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RESEARCH PAPER

Lamellar Liquid Crystalline Phases for Cutaneous Delivery of Paclitaxel: Impact of the Monoglyceride

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ABSTRACT

Purpose To develop liquid crystalline phases with monoglycerides, and assess whether the monoglyceride type favors cutaneous over transdermal paclitaxel delivery.

Methods BRIJ-based lamellar phases were prepared with 0.5% paclitaxel and 20% of either monocaprylin (LP-MC), monomyristolein (LP-MM) or monoolein (LP-MO). Skin electrical resistance, drug release and cutaneous delivery *in vitro* and *in vivo* were assessed. Viability of skin equivalents and release of IL-1α were assessed as indexes of irritation potential.

Results An inverse relationship between monoglyceride acyl chain length and amount of paclitaxel delivered was observed. Although the largest paclitaxel amounts were delivered by LP-MC, all formulations delivered higher levels of drug in the skin (56–64-fold) than across the tissue. The superiority of LP-MC seems related to a stronger decrease in skin resistance (as an index of permeability), and not to increased drug release. LP-MC displayed similar penetration-enhancing ability *in vivo,* and a much lower irritation potential than Triton-X100 (a moderate irritant), leading to 3-fold higher skin equivalent viability and release of 60-fold less IL-1α.

Conclusions Even though LP-MC delivered the largest amounts of paclitaxel, all formulations provided similar cutaneous/transdermal delivery ratios, suggesting that changing the monoglyceride acyl chain length did not affect the balance between cutaneous and transdermal delivery.

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ABBREVIATIONS

ED	viable epidermis and dermis
LP-MC	lamellar phase containing monocaprylin
LP-MM	lamellar phase containing monomyristolein
LP-MO	lamellar phase containing monoolein
SC	stratum corneum

INTRODUCTION

Paclitaxel is highly efficacious against multiple forms of cancer, including those that affect the skin, such as classical and HIV-associated forms of Kaposi's sarcoma and basal cell carcinoma (1–4). However, the usefulness of paclitaxel for the therapy of skin cancer is limited by the serious adverse effects associated with the systemic administration of the drug, which include hypersensitivity reactions, neutropenia, and thrombocytopenia (2, 5). Such adverse effects may be circumvented by the development of topical formulations that restrict the transdermal delivery of paclitaxel while promoting its delivery to viable skin layers. In spite of the obvious advantages associated with the cutaneous delivery of paclitaxel, there is currently no topical formulation commercially available.

Although nanoemulsions, liposomes, ethosomes and binary solvent mixtures have been studied as topical delivery systems for paclitaxel (6–9), only a few studies have attempted to design formulations to maximize paclitaxel cutaneous delivery while minimizing transdermal transport. We have previously developed and evaluated BRIJ-based liquid crystalline phases to improve cutaneous localization of paclitaxel (10). Compared to other topical delivery systems, use of liquid crystalline phases presents several advantages, including possibility to protect drugs from degradation and to slow down drug

release, in addition to hydrating properties (11–13). The lamellar phase in particular also presents considerable solubilizing capability for oil- and water-soluble compounds, suitable rheological properties (sheer-thinning behavior) and consistency for application to the skin, being more viscous than dispersed systems like microemulsions, but not as stiff and difficult to spread as other liquid crystalline phases (like the cubic phase) (12, 14, 15).

In our previous study, we compared the ability of BRIJ-based lamellar and hexagonal phase bulk gels containing various ratios of medium chain mono/diglycerides to localize paclitaxel within skin layers (10). The lamellar phase provided higher release rates than the hexagonal system, and an increase in drug localization into viable skin layers once the mono/diglycerides concentration was increased from 10 to 20%. Building upon this work, we now aim at assessing whether the length of the glyceride acyl chain added to lamellar phase formulations influences the balance between cutaneous and transdermal delivery of the drug. Because the penetration-enhancing ability of formulations containing glycerides seems to be inversely proportional to the number of acyl chains bound to glycerol (16, 17), only monoglycerides were evaluated in this study. Monoglycerides with acyl chain length ranging from 8 to 18 carbons were incorporated in lamellar phases and formulation effects on the barrier function of the skin, cutaneous delivery of paclitaxel *in vitro* and *in vivo*, and viability as well as release of IL-1α by bioengineered skin tissues (as indexes of irritation potential) were evaluated.

MATERIALS AND METHODS

Materials

BRIJ 97 (polyoxyethylene-10-oleoyl ether) was obtained from Sigma (St. Louis, MO, USA). Monocaprylin, monomyristolein and monoolein were kindly supplied by Abitec Corporation (Janesville, WI) or purchased from Nu-chek Prep (Elysian, MN). Myvacet oil (diacetylated monoglycerides from soybean oil) was obtained from Quest (Norwich, NY, USA). Acetonitrile, ethanol and methanol were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA), and paclitaxel from Polymed Therapeutics (Houston, TX, USA).

Methods

Formulation Development and Characterization

BRIJ-based bulk lamellar phases were used in this study. The systems are gel-like, and consist of a linear arrangement of bilayers and water (18, 19). The monoglycerides monocaprylin (MC, containing 8 carbons in the acyl chain), monomyristolein (MM, containing 14 carbons in the acyl chain) or monoolein (MO, containing 18 carbons in the acyl chain) were mixed with BRIJ, then water was added at various ratios. The concentration of the monoglyceride in all systems was fixed at 20% (*w*/*w*) since previous studies have demonstrated that this concentration improves cutaneous localization of lipophilic drugs while having little effect on transdermal delivery (10, 20). The content of BRIJ in the formulation was varied from 40 to 75% (*w*/*w*); the content of water was varied from 5 to 40% (*w*/*w*). Samples were prepared and stored for 4 days at room temperature (maintained at 25°C) for equilibration, after which they were visually inspected for fluidity and transparency, and observed under a polarized light microscope (Axiotop, Carl Zeiss, Oberkochen, Germany) for phase identification. Under a polarized light microscope, the lamellar phase displays a distinct woven structure and/or a mosaic or Maltese cross pattern (12). Phase diagrams were constructed to show the phase behavior of the samples.

Based on the partial phase diagrams obtained (see Results), lamellar phases containing 16% of water were chosen for further studies. This concentration of water was chosen for two reasons: first, knowing that 14% of water was necessary for lamellar phase formation, 16% was used to avoid phase transition induced by water evaporation, which can occur during application of topical formulations (21). Second, we have previously observed drug precipitation from liquid crystalline phases containing water above 20% (probably due to the drug lipophilic nature) (10); hence, for better stability, water content was limited to less than 20%. Paclitaxel was incorporated at a final concentration of 0.5% to obtain formulations containing BRIJ:monoglyceride:water:paclitaxel at 63.5:20:16:0.5 (*w*/*w*/*w*/*w*). The lamellar phases will be referred to as LP-MC (containing monocaprylin), LP-MM (containing monomyristolein) or LP-MO (containing monoolein). These formulations were prepared at room temperature (maintained at 25°C), and no phase transition was observed between 25 and 37°C (temperature increment of 1°C/min), as verified by polarized light microscopy equipped with a hot stage (Linkam, Tadworth, UK). A drug concentration of 0.5% was chosen since it was the highest concentration that could be incorporated without precipitation within 1 week. To determine this concentration, paclitaxel was incorporated at 0.25, 0.5, 0.65, 0.75 and 1% in lamellar phases containing each monoglyceride. The formulations were allowed to rest in closed vials for 1 week at room temperature. Even though the lamellar structure was maintained with drug incorporation up to 1%, drug precipitation (with formulations turning cloudy to milky and presence of drug crystals under light microscope) in all formulations was observed at 0.65% and above. For preparation of drug-loaded lamellar phases, paclitaxel was first dissolved in each monoglyceride using bath sonication for 15 min. Following, each solution was mixed with BRIJ and water. More specifically, to prepare 1,000 mg of the lamellar phases, 5 mg of paclitaxel was dissolved in

200 mg of each monoglyceride using bath sonication. The entire volume of the solution of monoglyceride and drug was then mixed with 635 mg of BRIJ prior to addition of 160 μL of water. Formulations were allowed to equilibrate for 4 days before use.

To evaluate drug incorporation and formulation stability, unloaded and paclitaxel- loaded LP-MC, LP-MM and LP-MO were stored at 4 ° C for 0, 1, 2 or 3 months. At each time point, samples were allowed to reach room temperature, observed under a polarized light microscope to assess phase transitions, and paclitaxel content was assessed by HPLC after dilution of the samples with methanol to obtain a final paclitaxel concentration of 25 μg/mL.

The rheological behavior of LP-MC, LP-MM and LP-MO was investigated using a R/S Plus controlled stress rheometer with cones RC75-1 and RC50-1 (Brookfield Engineering laboratories, Middleboro, MA), and a Julabo F26MA bath/circulator for temperature control (25°C). The experiments were performed with shear rates up to 500 s^{-1}. The relationship between the shear stress and the shear rate of each formulation was evaluated using the Power law equation $\tau = K\,\gamma^{n}$, where τ is the shear stress, γ is the rate of shear, K is the consistency index and n is the flow index (22).

In Vitro Skin Penetration Study

Skin penetration assays were conducted using Franz diffusion cells (diffusion area of 1 cm^2; Laboratory Glass Apparatus, Inc, Berkeley, CA) and porcine ear skin as the model tissue. Skin from the outer surface of a freshly excised porcine ear was carefully dissected, stored at −20°C, and used within a month. The receptor phase consisted of phosphate buffer (pH 7.4, 100 mM) with 20% ethanol, and was maintained at 37 ± 0.5 ° C with magnetic stirring at 350 rpm throughout the experiment. Ethanol was included to increase aqueous solubility of paclitaxel, which is very low (4 μg/mL) (23). Paclitaxel solubility was assessed by adding 1 mg of drug in 3 mL of receptor phase, followed by magnetic stirring for 24 h, filtration, and drug assay in the supernatant by HPLC (23). With 20% of ethanol, paclitaxel solubility increased to 52.1 ± 4.2 μg/mL; a further increase in ethanol to 30% resulted in very limited increase in solubility. Larger amounts were not considered due to the possibility of changes in barrier function of the skin (8). The maximum amount of paclitaxel quantified in the receptor phase after 12 h (approximately 0.5 μg/cm^2 of skin or 0.15 μg/mL, see Results) is 300-fold smaller than the solubility limit of the drug, which suggests that drug transdermal delivery was not restricted by its solubility in the receptor phase.

The paclitaxel-loaded lamellar phase formulations (100 mg), lamellar phases without paclitaxel (unloaded) or a control formulation (100 mg of paclitaxel in myvacet oil, 0.5%, *w/w*) were added to the donor compartment. Skin treatment with lamellar phases and myvacet oil without drug produced no peak at the same region of paclitaxel elution in the chromatograms, suggesting that the formulation components do not interfere with paclitaxel quantification. All lamellar phases remained unchanged during the skin penetration experiments, as verified by polarized light microscopy. The delivery of paclitaxel into the stratum corneum (SC), viable skin layers (viable epidermis and dermis, ED) and receptor compartment was assessed at 2, 4, 8 and 12 h as detailed elsewhere (10). In brief, skin sections were rinsed, and tape stripping was performed to separate the stratum corneum (SC) and viable skin layers (viable epidermis and dermis, ED). Paclitaxel was extracted from the SC-containing tapes and from the ED with methanol. Paclitaxel in SC and ED was an index of cutaneous delivery, whereas drug in the receptor phase was an index of transdermal delivery. The ratio $\Delta cutaneous/\Delta transdermal\ delivery$ at 12 h was calculated by dividing the difference of paclitaxel delivered into the skin by the transdermal delivery difference between each lamellar phase and control. This ratio was used to evaluate whether the type of monoglyceride affected the balance between cutaneous and transdermal delivery.

The content of paclitaxel in the extracts and in the receptor phase was determined by high performance liquid chromatography (HPLC) using an equipment consisting of a Shimadzu Prominence HPLC system equipped with a pump model LC-20AB, an autosampler model SIL-20A and a photodiodoarray detector model SPD-M20A. Separation was performed in a Phenomenex C_{18} column, using mobile phase composed of 1:1 acetonitrile:water (*v/v*) at a flow rate of 1.2 mL/min, and UV detection at 228 nm as previously described (10).

To investigate whether penetration of paclitaxel occurred throughout the surface of the skin or was limited to certain skin structures (such as hair follicles), the distribution of a fluorescent derivative of paclitaxel (an Oregon Green 488 conjugate, Invitrogen, Carlsbad, CA) in the skin was studied using fluorescence microscopy. One hundred milligrams of the paclitaxel-loaded lamellar phase, or control (myvacet oil) formulations were applied to the donor compartment of the Franz diffusion cells for 12 h. Then, the surface of the skin was carefully cleaned, and the diffusion area was frozen, embedded in Tissue-Tek OCT compound (Pelco International, Redding, CA, USA), and sectioned at 10, 14 or 18 μm using a cryostat microtome (Micron, Model HM505-E, Walldorf, Germany). The sections were analyzed under a 20× objective in a fluorescence microscope equipped with a filter for fluorescein isothiocyanate (Olympus, Center Valley, PA). Tissue autofluorescence and interference of formulation components was investigated by visualizing skin samples treated with plain PBS, myvacet oil and unloaded lamellar phases containing each monoglyceride. Time of exposure was fixed to minimize interference of autofluorescence. Based on the quality of sections, a tissue thickness of 14 μm was chosen.

Electrical Resistance of the Skin

Penetration enhancers and delivery systems containing such compounds can reversibly decrease the skin barrier function and its electrical resistance (24). Changes in skin electrical resistance were assessed as an index of the ability of lamellar phases to change skin permeability depending on the monoglyceride incorporated. This method was chosen as it has been described as a quick, robust and safe method to assess skin integrity, especially if compared to the standard tritiated water method, in which T_2O water flux across the skin is measured (25). Results are generally in good agreement with those obtained from the tritiated water and transepidermal water loss methods as well with results from standard permeation experiments (24–26). The effect of the lamellar phases was assessed by measuring the electrical resistance of skin before and after application of water (control) or the lamellar phases (100 mg) for 4 or 8 h using an LCR multimeter (Mod. 179, accuracy 0.8%, Fluke, Everett, WA). The change in resistance (Δ resistance) was calculated based on the initial resistance of skin sections and plotted as a function of time.

In Vitro Release of Paclitaxel

To evaluate whether differences in topical delivery might have resulted from differences in drug release, we determined the *in vitro* kinetics for paclitaxel release from LP-MC, LP-MM and LP-MO using cell culture inserts (area of 1 cm^2, with cellulose membrane of 1 μm pores) as support (donor compartment) for the formulations and 1.5% (*w/v*) hydroxypropyl cellulose gel (1.5 g with 20% ethanol) as receptor phase, as previously described (10). The hydroxypropyl cellulose gel was chosen as receptor phase for two reasons: (a) compared to a buffer solution, gels may simulate better the diffusion conditions in the skin (27, 28); and (b) gels delayed the absorption of water by the lamellar phases and the consequent transition to hexagonal phases for as long as 8 h, which was set as the maximal duration for this experiment (10). Paclitaxel was extracted from the receptor phase by gel acidification (with 20 μL of trifluoroacetic acid) to precipitate the polymer, followed by centrifugation and filtration. The supernatant was assayed by HPLC, as described above. The liquid crystalline structure of the formulations in the inserts was monitored at all time-points by polarized light microscopy.

In Vivo Cutaneous Delivery

Since LP-MC delivered the largest amounts of paclitaxel in the skin *in vitro*, the ability of this formulation to improve paclitaxel delivery *in vivo* was investigated in male hairless rats obtained from Charles River (~250 g, Wilmington, Massachusetts), under protocol approved by the Institutional Animal Care and Use Committee. The rats had free access to water and food, and were kept on a 12:12 h light–dark cycle (lights on at 7:00 AM). To minimize handling-associated stress during the experiment, all animals were handled daily for at least one week prior to the experiment. During an experiment, rats in their home cages were maintained inside of an environmental chamber (Environmental Growth Chambers, Chagrin Falls, OH) at constant ambient temperature of 26°C. The LP-MC or myvacet solution containing paclitaxel (0.5%, *w/w*) was applied inside a plastic ring (1 cm^2) secured with medical tape over a designated area of the nape. The ring prevented leaking and spreading of the formulations.

The formulations stayed in contact with the skin for 8 h (since the rate of skin penetration *in vitro* using LP-MC reached its maximum at 8 h, see Results). Following, the rats were euthanized with sodium pentobarbital (100 mg/kg, intraperitoneal route) and the treated skin area (with the subcutaneous tissue) was excised. The SC was separated from the viable ED by tape stripping; the viable ED was separated from the subcutaneous fat by blunt dissection. Paclitaxel was extracted from each layer using methanol and assayed by HPLC. While drug concentration in SC and ED were an index of cutaneous delivery, paclitaxel content in the subcutaneous tissue was used as an estimate of the ability of paclitaxel to penetrate deep enough for systemic absorption. We acknowledge that lipophilic drugs may penetrate to a great depth and accumulate in tissues under the skin instead of being largely subjected to clearance by the dermis blood supply (29). However, if a drug can penetrate deep enough to reach the subcutaneous tissue, it is more likely to be absorbed than drugs displaying poor penetration. Based on this rationale and because of difficulties in quantifying paclitaxel transdermal delivery (due to low plasma levels at time points as high as 48 h) (30), we quantified the drug in the subcutaneous tissue as a means to estimate its ability to penetrate deep enough for systemic absorption.

Evaluation of Irritation Potential of a Selected Formulation

The irritation potential of LP-MC was evaluated by assessing its effects on the viability of engineered skin equivalents (EpiDerm™, MatTek Corporation) after various exposure times in comparison to phosphate buffered saline (PBS, negative control) and Triton-X100 (considered a moderate irritant) (31, 32). The classification of Triton as a moderate irritant is based on its effects on the skin and eyes of rabbits as determined in Draize tests (32). Each of these agents (50 mg) was placed in contact with the stratum corneum of the skin equivalents and incubated at 37°C and 5% CO_2 for 2, 5, 12 and 18 h (33). A colorimetric assay that measures the reduction of a yellow tetrazolium component into an

insoluble purple formazan product by viable cells (MTT assay) was used to account for tissue viability. After treatment, tissues were thoroughly rinsed with PBS to remove the formulations, and incubated with 300 µL of MTT solution (1 mg/mL) for 3 h at 37°C and 5% CO_2. MTT was then extracted by immersing the tissues in 2 mL of extraction solution overnight. The optical density of the extracted samples was determined at 570 nm (background reading at 650 nm was subtracted from the readings). Tissue viability (%) was plotted as function of time. The time of exposure necessary to reduce cell viability to 50% (ET_{50}) was determined and compared among treatments.

To further differentiate the irritation potential of the formulation from the controls, extracellular IL-1α was assessed in the culture medium using ELISA (Invitrogen, Camarillo, CA, USA), since its rise is consistent with induction of an inflammatory cascade by irritants (34, 35). Briefly, the media from the bioengineered tissue, diluent buffer, incubation buffer, and anti-IL-1α biotin conjugate solution were placed into wells coated with the IL-1α capture antibody. After a 2 h incubation period, the wells were washed; streptavidin-HRP solution was added to the wells. After a second incubation period (30 min), the wells were washed and tetramethylbenzidine was added. The absorbance of each well was measured at 450 nm. To study the relationship between cytokine release, changes in cell viability and time, the difference in IL-1α release between two consecutive time-points (ΔIL-1α) was divided by the average cell viability at those same points.

Data Analysis

The results were reported as mean ± SD. Data were statistically analyzed using the ANOVA test followed by Tukey post-hoc test using Prism GraphPad software. Values were considered significantly different when $p<0.05$.

RESULTS

System Characterization and Partial Phase Diagrams

In this experiment, we evaluated the influence of water content on BRIJ-monoglyceride phase behavior. At room temperature, formulations of BRIJ, water, and monocaprylin (MC) formed lamellar phase when water content was greater than 14% (*w*/*w*, Fig. 1a). For formulations containing monomyristolein (MM), lamellar phases were formed with water concentrations greater than 10% (*w*/*w*). Lastly, for formulations containing monoolein (MO), lamellar phases were observed when water content ranged between 10 and 30% (*w*/*w*). A multiple phase system (exhibiting microscopic properties of both lamellar and hexagonal phases) was observed in the latter MO mixture when water content exceeded 30% (*w*/*w*). Formulations with either monocaprylin (LP-MC), monomyristolein (LP-MM) or monoolein (LP-MO) were prepared with BRIJ:monoglyceride:water:paclitaxel at 63.5:20:16:0.5 (*w*/*w*/*w*/*w*). Table I show their detailed composition, and Fig. 1b, their textures under the polarized light microscope at 25°C. Independently of the monoglyceride added, lamellar phases with similar textures under the microscope were formed with 16% of water.

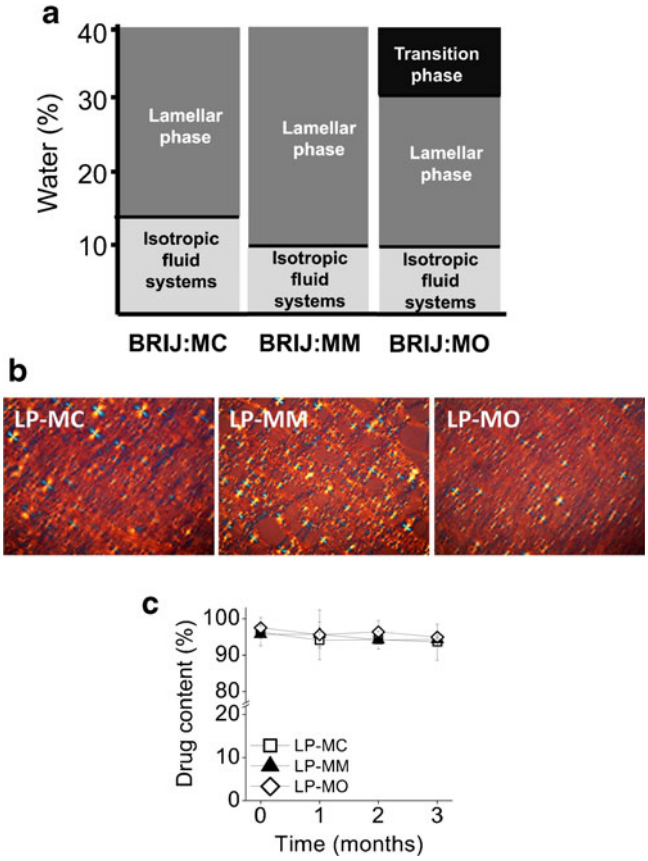

Fig. 1 Lamellar phase formation and stability. (**a**) Phase diagrams of formulations containing monocaprylin, monomyristolein or monoolein (each at 20%) with different ratios of BRIJ and water. (**b**) Textures of the selected lamellar phases obtained with each monoglyceride under the polarized light microscope (magnification=200×) at 25°C. (**c**) Paclitaxel content in each selected lamellar phase during 3 months of storage at 4°C.

Table I Composition of the Lamellar Phases

Components (%, *w*/*w*)	LP-MC	LP-MM	LP-MO
BRIJ	63.5	63.5	63.5
Water	16	16	16
Monocaprylin	20	–	–
Monomyristolein	–	20	–
Monoolein	–	–	20
Paclitaxel	0.5	0.5	0.5

Over 96% of the paclitaxel added was retained within the formulations right after their preparation (Fig. 1c, t=0). No phase transformation or drug precipitation was observed from the lamellar phases stored at 4°C over a 3-month period (Fig. 1c). Paclitaxel content did not vary significantly (p<0.05) during this period of time within the same formulation or among the formulations (Fig. 1c). This is consistent with previous reports from our group in which the stability of lamellar phases containing 10 and 20% of mono-diglycerides was demonstrated at room temperature and at 4°C (10).

The flow properties of the selected lamellar phases are displayed in Fig. 2 and Table II. All three lamellar phases displayed rheological behavior consistent with pseudoplastic systems, characterized by a non-linear relationship between shear rate and shear stress and a decrease in viscosity (shear-thinning behavior) with increases in the rate of shear. This suggests that the type of monoglyceride did not affect the type of rheological behavior. This behavior can be further supported by values of the flow index, which varied from 0.45 to 0.57. Flow is considered Newtonian when n=1, whereas n >1 or n <1 indicates shear-thickening or shear-thinning, respectively (22, 36). The pseudoplastic behavior is consistent with previous reports in the literature, and may be related to formation of multilamellar vesicles at high rates of shear (36–38). The consistency index increased from 7.75±1.56 to 12.57±2.46 from LP-MC to LP-MO (Table II), but this difference was not found to be significant (p>0.05). Additionally, within the studied range, viscosity of LP-MO was only 1.2–1.4-fold higher than LP-MC. At initial rates of shear (values up to 20 1/s were analyzed), we found no significant difference on viscosity among formulations.

In Vitro Skin Penetration Study

The amount of paclitaxel delivered into or across the skin increased up to 12 h post-application (Fig. 3). Using the control solution, 6.8±1.7 μg/mL of drug was delivered into the SC, and 0.57±0.06 μg/mL into ED (Fig. 3). Significantly higher (p<0.05) amounts of paclitaxel were delivered by LP-MC, LP-MM, and LP-MO into the SC (2.7-fold, 2-fold, and 1.6-fold higher, respectively) and into the ED (4.8-fold, 3.4-fold, and 2.8-fold, respectively). The maximum rate of cutaneous penetration of paclitaxel from LP-MM and LP-MO was achieved at 4 h post-application, whereas this effect was observed at 8 h using LP-MC (Fig. 4a). Transdermal delivery was much smaller than cutaneous retention, and reached significance (p<0.05) with LP-MC and LP-MM compared to control. It should be noted that the transdermal delivery of paclitaxel using LP-MC

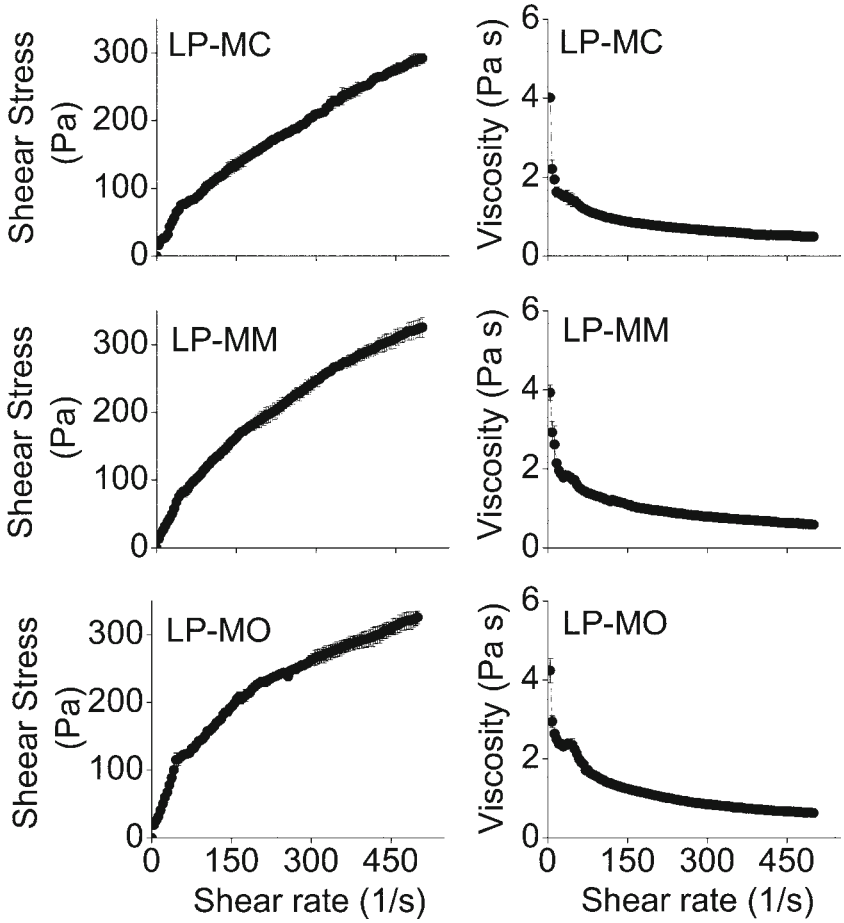

Fig. 2 Flow curves and shear-induced changes on viscosity of selected lamellar phases at 25°C. Each point represents means ± standard deviation of 3 replicates.

Table II Characteristics of the Lamellar Phases and Drug Release Provided

Formulation	Rheological parameters		Drug release rate (%/h)
	K (Pa.s)	n	
LP-MC	7.75 ± 1.56	0.53 ± 0.05	3.80 ± 0.66
LP-MM	8.45 ± 0.35	0.57 ± 0.02	3.55 ± 0.35
LP-MO	12.57 ± 2.46	0.45 ± 0.04	3.00 ± 0.34

The table depicts averages ± standard deviation of 3 replicates for rheological parameters and of 4–5 replicates for release rate

and LP-MM was smaller compared to a lamellar phase containing 10% of medium chain glycerides (10).

These results demonstrate that there was an inverse relationship between the chain length of the monoglyceride and paclitaxel delivery into or across the skin (Fig. 4b). LP-MC yielded the greatest increase in cutaneous drug delivery (into the whole skin, sum of SC + ED); however, it also increased transdermal delivery, suggesting that increases in drug delivery into the skin could not be maximized without some increase in the transdermal delivery. All systems displayed similar ratios $\Delta cutaneous/\Delta transdermal\ delivery$ (Fig. 4c) at the end of the experimental period (12 h), promoting the delivery of 56–64-times greater amounts of paclitaxel into the skin than across the tissue.

Distribution of the drug in the skin was studied using fluorescence microscopy. Treatment with unloaded (not containing paclitaxel) myvacet oil or lamellar phases did not result in fluorescent staining of the skin, suggesting that formulation components did not interfere with visualization of paclitaxel penetration. When paclitaxel was incorporated in the myvacet oil solution, fluorescence was present mostly in the SC (Fig. 5C). Drug incorporation in all lamellar phases resulted in a stronger fluorescent staining compared to control, especially in viable layers. The fluorescence seemed fairly homogenously dispersed in the skin surface, supporting the notion that, independently of the type of monoglyceride used in the lamellar phase, paclitaxel penetration occurs through the stratum corneum and was not restricted to hair follicles or other appendages.

Electrical Resistance of the Skin

Some delivery systems containing penetration enhancers can reversibly decrease the skin's barrier function, and

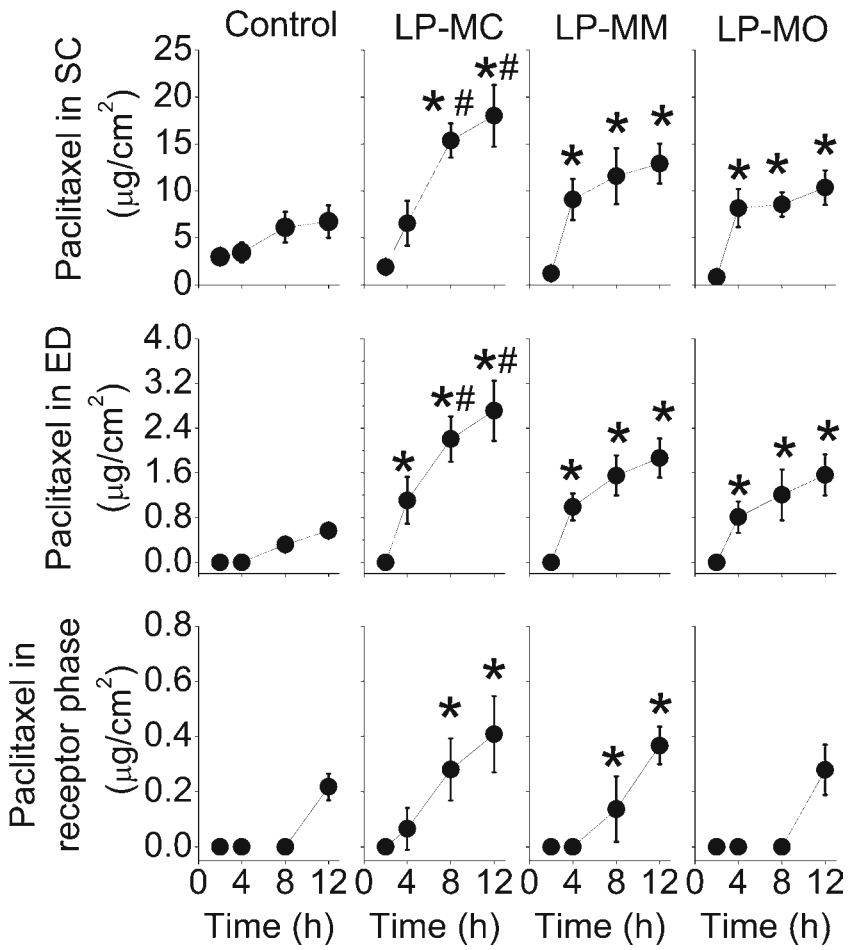

Fig. 3 Time-course of the *in vitro* skin penetration of paclitaxel from a control formulation, LP-MC, LP-MM and LP-MO (drug at 0.5% *w/w*). Each point represents means ± standard deviation of 4–6 replicates. Control solution: drug in myvacet oil (0.5% *w/w*). $^{*}p < 0.05$ compared to control formulation; $^{\#}p < 0.05$ compared to LP-MM and LP-MO.

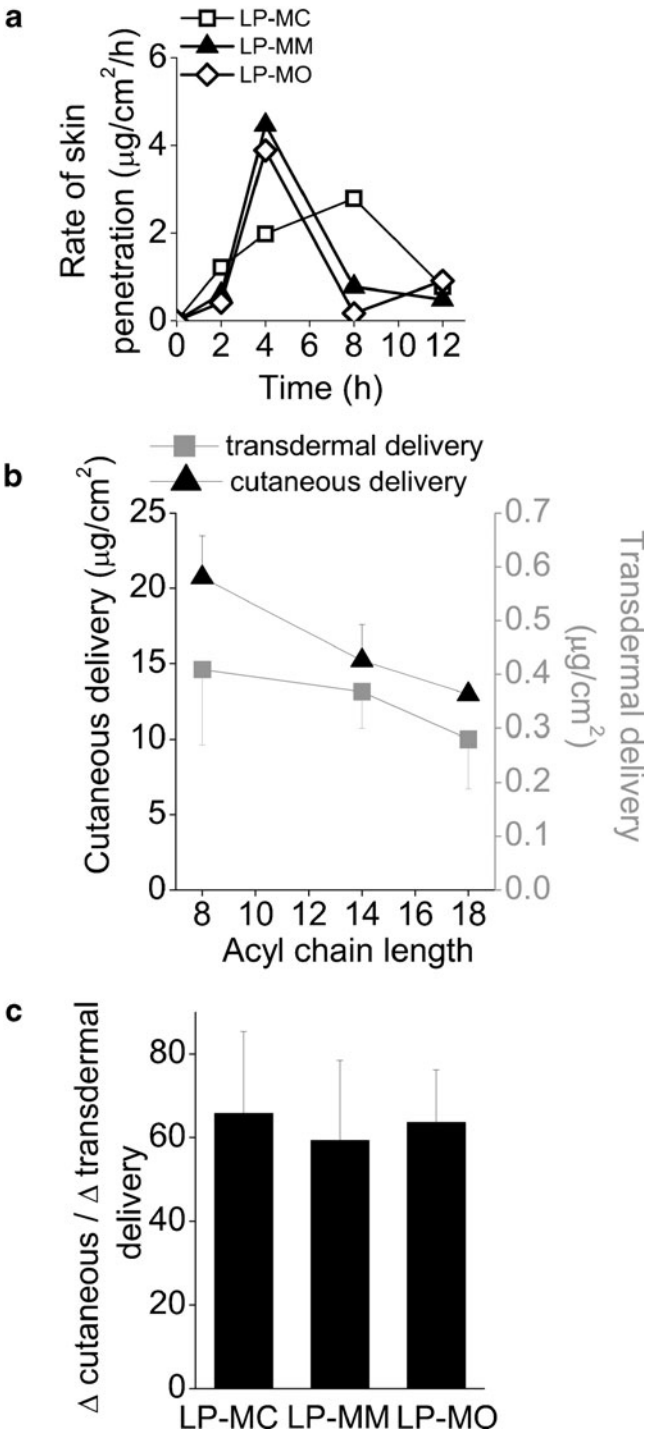

Fig. 4 Influence of the length of acyl chain of the monoglyceride on paclitaxel delivery. (**a**) Rate of drug penetration in the whole skin (SC + ED) as a function of time; (**b**) influence of the monoglyceride on cutaneous (SC + ED) and transdermal delivery of paclitaxel; (**c**) ratio *Δcutaneous/ Δtransdermal delivery* at 12 h, calculated as the difference of paclitaxel delivered by the formulation and control into the skin divided by the delivery difference across the tissue.

consequently, its electrical resistance (39). In this experiment, we evaluated whether the type of monoglyceride influenced the ability of the lamellar phase to alter skin's electrical resistance. Treatment with water (control) produced a mild decrease in the electrical resistance of the skin, which may result from tissue manipulation. Compared to water, all three liquid crystalline formulations significantly decreased skin resistance after 4 h (1.8 to 2.2-times, $p<0.05$, Fig. 6). After 8 h, the effect of LP-MC was more intense (3-fold decrease in resistance compared to control) than the other lamellar phases.

In Vitro Release of Paclitaxel

Since drug release may be dependent on the components of the formulation, we evaluated whether variations in drug release from the lamellar phases could parallel the differences observed in the cutaneous delivery study. Paclitaxel release from the lamellar phases is depicted in Fig. 7. No significant difference ($p>0.05$) in cumulative drug release (Fig. 7) or release rate (Table II) was found among the three lamellar phases studied, even though there was a trend towards higher release from LP-MC with time. Linear relationships between cumulative drug release *versus* time with coefficient of determination superior to 0.98 were obtained for all lamellar phases, suggesting that drug release during the studied period follows zero-order kinetics. This is in agreement with previous studies from our and other groups that evaluated drug release from lamellar phases (10, 19) and suggests that the type of monoglyceride did not interfere with the kinetics of drug release.

In Vivo Cutaneous Delivery

Because LP-MC delivered the largest amounts of drug into the skin *in vitro*, this formulation was selected for the *in vivo* study. The SC and ED of animals treated with LP-MC presented approximately 4- and 6-times more paclitaxel, respectively, than the tissue from control-treated animals, demonstrating the ability of the lamellar phase containing monocaprylin to improve paclitaxel delivery into the skin of live species (Fig. 8). Very small amounts (~200 ng/cm^2) of paclitaxel were detected in the subcutaneous tissue of animals treated with the lamellar phase (but not in control-treated skin), suggesting that only very small amounts of drug can penetrate deep enough and cross the skin using the lamellar phase.

Evaluation of Irritation Potential of a Selected Formulation

The irritation potential of LP-MC was compared to that of Triton-X100 and PBS in a bioengineered skin model. PBS is considered safe, and did not reduce tissue viability (Fig. 9a). Compared to PBS, the viability of the tissues treated with Triton, but not with LP-MC, was significantly reduced (to 78.1±9.9%, $p<0.05$) after 2 h; LP-MC caused a similar

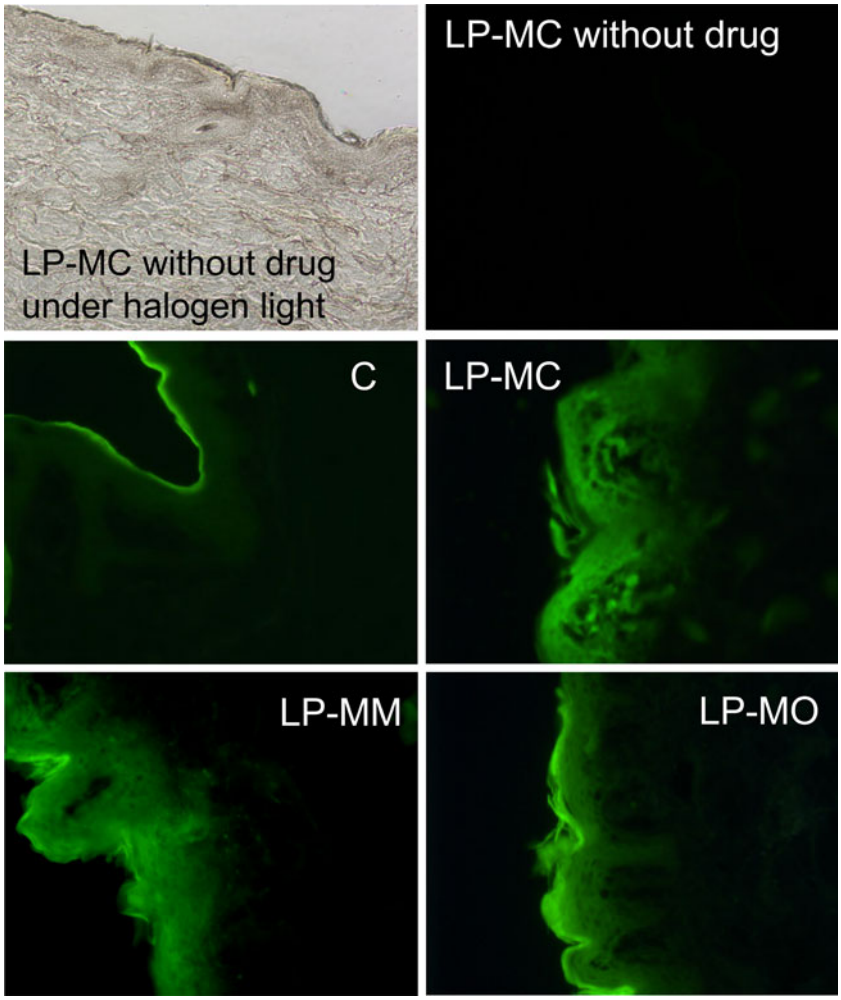

Fig. 5 Fluorescence microscopy of skin sections treated with a control solution, an unloaded lamellar phase (LP-MC), or the lamellar phases containing the fluorescent derivative of paclitaxel (0.5% *w/w*) for 12 h. The panels show skin treated with LP-MC observed under halogen and fluorescent light (as representative pictures demonstrating that treatment with liquid crystalline phases without drug produce no interfering fluorescence), skin treated with paclitaxel in myvacet oil (C), or with paclitaxel- loaded LP-MC, LP-MM, and LP-MO.

reduction in tissue viability after 5 h only, suggesting that LP-MC is better tolerated by the tissue than Triton. Additionally, the time of exposition necessary to reduce tissue viability to 50% (ET_{50}) was approximately 3 times higher for LP-MC than for Triton (4.7 and 15 h for Triton and LP-MC, respectively).

The levels of IL-1α in the culture medium were also assessed at 2, 5 and 12 h. Cytokine production at 18 h was

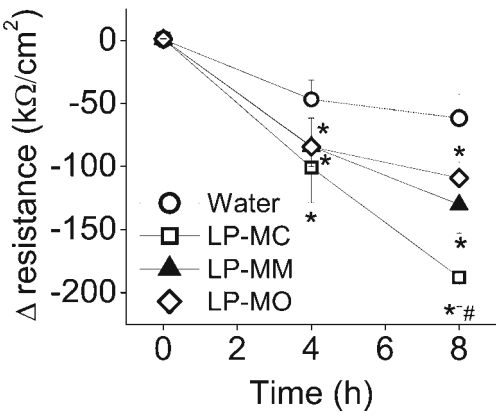

Fig. 6 Effect of treatment with water, LP-MC, LP-MM and LP-MO on skin electrical resistance as a function of time. The change in resistance was calculated based on the initial resistance of skin sections. Each point represents means ± standard deviation of 4 replicates *$p < 0.05$ compared to water; #$p < 0.05$ compared to LP-MM and LP-MO.

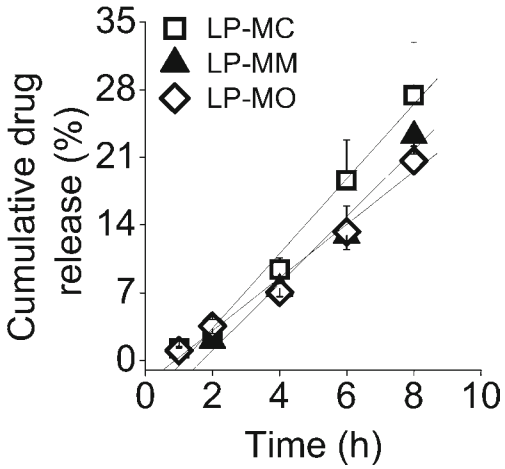

Fig. 7 *In vitro* cumulative release of paclitaxel from LP-MC, LP-MM and LP-MO as a function of time using the hydroxypropyl cellulose gel as receptor phase. Each point represents means ± standard deviation of 4–5 replicates.

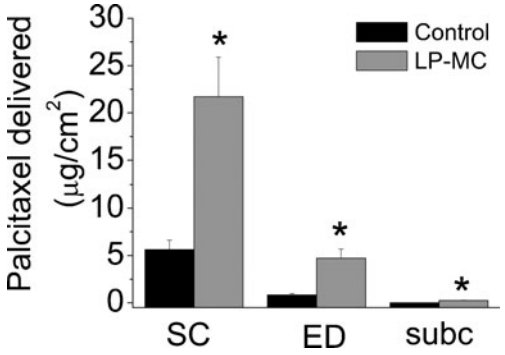

Fig. 8 Delivery of paclitaxel *in vivo* for 8 h. Each point represents means ± standard deviation of 6 replicates. *$p < 0.05$ compared to the control solution. SC: stratum corneum; ED: viable epidermis and dermis; subc: subcutaneous tissue.

not assessed since the viability of tissues treated with both Triton and LP-MC dropped below 50%. Compared to PBS, Triton increased the IL-1α release by viable cells at all time-points studied (Fig. 9b). The amount of cytokine produced by viable cells after Triton exposure for 12 h was over 200-fold higher than after PBS exposure. LP-MC-induced cytokine production by viable cells was 60-fold lower compared to Triton, although still higher (4-fold) than PBS.

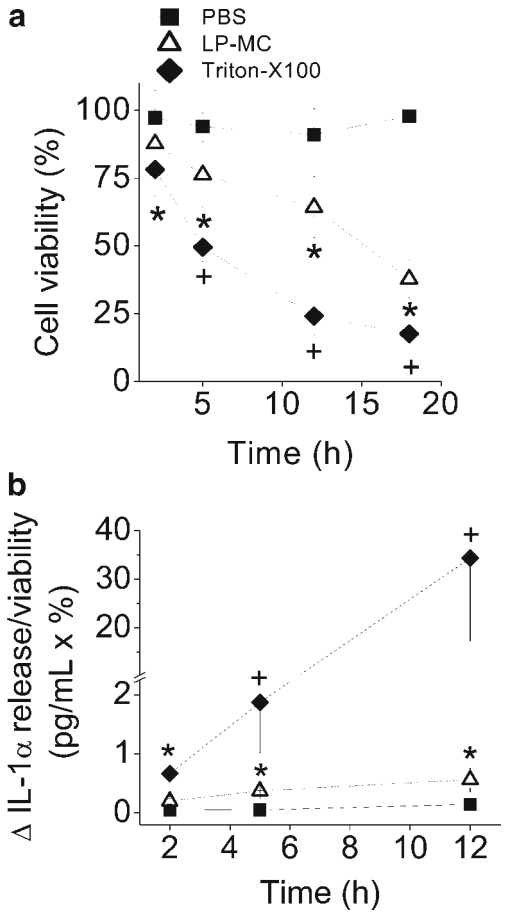

Fig. 9 Time-dependent effects of treatments (PBS, Triton-X100 and LP-MC) on the viability of bioengineered skin equivalents (**a**) and on the release of IL-α by the viable cells in the tissues (**b**). *$p < 0.05$ compared to PBS, +$p < 0.05$ compared to PBS and LP-MC. Each point represents means ± standard deviation of 3–5 replicates.

DISCUSSION

In this study, we developed BRIJ-based lamellar phases containing monoglycerides with various acyl chain lengths to assess their ability to maximize cutaneous over transdermal delivery. BRIJ forms a variety of structures upon self-assembly in aqueous environment; the type of structure formed is largely dependent on the surfactant's *critical packing parameter* (*CPP* = V/a_0L, where V is the hydrophobic chain volume, a_0 is the headgroup area, and L is the chain length of the molecule), which in turn, is influenced by the presence of other compounds (*e.g.*, monoglycerides), water content and temperature (40, 41). In general, *CPP* values below 1/3 and between 1/3 and ½ give rise to spherical and cylindrical structures, respectively, whereas values between ½ and 1, and above 1 result in formation of bilayes and inverted structures, respectively (40). The *CPP* value of BRIJ in aqueous solutions was estimated as 0.45 (40), which favors cylinders (42). As the surfactant increases (and water decreases below 40%), a decrease in a_0 and formation of lamellar phases can be expected (40). Addition of monoglycerides did not preclude formation of the lamellar phase, but increasing the chain length of the monoglyceride promoted formation of lamellar phases with approximately 4% less water probably due to an increase in the hydrophobic chain volume, and in *CPP* (40). Chain length-dependent phase transition was also observed by Amar-Yuli and Garti when triglycerides were added to monoolein-based systems due changes in the *CPP* of the structure-forming agent (43). In the case of monoglycerides, increases in their acyl chain length generally give the molecules an overall conical shape with larger *CPP* (44). The *CPP* of monoolein, for example, was described to be over 1.2 in water (and varies with water content and temperature) (45). Therefore, monoglycerides with longer chains like monoolein and monomyristolein may have a stronger influence at increasing *V* and thus *CPP* values of BRIJ, which seems to favor the lamellar phase at low amounts of water.

The acyl chain length influences transdermal delivery of small molecules when glycerides are used in binary solvent solutions (17) and the co-localization of antioxidants in the skin when monoglycerides are present in microemulsions (46), but the effects on skin localization *versus* transdermal delivery when incorporated in liquid crystals have not been assessed. This is especially relevant considering that the effect of a penetration modifier can change from vehicle to vehicle due to various interactions on the surface of stratum corneum that may lead to the enhanced penetration or retardation of drugs

(47). We observed an inverse relationship between the length of the monoglyceride acyl chain and the cutaneous delivery of paclitaxel. Transdermal paclitaxel delivery was also reduced as the monoglyceride acyl chain length increased, which is in accordance with previous studies showing that a 6- carbon chain maximized pentazocine flux, whereas further increases (up to 18 carbons) decreased it (17). In other words, an increase in paclitaxel cutaneous levels was associated with increased transdermal delivery; the latter could only be reduced at the expense of reducing the former. Nevertheless, drug levels in the whole skin (sum of SC and ED) were 56- to 64-times higher than in the receptor phase, demonstrating that all the formulations have a higher impact at improving cutaneous drug localization, possibly due to the affinity of the lipophilic paclitaxel with tissue components or with monoglycerides that partitioned in the skin (20, 48). Taken together, these results suggest that all formulations favored cutaneous retention but, even though the monoglyceride type affected the absolute amounts of drug delivered into the skin, it did not influence the balance between cutaneous and transdermal delivery.

In relative terms, a comparison of our *in vitro* results with the results obtained with other delivery systems reveals that the lamellar phases were indeed efficient at improving paclitaxel localization into the skin without a large transdermal delivery. Using ethosomes, Paolino *et al.* were able to deliver 103.5 μg/cm^2 of paclitaxel into human dermis *in vitro* after 24 h, and over 140 μg/cm^2 across epidermis membranes (9). Transdermal and cutaneous delivery of over 150 and 20 μg/cm^2 of paclitaxel, respectively, were observed in rat skin *in vitro* after 48 h using ethanol:isopropyl myristate (1:1) as vehicle (8). Improved cutaneous localization was obtained with nanostructured and nanoparticulate systems (7, 49): nanoemulsions (4 h, *in vivo* treatment in rats) and tyrosine-derived nanospheres (6 h treatment using human skin) delivered approximately 20 μg/4 cm^2 and 130 ng/cm^2 of paclitaxel into the skin (sum of epidermis and dermis), respectively, and less than 100 ng/mL and 10 ng/mL across the tissue (7, 49), respectively.

The superiority of LP-MC may result from two effects. First, since monocaprylin is smaller, its lipophilicity is less pronounced, which may avoid paclitaxel retention in the formulation (50, 51). Additionally, because decreases in acyl chain length and molecular weight are generally associated with a more fluid state of matter, addition of monocaprylin could result in a less viscous system with higher release rates compared to the other lipids. However no significant difference ($p > 0.05$) was observed on viscosity at initial shear rate values, on consistency indexes or on paclitaxel release (cumulative amount or rate) among formulations. The second possible explanation relates to the stronger effect of LP-MC on disrupting the skin barrier, which promotes penetration of larger amounts of drug (24, 52). This is supported by the formulation's ability to significantly reduce electrical skin resistance compared to the other phases at longer time points, and agrees with previous observations from our group describing the superiority of monocaprylin-containing microemulsions at increasing lycopene skin penetration (16, 46). Liquid crystalline phases can cause some barrier disruption possibly due to interactions between their nanostructure and the intercellular lipids in the stratum corneum, which yield a more fluid and permeable lipid packing (42, 53, 54), and to stratum corneum hydration by the hydrophilic domain of the systems, leading to an increase in the interlamellar volume of the lipid bilayers, and disorganization (53). The type of monoglyceride added seems to further influence this effect, as demonstrated by monoglyceride-dependent changes on skin electrical resistance. Compared to the other lamellar phases, the rate of cutaneous delivery of paclitaxel from LP-MC reached its maximum at 8 h post-application, 4 h later than the other formulations. This may result from the fact that the influence of LP-MC on skin permeability becomes significantly stronger than that of other phases after 4 h.

Consistent with our *in vitro* observations, paclitaxel cutaneous delivery by LP-MC was higher compared to the control formulation *in vivo*. In a general manner, the amount of drug retained in the skin was higher *in vivo* than *in vitro*, especially considering the viable layers. Paclitaxel delivery into the SC and ED *in vivo* was 1- to 1.5- times and 2- to 2.5- times higher, respectively, than *in vitro*. Differences in the content of drug delivered *in vitro* and *in vivo* have been observed in other studies and attributed to differences of skin models. Rat skin used *in vivo* is more permeable than porcine skin (*in vitro)* (55). Additionally, the lack of blood flow in the dermis *in vitro* may artificially hinder the skin absorption of lipophilic compounds in this water- rich environment (55, 56). Nevertheless, these results confirm the penetration-enhancing ability of LP-MC *in vivo*.

The irritation potential of LP-MC was assessed based on its time-dependent effects on human reconstructured tissue equivalents (57, 58). The ET_{50} for LP-MC was approximately 3 times longer than that of Triton, which suggests that this formulation should be better tolerated by the skin. In a previous study, Triton showed a stronger damaging effect to tissues compared to another surfactant of the BRIJ series (BRIJ 35, in which the oleyl chain was replaced by a lauryl chain) as determined by the Draize test (59), supporting our observation that BRIJ-based formulations are better tolerated. When evaluating the effects of nanostructured delivery systems formed by self-aggregation of surfactants (as the lamellar phase), one should consider that only monomers of surfactants are able to penetrate across bilayers and cause tissue damage, so the cytotoxicity of surfactants increases with their critical micelle concentration (CMC) (60). The concentration of Triton used (1% *w/v*) was below its CMC (129.4 μg/mL) (60, 61),

whereas BRIJ was used over its CMC, which is 2.2-times higher than that of Triton (60). So far, there is no well-defined value of ET_{50} that ensures formulation safety, but several marketed topical formulations with good tolerability have ET_{50} values 3 to 9-times longer than Triton (62). The fact that LP-MC increased the ratio of ΔIL-1α release/viability by 4 to 7-fold compared to PBS suggests that the formulation might cause minor irritation, which is expected due to the presence of a surfactant and a penetration enhancer (monocaprylin) that can change the skin permeability possibly by disrupting the barrier (16, 63, 64). However, the magnitude of the reaction was much smaller than that caused by Triton, which supports our previous observation that LP-MC is safer than the moderate irritant Triton.

In conclusion, the choice of monoglyceride influenced the penetration-enhancing ability of lamellar phases and the amount of paclitaxel delivered into and across the skin, but not the balance between cutaneous and transdermal delivery. An inverse relationship between paclitaxel penetration and acyl chain length of the monoglyceride was observed, with the lamellar phase containing the shortest monoglyceride (LP-MC) being the most effective. The superiority of LP-MC was not related to a higher drug release, but to its stronger effect on disruption of the skin barrier. The formulation was better tolerated by skin equivalents than the moderate irritant Triton.

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