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Research paper

Self-assembled mPEG-hexPLA polymeric nanocarriers for the targeted cutaneous delivery of imiquimod

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ABSTRACT

mPEG-hexPLA micelles have shown their ability to improve delivery and cutaneous bioavailability of a wide range of poorly water soluble and lipophilic molecules. Although poorly water soluble, imiquimod (IMQ) is only moderately lipophilic and it was decided to investigate whether mPEG-hexPLA polymeric micelles could be used as a drug delivery system for this "less than ideal" candidate for encapsulation. Nanosized IMQ micelles (d_n = 27 nm) were formulated and characterized. Moreover, the innovative use of size exclusion chromatography allowed the exact drug localization inside the formulation to be determined; it appeared that the use of acetic acid to solubilize IMQ led to a higher IMQ content outside the micelle than inside. IMQ micelles (0.05%) were formulated in a gel using carboxymethyl cellulose (CMC). *In vitro* application of this formulation to porcine and human skin led to promising delivery results. IMQ deposition in human skin was 1.4 ± 0.4 μg/cm^2 while transdermal permeation was only 79 ± 19 ng/cm^2: the formulation displayed > 17-fold selectivity for cutaneous deposition over transdermal permeation. The optimized 0.05% gel significantly outperformed Aldara® cream (containing 5% IMQ) formulation in terms of delivery efficiency to human skin (2.85 ± 0.74% vs 0.04 ± 0.01%). Despite IMQ being only partially incorporated in the micelles, the biodistribution profile showed that the optimized 0.05% gel delivered as much as 518.2 ± 173.3 ng/cm^2 (1.04 ± 0.35% of the applied dose) to the viable epidermis and 236.4 ± 88.2 ng/cm^2 (0.47 ± 0.18% of the applied dose) to the upper dermis where the target antigen presenting cells reside. In contrast, for Aldara® cream, the delivery efficiencies in those layers were less than 0.02%. The optimal 0.05% gel thus allowed therapeutically relevant drug levels to be achieved in target tissues despite a 100-fold dose reduction.

1. Introduction

Skin cancer is one of the most common and deadly cancers worldwide. The incidence of non-melanoma skin cancers (i.e. basal cell carcinoma and squamous cell carcinoma) has been strongly increasing in recent decades [1]. Among various therapeutic approaches to treat skin cancers, the topical application of imiquimod (IMQ), offers an acceptable, non-invasive option [2]. IMQ is an imidazoquinoline that has shown potent antiviral and antitumor activity in animal models [3]. It is thought that IMQ acts indirectly on cell proliferation as an immune response modulator. It binds to Toll-like receptor 7 (TLR7) in epidermal plasmacytoid dendritic and Langerhans cells, inducing increased production of interferon-α, interleukin-12, tumor necrosis factor-α and T-helper 1 cells, thus stimulating both innate and acquired immunity [4,5]. IMQ is available on the market as a 5% cream, Aldara® (3 M Pharmaceuticals, USA), which is mainly indicated in the treatment of pre-cancerous lesions such as actinic keratosis and basal cell carcinoma [6,7]. However, it can also be used to treat superficial squamous cell carcinoma [8], genital warts [9,10] and to prevent recurrence after melanoma resection [11]. Nevertheless, formulation of IMQ is

Abbreviations: ACN, Acetonitrile; ANOVA, Analysis of variance; BSA, Bovine serum albumin; CMC, carboxymethyl cellulose sodium; DLS, dynamic light scattering; EC50, Half maximal effective concentration; FDA, Food and Drug Administration; GPC, gel permeation chromatography; HPLC, High-Pressure Liquid Chromatography; HEC, hydroxyethyl-cellulose; ICH, International Conference on Harmonization; IMQ, Imiquimod; mPEG-hexPLA, methoxy-poly(ethylene glycol)-)-hexyl-substituted lactide block copolymer; NMR, Nuclear magnetic resonance; PBS, Phosphate-buffered saline; PS, polystyrene; RSQ, Resiquimod; TLR7, Toll-like receptor 7; TEM, transmission electron microscopy; UHPLC-MS/MS, Ultra High-Pressure Liquid Chromatography coupled to tandem mass detection; USP, United States Pharmacopeia

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challenging due to its lipophilicity (Log P: 3.0) [12] and its poor aqueous solubility (18 μg/ml; experimental value) – indeed, it is also poorly soluble in many organic solvents (e.g. 96 μg/ml in acetone, 120 μg/ml in propylene glycol and 472 μg/ml in ethanol).

Conventional vehicles, such as ointments and creams, used for formulation and delivery of lipophilic drugs are not usually optimized with respect to delivery efficiency; the active principle has a high affinity for the vehicle (i.e. low thermodynamic activity), which does not favor partitioning into the skin. Thus, although "large" amounts of drug are loaded in the formulation, only a small fraction actually enters the skin and only a fraction of that goes on to reach the target tissue, most of the drug remains in the formulation and is not delivered. This phenomenon has already been observed for commercial formulations, e.g. Protopic® [13] or Retin-A® [14]. Polymeric micelles are remarkable because of their stability, nanometer-scale size and ability to encapsulate significant amounts of lipophilic poorly water soluble drugs [15]. In previous studies it was demonstrated that micelles prepared using the block copolymer methoxy-poly(ethylene glycol)-)-hexyl-substituted lactide (mPEG-hexPLA) loaded with econazole nitrate [16], tacrolimus [13] or ciclosporin A [17] were able to enhance cutaneous drug delivery improving both topical bioavailability of the drug and delivery efficiency without yielding any undesired transdermal permeation [13,16,17]. Although IMQ is certainly poorly water soluble, it is less lipophilic than other molecules tested to-date with mPEG-hexPLA micelles and it was decided to investigate the potential of mPEG-hexPLA as a drug delivery system for this challenging molecule with sub-optimal physicochemical properties.

The aim of the present study was to develop a micelle formulation for IMQ using mPEG-hexPLA block copolymer and evaluate its delivery *in vitro*. IMQ loaded micelles were prepared and characterized. The exact location of the drug within the formulation was investigated for the first time using size exclusion chromatography. A hydrogel was subsequently formulated using the optimal micelle solution. Optimal formulations were evaluated with respect to their ability to deliver IMQ to porcine and human skins using Aldara® cream as a control formulation. The biodistribution of IMQ in the skin layers was investigated using the previously described technique [13,18].

2. Materials and methods

2.1. Materials

IMQ was purchased from Hangzhou Dayang Chem (Hangzhou, P.R.China). Acetone, acetonitrile (Chromasolv HPLC grade), ethanol, isopropanol, tetrahydrofuran, sodium and potassium chloride, sodium and potassium phosphate, formic acid and acetic acid (99–100%) were purchased from Sigma Aldrich (Buchs Switzerland). Bovine serum albumin (BSA) was purchased from Axon Lab (Baden-Dättwil, Switzerland). Nile red was purchased from TCI Europe (Eschborn, Germany), hydroxyethyl-cellulose (HEC) (203 mPa.s, 1% in H_2O; 20 °C) was purchased from Fluka (Buchs, Switzerland) and carboxymethyl cellulose sodium (CMC) (2000 mPa.s) was obtained from Hänseler AG (Herisau, Switzerland). Ultra-pure water (Millipore Milli-Q Gard 1 Purification Pack resistivity > 18 MΩ.cm, Zug, Switzerland) was used to prepare all solutions. All other chemicals were at least of analytical grade.

The block copolymer methoxy-poly(ethylene glycol)-)-hexyl-substituted lactide (mPEG-hexPLA), was synthesized in-house as described previously [13,17,19,20]. The copolymer was characterized with respect to its molecular weight (5333 g/mol) using gel permeation chromatography (GPC). The GPC setup was composed of a Waters 717 autosampler, Waters 515 HPLC pump and a Waters 410 differential refractometer (Waters; Baden-Dättwil, Switzerland) using Waters Styragel HR1-4 columns. The analysis was carried out with polystyrene (PS) of different molecular weights as calibration standards (PSS; Mainz, Germany). The copolymer was also characterized by ^{1}H NMR (Brüker 300 MHz). Aldara® cream (5%, 3 M Pharmaceuticals) was purchased from a local pharmacy. It contained IMQ, isostearic acid, benzyl alcohol, cetyl alcohol, stearyl alcohol, white soft paraffin, polysorbate 60, sorbitan monostearate, glycerol, methyl hydroxybenzoate, propyl hydroxybenzoate, xanthan gum, purified water.

2.2. Analytical methods

2.2.1. Quantification of IMQ by HPLC-Fluorescence.

The HPLC apparatus consisted of a P680A LPG-4 pump equipped with an ASI-100 autosampler, a TCC-100 thermostatted column compartment and a RF 2000 fluorescence detector (formerly Dionex AG, now ThermoFisher Scientific AG; Reinach, Switzerland). IMQ was detected at an excitation wavelength of 260 nm and an emission wavelength of 340 nm. Isocratic separation was performed using a LiChrospher®100, RP-8e, 5 μm, 125 × 4 mm column (BGB Analytik AG; Boeckten, Switzerland) that was maintained at 35 °C. Chromeleon® software was used for integration and data analysis. The mobile phase consisted of a mixture of acetonitrile and acetate buffer pH 4 (60:40 v/v). The flow rate and injection volume were 1.0 ml/min and 25 μl, respectively. A peak for IMQ was obtained at 4.2 min and the run time was 7.0 min. The HPLC-Fluorescence (HPLC-Fluo) method was validated (Supplementary Material).

2.2.2. Quantification of IMQ by UHPLC-MS/MS.

To increase sensitivity and specificity, UHPLC with tandem mass spectrometry detection was used to quantify the drug deposited in and permeated across the skin during the *in vitro* transport experiments. UHPLC-MS/MS analysis was carried out using a Waters Acquity UPLC® system (Baden-Dättwil, Switzerland) comprising a binary solvent pump and sample manager and a Waters XEVO® TQ-MS detector (Baden-Dättwil, Switzerland). Isocratic separation was carried out using a Waters XBridge™ BEH C18 2.1 × 50 mm column containing 2.5 μm particles. The column was thermostatted at 40 °C. The mobile phase consisted of water and acetonitrile (40:60 v/v) with addition of 0.1% of formic acid. The flow rate was set at 0.4 ml/min and the injection volume was 5 μl. As ion suppression was observed in the samples – presumably due to the presence of endogenous compounds released from the skin, resiquimod (RSQ) was used as an internal standard. Each injected sample contained the internal standard at a concentration of 50 ng/ml.

Mass spectrometric detection was performed with electrospray ionization in positive ion mode using multiple reaction monitoring (MRM). The detection settings for both IMQ and RSQ are presented in Table 1. The UHPLC-MS/MS method was also validated (Supplementary Material).

Table 1
MS/MS settings for detection of IMQ and RSQ.

	Imiquimod	Resiquimod
Nature of parent ion	$[M + H]^+$	$[M + H]^+$
Parent ion (*m/z*)	241.01	315.10
Daughter ion (*m/z*)	185.04	251.08
Collision energy (V)	26	26
Cone voltage (V)	42	32
Capillary voltage (kV)	1.49	1.49
Capillary temperature (°C)	200	200
Desolvation gas flow (L/h)	1000	1000
Cone gas flow (L/h)	50	50
Collision gas flow (L)	0.15	0.15
LM resolution 1	2.78	2.78
HM resolution 1	15	15
Ion energy 1(V)	0.7	0.7
LM resolution 2	2.74	2.74
HM resolution 2	14.88	14.88
Ion energy 2 (V)	0.6	0.6

2.3. Preparation of the micelle formulation

The solvent evaporation method was extensively used in previous studies [13,16,17]; however, it was not suitable for IMQ due to its poor solubility in Class 3 USP volatile, water miscible solvents like acetone (0.096 mg/ml). Therefore, the solubility of IMQ in this solvent was increased by the addition of 0.25% acetic acid to obtain a stock solution at 0.25 mg/ml of IMQ. 20 or 40 mg of copolymer (corresponding to a final copolymer content of 5 or 10 mg/ml) was solubilized in the appropriate volume of this solution (2, 4, 8, 12 or 16 ml corresponding to 0.5, 1, 2, 3 or 4 mg of IMQ to reach target drug loadings of 25, 50, 100 or 150 $mg_{drug}/g_{copolymer}$) and was added dropwise under magnetic stirring to 4 ml of water or 10 mM sodium acetate buffer. The organic phase was subsequently evaporated using a rotary evaporator (Büchi RE 121 Rotavapor). The final copolymer content was adjusted to 5 or 10 mg/ml. After overnight equilibration, the micelle solution was centrifuged at 10,000 rpm for 15 min (10,621*g*; Eppendorf Centrifuge 5804; Hamburg, Germany) to remove non-incorporated drug and the supernatant was collected.

2.4. Characterization of micelle formulations

2.4.1. Visual evaluation and pH

Formulations were inspected visually for transparency, absence of drug precipitation and the pH was measured. The residual solvent content was tested using NMR (Varian Gemini 300 BB, 300 MHz, Varian; Palo Alto, USA) against a control solution containing 5000 ppm (0.5%) of acetone and acetic acid. This concentration is prescribed as the Class 3 solvent content limit in both the US Pharmacopeia [21] and ICH Q3C Guidelines [22].

2.4.2. Size and morphology characterization

mPEG-hexPLA micelles were analyzed with respect to their size. The number-weighted diameter (d_n) and the hydrodynamic diameter (Z_{av}) were measured using dynamic light scattering (DLS) with a Zetasizer HS 3000 (Malvern Instruments Ltd; Malvern, UK). Analyses were performed at an angle of 90° at 25 °C. All measurements were obtained after three runs of ten measurements.

The morphology of mPEG-hexPLA micelles was determined by transmission electron microscopy (TEM) (FEI Tecnai™ G2 Sphera, Eindhoven, Netherlands) using the negative staining method. 5 µl of the micellar solution were dropped onto an ionized carbon-coated copper grid (0.3 Torr, 400 V for 20 s). The grid was then deposited for 1 s onto a 100 µl drop of a saturated uranyl acetate aqueous solution and then onto a second 100 µl drop for 30 s. Excess staining solution was removed and the grid was dried at room temperature prior to the measurement. The grid finally underwent TEM analysis.

2.4.3. Drug content determination

Drug content was quantified by HPLC-Fluo. To ensure complete micelle destruction and release of incorporated drug, 1:5, 1:10 1:20 dilutions in mobile phase were made for each formulation and injected in the HPLC system

The drug content and drug loading were calculated using Eqs. (1) and (2) respectively:

$$\text{Drug content } (mg_{drug}/ml_{formulation}) = \frac{amount\ of\ IMQ\ in\ the\ formulation\ (mg)}{Volume\ of\ the\ formulation\ (ml)} \quad (1)$$

$$\text{Drug loading } (mg_{drug}/g_{copolymer}) = \frac{[IMQ]\ in\ the\ formulation\ (mg/ml)}{[Copolymer]\ in\ the\ formulation\ (mg/ml)} \quad (2)$$

2.4.4. Physical stability

Formulations were stored at 4 °C for 3 months and sampled at 1, 7, 14, 28 and 90 days. Drug content in micelle formulations was quantified by HPLC-Fluo to assess the drug concentration as a function of storage time.

2.4.5. IMQ localization in the formulation

In order to elucidate the exact location of the IMQ inside the micelle formulation, i.e. discriminate between the entrapped IMQ and free IMQ, the optimal micelle formulation was subjected to size exclusion chromatography using 14.5 × 50 mm PD-10 Desalting Columns (GE healthcare, Glattbrugg, Switzerland) packed with 85–260 µm Sephadex G-25 cross-linked dextran particles. The exclusion limit of 5000 Da enabled the 240.3 Da free IMQ to be retained, whereas micelles eluted through the column in their intact form (Supplementary Material).

PD-10 columns were equilibrated with 25 ml of 10 mM sodium acetate buffer (pH 6.6). 2.5 ml of sample (optimal micelle formulation: Formulation F 0.05% (see below) or control buffer solution) was applied to the column and allowed to enter the bed. Elution was subsequently performed using 25 ml of 10 mM sodium acetate and subsequently 10 ml of 0.5% acetic acid solution in water to recover all loaded IMQ. Fractions of 500 and 1000 µl were collected and analyzed by HPLC-Fluo.

2.5. Gel formulation for IMQ using mPEG-hexPLA micelles

2.5.1. Formulation of the gel

The optimal micelle formulation containing 0.05% IMQ (Formulation F 0.05%) was gellified in order to enable an accurate skin application. Several cellulose derivatives such as CMC and HEC were tested at different concentrations.

To prepare the gel, the required amount of gelling agent was introduced into the micelle solution (Formulation F) stirred at 350 rpm for 2 h. The IMQ content was adjusted to 0.05% by adding 10 mM sodium acetate buffer. It was verified that micelles were intact after the gel preparation (Supplementary Material). To prepare the control gel, the required amount of gelling agent (carboxymethyl cellulose 2%) was introduced into pH 4 acetate buffer solution containing 0.05% of IMQ.

2.5.2. Characterization of the gel

The prepared gel was visually inspected for clarity, consistency, color, and transparency. The pH was also measured. The rheology of gels was determined using a Haake Rheostress 1 Rheometer (HAAKE GmbH., Germany). A C35/2° cone (plate diameter 35 mm; 2° angle) was used to study dynamic rheological properties of the gels. All measurements were done at 25 °C in triplicate.

2.6. Skin preparation

2.6.1. Porcine skin

Porcine ears were purchased from a local abattoir (CARRE; Rolle, Switzerland). After washing under running cold water, skin samples (thickness ~1.2 mm) were harvested. Hair was removed from the skin surface using clippers. Discs corresponding to the permeation area were punched out (Berg & Schmid HK 500; Urdorf, Switzerland). Skin samples were frozen at −20 °C and stored for a maximum period of 3 months. Prior to the experiment, skin samples were thawed at room temperature and placed for 15 min in 0.9% saline solution for rehydration.

2.6.2. Human skin

Human skin samples were collected immediately after surgery from the Department of Plastic, Aesthetic and Reconstructive Surgery, Geneva University Hospital (Geneva, Switzerland). The study was approved by the Central Committee for Ethics in Research (CER: 08-150 (NAC08-051); Geneva University Hospital). Hypodermis and fatty

Table 2
Compositions of formulations used for *in vitro* studies.

Formulation	Drug content	Copolymer content (mg/ml)	Excipients	pH
Aldara®	5%	n.a.	Isostearic acid, benzyl alcohol, cetyl alcohol, stearyl alcohol, white soft paraffin, polysorbate 60, sorbitan monostearate, glycerol, methyl hydroxybenzoate, propyl hydroxybenzoate, xanthan gum	n.a.
Micelle solution 0.05%(F 0.05%)	0.05%	5	mPEG-hexPLA copolymer sodium acetate buffer 10 mM	4.2
Micelle gel 0.05%	0.05%	5	mPEG-hexPLA copolymer sodium acetate buffer 10 mM carboxymethyl cellulose 2%	4.2
Control gel 0.05%	0.05%	n.a.	Sodium acetate buffer 10 mM carboxymethyl cellulose 2%	4.0

tissue were removed and discs corresponding to the permeation area were punched out (Berg & Schmid HK 500; Urdorf, Switzerland). The skin discs were subsequently horizontally sliced with a Thomas Stadie-Riggs slicer (Thomas Scientific; Swedesboro, NJ, US) to obtain a thickness of ~1 mm. The skin was stored in a biobank at −20 °C for a maximum period of 3 months.

2.7. Evaluation of IMQ skin delivery in vitro

Porcine or human skin samples were equilibrated in 0.9% NaCl solution for 15 min to allow them to thaw and to hydrate. Skin samples were mounted on standard Franz diffusion cells (area = 2 cm^2). The receptor compartment (volume 10 ml) was filled with sonicated phosphate buffered saline (PBS, pH 7.4) containing 1% bovine serum albumin (BSA). After equilibration, 200 mg of micellar formulation, micellar gel or 200 mg of Aldara® cream were applied to the donor compartment. The receptor compartment was stirred at 250 rpm at 33 °C for 8 h (recommended application time in the prescribing guidelines [23]) 1 ml of receptor phase was withdrawn at the end of the experiment to quantify IMQ permeation. The compositions of the formulations tested are summarized in Table 2. At the end of the permeation experiments, the diffusion cells were dismantled and the skin surface was washed with running water for 5 s. The amount of IMQ deposited in the skin was extracted by cutting the skin samples into small pieces and soaking in 2 ml of mobile phase (60% ACN, 40% pH 4 acetate buffer) for 2 h with continuous stirring at room temperature. After 2 h, the extraction media was removed and set aside; in order to maximize the extraction yield, the skin pieces underwent a second extraction with 2 ml of fresh mobile phase for 2 h. Finally, extraction media from both extractions were pooled. The extraction procedure was validated (Supplementary Material). The extraction samples were centrifuged at 10,000 rpm for 15 min prior to UHPLC-MS/MS analysis.

2.8. Determination of IMQ biodistribution profile in human skin

Aldara® cream, micelle gel or control gel were applied for 8 h to human skin using the conditions described above (n = 5). At the end of the experiment and after the wash procedure, a small area of 0.8 cm^2 was punched out from the 2 cm^2 skin samples. These small skin discs were snap-frozen in isopentane cooled to its freezing point (−160 °C) with liquid nitrogen and mounted in a cryotome (Microm HM 560 Cryostat, Walldorf, Germany) and sliced to obtain horizontal (XY-plane) 20 µm thick lamellae. A total of 20 lamellae was taken from each sample – going from the skin surface to a depth of 400 µm, providing tissue samples from the stratum corneum, viable epidermis and upper dermis. Each lamella, as well as the remaining tissue, was collected in an individual Eppendorf tube. IMQ deposited in each lamella was extracted overnight with 200 µl of acetonitrile containing 0.1% formic acid and 50 ng/ml of internal standard (RSQ) by agitation at room temperature. The remaining dermis layer was collected in a separate tube and extracted with 1 ml of extraction solvent. Each extract was subsequently subjected to UHPLC-MS/MS analysis.

2.9. Data analysis

Data were expressed as the mean ± SD. Outliers determined using the Dixon test were discarded. Results were evaluated statistically using either Student's Test or analysis of variance (ANOVA) followed by Student Newman Keuls test when necessary as a post-hoc procedure. The level of significance was fixed at $\alpha = 0.05$.

3. Results

3.1. Micelle formulation characterization and optimization

3.1.1. Drug content and physical stability

A first series of formulations (Formulations A-D) was prepared with constant copolymer content (5 mg/ml) but different target IMQ loadings: 25, 50, 100, 150 mg of IMQ per g of copolymer. All formulations appeared as clear/opalescent liquids. The actual drug loadings achieved, drug contents and incorporation efficiencies obtained for each formulation are given in Table 3. The micelles incorporated IMQ

Table 3
Drug contents of different micelle formulations.

Formulation	Copolymer content (mg/ml)	Target drug loading (mg_{IMQ}/g_{copo})	Additives	Drug loading ± SD (mg_{IMQ}/g_{copo})	Drug content ± SD ($mg_{IMQ}/ml_{formulation}$)	Incorporation efficiency ± SD (%)
A	5	25	n.a.	22.00 ± 1.63	0.110 ± 0.008	88.0 ± 6.5
B	5	50	n.a.	46.60 ± 3.70	0.233 ± 0.019	93.2 ± 7.4
C	5	100	n.a.	89.32 ± 3.36	0.447 ± 0.017	89.3 ± 3.4
D	5	150	n.a.	157.75 ± 5.17	0.789 ± 0.002	102.2 ± 3.4
E	5	100	sodium acetate 10 mM	95.76 ± 3.78	0.479 ± 0.019	95.8 ± 3.8
F	10	100	sodium acetate 10 mM	94.55 ± 1.76	0.994 ± 0.018	99.4 ± 1.8
F 0.05%*	5	100	sodium acetate 10 mM	94.55 ± 1.76	0.503 ± 0.020	99.4 ± 1.8

* Prepared by dilution of formulation F.

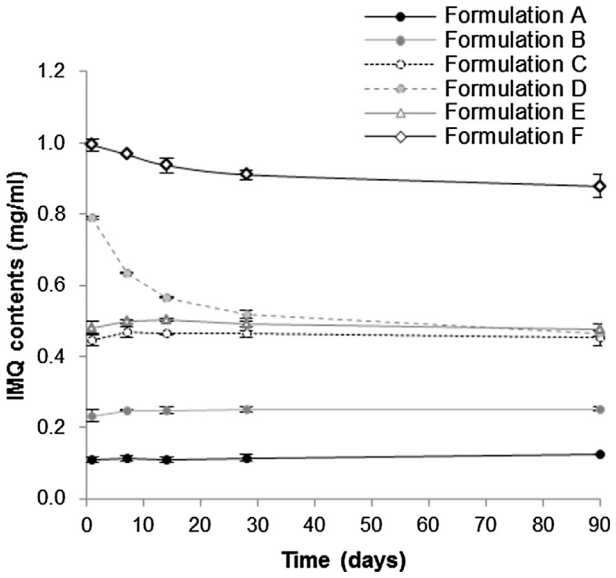

Fig. 1. Stability of IMQ loaded micelle formulations: evolution of drug content as a function of time during 3 months at 4 °C. (Composition of Formulation A-F is presented in Table 3).

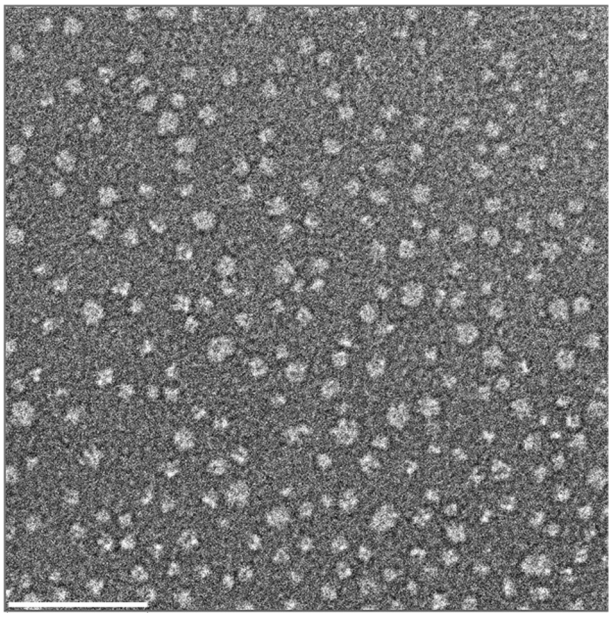

Fig. 2. TEM micrograph of micelles IMQ loaded micelles (Bar: 200 nm).

with efficiencies ranging from 88.0 ± 6.5% to 102.2 ± 3.4%. This resulted in drug contents ranging from 0.110 ± 0.008 mg/ml to 0.789 ± 0.002 mg/ml, the latter corresponding to a 43-fold increase in aqueous solubility.

As can be seen from Fig. 1, Formulation D was unstable showing drug precipitation after storage for 7 days and losing 40% of its IMQ load after the three month storage period. In contrast, Formulations A, B and C were more stable, retaining more than 95.4 ± 4.2% of the initially loaded IMQ. Formulation C was selected as the best of the stable formulations since it enabled an IMQ content of 0.447 ± 0.017 mg/ml to be achieved. It was further optimized by the addition of sodium acetate as pH corrector (Formulation E). The addition of the salt did not result in formulation instability since a drug content of 0.479 ± 0.019 mg/ml was achieved and was constant over three months.

Finally, in Formulation F, the copolymer content was increased to 10 mg/ml to incorporate a greater amount of IMQ. This resulted in a high and stable drug content of 0.994 ± 0.018 mg/ml.

3.1.2. Characterization of optimal IMQ micelle formulations.

Formulation F was considered to be the most stable formulation; its drug content was adjusted to 0.5 mg/ml for further experiments. The resulting Formulation F 0.05% was further characterized in terms of pH, residual solvent content, micelle size distribution and morphology. It presented a skin-friendly pH of 4.2 [24] and its acetone and acetic acid content were 0.52 ± 0.07% and 0.52 ± 0.33% respectively, which is below the 5000 ppm (0.05%) limit prescribed as Class 3 solvent content limit in both US pharmacopeia [21] and ICH Q3C guidelines [22]. A representative NMR spectrum is shown in the Supplementary Material.

DLS measurements revealed that the micelles presented nanometer-scale sizes with a hydrodynamic diameter of 181 nm, a polydispersity index of 0.112 and number weighed diameter (d_n) of 27 nm. The morphology of the micelles in Formulation F 0.05% was investigated by TEM – images of IMQ loaded mPEG-hexPLA micelles are shown in Fig. 2. According to the TEM observations micelles were spherical and of homogeneous size.

3.1.3. IMQ localization in the formulation

IMQ has a pKa of 6.3 [12]; thus, given that the pH of Formulation F 0.05% was 4.2, it can be assumed that IMQ was mostly present in its ionized form – therefore, was it localized inside the micelles? It was decided to use size exclusion chromatography to answer this question and to determine the exact location of IMQ in the formulation. This technique had already been used for the characterization and purification of colloidal entities such as liposomes [25], but had never been tried for micelles. Both Formulation F 0.05% and a control 0.05% aqueous solution were tested on the size exclusion column.

It appeared that micelles eluted with the first 4 ml of mobile phase (fractions 2–7; Fig. 3) as an opalescent solution: their integrity was verified by light scattering (Fig. 3b). The remaining fractions were clear (i.e. without micelles) and only contained free IMQ. It was hypothesized that micelles remained intact during the elution procedure since the particle size of dextran particles was ranging from 85 to 260 μm, i.e. 4,250 to 13,000-fold greater than the size of the micelles. Moreover, the low critical micelle concentration of the copolymer (1.6×10^{-6} M) [17] ensured the stability of the micelles with respect to dilution. This hypothesis was indeed verified using the Nile red encapsulation technique: Nile red loaded micelles appeared colored throughout the whole procedure demonstrating that no micelle disassembly and subsequent release of the dye occurred (Supplementary Material).

All fractions underwent IMQ quantification and Fig. 3a presents the amount of IMQ quantified in each fraction as a function of elution volume.

It was observed that for the control buffer solution, IMQ was retained by the size exclusion column and started eluting at 6 ml, whereas in the elution profile of the micelle containing sample there was a peak between 0 and 5 ml, corresponding to the micelle-entrapped IMQ since micelles co-eluted in these fractions (fractions 2–7). Thus, it was indeed possible to separate the entrapped drug from the free compound using size exclusion chromatography. The total amounts of IMQ recovered from tested formulations were 86–87% of the theoretical loading. The area under the curve indicated that in Formulation F 0.05%, 8.1% of IMQ was entrapped in the micelles and the rest was present as free drug in the continuous water phase presumably in its ionized state. Thus, IMQ in Formulation F 0.05% was only partially incorporated in the micelles due to the mild acidic pH of the aqueous phase; however, it is impossible to solubilize IMQ in an acceptable organic solvent without the addition of an acid. More importantly, the poly-lactic acid moiety of the copolymer is chemically more stable at mild acidic pH and even neutral conditions can lead to poly-lactic acid degradation [26]. Finally, skin itself presents an acidic pH at its surface [24], therefore application

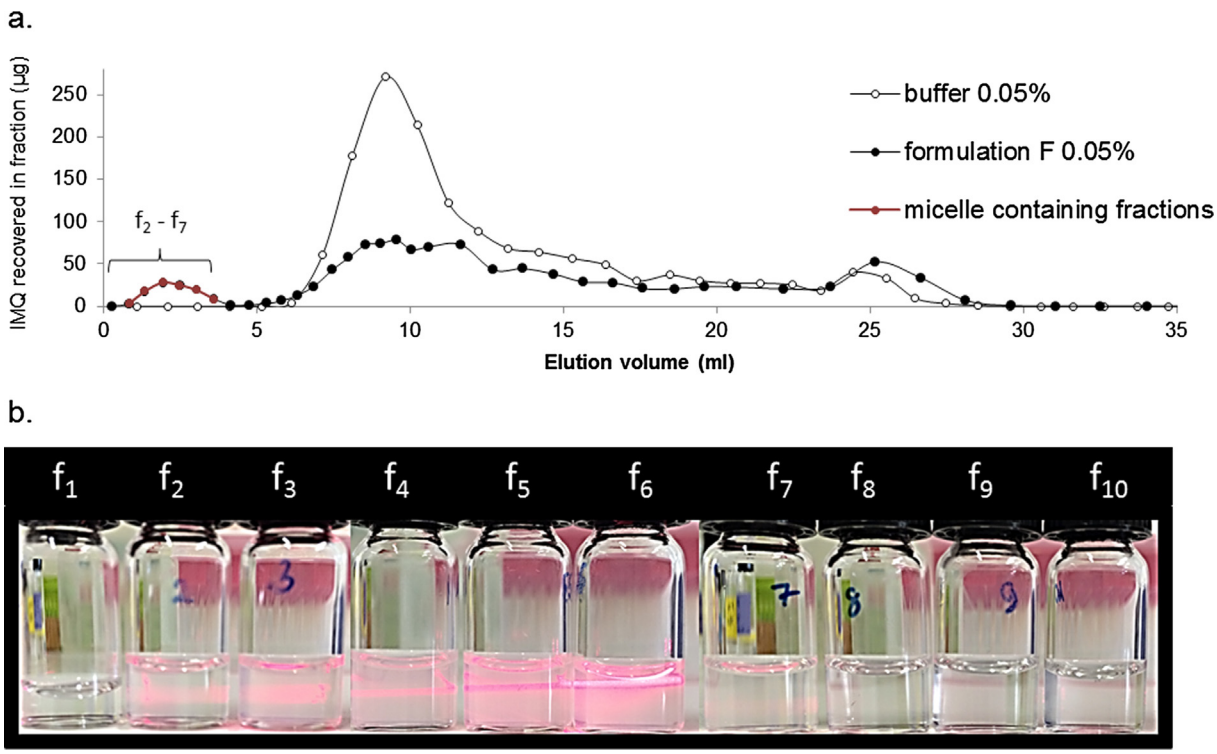

Fig. 3. (a) Amount of IMQ quantified in each fraction as a function of elution volume and (b) visualization of micelles in fractions 2–7 using laser diffraction.

of formulations with a pH of 4–5 is well tolerated.

3.1.4. Formulation of an optimal micelle based hydrogel

Micelle solutions have shown their potential as promising topical drug delivery systems; however, the application of a solution on skin is not practical because of the lack of viscosity and adhesion. Several gelling agents were screened to improve the texture of the formulation. Carbopol® (poly-acrylic acid) is a commonly used gelling agent in the pharmaceutical industry. However, this excipient could not be used in the present work since the neutralization step would influence the pH of the formulation and could trigger IMQ precipitation. Cellulose derivatives were thus considered as better candidates for the formulation of a micelle based gel. HEC (1%, 1.5% and 2%) and CMC (1.5%, 2% and 2.5%) placebo gels were formulated and their dynamic rheological behavior tested (Fig. 4).

All formulations showed shear-thinning behavior (Fig. 4). The highest viscosity profiles were yielded by the gels with highest cellulose contents (HEC 2% and CMC 2.5%). In contrast, gels with lowest cellulose content (HEC 1% and CMC 1.5%) yielded lowest viscosity profiles. An ideal topical formulation should have a low viscosity at high shear rate to enable an easy skin application and an intermediate viscosity at low shear rate to yield a good skin adherence without impairing the diffusion of the active inside the formulation. It appeared that high cellulose content led to viscosities that were too high at a low shear rate and could negatively influence micelle diffusion; low cellulose content resulted in viscosities that were too low at a low shear rate, which could prevent good adherence of the formulation to the skin. Thus, intermediate cellulose content gels (HEC 1.5% and CMC 2%) were considered as the best candidates for further development. Their viscosity at low shear rates was 2125 ± 236 mPa.s and 3179 ± 111 mPa.s at 1 s^{-1} shear rate, respectively; shear thinning behavior led to lower viscosities of 736 ± 11 mPa.s and 987 ± 16 mPa.s respectively at 46.4 s^{-1} shear rate. Despite a slightly higher viscosity profile, a preference was given to the CMC 2% gel as

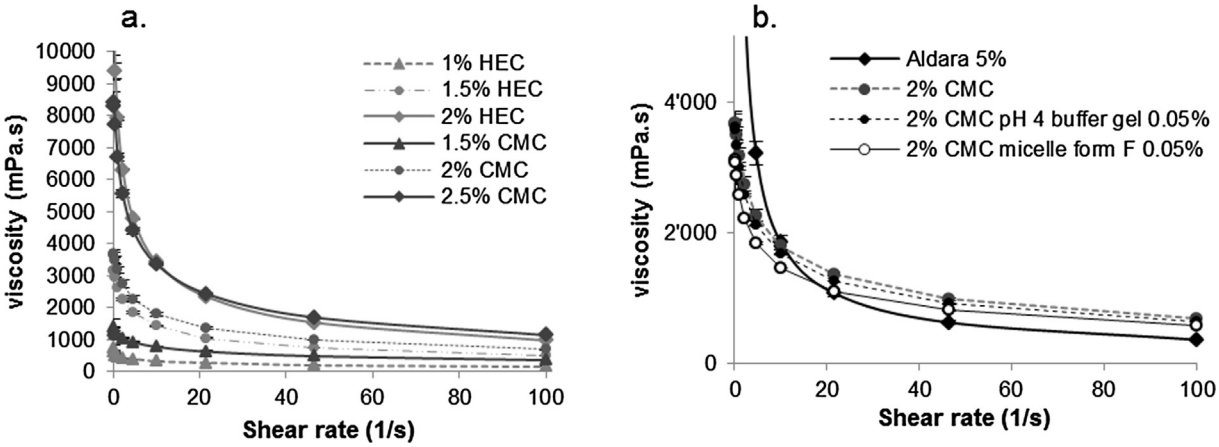

Fig. 4. Flow diagrams of (a) HEC and CMC gels; and (b) Aldara® 5% cream, the optimal 2% CMC placebo gel, the 2% CMC control 0.05% IMQ gel and micelle Formulation F 0.05% IMQ gel.

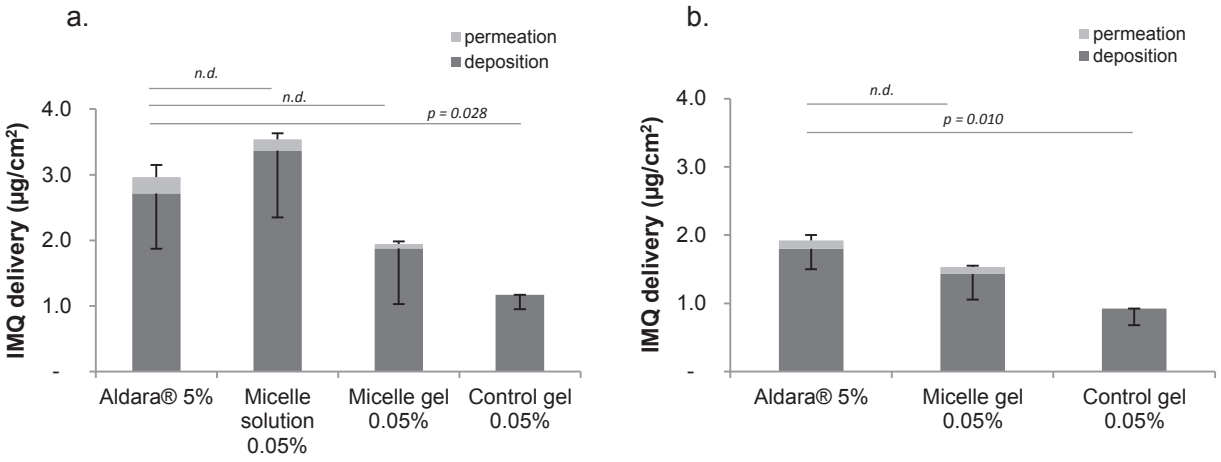

Fig. 5. Topical and transdermal delivery from different formulations to (a) porcine skin and (b) human skin. (*n.d.* – not different; *p* value is indicated when significantly different). Formulation excipients: Micelle solution 0.05% – mPEG-hexPLA copolymer; sodium acetate buffer 10 mM. Micelle gel 0.05% – mPEG-hexPLA copolymer; sodium acetate buffer 10 mM; carboxymethyl cellulose 2%. Control gel 0.05% – sodium acetate buffer 10 mM, carboxymethyl cellulose 2%.

the optimal vehicle because of its clearer appearance; indeed, the HEC gel had a yellowish color.

Aldara® 5% cream (Fig. 4b) had a high viscosity at low shear rate (9935 ± 602 mPa.s at 1 s^{-1} shear rate), which decreased significantly to 631 ± 29 mPa.s at 46.4 s^{-1} shear rate. It can be seen from the same figure that micelle incorporation in the gel led to a lower viscosity profile (3178 ± 47 mPa.s at 1 s^{-1} and 860 ± 8 mPa.s at 46.4 s^{-1}) whereas the modification of continuous phase from water to pH 4 buffer did not influence the rheological behavior.

The final optimal formulation was thus a 2% CMC gel with a 0.05% IMQ content. The micelle integrity in the gel was verified using the Nile red encapsulation technique (Supplementary Material). The gel was slightly opalescent due to the presence of micelles and presented a skin-friendly pH of 4.3.

3.2. In vitro IMQ delivery experiments

3.2.1. Skin delivery to porcine and human skins

IMQ deposition in porcine skin (Fig. 5a) from Formulation F 0.05% (3.4 ± 1.0 µg/cm^2) was statistically equivalent to that from Aldara® 5% cream (2.7 ± 0.8 µg/cm^2) – despite the 100-fold lower IMQ content.

The formulation of the micelle gel induced a slightly decreased skin deposition (1.9 ± 0.8 µg/cm^2) but was still statistically comparable to that of the commercial formulation. The lowest skin deposition was yielded by the control 0.05% gel (1.2 ± 0.2 µg/cm^2).

Application of Aldara® 5% cream and Formulation F 0.05% for 8 h resulted in a low but detectable skin permeation (249 ± 184 ng/cm^2 and 172 ± 89 ng/cm^2, respectively). The incorporation of the micelles in the CMC 2% gel decreased transdermal permeation to 70 ± 39 ng/cm^2, whereas transdermal IMQ was not detected for the control 0.05% gel. The 2% CMC gel (Micelle gel 0.05%) was selected for experiments on human skin because (i) it presented favorable rheological properties and (ii) the ability to selectively deliver IMQ to skin thus minimizing transdermal permeation.

Human skin experiments confirmed the results obtained with the porcine model (Fig. 5b) showing skin depositions of 1.8 ± 0.3 µg/cm^2; 1.4 ± 0.4 µg/cm^2 and 0.9 ± 0.2 µg/cm^2 for Aldara® 5% cream , micelle 0.05% gel and control 0.05% gel respectively and were not different from porcine skin data (T-test, $\alpha = 0.05$). Moreover, no statistical difference was observed between deposition yielded by Aldara® 5% cream and optimal micelle 0.05% gel nor between the latter and the control 0.05% gel. Permeation across human skin was in the nanogram range (123 ± 79 ng/cm^2 and 79 ± 19 ng/cm^2 for the commercial formulation and the micelle 0.05% gel respectively). As expected, the control 0.05% gel showed no transdermal delivery.

3.2.2. IMQ biodistribution in human skin.

Given that the different formulations were able to deliver IMQ to human skin, it was subsequently of great interest to know in which skin layer the drug was localized, keeping in mind that the target antigen presenting cells are situated in viable epidermis and can migrate to deeper dermal lymph nodes [27]. Therefore, IMQ was quantified in individual 20 µm thick skin layers after application of the different

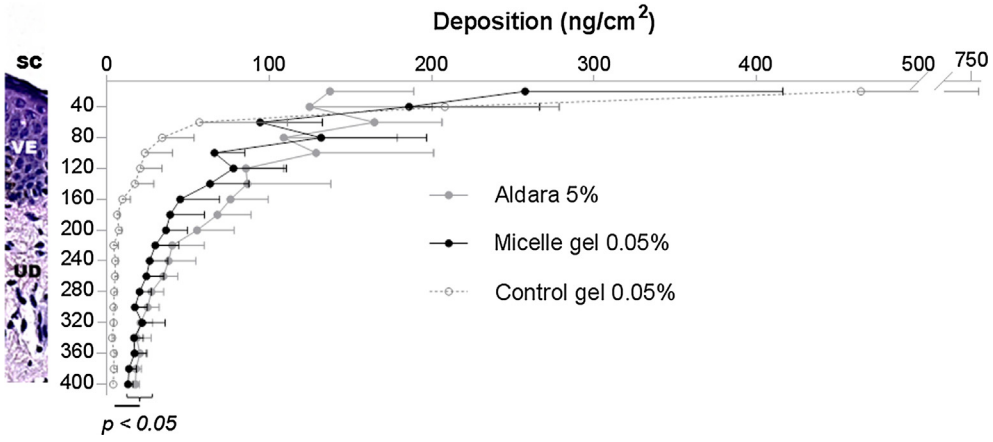

Fig. 6. Biodistribution profile of IMQ in human skin showing the amounts of IMQ deposited as a function of skin depth (SC; stratum corneum, VE; viable epidermis; UD: upper dermis). (*n.d.* – no significant difference; *p* value is indicated when significantly different). Formulation excipients: Micelle gel 0.05% - mPEG-hexPLA copolymer; sodium acetate buffer 10 mM; carboxymethyl cellulose 2%. Control gel 0.05% - sodium acetate buffer 10 mM, carboxymethyl cellulose 2%.

formulations and the biodistribution profile is presented in Fig. 6; Aldara® 5% cream and micelle 0.05% gel produced a very similar IMQ distribution skin profile, with high IMQ deposition in the first layer (137.4 ± 51.6 ng/cm^2 and 257.7 ± 158.7 ng/cm^2 respectively) gradually decreasing to 17.6 ± 2.3 ng/cm^2 and 13.1 ± 2.9 ng/cm^2, respectively in the last layer. No statistical difference could be evidenced between both profiles (point-by point T-test, $\alpha = 0.05$). The control 0.05% gel showed a higher accumulation in the first layer (464.3 ± 323.6 ng/cm^2) that rapidly decreased to reach significantly lower (3.0–9.0 ng/cm^2) levels in deeper layers.

4. Discussion

4.1. Formulation optimization

Stable IMQ loaded micelle formulations were successfully formulated and improved aqueous solubility by up to 43-fold. Formulation F was considered as the optimal stable formulation and its IMQ content was adjusted to 0.05%. The micelles presented homogeneous nanometer scale diameters (d_n of 27 nm) and spherical morphology. The pH of the final formulation, Formulation F 0.05%, was compatible with skin application and favored hexPLA stability.

Previous studies with mPEG-hexPLA micelle formulations suggested that drug loading was influenced by several molecular parameters such as the number of H-donors, number of H-acceptors, log P, and aqueous solubility [16]. It was noticed that the drugs that were efficiently loaded into micelles (*i.e.* drug loading > 100 mg/g) possessed a high number of H donors and H acceptors and intermediate log P (0.4–5.2). Drugs like clotrimazole and retinoic acid presented low drug loadings into mPEG-hexPLA micelles [14,16]. Their common features were few H-bond donors and H-bond acceptors, and high log P. The presence of an ionizable site could also negatively influence drug incorporation into micelles, e.g. retinoic acid [14]. Although IMQ possesses a moderate log P of 3.0, and 2 H donors and 4 H acceptors and intermediate aqueous solubility (0.018 g/l), it also contains an ionizable group with a pKa of 6.3 and this must have affected its incorporation.

The major practical difficulty encountered in the formulation process was the lack of IMQ solubility in volatile water miscible and immiscible solvents, which demanded an adjustment of the commonly used solvent evaporation method by the addition of an acid. Total drug loading in the formulation was quite high (up to 94.55 ± 1.76 mg $_{IMQ}$/g $_{copo}$); however, the investigation into the exact localization of IMQ in the formulation using size exclusion chromatography indicated that the drug was only partially incorporated in the nanocarriers. This phenomenon was probably due to the use of acetic acid in the organic phase. Thus, IMQ being in its ionized form already in acetone; its incorporation into the micelles was impaired, leaving it in the outer aqueous phase.

It is important to note that although size exclusion chromatography has been previously used for the purification of liposomes [25], it has never been applied to micelle solutions. Interestingly, IMQ-containing micelles were well separated from free IMQ, moreover it was shown that micelles eluted through the column in their intact form. Thus, size exclusion chromatography was proven to be an extremely useful technique in the analysis and purification of these colloidal carriers.

Micelles were incorporated into a gel in order to improve ease of application and pleasant esthetic appearance. The best gel-forming excipient was CMC at 2% as micelle integrity was not perturbed and the rheological behavior of the gel enabled its easy application on skin. The slight decrease in viscosity produced by the micelles may be due to the interaction between the outer micelle shell and polymeric network, facilitating their alignment under shear.

4.2. IMQ skin delivery in vitro

The IMQ deposition in porcine skin (Fig. 5a) from micelle Formulation F 0.05%, micelle 0.05% gel and Aldara® 5% cream were similar. The slight decrease in topical delivery yielded by the micelle gel formulation in comparison to the micelle solution can be attributed to impairment of drug and micelle diffusion through the three-dimensional cellulose network. However, it is important to emphasize that despite a 100-fold lower IMQ content, both micelle formulations produced a similar delivery to the commercial formulation. Thus, despite the complex composition of Aldara® cream (isostearic acid, benzyl alcohol, cetyl alcohol, stearyl alcohol, white soft paraffin, polysorbate 60, sorbitan monostearate, glycerol, methyl hydroxybenzoate, propyl hydroxybenzoate, xanthan gum, purified water) and the presence of excipients acting as penetration enhancers (e.g. polysorbate 60) it appeared that this formulation had a poor delivery efficiency (only 0.04 ± 0.01% of the applied dose was delivered topically to human skin). In contrast, the micelle 0.05% gel yielded much higher delivery efficiency of 2.85 ± 0.74%. Surprisingly, even a simple pH 4 aqueous gel able to solubilize 0.05% of IMQ was able to yield a delivery efficiency of 1.84 ± 0.49%.

Aldara® cream was developed by 3 M Pharmaceuticals and approved by the FDA in 1997 [28]. Later 3 M Pharmaceuticals published the formulation development process [29]. The formulation was required to be “water-washable”, “cosmetically elegant” and able to “provide local cytokine induction”. First efforts focused on the development of a micronized IMQ suspension in a gel at 1, 3, 5%, but this formulation failed to produce a cytokine response in rodents. Further work was conducted to formulate a 5% cream where isostearic acid was used as the main solubilizer for IMQ. This formulation was shown to induce effective cytokine production after topical application to hairless mice. Thus, the use of an emulsion system solved the problem of IMQ solubility; however, no further optimization of the delivery efficiency was undertaken. Thus, despite a very high drug content of 5% (50 mg/g), Aldara® cream suffers from poor delivery efficiency, it can be supposed that because of the formulation’s high solubilizing abilities, IMQ affinity for the vehicle is too high and thermodynamic activity too low, thereby preventing the drug from readily partitioning into skin. In contrast, in the micelle formulations and even in the aqueous gel, IMQ content is relatively low (0.05%), but close to saturation, thus increasing the thermodynamic activity of the system and driving drug into the skin.

The micelle 0.05% gel appeared as an optimal, easy to apply formulation, able to target the skin since undesired transdermal permeation was limited (less than 100 ng/cm^2) and lower than that from Aldara® cream. The non-significant difference in skin delivery between the micelle 0.05% gel and the control 0.05% gel was attributed to the partial incorporation of IMQ in the mPEG-hexPLA micelles.

Interestingly, investigation into the biodistribution profile of IMQ in human skin confirmed that micelle 0.05% gel was able to deliver IMQ as deep as the upper dermis in the same way as Aldara® cream. In contrast, the control 0.05% gel was not able to deliver substantial IMQ amounts to deep skin layers. Fig. 7 summarizes the IMQ amounts retrieved in different skin layers after application of different formulations (Stratum Corneum: 0–40 μm; viable epidermis 40–160 μm; upper dermis 160–200 μm; lower dermis 200–400 μm) [13,30].

Indeed it can be seen from Fig. 7a that IMQ from micelle 0.05% gel and Aldara® cream were both preferentially deposited in the viable epidermis (518.2 ± 173.3 ng/cm^2 and 719.0 ± 227.8 ng/cm^2 respectively) and upper dermis (236.4 ± 88.2 ng/cm^2 and 316.1 ± 85.2 ng/cm^2 respectively) whereas the control 0.05% gel could essentially only deliver IMQ to the stratum corneum (672.4 ± 267.4 ng/cm^2), thus indicating its inferiority in comparison with the other two formulations. It is important to note that Aldara® cream yielded a high but undesired deposition in lower dermis (627.6 ± 341.7 ng/cm^2), followed by higher transdermal permeation.

As mentioned above, no significant difference was evidenced between topical delivery to human skin yielded by the micelle gel and its micelle-free equivalent. However, a clear difference between those

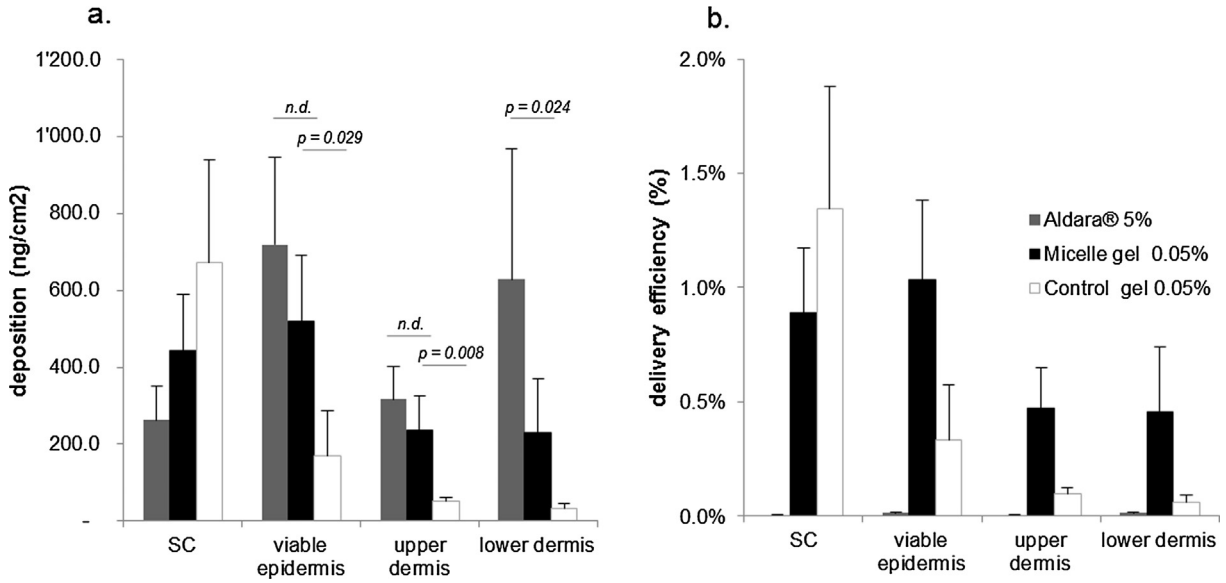

Fig. 7. (a) IMQ deposition from tested formulations in different human skin layers and (b) IMQ deposition in different human skin layers expressed as a percentage of applied dose (i.e. delivery efficiency). (*n.d.* – no significant difference; *p* value is indicated when significantly different).

formulations can be seen in the skin biodistribution: the micelle formulation could efficiently target the viable epidermis and upper dermis whereas the control gel could not. It consequently appears that even though IMQ is only partially incorporated in the micelles, the presence of the mPEG-hexPLA copolymer led to a targeted delivery to viable epidermis and dermis, where the antigen presenting cells reside, without an increased delivery to lower dermis and transdermal delivery, bringing further evidence to suggest that the copolymer may act as a mild penetration enhancer [13,17]. The micelle 0.05% gel also outperformed the other two formulations in terms of delivery efficiency as 1.04 ± 0.35% of the applied dose was delivered to the viable epidermis and 0.47 ± 0.18% to the upper dermis (for Aldara® cream the delivery efficiencies in those layers were less than 0.02% and for the control 0.05% gel, 0.34 ± 0.24% and 0.10 ± 0.02% could be found in those respective layers).

Recent efforts to improve local delivery of IMQ to skin have shown that nanoparticulate systems are indeed promising [31–35]. However, only a few studies [33–35] were comparable to the present work since other investigations were done on rodent skin. Ma et al. developed a nanoscale vesicular system (around 100 nm) composed of transethosomes (comprising phospholipids, ethanol and permeation enhancers) that was able to yield a substantial skin deposition (above 20 μg/cm^2) *and* permeation (above 3 μg/cm^2) into and across porcine tissue. Given that the Aldara® cream that served as the control also yielded very high permeation values (6.52 ± 1.53 μg/cm^2), it is supposed that the experimental conditions (presence of ethanol in the receiver compartment and 24 h exposition to the formulation) led to the elevated delivery observed. Indeed these data may result in poor correlation with what is seen in human subjects since systemic levels of IMQ after Aldara® cream application in patients were reported to be in the ng/ml range [23,36]. Two studies conducted by the Santi group [34,35] on the ability of microemulsions to deliver IMQ to porcine skin showed similar skin deposition to the present study for the commercial formulation (6 h application; 1.89 ± 0.77 μg/cm^2) [34]. An appreciable amount of formulation work yielded a series of bicontinuous microemulsions that were able to solubilize from 0.27% to 0.45% of IMQ [35]. The optimal ME44 (0.33% IMQ content) was able to deliver 0.31% of the applied dose to the skin: 0.23% was retrieved in the epidermis and 0.08% in the dermis. Considering this, the present micelle 0.05% gel, despite a lower drug content, outperformed ME44 in terms of delivery efficiency as 1.92 ± 0.51% of the applied dose was delivered to the epidermis (SC + VE) and 0.93 ± 0.43% to the dermis (UD + LD). Telo et al. showed that skin delivery directly depended on IMQ solubility in the excipients and their uptake by stratum corneum [34]. The pH of the aqueous phase was not determined; therefore, it was not possible to know the exact IMQ distribution within the microemulsion. Given the complexity of the microemulsion system and its variable and dynamic microstructure it is difficult to explain the differences in delivery efficiency from both formulations.

To conclude, it has been reported that the EC50 of IMQ towards the TLR 7 is 2.12 μM [37], the tissue levels yielded by the 0.05% micelle gel were at least 27.2 ± 6.0 μM, i.e. 12 times above the EC50 of IMQ indicating that therapeutically relevant drug levels can be achieved in skin.

5. Conclusion

IMQ micelle and gel formulations were successfully formulated and their application to porcine and human skin led to promising delivery results despite the fact that IMQ was only partially incorporated in the micelles. Micelles were included in an easy-to apply 0.05% gel, which outperformed the 5% Aldara® commercial formulation in terms of delivery efficiency and enabled therapeutically relevant drug levels to be achieved in viable epidermis and dermis. Finally, this work reports for the first time the possibility of using size exclusion chromatography to discriminate between drug incorporated in micelle nanocarriers and free drug present in solution.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.01.008.

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