Langmuir Article

pubs.acs.org/Langmuir

Dynamic Behavior of Giant Liposomes at Desired Osmotic Pressures

Masae Ohno,[†] Tsutomu Hamada,[‡] Kingo Takiguchi,*[,†] and Michio Homma[†]

[†]*Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan, and* [‡]*School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan*

Received March 4, 2009. Revised Manuscript Received August 13, 2009

To apply accurate and uniform osmotic pressures to liposomes, they can be formed using the spontaneous transfer method in solutions with different osmolarities. The majority of liposomes unexpectedly opened large holes (several micrometers in diameter) in response to the osmotic pressure regardless of its strength, that is, the difference between the outside and inside solute (sucrose or KCl) concentrations. However, the lag time for any response, including the opening of a hole, after the formation of the liposome decreased with increasing osmotic pressure.

1. Introduction

Biological membranes consist mainly of lipid bilayers and proteins. The morphology, topology, and features of lipid bilayers can change in response to various physiological signals and environmental cues. Osmotic pressure is the most familiar and one of the most important environmental factors involved in biological membrane homeostasis. Therefore, many studies have tried to evaluate the resistance of membranes to osmotic pressure, utilizing liposomes as the simplest artificially produced lipid bilayer vesicle.[1−11]

However, because to the best of our knowledge no procedure to expose liposomes to a uniform desired osmolarity has been reported, it has been impossible to elucidate the exact relationship between osmolarity and membrane stability. In most previous studies, liposomes made by the natural swelling or electroformation method were monitored in a concentration gradient of a solute that was formed by exchanging or mixing solutions or by dilution.[1−8,12−16]

Recently, it was shown that water-in-oil phospholipid-coated microdroplets (W/O droplets) could be used as precursors of liposomes.[17−19] W/O droplets at an oil/water interface were observed by microscopy to transform spontaneously into liposomes by crossing through the oil/water interface (the spontaneous transfer method). Liposomes obtained in that manner are several tens of micrometers in diameter, which is comparable to the size of living cells.[20] In addition, using that methodology, the concentrations of chemical species can be controlled both inside and outside the formed liposomes. Notably, it was reported that ATP and salts can be delivered inside liposomes through protein pores inserted in the membrane,[21,22] which supports the idea that the essential unilamellar bilayer structures occur in these liposomes.

In this study, we adapted the spontaneous transfer method to expose liposomes homogenously to a number of defined osmolarities. This approach enables us to create Milli-Q water encapsulating liposomes in solutions of specific concentrations of sucrose (0−1000 mM) or KCl (0−500 mM). Consequently, we discovered that individual liposomes may show five different types of behavior, the majority of them unexpectedly opening large holes (several micrometers in diameter), independent of the outside solute concentration. However, the lag time between the formation of the liposome and the start of any response decreased with increasing outside solute concentration.

2. Materials and Methods

Phosphatidylcholine (eggPC) was purchased from Sigma (St. Louis, MO). The preparation and observation of liposomes were performed as previously reported (the spontaneous transfer method, Figure 1).[19] A chloroform/methanol solution of eggPC was poured into a glass test tube, and the organic solvent of that solution was then evaporated under nitrogen flow and was dried under vacuum for more than 15 min to produce a dry film. The dry film was mixed with mineral oil (Nacalai Tesque, Kyoto, Japan) by ultrasonication for 60 min at 50 °C and vortex mixing. Briefly, 5 μL of Milli-Q water was emulsified in 100 μL of oil

*To whom correspondence should be addressed. Phone: +81-52-789-2993. Fax: +81-52-789-3001. E-mail: j46037a@nucc.cc.nagoya-u.ac.jp.

(1) Menger, F. M.; Angelova, M. I. *Acc. Chem. Res.* **1998**, *31*, 789–797.
(2) Peterlin, P.; Arrigler, V. *Colloids Surf., B* **2008**, *64*, 77–89.
(3) Taupin, C.; Dvolaitzky, M.; Sauterey, C. *Biochemistry* **1975**, *14*, 4771–4775.
(4) Sabín, J.; Prieto, G.; Ruso, J. M.; Hidalgo-Alvarez, R.; Sarmiento, F. *Eur. Phys. J. E* **2006**, *20*, 401–408.
(5) Quemeneur, F.; Rammal, A.; Rinaudo, M.; Pépin-Donat, B. *Biomacromolecules* **2007**, *8*, 2512–2519.
(6) Bernard, A. L.; Guedeau-Boudeville, M. A.; Jullien, L.; di Meglio, J. M. *Biochim. Biophys. Acta* **2002**, *1567*, 1–5.
(7) Menager, C.; Cabuil, V. *J. Phys. Chem. B* **2002**, *106*, 7913–7918.
(8) Viallat, A.; Dalous, J.; Abkarian, M. *Biophys. J.* **2004**, *86*, 2179–2187.
(9) Mui, B. L.; Cullis, P. R.; Evans, E. A.; Madden, T. D. *Biophys. J.* **1993**, *64*, 443–453.
(10) Shibata, A.; Ikawa, K.; Shimooka, T.; Terada, H. *Biochim. Biophys. Acta* **1994**, *1192*, 71–78.
(11) Levin, Y.; Idiart, M. A. *Physica A* **2004**, *331*, 571–578.
(12) Lasic, D. D. In *Structure and Dynamics of Membranes*; Lipowsky, R., Sackmann, E., Eds.; Elsevier Science: Amsterdam, 1995; Vol. *1a*, p 491.
(13) *Giant Vesicles: Perspectives in Supramolecular Chemistry*; Luisi, P. L., Walde, P., Eds.; Wiley: Chichester, U.K., 2000.
(14) Dimitrov, D. S.; Angelova, M. I. *Bioelectrochem. Bioenerg.* **1988**, *19*, 323–336.
(15) Angelova, M. I.; Soleau, S.; Meleard, P.; Faucon, J.-F.; Bothorel, P. *Prog. Colloid Polym. Sci.* **1992**, *89*, 127–131.
(16) Takeda, S.; Saitoh, A.; Furuta, M.; Satomi, N.; Ishino, A.; Nishida, G.; Sudo, H.; Hotani, H.; Takiguchi, K. *J. Mol. Biol.* **2006**, *362*, 403–413.
(17) Pautot, S.; Frisken, B. J.; Weitz, D. A. *Langmuir* **2003**, *19*, 2870–2879.
(18) Hase, M.; Yoshikawa, K. *J. Chem. Phys.* **2006**, *124*, 104903.
(19) Yamada, A.; Yamanaka, Y.; Hamada, T.; Hase, M.; Yoshikawa, K.; Baigl, D. *Langmuir* **2006**, *22*, 9824–9828.
(20) Hamada, T.; Miura, Y.; Komatsu, Y.; Kishimoto, Y.; Vestergaard, M.; Takagi, M. *J. Phys. Chem. B* **2008**, *112*, 14678–14681.
(21) Noireaux, V.; Libchaber, A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17669–17674.
(22) Pontani, L.-L.; van der Gucht, J.; Salbreux, G.; Heuvingh, J.; Joanny, J.-F.; Sykes, C. *Biophys. J.* **2009**, *96*, 192–198.

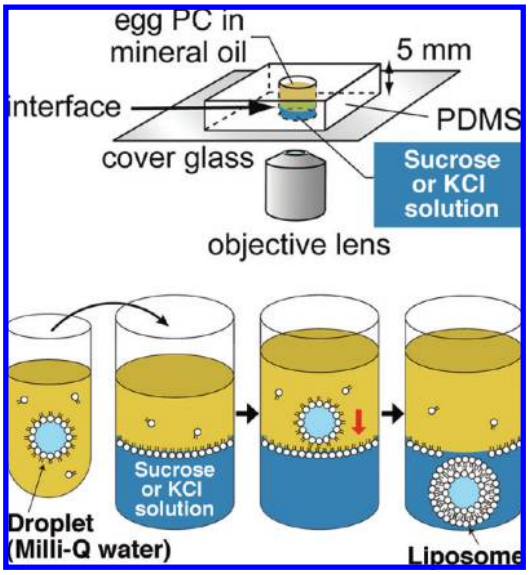

Figure 1. Schematic of the spontaneous transfer method and the formation and observation of liposomes.

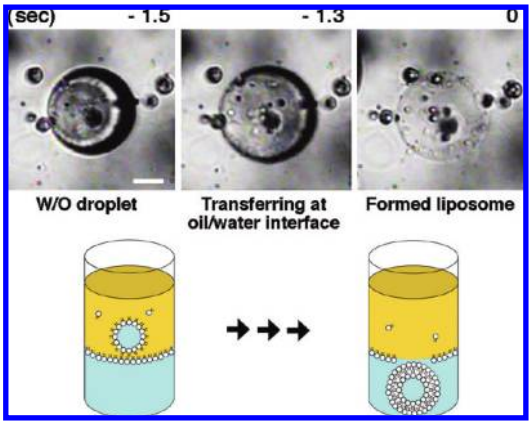

Figure 2. (Top) Process of liposome formation from a W/O droplet by the spontaneous transfer method. The solution surrounding the formed liposomes is Milli-Q water. Bar = 20 μm. We adjusted the microscopic focus position according to the motion of droplets or liposomes during the spontaneous transfer process. The number indicated at the top of each panel shows the elapsed time (in seconds) before the formation of the liposome by transferring a W/O droplet through the oil/water interface. The time when the liposome was formed is defined as 0 s. Transmission images show the existence of small oil droplets around and within the liposome, which were squeezed out of the oil phase. (Bottom) Model illustrating the liposomal formation process.

containing eggPC (0.5 or 1.0 mM) to obtain W/O droplets using the pipetting procedure. The oil containing the W/O droplets was then positioned on an oil phase (10 μL, containing 0.5 or 1.0 mM eggPC) that had been placed above an aqueous phase (10 μL, Milli-Q water with dissolved sucrose or KCl). The W/O droplets in the oil gradually fell onto the oil/water interface because of gravity. Interestingly, the droplets then spontaneously moved through the interface into the aqueous solution, keeping their spherical shape. Under our experimental conditions, the transferred droplets, or liposomes, were anchored to the interface, as schematically depicted at the bottom of Figure 1. Full details of the transformation of a droplet in oil into a liposome in water have already been reported.[19] We monitored the full process of the transfer of each liposome and observed the behaviors of liposomes in response to different osmolarities after their formation using a Zeiss Axiovert 135 M inverted microscope at 25 °C. The recorded images were analyzed using ImageJ software. The observation chamber consisted of a cylindrical hole in a poly(dimethylsiloxane) (PDMS) sheet (ca. 5 mm thick), which was obtained by mixing the base solution and a curing agent of Silpot 184 W/C (Dow Corning Toray, Tokyo, Japan) on a glass microscope slide (0.12−0.17 mm thick).

3. Results

3.1. Liposomes Produced by the Spontaneous Transfer Method. Figure 2 shows the process of liposome formation by the spontaneous transfer method. Previously, we successfully used the method to construct giant liposomes in the presence of salt, up to 20 mM $MgCl_2$ or 50 mM KCl.[18,19,23] The liposomes produced by this method were several tens of micrometers in diameter. Liposomes formed in a solution with the same osmolarity were spherical and stable and did not show any change after formation (Figure 2), although they occasionally spontaneously burst and disappeared (probably breaking into much smaller vesicles or some wreckage). It should be mentioned that this spontaneous breakage process was easily distinguishable from the membrane ruptures that accompanied the large hole openings described below.

(23) Takiguchi, K.; Yamada, A.; Negishi, M.; Tanaka-Takiguchi, Y.; Yoshikawa, K. *Langmuir* **2008**, *24*, 11323–11326.

3.2. Responses of Liposomes to Different Osmolarities. Figures 3 and 4 show the behavior of liposomes in response to different osmolarities after their formation from W/O droplets through the oil/water interface.

The most particular behavior was membrane rupture, the formation of a temporary, large opening, usually in one spot of the liposome membrane (opening of a hole, Figure 3). The sizes of those membrane holes were tens of micrometers in diameter, and their duration was around 1 s. Liposomes that opened a hole responded further to the osmolarity via one of three different types of processes: they broke completely and finally disappeared (Figure 3A), they transformed and/or shrunk (Figure 3B), or the hole resealed and they returned to their initial spherical shape (Figure 3C). The changes were complete within several seconds.

However, some liposomes transformed and/or shrank without opening a large hole (Figure 4). In these cases, the liposomes continued their response for up to several minutes, and thus the duration of any liposomal response was probably lengthened considerably when a membrane hole did not open. The duration of the response behaviors is understandable because the rate of movement of water and the exchange of solutions between the outside and the inside of a liposome should be much faster when the membrane opens a large hole than when the membrane remains intact.

Figure 5 summarizes the various liposomal behaviors. We observed five different types of responses (types I, II, III, IV, and V, see Figures 3 and 4), and they are classified into four categories according to two criteria: (1) whether there was an opening of a hole and (2) whether any shape changes were observed or their spherical shape was maintained.

It should be noted that there was no clear relation between the observed relative frequency of each type of response (types I−V and the cases where no response was observed within 5 min (type VI)) and the outside sucrose concentration (Figure 6). In the case of an accompanying opening of a hole (25−75%, total of three responses, types I−III), the maximum diameter of the hole was nearly proportional to the initial diameter of the liposome, and

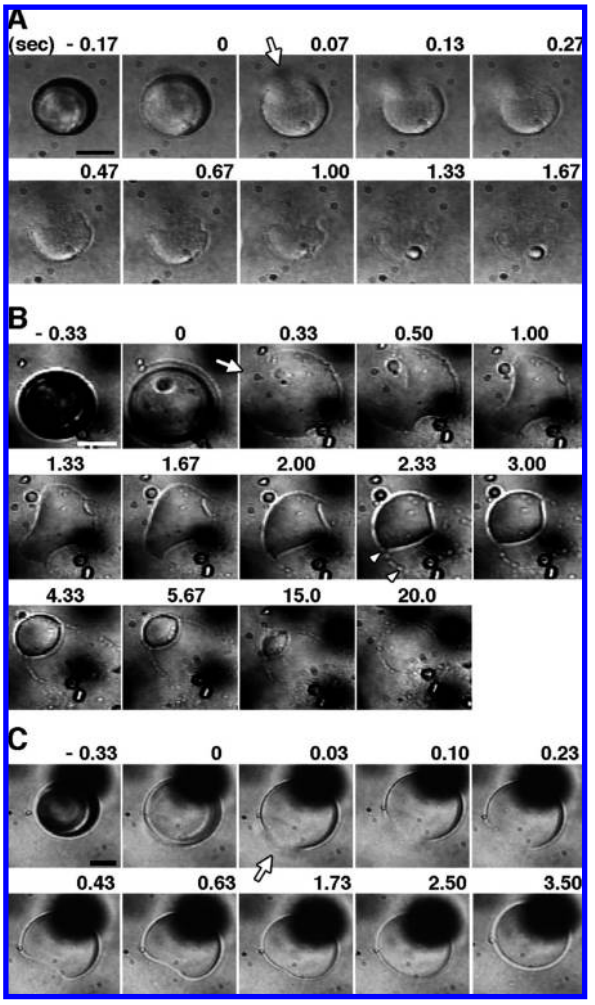

Figure 3. Time-lapse images of liposomes showing large membrane ruptures (opening of holes, indicated by arrows) in response to different osmolarities. The concentration of sucrose outside the formed liposomes is 1 M. Bars = 20 μm. We adjusted the microscopic focus position according to the motion of liposomes during the spontaneous transfer. The number indicated at the top of each panel shows the elapsed time (in seconds) after the formation of the liposome by transferring a W/O droplet through the oil/water interface. The time when the liposome was formed is defined as 0 s. (A) Liposome that burst and disappeared after the opening of a hole. This type of response is termed I. (B) Liposome that showed shrinkage and transformation simultaneously with or after the opening of a hole. Arrowheads indicate the tubular wreckage of the broken part of the transforming liposome often observed during liposomal transformation. This type of response is termed II. (C) Liposome that showed a resealing of the membrane after opening a hole. This type of response is termed III. In each case, the opening hole is easily distinguishable from invaginations of the liposomes and from breakage into much smaller aggregates by the movements of the surrounding small liposomes and debris (probably small oil droplets, which were squeezed out of the oil phase) (Supporting Information, Movies S1−S3). During the response, some liposomes are reduced in size. In such a case, the excess membrane material probably breaks into much smaller vesicles or some wreckage or the lipid density of the membranes increases. Note here that the black shadows that sometimes come into view are shadows of W/O droplets that happen to be nearby.

the relationship between the maximum diameter of the hole and the initial diameter of the liposome was independent of the outside sucrose concentration (Figure 7). Therefore, the average of the maximum hole diameter was also independent of the outside sucrose concentration and was constant because the size of the

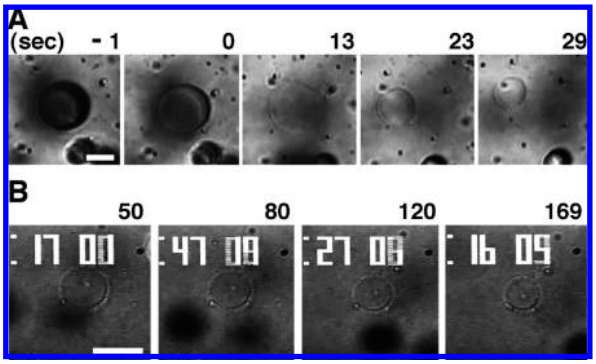

Figure 4. Time-lapse images of liposomes showing shrinkage, but not the opening of a hole, in response to a different osmolarity. During the shrinkage, some liposomes transformed their shape (A), but others did not (B). These responses are termed IV (A) and V (B), respectively. The concentrations of sucrose outside the formed liposomes are 500 (A) and 50 mM (B). Bars = 20 μm. We adjusted the microscopic focus position according to the motion of liposomes during the spontaneous transfer. The number indicated at the top of each panel shows the elapsed time (seconds) after the formation of the liposome by transferring a W/O droplet through the oil/water interface. The time when the liposome was formed is defined as 0 s.

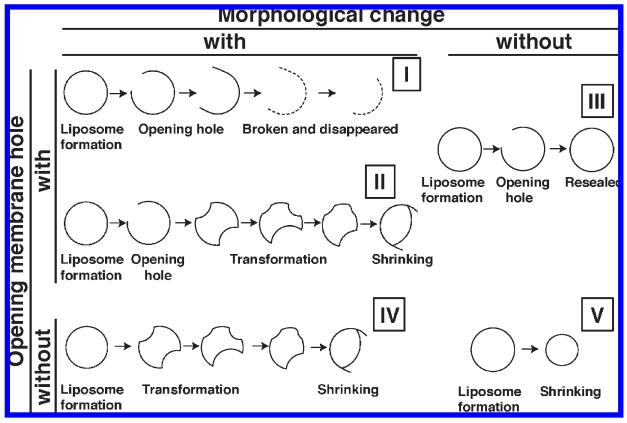

Figure 5. Summary of liposomal responses to osmotic pressure. There are five different types of responses, classified according to whether the opening of a hole is observed (vertical) and whether their spherical shape is altered (horizontal). They are termed type I to type V, respectively (Roman numbers in boxes; also see Figures 3 and 4).

liposomes formed is unaffected by the outside sucrose concentration (Figure 7, inset).

3.3. Lag Time for the Start of Liposomal Responses after Their Formation. The lag time for the liposomal response after its formation from a W/O droplet through the oil/water interface strongly depended on the outside sucrose or KCl concentration.

Figure 8 shows the relationship between the lag time and the concentration of sucrose or KCl ions and clearly shows that around 200 mM is the critical concentration that determined the lag time. When the outside solution concentration of sucrose was >250 mM, the response was induced in the liposome immediately after its formation. When the surrounding solution concentration of sucrose was <150 mM, a longer time period, sometimes more than 5 min, was required to detect any response in a liposome. The osmotic pressure on the liposomal membrane calculated from the concentration at 200 mM was about 0.5 pN/nm^2. The relationship between the lag time and the outside solute concentration is discussed further below.

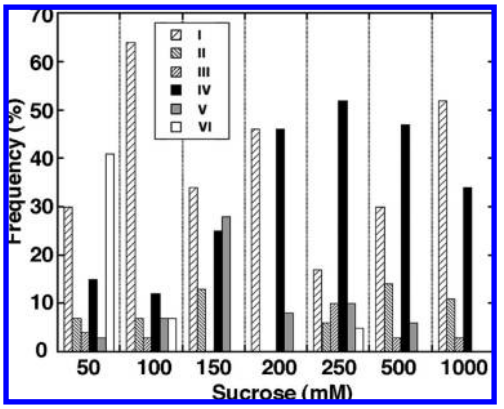

Figure 6. Dependence of liposomal responses on the outside sucrose concentration. The observed frequency (%) of each type of response at each sucrose concentration (mM) is shown. Roman numbers (I−V) indicate the respective response type (Figure 5), and VI (white bar) indicates cases where no response was observed within 5 min. Type VI could probably be designated as a type IV response with a much longer lag time. For each sucrose concentration, more than 20 liposomes, whose full response process could be monitored, were counted.

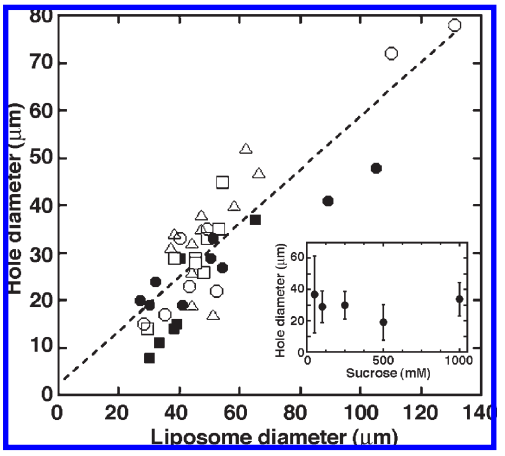

Figure 7. Dependence of the hole size on the initial size of the liposome. The maximum diameter of the membrane hole (micrometers) is plotted against the initial diameter of the liposome (micrometers). The outside concentrations of sucrose were 50 (○), 100 (●), 250 (□), 500 (■), and 1000 mM (△). For each sucrose concentration, more than eight liposomes, whose full response process could be monitored, were measured. The results include a total of three responses, types I−III. The straight dotted line is the result of the minimum square fitting to all plots. (The slope is 0.57, and the intercept of the perpendicular axis is 1.8, $R = 0.86$). (Inset) Average of the maximum diameter of the opened holes (micrometers) is replotted against the outside sucrose concentration (millimolar). Error bars indicate standard deviations.

Similar results were obtained when the liposomes encapsulated sucrose solution instead of water, as long as the difference in sucrose concentration between the outside and inside solutions of liposomes was the same (i.e., cases of creating liposomes encapsulating water in solutions of 100 mM sucrose and of creating liposomes encapsulating 100 mM sucrose in solutions of 200 mM sucrose; data not shown).

3.4. Liposomal Responses to Different Osmolarities of Salt. To confirm that our results were actually due to the osmotic effect, we also observed liposomal responses in solutions containing different concentrations of KCl and compared them with the results obtained using sucrose. The same liposomal behaviors were observed with different solutes. Liposomes responded to the different osmolarities through one of the five types of behaviors described above. Under all experimental conditions, the remarkable opening of a hole in the membrane was frequently observed. Furthermore, the relationship between the lag time after liposome formation for any response and the outside concentration of KCl ions (mM) was almost the same as that obtained when sucrose was used as the solute (Figure 8).

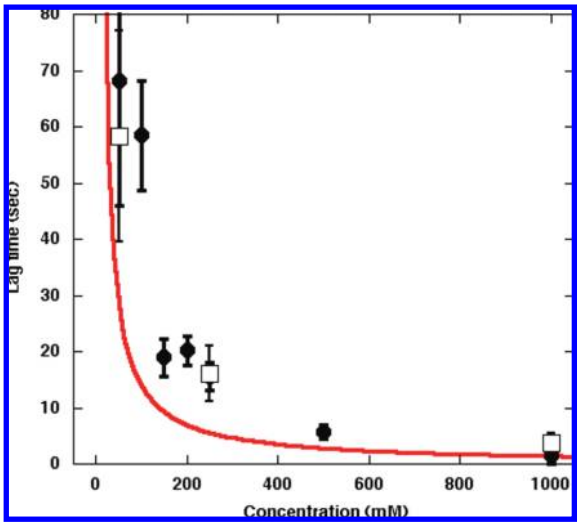

Figure 8. Lag times (seconds) between liposome formation and liposomal responses are plotted against the outside sucrose concentration (mM) (●) or twice that of KCl (□). For each point, more than 20 liposomes were observed and counted in triplicate experiments. Error bars indicate standard errors. The solid red line shows the theoretical curve given by eq 1.

4. Discussion

It was previously thought that, in response to osmotic pressure, a lipid bilayer membrane would allow water molecules to permeate its hydrophobic region. Therefore, when liposomes are subjected to high osmolarity, the water efflux across the membranes probably reduces the inner aqueous volume, leading to membrane shrinking.[5] Our results, however, clearly reveal that the vesicles are often broken and large holes, up to several micrometers in diameter, open in their membranes sometimes without membrane shrinking. In other words, the osmotic pressure, which is expected to force the vesicles to shrink, caused the membranes to break, including the opening of holes.

The remarkable membrane breaks observed at different osmolarities or in the presence of some additives are potentially very dangerous to living cells,[1,24,25] and cells have evolved various mechanisms to maintain their membrane stability. It seems likely that in vivo there are many proteins within or beneath the cellular membranes with activities that efficiently transport molecules through the membrane barrier, such as aquaporins (channels for water),[26] and that protect membranes from breakage due to physical stimuli, such as membrane-associated spectrin (fodrin) and actin filaments.[27] Thus, systems adopting the spontaneous transfer method evoked in this report should be a powerful tool

(24) Tamba, Y.; Yamazaki, M. *J. Phys. Chem. B* **2009**, *113*, 4846–4852.
(25) Saitoh, A.; Takiguchi, K.; Tanaka, Y.; Hotani, H. *Proc. Natl. Acad. Sci. U. S.A.* **1998**, *95*, 1026–1031.
(26) Agre, P.; Bonhivers, M.; Borgnia, M. J. *J. Biol. Chem.* **1998**, *273*, 14659–14662.
(27) Graham, H. T. *Bioessays* **2001**, *23*, 152–160.

for studying the effects of such proteins on the stability of membranes when they are subjected to a different osmolarity.

We previously observed the effect of changing osmolarity, which was caused by increasing the sucrose concentration of the outside solution using a mixing chamber, on giant liposomes that were made from eggPC by the natural swelling method.[16] That observation revealed that, in a sucrose gradient, the liposomes showed only shrinking transformations but not large membrane breaks such as the opening of holes found in this study (Figure S1). More essentially, with such a procedure we could not determine the exact osmolarity around the transforming liposomes.

In this study, however, we successfully applied an accurate, uniform osmotic pressure to liposomes using the spontaneous transfer method, and the results revealed that liposomes exhibit various types of behavior, including the opening of holes, in response to different osmolarities (Figures 3-6). In the case of an accompanying opening of a hole, the size ratio of the hole to the liposome was essentially constant (ratio of diameters, 0.57) and was independent of the sucrose concentration (Figure 7). To induce rapid responses to osmolarity, about 200 mM sucrose or 100 mM KCl was required (Figure 8).

The relationship between the lag time for liposomal response and the outside solute concentration can be interpreted in terms of membrane permeability. Under osmotic stress, the reduction of inner aqueous volume ΔV during time Δt is expressed as

$$\frac{\Delta V/v_{\mathrm{m}}}{\Delta t} = -APc \qquad (1)$$

where A is the membrane surface area, P is the membrane permeability (we use $P = 4 \times 10^{-3}$ cm s^{-1} as reported previously[28]), v_{m} is the water molar volume, and c is the difference in molar concentrations across the vesicle membrane. Here, we define the lag time as the time for which pressure moves the membrane wall in the micrometer range (microscopically observable scale). By substituting $\Delta V = A \times 1\ \mu$m into eq 1, we can obtain c (mM) $\times \Delta t$ (s) $= 1.4 \times 10^3$. The time Δt is proportional to the inverse of the molar concentration Δc, which is quantitatively in agreement with the experimental results (Figure 8). Thus, the permeability of the membrane to water molecules may play an important role in determining its behavior when placed in a different osmolarity.

In our system, however, the following questions remain to be investigated. (i) Only the case of water-containing liposomes formed in the solute-containing solution is shown in this report because the rapid membrane breakages, probably caused by expansion, made observations with reversed osmolarity much more difficult. Thus, cases when a reversed osmotic pressure is applied to liposomes need to be studied. It should be noted here that, in our experiments, the osmotic pressure from the surrounding solution (containing sucrose or KCl) forces the vesicles to shrink. Thus, the opening of the membrane holes found in this study is distinct from burst membranes, which are induced by the expansion of liposomes.[1] (ii) The effects on liposomal responses of biological or chemical factors thought to affect membrane stability or molecular transport across the lipid bilayer membrane should be studied. Fodrin/spectrin and aquaporins may be candidates for such biological factors. The former protein is well known to form a network underlying plasma membranes to reinforce them,[29] and the latter are water channels thought to regulate cellular osmotic conditions.[30,31] (iii) We used only eggPC to form liposomes because our main purpose was to investigate biological membrane homeostasis against osmotic pressure. EggPC is one of the most representatively used phospholipids obtained from a native source. Other lipid compositions, however, should be tested. For example, we could study the behavior of liposomes that are made from lipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) because those lipids frequently have been used to make giant liposomes by the natural swelling and electroformation methods.[2,32] (iv) It should be determined if additional factors may determine the site of the opening in a liposomal membrane and, if so, what factors are involved. The reason, along with the mechanism, as to why there are different possible behaviors of liposomes responding different osmolarities, even under the same experimental conditions, should also be characterized.

Even though it has been validated that the membranes obtained by the spontaneous transfer method are essentially unilamellar lipid bilayers (e.g., by the reconstructing activity of protein channels in the membrane[33]),[17,21,22] it is still possible that oil is contained in the inner hydrophobic part of the lipid bilayers. In addition, the content and distribution of the oil contained in the bilayers have remained unclear. Hence, the possible presence of oil in the lipid bilayers is an influential reason for the nonuniformity in the membrane of individual liposomes just formed. Such nonuniformity may induce the variations in the liposomal responses, including membrane ruptures such as the opening of holes, even under the same experimental conditions. The gap between the observed results and the model concerning the relationship between the lag time for liposomal responses and the outside solute concentrations (Figure 8) also might be explained by the possible presence of oil.

Alternatively, because the liposomal membranes used in this study were made from eggPC, which may have various hydrophobic tails even though the hydrophilic heads are uniform, it is possible that the liposomes had some nonuniformity in their membranes and that the stability throughout the liposomal membranes was not uniform, resulting in the observed variations in liposomal behavior. To evaluate this possibility, the behavior of liposomes made from a single synthetic phospholipid such as DOPC or POPC should be observed and compared with the case of eggPC. However, even if liposomes are formed by a single synthetic phospholipid, it is still possible that they will have some nonuniformity, such as a subtle difference in the curvature or density of lipid molecules, in their membrane, thus similar membrane ruptures, such as the opening of holes, could be observed.

In addition, in our procedure, the lipid headgroups of the initially formed monolayer (the inner leaflet of the bilayer liposome) are facing Milli-Q water whereas the lipid head groups of the subsequently formed monolayer (the outer leaflet of the bilayer liposome) are facing the sucrose or KCl solution. Therefore, it is possible that the lipid bilayer that forms the liposome membrane is asymmetrical because of specific interactions between the solute and the membrane lipids and that this results in inherent membrane instability and/or liposome transformations (i.e., the significant membrane burst and/or liposome shrinkage).[2] Moreover, because it is too difficult to decouple the effects of the

(28) Boroske, E.; Elwenspoek, M.; Helfrich, W. *Biophys. J.* **1981**, *34*, 95–109.
(29) Marchesi, V. T. *Annu. Rev. Cell Biol.* **1985**, *1*, 531–561.
(30) Agre, P.; Kozono, D. *FEBS Lett.* **2003**, *555*, 72–78.
(31) Kaldenhoff, R.; Fischer, M. *Acta Physiol.* **2006**, *187*, 169–176.
(32) Shimanouchi, T.; Umakoshi, H.; Kuboi, R. *Langmuir* **2009**, *25*, 4835–4840.
(33) Le Pioufle, B.; Suzuki, H.; Tabata, K.; Noji, H.; Takeuchi, S. *Anal. Chem.* **2008**, *80*, 328–332.

osmotic pressure itself and of the membrane properties of liposomes generated under osmotic stress, the above-mentioned problems, which originate in the methodology, still remain an important subject.

As previously mentioned, there still remain many subjects that should be clarified, including the problem of oil contamination. Regarding the differences in methodology, we are now trying to compare the response behavior to osmolarity of giant liposomes obtained using the spontaneous transfer method where the vesicles are obtained by natural swelling or electroformation. Nevertheless, we conclude that the liposome behaviors observed here might result from some effect of osmotic pressure because the same results were obtained regardless of whether sucrose or salt was used as the solute (Figure 8) and also because the liposome behaviors were seen only when the liposomes were subjected to a different osmolarity (Figure 2). Our system should at least be effective in characterizing the stability of biological membranes because they often contain constituents besides phospholipids, such as cholesterol, fatty acids, and/or other hydrophobic biological elements.

The remarkable membrane breaks observed in this study are potentially very dangerous to living cells, and cells have evolved various mechanisms to maintain their membrane stability, mainly utilizing various membrane-associated proteins such as cytoskeletal arrays beneath the cellular membranes.[27] The spontaneous transfer is a very convenient method of supplying cell-sized giant liposomes that contain the desired amounts of complex protein systems.[23] Thus, our system adopting the spontaneous transfer method should be a powerful tool for studying the mechanisms by which such proteins are used to maintain membrane stability.

5. Conclusions

In this study, using the spontaneous transfer method, we succeeded in putting accurate and uniform osmotic pressures on liposomes. The results show that (i) cell-sized giant liposomes containing water were successfully formed in solutions containing sucrose (up to 1000 mM) or KCl (up to 500 mM), (ii) individual liposomes randomly showed one of five different possible responses, (iii) the majority of the liposomes showed opening-of-a-hole behavior regardless of the difference between the inside and outside solute concentrations, (iv) the lag time for any response, including the opening of a hole, after liposome formation decreased with the increasing difference in concentration, and (v) liposomes responded to different osmolarities in the same way, whether surrounded by a sucrose or KCl solution.

Acknowledgment. We thank Dr. Kenichi Yoshikawa (Kyoto University, Japan) for critically reading the manuscript and making valuable comments, Mrs. Makiko Negishi (Kyoto University, Japan) and Mrs. Ayako Yamada (Ecole Normale Superieure, France) for invaluable encouragement and technical support, and Mr. Masato Ito (Nagoya University) for the preparation of the bead-containing liposomes. This study was supported in part by a Grant-in-Aid (Scientific Research of Priority Areas, System Cell Engineering by Multiscale Manipulation) from the MEXT of Japan.

Supporting Information Available: Liposome behavior observed in a concentration gradient of sucrose. Movies S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.