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Vesicles as tools for the modulation of skin permeability

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Human skin is a remarkably efficient barrier designed to keep our insides in and the outside out. The modulation of this efficient barrier's properties, including its permeability to chemicals, drugs and biologically active agents is the prime target for various dermal, transdermal, drug, antigen and gene delivery approaches. Therefore, several methods have been attempted to enhance the permeation rate of biologically active agents, temporarily and locally. One of the approaches is the application of drug-laden vesicular formulations. This review presents various mechanisms involved in increasing drug transport across the skin via different vesicular approaches, such as liposomes, elastic vesicles and ethosomes, along with compiling the research work conducted in this field.

Keywords: elastic liposomes, ethosomes, skin permeation, transdermal delivery

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1. Introduction

Advances in the understanding of physiological processes has led to the development of novel drug carriers that are capable of controlling drug release temporally or spatially, delivering drugs at the desired site at a pre-determined rate, thus overcoming the drawbacks of multi-dose therapy and hence providing enhanced patient compliance with improved safety and efficacy. However, most drugs are administered orally – a route which, because of the body's high metabolic activity, and changing chemical environment, limits the availability of a wide variety of drugs, including proteins and peptides. The search for alternative routes of drug delivery has led to a greater understanding of drug passage via various biological membranes, such as skin, stimulating further research in dermal and transdermal drug delivery systems. Dermal drug delivery generally signifies the topical application of drugs, thus targeting skin for treatment of various skin diseases. This has the advantage that high concentrations of drugs can be localised at the site of action, reducing systemic drug levels and, thus, reducing the systemic side effects. Transdermal drug delivery, on the other hand, utilises skin as the site for the administration of systemically active drugs. Following skin permeation, the drug first reaches the systemic circulation and is then transported to the target site, which could be relatively remote from the site of administration, to produce its therapeutic action. However, as the natural function of the skin is to protect the body from unwanted effects from the environment, the most important limitation in the dermal and transdermal application of drugs is the skin itself.

2. Skin – anatomical considerations, functions and penetration pathways

Skin has evolved to provide various advantages, including a barrier to the entry of toxins, microbes and unwanted materials into the body, and minimising water

loss, suggesting its role as a blanket to the underlying biological systems. The most prominent morphological feature of normal skin is its stratification (**Figure 1**) [1]. Skin is taken to consist of three layers, namely the epidermis (and its associated appendages, pilosebaceous units and sweat glands), the dermis, separated from the epidermis by the dermal–epidermal junction, and the hypodermis.

The very thin (1 – 10% of the total thickness) outermost skin layer contributes to > 80% of the resistance to transport across the skin. A unique hierarchical structure of lipid-rich matrix with embedded corneocytes in the upper strata (10 – 20 μm) of skin, the stratum corneum (SC), is responsible for this barrier. The SC layer, a composite of the corneocytes (terminally differentiated keratinocytes) and the secreted contents of the lamellar bodies that provides a bricks (keratin-filled keratinocytes) and mortar (intercellular matrix) type organisation, is dry and hence best suited for the purpose of the permeability barrier [2,3]. The arrangement of corneocytes and lamellar bodies creates a tortuous pathway through which substances have to traverse to in order to cross the SC. The lipid material between corneocytes is highly organised, and acts as intracellular 'glue' sealing the spaces between the cells in the skin. Intracellular lipids in the horny layer mainly encompass the relatively non-polar substances, such as free fatty acids, cholesterol and cholestryl esters, in addition to more than a dozen ceramides. This lipid composition and lipid-packing structure of the SC differentiates it from other biological membranes, thus contributing to its relative impermeability to various chemicals and drugs. Forslind [4] proposed a domain mosaic model to take account of the heterogeneity in the lipid packing of SC bilayers, and suggested that more fluid liquid crystalline domains present within the SC bilayers could provide an easy permeation pathway. Reduced diffusional resistance was also suggested, due to the presence of proteins and desmosomes within the lipid bilayers.

Skin appendages (hair follicles and sweat glands) could also be ascribed as possible macroroutes for drug permeation across intact skin [5]. However, the hair follicle is an invagination of the epidermis, which may allow for a much greater potential area for absorption. The role of these appendages became apparent when the rapid transport of large molecules and ions was proposed via follicular shunt. Early reports of a transient follicular pathway were based primarily on qualitative, histological studies of dye and stain localisation in hair follicles [6-8]. In additional studies, it was found that the greatest absorption of some compounds occurred at sites with the greatest follicular density. More recent studies yielded increasingly qualitative data, which characterised follicular transport as a complex phenomenon dependent upon compound and/or vehicle composition and may occur over several hours [9-11]. Recently, it has been proposed that even naked DNA – a large molecule – can immunise by topical application, suggesting follicular transport, and the hair follicle also promises to be a route for gene therapy [12,13].

Although the routes of penetration of molecules through the SC are still highly researched and debated, there is a consensus that small, uncharged molecules permeate via intercellular lipid routes, whereas highly hydrophilic molecules may follow a transcellular pathway, although the bilayered lipid regions traversed between keratinocytes remain a rate-limiting barrier [14]. Various strategies have been designed to modulate the skin's permeability, in order to transdermally deliver a wider range of drugs. The strategies include the optimisation of drug and vehicle properties, and modification of the SC by chemicals or electrical/external force methods [15]. One such strategy to modulate skin permeability is through the use of vesicles loaded with active agents [16,17].

3. The modulation of skin permeability by conventional liposomes

The origin of liposome can be traced back to the contribution of Bangham *et al.* in the mid-1960s. The description of lecithin dispersion as containing 'spherulites composed of concentric lamellae' [18] was followed by the observation that the diffusion of univalent cations and anions out of the spontaneously formed liquid crystals of lecithin is remarkably similar to the diffusion of ions across biological membranes [19]. Later, Gregory Gregoriadis and Demetrios Papahadjopoilous pioneered the utilisation of liposomes as the most versatile carrier yet known, which had found applications in all spheres of the targeted and controlled delivery of biologically active agents, including drugs, vaccines and genes [20].

Although liposomes have been investigated for the selective delivery of anticancer, antibiotic and antifungal agents for many years, only for approximately three decades have they been used for topical and transdermal delivery. The preliminary studies of using liposomes for topical drug delivery were conducted by Mezei and Gulasekharam, where they reported enhanced epidermal and dermal concentrations of radiolabelled triamcinolone acetonide via liposomal lotion and gel formulations, compared with conventional triamcinolone acetonide formulations [21,22]. Similar results were obtained by other investigators testing triamcinolone and hydrocortisone in animal and human experiments [23-25].

After initial works, which were encouraging, a number of studies followed, which sparked great interest in the use of liposomal formulations for topical drug delivery. As research proceeded further, the quest to establish the mechanism of the modulation of skin permeability via these carriers also amplified. Four general mechanisms have been reported, which include i) intact drug-laden vesicle penetration into the different strata of the skin; ii) liposomes acting as penetration enhancers via their skin lipid-fluidising property; iii) direct carrier–skin drug exchange by

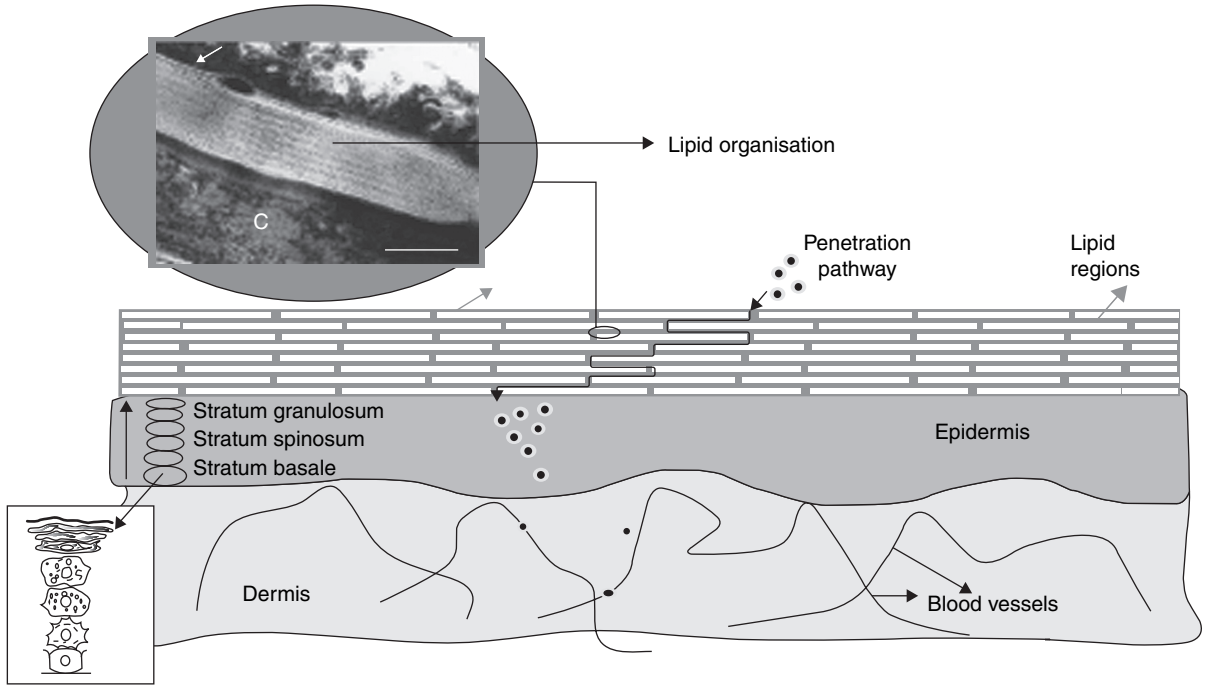

Figure 1. A schematic drawing of a skin cross section. The skin is composed of a dermis and an epidermis. In the basal layer of the epidermis, cells proliferate. Upon leaving the basal layer, cells start to differentiate and migrate in the direction of the skin surface. At the interface between the stratum granulosum and the stratum corneum, final differentiation occurs, during which the viable cells are transformed into dead keratin-filled cells (corneocytes). The corneocytes are surrounded by a cell envelope composed of cross-linked proteins and a covalently bound lipid envelope (see arrow). The corneocytes are embedded in lipid lamellar regions, which are orientated parallel to the corneocyte surface. Substances permeate mainly along the tortuous pathway in the intercellular lamellar regions. Bar = 100 nm.

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C: Corneocyte filled with keratin.

'collision complex transfer' between the drug intercalated in the liposomal bilayer and the surface phase of the SC; and iv) liposome-mediated enhanced transdermal drug delivery via appendegal pathways [26].

Mezei and Gulashekharan (1980, 1982) were the first to report intact vesicular penetration of the skin, reaching the vascular epidermis [21,22]. This controversial finding was well-addressed by Schreier and Bouwstra [27] and Knepp *et al.* [28], who commented that Mezei's apparent finding could be attributed to the experimental method employed (i.e., the use of alcohol swabs to remove the unabsorbed compound from the skin surface, which may have caused transient penetration enhancement and promoted transdermal delivery of the triamcinolone acetonide). Ganeshan *et al.* [29] and Ho *et al.* [30] designed a very elegant set of studies and concluded that, in an *in vitro* mouse skin system, neither liposomes nor phospholipid molecules diffuse across the intact skin. Consequently, various techniques were employed by researchers to study intact vesicular skin penetration. Foldvari *et al.* [31] utilised colloidal iron-loaded multilamellar liposomes for a guinea-pig model, and observed by electron microscopy that unilamellar liposomes were present in the dermis. The authors suggested that the loss of the external bilayer might have occurred during penetration, and further added that liposomes could have been adsorbed intact on the skin surface before penetration, with the possibility of the rupture of some vesicles.

Kirjavainen *et al.* [32] presented a clearer picture of liposome–skin interaction. They visualised liposome penetration into human skin via confocal laser scanning microscopy (CLSM) – another technique that has been widely acclaimed for studying vesicular penetration. The researchers prepared liposomes in different compositions and labeled them with a fluorescent lipid bilayer marker, *N*-Rh-PE (L-α-phosphatidyl ethanolamine-*N*-lissamine rhodamine B sulfonyl) and observed that fluorescently labelled liposomes were not able to penetrate the granular layer of the epidermis. The authors further studied and concluded that the interaction between liposomes and skin was highly dependent on the lipid composition of the liposomes, whereas surface charge density, acyl chain length or the presence of cholesterol did not play a major role. Kirjavainen *et al.* [32] further presented a view of enhanced liposomal skin delivery that was totally different from that proposed by Mezei and co-workers. They suggested that liposome lipids penetrate into the SC by adhering to the

surface of the skin and subsequently destabilising and fusing or mixing with the lipid matrix and, hence, act as a penetration enhancer.

Another mechanistic study conducted by Blume *et al.* [33], showing the interaction of phospholipids with liposomes composed of model mixtures resembling the composition of the SC, using the techniques of ^{2}H NMR and differential scanning calorimetry (DSC), suggested the possibility of local mixing of the two phospholipid vesicles either via monomer mixing or by direct fusion. This could further explain the interaction of liposomes with the skin, suggesting a mechanism of penetration enhancement with liposomes. Earlier, Abraham and Downing [34], and later Hofland *et al.* [35], also suggested the possibility of the adsorption and fusion of liposomes onto the skin surface, leading to the formation of lamellae and rough structures that could further enhance the driving force for the permeation of liberated molecules, although the formation of an additional lipid barrier, reducing the permeability of hydrophilic molecule, was not taken into account. Other reports are also available from various groups for improved skin deposition and penetration-enhancing activity [36,37].

The modulation of skin permeability with liposomes via an appendegeal pathway has also been studied. El Maghraby *et al.* monitored vesicular delivery through the epidermis and compared it with penetration through a sandwich of SC and epidermis – assuming that there was a negligible chance of superimposition of shunts in the two membranes, and that most of the shunts available in the bottom membrane were blocked. They concluded that the transappendegal route played a major role in the transdermal delivery of drug (estradiol) from liposomes [37]. Recently, Han *et al.* only achieved enhanced follicular delivery with the use of iontophoresis [38]. In addition, a recent review by El Maghraby *et al.* suggests that liposomes play no major role in transappendegal delivery [26].

The prospects of appendegal targeting have been extended to the follicular delivery of large hydrophilic proteins and peptides via liposomes. Leib *et al.* [39] encapsulated a small fluorescent hydrophilic dye, carboxyfluorescein (CF), in liposomes, and applied it *in vitro* to a hamster ear model. They observed that phospholipid-based liposomes were capable of selectively targeting and further depositing a greater amount of CF into the pilosebaceous units, compared with CF-containing HEPES buffer, propylene glycol (5%) in HEPES buffer, ethanol (10%) in HEPES buffer, and sodium lauryl sulfate (0.05%) in HEPES buffer. Du Plessis *et al.* [40] compared the *in vitro* deposition of aqueous IFN-γ and liposomal IFN-γ solution in the various strata of humans, hairless mice and hamsters. They observed that, for all three species, significantly greater deposition of IFN-γ was achieved with liposomes than with the aqueous solution. In addition, there are other reports that liposomes could be effectively targeted to skin appendages [41,42].

4. The modulation of skin permeability by elastic vesicles (elastic liposome/Transfersome®)

A novel class of modified liposome, containing an optimum amount of edge activator and providing it with a highly elastic nature, has been developed (Transfersome®; Idea AG). These carriers were first described by Cevc and Blume, and subsequently they have been the subject of numerous patent and literature reports [43-49,101]. Elastic liposomes (Transfersomes) have been claimed to penetrate and permeate the skin layers as intact vesicles to reach the systemic circulation. The elasticity possessed by these vesicles is the consequence of an edge activator (often a single-chain surfactant that enhances the deformability via lipid bilayer destabilisation) incorporated within the phospholipid-based system. In most cases, the phospholipid and edge activator contents have been optimised to attain the desired deformable nature of the elastic liposomes, to increase elasticity and penetrability.

In the present context, a number of Transfersome-based products are at an advanced clinical trial stage, such as IDEA-033 (Idea AG), which is expected to become the first truly effective topical analgesic for the effective management of osteoarthritis [101]. This advancement is the outcome of nearly 15 years of extensive research made in the field of elastic vesicles. The work in this area commenced when Cevc and Blume investigated the fate of radiolabelled lipids applied in the form of Transfersomes, where they observed that 30 ± 10% of applied lipids accumulated in the subdermis and ~ 6 – 8% in the blood [43]. Furthermore, Cevc and coworkers [44] investigated the role of lidocaine- and tetracaine-loaded Tranfersomes on rats and humans, respectively, and observed that, in the case of rats, there was a 130% increase in efficacy compared with the control. In humans, the pinprick sensation effectiveness was comparable with the subcutaneous injection bearing an equal amount of anaesthetic agent. Paul *et al.* [45,46] further reported the transdermal delivery of human serum albumin and gap junction proteins via Transfersomes, and observed a rise in specific antibody titres marginally higher than those elicited by subcutaneous injections of the antigens in Transfersomes, mixed micelles or liposomes. Epicutaneous antigen application via Transfersomes has also been reported to generate an unusually large amount of IgAs, which was not observed with subcutaneous injection.

Guo *et al.* [47] investigated both the *in vitro* and *in vivo* efficacy of ciclosporin A-loaded sodium cholate flexible liposomes, and observed that 1.88 ± 0.66 µg of the drug was delivered across Kunming mouse abdominal skin after a 24 h of study. Conventional liposomes showed no significant delivery of the drug after 24 h. The researchers also measured serum drug concentrations and found that 154.37 ± 27.15 ng/ml of ciclosporin A was delivered via flexible liposomes after 8 h in a mouse model,

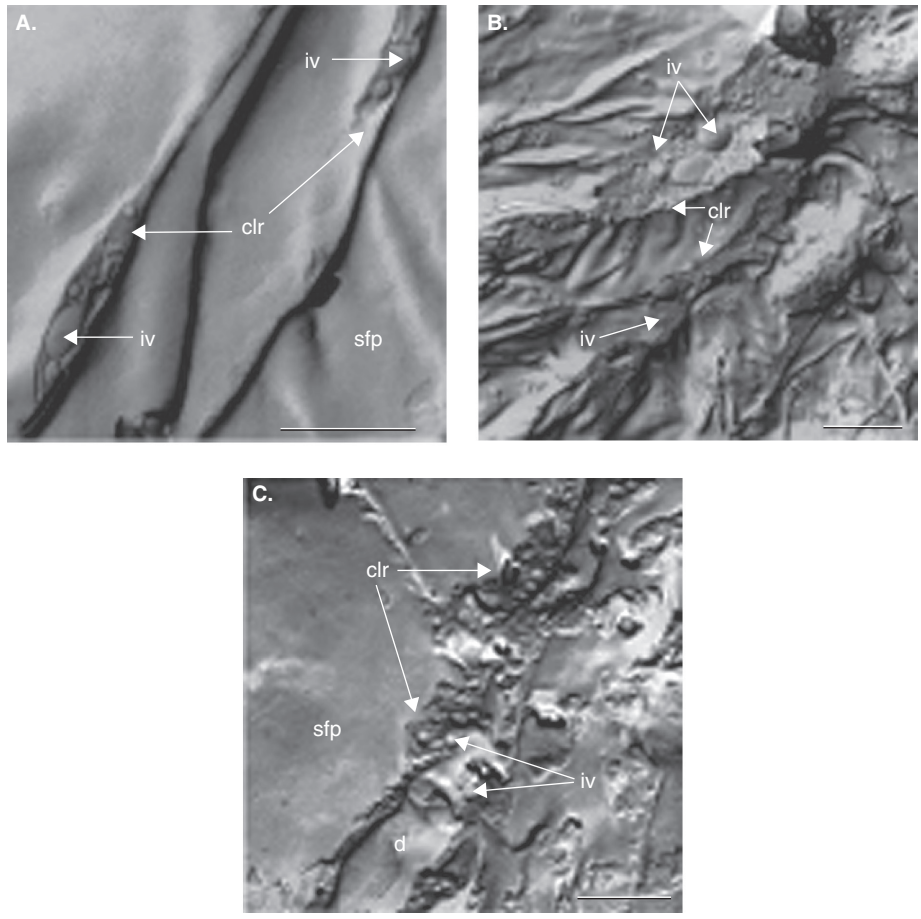

Figure 2. *In vivo* interactions between elastic vesicles and human skin in the deeper layers of the stratum corneum. **A.** Micrograph of the ninth tape strip of skin treated with L-595/PEG-8-L/sulfosuccinate (50:50:5) elastic vesicles. **B.** and **C.** Micrographs of the ninth tape strip of skin treated with L-595/Tween 20/sulfosuccinate (60:40:5) elastic vesicles. For both elastic vesicle compositions, channel-like regions can be seen containing vesicular structures. Although fused vesicle material is present, intact vesicles were also clearly seen. This strongly suggests that elastic vesicles can enter the deeper layers of the stratum corneum within 1 h of vesicle application.

Scale bar represents 1 µm.

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CLR: Channel-like regions; D: Desmosome; IV: Intact vesicles; SFP: Smooth fracture planes.

whereas no measurable amount was observed in the case of conventional liposomes.

Cevc *et al.* evaluated the hypoglycemic action of insulin-loaded Transfersomes and observed hypoglycemic efficacy comparable to subcutaneous insulin injection, but with 45 – 145 min lag time [48,49]. Guo *et al.* also reported a drop in blood glucose of 61.4 ± 8.9% at 5 h after the application of insulin elastic vesicles [50].

Cevc and coworkers further developed Transfenac® (Idea AG) – a topical diclofenac formulation based on the Transfersome approach – which provided a therapeutically meaningful drug concentration in target tissue following administration, compared with a hydrogel formulation containing a higher diclofenac dose; the average intramuscular concentration was threefold higher than the Transfersomal formulation [51].

A number of subsequent studies in mouse, human and other *in vivo* models, as well as on *in vitro* diffusion cells suggested the improved skin permeation of a number of drugs and biologically active agents [52-59]. El Maghraby *et al.* [37,60] investigated the effect of vesicular composition and characteristics on the skin (heat separated human epidermis) permeation of estradiol. They observed an enhanced flux of the drug that was ~ 17-fold after application of drug-loaded deformable liposomes bearing sodium cholate and Span-80, compared with the control. Tween-80-bearing drug-loaded deformable liposomes also provided improved flux (14- to 15-fold), compared with the control, which was not significantly different from other deformable formulations. The pretreatment of skin with empty deformable vesicles had minimal effects on drug flux. In addition, vesicular size did not affect the flux of estradiol.

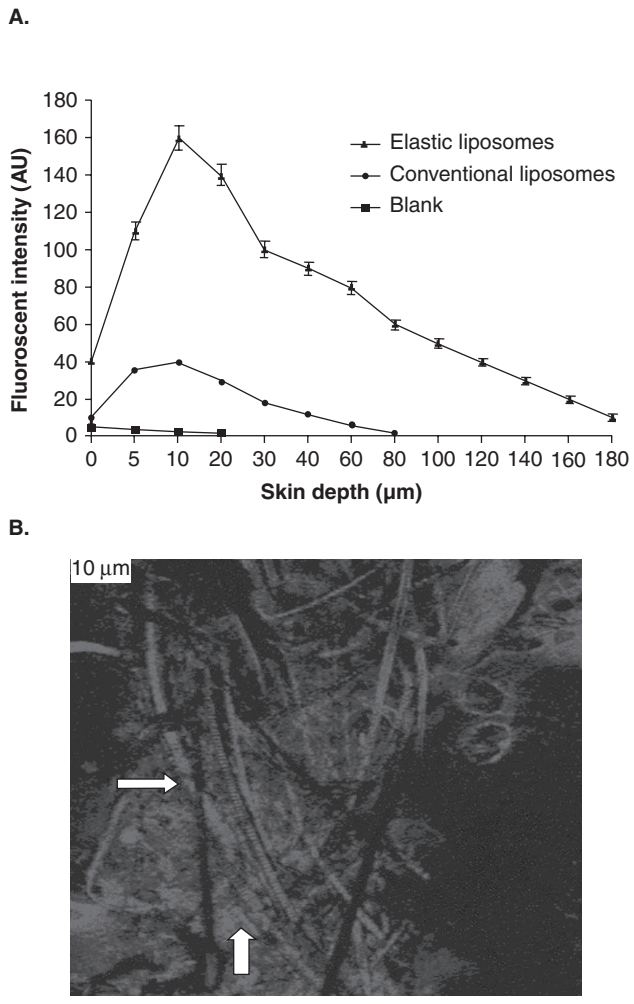

Figure 3. A. Fluorescent intensity (AU) versus skin depth (µm) studies revealing a comparative skin penetration profile of elastic liposomes and rigid liposomes. B. Virtual channel-like structures can be visualised (white arrows representing the stained channels) at a skin depth of 10 µm.
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AU: Arbitrary unit.

This study confirmed the role of the osmotic gradient, as, under occlusive conditions, estradiol transdermal flux was only six- to ninefold higher than the control. However no evidence of intact vesicle penetration was given.

Jain *et al.* have also added significantly to this area of elastic and deformable liposomes for transdermal drug delivery. They have extensively investigated dexamethasone-loaded elastic lipsomes after both *in vitro* and *in vivo* application [53]. In an *in vitro* study, they observed zero-order release across rat skin, with no lag phase on application of dexamethasone-loaded elastic lipsomes, compared with conventional drug formulations, which depicted a lag phase. Drug-loaded elastic lipsomes also significantly decreased edema in a caragennann-induced paw edema model, compared with other conventional formulations. In another extensive study using ethinyl estradiol-loaded elastic liposomes, they obtained a flux 15- to 18-fold greater than the control formulation. In an *in vivo* study on female Sprague-Dawley rats, a significant antiovulatory activity of ultraflexible liposomes was observed, as compared with traditional liposomes and plain drug solutions given orally and topically [57].

Recently, Jain *et al.* coworkers observed a substantially higher accumulation of zidovudine – an anti-HIV agent – in the target reticuloendothelial system, which is a major reservoir for HIV, when applied via an elastic liposomal formulation. The zidovudine-loaded elastic liposomal system presented a transdermal flux of 98.8 ± 5.8 µg/cm^2/h across rat skin, compared with 5.72 ± 0.30 µg/cm^2/h for free drug, and had an AUC (0 – 24 h) of nearly 12-fold higher than the control [61].

A provesicular approach proposed and developed by Jain *et al.* led to the formulation of levnorgestrel-loaded proultraflexible lipid vesicles, which provided an extended stability to the system, along with possessing greater skin permeation potential and higher entrapment efficiency [54]. Furthermore, they developed melatonin-loaded sodium deoxycholate elastic liposomes that presented an optimum flux of 51.2 ± 2.21 µg/cm^2/h across dermatomed abdominal human cadaver skin. The obtained flux was nearly 5.0- and 12.3-fold higher than conventional liposomal and plain drug solutions, and also achieved a decreased lag time of 1.1 h for melatonin, which was the lowest recorded melatonin release until that report [58].

The transcutaneous immunisation potential of a hepatitis B surface antigen (HBsAg)-loaded elastic liposomal system has been recently well established [62]. In this study, elastic liposomes induced robust systemic and mucosal antibody responses against HBsAg, compared with other formulations. In addition, the fluorescence microscopy study suggested prominent skin permeation and biodistribution, demonstrating the efficient delivery of antigens to immunocompetent Langerhans cells and lymphatics. The elastic liposomal formulation provided a higher entrapment efficiency, enhanced penetration and effective immuno-adjuvancy, demonstrating its potential for improved vaccine delivery. Recently, the dendritic cell uptake potential of HBsAg-loaded elastic liposomes, leading to the generation of a protective immune response, has been well demonstrated by Mishra *et al.* [63].

A new generation of elastic vesicles have been developed and evaluated by Van den Berg *et al.*, which consists of bilayer-forming surfactant L-595 (sucrose laurate ester) and the micelle-forming surfactant PEG 8-L (octaoxyethylene laurate ester). The group also suggested an improved drug transport of pergolide and rotigotine across the skin with these new vesicles [64,65].

Cevc and Blume proposed the mechanism of penetration of biologically active agent-laden elastic vesicles as them having high and stress-dependent adaptability, enabling them to squeeze intact between the SC cells and into the

Table 1. *In vivo* studies investigating the efficiency and application of ethosomes as carriers for the skin delivery of drugs.

Drug	Criteria under investigation	Subjects	Results	Ref.
Acyclovir	Clinical efficacy in the treatment of recurrent herpes labialis	Humans	Time to crusting of lesions and time to loss of crust were shorter with the ethosomal acyclovir than with the commercial cream (Zovirax®* cream)	[78]
Testosterone	Pharmacokinetics	Rabbits	After application for 5 days (new patch applied daily), AUC was 125% greater with ethosomal patch than with commercially available patch	[76]
Testosterone	Pharmacokinetics	Male Sprague-Dawley rats	AUC was ~ 64% greater with ethosomes than with commercial gel	[79]
Cannabidiol	Suppression of carrageenan-induced aseptic paw edema (anti-inflammatory action)	Male mice	Development of edema was prevented entirely only in pretreated (ethosomal patch) group of mice. Delta in paw thickness of pretreated mice was statistically different from that of the non-pretreated mice starting from 1-h post-carrageenan injection and lasting until the end of the inflammation course	[80]
Erythromycin	*In vivo* antibacterial efficiency	*Staphylococcus aureus* inoculated mouse skin	Ethosomal erythromycin resulted in complete inhibition of infection, and hydroethanolic erythromycin solution caused deep dermal and subcutaneous abscesses within 5 days after challenge	[81]
Ammonium glycyrrhizinate	Suppression of chemically induced erythema (anti-inflammatory action)	Human volunteers	Ethosomes reduced the erythema more rapidly with respect to drug solutions. Ethosomes also showed sustained effect	[82]

*GlaxoSmithKline.
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skin via a xerophobic driving force [43]. Using CLSM, Cevc and Schatzlein [66] further reported highly fluorescent interclusters and intercorneocyte pathways located within the intercellular lipid lamella of murine SC, which, according to them, could further act as virtual channels through which intact vesicles could penetrate. Van den Berg *et al.* [64], using transmission and freeze fracture electron microscopy, demonstrated morphological changes in the intercellular lipid lamellar structure, with no changes in the viable epidermis ultrastructure. They also reported much finer thread-like channels, compared with that reported by Schatzlein and Cevc; however, no fluorescence could be detected in the viable epidermis. Honeywell-Nguyen *et al.* [67] could not visualise any vesicles in the deepest layers of the SC in an *in vivo* microscopic study; however, in a freeze fracture electron microscopy study of tape-stripped human skin, they observed that after 1 h of non-occlusive application of elastic vesicles, followed by sequential tape stripping, vesicles could be found up to the ninth strip in the SC (**Figure 2**) in the channel-like regions [68], similar to the thread-like channels observed by Van den Berg *et al.* [64].

Jain and coworkers also observed some highly stained channel-like pathways on application of Rhodamine Red-loaded elastic liposomes, which could act as a permeability shunt, further lowering the permeability barrier. In a fluorescent intensity versus skin depth graph, the present authors have well demonstrated the highest fluorescent intensity to be near to 10 µm in depth, as well as detecting skin penetration up to 180 µm after an 8-h study [58], which demonstrated the skin-penetration potential of elastic liposomes (**Figure 3**).

Recently, a study by Verma *et al.* [69], utilising a CLSM technique, demonstrated that vesicular size greatly affects the transdermal potential of the prepared vesicular system. Their study indicated that larger vesicles (size ≥ 600 nm) were not able to deliver their contents into the deeper layers of the skin. These vesicles stay in/on the SC, and after drying they may form a layer of lipid, which may further strengthen the barrier property of

Table 2. A summary of ethosomes *in vitro* skin permeation/deposition studies.

Drug	Tissue used	Enhancement ratio		Ref.
		Permeation*	Deposition	
Trihexyphenidyl HCl	Male nude mouse dorsal skin	51.0‡, 4.5§, 87.0§	4.6‡, 1.4§, 1.4§	[77]
Minoxidil	Male nude mouse abdominal skin	45‡, 35¶, 10**	7§, 5¶, 2**	[76]
Minoxidil	Rat abdominal skin	1.2§ (addition of cholesterol significantly improved skin delivery from ethosomes)	Not determined	[83]
Testosterone	Rabbit pinna skin	30 (relative to commercial patch)	7 (relative to commercial patch)	[76]
Testosterone	Dermatomed cadaver skin	6.4 (relative to commercial gel)	Not determined	[79]
Azelaic acid	Synthetic membranes	Release rate was higher from ethosomes than from liposomes. Ethosomes having the highest ethanol concentration released the drug more rapidly	–	[84]
Zidovudine	Rat skin	15.1§, 10.9¶, 12.9§, 7.7¶	Not determined	[85]
Ammonium glycyrrhizinate	Human epidermis	Ethosomes improved cumulative drug permeated after 24 h and reduced lag time relative to aqueous solution, hydroethanolic solution, and mixture of empty ethosomes–hydroethanolic drug solution	Not determined	[82]
Ketotifen	Rabbit pinna skin	1.2‡, 1.4§, 1.2§	3.3‡, 6.2§, 1.7§	[55]
Melatonin	Dermatomed cadaver skin	2.6§, 5.4#	2.2§, 1.4#	[86]
Methotrexate	Dermatomed cadaver skin	2.5§, 3.9#	3.8§, 3.84#	[87]

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*Estimated based on cumulative amounts permeated at the end of the experiment or on flux data.

‡Relative to aqueous solution.

§Relative to hydroethanolic solution.

¶Relative to absolute ethanol.

#Relative to traditional liposomes.

**Relative to lipid ethanolic solution.

the SC. Liposomes with a size ≤ 300 nm were able to deliver to some extent into the deeper layers of the skin. However, liposomes with size a ≤ 70 nm seemed to achieve the maximum depth.

A possible penetration-enhancing mechanism of deformable liposomes could also be postulated based on the studies performed by a number of other groups [70,71], and only the improvement of drug deposition in the skin with deformable vesicles is supported by other studies [72,73]. Thus it could be summarised that the mechanism of skin modulation by intact vesicle penetration, as proposed by Cevc and coworkers, is just speculation that needs experimental corroboration.

5. The modulation of skin permeability by ethosomes

Ethanolic liposomes, or ethosomes, are non-invasive delivery carriers that enable biologically active agents to reach the deep skin layer and/or systemic circulation [74]. These systems are mainly composed of phospholipids, a relatively high concentration of ethanol (20 – 50%) and water. Before the proposal of ethosomes by Touitou *et al.* [75,102], it was generally thought that a high alcohol concentration lead to the destruction of lipid vesicular structure, owing to the interdigitating effect of alcohol on lipids. This research group demonstrated the coexistence of phospholipid vesicles with a high concentration of ethanol, leading to the formation of soft, malleable, highly fluid vesicles (ethosomes). They demonstrated the formation of vesicles using ^{31}P-NMR and paramagnetic ion NMR experiments [76]. Several studies have investigated the various physicochemical characteristics of ethosomal vesicles [76,55]. The shape and lamellarity of ethosomal structures was shown to depend on the system's composition: the size of ethosomal carriers decreased with increasing ethanol concentration, but increased with increasing phospholipid concentration [76]. The physicochemical characteristics and concentration of entrapped active agent also affected the vesicular system. In the case of trihexyphenidyl HCl-loaded ethosomes, researchers observed a systematic size reduction with increasing drug concentration, suggesting possible

Table 3. Thermal analysis of vesicles containing various concentrations of bacitracin as determined by DSC.

Bacitracin conc. (% w/w)	Ethosomes (T_m, °C)	Liposomes (T_m, °C)	ΔT_m (°C) [$T_{m\ liposomes}$–$T_{m\ ethosomes}$]
0	-9.5	14.9	24.4
1	-16.1	14.2	30.3
3	-19.2	14.3	33.5

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DSC: Differential scanning calorimetry; T_m: Transition temperature.

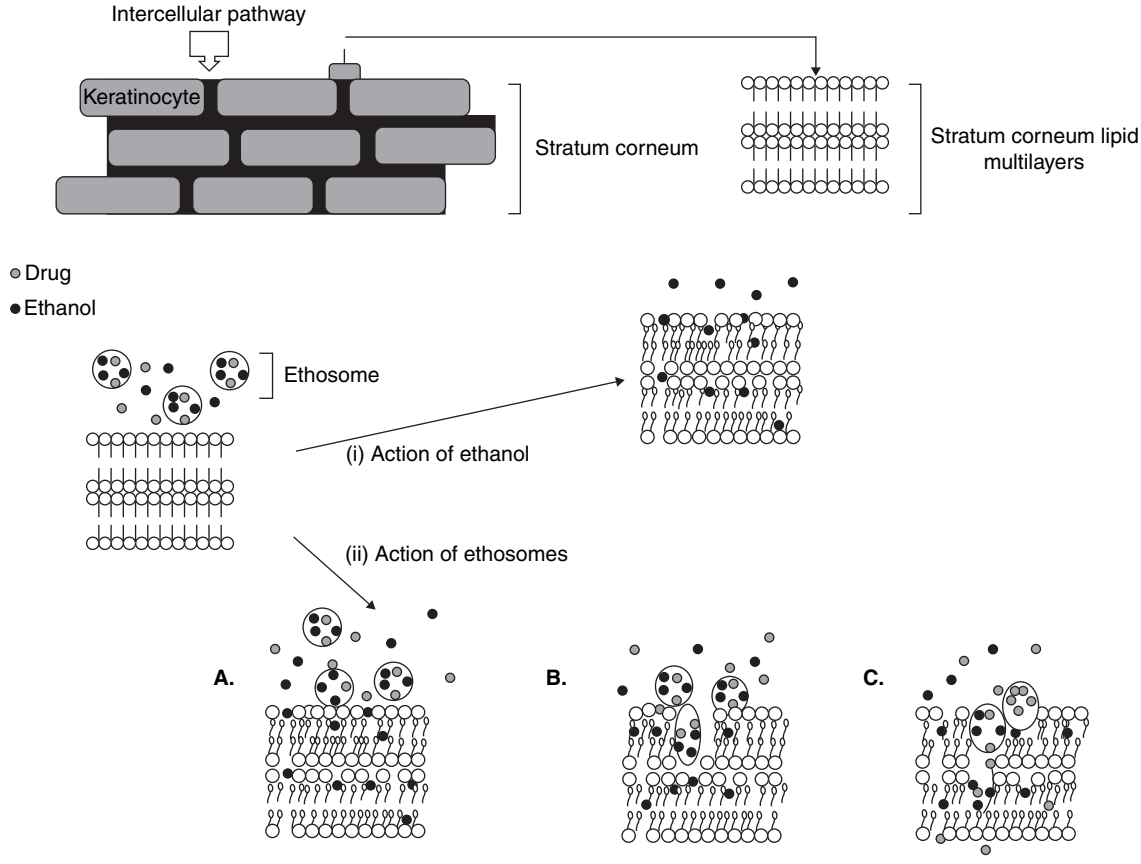

Figure 4. A proposed model for skin delivery by ethosomal carriers.
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surface activity of this molecule [77]. The entrapment efficiency of ethosomal carriers has been reported to be high for a wide range of molecules, including lipophilic drugs – a phenomenon that could be explained on the basis of the multilamellar structure of ethosomes and presence of ethanol that could improve the solubisation of many drugs. However, research in this field is limited, and a general correlation between physicochemical properties and transdermal delivery efficacy is yet to be established.

The first clinical study in humans using an ethosomal 5% acyclovir system demonstrated a shortened duration of time of crust formation, and time of loss of crust, compared with conventional 5% acyclovir formulation, suggesting the clinical efficacy of ethosomal acyclovir in treatment of recurrent herpes labialis [78].

Touitou *et al.* further evaluated the *in vitro* potential of testosterone-loaded ethosomes and observed an enhancement ratio of 30 compared with a commercial patch across rabbit pinna skin, whereas in a recent study they obtained an enhancement ratio of 6.4 from testosterone-loaded ethosomes, compared with a commercial gel across dermatomed human cadaver skin. In the *in vivo* studies, the group reported an AUC of 125% greater with the ethosomal patch than with a commercially available patch in a rabbit model [76]. Recently, using male Sprague-Dawley

Table 4. The effect of various formations on the C–H asymmetric and C–H symmetric stretching absorbance shifts on the acyl chains of stratum corneum lipids.

Treatments	C–H symmetric stretching	C–H asymmetric stretching
No treatment	2850.24 ± 1.1	2920.14 ± 1.12
Liposomes	2851.76 ± 0.88	2921.76 ± 0.88
30% Hydroethanolic solution	2852.14 ± 1.15	2924.42 ± 0.68
Ethosomes	2854.42 ± 1.19	2925.12 ± 1.11

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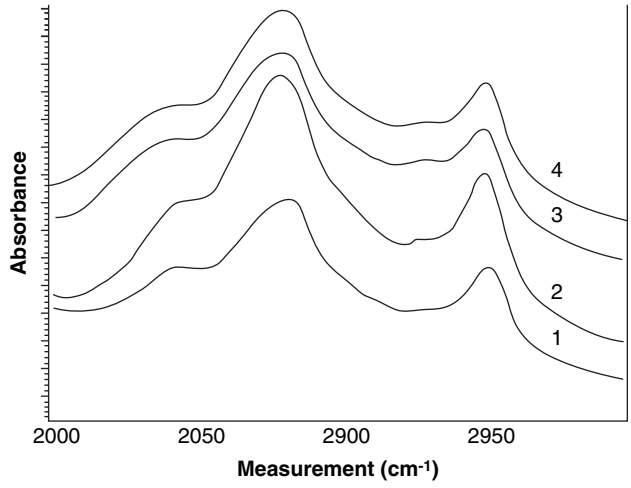

Figure 5. Fourier transform – infrared spectra of human cadaver skin after 6 h.
The adapted figure was published in [86], Copyright Elsevier.
1: Untreated skin; 2: Liposomes; 3: Hydroethanolic solution; 4: Ethosomes.

rats as subjects, Ainbinder and Touitou reported an AUC of 64% greater than a commercial gel [79].

These preliminary studies were followed with number of extensively worked experiments using various drug candidates applied for both *in vitro* and *in vivo* models. In nearly all cases, the various research groups observed an improved dermal and transdermal performance with ethosomal carriers, compared with conventional liposomes, hydroethanolic solution and aqueous drug solution (**Tables 1** and **2**).

Jain *et al.* also observed a greater transdermal flux of zidovudine across rat skin compared with that of hydroethanolic solution, absolute ethanol, lipid ethanolic solution and lipid ethanolic solution [85]. In a recent study, they observed a greater flux (59.2 ± 1.22 μg/cm^2/h) and a decreased lag time (0.9 h) of melatonin across human cadaver skin using ethanolic liposomal carriers, suggesting their suitability as a carrier for the transdermal delivery of melatonin [86].

Ethanol is a well-known penetration enhancer and is commonly believed to act by affecting the intercellular region of the SC, thus enhancing permeation. This penetration-enhancing effect of ethanol could be attributed to two factors: i) an increase in thermodynamic activity due to the evaporation of ethanol, known as 'push effect'; and ii) 'pull effect', in which the penetration of a drug molecule is increased due to a reduction by ethanol in the barrier property of the SC [88]. Ethanol encapsulated in lipid vesicles in the form of ethosomes provides fluidity to the ethosomal bilayers, and, when applied to the skin, it fluidises the SC lipids. This enhances the fluidity of ethosomal bilayers and has been well demonstrated by DSC (**Table 3**) [86].

Ethosomal carriers have been reported to be highly effective permeation enhancers compared with any of the components of the system alone. The enhanced delivery of biologically active agents/markers from ethosomal carriers has been well reported. Touitou and coworkers, using CSLM, showed the facilitation of probe penetration to a greater skin depth with ethosomal carriers, and also a relatively high fluorescence intensity, compared with hydroethanolic solution and conventional liposomes, using various probes of different nature, such as D-289 and Rhodamine Red [76,77]. In a recent study, the present authors' group demonstrated a

large degree of skin penetration of Rhodamine Red (up to 240 μm) from ethosomal carriers (2% phosphatidylcholine, 30% ethanol) [86].

In terms of the enhanced potential for the transdermal delivery of biologically active agents in ethosomal carriers, the exact mechanism of skin permeability modulation remains speculation. According to Touitou *et al.*, a synergistic mechanism between ethanol, vesicles and skin lipids exists, leading to an improved permeation profile [76]. As illustrated in **Figure 4**, the proposed mechanism of ethosomal skin modulation lies in the interaction of ethanol with lipid molecules in the polar head group region, resulting in a reduction in the transition temperature (T_m) of SC lipids, thus enhancing their fluidity, leading to a disordered SC, which provides a potential site for soft, malleable ethosomes to penetrate more easily within the skin layers. Recently, Jain and coworkers performed an Fourier Transform-Infrared spectral profile study that could be a measure of SC lipid fluidity [86]. The study compared untreated SC with SC treated with liposomes, hydroethanolic solution and ethosomes, and resulted in a shift to a higher frequency and an absorbance broadening for both C–H symmetric and asymmetric stretching, which provides a measure of skin lipid fluidity (**Table 4**, **Figure 5**). The results suggested that extensively applied phosphatidylcholine could disrupt the SC lipid structure, whereas ethanol in hydroethanolic solution may have increased the rotational freedom of lipid acyl chains, leading to an increased fluidity of skin lipids. Ethosomes caused a higher frequency shift and broadening compared with liposomes and hydroethanolic solution, suggesting greater mobility of SC lipids after the application of ethosomes. The study also suggested that ethosomes are not just an admixture of ethanol and phospholipids, but a system that intercalates ethanol within itself, which in turn provides its bilayer with enhanced fluidity and malleability. In a recent study, Elsayed *et al.* [56] observed that entrapping a drug (ketotifen) within a vesicle provided a greater flux compared with an admixture of drug and ethosomes. Dubey *et al.*, in a vesicle–skin interaction study, demonstrated the mild swelling of corneocytes after the application of ethosomes, suggesting the retention of fluids and, thus, provides an insight into the sustained drug delivery mechanism of ethosomes [87].

6. The modulation of skin permeability by other vesicles

Other vesicular approaches that find possible application in modulating skin permeability are niosomes, aspasomes and other vesicular approaches, comprising one or more penetration enhancers. Niosomes, in a general sense, are vesicles composed of non-ionic surfactants that have been studied and evaluated for a number of cosmetic and drug delivery applications [90]. These non-ionic vesicles are largely considered alternatives to liposomes, which further alleviate the problems of chemical instability, phospholipid impurity and high cost associated with liposomes [90-94]. The mechanism of the modulation of skin permeability by niosomes is specualeted to be quite similar to that of liposomes. Niosomes are reported to disrupt the barrier properties of SC, as well as directly fuse into the upper layers of the skin, thereby enhancing skin permeation [27].

Ascorbyl palmitate vesicles, or aspasomes, are combinations of ascorbyl palmitate – a bilayer forming antioxidant – with cholesterol and negatively charged dicetyl phosphate [95]. Gopinath *et al.* proposed and developed this system and further utilised it for the transdermal permeation of zidovudine [95]. They observed greater permeation of zidovudine via these carriers, and speculated that aspasomes, due to their lipophilicity, partition into lipids of the skin and, by their amphiphilic character, alter the intercellular space, thereby improving drug permeation. Studies involving a combination of vesicular and other penetration-enhancing approaches such as iontophoresis and electroporation have also been reported. These approaches represent future technologies, which could help the pharmaceutical scientists to obtain a predetermined flux and could provide support the treatment of various topical and systemic disorders [38,96-98].

7. Conclusion

The modulation of skin permeability by various vesicular approaches could be exploited as a possible mode of drug delivery via the skin. Conventional liposomes, Transfersomes and ethosomes represent the three basic classes of vesicular systems that could efficiently modulate skin permeability. Liposomes have been observed to remain in the upper strata of the skin, mixing with skin lipids and, thus providing a penetration-enhancing effect. Deformable vesicles and ethosomes are better vectors for modulating skin permeability and, thus provide deep skin and systemic access to a wide variety of molecules. The wide use of techniques such as CLSM, Fourier Transform-Infrared, NMR and others could unravel the mechanisms of enhanced dermal/transdermal permeation through these vesicular systems.

8. Expert opinion

The use of vesicles in delivery systems for dermal and transdermal applications has attracted great interest in recent decades. However, although classic liposomes are of little value for transdermal applications, novel vesicular approaches such as Transfersomes, ethosomes and others have proved to be better and more reliable.

Transfersomes, in general, offer several advantages with a lipid bilayer, and incorporating an edge activator permits elasticity and deformability of the bilayer structure, which results in facilitated permeation via narrow pores in the skin. Debating the mechanism of penetration of elastic vesicles may be worthless, when a wide number of

biologically active agents ranging from small molecules to large proteins have been delivered successfully by them. However, establishing the mechanism is desirable from a scientific standpoint. Virtual thread-like channels have been identified in the SC after the exposure of elastic liposomes. Reports suggest that the transdermal hydration gradient is the major driving force for the penetration of Transfersomes through the intact SC and into the epidermis. The presence of intact elastic vesicles well within the SC has also been demonstrated, although their passage from viable epidermis to the systemic circulation is still a matter of speculation. Furthermore, the requirement of an open (non-occlusive) protocol limits the applicability of these vesicles.

In our opinion, the full potential of elastic liposomes is yet to be explored. Our group is working on a novel approach of ligand-conjugated antigen-bearing elastic systems that could be a targeting vector for immunocompetent skin cells, such as Langerhans cells; these would further carry the payload to the lymph nodes, where it would be presented and an immune response for that antigen could be generated. In addition, we also plan to encapsulate various herbal biologically active agents to achieve their best possible utility, such as in wound healing.

Ethosomes, in our opinion, have come out well as an efficient dermal and transdermal delivery system. Although the permeation mechanism of ethosomes into the skin is still a matter of speculation, some insights are well documented in the literature. Various ethosome-based products are in the pipeline of a biopharmaceutical company, Novel Therapeutic Technology, Inc., including those for the treatment of alopecia, deep skin infections, herpes, hormone deficiencies, inflammation, post-operative nausea, atopic dermatitis and erectile dysfunction. Although a number of drug candidates have been successfully delivered via ethosomes, there are still no published reports on ethosomes being used as immunoadjuvants, delivering antigens to the immunological milieu of the skin. In our laboratory, we have obtained some encouraging results on this aspect of transcutaneous immunisation via ethosomal carriers. Furthermore, we have also obtained some good results with melatonin-loaded elastic ethanolic liposomes: a hybrid system generated by the intelligent combination of elastic liposomal and ethosomal approaches. Such hybrid systems possibly represent future platform technologies and may be useful for drug, antigen and gene delivery purposes. However, the vesicle deformability, fluidity and flexibility required for improved transdermal efficacy are associated with reduced physical stability and, hence, further insight in this area would be desirable in the near future so that the full potential of these systems can be utilised.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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