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Novel Asparagine-Derived Lipid Enhances Distearoylphosphatidylcholine Bilayer Resistance to Acidic Conditions

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Abstract

A novel asparagine-derived lipid analogue ($ALA_{11,17}$) bearing a tetrahydropyrimidinone head group and two fatty chains (*11* and *17* indicate the lengths of linear alkyl groups) was synthesized in high yield and purity. The thin film hydration of formulations containing 5 mol% or greater $ALA_{11,17}$ in distearoylphosphatidylcholine (DSPC) generated multilamellar vesicles (MLVs) that remained unaggregated according to optical microscopy, while those formed from DSPC only were highly clustered. The MLVs were processed into unilamellar liposomes via extrusion and characterized by dynamic light scattering (DLS), zeta potential, turbidity, and scanning electron microscopy (SEM) analysis. Results show that the presence of $ALA_{11,17}$ in DSPC liposomes significantly alters the morphology, colloidal stability, and retention of encapsulated materials in both acidic and neutral conditions. The ability of $ALA_{11,17}$-hybrid liposomes to encapsulate and retain inclusions under neutral and acidic conditions (pH < 2) was demonstrated by calcein dequenching experiments. DLS and SEM confirmed that $ALA_{11,17}$/DSPC liposomes remained intact under these conditions. The bilayer integrity observed under neutral and acidic conditions and the likely biocompatibility of these fatty amino acid analogues suggest that $ALA_{11,17}$ is a promising additive for modulating phosphatidylcholine lipid bilayer properties.

Keywords

Tetrahydropyrimidinone; lipid; DSPC liposomes; nanostructures; multilamellar vesicles; scanning electron microscopy

Introduction

Liposomes are spherical lipid bilayer vesicles that are under investigation as nanoscale capsules for transporting therapeutic[1,2] and non-therapeutic materials.[3–8] In medicinal applications, liposome encapsulation affords the potential for reduced toxicity,[9] improved

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Supporting Information Available. ^{1}H, ^{13}C, and 2D-COSY NMR spectra of $ALA_{11,17}$ lipid, zeta potential and size distribution analysis. These materials are available free of charge via the internet at http://pubs.acs.org.

bioavailability,[10,11] and tissue-targeted delivery.[12–17] An important challenge that remains to be addressed is the development of liposomes that are compatible with oral administration.[18,19] Such liposomes must survive harsh gastrointestinal environments including severe stomach acidity (pH 1–2) before reaching the small intestines where ingested substances are absorbed.[19b–c,20,21] Although liposomes have been fortified for enhanced stability with cholesterol,[22,23] triterpenoids,[24] polyelectrolyte coatings,[25,26] and lipid cross-linking, [19a, 27–29] the development orally-competent liposomes, which would be an important therapeutic platform, requires further advancement.[18]

Amino acid-derived amphiphiles have been prepared for diverse applications including liposome targeting and adjuvants for liposomal transfection.[30–32] Recently, Sorrells and Menger[33] reported that a serine-derived diketopiperazine lipid influenced the formation and stabilization of micellar aggregates. Asparagine-derived lipids have been reported[34] in liposomes for nucleic acid delivery with effective controlled release properties,[34a] for the preparation of long-circulating liposomes with drug-targeting capacities,[34b] and to trigger vesicle fusion.[34c] Our ongoing investigations on asparagine-derived heterocycles in asymmetric synthesis,[35] and those of others in peptide structure and drug discovery[36] inspired the design of the lipid material discussed here. In the aforementioned studies, the cyclocondensation of L-asparagine with an aldehyde or ketone followed by amine acylation generates stereochemically homogeneous, *cis*-1,3-disubstituted tetrahydropyrimidinonecarboxylates (Scheme 1). We reasoned that the employment of a fatty aldehyde (e.g., *n*-$C_{11}H_{23}$-CHO; Scheme 1) and fatty acyl chloride (e.g., *n*-$C_{17}H_{35}$-COCl; Scheme 1) in this synthesis would generate a novel asparagine-derived lipid ($ALA_{11,17}$)[35a,37] that bears an unprecedented pyrimidinone carboxylate headgroup, which can be employed to impart unique liposomal properties through the ligation of chemical and biological entities to the liposome surface. Herein, we disclose the preparation of a novel asparagine-derived tetrahydropyrimidinone lipid ($ALA_{11,17}$) and its use in liposome formation and stabilization in neutral and acidic conditions. DSPC, which forms uncharged bilayer vesicles that readily flocculate and are susceptible to facile acid degradation, was employed in these studies to facilitate examination of colloidal and bilayer stabilities associated with the incorporation of novel lipid in various proportions.

Experimental Section

Generals

The melting point was measured using a Mel-Temp® melting point apparatus and is uncorrected. The ^1H and ^{13}C NMR spectra were obtained using a Varian Inova™ NMR spectrometer operating at 500 and 125 MHz, respectively. All NMR spectra were recorded in $CDCl_3$ unless otherwise indicated using tetramethylsilane (TMS) at δ 0.00 for ^1H and residual $CDCl_3$ at δ 77.00 for ^{13}C internal standards. Specific rotation ($[\alpha]^{589}$; 9.0 mM solution in methanol) was determined at room temperature (22 °C) using an AUTOPOL® IV (Rudolph Research) automatic polarimeter at 589 nm. Particle size measurements were performed on a Delsa™ Nano C. zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter Inc., Fullerton, CA, USA). All liposome formulations were prepared using a Lipex™ stainless steel extruder (Northern Lipids Inc., Burnaby, Canada). Scanning electron microscopy (SEM) studies were performed on Hitachi S5500 cold field emission scanning electron microscope operating at 1–30 kV with 1.6–0.4 nm resolution. Optical microscopy was performed using Axio Scope 40 POL polarizing microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Spectrofluorimetry was performed using a Photon Technology International fluorometer (PTI Inc., London, Ontario, Canada).

Material

Distearoylphosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids, Alabaster, AL. All other chemicals (dodecanal, octadecanoyl chloride, L-asparagine, etc.) were obtained from either Sigma-Aldrich or Acros Organics. Dubecos phosphate buffer saline (PBS; pH 7.4), polycarbonate filters for extrusion, and Electron Microscopy Diatome copper grids with formvar/carbon Film (400 mesh) for electron microscopy were purchased from Fischer Scientific. All chemicals were reagent grade and were used as received.

Synthesis of (2*S*,4*S*)-6-oxo-3-stearoyl-2-undecylhexahydropyrimidine-4-carboxylic acid (*syn* and *anti conformers of* $ALA_{11,17}$)

Sodium hydroxide (1.0 mmol) and L-Asparagine (1.0 mmol) were sequentially added to methanol (10 mL) and the mixture was stirred for 15 min after each addition. To this clear solution, dodecylaldehyde (1.2 mmol in 10 mL of methanol) was added and the mixture stirred overnight at room temperature. The methanol was evaporated, and the residue was washed with hexane (3 × 25 mL) and dried to a white powder. The resulting white powder was suspended in THF (20 mL) and 2,6-lutidine (1.1 mmol), cooled to 0 °C, and stearoyl chloride (1.1 mmol in 10 mL of THF) was added slowly over 15 min (each of the above steps were performed in a loosely capped vessel to reduce pressurization and atmospheric exposure). After stirring overnight, the reaction mixture was poured into 10% HCl (50 mL), extracted with dichloromethane, and the combined organic extract was concentrated to a waxy solid. The solid was triturated with (ethyl acetate/hexanes) to yield a white amorphous powder. Yield 81%; mp: 105–109 °C; $[\alpha]^{589}$: −37.8 (5.08 g/L, MeOH); ^{1}H NMR (500 MHz): δ 0.88 (t, *J* = 6.8 Hz, 6H, 2*CH*$_3$), 1.22–1.39 (m, 46H, 23*CH*$_2$), 1.63 (tt, *J* = 6.8, 7.8 Hz, 2H, 2*H-3*), 1.62–1.71 (m, 0.67H, 2*anti-H-1*''), 1.78–1.89 (m, 1.33H, 2*syn-H-1*''), 2.34 (dd, *J* = 6.3, 7.8 Hz, 0.67H, 2*anti-H-2*), 2.35 (dt, *J* = 7.8, 15.6 Hz, 0.67H, *syn-H-2*), 2.44 (ddd, *J* = 6.4, 7.3, 14.6 Hz, 0.67H, *syn-H-2*), 2.77 (dd, *J* = 7.3, 16.6 Hz, 0.33H, *anti-H-5*), 2.81 (dd, *J* = 8.3, 17.1 Hz, 0.67H, *syn-H-5*), 2.98 (dd, *J* = 5.9, 16.6 Hz, 0.33H, *anti-H-5*), 3.07 (dd, *J* = 9.8, 17.1 Hz, 0.67H, *syn-H-5*), 4.73 (t, *J* = 6.4 Hz, 0.33H, *anti-H-4*), 4.97 (t, *J* = 8.8 Hz, 0.67H, *syn-H-4*), 5.09 (dt, *J* = 5.9, 7.8 Hz, 0.67H, *syn-H-2*), 5.74–5.80 (m, 0.33H, *anti-H-2*), 7.37 (br d, 0.33H, *anti-H-1*), 7.90 (br d, *J* = 4.4 Hz, 0.67H, *syn-H-1*); ^{13}C NMR (125 MHz): δ 14.3 (q), 22.9 (t), 25.1 (t, *syn*), 26.0 (t, *anti*), 29.27 (t), 29.34 (t), 29.37 (t), 29.54 (t), 29.57 (t), 29.60 (t), 29.69 (t), 29.73 (t), 29.79 (t), 29.83 (t), 29.90 (t), 29.92 (t), 29.93 (t), 29.96 (t), 31.2 (t), 31.9 (t), 32.2 (t), 33.4 (t, *syn*), 33.8 (t, *anti*), 35.9 (t, *anti*), 37.4 (t, *syn*), 51.4 (d, *syn*), 52.4 (d, *anti*), 63.2 (d, *anti*), 66.0 (d, *syn*), 170.0 (s, *anti*), 170.8 (s, *syn*), 172.6 (s, *anti*), 172.7 (s, *syn*), 174.2 (s, *syn*), 174.9 (s, *anti*); ATR-FTIR (ν_{max}, cm^{-1}): 3218, 2918, 2849, 1733, 1631, 1469, 1399, 1211; MS (ES, m/z): 588.6 (100, $MH+Na^+$), 587.7 (85, $M+Na^+$), 382.2 (20, MH^+-$C_{11}H_{22}CHO$); Exact mass analysis: calcd for $C_{34}H_{64}N_2O_4$ (MH^+) 565.4944, found 565.4927.

General procedure for preparation of liposomes

A mixture of DSPC and $ALA_{11,17}$ (0, 5, 10, 25, or 50 mol% of $ALA_{11,17}$; total moles of lipids: 1.26×10^{-4}) in a round bottom flask was dissolved in chloroform (5–10 mL). The solvent was evaporated under reduced pressure to obtain a thin film and was further stripped of solvent under house vacuum for 45–60 min before storage (4 °C). The thin film was hydrated with 4.0 mL of PBS (10 mM phosphate buffer and 150 mM NaCl, pH 7.4). The resulting emulsion was vortexed and incubated at 55–60 °C alternately until a cloudy suspension of multilamellar vesicles (MLVs) was formed. The MLVs were subjected to sequential extrusion with moderate pressure (200–700 psi) through polycarbonate filters of descending pore sizes (3× through each filter; pore sizes: 2.0, 1.0, 0.40, 0.20, and 0.10 μm) mounted in a stainless steel extruder connected to circulating warm water (55–60 °C) to obtain unilamellar liposomes with a mean diameter of 134 nm after the third extrusion through 0.10 μm filter (by DLS analysis). Repeated extrusions (11×) through the 0.10 μm

filter produced smaller liposomes (95 nm). For convenience, all liposomes were prepared by the triple extrusion protocol and used within 24 hours for all experiments unless otherwise noted.

Particle size analysis by dynamic light scattering (DLS)

Average particle size distribution and polydispersity index (PDI) were determined by photon correlation spectroscopy (PCS) using a Zeta Potential and Submicron Particle Size Analyzer. The liposomes dispersed in PBS 1.8 mL (200 μL, 33 mM; pH 7.4) were loaded to Beckman Coulter flow cell holder and counting was performed (90 accumulation times) at 25 °C. Each experiment was performed in duplicate and the measuring SOPs (standard operating procedures) were selected considering water as the medium for all experiments.

Zeta potential analysis

Particle zeta potentials were determined by measuring the electrophoretic mobility of the particles under an applied electric field using a Zeta Potential and Submicron Particle Size Analyzer. The instrument uses a zeta potential module equipped with dual-laser diodes operating at 35 mW (658 nm). Scattered light was detected at 90° (25 °C). The same samples from DLS experiments were used for zeta potential measurements. Ten data points were recorded for each electrophoretic velocity and each experiment was performed in duplicate. Zeta potential values were automatically calculated from measured velocities using the Helmholtz-Smoluchowski relationship.[38]

Sample preparation for scanning electron microscopy (SEM) studies

Liposome suspensions (50 μL)) obtained by the above protocols were diluted with an equal volume of 0.1 M non-saline phosphate buffer. The diluted sample was treated with an 11% (w/w; pH 7.2) solution of ammonium molybdate ($(NH_4)_6Mo_7O_{24}$; 30 μL) and allowed to stand (in the open air) at room temperature for at least 5 h. Samples were prepared by placing drops of this suspension on 300 mesh copper grids coated with either lacey carbon or Formvar and the excess liquid was carefully removed using a pointed filter paper. The copper grids were placed on a filter paper in a Petri dish and dried in air for 3 h before SEM analysis. To collect images under acidic conditions, each liposome sample was acidified to the desired pH (1.9), allowed to stand 45–60 min, and stained with $(NH_4)_6Mo_7O_{24}$. A drop of the stained solution was added to the grids, the excess liquid was removed with a pointed filter paper and images were collected immediately.

Turbidity measurements

Each of 0, 5, 10, 15, 25, and 50 mol% $ALA_{11,17}$/DSPC liposomes in PBS was treated with aliquots of 1% HCl (v/v), and the pH and optical density at 400 nm were recorded immediately after each addition. The data were normalized to the original optical density at pH 7.4 and plotted against pH.

Calcein leakage experiment

Calcein leakage studies were performed by spectrofluorometry using a Photon Technology International fluorometer. Calcein encapsulated liposomes were prepared following the same extrusion protocol described above. Calcein was encapsulated at self-quenching concentration (100 mM calcein in PBS; pH 7.4) at thin-layer hydration stage and calcein-encapsulated MLVs were exruded through polycarbonate filters of descending pore sizes (*vide supra*). The remaining extra-liposomal dye was freed from the bulk solution by size-exclusion filtration through Sephadex G50.[39] Dye leakage was determined by comparing the ratio of fluorescence at selected time points to the maxium sample fluorescence, which was determined after addition with one drop of 0.1% Triton X-100.

Results and Discussion

Lipid synthesis

The cyclocondensation of sodium L-asparaginate with dodecanal was examined in THF, 50/50 THF/water, DMF, and methanol. Methanol was the only solvent system that fully dissolved the amino acid salt and promoted complete heterocyclization over 4 h. After removal of solvent, the residue was acylated (stearoyl chloride, 2,6-lutidine, THF) at ice bath temperature. The waxy solid obtained after extractive work-up was triturated in ethylacetate/hexane (50/50) to afford a white powdery product (81% yield). The ^{1}H NMR and ^{13}C NMR ($CDCl_3$) indicated the presence of *N-3* amide anti and syn conformers (Scheme 2; note: for the following discussion the syn conformer *N3*-acyl oxygen is directed towards the carboxylate). The methine protons at C4 and C2 were assigned based on *^{1}H-^{1}H* COSY, which exhibited a diagnostic coupling interaction between *C4-H* with the diastereotopic *C5-H* methylene protons at 2.9 ppm. NOE experiments failed to identify the major conformer, however, the greater proportion of the minor $ALA_{11,17}$ conformer in more polar solvents is consistent with increasing intramolecular alkyl chain associations in the syn conformer. This reasoning also applies to the conformational difference observed for the sodium salt of $ALA_{11,17}$ in CD_3OD and DMSO-*d_6*. These studies demonstrate that the conformational equilibrium of $ALA_{11,17}$ is strongly solvent and ionization dependent. The syn conformer dominates for the sodium salt of $ALA_{11,17}$ in CD_3OD and DMSO-*d_6* (K_{eq} = 1.86 and 49, respectively; Scheme 2). Lipids with spectroscopically observable syn or anti conformations such as $ALA_{11,17}$ have not been reported, however the former is presumably desirable in the present application since it enables better packing of fatty chains in hydrophobic portion of the bilayer. The ^{1}H NMR signals of the sodium salt of $ALA_{11,17}$ in *D_2O and $CDCl_3$* were severely broadened under these conditions, presumably due to the formation of self-assembled structures.

Multilamellar vesicle (MLV) preparation

MLV suspensions of 0, 5, 10, 25, and 50 mol% $ALA_{11,17}$/DSPC in phosphate buffer saline (PBS, pH 7.4) were analyzed by optical microscopy (Figure 1). The optical micrographs of DSPC MLVs showed the presence of highly clustered vesicles (Figure 1a). In contrast, those prepared containing 5, 10, 25, and 50 mol% $ALA_{11,17}$/DSPC appeared as isolated vesicles (for example, see Figures 1b–e). The observation of isolated vesicles for ALA preparations is consistent with $ALA_{11,17}$ incorporation, since the lipid carboxylate endows MLV surfaces with repulsive anionic charges.

The observation that MLV preparations of $ALA_{11,17}$/DSPC appeared to form well defined morphologies compared to those generated from DSPC alone prompted an investigation of the MLV structures using scanning electron microscopy (SEM). Hence, both DSPC and 10 mol% $ALA_{11,17}$/DSPC MLVs were freshly prepared, negatively stained ($(NH_4)_6Mo_7O_{24}$), and visualized by SEM (Figure 2). The SEM image of DSPC MLVs exhibited diverse sizes of aggregated structures (Figure 2a), analogous to the MLV optical micrographs (Figure 1a). The SEM image of the sample containing 10 mol% $ALA_{11,17}$/DSPC (Figure 2b) exhibited a roughly bimodal size distribution of vesicles with sizes in the ranges of 100–200 and 400–500 nm, respectively. The electron micrograph of unextruded self-assemblies of 100 mol% $ALA_{11,17}$ as control, exhibited spherical structures but with less morphological uniformity (Figure 2c; the vesicles appear to be adhered to the grid in this image). The heterocyclic head group likely endows $ALA_{11,17}$ with conicity that templets bilayer curvature, which influences the observed size distribution and morphology of the vesicles.[40,41] However, this issue was not further examined in these studies.

Preparation and characterization of DSPC and $ALA_{11,17}$/DSPC liposomes

The MLV suspensions comprised of 0, 5, 10, 15, 25, 50 mol% $ALA_{11,17}$/DSPC and 100 mol % $ALA_{11,17}$ were sequentially extruded to generate liposome suspensions of each composition. DSPC liposomes at pH 7.4 displayed a wide size distribution according to dynamic light scattering (DLS) experiments (average particle size (APS): 850 nm; polydispersity index (PDI) > 1; figures 3a and 3b). This is consistent with the known colloidal instability of DSPC liposomes and suggests substantial vesicle aggregation. At 5 mol% $ALA_{11,17}$, the APS and PDI of the constituent liposomes decreased dramatically (APS: 350 versus 850 nm; PDI: 1.0 versus ~5.9), indicating reduced aggregation. The minimum values for these indices were recorded for the 10 mol% $ALA_{11,17}$/DSPC sample (Figure 3). There were no significant differences in APS and PDI for 10, 15, 25, and 50 mol % $ALA_{11,17}$/DSPC liposomes. Liposomes formulated with $ALA_{11,17}$ only, on the other hand, exhibited increased APS (300 nm) but similar PDI (0.4) values compared to 10 mol% $ALA_{11,17}$/DSPC. Notably, 10 mol% $ALA_{11,17}$/DSPC liposome sample maintained colloidal homogeneity, vesicle size distribution, and PDI for greater than one month at 23 °C and greater than 1 year at 4 °C. Thus, 10 mol% $ALA_{11,17}$/DSPC is sufficient to impart a maximum impact on APS and PDI, and particles with this formulation exhibited excellent shelf life upon extended storage.

Scanning electron micrographs were collected for DSPC and 10 mol% $ALA_{11,17}$/DSPC liposomal formulations prepared identically to those employed in DLS experiments (Figure 4). The electron micrographs showed size distributions similar to those obtained by DLS. The SEM image of DSPC liposomes at pH 7.4 showed clustered vesicles with wide polydispersity (Figure 3a), consistent with aggregation of phosphatidylcholine nanostructures bearing neutral surface charge.[42,43] In contrast, the SEM images of liposomes formulated with 10 mol% $ALA_{11,17}$/DSPC at pH 7.4 appeared predominantly as isolated spheres with narrow size distribution, an interpretation that is consistent with DLS analysis (Figures 4b–c). Though some vesicle association is apparent in SEM, it is likely that vesicle proximities are an artifact of suspension concentration upon drying that preceded SEM data collection. Thus, self-assembled vesicles constituted with $ALA_{11,17}$ resist aggregation or fusion resulting in stable colloid suspensions.

Zeta potential (ζ) values of $ALA_{11,17}$/DSPC liposome formulations were determined in PBS (pH 7.4) at constant ionic strength (150 mM NaCl). The zeta potential decreased linearly for liposomes with increasing $ALA_{11,17}$ proportion (Figure 5), indicative of the presence and miscibility of negatively charged $ALA_{11,17}$ in DSPC liposome bilayer. The negative surface imparted by $ALA_{11,17}$ is expected based on the rationale posited for the observed colloidal stability of these liposomes at pH 7.4.

Liposome pH dependent colloidal stability by turbidity experiments

Optical density (OD) measurements at 400 nm were employed to probe the colloidal integrity of DSPC and $ALA_{11,17}$/DSPC liposome formulations as a function of pH. The pH profiles of all liposome formulations show a steady, normalized OD at higher pH values (pH > 4.5; Figure 6a). At pH below 4.5, the pH profile of DSPC liposomes indicated a decrease in OD consistent with the low colloidal stability of phosphatidylcholine-based liposomes.[42–46] The 5 mol% $ALA_{11,17}$/DSPC liposomes exhibited steady OD to pH 3.5, after which it decreased, though less than that of the DSPC sample. In contrast, 10 mol% $ALA_{11,17}$/DSPC liposome sample maintained a steady OD over the entire pH range (7.4–1.9). The constant turbidity (constant normalized OD) throughout the titration exhibited by this sample suggests liposome persistence in this pH range. The scattering profiles for 15, 25, and 50 mol% $ALA_{11,17}$/DSPC liposomes versus pH, on the other hand, showed increasing turbidity below pH 4.0 with absorbance maxima at pH 3.8, 3.3, and 2.5 (pH_{max}),

respectively. At pH < pH_{max} (isoelectric point) the optical densities of each preparation decreased steadily, returning to the original turbidity near pH 2. These observations suggest that liposomes with $ALA_{11,17}$ proportions greater than 10 mol% undergo composition-dependent aggregation at pH < 4 as the vesicle surfaces are neutralized. Upon further acidification these samples become disaggregated as a consequence of positive charge accumulation at the liposome surfaces. Thus, DSPC liposomes constituted with 10 mol% $ALA_{11,17}$ are stabilized in acidic conditions and those with higher proportions of $ALA_{11,17}$ apparently impart reversible, pH-dependent aggregation behavior. These observations also suggest that the pI (isoelectric point) of the liposome formulations is strongly dependent on the composition of $ALA_{11,17}$ in the bilayer, consistent with prior reports that the pK_a of liposomal fatty acids are influenced by lipid composition (Figure 6b).[47] It was unclear from these data if the apparent colloidal stabilities correlated with lipid bilayer integrity for vesicles with $ALA_{11,17}$. Therefore, the influence of $ALA_{11,17}$ on the permeability of DSPC bilayers was examined by standard fluorescent-dequenching experiments.

Dye encapsulation and leakage experiment

The vesicle bilayer stabilities of $ALA_{11,17}$/DSPC liposome systems was interrogated by measuring the retention of encapsulated calcein in 0, 5, 10, and 25 mol% $ALA_{11,17}$/DSPC liposomes. In these experiments appropriate $ALA_{11,17}$/DSPC thin films were hydrated with collisionally self-quenched solutions of calcein dye (100 mM). After sequential extrusion through polycarbonate filters (2.0–0.10 μm) as previously described, the unencapsulated dye was removed by Sephadex G-50 gel-filtration. The calcein-encapsulated liposomes were diluted 10,000 fold and the dye release was monitored using the calcein fluorescence dequenching experiments.[39] A comparision of DSPC and $ALA_{11,17}$/DSPC liposomes showed that the presence of $ALA_{11,17}$ in DSPC bilayer significantly reduces the permeability of the constituent liposomes at pH 7.4 and at pH 1.9 as indicated by their relative calcein leakage rates (Figures 7a and 7b, respectively). No significant leakage was recorded among the samples containing various proportions of $ALA_{11,17}$ for 250 min time period at pH 7.4 (Figure 7a). In contrast, DSPC liposomes showed approximately 50% calcein dequenched at pH 7.4 within the same time interval, indicative of greater release rates.[48] In acidic conditions (pH 1.9) $ALA_{11,17}$/DSPC liposome formulations showed near identical results compared to those at pH 7.4, while DSPC liposomes at the lower pH leaked at a faster rate (~65% dequenched within 50 min; Figure 7b). At lower pH, additives such as cholesterol have been reported to impart liposome stability through the formation of localized rafts,[22,23] while fatty acids are known to be protonated and hence precipitated from the bilayer, thus decreasing bilayer stability.[46] In contrast, $ALA_{11,17}$ remains in the bilayer as suggested by the leakage rates, optical microscopy, and turbidity experiments. In addition, the imbedded $ALA_{11,17}$ maintains liposome bilayer integrity at low pH, suggesting that protonated $ALA_{11,17}$ also contributes to bilayer stability.

Additional studies of liposome acid stability

To further examine the effect of acidity on the integrity of these liposomes, DSPC and 10 mol% $ALA_{11,17}$/DSPC liposome samples were freshly prepared and portions of each were exposed to strongly acidic conditions (pH 1.9) for a period of 1.0 hour. Particle size analysis of all four suspensions by DLS (Figure 8) demonstrate that the integrity of 10 mol% $ALA_{11,17}$/DSPC liposomes were unperturbed before and after acid exposure (Figures 8b and 8d).

The morphologies of acidified samples of representative liposome formulations (DSPC and 10 mol% $ALA_{11,17}$/DSPC) were also examined by SEM to investigate the behavior inferred from turbidity experiments (Figure 6) and DLS analysis (Figure 8). Thus, DSPC and 10 mol % $ALA_{11,17}$/DSPC liposomes were freshly prepared at pH 7.4, acidified to pH 1.9, left to

stand at ambient conditions for one hour, and negatively stained (ammonium molybdate) before subjected to SEM analysis (Figure 9). The SEM image of DSPC liposomes at pH 1.9 revealed an array of highly polydispersed spherical and non-spherical lipid structures (Figure 9a) compared to clustered but unfused liposomes observed at pH 7.4 (Figure 4a), suggesting spontaneous vesicle degradation and reformulation under acidic conditions.[44] The SEM image of 10 mol% $ALA_{11,17}$/DSPC liposomes displayed intact structures at pH 1.9 (Figure 9b) that were similar in size distribution to those observed at pH 7.4 (Figures 8b). These studies support the interpretation of the results of the turbidity and DLS experiments, which showed that $ALA_{11,17}$/DSPC liposomes (≥ 10 mol%) remain stable at pH < 2.

The mechanism by which PC lipid bilayers are stabilized by $ALA_{11,17}$ is still to be established. During preparation, $ALA_{11,17}$ precipitates upon acidification suggesting strong propensity to aggregate in aqueous media. $ALA_{11,17}$ in DSPC liposomes apparently remain in the bilayer but may likewise become self-associated and accumulate within the DSPC bilayer, forming domains rich in $ALA_{11,17}$ that influence the permeability and colloidal stability of the nanoparticles.

Conclusions

We have synthesized a novel asparagine-derived lipid in high yield and purity. The novel lipid formed multilamellar structures with and without DSPC. The physical structures of the MLVs composed of 10 mol% $ALA_{11,17}$/DSPC appeared as well-dispersed spheres compared to DSPC MLVs. Self-assembled structures of the various formulations exhibited significant colloidal stability compared to DSPC liposomes as determined by optical microscopy, turbidity analysis, and particle size measurements. Calcein leakage studies demonstate that these liposomes formulated with the novel lipid retained chemical entities more effectively than DSPC vesicles under neutral and acidic conditions, suggesting enhanced bilayer integrity. Physical studies also demonstated that the integrity of the liposomes composed of $ALA_{11,17}$ and DSPC remained unperturbed after exposure to acidic conditions, while identically treated DSPC liposomes were dramatically altered. Thus, this additive has a robust, stabilizing effect on lipid DSPC-based nanoparticles and may find utility in applications where stable liposomes are required at neutral or acidic conditions. In addition, liposomes with greater than 10 mol% $ALA_{11,17}$ imparted a pH-sensitive aggregation behavior. Because $ALA_{11,17}$ is assembled from biogenic L-Asn it is likely to be biocompatible compared to fully synthetic lipids, and thus may be of broad interest in amphiphile applications. Ongoing studies are directed at examining the impact of $ALA_{11,17}$ structure on liposome morphology and stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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37. Abbreviations: ALA, asparagine-based lipid analogue; L-Asn, L-asparagine; DSPC, distearoylphosphatidylcholine; PBS, phosphate buffer saline; MLV, multilamellar vesicle; DLS, dynamic light scattering; SEM, scanning electron microscopy; OD, optical density; CID, chemical ionization detector; ATR, attenuated total internal reflection; DMF, dimethyl formmamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; MeOH, methanol; TEA, triethylamine; TMS, tetramethylsilane; Lutidine, 2,6-dimethylpyridine.
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43. The DSPC liposomes precipitated over a 2-hour period (pH 7.4) at ambient conditions and did not regain homogeneity even after vigorous agitation. This clustering of the DSPC liposomes is known to correlate with the neutral surface charge of phosphatidylcholine assemblies (see reference 42, p 253).
44. Among the four-ester bonds possessed by the phosphatidylcholine framework, the two acyl ester bonds are most labile to acid hydrolysis. At pH 4.5 the observed rate of hydrolysis (k_{obs}) is about 1×10^{7} s^{-1} at 40 °C: Reference 2b, p 110–111 and Zuidam, N. J.; Crommelin, D. J. J. Pharm. Sci. 1995, 84, 1113–1119.
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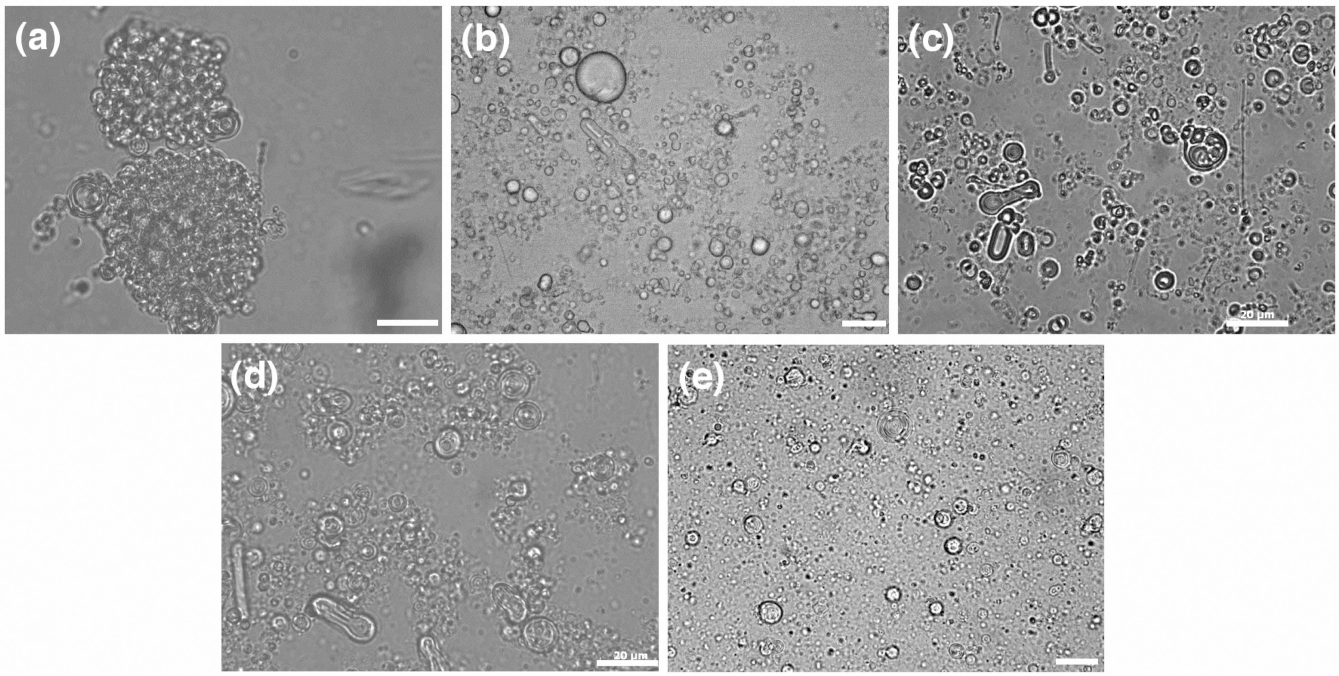


Figure 1.

The optical micrographs of $ALA_{11,17}$/DSPC MLVs (pH 7.4 PBS buffer) in several formulations: (a) DSPC; (b) 5 mol% $ALA_{11,17}$/DSPC; (c) 10 mol% $ALA_{11,17}$/DSPC; (d) 25 mol% $ALA_{11,17}$/DSPC; and (e) 50 mol% $ALA_{11,17}$/DSPC (Scale bar = 20 μm).

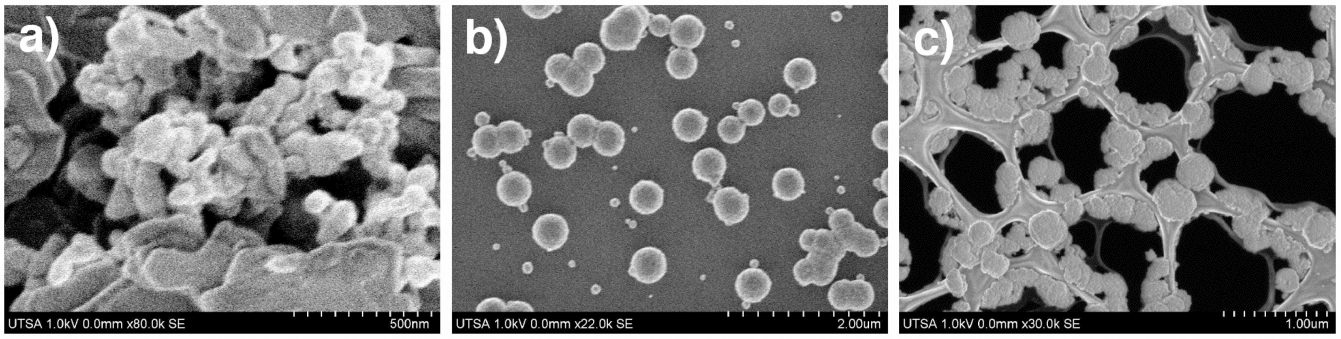


Figure 2.

Scanning electron micrographs of vortexed lipid self-assemblies after negative staining with ammonium molybdate at pH 7.4 (PBS buffer): (a) DSPC MLVs; (b) 10 mol% $ALA_{11,17}$/DSPC MLVs; and (c) $ALA_{11,17}$ MLVs.

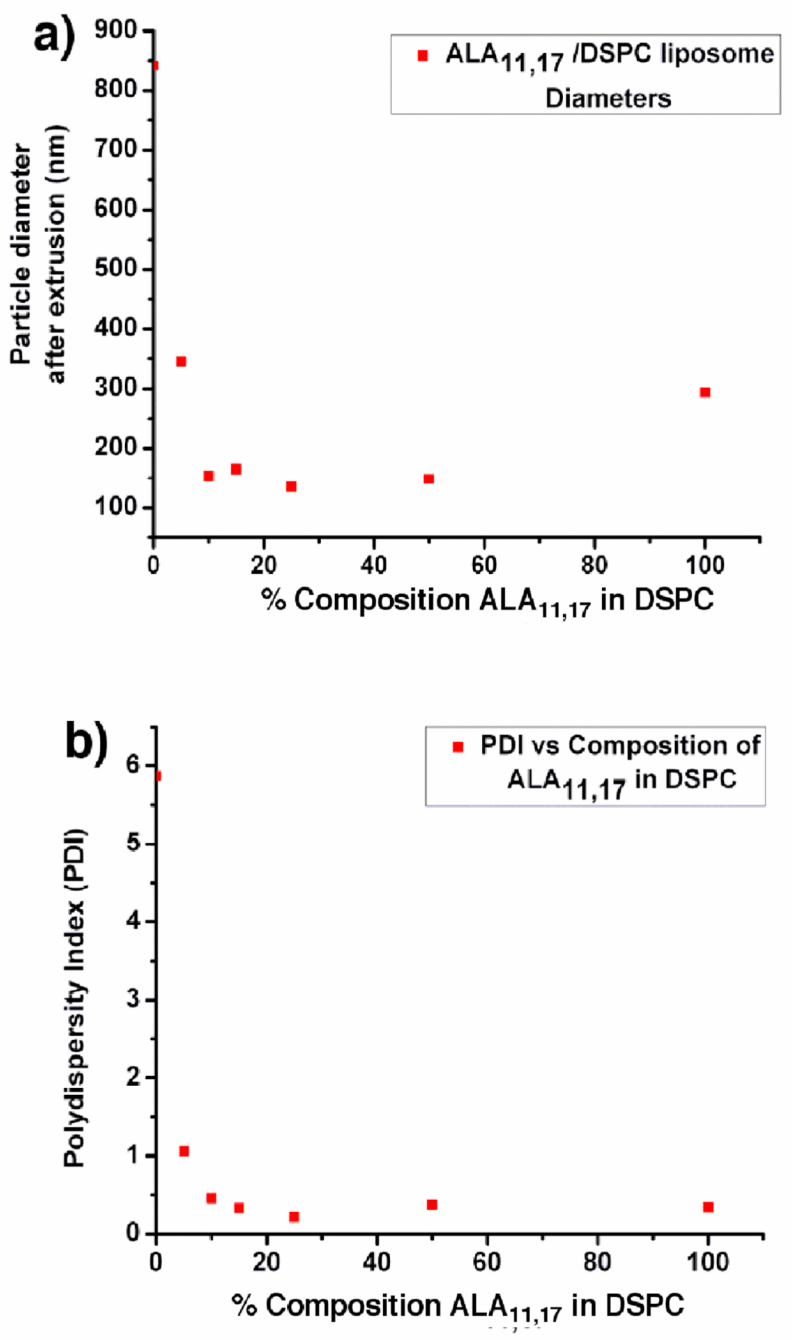


Figure 3.
Particle size analysis by DLS of extruded liposomes in PBS at pH 7.4: (a) Average particle size of $ALA_{11,17}$/DSPC liposomes versus lipid composition; (b) The corresponding polydispersity index (PDI) of $ALA_{11,17}$/DSPC liposomes versus lipid composition.

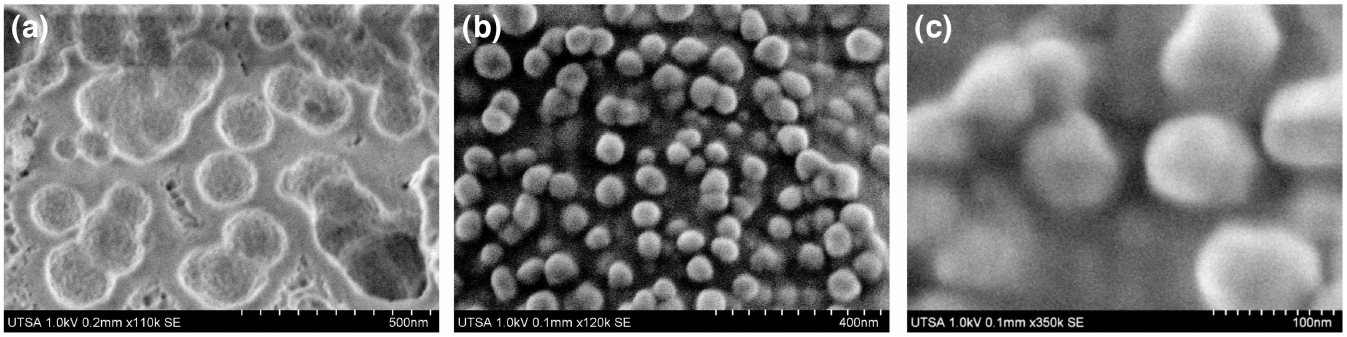


Figure 4.

Scanning electron micrographs of negatively stained liposome samples prepared by extrusion (3×) at pH 7.4 (PBS buffer): (a) DSPC; (b) 10 mol% $ALA_{11,17}$/DSPC; (c) 10 mol % $ALA_{11,17}$/DSPC at higher magnification.

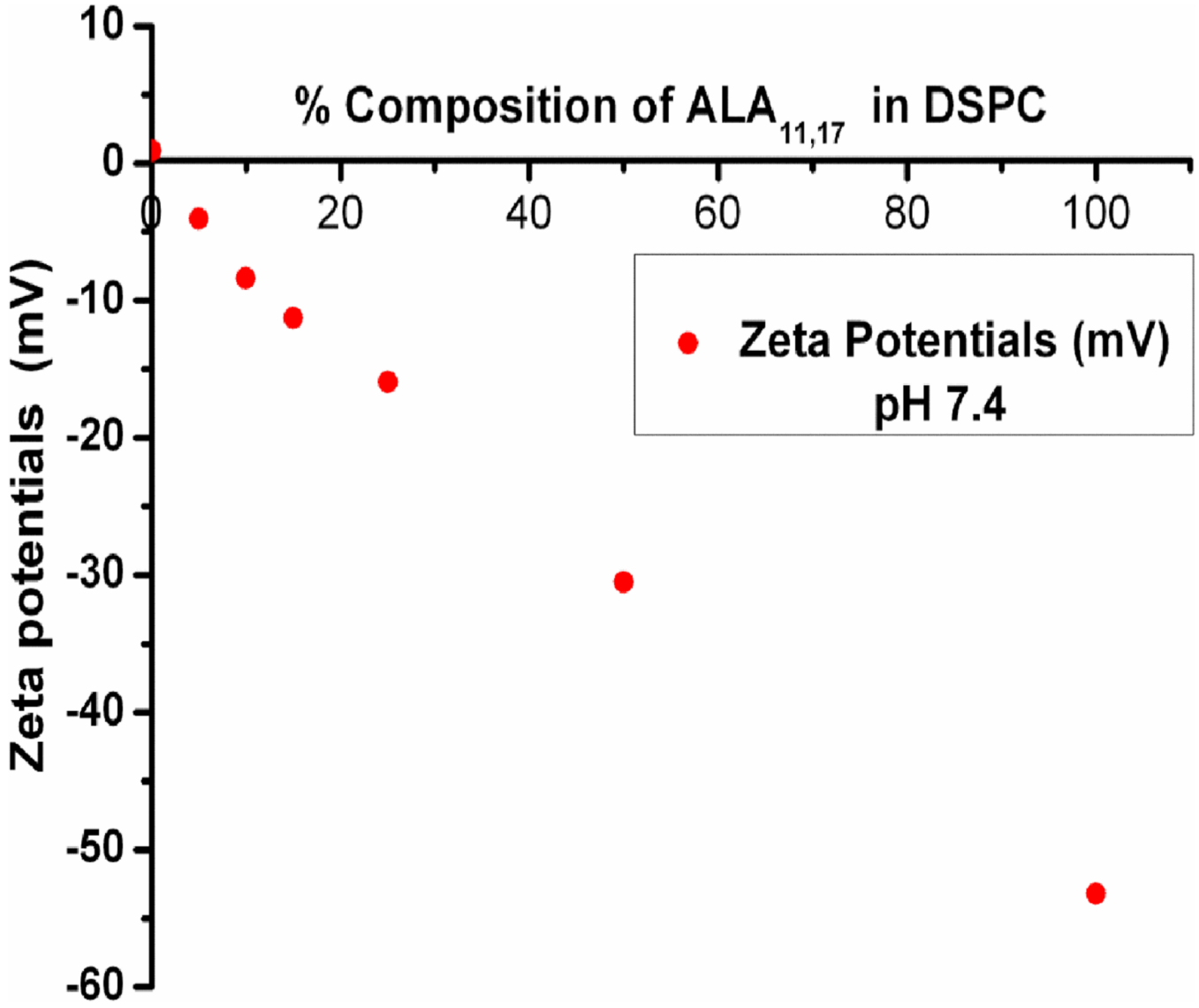


Figure 5.
Zeta potential (ζ) analysis $ALA_{11,17}$/DSPC liposomes in PBS (pH 7.4).

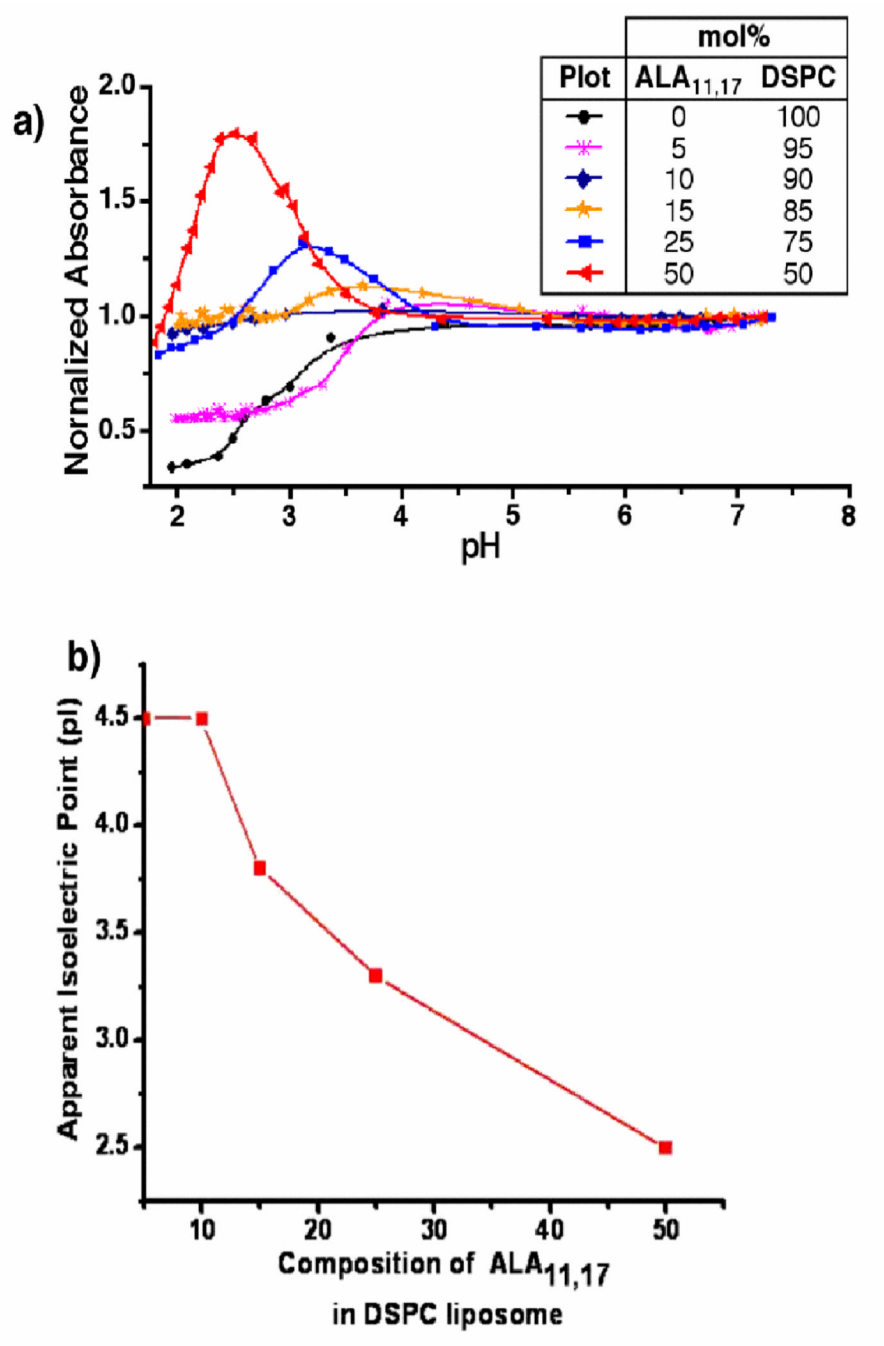


Figure 6.

(a) Normalized absorbances of liposomal solutions of 0, 5, 10, 15, 25, and 50 mol% $ALA_{11,17}$/DSPC in PBS at 400 nm in various pH. The pH was changed by the addition of appropriate aliquots of 1% HCl (v/v). (b) Correlation between membrance aggregation upon surface neutralization and apparent isoeletric points (pI).

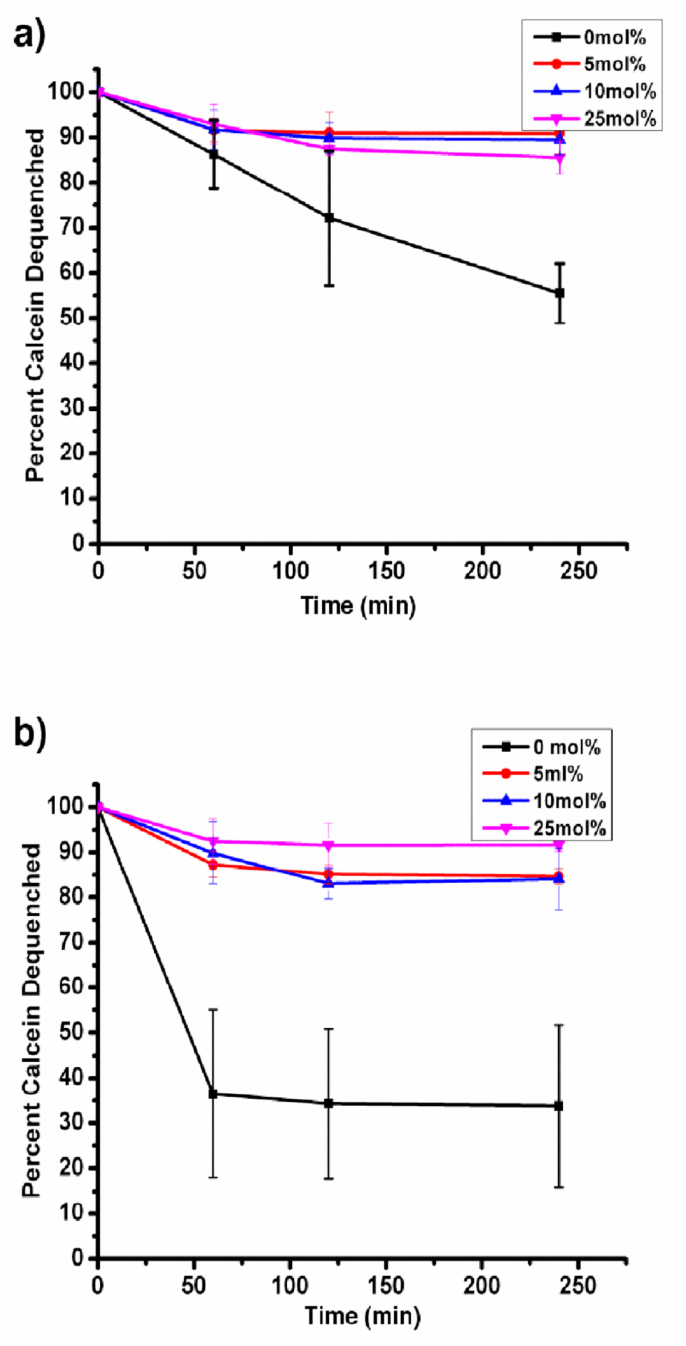


Figure 7.
Time dependent calcein leakage experiment of $ALA_{11,17}$/DSPC Liposomes in PBS determined by calcein fluorescence dequenching experiments: (a) at pH 7.4; (b) at pH 1.9

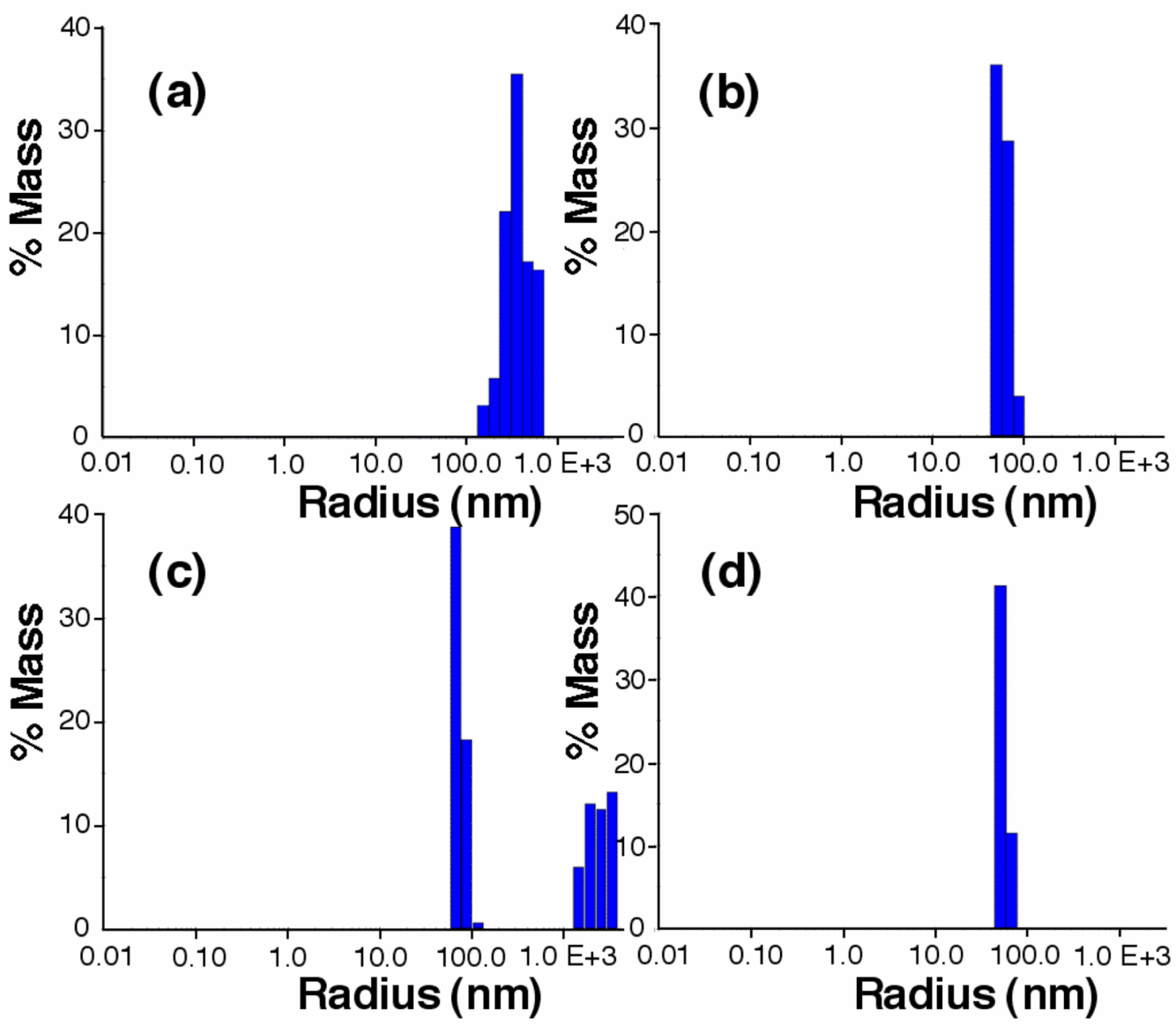


Figure 8.

Dynamic Light Scattering Analysis showing the radii and size distribution of liposome suspension at different pH: (a) DSPC at pH 7.4 (PBS); (b) 10 mol% $ALA_{11,17}$/DSPC at pH 7.4 (PBS); (c) DSPC at pH 1.9 (PBS/HCl); and (d) 10 mol% $ALA_{11,17}$/DSPC at pH 1.9 (PBS/HCl).

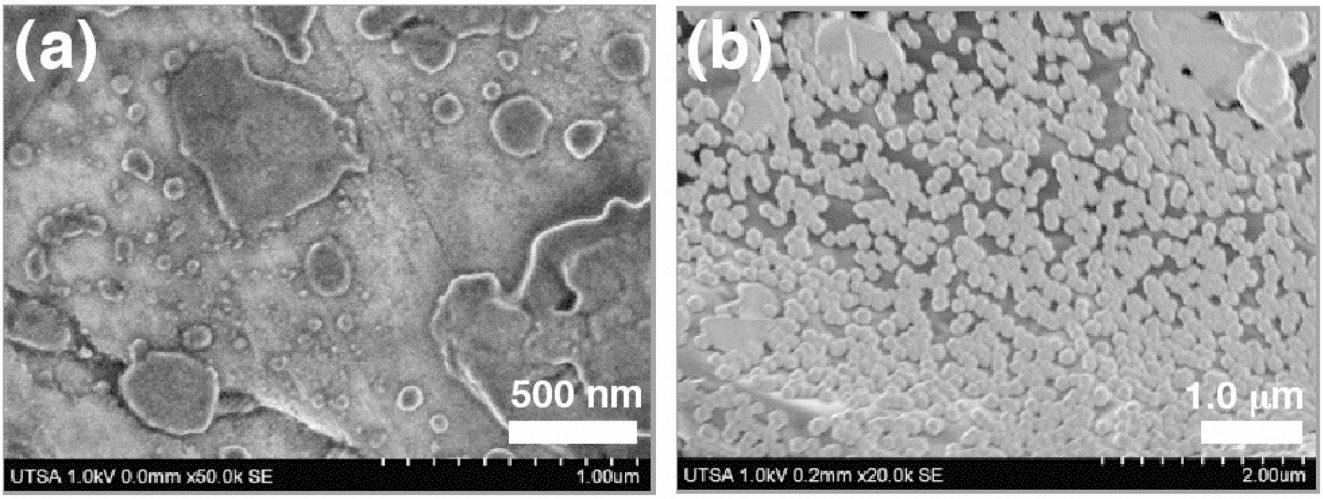
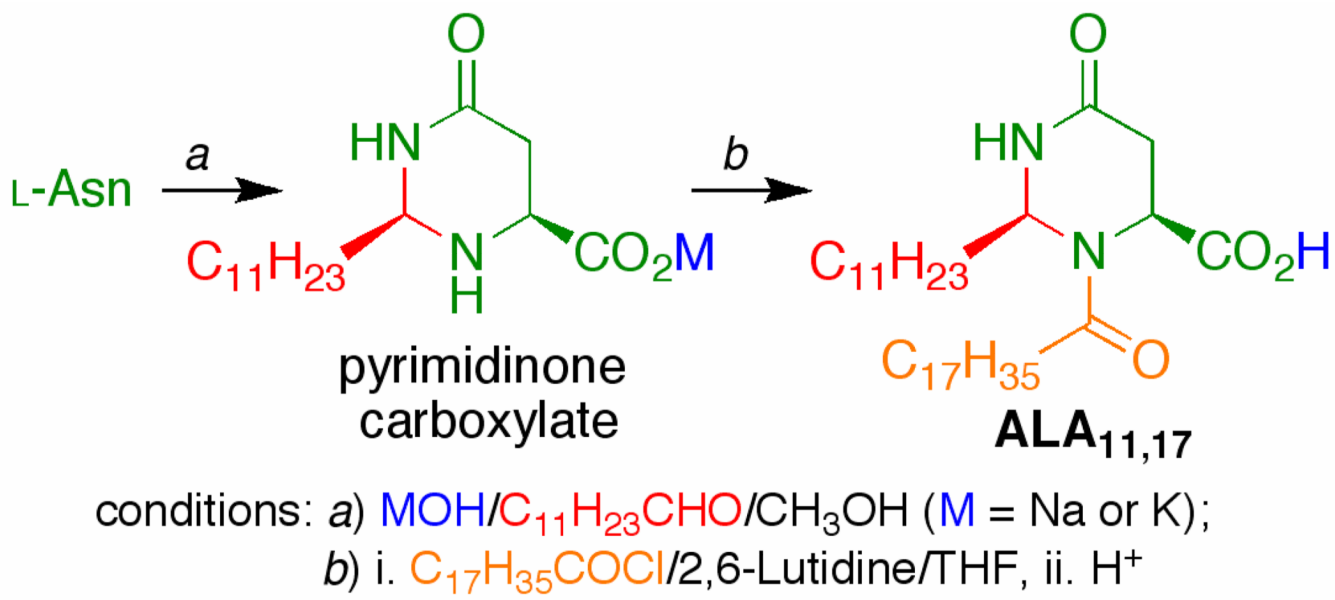
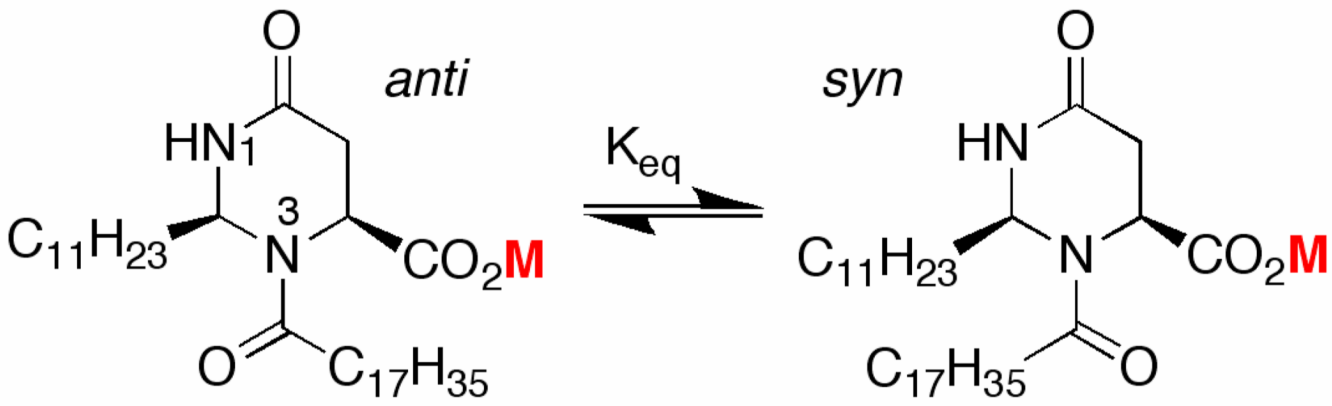


Figure 9.

Scanning electron microscopy images of (a) DSPC and (b) 10% $ALA_{11,17}$/DSPC liposomes at pH 1.9 (PBS/HCl). Both liposome suspensions were negatively stained by ammonium molybdate.



Scheme 1.
Preparation of 1,3-*cis*-substituted tetrahydropyrimidinones from L-asparagine



Solvents	K_{eq} M = H	K_{eq} M = Na
$CDCl_3$	0.50	insol
CD_3OD	0.74	1.86
DMSO-d_6	0.84	> 49

Scheme 2.
Conformers of $ALA_{11,17}$ in deuterated methanol, DMSO, and chloroform