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Review

Membrane protein synthesis in cell-free systems: From bio-mimetic systems to bio-membranes

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ABSTRACT

When taking up the gauntlet of studying membrane protein functionality, scientists are provided with a plethora of advantages, which can be exploited for the synthesis of these difficult-to-express proteins by utilizing cell-free protein synthesis systems. Due to their hydrophobicity, membrane proteins have exceptional demands regarding their environment to ensure correct functionality. Thus, the challenge is to find the appropriate hydrophobic support that facilitates proper membrane protein folding. So far, various modes of membrane protein synthesis have been presented. Here, we summarize current state-of-the-art methodologies of membrane protein synthesis in biomimetic-supported systems. The correct folding and functionality of membrane proteins depend in many cases on their integration into a lipid bilayer and subsequent posttranslational modification. We highlight cell-free systems utilizing the advantages of biological membranes.

1. Introduction

Membrane proteins (MPs) are of fundamental importance in signal transduction, energy metabolism, transport processes and a variety of additional functions vital to the survival of organisms. Thus, it is not a coincidence that they form the major group of pharmaceutical targets [1–3]. Nevertheless our knowledge about MPs, their structure and function is limited although scientists have developed many sophisticated experimental setups to analyze them in detail. Individual MP-species are usually of low abundance in their biological environment and bio-physical characterization of these proteins is often difficult, due to their hydrophobic nature. Additionally, cells strongly regulate MP synthesis and control the overall protein balance according to the crucial requirements to keep their membrane integrity. Thus, a major challenge in MP studies is the preparation of sufficient amounts of correctly folded fully functional target protein. Conventional cell-based methods focus on over-expression strategies and thus often lead to insufficient membrane insertion, precipitation of *de novo* synthesized MP or even cytotoxicity due to the extensive alterations in the host cell's metabolism. In this context, the transformation of the biological protein synthesis machinery into a cell-independent synthesis system seems to be valuable. However, the function and activity of a given MP is not simply correlated to its high-yield production in itself, but rather depends critically on the suitable membrane environment. Important parameters regulating the embedded MP's function are on the level of the membrane: lipid composition, phase, tension, fluidity as well as curvature. Furthermore, on the molecular level of the lipid, parameters as the hydrophobic chain length, head group geometry, charge, hydrogen bonding potential as well as hydration strongly affect the bio-physical properties of the system. These membrane/lipid properties provide the framework for the adjustment of protein structure and function on various scales. For example, the structure of a lipid head group could determine locally the structure of the corresponding protein region via hydrogen bonding [4]. On a larger scale, the full hydrophobic surface of a protein will adapt to the hydrophobic core of the membrane and vice versa. Finally, energetically costly protein–lipid interactions

Abbreviations: a, membrane anchored; c, multimeric protein complex; CF, cell-free; CMC, critical micelle concentration; EF, elongation factor; ER, endoplasmic reticulum; GUVs, giant unilamellar vesicles; IF, initiation factor; LUVs, large unilamellar vesicles; MP, membrane protein; n.d., not determined; PURE system, protein synthesis using recombinant elements system; Ref., reference; RF, release factor; RRL, rabbit reticulocyte lysate; *Sf*, *Spodoptera frugiperda*; TMR, transmembrane region; WG, wheat germ

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can drive protein aggregation to higher order oligomers [5,6]. Effectively, the ability of the environment to promote certain protein conformations regulates the activity of a protein in a given setting. A large number of reviews documents the growing interest in protein-lipid interactions [some examples for hydrophobic mismatch: [7]; channels: [8,9]; G-protein-coupled receptors: [5,10]; membrane lateral pressure and curvature: [11]; membrane elasticity: [12]; cholesterol: [6]. This demonstrates that besides the appropriate expression system, additionally the MPs' environment has to be carefully chosen in order to obtain a correctly folded and functional protein.

2. Cell-free synthesis of membrane proteins

Cell-free (CF) systems provide the protein translation machinery gained from cell lysates thus enabling the *in vitro* synthesis of various target proteins independent of a living cell's integrity. Historically, CF systems were initially employed to unravel the genetic code [13]. Further studies used CF systems to characterize translocation processes of proteins across membranous boundaries or alternatively directed proteins into biological membranes (among others [14–17]). Since that time, a variety of sophisticated CF systems have emerged as promising alternatives to classical cell-depended MP over-expression strategies.

Currently several prokaryotic synthesis systems based on *Escherichia coli* cell-extracts have been reported. The scope ranges from the "protein synthesis using recombinant elements" (PURE) system, a minimal synthesis system using a set of purified elements required for the translation reaction [18], up to the complex "Cytomim" system. The latter utilizes not only a crude cell extract but inverted inner membrane vesicles from *E. coli* to efficiently activate oxidative phosphorylation and to improve protein folding [19]. Eukaryotic CF protein synthesis systems are gained from wheat germ (WG), rabbit reticulocyte (RRL) or *Spodoptera frugiperda* (*Sf*) cell lines. Furthermore, systems based on Chinese hamster ovary cells [20], mouse embryonic fibroblasts [21] as well as HeLa cell lines [22] are reported. A general benefit of CF protein synthesis systems results from their independence of cell viability, thus enabling the synthesis of difficult-to-express MPs as well as cytotoxic proteins [23–25]. Open CF synthesis reactions can be easily supplemented with a variety of additives, so-called compatible solutes, supporting protein synthesis, stabilization and last but not least providing a hydrophobic environment for MP embedding. In this review we briefly summarize various strategies supporting MP synthesis in the presence of detergents and other chemical additives. We focus on lipid and biological membrane-assisted approaches as these methods contribute a fundamental prerequisite to target MPs in the more biological environment of a bilayer.

2.1. Chemical additives for cell-free synthesis of membrane proteins in membrane depleted systems

Systems prepared from *E. coli* or WGs are lacking significant amounts of biological membrane structures. They are frequently used to systematically screen for suitable detergents and other membrane mimetic components for efficient MP synthesis and solubilization (among others reviewed in [26]). A typical approach in this context is the synthesis of target MPs as precipitates in the absence of solubilizing agents, followed by subsequent protein purification and re-solubilization using detergents. Thus, additive mediated negative influences on protein yields are avoided, but re-solubilization protocols are required. Compared to that procedure, protein synthesis in the presence of an appropriate additive promoting MP solubility seems to be a more straight forward strategy. Reaction supplementation with a hydrophobic environment enables the co-translational MP solubilization by the formation of proteo-micelle complexes. Essential requirements for successful CF MP synthesis in the presence of membrane mimicking amphiphilic supplements are, firstly, the compatibility of the applied reagents with the protein synthesis reaction itself and secondly, the concentration of these agents needed to form micelles (critical micelle concentration-CMC) thus enclosing the target protein (see also [26]). Both factors have to correlate to efficiently generate soluble target MPs suitable for subsequent functional characterization of the target protein. Frequently used detergents such as variants from the Brij- or Tween-series, DDM as well as Digitonin and Triton X-100 are capable of MP synthesis in presence of detergents (among others screened in: [27–30]). The micelle integrity and protein incorporation are strongly dependent on the CMC. Thus, downstream processing and further protein characterization always requires the maintenance of the adequate additive concentration, detergent replacement or even reconstitution of the target MP into a lipid bilayer system. For instance the mechano-sensitive channel MscL was synthesized in presence of detergents and subsequently purified and reconstituted into liposomes for further studies of its electrophysiological characteristics [27]. This approach requires the complete removal of the detergent to prevent detergent–lipid interactions and membrane damage.

Besides the classical detergents, other synthetic surfactants like fluorinated surfactants consisting of fluorinated carbon chains [31,32], high molecular mass amphiphilic polymers called amphipols [31,33] and lipid-like peptide-detergents [34,35] represent synthesis-compatible supplements facilitating MP production in a soluble form. Lipid-like peptide-detergents are comparable to the different Brij variants with respect to effectiveness [34,35]. Amphipols and fluorinated surfactants have been reported to be compatible with lipid structures, supporting direct MP reconstitution into membranous structures [36,37].

So far, several publications are available screening for solutes that are appropriate for co-translational synthesis of MPs in a soluble and best-case functional manner. For more detailed insight in this topic we recommend the following reviews and the included references (among others in [26,28,38]). Results presented in this broad range of publications demonstrate that compatible solutes represent suitable model systems for MP characterization in an environment that is much simpler compared to native membranes. However, having stated the importance of lipids and membrane structures for MP folding and functionality, it is desirable to directly integrate target proteins into a lipidic environment. This is preferable not only in respect to the difficulties occurring while transferring MPs from micelle complexes into bilayers, but also to enable co-translational lipid–protein interactions. Another disadvantage of membrane-depleted *in vitro* systems is their inability to produce proteins that include more complex posttranslational modifications like signal peptide cleavage, lipid-modifications and glycosylation. The complementation of exogenous enzymes to reengineer glycosylation pathways [39] as well as the addition of biological membrane vesicles [40] might additionally contribute to CF production of posttranslationally modified proteins.

2.2. Cell-free synthesis of membrane proteins in lipidic environments

Since a lipidic environment is an essential prerequisite for proper MP folding and functionality, we will now present established methods for the combination of lipidic environments with CF synthesis systems. In this field, different approaches have been successfully applied to synthesize MPs in presence of biomimetic lipid–detergent-based systems, nanodiscs, liposomes or even biological membrane environments, graphically summarized in Fig. 1.

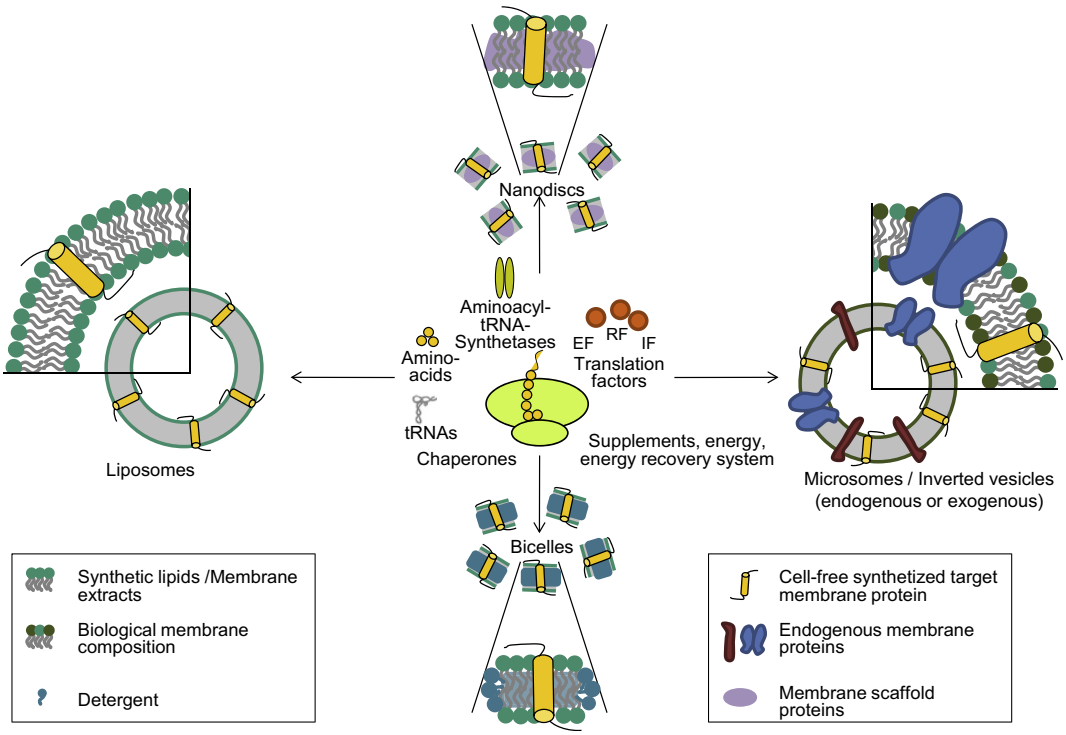

Fig. 1. Cell-free synthesis of membrane proteins in presence of different lipid based membrane structures. Pro- and eukaryotic cell extracts supply the entire translational machinery as well as chaperones. The membrane proteins synthesis can be supported by the supplementation of the reaction mixture with synthetic structures as liposomes, nanodiscs or bicelles. Alternatively, biological membrane structures as microsomes or inverted vesicles provide endogenous proteins thus enabling the biological process of co-translational translocation. EF: elongation factor, IF: initiation factor, RF: release factor.

2.2.1. Cell-free synthesis of membrane proteins supported by lipid–detergent systems

Bicelles are assemblies of phospholipids and detergents in a flattened, disc-like shape. The exact morphology depends critically on the lipid–detergent ratio (summarized by [41]). Usually the detergents surround the lipids in the center, like a rim and thus shelter the hydrophobic elements from water. Short-chain phospholipids can be used to replace the detergent rim in order to more closely mimic structures which resemble membranes. Thus, bicelles form an intermediate between micelles composed of pure detergents and lipidic membrane structures. Lyukmanova and coworkers demonstrated that lipid–detergent bicelles on one hand reduced the yield of various CF synthesized MPs compared to the investigated detergent micelles, but on the other hand supported synthesis of correctly folded proteins in some cases [30]. Moreover, membrane subunits of a prokaryotic ATP-synthase have been produced by CF synthesis in the presence of bicelles [42]. In this system, subunit *a* was shown to have a similar folding compared to the native protein extracted from bacterial cells. Nozawa and coworkers reported a methodology for the rapid screening of various structurally divergent MPs for their co-translational insertion into liposomes formed from phospholipids in presence of detergent in a WG CF system [43]. However, the presence of detergents in these systems might hamper their compatibility with membrane structures and have an effect on the protein's functionality. Consequently, for example the reconstitution of bacteriorhodopsin gained from lipid–detergent supplemented CF reactions into liposomes required the removal of the initially added detergent to asses MP function in the lipid bilayer [44].

2.2.2. Cell-free synthesis of membrane proteins in presence of nanodiscs

Nanodiscs are one of the membrane soluble supplements which could be added directly to the CF reaction mixture. They are nanoparticles consisting of a discoidal phospholipid bilayer encircled non-covalently by two copies of a membrane scaffold protein, which itself constitutes a modified apolipoprotein [45]. Depending on the length and type of the membrane scaffold protein, the diameters of the nanodiscs vary from 10 to 20 nm [46,47]. The size of nanodiscs can be measured by dynamic light scattering or transmission electron microscopy [48]. The MP will incorporate into the nanodiscs in a passive manner during CF synthesis, where the membrane scaffold protein additionally stabilizes the incorporated MP. Apart from providing stability to the MP, nanodiscs in general have several advantages over conventional solubilization agents [49,50]. Proteins can be extracted from nanodiscs in a native functional form. They are accessible from both sides which could help in studying the ligand binding interactions as well as binding of signaling molecules on the cytoplasmic side. Moreover, the membrane composition can be defined by a wide range of lipids which could influence the functionality of the protein [30,51]. Compared to other solubilization agents, protein decorated nanodiscs are monodisperse and homogenous [52]. A crucial advantage of nanodiscs is that, once formed, they sustain the soluble target MP in a detergent-free environment. Hence, the following purification and functional analysis are not restricted to detergent based limitations as in the case of bicelles. Furthermore, the protein purification procedure in this system is simple due to a His-tag introduced by the membrane scaffold protein. Nanodiscs have been explored for a wide range of applications (among others reviewed in [50,52]). Using the excellent solubilization properties of nanodiscs, their use as vaccine delivery platforms with increased immune stimulation was presented recently [53,54]. Due to their easy-to-handle characteristics, nanodiscs are now used as promising molecular tools to explore the functionality of G-protein-coupled receptors [55]. Very recently nanodiscs were doped with light converting-proteins. They were used for creating the first synthetic photo-electrochemical complexes capable of converting

solar energy into electrochemical currents [56]. One approach across the systems presented in this review is the combination of nanodisc- and bicell-technology to study MP oligomerization [57]. Apart from preserving the native configuration of MPs, nanodiscs create a detergent-free environment that is also suitable for mass spectrometry analysis [58]. Although nanodiscs are promising structures for the solubilization of MPs, they have their own limitations. As these particles are accessible from both sides and due to the orientation-independent statistical incorporation of the CF-produced MPs into these entities, nanodiscs are not suitable for applications regarding transporter assays or ion channel characterization. Additionally, they are limited in size and thus do not allow for the insertion of a complex cluster of MPs into the same disc.

2.2.3. Cell-free synthesis of membrane proteins in presence of liposomes

Liposomes are spherical membrane vesicles formed from either synthetic lipids or biological lipid extracts usually prepared by disintegrating biological membranes. After the integration of MPs into liposomal membranes, they are termed proteo-liposomes. Interestingly, it was already demonstrated in 1985 by Geller and Wickner that the pro-coat protein from the phage M13, is co-translationally translocated across the membrane of bare liposomes in absence of any additional MP [59]. Since that time, many other functionally and structurally divergent MPs have been synthesized in CF systems supported by liposomes (see Table 1). In most cases protein production and co-translational insertion into lipid-based structures was performed in *E. coli* and WG-derived synthesis systems.

Even to the point that some MPs display functionality in presence of detergents and other solutes, there are some proteins that are well known to require lipids to attain maximum function. For instance, the mitochondrial ADP/ATP carrier [60] and KcsA [61] are reported to contain lipid molecules in their crystal structures. The presence of specific lipids is essential for both proteins to gain their function ([62,63], respectively). Thus, these lipids act as co-factors for the proteins. Long and coworkers demonstrated in a WG system that ADP/ATP carrier integration into a liposome's bilayer is based on an obligatory co-translational mechanism, even in the absence of membrane-embedded translocation complexes. Moreover, it was found that beyond the functionality of the ADP/ATP carrier even the efficiency of protein synthesis itself as well as its membrane insertion are cardiolipin dependent in the utilized CF system [64]. Another example shows that membrane association and tetramerization of KcsA are supported by phosphatidylethanolamine and phosphatidylglycerol applied to the system in a ratio similar to the protein's native prokaryotic membrane environment [65]. Moreover, phosphatidylethanolamine acting as a molecular chaperone is reported to assist for example the proper protein folding of LacY [66].

The influence of the thickness of the lipid membrane as well as the effect of the membrane-spanning region of model MPs on the hydrophobic matching have been studied extensively in a liposome-supported CF system by Ridder and coworkers. It was found

Table 1
Examples for co-translational insertion of membrane proteins into lipid-based vesicular structures in cell-free systems in the absence of detergents.

Membrane protein (explanation)	TMR	Cell-free system + vesicular structure	Protein characterization	Ref.
α-bENaC (Na+ channel)	2	RRL + microsomes	Electrophysiology	[83]
ADP/ATP carrier (mitochondrial)	6	WG + liposomes	ATP transport + inhibition	[64]
Apo cytochrome b5-DHFR (dihydrofolate reductase chimera)	1	WG + giant liposomes	Enzymatic activity DHFR	[110]
Aquaporin Z (pore forming)	6	*E. coli* + liposomes	Water permeability	[72,111]
ATP synthase (complex)	c	*E. coli* + liposomes	Membrane incorporation	[102]
β2AR (β2-adrenergic receptor)	7	RRL + microsomes	Ligand binding	[81]
Bacteriorhodopsin (proton pump)	7	*E. coli* + liposomes	Photocurrent generation	[68]
Bak (pro-apoptotic)	1	*E. coli* + liposomes	Apoptosis induction in cells	[99]
BmOR1 (olfactory receptor)	7	Insect + microsomes in GUVs	Electrophysiology	[86]
Connexins (diverse variants - gap junctions)	4	RRL + microsomes	Electrophysiology	[80]
Connexin 43 (gap junctions)	4	PURE-system + liposomes	Membrane permeability	[69,97]
		RRL + liposomes		[98]
CrdS (curdlan synthase)	7	WG + liposomes	Enzymatic activity	[70]
CXCR4 (chemokine receptor)	7	Insect + endogenous microsomes	Diffusion in hybrid-GUVs	[87]
Cytochrome b5 (electron transport)	1	WG + liposomes	Enzymatic activity	[103]
Stearoyl-CoA desaturase (lipid biosynthesis)	4			
ETB (endothelin receptor)	7	Insect + endogenous microsomes	Diffusion in hybrid-GUVs	[87]
FtsQ (cell division)	1	PURE-system + inverted vesicles	Membrane incorporation	[73]
GPAT (phospholipid synthesis)	n.d.	PURE-system + liposomes	Enzymatic activity	[104]
LPAAT (phospholipid synthesis)	a			
Hb-EGF (growth factor)	1	Insect + endogenous microsomes	Diffusion in hybrid-GUVs	[87]
IP_3Rs (inositol trisphosphate receptors)	1/3	RRL + microsomes	Hetero-oligomerization	[84]
KcsA (potassium channel)	2	*E. coli* + LUVs	Tetramerization	[65]
		Insect + endogenous microsomes	Electrophysiology	[88]
MraY (translocase)	10	*E. coli* + liposomes	Enzymatic activity	[71]
MscL (mechano-sensitive channel)	2	*E. coli* + liposomes	Electrophysiology	[76,77]
MtlA (mannitol permease)	6	PURE-system/*E. coli* + inverted vesicles	Membrane incorporation	[73,75]
nAChR (nicotinic acetylcholine receptor)	4	RRL + microsomes	Electrophysiology	[85]
pOmpA (pore forming)	8	PURE-system + inverted vesicles	Membrane incorporation	[73]
PulD (outer membrane secretin)	n.d.	*E. coli* + liposomes	Multimerization	[112]
Shaker potassium channel	6	WG + liposomes	Electrophysiology	[100]
		RRL + microsomes		[82]
TetA tetracycline pump	12	*E. coli* + inverted vesicles	Substrate transport	[75]
VDAC (pro-apoptotic anion channel)	19	*E. coli* + liposomes	Apoptosis induction in cells	[99]

a: Membrane anchored; c: multimeric protein complex; GUV: giant unilamellar vesicles; LUV: large unilamellar vesicles; n.d.: not determined; PURE system: minimal synthesis system based on purified translation components from *E. coli* cell-extracts; Ref.: reference; TMR: transmembrane region; WG: wheat germ; RRL: rabbit reticulocyte lysate.

that the bilayer insertion of a single membrane-spanning region depends primarily on the membrane thickness, caused by the hydrophobic chain length. Furthermore, the hydrophobicity of the relevant amino acids and the overall-length of the transmembrane region also regulate this process [67]. For bacteriorhodopsin, the ramifications of the chain length and the transition temperature were evaluated and additional parameters like membrane curvature and lateral membrane tension were discussed [68]. The effect of different lipids on the protein integration and functionality was also studied by several other groups [69–71].

Even though integration of MP into liposomes is possible and may under certain conditions result in MP functionality, the entire biological set of membrane-attached and membrane-spanning proteins cannot be addressed using a uniform lipid-embedding technology. Moreover, the lipid complexity in many cases is partially reduced in these synthetic systems. The lack of complex biological multipliers doubtlessly has considerable impact on a given MP's insertion mechanism. Thus, MP integration into bare synthetic membranes is based on passive processes. In terms of combining synthetic membrane structures with CF synthesis, additional advantages resulting from the remaining complexity of the membrane-depleted cell lysates can be considered. These lysates do not only provide the entire translation machinery, but additionally soluble chaperones, signal recognition particles and further essential factors are present, depending on the lysate's pro- or eukaryotic origin. It is likely that these chaperones for example bind to the target MPs supporting their passive integration into liposomes even in the absence of membrane-embedded translocon complexes. These aspects are discussed in more detail by Long and coworkers in the context of the ADP/ATP carrier synthesis [64].

Surprisingly, the supplementation of the liposome-supported Cytomim system with the membrane-associated signal recognition particle and its receptor, only increased the yield of CF produced Aquaporin Z and its liposomal association, but did not affect the protein's activity [72]. This demonstrates that the insertion of target proteins into liposomes might in some cases be independent of the biological co-translational translocation process, as shown in the case of Aquaporin Z. Yet, these passive insertion processes do not necessarily trigger the protein's functionality.

In terms of the *E. coli* based minimal PURE system, soluble chaperones and other factors are depleted, thus enabling one to evaluate their effect on target protein folding and translocation by supplementation studies [73,74]. It is reported that different chaperones are required for the translocation of various MPs into biological membrane vesicles [73]. These and other results [75,76] claim the distinct influence of defined soluble chaperones on a given protein's translocation across membranes. The impact of membrane bound translocation components non-attendant in synthetic liposomes, but inherently present in biological membranes, additionally has to be considered for proper MP targeting [73,75,77].

2.2.4. Cell-free synthesis of membrane proteins utilizing biological membranes

Biological membranes are sophisticated entities mainly composed of lipids and proteins. Their structure depends basically on their pro- or eukaryotic origin as well as their sub-cellular localization, in particular in the case of membranes of eukaryotic origin. The biological process of MP translocation in viable cells is a complex mechanism recruiting a huge set of diverse soluble and membrane-integrated proteins. These mechanisms are highly conserved and the general procedure of co-translational insertion via a protein-conducting channel, in general termed translocon, is similar in pro- and eukaryotes. However, the proteins usually involved in this process are well known and the detailed translocation mechanisms are summarized in several reviews (among others in [78,79]). Taking advantage of these complex mechanisms in CF systems is an option to provide an optimal environment for the efficient synthesis of functional MPs that is even more biomimetic than all other systems we have described so far. Examples for successful applications have been included in Table 1 [73,75,80–88].

Regarding the PURE system and additional *E. coli*-based systems, inverted vesicles gained from *E. coli* inner membranes served as useful tools for proper targeting of proteins to their native environment due to the implementation of membrane-bound prokaryotic translocation components [73,75]. The inversion of these *E. coli* inner membranes enabled open access of CF systems to the MPs located at the cytosolic side, facilitating the process of co-translational translocation.

Microsomes present in eukaryotic CF systems are membranous vesicular structures derived from a eukaryotic cell's endoplasmic reticulum (ER). Common sources for microsomes supplemented to eukaryotic rabbit reticulocyte or WG CF systems are dog pancreas cells [80,84,89,90], oocytes [81,85] or oviduct cells [82]. The complex luminal and membranous proteome composition of dog pancreas microsomes has been intensively studied and well characterized [91–93].

In terms of CF systems derived from cultured insect cells, endogenous microsomes are generated during lysate preparation. As consequence, they do not have to be added separately [94]. This homogenous eukaryotic system facilitates posttranslational modifications such as glycosylation [94,95] and lipid modification [96]. Deploying the electroswelling process, the endogenous microsomes can be used to integrate the MP of interest into giant unilamellar vesicles (GUVs) [96]. Furthermore, the acceleration of this swelling methodology by lipid supplementation enables one to modify MP harboring microsomes with synthetic lipids thereby gaining so-called hybrid-GUVs [87]. Additional techniques for further functionalization and immobilization are available, either to embed GUV-membranes into technical processes, or to incorporate proteins in synthetically modified microsomal membranes.

Although microsomes are obtained from sub-cellular membranes, thus carrying endogenous MPs, they conveniently enable the analysis and functional characterization of the *de novo* synthesized proteins. For example, using microsomes as micro-containers, it is possible to introduce *de novo* synthesized ion channels into planar bilayers immediately followed by a detailed electrophysiological characterization on the single molecule level [80,82,83,85,88]. The presence of endogenous MPs, such as the inositol trisphosphate receptor, not only demonstrates the microsome's ER-based origin, but further enables the functional analysis of ER-resident proteins [88]. Additionally, endogenous channels can serve as internal control proteins to monitor the fusion of ER-derived microsomes to planar bilayers. Even the combination of endogenous MPs and additional CF-produced target proteins to build up complex signaling cascades is a challenging option. Although early approaches in the field of CF protein synthesis utilized biological membranes, only few publications focused on the application of this combination (among others summarized in Table 1). One aspect that has to be considered in this context is the increasing complexity that biological membranes introduce to a CF system, thereby impeding the potential to run the experiments under extremely defined conditions. Eukaryotic ER-derived microsomes however offer a window towards much more complex cellular functionality. Due to their sophisticated structure and composition they enable one to implement e.g. biological glycosylation processes into CF-systems and such glycosylations often determine protein functionality. Some representative examples of microsome mediated MP glycosylation are the human β-adrenergic receptor [81], the Shaker potassium channel [82], different

variants of connexins [80], inositol trisphosphate receptors [84] and the nicotinic acetylcholine receptor [85].

3. Conclusion and outlook

CF systems offer a huge variety of different reaction modes and recent advances have made possible cost-effective micro-scale to manufacturing-scale synthesis of complex MPs. The open systems offer a versatile environment for direct manipulation and optimization of parameters fitting the individual requirements of specific target MPs. In some cases the origin of the protein had to be considered to choose for instance the necessary lipids for the model membrane composition embedding the *de novo* synthesized MPs [64,70]. Due to the amazing range of options to control and adjust CF systems to the protein's requirements, a remarkable amount of diverse approaches to build up biomimetic environments for MP synthesis and analysis have been established. In general, the success of the different strategies seems to be strongly dependent on the individual MP properties. Additionally, the requirements for the follow-up protein characterization strategies have to be considered in terms of choosing a compatible CF system.

Here, we presented different strategies to gain the target MP in a membranous environment without applying any additional reconstitution steps. Due to their spherical shape, synthetic liposomes and microsomes directly allow to analysis of the transport activity [69,97,98]. Even the application of liposomes in or on living cells is reported [98–101]. Liguori and coworkers for example demonstrated the liposomes' ability to deliver cell-free synthesized apoptotic MPs to cells thus inducing apoptosis. This system was proposed to facilitate the delivery of therapeutic MPs for cancer treatment [99]. These publications demonstrate that the compatibility of proteo-liposomes harboring CF-synthesized MPs with cell culture systems provides a wide scope for interaction and delivery-studies on viable cells. Moreover, the simultaneous synthesis and proper assembly of the large heteromeric protein complex ATP-synthase in CF systems was demonstrated by Matthies and coworkers [102]. Additionally, the combination of various MPs into the same set of liposomes or microsomes was demonstrated for other complex protein combinations as well [87,103,104]. These developments demonstrate that CF protein synthesis in combination with membranous vesicles might be a route towards the building up e.g. entire signal transduction pathways for ligand detection or protein interaction studies. Utilizing natural membrane structures these systems may put a spin on the fundamental mechanistic understanding of biological processes connected to MPs. Compatible solute- or nanodiscs-based systems do not offer this opportunity, due to their intrinsic limitations.

Another promising application is the encapsulation of CF synthesis systems into phospholipid vesicles (among others reported by: [86,104–108]. Hence, these studies constitute an important first step towards the design of a minimal cell. These cell models even enabled the synthesis of α-hemolysins, their integration into the provided membrane and consequently the nutrient supply of the synthesis reaction by the formed pores [107,108]. The encapsulation of the CF reaction mixture further empowered the functional synthesis of MPs involved in the phospholipid synthesis pathway [104]. Nevertheless, the combination of the machineries required for sufficient DNA-replication, transcription and translation is rather challenging in terms of building up a minimal cell. Noteworthy is for instance the fact that these complex mechanisms have different requirements in terms of optimal reaction conditions or the coordination of the synthesis of different proteins has to be engineered (summarized by: [109]). A different approach to convey a cellular function into a cell model is based on the endogenous microsomes derived from an insect-based CF synthesis system [87]. The formation of tailored hybrid-GUVs harboring a variety of MPs may facilitate for instance the reconstitution and characterization of selected signal transduction pathways in a well-defined and cell-sized biomimetic environment.

Choosing the appropriate hydrophobic environment during CF MP synthesis is a critical point to ensure proper protein folding and high insertion efficiency. Since each MP of interest usually requires an individual hydrophobic mixture, detecting the optimum can be time-consuming when several lipids and other solutes have to be added simultaneously. Hence, eukaryotic synthesis utilizing biological membranes like the insect based endogenous microsomes or the addition of inverted vesicles to *E. coli* or WG systems provide a convenient alternative. They introduce additional options in terms of MP studies by their natural membrane elements. Thus, the toolbox of CF protein synthesis is now expanded by a sophisticated biological component. Given the exquisite capability to modify and adapt CF systems for high-throughput, cost-effective and high-level MP synthesis, this technology should resolve a growing number of applications in near future.

4. Conflicts of interest

The authors declare that there are no conflicts of interest.

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