

Ethosomal nanocarriers: the impact of constituents and formulation techniques on ethosomal properties, in vivo studies, and clinical trials

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Abstract: Ethosomal systems are novel lipid vesicular carriers containing a relatively high percentage of ethanol. These nanocarriers are especially designed for the efficient delivery of therapeutic agents with different physicochemical properties into deep skin layers and across the skin. Ethosomes have undergone extensive research since they were invented in 1996; new compounds were added to their initial formula, which led to the production of new types of ethosomal systems. Different preparation techniques are used in the preparation of these novel carriers. For ease of application and stability, ethosomal dispersions are incorporated into gels, patches, and creams. Highly diverse in vivo models are used to evaluate their efficacy in dermal/transdermal delivery, in addition to clinical trials. This article provides a detailed review of the ethosomal systems and categorizes them on the basis of their constituents to classical ethosomes, binary ethosomes, and transethosomes. The differences among these systems are discussed from several perspectives, including the formulation, size, ζ -potential (zeta potential), entrapment efficiency, skin-permeation properties, and stability. This paper gives a detailed review on the effects of ethosomal system constituents, preparation methods, and their significant roles in determining the final properties of these nanocarriers. Furthermore, the novel pharmaceutical dosage forms of ethosomal gels, patches, and creams are highlighted. The article also provides detailed information regarding the in vivo studies and clinical trials conducted for the evaluation of these vesicular systems.

Keywords: ethosomes, transdermal, lipid-based vesicles, delivery systems

Introduction

The skin is the largest and most easily accessible organ of the body; it serves as a potential route of drug administration for systemic effects. However, the outer layer of the skin, the stratum corneum, represents the most resistible barrier to drug penetration across the skin, which limits the transdermal bioavailability of drugs. Therefore, special carriers are required to combat the natural skin barrier to deliver drug molecules with different physicochemical properties to the systemic circulation.

Transdermal drug-delivery systems offer many advantages, such as avoidance of first-pass metabolism by the liver, controlled delivery of drugs, reduced dosing frequency, and improved patient compliance, as they are noninvasive and can be self-administered.^{1,2} The first transdermal patch containing scopolamine for the treatment of motion sickness was approved in the US in 1979.

A new era of research in this field was opened with the use of liposomes for the topical delivery of triamcinolone,³ and since then a wide range of novel lipid-based vesicular systems have been developed. Deformable or elastic liposomes, which are

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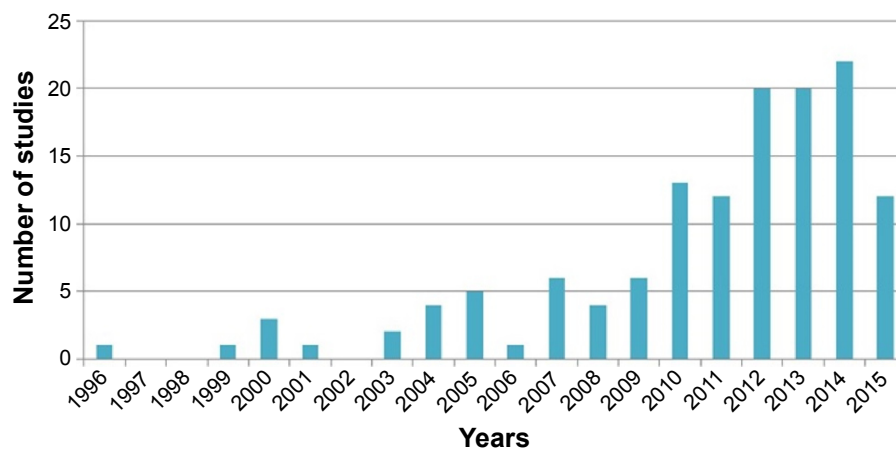


Figure 1 Ethosomal studies covered by this review, and their annual distribution from July 1996 to November 2015.

currently known as transfersomes, were introduced by Cevc and Blume in 1992⁴ and followed by the innovative work of Toubou et al, which led to the discovery of a novel lipid vesicular system called ethosomes. Ethosomal systems differ from liposomes because they contain relatively high concentrations of ethanol, in addition to phospholipids and water.^{5,6} New generations of ethosomal systems have been introduced since then by adding other compounds to the basic ethosomal formula in an attempt to enhance vesicular characteristics and skin permeation. However, to date, there has been no clear distinction among the classical ethosomes and their newer generations. This article provides a detailed review of ethosomal systems and identifies the different types of these vesicles based on the compounds used in their production and the impact of these compounds on ethosomal properties. Additionally, this article also highlights the ethosome preparation methods and pharmaceutical dosage forms, as well as the *in vivo* studies and clinical trials conducted on these promising nanocarriers for dermal/transdermal delivery. Figure 1 illustrates the number of published articles covered by this review and their annual distribution over a period of almost 2 decades since the development of ethosomes in 1996 till November 2015.

Ethosomal system types

Figure 2 illustrates the three types of ethosomal systems, classified on the basis of their compositions.

Classical ethosomes

Classical ethosomes are a modification of classical liposomes and are composed of phospholipids, a high concentration of ethanol up to 45% w/w, and water. Classical ethosomes

were reported to be superior over classical liposomes for transdermal drug delivery because they were smaller and had negative ζ -potential and higher entrapment efficiency. Moreover, classical ethosomes showed better skin permeation and stability profiles compared to classical liposomes.⁶⁻⁸ The molecular weights of drugs entrapped in classical ethosomes have ranged from 130.077 Da to 24 kDa.^{9,10}

Binary ethosomes

Binary ethosomes were introduced by Zhou et al.¹¹ Basically, they were developed by adding another type of alcohol to the classical ethosomes. The most commonly used alcohols in

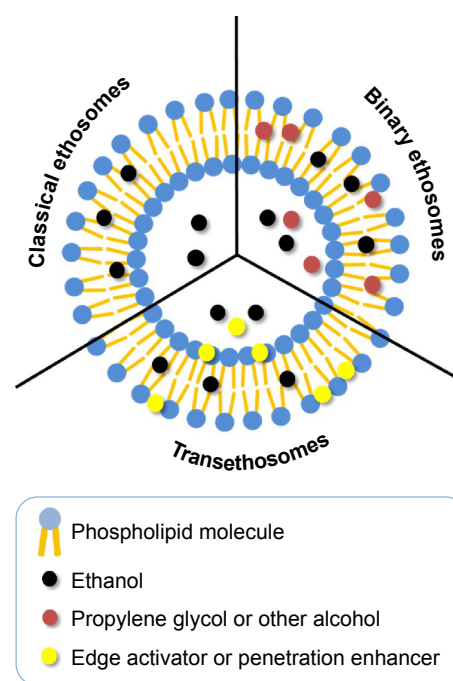


Figure 2 Schematic representation of the different types of ethosomal systems.

Table 1 Comparison of classical ethosomes, binary ethosomes, and transethosomes in their initial suspension form

Parameter	Classical ethosomes	Binary ethosomes	Transethosomes
Composition	1. Phospholipids 2. Ethanol 3. Stabilizer 4. Charge inducer 5. Water 6. Drug/agent	1. Phospholipids 2. Ethanol 3. Propylene glycol (PG) or other alcohol 4. Charge inducer 5. Water 6. Drug/agent	1. Phospholipids 2. Ethanol 3. Edge activator (surfactant) or penetration enhancer 4. Charge inducer 5. Water 6. Drug/agent
Morphology	Spherical	Spherical	Regular or irregular spherical shapes
Size	Smaller than the classical liposomes	Equal to or smaller than classical ethosomes	Size based on type and concentration of penetration enhancer or edge activator used
ζ -Potential	Negatively charged	Negatively charged	Positively or negatively charged
Entrapment efficiency	Higher than classical liposomes	Typically higher than classical ethosomes	Typically higher than classical ethosomes
Skin permeation	Typically higher than classical liposomes	Typically equal to or higher than classical ethosomes	Typically higher than classical ethosomes
Stability	Stabler than classical liposomes	Stabler than classical ethosomes	No particular trend determined

binary ethosomes are propylene glycol (PG) and isopropyl alcohol (IPA).^{12–16}

Transethosomes

Transethosomes are the new generation of ethosomal systems and were first reported by Song et al in 2012.¹⁷ This ethosomal system contains the basic components of classical ethosomes and an additional compound, such as a penetration enhancer or an edge activator (surfactant) in their formula. These novel vesicles were developed in an attempt to combine the advantages of classical ethosomes and deformable liposomes (transfersomes) in one formula to produce transethosomes. Many researchers have reported superior properties of transethosomes over classical ethosomes.^{17–30} Different types of edge activators and penetration enhancers have been investigated to produce ethosomal systems with better characteristics. Transethosomes were reported to entrap drugs with molecular weights ranging from 130.077 Da to 200–325 kDa.^{18,21} Table 1 shows the comparison of classical ethosome, binary ethosome, and transethosome properties in their initial suspension form.

Effects of materials used on ethosomal system properties

Ethanol

Ethanol is an efficient penetration enhancer.³¹ It plays an important role in ethosomal systems by giving the vesicles unique characteristics in terms of size, ζ -potential, stability, entrapment efficacy, and enhanced skin permeability. Concentrations of ethanol in ethosomal systems have been reported to be ~10%–50%.^{6,32} Many researchers concluded that when

the concentration of ethanol is increased, the size of the ethosomes would decrease.^{6,12,33–38} Bendas and Tadros found that the mean vesicle diameter of an ethosomal formulation containing 40% ethanol was 44.6% smaller than the classical liposomal formulation, which had no ethanol.³⁹ However, increasing ethanol concentration beyond the optimum level would cause the bilayer to be leaky and lead to a slight increase in vesicular size and severe decrease in entrapment efficacy, and by further increasing in ethanol concentration it would solubilize the vesicles. Some studies have suggested that high concentrations of ethanol causes interpenetration of the ethanol hydrocarbon chain, which leads to a reduction in vesicular membrane thickness and hence reduces vesicular size. Other researchers have proposed that ethanol modifies the net charge of the systems into some degree of steric stabilization, which could lead to a decrease in mean vesicle size.^{23,36,37,39–41}

Vesicular charge is an important parameter that can influence vesicular properties, such as stability and vesicle–skin interaction. The high ethanol concentration in ethosomes has shifted the vesicular charge from positive to negative.^{6,42} Dayan and Touitou found that the negative charge of the empty ethosomes increases with increasing ethanol concentration.⁴³ Ethanol acts as a negative charge provider for the surface of ethosomes, thereby avoiding aggregation of the vesicular system due to electrostatic repulsion. Additionally, ethanol was also reported to have stabilizing effects.^{40,44} Ethanol also has a significant effect on ethosomal system entrapment efficiency, and in general increasing ethanol concentration will increase entrapment efficiency.⁴⁵ This effect applies to molecules of varying lipophilicities, whereby ethanol increases the solubility of

the lipophilic and amphiphilic drugs and hence increases drug loading. This relationship was found to be linear, with ethanol concentrations between 20% and 40%.⁴⁶ For this reason, ethanol concentration should be optimized during the formulation process, as at low concentrations entrapment efficacy will be minimal, and at very high concentrations ethosomal membrane will be more permeable because phospholipids can easily be dissolved in ethanol, leading to a significant reduction in entrapment efficacy.

Phospholipids

Phospholipids from different sources have been used in ethosomal system formulation. The selection of phospholipid type and concentration for the formulation are important factors during the development of ethosomal system because they will influence the size, entrapment efficacy, ζ -potential, stability, and penetration properties of the vesicles. The different types of phospholipids used in the preparation of ethosomal systems are summarized in Table 2.

Prasanthi and Lakshmi used three phospholipid types (Phospholipon 90H and 80H and soy phosphatidylcholine) in the preparation of ethosomal systems of alfuzosin hydrochloride⁴⁶ and tolterodine tartrate⁴⁷ and found that the

phospholipid type had a significant effect on the ethosomal size, but not the entrapment efficiency. Shen et al found that the transethosomes prepared by either Lipoid S100 or SPC50 were smaller than those produced by Lipoid E80, and the phospholipids with higher phosphatidylcholine content led to the production of stabler vesicles.²⁷ Highly negatively charged vesicles were produced by the incorporation of DPPG (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol) in the ethosomal formulation,⁴⁸ while cationic ethosomal vesicles were produced by using a cationic lipid, such as DOTAP (1,2-dioleoyl-3-trimethylammonium-propane [chloride salt]).²²

In general, the concentration range of phospholipids in an ethosomal formulation is 0.5%–5%.²⁴ Increasing phospholipid concentration will increase vesicular size slightly or moderately,^{32,34,64} but will increase entrapment efficiency significantly. However, the relationship is true only until a certain concentration, whereby further increment in phospholipid concentration will have no effect on entrapment efficiency.³⁸

Cholesterol

Cholesterol is a rigid steroid molecule, and its incorporation in ethosomal systems enhances the stability and entrapment

Table 2 Phospholipids used in ethosomal formulations

Phospholipid name/brand	Composition and source	Reference(s)
Phospholipon 90G	Phosphatidylcholine from soybean (90%), granules	37, 49, 50
Phospholipon 90H	Hydrogenated phosphatidylcholine from soybean (90%), powder	51
Phospholipon 80H	Hydrogenated phospholipids from soybean with 70% phosphatidylcholine, powder	46
NAT 8539	Contained phosphatidylcholine (73%–79%), lysophosphatidylcholine (up to 6%), cephalin (up to 4%), and phosphatidic acid (up to 6%) of the dry residue; natural oils and sterol up to 6%; and ethanol (23%–27%)	52
Dipalmitoylphosphatidylcholine (DPPC)	1,2-Dipalmitoyl- <i>rac</i> -glycero-3-phosphocholine, ~99%, powder	53
Lipoid S100	Phosphatidylcholine from soybean, agglomerates	9, 17, 54, 55
Lipoid S75-3	Phosphatidylcholine content (70%–75%), from soybean	56
Lipoid S75	Phosphatidylcholine content (68%–73%), from soybean	16
Lipoid E80	Phosphatidylcholine content (81.7%), from egg yolk, agglomerates	27, 57
Phosphatidylethanolamine (PE)	3- <i>sn</i> -Phosphatidylethanolamine, $\geq 98\%$, from bovine/sheep brain, lyophilized powder	58
L- α -Phosphatidylcholine (PC)	1,2-Diacyl- <i>sn</i> -glycero-3-phosphocholine, $\geq 99\%$, from soybean/egg yolk, lyophilized powder	24, 59, 60
POPC (1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine)	1-Hexadecanoyl-2-(9Z-octadecenoyl)- <i>sn</i> -glycero-3-phosphocholine, >99%, synthetic, powder	61, 62
DPPG (1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphatidylglycerol)	1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phospho-(1'- <i>rac</i> -glycerol) (sodium salt), powder	48
Coatsome® FE-6081SU5 POPE-NHS	N-(Succinimidyl-oxy-glutaryl)-L- α -phosphatidylethanolamine, 1-palmitoyl-2-oleoyl	21, 22
DOTAP (1,2-dioleoyl-3-trimethylammonium-propane [chloride salt])	1,2-Dioleoyl-3-trimethylammonium-propane (chloride salt), powder or ethanol solution	22
Phospholipon 50	Lecithin from soy purified phosphatidylcholine, concentration 45%, rich in linoleic acid (65%) and palmitic acid (~20%), solid wax	63
SPC50	Phosphatidylcholine content (50.3%), from soybean	27

efficiency of drugs. It prevents leakage and reduces vesicular permeability and vesicular fusion. Generally, it is used at a concentration of <3%,⁵⁹ but in some formulations it was used up to 70% of the total phospholipid concentration in the formulation.^{24,33} Several studies have reported that cholesterol increased the vesicular size of ethosomal systems.^{24,33,39,65} López-Pinto et al found that ethosomal size increased from 136±42 nm to 230±27 nm when 25.87 mM of cholesterol was used in the formulation.³³ Another study reported that the increment of cholesterol concentration from 0% to 0.15% w/w caused vesicular size to increase from 102±13 nm to 152±12 nm.⁶⁵ However, only one study has found that cholesterol had no stabilization effects on the ethosomes. The authors used phosphatidylethanolamine and 15% ethanol in the formulation.²³ López-Pinto et al found that incorporation of cholesterol in the ethosomal formulation increased the vesicular stability and rigidity. However, in vitro studies using Franz diffusion cells and confocal laser-scanning microscopy showed that these multilamellar vesicles (MLVs) were not able to pass across the stratum corneum due to the higher rigidity, and hence it was more difficult for the drug to permeate across the skin.³³ The increased rigidity (ie, decreased elasticity) of the ethosomal vesicles upon the addition of cholesterol was also reported by other researchers.^{24,65}

Dicetyl phosphate

Dicetyl phosphate is commonly used to prevent aggregation of the vesicles and enhance the stability of the formula. It is used at concentrations between 8% and 20% of the total phospholipid concentration in the ethosomal formulation. Maestrelli et al reported that all ethosomal formulations containing dicetyl phosphate produced vesicles with sharply negative ζ -potential.⁶⁶ However, the effects of dicetyl phosphate on other ethosomal system properties are still unclear.

Stearylamine

Stearylamine is a positive-charge agent, and has been used in ethosomal formulations in two studies. The first study involved an ethosomal system consisting of phosphatidylcholine:cholesterol:stearylamine at a molar ratio of 2:1:1 and loaded with mycophenolic acid. The addition of stearylamine to the ethosomal formulation caused a great increase in vesicular size and decrease in entrapment efficiency, and change in the ζ -potential charge from negative to positive which lead to aggregation of the vesicles within 1 week. The authors attributed all these effects to the incompatibility of the positive charge of stearylamine with the negative charges of soy phosphatidylcholine and mycophenolic acid.²⁴ The

second study compared the in vitro permeation of two types of ethosome-encapsulated vancomycin hydrochloride with and without stearylamine. The quantity and the transdermal flux of the drug delivered after 12 hours of the negatively charged ethosomes was found to be significantly higher than the positively charged ethosomes. The lower permeation of the positively charged ethosomes was due to the shielding effect of stearylamine and the competition of the positively charged stearylamine with positively charged vancomycin hydrochloride. Stearylamine penetrates the skin easily because it has smaller molecular weight (296.5 Da) compared to vancomycin hydrochloride (1,458.7 Da). Therefore, stearylamine will cross the skin more easily than vancomycin hydrochloride.⁶⁷

Other alcohols

Other alcohols, such as PG and IPA, are also used in the preparation of binary ethosomes along with ethanol.

Propylene glycol

PG is a widely used penetration enhancer. It is used in the preparation of binary ethosomes at a concentration range of 5%–20% and found to influence the ethosomal properties of size, entrapment efficiency, permeation, and stability. Incorporation of PG in ethosomal systems will lead to further reduction in particle size in comparison to systems without PG. Significant decrease in particle size from 103.7±0.9 nm to 76.3±0.5 nm was achieved when PG concentration was increased from 0% to 20% v/v.¹³

Zhou et al observed that the entrapment efficiency of sophoridine, matrine, sophocarpine, and lehmamine extracted from *Sophora alopecuroides* increased significantly when PG was incorporated into the ethosomal system with a ratio of ethanol to PG of 1:1 (total alcohol 45%).¹¹ Other researchers have suggested that the presence of ethanol and PG in ethosomes provides better solubility of drugs, and hence higher entrapment efficiency and improved drug distribution throughout the vesicle.¹⁴ In vitro drug-permeation studies using Franz diffusion cells found that there was no significant difference between classical ethosomes and binary ethosomes in tacrolimus deposition in the stratum corneum. It was also reported that increasing PG concentration in ethosomes from 0% to 20% v/v decreased the amount of tacrolimus deposition in the epidermis from 2.23±0.10 $\mu\text{g}/\text{m}^2$ to 1.48±0.04 $\mu\text{g}/\text{m}^2$. Additionally, vesicles containing PG only at a concentration of 30% v/v would not increase tacrolimus disposition significantly in the epidermis. In contrast, ethosomes containing ethanol only at a concentration of 30% v/v had the highest drug disposition in the epidermis.

Therefore, this finding indicated that ethanol has stronger permeation-enhancing effects than PG on stratum corneum.¹² Zhang et al investigated the effects of different weight ratios of PG to ethanol of 10:0, 9:1, 7:3, 5:5, 3:7, 1:9, and 0:10 on the in vitro skin deposition of terbinafine hydrochloride ethosomes. The total percentage of ethanol and/or PG in the ethosomal system was 45% v/w. They observed that skin deposition of terbinafine hydrochloride was decreased by increasing PG percentage in the ethosomes. This could have been due to the lower concentration of ethanol in ethosomes, which affected phospholipid bilayer solubility. The highest skin deposition of terbinafine was obtained in ethosomes containing ethanol:PG at a ratio of 7:3.¹³ Therefore, it can be concluded that the ratio of ethanol:PG should be optimized in binary ethosomes in order to get higher drug permeation. Binary ethosomes were found to be stabler than classical ethosomes when stored at 4°C.¹¹

Therefore, it is suggested that PG enhances ethosome stability by increasing the viscosity and antihydrolysis property.^{12,13}

Isopropyl alcohol

Dave et al studied the influence of IPA on the entrapment efficiency and skin permeation of a diclofenac-loaded ethosomal system. Three types of formulations were prepared: classical ethosomes containing 40% ethanol, binary ethosomes containing 20% IPA and 20% ethanol, and a vesicular system containing 40% IPA. It was found that the vesicular system containing 40% IPA had better entrapment efficiency (95%) than the binary ethosomes (83.8%). However, this vesicular system had the lowest (83.2%) in vitro drug release in 8 hours compared to binary ethosomes (85.4%) and classical ethosomes (93%). Moreover, transdermal drug-flux values through mouse skin showed that the vesicular system had the lowest flux (146 µg/cm²/h) compared to binary ethosomes (159 µg/cm²/h) and classical ethosomes (226.1 µg/cm²/h). It was concluded that IPA had a pronounced effect on entrapment efficiency but less effect on drug release.¹⁵ Further studies are required to evaluate the influence of IPA or other alcohols on other ethosomal system properties.

Edge activators or penetration enhancers

The selection of a proper edge activator or penetration enhancer is a critical step in the formulation of transethosomes, as they have profound effects on the properties of the ethosomal system. Table 3 shows a list of edge activators and penetration enhancers and their concentration used in the preparation of transethosomes.

N-Decylmethyl sulfoxide and dimethyl sulfoxide

N-Decylmethyl sulfoxide is a nonionic surfactant known to increase the permeability of some amino acids and the pentapeptide enkephalin across hairless mouse skin.⁶⁸ Ainbinder and Touitou incorporated *N*-decylmethyl sulfoxide at a concentration of 0.35%–1% w/w in an ethosomal system of 5-fluorouracil and named it Tumorep DS (tumor-repressive delivery system). The prepared transethosomes were spherical MLVs with a size of 222.2±18.1 nm. In vitro dermal delivery of 5-fluorouracil from the transethosomes and classical ethosomes was compared using porcine ear skin. Higher drug accumulation in the porcine skin was obtained from transethosomes (7.98%) compared to classical ethosomes (3.39%). An in vitro study was also conducted to compare skin delivery of transethosomes with the commercially available skin product Efudex (Valeant Pharmaceuticals, Bridgewater, NJ, USA). Both Tumorep DS and Efudex contained 5-fluorouracil 5% w/w. The results revealed that 5-fluorouracil from transethosomes accumulated threefold more (10.17%) in the skin than Efudex (3.75%).¹⁸ Dimethyl sulfoxide is a well-established penetration enhancer used in topical pharmaceutical formulations.⁶⁹ Ainbinder and Touitou incorporated 10% dimethyl sulfoxide in transethosomes of 5-fluorouracil instead of 1% w/w *N*-decylmethyl sulfoxide in Tumorep DS. The in vitro study results showed that the transethosomes had 2.3 times lower drug accumulation in the skin and 2.9 times higher permeation (109.23±12.35 µg) of the drug across the skin than the classical ethosomes (38±8.86 µg).¹⁸

Tweens and Spans

Tween 80 is used at concentrations of 10%–50% of total phospholipid concentration in the ethosomal system. Incorporation of Tween 80 in ethosomal systems was reported to reduce vesicular size and enhance system stability and skin-permeation properties. The effects of Tween 80 on the ethosomal system are mainly due to its solubilizing property and the prevention of vesicle fusion.^{17,19,24,27,70} Shen et al used Tween 60 in the formulation of transethosomes of artesunate and febrifugine. However, the formulations were unstable, as needle crystals formed in the formula after 5 days.²⁷ Bragagni et al introduced Tween 20 in an ethosomal system of celecoxib at 15% of the total phospholipid concentration. It was found that Tween 20-containing transethosomes had smaller vesicular size (258.4±3.3 nm), higher entrapment efficiency (54.4%), and better ex vivo skin permeation through human skin compared to transethosomes containing Tween 80 or sodium cholate.¹⁹ In another study, addition of

Table 3 Edge activators or penetration enhancers used in the preparation of transethosomes

Edge activator/penetration enhancer	Type	Concentration	Reference(s)
N-Decylmethyl sulfoxide	Nonionic surfactant	0.35%–1% of the total ethosomal system	18
Dimethyl sulfoxide	Penetration enhancer	10% of the total ethosomal system	18
Tween 80	Nonionic surfactant	10%–50% of the total phospholipid concentration in the ethosomal system	17, 19, 24, 27, 70
Tween 60	Nonionic surfactant	Up to 50% of the total phospholipid concentration in the ethosomal system	27
Tween 20	Nonionic surfactant	15%–50% of the total phospholipid concentration in the ethosomal system	19, 27
Oleic acid	Penetration enhancer	0.5%–3% of the total ethosomal system	17, 25, 29
L-Menthol	Penetration enhancer	5% of the total ethosomal system	25
Sodium stearate	Anionic surfactant	Phosphatidylethanolamine:cholesterol:sodium stearate at a molar ratio of 2:1:2.5	23
Deoxycholic acid	Bile acid/anionic surfactant	Phosphatidylcholine:cholesterol:deoxycholic acid at molar ratios of 2:1:1 and 6:2:1	24
Sodium deoxycholate	Bile salt/anionic surfactant	0.8% w/v of the total ethosomal system	29
Sodium cholate	Bile salt/anionic surfactant	0.66% of the total ethosomal system	19
Sodium taurocholate	Bile salt/anionic surfactant	0.53% of the total ethosomal system	17
Polyethylene glycol 4000	Surfactant	Phosphatidylcholine:cholesterol:polyethylene glycol 4000 at molar ratios of 2:1:1 and 6:2:1	24
Hexadecyltrimethylammonium bromide	Cationic surfactant	1% of the total ethosomal system	20
Cremophor EL-35	Nonionic surfactant	0.5%–1.5% of the total ethosomal system	20
Cremophor RH-40	Nonionic surfactant	Up to 50% of the total phospholipid concentration in the ethosomal system	27
Spans 80, 60, 40, 20	Nonionic surfactant	Up to 50% of the total phospholipid concentration in the ethosomal system	19, 27, 30
Skin-penetrating and cell-entering (SPACE) peptide	Penetration enhancer	2–10 mg/mL	21, 22
Sodium dodecyl sulfate	Anionic surfactant	0.8% of the total ethosomal system	26, 29

Tween 20 formed an unstable formulation.²⁷ Spans 80, 60, and 40 were not successful in producing homogeneous and stable transethosomes.^{19,27} Only Span 20 was used successfully in the preparation of transethosomes of caffeine and vitamin E.³⁰

Oleic acid

Oleic acid influences vesicular size, elasticity, ζ -potential, and skin-permeation properties by increasing the fluidity of the stratum corneum.^{17,25,29} Song et al used oleic acid as a penetration enhancer at a concentration of 0.5% and found that transethosomes of voriconazole containing oleic acid were smaller and more elastic than transethosomes containing Tween 80 or sodium taurocholate. Moreover, the transethosomes containing oleic acid had higher negative ζ -potential, skin-permeation rate, and drug disposition in the epidermis/dermis.¹⁷ Ma et al found that the entrapment efficiency of imiquimod in transethosomes containing oleic acid (85.38%±1.4%) was significantly higher than classical ethosomes (41.93%±2.1%).²⁹

L-Menthol

L-Menthol was added as a penetration enhancer to transethosomes of ascorbic acid at a concentration of 5%. In vitro studies showed that the cumulative percentage of drug release after 24 hours through a human skin cadaver was higher for transethosomes containing L-menthol (36.5%) than classical ethosomes (33.55%). The authors attributed this effect to L-menthol, which formed a eutectic mixture with the drug, which in turn enhanced drug solubility and altered the barrier properties of the stratum corneum.²⁵

Sodium stearate

Sodium stearate was incorporated in an ethosomal system of 5-aminolevulinic acid consisting of phosphatidylethanolamine:cholesterol:sodium stearate at a ratio of 2:1:2.5. Sodium stearate was used in an attempt to raise the negative charge of the vesicular surface and increase system stability. The addition of sodium stearate caused three main effects: 1) reduced the vesicles from 132.6±1.9 nm to 126.4±2.8 nm, which suggested the insertion of carbon chains of sodium

stearate into the lipid bilayers; 2) increased the negative surface charge from -53.0 ± 0.8 mV to -70.06 ± 1.9 mV; and 3) increased the entrapment efficiency of 5-aminolevulinic acid from $9.09\% \pm 4.02\%$ to $66.42\% \pm 0.34\%$, which was due to the interaction between the drug and sodium stearate in the lipid bilayers.²³

Bile acids and salts

Deoxycholic acid is a bile acid used in the preparation of mycophenolic acid transethosomes and has been found to cause significant increase in vesicular size (ie, from 258 ± 5 to 546 ± 33 nm) and entrapment efficiency, which could be due to high interbilayer distance caused by electrostatic repulsive force. Moreover, it also improved ethosomal system stability, which could be due to an increase in the negative surface charge of the vesicle, and hence prevented vesicular aggregation.²⁴ The bile salt of sodium deoxycholate was used in formulating transethosomes of imiquimod at a concentration of 0.8% w/v. It was found to decrease ethosomal size significantly – from 190.6 ± 10 nm to 92.7 ± 8.7 nm – and to increase ζ -potential and entrapment efficiency from -19.9 ± 2.7 mV to -28 ± 2.4 mV and $41.93\% \pm 2.1\%$ to $70.86\% \pm 1.2\%$, respectively.²⁹ Sodium cholate was used for the preparation of transethosomes of vitamin E and caffeine. The system was composed of soy phosphatidylcholine:sodium cholate at a molar ratio of 3.75:1, and it was found to increase the value of the negatively charged ζ -potential of the vesicles, suggesting better stability.³⁰ In another study, it was used at a concentration of 0.66%, but physically unstable vesicles were formed in <24 hours.¹⁹ Sodium taurocholate was used at a concentration of 0.53% in the preparation of voriconazole-loaded transethosomes and shown to cause higher negative ζ -potential, stabler formulation, better vesicle elasticity, and in vitro permeability through hairless mouse skin compared to classical ethosomes, traditional liposomes, or transfersomes.¹⁷

Polyethylene glycol 4000

Only one study has used polyethylene glycol 4000 in transethosomes of mycophenolic acid: these consisted of phosphatidylcholine:cholesterol:polyethylene glycol 4000 at molar ratios of 2:1:1 and 6:2:1. The authors reported that it increased vesicular size, but no results were shown regarding entrapment efficiency, permeation properties, or stability of the ethosomal system.²⁴

Hexadecyltrimethylammonium bromide

Meng et al studied the effects of a cationic surfactant hexadecyltrimethylammonium bromide on transethosomes of

testosterone propionate. They found that increasing the concentration of this surfactant from 0% to 1% w/w would decrease vesicle size and change vesicular charge from negative (-1.22 ± 0.89 mV) to positive (9.39 ± 1 mV). It was noted that the addition of only 0.2% w/w of hexadecyltrimethylammonium bromide was sufficient to change the ζ -potential of ethosomal system.²⁰ They suggested that this positively charged ζ -potential may enhance the skin-permeation properties of the ethosomal system, due to attraction with the negatively charged skin surface.

Cremophor

Cremophor is the brand name of a range of nonionic polyethoxylated detergents. Cremophor EL-35 was used in an ethosomal system of testosterone propionate at concentrations of 0.5%–1.5% w/w. It was found to reduce vesicular size and to increase the solubility of the drug and entrapment efficiency.²⁰ Shen et al used Cremophor RH-40 in the formulation of transethosomes of artesunate and febrifugine; however, the vesicles were unstable and needle crystals had formed after 5 days.²⁷

Skin-penetrating and cell-entering peptide

Skin-penetrating and cell-entering (SPACE) peptide is a skin-penetration enhancer discovered through phage display and shown to deliver short interfering RNA (siRNA) and streptavidin into the skin after direct chemical conjugation.⁷¹ This penetration enhancer was incorporated in transethosomes for the delivery of hyaluronic acid and siRNAs.^{21,22} The authors named these transethosomes SPACE ethosomal system.

The in vitro human skin penetration of hyaluronic acid transethosomes was 7.8 \pm 1.1-fold higher than buffer solution, 5.9 \pm 2.5-fold higher than buffer–ethanol solution, and 3.2 \pm 0.6-fold higher than classical ethosomes. The penetration enhancement from the SPACE-peptide transethosomes was dependent on the concentration of the SPACE peptide in the formula.²¹

siRNA-loaded transethosomes were named SPACE peptide-decorated cationic ethosomes (DOTAP-SPACE ethosomal system). The formulation contained SPACE-conjugated siRNA encapsulated in a carrier made up of a cationic lipid (DOTAP), cholesterol, SPACE-conjugated lipids (SPACE peptide – POPE) and free SPACE peptide; all were suspended in buffer–ethanol solution 45% v/v. The SPACE peptide was at three places: direct conjugation to siRNA, direct conjugation to the vesicular surface, and in free form. In vitro porcine skin-penetration studies showed that the penetration of siRNA transethosomes (ie, DOTAP-SPACE

ethosomal system) was 6.3 ± 1.7 -fold higher than the penetration of siRNA aqueous solution.

Sodium dodecyl sulfate

Sodium dodecyl sulfate is an anionic surfactant and has been used at a concentration of 0.8% w/v in the preparation of transethosomes of ketoconazole²⁶ and imiquimod.²⁹ The results showed that sodium dodecyl sulfate significantly reduced the size, increased the entrapment efficiency and the ζ -potential, and enhanced the *in vitro* and *in vivo* skin-permeation properties of ethosomal systems.

Drug/agent effects on ethosomal system properties

The most important factor to consider in ethosomal formulations is the nature or physicochemical properties of the drug/agent that is going to be incorporated. This is because the drug/agent may affect the properties of the ethosomal systems, especially particle size and ζ -potential.

Lodzki et al observed the vesicular size of an ethosomal system decreased when trihexyphenidyl hydrochloride was incorporated in the system. The average diameter of the blank ethosomes was 154 ± 15 nm and decreased to 109 ± 6 nm after drug loading. The authors attributed this effect to the surface-active properties of the incorporated drug.⁴³ Reduction in vesicular size has also been reported in ethosomal systems loaded with buspirone hydrochloride,⁷² cromolyn sodium,³⁶ and diclofenac sodium.⁶³

In contrast, Paolino et al noticed a significant increase in vesicular size following the incorporation of paclitaxel in an ethosomal system, and this increment was profound with certain formulations. For example, the mean size of blank ethosomes was 131 ± 13 nm and increased to 751.3 ± 38.34 nm after loading the drug.⁵⁰

In general, ethosomal vesicles tend to have a negative charge. However, some researchers have observed that the drug entrapped in an ethosomal system can shift the vesicular charge from negative to positive. Shumilov and Touitou reported that the charge of an empty ethosomal system was negative (-8.8 mV) and shifted to positive (7.16 mV) after the incorporation of 30 mg buspirone hydrochloride into the ethosomal system.⁷² A similar finding was also observed with trihexyphenidyl hydrochloride 0.5% w/w, where the empty ethosomes' negative charge (-4.5 mV) shifted to a positive charge (4.8 mV). This effect was dependent on the concentration of the trihexyphenidyl hydrochloride added. Increasing the percentage of the drug to 1% and 3% w/w resulted in a corresponding increase in ζ -potential values of

7.2 mV and 10.4 mV, respectively.⁴³ The vesicular charge shifting from negative to positive was also reported with an ethosomal system of a synthetic lipophilic prodrug of acyclovir (ACV- C_{16}). This effect was attributed to the addition of a carbonyl group during the synthesis of the prodrug. The carbonyl group acted as an electron-withdrawal group, and hence resulted in ACV- C_{16} ethosomes being positively charged. On the contrary, the ζ -potential of the active acyclovir (nonprodrug)-loaded ethosomal system mentioned in the same study was negative (-22.3 ± 1.7 mV).⁴¹ Rakesh and Anoop reported that the incorporated drug could further increase the negative value of the ζ -potential. The ζ -potential of the blank vesicles was -9.11 ± 1.5 mV and increased to -63.42 ± 1.3 mV after the incorporation of 1% w/v cromolyn sodium.³⁶ A similar phenomenon was also observed between blank and paclitaxel-loaded ethosomal systems.⁵⁰

Preparation techniques and their effects on ethosomal properties

The classical cold method

This is the simplest and most widely used method for the preparation of ethosomal systems, and if required it could be done under nitrogen protection.⁶⁴ It was introduced by Touitou in 1996,⁵ and involves the preparation of the organic phase and aqueous phase separately. The organic phase is obtained by dissolving the phospholipids (in addition to surfactants or penetration enhancer for transethosomes) in ethanol or mixture of solvents (ethanol/PG) for the preparation of binary ethosomes,¹³ at room temperature,⁷³ or at 30°C .⁶ The aqueous phase used is either water,^{44,74,75} buffer solution,^{76,77} or normal saline solution.⁷⁸ The aqueous phase is added to the organic phase in a fine stream,⁶ dropwise,⁴¹ or using a syringe pump at a constant rate of 1^{75} or $200 \mu\text{L}/\text{min}$.⁶⁴ The mixture is stirred at a speed of 700 – $2,000$ rpm,^{6,75,79,80} using an overhead⁸¹ or magnetic⁸² stirrer. The mixing is done for 5 – 30 minutes^{75,83} to obtain the required ethosomal suspension. The drug to be incorporated in the ethosomal system will be dissolved in either the aqueous or the organic phase, depending on its physicochemical properties. Figure 3 presents the preparation steps of an ethosomal system using the classical cold method.

The ethanol injection–sonication method

In this method, the organic phase containing the dissolved phospholipid in ethanol is injected to the aqueous phase, using a syringe system,³⁸ at a flow rate of $200 \mu\text{L}/\text{min}$, then homogenized with an ultrasonic probe for 5 minutes.³⁴

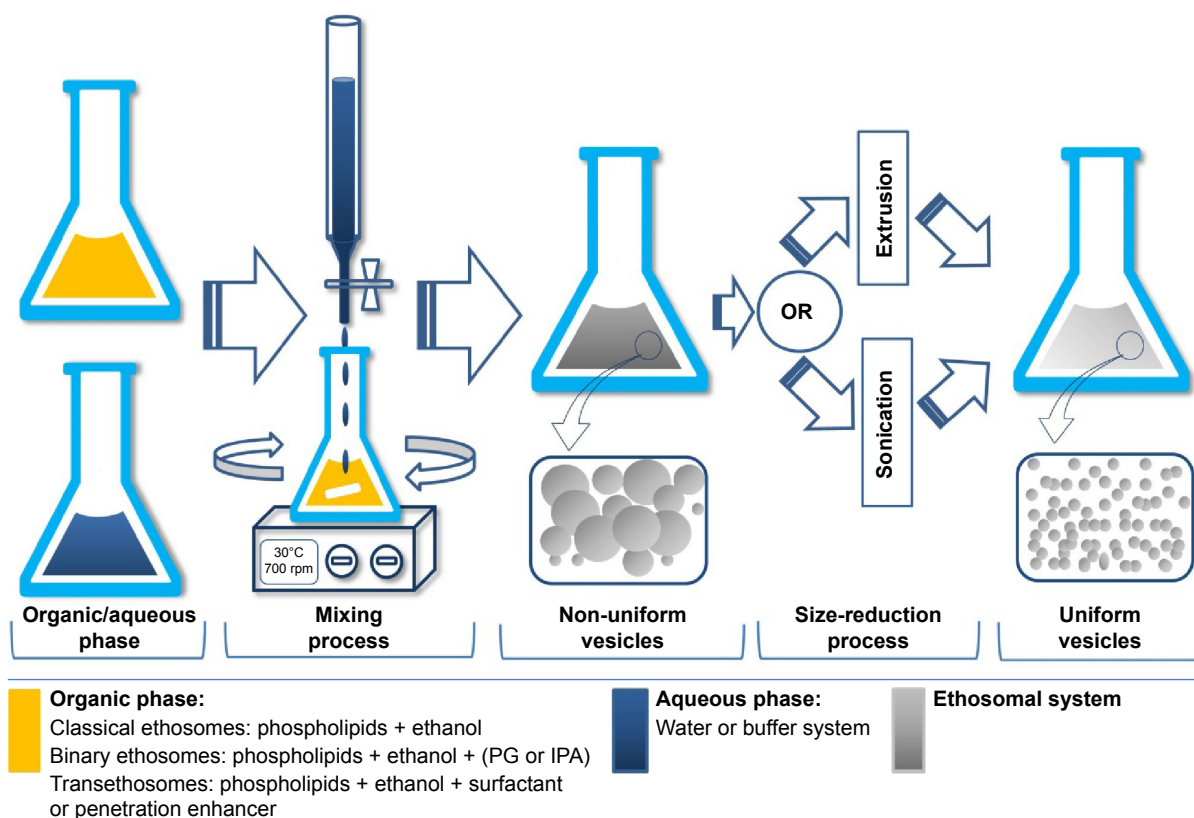


Figure 3 The classical cold method for the preparation of ethosomal systems.
Abbreviations: PG, propylene glycol; IPA, isopropyl alcohol.

The hot method

This method was first described by the inventor of ethosomes in 1996.⁵ In one vessel, phospholipid is dispersed in water and then placed in a water bath at 40°C until a colloidal suspension is obtained. In another vessel, ethanol is heated to 40°C and then added dropwise to the phospholipid dispersion under continuous mixing using a mechanical or magnetic stirrer.⁸⁴ The drug is dissolved in either the organic or aqueous phase, based on its hydrophilic/hydrophobic properties.

The thin-film hydration method

This represents the extension of the conventional liposome-preparation method, but in this method the lipid film is hydrated by a hydroethanolic solution. The phospholipid is first dissolved in chloroform only⁸⁵ or a chloroform–methanol mixture at ratios of 3:1¹⁰ or 2:1⁸⁶ in a clean, dry, round-bottom flask. Organic solvents are removed by a rotary vacuum evaporator at a temperature above the lipid-phase transition temperature. Then, the traces of the solvents are removed from the deposited lipid film under vacuum overnight. The lipid film is then hydrated with a water–ethanol solution³³ or phosphate buffered saline–ethanol solution.⁸⁷ During the hydration process, the lipid film is rotated and heated at the

required temperature, which depends on the phospholipid property, for 30 minutes,²³ 1 hour,⁸⁸ or 6 hours.⁸⁶

The reverse-phase evaporation method

This is the least used method and specially designed to produce large unilamellar vesicles. The organic phase is prepared by dissolving the phospholipid in diethyl ether and then mixing it with the aqueous phase at a ratio of 3:1 v/v in an ultrasonic bath at 0°C for 5 minutes to form a water-in-oil emulsion. The organic solvent is removed under reduced pressure to produce a gel, which turns into a colloidal dispersion upon vigorous mechanical agitation.⁶⁶

Transmembrane pH-gradient method

In all the methods just mentioned, the drug is added in either the organic or the aqueous phase, and it is spontaneously or “passively” loaded in the ethosomal system. In the transmembrane pH-gradient method, the drug is loaded “actively”, based on the pH-gradient difference between the acidic interior of the internal phase and the basic exterior of the external phase of the ethosomal system. The concept of this method was first applied in the preparation of liposomes,^{89,90} then it was used by Zhou et al¹¹ and Fan et al⁹¹

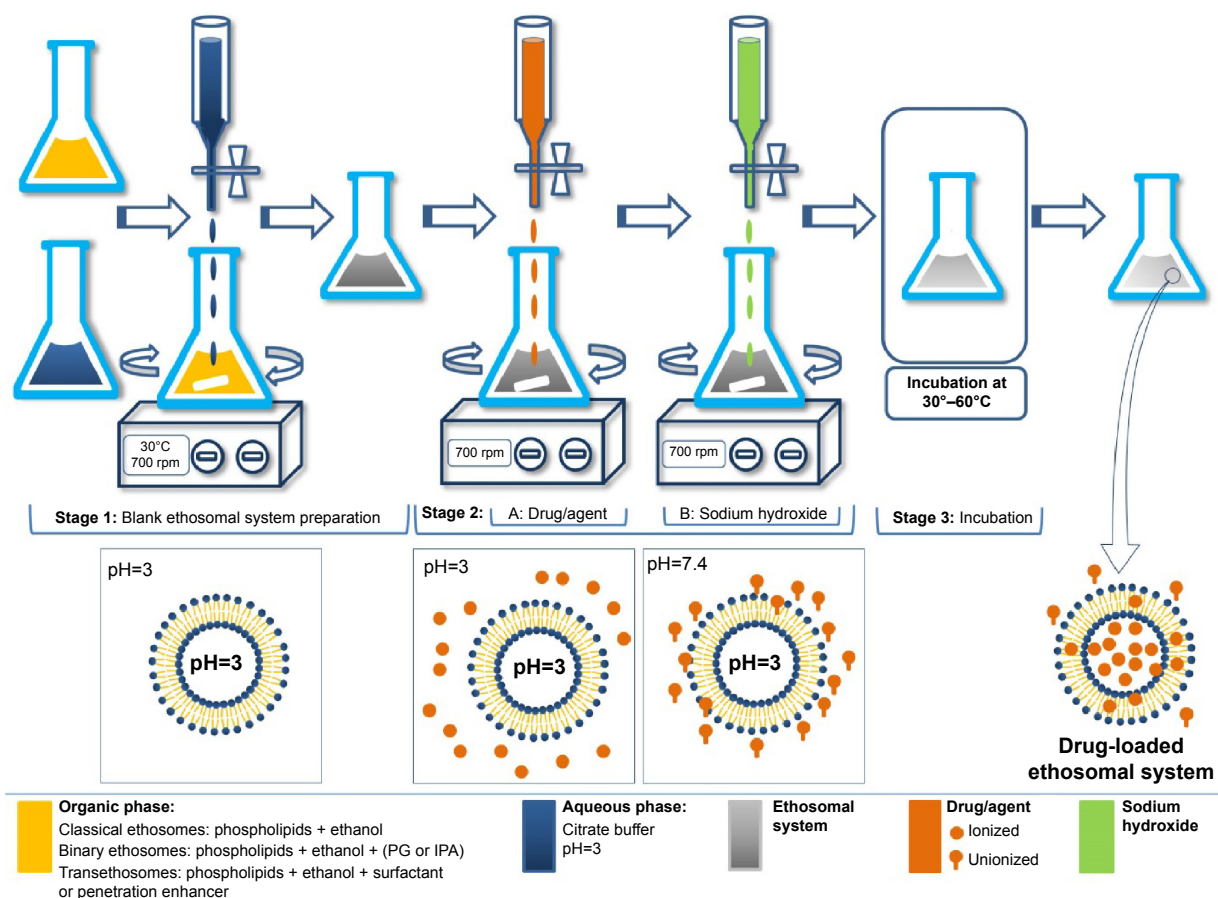


Figure 4 The transmembrane pH-gradient method for the preparation of ethosomal systems.
Abbreviations: PG, propylene glycol; IPA, isopropyl alcohol.

in the preparation of ethosomal systems of total alkaloid extracts of *S. alopecuroides* and tetrandrine, respectively. The method is suitable only for water-soluble drugs that have protonizable amine functions. This method involves three stages: preparation of the blank ethosomal system, active loading of the drug, and incubation (last stage).

In the first stage, the empty ethosomal suspension is prepared using any of the aforementioned methods, but the aqueous phase or the hydration process uses an acidic buffer (usually citrate buffer, pH 3). The second stage involves the active loading of the drug into the empty ethosomal suspension, followed by continuous stirring. In order to make the external phase more alkaline and to establish the pH gradient between the acidic internal (pH 3) and basic external phases of the ethosomal system, an alkali, usually a sodium hydroxide solution of 0.5 M, is added to make the external pH 7.4. In the third stage, the ethosomal system is incubated at a specified time and temperature (30°C–60°C) to give the opportunity for the unionized drug to actively pass the bilayer of the ethosomal vesicles and get entrapped.¹¹ Before preparation of ethosomal systems using this method,

some factors need to be taken into consideration, such as the physiochemical properties of the drug/agent to be incorporated, the pH of the internal and external phases, and the temperature and the duration of the incubation period. The different steps of ethosomal preparation using this method are illustrated in Figure 4.

Ethosomal system size and lamellarity

Ethosomal nanocarriers are especially designed for topical and transdermal drug delivery, and hence the size and lamellarity of these carriers are of high importance. The size of these vesicles should be reduced to <200 nm or 300 nm to be suitable for this route of administration.^{14,40,92} Therefore, in all the aforementioned methods except the reverse-phase evaporation method, the prepared ethosomal systems are subjected to different further processing to get the required size or lamellarity.

Generally, there are two main techniques used to reduce the size of ethosomal systems: extrusion and sonication processes. Homogenization is sometimes employed, but

practically it has no effect in reducing vesicular size. The extrusion process is usually done by sequentially extruding the ethosomal system under relatively low pressure for certain times or cycles through polycarbonate membranes of different pore sizes of 50–400 nm. The extrusion is done by either multiple extrusions through one membrane with one pore size or sequential extrusion of the ethosomal system through stacked membranes of different pore sizes. The smaller the pore size of the membrane, the smaller the diameter of the ethosomal system produced. Gu et al reported that the mean sizes of 5-fluorouracil ethosomes extruded through membranes with pore sizes of 50 nm and 100 nm were 60 ± 10 nm and 110 ± 13 nm, respectively.⁹³ Many commercially available extruders are used for the extrusion process, such as the Lipex[®] extruder (Transferra Nanosciences, Burnaby, BC, Canada), a hand extruder (EMD Millipore, Billerica, MA, USA),⁶⁵ the Gastight 1001 extruder (Hamilton, Reno, NV, USA),⁴⁸ the Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA),⁹⁴ and disposable filters.⁸⁰ The selection of membrane type, pore size, pressure, number of extrusions, filtration times, or cycles should be optimized according to the required properties of the ethosomal system. The result of this process is the production of more uniform ethosomal colloidal suspension of small unilamellar vesicles (SUVs) with lower polydispersity index values.

The sonication method uses either the probe sonicator (the most common)⁴⁰ or the ultrasonic bath.⁹ This method produces uniform ethosomal suspension of SUVs. Some parameters in this method, ie, sonicator type, voltage, temperature, probe types, time, and number of cycles, should be optimized according to the ethosomal system components and the properties required. Maestrelli et al observed that the size of SUVs obtained by sonication was smaller (189.3 ± 13.7 nm) compared to extrusion (295.8 ± 33.2 nm).⁶⁶ Furthermore, Chen et al reported that the entrapment efficiency of triptolide was increased by increasing the sonication time till it reached a point that had no significant effect.⁹⁵ However, the entrapment efficiency of SUVs obtained by extrusion was higher than that by sonication, which may be attributed to the bigger vesicles.⁶⁶

There is no clear rule for the preparation of ethosomal vesicles with specific lamellarity; generally, all the preparation techniques discussed previously lead to the production of vesicles with diverse sizes and lamellarities ie, MLVs, oligolamellar vesicles, large unilamellar vesicles, and SUVs. Application of size-reduction techniques lead mainly to the production of SUVs. Large unilamellar vesicles are produced by the reverse-phase evaporation method. Frozen and thawed MLVs, which

are bigger than MLVs, are produced by exposing MLVs to a number of alternative cycles (each of 30 seconds) of freezing using liquid nitrogen and thawing in a bath at 58°C. Frozen and thawed MLVs show lower entrapment efficiency than MLVs despite their size, and they become smaller during storage, because they are less stable.⁶⁶

Ethosomal dosage forms

The majority of the published articles have studied ethosomal systems in their initial suspension form. Ethosomal suspension contains a high concentration of alcohol, and thus further incorporation of the system in a suitable vehicle for dermal/transdermal delivery has some advantages, ie, preventing ethanol evaporation, prolonging contact time with the skin, enhancing the therapeutic efficacy of the entrapped drug, improving stability and shelf life of the final dosage form, and patient compliance. Ethosomal systems have been incorporated in different vehicles to produce novel pharmaceutical formulations, such as ethosomal gels, transdermal patches, and creams.

Ethosomal gels

Ethosomal gels are characterized for their pH, viscosity, spreadability, and extrudability. The most commonly used gel-forming agents for incorporating ethosomal systems are Carbopol and hydroxypropyl methylcellulose with all their related grades. These polymers have been shown to be compatible with ethosomal systems, providing the required viscosity and bioadhesive properties. The types of polymers used in the preparation of ethosomal gels and their concentrations are listed in Table 4.

Several researchers have studied the skin-permeation and disposition properties of drugs from ethosomal gels in comparison to the traditional or marketed gels or creams. Puri and Jain compared the *in vitro* skin-permeation properties of 5-fluorouracil from ethosomal gel and marketed cream using Franz diffusion cell and albino rat skin and found that the transdermal flux of 5-fluorouracil from ethosomal gels was 4.9-fold higher than the marketed cream. Moreover, the skin disposition of the drug from the ethosomal gel was 9.4-fold higher than the marketed cream.³² Other researchers found that *in vitro* transdermal flux of aceclofenac from ethosomal gel was higher ($226.1 \mu\text{g}/\text{cm}^2/\text{h}$) than Zynac gel ($131.1 \mu\text{g}/\text{cm}^2/\text{h}$).¹⁵ Some authors have reported the same superior properties of ethosomal gels for different drugs/agents over traditional gels.^{14,18,96} Interestingly, it was found that the drug-release rate from the ethosomal suspension was faster than from ethosomal gel, due to the high viscosity of the gel.^{97–99}

Table 4 Types and concentrations of gel-forming agents used in ethosomal gel preparations

Drug/agent	Gel-forming agent		Reference(s)
	Name	Concentration	
Amphotericin B	Carbopol 934	1% w/w	100
Econazole nitrate	Carbopol 934	1% w/w	40
Ketoconazole	Carbopol 934	2% w/w	84
Lopinavir	Carbopol 934	0.8% w/v	35
Losartan	Carbopol 934	0.75% w/v	96
Meloxicam	Carbopol 934	1% w/w	101
Testosterone	Carbopol 934	NM	102
Tretinoin	Carbopol 934	1.5% w/w	78
Valsartan	Carbopol 934	NM	51
5-Fluorouracil	Carbopol 934P	1.5% w/w	32
Aceclofenac	Carbopol 934P	0.75% w/v	15
Azelaic acid	Carbopol 934P	0.75% w/w	97
Methoxsalen	Carbopol 934P	2% w/w	103
Vinpocetine	Carbopol 934P	1% w/w	104
Clotrimazole	Carbopol 934LR	1% w/v	14
Psoralen	Carbopol 934LR	1% w/v	92
Benzocaine	Carbopol 940	0.5% w/w	66
Black-tea extracts	Carbopol 940	NM	28
Buspirone hydrochloride	Carbopol 940	0.7% w/w	72
Capsaicin and capsicum tincture	Carbopol 940	1% w/w	105
Ligustrazine phosphate	Carbopol 940	1% w/w	106
Repaglinide	Carbopol 940	1.5% w/w	53
Valsartan	Carbopol 940	1% w/w	107
Erythromycin	Carbopol 974	1% w/w	108
Erythromycin	Carbopol 974	NM	109
Cetirizine	Carbopol 980 NF	1.25%	98
Griseofulvin	Carbopol 980 NF	0.5% w/w	110
5-Fluorouracil	Carbopol ultrez	0.8% w/w	18
Ibuprofen	Carbopol (grade NM)	NM	111
Felodipine	Hydroxypropyl cellulose	1.5% w/v	112
Fluconazole	Hydroxypropyl methyl cellulose K4M	2% w/w	113
Mitoxantrone	Hydroxypropyl methylcellulose RG4T	10% w/v	114
Salbutamol sulfate	Pluronic F127	20% w/w	39
Granisetron hydrochloride	Lutrol F127	20% w/w	115
Metronidazole	Sodium alginate	8% w/v	116
Cannabidiol	NM	NM	117
Diclofenac	NM	NM	8

Abbreviation: NM, not mentioned.

Ethosomal patches

The preparation and evaluation of ethosomal patches are more complicated than for ethosomal gels, as molds are required for their preparation. Based on a literature search, only seven research articles had reported ethosomal patch formulations for several drugs, ie, testosterone,⁶ artesunate and febrifugine,²⁷ ligustrazine,^{34,118} valsartan,³⁷ tizanidine hydrochloride,¹¹⁹ and insulin.¹²⁰ Different polymers were used to prepare the ethosomal patches, including polyvinylpyrrolidone/vinyl acetate, acrylic resin, and hydroxypropyl methylcellulose E15. Triethyl citrate was added to the formulation as a plasticizer.

Touitou et al⁶ compared the in vitro and in vivo transdermal delivery properties from ethosomal patches and the nonethosomal patches of testosterone marketed as Testoderm; both patches had the same dimensions and drug contents. In vitro studies were done using Franz diffusion cells and rabbit pinna skin taken from the ear. The results showed that 24 hours after application, the amount of testosterone permeated from the ethosomal patches was 30 times higher ($848.16 \pm 158.38 \mu\text{g}$) than the Testoderm patches ($27.79 \pm 16.23 \mu\text{g}$), while drug deposition in the skin was seven times higher from the ethosomal patch than the Testoderm patch.

The *in vivo* studies of testosterone ethosomal patches and Testoderm were conducted in two stages using rabbits. In the first stage, the serum concentrations of the drug were measured at different time intervals up to 24 hours, after a single application of both patches in the central area of the rabbits' inner ears. In the second stage, the total serum concentrations of the drug were measured after multiple applications of both patches for a period of 5 days (a new patch was applied every 24 hours). The results of the first stage showed that there was no significant difference in serum drug concentration between the control and treated groups. However, in the second stage, the area under the concentration–time curve of the groups treated with ethosomal patches was significantly higher (125%) than the groups treated with Testoderm.⁶

Ethosomal patches have some advantages compared to ethosomal gels and creams because they offer the application of ethosomes under occlusive conditions, and hence more permeation is expected. Godin and Touitou studied the *in vitro* permeation behavior of bacitracin ethosomes using human cadaver skin under occlusive and nonocclusive applications. They found that there was no significant difference in drug permeation from ethosomes under either application method. It was concluded that the occlusion had almost no effect on the permeation behavior of the drugs from ethosomes.¹²¹ Similar findings related to this subject suggested that ethosomes are able to enhance the dermal delivery of drugs under occlusive and nonocclusive conditions.^{6,13,122}

Ethosomal creams

There have only been two studies reporting the formulation of ethosomal creams. Both of these involved the incorporation of *Curcuma longa* extract-loaded ethosomal systems in a cream base as a photoprotective and antiwrinkle agent.^{79,123} In both studies, *C. longa* extract-loaded ethosomal creams were applied to human volunteers and showed promising results as either a photoprotective⁷⁹ or an antiwrinkle agent.¹²³

Based on all the aforementioned studies, the incorporation of ethosomal systems in suitable vehicles such as gels, patches, and creams improves skin permeation of the drug/agent from the ethosomal systems. Among the vehicles discussed, gels are the most suitable vehicle for the incorporation of ethosomal systems, while ethosomal creams may be preferred for cosmetic preparations.

Mechanisms of ethosomal system skin permeation

Ethanol and phospholipids are reported to act synergistically to enhance the skin permeation of drugs in ethosomal

formulations. Ethanol fluidizes the lipid bilayers of the ethosomal vesicles and the stratum corneum simultaneously, changing the arrangement and decreasing the density of skin lipids. Therefore, the highly malleable and soft vesicles of an ethosomal system will penetrate the altered structure of the stratum corneum and create a pathway through the skin. The release of the therapeutic agent occurs by the fusion of these vesicles into cell membranes in the deeper layers of the skin.^{6,124–128}

It is suggested that transethosomes have superior skin-permeation properties over classical ethosomes. This is because transethosomes contain both ethanol and the edge activator or the penetration enhancer, which both act together to increase vesicular malleability and skin-lipid perturbation.^{19,30} However, the detailed mechanisms of ethosomal system skin penetration are still not fully understood. A proposed mechanism is illustrated in Figure 5.

In vivo studies

Highly diverse *in vivo* studies and models have been carried out by many researchers using humans, rabbits, rats, mice, and guinea pigs for the evaluation of ethosomal systems involving skin permeation, pharmacokinetics, pharmacodynamics, safety, and skin irritation studies.

The ability of ethosomal systems to penetrate intact skin, delivering molecules with diverse physicochemical properties in therapeutic amounts to the blood circulation, has been reported by several *in vivo* studies. For instance, Ahad et al^{37,107} studied the pharmacokinetic and pharmacodynamic effects of a valsartan-loaded ethosomal system in albino Wistar rats. The bioavailability of transdermal ethosomal valsartan was significantly higher (3.03 times) than the oral valsartan suspension; the area under the concentration–time profile curve from time 0 to ∞ for the transdermal ethosomal formulation was 177,298.82±665.01 ng/mL/h, while for the oral suspension it was 55,554.54±774.01 ng/mL/h. The dose of valsartan used in both routes was 3.6 mg/kg. The reported maximum drug concentration (C_{max}) and the time taken to reach maximum concentration (T_{max}) values for the oral administration were 13,100±101.12 ng/mL and 1±0.01 hours, respectively. The C_{max} and T_{max} values for the transdermal route were 7,944±134.32 ng/mL and 5.0±0.83 hours, respectively.³⁷ Ethosomal valsartan was shown to effectively reduce blood pressure by 34.11% for 48 hours in hypertensive rats.¹⁰⁷

In vivo safety studies on humans and animals have shown that classical ethosomes are highly safe with excellent skin tolerability.^{44,64,129} Further *in vivo* safety studies are much required to evaluate the short- and long-term effects

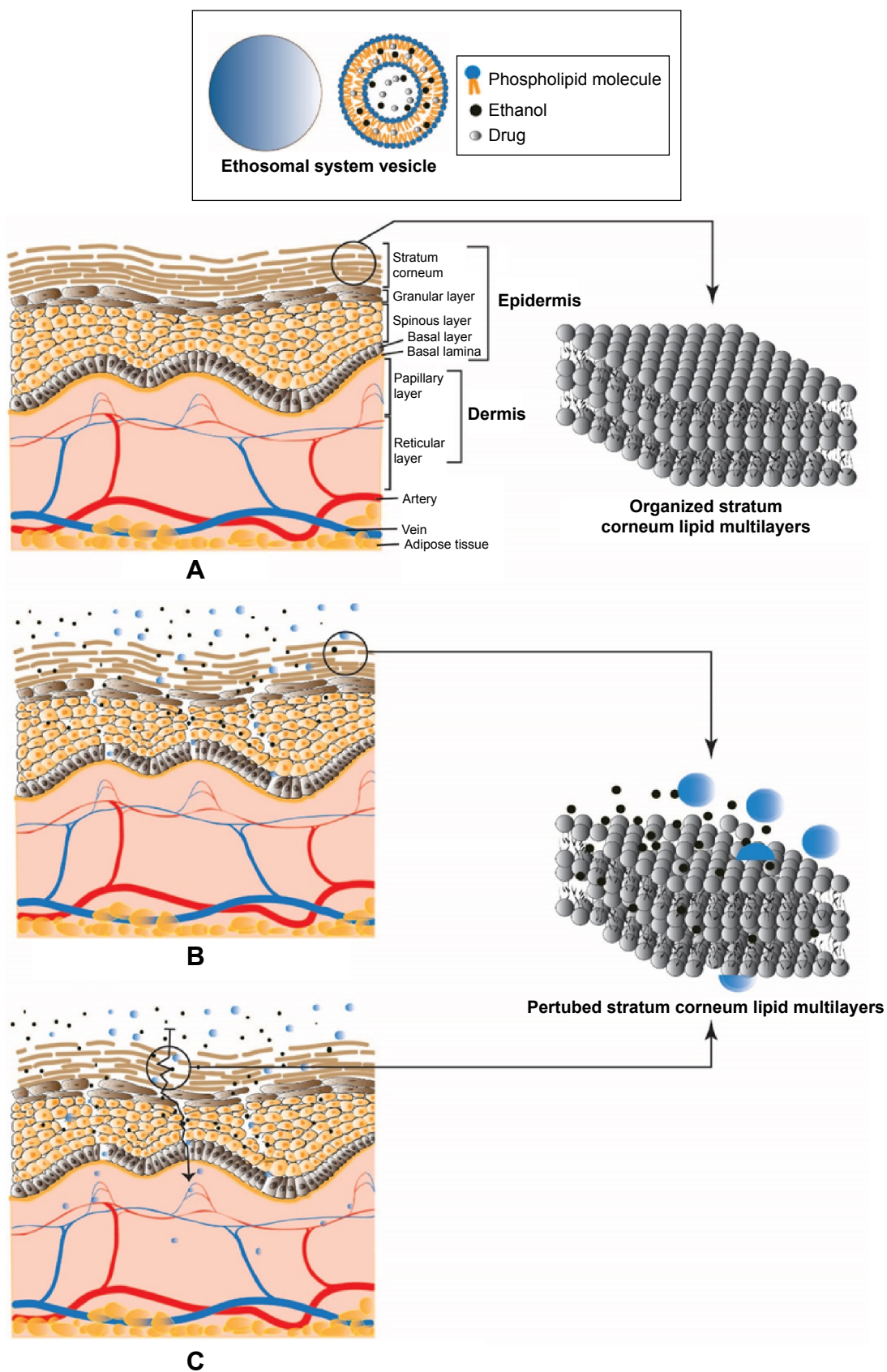


Figure 5 Proposed mechanism for permeation of molecules from ethosomal system through the skin.

Notes: (A) Normal skin; (B) Skin-lipid perturbation by ethanol effects; (C) Penetration of the soft malleable ethosomal system vesicles.

of repeated application of these nanocarriers on the skin, particularly for the other two classes of ethosomal systems (binary ethosomes and transethosomes), which contain penetration enhancers and/or edge activators in their formulation and thus may increase the possibility of developing adverse skin reactions, such as irritations or erythema. All the in vivo studies conducted on the ethosomal systems since their invention and a summary of results are shown in Table 5.^{130–134}

Clinical trials

Based on a literature searches, only three clinical trials have been conducted on ethosomal systems in human volunteers (Table 6). Horwitz et al carried out a pilot, double-blind, randomized clinical study to compare the efficacy of an ethosomal acyclovir preparation and commercially available acyclovir cream (Zovirax®) in treating recurrent herpes

labialis in 40 human volunteers. The results revealed that the ethosomal acyclovir preparation performed better than Zovirax cream and showed significant improvement in all the evaluated clinical parameters, such as the time of crust formation and disappearance and pain parameters.¹³⁵ The efficacy of ethosomal gel of clindamycin phosphate and salicylic acid was evaluated in a pilot clinical trial of 40 acne patients treated with the gel twice daily for 8 weeks. Volunteers treated with ethosomal gel showed considerable improvement in acne condition, with a decreased number of comedones, pustules, and total number of lesions compared to placebo.¹³⁶ Ethosomal preparation of prostaglandin E₁ was evaluated in a pilot clinical study in patients with erectile dysfunction. It was observed that 12 of 15 tested patients had improved peak systolic velocity and penile rigidity. Erection duration was 10–60 minutes.¹²⁵ There was no reported

Table 5 Summary of the in vivo studies

Drug/agent	Subjects/species	Aim	Model/method	Main results	DF	Reference(s)
5-Aminolevulinic acid	Nude mice	Skin penetration	Measurement of protoporphyrin IX (PpIX) by CLSM	Penetration ability of ethosomal systems was greater than that of liposomes	Susp	23
5-Aminolevulinic acid	Nude mice	Skin-penetration behavior	Hyperproliferative skin murine model/the tape-stripping technique	Significant improvement in the formation of PpIX in both normal and hyperproliferative skin	Susp	58
5-Fluorouracil	Nude mice	Evaluation of the antitumor effect	Two skin cancer models: 1. Intradermal (ID) injection of TE.354.T cells 2. ID injection of ES2 cells	Significant repression of tumor-development rate in both models compared to the commercial product	Gel	18
5-Fluorouracil	Albino rats, rabbits	Vesicle–skin interaction and drug-localization index	Fluorescence microscopy, CLSM	Ethosomal gel showed five- and tenfold deeper skin penetration than the lipogel and marker solutions, respectively	Gel	32
5-Fluorouracil	New Zealand White rabbits	Evaluation of laryngotracheal stenosis treatment	Airway-stenosis rabbit model	Ethosomal system resulted in less stenosis than the 5-fluorouracil solution alone	Susp	93
Ammonium glycyrrhizinate	Humans	Permeation, toxicity, and anti-inflammatory activity	Model of skin erythema	The ethosomal system showed very good skin tolerability, even when applied for a long period (48 hours)	Susp	64
Apigenin	SD rats + Kunming mice	Skin deposition, antiultraviolet effects	Ultraviolet B-induced skin inflammation	Ethosomes showed superior skin targeting in reducing COX-2 levels	Susp	16
Bacitracin	SD rats	Mechanisms of skin permeation	CLSM	The antibiotic delivered from ethosomes entered the skin between the corneocytes through the intercellular lipid domain	Susp	121

(Continued)

Table 5 (Continued)

Drug/agent	Subjects/species	Aim	Model/method	Main results	DF	Reference(s)
Benzocaine	Albino rabbits	Anesthetic activity	Conjunctival reflex test	The efficacy of ethosomal benzocaine was significantly improved in comparison to benzocaine solution	Gel	66
Buspirone	Wistar rats, SD rats	Pharmacokinetics, menopause syndromes	Ovariectomized rats	The drug was present in plasma for 12 hours, reaching C_{max} after 2 hours. Observed temperature alleviation ($1.6^{\circ}\text{C}\pm 0.7^{\circ}\text{C}$) to normal values after (3 hours) of administration	Gel	72
Cannabidiol	ICR mice	Anti-inflammatory effects	Carrageenan-induced inflammation	Significant accumulation of the drug in the skin and in the underlying muscle. Prevented inflammation and edema	Gel	117
Cetirizine	BALB/c-strain mice	Atopic dermatitis	Oxazolone-induced atopic dermatitis	Enhanced therapeutic performance of the ethosomal system	Gel	98
Combination of artesunate and febrifugine	Kunming mice	Antimalarial effects	Animals infected with <i>Plasmodium berghei</i>	Ethosomal cataplasm showed better antimalarial activity	Patch	27
Contact allergens: PD, DCB, and isoeugenol	CBA/Ca mice	Sensitization studies	Mouse local lymph node assay	Ethosomes were able to enhance the sensitizing capacity of the contact allergens	Susp	62
Contact allergens (isoeugenol and DCB)	CBA/Ca mice	Sensitization studies	Mouse local lymph node assay	Increased sensitizing potency compared with the allergens in solution	Susp	130
<i>Curcuma longa</i> extract	Humans	Photoprotective effects	Measuring skin hydration (Cutometer) and sebum content (Sebumeter)	Improvement in skin properties such as skin hydration and sebum content	Cream	79
<i>C. longa</i> extract	Humans	Antiwrinkle effects	Cutometer	The ethosomal cream delivered the antiwrinkle agent to the skin efficiently	Cream	123
Curcumin	SD rats	Anti-inflammatory effects	Carrageenan-induced paw edema	PG liposomes showed the highest and longest inhibition of the development of paw edema, followed by ethosomes and traditional liposomes	Susp	70
Diclofenac	SD rats	Anti-inflammatory effects	Carrageenan-induced paw edema	Significant inhibition of paw edema with ethosomal gel in comparison to liposomal and plain gels	Gel	8
DNA	CD1 nude mice	Gene delivery	CLSM	Good ethosomal gene delivery and expression in skin cells	Susp	120
Erythromycin	ICR mice	Antibacterial activities	<i>Staphylococcus aureus</i> -infected mouse skin	Therapy with ethosomal erythromycin was as effective as systemically administered drug	Gel	109

(Continued)

Table 5 (Continued)

Drug/agent	Subjects/species	Aim	Model/method	Main results	DF	Reference(s)
Erythromycin	ICR mice	Antibacterial efficiency	<i>S. aureus</i> -infected mice skin	Ethosomal system inhibited of the infection and stopped its development	Gel	108
Felodipine	Wistar albino rats	Drug release through the skin	Measuring drug plasma concentration	Enhanced transdermal delivery. Improved drug bioavailability	Gel	112
<i>Fraxinus angustifolia</i> leaf and bark extracts	CD1 mice	Antioxidant, anti-inflammatory, and wound-healing effects	12- <i>O</i> -Tetradecanoylphorbol-13-acetate-induced skin inflammation and ulceration	Highest antioxidant and anti-inflammatory effects were obtained from ethylene glycol containing vesicles rather than ethosomes and transcutol containing vesicles	Susp	131
Griseofulvin	Guinea pigs	Evaluation of antifungal activity	Guinea pig model for dermatophytosis	Complete cure of the fungal infection in 8 days for ethosomes and 14 days for liposomes	Gel	110
Hyaluronic acid	SKH1 ^{HR} strain hairless mice	Skin penetration	Fluorescence spectroscopy	A fivefold enhancement in penetration was found compared to PBS control	Susp	21
Ibuprofen	Mice, Wistar rats	Pharmacokinetics, analgesic and antipyretic effects	Tail-flick nociception mice, brewer's yeast-induced fever rat	The analgesic effect of ethosomal ibuprofen gel was comparable to oral treatment	Gel	111
Insulin	SD rats	Blood glucose levels lowering effectiveness	Diabetic rats	Up to 60% decrease in blood glucose levels	Patch	120
Imiquimod	SD rats	Skin deposition	Fluorescence spectrophotometer	Transethosomes showed significantly higher skin deposition of the drug than the marketed product Aldara [®]	Susp	29
Isoeugenol and MDG	Humans	Evaluation of the allergens induced responses	Patch-test conditions	Significantly enhanced patch-test reactions in comparison with the allergen hydroethanolic solution	Susp	61
Ketoconazole	Wistar rats	Antifungal activity	Rats with skin, induced fungal infection	Transethosomes enhanced the antifungal activity in a shorter duration of time than other vesicles	Susp	26
Lidocaine	Guinea pigs	Anesthetic activity	Pinprick tests	Shorter onset and longer duration than the liposomes or hydroethanolic solution	Susp	59
Ligustrazine	SD rats	Pharmacokinetics	Determination of plasma drug concentration by HPLC	Ethosomal system-enhanced drug absorption and bioavailability	Patch	34
Ligustrazine	SD rats	Pharmacokinetics, antimyocardial ischemic effects	Rat model of acute myocardial ischemia	Highest AUC was achieved by the ethosomal patch	Patch	118
Ligustrazine phosphate	SD rats	Anti-Alzheimer's disease effects	Scopolamine-induced amnesia model in rats	Ethosomal system is a potential alternative therapy for Alzheimer's disease	Gel	106

(Continued)

Table 5 (Continued)

Drug/agent	Subjects/species	Aim	Model/method	Main results	DF	Reference(s)
Low-molecular-weight heparin (LMWH)	Hairless mice	Pharmacokinetics	Measurement of anti-factor Xa activity as an indicator of LMWH bioactivity using Coates® colorimetric plasma assay	Better skin permeation and bioavailability of LMWH achieved with flexible liposomes than ethosomes	Susp	94
Lycopene extract from tomato	NMRI mice	Antioxidant and anti-inflammatory activities	Anthrakinone-induced ear edema animal model	Ethosomes were able to decrease the level of anthrakinone-induced ear swelling in a way that was highly comparable to the positive control	Susp	54
Matrine	SD rats	Anti-inflammatory activity	Reflection spectrophotometer	Improved anti-inflammatory activity	Susp	38
Melatonin	Albino rabbits	Skin irritation studies	Scoring degree of erythema	Good skin tolerability of the ethosomal system	Susp	44
Meloxicam	Albino Wistar rats	Anti-inflammatory activity	Carrageenan-induced rat paw-edema model	Significantly higher percentage inhibition of edema in comparison with the oral route	Gel	101
Methoxsalen	Wistar rats	Skin-photosensitization studies	UV-light exposure	Ethosomal methoxsalen showed profoundly less phototoxicity on the skin	Gel	103
Mitoxantrone	BALB/c nude mice, Wistar rats	Antimelanoma	Melanoma-bearing mice	Ethosomal mitoxantrone showed higher antimelanoma effect than the same drug solution	Gel	114
Mouse epidermal growth factor (mEGF)	C57BL/6 mice	Penetration mechanisms and biological effects	Penetration pathways and depth were observed by CLSM	Penetration was mainly through the pilosebaceous unit and partly through the intercellular domain	Susp	132
Psoralen	SD rats	Evaluation of drug release in the skin	Using skin microdialysis	Peak concentration and AUC of ethosomal psoralen were 3.37 and 2.34 times higher than psoralen tincture, respectively	Susp	55
Psoralen	SD rats	Skin deposition studies	Extracted drug determined by HPLC	Ethosomes showed enhanced psoralen permeability and targeting deep skin via intercellular and intracellular transport pathways	Susp	133
Psoralen	Rats	Investigating biocompatibility of the vesicles with the skin	Testing the skin using inverted fluorescence microscopy	Ethosomes and liposomes were found to be safe following daily application and for 7 days	Susp	129
Repaglinide	Albino Wistar rats	Antidiabetic activity	Alloxan solution-induced diabetes model	Prolonged antidiabetic effect of the drug over a significantly longer period of time in comparison with the equivalent oral dose	Gel	53
Silymarin	SD rats	Pharmacokinetics and tissue distribution of orally or IV-administered ethosomes	Using liquid chromatography with tandem mass spectrometry (LC-MS/MS)		Susp	134

(Continued)

Table 5 (Continued)

Drug/agent	Subjects/species	Aim	Model/method	Main results	DF	Reference(s)
Short interfering RNAs (siRNAs)	BALB/c mice	Drug penetration	Using the Micro BCA protein assay and KDAlert GAPDH assay kits	Tissue-distribution patterns of silymarin ethosomes and liposomes were similar, except that lung and spleen accumulation of silymarin ethosomes was higher than that of liposomes. Results confirmed the efficacy of transethosomes in delivering GAPDH siRNA into the skin.	Susp	22
Tacrolimus	BALB/c mice	Pharmacologic effects	Animal model of atopic dermatitis	Ethosomal tacrolimus displayed the lowest ear swelling compared to traditional liposomes and commercial ointment.	Susp	12
Testosterone	Rabbit pinnae	Skin permeation and blood levels	Using radioimmunoassay	Significantly higher testosterone blood levels from ethosomal patch than the marketed patch.	Patch	6
Testosterone propionate	SD rats	Bioavailability	Using a Cobas 411 analyzer	Improved absorption and bioavailability of the drug in comparison to liposomes and hydroethanolic solution.	Susp	21
Tetrandrine	Wistar rats	Antiarthritic activity	Freund's complete adjuvant-induced arthritis	Ethosomes significantly enhanced the therapeutic efficacy of tetrandrine in comparison to liposomes.	Susp	91
Tretinoin	LACA mice	Antipsoriatic activity	Mouse-tail model	Ethosomes were not of much utility for treatment of superficial skin disorders such as psoriasis.	Gel	78
Triptolide	SD rats	Anti-inflammatory activity	Methyl nicotinate-induced skin erythema model	Ethosomes reduced erythema rapidly and completely.	Susp	80
Valsartan	Albino Wistar rats	Pharmacokinetics	Drug plasma concentration determined by HPLC	A significant increase in the bioavailability of the transdermal ethosomal valsartan (3.03 times) in comparison to the oral suspension.	Patch	37
Valsartan	Albino Wistar rats	Antihypertensive activity	Methyl prednisolone acetate-induced hypertension	Better and prolonged antihypertensive activity compared to the orally administered suspension.	Gel	51
Valsartan	Albino Wistar rats	Antihypertensive activity	Methylprednisolone acetate-induced hypertension	Ethosomal gel was found to be effective, with a 34.11% reduction in blood pressure.	Gel	107
Vancomycin hydrochloride	SD rats	Antibacterial activity	Methicillin-resistant <i>S. aureus</i> -induced mediastinitis	No statistically significant difference between intramuscular vancomycin treatment and treatment conducted by combined ethosomes and iontophoretic method.	Susp	67

(Continued)

Table 5 (Continued)

Drug/agent	Subjects/species	Aim	Model/method	Main results	DF	Reference(s)
Vinpocetine	SD rats	Pharmacokinetics	Drug plasma concentration determined by HPLC	AUC and elimination half-life of transdermal administration were significantly higher than that by intragastric administration ($P < 0.01$)	Gel	104
Voriconazole	Hairless mice	Skin deposition studies	Extracted drug from the skin determined by HPLC	Enhanced skin deposition of the drug in the dermis/epidermis region compared to other formulations	Susp	17
Zidovudine	SD rats	Vesicle-skin interaction study	Histological studies using optical microscope	Ethosomes affected the ultrastructure of the stratum corneum	Susp	45

Abbreviations: AUC, area under the (plasma drug concentration–time) curve; CLSM, confocal laser-scanning microscopy; C_{max} , maximum concentration; DCB, dinitro-chlorobenzene; DF, dosage form; HPLC, high-performance liquid chromatography; IV, intravenous; MDG, methylidibromoglutaronitrile; PBS, phosphate-buffered saline; PD, potassium dichromate; SD, Sprague Dawley; Susp, suspension; UV, ultraviolet.

adverse skin reactions associated with the treatment in any of the aforementioned clinical trials.

Ethosomal systems for other applications

Some have studied ethosomal systems containing iodine for computed tomography as contrast agent for imaging applications.^{137,138} Coelho et al used ethosomes for the entrapment of new lipophilic excited-state intramolecular proton-transfer dyes for fluorescence spectroscopy.¹³⁹ In another study, a hair dye of transethosomes was developed and found to be more efficient in delivering and enhancing the adsorption of black tea extracts to the hair surface than a hydroalcoholic solution of the same extract.²⁸

Ethosomal systems for other administrations

Ethosomal systems have also been reported for other administrations, such as oral¹³⁴ and vaginal.¹¹⁶ Chang et al studied the ability of ethosomes and liposomes to enhance the oral bioavailability and tissue distribution of silymarin in an in vivo study. Pharmacokinetic results revealed that both

vesicular systems (ie, ethosomes and liposomes) were able to improve the bioavailability of silymarin, but better results were gained from liposomes. For tissue distribution, it was found that the silymarin-loaded ethosomes had high accumulation in the lung. The authors suggested that ethosomes may have the potential to be used as a lung-targeting drug carrier for the treatment of lung diseases.¹³⁴ Furthermore, the in vitro permeation of an ethosomal gel of metronidazole intended for vaginal delivery was evaluated by Mbah et al using Franz diffusion cells and regenerated cellulose semipermeable membrane. The ethosomal gel demonstrated sustained delivery of metronidazole, with a maximum flux of $143.67 \pm 2.73 \mu\text{g}/\text{cm}^2/\text{h}$.¹¹⁶ However, in vivo studies are required to evaluate the suitability of ethosomal systems for this route of administration.

Stability of ethosomal nanocarriers

Stability studies in terms of vesicular structure and size distribution of ethosomal systems containing various drug entities in their initial suspension form or after their incorporation in a particular dosage form, such as gels, patches, or creams, have been conducted by many

Table 6 Summary of the clinical trials

Drug/agent	Dosage forms/types	Volunteers, n	Clinical trial type	Reference(s)
Acyclovir	NM	40	Pilot clinical trial	135
Clindamycin phosphate and salicylic acid (CLSA) combination	Ethosomal gel	40	Pilot clinical trial	136
Prostaglandin E ₁ (PGE1)	NM	15	Pilot clinical trial	125

Abbreviation: NM, not mentioned.

researchers and shown promising results.^{9,11,13,20,27,140} However, few studies were continued for 1 year or more. Classical ethosome suspensions of minoxidil, testosterone,⁶ and trihexyphenidyl HCl⁴³ were found to be stable for 2 years, while a classical ethosome gel of erythromycin was found to be stable for ~1 year.¹⁰⁸ A stability study of a transethosome gel of 5-fluorouracil showed that there were no changes in appearance and vesicular size after 2 months under accelerated conditions and 11 months at room temperature.¹⁸ However, more studies are much required to evaluate the stability of ethosomal systems for long-term storage, particularly when they are incorporated in dosage forms.

Ethosomal system-based marketed products

A research development company (Yissum) in the Hebrew University of Jerusalem and Professor Elka Toutitou have worked together with Trima (pharmaceutical company) in the production of an ethosomal cream of acyclovir (Supravir). The preparation was used for the treatment of herpes simplex virus infections of the skin and mucous membrane.¹⁴¹ However, the product information is no longer available at the producing company's website. Other ethosomal system-based products reported by some authors include Nanominox, Cellutight EF, Skin Genuity, and Decorin cream.^{127,142} These products were produced a few years ago, but currently there is no detailed information available online about the products or their producing companies.

Conclusion

It has been almost 2 decades since the invention of ethosomes, and during this period these nanocarriers have proven their unique ability to deliver therapeutic agents of different physicochemical properties through the skin for local and systemic use. Continuous extensive research has led to the introduction of a new generation of ethosomal systems called transethosomes, which have been found to have better properties over the classical ethosomes in terms of vesicular properties and skin-permeation abilities. Transethosomes provide the highest flexibility to the formulator to change the ethosomal properties according to the required research criteria by changing the edge activators and/or penetration enhancers. The incorporation of ethosomal systems in suitable vehicles such as gels, patches, and creams represents an important step to get better skin-permeation and therapeutic results. However, more studies are required to enhance the stability of the ethosomal system. The results of the in

vivo studies and clinical trials are reflecting the potential of ethosomal systems in dermal and transdermal delivery of therapeutic and cosmetic agents.

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Disclosure

The authors report no conflicts of interest in this work.

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