Published in final edited form as:

J Am Chem Soc. 2013 November 20; 135(46): . doi:10.1021/ja409708e.

Lipid Directed Intrinsic Membrane Protein Segregation

Jesper S. Hansen[†], James R. Thompson[†], Claus Hélix-Nielsen[‡], and Noah Malmstadt^{*,†}

[†]Mork Family Department of Chemical Engineering and Materials Science, 925 Bloom Walk, Los Angeles, CA 90089, USA

[‡]DTU-Physics, Department of Physics, Technical University of Denmark, Fysikvej 309, 2800 Kgs. Lyngby, Denmark

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 alpha-helical protein co-segregates 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 electroformation⁸ 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 firstly 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 isn't 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 coworkers,^{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)

Corresponding Author: All correspondence should be addressed to Noah Malmstadt. malmstad@usc.edu. JSH and JRT contributed equally to this work.

Supporting Information Available

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 reconstituted aquaporins, calculations and supporting images. This material is available free of charge via the Internet at <http://pubs.acs.org>.

No competing financial interests have been declared.

of the detergent. Recent work by Mayer and coworkers,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 alpha-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 non-specifically 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 N₂ 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 N₂ gas stream (see also supporting information). 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 aqueous buffer (see also supporting Movie 1, Supporting 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 supporting Figure S3). Figure 2 shows a bright-field image of harvested giant vesicles and the corresponding size distribution (see also supporting Figure S2). The image shows homogenous and well-formed giant vesicles without smaller malformed lipid structures which are often seen in electro-formed 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 subsequent cooling to 25°C resulted in clear segregation of the protein into one of the liquid domains in a co-existing regime (Figure 3). The formation of co-existing 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), 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 supporting Figures S4 & S5). 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 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 ($\geq 50\%$). (2) 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 hr. 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 homogenous 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}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The aquaporins used in this study were a gift from Aquaporin A/S (Denmark). The authors thank Dr Jeff Bertram for his help with stopped-flow fluorescence measurements. This work was supported by the National Institutes of Health (award R01GM093279) and the Office of Naval Research (Young Investigator award N000141210620).

References

1. Bagatolli LA. *Biochim Biophys Acta*. 2006; 1758:1541–1556. [PubMed: 16854370]
2. Bernardino de la Serna J, Oradd G, Bagatolli LA, Simonsen AC, Marsh D, Lindblom G, Perez-Gil J. *Biophys J*. 2009; 97:1381–1389. [PubMed: 19720026]
3. Dietrich C, Bagatolli LA, Volovyk ZN, Thompson NL, Levi M, Jacobson K, Gratton E. *Biophys J*. 2001; 80:1417–1428. [PubMed: 11222302]
4. Lingwood D, Simons K. *Science*. 2010; 327:46–50. [PubMed: 20044567]
5. Plasencia I, Norlen L, Bagatolli LA. *Biophys J*. 2007; 93:3142–3155. [PubMed: 17631535]
6. Veatch SL, Keller SL. *Phys Rev Lett*. 2002; 89:268101. [PubMed: 12484857]
7. Li S, Hu P, Malmstadt N. *Anal Chem*. 2010; 82:7766–7771. [PubMed: 20722391]

8. Angelova, M.; Soléau, S.; Méléard, P.; Faucon, J.; Bothorel, P. Trends in Colloid and Interface Science VI. Helm, C.; Lösche, M.; Möhwald, H., editors. Steinkopff: Progress in Colloid & Polymer Science; 1992. p. 127-131.
9. Hansen JS, Vararattanavech A, Vissing T, Torres J, Emneus J, Helix-Nielsen C. *Chembiochem*. 2011; 12:2856–2862. [PubMed: 22069223]
10. Walde P, Cosentino K, Engel H, Stano P. *Chembiochem*. 2010; 11:848–865. [PubMed: 20336703]
11. Hu PC, Li S, Malmstadt N. *ACS Appl Mater Interfaces*. 2011; 3:1434–1440. [PubMed: 21449588]
12. Weinberger A, Tsai FC, Koenderink GH, Schmidt TF, Itri R, Meier W, Schmatko T, Schroder A, Marques C. *Biophys J*. 2013; 105:154–164. [PubMed: 23823234]
13. Renthall R. *Biochemistry*. 2006; 45:14559–14566. [PubMed: 17144649]
14. Scheve C, Gonzales P, Momin N, Stachowiak J. *J Am Chem Soc*. 2013; 135:1185–1188. [PubMed: 23321000]
15. Girard P, Pecreaux J, Lenoir G, Falson P, Rigaud JL, Bassereau P. *Biophys J*. 2004; 87:419–429. [PubMed: 15240476]
16. Bouvrais H, Cornelius F, Ipsen JH, Mouritsen OG. *Proc Natl Acad Sci U S A*. 2012; 109:18442–18446. [PubMed: 23093677]
17. Dezi M, Di Cicco A, Bassereau P, Levy D. *Proc Natl Acad Sci U S A*. 2013; 110:7276–7281. [PubMed: 23589883]
18. Doeven MK, Folgering JH, Krasnikov V, Geertsma ER, van den Bogaart G, Poolman B. *Biophys J*. 2005; 88:1134–1142. [PubMed: 15574707]
19. Sezgin E, Kaiser HJ, Baumgart T, Schwille P, Simons K, Levental I. *Nat Protoc*. 2012; 7:1042–1051. [PubMed: 22555243]
20. Baumgart T, Hammond A, Sengupta P, Hess S, Holowka D, Baird B, Webb W. *Proc Natl Acad Sci U S A*. 2007; 104:3165–3170. [PubMed: 17360623]
21. Lopez-Montero I, Rodriguez-Garcia R, Monroy F. *J Phys. Chem. Lett*. 2012; 3:1583–1588.
22. Stachowiak J, Hayden C, Sanchez M, Wang J, Bunker B, Voigt J, Sasaki D. *Langmuir*. 2011; 27:1457–1462. [PubMed: 21155607]
23. Manley S, Horton M, Lecszynski S, Gast A. *Biophys J*. 2008; 95:2301–2307. [PubMed: 18502811]
24. Hussain N, Siegel A, Ge Y, Jordan R, Naumann C. *Biophys J*. 2013; 104:2212–2221. [PubMed: 23708361]
25. Kahya N, Brown D, Schwille P. *Biochemistry*. 2005; 44:7479–7489. [PubMed: 15895991]
26. Holden M, Bayley H. *J Am Chem Soc*. 2005; 127:6502–6503. [PubMed: 15869249]
27. Heron AJ, Thompson JR, Mason AE, Wallace MI. *J Am Chem Soc*. 2007; 129:16042–16047. [PubMed: 18052065]
28. Leptihn S, Thompson JR, Ellory JC, Tucker SJ, Wallace MI. *J Am Chem Soc*. 2011; 133:9370–9375. [PubMed: 21591742]
29. Leptihn S, Castell OK, Cronin B, Lee EH, Gross LC, Marshall DP, Thompson JR, Holden M, Wallace MI. *Nat Protoc*. 2013; 8:1048–1057. [PubMed: 23640169]
30. Horger KS, Estes DJ, Capone R, Mayer M. *J Am Chem Soc*. 2009; 131:1810–1819. [PubMed: 19154115]
31. Tsai FC, Stuhmann B, Koenderink GH. *Langmuir*. 2011; 27:10061–10071. [PubMed: 21707043]
32. Tornroth-Horsefield S, Wang Y, Hedfalk K, Johanson U, Karlsson M, Tajkhorshid E, Neutze R, Kjellbom P. *Nature*. 2006; 439:688–694. [PubMed: 16340961]
33. Karlsson M, Fotiadis D, Sjovall S, Johansson I, Hedfalk K, Engel A, Kjellbom P. *FEBS Lett*. 2003; 537:68–72. [PubMed: 12606033]
34. Plasencia I, Survery S, Ibragimova S, Hansen JS, Kjellbom P, Helix-Nielsen C, Johanson U, Mouritsen OG. *PloS one*. 2011; 6:e14674. [PubMed: 21339815]
35. Veatch SL, Keller SL. *Biophys J*. 2003; 85:3074–3083. [PubMed: 14581208]
36. Veatch SL, Keller SL. *Biophys J*. 2003; 84:725–726. [PubMed: 12524324]
37. Villar G, Graham A, Bayley H. *Science*. 2013; 340:48–52. [PubMed: 23559243]
38. Merkle D, Kahya N, Schwille P. *Chembiochem*. 2008; 9:2673–2681. [PubMed: 18830993]

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 mins 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 $11.9 \pm 0.1 \mu\text{m}$ with a FWHM of $7.0 \pm 0.2 \mu\text{m}$ by least-squares fitting with a normal distribution.

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 phase transition temperature this vesicle clearly shows liquid-liquid domain co-existence and concomitant protein segregation. Scale bars are 10 μ m, 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 co-segregates 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 μ m, normalized min-max fluorescence intensity is shown by color calibration bar.