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In vitro skin penetration enhancement techniques: A combined approach of ethosomes and microneedles

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ABSTRACT

Dermal administration of different macromolecules, such as nucleic acids, remains a real challenge because of the difficulty of crossing the main skin barrier, the stratum corneum (SC). To overcome this barrier, the use of deformable lipid-based nanovectors were developed to increase topical penetration through the SC and to promote the intercellular delivery of drugs. The purpose of this study is to compare the skin penetration of different liposome formulations according to their composition. *In vitro* and *ex vivo* experiments using Franz diffusion cells were performed to highlight the effect of (i) lipid charge, (ii) edge activators (EA) and (iii) ethanol on the diffusion properties of nanovectors. We showed that all formulations were not able to cross the SC. However, on a tape stripped skin, we showed that cationic formulations containing an EA and ethanol improved the skin penetration. The use of microneedles was considered to bypass the SC. We have shown that sodium cholate and ethanol were necessary to ensure an appropriate diffusion of liposomes into the dermis when applied by means of microneedles. This could be a promising approach to further deliver efficiently macromolecules such as genes into the skin.

1. Introduction

The skin is the largest organ of the human body representing an alternative route of administration by providing an easy and pain-free drug delivery option. Cutaneous drug delivery is not associated with a significant drug loss compared to systemic administration due to low enzymatic degradation and a negligible clearance. Moreover, the systemic toxicity resulting from cutaneous application is limited. Thanks to these advantages there is a high interest in using the skin as a route of administration to deliver macromolecules such as nucleic acids or peptides. However, the major hurdle is the stratum corneum (SC) acting as an efficient barrier which protects the body from the external environment and therefore limits drug penetration. This phenomenon is due to the SC structure. Indeed, the SC is a layer composed of dead and anucleated cells, which are embedded in a lipid-matrix. This particular architecture makes the SC very hydrophobic and represents the most important barrier to cross for drugs and foreign compounds (Bolzinger et al., 2012).

As recently reviewed (Bellefroid et al., 2019), different strategies have been proposed to overcome this barrier and enable efficient gene therapy. Physical and active methods involve the disruption of the SC. For instance, microporation and sonoporation involve local electrical current for ablation of corneocytes and ultrasound for SC permeabilization, respectively (Zakrewsky et al., 2015). These methods are highly effective in terms of nucleic acids penetration but suffer from significant drawbacks. Indeed, they are invasive, painful and relatively expensive. As a result, patient compliance is impacted. Besides, other physical techniques such as microneedles (MNs) have been proposed to limit these main drawbacks. Indeed, MNs are simple and easy-to-use devices for crossing the SC without reaching the nerves which makes this system minimally invasive and painless (Waghule et al., 2019; Zhu et al., 2019). Although MNs are highly effective in crossing the SC barrier, other limitations to nucleic acid delivery, such as intracellular entry and endosomal escape, could be challenging (Chen et al., 2016). To overcome these limitations, MNs can be associated with passive methods (Lan et al., 2018; McCaffrey et al., 2016; Seok et al., 2017). Besides physical and active methods, passive methods using non-viral vectors, such as polymeric nanoparticles, dendrimers, peptides or lipid-

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based nanovectors were developed to increase topical penetration through the SC barrier and to promote the intercellular delivery of large molecules such as nucleic acids (Bellefroid et al., 2019). Lipid-based nanocarriers, especially liposomes have been studied due to their assets. Indeed, liposome specifications, such as biodegradability, versatility, inexpensive and easy to manufacture, make them attractive to increase gene delivery. It has, however, become obvious that conventional liposomes do not promote the skin penetration (El Maghraby et al., 2008). Liposomes have been described as acting as a "reservoir" in the upper layer of the skin and they are confined to the SC without penetrating deeper in the epidermis (Bibi et al., 2017). To improve skin penetration, the use of deformable liposomes using two strategies has been proposed. Firstly, the addition of ethanol known as a penetration enhancer is added to the lipid composition to form ethosomes which allow the alteration of the intercellular domains of the SC. This effect is achieved using concentrations of ~10–50% of ethanol (Abdulbaqi et al., 2016). Consequently, nanovectors can cross the skin more easily and can reach targeted cells in the viable epidermis or the dermis involved in dermatological pathologies. The second technique is to form deformable liposomes by adding edge activators (EAs) which are surfactant molecules inserted into the lipid bilayer. Transfersomes®, introduced by Cevc and Blume in 1992, are more flexible allowing them to sneak through the SC, and thus to increase skin penetration (Cevc and Blume, 1992). Polysorbate, sorbitan monooleate, bile salts as sodium cholate (Nachol) or sodium deoxycholate, octadecylamine or potassium glycyrrhizinate are among the widely used EAs.

Despite many efforts, the application of nucleic acids on skin is still limited. A new tendency to improve skin penetration is to associate different techniques (physical, active and passive methods) to combine their advantages. For example, Jose et al. have efficiently delivered cationic liposomes encapsulating curcumin and STAT3 siRNA into the skin. These nanocomplexes combined with the use of iontophoresis were able to inhibit the growth of cancer cells and led the cells to apoptosis (Jose et al., 2017). Alongside, several passive method improvements can be used simultaneously. For instance, flexible nanosomes (termed SECosomes) were developed and composed of a cationic lipid (DOTAP), cholesterol, Nachol and ethanol in the context of gene transfection. SECosomes were able to transfect keratinocytes of *ex vivo* human skin (Geusens et al., 2010).

In this study, we assessed different penetration enhancement techniques to develop a suitable liposome formulation. These liposomes were developed with the ultimate goal of subsequently allowing the complexation and transport of nucleic acids in the deepest layers of the skin, the dermis.

In order to improve liposomes ability to carry drugs into the dermis, the effect of the surface charge of liposomes and of the presence of an EA as well as ethanol on their capacity to diffuse in the skin was evaluated. In addition, to overcome the SC, a comparison between the use of tape stripping or MNs was investigated.

2. Materials and methods

2.1. Materials

Lipids (1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1-palmitoyl-2-{12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC)) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Nachol, polysorbate (Tween®80), sorbitan monooleate (Span®80) and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma Aldrich (Belgium). The tissue-TEK® O.C.T. compound was purchased from VWR (Belgium). DAPI (4′,6-diamidino-2-phenylindole) was purchased from Boehringer Mannheim (Paris, France). Corneofix®F20 were purchased from CK Electronic (Germany). Microneedles arrays were purchased from AdminMed® nanobioSciences LLC (Sunnyvale, CA, USA). Patch Test Chambers® were purchased from Van der Bend BV (Brielle, Netherlands). Ultrapure water was produced by a Milli-Q system (Millipore, Bredford, MA, USA). All other reagents were of analytical grade.

2.2. Nanocarrier formulation

Liposomes were made of a charged lipid (DOTAP or DOPC or DOPG), DOPE and NBD-PC at the weight ratio of 1/0.5/0.2. Nachol, Tween®80 or Span®80 (15% w/w) were used as EA. Liposomes were prepared using the thin film hydration method previously described (Gillet et al., 2009; Lechanteur et al., 2018). Briefly, the appropriate amount of lipids and the EA (if soluble in ethanol such as Span®80) were dissolved in ethanol in a round-bottomed flask. Then, the solution was dried at 30 °C under vacuum using a rotavapor for one hour. The thin lipid film was then rehydrated with ultrapure water and the EA (if soluble in water such as Tween®80 and Nachol) up to a volume of 2 mL. In case of ethosomes preparation, 25% v/v of ethanol was added to ultrapure water to rehydrate the lipid film. Then, suspensions were extruded through Nucleopore® polycarbonate membranes (Whatman, UK) five times on 0.4 µm membranes, three times on 0.2 µm membranes and then three times on 0.1 µm membranes.

2.3. Physicochemical properties

Nanocarriers in ultrapure water were sized in terms of Z-average size (nm) by Dynamic Light Scattering (DLS) at 25 °C with a fixed angle of 90° using the Malvern Zetasizer® (Nano ZS, Malvern Instrument, UK). The polydispersity index (PDI) was also obtained by this instrument. The zeta potential (mV) of neutral and charged liposomes was determined with the same instrument. All experiments were measured in triplicate ($n = 3$).

2.4. Stability

The stability of cationic formulations containing ethanol and Nachol was evaluated during two months of storage at 4 °C or 24 °C. The Z-average size, PDI and zeta potential were analyzed and compared with the initial parameters.

2.5. Statistical analysis

The significance of the differences between the different formulations was tested using one-way Anova (Graph Pad Prism Version 5) with a post-test Tukey. The differences are considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

2.6. Ex vivo skin penetration

2.6.1. Skin preparation

Pig ears were freshly excised on pigs at the slaughterhouse of Malmedy (Belgium). The ears were gently washed and the back skin was isolated from the cartilage. The skin was stored at −20 °C and used within six months. Before use, the skin was thawed at room temperature. After that, the hairs were gently removed with an electric razor. Punches were cut and mounted on Franz diffusion cells. If tape stripping was required to remove the SC, 20 Corneofix® strips were used. If MNs are used, they were pushed into the skin during 10 s to perforate the skin and formulations were directly applied on the perforated skin.

2.6.2. Skin penetration experiments

Skin penetration experiments were carried out using Franz diffusion cells. The excised skin was mounted onto the receiver chamber, epidermal side up. The receiver chamber was filled of a receptor phase composed of HEPES buffer containing 0.01% NaN_3 as the preservative.

The receptor chamber was kept under magnetic stirring (630 rpm) and the skin was thermostated at cutaneous temperature (32 ± 1 °C) throughout the experiment. A volume of 300 µL of each formulation was applied on the skin under non-occlusive condition. The diffusion area was 1.767 cm^2. At the end of the experiment, the skin was removed from the Franz diffusion cells and washed with 3 mL of HEPES buffer on each side to remove the residual sample and gently dried with paper. The treated area was punched out with scalpel and then embedded into an O.C.T compound (Tissue-Tek®, Sakura, The Netherlands) and frozen at −20 °C. After freezing, 7 µm slices were made, and cell nuclei were stained with DAPI. Moreover, other slices were stained with hematoxylin and eosin (HE).

2.7. Confocal laser scanning microscopy (CLSM)

The skin penetration of nanocarriers was observed under a Leica SP5 AOBS confocal microscope. Images were acquired using a 20x objective lens. Two excitation wavelengths were used: 405 nm for the DAPI (emission signals were shown in blue) and 488 nm for NBD-PC (emission signals were shown in green).

2.8. In vivo skin tolerance study

2.8.1. Liposome preparation

Liposomes and ethosomes (25% v/v) were made of DOTAP, DOPE and PC at the weight ratio of 1/0.5/0.2. Nachol (15% w/w) was used as EA. Nanocarriers were prepared and characterized as previously described (see Sections 2.2 and 2.3).

2.8.2. Volunteers

Seventeen healthy volunteers participated to the tolerance study (13 women and 6 men between 22 and 55 years old). The study, in which the formulations were classified as cosmetological forms, was approved by the Ethics Committee of the University of Liège, Belgium (B707201836746). The volunteers signed the information and consent document to participate to the study. They were asked not to use skin products on their forearms the day before and during the study.

2.8.3. Method of application of the formulation

The three application areas were precisely delimited on the anterior forearms avoiding nevus, scars and visible blood vessels. Two formulations were applied. One site remained untreated, one site received the liposomes preparation and one site received the ethosomes preparation. The areas receiving the formulations and the untreated area were covered by the Van der Bend Patch® test chambers containing 25 µL of each formulation. The application of the patches containing the preparations was done on day 0 and was left in contact with the skin for 48 h (D2).

2.8.4. Assessment of the irritative response

The irritative response was assessed using the chromameter (Konica Minolta® CR-400 chromameter, Osaka, Japan). The instrument was calibrated with the standard white plate and was qualified with the standard green plate (plates were provided with the chromameter) before each use.

At days 0, 2 and 3, the color parameters of the skin were measured using the chromameter. To determine the skin color, the chromameter provided three values: L^*, a^* and b^*. In this irritative study, the a^* value is the most appropriate parameter to assess since this value correspond to redness. Moreover, the Euclidean distances (ΔE) can be recorded by the chromameter. This value gives information about the general color change since (ΔE) takes in account the all data recorded (ΔL, Δa and Δb).

At day 0, after resting the forearm in a horizontal position for 3 min, the chromameter measurements of the baseline was recorded. After 48 h of application, the patch was removed and the skin color was recorded. To prevent a late allergic reaction, measurements were performed after 72 h. Each measure was recorded independently 3 times and the average value was calculated. The recorded Δa and ΔE values were calculated with subtracting the baseline (time zero). Moreover, the untreated area value (also baseline corrected) was deducted to acquire Δa and ΔE values:

$$\Delta \text{ value} = (T_{tx} - T_{t0}) - (U_{tx} - U_{t0}),$$

where T corresponds to the treated area (liposomes or ethosomes), U corresponds to the untreated area, tx corresponds to 48 h or 72 h and t0 to the baseline recorded at time zero.

2.8.5. Statistical analysis

The significance of the differences between the different formulations was tested using one-way Anova (Graph Pad Prism Version 5) with a post-hoc test Tukey. The differences are considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3. Results and discussion

3.1. Liposomes and ethosomes characterization

The physicochemical properties of cationic liposomes (DOTAP/DOPE 1/0.5 w/w) were evaluated (Fig. 1). Ethanol (25% v/v) was added to form ethosomes and/or EA such as Span®80, Tween®80 and Nachol were used to form flexible formulations.

All formulations, excepted those containing Span®80, had a size below 200 nm which is the maximum size reported by several authors as suitable for transdermal administration (Su et al., 2017; Verma and Pathak, 2012) (Fig. 1A). Even if differences are not statistically different ($p > 0.05$), ethanol induces a small decrease of the size compared to liposome formulations. The PDI shows the same trend (Fig. 1B). Indeed, PDI of all formulations (Span®80 excepted) was below 0.2 which means that suspensions were homogeneous. The surface charge of the formulations is around +50 mV; this positive charge is obviously induced by the cationic lipid (DOTAP) used (Fig. 1C). Once again, the surface charge resulting from ethosomes is slightly decreased.

It seems evident that the presence of ethanol affects the physicochemical properties (Z-average size, PDI and surface charge) of formulations. This effect has also been highlighted by several authors explaining that alcohol, especially ethanol, can modify the net surface charge of nanocarriers since ethanol acts as a negative charge donor. As a result, it confers some degree of steric stabilization due to electrostatic repulsions leading to a decrease of the vesicular size by avoiding aggregation (Abdulbaqi et al., 2016).

In this study, three EAs belonging to three different classes of surfactants (lipophilic surfactant such as Span®80, hydrophilic surfactant such as Tween®80 and a bile salt such as sodium cholate) were tested (Table 1). It is commonly accepted that the addition of an EA increases the deformability of the vesicles as a consequence of the reduction of the main phase transition temperature (T_m), which gives them the ability to cross the SC. Some authors report that the use of EA with low hydrophilic-lipophilic balance (HLB) results with smaller vesicle size (El Zaafarany et al., 2010). Their underlying hypothesis is that by increasing the hydrophobicity (low HLB), there is a decrease of surface energy and thus arising in small size. However, our results go against this hypothesis since formulations containing Span®80 exhibit the largest size. The hypothesis recently supported by Abdel-Messih et al. (2019), is that the increase of the hydrophobicity of the EA increases its interaction with phospholipid groups in the liposome bilayer. As a result, there is an increase in the packing density of the layer, which increases the free surface energy followed by a lipid bilayer fusion and therefore a bigger size (Abdel-Messih et al., 2019). These hypotheses can explain the larger size and PDI associated to Span®80 formulations, predicting a poor skin penetration. Therefore, the Span®80 was excluded from further experiments.

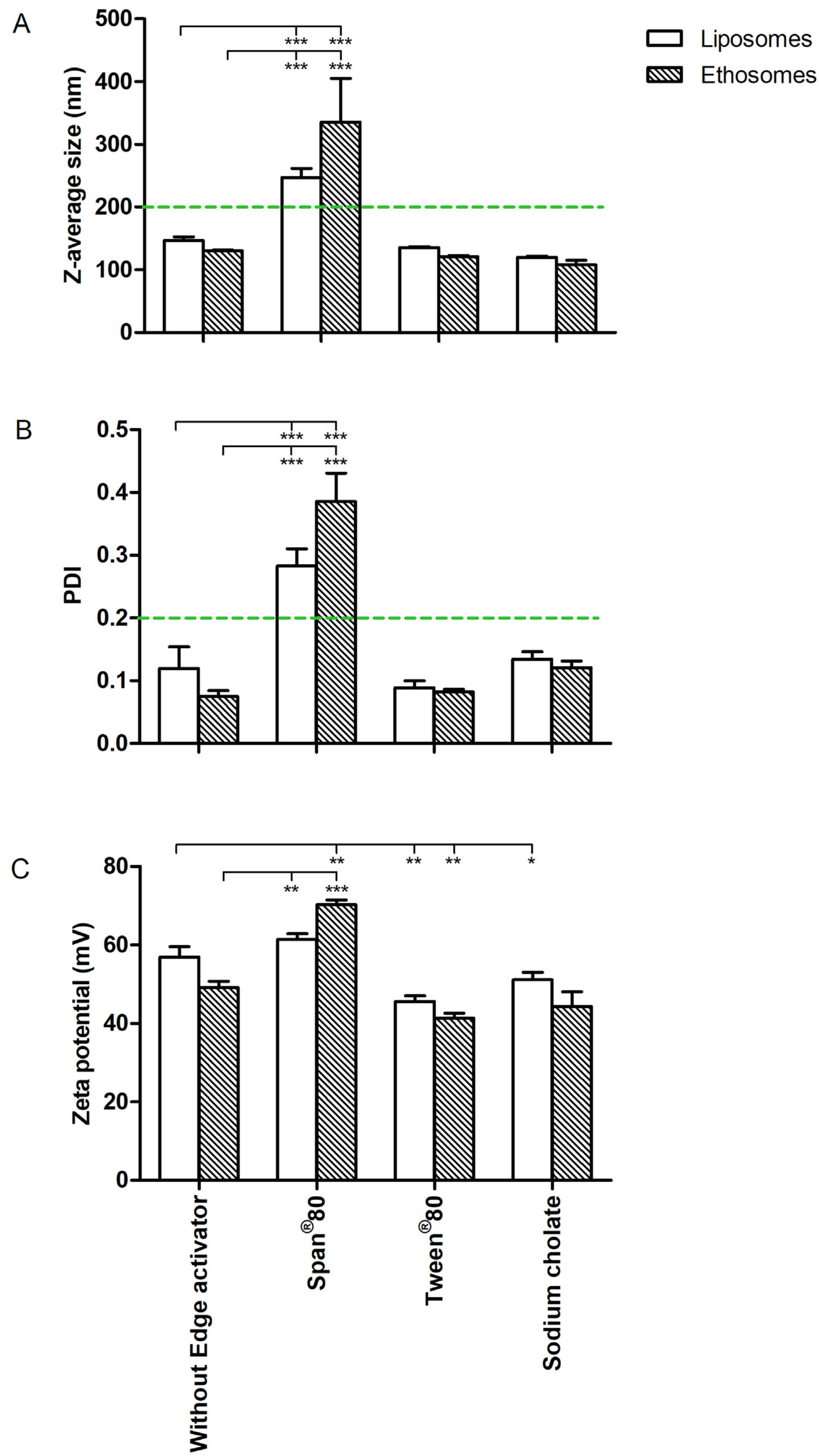

Fig. 1. Comparison of the physicochemical properties of formulations according to their composition. Liposomes and ethosomes (containing 25% v/v of ethanol) were formulated with different edge activators (EA) as Span®80, Tween®80 and sodium cholate. All formulations were characterized in terms of A: Z-Average size (nm), B: PDI, C: Zeta potential (mV). Green lines represent acceptable limits. n = 3.

Table 1
Features of edge activators (EA) used (Molecular weight, hydrophilic-lipophilic balance (HLB) and category of surfactant).

EA	Molecular weight (g/mol)	HLB	Category
Span®80	428.6	4.3	Non-ionic
Tween®80	1310	15	Non-ionic
Sodium cholate	430.6	16.7	Anionic

Although positively charged liposomes seem necessary to complex plasmid DNA in case of gene therapy, the effect of different lipid charge (neutral and anionic) on physicochemical properties (Fig. 2) and further on skin penetration was studied.

As shown in Fig. 2A and B, the charge of the principal lipid does not affect the size of liposomes. All the formulations have a size below 200 nm and a PDI below 0.2.

As expected, there were statistically significant differences in surface charge values (Fig. 2C). These differences are obviously due to the lipid used in the formulation: cationic (DOTAP), anionic (DOPG) or

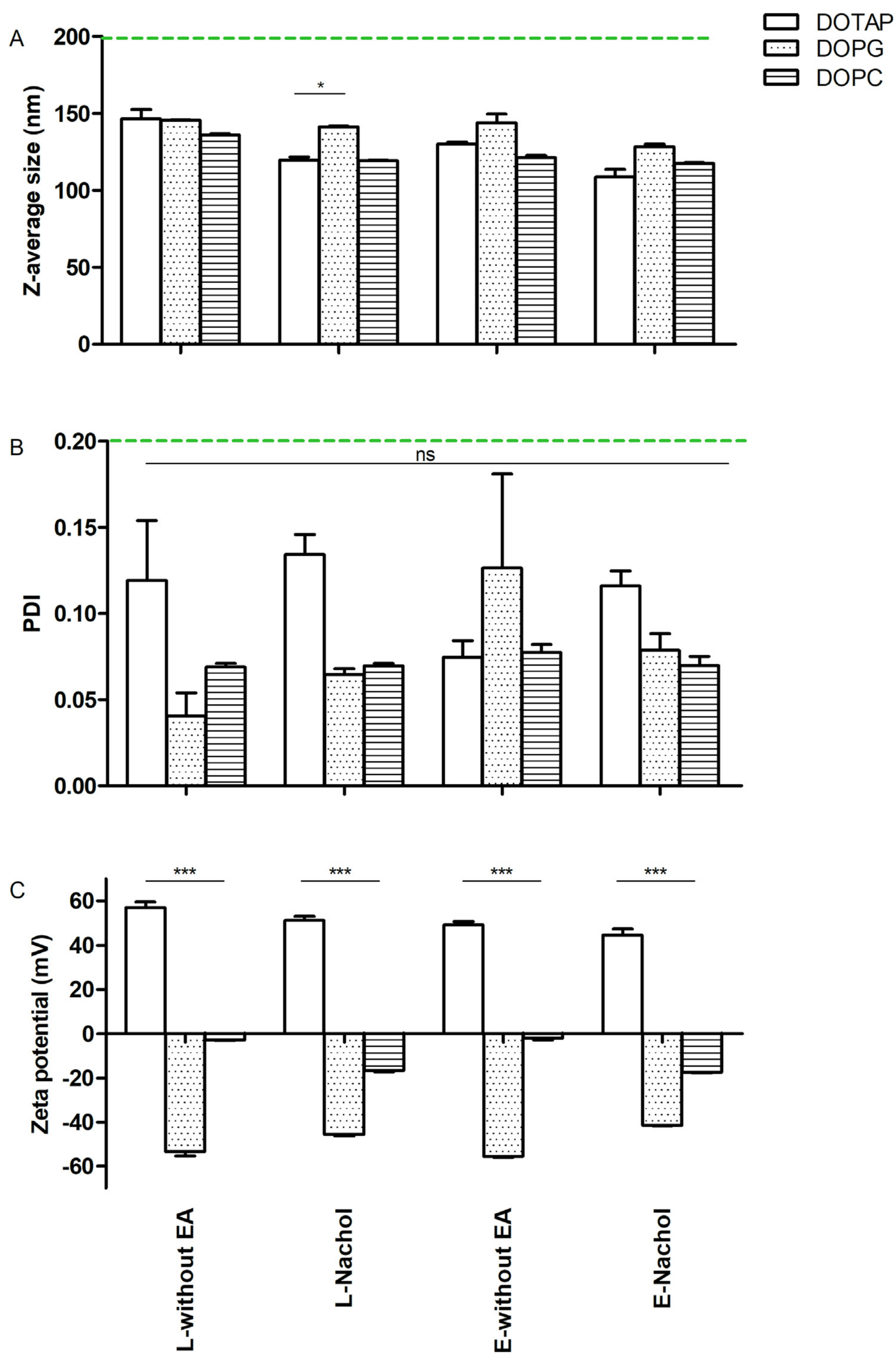

Fig. 2. Comparison of the physicochemical properties of liposomes (L) and ethosomes (E) with or without an edge activator (EA) such as sodium cholate (Nachol) according to the lipid charge (DOTAP: cationic, DOPC: neutral and DOPG: anionic) A: Z-Average size (nm), B: PDI, C: Zeta potential (mV). Green lines represent acceptable limits. n = 3.

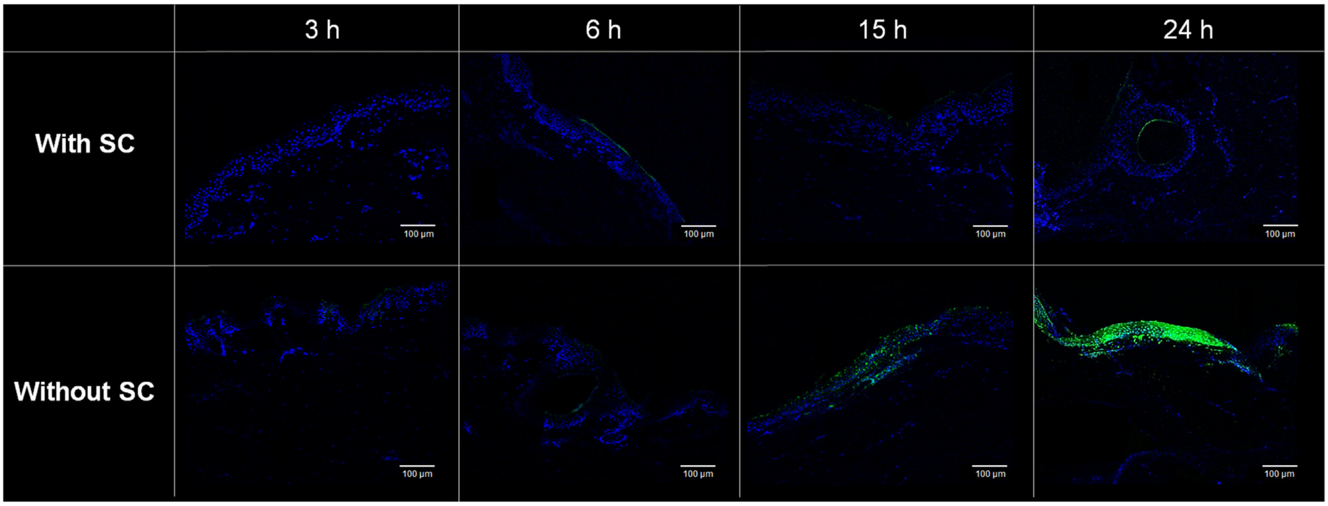

Fig. 3. Confocal images obtained after different time of application of ethosomes containing sodium cholate on a skin with or without the stratum corneum (SC) (removed by tape stripping). Scale bar represents 100 µm. n = 3.

neutral (DOPC). Moreover, the addition of Nachol decreased the surface charge of the formulations. This can be easily explained by the anionic feature of Nachol (Table 1).

3.2. *Ex vivo skin penetration evaluated by confocal microscopy*

3.2.1. *Kinetics*

To determine the optimal incubation time allowing a deep penetration, kinetics was performed up to 24 h. This experiment was performed with the most deformable expected formulation, ethosomes with Nachol (Fig. 3). The SC being the major barrier to cross to allow a penetration in the deeper layers of the skin, tape stripping (Ibaraki et al., 2018) was performed to compare the ability of liposomes to diffuse through the skin once SC was removed. The fluorescent lipid NBD-PC was incorporated in the formulation composition to follow its skin penetration. It should be noted that the physicochemical properties (Z-average size, PDI, zeta potential) of fluorescent formulations do not differ from non-fluorescent formulations. Since NBD-PC is an integral part of the vesicles, it could be assumed that the penetration of the whole liposome is comparable to the penetration of the fluorescent lipid. From this hypothesis, we consider that the fluorescence (green) observed on the confocal images is assimilated to the penetration of liposomes.

After 3 h and 6 h, no penetration can be perceived even if the SC was removed. After 15 h of application on a tape stripped skin, some fluorescence can be visualized. However, it appeared that the fluorescence was utmost after 24 h of application. The penetration of ethosomes seemed stronger and deeper since fluorescence was observed in the viable epidermis. Therefore, all experiments were applied on the skin for 24 h. Furthermore, Fig. 3 shows that the SC removing seems necessary to enable diffusion of the nanocarriers.

3.2.2. *Influence of ethanol on skin penetration*

Since ethanol is described as a penetration enhancer, several authors reported that the penetration of ethosomes was more efficient than the penetration of liposomes (Bibi et al., 2017). Fig. 4 compares liposomes and ethosomes skin penetration (with and without the SC). We have observed that penetration of liposomes could not be perceived on contrary to ethosomes (Fig. 4).

It is commonly accepted that conventional liposomes (without any penetration enhancers) do not promote the skin penetration. They are generally enclosed in the SC or accumulated into the first layers of the viable epidermis with minimal penetration ability due to a lack of deformability (Hussain et al., 2017). While liposomes remain confined in the first layers of viable epidermis on a tape stripped skin, Fig. 4 suggests that ethosomes can diffuse more deeply into this layer once SC was removed. This observation can be explained by the “ethanol effect” (Verma and Pathak, 2012). This phenomenon wherein ethosomes enhanced skin penetration has been described by Touitou and coworkers (Touitou et al., 2000). They hypothesized that ethanol contained in formulations could affect the conformation of the SC lipids which are deeply compacted and extremely ordered in physiological conditions. This could happen because of the interaction between ethanol and the lipid polar headgroup of SC, which would lead to a decrease in T_m thus inducing an increase of SC fluidity. Moreover, the incorporation of ethanol in vesicles makes ethosomes able to fuse with skin lipids due to their malleability resulting in new pathways opening which allow drug diffusion through the disturbed SC. This additional mechanism is referred to “ethosome effect” (Bibi et al., 2017; Elsayed et al., 2007).

The SC acts as an efficient barrier impeding drug penetration. The use of a SC removal technique as the tape stripping was mandatory to determine which formulation is able to diffuse more freely into the skin (Fig. 4). Therefore, the results of the next experiments will only be shown for skin without SC.

3.2.3. *Influence of EA on skin penetration*

The influence of EAs on skin penetration was then evaluated. Since ethanol appears as a penetration enhancer (Section 3.2.2), the aim of this experiment is to demonstrate whether the addition of an EA increases this skin penetration (synergistic effect) compared to formulations containing only ethanol and formulations containing only an EA. Cutaneous penetration of formulations with and without ethanol and/or EAs (Tween®80 or Nachol) is shown in Fig. 5.

Fig. 5A–C (corresponding to liposomes) display a fluorescence intensity lower than Fig. 5D–F (corresponding to ethosomes) which means that the liposomes do not diffuse as much as ethosomes. These results showed that the ethanol effect is anew highlighted. Furthermore, it is obvious that EAs favor a deeper penetration compared to formulations without EAs. Differences can be established depending of the EA used. Whereas liposomes containing Tween®80 remain in the first cell layers of viable epidermis, liposomes containing Nachol are able to penetrate more profoundly. It has been suggested that Nachol have a pK_a close to skin pH ~ 5.5. As a result, the protonation of Nachol occurs in the nanovectors. This protonation being exothermic, it could modulate the skin structure making it more permeable (Geusens et al., 2010). Interestingly, the formulation containing ethanol and Nachol allows the deepest penetration since fluorescence was localized across the viable epidermis until the upper dermis (Fig. 5). This synergy

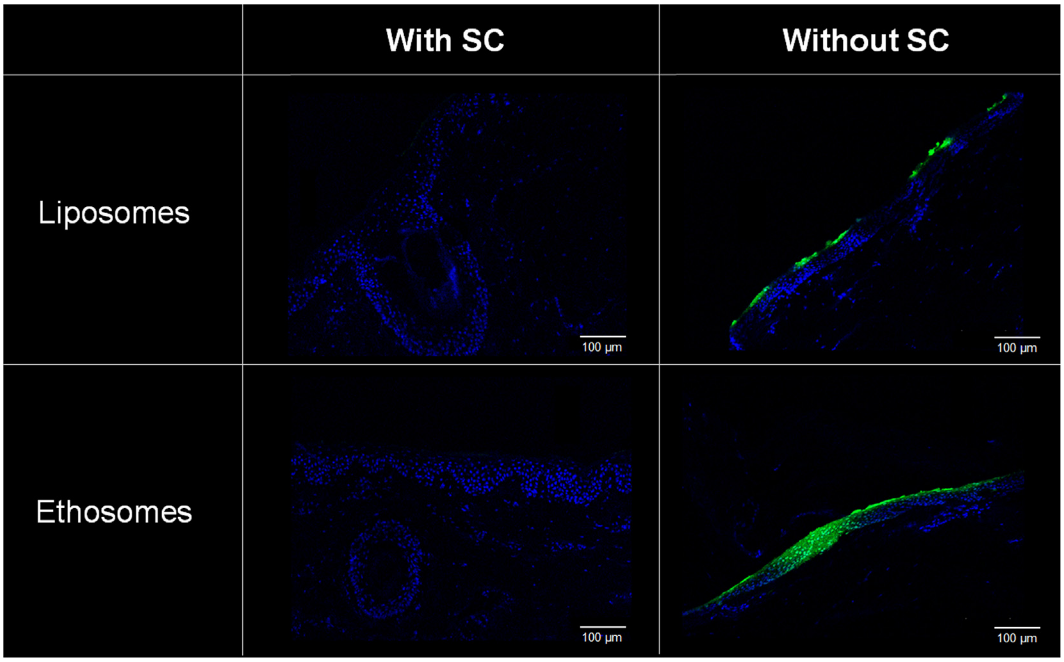

Fig. 4. Confocal images obtained after application of liposomes or ethosomes on skin with or without the stratum corneum (SC). Scale bar represents 100 µm. n = 3.

between ethanol and EA has also been noticed by several authors (Abdel-Messih et al., 2019).

3.2.4. Influence of the lipid charge on skin penetration

In order to check the influence of the main lipid charge, skin penetration of cationic, neutral or anionic liposomes with and without ethanol and/or Nachol was investigated (Fig. 6).

As shown in Fig. 6 and observed previously, fluorescence intensity associated to cationic nanocarriers (DOTAP) increases when ethanol is added to the formulation. On the contrary, fluorescence of neutral (DOPC) and anionic (DOPG) formulations could not be detected with or without ethanol in the formulation. These diffusion differences are probably due to the fact that the skin is considered as a negatively charged membrane. It therefore seems that an opposite charge formulation (containing DOTAP) is able to diffuse into the skin compared to neutral and anionic formulation with which charge repulsions can occur and prevent penetration. These results agreed with recent research on the effect of the surface charge and flexibility of liposomes allowing effective dermal penetration (Ibaraki et al., 2019). They found that penetration of cationic liposomes on tape stripped mouse skin is

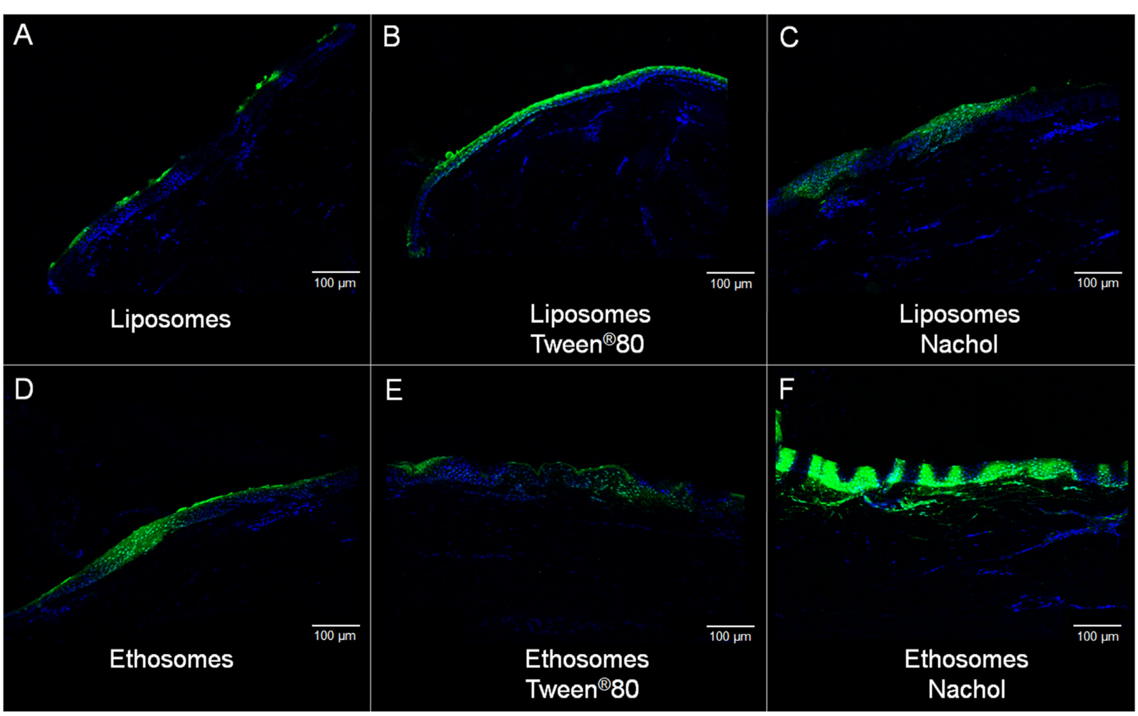

Fig. 5. Confocal images obtained after application of formulations with and without ethanol and/or Tween®80 and sodium cholate (Nachol) on a tape stripped skin. Scale bar represents 100 µm. n = 3.

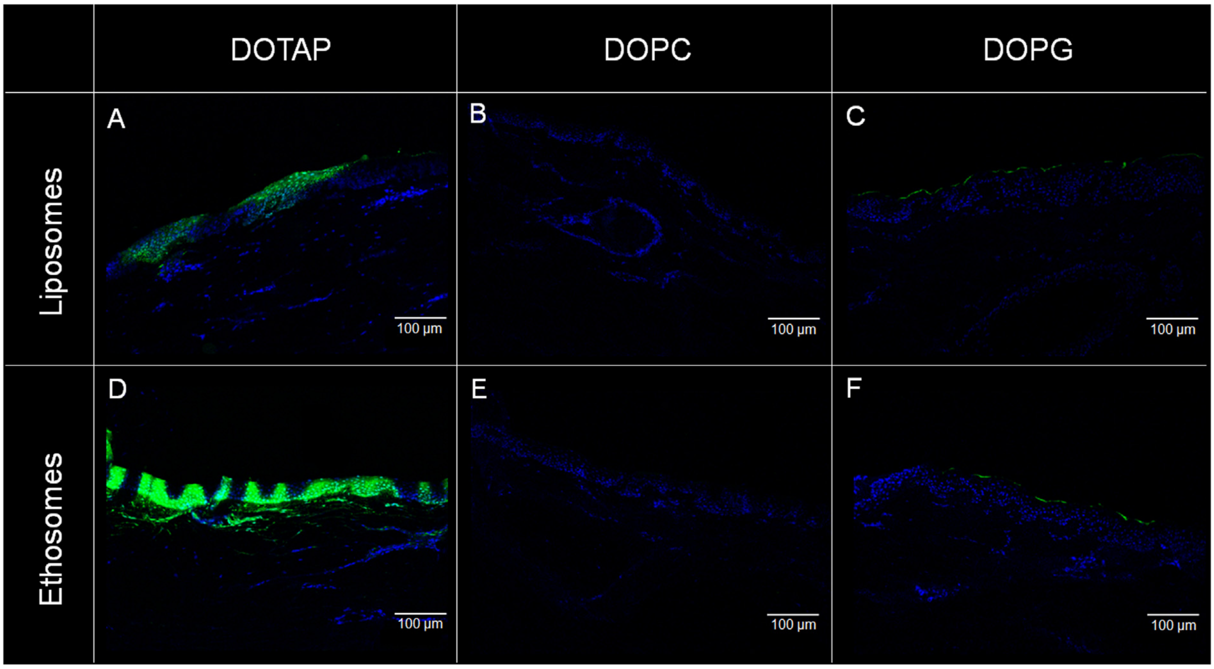

Fig. 6. Confocal images obtained after application of cationic, neutral or anionic formulations with and without ethanol and/or sodium cholate on a tape stripped skin. Scale bar represents 100 µm. n = 3.

higher than anionic ones even higher than neutral liposomes. These authors also demonstrated that cationic formulation showed higher damage of the skin caused by a collapsing of tight junctions in skin tissue due to the strong electrostatic interaction between cationic formulations and the skin.

Due to these observations and because our purpose is to complex plasmid DNA which is negatively charged, we decided to select the cationic formulation containing ethanol and Nachol.

3.2.5. Microneedles to bypass the stratum corneum

As evidenced by previous skin penetration tests, penetration of nanocarriers could only be observed if the SC had been removed. It is therefore obvious that even if the formulations are supposed to be deformable, none of them was able to cross the SC. Thenceforth, it is necessary to bypass the SC in a painless and easily achievable way for the patient (Chen et al., 2019; Jin et al., 2018). For this purpose, the use of MNs is appropriate. Indeed, MNs are simple and easy-to-use devices which allow the crossing of the SC without reaching the nerves which makes this system painless. Different needles lengths are available which allows different skin layers to be reached depending on their depth. Indeed, MNs are available from 150 to 1500 µm long. Longer MNs arrays could reach the nerves that lie in the dermis and cause pain (Waghule et al., 2019). Here, the *ex vivo* penetration experiments aim to reach the upper layer of the dermis hence maximum lengths will be used to reach this layer. Fig. 7 shows the different densities and needles lengths tested to define the length allowing a penetration into the dermis (AdminMed®).

The effectiveness of these MNs has been tested with the cationic formulation containing ethanol and Nachol (Fig. 8).

Fig. 8A–D shows the eosin hematoxylin (HE) and confocal microscopy images (E-H) obtained before and after the application of MNs. On HE images, the micro-holes created by needles can be visualized. Even if the impact of the MNs is observed, it seems that the micro-holes have collapsed. It could be explained by the skin recovery process by which the skin recovers after 2–40 h depending of the occlusion state (the recovery is faster under non-occlusive condition) (Waghule et al., 2019). On confocal microscopy images, there is a comparison between skin penetration without using MNs and skin penetration using MNs of 500 µm, 800 µm and 1400 µm of length. Rationally, the confocal images show that the depth of penetration increases with the length of the needles. With the longest needles (1400 µm), the nanovectors can even reach the dermis.

In order to evaluate if the ethanol and Nachol are favorable to penetration associated with MNs, Fig. 9 exhibits a comparison of skin penetration of formulations with and without ethanol and/or with and without Nachol on a skin pretreated with MNs of 1400 µm in length.

Fig. 9 shows that even if the SC is bypassed by MNs, the addition of ethanol and Nachol is necessary to promote a deeper penetration and skin diffusion. Although the mechanism remains unclear, a synergistic effect of the Nachol and ethanol could be involved in the improvement

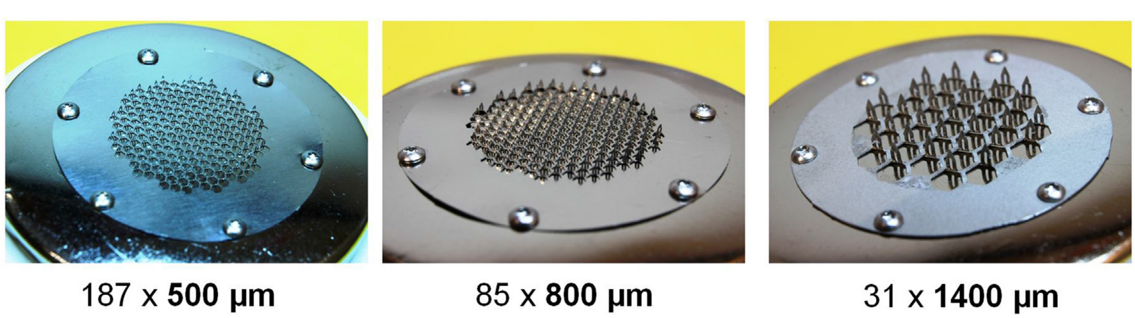

Fig. 7. Microneedles arrays tested (density cm^{-2} × length of the needles) (From AdminMed®, http://adminmed.com/adminstamp).

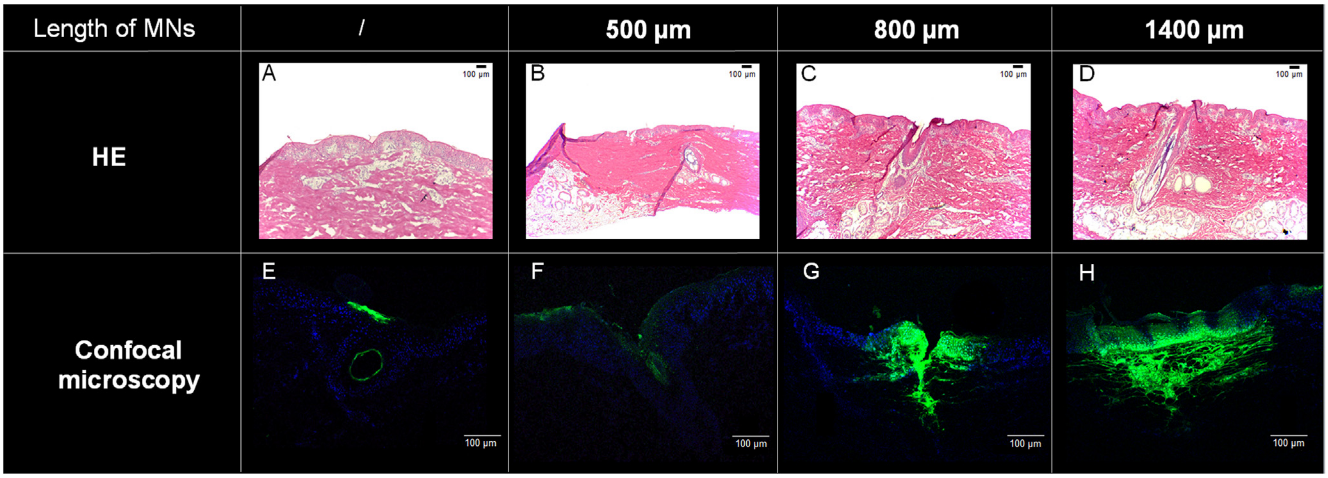

Fig. 8. HE staining (4× magnification) (A–D) and confocal images (20× magnification) (E–H) obtained after application of the cationic formulation containing ethanol and Nachol using different microneedles arrays (MNs). Scale bar represents 100 µm. n = 3.

of skin penetration and dermal diffusion of formulations applied by MNs.

Some authors reported that occlusion condition increases skin penetration induced by MNs by increasing the opening time of the microholes (Kalluri et al., 2011; Van Der Maaden et al., 2012). Therefore, to verify this hypothesis, the skin penetration under occlusion condition was tested. The skin penetration experiments under occlusion were performed (i) on an intact skin, (ii) on a tape stripped skin and (iii) on a perforated skin with MNs of 1400 µm of length (Fig. 10).

Fig. 10 shows that cutaneous occlusion on intact skin and a tape stripped skin does not enhance the penetration. As suggested by some authors (Cevc and Blume, 2001), the osmotic gradient is the main driving force to push nanocarriers into the skin. This hypothesis assumes that when applied on the skin under non-occlusive condition, liposomes and deformable liposomes are exposed to dehydration and therefore the concentration of liposomes increases on the top of the SC. Consequently, liposomes tend to penetrate the skin until reaching equilibrium between the concentration outside and inside the skin. In addition, since the deeper layer of the epidermis, the viable epidermis, contains a larger amount of water (~75%) than the SC (~10–30%), there is a transepidermal osmotic gradient between these two layers (Hussain et al., 2017). This gradient acts as pushing agent to drive liposomes to penetrate the skin. Under occlusive condition, this transepidermal hydration gradient is thus abolished owing to the hydration of the SC in this condition. Another point is that the water affinity of liposomes must be taken into account when penetration is considered. Indeed, liposomes have been described as xerophobia (Cevc and Blume, 1992); so they tend to reach an hydrated layer, in particular the viable epidermis. In consequence, many mechanisms can explain the fact that occlusion condition does not enhance the (deformable) liposomes skin penetration (Hussain et al., 2017).

In contrast to liposomes and deformable liposomes, it has been reported that ethosomes skin penetration is not affected by the occlusion state of the skin. For instance, Touitou and coworkers showed that the drug delivery is achieved under both non-occlusive and occlusive conditions (Dayan and Touitou, 2000; Elsayed et al., 2007; Godin and Touitou, 2004).

Since the formulation contains both ethanol and an EA, the resulting formulation penetrates preferentially into the non-occlusive condition (Fig. 10). Contrarily to previous observations from some researchers

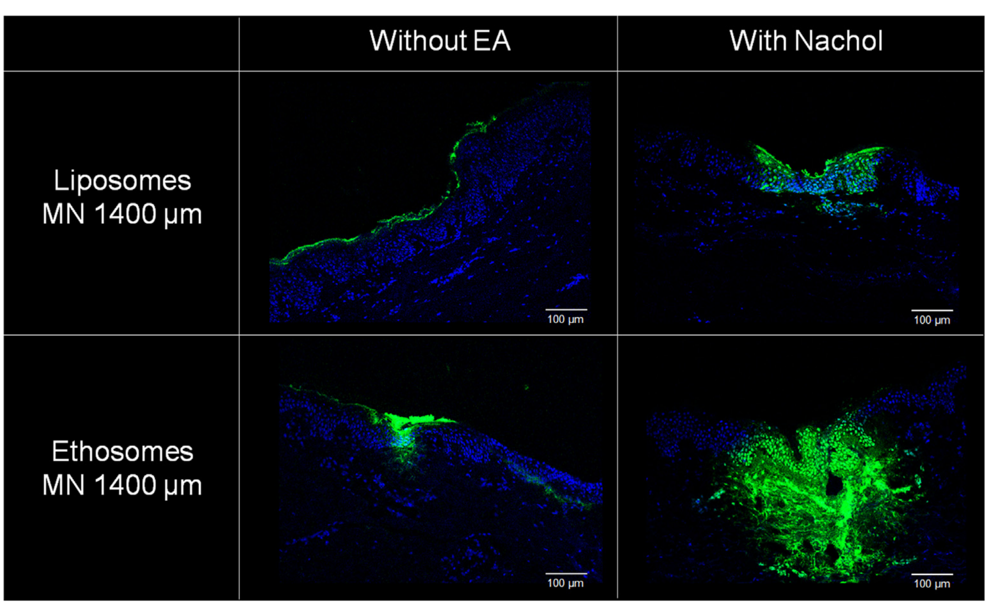

Fig. 9. Confocal images obtained after application of cationic formulations with and without ethanol and/or sodium cholate (Nachol) as edge activator (EA) on skin pretreated by application of microneedles (MN) of 1400 µm in length. Scale bar represents 100 µm. n = 3.

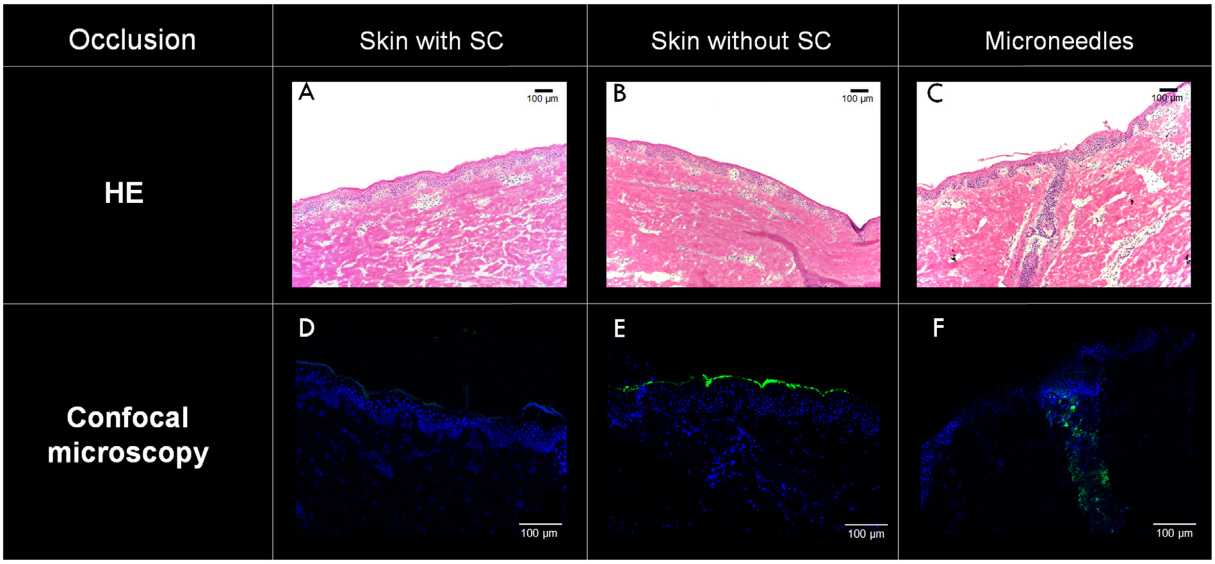

Fig. 10. HE staining (10× magnification) and confocal images (20 × magnification) obtained after application of cationic formulation containing ethanol and sodium cholate under occlusion on an intact skin, a tape stripped skin and perforated skin with microneedles of 1400 µm of length. Scale bar represents 100 µm. n = 3.

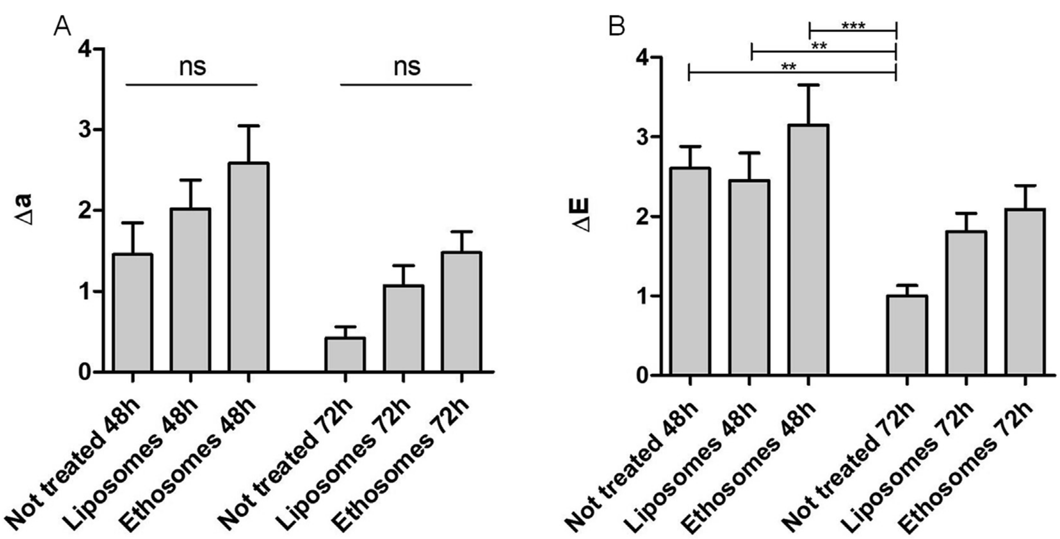

Fig. 11. (A) Δa (redness) recorded for different conditions after the patch removing (48 h) and the day after (72 h). (B) ΔE (general color change) recorded for different conditions after the patch removing (48 h) and the day after (72 h). n = 17.

(Qiu et al., 2008) that showed that the nanocarriers can penetrate deeper under occlusive condition on a pretreated skin with MNs, we could not observe that the micro-holes were opened longer allowing a stronger penetration of our formulation under occlusive condition. Moreover, this theory is not appropriate to an *in vivo* application. Indeed, the micro-channels created by MNs could increase the risk of infection due to the direct opening of the skin surface to the inner part of the skin. Therefore, we suggest that the application of cationic formulations containing ethanol and Nachol is more efficient in terms of skin penetration and safer in terms of risk infections under non-occlusive condition.

The physicochemical properties of the cationic formulation containing ethanol and Nachol (Z-average size, PDI and Zeta potential) were monitored over two months at 4 °C and 24 °C. The particle size stayed around 120 nm, the PDI remained under 0.2 and the Zeta potential remained constant (around + 45 mV) during two months at both 4 °C and 25 °C. This result can be explained by repulsive forces of bilayers which contain Nachol (anionic surfactant) and thus preventing ethosomes aggregation and instability (Limsuwan and Amnuaikit, 2012). In addition, the rigid bulky steroid-like structure of Nachol inserted in the lipid bilayer can stabilize the membrane and prevent its instability.

3.3. Skin tolerance study

A skin tolerance study was conducted to ensure the safety of the selected formulation. The irritative response induced by cationic ethosomes containing Nachol was evaluated and compared with a control formulation without ethanol (liposomes) and with an untreated area.

Fig. 11A and B represents the Δa and ΔE values, respectively. Since *a*-scale increases with the redness, a high value of Δa indicates an irritation probably due to the application of formulation. Fig. 11A shows that after removal of the patch containing the formulations, the Δa between not treated areas or treated with the formulations with and without ethanol are not statistically different. Even if differences are not statistically different, a trend can be noticed in function of the

Fig. 12. Pictures of areas tested on volunteers after 48 h of application. Application areas: top left: liposomes, top right: ethosomes, bottom right: untreated area. n = 17.

formulation complexity. Indeed, the irritation induced by ethosomes seems stronger than irritation caused by liposomes. Although Fig. 11A and B shows a tendency for irritation depending on the formulation, the volunteers felt no pain or itching during this study. When comparing pictures of treated areas on volunteers after 48 h of application (pictures were taken directly after removal of the patch), no irritation on treated areas could be perceived (Fig. 12). Even if data recorded the next day (72 h) seem to follow the same trend, *a*-scale after 72 h is weaker. This difference can be explained by the removal of the patch. Indeed, the adhesive tape could irritate the skin during the patch removing. The skin folds were irritated around the treated areas where the patch was adherent (Fig. 12). This irritation disappeared the day after and thus the Δa decreased. In a similar manner, the ΔE values are related to Δa values. Same trends are observed and ΔE values of treated areas do not differ from the untreated areas. Consequently, we could conclude that formulations, even ethanol containing formulations are not irritating.

4. Conclusion

In this study, different lipid-based nanovectors have been developed. In order to overcome the skin major barrier, the SC, different strategies have been implemented. The influence of several EAs and ethanol in the lipid composition as well as different lipid charges were studied by *in vitro* and *ex vivo* experiments.

Based on physicochemical properties, two EAs were chosen, the Tween®80 and the Nachol. *Ex vivo* skin penetration demonstrated that all the formulations were not able to cross the SC even deformable formulations. However, when the SC was removed, we showed that the presence of an EA in particular the Nachol improved the skin penetration. We also observed that the penetration is deeper when ethanol is added to the composition. Finally, we demonstrated that the neutral or anionic formulations were less effective in terms of skin penetration than cationic formulations. For these reasons the cationic formulation containing Nachol as EA and ethanol was chosen as the most promising formulation. Furthermore this formulation did not induce irritation during the skin tolerance study.

As the selected formulation was effective once the SC was removed by the tape stripping method, the use of MNs was considered. We have shown that longer MNs drive the nanovectors efficiently into the dermis and that the Nachol and the ethanol are necessary to the lipid composition to ensure an appropriate release into the dermis.

In conclusion, we developed lipid-based nanovectors containing a cationic lipid, Nachol and ethanol able to diffuse into the dermis when applied by means of MNs. This formulation could be a promising approach to deliver large macromolecules such as nucleic acids into the skin.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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