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CUBIC LIQUID CRYSTALLINE STRUCTURES IN DILUTED, CONCENTRATED AND HIGHLY CONCENTRATED EMULSIONS FOR TOPICAL APPLICATION: INFLUENCE ON DRUG RELEASE AND HUMAN SKIN PERMEATION

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ABSTRACT

Novel emulsions with a nanostructured continuous phase have been proposed as controlled drug delivery systems to enhance topical delivery of active ingredients avoiding systemic effects. In this study, oil-in-water (O/W) emulsions with two surfactant/water (S/W) weight ratios of 40:60 and 35:65, and oil concentrations of 10wt% (diluted emulsion), 40wt% (concentrated emulsion) and 85wt% (highly concentrated emulsion) have been investigated to identify the presence of liquid crystalline structures and their influence on drug release and skin permeation. The emulsions have been characterized in terms of visual appearance, rheology and drug release. The presence of cubic liquid crystalline structures in emulsions with S/W 40:60 was confirmed by small angle X-ray scattering (SAXS). Rheology results showed a markedly different behaviour in emulsions with S/W 40:60 compared with nonstructured emulsions. A model drug, diclofenac sodium (DS) was successfully incorporated in the emulsions. DS release was studied with hydrophilic and lipophilic membranes, and the amount of DS in the receptor solution was significantly lower in the formulations containing cubic liquid structures. An *in vitro* skin permeation study with dermatomed human skin showed that emulsions with a nanostructured continuous phase are suitable formulations for topical delivery with DS retention in skin layers. The results indicate that the amount of drug retained in skin structures may be tuned by modification of liquid crystal concentration and emulsion structure.

Keywords

Highly concentrated emulsion, cubic liquid crystalline phase, rheology, SAXS, skin permeation, controlled drug release, diclofenac sodium.

1. Introduction

Human skin is a complex organ with a barrier function [1] to protect against external factors, minimizing the entrance of substances and avoiding the loss of water [2]. The development of innovative topical dosage forms to facilitate drug penetration through the stratum corneum (SC) and avoiding to reach the systemic circulation is a challenge [3]. Highly concentrated emulsions also called high internal phase ratio emulsions (HIPREs) [4-5] constitute an interesting class of emulsions that have been reported in the literature as controlled drug delivery systems [6-7]. These emulsions present a large internal phase volume fraction, higher than 0.74, value corresponding to the highest packing density of monodisperse spherical droplets [8-9]. Very high internal phase ratios can be achieved if the droplets are deformed to a polyhedral shape and/or they are polydisperse in size. HIPREs with nanostructured continuous phase consisting of microemulsions [10-11], hexagonal liquid crystals [12-14] or cubic liquid crystals [12, 15-19] have been described and

the influence on the solubilization of active ingredients, drug release properties and emulsion stability have been studied [20-22].

There are only a few studies concerning the effect of the liquid crystalline (LC) structures on drug release and skin permeation. The most studied formulations are formed by monoolein or glyceryl monooleate (GMO). Lopes et al. [23] demonstrated that GMO-based bicontinuous cubic phases significantly enhance the penetration of Cyclosporine A (Cys-A) through the skin. The drug entrapped within these gel-based systems favored a retention of Cys-A in the skin layers, in comparison with reverse hexagonal phases from the same system that permeated down deeper skin layers with transdermal delivery. Yariv et al. [24] studied the permeation of diclofenac salts from GMO-lamellar and bicontinuous cubic liquid crystalline systems. The results showed that the potassium and diethyl amine salts prolonged the release compared with the sodium salts, which presented an accelerated transdermal delivery. Bender et al. [25] studied the distribution of sulforhodamine B in full-thickness human skin from two bicontinuous lipid cubic systems compared to a commercial ointment and a water solution used as references. The cubic phases were prepared from the monoolein/water and phytantriol/water systems, and higher amount of the compound was obtained in the outermost layer of the epidermis for samples containing cubic phases, compared with the references. The micro-fissures formed by microscopic clustering of the keratinocytes in the skin were proposed as the dominating delivery route for cubic phases.

There is a need to determine the influence of nanostructures in the continuous phase of emulsions on skin permeation and specially to investigate nanostructured formulations with low amount of surfactant, minimizing local toxicity. In this context, the aim of the present work is the formulation and characterization of O/W emulsions of the water/Cremophor RH40/Miglyol 812 system and the study of the influence of direct cubic liquid crystalline structures on the release and skin permeation of diclofenac sodium (DS) as a model drug [26]. The results obtained will allow to design a topical dosage form containing diclofenac sodium retained in skin layers with a minimal systemic absorption.

2. Material and methods

2.1. Materials

An ethoxylated non-ionic surfactant, Cremophor® RH40 (Polyoxyl-40 hydrogenated castor oil) from *BASF* (Ludwigshafen, Germany) was used. Miglyol® 812, a mixture of neutral esters of saturated coconut and palm kernel oil-derived caprylic and capric fatty acids and glycerin or propylene glycol, from *Fagron Ibérica S.A.V.* (Barcelona, Spain) was used as oil component. Water was deionized by *MilliQ®* filtration. Diclofenac sodium (DS) was purchased from *Aarti Drugs Ltd.* (Mumbai, India). A hydrophilic cellulose tubular membrane Cellu Sep® T3 with a nominal molecular weight cut-off (MWCO) of 12000-14000 Da, from *Orange Scientific* (Braine l'Alleud, Belgium) was used in the release studies. A lipophilic membrane, Strat-M®, from *Millipore* (Billerica, USA) was used in the *in vitro* permeation studies through synthetic membranes. Dermatomed human skin (0.4 mm), from *Biopredic International* (Saint-Grégoire, France) was used in the *ex vivo* permeation studies. The analytical products used for HPLC analysis were: citric acid, purchased from *Acofarma* (Barcelona, Spain), NaCl and NaOH, purchased from *Fagron Iberica S.A.V* (Barcelona, Spain) and acetonitrile, purchased from *Carlo Erba* (Barcelona, Spain).

2.2.Methods

2.2.1. Determination of phase diagrams

The phase diagram of the water/Cremophor RH40/Miglyol 812 system was determined at 25^{o}C by the stepwise addition method [27]. Surfactant/water (S/W) mixtures were prepared at different weight ratios. The mixtures were

heated at 60°C and stirred with a vibromixer until homogenization and then, the oil was added stepwise. The samples were characterized according to transparency, homogeneity, phase separation, fluency and presence of birefringence (anisotropy) under crossed polarizers (in order to identify lamellar or hexagonal (L_α or H) liquid crystalline phases [18]). Microemulsion regions were confirmed by preparing the mixtures by the direct weight method: all the components were weighted and stirred, and a transparent formulation was obtained.

2.2.2. Preparation of emulsions

O/W emulsions of the water/Cremophor RH40/Miglyol 812 system were prepared at 25°C with surfactant/water weight ratio of 40:60 or 35:65 and different oil content by stepwise addition of the oil phase to the surfactant/water mixture under continuous stirring with a vibromixer. The oil concentrations selected to prepare the emulsions are shown in Table 1.

2.2.3. Drug solubilisation

Emulsions containing 1 wt% of diclofenac sodium (DS) were prepared by adding DS to the initial Cremophor RH40/water mixtures. The samples were stirred with a vibromixer until a transparent appearance was obtained. Then, Miglyol 812 was added by stepwise addition and the formulations were kept at 25°C. To detect the presence of DS crystals in the emulsions, the samples were observed by optical microscopy under polarized light.

2.2.4. Optical microscopy

The morphology of emulsions (shape of droplets and polydispersity) was characterized by optical microscopy with a Nikon Eclipse 50i optical microscope and a Leica DFC295 camera at 25°C. The droplet size distribution was studied by the Image J software, using micrographs of all the emulsions at different magnifications (100x, 400x and 1000x). The diameters of approximately 300 droplets for each formulation were measured. Liquid crystalline phases (lamellar and hexagonal) were characterized by optical microscopy under polarized light at 25°C using an Olympus DX S1 TRF-6 connected to an Olympus DP73 camera.

2.2.5. Small-Angle X-Ray Scattering (SAXS)

Liquid crystals were characterized with S3 MICRO instrument (Hecus X-ray Systems) equipped with a GENIX micro-focus X-ray source (λ = 1.54 Å) operating at a 50kV and 1 mA and a FOX 2D point-focusing element. The scattered intensity was recorded using a position sensitive detector (HECUS). The samples were placed in flame sealed glass capillaries. For calculations of structural parameters derived from SAXS data, the position of the diffraction patterns, known as Bragg peaks, was used to identify the structure of the liquid crystals. Peaks position is related to the distance between the planes of the liquid crystalline structures [28].

2.2.6. Rheology

Rheological studies were conducted by using a rotational stress Haake MARS III rheometer (Thermo Scientific) with parallel cone-plate geometry ((Ø=35mm; cone angle 2°) and 1 mm gap in order to minimize slipping. The flow properties of emulsions were measured by means of the viscosity at low shear rates between 10^{-5} to 10 s^{-1} and 25°C. The viscoelastic properties of emulsions were determined at 25°C. Dynamic strain sweep measurements were performed to identify the linear viscoelastic region (LVE). In these assays, the strain amplitude was progressively increased. In the LVE region, the ratio of stress to strain is only a function of time or frequency and it is independent of

the magnitudes of stress and strain. The dynamic frequency sweep assays were determined in the LVE region. The storage moduli (G') and loss moduli (G'') were studied to establish the frequency dependence of the emulsions.

2.2.7. In vitro drug release and permeation studies through synthetic membranes

The release and permeation studies of DS from the previously characterized emulsions and micellar solutions were carried out by using a Microette Plus equipment (Hanson Research) containing 6 amber Franz diffusion cells [29] with a diffusion area of 1.77 cm^2. Experiments were performed in triplicate. The hydrophilic (Cellu Sep® T3) membrane was used to study the drug release, as the drug will present low interaction with this membrane. The lipophilic membrane (Strat-M®) was used in permeation studies through synthetic membranes, as the composition of the membrane attempt to emulate the structure and composition of the skin. The membrane was placed between the donor and the receptor compartment of the cell. Approximately 200 mg of each formulation were applied on the membrane, and a volume of 7ml of receptor medium (PBS, phosphate buffer solution pH=7.4) was placed in the receptor compartment, which was kept at 32±1 ^oC. The receptor solution was stirred by means of a magnetic stirrer at 600 rpm. Aliquots of 1 ml of receptor solution were withdrawn at predetermined times during 24 hours. The aliquot withdrawn was replaced with the same volume of receptor medium in order to maintain the volume of the receptor solution constant. The concentration of the drug in the receptor medium was not higher than that 10% of its solubility in the medium, ensuring sink conditions.

2.2.8. Ex vivo permeation studies through human skin

The permeation studies of DS from the formulations were conducted with the same equipment and experimental conditions as in the release studies but using as a membrane dermatomed human skin (0.4 mm) from the same donor. The skin was received frozen (Biopredic Int) and stored between -18^oC and -20^oC until its use. The skin was taken out of the freezer and after 30 minutes it was placed between the donor and the receptor compartment of the diffusion cell. Transepidermal water loss (TEWL) was measured before starting each experiment to ensure the integrity of the skin. Aliquots of 1 ml of receptor solution were withdrawn at predetermined times during 24 hours. At the end of the assay, the skin surface was washed to remove the formulation remaining in the donor compartment and skin layers were separated by heating. Skin layers were subjected to DS extraction with acetonitrile and quantified as described below.

2.2.9. Quantification of drug by HPLC

Quantification of DS from the *in vitro* release and skin permeation samples was carried out by HPLC (Shimadzu HPLC equipment) and UV detection (254 nm). A Waters Spherisorb® column (5µm and 4.6x150mm) was used. The system was operated with isocratic gradient using a mobile phase consisting of a mixture of acetonitrile and aqueous phase pH=2.5 at a phase ratio of 65:35 v/v pumped at a flow rate of 1.2mL/min at 25^oC. The sample was injected at a volume of 20µl. The DS retention time was approximately 1.99 min.

2.2.10. Statistical analysis

Amount of DS in the receptor solution and skin layers were compared by a non parametric assay (Mann-Whitney U-test) [30]. NCSS97 software was used and $p<0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1 Phase behavior

The phase behavior of water/Cremophor RH40/Miglyol 812 system at 25°C was investigated by means of phase diagram determinations. The results are shown in Figure 1(A).

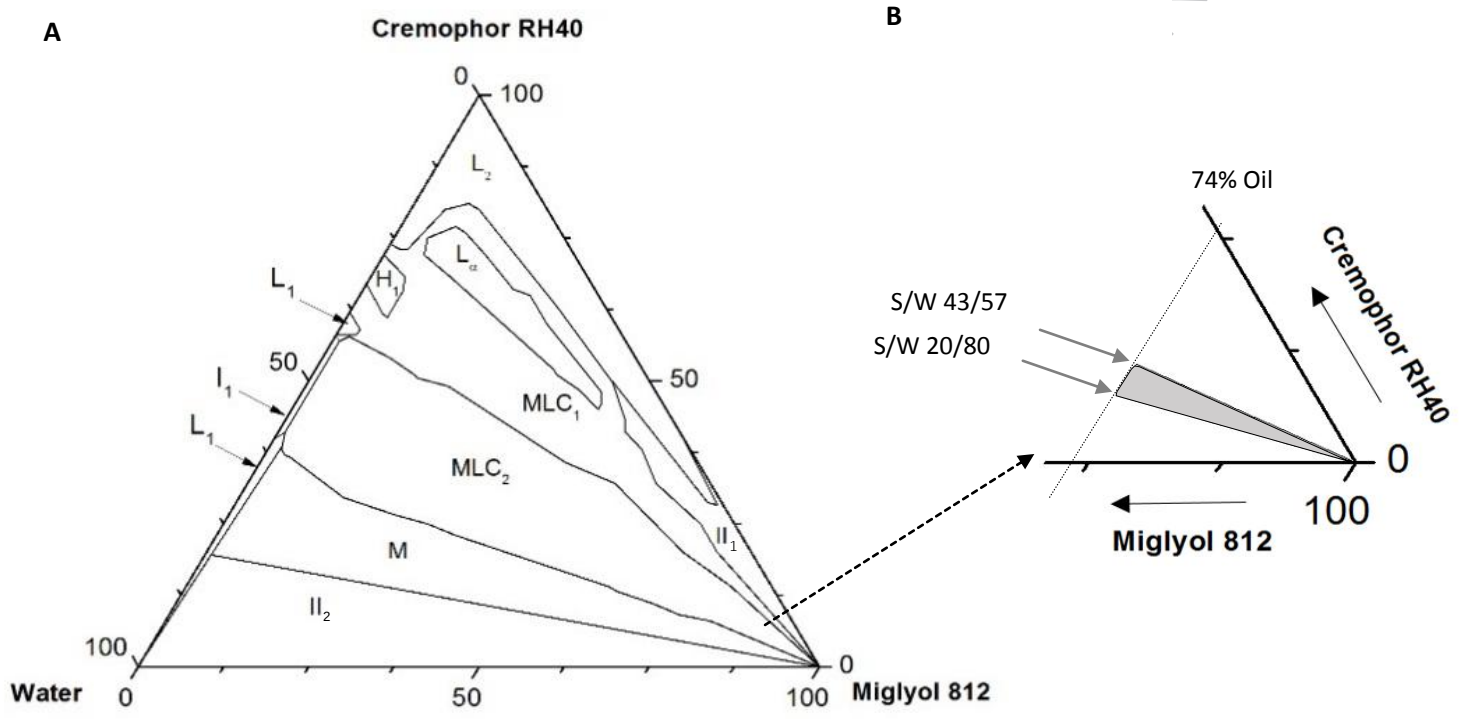

Fig. 1. *(A) Phase diagram of water/Cremophor RH40/Miglyol 812 system, at 25°C. L_1: isotropic liquid direct micellar solution or O/W microemulsion; L_2: isotropic liquid reverse micellar solution or W/O microemulsion; L_α: anisotropic lamellar liquid crystalline phase; H_1: anisotropic hexagonal liquid crystalline phase; I_1: isotropic and highly viscous cubic liquid crystalline phase; MLC_1: multiphase region with presence of anisotropic LC; MLC_2: highly viscous multiphase region with presence of isotropic LC; M: liquid multiphase region; II_1 and II_2: regions with two macroscopically separated liquid phases. (B) HIPRE formation region.*

Different monophasic regions were identified: three isotropic, transparent regions, designated as L_1, L_2 and I_1 (see caption of Fig. 1 for nomenclature); and two optically anisotropic and transparent regions, L_α and H_1. Two L_1 regions were observed along the Cremophor RH40/water axis from a surfactant/water weight ratio of 62/38 to 58/42 with a maximum oil solubilized of 2wt% and from 38/62 to the water vertex. The L_2 region forms up to a surfactant/water weight ratio of 73/28 with a maximum oil content of 65wt%. A transparent gel-like region, isotropic, highly viscous and non-birefringent designated as I_1, was identified as a cubic LC region along the Cremophor RH40/water axis from a surfactant/water weight ratio of 58/42 to 42/58. The maximum oil solubilized in this region is 1 wt%. Birefringent liquid crystalline (LC) phases were identified by using crossed polarizers and the LC structures were characterized by optical microscopy under polarized light. Optical microscopy images with polarized light of samples in the L_α and H_1 regions are shown in Table S1. L_α, a lamellar LC region, which was birefringent and fluid, extends perpendicular to the Cremophor RH40/water axis from a surfactant/water weight ratio of 87/13 to 78/22 and oil content between 5 wt% to 45 wt%. The other birefringent and highly viscous region corresponds to H_1, a hexagonal liquid crystalline region, which forms in the Cremophor RH40/water axis from a surfactant/water weight ratio of 72/28 to 65/35 and solubilizing a maximum of 6 wt% oil. The rest of the diagram corresponds to multiphase regions. Five different multiphase regions were determined: M, II_1 and II_2 are liquid multiphase regions. However, samples in region M show kinetic stability and samples in region II_1 and II_2 separate in two phases immediately. MLC_1 and MLC_2 are multiphasic regions with presence of liquid crystalline structures: MLC_1, birefringent; MLC_2, non-birefringent and nonfluid.

The phase behavior of the water/Cremophor EL/Miglyol 812 system at 25°C was studied by Sadurni et al. in 2005 [27]. Similarities in the L_1, H_1, L_α, L_2, II_1 and M regions were observed, although the L_2 region is slightly increased. However, some differences as new regions, I_1 and II_2 were found. In addition, MLC region described by Sadurni has been

subdivided in MLC_1 and MLC_2. Both surfactants (Cremophor RH40 and Cremophor EL) present similar structure as they are castor oil derivatives with ethylene oxide. Cremophor RH40 is hydrogenated and present approximately 40 moles of ethylene oxide (HLB=14-16) and Cremophor EL (Polyoxyl-35 castor oil) is non-hydrogenated and present approximately 35 moles of ethylene oxide (HLB=12-14). These differences could explain the phase behavior of both systems.

3.2 Emulsion formation

Formation of O/W highly concentrated emulsions was studied in the water/Cremophor RH40/Miglyol 812 system at 25°C by adding the oil phase under continuous stirring to surfactant/water mixtures. The boundaries of the HIPRE region (Figure 1(B)) extends between the Cremophor RH40/water ratios of 43/57 and 20/80 and oil content from 74wt% to 99wt%.

HIPREs with similar composition were selected to perform the experiments. Generally, diluted emulsions are fluid and the viscosity increases with the volume fraction of the inner phase [31]. In the pathway to prepare the HIPRE with a S/W weight ratio of 40:60, highly viscous emulsions were obtained even when the oil concentration was low, while when preparing the HIPRE with a S/W weight ratio of 35:65, emulsions were fluid until the HIPRE region was reached.

Two diluted emulsions containing 10 wt% of oil (E1 and E4), two concentrated emulsions containing 40 wt% of oil (E2 and E5) and two highly concentrated emulsions containing 85 wt% of oil (E3 and E6) were prepared and characterized (Table 1). For the two S/W selected (40:60 and 35:65), the initial mixtures were isotropic (non-birefringent), transparent and highly viscous. The addition of oil to the binary mixture of 40:60 increased the viscosity. After two days, E1 and E2 did not flow, in contrast to the diluted and concentrated emulsions, E4 and E5 which were fluid. The highly concentrated emulsions, E3 and E6 did not flow. The influence of the incorporation of a drug (1wt% of DS) on emulsion properties, was studied. All emulsions with DS showed the same visual appearance as the corresponding formulations without the drug.

Table 1. *Emulsions and micellar solutions: composition and description of the type of formulation.*

Notation	S/W	Composition			Description of the formulations
		Cremophor RH40 (wt%)	Water (wt%)	Miglyol 812 (wt%)	
E1	40:60	36	54	10	Diluted emulsion
E2		24	36	40	Concentrated emulsion
E3		6	9	85	Highly concentrated emulsion (HIPRE)
E4	35:65	31.5	58.5	10	Diluted emulsion
E5		21	39	40	Concentrated emulsion
E6		5.25	9.75	85	Highly concentrated emulsion (HIPRE)
M1	-	36	64	-	Micellar solution
M2		24	76	-	Micellar solution
M3		6	94	-	Micellar solution

S/W: surfactant/water weight ratio

3.3 Characterization and stability of O/W emulsions

The emulsions were observed after a week of preparation, to assess their stability. The samples were homogeneous and with no apparent phase separation. Optical microscopy, SAXS and rheology were used to characterize the formulations.

3.3.1 Optical microscopy

The structure of emulsions regarding the shape of the droplets, the droplet size and polydispersity is of a great importance, affecting the properties of emulsions such as the viscosity or the stability of the formulations among others. The micrographs (Figure 2) of the emulsions observed by optical microscopy show significant differences between emulsions at different oil concentrations.

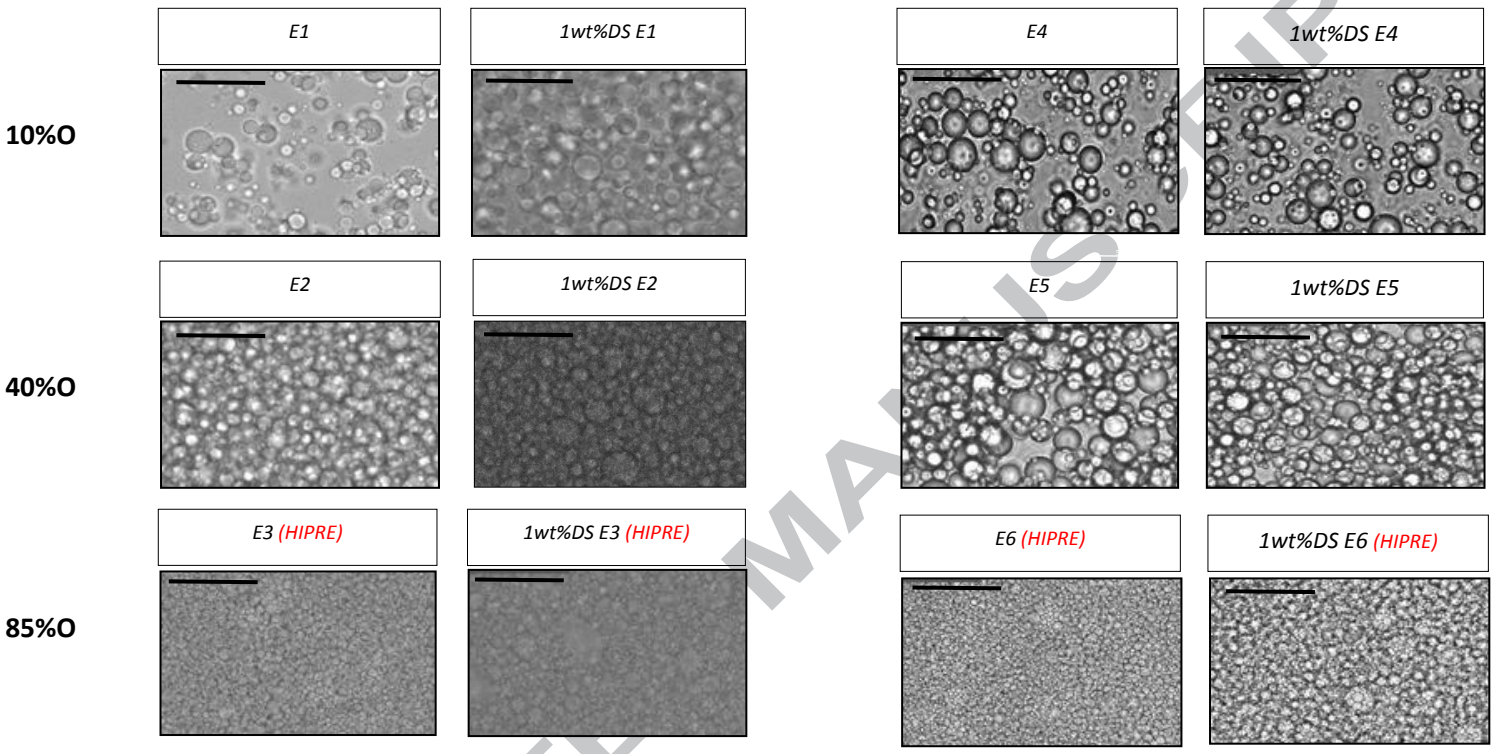

Figure 2. *Micrographs of emulsions with S/W=40:60 (E1, E2 and E3) and 35:65 (E4, E5 and E6) without and with 1%DS, at 25°C. The scale bars represent 50µm.*

Emulsions with 10wt% and 40wt% of oil presented the typical microscopic appearance of diluted and concentrated emulsions with spherical droplets, in contrast to the densely packed droplets with a polyhedral shape observed in HIPREs (Fig. 2, 85% oil, E3 and E6 without and with 1wt% DS), previously reported by Princen [32].

For each formulation three micrographs were selected and 100 droplets were measured in each micrograph. For HIPREs micrographs image magnification was used to measure the droplet sizes more accurately. Despite the high polydispersity typical of highly concentrated emulsions, in the samples studied we observed a higher polydispersity in the diluted and concentrated emulsions. The droplet size distributions of the samples are shown in Figure S1. It was found that incorporation of DS in the emulsions does not change significantly the droplet size distribution (E1 5.8±1.8 µm; E1 1%DS 5.1±2.1 µm; E2 5.3±1.5 µm; E2 1%DS 3.4±0.9 µm; E3 4.0±0.7 µm; E3 1%DS 3.1±0.8 µm).

3.3.2 SAXS

The high viscosities of emulsions E1 and E2 suggested the presence of mesophases. In order to evaluate the presence of liquid crystalline structures in the highly viscous emulsions, which may have an influence on stability and drug release and skin permeation, Small-Angle X-Ray scattering (SAXS) measurements were performed in diluted (E1), concentrated (E2) and highly concentrated (E3) emulsion at 25°C. Emulsion with the lowest and the highest oil content with 1wt%DS were also selected to study if the drug had any effect on the nanostructures formation (Fig. 3). SAXS patterns at various oil concentrations (10wt% and 40wt% of Miglyol 812) showed three peaks that together with absence of birefringence of the samples point to the presence of direct micellar cubic phases (I_1) with characteristic peak ratios [33]. In HIPRE E3, only one peak was resolved. The peak observed in the HIPRE E3 SAXS pattern suggests that liquid crystalline structures were present. However, at high oil concentrations, the fraction of structured phase is low. The addition of 1wt% of DS to emulsions (E1 and E3) barely modified the scattering patterns, suggesting that DS does not affect liquid crystalline structures. The Bragg spacing (*d*), indicates the separation between crystalline planes and it has been calculated from the first and most intense peak in all the formulations. The increase of *d* with oil content (Table 2) suggests swelling of the aggregates forming the cubic phase.

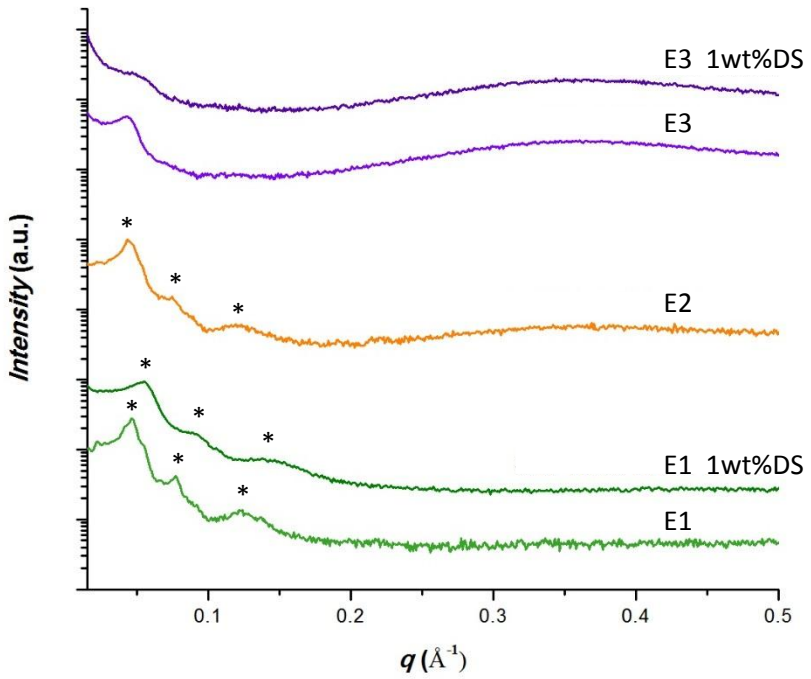

Figure 3. *SAXS scattering patterns (25°C) at Cremophor RH40: water weight ratio of 40:60 and various oil (Miglyol 812) concentrations. (*) represents the peaks from which the peak position ratios were calculated.*

Table 2. *Peak position ratios and Bragg distances, d (separation between planes) for different oil concentrations.*

Emulsion	Oil concentration	Peak position ratios (q_1:q_2:q_3)	*Bragg spacing* *d* (Å)
E1	10%	1: .1.7: 2.7	136
E1 1%DS	10%	1:1.6:2.6	114
E2	40%	1: .1.7: 2.7	141
E3	85%	-	143
E3 1%DS	85%	-	123

3.3.3 Rheology

The flow properties of emulsions are very important for applications. Diluted emulsions (volume fraction of dispersed phase below 0.2 approximately) show a Newtonian behavior. Concentrated and highly concentrated emulsions show Non-Newtonian behavior with different viscosities according to the volume fraction and the structure of the droplets. Moreover, the rheological properties could change when the continuous phase of emulsions is nanostructured with cubic liquid crystals. Contrary to what could be expected, diluted and concentrated emulsions, E1 and E2, were not fluid. In order to elucidate the cause of the increase in viscosity, rheological properties were studied. Figure 4(A) shows the shear viscosity as a function of shear rate for the selected emulsions (samples were measured in triplicate). Non-newtonian behavior and shear thinning after a critical shear stress were observed in all samples. To compare the viscosity between formulations a fixed value of shear rate was selected. At a fixed shear rate of 0.2 s^{-1} the viscosity of emulsion E1 was 1596.7±190.1 Pas, for E1 1%DS was 1840±480.1 Pas, for E2 was 688.7±164.1 Pas, for E2 1%DS was 891.7±80 Pas, for E3 was 260.3±51.7 Pas and for E3 1%DS was 195.3±20.4 Pas. Emulsion E1 followed by emulsion E2 presented the highest viscosity at low shear from 0.0001 to 0.01 s^{-1} evidencing a possible nanostructure in the continuous phase, while HIPRE, E3, presented the lowest viscosities (Fig. 4(A)).

Emulsions, E1 and E2, have a higher surfactant concentration in comparison to HIPRE (Table 1). The addition of oil to the initial binary mixture (S/W=40:60) could induce that part of the surfactant molecules may be placed at the interface of the oil droplets and the rest of the surfactant molecules could arrange in liquid crystalline phases increasing the viscosity, being greater in emulsion E1 as the surfactant concentration is higher. When the oil concentration increases, more molecules could diffuse to the interface of the droplets, stabilizing the emulsion and decreasing the number of surfactant molecules available to form nanostructures, therefore reducing the viscosity. For drug-loaded systems, values partly showed deviations at certain measurement points when compared to the drug-free formulations (Figure 4A), but no clear trend caused by drug incorporation could be determined.

Viscoelastic properties of emulsions were determined by dynamic oscillatory measurements. Firstly, the linear viscoelastic (LVE) region of emulsions was determined in triplicate by strain sweep assays at a constant frequency of 1Hz at 25^{o}C. The frequency sweep assays were determined at a fixed strain of 0.01. The values were obtained from a single measurement (Figure 4(B)). To compare the elastic (G') and viscous (G'') moduli between formulations a frequency value of 1 Hz was fixed. E1 presented a G' of 11550 Pa and G'' of 1998 Pa, E2 presented a G' of 7614 Pa and G'' of 1030 Pa and E3 presented a G' of 1524 Pa and G'' of 125.9 Pa. The results showed that the elastic moduli (G') for emulsion E1 and emulsion E2 were nearby 10^4 Pa, typical for cubic phases [18]. The highest moduli, G' and G'', were obtained for emulsion E1 and no cross points was observed between the elastic (G') and viscous (G'') moduli, indicating a gel-like behavior. When the oil concentration increases, both moduli decrease and HIPRE E3 showed significantly lower values of elasticity and plasticity at all shear frequencies (Fig. 4(B)). The viscoelastic properties of all emulsions with 1 wt%DS did not show significant differences compared with emulsions without drug.

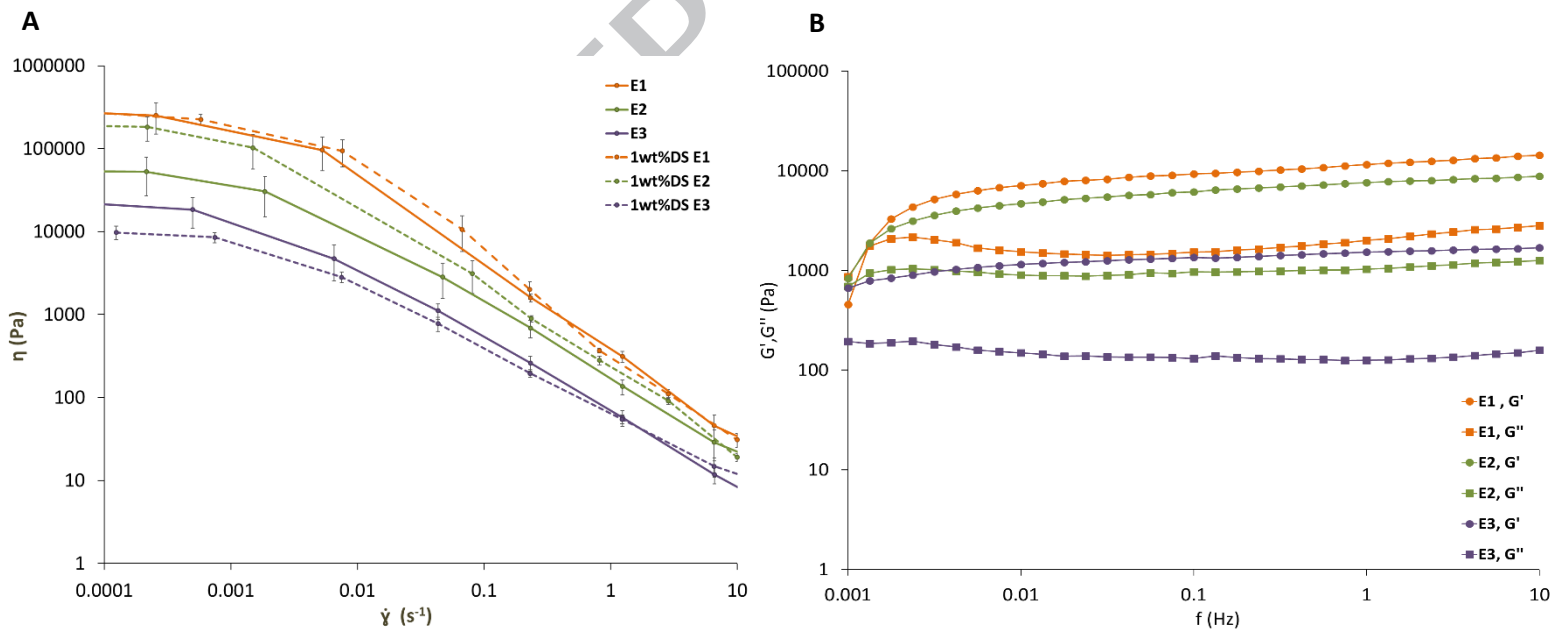

Fig. 4. (A) *Shear viscosity as a function of shear rate at 25^{o}C for emulsions E1, E2, E3 and E6 without and with 1wt%DS. Values are the mean for n=3 ± SD.* ***(B)*** *Oscillatory rheometry of emulsions E1, E2, E3 and E6. Values provided come from a single measurement.*

3.3.4 In vitro drug release and permeation studies through synthetic membranes

The release of DS from all the previously characterized emulsions was studied for 24 hours through a hydrophilic membrane (cellulose acetate). In addition, micellar solutions with 1wt%DS and the same surfactant concentration as emulsions (S/W=40:60), but without oil were assayed to study the influence of the formulation structure on drug release. Permeation studies were also performed through a lipophilic membrane (Strat-M) from the formulations with the lowest and the highest release of DS. Figures 5(A-B) show DS release through a cellulose membrane from emulsions (E1-E6). The results for E1, E2 and E3, with 1wt%DS and a PBS solution with 0.36%DS are shown in Figure 5(A). The PBS solution was assayed as a reference, with a concentration (0.36%), lower than the saturation concentration. According to the results, the diffusion of DS through acetate cellulose membrane was not rate limiting, and the release profile was only affected by the formulation characteristics. The PBS solution showed the highest release and a burst release was observed in the first hours of the experiment, typical of solutions. For the emulsions studied, HIPRE E3 showed a release percentage after 24h of 55.2±1.5%. Similar and lower profiles were observed for the diluted and concentrated emulsions (19.7±0.4% and 22.8±1.8% for the emulsions E1 and E2, respectively). The

release results obtained can be correlated to the rheological behavior and SAXS determinations of the investigated emulsions. The diluted emulsion (E1) has the higher amount of surfactant which could be arranged in nanostructures in the continuos phase of the emulsions. Thus, the more cubic liquid crystalline structures, the higher the viscosity. With the increase of oil concentration in emulsions, more surfactant molecules were placed at the interface and less nanostructures were formed in the continuous phase, reducing the peaks in the SAXS patterns, and conversely, the release of DS increased. The increase in the viscosity had been related to a decrease in the droplet diffusion [34] which could explain the decrease in the release rate of DS.

Emulsions E4, E5 and E6 (Figure 5(B)) with a S/W=35:65, showed higher release profiles than the corresponding E1, E2 and E3 (Figure 5(A)). The DS release percentage obtained was 41.4±2.7% for the diluted emulsion E4, 48.5±2.2% for concentrated emulsion E5 and 66.6±4.7% for HIPRE E6. It seems to be an influence of the structure of emulsions with S/W=40:60. We speculate that the surfactant could be located at the interface of the droplets but also in cubic liquid crystalline structures where the DS should be strongly retained.

For comparative purposes, micellar solutions with the same surfactant concentration as emulsions E1, E2 and E3, but without oil were also assayed (Figure 5(C)). Micellar solutions showed higher release after 24 hours compared to emulsions with the same surfactant concentration (E1-E3). The micellar solution M1 presented the lowest release of DS, 35.3±1.3% after 24 hours (19.7% for E1), followed by micellar solution M2 with a DS release of 47.8±1.5% (22.8% for E2) and micellar solution M3 presented the highest release of DS, 99.8±1.5% after 24 hours (55% for E3).This assay revealed the influence of formulation structure on drug release. Comparing the release from micellar solutions and emulsions with the same surfactant concentration (E1, E2 and E3), the lower release of the drug from emulsions would be related to the presence of oil.

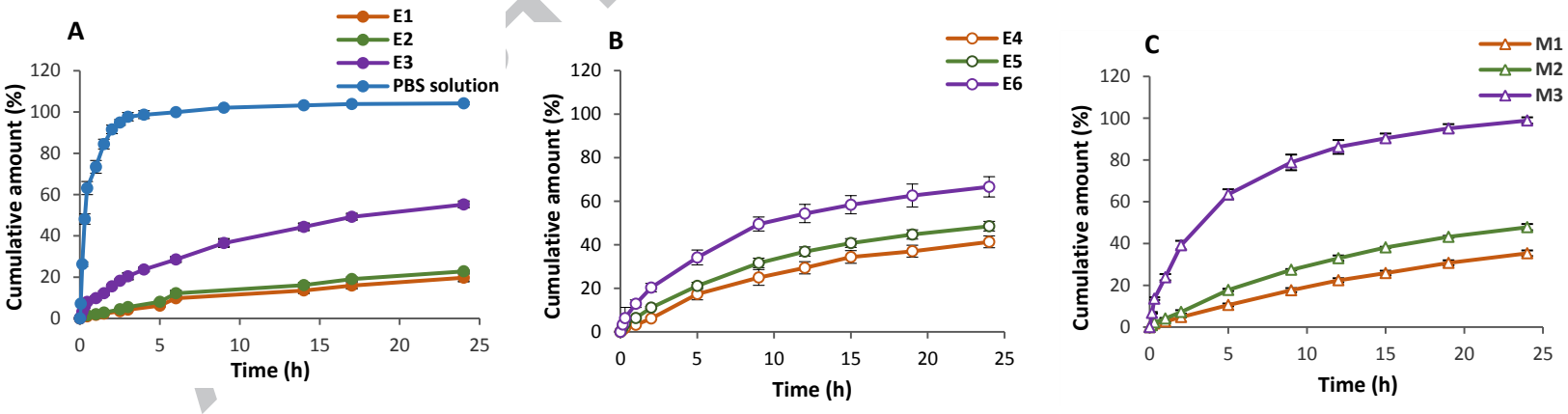

Fig. 5. (A) Percentage of DS released through cellulose acetate membrane from emulsions with 10 wt%, 40 wt% and 85 wt% of Miglyol 812 with S/W= 40:60 and a PBS solution 7.4 at 32°C. (B) Percentage of DS released from emulsions with 10 wt%, 40 wt% and 85 wt% of Miglyol 812 with S/W= 35:65 at 32°C. (C) Percentage of DS released from Cremophor RH40:water solutions at different surfactant concentrations at 32°C. Values are means of n=3 ± SD.

Formulations that presented the lowest release after 24 hours (E1, E2 and E3) and the aqueous micellar solutions (M1, M2 and M3) that presented the higher release after 24 hours were selected to study the *in vitro* permeation through synthetic membranes, Strat-M. Figures 6 (A) and (B) show DS permeation through the Strat-M® membrane from emulsions with S/W=40:60 (E1, E2 and E3) and aqueous micellar solutions (M1, M2 and M3) respectively. These results showed the same tendency as those obtained with cellulose acetate membranes. Emulsions present lower permeation in contrast to aqueous micellar solutions and the permeation of DS from emulsions decreases when the content of surfactant increases, which could be related to the higher fraction of nanostructures, that would retain the drug. After 24 hours, the percentage of DS permeated were 3.7±0.2% from diluted emulsion (E1), 16.3±1.8% from concentrated emulsion (E2) and 16.5±1.8% from highly concentrated emulsion (E3). The permeation percentage of DS from micellar solutions M1, M2 and M3 after 24 hours were 13.7±0.4%, 18.3±3.3% and 71.5±1.6%. Moreover, the diffusion of DS from formulations was lower when Strat-M® was used as a membrane respect to the results obtained with a cellulose acetate membrane, which can be attributed to the structure of Strat-M® membrane, since it is formed by two layers of polyethersulfone and one layer of polyolefine. The two layers of polyethersulfone contains a combination of lipids in a specific ratio similar to what is found in the human stratum corneum.

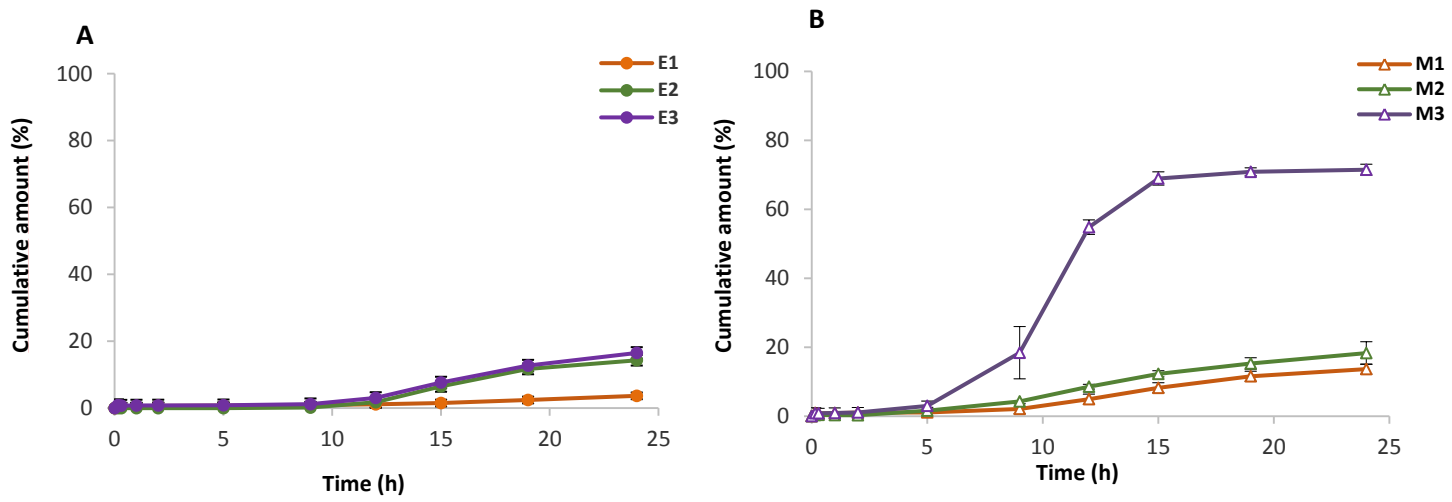

Fig. 6 (A) *Percentage of DS released through Strat-M® membrane from emulsions with 10 wt% (E1), 40wt% (E2) and 85 wt% (E3) of Miglyol 812 with a S/W=40:60 at 32°C.* ***(B)*** *Percentage of DS released from Cremophor RH40:water micellar solutions (M) with different surfactant concentrations at 32°C. Values are means of n=3 ± SD.*

3.3.5 Skin permeation

The surfactant liquid crystalline structures could promote an enhancer effect in drug percutaneous absorption [35]. Their characteristic high viscosity can be also responsible for controlled sustained release due to bioadhesive properties [36] and specific interactions of liquid crystals with the stratum corneum (SC) [37]. Furthermore, HIPREs have been studied as drug delivery systems due to the special structure with polyhedral droplets shape which may affect drug release [6]. To investigate if the emulsions with nanostructured continuous phase previously studied are suitable formulations for topical application, DS permeation studies through dermatomed abdominal human skin were carried out. In this sense, emulsions with nanostructured continuous phase with the lowest and the highest release after 24h through synthetic membranes (E1 and E3) and the aqueous micellar solution with the highest release after 24h (M3) were selected for the *ex vivo* skin permeation studies. It should be noted that only for the aqueous micellar solution, DS was quantified in receptor fluid (Fig. 7 (A)), while no DS was detected from both emulsions.

Regarding the amount of DS in skin layers, in epidermis (Fig. 7(B)), significant differences ($p<0.05$) were observed for diluted emulsion (E1), with the highest amount of DS in epidermis compared to HIPRE (E3) and aqueous micellar solution (M1) with lower retention of DS. Diluted and highly concentrated emulsions with cubic liquid crystalline (LC) structures presented different amount of DS in skin layers. In E1, more DS would be retained in LC structures and higher permeation could take place by interaction with microstructures of the stratum corneum lipids, enhancing the drug penetration. The HIPRE E3 allowed a lower permeation of DS, which could be influenced by a lower amount of cubic LC structures compared with E1, as shown by SAXS and rheology results. It should be noted that the structure of the HIPRE has an impact on drug release and permeation through synthetic membranes and skin permeation, due to the densely packed droplets and the high viscosity that reduce DS diffusion and penetration in the skin [7]. In the micellar solution M1, the high amount of water could promote the permeation of DS by hydration of skin [38], but also the drug could be solubilized in micelles, which vehiculate DS through the skin down to the receptor fluid.

The results obtained in dermis (Fig. 7(C)) showed no significant differences between E1 and M1, both presenting higher amount of DS retained with respect to E3.

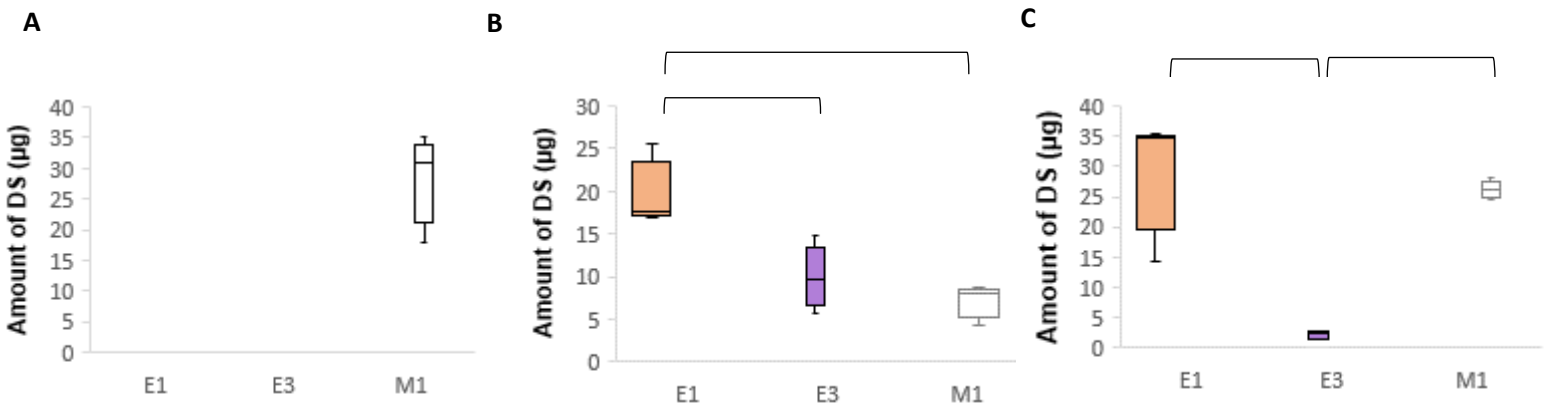

Fig. 7. *Amount of DS in receptor fluid (A), epidermis (B) and dermis (C) after 24h in skin permeation experiments for each formulation assayed. Each bar represents the median ± minimum and maximum values (n=3). Brackets between the bars indicate significant differences between groups in each graph (P<0.05, Mann-Whitney U test).*

Comparing the plots, a great amount of DS permeated down to the dermis from diluted emulsion with a higher fraction of cubic liquid crystal (E1). In the HIPRE (E3), DS penetrated the SC and the amount of DS in epidermis was higher than the amount quantified in dermis, where only a small amount of DS reached this deeper layer. With the micellar solution (M1), DS permeated down the skin, reaching the receptor fluid, but also a high amount of DS was retained in the dermis.

4. CONCLUSIONS

We have demonstrated the impact of formulation structure on DS release and skin permeation. Cubic liquid crystalline structures were identified by SAXS in O/W emulsions without and with 1%DS. The high stability of emulsions prepared with Cremophor RH40 with a S/W of 40:60 was attributed to the presence of liquid crystalline structures. Drug release and skin permeation can be tuned by judicious selection of the sample composition from the phase diagram. Drug release and permeation are not only influenced by surfactant concentration but also by the presence of liquid crystal phases. In addition, these results showed the HIPREs as promising formulations for sustained release of diclofenac sodium with topical application due to the low amount of surfactant required, low permeation and high skin retention. The present work is useful for the development of topical delivery systems with drug targeting in skin layers and avoiding possible systemic effects.

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Supplementary material for:

CUBIC LIQUID CRYSTALLINE STRUCTURES IN DILUTED, CONCENTRATED AND HIGHLY CONCENTRATED EMULSIONS FOR TOPICAL APPLICATION: INFLUENCE ON DRUG RELEASE AND HUMAN SKIN PERMEATION

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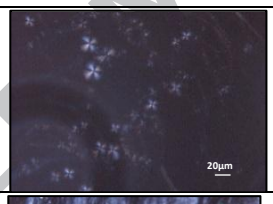
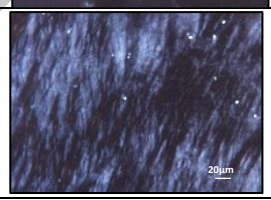
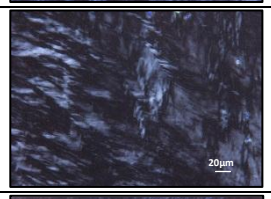
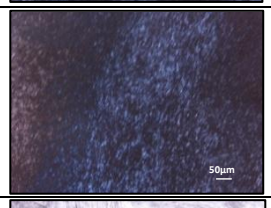
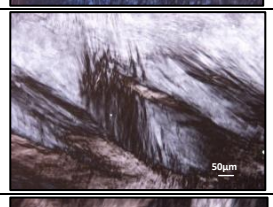
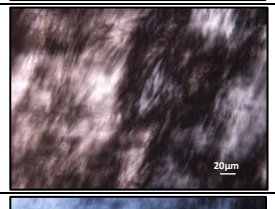
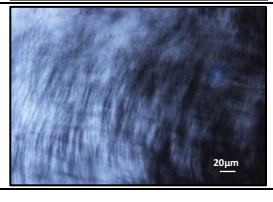
Table S1. *Characterization of liquid crystals by optical microscopy with polarized light.*

Identification	Composition	Micrographs	Description of the liquid crystals
1	S/W 80:20 15%O		L_α
2	S/W 75:25 10%O		H_1
3	S/W 72:28		H_1
4	S/W 72:28 5%O		H_1
5	S/W 70:30		H_1
6	S/W 70:30 5%O		H_1
7	S/W 65:35 5%O		H_1

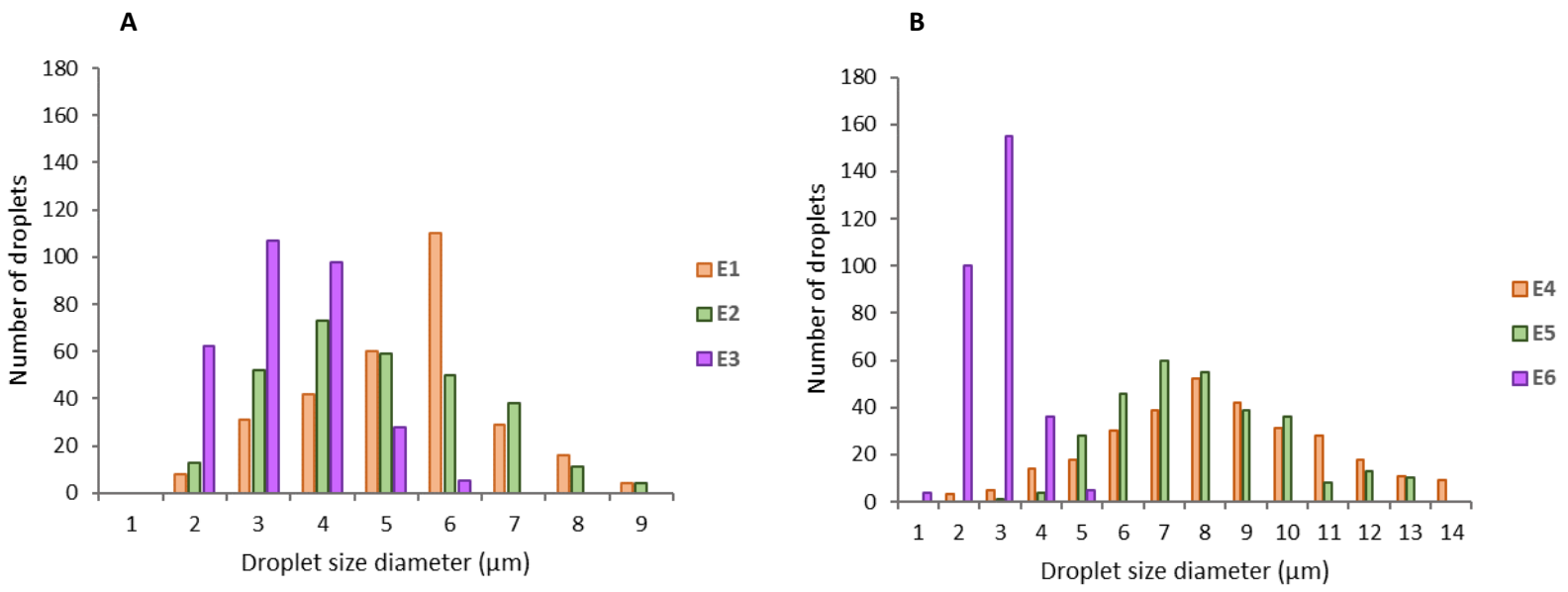


Figure S1. *Droplet size distribution of (A) emulsions with S/W=40:60 (E1, E2 and E3). (B) emulsions with S/W=35:65 (E4, E5 and E6).*

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: