Development of microemulsions of suitable viscosity for cyclosporine skin delivery

Development of microemulsions of suitable viscosity for cyclosporine skin delivery

Marta Benigni, Silvia Pescina, Maria Aurora Grimaudo, Cristina Padula, Patrizia Santi, Sara Nicoli*

Food and Drug Department, Parco Area delle Scienze 27/A, 43124 Parma, Italy

*Corresponding author:

Sara Nicoli
Food and Drug Department
University of Parma
Parco Area delle Scienze 27/A
43124 Parma, Italy
Tel: +39 0521 905065
Fax: +39 0521 905006
Email: sara.nicoli@unipr.it

KEYWORDS

Skin delivery

Microemulsion

Psoriasis

Thickening

Cyclosporine

Polysorbate 80; Tween®80

Vitamin E TPGS

Viscosity

ABSTRACT

Psoriasis is a widespread chronic disease affecting 2-4 % of the population in Western countries. Its mild-to-moderate form, representing approximately 80% of the total cases, is treated by topical application, with corticosteroid being the standard treatment. However, in case of psoriasis, no single treatment works for every patient and optimizing topical therapy is a key aspect. A possible alternative is represented by cyclosporine, an immunosuppressant cyclic peptide administered orally in the treatment of the severe form. Its topical application could avoid the problems related to systemic immunosuppression, but the unfavourable physico-chemical properties (MW:1202 Da; LogP≈3) hinder its permeation across the stratum corneum. The aim of the paper was the preparation, characterization and *ex-vivo* evaluation of cyclosporine loaded microemulsions using oleic acid as oil phase, either Tween® 80 or a soluble derivative of vitamin E (TPGS) as surfactants and either Transcutol®, propylene glycol or 1,3 propanediol as co-surfactants. The issue of formulation viscosity was also addressed 1) by evaluating the thickening of Tween®80-based microemulsions by direct addition of different rheological modifiers 2) by building pseudo-ternary phase diagrams using TPGS, to identify the water/oil/surfactants proportions resulting in viscous self-gelifying systems. Nine formulations (five Tween®80-based and four TPGS-based) were selected, characterized in terms of droplets size (low viscosity systems) or rheological properties (high viscosity systems), loaded with 6 mg/g cyclosporine and applied *ex-vivo* on porcine skin for 22 h. A relevant skin accumulation was obtained either with a low-viscosity Tween®80-based microemulsion (9.78±3.86 μg/cm^2), or with a high viscosity TPGS-based microemulsion (18.3±5.69 μg/cm^2), with an increase of about 3 and 6 times respectively for comparison with a control cyclosporine solution in propylene glycol. The role of water content, surfactant, co-surfactant and viscosity was also addressed and discussed. The kinetic of skin uptake from the best performing formulation was finally evaluated, highlighting a relatively quick skin uptake and the achievement, after 2 h of contact, of potentially therapeutic cyclosporine skin concentrations.

1. Introduction

Psoriasis is a chronic inflammatory and autoimmune skin condition affecting 2-4% of the population in Western countries. It is characterized by the presence of plaques of thickened and erythematous skin with white scales, due to the hyperproliferation of epidermal keratinocytes. This excessive proliferation is triggered by inflammatory mediators such as interferon- γ, interleukin (IL)-17 and IL-22, secreted by T lymphocytes[1]. The treatment of this condition differs depending on disease severity, assessed by the appearance and extension of the plaques. For severe psoriasis, the systemic treatment is necessary; together with the classic immunosuppressive agents, such as cyclosporine, retinoids, and methotrexate, new therapeutic options are now available, and include both "biologics" (anti T-cells and anticytokine) and small drugs (tofacitinib, apremilast). In the case of mild-to-moderate psoriasis, representing approximately 80% of the patients affected by this disease, topical treatment is recommended[2]. The main therapeutic options include corticosteroids, Vitamin D analogues, tazarotene, tacrolimus and picrolimus, with corticosteroid being the standard treatment for most patients. Indeed, corticosteroids are versatile drugs, being available on the market in different potency, strength and formulations. However, they have important local and systemic side effects and are not always efficacious for psoriasis. Indeed, no single treatment works for every patient and optimizing topical therapy is a key aspect of psoriasis treatment as witnessed by the development of new drugs for topical application[3, 4] and by the combinations used for added efficacy[5].

A considerable literature has grown up around the possibility of administering cyclosporine by topical application, as an alternative therapeutic tool. This administration route could avoid the problems related to systemic immunosuppression, but the unfavourable physico-chemical properties of cyclosporine (MW:1202 Da; LogP≈3; low water solubility) hinder its permeation across the stratum corneum. Indeed, up to now, topical cyclosporine for psoriasis treatment has been limited by the lack of formulations providing sufficient skin absorption [6,7]. Recently, a small therapeutic exploratory study highlighted the possibility of an efficient skin delivery: Kumar *et al*[8] demonstrated that 2% topical cyclosporine liposomal formulation included in a Carbopol® gel was effective in the treatment of limited chronic plaque psoriasis, contrarily to a traditional o/w emulsion, supporting the importance of the formulation on cyclosporine skin delivery.

Given the interest for the topical application of this molecule, several approaches have been evaluated by different researchers worldwide. Chemical enhancers were investigated such as

ethanol, ethyl oleate, Transcutol®, propyleneglycol, azone[9], decylmethylsulfoxide[10], monoolein[11] and skin penetrating peptides[12, 13]. Also physical enhancing technique were evaluated, in particular electroporation[14], and ultrasounds[15]. Among nanocarriers, nanoparticles[16-18], liposomes[19-21] and micelles[22] were investigated. Other authors have evaluated the possibility to deliver cyclosporine A by using nanoemulsions[18], microemulsions[23, 24] and/or liquid crystalline systems[25, 26]. Indeed microemulsions (ME) (i.e transparent single-phase systems generally composed of a blend of oil, water a surfactant and a co-surfactant) could be particularly advantageous for cyclosporine delivery, due to their capability to solubilize highly hydrophobic drugs. Furthermore, ME offer the advantage of spontaneous formation and thermodynamic stability and have widely demonstrated the capability to enhance drug uptake into the skin. However, a limitation in the use of ME for skin delivery is represented by the very low viscosity, that makes skin application difficult. It is worth underlying that rheological properties play a crucial role in dermal administration, since viscosity not only favours the retention of the formulation on the skin surface but can also slow down water evaporation maintaining for a longer period of time the ME structure and thus its peculiar enhancing properties. Additionally, the rheological properties of a dermal formulation have an impact on patient's acceptability and adherence, a very relevant issue in psoriasis treatment[27-29].

Different approaches can be followed to increase the viscosity of a microemulsion: 1. mix of the ME with an already prepared gel[30, 31] 2. direct addition of a thickening agent to the ME[32, 33]3. use of specific excipients able to give high viscosity systems, often associated to the formation of lamellar, cylindrical or worm-like structures[34-38].

The aim of this work was the development of ME of suitable viscosity for cyclosporine skin delivery using oleic acid as oil phase and either Tween® 80 or TPGS (D-α-Tocopheryl polyethylene glycol 1000 succinate) as surfactants. Tween® 80-based microemulsion, prepared using Transcutol® as co-surfactant, were simply added of different rheological agents. In case of TPGS-based systems, pseudo-ternary diagrams were built to assess the ratio between water, oil phase and surfactant - co-surfactant mixture (Smix), necessary to obtain self-gelifying formulation. In this case, three different co-surfactants, namely Transcutol®, propylene glycol and 1,3 propanediol, were evaluated. The formulations will be characterized and evaluated for cyclosporine skin uptake.

2. Materials and methods

2.1. Materials

Cyclosporine ($C_{62}H_{111}N_{11}O_{12}$, MW 1202.61 g/mol, crystalline solid) was from ThermoFisher Scientific (Karlsruhe, Germany). D-α-Tocopheryl polyethylene glycol 1000 succinate (Kolliphor® TPGS, MW 1513 g/mol) and poloxamer 407 (Pluronic® F127, MW 12.6 kDa) were a kind gift from BASF (Ludwigshafen, Germany). Trifluoroacetic acid (TFA, MW 114.02 g/mol), albumin from bovine serum (≈66 kDa, ≥96%), 1,3-propanediol (MW 76 g/mol) and alginic acid sodium salt were purchased from Sigma Aldrich (St. Louis, MO, USA). Oleic acid was from Alfa Aesar (Karlsruhe, Germany), Transcutol® was a gift from Gattefossè (Lyon, France). Sodium hyaluronate (MW 1000 kDa) was a gift of IBSA Farmaceutici S.p.A (Lodi, Italy). 1,2-propanediol (MW 76 g/mol) was purchased from A.C.E.F. S.p.A. (Fiorenzuola d'Arda, Italy). Aerosil® 200 Pharma (fumed silica) was from Evonik Industries (Essen, Germany). Carbopol® 940 was purchased from The Lubrizol Corporation (Wiekliffe, OH, Usa). For HPLC analysis, pure water (Purelab® Pulse, Elga Veolia, UK) and HPLC grade acetonitrile were used. Phosphate-buffered saline (PBS) composition was 0.19 g/l KH_2PO_4, 5.98 g/l Na_2HPO_4▪$12H_2O$, 8.8 g/l NaCl, pH 7.4

2.2. Cyclosporine quantification method.

Cyclosporine was quantified by HPLC-UV (Infinity 1260, Agilent Technologies, Santa Clara, CA, USA), with a reverse-phase Nova-Pack C_{18} cartridge (150*3.9 mm, 4 µm) (Waters, Milford, Massachussets, USA) and a C_{18} guard column (3.2*0.8 mm, Security Guard™ Cartridge, Phenomenex, Torrance, USA) both thermostatted at 65°C. The mobile phase, pumped at 1.6 ml/min, was a 65:35 (v/v) mixture CH_3CN: water with TFA 0.1%. The injection volume was 100 µl, and absorbance was monitored at 230 nm. In these conditions, cyclosporine retention time was about 5 min. The method was previously validated in the concentration interval 0.25-50 µg/ml[39].

2.3. Pseudo-ternary phase diagram construction

Pseudo-ternary phase diagrams were built to identify the microemulsion region in multiphasic systems. Oleic acid was used as oil phase, and a 1/1 (w/v; g/ml) mixture of TPGS and co-surfactant (either Transcutol®, 1,2-propanediol or 1,3-propanediol) was used as surfactant system (Smix). The diagrams were built using the aqueous tritation method: for fixed ratios oil/Smix (0.5/9.5, 1/9, 1.25/8.75, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, 9/1) increasing amounts of water, between 5 and 95%,

were added. After each addition, the mixture was vortexed and left 1 minute to rest, then by visual observation the viscosity and clearness of the system were evaluated. In case of highly viscous mixtures, the system was heated in a thermostatted bath at 50°C before each water addition in order to reduce the viscosity and favour the mixing by vortex to achieve homogeneity. The evaluation of the system was performed after cooling at room temperature. The formulation is clear and exhibits low viscosity in the microemulsion region, while it is clear and viscous in the microgel region where the formulation does not slide along the vial walls. The diagrams were built using OriginPro® 2016 (Originlab, Northampton, MA, USA).

2.4. Thickening of Tween®80-based microemulsions

Tw20T (composition in Table I) was prepared by mixing the different components into a glass vial, under magnetic stirring, in the following order: oil phase, co-surfactant, surfactant and water. Then, the thickener was added and the mixture was slowly magnetically stirred overnight. Type and concentration of the thickeners used are reported in Table II.

2.5. Rheological behavior

Rheology measurements were performed in oscillation mode with an Ares Rheometer (TA Instruments, New Castle, DE, USA) equipped with a plastic cone and plate fixture. Cone diameter was 50 mm and cone angle was 0.04 radian. Sample's linear viscoelastic region (LVE) was determined by strain sweep (10^{-2}-10^{+2} strain %) at 23°C. Frequency sweep was at 0.1% strain for samples TPGS 44 T, TPGS 25 PG1,2, TPGS 12 PG1,3 and Tw20T 8% silica and 1.3% strain for Tw 20T 1% Carbopol®. Temperature was set at 23°C. Rheological data were acquired using Orchestrator software and then processed using Trios (v 4.3.0.38388 free version; TA instruments, New Castle, DE, USA).

In order to roughly compare the viscosity of the different systems, a modified Erramreddy and Ghosh method was also employed[40]. A known volume of formulation (1 ml) was inserted in a 8 ml polypropylene tube. The tube was then placed onto a support with a 10° inclination; the formulation slide along the wall and the time necessary to reach the equilibrium position can be measured (Figure S2). Measures were always performed at 23±1 °C.

2.6. Polarized optical microscopy

To asses microemulsions optical properties, MEs were spread between glass slide plates, to prevent water loss. Samples were analysed at 4X, 10X and 20X magnification using a polarized optical microscope (Nikon, Shinjuku, Japan) and images were taken with a 13 megapixels camera (Samsung Galaxy S4, Seoul, South Korea).

2.7. Cyclosporine-loaded formulations

For cyclosporine loading into the microemulsions, the drug was dissolved in the oil phase (oleic acid) so as to obtain a 6 mg/g concentration in the final system. Then, the other components were added and the system was mixed on vortex until homogeneity. The composition of the formulations prepared are shown in Table I.

Table I. Composition of the cyclosporine-loaded formulations prepared. Cyclosporine concentration was 6 mg/g. For all the formulations, the oil/Smix ratio was 1.25/8.75

CODE*	Oleic acid(ml)	Surfactant		Co-surfactant (ml)		Water (ml)
Tw 20 T	10	Tween® (ml)	35	Transcutol®	35	20
Tw 35 T	8.2	Tween® (ml)	28.4	Transcutol®	28.4	35
Tw 44 T	7	Tween® (ml)	24.5	Transcutol®	24.5	44
TPGS 20 T	10	TPGS (g)	35	Transcutol®	35	20
TPGS 44 T	7	TPGS (g)	24.5	Transcutol®	24.5	44
TPGS 25 PG1,2	9.4	TPGS (g)	32.8	1,2 propanediol	32.8	25
TPGS 12 PG1,3	11	TPGS (g)	38.5	1,3 propanediol	38.5	12

**The code is given by the surfactant used (TPGS or Tween®=Tw), followed by the water percentage and by the co-surfactant used (T=Transcutol®, PG1,2=propylene glycol, PG1,3=1,3 propanediol)*

2.8. Skin accumulation and permeation experiments

For permeation experiments, porcine skin excised from the outer part of pig ears was used. The skin was separated from the underlying cartilage with a scalpel and frozen at -20°C until the day of experiment. All tissues were used within 3 months from freezing. On the day of the experiment, the skin was thawed and mounted on vertical diffusion cells (DISA, Milano, Italy; 0.6 cm^2 surface area) with the stratum corneum facing the donor compartment. The receptor compartment was filled with about 4 ml of PBS pH 7.4 (cyclosporine solubility: 22.8±2.6 µg/ml). Different formulations (composition in Table I) were evaluated together with a control solution (6 mg/ml cyclosporine in propylene glycol); microemulsion Tw 20 T was also tested after addition of either Carbopol® 1% w/w or silica 8% w/w. The formulations were applied for 22 h in the donor compartment at infinite dose (approximately 200 mg/cm^2). Each condition was replicated 3 to 6 times. Cyclosporine skin

deposition from TPGS 44 T was also evaluated as a function of time by applying the formulation for 0.5, 2, 4, 8 and 22 hours. In this case, in order to reduce the variability, all the experiments were performed the same day on the same skin batch and were replicated 3 times (2 times for the 22 h point). At the end of the experiments, the receptor solution was sampled, the donor formulation was carefully removed with absorbent paper, the tissue was rinsed with 10 ml of pure water and blotted dry with filter paper. Then, the skin was cut (0.6 cm^2), minced and extracted using a mixture CH_3CN/water/PBS (75/20/5) for 2 hours at room temperature. Extraction and permeation samples were analysed by HPLC. The extraction method was validated: no interfering peaks were present at cyclosporine retention time and the recovery efficiency was 95.9±6.0%. The skin cleaning procedure was validated as well, by applying a cyclosporine-loaded microemulsion and removing it immediately; the recovered amount was lower than LOQ.

2.9. Light scattering

Non-viscous microemulsions (Tw20T, Tw35T, Tw44T and TPGS20T) were analysed undiluted at room temperature using a Zetasizer® NanozetaS (Malvern Instruments, UK).

2.10. Statistical analysis

The significance of the results was assessed using ANOVA followed by Bonferroni post-hoc test. Differences were considered statistically significant when $p<0.05$. All data in text and figures are reported as mean value±SD.

3. Results and discussion

3.1. Cyclosporine skin accumulation from low viscosity Tween® 80-based microemulsions

At first, un-thickened microemulsions were evaluated. In particular, we tested a relatively common system prepared using oleic acid as oil phase, polysorbate 80 (Tween® 80) as surfactant and Transcutol® as co-surfactant, used in 1:1 ratio. The ternary diagram associated with these components was previously published by other authors[41]. Inside these diagram the oil/Smix 1.25/8.75 ratio was selected and three formulations containing 20, 35 and 44% of water (Tw20T, Tw35T and Tw44T in Table I) were loaded with 6 mg/ml cyclosporine. Cyclosporine was never recovered in quantifiable amounts in the receptor compartment, despite the relatively long application time and the presence of sink conditions (cyclosporine solubility in the receptor solution: 22.8±2.6 µg/ml; LOQ of the analytical method: 0.25 µg/ml), probably because of its considerable lipophilicity [42]

The results obtained in terms of skin accumulation after 22 h is reported in Figure 1 where it is compared with that obtained from a simple cyclosporine solution in propylene glycol at the same concentration.

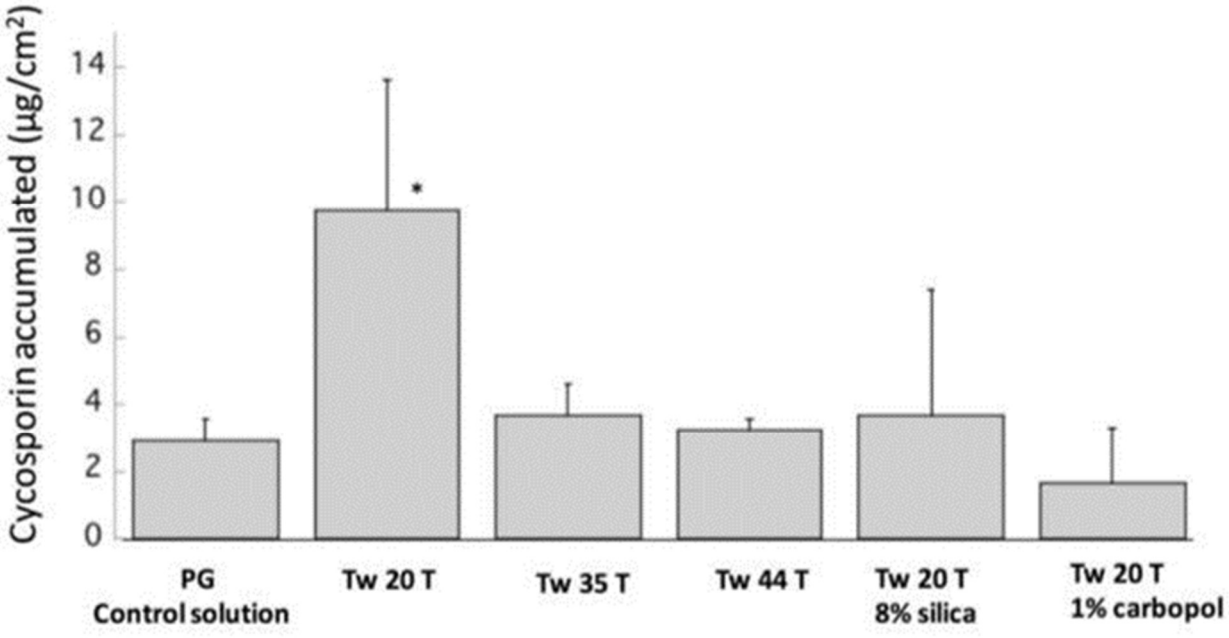

Figure 1. Cyclosporine skin deposition starting from low viscosity microemulsions made with oleic acid, Tween® 80/Transcutol® 1/1 as Smix and different water % (mean±sd). All the vehicles contain 6 mg/ml cyclosporine.
* statistically different from all the other conditions.

This solution was chosen as control since it was previously used in literature as control solution on pig skin (cyclosporine accumulation obtained: 0.9±0.5 µg/cm^2)[22]; indeed, in this work a reasonably similar skin accumulation was found (Figure 1). The use of Tw20T determined a significant ($p<0.05$) increase in the amount of cyclosporine accumulated, with an enhancement factor (calculated as the ratio between cyclosporine accumulated from microemulsion and cyclosporine accumulated from control solution) of 3.35±1.32. The increase in water content to 35 and 44% determined a statistically significant reduction of drug deposition, an opposite trend compared to data reported in the literature for lipophilic compounds where a higher accumulation/permeation is often associated to a higher water content[31, 43].

Dynamic light scattering (or photon correlation spectroscopy) of undiluted microemulsions was used to analyze droplet size via determination of hydrodynamic radius. The result (Figure 1S) did not help to explain the skin retention data, given the similar z-average found for Tw20T and Tw35T (106 and 138 nm respectively). Maybe, the presence of a small intensity peak at about 17 nm in Tw20T (reasonably attributable to surfactant micelles) could have contributed to the result obtained. In case of Tw44T, two peaks centered at about 600 and 60 nm and equally contributing to the total signal intensity, were found. It is however important to underline the limitation of this technique for microemulsion analysis, given the unknown viscosity of the samples.

3.2. Thickening of Tween® 80-based microemulsions

Given the need for viscous ME, attempts were made to improve the rheological properties of Tw20T, the best formulation previously evaluated. A possible approach is represented by the direct addition of a thickener. Different compounds were added to the blank microemulsion; the concentration used and the result obtained are summarized in Table II.

Table II. Type and (%w/w) concentration of the thickening agents evaluated to increase Tw20T viscosity

Thickening agent	Concentration % (w/w)	Observed characteristic
Pluronic® F127 (Poloxamer 407)	20	No viscosity increase
Sodium hyaluronate	1	No viscosity increase
	2	Slight viscosity increase Presence of a precipitate
Sodium alginate	5	No viscosity increase Presence of a precipitate
Crosslinked polyacrylic acid polymer (Carbopol® 940)	1	Viscosity increase Clear gel formation
Fumed silica (Aerosil® 200)	2-7	Concentration-dependent viscosity increase At 7%, formation of a viscous clear gel

Poloxamer 407 (Pluronic® F127) dissolved in the ME at 20% w/w without any viscosity increase neither at ambient temperature nor at 37°C, probably because the formation of micelles[44], responsible for the temperature-dependent viscosity increase, is here prevented, due to the low water percentage and the high surfactant content. Similarly, the poor performance of hydrocolloids (sodium hyaluronate and sodium alginate) could be put into relation with the limited water amount in the formulation. On the contrary, the addition of Carbopol® 940 (a crosslinked polyacrylic acid polymer) at 1% w/w significantly increased ME viscosity. Actually, polyacrylic acids are able to thicken also polar organic solvents such as alcohols and glycols, and can interact with ethoxylated surfactants (such as Tween® 80) via hydrogen bonding between the carboxylic groups and the oxygen of the ethyleneoxide chain[45]. The viscosity obtained is however lower than the one obtained in water for comparable concentrations, maybe due to the suboptimal pH. The addition of NaOH or ethanolamine for neutralization was evaluated, but phase separation occurred. Additionally, it was not possible to further increase the viscosity by increasing Carbopol concentration, due to its limited solubility in Tw20T.

After silica addition, the viscosity of Tw20T increased gradually for concentrations from 3 to 8% (w/w), than abruptly for higher concentrations (Figure S2, supplementary material). Literature data[46, 47] report an inverse correlation between the capability of silica particles to interact with the system and the gelation power. In fact, the higher the affinity for the system (with hydrogen bond formation), the lower the possibility of silica-silica interaction, necessary for the network formation and viscosity increase. Here, for silica concentration up to 8% the interaction with the polar component of the ME is predominant, while at higher percentage an indirect interaction between silica particles via the molecules in the liquid occurs[48].

Thickened microemulsions containing 1% Carbopol® and 8% silica were selected for further studies; thus, their rheological properties were evaluated using a cone and plate rheometer (Figure 2 and Figure 3S).

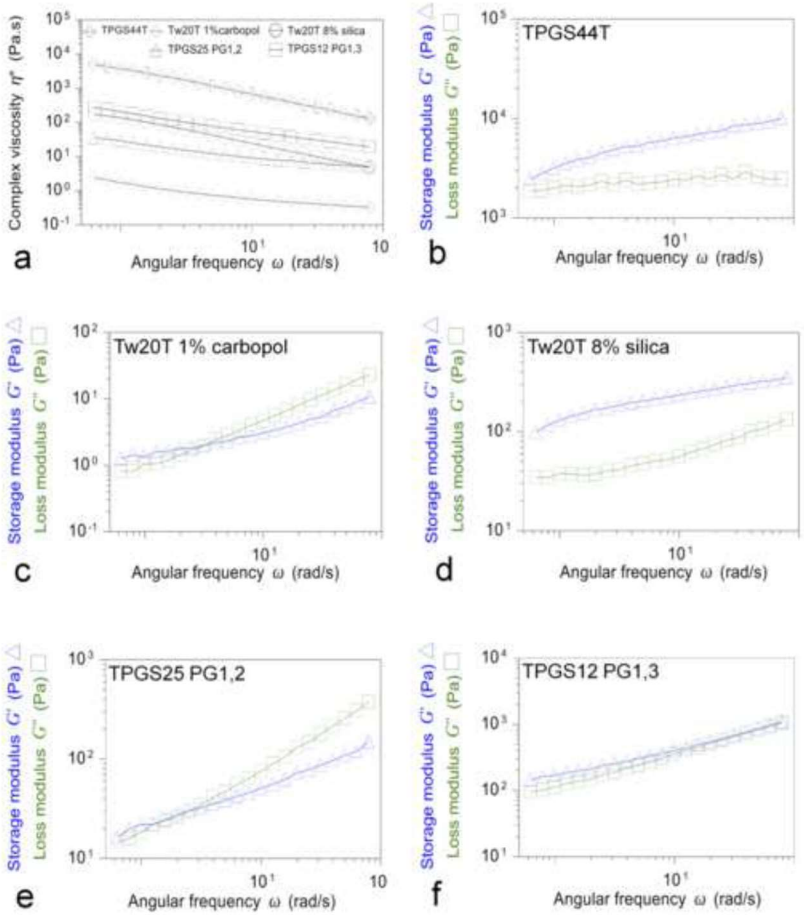

Figure 2. Rheological behavior of Tween®80-based thickened microemulsions and TPGS-based viscous systems. The curves relative to TPGS44T (panel b) is adapted with permission from35 Copyright 2018 American Chemical Society. In panel a) complex viscosity against angularfrequency is reported: all formulations tested show a pseudoplastic behaviour. Panel b)-f) storage (G'; triangle) and loss (G''; square) moduli of the different MEs. In panel b) and d) the storage modulus appears always larger than the loss one, identifying two MEs having a predominant elastic behaviour. G'' appears higher than G' in TPGS12 PG1,3 (f), at least until an angular frequency of 1 rad/s, when two moduli start to be superimposed; however, no crossover can be detected. Conversely, a crossover point between G' ad G'' can be observed in panel c) and e): in both cases material shows a transition from solid to liquid-like behaviour, due to the increase of angular frequency. (In all cases, the analysis was run at 23°C; strain 0.1% for Tw44T, Tw20T 8% silica, TPGS25 PG1,2 and TPGS12 PG1,3; strain 1.3% for formulation Tw20T 1% Carbopol).

Both formulations showed a pseudoplastic behavior with a significantly higher viscosity for the silica-thickened formulation (Figure 2a). The analysis of the storage (G') and loss (G'') moduli highlights a predominant elastic behavior for Tw20T thickened with 8% silica (loss modulus is always larger than the storage one, Figure 2d). In case of Tw20T thickened with 1% Carbopol®, a crossover point between G' ad G'' can be observed indicating a transition from solid to liquid-like behaviour, due to the increase of angular velocity (Figure 2c).

The two gels, loaded with 6 mg/ml cyclosporine were then applied to the skin. Both Carbopol® and silica thickening drastically reduced cyclosporine skin deposition (Figure 1) that dropped down to the level of the control solution. The reason of this low performance could be attributed to a more

difficult diffusion of the drug into the formulation, not so much due to the higher viscosity, but rather to a change of microemulsion structure. A very recent paper of Michniak-Kohn[49] showed, on the contrary, that the addition of 1% Carbopol® did not interfere with the skin delivery of a lipophilic drug (clotrimazole) from microemulsion, but the composition was different and, above all, the water percentage was much higher (from 50 to 65%).

Given the low and highly variable accumulation results obtained with these systems, a change in the surfactant was made, and a soluble derivative of vitamin E (TPGS, D-α-Tocopheryl polyethylene glycol 1000 succinate) was evaluated.

3.3. Self-gelifying TPGS-based microemulsions

Since TPGS is known to be able to form viscous microemulsions[35, 50-52], the following step was the construction of pseudo-ternary phase diagrams using oleic acid as oil phase and TPGS as surfactant. The co-surfactant selected (1:1 v:w ratio with TPGS) were glycols used in cosmetic and/or pharmaceutical products: 1,2 propanediol (propylene glycol), Transcutol® (diethylene glycol monoethyl ether) and 1,3 propanediol that, recently evaluated, demonstrated to be safe, showing a very low potential of skin reactivity[53]. Figure 3 illustrates the phase diagrams obtained.

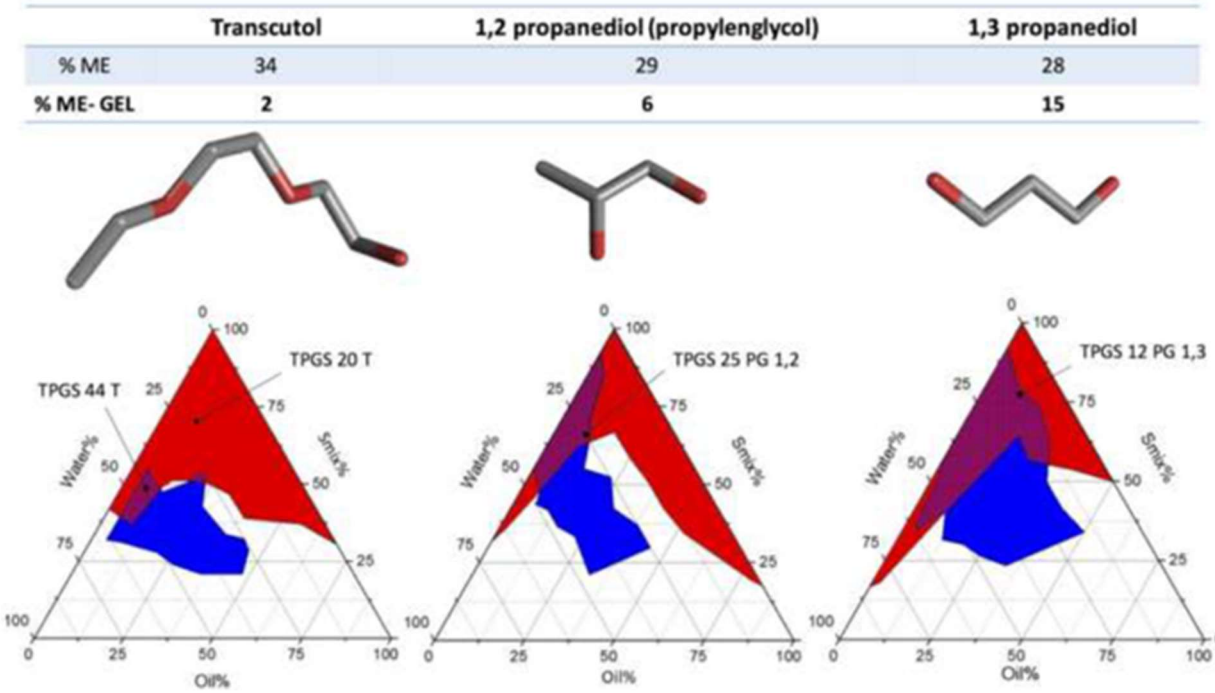

Figure 3. Pseudo-ternary phase diagram of the surfactant/co-surfactant (Smix) – Oil – Water systems. The oil phase is oleic acid, Smix is a mixture of TPGS/co-surfactant 1/1 (w/v). Cosurfactants used are Transcutol®, 1,2 propanediol (propylene glycol), and 1,3 propanediol. The diagram obtained with Transcutol® is adapted with permission from 35 Copyright 2018 American Chemical Society. The red region indicates transparent formulations, the blue region indicates

viscous formulations; the overlapping domains (clear and highly viscous formulations) are represented in dark violet. In the remaining region, low viscosity coarse turbid emulsions or phase separated systems were observed. The table in the figure reports the extension of the area corresponding to transparent formulations (%ME) and to transparent and viscous formulations (%ME-GEL) expressed as a percentage of the total diagram area. The 3D conformations of the cosurfactants are from PubChem (https://pubchem.ncbi.nlm.nih.gov); oxygen atoms are represented in red, hydrogens are not shown.

In each diagram, two different regions are highlighted: in red that of viscous formulations, in blue that of clear transparent formulations. The overlapping domains represent clear and highly viscous systems, linked to the formation of ordered lamellar structures[35]. In the remaining region, low viscosity coarse turbid emulsions or phase-separated systems were observed. In case of high viscosity systems obtained with 1,2 propanediol and 1,3 propanediol as co-surfactants, it was necessary to heat the components at 50°C in order to favour homogenization and mixing.

By comparing the three diagrams it is possible to highlight the role of the co-surfactant; first of all, using Transcutol® it is not possible to obtain viscous systems when the oil/Smix ratio is 0.5/9.5, suggesting that a certain percentage of oleic acid is necessary to obtain ordered structures. On the contrary, in the presence of propanediols, the gelification occurs with an oil/Smix ratio of 0.5/9.5 and even in the absence of oleic acid. To further comment the pseudo-ternary diagrams, some parameters can be calculated and compared such as the area of the ME in the phase diagram (ME%) and the area of the ME gel-transparent region; the values, reported in Figure 3, highlight that, while the extension of the ME region is comparable for the three co-surfactants –even if with different shapes - the ME-gel region is highly dependent on co-surfactant structure. Apparently the linear 1,3 propanediol (Figure 3) facilitates the formation of ordered phases if compared with 1,2 propanediol, and the higher steric hindrance of Transcutol® further reduces the possibility of formation of ordered lamellar structure.

Then, drug-loaded systems were prepared: cyclosporine was dissolved in the 1.25/8.75 oil/Smix and then water was added until the achievement of a viscosity suitable for cutaneous application but not so high as to need heating for homogenization (see method section, paragraph “Pseudo-ternary phase diagram construction”). This happened for 44 % of water in case of Transcutol®, 25% of water in case of PG 1,2 and 12% in case of PG 1,3 (see Figure 3). Analysis by polarized light microscopy reveals that these formulations are anisotropic and show peculiar patterns, typical of birifrangent ordered structures (Figure 4).

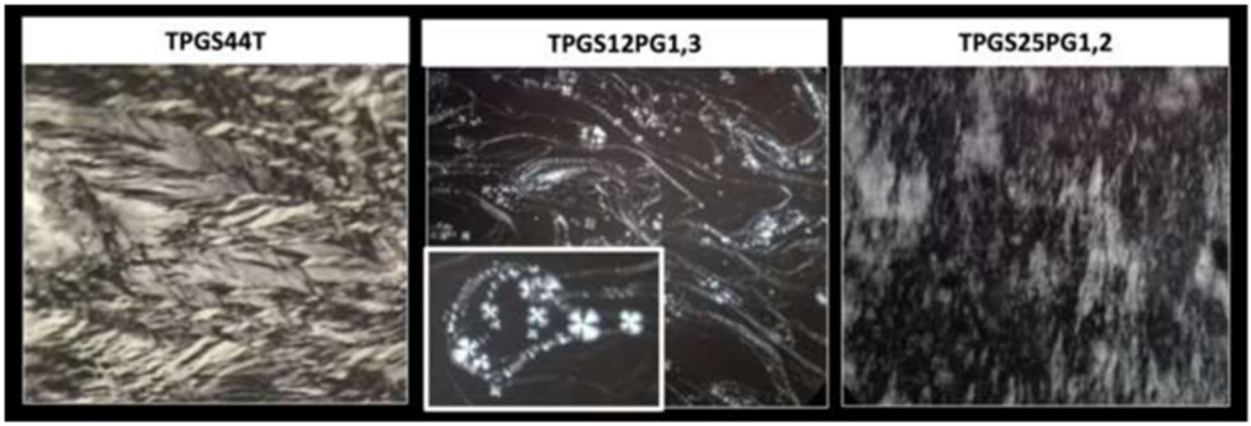

Figure 4. Polarized-light microscopy (10X) images of the TPGS-based microemulsions containing Transcutol®, PG1,3 or PG1,2 as co-surfactant. An enlargement of TPGS12PG1,3 image is shown to highlight the presence of Malta crosses.

The pattern changes as a function of temperature and sample manipulation, however, the presence of Maltese crosses is visible only in case of TPGS12 PG 1,3, indicating that – maintaining constant the Oil/Smix ratio- lamellae are differently organized as a function of co-surfactant type and/or water content. The formation of lamellar structure is responsible for the increased viscosity of the systems; the rheological properties are reported in Figure 2 and show a pseudoplastic behavior with a viscosity in the order TPGS44T>TPGS12PG1,3>TPGS25PG1,2 (panel a). The analysis of the storage (G') and loss (G'') moduli highlights a predominant elastic behavior for TPGS44T, the storage modulus being always larger than the loss one (Figure 2b). G'' is slightly higher than G' in TPGS12 PG1,3 (panel f), at least until an angular frequency of 1 rad/s, when the two moduli become superimposed, without ever reaching a crossover (G''= G'). On the contrary, a crossover point is observed in Figure 2e, where, by increasing angular frequency, TPGS23PG1,2 shifts from solid to liquid-like.

3.4. Cyclosporine skin accumulation from TPGS-based microemulsions

A low viscosity anisotrope TPGS-based microemulsion containing 20% water and Transcutol® (TPGS20T in Figure 3) was initially evaluated. This microemulsion was previously analysed by X-ray scattering and showed the structure of a dispersed system[35]. The DLS analysis highlighted the presence of two population of particles centered at about 20 and 5 nm, however the quality of the measure is always low (data not shown). The amount of cyclosporine accumulated (Figure 5a) was comparable to the control.

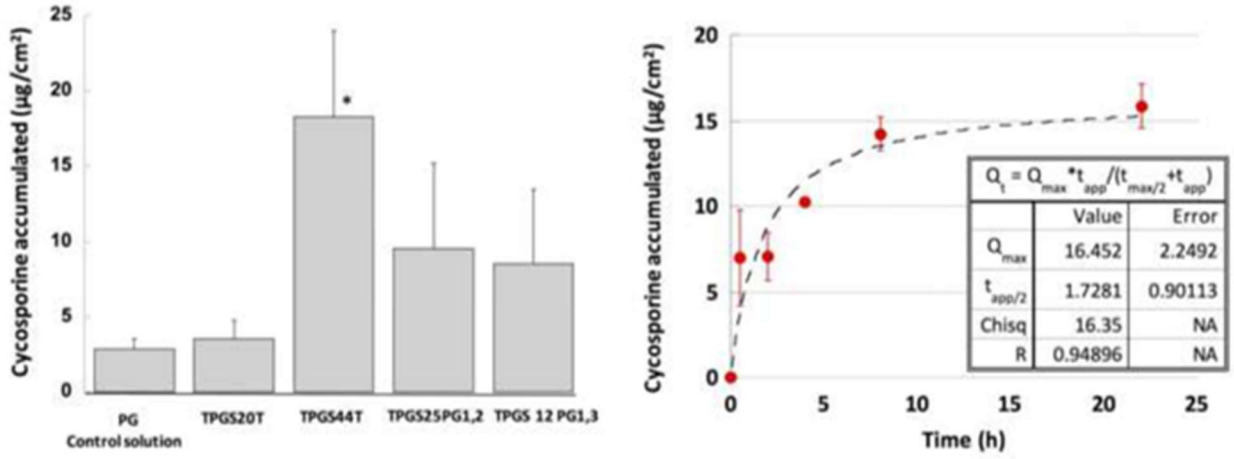

Figure 5. Panel a) Cyclosporine skin deposition (mean±sd) from microemulsions made with oleic acid as oil phase, TPGS as surfactant and different co-surfactants. The exact composition of the vehicles is illustrated in Table I. All the vehicles contain 6 mg/g cyclosporine. * statistically different from control solution, TPGS20T and TPGS12PG1,3 (for TPGS 25 PG 1,2, p=0.06). Panel b) Cyclosporine skin deposition from TPGS44T as a function of application time. All the experiments presented in panel b were performed the same day using the same skin batch.

The data collected using viscous lamellar systems highlight, on the contrary, a considerable increase in cyclosporine accumulation. The enhancement factor (i.e. the ratio between the amount of drug accumulated from the microemulsions and the amount of drug accumulated from the control solution) resulted 6.3±2.0, 3.3±2.0 and 2.9±1.7 µg/cm^2 for TPGS 44 T, TPGS 25 PG1,2 and TPGS 12 PG 1,3, respectively. The good results obtained with TPGS-based viscous microemulsion can be attributed to the lamellar structure that has previously demonstrated to enhance the skin retention of hydrophobic drugs[35]. However, in case of propanediol 1,2 and propanediol 1,3 as co-surfactants, the ANOVA highlighted the lack of statistical difference with respect to the control solution. On the contrary, TPGS 44 T is statistically higher than PG control solution, TPGS 20 T and TPGS 12 PG 1,3 (for TPGS 25 PG 1,2, p=0.06). Differences between the three TPGS-based gel-like formulations could be due to a different internal structure (see Figures 2 and 4). Indeed, Borghet-Cardoso *et al*[42] studying nicotine transdermal transport from liquid crystalline systems made with TPGS, isopropylmiristate and propylenglycol found a different drug release and skin permeation depending on the presence of a cubic *vs* lamellar phase.

To further discuss the role of surfactant and microemulsion structure on cyclosporine skin delivery, the data obtained from ME with the same co-surfactant (Transcutol®) and water content (20% and 44%) are compared in Figure 6. When water content is 20%, both microemulsions are characterized by the presence of disconnected droplets (Figure 1S and reference [35]) Given the similar structure, the same co-surfactant and the same water percentage, we can hypothesize that the surfactant

used affects the packing of the interfacial layer thus influencing drug release. Indeed, despite the similar MW (*approx.* 1500 Da for TPGS and 1300 for Tween® 80), the two molecules have different lipophilicity (Tween® 80 HLB=15; TPGS HLB=13) and, above all, a completely different geometry, with an extended polyethylene glycol headgroup in case of TPGS (Figure 6) that could support a closer packing. An additional explanation could be related to a different effect of the surfactants on stratum corneum organization and permeability[31, 54].

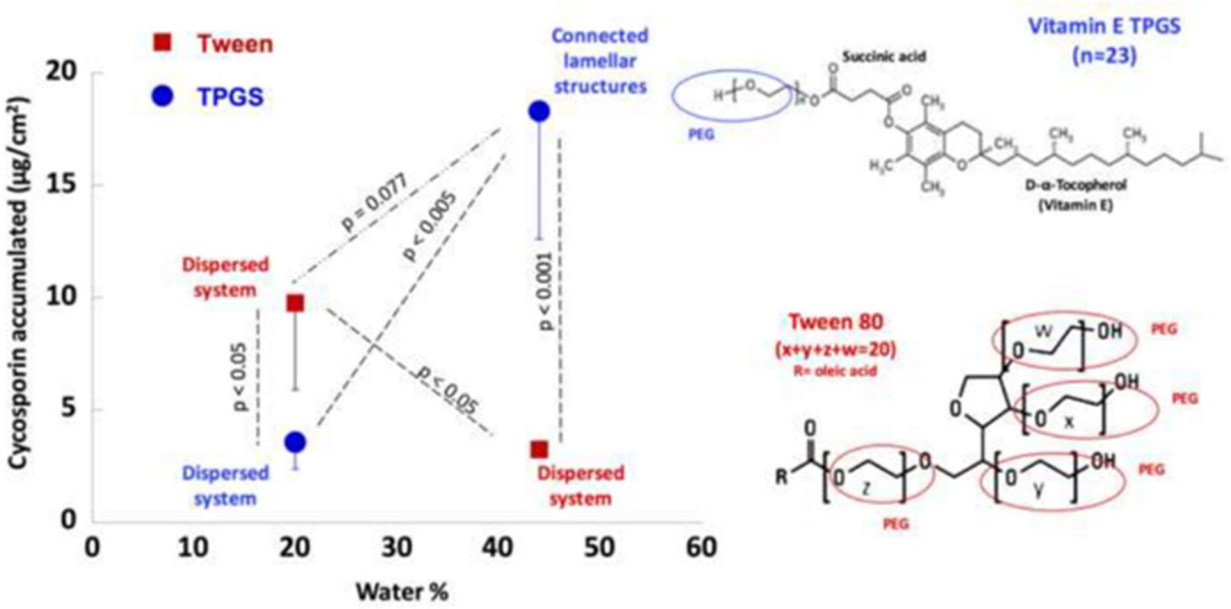

Figure 6. Cyclosporine skin deposition (mean±sd) as a function of ME water content. Microemulsions were made with oleic acid as oil phase, TPGS or Tween® 80 as surfactant and Transcutol ad co-surfactant. The exact composition of the vehicles is illustrated in Table I. All the vehicles contained 6 mg/g cyclosporine.

When the water content is increased to 44%, a reversal result is obtained, with a significant reduction of cyclosporine uptake in case of Tween®80 ME, and significant increase for TPGS systems, despite the much higher viscosity that could, at least in principle, slow down drug diffusion. In this case, microemulsion mesostructure plays the relevant role, being, in case of TPGS44T, characterized by interconnected lamellar structures, as previously demonstrated by X-ray scattering[35]. This bicontinuous mesh, in which both hydrophobic and hydrophilic regions are individually connected all over can enhance drug mobility and modify its tendency to partition into the stratum corneum.

The kinetic of cyclosporine skin uptake from TPGS 44 T, the best performing formulation, was then studied. The results obtained are reported in Figure 5b. After 30 minute's application, cyclosporine skin retention was equal to 7.0±2.8 µg/cm^2; the skin deposition increased as a function of time and reaches a plateau between 8 and 22 hours. By processing these data using a Michaelis-Menten

model, as previously done by other authors with flufenamic acid[55] and cyclosporine[22], it is possible to describe the deposition of cyclosporine as a function of formulation application time:

$$Q_t = \frac{Q_{max} * t_{app}}{t_{max/2} + t_{app}}$$

where Q_t is the observed amount of cyclosporine deposited (µg/cm^2), t_{app} is the application time (minutes), Q_{max} is the maximum deposited amount (µg/cm^2) and $t_{max/2}$ is the time needed to reach half of the maximum value giving information on the rate of cyclosporine skin uptake from the ME. From the fitting of the experimental data (Equation in Figure 5b) the calculated parameters highlight a maximum value of 16.5 µg/cm^2 and a relatively quick uptake with a $t_{max/2}$ of 1.7 h.

To evaluate if the drug amounts found in the skin are clinically relevant, it is possible to calculate the average skin concentration (weight of porcine skin: 236±24 mg/cm^2) that results about 30 ng/mg after 2 h and about 43 ng/mg after 4 h. These values are higher than the ones found in keratomes of psoriatic lesions after 1 week of oral daily cyclosporine administration of 5-14 mg/kg (from 1.5 to 2.3 ng/mg)[56] and they are comparable to the levels recovered by Burns *et al*[6] in the epidermis of psoriatic patients after cyclosporine intralesional administration. However, Burns *et al* found that the relation between intralesional dose, tissue levels and efficacy was not clear, due to a substantial variability among patients[6]. The high variability in tissue levels was attributed to possible variations in the depth of intralesional injection and/or to the possibility that biopsy specimens still contain formulation droplets, but it is also important to underline that patient sensitivity to cyclosporine is extremely variable[57] and this could contribute to the difficulties in establishing a clear correlation between skin concentration and efficacy.

Finally, it is necessary to consider a limitation of the present study, where drug distribution between the different skin layers (stratum corneum, viable epidermis, dermis) was not performed. This aspect surely deserves further investigation to figure out cyclosporine penetration depth. In analogy, it will be necessary to assess the tolerability of the systems prepared. In principle, the excipients selected should have a very low irritating potential at the concentrations used (*approx.* 7% for oleic acid and 24.5% for TPGS): cosmetic product formulations containing oleic acid at concentrations up to 13% are not irritants nor sensitizers[58] and TPGS resulted a moderate irritant only when applied daily for 9 days at 75% concentration[59]. The combination of the two compounds by repeated use should, however, be evaluated.

4. Conclusion

The data collected in the present paper underline the important role of microemulsion structure on cyclosporine uptake into the skin. In particular, the data suggest that surfactant type, co-surfactant type, water content and the presence of a rheological modifier are relevant in so far as they impact on microemulsion structure.

Using both Tween®80 and TPGS as surfactants, it was possible to prepare microemulsions potentially useful for cyclosporine skin delivery. These systems could, in principle, contribute to optimizing topical therapy, also in the framework of the new strategies for psoriasis treatment, involving combination, rotational and sequential therapy.

Acknowledgements

The authors want to thank Dott. Pierugo Cavallini and Macello Annoni S.p.A. (Busseto, Parma, Italy) for kindly providing porcine eyes and BASF for providing Kolliphor® TPGS. Former undergraduate students Greta Bellini and Noemi Frattini are gratefully acknowledged for the contribution in data collection and Massimiliano Rinaldi for support in rheological measurement.

REFERENCES

1. Di Meglio, P.; Villanova, F.; Nestle, F.O., *Psoriasis.* Cold Spring Harb Perspect Med, 2014. **4**(8).
2. Menter, A.; Korman, N.J.; Elmets, C.A.; Feldman, S.R.; Gelfand, J.M.; Gordon, K.B.; Gottlieb, A.; Koo, J.Y.; Lebwohl, M.; Lim, H.W.; Van Voorhees, A.S.; Beutner, K.R.; Bhushan, R., *Guidelines of care for the management of psoriasis and psoriatic arthritis. Section 3. Guidelines of care for the management and treatment of psoriasis with topical therapies.* J Am Acad Dermatol, 2009. **60**(4): p. 643-59.
3. Papp, K.A.; Bissonnette, R.; Gooderham, M.; Feldman, S.R.; Iversen, L.; Soung, J.; Draelos, Z.; Mamolo, C.; Purohit, V.; Wang, C.; Ports, W.C., *Treatment of plaque psoriasis with an ointment formulation of the Janus kinase inhibitor, tofacitinib: a Phase 2b randomized clinical trial.* BMC Dermatol, 2016. **16**(1): p. 15.
4. Chiricozzi, A.; Pitocco, R.; Saraceno, R.; Nistico, S.P.; Giunta, A.; Chimenti, S., *New topical treatments for psoriasis.* Expert Opinion on Pharmacotherapy, 2014. **15**(4): p. 461-470.
5. Koo, K.; Jeon, C.; Bhutani, T., *Beyond monotherapy: a systematic review on creative strategies in topical therapy of psoriasis.* J Dermatolog Treat, 2017. **28**(8): p. 702-708.
6. Burns, M.K.; Ellis, C.N.; Eisen, D.; Duell, E.; Griffiths, C.E.; Annesley, T.M.; Hamilton, T.A.; Birnbaum, J.E.; Voorhees, J.J., *Intralesional cyclosporine for psoriasis. Relationship of dose, tissue levels, and efficacy.* Arch Dermatol, 1992. **128**(6): p. 786-90.
7. Hermann, R.C.; Taylor, R.S.; Ellis, C.N.; Williams, N.A.; Weiner, N.D.; Flynn, G.L.; Annesley, T.M.; Voorhees, J.J., *Topical ciclosporin for psoriasis: in vitro skin penetration and clinical study.* Skin Pharmacol, 1988. **1**(4): p. 246-9.
8. Kumar, R.; Dogra, S.; Amarji, B.; Singh, B.; Kumar, S.; Sharma; Vinay, K.; Mahajan, R.; Katare, O.P., *Efficacy of Novel Topical Liposomal Formulation of Cyclosporine in Mild to Moderate Stable Plaque Psoriasis: A Randomized Clinical Trial.* JAMA Dermatol, 2016. **152**(7): p. 807-15.
9. Liu, H.; Li, S.; Wang, Y.; Yao, H.; Zhang, Y., *Effect of vehicles and enhancers on the topical delivery of cyclosporin A.* Int J Pharm, 2006. **311**(1-2): p. 182-6.
10. Choi, H.K.; Flynn, G.L.; Amidon, G.L., *Percutaneous absorption and dermal delivery of cyclosporin A.* J Pharm Sci, 1995. **84**(5): p. 581-3.
11. Lopes, L.B.; Collett, J.H.; Bentley, M.V., *Topical delivery of cyclosporin A: an in vitro study using monoolein as a penetration enhancer.* Eur J Pharm Biopharm, 2005. **60**(1): p. 25-30.
12. Chen, M.; Kumar, S.; Anselmo, A.C.; Gupta, V.; Slee, D.H.; Muraski, J.A.; Mitragotri, S., *Topical delivery of Cyclosporine A into the skin using SPACE-peptide.* J Control Release, 2015. **199**: p. 190-7.
13. Kumar, S.; Zakrewsky, M.; Chen, M.; Menegatti, S.; Muraski, J.A.; Mitragotri, S., *Peptides as skin penetration enhancers: mechanisms of action.* J Control Release, 2015. **199**: p. 168-78.
14. Wang, S.; Kara, M.; Krishnan, T.R., *Transdermal delivery of cyclosporin-A using electroporation.* J Control Release, 1998. **50**(1-3): p. 61-70.
15. Liu, H.; Li, S.; Pan, W.; Wang, Y.; Han, F.; Yao, H., *Investigation into the potential of low-frequency ultrasound facilitated topical delivery of Cyclosporin A.* Int J Pharm, 2006. **326**(1-2): p. 32-8.
16. Romero, G.B.; Arntjen, A.; Keck, C.M.; Muller, R.H., *Amorphous cyclosporin A nanoparticles for enhanced dermal bioavailability.* Int J Pharm, 2016. **498**(1-2): p. 217-24.

17. Frusic-Zlotkin, M.; Soroka, Y.; Tivony, R.; Larush, L.; Verkhovsky, L.; Bregegere, F.M.; Neuman, R.; Magdassi, S.; Milner, Y., *Penetration and biological effects of topically applied cyclosporin A nanoparticles in a human skin organ culture inflammatory model.* Exp Dermatol, 2012. **21**(12): p. 938-43.
18. Musa, S.H.; Basri, M.; Fard Masoumi, H.R.; Shamsudin, N.; Salim, N., *Enhancement of physicochemical properties of nanocolloidal carrier loaded with cyclosporine for topical treatment of psoriasis: in vitro diffusion and in vivo hydrating action.* Int J Nanomedicine, 2017. **12**: p. 2427-2441.
19. Niemiec, S.M.; Ramachandran, C.; Weiner, N., *Influence of nonionic liposomal composition on topical delivery of peptide drugs into pilosebaceous units: an in vivo study using the hamster ear model.* Pharm Res, 1995. **12**(8): p. 1184-8.
20. Verma, D.D.; Fahr, A., *Synergistic penetration enhancement effect of ethanol and phospholipids on the topical delivery of cyclosporin A.* J Control Release, 2004. **97**(1): p. 55-66.
21. Guo, J.; Ping, Q.; Sun, G.; Jiao, C., *Lecithin vesicular carriers for transdermal delivery of cyclosporin A.* Int J Pharm, 2000. **194**(2): p. 201-7.
22. Lapteva, M.; Santer, V.; Mondon, K.; Patmanidis, I.; Chiriano, G.; Scapozza, L.; Gurny, R.; Moller, M.; Kalia, Y.N., *Targeted cutaneous delivery of ciclosporin A using micellar nanocarriers and the possible role of inter-cluster regions as molecular transport pathways.* J Control Release, 2014. **196**: p. 9-18.
23. Liu, H.; Wang, Y.; Lang, Y.; Yao, H.; Dong, Y.; Li, S., *Bicontinuous cyclosporin a loaded water-AOT/Tween 85-isopropylmyristate microemulsion: structural characterization and dermal pharmacokinetics in vivo.* J Pharm Sci, 2009. **98**(3): p. 1167-76.
24. Liu, H.; Wang, Y.; Han, F.; Yao, H.; Li, S., *Gelatin-stabilised microemulsion-based organogels facilitates percutaneous penetration of Cyclosporin A in vitro and dermal pharmacokinetics in vivo.* J Pharm Sci, 2007. **96**(11): p. 3000-9.
25. Lopes, L.B.; Lopes, J.L.; Oliveira, D.C.; Thomazini, J.A.; Garcia, M.T.; Fantini, M.C.; Collett, J.H.; Bentley, M.V., *Liquid crystalline phases of monoolein and water for topical delivery of cyclosporin A: characterization and study of in vitro and in vivo delivery.* Eur J Pharm Biopharm, 2006. **63**(2): p. 146-55.
26. Lopes, L.B.; Ferreira, D.A.; de Paula, D.; Garcia, M.T.; Thomazini, J.A.; Fantini, M.C.; Bentley, M.V., *Reverse hexagonal phase nanodispersion of monoolein and oleic acid for topical delivery of peptides: in vitro and in vivo skin penetration of cyclosporin A.* Pharm Res, 2006. **23**(6): p. 1332-42.
27. Devaux, S.; Castela, A.; Archier, E.; Gallini, A.; Joly, P.; Misery, L.; Aractingi, S.; Aubin, F.; Bachelez, H.; Cribier, B.; Jullien, D.; Le Maitre, M.; Richard, M.A.; Ortonne, J.P.; Paul, C., *Adherence to topical treatment in psoriasis: a systematic literature review.* J Eur Acad Dermatol Venereol, 2012. **26 Suppl 3**: p. 61-7.
28. Marty, J.P.; Lafforgue, C.; Grossiord, J.L.; Soto, P., *Rheological properties of three different vitamin D ointments and their clinical perception by patients with mild to moderate psoriasis.* J Eur Acad Dermatol Venereol, 2005. **19 Suppl 3**: p. 7-10.
29. Alisa, E.; Jasmina, H.; Ognjenka, R.; Edina, V. *Measuring the feeling: correlations of sensorial to instrumental analysesof cosmetic products*. 2017. Singapore: Springer Singapore.
30. Zhao, L.; Wang, Y.; Zhai, Y.; Wang, Z.; Liu, J.; Zhai, G., *Ropivacaine loaded microemulsion and microemulsion-based gel for transdermal delivery: preparation, optimization, and evaluation.* Int J Pharm, 2014. **477**(1-2): p. 47-56.
31. Lopes, L.B., *Overcoming the cutaneous barrier with microemulsions.* Pharmaceutics, 2014. **6**(1): p. 52-77.

32. Sabale, V.; Vora, S., *Formulation and evaluation of microemulsion-based hydrogel for topical delivery.* Int J Pharm Investig, 2012. **2**(3): p. 140-9.
33. Chen, H.; Chang, X.; Du, D.; Li, J.; Xu, H.; Yang, X., *Microemulsion-based hydrogel formulation of ibuprofen for topical delivery.* Int J Pharm, 2006. **315**(1-2): p. 52-8.
34. Hosmer, J.M.; Shin, S.H.; Nornoo, A.; Zheng, H.; Lopes, L.B., *Influence of internal structure and composition of liquid crystalline phases on topical delivery of paclitaxel.* J Pharm Sci, 2011. **100**(4): p. 1444-55.
35. Telo, I.; Favero, E.D.; Cantu, L.; Frattini, N.; Pescina, S.; Padula, C.; Santi, P.; Sonvico, F.; Nicoli, S., *Gel-like TPGS-Based Microemulsions for Imiquimod Dermal Delivery: Role of Mesostructure on the Uptake and Distribution into the Skin.* Mol Pharm, 2017. **14**(10): p. 3281-3289.
36. Hosmer, J.M.; Steiner, A.A.; Lopes, L.B., *Lamellar liquid crystalline phases for cutaneous delivery of Paclitaxel: impact of the monoglyceride.* Pharm Res, 2013. **30**(3): p. 694-706.
37. Milak, S.; Zimmer, A., *Glycerol monooleate liquid crystalline phases used in drug delivery systems.* Int J Pharm, 2015. **478**(2): p. 569-87.
38. Lawrence, M.J.; Rees, G.D., *Microemulsion-based media as novel drug delivery systems.* Adv Drug Deliv Rev, 2000. **45**(1): p. 89-121.
39. Grimaudo, M.A.; Pescina, S.; Padula, C.; Santi, P.; Concheiro, A.; Alvarez-Lorenzo, C.; Nicoli, S., *Poloxamer 407/TPGS Mixed Micelles as Promising Carriers for Cyclosporine Ocular Delivery.* Mol Pharm, 2018.
40. Erramreddy, V.V.; Ghosh, S., *Influence of emulsifier concentration on nanoemulsion gelation.* Langmuir, 2014. **30**(37): p. 11062-74.
41. Laddha, P.; Suthar, V.; Butani, S., *Development and optimization of self microemulsifying drug delivery of domperidone.* 2014, 2014. **50**(1): p. 10.
42. Ruela, A.L.M.; Perissinato, A.G.; Lino, M.E.d.S.; Mudrik, P.S.; Pereira, G.R., *Evaluation of skin absorption of drugs from topical and transdermal formulations.* Brazilian Journal of Pharmaceutical Sciences, 2016. **52**: p. 527-544.
43. Telo, I.; Pescina, S.; Padula, C.; Santi, P.; Nicoli, S., *Mechanisms of imiquimod skin penetration.* Int J Pharm, 2016. **511**(1): p. 516-523.
44. Jiao, J., *Polyoxyethylated nonionic surfactants and their applications in topical ocular drug delivery.* Adv Drug Deliv Rev, 2008. **60**(15): p. 1663-73.
45. Barreiro-Iglesias, R.; Alvarez-Lorenzo, C.; Concheiro, A., *Poly(acrylic acid) microgels (carbopol® 934)/surfactant interactions in aqueous media: Part I: Nonionic surfactants.* Int J Pharm, 2003. **258**(1): p. 165-177.
46. Raghavan, S.R.; Walls, H.J.; Khan, S.A., *Rheology of Silica Dispersions in Organic Liquids: New Evidence for Solvation Forces Dictated by Hydrogen Bonding.* Langmuir, 2000. **16**(21): p. 7920-7930.
47. Karakatsani, M.; Dedhiya, M.; Plakogiannis, F.M., *The effect of permeation enhancers on the viscosity and the release profile of transdermal hydroxypropyl methylcellulose gel formulations containing diltiazem HCl.* Drug Dev Ind Pharm, 2010. **36**(10): p. 1195-206.
48. ; Available from: https://www.pcimag.com/articles/86041-successful-use-of-fumed-silica-in-liquid-systems.
49. Zhang, J.; Michniak-Kohn, B.B., *Investigation of microemulsion and microemulsion gel formulations for dermal delivery of clotrimazole.* International Journal of Pharmaceutics, 2018. **536**(1): p. 345-352.
50. Ke, W.T.; Lin, S.Y.; Ho, H.O.; Sheu, M.T., *Physical characterizations of microemulsion systems using tocopheryl polyethylene glycol 1000 succinate (TPGS) as a surfactant for the oral delivery of protein drugs.* J Control Release, 2005. **102**(2): p. 489-507.

51. Carvalho, V.F.; de Lemos, D.P.; Vieira, C.S.; Migotto, A.; Lopes, L.B., *Potential of Non-aqueous Microemulsions to Improve the Delivery of Lipophilic Drugs to the Skin.* AAPS PharmSciTech, 2017. **18**(5): p. 1739-1749.
52. Suppasansatorn, P.; Nimmannit, U.; Conway, B.R.; Du, L.; Wang, Y., *Microemulsions as topical delivery vehicles for the anti-melanoma prodrug, temozolomide hexyl ester (TMZA-HE).* J Pharm Pharmacol, 2007. **59**(6): p. 787-94.
53. Belcher, L.A., *Evaluating 1,3-Propanediol for Potential Skin Effects.* Cosmetics&Toleitries, 2010. **125**(5).
54. Montenegro, L.; Carbone, C.; Puglisi, G., *Vehicle effects on in vitro release and skin permeation of octylmethoxycinnamate from microemulsions.* Int J Pharm, 2011. **405**(1-2): p. 162-8.
55. Wagner, H.; Kostka, K.-H.; Lehr, C.-M.; Schaefer, U.F., *Drug Distribution in Human Skin Using Two Different In Vitro Test Systems: Comparison with In Vivo Data.* Pharmaceutical Research, 2000. **17**(12): p. 1475-1481.
56. Ellis, C.N.; Fradin, M.S.; Messana, J.M.; Brown, M.D.; Siegel, M.T.; Hartley, A.H.; Rocher, L.L.; Wheeler, S.; Hamilton, T.A.; Parish, T.G.; et al., *Cyclosporine for plaque-type psoriasis. Results of a multidose, double-blind trial.* N Engl J Med, 1991. **324**(5): p. 277-84.
57. Hirano, T.; Oka, K.; Umezawa, Y.; Hirata, M.; Oh-i, T.; Koga, M., *Individual pharmacodynamics assessed by antilymphocyte action predicts clinical cyclosporine efficacy in psoriasis.* Clin Pharmacol Ther, 1998. **63**(4): p. 465-70.
58. Liebert, M.A., *Final report on the Safety Assessment of Oleic Acid, Lauric Acid, Palmitic Acid, Myristic Acid, and Steraic Acid.* Journal of the American College of Toxicology, 1987. **6**(3): p. 321-401.
59. Zondlo Fiume, M., *Final report on the safety assessment of Tocopherol, Tocopheryl Acetate, Tocopheryl Linoleate, Tocopheryl Linoleate/Oleate, Tocopheryl Nicotinate, Tocopheryl Succinate, Dioleyl Tocopheryl Methylsilanol, Potassium Ascorbyl Tocopheryl Phosphate, and Tocophersolan.* Int J Toxicol, 2002. **21 Suppl 3**: p. 51-116.