Supporting Information

Ionic liquid-mediated ethosome for transdermal delivery of insulin

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1. Materials and Methods

1.1. Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and ethyl trifluoromethyl sulfonate were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Linoleic acid (Lin) from Sigma-Aldrich Chemical Co. (St. Louis, MO). Insulin, human, recombinant (Lot. CAM195, potency ≥ 27.5 units/mg), Fluorescein Isothiocyanate Isomer I (FITC-I), D-MEM (Low Glucose) with L-Glutamine and Phenol Red were procured from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ethanol (99.5%) was purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). PD-10 Sephadex™ G-25 M columns were purchased from Sankei Chemical Co., Ltd. (Kagoshima, Japan)

Fetal bovine serum, and antibiotic–antimycotic were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's phosphate buffered saline and trypsin/ ethylenediaminetetraacetic acid (0.25% trypsin/1mM ethylenediaminetetraacetic acid) were purchased from Nacalai Tesque (Kyoto, Japan). A WST-8 cell counting kit containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Mammalian cell lines, HeLa were provided by the RIKEN cell bank (Tsukuba, Japan).

All the other chemicals and solvents were of analytical grade and used without any further purification.

1.2. Synthesis of ionic liquid

In this experiment, [EDMPC][Lin] consisting of the phospholipid derivative EDMPC as the cation and Lin (18:2) as the anion, was synthesized and characterized following the protocols that were previously reported [1]. The synthesis was confirmed by Fourier transform infrared (FTIR) spectroscopy (Fig. S1). 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 – 4000 cm^{-1} with 20 scans accumulation. Spectral outputs were recorded in a transmittance mode as a function of wave number. ^{1}H-NMR of [EDMPC][Lin] was employed by dissolving it in the chloroform-d solution. Spectrum data were processed by the Delta-V software package (version 5.0.5.1, JEOL) where coupling constants (J) were in Hz units.

1.3. Fluorescein isothiocyanate (FITC) labeling of insulin

FITC labeling of Insulin was prepared following a previously reported protocol [3] with a minor modification. Briefly, Human-derived Insulin was dissolved in 0.1M HCl, and 0.1M Sodium Bicarbonate buffer (P^H 9.3) was added to create a 10mg/mL insulin solution. FITC was dissolved in DMSO to create a 5mg/mL solution, and both solutions were gently mixed with a molar ratio of FITC:insulin 3:1 while shielded from light for 2.5 hours at room temperature (RT) with gentle continuing stirring. The resulting FITC-labeled Insulin (FITC-Insulin) mixture was collected using a PD-10 SephadexTM G-25 column and subsequently freeze-dried to obtain the final powdered form of FITC-Ins. The quality of FITC-Ins was confirmed through Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Fig. S4).

1.4. Circular dichroism (CD) spectroscopy

CD spectroscopy was employed to assess the stability of insulin's secondary structure in both 20-40% ethanol and 25-35% ETs. The J-1500 CD spectrometer (JASCO, Tokyo, Japan) was utilized with the parameters presented in Table S1.

Table S1. Parameters for CD measurement

Cell	Path length (mm)	Wavelength range (nm)	Integral count	Temperature (˚C)
Quartz cell	0.1mm	190 - 250	5	25

The obtained CD spectra were subjected to an adaptive smoothing method and then converted to the absolute value of CD.

1.5. Physicochemical characterization and stability study of ETs

1.5.1. Particle size and zeta potential (ZP/ ζ) distribution

Particle size and ZP distribution were determined using a Zetasizer Nano ZSP (Malvern, Nano series, Worcestershire, WR14 1XZ, U.K.) through dynamic light scattering (DLS) measurements. These measurements aimed to calculate the average hydrodynamic radius (R_H) and ZP, ζ of the particles. Disposable cuvettes were positioned at an angle of 173°, and the temperature was maintained at 25 ± 0.1°C during the measurements. Five measurements were taken for each sample, and the mean values were calculated using the Zetasizer 7.03 software, which provided

Z-averaged values of R_H and polydispersity index (PDI). The R_H calculation was conducted by the Stokes-Einstein equation,

$$D = \frac{kT}{6\pi\eta R_H}$$

Here, D represents the translational diffusion coefficient, k is the Boltzmann's constant, T is the absolute temperature, and η is the solvent viscosity. ZP, ζ was calculated by the measurement of the electrophoretic mobility using the Hemholtz-Smoluchowski equation. [5] Stability assessments of the formulations were performed at different time points over a period of 12 weeks.

1.5.2. Morphology of ETs by TEM & CLSM

A JEOL JEM-2010 Transmission Electron Microscopy (TEM) was employed to examine the size and shape of the ETs. Sample preparation involved placing 5µL aliquots on a carbon film-coated copper grid, allowing them to air-dry for 2 minutes (min), and removing excess material with filter paper. Staining was performed by applying 2.5 µL of 2% uranyl acetate solution, followed by a 5 min incubation. Subsequently, the TEM grid was vacuum-dried in a desiccator, and TEM images were captured at an accelerating voltage of 120 kV.

For confocal laser scanning microscope (CLSM) examination, a 5 µL formulation was mounted between a glass slide and a cover glass for fixation. The observation was made using a Carl Zeiss LSM700 microscope, Oberkochen, Germany at 63X magnification. Image processing was performed using LSM software from Carl Zeiss

1.6. Entrapment efficiency of ETs

The Encapsulation efficiency (%EE) was determined through fluorescence spectrophotometry. Non-encapsulated drug quantification was determined using a centrifuge concentrator method: Formulations were centrifuged using the TOMY MX-307, HIGH SPEED REFRIGERATED MICRO CENTRIFUGE, at 14,000 rpm at 4 °C for 30 min. The resulting supernatant was transferred to a separate Eppendorf tube and subjected to a secondary centrifugation for 15 min at 14,000 rpm.

The total quantity of unentrapped FITC-Insulin was assessed using a microplate spectrophotometer (Bio-Rad, Tokyo, Japan) within the wavelength range of 485 to 535 nm. Each sample underwent three evaluations. [6,7]

The percentage of encapsulated drug amount was calculated using the following formula:

$$\text{EE (\%)} = \frac{Amount\ of\ drug\ used - uncapsulated\ drug}{Total\ amount\ of\ drug} \times 100$$

1.7. Cell viability assay

The WST-8 cell viability assay was conducted using HeLa mammalian cell lines following a protocol outlined in the literature with some modifications. [8] HeLa cells were cultured until they reached approximately 70-80% confluency and were harvested from the cell culture dish through trypsinization. Subsequently, the cells were seeded into 96-well flat-bottomed plates at a density of $1.5x10^4$ cells per well (100 µL, concentration: 1.5×10^4 cells per mL) and cultured in MEM (containing 10% fetal bovine serum and 1% antibiotic-antimycotic) for 24 hours at 37°C in a CO_2 incubator.

Next, 2 µL of ET1, ET2, ET3, and ET4 formulations were added to the respective wells. Dulbecco's Phosphate-Buffered Saline (D-PBS), 25% ethanol (E25), and 1 mg/mL insulin solution were used as positive controls, while 5% Sodium Dodecyl Sulfate (SDS) was used as the negative control. The cells were further incubated for 24 hours. Subsequently, 10 µL of the Cell Counting Kit-8 solution was added and incubated for 3 hours to initiate the color reaction. The absorbance was measured at 450nm using a microplate spectrophotometer (Bio-Rad, Tokyo, Japan). As a negative control, 10 µL of the WST-8 solution was added, and for the blank, 10 µL of MEM medium was added instead of the WST-8 solution to the wells. Cell viability was calculated using the following formula:

Cell viability [%] = $[(A_s-A_b)/(A_c-A_b)]\times 100$

Where: A_s = Absorbance of the sample; A_c = Negative control absorbance; A_b = Blank absorbance.

1.8. *In vitro* skin permeation study

Mouse skin penetration was evaluated for various formulations loaded with FITC-Insulin using a Franz diffusion cell (FDC) following a previously established procedure. [9] Mouse skin sections (Hos: HR-1) were purchased from Hoshino Laboratory Animals (Ibaraki, Japan) and stored at -

80^0C. Skin sections were dissected into 2 cm x 2 cm square pieces with a scalpel for the *in vitro* experiment. In the FDC setup, the receiver phase contained 4 mL of 1X PBS buffer and was equipped with a magnetic stirrer. Mouse skin pieces were affixed to the upper side of the receiver phase, with the stratum corneum (SC) facing outward, serving as the donor compartment. To eliminate air bubbles, 1 mL of PBS buffer was introduced through the branch tube into the FDC. FITC-Insulin formulations (1mg/mL) with varying compositions were applied in 200µL onto the mouse skin within the donor compartment. After a 6-hour incubation, 1mL samples were collected from the receiver phase of all FDCs using syringes.

To quantify the topically delivered drug amount, the skin was unclamped, cleansed with Milli-Q water and 20% ethanol, and then sectioned into >16 slices using a scalpel. The skin slices were immersed in an extraction solution comprising PBS-ACN-MeOH (2:1:1; v/v/v) at RT for 12 hours (Fig. 4(b)) and 15 hours (Fig. 4(d)) with continuous agitation. The insulin content in the collected samples was assessed using a microplate spectrophotometer (iMARK, Bio-Rad, Tokyo, Japan).

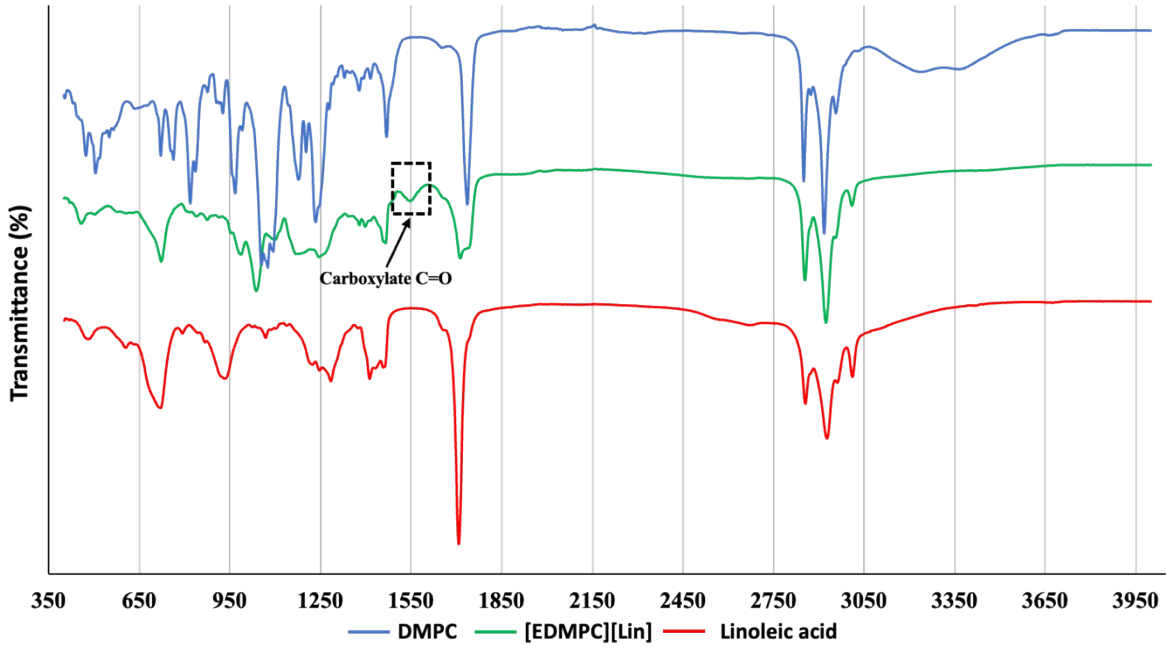
1.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (Version 6.05). One-way ANOVA with Dunnett's Multiple Comparison test was used to determine the statistical significance of the data. A P-value < 0.05 was considered statistically significant. All values shown here were carried out as the mean ± standard deviation.

2. Results and discussion

2.1. FTIR spectra of [EDMPC][Lin], Lin and DMPC and ^{1}H-NMR spectra of [EDMPC][Lin]

The FTIR spectra of [EDMPC][Lin], Lin, and DMPC were analyzed to confirm the synthesis of IL. The distinctive C=O stretching peak from carboxylate of the synthesized [EDMPC][Lin] was observed in the region of 1545 cm^{-1}. The detailed FTIR analysis along with the 1H NMR findings indicates the successful synthesis of [EDMPC][Lin] (Fig. S1 and Fig. S2).



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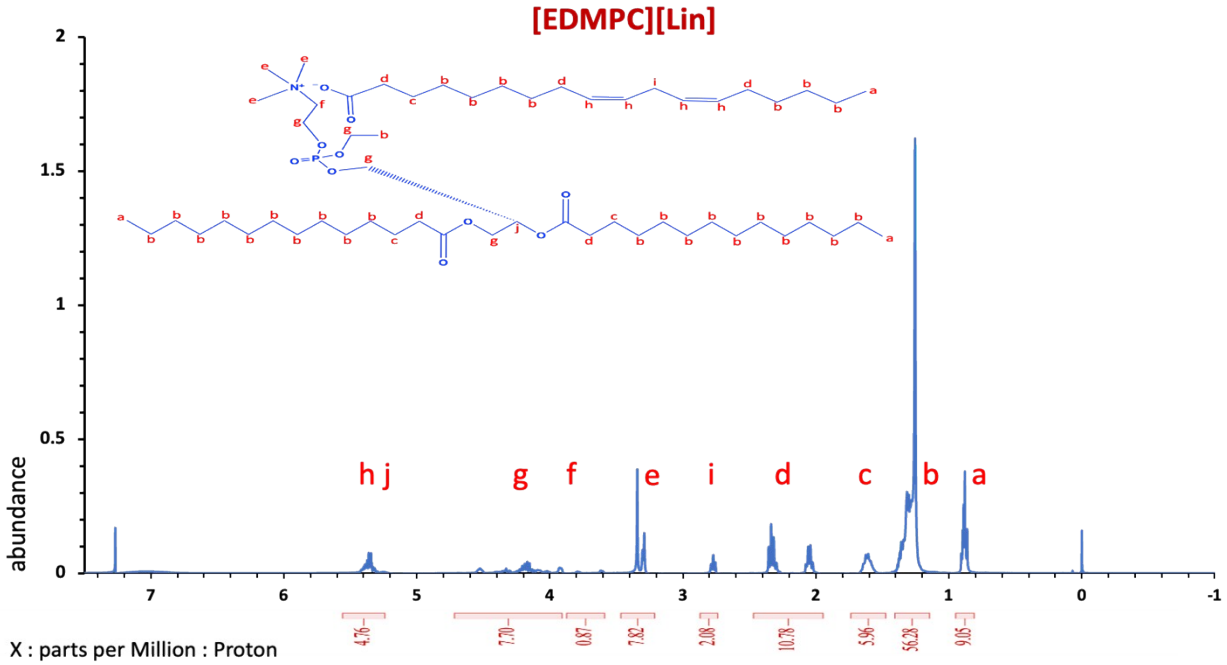
183 **Fig. S1** FTIR-spectra of [EDMPC][Lin], Lin, and DMPC



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185 **Fig. S2** ^{1}H-NMR spectra of [EDMPC][Lin]

2.2. CD spectra observation

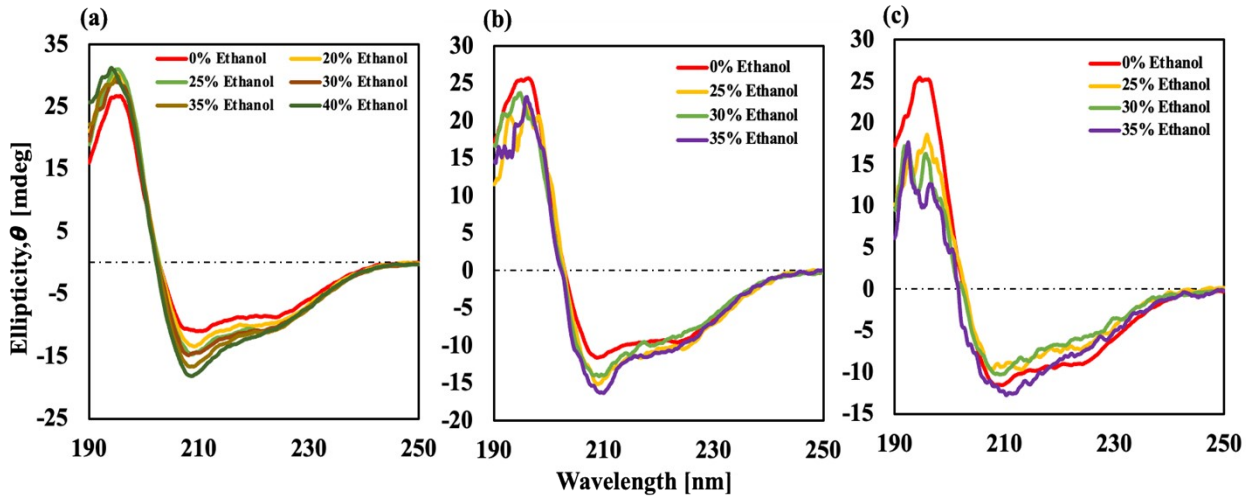

Fig. S3 CD spectra showing the conformational change of insulin in (a) 20-40% ethanol; ETs (b) after sonication, and (c) after homogenization in 25-35% ethanol

2.3. MALDI-TOF MS observation of FITC-Insulin

The MALDI-TOF MS analysis of FITC-Insulin revealed a notably high-intensity peak for the mono-conjugate of FITC-Insulin, with very minor peaks for di and tri-conjugated FITC-Insulin (Fig. S4).

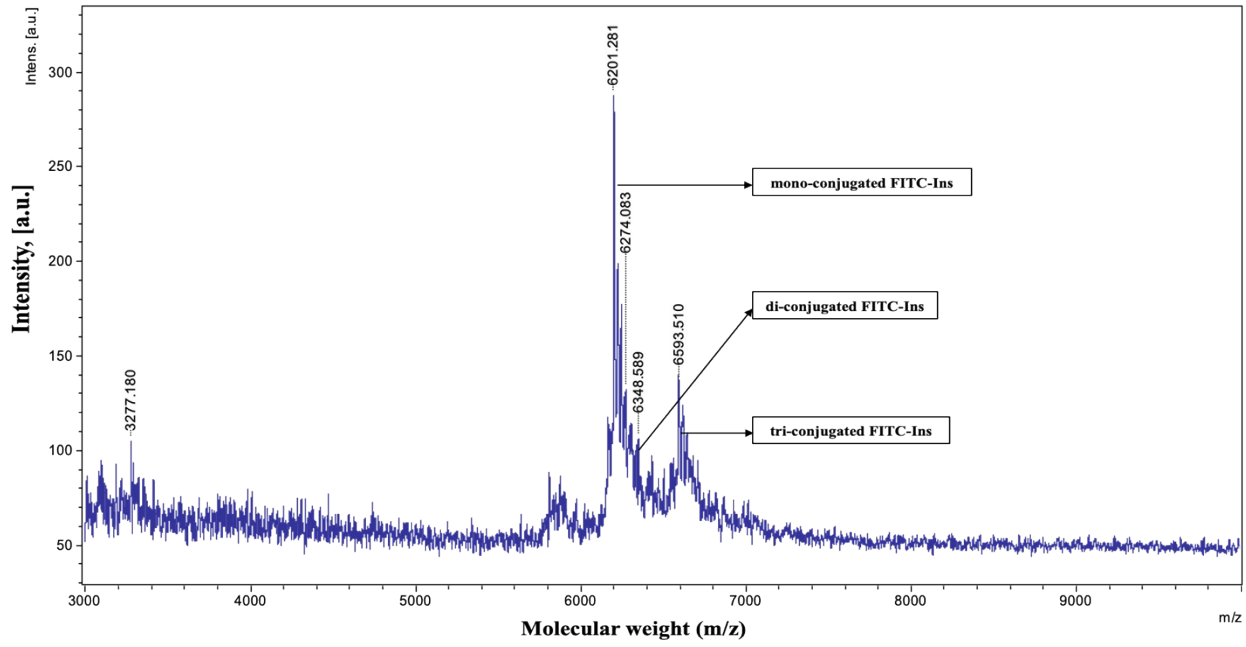

Fig. S4 MALDI-TOF MS spectrum of FITC-Insulin

2.4. Size distribution and ZP of ETs

ETs were prepared using DMPC and [EDMPC][Lin] with and without any drugs to assess successful vesicle preparation in the presence of ethanol. ETs were successfully synthesized using both DMPC and [EDMPC][Lin].

Table S2. Composition of LBIL ethosomal systems.

Samples	[EDMPC][Lin] (mM)	DMPC (mM)	Ethanol (w/w%)	Water (w/w%)
ET1	5	-	25.35	75.65
ET2	10	-	25.35	75.65
ET3	15	-	25.35	75.65
ET4	20	-	25.35	75.65
ET5	-	10	25.35	75.65
ET5	-	20	25.35	75.65

The size distribution of [EDMPC][Lin] and DMPC-mediated ETs without insulin was observed, revealing that the [EDMPC][Lin] ETs exhibited a larger vesicle size compared to that of DMPC ETs (**Table S3.**).

Table S3. Size distribution and ZP of ETs without drug

System	Z-Ave(nm)	PDI	ZP (mV)
ET2	377.3 ± 9.479	0.126 ± 0.043	45.9±2.68
ET4	374.1 ± 6.016	0.130 ± 0.047	53.8±2.51
ET5	230.0 ± 3.680	0.105 ± 0.058	-11.3±0.624
ET6	246.2 ± 5.658	0.134 ± 0.063	-8.66±0.499

2.4.1. FITC-Insulin loaded ETs

After loading with the FITC-Insulin drug, the DMPC ETs exhibited instability, characterized by significant variability in size and elevated PDI values (**Table S3.**). Within 24 hours, it became totally unstable to measure the DLS readings.

Table S4. Size distribution of DMPC-mediated ETs loaded with FITC-Insulin

System	Z-Avg (nm)	PDI
ET5	4678±442	0.584±0.332
ET6	4863±1359	0.714±0.330

With the loading of the FITC-Insulin drug the [EDMPC][Lin] ETs were stable with an increase in size.

Table S5. [EDMPC][Lin] ETs Z-Average and PDI values after loading with FITC-Insulin.

System	Z-Avg (nm)	PDI
ET2	604.2±34.9	0.324±0.054
ET4	609.8±47.2	0.402±0.130

2.4.2. Size reduction of ET formulations

The size distribution of nanovesicles is a critical factor in achieving effective drug delivery [10]. In our study, we utilized a combination of probe sonication and homogenization techniques to systematically reduce the size of 10mM [EDMPC][Lin] ETs at varying ethanol concentrations. Specifically, the application of a sonicator led to successful and uniform size reduction.

Table S6. Z-average and PDI values for ET2 formulation

Ethanol conc. (vol/vol)	Sonicator		Homogenizer	
	Z-Avg (nm)	PDI	Z-Avg (nm)	PDI
35%	384.23±6.04	0.255±0.03	683.53±23.95	0.349±0.06

2.5. Stability study of ETs

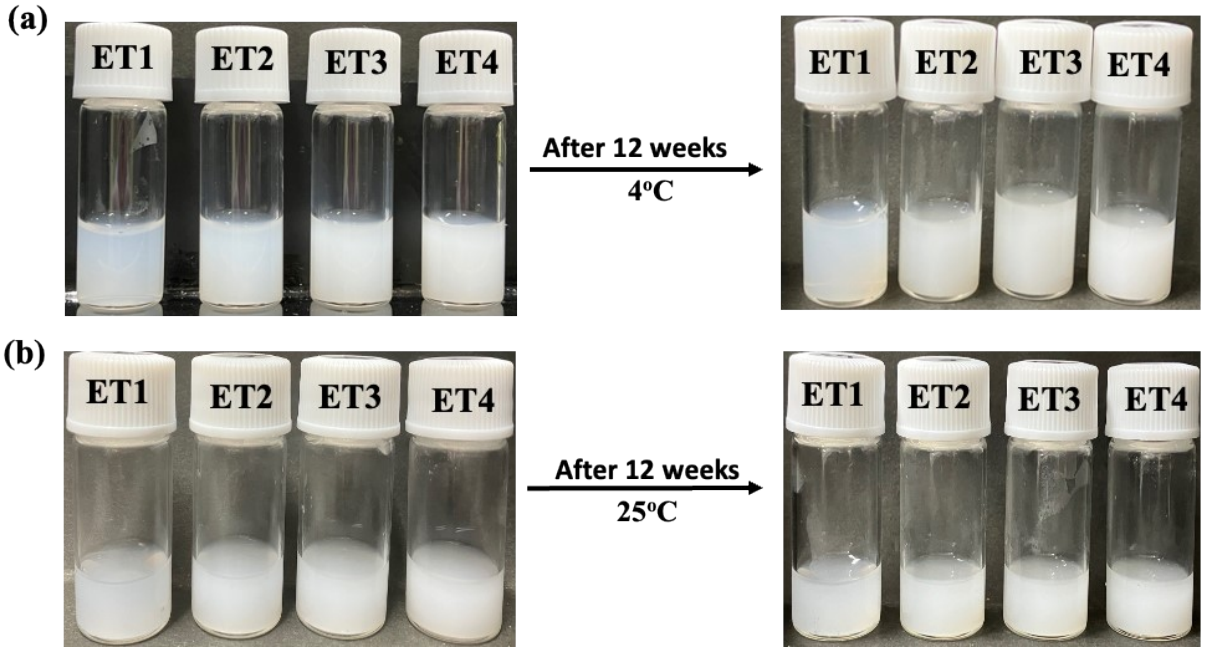

Fig. S5 Physical appearances of LBIL ETs before and after 12 weeks; (a) at 4°C; (b) at 25°C.

2.6. Entrapment efficiency of LBIL ETs

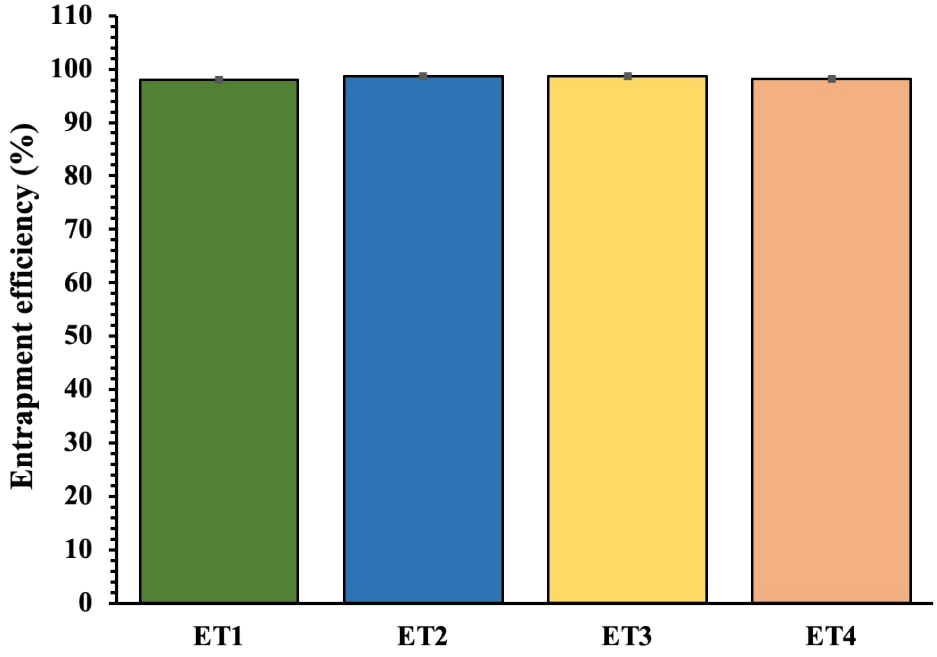

Fig. S6 Percent entrapment efficiency of ET formulations. All the data represent the average of the three experiments, and the error bars indicate the standard deviation.

2.7. Drug distribution by CLSM observation

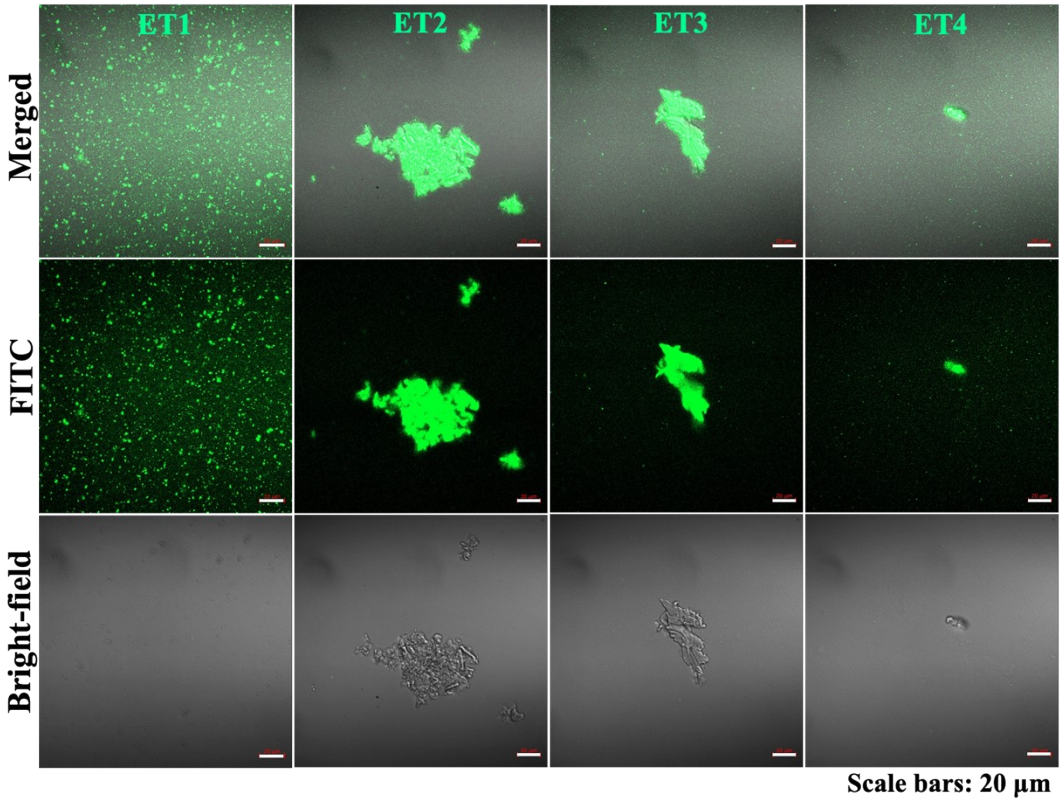

Fig. S7 Drug distribution observation of ET1, ET2, ET3 & ET4 encapsulated with FITC-Insulin by CLSM visualization through a 63X lens.

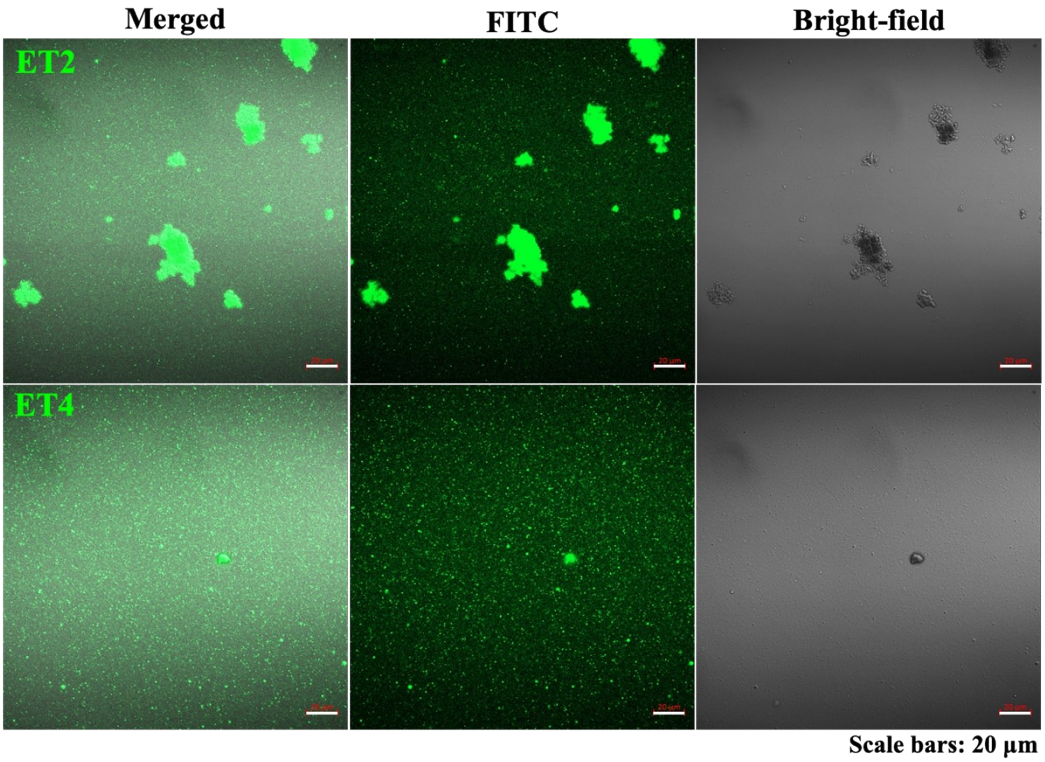

Fig. S8 Drug distribution observation of ET2 and ET4 prepared with 35% ethanol encapsulated with FITC-Insulin by CLSM visualization through a 63X lens.

2.8. Cell viability assay

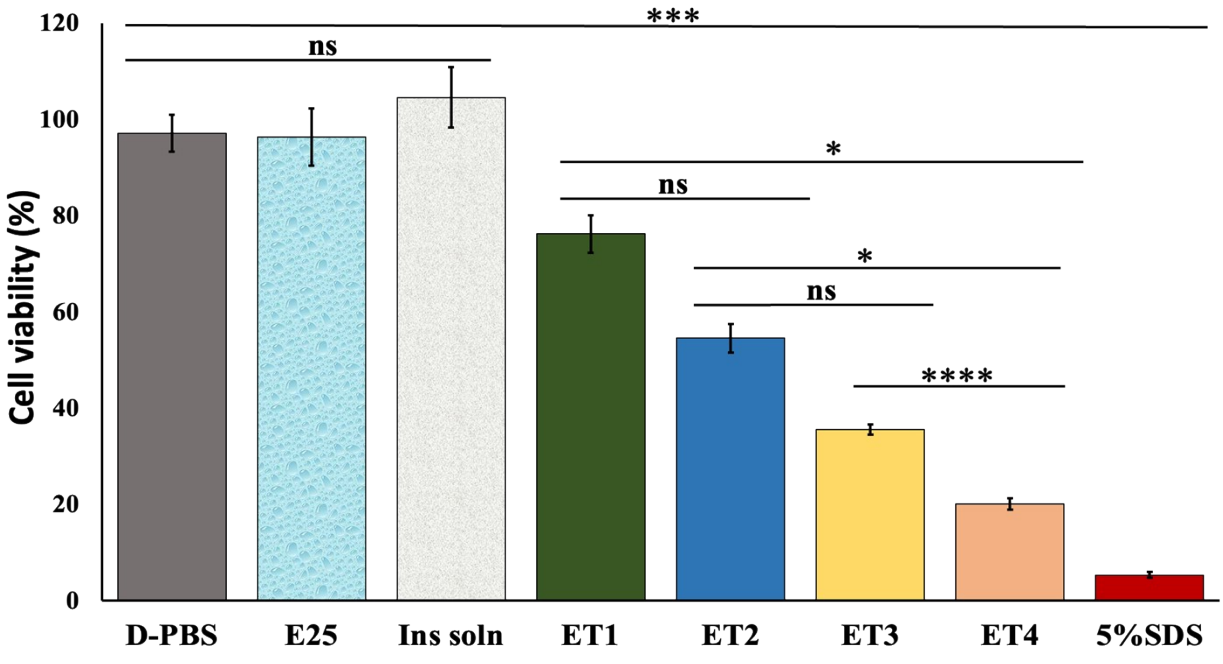

Fig. S9 Cytotoxicity of IL ETs towards HeLa cell lines, N = 10; mean ± SD; ns means, nonsignificant, *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ (Tukey multiple comparison test)

2.9. *In vitro* skin permeation study

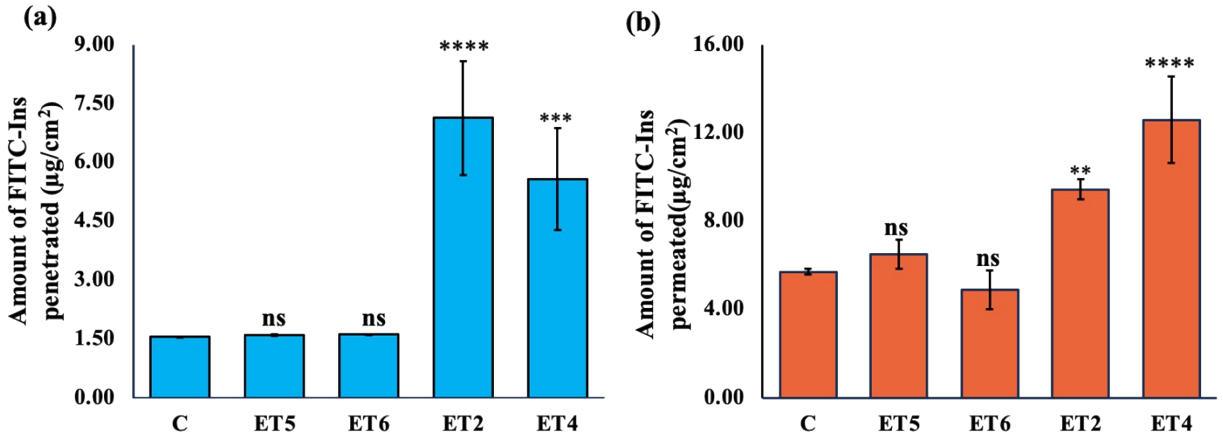

Fig. S10 In vitro skin permeation study, (a) drug penetration and (b) drug permeation by ETs in mouse skin. Here, C, control FITC-insulin solution (1mg/mL); ns, non-significant; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$ (Dunnett's multiple comparison test).

3. References

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