containing the expressed MP (Proverbio et al. 2013). Feedback from first quality evaluations should then be considered for re-optimization, fine-tuning of compound concentrations, and for analyzing cocktails of beneficial compounds for synergistic effects. In many cases, tremendous variations in the quality of MP samples produced at different CF conditions have been observed. A striking example is the MraY translocase, a membrane-embedded enzyme responsible for lipid-I precursor formation in the bacterial cell wall biosynthesis pathway. The *Bacillus subtilis* MraY can be functionally synthesized in a large variety of CF conditions implementing detergents and lipids. In contrast, the *E. coli* MraY enzyme was only functional if L-CF synthesized in the presence of nanodiscs containing anionic lipids (Roos et al. 2012; Ma et al. 2011).

If a short list of few promising reaction conditions has been determined, more time-consuming assays using purified samples such as size exclusion chromatography, multi-angle light scattering, or circular dichroism spectroscopy can be implemented in order to analyze homogeneity, folding, and oligomeric states of the MP. Functionality of MPs is often more difficult to analyze and currently available case studies are compiled in Tables 2.2–2.4. As some examples, ligand binding of GPCRs and transporters were shown by radioassays (Gao et al. 2012; Ishihara et al. 2005; Sansuk et al. 2008; Yang et al. 2011), fluorescence anisotropy measurement (Junge et al. 2010), SPR (Kaiser et al. 2008; Proverbio et al. 2013), or thermophoresis (Corin et al. 2011). Functional samples of channels and transporters after co-translationally or posttranslationally reconstitution into lipid bilayers were obtained from MscL (Berrier et al. 2004, 2011), EmrE (Elbaz et al. 2004), TetA (Wuu and Swartz 2008), PorA and PorH (Rath et al. 2011), eukaryotic organic ion transporters (Keller et al. 2008, 2011), as well as from aquaporins (Hovijitra et al. 2009; Kai et al. 2010; Müller-Lucks et al. 2013).

2.8 Perspectives for Structural Approaches

The possibility to produce pure and concentrated samples of even very difficult MPs in a short time by CF expression is certainly of major interest for structural studies. Efficient incorporation of selenomethionine is of value for X-ray crystallography. In addition, MPs could already co-translationally be stabilized by inhibitors or other ligands. X-ray structures of CF-expressed MPs are still limited to the multidrug transporter EmrE (Chen et al. 2007) and *Acetabularia* proteorhodopsin

(Wada et al. 2011) synthesized in a mixture of detergents and lipids. Crystallization was furthermore successful with the CF-expressed human voltage-gated anion channel VDAC1 (Nguyen et al. 2010; Deniaud et al. 2010). Despite some success, the still relatively low number of X-ray structures derived from CF-expressed MPs indicates that reaction conditions obviously may often not have been optimal in order to obtain crystallization grade MP samples. Systematic case studies are needed in order to identify the key parameters to be considered by choosing CF expression approaches. Sample homogeneity might be optimized by (1) intensive screening of reaction modes and hydrophobic supplements, (2) providing detergents in cocktails with some stabilizing lipids, (3) adjusting redox conditions for proper disulfide bridge formation, and (4) fine-tuning of solubilization conditions of P-CF samples.

The efficient and cost-effective labeling opportunities of CF expression are excellent prerequisites for structural approaches by NMR spectroscopy and a variety of sophisticated labeling tools and schemes have been developed and are already established standards (Klammt et al. 2012; Reckel et al. 2008; Ozawa et al. 2006). Nevertheless, liquid-state NMR of MPs is still a challenging task. MPs in micellar as well as in lipid environment are prone to signal broadening due to the large size and slow rotational tumbling. Furthermore, amino acids in α-helical structures tend to display narrow-range chemical shifts, resulting in severe peak overlaps. Numerous liquid-state NMR structures of P-CF (Klammt et al. 2012; Maslennikov et al. 2010; Sobhanifar et al. 2010) or D-CF (Reckel et al. 2008) expressed MPs have been reported (see also chapter by L. Catoire and D. Warschawski in this volume). For solid-state NMR, P-CF precipitates, samples posttranslationally reconstituted into liposomes, or L-CF samples by additions of liposomes or nanodiscs can be used (see also chapter by L. Catoire and D. Warschawski in this volume). Examples of successfully analyzed MPs are the mechanosensitive channel MscL (Abdine et al. 2010) and the multidrug transporter EmrE (Lehner et al. 2008). In particular, L-CF expression in the presence of nanodiscs can become attractive as shown in initial studies with proteorhodopsin (Mörs et al. 2013).

2.9 Conclusions

Besides exploring new approaches in particular for drug screening or single-molecule approaches, some major current challenges are the scale-up of CF reactions to industrial dimensions, the streamlined determination of expression conditions for crystallization grade MP samples, and the production of larger assemblies and MP complexes. Initial milestones have already been achieved and give promising perspectives. The manufacturing of multigram to kilogram scales in 100 L or even higher CF reaction volumes appears to become feasible (Zawada et al. 2011). Providing complex and more elaborated hydrophobic expression environments might be a direction for producing homogeneous samples suitable for crystallization (Wada et al. 2011). Tuning expression and template design might be a prerequisite for successful synthesis and assembly of MP complexes as recently been shown for the 542-kDa ATP synthase complex (Matthies et al. 2011).

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References

Abdine A, Verhoeven MA, Park K-H, Ghazi A, Guittet E, Berrier C, Van Heijenoort C, Warschawski DE (2010) Structural study of the membrane protein MscL using cell-free expression and solid-state NMR. J Magn Reson 204:155–159

Ahn J-H, Chu H-S, Kim T-W, Oh I-S, Choi C-Y, Hahn G-H, Park C-G, Kim D-M (2005) Cell-free synthesis of recombinant proteins from PCR-amplified genes at a comparable productivity to that of plasmid-based reactions. Biochem Biophys Res Commun 338:1346–1352

Ahn J-H, Hwang M-Y, Lee K-H, Choi C-Y, Kim D-M (2007) Use of signal sequences as an in situ removable sequence element to stimulate protein synthesis in cell-free extracts. Nucleic Acids Res 35:e21

Arduengo M, Schenborn E, Hurst R (2007) The role of cell-free rabbit reticulocyte expression systems in functional proteomics. In: Kudlicki WA, Katzen F, Bennett RP (eds) Cell-free protein expression, Landes Bioscience, Austin, pp 1–18

Arslan Yildiz A, Kang C, Sinner E-K (2013) Biomimetic membrane platform containing hERG potassium channel and its application to drug screening. Analyst 138:2007–2012

Basu D, Castellano JM, Thomas N, Mishra RK (2013) Cell-free protein synthesis and purification of human dopamine D2 receptor long isoform. Biotechnol Prog 29:601–608

Bazzacco P, Billon-Denis E, Sharma KS et al (2012) Nonionic homopolymeric amphipols: application to membrane protein folding, cell-free synthesis, and solution nuclear magnetic resonance. Biochemistry 51:1416–1430

Beebe ET, Makino S-I, Nozawa A, Matsubara Y, Frederick RO, Primm JG, Goren MA, Fox BG (2011) Robotic large-scale application of wheat cell-free translation to structural studies including membrane proteins. N Biotechnol 28:239–249

Berrier C, Park K-H, Abes S, Bibonne A, Betton J-M, Ghazi A (2004) Cell-free synthesis of a functional ion channel in the absence of a membrane and in the presence of detergent. Biochemistry 43:12585–12591

Berrier C, Guilvout I, Bayan N, Park K-H, Mesneau A, Chami M, Pugsley AP, Ghazi A (2011) Coupled cell-free synthesis and lipid vesicle insertion of a functional oligomeric channel MscL MscL does not need the insertase YidC for insertion in vitro. Biochim Biophys Acta 1808:41–46

Blesneac I, Ravaud S, Juillan-Binard C, Barret L-A, Zoonens M, Polidori A, Miroux B, Pucci B, Pebay-Peyroula E (2012) Production of UCP1 a membrane protein from the inner mitochondrial membrane using the cell free expression system in the presence of a fluorinated surfactant. Biochim Biophys Acta 1818:798–805

Cappuccio JA, Blanchette CD, Sulchek TA et al (2008) Cell-free co-expression of functional membrane proteins and apolipoprotein, forming soluble nanolipoprotein particles. Mol Cell Proteomics 7:2246–2253

Cappuccio JA, Hinz AK, Kuhn EA et al (2009) Cell-free expression for nanolipoprotein particles: building a high-throughput membrane protein solubility platform. Methods Mol Biol 498:273–296

Chae PS, Rasmussen SGF, Rana RR et al (2010) Maltose-neopentyl glycol (MNG) amphiphiles for solubilization, stabilization and crystallization of membrane proteins. Nat Methods 7:1003–1008

Chalmeau J, Monina N, Shin J, Vieu C, Noireaux V (2011) α-Hemolysin pore formation into a supported phospholipid bilayer using cell-free expression. Biochim Biophys Acta 1808:271–278

Chen Y-J, Pornillos O, Lieu S, Ma C, Chen AP, Chang G (2007) X-ray structure of EmrE supports dual topology model. Proc Natl Acad Sci U S A 104:18999–19004

Corin K, Baaske P, Ravel DB et al (2011) Designer lipid-like peptides: a class of detergents for studying functional olfactory receptors using commercial cell-free systems. PLoS One 6:e25067. doi:10.1371/journal.pone.0025067

Craig D, Howell MT, Gibbs CL, Hunt T, Jackson RJ (1992) Plasmid cDNA-directed protein synthesis in a coupled eukaryotic in vitro transcription-translation system. Nucleic Acids Res 20:4987–4995

Deniaud A, Liguori L, Blesneac I, Lenormand JL, Pebay-Peyroula E (2010) Crystallization of the membrane protein hVDAC1 produced in cell-free system. Biochim Biophys Acta 1798:1540–1546

Elbaz Y, Steiner-Mordoch S, Danieli T, Schuldiner S (2004) In vitro synthesis of fully functional EmrE, a multidrug transporter, and study of its oligomeric state. Proc Natl Acad Sci U S A 101:1519–1524

Endo Y, Sawasaki T (2005) Advances in genome-wide protein expression using the wheat germ cell-free system. Methods Mol Biol 310:145–167

Endoh T, Kanai T, Imanaka T (2008) Effective approaches for the production of heterologous proteins using the *Thermococcus kodakaraensis*-based translation system. J Biotechnol 133:177–182

Ezure T, Suzuki T, Shikata M, Ito M, Ando E, Nishimura O, Tsunasawa S (2007) Expression of proteins containing disulfide bonds in an insect cell-free system and confirmation of their arrangements by MALDI-TOF MS. Proteomics 7:4424–4434

Ezure T, Suzuki T, Shikata M, Ito M, Ando E (2010) A cell-free protein synthesis system from insect cells. Methods Mol Biol 607:31–42

Gao T, Petrlova J, He W, Huser T, Kudlick W, Voss J, Coleman MA (2012) Characterization of de novo synthesized GPCRs supported in nanolipoprotein discs. PLoS One 7:e44911

Genji T, Nozawa A, Tozawa Y (2010) Efficient production and purification of functional bacteriorhodopsin with a wheat-germ cell-free system and a combination of Fos-choline and CHAPS detergents. Biochem Biophys Res Commun 400:638–642

Goerke AR, Swartz JR (2008) Development of cell-free protein synthesis platforms for disulfide bonded proteins. Biotechnol Bioeng 99:351–367

Goren MA, Fox BG (2008) Wheat germ cell-free translation, purification, and assembly of a functional human stearoyl-CoA desaturase complex. Protein Expr Purif 62:171–178

Gourdon P, Alfredsson A, Pedersen A et al (2008) Optimized in vitro and in vivo expression of proteorhodopsin: a seven-transmembrane proton pump. Protein Expr Purif 58:103–113

Guarino C, DeLisa MP (2012) A prokaryote-based cell-free translation system that efficiently synthesizes glycoproteins. Glycobiology 22:596–601

Guilvout I, Chami M, Berrier C, Ghazi A, Engel A, Pugsley AP, Bayan N (2008) In vitro multimerization and membrane insertion of bacterial outer membrane secretin PulD. J Mol Biol 382:13–23

Haberstock S, Roos C, Hoevels Y, Dötsch V, Schnapp G, Pautsch A, Bernhard F (2012) A systematic approach to increase the efficiency of membrane protein production in cell-free expression systems. Protein Expr Purif 82:308–316

Hovijitra NT, Wuu JJ, Peaker B, Swartz JR (2009) Cell-free synthesis of functional aquaporin Z in synthetic liposomes. Biotechnol Bioeng 104:40–49

Isaksson L, Enberg J, Neutze R, Göran Karlsson B, Pedersen A (2012) Expression screening of membrane proteins with cell-free protein synthesis. Protein Expr Purif 82:218–225

Ishihara G, Goto M, Saeki M, Ito K, Hori T, Kigawa T, Shirouzu M, Yokoyama S (2005) Expression of G protein coupled receptors in a cell-free translational system using detergents and thioredoxin-fusion vectors. Protein Expr Purif 41:27–37

Junge F, Luh LM, Proverbio D, Schäfer B, Abele R, Beyermann M, Dötsch V, Bernhard F (2010) Modulation of G-protein coupled receptor sample quality by modified cell-free expression protocols: a case study of the human endothelin A receptor. J Struct Biol 172:94–106

Junge F, Haberstock S, Roos C, Stefer S, Proverbio D, Dötsch V, Bernhard F (2011) Advances in cell-free protein synthesis for the functional and structural analysis of membrane proteins. N Biotechnol 28:262–271

Kai L, Kaldenhoff R, Lian J, Zhu X, Dötsch V, Bernhard F, Cen P, Xu Z (2010) Preparative scale production of functional mouse aquaporin 4 using different cell-free expression modes. PLoS One 5:e12972

Kai L, Dötsch V, Kaldenhoff R, Bernhard F (2013) Artificial environments for the co-translational stabilization of cell-free expressed proteins. PLoS One 8:e56637

Kaiser L, Graveland-Bikker J, Steuerwald D, Vanberghem M, Herlihy K, Zhang S (2008) Efficient cell-free production of olfactory receptors: detergent optimization, structure, and ligand binding analyses. Proc Natl Acad Sci U S A 105:15726–15731

Kalmbach R, Chizhov I, Schumacher MC, Friedrich T, Bamberg E, Engelhard M (2007) Functional cell-free synthesis of a seven helix membrane protein: in situ insertion of bacteriorhodopsin into liposomes. J Mol Biol 371:639–648

Kaneda M, Nomura SM, Ichinose S, Kondo S, Nakahama K, Akiyoshi K, Morita I (2009) Direct formation of proteo-liposomes by in vitro synthesis and cellular cytosolic delivery with connexin-expressing liposomes. Biomaterials 30:3971–3977

Katzen F, Kudlicki W (2006) Efficient generation of insect-based cell-free translation extracts active in glycosylation and signal sequence processing. J Biotechnol 125:194–197

Katzen F, Chang G, Kudlicki W (2005) The past, present and future of cell-free protein synthesis. Trends Biotechnol 23:150–156

Katzen F, Fletcher JE, Yang J-P et al (2008) Insertion of membrane proteins into discoidal membranes using a cell-free protein expression approach. J Proteome Res 7:3535–3542

Kawashima Y, Miyazaki E, Müller M, Tokuda H, Nishiyama K (2008) Diacylglycerol specifically blocks spontaneous integration of membrane proteins and allows detection of a factor-assisted integration. J Biol Chem 283:24489–24496

Keller T, Schwarz D, Bernhard F, Dötsch V, Hunte C, Gorboulev V, Koepsell H (2008) Cell free expression and functional reconstitution of eukaryotic drug transporters. Biochemistry 47:4552–4564

Keller T, Egenberger B, Gorboulev V et al (2011) The large extracellular loop of organic cation transporter 1 influences substrate affinity and is pivotal for oligomerization. J Biol Chem 286:37874–37886

Khabibullina NF, Liukmanova EN, Kopeina GS, Shenkarev ZO, Arsen'ev AS, Dolgikh DA, Kirpichnikov MP (2010) [The development and optimization of coupled cell-free expression system for production of the transmembrane domain of the receptor tyrosine kinase ErbB3]. Bioorg Khim 36:654–660

Kigawa T, Yokoyama S (1991) A continuous cell-free protein synthesis system for coupled transcription-translation. J Biochem 110:166–168

Kim D-M, Swartz JR (2004) Efficient production of a bioactive, multiple disulfide-bonded protein using modified extracts of *Escherichia coli*. Biotechnol Bioeng 85:122–129

Klammt C, Löhr F, Schäfer B, Haase W, Dötsch V, Rüterjans H, Glaubitz C, Bernhard F (2004) High level cell-free expression and specific labeling of integral membrane proteins. Eur J Biochem 271:568–580

Klammt C, Schwarz D, Fendler K, Haase W, Dötsch V, Bernhard F (2005) Evaluation of detergents for the soluble expression of alpha-helical and beta-barrel-type integral membrane proteins by a preparative scale individual cell-free expression system. FEBS J 272:6024–6038

Klammt C, Schwarz D, Eifler N, Engel A, Piehler J, Haase W, Hahn S, Dötsch V, Bernhard F (2007) Reprint of "cell-free production of G protein-coupled receptors for functional and structural studies" [J Struct Biol 158 (2007) 482–493]. J Struct Biol 159:194–205

Klammt C, Perrin MH, Maslennikov I, Renault L, Krupa M, Kwiatkowski W, Stahlberg H, Vale W, Choe S (2011) Polymer-based cell-free expression of ligand-binding family B G-protein coupled receptors without detergents. Protein Sci 20:1030–1041

Klammt C, Maslennikov I, Bayrhuber M et al (2012) Facile backbone structure determination of human membrane proteins by NMR spectroscopy. Nat Methods 9:834–839

Kovtun O, Mureev S, Jung W, Kubala MH, Johnston W, Alexandrov K (2011) Leishmania cell-free protein expression system. Methods 55:58–64

Kralicek AV, Radjainia M, Mohamad Ali NAB, Carraher C, Newcomb RD, Mitra AK (2011) A PCR-directed cell-free approach to optimize protein expression using diverse fusion tags. Protein Expr Purif 80:117–124

Kuruma Y, Nishiyama K-I, Shimizu Y, Müller M, Ueda T (2005) Development of a minimal cell-free translation system for the synthesis of presecretory and integral membrane proteins. Biotechnol Prog 21:1243–1251

Kuruma Y, Suzuki T, Ueda T (2010) Production of multi-subunit complexes on liposome through an *E. coli* cell-free expression system. Methods Mol Biol 607:161–171

Langlais C, Guilleaume B, Wermke N, Scheuermann T, Ebert L, LaBaer J, Korn B (2007) A systematic approach for testing expression of human full-length proteins in cell-free expression systems. BMC Biotechnol 7:64

Lehner I, Basting D, Meyer B, Haase W, Manolikas T, Kaiser C, Karas M, Glaubitz C (2008) The key residue for substrate transport (Glu14) in the EmrE dimer is asymmetric. J Biol Chem 283:3281–3288

Liguori L, Marques B, Lenormand J-L (2008) A bacterial cell-free expression system to produce membrane proteins and proteoliposomes: from cDNA to functional assay. Curr Protoc Protein Sci, Chapter 5: Unit 5.22

Liguori L, Blesneac I, Madern D, Vivaudou M, Lenormand J-L (2010) Single-step production of functional OEP24 proteoliposomes. Protein Expr Purif 69:106–111

Lyukmanova EN, Shenkarev ZO, Khabibullina NF et al (2012) Lipid-protein nanodiscs for cell-free production of integral membrane proteins in a soluble and folded state: comparison with detergent micelles, bicelles and liposomes. Biochim Biophys Acta 1818:349–358

Ma Y, Münch D, Schneider T, Sahl H-G, Bouhss A, Ghoshdastider U, Wang J, Dötsch V, Wang X, Bernhard F (2011) Preparative scale cell-free production and quality optimization of MraY homologues in different expression modes. J Biol Chem 286:38844–38853

Madin K, Sawasaki T, Ogasawara T, Endo Y (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. Proc Natl Acad Sci U S A 97:559–564

Marques B, Liguori L, Paclet M-H, Villegas-Mendéz A, Rothe R, Morel F, Lenormand J-L (2007) Liposome-mediated cellular delivery of active gp91(phox). PLoS One 2:e856

Martín M, Albanesi D, Alzari PM, De Mendoza D (2009) Functional in vitro assembly of the integral membrane bacterial thermosensor DesK. Protein Expr Purif 66:39–45

Maslennikov I, Klammt C, Hwang E, Kefala G, Okamura M, Esquivies L, Mörs K, Glaubitz C, Kwiatkowski W, Jeon YH, Choe S (2010) Membrane domain structures of three classes of histidine kinase receptors by cell-free expression and rapid NMR analysis. Proc Natl Acad Sci U S A 107:10902–10907

Matthies D, Haberstock S, Joos F, Dötsch V, Vonck J, Bernhard F, Meier T (2011) Cell-free expression and assembly of ATP synthase. J Mol Biol 413:593–603

Mikami S, Masutani M, Sonenberg N, Yokoyama S, Imataka H (2006) An efficient mammalian cell-free translation system supplemented with translation factors. Protein Expr Purif 46:348–357

Mikami S, Kobayashi T, Masutani M, Yokoyama S, Imataka H (2008) A human cell-derived in vitro coupled transcription/translation system optimized for production of recombinant proteins. Protein Expr Purif 62:190–198

Mineev KS, Khabibullina NF, Lyukmanova EN, Dolgikh DA, Kirpichnikov MP, Arseniev AS (2011) Spatial structure and dimer—monomer equilibrium of the ErbB3 transmembrane domain in DPC micelles. Biochim Biophys Acta 1808:2081–2088

Miot M, Betton J-M (2011) Reconstitution of the Cpx signaling system from cell-free synthesized proteins. N Biotechnol 28:277–281. doi:10.1016/j.nbt.2010.06.012

Moritani Y, Nomura SM, Morita I, Akiyoshi K (2010) Direct integration of cell-free-synthesized connexin-43 into liposomes and hemichannel formation. FEBS J 277:3343–3352

Mörs K, Roos C, Scholz F, Wachtveitl J, Dötsch V, Bernhard F, Glaubitz C (2013) Modified lipid and protein dynamics in nanodiscs. Biochim Biophys Acta 1828:1222–1229

Müller-Lucks A, Bock S, Wu B, Beitz E (2012) Fluorescent in situ folding control for rapid optimization of cell-free membrane protein synthesis. PLoS One 7:e42186

Müller-Lucks A, Gena P, Frascaria D, Altamura N, Svelto M, Beitz E, Calamita G (2013) Preparative scale production and functional reconstitution of a human aquaglyceroporin (AQP3) using a cell free expression system. N Biotechnol. doi:10.1016/j.nbt.2013.03.007

Nakashima N, Tamura T (2004) Cell-free protein synthesis using cell extract of *Pseudomonas fluorescens* and CspA promoter. Biochem Biophys Res Commun 319:671–676

Nguyen TA, Lieu SS, Chang G (2010) An Escherichia coli-based cell-free system for large-scale production of functional mammalian membrane proteins suitable for X-ray crystallography. J Mol Microbiol Biotechnol 18:85–91

Nishiyama K, Ikegami A, Moser M, Schiltz E, Tokuda H, Müller M (2006) A derivative of lipid A is involved in signal recognition particle/SecYEG-dependent and -independent membrane integrations. J Biol Chem 281:35667–35676

Nomura S-IM, Kondoh S, Asayama W, Asada A, Nishikawa S, Akiyoshi K (2008) Direct preparation of giant proteo-liposomes by in vitro membrane protein synthesis. J Biotechnol 133:190–195

Nozawa A, Nanamiya H, Miyata T, Linka N, Endo Y, Weber APM, Tozawa Y (2007) A cell-free translation and proteoliposome reconstitution system for functional analysis of plant solute transporters. Plant Cell Physiol 48:1815–1820

Nozawa A, Ogasawara T, Matsunaga S, Iwasaki T, Sawasaki T, Endo Y (2011) Production and partial purification of membrane proteins using a liposome-supplemented wheat cell-free translation system. BMC Biotechnol 11:35

Ozawa K, Wu PSC, Dixon NE, Otting G (2006) N-Labelled proteins by cell-free protein synthesis. Strategies for high-throughput NMR studies of proteins and protein-ligand complexes. FEBS J 273:4154–4159

Park K-H, Billon-Denis E, Dahmane T, Lebaupain F, Pucci B, Breyton C, Zito F (2011) In the cauldron of cell-free synthesis of membrane proteins: playing with new surfactants. N Biotechnol 28:255–261

Pédelacq J-D, Cabantous S, Tran T, Terwilliger TC, Waldo GS (2006) Engineering and characterization of a superfolder green fluorescent protein. Nat Biotechnol 24:79–88

Pedersen A, Wallgren M, Karlsson BG, Gröbner G (2011) Expression and purification of full-length anti-apoptotic Bcl-2 using cell-free protein synthesis. Protein Expr Purif 77:220–223

Periasamy A, Shadiac N, Amalraj A, Garajová S, Nagarajan Y, Waters S, Mertens HDT, Hrmova M (2013) Cell-free protein synthesis of membrane (1,3)-β-d-glucan (curdlan) synthase: co-translational insertion in liposomes and reconstitution in nanodiscs. Biochim Biophys Acta 1828:743–757

Proverbio D, Roos C, Beyermann M, Orban E, Dötsch V, Bernhard F (2013) Functional properties of cell-free expressed human endothelin A and endothelin B receptors in artificial membrane environments. Biochim Biophys Acta. doi:10.1016/j.bbamem.2013.05.031

Rath P, Demange P, Saurel O, Tropis M, Daffé M, Dötsch V, Ghazi A, Bernhard F, Milon A (2011) Functional expression of the PorAH channel from *Corynebacterium glutamicum* in cell-free expression systems: implications for the role of the naturally occurring mycolic acid modification. J Biol Chem 286:32525–32532

Reckel S, Sobhanifar S, Schneider B, Junge F, Schwarz D, Durst F, Löhr F, Güntert P, Bernhard F, Dötsch V (2008) Transmembrane segment enhanced labeling as a tool for the backbone assignment of alpha-helical membrane proteins. Proc Natl Acad Sci U S A 105:8262–8267

Roos C, Zocher M, Müller D et al (2012) Characterization of co-translationally formed nanodisc complexes with small multidrug transporters, proteorhodopsin and with the *E. coli* MraY translocase. Biochim Biophys Acta 1818:3098–3106

Roos C, Kai L, Proverbio D, Ghoshdastider U, Filipek S, Dötsch V, Bernhard F (2013) Co-translational association of cell-free expressed membrane proteins with supplied lipid bilayers. Mol Membr Biol 30:75–89

Sansuk K, Balog CIA, Van der Does AM, Booth R, De Grip WJ, Deelder AM, Bakker RA, Leurs R, Hensbergen PJ (2008) GPCR proteomics: mass spectrometric and functional analysis of histamine H1 receptor after baculovirus-driven and in vitro cell free expression. J Proteome Res 7:621–629

Savage DF, Anderson CL, Robles-Colmenares Y, Newby ZE, Stroud RM (2007) Cell-free complements in vivo expression of the *E. coli* membrane proteome. Protein Sci 16:966–976

Sawasaki T, Morishita R, Gouda MD, Endo Y (2007) Methods for high-throughput materialization of genetic information based on wheat germ cell-free expression system. Methods Mol Biol 375:95–106

Schneider B, Junge F, Shirokov VA, Durst F, Schwarz D, Dötsch V, Bernhard F (2010) Membrane protein expression in cell-free systems. Methods Mol Biol 601:165–186

Schwarz D, Junge F, Durst F, Frölich N, Schneider B, Reckel S, Sobhanifar S, Dötsch V, Bernhard F (2007) Preparative scale expression of membrane proteins in *Escherichia coli*-based continuous exchange cell-free systems. Nat Protoc 2:2945–2957

Schwarz D, Dötsch V, Bernhard F (2008) Production of membrane proteins using cell-free expression systems. Proteomics 8:3933–3946

Schwarz D, Daley D, Beckhaus T, Dötsch V, Bernhard F (2010) Cell-free expression profiling of *E. coli* inner membrane proteins. Proteomics 10:1762–1779

Sevova ES, Goren MA, Schwartz KJ, Hsu F-F, Turk J, Fox BG, Bangs JD (2010) Cell-free synthesis and functional characterization of sphingolipid synthases from parasitic trypanosomatid protozoa. J Biol Chem 285:20580–20587

Shao S, Hegde RS (2011) Membrane protein insertion at the endoplasmic reticulum. Annu Rev Cell Dev Biol 27:25–56

Shenkarev ZO, Lyukmanova EN, Butenko IO, Petrovskaya LE, Paramonov AS, Shulepko MA, Nekrasova OV, Kirpichnikov MP, Arseniev AS (2013) Lipid-protein nanodiscs promote in vitro folding of transmembrane domains of multi-helical and multimeric membrane proteins. Biochim Biophys Acta 1828:776–784

Shimada Y, Wang Z-Y, Mochizuki Y, Kobayashi M, Nozawa T (2004) Functional expression and characterization of a bacterial light-harvesting membrane protein in *Escherichia coli* and cell-free synthesis systems. Biosci Biotechnol Biochem 68:1942–1948

Shimizu Y, Inoue A, Tomari Y, Suzuki T, Yokogawa T, Nishikawa K, Ueda T (2001) Cell-free translation reconstituted with purified components. Nat Biotechnol 19:751–755

Shimono K, Goto M, Kikukawa T, Miyauchi S, Shirouzu M, Kamo N, Yokoyama S (2009) Production of functional bacteriorhodopsin by an *Escherichia coli* cell-free protein synthesis system supplemented with steroid detergent and lipid. Protein Sci 18:2160–2171

Shin J, Noireaux V (2010) Efficient cell-free expression with the endogenous *E. Coli* RNA polymerase and sigma factor 70. J Biol Eng 4:8

Sobhanifar S, Reckel S, Junge F, Schwarz D, Kai L, Karbyshev M, Löhr F, Bernhard F, Dötsch V (2010) Cell-free expression and stable isotope labelling strategies for membrane proteins. J Biomol NMR 46:33–43

Spirin AS, Baranov VI, Ryabova LA, Ovodov SY, Alakhov YB (1988) A continuous cell-free translation system capable of producing polypeptides in high yield. Science 242:1162–1164

Stech M, Merk H, Schenk JA, Stöcklein WFM, Wüstenhagen DA, Micheel B, Duschl C, Bier FF, Kubick S (2012) Production of functional antibody fragments in a vesicle-based eukaryotic cell-free translation system. J Biotechnol 164:220–231

Takai K, Sawasaki T, Endo Y (2010) The wheat-germ cell-free expression system. Curr Pharm Biotechnol 11:272–278

Uhlemann E-ME, Pierson HE, Fillingame RH, Dmitriev OY (2012) Cell-free synthesis of membrane subunits of ATP synthase in phospholipid bicelles: NMR shows subunit a fold similar to the protein in the cell membrane. Protein Sci 21:279–288

Umakoshi H, Suga K, Bui HT, Nishida M, Shimanouchi T, Kuboi R (2009) Charged liposome affects the translation and folding steps of in vitro expression of green fluorescent protein. J Biosci Bioeng 108:450–454

Uzawa T, Yamagishi A, Oshima T (2002) Polypeptide synthesis directed by DNA as a messenger in cell-free polypeptide synthesis by extreme thermophiles, *Thermus thermophilus* HB27 and *Sulfolobus tokodaii* strain 7. J Biochem 131:849–853

Wada T, Shimono K, Kikukawa T et al (2011) Crystal structure of the eukaryotic light-driven proton-pumping rhodopsin, acetabularia rhodopsin II, from marine alga. J Mol Biol 411:986–998

Wang Z (2006) Controlled expression of recombinant genes and preparation of cell-free extracts in yeast. Methods Mol Biol 313:317–331

Wang X, Liu J, Zheng Y, Li J, Wang H, Zhou Y, Qi M, Yu H, Tang W, Zhao WM (2008) An optimized yeast cell-free system: sufficient for translation of human papillomavirus 58 L1 mRNA and assembly of virus-like particles. J Biosci Bioeng 106:8–15

Wang X, Corin K, Baaske P, Wienken CJ, Jerabek-Willemsen M, Duhr S, Braun D, Zhang S (2011) Peptide surfactants for cell-free production of functional G protein-coupled receptors. Proc Natl Acad Sci U S A 108:9049–9054

Wang X, Cui Y, Wang J (2013) Efficient expression and immunoaffinity purification of human trace amine-associated receptor 5 from *E. coli* cell-free system. Protein Pept Lett 20:473–480

Witherell G (2001) In vitro translation using HeLa extract. Curr Protoc Cell Biol. Unit 11:8

Wulfetange K, Saenger W, Schmülling T, Heyl A (2011) *E. coli*-based cell-free expression, purification and characterization of the membrane-bound ligand-binding CHASE-TM domain of the cytokinin receptor CRE1/AHK4 of *Arabidopsis thaliana*. Mol Biotechnol 47:211–219

Wuu JJ, Swartz JR (2008) High yield cell-free production of integral membrane proteins without refolding or detergents. Biochim Biophys Acta 1778:1237–1250

Yabuki T, Motoda Y, Hanada K, Nunokawa E, Saito M, Seki E, Inoue M, Kigawa T, Yokoyama S (2007) A robust two-step PCR method of template DNA production for high-throughput cell-free protein synthesis. J Struct Funct Genomics 8:173–191

Yang J-P, Cirico T, Katzen F, Peterson TC, Kudlicki W (2011) Cell-free synthesis of a functional G protein-coupled receptor complexed with nanometer scale bilayer discs. BMC Biotechnol 11:57

Yildiz AA, Knoll W, Gennis RB, Sinner E-K (2012) Cell-free synthesis of cytochrome bo(3) ubiquinol oxidase in artificial membranes. Anal Biochem 423:39–45

Yin G, Swartz JR (2004) Enhancing multiple disulfide bonded protein folding in a cell-free system. Biotechnol Bioeng 86:188–195

Zawada JF, Yin G, Steiner AR, Yang J, Naresh A, Roy SM, Gold DS, Heinsohn HG, Murray CJ (2011) Microscale to manufacturing scale-up of cell-free cytokine production—a new approach for shortening protein production development timelines. Biotechnol Bioeng 108:1570–1578

Zhou Y, Asahara H, Gaucher EA, Chong S (2012) Reconstitution of translation from *Thermus thermophilus* reveals a minimal set of components sufficient for protein synthesis at high temperatures and functional conservation of modern and ancient translation components. Nucleic Acids Res 40:7932–7945