Supporting Information

for

Lipid based biocompatible ionic liquids: synthesis, characterization and biocompatibility evaluation†

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1. Materials and Methods

1.1 Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), ethyl trifluoro-methane sulfonate (EtFMS) and Isopropyl alcohol (IPA) were obtained from Tokyo Chemical Industry Co. ltd (Tokyo, Japan). Dehydrated chloroform; ethanol (EtOH); acetonitrile; methanol; dimethyl sulfoxide (DMSO); n-hexane, cyclo-hexane; octane, dodecane and chloroform-d (NMR grade) were purchased from Fujifilm Wako Pure Chemical Corporation, (Osaka- Japan). Choline, stearic acid (Ste), oleic acid (Ole), linoleic acid (Lin), octane and sodium dodecyl sulfate (SDS) and isopropyl myristate (IPM) were purchased from Sigma Aldrich (Steinheim, Germany). 1-ethyl-3-methylimidazolium bis (trifluoro-methyl sulfonyl) imide (emim -TFSA) was purchased from Kanto Chemical Co. INC. (Tokyo, Japan), and Lab-Cyte^{TM} EPI-MODEL human artificial cell line was purchased from Japan Tissues Engineering Co. Ltd (Aichi, Japan).

1.2 Cationic lipid synthesis, purification and characterizations

1,2-Dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) lipid powder was mixed with ethyl trifluoro methane-sulfonate at 1:1 mole ratio.[1] The reaction was carried out at 45°C for 12hr with a continuous flow of nitrogen gas (N_2). The product 1,2-dimyristoyl- sn-glycero- 3-ethyl-phosphatidylcholine (EDMPC) trifluoro salt was verified with thin-layer chromatography (TLC) using $CHCl_3$: MeOH (9:1) v/v mixture as an eluting solution. The *p*-anisaldehyde identical coloring solution consisting 13mL of P-anisaldehyde, 5mL of acetic acid, 18mL of 10M concentrated sulfuric acid and 478mL of ethanol was used to visualize the EDMPC trifluoro salt's spot-on TLC plate. Shifting ethyl part from EtFMS to DMPC was confirmed by measuring ^{1}H-NMR (JEOL ECZ400S 400MHZ NMR, Tokyo, Japan) using chloroform-d as solvent. Synthesized EDMPC trifluoro salt was dissolved in chloroform followed by addition of 0.2N HCl with shaking to form EDMPC-Cl. The byproduct, trifluoromethyl sulfonate, and unreacted HCl were removed from the transparent aqueous phase. The nonaqueous phase consisted of EDMPC-Cl was further purified with Milli-Q water. Chloroform and Milli-Q water were removed out by freeze-drying. Finally, the purity of EDMPC-Cl was investigated with TLC, ^{1}HNMR, fourier transform infrared (FTIR), Mass spectrometric (MS) and chemical elementary analysis (EA) etc.

1.3 Lipid based ionic liquids preparation

EDMPC-Cl and fatty acid were reacted at equimolar ratio in chloroform for overnight

(~12hr) at 45°C, with a continuous flow of N_2 in a light protective environment. The reaction solution was freeze-dried to obtain the synthesized lipid based ionic liquid (LBIL) that was further characterized by TLC, ^{1}HNMR, FTIR, DSC, UV and EA etc.

1.4 Characterization procedures of cationic lipid and LBILs

Thin-layer chromatography (TLC): DMPC, EDMPC-SO_3CF_3, EDMPC-Cl and LBILs ([EDMPC][Lin], [EDMPC][Ole] & [EDMPC][Ste]) in chloroform were spotted on the TLC plate separately, using 10% methanol in the chloroform (v/v) as eluting solution. The TLC plate was dried and stained with phosphomolybdic acid (PMA) solution (10% in ethanol). After drying a white identical spot for each sample was found on the TLC plate. The distance of the white identical spots was measured to calculate the retention factor (R_f), where R_f is the migration distance of the analyte/the migration distance of the solvent front value.

Nuclear magnetic resonance (NMR): The ^{1}HNMR of EDMPC-SO_3CF_3, EDMPC-Cl and LBILs were recorded in the chloroform-d solution. Coupling constants (J) in Hz units and spectrum data were processed by the Delta-V software package (version 5.0.5.1, JEOL).

Fourier transform infrared spectroscopy (FTIR): The FTIR spectra of synthesized LBILs were recorded by a Perkin Elmer FTIR spectrophotometer with a diamond crystal reflection sampler. All samples were analyzed in the range of 400-4000cm^{-1} with 20 scans accumulation. Spectral outputs were recorded in transmittance mode as a function of wave number.

MALDI-TOF mass spectrometry (MS): Molecular weight of EDMPC-Cl was investigated using a MS (Bruker MADI-TOF MS Autoflex II) operated in the positive ion reflection mode. On the MTP anchor chip plate (Bruker Dalton-ics), around 1μL of matrix solutions (hydroxy picolinic acid [3-HPA], 10 g/L and di-ammonium citrate, 1 g/L) was spotted and dried at room temperature. Approximately 1μL of sample (2mg/mL in ethanol) was spotted onto the previously dried matrix solution and allowed to dry again in room temperature, and measured by the MS.

Water content determination: The moisture contents in LBILs were determined by the Karl fisher moisture analyzer (MKV-710S, Kyoto, Japan). The IBILs samples were prepared at 20% (w/w) in hexane for titration.

Differential scanning calorimetry (DSC): The thermal phase behavior of LBILs was investigated using DSC (Hitachi High Technology TG/DTA 7300, Tokyo, Japan) with continuous flowing of N_2 30mL/min. Three cycles for each sample were measured and the empty aluminum pan

was sealed with the same procedure used as the reference.

1.6 Solubility of LBILs

The solubility of LBILs was investigated in different polar and nonpolar solvents. Briefly, excess LBIL (up to 50:50, w/w) was added in different solvents, vortexed for 5-10min and observed visually. For PBS and Milli-Q water, LBILs were used at maximum of 20mg/mL concentration. The absence of any particle/precipitation was considered as the solubility of LBILs in each solvent. The solutions were kept standing for two weeks and visually observed to detect any precipitation occurred. The solubility was further investigated using UV spectrophotometer (JASCO V-750, Japan) and Dynamic Light Scattering (DLS, Zetasizer, Malvern, UK), respectively. For DLS, the measurements scatting angel was maintained at 173° with 1 cm length capped quartz cell in 25 ± 0.1°C. Each sample was measured at 10times and the average value was considered as the experimental output.

1.7 pH of LBILs

The LBILs were dissolved in Milli-Q water at a concentration of 20mg/mL (w/w) to measure the pH of LBIL in water solution using a pH meter (TOA, HM-30R. DKK TOA Corporation, Japan). The data represents the average of the three experiments.

1.8 Biocompatibility of LBILs

The biocompatibility of LBILs were investigated using a skin irritation test on human artificial epidermis model (LabCyte^{TM}, EPI-MODEL). The procedures were performed as recommended by supplier in accordance with published literature with some modification.[3] LBIL at different concentrations in IPM (For [EDMPC][Lin] : 5%, 10%, 20%, 50%, and 100%, w/w) were used for this study. Briefly, cells containing cups were transferred into 24-well plate with previously added 500µL of assay medium and incubated for overnight (24hr) at 37°C, 5% CO_2 humidified atmosphere. Then, 25µL of each sample (LBILs in IPM) was added to the cell that covered the whole surface on the tissue and incubated for 24hr. After that, the cells were washed carefully with D-PBS to remove any remaining sample. A 500µl of freshly prepared MTT assay medium was added in a 24-well plate where previously washed cells were transferred and incubated for 3hr. The treated tissues were taken out and transferred into a 300µl of 2-propanol containing tube and allowed to stand for 48hr at room temperature in dark condition to extract the pigment. A 100µl of extracted solution was placed to a 96-well plate to measure the optical density with a microplate reader at 650nm and 570nm. The (%) percentage of cell viability was calculated by considering the untreated cell as control. The data was reported as average of three repeated measures. Finally, the biocompatibility of three LBILs was compared with commercially available moderately toxic ionic liquids such as [emim][TFSA] and [bmim][TF_2N], and a well-known pharmaceutically available surfactant, SDS.

2. Figures and Tables

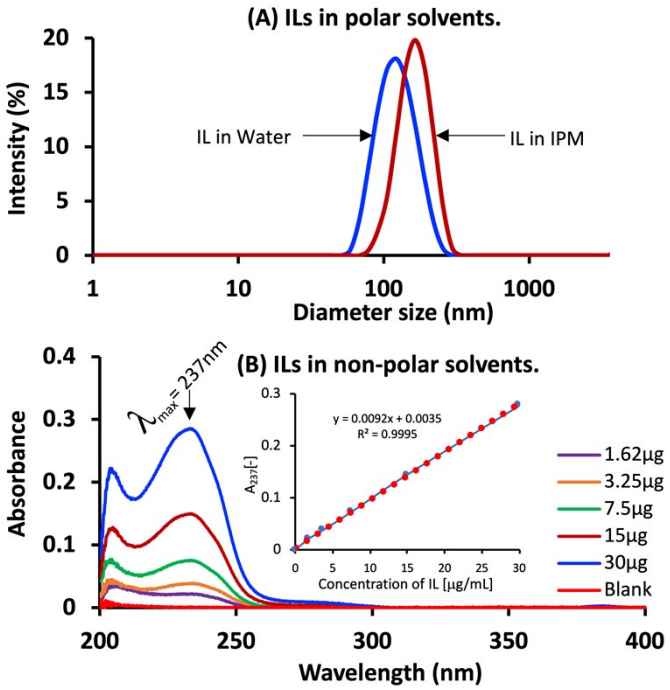


Fig. S1: (A) The particle size distributions of [EDMPC][Lin] after dispersed in water and IPM by DLS analysis. (B) The UV-visible spectrum of [EDMPC][Lin] after dissolved in hexane with correlation of absorbance vs concentration (Correlation coefficient, R^2= 0.9995).

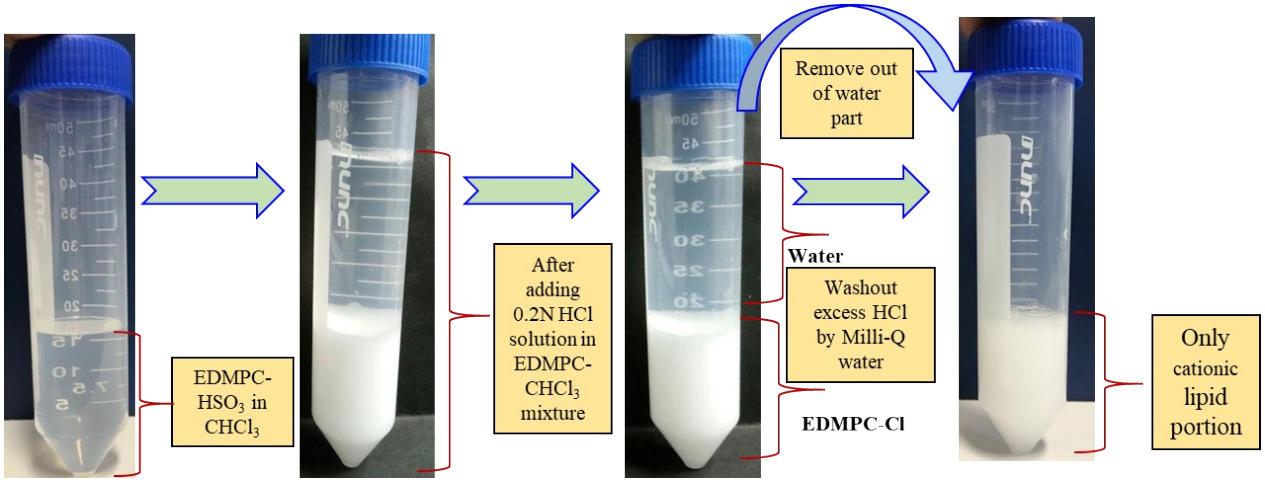


Fig. S2: EDMPC-Cl synthesis and purification process by phase separation process.

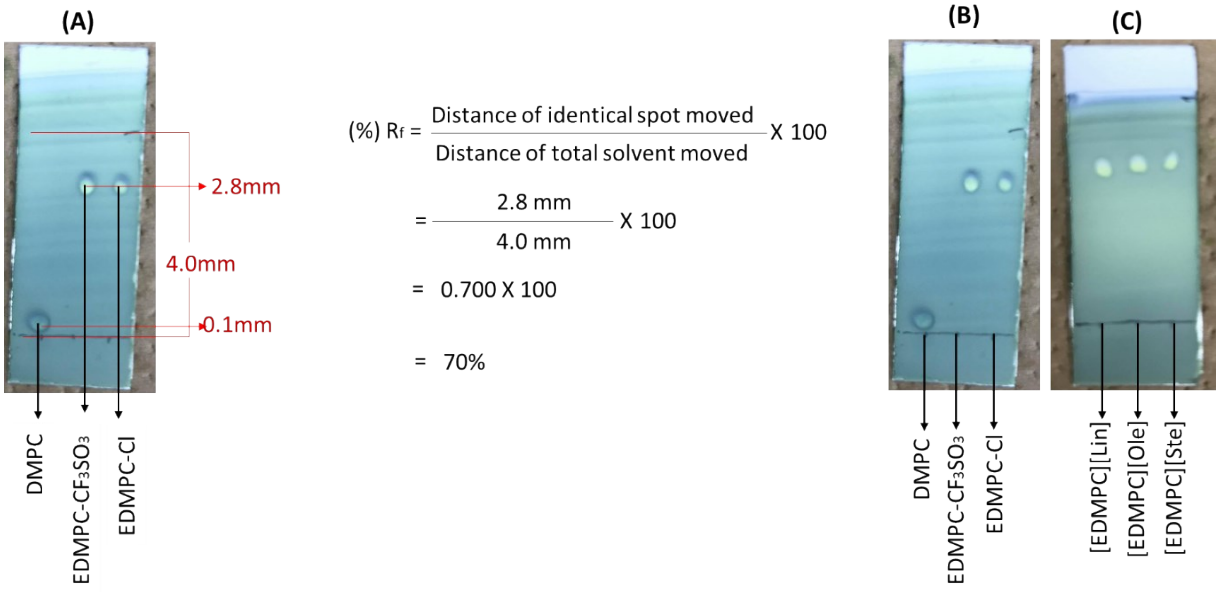


Fig. S3: Thin layer chromatographic image of DMPC, EDMPCSO3CF3, EDMPC-Cl and three LBILs. The retention factor (Rf) value was calculated.

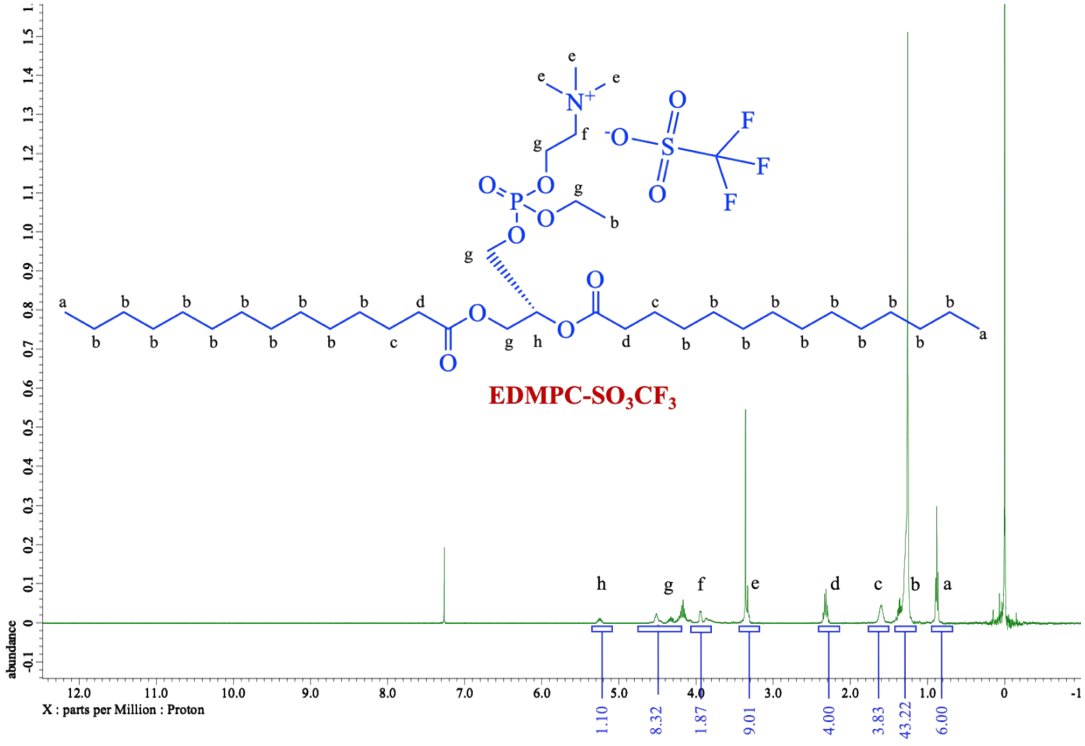


Fig. S4 (A): ^{1}H-NMR of **EDMPC-SO_3CF_3**; (400 MHz, chloroform-d) δ 5.24 (t, J = 5.3 Hz, 1H), 3.66-4.49 (m, 10H), 3.27-3.30 (m, 9H), 2.28-2.34 (m, 4H), 1.54-1.59 (m, 4H), 1.24-1.39 (m, 43H), 0.85 (t, J = 6.4 Hz, 6H). Chloroform (7.2-7.5 ppm).

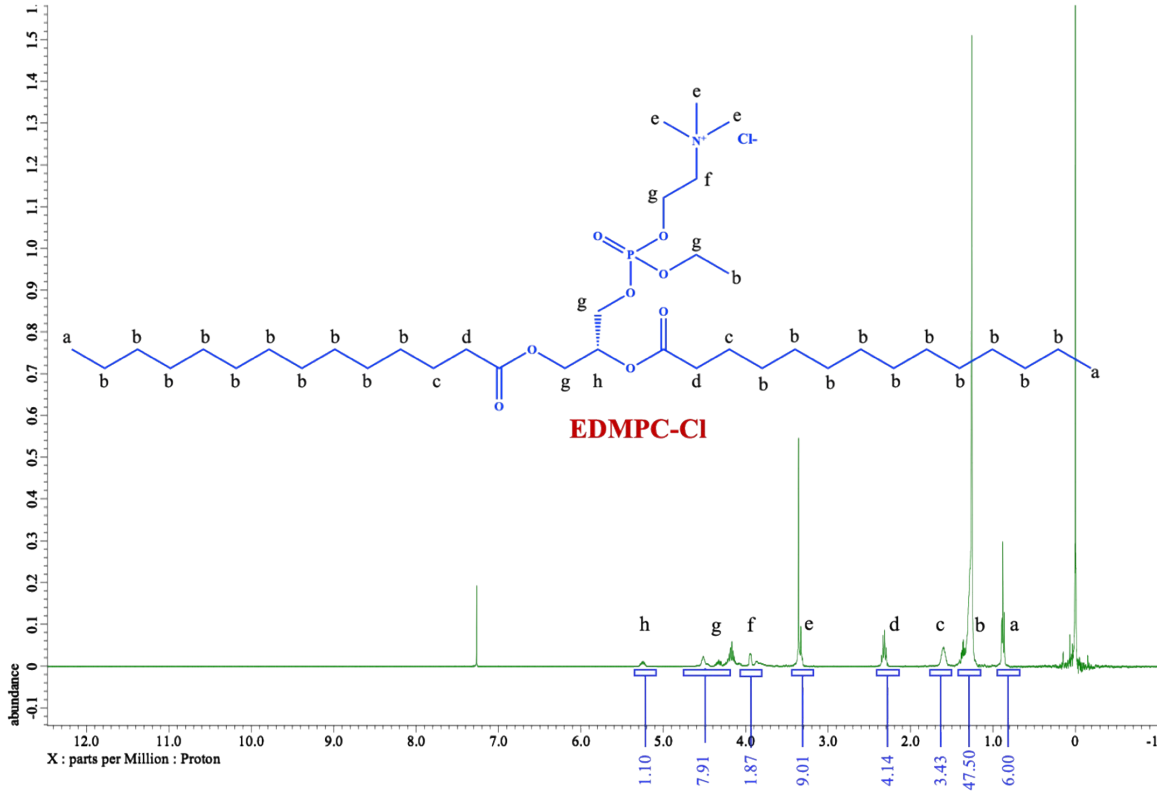


Fig. S4 (B): ^{1}H-NMR of **EDMPC-Cl**; (400 MHz, chloroform-d) δ 5.24 (s, J = 5.3 Hz, 1H), 3.85-4.50 (m, 10H), 3.29-3.38 (m, 9H), 2.26-2.34 (m, 4H), 1.15-1.59 (m, 47H), 1.59-1.61 (s, 4H), 0.86 (t, J = 6.9 Hz, 6H). Chloroform (7.2-7.5 ppm). Some excess amount of H found between 1.15-1.56ppm, those H derived from unexpected water of synthesized EDMPC-Cl.

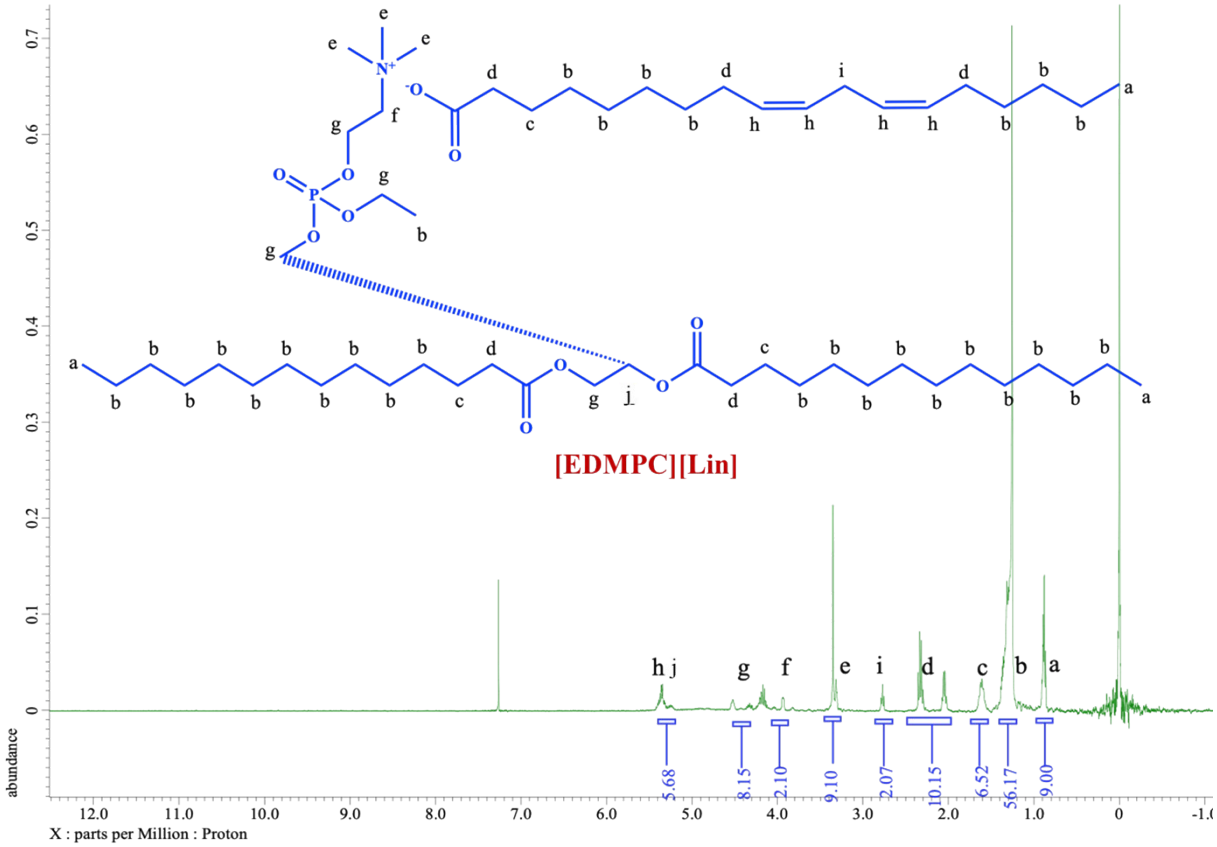


Fig. S4 (C): ^{1}H-NMR of Linoleic acid base LBIL **[EDMPC][Lin];** (400 MHz, chloroform-d) δ 5.21 (s, 1H), 5.34 (m, 4H), 4.11-4.50 (m, 6H), 3.92 (s, 1H), 3.32 (m, J = 14.2 Hz, 8H), 2.75 (t, J = 6.6 Hz, 2H), 2.31 (m, J = 7.6 Hz, 6H), 2.03 (m, J = 6.9 Hz, 4H), 1.14-1.61 (m, 70H), 0.87 (t, J = 11.0, 6.9 Hz, 9H). Chloroform (7.2-7.5 ppm).

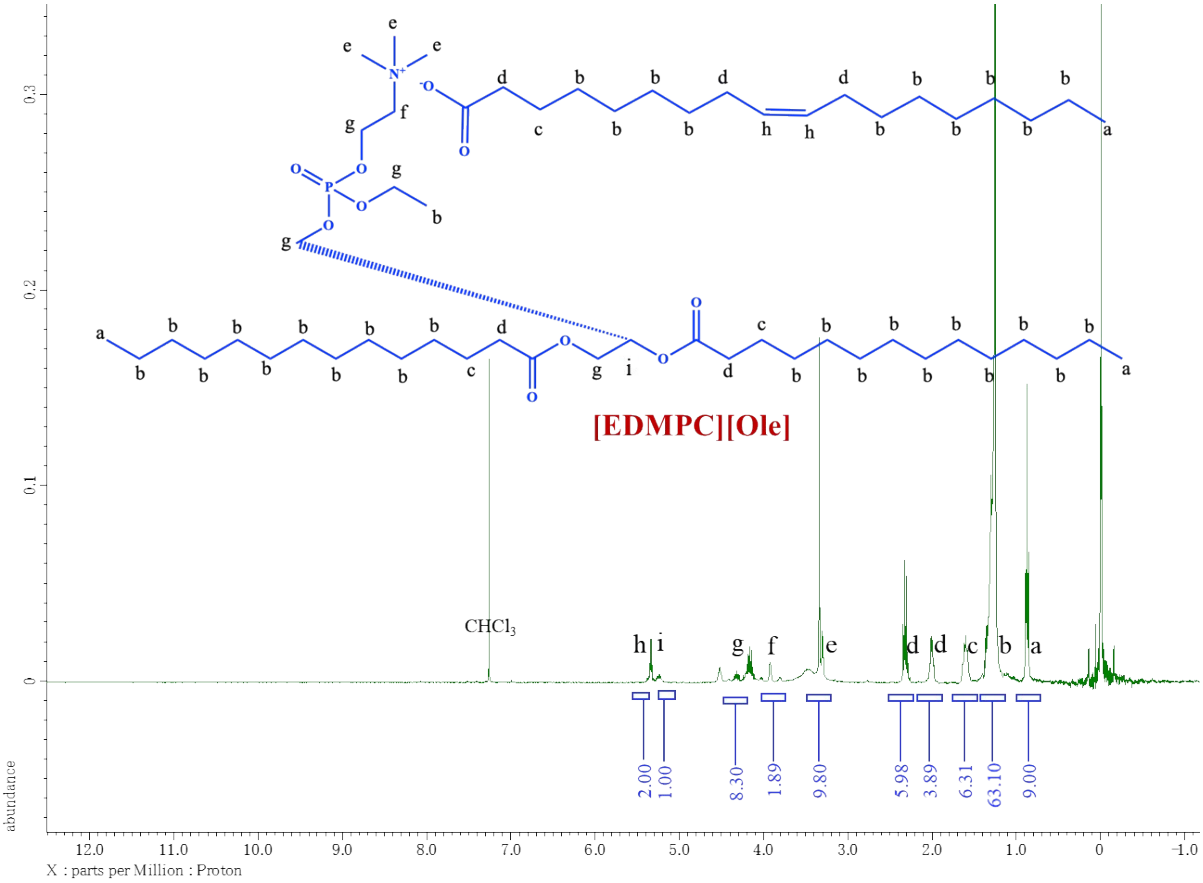


Fig. S4 (D): ^{1}H-NMR of Oleic acid base LBIL **[EDMPC][Ole];** (400 MHz, chloroform-d) δ 5.1 (s, 1H), 5.34 (m, 2H), 3.91-4.51 (m, 7H), 3.29-3.51 (m, 12H), 2.31 (m, J = 7.6 Hz, 6H), 1.99 (d, J = 5.9 Hz, 4H), 1.19-1.63 (m, 66H), 1.51-1.65 (s, 6H), 0.86 (t, J = 6.9 Hz, 9H). Chloroform (7.24 ppm).

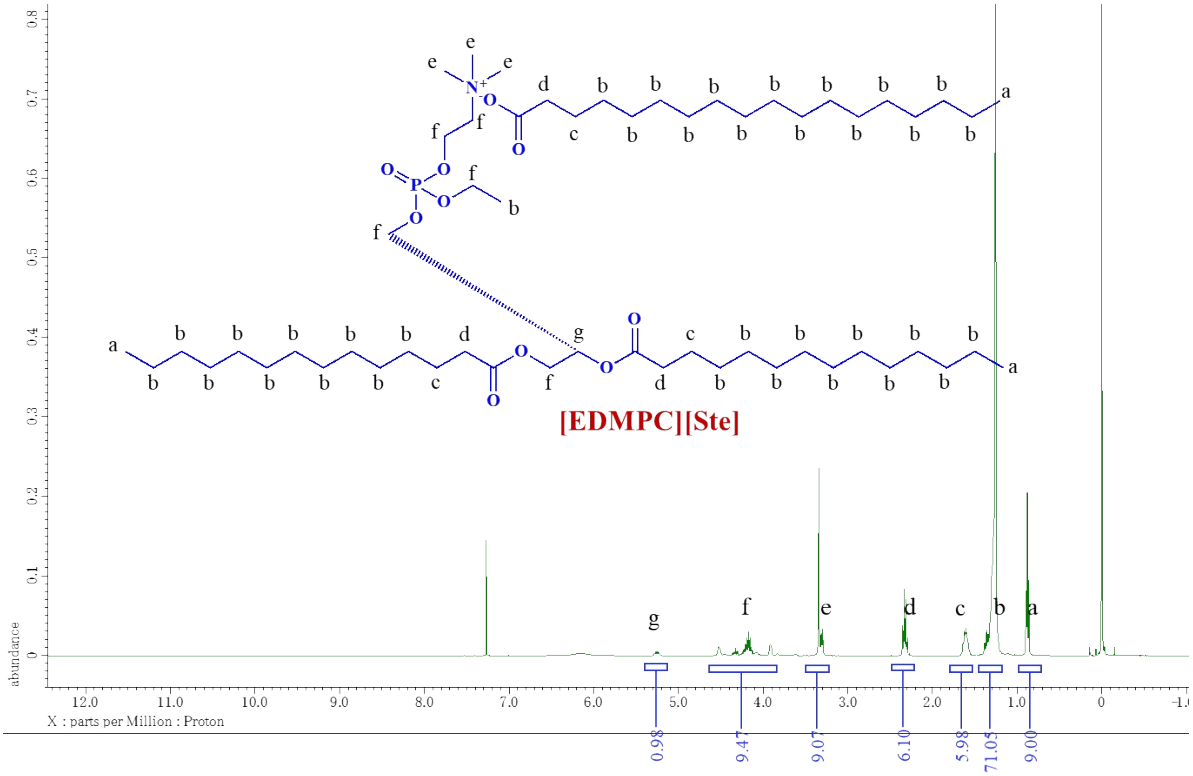


Fig. S4 (E): ^{1}H-NMR of Stearic acid base LBIL **[EDMPC][Ste];** (400 MHz, chloroform-d) δ 5.27 (s, 1H), 3.91-4.58 (m, 9H), 3.30-3.34 (m, 9H), 2.32 (m, J = 7.5 Hz, 6H), 1.60-1.70 (m, 6H), 1.26-1.39 (m, 75H), 0.88 (t, J = 6.9 Hz, 9H). Chloroform (7.2-7.5 ppm).

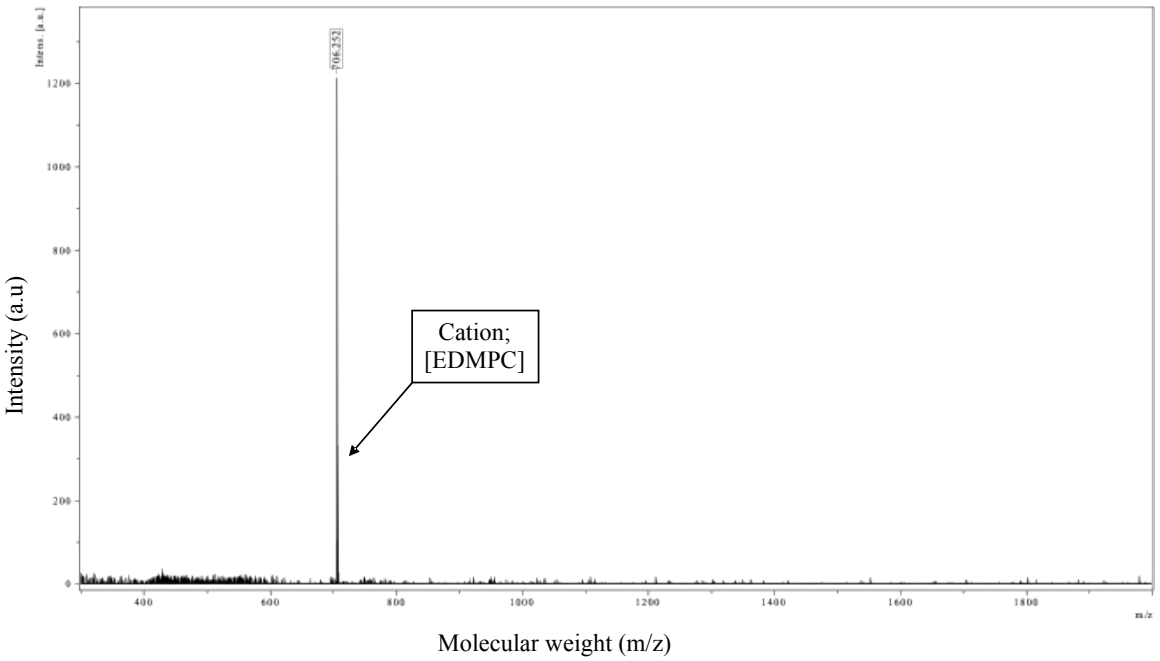


Fig. S5: MALDI-TOF MS measurement spectrum of cationic lipid EDMPC showed an identical peak at 706.252 m/z.

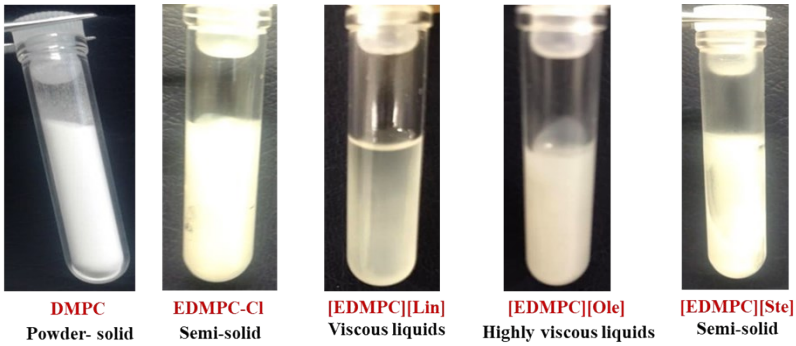


Fig. S6: Physical appearances of DMPC, EDMPC-Cl and three LBILs at room temperature (25°C) were represented in this figure.

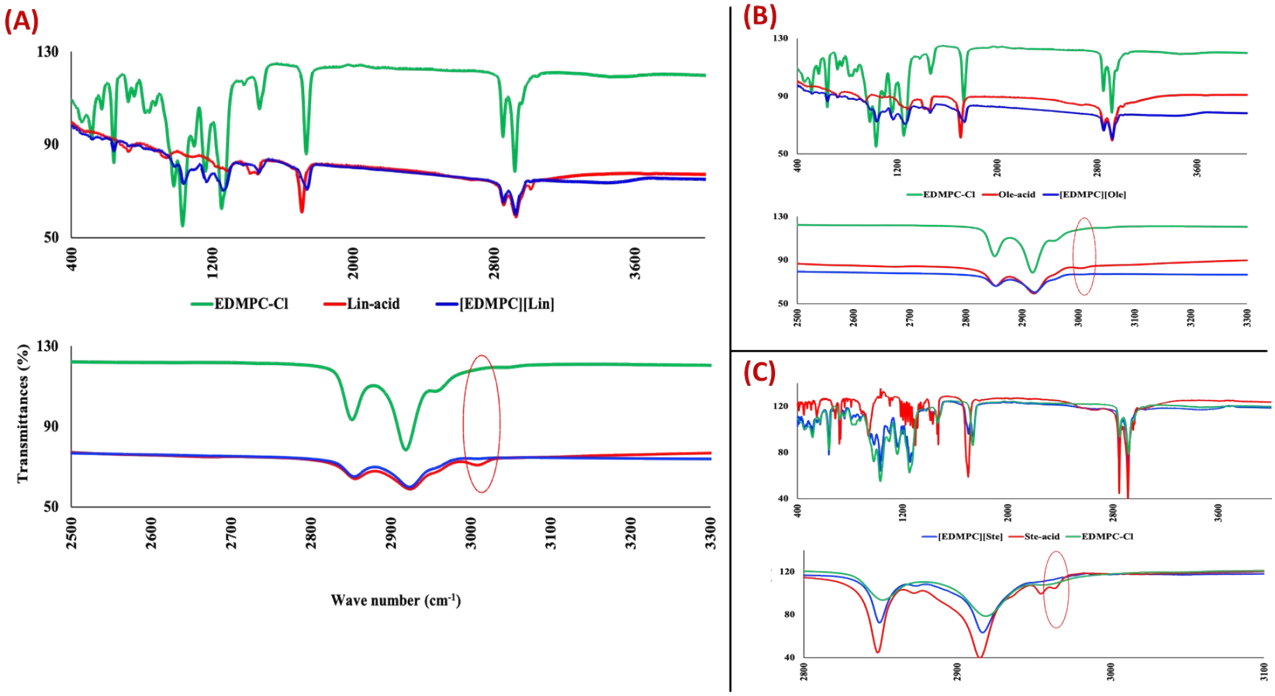
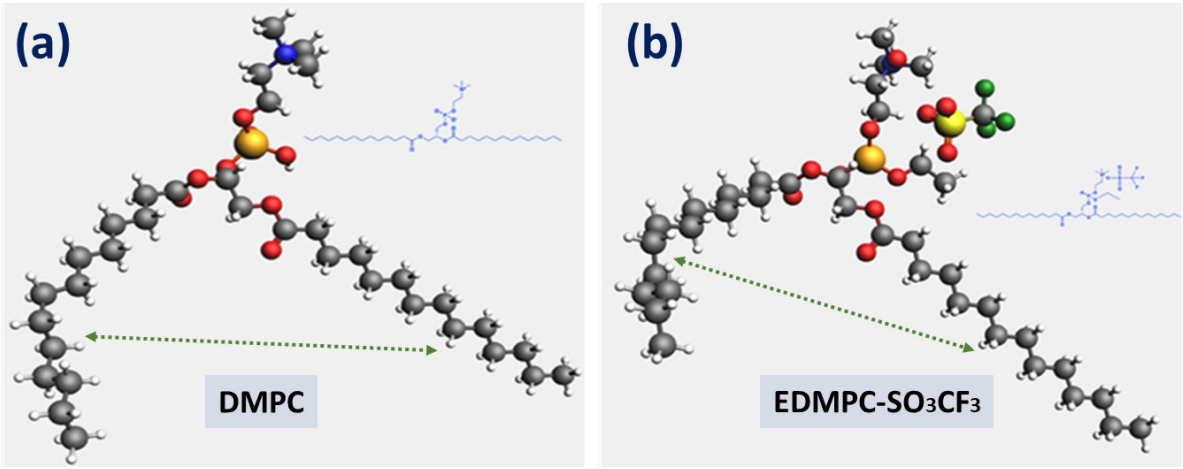


Fig. S7: The FTIR spectra of LBILs were represented in the figure (A) [EDMPC][Lin], (B) [EDMPC][Ole], and (C) [EDMPC][Ste].



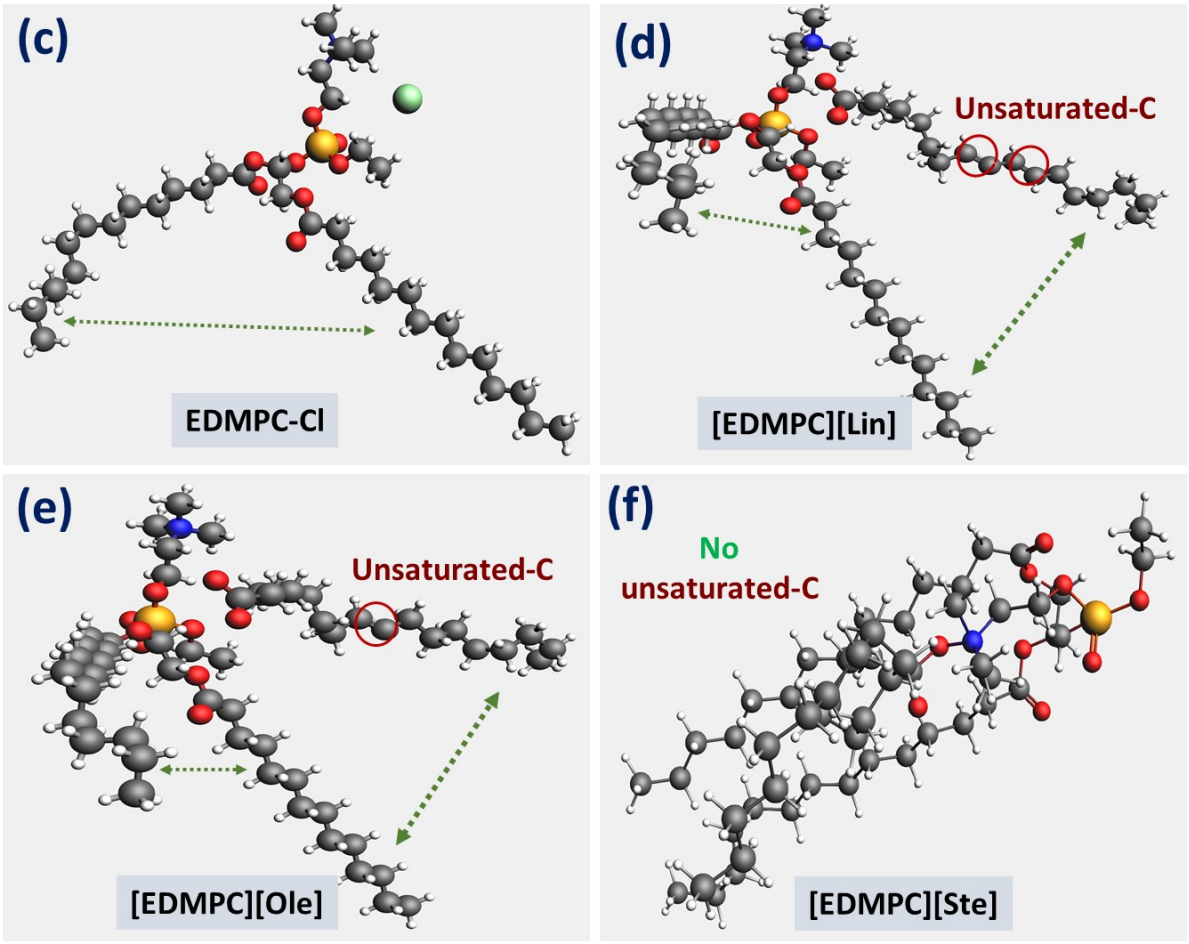


Fig. S8: Chemical structure of LBILs- predicted by ADF cosmo-RS ADF:2019-301. Physical appearance, combining statistical thermodynamics approach, charge distribution, surface, and chemical potential distribution, intermolecular interaction and the molecular structure of the molecules can be predicted by the COSMO-RS analysis.[4]

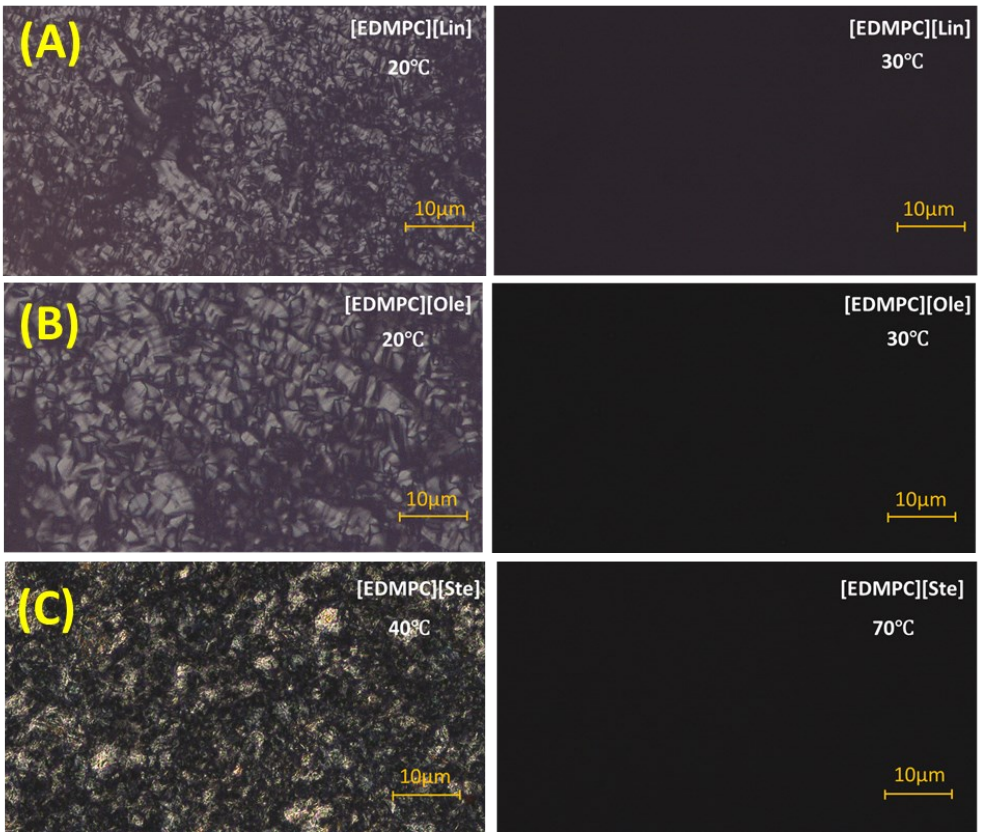


Fig. S9: Temperature-dependent optical polarized microscopic images of LBILs. (A) [EDMPC][Lin], (B) [EDMPC][Ole], and (C) [EDMPC][Ste].

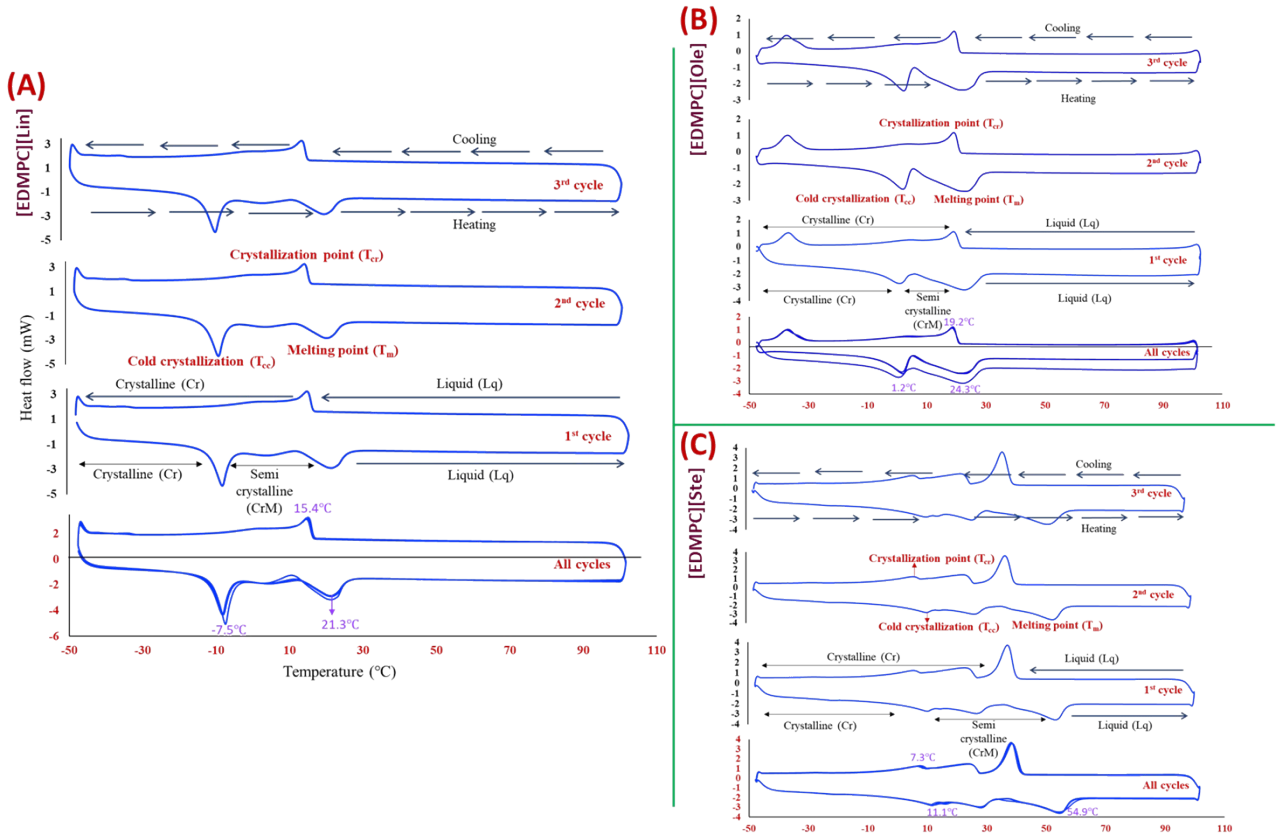


Fig. S10: The thermal phase behavior of LBILs was investigated using DSC. (A) represented the [EDMPC][Lin] IL, (B) represented the [EDMPC][Ole] IL, and (C) represented the [EDMPC][Ste] IL.

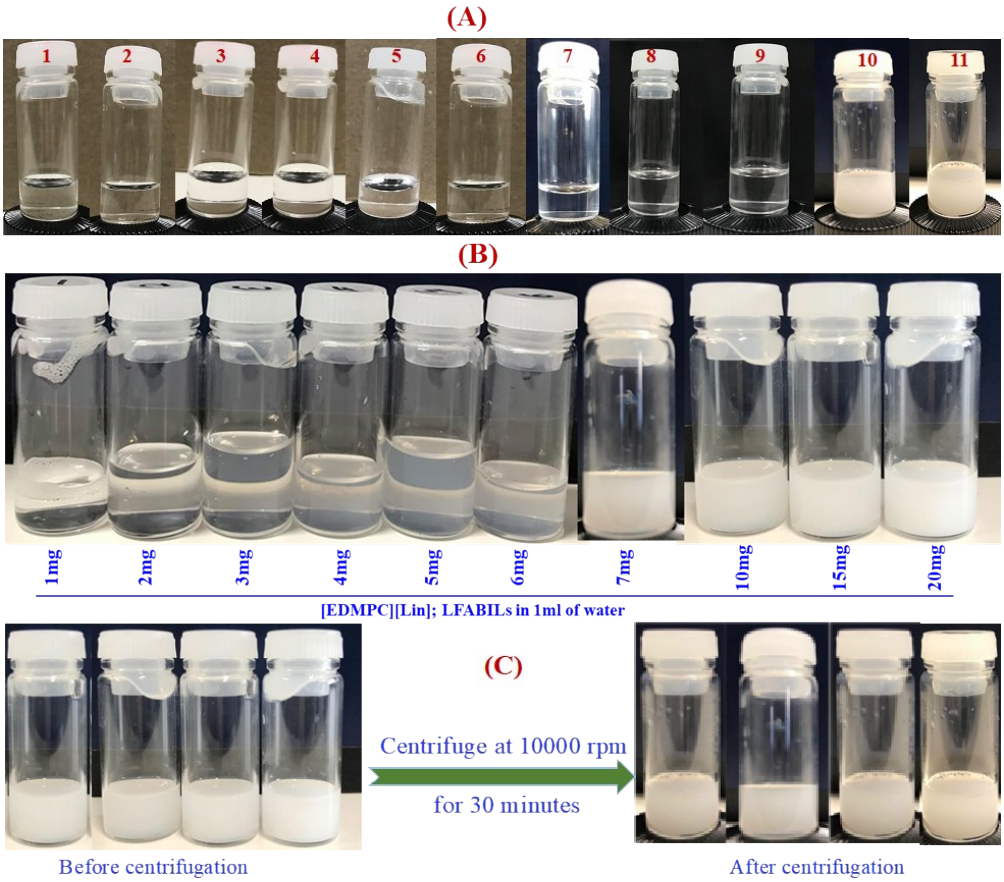


Fig. S11: (A) Visual observation of the solubility of [EDMPC][Lin] in various solvents, (1) cyclohexane, (2) hexane, (3) heptane, (4) DMSO, (5) ethanol, (6) methanol, (7) IPA, (8) IPM, (9) toluene, (10) PBS and (11) water. (B) [EDMPC][Lin] IL was dispersed in water at different concentrations (w/v). (C) Dispersibility was observed by centrifugation.

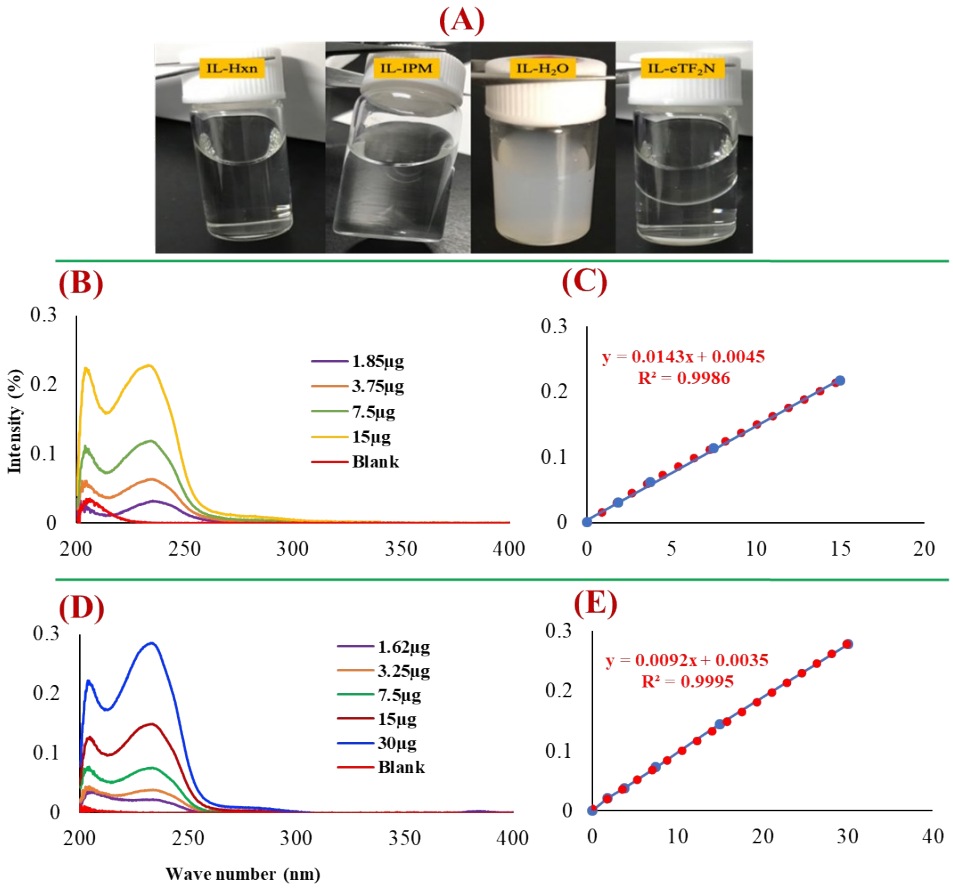


Fig. S12: Solubility of LBILs in nonpolar solvents. (A) [EDMPC][Lin] IL dissolved in hexane, IPM, water, and bmim-Tf_2N. (B) [EDMPC] [Lin] IL solubility in methanol was investigated using UV (High peak at 240 nm) and (C) Correlation coefficient R^2 in methanol. (D) [EDMPC] [Lin] IL solubility in hexane was investigated using UV (High peak at 237 nm) and (E) Correlation coefficient R^2 in hexane.

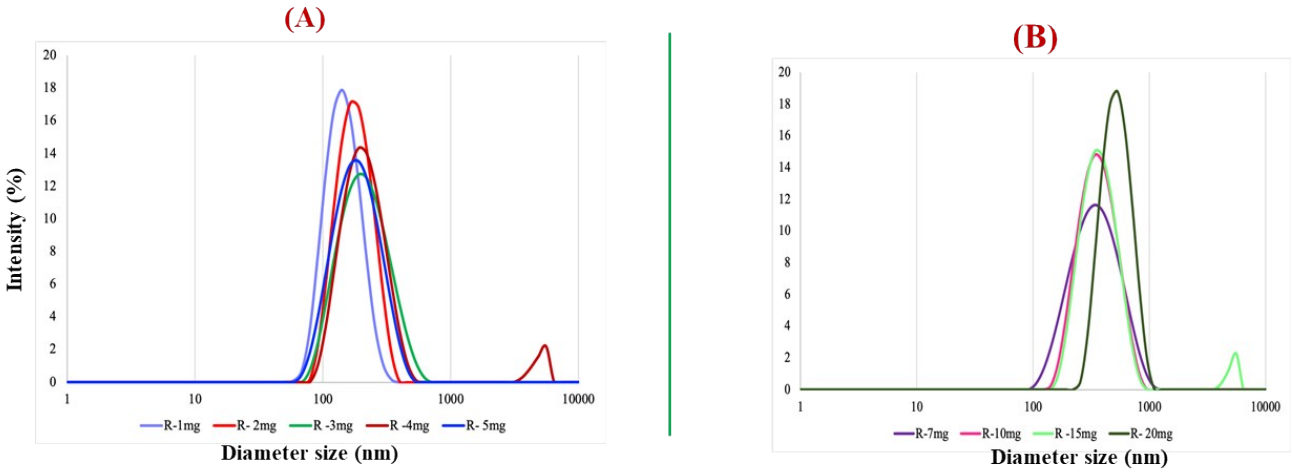


Fig. S13: [EDMPC][Lin] solubility in water was investigated using DLS.

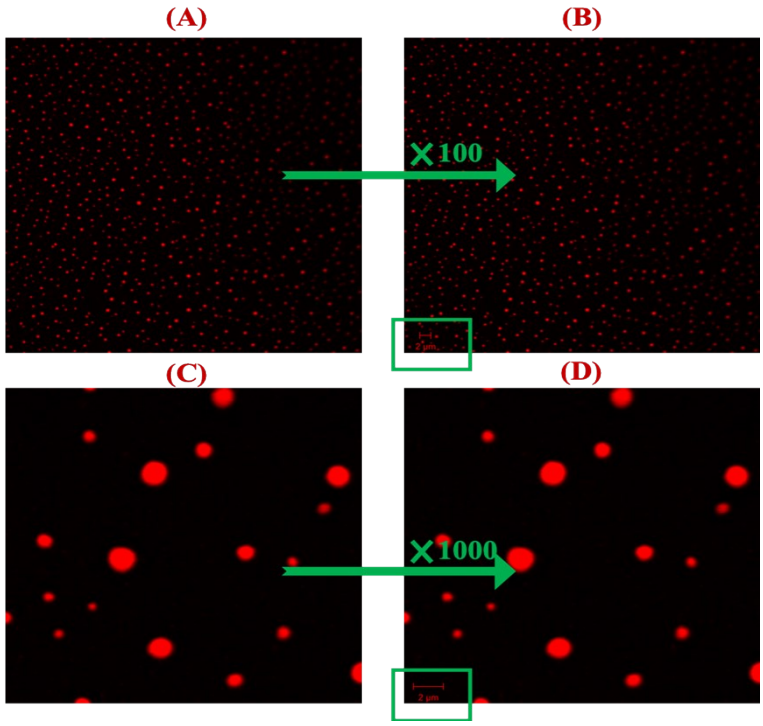


Fig. S14: Confocal Laser Scanning Microscopy (CLSM) image of LBILs solubility in water.

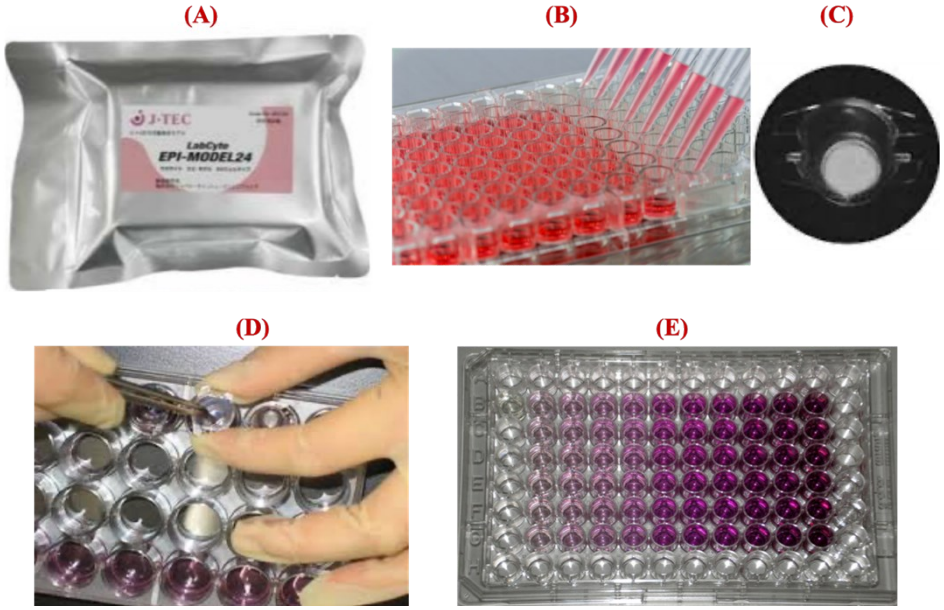


Fig. S15: LabCyte EPI-MODEL 24 cells were used to check the biocompatibility of LBILs.

Table S1: Elementary analysis of LBILs and moisture contents are reported in the table.

Ionic Liquids	Formula (molecular weight)		Elementary analysis (%) C	H	N	Moisture uptake
[EDMPC][Lin]	$(C_{56}H_{108}NO_{10}P)_n$ $(985.8)_n$	Calc.	68.19	11.04	1.42	
		Found	65.72	10.94	1.36	3.92%
		Corrected	65.75	10.96	1.37	
[EDMPC][Ole]	$(C_{56}H_{110}NO_{10}P)_n$ $(987.8)_n$	Calc.	68.05	11.22	1.42	
		Found	65.61	11.12	1.36	3.86%
		Corrected	65.63	11.13	1.37	
[EDMPC][Ste]	$(C_{56}H_{112}NO_{10}P)_n$ $(989.9)_n$	Calc.	67.91	11.4	1.41	
		Found	65.48	11.32	1.35	3.12%
		Corrected	65.50	11.31	1.36	

Table S2: pH observation of the 20mg/mL concentrated LBILs in water. The data represents the average of the three experiments.

Name of LBILs	**Concentration of ILs**	**Average pH of 3 samples**
[EDMPC][Lin]	20mg ILs in 1mL water	5.8
[EDMPC][Ole]	20mg ILs in 1mL water	6.1
[EDMPC][Ste]	20mg ILs in 1mL water	6.0

Table S3: USP and BP quantitively solubility criteria of solutes in solvents.

USP and BP Solubility Criteria		
Term	**Parts of solvents required for 1 parts of solute**	**Solubility defined in mg/mL**
Very soluble	Less than 1 part	>1,000 mg/mL
Freely soluble	1-10 parts	100-1,000 mg/mL
Soluble	10-30 parts	33-100 mg/mL
Sparingly soluble	30-100 parts	10-33 mg/mL
Slightly soluble	100-1,000 parts	1-10 mg/mL
Very slightly soluble	1,000-10,000 parts	0.1-1 mg/mL
Insoluble	>10,000 parts	< 0.1 mg/mL

Table S4: Qualitative solubility studies of LBILs in various solvents at room temperature (25°C). Here, (++) very soluble, (±) sparingly soluble, and insoluble (-).

Solvents	Solutes			
	EDMPC-Cl	[EDMPC][Lin]	[EDMPC][Ole]	[EDMPC][Ste]
IPM	++	++	++	++
IPA	++	++	++	++
Ethanol	++	++	++	++
Methanol	++	++	++	++
DMSO	++	++	++	++
Water	-	±	±	±
PBS	-	±	±	±
Hexane	-	++	++	++
Heptane	-	++	++	++
Octane	-	++	++	++
Dodecane	-	++	++	++
Toluene	-	++	++	++

Table S5: DLS observation of [EDMPC][Lin] IL in water at different concentrations.

Conc. of LBILs in water	Diameter Average (nm)	PDI	Derived count rate
1 mg/mL	135	0.387	34401
2 mg/mL	153	0.220	66922
3 mg/mL	173	0.334	97089
4 mg/mL	156	0.202	257390
5 mg/mL	154	0.149	302926
7 mg/mL	255	0.345	340552
10 mg/mL	372	0.330	611252
15 mg/mL	393	0.314	800867
20 mg/mL	485	0.297	957960

Table S6: The refractive (RI) index of LBILs. The data represents the average of the three experiments.

Ionic Liquids	**Chemical formula**	**Average refractive Index (RI)**
[EDMPC][Lin]	$C_{56}H_{108}NO_{10}P$; $\mathbf{C_{18:2}}$	1.4506
[EDMPC][Ole]	$C_{56}H_{110}NO_{10}P$; $\mathbf{C_{18:1}}$	1.4630
[EDMPC][Ste]	$C_{56}H_{112}NO_{10}P$; $\mathbf{C_{18:0}}$	1.4802

Table S7: Quantity of the cell viability of LBILs formulated sample in skin irritation tests were represented in this table.

Concentration base Toxicity Studies		
Concentration of IL (w/w)	**Average cell viability**	**Standard Deviation (SD) ±**
PBS	100.0%	6.9
IPM	101.3%	4.4
5% [EDMPC][Lin]	100.5%	3.2
10% [EDMPC][Lin]	101.4%	5.1
20% [EDMPC][Lin]	100.4%	5.2
50% [EDMPC][Lin]	82.6%	3.7
100% [EDMPC][Lin]	51.8%	6.8
100% [EDMPC][Ole]	50.4%	5.4
Comparative Toxicity Studies		
20% Conc. of IL (w/w)	**Average cell viability**	**Standard Deviation (SD) ±**
PBS	100.00%	6.9
IPM	101.34%	4.4
[EDMPC][Lin]	101.4%	5.2
[EDMPC][Ole]	98.3%	1.7
[EDMPC][Ste]	97.8%	2.6
emim TFSA	69.4%	4.2
bmim-TF_2N	27.2%	4.1
SDS	1.9%	1.0

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