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Novel micelle formulations to increase cutaneous bioavailability of azole antifungals

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ABSTRACT

Efficient topical drug administration for the treatment of superficial fungal infections would deliver the therapeutic agent to the target compartment and reduce the risk of systemic side effects. However, the physicochemical properties of the commonly used azole antifungals make their formulation a considerable challenge. The objective of the present investigation was to develop aqueous micelle solutions of clotrimazole (CLZ), econazole nitrate (ECZ) and fluconazole (FLZ) using novel amphiphilic methoxy-poly(ethylene glycol)-hexyl substituted polylactide (MPEG-hexPLA) block copolymers. The CLZ, ECZ and FLZ formulations were characterized with respect to drug loading and micelle size. The optimal drug formulation was selected for skin transport studies that were performed using full thickness porcine and human skin. Penetration pathways and micellar distribution in the skin were visualized using fluorescein loaded micelles and confocal laser scanning microscopy. The hydrodynamic diameters of the azole loaded micelles were between 70 and 165 nm and the corresponding number weighted diameters (d_n) were 30 to 40 nm. Somewhat surprisingly, the lowest loading efficiency (<20%) was observed for CLZ (the most hydrophobic of the three azoles tested); in contrast, under the same conditions, ECZ was incorporated with an efficiency of 98.3% in MPEG-dihexPLA micelles. Based on the characterization data and preliminary transport experiments, ECZ loaded MPEG-dihexPLA micelles (concentration 1.3 mg/mL; d_n<40 nm) were selected for further study. ECZ delivery was compared to that from Pevaryl® cream (1% w/w ECZ), a marketed liposomal formulation for topical application. ECZ deposition in porcine skin following 6 h application using the MPEG-dihexPLA micelles was >13-fold higher than that from Pevaryl® cream (22.8 ± 3.8 and 1.7 ± 0.6 μg/cm^2, respectively). A significant enhancement was also observed with human skin; the amounts of ECZ deposited were 11.3 ± 1.6 and 1.5 ± 0.4 μg/cm^2, respectively (i.e., a 7.5-fold improvement in delivery). Confocal laser scanning microscopy images supported the hypothesis that the higher delivery observed in porcine skin was due to a larger contribution of the follicular penetration pathway. In conclusion, the significant increase in ECZ skin deposition achieved using the MPEG-dihexPLA micelles demonstrates their ability to improve cutaneous drug bioavailability; this may translate into improved clinical efficacy *in vivo*. Moreover, these micelle systems may also enable targeting of the hair follicle and this will be investigated in future studies.

1. Introduction

The incidence of mycoses especially superficial fungal infections is increasing and according to a recent report more than 25% of the world's population is affected [1,2]; disease progression is more rapid and severity increased in patients with compromised immune function [3]. Host immunity can be impaired during infancy, in old age, by pregnancy, by disease, e.g. diabetes mellitus, or through the administration of antibiotics and glucocorticoids [4]. Azole antifungals such as clotrimazole (CLZ), econazole nitrate (ECZ) and fluconazole (FLZ) are the first line treatments for various fungal infections [5]. Topical therapy is desirable since, in addition to targeting the site of infection, it reduces the risk of systemic side effects. In general, azole antifungals tend to be highly lipophilic (although there are exceptions (e.g., FLZ)) and they can readily partition into the lipid-rich intracellular space in the stratum corneum; the challenge is to develop a simple stable formulation that facilitates drug release into the skin [6]. Given the desirable properties of aqueous formulations and the lipophilic character and poor water solubility of azoles, it was decided to investigate polymeric micelles as a drug carrier system. Due to their stability, size and ability to incorporate significant amounts of hydrophobic drugs in their core, these systems seem to be well-suited for use with azole antifungals. In previous studies, micelle formulations using two novel amphiphilic methoxy-poly(ethylene glycol)-hexyl-substituted poly(lactides) (MPEG-hexPLA) block copolymers, mono- and di-hexyl-substituted (MPEG-monohexPLA and –dihexPLA, respectively) demonstrated their ability to incorporate several poorly water soluble drugs with high loading efficiencies [7–9]. The present

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study investigated the micelle formulations of three azole antifungals, clotrimazole, econazole nitrate and fluconazole, possessing different physicochemical properties (Table 1).

The specific objectives were (i) to develop and to characterize micelle formulations for CLZ, ECZ and FLZ using the novel excipients – MPEG-monohexPLA and –dihexPLA – and to compare them with formulations using standard MPEG-polylactide (MPEG-PLA), (ii) to select the best drug candidate (as determined by incorporation efficiency and micelle properties) and to optimize the formulation for skin deposition, (iii) to quantify drug deposition in full thickness porcine and human skin and to compare delivery to that from a commercial formulation and (iv) to visualize micellar transport pathways using fluorescein loaded micelles and confocal laser scanning microscopy.

2. Materials and methods

2.1. Materials

Clotrimazole (CLZ), econazole nitrate (ECZ), fluconazole (FLZ), dipotassium hydrogen phosphate, monobasic ammonium phosphate, acetone, and fluorescein acid were purchased from Sigma Aldrich (Buchs, Switzerland). Methanol and acetonitrile (Chromasolv HPLC grade) and nylon membrane filters (0.22 μm) were purchased from VWR (Nyon, Switzerland). Pevaryl® cream (1% w/w ECZ) was purchased from Janssen-Cilag (7HB5P01); it contains PEG-6 stearate, glycol stearate and PEG-32 stearate (Téfose 63), liquid parafin, a polyoxyethylated kernel oil (Labrafil M 1944 CS), benzoic acid (E 210), perfumes (essential oils of rose, jasmine, iris, sandalwood, coriander, ylang-ylang, vétyver, linalol, cinnamic alcohol, cinnamic aldehyde), butylhydroxyanisole (E 320), purified water.

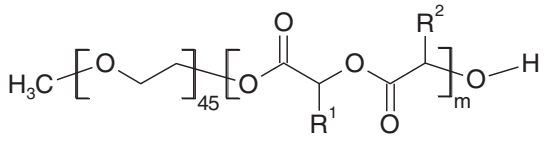
The block copolymers, methoxy-poly(ethylene glycol)-di-hexyl-substituted lactide (MPEG-dihexPLA), -mono-hexyl-substituted lactide (MPEG-monohexPLA) and -polylactide (MPEG-PLA) were synthesized with $MPEG_{2000g/mol}$ as initiator as described previously [7,9].

The structures of these three block copolymers are presented in Scheme 1 and their molecular weights and polydispersity indices are shown in Table 2.

Please note that in the following text, the term "MPEG-hexPLA" is used to refer collectively to the MPEG-monohexPLA and MPEG-dihexPLA polymers.

2.2. Preparation of drug loaded MPEG-hexPLA and MPEG-PLA micelles by stirring (Method 1)

The micelles were prepared by a co-solvent evaporation method. Briefly, 6 mg of drug was dissolved in 1 mL acetone and mixed with 1 mL copolymer solution (20 mg/mL) in acetone. The organic solution was added dropwise every 5 s, using a peristaltic pump, into 4 mL of ultra-pure water under continuous stirring. Acetone was then slowly removed by evaporation (with stirring) in a desiccator under vacuum (2 h, 200 mbar). The final micelle concentration was adjusted by adding ultra-pure water in order to reach a copolymer concentration of 5 mg/mL. After overnight equilibration, the solution was centrifuged at 9500×*g* for 15 min to remove non-incorporated drug.



R^1, R^2 $=CH_3$: $MPEG_{2000g/mol}$-$PLA_{3000g/mol}$ for m=21
$R^1=C_6H_{13}$, $R^2=CH_3$: $MPEG_{2000g/mol}$-monohexPLA$_{3000g/mol}$ for m=14
R^1, R^2 $=C_6H_{13}$: $MPEG_{2000g/mol}$-dihexPLA$_{3000g/mol}$ for m=11

Scheme 1. Structure of MPEG-hexPLA and MPEG-PLA copolymers.

2.3. Preparation of ECZ loaded MPEG-dihexPLA micelles by sonication (Method 2)

Econazole nitrate (ECZ) loaded MPEG-dihexPLA micelle formulations with copolymer concentrations of 5 and of 10 mg/mL were also prepared by the co-solvent evaporation sonication method [7]. ECZ and MPEG-dihexPLA copolymer were dissolved in 2 mL acetone and added dropwise every 5 s into 4 mL ultra-pure water under sonication. The remaining acetone was removed by evaporation with a rotavapor at 15 mbar. The concentration of copolymer was adjusted and the non-incorporated drug was removed by centrifugation.

2.4. Preparation of fluorescein loaded MPEG-dihexPLA micelles

Fluorescein loaded MPEG-dihexPLA micelles were prepared by Method 2.

2.5. Size determination of drug loaded micelles

Dynamic light scattering measurements (at 25 °C at an angle of 90°) using a Zetasizer HS 3000 (Malvern Instruments Ltd; Malvern, UK) were made to determine the hydrodynamic diameter (Z_{av}), the number-weighted diameter (d_n) and the percentage of micelles having the number-weighted diameter ($[\%]_{dn}$). All measurements were done in triplicate.

2.6. Morphology determination of drug loaded micelles

The morphology of drug loaded micelles was determined by transmission electron microscopy (TEM) (EM 410, Philips, 60 kV) using the negative staining method. Briefly, 30 μL of the micellar solution were dropped onto an ionised 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 uranyl acetate solution (400 μL of a saturated uranyl acetate solution dissolved in 600 μL of distilled water) and afterwards

Table 1
Physicochemical characteristics of the three antifungal agents.

	MW[a] (g/mol)	log $P_{o/w}$[b]	Aqueous solubility (g/L)	pKa
Clotrimazole (CLZ)	344.84	5.9 [10]	0.030 [11]	5.83 [12]
Fluconazole (FLZ)	306.27	0.4[c]	0.001[c]	–
Econazole nitrate (ECZ)	444.70	5.2[d] [10]	0.800[e]	6.65[d] [10]

[a] Molecular weight.
[b] Experimental partition coefficient between octanol and water.
[c] Data taken from http://www.drugbank.ca/drugs.
[d] Data taken from econazole.
[e] Determined experimentally by the shake-flask method (24 h at 25 °C).

Table 2
Characteristics of MPEG-hexPLA and MPEG-PLA copolymers.

Copolymer	MW[a] (g/mol)	P.I.[b]
MPEG-PLA	5050	1.17
MPEG-monohexPLA	5040	1.13
MPEG-dihexPLA (1)	5554	1.13
MPEG-dihexPLA (2)	4881	1.11

(1) Copolymer used only for comparison of CLZ, ECZ and FLZ micelle formulations prepared by Method 1.
(2) Copolymer used for the optimization of ECZ MPEG-dihexPLA micelle formulations, skin experiments and for the fluorescein micelle formulation.
[a] Determined by ^{1}H NMR (Bruker, 300 MHz).
[b] Polydispersity index (P.I.) determined by GPC.