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Cholesteryl hemisuccinate exhibits pH sensitive polymorphic phase behavior

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Abstract

Cholesteryl hemisuccinate (CHEMS) is an acidic cholesterol ester that self-assembles into bilayers in alkaline and neutral aqueous media and is commonly employed in mixtures with dioleoylphosphatidylethanolamine (DOPE) to form 'pH sensitive' fusogenic vesicles. We show here that CHEMS itself exhibits pH sensitive polymorphism. This is evident from the fusogenic properties of large unilamellar vesicles (LUV) composed of CHEMS and direct visualization employing freeze-fracture electron microscopy. Below pH 4.3, LUV composed of CHEMS undergo fusion as monitored by lipid mixing assays and freeze-fracture electron micrographs reveal the characteristic striated signature of H_{II} phase lipid. It is suggested that the pH dependent phase preferences of CHEMS contribute to the pH sensitivity of LUV composed of mixtures of CHEMS and DOPE.

Keywords: Hexagonal (H_{II}) phase; pH sensitive liposome; Fusogenic liposome

1. Introduction

Cholesteryl hemisuccinate (CHEMS) consists of succinic acid esterified to the β-hydroxyl group of cholesterol (Fig. 1A). This chemical modification results in the ability of CHEMS to adopt a lamellar organization upon hydration in neutral or alkaline aqueous media [1,2], whereas cholesterol forms monohydrate crystals in an aqueous environment [3].

CHEMS can stabilize dioleoylphosphatidylethanolamine (DOPE), a lipid which preferentially adopts the inverted hexagonal (H_{II}) phase above 10°C [4], into the lamellar phase at pH 7.4 [5–7]. Lamellar CHEMS/DOPE systems can be prepared at neutral or slightly alkaline pH but these systems become unstable and fuse at acidic pH [5]. In these 'pH sensitive liposomes' the ionization state of

Abbreviations: CHEMS, cholesteryl hemisuccinate; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; H_{II}, inverted hexagonal phase; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; Lα-H_{II}, lamellar to inverted hexagonal phase transition; LPC, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; LUV, large unilamellar vesicles; MES, 2-[*N*-morpholino]ethanesulfonic acid; MLV, multilamellar vesicles; NBD-PE, 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Rh-PE, 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-lissamine rhodamine b sulfonyl; Triton X-100, *t*-octylphenoxypolyethoxyethanol; TNS, 2-*p*-toluidinyl naphthalene 6-sulfonic acid

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CHEMS dictates the phase behavior, and thus the fusogenic behavior, of the lipid ensemble. This behavior is usually rationalized as an ability of the anionic form of CHEMS to stabilize DOPE into a lamellar phase, whereas when CHEMS is in the neutral form this stabilizing property is reduced and DOPE can adopt the H_{II} phase it prefers in isolation, thus promoting fusion. However, in mixtures of CHEMS with phosphatidylethanolamine (PE) exhibiting higher lamellar to inverted hexagonal ($L\alpha$-H_{II}) phase transition temperatures, it has been shown that CHEMS can modulate the $L\alpha$-H_{II} phase transition temperature depending on its state of ionization. In the anionic form CHEMS stabilizes against H_{II} phase formation as indicated by an increase in the $L\alpha$-H_{II} phase transition temperature while the neutral form of CHEMS induces a marked reduction in the temperature of the $L\alpha$-H_{II} phase transition [6,8]. Cholesterol and other neutral sterols can also reduce the $L\alpha$-H_{II} transition temperatures in mixtures with PE [9,10], but to a much lesser extent than protonated CHEMS [6,8].

In this work we investigate the molecular mechanism of the strong H_{II} phase inducing activity of CHEMS at acidic pH. We show that large unilamellar vesicles composed of CHEMS undergo membrane fusion upon acidification as indicated by lipid mixing, consistent with a preference of the neutral form for the H_{II} phase. This conclusion is supported by freeze-fracture electron microscopy studies.

2. Materials and methods

2.1. Materials

CHEMS (morpholine and Tris salt) was obtained from Sigma Chemical Company (St. Louis, MO) and was pure as assayed by thin layer chromatography. 2-*p*-Toluidinyl naphthalene 6-sulfonic acid (TNS), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), 2-[*N*-morpholino]ethanesulfonic acid (MES), *t*-octylphenoxypolyethoxyethanol (Triton X-100) and glacial acetic acid were obtained from Sigma. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC), 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-lissamine rhodamine b sulfonyl (Rh-PE) and 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). The concentration of phospholipid solutions was determined by phosphate assay [11].

2.2. Large unilamellar vesicle (LUV) preparation

CHEMS morpholine salt, POPC, NBD-PE and Rh-PE were dissolved separately in chloroform and stored at −20°C. CHEMS Tris salt was stored at −20°C in its powder form. CHEMS LUV were either prepared by hydration of dried lipid films of CHEMS morpholine salt or by direct hydration of the CHEMS Tris salt. Both CHEMS salts gave identical results. For the p*K* determination, POPC was mixed with an equimolar amount of CHEMS morpholine salt in $CHCl_3$. For the lipid mixing assays, CHEMS morpholine salt was mixed with NBD-PE and Rh-PE (1 mol% each) in chloroform. The chloroform solutions were dried to a thin film under a stream of N_2 gas. The lipid films were further dried in the dark for at least 1 h under high vacuum (30–60 mTorr) to remove residual organic solvent. Dry lipid was hydrated using the appropriate buffer by vortex mixing to produce multilamellar vesicles (MLV). LUV were prepared by a freeze-thaw and extrusion technique [12]. The MLV were subjected to five freeze-thaw cycles (liquid nitrogen/room temperature) and extruded 10 times through two stacked 0.1 μm polycarbonate filters using an extrusion device (Lipex Biomembranes, Vancouver, BC). Depending on the lipid formulation the mean diameter of the LUV was 85–110 nm as determined by a quasi elastic light scattering device (Nicomp 270 submicron particle sizer) operating in the vesicle sizing mode.

2.3. pK determination

The p*K* of CHEMS was determined employing TNS, a surface potential probe and pH stable CHEMS/POPC LUV. It was not possible to perform the TNS assay on vesicles composed solely of CHEMS due to precipitation of the lipid at acidic pH. CHEMS/POPC (1/1 mol ratio) LUV were prepared in 10 mM HEPES, 150 mM NaCl, pH 7.8. TNS was prepared as a 100 μM stock solution in

distilled water. Lipid vesicles were diluted to 100 μM lipid in 2 ml of buffered solutions containing 1 μM TNS, 10 mM HEPES, 10 mM MES, 10 mM acetate, 140 mM NaCl, where the pH ranged from 2.5 to 11. Fluorescence intensity was monitored in a thermostated cuvette (25°C) in a Perkin Elmer LS-50 Spectrophotometer using excitation and emission wavelengths of 321 nm and 445 nm. The pH of each solution was measured after fluorescence measurements.

2.4. Lipid mixing assay

Fusion of CHEMS vesicles was assayed by a lipid mixing assay employing fluorescence resonance energy transfer [13]. Labeled CHEMS LUV were prepared to contain NBD-PE and Rh-PE (1 mol% each) in 10 mM HEPES, 150 NaCl, pH 7.8. Labeled and unlabeled CHEMS vesicles were mixed in a 1:5 mol ratio respectively, and introduced together at a final concentration of 150 μM lipid by pipette injection into a stirred cuvette containing 2 ml of 10 mM HEPES, 10 mM MES, 10 mM acetate, 140 mM NaCl equilibrated to the desired pH values. Addition of the lipid did not alter the pH of the buffer. An increase of NBD-PE fluorescence indicates dilution of the membrane bound probes. NBD-PE fluorescence measurements were obtained at 25°C in a Perkin Elmer LS-50 using an excitation wavelength of 467 nm and emission wavelength of 540 nm using an emission filter at 530 nm. Lipid mixing was monitored for approximately 300 s, after which an aliquot of Triton X-100 (10% v/v) was added to a final concentration of 0.1% v/v. Lipid mixing as a percentage of infinite probe dilution was determined using the equation: Lipid mixing $(\%) = (F_t\text{-}F_0)/(F_{max}\text{-}F_0) \times 100\%$, where F_t is the fluorescence intensity of NBD-PE (at 540 nm) during the assay, F_0 is the initial value of NBD-PE fluorescence, and F_{max} is the maximum fluorescence possible given by infinite probe dilution in the presence of 0.1% v/v Triton X-100.

2.5. Effect of lysophosphatidylcholine on the stability of CHEMS bilayers

LPC was dissolved in ethanol (8.0 mM) and stored at −20°C. Prior to each lipid mixing experiment, the ethanol stock of LPC was diluted with distilled water to a concentration of 0.5, 1.0 or 2.0 mM. Labeled and unlabeled CHEMS LUV (1:5 mol ratio) were added to 2 ml of 5 mM HEPES, 150 mM NaCl, pH 7.5 to obtain a final lipid concentration of 150 μM. Addition of LPC to preformed LUV composed of CHEMS should lead to LPC incorporation only in the outer membrane leaflet owing to the slow transbilayer flip-flop of LPC [14]. Fluorescence measurements were made at 25°C using excitation and emission wavelengths of 467 nm and 540 nm, respectively. After a stable signal was obtained, 20 μl of LPC was added from the 0.5, 1.0, or 2.0 mM LPC solutions to obtain a final LPC concentration of 5, 10 or 20 μM. The amount of LPC incorporation into CHEMS LUV was estimated by fluorescence dequenching of NBD-PE in NBD-PE/Rh-PE labeled vesicles using the methodology of Chernomordik et al. [15]. The extent of NBD-PE fluorescence dequenching following the addition of LPC was used as a direct indication of LPC incorporation. Following the addition of LPC, the HEPES buffered saline was acidified with an aliquot of 1 M acetic acid to obtain the desired final pH.

Light scattering measurements were made on a Perkin Elmer LS-50B spectrophotometer using excitation and emission monochromators set to 400 nm under continuous stirring at 25°C. This technique was used to assess dynamic changes in size of CHEMS LUV in response to acidification. CHEMS vesicles were diluted to 150 μM lipid in 2 ml of 10 mM HEPES, 150 mM NaCl, pH 7.5. Addition of an aliquot of 1 M acetic acid was added to acidify the HEPES buffered saline. Measurements were made in the presence and absence of LPC.

2.6. Freeze-fracture electron microscopy

The structure of the CHEMS dispersions was analyzed using freeze-fracture electron microscopy. MLV and LUV samples were prepared from 100 mM CHEMS Tris in 150 mM NaCl, pH 8.1. MLV and LUV were mixed with glycerol to a final concentration of 30% (v/v). LUV samples were also mixed at room temperature with acidic buffer containing 50 mM NaCl, 300 mM acetate, pH 2.7. Acidification of LUV induced rapid aggregation and precipitation of the lipid. Glycerol was included to a final concentra-

tion of 30% (v/v). The time interval between sample acidification and freezing was approximately 2 min. Samples were dispensed onto gold cups and rapidly frozen in freon 22 cooled in liquid nitrogen. Platinum/carbon replicas were prepared using a Balzers Freeze-etching unit BAF 400D and observed by transmission electron microscopy as described elsewhere [16].

3. Results and discussion

The p*K* of CHEMS in LUV was measured using TNS to probe for changes in membrane surface charge in response to pH. TNS is a lipophilic anion that is essentially non-fluorescent in aqueous solution but exhibits an increase in fluorescent quantum yield upon membrane binding. TNS fluorescence has been used to assay the transmembrane distribution of acidic phospholipids [17,18], and to determine p*K* values for ionizable lipid species in a membrane [19]. The data in Fig. 1B shows the change of TNS fluorescence in response to pH in the presence of pH stable LUV composed of equimolar amounts of POPC/CHEMS. Fitting the data to the Henderson-Hasselbach equation gives an apparent p*K* value of 5.8 for CHEMS.

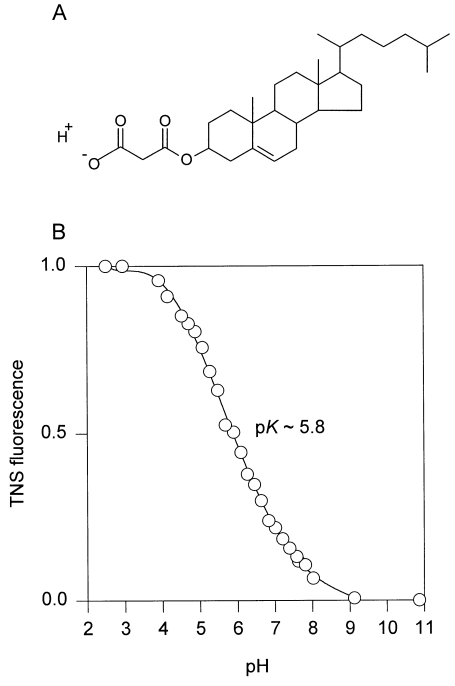

Fig. 1. Structure and p*K* of CHEMS. The p*K* of CHEMS was determined from a pH titration of POPC/CHEMS (1/1 mol ratio) LUV using the TNS assay. POPC/CHEMS LUV (100 μM lipid) were prepared in media containing 1 μM TNS, 10 mM HEPES, 10 mM MES, 10 mM acetate and 140 mM NaCl. The pH was adjusted from 2.5 to 11. TNS fluorescence was monitored at excitation and emission wavelengths of 321 nm and 445 nm and expressed in relative fluorescence units.

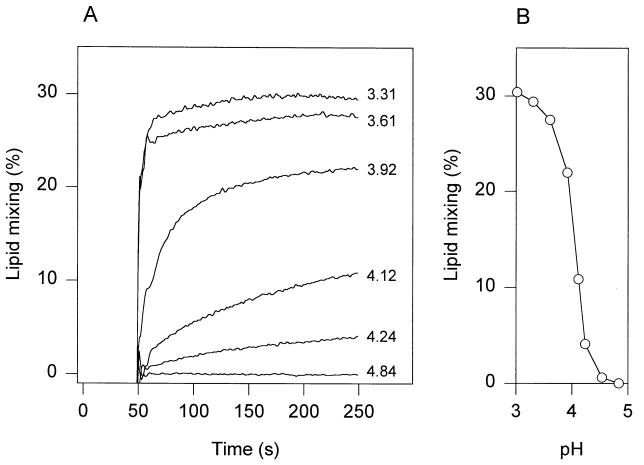

Fig. 2. Acid induced fusion of CHEMS LUV. (A) Lipid mixing of CHEMS LUV in response to acidic pH. (B) Extent of lipid mixing at 250 s as a function of pH. Labeled CHEMS LUV containing Rh-PE and NBD-PE (1 mol% each) were mixed with unlabeled CHEMS LUV (1:5 mol ratio) and introduced at a final concentration of 150 μM lipid (at 50 s) into solutions containing 10 mM HEPES, 10 mM MES, 10 mM acetate and 140 mM NaCl equilibrated to desired pH values. NBD-PE fluorescence was monitored at excitation and emission wavelengths of 467 nm and 540 nm.

CHEMS is commonly used in concert with DOPE to prepare pH sensitive LUV [20,21]. The pH dependent fusogenic properties of LUV composed of CHEMS alone were determined employing the lipid mixing assay [13]. Lipid mixing of CHEMS LUV occurred below pH 4.3 with extensive lipid mixing at pH 4.2 and below (Fig. 2A). Half-maximum lipid mixing of CHEMS LUV occurs at pH 4.1 (Fig. 2B). Fusion of CHEMS/DOPE (3:7 mol ratio) LUV occurs at slightly higher pH values (between pH 4 and 5) [5], consistent with the H_{II} phase preference of DOPE.

LPC can inhibit membrane fusion by inhibiting the formation of fusion intermediates with a negative membrane curvature [22]. Fig. 3A shows that acid promoted lipid mixing of CHEMS LUV is increasingly blocked in the presence of up to 20 μM LPC.

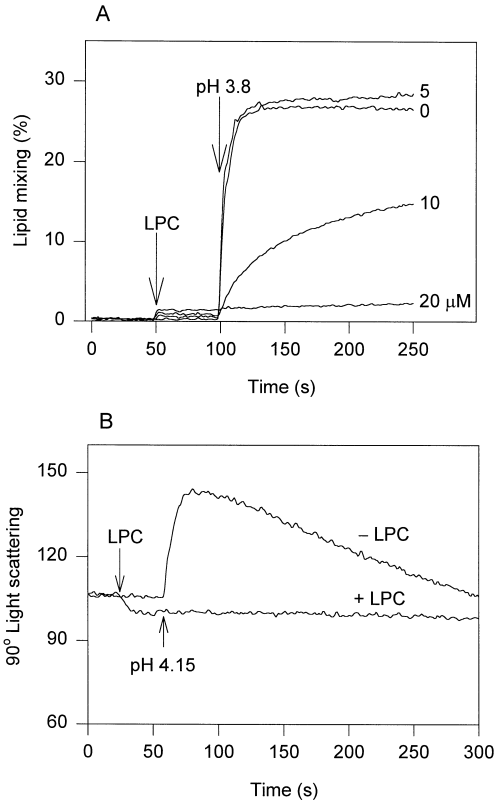

Fig. 3. LPC inhibits acid induced fusion of CHEMS LUV. (A) LPC dependent inhibition of acid induced lipid mixing of CHEMS LUV. (B) Acid induced 90° light scattering changes in CHEMS LUV in the presence and absence of LPC. CHEMS LUV were diluted to 150 μM lipid in 10 mM HEPES, 150 mM NaCl, pH 7.5. For lipid mixing experiments LPC was added prior to sample acidification. Lipid mixing was monitored by measuring NBD-PE fluorescence (467 nm excitation and 540 nm emission). 90° light scattering was observed using excitation and emission monochromators set to 400 nm. Samples were acidified to desired pH with an aliquot of 1 M acetic acid.

Incorporated LPC is estimated to be 2 mol% of the outer membrane leaflet in CHEMS LUV at a final concentration of 20 μM LPC based on the dequenching of NBD-PE after LPC addition.

The ability of LPC to inhibit fusion of CHEMS LUV was also analyzed by 90° light scattering. In the absence of LPC, acidification (pH 4.15) caused an immediate increase in light scattering followed by a gradual decrease (Fig. 3B, LPC). The solution initially became cloudy, followed by the precipitation of lipid aggregates in the buffer. Acidification in the presence of 20 μM LPC did not alter light scattering, indicating that the vesicle size remained unchanged (Fig. 3B, +LPC). The stabilization of CHEMS LUV against fusion by trace amounts of LPC at low pH is consistent with the inhibition of the formation of non-bilayer structures required for the Lα-H_{II} phase transition to proceed. Detergents such as LPC can stabilize DOPE, an H_{II} phase forming lipid, into a bilayer configuration [23] by shape complementarity. The stabilization of acidified CHEMS bilayers by LPC is compatible with a preference of the neutral form of CHEMS for the H_{II} phase.

Freeze-fracture electron microscopy was used to identify the structure of the precipitates formed upon acidification of CHEMS LUV. CHEMS forms MLV upon hydration in alkaline solution as observed by the cross-fracture of multiple lamellae (Fig. 4A). This confirms earlier reports of the self-assembly of CHEMS into closed bilayers in aqueous media [1,2]. Freeze-fracture micrographs of the LUV produced by freeze-thaw and extrusion of CHEMS MLV are shown in Fig. 4B. Incubation of these LUV in acidic media results in structures that display the characteristic striated H_{II} phase freeze-fracture pattern at pH 4.3 (Fig. 5A) and at pH 3.7 (Fig. 5B,C). The diameter of the hexagonal cylinders is ca. 6 nm as measured from Fig. 5C.

The results presented demonstrate an ability of CHEMS to adopt the H_{II} phase upon exposure to acidic pH. It is likely that the phase behavior of CHEMS at low pH is related to effects observed in mixtures with PE. For example, the preference of H_{II} phase by CHEMS at or below pH 4.3 is consistent with the pronounced reduction of the Lα-H_{II} phase transition temperature induced by CHEMS at acidic pH in mixtures with PE. Cholesterol levels of 50 mol% in DOPE membranes lower the Lα-H_{II} phase transition temperature by 10–20°C [9], while only 25 mol% CHEMS at pH 4.5 reduces the Lα-H_{II} phase transition of egg PE by 30°C [6]. This is consistent with an ability of the neutral CHEMS to actively induce the H_{II} phase.

The polymorphism of CHEMS is closely related to that observed for dioleoylphosphatidylserine (DOPS) and α-tocopherol hemisuccinate (THS). DOPS and THS form bilayers under alkaline conditions, but undergo a phase transition to the H_{II} phase upon protonation of their acidic headgroups. THS forms a lamellar phase above pH 7, but below this pH, H_{II} phase and inverted phase intermediates are favored

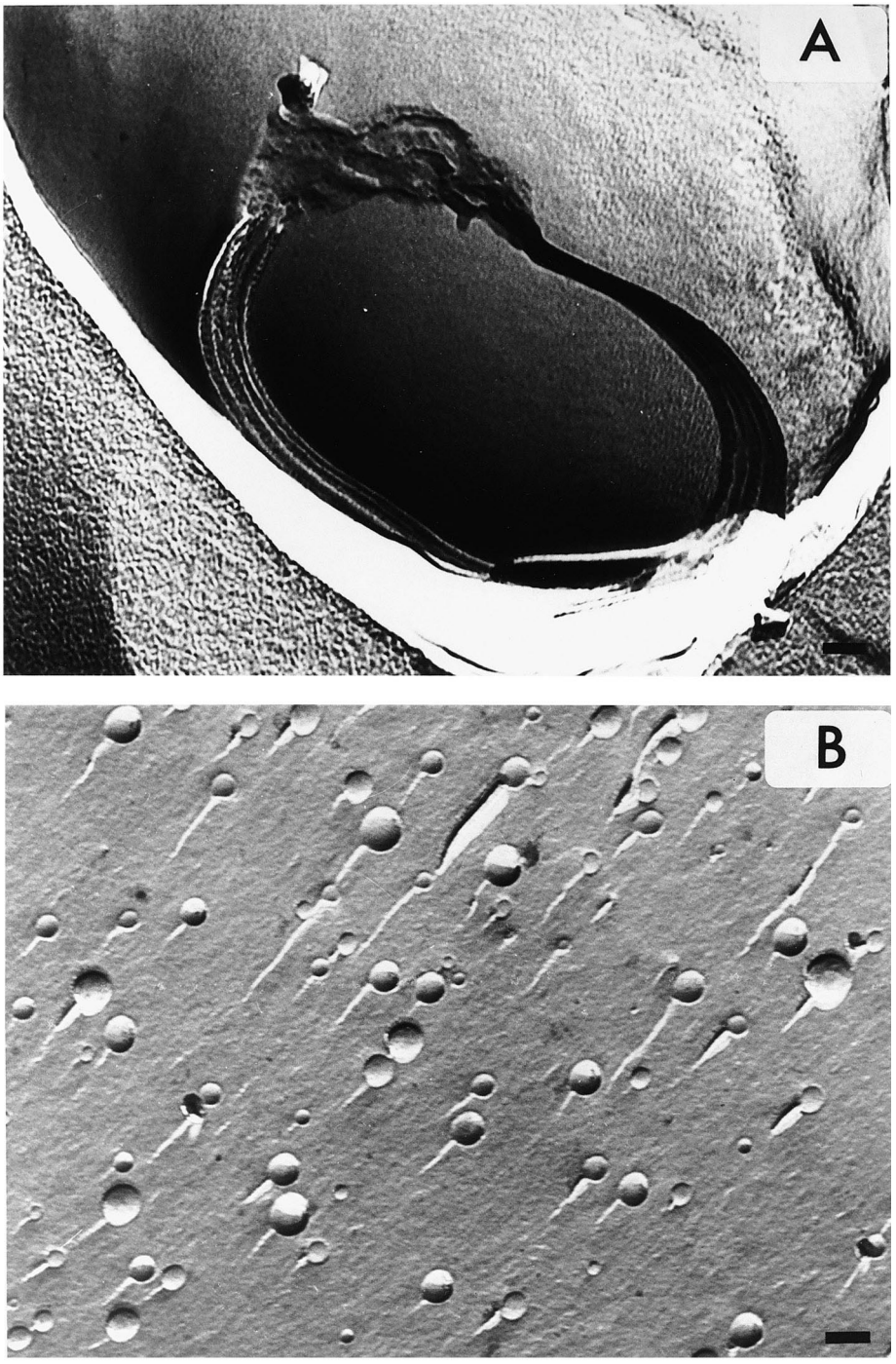

Fig. 4. CHEMS adopts a lamellar phase on hydration at alkaline pH. Transmission electron micrographs of platinum/carbon freeze-fracture replicas of CHEMS MLV, pH 8.1 (A) and extruded CHEMS LUV, pH 8.1 (B). Scale bars indicate 100 nm.

[24]. DOPS also forms a lamellar organization above pH 4.0 but below pH 3.5 DOPS adopts the H_{II} phase [25,26]. Qualitative molecular shape arguments have been used to rationalize such phase behavior, and similar arguments can be used to rationalize the phase behavior of CHEMS. In particular, protonation of the CHEMS headgroup may be expected to lead to a reduction of the headgroup area at the lipid-water interface due to reduced electrostatic repulsion between headgroups and reduced hydration.

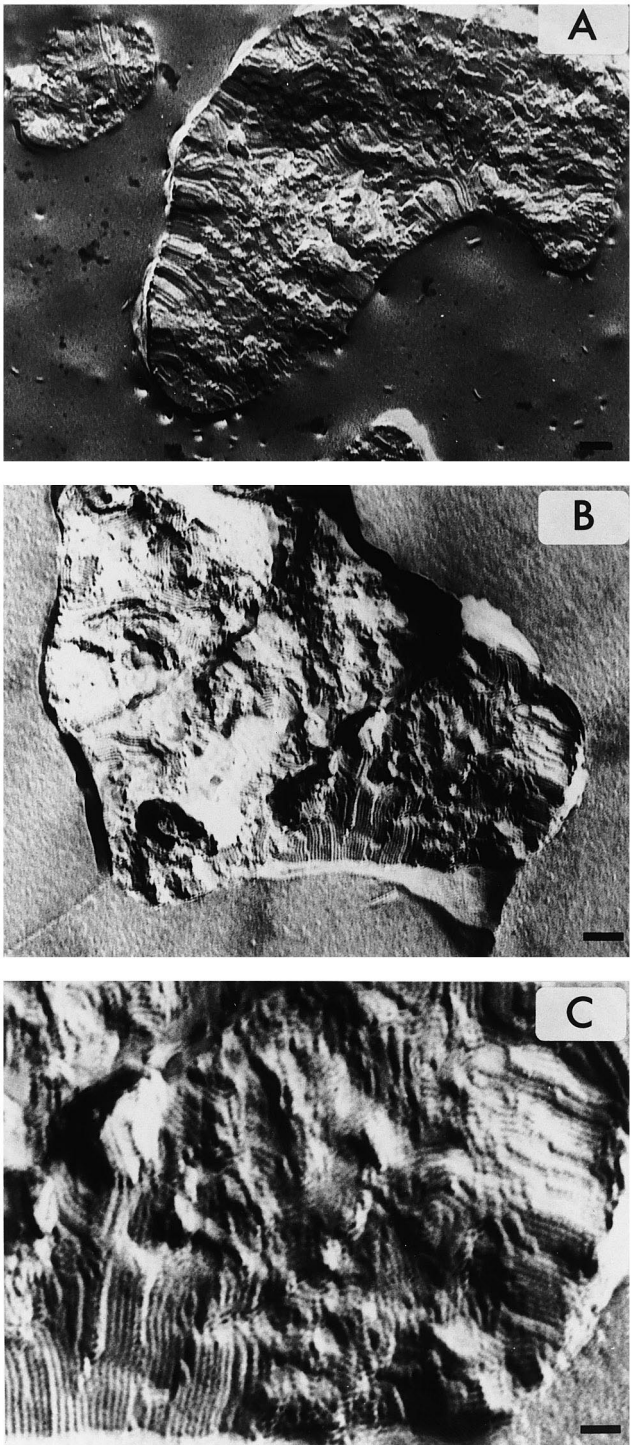

Fig. 5. Acidified CHEMS bilayers adopt the hexagonal phase at acidic pH. Transmission electron micrographs of platinum/carbon freeze-fracture replicas of CHEMS LUV incubated at (A) pH 4.3 and (B and C) pH 3.7. Scale bars represent 100 nm in (A), 87 nm in (B) and 36 nm in (C).

Since the area of the hydrophobic sterol domain presumably remains unaltered upon headgroup neutralization, the effective molecular shape may be modeled as changing from a bilayer forming cylinder to that of an inverted cone that preferentially adopts the H_{II} phase.

In summary, the results presented here demonstrate that CHEMS adopts the H_{II} organization at pH values below the pK of the succinate headgroup. This is consistent with an ability of the neutral form of CHEMS to actively induce the H_{II} phase in mixtures with DOPE, contributing to the pH sensitive fusion of such systems.

Acknowledgements

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