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SNAP-Tag-Reactive Lipid Anchors Enable Targeted and Spatiotemporally Controlled Localization of Proteins to Phospholipid Membranes

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Supporting Information

ABSTRACT: The natural mechanisms that direct proteins to membranes are typically complex, requiring multiple steps and accessory components. It would be advantageous to develop simplified methods to direct proteins of interest to phospholipid membranes in a single step. Here we report a modular method for membrane localization of proteins by using chemically modified phospholipid anchors capable of covalent attachment to O^6-methylguanine DNA methyltransferase (SNAP-tag) fusion proteins. To our knowledge, this is the first use of SNAP-tag reactions to modify benzylguanine-functionalized lipid membranes. We demonstrate that photocaged lipid precursors enable light-triggered spatial and temporal control over protein localization. The anchoring system is compatible with cell-free expression, allowing for genetic targeting of proteins to lipid membranes of giant unilamellar vesicles. This technique can be used to control membrane curvature effects, similar to what has been previously observed with certain membrane-bound proteins. This work addresses a current need in synthetic biology for simplified and robust methods to control membrane localization of expressed proteins and shows promise as a general tool for protein targeting to lipid vesicles and cellular membranes.

Membrane-bound proteins are critical for regulating signaling, transport, binding interactions, and curvature.[1−3] Thus, controlled localization of proteins to discrete regions of the cellular membrane is essential for all organisms. Cells accomplish this task through several transport and lipidation pathways, often requiring enzymes specific to the target protein.[4,5] Current techniques for incorporating membrane proteins into phospholipid membranes involve the growth of isolated cellular membranes[6] or require the use of proteoliposomes and detergent,[7,8] making *in situ* protein localization difficult. Approaches to facilitate membrane localization of proteins of interest include the use of transmembrane domain and isoprenylation sequence fusions,[9−11] membrane-localizing ligands,[12] optogenetics,[13] or supramolecular interactions.[14]

These techniques, while useful, have several drawbacks, including a lack of covalent membrane attachment and the inability to control interactions in a spatiotemporal manner. A tool that overcomes these hurdles would allow for controlled studies on topics such as protein signaling and induced membrane curvature, where efficient and localized protein attachment is required to elicit specific responses.[1,2] Furthermore, such tools would be capable of guiding *in situ* synthesized proteins to membranes for synthetic biology applications. Here we present a simplified approach to membrane localization that results in the selective and covalent linkage of proteins to the lipid bilayer. This approach utilizes benzylguanine (BG)-modified lipid anchors and the well-established mutant O^6-methylguanine-DNA methyltransferase, SNAP-tag,[15] to tether proteins to phospholipid membranes. Additionally, it allows for photoactivation of protein to defined vesicles or portions of a single vesicle membrane with spatial resolution on the order of micrometers. To our knowledge, this is the first demonstration of the use of the SNAP-tag reaction to anchor proteins to BG-functionalized phospholipid membranes.

SNAP-tag technology has seen increasing use in biomolecular imaging due to its several attractive features, which include the ability to access a broad range of bright and stable synthetic fluorophores, the availability of fluorogenic probes, and the relatively small size of the reactive protein (∼20 kDa).[16−19] The remarkable versatility of SNAP-tag has enabled numerous applications beyond imaging, such as the detection of drug−protein interactions,[20] conjugation of biomolecules,[21,22] proximity ligation of oligonucleotides[23] and immobilization of protein on self-assembled monolayers.[24] However, despite these past results, SNAP-tags have not been explored for lipid membrane modification. We thus set out to investigate whether reactive lipid anchors on phospholipid membranes could capture SNAP-tag proteins.

To determine if SNAP-tags could be used for lipid membrane modification, we synthesized several SNAP-tag reactive BG lipid anchors. Interestingly, initial experiments without a linker and with a short 7-member poly(ethylene glycol) (PEG) linker between the BG and lipid headgroup failed to promote localization of SNAP-tag to model phospholipid membranes (compounds **7** and **9**, Supporting Information). Reasoning that the phospholipid membrane-bound BG group may have hindered access to the active site of the SNAP-tag protein,[25] we chose to explore longer and more flexible linkers and found that 17- and 45-member PEG linkers (DOPE-PEG17-BG and DSPE-PEG45-BG) were able to maintain reactivity of membrane-bound BG with SNAP-tag proteins (Figure S1). To confirm the covalent reactivity of DSPE-PEG45-BG, the lipid

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was incubated with a purified SNAP-tag protein at 37 °C overnight. SDS-PAGE gel analysis of the completed reaction showed an expected increase in protein mass, indicating covalent linkage of SNAP-tag and the lipid anchor (Figure S2). The sensitivity of the membrane reaction to linker structure offers a possible explanation for why past attempts to extend SNAP-tags to phospholipid membrane modification have not met with success.[26] PEG linkers are an attractive solution to this problem because they increase the flexibility of the reactive substrate while still maintaining a short end-to-end distance. Modeling experiments suggest that 18-member poly(ethylene oxide) molecules, very similar in length and structure to the linker region of DOPE-PEG17-BG, have an average length of 25.3 Å.[27]

To investigate the utility of these anchors for phospholipid membrane modification (Figure 1B), dioleoylphosphatidyl-

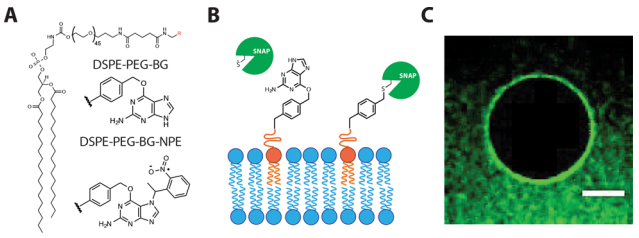

Figure 1. (A) DSPE-PEG lipid anchor with benzylguanine (BG) and photocaged benzylguanine (BG-NPE) head groups. (B) Proposed reaction of SNAP-tag protein with a membrane embedded DSPE-PEG lipid-anchored BG substrate. Transfer of the benzyl group to the active site of the SNAP-tag results in covalent attachment of the protein to the phospholipid membrane. (C) Visualizing membrane localization of a fluorescently tagged (AF-488) SNAP-tag protein on a giant unilamellar vesicle. The vesicle was formed with 5 mol % DSPE-PEG45-BG lipid anchors, which captures the SNAP-tag protein (scale bar 10 μm).

choline (DOPC) giant unilamellar vesicles (GUVs) containing 5 mol % DSPE-PEG45-BG were formed by adapting a previously published inverse emulsion technique.[28] Formed vesicles were incubated with 3 μM Alexa Fluor 488 (AF488) dye-labeled SNAP-tag for 1 h at 37 °C. Fluorescent microscopy showed excellent membrane localization of SNAP-tag to DSPE-PEG45-BG containing membranes (Figure 1C). To confirm that the localization we observed was due to reaction with the lipid anchor and not other nonspecific interactions, we incubated AF488-labeled bovine serum albumin with DSPE-PEG45-BG vesicles and observed no membrane localization. Additionally, AF488-SNAP-tag incubated with DOPC vesicles lacking the reactive lipid anchor showed no localization to the membrane, indicating that the fluorescent SNAP-tag protein does not bind due to inherent phospholipid membrane affinity (Figure S3).

Having determined that DSPE-PEG45-BG is capable of reacting with SNAP-tag protein, we next explored whether the lipids could be used to target specific microdomains formed on mixed phospholipid vesicles. Previous studies have shown that mixtures of saturated and unsaturated phospholipids, along with cholesterol, can produce vesicles with two distinct lipid phases.[29,30] Using a 1:1:1 mixture of DOPC:distearoylphosphatidylcholine (DSPC):cholesterol (Chol) with 5 mol % DSPE-PEG45-BG, we electroformed GUVs with two lipid phases. After introduction of AF488-labeled SNAP-tag, specific labeling of the less ordered lipid domains occurred, evidenced by colocalization with the fluorescent lipid Texas Red DHPE (Figure S4 and Movie M1). These results suggest that BG-modified lipids could serve as a valuable tool for studying the effect of lipid domains on the function and structure of embedded proteins.

A major advantage of using SNAP-tag reactive lipids is the potential to spatiotemporally control membrane protein modification. Elegant work has established that photocaged alkylguanosines can be activated with light to trigger binding with SNAP-tagged proteins.[31] With this in mind, we explored the feasibility of light-activated protein immobilization using a photocaged lipid anchor (DSPE-PEG45-BG-NPE) (Figure 1A). Kinetic studies showed rapid uncaging of the lipid in vesicles ($t_{1/2}$ = 80 s) upon irradiation with 360 nm light. To test the reactivity of the DSPE-PEG45-BG-NPE vesicles to SNAP-tag, DOPC GUVs were synthesized containing 5 mol % DSPE-PEG45-BG-NPE. Obtained vesicles were irradiated with 360 nm light for 10 min and then incubated with AF488-labeled SNAP-tag protein for 1 h (see Supporting Information for full experimental details). The UV activated vesicles showed marked localization of protein to the membrane compared to non-activated vesicles, which lacked any discernible binding even after 2 h of incubation with SNAP-tag proteins (Figure S5). An exciting feature of this technique is its compatibility with laser scanning confocal microscopy. By controlling the region of light exposure with a 405 nm diode laser, we were able to activate a selected vesicle of a population without triggering protein localization in neighboring vesicles. Activation was rapidly accomplished using 4.5 s of light exposure. After deprotection, SNAP-tag protein localization occurred within minutes (Figure 2B). We next synthesized DPPC GUVs containing 5 mol % DSPE-PEG45-BG-NPE using a solvent evaporation method.[32] By employing DPPC (T_m = 41 °C) as the main component of the phospholipid membrane, we were able to produce vesicles possessing a gel phase membrane at ambient temperature. Because the vesicle membranes are in the gel phase, lateral diffusion of constituent phospholipids is extremely slow, thus preventing lipid anchors from translating throughout the membrane.[33] This characteristic allowed us to photo-uncage lipid anchors on only a portion of the membrane. Vesicles were incubated with 230 μM SNAP-tag GFP and then irradiated for 2 s on one side of a vesicle membrane. After exposure, protein localization was observed within 90 s and after 10 min no further binding was evident. This unique capability may allow for the controlled decoration of membranes with several different fusion proteins at specific locations on a vesicle, an area that is of interest for drug delivery applications.[34,35]

Recently there has been increasing interest in the use of vesicle encapsulated cell free expression systems as cell mimics. Applications include fundamental studies on the essential elements for cellular function, minimal cells for synthetic biology, and drug delivery.[36,37] Seminal work in this area has demonstrated the efficient expression of protein in GUVs that encapsulate either *E. coli* lysate or the recombinant PURE system.[10,38] A challenge in this area has been to target *in situ*-synthesized proteins to the GUV membrane. Having this capability is essential for the reconstitution of natural membrane protein function and the design of new artificial systems in self-contained minimal cells. While there have been elegant demonstrations using spontaneous incorporation of certain integral membrane proteins,[12] and reconstitution of the Sec translocon to create lipid/protein membranes,[39] the use of SNAP-tag reactive lipids could offer a simple method to genetically target a much wider range of proteins to the surrounding lipid bilayer (Figure 3A). We therefore examined whether DSPE-PEG45-BG lipids could be utilized in con-

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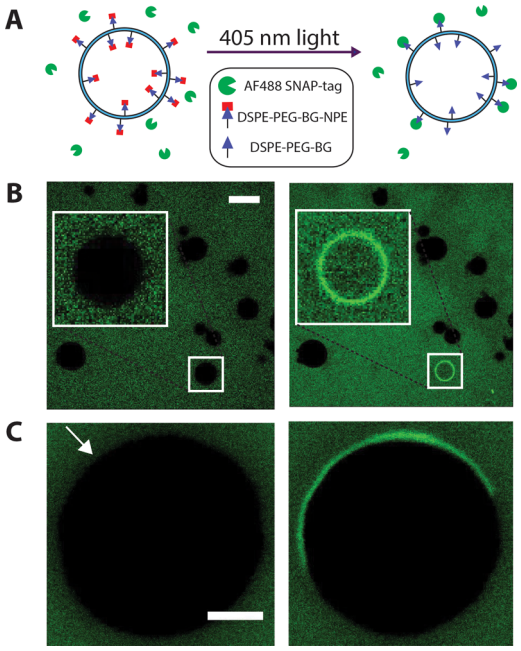

Figure 2. (A) Exposure of DSPE-PEG-BG-NPE containing vesicles to a 405 nm laser results in the loss of the NPE caging group and spatiotemporally activates binding of SNAP-tag proteins to the membrane. (B) Left: 20:1 DOPC:DSPE-PEG-BG-NPE vesicles incubated with dye (AF488)-labeled SNAP-tag before photoactivation (scale bar 30 μm).: A single vesicle of the population was irradiated with the 405 laser line for 4.5 s (white box). Right: After 10 min, protein has bound specifically to the uncaged vesicle. (C) Left: 20:1 DPPC:DSPE-PEG45-BG-NPE vesicles incubated with SNAP-tag GFP fusion before photoactivation (scale bar 2 μm). A portion of the vesicle was exposed to the 405 laser line (white arrow) for 2 s. Right: after 10 min protein binding is observed only on the selected portion of the gel phase membrane.

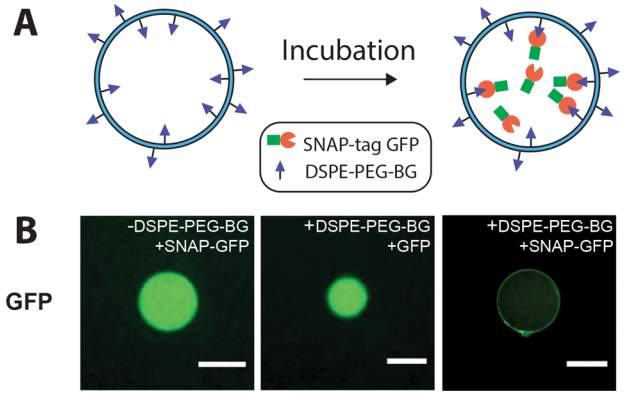

Figure 3. (A) Expression by *E. coli* lysate encapsulated in 5 mol % DSPE-PEG-BG vesicles leads to subsequent membrane localization of SNAP-tag GFP fusion protein. (B) GUVs expressing fluorescent protein (green channel, bottom row). Left: DOPC GUVs with no DSPE-PEG-BG expressing SNAP-tag GFP fusion (scale bar 15 μm). Middle: 5 mol % DSPE-PEG45-BG vesicles expressing GFP (scale bar 10 μm). Right: 5 mol % DSPE-PEG45-BG vesicles expressing SNAP-tag GFP fusion (scale bar 25 μm).

junction with cell free expression systems to genetically trigger membrane localization of a SNAP-tag fusion protein. Plasmid encoding for a SNAP-tag GFP fusion protein and an S30 T7 protein expression system were encapsulated in GUVs containing 5 mol % DSPE-PEG45-BG using a modified inverse emulsion method. After protein expression at 37 °C for 1 h, vesicles containing lipid anchor showed excellent localization of expressed fusion protein to the vesicle membrane (Figure 3B). Control experiments with vesicles lacking reactive lipid or using a control GFP resulted in no localization of membrane protein. This result demonstrates the ability of the SNAP-tag reactive lipid system to effectively emulate cellular protein trafficking and allow for genetically targeted control over membrane localization.

Having demonstrated the wide range of capabilities and control offered by SNAP-tag reactive lipid anchors, we wished to explore if this technique could be used to recreate membrane effects caused by natural phospholipid-bound proteins. Recently it has been shown that some natural membrane-bound proteins such as epsin1 are able to induce membrane curvature changes due to crowding effects alone and that even tethering GFP to the membrane at high concentration can result in membrane tubulation. We therefore reasoned that our membrane anchor could be used to induce membrane curvature via crowding by tethering protein near the membrane in high concentration.[40] Electroformed vesicles possessing disordered microdomains were synthesized containing 5 mol % DSPE-PEG45-BG. Upon incubation with 185 μM SNAP-GFP, membrane tubulation and deformation was observed within 90 s (Figure 4 and Movie M2),

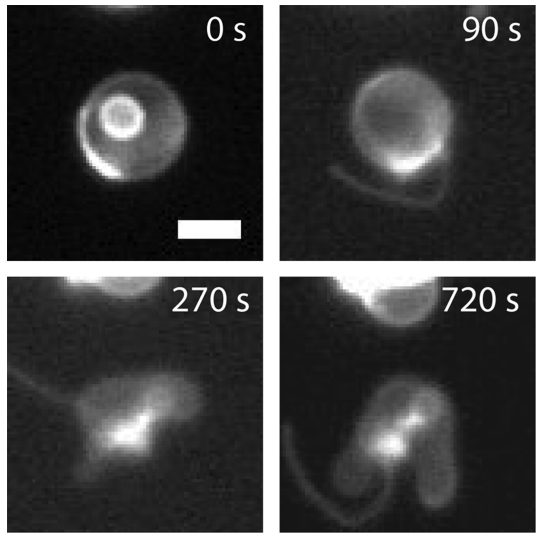

Figure 4. Introduction of an excess of SNAP-tag GFP fusion protein to microdomain containing GUVs results in significant membrane deformation. Images show a representative GUV, labeled with 0.1 mol % Texas Red DHPE, undergoing tubulation and blebbing events on the minute time scale. Time noted in seconds after introduction of protein (scale bar 5 μm).

while controls lacking membrane anchor displayed no noticeable change in membrane morphology (Figure S6). Membrane curvature phenomenon could also be observed with lower concentrations of SNAP-GFP, down to 10 μM (Figure S7). These results demonstrate that the protein is anchored close enough to the membrane to allow for collective protein behavior to be transmitted to the lipid anchor and result in a dramatic change in membrane curvature.

While this technology offers several opportunities for modifying model phospholipid membranes, there is also tremendous interest in directing proteins to living cellular membranes for applications ranging from live cell imaging, drug

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delivery, and biochemical studies. Given that the SNAP-tag technology has seen extensive use for live-cell imaging applications, we reasoned that our lipid anchors would be suitable for directing proteins to the membranes of living cells. Cells treated with lipid anchor showed strong surface staining by fluorescently tagged protein, while control cells lacking the lipid anchor showed minor background (Figure S8). In this capacity, DSPE-PEG45-BG could be useful in cell signaling or adhesion studies by offering a simple method for membrane tethering of proteins or polypeptides.[41] Unfortunately, due to the charged and relatively large nature of the lipid linker and SNAP-tag protein, studies would be limited to the outer leaflet of the cellular membrane.

In summary, we have demonstrated a straightforward and robust method for targeting proteins to phospholipid membranes in a controlled and homogeneous fashion using SNAP-tags. With photocaged lipids, this technique enables light-driven spatiotemporal control over the formation of protein-modified membranes. Additionally, the use of native phospholipids as building blocks suggests that various natural lipids could be used to direct SNAP-tag reactive anchors to defined regions of cells or multiphasic artificial membranes. SNAP-tag reactive lipid anchors serve as a versatile tool for the controlled localization of proteins to phospholipid membranes and may have potential therapeutic, synthetic biology, and cellular biology applications, which we are currently exploring in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary data, experimental procedures, and characterization data; Movie M1, showing localization of AF488-labeled SNAP-tag to DSPE-PEG-BG-containing membrane microdomains; Movie M2, showing membrane deformation upon introduction of a high concentration of SNAP-tag GFP fusion protein to microdomain containing GUVs. 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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■ REFERENCES

(1) Hartman, N. C.; Groves, J. T. *Curr. Opin. Cell Biol.* **2011**, *23*, 370.
(2) Baumgart, T.; Capraro, B. R.; Zhu, C.; Das, S. L. *Annu. Rev. Phys. Chem.* **2011**, *62*, 483.
(3) Hancock, W. O. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 615.
(4) Nadolski, M. J.; Linder, M. E. *FEBS J.* **2007**, *274*, 5202.
(5) Hicke, L.; Dunn, R. *Annu. Rev. Cell Dev. Biol.* **2003**, *19*, 141.
(6) Mikelj, M.; Praper, T.; Demič, R.; Hodnik, V.; Turk, T.; Anderluh, G. *Anal. Biochem.* **2013**, *435*, 174.
(7) Girard, P.; Pécréaux, J.; Lenoir, G.; Falson, P.; Rigaud, J.-L.; Bassereau, P. *Biophys. J.* **2004**, *87*, 419.
(8) Varnier, A.; Kermarrec, F.; Blesneac, I.; Moreau, C.; Liguori, L.; Lenormand, J. L.; Picollet-D'hahan, N. *J. Membr. Biol.* **2010**, *233*, 85.
(9) von Nickisch-Rosenegk, M.; Teschke, T. J.; Bier, F. F. *Nanobiotechnology* **2012**, *10*, 1.
(10) Noireaux, V.; Libchaber, A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17669.
(11) Erhart, D.; Zimmermann, M.; Jacques, O.; Wittwer, M. B.; Ernst, B.; Constable, E.; Zvelebil, M.; Beaufils, F.; Wymann, M. P. *Chem. Biol.* **2013**, *20*, 549.
(12) Ishida, M.; Watanabe, H. *J. Am. Chem. Soc.* **2013**, *135*, 12684.
(13) Kennedy, M.; Hughes, R.; Peteya, L.; Schwartz, J.; Ehlers, M.; Tucker, C. *Nat. Methods* **2010**, *7*, 12.
(14) Uhlenheuer, D. A.; Wasserberg, D.; Haase, C.; Nguyen, H. D.; Schenkel, J. H.; Huskens, J.; Ravoo, B. J.; Jonkheijm, P.; Brunsveld, L. *Chemistry* **2012**, *18*, 6788.
(15) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2003**, *21*, 86.
(16) Hinner, M. J.; Johnsson, K. *Curr. Opin. Biotechnol.* **2010**, *21*, 766.
(17) Johnsson, K. *Nat. Chem. Biol.* **2009**, *5*, 63.
(18) Lukinavičius, G.; Umezawa, K.; Olivier, N.; Honigmann, A.; Yang, G.; Plass, T.; Mueller, V.; Reymond, L.; Corrêa, I. R.; Luo, Z.-G.; Schultz, C.; Lemke, E. A.; Heppenstall, P.; Eggeling, C.; Manley, S.; Johnsson, K. *Nat. Chem.* **2013**, *5*, 132.
(19) Sun, X.; Zhang, A.; Baker, B.; Sun, L.; Howard, A.; Buswell, J.; Maurel, D.; Masharina, A.; Johnsson, K.; Noren, C. J.; Xu, M.-Q.; Corrêa, I. R. *ChemBioChem* **2011**, *12*, 2217.
(20) Chidley, C.; Haruki, H.; Pedersen, M. G.; Muller, E.; Johnsson, K. *Nat. Chem. Biol.* **2011**, *7*, 375.
(21) Chidley, C.; Mosiewicz, K.; Johnsson, K. *Bioconjugate Chem.* **2008**, *19*, 1753.
(22) Lemercier, G.; Gendreizig, S.; Kindermann, M.; Johnsson, K. *Angew. Chem., Int. Ed.* **2007**, *46*, 4281.
(23) Gu, G. J.; Friedman, M.; Jost, C.; Johnsson, K.; Kamali-Moghaddam, M.; Plückthun, A.; Landegren, U.; Söderberg, O. *Nat. Biotechnol.* **2013**, *30*, 144.
(24) Engin, S.; Fichtner, D.; Wedlich, D.; Fruk, L. *Curr. Pharm. Des.* **2013**, *19*, 5443.
(25) Mollwitz, B.; Brunk, E.; Schmitt, S.; Pojer, F.; Bannwarth, M.; Schiltz, M.; Rothlisberger, U.; Johnsson, K. *Biochemistry* **2012**, *51*, 986.
(26) Erhart, D. Control of Cellular Signals in Time and Space. Ph.D. Thesis, University of Basel, 2012.
(27) Lee, H.; Venable, R. M.; Mackerell, A. D.; Pastor, R. W. *Biophys. J.* **2008**, *95*, 1590.
(28) Pautot, S.; Frisken, B.; Weitz, D. *Langmuir* **2003**, 2870.
(29) Veatch, S. L.; Keller, S. L. *Biophys. J.* **2003**, *85*, 3074.
(30) Feigenson, G. W.; Buboltz, J. T. *Biophys. J.* **2001**, *80*, 2775.
(31) Banala, S.; Arnold, A.; Johnsson, K. *ChemBioChem* **2008**, *9*, 38.
(32) Moscho, A.; Orwar, O. W. E.; Chiu, D. T.; Modi, B. P.; Zare, R. N. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11443.
(33) Kahya, N.; Scherfeld, D.; Bacia, K.; Schwille, P. *J. Struct. Biol.* **2004**, *147*, 77.
(34) Mufamadi, M. S.; Pillay, V.; Choonara, Y. E.; Du Toit, L. C.; Modi, G.; Naidoo, D.; Ndesendo, V. M. K. *J. Drug Delivery* **2011**, *2011*, 939851.
(35) Torchilin, V. P. *Nat. Rev. Drug Discovery* **2005**, *4*, 145.
(36) Noireaux, V.; Maeda, Y. T.; Libchaber, A. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 3473.
(37) Shin, J.; Noireaux, V. *ACS Synth. Biol.* **2012**, *1*, 29.
(38) Nourian, Z.; Roelofsen, W.; Danelon, C. *Angew. Chem.* **2012**, *124*, 3168.
(39) Dezi, M.; Cicco, A. Di. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 7276.
(40) Stachowiak, J. C.; Schmid, E. M.; Ryan, C. J.; Ann, H. S.; Sasaki, D. Y.; Sherman, M. B.; Geissler, P. L.; Fletcher, D. A.; Hayden, C. C. *Nat. Cell Biol.* **2012**, *14*, 944.
(41) Song, W.; Yu, Z.; Madden, M. M.; Lin, Q. *Mol. Biosyst.* **2010**, *6*, 1576.

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