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ARTICLE *in* LANGMUIR · AUGUST 2012

Impact Factor: 4.46 · DOI: 10.1021/la302278q · Source: PubMed

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Formation of pH-Sensitive Cationic Liposomes from a Binary Mixture of Monoalkylated Primary Amine and Cholesterol

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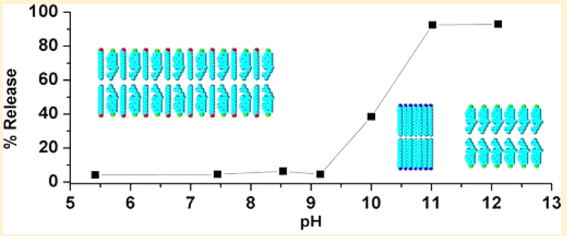
ABSTRACT: It has been shown that mixtures of monoalkylated amphiphiles and sterols can form liquid-ordered (lo) lamellar phases. These bilayers can be extruded using conventional methods to obtain large unilamellar vesicles (LUVs) that have very low permeability and a specific response to a given stimulus. For example, pH variations can trigger the release from LUVs formed with palmitic acid and sterols. In the present work, the possibility to form non phospholipid liposomes with mixtures of stearylamine (SA) and cholesterol (Chol) was investigated. The phase behavior of these mixtures was characterized by differential scanning calorimetry, infrared, and ^{2}H NMR spectroscopy. It is found that this particular mixture can form a lo lamellar phase that is pH-sensitive as the system undergoes a transition from a lo phase to a solid state when pH is increased from 5.5 to 12. LUVs have been successfully extruded from equimolar SA/Chol mixtures. Release experiments as a function of time revealed the relatively low permeability of these systems. The fact that the stability of these liposomes is pH dependent implies that these LUVs display an interesting potential as new cationic carriers for pH-triggered release. This is the first report of non phospholipid liposomes with high sterol content combining an overall positive charge and pH-sensitivity.



1. INTRODUCTION

Liposomes have been attracting considerable interest since British hematologist Dr. Alec Bangham et al. first reported their discovery in 1964.[1] Over the last 50 years, liposomal nanotechnology has significantly evolved, and now liposomes have found applications in many fields including nanopharmaceutics,[2] cosmetics,[3] food,[4] and textile[5] industries. Liposomes are essentially used as nanocontainers for protecting, transporting, and targeting solutes. Molecular features can be introduced in liposomes to craft some beneficial properties. For example, pH-sensitive liposomes are of interest for the release of encapsulated solutes at a specific location in cells or organisms where a distinct local pH prevails.[6] Similarly, cationic liposomes display distinct advantages for some applications related to vectorization and delivery. For instance, in gene therapy, cationic liposomes are reported to interact and complex with DNA or oligonucleotides, markedly prevent nuclease degradation, enhance cellular uptake rate of oligonucleotide, and result in a better intracellular distribution.[7−9] Cationic liposomes were also shown to enhance the stability of some anticancer drugs, such as paclitaxel,[10] to improve cancer cell uptake and to target tumor microvasculature endothelial cells.[11−13] Cationic liposomes were shown to be useful for dermal and transdermal drug delivery,[3,14,15] and were found to have high affinity to bacteria biofilms.[16]

Over the past decade, it has been reported that cholesterol (Chol) and other sterols can induce the formation of fluid lamellar phases when mixed with monoalkylated amphiphiles, such as palmitic acid (PA),[17−19] *N*-acylethanolamine,[20] lysopalmitoylphosphatidylcholine (lyso-PPC),[21] cetylpyridinium chloride (CPC),[22] octadecyl methyl sulfoxide (OMSO),[23] and cetyltrimethylammonium bromide (CTAB).[24] Even though these monoalkylated amphiphiles or sterols do not form fluid lamellar phases once hydrated individually, their mixtures lead to stable liquid-ordered (lo) bilayers. Typically, these fluid bilayers include a high sterol content, varying between 50 and 75 mol %. At this point, some molecular prerequisites for fluid-bilayer formation have been identified. The hydrophobic match between the length of the alkyl chain (14−18 carbon atoms) of the monoalkylated amphiphiles and that of the hydrophobic segment of cholesterol is essential for the formation of lo bilayers.[25] The stability of those bilayers has been found to be intimately associated with the bilayer surface electrostatics. The presence of bilayer interfacial charges is suggested to be a key element in the intermolecular interactions between the bilayer components and is believed to contribute to the proper hydration of the interface. It has been established that large unilamellar vesicles (LUVs) can be obtained from these fluid bilayers using conventional extrusion methods. As the stability of PA/sterol bilayers is dictated by the protonation state of the fatty acid, the resulting liposomes are found to be pH-sensitive, the modulation of the bulk pH resulting in switching between the protonation/deprotonation states of PA molecules.[18,26] Moreover, it has been shown that one can control the pH at which the release is triggered by using PA derivatives with different pK_a.[27]

Received: June 5, 2012
Revised: August 22, 2012
Published: August 29, 2012

   dx.doi.org/10.1021/la302278q | *Langmuir* 2012, 28, 13668−13674

In this Article, we report the development of non phospholipid liposomes that are both cationic and pH sensitive, two features that have never been jointly crafted in a liposome. This investigation aims essentially at establishing that specific properties can be introduced in liposomes based on the current understanding of the rules dictating the formation of stable self-assemblies. We examined the possibility of forming liposomes using stearylamine (SA) as the monoalkylated amphiphile, and cholesterol. The primary amine is a functional group whose protonation/deprotonation state can be exploited to control the surface charge density. The pK_a of monomeric stearylamine is 10.6,[28] while its apparent pK_a when inserted in phosphatidylcholine bilayers is approximately 9.5.[29] Stearylamine was also selected because the length of its 18 carbon alkyl chain matches the hydrophobic section of cholesterol.[25] This fatty amine has been previously used to provide a cationic charge to phospholipid liposomes.[14] First, the phase behavior of SA/Chol mixtures in different proportions was characterized by differential scanning calorimetry (DSC), infrared (IR) spectroscopy, and nuclear magnetic resonance spectroscopy of deuterium (^{2}H NMR), to identify the conditions leading to the formation of lo phase bilayers. Second, we examined the possibility to extrude the SA/Chol mixtures forming fluid bilayers to obtain LUVs and characterized the stability and the permeability of these resulting liposomes. Finally, the pH-sensitivity of those cationic liposomes was assessed.

2. MATERIALS AND METHODS

Cholesterol (>99%), stearylamine (99%), tris(hydroxymethyl)-aminomethane (TRIS) (99%), 2-[*N*-morpholino]ethanesulfonic acid (MES) (>99%), ethylenediaminetetraacetic acid (EDTA) (99%), NH_4Cl (>99%), NaCl (>99%), and cholesterol-2,2,4,4,6-d_5 (Chol-d_5) were supplied by Sigma Chemical Co. (St. Louis, MO). 1-palmitoyl-2-oleyl-*sn*-glycero-phosphocholine (POPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). ^{3}H-glucose was obtained from Perkin-Elmer (Boston, MA). Sephadex G-50 Medium was purchased from Pharmacia (Uppsala, Sweden). Methanol (spectrograde) and benzene (high purity) were obtained from American Chemicals Ltd. (Montreal, QC, Canada) and BDH Inc. (Toronto, ON, Canada), respectively. All solvents and products were used without further purification.

Mixtures of stearylamine and cholesterol were prepared by dissolving weighed amounts of the solid chemicals in a mixture of benzene/methanol 90/10 (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 by cholesterol-d_5 for the ^{2}H NMR experiments. The freeze-dried lipid mixtures were hydrated with a MES/TRIS buffer (TRIS 50 mM, MES 50 mM, NaCl 130 mM, EDTA 0.5 mM) providing a buffered range between pH 5 and 9, or with a TRIS/NH_4Cl buffer (TRIS 50 mM, NH_4Cl 50 mM, NaCl 130 mM, EDTA 0.5 mM) providing a buffered range between pH 7 and 11. The buffers were prepared with Milli-Q water for DSC and IR spectroscopy. Deuterium-depleted water was used for ^{2}H NMR experiments. The final lipid concentration was 20 mg/mL for DSC and 30 mg/mL for IR and ^{2}H NMR spectroscopy experiments. To ensure a good hydration of the samples, the suspensions were subjected to five temperature cycles from liquid nitrogen temperature to ~70 °C, and were vortexed between successive cycles. After hydration, the pH was measured and readjusted, if necessary, by the addition of an aliquot of diluted HCl or NaOH solution.

DSC was performed with a VP-DSC microcalorimeter (MicroCal, Northampton, MA). The reference cell was filled with the corresponding buffer. Data acquisition was performed from 25 to 80 °C, at a heating rate of 40 °C/h. Data acquisition and treatment were performed with the Origin software (MicroCal software, Northampton, MA).

IR spectra were recorded on a Thermo Nicolet 4700 spectrometer, equipped with a KBr beam splitter and a DTGS-L-alanine detector. An aliquot of the sample was placed between two BaF_2 windows separated by a 5 μm-thick Teflon ring. This assembly was inserted into a brass sample holder, whose temperature was controlled by Peltier thermopumps. Each spectrum was the result of 60 scans with a nominal 2 cm^{-1} resolution, Fourier transformed using a triangular apodization function. The temperature was varied from low to high, and there was 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.

^{2}H NMR spectra were recorded on a Bruker AV-400 spectrometer, using a Bruker static probe equipped with a 10 mm coil. The hydrated lipid suspension was transferred into a homemade Teflon holder that filled the coil. A quadrupolar echo sequence was used with a 90° pulse of 5.5 μs and an interpulse delay of 30 μs. The recycling time was 30 s. In the absence of a slow-relaxation 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, and the data acquisition was carried out as a function of increasing temperature.

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 room temperature. The chemical composition of the extruded liposomes corresponded to the initial SA/Chol proportions, as assessed by mass spectrometry. The hydrodynamic diameter and the zeta potential of resulting LUVs were measured at 25 °C, using a Malvern Zetasizer.

The glucose passive permeability of the LUVs was evaluated. Glucose (240 mM and an aliquot of ^{3}H-glucose) was added to the SA/Chol mixtures, hydrated with a TRIS/NH_4Cl buffer (TRIS 50 mM, NH_4Cl 50 mM, NaCl 10 mM, EDTA 0.5 mM), pH 7.5, before the freeze-and-thaw cycles. The lipid concentration was 30 mg/mL, while the radioactivity was ~43 μCi/mL. After extrusion, the glucose-containing vesicles were isolated at room temperature from the free glucose by gel permeation chromatography using Sephadex G-50 (column diameter, 1.5 cm; length, 25 cm), equilibrated with a TRIS/NH_4Cl buffer (TRIS 50 mM, NH_4Cl 50 mM, NaCl 130 mM, EDTA 0.5 mM), isoosmotic with the glucose-containing buffer. The lipid concentration was then ~3 mg/mL. The glucose passive release was determined as a function of time by isolating the entrapped glucose of a 100 μL aliquot of the vesicle suspension using a Sephadex G-50 spin column (0.4 cm in diameter, 7 cm in length). The radioactivity of an aliquot of the harvested fraction was measured. To take into account the sample dilution on the spin column, an aliquot of POPC liposomes, used as an internal standard, was added to the sample right before the spin column. POPC concentration in the harvested fraction was quantified using the Bartlett method.[30] The potential pH-triggered leakage of glucose was also examined. The external pH was modified by adding an aliquot of diluted HCl or NaOH solution to the LUVs suspension. Spin columns were used to isolate the released glucose, and % release was calculated as described above. The sample radioactivity was measured on a Beckman LS6500 counter.

3. RESULTS AND DISCUSSION

3.1. Thermal Phase Behavior of SA/Chol Mixtures. We first explored the phase behavior of the SA/Chol mixtures to identify whether this mixture can lead to the formation of a fluid lamellar phase, a prerequisite to the formation of liposomes. The thermal behavior of SA/Chol mixtures with various compositions was examined by DSC. Figure 1 presents the thermograms of these mixtures hydrated at pH 5.5, a low pH ensuring the protonation of the amine groups. For pure stearylamine, the thermogram showed a sharp peak at about 58 °C, corresponding to the melting of the amphiphile. The presence of cholesterol, up to 30% molar ratio, led to a small downshift of the peak, while its width increased. When stearylamine and cholesterol were in equal molar proportion,

dx.doi.org/10.1021/la302278q | *Langmuir* 2012, 28, 13668−13674

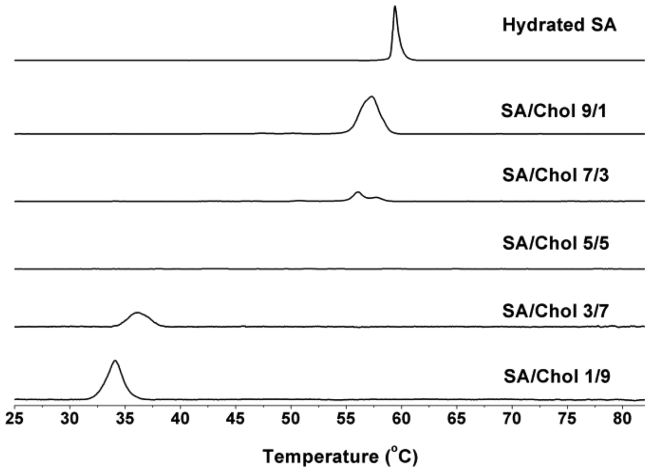


Figure 1. Thermograms of hydrated pure SA and of SA/Chol mixtures of various molar ratios, pH 5.5.

no peak could be observed, suggesting the absence of transition over the whole investigated temperature range. For SA/Chol mixtures with larger cholesterol contents, a broad endothermic peak was observed at ~35 °C. This endotherm is believed to be associated with excess solid cholesterol experiencing the transition from the anhydrous form I to the anhydrous form II.[31] During the cooling, a transition was observed at 24 °C (data not shown), corresponding to the conversion of cholesterol anhydrous form II into its form I with a hysteresis of ~10 °C, in agreement with a previous report.[31] Therefore, it is inferred that proportions of cholesterol larger than 50 mol % led to solid cholesterol, suggesting that the solubility limit of cholesterol in the SA/Chol system had been reached.

The thermal behavior of SA/Chol mixtures was also examined by IR spectroscopy. The position of the symmetric C−H stretching (ν_{C-H}) mode, associated with the stearylamine alkyl chain, is mainly influenced by the trans−gauche chain isomerization and by the interchain coupling. As a consequence, it constitutes a sensitive probe for transitions involving a change in chain conformational order.[32−34] The melting of pure hydrated stearylamine was easily detected at about 58 °C by the abrupt shift of the ν_{C-H} band from ~2849.7 to ~2852.7 cm^{-1} (Figure 2). These positions are representative of highly

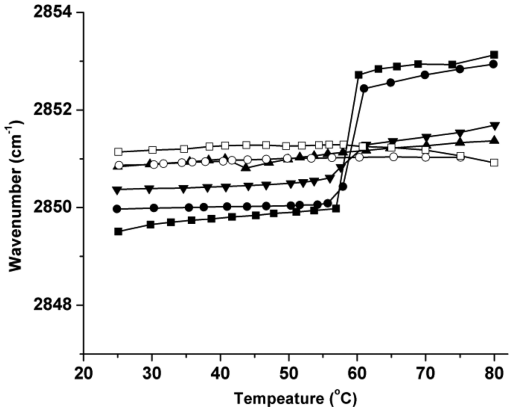


Figure 2. Thermotropism, reported by the ν_{C-H} band position of the IR spectra, of hydrated SA (■), and of SA/Chol mixtures with various compositions: 1/9 (□), 3/7 (○), 5/5 (▲), 7/3 (▼), and 9/1 (●) (molar ratio), pH 5.5.

ordered and disordered chains, respectively. The presence of cholesterol induced an increase of the ν_{C-H} band wavenumber below the transition temperature and a reduction above the transition temperature; these changes led to the disappearance of the phase transition. This impact of cholesterol is reminiscent of its well-established effect on phospholipid bilayers,[35,36] as cholesterol decreases and ultimately abolishes the gel to liquid crystalline phase transitions of phospholipids. An analogous effect is also observed for the mixtures of cholesterol with some monoalkylated amphiphiles, including palmitic acid and octadecyl methyl sulfoxide.[23,26] In these cases, however, in contrast with phospholipid/cholesterol bilayers, the monoalkylated amphiphile and the sterol exist in solid form below the transition temperature. At low temperatures, cholesterol prevents the formation of a solid phase by the monoalkylated amphiphiles, introduces fluidity in the self-assembly, and promotes the formation of a lo lamellar phase. At temperatures higher than the transition temperature, it orders the amphiphile alkyl chain, favoring again the formation of lo lamellar phases. The overall effect is a reduction of the frequency shift associated with the transition, consistent with the DSC results. For mixtures containing 50 mol % or more cholesterol, the ν_{C-H} position was ~2851 cm^{-1} over the investigated temperature range, a value observed for lo lamellar phases.[32,37]

The ν_{C-H} position of the IR spectra essentially reported the behavior of stearylamine alkyl chain in the mixtures. To investigate the phase behavior from the cholesterol point of view, ^2H NMR spectroscopy of mixtures including deuterated cholesterol was carried out. Figure 3 presents ^2H NMR spectra of equimolar SA/Chol-d_5 mixtures. At pH 5.5, the spectra displayed three fairly well-resolved powder patterns, typical of cholesterol solubilized in fluid bilayers. These spectra were very similar to those obtained for Chol-d_5 inserted in other fluid bilayers formed by monoalkylated amphiphiles and cholesterol, such as PA/Chol[17] and OMSO/Chol[23] systems, in phosphatidylcholine bilayers,[38,39] and even in biological membranes such as human red blood cell membranes,[40] and membranes of mycoplasma *Acholeplasma laidlawii* (strain B).[41] The quadrupolar splittings measured between the maxima are reported in Table 1. Using a model previously described,[17,39] it was possible to reproduce the experimental values of quadrupolar splittings, assuming that cholesterol had axially symmetric motions in the bilayers, and that the orientation of this axis of rotation in cholesterol molecule was similar to that determined for phospholipid bilayers. β is the fixed angle between the C−D bond and the cholesterol long axis, defined as the rotation axis. The values that we used (Table 1) were those previously determined for cholesterol embedded in a phosphatidylcholine matrix.[39] Therefore, the only fitting parameter in this approach is the molecular order parameter, S_{mol}, describing the whole-body motion of cholesterol. The fitted value for S_{mol} in the SA/Chol-d_5 equimolar mixture was 0.95, indicating that the axis of rotation of cholesterol is practically parallel to the bilayer normal and that the molecules experience practically no wobbling. Such structure and dynamics were also proposed for the other cholesterol-containing systems mentioned above, and this behavior seems to be rather general. Combining DSC, IR, and ^2H NMR results, we conclude that an equimolar SA/Chol mixture forms a fluid bilayer phase over the investigated temperature range.

3.2. Effect of pH on the Phase Behavior of SA/Chol Mixtures. The selection of stearylamine as the monoalkylated

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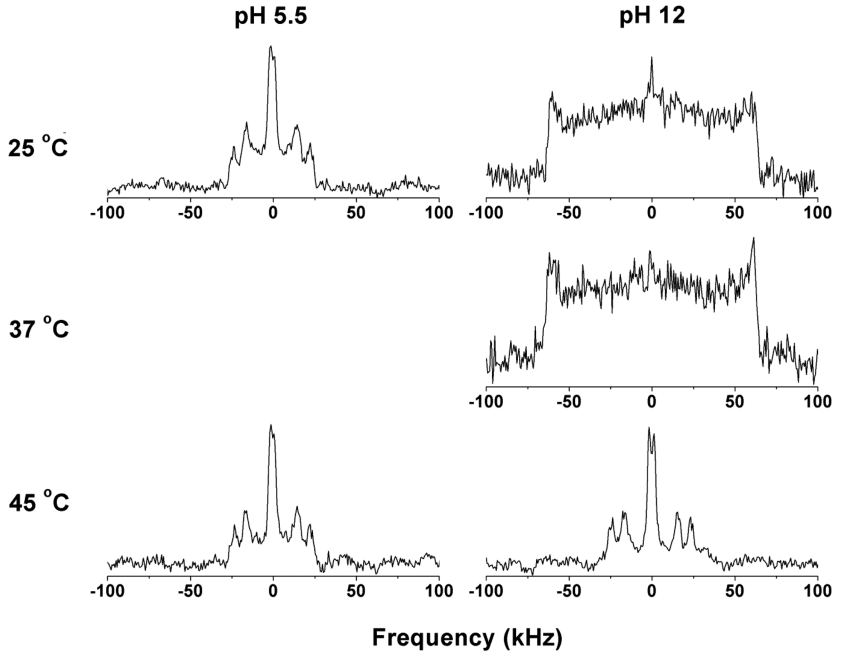

Figure 3. ^{2}H NMR spectra of an equimolar SA/Chol-d_5 mixture.

Table 1. ^{2}H NMR Parameters Associated with Cholesterol in Equimolar SA/Chol-d_5 Mixtures, at 45 °C

pH	position	exp $\Delta\nu_Q$ (kHz)	β obtained from ref 39	calcd S_{mol}	calcd $\Delta\nu_Q$ (kHz)
5.5	2,4-$^2H_2$ ax	46.8	74.1	0.95	46.9
	2,4-$^2H_2$ eq	33.6	66.2		31.0
	6-^{2}H	3.0	55.8		3.2
12	2,4-$^2H_2$ ax	50.0	74.1	1	49.4
	2,4-$^2H_2$ eq	34.2	66.2		32.6
	6-^{2}H	4.3	55.8		3.3

amphiphile was based on the hypothesis that the primary amine group could introduce in the system a pH sensitivity that may be exploited to trigger the release from the derived liposomes. Therefore, we examined the impact of pH on the phase behavior of the equimolar SA/Chol mixture, using DSC, IR, and ^{2}H NMR spectroscopy. No transition was observed by DSC for the equimolar SA/Chol mixture when the bulk pH was between 5.5 and 9 (Figure 4). This observation is consistent with the FTIR results as the ν_{C-H} position of the mixture spectra was, for these pH values, around 2851 cm^{-1} over the whole investigated temperature range (Figure 5). These findings indicate the formation of a stable lo lamellar phase in these conditions.

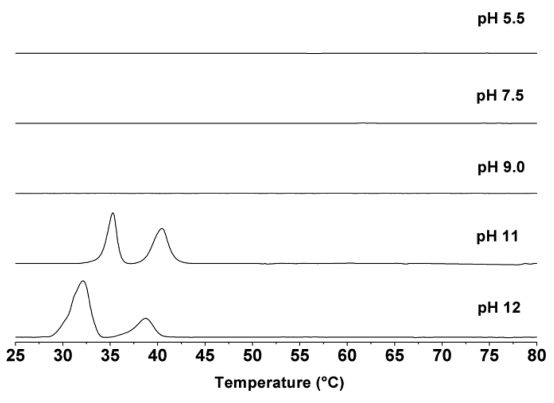

Figure 4. Thermograms of equimolar SA/Chol mixtures at various pH values.

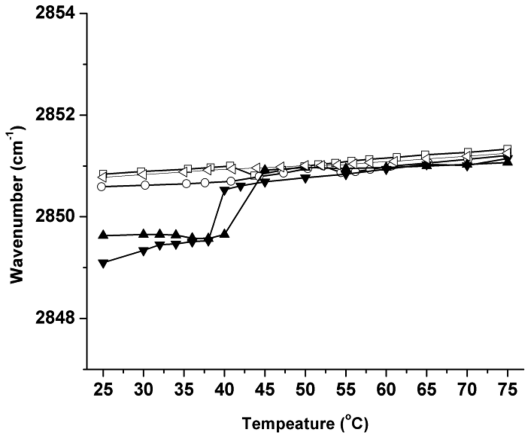

Figure 5. Thermotropism, reported by the ν_{C-H} band position of the IR spectra, of equimolar SA/Chol mixtures at pH 5.5 (□), 7.5 (◁), 9.0 (○), 11 (▼), and 12 (▲).

When the pH was increased to 11 and 12, the thermograms revealed two maxima, at around 34 and 40 °C. The transition at around 40 °C was also detected by IR spectroscopy from the shift of the ν_{C-H} frequency, going from lower than 2850 cm^{-1} at low temperatures to ~2851 cm^{-1} above the transition temperature. These values suggest that stearylamine chains underwent a transition from a solid to a lo lamellar phase. At pH 12, 25 °C, a single broad powder pattern was observed in the ^{2}H NMR spectrum of the SA/Chol-d_5 mixture (Figure 3). The quadrupolar splitting between the two maxima was 125 kHz. These spectra are identical to that obtained from solid cholesterol, all five deuterated positions giving rise to this single and maximum quadrupolar splitting, suggesting that the cholesterol molecules are immobile.[17,42] ^{2}H NMR spectra

dx.doi.org/10.1021/la302278q | Langmuir 2012, 28, 13668−13674

indicated that, at pH 12, cholesterol remained in a solid phase up to 37 °C. At 45 °C, the characteristic profile of cholesterol solubilized in fluid bilayers was obtained, and the solid-cholesterol contribution could no longer be observed. The quadrupolar splittings associated with this mobile cholesterol were very similar to those measured at pH 5.5 (Table 1). Therefore, the transition observed at 40 °C was assigned to a transition from a solid to a lo lamellar phase in the equimolar SA/Chol mixture. The transition at about 35 °C observed in the thermograms recorded at high pH (Figure 4) is suggested to be associated with a crystalline reorganization of stearylamine. This transition was observed in the thermogram of pure hydrated stearylamine at pH 12, whereas the chain melting temperature of stearylamine was 66 °C in these conditions (data not shown). Furthermore, at pH 12, X-ray diffraction patterns obtained from pure hydrated stearylamine showed different crystalline features at 25 and 45 °C.

These results altogether established that, in the equimolar mixture at pH < 9, protonated stearylamine is well mixed with cholesterol molecules, leading to the formation of stable lo lamellar phases. However, pH values greater than 9 lead to a phase separation giving rise to solid cholesterol as well as solid stearylamine; this behavior is associated with the deprotonated state of the amine group. Cholesterol however reduces the chain melting transition of stearylamine to ~40 °C, and, above this transition temperature, lo lamellar phases formed with stearylamine and cholesterol were observed. We can conclude that the bilayers formed by an equimolar SA/Chol mixture at pH ≤ 9 are positively charged and pH-sensitive. Therefore, we examined whether they represent an opportunity to create novel pH-sensitive non phospholipid liposomes.

3.3. Liposome Properties. The phase behavior of an equimolar SA/Chol mixture guided our selection of conditions that could lead to the formation of liposomes. It was shown previously that lo bilayers made of monoalkylated amphiphiles and sterols can be extruded despite their high sterol content.[22,23,43] The SA/Chol system displayed a similar behavior, and LUVs could be obtained by extrusion from this mixture, at pH ≤ 9.5. Table 2 reports the size and the zeta potential of the resulting liposomes. The measured liposome diameters were, as expected, slightly larger than the pore size of the extrusion filters, and these sizes remained constant over at least 1 month. However, at pH 10.5 and 11.5, the extrusion process was impossible as solid lipids would simply obstruct the filter pores. The liposomal surface charge density can be conveniently investigated by microelectrophoresis and characterized by the zeta potential. The zeta potential of 100 nm liposomes made from equimolar SA/Chol mixtures (Table 2) was 43 and 33 mV at pH 7.5 and 8.5, respectively, indicating that the suspension is stable.[44] The size increase and the small decrease of the LUV zeta potential observed with the pH increase are likely associated with the progressive deprotonation of the amine groups.

Table 2. Characteristics of Extruded LUVs for Equimolar SA/Chol Mixtures at Various pH Values[a]

pH	d_{LUVs} (nm)	zeta potential (mV)
7.5	101 ± 5	43 ± 3
8.5	113 ± 10	33 ± 2
9.5	170 ± 7	24 ± 2

[a]The samples were hydrated in TRIS/NH_4Cl buffers with pH varying from 7.5 to 9.5.

We have determined the permeability of the liposomes formed with an equimolar SA/Chol mixture, at pH 7.5. The passive leakage of glucose from these vesicles is presented in Figure 6. A progressive release was observed, and it took approximately 30 days to have about 90% of the entrapped glucose released. As a comparison, more than 90% of encapsulated glucose leaked out from phosphatidylcholine liposomes containing between 40 and 50 mol % cholesterol within 10 days.[43,45] Therefore, we can conclude that the passive permeability of the SA/Chol liposomes is considerably more limited than that of cholesterol-containing phospholipid liposomes. It is proposed that the high cholesterol content makes the bilayers more rigid and thicker, resulting in a lower permeability. This cationic formulation seems however to be somewhat more permeable than PA/Chol liposomes. Only 10% of entrapped glucose was released after 30 days from the latter.[43] This increased impermeability may be associated with the higher cholesterol content as these LUVs were formed with PA/Chol mixtures in a molar ratio of 30/70.

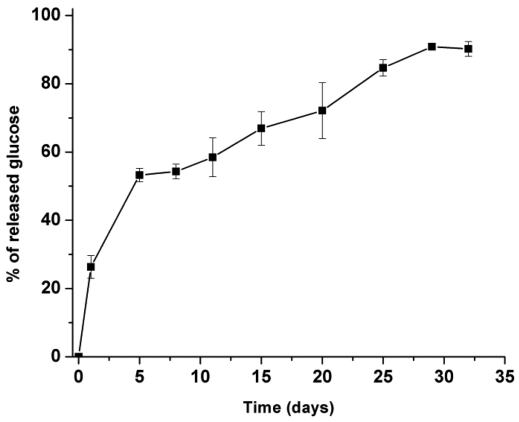

Figure 6. Passive release of equimolar SA/Chol LUVs, at room temperature, pH 7.5. (n = 3)

Because of the impact of the amine protonation state on the phase behavior of the SA/Chol mixture reported above, the LUVs formed with this mixture were expected to be pH-sensitive. To demonstrate the pH-triggered release, we modified the external pH of a suspension of glucose-loaded liposomes initially prepared at pH 7.5 from an equimolar SA/Chol mixture, and the glucose release was measured. Figure 7 shows that the content release was strongly dependent on the external pH. For pH < 9.5, practically no leakage was observed. Conversely, for pH ≥ 11, the entrapped glucose was completely released within 10 min. The release profile as a function of pH is reminiscent of a titration curve with an inflection point at pH 10, corresponding to the pK_a of the amine group.[29] The rapid pH-triggered release is associated with the fact that neutral stearylamine and cholesterol molecules phase separate at high pH and exist in solid forms in these conditions. As pH was increased above 9.5, macroscopic aggregates could be observed in the samples, and vesicles could no longer be detected by dynamic light scattering. The formation of solid phases by the components would indeed be accompanied by the release of glucose that was initially entrapped in the liposomes. The reported behavior is reminiscent of PA/sterol LUVs, which are also pH-sensitive. A sudden burst of release of the entrapped

dx.doi.org/10.1021/la302278q | Langmuir 2012, 28, 13668−13674

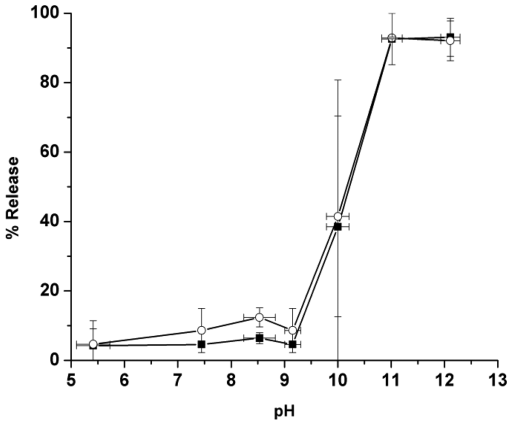

Figure 7. pH-triggered release of glucose from LUVs obtained from an equimolar SA/Chol mixture. The measurements were carried out at room temperature, ~10 min (■) and ~1 h (○) after the external pH change. (n = 3)

materials was observed when the pH was lowered and PA became neutral (i.e., protonated).[18,43] The protonated/deprotonated state of the pH-sensitive group appears to be the crucial parameter leading to the triggered release.

These new findings reinforce the hypothesis that interfacial charges play a pivotal role in the stability of the fluid bilayers formed by binary mixtures of a monoalkylated amphiphile bearing a small polar headgroup (e.g., carboxylic, ammonium, etc.) and a sterol. The binary mixtures that could form lo lamellar phases include unprotonated PA/Chol,[26] protonated PA/cholesterol sulfate (Schol),[19] CPC/Chol, CPC/Schol,[22] CTAB/Chol,[24] *N*-alkylethanolamine/Chol,[20] and lyso-PPC/Chol[21] systems. All of these mixtures include at least one charge at the headgroup level; it could be a zwitterionic headgroup like in the case of lyso-PPC. The stability associated with the presence of an interfacial charge is exemplified by the mixtures that include an ionizable group such as a carboxylic group[19,26] or an amine group (this work). In the presence of cholesterol, stable lo lamellar phases are obtained at room temperature when the monoalkylated amphiphile bears a charge: a negatively charged unprotonated carboxylic group in the case of fatty acids[17,19,26] and a positively charged protonated amine group in the case of stearylamine (this work). When the pH is modified in such a way that the monoalkylated amphiphile becomes neutral (a pH decrease in the case of PA to provide the protonated form and a pH increase in the case of stearylamine to provide the unprotonated form), the mixtures with cholesterol lead to a phase separation and to the disruption of the fluid bilayers.[19,26] It was found that the charge can also be carried by the sterol molecule as negatively charged cholesterol sulfate forms stable lamellar phases in the presence of neutral protonated PA.[19] The importance of the interfacial charges in the stability of these fluid bilayers is proposed to arise from two phenomena. First, the lipid mixing is promoted by the presence of a charged species as the mixing would lead to a reduction of the unfavorable electrostatic repulsion. Such effect would exist for mixtures formed by a charged and a neutral species as well as in the cases where the two species bear charges of opposite sign such as CPC and Schol.[22] Indeed, electrostatic stabilization would not be observed if the two species both carry negative charges such as in the case of unprotonated PA and Schol, which do not mix and do not lead to the formation of a lo lamellar phase.[19] Second, the presence of interfacial charges is proposed to provide an appropriate hydration of the bilayer interface, a feature that is critical for the formation of a fluid bilayer.[23]

In conclusion, we discovered the possibility to form a lo lamellar phase with a binary mixture including stearylamine and cholesterol. These fluid bilayers were stable at room temperature only for pH lower than the pK_a of the amine group (pH < 9.5). We also demonstrate that it is possible to extrude liposomes from the equimolar SA/Chol mixtures, leading to LUVs that display a positive zeta potential, are relatively impermeable, and are pH-sensitive. This new formulation not only extends the family of non phospholipid liposomes with high sterol contents, but it also establishes that the identified molecular requirements allow designing tailored liposomes from monoalkylated amphiphiles and sterol. In this Article, we targeted the formation of cationic liposomes with a simple and cheap amphiphile. On the basis of the same rationale, we believe that it is possible to create novel cationic formulations using analogous molecules such as alkylated imidazole and glucosamine that would offer the opportunity to tune the pH-triggered release of the liposome content, based on their respective pK_a. Such advances present a new avenue for making potentially useful nanovectors.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Z.-K.C. is grateful to the China Scholarship Council and Université de Montréal for his scholarship. We would like to thank the Natural Sciences and Engineering Research Council of Canada for financial support. This work was also funded by Fonds Québécois de la Recherche sur la Nature et les Technologies through its financial support to the Center for Self-Assembled Chemical Systems (CSACS). We thank Guillaume Bastiat for carrying out preliminary experiments.

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