Synthetic Mimics of Bacterial Lipid A Trigger Optical Transitions in Liquid Crystal Microdroplets at Ultralow Picogram-per-Milliliter Concentrations

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Supporting Information

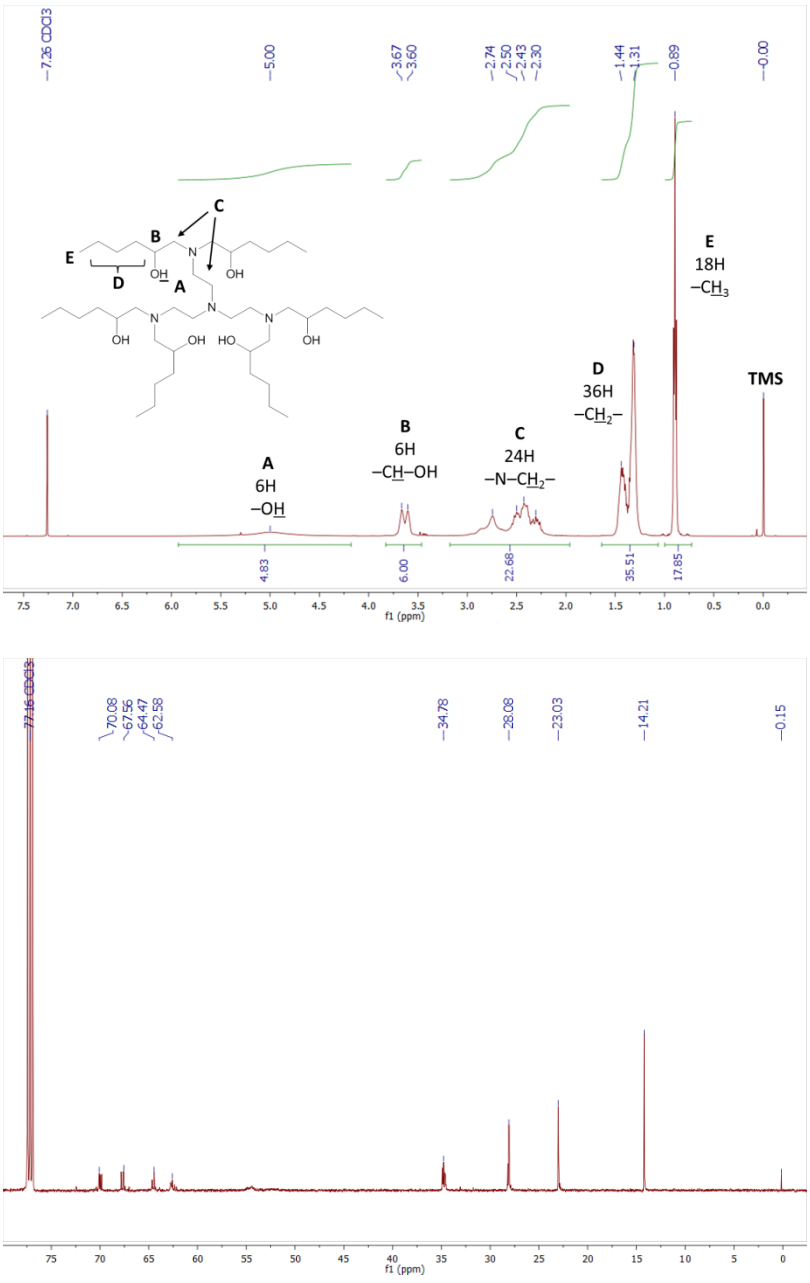
Materials and Methods

Materials and General Considerations. Tris(2-aminoethyl)amine (TREN, 96%) was purchased from Acros Organics (Morris Plains, NJ). 1,2-Epoxyhexane (>96%) and 1,2-epoxyhexadecane (>80%) were purchased from TCI America (Philadelphia, PA); 1,2-epoxyoctane (99%), 1,2-epoxydecane (97%) and 1,2-epoxydodecane (95%) were purchased from Alfa Aesar (Radnor, PA). Lipid A from *Escherichia coli F583* (Rd mutant, 1800 Da), sodium dodecyl sulfate (SDS), triethylamine (TEA), sulfuric acid (H_2SO_4) and phosphate-buffered saline (PBS) pouches (used to make PBS buffer as the sheath fluid for flow cytometry), dichloromethane (HPLC grade, >99.8%), chloroform ($CHCl_3$; HPLC grade, >99.8%), aqueous ammonium hydroxide solution (ACS grade, 28-30%), deuterated chloroform ($CDCl_3$, 99.8%) and deuterated methanol (MeOD, 99.8%) were purchased from Sigma-Aldrich (Milwaukee, WI). Methanol (MeOH; ACS grade) was purchased from Avantor Performance Materials (Center Valley, PA). 4'-Pentyl-4-cyanobiphenyl (5CB) was obtained from EM Sciences (New York, NY). EndoTrap® Red Equilibration Buffer [10 mM Na_2HPO_4/NaH_2PO_4, 80 mM NaCl, pH 7.2; certified to contain a concentration of endotoxin of less than 0.02 EU/mL (2 pg/ mL)] was purchased from Hyglos GmbH (Regensburg, Germany). Iron (II) sulfate ($FeSO_4$), copper (II) sulfate ($CuSO_4$), and

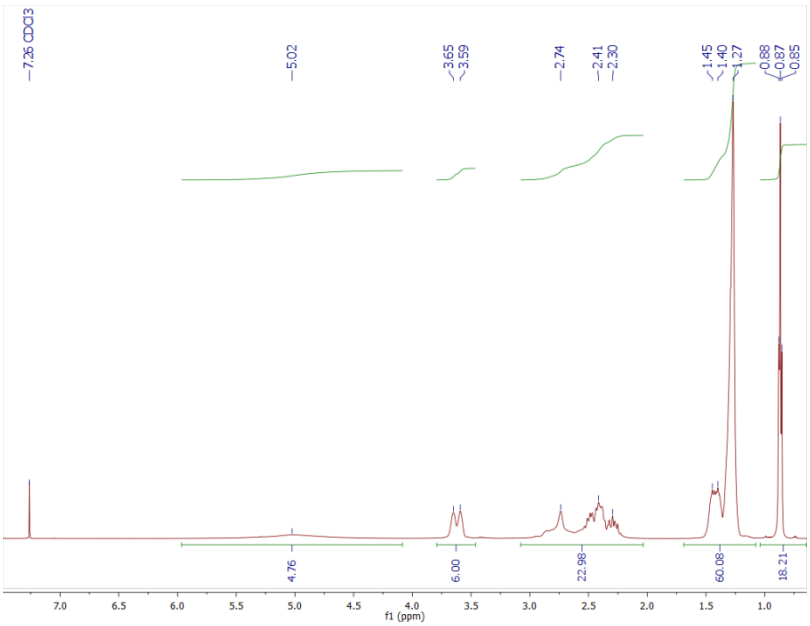
manganese (II) chloride ($MnCl_2$) were purchased from Sigma-Aldrich ($FeSO_4$ and $CuSO_4$) and Fisher Scientific ($MnCl_2$; Pittsburgh, PA). Neptune pipette tips (no detectable endotoxin) were purchased from Continental Lab Product, Inc. (San Diego, CA). Fisher's Finest PremiumGrade cover glass, Wheaton glass 20 mL scintillation vials, 1.5 mL FisherBrand micro-centrifuge tubes, 12 x 75 mm disposable glass culture tubes, and a 10 μL glass Hamilton blunt-tipped syringe were obtained from Fisher Scientific. Quartz capillary tubes (1.5 mm diameter) used for SAXS experiments were obtained from Hampton Research (Aliso Viejo, CA). Deionization of a distilled water source was performed with a Milli-Q system (Millipore, Bedford, MA) to give water with a resistivity of 18.2 MΩ cm (referred to as Milli-Q water below). SilicaFlash P60 silica gel (230-400 mesh) was purchased from Silicycle (Quebec City, QC). Silica gel TLC plates were purchased from Sigma-Aldrich (Milwaukee, WI); spots were visualized using iodine and anisaldehyde stains. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 500 spectrometer. NMR chemical shift values are given in ppm and are referenced to the residual protons of $CDCl_3$ (δ 7.26, 77.2) or deuterated methanol (δ 3.31). Mass spectrometry was performed on a Waters LCT electrospray ionization time-of-flight mass spectrometer using 10 mM NH_4OAc/CH_3CN in methanol at a flow rate of 40 uL/min. Unless otherwise noted, materials were used as received without further purification.

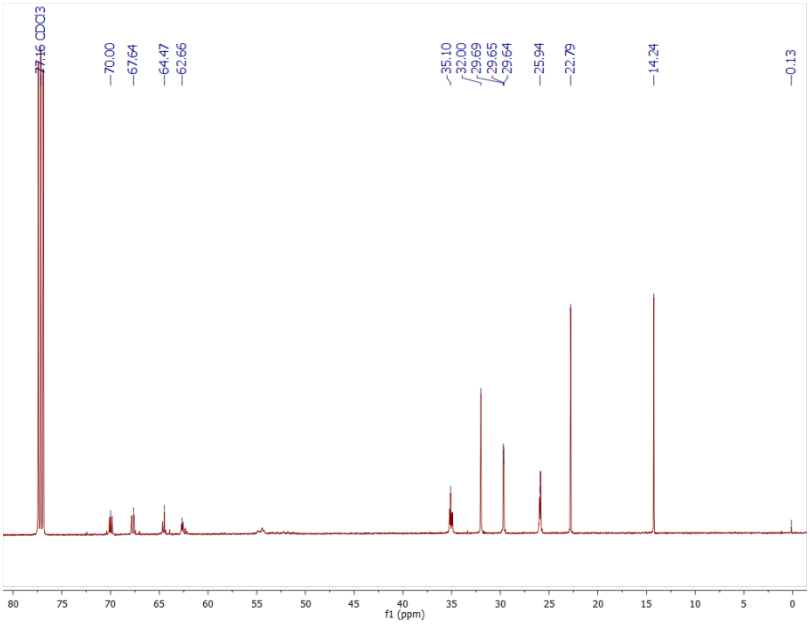
Synthesis of Six-Tailed Amphiphiles. Amphiphiles $\mathbf{1_6}$ – $\mathbf{1_{16}}$ were synthesized using the following general procedure. TREN (1.0 eq) and an epoxide having the desired aliphatic chain length (6.6 eq) were weighed into a 6 mL glass vial equipped with a magnetic stir bar. The vial was briefly purged with nitrogen and then sealed with a Teflon-lined cap and Parafilm, and placed into an oil bath at 90 °C for 4 days. Crude reaction products were diluted by

approximately half with DCM and then purified by flash chromatography on silica gel using gradient elution from 100% DCM to 75:22:3 DCM/MeOH/$NH_4OH_{(aq)}$. (In 100% DCM: $R_{f\ (1,2\text{-epoxydecane})} = 0.54$; $R_{f\ (\mathbf{1}_{10})} \sim 0$. In 75:22:3 DCM/MeOH/$NH_4OH_{(aq)}$: $R_{f\ (1,2\text{-epoxydecane})} = 1.00$; $R_{f\ (\mathbf{1}_{10})} = 0.95$). Solvent was evaporated under reduced pressure and purified amphiphiles were dried under high vacuum overnight. Representative ^{1}H and ^{13}C NMR spectra for each amphiphile are given below.

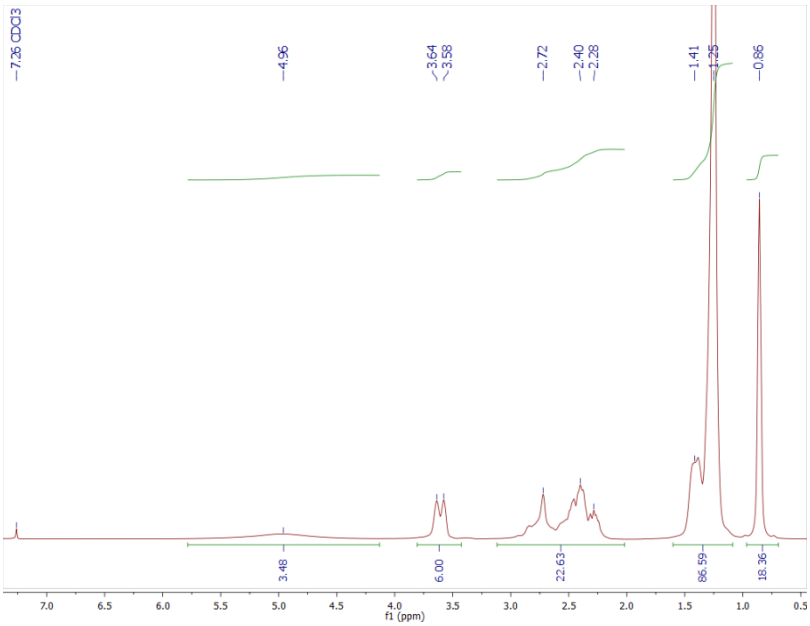


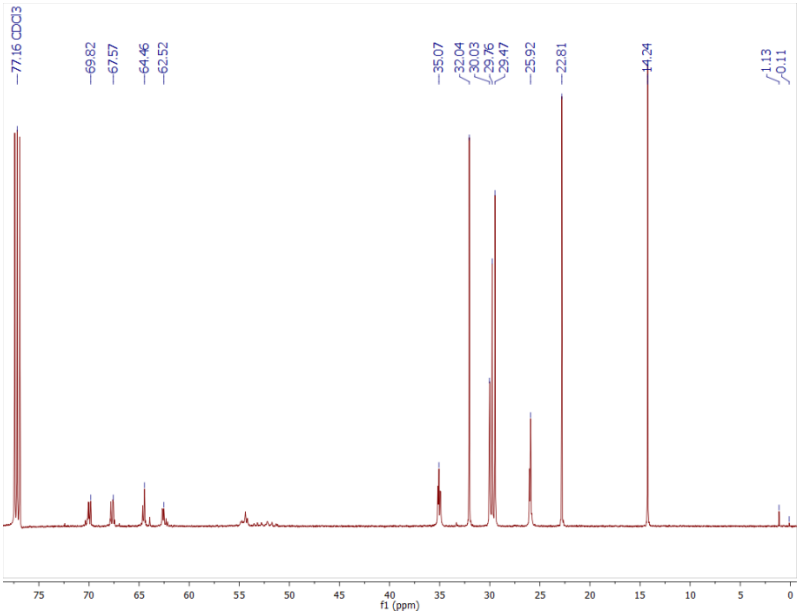
Amphiphile 1_6: Yield: 90.7%, viscous yellow oil. ^{1}H NMR (500.022 MHz, $CDCl_3$): δ 0.89 (t, J = 6.92 Hz, 18H), 1.22-1.53 (m, 36H), 2.08-3.04 (m, 24H), 3.65 (d, J = 30.63 Hz, 6H), 5.00 (s, 6H). ^{13}C NMR (125.74 MHz, $CDCl_3$): 14.21, 23.03, 28.08, 34.78 (m), 62.58 (m), 64.57 (m), 67.56 (m), 70.08 (m). MS (ESI+) found: $[M+H]^+$ 747.6; $[M+2H]^{2+}$ 374.3



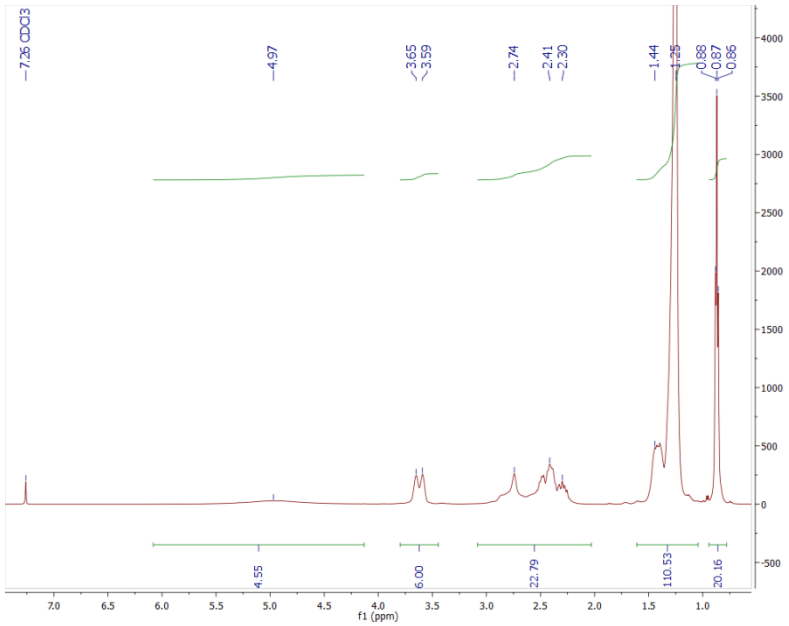


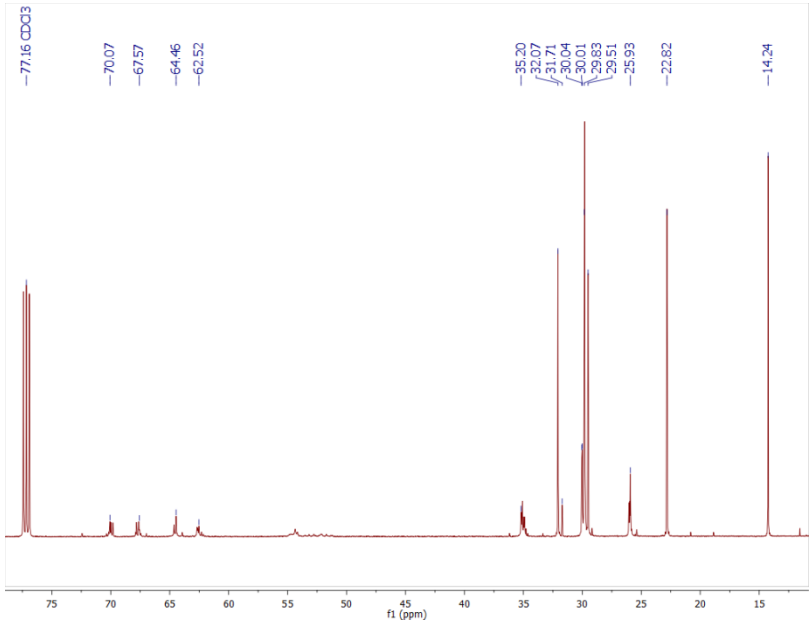
Amphiphile 1_8: Yield: 91.0%, viscous yellow oil. ^{1}H NMR (500.022 MHz, $CDCl_3$): δ 0.87 (t, J = 6.60 Hz, 18H), 1.10-1.60 (m, 60H), 2.06-3.05 (m, 24H), 3.63 (d, J = 28.83 Hz, 6H), 5.02 (s, 6H). ^{13}C NMR (125.74 MHz, $CDCl_3$): 14.24, 22.79, 25.94 (m), 29.64 (m), 32.00, 35.10 (m), 62.66 (m), 64.47 (m), 67.64 (m), 70.00 (m). MS (ESI+) found: $[M+H]^+$ 915.9; $[M+2H]^{2+}$ 458.5



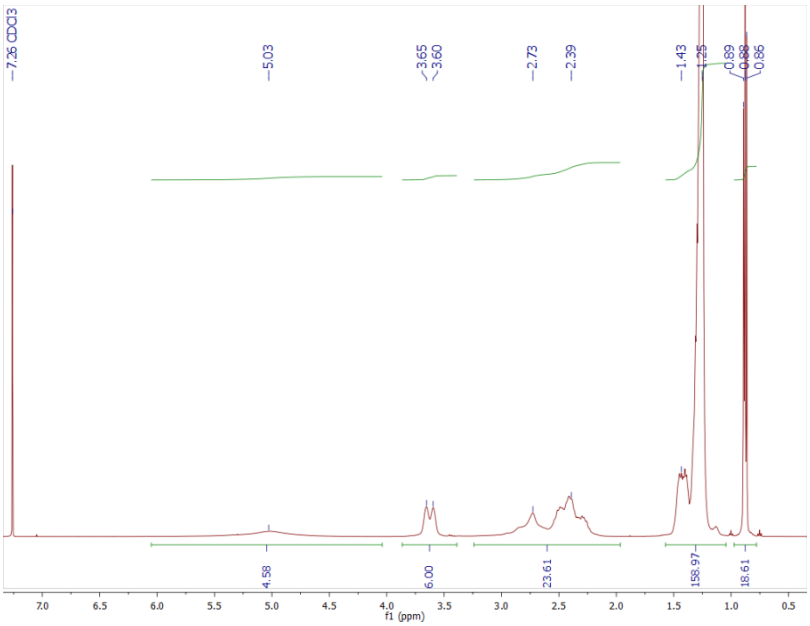


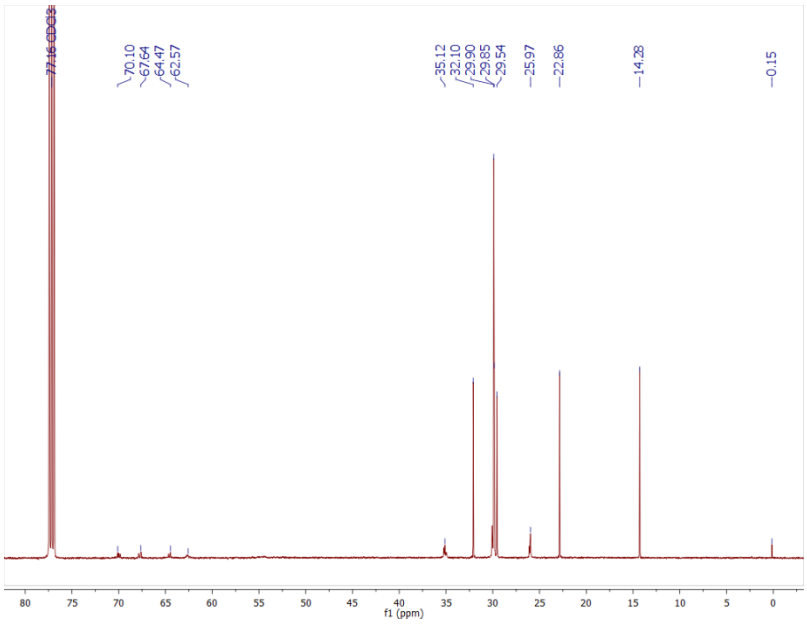
Amphiphile 1_{10}: Yield: 88.2%, viscous yellow oil. ^{1}H NMR (500.022 MHz, $CDCl_3$): δ 0.87 (t, J = 6.80 Hz, 18H), 1.05-1.57 (m, 84H) 2.16-3.05 (m, 24H), 3.62 (d, J = 28.95 Hz, 6H), 4.99 (s, 6H). ^{13}C NMR (125.74 MHz, $CDCl_3$): 14.24, 22.841, 25.92 (m), 29.47, 29.76, 30.03, 32.04, 35.07 (m), 65.52 (m), 67.57 (m), 67.57 (m) 69.82 (m). MS (ESI+) found: $[M+H]^+$ 1084.2; $[M+2H]^{2+}$ 542.6





Amphiphile 1_{12}: Yield: 95.3%, viscous yellow oil. ^{1}H NMR (500.022 MHz, $CDCl_3$): δ 0.87 (t, J = 6.66 Hz, 18H), 1.08-1.56 (m, 108H) 2.15-3.00 (m, 24H), 3.58 (d, J = 29.19 Hz, 6H), 4.97 (s, 6H). ^{13}C NMR (125.74 MHz, $CDCl_3$): 14.24, 22.82, 25.93 (m), 29.51, 29.83, 30.03 (d, J = 3.77 Hz), 31.71, 32.0, 35.20 (m), 62.52 (m), 64.46 (m), 67.57 (m), 70.07 (m). MS (ESI+) found: $[M+H]^+$ 1252.0; $[M+2H]^{2+}$ 626.5





Amphiphile 1_{16}: Yield: 81.0%, light brown paste. ^{1}H NMR (500.022 MHz, $CDCl_3$): δ 0.88 (t, J = 6.96 Hz, 18H), 1.06-1.54 (m, 156H) 2.12-3.19 (m, 24H), 3.63 (d, J = 29.15 Hz, 6H), 5.03 (s, 6H). ^{13}C NMR (125.74 MHz, $CDCl_3$): 14.14, 22.71, 25.82 (m) 29.40, 29.70 (m), 31.95, 34.98 (m), 62.48 (m), 64.32 (m), 67.50 (m), 69.96 (m). MS (ESI+) found: $[M+H]^+$ 1588.9; $[M+2H]^{2+}$ 794.4

Preparation of Aqueous Dispersions of Lipid A. Powdered Lipid A (1 mg) was dissolved in 1 mL of a 10 μM solution of SDS in EndoTrap® Red Equilibration Buffer [also containing 0.2% (v/v) TEA] at room temperature. The resulting solution was then subjected to two cycles of sonication for 5 s using a Branson Sonifier 250 (Emerson Electric Company, St. Louis, MO) and vortex mixing at 2,500 rpm for 1 minute. Following mixing, the 1 mg/mL stock solution was divided into 80 μL aliquots and stored -80 °C for future use. Serial dilutions were performed by first removing an 80 μL aliquot of 1 mg/mL Lipid A from the freezer and allowing the solution to warm to room temperature. This stock was then vortexed at 2,500 rpm for 30 s, and serial dilutions were performed to reach final desired concentrations.

Preparation of Aqueous Dispersions of Synthetic Amphiphiles. The desired amphiphile (~1 mg) was weighed into a 12 x 75 mm disposable glass culture tube and a desired volume of a 10 μM solution of SDS in EndoTrap® Red Equilibration Buffer [also containing 0.2% (v/v) TEA] was added at room temperature to reach a final concentration of 1 mg/mL. The resulting dispersion was then subjected to two cycles of vortex mixing at 3,000 rpm for 30 s followed by sonication for 15 min in a water bath at 55 °C, and serial dilutions were performed to reach final desired concentrations.

Preparation of LC Emulsions and Dilution into Aqueous Dispersions of Amphiphiles. LC-in-water emulsions were formed by adding 6 μL of 5CB to the bottom of a 12 x 75 mm disposable glass culture tube using a 10 μL blunt-tipped syringe. Then, 3 mL of a 10 μM solution of SDS in EndoTrap® Red Equilibration Buffer was added to the tube, and the mixture was vortexed for 30 s at 3,000 rpm. A concentration of 10 μM SDS was selected because it led to

reproducible emulsions without influencing the configuration of the 5CB droplets. The emulsions were allowed to incubate for 1 hour prior to dilution to allow measurable coalescence to arrest. As described in past studies,[1] this emulsification procedure resulted in relatively polydisperse emulsions with droplet sizes ranging between 1 and 20 μm in diameter. Finally, 70 μL aliquots of the stock emulsions were diluted into 700 μL of aqueous dispersions of either Lipid A or the desired amphiphile, and the emulsion was allowed to incubate for at least 3 hours prior to characterization by light scattering by flow cytometry (a 3 hour incubation was found to be optimal for the ordering transitions induced by the amphiphiles to reach completion; see below for additional details). We measured this emulsion-to-sample volume ratio to result in an average droplet concentration of 5,600 ± 1,000 LC droplets/μL.

Characterization of LC Emulsions by Flow Cytometry. Frequency histograms of the intensity of forward light scattering (FSC) were obtained for LC emulsions using a BD Accuri C6 flow cytometer (Ann Arbor, MI). FSC was measured at a detection angle of 0° ± 15°; all flow cytometry measurements were performed at room temperature. Emulsions were pumped through the flow cytometer at a flow rate of 14 μL/min and histograms were constructed from data collected from the measurement of 5,000 LC droplets.

Calculation of the Percentage of Radial Droplets Contained in a LC Emulsion from Frequency Histograms of FSC. The percentage of radial droplets contained in a LC emulsion was quantified according to our previously reported procedure.[2, 3] Briefly, for a given emulsion, the number of light scattering events measured between 30,000 a.u. and 60,000 a.u. was compared to the number of events measured within this region for an emulsion of LC droplets

dispersed in 100 ng/mL Lipid A (positive control), because this concentration of Lipid A was found to lead to a pure population of radial droplets. The following equation was used to calculate the percentage of radial LC droplets contained in an emulsion from the peak occurring between 30,000 a.u. and 60,000 a.u in frequency histograms of FSC values:

$$\text{Percent Radial} = \frac{\sum_{FSC=30,000}^{60,000} Count|_{emulsion} - \sum_{FSC=30,000}^{60,000} Count|_{10\mu MSDS}}{\sum_{FSC=30,000}^{60,000} Count|_{\frac{100ng}{mL}} - \sum_{FSC=30,000}^{60,000} Count|_{10\mu MSDS}} \times 100\% \qquad (1)$$

In Equation 1, the area below the peak measured for an emulsion of LC droplets dispersed in a 10 μM aqueous solution of SDS (negative control) is subtracted from the integrated area under each peak as a baseline. Then, the percentage of the corrected area under each peak relative to the area under the corrected peak measured for the positive control is calculated.

Optical Characterization of LC Emulsions by Polarized Light Microscopy. LC emulsions were characterized by polarized light microscopy according to previously published methods.[4] Briefly, a 40 μL volume of LC emulsion was dispensed onto a glass coverslip, and the configurations of the LC within the emulsion droplets were determined by observation of the droplets using an Olympus IX71 inverted microscope (Center Valley, PA) with an objective magnification power of 100x (oil-immersion lens). Polarized light micrographs of the LC emulsions were collected with a Hamamatsu 1394 ORCAER CCD camera (Bridgewater, NJ) connected to a computer and controlled through SimplePCI imaging software (Compix, Inc., Cranberry Twp., NJ). We characterized LC droplets that were located at least 50 μm above the surface of the coverslip and translating with velocities greater than 1 μm/s to avoid observation of LC droplets interacting with the surfaces of the coverslips.[5] Because the droplets were

diffusing (translating and rotating), radial droplets exhibited an optical appearance that was invariant with time when viewed between crossed polarizers, whereas bipolar droplets had a distinct, time-varying optical appearance.[4] Multiple images of each droplet were taken to determine the time-dependent optical appearances of the droplets, and thus the director configurations.

Characterization of the Interactions of LC Droplets with Amphiphiles in the presence of Divalent Metal Ions. LC-in-water emulsions containing the desired concentrations of amphiphiles were prepared as described above with the following modification. Solutions of metal ions were prepared in 10 μM SDS in EndoTrap® Red Equilibration Buffer [also containing 0.2% (v/v) TEA] by direct dissolution at room temperature. Solutions containing 500 pg/mL amphiphile and 1 mM metal ion were obtained by mixing 750 μL of 1 ng/mL amphiphile and 750 μL of 2 mM metal ion solutions.

Measurement of Langmuir Isotherms. Langmuir films of Lipid A or synthetic amphiphiles were prepared on a Nima 602A film balance (Coventry, England) equipped with a filter paper Wilhelmy plate for surface pressure measurements. The sub-phase was Milli-Q water and was maintained at a temperature of 25.0 ± 0.1 °C by circulating water at a constant temperature beneath the trough. Surface pressure/area isotherms (Langmuir isotherms) were measured by spreading a solution of Lipid A (1 mg/mL in $CHCl_3$/MeOH/water (96.6/3/0.4 %v/v)) or amphiphile $\mathbf{1_n}$ (1 mg/mL in $CHCl_3$) onto the air/water interface, allowing the solvent to evaporate for 20 minutes, and compressing the barriers at a rate of 35 cm^2/min. Isotherms for all amphiphiles are given in Fig. S1 below.

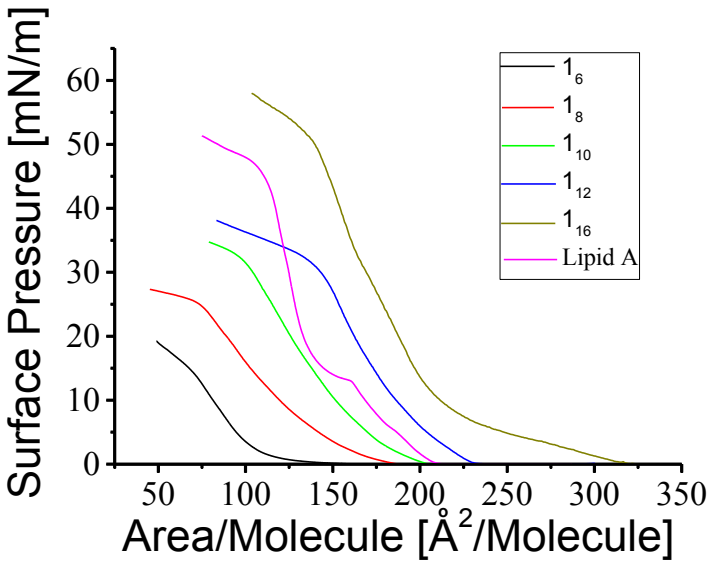


Figure S1. Langmuir isotherms measured for synthetic amphiphiles and Lipid A at an air-water interface at 25 °C. The estimated areas for each molecule, in Å^2, are follows: $\mathbf{1_6}$: 105, $\mathbf{1_8}$: 145, $\mathbf{1_{10}}$: 175, $\mathbf{1_{16}}$: 200, $\mathbf{1_{16}}$: 225, Lipid A: 150.

Small-Angle X-Ray Scattering (SAXS). SAXS measurements were performed in the Materials Science Center at the University of Wisconsin-Madison using a Bruker D8 Discover diffractometer. Amphiphile $\mathbf{1_6}$ and $\mathbf{1_{10}}$ (~15 mg) were weighed into a glass vial to which 20 mL of 1 M H_2SO_4 was added such that the final concentration of amphiphile was 0.133 wt%. The solid, gel-like samples were subjected to repeated vortex mixing and sonication at room temperature before being transferred to a quartz capillary tube. After addition of 1 M H_2SO_4, the tube was sealed with Parafilm and the samples were allowed to equilibrate in a humid environment at room temperature overnight prior to analysis by SAXS. Analysis of a sample of $\mathbf{1_{10}}$ incubated in 1 M H_2SO_4 for 1 week by ^{1}H NMR and mass spectrometry revealed no chemical degradation (Figure S2). SAXS analysis was performed with Cu-K_α X-rays produced from a micro x-ray source with a Montel mirror passed through a 0.5 mm pinhole to collimate the beam. Each sample was irradiated for 5 min at room temperature. Two-dimensional scattering patterns

were collected on a VANTEC-500 detector (140 mm in diameter, at a sample-to-detector distance of 22.26 cm) calibrated with a silver behenate standard (d = 58.38 Å). The resulting 2D SAXS patterns were azimuthally integrated using the DataSqueeze software package to obtain one-dimensional scattering profiles. Cubic diffraction lines were fit to peaks in the one-dimensional profile using the DataSqueeze software package and procedures written for use with Igor Pro. [6]

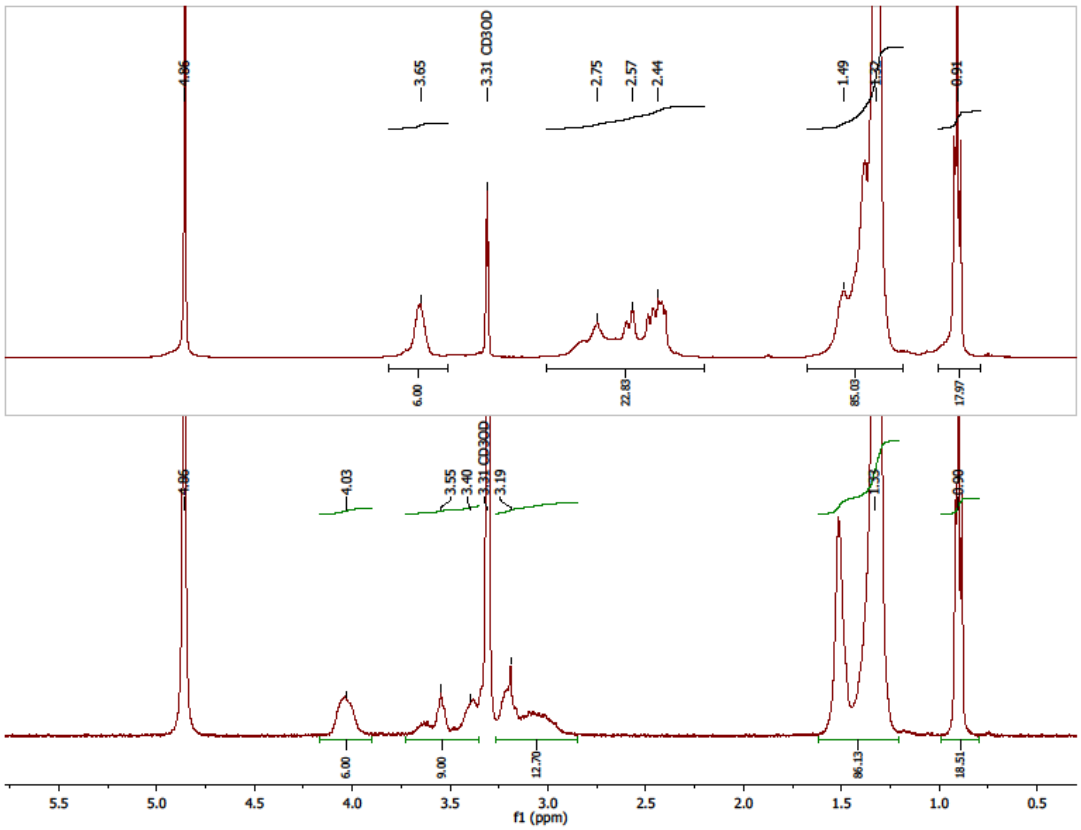


Figure S2. ^{1}H NMR spectrum (top) of untreated amphiphile $\mathbf{1_{10}}$ and (bottom) amphiphile $\mathbf{1_{10}}$ after incubation in 1 M H_2SO_4 for one week; both spectra were obtained in deuterated methanol (CD_3OD, δ 3.31 ppm). Mass spectrometry analysis (EI, positive ion mode) of the acid-treated amphiphile in 10 mM NH_4OAc/CH_3CN gave $[M^{2+}OAc^-]$ = 1144 and $[M^{3+}OAc^-]$ = 527.5, further suggesting the amphiphile was not chemically degraded by treatment in H_2SO_4 under these conditions.

Frequency histograms of forward light scattering (FSC) intensities measured for dispersions of bipolar and radial droplets and mixtures of radial and escaped radial droplets. Recently, we showed that measurement of low angle light scattering [so-called forward scattering (FSC)] in the flow focusing device of a flow cytometer can be used distinguish between LC droplets in a bipolar or radial configuration.[2] However, light scattering measurements for dispersions of escaped radial LC droplets, a configuration of 5CB droplets that persists at long times after interaction with amphiphiles (see main text), have not been reported to be independently distinguishable using this approach. We note here that the distinct light scattering from bipolar droplets and radial droplets, the latter of which closely resemble escaped radial droplets (see Figure 1D-I of the main text), stems from differences in the rotational symmetry of the refractive index environments of the droplet configurations.[2] Specifically, radial droplets possess a centrosymmetric refractive index environment, and thus scatter light as isotropic oil droplets. In contrast, bipolar droplets possess a rotationally-dependent refractive index environment, which leads to broadening of the distribution of FSC intensities measured by flow cytometry. Based on these previous results for bipolar and radial LC droplets and the nearly centrosymmetric refractive index environment of escaped radial droplets (see Figure 1E,H of the main text), we reasoned that the light scattering from escaped radial droplets would be similar to that from radial droplets. Consistent with our hypothesis, frequency histograms of FSC intensities for 5CB droplets dispersed in 500 pg/mL solutions of amphiphile $\mathbf{1_{10}}$ (see Figure 1 of the main text and surrounding discussion) were similar to those measured for a dispersion of droplets entirely in the radial configuration as a result of interaction with Lipid A (100 ng/mL Lipid A; Figure S3). From this result, we conclude that radial and escaped radial configurations of LC droplets lead to similar distributions of FSC intensities that cannot be distinguished from

each other, but both of which are readily distinguishable, alone or in combination, from distributions measured for bipolar droplets (Figure S3).

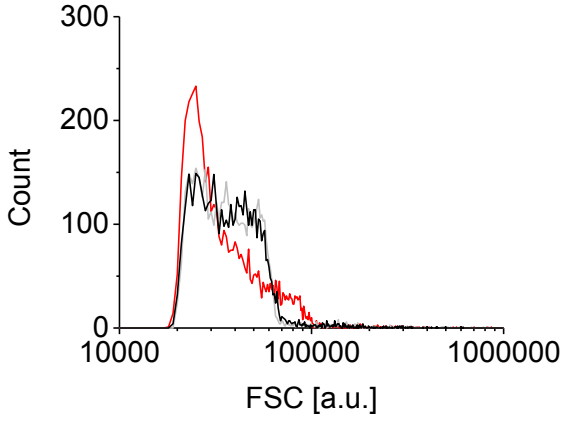


Figure S3. Frequency histogram of the intensity of forward light scattering (FSC) events generated by flowing droplets of nematic 5CB microdroplets dispersed in 10 μM aqueous solutions of SDS to which no lipid (red), 100 ng/mL of Lipid A (grey), or 500 pg/mL of amphiphile $\mathbf{1_{10}}$ (black) were added. The droplets dispersed in 10 μM SDS without added lipid were bipolar, whereas those dispersed in 100 ng/mL Lipid A were radial, and those dispersed in 500 pg/mL amphiphile $\mathbf{1_{10}}$ were a combination of escaped radial and radial. The concentration of droplets was ~5,000 droplets/μL and the histograms were constructed from an analysis of 5,000 droplets.

References

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