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Reconstitution of SNARE proteins into solid-supported lipid bilayer stacks and X-ray structure analysis

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ABSTRACT

SNAREs are known as an important family of proteins mediating vesicle fusion. For various biophysical studies, they have been reconstituted into supported single bilayers via proteoliposome adsorption and rupture. In this study we extended this method to the reconstitution of SNAREs into supported multilamellar lipid membranes, i.e. oriented multibilayer stacks, as an ideal model system for X-ray structure analysis (X-ray reflectivity and diffraction). The reconstitution was implemented through a pathway of proteomicelle, proteoliposome and multibilayer. To monitor the structural evolution in each step, we used small-angle X-ray scattering for the proteomicelles and proteoliposomes, followed by X-ray reflectivity and grazing-incidence small-angle scattering for the multibilayers. Results show that SNAREs can be successfully reconstituted into supported multibilayers, with high enough orientational alignment for the application of surface sensitive X-ray characterizations. Based on this protocol, we then investigated the effect of SNAREs on the structure and phase diagram of the lipid membranes. Beyond this application, this reconstitution protocol could also be useful for X-ray analysis of many further membrane proteins.

1. Introduction

N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) have been identified as a family of proteins which promote vesicle fusion. They mediate almost every individual trafficking step of the secretory pathway [1]. Although there are some other SNAREs and non-SNARE proteins involved in synaptic vesicle fusion, its driving force for the actual merger mainly originates from syntaxin (Syx) and SNAP25 which locate on the presynaptic plasma membrane, and synaptobrevin (Syb) which anchors itself in the synaptic vesicle membrane [2]. The Syx/SNAP25 complex and Syb bind with high affinity and together form a 4-helix bundle from the N-terminal to the C-terminal in a zipper like fashion [3]. It is commonly believed that this self-assembly supplies enough energy to overcome the repulsion between opposing membranes, pulls them into close contact and thus facilitates fusion [1,4].

To gain a clear insight into complicated SNARE-SNARE and SNARE-lipid interactions, model lipid membranes can be used and are amenable to structural characterizations, in particular to X-ray and neutron scattering [5]. Various model membrane systems have been developed ranging from black lipid membranes [6], vesicles [7], nanodiscs [8] and supported lipid bilayers [9,10]. For scattering studies, one can choose between several different scattering geometries and sample preparation methods, depending on the experimental constraints and the information required. Small-angle X-ray scattering (SAXS) of either unilamellar or multilamellar vesicles [11,12], can be used to deduce the bilayer electron density profiles from least-square fitting of the form factor.

The classical SAXS characterization is limited by the loss of structural information inherent in powder averaging. Provided suitable preparation techniques for highly aligned membranes, reflectivity and grazing incidence small-angle scattering (GISAXS) circumvent this loss of information. In these interface sensitive scattering techniques, the momentum transfer parallel ($q_{\parallel}$) and perpendicular (q_z) to the surface of the membranes can be well distinguished. Furthermore, the scattering volume can be precisely tuned by the angle of incidence α. To this end, the membrane mosaicity as quantified by the tilt distribution of membrane normal vectors ω, has to be smaller than the critical angle α_c of total external X-ray reflection. Preparation of such oriented single

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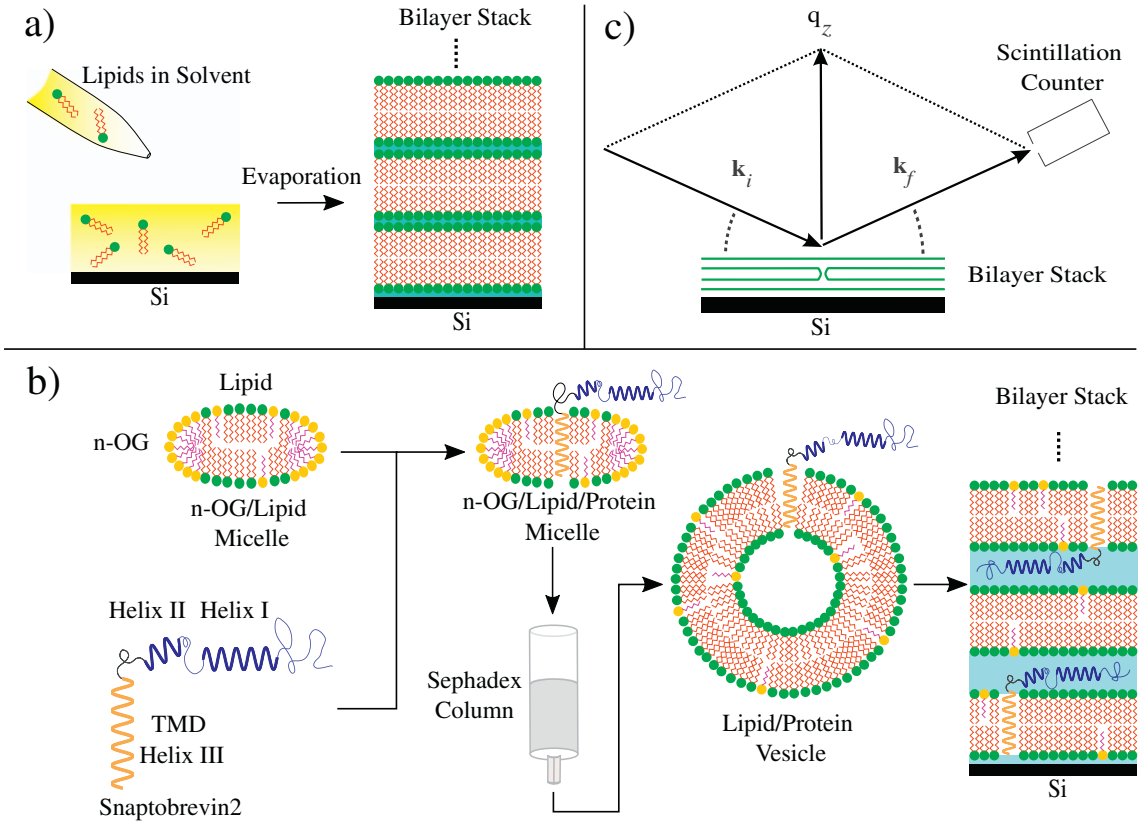

Fig. 1. (a) Cartoon of the conventional spreading organic solution (sOS) method. (b) Cartoon flow chart of the spreading vesicle suspension (sVS) method. (c) Sketch of the reflectivity experiment used as one of the characterization approaches of the multilamellar bilayers.

bilayers [13] or bilayer stacks [14,15] then offers the opportunity to map the two-dimensional reciprocal space. X-ray reflectivity, which probes the structure factor along q_z typically offers strong signal, and thus provides the electron density profile ρ(z) as well as the inter-bilayer correlations down to the Angstrom scale [16,17]. Contrarily, lateral correlations and structure in particular regarding the proteins which can be monitored by GISAXS, exhibit much weaker signal. While synchrotron radiation is brilliant enough to still pick up the diffraction signal of a single monolayer or bilayer, it is often advisable to amplify the diffraction signal by increasing the number of bilayers *N*. Aside from the higher signal, a further advantage of studying membrane proteins using bilayer stacks (i.e. multibilayers) is that structural alterations and artifacts induced by the substrate surface can be significantly reduced [18]. Adding a soft cushion or tethering can overcome this problem also for single supported bilayers [19], but at the expense of extra complexity and additional structural parameters.

However, membrane proteins including SNAREs cannot be reconstituted into oriented bilayer stacks with the rather convenient and most frequently used solvent method which achieves film deposition by spreading organic solution (sOS) onto solid-supports (Fig. 1a) [20,21]. In the sOS protocol, the lipid molecules gradually self-assemble into lipid bilayers during solvent evaporation, and in the end form well-aligned, homogeneous bilayer stacks on the substrates. Although the use of organic solvent is not a concern and even desired for optimized mixing in many lipid-peptide systems, it is prohibitive for membrane proteins. Alternatively, membrane proteins (e.g. glycophorin, porin and bacteriorhodopsin [22]) can be reconstituted into lipid bilayer stacks by depositing proteoliposome suspensions onto solid-supports. This approach is similar to the deposition of supported single bilayers with vesicle suspensions [23], which has already been extensively applied to the reconstitution of SNAREs [24,25]. The main difference between these two is that for single bilayers only a single layer of vesicles adsorb onto the solid-supports and slowly rupture into single bilayers, while for bilayer stacks bulk vesicle suspensions are forced to dry and to form multibilayers during water evaporation. A related solvent-free approach, which did not include reconstituted proteins, was presented by Kucerka et al. [26].

In this work we show how reconstituted SNAREs can be incorporated into oriented lipid bilayer stacks enabling X-ray analysis without powder averaging. This is required to unambiguously identify the stalk structures from the two-dimensional GISAXS diffraction pattern, as previously shown for pure lipid systems [27,28]. A first goal is then to verify the structural integrity of the multi-bilayer stacks, and to quantify how the inter-membrane distance, i.e. the lamellar repeat distance *d*, as well as the lamellar ordering and electron density changes with protein reconstitution. Note that the X-ray measurements are dominated by the indirect collective response of the bilayer to protein insertion, not by proteins directly. Another important goal which motivates this study is to find out how the phase diagram changes in the presence of SNAREs, and whether reconstituted SNAREs promote the equilibrium stalk phase, i.e. are able to lower the critical osmotic pressure at which the phase appears. Finally, a long term goal is the reconstruction of the three-dimensional electron density distribution ρ(**r**) of model membranes containing SNAREs in the rhombohedral (*R*) phase.

To obtain oriented membrane stacks with reconstituted SNAREs, vesicles containing v- and t-SNAREs were first separately prepared by eluting SNARE-containing micelles through size-exclusion columns [29], and then spread onto Si substrates, as shown in Fig. 1b. This SNARE reconstitution protocol was carefully monitored and validated by verifying the structural transitions in each step (i.e. proteomicelle to proteoliposome to multibilayer). SNARE-reconstituted proteomicelles and proteoliposomes were studied by SAXS to reveal the effective structural changes. The supported multibilayers were characterized by X-ray reflectivity and GISAXS, which together provided a detailed view of the effects of SNARE reconstitution on the multibilayers. This