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Review

Large-scale production of functional membrane proteins

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Abstract. The preparation of sufficient amounts of high-quality samples is still the major bottleneck for the characterization of membrane proteins by *in vitro* approaches. The hydrophobic nature, the requirement for complicated transport and modification pathways, and the often observed negative effects on membrane properties are intrinsic features of membrane proteins that frequently cause significant problems in overexpression studies. Establishing efficient protocols for the production of functionally folded membrane proteins is therefore a challenging task, and numerous specific characteristics have to be considered. In addition, a variety of expression systems have been developed, and choice of appropriate techniques could strongly depend on the desired target membrane proteins as well as on their intended applications. The production of membrane proteins is a highly dynamic field and new or modified approaches are frequently emerging. The review will give an overview of currently established processes for the production of functionally folded membrane proteins.

Keywords. Membrane proteins, expression systems, detergents, posttranslational modification, cell-free expression.

Introduction

Membrane proteins (MPs) represent the vast majority of clinical drug targets and are a focus of pharmaceutical and biotechnological interests. Despite significant and considerable recent improvements, the expression of functionally folded MPs in sufficient amounts for functional and structural studies is still a challenging task. If compared with soluble cytoplasmic proteins, the expression of MPs requires a variety of special considerations. (I) MPs are not just released into the cytosol but must rather be targeted and translocated to their final destinations in membranes [1–4]. In particular in eukaryotic cells, directed targeting through the endoplasmatic reticulum and Golgi apparatus requires sophisticated recognition and sorting mechanisms. (II) Insertion of MPs into membranes usually does not occur spontaneously. It often requires translocation machineries like the prokaryotic Sec machinery or the eukaryotic Sec61 complex. Copy number and capacity of those systems are limited, and translocation can be selective only for distinct groups of MPs [1, 3, 5]. (III) For structural studies and many other purposes, MPs have to be extracted out of the cellular membranes after expression, followed by transfer into artificial and defined hydrophobic environments like micelles or liposomes. This procedure can become highly critical as membranes have to be disintegrated by relatively harsh detergents. Contact with these detergents concomitant with destabilization by the sudden removal of

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Table 1. General features of MP expression system.[1]

	E. coli	*L. lactis*	Yeast	Insect	Mammalian	E-CF/W-CF
Basic properties[2]						
Setup requirements	1	1	1	3	3	1/1
Time investment	1	1	2	3	3	1/3
Costs	1	1	2	3	3	2/2
Robustness	3	3	3	1	1	2/2
Preparative scale	3	3	3	2	1	3/3
Bio-safety risk	1	1	1	1–2	1–3	1/1
Characteristics						
Throughput option	+	+	+	–	–	+/+
Expression environment						
Native membranes	+	+	+	+	+	?/?
Micelles	–	–	–	–	–	+/+
Artificial liposomes	–	–	–	–	–	+/+
IBs/precipitate	+	?	(+)	(+)	–	+/+
MP labelling						
Uniform	+	+	+	–	–	+/+
Specific	+	+	+	–	–	+/+
Combinatorial	(+)	(+)	(+)	–	–	+/+
PTMs						
Glycosylation	–	–	+	+	+	–/?
Prenylation	–	–	+	+	+	–/?
Disulfide bonds	(+)	–	+	+	+	+/?

[1] IBs, inclusion bodies; PTMs, posttranslational modifications; ?, not analysed yet.
[2] 1, low; 2, medium; 3, high.

lateral pressure can result in conformational aberrations or in unfolding of MPs. (IV) The hydrophobic nature of MPs makes general handling and sample preparation problematic as arrays of diverse detergents, lipids and mixtures thereof have to be analysed in order to provide optimal conditions for the stability and functional folding of a particular MP.

In general, significant problems upon overexpression of MPs in living cells arise from overloading and blocking of essential components of the targeting and translocation systems. Changes in membrane properties after massive insertion of recombinant MPs concomitant with the ongoing activity of the heterologously expressed channels, transporters or porins can further cause cytotoxic effects [6]. It is therefore not surprising that the structural information obtained from MPs with some 150 unique structures and only approx. 7% entries in the Protein Data Bank is lacking far behind the data obtained from soluble proteins. The main techniques for high-resolution structure determination – X-ray, nuclear magnetic resonance (NMR) spectroscopy and electron microscopy – require substantial amounts of high-quality and stable protein samples. Based on the enormous potential of MPs for industrial and medical applications, expression technologies of MPs are a highly dynamic field, and optimized protocols, modified systems or even new approaches have frequently emerged (Fig. 1). A diverse array of options and protocols is available for the expression of MPs (Table 1). The careful evaluation and determination of strategies suitable for the production of an individual MP is therefore crucial and can depend on many parameters, such as the nature and origin of the MP and its desired application.

This review is intended to give an overview of promising approaches for obtaining polytopic MPs in functionally folded conformations. We will primarily focus on expression systems efficient enough to produce amounts of MPs sufficient even for structural analysis. We further emphasize parameters relevant for solubilization, posttranslational processing, maturation and modification of expressed MPs as potentially important components for the stability and activity of MPs. Commonly employed general approaches for a first evaluation of MP sample quality will additionally be considered, as those techniques are essential prerequisites for expression optimization strategies.

General considerations for functional MP expression

Optimization of expression protocols is demanding for MP production in any system. Modifications of each little step, such as vector design and transfer, cultivation conditions, expression strategies, MP extraction, choice of detergents and buffer conditions, can have a significant impact on the final yield and sample quality. Expression protocols must thus be

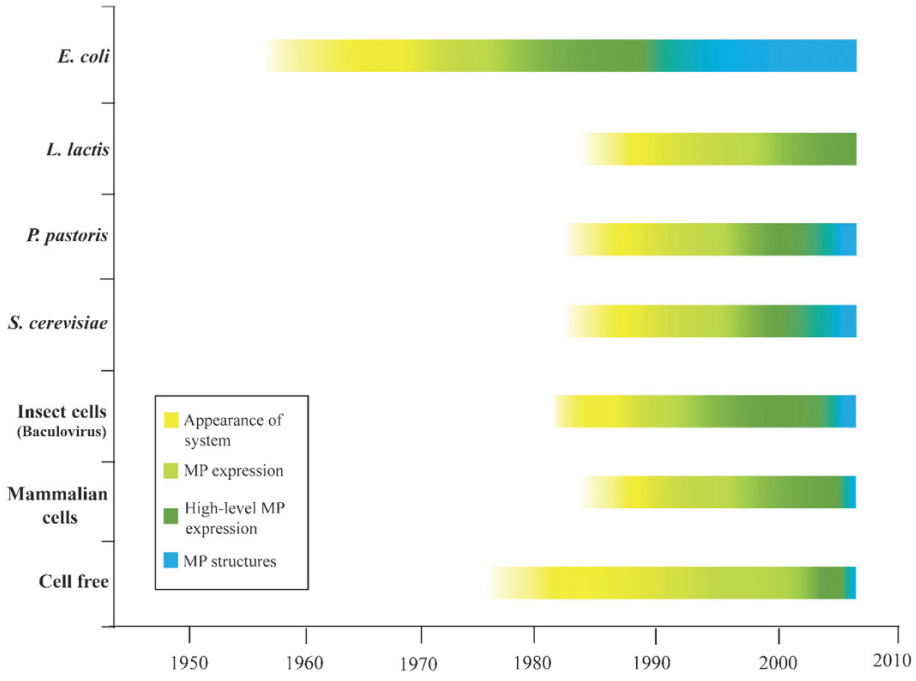

Figure 1. Development of MP expression systems. Comparison of the currently most popular MP expression systems with respect to emerging applications for general protein expression (yellow), initial applications to MP production (light green), improvement for preparative-scale MP production (dark green) and first appearance of MP structures (blue).

individualized according to the requirements of each particular MP. It should be realized that the highest-yield expression and best sample quality are often mutually exclusive, and protocols allowing satisfying compromises must be established. Optimization strategies are usually based on iterative processes of trial and error and can take many months. In this section, general parameters that are important for the optimization of MP production are discussed, while specific characteristics of individual systems will be considered in the next sections.

Choosing a cellular background

Central factors that could determine the yield, integrity, activity and stability of synthesized MPs are the availability of highly processive transcription and translation machineries, suitable folding environments, the lipid composition of cellular membranes, the presence of efficient targeting systems and appropriate pathways for posttranslational modifications (PTMs). Strong biases between the codon usage of the target gene and the host's translation machinery could result in premature termination of the polypeptide chain or in mis-incorporation of amino acids, both having severe effects on sample quality and functionality of the final MP [7, 8]. Most PTMs of recombinant eukaryotic MPs are unlikely to occur in prokaryotic expression hosts such as *Escherichia coli* and *Lactococcus lactis*, which sometimes may hamper functional folding but in other cases could be beneficial for crystallization studies. In addition, bacterial MPs typically have a cytoplasmic location for both the N- as well as the C-terminus [9]. The prevalent requirement of an N-terminus-out topology for eukaryotic MPs like G-protein-coupled receptors (GPCRs) could therefore cause problems in correct targeting and membrane insertion.

Specific lipids or hydrophobic environments can play important roles in the functional folding or stabilization of MPs [10–12]. Many crystallized MP complexes show defined protein-lipid contacts. Especially sterol derivatives such as cholesterol appear to influence the function of distinct mammalian MPs. Also, folding and function of bacterial MPs can depend on the presence of specific lipids, as reported for the lactose permease in *E. coli* [13]. Most observed lipid effects on MPs are still difficult to interpret. They could result from altered membrane properties like changes in fluidity, bilayer thickness and lateral pressure, from positioning MPs in correct orientations within the membrane or from structural stabilization by direct lipid-MP interactions. While specificity could be shown in some cases, it is not clear for many identified interactions whether they are promiscuous and whether the lipids could be replaced by other structurally similar compounds. The lipid composition of host cells from the different expression systems varies considerably, and an overview is given in reference [10]. Bacterial membranes are generally devoid of sterols, sphingolipids and polyunsaturated side chains, which are prevalent in eukaryotic membranes. However, insect cells also have much lower cholesterol levels compared with mammalian cells, and they completely lack phosphatidyl-serine. The selection of an expression

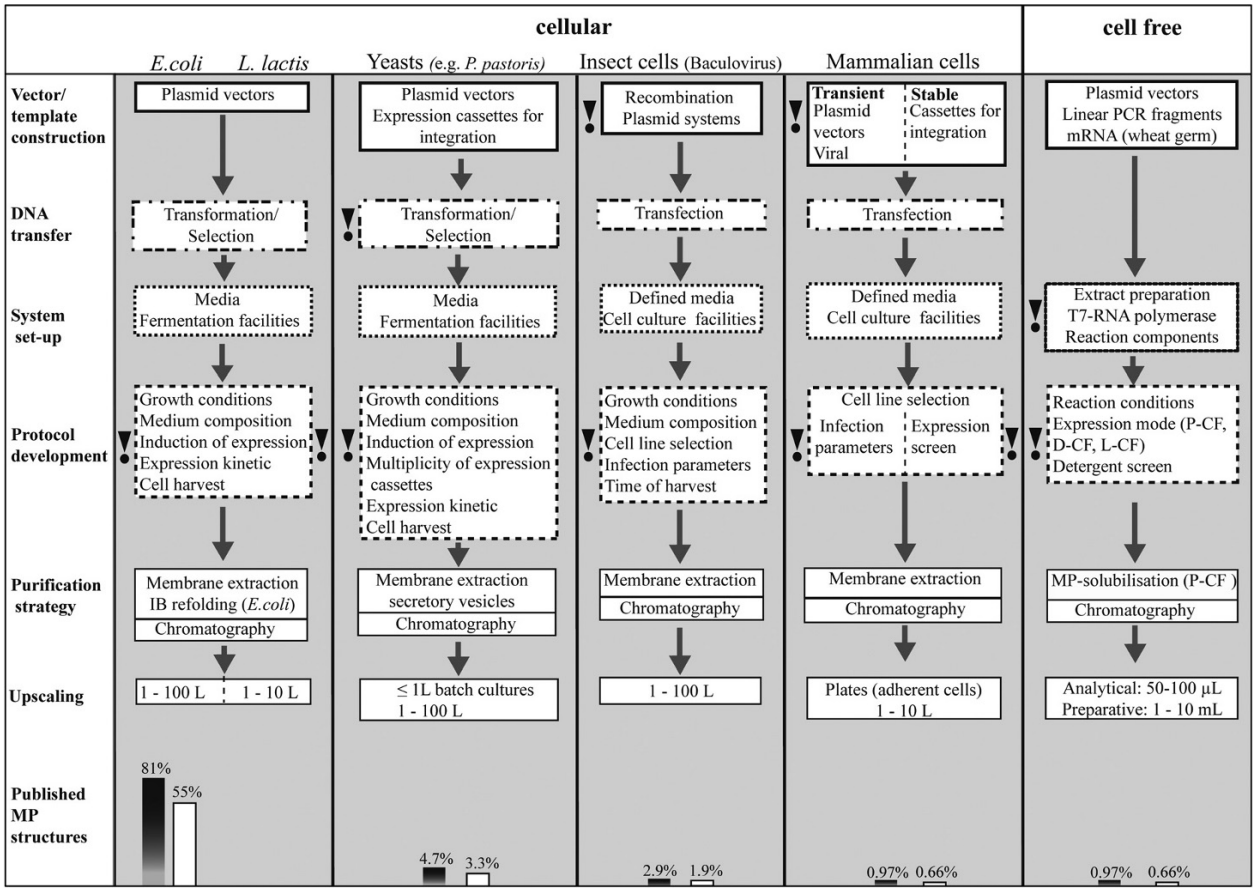

Figure 2. Process design of MP expression systems. Overview of protocol development for the most popular MP expression systems. Basic optimization parameters characteristic for the individual expression systems are illustrated, and the most critical parts are indicated. Not considered are optimization parameters involved in vector or target design, e.g. fusion technologies or modifications of the expression cassettes. Grey bars illustrate the success in obtaining MP structures after using the individual expression systems, while white bars correspond to those MP structures obtained only after recombinant expression.

system or cellular background should therefore be of major concern for the lipid composition of the host cell membranes in addition to specific codon usages and prevalent glycosylation patterns. Expression of MPs in cellular backgrounds as closely related to their origin as possible thus appears to be the most reasonable strategy. However, the efficiency and workload of the individual expression systems are quite different, and preparative amounts of MPs are often only obtained with bacterial, yeast or cell-free systems (Fig. 2). Therefore, compromises must be made, and if no specific requirements for the synthesis, folding or transport of an MP are evident, heterologous expression even in unrelated backgrounds might still provide the greatest success, especially in view of obtaining higher yields.

PTMs

Genomes of more complex species increasingly encode for proteins requiring PTMs in order to expand their structural and functional diversity in an economic way. Common PTMs of eukaryotic MPs are proteolytic processing, phosphorylation, prenylation, disulfide bond formation and glycosylation (Fig. 3). Proteolytic processing of MPs usually takes place in the endoplasmic reticulum (ER) and comprises the removal of N-terminal methionines, the cleavage of signal peptides by signal peptidases and the proteolytic activation of proteins like membrane-integrated proteases. Hydroxyl groups of the amino acids serine, threonine and tyrosine can be phosphorylated by specific kinases (Fig. 3). This reversible process is mostly involved in signalling pathways and occurs in prokaryotes as well as in eukaryotes. For GPCRs, phosphorylation of intracellular domains leads to downregulation of the receptors. Association for example of GPCRs with the plasma membrane is frequently stabilized by prenylation. Isoprenoid precursors for prenylation are derived from the cholesterol biosynthetic pathway representing either 15-carbon farnesyl groups or 20-carbon geranylgeranyl groups. The residues are attached via thioether link-

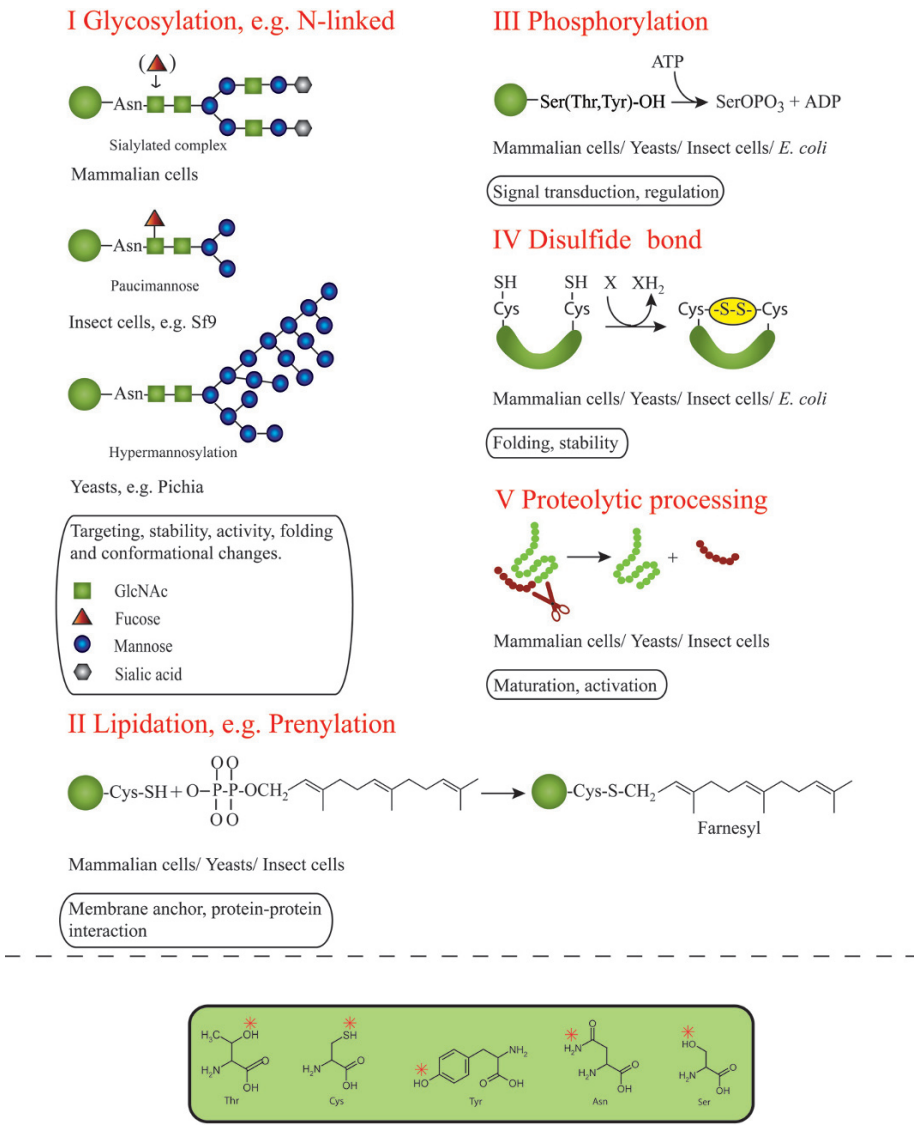

Figure 3. Posttranslational modification pathways in MP expression systems. Schematic overview of most frequent PTMs and their impact on structure and function of MPs. MP expression systems where the specific PTMs in principle can be expected are indicated.

age by specific enzymes to a cysteine residue in the C-terminus of MPs typically at the consensus sequence CAAX, where A is any aliphatic amino acid except alanine [14]. Bacteria are unable to perform most PTMs of eukaryotic MPs due to the absence of the required enzyme systems [15]. Less problematic might be disulfide bond formation, which often occurs during MP folding in an autocatalytic process in the oxidizing environment of the eukaryotic ER [16] or in the periplasmic space of bacterial cells. Disulfide bonds in MPs are typically found in exoplasmic domains, and they can significantly affect structural folding and protein stability.

More complex is the mechanism of glycosylation, which is required for about 1% of the proteins in higher organisms [17]. Glycosylation of MPs can become crucial for folding and stability, and it occurs co- and posttranslationally in the ER and Golgi apparatus. Glycosylation patterns differ in composition as well as in the quantity of glycan moieties, and they are specific for individual eukaryotic organisms. The most frequent compounds are glucose, galactose, mannose, fucose, N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc) and sialic acid. N-linked glycosylation takes place at specific asparagines within the recognition sequence Asn-X-Ser/Thr, with GlcNAc added as the first moiety [18]. O-linked glycosylation typically starts with a GalNAc attachment at the hydroxyl groups of serine and threonine, while no common recognition sequences are known. Glycosylated proteins in yeast, insect and mammalian cells have a common sugar core containing two GlcNAc and three mannose residues. MPs in mammalian cells are further modified with terminal fucose, sialic acid or galactose residues by the enzymes sialyltransferase and galactosyltransferase [19, 20].

Glycosylation patterns in insect cells depend on the expressed MP and on the host cell line. Typical patterns are the high mannose type or the insect-specific paucimannose type [19]. Mammalian cells further modify the high mannose intermediate into a complex glycosylation type [21]. Yeast as a lower eukaryote only adds mannose residues to the common sugar core, while in particular in *Saccharomyces cerevisiae* hypermannosylation of heterologously expressed MPs can occur [20].

Efficient PTMs of recombinantly expressed MPs clearly depend on the rate of synthesis and on the availability of the modifying enzymatic pathways in the selected expression system. MP production in analytical scales in appropriate systems can result in almost homogenous native-like PTM patterns, while preparative-scale expression of MPs in even homologous systems often oversaturates the modifying capacities, yielding poor or incomplete PTMs. This might not necessarily be critical for MP activity, but it could cause significant problems e.g. in crystallization studies [15, 22]. The use of host mutants defective in central glycosylation enzymes such as N-acetylglucosaminyltransferase-I negative HEK 293S cells [23] or *in vitro* deglycosylation of expressed glycoproteins by endoglycosidases could be considered in order to prevent heterogeneous glycans. Tailoring of expression systems by co-expression of individual modifying enzymes could be helpful in individual cases, but it is unlikely to become a general approach. Too many different compounds are involved in MP assembly, many of them also being MPs whose successful expression would be quite uncertain. Side effects of the cell physiology and restrictions due to substrate specificities could further prevent the assembly of heterologous MPs.

Promoters and strength of expression

Modulating the strength of expression is a key issue in protocol development for MP production. In contrast to soluble proteins, for which often strong and fast expression is possible, the capacity of cells to synthesize functionally folded MPs and to accommodate them into their membranes is much more restricted. Folding, transportation, modification and membrane insertion of MPs takes significantly longer, and these processes are often rate-limiting steps of expression [24]. Space in the two-dimensional membrane is also limited, and in *E. coli* membranes MPs and lipids are estimated to cover already equal surfaces under native conditions [25]. It is therefore evident that problems with overloading or improper function of membranes as well as with transport and modification systems are likely to occur sooner or later as physiological consequences of MP overexpression [1, 22, 26, 27]. The results can be toxic effects to the host cells as well as improper folding, incomplete PTMs, proteolytic digestion and precipitation of the synthesized MPs. Reasonable compromises between expression yield and sample quality must therefore be evaluated. Expression rates could be triggered by choosing tightly regulated moderately strong or weak promoters, by low copy number vectors or by reducing concentrations of transcriptional inducers. Incubation of producer cells at lower temperatures and for shorter time periods and induction of expression at high cell densities at late growth stages further modulate the expression kinetics. Approaches to produce MPs in cells with proliferated membranes and increased MP assembly capacities could further enhance expression yields while maintaining good quality [28, 29].

Expression strategies resulting in lower efficiencies and reduced synthesis rates may generally be more likely to yield membrane-integrated functionally folded MPs. In yeasts, systematic optimization of fermentation conditions resulted in a significant quality improvement of recombinant GPCRs, while the overall expression yields have not been increased [30, 31]. This overall tendency is an obvious dilemma for structural approaches, for which preparative amounts of MPs are required. Large fermentation volumes up to several hundreds of litres and extended incubation times of days or even weeks are therefore common.

Target gene design and construction of fusion proteins

A first and general consideration for MP expression is to ensure optimal transcription and translation in the chosen host cells. Extended palindromic sequences or repetitive rare codons in the reading frame should be eliminated, as they could cause prolonged pausing of the ribosomes with risk of premature termination or misincorporation of amino acids. Silent mutagenesis or even optimally designed synthetic genes could be used in order to avoid basic problems with expression. Expression of thermophilic homologues is sometimes preferred because of potential benefits in protein stability.

Similar to expression strategies for cytosolic proteins [32], fusion technologies are often employed in order to optimize expression, detection and purification of the synthesized MPs. Some popular larger fusion partners used for increased expression are the maltose binding protein (MBP), glutathione-S-transferase (GST) and thioredoxin (Trx) (Table 2). Specific antibodies directed against the fusion partners are commercially available and can be used for the detection and quantification of the expressed fusion protein. Furthermore, the specific substrate binding properties

Table 2. Fusion partners for MP expression.

Fusion	Size [kDa]	Localization[1]	Properties[2]	Source[3], reference or sequence
Proteins:				
MBP (*malE*)	42	N-terminal	Expression/Detection Stabilization Affinity purification Targeting	New England Biolabs (pMal vectors)
GST	24	N-terminal	Expression/Detection Affinity purification IB formation	GE Healthcare (pGEX vectors)
Trx	12	N/C-terminal	Expression/Detection MP Reduction	Invitrogen (ThioFusion system)
GFP	35	C-terminal	Detection/Monitoring	[33, 34]
NusA	58	N-terminal	Expression/Detection Stabilization	Novagen (pET vectors)
Protein D	10	N-terminal	Expression/Detection	[35]
Protein GB1	5	N-terminal	Expression/Detection	[36]
Mistic	9	N-terminal	Translocation	[37]
Peptide tags:				
StrepII	1.0	N/C-terminal	Affinity purification Detection Expression	NWSHPQFEK IBA (pASK vectors)
$Poly(His)_{6-10}$	0.8–1.2	N/C-terminal	Affinity purification Detection Expression	Qiagen (pQE vectors) Novagen (pET vectors)
T7	1.4	N-terminal	Expression/Detection	MASMTGGQQMG Novagen (pET vectors)
FLAG	0.9	N/C-terminal	Expression/Detection	DYKDDDDK
MYC	1.2	N/C-terminal	Expression/Detection	EQKLISEEDL
HA	1.0	N/C-terminal	Expression/Detection	YPYDVPDYA

[1] Preferred or recommended localization in expression constructs with respect to the target MP.
[2] Potential benefits, positive effects on expression are usually only observed if fusion partners are attached N-terminally to the target MP.
[3] Popular commercial sources: New England Biolabs, Ipswich, MA, USA; GE-Healthcare (Pharmacia), München, Germany; Invitrogen, Carlsbad, CA, USA; IBA, Göttingen, Germany; Qiagen, Hilden, Germany; Merck Biosciences (Novagen), Darmstadt, Germany.

of e.g. GST or MBP are valuable for affinity purification of the fusion proteins. An additional characteristic benefit of using MBP as fusion partner is its high potential in stabilization of proteins fused to its C-terminus [38]. N-terminal fusion of an MP target to a reporter protein like green fluorescent protein (GFP) can help to monitor and optimize expression directly in the host cell [33, 34]. Fusions to the mistic protein from *Bacillus subtilis* could facilitate the efficient translocation of recombinant MPs [37]. The C-terminal GFP moiety only folds into a functional and fluorescent conformation if the MP-GFP construct is successfully expressed in a soluble form and inserted into membranes or micelles. GFP quantification could thus be used to optimize expression conditions directly in the cell or in the reaction mixture in case of cell-free expression. However, the relatively large GFP moiety might sometimes cause problems in expression, targeting, translocation or folding processes of the attached MP. Larger fusion partners often have to be removed from the MPs after purification in order to proceed with structural or functional studies. Recognition sites for specific restriction proteases (e.g. tobacco etch virus protease, enterokinase, thrombin or factor Xa) should therefore be inserted within the linker in between fusion partner and target MP. This processing is a major disadvantage of MP expression with fusion strategies as it is associated with significant losses in yield due to inefficient proteolytic cleavage and additional purification steps.

Alternatively, small functional peptide tags containing only few amino acid residues like the Strep-tag or $poly(His)_n$-tags are frequently considered instead of or in addition to larger fusion partners (Table 2). Strep- and $poly(His)_n$-tags are well known for their potential in the efficient affinity purification of attached proteins. Similar to fusion proteins, such tags can be used as antigenic epitopes for the immunodetection of the expressed MPs. Expression approaches often start with low yields, and the detection of recombinant MPs in Coomassie-Blue-stained gels can only be achieved after intensive optimization processes. Specific antibodies for the expressed MPs are often not available. In addition, MPs are frequently not separated according to their calculated molecular mass in SDS polyacrylamide gels, most likely due to improper denaturation. The immunodetection of terminal tags is therefore a valuable tool in order to verify expression of the full-

length MP. A critical step in protein expression can be the efficient initiation of translation, which primarily depends on the first codons of a reading frame. Inappropriate codon structure can cause failure of expression, in particular of eukaryotic MPs in prokaryotic backgrounds. Codon-optimized N-terminal tags can help to avoid this problem. N-terminal T7-tags for example have been essential for the efficient expression of GPCRs in cell-free systems [39]. The functional folding of MPs in general is not assumed to be prevented by terminal fusion tags [40, 41]. However, the length and position of a tag at either end of an MP could influence its expression level and may be a subject of optimization [42]. The cumulative positive charges of poly(His)$_n$-tags could furthermore inhibit MP targeting in particular cases [43]. Even small tags can become problematic for crystallization, and subsequent removal by restriction proteases might be necessary.

Extracytoplasmic proteins in *E. coli* such as outer membrane proteins typically require cleavable amino-terminal signal peptides of characteristic structures [44]. The addition of prokaryotic OmpT and PelB leaders to the N-termini could direct MPs into the periplasmic space or into the outer membrane. However, most prokaryotic MPs inserted into the inner membrane are synthesized without detectable signal sequences, probably as consequence of the frequent localization of the amino-termini in the cytoplasm. For eukaryotic MPs, signal sequences are often essential. The addition of the signal sequences of prepromelittin [45] or the alpha-factor prosequence [46] to the N-terminus of the dopamine D2S receptor increased expression levels severalfold in insect cells and also supported its insertion into the plasma membrane. A modified influenza hemaglutinin signal sequence at the N-terminus of the β_2-adrenergic receptor increased its functional expression due to better translocation into the ER membrane [47].

Sample quality control

Fast and reliable quality control of expressed MPs is a fundamental prerequisite in order to obtain the necessary feedback information for the optimization of expression protocols for functionally folded proteins [4]. Detergents and lipids used for solubilization or reconstitution procedures are primary determinants of MP quality, and a variety of compounds with different physical properties should be evaluated. Appropriate conditions are hardly predictable and depend very much on the characteristics of the protein target [48]. It is thus essential to generally combine optimization processes with quality control assays. However, the functional folding of MPs is often difficult to assess, as in many cases activity assays have not been developed or specific functions are not even known. Therefore, ensuring the purity and monodispersity of MP samples often remains the only amenable parameter in particular for structural approaches. A selection of frequently employed techniques in order to receive information relevant for a preliminary evaluation of MP quality is compiled in Table 3.

It should be emphasized that 'quality' is certainly determined by a number of different properties: (I) Yield and MP integrity will always be of primary concern, and they can be analysed already in crude samples without prior purification and with any MP sample. Most popular is the separation of samples by SDS-polyacrylamide gel electrophoresis (PAGE) and detection of the expressed MPs by staining with e.g. Coomassie Blue or by the more sensitive immunostaining, if appropriate antibodies are available. Full-length expression and integrity of MPs, which often do not migrate according to their predicted molecular mass by SDS-PAGE, can be verified by detection of attached terminal immunotags. (II) Sample homogeneity is often analysed by size exclusion chromatography (SEC). Elution profiles and evenly shaped Gaussian peaks give information about purity, complex formation and a potential aggregation of MPs. Mass spectrometry could in addition be employed to detect small molecular heterogeneities like improper processing of signal sequences or misincorporation of amino acids [56]. (III) Initial evidence of the secondary structure of MPs can be obtained by circular dichroism (CD) spectroscopy. Purified α-helical MPs give characteristic spectra with maximal absorption at 190 nm and with two minima at 208 nm and 222 nm, respectively. CD spectroscopy is relatively tolerant against detergents and even mixed micelles, making it suitable for screening of optimal hydrophobic environments [56]. Fluorescence spectroscopy can be used to analyse the incorporation of MPs into micelles. Absorption maxima of Trp residues in between 320–330 nm correspond to hydrophobic environments, while at 350 nm Trp has access to hydrophilic solvents, indicating potential misfolding. Recording NMR spectra could be considered in special cases where ^{15}N-labelled MP samples can be generated, e.g. by CF expression. The chemical shift dispersion in the ^{1}H-dimension of NMR spectra can give first evidence of the structural folding of an MP [57, 83]. (IV) Formation of oligomeric complexes is a characteristic of many MPs, and a specific association of monomers could be considered as evidence of adopting native conformations. Native PAGE, SEC or pull-down assays with differentially tagged MP derivatives are some initial and general approaches

Table 3. Primary approaches for the evaluation of MP sample quality.[1]

Evaluation parameters	Method	Sample				Requirements	Ref.
I. Overall quality							
Yield and purity	SDS-PAGE/immunodetection	IB	PM	PL	NM	Specific antibodies	[49]
Integrity	SDS-PAGE/immunodetection	IB	PM	PL	NM	PP, terminal immuno-tags	[50]
Homogeneity	SEC, peak shape		PM			PP	[51]
	Mass spectrometry		PM			PP, electrophoresis	[52, 53]
	Light scattering (dynamic)		PM			PP	[54, 55]
II. Structural characteristics							
Overall folding	CD spectroscopy		PM			PP	[56]
	Fluorescence spectroscopy		PM			PP, Trp res. in TMSs	[56]
	NMR (HSQC)		PM			^{15}N-lab. small MPs	[57]
Oligomerization	Native PAGE		PM			PP	[58]
	SEC		PM			PP	[59]
	Pull-down assays		PM	PL		Immobilization	[60]
	Two-hybrid experiments				NM	Yeast expression	[61]
	Ultracentrifugation		PM	PL		PP	[62, 63]
	Electron microscopy		PM	PL	NM	PP, larger MPs	[64]
	FRET / BRET		PM	PL	NM	Fusion proteins	[65, 66]
Glycosylation	Enzymatic detection		PM	PL	NM	Eukaryotic MPs	[50]
	SDS-PAGE/mobility shifts		PM	PL	NM	Reference	[49]
III. Functional assays							
Membrane insertion	Freeze-fracture EM			PL		PP	[67]
	Immunodetection			PL	NM	Specific antibodies	[68]
	Fluorescence spectroscopy			PL		PP	[56]
	Reporter protein activity			PL	NM	Fusion proteins	[9]
						(e.g. GFP, PhoA)	[69]
	Single cysteine labelling			PL		PP, site-directed mutants	[70, 71]
	Protease protection assay			PL	NM	PP	[72]
Transport activity	Solid supported membranes			PL		PP	[68]
	Planar lipids			PL		PP	[73]
	IR spectroscopy			PL		PP	[74]
	Fluorescence spectroscopy			PL		PP	[75, 76]
	Patch clamp				NM	*In vivo* expr. system	[77, 78]
Ligand binding	Radioligand assays		PM	PL	NM	Radiolabelled ligands	[31, 79, 80]
	Co-elution, pull-down assays		PM			PP	[81]
	Surface plasmon resonance		PM			PP	[82]

[1] IB: inclusion bodies; PM, proteomicelles; PL, proteoliposomes; NM, native membranes; PP, purified protein.

in addition to a number of further elaborated techniques (Table 3). (V) Inefficient glycosylation can cause heterogeneous samples following MP expression in eukaryotic hosts, and incomplete glycosylation might even result in misfolding of MPs [49, 84]. Enzymatic detection or migration patterns of MPs by SDS-PAGE showing shifted additional bands could give a first hint of glycosylation. (VI) Correct membrane integration of MPs is often a prerequisite for functional activity, e.g. in the case of transporters. General membrane integration can be analysed by ultracentrifugation, freeze-fracture analysis or immunodetection, while protease protection assays or MP fusions with reporter proteins like GFP or PhoA could in addition give topological information. (VII) MP activity and functional folding is certainly the most difficult parameter to determine, and target-specific assays often have to be developed. A lack of functional assays especially for the *in vitro* analysis of proteomicelles, unknown ligands and complicated technical setups are major problems in analysing MP function. In addition, established assays are often specific to distinct MPs and cannot be applied even to related proteins. Due to their high sensitivity, radioassays are frequently used for the analysis of transport activity and ligand binding, and those assays might in be particular applicable for screening processes, as they can be used with crude sample preparations. Ligand affinity columns during purification could enable the separation of active from non-functional material, allowing quantitative analysis [85].

High-level MP production for structural approaches

Structural studies require that purified MPs should be chemically and conformationally homogeneous, stable at relatively high concentrations for a long period and available in milligram amounts. High expression rates are therefore necessary, and only the purest fractions of subsequent purification steps should be used in order to address the high demand for sample homogeneity. Few expression systems are successful in producing milligram quantities of MPs, and it is therefore not surprising that a significant part of the

Table 4. High-level MP production for structural analysis.[1]

Protein (origin)	Source or host	Size [kDa]	TMS	Res [Å]	Ref
I: Isolation from native source[2]					
Bacteriorhodopsin (*H. salinarium*)	n.a.	24	7	1.5	[86]
Fumarate reductase (*W. succinogenes*)	n.a.	73	5	1.8	[87]
Formate dehydrogenase (*E. coli*)	GL101	170	5	1.6	[88]
Cytochrome C oxidase (*B. taurus*)	heart muscle cells	205	26	1.8	[89]
Rhodopsin (*B. taurus*)	rod outer segments	40	7	2.2	[90]
Ca^{2+} ATPase (*O. cuniculus*)	muscle cells	109	10	2.3	[91]
Glutathione-S-transferase (*R. norvegicus*)	liver cells	17	4	3.2	[92]
Aquaporin-1 (*H. sapiens*)	red blood cells	29	6	3.8	[93]
II. Recombinant – homologous expression[2]					
Halorhodopsin (*H. salinarium*)	n.a.	27	7	1.8	[94]
Nitrate reductase NarGHI (*E. coli*)	LCB2048	223	5	1.9	[95]
GlpF glycerol channel (*E. coli*)	n.a.	30	6	2.2	[96]
YiiP zinc transporter (*E. coli*)	BL21 x pLys	33	6	3.8	[97]
BtuCD vitamin B12 transporter (*E. coli*)	BL21	28	10	3.2	[98]
EmrD MDR transporter (*E. coli*)	BL21	42	12	3.5	[99]
OmpG (*E. coli*)	C41	33	β	2.4	[100]
PagP (*E. coli*)	BL21 x pLys	20	β	n.a.	[101]
III. Recombinant – heterologous expression					
Ammonium transporter Amt-1 (*A. fulgidus*)	*E. coli* C43	42	11	1.5	[102]
Leucine transporter LeuTaa (*A. aeocelius*)	*E. coli* C41	58	12	1.6	[103]
Aspartate transporter (*P. horkoshii*)	*E. coli* TOP10	45	8	2.9	[104]
KcsA potassium channel (*S. lividans*)	*E. coli* XL-1 Blue	13	2	2.0	[36]
SAV1866 ABC transporter (*S. aureus*)	*E. coli*	65	12	3.0	[105]
Leukotriene C4 synthase (*H. sapiens*)	*P. pastoris*	18	4	2.1	[106]
FLAP (*H. sapiens*)	*E. coli* BL21	18	4	4.2	[107]
ß2-adrenergic receptor (*H. sapiens*)	*S. frugiperda*	47	7	2.4	[108]

[1] Representative overview of MP structures obtained by different preparation strategies. Most eukaryotic as well as selected prokaryotic examples are listed. Preference has been given to systems resulting in high-resolution structures.
[2] Bacterial host strains are indicated; n.a., not announced.
[3] Host strain not specified.

current MP structures represents proteins with a relatively high natural abundance, allowing purification from their native source (Table 4, Fig. 4).

Prominent examples are the opsin derivatives, such as bovine rhodopsin, which can be isolated from the rod outer segments of bovine lenses [109], or human aquaporin-1, which gives high yields when extracted from red blood cells [110]. A major disadvantage of isolating proteins from their native source is, however, that no affinity tag is attached to the protein to facilitate purification. Moreover, production of mutants and the labelling of the proteins for NMR analysis is hardly feasible. Efficient recombinant expression systems are therefore highly desirable, and among the reported structures, *E. coli* is still the most commonly used system for homologous as well as heterologous expression of MPs (Fig. 4). However, the majority of currently known structures comprise prokaryotic proteins, while examples of eukaryotic MPs such as the KcsA potassium channel from *Streptomyces lividans* [36] and human 5-lipoxygenase activating protein (FLAP) [107] are still the exception (Table 4). FLAP is thus far the only example of a human MP that has been expressed successfully in standard *E. coli* BL21 cells and crystallized in its functional, trimeric form. Two other recent human crystal structures are available – the leukotriene C4 synthase (LTC4) and the β2-adrenergic receptor. While LTC4 was expressed and isolated from the fission yeast *Schizosaccharomyces pombe* [111] as well as *Pichia pastoris* [106], with yields reaching 2.5 mg per milliliter, β2-adrenergic receptor samples were prepared from Sf9 insect cells [112]. A further structure of bovine rhodopsin was recently obtained after being recombinantly expressed in mammalian COS-1 cells [113]. In particular, eukaryotic MP expression for structural approaches is still challenging, and no universal guideline for MP expression is evident [114].

There is a clear tendency towards only a few detergents that are regularly used for structural studies (Fig. 5). Approx. 80 % of the current MP structures have been solved using X-ray crystallography, while NMR structures are clearly the minority [115]. The list is thus mostly dominated by detergents optimized for crystallography, while detergents more suitable for NMR, such as DPC and SDS, are underrepresented. In crystallography, proteins with large extra-membranous domains are favoured because protein-protein contacts which form the scaffold of the crystal lattice

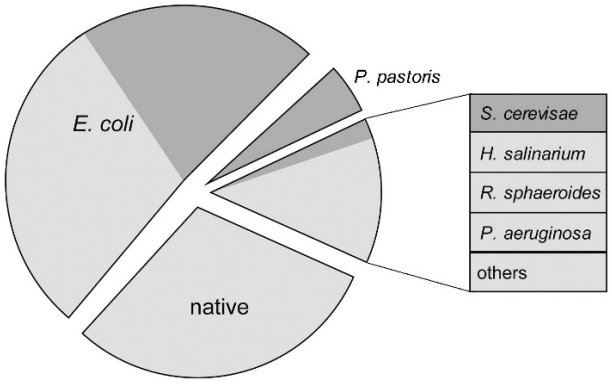

Figure 4. Expression hosts used for structural studies of MPs. The graphic illustrates the frequency of the expression hosts involved in successful structural approaches. Data are based on the Membrane Protein Data Bank, and only unique protein structures were considered. The native section comprises different hosts used for the non-recombinant isolation of naturally overproduced MPs. Recombinant systems resulting in only 1% or less of the MP structures are summarized and comprise *W. succinogenes*, *C. rheinhardtii, N. pharaonis, R. capsulatus, S. typhimurium*, *T. thermophilus*, Sf9 cells, COS-1 cells and CF extracts of *E. coli*. Homologous expressions are in light grey, while heterologous expressions are given in dark grey.

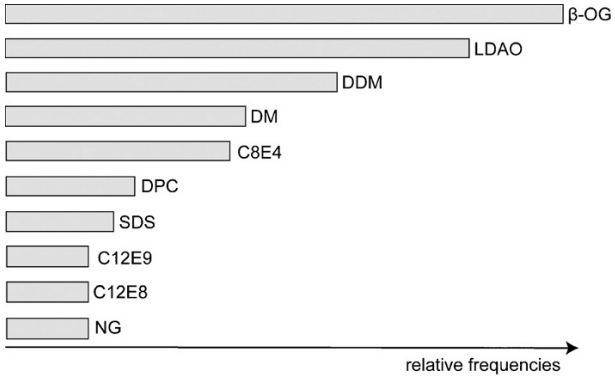

Figure 5. Detergents suitable for structural analysis of MPs. The evaluation is based on information deposited in the Membrane Protein Data Bank. The statistics include an initial number of 1211 structures, while for the figure only detergents with a frequency of >2% and representing a total of 535 cases have been further considered. Octyl-β-D-glucopyranoside (β-OG), dimethyldodecylamineoxide (LDAO), dodecyl-β-D-maltopyranoside (DDM), decyl-β-D-maltopyranoside (DM), octyltetraoxyethylene (C8E4), dodecylphosphocholine (DPC), sodiumdodecylsulfate (SDS), dodecyloctyoxyethylene (C12E8), dodecylnonaoxyethylene (C12E9) and nonyl-β-D-glucopyranoside (NG).

are found predominantly between the exposed, polar surfaces, while the transmembrane parts remain buried from the detergent micelle. This also explains why detergents that assemble into smaller micelles, such as β-OG or LDAO, are preferred in crystal trials [116]. Nonetheless, DDM, a detergent that forms large micelles, has given rise to quite a few structures as e.g. the aspartate transporter [117] as well as human FLAP. In order to study a MP with NMR, it must be solubilized with a detergent that allows fast isotropic tumbling in solution as do DPC and SDS. The structures of the two outer membrane β-barrel proteins, OmpG [118] and PagP [101], were solved in DPC. A few other detergents have shown beneficial properties with regard to sample lifetime and spectral quality, such as the lysolipids 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (LMPG) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (LPPG) [119], for which, however, no structures have been published yet.

The relatively strict requirement that the selected detergent must be compatible with the applied biophysical method could interfere with the attempt to maintain native folding of the MPs. Finding a suitable detergent that ensures protein homogeneity and stability but is also suitable for the chosen structural approach can become a limiting step. Addition of lipids or preparation of bicelles could support and stabilize the correct folding of MPs by providing a more natural environment if compared with pure detergent micelles. Bicellar environments are more or less compatible with X-ray and NMR, and novel approaches have been successfully applied [120] as demonstrated by the crystal structure of the β2-adrenergic receptor [108] and the NMR studies on the *Staphylococcus* multidrug resistance transporter [121].

Size and number of transmembrane segments do not seem to be primary determinants for successful structural analysis of MPs, at least by crystallization (Table 4). Apparently, the intrinsic secondary structure plays a basic role in the stability of a MP. This is e.g. illustrated by the fact that on average more β-barrel MP structures have been solved at high resolution than those of α-helical MPs. The β-barrel structure is characterized by a high degree of stability, as amino acid residues are completely connected by main-chain hydrogen bonds and thus form a stable scaffold [122]. On the other hand, α-helical MPs possess a high internal mobility with loosely packed helices, which reduces stability. The addition of specifically bound ligands could support and stabilize the globular and functional fold of MPs, and it might be advantageous in some cases to perform all purification and crystallization steps, or even the expression, in the case of CF systems, in the presence of ligands. Another difficulty in obtaining well-ordered crystals is the presence of only minimal hydrophilic, extra-membranous protein parts that are not capable of forming enough crystal contacts. To overcome this problem, strategies have been developed to enlarge the polar surface of the protein which include antibody fragments [112, 123] as well as addition of

internal polar fusions [108]. For NMR analysis, protein flexibility is less critical, but the limited chemical shift dispersion of α-helical structures results in a large signal overlap between 7.5 and 8.5 ppm proton chemical shift. In addition, the surrounding detergent micelles contribute to the overall molecular weight of the protein, causing slow tumbling rates and line broadening, which further impairs spectral quality. The development of selective isotope labelling strategies in combination with cell-free protein expression provides a promising way to overcome these overlap problems [57].

Cellular expression systems

Isolation of naturally highly abundant proteins will certainly be among the first methods of choice if such a system has been identified. In some cells or organisms, one or a few MPs are highly abundant, e.g. certain rhodopsin derivatives in *Halobacterum salinarium* and bovine eye retina. It has been attempted to use those naturally evolved high-level expression systems also for the production of closely related MPs. However, the efficiency of the expression pathway in *H. salinarium* appeared to be rather specific for bacteriorhodopsin and some of its derivatives. Other eukaryotic MPs even under control of the bacteriorhodopsin promoter were expressed only in spurious amounts [124, 125].

Consequently, the homologous expression of MPs in their natural cellular environments, preferentially with increased copy numbers after genetic recombination, appears promising in order to achieve optimal folding conditions, targeting and translocation of the target MPs, as all necessary machineries should in principle be present. Almost all established expression systems with clear emphasis on *E. coli* have been used for homologous expression. In addition, in a number of further not commonly used prokaryotes or lower eukaryotes, MPs in crystallization-grade quality could be obtained by homologous expression (Fig. 4) [126–133]. A promising option could also be the expression of MPs in hosts that are very closely related to their origin. The sucrose-specific porin ScrY was poorly expressed in *E. coli*, probably due to toxic effects. Its expression in the homologous host *Salmonella typhimurium* resulted in its structural determination by X-ray crystallography [134]. Overall, an estimate of 45% of the currently available MP structures originates from homologous expression approaches.

More critical, in particular with regard to the availability of efficient accessory helper systems, are heterologous expression approaches of MPs in non-related hosts or cellular backgrounds. However, powerful and efficient expression systems are difficult to establish, and the necessary technical developments, e.g. the construction of strains, vectors and controllable regulation mechanisms, are very demanding. Few elaborated and well-documented systems like expression in *E. coli*, yeasts or in insect cells are therefore prevalent for MP production, as their versatility, efficiency and the availability of reliable protocols dominates over potential disadvantages of not providing the optimal expression background (Table 1). Emerging systems such as expression in *L. lactis* or cell-free systems might become interesting options in the near future. In particular, cell-free expression systems represent distinct alternatives, as they enable completely new strategies for the production of MPs directly into detergent micelles (Table 1). Expression in *E. coli* is frequently the first and only choice for prokaryotic MPs, while eukaryotic MPs are often attempted to be produced in prokaryotic as well as in eukaryotic backgrounds. Reasons to switch to the latter more expensive and time-consuming systems are almost exclusively the intention to improve functionality or the overall quality of the MP samples. The following paragraphs intend to give an introduction to the production of functional MPs with the most common expression systems.

MP expression in *E. coli*

Based on its long tradition as a genetic model system resulting in a wealth of sophisticated and efficient genetic manipulation techniques in combination with simplicity and low cultivation cost, *E. coli* can be considered as the oldest and still most popular host for the overproduction of MPs (Fig. 1). A remarkable feature is its high versatility, providing numerous options for the optimization of expression protocols [135]. (I) A variety of vector and regulation systems can be selected in order to modulate the strength of expression and the copy numbers of the product and to induce expression at desired time points. Commonly employed promoters are among others the moderately strong arabinose promoter P_{BAD} [136], the strong hybrid *lac*/T5 promoter and the bacteriophage T7 promoter [137]. Expression is frequently controlled by insertion of *E. coli lac* operator sequences into the promoters and by releasing the bound LacI repressor by addition of isopropyl-β-D-thiogalactopyranoside at the desired time points of induction [138]. Increased *lacI* copy numbers either of chromosomal or episomal origin can help to strongly suppress background expression in non-induced conditions in case of e.g. toxicity problems. (II) A number of expression strains are available harboring specific mutations that could

boost MP expression. Strains such as C41 and C43 have been selected due to their tolerance for high-level expression of some toxic MPs [28]. Expression of certain MPs in particular in C43 (DE3) appears to be accompanied by proliferation of intracellular membranes, thus simply providing more space for MP insertion [139]. Strains devoid of abundant non-essential outer membrane proteins like OmpF and OmpC can be used for the expression and purification of other outer membrane proteins. (III) Co-expression of limiting factors required for folding or directing MPs to the membrane by vectors compatible with the expression vector is a powerful option [135]. Objects for co-expression could be genes encoding rare codon tRNAs [8, 140], chaperone systems [141, 142] or enzymes involved in PTMs. (IV) Proteolysis of MPs can be suppressed by changing to expression strains generally low in proteolytic activity such as BL21 derivatives. (V) *E. coli* tolerates a wide range of cultivation conditions covering media composition, temperatures and oxygen supply. The operator has therefore extended possibilities to modulate yield and folding of synthesized MPs.

In *E. coli* generally two principal strategies for MP production are possible, the expression as soluble MP inserted into the membrane or the expression as inclusion bodies (IBs) representing cytoplasmic aggregates. IB formation is mostly observed upon expression in *E. coli*, while probably yeast [143] and insect cells [144] are able to form similar structures. MPs deposited in IBs initially adopt non-native conformations, but their refolding by elaborated denaturation/renaturation procedures has been possible in several cases [122, 145]. The IB-based expression strategy can therefore be considered as an alternative approach for the production of functionally folded MPs. Clear benefits are that IBs are non-toxic to the cell and the precipitated MPs are protected against degradation by proteolysis, as reported e.g. for the expression of a porin from *Rhodopseudomonas blastica* [146]. In many cases, IB formation dramatically boosts MP expression up to several thousand-fold, and it is therefore particularly interesting for structural approaches. IB formation can be promoted by omitting signal sequences in the case of OMP expression [122] or by fusing MPs to distinct partners like GST [147]. This results in prevention of translocation and increased intracellular concentrations of the expressed MPs, both favouring aggregation and IB formation. The bottleneck of this strategy for obtaining functional MPs is to establish efficient refolding protocols. While for soluble proteins the refolding from IBs is routinely used and protocols have been developed employing dilution techniques as well as on column refolding, the information about MP refolding is sparse and mostly dominated by β-barrel proteins, as e.g. OmpG [118]. In principle, refolding procedures employ the following steps: (1) solubilisation in either a strong detergent, a chaotrope or in organic solvents, (2) transfer into a strong detergent such as SDS or N-lauroylsarcosinate with subsequent (3) reconstitution or exchange into a mild detergent for functional analysis as described by [145]. Refolding success could be monitored via migration behaviour by SDS-PAGE, and examples of β-barrel protein refolding are reviewed in [148]. In the case of α-helical MPs, IB refolding conditions have, for instance, been established for the light-harvesting complex (LHC) [149] and diacylglycerol kinase (DAGK) [150].

For the production of α-helical MPs, functionally folded expression and targeting into the cytoplasmic membrane are usually anticipated. An immense and rapidly increasing literature reporting successful MP expression in *E. coli* in levels sufficient even for structural studies is available (Table 4). However, despite the high number of reports, general guidelines are still difficult to conclude. For almost each new target, expression protocols were modified and optimized. Localization and choice of terminal tags and fusion partners, cultivation conditions and in particular tight control of expression appear to be the prime optimization parameters [151]. Weak or moderate promoters like P_{BAD} might be preferred to the strong T7 promoter [27, 152]. Fusions C-terminal to MBP appear to be a more common and promising strategy, but even multiple fusions of e.g. MBP and Trx with the neurotensin or neurokinin-2 receptors can finally succeed in producing functionally folded GPCRs and other MPs [43, 152–155]. Detailed expression conditions and yields of a larger number of secondary transporters of Gram-negative but also Gram-positive bacteria are compiled in [27], while successfully expressed GPCRs are listed in [43]. Which MP characteristics might be most important for successful expression is still being discussed. Large size and a high number of transmembrane segments do not seem to be as critical for successful production as assumed. However, the synthesis of smaller proteins was clearly preferred in an expression screen of 70 *Mycobacterium tuberculosis* integral MPs [156]. Bacteria often have difficulties with the expression, folding, stability and assembly of eukaryotic MPs. Success rates of throughput studies of eukaryotic MP expression are in between 10 and 30% if compared with 40–70% of eukaryotic systems [157, 158]. Nevertheless, *E. coli* is of high value for the functional high-level production of an already considerable variety of eukaryotic MPs [6, 159].

MP expression in Lactococcus lactis

L. lactis belongs to the group of aerotolerant Gram$^+$ lactic acid bacteria. At optimal growth conditions of approx. 30°C and at pH 6.8, the doubling times of 35–60 min are considerably fast. *L. lactis* has been well known from the food industry for almost 3 decades, and its potential as host for the overexpression of homologous and heterologous proteins has been explored [160, 161]. Easy and inexpensive cultivation, well-established genetic methods and vector systems, moderate proteolytic activity and efficient targeting of MPs into the cytoplasmic membrane explain why *L. lactis* is an interesting alternative expression host. General overviews are given in [80, 162]. However, the relatively small genome size of only 50% compared to *E. coli* results in deficiencies of several chaperone systems and other auxiliary factors that could be necessary for targeting and functional folding of particular MPs [80]. Furthermore, the *L. lactis* codon usage has an approx. 65% bias for AT base pairs. This may cause limitations, especially for the high-level expression of larger mammalian MPs as shown for the human KDEL receptor [163]. Codon optimization is therefore a first approach for improving expression efficiencies.

Commonly used *L. lactis* strains such as MG1363 or its derivative NZ9000 containing chromosomal copies of *nisRK* are multiple amino acid auxotrophs, making this host well suited for the labelling of proteins in defined media [80]. The tightly regulated *nisA* promotor derived from the bacteriocin nisin A causes no or only spurious background expression, allowing even the expression of toxic proteins when induced by the peptide nisin [76, 80, 163, 164]. The *nisA* promotor in combination with the nisin-sensing NisRK two-component signal transduction system is relevant for the NICE system expression strategy. Given high expression levels with recombinant MPs, >5% of total membrane proteins can be obtained after only a few hours of induction [80, 163].

Prokaryotic as well as eukaryotic MPs have been functionally expressed in *L. lactis,* while the total number of targets is still relatively low. The list comprises members of different MP families such as ABC transporters, major facilitators, mechanosensitive channels, peptide transporters, mitochondrial carriers and the human KDEL receptor. The activity of the MPs was evaluated by various transport or binding assays [80, 162]. The achieved production levels depend on the target proteins and range from <0.1% of total membrane protein for the human KDEL receptor, 1–2% for LacS from *Streptococcus thermophilus*, 5% for different yeast mitochondrial carriers and up to 5–30% for the homologously expressed secondary multidrug transporter LmrP and the ABC efflux pump LmrA, respectively [76, 165]. N-terminal truncations or modifications of the target proteins may be considered from the perspective of optimizing expression levels as well as functional folding as exemplified for several yeast mitochondrial carriers [80, 162, 163].

E. coli and *L. lactis* are currently the major prokaryotic hosts for the overproduction of MPs, and it is questionable whether they are alternative or complementary hosts. The overproduction of 37 transporters from 14 distinct families and originating from mesophilic and hyperthermophilic bacteria as well as from archaea was compared in the two hosts. For the chosen target proteins, the *E. coli* system appeared to be more reliable than *L. lactis* in cloning as well in expression. Of the successfully cloned targets, only 40% could be produced in *L. lactis* as compared to 84% in *E. coli.* This could indicate that *L. lactis* might be more sensible to the expression of toxic target proteins, while *E. coli* appears to be a more robust system. However, the implementation of more elaborated protocols, such as the use of plasmids encoding for rare codon tRNAs in case of the *E. coli*-based expressions, might have also contributed to this bias in successful expression [166].

MP expression in yeasts

Yeast expression systems combine prokaryotic as well as eukaryotic characteristics. Single cells, fast growth rates, inexpensive media, high cell densities and the well-developed variety of genetic tools make yeasts to an attractive eukaryotic host [167–169]. In recent years, yeasts have become the second most popular MP expression system after *E. coli*, with more cost effective than expression in mammalian or insect cell lines and often higher yields. Targeting and translocation systems and many of the second-messenger signalling pathways are similar to those in higher eukaryotes [170]. However, significant differences exist in membrane composition, in particular due to the absence of cholesterol, which might influence the functionality of mammalian MPs [10] as well as the success of crystallization trials [171]. Some eukaryotic-specific PTMs such as proteolytic processing and lipidation can be performed in yeasts [172]. However, glycosylation patterns are significantly different from their mammalian counterparts, and a common feature in yeast is hypermannosilation (Fig. 3), which can affect the proper folding and activity of heterologously expressed MPs [168]. In some cases, yeast-specific glycosylation pathways could be manipulated successfully in order to produce complex human glycoproteins with uniformally 'humanised' glycosylation patterns [173].

Widely used yeasts for the production of functional MPs are *P. pastoris*, *S. cerevisiae* and *S. pombe,* besides a few less frequently used hosts such as *Yarrowia lipolytica*, *Klyveromyces sp.* and *Hansenula polymorpha.* Since beginning of the 1990 s, the methylotrophic yeast *P. pastoris* has become one of the standard tools in molecular biology for the generation of recombinant proteins (Fig. 1). Its popularity is mainly based on its tightly regulated strong alcoholoxidase 1 (AOX1) gene promotor, its potential to grow to cell densities of OD_{600} ~ 500, and its availability in commercial kits [174]. The AOX1 promoter is repressed by glucose and can be induced more than 1000-fold by methanol used as a sole carbon source.

Complementation of yeast mutants represents an elegant option for the expression of functional transporters. This strategy has been used with particular success in the identification of various plant transporter genes [175]. The activity of homologous or heterologous transporters can be studied in cells lacking the corresponding endogenous transporters [168, 176]. For example, the identification of cells with a drug resistance phenotype then indicates functional production of efflux pumps [177]. Ion channels can be investigated by patch clamping with whole yeast cell membranes in order to study ionic currents and additionally offering the opportunity of directly comparing the activity of mutant proteins [178]. In the case of the water channel AQP1, the protein was correctly localized and fully functional in yeast secretory particles in contrast to expression in oocytes [179]. Already in the early 1990 s the human M1 muscarinic receptor [180], the rat M5 muscarinic receptor [181] and the human dopamine D2 receptor were produced in a functional conformation as analysed by radioassays of whole cells or isolated cell membranes. Activity of the human β_2-adrenergic receptor could be observed after co-expression with a mammalian G-protein subunit in *S. cerevisiae* by partial activation of the yeast pheromone-response pathway [182]. Expression could be improved by (I) replacing the 5′ untranslated region, (II) use of the strong GAL1 promotor and co-expression of its transcriptional activator and (III) induction in the presence of its ligand up to final amounts of 115 pmol of β_2-adrenergic receptor per milligram of total MP [182].

Yeasts are a preferred host for the production of GPCRs [183]. In a concerted approach to express 100 different GPCRs, more than 90 % of the targets were successfully produced in *P. pastoris* [158]. A systematic optimization screen was used to increase the production levels of 20 selected GPCRs in protease-deficient *P. pastoris* mutants [31]. Lowered expression temperatures, addition of specific ligands and the supplementation of other compounds such as histidine or DMSO were analysed for effects on yield and MP activity. Combinations of optimization parameters were successful, and 8 of the 20 tested GPCRs were finally obtained in amounts of more than 0.35 mg/l culture (20 pmol per milligram of total membrane) with a variation in specific binding constants from 50 pM to 38 nM [31]. Oxygen supply and defined pH values were further found to be valuable for the expression of functional GPCRs [30]. In addition, optimization of the expression conditions resulted in a severalfold difference in the production of the glycerol channel Fps1p [184]. In a comprehensive expression study of 25 human ABC transporters in *P. pastoris*, amounts between 3 and 5 mg/100 g of cells have been produced. In one example, the effect of supplied bile acids, lipids, glucuromides and modified pH values on the specific transport activity was evaluated [185].

Currently some 30 MP crystal structures have been solved after expression in yeasts with a clear bias on MPs isolated from *P. pastoris*, including the two best-resolved structures of mammalian MPs. The X-ray structure of the human leukotriene C4 synthase, a homotrimer with 4 TMS per subunit, was solved at 2.0 Å resolution for the apo form and at 2.15 Å for the complexed form [106]. In 2005 the crystal structure of the tetrameric mammalian *Shaker* family Kv channel Kv1.2 in complex with its β2 subunit was published, giving a deeper view into possible mechanistic properties, such as voltage sensors as self-contained domains [171, 186]. The complexed subunits were obtained by heterologous co-expression in *P. pastoris* with special concern for the supplied detergents and lipids as well as keeping a reducing environment during purification and crystallisation. For NMR analysis, yeasts represent an expression host with high potential, and structures of at least membrane-embedded domains or the analysis of glycosylation patterns might become feasible in future. Yeasts also grow on minimal media to high cell densities, thus allowing efficient ^{13}C and ^{15}N incorporation even in combination with deuteration [187].

MP expression in insect cells

Since its development in the early 1980s [188–190], cultivated insect cells in combination with vectors derived from the baculovirus family represent a major system for the expression of MPs (Fig. 1) [191]. Wild-type baculovirus particles have a narrow host range comprising only a few insect species. The most popular vectors are derivatives of the baculovirus species *Autographa californica* multiple capsid nucleopolyhedrovirus (AcMNPV), a double-stranded DNA virus surrounded by a membrane [21, 192]. After infection,

the virus uses the cellular protein biosynthesis machinery, and cell lysis occurs 4–5 days post-infection. Baculoviruses have a genome with approximately 130 kb, which also includes non-essential areas that can be replaced with even larger fragments of foreign DNA [20]. Expression is based on the construction of recombinant baculoviruses by replacing non-essential areas such as the polyhedrin gene with genes of interest by homologous recombination. Expression of the target genes is usually controlled by the very strong polyhedrin and p10 promoters, which both are very late promoters (8–24 h post-infection). This could be an advantage, as MP overexpression is often toxic for the host cell. The 'basic' protein is a structural core protein which is tightly associated with the virus DNA in the nucleocapsid, and its promoter is activated already in the late phase (6–8 h after infection) [193]. It has been used for the successful expression of correctly assembled MPs accompanied by reduced expression levels [194, 195].

Different insect cell lines are available as hosts for baculovirus. Amongst those are the most commonly used Sf9 and Sf21 cells derived from *Spodoptera frugiperda* ovarian tissue, high-five cells derived from egg cells of *Trichoplusia ni* and a *Mammestra brassicae* (Mb) cell line. High-five cells are assumed to increase the expression of MPs due to their higher capacity for protein secretion [196]. They are also bigger than Sf9 cells, thus providing more membrane space, and higher cell densities can be reached [40, 197]. Insect cells grow at optimal temperatures between 25 and 27 °C and do not require CO_2 as do mammalian cells. They have a relatively long generation time of 18–24 h and require complex growth media. Insect cells are able to grow in suspension as well as in monolayers and they can be adapted to serum-free media [196]. Scale-up procedures from initial 100 ml flasks are non-linear processes and several conditions like aeration and pH might have to be changed in order to preserve growth kinetics and expression levels. However, even 100 l fermentors are possible [198], and the MP bovine rhodopsin was functionally expressed in a 10 l bioreactor at amounts of 30–40 mg [199] (Fig. 2).

The construction of recombinant baculovirus vectors for protein expression is relatively time consuming and requires recombination events induced by co-transfection of recombinant transfer plasmids together with attenuated virus genomes into insect cells. The target DNA has first to be placed within a transfer plasmid under control of e.g. the polyhedrin regulatory region [200]. Recombination efficiencies after cotransfection can be improved by either using linearized baculovirus DNA [19] or mutant viruses containing deletions in essential genes like the commercially available BD BaculoGold derivative, where virulence can only be restored by recombination. Recombinant virus particles identified by plaque assays are isolated, and the infection/isolation cycle is repeated twice in order to obtain homogeneous recombinant virus particles. The whole process of vector construction can take several months (Fig. 2). Modified systems like the commercial Bac-to-Bac or BaculoDirect systems (Invitrogen) might accelerate the screening process by taking advantage of resistance markers and blue/white selection [19]. Post-translational modifications in insect cells including proteolytic processing, O-glycosylation, prenylation and phosphorylation are similar to those of mammalian cells (Fig. 3). One exception is N-glycosylation, which remains at the high mannose type [196]. Mammalian cell-specific glycosylation patterns could be achieved by using Mimic SF9 cells, which stably express a variety of mammalian glycosyltransferases, thus allowing the formation of bifurcated, terminally sialylated N-glycans in insect cells.

Expression protocols must be adapted to the individual MP targets by optimizing the insect cell type, the initial cell density at infection, the multiplicity of infection and the incubation time after infection [40] (Fig. 2). For 16 expressed human GPCRs, an extended incubation time after infection from 24 h up to 72 h resulted in increased protein production [197]. An interesting option might be to enhance functional MP expression by cotransfection of baculovirus-expressing chaperones [194]. Expression in insect cells was successful for the production of many MPs such as GPCRs, glycoproteins, aquaporins and members of the cytochrome p450 enzyme family [19, 195, 201]. Under optimized conditions, numerous GPCRs can be produced in functional form and in considerable amounts of several hundreds of picomoles per milligram of total membrane protein, which is sufficient for structural studies [22, 197, 199]. Screening for functional GPCR expression in SF9 insect cells by ligand binding assays is supported by low background due to low levels of endogenous G-proteins and GPCRs [202]. A very recent success in insect cell expression is the crystal structure of the β_2-adrenergic receptor at 2.4 Å resolution [108].

Expression in mammalian cells

Mammalian cells provide the most authentic cell environment for human MPs, and they are able to perform specific post-translational modifications (Fig. 3) [195, 203]. However, while the majority of proteins can be expressed at high quality in functional conformation, the obtained yields of MPs in mammalian cells are usually relatively low [15, 20]. Typical applications that employ mammalian cell expression systems are therefore functional characterizations of

MPs that can be carried out with relatively small amounts of protein.

Commonly used cell lines for protein expression are derived from e.g. monkey kidney (COS), chinese hamster ovary (CHO), baby hamster kidney (BHK)-21, human embryonic kidney (HEK)293, human epitheloid carcinoma (HeLa) as well as GH3 cell lines. The cells typically have an optimal growth temperature of about 37°C and require about 5% CO_2. Two major expression strategies are established, the transient and the stable gene expression (Fig. 2). For transient gene expression, the DNA template has to be transferred into the cell either by transfection of a plasmid containing the gene of interest or by infection of the cell with a recombinant virus e.g. vaccinia virus, simian virus 40 or Semliki Forest virus. Transfected cells are not selected, and the highest expression levels are usually achieved after 48–72 h. The highest expression yields and most efficient gene delivery are typically obtained by infection of cells with recombinant viruses, amongst others resulting from the strong promoters used in viral systems like the 11K promoter [204]. The disadvantages of this approach are cell death after some days and problematic scaling up, as efficient transfection occurs only when the cells grow in monolayers and not in suspension. Nevertheless, transient gene expression systems are faster to develop than expression in stable cell lines, and results can usually be obtained in 3–4 days. Transient expression is therefore suitable for initial expression screens, while higher protein quantities might later be obtained by fermentation of larger volumes of stably transformed cell lines [203]. Stable integration of foreign DNA into the cellular genome is a relatively rare event that can be identified by using a selection marker, e.g. antibiotic resistance or dihydrofolate reductase. Identified recombinant cells then have to be screened for best expression rates, as high rates of variation exist from cell to cell (Fig. 2). An interesting selection approach involves the co-expression of reporter proteins, e.g. GFP or antibiotic-resistant marker. An internal ribosome entry site (IRES) allows the translation of two open reading frames under the control of the same promoter. The IRES element is located downstream of the gene of interest, followed by e.g. the GFP reading frame. Co-expression of GFP can thus be correlated with the expression level of the target protein, and selection processes can rely on e.g. fluorescence measurements [205].

Optimization strategies for MP expression first focus on the choice of the host cell line, as they determine the efficiencies of the translocation machineries that support the correct trafficking of MPs to the membrane. For the expression of GPCRs by viral infection, BHK and COS cells have been shown to result in the highest yields, while stable expression was best in HEK293 and CHO cells [43]. Mammalian cell lines can be a good choice for the analysis of GPCRs because they provide endogenous G-proteins for interaction studies. However, e.g. CHO cells lack some of the G protein subtypes like $G_{i\alpha1}$ and $G_{0\alpha}$, which could lead to misinterpretation of coupling results with expressed GPCRs [206, 207]. Optimization of incubation temperature and pH could further be of importance. A small change in pH from pH 6.9 to pH 7.4 during the expression phase yielded a 2.5-fold increase in the serotonin receptor 5-HT_3 [208]. Several cell lines, such as HEK293, BHK-21, CHO-K1 and rat C6 glioma cells, can be cultured in serum-free medium and even grown in biofermentors [196, 204]. Exceptional are the preparative scale expression of different rhodopsin derivatives in stably transformed HEK293 cells at 3–9 mg/l culture [23] and the expression of COX2 in BHK cells with 16 mg/l active protein [204]. Sufficient amounts of a recombinant rhodopsin mutant were purified from COS-1 cells to determine its crystal structure [113].

Cell-free (CF) expression systems

CF expression is an emerging technique that offers promising new perspectives for the efficient production of MPs for functional and structural applications [51, 83, 209, 210]. Obvious advantages are that several principal problems of MP overproduction commonly occurring in living host cells are eliminated by using CF extracts (Fig. 2). Most toxic or growth inhibitory effects upon MP overproduction can be avoided. CF expression reactions are open systems allowing easy access at any time point. Stabilizers, protease inhibitors, ligands and virtually any other compound that might improve the expression or folding of a particular MP can be directly added into the expression reaction. No transport or selection mechanisms through cellular membranes need to be considered. Moreover, the cellular metabolism pathways are largely reduced in cell extracts, and metabolic conversion problems of supplemented compounds are minimized. Cellular membranes are eliminated during CF extract preparation, and therefore targeting and translocation problems of the recombinant MPs cannot occur. In contrast, the addition of detergents into CF expression reactions offers the unique possibility to synthesize MPs from the ribosomes directly into micelles. This expression approach is interesting in particular for structural approaches, as procedures for the

extraction of MPs out of cellular membranes can be avoided.

A remarkable characteristic of CF expression systems is their small volume of only few milliliters of reaction. Batch systems contain only one compartment, while the highest levels of MP production can be obtained with continuous-exchange cell-free (CECF) systems. In the CECF setup, the reaction is divided into two compartments holding a reaction mixture (RM) and a feeding mixture (FM) with constant volumes. The two compartments are separated by a semipermeable membrane with a molecular weight cutoff of 10–50 kDa. The RM contains all the high molecular weight compounds necessary for transcription and translation such as ribosomes, nucleic acids and enzymes, whereas the FM contains low molecular weight precursors like amino acids and nucleotides. Intensive stirring, rolling or shaking of the reaction device during incubation ensures an efficient exchange of fresh precursors from the FM against breakdown products from the RM through the membrane. CECF systems reach expression times of approx. 20 h and can yield amounts of up to several milligrams of target protein per 1 ml of RM.

Extracts of *E. coli* cells and of wheat germs have been used for the CF production of MPs and efficient protocols for cell extract preparation have been published [209, 211, 212]. The excessive dialysis of the freshly prepared extracts removes endogenous low molecular weight substances such as amino acids. Later in the reaction, the operator can therefore provide specified amino acid mixtures with regard to amino acid biases of the target MP or in view of combinatorial labelling approaches. The easy implementation of labelled amino acids in any combination is of particular value for structural approaches by NMR spectroscopy and by X-ray crystallography [213, 214]. CF extracts already contain all the important factors for translation such as ribosomes, aminoacyl-tRNA synthetases and translation factors. Precursors for transcription and translation, such as amino acids, tRNAs, NTPs and template nucleic acids, as well as efficient energy regeneration systems have to be supplied to the CF reactions [209, 211, 215, 216]. Numerous modifications of *E. coli* CF reaction protocols have been published in recent years that mainly address variations in energy precursors and strategies for the extension of reaction times in batch systems [217–219].

E. coli systems are coupled transcription/translation systems that are based on transcription by the highly processive T7 RNA polymerase. The target gene has therefore to be cloned under control of an appropriate T7 promoter [209]. Popular expression vectors suitable for CF systems are pET derivatives or vectors of the pIVEX series. Efficient protocols for the production of T7-RNA polymerase as a major constituent of the CF reaction are available [209, 220]. CF expression based on wheat germ extracts are usually operated as translation system by providing purified mRNA of the target protein as template [216].

Several strategies are described for the overexpression of MPs in CF systems. The target proteins can be expressed either as precipitate (P-CF) without providing hydrophobic environments or in the presence of supplied detergents (D-CF) or lipids (L-CF) [72, 209, 221–226]. In D-CF or L-CF systems, the operator creates artificial hydrophobic environments of defined composition directly in the reaction chamber, in which the nascent protein chain can be integrated. The final full-length MPs can then stay in a soluble form. The solubilization properties in D-CF reactions depend on the type of supplied detergent. Only a few detergents such as β-OG, DPC and sodium cholate appear to inhibit protein expression in D-CF systems already at low concentrations that hardly exceed their critical micellar concentration (CMC). Other commonly used detergents such as diC_8PC, DDM and Triton X-100 are tolerated by CF systems up to 10 times their CMC [227]. Exceptionally suitable for the soluble expression of even larger α-helical MPs like GPCRs are Brij detergents, relatively mild polyoxyethylene alkyl ethers, and the steroid derivative digitonin [225, 228]. The tolerance of CF systems for Brij detergents is very high and exceeds 100 times CMC. CF expression in P-CF systems is followed by detergent solubilization of the generated MP precipitates. Suitable for the resolubilization process are LMPG, but also DPC, DDM or SDS [83, 222, 225]. P-CF-generated MP precipitates appear to be different from the IBs already described from expression in *E. coli.* Instead of elaborated refolding processes, the simple solubilization of P-CF-generated precipitates in relatively mild detergents like DDM or DPC resulted in functionally active protein as shown for the multidrug transporter EmrE [83, 222]. However, only D-CF expression in presence of the detergent Triton X-100 yielded protein with high activity in the case of the nucleoside transporter Tsx [225]. Evaluating the most appropriate CF expression mode for particular MP targets in combination with the most suitable type of detergent is therefore an important optimization step [209]. An important characteristic of D-CF expression is that the synthesized MPs are instantly available in detergent solubilized form. The critical and often inefficient step of MP extraction out of cellular membranes is therefore completely avoided (Fig. 2). As soon as the reaction is terminated, the CF RM can be applied directly to the first purification column without further processing.

In the L-CF expression mode the protein synthesis is accomplished in the presence of liposomes that create a hydrophobic and probably more membrane-mimicking environment than detergents. A first systematic analysis of the CF expression of an MP into unilamellar liposomes of defined composition was given in 2007 with bacteriorhodopsin. One clear advantage of the L-CF mode is the complete inside-out orientation of the target MP. This simplifies functionality assays and enhances their sensitivity. Liposomes consisting of various bilayer-forming phospholipids such as dimyristoyl-, dipalmitoyl-, distearoyl- and dioleyl-phosphatidylcholine were tested on their effect of yield and ability to integrate bacteriorhodopsin. The thickness of the lipid bilayer, the phase transition temperature, the length of the acyl chains of the chosen phospholipids and their concentration seem to affect protein synthesis and integration. Dioleyl-phosphatidylcholine-integrated bacteriorhodopsin showed wild-type comparable photocycle and light-induced proton pump activity [72].

MP expression in *E. coli* CF extracts

Extract sources can be various *E. coli* strains such as BL21 derivatives or strains deficient in endogenous RNAses such as A19 or D10. MP targets that so far have been CF-expressed in functional forms comprise a variety of prokaryotic multidrug transporters such as EmrE [83, 214, 222, 229], β-barrel proteins such as the nucleoside transporter Tsx [225], the bacterial light-harvesting protein α-apoprotein of LH1 [223] and the mechanosensitive channel MscL [221]. The MPs could be expressed either in commercial or individual CF extracts in levels ranging from 0.5 to 4 mg/ml of RM depending on the chosen expression mode. Their functionality and quality were assessed by a variety of approaches, including patch clamp assays, oligomer formation and specific transport assays.

A putatively high potential resides in the CF expression of α-helical GPCRs, which have become one of the most important drug targets. Several GPCRs have already successfully been overexpressed in considerable quantity [224, 226, 230, 231]. A Trx fusion with the β2-adrenergic receptor also fused to its cognate $G_s\alpha$ protein yielded up to 1.1 mg/ml of RM within 8 h of expression, and its ligand binding was demonstrated by specific radioassays [224]. A further Trx fusion with the human histamine H1 receptor was produced in amounts of 3–5 mg/ml of RM and also showed specific ligand binding activity after reconstitution into liposomes [226]. ETB is another example of the rhodopsin-like family of GPCRs that has been overproduced in P-CF and D-CF systems in yields between 1 and 3 mg/ml of RM. The produced sample quality was evaluated by electron microscopy and by different types of ligand binding assays [81, 225, 231]. Evenly distributed particles of ETB putatively representing dimers were observed in negative stain electron microscopy, giving evidence of a sample homogeneity.

MP expression in CF extracts of eukaryotic cells

CF systems based on prokaryotic sources such as *E. coli* most likely lack the ability to introduce co- and posttranslational modifications in eukaryotic proteins which may be necessary for their functional folding or stability in membranes. In addition, the absence of eukaryotic chaperone systems might prevent the correct folding of particular targets. Approaches to address these potential problems are to supplement extracts with chaperones or microsomal fractions isolated from eukaryotic cells [232–234]. On the other hand, CF systems based on eukaryotic cell extracts like wheat germ or rabbit reticulocytes might be employed as further alternative for the production of MPs in the near future.

The wheat germ CF system is relatively robust, and protocols for the preparation of wheat germ extracts are well documented [212, 216, 235, 236]. The quality of wheat germ extracts depends highly on the origin of the wheat embryos and on the complete removal of the endosperm tissue, which contains high amounts of degradation enzymes and translation inhibitors. The stability of mRNA is relatively high in wheat germ extracts due to low endogenous nuclease content concomitant with polysome display, thus keeping protein expression ongoing for several days [212]. In contrast to the *E. coli* system, the wheat germ CF system does not work well as a coupled transcription/translation system, since magnesium optima for transcription are higher (~15–18 mM Mg^{2+}) than those for translation (around 3 mM Mg^{2+}) [237]. Translation systems are thus preferred with supplied mRNA as a template (Fig. 2). The basic components of wheat germ systems are similar to those of the *E. coli* CF systems with only minor differences [212, 238, 239]. Polyethylene glycol, which is used in *E. coli* CF systems in order to mimic the viscosity of the cytoplasm, is often replaced by polyamines like spermidine that may also be beneficial for the stabilization of DNA and RNAs and for the efficiency of the translational process [212, 240]. The translation efficiency of mRNA templates in wheat germ systems strongly depends on the nature of the 5′ and 3′ untranslated regions (UTRs), and they represent prime targets for optimization strategies [241–243]. Suitable expression vectors for wheat germ CF systems are the pSP65-derived plasmid pEU containing the SP6 polymerase promotor [242–244] or

pUC18-derived vectors containing the T7 RNA polymerase promotor. Adequate cloning protocols are available in the literature [244].

While high yields of cytoplasmic proteins can routinely be obtained by CF expression in wheat germ systems [212, 243, 245], the production of MPs is just emerging. The recent functional expression and reconstitution of plant solute transporters into liposomes based on experiments with *Arabidopsis thaliana* phosphoenolpyruvate/phosphate translocator 1 is one of the first reports of successful MP production in wheat germ CF systems [210]. A special bilayer technique was used representing a modification of common CECF systems [242]. The wheat germ CF system thus might develop to become a suitable alternative for eukaryotic MP expression.

In lysates derived from rabbit reticulocytes, a variety of co- and posttranslational modifications have been shown even in absence of microsomal membranes [246–249]. Besides N- and O-linked glycosylation [250, 251], observed modifications include lipid modification and acetylation [252, 253], signal peptide cleavage and proteolytic processing [249]. Rabbit reticulocyte extracts are usually prepared from lysed reticulocytes of rabbits pre-treated with phenylhydrazine. One advantage of this system is determined by its mammalian nature and consequently its close mimic of human cell environments [249]. However, CF expression in reticulocyte extract is characterized by low efficiency, and only a few MPs have so far been produced on analytical scale.

Perspectives of CF expression systems for MP production and analysis

Preparative-scale CF expression of MPs is a relatively new approach with first reports starting in 2004 (Fig. 1) [83, 222]. Nevertheless, a preliminary X-ray structure involving a CF-produced MP has already been presented with the multidrug transporter EmrE [214]. The absence of PTMs like glycosylation or lipidation could in principle contribute to sample homogeneity and might in general beneficial for successful crystallization. Disulfide bond formation as a common prerequisite for maintaining functional three-dimensional structures could easily be triggered by modulating the oxidizing conditions in CF systems. One concern is that CF-produced MPs do not pass distinct cellular quality control mechanisms, and this might cause sample heterogeneity in individual cases. Optimized protocols as well as new approaches and modifications like the recently published L-CF expression of MPs are rapidly emerging [72], indicating that CF expression technologies are currently a highly dynamic field.

The fast and highly efficient labelling of CF-produced MPs with stable isotopes in virtually any combination opens interesting perspectives for structural approaches by NMR spectroscopy. Combinatorial labelling schemes together with standard triple resonance measurements can now result in the fast backbone assignment and identification of consecutive amino acid pairs of even more complex α-helical MPs by measuring two-dimensional versions of HNCO experiments [57, 254]. The elimination of background labelling by CF production offers in addition the opportunity to measure labelled MPs directly in the RM without prior purification [255]. Amino acid specifically labelled MP samples even in deuterated form and ready for NMR analysis can thus be obtained in less than 2 days [256]. These significant advantages will enable the fast development of new techniques and modifications for structural approaches of α-helical MPs by solution or solid-state NMR [57, 227]. A similar advantage offers the efficient labelling of MPs with the non-natural amino acid selenomethionine for X-ray analysis [214]. The labelling properties offered by the CF expression technique could further be extended beyond stable isotopes to a higher variety of labels, i.e. fluorescent dyes or biotinylated moieties, and the incorporation of artificially labelled amino acids could be facilitated by modified versions of the suppressor tRNA/stop codon approach [257–259].

The absence of living organisms, reduced sterility problems and short reaction times recommend CF expression systems for throughput applications based also on robotic processes. While the automated expression and structural calculation of cytoplasmic proteins by CF expression is already a routine process [245, 260], a similar application for MPs has just been started [51]. Large libraries of clones could be screened for expression, solubility and function. The CECF system is a productive approach, but its setup is relatively complicated and cannot easily be transferred to microplate formats necessary for throughput applications. However, the productivity of batch mode CF systems has been considerably improved in recent times, and yields of several hundreds of micrograms of protein per millilitre of reaction are already possible [261, 262]. Further optimized reaction protocols and probably also additional extract sources might thus become more and more interesting to expand the applications for CF MP expression.

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