

Drug-in-micelles-in-liposomes (DiMiL) systems as a novel approach to prevent drug leakage from deformable liposomes.

Silvia Franzè*, Umberto M. Musazzi, Paola Minghetti, Francesco Cilurzo

Department of Pharmaceutical Sciences, University of Milan, via G. Colombo 71, 20133 Milan

* Correspondence: silvia.franze@unimi.it; Tel.: +39-02-5032-4663

Abstract

Deformable liposomes (DL) are successfully exploited to enhance the skin penetration of several compounds. Nevertheless, the “soft” nature of the bilayer favors the drug leakage, mainly in the case of hydrophobic compounds. This work aimed to develop a suitable strategy to stabilize the lipid bilayer, without compromising the deformability properties of DL. The approach relied on the design of a “matryoshka” system, namely a drug in micelles in deformable liposomes (DiMiL) system. The performances (drug leakage, deformability and *in vitro* skin penetration profile) of DiMiLs were tested using nifedipine and piroxicam as model compounds and compared to those of traditional DL. The micelles were made of Kolliphor HS15 whereas the lipid vesicles were composed of egg-phosphatidylcholine and Tween 80 (T80) at 95:5 or 85:15 w/w ratios. As expected, the drug leakage from DL was high after only one month of storage (almost 50% in the case of nifedipine and in the range of 39-79 % in the case of piroxicam loaded DL, depending on T80 content). Optimized DiMiL formulations retained instead the drug content up to two-months storage period. Moreover, the constant of deformability of DiMiLs fell in the acceptance range for deformable vesicles intended for cutaneous application and the skin permeated amount of the delivered drugs was increased of at least 4 times. In conclusion, DiMiL reveals to be a suitable approach to avoid the leakage of hydrophobic compounds and an attractive transdermal drug delivery system for poorly permeable drugs.

Keywords:

Transdermal, elastic vesicles, transethosomes, piroxicam, nifedipine, ethosomes, transfersomes

1 Introduction

The use of nanotechnology in transdermal drug delivery field is growing in interest and several nanocarriers (e.i. polymeric and metallic nanoparticles, liposomes, dendrimers, nanogels etc) have been studied for breaching the skin barrier (Roberts, 2017; Jijie, 2017). This interest born with the hope to develop a shell able to maintain the drug payload whilst squeezing through the pores resulting from the imperfect overlapping of the cell membranes of corneocytes. However, considering that each nanosystem has an its own structure and a diameter of 100-200 nm and that the pores of stratum corneum have a maximum opening of 20-30 nm, as a matter of fact almost all nanocarriers used in drug delivery suffer of limited skin penetration (Cevc, 2003; Cevc, 2004; Cevc and Vierl, 2010). To overcome this issue deformable liposomes (DL) (introduced as TransfersomesTM), ethosomes and transethosomes have been proposed (Ascenso, 2015). The first class consists of liposomes formed by the combination of phospholipids and single chain surfactants, that act as destabilizing agents of the lipid bilayer. In fact, owing to their natural affinity for curved configurations, the surfactants may relocate in the areas of maximum curvature when the vesicles undergo an anisotropic stress (Sala, 2018). This behavior should allow the vesicle to reversibly modify its morphology elongating in the narrow pores of the stratum corneum (Cevc and Vierl, 2010). Although this mechanism of action is still matter of debate, several evidences are reported in literature and in a previous work we found some groups of isolated, intact vesicles, in the deep epidermis layers (Franzè, 2017). The other two classes of elastic vesicles, namely ethosomes and transethosomes, are instead characterized by a high ethanol content that decreases both the young modulus of the lipid bilayer and the tightness of the stratum corneum barrier (Touitou and Godin, 2007; Pandey, 2015). Elastic vesicles have been exploited to deliver in the skin several molecules and their efficiency has been widely proven both in *in vitro* and *in vivo* studies (Cevc, 1998; Touitou and Godin, 2007; Nava, 2011; Godin, 2005). One of the major drawbacks of these systems is the difficile balance between the flexibility, required for skin penetration, and the stability. In fact, mainly in the case of hydrophobic compounds, the lack of cholesterol along with the fluidity of the lipid chains of the main constituents of deformable liposomes, favor the leakage of the drug out from the bilayer (Bryant, 2018; Hussain, 2017). This decreases the shelf life of the product and therefore limits the possibility to reach the market. The steric stabilization of these systems cannot easily be achieved since, as demonstrated using hyaluronan moieties grafted on the surface of liposomes, it may

cause an increase of the packing order of the bilayer abolishing the ability of the vesicles to penetrate the skin (Franzè, 2018).

Therefore, there is the need to use a different approach that allows to improve the chemical stability of the systems without affecting the overall elasticity properties of deformable liposomes. In this work, we propose a novel drug-in-micelles-in-liposomes system (DiMiL) to slow down the unwanted drug release from deformable liposomes, thus avoiding the drug leakage. Basically, micelles made of a hydrophilic surfactant were used to solubilize hydrophobic model compounds in the aqueous core of the deformable liposomes. In particular, PEG-hydroxystearate (Kolliphor HS 15) was selected as surfactant for micelles formation since it is very well tolerated and already proposed as solubilizer in aqueous parenteral preparations (Tolva, 2016; Liu, 2016). Nifedipine (NIF) and piroxicam (PRX) were selected as model hydrophobic drugs because the drug leakage was expected (Franzè, 2018). Moreover, they are not able to massively permeate the skin and could be used in the treatment of Raynaud's syndrome (Franzé, 2018) and non-melanoma skin cancer (Musazzi, 2018), respectively.

The structure of DiMiL system was investigated by asymmetrical flow field-flow fractionation (aF4) and the performances of the DiMiL systems were compared to those of conventional deformable liposomes (DL) in terms of physico-chemical stability over a period of storage of two months, drug release, deformability properties and skin permeability performances.

2 Materials and Methods

2.1 Materials

Egg phosphatidylcholine (e-PC) was kindly gifted by Lipoid (Steinhausen, Switzerland); Kolliphor HS 15 was provided by BASF (Cesano Maderno, Italy); nifedipine was obtained from Prosintex Industrie Chimiche Italiane SRL (Settimo Milanese, Italy); piroxicam (PRX) was purchased from Cameo Healthcare (Thane, India) and Tween® 80 from Crodachocques (France); ascorbic acid (VWR Prolabo chemicals, Belgium); ammonium molybdate (Sigma- Aldrich Italy); sodium dihydrogen phosphate (Emsure®, Merck, Germany). Propylene glycol was bought from Carlo Erba (Italy). HPLC grade and analytical-grade organic solvents were purchased from Sigma-Aldrich (Milan, Italy).

2.2 Methods

2.2.1 Solubility assay

Saturated solutions of both drugs in kolliphor at 2.5, 5 and 10% w/v in water were prepared and let under stirring at 25 ± 1 °C for 24 hours, prior to be filter with a 0,45 µm PVDF filter. The maximum solubility of the two drugs in each kolliphor solution was determined by HPLC according to the analytical methods reported below.

2.2.2 Preparation of the formulations

Drug loaded micelles were prepared by adding a proper amount of drug to the kolliphor solution (**Table 1**). Then, the mixture was let under stirring for 24 hours prior to be used for liposomes preparation. Liposomes were prepared by thin film hydration method. Briefly, e-PC and Tween® 80 (T80) dissolved in chloroform/methanol (2:1 v/v) were mixed in the proper weight ratios (**Table 1**) in a round bottom flask. The organic solvent was evaporated for 1 hour at 40°C under reduced pressure using a rotatory evaporator (R11, Buchi, Italy), to obtain a thin lipid film. The film was rehydrated with the micellar solution (**Table 1**) for 1 h to obtain a final lipid concentration of 30 mg/mL. The reference formulations were obtained by rehydrating the lipid film with ultrapure water. In both cases, to homogenize the particle size distribution of the vesicles, the liposome dispersion was extruded (Avanti® Mini-Extruder, Avanti Polar Lipids, Inc.) through sequential passages through 0.2 µm (5 passages) and 0.1 µm (6 passages) polycarbonate membranes. Finally, the formulations were purified from free materials (drug and/or micelles) by molecular size exclusion chromatography on Sepharose CL-4B columns, eluting with ultrapure water.

2.2.3 Physico-chemical characterization

2.2.3.1 Determination of the main physical properties

The particle size distribution was determined both by dynamic light scattering (DLS), using a Zetasizer (Nano-ZS, Malvern Instrument, UK), and Nanoparticle Trafficking Analysis (NTA) using a Nanosight NS 300 (Malvern Instrument, UK). In the first case particle size measurements were carried out inserting the sample in a disposable cuvette after a 1:10 dilution in 0.22 µm filtered milliQ® water, with a detection angle of 173°.

ζ-potential was assessed on the diluted samples inserted in a capillary cell. Three measurements were taken for each sample and the results are expressed as the mean and standard deviation.

For NTA analysis the samples were diluted 5×10^5 times with ultrapure water and 6 sequential measurements at 25 °C were performed using a Blue488 laser.

2.2.3.2 Determination of the encapsulation efficiency

Drug encapsulation was measured by diluting the liposomes 1:100 in methanol to break the vesicles and release the free drug. The amount of drug was then determined by a HPLC system equipped with a diode array detector (HPLC HP 1100 Chemstations, Agilent Technologies, Waldbronn, Germany). For NIF quantification, the samples were eluted through a Lichrospher 100 RP-18E column (CPS Analytica, Milan, Italy) with a mixture of acetonitrile:methanol:Milli-Q® water (25:25:50 v/v), that was used as mobile phase; the flow rate was 1.3 mL/min, the thermostat was set at 37°C and the UV lamp at 230 nm. NIF quantification was done on the basis of a calibration curve of drug in mobile phase in the concentration range 5–100 µg/mL.

PRX quantification was performed using a 0.03 M phosphate pH 3.0 buffer/acetonitrile (60/40% v/v) mixture as mobile phase. The flow rate was 1.5 mL/min, and the UV lamp was set at two wavelengths (i.e., 248 nm, 360 nm). Sample concentrations were calculated basing on calibration curves built between 0.05 and 20 µg/mL.

The encapsulation efficiency (EE, %) was expressed as the percentage ratio between the amount of drug (mg/mL) encapsulated in the vesicles and the cumulative amount of the drug (mg/mL) loaded in the liposomes at the time of the preparation.

2.2.3.3 Investigation of micelles-bilayer interactions

To evaluate the possible distribution of the kolliphor micelles in the bilayer with following formation of mixed aggregates the trend of vesicle size and turbidity of the liposome dispersion in presence of increasing concentrations of surfactant was monitored in accordance to the model recently proposed by Elsayed and co-workers (Elsayed, 2018).

Briefly, DL 85:15 and DL 95:5 formulations were prepared and diluted with sterile ultrapure water up to a final ePC concentration of 8mM (Elsayed, 2011). Then, the DL dispersion was mixed with an equal volume of kolliphor dispersion in a final concentration range of 0.5-40% w/v. The mixture was incubated under agitation for 18 hours to reach the equilibrium. The particle size of the resultant dispersion was measured by DLS with a scattering angle of 173°. Finally, the optical density spectra of all dispersions were recorded between 400 and 600 nm using a lambda 25 spectrophotometer (PerkinElmer, USA).

Moreover, structural investigation of DL and DiMiL compositions was deepened by asymmetrical flow field-flow fractionation (aF4) analyses. AF4 measurements were performed on an AF2000 MT system (Postnova Analytics, Landsberg, Germany) coupled to a refractive index (RI, PN 3150), a

multi-angle laser light scattering (MALS, PN3621), a dynamic light scattering (DLS, PN3704) and a UV detector (UV, PN3211) set at two different wavelengths, namely 254 and 280 nm. The separation channel was 350 µm tick and a membrane of regenerated cellulose with a MWCO of 10 kDa served as accumulation wall. All samples were diluted in PBS buffer (1:10 and 1:3 for DL and DiMiL, respectively) at pH 7.4 and filtered through a 0.1 µm membrane prior to be injected. The injected volume was 20 µL and the flow rate was 0.5 mL/min.

2.2.3.4 *Deformability assay*

The deformability properties of the vesicles were determined using a dynamometer assisted extrusion assay previously developed by our research group (Franzè, 2017).

Briefly, all liposomal formulations were diluted to the same lipid concentration (0.23 mM) and loaded in a gas tight syringe. Lipid concentration was determined using an adaptation of the Rouser method, as described elsewhere (Franzè, 2018). The syringe plunger was put in contact with a 50 N load cell of a dynamometer (INSTRON 5965, ITW Test and Measurement Italia Srl, Italy) and forced to move at a constant speed of 1 mm/s, forcing the liposomal dispersion through a 50 nm polycarbonate membrane fixed in an extruder casing. The force (N) required to move the syringe plunger (dependent on the resistance opposed to vesicle penetration through the pores) was registered as a function of the plunger displacement (mm). The slope of this plot, namely the constant of deformability (k), was then derived. The higher the k value, the lower the deformability of the carriers (Franzè, 2017).

2.2.4 *Physical stability*

The physical stability of the prepared liposomes after storage at 4 °C under nitrogen atmosphere was evaluated monitoring over time the eventual changes in particle size distribution, ζ-potential and drug content of the DL and DiMiL systems. The drug leakage was determined by purifying the liposomal and DiMiL dispersions from leached drug by low speed (5000 rpm) centrifugation for 10 min at 25 °C (Universal 30RF, Hettich Zentrifugen, Germany). In these conditions, the free drug precipitated in the crystalline form. The supernatant instead was recovered and diluted with pure methanol to break the vesicles. The amount of drug encapsulated in the liposomes was determined after 1 and 2 months of storage and the percentage of drug leakage was calculated according to the following equation:

$$\text{Drug leakage \%} = \frac{(E_{t0} - E_{tx})}{E_{t0}} \times 100$$

were E_{t0} is the drug amount in mg/mL encapsulated in the liposome or DiMiL system at the time of preparation and E_{tx} is the amount of drug still encapsulated after 1 or 2 months of storage.

Moreover, the growth of drug crystals in the formulation during storage was monitored by light microscopy using a stereomicroscope (Nikon, Italy). An aliquot of formulation was spread on a glass slide. The micrographs were acquired at 20X magnification with a digital camera of 3.1 Mpx (CCD 3, ToupView, ToupTek, China).

2.2.5 *In vitro* drug release studies

In vitro drug release studies were performed using Franz diffusion cells having a receiving volume of about 3.0 mL and a surface area of 0.636 cm². An inert synthetic membrane of regenerated cellulose (Cuprophane, Millipore HAWP) was placed between the donor and receptor compartments that were sealed together by means of a clamp. Three hundred μ L of each formulation diluted to the same drug concentration were loaded in the donor chamber of the Franz diffusion cell. The loading drug concentration was established to be over the drug solubility to mime the storage conditions and at the same time to guarantee a complete solubilization on the receiver compartment. The release medium was an acetate buffer (pH 4.6)/polyethylene glycol 400 (PEG 400) (16% w/v) mixture and a water/propylene glycol (50/50 v/v) for NIF and PRX, respectively. The medium was maintained under stirring at 600 rpm and at a controlled temperature of 37 ± 1 °C by means of a circulating water bath. At fixed time intervals (15 min, 30 min, 1, 1.5, 2, 3, 4, 7, 10 and 24 h) 200 μ L of release medium were withdrawn and replaced with an equal volume of fresh medium.

2.2.6 *In vitro* skin permeability studies

In vitro skin permeability studies were carried out using the Franz diffusion cells method and human skin as membrane, which is obtained from healthy, informed volunteers undergoing abdominoplasty. The skin samples were prepared according to an internal procedure (Franzè, 2015). Briefly, at the arrival of the tissue in the lab, the excess fat is carefully removed and full-thickness skin is cut into squares, sealed in evacuated plastic bags and stored at -20 °C until their use. Epidermis sheets are obtained through mechanical separation from the remaining tissue with forceps, after skin immersion in water at 60 ± 1 °C for 1 min.

Prior to use the tissue samples for the experiment, the integrity of the skin was evaluated by measuring the electrical impedance of the epidermis sheets (Agilent 4263B LCR Meter, Microlease, Italy) (Musazzi, 2018).

The epidermis sheets were mounted on the lower half of the Franz diffusion cells with the stratum corneum facing upward. The upper and lower parts of the cell were sealed with parafilm and fastened together with a clamp. The receiver compartment was filled with an acetate buffer (pH 4.6)/ PEG 400 mixture in the 84/16 v/v ratio for NIF to assure the sink conditions and **physiologic solution** for PRX. The receiver phase was continuously stirred with a magnetic bar at 1500 rpm. The donor compartment was filled with 300 μ L of each formulation and maintained under non-occlusive conditions. The micellar solutions were tested in parallel as control. The system was kept at 37 ± 1 °C by means of a circulating water bath so that the epidermis surface temperature was at 32 ± 1 °C throughout the experiment. At fixed time intervals (1, 3, 5, 7 and 24 h) 200 μ L of receiver phase was withdrawn and replaced with an equal volume of fresh medium.

At the end of the experiment, the cells were dismantled and the skin samples were recovered and washed on both sides with fresh methanol to remove any residues of formulation/solution. Epidermis samples were let to dry and weighted prior to be cut in small pieces and immersed in 5 mL of methanol to extract the drug retained in the tissue. Drug retained and permeated through the skin was quantified through HPLC analysis in the analytical conditions described in the section 2.2.3.2. For the quantification of NIF during these experiments a calibration curve ranging between 0.2 and 20 μ g/mL was built.

3 Results and Discussion

3.1 Solubility assays

Table 2 reports the maximum solubility of both NIF and PRX in the different Kolliphor solutions tested.

Basing on these results, the micellar solutions used for the rehydration of the lipid film were prepared by adding 0.12% NIF or 0.03% PRX to the Kolliphor 10% aqueous dispersion (**Table 1**).

3.2 Physico-chemical characterization of vesicles

All DL and DiMiL compositions were monodisperse with a mean hydrodynamic diameter comprised between 110 and 150 nm and a slightly negative ζ -potential (**Table 3**). Although it is recognized that the allocation of a hydrophobic drug within the fatty acid chains of phospholipids causes a certain distension of the lipid chains, thus increasing the overall vesicle diameter, neither NIF or PRX provoked a significant modification of the main physico-chemical characteristics of the vesicles. As expected, instead, the hydrodynamic diameter of DL slightly decreased increasing the T80 concentration because of the reduction of the interfacial surface free energy and the increased curvature (Bnyan, 2018). The presence of Kolliphor micelles (mean particle size of 12.56

± 0.00 nm) in the hydration medium during DiMiLs formation led to a further reduction of the mean diameter, as determined by DLS (**Table 3**). The effect of the surfactant on the encapsulation efficiency (EE) of DL is more controversial. It has been hypothesized that the incorporation of surfactants into lipid-based vesicles lowers the encapsulation of hydrophobic drugs (Bnyan, 2018; Jain, 2003; El Maghraby, 2000). Nevertheless, this effect seems to be strongly dependent on the chemical characteristics of the drug. Indeed, only in the case of PRX loaded DL, it was observed a decrease of EE% moving from 95:5 to 85:15 compositions. This behavior may be ascribed to the higher solubility of PRX ($\log P = 0.588 \pm 0.858$; ACD/Labs Software) with respect to NIF ($\log P = 3.582 \pm 0.588$; ACD/Labs Software) which favors the partition of the drug towards the hydration medium during liposome formation. The solubilization of the drug in micelles led to have an EE% of PRX in DiMiLs quite high and similar to that obtained with the other carriers (**Table 3**), that was satisfactory (almost 70%).

Since both drugs did not significantly influence the overall physico-chemical properties of the systems according to DLS data, the structural analyses aimed to elucidate the possible interactions among the excipients of DiMiL formulations were carried out only with the placebo formulations. In particular, the effect of the secondary component (kolliphor micelles) on the properties of deformable liposomes was deepened by Nanoparticle Trafficking Analysis (NTA) that, with respect to DLS, allows the direct visualization of the particles and discriminates different populations basing on size, with more accuracy with respect to DLS.

The particle size distribution of DiMiLs registered by NTA evidenced the presence of a small percentage of slightly larger vesicles (Figure 1a and 1b), probably resulting from the interaction of kolliphor micelles with the bilayer. To further investigate such hypothesis the turbidity and particle size variation of preformed liposomes after exposure to kolliphor micelles was evaluated, since those parameters are sensitive to additives able to interact with lipid bilayers altering the fluidity of the membrane (Elsayed, 2018). Regardless of the composition of DL (95:5 or 85: 15), the complete solubilization of the liposomal membrane was found at a concentration of kolliphor nearby the 35% w/v (**Figure 2**). In fact, at this concentration the membrane was broken definitively as demonstrated by the plunge of both the percentage intensity of the main population of vesicles and turbidity of the dispersion (Elsayed, 2018). Moreover, it was observed a new population of very small micelles (around 27 nm) and very larger aggregates with a mean diameter higher than 5000 nm resulting from the reassembly of the membrane fractions in big aggregates. In contrast, at the concentration value of the formulations in study (10% w/v) only a

small fraction of free micelles (12.4 ± 0.35 nm) added in the external medium in the experimental conditions used was revealed (**Figure 2**). However, the larger diameter of the main vesicles (206.8 ± 3.35 nm) along with the increased Pdl of the dispersion (0.444 ± 0.019) suggested the **merger** of the kolliphor micelles with the liposome bilayers with following enlargement of the vesicles.

This result is in line with the AF4 data. Representative fractograms for compositions containing 15% of T80 in the bilayer, considered as the most complex formulations, are reported in **Figure 3**. The obtained radius calculated by the MALS angular data and the hydrodynamic radius obtained by the flow DLS data demonstrated that the main fraction separated in the DiMiL sample corresponds to the liposomal vesicles found in the DL samples (the UV peak maximum was equal to 48 nm for DiMiL and 56 nm for DL, whereas the hydrodynamic radii, obtained by the flow DLS data, were 40 nm and 56 nm respectively, in agreement with the size obtained from MALS data). It is worth noting that only a small unquantifiable fraction of free micelles was found in the DiMiL samples due to residuals of the purification process. Basing on aF4 data and according to the DLS results, the presence of kolliphor micelles caused a reduction of the particle size of the vesicles. To verify if this behavior was accompanied by a change of particle morphology, the shape factor (ρ) was calculated for both compositions (DL and DiMiLs). This parameter is given by the ratio between the radius of gyration (R_g) and hydrodynamic radius (R_H) and for a hollow sphere like an empty liposomes is close to 1 (Patterson, 2014). Accordingly, the calculated ρ value for DL composition was 1.04 whereas for DiMiL system was slightly higher, namely 1.22. This result confirmed that a partial interaction between kolliphor and the lipid bilayer takes place, with an increase of the curvature of the bilayer. This change however is not sufficient to support the hypothesis of a complete intercalation of the micelles in the bilayer. Indeed, in accordance to literature data, materials able to establish strong molecular interactions with lipid bilayers move the ρ parameter towards higher values (~ 2) (Maherani, 2017).

To verify definitively the effect of the micelles on the deformability properties of the vesicles under stress, the constant of deformability, k , was determined by a modified extrusion assay that allows to study the forces involved in liposome penetration through narrow pores and resulted to be sensitive to discriminate also little changes in the deformability properties of lipid based systems (for further details see Franzè, 2017).

As expected, in the case of DL, the value of k decreased increasing the T80 content (**Table 3**). Comparing the DL/DiMiL pairs with different T80 content, one may note that in the case of 95:5 formulations, DiMiL systems showed a higher degree of flexibility with respect to DL. This data is in

line with the previous findings about a possible partial distribution of the micelles in the liposome bilayer that causes a further destabilization of the membrane with following decrease of the k value. Instead, in the case of 85:15 formulations, the behavior was opposite, namely the presence of micelles had a stiffening effect rather than a fluidizing one. In fact, at high concentrations T80 tends to assemble itself in the bilayer (Bnyan, 2018) therefore there is a competition with Kolliphor micelles. As result, in the purified formulation the interaction between micelles and liposome bilayer may be hindered. However, it is worth noting that all the k values registered for DiMiL formulations felt in the acceptance range for elastic vesicles, according to our experience (Franzè, 2017; Franzè, 2018). This means that the DiMiL approach does not change the overall mechanical properties of the carriers.

3.3 *In vitro drug release*

Drug release can be considered as a surrogate of the stability of the bilayer against the loss of the entrapped drug. In general, the extent of drug release from a liposomal formulation strongly depends on the bilayer composition and on the nature of the drug. Vesicles containing a softener agent release the drug faster than rigid ones because of the leakier membrane (El Maghraby, 2000). The rate limiting step of course in this type of assays is the release of the drug from the bilayer because only free drug can cross the cuprophane[®] membrane. In the case of NIF, the diffusion of drug through the membrane of DL 95:5 formulation takes only hour to start and the release curve presents the classical sigmoidal shape typical of DL (**Figure 4a**). The percentage of drug release did not go over the 30% because as the concentration of free drug leaching out from the bilayer increases the drug crystalizes (because the loading dose is **close to its solubility**); the same event occurs when the saturated solution of drug is used (data not shown). **Moreover**, in the case of the DL 85:15 composition the mean release data of NIF could not be even plotted because the release kinetic was highly variable also within the same set of experiment (**Figure 4c**). This behavior again was ascribed to the high tendency of nifedipine to convert into the crystalline form when released as free drug. This hypothesis was confirmed by monitoring the changes occurring in the donor phase by light microscopy (stereomicroscope Nikon, Italy). It was found that the drug release curve reached a plateau at different time points depending on when nucleation and crystal growth occurred (**Figure 4c**).

As awaited, DiMiL systems showed a better control of the drug release, regardless of the extent of primary surfactant (T80) in the vesicle bilayer (**Figure 4a**). This is perceivable because, as previously demonstrated in the case of drug-cyclodextrin complexes loaded in liposomes (Gillet,

2009), the encapsulation of the drug in micelles significantly reduces the concentration gradient of the drug towards the bulk medium. Accordingly, the use of DiMiL allows to overcome the issue related to the variability of the release data of NIF by reducing the extent of free drug in the medium and avoiding the possible crystallization.

In the case of PRX, the release from DL was even faster with respect to NIF since it was observed already after the first hour of experiment (Figure 4b). This could be related to both the higher aqueous solubility and a possible competition with T80 in the bilayer that probably impedes a strong intercalation of the drug in the bilayer, causing a burst effect in the release curve. DiMiL system, again, allowed to slow down the drug release rate.

3.4 Physical stability

In a previous work we reported about the advantage of using DL to enhance the nifedipine penetration in the epidermis and we already highlighted the issue of the high extent of drug leakage associated to these drug delivery systems (Franzé, 2018). In the previous paper, we referred only to the 85:15 formulation. The decrease of the concentration of T80 did not change in a significant manner this behavior. In fact, after one month of storage a high amount of drug crystals leached out from the bilayer was observed in both DL formulations and the drug leakage percentage was quite high as well (**Table 3**). In contrast, any drug leakage was observed from DiMiL systems after one month of storage and the entrapped drug amount did not change also over a two-month storage period (data not shown). These results are in line with the in vitro drug release data, that are predictive of the reduced repartition of the drug out of the liposomes. PRX loaded DiMiL systems showed also improved stability over time. In this case the phenomenon of drug leakage was even more stressed as expected basing on drug release data, with almost 80% of drug leakage from 85:15 DL after one month (**Table 3**). The leakage was lower in the case of 95:5 formulations because of the slight higher stability of the bilayer. Both DiMiL systems were able to improve the retention of the drug inside the vesicles but this time only DiMiL 95:5 system allowed to completely abolish the drug leakage from the liposomes (**Table 3**), even after two months of storage.

From a physical point of view, instead, all formulations prepared resulted stable over a period of two months since any variation of particle size and/or ζ -potential was observed.

3.5 *In vitro skin permeability studies*

NIF is a good candidate for (trans)dermal delivery due to the low molecular weight and the suitable logP value. Nevertheless, its passive diffusion through the skin is negligible. The encapsulation of NIF in DL favors the penetration of the drug through human skin (Franzè, 2018). Nevertheless, surprisingly, DiMiLs led to a drastic increase of NIF permeation that moved from $2.73 \pm 0.06 \mu\text{g}/\text{cm}^2$ in 24 h (DL) to $120.72 \pm 41.71 \mu\text{g}/\text{cm}^2$ after 7 h (DiMiL 85:15) when the equilibrium was reached (**Figure 5a**). Moreover, DiMiLs seemed to favor the partition of the drug in the skin since the lag time was significantly reduced and the drug permeation was observed already at the first hour of experiment. The ameliorant of NIF skin permeation cannot merely be ascribed to the effect of the additional surfactant since the drug amount permeated after 24 h of application of NIF micelles was not quantifiable. Therefore, the higher permeation pattern of nifedipine when carried in DiMiLs should be ascribed to the whole system. This result is in line with the data reported in literature about the effect of surfactants on skin permeation, which evidenced that the increase of the micelle strength decreases the skin permeation (James-Smith, 2011). It was hypothesized that the perturbation effect exerted by surfactants on the skin barrier depends on the presence of monomers and sub-micellar aggregates rather than micelles (James-Smith, 2011).

As far as PRX is concerned, following the results of the stability study, the formulations 95:5 were selected for the in vitro skin permeability assays (**Figure 5b**). It is well known that PRX scantily permeates the human skin when administered in form of saturated solution or topical products (Musazzi, 2018). This is due to the unfavorable physicochemical properties of the drug, namely the low solubility in the medium that decreases the thermodynamic activity, the high melting point and the zwitterionic nature. It is in fact reported that zwitterionic drugs present a very low skin permeability due to the large number of oppositely charged groups. Preparation of PRX salts resulted in fact in improved physicochemical properties and skin penetration ability (Cheong and Choi, 2002). The encapsulation of piroxicam in surfactant based micelles on one hand increases the solubility of the drug and, then, both the loaded dose in the donor compartment and the concentration gradient. In addition, when the drug is entrapped in micelles the charges are masked, therefore the drug partition in the skin is favored. As a consequence, free micelles in this case allowed a slight permeation of PRX, even if the drug was quantifiable only after 24 hours (**Figure 5b**). However, the amount of PRX permeated through human skin after 24 hours was higher after application of DL than micelles ($0.56 \pm 0.08 \mu\text{g}/\text{cm}^2$ versus $0.32 \pm 0.09 \mu\text{g}/\text{cm}^2$).

Indeed, deformable liposomes add to the solubilizing and charge masking effect of micelles the flexibility of the carrier, that gains easily access to the skin layers. Nevertheless, again only DiMiLs increased significantly the drug permeation (6.5 folds higher than that of pure micelles) along with a decrease of lag time, confirming a rapid partitioning of the delivery system itself in the skin (**Figure 5b**). Moreover, it is worth to note that both the loaded dose and the amount of PRX permeated through the skin after application of DiMiLs were comparable to those obtained with commercially available medicated plasters (Cilurzo, 2015).

The efficacy of the DiMiL systems to enhance the skin permeation was further evaluated using as descriptor the R_{24}/Q_{24} ratio, namely the relative amount of drug retained (R_{24}) and permeated through human skin after 24 hours (Q_{24}) of application of a certain vehicle. The lower the ratio, the greater the ability of the vehicle to enhance the flux of a compound (Franzé, 2015). As expected, this ratio resulted at least one order of magnitude higher in the case of DL with respect to the DiMiLs (PRX: DL $R_{24}/Q_{24}= 2.78$; DiMiL $R_{24}/Q_{24}= 0.01$; NIF: DL $R_{24}/Q_{24}= 0.41$; DiMiL $R_{24}/Q_{24}= 0.01$). It is well accepted in fact that deformable liposomes favor in general the retention of drugs in the skin rather than their permeation, in contrast to other lipid-based vesicles such as ethosomes and transethosomes (Ascenso, 2015, Campani, 2016). Thus, the overall data suggested DiMiL systems behaves similarly to ethanol containing lipid carriers even if the penetration mechanism has to be clarified.

4 Conclusions

The interest in exploiting deformable lipid carriers to increase the distribution of drugs in the skin is growing over years. Nevertheless, the development of a successful dosage form based on these carriers is limited by the low stability of deformable vesicles. The proposed DiMiL system appears an attractive solution to avoid drug leakage of lipophilic compounds. The overall results obtained in this work suggest that the hydration of phospholipids/surfactant blend with a micellar solution made of a secondary surfactant leads to a partial merger of the auxiliary surfactant with the vesicle bilayer, with further improvement of liposome fluidity, as demonstrated by turbidity, NTA and aF4 studies. Nevertheless, the slowed drug release rate along with the avoidance of the drug leakage phenomenon, suggest that reasonably some of the drug containing micelles remain entrapped in the inner core of the liposomes. Finally and unexpectedly, DiMiL composition significantly improved the diffusion through the skin of poorly permeable compounds, with respect to both deformable liposomes and pure micelles. Thus, the DiMiL system appears worth of further investigation for the development of transdermal delivery systems.

Acknowledgments

Authors thank Alfatest s.r.l. for giving access to the NanoSight for the Nanoparticle Trafficking Analyses and Dr. Santoliquido for the support in aF4 analyses.

References

- Ascenso, A., Raposo, S., Batista, C., Cardoso, P., Mendes, T., Praça, F., Bentley, V., Simões, S. 2015. Development, characterization, and skin delivery studies of related ultradeformable vesicles: transfersomes, ethosomes, and transethosomes. *Int J Nanomedicine*, 10, 5837–5851.
- Bryan, R., Khan, I., Ehtezazi, T., Saleem, I., Gordon, S., O'Neill, F., Roberts M. 2018. Surfactant Effects on Lipid-Based Vesicles Properties. *J Pharm Sci* 107, 1237-1246.
- Campani, V., Biondi, M., Mayol, L., Cilurzo, F., Franzé, S., Pitaro, M., De Rosa, G., 2016. Nanocarriers to enhance the accumulation of vitamin K1 into the skin. *Pharm. Res.* 33, 893–908.
- Cevc, G. 2004. Lipid vesicles and other colloids as drug carriers on the skin. *Adv Drug Deliv Rev* 56, 675–711.
- Cevc, G., Gebauer, D., Stieber, J., Schätzlein, A., Blume, G. 1998. Ultraflexible vesicles, Transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. *Biochim Biophys Acta* 1368, 201–215.
- Cevc, G., Schätzlein, A.G., Richardsen, H., Vierl U. 2003. Overcoming Semipermeable Barriers, Such as the Skin, with Ultradeformable Mixed Lipid Vesicles, Transfersomes, Liposomes, or Mixed Lipid Micelles. *Langmuir* 19, 10753-10763.
- Cevc, G., Vierl, U. 2010. Nanotechnology and the transdermal route. A state of the art review and critical appraisal. *J Control Release* 141, 277–299.
- Cheong, H., Choi, H. 2002. Enhanced Percutaneous Absorption of Piroxicam via Salt Formation with Ethanolamines. *Pharm Res*, 19 (9), 1375-1380.
- Cilurzo, F., Gennari, C.G.M., Selmin, F., Franzè, S., Musazzi, U.M., Minghetti, P. 2015. On the characterization of medicated plasters containing NSAIDs according to novel indications of USP and EMA: adhesive property and in vitro skin permeation studies. *Drug Dev. Ind. Pharm.*, 41, 183–189.
- El Maghraby, G.M.M., Williams, A.C., Barry, B.W. 2000. Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration. *Int. J. Pharm.* 196, 63–74.
- Elsayed M.M.A, Cevc, G. 2011. Turbidity spectroscopy for characterization of submicroscopic drug carriers, such as nanoparticles and lipid vesicles: size determination. *Pharm Res* 28, 2204-2222.
- Elsayed M.M.A., Ibrahim M.M., Cevc G. 2018. The effect of membrane softeners on rigidity of lipid vesicles bilayers: derivation from vesicle size change. *Chem Phys Lipids* 210, 98-109.
- Franzé S., Gennari, C. G. M., Minghetti, P., Cilurzo, F. 2015. Influence of chemical and structural features of low molecular weight heparins (LMWHs) on skin penetration. *Int. J. Pharm.* 481 (1–2), 79– 83.
- Franzé, S., Donadoni, G., Podestà, A., Procacci, P., Orioli, M., Carini, M., Minghetti, P., Cilurzo, F. 2017. Tuning the extent and depth of penetration of flexible liposomes in human skin. *Mol. Pharmaceutics*, 14 (6), 1998–2009.
- Franzé, S., Marengo, A., Stella, B., Minghetti, P., Arpicco, S., Cilurzo, F. 2018. Hyaluronan-decorated liposomes as drug delivery systems for cutaneous administration. *Int J Pharm*, 535, 333-339.

Gillet, A., Grammenos, A., Compère, P., Evrard, B., G. Piel. 2009. Development of a new topical system: Drug-in-cyclodextrin-in-deformable liposome. *Int. J. Pharm.* 380, 174–180.

Godin, B., Touitou E., Rubinstein, E., Athamna, Athamna, M. 2005. A new approach for treatment of deep skin infections by an ethosomal antibiotic preparation: an in vivo study. *J Antimicrob Chemother* 55, 989–994.

Hussain, A., Singh, S., Sharma, D., Webster, T.J., Shafaat, K., Faruk, A. 2017. Elastic liposomes as novel carriers: recent advances in drug delivery. *Int J Nanomedicine* 12, 5087–5108.

Jain, S., Jain, P., Umamaheshwari, R.B., Jain, N.K. 2003. Transfersomes—A Novel Vesicular Carrier for Enhanced Transdermal Delivery: Development, Characterization, and Performance Evaluation. *Drug Dev Ind Pharm* 29 (9), 1013-1026.

James-Smith, M.A., Hellner, B., Annunziato, N., Mitragotri, S. 2011. Effect of Surfactant Mixtures on Skin Structure and Barrier Properties. *Ann Biomed Eng.* 39 (4), 1215–1223

Jijie, R., Barras, A., Boukherroub, R., Szunerits, S. 2017. Nanomaterials for transdermal drug delivery: beyond the state of art of liposomal structures. *J. Mater. Chem. B.* 5, 8653-8675.

Liu, L., Mao, K., Wang, W., Pan, H., Wang, F., Yang, M., Liu H. 2016. Kolliphor® HS 15 Micelles for the Delivery of Coenzyme Q10: Preparation, Characterization, and Stability. *AAPS PharmSciTech* 17 (3), 757-766.

Maherani, B., Wattraint, O. 2017. Liposomal structure: a comparative study on light scattering and chromatography techniques. *J. Dispersion Sci. Technol.* 38 (11), 1633-1639.

Musazzi, U.M., Cencetti, C., Franzé S., Zoratto, N., Di Meo, C., Procacci, P., Matricardi, P., Cilurzo, F. 2018. Gellan Nanohydrogels: Novel Nanodelivery Systems for Cutaneous Administration of Piroxicam. *Mol. Pharmaceutics* 15 (3), 1028-1036.

Nava, G., Piñón, E., Mendoza, L., Mendoza, N., Quintanar, D., Ganem, A. 2011. Formulation and in Vitro, ex Vivo and in Vivo Evaluation of Elastic Liposomes for Transdermal Delivery of Ketorolac Tromethamine. *Pharmaceutics* 3, 954-970.

Pandey, V., Golhani, D., Shukla, R. 2015. Ethosomes: versatile vesicular carriers for efficient transdermal delivery of therapeutic agents. *Drug Delivery* 22(8), 988–1002.

Patterson, J.P., Robin, M.P., Chassenieux, C., Colombani, C., O'Reilly, R.K. 2014. The analysis of solution self-assembled polymeric nanomaterials. *Chem. Soc. Rev.*, 43, 2412-2425.

Roberts, M.S., Mohammed, Y, Pastore, M.N., Namjoshi, S., Yousef, S., Alinaghi, A., Haridass, I.N., Abd, E., Leite-Silva, V.R., Benson, H.A.E., Grice J.E., 2017. Topical and cutaneous delivery using nanosystems. *J Control Release* 247, 86–105

Sala, M., Diab, R., Elaissari, A., Fessi, H. 2018. Lipid nanocarriers as skin drug delivery systems: Properties, mechanisms of skin interactions and medical applications. *Int J Pharm*, 535, 1-17.

Tolva, V., Mazzola, S., Zerbi, P., Casana, R., Albertini, M., Calvillo, L., Selmin, F., Cilurzo, F. (2016) A successful experimental model for intimal hyperplasia prevention using a resveratrol-delivering balloon *Journal of Vascular Surgery*, 63 (3), 788-794.

Touitou, E., Godin, B. 2007. Dermal drug delivery with ethosomes: therapeutic potential *Therapy* 4(4), 465–472.

<https://scifinder.cas.org> (accessed 2018, December 13th); logP calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2018 ACD/Labs).

Figure Captions:

- **Figure 1-** Particle size distribution of placebo DL and DiMiL obtained by Nanoparticle Trafficking Analysis (NTA). The data is the mean of five measurements. The two panels show the particle size distribution of: A) DL95:5 and DiMiL95:5 systems; B) DL85:15 and DiMiL85:15 systems.
- **Figure 2-** Effect of kolliphor on preformed empty deformable liposomes (total ePC concentration 4mM). Panel A reports the average hydrodynamic diameter of the vesicles. The asterisks indicate detection of very larger aggregates (>5000 nm). Panel B shows the variation of the scattering intensity of the main vesicle population. Panel C represents the normalized turbidity ($\lambda = 450$ nm) of the DL dispersions (both DL95:5 and DL85:15).
- **Figure 3-** Fractogram of A) DL 85:15 and B) DiMiL 85:15 compositions obtained by aF4 analysis. Blue line represents the trend of the radius of gyration (R_g) versus the concentration signal and the red line represents the trend of the hydrodynamic radius (R_h) versus the concentration signal.
- **Figure 4-** In vitro drug release profiles of the compositions of deformable liposomes (DL) and drug-in-micelles-in liposomes (DiMiLs) systems containing (A) nifedipine and (B) piroxicam. Panel C reports three representative release curves obtained within the same experiment after repartition of a batch of DL 85:15 composition in the donor chamber of three Franz diffusion cells. The different trend of the three curves is explicative of the high intra-experimental variability of the data obtained using DL 85:15 loaded with nifedipine. The insert of panel C instead is one of the micrographs recorded by light microscopy on the donor phases at different time points and demonstrates the formation of drug crystals in the donor compartment as result of the low solubility of free nifedipine.
- **Figure 5-** In vitro permeation profile of a) nifedipine and b) piroxicam through human epidermis after application of deformable liposomes (DL), DiMiLs and kolliphor micelles.

Tables

Table 1- Quali-quantitative composition of the prepared liposomal formulations.

		Vesicle bilayer composition (%w/w)				Rehydration solution (%w/v)			
	Formulation	e-PC	T80	NIF	PRX	Kolliphor	NIF	PRX	water
PLACEBO	DL 95:5	95	5	-	-	-	-	-	100
	DiMiL 95:5	95	5	-	-	10	-	-	90
	DL 85:15	85	15	-	-	-	-	-	100
	DiMiL 85:15	85	15	-	-	10	-	-	90
NIF	DL 95:5	89.06	4.69	6.25	-	-	0.12	-	99.88
	DiMiL 95:5	95	5	-	-	10	0.12	-	89.88
	DL 85:15	79.69	14.06	6.25	-	-	0.12	-	99.88
	DiMiL 85:15	85	15	-	-	10	0.12	-	89.88
PRX	DL 95:5	94.06	4.95	-	0.99	-	-	0.03	99.97
	DiMiL 95:5	95	5	-	-	10	-	0.03	89.97
	DL 85:15	85	15			-	-	0.03	99.97
	DiMiL 85:15	84.16	14.85	-	0.99	10	-	0.03	89.97

Abbreviations: DL: conventional deformable liposomes; DiMiL: drug-in-micelles-in-deformable liposomes; e-PC: egg-phosphatidylcholine; T80: Tween® 80 ; NIF: nifedipine; PRX: piroxicam.

Table 2- Maximum solubility of nifedipine and piroxicam in kolliphor HS solutions (mean \pm standard deviation, n=3)

Kolliphor (% w/v)	Nifedipine ($\mu\text{g/mL}$)	Piroxicam ($\mu\text{g/mL}$)
0	5.9	16
2.5	283.7 \pm 10.0	93.19 \pm 1.15
5	612.2 \pm 6.0	175.79 \pm 0.44
10	1284.5 \pm 19.54	345.2 \pm 3.4