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Acid-responsive nanospheres from an asparagine-derived amphiphile

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Electronic Supplementary Information (ESI)

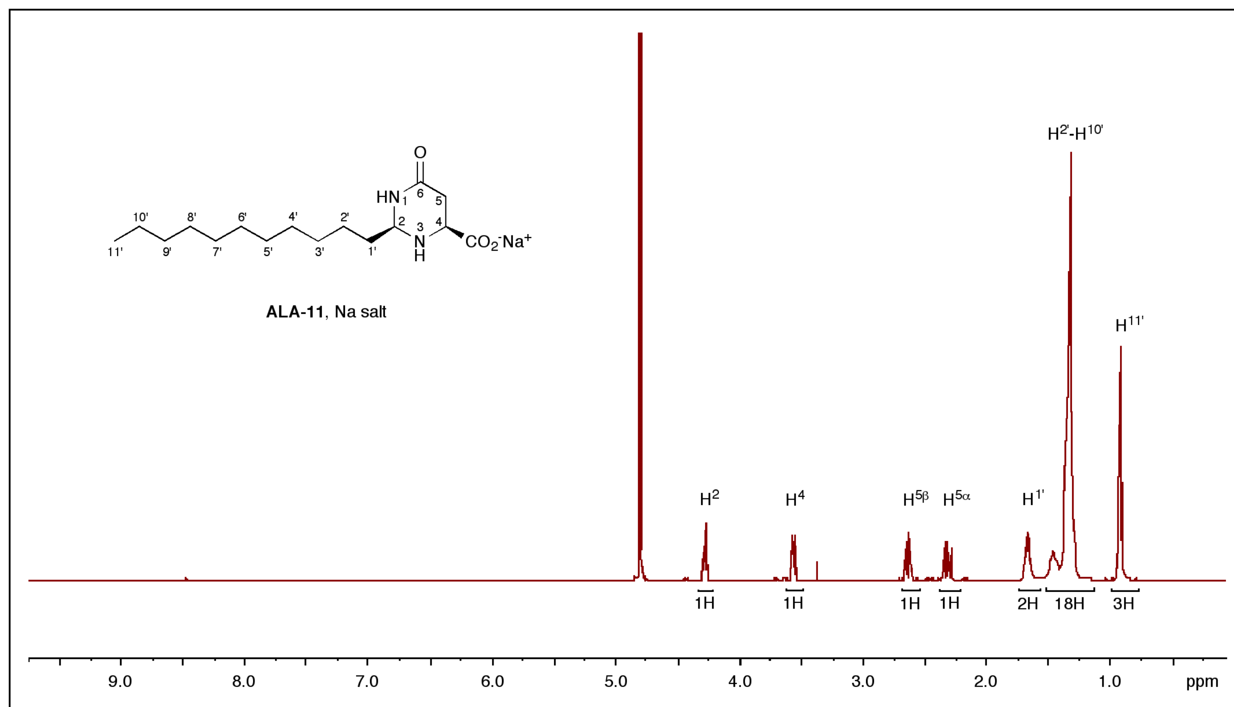
S1. Materials and Methods:

All chemicals including dodecanal and L-asparagine were obtained from either Sigma-Aldrich or Acros Organics. Dulbecco's phosphate buffer saline (PBS; pH 7.4) and electron microscopy diatome copper grids with Formvar/carbon film (400 mesh) were purchased from Fisher Scientific. All chemicals were reagent grade and were used as received. The ^{1}H and ^{13}C NMR spectra were obtained using a Varian Inova™ 500 NMR spectrometer operating at 500 and 125 MHz, respectively. All NMR spectra were recorded in either D_2O or deuterated phosphate buffer prepared by titration of a solution of sodium phosphate tribasic in D_2O with DCl to the appropriate *pD (pD = pH meter reading + 0.4 units).*[1] All chemical shifts (δ values) and ^{1}H-^{1}H coupling constants (*J* values) are given in ppm and Hz, respectively. All optical density (OD) measurements were carried out using a dual diode array UV-visible spectrophotometer (Varian CARY 5000). 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 and transmission electron microscopy was carried out using JEOL 2010-F

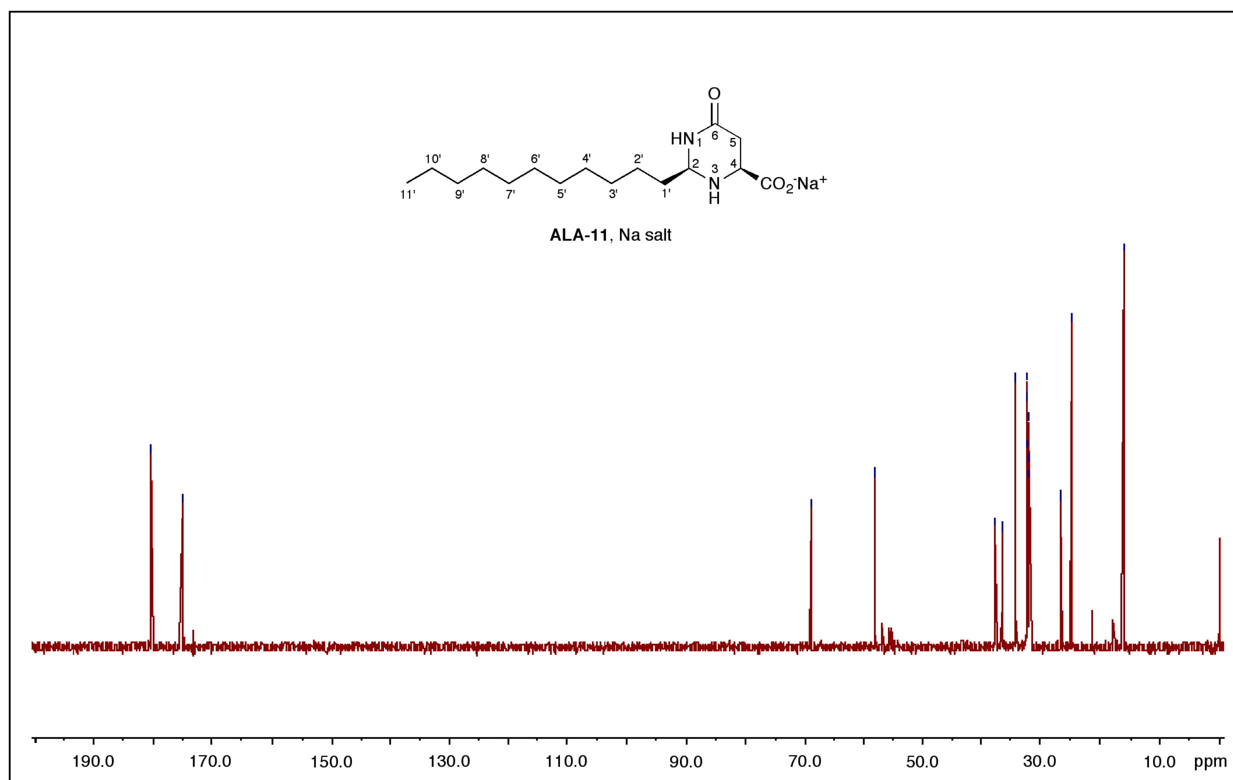
field emission TEM with magnification capabilities of up to 8,000,000X. Fluorescence measurements were carried out on an Aminco Bowman *Series 2* luminescence spectrometer. Average particle size distribution, zeta potential (ζ) measurements, and polydispersity index (PDI) were determined by photon correlation spectroscopy (PCS) using a Delsa™ Nano C. Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter Inc., Fullerton, CA, USA).

S2. Synthesis of sodium (2*R*,4*S*)-6-oxo-2-undecylhexahydropyrimidine-4-carboxylate (ALA-11):

To a solution of sodium hydroxide (1.0 mmol) in methanol (10 mL) was added L-asparagine (1.0 mmol) and the mixture was gently stirred for 15 min. To this clear solution, *n*-dodecanal (1.2 mmol) dissolved in methanol (10 mL) was added dropwise over 15 min and the mixture was stirred overnight at room temperature. The methanol was evaporated *in vacuo* and the residue was washed with hexane (3×25 mL), and upon drying, afforded ALA-11 (98%) as a white powder. δ_H (500 MHz; D_2O) 0.92 (3 H, t, J = 6.8, CH_3), 1.24–1.40 (16 H, m, 8 CH_2), 1.40–1.52 (2 H, m, CH_2), 1.67 (2 H, dt, J = 6.3, 7.7 Hz, CH_2), 2.32 (1 H, dd, J = 11.7, 17.6, C(5)H_{ax}), 2.64 (1 H, dd, J = 4.9, 17.6, C(5)H_{eq}), 3.56 (1 H, dd, J = 4.9, 11.2, C(4)H_{ax}), 4.28 (1 H, t, J = 5.9, C(2)H_{ax}). δ_C (125 MHz; D_2O; DSS): 16.1, 24.9, 26.6, 31.9, 31.97, 32.04, 32.25, 32.26, 32.3, 34.3, 36.7, 37.6, 58.0, 69.0, 175.3, 180.3. ATR-FTIR (ν_{max}, cm^{-1}): 3309.6, 2915.1, 2847.7, 1671.4, 1638.9, 1599.7, 1468.3, 1391.0, 1336.0.



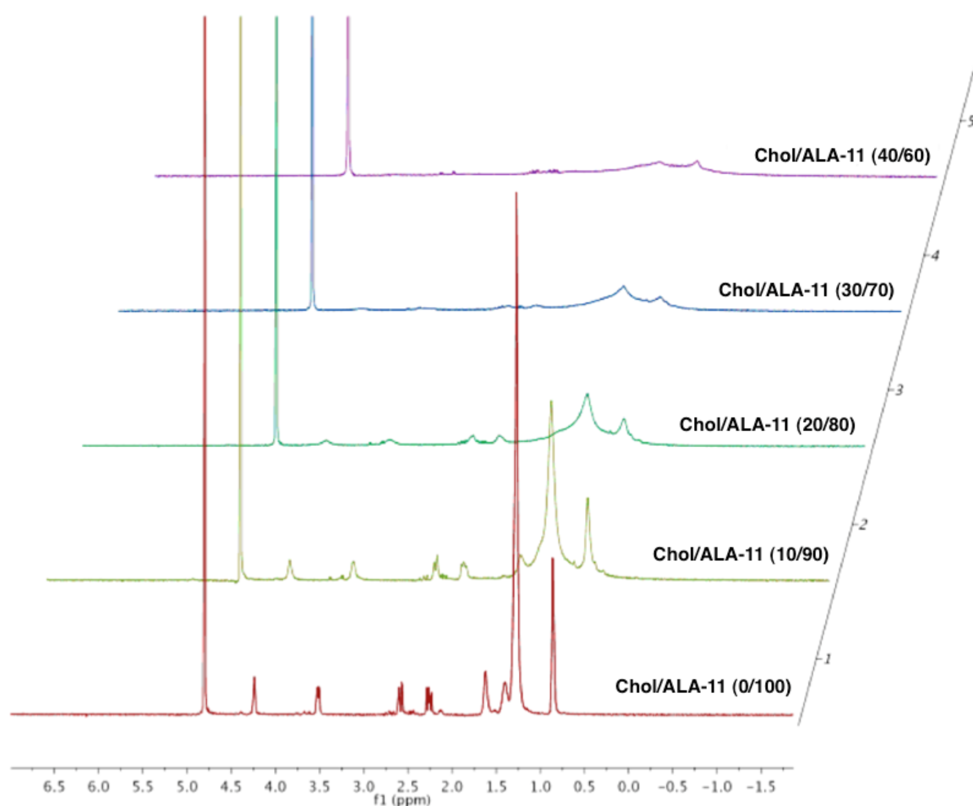
S3. ^{1}H NMR of sodium (2*R*,4*S*)-6-oxo-2-undecylhexahydropyrimidine-4-carboxylate (ALA-11) in D_2O.



S4. ^{13}C NMR of sodium (2*R*,4*S*)-6-oxo-2-undecylhexahydropyrimidine-4-carboxylate (ALA-11) in D_2O containing sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

S5. General procedure for self-assembly

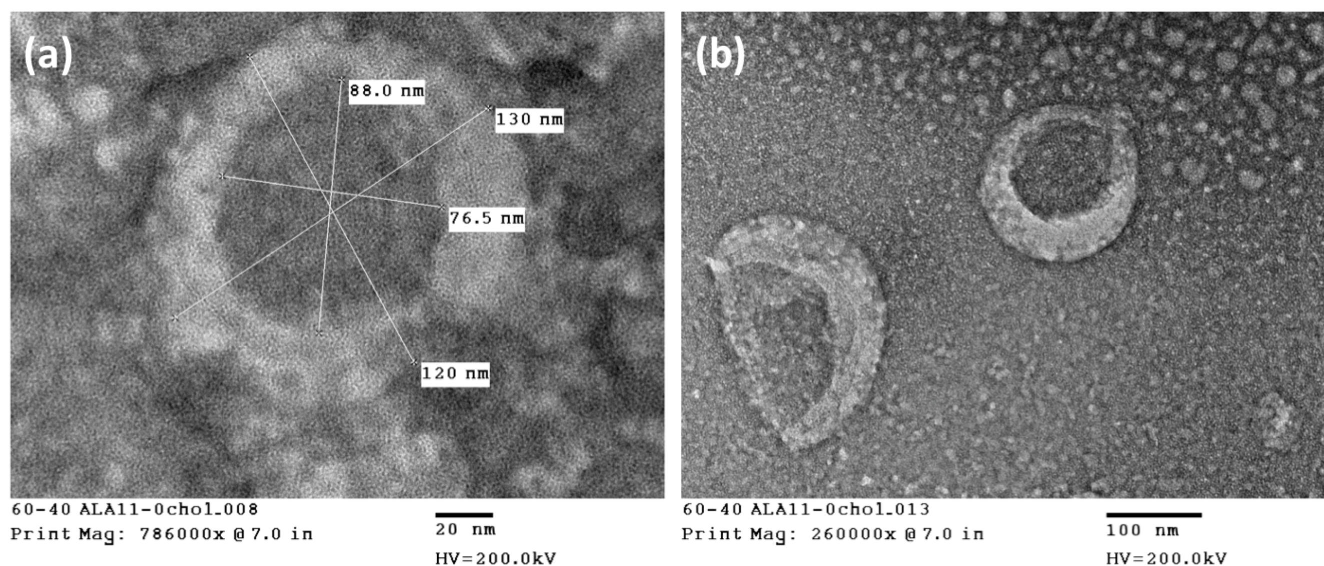
To ensure the formation of uniform mixtures, different proportions of ALA-11 and cholesterol (mol ratios of cholesterol/ALA-11: 0/100, 10/90, 20/80, 30/70, 40/60, and 50/50; total moles of lipids: 1.26×10^{-4}) were dissolved in round bottom flasks using (50:50) methanol/chloroform. The solvent of each preparation was evaporated under reduced pressure to generate uniform thin films that were stored at 4 °C until needed. To assemble the nanocarriers, the cholesterol/ALA-11 thin film was hydrated with 4.0 mL of PBS (10 mM phosphate buffer, 137 mM NaCl, pH 7.4) and sequentially vortexed and incubated at 55–60 °C until all the material was suspended in the turbid dispersion.



S6. Stacked ^{1}H NMR spectra showing the influence of cholesterol on the formation of supramolecular aggregates of ALA-11.

S7. Sample preparation for SEM and TEM studies

An aliquot of the suspension (100 µL) obtained by the above protocol (section 1.3) was diluted with an equal volume of deionized water. The diluted samples were dispensed on 400-mesh copper grids coated with either lacey carbon or Formvar films, and the excess liquid was removed using a pointed filter paper. Three drops of uranyl acetate solution (2% wt/vol) were added to the copper grids, and again the excess fluids were removed with a pointed filter paper. The grids were air dried for 1 h before EM analysis.



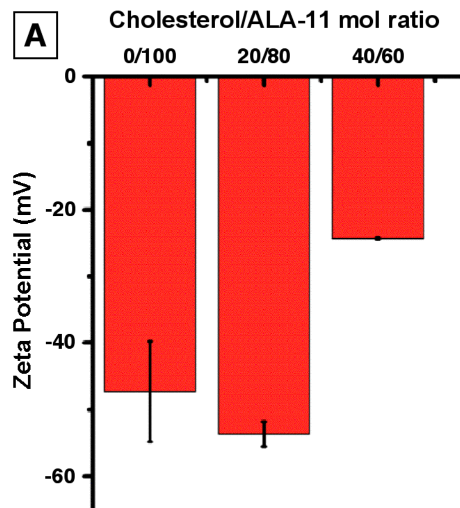
S8. Transmission electron micrographs (TEM) of 40/60 cholesterol/ALA-11 after negative staining with uranyl acetate: (a) vesicle showing the inner and outer diameter with an approximate membrane thickness of 44 nm and (b) Vesicles and micellar population.

S9. Dynamic light scattering (DLS) measurements

Aliquots (200 μL) of each of the dispersions of cholesterol/ALA-11 (0/100, 20/80, 40/60 mol%; 33 mM) were diluted in 1.8 mL of PBS (pH 7.4, 120 mM NaCl) and transferred to a disposable cell. Size determinations were carried out by Dynamic Light Scattering on the submicron particle size analyzer and counting was performed (20 accumulation times) at 25 °C using a deconvolution algorithm based on the CONTIN program. In these data collections, the measuring standard operating procedures were adjusted considering water as the medium for all experiments. Each experiment was performed using five independent runs.

S10. The zeta potential (ζ) measurements

The zeta potentials were determined by measuring the electrophoretic mobilities under an applied electric field of the same samples from DLS measurements loaded in the flow cell. The instrument uses a zeta potential module equipped with a 35 mW dual-laser diode (658 nm). Scattered light was detected at a 90 degrees angle at a temperature of 25 °C. Ten data points were recorded for each electrophoretic velocity and each experiment was performed in triplicate. Zeta potential values were automatically calculated from measured velocities using the Helmholtz-Smoluchowski relationship.[2]



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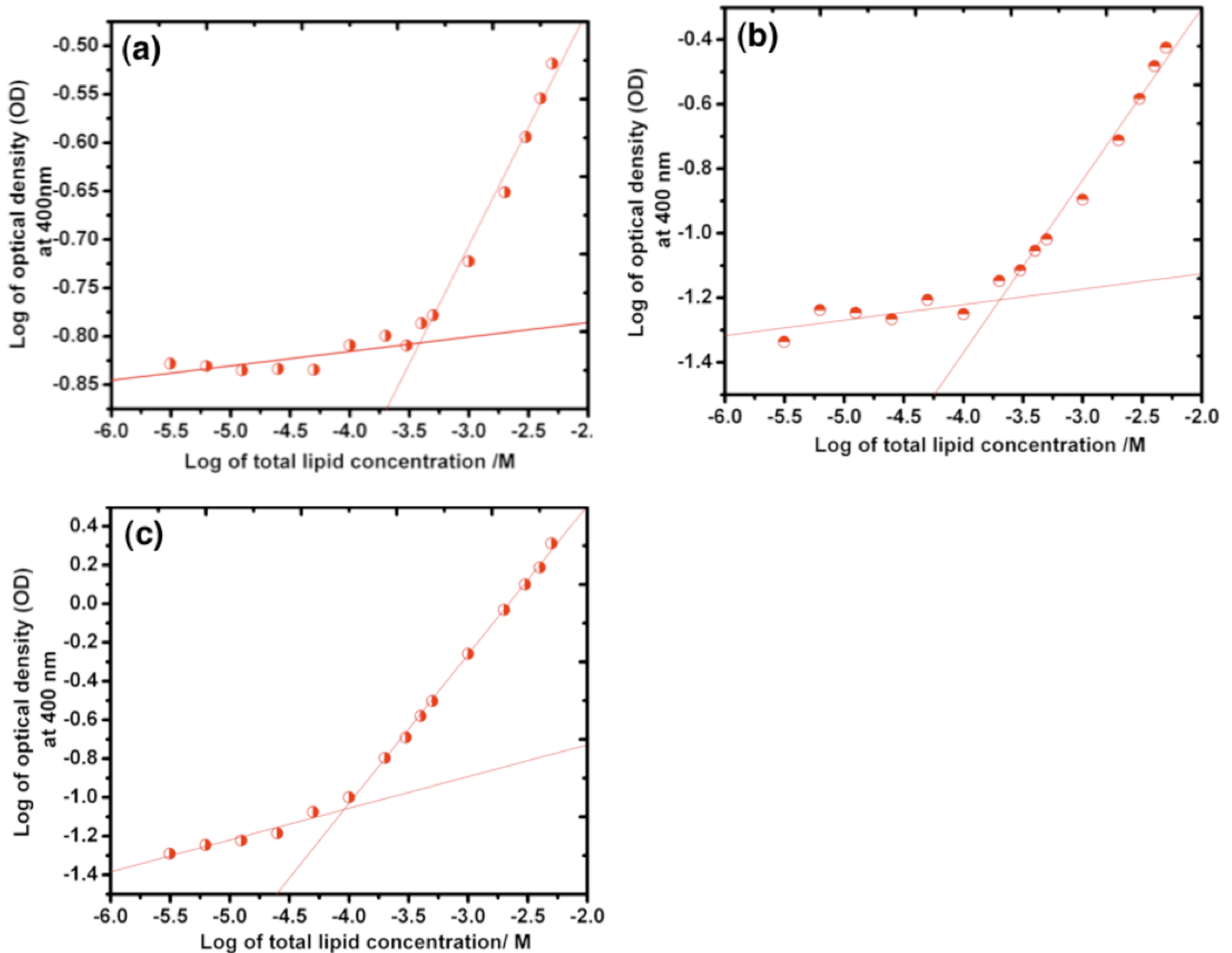
mol ratio*	size (nm)	PDI	ζ (mV)†
0/100	162 ± 2	0.28 ± 0.02	-47.4 ± 7.6
20/80	259 ± 5	0.29 ± 0.01	-55.2 ± 3.5
40/60	287 ± 4	0.25 ± 0.01	-24.3 ± 0.04

*Cholesterol/ALA-11 mol ratio; †Zeta-potential of vesicles

S11. (A) Zeta potential analysis of cholesterol/ALA-11 formulations in PBS at pH 7.4; (B) Size distribution, polydispersity indices (PDI), and zeta potentials (ζ) of selected cholesterol/ALA-11 formulations in PBS (pH 7.4)

S12. Determination of critical aggregation concentration (CAC) by UV-spectrophotometry

Vesicle suspensions comprising ALA-11 and cholesterol (0, 20, 40 mol% of cholesterol/ALA-11; 33 mM, 1.26×10^{-4} total moles in 4 mL of PBS) were prepared and sequentially diluted with PBS to generate a series of suspensions with known lipid concentrations. The optical densities at 400 nm of the series of suspensions of each formulation were measured and the CACs were estimated from the intersection of the lines of best fit from a log/log plot of optical density vs concentration.



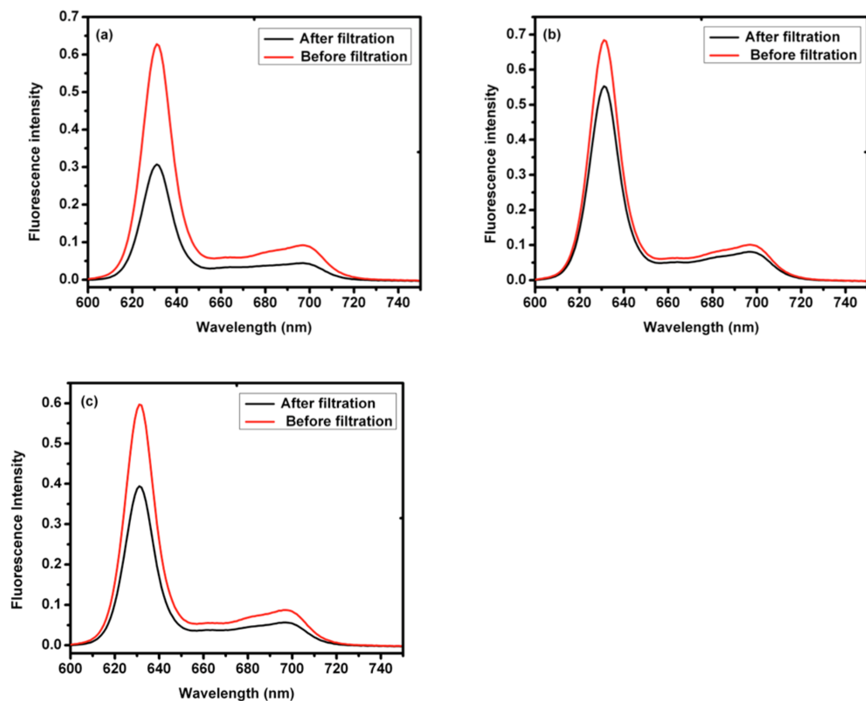
S13. Dependence of light scattering intensities at $\lambda = 400$ nm on CACs of cholesterol/ALA-11 self-assemblies: (a) 0/100 mol% cholesterol/ALA-11; (b) 20/80 mol% cholesterol/ALA-11; and (c) 40/60 mol% cholesterol/ALA-11.

S14. Sample preparation for encapsulation studies

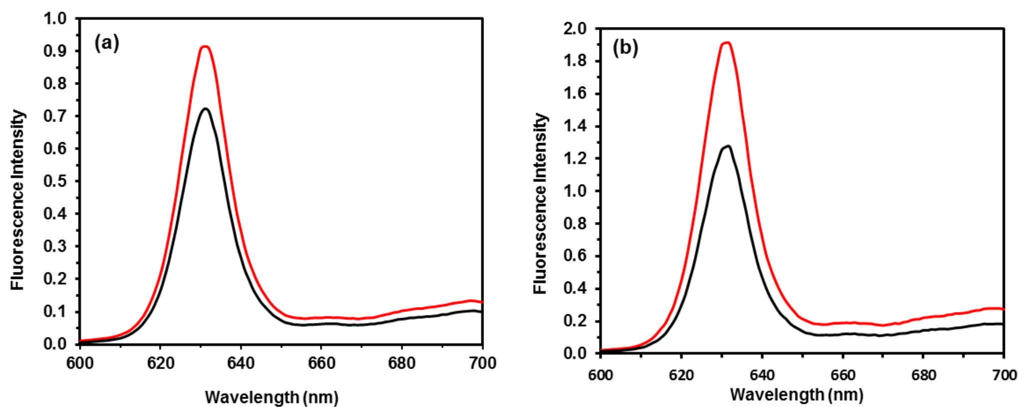
To include a selected hydrophobic agent within the nanocarriers, the thin films were made using a homogeneous solution of cholesterol/ALA-11 containing the compound to be encapsulated in 50:50 methanol/chloroform. The thin films were processed as described above (section 1.3) into nanocarriers and unencapsulated (undissolved) material was removed by passage through 0.45 μm filters or by Sephadex G-75 size exclusion chromatography.[2]

S15. Encapsulation of protoporphyrin IX dimethyl ester (PPIX-Me_2) in cholesterol/ALA-11

Various vesicle formulations of cholesterol/ALA-11 (0/100, 20/80, and 40/60) containing 2 mol% protoporphyrin IX dimethyl ester (PPIX-Me_2) were made by the procedure described above (1.26×10^{-4} moles of lipid and 2.52×10^{-6} moles PPIX-Me_2 in 4 mL PBS). The mixtures were alternately vortexed and incubated at 60 °C. Each of incubated formulations was devided to two parts and one part was directly used to obtain the fluorescence spectra (λ_{ex} = 400 nm and λ_{em} = 630 nm) after it was diluted 1000-fold in methanol (dilution procedure: 10.0 μL of each sample was made up to 10.0 mL with methanol). The known aliquots (600 μL) of second part of each formulation were processed through 0.45 μm filter or Sephadex G-75 column to remove non-encapsulated PPIX-Me_2 before the dilution with methanol and spectra were taken. The dilution in methanol was necessary to minimize the effect of light scattering and to dissolve the dye completely in methanol as well (the relavent spectra were illustrated in **S16** and **S17**). The encapsulation efficiencies were estimated from the ratio of the aliquot fluorescence before and after filtration. The loading was calculated as mass of solute encapsulated per total mass of lipids used in the formulations.

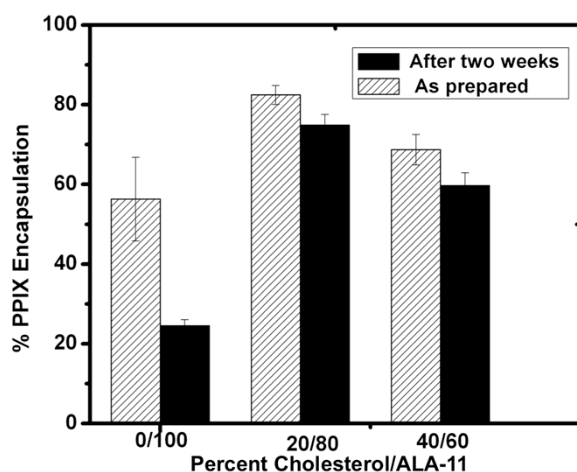


S16. Emission spectra of protoporphyrin dimethyl ester (PPIX-Me_2) when the non-encapsulated dye was removed by passage through 0.45 μm filter in **S15**, which were obtained by measuring the fluorescence at λ_{ex} = 412 nm (**red**: before PPIX-Me_2 removal; and **black**: after PPIX-Me_2 removal). The various mole ratios of cholesterol/ALA-11 used to study the association of PPIX-Me_2 with nanoparticles were: (a) 0/100; (b) 20/80; and (c) 40/60.



S17. Emission spectra of protoporphyrin dimethyl ester (PPIX-Me_2) when the non-encapsulated dye was removed by Sephadex G-75 size exclusion in **S15**, which were obtained by measuring the

fluorescence at λ_{ex} = 412 nm (**red**: before PPIX-Me_2 removal; and **black**: after PPIX-Me_2 removal). The two mole ratios of cholesterol/ALA-11 used to study the association of PPIX-Me_2 with nanoparticles were: (a) 20/80 and (b) 40/60.



S18. Stability of cholesterol/ALA-11 nanocarrier formulations after encapsulation of protoporphyrin IX dimethyl ester for two week period.

S19. Nile red encapsulation and controlled release under different pH conditions

Vesicle suspensions comprising cholesterol in ALA-11 (0 and 40 mol%) were prepared with Nile red (NR; 0.25 mol%) as the hydrophobic guest following the procedure described above. Aliquots of the respective suspensions (200 µL) were diluted in a curvette with 3 mL of the media under studies (acetate buffer pH 5.0; phosphate buffer pH 6.0, 7.4). The samples were immediately transferred to the spectrofluorometer and timecourse fluorescence intensities were recorded (λ_{ex} = 400 nm and λ_{em} = 580, 600, or 630 nm, depending on the solvatochromic property of the dye in each environment under consideration). The percent NR retained in the nanoparticles was calculated using the formula,

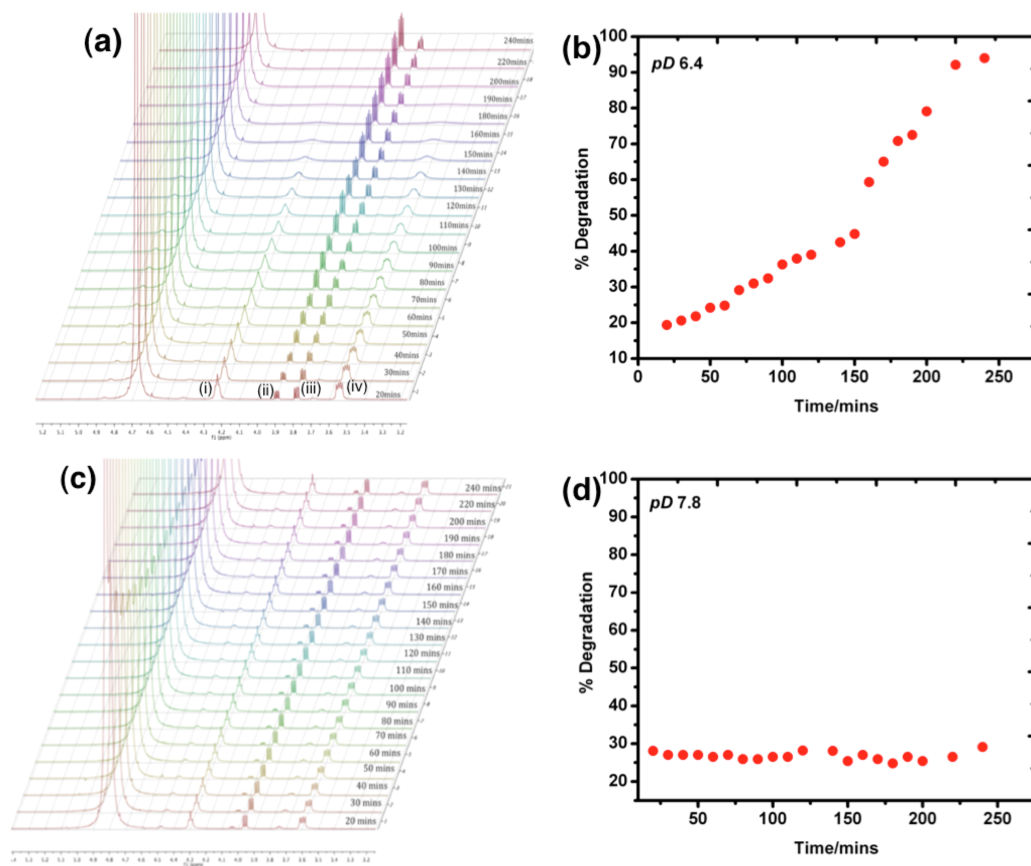
$$\% \text{ Nile red retained} = (I_t - I_f)/(I_o - I_f) \times 100$$

where I_t is the fluorescence at time (t), I_o is the initial fluorescence of the sample and I_f is the final

fluorescence after complete degradation of the sample. The percentage of NR retained versus time (t) was plotted for the various formulations and pH (the graphical representation of the results was illustrated in Fig. 6).

S20. The pH dependent degradation of ALA-11 and hence the aqeueous ALA-11 formulations

Deuterated aqeueous formulation of ALA-11 in a deuterated phosphate buffer at *pD* 7.8 (pH 7.4; 33 mM) was prepared (1.26×10^{-4} moles of lipid in 4 mL of buffer) and an aliquot of the solution (800 μL) was diluted 50% with deuterated phosphate buffer at *pD* 7.8 (1:1 dilution). The ^{1}H NMR of this sample was immediately obtained and subsequent spectra were obtained as a function of time at 10 min intervals. The pH dependency of the ALA-11 formulations were tested by repeating this experiment at pH 5.0 and 6.0 using deuterated formulations of ALA-11 at *pD* 5.4 and 6.4, respectively. The percentages of the degradation were calculated from the changes in the integral values of representative peaks and they were then ploted against time as shown below (**S21**).



S21. Stacked ^{1}H NMR spectra of the hydrolysis profile of ALA-11 amphiphile: (a) Stacked ^{1}H NMR spectra at *pD* 6.4; (b) corresponding plot of percentage degradation vs time at *pD* 6.4; (c) Stacked ^{1}H

NMR spectra at *pD* 7.8; and (d) corresponding plot of percentage degradation vs time at *pD* 7.8. The time between sample preparation and first data acquisition was 20 min after which the rest of the data were collected at 10 min intervals. The peaks labeled (i) and (iv) belong to the corresponding *N,N*-acetal proton and the methine proton alpha to the carboxylate function, respectively while (ii) and (iii) represent the protonated and unprotonated asparagine signals from the heterocycle after degradation.

[1] P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, 1960, **64**, 188–190.

[2] (a) R. F. Probstein, *Physicochemical Hydrodynamics: An Introduction*, John Wiley and Sons, Inc., Hoboken, NJ, 2nd edn., 2003. (b) B. De Meulenaer, P. van der Meeren and J. Vanderdeelen, in *Encyclopedia of Surface and Colloid Science*, ed. P. Somasundaran, Taylor & Francis Group, Boca Raton, FL, 2nd edn., 2006, vol. 3, pp. 2372–2384.