Supporting Information for:

Preparation, characterization, and formulation optimization of ionic liquid-in-water nanoemulsions towards systemic delivery of Amphotericin B

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MATERIALS. Normal saline (AirLife sterile 0.9% sodium chloride for irrigation USP) and Amphotericin B for injection USP (Gen-X pharmaceuticals) were purchased from the University of Wisconsin-Madison Hospital Pharmacy. Amphotericin B powder was purchased from Sigma-Aldrich Co, stored at 4°C, and shielded from light during storage. Rabbit red blood cells were purchased from Lampire Biologic Laboratories, Inc (Pipersville, PA) and stored at 4°C. The rabbit red blood cells were used within 2 weeks of receipt. Phosphate buffered saline (pH 7.4) (PBS) was purchased from Cellgro (Mediatech Inc., Manassas, VA). N-(Methylpolyoxyethyleneoxycarbonyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (DSPE-PEG, 2000 g/mol average molecular weight PEG) and 1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene (DSG-PEG, 2,000 g/mol average molecular weight of PEG) were purchased from NOF America Corporation (White plains, NY) and stored at -20°C. 1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene (DSG-PEG, 2,000 g/mol average molecular weight of PEG) was also purchased from Avanti® Polar Lipids, Inc (Alabaster, AL) and stored at -20°C. 1,7-dibromohexane, 98% was purchased from Fisher Scientific (Hampton, NH). Solvents and all other reagents were purchased from Sigma-Aldrich Co. (Milwaukee, WI) and Spectrum (Gardena, CA) and used as received, unless otherwise mentioned**.**

SYNTHETIC METHODS.

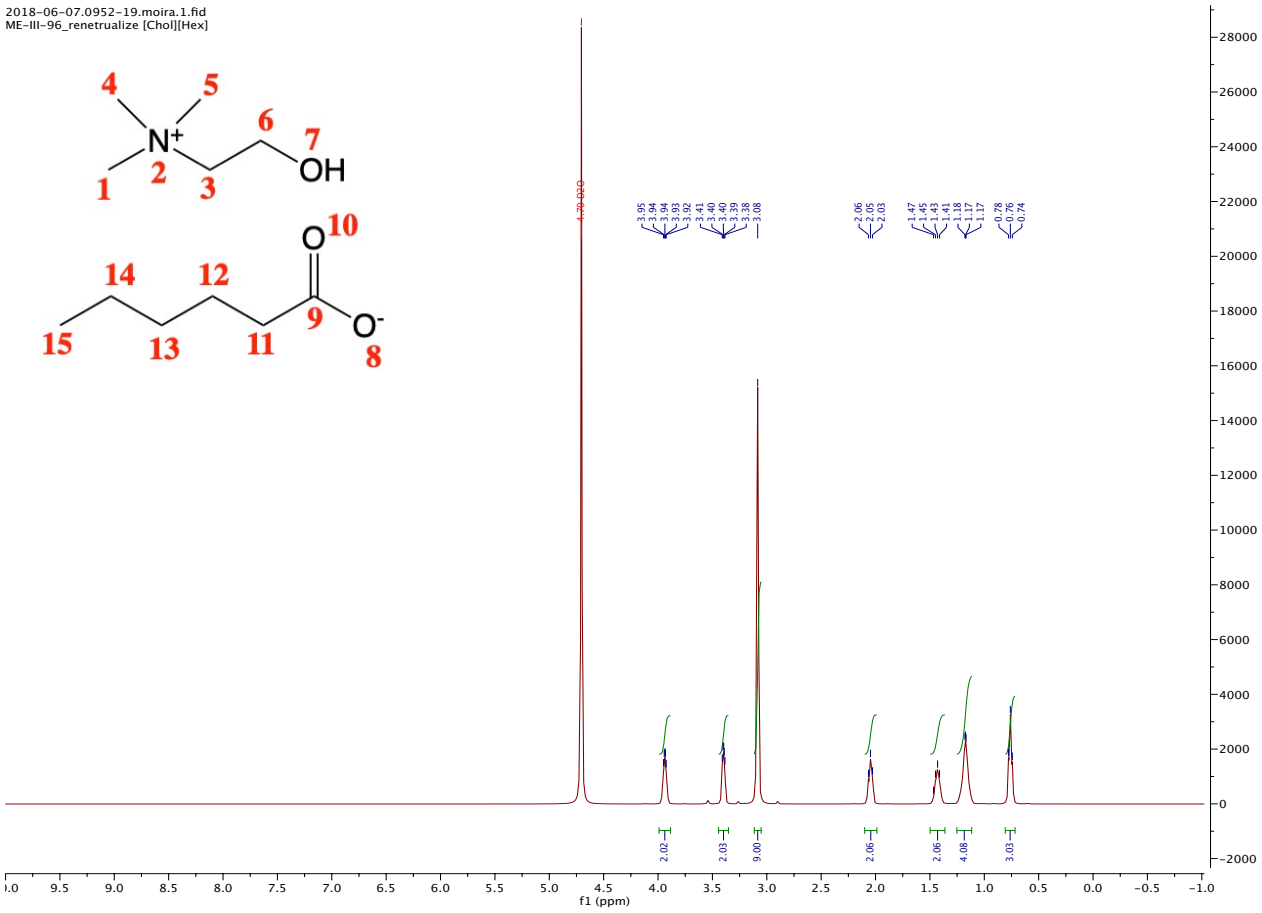
Synthesis of cholinium hexanoate. Cholinium hexanoate ([Chol][Hex]) was synthesized via an acid-base neutralization reaction between choline bicarbonate (~80% in water) and hexanoic acid. Hexanoic acid was added dropwise to the aqueous solution of choline bicarbonate. The reaction mixture was stirred at room temperature open to atmosphere for 24 hours. Water was subsequently removed using rotary evaporation under reduced pressure at 60°C. The isolated ionic liquid was further dried under high vacuum for 2 hours at 60°C. The ionic liquid was then further dried under high vacuum for 48 hours at room temperature.

Synthesis of dicationic cholinium-based bromide salt. Dicationic cholinium-based bromide salt ([DC-7][2Br]) was prepared via a quaternization reaction. Dimethylaminoethanol (0.078 mol) and alkyl dibromide (0.039 mol) were added to a glass reaction flask containing acetonitrile (100 mL). The reaction mixture was refluxed overnight. After this time, the reaction was cooled to room temperature. The white precipitate was filtered off and rinsed three times with acetone (60 mL acetone). The salt was dried under reduced pressure for a minimum of 24-hours before use.

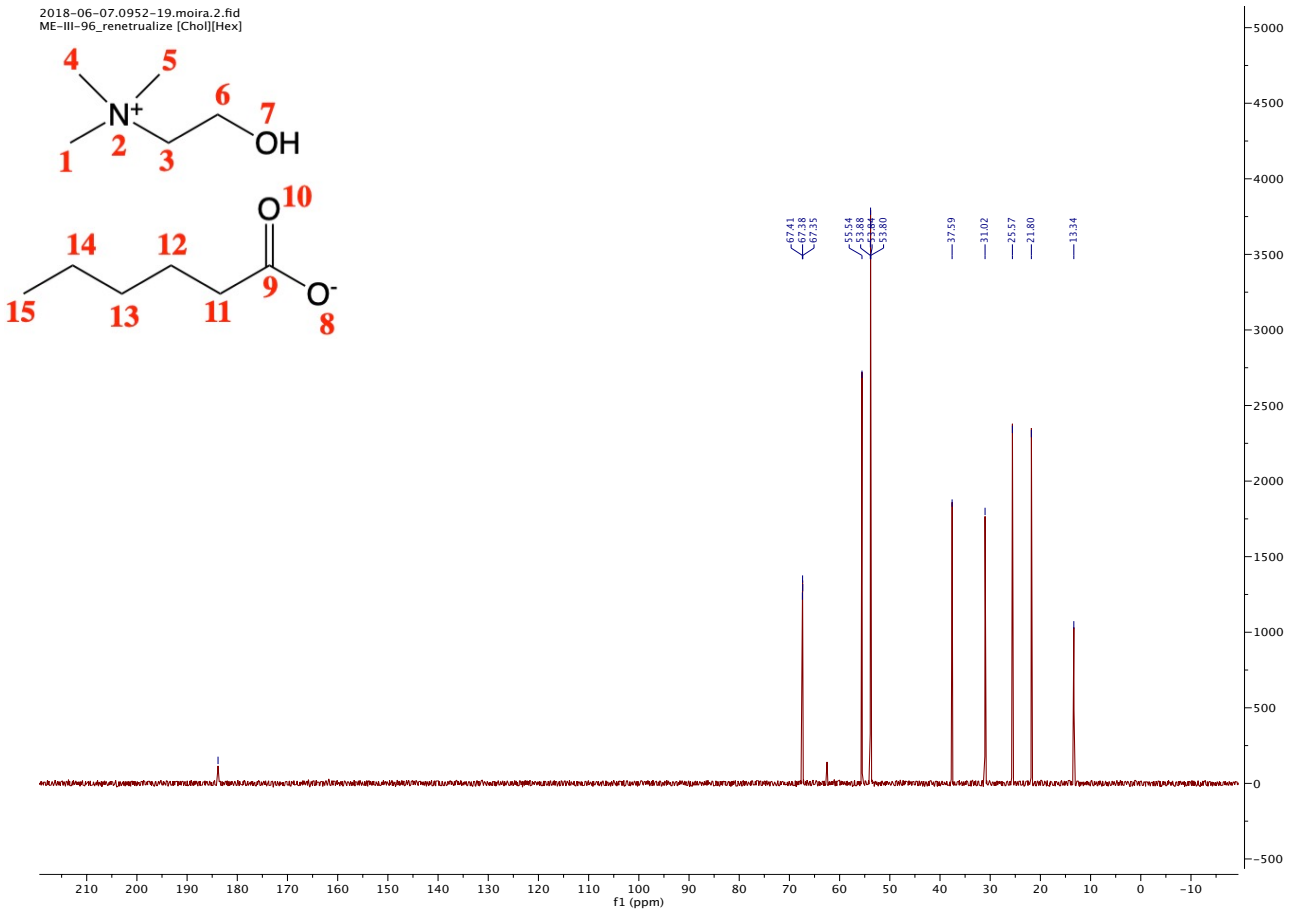
Synthesis of cholinium-based dicationic ionic liquid. Dicationic cholinium-based ionic liquid ([DC-7][2NTf_2]) was prepared via an anion-exchange metathesis reaction. Cholinium-based dicationic bromide salt (0.034 mol) was dissolved in Millipore water (500 mL). Lithium bis(trifluoromethylsulfonyl)imide (0.068 mol) was then added to the reaction mixture. The reaction mixture was stirred until phase separation was observed (between 6 and 12 hours). The ionic liquid layer was isolated and washed with deionized water until no bromide was observed using the silver nitrate test (1M silver nitrate in water).

CHARACTERIZATION METHODS

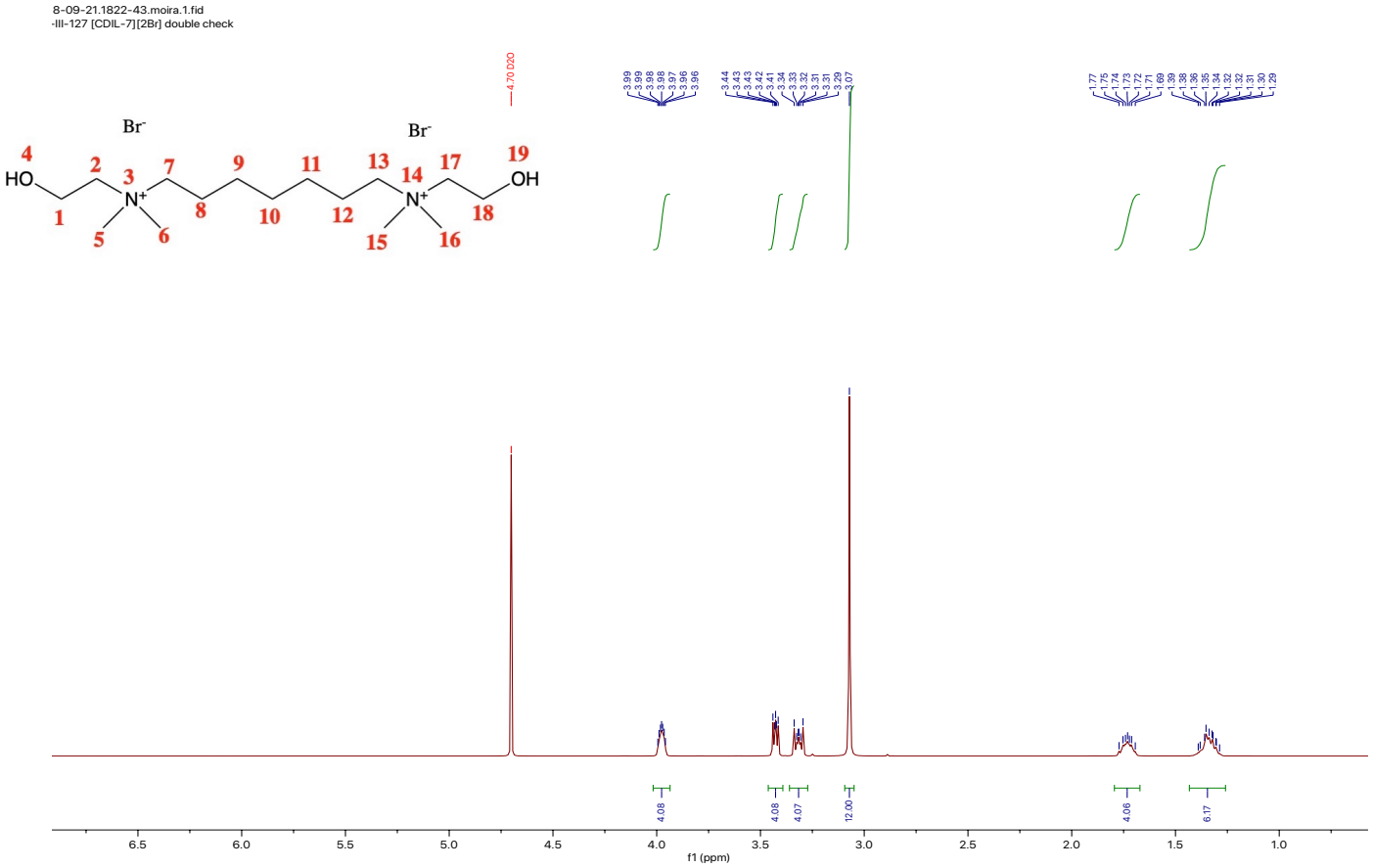
^{1}H, ^{13}C, and ^{19}F NMR spectroscopy. ^{1}H, ^{13}C, and ^{19}F NMR spectra were measured using a Bruker Avance III HD 400 MHz spectrometer or the Varian UI 500 MHz spectrometer. All spectra were measured with either $(CD_3)_2CO$, CD_3OD, or D_2O as the solvent.



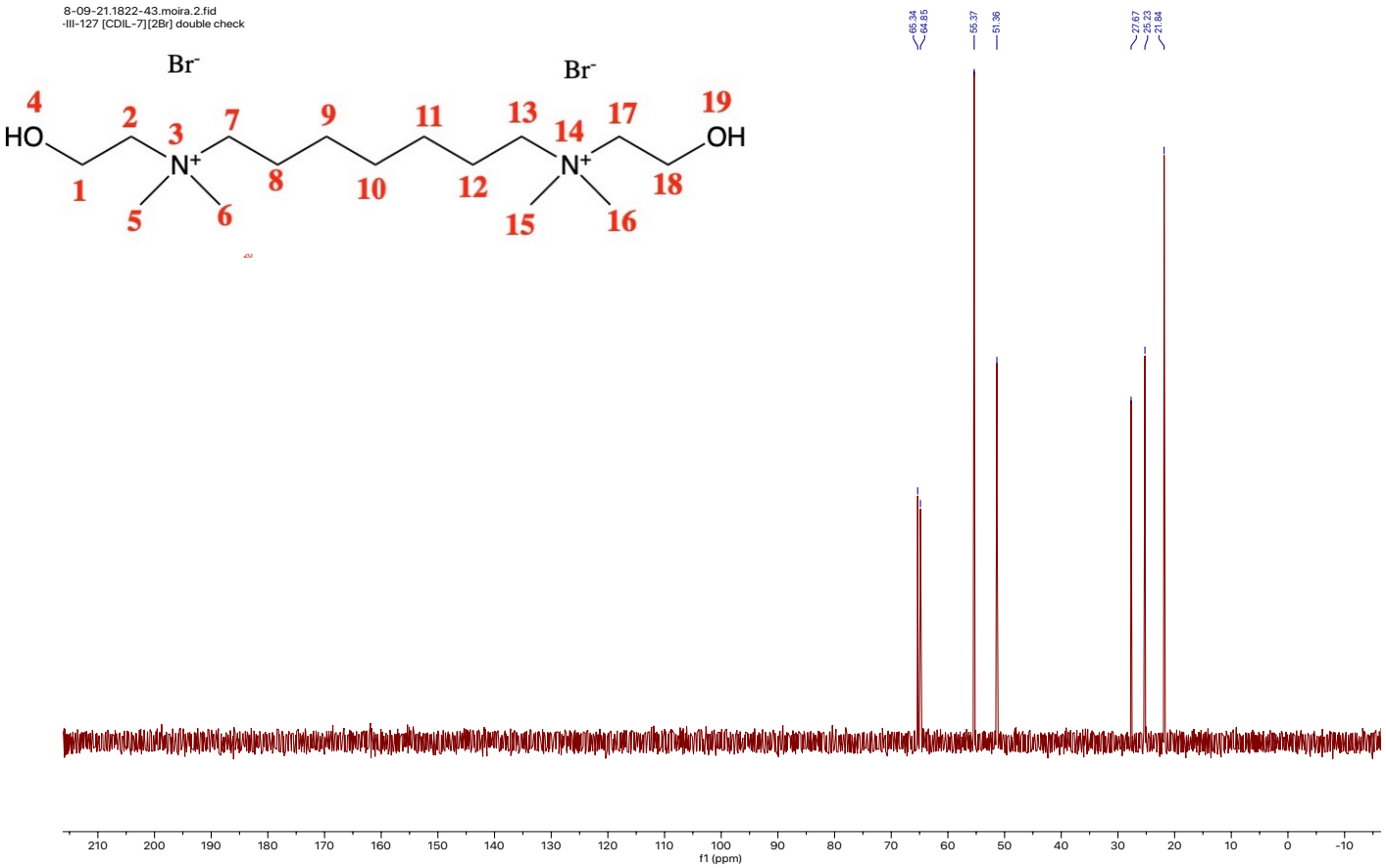
Spectra S1. ^{1}H NMR of [Chol][Hex] (**1**) hydrophilic ionic liquid in D_2O.
^{1}H NMR (D_2O, 400 MHz): δ/ppm= 0.76 (15 - t, *J* = 7.2 Hz, 3H); 1.17 (13,14 - m, 4H); 1.43 (12 - m, 2H); 2.05 (11 - t, *J* = 7.4 Hz, 2H); 3.08 (1, 4, 5 - s, 9H); 3.40 (6 - m, 2H); 3.94 (3 - m, 2H).



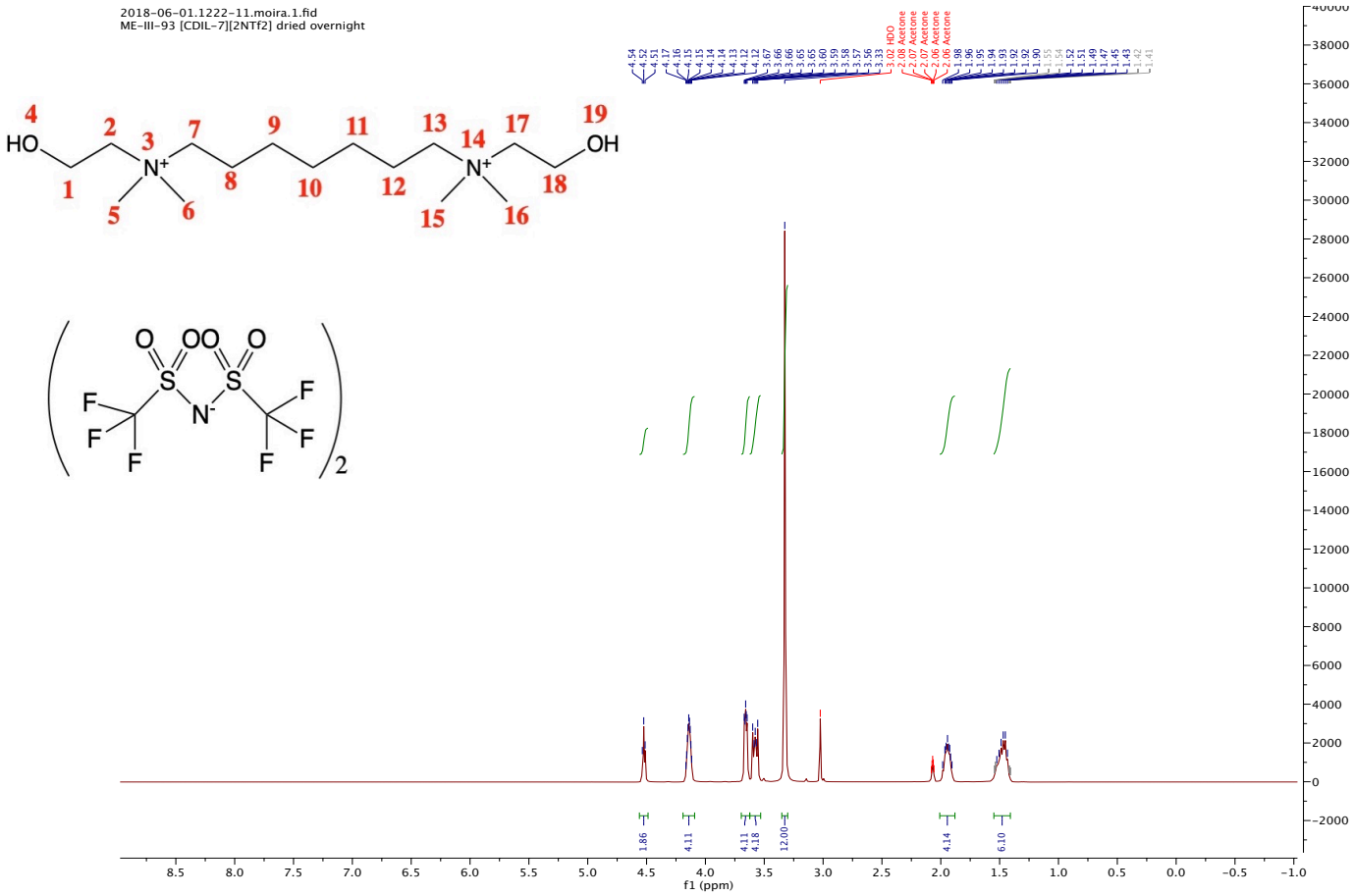
Spectra S2. ^{13}C NMR of [Chol][Hex] (**1**) hydrophilic ionic liquid in D_2O.
^{13}C NMR (D_2O, 400 MHz): δ/ppm = 13.3 (15); 21.8 (14); 25.5 (13); 31.0 (12); 37.5 (11); 53.8 (t, 1, 4, 5) ; 55.5 (6); 67.3 (t, 3); 183.8 (9)



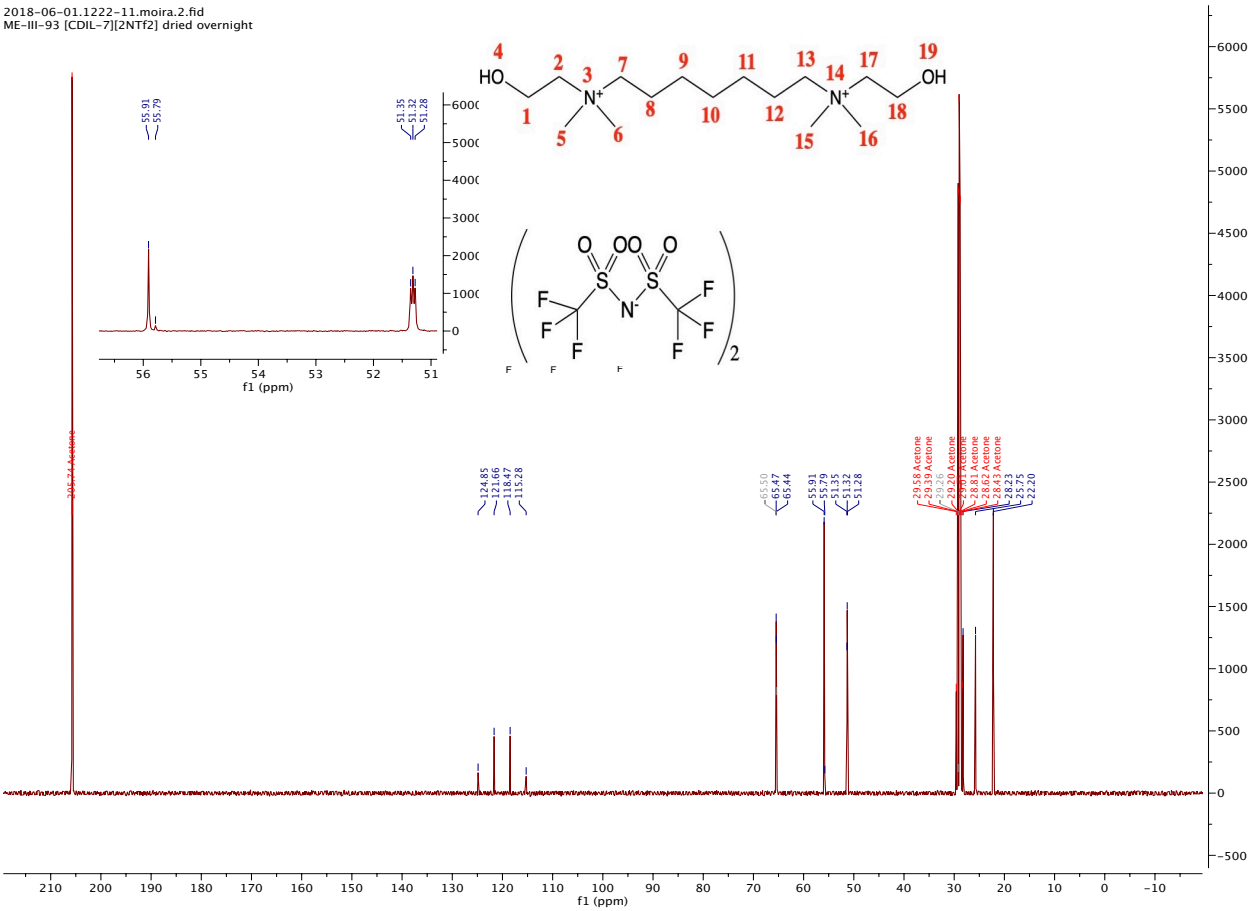
Spectra S3. ^{1}H NMR of [DC-7][2Br] (**2**) hydrophobic ionic liquid precursor in D_2O. ^{1}H-NMR (400 MHz, D_2O): δ/ppm 3.98 (2, 17 - m, 4H), 3.43 (7, 13 - m, 4 H), 3.32 (1, 18 - m, 4H), 3.07 (5, 6, 15, 16 - s, 12H), 1.73 (8, 12 - m, 4H), 1.32 (9, 10, 11 - m, 6H).



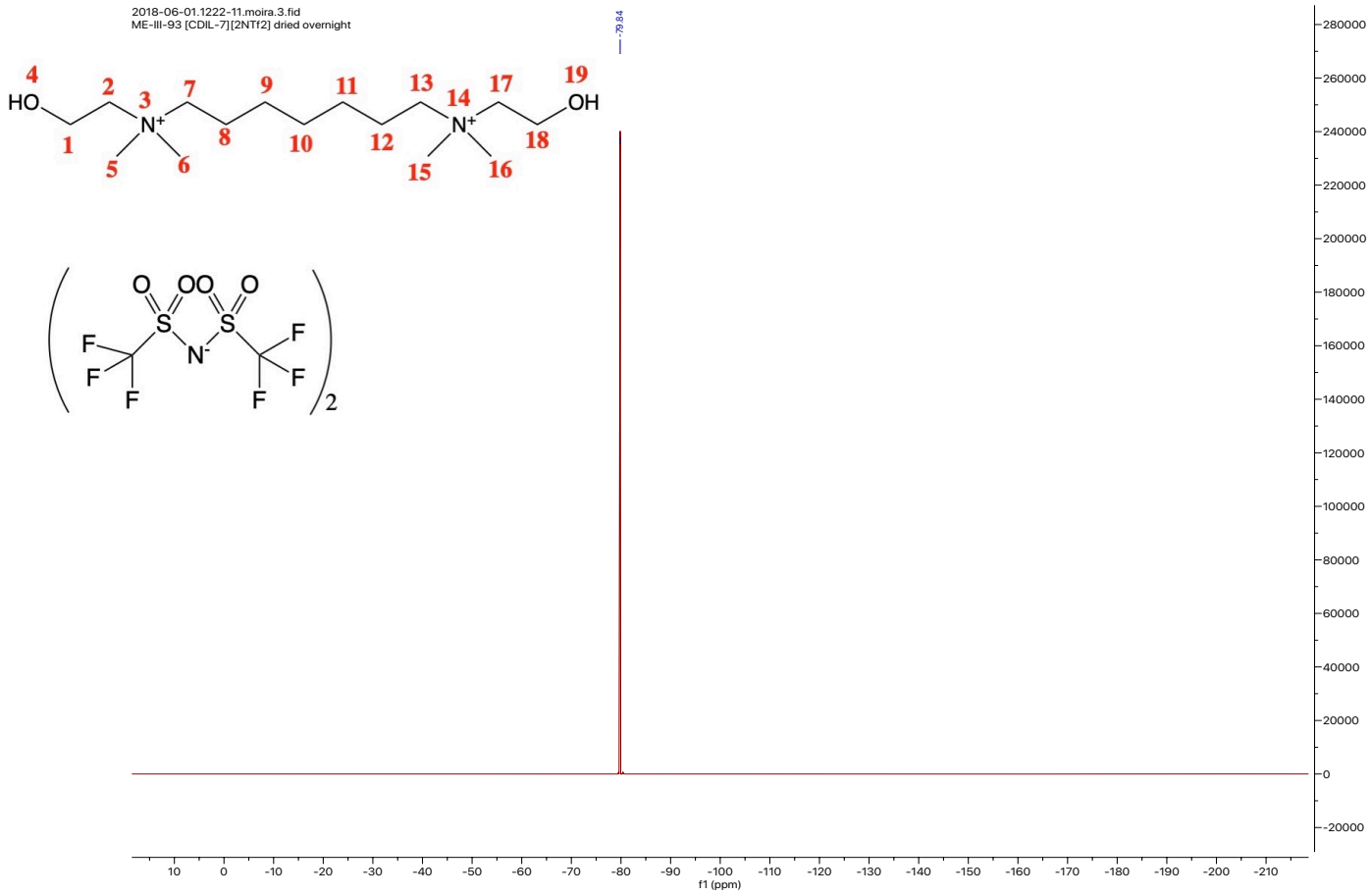
Spectra S4. ^{13}C NMR of [DC-7][2Br] (**2**) hydrophobic ionic liquid precursor in D_2O. ^{13}C NMR (101 MHz, D_2O): δ/ppm 65.34 (2, 17), 64.85 (7, 13), 55.37 (5, 6, 15, 16), 51.36 (1, 18), 27.67 (8, 13), 25.23 (9, 11), 21.84 (10).



Spectra S5. ^{1}H NMR of [DC-7][2NTf$_2$] (**3**) hydrophobic ionic liquid in $(CD_3)_2CO$. ^{1}H NMR (400 MHz, Acetone-d^6: δ/ppm 4.52 (4, 19 - t, 2H), 4.14 (2, 17 - m, 4H), 3.65 (7, 13 - m, 4H), 3.59 (1, 18 - m, 4H), 3.33 (5, 6, 15, 16 - s, 12H), 1.93 (8,12 - m, 4H), 1.51 (9, 10, 11 - m, 6H).



Spectra S6. ^{13}C NMR of [DC-7][2NTf$_2$] (**3**) hydrophobic ionic liquid in $(CD_3)_2CO$. ^{13}C NMR (101 MHz, Acetone-d^6): δ/ppm 120.0 (two CF_3 groups q, J_{C-F} = 320 Hz), 65.48 (2, 17), 55.91 (7, 13), 55.79 (5, 6, 15, 16), 51.32 (t, J_{C-H} = 3.8 Hz) (1, 18), 28.24 (8, 13), 25.75 (9, 11), 22.20 (10).



Spectra S7. ^{19}F NMR of [DC-7][2NTf$_2$] (**3**) hydrophobic ionic liquid in $(CD_3)_2CO$. ^{19}F NMR (376 MHz, Acetone-d^6): δ/ppm -79.84 (two CF_3 groups).

Quantitative ^{19}F NMR spectroscopy (QNMR) for determination of concentration of hydrophobic ionic liquid in a water-solution. ^{19}F NMR spectra was measured using a Varian UI 500 MHz spectrometer equipped with a Nalorac Quad Nucleus DD probe (qn6121, 5 mm). The internal temperature of the NMR spectrometer was maintained at 25°C. Sodium trifluoroacetate was used as an internal standard was employed in order to determine the concentration of [DC-7][2NTf$_2$]. The internal standard was prepared in D_2O and the stock solution was allowed to sonicate at 30°C for 4 hours prior to use in order to ensure complete dissolution of sodium trifluoroacetate. Prior to quantitative study, the T_1 value of both [DC-7][2NTf$_2$] and sodium trifluoroacetate were determined using an inversion recover experiment acquired with 12 independent quadratically spaced variable (tau) values (values from 0.06944 – 10). The T_1 value was calculated in order to ensure that both signals fully relaxed between pulses. The following parameters were employed for acquisition of T_1 spectra: 500 MHz; spectra width, 107962.2 Hz ; acquisition time, 1.004 s; number of points, 216688; relaxation delay, 18.996 s; number of transients, 1; 90° pulse width, 17.1 µs. X-Win32 software was used to calculate the T_1 value and processing included a line broadening of 1 Hz. The T_1 value of the sodium trifluoroacetate and [DC-7][2NTf$_2$] was determined to be 2.76 ± 0.172 seconds and 2.69 ± 0.0885 seconds, respectively. After determination of T_1, the QNMR study was completed on an optimized spectrum. The following parameters were employed for acquisition of QNMR spectrum: 500 MHz; spectral width, 107962.2 Hz; acquisition time, 2.00 s; number of points, 431848; relaxation delay, 28.00 s; number of transients, 32; 90° pulse width, 17.1 µs. The acquired spectrum has a signal to noise ratio of 4700, which exceeded the desired 3000 needed for quantitation. Sample preparation was as follows and exactly mimicked samples preparation for *in vivo* toxicity study to ensure accurate representation of concentration: 1.5 mL of [DC-7][2NTf$_2$] was added to a 15 mL conical centrifuge tube followed by 1.5 mL of D_2O. The sample was allowed to sit for 6 days prior to use. Each day, the sample was sonicated at 40°C for 6 hours and then allowed to remain at room temperature for 18 hours. After 6 days, the sample was centrifuged at 2000 rpm for 5 minutes to ensure complete sedimentation of the ionic liquid. 0.5 mL of D_2O was removed and then subsequently analyzed. Integration of the internal standard and [DC-7][2NTf$_2$] allowed for quantitation of the concentration of [DC-7][2NTf$_2$] present in the stock solution used for the zebrafish viability assay. The concentration of [DC-7][2NTf$_2$] in the D_2O sample was found to be 10.66 mM using the following formula:

$$\textbf{Concentration IL} = \frac{\left(\frac{\boldsymbol{Integration\ DC{-}7}}{\mathbf{12}}\right)\left(\boldsymbol{Concentration\ Standard}\right)}{\left(\frac{\boldsymbol{Integration\ Standard}}{\mathbf{3}}\right)}$$

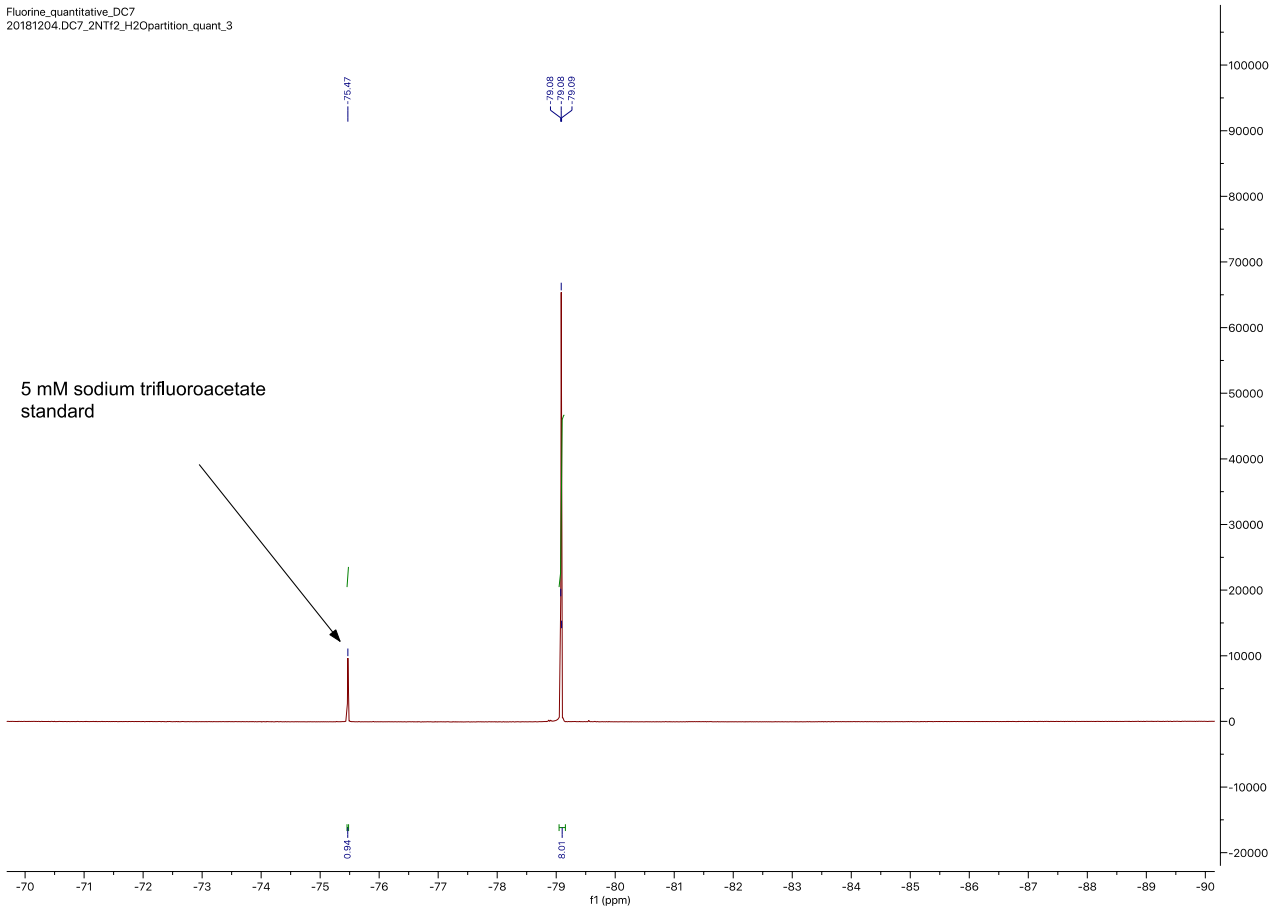


Figure S8. ^{19}F NMR of [DC-7][2NTf$_2$] (**3**) in D_2O.

Electrospray Ionization Mass Spectrometry (ESI). Sample was analyzed using ESI ultra high resolution QTOF MS (MaXis 4G) with ESI infusion on positive ion mode and negative ion mode. Sample was prepared with a 1:1,000 dilution in methanol for the positive ion mode and 1:100,000 in methanol for the negative ion mode.

Compound formula	Theoretical (monoisotopic) mass	5 ppm	Range	Observed Mass	Mass Accuracy (ppm)	Resolution (FWHM)	Intensity
$[C_{15}H_{36}N_2O_2]^{2+}$	138.138291	138.1376003	138.1389817	138.13770	-4.278	16,189	1,539,372
$[C_2F_6N_1O_4S_2]^-$	279.916745	279.9153454	279.9181446	279.91606	-2.447	49,005	1,065,709

Table S1. Analysis report of [DC-7][2NTf$_2$] using electrospray ionization mass spectrometry.

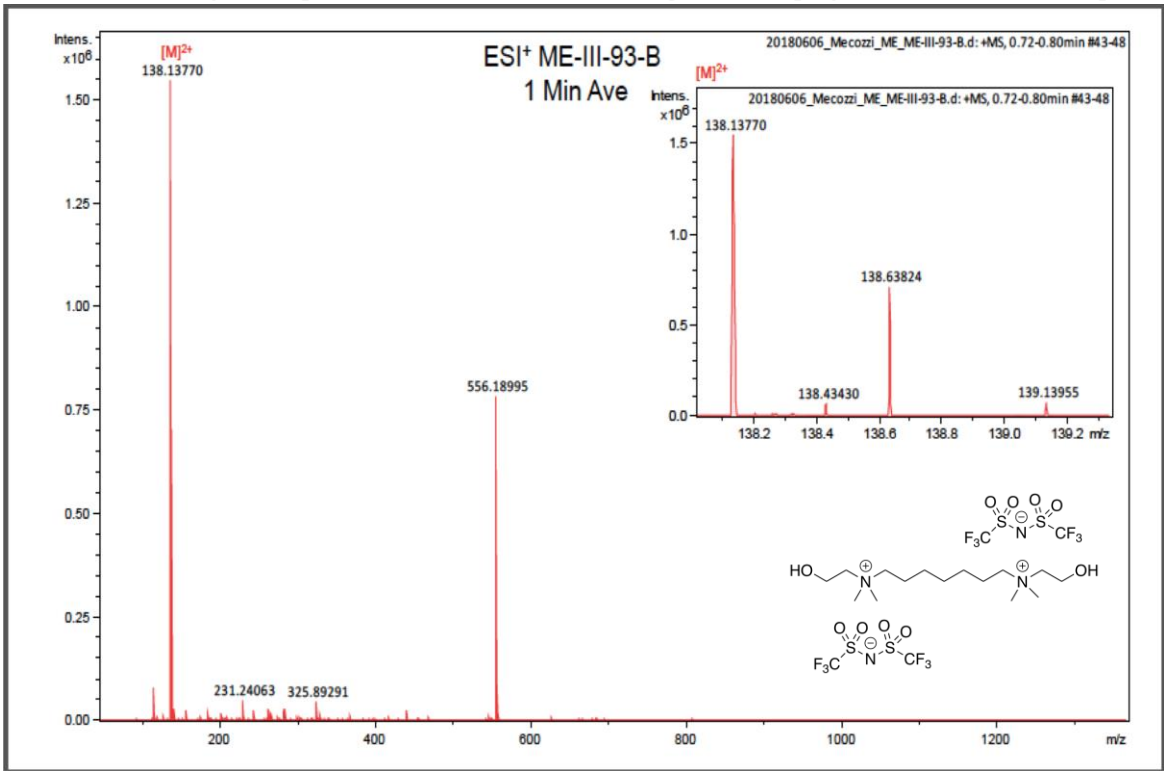


Figure S9. ESI-MS spectrum of [DC-7][2NTf$_2$] (**3**) hydrophobic ionic liquid in positive ion mode.

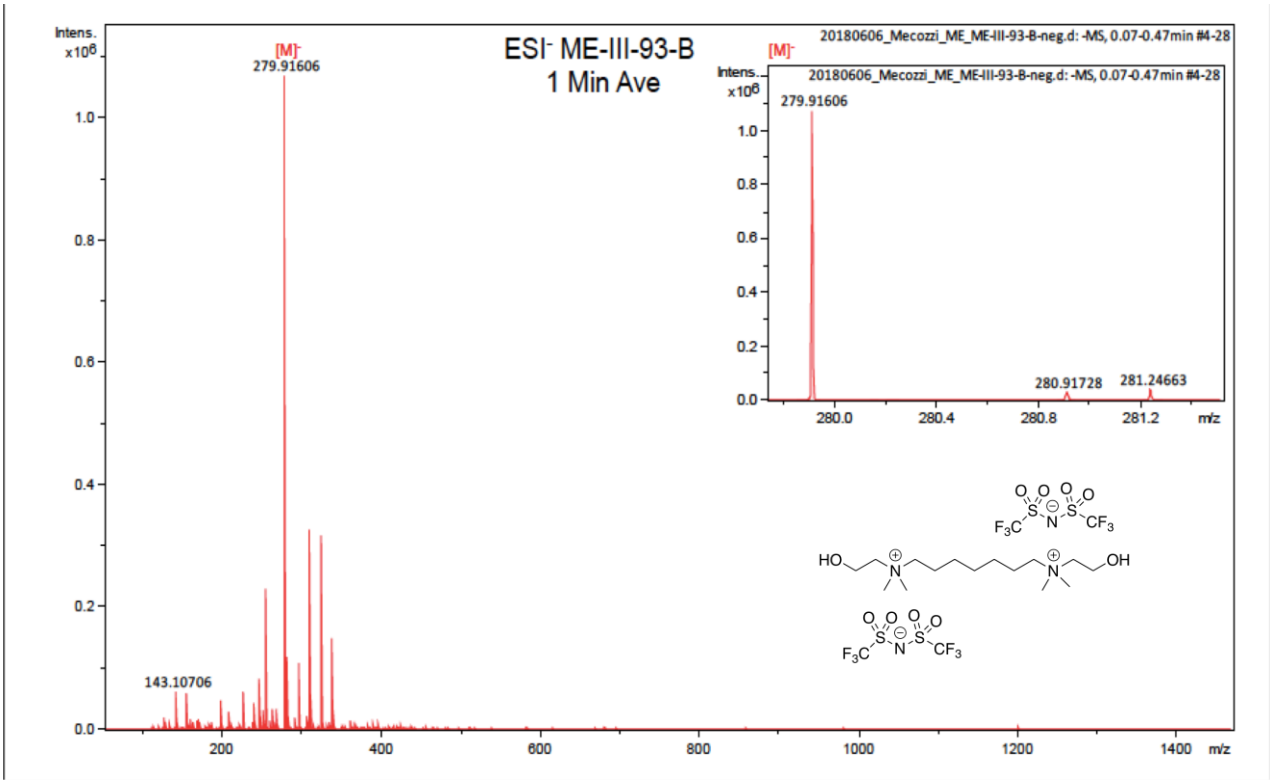


Figure S10. ESI-MS spectrum of [DC-7][2NTf$_2$] (**3**) hydrophobic ionic liquid in negative ion mode.

Differential Scanning Calorimetry. Thermograms in a temperature range from -80°C to 100°C were recorded using a differential scanning calorimeter from Perkin Elmer, model DSC 7. The heating-cooling rate was 10°C min^{-1}. The sample was equilibrated at 25°C followed by heating to 100°C. An isothermal hold occurred for 10 minutes and then the sample was cooled to -80°C, followed by another isothermal hold for 10 minutes. The sample was then heated to 100°C. The melting point of [DC-7][2NTf_2] was determined to be 40°C.

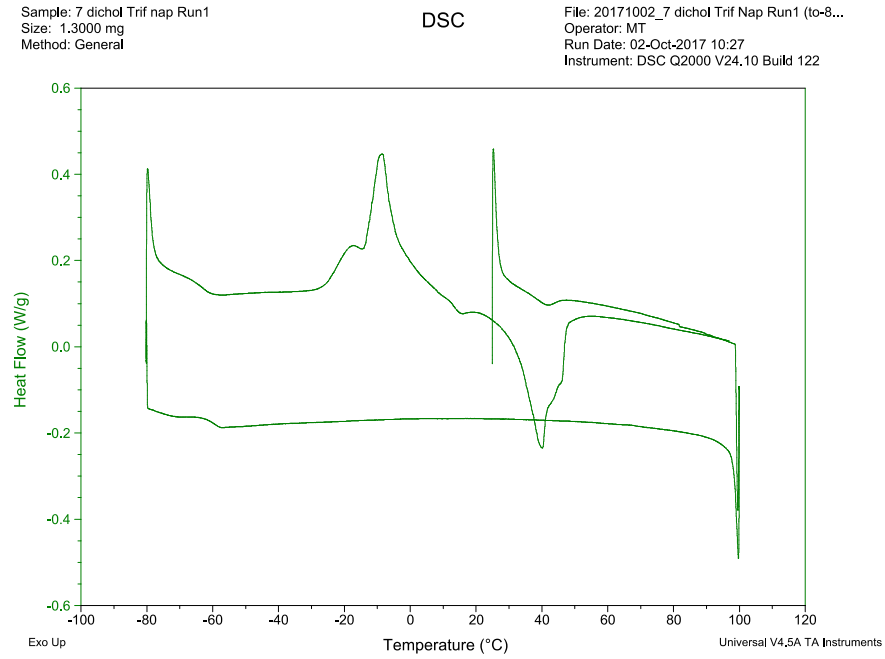


Figure S11. Differential scanning thermographs with heat flow vs. temperature.

***In Vivo* Developmental Toxicity Study.** An embryo − larval zebrafish (*Danio rerio*) model was used to evaluate the toxicity of the novel dicationic cholinium-based ionic liquid. The following protocol was taken from previously published protocol, analyzing the toxicity of a novel triphilic polymers for use as a component in a nanoemulsion.[1] Zebrafish of the AB strain were obtained from Dr. Michael Taylor at the University of Wisconsin-Madison School of Pharmacy, where the fish were cultured until sexual maturation. Zebrafish were maintained in a light/ dark cycle of 14:10 h at 28.5 °C in egg water (0.03% Instant Ocean, Blacksburg, VA, USA).The adult fish were fed *Artemia nauplii* twice daily. Embryos were obtained from adult fish with a ratio of 1:2 for female to male. Six breeding groups were placed in separate spawning aquariums, equipped with a mesh bottom to prevent the eggs from being cannibalized. Crossing was induced in the morning. After one hour, eggs free of macroscopically discernible symptoms of infection and disease were collected, rinsed with egg water, and transferred into Petri dishes until chemical exposure. The embryo−larval toxicity assay was subsequently carried out. 8 fertilized eggs at 2 hours post fertilization (hpf) stage were added to a 24-well plate. Each well was filled with eggs and contained 600 µL of egg water. Six concentrations plus two controls (negative and positive) were plated. For analysis of [DC-7][2Br], sodium bis(trifluoromethylsulfonyl)imide, and [DC-7][2NTf_2] containing nanoemulsion the concentrations analyzed were 1 mM, 333 µM, 111 µM, 37 µM, 12.3 µM, and 4.1 µM. 1.5 mL of [DC-7][2NTf_2] was added to a 15 mL conical centrifuge tube followed by 1.5 mL of egg water. The sample was allowed to sit for 6 days prior to use. Each day, the sample was sonicated at 40°C for 6 hours and then allowed to remain at room temperature for 18 hours. After 6 days, the sample was centrifuged at 2000 rpm for 5 minutes to ensure complete sedimentation of the ionic liquid. 1.2 mL of egg water was removed and served as the stock solution. This served as the highest concentration sample. Integration of the internal standard and [DC-7][2NTf_2] in the QNMR sample allowed for quantitation of the concentration of [DC-7][2NTf_2] present in the stock solution used for the zebrafish viability assay. The concentrations analyzed for [DC-7][2NTf_2] sample were 7.11 mM, 3.56 mM, 1.78 mM, 889 µM, 444 µM, and 222 µM.

The plates were covered and incubated at 28.5 °C in a light/dark cycle of 14:10 throughout the 96 hpf exposure period. The observations of zebrafish development were made directly in the well using a stereomicroscope (Nikon SMZ18) every 24 hours. The end points that were selected to monitor the effects effects of [DC-7][2Br], sodium bis(trifluoromethylsulfonyl)imide, [DC-7][2NTf_2] saturated water solution and nanoemulsion included mortality, spontaneous movement, hatching success, pericardial edema, and curved body axis. Embryos and larvae were considered dead when no heartbeat was observed. The number of hatched embryos and a cumulative mortality tally were recorded every 24 h after 2 hpf. At 96 hpf, following the final observations, representative larvae for [DC-7][2Br] and [DC-7][2NTf_2] saturated egg water were anesthetized with 0.4% tricaine mesylate solution and mounted on Petri dishes using low melting point agarose. These larvae were then photographed using a high-definition color microscope camera (Nikon DSFi2). After photographing, all larvae used for the analysis were euthanized.

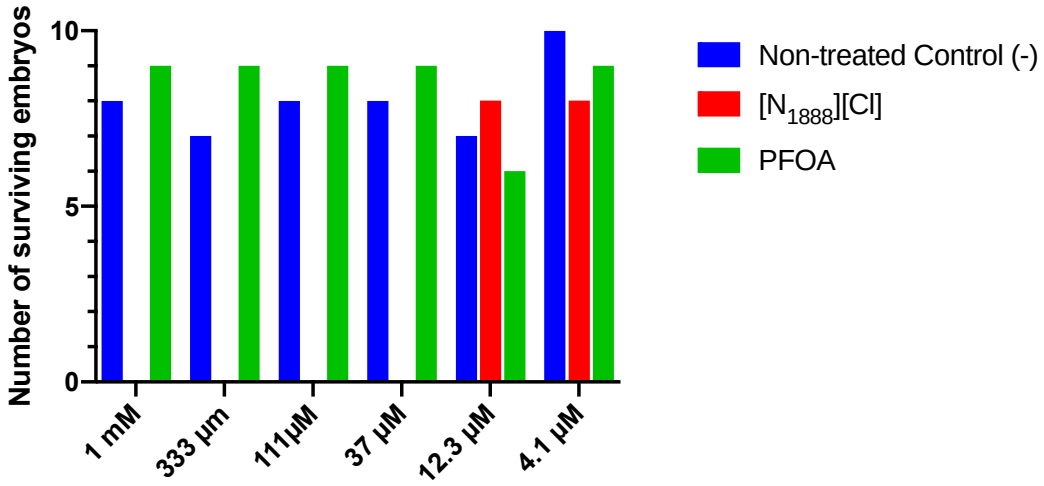


Figure S12. Zebrafish survival after exposure to methyltrioctylammonium chloride 0 hours post-fertilization Average number of embryos surviving after exposure to methyltrioctylammonium chloride 0 hours post-fertilization. All embryos tested with a specific solution either survived or died. Non-treated control is represented in blue, methyltrioctylammonium chloride is represented in red, and perfluorooctanoic acid is in green.

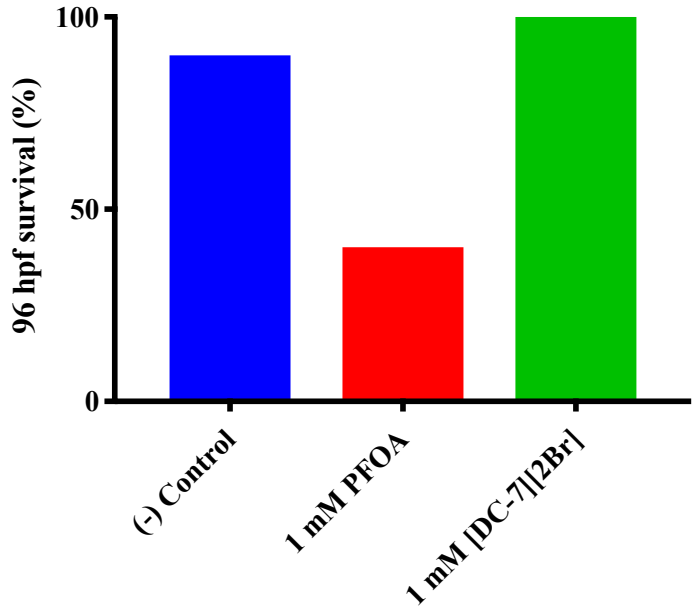


Figure S13. Zebrafish survival 96 hours post-fertilization of water-soluble DC precursor ([DC-7][2Br], green), non-treated control (blue), and positive control perfluorooctanoic acid (PFOA, red).

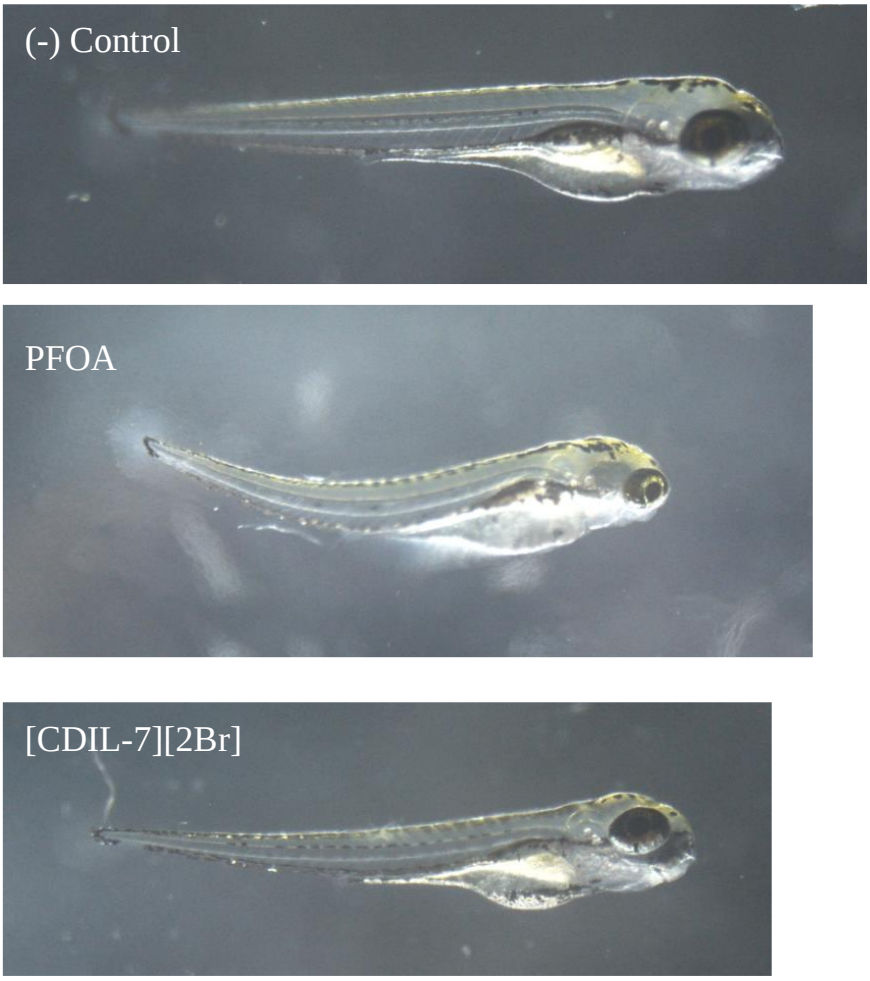


Figure S14. Microscope images for malformation analysis of zebrafish 96 hours post-fertilization of water-soluble DC precursor ([DC-7][2Br], bottom labeled [CDIL-7][2Br]), non-treated control (top), and positive control perfluorooctanoic acid (PFOA, middle). Both the non-treated control and [DC-7][2Br] exhibit no malformations, in comparison to PFOA which exhibits spinal curvature.

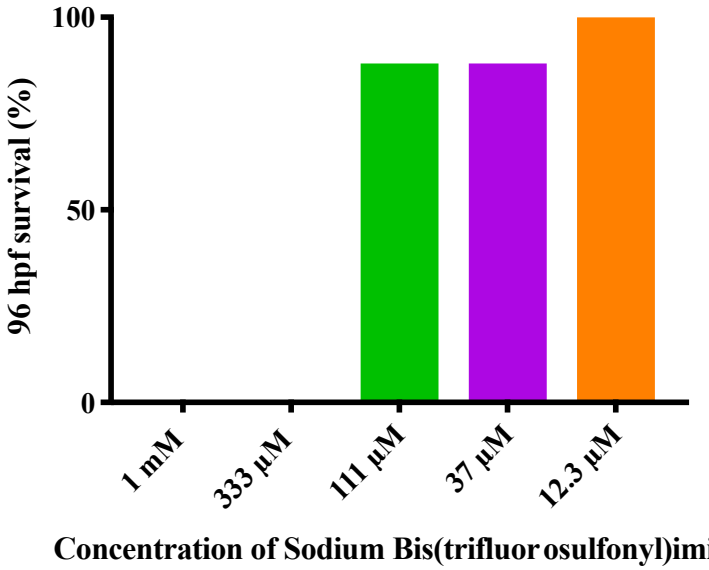


Figure S15. Zebrafish survival 96 hours post-fertilization of water-soluble anion salt, sodium bis(trifluoromethylsulfonyl)imide.

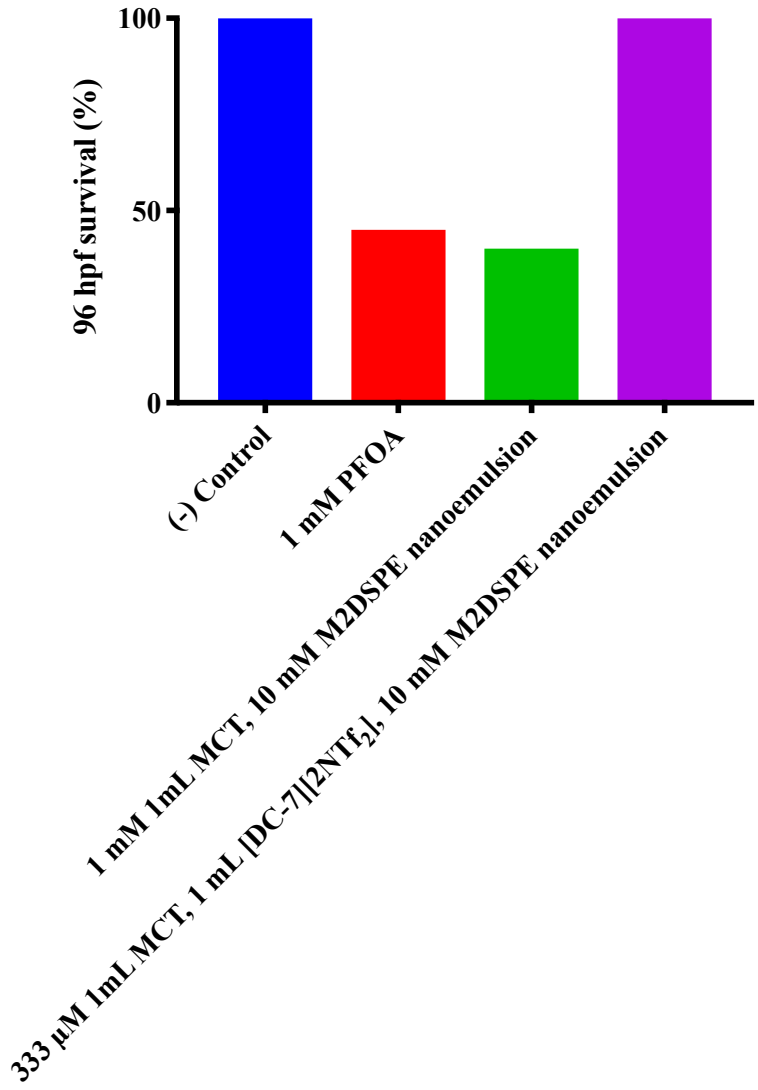


Figure S16. Zebrafish survival 96 hours post-fertilization of nanoemulsion prepared with [DC-7][2NTf$_2$] (purple), non-treated control (blue), positive control perfluorooctanoic acid (PFOA, red), and an additional nanoemulsion to serve as control (green).

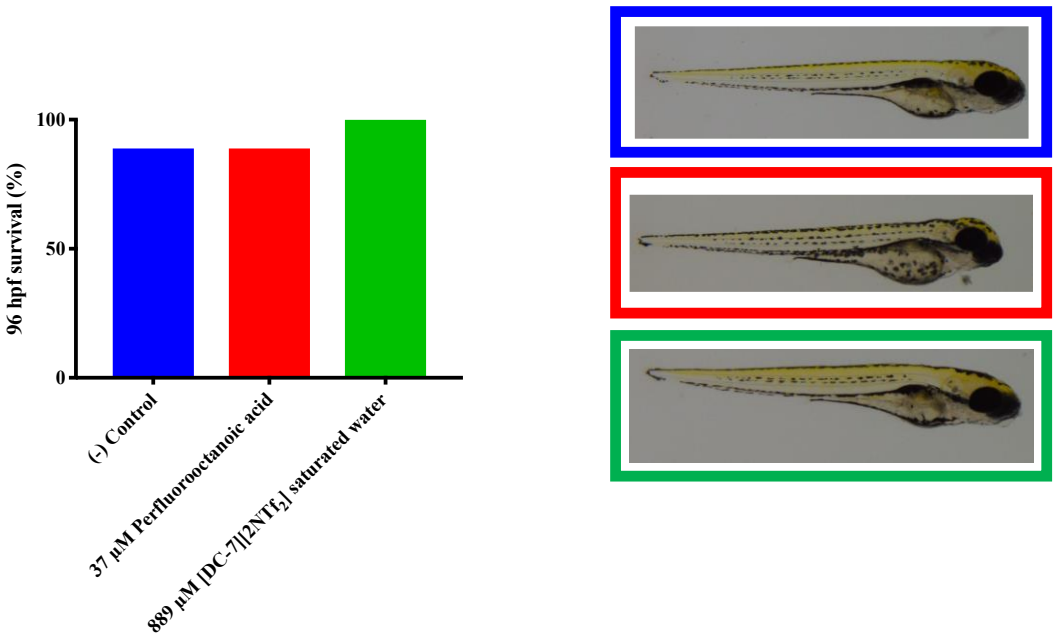


Figure S17. Zebrafish survival 96 hours post-fertilization after treatment with [DC-7][$2NTf_2$] saturated water: nontreated control (blue), perfluorooctanoic acid positive control (red), and [DC-7][$2NTf_2$] saturated water (green).

UV-visible Spectroscopy. Measuring absorbance of Amphotericin B (AmB) at distinct wavelengths using UV/vis spectroscopy provides insight into its degree of self-aggregation. Absorption spectra were obtained on a Varian Cary 100Bio UV-vis spectrometer (Varian, Palto Alto, CA) at 25°C using a quartz coverslip with a 0.1 mm pathlength (Starna Cells, Inc., Atascadero, CA) or a 1 mm pathlength quartz cuvette (Starna Cells, Inc., Atascadero, CA). Absorbance was measured from 300 nm to 500 nm. Samples were prepared by direct addition of AmB powder to neat ionic liquids. Ionic liquids solid at room temperature were first melted using a water bath at 60°C prior to addition of AmB powder. After addition of solid drug, (1) 100 µL of methanol was then added to the IL mixture and the mixture was stirred at room temperature for 1 hour. (2) The drug/IL mixture was then placed under rotary evaporation in a water bath with at a temperature of 60°C for 1 hour. (3) The drug/IL mixture was then placed under high vacuum for 1 hour. (4) The drug/IL mixture was then allowed to stir for 1 hour. This process (steps 1-4) was repeated until all drug visibly was solubilized. The drug/IL mixture was then placed under rotary evaporation at 60°C for an additional hour and then under high vacuum for 24 hours prior to use to ensure removal of all methanol. For samples containing [DC-7][2NTf$_2$], if the IL solidified while under vacuum, the sample was placed under rotary evaporation at 60°C for an additional hour prior to analysis to ensure the sample was liquid. Samples were shielded from light prior to analysis. Samples were shielded from light prior to analysis. Neat ionic liquids served as the baseline for AmB samples solubilized in ionic liquid. Dilution samples were performed using 1x PBS for dilution. Analysis of AmB nanoemulsion required a nanoemulsion without drug as the baseline to correct for inherent scattering of the prepared nanoemulsion.

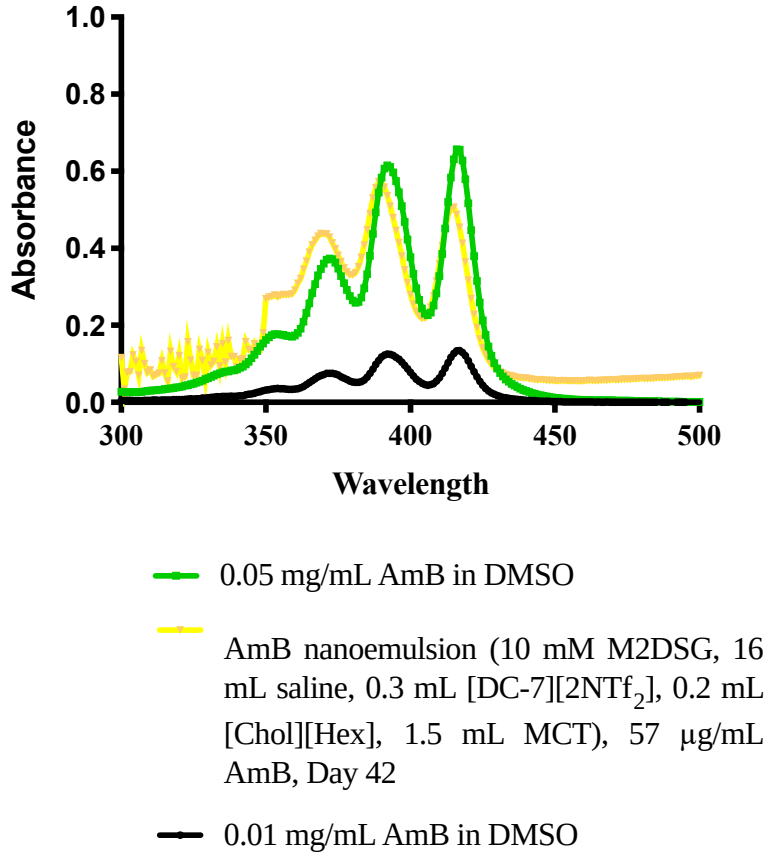


Figure S18. UV/vis graph of AmB containing nanoemulsion (yellow) prepared 42 days prior to analysis with a concentration of 57 µg/mL AmB in comparison to monomeric AmB in DMSO at a concentration of 0.01 mg/mL (black) and 0.05 mg/mL (green). The prepared AmB nanoemulsion exhibits excellent monomerization and long-term stability.

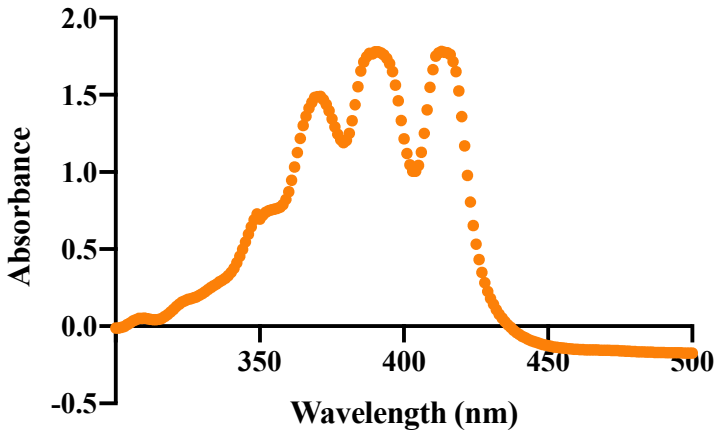


Figure S19. UV/vis graph of AmB solubilized in a 1.5:1 v/v ratio [DC-7][$2NTf_2$]:[Chol][Hex] prior to emulsification. AmB present in the ionic liquid mixture exhibits excellent monomerization.

UV/visible spectroscopy Amphotericin B water partition study. The ionic liquid mixture was prepared by direct addition of AmB powder to neat ionic liquids. Ionic liquids solid at room temperature were first melted using a water bath at 60°C prior to addition of AmB powder. After addition of solid drug, (1) 100 µL of methanol was then added to the IL mixture and the mixture was stirred at room temperature for 1 hour. (2) The drug/IL mixture was then placed under rotary evaporation in a water bath with at a temperature of 60°C for 1 hour. (3) The drug/IL mixture was then placed under high vacuum for 1 hour. (4) The drug/IL mixture was then allowed to stir for 1 hour. This process (steps 1-4) was repeated until all drug visibly was solubilized. Prior to the partition study, the IL mixture was stirring for 5 minutes, followed by 25 minutes of rotation on rotary evaporator at 60°C to remove any residual water. 200 µL of this sample was removed and saved for later UV/vis analysis. 500 µL of water was added to the remaining 500 µL of the IL mixture (at a temperature of ~60°C as was used immediately after stirring) in a 1.5 mL centrifuge tube. The sample was vortexed for 30 seconds, and sonicated for 1 min. The sample was then centrifuged at 2000 rpm for 2 minutes. The water layer was immediately removed after centrifugation. Water of a mixture of [DC-7][2NTf$_2$] and [Chol][Hex] was used as the baseline. Samples were analyzed using a quartz microscope slide with a 0.1 mm pathlength and were analyzed from 300 nm to 500 nm. 200 µL of sample was placed on the microscope slide for each analysis for uniformity.

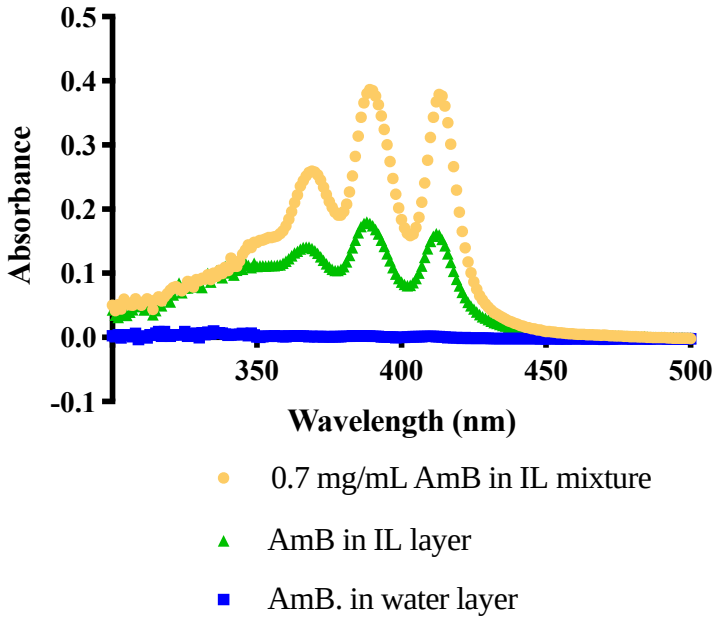


Figure S20. UV/vis graph of AmB water partition analysis. AmB was solubilized in an ionic liquid mixture of 1.5:1 v/v [DC-7][2NTf$_2$]: [Chol][Hex] (yellow). AmB remaining in the IL mixture (green) was compared to AmB partitioned into water (blue). While a small amount of AmB may partition into the aqueous phase during the emulsification process, the AmB largely remains in the IL mixture.

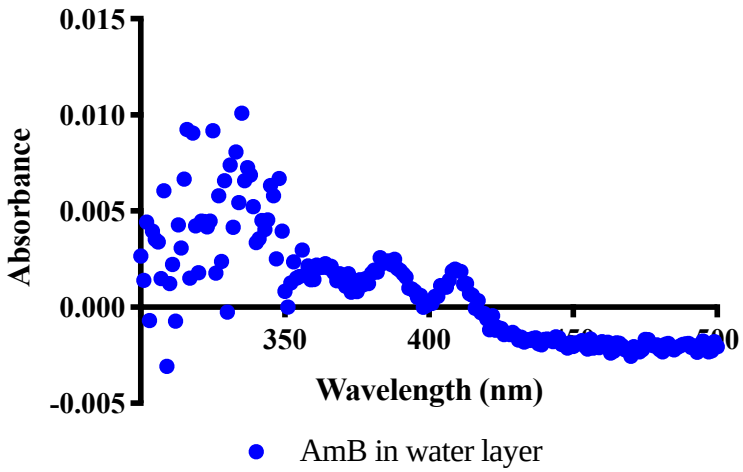


Figure S21. UV/vis graph of AmB aqueous layer in water partition analysis. AmB partitions into the aqueous layer in both monomeric and aggregated form.

SAMPLE PREPARATION.

Nanoemulsion preparation. Aqueous polymer solutions were prepared freshly (10 mM or 15 mM) in sterile, normal saline. The polymer solutions were sonicated at 40°C to ensure complete dissolution of polymer (approximately 2 hours). Saline was composed of 0.9% (w/w) sodium chloride USP. Amphotericin B/IL mixture solutions were prepared freshly, being allowed to sit for no more than 48 hours. Solid drug was directly added to the IL mixture and solubilization of AmB was completed by: (1) 100 µL of methanol was then added to the IL mixture and the mixture was stirred at room temperature for 1 hour; (2) The drug/IL mixture was then placed under rotary evaporation in a water bath with at a temperature of 60°C for 1 hour; (3) The drug/IL mixture was then placed under high vacuum for 1 hour. (4) The drug/IL mixture was then allowed to stir for 1 hour. This process (steps 1-4) was repeated until all drug was visibly dissolved. The drug/IL mixture was then placed under rotary evaporation at 60°C for an additional hour and then under high vacuum for 24 hours prior to use to ensure removal of all methanol. During solubilization, the IL mixture was shielded from light to prevent degradation of AmB. The homogenizer and microfluidizer were first cleaned with 100% and 70% ethanol followed by 100% and 70% methanol and finally three rinses with Millipore water to remove all traces of any previous nanoemulsions. Drug/IL mixture solution and MCT were added to the polymer solution. To ensure all of the drug/IL mixture was added to the polymer solution, the drug/IL mixture was warmed to 60°C to allow for ease of pouring. The hydrophobic media and polymer solution were then homogenized at room temperature with the high-speed homogenizer (Power Gen 500, Fisher Scientific, Hampton, NH) for 1 min at 21,000 rpm. The resulting crude emulsion was then added to a microfluidizer (model M-110S, Microfluidics Corp., Newton, MA) for 1 min at 5,000 psi with the cooling bath kept at 0 °C in order to reduce particle sizes. The final emulsion was then filtered with a 0.45 µm nylon filter and stored in a sterile, plastic centrifuge tube (Corning Inc., Corning, NY) at 4 °C. Nanoemulsions prepared with Amphotericin B were shielded from light during storage. Successful emulsification was verified using dynamic light scattering.

Particle Size Determination by dynamic light scattering (DLS). Particle sizes of nanoemulsions were analyzed by dynamic light scattering (NICOMP 380ZLS, Particle Sizing Systems, Santa Barbara, CA). The emulsions were diluted to an intensity factor of 500 KHz by adding 100 µL of the emulsion to 3.00 mL of Millipore water. Each particle size analysis was completed in a disposable cuvette and was repeated in triplicate. The total data acquisition time was 15 minutes. All data was acquired at room temperature. The data was analyzed using Gaussian analysis and reported as volume weighted average diameters.

Table S2. Nanoemulsion particle size and standard deviation monitored over time using dynamic light scattering for ionic liquid nanoemulsions with Amphotericin B and no drug.

10 mM M2DSG, 16 mL saline, 0.3 mL [DC-7][2NTf$_2$], 0.2 mL [Chol][Hex], 1.5 mL MCT, 57 µg/mL AmB			15 mM M2DSG, 14 mL saline, 0.6 mL [DC-7][2NTf$_2$], 0.4 mL [Chol][Hex], 3 mL MCT, 128 µg/mL			10 mM M2DSG, 16 mL saline, 0.3 mL [DC-7][2NTf$_2$], 0.2 mL [Chol][Hex], 1.5 mL MCT		
Day	Particle Size (nm)	Standard Deviation (nm)	Day	Particle Size (nm)	Standard Deviation (nm)	Day	Particle Size (nm)	Standard Deviation (nm)
0	155.2	58.7	0	123.4	37.5	0	156.6	52.2
1	158.4	54.3	1			1	157.1	55.6
2	158.1	58.2	2	125.3	32.3	2		
3	159.2	53.0	3	126.7	30.4	3	158.4	58.1
4	160.8	51.6	4	128.1	39.4	4	158.7	54.1
5			5	128.6	32.3	5		
6			6	130.1	35.8	6		
7	160.8	57.9	7	131.5	34.7	7	159.6	56.821
14	166.1	57.5	14	132.8	40.2	14	165.3	47.3

21	166.7	56.7	21			21	164.2	56.7
28			28	139.3	40.8	28		
32	172.0	55.4	35	142.0	40.9			
42	175.1	61.5				42	172.8	53.7
56	181.6	51.8						
70	180.4	54.7						
84	183.6	44.1						
98	179.8	62.0						

***In Vitro* Drug Release.** A 3 mL capacity SlideA-Lyzer Dialysis cassette (G2 2,000 MWCO from Thermo Fisher Scientific Inc., Fitchburg, WI) was hydrated prior to use by stirring for 12 h in a 3 L PBS bath (300 mL of 10× PBS and 2,700 mL of Millipore Milli-Q water) at 37 °C. After this time, 2.5 mL nanoemulsion was directly added to the cassette. A time-zero time point was established by diluting 100 μL of the nanoemulsion mixture above in 900 μL of methanol (MeOH). 100 μL of PBS was then added to the cassette, which was then returned to the PBS bath and stirred for 201 hours at 37 °C. Three cassettes were used in the experiment. Time points were taken at 0.5, 2, 3, 6, 9, 12, 24, 36, 48, 72, 96, 120, 144, 153, and 201 h. To ensure uniform analysis, at each time point a long-stemmed glass pipet was used to mix the contents of the cassette three times before the nanoemulsion aliquot was removed. Then a 100 μL aliquot of nanoemulsion was removed from the cassette and diluted with 900 μL of MeOH. The nanoemulsion aliquot was then replaced in the cassette by 100 μL of PBS. Sink conditions were maintained by replacing the 3 L PBS baths at the 3, 6, 9, and 12 h time points and every 12 h thereafter. To ensure no degradation of Amphotericin B in the methanol, 20 μg/mL propyl gallate was added to time point samples 0, 0.5, 2, 3, 6, 9, 12, 24, 36, 48, 72, 96 and 120 h. 200 μg/mL propyl gallate was added to time point samples 144, 153 and 201 h.

The Amphotericin B concentration remaining in the nanoemulsion was quantified by reverse phase HPLC. The HPLC system used was a Shimadzu prominence HPLC system (Shimadzu, Japan) equipped with an LC-20AT pump, SIL-20 AC HT autosampler, CTO-20 AC column over, and an SPD-M20A diode array detector. For each time point sample, 20 μL was injected into a C8 column (Agilent XDB-C8, 4.6 Å x 150 mm) and eluted with an isocratic mixture of 10% water (with 5% acetic acid) and 90% MeOH (with 5% acetic acid). The for samples 0, 0.5, 2, 3, 6, 9, 12, 24, 36, 48, 72, 96 and 120 h the run time was 4 minutes, the flow rate was 0.8 mL min^{-1}, and the detection was set at 406 nm. Amphotericin B eluted at 2.4 minutes. For samples 144, 153 and 201 h the run time was 7 minutes, the flow rate was 0.8 mL min^{-1}, and the detection was set at 406 nm. Amphotericin B eluted at 5.1 minutes. Concentration of Amphotericin B was determined by integrating the area of the peak and extrapolation from a standard calibration curve (500, 100, 50, 25, 10, 5, 1 μg mL^{-1}). Curve fitting analysis using a one-phase exponential association was used to calculate the first order rate constant (half-life, $t_{1/2}$).

Hemolysis Assay. A method to one previously described was adapted.[2] To determine the hemolytic activity of the ionic liquid nanoemulsion with amphotericin B, 10% suspended rabbit red blood cells (Lampire Biologic Laboratories, Inc. Pipersville, PA), were washed three times with PBS. For each wash, the cells were centrifuged at 2000 rpm for 4 min at 15°C. Cells were diluted 200-fold in PBS prior to analysis. The cell suspension was then further diluted 50:50 v/v with AmB-containing formulations. Samples (three samples per concentration analyzed) were incubated at 37°C with 200 x g shaking for 1 hour. Samples were then removed and set on ice for 5 minutes to halt hemolysis. The samples were centrifuged at 2000 rpm for 4 minutes at 15°C to settle intact cells and cell debris. 100 µL portions of supernatant from each sample were transferred to a 96 well plate in triplicate. Sample hemoglobin absorbances were measured at 540 nm. The percentage of hemolysis was calculated as: $100 \times \frac{Abs_{sample} - Abs_{negative}}{Abs_{positive} - Abs_{negative}}$, where Abs_{sample}, $Abs_{negative}$, and $Abs_{positive}$ refer to the absorbances of sample supernatants, negative control supernatant, and positive control supernatants incubated with rabbit red blood cells, respectively. The concentrations of AmB-containing formulations were 0.1 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 2 µg/mL, 6 µg/mL, 10 µg/mL, and 25 µg/mL. The positive control sample, in which total lysis of cells was achieved was prepared with the rabbit red blood cells in the presence of 25 µg/mL AmB sodium deoxycholate (Amphotericin B for Injection USP, X-GEN Pharmaceuticals, Horseheads, NY), also referred to as Fungizone®. Amphotericin B for Injection USP was rehydrated according to the manufacturer's instructions. Nanoemulsion samples were prepared by dilution of nanoemulsion with phosphate buffered saline in sterile conditions. Incubation of red blood cells with phosphate buffered saline served as the negative control. All AmB containing samples were shielded from light during the analysis. To correct for scattering of nanoemulsion, nanoemulsion without drug was used as the background. This nanoemulsion was incubated at 37°C with 200 x g shaking for 1 hour but did not have red blood cells present during incubation. The control analysis of the commercial formulation, Fungizone®, is consistent with previously analyzed hemolytic activity of Fungizone®.[3]

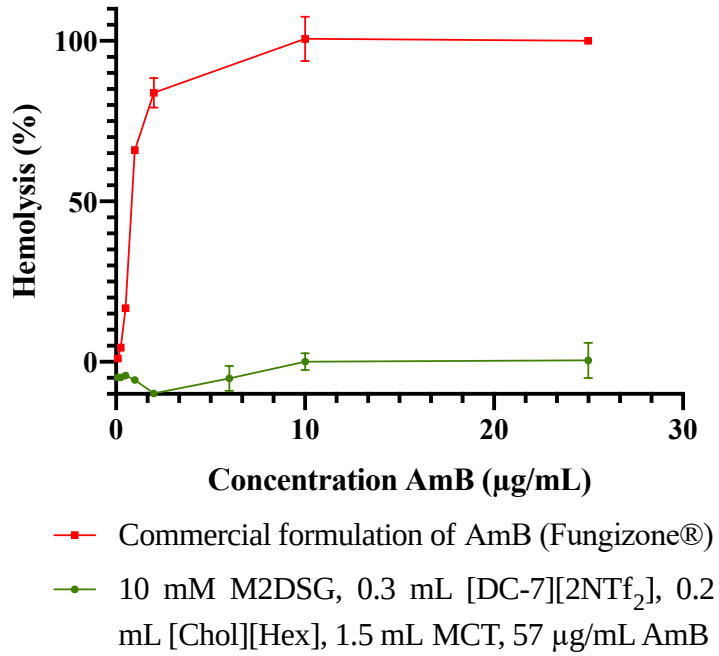


Figure S22. IL-in-water nanoemulsion with AmB (green) exhibits no hemolytic toxicity after 1-hour incubation with rabbit red blood cells at 37°C as measured by UV/vis spectroscopy in comparison to AmB commercial formulation (red).

Minimum Inhibitory Concentration (MIC) analysis. The following method was adapted from previously analyzed MIC studies of Amphotericin B.[2] A fluconazole resistant strain of *Candida Albicans*, K1, was used in antifungal efficacy studies. The strain was subcultured on Sabouraund dextrose agar (SDA) plates and grown in YPD medium. To prepare the inoculum, fungal strains were cultured for approximately 24 hours on SDA plates. SDA plates were adjusted to 1 x 10^3 CFU/mL in RPMI 1640 buffered to pH 7.0 with MOPS. Fungi were added to serial dilutions of emulsion with drug concentrations of 55 x 10^{-3} – 28.5 µg/mL in 96-well plates using the microdilution method. Plates were incubated at 37°C throughout the study. Plates were analyzed by absorbance (OD600, 530 nm) at 24 h and 48 h using a Synergy H1 microplate reader (BioTek). The minimum inhibitory concentration is identified as the concentration range in which a low absorbance value (corresponding to minimal fungi) increases, indicating increased fungal growth. As **1** has previously been shown to exhibit antifungal activity, we conducted a control experiment with a nanoemulsion containing no drug. This did not exhibit any antifungal activity, demonstrating that all antifungal activity observed corresponded solely to the AmB.

Note: The increased absorbance values for higher concentrations of AmB with the AmB nanoemulsions and Fungizone® can be attributed to the scattering of the emulsion or sodium deoxycholate aggregated, increasing the absorbance.

	No fungi control	**28.5 µg/mL AmB**	**14.25 µg/mL AmB**	**7.125 µg/mL AmB**	**3.5625 µg/mL AmB**	**1.78 µg/mL AmB**	**0.89 µg/mL AmB**	**0.44 µg/mL AmB**	**0.22 µg/mL AmB**	**0.11 µg/mL AmB**	**0.055 µg/mL AmB**
AmB nano	0.825	1.579	0.992	0.524	0.327	0.197	0.137	0.087	0.075	0.067	0.076
AmB nano	0.134	1.573	1.017	0.599	0.349	0.209	0.132	0.097	0.077	0.069	0.092
AmB nano	0.045	1.47	0.988	0.605	0.381	0.223	0.147	0.104	0.081	0.071	0.066
No Drug nano	0.038	1.488	0.782	0.453	0.271	0.167	0.119	0.209	0.269	0.267	0.26
No Drug nano	0.046	1.411	0.828	0.481	0.293	0.187	0.129	0.164	0.282	0.275	0.266
No Drug nano	0.056	1.45	0.852	0.484	0.294	0.179	0.13	0.155	0.275	0.263	0.268
Fungizone control	0.037	0.383	0.253	0.218	0.139	0.095	0.077	0.063	0.055	0.052	0.05
Fungizone control	0.037	0.359	0.239	0.249	0.153	0.106	0.083	0.067	0.058	0.054	0.052

Table S3. Absorbance at 530 nm of formulations incubated with K1 strain *Candida Albicans* at 37°C for 24 h.

	No fungi control	**28.5 µg/mL AmB**	**14.25 µg/mL AmB**	**7.125 µg/mL AmB**	**3.5625 µg/mL AmB**	**1.78 µg/mL AmB**	**0.89 µg/mL AmB**	**0.44 µg/mL AmB**	**0.22 µg/mL AmB**	**0.11 µg/mL AmB**	**0.055 µg/mL AmB**
AmB nano	0.793	1.526	1.014	0.535	0.332	0.198	0.138	0.088	0.088	0.21	0.299
AmB nano	0.173	1.592	1.041	0.598	0.342	0.207	0.131	0.098	0.091	0.236	0.276
AmB nano	0.057	1.467	1.01	0.645	0.47	0.22	0.154	0.103	0.086	0.227	0.255
No Drug nano	0.188	1.099	0.88	0.522	0.307	0.208	0.223	0.347	0.411	0.392	0.364
No Drug nano	0.123	1.247	0.924	0.551	0.391	0.225	0.274	0.311	0.39	0.379	0.366

No Drug nano	0.166	1.295	0.938	0.584	0.328	0.216	0.236	0.305	0.386	0.369	0.352
Fungizone control	0.038	0.376	0.242	0.21	0.133	0.092	0.075	0.061	0.054	0.05	0.052
Fungizone control	0.036	0.359	0.233	0.246	0.149	0.103	0.08	0.065	0.057	0.051	0.053

Table S4. Absorbance at 530 nm of formulations incubated with K1 strain *Candida Albicans* at 37°C for 48 h.

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