Prolonged-Skin Retention of Clobetasol Propionate by Bio-Based Microemulsions: A Potential Tool for Scalp Psoriasis Treatment

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Abstract

Novel effective and cosmetically acceptable formulations are needed for the treatment of scalp psoriasis, due to the poor efficacy of the current products. The challenge in developing safe, efficient and convenient delivery systems for this drug was addressed in the present work by formulating clobetasol propionate loaded W/O microemulsions. Pseudo-ternary phase diagrams were constructed by using a combination of biocompatible and biodegradable excipients. Characterization studies demonstrated that selected microemulsions had suitable technological features such as: being Newtonian fluids, possessing low viscosity and high thermodynamic stability. Photomicrographs showed a significant alteration of the skin structure after treatment with microemulsions, and a preferential concentration of these in the *stratum corneum* and epidermis. These data, together with *ex vivo* permeation results, suggested an enhanced topical targeted effect due to an increased drug retention efficacy in the upper skin layers, as desired. Moreover, the bio-based excipients selected could contribute to the healing of the psoriatic scalp. In this way, the improvement of clobetasol efficacy is combined with the useful properties of the microemulsion components and with environmental safety.

Keywords: microemulsion, corticosteroid, biocompatible formulation, topical delivery, scalp psoriasis.

Introduction

Psoriasis is a common skin disorder associated with both physiological and psychological distress [1]. Although psoriasis can affect the entire body, the scalp and extremities are most commonly involved. The clinical presentation of scalp psoriasis can be highly variable, ranging from mild disease with light desquamation, to more severe and untreatable forms with thickened crusted plaques that may affect the entire scalp [2]. Different therapeutic agents are presented in the literature for the treatment of scalp psoriasis, from corticosteroids and vitamin D analogues to phototherapy. Despite a wide range of therapy options and an extensive literature base, scalp psoriasis remains difficult to treat as the surface is relatively non-accessible due to the hair and because of its proximity to the facial skin [3]. Scalp psoriasis, therefore, can be a major therapeutic challenge. Most topical treatments have low efficacy and are considered time-consuming by the patients, resulting in poor compliance [4]. Topical corticosteroids (TC) are found to be the most effective of the currently available topical therapies [5]. The safety profile of TC depends both on the type of corticosteroid and the formulation used. Moreover, the scalp is a highly vascularized area with high opportunities for an active substance to enter into circulation, so that TC should be applied with special care on the scalp [6]. Among TC, clobetasol-17-propionate (CP) has proved to be the most potent in the treatment of inflammatory manifestations of scalp psoriasis in few dosage forms, such as solutions, shampoos and foams [7-9] due to its vasoconstrictive, anti-inflammatory, immunosuppressive and anti-proliferative effects [10]. However, traditional dosage forms are safe only for short-term therapies (up to 4 weeks), as topical side effects are also associated to CP treatment, i.e. skin atrophy and

telangiectasia. For all these reasons, this research has focused on a strategy to optimize the potency of CP while minimizing adverse effects, thus improving the CP benefit-risk ratio. This has led to study new topical vehicles for CP delivery to overcome the limits of the drug profile combined to the needs of the pathology.

Colloidal and innovative formulations such as microemulsions (MEs) have been investigated as drug delivery and targeting systems [11, 12] as they offer several advantages over traditional formulations [13-17]. Therefore, the goal of this study was to develop CP-loaded MEs for the potential treatment of scalp psoriasis. In the W/O MEs prepared, the solubility of the drug was improved by using a mixture of oil, surfactant and co-surfactant (i.e lecithin, olive oil, isopropyl myristate). Furthermore, the microemulsions studied in this work were developed paying special attention to the biocompatibility and biodegradability of the excipients employed. Lecithin, used as main surfactant, presents a notable affinity with cellular membranes, thus leading to an increased absorption of several drugs [18, 19]. Isopropyl myristate is a non-toxic ester with good systemic and local tolerance [20] and is pharmaceutically acceptable as the oil component in MEs. Olive oil has been used as a promising excipient for dermatological products, due to its great affinity for the skin and its moisturizing activity [21-23]. The high amount of oil and surfactant phases selected for our systems, as well as their bio-based nature, might be complementary to the drug activity in promoting the healing of psoriatic scalp. This fact, together with the liquid form of MEs, allows for an easy application and a suitable residence time at the target site compared to traditional formulations (mainly shampoos and solutions) or to water-based CP delivery systems already developed [24, 25]. Consequently, formulations proposed have the advantage to improve prolonged CP skin retention (due to both enhancement

effect of microsystems and the nature of the ingredients chosen) without increasing its transdermal permeation. Such a system can also satisfy the increasing need for eco-sustainability of cosmetic and pharmaceutical products and it might improve the compliance of the patient by optimizing the therapeutic efficacy of the CP, reducing side effects.

Materials and Methods

Materials

Clobetasol-17-propionate (CP) (99.5%, batch 9) and egg lecithin (80% phosphatidylcholine from egg, Lipoid E80) were a kind gift from GlaxoSmithKline (Turkey) and Lipoid GmbH (Ludwigshafen, Germany), respectively. Isopropyl myristate (IPM) was purchased from Yilmaz Kimya A.S (Istanbul, Turkey). Extra virgin olive oil (straw yellow with green colour, free acidity = % oleic acid <0.6; peroxide value <15) was obtained from Secchi S.R.L (Italy). Ethanol (96%), 2-propanol (Reag. Ph. Eur.) and acetonitrile (chromatographic grade) were purchased from Merck (Darmstad, Germany). HPLC grade water was obtained using a Milli-Q system from Millipore (Bedford, CA, USA). Paraformaldehyde powder (95%) and fluorescein free acid (Reag. Ph. Eur.) were purchased from Sigma Aldrich (Italy). All other chemicals and reagents were of analytical grade.

Construction of pseudo-ternary phase diagrams

In order to determine the concentration ranges of components for the existing range of MEs, pseudo ternary phase diagrams were constructed using the water titration method at room temperature [11, 26-29]. Two different phase diagrams were prepared with 1:1 and 1:2 weight ratio (K_m) of Lipoid E80/2-propanol and Lipoid E80/ethanol, used as surfactant/co-surfactants mixture. For each phase diagram at a specific weight ratio, the ratios of oil to the mixture of surfactant and co-surfactant (*Smix*) were varied as 0.5:3.5, 1:3, 1.5:2.5, 2:2, 2.5:1.5, 3:1 and 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1. These ratios were taken by previous literature findings (27-29); other ratios were also preliminarily tried (data not reported), but discarded due to the unsuitable appearance of MEs obtained. Distilled water was added dropwise to the mixture of oil and *Smix* under moderate magnetic stirring until the solution became cloudy or turbid. The amount of water required for each procedure was recorded. The pseudo-ternary phase diagram was constructed by plotting the amount of water, oil and *Smix* used in each experiment. The MEs region (transparent solution) was identified as shown in Figure 1.

Preparation of microemulsions

According to the ME regions in the phase diagrams, different ME formulations were prepared by varying the ratio between surfactant/co-surfactant, thus mixing the appropriate amounts of oil phases with *Smix* (Table 1). Olive oil and 2-propanol were chosen as oil phase and co-surfactant for ME1, respectively, while IPM and ethanol were selected for ME2. Egg lecithin was used as surfactant for both formulations. Then, appropriate amounts of water were added with continuous stirring, to obtain a clear isotropic solution. Drug loaded MEs were prepared by adding CP (0.05% w/w) to the

mixture of oil, surfactant and co-surfactant.

Physicochemical characterization of MEs

The pH of ME1 and ME2 was measured by a pH meter (pH 720inoLab, Germany). All measurements were carried out in triplicate at room temperature (n = 3).

Electrical conductivity (EC) of formulations was measured by using a conductivity meter (Cond 3110 SET1, Germany), dipping the electrode in the MEs sample until equilibrium was reached. Before measurements, the conductivity probe was calibrated using standard KCl solution (n = 3).

The average droplet size and polydispersity index (PDI) were evaluated by dynamic light scattering (Zeta sizer, Malvern instruments, UK). All samples were analysed in triplicate in a thermostatic chamber at 25°C without dilution. Refractive indexes (RI) were calculated using a digital refractometer (DR301-95, Germany).

Formulations were centrifuged (Mikro 120 Hettich Zentrifugen, Germany) at 13,000 rpm for 30 min to assess their physical stability, as previously reported [26]. Moreover, storage conditions were tested at room temperature and at 2-8°C to evaluate any macroscopic change in the ME system.

Rheological studies and viscosity measurements

The rheological analysis of the formulations was performed at 25 ± 0.1°C using an AR 2000 controlled stress/controlled rate rheometer (Haake MARS, plate C60/1 Ti, plate cover MPC 60/S QF, Karlsruhe, Germany). In continuous shear analysis, the upward and downward flow curves for each formulation were measured over shear rates ranging

from 10 to 1000 s^{-1}. All experiments were replicated five times for each sample. Results were reported as flow curves (shear stress/shear rate) and viscosity curves as function of shear rate.

Furthermore, formulations were evaluated for their viscosity by using a Vibro Viscosimeter (SV-10 Series, Tokyo, Japan), by placing 10 ml of each sample into a bowl and subjecting it to a vibrating motion for 5 min.

Differential Scanning Calorimetry (DSC) measurements

DSC investigations were carried out by a DSC 214 Polyma (Netzsch, Germany) equipped with refrigerated cooling system; thermoanalytical parameters were obtained by using Proteus v7.0 Software. Approximately 10 mg of each microemulsion, both unloaded and CP-loaded, as well as every single component, were weighted into hermetic aluminium pans and quickly sealed. An empty sealed aluminium pan was used as reference. DSC curves were generated by cooling the samples from 25°C to -100°C (cooling rate 10°C/min) in a nitrogen atmosphere with a flow rate of 40 ml/min [30, 31].

Atomic force microscope (AFM) studies

Morphology of unloaded and CP-loaded MEs was observed using AFM. An aliquot of a solution (5 µl) was placed on a surface of freshly-cleaved muscovite mica (1 cm^2; Agar Scientific, Stansted, Essex, UK), dried in the desiccator, and further dried in a N_2 stream when needed. The surface was then attached to a nickel disk mounting assembly (1 cm^2) using double-sided adhesive tape and placed on top of the AFM scanner. AFM studies were carried out using a Multi-Mode/NanoScope IV scanning probe microscope, Bruker, Santa Barbara, CA, USA and were performed in air under ambient conditions

(T = 22°C, RH = 64%) using the J-scanner (max. xy = 200 µm). Scanning was performed in tapping mode using Si cantilevers with integrated tips (t = 3.2–4.2 µm, l = 145–175 µm, w = 38–42 µm, υ_0 = 200–400 kHz, k = 8.4–57 N m^{-1}, R<10 nm; model: OTESPA-R3, Bruker, France), and an RMS amplitude of 2.0 V. The images were subsequently processed and dimensions measured using NanoScope Analysis software (V 1.2, Bruker).

Determination of CP concentration in MEs

CP content in the formulations was determined using a modified rapid and sensitive high performance liquid chromatographic (HPLC) method [32]. The filtered binary mobile phase, consisting of acetonitrile and water (70:30), was pumped isocratically at a flow rate of 1.0 ml/min at room temperature. The injection volume was 20 µl and the analysis time was 4 min per sample. The retention time of CP was 2.83 min. Peak heights rather than areas in the chromatography were recorded and measured at 240 nm. Concentrations of CP were calculated by interpolation of its respective standard curve. A stock standard solution of CP in acetonitrile (final concentration of 1000µg/ml) was prepared. Working standards of CP were prepared freshly for each assay in the range of 5 -100 µg/ml.

CP loaded MEs (200 µl) were mixed with 1 ml of acetonitrile: water (70:30), vortexed for 2 min and centrifuged at 14,000 rpm for 3 min. Thus, the supernatant solutions were withdrawn and filtered through nylon filters (0.22 µm) before starting HPLC analysis. CP content in formulations was determined using the calibration curve (y=191112x-17056, R^2 = 0.9999). Drug content percentage (n = 3) was calculated from the ratio

between the real drug content and the theoretical one.

Skin treatment with MEs: morphological and penetration studies

Preparation of the skin

Pig ear skin was a kind gift from a local slaughterhouse (Milia S.r.L, Approval Number CE IT 1856 M (Regulation EC 853/2004)). Since porcine and human skins have similar surface lipids, barrier thickness and morphological aspects, pig skin is a preferred model for human skin for topically applied substances [32,33]. Specifically, several works aimed at studying scalp diseases reported the use of porcine ear skin showing comparable results with *in vivo* studies, particularly when considering follicular uptake [25, 34-36]. The pig ears were shaved and samples were treated as previously reported [37].

Histological examination and ultra-structural analysis by SEM

The effect of loaded formulations on treated skin was evaluated in comparison with untreated skin (controls) by histological examination and ultra-structural analysis by SEM. The skin was mounted on a Franz cell system and placed in a water bath at 37°C; approximately 200 µl of each formulation was applied on the skin (surface area: 0.87 cm^2) for 24 h. Two hundred µl of phosphate buffer pH 7.2 was used for control group (CTR). After the time of exposure, the skin samples were removed and fixed using 4% paraformaldehyde solution for 15 h. Samples were treated as previously reported [24,38] and slides observed under light microscope (GX microscopes L1500 BHTG) after staining them with haematoxylin and eosin.

The same procedure was used to treat skin samples for SEM investigations. In this case, after the application time, samples were fixed overnight in 4% paraformaldehyde solution at 4°C. Subsequently, they were washed in 7% sucrose buffer, further fixed with 2% osmium tetroxide for 1h and finally dehydrated in graded concentrations of ethanol. Specimens were processed via 'critical point drying' (CPD) to avoid artefacts such as shrinkage and collapse of surface structures during the final drying. The dried samples were fixed with gold using an ion coater, thus examined by SEM (JEOL-JSM 6060LV).

Confocal laser scanning microscopy (CLSM)

Visualization of the skin penetration was performed by using excised pig ear skin, as previously described. Formulations were labelled with fluorescein as marker and applied to the skin for 24 h. A fluorescein ethanol solution was used as a reference. After the treatment, samples were frozen with liquid nitrogen and isopentane, then sliced perpendicularly to the surface plane (10-20 µm thickness) by using a Cryostat (Leica CM 3050S,Wetzlar, Germany). Slides were examined using a Confocal Laser Scanning Microscope LSM TPMT (Zeiss, Germany): fluorescein was excited using the 488 nm laser and excitation emission was collected between 493 - 625 nm. Moreover, few images were taken by sealing slides using Vectashield® Mounting Medium with DAPI, as a nuclear counterstain.

Ex vivo *permeation studies*

Pig ear skin was cut and mounted on the bottom of a cylindrical plastic support connected to a drive shaft of the dissolution apparatus (Erweka DT 70; Erweka GmbH,

Heusenstamm, Germany) as previously reported [39].

MEs (400 mg) and marketed product (Clobetasolo, ISDIN, 500 μg/ml, aqueous solution) REF equivalent to 0.2 mg CP were uniformly spread on the surface of the skin (area 3.46 cm^2), keeping the epidermis side in contact just with the fluid. The system was then inserted into the vessel containing an ethanol–water (30:70 v/v) solution (100 ml) as receptor medium [32]. Samples of 1 ml were taken from the receptor medium at specified time intervals (1, 2, 3, 4, 5 and 6 h), and immediately refilled with the same volume of fresh solution. CP concentration was determined using HPLC, as previously described. At the end of the 6 h of permeation experiments, the excess of formulations was recovered by washing membranes 3-4 times with the mobile phase. Afterwards, samples were quickly vortexed, centrifuged at 13,000 rpm for 5 min, filtered and analysed in order to calculate the amount of CP not permeated. Successively, the skin was cut into small pieces and kept in the mobile phase for 15 min, under magnetic stirring. Samples were taken and filtered before analysis. Each experiment was replicated three times. The total CP content permeated, retained and remained on the skin was expressed as percentage.

Statistical analysis

Data were analysed using unpaired t-test and the analysis of variance (one-way ANOVA) followed by a Tukey's multiple comparison test (GraphPad Prism, version 6.02; GraphPad Software Incorporated).

Data are shown in both *in vitro* as well *ex vivo* cases as mean ± standard deviation, SD; at least triplicates were performed.

Results

Preparation of microemulsions

Pseudo-ternary phase diagrams were constructed to determine the concentration range of components in the MEs existence range. Areas of the phase diagram containing one-phase systems were identified and samples therein characterized as MEs. Figure 1 shows the pseudo-ternary phase diagrams of the different W/O MEs composed of olive oil, IPM, egg lecithin, 2-propanol, ethanol and distilled water. As shown in Figure 1b, the existing region for ME2 is larger than the area for ME1 (Figure1a), probably due to a higher ability of IPM to include water into the system, compared to olive oil. Moreover, the phase diagrams indicate the exact point corresponding to the optimal concentration for each formulation in the existing area of MEs. The other areas represent the non-microemulsion region with an opaque appearance. According to these results, ME1 and ME2 were easily prepared using the optimal composition of oils, *Smix* and water (Table 1). ME1 was prepared with an optimal surfactant/co-surfactant ratio of 1:1, while 1:2 ratio was used for ME2. Both prepared MEs were homogeneous, transparent, without any precipitates, optically isotropic and yellow coloured in the case of ME1.

Physicochemical characterization of MEs

CP-loaded and unloaded formulations exhibit similar pH values (Table 2). Incorporation

of the drug does not significantly affect the pH values of each microemulsion ($P>0.05$) although some statistical differences can be observed between ME1 and ME2 (Table2). As expected, EC values obtained are quite low, due to the oily nature of MEs. However, a significant difference ($P<0.05$) can be highlighted between the two types of MEs and between ME2 (unloaded and CP-loaded). The average droplet size and PDI values are reported in Table 2. The mean droplet diameters were found very low for both formulations, without relevant differences between drug loaded and unloaded MEs ($P>0.05$). The small PDI of 0.1-0.2 indicates the narrow distribution of the globule size, approaching thus a monodisperse system. The CP content registered after HPLC analyses revealed a high drug loading for both MEs prepared, as the recovery percentages were 94.75 ± 0.0047% for ME1 and 95.73 ± 0.0034% for ME2. The centrifuge test demonstrated that the investigated formulations had good physical stability. All MEs exhibited no phase separation, breaking or drug precipitation. Preferable storage conditions are at 2-8°C, due to Lipoid E80 stability at these temperatures.

Rheological studies and viscosity measurements

Representative flow curves and viscosity *versus* shear rate are graphically presented in Supplementary FigureS1. The shear stress changes upon shear rates increase have been used to determine whether the rheological behaviour of the formulation is Newtonian or non-Newtonian. In continuous shear rheometry, both types of MEs exhibit Newtonian behaviour, as expected from this kind of formulations. Investigated MEs showed proportionality between shear stress and shear rate ($R^2= 0.999$). Moreover, viscosity did not change with increasing shear rate.

Differential Scanning Calorimetry (DSC) measurements

DSC results provide useful information about MEs microstructure and water behaviour. There were no differences between unloaded and drug loaded formulations curves (Supplementary Figure S2), as the thermograms did not reveal any significant effect of the drug on the whole system within the range of temperature used. The clear exothermic peak for ME2 at around -16°C can be attributed to water bound to the surfactant at the interface [30]. No 'bulk' (free) water peak (around 0°C) was observed. However, in the case of ME1 a stronger interaction between water and surfactant at the interface can be hypothesized, this causes the freezing point to move to very low temperatures (-60°C), although with minor intensity, due to a lower water content than ME2. This water behaviour has been already found for W/O MEs in previous works [31, 40]. By comparing the curves of the single components (data not shown), a characteristic peak for ME1 (around -40°C) attributed to olive oil can be also observed. The heating thermograms (data not shown) do not reveal any interesting aspect but presented evident peaks related to the melting point of water.

Atomic force microscope (AFM) studies

Besides delivering a general size overview, AFM was used to characterize the shape and surface structure of the investigated samples [41]. The results are shown in Supplementary Figure S3. AFM images obtained serve as an additional method to further demonstrate that the prepared microemulsions can be classified as nano-scaled drug delivery systems (dimension range 1-10 nm). Overview images showed distinct ME droplets with spherical shape and a good size homogeneity (Supplementary Figure

S3b,c) which can be clearly attributed to the W/O systems herein developed. In this case, we did not report images of drug loaded formulations since it has been already demonstrated that the addition of the drug did not significantly influence the vehicles nanostructure [31]. This assumption is also in accordance to most of experimental parameters above reported.

Skin treatment with MEs: morphological and penetration studies

Histological examination and ultra-structural analysis by SEM

The ultrastructure and the surface of the skin were investigated by observing the photomicrographs and SEM pictures of the pig tissue after treatment with MEs, in comparison with a control sample. The microstructure of untreated skin (CTR) was observed to have a highly packed *stratum corneum* (SC) intercellular domain, with a tight multilayer organization (Figure 2a). SEM images (Figure 3) also showed a homogeneous structure, tight cell junction and minimal keratin fragments. Histological examination of samples treated with ME1 (Figure 2b) showed loose and scattered SC. This pattern was more evident in the case of ME2 (Figure 2c) where a further increase of cell gaps and a flaky appearance of keratin could be evidenced. Furthermore, normal cell junctions were broken, cell nucleus appeared to be wider than ME1 and, obviously, the control. SEM images of skin treated with MEs showed significant modifications of the skin surface compared to untreated skin (Figure 3). The surfaces of (b) and (c) appeared rougher than the control: a separation of corneocytes, thus a weakening of the SC was the phenomenon of the skin desquamating process that can be highlighted for these samples (Figure 2-3b, c). All these results are consistent with previous literature

findings [24,38].

Confocal laser scanning microscopy (CLSM)

The distribution and penetration of MEs into skin layers have been evaluated. From Figure 4a,it can be seen that a clear uptake of the fluorescent dye was revealed along the SC, going towards the epidermal layer, despite a fading of the fluorescence signal. This behaviour was similar for the two MEs, although a greater alteration of the skin could be observed for ME2, due to the presence of ethanol as co-surfactant, leading to a deeper penetration of the fluorescent marker in the skin strata. This result was also in accordance with the histology studies, previously reported. On the other hand, the ethanol fluorescein solution used as control clearly demonstrated an over modification of the untreated skin structure, with massive cell gaps and extremely large cell nucleus (Figure 4c). It can be seen that the fluorescein spread everywhere, without binding to any cellular structure or appendices. This demonstrates that such colloidal drug delivery system can target the absorption and penetration of a drug in specific skin layers, i.e the SC and the epidermis, as desired [42].

Fluorescence images taken with DAPI indicated selective localization of fluorescein along cell membranes, in particular around the nucleus. This can be clearly observed in Supplementary Figure S4 that reports as example an image detail of skin treated with ME1. The fluorescence from DAPI was pseudo coloured as red and fluorescein as green; the image indicates that a more targeted accumulation and retention of the dye into the skin strata was achieved than the control. Fluorescein was detected more weakly in the control sample than MEs (data not shown) demonstrating that the free dye was not able to be properly retained in the skin layers, further according to

Supplementary Figure S4c.

Ex vivo *permeation studies*

The *ex vivo* permeation was exploited to quantitatively assess the behaviour of applied formulations in terms of drug permeated through or accumulated into or on the skin. The permeation profiles of both MEs demonstrate that irrelevant amounts of drug permeate the tissue after 6 h of experiment (Figure 5), a quantity statistically different from either percentages on the skin and inside the skin ($P<0.05$). Nevertheless, around 22% and 12% of the drug was found inside the skin for ME2 and ME1, respectively. A consistent portion was still in the residual formulations, although with statistical differences between ME1 and ME2 ($P<0.05$) (Figure 5). On the contrary, REF showed a different behaviour: after 6 h, most of the drug was accumulated into the skin (75%) while 9.5% of CP permeated (Figure 5).

Discussion

Microemulsions are formed instantaneously when interfacial tension between oil and water is reduced close to zero. Most works in the literature reported pharmaceutically unacceptable components to prepare MEs. There is indeed an increasing trend to the development of innovative and eco-sustainable formulations [43,44]. In this work, the various MEs ingredients were chosen taking into account their biocompatibility in order to comply with environmental safety and improve the acceptability of the final formulation. The use of biodegradable excipients was also aligned with the valorisation

of natural sources, taking advantage over traditional excipients in terms of safety, wide availability and affordability. Although lecithin based-MEs were already known in the literature [45], the combination of this natural phospholipid with other "green" components was successful in this work for the construction of stable W/O MEs, instead of the most common counterparts O/W. However, due to the physicochemical properties of lecithin, MEs are not generally formed using it as primary surfactant: a short chain alcohol as co-surfactant is needed [19]. In this study, 2-propanol was found the most suitable for ME1, while ethanol was selected for ME2, based on pre-formulative studies. Despite well- known as innovative drug delivery systems, microemulsions are suggested in this work for the first time for the topical treatment of scalp psoriasis, due to their advantages in terms of suitable dosage form, green composition and appropriate technological properties. Indeed, other type of CP-loaded MEs with a different topical target have been recently studied, with interesting results [24]. The two prepared MEs showed different abilities to include water into the system (Table 1, Figure 1b). From a technological viewpoint, characterization results demonstrated that MEs prepared had suitable features for topical administration (Table 2), with acceptable pH values for dermal delivery, as already reported [46]. EC measurements generally exhibited a continuously increasing trend with increasing water fraction [47]. Results obtained confirmed this assumption, since higher values were registered for ME2 formulation, due to its higher water content than ME1.

Data from droplet size and PDI can be explained by surfactant levels that can lead to a decrease in surface tension, thus decreasing their sizes [48]. It has already been reported that the use of lecithin alone as main surfactant leads to reduced droplet size (<10 nm), as in this study [49]. In addition, MEs were found to have a high thermodynamic

stability, as already reported [14,24]. Furthermore, the incorporation of CP did not significantly influence any parameter ($P>0.05$), demonstrating a good ability of the formulations to act as vehicle for the steroid. Despite both formulations displaying a Newtonian behaviour, ME2 showed lower values of shear stress and viscosity compared to ME1 (Supplementary Figure S1a,b); this was probably related to the different viscosity of the oily phases used, as well as to the increase in water content in ME2 [29]. Moreover, the higher hydrophilicity of ethanol compared to 2-propanol, as well as the higher ethanol-lecithin ratio, can be a further explanation of this result.

DSC studies confirmed that MEs with a W/O microstructure had a water behaviour altered due to the strong interaction with the surfactant layer becoming bound water molecules, moving its freezing point to low temperatures [50]. However, this trend was more evident in ME1 where the water content is lower than ME2, demonstrating that the type of the oil, the co-surfactant and the nature of the surfactant affected the binding capacity of water [51]. From AFM micrographs, it can be also hypothesized that the ME droplets did not exhibit a clear contour between core and shell, because of the interfacial location of the emulsifier [52]. The combination of these data, together with droplet size results, indicated that our delivery systems can be classed as type II microemulsions with a confinement in water droplets coated by surfactant film within the continuous oil phase [50]. It is known that SC plays an important part in preventing penetration of drugs. Researchers have tried various approaches to either disrupt or weaken the *stratum corneum* to improve skin delivery. The development of nanocarriers and lipid-based delivery systems has been an interesting approach since they can increase skin transportation by improving drug solubilisation in the formulation, drug partitioning into the skin, and by fluidizing the skin lipid [53]. Further, psoriasis is normally

associated to hyperkeratosis; this abnormal growth can be controlled by novel topical medications, such as W/O MEs, that, unlike traditional formulations, are able to manipulate the functions of skin barrier [54]. Despite most O/W MEs reported in literature, in this work W/O MEs were prepared in order to enhance drug penetration and at the same time, limit its permeation to the derma. The drug permeation, in fact, increases with water content being enhanced for O/W morphology with respect to W/O MEs [55].

Histology, SEM and CLSM images obtained showed that MEs might increase the fluidity of the intercellular lipids of the SC, separating corneocytes from each other and desquamating from the intact SC, thus weakening its barrier function. These results agreed with the findings of other studies on topical formulations [24,56]. Slight irritation occurs but it could be an advantage of these systems in the psoriasis treatment because drug penetration (not permeation) could increase. The observed relevant changes induced by both MEs on the skin could be also related to their composition. Ethanol, particularly, is widely used as a penetration enhancer for many drugs. IPM is an excellent enhancer too [57]. The combination of these two components in ME2 should have the consequence of a greater penetration into the SC than ME1, as already reported [58]. Nevertheless, transdermal permeation should be avoided to ensure drug topical effects within the scalp surface only, thus the aim of the work. *Ex vivo* permeation test confirmed this feature, as the released drug scarcely crossed the biological membrane. The observed low permeation of CP through the skin would be beneficial because less free drug was available to deeply cross the skin and cause adverse effects. Secondly, the residual formulation resided in the upper epidermal layers (especially SC) where it might continuously release the drug over time, thus exerting

the therapeutic activity for a long period of time. This profile may be due to the high amount of lipids in our formulations, which delayed the drug release from formulation by increasing the diffusional layer [59]. It is also likely that the lipophilicity of the drug, associated with the lipid character of MEs, was the reason of its retention. This is confirmed by the behaviour of REF: the amount of drug on the skin remained limited due to the aqueous vehicle.

Although most papers suggest the incorporation of MEs into more viscous systems [60] to ensure an appropriate topical application, in this work the low viscous solution form of MEs was proposed as the most suitable for the treatment of scalp psoriasis, due to the difficulty to apply semisolid products in this area. The topical delivery of lipoid carrier systems into the psoriatic skin can solve the problem of lipid imbalance and the absence of normal moisturizing factors, restoring normal skin conditions. Hence, the lipid character of our MEs, the moisturizing properties of olive oil and lecithin, suggested a greater affinity with natural cutaneous lipids in comparison with traditional products that may lead to a better interaction with the SC. On the other hand, a simple oily formulation, although similar to skin lipids, would be unpleasant for patients and lose the MEs penetration properties.

Conclusions

Biocompatible and biodegradable (W/O) microemulsions can be obtained using olive oil or IPM as oils, water, and a mixture of lecithin as surfactant and either 2-propanol or ethanol as co-surfactants. The dermal drug targeting potential has been confirmed by *ex vivo* permeation studies and visualization of skin microstructure via histology as well as

SEM and CLSM techniques. *Stratum corneum* of the skin treated with W/O MEs appeared deeply altered as compared to the control, assuming a CP penetration and drug concentration enhancement in the various upper skin strata. The combination of all elements lead us to consider such bio-based formulations as a promising controlled CP delivery system for the treatment of scalp psoriasis also due to be non-invasive, easily applied, able to enhance patient compliance; furthermore, W/O MEs improve topical delivery and skin retention efficacy of CP, prolong drug release and simultaneously reduce the corticosteroid side effects.

Acknowledgments

Dr Rita Langasco gratefully acknowledges Sardinia Regional Government for the financial support of her PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007-2013 - Axis IV Human Resources, Objective l.3, Line of Activity l.3.1.).

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Conflict of Interest

The authors have no conflict of interest to declare

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Table 1.

Percentage composition (% w/w) of the prepared MEs. Loaded formulations contain CP 0.05% w/w.

Code of Formulations	Oil	*Smix*	Distilled water
ME1	29.47	63.52	7.01
ME2	29.51	59.74	10.75

Table 2.

Physicochemical characterization of MEs

Formulation code	Droplet size (nm ± SD)	PDI	RI	pH	EC (µs/cm ± SD)
ME1	2.25 ± 0.71	0.228 ± 0.08	14.169 ± 0.02	5.74 ± 0.08[a]	47.17 ± 3.20[b]
ME1-CP	3.86 ± 1.31	0.197 ± 0.05	14.141± 0.01	5.78 ± 0.02[c]	40.27 ± 2.77[d]
ME2	2.48 ± 0.60	0.139 ± 0.09	14.452 ± 0.05	6.17 ± 0.06 [a]	170.47 ± 5.84[b,e]
ME2-CP	2.41 ± 0.54	0.133 ± 0.13	14.450 ± 0.03	6.21 ± 0.02 [c]	188.23 ± 2.65[d,e]

PDI= polydispersity index; RI= refractive index. P< 0.05:[a,b]ME1*versus* (*vs*) ME2; [c,d]ME1-CP *vs*ME2-CP; [e]ME2 *vs* ME2-CP.

Figure captions

Figure 1a,b. Pseudo ternary phase diagrams of ME1 (a) and ME2 (b).

Figure 2a-c. Histology sections. Photomicrographs of untreated skin (a), skin treated with ME1 (b) and ME2 (c) after 24h application.

Figure 3a-c. SEM images. Untreated skin surface (a), skin treated with ME1 (b) and ME2 (c) after 24h application.

Figure 4a-c. Confocal laser scanning microscopy images showing the distribution of ME1 (a), ME2 (b) and the control (c).

Figure 5. *Ex vivo* experiments. CP distribution after the permeation test from microemulsions, through porcine ear skin (N=3±SD). $^{\S}P<0.05$: CP permeated *vs* CP into the skin, and *vs* CP on the skin for both MEs; $^{£}P<0.05$: CP into the skin *vs* CP on the skin for REF. $P<0.05$: *ME1 *vs* ME2 and *vs* REF into the skin, #ME1 *vs* ME2 and *vs* REF on the skin.

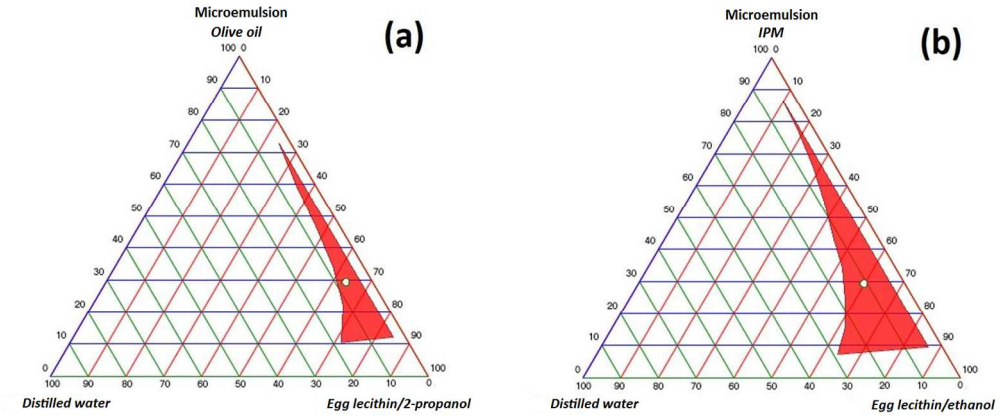

Figure 1a,b. Pseudo ternary phase diagrams of ME1 (a) and ME2 (b).

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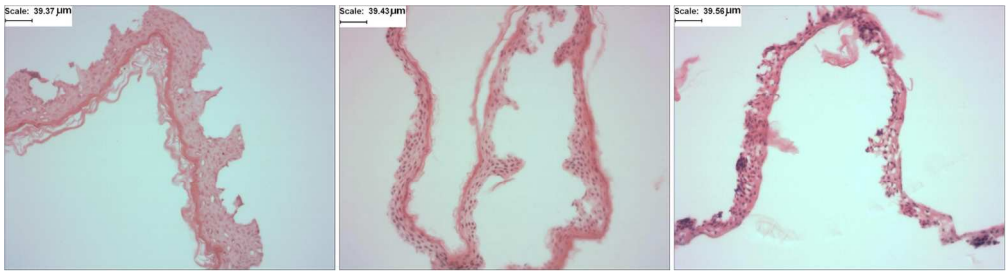

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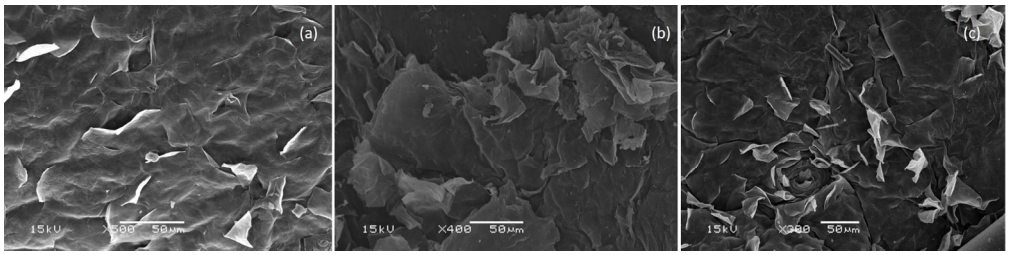

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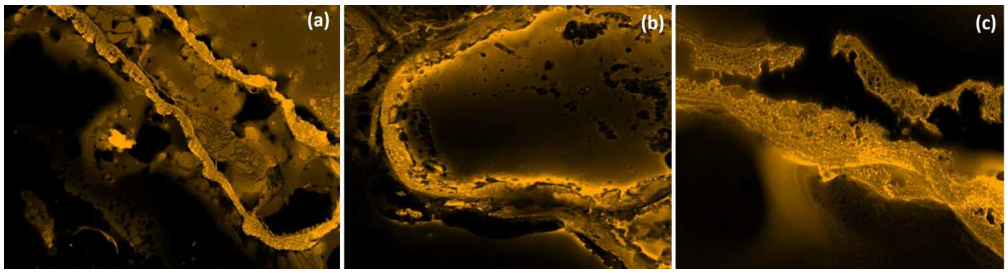

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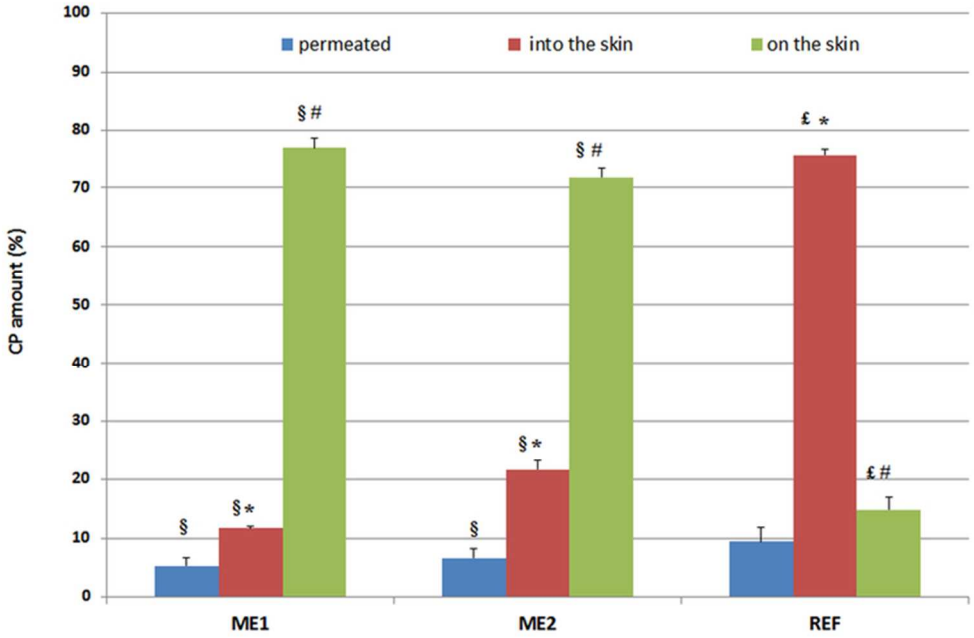

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65x42mm (300 x 300 DPI)

Supporting Information

Prolonged Skin Retention of Clobetasol Propionate by Bio-Based Microemulsions: A Potential Tool for Scalp Psoriasis Treatment

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Figure S1 a, b. Rheological studies. Flow curves (a) and viscosity versus shear rate at 25°C (b) of unloaded and CP-loaded MEs.

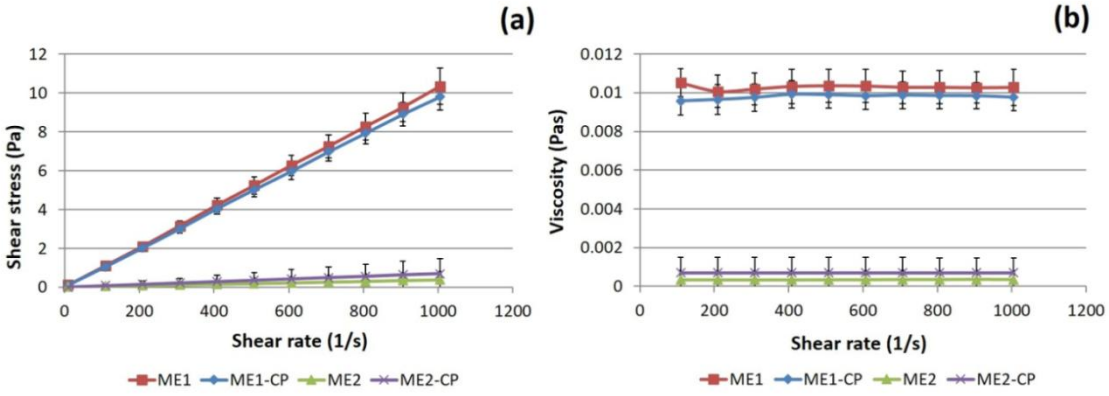

Figure S2. DSC studies. Cooling curves of unloaded and CP- loaded microemulsions.

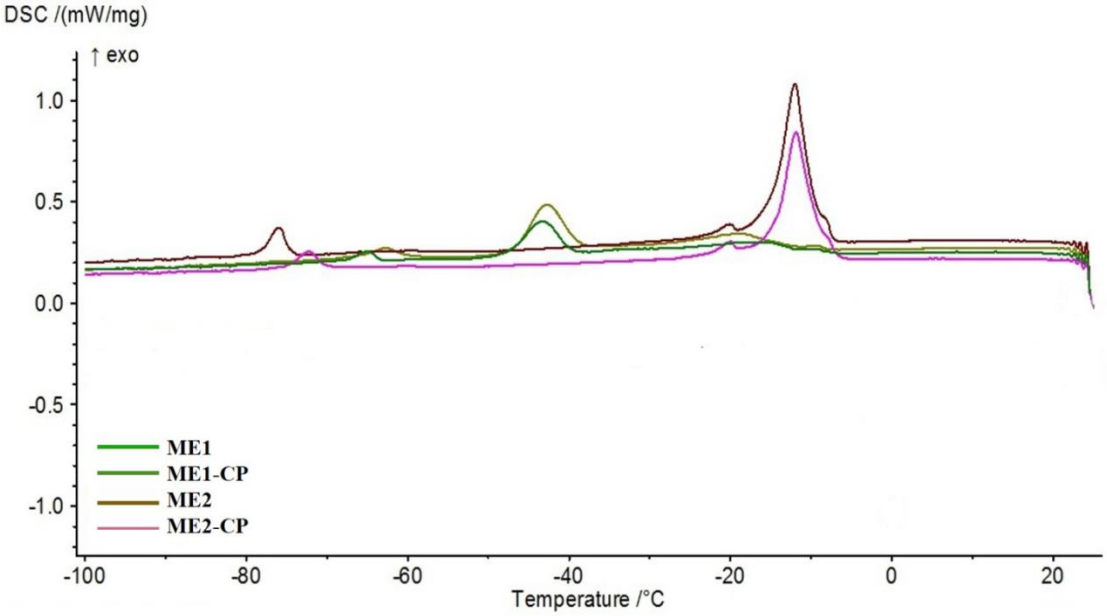

Figure S3 a-c. Example of AFM error signal (a), 2D (b) and 3D topography (c) images of unloaded ME1.

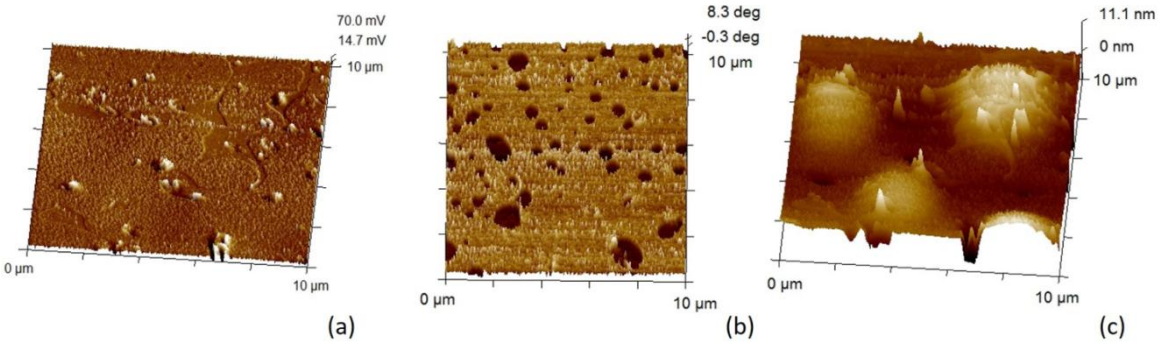

Figure S4 a-c. Confocal laser scanning microscopy images showing the distribution of DAPI (red) as nucleus marker (a), fluorescein (green) around the nucleus (b). Merged images of A and B (c).