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Article

Micelles-in-Liposome Systems Obtained by Proliposomal Approach for Cannabidiol Delivery: Structural Features and Skin Penetration

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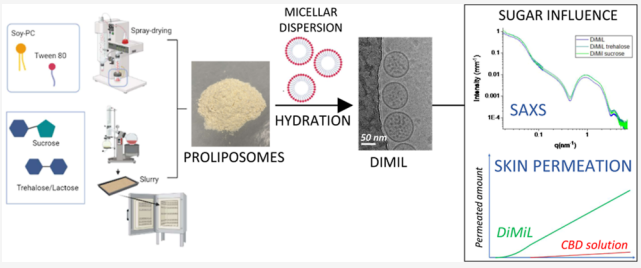
ABSTRACT: Deformable liposomes represent valuable drug carriers for cutaneous administration. Nevertheless, the fluid lipid membrane can favor the drug leakage during storage. Proliposomes may represent a suitable strategy to solve this issue. As an alternative, a novel carrier, which encloses hydrophobic drugs in the inner core of vesicles, namely, a drug-in-micelles-in-liposome system (DiMiL), has been proposed. In this work, we investigated the possible advantages of combining these two approaches to obtain a formulation able to enhance the skin penetration of cannabidiol (CBD). Proliposomes were prepared by spray-drying or slurry method testing lactose, sucrose, and trehalose as carriers at different sugar/lipid weight ratios. The ratio between soy-phosphatidylcholine (main lipid) and Tween 80 was instead fixed at 85:15 w/w. DiMiL systems were extemporaneously obtained by the hydration of proliposomes with a Kolliphor HS 15 micellar dispersion (containing CBD, when appropriate). Based on the technological properties, sucrose and trehalose at 2:1 sugar/lipid ratio resulted in the best carriers for spray-dried and "slurried" proliposomes, respectively. Cryo-EM images clearly showed the presence of micelles in the aqueous core of lipid vesicles and the presence of sugars did not alter the structural organization of DiMiL systems, as demonstrated by SAXS analyses. All formulations were highly deformable and able to control CBD release regardless of the presence of sugar. The permeation through human epidermis of CBD carried by DiMiL systems was significantly improved if compared to that obtained loading the drug in conventional deformable liposomes with the same lipid composition or in an oil solution. Furthermore, the presence of trehalose led to a further slight increase of the flux. Altogether, these results demonstrated that proliposomes may be a valuable intermediate for the preparation of deformable liposome-based cutaneous dosage forms, improving the stability without compromising the overall performances.



KEYWORDS: *proliposomes, deformable liposomes, cannabidiol, trehalose, SAXS, skin penetration*

1. INTRODUCTION

The skin is an excellent barrier evolved to protect the body from the external environment. In particular, the outermost layer of the skin, the stratum corneum, is responsible for this function thanks to its complex and particular organization, being composed of several layers of overlapped dead cells, namely, the corneocytes, embedded in a lipid matrix. This so called "brick and mortars" structure prevents the passive diffusion of xenobiotics, including most of the active ingredients applied on the skin. This is the reason why still today (trans)dermal administration is limited to very few compounds with unique physico-chemical properties. These are mainly low molecular weight (<500 Da), oil/water partition coefficient ranging from 1 to 3, a certain solubility both in oil and in water and, when a systemic effect is desired, a low daily dose.[1]

Several strategies have been developed to breach the skin barrier and one of the most studied is the use of nanotechnology and in particular, deformable liposomes. These are lipid vesicles that seem to be able to undergo a reversible deformation under stress to pass through the nanosized pores (20−40 nm) physiologically present among corneocytes, thus delivering the cargoes in the deeper layers of the skin.[2,3] This behavior is ascribed to the composition of these vesicles that are appositely designed to have a very fluid membrane. For this reason, deformable liposomes are generally composed of lipids with a very low transition temperature (lower than 25 °C), they do not contain stabilizing agents such as cholesterol, but instead contain some softening agents, namely, compounds that, sitting in the bilayer or coming into contact with it, decrease the Young modulus of the vesicles.

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Examples of softening agents are single chain surfactants and ethanol.[4] Deformable liposomes have been demonstrated to improve the skin penetration of several compounds regardless of the physico-chemical characteristics.[5,6] Nevertheless, the fluid membrane of deformable liposomes may be responsible for instability phenomena decreasing the shelf-life of the product and then the possibility to reach the market. The main stability issue relies on the easier diffusion of loaded drugs through the leaky membranes that, depending on the strength of the affinity between drug and lipids, can result in a great extent of drug leakage.[7]

Proliposomes are provesicular products that can be successfully exploited to overcome these instability issues. The production of proliposomes involves the coating of phospholipids on a soluble carrier with high surface area and porosity (e.g., sugars) to form a dry powder that effectively forms liposomes upon contact with water.[8] Although the proliposome approach has been proposed several years ago, less than 250 papers dealing with the design of proliposomes can be found in literature and many of them are related to the development of dry powders for inhalation or oral solid dosage forms. Very few data are available on the design of proliposomes to be applied on the skin, either in the form of powders (to be converted into liposomes directly after contact with the skin moisture)[9] or after loading in semisolid preparations, such as gels and creams.[10,13] Moreover, none of the previous works reported the use of proliposomes as precursors of deformable liposomes and, therefore, a systematic study on the impact of the production method and selected excipients on the properties of these final vesicles (e.g., deformability) is missing. This lack of information limited the possibility to identify a consistent formulation space and the critical quality attributes required for the design and development of an optimal formulation.

An alternative approach to limit the drug leakage while maintaining deformable liposomes in aqueous dispersion is the use of DiMiL, a novel carrier recently proposed, which is basically a dual carrier in which hydrophobic drugs are encapsulated in the aqueous core of the liposomes through micellar solubilization. With this expedient the concentration gradient of the free drug is decreased, limiting the drug leakage phenomenon. Along with the better stability, the DiMiL system was demonstrated to have greater efficiency in enhancing the permeation of poorly permeable compounds through human skin with respect to "conventional" deformable liposomes with a similar lipid composition.[14]

To facilitate the industrial production and further improve the stability over time of deformable liposomes, in this work. the feasibility to prepare DiMiL systems by proliposome approach was investigated, studying the effect of the manufacturing process and excipients choice on the technological (i.e., vesicle deformability) and biopharmaceutical properties (i.e., drug release and drug skin permeation) of DiMiL. The attention was focused mainly on the role of sugar carriers on the structure and skin permeation performances of DiMiL systems, also considering the fact that sugars, such as trehalose, might have a skin hydration effect that can in turn change the overall skin penetration behavior of the system.[15]

Proliposomes were prepared by both spray-drying and slurry method,[16,17] testing three different carriers, namely, lactose, sucrose, and trehalose, at different weight ratios with respect to phospholipid, namely, soy-phosphatidylcholine (s-PC). DiMiL systems obtained following the hydration with the micellar dispersion of the prepared proliposomal powders (*p*-DiMiL) were then characterized for their main physico-chemical properties (i.e., particle size distribution and deformability), in comparison with analogous formulations prepared by conventional methods (i.e., thin lipid film hydration method) and, therefore, differing only for the presence of the carrier. Details on the structure and on the internal arrangement of components were obtained by cryo-EM and small-angle X-ray scattering (SAXS). The latter technique can provide important and novel information on the DiMiL ultrastructure, meanwhile clarifying the role of micelle concentration and sugars on the particular micelles-in-liposome organization. The skin penetration performances were assessed using cannabidiol (CBD) selected as the growing interest in inflammation-based skin diseases.[18,19]

2. MATERIALS AND METHODS

2.1. Materials. Soy-phosphatidylcholine (s-PC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were kindly provided by Lipoid (Steinhausen, Switzerland); Kolliphor HS 15 (critical micellar concentration in the 0.005−0.02% range) was provided by BASF (Cesano Maderno, Italy); Tween 80 (T80) was purchased from Croda Chocques (France); vaseline oil, trehalose dihydrate, and ascorbic acid were obtained from VWR International (I); and ammonium molybdate, sodium dihydrogen phosphate, lactose, sucrose, and analytical-grade organic solvents were obtained from Merck Life Science (Milan, Italy). Cannabidiol (CBD) was kindly gifted by Indena (Italy).

2.2. Preparation of Proliposomes. Proliposomes were composed of s-PC and T80 in weight ratio of 85:15; trehalose, sucrose, or lactose were added in sugar/lipid weight ratio ranging from 1:1 to 1:3. Powder formulations were obtained by the following methods:

Spray-drying (SD)—the main formulative and process variables were studied to obtain powders from feeds containing at least 10% w/w solid, having a water content lower than 5%, a yield higher than 80% and in which the main chemical status of lipids was preserved. The feeds were prepared by suspending the sugar in the lipid organic solutions or dissolving the sugar and the lipid components in water/organic solvent mixtures. In particular, different solvents miscible with water (i.e., ethanol and methanol) as well as different concentrations of the components were screened.

Feeds were spray-dried by a 4M8 spray-drier (Procept, Netherlands) equipped with a 0.6 mm nozzle with an inlet temperature of 150 °C (measured chamber temperature: 57° ± 1 °C) and a feed flux of 10 mL/min.

Slurry method (SL)—initially, sugars were micronized to increase the surface area using an ultra-centrifugal mill (Ultra Centrifugal Mill ZM 200, Retsch, Germany) equipped with a 120 μm mesh size sieve. Samples were mixed with dry ice in the mill hopper and rotor speed was fixed at 6000 rpm.

For the preparation of proliposomes, s-PC and Tween 80 were dissolved in ethyl acetate in a 100 mL round-bottom flask. After adding the micronized carrier to reach a final solid content of 12% w/v, the solvent was mostly evaporated at 40 °C under reduced pressure by using a rotary evaporator HB10 (IKA, Germany), and then, the slurry was completely dried at 70 °C overnight. The resulting dry powder was collected and the granulometry was uniformed by sieving the powder through a 355 μm sieve.

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Table 1. Composition and Main Characteristics of DiMiL Systems[a]

form. code	micelle content (%, w/v)	sPC/sugar ratio	sugar	D (nm)	PdI	K (mN/mm)	EE %
S1	10	1/1	sucrose	118	0.4	10 ± 2	
S1.5	10	1/1.5	sucrose	109	0.3	10 ± 3	
S2	10	1/2	sucrose	108	0.3	10 ± 2	
S3	10	1/3	sucrose	117	0.3	13 ± 3	
S2-C	10	1/2	sucrose	95	0.3	1 ± 0	49.4 ± 4.8
T2	10	1/2	trehalose	112	0.3	11 ± 4	
T3	10	1/3	trehalose	129	0.3	9 ± 2	
T2-C	10	1/2	trehalose	129	0.3	4 ± 3	45.5 ± 2.4
FH0				117	0.1	8 ± 1	
FH4	4			122	0.1	2 ± 1	
FH10	10			125	0.1	7 ± 3	
FH0-C				106	0.1	3 ± 0	52.8 ± 3.0
FH4-C	4			138	0.2	5 ± 1	35.8 ± 1.2
FH10-C	10			140	0.2	2 ± 0	45.8 ± 1.3

[a]FH: liposomes prepared by the thin lipid film hydration method; S: sucrose (spray-drying method); T: trehalose (slurry method); and C/CBD. The standard deviation on D is lower than 1 nm, on the PDI is lower than 0.05.

2.3. Preparation of DiMiL Formulations. Blank DiMiL formulation was obtained by hydrating proliposomal powders with a 4 or 10% w/v Kolliphor micellar dispersion for 1 h at 40 °C using an overhead paddle stirrer at 350 rpm (RW-20, DZM Jankel & Kunkel, Germany). In case of drug-loaded formulations, CBD at the concentration of 3.5 mg/mL was preliminarily loaded in micelles and then CBD micellar dispersion was used as hydration medium. Particle size was uniformed by 5 min sonication (30 s on and 30 s off) using a probe sonicator UP200St (Hielscher, Germany). The formulation composition is summarized in Table 1.

In both cases, DiMiL were purified by un-entrapped micelles by size-exclusion chromatography using Sepharose-4CL columns and eluting with ultrapure water.

As the control, DiMiL systems were also prepared by the conventional thin lipid film hydration method (FH-formulations, Table 1).[14] The final concentration of bilayer components was set at 30 mg/mL for all formulations independently of the preparation method.

2.4. Characterization of Proliposomes. In the first instance, proliposomal powders were analyzed in terms of granulometry, water content, and flowability. The granulometry was assessed directly on the dry proliposomal powders using the laser diffraction particle size analyzer Mastersizer 3000 (Malvern, UK).

The moisture content (MC) was measured by a Volumetric Karl Fischer titration V20 (Mettler Toledo, Italy). Samples were prepared by dissolving 10 mg of proliposomes in 1 mL of anhydrous methanol. Afterward, samples of 500 μL of methanol were injected into the titration vessel. The water content was measured in triplicate.

Powder flowability was assessed by measuring the angle of repose and the compressibility index according to the European Pharmacopeia. To assess the angle of repose, about 5 g of powder was allowed to drain from a funnel of 12 mm with a height of 5 cm from the plane basis. The angle of repose (α) was calculated according to the following equation

$$\tan(\alpha) = \frac{\text{height}}{0.5 \times \text{base}}$$

To assess the compressibility index (I_c), about 100 g of powder was put in a 250 mL volumetric cylinder and tapped 100 times. I_c was calculated as follows

$$I_c = 100 \times \frac{V_0 - V_f}{V_0}$$

where V_0 is the unsettled apparent volume and V_f is the final tapped volume.

Finally, phospholipid oxidation in proliposomal powders was studied according to the method described by Goldbach et al.[20] Briefly, raw s-PC and proliposomes were dissolved in absolute ethanol to get the s-PC concentration of 1 mg/mL. The absorbance values measured at 215 and 233 nm (Lambda 25, PerkinElmer—USA) were used to calculate the oxidation index (R) as the ratio A_{233nm}/A_{215nm}.

2.5. Characterization of DiMiL System. *2.5.1. Physico-Chemical Characterization.* DiMiL systems obtained after rehydration of the proliposomal powders with Kolliphor dispersions were characterized in terms of particle size distribution and ζ-potential by dynamic light scattering (DLS) by using Nano-ZS Zetasizer (Malvern Instrument, UK). Particle size measurements were carried out by 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° for Zetasizer analyses. For ζ-potential determination, the samples were inserted in a capillary cell after 1:10 dilution. Three measurements were taken for each sample and the results were expressed as the mean and standard deviation.

Encapsulation efficiency (EE, %) was determined on purified lipid vesicles after breakage of the vesicles in methanol. The quantification of CBD was carried out by using an HPLC system equipped with a diode array detector (HLPC HP 1100 Chemstations, Agilent Technologies, Germany). CBD concentration was determined by using a C8 column (150 × 4.6 mm, 5 μm; PhenoSphere-NEXTTM, Phenomenex) with a mixture of pH = 3 phosphate buffer and acetonitrile in the ratio 75/25 v/v as mobile phase. The flux was fixed at 1.5 mL/min and wavelength at 215 nm. Analyses were performed at 25 °C and the injection volume was 20 μL.[11,12]

2.5.2. Determination of the Deformability Constant (k). The lipid concentration of the selected formulations was measured using an adaptation of the Rouser method, as described elsewhere[21] and formulations were diluted to a total lipid concentration of 0.23 mM. The deformability was studied by using a dynamometer-assisted extrusion assay, previously developed.[22] An aliquot of 1 mL liposome dispersion was

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loaded in a gas tight syringe which was inserted in an extruder casing and put in contact with a 50 N load cell of a dynamometer (INSTRON 5965, ITW Test and Measurement Italia Srl, Italy). The syringe plunger was moved at a constant speed of 1 mm/s, forcing the liposomal dispersion through a 50 nm polycarbonate membrane fixed in the extruder casing. The resistance opposed to the passage of liposomes through the membrane was registered by the load cell and the force values (mN) were plotted versus the plunger displacement (mm). The constant of deformability (k) was calculated as the slope of the linear part of the plot. The higher the k value, the lower the deformability of liposomes. The assay was carried out at least on three different samples for each formulation.

2.5.3. Structural Characterization. 2.5.3.1. Cryo-EM. Sample vitrification was carried out with a Mark IV Vitrobot (Thermo Fisher Scientific). An aliquot of 4 μL sample was applied to a Quantifoil R2/1 Cu 300-mesh grid previously glow-discharged at 30 mA for 30" in a GloQube (Quorum Technologies). Immediately after sample application, the grids were blotted in a chamber at 4 °C and 100% humidity and then plunge-frozen into liquid ethane.

Vitrified grids were transferred to a Talos Arctica (Thermo Fisher Scientific) operated at 200 kV and equipped with a Falcon 3 direct electron detector (Thermo Fisher Scientific). Micrographs were acquired at a nominal magnification of 73'000×, corresponding to a pixel size of 1.43 Å/pixel, in linear mode, with a defocus of −3.0 μm and with a total dose of 40 e−/Å2.

2.5.3.2. mall-Angle X-ray Scattering. Experiments were carried out at the ID02 beamline at ESRF (Grenoble, F) (DOI 10.15151/ESRF-ES-653835676). Liposome dispersions and reference solvents were put in 2 mm capillaries (ENKI, Italy) mounted on a horizontal sample holder to be irradiated by a monochromatic X-ray beam (λ = 0.995 nm). The scattered intensity was collected on a 2D detector at two different sample-to-detector distances (1 m and 10 m) to investigate a wide range of $q = 4\pi\sin(\theta/2)/\lambda$, where θ is the scattering angle. After angular regrouping and careful background subtraction, the intensity profiles $I(q)$ give information on the structural properties of the liposomes on different length scales, from the hundreds of nms (mesoscale) to the nms (local scale). For micellar systems, the profiles were reconstructed with the SasView application (version 4.2.0, 2019).

2.5.4. Differential Scanning Calorimetry. Thermal analyses were performed using a differential scanning calorimetry (DSC) 1 Stare System (Mettler Toledo, Novate Milanese, Italy), equipped with an intracooler. Samples of 40 μL of liposome dispersions were sealed in a pin holed aluminum pan and subject to a cooling cycle from 25 to 0 °C at a cooling rate of 1 K min^{-1} and after 5 min at 0 °C, samples were heated up to 60 °C at a heating rate of 2 K min^{-1}. The DSC cell was purged with dry nitrogen at 80 mL/min.

2.6. In Vitro Drug Release and Skin Permeability Studies. The in vitro drug release and skin permeability studies were carried out using the Franz diffusion cells with a receiving volume of about 3.0 mL and a surface area of 0.636 cm^2. The drug release of CBD from selected formulations was studied as detailed elsewhere[23] by using an artificial membrane of nitrate cellulose.

The skin permeability studies were carried out using human epidermis obtained from healthy volunteers undergoing abdominoplasty and prepared according to an internal procedure.[24] Briefly, the excess of fat was 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 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 for the experiment, the integrity of the skin was evaluated by measuring the electrical impedance using an Agilent 4263B LCR Meter (Microlease, Italy).[24] 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. Three hundred microliters of each formulation were loaded into the donor compartment. For CBD, vaseline oil solution was used as the control.

The receiver compartment was filled with a mixture of ultrapure water and ethanol (50:50 v/v) under magnetic stirring at 1500 rpm. 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, which was carried out in non-occlusive conditions. At fixed time intervals (i.e., 1, 3, 5, 7, and 24 h) 200 μL of receiver phase was withdrawn and replaced with an equal volume of fresh medium. The cumulative amount permeated through the skin per unit area (Q_i) was calculated from the drug concentration in the receiving medium and plotted as a function of time. The maximal flux (J_{max}) was determined as the slope of the linear portion of the plot Q_i versus time.

2.7. Statistical Analysis. The differences of the performance of the formulations in study were evaluated by analysis of the variance followed by Tukey post hoc analyses (OriginPro 2021b). The level of significance was taken as $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Characterization of Proliposomal Powders. In the attempt to prepare proliposomes by spray-drying, the use of co-solutions was preferred to the suspensions due to issues related to the clogging of the tubing and nozzle. In particular, the use of sucrose and a methanol/water solution in 3.4:1 ratio allowed the preparation of physically stable feeds with a total solid content of 12% w/v.

Regarding the ratio between s-PC and sucrose, the process yield increased with the sucrose content because the carrier limited the lipid adherence to the glass wall of the drying chamber. Indeed, the yield was improved from about 60% to about 90% for s-PC/sucrose ratio 1:1 and 1:3, respectively. The moisture content of the spray-dried powders ranged between 2 and 4%. Proliposomes had a diameter smaller than 10 μm (Figure 1) and, consequently, the powder did not satisfy the flowability test.

In the case of the slurry method, mixtures at the lowest s-PC/sugar ratio led to wax-like and unprocessable materials. Moreover, only trehalose provided suitable powders with a mean diameter of about 330 μm (Figure 1). Even if the moisture content was about 10% due to trehalose's hygroscopicity, the powders obtained showed an angle of repose of 36 ± 3° and a compressibility index, I_c = 23.5, showing an acceptable flowability according to European Pharmacopeia specifications. It should also be mentioned that the good flowability of proliposomes prepared by slurry method is desirable since powders should be poured in the mixing vessel through a hopper.

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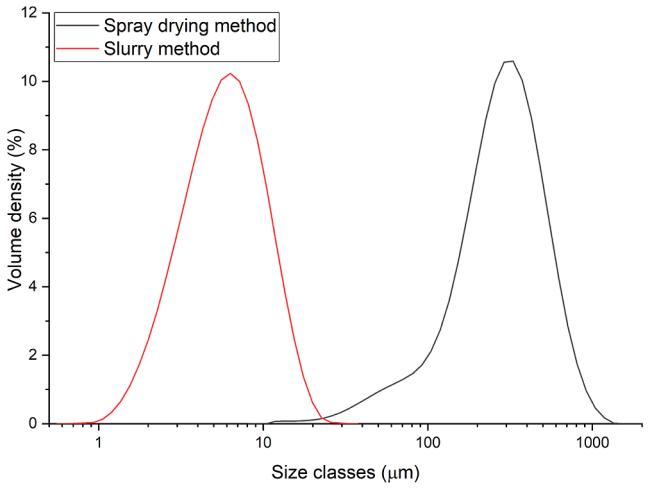

Figure 1. Granulometry of proliposomes at 1:2 sPC-sugar ratio obtained by spray-drying and slurry method. As an example, the distribution of the powders is reported in both cases.

Interestingly, both drying methods did not cause phospholipid oxidation since no significant variations of the oxidation index ($0.18 < R_{A233/A215} < 0.22$) were observed with respect to raw s-PC ($R_{A233/A215} = 0.19 \pm 0.01$).

Proliposomes with a lipid/sugar ratio of 1:3 were discarded because of the tackiness of the obtained powders that affect the handling of the intermediate product. Since the surface area of the powder and the type of sugar may be critical for the formation of unilamellar liposomes with a narrow polydispersity index, all proliposomes were used for the further studies independently of their physical features.

3.2. Physico-Chemical Characterization of DiMiL Systems. The hydration of proliposomes with micelle dispersion allowed to obtain a monodisperse population of liposomes with the desired size (Table 1).

The presence of the sugar in the aqueous core of liposomes, as a result of the hydration process (not removable by purification), affected neither the encapsulation efficiency of CBD nor the deformability of the DiMiL systems which were comparable to those obtained in liposomes prepared by conventional thin lipid film hydration method (Table 1). The deformability of DiMiL systems was high[16] and seemed to slightly decrease upon CBD loading. This variation could be attributed to a partial fusion of CBD carrying micelles into the external bilayer. In agreement with SAXS data detailed below, it might be supposed that a fraction of the loaded CBD enters in the bilayer and modifies the fluidity of the membrane. In fact, thermal behavior of a model membrane made of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) significantly changed in presence of CBD since after drug encapsulation the main transition temperature of DPPC shifted from 42.2 ± 0.1 to 37.3 ± 0.5 °C with a concomitant reduction of the transition enthalpy from 37.0 ± 0.5 to 16.3 ± 1.0 J/g. These results confirm the fluidizing effect exerted by CBD, already verified in a previous work.[23] The positive effect of CBD on the *k* value was not evidenced for the FH4 pair. This behavior might be justified considering the lower *k* value registered for the placebo formulation, FH4, and again in light of the results of the SAXS analyses that evidenced a greater presence of surfactant-based micelles in the bilayer for FH4 with respect to FH10 DiMiL systems, which can contribute to the overall fluidity of the membrane.

3.3. Structural Analysis of DiMiL Systems. Cryo-EM clearly evidenced the micelles-in-liposomes structure of DiMiL systems (Figure 2). Micelles were found in the aqueous core of both FH4 and FH10 liposomes. However, in the case of FH4, micelles were not uniformly distributed and empty liposomes coexist with liposomes encapsulating a variable number of micelles (Figure 2B). Increasing the concentration of micelles in FH10, micelles were uniformly present in the core of analyzed DiMil systems (Figure 2C). Moreover, the mean diameter of micelles was in agreement with DLS data (d: 12 ± 0 nm; PDI: 0.06 ± 0.00). Finally, no trace of micelles was revealed in the dispersing medium suggesting the stability of the formed system after preparation.

The structure of the liposomes at the nanoscale was deepened by SAXS experiments. The comparison among SAXS spectra of conventional deformable liposomes (FH0, without micelle in the inner core), FH4 and FH10 is reported in Figure S1. While the similarities in the overall features of the intensity profiles confirm the liposomal structure of FH4 and FH10, DiMil systems are clearly distinguishable from the conventional liposomes (FH0). The low and medium *q* behavior of the curves indicates a slightly smaller radius of the liposomes in the presence of micelles (from about 120 to 100 nm) and a different contrast profile of the bilayer. This latter

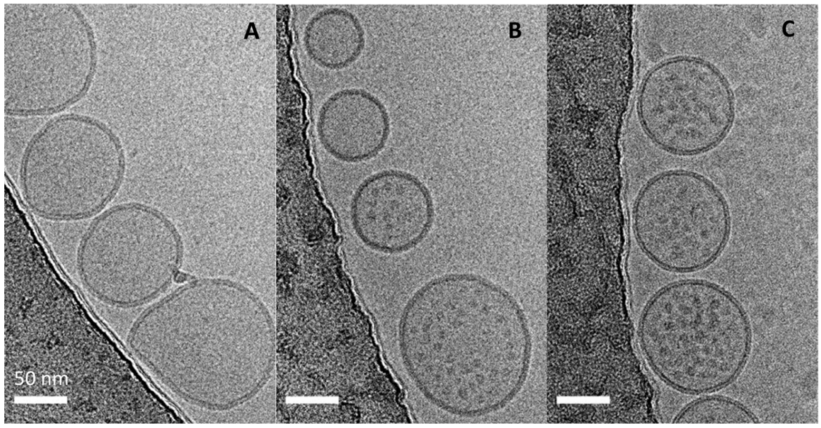

Figure 2. Cryo-EM of (a) conventional deformable liposomes, FH0; (b) DiMiL at 4% micelles concentration, FH4; and (c) DiMiL at 10% micelles concentration, FH10.

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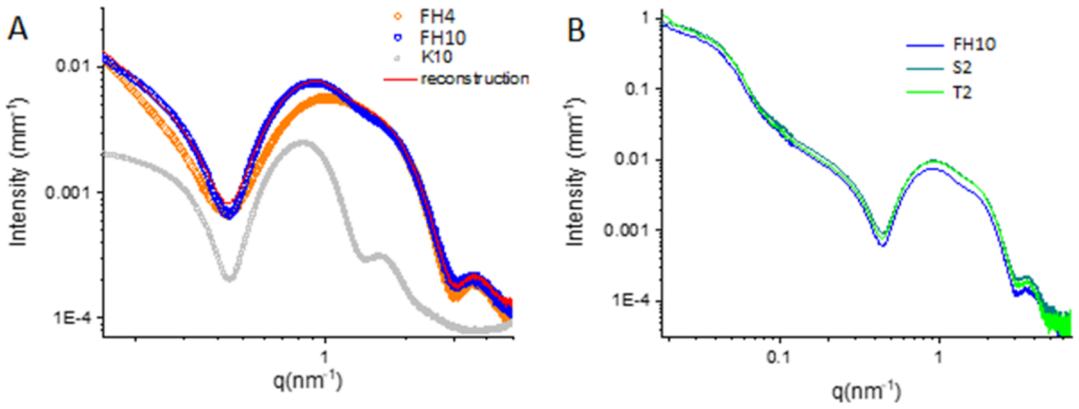

Figure 3. Effect of micelle and sugars on SAXS spectra of placebo formulations. Panel A: FH10 experimental spectrum (blue dots) and the reconstruction (red line) obtained by summing a proper fraction (0.15) of 10% Kolliphor micellar dispersion spectrum (K10, gray points) to the FH4 spectrum (orange diamonds). Panel B: spectra recorded on S2 (dark green) and T2 (light green) which evidence the lack of contribution of trehalose and sucrose, respectively.

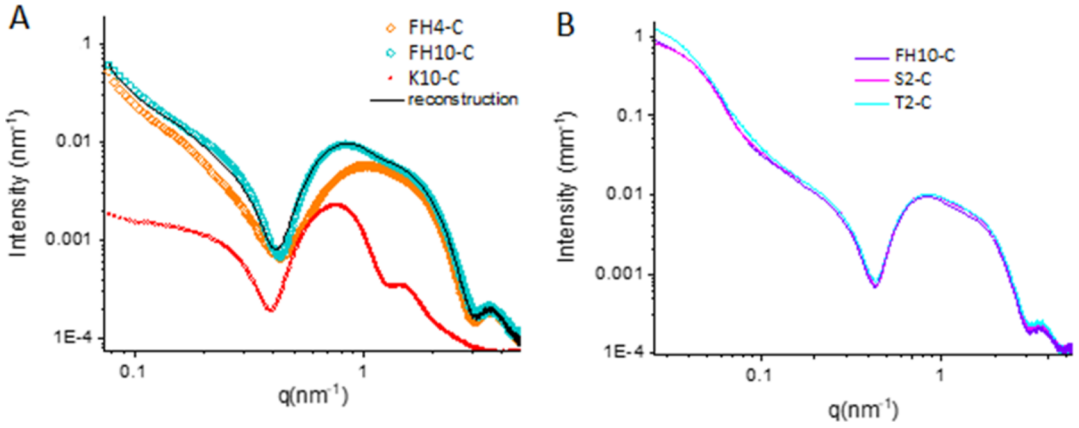

Figure 4. Effect of sugars on SAXS spectra of CBD-loaded formulations. Panel A: FH10-C experimental spectrum (light blue dots) and the reconstruction (black line) obtained by summing a proper fraction (0.17) of the CBD micellar dispersion spectrum (K10 CBD, red points) to the FH4-C spectrum (orange diamonds). Panel B: spectra recorded on T2-C (light blue) and S2-C (magenta) which confirm the evidence of the lack of contribution of trehalose and sucrose, respectively.

feature suggests a partial insertion of Kolliphor molecules in the bilayer of the liposomes.

The presence of micelles in the purified DiMiL was verified by SAXS spectra reconstruction.

The SAXS spectra of placebo formulations FH4 and FH10 are reported in Figure 3A along with the 10% w/v Kolliphor micellar dispersion (K10). The structural characterization of Kolliphor micelles at different concentrations is reported in Figure S2. As observed before, the intensity profiles of DiMiL systems were different from those of conventional liposomes (Figure S1), reinforcing the hypothesis of the partial insertion of some Kolliphor molecules in the bilayer, independently of the micelle concentration of the hydration medium. Moreover, an undulation is visible at $q \sim 0.85$ nm^{-1} in FH10 spectrum (Figure 3A, blue dots), being almost undetectable in the case of FH4. This intensity broad maximum is at the same q position as the one observed in the spectrum of Kolliphor micelles (Figure 3A, gray squares). An excellent reconstruction of the FH10 intensity profile was obtained by summing a proper fraction of the spectrum of 10% Kolliphor micellar dispersion (0.15) to the FH4 spectrum, as reported in Figure 3A, red line. The fraction of the micellar spectrum and the internal aqueous volume of liposomes (size 100−120 nm) are comparable, as calculated for a 30 mg/mL concentration of lipid components used in this work. This result indicates that micelles are homogeneously distributed in all liposomes. Notably, a good reconstruction cannot be obtained by summing an intensity contribution of Kolliphor micelles at lower concentration (2.5−4% w/v), whose spectra differ from that of Kolliphor at 10 and 4 w/v %, as discussed in the Supporting Information section (Figure S2). This result reveals that Kolliphor micelles in the core of FH10 are close to theoretical value. These data were in agreement with the cryo-EM results that showed a higher number of micelles in the core of FH10 with respect to FH4.

The encapsulation of CBD in the systems did not affect their key structural properties. Figure 4A reports the spectrum of CBD-loaded Kolliphor micelles and the spectra of CBD-loaded DiMiL systems (the comparison with unloaded systems, micelles, and placebo DiMil, are reported in Figures S3 and S4), The FH10-C reconstruction has been obtained by summing the intensity contribution of CBD-loaded micelles (0.17) to the FH4 spectrum, similarly to the unloaded system. Results show that DiMiL peculiar micelles in liposomes structure is preserved in the presence of CBD: the bilayer of the liposomes contains Kolliphor and possibly CBD, while the core contains stable micelles, which in the case of FH10-C are uniformly distributed at a concentration of about 10%.

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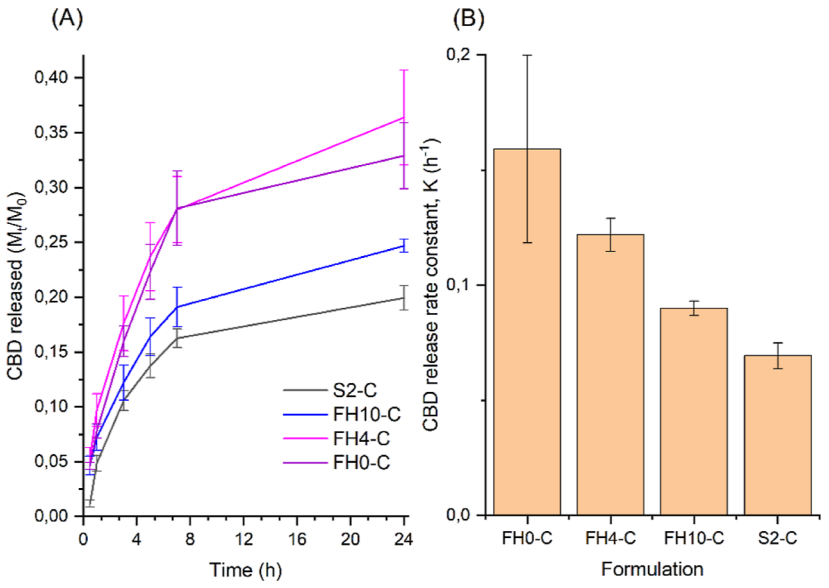

Figure 5. In vitro CBD release profiles of DiMiLs at different Kolliphor concentrations (hydrated without micelles, FH0-C; 4% w/v, FH4-C; 10% w/v FH10-C) against deformable liposomes prepared by hydration of proliposomes made of sucrose (S2-C). Panel A: the CBD release profiles; Panel B: release rate constants calculated in the 30 min−7 h range.

Table 2. Effect of Surfactants on the Main Transitions of DPPC Bilayers (n = 3)

	peak 1 pre-transition[a]	peak 2 main phase transition		peak 3	
formulation	T_p (°C)	T_m (°C)	enthalpy (J/g)	T (°C)	enthalpy (J/g)
DPPC	39.93 ± 0.11	42.2 ± 0.07	37.0 ± 0.5		
DPPC/T80	32.16 ± 0.13	41.6 ± 0.01	28.5 ± 0.4		
DiMiL (4)	31.42 ± 0.65	40.2 ± 0.08	9.9 ± 1.2		
DiMiL (10)	32.33 ± 0.90	39.8 ± 0.04	6.3 ± 0.9	46.0 ± 0.02	1.6 ± 0.1

[a]Inflection point.

Moreover, the additional presence of sugars did not affect the DiMiL systems since the intensity spectra in Figure 4A,B displayed an identical profile, the shift in intensity being ascribable to a small contrast variation due to sugars. Results excluded any structural modifications in the presence of sugars for both unloaded and CBD-loaded DiMiLs.

3.4. In Vitro Drug Release. The release rate constant of CDB from the micellar dispersions was about 0.25 h independent of Kolliphor concentration (4% or 10% w/v)[1] and the amount of CBD diffused through the membrane after 5 h was up to 40%. This value was twice with respect to those measured on the DiMiL system, confirming that the encapsulation of CBD-loaded micelles in the inner core of the liposomes assures a better control of the drug release.

The release rate constants FH10-C and S2-C were significantly lower ($p < 0.05$) with respect to those of both conventional deformable liposomes (FH0-C) and FH4-C, regardless of the preparation method (Figure 5). In contrast, the release rate of FH4-C was not different from that of conventional deformable liposomes. These data confirm the SAXS evidence on the insertion of Kolliphor and CBD molecules in the liposome bilayer, leading to a prevalent distribution of the drug in the bilayer rather than in the aqueous core of liposomes.

At higher micellar concentration, a threshold might be reached, above which micelles as a whole could fuse with the bilayer forming Kolliphor-rich domains in the liposome bilayer. To verify this hypothesis, the thermotropic behavior of liposome bilayers in the presence of 4 and 10% micelles was studied by DSC. For the thermal analyses, s-PC was replaced by DPPC because the low transition temperature, T_m, at about −20 °C would have made the analysis difficult. As summarized in Table 2, DPPC bilayers showed the characteristic thermogram consisting of enthalpy event corresponding to the pre-transition temperature, T_p, at 39.93 ± 0.11 °C and a sharp endothermic event, namely, the main transition temperature, T_m, at 42.2 ± 0.07 °C. The addition of 15% T80 to the bilayer led to a shift of both T_p and T_m toward lower values (Table 2). At the concentration of T80 used in the study, the shape of the peak of the main transition did not change, whereas the enthalpy slightly decreased. These data are in agreement with those reported in literature, confirming the insertion of T80 within the lipid bilayer with an overall fluidizing effect, but without the formation of new species.[25]

The hydration of DPPC-T80 bilayers with a 4% Kolliphor dispersion (DiMiL 4) caused a more pronounced depression of the T_m peak that shifted to 40 °C and became very broad and with a very low enthalpy. This pattern suggests that the fusion of micelles with the bilayer, leads to a further fluidification of the membrane, as evidenced in the deformability test. At higher Kolliphor concentrations (i.e., 10% w/v), T_m did not undergo to further decrease confirming the saturation of the Kolliphor solubilization within the liposome bilayer. Surprisingly, a third endothermic event centered at about 46 °C was evident in the DSC traces (Table 2). This event at $T > T_m$ may be ascribed to a phase separation

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within the membrane with the formation of regions where lipid chains are more rigid and resistant to a transition, as could occur in the case of interdigitated lipid chains.[26−28] The simultaneous presence in the bilayer of mixed lipid/surfactant islands and rigid regions, may improve the stability of the DiMiL system despite the high content of surfactants.

3.5. In Vitro Skin Permeability Studies. The main limitation in CBD permeation study is related to the poor drug solubility; therefore, a solution of CBD in vaseline oil was used as control. The encapsulation of the drug in the DiMiL system improved the in vitro skin permeation of CBD with respect to both control solution and conventional deformable liposomes (FH0-C), as already demonstrated with other drugs.[14] Moreover, DiMiL increased the CBD permeation flux of about 5 and 3 times with respect to solution and FH0-C, respectively. Furthermore, the lag time was significantly shortened since FH10-C and S2-C allowed the detection and quantification of the drug in the receiver solution already after 3 and 5 h, respectively (Figure 6). Interestingly, it was

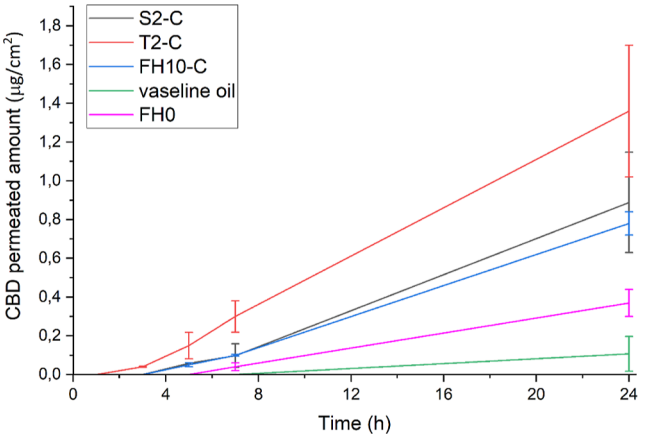

Figure 6. Skin permeation profile through human epidermis of CBD delivered by DiMiL deriving from hydration of proliposomes obtained by spray-drying (S2-C) and slurry method (T2-C) or DiMiL prepared by conventional thin lipid film hydration method (FH10-C). Vaseline oil and liposomes without micelles (FH0) were used as controls.

observed that the type of sugar used for the preparation of proliposomes had a role in defining the skin permeation properties of the system since the presence of residual trehalose in the formulation T2-C resulted in a significant improvement of CBD permeation (lower lag time and increased permeated amounts) with respect to FH10-C and S2-C (Figure 6). These data can be justified in light of the recent findings of Greco and co-workers[15] who suggested that the addition of trehalose to cream products increases the skin hydration levels and significantly decreases the mean basal values of transepidermal water loss (TEWL).[18] As a consequence, the improvement in skin hydration may cause an enlargement of the hydrophilic pores of the stratum corneum favoring the drug diffusion. This aspect is particularly interesting considering the final therapeutic indication of the topical preparation of CBD, namely as anti-inflammatory drug for which the onset of action is a key parameter.[29] Finally, it is also important to remark that CBD has a very strong affinity for stratum corneum lipids, mainly ceramides; therefore, it has a very poor tendency to diffuse toward the viable epidermis.[30] Instead, the amount of CBD found in the stratum corneum at the end of the in vitro permeability studies was almost 10-fold lower after application of DiMiL systems than vaseline oil (S2-C: 4.67 ± 1.23 $\mu g/cm^2$; vaseline oil: 39.13 ± 6.81 $\mu g/cm^2$), confirming the potentiality of DiMiL as drug carrier for the skin delivery of poorly permeable compounds.

4. CONCLUSIONS

The use of proliposomes as carriers for (trans)dermal delivery has been scantly investigated. Consequently, there is a lack of information about the effect of the preparation process and excipient choice on the quality attributes of the final system. In this work, it was made an attempt to deepen these aspects focusing on the selection of sugar carriers as function of the preparation method and their effect on the structure and functionality of deformable liposomes.

Among the preparation methods tested, spray-drying resulted more versatile since it allowed to obtain suitable proliposomal powders also using the lowest sugar/lipid ratio (1:1), even if the resulting powders were unable to flow. The slurry method instead required higher amounts of carrier (2:1/3:1 sugar/s-PC ratio) and gave free-flowing powders only using trehalose as a carrier.

It is worth noting that both selected sugars (sucrose and trehalose) did not affect the structure of deformable liposomes as supported by SAXS data. This was unexpected since it is well known that disaccharides as sucrose and trehalose strongly interact with the polar heads of the phospholipids. This interaction is generally exploited to stabilize the structure of liposomes, for example, during freeze-drying. Nevertheless, according to our experimental data, this stabilization does not cause a loss of deformability of the carrier, which could in turn affect the skin penetration behavior. The latter seemed instead to be affected by the nature of the sugar used during proliposomes preparation. In fact, it was found that the addition of trehalose led to an improvement of the permeation flux of CBD as result of the increase of skin hydration level, even if further data are required to confirm this suggestion.

Finally, in this work, the internal structure of the recently proposed DiMiL carrier was deepened. Information provided by cryo-EM and SAXS analyses not only confirmed the micelles-in-liposomes structure but evidenced for the first time the key role of the Kolliphor concentration in defining the final organization of the DiMiL system. In fact, it was definitively proven that 10% w/v Kolliphor concentration guarantees a complete and homogeneous distribution of micelles in the inner core of all vesicles of DiMiL systems and an enough stable membrane to assure a controlled drug release rate. More in general, deep experimental characterization provides useful information about the structure of deformable liposomes, highlighting, as for DiMiL, that a change in the manufacturing process does not always imply a significant modification in the structure and function of liposomes. These structural studies may represent then a helpful tool to rationalize the design of the production process and to define the formulation space of liposomal based products to be applied on the skin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.3c00044.

SAXS data; spectra of conventional liposomes prepared by the film hydration method, FH4 and FH10; Kolliphor

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micelles at different concentrations; Kolliphor micelles blank and encapsulating CBD; and FH10 and FH10-C systems (PDF)

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Notes

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