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Lipid Directed Intrinsic Membrane Protein Segregation

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Supporting Information

ABSTRACT: We demonstrate a new approach for direct reconstitution of membrane proteins during giant vesicle formation. We show that it is straightforward to create a tissue-like giant vesicle film swelled with membrane protein using aquaporin SoPIP2;1 as an illustration. These vesicles can also be easily harvested for individual study. By controlling the lipid composition we are able to direct the aquaporin into specific immiscible liquid domains in giant vesicles. The oligomeric α-helical protein cosegregates with the cholesterol-poor domains in phase separating ternary mixtures.

Giant vesicles are a well-established tool for the study of lipid bilayer membranes.[1−7] Typically they are formed by electroformation[8] and by solvent exchange methods using hydrocarbons.[9−11] However, both approaches are cumbersome and time-consuming.[12] In addition giant vesicle studies with intrinsic membrane proteins are difficult due to the necessary detergent exchange procedures and the large quantities of protein required for reconstitution.[13,14] Previous strategies have first revolved around exchanging detergent micelle stabilized membrane proteins into liposomes.[15] Thereafter either proteoliposome swelling by electroformation[16−18] or addition of proteoliposomes to preformed giant vesicles is performed.[15] Giant vesicles can also be prepared by blebbing plasma membranes; however in this case control of lipid constituents is not possible.[19−21] These hindrances have meant that to date relatively few studies of intrinsic membrane protein interactions with the lipid bilayer in giant vesicles have been performed versus other lipid bilayer approaches. Functionalized lipids with bound protein and adherent proteins have served as intrinsic membrane protein replacement models in planar bilayers and giant vesicles.[14,22−24] This work specifically aims to demonstrate that obligate intrinsic membrane proteins, i.e. those which are expressed directly into the lipid bilayer, are straightforwardly and robustly reconstituted into giant vesicles while avoiding many of the laborious challenges of detergent removal and exchange between different amphiphiles.[25]

Following on from the work by Bayley, Wallace and co-workers,[26−29] we hypothesized that it might be possible to adapt the droplet bilayer method of encapsulating detergent-solubilized membrane proteins in a hydrogel below the critical micelle concentration (CMC) of the detergent. Recent work by Mayer and co-workers,[30] and others,[12,31] has shown that it is straightforward to form giant vesicles by swelling a lipid film from a partially dehydrated hydrogel in aqueous buffer. By combining these approaches for lipid bilayer formation and membrane protein reconstitution we would circumvent the problem of lipid bilayer formation in a hydrocarbon medium,[27−29] while directly incorporating membrane protein into the nascent lipid bilayers.

The work we present here demonstrates that the adaptation of these two approaches enables efficient reconstitution of the spinach aquaporin SoPIP2;1 into giant vesicles during formation. SoPIP2;1 is oligomeric and α-helical in structure and represents an ideal model protein with which to test this new method.[32] By fluorescently labeling the protein we are able to image the reconstitution directly. We demonstrate that the reconstituted protein is functional using stopped-flow kinetic measurements of vesicle swelling (see Supporting Information, Figure S8).[33] In addition, we demonstrate that by controlling ternary mixtures of phospholipids and cholesterol we are able to segregate incorporated aquaporin into a specific immiscible liquid domain.

We nonspecifically labeled the aquaporin in detergent-stabilized aqueous solution with NHS-rhodamine and separated the unreacted dye from the protein. The labeled protein was diluted into warm molten agarose. Dilution below the CMC did not result in any noticeable aggregation or precipitation, as has been noted previously.[28,29,34] The molten gel was then spread onto a glass coverslip and partially dehydrated in a gentle N_2 gas stream until apparently dry to the naked eye. Mayer and co-workers previously noted that agarose retains a high water content, even after substantial heating in an oven.[30] We speculate that this environment is therefore not denaturing for the membrane protein. 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) dissolved in chloroform was deposited in small drops onto the partially dried hydrogel, and residual solvent was immediately evaporated in a N_2 gas stream (see also Supporting Information, Figure S4). This resulted in formation of a thin lipid film on top of the protein-containing hydrogel. Figure 1 shows the results of rehydrating this film in an aqueous buffer (see also supporting Movie 1, Figure S1). A densely packed film of giant vesicles is formed from the hydrogel surface. The giant vesicle membranes are clearly fluorescent. We crudely estimate that the protein incorporation efficiency from the hydrogel is greater than 50% by measuring spatial fluorescence intensity (see Figure S3). Figure 2 shows a bright-field image of harvested giant vesicles and the corresponding size distribution (see also Figure S2).

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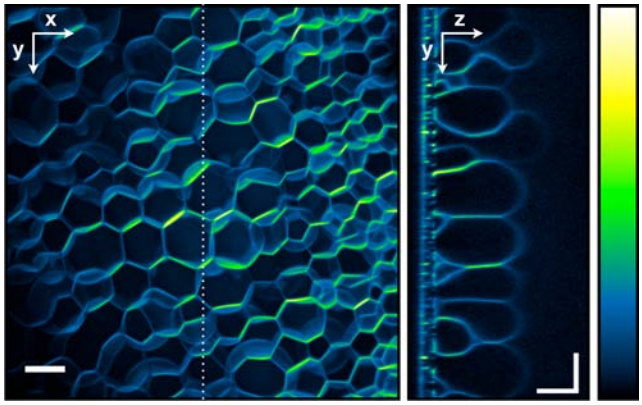

Figure 1. Rehydrating a DPhPC-coated partially dried hydrogel containing pure rhodamine-labeled SoPIP2;1 aquaporin. (A) False-color fluorescence micrographs showing a top-down standard deviation projection of the hydrated lipid/gel film from a z-stack (left) and z-reslice through the white dotted line (right). All scale bars are 10 μm; normalized min−max fluorescence intensity is shown by color calibration bar. Giant vesicles are densely packed upon the rehydrated gel surface after 30 min of incubation in buffer.

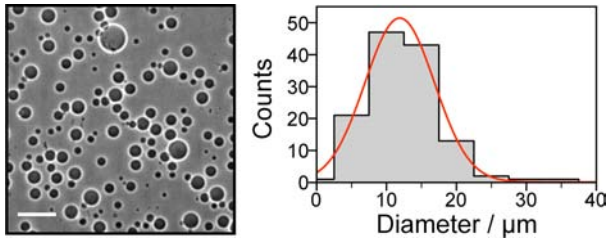

Figure 2. Harvested giant vesicles are homogeneous and well-formed spheres. (Left) A bright-field image showing different harvested giant vesicles (DPhPC + 1 mol % biotinyl-DPPE) stuck to a coverslip through streptavidin−biotin-BSA binding (scale bar = 50 μm). (Right) A histogram of vesicle diameters from the image. Mean diameter is 11.9 ± 0.1 μm with a fwhm of 7.0 ± 0.2 μm by least-squares fitting with a normal distribution.

The image shows homogeneous and well-formed giant vesicles without smaller malformed lipid structures which are often seen in electroformed giant vesicles.

Repeating this procedure but with a ternary mixture of lipids (DPhPC:brain sphingomyelin (BSM):cholesterol (Chol) 2:2:1), at a temperature above the phase transition point and with subsequent cooling to 25 °C, resulted in clear segregation of the protein into one of the liquid domains in a coexisting regime (Figure 3). The formation of coexisting immiscible liquid−liquid domains in giant vesicles is a well-characterized phenomenon.[6,35,36] SoPIP2;1, aquaporin-Z, and bacteriorhodopsin reconstituted by this method all show that the protein segregates into the liquid-disordered phase (see Supporting Information, Figure S7), in accordance with what has been observed previously for bacteriorhodopsin in giant vesicles.[25]

By modulating the phospholipid to cholesterol ratio we are able to control the protein accumulation in topologically distinct domains. Figure 4 shows the results of altering the DPhPC to BSM or DPPC to cholesterol ratio (see also Figures S5 and S6). At both mixture ratios of 2:2:1 and 1:1:2 the protein apparently localizes in cholesterol-poor domains. In the 2:2:1 case this is apparent as the bulk region of the giant vesicles (Figure 4A, C). In the 1:1:2 case the protein is seen concentrated in the smaller domains (Figure 4B, D). The phase

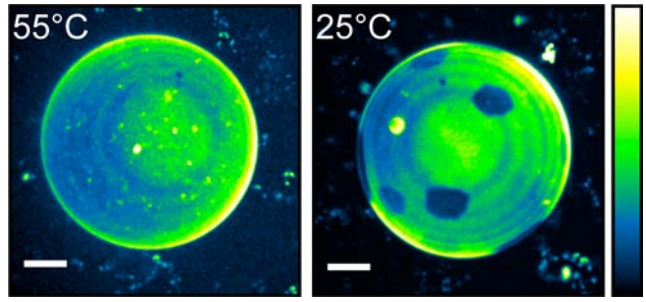

Figure 3. Imaging the condensation process during cooling of a ternary mixture giant vesicle. Standard deviation projections from 3D z-stacks of an aquaporin-swelled single vesicle (DPhPC:BSM:Chol, 2:2:1) undergoing cooling on the microscope stage. Upon cooling below the miscibility transition temperature this vesicle clearly shows liquid−liquid domain coexistence and concomitant protein segregation. Scale bars are 10 μm; normalized min−max fluorescence intensity is shown by color calibration bar.

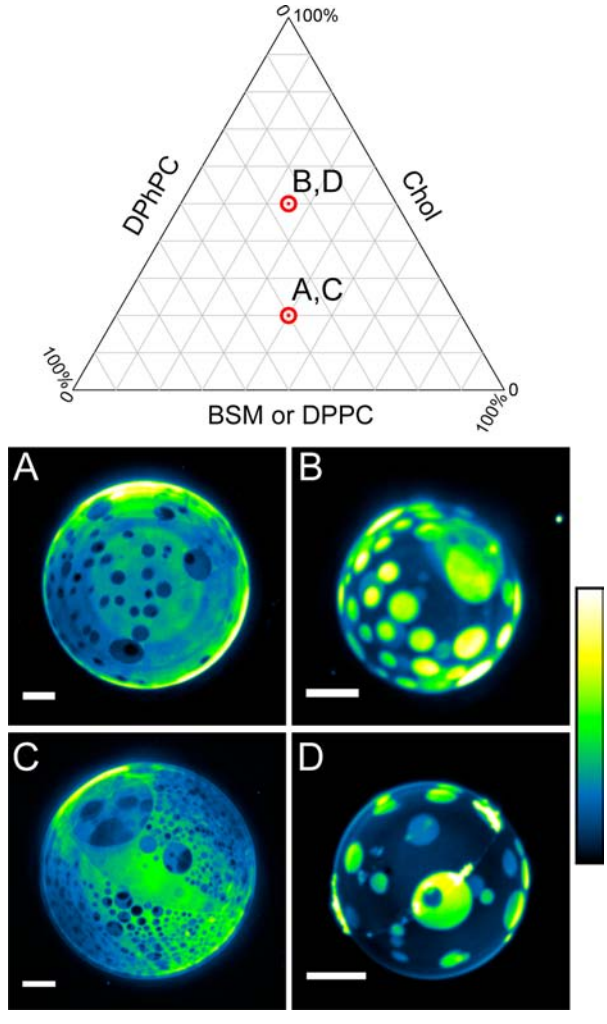

Figure 4. Lipid phase directed protein segregation. SoPIP2;1-swelled giant vesicles show that the protein cosegregates with topologically distinct cholesterol-poor domains. Standard deviation projections from 3D z-stacks of individual vesicles at 25 °C. (A) DPhPC:BSM:Chol, 2:2:1; (B) DPhPC:BSM:Chol, 1:1:2; (C) DPhPC:DPPC:Chol, 2:2:1; (D) DPhPC:DPPC:Chol, 1:1:2. Scale bars are 10 μm; normalized min−max fluorescence intensity is shown by color calibration bar.

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behavior is exactly in accordance with the immiscibility behavior of protein-free lipid bilayers.[35]

The approach we describe is advantageous in a number of ways: (1) Protein incorporation efficiency is high (≳50%). (2) The total quantity of membrane protein required for the formation of protein-swelled vesicles is considerably lower than other approaches (picomolar vs micromolar regimes). (3) The time required to prepare and form vesicles is on the order of 1 h. This is in stark contrast to lengthy dialysis-type detergent exchange and electroformation protocols. (4) Giant vesicle formation proceeds efficiently even in the presence of high ionic strength buffers. Hypothetically this means that these vesicles might be ideally suited for electrical measurements of reconstituted ion channels. (5) Giant vesicles appear to be uniform and homogeneous in these preparations, with little contamination, e.g. protein−lipid aggregates. (6) Reconstituted protein is functionally active following reconstitution (see Supporting Information).

We believe this approach by virtue of its increased efficiency of reconstitution from small quantities of material might enable in vitro studies of dilute or poorly expressing membrane proteins in controlled lipid environments, circumventing the need to express and handle unstable membrane proteins in large quantities.

The protein-reconstituted and densely packed giant vesicle films that we demonstrate in this work resemble a tissue-like material. Protocells and artificial tissues mimicking real biological phenomena are increasingly being sought for various applications.[37] For example artificial tissues could be used as replacement therapeutics in the future, albeit with careful control of constituents used (e.g., without chloroform). In addition engineered bottom-up mimics of complicated biological systems are important for basic scientific research. In pharmaceutical research these materials could aid in the understanding of the biological action of drugs in screens. We believe it will be possible to adapt the approach described in this work to create a basic functioning prototissue with functionally active membranes and cytoskeletons for basic research.[31,38]

■ ASSOCIATED CONTENT

Supporting Information

A movie showing giant vesicle film formation, a movie showing a 3D representation of a protein-swelled phase-separated giant vesicle, supporting methods, image analysis procedures, functional assay of reconsituted aquaporins, calculations, and supporting images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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