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Influence of formulation factors on the deposition of liposomal components into the different strata of the skin

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Synopsis

The effects of lamellarity and total lipid concentration on the deposition of liposomal components into the various skin strata were determined using *in vitro* diffusion experiments. Large unilamellar vesicles, multilamellar vesicles, and dehydration/rehydration vesicles composed of egg lecithin, cholesterol, and cholesteryl sulfate at lipid concentrations of 10, 25, and 50 mg/ml were tested. The results suggest that mixing and interaction of liposomal bilayers with the stratum corneum is extensive and that the skin is capable of incorporating large amounts of lipids when they are applied in a bilayer configuration. For each type of liposome tested, the amount of lipid deposited in the deeper skin strata of pig skin was at least ten times higher than that deposited in mouse skin. Lamellarity had little effect on the deposition of liposomal components into the skin strata when the formulations were compared at equal lipid concentrations.

INTRODUCTION

Liposomes are microscopic vesicles composed of one or more lipid bilayers arranged in concentric fashion, enclosing an equal number of aqueous compartments (1). Various amphipathic molecules have been used to form the liposomes, and the method of preparation can be tailored to control their size and morphology. The classification of liposomes is often confusing and can be based on whether they contain only one (unilamellar) or more (multilamellar) bilayers, their size, or their method of preparation.

Recently, a great deal of interest in the use of liposomes in skin gels or skin creams has been generated in the field of cosmetics. Phospholipids are widely used for topical applications in cosmetics and dermatology, since they have a high content of esterified essential fatty acids, the proper blend of which is believed to increase the barrier function of the skin and decrease water loss within a short period of time after application (2,3).

The key ingredient that keeps the human skin soft and flexible is water. Skin, particularly the horny layer, performs a significant protective role by providing, in addition to mechanical protection, a barrier against extraneous substances. This function of the horny layer is dependent on its elasticity, determined by the content of fats and inorganic salts, as well as by the hydration state. Increasing the skin humidity leads to an increase in its elasticity (5).

Skin humidity is regulated to a large extent by lipids in the skin's horny layer. This complex lipid mixture is oriented, at least in part, as bimolecular leaflets (4). Liposomes have been employed in cosmetics and skin care products for several years with great success. However, few reports on the deposition of liposomal lipids into skin after topical application have been published (5–7).

The objectives of the present study were to understand the nature of interaction of liposomal components with the skin by investigating the effect of liposome type and lipid concentration on deposition into the various strata of hairless mouse and pig skin using *in vitro* diffusion studies.

MATERIALS AND METHODS

Cholesterol (CH), cholesteryl sulfate (CS), and HEPES free acid were obtained from Sigma (St. Louis, MO). Egg lecithin (PC) was obtained from Avanti Polar Lipids (Birmingham, AL). α-Tocopheral (α-T) was obtained from Eastman Kodak (Rochester, NY). ^{14}[C]-CS and ^{3}[H]-CH were obtained from Amersham (UK). All other chemicals were of analytical grade.

PREPARATION OF LIPOSOMES

Multilamellar liposomes (MLV) containing PC:CH:CS at a molar ratio of 1:0.5:0.1 were prepared using the conventional film method (1). Briefly, the lipid mixtures were dissolved in a 2:1 (v/v) mixture of chloroform and methanol. Trace amounts of ^{3}[H]-CH and ^{14}[C]-CS were incorporated in the phospholipid-based liposomes. The lipids and markers were deposited as a thin film in a round-bottomed flask by rotary evaporation under nitrogen. The flask containing the lipid film was stored in vacuum overnight to facilitate removal of residual solvents. The films were hydrated by the addition of an isotonic 0.05 M HEPES buffer, pH 7.4, with mild agitation at 45°C. The final concentrations of lipid were 50 mg/ml, 25 mg/ml, and 10 mg/ml. All of the liposomal preparations were examined with a Nikon Diaphot Light microscope to ensure liposomal quality and integrity.

Large unilamellar vesicles were prepared by a modification of the reverse-phase evaporation method (REV) of Szoka and Papahadjopoulos (8). Appropriate amounts of the lipid mixtures, with trace amounts of radiolabeled CH and CS, were dissolved in 10 ml of a chloroform-methanol mixture (2:1; v/v). Five ml of 0.05 M HEPES buffer (pH 7.4) and enough additional methanol (up to 1.5 ml) were added to yield a clear solution after brief sonication. The organic solvents and a small amount of water were then removed under nitrogen at 45°C. Solvent removal was continued until all foaming ceased. The resulting liposomal suspension was stored at 4°C overnight before use in the diffusion experiments.

Dehydration/rehydration liposomes (DRV) were prepared by a modification of the method reported by Kirby and Gregoriadis (9). Briefly, appropriate amounts of the various lipids, along with the radiolabeled lipid markers, were dissolved in chloroform/methanol (2:1; v/v) in a round-bottomed flask. The solvents were removed using a rotoevaporator under vacuum, and the flask containing the film was dried overnight in a desiccator to remove residual solvent. An appropriate aliquot of 0.05 M HEPES buffer was then added, and the mixture was hydrated at 45°C. Intermittent vortexing was required for complete hydration. The resultant dispersion was then dehydrated at 50°C under vacuum using the rotoevaporator. When the liposomal suspension became very viscous, an amount of water, equivalent to that removed, was reintroduced into the viscous suspension. The rehydrated liposomes were allowed to equilibrate for about 45 min at 45°C, and the dispersion was stored at 4°C overnight before use in the diffusion experiments.

DEPOSITION EXPERIMENTS

Full-thickness hairless mouse skin was excised from fresh carcasses, and subcutaneous fat was carefully removed using a scalpel. Pig skin was obtained from a local abattoir and cleaned of any subcutaneous fat. The skin sections were mounted on Franz diffusion cells with a nominal surface area of 2 cm^2 and a receiver compartment with a 7-ml capacity (Crown Glass, Somerville, NJ). The epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed by a 0.05 M isotonic HEPES buffer. The receiver solution was stirred continuously using a small Teflon-covered magnet. Care was exercised to remove any air bubbles between the underside of the skin and solution in the receiver compartment. The temperature of the receiver was maintained at 37°C. Following mounting of the section of skin, 200 μl of the test formulation were applied to the epidermal surface of the hairless mouse skin and 400 μl of the test formulation were applied to the pig skin. A smaller amount of formulation was found to be insufficient to ensure uniform spreading across the entire exposed surface of the skin in the cell. A minimum of two cells was used for each formulation, using sections of skin from different skin specimens for each formulation. All experiments were carried out with non-occluded donor compartments. After 24 hr, the experiments were stopped and the diffusion set-up was dismantled for assay of radiolabeled lipids.

ASSAY OF RADIOLABELED MARKERS

Upon dismantling, the donor compartment of the cell was rinsed carefully five times with 0.5 ml buffer; the skin was removed, and it too was rinsed twice with 3 ml of buffer. The washing procedure was found to be sufficient to remove more than 99 percent of the formulation when determined at time zero. All washings were collected and assayed for radiolabel. Following the rinsing procedure, the skin patch was mounted on a board, and a piece of adhesive tape (Scotch Magic Tape, 810, 3M Commercial Office Supply Division, St. Paul, MN), 1.9 cm wide and about 6 cm long, was used to strip the skin. The tape was of sufficient size to cover the full area of skin that was in contact with the formulation. Based on extensive investigations of the extent of stripping of the stratum corneum, as monitored using TEWL (transepidermal water loss) measurements (14), it was determined that nine strippings were required for complete removal of mouse stratum corneum. The skin after nine strippings appears glossy. A total of 18 strippings was needed for pig skin in order to remove the stratum corneum, as judged by the glossy appearance. Nine such strippings were carried out for mouse skin and 18 strippings for pig skin, and each strip was analyzed separately for radiolabeled lipid. The remaining skin, as well as the receiver compartment solution, was also assayed for lipid content. Assay of the donor, skin rinses, and receiver solutions were carried out after addition of about 15 ml of Ecolite + (ICN Biomedical, Inc., Irvine, CA) to each system. The tape strippings and remaining skin were assayed as follows: Each sample was placed in a combustion cone and burned in a tissue oxidizer (Model 306, Packard Instrument Co., Downers Grove, IL). The separated radionuclides were assayed using a scintillation counter.

RESULTS AND DISCUSSION

Although the use of liposomal formulations for topical application has been steadily increasing, few studies have been undertaken to explain the mechanism by which liposomes deposit their lipids into the skin and the depth to which these lipids are deposited. Most *in vitro* transport studies concern themselves with permeation of drug through the skin and do not focus on the accumulation of lipid and entrapped ingredients in the various skin strata. In the cosmetic industry, it is essential to determine the extent to which the components of the formulation accumulate in the stratum corneum and, more importantly, to determine whether or not they are transported to the living epidermis and beyond.

There is a vast literature dealing with the mechanisms by which liposomes interact with cell membranes and deposit their entrapped materials in the cell interior. However, little work has been done on the interactions between topically applied liposomes and the skin. The barrier function of the skin resides mainly in the stratum corneum, which lacks nuclei and organella but contains keratin fibers and a complex mixture of lipids.

The stratum corneum of humans, mice, and pigs has been shown to be essentially devoid of phospholipids. Its lipid composition is rather non-polar and consists primarily of ceramides, triglycerides, cholesterol, fatty acids, and cholesteryl sulfate. These lipids are arranged in bilayer structures that fill the intercellular space in the stratum corneum. The primary pathway to the transport of water and other molecules is believed to reside mainly in these structures. The removal of these bilayer sheets either by solvent treat-

Table I

Distribution of Cholesterol (expressed as percent formulation applied ± standard deviation) in Various Strata of Hairless Mouse and Pig Skin 24 hr After *In Vitro* Topical Application of Various PC/CH/CS Liposomal Formulations Onto Full-Thickness Skin (n = 4–5)

	REV		DRV		MLV	
Compartment	Mouse	Pig	Mouse	Pig	Mouse	Pig
Total donor	29.6 ± 0.03	41.4 ± 8.03	64.3 ± 5.6	42.0 ± 7.6	49.0 ± 8.9	46.6 ± 4.2
Surface stratum corneum	48.2 ± 6.8	25.3 ± 4.9	18.6 ± 3.9	26.4 ± 2.8	27.1 ± 3.3	22.9 ± 3.8
Deeper stratum corneum	21.6 ± 4.2	27.8 ± 5.8	16.7 ± 4.3	25.7 ± 5.8	22.2 ± 6.5	22.0 ± 0.8
Deeper skin strata	1.2 ± 0.3	5.5 ± 0.7	1.4 ± 1.3	5.8 ± 0.5	1.6 ± 0.2	8.3 ± 1.6
Total skin	70.3 ± 2.4	58.6 ± 11.5	35.6 ± 6.2	57.9 ± 8.0	50.9 ± 9.6	53.2 ± 6.2
Receiver	0.1 ± 0.05	0.05 ± 0.05	1.1 ± 0.02	0.1 ± 0.1	1.2 ± 0.03	0.2 ± 0.06

All values were corrected to 100%.

ment (10) or by successive tape stripping (11) increases the permeability of water, suggesting a decreased barrier function.

In an effort to understand effects of liposomal compositions and the method of preparation on the deposition of lipids into the stratum corneum and deeper strata of the skin, the topical delivery of several liposomal formulations was evaluated using *in vitro* diffusion techniques. Table I shows the degree of deposition of cholesterol into the various strata (surface stratum corneum, deeper stratum corneum, and deeper skin strata) of hairless mouse and pig skin 24 hr after *in vitro* topical application of the liposomal formulations with different lamellarities having a total lipid concentration of 10 mg/ml. The amount of lipids adhering to the stratum corneum surface was defined as that determined by analysis of the first two tape strippings. The amount of lipids penetrating the deeper stratum corneum was defined as that determined by the analysis of tape strippings 3 through 9 for hairless mouse skin and 3 through 18 for pig skin. The amount of lipids penetrating the deeper skin strata was defined as that determined by analysis of the remainder of the full-thickness skin. A mass balance of >96% was achieved after the donor compartment and the skin rinses were accounted for. No lipid could be detected in the receiver compartment for any of the liposomal systems tested. It was found that application of PC/CH/CS MLV resulted in almost the same degree of lipid deposition in the deeper stratum corneum and the deeper skin strata as did

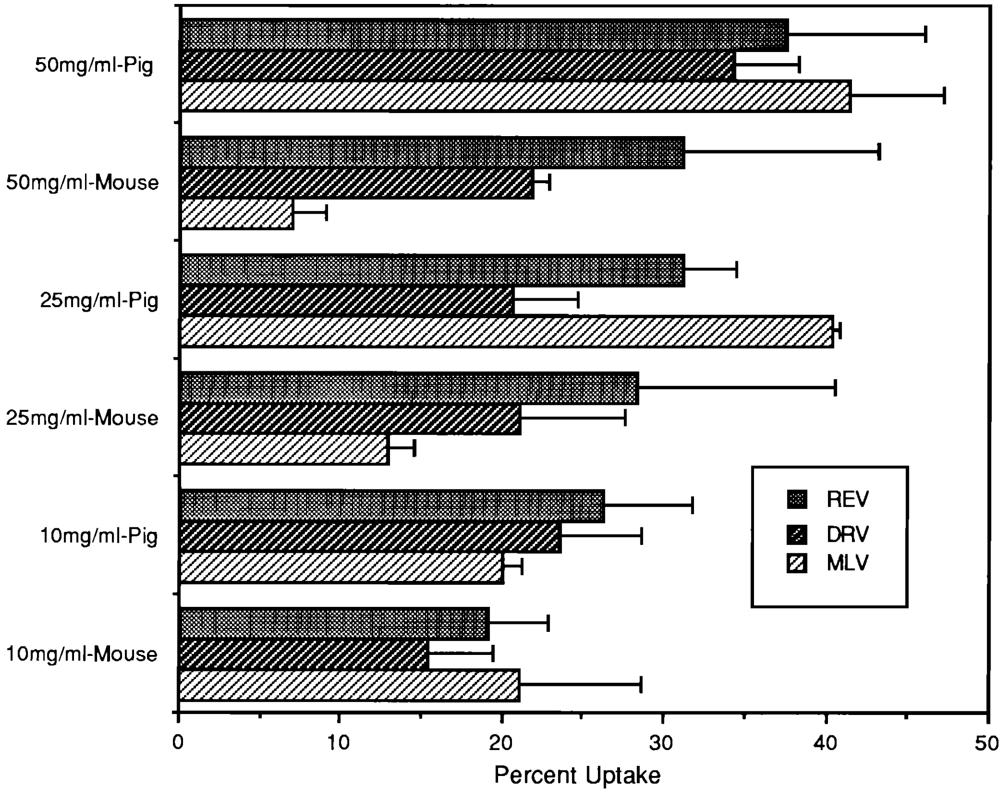

Figure 1. Comparison of the 24-hr *in vitro* uptake of ^{14}[C]-cholesteryl sulfate from PC/CH/CS DRV, MLV, and REV with total lipid concentrations of 10, 25, and 50 mg/ml in the deeper stratum corneum of hairless mouse and pig skin (n = 4–5).

application of PC/CH/CS DRV or PC/CH/CS LUV of the same lipid concentrations. Figures 1 and 2 show representative plots of the percent cumulative uptake of cholesteryl sulfate from different liposomal formulations with various lamellarities and lipid concentrations in the deeper stratum corneum and deeper skin strata. These results suggest that the interaction of liposomes with skin, leading to lipid deposition in the deeper skin strata, does not depend on the degree of lamellarity. It should be pointed out, however, that lamellarity may play an important role in the deposition of entrapped hydrophilic drugs. For example, we have shown the pronounced advantage of DRV over LUV for the deposition of entrapped interferon into deeper skin strata (12). These data are not conflicting, since the amount of drug associated with the lipid bilayers transported into the deeper skin strata would depend, to a large extent, on the lamellarity and other factors associated with the method of liposomal preparation (13).

We also examined the effect of skin species on deposition of lipids after topical application of liposomes as a function of method of preparation and lipid concentration. These differences could be related to either species differences in stratum corneum lipid structure or to the virtual absence of the follicular route in the mouse skin as compared to the pig skin.

The effect of liposomal lipid concentration on *in vitro* permeation of liposomal lipid into pig skin was evaluated using lipid concentrations 10 mg/ml and 50 mg/ml for three

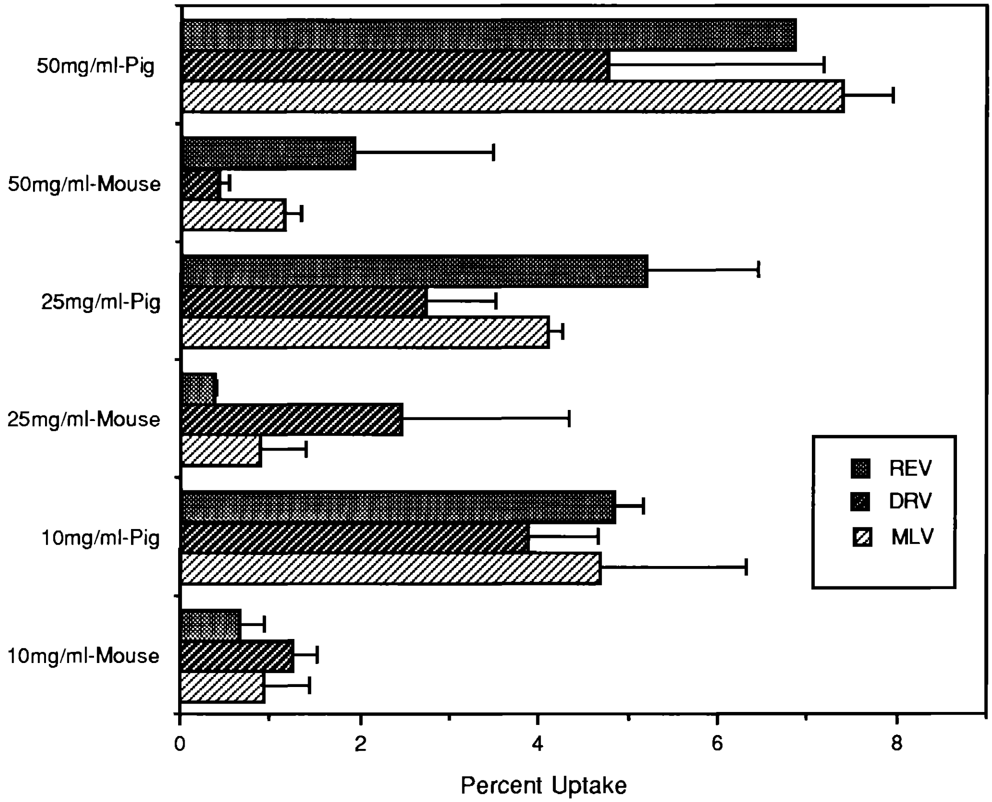

Figure 2. Comparison of the 24-hr *in vitro* uptake of ^{14}[C]-cholesteryl sulfate from PC/CH/CS DRV, MLV, and REV with total lipid concentrations of 10, 25, and 50 mg/ml in the deeper skin strata of hairless mouse and pig skin (n = 4–5).

liposomal systems (MLV, DRV, and LUV). Table II shows the degree of deposition of cholesteryl sulfate into the various strata of pig skin 24 hr after *in vitro* topical application of the various liposomal formulations with lipid concentrations of 10 and 50 mg/ml. The data shown in Table II indicate that the amount of lipid deposited in the deeper skin strata of pig skin was ten times higher than that found with mouse skin. These differences could be related to either species differences in stratum corneum lipid structure or to the virtual absence of the follicular route in the mouse skin as compared to the pig skin.

The CH/CS ratios in the different mouse skin strata 24 hr after topical application of the liposomal formulations are shown as a function of liposomal type and lipid concentration in Table III. The molecular ratio of radiolabeled lipids of the liposomal preparations was maintained in the surface stratum corneum and the deeper stratum corneum following topical liposomal MLV, DRV, and LUV systems. The results in Table III suggest that the two lipid components are diffusing into the surface and the deeper stratum corneum at the same rate despite a large difference in their polarities. The ratios of the two lipids in these strata are the same as that in the original liposomal suspension, indicating that the lipid components are transported as bilayer units. The fact that the ratio of the

Table II

Distribution of Cholesteryl Sulfate (expressed as percent formulation applied ± standard deviation) in Various Strata of Pig Skin 24 hr After *In Vitro* Topical Application of Various PC/CH/CS Liposomal Formulations Having Different Lipid Concentrations Onto Full-Thickness Skin (n = 4–5)

	10 mg/ml			50 mg/ml		
Compartment	REV	DRV	MLV	REV	DRV	MLV
Total donor	43.5 ± 9.4	47.0 ± 6.7	52.9 ± 2.0	27.5 ± 1.1	23.7 ± 0.3	18.1 ± 0.8
Surface stratum corneum	24.1 ± 5.5	27.2 ± 0.1	21.9 ± 4.1	27.8 ± 4.9	36.8 ± 2.2	32.6 ± 0.9
Deeper stratum corneum	26.2 ± 5.4	23.7 ± 5.3	20.1 ± 1.2	37.5 ± 8.5	34.3 ± 3.9	41.4 ± 5.8
Deeper skin strata	4.9 ± 0.3	3.9 ± 0.8	4.7 ± 1.6	6.9 ± 0.0	4.8 ± 2.4	7.4 ± 0.6
Total skin	55.3 ± 9.7	54.8 ± 4.5	46.6 ± 6.9	72.2 ± 3.6	75.9 ± 0.7	81.4 ± 5.4
Receiver	1.3 ± 0.2	0.6 ± 0.06	0.5 ± 0.1	0.4 ± 0.03	4.1 ± 0.5	0.6 ± 0.1

All values were corrected to 100%.

Table III

The Cholesterol:Cholesteryl Sulfate Ratio in Various Strata of Hairless Mouse Skin 24 hr after *In Vitro* Topical Application of PC/CH/CS MLV, DRV, and REV With Different Lipid Concentrations Onto Full-Thickness Skin (n = 4–5)

	MLV			DRV			REV		
Compartment	10 mg/ml	25 mg/ml	50 mg/ml	10 mg/ml	25 mg/ml	50 mg/ml	10 mg/ml	25 mg/ml	50 mg/ml
Surface stratum corneum	1:0.98	1:1.02	1:1.09	1:0.95	1:0.96	1:0.97	1:0.96	1:0.87	1:0.99
Deeper stratum corneum	1:0.95	1:0.83	1:0.50	1:0.92	1:0.97	1:0.94	1:0.88	1:1.44	1:0.94
Deeper skin strata	1:0.61	1:0.53	1:0.65	1:0.93	1:0.96	1:0.32	1:0.58	1:0.20	1:0.82

radiolabeled lipids is not maintained in the deeper skin strata suggests that the liposomal components are diffusing independently in these regions. Cholesteryl sulfate is more polar than cholesterol, and the two molecules should diffuse at independent rates once they are no longer associated in a bilayer configuration.

The combined results suggest that topically applied liposomal formulations could be an effective delivery system for the treatment of skin. Since these liposomal formulations provide sustained, enhanced levels of lipids in the skin, they may be of value to cosmetic products by encapsulating a variety of ingredients such as moisturizers and skin care agents. Furthermore, these formulations can be optimized, with respect to liposomal type and lipid concentration, to release material to the various layers of the skin at defined rates.

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