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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.