Hyaluronan-decorated liposomes as drug delivery systems for cutaneous administration

HYALURONAN-DECORATED LIPOSOMES AS DRUG DELIVERY SYSTEMS FOR CUTANEOUS ADMINISTRATION

Silvia Franzé[1], Alessandro Marengo[2], Barbara Stella[2], Paola Minghetti[1], Silvia Arpicco[2], Francesco Cilurzo[1]*

[1]Department of Pharmaceutical Science – Università degli Studi di Milano

[2]Department of Drug Science and Technology - Università degli Studi di Torino

Corrispondence to:

Prof. Francesco Cilurzo, PhD
Department of Pharmaceutical Sciences
Università degli Studi di Milano
via Giuseppe Colombo, 71 – 20133 Milano (Italy)
phone: +39 02 503 24635
fax: +39 02 503 24657
e-mail address: francesco.cilurzo@unimi.it

Abstract

The work aimed to evaluate the feasibility to design hyaluronic acid (HA) decorated flexible liposomes to enhance the skin penetration of nifedipine. Egg phosphatidylcholine (e-PC) based transfersomes (Tween 80) and transethosomes (ethanol) were prepared. HA was reacted with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (HA-DPPE) and two molar ratios (0.5 and 3%) of conjugate with respect to e-PC were tested. The presence of HA significantly increased the packing order of the bilayer (as verified by differential scanning calorimetry), reducing both the encapsulation efficiency and the flexibility of the decorated liposomes in a dose-dependent manner. In fact, at the highest HA content the constant of deformability (K, N/mm) increased and the carriers remained on the skin surface after topical application. The stiffening effect of HA was counterbalanced by the addition of ethanol as fluidizing agent that allowed to maintain the highest HA concentration, meanwhile reducing the K value of the vesicles. HA-transethosomes allowed a suitable nifedipine permeation (J ~30 ng/cm^2/h) and significantly improved the drug penetration, favouring the formation of a drug depot in the epidermis. These data suggest the potentialities of HA-transethosomes as drug delivery systems intended for the treatment of cutaneous pathologies and underline the importance of studying the effect of surface functionalization on carrier deformability to rationalize the design of such systems.

Keywords: surface functionalization, transethosomes, flexibility, DSC, human skin permeability, coated liposomes.

1. Introduction

Primary Raynaud's syndrome is an idiopathic condition characterized by episodic vasospasm induced by environmental or emotional stress, restricting blood flow, leading to ischaemia, particularly in the extremities. The most common symptom is a change in colour, due to the deoxygenation and subsequent perfusion of the vasculature, accompanied to numbness, tingling sensation and pain (Hughes et al. 2016).

The first line treatment generally consists of life-style advice. In the case of severe conditions, the pharmacological approach relies on the administration of calcium-channels blocker drugs, mainly nifedipine (Sinnathuraiand et al., 2013; Ennis et al., 2016). However, licensed conventional oral dosage forms of nifedipine have some limitations owing to the low bioavailability and the short half-life of the drug that leads to the need of multiple dose administrations per day. Moreover, also in the case of prolonged-release tablets, the treatment is non-selective and has many off target effects. Interestingly, previous works demonstrated that even if nifedipine is a good candidate for transdermal delivery owing to the low molecular weight and the suitable octanol-water partition coefficient (log P, 2.2), its passive diffusion through human epidermis is negligible (Yasam et al., 2016).

The encapsulation of the drug in liposomes can overcome these issues. Indeed, on one hand the use of such lipid vesicles is a well-consolidated approach to solubilise and deliver highly hydrophobic compounds (Sauvage et al. 2016); on the other hand liposomes have been employed as facilitators to the delivery of molecules in the deep layers of the skin, where they may form a depot, for the slow, sustained release of the drug, to reduce the frequency of administration (Campani et al., 2016). In particular, flexible liposomes, ethosomes and transethosomes have been resulted as the most suitable carriers for breaching the skin barrier (Ashtikar et al., 2016; Abdulbaqi et al., 2016)

The common denominator of such systems is the membrane fluidity (Morilla & Romero, 2016). In the case of flexible liposomes, the increased bilayer fluidity is due to the addition of an edge activator, usually a surfactant, in the phospholipid bilayer of the vesicles, that lends to a stress dependent adaptability. When deformable liposomes undergo a stress, the liposome bilayer is rearranged, and the surfactant relocates to zones of higher vesicle curvature and hydrophobic regions to the least vesicle curvature/stress (Cevc & Vierl, 2010). This rearrangement requires minimal energy and spontaneous permeation is driven, under non-occlusive conditions, by the natural transdermal osmotic gradient (Cevc & Blume, 1992). A further improvement of the performances of lipid-based carriers such as flexible liposomes and ethosomes may be obtained grafting on their surface a targeting moiety, which may drive the penetration of the carrier in the skin, as already proven in the case of some peptides (Chen et al., 2014a; Chen et al., 2014b).

Hyaluronic acid (HA) may be a suitable moiety since this polysaccharide demonstrated to be retained in the epidermis (Cilurzo et al., 2014) and to be able to improve the dermal delivery of different molecules, including high molecular weight proteins such as bovine serum albumin (Witting et al., 2015). However, the grafting of HA on the surface of vesicles may cause a significant stiffening of the bilayer, thus altering the overall flexibility properties of the drug delivery systems, compromising their efficiency (Franzè et al., 2017).

This work aimed to evaluate the feasibility to design HA-decorated flexible liposomes to enhance the skin delivery of nifedipine.

For the coating of HA on the surface of liposomes the polysaccharide was conjugated to the phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE). A 4800 Da HA was used in this study since this low molecular weight HA provided the best performances in terms of dermal delivery of co-administered compounds (Witting et al., 2015). Moreover, since the effect of HA applied on the skin seems to be dose-dependent, different molar ratios of HA-DPPE conjugate were tested. Liposome formulations were characterised for the main physicochemical properties, with focus on the effect of the surface coating on drug encapsulation and, mainly, carrier deformability. Indeed, this result could be indicative of whether the formulation can enhance the transdermal permeation of nifedipine.

2. Materials and Methods

2.1 Materials

DPPE was purchased from Avanti Polar Lipids (Alabaster, United States); egg phosphatydilcholine (e-PC) was kindly gifted by Lipoid (Steinhausen, Switzerland); hyaluronic acid 4800 Da was purchased from Lifecore Biomedical (Chaska, MN); Tween® 80 (Crodachocques, France); carbazole was purchased from Merck (Darmstadt, Germania); nifedipine (Siegfried Ltd, Switzerland);4-(2-hydroxy- ethyl)piperazine-1-ethanesulforic acid (HEPES) (Amresco, USA); ascorbic acid (VWR Prolabo chemicals, Belgium); ammonium molybdate (Sigma- Aldrich Italy); sodium dihydrogen phosphate (Emsure®, Merck, Germany). HPLC grade and analytical-grade organic solvents were purchased from Sigma-Aldrich (Milan, Italy).

2.2 Synthesis of HA-DPPE conjugate

HA-DPPE conjugate was obtained by the synthetic procedure described by Arpicco et al. (2013) with minor modifications. Briefly, 0.060 mmol of HA (0.3 g) was quite completely dissolved in 20 mL of methanol and dimethyl sulfoxide (1:1 v/v) and stirred at 60 °C. After 30 min, DPPE (0.038 g, 0.060

mmol) previously dissolved in 2 mL of methanol and chloroform (1:1 v/v) was added to the HA solution. Then, acetic acid was added and the pH was adjusted at 4.5 and the mixture was stirred for 2 hours at 60 °C. Subsequently, sodium triacetoxyborohydride [$NaBH(OAc)_3$] (0.038 g, 0.180 mmol) dissolved in 2 mL of methanol and chloroform (1:1 v/v) was added dropwise. The reaction proceeded for 96 hours at 60 °C under magnetic stirring. After removal of the solvent under reduced pressure, the crude product was resuspended in a modest amount of water and purified by dialysis at 4°C against distilled water using a Spectra/Por regenerated cellulose membrane (Spectrum, Breda, The Netherlands) with a molecular cut-off of 3500 and then freeze-dried. Finally, to completely remove the unreacted phospholipid, extraction process with dichloromethane was performed; in this way, the unreacted DPPE was solubilised in the organic phase and thus separated from the conjugate. The aqueous phase was analyzed by TLC to monitor the disappearance of free DPPE from the conjugate and the extraction with dichloromethane was repeated until complete removal of the free phospholipid.

2.3 Preparation of liposomes

Liposome formulations were prepared using the conventional "Thin Film Hydration Method". e-PC, Tween® 80 and nifedipine, in the proportions shown in Table 1, were dissolved in a chloroform/methanol (2:1 v/v) mixture and put into a round flask. The organic solvent was evaporated under reduced pressure (80 mbar), at 40°C and 60 rpm for 1 hour using a rotatory evaporator (RII, Buchi, Italy). The lipid film was re-hydrated for 1 hour with 20mM HEPES buffer (pH 7.4) to reach a 3% w/v lipid suspension.

In the case of HA-decorated liposomes, the formulations were prepared according to the same procedure but the lipid film was hydrated with a solution of HA-DPPE conjugate (3 or 0.5 molar ratio) in HEPES buffer.

In all cases, after the re-hydration process, the formulations were extruded (Avanti® Mini-Extruder, Avanti Polar Lipids, Inc.) 5 times through 0.2 μm then 6 times through 0.1 μm polycarbonate membranes to obtain unilamellar vesicles.

The formulations were then purified to remove the non-incorporated materials from the liposomes, by molecular exclusion chromatography on Sepharose CL-4B columns, eluting with HEPES buffer.

Transethosomes were prepared according to the same method, but rehydrating the lipid film with a mixture of HEPES buffer/ethanol (60:40 % v/v). For each formulation containing the HA-DPPE conjugate, the corresponding liposome carrying the same amount of DPPE were prepared and tested as a control (Table 1) to assess the contribution of the addition of a lipid with a high transition temperature, T_m (around 46°C, as determined by DSC analysis), on the stiffness of the bilayer. It may

be plausible that, mainly at the highest molar ratio, the presence of lipid domains in the gel state during the experiment, would obstacle the movements of the lipid chains. As a consequence, the lipid structure would be stiffer and more fragile, meaning it is more likely to break under stress.

2.4 Liposome physiochemical characterisation

2.4.1 Particle size and ζ-potential

The particle size distribution and ζ-potential of prepared liposomes were assessed by dynamic light scattering (DLS) using a Zetasizer (Nano-ZS, Malvern Instrument, UK), after a 1 to 10 dilution of the samples with purified Milli-Q® water (refractive index:1.345; absorption: 0.010). Particle size measurements were carried out using a disposable cuvette and a detection angle of 173°. ζ-potential determination was performed on the diluted sample inserted in a capillary cell. Three measurements were taken for each sample and the results are expressed as the mean and standard deviation.

2.4.2 Lipid concentration

The concentration of the phospholipids in the vesicles was estimated by an adaptation of the Rouser method (Rouser et al., 1970). This method quantifies the content of phosphate cleaved from the phospholipids with perchloric acid and concentration is calculated based on a known concentration of standard (sodium dihydrogen phosphate). A control (empty tube), the formulation samples and the standard were heated in separate tubes to 100°C until they had completely evaporated. Three hundred microliters of perchloric acid (70%) was added to each tube, covered to prevent further evaporation and heated at 200°C for 20 minutes. During this step, phosphate is liberated from the phospholipids and standard. The samples were cooled and 1 mL of purified water and 400 µL ammonium molybdate (1.25% w/v) was added to each tube and vortexed. Four hundred microliters of ascorbic acid (5% w/v) was added and mixed and the tubes were heated at 100°C for another 5 minutes. On heating, the samples turn blue in the presence of phosphate and the absorbance at 820 nm was read. The concentration of lipid is calculated proportional to the absorbance of the 40 nmol/µL standard.

2.4.3 HA determination

The determination of the amount of HA was assessed using the carbazole assay (Bitter & Muir, 1962). A calibration curve was built in the range of concentration 10-80 µg/mL starting from a stock solution of raw HA in milli-Q® water and following dilutions (Figure S1). A 'blank', containing only HEPES buffer was used as a control. Moreover, since there is a possible interference of lipids in this test, in each assay the formulation having the same composition of the tested one (except that for the absence of HA) was analyzed in parallel. The samples were diluted 20 times up to 1 mL with purified water. Three milliliters of a 25 mM solution of sodium tetraboratedecahydrate in sulfuric acid 96% w/v was added to each tube, vortexed and placed in a water bath at 100°C for 10 minutes. The samples were then cooled under running water for 15 minutes, then 100 µL of a 0.125 % w/v carbazole solution in absolute ethanol was added, vortexed and heated once more to 100°C for 10 minutes. Once the samples had cooled in running water for another 15 minutes, the absorbance was read at 530 nm using an UV spectrophotometer. The absorbance reading of the blank and reference formulation was subtracted from the absorbance of each sample. Three different samples for each formulation batch were assayed each time to get the mean HA content.

2.4.4 Encapsulation efficiency

Drug encapsulation was determined by breaking the purified vesicles with methanol to release the incorporated nifedipine. The samples were filtered through 0.45-µm polycarbonate filters and analyzed by a HPLC system equipped with a diode array detection (HLPC HP 1100 Chemstations,

Agilent Technologies, Waldbronn, Germany). A lichrospher 100 RP-18E column (CPS Analitica, Milan, Italy) was used as the stationary phase. A mixture of acetonitrile:methanol:Milli-Q® water (25:25:50 v/v) 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. The retention time of nifedipine under these conditions was 4.1 min. The amount of nifedipine incorporated in the liposome bilayer was determined from a calibration curve of nifedipine in mobile phase (5 µg/mL – 100 µg/mL, Figure S2). Then the encapsulation efficiency was expressed as the concentration of µg of nifedipine per nmol of lipid. The encapsulation efficiency % was instead calculated from the percentage ratio of the drug concentration in the liposomes and the total concentration added during the liposome preparation.

2.5 Physical stability

The physical stability of the prepared liposomes in the storage conditions (4°C, under nitrogen atmosphere) was evaluated monitoring over time the particle size distribution, ζ-potential of the vesicles, along with the drug leakage. In the last case, an aliquot of each formulation was passed again through the Sepharose CL-4B column to remove the released drug. Then, the drug content was determined as described above and compared to the drug amount found in the vesicle bilayer the day of preparation of the liposomes.

2.6 DSC analyses

Thermal analyses on liposomal formulations were performed with a TOPEM modulated temperature DSC method using a DSC 1 Stare System (Mettler Toledo, Novate Milanese, Italy), equipped with an intracooler.

Samples of 10 mg of liposome suspension were sealed in a pin holed aluminum pan and subject to a heating cycle from -20 to 10°C at the heating rate of 1 $K \cdot min^{-1}$. The pulse height was 1 K and the period was 30 s. The DSC cell was purged with dry nitrogen at 80 mL/min.

To point out the main transitions of pure ePC in aqueous suspension and then the effect of the addition of the drug and HA to the vesicles, in first instance the thermotropic behavior of multilamellar phases of ePC obtained from thin films of pure lipid rehydrated in HEPES buffer was studied by TOPEM-DSC. Unilamellar vesicles of ePC in HEPES were analysed as well. Moreover, to minimize the interference of the buffer (because of the e-PC transition temperature, Tm, which is very close to freezing point of water), a same amount exactly weighted HEPES was inserted in the reference pan during the analysis. Nonetheless, modulated DSC allows to completely discriminate between irreversible events (i.e. water fusion and crystallization) and reversible events.

2.7 Deformability assay

The deformability of the liposomes was assessed by a dynamometer assisted extrusion assay previously developed (Franzè et al., 2017). Briefly, the test relies on forcing the formulation to pass through a 50 nm polycarbonate membrane (track-etch membrane, Nucleopore®), just over half the liposome size, monitoring the forces involved during such process. All liposome samples were diluted to the same lipid concentration (0.23 nM) and the weight and size were accurately recorded prior to extrusion.

The force (N) required to displace the plunger was measured and plotted as a function of plunger displacement (mm) and from the slope of such graph the constant of deformability (k, N/mm) was derived.

At the end of the test, the diameter (d_H) of the extruded vesicles was measured by DLS to calculate the percentage variation in vesicle size (Δd_H), according to the following equation:

$$\Delta d_H = \frac{(Rvi - Rve)}{(Rvi)} * 100 \qquad \text{Eq. (1)}$$

Where R_{vi} and R_{ve} is the diameter of the particles before and after extrusion respectively.

Moreover, the lipid concentration in the extruded suspension was measured again to calculate the lipid recovery as the percentage of lipid that passes through the 50 nm membrane with respect to the starting lipid concentration of the suspension:

$$\text{Lipid Recovery (\%)} = (\mu g \text{ lipid extruded})/(\text{initial } \mu g \text{ lipid}) \times 100 \qquad \text{Eq. (2)}$$

2.8 In vitro skin permeability studies

In vitro skin permeability studies were carried out using Franz diffusion cells method and human skin samples, which were obtained from healthy volunteers who underwent abdominoplasty and signed an informed consent. The excess fat was carefully removed and full-thickness skin was cut into squares, sealed in evacuated plastic bags and stored at −20 °C until their use. Epidermis sheets were 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) (Franzè et al., 2015).

Modified Franz diffusion cells having a diffusion area of 0.636 cm^2 and a receiver compartment volume of about 3 mL were used for the studies. The human 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)/polyethylene glycol 400 (PEG 400) (16%

w/v) mixture containing 100 µg/mL sodium azide as a preservative, and continuously stirred by a magnetic bar. The donor compartment was loaded with 300 µL of liposome suspension under non-occlusive conditions or a saturated solution of nifedipine in acetate buffer/PEG 400 mixture. 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 µL of receiver phase was withdrawn and replaced with an equal volume of fresh medium.

At the end of the experiment, the cells were dismounted 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 nifedipine retained in the tissue. The amount of nifedipine permeated and retained in the skin was quantified according to the analytical method reported in section 2.4.4 basing on a calibration curve built in the range 0.2-20 µg/mL (Figure S3).

2.9 Statistical Analysis

The comparison among the samples were performed by analysis of the variance followed by Tuckey test (OriginPro 2015, OriginLab, USA). The level of significance was taken as $p < 0.05$.

3. Results and discussion

3.1 Liposome physico-chemical characterization

The main physicochemical properties of all formulations are summarized in Table 2. All liposome suspensions were monodispersed, with a negative ζ potential and a particle size ranging between 120 and 150 nm, which is considered suitable for transdermal delivery.

The main differences among the formulations are mainly ascribable to the presence of HA. In fact, for HA decorated liposomes it was observed an increase of negativity in a dose dependent manner (given the greater change seen in F3 than F6, Table 2). This result was expected considering the polyanionic nature of HA polysaccharide and its molecular volume and it was considered as a first proof on the presence of HA on the surface of the vesicles.

The lipid concentration in the formulations was about 20 nmol/µL with the exception of the F3/F4 pair which presents a significant lower lipid amount (Table 2). This decrease in lipid content is explained by the stiffening of the bilayer caused both by DPPE and HA-DPPE conjugate at the highest molar ratio (as it will be described deeply in the following sections), which made difficult to extrude the liposome suspension through the 0.1 µm polycarbonate membrane. It was supposed that part of the formulation, and therefore of lipids, was lost during the downsizing process. The presence of

DPPE and HA-DPPE conjugate in the bilayer had also an impact on the encapsulation efficiency of nifedipine. Indeed, the presence of the additional phospholipid (DPPE) or of the HA-DPPE conjugate reduced the nifedipine encapsulation efficiency (EE%) independently of their strength (Table 2). This effect may be due to a different packing of the lipids in the bilayer following the addition of DPPE that leads to a reduction of the capability of the hydrophobic drug to be incorporated in the bilayer. It should be also underlined the effect of HA at the highest concentration on the drug content expressed as a function of lipid content (F3 and F7; Table 2).

Nonetheless, the encapsulation of the drug in liposomes led to an increase of the apparent solubility of nifedipine in a range of 70-128 times compared to the solubility in pure HEPES buffer (7.38 ± 0.08 µg/mL) and of 10-18 times compared to the solubility in HEPES/PEG 400 mixture used as receiver medium in *in vitro* skin permeation study (55.40 ±8.86 µg/mL).

Finally, all liposome formulations did not show any appreciable changes of particles size and ζ potential in the storage conditions over one month period, excluding any phenomenon of aggregation and precipitation of the vesicles (data not shown). However, the drug leakage was important in all formulations tested. This is one of the major drawbacks of the encapsulation of hydrophobic drugs in highly fluid lipid bilayers. It should be mentioned that the presence of the DPPE led to a better retention of the drug inside the liposomes. In fact, after two months of storage the loss of drug was 72% in the case of the formulation F2 and 40% for the formulation F5. No significant modification occurred at prolonged period of times. On the contrary, the presence of HA-DPPE conjugate did not further reduce the drug leakage independently of its concentration. As an example, the nifedipine leakage after 8 months of storage resulted slightly higher than 40% of the initial content. Thus, conversely to our predictions, HA chains did not permit a significant steric stabilization of the systems and the drug leakage of a lipophilic compound such as nifedipine from flexible vesicle remains an open concern.

The flexibility is a requirement for the liposomes to be applied on the skin (Morilla & Romero, 2016), therefore the effect of all additives on bilayer elasticity was studied by the extrusion assay measuring the constant of deformability, the loss of lipids and the variation of vesicle size after extrusion.

As expected, the F(-) exhibited the typical pattern of a flexible liposome being the K value almost negligible (Franzé et al. 2017). Moreover, the concentration of phospholipids before and after extrusion did not change significantly ($p=0.22$). The presence of nifedipine in the liposome bilayer instead led to a slight increase of the constant of deformability, K (Table 3). Anyway, formulation F2 still presents a suitable bilayer deformability. Following the addition of the DPPE at 3% molar ratio in the liposomes composition (F4, Table 3), the K value increased of one order of magnitude with respect to empty liposomes. A further stiffness of the bilayer was observed after the addition of HA

on the surface, with the mean K value which was almost the double of that obtained in similar experimental conditions with conventional liposomes prepared using DOTAP as main lipid (Franzé et al. 2017). It may be deduced that the presence of a large molecule on the liposome surface interferes with the vesicle bending, preventing deformation and subsequently rupturing the vesicles during extrusion. This hypothesis seems to be reinforced by the lipid recovery data which resulted significantly different from formulation F(-) only in the cases of F3 and F4 (Tuckey test, $p<0.05$). This reduction to a half of the total lipid amount loaded in the syringe suggested a rupture of the vesicles and an occlusion of the pores by free lipids, with following increase of the forces involved in the pore penetration. Coherently, F3 and F4 showed also the highest variation of the particle size after extrusion (Table 3). This behavior is concentration dependent since when the concentration of the HA- DPPE conjugate is reduced up to 0.5% molar ratio the stiffness of the bilayer significantly decreases with respect to F3 and F4 and becomes close to the values registered for F2 (F6, Table 3). Regardless, surface decoration with HA does appear to impact deformability which is essential for penetration into the skin, and furthermore, the promotion of drug delivery deeper into the tissue. Looking for a compromise between bilayer flexibility and the need to have a large amount of HA on the liposome surface to have an effective localizing effect after skin application, it was thought to maintain the molar ratio of the conjugate at 3% molar and to counterbalance the stiffening effect of HA moiety by adding a quite high ethanol content, preparing those that are more properly called "transethosomes". Indeed, it is known that the ethanol, interacting with the polar heads of phospholipids, exerts a fluidizing effect on the lipid membranes. Moreover, thanks to the same mechanism of action, it may act as skin penetration enhancer, creating some gaps in the stratum corneum region through which the fluid vesicles may find access to the deeper skin layers (Song et al., 2012). As can be deduced from Table 3, this strategy led to the desired result, since the K value obtained during the extrusion of F7 moved towards that of F2, allowing to have suitable deformable carriers carrying a higher amount of polysaccharide on the surface. Moreover, the fluidizing effect exerted by ethanol on liposome bilayer counteracted also the contribution of DPPE to the binding rigidity of the vesicles, as demonstrated by the lower K value of F9 compared to that obtained with F4 formulation carrying the same amount of the high T_m phospholipid (Table 3).

Since the flexibility of the vesicles is related to the rigidity of the bilayer, which is in turn determined by the thermotropic pattern of the constituent lipids, the thermic behavior of the vesicle suspension was analyzed by DSC. In particular the effect of the addition of the surfactant, DPPE and HA- DPPE conjugate at the lowest concentration were analyzed focusing the attention on the lipid transition on the reversible curve retrieved by the DSC data.

As shown in Figure 1, the reversible thermogram of pure e-PC unilamellar vesicles (LUV) shows two main events, namely the main phase transition, T_m, which occurs at -0.85°C and a lower transition at about -4°C, that may coincide with pre-transition (T_p) from the gel to a ripple phase (Biltonen & Lichtenberg, 1993). In case of pure C16:0 phospholipids, such as DPPC, this event is observable generally at 5-10 °C below the T_m. However, egg lecithin is a mixture of saturated and unsaturated lipid chains having different chain lengths with the prevalence of C18 lipid chains. It is reported that as the lipid chain length increases, the T_p value progressively moves toward the T_m and, in our case, it appeared almost as a shoulder of the main transition peak (El Maghraby et al., 2004).

The addition of the Tween 80® [F(-)] causes a destabilization of the bilayer, by reducing its packing order and increasing the fluidity. Indeed, the surfactant determined a significant reduction of the enthalpy associated to the T_m [LUV: $\Delta H \approx 130$ μJ/nmol lipids; F(-):$\Delta H \approx 2$-3 μJ/nmol lipids] along with the presence of shoulder at about +2°C, suggesting the formation of two different lipid domains induced by the addition of the surfactant. The T_p is not completely abolished at such surfactant concentration, but it moves towards lower temperature (~ -3°C), accordingly to data already reported in literature for DPPC (El Maghraby et al., 2004). All these modifications resulted consistent with the significant improvement of flexibility of the vesicles.

The encapsulation of nifedipine in the bilayer increased the enthalpy of T_m peak (F2: $\Delta H \approx 9$ μJ/nmol lipids) and abolished the T_p by reducing the cooperativity of the transition. Moreover, the shoulder at about +2°C was not still evident and this may be a confirmation of the changed packing of the lipid membrane. The addition of the DPPE at the 0.5% mol/mol did not significantly modify the pattern of the thermogram and the T_m intensity. The addition of HA- DPPE conjugate gave the most significant modification in the series of "flexible" liposomes. In this case, the T_p was no more detectable and the main T_m peak resulted sharper, confirming the increased order of the lipid domains. Finally, also the T_m enthalpy (F5: $\Delta H \approx 70$ μJ/nmol lipids) increased of about 30 fold with respect to F(-). All these findings agree with the flexibility scale established by the measurements of K values. Indeed, F(-) was the most flexible formulations, the data generated by F2 and F5 overlapped while the K value of F6 was found 3 fold higher than that of F5.

3.2 In vitro skin permeability studies

The permeability properties of the liposomal formulations were tested on *in vitro* human skin against the saturated solution of nifedipine, used as control. After application of the drug solution, the permeated and retained amount of nifedipine was negligible for each time sampling. The encapsulation of nifedipine in flexible liposomes allowed a slight permeation of the drug, but only after 24 hours experiment, therefore it was not possible to calculate the flux. These results are different

to those obtained by Yasam et al. (2016) using niosomes. This discrepancy can be justified by the nature of the membrane, being the human skin highly less permeable than the rat skin used in the mentioned studies.

As expected, when F3 and F4 were tested at the end of the experiment yellow agglomerates were found on the skin surface, supporting again the hypothesis that only fluid or elastic vesicles are able to cross the tight stratum corneum barrier. A significant increase of the permeation parameters was obtained with the two transethosomal formulations, F7 and F8. It is recognized that transethosomes have superior permeation characteristics of both ethosomes and flexible liposomes owing to the synergistic effect of ethanol, surfactant and vesicles themselves (Song et al., 2012; Ascenso et al., 2010).

Both F7 and F8 formulations led to a decrease of the lag time compared to the flexible liposomes F2, since nifedipine permeation was observed after 5 hours application of liposomes. Overall, the formulation F8 carried an almost double amount of nifedipine throughout the skin after 24 hours experiment (Q_{24}) with respect to the HA-decorated vesicles F7 (2.17± 0.22 vs 1.27 ± 0.69 μg/cm^2), although the flux of the drug was the same after application of F7 and F8 (30 ± 10 and 40 ± 30 ng/cm^2/h, respectively). Nevertheless, only the formulation F7 allowed the formation of the drug depot desired for the sustained release of nifedipine, since the retention of the drug in the skin after application of F8 was completely negligible (Figure 2). This result confirms that HA is a suitable localizing agent of nifedipine into the skin. In first instance, this effect may be ascribable to the well-known hydration properties of HA, that may act simply widening the hydrophilic pores of the stratum corneum. To test this hypothesis, a transethosomal formulation with 3% molar DPPE, namely F9, was prepared (Table 1) and the same amount of HA found to be present in F7 liposomes (as determined by the carbazole assay) was added in the liposome suspension. The effect of the co-administration of HA and lipid vesicles on the permeability properties of nifedipine was assessed *in vitro*. However, following the co-administration of HA and liposomes, any permeation/retention of nifedipine was observed, suggesting that HA limits the penetration of the transethosomes into the skin and it is effective in promoting the drug absorption only when covalently linked to the phospholipid. This behavior may be explained considering the penetration pathways of HA. In a previous work, we found that HA presents high affinity for keratinocytes with a homogeneous distribution of the polysaccharide in the stratum corneum within 2 hours after application (Cilurzo et al. 2014). Therefore, when HA is added to vesicle suspensions in co-administration, it will tend to penetrate in the superficial layers of the skin first, filling the holes for the liposomes passage. Moreover, the moisturizing effect of free HA once applied on the skin surface may actually reduce

the penetration of the lipid vesicles because of the increase of the water content of the stratum corneum.

4. Conclusions

This work provided a proof of concept of the potentiality of using HA-decorated liposomes to design drug delivery systems suitable for enhancing the cutaneous administration of drugs.

The presence of the polysaccharide on the vesicle surface resulted to be essential for an efficient penetration of the liposomes into the skin, which was found to be dependent on HA concentration. However, the amount of the HA moiety on the vesicle surface significantly affected the flexibility of the vesicles by increasing the packing of the lipid chains and then limiting the structural rearrangement of the vesicles. The overall data obtained in this study further confirm the key role exerted by vesicle fluidity on skin penetration and the need to find a counterbalance between a targeting moiety and the stiffening of the bilayer. It should be underlined that the system proposed in this work, improving the skin penetration either with respect to the nifedipine saturated solution and the undecorated vesicles, presents similar effects to those already described for skin penetrating peptides grafted on the surface of liposomes. Finally, the targeting agent here proposed is a natural polysaccharide and a physiologic component of the extracellular matrix and the skin, therefore it assures a high level of safety and biocompatibility.

Table 1- Quali-quantitative composition (%w/w) of liposomes

	NIFEDIPINE	e-PC	HA-DPPE	DPPE	TWEEN 80	HEPES	HEPES/EtOH
F(-)	-	85	-	-	15	+	-
F2	2*	85	-	-	15	+	-
F3	2*	69.6	15.4 [a]	-	15	+	-
F4	2*	69.6	-	15.4[a]	15	+	-
F5	2*	84.62	-	0.38[b]	15	+	-
F6	2*	84.62	0.38[b]	-	15	+	-
F7	2*	69.6	15.4 [a]	-	15	-	+
F8	2*	85	-	-	15	-	+
F9	2*	69.6	-	15.4 [a]	15	-	+

**mg/mL theoretical value calculated on the final suspension; corresponding to a molar ratio of: [a] 3%; [b] 0.5% with respect to e-PC*

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Table 2-Main physicochemical properties of liposomal formulations. Each parameter results from the analysis of three different formulations and all values are expressed as mean ± standard deviation (N=3).

Form	**diameter**	**PdI**	**ζ**	**lipids**	**EE**		**HA amount**	
	(nm)		**(mV)**	**(nmol/µL)**	(µg/nmol lipid)	(%)	(mg/mL)	(mg/nmol lipid)
F(-)	122.3 ± 0.5	0.07 ±0.01	- 4.0 ± 0.4	28.2 ± 3.5	-	-	-	-
F2	113.3 ± 6.5	0.12 ± 0.20	- 6.5 ± 1.8	21.9 ± 3.9	43.8±14.7	47.8 ±12.0	-	-
F3	123.2 ± 4.6	0.10± 0.03	-18.2± 3.7	13.7 ± 1.8	51.5 ± 0.5	28.5 ± 5.0	0.8±0.1	54.7±7.1
F4	121.3 ± 1.4	0.15± 0.02	-8.4± 0.7	15.1 ± 0.1	41.6 ± 1.4	31.4 ± 1.0	-	-
F5	120.7 ± 2.6	0.07 ± 0.01	-3.6±0.6	23.6 ± 3.2	27.2± 1.0	32.0 ± 1.2	-	-
F6	118.1± 1.3	0.10 ± 0.02	-9.2 ± 2.6	20.7 ± 2.5	29.6 ± 2.7	25.1 ± 9.9	0.4±0.1	20.1±6.0
F7	150.3 ±0.8	0.11 ± 0.01	- 17,7±0.7	20.8 ± 2.2	53.1 ± 10.0	28.7± 6.7	1.4±0.3	64.7±17.3
F8	134.4 ±0.2	0.10 ± 0.01	- 3.8 ± 0.2	27.2 ± 3.6	20.0 ± 0.9	27.2 ± 1.1	-	-
F9	116.0 ±1.3	0.06 ± 0.00	- 3.4 ± 0.4	21.0 ± 1.2	36.5 ± 3.8	38.3 ± 3.9	-	-

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Table 3- Mechanical properties of liposomal formulations as measured by the dynanometer-assisted extrusion assay. The data are expressed as mean ± standard deviation (N= 3)

	K (N/mm)	Lipid recovery (%)	Δd_H
F(-)	0.02 ± 0.00	85.7 ± 16.70	14.76 ± 3.87
F2	0.06 ± 0.02	80.9 ± 7.50	13.18 ± 2.80
F3	0.46 ± 0.09	49.0 ± 6.50	17.21 ± 3.00
F4	0.18 ± 0.05	50.9 ± 2.00	31.8 ± 4.50
F5	0.03 ± 0.01	65.8 ± 16.50	10.94 ± 2.58
F6	0.10 ± 0.01	80.4 ± 30.60	11.59 ± 2.99
F7	0.08 ± 0.04	67.3 ± 5.00	16.07 ± 4.29
F8	0.03 ± 0.01	60.3 ± 0.22	11.39 ± 3.05
F9	0.02 ± 0.01	70.9 ± 11.24	9.21 ± 0.40

K: constant of deformability (N/mm); Δd_H: percentage variation of particle size after extrusion

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Figure captions:

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Figure 1- Reversing thermograms of flexible liposomes: phase transition temperature (T_m) region of ePC.

Figure 2- Amount of nifedipine permeated (Q_{24}) and retained (R_{24}) into the skin after 24 hours of
490 application of the most fluid formulations against the saturated solution of the free drug (* and #: significant different from the saturated solution, $p < 0.05$).

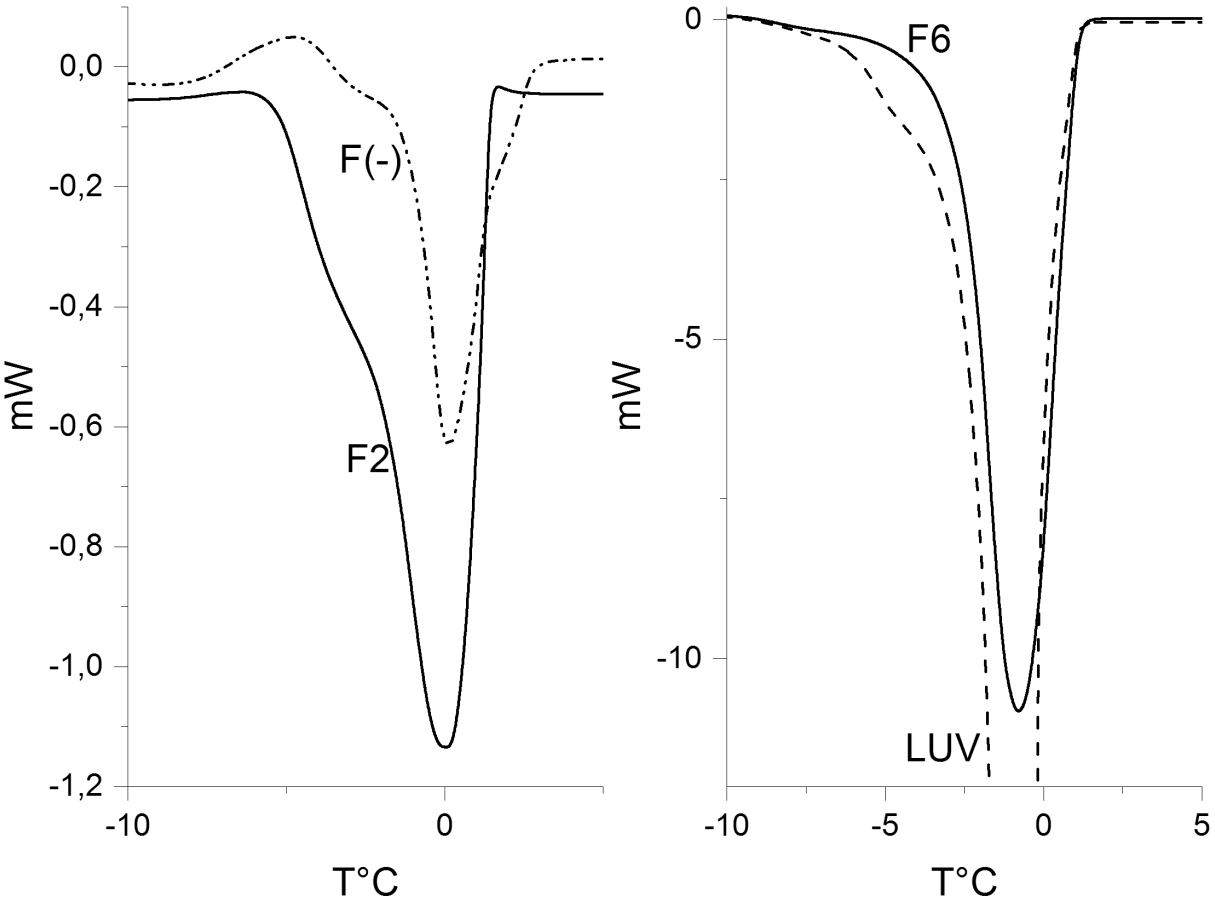

Figure 1

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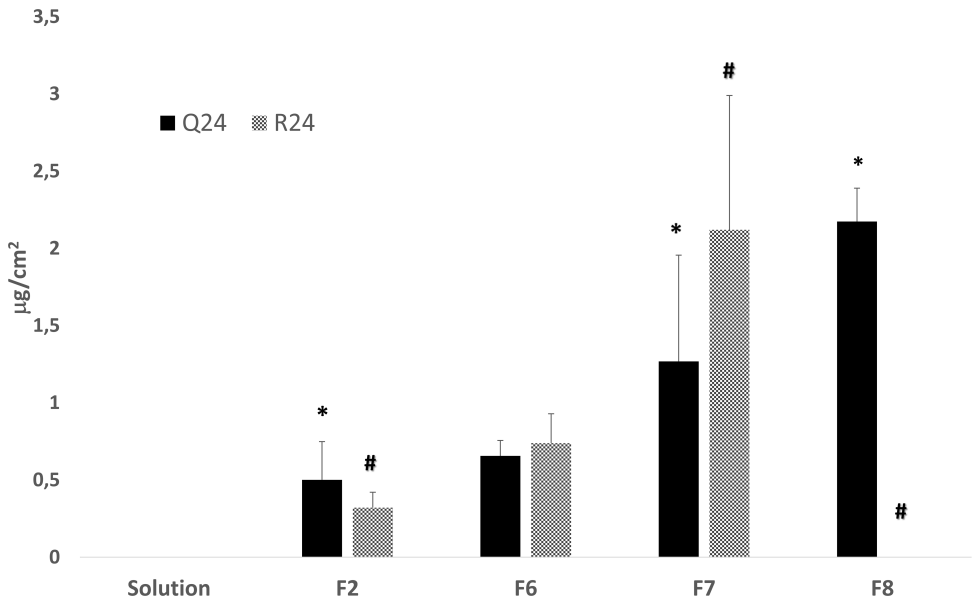

Figure 2