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Cell-free synthesis of membrane proteins: Tailored cell models out of microsomes ☆

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ABSTRACT

Incorporation of proteins in biomimetic giant unilamellar vesicles (GUVs) is one of the hallmarks towards cell models in which we strive to obtain a better mechanistic understanding of the manifold cellular processes. The reconstruction of transmembrane proteins, like receptors or channels, into GUVs is a special challenge. This procedure is essential to make these proteins accessible to further functional investigation. Here we describe a strategy combining two approaches: cell-free eukaryotic protein expression for protein integration and GUV formation to prepare biomimetic cell models. The cell-free protein expression system in this study is based on insect lysates, which provide endoplasmic reticulum derived vesicles named microsomes. It enables signal-induced translocation and posttranslational modification of *de novo* synthesized membrane proteins. Combining these microsomes with synthetic lipids within the electroswelling process allowed for the rapid generation of giant proteo-liposomes of up to 50 μm in diameter. We incorporated various fluorescent protein-labeled membrane proteins into GUVs (the prenylated membrane anchor CAAX, the heparin-binding epithelial growth factor like factor Hb-EGF, the endothelin receptor ETB, the chemokine receptor CXCR4) and thus presented insect microsomes as functional modules for proteo-GUV formation. Single-molecule fluorescence microscopy was applied to detect and further characterize the proteins in the GUV membrane. To extend the options in the tailoring cell models toolbox, we synthesized two different membrane proteins sequentially in the same microsome. Additionally, we introduced biotinylated lipids to specifically immobilize proteo-GUVs on streptavidin-coated surfaces. We envision this achievement as an important first step toward systematic protein studies on technical surfaces.

1. Introduction

Cells are complex entities with a large variety of active and passive components that serve tasks from defining the structural integrity of the cell to the emergence of cell decision-making through complex and intertwined signaling pathways. As of the sheer complexity our knowledge about the detailed functioning of a cell is still at its infancy. Following Feynman's challenge of "what I cannot create, I do not understand" [1] the construction of cellular functionality into an artificial system is a major challenge that will supposedly lead us to the learned understanding of cellular behavior. In this endeavor well-controlled experiments on biomimetic systems are the essential steps towards a mechanistic understanding of cellular function (for reviews see [2–5]).

One of the simplest cell models, a giant unilamellar vesicle (GUV), consists only of a spherical lipid bilayer enclosing an aqueous buffer that has a size of up to several tens of micrometers in diameter. While GUVs already extensively served as biomimetic models to study lipid-based membrane processes [5], a whole new set of cellular functionality will become accessible upon introduction of functional proteins that are part of cell-signaling pathways into this well-characterized model system. Here, we

Abbreviations: GUVs, Giant unilamellar vesicles; GPCR, G protein-coupled receptor; eYFP, Enhanced yellow fluorescent protein; ETB, Endothelin B receptor; CXCR4, C-X-C chemokine receptor type 4; Hb-EGF, Pro-Heparin-binding epithelial growth factor like factor; ER, Endoplasmic reticulum; E-PCR, Expression polymerase chain reaction; TCA, Trichloroacetic acid; TMD, Transmembrane domain; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; ITO, Indium tin oxide; DOPE biotin, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl); msd, Mean squared-displacement; *D*, Diffusion coefficient; *E. coli*, *Escherichia coli*; exp., Experimental value; theo., Theoretical value; CFP, Cyan fluorescent protein; PDMS, Polydimethylsiloxane; δ, One-dimensional localization precision; D, Diffusion coefficient; n, Number of integrated target membrane proteins; N, Number of single molecules detected per movie; μ′, Medium's viscosity; μ, Membrane's viscosity; k_B, Boltzmann's constant; T, Temperature; b, mobility; h, Membrane thickness; ε, Reduced radius

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concentrate on controlled incorporation of various membrane-anchored and transmembrane proteins with an emphasis on G protein-coupled receptors (GPCRs). GPCRs are membrane proteins of interest for both academia and the pharmaceutical industry as primary drug targets.

A protocol to produce pure lipid GUVs in sucrose solution has been introduced in 1986 [6] and has become a standard preparation technique since. Incorporation of transmembrane proteins into GUVs however is non-trivial and requires specialized protocols that vary for each protein. Early attempts to address a more ubiquitous approach include detergent assisted protein insertion in the membrane of large liposomes [7] in combination with fusogenic peptides [8], and the electroswelling of preformed large proteo-liposomes [9,10]. Only recently, cell-free protein expression systems based on *Escherichia coli* (*E. coli*) were used to prepare small proteo-liposomes that spontaneously fuse with giant liposomes to achieve giant proteo-liposomes [11]. More research on the combination of both techniques, GUV preparation and cell-free protein synthesis, so far focused on the role of vesicles as reaction containers to encapsulate the protein synthesis machinery in synthetic lipid or polymer membranes [12–14]. However, such *E. coli*-based *in vitro* systems do not permit to produce mammalian proteins that include posttranslational modifications like glycosylation without e.g. the addition of exogenous enzymes to reengineer glycosylation pathways [15].

In recent years manifold pro- and eukaryotic *in vitro* expression systems have been established providing the opportunity of well-defined protein synthesis in a viable cell-independent manner. With these cell-free techniques, the expression of a variety of cytotoxic [16,17] as well as membrane spanning proteins (amongst others [18–22]/reviewed in [23]) can be performed within a short time in a versatile fashion. In comparison to the common eukaryotic rabbit reticulocyte lysate supplemented with canine pancreatic microsomal membranes, the insect cell-free system used here provides endogenous microsomes. These are endoplasmic reticulum (ER) derived vesicular structures, enabling the co-translational translocation of membrane proteins into the biological membrane [24–26] in a well-oriented fashion. Furthermore, the methodology allows for posttranslational modification such as glycosylation [22] and lipid modification [27]. Compared to common methods of membrane protein integration into synthetic membranes or micelles, this method does not require any detergent solubilization step.

Here we present the insect based cell-free system and its endogenous microsomes in combination with a tailored GUV formation process as flexible tools for the expression of a variety of membrane proteins in a biological environment. Thus, we are able to build advanced model cells that become accessible to quantitative biophysical interrogation such as single-molecule microscopy.

2. Material and methods

2.1. DNA templates

Expression of the target protein Pro-Heparin-binding epithelial growth factor like factor fused to an enhanced yellow fluorescent protein (Hb-EGF-eYFP) was performed using a pIX3.0 based plasmid template, whereas C-X-C chemokine receptor type 4 (CXCR4-eYFP) and cyan fluorescent protein fused to a CaaX sequence (CFP-CaaX) were synthesized via pcDNA3-vectors. The endogenous signal sequence of Hb-EGF-eYFP was substituted by the Melittin signal sequence (for DNA and peptide sequence see [28]).

The linear expression polymerase chain reaction (E-PCR) product of Endothelin B receptor (ETB) was fused to an eYFP encoding gene sequence. Regulatory sequences obligatory for cell-free expression were added to the gene using three consecutive PCR reactions. First an overlapping sequence for the eYFP fusion was added at the 3′ end of the ETB-encoding sequence and the 5′ complementary sequence for the addition of the regulatory sequences was introduced. In the second PCR step, ETB was fused to the eYFP sequence and the sequence for the addition of 3′ regulatory sequences was introduced. Finally, the E-PCR product was completed in a third amplification reaction by the addition of the final 5′ and 3′ regulatory sequences. PCR primers containing the gene specific and regulatory sequences are listed in Table S1. The PCR reactions were performed using High-Fidelity polymerase and appropriate puffer components from New England Biolabs according to the manufacturer's instructions.

Linear PCR products and circular plasmid cDNA templates were suitable for direct transcription and translation reactions.

2.2. Cell-free protein synthesis

Protein expression was performed in a linked transcription/translation system [28]. Transcription reaction mixes contained 60 μg/mL plasmid DNA or 8 μg/mL E-PCR product, respectively. Transcription was performed for 2 h at 37 °C in case of Hb-EGF-eYFP and for 12 h in case of CXCR4-eYFP and ETB-eYFP. Transcription reaction components were purchased from Qiagen (EasyXpress Insect Kit II). The generated mRNA was purified using DyeEx spin columns (Qiagen) according to the manufacturer's protocol. Lysates generated from *Spodoptera frugiperda* (*Sf*21) cells were used for cell-free protein synthesis in a batch-based mode. Standard lysate preparation was performed as published previously [29]. The gentle disruption of the cells during this procedure leads to the rearrangement of endogenous membranous structures as the ER and their reconstitution as small vesicular structures, called microsomes. The translation reaction contained 25% (v/v) lysate, approximately 250 μg/mL protein encoding mRNA, canonical amino acids (200 mM), ATP (1.75 mM) and GTP (0.45 mM). Reaction mixes for radioactive labeling contained additionally ^{14}C leucine with a final specific radioactivity of 46.2 dpm/pmol. Each protein of interest was expressed separately for 90 min at 27 °C and analyzed as described in the following passage. Fluorescent proteins for microscopic investigations were prepared in the absence of radiolabeled amino acids.

The synthesis of two different types of proteins was performed in a sequential manner. After the initial translation of the first membrane protein, the reaction mixture was separated into vesicular and supernatant fraction by centrifugation (16,000 ×g, 4 °C, 10 min). The vesicular fraction contained the co-translationally translocated protein. The second translation step was prepared by resuspending the vesicular fraction from the initial translation reaction in a vesicle depleted translation reaction mix. Protein synthesis was completed using mRNA coding for the second membrane protein of interest. The translation reaction was performed using standard conditions as previously described.

2.3. Protein separation and autoradiography

Membrane proteins were separated according to their molecular mass using a Nu-PAGE SDS-PAGE system purchased from Life Technologies. 5 μL aliquots of standard translation mixes were acetone precipitated at 4 °C and precipitates were centrifuged at 20,000 ×g for 10 min Protein containing pellets were resuspended in 20 μL of 1× SDS sample buffer. Subsequently samples were separated electro-phoretically using 10% Bis-Tris gels for 35 min at 200 V. Bis-Tris gels were dried for 60 min at 70 °C (Unigeldryer 3545D, Uniequip) and ^{14}C leucine labeled membrane proteins were visualized using the phosphor imaging technique (Typhoon Trio+, Image Eraser, storage phosphor screens and cassettes, ImageQuant TL software, GE Healthcare).

2.4. Quantification of de novo synthesized membrane proteins

Protein yield was determined using hot trichloroacetic acid (TCA) precipitation and scintillation quantification. 3 mL of a 10% (v/v) TCA solution containing 2% (w/v) casein hydrolysate were added to 5 μL of the translation reaction mix containing ^{14}C leucine labeled membrane proteins. The translation mix was heated for 15 min at 80 °C followed by subsequent cooling for 30 min at 4 °C. The precipitated proteins