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Research paper

Ex vivo skin delivery of diclofenac by transcutol containing liposomes and suggested mechanism of vesicle–skin interaction

Maria Manconi [a], Carla Caddeo [a], Chiara Sinico [a], Donatella Valenti [a], Maria Cristina Mostallino [b], Giovanni Biggio [b,c], Anna Maria Fadda [a,*]

[a] Department Farmaco Chimico Tecnologico, University of Cagliari, Cagliari, Italy
[b] CNR, Institute of Neuroscience, Monserrato, Italy
[c] Department of Experimental Biology, University of Cagliari, Monserrato, Italy

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ABSTRACT

Recently, we described a novel family of liposomes, the Penetration Enhancer-containing Vesicles (PEVs), as carriers for enhanced (trans)dermal drug delivery. In this study, to go deeply into the potential of these new vesicles and suggest the possible mechanism of vesicle–skin interaction, we investigated transcutol containing PEVs as carriers for diclofenac, in the form of either acid or sodium salt. PEVs, prepared with soy phosphatidylcholine and aqueous solutions containing different concentrations of transcutol, were characterized by size distribution, zeta potential, incorporation efficiency, thermotropic behavior, and stability. (Trans)dermal diclofenac delivery from PEVs was investigated *ex vivo* through new born pig skin using conventional liposomes and a commercial gel as controls. The mode of action of the vesicles was also studied by performing a pre-treatment test and confocal laser scanning microscopy (CLSM) analyses. Results of the all skin permeation experiments showed an improved diclofenac (both acid and sodium salt) delivery to and through the skin when PEVs were used (especially in comparison with the commercial gel) thus suggesting intact PEVs' penetration through the pig skin. Images of the qualitative CLSM analyses support this conclusion. Thus, this work shows the superior ability of the PEVs to enhance *ex vivo* drug transport of both hydrophilic and lipophilic diclofenac forms.

1. Introduction

During last decades, there has been a great deal of interest in lipid vesicles as a tool to improve dermal and transdermal drug delivery. However, the lack of ability of conventional liposomes to deliver drugs across the skin has led to intensive research with the introduction and development of new classes of lipid vesicles. Several authors have been showing that modification of lipid vesicle composition can lead to elastic, deformable and/or soft vesicles that have superior capability to enhance dermal and transdermal drug delivery with respect to conventional liposomes. Therefore, a great deal of innovative lipid vesicles has been introduced such as transfersomes and ethosomes, which are reported to have superior skin penetration ability. Transfersomes, introduced by Cevc and Blume [1], are ultradeformable lipid vesicles made from surfactant addition to phospholipid bilayers. Their high deformability enhances drug delivery through the skin barrier, if compared to conventional liposomes and niosomes [2,3]. Ethosomes are soft and malleable vesicles composed mainly of phospholipids, ethanol and water. Unlike conventional liposomes, which are known to deliver drugs mainly to the outermost skin layers, ethosomes have been shown to transport active substances more efficaciously through the stratum corneum and to the deeper skin layers [4,5]. Successively, new deformable vesicles were developed by using penetration enhancer molecules (e.g. oleic acid, terpenes) as edge activators [6,7]. In the last years, we studied the influence of different penetration enhancer (PE) molecules in the composition of vesicular formulations on vesicle deformability and (trans)dermal delivery of different drugs. To this purpose, several lipophilic and hydrophilic PE (i.e. labrasol, cineole, and transcutol) were tested and PEVs (Penetration enhancer-containing vesicles) have been introduced [8,9]. In particular, diethylene glycol monoethyl ether (Transcutol® P, Trc) containing PEVs were tested as carriers for topical delivery of minoxidil and diclofenac [8,9]. Trc is a well known and efficient permeation enhancer, non-toxic and biocompatible, soluble both in water and in oil ($K_{O/A} = 0.7$) and used in several dosage forms. It is also a powerful solubilizing agent capable of reversibly compromising the skin barrier function, thus allowing the drug to penetrate into the skin.

Hence, to go deeply into the potential of these new vesicles as carriers for enhanced (trans)dermal drug delivery and investigate

* Corresponding author. Department Farmaco Chimico Tecnologico, Faculty of Pharmacy, University of Cagliari, Via Ospedale 72, 09124 Cagliari, Italy. Tel.: +39 070 6758565; fax: +39 070 6758710.
E-mail address: mfadda@unica.it (A.M. Fadda).

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the possible mechanism of their interaction with skin, in this study we focused on the influence of different amounts of transcutol on both physico-chemical and (trans)dermal drug delivery properties of these vesicles. Indeed, the mode of action by which vesicles are able to transport molecules into and through the skin still represents one of the most controversial issues in skin research [10 and references therein]. To this purpose, the transcutol containing PEVs were prepared from a mixture of enriched soy phosphatidylcholine (Phospholipon 90, P90G) and different amounts of Trc. Moreover, they were loaded with diclofenac either in the acid (DCF) or in the sodium salt form (DCF_{Na}) in order to obtain from the *ex vivo* skin permeation experiments additional information regarding the role of the used carriers (PEVs and conventional liposomes). In fact, it is well known that physico-chemical drug properties (i.e. solubility) play a fundamental role in the diffusion process across the skin, a heterogeneous multilayer tissue consisting of stratum corneum (SC), viable epidermis, and dermis.

Diclofenac acid (2-[(2,6-dichlorophenyl)amino]phenylacetic acid; DCF) is one of the most potent and commercially successful non-steroidal anti-inflammatory drugs (NSAIDs) [11,12]. Due to several drawbacks when orally given, topical administration is usually used for diclofenac in long-term cures, such as treatment of local muscle inflammation, although the drug shows low skin permeability. Different penetration enhancers have been employed to increase its percutaneous absorption because they reversibly reduce the skin barrier resistance [12,13]. However, they can be irritating and cause permanent epidermal damage that can be repaired only by the regeneration of the stratum corneum. As a promising alternative, DCF inclusion within nanocarrier systems (e.g., liposomes, transfersomes, and ethosomes) has been proved to be a highly efficient method to enhance its transdermal delivery [11,14]. Indeed, diclofenac is a good candidate for incorporation and delivery by vesicles, because of its poor water solubility (0.02 mM at 25 °C) and skin permeability [15]. For these reasons, it is administered as sodium salt in several commercial topical formulations, which also contain a permeation enhancer to facilitate the transdermal delivery. However, only approximately 10% of the applied diclofenac is biologically available from topical formulations [8].

The DCF and DCF_{Na}-loaded PEVs were prepared, characterized, and tested *ex vivo* to evaluate the influence of the carrier on diclofenac delivery to and through new born pig skin. A commercial gel formulation (Diclofenac Sandoz®, 1% gel) and conventional liposomes with the same lipid composition of the PEVs were used as controls. The potential mechanisms of DCF delivery from PEVs to and through the skin were also investigated by rheological and confocal laser scanning microscopy (CLSM) studies.

2. Materials and methods

2.1. Materials

Enriched soy phosphatidylcholine (Phospholipon® 90G, P90G) and hydrogenated soy phosphatidylcholine (Phospholipon® 90H, P90H) were kindly supplied by AVG S.r.l. (Garbagnate Milanese, Milan, Italy), and Lipoid GmbH (Germany). 2-(2-Ethoxyethoxy)ethanol (Transcutol® P) was a gift from Gattefossè (Saint Priest, France). Phosphate buffer solution (PBS, pH 7) was purchased from Carlo Erba Reagents (Rodano, Italy). Diclofenac sodium (DCF_{Na}), oleic acid (OA), and all the other products were of analytical grade and were purchased from Sigma–Aldrich (Milan, Italy). Diclofenac free acid (DCF) was obtained by acidic precipitation from a solution of diclofenac sodium. Diclofenac Sandoz® gel 1% is a commercial formulation of DCF_{Na}, produced by Sandoz S.p.A. (Origgio, Varese, Italy).

2.2. Vesicle preparation

Liposomes and PEVs, empty or loaded with DCF or DCF_{Na} (1% w/v), were prepared according to the thin film hydration method using P90G and OA in the appropriate ratio (90 and 10 mg/ml, respectively). Hydration of the film was performed in two steps: first, a 5 ml aliquot of PBS (to obtain liposomes) or 10%, 20%, 30% Trc/PBS solution (to obtain PEVs) was added to the flask and the concentrated dispersion was mechanically shaken for 1 h at room temperature. Then, a second 5 ml aliquot was added and the dispersion shaken for another hour. The obtained suspensions were then sonicated (10 s on and 10 s off; 13 μm of probe amplitude) with a Soniprep 150 ultrasonic disintegrator (MSE Crowley, UK), until a clear opalescent dispersion was obtained. The Small Unilamellar Vesicle (SUV) dispersions were purified from the non-incorporated drug by exhaustive dialysis. Dispersions were loaded into dialysis tubing (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., USA) and dialyzed against distilled water at 5 °C. Dialysis of each sample (2 ml) was carried out in 1000 ml of water for 2 h. Drug loading efficiency (*E%*), expressed as the percentage of the amount of drug initially used, was determined by high performance liquid chromatography (HPLC) after disruption of vesicles with 0.025% non-ionic Triton X-100. Diclofenac content was quantified at 227 nm using a chromatograph Alliance 2690 (Waters, Italy). The column was a Symmetry C18 (3.5 μm, 4.6 × 100 mm, Waters). The mobile phase was a mixture of 30% water and 70% acetonitrile (v/v), delivered at a flow rate of 0.5 ml/min. A standard calibration curve (peak area of diclofenac versus known drug concentration) was built up by using working, standard solutions (1.0–0.01 mg/ml). Calibration graphs were plotted according to the linear regression analysis, which gave a correlation coefficient value (R^2) of 0.998. The diclofenac retention time (t_r) was 1.5 min, and the minimum detectable amount was 2 ng/μl.

2.3. Vesicle characterization

Vesicles were characterized by Transmission Electron Microscopy (TEM) for vesicle formation and morphology. Samples, stained with a 1% phosphotungstic acid, were examined with a JEM-1010 (Jeol Europe, France) transmission electron microscope equipped with a digital camera MegaView III and Software "AnalySIS", at an accelerating voltage of 80 kV.

The average diameter and polydispersity index (PI) of the samples were determined by Photon Correlation Spectroscopy (PCS) using a Zetasizer nano (Malvern Instrument, UK). Zeta potential was estimated using the Zetasizer nano by means of the M3-PALS (Phase Analysis Light Scattering) technique. All the samples were analyzed 24 h after their preparation.

A stability study was performed by monitoring the vesicle average size and zeta potential over 90 days at 4 ± 1 °C.

2.4. Rheological study

The rheological study was performed in the oscillation mode using a Gemini 150 HR Nano controlled stress rheometer (Malvern Instruments, UK) equipped with a Bohlin data acquisition software. Samples were allowed to rest for at least 300 s prior to analysis. Single frequency stress controlled with temperature ramp tests were performed using a plate-plate geometry (20 mm diameter), at constant shear stress (1 Pa) and frequency (1 Hz) over an appropriate temperature range (from 20 to 80 °C, with a heating rate of 1.167 °C/min) for all studied systems. For each sample, three replicates were tested.

The main oscillatory parameter used to compare the rheological features of the different vesicular dispersions was the complex

viscosity ($\eta^* = G^*/\omega$, where G^* is the complex modulus equals to $(G'^2 + G''^2)^{1/2}$ and ω is the angular frequency). As it expresses the contributions of both the storage (G', relative to elastic characteristics) and loss (G'', relative to viscous characteristics) moduli to the overall resistance to deformation of a material, it provided useful information concerning the structure of the tested systems.

2.5. Deformation index determination

Comparative measurement of liposome and PEV bilayer deformability was carried out by the extrusion method. Each vesicle dispersion was extruded at constant pressure through 19-mm polycarbonate filters of definite pore size (50 nm), using an extrusion device Liposofast® (Avestin, Canada). The vesicle deformability was expressed in terms of deformation index (DI) according to Eq. (1):

$$\mathrm{DI} = J\left(\frac{d_0}{p}\right)\left(\frac{d_0}{|d_0 - d_1|}\right) \quad (1)$$

where J is the fraction of suspension recovered after extrusion (ranging from 0 to 1, with 1 representing 100% of the dispersion loaded in the extruder 0.5-ml syringe); d_0 and d_1 are vesicle mean diameters before and after extrusion; p is the pore size of the extruder membrane [9].

2.6. Ex vivo skin penetration and permeation studies

Experiments were performed non-occlusively by means of Franz diffusion vertical cells with an effective diffusion area of 0.785 cm^2, using new born pig skin. One-day-old Goland–Pietrain hybrid pigs (~1.2 kg), died by natural causes, were provided by a local slaughterhouse. The skin, stored at 80 °C, was pre-equilibrated in PBS solution at 25 °C, two hours before the experiments. Skin specimens ($n = 6$ per formulation) were sandwiched securely between donor and receptor compartments of the Franz cells, with the stratum corneum (SC) side facing the donor compartment. The receptor compartment was filled with 5.5 ml of PBS solution, which was continuously stirred with a small magnetic bar and thermostated at 37 ± 1 °C throughout the experiments to reach the physiological skin temperature (i.e. 32 ± 1 °C). Hundred microliters of the tested vesicle suspensions or the commercial formulation Diclofenac Sandoz® gel was placed onto the skin surface. At regular intervals of 2 h, up to 8 h, the receiving solution was withdrawn and analyzed by HPLC for drug content (as described in Section 2.2).

After 8 h, the skin surface of specimens was washed and the SC was removed by stripping with adhesive tape Tesa® AG (Hamburg, Germany). Each piece of the adhesive tape was firmly pressed on the skin surface and rapidly pulled off with one fluent stroke. The epidermis was separated from the dermis with a surgical sterile scalpel. Tape strips, epidermis, and dermis were placed each in methanol, sonicated to extract the drug and then assayed for drug content by HPLC (see Section 2.2).

Pre-treatment studies were performed applying overnight 100 μl of empty PEVs and liposomes (control) onto the skin surface. After incubation, the skin surface was washed with 1 ml of distilled water and 100 μl of DCF_{Na} in PBS solution (10.74 mg/ml) was applied on the skin samples. The permeation study was carried out for 8 h as described earlier.

2.7. Confocal laser scanning microscopy (CLSM)

Sections of new born pig skin, used for the permeation studies, were examined under the confocal laser scanning microscope. For this purpose, a lipophilic and a hydrophilic fluorescent markers, namely β-carotene (0.025% w/v; βC) and 5(6)-carboxyfluorescein (0.025% w/v; CF), were added during vesicle preparation. Vesicles were purified from the non-entrapped markers by dialysis. After an 8-h incubation period at 37 °C with these preparations (placed on the skin surface) in the Franz diffusion cell, pig skin was washed and then rapidly frozen at −80 °C.

Sections of skin (25 μm thickness) were cut with a cryostat microtome (Microm HM 560, Bio-Optica, Milan, Italy) parallelly (in the x–y plane) to the surface, mounted on a glass microscope slide with a permanent aqueous mounting medium (Sigma, Milan, Italy) and examined to investigate the fluorescent probe distribution in the different skin strata.

Analyses were carried out using a Leica TCS SP5X Inverted Supercontinuum Confocal Laser Scanning microscope (Leica Microsystems, Heidelberg, Germany) equipped with a white laser. Using a Plan Apo 40× oil immersion objective NA 1.25, images with a field size of 512 × 512 μm were generated. In our experiments for excitation of the hydrophilic fluorescent probe, the 488 nm wavelength was used and the emission was detected in the 515–535 nm range, while for the lipophilic probe the 514 nm wavelength and the 660–1250 nm range were used, respectively, for excitation and emission. The instrument allowed simultaneous fluorescence and differential interference contrast (DIC) imaging, which enabled the acquisition of images showing the distribution of the markers among skin structures. Control images were obtained using new born pig skin incubated with PBS alone and tested for autofluorescence studies.

2.8. Statistical analysis of data

Data analysis was carried out with the software package R, version 2.10.1. Results are expressed as the mean ± standard deviation. Multiple comparisons of means (Tukey test) were used to substantiate statistical differences between groups, while Student's t-test was used for comparison between two samples. Significance was tested at the 0.05 level of probability (p).

3. Results and discussion

3.1. Vesicle formation and characterization

In the present work, new vesicular formulations, PEVs, were prepared by hydrating the lipid film with buffered solutions of transcutol at three different concentrations (10%, 20%, 30% v/v) in order to enhance drug solubility, vesicle elastic properties, and skin permeation ability. Conventional phospholipid liposomes were also prepared using the same lipid composition (i.e. soy phosphatidylcholine and oleic acid) and used as a control. For each formulation type (liposomes and PEVs), unilamellar vesicles (SUVs), empty or DCF and DCF_{Na}-loaded (1% w/v), were prepared and compared in terms of morphology, size, surface charge (Table 1), encapsulation efficiency (Fig 1) and stability.

TEM provided the evidence of vesicle formation and their morphological evaluation analysis showed small, spherical, and unilamellar vesicles (Fig. 1). The investigated formulation composition did not influence the vesicle morphology. PCS confirmed these findings, showing that all liposomes and PEVs were small in size, with mean diameter between 87 and 146 nm (Table 1). They were generally homogeneously dispersed (being 0.3 the highest PI value), and the zeta potential was always highly negative (between −53 and −78 mV) because of the presence of oleic acid, specifically added to stabilize the vesicle dispersions. DCF_{Na} in liposomes and in PEVs caused a decrease in size and zeta potential values, with respect to empty and DCF vesicles. Ten and 20% Trc-PEVs, empty or drug loaded, were always smaller and showed a more negative

Table 1
Characteristics of empty, diclofenac acid (DCF) and sodium salt (DCF_{Na}) control liposomes (0% Transcutol – Trc) and 10%, 20%, 30% Trc-PEVs: average size, polydispersity index (PI) and Zeta potential. Each value is the mean ± standard deviation of at least six experimental determinations.

Sample	Trc %	Size	PI (nm)	Zeta potential (mV)
Empty	0	132 ± 2	0.195	−66 ± 3
	10	109 ± 2	0.178	−70 ± 2
	20	110 ± 5	0.246	−71 ± 3
	30	146 ± 21	0.281	−59 ± 6
DCF	0	132 ± 6	0.228	−53 ± 2
	10	98 ± 5*	0.327	−56 ± 1
	20	110 ± 4	0.246	−57 ± 2
	30	131 ± 7	0.331	−55 ± 6
DCF_{Na}	0	110 ± 3	0.291	−72 ± 3
	10	87 ± 6*	0.321	−78 ± 4
	20	95 ± 6	0.244	−74 ± 3
	30	114 ± 5	0.352	−71 ± 7

* $p < 0.05$.

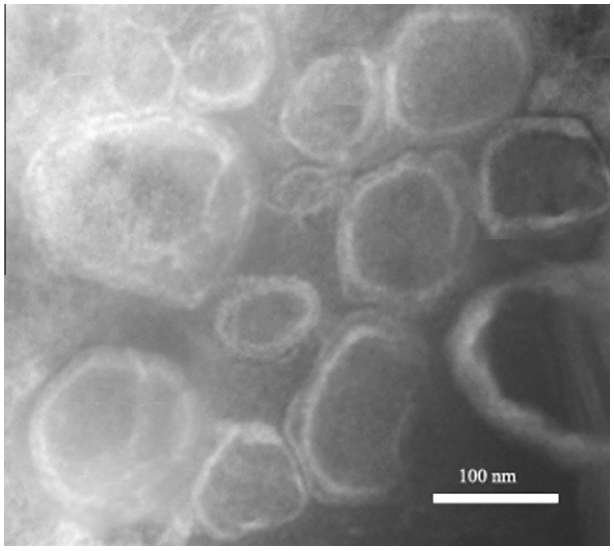

Fig. 1. Negative stain electron micrograph of diclofenac-loaded PEVs prepared from soy phosphatidylcholine and 20% transcutol; bar: 100 nm.

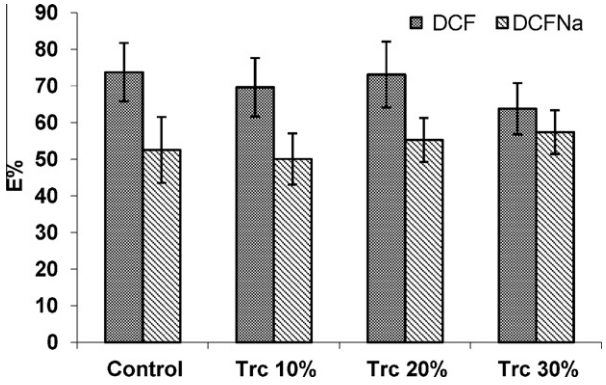

Fig. 2. Entrapment efficiency (*E*%) of diclofenac acid (DCF) and sodium salt (DCF_{Na})-loaded liposomes (control) and 10%, 20%, 30% transcutol (Trc) PEVs. Error bars represent standard deviation, $n = 6$.

zeta potential value than the corresponding liposomes. This is probably due to the Trc capability of interpenetrating the phospholipid, with a consequent modification of the bilayer packing (see below). Thirty percent Trc-PEVs were similar in size to the corresponding conventional liposomes.

All vesicular formulations showed a highly negative zeta potential that is known to prevent vesicle aggregation and fusion and, therefore, is indicative of a good stability. In particular, DCF_{Na}-loaded PEVs showed the highest negative values, ranging from −71 to −78 mV, in contrast to DCF PEVs that had the lowest ones (around −53 mV). As a consequence, during 3 months on storage at 4 ± 1 °C, all studied vesicular formulations did not demonstrate any appreciable modification in mean size and zeta potential values. In particular, the mean size of DCF_{Na}-loaded vesicles showed a very small increase (<10%) while that of the DCF-loaded ones increased up to a maximum of 30%. The negative zeta potential values were always constant thus proving that no aggregation or fusion had occurred during storage.

All vesicles, control liposomes and PEVs, were able to incorporate DCF in good yields (65–75%), always higher than those of the corresponding DCF_{Na} vesicles, whose entrapment efficiency ranged from 50% to 57% (Fig. 2). The presence of the different amounts of Trc did not significantly affect *E*% ($p < 0.05$).

The deformability of the vesicular membrane was evaluated by extruding the dispersions through polycarbonate filters with pores smaller than vesicle mean size. It is well known that vesicle ability to squeeze through these pores depends on the deformability of the vesicular bilayers and, for Trc-containing vesicles, on the capability of the co-solvent (Trc) of destabilizing the phospholipid packing in the vesicular bilayer. Results, listed in Table 2, indicate that control liposomes and PEVs (empty and DCF_{Na}-loaded) had roughly the same elasticity (DI ≈ 5), whereas DCF PEVs appeared approximately up to 3-fold (DI ∼ 16) more elastic as a function of the Trc concentration. Most likely, the simultaneous presence of the hydrophobic diclofenac acid, which is supposed to be intercalated in the bilayer and Trc at high concentration (20–30%), led to a decrease in lamellar phase stability with a consequent increase in vesicle elasticity. However, results indicate a low deformability of these new Trc-containing vesicles in comparison with other previously tested PEVs [9].

Table 2
Deformation index (DI) of empty, diclofenac acid and sodium salt (DCF and DCF_{Na}) control liposomes (0% Transcutol – Trc) and 10%, 20%, 30% Trc-PEVs. Each value represents the mean ± SD, $n = 3$.

Trc %	DI		
	Empty	DCF	DCF_{Na}
0	6.21 ± 1.24	5.33 ± 2.21	4.17 ± 1.73
10	5.71 ± 1.90	5.65 ± 1.72	5.79 ± 0.90
20	6.52 ± 3.04	11.87 ± 3.84	5.04 ± 1.38
30	10.16 ± 3.05	16.42 ± 2.41	5.16 ± 0.23

3.2. Thermotropic behavior

In order to study in depth the physico-chemical properties of the PEVs, the influence of Trc on the phase transition temperature (T_c), from gel (also called "solid-ordered") to liquid–crystalline ("liquid-disordered") phase of the vesicle bilayers was assessed by a rheological study. As well known, above the T_c, vesicle bilayers change from gel to liquid–crystalline phase becoming more fluid and disordered and the transition is accompanied by a lateral expansion and a decrease in the bilayer thickness. Thermal phase transition of phospholipid vesicles has been exhaustively examined by various physical methods such as differential scanning calorimetry [16], measurements of membrane fluidity by fluorescence polarization [17], determination of changes in turbidity and fluorescence properties [18].

Recently, rheometry studies have also been shown to be of great value in determining the phase transition temperature of liposomal bilayers: temperature-controlled rheometry in the oscillation mode has been demonstrated to be a valuable alternative to DSC. Moreover, this method was used to correlate the thermotropic behavior with superstructural arrangements showing its capability of determining structural defects or special features of semisolid liposomal preparations [19].

Therefore, in the present study, we measured T_c as a function of the complex viscosity variation. The phase transition temperature of P90G has been reported in the range of −15/−7 °C, depending on the length and saturation degree of the fatty acyl chains [16]. Consequently, hydrogenated phosphatidylcholine (P90H), which has fully saturated acyl chains and T_c = 51 °C, was selected for the rheological experiments, rather than P90G, to disclose easily and more accurately how Trc could affect the vesicular bilayer organization. Complex viscosity η^* is a frequency-dependent viscosity function, which describes total strengths versus dynamic shear stress, and was determined during forced harmonic oscillation of shear stress. Fig. 3 shows the experimental slopes of the complex viscosity versus temperature for empty vesicles (liposomes and PEVs). As can be seen, as the temperature increased, the complex viscosity decreased until a minimum was reached at approximately 45 °C. Then, it increased again reaching a maximum from where it abruptly decreased. Clearly, as the temperature increases the viscoleastic character of the gel-state bilayers decreases because of its enhanced fluidity. On approaching T_c, the transition involves mainly a disordered phase that increases viscosity, followed by a thermodynamically favored reorientation of phospholipid molecules to liquid–crystalline membrane phase at their T_c, which can

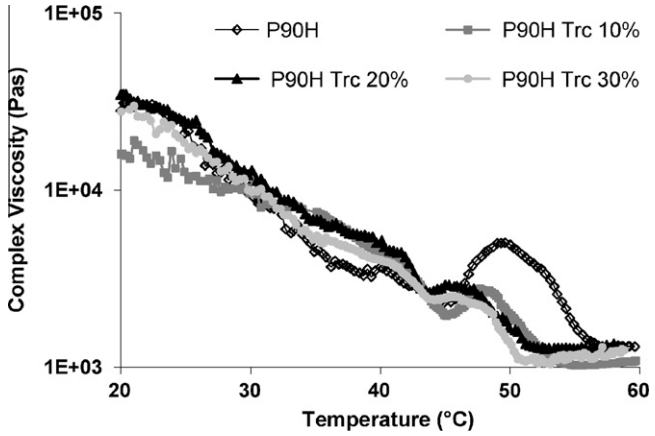

Fig. 3. Complex viscosity spectra for P90H (hydrogenated soy phosphatidylcholine) liposomes and 10%, 20%, 30% transcutol (Trc) PEVs from single frequency with temperature ramp tests. Each single spectrum represents the average of three determinations.

Table 3
Influence of transcutol (Trc) on vesicular lipid phase transition temperature (T_c). The width (temperature range) and the area under the peak of the complex viscosity spectra of the formulations are presented. Values represent the means ± SD, n = 3.

Trc %	T_c (°C)	Temperature range (°C)	Peak area (au)
0	51.31 ± 0.056	11.24 ± 0.411	622 ± 36
10	49.78 ± 0.321	9.43 ± 0.416	302 ± 24
20	48.55 ± 0.926	7.78 ± 0.551	152 ± 15
30	46.62 ± 0.275	7.83 ± 0.568	76 ± 12

lead to a lower viscosity values. The maximum value of the complex viscosity variation range was taken as T_c (Table 3). It was 51.31 °C for control liposomes with a large temperature range (TR ∼ 11 °C) and a high peak area (PA, 622 au). PEVs had dose-dependent, lower T_c and PA, since the transition was shifted to lower temperatures (Table 3, Fig. 3). Each 10% increase in Trc produced an average ΔT_c of ∼1–2 °C. Similarly, it was previously shown that ethanol in ethosomes might reduce the temperature of the main transition and increase bilayer fluidity [5,20]. The presence of Trc in PEVs reduced the peak area, thus shortening the transition temperature range in comparison with control liposomes. It can be supposed that Trc interacts with the lipid packing, and the linear decrease in T_c at increasing concentrations of the PE can be explained as a freezing-point depression phenomenon of the solvent by a solute, caused by its preferential partitioning into the liquid–crystalline phase rather than into the gel phase, as reported for ethanol [21].

The decrease in T_c and peak area values may indicate that the co-solvent perturbs, in a dose-dependent manner, the phospholipid packing characteristics and, thus, fluidizes the vesicle bilayer. Trc at low concentrations is water miscible and, therefore, preferably localized in the aqueous inner/outer phase of the vesicles, poorly interacting with the lipid membranes. At higher concentrations, it may be partially incorporated into the bilayers, producing significant changes in their organization, such as a diminution of their cooperativity [22]. We can assume that Trc does not insert deeply in the bilayer but interacts just with the initial portion of the phosphatidylcholine acyl chains, because of the steric hindrance of Trc polar head and its short lipophilic chain. The hydrophilic polar head sticks out from the outer layer of the vesicles and interacts with the water molecules.

Our findings imply that PEVs are in a more fluid state in comparison with the conventional liposomes. Superior PEVs' fluidity, undoubtedly due to the Trc presence, might facilitate the interaction between vesicles and skin, and even favor penetration of intact vesicles through the skin lipid bilayers, disorganized by the presence of the "free" transcutol. Indeed, *in vitro* and *in vivo* results have shown that liquid-state vesicles have higher capability of interacting with the skin than gel-state vesicles [23–26].

To give evidence to this theory, *ex vivo* skin permeation studies were carried out. Liposomes and PEVs loaded with DCF and DCF_{Na} were used to study the drug (trans)dermal delivery.

3.3. Ex vivo transdermal diclofenac delivery

In order to clarify the influence of PEVs on the diclofenac permeation process, DCF and DCF_{Na} permeation studies through new born pig skin were carried out. Human skin is the membrane of choice in *ex vivo* transdermal experiments, but it is not easily available. Therefore, many efforts have been made to select a suitable substitute. Although animal skin is different from the human one in several features, it is well known that pig skin is a good substitute in *ex vivo* permeation experiments thanks to the similarity of its stratum corneum in terms of lipid composition, even if it presents a marked difference in terms of thickness [27,28]. New born pig SC is considerably thinner than that of adult pigs, and more

similar to that of the human skin, even if the number of hair follicles is higher. Several studies have used new born pig skin in *ex vivo* skin permeability experiments, e.g. [8,9,26,29,30], while a limited number of investigations, aiming at comparing the performances of new born pig skin with respect to excised human epidermis, are available in the literature [28,31]. Nevertheless, these works confirm the suitability of new born pig skin in preliminary skin permeation screenings. In addition, the *ex vivo* experiments have the advantages to give information regarding single factors that may affect drug penetration even if the method does not exactly replicate the behavior of living tissue in skin.

Franz diffusion cells were used, and experiments were carried out in non-occlusive conditions. Results are shown in Fig. 4, where the DCF and DCF_{Na} accumulated in and permeated through the whole skin are reported. As can be seen, DCF and DCF_{Na} delivery was enhanced by PEVs in comparison with both controls. In particular, the lowest drug delivery into and through the new born pig skin was found when the commercial DCF_{Na} gel formulation was tested. Moreover, at the same Trc concentration, PEVs led to a DCF accumulation into the skin greater than or equal to DCF_{Na}, except for 30% Trc-PEVs that provided a larger amount of DCF_{Na} into the SC and Epidermis. For DCF, the highest drug accumulation was always found in the epidermis where 10 and 20% Trc-PEVs enhanced drug deposition three times more than conventional liposomes. DCF and DCF_{Na} permeation also increased but the transdermal delivery was always higher for the hydrosoluble form of the drug. Moreover, results showed that drug accumulation and permeation were closely related to Trc concentration. Drug deposition into the dermis, in particular DCF amount, and diclofenac delivery through the skin improved by increasing Trc concentration. Therefore, PEVs showed to be better carriers than liposomes and commercial gel formulation for the delivery of diclofenac (acid or sodium salt) locally to the skin layers.

Cumulative amounts of permeated drug ($\mu g/cm^2$) were calculated and plotted against time (Fig. 5). The flux (J) was determined as the slope of the linear portion of the plot (Table 4). Profiles obtained when control liposomes and gel formulation were used, showed a linear trend with a short initial lag time (2.1 h for DCF and 2.4 h for DCF_{Na} in liposomes and 2.1 h for DCF_{Na} in gel). The mean amount of the drug permeated after 8 h from conventional liposomes was 16.6 and 7.2 $\mu g/cm^2$ for DCF and DCF_{Na}, respectively, while 2.1 $\mu g/cm^2$ of DCF_{Na} was delivered by the commercial

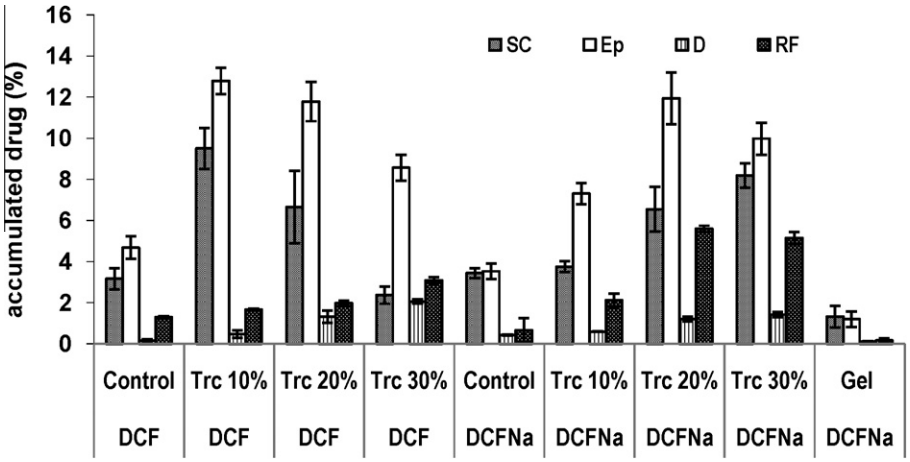

Fig. 4. Cumulative amount of diclofenac, acid (DCF) and sodium salt (DCF_{Na}), retained into and permeated through pig skin layers after 8 h non-occlusive treatment with PEV suspensions, and controls (conventional liposomes and commercial DCF_{Na} gel). SC, stratum corneum; Ep, epidermis; D, dermis; RF, receptor fluid. Each value is the mean ± standard deviation of at least six experimental determinations.

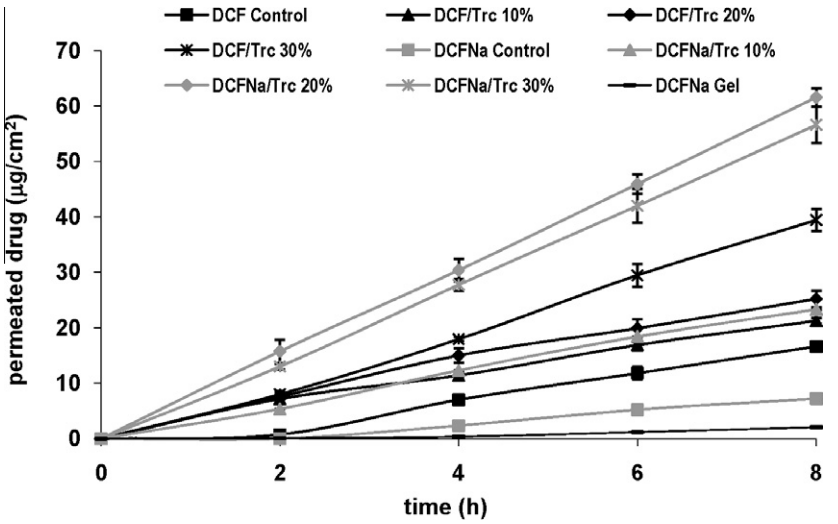

Fig. 5. *Ex vivo* diffusion of diclofenac acid (DCF) and sodium salt (DCF_{Na}) through pig skin from PEVs in comparison with control liposomes and commercial DCF_{Na} gel. Data represent the mean ± standard deviation of at least six experimental determinations.

Table 4
Results of *ex vivo* permeation study from PEVs (10%, 20%, 30% Transcutol – Trc), control liposomes (0% Trc) and commercial DCF_{Na} gel. Amount of diclofenac acid (DCF) and sodium salt (DCF_{Na}) accumulated into the whole skin and delivered through the pig skin at the end of the experiments (8 h); Local Accumulation Efficiency (LAC) values: drug accumulated into the skin/drug delivered through the skin ratio; and transdermal flux (*J*).

Composition		Accumulated (μg/cm²)	Permeated (μg/cm² ± SD)	LAC	*J* (μg/cm²/h ± SD)
Trc (%)	DCF				
Gel	DCF_{Na}	33.5	2.1 ± 0.1	16.4	0.3 ± 0.1
0	DCF	102.1	16.7 ± 0.5	6.1	2.6 ± 1.1
10		290.9	21.2 ± 0.5	13.7	2.8 ± 0.7
20		252.1	25.2 ± 1.5	10.0	3.3 ± 0.6
30		165.9	39.5 ± 2.0	4.2	4.8 ± 1.2
0	DCF_{Na}	81.3	7.2 ± 0.1	11.3	1.2 ± 0.6
10		128.2	23.3 ± 0.1	5.5	3.0 ± 0.9
20		216.5	61.9 ± 1.7	3.5	7.7 ± 0.9
30		215.5	56.7 ± 3.3	3.8	7.1 ± 0.8

gel. Using PEVs, the permeation profiles were approximately linear with a much shorter lag time (≈10 min) as long as sink conditions were maintained, indicating nearly zero-order release kinetics.

PEVs provided the highest drug permeation through the skin with respect to the control, and the permeation enhancement was directly proportional to the Trc increase. DCF_{Na} permeation from 20% and 30% Trc-PEVs increased dramatically, reaching 60 μg/cm² after 8 h.

To have an estimation of the main target of the topically applied drug by using PEVs, it is interesting to compare the ratio of DCF accumulated into the whole skin versus DCF permeated through the skin, which gives a dimensionless number for the quantification of the Local Accumulation Efficiency (LAC) of the formulations (Table 4). The highest LAC value was shown by the commercial gel formulation (LAC = 16.4) followed by the 10% Trc DCF-loaded PEVs (LAC = 13.7), the 20% DCF_{Na}-loaded liposomes (LAC = 11) and the 20% Trc DCF-loaded PEVs (LAC = 10). However, the high LAC values obtained from the commercial gel and the conventional liposomes are the consequence of a poor delivery of DCF_{Na} into and through the pig skin strata. Indeed, when DCF_{Na} was delivered by the commercial gel, the mean amount of drug accumulated and permeated into and through the whole pig skin was, respectively, 4–6.5-fold and 11–30-fold lower than those obtained from the DCF_{Na}-loaded PEV formulations. Regarding conventional liposomes, both accumulation and flux were 3-fold lower than those obtained from PEVs, where the high LAC was due to high accumulation and low permeation. In the case of 20% and 30% Trc DCF_{Na}-loaded PEVs, the accumulated drug was high (∼215 μg/cm²) as well as the permeated amount (∼60 μg/cm²); therefore, LAC values were the lowest ones. Although the amount of the accumulated drug is similar for all PEVs, the permeated DCF_{Na} was higher than DCF, due to its hydrophilic nature.

Therefore, by comparing LAC and drug accumulation and permeation values it can be inferred that 10 and 20% Trc DCF-loaded PEVs are the optimal carriers for the local accumulation of diclofenac. The high deposition of both drug forms (in particular of DCF, more lipophilic) into the epidermis seems to indicate that PEVs are capable of penetrating intact the skin, reaching the epidermis where they form a depot from which the drug can be released. Then, diclofenac may independently penetrate deeper and exert its pharmacological activities. This is probably due to a dual effect of transcutol that improves vesicular bilayer fluidity (see above) and also acts as a penetration enhancer by reducing the barrier function of SC transiently, thus creating an easier pathway for the highly fluidized vesicles.

To assess this assumption and to elucidate the mode of action of PEVs, new born pig skin was pre-treated with empty vesicle dispersions for 8 h in Franz cells, followed by application on skin surface, of DCF_{Na} in PBS solution. The pre-treatment method allowed vesicle–skin interaction, but obviously avoided drug delivery from the vesicles. Results (Fig. 6) showed that the pre-treatment generally reduced drug accumulation in the skin strata, as well as transdermal delivery, with respect to conventional treatment with vesicular DCF_{Na}. More specifically, the pre-treatment with empty liposomes led to a loss of drug accumulation in epidermis and dermis while with empty PEVs it resulted in a 2-fold (with 10% and 20% Trc) and a 4-fold (with 30% Trc) decrease in the drug deposition into the whole skin. Therefore, these results demonstrate that vesicle carriers are needed to deliver diclofenac to and through the skin, and further support the hypothesis that PEVs do not simply behave as enhancers but they act as drug carriers, as also reviewed by Elsayed et al. for surfactant-based elastic vesicles [32].

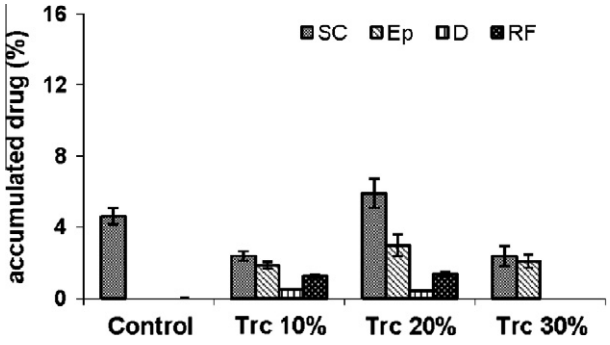

Fig. 6. Results of *ex vivo* permeation study after pre-treatment of pig skin with empty PEVs and control liposomes followed by treatment with diclofenac sodium solution. Amount of drug accumulated into and delivered through the skin at the end of the experiments (8 h). Each value is the mean ± standard deviation of at least six experimental determinations.

To additionally elucidate the mode of action of PEVs, we evaluated the diffusion and accumulation of the colloidal dispersions into the skin strata using CLSM. To this purpose, penetration extent and localization of lipophilic βC/hydrophilic CF co-labelled vesicles were visualized. Fig. 7 illustrates images of conventional liposomes (control) and 20% Trc-PEVs applied onto the new born pig skin in the same experimental conditions. These images were taken 8 h after the non-occlusive application of the vesicles containing the green fluorescent CF and the red fluorescent βC labels.

The confocal pictures presented in this work derived from merging images obtained in transmission mode plus those in fluorescence mode (green and red) into a single colour image. The amount of markers used to treat the skin was low (25 μg each per 100 μl of vesicular dispersion), producing dim fluorescence that was not adjusted or manipulated (e.g. intensified) with any image processing software to achieve brighter pictures.

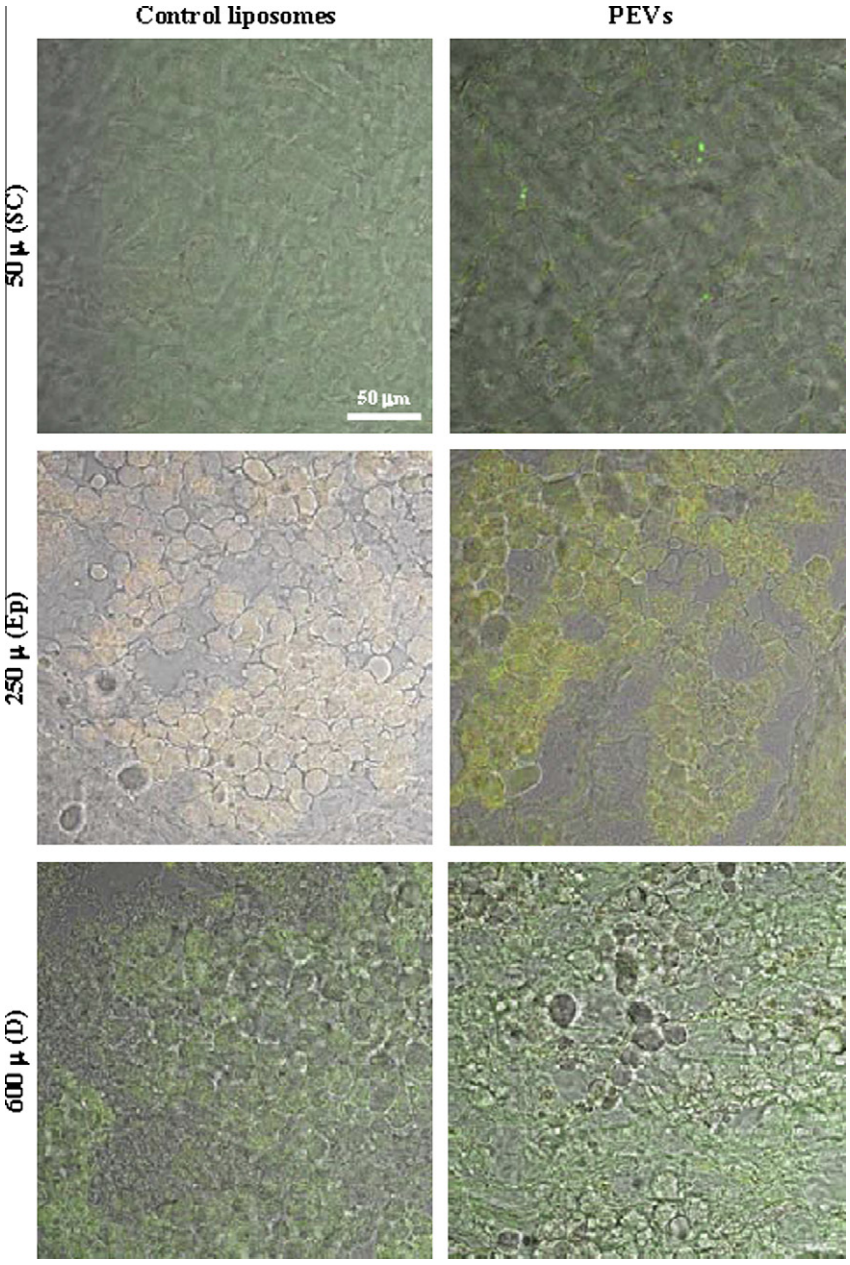

Fig. 7. Confocal laser scanning microscopy images of sections parallel to the surface of pig skin incubated for 8 h with fluorescent co-labelled conventional liposomes (control) and 20% Trc-PEVs. Images show the distribution of green and red fluorescence corresponding, respectively, to 5(6)-carboxyfluorescein and β-carotene, into stratum corneum (SC), epidermis (Ep) and dermis (D).

Nevertheless, the cytoarchitecture is visible, together with the distribution of the fluorophores. As can be seen from Fig. 7, a different distribution of the two labels was observed depending on the used carrier. In the SC, there was a prevalence of the green fluorescence (CF) for both control and PEVs, presumably due to the "free" label. However, the fluorescence was diffused all over the surface when the skin was treated with liposomes, while it was more intense and predominantly accumulated in the inter-corneocytes spaces in the case of PEVs.

Images of epidermis revealed the highest difference in the liposomes and PEVs behavior. Indeed, both images show a superposition of the green (CF) and red (β-C) fluorescence. However, this is particularly evident and intense for PEVs-treated skin, where large regions show a yellow fluorescence that clearly indicates that the hydrophilic CF and the lipophilic β-C reached 250 µm depth at the same time. Conventional liposomes also gave a superposition but the faded red fluorescence widespread all over this layer indicates a prevalence of the lipophilic marker deposition probably because the hydrophilic CF permeates more quickly towards the deeper skin strata. Indeed, in the dermis, the fluorescence of both markers is still evident with a prevalence of the green fluorescence of the hydrophilic label. Once more, the intensity of the fluorescence as well as its diffusion all over the surface was greater for PEVs.

Therefore, at the end of the permeation experiments, the extent of drug distribution into the skin was diverse due to the physicochemical properties and bilayer fluidity of the vesicles, primarily related to the Trc presence in PEVs. CLSM images indicate a penetration ability of PEVs greater than that of liposomes.

Therefore, CLSM images would support our assumption regarding the mode of action of PEVs. In particular,these vesicles, or most of them, penetrate intact down to the epidermis, due to

a synergistic mechanism among the penetration enhancer (Trc), PEVs, and intercellular pig skin lipids, as suggested by Touitou et al. for ethosomes [4]. Trc enhances PEVs' bilayer fluidity and perturbs intercellular skin lipid pathway, allowing transport and accumulation of loaded lipophilic and hydrophilic diclofenac to deep layers of the skin. This phenomenon was attenuated when DCF and DCF_{Na} were vehiculated in conventional liposomes, since these presumably disintegrated and fused with SC lipids [26], enhancing the penetration of the incorporated and/or encapsulated compound mainly at that level. Once vesicles disintegrate, a depot of the drugs is formed and each of them continues penetrating, free from the carrier, depending on its solubility. Hence, it is not surprising that a prevalence of DCF_{Na} (as well as the hydrophilic probe) deposition was observed in the dermis.

4. Conclusions

The physico-chemical stability, in terms of mean particle size and zeta potential, of the novel liposomes was significantly improved by the addition of transcutol. Although these PEVs cannot be considered deformable vesicles, the rheological experiments have revealed that Trc was able to improve bilayer fluidity. This property together with the synergistic activity of the "free" penetration enhancer can explain the superior ability of the PEVs to improve *ex vivo* drug transport through intact new born pig skin. Overall, the present results seem to indicate that PEVs can be a promising formulation for (trans)dermal delivery of both hydrophilic and lipophilic drugs.

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