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Research paper

Liposomes increase skin penetration of entrapped and non-entrapped hydrophilic substances into human skin: a skin penetration and confocal laser scanning microscopy study

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Abstract

Liposomes have been extensively studied and suggested as a vehicle for topical drug delivery systems. However, the mechanism by which liposomes deliver drugs into intact skin is not fully understood. In the present study, we have tried to understand the mechanism of transport of hydrophilic drugs into the skin using liposomes. The effect of separation of the non-entrapped, hydrophilic fluorescent compound, carboxyfluorescein (CF), from liposomally entrapped CF was investigated by measuring the penetration of CF across human skin under non-occlusive conditions in vitro using Franz diffusion cells. The fluorescent dye, CF, was incorporated into the liposomes and applied onto the skin. After a 6 and 12 h incubation period, the amount of CF in the epidermal membrane and the full thickness skin was determined by fluorescence spectroscopy or by confocal laser scanning microscopy (CLSM). The liposomal formulation containing CF both inside and outside the vesicles showed statistically enhanced penetration of CF into the human stratum corneum (SC) as compared to the formulations containing CF only outside of the liposomes and CF in Tris buffer. The CLSM results revealed that the formulation in which CF was present outside the liposomes showed bright fluorescence intensity in the SC and very weak fluorescence in the viable epidermis. However, the CF in Tris buffer failed to show any fluorescence in the viable epidermis. The results indicated that phospholipid vesicles not only carry the entrapped hydrophilic substance, but also the non-entrapped hydrophilic substance into the SC and possibly into the deeper layers of the skin.

Keywords: Topical delivery; Liposomes; Entrapment; Carboxyfluorescein; Skin penetration

1. Introduction

Mezei and his group initiated research in the use of liposomes for topical skin application in the early 1980s. Two in vivo studies in rabbits documented comparisons between liposomal and conventional formulations of triamcinolone acetonide [1,2]. In both studies, the application of the liposomal preparations was associated with enhanced steroid concentrations in the epidermis, as well as in the dermis, and a lower systemic absorption than the regular formulations. Further bio-deposition studies in animals have demonstrated that liposomal encapsulation can improve the penetration of various molecules. Enhanced delivery into the skin has been reported for caffeine in hairless rats [3] and lidocaine in rats [4]. In these systems, liposomal delivery resulted in the formation of a large drug reservoir in the skin, which could be used for local treatment. The penetration kinetics of molecules from liposomes has also been assessed using in vitro skin studies. Egbaria and co-workers found that incorporation into liposomes resulted in the increased uptake of hydrocortisone, fluocinolone acetonide and ciclosporin [5] into the cornified layer of hairless mice and guinea pigs.

Further research focusing on the use of liposomes for targeting drugs into the pilosebaceous structures, has indicated that liposomal encapsulation could be beneficial for treating hair follicle-associated disorders, such as acne, alopecia, as well as for various tyopes of skin cancer. In addition lipsomal formulations may mediate accelerated

Abbreviations: CF, carboxyfluorescein; SC, stratum corneum; PI, polydispersity index; MLV, multilamillar liposomes; CLSM, confocal laser scanning microscopy.

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systemic delivery via transport through the shunt pathway, also called the follicular pathway [6]. Experiments with the Syrian hamster ear model have demonstrated that carboxyfluorescein (CF)-loaded liposomes delivered much higher drug concentrations into the sebaceous glands as compared to conventional CF formulations [7]. Li and co-workers found that liposomal entrapment of calcein [8], melanin [9] and DNA [10] resulted in specific delivery into the hair follicles of histocultured mouse skin, while aqueous control solutions of these molecules showed no drug localization within the follicle.

An important contribution to the understanding of the interactions between vesicles and human skin was made by Junginger and his group [11,12] with the use of freeze fracture electron microscopy and small angle X-ray scattering to study the effects that vesicle formulations have on the stratum corneum (SC). They identified two types of liposome–skin interactions: (1) adsorption and fusion of loaded vesicles on the surface of the skin leading to increased thermodynamic activity and enhanced penetration of lipophilic drugs; (2) interaction of the vesicles within the deeper layers of the SC promoting impaired barrier function of these layers to the drug.

Liposomal carriers have been used successfully in enhancing the clinical efficacy of a number of drugs. These have included tretinoin for the treatment of acne [13], glucocorticoids for the treatment of atopic eczema [14], as well as the anesthetics lidocaine and tetracaine [4,15]. For more information, a review on enhanced topical delivery by liposomal carriers was published in 1995 [16]. The first marketed topical liposomal preparation, Pevaryl Lipogel, produced by Cilag A.G. became available in Switzerland in 1988. The product contains 1% econazole in liposomes formulated in a gel.

Recent approaches in modulating drug delivery through the skin has resulted in the design of two novel vesicular carriers, ethosomes and transfersomes. These vesicles have been shown to deliver molecules through the skin and into the deeper layers. The ethosomes are soft phospholipid vesicles whose size can be modulated from tens of nanometers to microns [17]. These vesicular systems have been found to be very efficient for enhanced delivery of molecules with different physico-chemical characteristics to/through the skin. Transfersomes have also been shown to be versatile carriers for the local and systemic delivery of various steroids, proteins and hydrophilic macromolecules [18]. The mechanism proposed by the authors is that these transfersomes are highly deformable and this property facilitates their rapid penetration through the intercellular lipid pathway of the SC. The osmotic gradient, caused by the difference in water concentrations between the skin surface and skin interior, has been proposed as the major driving force for the penetration of transfersomes [19]. Although the precise mechanism by which vesicular carriers achieve their enhancement effects is yet to be elucidated, current data point towards their great potential in the design of improved delivery systems.

Confocal laser scanning microscopy (CLSM) has been used extensively to visualize the intensity of hydrophilic and lipophilic markers in the skin. The major advantage of CLSM is that the distribution of the model fluorescent compound in the sample can be visualized without cryofixing. However, in the case of the penetration studies with liposomes, CLSM does not provide information about the permeation of the entire liposome. Instead, CLSM can only provide evidence about the localization of the fluorescent label [20].

We assumed that the topically applied liposomes, prepared from phospholipids, can carry both entrapped as well as non-entrapped, hydrophilic drugs into the skin. When the model hydrophilic compound is present both in the bulk of the formulation and in the core of the liposomes, the vesicles are not under osmotic stress and may transfer their payload more easily into the SC. In the present study, we evaluated the penetration behavior of liposomes, which has CF both inside and outside of the liposomes (liposomes CF_{in-out}), i.e. no separation of the non-entrapped CF; liposomes having CF only inside (liposomes CF_{in}), i.e. liposomes separated from free CF by dialysis; and empty liposomes containing CF outside only (liposomes CF_{out}), i.e. CF solution was added after making liposomes and the CF solution in Tris buffer.

2. Materials and methods

2.1. Materials

2.1.1. Lipids

Phospholipon 90 was purchased from Nattermann GmbH (Cologne, Germany) and α-tocopherol from Merck (Darmstadt, Germany).

2.1.2. Other chemicals and instruments

CF was purchased from Fluka Biochemika (Steinheim, Germany). Sodium cholate, chloroform and methanol were purchased from Merck ((Darmstadt, Germany). Tris ultra pure was purchased from ICN Biomedicals (Aurora, Ohio). All other chemicals were of analytical grade and the water used was demineralized by means of a Milli-Q plant (Millipore, Darmstadt, Germany). Mini extruder Lipofast was from Avestin (Ottawa, Canada). Mini Lipoprep was purchased from Dianorm (München, Germany). The fluorescence spectrometer was a Perkin Elmer-LS50B luminescence (Langen, Germany). The dialysis membrane with a diameter of 63 mm was purchased from Diachema, Germany. The cryotome cuts were carried out with a Vogel Cryotome AS 620 (Anglia-Scientific, UK).

2.2. Liposome preparation and characterization

2.2.1. Liposome preparation

Liposomes were prepared by a conventional rotary evaporation method. Phospholipon 90 (10% wt), α-tocopherol (1% wt) and sodium cholate (wt ratio of surfactant and lipid = 0.28) [21] were dissolved in methanol and chloroform (1:1). This mixture was dried to a thin film in the presence of nitrogen and under vacuum. This film was kept under high vacuum for 2 h to remove the traces of organic solvents. The film was then hydrated with Tris buffer pH 7.0 containing CF to make a lipid coarse suspension. These multilamillar vesicles (MLV) were pressed through 400, 200, 100 and 50 nm pore size polycarbonate membranes with the help of an Avestin mini extrusion device [22]. For preparation of liposomes CF_{out}, the blank liposomes were first made from Phospholipon 90, α-tocopherol, sodium cholate and Tris buffer pH 7.0. Afterwords a pre-calculated amount of CF was added to make up the final volume.

2.2.2. Liposomal size

The diameter of vesicles was measured by a Zetasizer instrument (Malvern Instruments, Malvern, UK). The polydispersity index (PI) was used as a measure of an unimodal size distribution. A small value of PI (<0.1) indicates a homogenous population, while a PI > 0.3 indicates a higher heterogeneity.

2.2.3. Separation of non-entrapped CF from liposomes

As liposomes are much larger in size than the materials being entrapped, separation of non-entrapped material can be achieved on the basis of size differences either by gel filtration column chromatography or by dialysis. In the present study, we used dialysis techniques for separating non-entrapped CF. The non-entrapped CF was separated from entrapped CF by using the Mini Lipoprep device with a dialysis membrane of 63-mm diameter. The membrane was submerged for 20 min in water. The water was replaced with fresh water every 5 min. This was followed by soaking the membrane for 10 min in Tris buffer pH 7.0. The membrane was then fixed in the Teflon chamber of the device. The system was placed into 2 liters of Tris buffer in an angular position. The dialyzer was filled with 1 ml of CF liposomes and rotated at the speed of 10 rpm. The Tris buffer was replaced with fresh buffer after 0.5, 1, 2 and 3 h. Samples were taken from the receiver solution at predetermined time periods and were analyzed for their CF content. After 6 h, the experimental setup was dismantled and purified liposomes were taken out of the Teflon chamber. The CF concentration was analyzed by adding a small amount of Triton X-100. The liposomes were immediately used for skin penetration studies.

2.3. The skin

2.3.1. Skin dissection

Excised human skin from female patients who had undergone abdominal plastic surgery was used. Immediately after excision, the subcutaneous fatty tissue was removed using a scalpel. The skin was wrapped in aluminum foil and stored in polyethylene bags at −25°C until use. Under these conditions, the skin is stable with regard to the penetration of drugs, as well as the thickness of the SC, over a time period of 3 and 6 months, respectively [23–25]. For penetration experiments, skin disks of 35 mm in diameter were punched out, thawed, cleaned with cotton, which was soaked with Ringer solution and transferred onto the Franz diffusion cell.

2.3.2. Determination of the thickness of the SC and the skin

For determination of the thickness of the SC, the adhesive tapes were accurately weighed before and after stripping. The resulting layer thickness was calculated according to the following equation [26]:

$$T = d/ap$$

where T represents the thickness of SC removed (μm), d is the differences in strip weight after and before stripping (μg), a denotes the area of the strip (μm^2) and p is the density of the SC (1×10^3 μg/1×10^9 μm^3).

The stripped skin was sliced into surface parallel sections and pooled according to the following schemes: # 1 = first slice; # 2–5 = 6 × 20 μm sections; # 6–9 = 12 × 20 μm sections; # 10 = rest of the residual tissue. The thickness of the first and last sample was calculated from the total weight of the full thickness slices. The slices were pooled for analytical reasons.

2.4. Penetration studies

2.4.1. Dosage regime and incubation times

Ten microliters of the liposomal formulations or CF solution, were non-occlusively applied per cm^2 onto the skin surface. The duration of the penetration studies lasted for 6 or 12 h on a Franz diffusion cell.

2.4.2. Franz diffusion cell

On the Franz diffusion cell (Gauer Glas, Püttlingen, Germany), the skin sections were mounted with nominal surface areas of 3.14 cm^2 and a receiver compartment of a 12 ml capacity. The epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed by phosphate buffer saline pH 7.4. The receptor fluid was mixed with a magnetic stirring bar at 500 rpm. Buffer was kept at 37 ± 1°C by a water jacket. All bubbles were carefully removed between the underside of the skin and solution in the receiver compartment. To achieve higher reproducibility, the skin was pre-hydrated with the basolateral receptor medium for 60 min before applying the

formulation. The liposomal formulations were applied onto the skin surface area of 3.14 cm^2 by help of a micropipette. A minimum of three diffusion cells was used for each formulation. All experiments were carried out with non-occluded donor compartments. After 6 or 12 h, the experiments were stopped and the diffusion setup was dismantled.

2.4.3. Skin stripping and skin cryosectioning

After incubation for a predetermined time period, the liposomal formulation was removed by wiping the skin with cotton. The skin was transferred to a special apparatus where it was mounted on cork discs using small pins. The stretching of the skin was important as it helped to overcome problems of furrow in the subsequent tape-stripping procedure. For this procedure, the surface of the skin was covered with a Teflon mask containing a central hole of 15 mm in diameter. Proceeding from this hole, the skin was stripped with 20 pieces of adhesive tape (size = 15 × 20 mm, Tesa®, Beiersdorf, Hamburg, Germany). The tape was of sufficient size to cover the full area of the skin, which was in contact with the formulation. In a standardized procedure, each tape was charged with a weight of 2 kg for 10 s and, thereafter, removed rapidly. The adhesive tape was weighed before and after stripping to determine the weight of SC removed by each strip.

After the tape stripping, the skin was rapidly frozen liquid nitrogen, a specimen with a diameter of 13 mm was taken out of the stripped area and frozen on a metal block. The metal block was transferred into a cryomicrotome for slicing the stripped skin as explained in Section 2.3.2.

2.4.4. Skin cryosectioning for CLSM

After an incubation period of 6 h on Franz diffusion cell, the skin pieces were sliced in sections of 7-μm thickness, perpendicular to the skin, with the help of cryomicrotome. These cross-sections were investigated for the amount of CF in the different skin layers by using a laser scanning confocal imaging system ('True confocal Scanner', Leica TCS 4D, Microscope-Leitz DM R XE upright, Laser: argon krypton emission wavelengths of 488, 578 and 647, Filters: OG 590 for DiI and BP-FITC for CF and Programme Scanware 5.10).

2.5. Extraction of CF from the strips and skin samples

The CF was extracted from the adhesive tape and skin cuts with a mixture of ethanol and water pH 7.0 in 1:1 ratio. For extraction of CF from the adhesive tape, 2 ml of the extraction solvent was added to each sample and was shaken for 1 h. For extraction of CF from the skin cuts, 2 ml of extraction solvent was added to each sample and this mixture was vortexed for 2 min, followed by probe sonication for 2 min. The samples were then shaken on a vibrator (MLW Labor Technik, Ilmenau, Germany) for 2 h. After shaking, the skin samples were centrifuged at 5000 rpm for 30 min. All samples were quantitatively analyzed by spectrofluorometry at an excitation wavelength of 470 nm and emission wavelength of 520 nm.

Table 1
The diameter of the formulations with PI value

Type of formulation	Average diameter (nm)	Polydispersity index
CF in and outside liposomes	85.9	0.261
CF inside liposomes	73.3	0.289
CF outside liposomes	86.7	0.273

2.6. Statistical data analysis

Data analysis was carried out with the software package Microsoft Excel, Version 97. Results are expressed as mean ± standard error ($n = 3$ independent samples). Statistically significant differences were determined using the Student's t-test and analysis of variance (ANOVA) with $P < 0.05$ as a minimal level of significance.

3. Results and discussion

All liposomal formulations showed a sufficiently good PI below 0.3, which indicates reasonable size homogeneity of the liposomes. The mean diameter of the various liposomal suspensions ranged from 70 to 90 nm with PI between 0.26 and 0.29 (Table 1).

The various formulations with liposomes can be compared with regard to the penetration depth of the label and the intensity of the fluorescence in the various skin layers. Table 2 represents the amount of CF delivered from the different formulations into the different layers of the human abdominal skin. Figs. 1 and 2 represent the SC strip profile and deeper skin depth profile, respectively. Fig. 3 depicts the cumulative amount of CF delivered into the SC at an average depth of 9.4 μm.

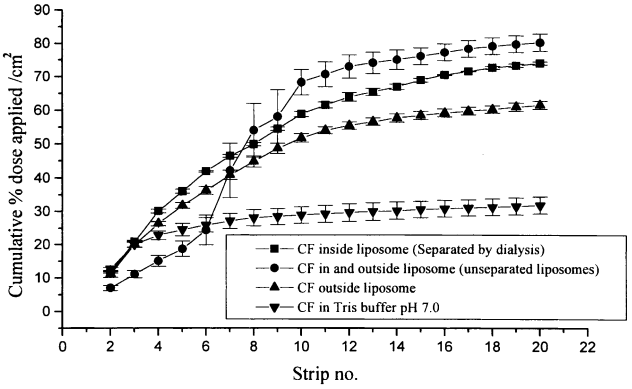

Fig. 1. SC strip profile of CF liposomes from different formulations into the human abdominal skin in vitro after 12 h of non-occlusive application of different formulations (expressed as % dose applied ± SE, $n = 3$).

Table 2
The amount of CF (expressed as % dose applied ± SE) delivered from different formulations into the different layers of human abdominal skin using a Franz diffusion cell in vitro after 12 h non-occlusive application ($n = 3$)

Type of formulation	Stratum corneum	Deeper skin	Receiver compartment
CF in and outside liposomes	80.42 ± 2.57	0.16 ± 0.03	0.02 ± 0.01
CF inside liposomes	74.07 ± 0.47	0.20 ± 0.01	0.11 ± 0.01
CF outside liposomes	61.60 ± 1.23	0.14 ± 0.01	0.01 ± 0.01
CF in Tris buffer pH 7.0	31.87 ± 2.57	0.07 ± 0.01	0.00 ± 0.00

Liposomal formulations showed a 2.52-, 2.32- and 1.93-fold higher deposition of CF in the SC for CF_{in-out}, CF_{in} and CF_{out}, respectively, as compared to the CF in buffer solution. The liposomal formulation, CF_{in-out}, showed statistically enhanced penetration of CF into the human SC as compared to the CF_{out} formulation. However, in the viable epidermis, the liposomes CF_{in} showed the highest fluorescence with a 1.21-fold higher deposition than liposomes CF_{in-out}, a 1.42-fold higher deposition than liposomes CF_{out} and a 2.8-fold higher deposition than CF Tris solution. The liposomes CF_{in} also showed the maximum amount, as compared to all other formulations, in the receiver compartment of the Franz diffusion cell. Other types of carrier systems (transfersomes) also seem to pass intact through the SC (personal communication of Cevc G., Idea AG, Munich, Germany). Intact elastic liquid state vesicles were also seen in the deeper SC layers by freeze fracture electron microscopy studies [27]. Surprisingly, the empty liposomes, with CF_{out}, were able to deliver almost a 2-fold higher amount as compared to the CF Tris solution. These results indicate that phospholipid vesicles not only carry the entrapped hydrophilic substance, but also the non-entrapped hydrophilic substance into the SC and possibly to the deeper layers of the skin.

Fig. 4 depicts the images of the CLSM experiment. All formulations used were able to deliver CF fairly homogeneously into the SC regardless of the location of the CF in liposomes and Tris buffer. The liposomal formulation CF_{in} exhibited a homogeneously distributed fluorescence in the viable epidermis (Fig. 4A). Liposomes CF_{in-out} showed a less homogeneously distributed fluorescence (weak to medium) in the viable epidermis as compared to the separated one (Fig. 4B). In the case of empty liposomes, CF_{out}, there was very weak fluorescence in the viable epidermis as compared to no fluorescence observed in the skin treated with CF Tris buffer solution.

The penetration study and CLSM images showed that the liposomal formulation containing CF both inside and outside exhibited maximum deposition of CF in the SC, whereas the liposomes CF_{in} exhibit a higher penetration into deeper skin layers, such as the viable epidermis, and through the skin to the receiver compartment of Franz diffusion cell. This study supports our assumption that the liposomes CF_{in-out} are not under osmotic stress and will, therefore, transfer themselves more easily into the SC. The CLSM images do not provide the visualization of single liposome in the skin sections, so the penetration of intact liposomes through the SC still remains an unsolved question. There may be three mechanisms by which the fluorescence label can penetrate into skin: (i) the label penetrates associated with the liposomal bilayer (penetration of intact vesicles), (ii) the fluorescence label penetrates associated with a liposomal bilayer fragment or (iii) the label penetrates solitarily [20].

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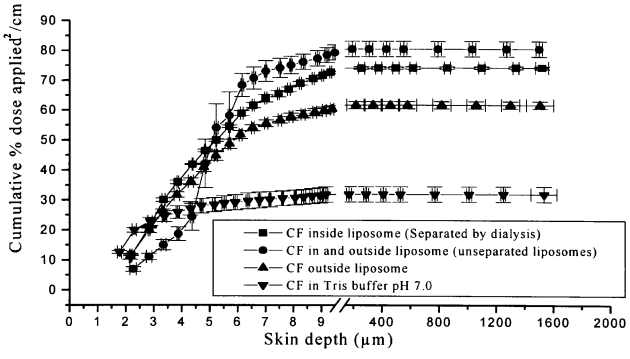

Fig. 2. Skin depth profile of CF from different formulations into the human abdominal skin in vitro after 12 h of non-occlusive application (expressed as % dose applied ± SE, $n = 3$).

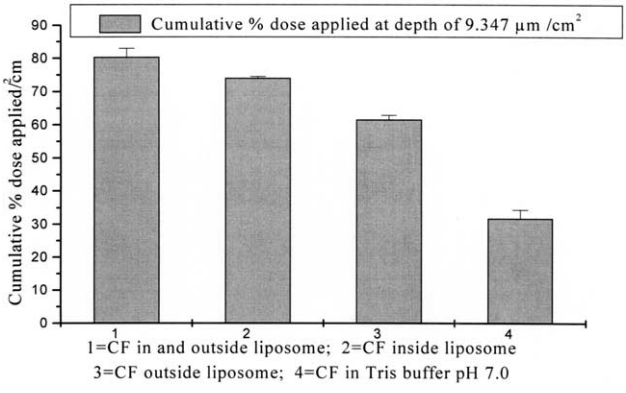

Fig. 3. The amount of CF delivered into the SC in vitro at an average depth of 9.4 µm after 12 h of non-occlusive application (expressed as % dose applied ± SE, $n = 3$).

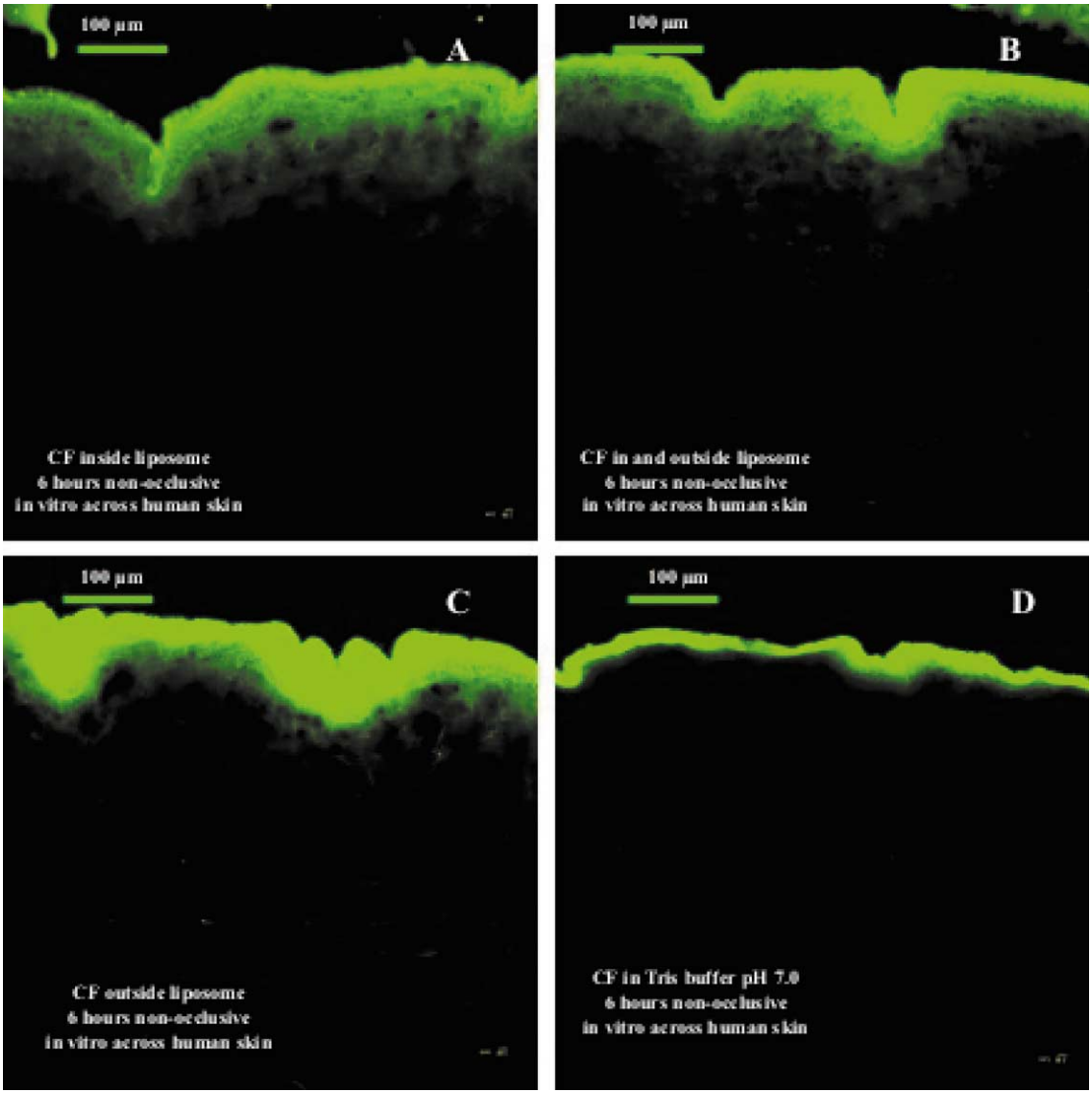

Fig. 4. CLSM images of a cross-section of human abdominal skin incubated on a Franz diffusion cell with different formulations containing CF. The liposomes were applied non-occlusively for 6 h: (A) liposomes CF_{in}, (B) liposomes CF_{in-out}, (C) liposomes CF_{out} and (D) CF in Tris buffer. Scale bar represents 100 µm.

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