# Evaluation of Skin Viability Effect on Ethosome and Liposome-Mediated Psoralen Delivery via Cell Uptake

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**ABSTRACT:** This study investigated the effect of skin viability on its permeability to psoralen delivered by ethosomes, as compared with liposomes. With decreasing skin viability, the amount of liposome-delivered psoralen that penetrated through the skin increased, whereas skin deposition of psoralen from both ethosomes and liposomes reduced. Psoralen delivery to human-immortalized epidermal cells was more effective using liposomes, whereas delivery to human embryonic skin fibroblast cells was more effective when ethosomes were used. These findings agreed with those of *in vivo* studies showing that skin psoralen deposition from ethosomes and liposomes first increased and then plateaued overtime, which may indicate gradual saturation of intracellular drug delivery. It also suggested that the reduced deposition of ethosome- or liposome-delivered psoralen in skin with reduced viability may relate to reduced cellular uptake. This work indicated that the effects of skin viability should be taken into account when evaluating nanocarrier-mediated drug skin permeation. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3120–3126, 2014
**Keywords:** nanotechnology; liposomes; transdermal; *in vitro* models; transcellular transport

## INTRODUCTION

Ethosomes area novel type of liposome, first developed by Prof. Touitou E.[1] In recent years, ethosomes have been employed extensively in transdermal drug delivery studies because of their excellent enhancement of transdermal penetration and deep skin targeting of their drug cargo. Ethosomes have been used to deliver drugs for systemic therapy, including indinavir for HIV infections, trihexyphenidyl HCl for shaking palsy, ferulic acid for angiocardiopathy, and various drugs for topical administration.[2–6] Because of their enhanced cellular uptake in deep skin, ethosomes have been developed as drug carriers for the treatment of skin diseases, for example, cutaneous carcinoma and psoriasis.[7,8] Two features of ethosomes reportedly improve cutaneous drug absorption. First, the ethanol in ethosomes enhances penetration by increasing cell membrane lipid fluidity and therefore reducing epidermal membrane density, and second, their high deformability facilitates deep skin penetration. These features of ethosomes have been demonstrated using cellulose nitrate membranes simulating the epidermal barrier: skin from rat, rabbit, nude mouse, pig, and human; and human skin with scar tissue.[9,10] These studies usually employed laser confocal microscopy to observe skin penetration or cellular uptake of fluorescently labeled ethosomes into mouse skin fibroblasts cells (3T3) or human dendritic cells *in vitro*. Results showed that ethosome dispersal into deep skin, as well as 3T3 and human dendritic cells, was greater than that of conventional liposomes and other formulations, indicating ethosome drug delivery via both intercellular and intracellular channels.[7,11–13]

The present study extended the findings of previous *in vitro* transdermal penetration studies performed using excised skin or cells studies by investigating the effect of skin viability on ethosome permeability and drug delivery.[7,11,13] Psoralen is a small molecule that has been used to treat vitiligo and psoriasis through exposure to sunlight or ultraviolet radiation, and psoralen ultraviolet A therapy has been approved by the US Food and Drug Administration for clinical use.[14] In the present study, psoralen was loaded into ethosome vesicles and psoralen-loaded conventional liposomes were also prepared for comparison. The permeability of excised rat skins to these formulations was studied using a Franz-type diffusion cell following storage for various periods at room temperature (25°C) to evaluate the influence of skin viability on cutaneous absorption of psoralen. Cellular uptake of ethosomes and liposomes was compared *in vitro* in a human-immortalized epidermal cell line (HaCaT) and in a human embryonic skin fibroblast cell line (CCC-ESF-1). The *in vivo* skin deposition of the active molecules was also determined in rats.

## MATERIALS AND METHODS

### Materials

Lipoid S 100 [phosphatidylcholine (PC) from soybean lecithin] containing 95.8% PC was purchased from Lipoid GmbH (Ludwigshafen, Germany). Psoralen with purity not less than 98.0% (isolated from the fruit of *Psoralea corylifolia* L.) was kindly supplied by Ze-lang BioScience (Nanjing, China). Methyl thiazolyl tetrazolium (MTT), high-glucose Dulbecco's modified Eagle's medium [DMEM/high; Fisher Scientific Worldwide (Shanghai) Company Ltd., Shanghai, China), 0.25% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), Zymine solution, phosphate-buffered saline (PBS), normal saline, and culture dish were obtained from Shanghai Usen Biotechnology (Shanghai, China). All other chemicals were obtained from Sinopharm Chemical Reagent Company Ltd. (Shanghai, China) and were of HPLC or analytical grade.

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### Animals and Cell Lines

Male Sprague–Dawley rats weighing 180–220 g were used. The animal study was approved by the Animal Ethical Committee, Shanghai University of Traditional Chinese Medicine. Animals were kept in an agreeable environment with free access to rodent diet and water according to the related guidance provided in the "OECD GUIDELINE FOR THE TESTING OF CHEMICALS," and were acclimatized for at least 1 week before the start of the study.

The HaCaT cell line was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia) and the CCC-ESF-1 cell line was obtained from the Cell Culture Center of the Chinese Academy of Medical Sciences (CAMS, Beijing, China).

### HPLC Assay

The LC-2010A HT Liquid Chromatography System (Shimadzu Corporation, Kyoto, Japan) was used to determine the concentration of psoralen in samples. A Diamonsil C18 reverse-phase column (5 μm, 4.6 mm inner diameter × 25 cm; Dikma Technologies, Inc., Beijing, China) was used, and the mobile phase was methanol–water (55:45, v/v) with a flow rate of 1 mL/min. The column temperature was constant at 35°C and the detection wavelength was 246 nm. Samples from *in vitro* experiments were filtered through a nylon 0.45-μm pore disposable syringe filter (diameter: 13 mm; Shanghai Anpel Scientific Instrument Inc., Shanghai, China) before automatic injection into the HPLC unit.

### Preparation of Ethosomes and Other Formulations

Touitou's method was used to prepare the colloidal ethosome suspension.[11] In a closed environment, 5.0% (w/v) Lipoid S 100 and 0.2% (w/v) psoralen were dissolved in ethanol. The required amount of purified water was then injected at a flow rate of 12 mL/h with a WZ-50C6 Micro Infusion pump (Smiths Medical, Norwell, Massachusetts) into this solution with constant mixing at 700 rpm by a magnetic stirrer (IKA Works GmbH and Company., Staufen, Germany) till the ethanol concentration to 40% (v/v). After water injection, magnetic stirring was continued for 5 min at 300 rpm to ensure uniform mixing. The ethosome suspension was probe sonicated for 5 min (power: 900 Hz, quiescent interval: 3 s) in an ice-water bath using an ultrasonic cell disruption system (Ningbo Scientz Biotechnology Company Ltd., Ningbo, China).

Liposomes-loaded with psoralen was prepared by the conventional mechanical dispersion method. Briefly, 5.0% (w/v) Lipoid S 100 and 1.5% (w/v) cholesterol (CH) were dissolved in 40 mL of chloroform at a Lipoid S 100–CH ratio of 10:3 (w/w). Psoralen was added to produce a final preparation concentration of 0.2% (w/v). Chloroform was then removed by rotary evaporation (Rotavapor, Buchi, Germany) at 37°C, followed by overnight vacuum drying. The deposited lipid film was hydrated by rotation with 40 mL of distilled water at 100 rpm for 30 min at 37°C. The liposomal suspension was then sonicated for 5 min, as described above for ethosome preparation.

Rhodamine 110-labeled ethosomes and liposomes were prepared by replacing psoralen with rhodamine 110 dissolved in ethanol (1.0 mg/mL). The concentration of rhodamine 110 in the prepared ethosomes and liposomes was 10 μg/mL.

### Characteristics of Ethosomes and Liposomes

The average particle sizes of the prepared ethosomes and liposomes were measured by dynamic light scattering using a computerized Malvern Autosizer Nano ZS90 inspection system (Malvern Instruments Ltd., Malvern, UK). Measurements were performed in triplicate.

The entrapment efficacy (EE) of ethosomes and liposomes was determined using dialysis.[15] A dialysis bag with a molecular weight cut-off of 10,000 Da (Shanghai Qingyang Biological Technology, Shanghai, China) was soaked in boiling purified water for 30 min. Ethosome or liposome suspension (0.5 mL) was placed in the dialysis bag, which was then closed using dialysis tubing closures and immersed into 200 mL of 20% polyethylene glycol 400 aqueous solution (v/v). This receiver medium was stirred at 300 rpm using a magnetic stirrer for approximately 5 h. It was then assayed for psoralen using HPLC, and EE was calculated using Eq. (1).[5]

$$\mathrm{EE} = (D_\mathrm{t} - D_\mathrm{d})/D_\mathrm{t} \tag{1}$$

where $D_\mathrm{t}$ is the total amount of psoralen in the ethosome or liposome suspension and $D_\mathrm{d}$ is the amount of psoralen that had diffused into the receiver medium. The results reported represented the mean of three independent experiments, performed in triplicate.

### Preparation of Excised Rat Skin

A rat was humanely sacrificed after being anesthetized, its abdominal fur was shaved off with a razor, and the skin was excised carefully and washed with normal saline. Skins were wetted with 10 mL of normal saline, packaged with polyethylene preservative film, and stored at 25°C for the time periods indicated. Five pieces of skin from different rats were stored for each time period.

### Assessment of Excised Rat Skin Viability

Skin viability was measured using the MTT method.[16] Following the storage of rat abdominal skin at 25°C (section *Preparation of Excised Rat Skin*), triplicate circular samples of skin with a diameter of 6 mm were weighed precisely before adding each sample to 1 mL of MTT solution (0.5 mg/mL MTT in PBS, pH 7.20 ± 0.05). The sample was placed in an incubator at 37°C (5% $CO_2$) for 1 h before removing the liquid, adding 2.5 mL of dimethyl sulfoxide (DMSO), and shaking in a water bath at 20°C, 100 rpm for 2 h. The skin sample was removed and leach liquor from each tube was then pipetted into six wells of a 96-well plate (200 μL per well). Negative control blank wells contained 0.5 mg/mL MTT in PBS and the same content of DMSO as mentioned above but without adding skin samples. Optical density (OD) was determined at 570 nm with a multiskan plate reader (Synergy™ HT; BioTek, Winooski, Vermont). All the samples were assayed in triplicate. Data were obtained from 15 skin samples at each time point (five pieces of skin stored, three samples of each) and used to calculate skin viability (SV) using Eq. (2):

$$\mathrm{SV} = (\mathrm{OD_s} - \mathrm{OD_{bc}})/W_\mathrm{s} \tag{2}$$

where $\mathrm{OD_s}$ is the mean OD value (six wells) for the skin sample, $\mathrm{OD_{bc}}$ is the mean blank control OD, and $W_\mathrm{s}$ is the weight of the

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