Construction of Asymmetric Cell-Sized Lipid Vesicles from Lipid-Coated Water-in-Oil Microdroplets

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · DECEMBER 2008

Impact Factor: 3.3 · DOI: 10.1021/jp807784j · Source: PubMed

6 AUTHORS, INCLUDING:

Masahiro Takagi
Japan Advanced Institute of Science and T…

253 PUBLICATIONS **4,995** CITATIONS

SEE PROFILE

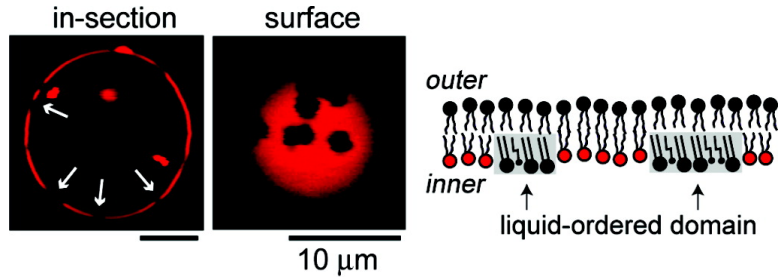
THE JOURNAL OF PHYSICAL CHEMISTRY B

Letter

Construction of Asymmetric Cell-Sized Lipid Vesicles from Lipid-Coated Water-in-Oil Microdroplets

Tsutomu Hamada, Yoko Miura, Yuuki Komatsu, Yuko Kishimoto, Mun'delanji Vestergaard, and Masahiro Takagi

J. Phys. Chem. B, **2008**, 112 (47), 14678-14681 • Publication Date (Web): 05 November 2008



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

THE JOURNAL OF PHYSICAL CHEMISTRY B LETTERS

2008, *112,* 14678–14681
Published on Web 11/05/2008

Construction of Asymmetric Cell-Sized Lipid Vesicles from Lipid-Coated Water-in-Oil Microdroplets

Tsutomu Hamada, Yoko Miura, Yuuki Komatsu, Yuko Kishimoto, Mun'delanji Vestergaard, and Masahiro Takagi*

School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan

Received: September 2, 2008; Revised Manuscript Received: October 22, 2008

We present a simple, rapid, and robust method for preparing asymmetric cell-sized lipid bilayer vesicles using water-in-oil (W/O) microdroplets transferred through an oil−water interface. The efficiency for producing cell-sized model membranes is elucidated in relation to the vesicular size and the weight of contained water-soluble molecules. We demonstrate the biological asymmetric nature and the formation of lipid raft microdomain structures using fluorescence microscopy.

Introduction

An asymmetric lipid distribution in the two leaflets of plasma membranes serves important biological functions, such as signal reception, membrane transport, and recognition of apoptotic cells.[1,2] Recently, much attention has been given to the coupling between trans-bilayer asymmetry and membrane lateral microclusters, such as lipid raft domains, that concentrate signaling proteins.[3,4] Each leaflet of plasma membranes exhibits different lateral domain structures with regard to size and stability, which corresponds to their lipid compositions.[5] During signaling events, it is believed that such microdomain structures associate with both leaflets, triggering intracellular signaling response.[6] However, the mechanism for the interleaflet interactions of heterogeneous bilayer membranes is far from understood. Very recently, studies on the interaction between heterogeneous leaflets using "planar" asymmetric bilayer membranes have reported that one leaflet can affect the lateral organization in another leaflet.[7,8] However, such a microdomain pattern is strongly coupled with membrane spatial structures, and this coupling promotes biological functions, such as domain-mediated endocytosis.[9−11] An asymmetric cell-sized model membrane with a vesicular structure would be of profound value in studies on the biochemical/biophysical functions of lipid organization structures.[12] Cell-sized liposomes possess, as their main strength, the ability to enable the researcher to manipulate a "biological" microvesicle under a controlled environment. Our current efforts have been directed at developing a robust, yet simple, method for the preparation of cell-sized lipid vesicles with an asymmetric lipid bilayer.

Some of the major outstanding challenges in the preparation of model membranes include controlling the size of vesicles and capturing the asymmetric nature of lipid bilayers.[13] With conventional methods for the preparation of cell-sized liposomes that are based on the hydration of dry lipid films,[14] it can be difficult to produce biomimetic trans-bilayer asymmetry. It has been suggested that asymmetric vesicles could be prepared by transferring water-in-oil (W/O) droplets coated by phospholipids from an oil phase to a water phase using an external force such as centrifugation.[15,16] In this procedure, independently prepared monolayers are used to construct an asymmetric liposome with hybrid bilayer structures between inner and outer leaflets. However, in these earlier studies typical liposomes were on a submicron scale.[17] Previously, we developed a centrifuge-independent method for the preparation of cell-sized liposomes by transferring equi-sized W/O droplets.[18] This technique allows for real-time observation of the transfer process, including the direct observation of events at the interface, and has improved sample economy by 1−2 orders of magnitude. In the current study, we devised a method for liposome formation that does not require centrifugation but instead utilizes a sugar weight gradient for the efficient transfer of W/O microdroplets. The sugar gradient significantly increased the yield of cell-sized liposomes compared to the previous report.[18] We observed and characterized membrane properties such as vesicular size and morphology. The effectiveness for the preparation of asymmetric bilayer lipid vesicles was evaluated using conventional methods. Finally, we demonstrated that this method can successfully capture the known biochemical phenomenon of lipid raft microdomain formation within asymmetric bilayer vesicles.

Experimental Section

Materials. Dioleoyl-L-α-phosphatidylcholine (DOPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), and cholesterol were obtained from Avanti Polar Lipids. Egg yolk L-α-phosphatidylcholine (egg PC) was purchased from Nacalai Tesque. The fluorescent phospholipid, *N*-(rhodamine red-X)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-PE) (λ_{ex} = 560 nm, λ_{em} = 580 nm), was obtained from Invitrogen, and 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoethanolamine (NBD-PE) (λ_{ex} = 460 nm, λ_{em} = 534 nm) was from Avanti Polar Lipids. Mineral oil, D(+)-glucose, sucrose, and sodium chloride were purchased from Nacalai

* Corresponding author. E-mail: takagi@jaist.ac.jp. Phone: +81-761-51-1650. Fax: +81-761-51-1525.

10.1021/jp807784j CCC: $40.75

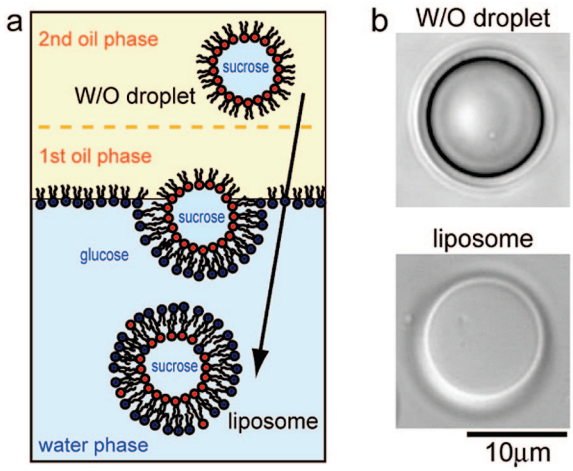


Figure 1. (a) Transfer process from water-in-oil (W/O) droplets into liposomes through an oil−water interface. (b) Typical microscopic images of W/O droplets in the oil phase and the formed cell-sized liposomes in the water phase.

Tesque and were of analytical grade. Deionized water obtained from a Millipore Milli-Q purification system was used to prepare buffers and reagents.

Preparation and Observation of Liposomes Transferred from W/O Droplets. First, phospholipids dissolved in chloroform/methanol (2:1, v/v) were poured into a glass test tube. The organic solvent was then evaporated under nitrogen flow and dried under vacuum to produce a dry film at the bottom of the test tube. Mineral oil was then added to the test tube prior to ultrasonication for 60 min at 50 °C and vortex mixing (final lipid concentrations in oil were 1 mM for DOPC and 0.35 mg/mL for egg PC). The observation chamber consisted of a cylindrical hole (ca. 4 mm in diameter) in a PDMS (poly(dimethylsiloxane)) sheet (ca. 4 mm thick) on a microscope glass slide (see Supporting Information). Water (10 μL) containing 0.1−1 M glucose was introduced at the bottom of the cylinder and covered with 7 μL of oil containing DOPC (first oil phase). The chamber was then incubated for about 2 h at ambient temperature (24 °C). To obtain W/O droplets, we added 5 vol % of 0.1−1 M sucrose solution (equimolar to glucose in the water phase) to the oil phase containing egg PC and then emulsified the mixture by tapping. 5 μL of the W/O droplet solution was added to the first oil phase within a couple of seconds after emulsification to form a second oil layer. The W/O droplets in the second oil phase spontaneously crossed the interface between the oil phase to the water phase, driven by the difference in molar density between sucrose (MW = 342) and glucose (MW = 180). During transfer, the W/O microdroplets were converted into bilayer lipid vesicles with the prepurposed asymmetry (Figure 1a), where the DOPC monolayer at the oil−water interface becomes the outer leaflet, and the egg PC monolayer covering W/O microdroplets becomes the inner leaflet. The spontaneously assembled liposomes were observed using phase-contrast microscopy (TE2000, Nikon). The images were recorded at 30 frames/s.

Fluorescent Observation of the Asymmetric Trans-bilayer. We prepared an oil phase containing lipids and fluorescent probes using the same procedure as described above (final lipid concentrations in oil were 1 mM for DOPC with or without 2 mol % NBD-PE and 0.35 mg/mL for egg PC with or without around 1 mol % rhodamine-PE). We then used the DOPC-oil as a first oil phase (outer leaflet) and egg PC-oil as a second oil phase (inner leaflet), where the probes mixed in only DOPC, only egg PC, or both lipids. The asymmetric colored liposomes were observed using a fluorescent microscope (IX71; Olympus) with 100 W high-pressure Hg lamp. Standard filter sets (U-MWIG3: ex 530−550 nm, dichroic mirror 570 nm, em 575 nm; U-MNIBA3: ex 470−495 nm, dichroic mirror 505 nm, em 510−550 nm) were used to monitor the fluorescence of rhodamine-PE and NBD-PE, respectively.

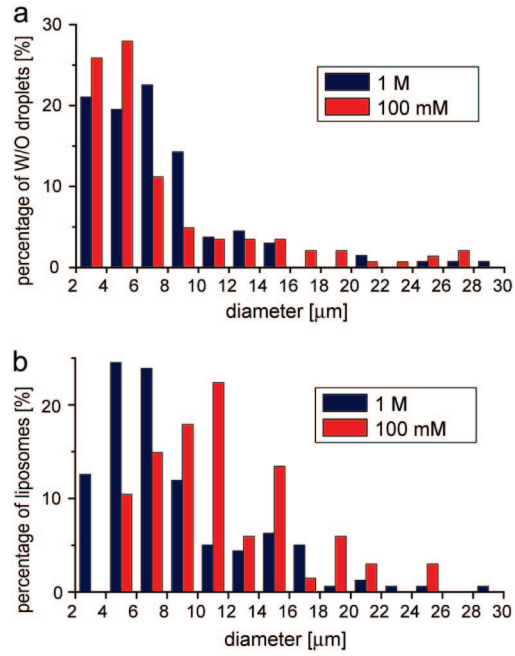


Figure 2. Size distribution of (a) W/O droplets before transfer (N = 133 (1 M), 143 (100 mM)) and (b) transferred liposomes (N = 159 (1 M), 67 (100 mM)) dependent on the concentration of sugar molecules contained in aqueous buffer solution.

Lipid Organization within Asymmetric Bilayer Liposomes. An oil phase containing lipids was prepared using the same procedure as described above (final lipid concentrations in oil were 1 mM for DOPC and 0.1 mM for the ternary lipid mixture, DOPC/DPPC/cholesterol = 30/30/40 molar ratio). To monitor the fluorescence from each leaflet of asymmetric trans-bilayer membranes, rhodamine-PE was mixed with DOPC or the ternary lipid at a molar ratio of 1/100. We used the DOPC-oil as a first oil phase (outer leaflet) and ternary-oil as a second oil phase (inner leaflet). The structures of lipid organization in the membrane surface were observed using a laser scanning microscope (FV500; Olympus). A HeNe laser (543 nm) was employed to excite the rhodamine-PE.

Results and Discussion

As shown in Figure 1b, a W/O microdroplet in the oil phase and a liposome transferred from the W/O droplets in the water phase are spherical, with no observable fluctuations, and exhibit relatively large sizes, comparable to typical eukaryotic cells (~10 μm). An optical image of the liposomes shows evidence of far less oil residue, since phospholipid W/O/W droplets with oil between two monolayers show much higher contrast.[19] To investigate the effect of droplet size on transfer behavior, we measured the diameter distribution of W/O droplets (Figure 2a) and formed liposomes (Figure 2b) under 1 M and 100 mM sugar gradient. No apparent difference in the size distribution of W/O droplets was noted between these two different sucrose molar concentrations (Figure 2a). We could successfully control the size of liposomes during the transportation simply by changing the concentration of the sugar molecules (Figure 2b). Trans-

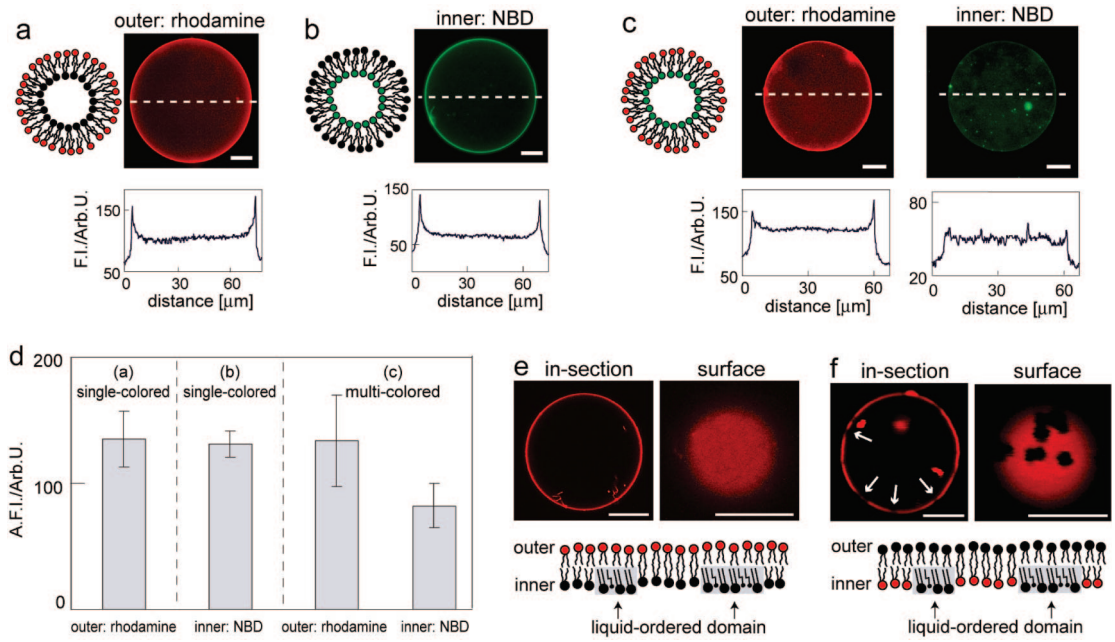


Figure 3. (a−c) Typical fluorescent images of asymmetric liposomes. (a) The outer leaflet is tagged with rhodamine-PE, (b) the inner leaflet is tagged with NBD-PE, and (c) the inner and outer leaflets are simultaneously tagged with rhodamine-PE and NBD-PE, respectively. Cross-section profiles of the respective microscopic images are shown at the immediate bottom of the fluorescence images. F.I. = fluorescence intensity. (d) Relative fluorescent intensities averaged for the peak of the cross-section profiles for many liposomes (N = 32 (a), 8 (b), 25 (c)). A.F.I. = average fluorescence intensity. (e, f) Typical fluorescent images (up) and schematic representations (bottom) of asymmetric liposomes with a single-component outer leaflet and three-component inner leaflet. Only the outer (e) or inner (f) leaflet was stained by rhodamine-PE, which preferentially partitions into the liquid-disorder phase. White arrows represent microdomain regions on the perimeter of the in-section image. Scale bars are 10 μm (a−c, e) and 5 μm (f).

ported liposomes under high sugar gradient (1 M) showed a similar trend in the size distribution to the added W/O droplets, indicating that almost all of the W/O droplets transferred into liposomes. Lowering the concentration of the sugar molecules by an order of magnitude resulted in a decrease in the number of smaller liposomes, thus selectively producing cell-sized liposomes. The mechanism behind size control in our study can be interpreted in terms of the free energy of transportation. The lipid monolayer was forced to curve around the droplet during the incorporation of the outer leaflet into a bilayer liposome, which leads to an increase in bending energy. In general, the competition between gravitational and bending energies determines the transportation behavior. The theoretically calculated transition size is in the micrometer range, in agreement with our experimental results (see Supporting Information). Vesicles with a lower molar sugar concentration show a larger transition size, indicating that small-sized vesicles are not readily transported to form liposomes. Technologies such as micromanipulation and microfluidics may also be useful for controlling liposome size by making it possible to prepare monodispersed microdroplets.[20−22] Additionally, we evaluated the characteristics of the obtained liposomes by subjecting them to osmotic stress and subsequently observing their morphological changes over time (see Supporting Information). The liposome showed essentially the same response to osmotic stress as conventional liposomes,[14] which validates this preparation method as suitable.

Next, the asymmetric trans-bilayer nature of the membrane was examined by monitoring the fluorescence from each leaflet. Figure 3a shows a typical fluorescent image of liposomes with rhodamine-PE on the outer leaflet, where the DOPC monolayer of oil/water interface was tagged. The inner leaflet composed of egg PC was monitored using NBD-PE (Figure 3b). The clear intensity from the vesicular membranes with each stained leaflet indicates that W/O droplets as the inner leaflet successfully captured the outer leaflet to become liposomes, i.e., bilayer structure, while passing across the monolayer. In addition, we observed multicolored liposomes whose leaflets were simultaneously stained with rhodamine-PE (outer) and NBD-PE (inner) probes, respectively (Figure 3c). The decrease in fluorescent intensity from NBD-PE can be attributed to the transfer of fluorescence energy from NBD-PE ($\lambda_{ex/em}$ = 460/534 nm) to rhodamine-PE ($\lambda_{ex/em}$ = 560/580 nm) across the bilayer membrane (Figure 3d).

We demonstrated that the prepared asymmetric vesicles could exhibit the biological phenomenon of microdomain formation in membranes. This event was studied by replacing egg PC with a ternary mixture of DOPC, DPPC, and cholesterol in the inner leaflet (molar mixing ratio of 3:3:4). The ternary system adapted to the inner leaflet shows phase separation between a biological raft microdomain (liquid-ordered phase), composed largely of DPPC and cholesterol, and the surrounding fluid membrane (liquid-disordered phase), which is rich in DOPC.[9] Fluorescent images for each leaflet unequivocally show a lateral lipid structure organization only in the inner leaflet (Figure 3e,f). In the one-component outer leaflet, fluorescence was observed over the entire membrane surface (Figure 3e), indicating that the lipid molecules are uniformly mixed. Conversely, the ternary inner leaflet surface was covered by several microdomains (Figure 3f). We also observed such microdomain structures with other ternary mixing ratios (see Supporting Information). Liposomes prepared by simple hydration methods have symmetric bilayer structures, where the domains in each leaflet are observed in perfect registration.[9] In contrast, our new W/O microdroplet method successfully produced heterogeneous and asymmetric cell-sized liposomes with microdomain structures. Further experimental developments intended to unravel the possible effect of oil residues on the lipid organization behavior and improvement on any weaknesses the current methodology may have are underway.

Conclusions

To the best of our knowledge, this is the first report on the preparation of cell-sized asymmetric lipid vesicles, the size of which can be controlled simply by adjusting the involved sugar gradient. We also demonstrated lateral lipid organization which resulted in the formation of lipid raft microdomains. Raft microdomains are essential components of physiological functions such as cell adhesion, cell sorting, and membrane traffic and as docking sites for cellular signaling.[13,23,24] Their successful self-organization in asymmetric cell-sized model membranes opens up opportunities for the real-time observation of membrane events such as signal-receptor interactions.

Acknowledgment. We thank Professor K. Yoshikawa, Dr. A. Yamada, and Mr. T. Yamanaka (Kyoto University) and Mr. M. Hase for their fruitful discussions. This work was supported by a KAKENHI Grant-in-Aid for Scientific Research (B) (No. 20360370) from the Japan Society for the Promotion of Science (JSPS) and on Priority Areas "Soft Matter Physics", "Bio Manipulation", and "Life Surveyor" from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT). M.V. gratefully acknowledges financial support from JSPS.

Supporting Information Available: Illustration of the observation chamber, free energy of transportation of W/O droplets into liposomes, osmotic deformation of liposomes, and microdomain structures in asymmetric vesicles. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Rothman, J. E.; Lenard, J. *Science* **1977**, *195*, 743–753.
(2) Verkleij, A. J.; Post, J. A. *J. Membr. Biol.* **2000**, *178*, 1–10.
(3) Devaux, P. F.; Morris, R. *Traffic* **2004**, *5*, 241–246.
(4) Wagner, A. J.; Loew, S.; May, S. *Biophys. J.* **2007**, *93*, 4268–4277.
(5) Wang, T.-Y.; Silvius, J. R. *Biophys. J.* **2001**, *81*, 2762–2773.
(6) Kusumi, A.; Koyama-Honda, I.; Suzuki, K. *Traffic* **2004**, *5*, 213–230.
(7) Kiessling, V.; Crane, J. M.; Tamm, L. K. *Biophys. J.* **2006**, *91*, 3313–3326.
(8) Collins, M. D.; Keller, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 124–128.
(9) Hamada, T.; Miura, Y.; Ishii, K.; Araki, S.; Yoshikawa, K.; Vestergaard, M.; Takagi, M. *J. Phys. Chem. B* **2007**, *111*, 10853–10857.
(10) Hanzal-Bayer, M. F.; Hancock, J. F. *FEBS Lett.* **2007**, *581*, 2098–2104.
(11) Yanagisawa, M.; Imai, M.; Taniguchi, T. *Phys. Rev. Lett.* **2008**, *100*, 148102.
(12) Laradji, M.; Kumar, P. B. S. *Phys. Rev. E* **2006**, *73*, 040901.
(13) Vestergaard, M.; Hamada, T.; Takagi, M. *Biotechnol. Bioeng.* **2008**, *99*, 753–763.
(14) Hotani, H.; Nomura, F.; Suzuki, Y. *Curr. Opin. Colloid Interface Sci.* **1999**, *4*, 358–368.
(15) Xiao, Z.; Xu, M.; Li, M.; Lu, Z.; Wei, Y. *Supramol. Sci.* **1998**, *5*, 619–622.
(16) Pautot, S.; Frisken, B. J.; Weitz, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10718–10721.
(17) Pautot, S.; Frisken, B. J.; Weitz, D. A. *Langmuir* **2003**, *19*, 2870–2879.
(18) Yamada, A.; Yamanaka, T.; Hamada, T.; Hase, M.; Yoshikawa, K.; Baigl, D. *Langmuir* **2006**, *22*, 9824–9828.
(19) Hase, M.; Yamada, A.; Hamada, T.; Yoshikawa, K. *Chem. Phys. Lett.* **2006**, *426*, 441–444.
(20) Utada, A. S.; Lorenceau, E.; Link, D. R.; Kaplan, P. D.; Stone, H. A.; Weitz, D. A. *Science* **2005**, *308*, 537–541.
(21) Yobas, L.; Martens, S.; Ong, W.-L.; Ranganathan, N. *Lab Chip* **2006**, *6*, 1073–1079.
(22) Shum, H. C.; Lee, D.; Yoon, I.; Kodger, T.; Weitz, D. A. *Langmuir* **2008**, *24*, 7651–7653.
(23) Simons, K.; Ikonen, E. *Nature (London)* **1997**, *387*, 569–572.
(24) Brown, D. A.; London, E. *J. Biol. Chem.* **2000**, *275*, 17221–17224.

JP807784J