FORMULATION AND IN VITRO EVALUATION OF TAMOXIFEN ETHOSOMAL GELS FOR TRANSDERMAL DELIVERY

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FORMULATION AND IN VITRO EVALUATION OF TAMOXIFEN ETHOSOMAL GELS FOR TRANSDERMAL DELIVERY

by

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LIST OF ABBREVIATIONS

%	Percent
ANOVA	Analysis of Variance
Breast cancer	BC
°C	Celsius
µl	Microliter
cm	Centimetre
cm^2	Squared centimetre
USFDA	United States Food and Drug Administration
T20(12)	Ethosomal system with span 80®; Transethosome
SP20(7)	Ethosomal system with span 20®; Transethosome
SP80(7)	Ethosomal system with tween 20®; Transethosome
NaTC(3)	Ethosomal system with sodium taurocholate; Bilosome
NaCH(7)	Ethosomal system with sodium cholate; Bilosome
Lim(13)	Ethosomal system with limonene; Invasome
Iso(12)	Ethosomal system with isophytol; Invasome
HLB	Hydrophilic Lipophilic Balance
HPLC	High-Performance Liquid Chromatography
HPMC	Hydroxypropyl Methylcellulose
hr	Hour
ISO	Isophytol
LOD	Limit of Detection
LOQ	Limit of Quantification
LIM	Limonene
MCF-7	Michigan Cancer Foundation-7
mg	Milligram

mg/ml	Milligram per millilitre
min	Minute
ml	Millilitre
ml/min	Millilitre per minute
mM	Millimole
mV	Millivolt
N	Theoretical plate number
NE gel	Non-ethosomal gel
nm	Nanometer
Đ	Dispersity
PTFE	Polytetrafluoroethylene
RH	Relative Humidity
rpm	Revolutions per minute
RSD	Relative Standard Deviation
SD	Standard deviation
Span 20	Span 20®
Span 80	Span 80®
Sec	Second
NaTC	Sodium taurocholate
NaCH	Sodium cholate
TXN	Tamoxifen
TEM	Transmission Electron Microscopy
T20	Tween 20®
UK	United Kingdom
USA	United States of America
USP	United States Pharmacopoeia
UV	Ultraviolet

WHO	World Health Organisation
w/v	Weight per volume
w/w	Weight per weight
η	Viscosity
ZP	Zeta potential

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FORMULASI DAN PENILAIAN IN VITRO TAMOXIFEN GEL ETHOSOM UNTUK PENYAMPAIAN TRANSDERMAL

ABSTRAK

Kanser payudara menimbulkan kebimbangan kesihatan global yang ketara, menyumbang kepada 2.3 juta kes dan 685,000 kematian pada 2020. Antara kes ini, dua pertiga melibatkan tumor positif reseptor hormon dicirikan oleh reseptor estrogen yang mendorong pertumbuhan kanser. Tamoxifen merupakan terapi konvensional dan langkah pencegahan untuk wanita yang berisiko atau sudah mengalami kanser payudara positif reseptor hormon. Akan tetapi, tamoxifen oral menghadapi halangan seperti penyerapan terhad akibat hidrofobisiti dan kesan laluan pertama hati, bersama dengan kesan sampingan yang teruk termasuk penyakit hati berlemak, darah beku dan peningkatan risiko kanser endometrium. Untuk mengurangkan kesan buruk ini, kajian ini menganalisis penghantaran transdermal tamoxifen setempat menggunakan sistem etosomal sebagai laluan alternatif kepada pengambilan oral. Kerja penyelidikan ini melibatkan kajian tiga kelas ethosom yang berbeza: transethosom yang menggunakan Tween 20®, Span 20®, dan Span 80®, bilosom yang menggunakan natrium taurocholate dan natrium kolat, dan invasom yang menggabungkan limonene dan isophytol. Kajian ini merangkumi tujuh reka bentuk faktorial 2^4 yang berasingan untuk menilai kesan lipid, etanol, kepekatan penambah penembusan dan masa penyemperitan kepada parameter utama seperti saiz zarah (PS), penyebaran ($Đ$), dan potensi zeta (ZP). Hanya formulasi yang memenuhi kriteria tertentu disambung ke kajian seterusnya iaitu keberkesanan perangkap. Antara kepekatan tamoxifen (0.6, 1, dan 1.4 mg/ml) yang dikaji, 1 mg/g telah dipilih. Kepekatan ini dikenal pasti sesuai untuk menghasilkan ciri-ciri formulasi yang padan untuk penghantaran ubat

transdermal dan dicalonkan untuk kajian yang selanjutnya. Sistem etosomal yang optimum telah dibangunkan menjadi bentuk gel dan bereksperimen dengan pelbagai asas polimer, termasuk Carbopol 940®, Poloxamer 407®, dan HPMC. Antaranya, hanya HPMC 6% w/w berjaya menghasilkan gel dalam julat PS, *Đ* dan ZP yang dikehendaki dan mengekalkan tahap pH mesra kulit. Imej TEM telah mengesahkan integriti struktur vesikel etosomal dalam matriks gel. Penilaian reologi kelikatan dan kebolehtebaran memenuhi syarat gel yang berjaya bagi sistem etosomal. Kajian resapan kulit Tamoxifen *ex vivo* menunjukkan bahawa gel yang diformulasi dengan Span 20® (transethosomal) mempamerkan prestasi yang unggul, diikuti oleh gel berasaskan limonene (invasomal) dan gel berasaskan Tween 20® (transethosomal). Gel bilosomal secara perbandingan adalah kurang berkesan dalam menggalakkan penghantaran transdermal tamoxifen. Pada peringkat selular, ujian MTT pada garisan sel MCF-7 mendedahkan bahawa gel berasaskan Span 20® mempunyai sitotoksisiti dan nilai IC50 yang tertinggi, diikuti oleh gel berasaskan limonene (invasomal) dan gel berasaskan Tween 20® (transethosomal). Mengenai aktiviti antioksidan, hanya gel invasomal yang mengandungi bahan eksipien terpene seperti limonene dan isophytol telah menunjukkan potensi antioksidan. Akhir sekali, kajian kestabilan menunjukkan bahawa keadaan penyimpanan secara penyejukan adalah optimum untuk mengekalkan kestabilan fizikal gel, terutamanya dari segi PS, *Đ*, dan kandungan ubat yang sejajar dengan keperluan untuk penghantaran ubat transdermal. Penyelidikan ini menggariskan potensi gel transethosomal dan invasomal, terutamanya dengan Span 20® dan limonene dalam meningkatkan kebolehtelapan ubat dan sitotoksisiti terhadap sel-sel kanser. Kajian ini telah menandakan satu langkah besar ke arah pengurusan kanser payudara yang lebih selamat dan berkesan.

FORMULATION AND IN VITRO EVALUATION OF TAMOXIFEN ETHOSOMAL GELS FOR TRANSDERMAL DELIVERY

ABSTRACT

Breast cancer (BC) poses a significant global health concern, accounting for 2.3 million cases and 685,000 deaths in 2020. Among these cases, two-thirds involve hormone receptor-positive tumours, characterised by estrogen receptors that fuel cancer growth. Tamoxifen stands as the conventional therapy and preventive measure for women at risk or already afflicted by hormone receptor-positive BC. However, oral tamoxifen encounters obstacles such as limited absorption due to hydrophobicity and liver first-pass effect, along with severe side effects including fatty liver disease, blood clots, and a heightened risk of endometrial cancer. To mitigate these adverse effects, this work investigated the localised tamoxifen transdermal delivery utilising the ethosomal system as an alternative administration route to oral intake. The current research work has involved studying three distinct classes of ethosomes: transethosomes employing Tween 20®, Span 20®, and Span 80®, bilosomes using sodium taurocholate and sodium cholate, and invasomes incorporating limonene and isophytol. The study encompassed seven separate 2^4 factorial designs to assess the impact of lipid, ethanol, penetration enhancer concentrations, and extrusion times on key parameters like particle size (PS), dispersity (*Đ*), and zeta potential (ZP). Only formulations meeting specific criteria proceeded to entrapment efficacy studies. Among the investigated incorporated concentrations of tamoxifen (0.6, 1, and 1.4 mg/ml), 1 mg/g was selected. The concentration was identified as suitable for producing ideal formulation characteristics for transdermal drug delivery and nominated for further investigation. The optimised ethosomal systems developed into

a gel dosage form by experimenting with various polymer bases, including Carbopol 940®, Poloxamer 407®, and HPMC. Among these, only HPMC 6% w/w yielded gels within the desired PS, *Đ*, and ZP ranges, maintaining skin-friendly pH levels. TEM images confirmed the structural integrity of the ethosomal vesicles within the gel matrix. Rheological assessments of viscosity and spreadability met the requisites for successful gels of the ethosomal systems. Tamoxifen *ex vivo* skin permeation studies indicated that the gel formulated with Span 20® (transethosomal) exhibited superior performance, followed by the limonene-based gel (invasomal) and the Tween 20®-based gel (transethosomal). Bilosomal gels were comparatively less effective in promoting tamoxifen transdermal delivery. At the cellular level, MTT assay on MCF-7 cell lines revealed that the Span 20®-based gel had the highest cytotoxicity and IC50 value, followed by the limonene-based gel (invasomal) and the Tween 20®-based gel (transethosomal). Regarding antioxidant activities, only invasomal gels containing terpene excipients like limonene and isophytol exhibited antioxidant potential. Lastly, stability studies indicated that refrigeration storage conditions were optimal for maintaining the physical stability of ageing gels, particularly in terms of PS, *Đ*, and drug content aligning with the requirements for transdermal drug delivery. The research underscores the potential of transethosomal and invasomal gels, particularly with Span 20® and limonene, in improving drug permeability and cytotoxicity against cancer cells, heralding a significant stride towards safer and more effective breast cancer management.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Breast Cancer

Breast cancer (BC) is currently the most commonly diagnosed cancer worldwide, and its burden has been rising over the past decades. BC affects women in every country around the world at any age after puberty but at an increasing rate in later life (Figure 1.1). Women lose more disability-adjusted life years to BC than any other type of cancer worldwide. According to the World Health Organization (WHO), as of the end of 2020, 7.8 million women had been diagnosed with breast cancer in the previous five years, with 2.3 million diagnosed cases and 685000 deaths in 2020 alone (World Health Organization, 2020). According to the Malaysian Study on Cancer Survival (MySCan) 2018, BC in Malaysia has the lowest survival rate among other countries such as Japan, USA, Korea, Australia and China (National Cancer Registry, 2018). By 2040, the burden of breast cancer is predicted to increase to over 3 million new cases and 1 million deaths every year due to population growth and ageing (Arnold et al., 2022).

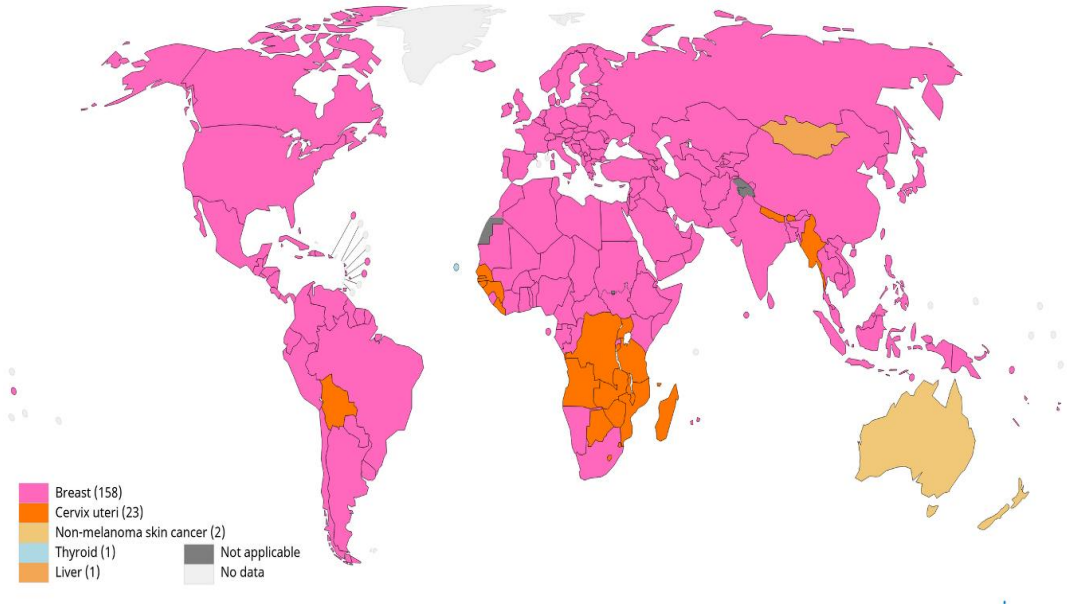

Figure 1.1 Top cancers affecting females globally per number of countries. Adopted online from The International Association of Cancers Registries, link: https://gco.iarc.fr/today/home

1.1.1 Breast Cancer Classification

Breast cancer can be classified based on hormone receptor status. Hormone receptor-positive (HR+) breast cancer cells have either estrogen (ER) or progesterone (PR) receptors or both. Thus, cancer cells are fueled by estrogen and/or progesterone due to special proteins inside the tumour cells called hormone receptors. When hormones attach to hormone receptors, the cancer cells grow. Hormone receptor-positive cancers tend to grow slower than those that are hormone receptor-negative. Five subtypes of breast cancer are widely recognised based on hormone receptor status: luminal A, normal-like, luminal B, HER2-positive, and triple-negative as shown in Figure 1.2.

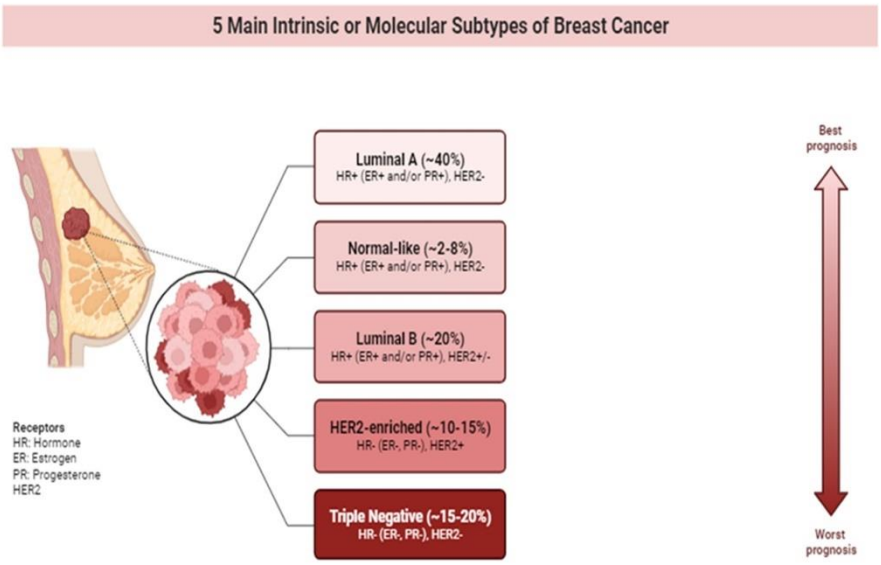

Figure 1.2 The classification of breast cancer. Adapted from Hanusek et al., 2022.

Approximately two out of every three BC cases (80%) are estrogen hormone receptor-positive (Özdemir et al., 2018; Zattarin et al., 2020). In 1936, Professor Antoine Lacassagne was the first researcher who suggested that breast cancer might be caused by a special sensitivity to estrogen (Bentrem et al., 2003). He conducted laboratory experiments to prevent estrogen replacement to enhance tumorigenesis in strains of mice with a high incidence of mammary cancer. Such an approach led to the idea of using a chemical to prevent breast cancer (Maximov et al., 2013). The timeline of the major landmarks in estrogen action and antiestrogens for the treatment and prevention of breast cancer is illustrated in Figure 1.3.

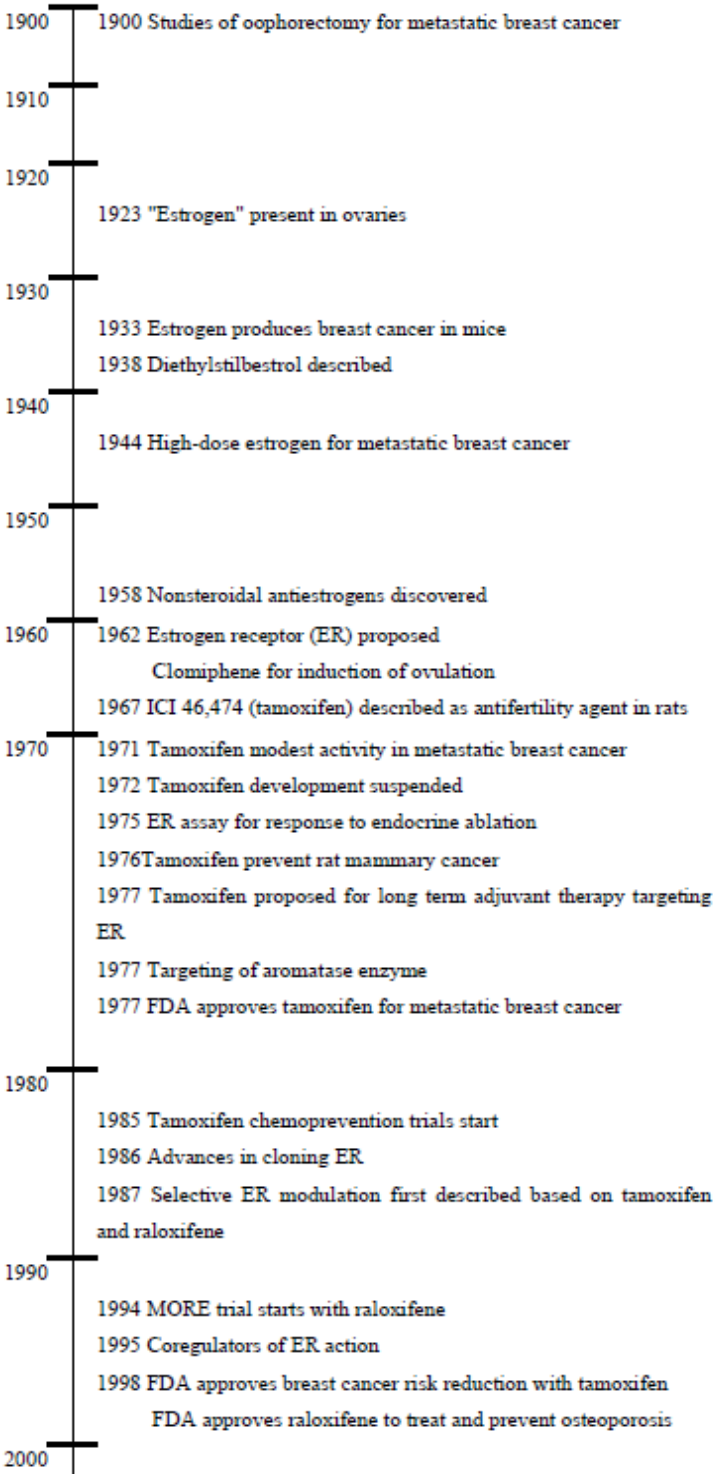

Figure 1.3 The timeline of the major landmarks in estrogen action and antiestrogens for the treatment and prevention of breast cancer. Adapted from Maximov et al., 2013.

1.1.2 Development of Estrogen Receptor (ER) Positive Breast Cancer

To understand the development of ER-positive breast cancer, it is important to start with normal breast tissue. In the female breast, estrogen plays a critical role in the development and maintenance of the breast ducts and lobules. Estrogen receptors are naturally present in these tissues (Elliott & Cescon, 2022). The development of ER-positive breast cancer is strongly influenced by hormonal factors. Estrogen, a female sex hormone, can bind to the estrogen receptors on breast cells and trigger various cellular processes. Under normal circumstances, this hormonal signalling helps maintain healthy breast tissue (Osborne & Schiff, 2011).

The development of ER-positive breast cancer is influenced by both genetic and environmental factors. Up to 25% of BC hereditary cases are due to a mutation in one of the few identified rare, but highly penetrant genes (BRCA1, BRCA2, PTEN, TP53, CDH1, and STK11), which confer up to an 80% lifetime risk of breast cancer. Notably, BRCA1 and BRCA2 mutations are particularly implicated in estrogen-dependent subtypes of breast cancer (Shiovitz & Korde, 2015). Additionally, exposure to environmental factors like diet, lifestyle and endocrine-disrupting chemicals may affect estrogen signalling and contribute to cancer development (Guo et al., 2017; Martin & Weber, 2000).

The mechanisms underlying the initiation of ER-positive breast cancer are complex and typically involve genetic mutations or alterations that lead to uncontrolled cell growth. These mutations can affect the estrogen receptors themselves or other genes involved in the regulation of cell growth. Once initiated, ER-positive breast cancer cells continue to grow and divide in response to estrogen stimulation. Such a reaction leads to the formation of a tumour within the breast tissue. The cancer

can progress from early stages to more advanced stages if left untreated (Scabia et al., 2022). ER-positive breast cancer tends to have a relatively favourable prognosis compared to some other breast cancer types, as it responds well to hormone therapy. However, the prognosis can vary depending on factors such as the stage of diagnosis and the response to treatment (Bae et al., 2015).

1.1.3 Drug of Choice for Estrogen Receptor (ER)-Positive Breast Cancer

The model drug for treating estrogen receptor (ER)-positive breast cancer is tamoxifen. Tamoxifen (TXN) is a selective estrogen receptor modulator (SERM), and it has been widely used for decades in the treatment of ER-positive breast cancer (Farrar & Jacobs, 2018). Besides, oral tamoxifen dosage form (tablet or solution) action and role in the treatment and prevention of this type of breast cancer is addressed as follows:

i. Anti-Estrogenic Action: Tamoxifen works by binding to the estrogen receptors on breast cancer cells. However, unlike natural estrogen, tamoxifen does not stimulate cell growth. Instead, it acts as a competitive inhibitor, blocking estrogen from binding to its receptors. Such anti-estrogenic action helps to slow down or halt the growth of ER-positive breast cancer cells (Ali et al., 2016).

ii. Adjuvant Therapy: Tamoxifen is often used as adjuvant therapy, which means it is given after the primary treatment of breast cancer, such as surgery or radiation therapy. It helps to reduce the risk of recurrence or the development of new breast cancers in the same or contralateral breast (Plowman, 1993).

iii. Treatment Duration: The duration of tamoxifen therapy can vary but is typically prescribed for 5 to 10 years. The decision on the duration of treatment is made based on individual factors and the specific characteristics of the breast cancer (Gupta et al., 2018).

iv. Preventive Use: Tamoxifen may also be considered for women at high risk of developing breast cancer, as it has been shown to reduce the risk of developing ER-positive breast cancer in some cases (Cuzick et al., 2015).

1.2 Skin as Route of Drug Administration

The skin, as a route of drug administration, plays a pivotal role in the field of pharmaceuticals and healthcare. Understanding how drugs interact with the skin is of paramount importance, as it offers a non-invasive and convenient means of delivering medications for various therapeutic purposes (Jeong et al., 2021). The skin, our body's largest organ, serves as a protective barrier against external physical, chemical, and biological agents, making it a formidable yet versatile interface for drug absorption (Sengar et al., 2018).

Such a natural barrier comprises three distinct layers: the epidermis, dermis, and subcutaneous tissue. The epidermis, the outermost layer, consists primarily of keratinocytes that synthesis keratin, a protective protein. Beneath the epidermis lies the dermis, primarily composed of collagen, a structural protein that imparts strength and resilience to the skin. The dermis houses essential structures such as blood vessels, nerves, and hair follicles. Finally, the subcutaneous tissue, also known as the panniculus, contains adipose tissue or fat cells, contributing to energy storage (Yousef et al., 2017). These three layers together create a dynamic environment that influences

drug permeation (Y.-Q. Yu et al., 2021) and, therefore, plays a critical role in transdermal drug delivery (Alkilani et al., 2015; Prausnitz & Langer, 2008).

The stratum corneum is the outermost layer of the epidermis, and it is made up of 10 to 30 thin layers of continually shedding, dead cells named keratinocytes and corneocytes (Murphrey et al., 2018). As illustrated in Figure 1.4, drug penetration across the stratum corneum is limited primarily by the lipids organised in bilayer structures (L) that fill the intercellular spaces between corneocytes (C). Thus, every drug molecule that is intended for transdermal drug delivery must pass through the stratum corneum layer, to reach the localised application site, and/or the systemic circulation (Prausnitz & Langer, 2008).

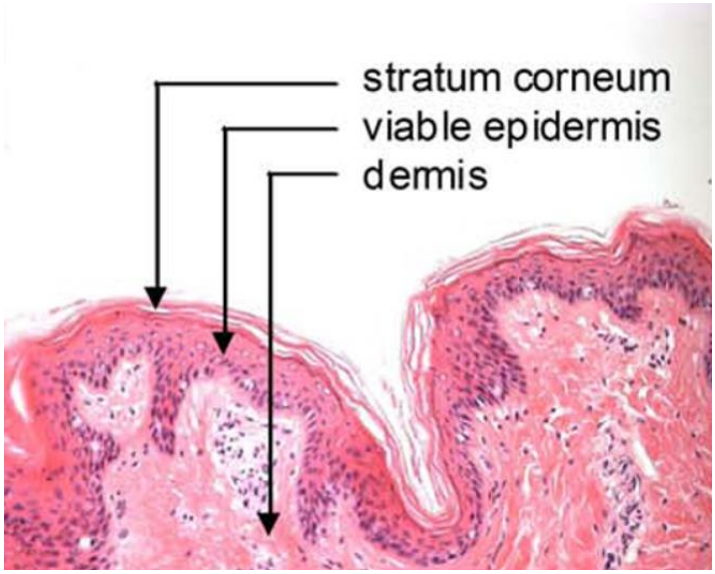

Figure 1.4 The histological structure of mammalian skin demonstrates the skin structure. Adopted from Prausnitz & Langer, 2008.

Various routes exist for delivering drugs via the skin, each offering a distinct path for medications to enter the body as shown in Figure 1.5. One such route is the transappendageal route, which involves drug absorption through hair follicles (follicular) and sweat glands. Such a route is often referred to as the "shunt route"

because it allows drugs to bypass the tough outer layer of the skin, the stratum corneum, and directly access the bloodstream (N'Da, 2014; Y.-Q. Yu et al., 2021).

Additionally, drugs can take the intracellular route, diffusing through and across the corneocytes, which are the specialised skin cells that make up the epidermis (Pizzimenti et al., 2016). Lastly, there is the intercellular route, where drugs permeate through the organised regions of intercellular skin lipids, effectively navigating the spaces between skin cells (Szunerits & Boukherroub, 2018). These various routes offer pharmaceutical researchers and formulators valuable options for designing drug delivery systems tailored to specific therapeutic needs, whether for localised treatments or systemic drug administration.

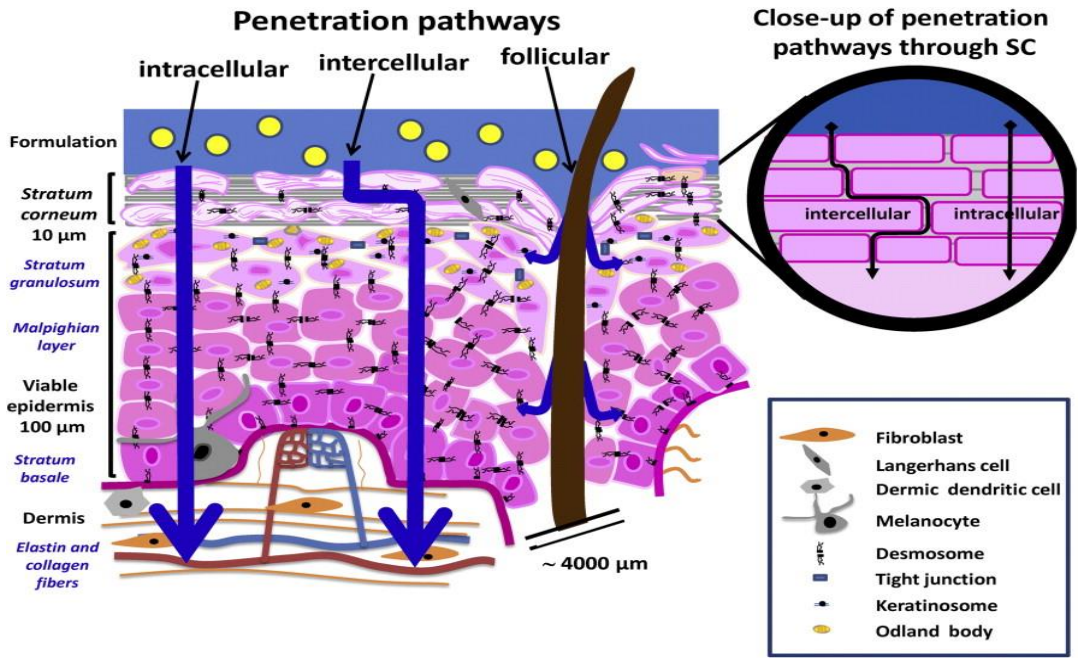

Figure 1.5 The mechanisms of penetration of drugs through the skin. Adapted from Bolzinger et al., 2012.

1.3 Technologies of Transdermal Drug Delivery Formulations

Transdermal drug delivery is a pharmaceutical approach designed to administer medications through the skin's surface, allowing for systemic absorption into the bloodstream. Its primary purpose is to provide a non-invasive and controlled means of delivering drugs over an extended period, ensuring steady and sustained therapeutic levels (Alkilani et al., 2015). Unlike other drug delivery methods like oral ingestion, transdermal delivery avoids the gastrointestinal tract and liver's first-pass metabolism, reducing the risk of digestive side effects, and improving bioavailability (Jeong et al., 2021).

1.3.1 First Technology

The concept of transdermal drug delivery dates back to ancient times when various cultures used plant extracts and ointments for medicinal purposes. However, it was not until the 20^{th} century that modern transdermal drug delivery systems emerged. The first significant development came in the initial technology form of transdermal delivery products of patches in the 1970s. Precisely, this was with the introduction of the scopolamine patch to combat motion sickness (Pastore et al., 2015). Such technology offered limited transdermal effect and was applicable only for certain molecules of specific characteristics such as very low molecular weight. Thus there was a need to develop the second technology (Prausnitz & Langer, 2008).

1.3.2 Second Technology

The second Technology of transdermal drug delivery systems is characterised by a shift towards the utilisation of small molecules as nanocarriers. Such innovative approach is used to revolutionise drug delivery, finding applications in localised

treatments, dermatological solutions, cosmetic formulations, and even some systemic therapies (Prausnitz & Langer, 2008). The key factor in this category of formulation is the employment of penetration enhancers. Such technology had limited transdermal delivery for macromolecules (Guy, 2010; Prausnitz & Langer, 2008)

By employing nanocarriers, these advanced systems enhance the efficiency and precision of drug delivery through the skin. These nanocarriers are carefully designed to facilitate the transport of therapeutic agents across the skin barrier while minimising systemic absorption, thus reducing the risk of unwanted side effects (Z. Yu et al., 2021).

Various types of nanocarriers have been developed for skin permeation enhancement and targeted delivery to skin organelles. Such types include polymeric nanoparticles, metallic nanoparticles, dendrimers, micelles, lipid-based nanoparticles, and quantum dot nanocarriers (Liu et al., 2023). Their small size and tailored properties make them ideal for encapsulating drugs and releasing them at specific sites within the skin or even deeper into the body when necessary. Such precision allows for the development of highly effective dermatological treatments, cosmetic products, and localised therapies (Zeb et al., 2019).

1.3.3 Third Technology

The third technology for transdermal delivery systems involves the use of medical devices and novel microneedle technologies to enhance drug delivery. Such technology offered a new approach to delivering macromolecules (including proteins and DNA) and vaccines. It also includes device development for enhancing transdermal drug delivery (Peña-Juárez et al., 2022). Examples of third technology for transdermal delivery systems include microneedle patches and iontophoretic devices

as well as technologies of thermal ablation, microdermabrasion, electroporation and cavitational ultrasound (Joshi et al., 2023; Prausnitz & Langer, 2008).

Examples of milestones achieved by each technology as a form of transdermal delivery systems are shown in Table 1.1.

Table 1.1 Examples of main formulations achieved by first, second, and third technologies of transdermal drug delivery systems.

Transdermal Drug Delivery Technology	Name of Formulation/Nanocarrier/Device	Drug	Application	Reference
First technology	Patch	Nicotine	Smoking cessation	(Mancuso et al., 1999)
		Estradiol	Hormone replacement	(Guichard et al., 1999)
		Fentanyl	Pain management	(Bhatt, 2005)
		Lidocaine	Pain management	(Gammaitoni et al., 2003)
		Nitroglycerin	Angina treatment	(Peppas & Robinson, 1995)
		Rivastigmine	Anti-Alzheimer's Disease	(Winblad & Machado, 2008)
Second technology	Liposomes	Amphotericin B	Fungal infections treatment	(Akbarzadeh et al., 2013)
		Lidocaine	Local Anesthesia	(Sinico & Fadda, 2009)
	Ethosomes	5-Fluorouracil	Skin cancers treatment	(Gu et al., 2015)
		Minoxidil	Anti-hair loss (Alopecia)	(Builders et al., 2014)
	Microemulsions	Clonidine	Anti-Hypertension	(Paudel et al., 2010)
		Ketoprofen	Pain Management	(Date & Patravale, 2007)
	Nanoparticles	Doxorubicin	Skin cancers treatment	(Huber et al., 2015)
		Sumatriptan	Migraine relief	(Ahmed Kassem, 2016)
Third technology	Dermal Microneedles	Insulin	Diabetes management	(Darvishha & Amiri, 2019)
		Sumatriptan	Migraine relief	(Ita, 2015)
	Microparticles	Vaccine	Breast cancer control	(Zaman et al., 2022)
		Vaccine	Melanoma cancer control	(Bhowmik et al., 2011)
	Ultrasound-based transdermal drug delivery	Insulin	Diabetes management	(Seah & Teo, 2018)
	Ablative laser	Vaccine	Measles prevention	(Joshi et al., 2021)

1.3.4 Advantages and Disadvantages of Transdermal Delivery Route

Transdermal administration presents a range of advantages in comparison to the oral route. It is particularly utilised when there exists a substantial first-pass effect by the liver, which can lead to the premature metabolism of drugs. Additionally, transdermal delivery holds benefits over hypodermic injections, which can be painful, generate hazardous medical waste, and pose the risk of disease transmission through needle reuse (Sabbagh & Kim, 2022). Furthermore, transdermal systems can be cost-effective by offering options that can reduce healthcare expenses. Such characteristics can be observed through the transdermal formulations that are non-intrusive, self-administered, can offer sustained release for extended durations (up to a week), or short-term release for a few days (e.g., patches), and enhance patient adherence (Hou et al., 2023; Sabbagh & Kim, 2022).

1.3.5 Drugs Compatibility with Transdermal Delivery Route

When looking forward to utilising the transdermal route of administration for any drug, this drug should fulfil a list of terms and conditions related to its physicochemical properties and dosage requirements respectively. For example, the molecular weight of a drug is a significant factor. For some nanocarriers drugs with lower molecular weights (500-600 g/mol and below) tend to be more suitable for transdermal delivery (Alkilani et al., 2015). Furthermore, lipophilic drugs (log P from 1 to 5) can more readily penetrate the stratum corneum and are preferred for transdermal delivery (Bird & Ravindra, 2020). Besides, drugs with lower melting point are more suitable for the transdermal route as it will have greater solubility in the stratum corneum, potentially resulting in a higher amount of drug permeating into the skin (Ramadon et al., 2022).

Moreover, the required effective dose for the drug intended to be targeted transdermally should be within a few milligrams per day, ideally < 25 mg (Benson & Roberts, 2021; Hasan & Farooqui, 2021). Drugs with high doses may not be suitable for transdermal delivery due to the difficulty in formulating nanocarriers or dosage forms that contain enough doses at the effective level (Prausnitz & Langer, 2008; Margetts & Sawyer, 2007).

1.4 Lipid-Based Formulation for Transdermal Drug Delivery

Lipid-based formulations are a type of drug delivery system that uses lipids as the main component to improve the solubility, stability, and bioavailability of drugs for transdermal drug delivery (Akombaetwa et al., 2023). Lipid-based formulations can be used to deliver drugs through the skin and/or into the bloodstream, providing a non-invasive and controlled means of drug delivery (Stefanov & Andonova, 2021). Lipid-based formulations for transdermal drug delivery represent a versatile approach that can be applied to a wide range of drugs, from hydrophobic molecules to hydrophilic compounds. Their ability to enhance drug permeation through the skin makes them valuable tools in the development of effective and patient-friendly transdermal delivery systems (Sguizzato et al., 2021).

In a lipid-based delivery system, there are main parent classes namely liposomes, lipid nanocapsules, polymeric nanoparticles, solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs) and microemulsion drug delivery systems. From these parent classes (Figure 1.6) different subclasses have emerged based on the various excipients used, and the method of preparation employed. In terms of lipid-based formulation, this thesis will focus on the liposomes subclass namely ethosomes. The subclassing is further discussed in the next section

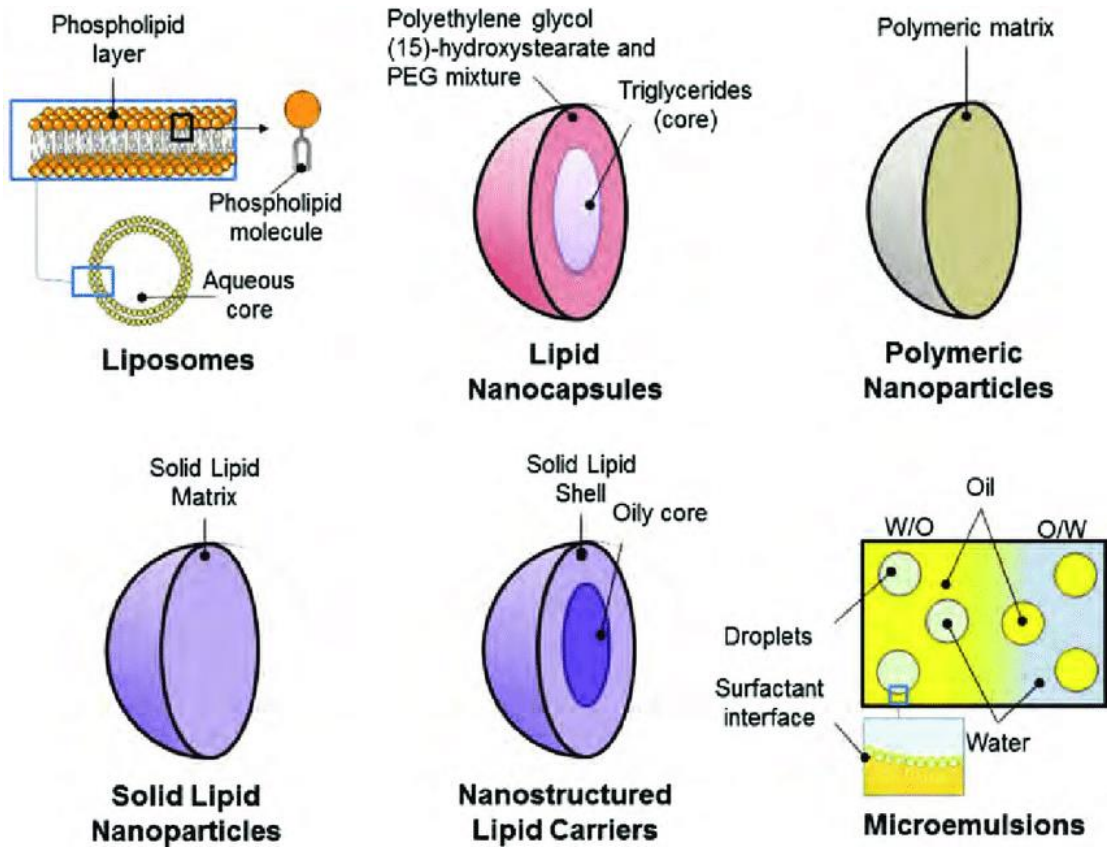

Figure 1.6 The schematic illustration of the lipid-based formulations parent classes. Adapted from de Almeida et al., 2017.

1.4.1 Ethosomal Systems

Liposomes, which belong to one of the parent categories of lipid-based formulations, were initially introduced in the 1960s. They predominantly consist of phospholipids and an aqueous medium (Nsairat et al., 2022). In 1995, a novel class emerged from liposomes known as “ethosomes”, by the addition of an ethanol (etho) phase to the liposomal vesicular carrier (somes). Since then ethosomes have led to a revolutionary development in topical and transdermal drug delivery (Pilch & Musiał, 2018; Touitou, 1995). Figure 1.7 shows a simple representation of the structure of ethosomes. Precisely, ethosomes consist of lipid bilayer vesicles, similar in structure to cell membranes. Such lipid bilayer is composed of phospholipids. Within the lipid

bilayer, there is an aqueous or water core. The core can encapsulate drugs, both lipophilic and hydrophilic, allowing them to be carried within the vesicles. Ethanol is typically a key component of ethosomes. It is present in the aqueous core and also interacts with the lipid bilayer. Ethanol acts as a penetration enhancer, disrupting the stratum corneum's lipid structure and promoting drug permeation through the skin (Verma & Pathak, 2010).

Ethosomes are nanometer-sized vesicles, which means they are very small structures. Their small size contributes to their ability to penetrate the skin effectively. Ethosomes are known for their flexibility and deformability. Such property is due to the inclusion of ethanol in their composition. Such key component alters the properties of ethosomes, making them highly flexible and deformable. Ethanol interacts with the phospholipid bilayers in ethosomes, leading to a fluidising effect that enhances their flexibility. The fluidising impact of ethanol on the vesicles' structure allows ethosomes to adapt and deform easily, enabling better penetration through the skin and enhancing their stability (Abu-Huwaij & Zidan, 2024). Such property allows them to adapt to the skin's surface and squeeze through the narrow gaps between skin cells, enhancing drug delivery (Niu et al., 2019).

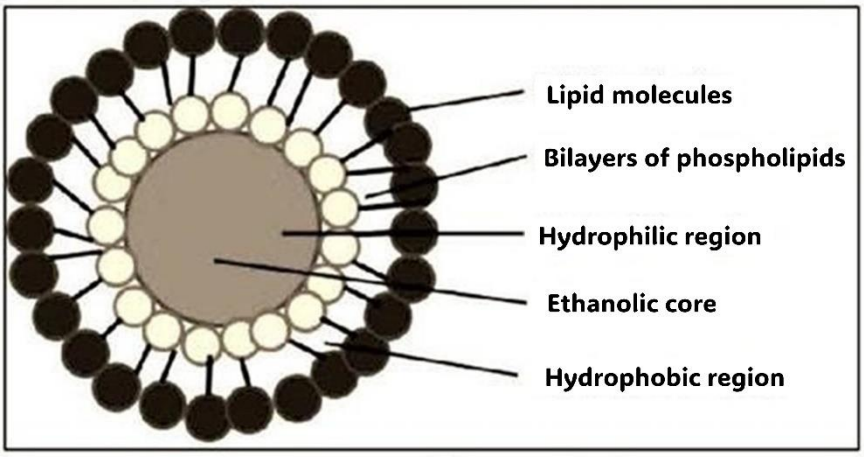

Figure 1.7 The diagram of ethosomes. Adapted from Misbah Ul Haq et al., 2021

The mechanism of skin permeation for ethosomes is illustrated in Figure 1.8 and involves several key steps that facilitate the passage of drug-loaded ethosomes through the skin's barrier, primarily the stratum corneum. Ethanol acts as a penetration enhancer by disrupting the tightly packed lipid structure of the stratum corneum by interacting with the lipid molecules in the skin barrier. Ethanol's small and hydrophilic nature allows it to diffuse into the core of the ethosomes and influence the lipid bilayer. By disrupting the packing of phospholipids within the bilayer, ethanol increases the fluidity of the membrane. Such appraoch enhanced fluidity contributes to the flexibility and deformability of ethosomes, enabling them to adapt to the skin surface more effectively and improve drug delivery (Emanet & Ciofani, 2023). Besides, ethanol's permeation-enhancing effect also arises from its superior ability to form hydrogen bonds with headgroup atoms of skin lipids (Gupta et al., 2020). Such disruption creates temporary gaps or fluidizes the lipids, increasing the permeability of the stratum corneum (Niu et al., 2019). Once within the stratum corneum, ethosomes can transport drug molecules across the skin layers by diffusing through the

intercellular spaces between skin cells. The lipid bilayer of the ethosomes interacts with the skin lipids, facilitating drug transfer (Y.-Q. Yu et al., 2021). Ethosomes continue to transport the encapsulated drug through the various skin layers until they reach the target site, such as the dermis or deeper tissues, where drug absorption occurs. The drug can then be released and enter the bloodstream or exert its therapeutic effect locally (Kesharwani et al., 2015).

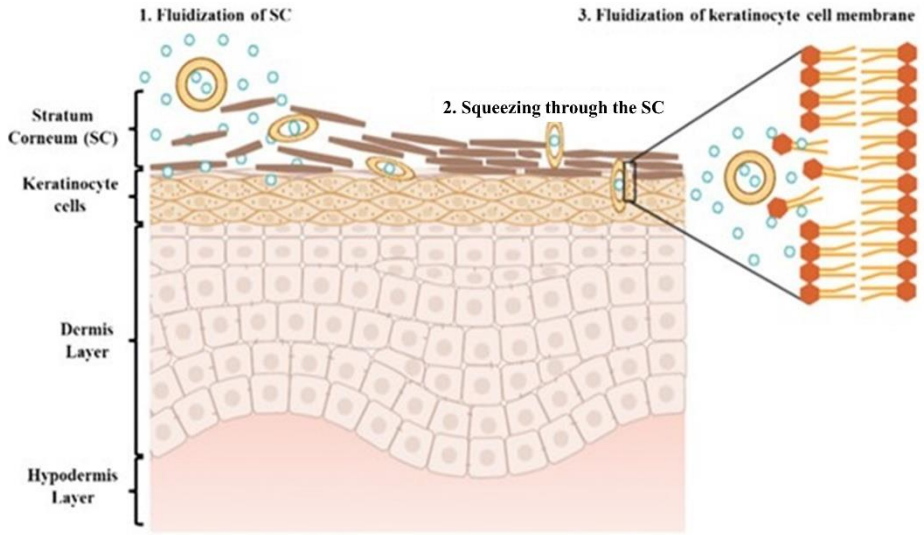

Figure 1.8 Schematic representation of ethosome penetration through stratum corneum. Adapted from Emanet & Ciofani, 2023.

1.4.1(a) Ethosomal Classification

The development of the basic ethosomal formulation which was initially composed of phospholipid, ethanol and water (current Chapter, Section 1.4.1) relies on the addition of the penetration enhancer. Ethosomal systems can be classified based on the penetration enhancer type used, generating emerging classes of this nanocarrier. These subclasses of ethosomes are developed to address specific challenges or requirements in drug delivery. These classes vary in terms of their ability to encapsulate different types of drugs, their skin penetration capabilities, and their

release kinetics. The ethosomal classes that are of interest in this research work scope are illustrated below.

Transethosomes

Transethosomes have the same composition as the basic ethosomal system in addition to penetration enhancers, mostly non-ionic surfactants (Ascenso et al., 2015). Transethosomes class was first reported in 2012 for the enhanced skin delivery of voriconazole (Song et al., 2012). Transethosomes are ultra-deformable nanovesicles that can deliver drugs into deeper tissues and have particle sizes (PS) ranging from 69 nm to 250 nm (Allam et al., 2022; Bajaj et al., 2021). Transethosomes have a dispersity (*Đ*) of less than 0.3, indicating a narrow size distribution (Ferrara et al., 2022). The zeta potential (ZP) of transethosomes ranges from -11.6 to -26.5 mV, indicating good stability of the colloidal dispersions (e.g., transethosomes) (Guillot et al., 2023; Mita et al., 2022). A higher magnitude of zeta potential (either positive or negative) indicates greater electrostatic repulsion between particles, which helps prevent aggregation and enhances the stability of the formulation (Cristiano et al., 2021). Based on the physicochemical nature of the penetration enhancer (aka, non-ionic surfactant), increasing its concentration can impact the PS and ZP values respectively.

Transethosomes improve the stability of the drug inside the tissues and have a high content of ethanol (up to 30%) together with a penetration enhancer (Raj et al., 2023). The presence of such a concentration of ethanol in transethosomes confers greater elasticity to the vesicles, allowing them to interact effectively with both skin and vesicle lipids. Such interaction promotes the passage of the entrapped drug through the stratum corneum, the outermost layer of the skin. Additionally, ethanol improves the thermodynamic stability and loading capacity of lipophilic drugs within

transethosomes, making them more efficient carriers for drug delivery (Esposito et al., 2022). Thus, transethosomes are very flexible, resulting in a high flux rate and skin permeation rate compared to other vesicular systems such as liposomes. They can deliver drugs ranging from low molecular weight to high molecular weight into deeper layers of the skin (Adnan et al., 2023).

Transethosomes are mainly used for the delivery of a wide range of hydrophobic drugs (e.g., olmesartan, voriconazole and apigenin) but it was reported in delivering hydrophilic drugs too (e.g., valsartan). Such nanocarrier offers entrapment efficiency of up to 90.5% where the drug is entrapped in either the hydrophilic or hydrophobic region of the nanocarrier as illustrated in Figure 1.7 (Albash et al., 2019; Farooq et al., 2022; Ahad et al., 2013). They are a promising approach for both topical and transdermal drug delivery, with a wide range of applications such as psoriasis and acne treatment respectively (Adnan et al., 2023; Munir et al., 2023).

Bilosomes

Bilosomes have the same composition as the basic ethosomal system in addition to penetration enhancer, which is a bile salt. Bilosomes were first reported in 2001 for the enhancement of oral drug solubility and bioavailability (Conacher et al., 2001). It was not until 2015 that the bilosomes were used for the transdermal delivery of tenoxicam (Al-mahallawi et al., 2015). Bilosomes have PS ranges from 90 nm to 3 µm (Palekar-Shanbhag et al., 2020), with a low $Đ < 0.2$ (Kharouba et al., 2022; Waglewska et al., 2022). The zeta potential of bilosomes is reported to be negatively charged with values close to - 30 mV. Due to the anionic nature of the bile salts, increasing their concentration in the formulation will lead to the increment of ZP and PS (Zarenezhad et al., 2023). In terms of entrapment efficiency, bilosomal systems'

entrapment efficacy (EE%) is usually not less than 86% but also reaches higher values of > 90% (Kharouba et al., 2022; Salem et al., 2022).

Overall, the anionic nature of bile salts is a key characteristic that allows them to enhance transepithelial permeability for different drugs (e.g., salmon calcitonin and insulin) (Moghimipour et al., 2015). However, the transdermal application of bile salts is still an area of active research and investigation (Alvarez-Figueroa et al., 2019). Bilosomes offered successful transdermal delivery mostly for enhancing the anti-inflammatory effects of lornoxicam (Ahmed et al., 2020), and tenoxicam (Al-mahallawi et al., 2015), as well as the antidiabetic transdermal delivery of metformin hydrochloride (Salem et al., 2022).

Invasomes

Invasomes vesicular system contains phospholipids, ethanol, and terpenes in their structures, which confer suitable transdermal penetration properties to the vesicles. Invasomes were reported in 2002 for their abilities to enhance topical drug delivery (Verma, 2002). Later in 2009, they were explored for transdermal delivery by using radiolabeled mannitol (Badran et al., 2009). Such class of ethosomal system offers a PS ranging from 100 nm to 819 nm (Amnuaikit et al., 2018; Jain et al., 2021), and $Đ < 0.3$ (Nangare & Dugam, 2020). In terms of ZP values, invasomes are known for their ZP values that can start from - 20 mV (Nangare & Dugam, 2020) and reach up to – 73 mV (Tawfik et al., 2020), based on the employed drug. The entrapment efficiency of invasomes can vary depending on the type of terpene used in the formulation, and their physicochemical compatibility with the drug used (Nangare & Dugam, 2020).

The main advantage of invasomes is their ability to increase the permeability of the drug into the skin, with the opportunity for a localised transdermal drug delivery profile (e.g. into the breast tissue or glands) (Babaie et al., 2020). Furthermore, the advanced performance of invasomes in systemic transdermal drug delivery has opened doors to new approaches in treating cardiovascular (Babaie et al., 2020), and autoimmune diseases (Verma & Pal, 2022), as well as skin and colorectal cancers (Dragicevic-Curic et al., 2010; Dragicevic-Curic et al., 2009). Besides, invasomes also offer cosmetic solutions for acne with their high skin deposition properties (El-Nabarawi et al., 2018).

1.4.1(b) Ethosome Preparation Methods

Different techniques can be utilised in the formulation of ethosomes, and they vary in terms of processing duration, the employment of organic solvents, complexity, and cost, depending on the specific requirements and optimisation studies of the research. These methods are delineated below.

Thin-Film Hydration Method

In this method, the lipid mixture is dissolved in an organic solvent (e.g., chloroform) to form a thin lipid film on the walls of a round-bottom flask via a rotary evaporator. The solvent is then removed under reduced pressure to create a dry lipid film. Ethanol or an aqueous solution (Figure 1.7) containing the drug is then added to hydrate the film and form ethosomes (Jain et al., 2007).

Cold Method

Such technique involves the direct mixing of preformed ethosome components at room temperature, thus reducing the risk of drug degradation due to heat exposure.

Phospholipids, ethanol, and water are blended under stirring at a speed of 700–2000 rpm for 5–30 minutes to create ethosomes (Satyam et al., 2010).

Hot Method

Here, the lipids, water, and the drug are heated together at an elevated temperature (40 °C) to form a homogenous mixture. The mixture is then hydrated with an aqueous solution, typically containing ethanol, while maintaining the elevated temperature under stirring. The heating helps in the incorporation of the drug into the lipid bilayers (Aute et al., 2012).

Sonication Method

Ultrasonic energy is applied to a mixture of lipids and drugs to generate ethosomes. The mechanical energy produced by ultrasonic waves disrupts the lipid bilayers and forms vesicles. This method is useful for producing small-sized ethosomes (Patrekar et al., 2015).

Reverse Phase Evaporation Method

Such method, designed specifically for generating large unilamellar vesicles, is the least commonly employed. It involves the formation of a water-in-oil (W/O) emulsion by adding an aqueous phase containing the drug to a lipid phase and the organic phase (e.g., diethyl ether, chloroform, ethanol). The emulsion (W/O) is then converted into ethosomes by evaporation of the organic solvent under reduced pressure (e.g., rotary evaporator instrument) (Esposito et al., 2004).