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Skin structure and mode of action of vesicles

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Abstract

The natural function of the skin is to protect the body for unwanted influences from the environment. The main barrier of the skin is located in the outermost layer of the skin, the stratum corneum. Since the lipids regions in the stratum corneum form the only continuous structure, substances applied onto the skin always have to pass these regions. Therefore, in the first part of this paper, the barrier function has been explained, focusing on the lipid composition and organisation. The major obstacle for topical drug delivery is the low diffusion rate of drugs across the stratum corneum. Several methods have been assessed to increase the permeation rate of drugs temporarily. One of the approaches is the application of drugs in formulations containing vesicles. In order to unravel the mechanisms involved in increasing the drug transport across the skin, information on the effect of vesicles on drug permeation rate, the permeation pathway and perturbations of the skin ultrastructure is of importance. In the second part of this paper, the possible interactions between vesicles and skin are described, focusing on differences between the effects of gel-state, liquid-state, and elastic vesicles.

Keywords: Skin lipids; Vesicles; Liposomes; Delivery; Visualisation; Stratum corneum; Percutaneous permeation

Contents

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1. General introduction

The natural function of the skin is to protect the body against the loss of endogenous substances. The main barrier for diffusion through the skin is the outermost layer of the skin, the stratum corneum (SC) [1]. The SC consists of keratin-filled dead cells, the corneocytes, which are entirely surrounded by crystalline lamellar lipid regions. The cell boundary, the cornified envelope, is a very densely crosslinked protein structure, which reduces absorption of drugs into the cells, see Fig. 1. For these reasons most of the active substances applied onto the skin are diffusing along the lipid lamellae in the intercellular regions [2]. In the past 20 years, many studies have been focused on the lipid composition and organisation in the SC and the changes involved in this lipid organisation as a consequence of topical application of drugs in formulations and patches.

1.1. Lipid composition

The composition of the SC lipids strongly differs from that of cell membranes of living cells. The major lipid classes [3–5] in the SC are ceramides (CER), cholesterol (CHOL) and free fatty acids

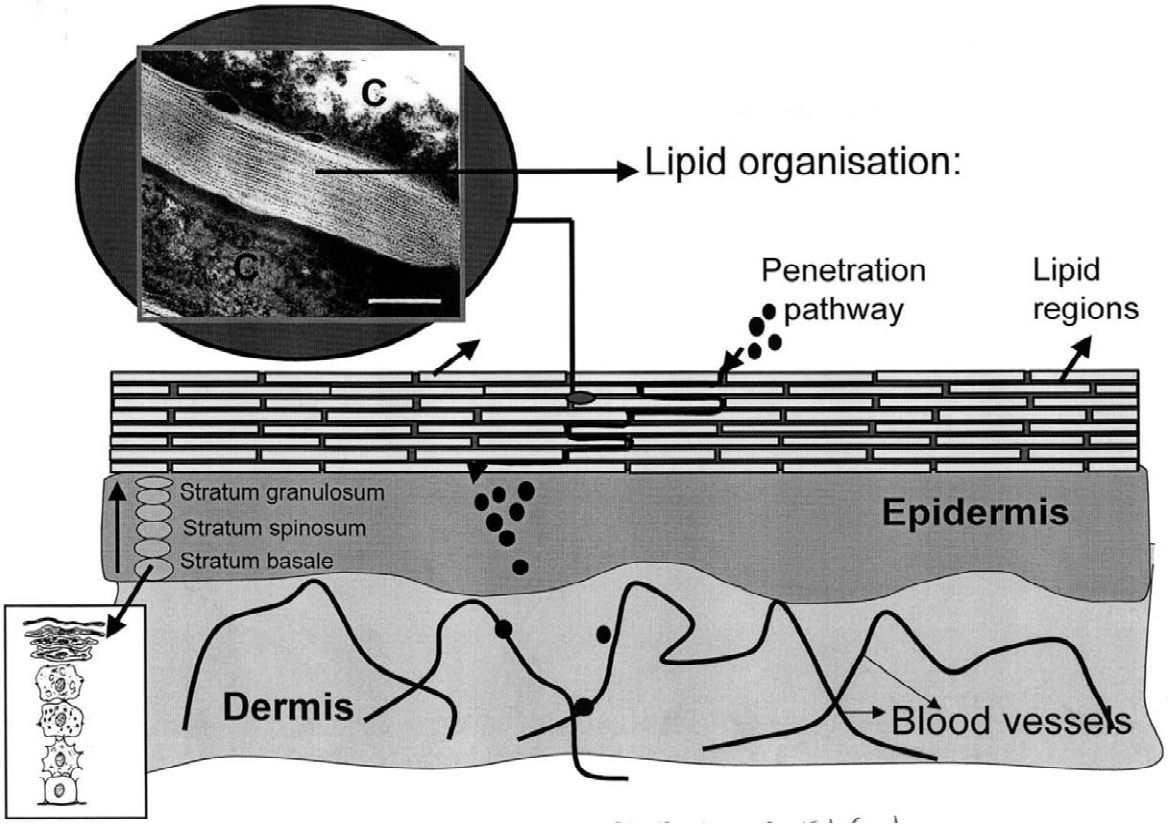

Fig. 1. A schematic drawing of a skin cross-section. The skin is composed of a dermis and an epidermis. In the basal layer of the epidermis cells proliferate. Upon leaving the basal layer cells start to differentiate and migrate in the direction of the skin surface. At the interface between stratum granulosum and 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. In stratum corneum the corneocytes are embedded in lipid lamellar regions, which are oriented parallel to the corneocyte surface. Substances permeate mainly along the tortuous pathway in the intercellular lamellar regions. C = corneocyte filled with keratin. Bar = 100 nm.

(FFA). The length of the acyl chains in the CER varies between 16 and 33 carbons, while the most abundantly chain lengths of FFA are C22 and C24. These chain lengths are much longer than those of phospholipids that are present in plasma membranes. Furthermore, the CER head groups are very small and contain several functional groups that can form inter and intra molecular hydrogen bonds. There are at least eight subclasses of ceramides (HCER) present in human stratum corneum, which are described elsewhere [6,7]. These HCER, often referred to as HCER 1–8, differ from each other by the head-group architecture (sphingosine, phytosphingosine and a 6-hydroxysphingosine base linked to a fatty acid or an α-hydroxy fatty acid) and the hydrocarbon chain length. HCER 1 and HCER 4 have an exceptional molecular structure: a linoleic acid is linked to a ω-hydroxy fatty acid with a chain length of approximately 30–32 C atoms.

1.2. Lipid organisation

Many efforts have been undertaken to characterize the lipid lamellar regions. In the 1970s, however, freeze fracture electron microscopic studies revealed that the lipids are organized in lamellae [8,9] located in the intercellular regions in the SC. More recently RuO_4 has been introduced as a post-fixation agent to preserve the saturated lipids in the SC. These electron microscopic studies revealed an unusual lamellar arrangement of a repeating pattern with electron translucent bands in a broad–narrow–broad sequence as shown by various authors [10–15]. The lipid lamellae follow the contours along the surfaces of the cells. Biophysical methods such as differential scanning calorimetry or Fourier-transformed infrared studies all indicated that the lipids are arranged in a crystalline sublattice and that only a small proportion of lipids formed a liquid phase [16–18]. In 1988, X-ray diffraction was used by White et al. [19] to determine the lipid organisation in murine SC. A lamellar phase with an unusual long periodicity of 13 nm was observed (referred to as the long periodicity phase). In addition, it was reported that the lipids in the lamellae are mainly organized in a crystalline sublattice, which is in agreement with the FTIR studies. In subsequent studies, the lipid organisation in pig SC and in human SC was assessed. From these studies it was concluded that the lipids in pig and human SC were organized in two lamellar phases with periodicities of approximately 13 and 6 nm, respectively [20,21]. The 6-nm phase is referred to as the short periodicity phase. Since the 13-nm phase has been observed in all the species studied so far, and is very characteristic for the SC lipid phase behavior, this phase is most probably crucial for the skin barrier function [22].

1.3. Lipid mixtures

Information on the relationship between lipid organisation and composition is of great importance for understanding the skin barrier function. This is demonstrated in diseased skin, in which an impaired barrier function often is related to an altered lipid composition and organisation [23,24]. To examine the role individual lipid classes play in the stratum corneum, CER were isolated from human stratum corneum. In this paper we will only summarize the phase behavior of equimolar CHOL:CER:FFA mixtures observed at pH 5, which approximates the pH at the skin surface.

1.3.1. Lamellar phases

In equimolar mixtures prepared from CHOL and CER the major fraction of lipids forms a lamellar phase with periodicities of 12.8 nm [25]. Only a very small fraction of lipids forms the short periodicity phase with a spacing of approximately 5.5 nm. Variation of the CHOL:CER molar ratio between 0.2 and 1.0 does not change the lipid phase behavior strongly suggesting that the phase behavior is insensitive to the CHOL:CER molar ratio. This has also been observed in lipid mixtures prepared from pig ceramides by Bouwstra et al. [26]. Addition of FFA promotes the presence of the short periodicity phase while the peaks attributed to the long periodicity phase weakened.

1.3.2. Lateral packing

In the absence of FFA, a diffraction pattern indicating a hexagonal lateral packing has been observed. The lateral packing is insensitive towards changes in CHOL:CER ratio, similarly as observed for the formation of lamellar phases. Addition of FFA induces a transition from a hexagonal to an

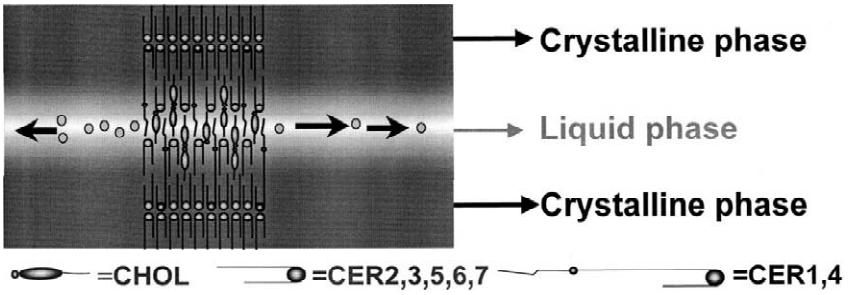

Fig. 2. The molecular arrangement of the LPP in HCER:CHOL mixtures. Following this model, the repeating unit in the structure consists of three lipid layers. In the central layer the unsaturated linoleic moiety of HCER1 and HCER4 are located. Based on the molecular model we proposed the 'sandwich model'. The liquid phase is located in domains or in continuous channels in the central lipid layer of this phase. In the sublattice adjacent to the central layer, a gradual change in lipid mobility occurs due the presence of less mobile long saturated hydrocarbon chains. This gradual change to crystalline packed lipid layers on both sides of the central layer avoids the formation of new interfaces. The highest permeability is expected to be located parallel to the basal plane of the lamellae in the fluid domains.

orthorhombic lateral packing. Furthermore, the presence of a liquid phase is more obvious [22]. The formation of the orthorhombic lateral packing, a very densely packed structure, demonstrates the important role of FFA for formation of a competent barrier.

1.3.3. CHOL:CER mixtures prepared in the absence of CER1

When CER1 is absent, as in CHOL:CER(2–8) mixtures, the phase behavior changes dramatically [25,27,28], namely only a very small population of lipids forms the 12-nm lamellar phase. This is observed for mixtures prepared with pig CER as well as with human CER.

1.3.4. CHOL:CER:FFA mixtures in which natural CER1 is substituted by either synthetic ceramide 1-stearate, synthetic ceramide 1-oleate or synthetic ceramide 1-linoleate

To elucidate the role of fatty acid linked to ω-hydroxy acid, natural CER1 was replaced by either synthetic CER1-linoleate (CER1-lin), by synthetic CER1-oleate (CER1-ol) or by synthetic CER1-stearate (CER1-ste). The following changes in lipid phase behavior were noticed [28]. (i) No liquid phase could be detected when CER1 was substituted for CER1-ste. (ii) Substitution of natural CER1 by either CER1-ol or CER1-lin revealed the presence of the liquid phase, being less prominent in mixtures with CER1-lin. (iii) As far as the lamellar phases are concerned, the long periodicity phase was not present in CER:CHOL mixtures in which CER1 was substituted for CER1-ste. (iv) The long periodicity phase was dominantly present in mixtures in which CER1 was substituted either by CER1-lin or by CER1-ol.

In conclusion, the liquid phase and the long periodicity phase were not present when substituting natural CER1 by CER1-ste. This observation indicates that for the formation of the long periodicity phase a certain (optimal) fraction of lipids has to form a liquid phase.

Having obtained information on the lipid phase behavior in stratum corneum and in lipid mixtures, the next step is to create a model for the molecular arrangement in the lipid lamellae based on the experimental data. In this respect, we like to focus mainly on the molecular arrangement of the long periodicity phase, since that phase has been suggested to be crucial for the skin barrier function. In Fig. 2, the molecular model based on the composition of human CER is provided referred to as the 'sandwich model'. However, a similar molecular model can be derived from the mixtures prepared with pig CER. As can be deduced from this figure, we propose a trilayer arrangement for the long periodicity phase. A trilayer arrangement has also been suggested by Swarzendruber et al. [29] and Kuempel et al. [30]. The trilayer arrangement is based on (a) the broad–narrow–broad sequence of electron lucent layers seen in RuO_4 fixed stratum corneum and (b) the broad narrow–broad sequence

of low electron density regions within the repeating structure of the long periodicity phase. This sequence is based on electron density calculations from the intensities of the diffraction peaks attributed to the long periodicity phase in mixtures prepared from CHOL and pig CER. The molecular model contains only CER and CHOL, since these two classes of lipids are important for the formation of the long periodicity phase. Incorporation of FFA into the molecular model would not change its basic features. The model and its experimental evidence is explained in more detail below:

(I) The linoleate moiety of CER1 and of CER4 are located in the narrow central layer of the long periodicity phase and link the trilayers together. With an equimolar CHOL:CER mixtures in the absence of CER1, the long periodicity phase was only weakly present (see above), indicating that the CER1 plays a crucial role in the formation of the long periodicity phase.
(II) The liquid sublattice is located in the central lipid layer of this phase. In the intercellular domain of the stratum corneum the orthorhombic phase coexists with a liquid phase (see above). In the sandwich model it is proposed that the liquid phase is located in the central narrow layer, in which unsaturated linoleate linked to ω-hydroxyacid of CER1 and CER4 is present. Recently, the relationship between the linoleate/oleate moiety and the presence of a liquid phase has been confirmed by a study [28], in which natural HCER1 was replaced by either synthetic CER1-linoleate, by CER1-oleate or by CER1-stearate (see above).
(III) In the sublattice adjacent to the central layer a gradual change in lipid mobility occurs due the presence of less mobile long saturated hydrocarbon chains. This results in densely packed lipid layers on both sides of the central layer and avoids the formation of new interfaces. The latter would be energetically unfavorable. The presence of a crystalline orthorhombic packing and of a liquid phase has been demonstrated by wide angle X-ray diffraction, FTIR spectroscopy and electron diffraction (see above).
(IV) Only a small fraction of lipids forms a fluid phase in the stratum corneum. Therefore, one can assume that this central lipid layer is not a continuous fluid phase, but crystalline domains coexist. In this respect, cholesterol may play a role as a line active substance between crystalline and fluid domains thereby avoiding phase boundaries [31].
(V) Lamellae are mainly oriented parallel to the surface of the corneocytes as demonstrated by electron microscopy studies. By passing the stratum corneum lipid regions in the direction perpendicular to the basal plane, substances have to pass the crystalline lipid lamellar region and can only partly diffuse through the less densely packed lipid regions parallel to the basal planes. In this way, a tortuous route is created, providing an excellent barrier.
(VI) The presence of fluid regions may facilitate the deformation of the lipid lamellae in the stratum corneum especially in case of shear stresses perpendicular to the stacking direction.

As far as the short periodicity phase is concerned, a 'classical' bilayer arrangement is the most likely arrangement. In this arrangement the hydrocarbon chains deeply interdigitate in the lipid bilayer. The suggestion that ceramides can be arranged in bilayers which is of particular interest for biological membranes has already been suggested by Dahlen and Pascher in 1979 [32].

2. Vesicles affect drug transport across skin

2.1. In vitro diffusion studies

To increase drug transport across the skin, penetration enhancers as well as other chemical methods have been used. One of the most controversial methods to increase drug transport across the skin is the use of vesicles. Although it has been generally accepted that the use of vesicles with proper composition should result in increased drug transport across the skin, many questions arise about the mechanism of action of these vesicular formulations. The first publications on interactions between liposomes and skin appeared in 1980 and 1982 [33,34]. In these publications, it was reported that liposomes applied to white rabbits skin in vivo favored the

deposition of drugs in the epidermis and dermis, while the amount of drug found in the various organs was reduced. Although in these studies it was strongly suggested that vesicles penetrated the skin, this suggestion was received with a lot of skepticism. After the first papers by Mezei and Gulasekharam [33,34], a large number of studies were initiated; a brief description of the most important studies will be provided below.

After the introduction of liposomes as drug delivery systems for the transdermal route Ganesan and Ho [35,36] published two studies. In these studies liposomes were prepared from DPPC (dipalmitoyldiphosphatidylcholine) that forms gel-state bilayers. The authors proposed that the drug could be transported either (i) as free solute, (ii) as free solute but also associated with the liposomes or (iii) by a direct transfer of the drug from liposomal bilayer to SC without partitioning into the water phase. They assumed that the vesicles neither absorbed intact nor fused with the SC surface. The permeation studies were performed in vitro with mouse skin using glucose, progesterone and hydrocortisone. The results of these studies did not confirm intact liposome penetration as suggested by Mezei and Gulukssaharam.

In a study by Knepp and co-workers [37,38], the vesicles were suspended in an agarose gel. They reported that progesterone release from an agarose gel alone was very fast compared to the release from liposomes embedded in the agarose gel and that vesicles reduced the progesterone transport rate across hairless mouse skin compared to application in an agarose gel alone. As far as the lipid composition is concerned progesterone was applied in liquid-state and gel-state liposomes. The gel-state vesicles resulted in a lower skin permeation rate of progesterone than the liquid egg-phosphatidylcholine liposomes. In two more recent papers [39,40], it has been reported that a gel immobilizes the liposomes and therefore might affect the interactions between the liposomes and the skin. However, the trend observed in the studies of Knepp and co-workers [35,36], in which incorporation of drugs in liquid-state liposomes results in a higher skin permeation rate than when incorporated in gel-state liposomes, was also observed for gel-free formulations (see below).

Several other studies were carried out to evaluate whether liposome composition affects skin penetration of drugs. In the early 1990s, Dowton et al. [41] compared the effect of liposomal composition on the disposition of encapsulated cyclosporine A in mouse skin when applied non-occlusively in vitro. The various liposomes were saturated with cyclosporin A keeping the thermodynamic activity of cyclosporin A equal in all formulations. They observed that application of the drug in non-ionic surfactant vesicles prepared from glyceryl dilaurate/cholesterol/polyoxyethylene-10 stearyl ether, the amount of cyclosporin A in deeper skin strata and receiver compartment was highest compared to the other formulations confirming the results of the studies of Knepp and co-workers [35,36]. The findings of Knepp and colleagues [35,36] were also confirmed by studies of Hofland et al. [42]. They found that estradiol incorporated in gel-state non-ionic surfactant resulted in a low drug transport rate through human SC compared to estradiol in liquid-state bilayers. These results were obtained with saturated estradiol formulations. In contrast to the studies of Knepp and colleagues, the studies of Hofland et al. [42] revealed that a drug applied in liquid-state vesicles resulted in higher penetration rates than when applied in a phosphate-buffered saline solution. This might be caused by a difference in the study design, since Hofland and colleagues suspended the vesicles in a buffer solution and used an equal estradiol thermodynamic activity. In the latter, an equal driving force from formulation to stratum corneum has been achieved. This difference in study design has a great impact when comparing drug permeation in vesicles compared to the control, such as a gel or buffer solution. In a subsequent study, van Hal et al. [43] reported that a decrease in cholesterol content in liquid state bilayers, which increases the fluidity of the vesicles bilayers, resulted in an increase in estradiol transport across SC. Meuwissen et al. [44] examined the diffusion depth of the bilayer label fluorescein–dipalmitoylphosphatidylethanolamine (-fluorescein–DPPE) in the skin when applied in gel-state liposomes and in liquid-state liposomes using confocal laser scanning microscopy. They reported that fluorescein–DPPE applied in liquid-state liposomes penetrated deeper in the skin than the label applied in gel-state liposomes. Recently, Fresta and

Puglisi [45] reported that corticosteroid dermal delivery with skin-lipid liposomes was more effective than delivery with phospholipid vesicles. This concerned the higher drug concentrations in deeper layers of the skin as well as the therapeutic effectiveness. From all the above mentioned studies it seems very clear that the thermodynamic state of the bilayers of the vesicles plays a crucial role in the effect of vesicles on drug transport rate across skin in vitro.

In a few studies occlusive application was compared to non-occlusive application. These studies revealed that occlusive application of vesicles suspension was less effective than non-occlusive application [46,47], but most probably also depends on the physical properties of the active agents. The results were somewhat unexpected, since it has been reported that water is an effective permeation enhancer [48,49]. However, in case of non-occlusive application the increased drug transport can be caused by (i) a more profound interaction between the liposomal constituents and the skin and/or (ii) the presence of a hydration gradient in the skin. According to Cevc et al. [46], the water gradient is an important driving force for drug diffusion. They claim an intact vesicle penetration through the skin, as long as flexible vesicles, Transferosomes®, have been used (see below). In a few studies, pretreatment of vesicles was compared to application of the drug associated with the vesicles. In all studies, it was found that drug association with vesicles was more effective than pretreatment with vesicles. These findings strongly suggest that at an equal thermodynamic driving force the vesicles do not act just as penetration enhancers [42], but when the proper vesicle composition is chosen an additional effect can be expected.

As reported by du Plessis et al. [50], the effect of vesicle size and lamellarity on drug deposition was minimal suggesting that intact vesicle transport does not occur. The authors concluded that intact penetration of liposomes does not occur. It seems that the physical parameters as vesicle size and lamellarity are less important than the application method and the thermodynamic state of the bilayers. These findings are in favor of the absence of intact vesicle penetration across the skin.

There are a number of studies [51–55] in which liposomes have been compared to lotion, creams or penetration enhancers. These studies are not discussed in this paper, since no information can be obtained about the mechanisms involved in vesicle transport. Touitou et al. [56] compared penetration enhancers with liposomes. Interestingly, they observed that liposomes can act as an excellent reservoir, while the penetration enhancers increased the drug transport through the skin. This group developed also a new system in which liposomes are combined with ethanol. Most probably the ethanol decreases the interfacial tension of the vesicles and makes the vesicles more elastic. These systems are referred to as ethasomes [57–59].

2.2. In vivo diffusion studies

In 1986, Komatsu et al. [60] applied butylparaben to the skin in a liposome formulation prepared from egg phosphatidylcholine, cholesterol and dicetylphosphate. The liposomes were applied occlusively to the dorsal skin of guinea pig in vivo. While the radioactive DPPC (dipalmitoylphosphatidylcholine) remained on the application site, even after 48 h, the applied ^{14}C-butylparaben radioactivity was present in small intestine, faeces, gall bladder and urinary bladder. In an additional in vitro study [61], the mechanisms involved in ^{14}C-butylparaben penetration was examined very systematically using a flow through diffusion cell. The amount of lipids as well as the amount of ^{14}C-butylparaben was varied. They observed that copenetration of butylparaben and phospholipids to deeper layers in the skin did not occur, which confirmed the results of their in vivo studies [60]. Again it was concluded that most likely the liposomes do not penetrate as intact entities across the skin. In another more recent study [62], double labeling was carried out. ^{3}H-phosphatidylcholine and ^{14}C-tretinoin were intercalated in soybean lecithin. It was examined that the ratio of the labels in SC was approximately constant, and that the ^{3}H/^{14}C ratio in epidermis was lower and decreased steeply until a skin depth of approximately 200 μm was reached. The authors concluded that co-penetration of a drug–liposome bilayer is possible in the SC, but that based on the reduced ^{3}H/^{14}C ratio in deeper skin strata, a

separate diffusion of the drug and liposomal bilayers in these layers occurs.

Cevc's group introduced Transfersomes®, which are elastic vesicles prepared from lipids and an edge activator, such as a single-chain lipid or surfactant. Only at the optimal balance between the amount of edge activator and the amount of bilayer forming lipid, are the vesicles elastic. If the edge activator level in the vesicles is too low, the vesicles are rigid and if the concentration edge activator is too high, the vesicles turn into micelles. It has been suggested that as a result of the hydration force in the skin, transfersomes due to their elastic nature can be squeezed through SC lipid lamellar regions [46]. Cevc's group compared occlusive with non-occlusive application and observed non-occlusive application to be more effective than occlusive application (see above). This finding is in line with their theory on lipid transport driven by an osmotic gradient across the skin. In order to detect the mass flow across the skin and the deposition in the animal, they used tritiated DPPC as a radioactive compound and reported that Transfersomes were much more effective than the 'conventional' rigid liposomes when applied non-occlusively. After 8 h of Transfersomes application in mice, significant amounts were found in blood (approximately 8% of the applied dose) and liver (20% of the recovered dose). They claimed that 50–90% of the dermally deposited lipids can be transported beyond the level of the SC. Although there is no doubt that their Transfersomes have clearly advantages with respect to increase the transport of active material across the skin, in these studies it was claimed that the elastic Transfersomes penetrated intact through SC and viable skin into the blood circulation. This statement especially resulted into a lot of skepticism in the research field. In the first published study on drugs associated with Transfersomes [63], the Transfersomes suspension contained relatively high amounts of lidocain or tetracain. The Transfersomes were tested on rats and on humans and in both studies the Transfersomes appeared to be more efficient than the conventional liposomes or solutions. The differences found using corticosteroids were somewhat less encouraging [64]. In another report diclofenac has been studied and appeared to be better deposited in skin when applied in Transfersomes [65]. Larger molecules such as insulin were also associated with Transfersomes [66,67]. Blood glucose levels could be lowered after about 3 h of application. A clear difference in glucose level in blood was observed between application of insulin associated with micelles, conventional liposomes or Transfersomes. The latter resulted in significant lower glucose levels. This was first observed in mice, but in more recent studies also in humans a decrease in glucose level was observed [68]. According to the results of Cevc et al. [69], even molecules such as FITC-BSA or I-BSA can be transported across the skin when associated with Transfersomes. These results are very encouraging and certainly show that Transfersomes have important advantages over vesicles prepared from only double chained phospholipids and cholesterol. It would be of extreme interest to study the mechanisms involved in the action of these vesicles in more detail.

In a study by Ogiso et al. [70], gel systems in which D-limonene or laurocapram was present as penetration enhancer were compared. The transport of betahistine in the presence or absence of liposomes in the gels was studied. The liposomes were prepared from either egg phosphatidylcholine or hydrogenated soybean phosphatidylcholine. The bioavailability of the drug was higher in the presence of the egg PC liposomes than in the absence of the egg phosphatidylcholine liposomes confirming earlier reports. It seems that the fluidity of the membranes is an important factor for the penetration enhancement, which confirmed the results of in vitro studies (see above).

2.3. Visualisation studies

2.3.1. Occlusive application in vitro

One of the first studies in which vesicle–skin interactions were visualized has been performed with isolated human stratum corneum incubated for 48 h with vesicles prepared from CHOL and polyoxyethylenealkylether surfactants. Hofland et al. [71] reported that after this long incubation time liquid as well as gel-state vesicles fused at the superficial layer of the stratum corneum, but that only liquid-state vesicles induced perturbations in lipid organization and formation of water pools within the stratum corneum. In these water pools, vesicular structures

were observed. Because the frequency of appearance of water pools was not determined and in a recent study presence of water pools has also been observed after pretreatment with a phosphate-buffered saline solution [72], it is not clear whether vesicles indeed promote the formation of water pools.

In another study the effect of composition of liquid-state liposomes on stratum corneum lipid organisation was studied ([73]) with three different phospholipid vesicles prepared according to Gehring [74]. The compositions of the vesicles are referred to as NAT106, NAT50 and NAT89. In these formulations the phosphatidylcholine content varied dramatically. Furthermore, phosphatidylcholine fraction contained also lysophosphatidylcholine (single-chain phospholipid). NAT50 (low phosphatidycholine content) liposomes only fused on the stratum corneum surface and did not cause perturbations in stratum corneum lipid organisation. When the stratum corneum was treated with the NAT89 liposomes (medium phosphatidylcholine content), rough structures were formed in the outermost four layers of the stratum corneum, indicating either intrusion of rough ultrastructures formed by fusion of vesicles or alteration of the stratum corneum lipid lamellae. The third formulation, NAT106, containing a high fraction of phosphatidylcholine induced marked changes in the stratum corneum ultrastructure. The corneocytes were considerably swollen and the ultrastructure of intercellular lipid lamellae showed flattened spherical structures. It seems that substantial amount of liposome material was incorporated in the stratum corneum intercellular regions. These changes were present throughout the stratum corneum. The reason for this strong interaction is not clear. A possible explanation is the presence of lysophosphatidylcholine in the phosphatidylcholine fraction. Since this is a single chain lipid, it might act as an edge activator making the vesicles elastic (see below).

The results described above clearly illustrate that liquid-state vesicles might act not only in superficial stratum corneum layers, but may also induce lipid perturbations in deeper layers of the stratum corneum, while gel-state vesicles interact only with the outermost layers in the stratum corneum. This might explain the difference in drug permeation enhancement between gel-state and liquid-state vesicles. In addition, fusion of gel-state vesicles on top of the stratum corneum might also act as an additional barrier for diffusion of drugs and therefore inhibit skin permeation.

2.3.2. Non-occlusive application in vitro and in vivo

Since it has been postulated that elastic vesicles require a hydration gradient in the stratum corneum to exert a maximal effect on its ultrastructure, several studies have been performed in which the effect of elasticity of vesicle bilayers on either penetration pathway or ultrastructure of the skin was investigated. Focused on the comparison between Transfersomes and conventional liposomes, recently studies have been performed in which the penetration of fluorescent labels across mice skin was visualized. For this purpose Schätzlein and Cevc [75] intercalated rhodamine-DHPE (1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-*N*-lissamine rhodamine B sulfonyl, triethylammonium salt) in the bilayers of Transfersomes prepared from soya phosphatidylcholine and sodium cholate. The suspensions were applied on mice skin for 4–12 h. Thereafter, the skin was examined ex vivo using confocal laser scanning microscopy. The confocal images revealed the existence of an inter-cluster pathway between groups of cells. These pathways appeared as high fluorescence intensity pathway in the intercellular lipid lamellar regions. The authors interpreted these regions as being virtual pores between the corneocytes through which vesicles can penetrate. Very recently the interaction between skin and elastic vesicles prepared from L-595 (sucrose ester surfactant, bilayers former) and PEG-8-L (octaethyleneglycol lauryl ester, micelle former) has been studied. The vesicles were applied onto human skin in vitro and mouse skin in vivo [76,77] and the ultrastructure of the skin was visualized using RuO_4 post-fixation in combination with transmission electron microscopy.

The fate of elastic vesicles after application onto excised human skin is shown in Fig. 3. Besides the presence of spherical lipid structures, large areas containing lipids, surfactants and electron dense spots and vesicle bilayers were observed deeper down into the stratum corneum. Furthermore, large areas of lamellar stacks were present throughout the entire stratum corneum. Occasionally, it was ob-

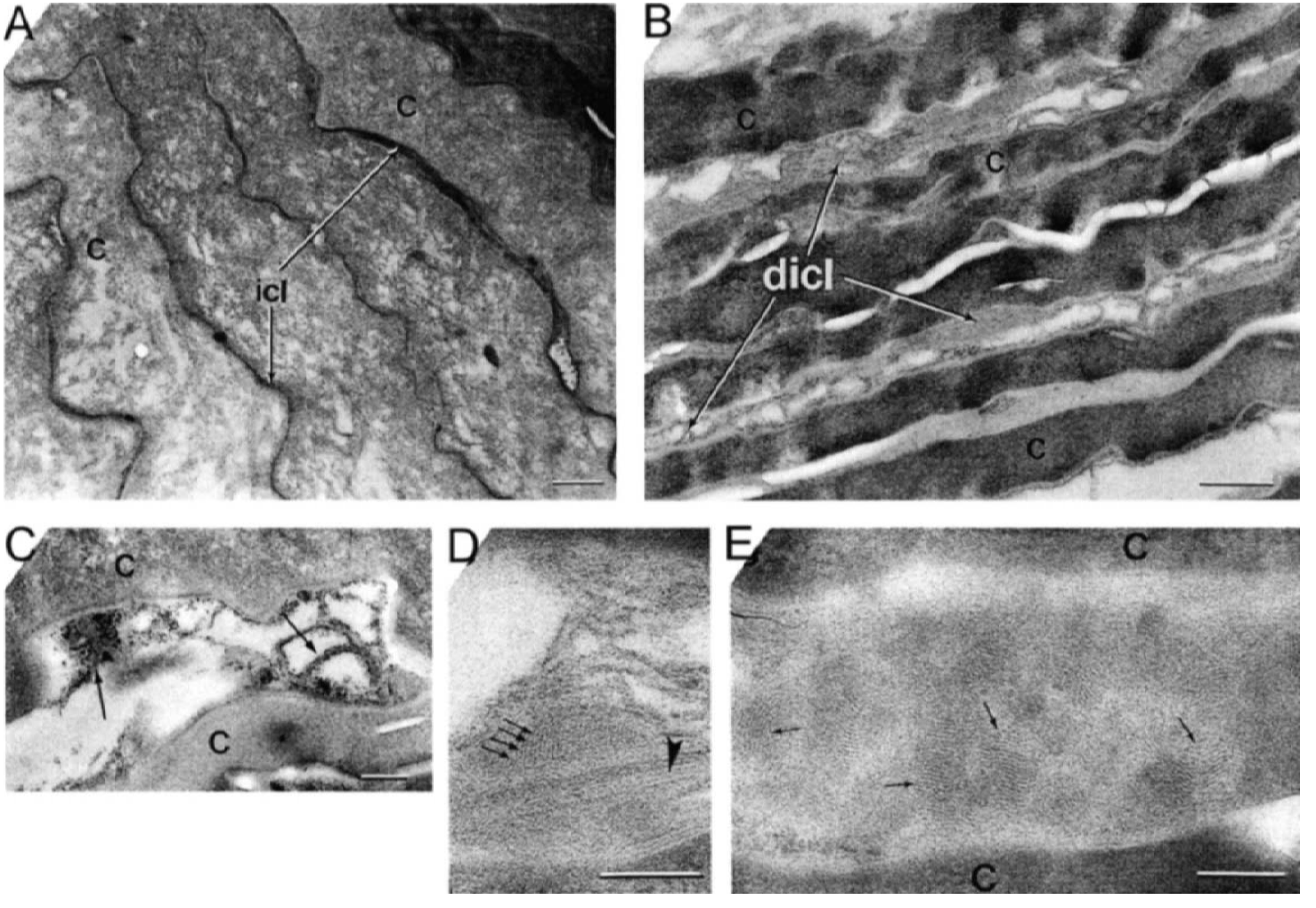

Fig. 3. Elastic vesicles prepared from L-595 and PEG-8-L changes the ultrastructure of human stratum corneum. In the electron micrographs, typical examples of the ultrastructural changes are depicted. (A) Transmission electron micrograph of phosphate-buffered saline in vitro treated human skin non-occlusively for 16 h. Between the corneocytes (C) the intercellular lipid lamellae (icl) are located. Bar = 100 nm. (B) Transmission electron micrograph of human skin treated by surfactant-based elastic vesicles non-occlusively for 16 h. In the four or five outermost layers in the stratum corneum, the lipid organisation has locally been perturbed (dicl). Frequently single lipid lamellae are visualized most probably originating from the vesicles. Fusion of vesicles has also been observed. C = corneocyte. Bar = 100 nm. (C) Transmission electron micrograph of human skin treated by surfactant-based elastic vesicles non-occlusively for 16 h. Single lamellae and dark spots are observed (see arrow) between the lamellae not observed in the control. C = corneocyte. Bar = 100 nm. (D) Transmission electron micrograph of human skin treated by surfactant-based elastic vesicles non-occlusively for 16 h. In the intercellular regions, characteristic domains are found consisting an array of narrow small lamellae (see arrows). The stacks are oriented perpendicular to the basal plane of the stratum corneum lamellae similarly as in mouse stratum corneum. The arrowhead indicates a Landman human characteristic for the skin lipid lamellae. Bar = 100 nm. (E) Transmission electron micrograph of human skin treated by surfactant-based elastic vesicles non-occlusively for 16 h. A large region of aggregated isolated domains consisting of narrow small lamellae (see arrows) has been visualized. The orientation of the lamellar stacks is randomly. Bar = 100 nm.

served that these lamellar stacks disorganized the intercellular skin lipid bilayers stacks similarly as observed in mice stratum corneum in vivo. The bilayers in these stacks were frequently oriented perpendicularly to the lamellae of the stratum corneum (Fig. 3C). Only when the surfactant ingredients accumulated in large regions, the lamellar stacks were oriented randomly (Fig. 3D). The formation of islands of lamellar stacks was similar to that observed frequently in elastic vesicles in stratum corneum of hairless mouse skin treated with elastic vesicles [76]. Treatment with conventional rigid gel-state vesicles affected the most apical corneocytes only to some extent. None of the vesicle formulations affected the viable epidermis or dermis indicating that the vesicle ingredients remained mainly in the stratum corneum. In addition to these extraordinary features observed in human and mice skin, the penetration pathway of a fluorescent label intercalated in the bilayers of elastic or rigid vesicles was visualized using two photon excitation microscopy. When the label was intercalated in the bilayers of the elastic vesicles, an inhomogeneous label distribution was observed (Fig. 4). Thread-like channels were visualized that might serve as penetration pathways for the dyes. However, without changing the settings of the microscope no dye could be detected in the viable epidermis. The distribution of the label in stratum corneum was different from that observed with Transfersomes, indicating that these surfactant-based elastic vesicles exert another interaction with the stratum corneum than Transfersomes do. Intercalation of the label in the bilayers of rigid vesicles resulted in an almost homogeneous label distribution in the intercellular regions (Fig. 4). Furthermore, after application in rigid vesicles the label could only be detected in the superficial stratum corneum layers. In case of micelles a similar label distribution was observed as seen with rigid vesicles. These results clearly demonstrate that elastic vesicles induce another penetration pathway for substances through the stratum corneum than the conventional (rigid) vesicles. Based on the results from fluorescent spectroscopy and transmission electron microscopy, it became clear that there is no evidence that material strongly associated with vesicles or the vesicle ingredients themselves penetrate fast into the viable epidermis. According to Cevc [46], the osmotic force resulting from the hydration gradient in the skin is the dominant force for partitioning of vesicles into the stratum corneum. However, recently it has been noticed (unpublished results) that even in fully hydrated state the water content in the lowest stratum corneum layers close to the viable epidermis is much

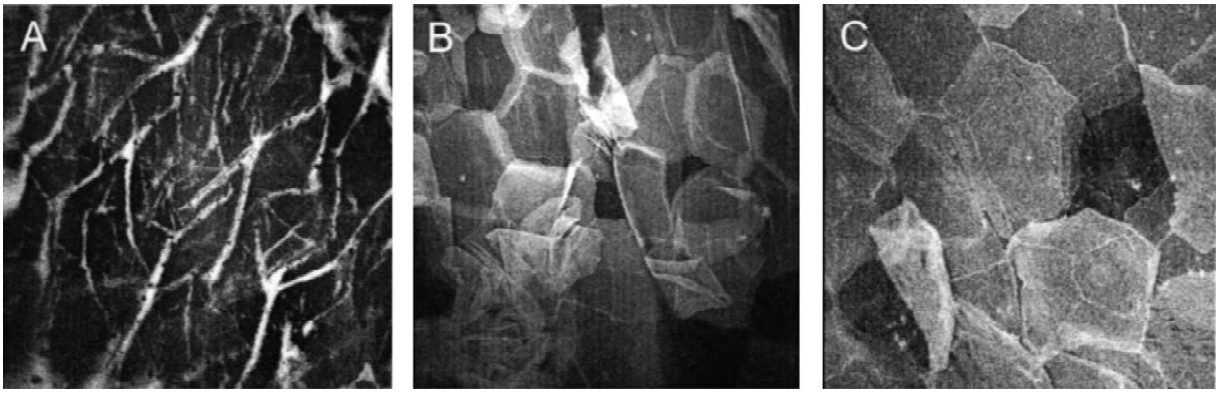

Fig. 4. Penetration of fluorescein diacylphosphatidylethanolamine across human stratum corneum. Fluorescein diacylphosphatidylethanolamine was applied onto human skin in either rigid vesicles (A), elastic vesicles (B) or micelles (C) after which the stratum corneum has been the penetration of the label was monitored by two photon excitation fluorescence spectroscopy. The *XY*-images (95×95 μm) have been taken approximately parallel to the skin surface. Application in rigid vesicles and micelles (A,C) results in a homogenous distribution of the fluorescent label in the intercellular space resulting in the visualization of the hexagonal shape of the cells in stratum corneum. In contrast application of the fluorescent label in elastic vesicles (B) results in an inhomogeneous label distribution: thread-like channels are visualized indicating that the penetration pathway is localized in channels.

lower than in the central regions of the stratum corneum. Therefore, it is expected that as a result of the osmotic force, the vesicle ingredients will not penetrate beyond the level of the lowest layers in the stratum corneum.

Whether the interactions observed in vitro with excised human skin can be extrapolated to the in vivo situation, has recently been studied [78] using freeze fracture electron microscopy. Elastic and rigid vesicles were applied non-occlusively onto human skin for 1 h after which the stratum corneum was stripped sequentially. The stripped stratum corneum was freeze-fractured approximately parallel to the corneocytes along the lipid lamellae. Surprisingly, the presence of vesicles was noticed at least up to the ninth strip in the stratum corneum in channel like regions (Fig. 5). These channel-like regions have a very similar appearance as the thread-like channels observed with two photon excitation fluorescent spectroscopy (Fig. 4) in vitro. This indicates that the label distribution in vitro is similar to the vesicle distribution in vivo. Furthermore, when substances remain strongly associated with the vesicles, elastic vesicles can be used to transfer substances rapidly into the deeper layers of the stratum corneum, after which the substances can permeate into the viable epidermis. Although these are very encouraging results, still many questions remain to be solved and further research is required to fully understand the mechanism of action of elastic vesicles. However, most of the studies clearly demonstrate that elastic vesicles have superior characteristics compared to rigid conventional vesicles, both in terms of drug permeation and skin interaction.

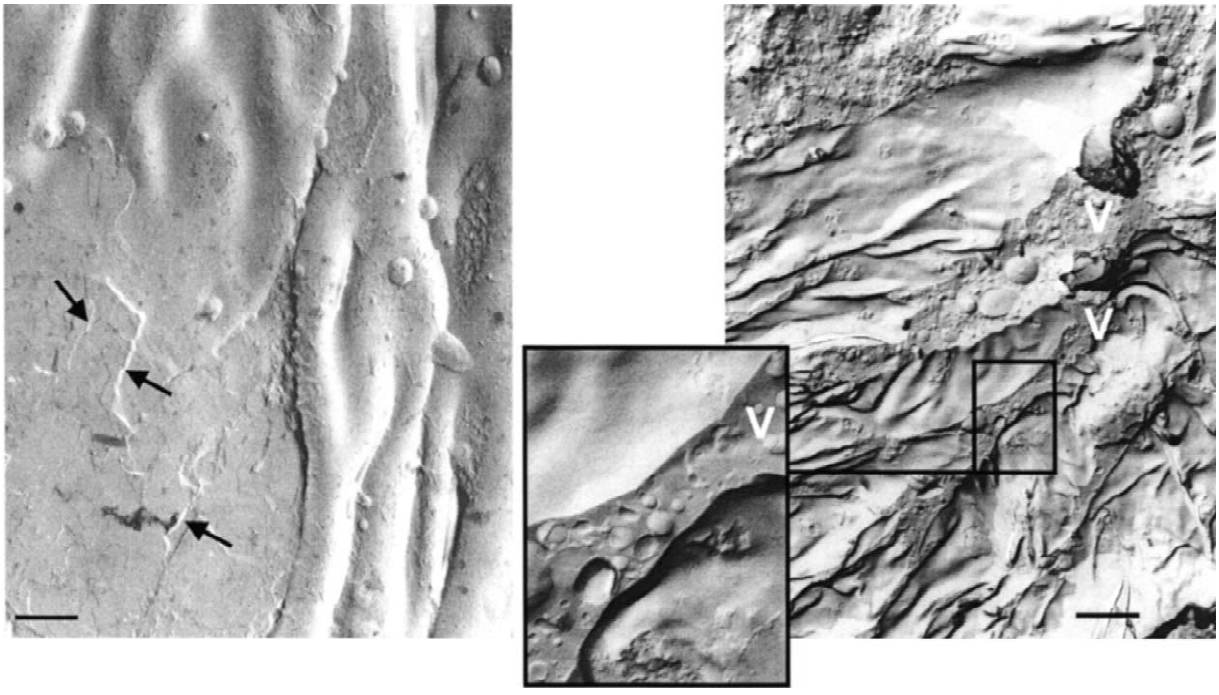

Fig. 5. Effect of elastic vesicles prepared from L-595 and PEG-8-L on human stratum corneum lipid organization. (A) Phosphate-buffered saline treated human skin in vivo. Freeze fracture electron micrograph of human stratum corneum using the stripping method. Shown is the fracture oriented parallel to the skin surface along the lipid lamellae. Smooth areas with desmosomes have been visualized (asterisks). Occasionally a fracture across the lipid lamellae is depicted (arrow). (B) Human skin treated in vivo for 1 h with elastic vesicles. Freeze fracture electron micrograph of human stratum corneum using the stripping method. Shown is the stratum corneum lipid organisation of the ninth strip. Channel-like regions are observed with vesicular structures (V). This strongly suggests that vesicles are present in the deeper layers of the stratum corneum.

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