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Membrane protein reconstitution into liposomes guided by dual-color fluorescence cross-correlation spectroscopy

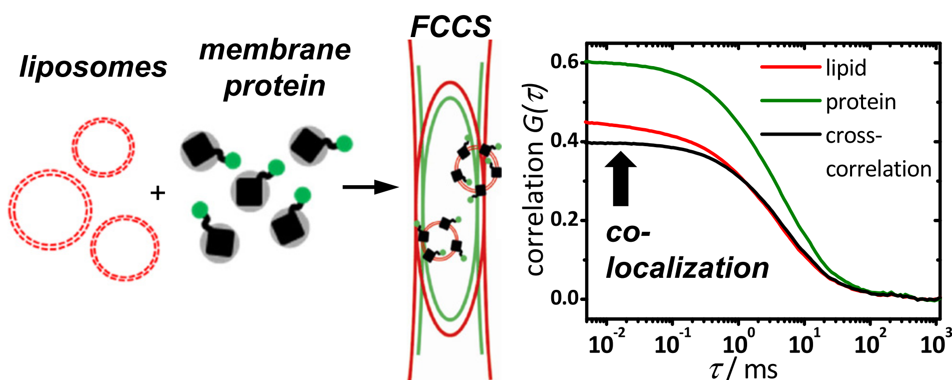
Peter Simeonov [a,1], Stefan Werner [b,1], Caroline Haupt [a,1], Mikio Tanabe [a,*], Kirsten Bacia [b,**]

[a] Membrane Protein Biochemistry, HALOmem, University of Halle, Kurt-Mothes-Str. 3, D-06120 Halle (Saale), Germany
[b] Biophysical Chemistry of Membranes, HALOmem, University of Halle, Kurt-Mothes-Str. 3, D-06120 Halle (Saale), Germany

HIGHLIGHTS

- Reconstitution into liposomes facilitates studies of membrane protein function.
- We show that FCCS tests protein and lipid co-localization in diffusing particles.
- FCCS can be used to guide a membrane protein reconstitution process.
- The multidrug resistance transporter NorA was functionally reconstituted.

GRAPHICAL ABSTRACT



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ABSTRACT

Proteoliposomes represent nanoscale assemblies of indispensable value for studying membrane proteins in general and membrane transporters in particular. Since no universal protocol exists, conditions for proteoliposome formation must be determined on a case-by-case basis. This process will be significantly expedited if the size and composition of the assemblies can be analyzed in a single step using only microliters of sample. Here we show that dual-color fluorescence cross-correlation spectroscopy (FCCS) is of great value for optimizing the reconstitution process, because it distinguishes micelles, liposomes and aggregates in heterogeneous mixtures and permits direct monitoring of the co-localization of proteins and lipids in the diffusing assemblies. As proof-of-principle, liposomes containing the functional multidrug resistance transporter NorA from *Staphylococcus aureus* were prepared, demonstrating that FCCS is an excellent tool to guide the development of reconstitution protocols.

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1. Introduction

Membrane proteins play vital roles in all organisms ranging from bacteria to higher eukaryotes. From an analytical point of view, a high quality reconstitution of a purified membrane protein into a lipid bilayer is a prerequisite for various biochemical and biophysical studies of membrane protein function. The need for reconstitution of the protein

* Corresponding author. Tel.: +49 345 55 24923.
** Corresponding author. Tel.: +49 345 55 24924; fax: +49 345 55 27408.
E-mail addresses: mt@halomem.de (M. Tanabe), kb@halomem.de (K. Bacia).
[1] These authors contributed equally to this work.

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into a lipid bilayer is particularly obvious for membrane transporters, because they – unlike for instance membrane receptors or enzymes – are only able to perform their transport function in the membrane-embedded form. Membrane transporters are universally found, integral membrane proteins that translocate substances actively (against an electrochemical gradient) or passively (by facilitated diffusion) across membranes [1].

Phospholipid vesicles (liposomes) have been shown to be an excellent tool for investigating the function of membrane transporters. Proteoliposomes consist of a self-closed phospholipid bilayer, into which the purified membrane transporter is incorporated [2]. Several methods are employed to insert the protein into the liposome, among which the detergent-mediated pathway is the most prominent one [3–5]. The membrane transporter, which is solubilized by detergent during purification, can be directly mixed with detergent-destabilized liposomes to form mixed phospholipid–protein–detergent complexes. Removal of the detergent forces the protein to associate with the phospholipid membrane, resulting in the desired proteoliposomes (Fig. 1A). The destabilization of the preformed liposomes by the addition of detergent and the subsequent removal of the detergent are critical steps during the reconstitution procedure.

Despite the usefulness and versatility of detergent-mediated reconstitution, optimizing the reconstitution conditions to obtain a homogeneous proteoliposome preparation with functionally integrated protein, with the desired protein-to-lipid ratio, and with the desired size and liposome integrity remains a major challenge. Several experimental techniques are employed for supporting detergent-mediated reconstitution [6,7]. Due to their ease of use, turbidimetry and light scattering are routinely performed. A marked decrease in the turbidity or light scattering signal indicates membrane solubilization on the conversion of liposomes to micelles [4,8,9]. Nuclear magnetic resonance (NMR) spectroscopy is best suited to analyze the starting point of the solubilization process [10–13]: as opposed to the broadened NMR signal of a phospholipid bilayer, a narrow isotropic signal is generated when micelles appear, making the vesicle–micelle transition easily detectable. One major disadvantage of NMR spectroscopy is its low sensitivity and hence the necessity for large amounts of highly concentrated samples. Other methods for analyzing membrane solubilization comprise infrared (IR) spectroscopy [14,15], fluorescence spectroscopy [6,16,17] including fluorescence energy transfer [18,19], isothermal titration calorimetry (ITC) [20,21], electron spin resonance (ESR) [22], X-ray diffraction [23], atomic force microscopy (AFM) [24,25] and electron microscopy (EM) [26–28].

One drawback with several techniques used to assist with membrane protein reconstitution is that they permit to analyze the solubilization of the liposomes but not the reconstitution of the protein itself. Another drawback with many techniques is that sample-averaged parameters are obtained which are not informative about potential sample inhomogeneity resulting from the solubilization and assembly processes. In particular, IR spectroscopy, fluorescence spectroscopy and ITC are limited to bulk observations. Although AFM and EM permit visualization of individual particles, the results can be biased, as some

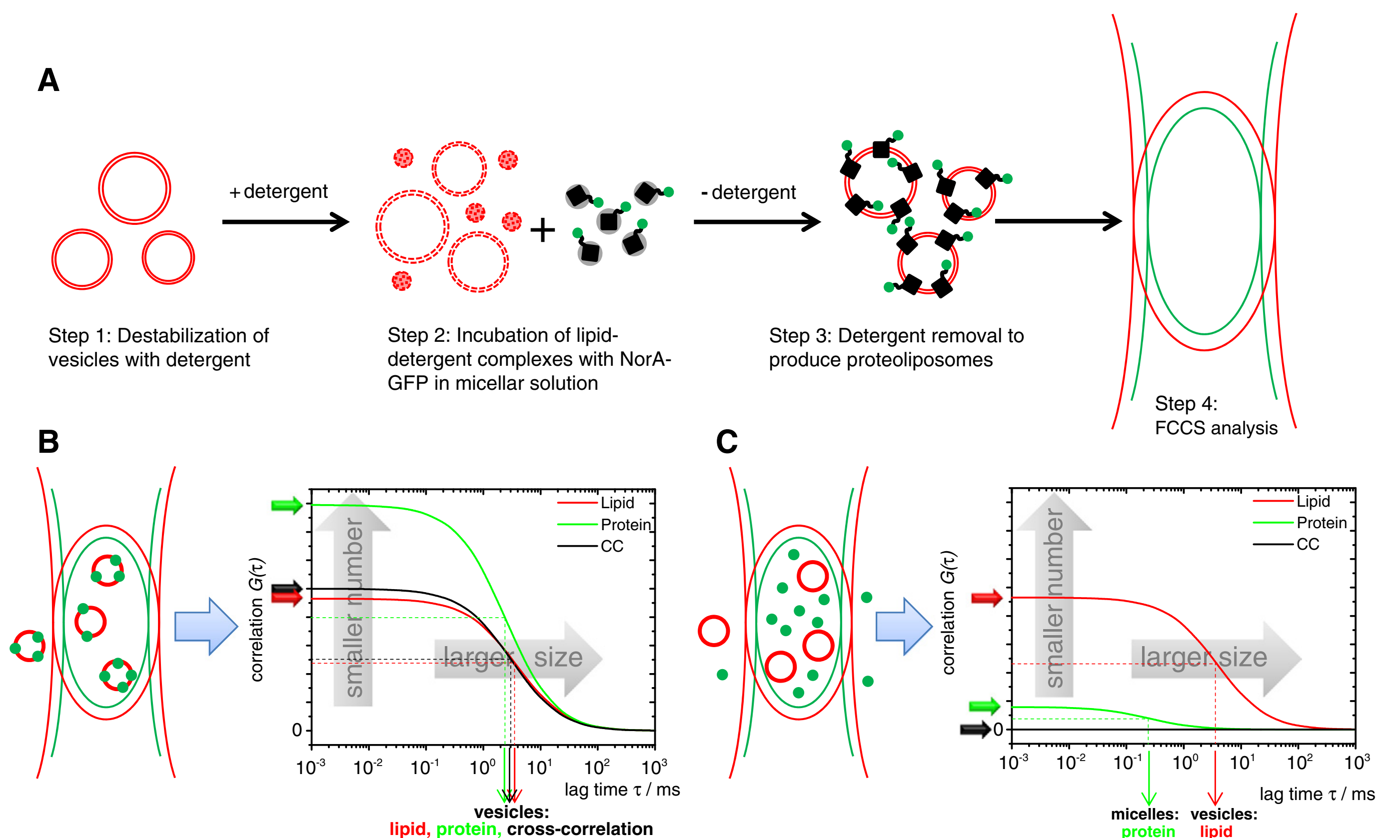

Fig. 1. (A) Schematic overview of the reconstitution procedure. Destabilization of liposomes with detergent yields detergent-containing liposomes and/or lipid-containing micelles (Step 1). Detergent-solubilized protein is added (Step 2). After detergent removal, FCCS is used to assess the particle numbers, particle types, relative particle contributions and the relative co-localization of protein and lipid (Steps 3 & 4). (B) Simulated auto- and cross-correlation curves for an ideal proteoliposome sample (concentrations of red and green particles are identical). The red autocorrelation amplitude is lower than the green one, because the red detection volume is larger. The cross-correlation amplitude is in between the two autocorrelation amplitudes, demonstrating maximal co-localization. All three curves exhibit a diffusion time typical of liposomal particles. (C) Simulated dual-color FCCS curves representing separate diffusion of liposomes (red) and protein (green). Total concentrations of red and green dye are the same as in panel B. As the protein molecules diffuse as individual entities, the number of green particles is larger than in panel B, causing a lower autocorrelation amplitude. The cross-correlation amplitude is zero, demonstrating the absence of co-localization between protein and liposomes. Lipid particles exhibit a diffusion time typical of liposomes, whereas protein molecules exhibit a diffusion time typical of micelles.

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