New Nanoscale Pulsatile Drug Delivery System

Xin-Ming Liu,[†] Bin Yang,[§] Yu-Lu Wang,*[,‡] and Jin-Ye Wang*[,†,§]

Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 354 Fenglin Road, Shanghai 200032, China, College of Chemical and Environmental Science, Henan Normal University, Xinxiang 453002, China, and Biomedical Engineering, Shanghai Jiao Tong University, 1954 Huashan Road, 200030, China

Received November 25, 2004
Revised Manuscript Received April 15, 2005

Since their discovery in the early 1960s by Bangham and co-workers,[1] liposomes have stimulated ongoing interest in applications in drug delivery and controlled release. This is because liposomes with diameters approximately of 100 nm can be delivered to tumor tissues at high concentrations and liposomes can encapsulate a wide range of drugs.[2,3] However, the passive release of encapsulated drugs from liposomes through adsorption, fusion, or endocytosis by cells at the target site may not achieve good bioavailability because of low liposome stability (due to easy fusion) and high spontaneous drug release, which limit their applications. Finding new materials to increase drug efficacy and decrease the spontaneous release and fusion of liposomes is important to making progress in this area. Several systems employing pH, thermal, ultrasonic, and photochemical triggering to achieve pulsatile release have been studied.[4−7] Phototriggering offers an attractive alternative to the temperature- or pH-driven modulation of drug release, as it provides a broad range of adjustable parameters (e.g., wavelength, duration, intensity) that can be optimized to suit a given application. Azobenzene derivatives were studied as phototriggers because they undergo photoisomerization upon UV and visible light irradiation. Morgan et al. incorporated bis-azo phosphatidylcholine (bis-azo PC) into liposomes as a phototrigger. The photoisomerism of azobenzene derivatives caused phase separation and fusion of liposomes, which induced rapid release of drug (total release within a few minutes),[8−14] but achieving multi-pulsatile release was difficult. Furthermore, bis-azo PC destabilized liposomes and promoted the spontaneous release of liposomes. The Yonezawa[15] and Hurst[16] groups incorporated single-chain azobenzene derivatives into liposomes and realized multi-pulsatile release. However, single chain azobenzene derivatives promoted the phase separation and fusion of liposomes,[17,18] and promoted spontaneous release of their contents.

It is well-known that cholesterol is an important component of biomembranes and that it can interact or form complexes with phospholipids.[19] Owing to this, cholesterol's functions include modulating the fluidity of biomembranes, increasing their mechanical strength, and decreasing their permeability.[20,21] Cholesterol derivatives containing an azobenzene moiety should not only serve as phototriggers, but should retain the intrinsic biophysical properties of cholesterol. This study describes a novel cholesterol lipid containing an azobenzene moiety.

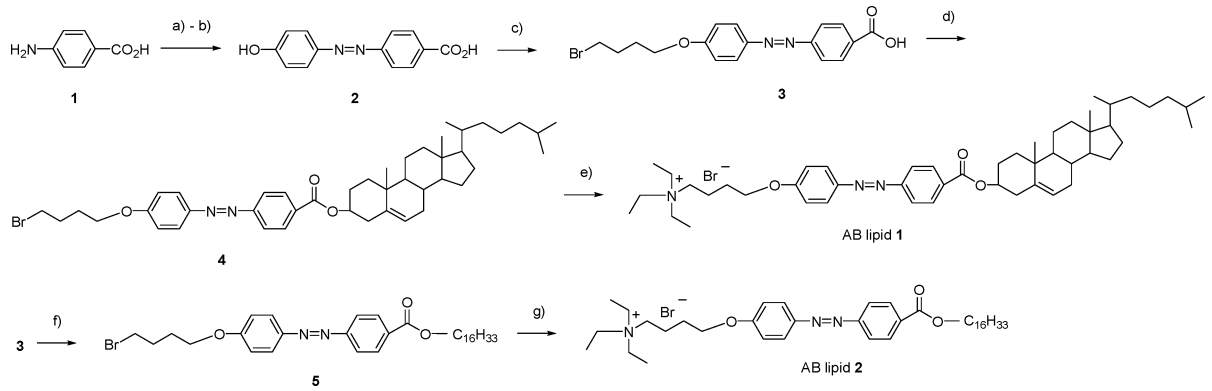
AB lipid **1** was synthesized and incorporated into liposomes to investigate its photocontrolled release of interior material from liposomes, and its effects on liposome stability and cytotoxicity. AB lipid **2** was also synthesized and compared with AB lipid **1** in liposomes, because its effective chain length is estimated to correspond to that of cholesterol.[22] The synthetic pathways for preparation of AB lipid **1** and AB lipid **2** are illustrated in Scheme 1. AB lipid **1** was incorporated into egg PC liposomes to investigate the photocontrolled release of liposomal contents. The diameter of the liposomes was about 100 nm (Figure 1A). Particle size analysis showed that the liposomes, with an average diameter of 99.7 nm, were rather homogeneous in size, and distributed between 80 and 120 nm (Figure 2). It is well-known that growing solid tumors have abnormal blood vessels with loose junction and insufficient lymphatic drainage, so that liposomes that have diameters in the range of approximately 100 nm can escape from tumor vasculature but not from normal vasculature and accumulate in tumors

* Authors to whom correspondence should be addressed. J.-Y.W. E-mail: jywang@mail.sioc.ac.cn. Y.-L.W. E-mail: wangyulu_youji01@sina.com.
[†] Chinese Academy of Sciences.
[‡] Henan Normal University.
[§] Shanghai Jiao Tong University.

(1) Bangham, A. D.; Flemans, R.; Heard, D. H.; Seaman, G. V. F. *Nature* **1958**, *182*, 642−644.
(2) Gerasimov, O. V.; Boomer, J. A.; Qualls, M. M.; Thompson, D. H. *Adv. Drug Delivery Rev.* **1999**, *38*, 317−338.
(3) Yuan, F.; Leunig, M.; Huang, S. K.; Berk, D. A.; Papahadjopoulos, D.; Jain, R. K. *Cancer Res.* **1994**, *54*, 3352−3356.
(4) Connor, J.; Yatvin, M. B.; Huang, L. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1715−1718. Yatvin, M. B.; Krentz, W.; Horwitz, M.; Shinitzky, M. *Science* **1980**, *210*, 1253−1255.
(5) Needham, D.; Dewhirst, M. W. *Adv. Drug Delivery Rev.* **2001**, *53*, 285−305.
(6) Lin, H. Y.; Thomas, J. L. *Langmuir* **2003**, *19*, 1098−1105.
(7) Shum, P.; Kim, J.-M.; Thompson, D. H. *Adv. Drug Delivery Rev.* **2001**, *53*, 273−284.
(8) Morgan, C. G.; Thomas, E. W.; Yianni, Y. P.; Sandhu, S. S. *Biochim. Biophys. Acta* **1985**, *820*, 107−114.
(9) Morgan, C. G.; Thomas, E. W.; Sandhu, S. S.; Yianni, Y. P.; Mitchell, A. C. *Biochim. Biophys. Acta* **1987**, *903*, 504−509.
(10) Morgan, C. G.; Bisby, R. H.; Johnson, S. A.; Mitchell, A. C. *FEBS Lett.* **1995**, *375*, 113−116.
(11) Bisby, R. H.; Mead, C.; Morgan, C. G. *FEBS Lett.* **1999**, *463*, 165−168.
(12) Bisby, R. H.; Mead, C.; Mitchell, A. C.; Morgan, C. G. *Biochem. Biophys. Res. Commun.* **1999**, *262*, 406−410.
(13) Bisby, R. H.; Mead, C.; Morgan, C. G. *Biochem. Biophys. Res. Commun.* **2000**, *276*, 169−173.
(14) Bisby, R. H.; Mead, C.; Morgan, C. G. *Photochem. Photobiol.* **2000**, *72*, 57−61.
(15) Sato, T.; Kijima, M.; Shiga, Y.; Yonezawa, Y. *Langmuir* **1991**, *7*, 2330−2335.
(16) Lei, Y.; Hurst, J. K. *Langmuir* **1999**, *15*, 3424−3429.
(17) Shimomura, M.; Kunitake T. *J. Am. Chem. Soc.* **1987**, *109*, 5175−5183.
(18) Shimomura, M.; Kunitake T. *Chem. Lett.* **1981**, 1001−1004.
(19) Ohvo-Rekila, H.; Ramstedt, B.; Leppimaki, P.; Slotte, J. P. *Prog. Lipid Res.* **2002**, *41*, 66−97.
(20) Yeagle, P. L. *Biochim. Biophys. Acta* **1985**, *822*, 267−287.
(21) McIntosh, T. J. In *Membrane Permeability: 100 Years Since Ernest Overton*; Vol. 48 of Current Topics in Membranes; Deamer, D. W., Kleinzeller, A., Fambrough, D. M., Eds.; Academic Press: San Diego, 1999; pp 23−47.
(22) Wu, W.-G.; Chi, L.-M. *J. Am. Chem. Soc.* **1991**, *113*, 4683−4685.

10.1021/cm0479335 CCC: $30.25 © 2005 American Chemical Society
Published on Web 04/29/2005

Scheme 1. Synthesis of AB Lipids 1 and 2[a]



[a] Reagents and conditions: (a) HCl, $NaNO_2$, 0 °C; (b) NaOH, phenol, 0 °C, 75%; (c) 1,4-dibromobutane, K_2CO_3, 18-crown-6, reflux, 80%; (d) DCC, DMAP, cholesterol, r.t., 55%; (e) Et_3N, reflux 50%; (f) DCC, DMAP, 1-hexadecanol, r.t., 60%; (g) Et_3N, reflux 53%.

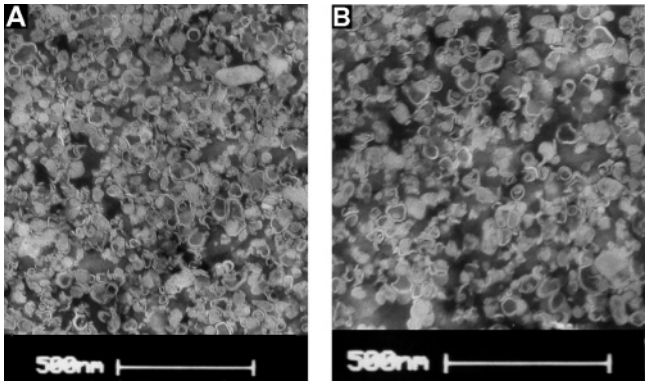


Figure 1. Transmission electron micrographs of liposomes (1:1 molar ratio, egg PC:AB lipid) before (A) and after (B) five cycles of periodic UV and visible light irradiation. Phosphotungstic acid negative stain (2% w/v); the scale bar is 500 nm.

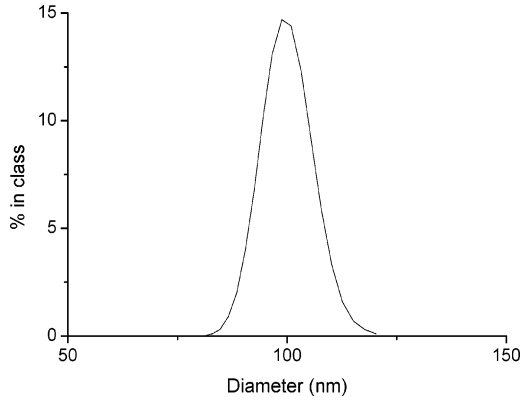


Figure 2. Particle distribution of the liposomes (1:1 molar ratio, egg PC: AB lipid).

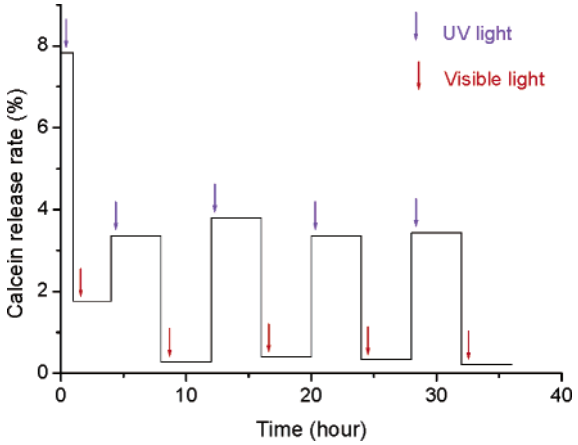


Figure 3. Photocontrolled release of calcein as a function of time from liposomes (1:1 molar ratio, egg PC:AB lipid **1**) after periodic UV and visible light irradiation in PBS, pH 7.4, 15 °C. Each point represents the mean of triplicates.

to achieve passive targeting under proper conditions such as hyperthermia (42 °C).[23] Moreover, incorporation of cholesterol into the lipid membrane of liposomes has been known to increase their circulating time (in DaunoXome long-circulating liposomes, for example).[24] By combining this with other effective techniques such as modifying the liposomal surface with poly(ethylene glycol) (PEG), our liposomes containing cholesterol derivatives may be used as passive targeting drug delivery systems to deliver anticancer drugs to tumor tissues.[25]

Calcein was chosen as a low-molecular-weight model drug with which to investigate photocontrolled release from PC/AB lipid liposomes. The fluorescence of calcein is self-quenched in liposomes at high concentration (ca. 100 mM). Calcein begins to exhibit strong fluorescence when released from the liposomes and diluted. Briefly, the liposome dispersion was added to phosphate-buffered saline (PBS). After UV or visible light irradiation for 10 min every 4 h (see Figure 3), the release of calcein from the liposomes was measured by fluorescence spectroscopy (Perkin-Elmer LS 550, excitation at 494 nm, emission at 514 nm). The fluorescence of 100% release of calcein was estimated by the fluorescence intensity after the complete destruction of the liposomes by the addition of Triton X-100 (final concentration: 0.3%). The amount of calcein released (%) from liposomes was calculated by the following equation: calcein released (%) = 100 $(I_t - I_o)/(I_{max} - I_o)$, where I_0 is the fluorescence intensity of the liposome dispersion containing calcein at the initial time, I_{max} is the maximum fluores-

(23) Kong, G.; Braun, R. D.; Dewhirst, M. W. *Cancer Res.* **2000**, *60* (16), 4440−4445.
(24) Forssen, E. A.; Ross, M. E. *J. Liposome Res.* **1994**, *4*, 481−512.
(25) Papahadjopoulos, D.; Allen, T. M.; Gabizon, A.; Mayhew, E.; Matthay, K.; Huang, S. K.; Lee, K. D.; Woodle, M. C.; Lasic, D. D.; Redemann, C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11460−11464.

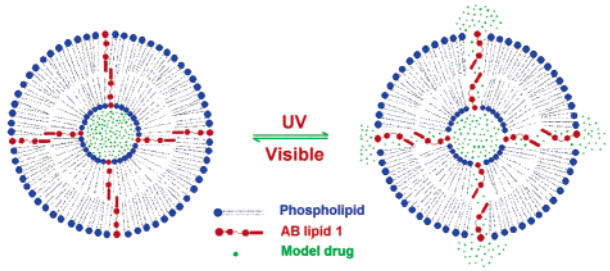


Figure 4. Possible photocontrolled release mechanism of liposomes.

cence intensity after Triton X-100 addition, and I_t is the measured fluorescence intensity at certain period.

Figure 3 shows the release behavior of calcein from liposomes during periodic UV irradiation to convert AB lipid **1** to its cis isomer and visible light irradiation to convert AB lipid **1** to its trans isomer. The calcein release rate was greatly increased after UV light irradiation and the drug release rate was greatly suppressed after visible light irradiation. This photocontrolled release could be repeated several times. The release rate with every UV light irradiation became coincident after a burst release on the first UV irradiation. Thus, this drug delivery system can achieve multipulsatile drug release through UV and visible light irradiation. Furthermore, the diameter of the liposomes and the UV−visible spectrum of AB lipid **1** in the liposomes did not change after periodic UV and visible light irradiation (Figure 1B), implying that photoisomerization of AB lipid **1** did not induce phase separation and fusion of liposomes, and that the photocontrolled release mechanism of this drug delivery system is not the phase separation and fusion of the liposomes. Mechanistically, calcein release across the lipid bilayer may be explained by diffusion of calcein through the pores formed by photoisomerization of AB lipid **1** to the cis isomer, which has a bulkier structure than the trans isomer. The diffusion rate of calcein will increase/decrease when AB lipid **1** is in the form of cis/trans isomer (Figure 4).

To further investigate the mechanism of photoinduced release of calcein from liposomes, liposomes were irradiated periodically with UV light for 5 min every 4 h to restrict AB lipid **1** to the cis isomer. The calcein release profile was biphasic and characterized by an initial burst release stage, followed by a constant rate release stage (zero-order release stage, Figure 5). This phenomenon was found both below and above the phase transition temperature of the liposomes. The longer AB lipid **1** was held in its cis isomer, the more drug was released from liposomes. Moreover, incorporated AB lipid **1** could be as much as 50% (mol:mol) of the total lipids in our system, which is much higher than the incorporation of phototriggers reported for other systems (less than 10% of the total lipids), implying that the cholesterol moiety of AB lipid **1** functions much as cholesterol itself does in, for example, stabilizing liposomes. Particle size analysis showed that the average diameter of the liposomes increased slightly, from 99.7 to 125 nm, and was distributed between 110 and 140 nm after storage for six months at room temperature, while normal liposomes lacking AB lipid **1** were mostly fused and precipitated within one month at room temperature. AB lipid **1** thus inhibits the fusion of liposomes.

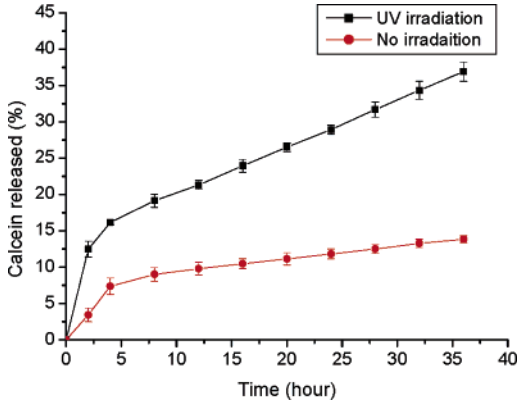


Figure 5. Release profiles of calcein as a function of time from liposomes (1:1 molar ratio, egg PC:AB lipid **1**) after periodic UV light irradiation for 5 min every 4 h in PBS, pH 7.4, 15 °C (■ UV irradiation; ●, no light irradiation). Each point represents the mean ± SD ($n = 3$).

This may be due to its rigid structure and intermolecular cationic repulsion. The release rate could also be controlled over a wide range by altering the incorporation ratio of AB lipid **1**; the low incorporation ratio of other azobenzene derivatives has made regulating the photocontrolled release rate difficult. Surprisingly, only 35% of encapsulated calcein was released within 36 h at a constant release rate when AB lipid **1** constituted 50% of the lipid (Figure 5). We can expect that this system can release a drug for a much longer period with a lower incorporation ratio. The photocontrolled release of other systems was so rapid (on the scale of minutes) that a sustained zero-order release could not be achieved.

The ability of cholesterol to inhibit leakage from liposomes may contribute to the mechanism of sustained release. To verify this, we compared the spontaneous release of calcein from liposomes incorporated with AB lipid **1** and with AB lipid **2** (Figure 6). The leakage from the AB lipid **1** liposomes was less than that from the AB lipid **2** system. This was much more significant when the AB lipid **1** content of the liposome was higher (Figure 6B), indicating that the cholesterol moiety of AB lipid **1** enables it to inhibit the spontaneous release of drug from liposomes more effectively than other azobenzene phototriggers reported, further contributing to the sustained release.

The cytotoxicity of this drug delivery system (AB lipid **1**/egg PC = 1:1 molar ratio) was examined using NIH3T3 cells. DOTAP [*N*-(1-(2, 3-dioleoyloxy)propyl-*N*,*N*,*N*-trimethylammonium mesylate) was used as the control. The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and incubated at 37 °C with 5% CO_2. The cells were seeded onto 96-well plates at a density of 20 000 cells per well and incubated for 1 day. Final concentrations of AB lipid **1** and DOTAP were adjusted to 25, 50, 100, 200, 400, 600, and 800 μg/mL. The blank was RPMI 1640 medium. A 200-μL portion of fresh growth medium and 20-μL aliquots of MTT solution were used to replace the mixture in each well after 48 h. The plates were then returned to the incubator and maintained in 5% CO_2 at 37 °C for a further 4 h. After removing the medium, 200 μL of dimethyl sulfoxide per well was added to dissolve the internalized purple formazan crystals. An aliquot of 100 μL was taken from each well and transferred to a fresh 96-well plate. Each sample was tested in six replicates per plate. The plates were

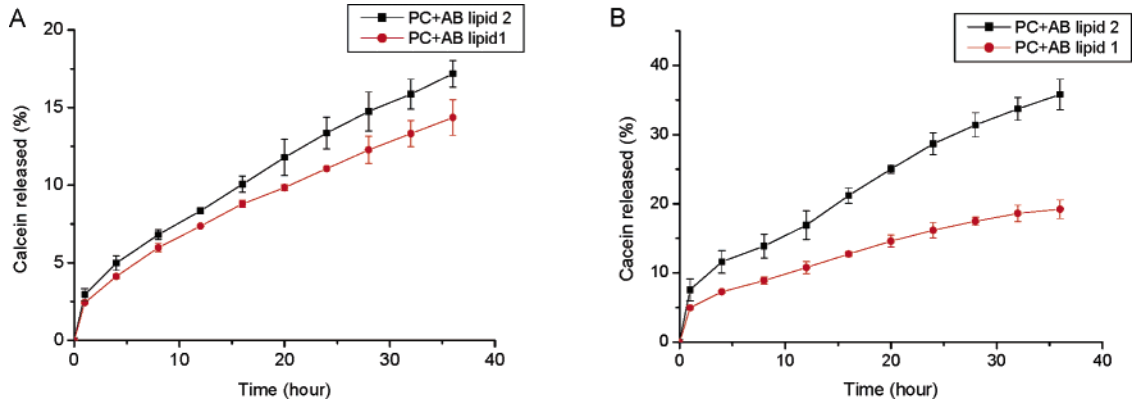


Figure 6. In vitro spontaneous calcein leakage profiles as a function of time, from liposomes incorporated with different ratios of synthesized cholesterol lipid containing an azobenzene moiety in PBS, pH 7.4, 25 °C. (A) 10:1 molar ratio, egg PC:AB lipid **1** or **2**; (B) 5:1 molar ratio, egg PC:AB lipid **1** or **2**. Each point represents the mean ± SD ($n = 3$).

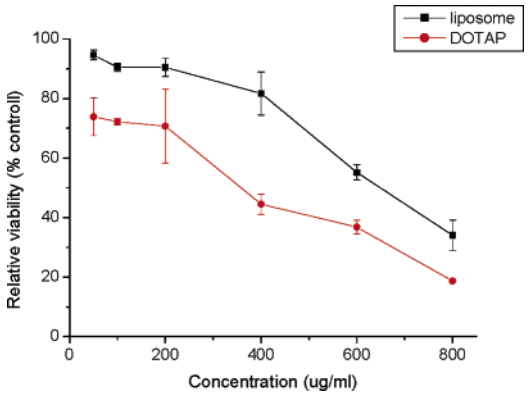


Figure 7. Cytotoxicity of liposomes incorporated with AB lipid **1** (■, liposomes; ●, DOTAP). Each point represents the mean ± SD ($n = 6$).

then assayed at 590 nm. The results were expressed as a percentage of the absorbance of the blank control. The IC_{50} values of our drug delivery system and DOTAP were 650 μg/mL and 350 μg/mL, respectively (Figure 7). Our drug delivery system had a lower cytotoxicity than DOTAP, which has been widely used in gene transfer and has the advantages of low toxicity and high safety.[26] We conclude that our new drug delivery system is rather safe.

(26) Liu, Y.; Liggit, D.; Zhou, N.; Gaensler, K.; Debs, R. *J. Biol. Chem.* **1995**, *270*, 24864−24870.

In conclusion, we have described a new type of photocontrolled, liposomal drug delivery system with an average diameter of 99.7 nm that can achieve zero-order, multipulsatile release, and sustained release. The synthetic phototrigger (AB lipid **1**) decreases the spontaneous release of drug and increases the stability of liposomes beyond what has been previously reported. The amount of drug released from liposomes can be controlled by maintaining the concentration of the cis isomer of AB lipid **1** by UV irradiation, and the release rate can be controlled by altering the incorporation ratio of AB lipid **1**. Furthermore, this drug delivery system has low cytotoxicity.

Acknowledgment. This study was supported by the Chinese Academy of Sciences (BaiRenJiHua, KGCX2-SW-602-5), the Committee of Shanghai Science and Technology (022261018), the National Hi-Tech Research and Development Plan of China (863 Project, 2002AA327100), and the National Natural Science Foundation of China (30470477).

Supporting Information Available: Materials and methods for the preparation of AB lipids, and experimental protocol for preparation of liposomes (pdf). This material is available free of charge via the Internet at http://pubs.acs.org.

CM0479335