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Development and characterization of skin permeation retardants and enhancers: A comparative study of levothyroxine-loaded PNIPAM, PLA, PLGA and EC microparticles

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ABSTRACT: Polymeric microparticles suitable for topical and transdermal delivery systems were studied using poly D,L lactide (PLA), poly D,L lactide co glycoside (PLGA), poly (*N*-isopropylacrylamide) (PNIPAM) and ethyl cellulose (EC). Drug encapsulation efficacy, microparticle stability and skin permeation studies of levothyroxine loaded microparticles were carried out using excised human skin, and the skin permeation pattern was observed using confocal laser scanning microscopy.

It was found that ethyl cellulose microparticles had the highest drug encapsulation and minimal drug leakage during the 14week storage period. The PNIPAM microparticles had the lowest drug encapsulation efficiency and a fast degradation rate. The PLGA microparticles exhibited a temperature dependent drug leakage. Permeation studies using a flow-through diffusion cell indicated that the polymer transition temperature (T_g) may influence the skin permeation rate of levothyroxine. Polyesters (PLA and PLGA) and PNIPAM acted as a skin penetration retardant and caused skin accumulation of the drug. These microparticles have potential use in skin formulations containing sunscreens and other active ingredients that are meant to be concentrated on the skin surface. However, skin permeation was observed from EC microparticles, therefore such polymers may be used as carriers in transdermal formulations to help achieve therapeutic concentrations of the drug in the plasma. Copyright © 2011 John Wiley & Sons, Ltd.

Key words: penetration enhancers; penetration retardants; ethyl cellulose; poly D, L lactide; poly (*N*-isopropylacrylamide)

Introduction

Polymeric microspheres are being used widely in drug delivery systems. Biodegradable polymers can be designed to control and prolong drug release by adjusting the degradation rate of the polymer. Ethyl cellulose (EC), a non-toxic, inexpensive and biocompatible polymer, has been used in a variety of applications in pharmaceutical dosage forms such as sustained release and controlled delivery of drugs [1–3]. The hydrophilic nature of EC can help to increase the bioavailability of poorly water-soluble compounds [2]. Aliphatic polyesters such as poly D,L lactide (PLA) and its copolymers with glycolic acid (PLGA) are remarkable for sustained drug delivery and targeting drugs to

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specific sites. These biocompatible and biodegradable polymers undergo bulk hydrolysis thereby providing sustained delivery of the therapeutic agent [4,5]. Smart polymers can release the drug by external stimuli such as a change in temperature, pH or ionic composition [6,7]. Thermosensitive polymers, such as poly (*N*-isopropylacrylamide) (PNIPAM), exhibit a temperature-dependent shrinking at lower critical solution temperature (LCST). These changes corresponding to swelling or de-swelling of the polymer can be controlled to release the encapsulated drugs in response to external temperature changes [8,9].

Levothyroxine (T_4), a model drug, is a synthetic hormone administered orally for the treatment of hypothyroidism and goitre [10,11]. Topical administration of T_4 has been used in cosmetic creams to reduce deposits of adipose tissue on skin [12,13]. However, the high concentrations of T_4 in cosmetic products may raise concerns about its systemic effects.

The aim of the present study was to determine whether the topical administration of T_4 can result in transdermal penetration. Four types of polymers of different molecular weights and different hydrophobicities were used to encapsulate T_4. The microspheres were characterized for drug entrapment efficiency, storage stability, *in vitro* drug release and skin penetration. The skin permeation pattern was observed using confocal laser scanning microscopy.

Materials and Methods

Materials

L-Levothyroxine, poly vinyl alcohol (MW 31000), poly (*N*-isopropylacrylamide) (MW 20000–25000), fluorescein, 4′,6-diamidino-2-phenylindole (DAPI) and ethyl cellulose were purchased from Sigma-Aldrich (Singapore). The PLA (R 203H), density of 0.34 dl/g, and PLGA 48/52 (RG 503H) with a density of 0.52 dl/g were gifts from Boehringer Ingelheim (Germany).

Preparation of microparticles

Levothyroxine microparticles were prepared by an emulsification–solvent evaporation technique. The organic phase consisted of 50 mg of polymer (EC, PLA, PLGA or PNIPAM) dissolved in 1 ml of dichloromethane, which was then emulsified in a PVA aqueous solution (5 ml, 5% w/v PVA, 2 mg/ml T_4). The system was stirred continuously at 700 rpm for 5 h to allow the evaporation of the organic solvent [14].

Encapsulation efficacy and stability studies

The drug-loaded microparticles were centrifuged at 17000 rpm for 45 min at 20°C. The free levothyroxine in the supernatant was determined by the HPLC method and the encapsulation efficacy (EE %) was calculated from the following expression:

$$EE\% = \frac{[total\ \ drug] - [free\ \ drug]}{[total\ \ drug]} \times 100$$

As the structure of polymeric particles can significantly change upon storage, the encapsulation efficacy was investigated after a 14 week storage at room temperature (20°C) and in the fridge (4°C).

Field emission scanning electron microscopy (FESEM)

The surface morphology and appearance of microparticles were examined. The prepared microspheres were coated by a sputtering unit (Jeol JFC-1600 auto fine coater; Japan) under vacuum. The coated samples were then examined using a field emission scanning electron microscope (Jeol JSM-6701F; Japan) operating at an accelerating voltage of 5 kV.

HPLC analysis

The concentration of T_4 was determined by modifying a previously developed HPLC method [15]. The analysis was carried out using HPLC with an Agilent HP 1100 Series (USA) and an X-bridge column (3.5 μm, 4.6 mm × 100 mm; USA). The mobile phase (70:30 acetonitrile and 0.05 M phosphate buffer adjusted to pH 3 using phosphoric acid) was delivered at a rate of 0.6 ml/min. The UV detection at a wavelength of 220 nm and an injection volume of 100 μl gave a retention time of 9 min. Standard solutions of T_4 (0.05–2 μg/ml) in 40% v/v ethanol were prepared. Coefficients of variation of intra- and inter-day measurements were < 5%.

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In vitro drug release

In vitro drug release from the drug-loaded beads was studied in phosphate buffer saline (PBS; pH 7.4) at 37°C in a horizontal shaker. At specific intervals, 1 ml samples were taken and the microparticulate dispersions were centrifuged to remove impurities and assayed for drug content via the HPLC method. An equal volume of fresh PBS was added immediately to the receptor cell after each sampling.

Skin preparation

Abdominal skin from a female adult was obtained with patient consent and ethics approval post plastic surgery. This study was approved by the Institutional Review Board (IRB) of Singapore General Hospital, Republic of Singapore (IRB Reference Number 196/2006). This IRB operates in accordance with the International Conference on Harmonization/Singapore Guideline for Good Clinical Practices, and with the applicable regulatory requirements.

Epidermal membranes were prepared by the commonly used heat separation technique. Subcutaneous fat was separated carefully from the epidermis after immersing the whole skin in distilled water at 60±5°C for 2 min. Skin samples were stored at −80°C until use. Prior to permeation studies, the skin samples with stratum corneum side facing upwards, were equilibrated for 2 h in 0.9% w/w sodium chloride solutions containing 1% v/v antibacterial antimycotic solution [16].

In vitro skin penetration studies

Permeation studies of the drug-loaded microparticles were performed using a flow-through diffusion cell apparatus. The flow-through diffusion cell consists of an upper donor cell and a lower receiver compartment through which a continuous supply of phosphate buffer saline, pH 7.4, flows to help maintain the system in sink condition. The flow is directed to an automatic fraction collector where samples are collected for certain time intervals. The donor compartment was filled with 1 ml of aqueous polymeric microparticle solution and the receptor compartment was phosphate buffer saline, pH 7.4. The exposed surface area of the skin for the permeation of the drug was 0.785 cm^2. Samples from the receptor compartment were collected at predetermined time points over a 24 h period, and the amount of T_4 permeated was analysed by HPLC. The cell temperature was kept at 37±0.5°C throughout the experiment. All experiments were carried out in triplicates.

Confocal laser scanning microscopy (CLSM)

To study the effect of polymeric microparticles on skin penetration, the confocal study was carried out using a Nikon A1R laser scanning confocal and digital camera from Japan. An objective lens of ×60 was used to view the samples. Skin samples were treated with polymeric microparticles using the flow-through diffusion cell, then an aqueous solution of 0.03% w/v fluorescein dye was applied to the skin surface. Following 24 h *in vitro* skin permeation studies, the skin samples were washed and placed on a glass slide and covered with a glass cover-slip. The slides were inverted and images were captured through the cover-slip side of the prepared samples.

Fourier transform infrared spectroscopy (FTIR) of skin sample

The FTIR spectra of the skin samples treated with polymeric particles were obtained with a Perkin Elmer Spectrum 100 (USA) in the wavenumber region of 500–4000 cm^{-1} at ambient temperature. After application of the formulation on the epidermis layers for 48 h, the skin samples were washed three times with PBS and vacuum-dried at room temperature. The skin samples were placed on a KBr holder and mounted in the enclosed sample chamber, and then subjected to FTIR measurement.

Statistical analysis

The results were expressed as the mean±SD of at least three experiments. Analysis of variance (ANOVA) was carried out (Graph Pad Prism, Version 2.0) followed by the Tukey post-hoc test to determine the differences between treatment groups. A value of $p<0.05$ was considered statistically significant.

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Results and Discussion

Field emission scanning electron microscopy

The FESEM images of the microparticles are shown in Figure 1. It can be seen that the appearance of the microparticles clearly varied with the polymer type. EC microspheres had a uniform microporous and sponge-like structure. No considerable difference was observed between the microstructures of the PLA and PLGA microparticles. Cracks in the surface of the PLGA microparticles were probably artifacts due to the high energy of the electron beam at higher magnifications. The PLGA has a low glass transition temperature (T_g), therefore the polymer transforms from a glassy to a rubbery state that is more susceptible to the vacuum pressure of FESEM [17]. The PNIPAM microcapsules were fragmented but not deformed.

The rate of solvent evaporation, polymer precipitation and stability of the inner aqueous phase play a major role in microcapsule morphology [18]. The surface tension of the solution greatly affects the microparticle structure. A reduction in surface tension of the solution will lead to fast and rapid solvent evaporation that will result in fewer pores on the particle surface [19]. In our study, parameters such as the composition of the solvent system and aqueous phase were kept constant, therefore any difference in the morphology or structure of the particles is likely to be related to the intrinsic properties of the polymers.

Encapsulation efficacy and stability

The encapsulation efficiency of T_4-loaded microparticles is displayed in Figure 2. It was found that irrespective of the storage temperature, ethyl cellulose microparticles remained stable during the 14 week storage period without significant drug leakage ($p > 0.05$). The degradation rate of PNIPAM microparticles was faster than the PLA and PLGA microparticles. The PLGA microparticles stored at 4°C did not show any significant drug loss over the study period ($p > 0.05$), however, storage at 20°C resulted in significant drug leakage ($p < 0.05$). After 14 weeks at 20°C and 4°C, the T_4 contents of PLA and PNIPAM microparticles were significantly lower than the original ($p < 0.05$).

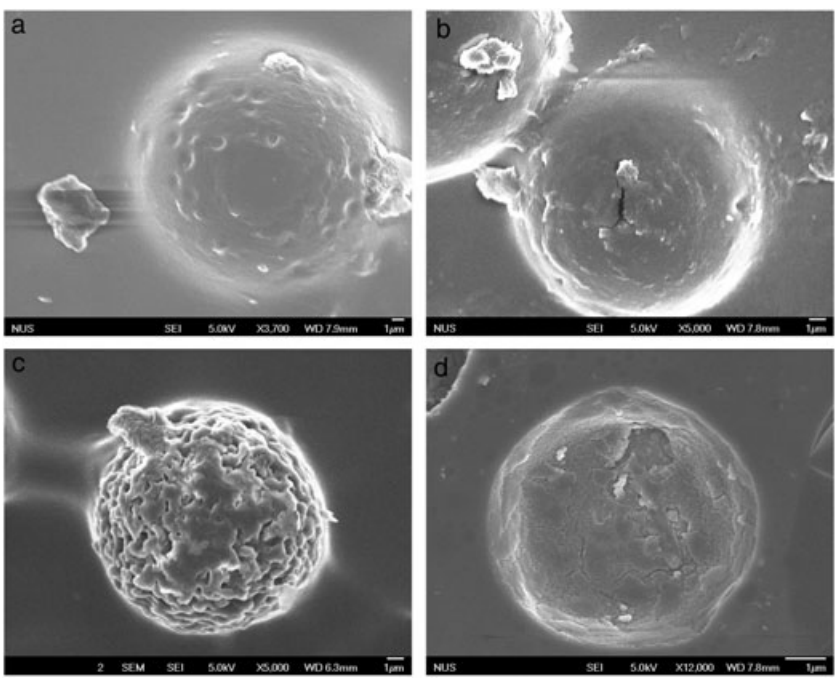

Figure 1. Field emission scanning electron microscope images of T_4 loaded in (a) PLA, (b) PLGA, (c) EC and (d) PNIPAM microparticles

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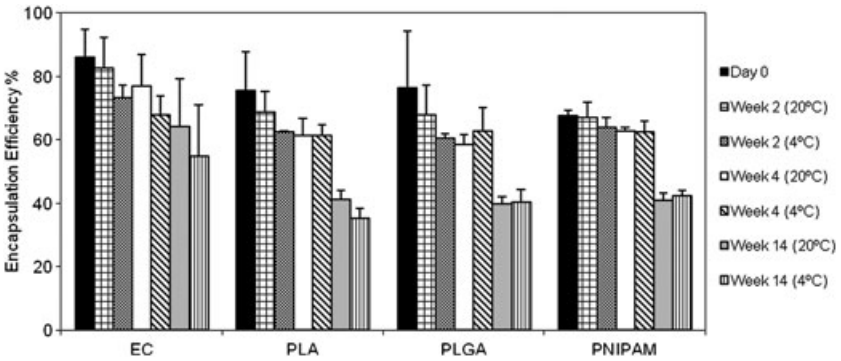

Figure 2. Stability of microparticle suspensions: Encapsulation efficacy of the vesicles over time at room temperature (20°C) and fridge temperature (4°C)

In vitro release of levothyroxine

The release rates of T_4 from microspheres of EC, PLA, PLGA and PNIPAM are shown in Figure 3. The profiles show the influence of polymer type on the *in vitro* release of T_4. It was found that T_4 exhibited a burst release from all formulations irrespective of polymer type. The drug release from microparticles seems to occur in two phases: an initial rapid release followed by a slow release. This first initial burst effect is probably due to the adsorption of the drug onto the wall of the microparticles which would be immediately released. After which, the drug release profile displayed a delayed release that may be attributed to diffusion of the drug entrapped within the core of the microparticles.

Skin penetration studies

In vitro skin permeation studies were performed to evaluate the skin absorption of T_4 from these preparations. Figure 4 depicts the permeation profile of T_4 from the polymeric particles. Drug retention in the epidermis was increased from systems that contained PLA, PLGA or PNIPAM microparticles; however, EC microparticles showed some drug penetration across the skin. By comparing the *in vitro* drug release and the skin permeation results it is concluded that rapid and immediate drug release from these particles does not guarantee skin permeation. These results are consistent with the previous findings where PNIPAM microgels and PLGA microparticles were able to increase drug retention in the epidermis and decrease the drug permeation through the skin; however, the mechanism for this effect remains unclear [20,21]. Several studies have shown sustained and controlled release of drugs from transdermal patches that contained EC [22–24].

Amorphous polymers exhibit glass transition temperature (T_g). Below this temperature, the

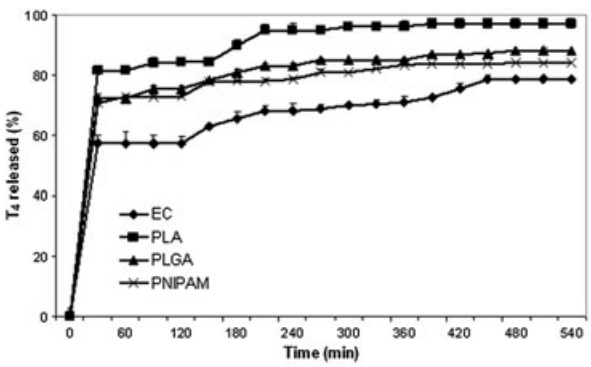

Figure 3. *In vitro* release profile of T_4 from microparticles in phosphate buffer (pH 7.4) at body temperature (37°C), $n=3$

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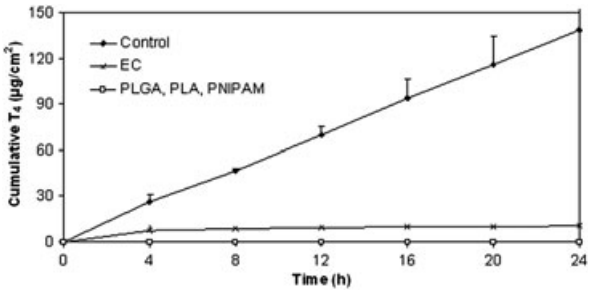

Figure 4. Permeation profile of T_4 across human epidermis

polymer is in a glass-like state. Above this temperature, the polymer passes from a glass state to a rubber-like state that may cause coalescence and precipitation of the polymer network [17,25,26]. The T_g may be lowered by loading polymers with other molecules. This could be critical with respect to storage stability and drug release profile. Water has a plasticizing effect on polyesters and lowers the T_g from >40°C to around 37°C [17,26–28]. Ethyl cellulose has a high T_g of >100°C [29–31]. Aqueous PNIPAM solutions exhibit a LCST of 32°C. At a temperature below the LCST, PNIPAM is hydrophilic and exists in a random coil form; however, above the LCST, its conformation is transformed into a globule and it becomes insoluble and precipitates out from the aqueous solution. Furthermore, this precipitation will delay the drug release by acting as an additional diffusion barrier [32,33].

It is possible to hypothesize that the low T_g of PLA and PLGA, and low LCST of PNIPAM may cause precipitation of the rubber-like, insoluble polymer on the skin surface. This could create an impermeable barrier that may prevent drug penetration across the epidermis. The T_4 skin penetration observed for EC microparticles may be due to its high T_g, where the polymer remains in the glassy state which is soluble and does not precipitate.

Luengo and coworkers studied the effect of PLGA nanoparticles on the skin permeation of flufenamic acid. At shorter incubation times there was no significant difference in the permeated amount of drug, however, after a long incubation time due to the degradation of the polymer to lactic and glycolic acid and the reduction of the pH of the donor compartment, skin permeation was enhanced [34]. The authors could not explain the non-enhancing effect of nanoparticles at short incubation time, however, this phenomenon might be due to the change in the physical state of the polymer with regard to its low transition temperature.

An alternative view is that the presence of oxygen atoms in the polymer molecule could facilitate the formation of hydrogen bonds with the skin lipids thus decreasing the diffusion of topically applied actives. This could have stabilized the rigidity of the solid-lipid state of the skin structure by increasing the skin T_g. Consequently, this could retard skin penetration [35,36].

Confocal images of the skin samples treated with the polymeric microparticles are shown in Figure 5. The presence of green fluorescence on the skin sample implies a retarding effect of the polymer, whereas no fluorescence on the skin sample states that all of the dye has penetrated across the skin. It can be seen that the fluorescein dye easily penetrated through the control skin samples. Skin samples treated with EC microparticles showed some skin penetration of fluorescein. However, it can be seen that the fluorescein dye could not penetrate the skin samples treated with polyesters and PNIPAM microparticles and the dye was mainly focused on the outer layer of the skin. This may be due to the impermeable barrier of the polymer on the skin surface which prevents the penetration of the fluorescein. Skin cell nuclei were counter-stained with DAPI, blue signal, for easier visualization.

FTIR of human skin samples

Figure 6 presents polymer-induced changes in the skin structure monitored through FTIR. The spectra of the skin samples were recorded at the

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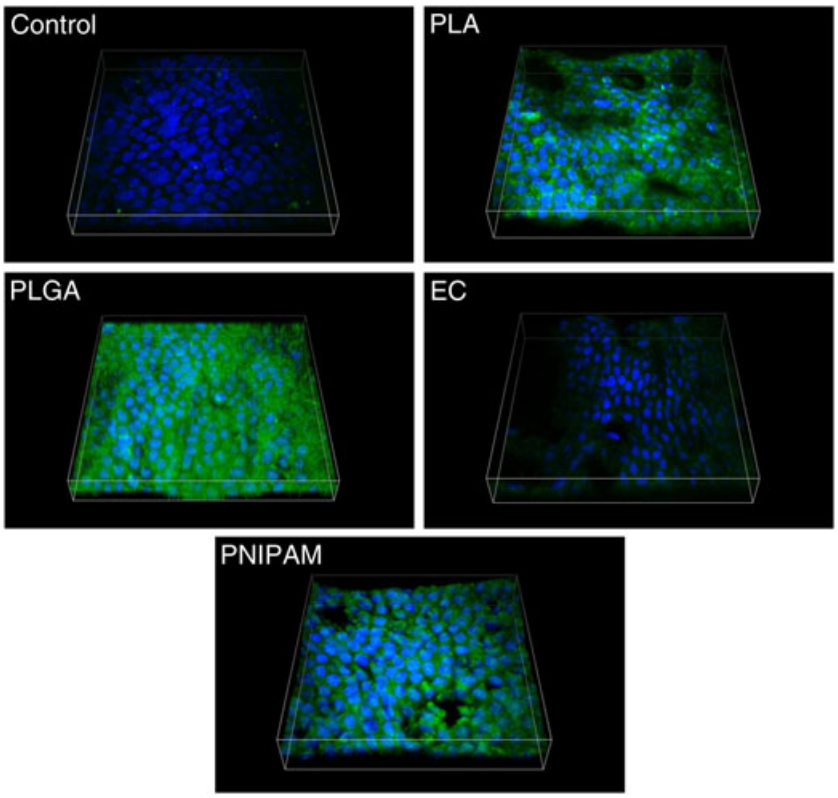

Figure 5. Binary image of the epidermis and localization of green fluorescence on the skin after treatment with the various polymeric microparticles. For better visualization cell nuclei were counter-stained with DAPI

end of the *in vitro* permeation study. The CH_2 asymmetric and symmetric stretching vibrations were observed at 2920 and 2851 cm^{-1}, respectively. These peaks indicate that the majority of the stratum corneum lipids are in the solid gel state. The shift of these peaks to a higher wavenumber after skin treatment would suggest increased lipid fluidity of the skin structure. Peaks typical of SC proteins were those occurring in the region of 1500–1700 cm^{-1}. The carbonyl stretching observed at 1743 cm^{-1} is due to C-O stretching. The amide I band at 1650 cm^{-1} is associated with α-helix conformation of the protein backbone [37–39]. After the exposure of the skin samples to

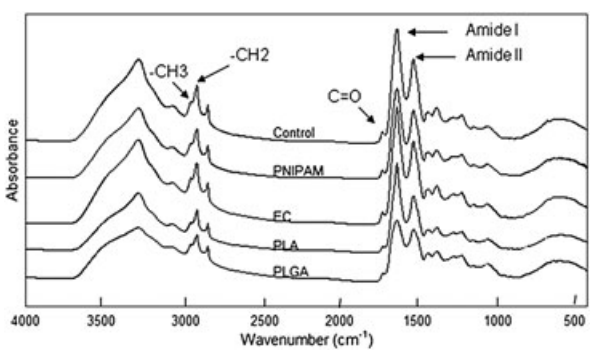

Figure 6. Fourier transform infrared (FTIR) spectral of the skin samples treated with polymeric particles

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microparticle solutions, no peak shift in the CH stretching area and the protein domain was observed. These polymers did not alter the lipid fluidity of the SC, and did not interact with the SC proteins.

Conclusion

Polymer selection and design are of prime importance in the development of topical or transdermal delivery systems. Penetration retardants have many applications in cosmetic and pharmaceutical formulations and are gaining more attention. From this study it was found that polyesters and PNIPAM could be penetration retardants under the described experimental conditions. These microparticles have potential uses in formulations containing sunscreens and insect repellants. The accumulation of the active compounds on the skin surface can minimize adverse side effects, if any, which may be caused by systemic absorption. The EC microparticles may be used in transdermal formulations and can help to achieve therapeutic concentrations of the drug in the plasma.

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