Double Emulsion Templated Monodisperse Phospholipid Vesicles

Ho Cheung Shum,[†] Daeyeon Lee,[†] Insun Yoon,[§] Tom Kodger,[†] and David A. Weitz*

School of Engineering and Applied Sciences and Department of Physics, Harvard University, Cambridge, Massachusetts 02138

Received June 11, 2008

Ⓦ **This paper contains enhanced objects available on the Internet at http://pubs.acs.org/Langmuir.**

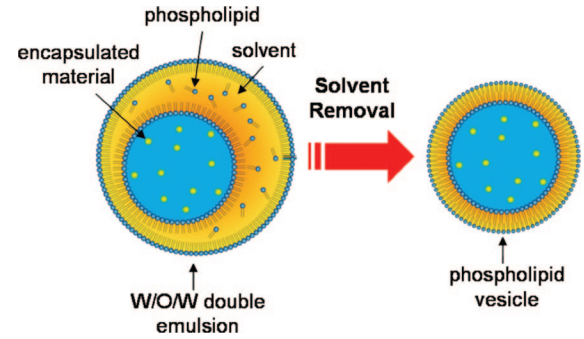
We present a novel approach for fabricating monodisperse phospholipid vesicles with high encapsulation efficiency using controlled double emulsions as templates. Glass-capillary microfluidics is used to generate monodisperse double emulsion templates. We show that the high uniformity in size and shape of the templates are maintained in the final phospholipid vesicles after a solvent removal step. Our simple and versatile technique is applicable to a wide range of phospholipids.

Introduction

Vesicles, also known as liposomes, are phospholipid bilayer membranes which surround aqueous compartments. They are promising delivery vehicles for drugs,[1] enzymes,[2] and gases,[3] and bioreactors for biomedical applications.[4] Since phospholipids are an integral component of biological membranes, phospholipid vesicles also provide ideal platforms for the study of the physical properties of biomembranes.[5] Conventional vesicle formation techniques such as hydration and electroformation rely on the self-assembly of phospholipids in an aqueous environment under shear and electric field, respectively.[6] Due to the random nature of the bilayer folding, these methods typically lead to the formation of vesicles that are nonuniform in both size and shape. Moreover, the encapsulation efficiency of these processes is quite low, generally less than 35%.[7,8]

A new strategy to form phospholipid vesicles with high uniformity in size and high encapsulation efficiency is to utilize templates to generate the vesicles. An example is the use of simple water-in-oil (W/O) emulsions as templates.[10]Monodisperse emulsion templates can be generated using microfluidics.[8] Another strategy is to apply pulsed microfluidic jets to deform planar lipid membranes into vesicle-like compartments[11] and vesicles.[12] However, it is difficult to precisely control such processes for vesicle formation.

Scheme 1. Preparation of Phospholipid Vesicles Using Double Emulsion As Templates



Here, we present a novel technique for forming phospholipid vesicles using monodisperse double emulsions with a core−shell structure as templates. Because of the resemblance of core−shell structures to vesicular structures, techniques that rely on double emulsion templates should be robust and straightforward. In our approach, phospholipids are dissolved in a mixture of volatile organic solvents that is immiscible with aqueous phases. The phospholipid solution forms the shell of water-in-oil-in-water (W/O/W) double emulsions. Such phospholipid-stabilized W/O/W double emulsion drops have previously been used as vesicle-like compartments for encapsulation;[11] however, in our work, we use them as templates to direct the formation of phospholipid vesicles by removing the solvent in oil phase through evaporation, as illustrated in Scheme 1. This approach has been applied to form polymersomes, vesicular structures composed of a bilayer of amphiphilic diblock copolymers.[13] However, phospholipid bilayers are too fragile to undergo the solvent removal step without

* To whom correspondence should be addressed. Telephone: 617-496-2842. Fax: 617-495-2875. E-mail: weitz@seas.harvard.edu.
† School of Engineering and Applied Sciences.
‡ Department of Physics.
§ Current affiliation: Mount Holyoke College, Amherst, Massachussetts.

(1) (a) Cevc, G. *Adv. Drug Delivery Rev.* **2004**, *56*, 675–711. (b) Gregoriadis, G.; Florence, A. T. *Drugs* **1993**, *45*, 15–28. (c) Schreier, H.; Bouwstra, J. *J. Controlled Release* **1994**, *30*, 1–15.
(2) Walde, P.; Ichikawa, S. *Biomol. Eng.* **2001**, *18*, 143–177.
(3) Sakai, H.; Tsuchida, E. *J. Liposome Res.* **2007**, *17*, 227–235.
(4) (a) Pohorille, A.; Deamer, D. *Trends Biotechnol.* **2002**, *20*, 123–128. (b) Monnard, P. A. *J. Membr. Biol.* **2003**, *191*, 87–97. (c) Noireaux, V.; Libchaber, A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17669–17674.
(5) (a) Hanczyc, M. M.; Szostak, J. W. *Curr. Opin. Chem. Biol.* **2004**, *8*, 660–664. (b) Noireaux, V.; Bar-Ziv, R.; Godefroy, J.; Salman, H.; Libchaber, A. *Phys. Biol.* **2005**, *2*, P1−P8. (c) Angelova, M. I.; Tsoneva, I. *Chem. Phys. Lipids* **1999**, *101*, 123–137. (d) Fischer, A.; Oberholzer, T.; Luisi, P. L. *Biochim. Biophys. Acta* **2000**, *1467*, 177–188. (e) Jahn, A.; Vreeland, W. N.; Gaitan, M.; Locascio, L. E. *J. Am. Chem. Soc.* **2004**, *126*, 2674–2675. (f) Jahn, A.; Vreeland, W. N.; DeVoe, D. L.; Locascio, L. E.; Gaitan, M. *Langmuir* **2007**, *23*, 6289–6293.
(6) (a) Lasic, D. D. *Biochem. J.* **1988**, *256*, 1–11. (b) Dimova, R.; Aranda, S.; Bezlyepkina, N.; Nikolov, V.; Riske, K. A.; Lipowsky, R. *J. Phys.: Condens. Matter* **2006**, *18*, S1151–S1176.
(7) (a) Sun, B. Y.; Chiu, D. T. *Anal. Chem.* **2005**, *77*, 2770–2776. (b) Glavas-Dodov, M.; Fredro-Kumbaradzi, E.; Goracinova, K.; Simonoska, M.; Calis, S.; Trajkovic-Jolevskaa, S.; Hincal, A. A. *Int. J. Pharm.* **2005**, *291*, 79–86.
(8) (a) Tan, Y. C.; Hettiarachchi, K.; Siu, M.; Pan, Y. P. *J. Am. Chem. Soc.* **2006**, *128*, 5656–5658. (b) Sugiura, S.; Kuroiwa, T.; Kagota, T.; Nakajima, M.; Sato, S.; Mukataka, S.; Walde, P.; Ichikawa, S. *Langmuir* **2008**, *24*, 4581–4588.
(9) (a) Taylor, P.; Xu, C.; Fletcher, P. D. I.; Paunov, V. N. *Chem. Commun.* **2003**, 1732–1733. (b) Taylor, P.; Xu, C.; Fletcher, P. D. I.; Paunov, V. N. *Phys. Chem. Chem. Phys.* **2003**, *5*, 4918–4922. (c) LeBerre, M.; Yamada, A.; Reck, L.; Chen, Y.; Baigl, D. *Langmuir* **2008**, *24*, 2643–2649.
(10) (a) Pautot, S.; Frisken, B. J.; Weitz, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10718–10721. (b) Yamada, A.; Yamanaka, T.; Hamada, T.; Hase, M.; Yoshikawa, K.; Baigl, D. *Langmuir* **2006**, *22*, 9824–9828.
(11) Funakoshi, K.; Suzuki, H.; Takeuchi, S. *J. Am. Chem. Soc.* **2007**, *129*, 12608–12609.
(12) Stachowiak, J. C.; Richmond, D. L.; Li, T. H.; Liu, A. P.; Parekh, S. H.; Fletcher, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 4697–4702.

10.1021/la801833a CCC: $40.75
Published on Web 07/10/2008

breakage; thus, this method has not been successfully applied to phospholipid vesicles. Here, we introduce new strategies to improve the stability of phospholipid vesicles during solvent removal. Our technique can be used to create phospholipid vesicles with different composition while maintaining high size uniformity and encapsulation efficiency.

Experimental Section

Materials. The inner phase of the water-in-oil-in-water (W/O/W) double emulsion droplets was made of 0−5 wt % poly(vinyl alcohol) (PVA; M_w: 13 000−23 000 g·mol^{-1}, 87−89% hydrolyzed, Sigma-Aldrich Co.) and ~0.02 wt % 1 μm yellow-green sulfate-modified microspheres (Fluosphere, Invitrogen, Inc.). Unless otherwise noted, the middle organic phase consisted of 5−10 mg·mL^{-1} lipids with 0.02 mol % Texas red labeled 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE) for fluorescent visualization in an organic solvent mixture of toluene (EMD Chemicals, Inc.) and chloroform (Mallinckrodt Chemicals, Inc.) in 1.8:1 volume ratio. Experiments were conducted with the following lipids: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phoscholine (POPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (DPPS), and Texas red labeled 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE). All lipids were purchased in powder form from Avanti Polar Lipids, Inc. The outer phase was either a 10 wt % PVA solution or a 40 vol % glycerol and 2 wt % PVA solution. Solutions and solvents were all filtered before introduction into glass microcapillary devices. Water with a resistivity of 18.2 MΩ·cm^{-1} was acquired from a Millipore Milli-Q system.

Generation of Double Emulsions. Monodisperse W/O/W double emulsions were prepared in glass microcapillary devices, which have been described previously.[14] The round capillaries, with inner and outer diameters of 0.58 mm and 1.0 mm, were purchased from World Precision Instruments, Inc. and tapered to desired diameters with a micropipet puller (P-97, Sutter Instrument, Inc.) and a microforge (Narishige International USA, Inc.). The tapered round capillaries were fitted into square capillaries (Atlantic International Technology, Inc.) with an inner dimension of 1.0 mm for alignment. The outer radii, R_o, of the double emulsions varied from 60 to 100 μm, while the inner radii, R_i, varied from 40 to 60 μm. These values were controlled by the size of the capillaries used and the flow rates of the different phases.[14] A typical set of flow rates for the outer, middle, and inner phase was 3500, 800, and 220 μL/hr, respectively, and the droplet generation frequency was about 500 Hz. The formation of lipid vesicles was monitored with optical microscopy for samples placed between a coverslip and a glass slide separated by a 0.5 mm thick silicone isolator (Invitrogen, Inc.) alone or by two silicon isolators separated by an anodized alumina membrane filter (Anodisc 25, 0.2 μm, Whatman plc.).

Characterization of Phospholipid Vesicles. Bright-field, phase-contrast, and fluorescence images were obtained with 5×, 10×, 20×, and 40× objectives at room temperature using an inverted fluorescence microscope (Leica, DMIRB or DMIRBE) or an upright fluorescence microscope (Leica, DMRX) equipped with a high speed camera (Phantom V5, V7, or V9) or a digital camera (QImaging, QICAM 12-bit). All double emulsion generation processes were monitored with the microscope using a high speed camera. The process of lipid vesicle formation from double emulsions and the resulting lipid vesicles were imaged with a digital camera.

Results and Discussion

Monodisperse double emulsions are generated with a glass microcapillary microfluidic device that combines a coflow and a flow focusing geometry shown in Figure 1a.[14] The inner phase (green arrow) is an aqueous solution of model encapsulant, while the outer phase (blue arrow) is an aqueous solution of poly(vinyl alcohol) (PVA) and glycerol. The middle phase (white arrow) is a solution of phospholipids dissolved in a mixture of toluene and chloroform. Hydrodynamically focused inner and middle fluid streams break up at the orifice of the collection tube to form monodisperse W/O/W double emulsion drops, as shown in Figure 1a. Typical droplet generation frequency is about 500 Hz. The overall size and the thickness of the shell of the double emulsions can be adjusted by tuning the flow rates of each fluid phase and the diameters of each capillary in the device.[14] The uniformity in size and shape of the collected double emulsion drops, shown in Figure 1b, makes them ideal templates for the generation of uniform phospholipid vesicles. In the absence of phospholipids, the double emulsions are unstable, suggesting that phospholipids adsorb at the W/O and O/W interfaces and stabilize the structures.

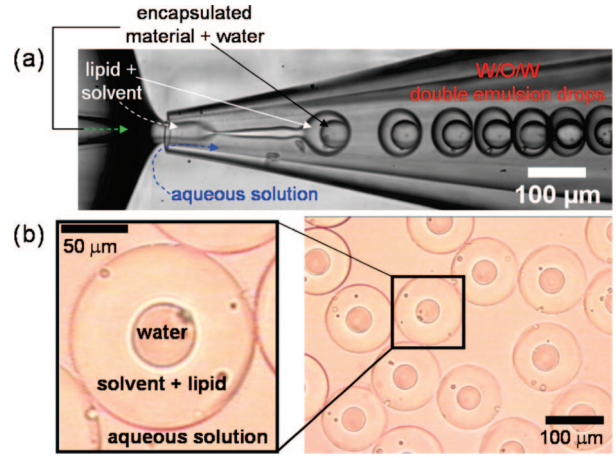


Figure 1. (a) Formation of phospholipid-stabilized W/O/W double emulsion in a glass microcapillary device. (b) Optical micrograph of the double emulsion collected. The double emulsion drops have an aqueous core surrounded by a solvent shell containing phospholipid.

Phospholipid vesicles are obtained from the double emulsions by removing the solvent from the hydrophobic layer of W/O/W double emulsions (Scheme 1). We use a mixture of volatile organic solvents, toluene and chloroform, to facilitate phospholipid dissolution and subsequent solvent evaporation. As the solvent layer becomes thinner during evaporation, the phospholipids are concentrated and then forced to arrange on the double emulsion templates, thereby forming vesicles. At the later stage of evaporation, the remaining solvent containing the excess phospholipids accumulates on one side of the vesicle, as shown in the top panel of Figure 2. Such a dewetting phenomenon has also been observed when amphiphilic diblock copolymers are used for the generation of polymersomes from double emulsions.[15] The depletion force generated by excess phospholipid molecules in the solvent induces the dewetting.[15] Due to the fragile nature of the phospholipid bilayers, the vesicles often destabilize and rupture during the evaporation process. To avoid this, we find that slow evaporation of the organic solvent is critical; thus, we use a loosely sealed container to slow the evaporation. We also remove solvent by dialysis; an anodized alumina filter was used as the dialysis membrane. The rate of the solvent removal can then be controlled by adjusting the solvent concentration of the adjacent solution. A typical removal rate is about 0.35 pL·min^{-1} (see Figure S1 in the Supporting Information). The reproducibility

(13) (a) Lorenceau, E.; Utada, A. S.; Link, D. R.; Cristobal, G.; Joanicot, M.; Weitz, D. A. *Langmuir* **2005**, *21*, 9183–91861. (b) Hayward, R. C.; Utada, A. S.; Dan, N.; Weitz, D. A. *Langmuir* **2006**, *22*, 4457–4461.

(14) Utada, A. S.; Lorenceau, E.; Link, D. R.; Kaplan, P. D.; Stone, H. A.; Weitz, D. A. *Science* **2005**, *308*, 537–541.

(15) Hayward, R. C.; Utada, A. S.; Dan, N.; Weitz, D. A. *Langmuir* **2006**, *22*, 4457–4461.

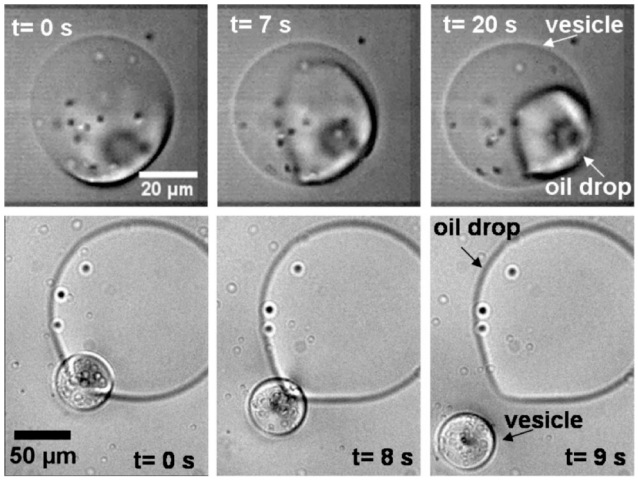


Figure 2. Ⓦ (Top) Vesicle formation (see video in AVI format) through solvent drying on the vesicle surface. Excess phospholipid is concentrated in the remaining oil drop attached to the resulting vesicle. (Bottom) Release of vesicle (see video in AVI format) from a double emulsion drop pinned on a glass slide. The oil drop that contains excess phospholipids remains on the glass slide. Fluorescently labeled latex particles, which were added to the inner aqueous phase during double emulsion formation, are encapsulated in the vesicles.

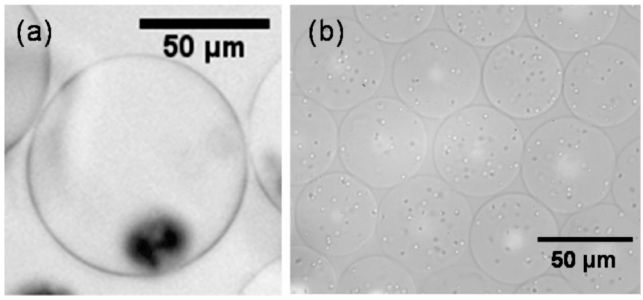


Figure 3. (a) Optical micrograph of a DPPC/DPPS (10:1 w/w) vesicle formed by solvent drying. Excess phospholipids remain on the vesicle, forming the dark spot after drying. (b) Optical micrograph of an array of homogeneous POPC vesicles, encapsulating 1 μm fluorescent latex particles that have been added to the inner aqueous phase.

of the technique is further improved by carrying out the evaporation step in highly concentrated glycerol solutions (typically above 80 wt %). We believe glycerol plays an important role in reducing the line tension incurred in the solvent removal step;[16] however, the exact stabilization mechanism is yet to be established. After the complete removal of the solvent, the excess phospholipids remain on the vesicle, leaving a thicker patch, as seen in Figure 3a as a dark spot. The size of this patch is minimized when the amount of excess phospholipid in the oil phase is reduced either by reducing the phospholipid concentration in the middle fluid or by forming a thinner shell when generating the double emulsion.

Phospholipid vesicles can also be formed through another mechanism. When the double emulsion droplets wet the substrate, they can become pinned to it, and the inner drops can be released as vesicles into the continuous phase. Upon release of the inner drops, the middle organic solvent layer remains pinned to the substrate, as shown in the bottom panel of Figure 2. This process resembles a method where phospholipid-stabilized water droplets

(16) (a) Sandre, O.; Moreaux, L.; Brochard-Wyart, F. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10591–10596. (b) Karatekin, E.; Sandre, O.; Guitouni, H.; Borghi, N.; Puech, P. H.; Brochard-Wyart, F. *Biophys. J.* **2003**, *84*, 1734–1749.

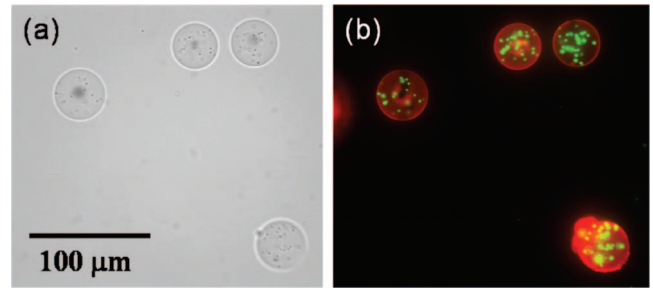


Figure 4. (a) Optical micrograph of yellow-green fluorescent latex microspheres encapsulated inside DPPC vesicles stained with 0.02 mol % Texas red labeled DHPE for visualization. (b) Overlay of two fluorescent images of the same vesicles as in (a). The microspheres remain encapsulated within the vesicles.

are formed in oil and subsequently transported through an oil/water interface that is covered with a monolayer of phospholipids, resulting in the generation of vesicles.[10] In our case, the inner drops of the pinned double emulsion, stabilized by phospholipids, move across the interface between the oil and the continuous aqueous phase. Phospholipids adsorbed at this water/oil interface stabilize the escaping inner drop by completing the bilayers. This second route to phospholipid vesicle generation offers a simple and effective way of obtaining homogeneous vesicles if the double emulsions can be controllably pinned on a substrate. An array of monodisperse phospholipid vesicles that have been formed through this second mechanism is shown in Figure 3b. Using the same approach, vesicles have been generated using a variety of phospholipids including both saturated (DPPC, DMPC, and DSPC) and unsaturated (DOPC and POPC) phosphocholines used alone or mixed with a phospho-L-serine (DPPS). Typical size of the vesicles ranges from 20 to 150 μm, a size where monodisperse vesicles are difficult to obtain otherwise.

To demonstrate the high encapsulation efficiency of our approach, we encapsulate 1 μm yellow-green fluorescent latex microspheres inside phospholipid membranes which are labeled with a small amount (0.02 mol %) of Texas Red-labeled 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE). Optical and fluorescence microscopy images of four DPPC vesicles encapsulating microspheres are shown in Figure 4. Very few microspheres are observed in the continuous phase, confirming that the high encapsulation efficiency of the double emulsion generation stage is retained even after the emulsion drops are converted to vesicles.

In conclusion, we present a general method for fabricating monodisperse phospholipid vesicles using controlled double emulsions as templates. Our simple and versatile technique offers a novel route to generate monodisperse phospholipid vesicles with high encapsulation efficiency for biomedical applications and for fundamental studies of biomembrane physics.

Acknowledgment. This work was supported by the NSF (DMR-0602684) and the Harvard MRSEC (DMR-0213805). H.C.S. and D.A.W. were supported in part by BASF Ludwigshafen in Germany. We thank Andrew S. Utada, Raphael Bruckner, Christian Holtze, Jin-Woong Kim, and Amy Rowat for helpful discussions.

Supporting Information Available: Estimation of the solvent removal rate. This material is available free of charge via the Internet at http://pubs.acs.org.

LA801833A