

Development of Lipid-based Biocompatible Ionic Liquids to Enhance Transdermal Drug Delivery Systems for Cancer Treatment.

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Development of Lipid-based Biocompatible Ionic Liquids to Enhance Transdermal Drug Delivery Systems for Cancer Treatment.



**A Thesis Submitted to Kyushu University for the Degree of
Doctor of Philosophy in Engineering**

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*In the Name of ALLAH,
The Most Beneficent and The Most Merciful.*

*This Thesis is Fully Dedicated
To
My Beloved Parents and Family.*

CHAPTER 1

GENERAL INTRODUCTION

1.1 Ionic Liquids (ILs) and Their Potential Applications in Drug Delivery System.

1.1.1 Definition of ionic liquids (ILs)

Ionic liquids (ILs) are defined as liquid stable organic compounds that are composed of a set of cationic and anionic counterpart ions with moderated melting points that are usually below 100 °C (212 °F).¹ ILs have been the most appealing and innovative fluids in modern science over the last few decades, especially since the development of room temperature ILs (RTILs). Since the mid-1900s, the importance of ILs has drawn the attention of many researchers, and their use has increased not only in the research field, but also in industrial areas.² ILs have received wide attention only during the last three decades, because of their excellent physio-chemical properties, such as physio-chemical thermal stability, wide range of solubility through organic and inorganic solvents, low flammability, high ionic conductivity, non-volatility, recyclability, low vapor pressure, and low or non-toxicity.³ ILs contain a bulky cationic molecules like alkyl-imidazolium ion $[R_1R_2IM]^+$; alkyl-phosphonium ion $[PR_4]^+$; cholinium $[OH-R_1NR_3]^+$; alkyl Pyridium $[RPy]^+$; alkylammonium $[NR]^+$, and its counter anion such as $[Br]^-$, $[Cl]^-$, $[BF_4]^-$, $[AlCl_4]^-$, $[Al_2Cl_7]^-$, $[RSO_4]^-$, $[(CF_3SOO)_2N]^-$, $[CF_3(CF_2)_3COO]^-$, $[NO_3]^-$, $[CH_3SOO]^-$, $[CH_3COO]^-$, $[CF_3COO]^-$ or $[PF_6]^-$, fatty acids and amino acids.⁴ As a result, a thousand cations and anions can prepare for a billion ILs, the synthesis process is not so difficult even on a large scale, and their physiochemical properties depend on the nature of the cations and anions.⁵ Some cases, low temperature ILs contain both ions and neutral molecules, and

called as deep eutectic solutions.⁶ Combination of non-ionic and ionic substances which have much lower melting temperature than that of the pure molecules.¹

1.1.2 History of ILs

Modern IL-chemistry can also demonstrate the chemistry of salts with a melting point of below 100°C. Scientists discovered a liquid known as "red oil" in the back to the second half of the nineteenth century. Fried and Craft reported in 1887 that when a small amount of ammonium chloride was mixed with amyl chloride, two layers formed in a liquid.⁷ In 1888, Gabriel first reported that Protic IL had a melting point of below 100 °C.⁵ At the end of the 19th century, Trowbridge and co-workers developed Pyridium salts, which had a low melting point.⁸ In 1914, Walden synthesized ethyl-ammonium nitrate and declared it as a room temperature ionic liquid (RTILs). Some RTILs have been developed since 1998, and various ILs are currently being developed.⁹ The explosion and interest in IL developments significantly increased in the 20th century.¹⁰ Based on their physiochemical properties and developmental periods, ILs are classified into three categories. These are known as 1st generation, 2nd generation and 3rd generation ILs.

1st Generation ILs: First generation ILs are considered as the earliest developed ionic liquids, such as halo-aluminate ILs in 1951, the earliest RTILs in 1982.⁹ 1st generation ILs have some unique physiochemical properties, such as a low melting point, no or low volatility, a large scale of liquidity, low vapor pressure, and high thermo stability.^{11,12} The majority of the first generation ILs, such as dialkyl-imidazolium chloroaluminates, [C4MIM] [BF₄], and [C4MIM] [PF₆], are either toxic to the environment or poorly biodegradable.² Some anions, such as [BF₄] and [PF₆] are hydrolyzed when they come into contact with toxic volatile moisture (HF, phosphorous

oxyfluoride). Moreover, some other anions such as $[\text{FeCl}_4]^-$, TFSA, triflate, TFA, alkyl-sulfate are also associated with the 1st generation of ILs.^{11,13,14}

2nd Generation ILs: Research interest is increasing in functional materials science with the development of the second generation of ILs, because of their tunable physical and chemical properties (Fig. 1), such as in energetic materials, ionic metal complexation, and lubricants.^{12,15} The second generation ILs have a wide range of architectural platforms on which both cations and anions can be modified independently and their tunability helps to make new active materials, without changing any core desired properties.^{11,16} The chemical features of the 2nd generation ILs, such as highly energetic density, chemical reactivity, coordination, solubility, flammability, chiral induction and electro chemical windows, help to choose desired physical properties.¹² Many second generation ILs have recently been reported as biocompatible ionic liquids containing both biocompatible cations and anions, resulting in the production of thousands of environmentally friendly, greener solvents. The biocompatibility of the 2nd generation ILs is widely used in biological applications, such as in oral, transdermal, and topical drug delivery systems (DDS).¹⁷

3rd Generation ILs: The third generation ionic liquids are considered to be biological active ionic liquids along with tunable physical and chemical properties for pharmaceuticals and biological applications.¹⁸ The main source of the 3rd generation IL synthesis is biologically active biomaterials, used to make suitable biological compounds such as antibacterial, antifungal, anticholinergic and local anesthetic agents (Fig. 1). The cations or anions or both ions of active pharmaceutical ingredients ILs (API-ILs) are derived from active ingredients or their precursors.¹⁹ The 3rd generation ILs are also

Evolution of Ionic Liquids

Cation

+

↓

New, functionalized ionic liquid material

Anion

—

Generation 1:

ILs with unique tunable physical properties

Physical property

$$\begin{matrix} R^1 \\ | \\ R^4-N^+-R^2 \\ | \\ R^3 \end{matrix} \quad \begin{matrix} R^1 \\ | \\ R^4-P^+-R^2 \\ | \\ R^3 \end{matrix}$$

- melting point
- density
- viscosity
- thermal stability
- conductivity
- hydrophobicity
- refractive index

Physical property

$$\begin{matrix} F_3C-S(=O)(F_3)-N^+-S(=O)(F_3)-F_3C \\ || \quad || \\ O \quad O \end{matrix}$$

lower melting point / hydrophobicity

$$\begin{matrix} C_6H_{13} \\ | \\ C_6H_{14}-P^+-C_{14}H_{29} \\ | \\ C_6H_{14} \end{matrix} \quad NC-N^+-CN$$

hydrophobicity / lower viscosity

high thermal stability / hydrophilicity

Physical property

$$\begin{matrix} O & O \\ || & || \\ F_3C-S-N-S-F_3 \\ || & || \\ O & O \end{matrix} \quad Cl^-$$

$$NC-N^+-CN \quad BF_4^- \quad FeCl_4^- \quad HO-S(=O)_2-O^-$$

- melting point
- density
- viscosity
- thermal stability
- conductivity
- hydrophobicity
- refractive index

Generation 2:

ILs with targeted chemical properties combined with chosen physical properties

Chemical property

- chemical reactivity
- high energy density
- electrochemical window
- flammability
- coordination
- solvation
- chiral induction

Chemical property

energy density / oxygen balance

lower density / solvation

chiral induction/ hydrophobicity

Chemical property

- high energy density
- electrochemical window
- flammability
- oxygen balance
- UV blocker
- chiral induction
- solvation

Generation 3:

ILs with targeted biological properties combined with chosen physical and chemical properties

Biological property

- antibacterial
- local anesthetic
- anticholinergic
- antifungal

Biological property

local anesthetic / emollient

anti-bacterial / UV blocker

lower melting, antibacterial / NSAID

Biological property

- emollient
- anti-acne
- antibiotic
- non-steroidal anti-inflammatory drug (NSAID)
- vitamin

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1.1.3 Physicochemical properties of ILs

In recent years, ILs have been the leading edge in the most advanced sciences and technologies because of their multidimensional properties. The major properties of ILs are described below.

Liquid range and thermo stability: ILs have a wide range of liquidus properties, including melting temperature (T_m) or glass transition temperature (T_g) and boiling (T_b) or thermo-decomposition (T_d) temperature, such as the T_g of 1-alkyl 3-methyl imidazolium salt is -70 °C to -90 °C and its T_d ranges between 250 °C to over 450 °C therefore, a wide range of liquid states is 300 °C. Where the liquid temperature of water ranges from 0°C to 100°C. Because ILs have a wide range of thermo-decomposition temperatures, they can be used to conduct chemical experiments at high temperatures without degradation. ILs are thermally stable even after converting from their original sources (cations and anions). Many bioactive ILs such as API-ILs have been reported to be thermostable compounds where their original sources (API) were unstable, so they were converted into ILs to make them thermo stable.⁴ The development of thermostable API-ILs has paved the way for ILs to be used in pharmaceutical applications.^{6,17}

Melting point: Melting temperature is the fundamental property of ILs because it has a wide range of liquid states with a low melting temperature as well as a high decomposition temperature.²¹ The wide range of solubility of ILs in organics and inorganics is directly correlated with the melting temperature as well as the freezing temperature.^{22,23} The melting point of ILs is significantly influenced by their structural symmetric cations or by the length or mixture of the eutectic solvents.²¹ For example, the melting point of imidazolium based room temperature ILs (RT-ILs) of [C₂mim] Cl is 87 °C and [C₂mim]

[AlCl₄] is 7 °C. A small change in the alkyl chain can significantly change the melting temperature of ILs, such as [C₄mim] Cl, which has a melting point of 65 °C, which is 22 °C lower than [C₂mim] Cl.

Vapor pressure: ILs have a negligible or very low vapor pressure. In 2005, Rebelo et al, reported that there was no reliable data on the IL vapor pressure and its relationship with temperature.²⁴ Notably, the vapor pressure of some imidazolium ILs with a short alkyl chain containing the cation based ILs is negligible. Consequently, many ILs show very negligible or no evidence of distillation below its decay temperature.

Heat capacity and heat transfer: ILs have a high capacity for heat storage and transfer. Based on this property, ILs are widely used in refrigerators, pumps, chemical reactors, solar systems, and other electrochemical industries. This property also includes the melting points, boiling points, heat capacity, and thermostability of ILs.

Viscosity: The viscosity of IL can be defined as the fluidity or flow of ILs, and it is the inverse of liquidity. At room temperature, ILs are generally more volatile than molecular solvents.²⁵ The viscosity of ILs mainly depends on the strength of hydrogen bonding capacity and the Vander-Walls interaction capacity between the cation and anion of the ILs structure.²³ It is reported that both the structure and nature of cations and anions can influence the viscosity of ILs, such as the viscosity of [C₂mim] [BF₄] is 34 centipoise (cP), [C₂mim] [PF₆] is 270 cP, [C₆mim] [BF₄] is 314 cP , and [C₆mim][NO₃] is 804 cP room temperature. Whereas the viscosity of water, acetone and ethanol is 0.89, 0.31 and 1.07 cP, respectively, at room temperature.²⁶

Density: ILs have a higher density than water because when water insoluble ILs are mixed with water, two identical phases form, with ILs in the lower phase and water in the upper phase. Both structural cations and anions influence the IL's density. The density of ILs is reverse-proportional to the bulkiness of their cations. Less-bulky or shorter alkyl length cations have a higher density than that of ILs containing large or higher alkyl length cations. Poor interaction of ILs forming bulky cations and weakly complex symmetrical anions may be the reason behind this. In the binary mixing, the density of halo-aluminate ILs increases as the proportion of aluminium halide increases. It has been reported that temperature has an effect on density. When the temperature increased from 293 K to 313 K, the density decreased linearly.^{22,26}

Solubility: Depending on the nature of ILs, forming cations and anions, ILs have a wide range of solubility. Generally, ionic liquids are polar solvents, as the resulting ILs are easily soluble in water and polar solvents.²⁷ Anions of IL structure are more effective than cations in increasing the solubility of ILs in various mediums. Moreover, ILs are soluble in various types of solvents, such as in organic solvents,²⁸ in inorganic solvents,²⁹ and in gaseous solvents.³⁰ Recently reported hydrophobic ionic liquids are soluble in nonpolar solvents which explore the range of their solubility.³¹

Conductivity: Conductivity of ILs can be defined as the liquid's ability to conduct an electric current where the ions act as the charge carrier. The conductivity of ILs mainly depends on their ion charge and their viscosity. The conductivity of the ILs is increased by more delocalized ions. Furthermore, the low mobility of the ions in ILs is responsible for the ILs' decreased conductivity. The measurement unit of conductivity is siemens per

meter (S m^{-1}). It has been reported that the small molecular cations or heterocyclic cations of the IL structure increase the conductivity of ILs..³²

Electrochemical potentials windows: Electrochemical windows are the thermodynamical measurement of a material and represent the voltage range over where it is reduced or oxidized and therefore electrically inert at an electrode. The electrochemical windows of an IL are primarily determined by cations (resistance to reduction) and anions (resistance to oxidation). ILs have a wide range of electrochemical properties, generally in the range of between 2.0-6.0 V. Impurities in the IL structure can have an impact on the electrochemical windows.

Surface tension: Surface tension is an important physical property of ILs. At a given temperature, the surface tension of an IL decreases as the anion size and increases and as the alkyl chain length of the cation increases.³³

Refractive index: The Refractive index (RI) or refraction index of a material is represented by a dimensionless number of how fast the light beam can pass or travel through the materials. The RI of ILs is primarily determined by the nature of both cations and anions. The RI increases as the number of branches, length, and cations increases.³⁴

Impurities effect: A trace amount of impurities (additives, chloride, water, inorganic/organic solvents or co-solvents) in the IL structure can affect physical, chemical, and spectroscopic properties.³⁵ The melting points and viscosity can increase significantly due to the presence of impurities. Sometimes the color of ILs is changed by the presence of any known and unknown impurities in the ILs' structure. In all cases, impurities are not harmful to ILs, and in some cases, the benefits of new compounds, such as intermediate water, can improve the biocompatibility of the developmental compounds.³⁶

Biodegradability: Biodegradability is a major and advanced property of ILs directly related to toxicity. Researchers have recently focused on the development of biodegradable ILs. ILs with the same cations containing a shorter chain exhibited lower biodegradability than that of a longer alkyl side chain. In addition, the biodegradability of ILs also depends on the nature of cations and anions, such as ILs containing short chain anions ($C < 3$; ethanoate, propanoate) were not rapidly biodegradable, but some anions with longer chain ($C > 3$; butanoate to octanoate) were completely biodegradable.³⁷

1.1.4 Biocompatible ILs (Bio-ILs)

When in contact with cellular tissues, bio-ILs are referred to as an eco-friendly, greener solvent family, ILs that are not toxic or avoid any types of undesirable cellular responses. Because IL biocompatibility is a major concern in the twenty-first century, researchers are focusing on developing new Bio-ILs. Biocompatibility is the major concern for drug delivery systems (DDS) or the application of ILs in a life science (cosmetics, pharmaceuticals, or health-care areas). According to the ISO 10933 standard, biocompatibility can be evaluated by assessing irritation or intracutaneous activity, cytotoxicity, sensitization, degradation, hemocompatibility, genotoxicity, acute or chronic or sub-chronic toxicity, carcinogenicity, implantation, pyrogenicity and developmental or reproductive toxicity.³⁹

1.1.5 Strategy of Bio-ILs synthesis

The ideal strategy for developing Bio-ILs is to derive the cations, anions, or both cations and anions of the IL structure from biocompatible sources. The main biocompatible sources of IL synthesis can be described as amino acids (AAs), fatty acids (FAs), glucose, isosorbide, arabinose, lactates, naturally occurring sugar, fructose, fructose derivatives

such as isomannide, acesulfamates, ester salts, levulinate, lignin, and carboxylic acids. Bio-renewable and natural sources are the other promising approaches to developing Bio-ILs, but in some cases, the resulting ILs are not considered as fully Bio-ILs.⁴⁰ In 2005, the first RTILs were developed by using 20 different natural amino acids coupled with imidazolium based cations.⁴² In recent decades, a remarkable number of cholinium based Bio-ILs have been developed with low toxicity and biodegradability and these Bio-ILs are significantly used in drug delivery systems.¹⁸ Cholinium based cations are coupled with different APIs (phenytoin, salicylic acids, ampicillin, niflumic acids, picolinic acids, nalidixic acids, and pyrazinoic acid) and explored API-ILs along with their biological activity.^{40,43} Recently, potentially cost-effective glycine betatine-based Bio-ILs were synthesized as an alternative to choline-based ILs.⁴⁴ and very recently, lipid based Bio-ILs were synthesized where phosphatidyl choline derivative was used as cations and fatty acids (linoleic acids, oleic acids and stearic acids) were used as its counter anions.³⁸

1.1.6 Applications of ILs in different fields

The applications of ILs are not only limited to the classical area of knowledge and in the research area, but also increasing in applications in industrial areas too. The applications of ILs have been found in clean and efficient energy, materials, fuel, lubricants, electrical and electronics, cosmetics, medical, and pharmaceuticals or healthcare industries.⁴ The use of ILs in different fields is mentioned below.

Chemical processing: It is reported that ILs act as a promising solvent to enhance many chemical reactions, such as many coupling reactions, Heck reactions, isomerization reactions, esterification reactions, Bails-Hillman reaction and Diels-Alder reaction.^{45,46}

ILs in extraction technology: Ionic liquids have recently been used to separate, extract, and purify new material.⁴⁶ ILs are used for the extraction and separation of bioactive materials from plant sources. ILs are also used to extract heavy metals and inorganic nanomaterials.

Designer solvents: ILs carry tunable cations and anions, as they can easily be modified into a new compound without changing its identical properties. Due to the highly tunable characteristics of ILs, they are considered as deep eutectic and designer solvents.

ILs as a green credentials: Nowadays, ILs are considered as a green solvent due to their excellent properties, such as low vapor pressure, high thermal stability, wide range of liquidity, high polarity, large electro chemical windows and low toxicity. ILs are regarded as greener solvents because they provide numerous excellent benefits, including environmental friendliness, biocompatibility, outstanding designability, low cost, and exceptional solubilization power.

ILs in electrochemistry: Ionic liquids are perfectly used in electrochemical fields such as lithium ion batteries, electroplating, electrochemical capacitors, polymer fuel cells, solar cells, solar thermal energy, and electrolytes.⁴⁷

Gas handling: ILs have low vapor pressure, high thermal stability, and high solvation properties. These IL properties are also used in gas handling.

ILs as catalyst: ILs are a new reaction medium that helps to resolve the catalyst recycling problem. It acts as a solvent for polymerization reactions.

ILs in waste recycling: ILs are used in the recycling of synthetic goods, metals, polymers, and plastics.

Chemical/ organic reactions: ILs are used in analytical chemistry, such as new compound synthesis, reaction enhancers, chromatographic electrophoretics and detection.

ILs in biotechnology: ILs are used in biotechnological techniques such as biomass pretreatments, extractions, biofuel production, biocatalysts, antimicrobial agents, clinical therapy, nutraceutical products, cosmetics, pharmaceutical and healthcare products.⁴⁸

ILs in pharmaceutical applications: ILs are considered as an auspicious applicant in the pharmaceutical areas. Many scientific reports have documented the advanced use of ionic liquids in pharmaceuticals.³⁷ Approximately 50% of commercial pharmaceutical materials are organic salt. Nowadays, ILs have more advantages than organic salts, and they are becoming a more promising and cost-effective alternative source of pharmaceutical solvents. Moreover, ILs can couple with dual APIs and develop dual active API-ILs, thus exploring the applications of ILs in the pharmaceutical industry.⁴

ILs in drug delivery system: ILs are used in drug delivery to enhance therapeutic effects. Recently, ILs have facilitated different routes of drug delivery systems, including oral, topical, transdermal, injection and nasal.

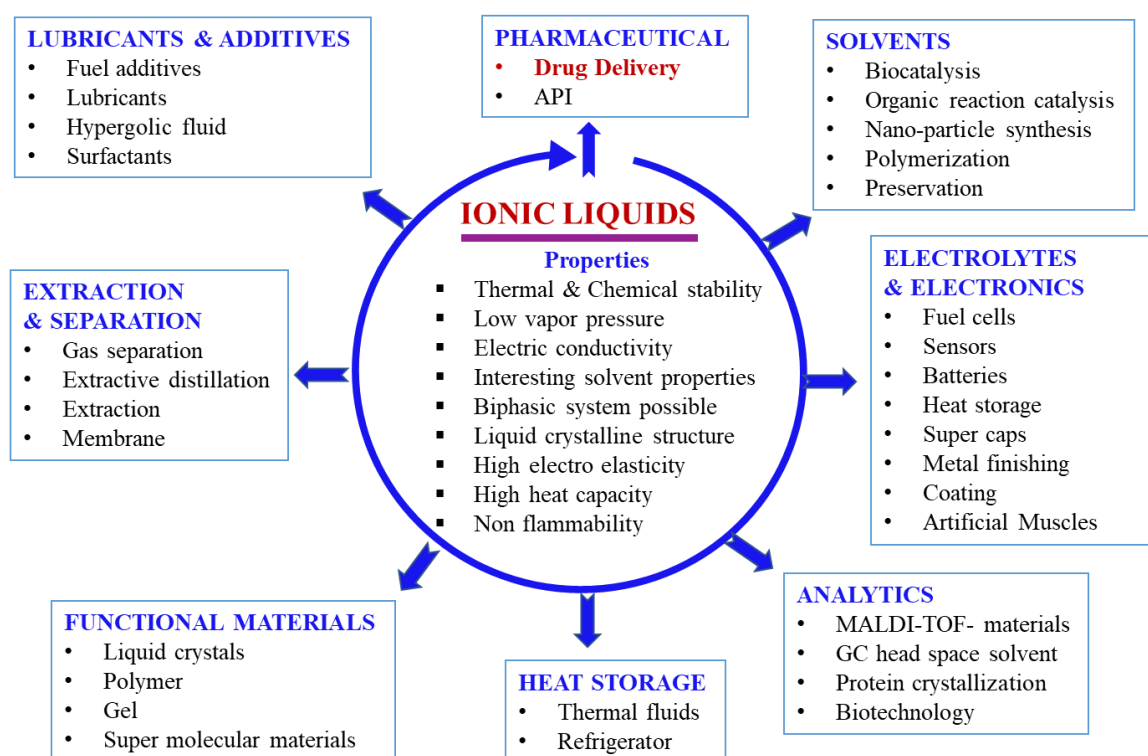


Figure. 2 Common properties and applications of ILs in different field.

The main target of this thesis is the development of lipid based novel biocompatible ionic liquids (LBILs) and to evaluate the effect of the synthesized ILs on drug delivery systems. So, the use of ILs in drug delivery systems is our main target.

1.1.7 Uses of ILs in drug delivery system (DDS)

ILs are reported as a multifunctional compound that has multiple applications in different areas of modern science. In nature, ILs have extraordinary physiochemical properties that are highly tunable. Due to the low solubility, low bioavailability, and low stability of a drug or API, the pharmaceutical industry faces numerous challenges in the development of new drugs or APIs and their delivery systems.¹¹

In recent years, ILs have played a significant role in drug developments as API-ILs and their delivery systems, including oral, transdermal, and topical drug delivery systems. After the development of Bio-ILs and Bio-API-ILs, the use of ILs has remarkably increased in drug development and delivery systems.

- i. **API-ILs:** The development of API-ILs was a significant accomplishment in the pharmaceutical industry. Anions or cations, even both ions, may derive from active pharmaceutical ingredients (APIs) or biological active compounds to develop API-ILs, which are referred to as Bio-API-ILs. The main issues for the next generation of drug delivery systems are biocompatibility and biodistribution, but Bio-API-ILs are the most advanced compounds to solve those problems.
- ii. **Solubility:** Several drug molecules are not soluble in water and common organic solvents; they demonstrate a low dissolution rate. Recently, ILs have been shown to effectively improve the solubility of poorly or sparingly soluble drug molecules in a pharmaceutically acceptable organic medium. The drug solvation capacity of ILs mainly depends on their structural cations and anions. Normally, IL structural anions have a larger effect on drug solubility than their cations.³⁷ Furthermore, the hydrophilicity and hydrophobicity of ILs is an intriguing aspect of drug solubility. It is clear that the solubility of drug molecules mainly depends on the H-bond formation between the drug molecules and IL anions.⁴⁹
- iii. **Stability:** Several drug molecules are not thermo-chemically stable for long periods of time. This is a major source of concern in the pharmaceutical industry. Recently, ILs have been actively used to increase several types of protein and peptide drugs' stability and to enhance their delivery systems.⁵⁰ ILs also help to develop thermostable API-ILs, thus enhancing drug stability and drug release..⁵¹

iv. **Drug wrapping techniques:** In the last few decades, ILs have mediated new, effective, and unique formulations used in drug delivery. These methods have significant pharmacokinetic and pharmacodynamic effects. ILs are used as surface active components in drug formulations where ILs wrap the drug molecules by forming nanoparticles. As illustrated below, there are several popular techniques used in IL-mediated drug delivery systems such as

- ❖ Micelles
- ❖ Reverse micelles
- ❖ Microemulsion
- ❖ Self-assembly
- ❖ Nano drug formulations:
 - S/O nanodispersion,
 - W/O nanodispersion.
- ❖ Liposome
- ❖ Invasome

1.1.8 Application of ILs in transdermal drug delivery system (TDDS)

The focus of this thesis was the development of lipid-based biocompatible ionic liquids and finding their potential application in TDDS. In the last three decades, different drug delivery target routes have emerged, among them TDDS, considered as a safe administrative route for drug delivery. TDDS has several advantages over oral, injection, and nasal delivery, including avoiding first-pass mechanisms (pH sensitivity, lysozyme degradation, and hepatic metabolism), being noninvasive, avoiding parental therapy, patient-friendly, and having fewer side effects, and increasing drug bioavailability.³⁷ There are also some disadvantages of TDDS, such as sometimes causing rashes, erythema,

suitable for hydrophilic and lipophilic nature, barrier of SC function from patient to patient. A remarkable research article noted that ILs are used as solvents for drug solubility to enhance TDDS. Several sparingly or poorly soluble drugs such as acyclovir, acetaminophen, caffeine, danazol, itraconazole, albendazole, coumarin, glibenclamide, isoniazid, ibuprofen, erythromycin, curcumin, methotrexate, dantrolene sodium, and paclitaxel can be delivered by an ILs mediated drug delivery system.⁴ ILs in oil microemulsion is a well-known technique for TDDS to overcome the skin's outermost barrier. Although the skin is a safer and an alternative route of drug delivery system, but it has some limitations that the skin prohibited the drug diffusion into the body is known as the main obstacle to the TDDS.

1.1.10 Main obstacle of TDDS

The first barrier to TDDS is the stratum corneum (SC) layer of the skin, the body's outermost layer, which is 10 to 20 m thick, hydrophobic in nature, and accounts for approximately 15% of total body weight. The SC is composed of lipids and proteins structurally organized as a brick and mortar model. The 'bricks' embedded the hydrated keratin and 'mortar' composed of multiple lipid bilayer of ceramides, cholesterol, cholesterol ester and fatty acids.⁵² Another major source of concern for the TDDS is SC structural lipid.¹⁷ The structural lipids are organized as multiple lamellar bilayers which actively prevent the loss of water from the body and likewise hinder the entry of any foreign particles into the body such as drug molecules, but it gives permission to enter into the lipid soluble or low molecular weight and small molecular size particles..⁵³ To overcome this barrier is the first challenge to administering medication via skin to get local effects or systemic therapeutic effects follow its entry into dermal capillaries

Ionic liquids (ILs) in cancer treatments

1.1.1 Cancer

Cancer is the world's most serious public health problem. Cancer is a disease characterized by uncontrollable or abnormal cell proliferation. The normal process in the body is that one cell is divided into two new daughter cells at a regulated frequency, but when this regulation is not under control, it is known as uncontrolled proliferation of cells or tissues and is the cause of cancer. Cancer is not a single disease; over 200 different types of cancer have been reported based on the location of the body.⁵⁴ Cancer that arises in the epithelium cell line in the internal organs or glands is called carcinoma cancer, such as the breast, bone, liver, lung, brain, peritoneum, colon, stomach, chest wall, skin, adrenal gland, ovary gland and prostate gland. Another class of cancer known as sarcoma cancer the tissues of the body such as bone, muscle, blood vessels, white blood cells (leukemias), and lymph glands (lymphomas). Melanoma is a type of cancer that develops in the skin. Cancer can also lead to various genetic (mutation), epigenetics (loss of heterozygosity) effects and cause exposure to various carcinogenicities, such as tobacco, viruses, bacterial infection, and ultra violet radiation.⁵⁵ It has been reported over the last decade that scientists and health care professionals are very concerned about controlling or preventing cancer. According to reports, the death rate from cancer diseases will rise by about 45% by 2030.⁵⁶

By the way, cancer is a complex collection of multiple diseases; each cancer has its own pattern of development. All cells have a set of behaviors that characterize them, such as,

Uncontrol growth: Normally a cell divided into two new daughter cells by meiosis or mitosis process. A normal cell has a controlled cell cycle, but cancerous cells lose

regulation of their own cell cycle division and can jump -start growth by themselves in multiple copies within a short time and thus develop a tumor. Tumors are the primary cause of cancer, and cells continue to proliferate within the tumor.

Immortality: Almost all cells have a programmed cell cycle to end the functioning, stop proliferation. Cells are committed to suicide when cellular machinery is damaged, infected by a virus, when the number of cells is significantly increased or when it is needed. Aging is a system in which cells stop dividing or programmed cell death occurs, also known as apoptosis. Cancer cells are ignored in order to prevent them from dividing, and as a result, they become immortal and proliferate indefinitely.

Ability to divide infinitely: Normal cells in the body can stop dividing while still alive, and their DNA signals remain programable as usual. DNA can send signals to other cells and can replicate or stop replication based on its own needs. A cancerous cell avoids this cell cycle by stopping signaling in a different way, resulting in the division of the cell into multiple unnecessary cells and the formation of many new vessels in the tumor. When precancer cells in a tumor cross the line and become a true cancer, they become a true cancer.

Recruiting a food supply: In the body, normal cells feed oxygen and nutrients from the blood circulation system and the body carefully regulates this system. Oxygen and nutrients are transported from the blood to the cells via vessels, and if additional oxygen is required, new vessels are formed, a process known as angiogenesis. Cancer cells avoid this system and independently make signals to the body to feed it, as the demand for new blood vessels is growing in the tumor. These numerous vessels aid in the rapid proliferation of tumor cancerous cells, and when specific cells cross the limited line, it is known as true cancer.

Random migration: Cells in the body are adhere in a particular tissues side and assist in its function. In contrast, some cancerous cells spread to new areas of the body from their own sources in the bloodstream or lymphatic system is known as metastasis. A metastatic tumor, or metastatic cancer, is one that cancerous cells are spread to the different or secondary sites of the host's body or tissues or organs from its primary origin site and assist in the tumor growth. Metastatic cancer is also known as advanced cancer or 4th stage cancer and it's more difficult to cure than other types of cancer, because the primary origin site is unknown. The primary cause of metastatic cancer is the random migration of cancerous cells or pathogenic agents.

1.1.2 Treatments for cancer

There are many types of cancer treatments reported, such as surgery, radiation (external and internal beam) therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell or bone transplant therapy, hyperthermia therapy, photodynamic therapy, cryotherapy, laser treatment therapy, and biomarker testing for cancer therapy.⁵⁷

The type of treatment mainly depends on the type of cancer and how advanced it is. Some patients will only receive one treatment, but the majority will receive a combination of treatments to cure their cancer.⁵⁴ The main target of cancer medications is to destroy, kill, shrink, or slow the cancer cells' growth. There are more than 100 different drugs currently used in cancer therapy. The cancer drugs can be given through intravenous, orally, injection or as cream and could be given daily, weekly, and continuously by an at-home pump.¹³ Broadly, cancer drugs can be classified into four categories, such as, (i) cytotoxic chemotherapy (alkylating agents, antimetabolites, anti-microtubule agents or mitotic inhibitors, and topoisomerase inhibitors I and II), (ii) hormonal therapies (anti-estrogen,

aromatase inhibitors or AIs, anti-androgens, luteinizing hormone releasing hormone (LHRH) agonist and antagonist, androgen biosynthesis inhibitors, androgens, corticosteroids, somatostatin analogues, progestins, and prolactin lowering agents), (iii) immune therapies (cytokines, vaccine therapy, immunomodulatory drugs or IMiDs, and differentiating agents), and (iv) target therapies (targeted therapeutic receptors, ligand or intracellular molecules).⁵⁷ Cancer treatments may cause a lot of complications that are known as the side effects of cancer, such as anemia, appetite loss, bleeding and bruising, constipation, delirium, diarrhea, edema or swelling, fatigue, fertility, flu-like symptoms, hair loss or alopecia, infection and neutropenia, lymphedema, memory and concentration problems, mouth and throat problems, nausea, vomiting, nerve problems, immunotherapy or organ related infections, pain, sexual health issues, skin and nail problems, insomnia, and urinary and bladder problems.^{54,57,58} Researchers are working hard to develop an alternative, effective drug or drug delivery system to improve cancer treatments in order to reduce the side effects of cancer treatments.

1.1.3 Major obstacles in cancer treatments

Anticancer drug development has been a progressive achievement in cancer treatments, but most anticancer drugs are water insoluble, delaying the drug's effectiveness.^{55,59} The lipophilicity, polarity and instability of anticancer drugs make it difficult to achieve the development of effective formulations.^{54,59} There are various solvents or co-solvents used to solubilize anticancer drugs and make effective formulations, such as methanol, ethanol, DMSO, acetonitrile, isopropanol, and water insoluble organic (oils, triglycerides, vitamins, or their associations). Different types of formulations, such as liposomes, nano and micro capsules, nano/micro emulsions, dendrimers, are largely used to increase the apparent water solubility of anticancer drugs.^{52,54,55,57,59} There are several challenges to

developing cancer treatments, including (i) understanding what clinical trials entail, (ii) challenges to improving early detection, (iii) improved access to resources, (iv) developing better models for cancer treatments, and (v) opportunities for advances in genomics and systems biology.⁶⁰

1.1.4 ILs in the cancer treatments

In recent years, ILs have become a potential green candidate to develop effective formulations for cancer treatments. ILs act as a biocompatible tool to enhance the drug delivery system by increasing the drug's efficacy and stability.¹³ Several studies have reported that ILs are used in biomedical applications such as antitumor agents, which are therapeutic agents with tunable properties that aid in the design of anticancer drugs or agents.⁶¹ According to reports, only 10% of anticancer agents are sold on the market, but toxicity remains a major issue. Despite their toxicity, the main cancer treatment approaches are immunotherapy, chemotherapy, and irradiation. One of the major goals of the pharmaceutical industry is the development of low or nontoxic new antitumor agents.⁶² The benefits of ILs-mediated anticancer agents include the ability to tune biocompatibility, while pharmacological and physiochemical properties are required for therapeutic effects.¹³ Ana Rita Dias et al. briefly described in a review article the use of ILs in anticancer treatments as solvents, co-solvents or API-ILs with increasing biocompatibility.¹³

1.3 Aims and output of this thesis

ILs are now used as an alternative source for various organic and inorganic solvents. Because of their tunable properties, ILs are a superior solvent for developing various pharmaceutical formulations to improve drug delivery systems. The use of ILs in

pharmaceutical and bio-medical fields has been increasing day by day. Moreover, toxicity is still a challenging issue, so scientists are very much concerned about developing novel biocompatible ILs (bio and eco-friendly family ILs) with a broad range of solubility, as the demand for 2nd generation ILs and 3rd generation ILs have developed. The most promising strategy to develop biocompatible ILs is that both structural cationic and anionic counter parts will be derived from biomaterial sources. Therefore, the aim of this study is the synthesis of lipid-based novel biocompatible ionic liquids that are nontoxic and biodegradable for the development of novel drug delivery systems for peptides and proteins as anticancer drugs. In particular, the characterizations of new lipid-based ILs, biocompatibility of ILs and their capability to develop nanoparticles, stability of nanodrug formulations, *in-vitro* & *in-vivo* transdermal drug penetration, pharmacokinetics, and anticancer effects were investigated in this research. Cellular *in-vitro* and *in-vivo* studies have been carried out to evaluate the potential applications of new lipid-based biocompatible ionic liquids as an effective drug delivery system of protein and peptide drugs for cancer treatments.

In **chapter 2**, we synthesized a series of lipid-based biocompatible ILs (LBILs), which contained a long chain phosphatidyl choline derivative as a cation (1,2-dimyristoyl-sn-glycero-3-ethyl-phosphatidylcholine; EDMPC) and a series of long chain fatty acids (linoleic acids; C_{18:2}, oleic acids; C_{18:1}, and stearic acids; C_{18:0}) as its structural counter anion. Physiochemical properties of synthesized ILs were characterized by thin layer chromatography (TLC), proton nuclear magnetic resonance (¹H NMR), Fourier transform infrared (FTIR), elementary analysis (EA), Karl-fisher moisture analyzer, TOA pH meter, and mass spectrometry (MS). Thermal properties were confirmed by dynamic scanning

calorimetry (DSC) and temperature-dependent optical polar microscopy (OPM). Solubility and particle size in different polar and nonpolar solvents was investigated by UV-visible spectrophotometric analysis and dynamic light scattering (DLS), respectively. Finally, the biocompatibility of LBILs was confirmed in the human artificial LabCyte EPI-Model cell line by MTT assay methodology.

In **chapter 3**, we developed the LBILs mediated nanodispersion (ILs in oil nanodispersion; IL/O-NDs) formulations containing leuprolide acetate peptide as a model peptide drug and Span-20 at a specific ratio as a cosurfactant to increase the stability of drug-ILs formulations. The physiochemical properties of IL/O-NDs were characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM) confocal laser scanning microscopy (CLSM), and the high-pressure liquid chromatography (HPLC) system. We observed the *in-vitro* transdermal drug delivery using Franz-cell and quantified by HPLA. Moreover, we investigated the pharmacokinetic effect on BALB/C mice blood *in-vivo* using ELISA enzyme linked immunosorbent assay (ELISA) methodology. We also observed the mechanisms of transdermal drug delivery into the SC layer by FTIR analysis. Finally, *in-vivo* and *in-vitro* biocompatibility of IL/O-NDs were observed on LabCyte EPI-Model and BALB/C mice skin histopathology, respectively. LBILs act as a promising biocompatible carrier to increase the stability of nano-drug formulation and enhance the transdermal delivery of a peptide drug, leuprolide acetate.

In **chapter 4**, we developed a LBIL; [EDMPC][Lin]mediated protein antigenic drug in oil phase nano particles (LPNPs) formulations. LPNPs contain a bulky antigenic protein, ova albumin from chicken egg white (OVA) with a molecular weight of 42.7 kDa as a

model anticancer drug and Tween-80 is used as a co-surfactant to increase the stability of LPNPs. The formulations were characterized by DLS and CLSM, and *in-vivo* and *in-vitro* biocompatibility was confirmed by the human artificial 3D reconstructed Lab-Cyte Epi-Model cell and the mice's skin H & S staining histopathological observation. In vitro skin permeation of protein via the LPNPs system was investigated by the Franz cell system. CLSM and FTIR analysis were performed to investigate the mechanism of intercellular routes of drug permeation via the SC layer of the skin. The effect of LPNPs against tumor growth and development was confirmed on C57/BL6N by inoculation of tumor OVA responsible cancerous cells, E.G7-OVA for the tumor development. The anti-cancerous antigen uptake of skin dendritic cells (DCs) and other antigen presenting cells (APCs) by flow cytometric and CLSM analysis also revealed the cancer immune response of LPNPs. Finally, we observed CD8⁺ T-cell inflammation in the tumor microenvironment. We found that LPNPs have an excellent anti-cancerous immune therapeutic effect compared with conventional chemical permeation enhancers and aqueous injection formulations. The effective application of LBIL-mediated LPNPs has indicated that LBIL will be a potential candidate for the platform of a nano-drug delivery system for cancer treatment.

In **chapter 5**, We summarized the findings of this research and briefly discussed further research directions using LBILs related to this research.

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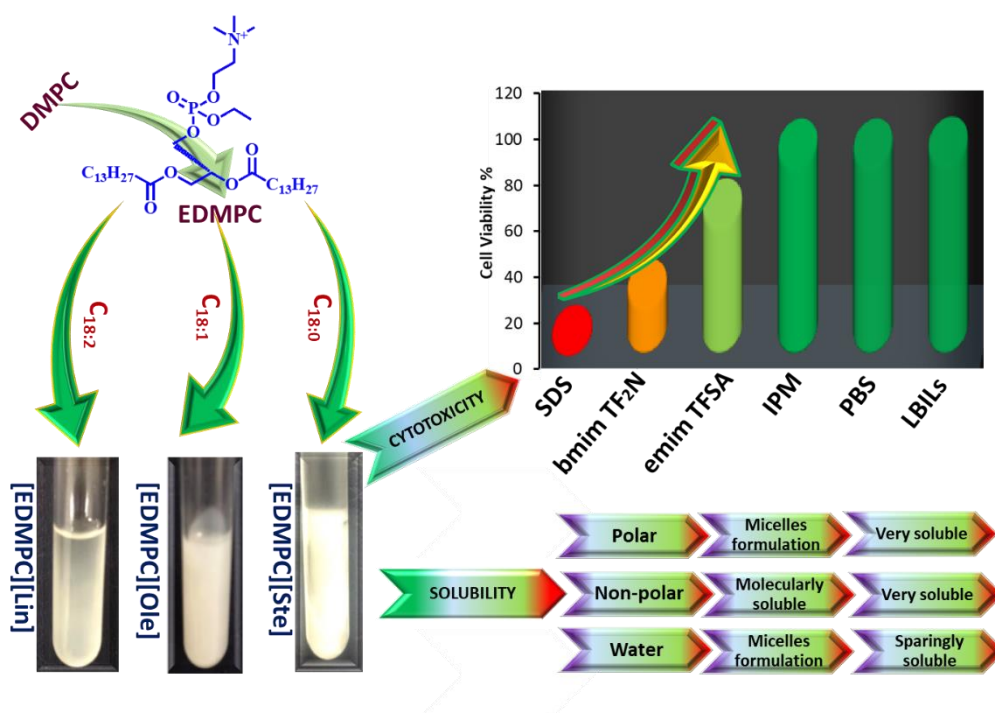
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CHAPTER 2

LIPID BASED BIOCOMPATIBLE IONIC LIQUIDS: SYNTHESIS, CHARACTERIZATION AND BIOCOMPATIBILITY EVALUATION.

Graphical Abstract



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2.1 Abstract

We report a new series of lipid-based biocompatible ionic liquids (LBILs) consisting of the long-chain phosphonium compound 1,2-dimyristoyl-sn-glycero-3-ethylphosphatidylcholine as the cation and the long-chain fatty acids stearic acid, oleic acid, or linoleic acid as anions. These materials were found to be completely miscible with many polar and nonpolar organic solvents as well as dispersible in water. These LBILs also exhibited excellent biocompatibility with an artificial three-dimensional human epidermis model.

2.2 Introduction

Over the last two decades, ionic liquids (ILs) have been increasingly employed as solvents, co-solvents or reagents in numerous applications, including in drug delivery systems (DDSs).^{1,2} In these applications, ILs have been used as active pharmaceutical ingredients (APIs),³ or as solvents,⁴ co-solvents⁵, or additives⁶ for pharmaceutical formulations including low molecular weight drugs⁷, proteins or enzymes, peptides⁶ and other biological substances.⁸ Recently, the use of biocompatible ILs (bio-ILs) in DDSs has received significant attention as a means of avoiding the adverse effects of the organic solvents commonly used as drug delivery carriers.⁹ Bio-ILs can be easily synthesized from cationic and anionic counterparts sourced from biocompatible substances, such as amino acids,¹⁰ carbohydrates¹¹ and carboxylic acids.¹² To date, several bio-ILs for use in DDSs have been obtained in this manner, with the aim of delivering therapeutic molecules in targeted treatment processes.^{7,9,13}

Transdermal drug delivery systems (TDDSs) are one of the most widely studied applications for ILs. In TDDSs, these compounds are used as drug solubilizing/stabilizing

agents as well as skin penetration enhancers to achieve effective therapeutic action.¹⁴ As an example, a recent study reported the efficient delivery of small interfering RNA (siRNA) via the skin using hydrophobic cations to cover the siRNA and a choline geranate (CAGE) IL to significantly enhance dermal penetration.⁸ In another study, several cyclic onium based ILs, such as N-dodecyldabco bromide ([C₁₂dabco]Br), N,N-dihexyldabco bromide ([C₆C₆dabco]Br), N-butyldabco bromide ([C₄dabco]Br), N-methyl-N-decylmorpholinium bromide ([C₁C₁₀mor] Br) and N-methyl-N-(4-butylsulfonic acid) pyrrolidinium hydrogen sulphate ([C₁C₄SO₃Hpyrr][HSO₄]), were used to evaluate the effects of ILs on the transdermal delivery of diltiazem. The data indicated that dermal permeation of this pharmaceutical was improved when an IL was included in the formulation, although the potential toxicity of these ILs was a concern.¹⁵ The dermal delivery of hydrophilic macromolecules was also improved following the addition of a choline malate IL, with no apparent toxicity to human epidermal cells (HEK-A).¹⁶ The greatly increased efficiency of TDDSs obtained from the use of ILs has been attributed to the replacement of natural skin lipids with the IL, which facilitates the drug delivery process.¹⁴ It has also been suggested that ILs may have non-localized enhancement effects that are not associated with geometrically-confined transport pathways.¹⁴ Specifically, hydrophobic ILs may modify lipid bilayers in the skin in IL-mediated TDDSs. For this reason, the incorporation of lipid moieties in an IL, as cations or anions or both, could be an innovative approach to developing novel lipid-lipid ILs that might provide benefits in TDDSs. Of course, the development of such ILs will require special attention to the biocompatibility of these moieties.

Herein we report the first-ever development of three novel lipid-based bio-ILs (LBILs), each comprising a long chain phosphonium cation (the phosphatidylcholine derivative

1,2-dimyristoyl-sn-glycero-3-ethyl-phosphatidylcholine; EDMPC) and a long chain fatty acid (FA). The FAs examined in this work as anions were stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$), EDMPC has been reported to exhibit excellent biocompatibility because it is similar to naturally occurring lipids in the skin,¹⁷ and has numerous biological applications.¹⁸ The long alkyl chain of the phosphonium cation also allows these LBILs to have both hydrophobic and hydrophilic parts.¹⁹ The LBILs were characterized by proton nuclear magnetic resonance (1H NMR) and Fourier transform infrared (FTIR) spectroscopy as well as by matrix assisted laser desorption time of flight mass spectrometry (MS) and thin layer chromatography (TLC). The solubility and miscibility of these substances were investigated by dynamic light scattering (DLS) and UV-visible spectrophotometric analysis. Their thermal properties, including melting, crystallization, and phase transition temperatures, were also determined using differential scanning calorimetry (DSC). Finally, the biocompatibility of the LBILs were assessed based on a well-established skin irritation test employing an artificial three-dimensional human epidermis model (Lab-CyteTM EPI-MODEL). The extraordinary physicochemical properties and excellent biocompatibility of these LBILs may permit their use in DDSs and suggest a new approach to the in IL-mediated transdermal delivery of therapeutic molecules.

2.3 Materials and Methods

2.3.1: 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-CyteTM EPI-MODEL human artificial cell line was purchased from Japan Tissues Engineering Co. Ltd (Aichi, Japan).

2.3.2 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.²⁰ The reaction was carried out at 45 °C for 12 hr with a continuous flow of nitrogen gas (N₂). The product 1,2-dimyristoyl-sn-glycero- 3-ethyl- phosphatidylcholine (EDMPC) trifluoro salt was verified with thin-layer chromatography (TLC) using CHCl₃: MeOH (9:1) v/v mixture as an eluting solution. The *p*-anisaldehyde identical coloring solution consisting 13 mL of P-anisaldehyde, 5 mL of acetic acid, 18 mL of 10M concentrated sulfuric acid and 478 mL 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 ¹H-NMR (JEOL ECZ400S 400MHZ

NMR, Tokyo, Japan) using chloroform-d as solvent. Synthesized EDMPC trifluoro salt was dissolved in chloroform followed by addition of 0.2 N 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 (**Figure 1**). Finally, the purity of EDMPC-Cl was investigated with TLC, ^1H NMR, Fourier transform infrared (FTIR), Mass spectrometric (MS) and chemical elementary analysis (EA) etc.

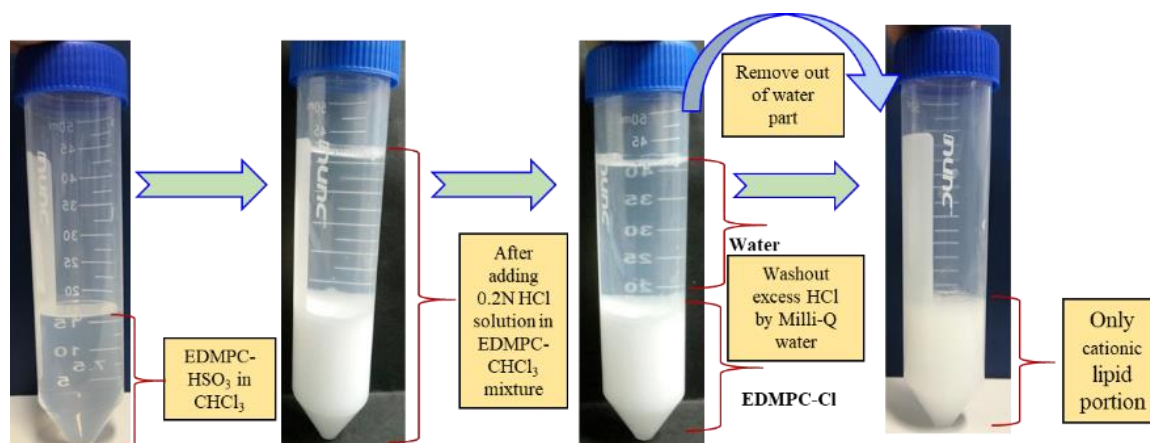


Figure 1: EDMPC-Cl synthesis and purification process by phase separation process.

2.3.3 Lipid based ionic liquids (LBILs) preparation

EDMPC-Cl and fatty acid were reacted at equimolar ratio in chloroform for overnight (~12hr) at 45°C, with a continuous flow of N₂ in a light protective environment (**Figure 2**). The reaction solution was freeze-dried to obtain the synthesized lipid based ionic liquid (LBIL) that was further characterized by TLC, ^1H NMR, FTIR, DSC, UV, and EA etc.

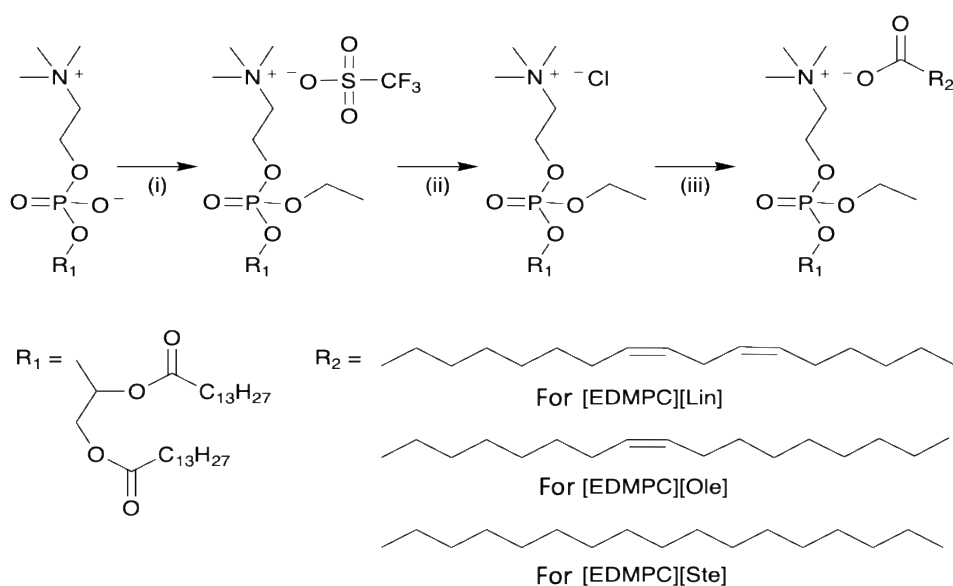


Figure 2: Schematically presented synthetic pathway of LBILs. (i) ethyl trifluoro methane sulfonate, CHCl_3 , 45°C , 12 h. (ii) 0.2N HCl; (iii) fatty acid/ CHCl_3 , 45°C , 12 h.

2.3.4 Characterization 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 ^1H NMR 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 400-4000 cm^{-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 1 μL 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 1 μL of sample (2 mg/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 30 mL/min. Three cycles for each sample were measured and the empty aluminum pan was sealed with the same procedure used as the reference.

2.3.5 Solubility study of synthesized 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 5-10 min

and observed visually. For PBS and Milli-Q water, LBILs were used at maximum of 20 mg/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 scattering angel was maintained at 173° with 1 cm length capped quartz cell in 25 ± 0.1 °C. Each sample was measured at 10 times and the average value was considered as the experimental output.

2.3.6 pH observation of LBILs

The LBILs were dissolved in Milli-Q water at a concentration of 20 mg/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.

2.3.7 Biocompatibility evaluation of LBILs

The biocompatibility of LBILs were investigated using a skin irritation test on human artificial epidermis model (LabCyte, EPI-MODEL). The procedures were performed as recommended by supplier in accordance with published literature with some modification.²¹ 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 500 μ L of assay medium and incubated for overnight (24 hr.) at 37 °C, 5% CO₂ humidified atmosphere. Then, 25 μ L of each sample (LBILs in IPM) was added to the cell that covered the whole surface on

the tissue and incubated for 24 hr. After that, the cells were washed carefully with D-PBS to remove any remaining sample. A 500 μ l of freshly prepared MTT assay medium was added in a 24-well plate where previously washed cells were transferred and incubated for 3 hr. The treated tissues were taken out and transferred into a 300 μ l of 2-propanol containing tube and allowed to stand for 48hr at room temperature in dark condition to extract the pigment. A 100 μ l of extracted solution was placed to a 96-well plate to measure the optical density with a microplate reader at 650 nm and 570 nm. The amount of cell viability (in %) 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][TF₂N], and a well-known pharmaceutically available surfactant, SDS.

2.4 Results and Discussion

2.4.1 Synthesis of LBILs: The LBILs were synthesized following the reaction scheme illustrated in **Figure 2**. Briefly, 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) was reacted with an equimolar amount of ethyl trifluoromethanesulfonate in CHCl₃ under a continuous flow of N₂ to obtain EDMPC-SO₃CF₃.²⁰ EDMPC-Cl for use in the LBILs was prepared using a phase separation process in 0.2 N HCl. The EDMPC-SO₃CF₃ was first purified by washing with deionized Milli-Q water.

2.4.2 Characterization of LBILs: The O-alkylation of the DMPC and synthesis of the EDMPC salt were confirmed by TLC, ¹H-NMR, FTIR and MS analyses. The TLC plate showed a single spot indicating the purity of the EDMPC, with a R_f (refractive index)

value approximately 0.70 when 10% methanol in chloroform was used as a mobile phase, demonstrating significant movement of the EDMPC by o-alkylation (**Figure 3**).

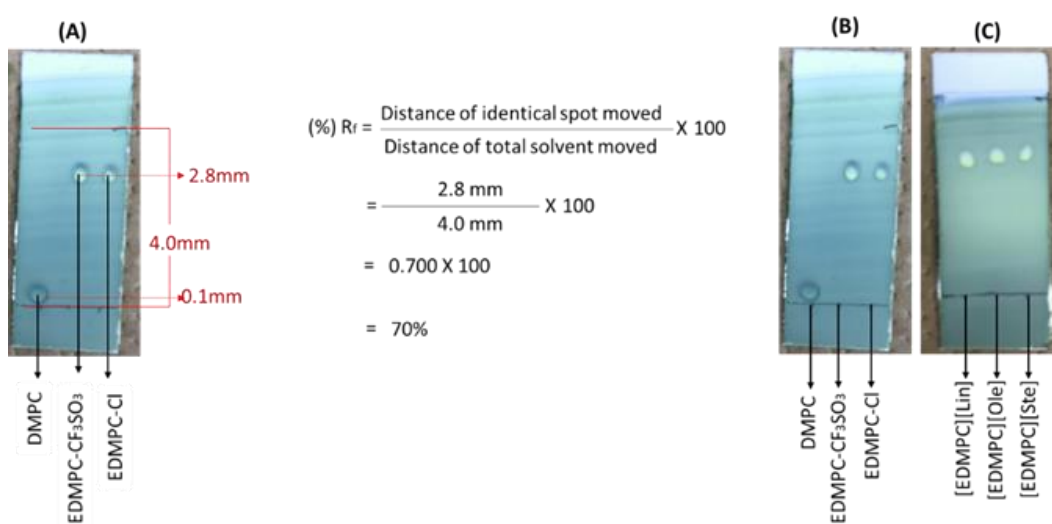


Figure 3: Thin layer chromatographic image of DMPC, EDMPCSO₃CF₃, EDMPC-Cl and three LBILs. The retention factor (R_f) value was calculated.

The transition of the O-alkyl derivative to EDMPC was established by examining the ¹H-NMR spectrum of the product (**Figure 4**). This spectrum showed ethyl protons at 1.24–1.39 ppm (m, 43 H). The spectrum of the EDMPC-Cl contained a more intense peak in the range of 1.59 to 1.61 ppm (s, 4 H) compared to the EDMPC-SO₃CF₃, representing the presence of water as a contaminant (**Figure 4**). According to the literature, water in CDCl₃ generates a peak at 1.5–1.6 ppm.²²

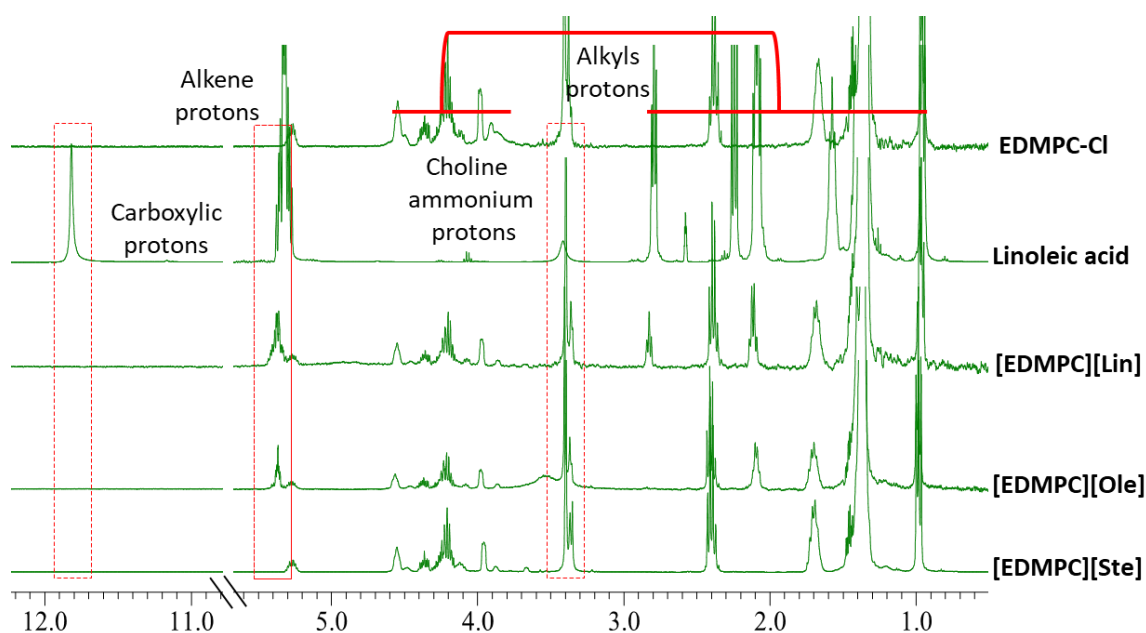


Figure 4: Stacked ^1H -NMR spectra of EDMPC-Cl, linoleic acid and three LBILs (400 MHz, Chloroform- d).

Here; ^1H -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).

^1H -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.

^1H -NMR of [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).

^1H -NMR of [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).

And

^1H -NMR of [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).

The purity and molecular weight of the EDMPC-Cl were assessed using MS and only a strong signal at an m/z of 706.252 was observed, in very good agreement with the calculated value of 706.856). There weren't visualized any unwanted peak as an impurity (Figure 5).

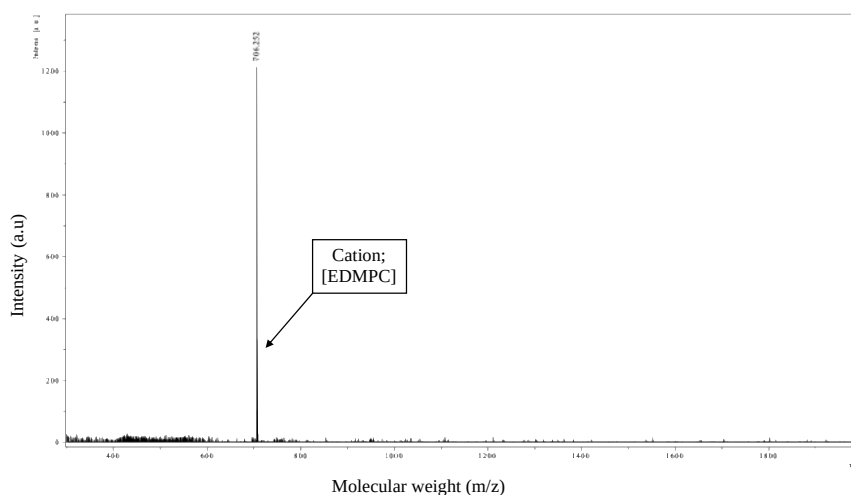


Figure 5: MALDI-TOF MS measurement spectrum of cationic lipid EDMPC showed an identical peak at 706.252 m/z .

Equimolar amounts of either linoleic, oleic, or stearic acid were combined with the EDMPC-Cl using the same reaction conditions originally employed to generate the cationic lipid. These mixtures yielded the [EDMPC][Lin], [EDMPC][Ole] and [EDMPC][Ste] LBILs, respectively (Figure 2). The HCl generated as a by-product readily evaporated from the reaction medium due to its volatility²³ and the absence of Cl^- ions in each product was confirmed by silver nitrate test.²⁴ Unbound solvents were removed from the LBILs by evaporation and freeze drying. The physiochemical properties and solubilities of the LBILs were investigated using NMR, FTIR, DSC, elemental analysis (EA), DLS and UV-visible spectroscopy. The [EDMPC][Lin],

[EDMPC][Ole] and [EDMPC][Ste] were found to be a viscous liquid, a highly viscous liquid and a semi-solid at ambient temperature, respectively (**Figure 6**).

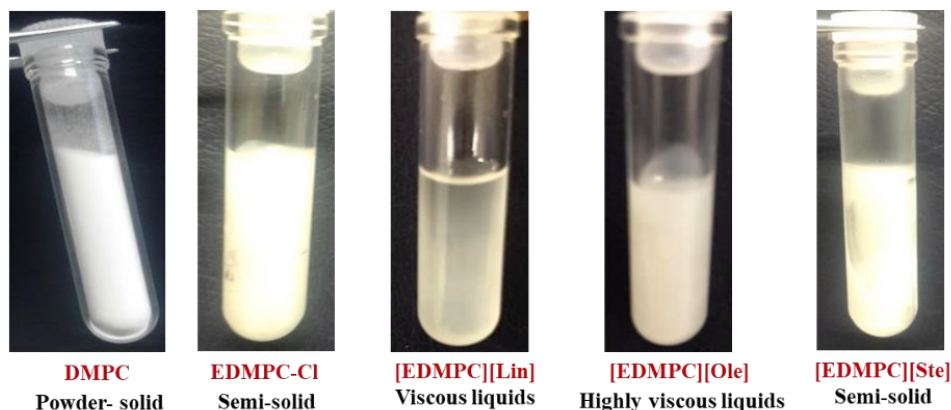


Figure 6: Physical appearances of DMPC, EDMPC-Cl and three LBILs at room temperature (25°C) were represented in this figure.

The interactions between the cation and the various counter anions when combined in a 1:1 molar ratio to form the LBILs structures were assessed by acquiring ^1H -NMR spectra in CDCl_3 . The fatty acid $-\text{CH}_3$ groups in the LBILs generated peaks at δ 0.86–0.88 (dd, $J = 6.9$ Hz, 9 H) while the $-\text{CH}_2-$ protons produced peaks in the range of 1.14–1.63 ppm (m, 71 H). None of the specimens produced peaks related to free carboxylic acids in the range of 11.68–11.97 ppm (s, 1 H), confirming the interaction between the carboxylate anions and the $-\text{N}^+$ of the cations.²⁵ Peaks related to unsaturated protons ($-\text{CH}=\text{CH}-$) were observed in the ranges of 5.28–5.37 ppm (m, 4 H) for the [EDMPC][Lin] and 5.31–5.34 ppm (m, 2 H) for the [EDMPC][Ole], while these peaks were not generated by the [EDMPC][Ste]. Each LBILs produced a signal for $-\text{NCH}$ protons in the range of 3.3–3.9 ppm. Each sample also showed a peak in the range of 1.5–1.6 ppm related to the presence of water as an impurity.²² All others peaks in the spectra represented long chain alkyl

groups (that is, $-\text{CH}_2-$), as depicted in **Figure 4**. The FTIR spectra also provided useful information about the structures of the LBILs. Carboxylic groups ($-\text{COOH}$) typically appear in the range of $2500\text{--}3300\text{ cm}^{-1}$, often centered at 3000 cm^{-1} .²⁶ These LBILs did not produce such peaks, suggesting strong interactions between the carboxylic groups and the cationic ammonium groups of the EDMPC-Cl (**Figure 7**).

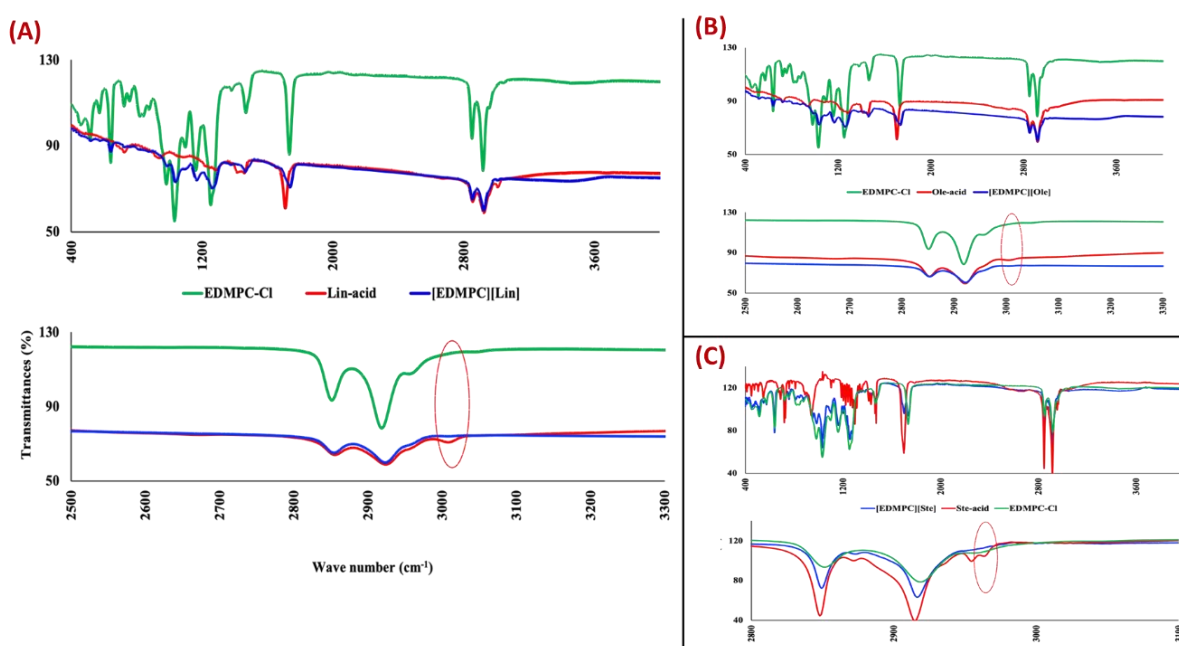


Figure 7: The FTIR spectra of LBILs were represented in the figure (A) [EDMPC][Lin], (B) [EDMPC][Ole], and (C) [EDMPC][Ste].

The thermal behaviors of these LILs, such as their phase transition temperatures, are very important in defining their physiochemical properties and can be affected by hydrocarbon chain length, charge, head groups and unsaturation.²⁷ Long hydrocarbon chains increase the Van der Waals interactions and hydrogen bonding, such that molecules are closely packed and more energy is required to disrupt the molecules. In addition, the presence of double bonds tends to result in the random orientation of molecules and increased fluidity, so that much lower temperatures are required to disrupt the molecules.²⁸ The structures

of these LBILs were simulated using the Cosmo RS 2019-3 and ADF:2019-301 software packages, using models in which the [EDMPC][Lin], [EDMPC][Ole] and [EDMPC][Ste] contained two, one and no double bonds (**Figure 8**).

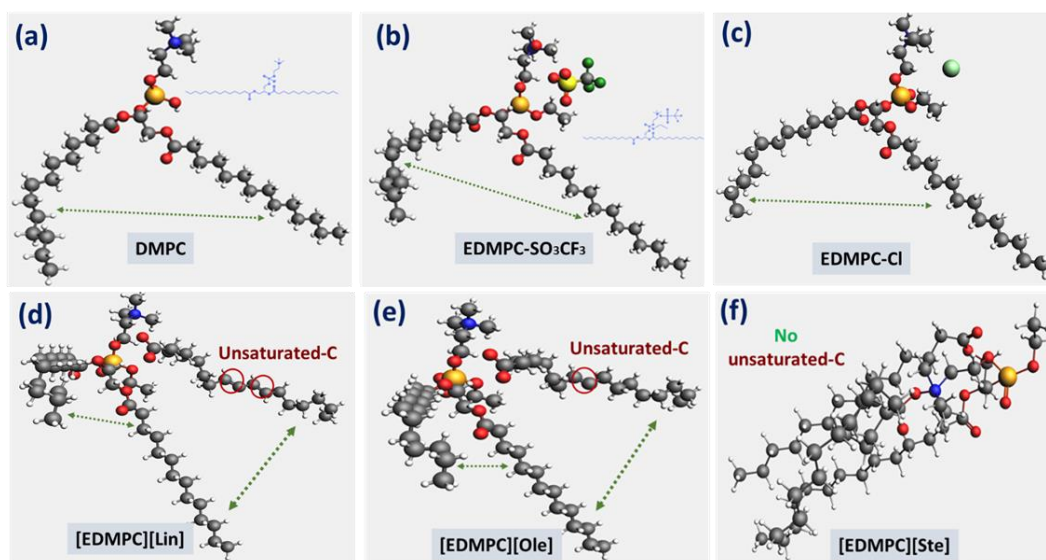


Figure 8: Chemical structure of LBILs- predicted by ADF Cosmo-RS ADF:2019-301. Physical appearance, combining statistical thermodynamics approach, charge distribution, surface, and chemical potential distribution, intermolecular interaction and the molecular structure of the molecules can be predicted by the COSMO-RS analysis.⁴

The smallest extent of hydrogen bonding was found in the case of the [EDMPC][Lin] while the strongest inter/intramolecular interactions and hydrogen bonding appeared in the [EDMPC][Ste]. As a result, more energy was required to disrupt the [EDMPC][Ste] and its phase transition temperature was higher (**Table 1**). The physical appearances of the LBILs at room temperature (**Figure 6**), the phase transition temperatures obtained by DSC (Table 1) and the temperature depended optical polarized microscopic observations (**Figure 9**) were all in good agreement. The thermal transitions of the LBILs during

heating and cooling were evidently associated with intermolecular interactions, double bonds (C=C), functional groups and the hydrocarbon chain lengths.

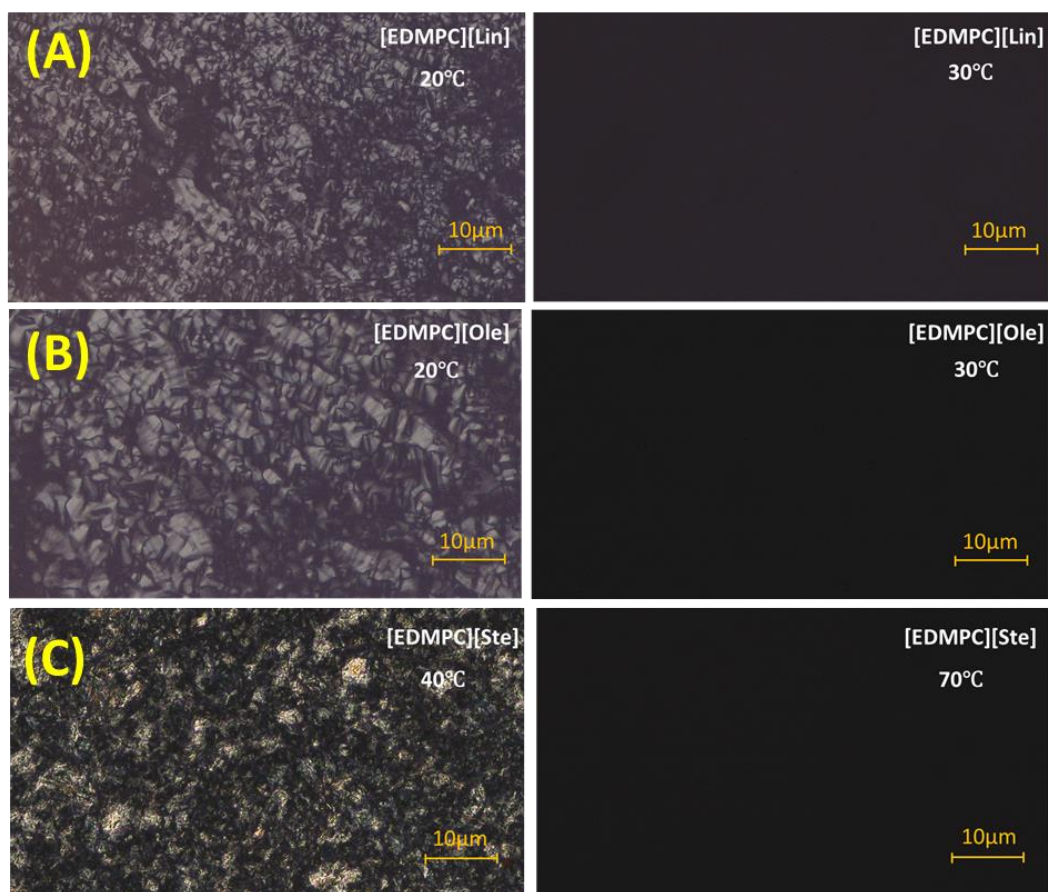


Figure 9: Temperature-dependent optical polarized microscopic images of LBILs. (A) [EDMPC][Lin], (B) [EDMPC][Ole], and (C) [EDMPC][Ste].

Table 1: The phase transition temperatures of the LBILs as determined by DSC.

Name of LBILs	Chemical formulation	Melting point ^a	Crystal point ^b	Phase transitions
[EDMPC][Lin]	C ₅₆ H ₁₀₈ NO ₁₀ P; C _{18:2}	20.8°C	15.4°C	Cr, CrM & Lq
[EDMPC][Ole]	C ₅₆ H ₁₁₀ NO ₁₀ P; C _{18:1}	24.3°C	19.2°C	Cr, CrM & Lq
[EDMPC][Ste]	C ₅₆ H ₁₁₂ NO ₁₀ P; C _{18:0}	54.9°C	7.3°C	Cr, CrM & Lq

Here, ^a Transition temperature from semi-crystalline to isotropic liquid; ^b Transition temperature from crystalline to semi-crystalline.; Cr = crystalline phase, CrM = semicrystalline phase and Lq = liquids phase.

During DSC analysis, there were three phase transition states; crystalline (Cr), semi-crystalline (CrM) or solid-liquid and liquid phase (Lq) were observed.¹² The crystallization temperatures (T_{cr}) of the three ILs were found to be in the range of 7–20 °C, and so were significantly lower than the melting points (T_m), which were in the range of 21–55 °C. These results reflect the abrupt transformation of the LBILs from the Cr to the CrM or Lq states (**Figure 10**). The DSC data also demonstrated a significant correlation between the T_m and the degree of unsaturation of the carbon backbone of the anion, even though all anions contained the same number of carbons. The purities of the LBILs were assessed using elementary analysis (EA) and the resulting C, H and N proportions (**Table 2**) were in very good agreement with the theoretical values.

Table 2: 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	

The moisture contents of the LBILs were also determined by Karl-fisher titration. pH regulates many physiological properties of biological systems and can also affect the performance of a DDS,²⁹ and so the pH values of 20 mg/mL solutions of the LBILs in water were determined. These values were [EDMPC][Lin]; 5.8, [EDMPC][Lin]; 6.1, and [EDMPC][Lin]; 6.0 respectively, indicated that these substances could be suitable for use in topical or TDDSs.

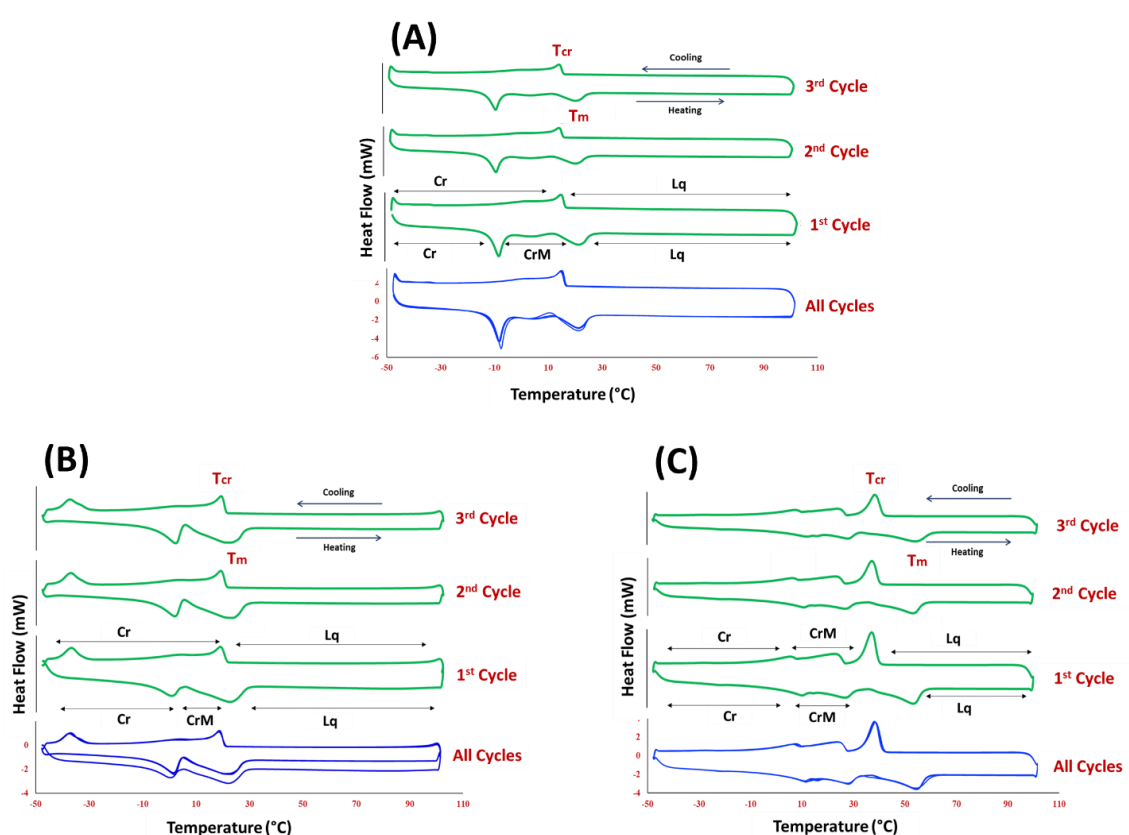


Figure 10: The thermal phase behavior of LBILs was investigated using DSC. (A) represented the [EDMPC][Lin] IL, (B) represented the [EDMPC][Ole] IL, and (C) represented the [EDMPC][Ste] IL.

2.4.3 Solubility evaluation of LBILs: The solubility of an IL is one of its most important properties, because these compounds might be used to improve the dissolution and

permeation characteristics of drugs.^{30,31} In addition, the solubilizing properties of ILs are directly related to the pharmacokinetics, toxicity, efficacy and pharmacodynamics of a DDS. In the present work, the LBILs were dissolved at equal ratio (50:50) in several polar solvents (IPM, IPA, methanol, ethanol and dimethyl sulfoxide-DMSO) and nonpolar solvents (hexane, heptane, octane, dodecane and toluene). Visual observations indicated that clear, transparent solutions were obtained, whereas dispersions of the compounds in water or PBS at 20 mg/mL gave cloudy suspensions (**Figure 11**).

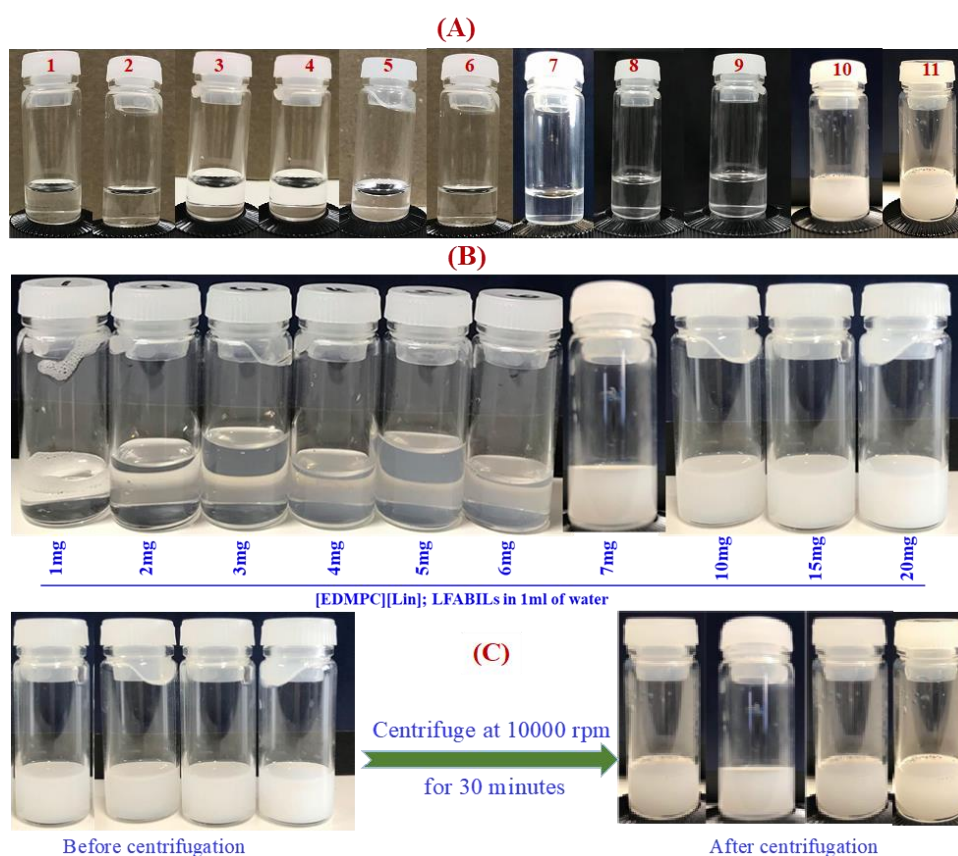


Figure 11: (A) Visual observation of the solubility of [EDMPC][Lin] in various solvents, (1) cyclohexane, (2) hexane, (3) heptane, (4) DMSO, (5) ethanol, (6) methanol, (7) IPA, (8) IPM, (9) toluene, (10) PBS and (11) water. (B) [EDMPC][Lin] IL was dispersed in water at different concentrations (w/v).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

According to the United States Pharmacopeia and British Pharmacopeia standards, these LBILs were judged to be very/freely soluble in both polar and nonpolar solvents but sparingly soluble in water (**Table 3**).

Table 3: 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	-	++	++	++

The dissolution of the [EDMPC][Lin] in a polar solvent (IPM), a nonpolar solvent (hexane) and in water were also investigated by DLS and UV-visible spectrophotometric analysis. The DLS results suggested that LBILs were dissolved in IPM and water by forming micelles, while the UV data indicated dissolution on a molecular level in hexane. The absorbance values at 237nm, exhibited very good correlation with concentration, giving a correlation coefficient of $R^2 = 0.9995$ for [EDMPC][Lin], $R^2 = 0.9986$ for

[EDMPC][Ole], and $R^2 = 0.9995$ for [EDMPC][Ste], indicated the solubility of LBILs in cyclo-hexane (**Figure 12**).

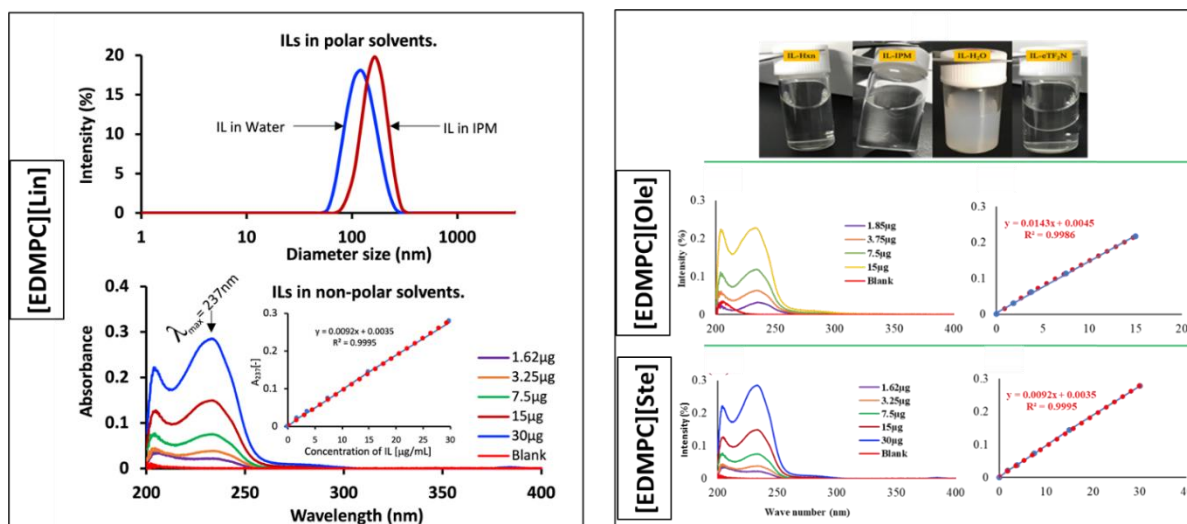


Figure 12: The particle size distributions of [EDMPC][Lin] after dispersed in water and IPM by DLS analysis and the UV-visible spectrum of [EDMPC][Lin] after dissolved in hexane with correlation of absorbance vs concentration (Correlation coefficient, $R^2 = 0.9995$). Solubility of LBILs in nonpolar solvents. In where [EDMPC][Lin] dissolved in hexane, IPM, water, and bmim-Tf₂N. [EDMPC][Ole] IL solubility in hexane was investigated using UV (High peak at 240 nm) where correlation coefficient R^2 is 0.9986. and [EDMPC][Ste] IL solubility in hexane was investigated using UV (High peak at 237 nm), where correlation coefficient R^2 is 0.9995.

This result confirmed the molecular-level solubility of the LBILs in hexane. The solubility of these LBILs in water was examined by preparing dispersions at concentrations up to 20 mg/mL. Visual observations indicated that, up to 5 mg/mL, the solutions were transparent, whereas concentrations of 7–20 mg/mL generated cloudy suspensions and the degree of cloudiness increased with increases in the ILs concentration. DLS results showed that the micelle size did not change over the range of 1–5 mg/mL but the derived count rates were increased significantly along with the LBILs concentration (**Table 4**).

Table 4: DLS observation of [EDMPC][Lin] IL in water at different concentrations. The particle size was increased with increasing the LBIL concentration in oil phases.

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

With the DLS observation, it was observed that the concentration range of LBIL; [EDMPC][Lin] from 1–20 mg/mL, both the micelle size and derived count rate was remain unchanged from 1-5 mg/mL (**Figure 13 B**) but when the LBILs micelles size was increased with increasing the concentration of ionic liquids in oil phases (**Figure 13 B**). The results indicated that, the LBILs were made a nano-particle in oil phases and solubilized with forming micelles.

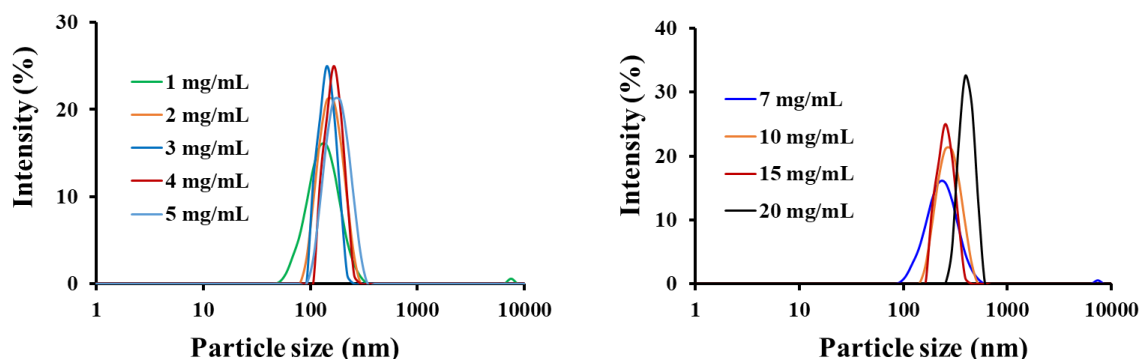


Figure 13: [EDMPC][Lin] solubility in water was investigated by using DLS analysis.

Cloudy solutions were centrifuged at 10,000 rpm for 30 minutes, but no precipitation was observed and there were no significant variations between the solution spectra before and after centrifugation. The refractive index (RI) values of the LBILs were determined and found that the RI value of [EDMPC][Lin] was 1.4506, [EDMPC][Ole] was 1.4603, and [EDMPC][Ste] was 1.4802, respectively. The formation of micelles by the LBILs in water was also confirmed using confocal laser scanning microscopy (CLSM). These analyses established the presence of micelles with sizes in the range of 150–200 nm, in agreement with the DLS results (**Figure 14**).

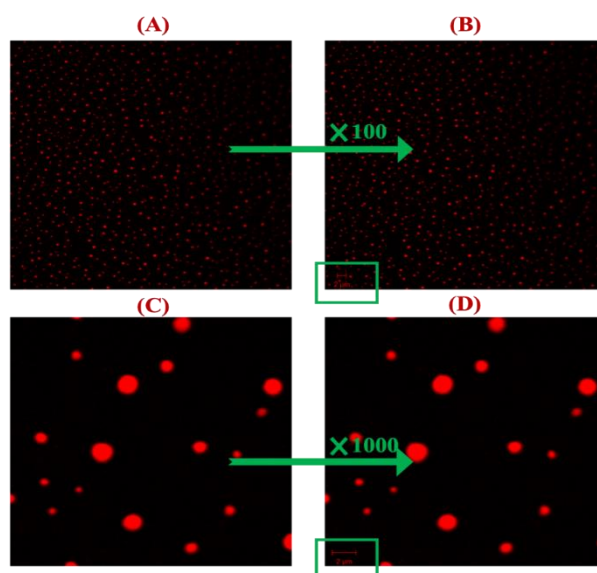


Figure 14: Confocal Laser Scanning Microscopy (CLSM) image of LBILs solubility in water.

2.4.4 Biocompatibility evaluation of LBILs: Biocompatibility refers to the ability of a substance to avoid any undesirable cellular response when in contact with tissue. According to the ISO 10933 standard, biocompatibility can be evaluated by assessing irritation or intracutaneous activity, cytotoxicity, sensitization, degradation, hemocompatibility, genotoxicity, acute or chronic or sub-chronic toxicity, carcinogenicity, implantation, pyrogenicity and developmental or reproductive

toxicity.^{32,33} In the present work, the biocompatibility characteristics of the LBILs were examined by performing a skin irritation test using artificial human epidermal LabCyte™ EPI-MODEL 24 cells. The test procedure was based on the recommended protocol for these cells and used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay method as reported in the literature with slight modifications.²¹ *In vitro* skin irritation tests were performed using both comparative and concentration-based studies. In comparative studies, the maximum cell viability was observed in 20% w/v LBILs formulation and the amount of cell viability were as like as IPM and PBS. The LBILs were determined to show better biocompatibility than the commercial IL; 1-butyl 3-methylimidazolium bis (trifluoromethylsulfonate) amide (bmim-Tf₂N), 1-Dodecyl-3-methylimidazolium bis (trifluoro-methyl sulfonyl) imide (emim-TFSA), and the conventional surfactant SDS (**Figure 15-A**). In the concentration-based studies, we observed that up to 50% w/v concentrated LBILs in IPM formulations did not significantly change the cell viability compared with negative control such as IPM (**Figure 15-B**). The cell viability results are summarized in **Table 5**, which shows that the cell viability was primarily determined by the LBILs concentration. Both the cationic and anionic parts of these LBILs were derived from biomaterials and the present skin irritation tests provide evidence for the biocompatibility of these materials.

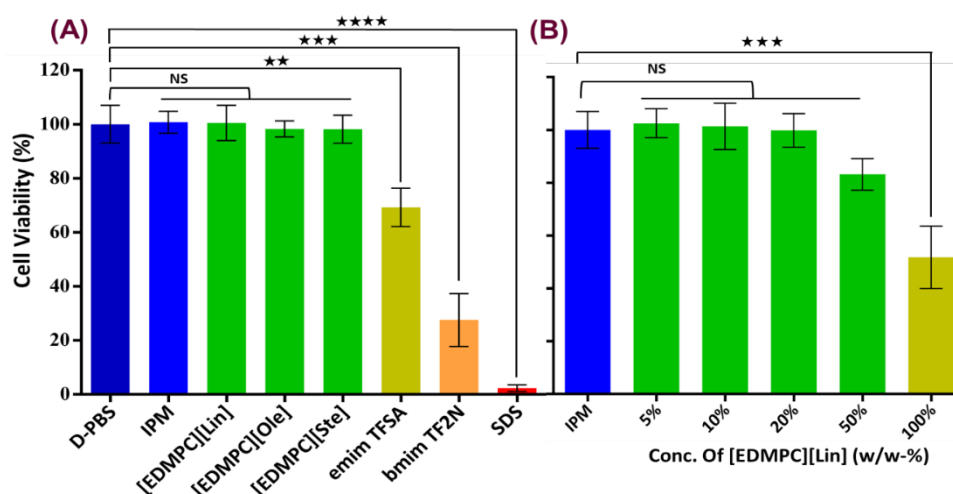


Figure 15: (A) The comparative cytotoxicity of the LBILs as determined using a skin irritation test with an artificial three-dimensional human epidermis model (B) The concentration-dependent skin irritation profile of [EDMPC][Lin]. Here, IPM and SDS were employed as negative and positive controls, respectively. NS represented as non-significant value.

Table 5: 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 ₂ N	27.2	4.1
SDS	1.9	1.0

2.4.5 Statistical Analysis: The statistical analysis was carried out using the two-way ANOVA multiple comparison test and differences were considered significant at $p < 0.05$, where $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$ based on Dunnett's multiple comparison test.

2.5 Conclusions

In summary, several new LBILs consisting of a long chain phosphonium cation (EDMPC) and a long chain fatty acid (such as linoleic acid, oleic acid, or stearic acid) were successfully synthesized and investigated. These compounds are the first examples of ILs containing long chain lipid moieties as both cation and anion. The LBILs were completely soluble in both polar and nonpolar solvents and somewhat soluble in water. These compounds exhibited excellent biocompatibility in a well-established skin irritation test using an artificial three-dimensional human epidermis model. The extraordinary physicochemical properties and excellent biocompatibility of these LBILs may permit their use in DDSs and provide new approaches to the IL-mediated transdermal delivery of therapeutic molecules.

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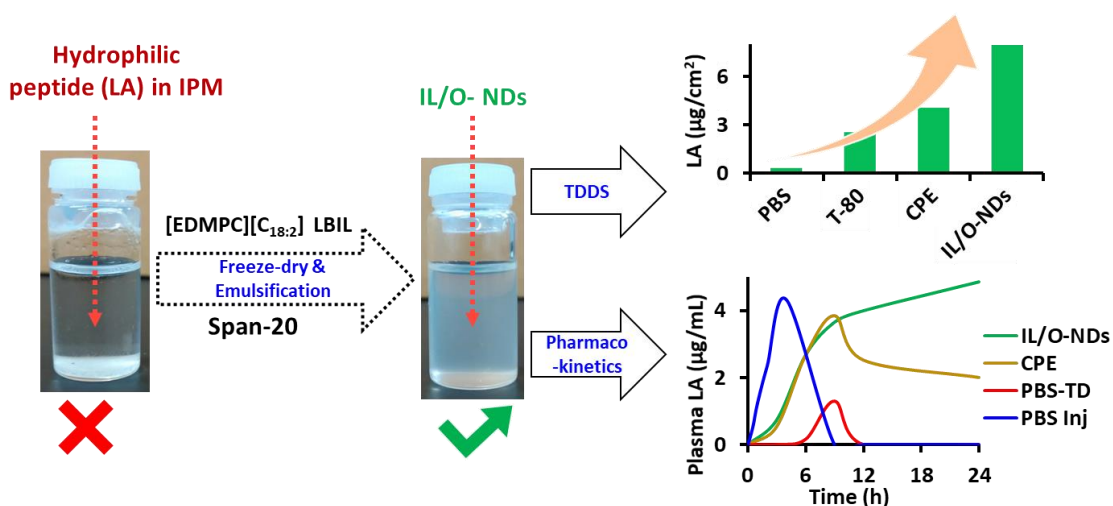
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CHAPTER 3

LIPID-BASED IONIC LIQUIDS MEDIATED NANODESPERSIONS AS BIOCOMPATIBLE CARRIER FOR THE ENHANCE TRANSDERMAL DELIVERY OF A PEPTIDE DRUG.

Graphical Abstract



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3.1 Abstract

Lipid-based biocompatible ionic liquids (LBILs) have attracted attention as carriers in transdermal drug delivery systems (TDDSs) because of their lipophilic character. In this study, we report a formulation of a peptide-LBIL complex microencapsulated in an oil phase as a potential carrier for the transdermal delivery of leuprolide acetate as a model hydrophilic peptide. The peptide-LBIL complexes were prepared via a water-in-oil emulsion composed of 1,2-dimyristoyl-sn-glycerol-3-ethyl-phosphatidylcholine (EDMPC), a fatty acid (stearic, oleic, and linoleic acid)-based LBIL, and cyclohexane followed by freeze drying to remove the water and cyclohexane. Then, the peptide-LBIL complexes were nanodispersed and stabilized in isopropyl myristate (IPM) using sorbitol laurate (Span-20). Ionic liquid-in-oil nanodispersions (IL/O-NDs) were prepared with varying weight ratios of LBILs and Span-20 as the surfactant and co-surfactant, respectively. Keeping the overall surfactant constant at 10 wt.% in IPM, an equal ratio of surfactant (IL) and cosurfactant (Span-20) in the IL/O-NDs significantly ($p < 0.0001$) increased the physiochemical stability, drug loading capacity, and drug encapsulation efficiency. The *in vitro* and *in vivo* peptide delivery across the skin was increased significantly ($p < 0.0001$) using IL/O-NDs, compared with non-IL-treated groups. Of all the LBIL-based formulations, [EDMPC][Linoleate]/O-ND was considered the most preferable for a TDDS based on the pharmacokinetic parameters. The transdermal delivery flux with [EDMPC][Linoleate]/O-ND was increased 65-fold compared with the aqueous delivery vehicle. The IL/O-NDs were able to deform the lipid and protein arrangements of the skin layers to enhance the transdermal permeation of the peptide. *In vitro* and *in vivo* cytotoxicity studies of the IL/O-NDs revealed the biocompatibility of

the LBIL-based formulations. These results indicated that IL/O-NDs are promising biocompatible carriers for lipid-peptide TDDSs.

KEYWORDS: Lipid-based ionic liquids, Leuprolide acetate, Ionic liquid-in-oil nanodispersion, Stability, Transdermal peptide delivery, Pharmacokinetics and Biocompatibility.

3.2 Introduction

Over the past few decades, transdermal drug delivery systems (TDDSs) have attracted attention for the delivery of not only cosmetic compounds but also therapeutic agents, such as small molecule drugs, peptides and peptide amphiphiles, and proteins, to elicit both local and systemic actions.^{1–3} The main advantages of TDDSs over oral and injection routes are the avoidance of the first-pass metabolic effect of the liver, diaphragm, the ability to bypass enzymatic degradation, the prolonged clearance of therapeutics, and increased patient compliance.^{4,5} However, TDDSs are not problem-free delivery systems, especially for hydrophilic and high molecular weight drugs, such as peptides and proteins, because of the barrier created by the strong hydrophobic outer layer of the skin, the stratum corneum (SC).^{6,7} It has been reported that only drugs that have a molecular weight < 600 Da, a water/octanol partition coefficient in the range of 1–3, and a low melting point are suitable for TD, indicating the limited number of drugs that are applicable to TDDSs.^{8,9} To overcome this limitation, several strategies have been reported, including the use of chemical permeation enhancers (CPEs), microneedle or laser ablation to reduce the barrier physically, and the incorporation of ionic liquids (ILs) in the delivery system to modulate the barrier function of the SC to aid the penetration non-invasively.¹⁰ ILs are

described as a liquid salt with a melting point less than 100 °C (212 °F).¹¹ ILs have been reported to enhance the transdermal transcellular and paracellular transportation of therapeutics by decreasing the barrier function of the SC by mechanisms, such as cellular integrity disruption, fluidization, and lipid extraction in the SC.^{4,12,13}

ILs are considered to be an alternative to conventional organic solvents and surfactants, particularly in drug delivery systems (DDSs).^{2,14,15} The use of ILs in the biopharmaceutical industry is increasing rapidly. In drug delivery systems (DDSs), ILs are mainly used as solvents/co-solvents,^{16,17} surfactants/co-surfactants,⁷ adjuvants,^{18,19} and API-ILs^{20–23} (API: active pharmaceutical ingredient) in IL-mediated formulations, such as IL-in-oil nanodispersions (IL/O-NDs),⁷ IL/O microemulsions,^{16,17} IL-drug complexes,²⁴ or simply IL-water mixtures²⁵ of very poorly water soluble or practically water insoluble drugs.^{26–28} In the pharmaceutical industry, the combination of ILs and API to develop API-ILs with task-specific bio-active compounds was a promising achievement. API-ILs increase the thermostability of a drug molecule by increasing its biocompatibility and its tunable biological activity without changing any desired properties.^{11,27,28} For instance, choline–amino acid and choline–fatty acid ILs have been shown to be able to solubilize poorly soluble drugs, such as paclitaxel and acyclovir,²⁹ as well as facilitate the delivery of proteins and peptides,³⁰ and small interfering RNA (siRNA)³¹ through and across the skin with considerably improved permeation rates. In several studies, ILs, such as 1-dodecyl-3-methyl imidazolium bis(trifluoromethyl sulfonyl)-amide ([C₁₂mim][Tf₂N]), *N*-dodecyl-dabco bromide, ([C₁₂dabco]Br), *N,N*-dihexyl-dabco bromide ([C₆C₆dabco]Br), 1-*N*-butyl-dabco bromide ([C₄dabco]Br), and *N*-methyl-*N*-(4-butylsulfonic-acid) pyrrolidinium hydrogen sulphate ([C₁C₄SO₃Hpyrr][HSO₄]), have been reported to act as excellent skin permeation enhancers when used in

TDDSs because they have a hydrophobic anion group.^{26,32} The high affinity of hydrophobic anions with the SC has been suggested to boost the TD of hydrophilic and hydrophobic drugs to overcome the skin barrier function.²⁶ ILs have been reported to be an alternative to CPEs, but their biosafety and toxicity remain concerning issues.³³ The third generation ILs may solve this issue as their precursors are mainly derived from natural and biocompatible sources. The 1st generation ILs generally composed of an imidazolium cation and a non-coordinating anion, such as tetrafluoroborate and hexafluorophosphate. Most of these ILs are poorly biodegradable and toxic. The 2nd generation ILs contain more biocompatible cations (e.g., cholinium salts) and anions, such as amines and amino acids, which can be derived from relatively inexpensive and eco-friendly natural products. The 3rd generation ILs comprises with the addition of a biological property along with tunable chemical and physical properties for biological applications. Compared to 1st and 2nd generation ILs, the 3rd generation ILs provide various advantages including low production cost, simple synthesis and purification process, the structural and functional integrity of API, and controllable polymorphism.² Recently, cholinium cation based ILs with coordinating anions, such as carboxylic acids, amino acids, and artificial sweeteners, have been reported to be readily biodegradable and biocompatible with minimal toxic effects. In our previous study, we reported a series of lipid-based biocompatible ILs (LBILs), consisting of the phospholipid derivative 1,2-dimyristoyl-sn-glycero-3 ethyl-phosphocholine (EDMPC) as the cation and C₁₈ based fatty acids (C_{18:0}, C_{18:1}, or C_{18:2}) as the counter anions. These LBILs showed excellent biocompatibility in the three-dimensional (3D) reconstructed human skin model, LabCyte EPI-MODEL.³⁴ Notably, these LBILs were completely miscible with the emollient ester, isopropyl myristate (IPM). The polar emollient IPM is widely used and well accepted for

use in cosmetics and topical and transdermal medicinal applications.^{9,20} The incorporation of fatty acids, which have anti-carcinogenic, anti-oxidative, anti-inflammatory, and immune stimulatory effects, in LBILs would be beneficial for therapeutic applications.⁷ Therefore, the combination of IPM and an LBIL could have promise in pharmaceutical formulations, especially for the TD of high molecular weight peptides and proteins.

In the present study, a hydrophilic peptide, leuprolide acetate, was incorporated into a LBIL-mediated IL/O-NDs for TDDs to the systemic circulation. The therapeutic peptide leuprolide acetate, which is a gonadotrophin-releasing hormone analog, is currently administered by intracutaneous injection and is clinically used for the treatment of many disorders, including prostate cancer, breast cancer, early puberty, uterine fibroids, and endometriosis.³⁵ The injection formulations have been reported to generate clinical side effects, including flashes, acne, and clammy skin, as well as itching, diarrhea, stomach and muscle pain, impotence, and memory problems.^{35,36} Therefore, an alternative delivery system that could avoid these limitations is highly desirable to deliver leuprolide acetate to the systemic circulation. Here, we report for the first-time LBIL-based formulation strategies using leuprolide acetate in IL/O-NDs and the optimization of the formulation by measuring the thermodynamic stability using dynamic light scattering (DLS). The preparation strategies, characterization, *in vitro* skin permeation, pharmacokinetics, and *in vitro* and *in vivo* biocompatibility of LBIL-based leuprolide acetate -IL/O-NDs were investigated in detail.

3.3 Materials and Methods

3.3.1 Materials: 1,2- dimyristoyl-sn-glycero-3-phosphocholine (DMPC), leuprolide acetate, ethyl trifluoro-methyl sulfonate; 1-ethyl-3- methyl-imidazolium bis-(trifluoro-

methyl sulfonyl) imide (e-TFSA), 1-dodecyl-3-methyl-imidazolium bis-(trifluoro methyl sulfonyl) imide (e-Tf₂N), and diethylene glycol monoethyl ether (DGME) a chemical permeation enhancer (CPE) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Tween-80, IPM, sodium dodecyl sulfate, oleic acid, linoleic acid, stearic acid, and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium (HEPES) salt were purchased from Sigma Aldrich Chemical Co (St. Louis, MO, USA). The human artificial tissue LabCyte EPI model cell line and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay medium was provided by Japan Tissues Engineering Co. Ltd. (Aichi, Japan). The mouse skin (Hos-HR-1) and Yucatan micropig (YMP) skin were obtained from the Hoshino animal laboratories (Ibaraki, Japan). LHRH (leuprolide) (enzyme-linked immunosorbent assay (ELISA) kit; lot number A18102) was purchased from BMA Biomedicals, Peninsula Laboratories, (Bubendorf, Switzerland). Female 6-week-old BALB/C mice were obtained from the Kyudo (Saga, Japan). *In vivo* experiments were performed using BALB/C mice and were approved by the Ethics Committee for the Animal Experiments of Kyushu University. All other laboratory reagents were of analytical research grade.

3.3.2 Preparation of IL/O-NDs formulations: First, LBILs, consisting of the phospholipid derivative EDMPC as the cation and C₁₈ based fatty acids (C_{18:0}, C_{18:1}, or C_{18:2}) as the anion were synthesized and characterized as previously reported.³⁴ Then, LBIL-mediated nanodispersions-in-oil (IL/O-NDs) using IPM as the medium were prepared according to the literature with some modification.³⁷ In brief, 1 mL of peptide aqueous solutions (3.0 mg/mL) and 4 mL of LBIL in cyclohexane solution (12.5 mg/mL) were homogenized with a mechanical homogenizer (POLYTRON, high-speed

homogenizer, PT2500E) at 26,000 rpm for 2 min. The resulting solutions were freeze dried (FD5N; YELA, Tokyo, Japan) to remove the water and cyclohexane, and an IL-peptide complex was prepared. This complex containing 3 mg of peptide and 5 wt.% LBIL was stirred for 12 h with 5 wt.% Span-20 in 1 mL of IPM solution to give an IL/O-NDs formulation (**Figure 1**).

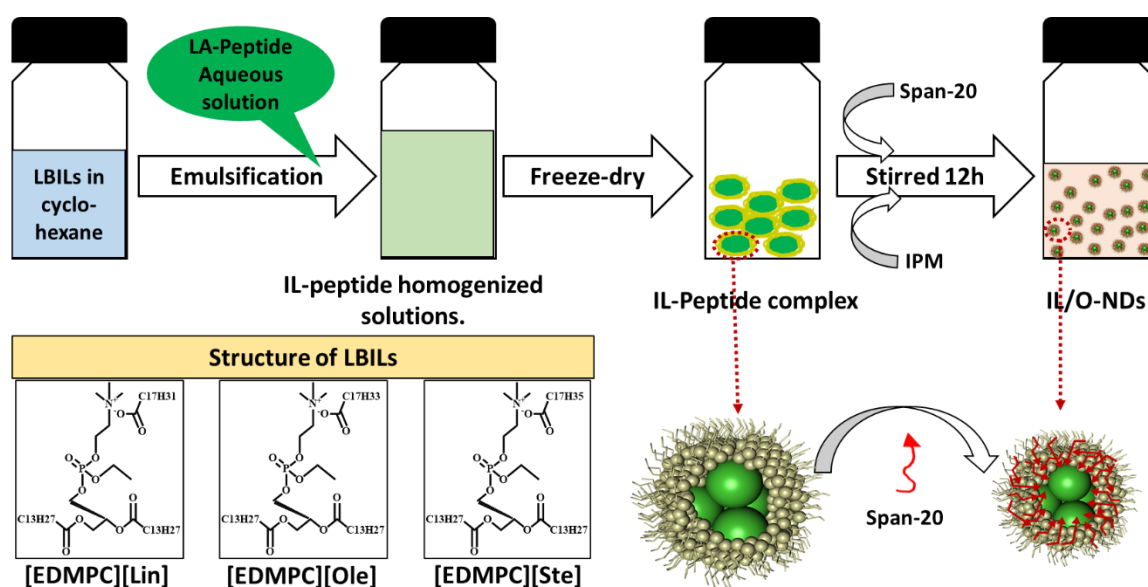


Figure 1: Schematic representation of the IL/O-NDs preparation technique. Structure of three LBILs were showed in the scheme.

3.3.3 Characterization of the IL/O-NDs: The particle size and polydispersity index (PDI) of the peptide-loaded IL/O-NDs were investigated by DLS (Malvern, Nano series, Worcestershire, WR14 1XZ, UK), maintained at 173° in a 1-cm length capped quartz cell at a temperature of 25 ± 0.1 °C. The mean diameters of 10 replicated measurements of each sample were taken as the particle size. The particle sizes and shapes were further characterized by TEM analysis.⁸ In brief, 2 μ L samples of the test solutions were placed on a carbon copper film TEM grid. After 2-min incubation, the solutions were absorbed by the carbon-coated film. The oil phase (IPM) of the formulation was washed with

cyclohexane and again incubated for 2 min. The deposited complexes were stained with 2% uranyl acetate solution and finally, the images of the IL/O-NDs were observed on a TEM-2010 (JEOL, Tokyo, Japan) at 120 kV. Moreover, the morphological analysis of the droplet sizes and shapes of the IL/O-NDs was confirmed by CLSM (LSM700, Carl Zeiss, Germany) using bright-field analysis.

3.3.4 Stability of the IL/O-NDs: The appropriate ratio of surfactant (LBIL) and co-surfactant (Span-20) was selected based on the stability of the IL/O-NDs at room temperature. The stability of different formulations was assessed by visual observations and DLS analysis. Different IL/O-NDs formulations were prepared by changing the ratio of the wt.% of the surfactant and co-surfactant (1:1, 1:2, and 2:1). The physical stability of the peptide-loaded IL/O-NDs formulations was investigated by visual observation over regular time (7 days) intervals until precipitation or any abnormalities were observed under different thermal conditions. The stability of the particle size of the leuprolide acetate peptide in the IL/O-NDs was investigated with different time intervals by DLS analysis. The chemical stability of the peptide was quantified using a high-pressure liquid chromatography (HPLC) analytical system at regular time intervals. The stability of the peptide-loaded IL/O-NDs was investigated for up to 90 days under different thermal conditions: low temperature (4 °C); room temperature (25 °C); and high temperature (37 °C). For HPLC analysis a linear correlation curve of a leuprolide acetate standard solution was constructed between 0.01 and 0.1 µg/mL concentration in a diluent (1% TFA and 25% acetonitrile in aqueous solution). The tested samples were prepared as below: IL/O-NDs formulations were centrifuged at 10,000 rpm for 10 min and the peptide-containing upper transparent solution was quantified using HPLC to check the drug stability. The

concentration of samples was adjusted with the control standard solution 0.1 µg/mL in diluent.

HPLC system: HPLC was used to quantify the amount of leuprolide acetate-peptide in the IL/O-NDs. HPLC (JASCO International Co. Ltd. Hachioji, Tokyo, Japan) was performed as described previously with a slight modification.³⁶ The mobile phase was a mixture of 77% v/v of 0.087 M monobasic ammonium phosphate aqueous solution (pH adjusted to 6.5 with NaOH/HCl) and 23% v/v of acetonitrile. The chromatographic separations were performed using an Inert sustain ODS column (150 × 4.6 mm, 5 µm, GL Sciences Inc., Shinjuku, Tokyo, Japan) at 30 °C. The injected sample solutions (100 µL) were separated using a flow rate of 1.0 mL/min with detection at 220 nm.

3.3.5 *In-vitro* transdermal peptide delivery study of peptide loaded-IL/O-NDs: A skin permeation study of various IL/O-NDs formulations (**Table 1**) was conducted on mouse skin using Franz diffusion cells with slight modification of the method described in a previous report.⁸ In brief, mouse skin segments (2 × 2 cm²) were placed on Franz diffusion cells, and 5 mL of HEPES buffer pH 7.4 (31 M concentrated HEPES salt was dissolved in milli-Q water then the pH was adjusted to pH 7.4 with NaOH or HCl solution) was placed in the receiver chambers. Test samples (250 µL) were added onto the hairless mouse skin area considered to be the donor compartment and the system was maintained at 32.5 °C using a circulating water bath with magnetic stirring. A 300-µL sample of medium was withdrawn and replaced with the same amount of fresh medium in the receiver phase at 1, 3, 6, 9, 12, 24, and 36 h. The transdermal (across the skin) and topical (into the skin) peptide delivery was quantified by HPLC. The topical drug delivery was estimated as follows: after 36 h, the donor compartments were unclamped, and skin

surface was washed several times with 20% ethanol solution, then the skin was cut into 16 pieces and the peptide was extracted from the skin by continuous shaking for 12 h in the diluent (1% TFA and 25% acetonitrile in aqueous solution). The extracted solutions were diluted 10–100 fold with diluent and the concentration of leuprolide acetate was quantified by HPLC.

Table 1: Composition of the various formulations used as TDDSs. In all formulations the total amount of surfactants (5 wt.%), cosurfactant (5 wt.%), and amount of leuprolide acetate peptide drug (3.00 mg/mL) were remained constant.

Formulations name	Symbol of formulations*	Compositions		
		Surfactants (5 wt.%)	Cosurfactants (5 wt.%)	Base liquids (1 mL)
[EDMPC][Lin]/O-NDs	F-1	[EDMPC][Lin]	Span-20	IPM
[EDMPC][Ole]/O-NDs	F-2	[EDMPC][Ole]	Span-20	IPM
[EDMPC][Ste]/O-NDs	F-3	[EDMPC][Ste]	Span-20	IPM
CPE/O-NDs	F-4	DGME (CPE)	Span-20	IPM
T-80/O-NDs	F-5	T-80	Span-20	IPM
Peptide in PBS	F-6	-	-	PBS
[EDMPC][Lin]/PBS-NDs	F-7	[EDMPC][Lin]	Span-20	PBS
e-TFSA/O-NDs	F-8	e-TFSA	Span-20	IPM
e-TF ₂ N/O-NDs	F-9	e-TF ₂ N	Span-20	IPM
SDS/PBS-NDs	F-10	SDS	SDS	PBS
[EDMPC][Lin] in IPM	F-11	[EDMPC][Lin]	-	IPM
Span-20 in IPM	F-12	Span-20	-	IPM

Here, DGME (CPE): Diethylene glycol monoethyl ether (used as the chemical permeation enhancer). T-80: Tween-80. e-TFSA: 1-Ethyl-3-methylimidazolium bis (trifluoromethyl sulfonyl) imide. e-TF₂N: 1-Dodecyl-3-methylimidazolium bis (trifluoromethyl sulfonyl) imide. SDS: Sodium dodecyl sulfate. *We used the symbol of the formulations in all relevant figures.

3.3.6 Calculation of skin permeation parameters: Skin permeation kinetic parameters were calculated using lag-time and least-square techniques. The transdermal flux (J) and co-efficient (K_p) were calculated according to the equation $K_p = J/C_d$, where C_d is the concentration of leuprolide acetate peptide ($\mu\text{g/mL}$) in the donor formulation. The lag time (t_L) was obtained from the intercept on the X-axis, and the diffusion coefficient (D)

was calculated according to the equation $D = I^2/6t_L$, where I is the thickness of the mouse skin (0.041 cm). The coefficient of skin partition (K_{skin}) was calculated according to the equation $K_{skin} = (J \times I) / (D \times C_d)$.

3.3.7 Effect of the IL/O-NDs on the SC layer of the skin: The SC was prepared in accordance with a previously published report.¹⁶ In brief, full-thickness frozen YMP skin was defrosted at room temperature. Then, the moisture-free skin was warmed at 60 °C for 120s and the epidermis layer were peeled off from the full-thickness skin. The extracted epidermis sheet was placed into a 0.25% trypsin and 1 mM ethylene-diamine-tetra acetic acid (EDTA) solutions with the SC side facing up for 24h at room temperature. The isolated SC was washed with water and dried for another 24h. The SC pieces were soaked in the test formulations (peptide-loaded LBIL, Tween-80 (T-80), and CPE in oil-based formulations and peptide in phosphate buffer saline (PBS) formulations) for 30min at RT in glass tubes. The SC sheets were washed with 20% ethanol and dried well for 1h. Finally, the SC sheets were analyzed using Fourier transform infrared (FTIR) spectroscopy.

3.3.8 Pharmacokinetic effects of the IL/O-NDs: Pharmacokinetic studies of the IL/O-NDs were performed using BALB/C female mice (6 weeks old, 20 ± 2 g). The mice were randomly divided into six groups of five mice. In brief, the dorsal hair of all the mice was removed and after 2days, 300 μ L (900 μ g peptide) of the test samples per mouse were administrated on the clean skin using a home-made patch composed of three layers of gauze (approximately 1×1 cm², Hakujuji, Japan) and Cathereep FS dressing tape (Nichiban, Tokyo). The same amount of peptide in PBS solution was injected subcutaneously as a positive control. Retro-orbital blood was collected from the eyes of the treated mice (approximately 200 μ L was collected to obtain 100 μ L of plasma) at 3,

6, 9, 12, 24, and 36 h time intervals. Blood was collected from the injection-administered groups at 30 min and 1, 3, 6, 9, 12, and 24 h time intervals. Subsequently, the serum was collected by centrifuging the blood samples at 10,000 rpm for 20 min and the supernatant was collected for further analysis. The concentrations of the peptide in the blood plasma were quantified by ELISA. In brief, The ELISA samples were prepared as below. 100 μ L of plasma vortexed with 100 μ L of 4% phosphoric acid and applied on WCX SPE plate. The elution WCX SPE plate was washed twice by 200 μ L of 5% ammonium hydroxide then 20% of acetonitrile and finally eluted the leuprolide acetate on 96-well with 100 μ L of 1% TFA in acetonitrile/water at 75/25 solutions. Eluting LA solutions were evaporated with a micro evaporator and diluted in 100 μ L of water and samples were ready for ELISA analysis as per the supplier provided protocol [Protocol V (Ab1hr.Std2hr.BtON)].

3.3.9 Biocompatibility evaluations: *In vitro* cytotoxicity study: The cytotoxicity study of the IL/O-NDs was performed on human artificial 3D cultured epidermal skin tissue (LabCyte EPIMODEL-24 well) using an MTT assay for a skin irritation test as described previously with some modifications.³⁴ In briefly, the Lab Cyte Epi-model cell-containing cups were placed into 24-well plates with 0.5mL of assay medium and incubated for overnight (24 h) at 37°C, 5% CO₂ humidified atmosphere. Then, 25 μ L of each formulated tested sample was applied on the tissue 24-well surface and incubated for 24 hrs. After incubation, the tissues were washed carefully at 15 times with D-PBS to remove any remaining samples and soaked in 0.5 mL of MTT medium then incubated for 3hrs. Each sample was immersed in 0.3 mL of propanol-2 and stand it for 48 hr. at 4 °C in the dark to extract the dye. Subsequently, the solutions were taken out into 96-well plates, then measured the absorbance using a micro plate reader at 570 and 650 nm. Propanol

was used as a blank, IPM and PBS were used as the negative control. Cell viability was calculated by considering the relative viability of the negative control IPM (**Table 2**).

Table 2: *In-vitro* cytotoxicity study of IL/O-NDs formulations. The cytotoxicity study of IL/O-NDs was performed on the human artificial 3D cultured epidermal skin tissue (LabCyte EPIMODEL-24 well) using skin irritation test, as described previously with some modifications.

Formulations name	Surfactants (5 wt.%)	Cosurfactant (5 wt.%)	Base-liquid	Cell viability (%)	SD (±)
PBS	-	-	PBS	99	4.84
IPM	-	-	IPM	100	4.81
F-1	[EDMPC][Lin]	Span-20	IPM	98	6.61
F-4	DGME	Span-20	IPM	59	3.29
F-5	Tween-80	Span-20	IPM	99	7.22
F-8	e-TFSA	Span-20	IPM	53	5.88
F-9	e-TF ₂ N	Span-20	IPM	18	5.14
F-10	SDS	-	PBS	6	3.97

Here, PBS: Phosphate buffer saline, IPM: Iso-propyl myristate, DGME (CPE): Diethylene glycol monoethyl ether (Chemical permeation enhancer). T-80: Tween-80. e-TFSA: 1-Ethyl-3-methylimidazolium bis (trifluoro-methyl sulfonyl) imide. e-TF₂N: 1-dodecyl-3- methylimidazolium bis (trifluoromethyl sulfonyl) imide. SDS: Sodium dodecyl sulfate.

In vivo biocompatibility observations: The *in vivo* biocompatibility study was performed on female BALB/C mice. To check the *in-vivo* biocompatibility, the mice were divided into six groups and each group contained five mice (6 weeks old, 20 ± 2 g). Test samples (300 μ L/mouse) were applied on the skin using a home-made patch. The doses were applied on the skin three times at 7-day intervals and each time the patch was left for 24 h. Body weight was observed regularly (one day interval), and at the end of the experiment, after three consecutive doses, the area of the tested skin was collected for histological observation according to the previous published report with slide modification.¹⁶ Desired skin area was rinsed with water and 50% propanol then rinsed in 4% formaldehyde solutions for 2h and frozen at -80 °C. The 20 μ m skin cryosection was fixed on the slide and washed with acetone, ethanol, and water. Desired skin samples

were stained with a hematoxylin solution for 6 minutes and washed with water and again stained with an eosin solution for 30 seconds. After washing with water and dehydrated by ethanol, the skin was ready to observe by a BZ-9000 color microscope (Keyence Co., Osaka, Japan).

Data Analysis: The statistical data were analyzed using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) based on a two-way ANOVA Tukey's and Dunnett's multiple comparison test. The comparisons were significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3.4 Results and Discussion

3.4.1 Selection of ingredients for the IL/O-NDs: The selection of ingredients for pharmaceutical formulations is crucial to construct a stable vehicle to ensure maximum drug solubility together with enhanced drug permeation capacity. Additionally, to develop a potential ILs/O-ND formulation for TDDS, all the selected elements should be biocompatible, and permeation enhancing hydrophobic solutions are preferable.²⁰ Leuprolide acetate itself is not soluble in IPM, but was completely miscible with the LBIL-mediated formulations (**Figure 2**).

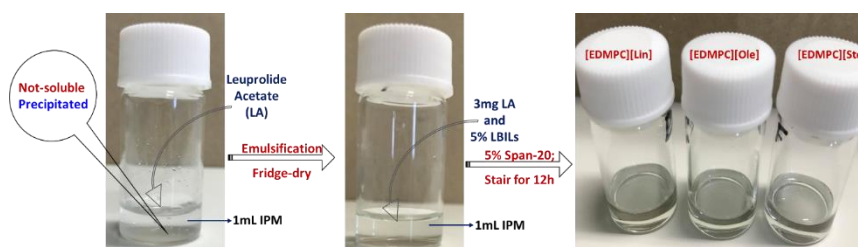


Figure 2: Solubility of leuprolide acetate in IPM by LBILs. Leuprolide acetate hydrophilic peptide is not soluble in IPM, but its soluble in oil phase IPM with 5% wt./V LBILs mediated formulation and make a nanodispersion solutions.

However, only LBILs in oil-based formulations were not stable; therefore, Span-20 was added to increase the stability of the drug-loaded formulations. Hydrophobic LBILs such as [EDMPC][Lin], [EDMPC][Ole], and [EDMPC][Ste] have a considerable solubilizing potential in both polar and non-polar solvents, making it easier to develop a stable IL/O-NDs.^{38,39} Here, IL/O-NDs were prepared using different weight ratios (w/w) of LBILs and Span-20 (1:1, 1:2, and 2:1) in IPM, where the total amount of surfactant and co-surfactant was kept constant at 10% w/w (**Table 3**).

Table 3: Initial assessment of various formulations used in this study and choose the appropriate and sustainable formulations for TDDS.

Surfactant amount (Wt./V)	Co-surfactant amount (Wt./V)	Total surfactant in 3mg/mL LA in IPM formulations	Observation	Droplet size; m/z (nm)
[EDMPC][Lin]: 5%	-	5% [EDMPC][Lin]	Precipitated after 13days	627
[EDMPC][Ole]: 5%	-	5% [EDMPC][Ole]	Precipitated after 9days	579
[EDMPC][Ste]: 5%	-	5% [EDMPC][Ste]	Precipitated after 7days	650
[EDMPC][Lin]: 10%	-	10% [EDMPC][Lin]	Precipitated after 6days	781
[EDMPC][Ole]: 10%	-	10% [EDMPC][Ole]	Precipitated after 5days	779
[EDMPC][Ste]: 10%	-	10% [EDMPC][Ste]	Precipitated after 3days	815
[EDMPC][Lin]: 5.0%	Span-20: 5.0%	F-1	Clear transparent solution & stable	251
[EDMPC][Lin]: 6.7%	Span-20: 3.3%	10% ([EDMPC][Lin]: Span-20=2:1)	Clear but irregular particle size	N/A
[EDMPC][Lin]: 3.3%	Span-20: 6.7%	10% ([EDMPC][Lin]: Span-20=1:2)	Clear but irregular particle size	N/A
[EDMPC][Ole]: 5.0%	Span-20: 5.0%	F-2	Clear transparent solution & stable	220
[EDMPC][Ole]: 6.7%	Span-20: 3.3%	10% ([EDMPC][Ole]: Span-20=2:1)	Clear but irregular particle size	N/A
[EDMPC][Ole]: 3.3%	Span-20: 6.7%	10% ([EDMPC][Ole]: Span-20=1:2)	Clear but irregular particle size	N/A
[EDMPC][Ste]: 5.0%	Span-20: 5.0%	F-3	Clear transparent solution & stable	265
[EDMPC][Ste]: 6.7%	Span-20: 3.3%	10% ([EDMPC][Ste]: Span-20=2:1)	Clear but irregular particle size	N/A
[EDMPC][Ste]: 3.3%	Span-20: 6.7%	10% ([EDMPC][Ste]: Span-20=1:1)	Clear but irregular particle size	N/A

Here, F-1; 10% ([EDMPC][Lin]: Span-20=1:1), F-2; 10% ([EDMPC][Ole]: Span-20=1:1), and F-3; 10% ([EDMPC][Ste]: Span-20=1:1),

In the IL/O-NDs, the ILs act not only as a surfactant, but also play a key role in improving the drug solubility and the stability of the formulations through covalent or electrostatic interactions between the ILs and the drugs.^{40,41} The IL/O-NDs were unstable at ratios of 1:2 and 2:1 but were stable at a ratio of 1:1, and no visual abnormalities or precipitation were observed. The formulation with an equal ratio of surfactants and co-surfactants was more stable than those with unequal ratios. Here, Span-20 acts as a stabilizer for the hydrophilic–lipophilic balance of the surfactant and co-surfactant and increased the drug solubility to give emulsions with a smaller droplet size.⁴² Based on the visual observations and DLS data, an equal ratio of surfactant and co-surfactant was used for all further formulations. Interestingly, only LBILs (without Span-20)-based formulations could encapsulate the peptide but this formulation was less stable than IL/O-NDs ([EDMPC][Lin]/O-NDs, [EDMPC][Ole]/O-NDs, and [EDMPC][Ste]/O-NDs) and had a larger particle size (≥ 600 nm) (**Figure 2A**). The particle size increased when the drug was loaded into the formulation, indicating that the drug was incorporated into the core side of the nanoparticle. Here, we measured the particle size both in drug loaded and without drug loaded formulations (**Figure 3**).

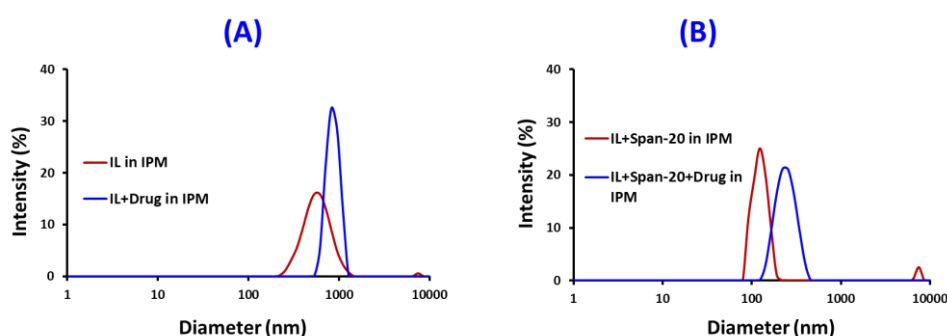


Figure 3: The particle size of drug loaded and without drug loaded formulations was observed by DLS. The particle size of drug-loaded formulations is increased, indicating that the drug has been incorporated into the nanoparticles.

Span-20 (5 wt.%) together with a LBIL (5 wt.%) in IPM resulted in IL/O-NDs that were more stable, had significantly increased ($p < 0.0001$) drug loading capacity, and more compact particles < 300 nm (**Figure 4A**). It has been reported that using Span-20 as a co-surfactant enhanced the drug solubility in microemulsion formulations.⁴² Higher amount of drug loading capacity and stability of IL/O-NDs formulations were supported the presence of strong H-bonds and electrostatic bonds between the LBILs and Span-20 to make a IL/O-NDs. These interactions may act as adhesive forces in the LBIL-peptide-Span-20 or IL/O-NDs systems, resulting in the development of a stable and more compact pharmaceutical formulation.⁴³ The [EDMPC][Lin]/O-NDs could load a larger concentration of drug compared with the other formulations because of the π - π interactions of linoleic acid, which played an important role in dissolving more drug molecules in the inner core of the nanoparticles (**Figure 4B**).³⁸

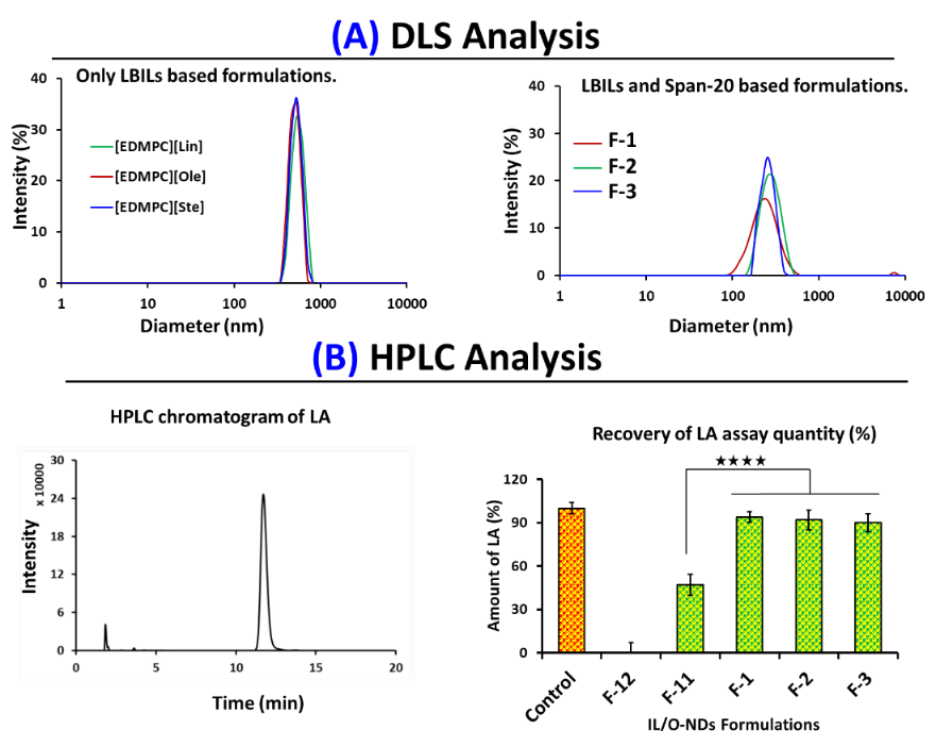


Figure 4: (A) The particle sizes of IL/O-NDs. (B) LA peptide recovery from IL/O-NDs. Here, the mean $\pm$ SD ($n = 3$); and * $p < 0.05$, **** $p < 0.0001$ using Tukey's multiple comparison test.

3.4.2 Characterization of the IL/O-NDs: LBIL-based IL/O-NDs formulations were characterized by DLS, TEM, and CLSM analytical processes. The observed particle size of the IL/O-NDs was significantly smaller and the particles were more stable than those of formulations consisting of only LBILs (**Figure 5A**); this was because the Span-20 increased the H-bonding to form more stable and compact particles. The particle sizes and polydispersity index (PDI) values of the peptide-loaded IL/O-NDs are summarized in **Table 4**.

Table 4: DLS observation of LBILs-based IL/O-NDs formulations.

Formulations name	Formulations (LA; 3mg/mL)			Particle size (nm)	PDI	Peak intensity
	Surfactants	Co-surfactant	Based solution			
F-1	[EDMPC][Lin]	Span-20	1mL IPM	251±21	0.257	99%
F-2	[EDMPC][Ole]	Span-20	1mL IPM	220±29	0.436	100%
F-3	[EDMPC][Ste]	Span-20	1mL IPM	265±36	0.389	98%

Here, F-1: [EDMPC][Lin]/O-NDs, F-2: [EDMPC][Ole]/O-NDs, and F-3: [EDMPC][Ste]/O-NDs.

It has been reported that the particle size and shape of pharmaceutical formulations play a key role in TDDSs.⁴⁴ Therefore, the particle sizes and shapes were further observed by TEM and found that the particles size and shape were near to same in all LBILs mediated IL/O-NDs. The particles had slightly deformed and irregular shapes was observed in the TEM images. During the sample preparation, the IPM in the IL/O-NDs was washed out by cyclohexane as described in a previous report,⁸ but the LBILs are the first reported ILs that are completely soluble in cyclohexane, and as a result the droplets were slightly deformed. A few particles had a defined shape, but most of the particles had lost any organized structure. Finally, the particle size and shape of the IL/O-NDs were characterized by CLSM. Using bright-field CLSM, clear and regular particle images were

visualized, and the nano-sized particles were moving freely in the IL/O-NDs. The size of the particles was in the range 200–300 nm, which was in strong agreement with the DLS and TEM observations (**Figure 5**). It has been reported that a particle size of between 200 and 300 nm for IL/O-NDs is very suitable for TDDSs.^{45,46}

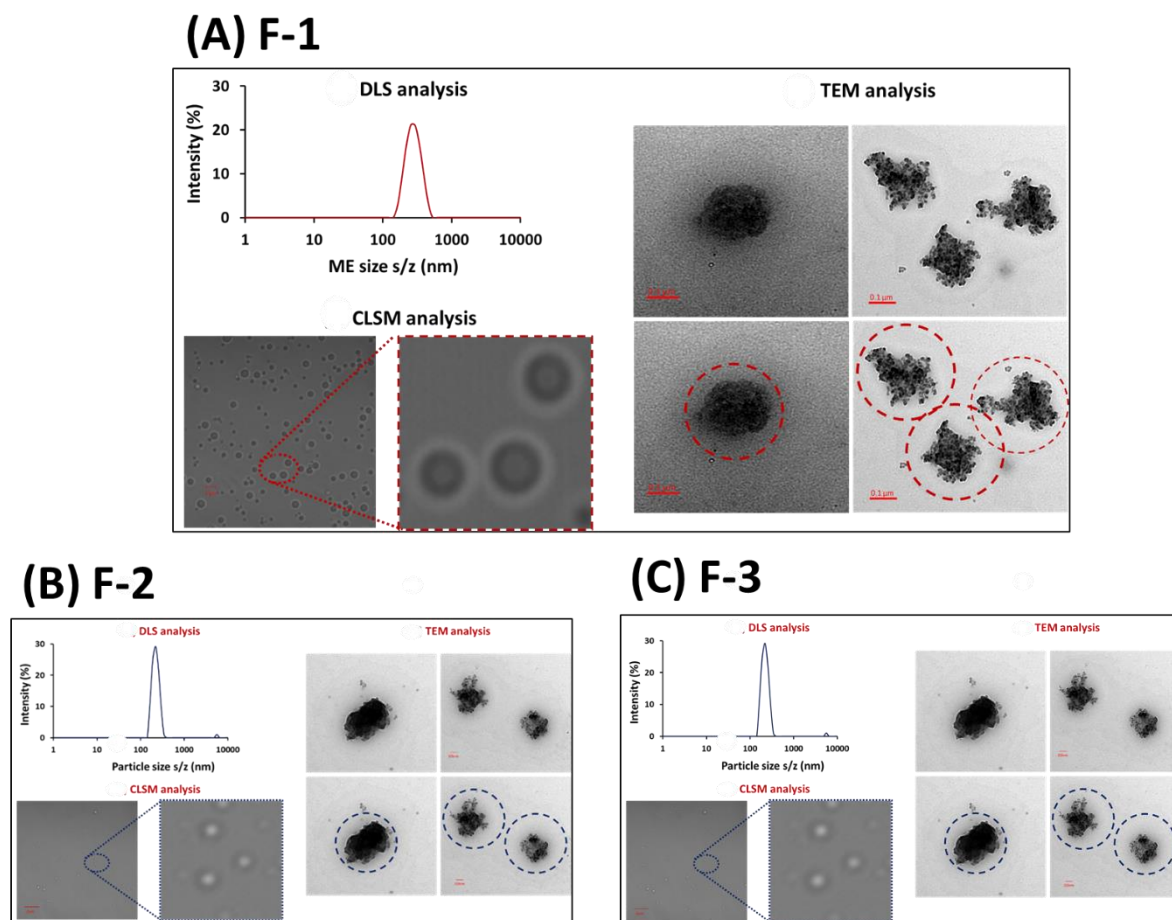


Figure 5: Particle sizes and shapes of IL/O-NDs was observed by DLS, TEM, and CLSM analysis. (A) F-1; ([EDMPC][Lin])/O-NDs, (B) F-2; ([EDMPC][Ole])/O-NDs, and (C) F-3; ([EDMPC][Ste])/O-NDs.

3.4.3 Stability of the IL/O-NDs: The inherent stability of an IL/O-ND is a prerequisite to develop a potential pharmaceutical formulation. Therefore, we investigated the physiochemical and thermodynamic stability of the developed IL/O-NDs formulations using DLS and HPLC. According to the DLS observations, the particle sizes of the IL/O-

NDs remained constant (200–300 nm) at RT and did not change significantly for up to 90 days. However, the particle size was decreased (50–60 nm) at high temperature (37 °C) and was increased (700–1000 nm) at low temperature (4 °C) (**Figure 6A**). These results indicated the stability of the IL/O-NDs under different thermal conditions. The particle sizes and stability of the [EDMPC][Ole] and [EDMPC][Ste]-based formulations were shown in **Figure 7**.

Table 5: Amount of leuprolide acetate in IL/O-NDs formulation was observed by HPLC system.

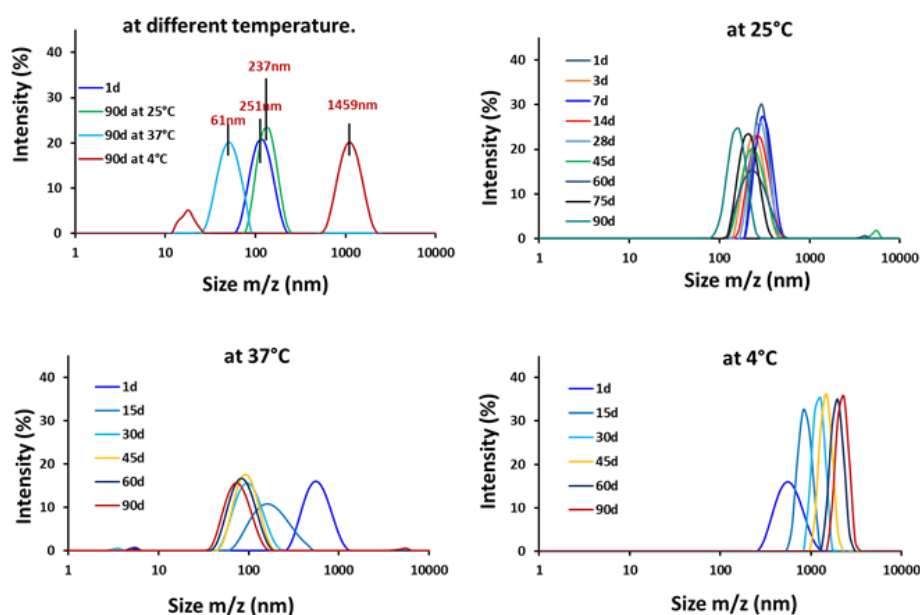
Formulations name	Storage temperature	Leuprolide acetate (%)				
		0d	15d	30d	60d	90d
F-1	25°C	94	92	92	91	93
	37°C	94	90	92	90	92
	-4°C	94	87	88	86	87
F-2	25°C	92	90	89	87	91
	37°C	92	91	90	89	90
	-4°C	92	86	85	84	86
F-3	25°C	90	88	87	88	89
	37°C	90	90	89	90	88
	-4°C	90	84	86	83	84

Here, F-1: [EDMPC][Lin]/O-NDs, F-2: [EDMPC][Ole]/O-NDs, and F-3: [EDMPC][Ste]/O-NDs.

Lipid molecules have a tendency to self-aggregation and lipid based ionic liquids (LBILs) become freeze in low temperature due to its high melting temperature (T_m).³⁴ These results indicated that the particles of IL/O-NDs acted as noninteracting hard spheres and were free from any unwanted fragments. The chemical stability of the leuprolide acetate in the IL/O-NDs was confirmed by HPLC system using a leuprolide acetate assay method.³⁶ The amount of leuprolide acetate remained unchanged at all the temperatures investigated although the amount was slightly decreased at low temperature but this decrease was not significant (**Figure 6B**). LBILs have a self-freezing property at low temperature; thus, it is logical that LBIL-based formulations will precipitate or will freeze at low temperature. Therefore, the amount of leuprolide acetate was decreased at low

temperature (Table 5). More studies are needed to find out the molecular mechanism of the decreasing amount of leuprolide acetate in low temperature.

(A) Stability observed by DLS analysis



(B) Stability was observed by HPLC analysis

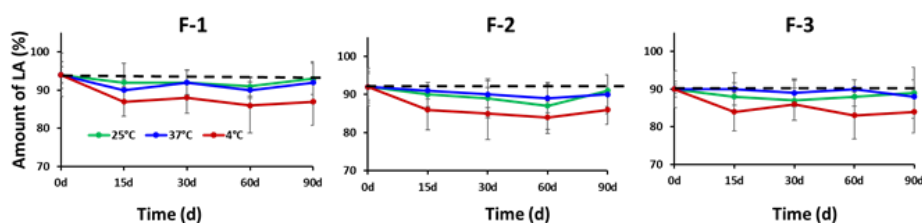
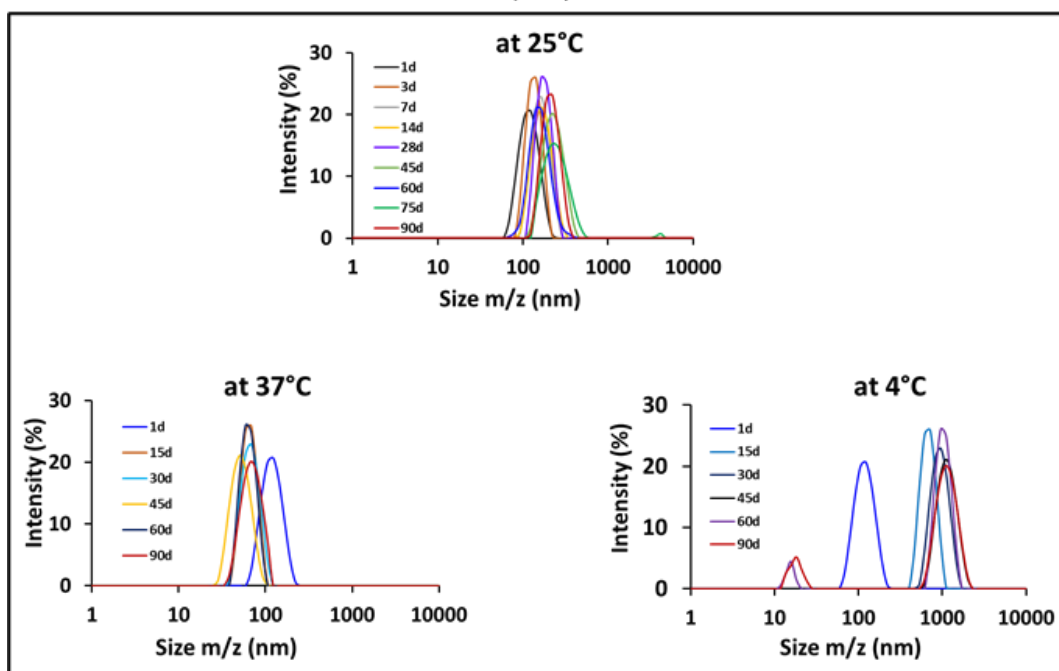


Figure 6: (A) Stability of the LA in [EDMPC][Lin]/O-NDs was observed by DLS. (B) Chemical stability of LA in LBILs/O-NDs were observed by HPLC. Results are presented as the mean; $\pm$ SD (n = 3).

(A)



(B)

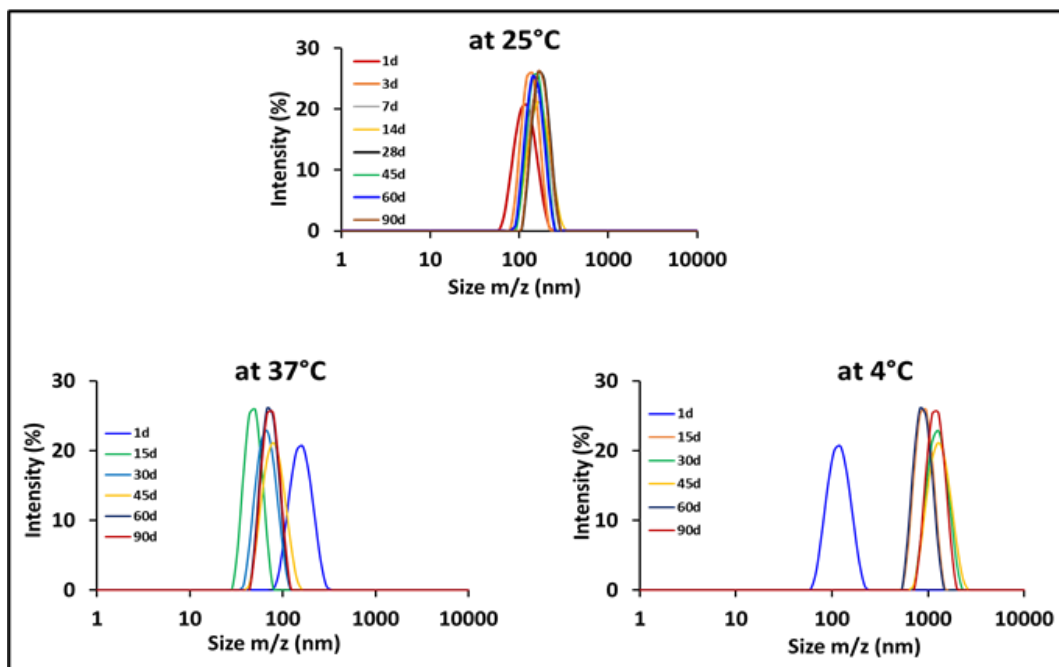


Figure 7: Stability observation of leuprolide acetate in (A) [EDMPC][Ole] mediated IL/O-NDs; F-2 and (B) [EDMPC][Ste] mediated IL/O-NDs; F-3 by using DLS at different thermal condition at different time interval.

3.4.4 Transdermal peptide delivery profiles of the IL/O-NDs: *In vitro skin-permeation:* The concentration of the leuprolide acetate peptide in the receiver-phase was the highest at 24 h and then the concentration gradually decreased in all the formulations, except for [EDMPC][Lin]/O-ND, in which the drug concentration gradually increased even after 36 h. The peptide delivery was the highest for the [EDMPC][Lin]/O-ND and was significantly higher than that of the other formulations, and the lowest peptide delivery was observed for the PBS aqueous formulation (**Figure 8**). Both topical and transdermal deliveries were significantly higher with the [EDMPC][Lin]-based formulation compared with the other IL/O-NDs and the conventional skin permeation enhancer, which indicated that the [EDMPC][Lin] formulation was the best for leuprolide acetate transdermal delivery. Furthermore, the skin permeability was investigated by calculating the permeation kinetic parameters using the lag-time method.⁸

Table 6: Skin permeation kinetic parameters of the IL/O-NDs.

Formulations	Cumulative Amount, Q_{36h} ($\mu\text{g}/\text{cm}^2$)	Transdermal Flux, J ($\times 10^{-2} \mu\text{g}/\text{cm}^2/\text{h}$)	Permeability Coefficient, K_p ($\times 10^{-4} \text{cm}/\text{h}$)	Diffusion Coefficient, D ($\times 10^{-4} \text{cm}^2/\text{h}$)	Skin Partition Coefficient, K_{skin} ($\times 10^{-2}$)
F-1	4.07 $\pm$ 0.39 ^a	14.84 $\pm$ 1.02 ^a	4.95 $\pm$ 1.23 ^a	0.79 $\pm$ 0.35 ^b	25.63 $\pm$ 1.19 ^a
F-2	2.67 $\pm$ 0.58 ^a	12.55 $\pm$ 0.98 ^a	4.18 $\pm$ 0.98 ^a	0.76 $\pm$ 0.21 ^b	22.59 $\pm$ 2.11 ^a
F-3	2.70 $\pm$ 0.82 ^a	11.74 $\pm$ 0.83 ^a	3.91 $\pm$ 1.20 ^a	0.77 $\pm$ 0.33 ^b	20.96 $\pm$ 0.98 ^a
F-4	1.31 $\pm$ 0.72 ^b	10.29 $\pm$ 1.12 ^a	3.43 $\pm$ 0.61 ^a	0.72 $\pm$ 0.52 ^b	19.63 $\pm$ 1.52 ^a
F-5	1.73 $\pm$ 0.65 ^b	9.79 $\pm$ 1.21 ^a	3.26 $\pm$ 1.35 ^a	0.74 $\pm$ 0.64 ^b	18.05 $\pm$ 1.35 ^a
F-6	0.13 $\pm$ 0.51	0.23 $\pm$ 0.69	0.05 $\pm$ 0.42	2.80 $\pm$ 0.96	0.07 $\pm$ 0.01

Here; **** for a and *** for b. Values were significant for ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by Dunnett's multiple comparison test. Data shown as the mean $\pm$ SD, n = 3.

The skin permeation kinetic parameters of the IL/O-NDs are summarized in **Table 6**. The transdermal flux, cumulative amount of leuprolide acetate after 36 h, and permeability coefficient of the [EDMPC][Lin]/O-ND were the highest compared with all the other

LBILs/O-NDs and were the best values for a TDDS. Linoleic acid has two unsaturated carbons (C_{18:2}) that can form a π - π interactions with the SC layers and facilitate the drug delivery across the skin.^{16,47,48} All the LBIL/O-NDs elicited better skin permeation compared with the conventional skin permeation enhancers CPE and Tween-80. These results indicated that all the LBILs have the capacity for drug delivery via the skin and [EDMPC][Lin] was the best potential candidate for a TDDS.

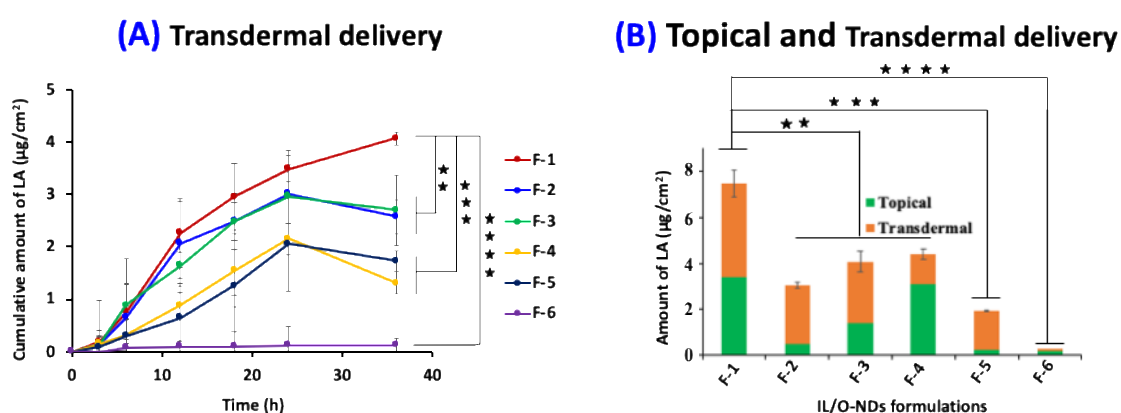


Figure 8: (A) *In vitro* peptide permeation observed using a Franz cell. (B) Transdermal and topical peptide delivery observations. The mean: $\pm$ SD ($n = 3$), values were significant for $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ by Dunnett's multiple comparison test.

3.4.5 Effect of the IL/O-NDs on the skin SC layer: The organization of the lipid matrixes (cholesterol, fatty acids, and ceramides of lamellar structures) and the keratin protein structure of the SC layer are the main barriers for transdermal drug delivery and govern the diffusion of a drug into the deeper parts of the skin.^{8,16} The FTIR spectrum of the SC layers showed a lipid stretching area at 2825–2975 cm^{-1} and stretching of the amide bonds of the proteins at 1475–1725 cm^{-1} .⁴⁹ The effect of the IL/O-NDs systems on the skin layers is shown in **Figure 9** and summarized in **Table 7**.

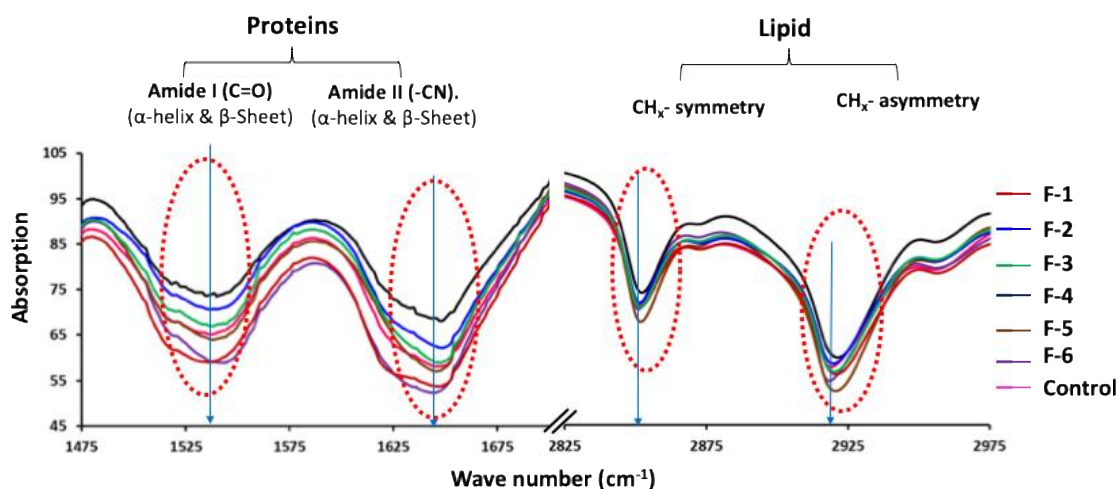


Figure 9: FTIR observations of the effect of the IL/O-NDs on the SC layer. Formulations name were defined in Table-1. Not treated group considered as control group.

The spectra showed that the absorptions of the amide bonds of the keratin protein and the carbohydrate groups of the lipids for the CH_2 -symmetric and CH_2 -asymmetric bands were shifted the most for the [EDMPC][Lin]-based IL/O-NDs was described as [EDMPC][Lin]/O-NDs. The disorganization of the SC layer is directly related to the molecular diffusion of a drug across the skin. The deformation of the α -helical and β -sheet structures of keratin proteins facilitates the drug penetration.⁴⁹ These shifts in the FTIR spectra were a result of the LBIL-based formulation because of the lipophilic cation and fatty acidic anion of the LBILs.²⁶ The double unsaturated carbons of the linoleic acid ($\text{C}_{18:2}$) in [EDMPC][Lin] had a greater effect in facilitating the deformation of the H-bonds in the SC layer, compared with the other fatty acids.¹⁶ This deformation is related to the gauche/trans conformers of linoleic acid based LBIL; [EDMPC][Lin], which deformed the SC lipid organization and resulted in a reduction in the lipid barrier function.⁸ The results suggested that [EDMPC][Lin] was the most suitable formulation to

disrupt the SC lipid barrier, compared with the other formulations, and the most suitable for a TDDS.

Table 7: FTIR peak shifts of SC after treatment with different IL/O-NDs formulation. Non-treated SC was considered as control group.

Formulations name	SC Components (Lipid)				SC Components (Proteins)			
	Absorption; CH ₂ Asymm. (cm ⁻¹)	Δ_{shift}	Absorption; CH ₂ Symm. (cm ⁻¹)	Δ_{shift}	Absorption; -C=O (cm ⁻¹)	Δ_{shift}	Absorption; HN-C=O (cm ⁻¹)	Δ_{shift}
F-1	2922.31	4.19	2852.76	2.02	1649.63	5.10	1538.41	2.10
F-2	2919.96	1.84	2851.98	1.24	1649.23	4.70	1538.57	2.26
F-3	2920.31	2.19	2851.45	0.71	1648.07	3.54	1538.11	1.80
F-4	2920.21	2.09	2852.02	1.28	1646.74	2.21	1536.93	0.62
F-5	2920.74	2.62	2852.14	1.40	1645.91	1.38	1538.43	2.12
F-6	2918.13	0.01	2851.01	0.27	1644.53	0.00	1538.49	2.18
Control	2918.12	0.00	2850.74	0.00	1644.53	0.00	1536.31	0.00

Here; F-1: [EDMPC][Lin]/O-NDs, F-2: [EDMPC][Ole]/O-NDs, F-3: [EDMPC][Ste]/O-NDs, F-4: CPE/O-NDs, F-5: T-80/O-NDs, F-6: Peptide in PBS and non-treated group was considered as control group.

3.4.6 Pharmacokinetics study: In recent years, pharmacokinetics (PK) evaluations have become an integral part of developing a DDS, especially when developing a new drug formulation.⁵⁰ Pharmacokinetics is concerned with the effects of the body organism on the drug (absorption, distribution, metabolism, and excretion).⁵¹ To determine the *in vivo* drug delivery profile of the IL/O-NDs (**Table 8**), we performed a PK study using BALB/C mice.

Table 8: Summary of IL/O-NDs formulations used in pharmacokinetics study and its delivery process. All formulations contain 3.0 mg/mL concentrated leuprolide acetate.

Formulations	Surfactants (5 wt.%)	Cosurfactants (5 wt.%)	Base- liquids	Delivery process
F-1	[EDMPC][Lin]	Span-20	IPM	TD
F-5	Tween-80	Span-20	IPM	TD
F-4	DGME	Span-20	IPM	TD
F-6 (TDDS)	-		PBS	TD
F-6 (Inj.)	-	-	PBS	SI

Here, F-1: [EDMPC][Lin]/O-NDs, F-5: Tween-80/O-NDs, F-4: CPE/O-NDs, F-6 (TDDS): Peptide in PBS for TDDS, and F-6 (Inj.): Peptide in PBS for subcutaneous injection. TD: Transdermal delivery by homemade patches, and SI: subcutaneous injection,

Leuprolide acetate was separated from retro-orbitally collected blood plasma by a Whatman compression sealing- solid phase extraction (WCS-SPE) column and quantified by ELISA. Briefly, 25 μ L of antiserum were placed in 96-well plate and incubated for 1h, then 50 μ L of desired samples were added in well and again incubated for 2 h. After incubation, 25 μ L of rehydrated biotinylated leuprolide Bt-tracer was added and incubated at 4°C for overnight (12 h). Carefully washed at five times with 300 μ L of 20 times diluted ELISA buffer then 100 μ L of streptavidin-HRP was added and incubated for 1 h. Again, washed the sample as same and add 100 μ L freshly prepared TMB chromogenic solution then incubated 10-30 min for color absorbance. Terminated the LA enzymatic reaction with 100 μ L of 2N HCl and read the absorbance at 450 nm (**Figure 10**).

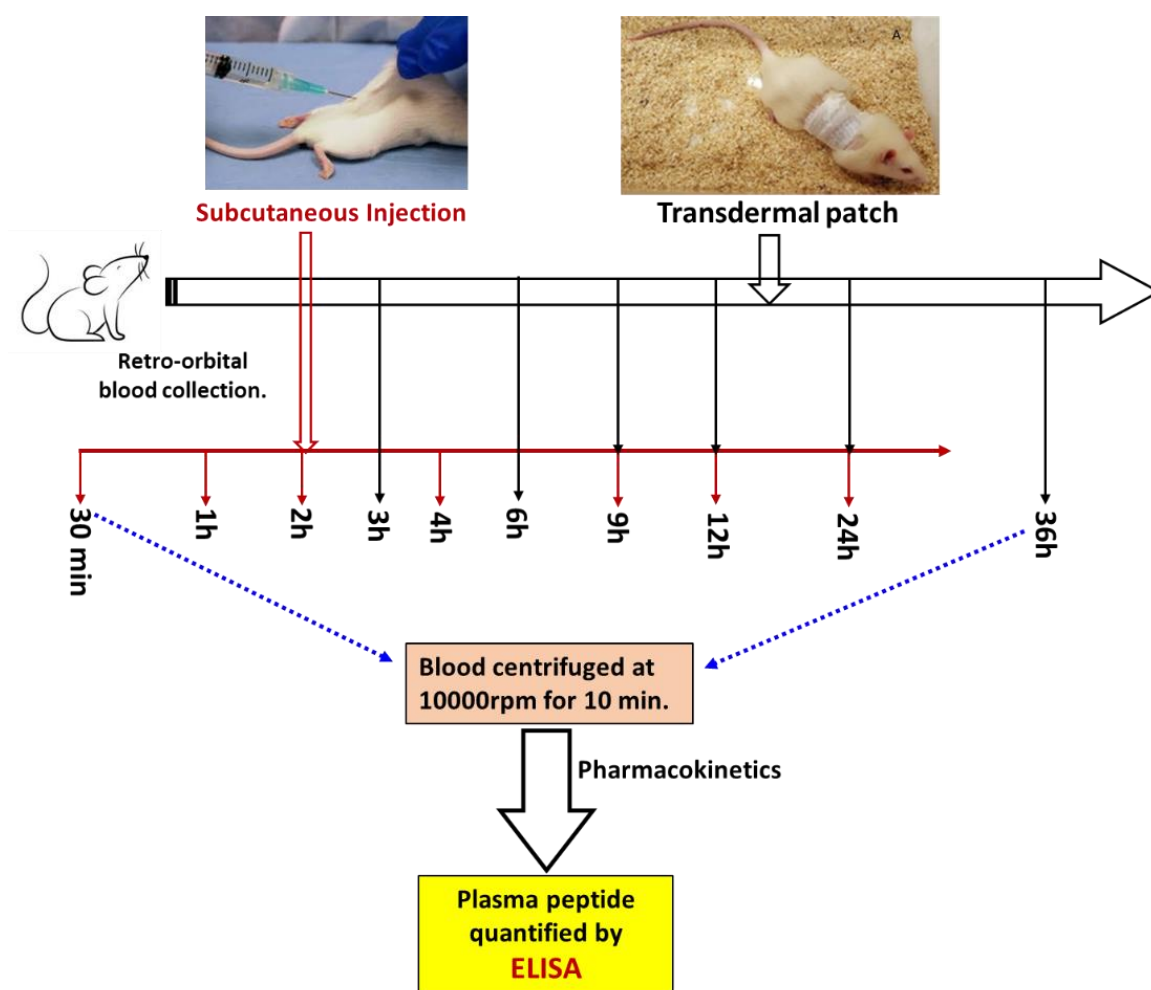


Figure 10: Schematically represented the pharmacokinetics study of IL/O-NDs on BALB/C mice to measure the concentration of plasma peptide by ELISA.

The PK parameters were compared between the transdermal delivery and the corresponding subcutaneous injection administration. Surprisingly, the results showed that the leuprolide acetate delivery to the plasma was more sustained using a TDDS than administration by injection. In the injection-treated group, the drug delivery rate in the blood increased within 30 min and decreased after 4 h; however, in the trans-dermally treated groups, the delivery rate gradually increased up to 36 h (**Table 9**). Based on the presence of leuprolide acetate in blood plasma, the IL/O-NDs could be used as a

sustainable drug delivery system using transdermal patches and showed that the LBILs mediated nanodispersions carried the highest amount of peptide in the circulation system via TDDSs (**Figure 11**).

Table 9: Concentration of Leuprolide acetate in blood plasma was observed by pharmaco-kinetics study of IL/O-NDs formulations.

Time (h)	Formulations and Conc. of LA						Time (h)
	F-1 (µg/mL)	F-5 (µg/mL)	F-4 (µg/mL)	F-6 (TDDS) (µg/mL)	Untreated (µg/mL)	F-6 (Inj.) (µg/mL)	
0	0.00	0.00	0.00	0.00	0.00	1.34	0.5
3	0.93	0.00	0.54	0.00	0.00	3.75	1
6	3.15	2.01	3.62	0.16	0.00	4.05	2
9	3.31	4.00	4.22	1.72	0.00	4.81	4
12	3.99	4.59	2.68	0.00	0.00	0.00	9
24	4.76	4.61	2.18	0.00	0.00	0.00	12
36	5.90	4.10	1.99	0.00	0.00	0.00	24

Here, F-1: [EDMPC][Lin]/O-NDs, F-5: Tween-80/O-NDs, F-4: CPE/O-NDs, F-6 (TDDS): Peptide in PBS for TDDS, and F-6 (Inj.): Peptide in PBS for subcutaneous injection.

The PK parameters were calculated based on the plasma leuprolide acetate concentration. The bioavailability (AUC_{0-t}) and maximum concentration (C_{max}) were the highest in the LBIL/O-ND-treated group, whereas the rate constant (K) was the lowest compared with all other formulations (**Table 10**). These results indicated the suitability of [EDMPC][Lin] for transdermal peptide delivery via an IL/O-NDs system.

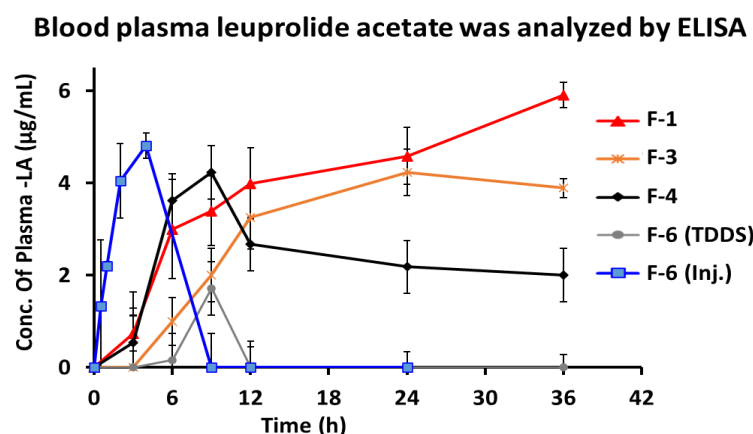


Figure 11: Plasma Leuprolide acetate concentration was observed by ELISA. Here; $\pm$ SD (n=5).

Table 10: Pharmacokinetics parameter of IL/O-NDs formulations.

Formulations	F-1	F-5	F-4	F-6(TDDS)	F-6(Inj.)
Surfactants	[EDMPC][Lin]	Tween-80	DGME	PBS	PBS
Co-surfactants	Span-20	Span-20	Span-20	-	-
Base-liquids	IPM	IPM	IPM	PBS	PBS
Conc. of LA	3.00	3.00	3.00	3.00	3.00
AUC_{0-t} ; ($\mu\text{g}\cdot\text{hr}/\text{mL}$)	85.58	67.67	64.01	5.22	28.21
$C_{\max}$; ($\mu\text{g}/\text{mL}$)	5.91	4.61	4.22	1.72	4.81
$C_{\max}$; time (h)	36	24	12	9	4
Constant rate, K (h^{-1})	0.01	0.06	0.07	0.18	0.75

Here, F-1: [EDMPC][Lin]/O-NDs, F-5: Tween-80/O-NDs, F-4: CPE/O-NDs, F-6 (TDDS): Peptide in PBS for TDDS, and F-6 (Inj.): Peptide in PBS for subcutaneous injection.

3.4.7 Biocompatibility evaluations: Biocompatibility evaluations of pharmaceutical formulations are required to indicate the safety profile of the final product and confirm that a formulation is unlikely to cause undesirable cellular responses.⁵² Here, we evaluated the *in vivo* and *in vitro* biocompatibility of the IL/O-NDs using IPM as a control. Furthermore, we compared the toxic effect of the IL/O-NDs with PBS, the conventional toxic IL; e-TF₂N or moderately toxic IL; e-TFSA, the pharmaceutically accepted

conventional surfactant; SDS, the skin penetration enhancer; CPE (diethylene glycol monoethyl ether; DGME), and the biocompatible conventional surfactant; Tween-80.³⁴ The results of the *in vitro* skin irritation test on human artificial Lab-Cyte EPI-MODEL indicated that the cell viability in PBS-, LBIL; [EDMPC][Lin]-, and Tween-80-based formulations were nearly 100%, but in CPE-, e-TFSA-, e-TF₂N-, and SDS-based formulations the cell viability values were 59%, 53%, 18%, and 6%, respectively, compared with IPM (**Figure 13-A and Table 2**).

Therefore, these *in vitro* experiments indicated the biocompatibility of the LBIL/O-NDs. Additionally, the *in vivo* biocompatibility was evaluated in BALB/C mice by assessing body weight changes and histopathological observation of the skin layers. The body weight was not changed significantly up to day 15 of the experimental period (**Figure 13-B and Table 11**) whereas the TF₂N-treated mice died after the first dose and the skin turned a blackish color (**Figure 12**).

Table 11: Average body weight of different formulations treated groups were observation during *in-vivo* cytotoxicity study of IL/O-NDs formulations.

Formulations	Body weights (g)							
	1d	3d	5d	7d	9d	11d	13d	15d
PBS	19.7	19.4	19.74	20.14	19.96	20.1	20.2	20.1
IPM	20.02	19.82	19.9	20.2	20.01	20	20.12	19.96
F-1	19.96	19.82	19.98	20.38	20.2	20.1	20.24	19.89
F-4	19.98	19.12	19.6	19.9	19.8	19.62	19.73	19.25
F-5	19.8	19.08	19.38	19.92	19.57	19.21	19.32	19.02
F-8	19.86	18.68	19.08	19.96	19.82	19.79	19.83	18.93
F-9	20.01	16.59	Death					
F-10	19.98	18.9	19.46	19.69	19.48	19.41	19.12	17.02

Here, F-1= [EDMPC][Lin]/O-NDs, F-4= CPE/O-NDs, F-5= T-80/O-NDs, F-8= e-TFSA/O-NDs, F-9= e-TF₂N/O-NDs, and F-10= SDS/PBS-NDs.

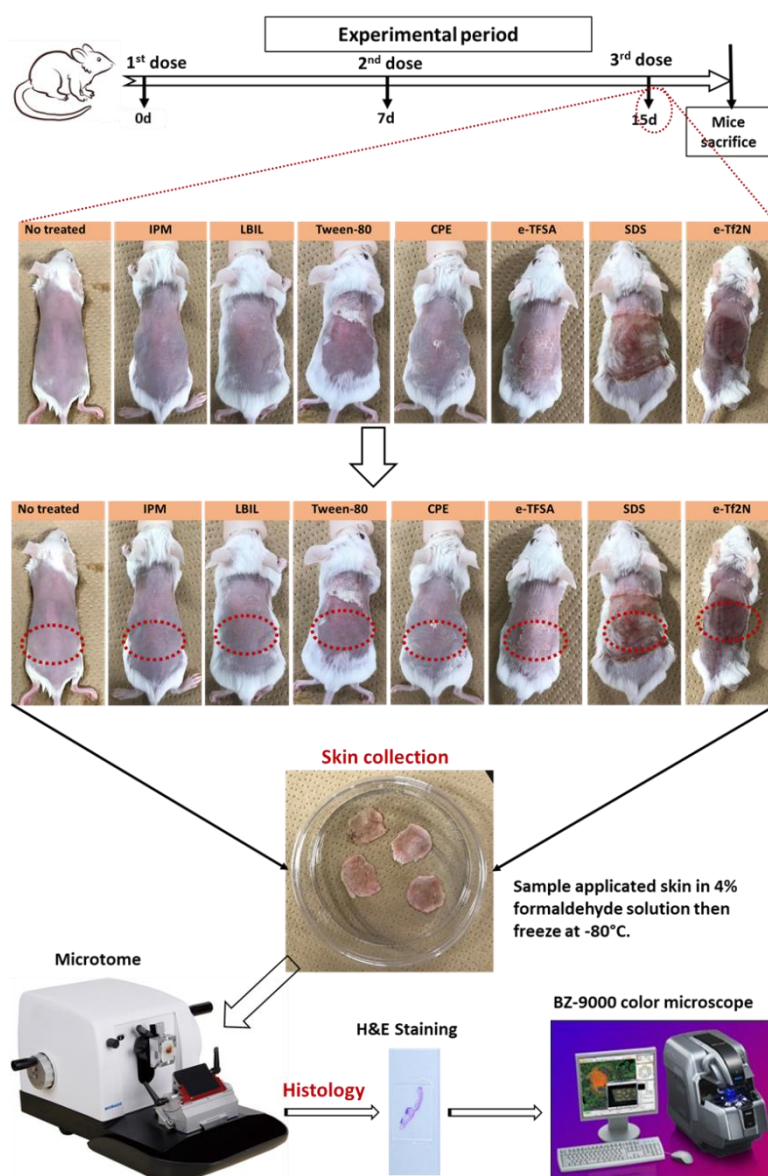


Figure 12: Schematically represented the *in-vivo* biocompatibility experimental process and histopathological observation.

The effect of the IL/O-NDs on the skin layers was investigated by the H&E staining histological process and finally was observed using a florescence microscope with 40-fold magnification. The histological images indicated that there were no defects present in the control (not treated) and IPM-, LBIL/O-NDs and Tween-80-treated groups, but slight defects were present in the CPE- and e-TFSA-treated groups, and both the epidermis and dermis layers of the skin were disrupted in the SDS- and TF₂N-treated

groups (**Figure 8C**). These *in vivo* and *in vitro* results suggested that the LBIL/O-NDs are safe for use as pharmaceutical formulations.

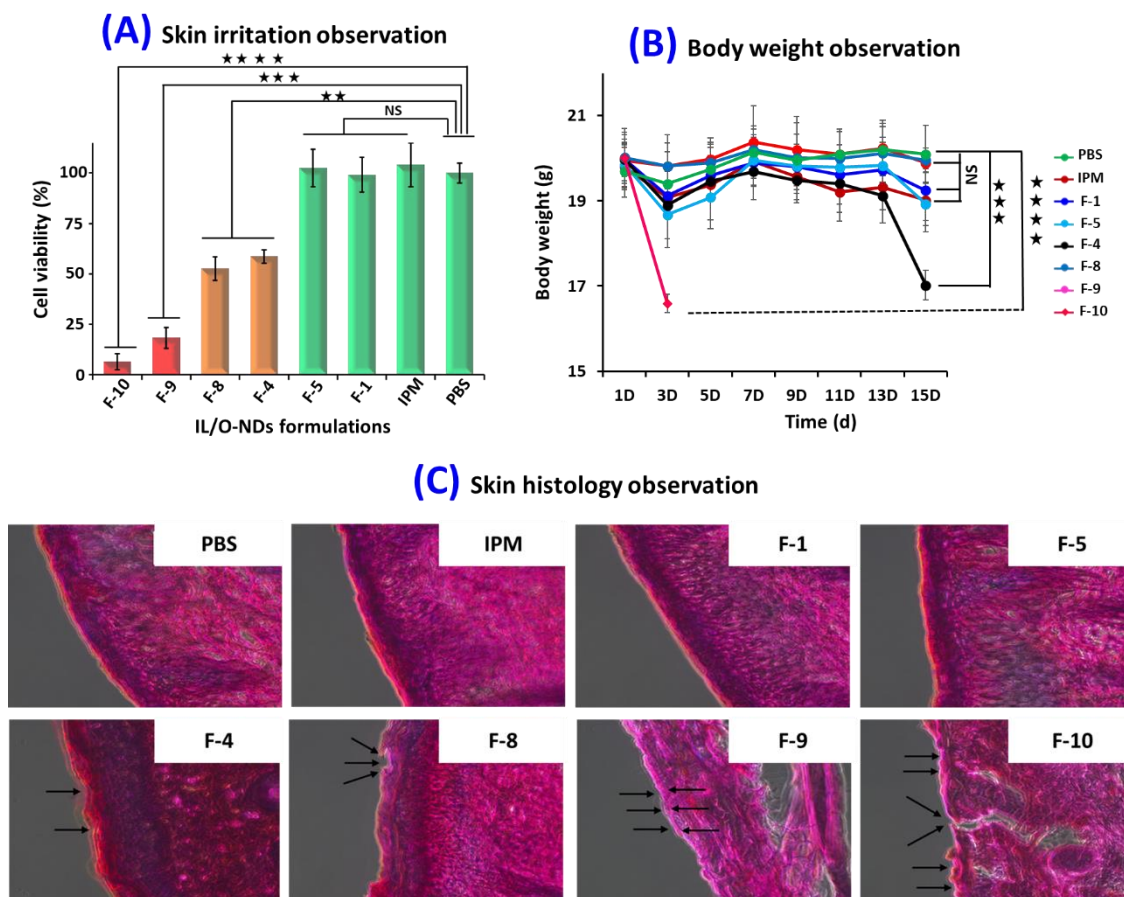


Figure 13: (A) Cell viability of IL/O-NDs carrier was observed on human LabCyte epi-model. (B) Body weight of mouse during *in-vivo* biocompatibility study. and (C) Skin histopathological experiment. The mean $\pm$ SD [$n = 3$ for (A) and $n = 5$ for (B) and (C)], values were significant for $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ using two-way ANOVA.

3.5 Conclusion

The synthesized LBILs could encapsulate a hydrophilic therapeutic peptide and dispersed the peptide in an oil medium (IPM) by forming IL/O-NDs. Among the various formulations, the HPLC and DLS data indicated that the [EDMPC][Lin] LBIL was the most preferable formulation in terms of the ability to form a thermodynamically stable formulation with the maximum drug recovery. The *in vitro* transdermal delivery of

leuprolide acetate was significantly increased with the [EDMPC][Lin]/O-NDs compared with the known chemical permeation enhancers DGME and Tween-80. The serum leuprolide acetate concentration was also significantly enhanced in the [EDMPC][Lin]/O-ND system and this system provided more suitable and sustained peptide delivery, compared with a subcutaneous injection. The mechanism of peptide delivery across the deeper part of the skin was facilitated by the LBILs through disruption of the lipids and keratin proteins of the SC layer. Among the fatty acids with different degrees of saturation, linoleic acid (C_{18:2})-based LBIL [EDMPC][Lin] resulted in the most skin penetration enhancer. Both the *in vitro* and *in vivo* results indicated the biocompatibility of the LBIL-based formulations. From all the results of the present study, we can conclude that the LBIL-based formulations, IL/O-NDs, are potential biocompatible candidates for drug delivery enhancers for future application in the pharmaceutical industry for the transdermal delivery of peptides, proteins, and hormones.

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**BIOCOMPATIBLE INONIC LIQUIDS MEDIATED NANOPARTICLE
ASSISTED TRANSDERMAL DELIVERY OF ANTIGENIC PROTEIN FOR
CANCER TREATMENT.**

Antigenic Protein

LPNPs

Skin Barrier Mechanism

APCs

Transdermal Protein Delivery

Protein delivery ($\mu\text{g}/\text{cm}^2$)

Nano particles formulations

Topical

Transdermal

PBS

CPE

LPNPs

Anti-tumor Immunity

Tumor Microenvironment

CD8+ T cells

Cancer Cells

4.1 Abstract

The use of transdermal drug delivery systems (TDDSs) is a promising strategy for preventing various diseases including cancer. However, the stratum corneum (SC) inhibits the permeation of foreign particles into the skin. To obtain an effective TDDS, we developed lipid-based ionic liquid-mediated protein nano particles (LPNPs) comprising a biocompatible ionic liquid—i.e., 1,2-dimyristoyl-sn-glycerol-3-ethylphosphatidylcholine linoleate ([EDMPC][Lin])—Tween-80, and ovalbumin (OVA) as an antigenic protein in isopropyl myristate. The LPNPs were physiochemically stable and biocompatible—both *in vitro* and *in vivo*—for the therapeutic purposes of TDDS systems. Both the *in vitro* and *in vivo* results demonstrated that the LPNPs significantly ($p < 0.0001$) enhanced the transdermal delivery of OVA and improved its pharmacokinetic parameters, compared with those of an aqueous formulation. The LPNPs increased the lipid fluidity of the SC layer and facilitated TDD via intercellular routes. Moreover, the LPNPs had effective anti-tumor activity, significantly ($p < 0.001$) inhibited tumor growth, and increased the survival rate of mice, compared with aqueous solutions and subcutaneous injection formulations. The LPNPS also increased the number of cytotoxic T-cells expressing CD8 antibody on their surfaces (CD8⁺ T-cells) in the tumor microenvironment. These findings indicate that lipid-based ionic liquids are promising biocompatible enhancers of the nano-drug delivery platform for cancer treatment.

KEYWORDS: Antigenic protein; Biocompatible ionic liquid; Transdermal drug delivery system; Biocompatibility; Cancer immunotherapy.

4.2 Introduction

Cancer is caused by uncontrolled cell proliferation that spreads rapidly throughout the body. It results in a million deaths worldwide each year, and is regarded as the most critical global health issue.¹ Many conventional approaches to cancer treatment—such as medicine, chemotherapy, and radiation therapy—have been successful, and there have been many improvements to cancer immune therapy.² The development of new immunization strategies—such as lipid-based nanomedicines or nanoparticles—is highly desirable.³ Nanoparticle drug delivery systems are thought of as magic bullets with regard to their molecular therapeutic effects on diseases.⁴ In recent years, the use of nanoparticles or nano-drug formulations has become the most promising and advanced approach in the pharmaceutical industry, particularly with regard to agents for transdermal drug delivery systems (TDDSs).⁵ Human skin contains various types of antigen-presenting cells (APCs), such as dendritic cells (DCs), T-cells, T-lymphocytes, fibroblast cells, keratinocytes, microphage cells (MCs), and Langerhans cells (LCs).⁶ The presence of these cells in the dermis and epidermis layers of the skin makes them a good target for immunization and therapeutic treatment.⁷ To evoke a skin-targeted, tumor-specific immune response, a cancer antigen must be reached in the DCs, APCs, and—in some specific cases—in the blood circulation system via the skin. This immune response results in the production of CD8⁺ T-cells, cytokines, interferon (IFN γ), and immunoglobulin-G (IgG-1, IgG-2a).^{1,8,9} The major obstacle to the delivery of drugs through the skin is the stratum corneum (SC) layer, which is the outer layer of the skin and a strong physical barrier to TDDSs.¹⁰

The SC layer is oriented towards highly hydrophobic phospholipids, fatty acids, corneocytes, and cholesterol, which hinder the permeation of hydrophilic and large-

molecular-weight drugs (> 500 Da) across the skin.¹¹ Various transdermal penetration enhancers—such as microemulsions,¹² liposomes and invasome,¹³ and hydrogel and nano-drug particles,¹⁴ are used for cancer immune treatments. Among these, the use of nano-drug delivery systems is a prominent technique for TDDS. However, stability and biocompatibility remain challenges for the new generation of nano-DDSs.^{15,16} In a previous study, we reported the synthesis of a lipid-based ionic liquid (LBIL)—i.e., 1,2-dimyristoyl-sn-glycerol-3-ethyl-phosphatidylcholine linoleate ([EDMPC][Lin])¹⁷ and its use in TDDSs for the production of a peptide drug leuprolide acetate. The LBIL-based formulation significantly enhanced peptide delivery. Encouraged by our previous findings, we hypothesized that the LBIL was a potential candidate for TDDSs of larger molecular weight drugs or proteins. Therefore, we selected the antigenic protein ovalbumin (OVA; MW 42.7 kDa) as a model drug in the present study.

In the present study, we developed LBIL-mediated protein nano particles (LPNPs) comprising an inner core containing hydrophilic OVA covered with LBIL, and a cosurfactant (Tween-80) in an outer oil phase of isopropyl myristate (IPM) as a novel pharmaceutical formulation. The oil phase loosens the rigidity of the SC barrier and the OVA-loaded LBILs enhance drug penetration through the lamellar lipidic barrier of the SC layer.¹⁸ To the best of our knowledge, this is the first report of a successful high-molecular-weight antigen delivery system comprising the LBIL-mediated nano-carrier. We used LBIL ([EDMPC][Lin])—which is biocompatible—as a skin permeation enhancer (SPE) to develop the LPNPs. The LPNPs significantly enhanced transdermal antigen delivery to the APCs and DCs compared with a conventional chemical permeation enhancer (CPE) and its aqueous formulations.¹⁹ The LPNPs were highly effective at disrupting the SC lipid barrier, and facilitated drug delivery via intercellular

routes without any toxicological effect on the skin.¹⁸ Moreover, the LPNP formulation produced a favorable immune response against melanoma and the growth and development of tumors and promoted the infiltration of CD8⁺ T-cells into the tumor microenvironment.

4.3 Methods and Materials

4.3.1 Materials: The Sigma Aldrich Chemical Co. (St. Louis, MO, USA) provided OVA from chicken egg white, polysorbate-80 (Tween-80), and linoleic acids (Lin). The Tokyo Chemical Industry Co. Ltd. (TCI, Tokyo, Japan) provided the IPM, isopropyl alcohol (IPA), 1,2- dimyristoyl-sn-glycerol-3-phosphocholine (DMPC), ethyl trifluoro methyl sulfonate (ETFMS), 1-dodecyl-3-methyl imidazolium bis(trifluoromethyl sulfonyl) amide (TF₂N), and diethylene glycol monoethyl ether (DGME)—which was used as the CPE—0.4% paraformaldehyde, and chloroform super-dehydrate. Fujifilm, Wako Pure Chemicals Industries. Ltd. (Osaka, Japan) provided hematoxylin, eosin, acetic acid, methanol, ethanol, and acetonitrile. The fluorescein isothiocyanate (FITC) and trypsin–ethylenediaminetetraacetic acid (EDTA) were from Thermo Fisher Scientific (Waltham, MA, USA). All the other chemical reagents were of analytical grade and were used as received.

4.3.2 Cells: The Japan Tissues Engineering Co. Ltd. (Aichi, Japan) provided the LabCyte EPI-Model human artificial 3D cell line and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay medium. Thermo Fisher Scientific (Waltham, Massachusetts, USA) provided research grade mouse antiserum monoclonal antibody CD8⁺ cells and mouse antiserum monoclonal antibody CD4⁺ cells, and all other related reagents, as per the protocols. Mouse DC2.4 dendritic cells and mouse EG7-OVA OVA-specific melanoma cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan).

The cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI 1640) and fetal bovine serum from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

4.3.3 Animals: Hoshino Laboratory Animals (Ibaraki, Japan) provided the mouse skin and stored it at -80°C . Kyudo Co. Ltd. (Saga, Japan) was supplied with 6-week-old female C57/BL6N mice (weight: 20 ± 2 g), which they raised in a controlled environment. The *in vivo* experiments were performed according to the guidelines of the ethics committee of Kyushu University, and the animal experiments and were carried out according to the guidelines of the Science Council of Japan.

4.3.4 Preparation of LBILs mediated protein nanoparticles (LPNPs): The LBIL [EDMPC][Lin] was synthesized and characterized according to the method described in our previous article.¹⁷ LBIL [EDMPC][Lin]-mediated OVA protein nanoparticles (LPNPs) in IPM were prepared according to the method described in a previous report with some modifications.²⁰ Briefly, a cyclohexane solution of LBIL (25 mg), an aqueous solution of FITC-OVA antigenic protein (4 mg), and Tween-80 (25 mg) were homogenized for 2 min at 26000 rpm using a mechanical homogenizer (Polytron; PT2500E). The homogenized mixture was immediately frozen in liquid nitrogen, and all excess water and cyclohexane were removed from the mixture using a freeze dryer (FD5N; YELA, Tokyo, Japan). The resulting mixture was dispersed in IPM by stirring for 12 h at 600 rpm. For comparison, we made a commercial CPE-based formulation using the same procedures described above, in which the LBIL was replaced by an equivalent amount of DGME and an aqueous formulation of FITC-OVA in phosphate-buffered saline (PBS).

4.3.5 Characterizations and physiochemical stability of LPNPs: The particle size distribution, polydispersity index (PDI), light intensity, and derived count rate (DCR) of the LPNPs were determined by dynamic light scattering (DLS) (Zetasizer nano series ZS, Malvern WR141XZ, Worcestershire, UK) using 1 cm long capping quartz cells at an angle of 173° and a temperature of 25 ± 0.1 °C. The mean values of 10 replicated measurements were taken as the true particle sizes. The morphological sizes and shapes of the LPNPs were investigated by confocal laser scanning microscopy (CLSM) (LSM700; Carl Zeiss, Germany) at 63X resolution. During the CLSM investigations, the LPNPs were diluted 40 times to determine the particle sizes in a particular orientation in the medium. Therefore, the tested samples contained a large number of particles that were very difficult to distinguish from each other.

The physical stability of the LPNPs was assessed visually, and the particle sizes were investigated by DLS for 3 months at room temperature (RT)—i.e., 25 ± 0.5 °C. The chemical stability of OVA in the LPNPs was quantified using a microplate reader (Mark, Bio-Rad Laboratories, CA, USA), wherein the absorbance was measured at a wavelength of 485–535 nm.²¹ The tested sample was centrifuged at 10000 rpm for 15 min, and transparent FITC-OVA solutions were diluted to 0.1 mg/mL in a diluent (PBS: acetonitrile: methanol = 2:1:1) to a concentration equal to that of a standard FITC-OVA solution in the diluent.

4.3.6 *In-vitro* and *in-vivo* biocompatibility of LPNPs: The LPNP carrier was investigated by a skin irritation test on the LabCyte EPI-MODEL artificial human 3D cell line using MTT assay methodology, as described in a previous report.²² The toxicity of the LPNP carrier was assessed in 6-week-old female C57/BL6N mice. Briefly, the mice were divided into five groups of five mice each, and the hair was removed from each

mouse using hair removal ointment. A homemade patch (2 cm × 2 cm) was used to apply 200 µL of the LPNP carrier to the dorsal side of each mouse. Three consecutive doses were applied to the skin for 24 h, with a 5-day interval between each application. During the experimental periods, physical abnormalities and body weight were investigated within a 1-day interval. At the end of the experimental period, the mice were sacrificed, and the treated areas of skin were collected for histopathological skin cryosection. After skin cryosection using a microtome (CM1860UV; Leica Biosystems, Germany) and hematoxylin and eosin (H&E) staining, the skin tissue was investigated using a BZ-9000 color microscope (Keyence Co. Osaka, Japan).

4.3.7 Evaluation of *in-vitro* skin permeability of LPPNs: The mouse skin was frozen at −80 °C and thawed at room temperature. It was then cut into appropriate segments (2 × 2 cm²) for use in the Franz cells. The receiver chamber was filled with 5 mL of PBS maintained at 32.5 °C. The tested samples (F-LPPNs, F-CPE, and F-PBS; 150 µL) were placed on the donor part of the hairless mouse skin area. Samples (300 µL) were collected from the receiver chamber and PBS was added at 2, 4, 6, and 8 h. The transdermal (across the skin) delivery of FITC-OVA from the receiver phases, and the topical (into the skin) delivery of concentrated FITC-OVA from the skin extraction tissue were determined by measuring fluorescence intensity at a wavelength of 485–535 nm using a multiplate microplate reader. To investigate topical delivery, the cells were unclamped and the treated skin area was washed several times with 20% ethanol, cut into 8 pieces, and extracted with a diluent (a mixture of 50 v% PBS, 25 v% methanol, and 25 v% acetonitrile) for 12 h while shaking continuously. Subsequently, FITC-OVE delivery was also investigated by CLSM. To check it, the applied skin was unclamped from the Franz cells after 6 h and cut into the desired area, then washed several times with 20% ethanol. Each washed skin

sample was submerged in 4% paraformaldehyde for 6 h and sliced into 20 μm sections using a microtome equipped with a cryostat. The skin sections were adjusted on a glass slide and the extent of OVA delivery was assessed by CLSM at 20 $\times$ time resolution. The permeation depth was calculated using ImageJ software (National Institute of Health, MD, USA).

4.3.8 Calculation of skin permeation parameters: The ability of the LPNPs to permeate mouse skin was determined using Franz cell experiments lasting for 2 h with intervals of up to 8 h. The concentration of the delivered antigenic protein was determined from the fluorescence intensity of the FITC in the receiver phases measured using a multiplate bio-reader. The skin permeation parameters were measured and calculated using lag-time methods. Least-squares methods were used to determine the permeation profiles.²³ The skin permeation partition coefficient from the donor phase to the receiver phase through the SC (K_{SC}), and the diffusion coefficient through the SC (D_{SC}) were calculated using the following flow equations: $K_{SC} = (Jl) / (D_{SC} \times C_d)$ and $D_{SC} = l_{SC}^2 / 6t_d$, respectively. Here, l_{SC} represents the thickness of the mouse skin layer (0.041 cm), (t_d) is the lag time, J is the transdermal flux, and (C_d) is the drug concentration in the tested formulations. The diffusion coefficient (D_{SC}) and the value of J are equal to the intercept of the x-axis and the approximate slope of the line, respectively.

4.3.9 Mechanisms of skin permeation routes of LPNPs: To determine the skin permeation routes of the LPNPs, the ears were harvested from the C57BL/6N mice, then 10 μL of the test sample was applied dropwise to the back (inner) side of the ear skin for 10 min. Subsequently, the ear auricles were washed with 20% ethanol until the residual solutions had been fully removed. They were then placed on a glass slide. The

experimental skin parts were treated with mounting solutions and the drug permeation routes of the skin surface were determined by CLSM.

4.3.10 Effect of LPNPs on the SC layer of the skin: The SC skin samples were prepared as previously reported with some slight modifications ²⁴. Frozen pig skin was heated at 60 °C for 2 min, then the epidermis layer was peeled from the whole skin. The epidermal parts were soaked in EDTA for 24 h at room temperature, then washed with ethanol and water. The SC sheet was again soaked in the test solutions for 20 min, then washed and dried. The effect on the SC layer was investigated by Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer; 64 scans; 1 cm⁻¹ resolution; 400–4000 cm⁻¹ wavelength).

4.3.11 Evaluation of *in-vivo* skin permeability of OVA: The test solution (50 µL) containing 200 µg of OVA was applied to the dorsal skin of the C57BL/6N mice via homemade patches (1 cm × 1 cm; three layers of gauze; Hakujuji, Japan) and tape (CATHEREETTM FS dressing tape, Nichiban, Tokyo). After 6 h, the mouse skin was collected and washed with 20% ethanol for cryosection with a microtome, as described in Section 2.6. The skin was investigated by CLSM, and the depth of FITC-OVA penetration as determined by fluorescence intensity was calculated using ImageJ software.

4.3.12 Therapeutic effect of LPNPs against melanoma: To develop tumors in the C57BL/6N mice, 100 µL of an EG7-OVA cell suspension (1.0 × 10⁶ cells/mL) was subcutaneously injected into the dorsal side of each mouse to inoculate it with tumors. On Days 5, 10, and 15 after tumor development, the mice were immunized transcutaneously with 50 µL (equivalent to 200 mg OVA) of the test samples, and subcutaneously with 100 µL of an aqueous solution containing an equivalent amount of the drug in the test samples, as described in Section 2.11. Tumor volume and body weight

were measured at 1-day intervals using slide calipers and a weighing scale. The tumor volume was calculated up to the point when it exceeded 10000 mm³ using the following equation: tumor volume [mm³] = (length [mm]) × (width [mm])² × 0.5. When the volume of the tumor exceeded 5000 mm³, the mouse was considered clinically dead.

4.3.13 Observation of Tumor Microenvironment: To investigate the tumor microenvironment—particularly the prevalence of APCs (cytokine T-cells; CD8+ T-cells) inside the tumor—we developed the tumor by inoculation with EG7-OVA cells, as described above. The immunization of the mice with the test samples is described in Section 4.3.12. After the third immunization, each mouse was euthanized for tumor collection. The tumor was bisected, and tumor tissue sections were prepared by cryosection, as described in Section 4.3.7. The desired tumor cryosections were incubated with the APC anti-CD8 antibody (BioLegend) to stain the CD8+ T-cells, following the protocol for staining nuclei with the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI), which binds strongly to adenine–thymine-rich regions in DNA. The tumor sections were investigated by microscopic fluorescence analysis (BZ-900; Keyence, Japan). The number of CD8+ T-cells in the tumor sections was determined using ImageJ software. The CD8+ T-cells in the tumor microenvironment images were optimized using ImageJ software trif-(red); 1360 × 1024.

4.3.14 Statistical Analysis: The statistical analysis was performed with Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was calculated by one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons. Significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

4.4 Results and Discussion

4.4.1 Preparation, Characterization and Stability of LPNPs: We developed a new LBIL-based formulation using [EDMPC][Lin] as the surfactant for the antigenic OVA protein. To increase the stability of the formulations, we prepared the LPNPs using a conventional biocompatible and pharmaceutically acceptable cosurfactant—i.e., Tween-80—with the hydrophobic IPM medium (**Figure 1-A** and **B**). The sizes of the nanoparticles were determined using a DLS particle size analyzer, and the size distribution was confirmed by CLSM. The FITC-OVA was not directly dispersed in the oil medium (IPM) itself, but when it was covered by LBIL, it formed a dispersion solution in the IPM (**Figure 1-C**). Only the LBILs were able to enclose the OVA particles, but the particle size was larger than 500 nm, and there was precipitation after 1 week. Tween-80 was used to increase the stability of the formulation. The Tween-80 cosurfactant helps to build stronger hydrogen bonds between the drug and the surfactant (LBIL), resulting in a reduction in particle size (**Figure 1-D**). The particles in the LBIL-based nanodispersion were 886 nm in size, but when combined with Tween-80, the particle size decreased to 279 nm. In the present study, Tween-80 acted as a cosurfactant and co-binder because Tween-80 itself is unable to form stable nanodispersions where LBILs play a key role in the formation of LPNPs. The mean particle sizes and PDIs of the nanoparticles in the LPNPs from 10 replicated measurements are summarized in **Table 1**. The morphological structure of the LPNPs was investigated by CLSM; the slight green fluorescence of the FITC-OVA drug nanoparticles revealed their free movement in the solutions, indicating that the antigenic protein had been encapsulated in the nanoparticles (**Figure 1-E**). The nanoparticle size obtained from CLSM agreed well with that derived from the DLS measurements.

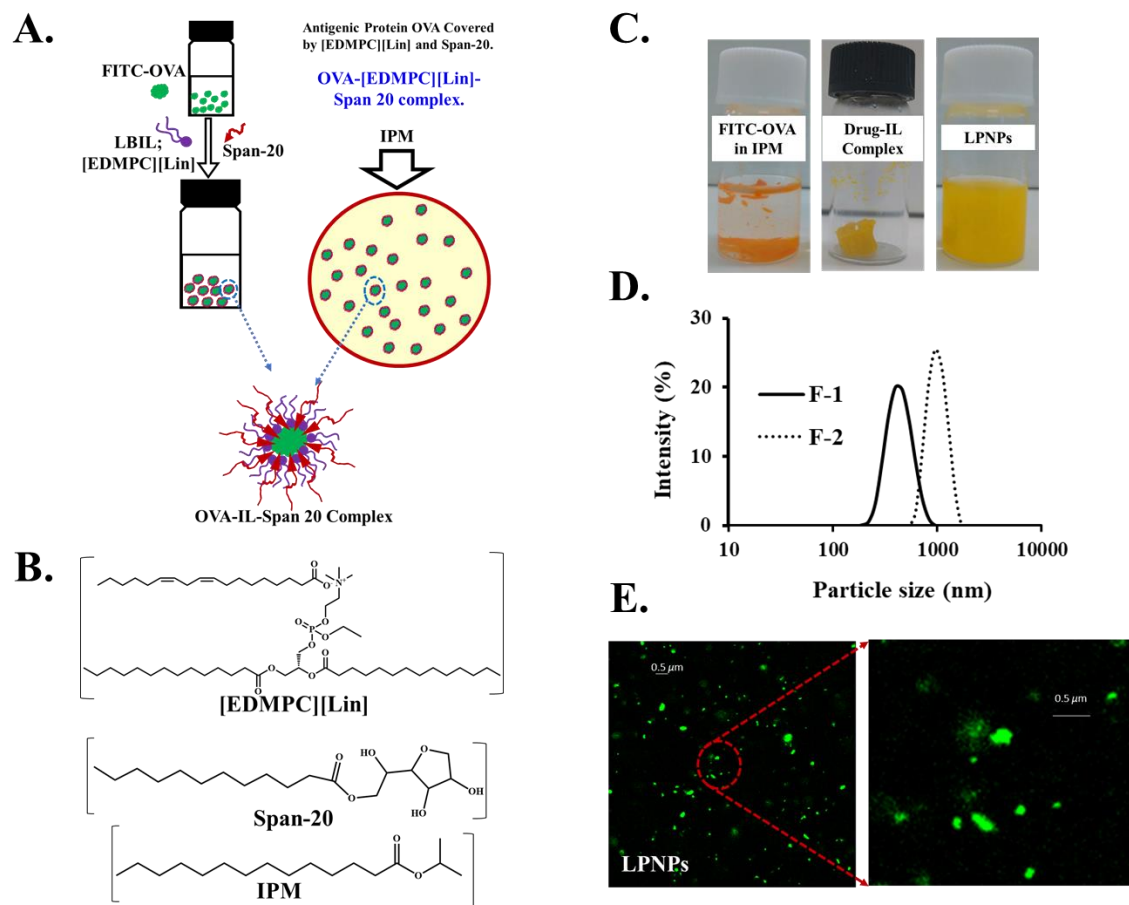


Figure 1. A) Schematic representation of the lipid-based ionic liquid-mediated protein nano particle (LPNP) formulation; B) biocompatible materials used in the preparation of the LPNPs; C) physical appearance of (i) fluorescein isothiocyanate–ovalbumin (FITC-OVA) in isopropyl myristate (IPM), (ii) FITC-OVA–ionic liquid (IL)–Tween-80 complexes, and (iii) LPNPs; D) particle size distributions of the LPNPs alone and the IL-enclosed formulation determined by dynamic light scattering (DLS); and E) confocal laser scanning microscopy (CLSM) images of the FITC-OVA-loaded LPNPs obtained using an oil-immersed lens at 63 $\times$ resolution.

Table 1. Particle size, polydispersity index (PDI), and derived count rate (DCR) of the lipid-based ionic liquid-mediated protein nano particles (LPNPs). Data represent the mean $\pm$ standard deviation of three replicated measurements (n = 3).

Formulations name	Symbol of formulations *	Surfactant (2.5 wt.%)	Cosurfactant (2.5 wt.%)	Particle size (nm)	PDI
LPNPs	F-1	[EDMPC][Lin]	Tween-80	279 $\pm$ 26	0.22 $\pm$ 0.06
ILNPs	F-2	[EDMPC][Lin]	-	886 $\pm$ 59	0.17 $\pm$ 0.09
CPENPs	F-3	DGME	Tween-80	465 $\pm$ 68	0.46 $\pm$ 0.12
PBSNPs	F-4	-	-	-	-
b-TF ₂ NNPs	F-5	b-TF ₂ NNPs	Tween-80	-	-
SDSNPs	F-6	SDS	Tween-80	-	-

DGME (CPE): Diethylene glycol monoethyl ether (used as the chemical permeation enhancer); ^aTF₂N: 1-dodecyl-3-methyl imidazolium bis (trifluoromethyl sulfonyl) amide (a conventional ionic liquid).

*We used the symbols of the formulations in all relevant figures in the manuscript.

The stability of the new pharmaceutical formulations is an important feature. Therefore, we determined the physicochemical stability of FITC-OVA in the LPNPs by DLS and quantified the concentration of FITC-OVA with a microplate reader. The physical stability of OVA at room temperature was investigated for up to 3 months with regular inspections; the particle size did not change over time (**Figure 2-A**). The concentration of OVA protein in the LPNPs did not decrease significantly from its initial concentration (Day 0) during the entire storage period, indicating the excellent chemical stability of the LPNPs (**Figure 2-B**). There was no precipitation during this experimental period.

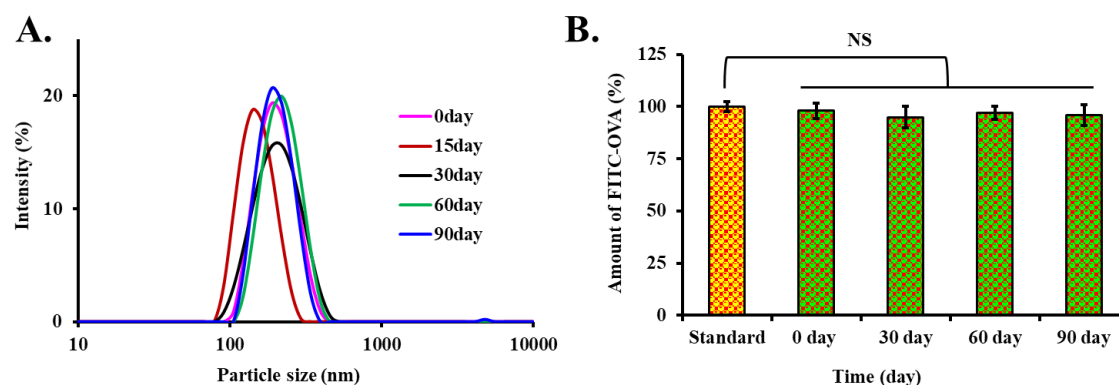


Figure 2: A.) Physical stability of OVA particle size in LPNPs formulation observed by DLS. and B.) Chemical stability of FITC-OVA was quantified the fluorescence intensity of FITC bind with OVA.

4.4.2 Biocompatibility of LPNPs: To estimate the safety profile of the LPNPs, we evaluated the skin irritation caused by them using the human artificial reconstituted epidermal model (HREM). The HREM comprises human melanocytes and keratinocytes, which are promising candidates for *in vitro* biocompatibility testing for topical and transdermal drugs ²⁵. The LPNPs contained 2.5 wt.% [EDMPC][Lin] and 2.5–5 wt% Tween-80 in IPM, which was safe and nontoxic for HREM. The cell viability was approximately 99% (considering cells in PBS to be 100% viable). To compare the toxic effects of our new formulation, we used 1-butyl-3-methylimidazolium bis(trifluoromethyl-sulfonyl) imide (a well-known toxic IL [bmim][TF2N]) and sodium dodecyl sulfate (SDS; a conventional anionic surfactant). IL [bmim][TF2N] and SDS were associated with significantly lower cell viability than IPM and the LPNPs, indicating that the LPNPs and IPM are as safe as other biocompatible solvents (**Figure 3-A**). We also carried out a histopathological skin test on the C57BL/6N mice to assess *in vivo* biocompatibility. After the application of the first dose, the mice in the SDS-treated groups died owing to severe toxicity, and after the second dose, the mice died from the

toxicological effect of the bmim-TF2N ILs. The mice in the PBS-, CPE-, IPM-, and LPNPs-treated groups remained alive and exhibited no visible adverse effects, indicating the biocompatibility of the LPNPs with IPM and PBS. When histopathological experiments (H&E staining) were performed on treated cryosections of mouse skin, no adverse effects were observed on the SC layer in the PBS-, IPM-, and LPNPs-treated groups, confirming that the LPNPs were as safe as IPM and PBS. The SC layers in the skins of the CPE-treated mice were slightly damaged, but they were severely ruptured in the SDS and bmim-TF2N-treated groups (**Figure 3-C**), indicating the toxicological effects of bmim-TF2N and SDS. The toxicological effects of the LPNPs were also reflected in the changes in body weight of the mice in the treated groups over 1-day intervals. The body weight variation rate of the LPNPs in the treated group was similar to that in the control- (PBS- and IPM-) treated groups, confirming the biocompatibility of the LPNPs *in vivo* (**Figure 3-B**).

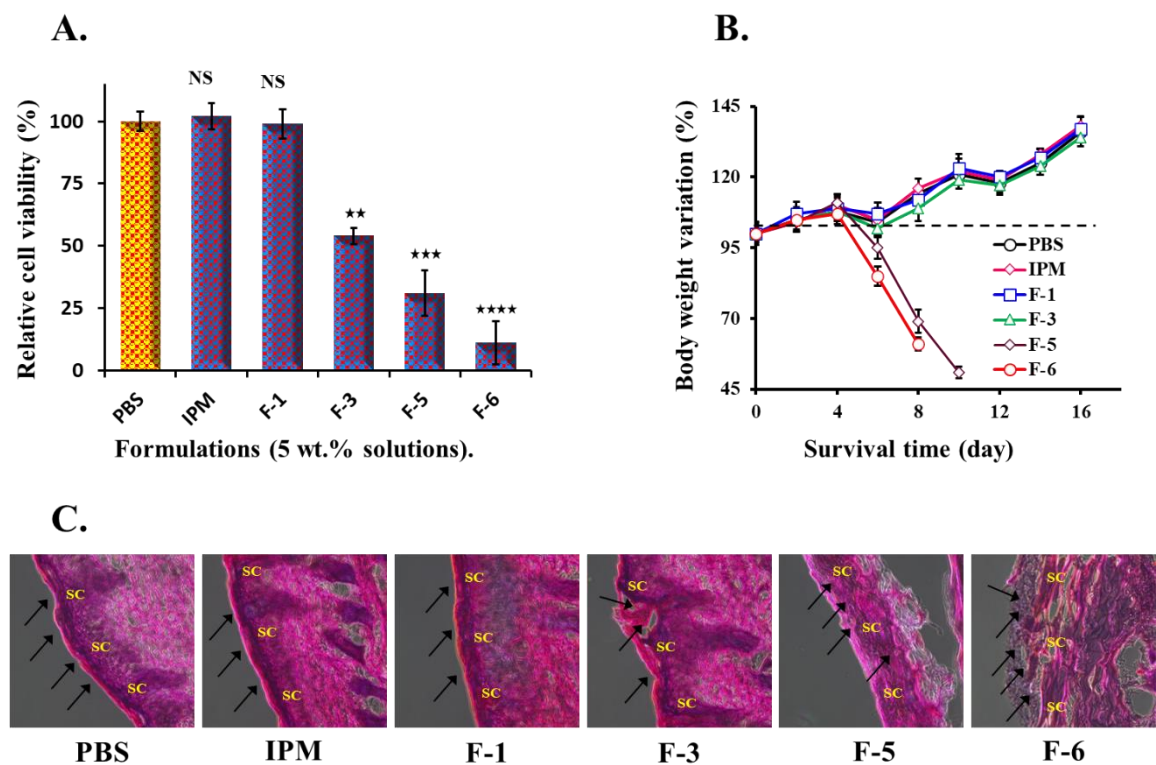


Figure 3. A) *In vitro* cell viability in the human epidermis LabCyte EPI-Model cell line, mean $\pm$ SD (n = 3); B) body weight variation (%) following transdermal application of the lipid-based ionic liquid-mediated protein nano particles (LPNPs) to the C57BL/6N mice to obtain safety and toxicity measurements, mean $\pm$ SD (n = 5); C) histopathological images of hematoxylin and eosin (H&E)-stained cryosections of mouse skin. *p < 0.1, **p < 0.01, ***p < 0.001, ****p < 0.0001, and NS: not significant.

4.4.3 In-vitro Skin Permeability Study of LPNPs: The skin permeability of FITC-OVA-loaded LPNPs was evaluated using the Franz cell diffusion system (FCDS). To achieve the best OVA delivery rate, we developed various LPNP formulations based on the ratio of the surfactant (LBIL) to the cosurfactant (Tween-80) in the IPM. We discovered that formulations in which the proportion of the surfactant was equal to that of the cosurfactant (IL = 2.5 wt.%; Tween-80 = 2.5 wt.%) resulted in optimum OVA delivery (**Figure 4**). The correct balance between the main surfactant and the cosurfactants in the LPNPs results in favorable correlation and a critical interaction between the drugs and the surfactants. This results in a stable carrier that is suitable for the delivery of OVA formulation (LPNPs). The antigenic protein sample was placed in the donor chamber of the FCDS to investigate permeation for 8 h at 2-h intervals. The LPNPs facilitated the permeation of high-molecular-weight hydrophilic antigenic proteins across the skin (transdermal delivery) within 2 h, and the delivery rate increased continuously (**Table 2**).

Table 2: FITC-OVA delivery across the mouse skin using Franz cells diffusions system (FCDS).

Formulations names	Surfactant (2.5 wt.%)	Cosurfactant (2.5 wt.%)	Base-liquid	Drug Delivery ($\mu\text{g}/\text{cm}^2$)				
				0h	2h	4h	6h	8h
LPNPs	[EDMPC][Lin]	Tween-80	IPM	0	3.09	12.15	24.21	42.56
CPE	DGME	Tween-80	IPM	0	1.19	3.28	7.02	14.67
PBS	-	-	PBS	0	0.1	0.45	1.96	2.72

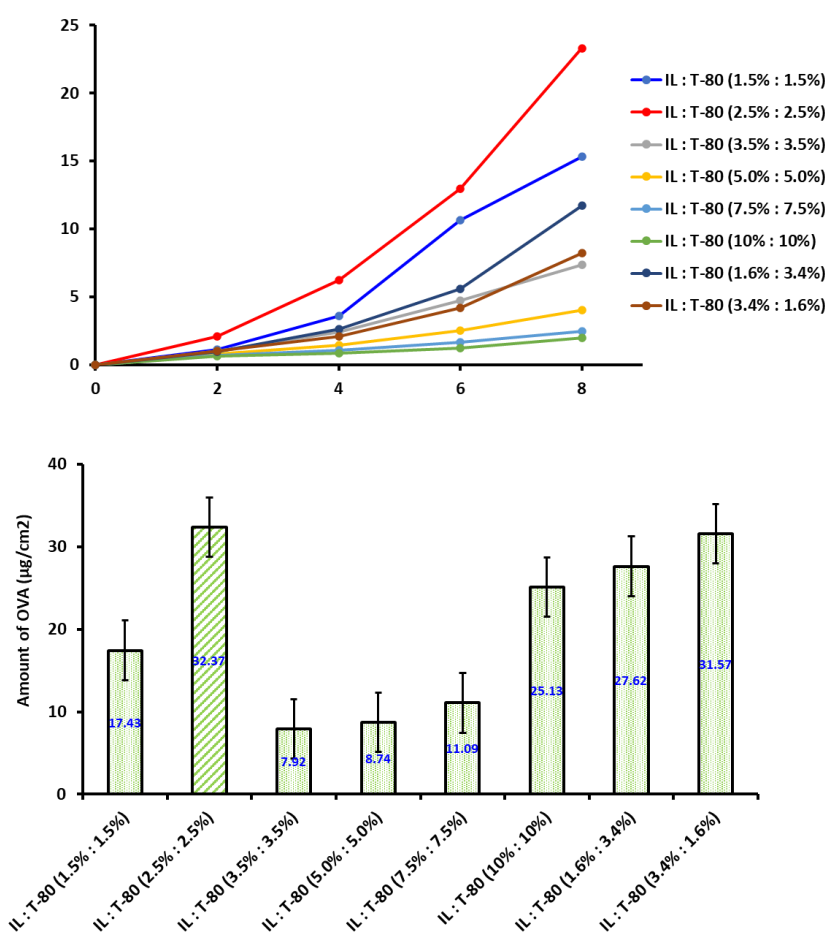


Figure 4: Transdermal OVA delivery with different LPNPs formulations.

The permeation of FITC-OVA was significantly higher with the LPNPs than with PBS and commercially available skin permeation enhancer-based formulations (**Figure 5-A**). Antigen permeation into the skin (topical drug delivery) after application for 8 h was quantified by the extraction of OVA from the skin. The amount of OVA that permeated the skin was significantly higher in the LPNPs-treated group ($49.9 \mu\text{g}/\text{cm}^2$) than in the CPE- and PBS-treated groups (29.7 and $4.15 \mu\text{g}/\text{cm}^2$, respectively) (**Figure 5-B**). However, in the absence of surfactants, the formulations (CPE and PBS) were unstable, and their drug delivery rates were significantly lower than those of the LPNPs, indicating

the importance of [EDMPC][Lin]-IL in LPNPs. The extent to which FITC-OVA permeated the samples of mouse skin was determined over 6 h. Cryosections of skin were investigated by CLSM using 20X lenses. FITC intensity was higher in the LPNPs-treated mice than in the PBS- or CPE-treated mice. The untreated mouse skin was considered a positive control (**Figure 5-C**). We investigated the mechanisms by which drugs can be delivered using LPNPs, as described later, and found that LBILs can play an important role in enhancing TDD. The results indicate that the surface molecule [EDMPC][Lin]-IL governed the permeability of FITC-OVA, and that the LPNPs had better permeation capability than the non-IL-mediated formulations.

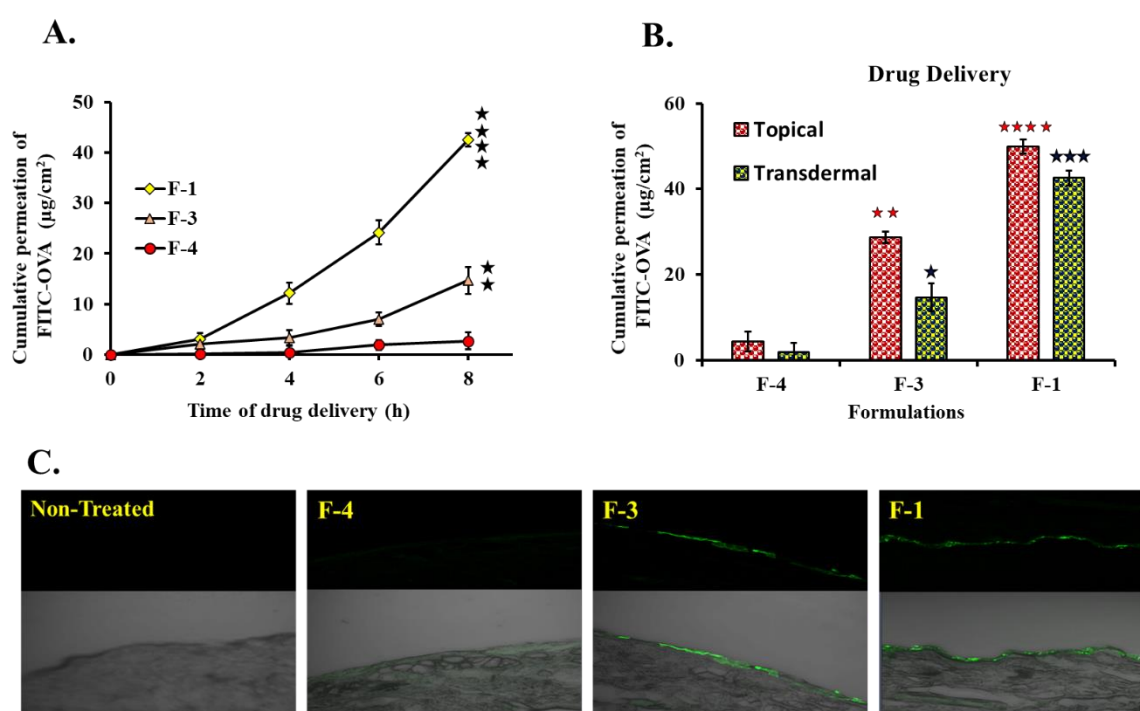


Figure 5. A) Permeation of fluorescein isothiocyanate–ovalbumin (FITC-OVA) through the skin into the receiver phase of the Franz cell diffusion system (FCDS); B) FITC-OVA delivery into the mouse skin; C) confocal laser scanning microscopy (CLSM) images of the mouse skin cryosections at 20X resolution. The green fluorescence indicates FITC-OVA penetration into the skin (the upper images are dark field, and the lower images are bright field). Mean $\pm$ SD (n = 3), *p < 0.1, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Based on the *in vitro* FITC-OVA delivery, we calculated the skin permeation parameters using lag-time methodology ²⁶. The lag-time method is commonly used to analyze drug permeability kinetics across biological barriers such as the skin, the gastrointestinal mucosa, the diaphragm, and the buccal mucosa ^{26,27}. When the LPNPs were used, all the pharmacokinetic parameters were significantly higher than when the CPEs and aqueous solutions were used. The LPNPs exhibited the highest transdermal flux (28 times higher than that of the aqueous formulation) (**Table 3**). The relatively high transdermal flux (J), diffusion coefficient (D_{SC}), and skin partition coefficient (K_{SC}) indicate the suitability of the LPNPs for transdermal drug delivery systems.

Table 3: Skin permeation parameters of the lipid-based ionic liquid-mediated protein nano particles (LPNPs) calculated using lag-time squared methodology.

Formulation	Transdermal flux; J ($\mu\text{g}/\text{cm}^2/\text{h}$)	Lag time; t_L (h)	Permeation coefficient; K_p (cm/h)	Diffusion coefficient; D ($\times 10^{-3}$ cm ² /h)	Skin-partition coefficient; K_{skin}
F-1	7.6 ± 0.6	2.5 ± 0.4	0.19 ± 0.07	1.12 ± 0.04	0.72 ± 0.16
F-3	4.9 ± 0.7	3.8 ± 0.6	0.12 ± 0.02	0.07 ± 0.01	0.67 ± 0.12
F-4	0.3 ± 0.1	4.5 ± 0.5	0.01 ± 0.01	0.06 ± 0.01	0.05 ± 0.02

4.4.4 In-vivo transdermal Delivery of LPNPs: *In vivo* FITC-OVA delivery was further investigated in C57BL/6N mice using a home-made patch, which was applied as described above. Tissue cryosection was used to investigate antigen delivery into the skin after 6 h. Skin penetration was significantly higher in the LPNPs-treated group than in the PBS- and CPE-treated groups. The lipophilic properties of LBILs in the LPNPs facilitate drug penetration into the skin. The depth of drug penetration was also investigated by CLSM and was analyzed using ImageJ software. The LPNPs had the highest drug penetration capability, indicating the surface activity of the ionic liquid drug

permeation enhancer [EDMPC][Lin] (**Figure 6**). Lipophilic particles can enter the skin through the intercellular spaces of the SC layer. The results confirmed the encapsulation of hydrophilic proteins by lipophobic ILs, resulting in a lipophilic nanodrug particle suitable for skin penetration. We also investigated the mechanism of transdermal drug delivery.

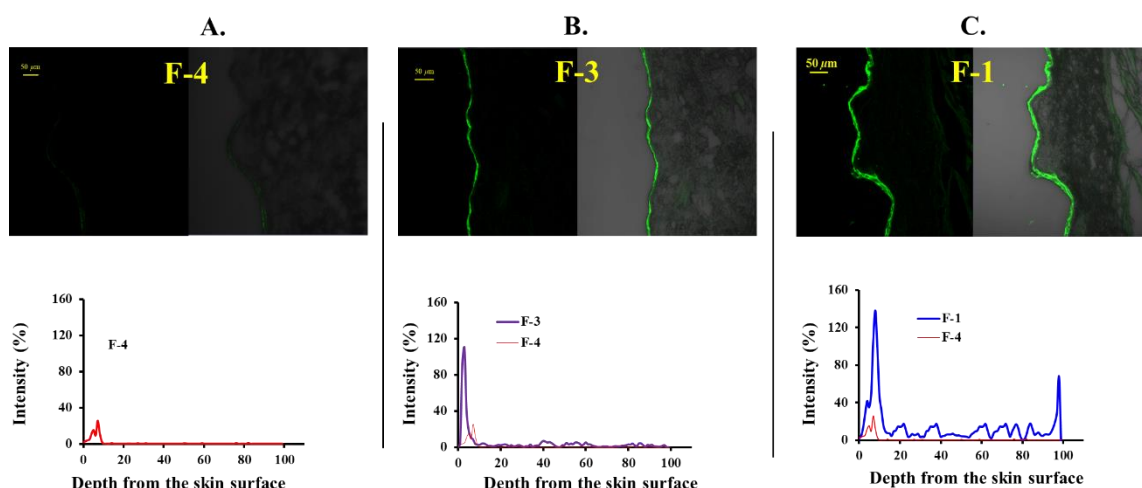


Figure 6. *In-vivo* antigenic protein penetration into mouse skin. Upper part is the CLSM image (left is dark field image and right is bright field image) and down part represented the depth of antigen delivery from the skin surface area was analyzed by ImageJ software. A.) Skin permeation of PBS aqueous solution. B.) Skin permeation of CPE-based formulation, and C.) The in-vivo skin permeation study of LPNPs.

4.4.5 Mechanisms of Drug Delivery Routes of LPNPs: Identifying the drug delivery route is critical to determining the efficacy of any pharmaceutical formulation²⁸. Many organs, tissues, cells, and circulation systems are targeted by drugs, so it is necessary that the drug molecules reach the desired binding sites¹. If a drug is to reach its intended functional site, it must first overcome the obstacle presented by the skin. There are three possible routes for drug delivery through the SC layer: intercellular, intracellular, and transcellular. To determine which route was involved in our current study, we applied the mouse ear skin test to all the samples and investigated the delivery routes by CLSM (**Figure 7-A**). The fluorescent green FITC-OVA accumulated in and passed through

intercellular routes, indicating that permeation predominantly occurred that way. The intercellular space in skin is lipophilic in nature, so it is logical that lipid-mediated particles should pass through the skin via that route. However, that pathway was predominantly facilitated by the lipid-based IL [EDMPC][Lin] of the LPNPs owing to the lipophilic nature and surface activity of IL. The mechanism was further evaluated by FTIR analysis of the intercellular lipids and fatty acids of the SC—such as cholesterol and ceramides—which predominantly hinder drug diffusion.³³ We evaluated the effect of the LPNPs on the (symmetrical and asymmetrical) alkyl chains of the structural lipids of the SC. We used untreated mouse skin as a control. The peaks at 2850 cm^{-1} and 2920 cm^{-1} can be attributed to the symmetrical ($\nu\text{CH}_2_{\text{sym}}$) (**Figure 7-B**) and asymmetrical ($\nu\text{CH}_2_{\text{asym}}$) (**Figure 7-C**) CH_2 alkyl chain vibration modes of the lipids, respectively. The shifts in the positions of the peaks can be attributed to gauche/trans conformational transitions. The kinked gauche conformers indicate that the decrease in SC lipid arrangements is a reason for the decline in barrier function. Both symmetric and asymmetric shifts were significantly higher in the LPNPs-treated group than in the PBS-, IPM-, and CPE-treated groups. This indicates that both the symmetric and asymmetric alkyl chains of the SC lipid structure were dissociated by the LPNPs, which facilitated the permeation of the OVA molecules through the skin by reducing the SC barrier function.

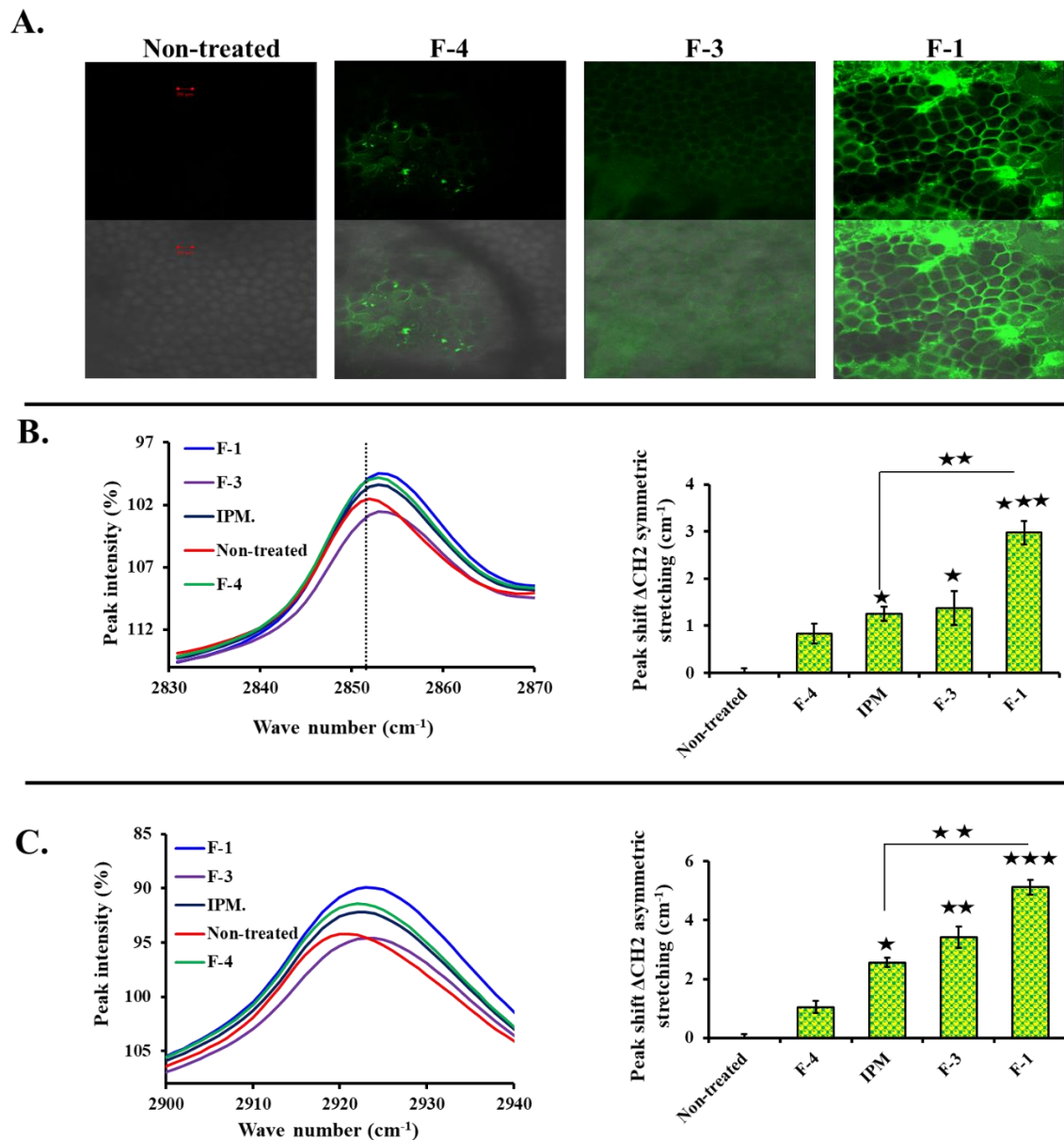


Figure 7: Evaluation of the drug permeation mechanism. A) Z-axis projection confocal laser scanning microscopy (CLSM) images of the mouse ear skin (20 μm scale bar; the upper parts are the dark field images and the lower parts are the bright field images). Effects of the test formulation on the stratum corneum (SC) layer, and peak shifts attributable to B) symmetric and C) asymmetric vibrations. * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$.

4.4.6 Therapeutic effect of LPNPs on Melanoma: To evaluate the therapeutic antitumor effect of the LPNPs, we inoculated OVA-EG7 cells (1×10^6) into each mouse and after 7–10 days, tumor budding occurred. The volume of the tumor that developed on the dorsal side of each mouse was measured at regular intervals using slide calipers. When the tumor volume reached 5000 mm^3 , the mouse was deemed clinically dead. Each mouse was immunized for 24 h with homemade patches containing $200 \text{ }\mu\text{g}$ of OVA 7 and 14 days after finding the tumor. To determine the antitumor activity of the LPNPs, all the test samples were applied to the skin of the mouse and the tumor volume was measured. Body weight was calculated during the toxicological study, and the survival rate of the immunized mice was also investigated during this study period. The order of the tumor growth inhibition efficacy of the treatment formulations was as follows: LPNPs > PBS-Inj. > CPE > PBS-TDDS > untreated group (the control group) (**Figure 8-A**). It was clear that LPNPs had superior tumor growth inhibition activity and anti-tumor activity compared with the OAV-in PBS subcutaneous injections, although each formulation contained the same amount of antigenic protein. No significant abnormality of body weight compared with the control group was observed during the present study (**Figure 8-B**). The survival rate of the mice in the LPNPs-treated group was also compared with the survival rates in all the other treatment groups (**Figure 8-C**). The survival rate was higher in the LPNPs-treated group than in the subcutaneous injection group. Although it did not differ significantly from the injection group, the LPNPs-treated group demonstrated greater antitumor efficacy. Further anticancer immunochemistry investigations of LPNPs—such as cytometric flow analyses of antigen uptake by skin dendritic cells, tumor antigen pharmacokinetic studies, tumor microenvironment

observations, and antigen-specific immune response studies—are ongoing and will be reported on in our next study.

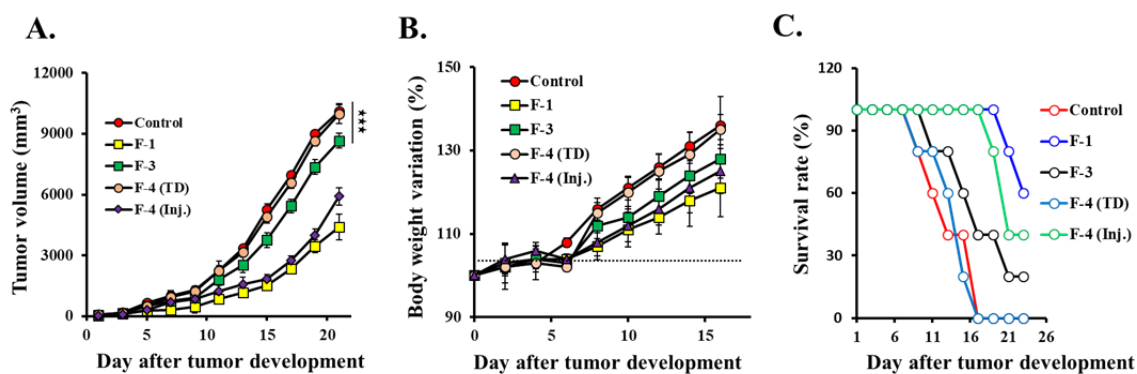


Figure 8: Therapeutic antitumor effect of lipid-based ionic liquid-mediated protein nano particles (LPNPs) against ovalbumin (OVA)-expressing cells OVA-EG7. A) Tumor growth inhibition observation; B) body weight variation (%) in immunized mice during antitumor effect study; and C) survival rate of tumor-growing mice. Mean $\pm$ SD ($n = 5$) and *** $p < 0.001$.

4.4.7 CD8⁺ T-cell infiltration of the tumor microenvironment: To confirm the anti-tumor immune response of the LPNPs, we investigated CD8⁺ T-cell infiltration of the tumor microenvironment (TME). CD8⁺ T-cells play a central role in mediating anti-tumor immunity, and can kill tumor cells.²⁹ Captured DC antigens activate CD8⁺ T-cell-specific antigens in the lymphoid organs, activating CD8⁺ T-cells, which can destroy tumor cells by secreting perforin and granzyme.²¹ The number of CD8⁺ T-cells in the TME provides conclusive evidence of the activation of tumor-specific adaptive immunity³⁰. The mice in the LPNPs-treated groups produced the highest number of CD8⁺ T-cells according to DAPI staining (**Figure 9-A**). The number of CD8⁺ T-cells in the LPNPs-treated group was significantly higher than the number of CD8⁺ T-cells in the aqueous formulation or in the conventional CPE-based formulation. The infiltration rate of the CD8⁺ T-cells in the TME was slightly higher than that in the subcutaneous injection

treatment groups, but the difference was not significant (**Figure 9-B**). The dark field images representing only the CD8⁺ T-cells were optimized using ImageJ software (**Figure 9-A down**), and the number of CD8⁺ T-cells that infiltrated the TME was also calculated using ImageJ software. These results confirmed the superior immune response against cancer associated with the LPNPs.

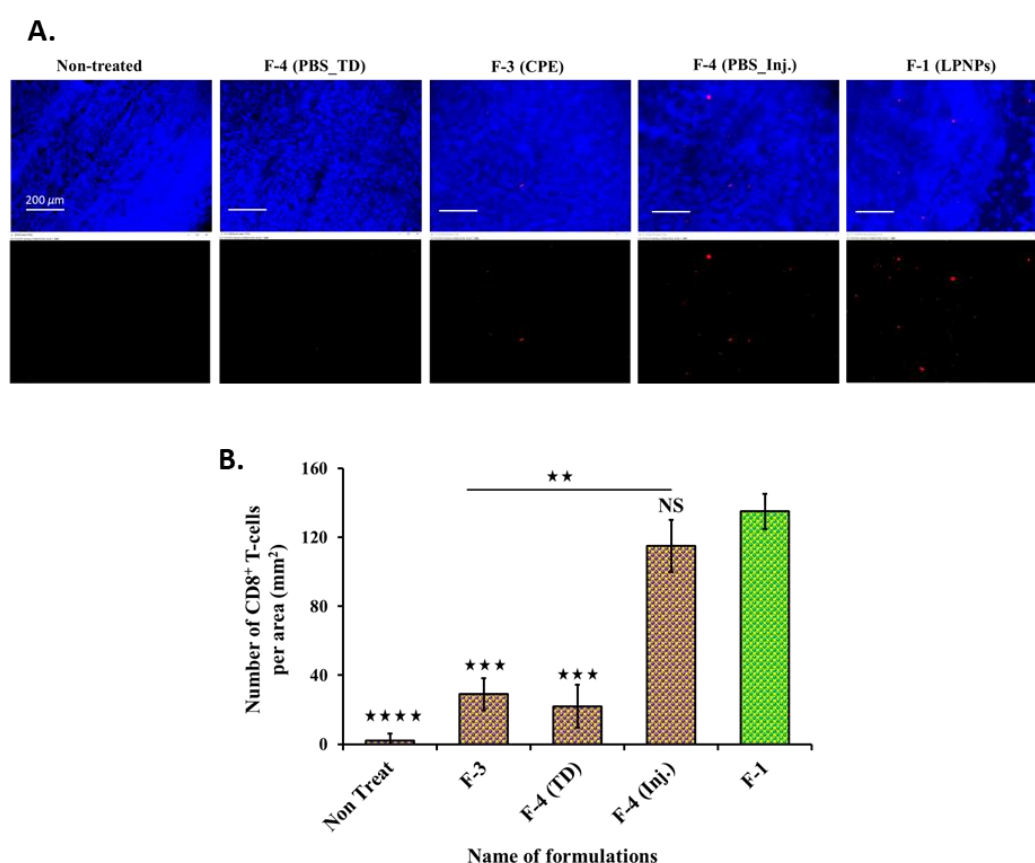


Figure 9: CD8⁺ T cell infiltration of the tumor microenvironment. (A) Fluorescence images of the tumor microenvironment. Antigen-presenting cell (APC)-anti CD8 antibody (red) reveals the CD8⁺ T-cells, and 4',6-diamidino-2-phenylindole (DAPI) (blue) reveals the cell nuclei. The upper images were obtained using a Keyence microscope and the lower images were optimized with ImageJ software, wherein only the CD8⁺ T-cells were visualized in the dark field. (B) The number of CD8⁺ T-cells per area in the tumor cross-sections harvested from the mice were calculated using ImageJ software. Mean $\pm$ SD (n = 5), **p < 0.01, ***p < 0.001, ****p < 0.0001, and NS: not significant.

4.5 Conclusions

Lipid-based ionic liquid-mediated protein nano particles (LPNPs) were successfully developed for the transdermal delivery of an antigenic protein (OVA). The LPNPs were physically and chemically stable at room temperature and were suitable for the TDD of an antigenic protein for cancer treatment. The LPNPs were biocompatible, and therefore suitable for transdermal or topical drug delivery systems. They included a lipid-based IL as the key surfactant to increase drug stability and enhance the transdermal drug permeability through the intercellular routes of the SC layer. The LPNPs interacted with the lipids of the SC layer to dissociate symmetric and asymmetric alkyl hydrogen bonds, facilitating TDD. An anti-tumor study indicated that the LPNPs demonstrated a strong therapeutic anticancer effect and significantly inhibited tumor growth when compared with a commercial CPE-based formulation. Based on these findings, we believe that the LPNPs are promising candidates for the development of a nano-drug delivery system for transdermal anticancer treatment

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CHAPTER 5

SUMMARY AND FUTURE WORK

5.1 Summary

In the past several years, ionic liquids (ILs) have been at the cutting edge of the most promising research and technology in the scientific field. The applications of ILs have been discovered to not only cover traditional areas of knowledge, but also to be an important candidate for solving critical problems such as clean and efficient energy. In particular, ILs have been found to play a special role in the pharmaceutical industry as clean and multifunctional solvents for a variety of applications. This neoteric class of solvents, ILs, generally presents interesting properties, namely, negligible vapor pressure at relatively ambient conditions, high thermal, chemical, and electrochemical stability, and broadly tunable properties regarding polarity, hydrophobicity, and solvent miscibility. These properties result from a matchless combination of molecular characteristics of their constitutive ions. Moreover, many types of ILs can be regarded as nano-segregated fluids with polar networks permeated by apolar domains, which enables the understanding of their peculiar solvent behavior at a molecular level and the numerous applications to solve classical problems addressing today's societal challenges. One of the major challenges of pharmaceutical research is the increasing number of active pharmaceutical ingredients (APIs) possessing limited aqueous solubility in biopharmaceutical classification system class II drugs. The challenging aspects of new formulations of such drug molecules are associated with their slow dissolution in biological fluids, and thus insufficient and inconsistent systemic exposure and consequent suboptimal clinical efficacy. ILs have been considered to be solvents and/or materials in the field of pharmaceuticals with the possibility of improved performance, such as improved solubility, stability, and drug

delivery. In fact, due to their tailor-made properties, ILs can dissolve complex molecules, including biologically active compounds, and it is highly desirable for ILs to be potential solvents for overcoming the problems concomitant with the pharmaceutical industries.

In **chapter 2**, I synthesized a series of lipid-based biocompatible ILs (LBILs), which contained a long chain phosphatidyl choline derivative as a cation (1,2-dimyristoyl-sn-glycero-3-ethyl-phosphatidylcholine; EDMPC) and a series of long chain fatty acids (linoleic acids; C_{18:2}, oleic acids; C_{18:1}, and stearic acids; C_{18:0}) as its structural counter anion. Physiochemical properties of synthesized ILs were characterized by thin layer chromatography (TLC), proton nuclear magnetic resonance (¹H NMR), Fourier transform infrared (FTIR), elementary analysis (EA), Karl-fisher moisture analyzer, TOA pH meter, and mass spectrometry (MS). Thermal properties were confirmed by dynamic scanning calorimetry (DSC) and temperature-dependent optical polar microscopy (OPM). Solubility and particle size in different polar and nonpolar solvents was investigated by UV-visible spectrophotometric analysis and dynamic light scattering (DLS), respectively. Finally, the biocompatibility of LBILs was confirmed in the human artificial LabCyte EPI-Model cell line by the MTT assay methodology.

In **chapter 3**, I developed the LBILs mediated nanodispersion (ILs in oil nanodispersion; IL/O-NDs) formulations containing leuprolide acetate peptide as a model peptide drug and Span-20 at a specific ratio as a cosurfactant to increase the stability of drug-ILs formulations. The physiochemical properties of IL/O-NDs were characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM) confocal laser scanning microscopy (CLSM), and the high-pressure liquid chromatography (HPLC) system. We observed the *in-vitro* transdermal drug delivery using Franz-cell and

quantified by HPLA. Moreover, we investigated the pharmacokinetic effect on BALB/C mice blood *in-vivo* using ELISA enzyme linked immunosorbent assay (ELISA) methodology. We also observed the mechanisms of transdermal drug delivery into the SC layer by FTIR analysis. Finally, *in-vivo* and *in-vitro* biocompatibility of IL/O-NDs were observed on LabCyte EPI-Model and BALB/C mice skin histopathology, respectively. LBILs act as a promising biocompatible carrier to increase the stability of nano-drug formulation and enhance the transdermal delivery of a peptide drug, leuprolide acetate.

In **chapter 4**, I developed a LBIL; [EDMPC][Lin]mediated protein antigenic drug in oil phase nano particles (LPNPs) formulations. LPNPs contain a bulky antigenic protein, ova albumin from chicken egg white (OVA) with a molecular weight of 42.7 kDa as a model anticancer drug and Tween-80 is used as a co-surfactant to increase the stability of LPNPs. The formulations were characterized by DLS and CLSM, and *in-vivo* and *in-vitro* biocompatibility was confirmed by the human artificial 3D reconstructed Lab-Cyte Epi-Model cell and the mice's skin H & S staining histopathological observation. In vitro skin permeation of protein via the LPNPs system was investigated by the Franz cell system. CLSM and FTIR analysis were performed to investigate the mechanism of intercellular routes of drug permeation via the SC layer of the skin. The effect of LPNPs against tumor growth and development was confirmed on C57/BL6N by inoculation of tumor OVA responsible cancerous cells, EG7OVA for the tumor development. The anti-cancerous antigen uptake of skin dendritic cells (DCs) and other antigen presenting cells (APCs) by flow cytometric and CLSM analysis also revealed the cancer immune response of LPNPs. Finally, we observed CD8⁺ T-cell inflammation in the tumor microenvironment. We

found that LPNPs have an excellent anti-cancerous immune therapeutic effect compared with conventional chemical permeation enhancers and aqueous injection formulations. The effective application of LBIL-mediated LPNPs has indicated that LBIL will be a potential candidate for the platform of a nano-drug delivery system for cancer treatment.

5.2 Future work

The development of pharmaceutical applications using IL-based methodologies requires a deep understanding of ILs both in terms of their macroscopic properties and at the molecular level, because structural aspects have been shown to play a crucial and unexpected role in a large number of situations. Even though ILs can no longer be considered a new field, the large diversity of combinations of cations and anions producing novel ILs with new specific properties is astonishing. The use of ILs should be promoted since it may be useful for many medicines that have been placed on hold owing to their low water solubility or polymorphic conversion. This may give these products a new market value, resulting in a new revenue stream for the pharmaceutical sector. Further attention should be focused on the large-scale production of biocompatible and biodegradable ILs. Research into these issues is underway, and we confidently believe that new types of biocompatible and biodegradable ILs will be available to promote the IL-based drug delivery technology.

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