Estradiol Permeation from Nonionic Surfactant Vesicles Through Human Stratum Corneum *in Vitro*

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The permeation of estradiol from vesicular formulations through human stratum corneum was studied *in vitro*. The vesicles were composed of nonionic *n*-alkyl polyoxyethylene ether surfactants (C_nEO_m). The thermodynamic activity of estradiol present in each formulation was kept constant by saturating all formulations with estradiol. The effects of both the particle size and the composition of the formulation on estradiol permeation across excised human stratum corneum were investigated. Stratum corneum that was pretreated with empty surfactant carriers allowed for significantly higher estradiol fluxes compared with untreated stratum corneum. However, estradiol fluxes obtained in these pretreatment experiments appeared to be significantly lower than those obtained by the direct application of the estradiol-saturated carrier formulation on top of the stratum corneum. Furthermore, in the case of pretreatment of the stratum corneum, an increase in carrier size resulted in a decrease in estradiol flux. For direct application the opposite was found. Two mechanisms are proposed to play an important role in vesicle–skin interactions, i.e., the penetration enhancing effect of surfactant molecules and the effect of the vesicular structures that are most likely caused by adsorption of the vesicles at the stratum corneum–suspension interface.

KEY WORDS: niosomes; nonionic surfactant vesicles; estradiol; transdermal delivery; stratum corneum.

INTRODUCTION

To study the physical barrier of the skin for transdermal drug delivery, a variety of formulations has been tested (1). Drugs incorporated in vesicles are promising formulations to control transdermal drug delivery (2). Vesicular drug formulations are also useful for investigating the limits of drug transport, permeability, and integrity of the skin (3–5). Although the use of vesicles in dermal or transdermal drug delivery has been studied (6–9), little is known about the mechanisms of the interactions between drug-loaded vesicle bilayers and human stratum corneum, and the influence of this interaction on penetration of lipophilic compounds through human stratum corneum *in vitro* and *in vivo*.

Nonionic surfactant vesicles (NSVs) were introduced in 1978 by Vanlerberghe *et al.* (10). NSVs were found to enhance the penetration of sodium pyrrolidone-carboxylate through stratum corneum. NSVs offer the advantage of a high chemical stability and intrinsic penetration enhancing properties concerning skin permeation of drugs. Nonionic surfactants are reported to be less irritating to the skin than ionic surfactants (1). Furthermore, nonionic surfactants can be tailor-made according to certain desired characteristics.

This report analyzes the mechanisms of estradiol permeation from NSVs through human stratum corneum *in vitro*. Estradiol was chosen as a model drug because of its high lipophilicity and therapeutic potency. The vesicles were composed of *n*-alkyl polyoxyethylene ether surfactants (C_nEO_m) and were characterized in previous studies (11). All formulations were saturated with estradiol to keep the thermodynamic activity of the estradiol present in each donor formulation identical. Therefore, differences in estradiol fluxes were caused by differences in interaction between the drug carrier system and the skin, and not by differences in the solubility of estradiol in the donor formulation. To separate vehicle–drug interactions from vehicle–skin interactions as possible causes of changes in drug flux, experiments were carried out according to two essentially different protocols: first, *direct application*, implying immediate application of a drug-loaded formulation to untreated stratum corneum, followed by flux measurements; and second, *pretreatment experiments*, implying a 6-hr pretreatment of stratum corneum with drug-free formulations, followed by a rinse, application of a saturated solution of the drug, and subsequent flux measurements. Using these two protocols the effects of particle size and composition of the formulation on estradiol permeation across excised human stratum corneum were investigated.

MATERIALS AND METHODS

Materials

Polyoxyethylene monoalkyl ether-type surfactants, C_nEO_m, were used in this study. The number of oxyethylene units, *m*, was 3 and 10, and the number of carbon atoms, *n*, was 12 and 18. Both $C_{12}EO_3$ and $C_{18}EO_3$ were purchased from Servo (Delden, The Netherlands). $C_{9=9}EO_{10}$ (Brij96) was a gift from Atlas Chemie (Essen, Germany). Cholesterol was purchased from J. T. Baker Chemicals B.V. (Deventer, The Netherlands). Estradiol was a generous gift from Organon International BV (Oss, The Netherlands).

Preparation of Surfactant Formulations

The effects of particle size and composition on estradiol permeation across excised human stratum corneum were investigated. For studying size effects a number of preparations were made of virtually equal compositions but varying in size. The formulations were based on $C_{9=9}EO_{10}$ and contained either micelles (<10 nm), small unilamellar vesicles (SUVs; 100–200 nm), or multilamellar vesicles (MLVs; >1 μm). Composition effects were studied using a series of MLV formulations based on either $C_{12}EO_3$, $C_{18}EO_3$, or $C_{9=9}EO_{10}$. To be able to prepare vesicles from these surfactants, 15 mol% cholesterol was added (11). The composition and size of the carriers are given in Table I. The carrier size was determined using dynamic light scattering (11).

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Table I. Composition of the Various Surfactant Formulations After Saturation with Estradiol[a]

Formulation	Size (μm)	Surfactant (m*M*)	Cholesterol (m*M*)	Estradiol (m*M*)
PBS	—	—	—	0.007
$C_{9=9}EO_{10}$ micelles	≤0.01	24	—	0.75
$C_{9=9}EO_{10}$ SUVs	0.3 (0.2)	24	4.5	1.5
$C_{9=9}EO_{10}$ MLVs	>1	24	4.5	1.5
$C_{12}EO_3$ MLVs	>1	24	4.5	1.5
$C_{18}EO_3$ MLVs	>1	24	4.5	0.3

[a] Sizes were determined by dynamic light scattering (12). The numbers in parentheses indicate the polydispersity index.

Both NSVs and micelles were prepared using the sonication method as described by Baillie *et al.* (12). The aqueous phase consisted of a phosphate-buffered saline (PBS) solution: 139 m*M* NaCl, 2.5 m*M* KCl, 8 m*M* Na_2HPO_4, and 1.5 m*M* KH_2PO_4; with the pH adjusted to 7.4. The preparations were carried out at 80°C, which was above the gel-liquid transition temperature of the surfactants. A mixture of 24 m*M* surfactant and 4.5 m*M* cholesterol was sonicated for 45 sec in 5 mL PBS, using a Branson Sonifier 250 (Danbury, UK), with an ⅛-in. microtip at a 60-W energy output. In the micelle formulation, cholesterol was omitted. SUVs (vesicles consisting of only one bilayer) were obtained after resonication of the formulation at room temperature (11). MLVs (vesicles consisting of more than one concentric bilayers) were prepared by heating the SUV suspension at 80°C for 5 min (11). Vesicle formation and the presence of cholesterol crystals were checked using polarization microscopy. Multilamellar vesicles were easily identified by the presence of so-called Maltese crosses.

The formulations were saturated with estradiol. Saturation of the formulations with estradiol was carried out at 28°C, overnight. A volume of 15 mL of PBS or surfactant formulation was dialyzed (Visking cellulose dialysis tubing, Visking Co., Chicago, IL) against 1 mL of saturated estradiol solution, containing an excess amount of estradiol, while shaking gently. The exchange of estradiol between the inner and the outer phase of the dialysis tubing was monitored to check the saturation process. The saturated formulations were checked for the presence of estradiol crystals with polarization microscopy. When crystals were found the sample was excluded from the experiment.

Skin Preparation

Within 24 hr after surgery, the human stratum corneum was isolated from the excised abdominal skin, obtained from two donors (A and B), using the following protocol: The subcutaneous fat was removed and the skin was dermatomed to a thickness of approximately 200 μm (Padgett Dermatome, Kansas City Ass. Co., Kansas City). The dermatomed skin was placed on filter paper soaked in 0.1% trypsin solution in PBS at 37°C, overnight. The stratum corneum was separated from the epidermis, washed with a 1.0% antitrypsin solution to block trypsin activity, and rinsed twice with distilled water. The stratum corneum was dried and stored in a desiccator over silica gel at room temperature. Before use, the stratum corneum was rehydrated by placing it carefully on the surface of a PBS solution in a petri dish and allowing it to equilibrate for approximately 10 min. Then 355 medical silicon adhesive (Dow Corning, Midland, MI) was used to attach the stratum corneum to a 0.127-mm supporting Silastic membrane (Dow Corning 500-1, nonsterile, nonreinforced) according to Tiemessen *et al.* (13).

Design of the Diffusion Experiments

Experimental Setup

The stratum corneum/Silastic sheeting membrane combination was mounted in a Franz-type diffusion cell. The effective diffusion area was 0.8 cm^2. The acceptor medium was kept at 32°C. The flow rate of the acceptor medium was 2.5 mL/hr and the effluent was collected in glass tubes at 1-hr intervals. Before starting the experiment the system was allowed to equilibrate for 1 hr.

Skin Incubation Protocols

Two essentially different skin incubation protocols were carried out to separate effects caused by vesicle–drug interactions from those caused by vesicle–skin interactions.

Direct Application

Estradiol flux was measured immediately after placing 3 mL of a saturated estradiol formulation in the donor chamber of the diffusion cell. As a control an estradiol saturated PBS solution was used.

Control experiments were performed to check the equivalence of the thermodynamic activities of estradiol, and hence the driving force for transport across the membrane, in two different donor formulations. These control experiments were performed using a reversed membrane: Silastic sheeting/medical adhesive/stratum corneum, i.e., the Silastic sheeting was facing the donor compartment.

Pretreatment

Three milliliters of an estradiol-free formulation was placed in the donor compartment of the diffusion cell. After 6 hr of incubation with this formulation, the stratum corneum was carefully rinsed twice with 3 mL PBS. The drug was applied to the pretreated stratum corneum only as a

saturated solution in PBS. Subsequently the estradiol flux was measured. The controls were pretreated with PBS.

Estradiol Analysis

The total amount of estradiol present in the saturated surfactant formulations was determined using reversed-phase HPLC according to Dohji *et al.* (14). The presence of the micelles or vesicles, containing surfactants and cholesterol, did not interfere with the HPLC analysis of estradiol. Hence, 100 μL of the intact formulation was simply injected into the reversed-phase column.

After the diffusion experiments estradiol was analyzed by radioimmunoassay (Coat a Count, DPC, Apeldoorn, The Netherlands). The data were analyzed using a logit-log transformation procedure according to Dudley *et al.* (15).

Calculations

Steady-state fluxes were determined from graphs in which the cumulative amount of penetrated estradiol was plotted against time. Fluxes were calculated from the slope of the steady-state portion of these curves. Whether steady-state fluxes were significantly different or not was tested using a one-way ANOVA.

The permeability of the stratum corneum was calculated using Eq. (1). The stratum corneum permeability can be determined only from results obtained after pretreatment experiments, since the drug distribution in the formulation is not homogeneous (either encapsulated or not) and the exact donor concentrations at the stratum corneum–formulation interface can not be determined accurately due to adsorption phenomena of the vesicles on the stratum corneum. The steady-state values were corrected for the resistance of the Silastic sheeting and medical adhesive, using Eqs. (2) and (3) (16).

$$P = J/C_d \quad (1)$$

$$R = 1/P \quad (2)$$

$$T_{tot} = R_{ss} + R_{sc} \quad (3)$$

R is the resistance; subscript ss, Silastic sheeting with medical adhesive; subscript sc, stratum corneum; J, the flux of the drug through the membranes at steady state; P, the permeability; and C_d, the drug concentration in the donor compartment. The membrane permeability, P_m, is expressed as

$$P_m = (D_m \cdot K_{m/d})/h_m \quad (4)$$

where D_m is the diffusion coefficient of the drug in the membrane, h_m the path length of the drug in the membrane, and $K_{m/d}$ the partition coefficient of the drug between the membrane and the donor solution. The enhancement factor (EF) of a formulation based on the permeability coefficient is defined as

$$EF = (P_{pf})/(P_{pPBS}) \quad (5)$$

where P_{pf} indicates the permeability of the stratum corneum after pretreatment with the drug-free surfactant formulation, and P_{pPBS} the permeability after PBS pretreatment.

RESULTS

Preparation of the Surfactant Formulations

A steady-state concentration of 2 mg/L (7.34 μ*M*) in PBS was reached for estradiol after saturation, overnight (16 hr). The surfactant formulations also reached a steady state (i.e., saturation) after 16 hr of dialysis. The final compositions of the surfactant formulations that were used for the diffusion experiments are given in Table I. Both the $C_{12}EO_3$ and the $C_{9=9}EO_{10}$ vesicle formulations appeared to have the highest estradiol concentration: 1.5 m*M* estradiol. Using the $C_{9=9}EO_{10}$ micellar solution 0.75 m*M* estradiol was incorporated, whereas the $C_{18}EO_3$ MLVs could incorporate 0.3 m*M* estradiol.

Diffusion Experiments

The steady-state fluxes obtained using the two application protocols for the various formulations are summarized in Table II.

Direct Application

It appeared that MLVs have a significantly greater effect ($P < 0.05$) on estradiol permeation, resulting in a flux of 64 ± 16.9 ng/cm²*hr, compared with the SUV and micellar formulations, giving fluxes of 45 ± 15.1 and 41 ± 2.0 ng/cm²*hr, respectively. The steady-state fluxes obtained with the SUVs and the micelles were not significantly different ($P > 0.05$).

Fluxes obtained with $C_{9=9}EO_{10}$ and $C_{12}EO_3$ MLVs—64 ± 17 and 55 ± 3 ng/cm²*hr, respectively—were not significantly different ($P > 0.05$). The results obtained with the $C_{12}EO_3$ MLVs were reproducible. $C_{18}EO_3$ MLVs appeared

Table II. Average Estradiol Fluxes, from Triplicate Experiments, Through Stratum Corneum Plus or Minus the Data Range[a]

	J_{tot} (ng/cm² · hr)	R_{sc} (hr/cm)	EF
SC[A]	0.9 ± 0.6	2127	1
SC[B]	3.1 ± 0.6	628	1
Pretreatment			
$C_{9=9}EO_{10}$ MLV[A]	2.3 ± 0.9	834	2.5
$C_{9=9}EO_{10}$ SUV[A]	4.5 ± 1.0	426	5.0
$C_{9=9}EO_{10}$ Mic[A]	5.6 ± 0.1	331	6.4
$C_{12}EO_3$ MLV[A]	8.3 ± 0.9	228	9.3
$C_{18}EO_3$ MLV[B]	1.0 ± 0.2	1859	0.3
Reverse membrane			
$C_{9=9}EO_{10}$ MLV[B]	3.6 ± 0.4		
$C_{9=9}EO_{10}$ Mic[B]	4.1 ± 0.3		
Direct application			
$C_{9=9}EO_{10}$ MLV[A]	64 ± 16.9		
$C_{9=9}EO_{10}$ SUV[A]	45 ± 15.1		
$C_{9=9}EO_{10}$ Mic[A]	41 ± 2.0		
$C_{12}EO_3$ MLV[A]	55 ± 3.0		
$C_{18}EO_3$ MLV[B]	2.8 ± 1.2		

[a] SC = the flux through stratum corneum that is supported by Silastic sheeting and medical adhesive. Experiments performed using stratum corneum obtained from either donor A or donor B are indicated by the superscripts.

to have no significant effect on the estradiol penetration ($P > 0.05$) compared to the control.

Control experiments involving estradiol diffusion from both a saturated $C_{9=9}EO_{10}$ micellar solution and a MLV suspension through a reversed-membrane were performed. There was no significant difference in the fluxes obtained from the $C_{9=9}EO_{10}$ formulations and the saturated PBS solution ($P > 0.05$) through this reversed membrane.

Pretreatment Experiments

After pretreatment of the stratum corneum with the $C_{9=9}EO_{10}$ formulations, an inverse relation was found between the size of the particles and the penetration enhancement. Pretreatment with MLVs resulted in the lowest estradiol fluxes compared with both SUV and micelle pretreatment. Differences after pretreatment with either SUVs or micelles were not significant ($P > 0.05$). The reproducibility of results obtained after micelle pretreatment was good, compared to that of results obtained using vesicles.

The $C_{12}EO_3$ MLV pretreatment increased the flux by a factor of 9.3; pretreatment with $C_{9=9}EO_{10}$ MLVs resulted in an enhancement of the estradiol flux by a factor of 2.5. These differences appeared to be significant ($P < 0.001$). An impairment of the estradiol transport by a factor of 0.3 (significant: $P < 0.001$) was found after pretreatment with $C_{18}EO_3$ MLVs.

DISCUSSION

Incorporation Characteristics

The ability to incorporate estradiol appeared to be dependent both on the formulation type (i.e., micelles, SUVs, or MLVs) and on the formulation composition (i.e., $C_{9=9}EO_{10}$, $C_{12}EO_3$, or $C_{18}EO_3$). The total amount of estradiol in the $C_{9=9}EO_{10}$ micellar formulation (being 0.75 m*M*) was different from the total amount of estradiol in the $C_{9=9}EO_{10}$ vesicular formulations (both being 1.5 m*M*). Furthermore, the amount of estradiol in saturated MLV formulations consisting of liquid-state bilayers $C_{9=9}EO_{10}$ and $C_{12}EO_3$ (both being 1.5 m*M*), on the one hand, and gel-state bilayers $C_{18}EO_3$ (0.3 m*M*), on the other, appeared to be quite different. It appeared that the incorporation of estradiol in gel-state MLV formulations was impaired by a factor of 5. Based on the lipophilic nature of the estradiol, it is expected that most of the estradiol is intercalated in the bilayers of the NSVs.

Validation of the Design of the Diffusion Experiments

Estradiol fluxes from saturated estradiol solutions through Silastic sheeting/medical adhesive without stratum corneum were at least two orders of magnitude higher than fluxes through Silastic/medical adhesive with stratum corneum. Thus it can be concluded that the stratum corneum, and not the supporting membrane, was rate limiting for estradiol diffusion in the experiments.

The fact that the driving force for estradiol transport was kept constant was demonstrated with the reverse membrane experiments. Since the Silastic sheeting, which is facing the donor compartment, is almost impermeable to surfactants (13), penetration enhancement across this membrane cannot take place. Hence, using this reversed membrane setup, only differences in fluxes caused by differences in estradiol solubility would be revealed. It was demonstrated that the permeation rates of estradiol from three different saturated formulations through this reversed membrane were not significantly different ($P > 0.05$). Therefore, the estradiol activities of these formulations were considered identical.

During pretreatment the stratum corneum was allowed to equilibrate for 6 hr. Flux data obtained with the direct application protocol showed that the estradiol diffusion attained steady state after 6 hr. Longer pretreatment periods were not tested since no additional effect of the surfactants was expected to occur after reaching steady state, and extended hydration damages the stratum corneum.

The differences found in estradiol fluxes obtained with MLVs and SUVs or micelles cannot be explained by differences in NSV density in the bulk suspension, caused by pelleting or by creaming effects. Spectrophotometric measurements were performed to check the homogeneity of NSV formulations during the experiment. It appeared that the NSV suspensions were stable during the diffusion experiments. Neither pellet formation nor creaming of the vesicles occurred during the diffusion experiments.

Formulation–Skin Interaction

Direct Application

The estradiol fluxes after direct application were very high compared with those obtained with pretreatment or control experiments. Freeze-fracture electron microscopy revealed that NSVs tend to adsorb on the outermost layers of the stratum corneum (20,21). Vesicles fuse at the adsorption site, thus forming stacks of bilayers on top of the stratum corneum (Fig. 1). However, since all formulations establish the same initial activity gradients across the membrane (equal thermodynamic activity of the formulations), no differences in estradiol fluxes are expected. The high fluxes that were found after direct application may be explained by differences in skin–formulation interactions, i.e., not a mere adsorption of bilayers, but an exchange of material between the formulation and the skin may take place. Previous studies (20,21), in which skin–NSV formulation interactions were studied using freeze-fracture electron mi-

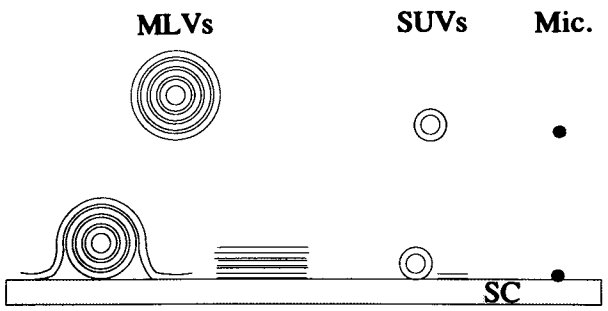

Fig. 1. Schematic representation of size-dependent interactions at the formulation–skin interface. Formulations containing MLVs, SUVs, or micelles applied on stratum corneum.

croscopy, revealed that the lipid bilayer structure of the stratum corneum can be disrupted by the NSV formulations. Other studies (24) showed that liposomes (phospholipid vesicles) are able to disrupt the bilayers of the stratum corneum in a similar manner. In addition, small-angle X-ray scattering studies (24) revealed that a mixing of liposomal components (phospholipids) and skin lipids occurs. Analogous to these skin–liposome interactions, it is very likely that a mixing of skin lipids and nonionic surfactants also occurs after application of NSVs. A mixing of gel-state lipids (from the stratum corneum) with liquid-state NSV formulations (saturated with estradiol) will result in a decrease in solubility of estradiol. Thus, the thermodynamic activity of the estradiol at the skin–formulation interface will increase, which causes the high fluxes that were observed.

Estradiol fluxes after direct application of estradiol-saturated $C_{9=9}EO_{10}$ formulations suggest a positive relation between particle size and estradiol permeation through human skin *in vitro*. This phenomenon, although not yet fully understood, may also be caused by differences in skin–formulation interactions.

Differences in effect on drug transport between the liquid-state $C_{12}EO_3$ and $C_{9=9}EO_{10}$ MLVs and the gel-state $C_{18}EO_3$ MLV formulation are thought to be either due to the lack of interaction between the rigid gel-state vesicle bilayers and the stratum corneum or due to the partition kinetics of the estradiol between the gel-state vesicles and the stratum corneum. This explains the fact that the estradiol permeation from the $C_{18}EO_3$ MLV formulation was not significantly different from that of the control.

Pretreatment Experiments

The surfactant molecules are thought to permeate into the intercellular lipid bilayers (where the barrier function of the skin is located), thereby reducing the crystallinity of the intercellular lipid bilayers and thus increasing the permeability of these bilayers. Kadir *et al.* (22) called this the "pull" effect of the membrane. This pull effect is observed after pretreatment with liquid-state formulations. The surfactant molecules in the rigid gel-state $C_{18}EO_3$ bilayers can neither be readily exchanged with the environment nor penetrate into the stratum corneum. Therefore, this surfactant will not be able to induce a penetration enhancing effect. Although this pull effect seems to be present in case of the liquid-state surfactant formulations, it does not account for the total penetration enhancement found for the saturated formulations, which is an order of magnitude higher. This indicates that a direct contact between the vesicles and the skin is necessary to obtain a maximal effect.

Since an inverse effect of particle size on the estradiol flux was observed in the pretreatment experiments, the stacks of empty bilayers deposited on top of the stratum corneum may introduce an additional diffusion barrier (Fig. 1). This is most apparent in the case of the $C_{18}EO_3$ formulations, where the rigid gel-state bilayers of the vesicles form an additional diffusion barrier for the estradiol diffusing from the saturated solution, resulting in an impaired estradiol transport.

CONCLUSION

Effects of pretreatment with MLVs based on $C_{12}EO_3$ surfactants were significantly higher and better reproducible compared with $C_{9=9}EO_{10}$ surfactants. This may be explained by the fact that the $C_{12}EO_3$ formulations are better defined; since this surfactant does not form micelles, virtually all surfactant molecules are present in bilayers.

Electron microscopy studies have demonstrated that neither $C_{9=9}EO_{10}$ nor $C_{18}EO_3$ NSVs have a detectable influence on the ultrastructure of the stratum corneum. This in contrast to $C_{12}EO_3$ NSVs (20,21), which induced ultrastructural changes of the intercellular lipid regions of the stratum corneum down to a depth of 10 μm. These changes in ultrastructure may account for the very fast and high penetration enhancement found after pretreatment with $C_{12}EO_3$ MLVs. Walters *et al.* (23) found a relation between methyl nicotinate transport through hairless mouse skin and both the alkyl chain and the polyoxyethylene headgroup length of the surfactants. For both EO_{10} and EO_{16} an optimum alkyl chain length of C_{12} was found.

In conclusion, it may be stated that direct contact between liquid-state MLV formulations and skin is imperative to exert the highest effect on drug transport. To maximize the effect of the formulation the driving force of drug transport across the stratum corneum should be maximized, hence the formulation should be saturated with the drug.

Since the effect of NSVs on drug transport through the skin can be either impairment (gel-state vesicles) or enhancement (liquid-state vesicles), NSVs appear to have potential as a novel drug carrier system for both dermal (gel-state vesicles) and transdermal (liquid-state vesicles) delivery of drugs.

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