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Formation of Fluid Lamellar Phase and Large Unilamellar Vesicles with Octadecyl Methyl Sulfoxide/Cholesterol Mixtures

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Systems composed of a monoalkylated amphiphile and a sterol have been shown to form stable liquid-ordered (lo) lamellar phases; these include negatively charged mixtures of unprotonated palmitic acid/cholesterol (Chol) or cholesterol sulfate (Schol) and mixtures of positively charged cetylpyridinium chloride/Schol. Large unilamellar vesicles (LUVs) could be formed by these systems, using conventional extrusion methods. The passive permeability of these LUVs was drastically limited, a phenomenon associated with the high sterol content. In the present paper, we showed that octadecyl methyl sulfoxide (OMSO), a neutral monoalkylated amphiphile, can form, in the presence of cholesterol, LUVs that are stable at room temperature. Differential scanning calorimetry, infrared spectroscopy, and nuclear magnetic resonance spectroscopy of deuterium were used to characterize the phase behavior of OMSO/Chol mixtures. A temperature−composition diagram summarizing the behavior of the OMSO/Chol system is proposed; it includes a eutectic with an OMSO/Chol molar ratio of 5/5. It is found that the fluid phase observed at temperature higher than 43 °C is metastable at room temperature, and this situation allows extruding these mixtures to form stable LUVs at room temperature. This distinct behavior is associated with the strong H-bond capability of the sulfoxide group. The properties associated with this neutral formulation expand the potential of these non-phospholipid liposomes for applications in several areas such as drug delivery.

Introduction

It is now established that mixtures of monoalkylated amphiphiles with high sterol contents can form fluid bilayers. For example, mixtures of palmitic acid (PA) with various sterols, including cholesterol (Chol), and cholesterol sulfate (Schol), are found to self-assemble to provide stable lamellar structure in the liquid ordered (lo) phase; in this phase, the lipids experience fast lateral and rotational diffusion but the PA alkyl chain displays high conformational order.[1−5] Similar structures are reported with cetylpyridinium chloride (CPC)/Schol,[6] and lyso-palmitoylphosphatidylcholine (lyso-PPC)/Chol,[7] whereas an analogous behavior is proposed for N-acylethanolamine (NAE)/Chol[8] mixtures. Typically, the monoalkylated amphiphile/sterol molar ratio of these bilayer-forming mixtures is between 5/5 and 3/7. Even though these monoalkylated amphiphiles or sterols do not form fluid lamellar phases once hydrated individually, their mixtures lead to stable lo-phase bilayers. These have a sterol/alkyl chain content considerably higher than typical phospholipid bilayers, considering that a phospholipid bears typically two alkyl chains.[9−11]

The main driving force associated with the formation of these fluid non-phospholipid bilayers is indeed the hydrophobic interactions, but the molecular prerequisites leading these molecules to self-assemble to form a fluid bilayer with alkyl chains highly ordered are not clearly established. The hydrophobic match between the apolar parts of the molecular constituents was shown to be critical for the formation of lo lamellar phases.[2] These bilayers are destabilized when the alkyl end chain of the sterol is bulky,[5] a feature that limits the ordering of the monoalkylated amphiphile and, consequently, the van der Waals interactions between the molecular species. Electrostatic interactions appear also to play an essential role in the stability of these bilayers and of the resulting LUVs. So far, all the reported monoalkylated amphiphile/sterol mixtures forming stable fluid bilayers at room temperature include at least one molecular species bearing a charge. For example, it is striking that, at room temperature, stable fluid lamellar phases are obtained with unprotonated PA and cholesterol, with protonated PA and Schol, but not with protonated PA and cholesterol.[12] In fact, it has been shown that the stability of these bilayers as a function of pH is intimately related to the pK_a of the fatty acid used to form the bilayers.[13] Similar fluid lamellar phases are obtained with cholesterol mixed with CPC, a cationic monoalkylated amphiphile.[6] It was also reported that mixtures of lyso-PPC and cholesterol can form fluid bilayers;[7] in this case, even though the overall charge is nil, the zwitterionic nature of lyso-PPC provides a local negative charge, on the phosphate group, and a positive one, on the quaternary ammonium group. Unprotonated PA/Chol, protonated PA/Schol, and CPC/Schol systems, which form these fluid bilayers at room temperature, can be extruded using standard extrusion procedures to form large unilamellar vesicles (LUVs).[4,6,12] Interestingly, the permeability of these LUVs is drastically reduced compared with the conventional

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(1) Paré, C.; Lafleur, M. *Langmuir* **2001**, *17*, 5587–5594.
(2) Ouimet, J.; Lafleur, M. *Langmuir* **2004**, *20*, 7474–7481.
(3) Ouimet, J.; Croft, S.; Paré, C.; Katsaras, J.; Lafleur, M. *Langmuir* **2003**, *19*, 1089–1097.
(4) Bastiat, G.; Oliger, P.; Karlsson, G.; Edwards, K.; Lafleur, M. *Langmuir* **2007**, *23*, 7695–7699.
(5) Cui, Z.-K.; Bastiat, G.; Jin, C.; Keyvanloo, A.; Lafleur, M. *Biochim. Biophys. Acta* **2010**, *1798*, 1144–1152.
(6) Phoeung, T.; Morfin Huber, L.; Lafleur, M. *Langmuir* **2009**, *25*, 5778–5784.
(7) Gater, D. L.; Seddon, J. M.; Law, R. V. *Soft Matter* **2008**, *4*, 263–267.
(8) Ramakrishnan, M.; Tarafdar, P. K.; Kamlekar, R. K.; Swamy, M. J. *Curr. Sci.* **2007**, *93*, 234–238.
(9) Bach, D.; Wachtel, E. *Biochim. Biophys. Acta* **2003**, *1610*, 187–197.
(10) Huang, J.; Buboltz, J. T.; Feigenson, G. W. *Biochim. Biophys. Acta* **1999**, *1417*, 89–100.
(11) Huang, J.; Feigenson, G. W. *Biophys. J.* **1999**, *76*, 2142–2157.
(12) Bastiat, G.; Lafleur, M. *J. Phys. Chem. B* **2007**, *111*, 10929–10937.
(13) Phoeung, T.; Aubron, P.; Rydzek, G.; Lafleur, M. *Langmuir*, accepted.

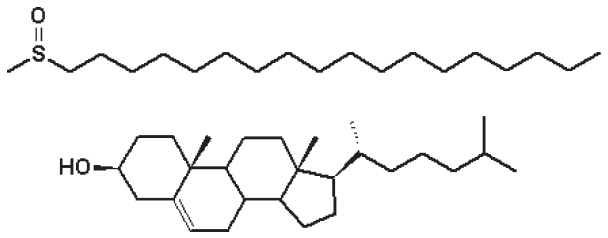

Figure 1. Chemical formula of OMSO and cholesterol.

liposomes made by phospholipids, likely due to their high sterol content. The protonated PA/Chol system, which is completely neutral, can form fluid bilayers only above 50 °C, while the components remain solid at room temperature.[1] In this case, it is impossible to extrude the mixture to form LUVs. Therefore, it appears that interfacial electrostatics plays a significant role in the stability of the lo-phase bilayers made of a monoalkylated amphiphile and a sterol.

In the present paper, we examine the possibility of using a neutral monoalkylated amphiphile and cholesterol to form completely neutral fluid bilayers. From an application point of view, neutral liposomes have been shown to display some distinct advantages. For example, it has been shown that liposomes used as biocompatible drug carriers can present improved behavior for the immune-mediated clearance and the inflammatory response.[14−16] Moreover, neutral liposomes were shown to display a distinctly uniform brain distribution, insensitive to the entrapped chemical, making them attractive nanovectors for predictable drug delivery and for imaging agents of brain.[17] We used octadecyl methyl sulfoxide (OMSO) (Figure 1), a dimethyl sulfoxide analogue bearing an 18 carbon atoms alkyl chain; its alkyl chain length matches the hydrophobic section of cholesterol.[2] OMSO is a neutral molecule, but the sulfoxide group is a very good H-bond acceptor leading to strong H-bonds.[18−21] For example, it is proposed that the H-bond interactions involving dimethyl sulfoxide (DMSO) are strong enough to induce the formation of a eutectic complex with water,[18,22] as well as with acetic acid.[19] In this work, we show that the particular H-bonding properties of the sulfoxide group lead to the possibility of forming fluid bilayers with OMSO and cholesterol mixtures, and these can be extruded to form, at room temperature, neutral LUVs. The phase behavior of OMSO/Chol mixtures was investigated by differential scanning calorimetry (DSC), infrared (IR) spectroscopy, and nuclear magnetic resonance spectroscopy of deuterium (^{2}H NMR). A temperature−composition diagram summarizes the behavior of the OMSO/Chol mixtures. This diagram indicated the suitable conditions to prepare non-phospholipid liposomes, and we examined the possibility to extrude the OMSO/Chol mixtures to form LUVs and characterized the stability and the permeability of these resulting liposomes.

Materials and Methods

Octadecyl methyl sulfoxide (OMSO) was obtained from Narchem Corporation (Chicago, IL, USA). Cholesterol (Chol) (> 99%), cholesterol-2,2,4,4,6-d_5 (Chol-d_5) (97 atom % D), tris(hydroxymethyl)aminomethane (TRIS) (99%), 2-[*N*-morpholino]ethanesulfonic acid (MES) (> 99%), ethylenediaminetetraacetic acid (EDTA) (99%), NaCl (> 99%), Triton X-100 (99%), and deuterium-depleted water (> 99.99%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Calcein (high purity) was obtained from Invitrogene (Burlington, ON, Canada), and Sephadex G-50 Medium from Pharmacia (Uppsala, Sweden). Methanol (spectrograde) was purchased from American Chemicals Ltd. (Montréal, QC, Canada), whereas benzene (high purity) was from BDH Inc. (Toronto, ON, Canada). All the chemicals were used without further purification.

Mixtures of OMSO and cholesterol were prepared by dissolving weighed amounts of the solids in a mixture of benzene/methanol 95/5 (v/v). The solutions were then frozen in liquid nitrogen and lyophilized for at least 16 h to allow complete sublimation of the organic solvent. Cholesterol was substituted with Cholesterol-d_5 for the ^{2}H NMR experiments.

DSC, ^{2}H NMR, and IR Spectroscopy. The freeze-dried lipid mixtures were hydrated with a MES/TRIS buffer (MES 50 mM, TRIS 50 mM, NaCl 10 mM, EDTA 5 mM) at pH 7.4. The buffer was prepared with Milli-Q water for DSC and IR spectroscopy and deuterium-depleted water for ^{2}H NMR experiments. The final lipid concentration was 20 mg/mL for DSC, and 30 mg/mL for IR spectroscopy and ^{2}H NMR experiments. The suspensions were subjected to five cycles of freezing-and-thawing (from liquid nitrogen temperature to ∼70 °C) and vortexed between successive cycles to ensure good hydration of the samples. The pH was measured and readjusted by the addition of HCl or NaOH diluted solution, if necessary. The samples were then incubated for at least one week at room temperature.

The DSC was performed with a VP-DSC microcalorimeter (MicroCal, Northampton, MA). The reference cell was filled with the buffer. The data acquisition was performed from 20 to 90 °C, at a heating rate of 20 °C/h. Data acquisition and treatment were performed with the *Origin* software (MicroCal software, Northampton, MA).

The ^{2}H NMR spectra were recorded on a Bruker AV-600 spectrometer, using a Bruker static probe equipped with a 5 mm coil. A quadrupolar echo sequence was used with a 90° pulse of 3.0 μs and an interpulse delay of 24 μs. The recycling time was 30 s. In absence of a slow-relaxation component, namely, a solid phase, the recycling delay was reduced to 0.3 s. Typically, 5000 FIDs were coadded. The temperature was regulated using a Bruker VT-3000 controller, varying from low to high and then back to the initial temperature. In order to characterize the kinetics of the solid phase formation, FIDs with the long (30 s) and short (0.3 s) relaxation delay were acquired in an alternating manner from a sample heated to 55 °C and then rapidly cooled down to 30 °C.

The IR spectra were recorded on a Thermo Nicolet 4700 spectrometer, equipped with a KBr beam splitter and a DTGS-L-alanine detector. Briefly, an aliquot of the sample was placed between two CaF_2 windows separated by a 5-μm-thick Teflon ring. This assembly was inserted in a brass sample holder, whose temperature was controlled using Peltier thermopumps. Each spectrum was the result of 60 coadded scans acquired with a nominal 2 cm^{-1} resolution and Fourier transformed using a triangular apodization function. The temperature was varied from low to high, with 2 °C steps and a 5 min incubation period prior to the data acquisition. The reported band positions correspond to the centers of gravity calculated from the top 5% of the band.

Permeability Measurements. The permeability of LUVs was measured using a standard procedure based on the self-quenching property of calcein at high concentration.[23,24] OMSO/Chol mixtures were hydrated, at a lipid concentration of 85 mM, with the MES/TRIS buffer, pH 7.4, containing calcein (80 mM). The

(14) Lian, T.; Ho, R. J. Y. *J. Pharm. Sci.* **2001**, *90*, 667–680.
(15) Immordino, M. L.; Dosio, F.; Cattel, L. *Int. J. Nanosci.* **2006**, *1*, 297–315.
(16) Ngo, K. X.; Umakoshi, H.; Shimanouchi, T.; Kuboi, R. *Colloids Surf., B* **2009**, *73*, 399–407.
(17) Saito, R.; Krauze, M. T.; Noble, C. O.; Tamas, M.; Drummond, D. C.; Kirpotin, D. B.; Berger, M. S.; Park, J. W.; Bankiewicz, K. S. *J. Neurosci. Methods* **2006**, *154*, 225–232.
(18) Cowie, J. M. G.; Toporowski, P. M. *Can. J. Chem.* **1961**, *39*, 2240–2243.
(19) Saleh, M. A.; Ahmed, O.; Ahmed, M. S. *J. Mol. Liq.* **2004**, *115*, 41–47.
(20) Wang, N.-N.; Li, Q.-Z.; Yu, Z.-W. *Appl. Spectrosc.* **2009**, *63*, 1356–1362.
(21) Abraham, M. H.; Platts, J. A. *J. Org. Chem.* **2001**, *66*, 3484–3491.
(22) Rasmussen, D. H.; Mackenzie, A. P. *Nature* **1968**, *220*, 1315–1317.
(23) Allen, T. M. Calcein as a tool in liposome methodology. In *Liposome technology*, Gregoriadis, G., Ed.; CRC Press: Boca Raton, FL, 1984; pp 177−182.
(24) Benachir, T.; Lafleur, M. *Biochim. Biophys. Acta* **1995**, *1235*, 452–460.

LUVs were prepared by extrusion using a hand-held Liposofast extruder (Avestin, Ottawa, Canada). The dispersions were passed 15 times through two stacked polycarbonate filters (100 nm pore size) at ~65 °C. Calcein-containing LUVs were separated at room temperature from free calcein by exclusion chromatography, using a column (diameter 1.5 cm, length 25 cm) filled with Sephadex G-50 medium gel and an isoosmotic MES/TRIS buffer (MES 50 mM, TRIS 50 mM, NaCl 130 mM, EDTA 5 mM, pH 7.4) as eluent. The collected LUV fraction was diluted 100 times with this isoosmotic buffer to perform the measurements.

Immediately after the isolation and dilution of the calcein-loaded vesicles, the fluorescence intensity was measured from an aliquot of this stock LUVs suspension prior to (I_i) and after (I_{i+T}) the addition of 10 μL of Triton X-100 solution (10% (v/v) in the external MES/TRIS buffer); these intensities were referred to as initial values, i.e., obtained at $t = 0$. After a given time, the calcein fluorescence intensity was measured on another aliquot of the LUV stock suspension before (I_f) and after (I_{f+T}) the addition of Triton X-100. The fluorescence intensity, measured after addition of the detergent, corresponded to the complete release of calcein and was used to normalize the leakage. The percentage of encapsulated calcein remaining at that time in the LUVs was calculated as follows:

$$\%\ \text{encapsulated calcein} = \left(\frac{(I_{f+T} - I_f)/I_{f+T}}{(I_{i+T} - I_i)/I_{i+T}}\right) \times 100 \quad (1)$$

The % of release corresponded to (100 − % of encapsulated calcein).

The potential pH-triggered leakage of calcein was also examined. The pH was modified by adding an aliquot of diluted NaOH or HCl solution directly to an aliquot of the LUV suspension. The calcein fluorescence intensities were measured after the stabilization of pH (typically after ~2 min). The percentage of released calcein was calculated with the eq 1, except I_i and I_{i+T} corresponded to the measurements at initial time and also initial pH (pH 7.4), whereas I_f and I_{f+T} were obtained from an aliquot at a modified pH, before and after the addition of Triton X-100, respectively. The pH effect was examined for both increasing and decreasing pH values. The calcein fluorescence intensity was relatively constant over the investigated pH range.[23]

The calcein fluorescence intensity was measured at excitation and emission wavelengths of 490 and 513 nm, respectively, using a SPEX Fluorolog spectrofluorimeter. All the leakage experiments were carried out at room temperature.

The hydrodynamic diameters of the LUVs were measured at 25 °C using a Coulter N4 Plus quasi-elastic light scattering apparatus coupled with a Malvern autocorrelator. The scattering intensity was adjusted by the dilution of the dispersion with the external MES/TRIS buffer. The fluorescence and quasi-elastic light scattering measurements were carried out as closely as possible.

Results and Discussion

DSC Experiments. Figure 2 presents thermograms of OMSO/Chol mixtures with various compositions, hydrated at pH 7.4. For pure OMSO, a phase transition is observed at about 60 °C, corresponding to its melting. For an OMSO/Chol mixture with a molar ratio of 7/3, two maxima are observed, at about 44 and 58 °C (Figure 2b). The shape of the endotherm is associated with the shape of the phase coexistence region in the phase diagram, as discussed below. The first maximum corresponds to the beginning of the formation of a fluid phase, crossing a three-phase line from solid phases to a region where there is coexistence of lo phase and solid OMSO. The rest of the endotherm corresponds to crossing the phase coexistence region. The OMSO/Chol 5/5 mixture shows only one endothermic sharp peak at 43 °C, indicative of the formation of a eutectic mixture. For OMSO/Chol molar ratios of 3/7 and 1/9, the maximum at 43 °C is still present, but another broader endothermic peak is observed at 70 °C for the 3/7 molar ratio and at 85 °C for the 1/9 molar ratio. A dehydration transition is observed for the solid cholesterol in excess water at ~72 °C,[25,26] and the observed endotherm is believed to be associated with excess solid cholesterol experiencing such a transition. This behavior is reminiscent of that previously observed by Ouimet et al.[3] for PA/Chol mixtures. That system showed, at pH 5.5, a eutectic behavior at similar proportions as a sharp transition from solid to fluid lamellar phase was observed at 53.5 °C, about 8 °C lower than the melting point of hydrated PA.[3] On the basis of these similarities, and the spectroscopic features reported below, a similar behavior is proposed for the OMSO/Chol mixtures. In this system, the eutectic composition corresponds to a 5/5 molar ratio and the solid to lo phase transition is observed at 43 °C.

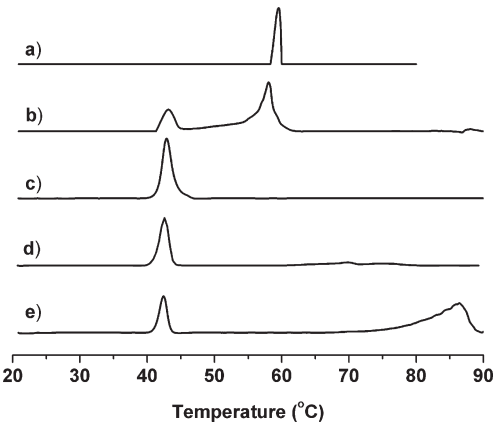

Figure 2. Thermograms of (a) pure OMSO and of OMSO/Chol mixtures with molar ratio of (b) 7/3, (c) 5/5, (d) 3/7, and (e) 1/9; pH 7.4.

No peak is observed upon cooling the eutectic mixture from 90 to 20 °C. In addition, no endotherm is observed during a second heating. The endotherm associated with the solid to lo phase transition reappears after ~3 days of incubation at room temperature. These results are indicative of the metastability of the phase formed at high temperature, and this aspect is discussed in the next section.

^{2}H NMR Experiments. Figure 3 column A presents the evolution of the ^{2}H NMR spectrum of an equimolar OMSO/Chol-d_5 mixture, at pH 7.4, as a function of increasing temperature. At 25 and 35 °C, a single broad powder pattern is observed and its quadrupolar splitting measured between the two maxima corresponds to 124 kHz. This profile is typical of solid cholesterol.[1,27] The five deuterated positions give rise to similar splittings when the cholesterol molecules are immobile, the chemical shifts and the static quadrupolar constants of the various deuterated groups being very similar.[28,29] At 45 °C and above, the ^{2}H NMR spectrum is drastically modified: three reasonably well resolved powder patterns with reduced quadrupolar splittings are observed. No contribution of a solid phase can be observed. This transition between solid cholesterol and cholesterol solubilized in fluid bilayers is consistent with the results obtained from the DSC experiments. For cholesterol-d_5 in the OMSO/Chol mixture at 55 °C, the quadrupolar splittings measured between the maxima (the 90° orientation) are reported in Table 1. These values are very

(25) Loomis, C. R.; Shipley, G. G.; Small, D. M. *J. Lipid Res.* **1979**, *20*, 525–535.
(26) Epand, R. M.; Bach, D.; Borochov, N.; Wachtel, E. *Biophys. J.* **2000**, *78*, 866–873.
(27) Fenske, D. B.; Thewalt, J. L.; Bloom, M.; Kitson, N. *Biophys. J.* **1994**, *67*, 1562–1573.
(28) Seelig, J. *Q. Rev. Biophys.* **1977**, *10*, 353–418.
(29) Dufourc, E. J.; Parish, E. J.; Chitrakorn, S.; Smith, I. C. P. *Biochemistry* **1984**, *23*, 6062–6071.

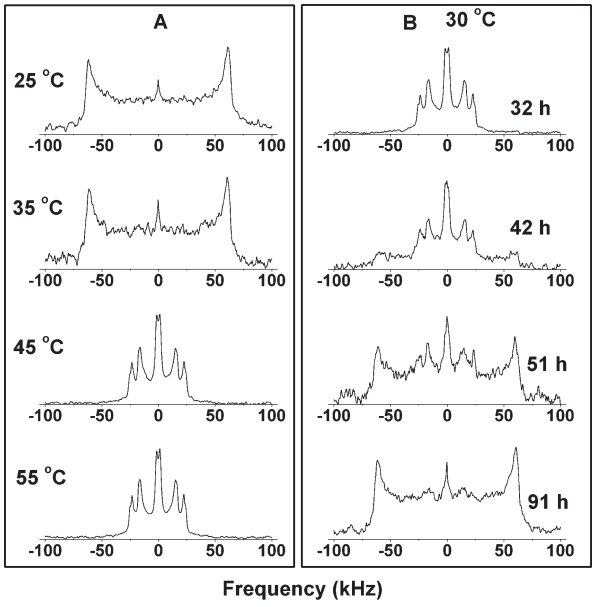

Figure 3. ^{2}H NMR spectra of an equimolar OMSO/Chol-d_5 mixture, pH 7.4. Column A, from top to bottom, represents the thermal evolution of the spectrum as a function of increasing temperature. Column B presents the progressive solidification of cholesterol in OMSO/Chol metastable bilayers that were obtained from a rapid cooling of the sample from 55 to 30 °C.

Table 1. ^{2}H NMR Parameters Associated with Cholesterol in Equimolar OMSO/Chol-d_5 Mixtures

position	exp $\Delta\nu_Q$ (kHz)	β obtained from ref 31	calcd S_{mol}	calcd $\Delta\nu_Q$ (kHz)
2,4-$^2H_2$ ax	45.4	74.1	0.92	46
2,4-$^2H_2$ eq	33.4	66.2		30
6-^{2}H	4.0	55.8		3

similar to those previously obtained for cholesterol-d_5 in various fluid bilayers including PA/Chol system,[1] phosphatidylcholine bilayers,[30,31] model mixtures of stratum corneum lipids,[27] and biological membranes such as human red blood cell membranes,[32] and membranes of mycoplasma *Acholeplasma laidlawii* (strain B).[33] This correspondence strongly suggests a similar orientation and dynamics of cholesterol in the OMSO/Chol fluid lamellar phase. The molecular design of cholesterol appears to strongly dictate its orientation in lipid bilayers with no considerable dependence on the composition of the matrix.

Using an approach previously described,[1,31] the residual quadrupolar splittings, $\Delta\nu_Q$, of the ^{2}H NMR powder pattern could be described by the fast rotation of cholesterol along its long axis. In this case, $\Delta\nu_Q$ is related to[28,34]

$$\Delta\nu_Q = \frac{3}{2} A_Q \left(\frac{3\cos^2\theta - 1}{2} \right) S_{mol} S_{C-D} \qquad (2)$$

where A_Q is the static quadrupolar constant (170 kHz for the C−D bonds[29]) and θ is the angle between the bilayer normal and the magnetic field. S_{mol} denotes the fluctuations of the whole cholesterol molecule with respect to the bilayer normal, whereas S_{C-D} represents the averaged orientational intramolecular order parameter. For labeled cholesterol, the C−D bonds are linked to the steroid rigid rings, and consequently, S_{C-D} should be essentially representative of the fixed angle (β) between the C−D bond and the cholesterol long axis, defined as the rotation axis. The specific assignment of the powder patterns to the various deuterated positions and the resulting specific orientation of the C−D bonds were reported for DMPC/Chol-d_5 systems[29] (Table 1). Using these β values, we could reproduce, within 3 kHz, the three measured quadrupolar splittings for OMSO/Chol system, using S_{mol} as the only adjustable parameter. The resulting S_{mol} is 0.92, a value similar to those previously obtained for cholesterol in lo phase bilayers formed with CPC/Chol,[6] PA/Chol,[1] and DMPC/Chol.[31] As S_{mol} is close to 1, it is concluded that the sterol long axis is essentially aligned parallel with the bilayer normal and it experiences very limited wobbling.

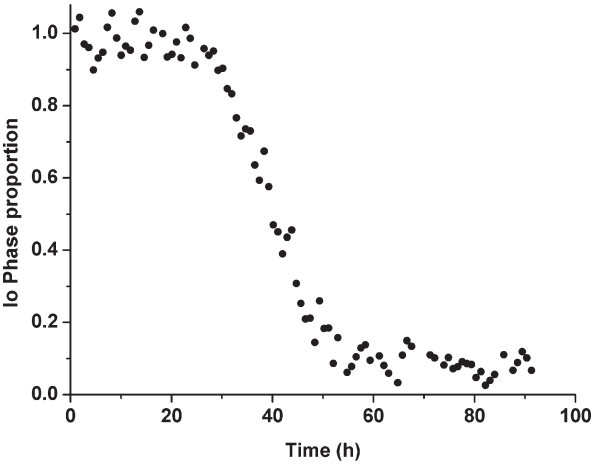

Figure 4. Kinetics of the crystallization of cholesterol in the metastable liquid ordered phase of an equimolar OMSO/Chol-d_5 mixture at 30 °C.

^{2}H NMR spectroscopy allowed us to discover that the high-temperature structure was metastable at room temperature. The irreversibility of the transition can be assessed, for example, by cooling rapidly the OMSO/Chol-d_5 sample from 55 to 30 °C and by recording the ^{2}H NMR spectrum as a function of time (Figure 3 column B). After rapid cooling to 30 °C, no solid phase is observed. The spectrum is practically identical to that measured at 55 °C, including essentially identical quadrupolar splittings. Progressively, the contribution of the solid phase appears and the spectrum becomes a combination of the spectra representative of solid cholesterol and cholesterol solubilized in fluid bilayers. After 91 h at 30 °C, cholesterol exists exclusively under the solid phase. We can describe the kinetics of this solidification of cholesterol in an OMSO/Chol-d_5 mixture using ^{2}H NMR (Figure 4). Spectra, obtained from 100 scans, were recorded as a function of time using alternatively a long (30 s) and short (0.3 s) relaxation delay. The relaxation time of solid cholesterol-d_5 is about 4 s,[33] whereas it is less than 14 ms for cholesterol solubilized in a fluid bilayer.[35] As a consequence, essentially only the cholesterol molecules solubilized in the lo bilayers contribute to the spectra acquired with the short relaxation delay, while the spectra obtained with the long relaxation delay are representative of all the sterol molecules in the sample. The proportion of the lo phase is calculated from the ratio of the area of the short-delay spectrum

(30) Douliez, J.-P.; Léonard, A.; Dufourc, E. J. *J. Phys. Chem.* **1996**, *100*, 18450–18457.
(31) Marsan, M. P.; Muller, I.; Ramos, C.; Rodriguez, F.; Dufourc, E. J.; Czaplicki, J.; A., M. *Biophys. J.* **1999**, *76*, 351–359.
(32) Kelusky, E. C.; Dufourc, E. J.; Smith, I. C. P. *Biochim. Biophys. Acta* **1983**, *735*, 302–304.
(33) Monck, M. A.; Bloom, M.; Lafleur, M.; Lewis, R. N. A. H.; McElhaney, R. N.; Cullis, P. R. *Biochemistry* **1993**, *32*, 3081–3088.
(34) Davis, J. H. *Biochim. Biophys. Acta* **1983**, *737*, 117–171.
(35) Dufourc, E. J.; Smith, I. C. P. *Chem. Phys. Lipids* **1986**, *41*, 123–135.

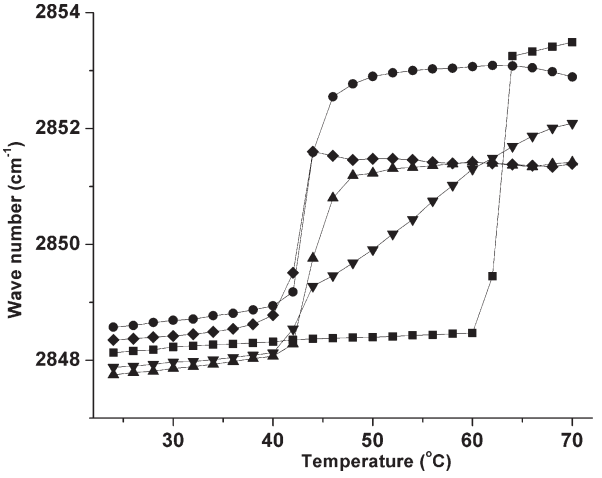

Figure 5. Lipid thermotropism, reported by the ν_{C-H} band position from IR spectra, of hydrated OMSO (■), and of OMSO/Chol mixtures with various compositions: 1/9 (●), 3/7 (♦), 5/5 (▲), and 7/3 (▼) (molar ratio), pH 7.4.

and that of the following long-delay spectrum. The suspension exists under a lo phase for the first 30 h following its cooling; consequently, the spectra acquired with long and short relaxation delays have the same area, leading to a lo phase proportion of 1. Afterward, a transition toward the solid phase occurs, and finally, cholesterol exists essentially under the solid form after ~55 h. A residual proportion of lo phase is reported (~0.08), but it corresponds in fact to cholesterol in an isotropic phase, which shows up as a narrow peak right in the middle of the ^{2}H NMR spectrum (Figure 3 column B). This phase is observed after the heating−cooling cycle but remains a minor proportion of cholesterol in the sample.

IR Spectroscopy Experiments. The thermal behavior of OMSO/Chol mixtures was examined by IR spectroscopy. The position of the symmetric C−H stretching (ν_{C-H}) mode, associated with OMSO alkyl chain, is mainly sensitive to the trans−gauche chain isomerization, and to the interchain coupling, providing a sensitive probe for transitions involving the introduction of chain conformational disorder.[36−38] The melting of pure hydrated OMSO is easily detected by the abrupt shift of the ν_{C-H} band from 2848 to about 2853.5 cm^{-1}, at about 60 °C (Figure 5). This temperature corresponds to the transition identified by DSC. The two extreme ν_{C-H} positions are representative of highly ordered and disordered chains, respectively.[36,39] A transition between 42 and 47 °C was observed for equimolar OMSO/Chol system, in agreement with the thermogram presented in Figure 2c. At low temperatures, the position of the ν_{C-H} band is about 2848 cm^{-1}; values below 2850 cm^{-1} are generally associated with all-trans alkyl chains for molecules under a solid form.[36,39] Therefore, those results are indicative of highly ordered alkyl chains of solid OMSO. At temperatures higher than the transition temperature, the position of the ν_{C-H} band was shifted to ~2851 cm^{-1}, a value lower than that observed for OMSO in the fluid state. This intermediate position corresponds to that observed for cholesterol-rich bilayers.[12,36,40,41] The thermal behavior, as probed by IR spectroscopy, was also determined for other OMSO/Chol mixtures (3/7, 7/3, and 1/9 (mol/mol)). At low temperature, the position of the ν_{C-H} band is ~2848 cm^{-1}, which indicates that OMSO alkyl chains are highly ordered, likely under a solid form.[36,39] Upon heating above 43 °C, the position of the ν_{C-H} band was abruptly shifted to ~2853 cm^{-1} for the OMSO/Chol mixture (1/9); this value is similar to that observed for OMSO in the fluid state and is indicative of highly disordered alkyl chains. The complete disordering of OMSO alkyl at 43 °C is then inferred. The thermal evolution of the position of the ν_{C-H} band for the OMSO/Chol (3/7) mixture is similar to that of the eutectic composition. In the case of the OMSO/Chol (7/3) mixture, a shift of the ν_{C-H} band by about 1 cm^{-1} is observed at about 43 °C, followed by a more progressive shift of the band position between 43 and 60 °C, indicating the progressive disordering of OMSO alkyl chain.

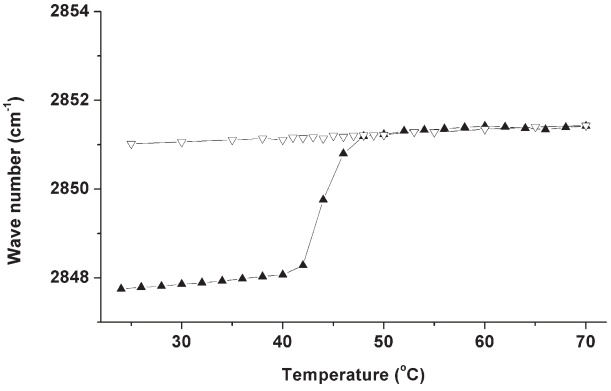

Figure 6. Lipid thermotropism, reported by the ν_{C-H} band position from IR spectra, of an equimolar OMSO/Chol mixture: heating (▲) and cooling (▽), pH 7.4.

The metastability of the fluid phase was also investigated by IR spectroscopy. Figure 6 includes the variation of the ν_{C-H} band position upon the cooling of an equimolar OMSO/Chol mixture, right after heating up to 70 °C. The ν_{C-H} band position remains at ~2851 cm^{-1} even at 25 °C, i.e., nearly 20 °C below the solid−lo phase transition observed upon heating. This observation is consistent with the bilayers remaining in a metastable lo phase. The CH_2 deformation mode $\delta(CH_2)$, between 1450 and 1480 cm^{-1}, was also examined to confirm the lo phase metastability, because this mode is sensitive to the chain packing of the OMSO (Supporting Information Figure S1). At the beginning of the experiment, when the sample has been incubated for at least one week at room temperature, the $\delta(CH_2)$ mode gives rise to two components located at 1464 and 1472 cm^{-1}. This splitting is typical of a solid phase for which the alkyl chains pack with an orthorhombic symmetry.[42] When the sample is cooled down to 30 °C after being heated at 70 °C, a single component at 1468 cm^{-1} is observed, confirming the existence of a metastable phase. The band splitting reappears as a function of time when the mixture is incubated at 30 °C.

OMSO/Chol Temperature−Composition Diagram. In order to summarize the phase behavior revealed by the calorimetric and spectroscopic studies, an OMSO/Chol temperature−composition diagram is proposed (Figure 7). Below 43 °C, OMSO (as inferred from the low ν_{C-H} band position) (Figure 5) and cholesterol (as inferred from the ^{2}H NMR spectra) form solid phases. Upon heating, there is the formation of a lo lamellar phase, observed from the thermograms, the shift of the ν_{C-H} position in IR spectroscopy, and the modification of the ^{2}H NMR spectrum of cholesterol-d_5 that is associated with the introduction of molecular

(36) Mantsch, H. H.; McElhaney, R. N. *Chem. Phys. Lipids* **1991**, *57*, 213–226.
(37) Kodati, R. V.; Lafleur, M. *Biophys. J.* **1993**, *64*, 163–170.
(38) Kodati, V. R.; El-Jastimi, R.; Lafleur, M. *J. Phys. Chem.* **1994**, *98*, 12191–12197.
(39) Moore, D. J.; Rerek, M. E.; Mendelsohn, R. *J. Phys. Chem. B* **1997**, *101*, 8933–8940.
(40) Paré, C.; Lafleur, M. *Biophys. J.* **1998**, *74*, 899–909.
(41) Chen, H.-C.; Mendelsohn, R.; Rerek, M. E.; Moore, D. J. *Biochim. Biophys. Acta* **2001**, *1512*, 345–356.
(42) Snyder, R. G.; Strauss, H. L.; Cates, D. A. *J. Phys. Chem.* **1995**, *99*, 8432–8439.

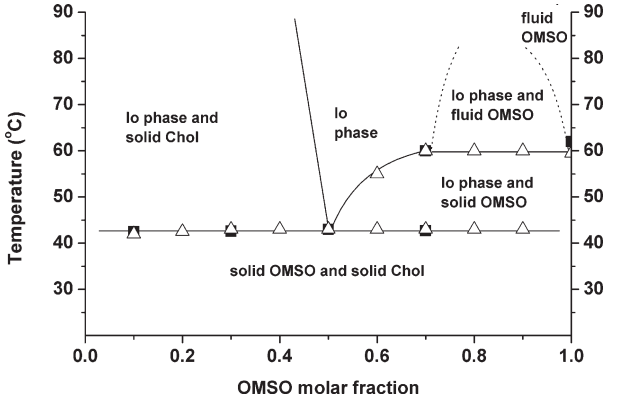

Figure 7. Proposed OMSO/Chol temperature−composition diagram. The phase boundaries identified by ■ were obtained from the shift of the ν_{C-H} band position (see Figure 5). Those identified with Δ were determined from the DSC thermograms (Figure 2). The borders of the lo−solid OMSO coexistence region are defined from both DSC and IR spectroscopy. The lo−fluid OMSO coexistence region (dotted lines) has been tentatively introduced to reconcile the formation of the lo phase by the eutectic mixture and the melting of OMSO.

rotational freedom. The OMSO/Chol 5/5 molar ratio corresponds roughly to a eutectic composition, as the DSC endotherm is fairly narrow. Only the lo phase is present for $T > 43$ °C on the basis of the fluid cholesterol ^{2}H NMR spectra and the ν_{C-H} band position consistent with alkyl chain in a lo phase. At $T > 43$ °C, higher cholesterol proportions appear to lead to the coexistence of the lo phase and solid cholesterol. For these samples, the variation of the ν_{C-H} band position (Figure 5) is consistent with the fluidification of the OMSO alkyl chain at 43 °C, crossing the three-phase line. The presence of solid cholesterol is suggested by the small endotherms at about 80 °C that could be attributed to the solid−solid dehydration of the sterol in excess. In addition, the impossibility to extrude the OMSO/Chol 3/7 mixture is compatible with solid cholesterol obstructing the filters. For OMSO proportions greater than the eutectic composition, the variation of the ν_{C-H} band position (Figure 5) and the DSC curves are indicative of a two-phase region where there is the coexistence of OMSO solid phase with the lo phase. It can be observed that the OMSO chain disordering undergoes a fluidification over ∼20 °C, between 43 °C (the fluidification temperature of the eutectic mixture) and 60 °C (the fluidification temperature of pure OMSO). Beyond 60 °C, pure OMSO becomes fluid according to the results from IR spectroscopy and DSC. The exact structure of these self-assemblies is not known. Because it is a monoalkylated amphiphile, OMSO likely forms micelles. In this case, there should be a region where two fluid phases (the lo phase and an OMSO-rich nonlamellar phase) coexist. Such region has been tentatively introduced in the temperature−composition diagram. However, it cannot be excluded that pure OMSO could form hydrated lamellar phases above 62 °C. In that case, a region where a single lamellar phase would continuously evolve from pure OMSO disordered lamellar phase to lo phase cholesterol-containing bilayers would be present in the temperature−composition diagram. Additional experiments are required to identify the details of this fluid phase region.

OMSO/Chol LUVs. It has been shown that, despite their very high cholesterol content, it is possible to extrude several mixtures of sterols and monoalkylated amphiphiles when they form a lo phase.[4,6,12] Despite the fact that OMSO/Chol systems exist under a solid phase at room temperature, we have examined the possibility to prepare LUVs from the OMSO/Chol mixtures as

Table 2. Hydrodynamic Diameter of LUVs d_{LUVs} and Initial Self-Quenching of Calcein for Various OMSO/Chol LUVs at pH 7.4

OMSO/Chol molar ratio	d_{LUVs} (nm)	initial self-quenching
3/7	impossible to extrude	
5/5	112 ± 8	0.93 ± 0.04
7/3	106 ± 11	0.91 ± 0.02

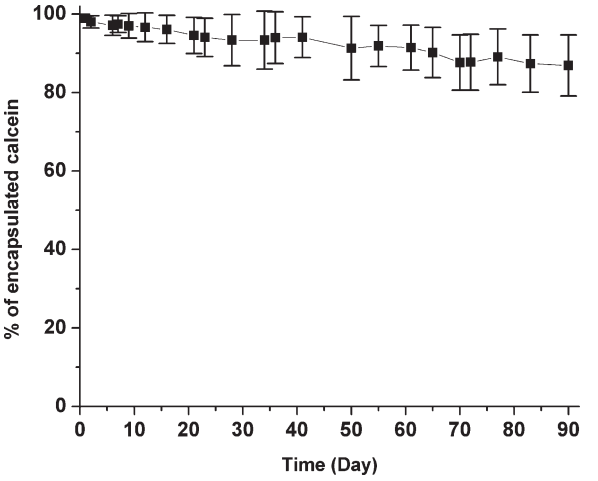

Figure 8. Passive calcein leakage from LUVs prepared from an equimolar OMSO/Chol mixture, at pH 7.4 and room temperature.

the lo phase existing above 43 °C was found to be metastable at room temperature. Mixtures with various OMSO/Chol molar ratios (3/7, 5/5 (the eutectic composition), and 7/3) were tentatively extruded at ∼65 °C, the LUVs being collected at room temperature. Table 2 reports the results of these attempts. It is impossible to extrude the 3/7 molar ratio OMSO/Chol mixture. It seems that the excess of the solid cholesterol plugs the pores of the polycarbonate filters. Conversely, extruded LUVs can be obtained from the OMSO/Chol mixtures with a 5/5 or 7/3 molar ratio. The quasi-elastic light scattering measurements indicate that the LUVs have, as expected, a hydrodynamic diameter consistent with the pore size of the filters (Table 2). The initial self-quenching of calcein entrapped in the LUVs was greater than 0.85, a typical value for entrapped calcein at a 80 mM concentration.[43] These results demonstrate the formation of LUVs with OMSO/Chol mixtures and their ability to encapsulate hydrophilic molecules. The passive release from calcein-loaded vesicles was determined (Figure 8). The leakage from OMSO/Chol LUVs (equimolar mixture) is very limited: even after 90 days, no significant release could be detected. Such limited passive permeability has been observed for LUVs prepared from sterol and palmitic acid.[4,12]

Considering the lifetime of the metastable lo phase, it is inferred that these LUVs would exist under a solid form. We have carried out the IR spectroscopic analysis of OMSO/Chol LUVs, calcein-free or loaded with calcein; their behavior (Supporting Information Figure S2) is similar to that observed for multilamellar dispersions (Figure 6). Freshly prepared LUVs, which have been extruded at ∼65 °C, show, at room temperature, a ν_{C-H} band position at around 2851 cm^{-1}; this value remains relatively constant up to 60 °C. Upon incubation at room temperature, the ν_{C-H} band frequency decreases to ∼2848 cm^{-1}, implying the rigidification of OMSO alkyl chains. An increase of the frequency of the ν_{C-H} mode is observed upon heating, in agreement with

(43) El Jastimi, R.; Lafleur, M. *Biospectroscopy* **1999**, *5*, 133–140.

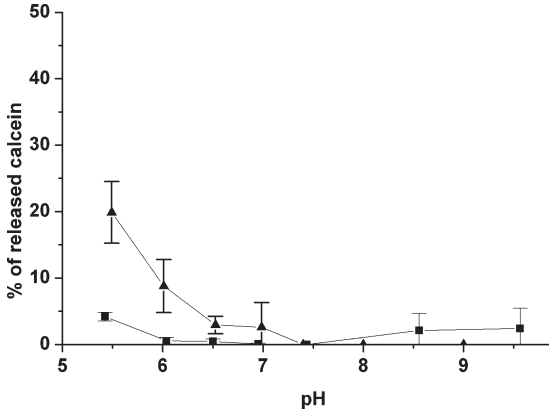

Figure 9. Effect of the external pH on the calcein release, for OMSO/Chol 5/5 (■) and PA/Chol 3/7 (▲) LUVs. The measurements were carried out at room temperature, ~2 min after the pH change.

thermal disordering of the chain. This behavior is not influenced by the presence of entrapped calcein.

The stability of OMSO/Chol (5/5 molar ratio) LUVs was assessed as a function of pH (Figure 9), and the pH dependence of its calcein release is compared with that observed for PA/Chol (3/7 molar ratio) LUVs, a pH-sensitive system.[4] The pH was modified by adding an aliquot of diluted NaOH or HCl solution directly to an aliquot of an LUV suspension and the leakage measurements were carried out after the stabilization of pH (typically after ~2 min), at 25 °C. There is practically no impact of pH on calcein release from OMSO/Chol LUVs when the external pH is modified between 5.5 and 10. Because the OMSO/Chol system does not include any pH-sensitive group, it was expected that pH would have little influence on their content release. This behavior contrasts with that of PA/Chol LUVs, for which no calcein release is observed between pH 6.5 to 10 but the calcein release is triggered at pH below 6.5; this pH-induced release was associated with the protonation of PA carboxylic group.[4] In addition, we have examined the influence of the pH on the LUV size, using quasi-elastic light scattering. The size of OMSO/Chol LUVs remains constant at about 100 nm between pH 2 and 12. This behavior also contrasts with the previous aggregation/fusion observed below pH 6 for PA/Chol LUVs,[4] an observation that was accompanied by the release of the entrapped calcein. In the case of OMSO/Chol LUVs, the absence of effect of pH on the LUV size and permeability illustrates the stability of these LUVs and is consistent with the hypothesis that the pH-triggered release is intimately associated with the change of surface charge density caused by the protonation/deprotonation of the carboxylic group of the fatty acid.[13]

Concluding Remarks

This work extends the compositions of non-phospholipid liposomes formed with a monoalkylated amphiphile and a very high sterol content. It is possible to prepare fluid ordered bilayers when neutral OMSO and cholesterol are mixed. Because of the metastability of the lo phase at room temperature, the equimolar OMSO/Chol mixture can be extruded by conventional methods and form LUVs whose passive permeability is very limited. It is, to our knowledge, the first neutral system to form stable non-phospholipid liposomes with such high cholesterol content. This unique behavior is directly associated with OMSO properties. OMSO has an 18 carbon atom alkyl chain, providing a hydrophobic match with cholesterol. This equivalence is a contribution to the free energy favoring the mixing of the two species and was shown to be a prerequisite for the formation of stable lo-phase bilayers.[2] At the interfacial level, the sulfoxide group provides a strong H-bond acceptor, a feature that appears to be critical for the distinct behavior of its mixtures with cholesterol. Two aspects of the intermolecular interactions could contribute to this behavior. First, S═O forms H bonds with hydroxyl groups that are stronger than those formed with C═O;[20,21] those between OMSO and cholesterol could be sufficient to promote the mixing of the two species resulting in the formation of fluid bilayers. The strong H-bond network at the interface could be associated with the formation of the metastable phase if the solidification requires a significant rearrangement of this network. Actually, seminal work from Hargreaves and Deamer[44] suggested, for analogous mixtures of alkylated soaps and homologous fatty alcohols, that the stabilizing interaction required to form fluid bilayers was an interfacial H-bond network between the constituents. Second, the sulfoxide group interacts also strongly with water,[18,22] and these interactions may provide the suitable hydration of the interface for forming fluid bilayers. In the other monoalkylated amphiphile/sterol systems forming fluid lamellar self-assemblies at room temperature, the presence of a charged functional group may play the role of ensuring proper interfacial hydration. The solidification of the lipid species would require the dehydration of the head groups; the energy required to break the interacting S═O/H_2O pair (or even complex) could be considerable, leading to a slow interface dehydration and, as a consequence, the existence of the metastable phase. These two phenomena can both contribute to the behavior of the OMSO/Chol system. The properties associated with this distinct behavior expand the potential of these liposomes for applications in several areas such as drug delivery.

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Supporting Information Available: Figure S1: the profile of the $\delta(CH_2)$ region of the IR spectrum of an equimolar OMSO/Chol mixture, supporting the formation of the metastable phase. Figure S2: the thermal behavior of OMSO/Chol Large Unilamellar Vesicles, as determined by the ν_{C-H} band position of the IR spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

(44) Hargreaves, W. R.; Deamer, D. W. *Biochemistry* **1978**, *17*, 3759–3768.