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RESEARCH ARTICLE

Development and characterization of minoxidil-loaded liposomal system for delivery to pilosebaceous units

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Abstract

The current study aimed to deliver minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide; MXD), a potent hypertrichotic agent, into the pilosebaceous units, exploring the potential of the liposomal system. MXD-loaded liposomes of different compositions were prepared by a thin-film hydration technique and subsequently characterized for various vesicle-specific attributes (i.e., size, shape, lamellarity, and entrapment efficiency). Comparative analysis among these compositions was conducted with reference to their vesicle-specific parameters, drug deposition, and drug-delivery mechanism toward pilosebaceous units. The latter may bring about a distinct change in MXD therapy for various aliments related to pilosebaceous units, such as alopecia. The *in vitro* drug release, *ex vivo* skin permeation, and drug-retention behavior of the prepared formulation were evaluated by employing rat skin (normal as well as pilosebaceous free) and semipermeable membrane. The results revealed that the neutral liposomes (mean vesicle size, 3.83 ± 0.18 μm) showed maximum drug deposition in the pilosebaceous units among all the other tested formulations. A quantitative estimation of pilosebaceous delivery revealed that the concentration of MXD in each pilosebaceous unit decreased in the following order: neutral liposomal formulation (5.8×10^3 to 7.25×10^3 μg) > positively charged liposomal formulation (4.7×10^3 to 5.87×10^3 μg) > negatively charged liposomal formulation (4.2×10^3 to 5.25×10^3 μg) > nonliposomal formulation (1.6×10^3 to 2.0×10^3 μg). Stability studies construed the need to store the liposomal formulation at lower temperatures. The results of the current work indicate that the neutral liposomes can deliver the drug molecules into pilosebaceous units more effectively than the other studied formulations.

Keywords: *Liposomes; pilosebaceous unit; minoxidil; drug delivery; alopecia*

Introduction

Alopecia areata is an autoimmune, psychologically devastating, and socially stigmatic skin disorder resulting in patchy loss of hair on the scalp and elsewhere on the body (Amos and Richard, 2006). This common, but very challenging, and capricious disease affects the quality of life of both men and women. Currently, many drugs, such as corticosteroids, retinoic acid, zinc, tricome, and anthraline, etc., are being used for the treatment of alopecia (Virginia and Samer, 1996). However, minoxidil (MXD), as a topical solution, to date, outperforms all the other drugs.

Known to exert direct vasodilator activity, MXD (2,4-diamino-6-piperidinopyrimidine 3-oxide) was initially introduced as an antihypertensive drug in the early 1970s (Zappacosta, 1980). Its action as a potent hair-growth promoter became evident with the reports of hypertrichosis associated with its oral administration in hypertension (Burton and Marshall, 1979). These findings led to the development of a topical formulation of MXD for the treatment of alopecia areata and alopecia androgenetica (Wong and Sefect, 1994). As on today, a topical formulation with 2% (w/v) MXD is the first U.S. Food and Drug Administration (FDA)-approved medication for stimulating hair growth. Besides, the MXD

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solution is also currently available with higher drug-dose concentrations. Twice-daily applications of MXD, as recommended, leads to several adverse effects (i.e., fluid retention, tachycardia, dyspnea, gynecomastia, fatigue, nausea, and cardiotoxicity). It has been reported that MXD, when applied topically, gets absorbed into the systemic circulation, leading to undesirable cardiovascular effects (Pavithran, 1993; Rumsfield et al., 1987).

Unlike other skin diseases, alopecia is specifically restricted to pilosebaceous units. Heightened interest in the pilosebaceous unit as a potential drug-delivery target is based upon the fact that the etiology of several dermatological abnormalities relate to the hair follicle. For the treatment of these pathological conditions, it is, therefore, important to increase the distribution of drugs in the hair follicles. Nevertheless, the problem associated with MXD can certainly be addressed by employing a drug-delivery approach, using a suitable carrier.

In the present study, the liposomal vesicular system was selected to investigate the pilosebaceous targeting of MXD. The liposomal system was preferred over other carrier systems, as this is a biodegradable, biocompatible, and nontoxic carrier with excellent potential for skin penetration and prolongation of drug release by acting as a depot in the deeper layers of skin (Uchegbu and Vyas, 1998; Mezei, 1993). The present work also aimed to study the effect of size, surface charge, formulation composition, etc., on the targeting potential of these carriers toward the pilosebaceous units.

Materials and methods

Materials

MXD was obtained as a free gift sample from Regrowth LLC (Whittier, California, USA). Phospholipon 90H (PC), having 97.3% phosphatidyl choline content, stearylamine (SA), dicetylphosphosphate (DCP), and Sephadex G-50 (medium grade) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) and used without further purification. Cholesterol (CHOL) was obtained from Hi-Media (Mumbai, India). Methanol (MTHOL) and other organic solvents employed in the study were obtained from E. Merck (India) Ltd., (Mumbai, India). Rhodamine 123 was purchased from Sigma. Double-distilled water, obtained by using an all-glass still (Scientific Instruments, New Delhi, India), was used throughout the experiments.

Preparation of MXD-loaded liposomes

Liposomes were prepared as multilamellar vesicles (MLVs) by the thin-film hydration method, as reported in the literature (Bangham et al., 1965). Accurately weighed quantities of the PC, CHOL, and charge-imparting agent (SA or DCP), in varying variable ratios (Table 1), were dissolved in a minimum amount of CHOL:MTHOL ratio (3:1 v/v) mixture in a round-bottomed flask. The organic solvent was removed by using a rotary flask evaporator under reduced pressure to form a thin lipid film on the wall of the flask. Following complete evaporation of the solution, the flask was kept under a vacuum overnight under a nitrogen atmosphere to remove the residual solvent. The dried film was then hydrated with MXD solution (1 mg/mL) in distilled water for 20 minutes. The homogenous suspension of MLVs was also subsequently sonicated (titanium microtip ¼"; Probe sonicator, Misonix S-3000 (Misonix, Inc. 1938 New Highway Farmingdale, NY 11735) USA; 4°C; 36 W; 10 minutes with 5 cycles of 2 minutes each) to obtain smaller MLVs. Larger MLVs were prepared by increasing the hydration time of the dried film correspondingly. Vesicular

Table 1. Liposomal formulations with different molar ratios of phosphatidyl choline (PC), cholesterol (CHOL), and charge-imparting agents.

Sample no.	Formulation code	Composition of system	Molar ratio			Entrapment efficiency (%)	Average vesiclesize (μm)	Zeta potential (mV)
			PC	CHOL	Charge			
1	LF1	PC/CHOL	9	1	—	54.5±1.22	—	—
2	LF2	PC/CHOL	7	3	—	61.0±1.24	—	—
3	LF3	PC/CHOL	5	5	—	56.3±1.51	—	—
4	LF2-S	PC/CHOL	7	3	—	57.3±1.31	1.84±0 .15	—
5	LF2-M	PC/CHOL	7	3	—	61.0±1.24	3.83±0.18	–7.2±0.03
6	LF2-L	PC/CHOL	7	3	—	62.6±1.16	5.52±0.27	—
7	LF2-0	PC/CHOL	7	3	—	—	Without vesicle	—
8	LF2-M-D1	PC/CHOL/DCP	7	3	0.5	61.52±0.28	—	—
9	LF2-M-D2	PC/CHOL/DCP	7	3	1.0	63.23±0.49	4.21±0.11	–29.11±0.09
10	LF2-M-D3	PC/CHOL/DCP	7	3	1.5	62.73±0.31	—	—
11	LF2-M-S1	PC/CHOL/SA	7	3	0.5	61.73±0.54	—	—
12	LF2-M-S2	PC/CHOL/SA	7	3	1.0	63.28±0.62	4.98±0.09	22.1±0.05
13	LF2-M-S3	PC/CHOL/SA	7	3	1.5	62.35±0.35	—	—

SA, stearyl amine; DCP, dicetyl phosphate.

formulations loaded with SA or DCP were also prepared analogously.

Additionally, one nonvesicular formulation was prepared by mixing drug and PC in water. This formulation was used to study the effect of vesicle on drug delivery.

Determination of drug-entrapment efficiency

Drug-entrapment efficiency of the formulated vesicles was determined by using the minicolumn centrifugation method (New, 1990). The liposomal vesicular suspension (1 mL) was placed in the Sephadex G-50 minicolumn (presaturated with empty vesicles) and centrifuged at 2,000 rpm (626×*g*) for 8 minutes. Elutes containing drug-loaded vesicles were collected and observed under optical microscope to ensure the absence of unentrapped drug particles. Intact vesicles, free from unentrapped drug, were collected after 2 washings with 0.9% (w/v) saline solution. Entrapment efficiency of vesicles was determined by lysing them by using 50% n-propanol. Following suitable dilutions, the samples were analyzed at a λ_{max} of 228.5 nm spectrophotometrically (Systronic 119 UV/VIS spectrophotometer Jona Wadaj, Ahmedabad, Gujarat, India). Percent drug entrapment (PDE) for the prepared liposomes was calculated by using Equation 1:

$$\text{PDE} = \frac{\text{Entrapped drug (mg)}}{\text{Total drug added (mg)}} \times 100 \quad (1)$$

Structure and morphology

Liposomal vesicles were visualized by using a transmission electron microscope (TEM Sophisticated Instrumentation Facility (SIF), AIIMS-New Delhi; Philips, Holland) with an accelerating voltage of 100 KV. A drop of the sample was placed on a carbon-coated copper grid to leave a thin film. Subsequently, the film was negatively stained with 1% phosphotungstic acid (PTA). Excess PTA was drained off with a filter paper. The grid was allowed to dry under the air thoroughly, and the sample was viewed under TEM to obtain the photomicrographs.

Size and size distribution

Preliminary examinations of liposomal vesicles were done under an optical microscope (Leitz-Biomed, Wetzlar, Germany). The vesicle-size and zeta-potential analysis of liposomes were carried out by the dynamic light scattering (DLS) technique by using a Malvern Zetasizer 2000 HS (Malvern Instruments Limited, Malvern, UK, NIPER, SAS Nagar, Punjab).

In Vitro *drug-release profile*

In vitro release of MXD-loaded liposomal formulations was studied by using an artificial cellophane membrane. The treated cellophane membrane was prewashed with distilled water before use. One milliliter of drug-loaded suspension was taken in a cellophane dialysis bag and placed in a beaker containing 100 mL of phosphate-buffered saline (PBS) (pH 6.5, 32 ± 1^{0}C), using a thermostatically calibrated magnetic stirrer (York, New Delhi, India). Aliquots of 1 mL were withdrawn at periodic time intervals and measured at a λ_{max} of 228.5 nm for estimating the drug content.

Skin permeation studies

Albino rats (5–6 weeks old), weighing 60–80 g, were used for the *ex vivo* skin permeation study. All the rats used in the study were treated according to the institutional guidelines.

Preparation of rat skin

Normal skin (NS)

To obtain animal skin, albino rats were anesthetized with diazepam. The dorsal surface of the animals was inversed for 30 seconds in water and maintained at 60°C. The hair of the animals were carefully shaved off with a pair of scissors, and the abdominal skin was separated from the underlying connective tissue by using a scalpel. The excised skin was placed on a piece of aluminium foil, and the dermal side of the skin was carefully wiped off to remove any adhering fat. Subsequently, skin was washed with saline solution and carefully observed by using a magnifying glass to ensure that the skin samples, to be employed for transdermal permeation studies, were free from any surface irregularities, such as tiny holes, cervices, etc.

Preparation of pilosebaceous unit–free skin (PFS)

A truly follicle-free PFS was created by a method reported by Behl et al. (1981). First, the experimental wounds were developed and the rats were anesthetized by using an intraperitoneal injection of diazepam. The dorsal surface of the animals was immersed in water for 30 seconds and maintained at 60°C. A small section of the abdominal skin was removed, and the animals were kept for healing with the regular application of an antiseptic to prevent any infection. Complete healing of the skin was observed after 45 days. Healed skin surface appeared as smooth and pinkish, typical of a scarred surface. This scarred portion of PFS was then employed for skin permeation studies.

Skin permeation study

For *ex vivo* studies, a Franz diffusion cell, fabricated in-house, was employed. The effective permeation area of the diffusion cell and receptor-cell volume was 2.5 cm^2 and 10 mL, respectively. Rat skin (NS as well as PFS) was tied between the donor and receptor compartments. A volume of 0.1 mL of the formulation was taken in the donor compartment. Ten milliliters of PBS (pH 6.5) was filled in the receptor compartment, and the temperature was maintained at 32 ± 1°C. Samples were withdrawn after regular periodic intervals from the receptor compartment and replaced with an equal volume of fresh PBS (pH 6.5). The study was conducted in triplicate.

Determination of drug retention into the pilosebaceous unit (APU)

Following the permeation study, skin (NS or PFS), mounted on the Franz cell, was removed carefully. The remaining formulation adhering to the skin was scraped with a spatula and then wiped with a tissue paper. Ten milliliters of methanol was added to the cleaned skin piece and put in a water-bath shaker for 24 hours at 32 ± 1°C. The solution was filtered, and the filtrate was precisely measured for drug content. Subsequently, the amount of MXD present in the pilosebaceous units (APUs) was calculated, as in Equation 2:

$$\text{APUS} = \text{NS} - \text{PFS} \tag{2}$$

The amount of drug in each APU was calculated, as in Equation 3:

$$\text{APU} = \frac{\text{APUS}}{800} - \frac{\text{APUS}}{1000} \tag{3}$$

Fluoresence microscopy

Rhodamine 123 was used as a fluorescent marker and rhodamine 123–loaded liposomes (LF2-M) were prepared according to the method described under Materials and Methods. Rhodamine 123–loaded formulation was applied topically to Albino rats. After 2 hours, the rats were sacrificed, skin was removed, cut into pieces, and washed with Ringer's solution. Skin was blotted and wiped with tissue paper. The wiped tissues were fixed in Carnay's fluid for 3–4 hours. The tissues were then dehydrated by using absolute alcohol. After dehydration, the tissues were passed through various mixtures of absolute alcohol:xylene for different time intervals and finally put in xylene. Wax scrapings were added to xylene up to the saturation level and kept undisturbed for 24 hours. The paraffin blocks were prepared by embedding the tissues in hard paraffin followed by maturation at 62–64°C. Then, microtomy was performed, and a ribbon of sections was fixed on slides, using egg albumin solution as a fixative. The sections were viewed under a fluorescence microscope (Leitx-Biomed), and photomicrographs were taken.

Stability studies

The liposomal formulation was tested for storage stability at 4 ± 1 and 37 ± 1°C for a period of 4 weeks. The MXD-loaded formulation (30 mL) was stored in amber-colored glass bottles. Samples from each batch were withdrawn at three periodic intervals of 10 days each. Stability parameters that were studied included vesicle size, fusogenecity (i.e., decrease in vesicle number), and percent residual drug in vesicles. The vesicle size was determined by using an optical microscope. For the determination of fusogenecity, an appropriate amount of liposomal formulation was suitably diluted with PBS (pH 6.5) and placed in a hemocytometer. The vesicles were counted by using an optical microscope (Leitx-Biomed), and the total number of vesicles per cubic millimeter was calculated, as shown in Equation 4:

$$\frac{\text{Number of vesicles}}{\text{per cubic mm}} = \frac{\text{Number of vesicles} \times \text{dilution factor} \times 4000}{\text{Number of small squares counted}} \tag{4}$$

The percent residual drug in vesicles was measured by using the centrifugation method. Samples were withdrawn and analyzed for MXD content.

Results and discussion

Preparation and characterization of MXD-loaded liposomes

The requisite formation of vesicles was ratified by using TEM studies. Distinct black and white contrast of prepared vesicles, observed in the TEM photomicrograph (Figure 1A), was ostensibly due to the alternative aqueous and nonaqueous (i.e., lipidic) compartments (i.e., lamellae), confirming the formation of MLVs. The lamellae of vesicles were evenly spaced to the core, ascribing high entrapment efficiency of drug in the liposomes.

During our studies, the uniform-shaped liposomes of the homogenous population were obtained by employing TEM, as confirmed by optical photomicrograph (Figure 1B). The absence of drug crystals in the optical photomicrograph indicates the lack of unentrapped

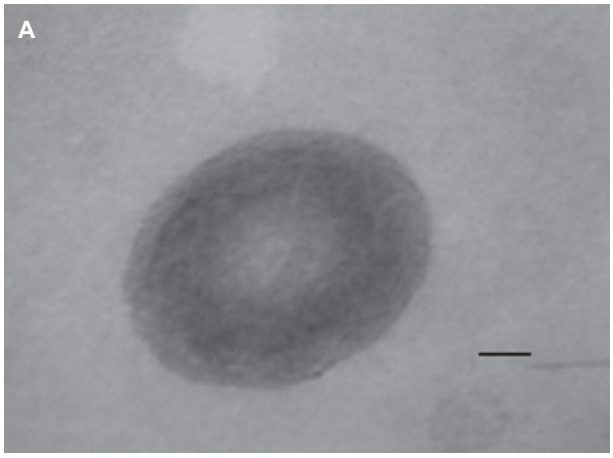

Figure 1A. Photomicrograph of the optimized liposomal formulation (LF2-M) by transmission electron microscopy (X60,000). Scale bar = 10 μm.

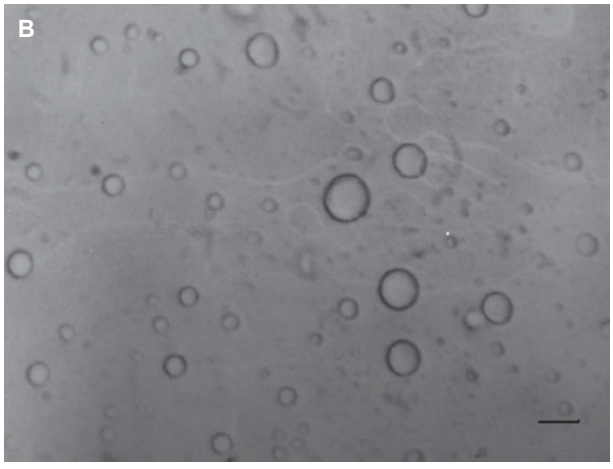

Figure 1B. Optical photomicrograph of optimized liposomal formulation LF2-M (X1,250). Scale bar = 10 μm.

drug and thus justifies the high entrapment efficiency of vesicles.

A myriad of factors govern the delivery of drugs into the pilosebaceous units from the topically applied formulations. Specifically, in the case of vesicular systems, these factors include the lipid composition, the size of the molecules, and the charge on the surface. Lipid composition and total lipid concentration have been proven to influence drug deposition into the skin layers. To study the abstractive effect of these factors on drug deposition of MXD into PU, various process variables (i.e., molar ratio, vesicle size, and surface charge) were optimized, as explained below.

MXD-loaded liposomes were prepared by employing different drug concentrations and types of ingredients. Liposomes containing DCP and SA were also prepared in an analogous manner as charge-inducing agents. Table 1 summarizes the effect of composition on PDE and vesicle size of the prepared formulations.

Optimization of molar ratio

Liposomes with different molar ratios of PC and CHOL (LF_1 to LF_3; Table 1) were prepared to obtain optimum composition in terms of PDE, as this would effect the *in vitro* and *ex vivo* performance of vesicles and impact drug delivery to a particular site simultaneously (Sinico et al., 2005; Mezei, 1998).

Maximum entrapment was recorded for formulation LF_2 (61.0 ± 1.24%). Low level of CHOL (i.e., in LF_1) makes the vesicles unstable and vulnerable to leakage of drug, leading to lower PDE. As the concentration of CHOL increases, PDE tends to exhibit an augmenting trend. This can be attributed ostensibly due to the ability of CHOL to cement the leaking space in the bilayer membranes, allowing enhanced drug entrapment in the prepared liposomes. However, with further increase in CHOL beyond a certain limit (i.e., in LF_3), the PDE tends to decrease, probably due to diminished hydration of the bilayer surface. The findings are in close agreement with the studies reported earlier (Du Plessis et al., 1992). Based on these results, formulation LF_2 was chosen for further optimization studies.

Optimization of vesicle size

Size of the vesicle is a vital parameter for optimization of drug delivery in PU. However, only a few dedicated studies have been performed, to date, on this aspect. Esposito et al. (1980) reported that the permeability coefficient of methyl nicotinate is inversely related to the liposome size. Analogously, Du Plessis et al. (1994) reported that topical drug delivery is significantly influenced by the size of liposomes.

Thus, for the selection of optimum vesicle size for drug delivery, three MXD liposomal formulations (i.e., LF2-S, LF2-M, and LF2-L) (Table 1) with different vesicle sizes were prepared. To obtain smaller vesicles, sonication was employed, while larger vesicles were obtained on increasing hydration time. Results indicate that with an increase in vesicle size, the PDE also increased. With increase in size, the number of lamellae in the vesicular structures also increases, resulting eventually in higher entrapment of hydrophilic drug in the prepared vesicles.

Further, the results of *in vitro* drug-release studies through cellophane membrane indicated that bigger size vesicles yielded higher drug release (i.e., 11.4 ± 0.49%), as compared with the smaller vesicles (i.e., 6.7 ± 0.35%). Contrary to the *in vitro* studies, the *ex vivo* studies showed (Figure 2B) that maximum drug was released by smaller size liposomes. Figure 2A and 2B corroborate the same graphically. Release flux of these formulations was found to follow the order: LF2-S > LF2-M > LF2-L. The bar diagram (Figure 3) indicates that medium-sized liposomes (LF2-M) showed maximum skin retention (i.e.,

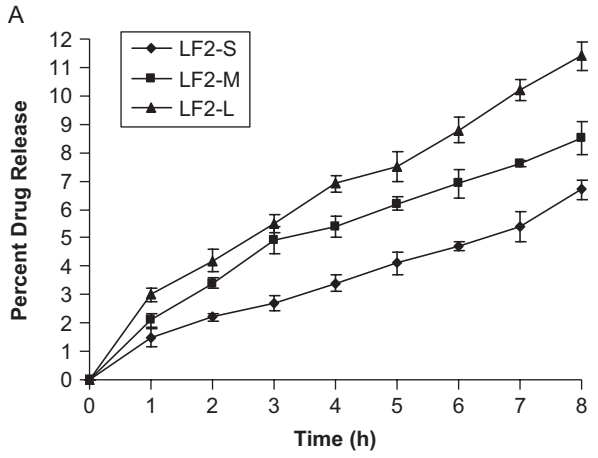

Figure 2A. *In vitro* drug-release profile of minoxidil from various liposomal formulations across the cellophane membrane. Each point represents mean ± standard deviation of three formulations.

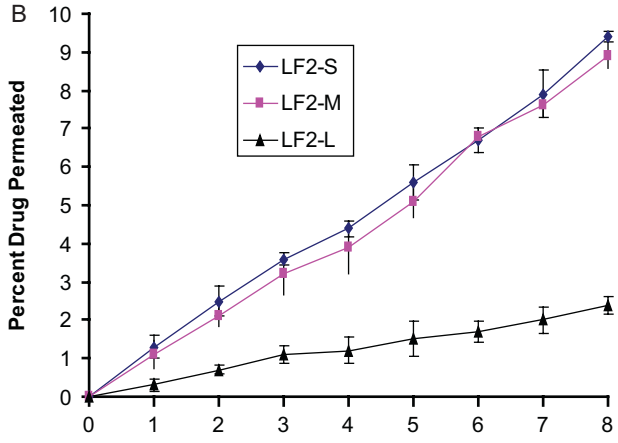

Figure 2B. *Ex vivo* excised drug permeated profile of minoxidil from various liposomal formulations through the NS. Each point represents mean ± SD of 3 formulations (n = 3).

7.5 ± 0.12 μg), in comparison to other formulations. The smaller liposomes (LF2-S) showed maximum amount of drug in the receiver compartment, while the larger ones (LF2-L) showed maximum drug in the donor compartment. These results connote that the smaller vesicles settle down on the skin surface close to the outermost corneocytes layer and disintegrate faster on the skin surface (Verma et al., 2003a). In these conditions, they easily make close contact between skin lipids and vesicles. Therefore, the material exchange between vesicles and the intercellular skin lipids may occur, leading to the diffusion of the drug across the stratum corneum (SC). Large-size vesicles settle down on the skin surface faster and stay on the SC surface, forming a thicker layer and increasing the diffusion pathway. These findings are in close agreement with previous reports (Verma et al., 2003b; Lauer et al., 1995) indicating optimum liposome size to be a requisite for the optimal drug deposition into the skin.

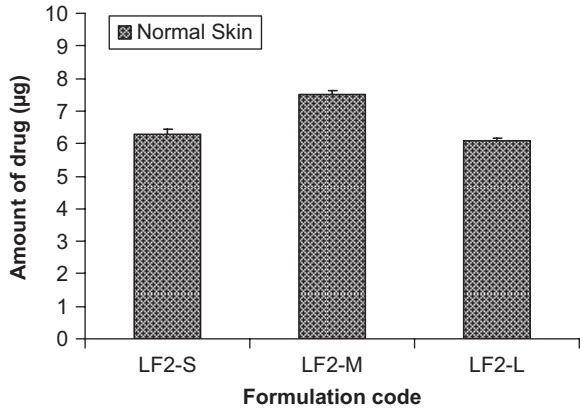

Figure 3. Amount of mean drug ($n = 3$) retained in the NS by various liposomal formulations of MXD. The cross-bars indicate ± standard deviation.

Overall, the results of *ex vivo* studies indicate that the intermediate-size vesicle (LF2-M) show maximum MXD deposition in the skin, as compared to larger or smaller size vesicles. It was concluded that LF2-M (3.83 ± 0.18 μm) might be the optimum vesicle size for topical drug delivery. Hence, the formulation, LF2-M, was selected for further studies. From the above results, it can also be concluded that the topical delivery of drug is remarkably influenced by the vesicle size of the prepared formulation

Optimization of surface charge

Two kinds of charge-inducing agents (SA; for positive charge) and (DCP; for negative charge) were chosen to investigate the effect of surface charge on pilosebaceous targeting. Three formulations of each type (i.e., positive, negative, and neutral), with various PC, CHOL, and charge molar ratios (shown in Table 1), were prepared and their entrapment efficiency determined. It was found that the liposomal formulation with a molar ratio of PC:CHOL:charge (7:3:1) exhibited better entrapment efficiency. The PDE of LF2-M-S2 was found to be 63.28 ± 0.62, while for LF2-M-D2, it was 63.23 ± 0.49. These two formulations were selected for further studies to evaluate the effect of different types of surface charge.

For quantitative estimation of pilosebaceous targeting, the optimized formulation (i.e., LF2-M), which have zeta potential near neutral (slightly negative due to MXD), was taken as the neutral liposomal formulation, while the formulations, LF2-M-S2 and LF2-M-D2 (which have zeta potential positive and negative), were the positively and negatively charged liposomal formulations, respectively (Table 1). Additionally, another lipidic formulation (LF2-0), a drug-phospholipid solution, was taken as the nonvesicular formulation. These four formulations were selected to compare the effect of vesicular and nonvesicular formulation on pilosebaceous targeting.

Results of the studies showed that the neutral liposomes have a vesicle size 3.83 ± 0.18 μm and charged liposomes result in increased average size, as shown in Table 1. It was noted that the inclusion of charged lipids caused an enhancement in average vesicle size. The increase in average size can be ascribed to increased intralamellar spacing due to the same charge on the bilayer.

The inclusion of charged lipid in the formulation indicates that drug entrapment was higher in the case of positively (63.28 ± 0.62%) and negatively charged (63.23 ± 0.49%) than that in the neutral (61.0 ± 1.24%) ones. This phenomenon can be attributed to the fact that the inclusion of charge increased the interlamellar space, leading to increased PDE.

No significant difference in the *in vitro* drug-release behavior of MXD from all the three vesicular liposomal formulations was observed (Figure 4). Maximum drug release was, however, observed with the nonvesicular lipidic solution; this could be due to the absence of vesicles in the formulation. Among the neutral, positive, and negatively charged vesicles, the former showed the highest drug-release extent.

The specific role of the hair follicle in drug transport to the pilosebaceous unit remains difficult to elucidate owing to the nonavailability of an appropriate animal model. The latter should enable one to distinguish the transfollicular from the transepidermal percutaneous absorption. The stump-tailed macaque monkey has extensively been used as an animal model for *in vivo* studies of alopecia (Uno, 1988). These macaque monkeys exhibit a species-specific frontal scalp baldness that coincides with puberty in both the sexes, thus representing a pertinent animal model for studying human androgenetic alopecia. However, the species being rare, it is usually quite difficult to trace the animals, especially in India. A number of rodent models have also been explored as alternative substitutes (Uno, 1989). However, this model cannot represent exclusively the transepidermal pathway of absorption, since the rodents are not follicle free. Nevertheless, a truly follicle-free skin has been created by a method reported by Behl et al. (1981).

In order to quantitatively investigate the involvement of transfollicular route, development of an appendage-free (pilosebaceous-free) model of skin is obligatory (Figure 5A–5C) An appendage-free structure, such as scar tissue, is useful for sorting out transfollicular and -epidermal contributions to percutaneous absorption by studying it simultaneously with normal skin. Therefore, *ex vivo* skin permeation studies were conducted by comparing the pilosebaceous compartment-free skin to normal skin of the rat, as represented in Figure 6.

The result indicates that the release flux of MXD in all the formulations (Table 2) was higher from the NS than from the PFS, plausibly due to the presence

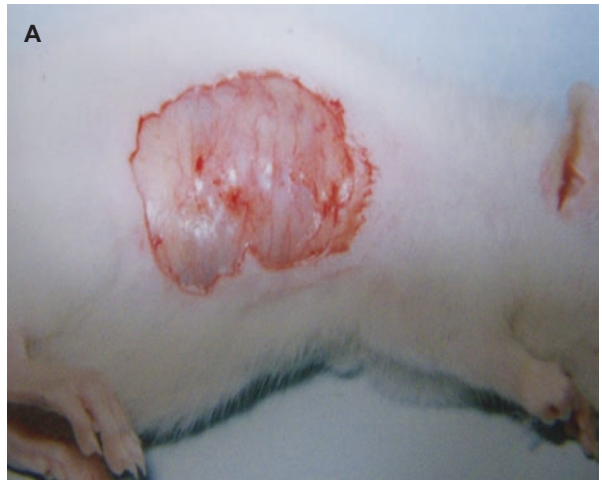

Figure 5A. Photograph of rat skin from abdomen side immediately after the skin removal from that side.

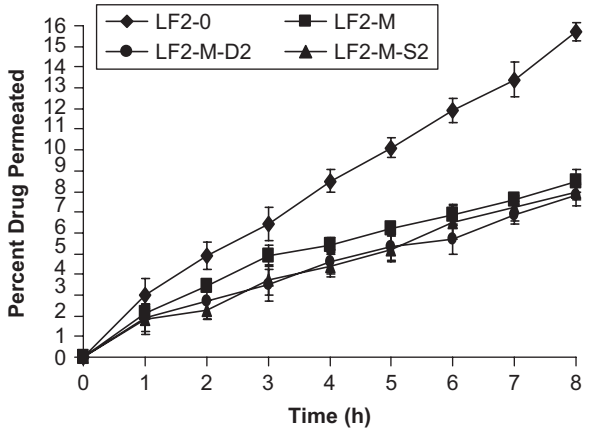

Figure 4. *In vitro* drug-release profile of minoxidil from nonliposomal and various liposomal formulations across the cellophane membrane. Each point represents mean ± standard deviation of three formulations.

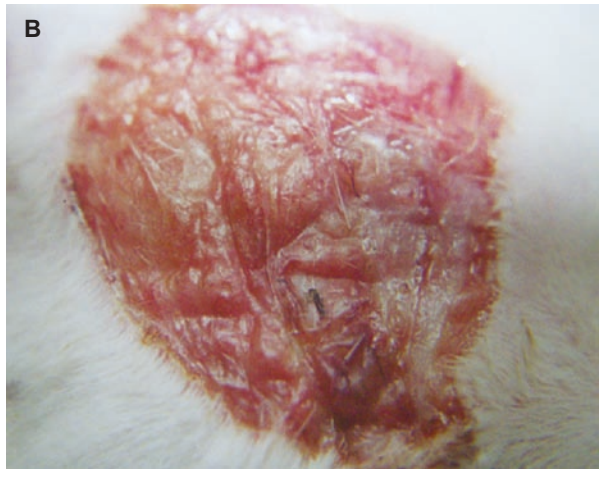

Figure 5B. Photograph of rat skin from abdomen side 15 days after the skin removal from that side.

of hair follicles in the normal skin providing a lane for drug delivery. The PFS, however, is devoid of such hair follicles. The potential role of hair follicles in the percutaneous absorption, observed in the current studies, is in consonance with literature studies (Hoffman, 1998). This indicates that hair follicles act as a promising target for transcutaneous drug delivery.

The results (Table 2) of *ex vivo* studies revealed that the vesicular formulations showed more than 2-fold higher permeation of MXD through NS than through PFS. No significant difference in drug permeation was, however, observed between NS and PFS, using the nonvesicular formulation. This can be ascribed to the fact that the vesicular carriers can make the drug molecules accessible within the skin layers and also increase the action through the transfollicular route. Further, these results also justify the ability of liposomal carriers to facilitate drug delivery for follicular targeting (Vogt et al., 2005).

The surface charge plays an important role in the penetration behavior of a liposomal formulation. This can be corroborated from the current experimental studies on the vesicular formulations (Figure 6), that the drug release was much higher from the positively charged liposomes than from the negatively charged ones. Neutral liposomes, as anticipated, showed the lowest percentage of drug release. Moreover, a positive charge seems to be quite helpful for drug penetration across the NS. This is ostensibly because of the fact that the skin and hair follicles have a negatively charged surface, and the positively charged particles will bind in an ion-exchange manner and, consequently, penetrate in higher concentrations. This perspective might be helpful in attributing lower penetration from the neutral liposomes because of binding to both the follicular ducts and the hair root (Ogiso et al., 2001).

Figure 5C. Photograph of PFS having developed after 45 days.

Determination of drug retention into the PU

Ability of the vesicles in retaining the drug within the skin milieu (i.e., depot effect) was investigated by determining the amount of drug deposited in the

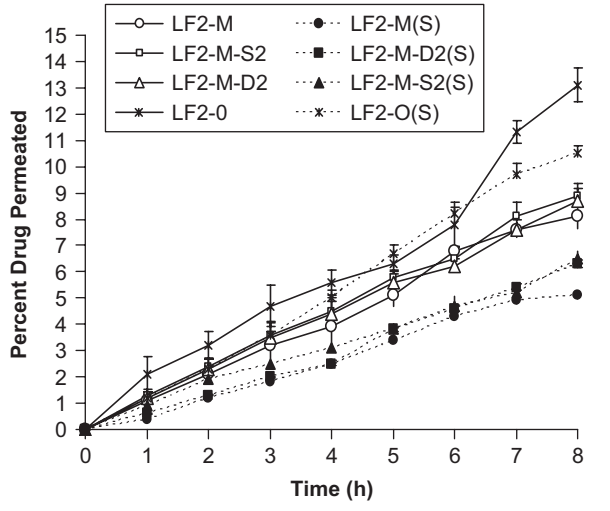

Figure 6. *Ex vivo* drug permeation of minoxidil from nonliposomal and various liposomal formulations through NS (dark lines) and through PFS (dotted lines). Each point represents mean ± standard deviation of three formulations ($n = 3$).

Table 2. Quantitative estimation of minoxidil delivered to pilosebaceous units.

Sample no.	Formulation code	DLD		DRR		DPS		APUS	Ff		APU[1]	APU[2]
		NS	PFS	NS	PFS	NS	PFS		NS	PFS		
1	LF2-S	36.0±0.21	—	7.7±0.15	—	6.3±0.14	—	—	0.32±0.08	—	—	—
2	LF2-M	36.4±0.14	—	6.1±0.03	—	7.5±0.12	—	—	0.25±0.06	—	—	—
3	LF2-L	38.7±0.26	—	5.2±0.24	—	6.1±0.06	—	—	0.21±0.04	—	—	—
4	LF2-M-S2	34.8±0.4	44.0±0.23	8.5±0.15	4.0±0.04	6.7±0.06	2.0±0.04	4.7±0.19	0.35±0.10	0.16±0.11	5.87×10^3	4.7×10^3
5	LF2-M-D2	36.1±0.25	44.4±0.26	8.0±0.17	3.9±0.18	5.9±0.05	1.7±0.04	4.2±0.11	0.33±0.03	0.16±0.14	5.25×10^3	4.2×10^3
6	LF2-M	36.4±0.14	44.5±0.09	6.1±0.03	3.8±0.25	7.5±0.12	1.7±0.01	5.8±0.10	0.25±0.06	0.15±0.10	7.25×10^3	5.8×10^3
7	LF2-O	35.9±0.13	41.6±0.14	10.1±0.29	6.0±0.23	4.0±0.06	2.4±0.03	1.6±0.15	0.42±0.24	0.25±0.18	2.0×10^3	1.6×10^3

Total amount of drug taken in the donor compartment was 50 μg.

DLD, amount of drug (μg) left in the donor compartment after 24 hours; DRR, amount of drug (μg) release after 24 hour (present in the receiver compartment); DPS, amount of drug (μg) present in the skin after 24 hours; APUS, amount of drug (μg) delivered to pilosebaceous units; Ff, flux of formulation (μg $cm^{-2}hr^{-1}$); APU[1], amount of drug in pilosebaceous units (when 800 units); APU[2], amount of drug in pilosebaceous units (when 1,000 units); NS, normal skin; PFS-scar (pilosebaceous-free skin).

skin samples. The diagram (Figure 7) unequivocally depicts that the amount of drug retained in the skin (i.e., containing pilosebaceous units of 1 cm^2) was considerably decreasing in the following order: neutral liposomes (5.8 ± 0.10 μg) > positively charged liposomes (4.7 ± 0.19 μg) > negatively charged liposomes (4.2 ± 0.11 μg) > nonliposomal formulations (1.6 ± 0.15 μg). Higher retention and less permeation of drug in vesicular systems, as compared to nonvesicular formulations can be attributed to intrinsic liposome-skin interaction behavior (Junginger et al., 1991). This observation may be ascribable to the fact that the liposomal PL may mix with the intercellular lipids, thereby causing the swelling of intercellular lipids. These swollen lipids subsequently serve to provide for local accumulation of the drug and the consequent formation of intracutaneous drug depots (Agarwal et al., 2001).

For skin-permeation studies, each square centimeter was taken as surface area, which contained approximately 800–1,000 pilosebaceous units. The amount of drug retained in each pilosebaceous unit, after the application of various formulations was also determined, as reported in Table 2. Results unequivocally show that the formulation composed of neutral liposomes is somewhat better than other formulations.

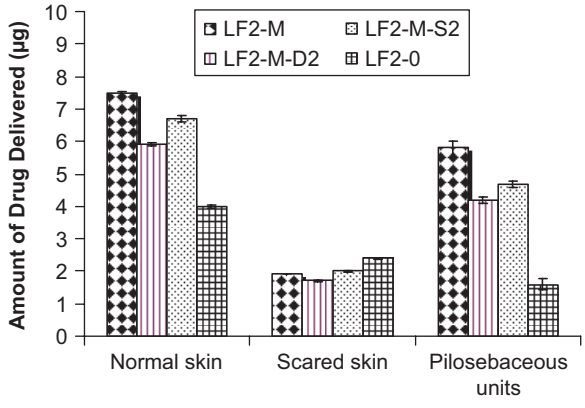

Figure 7. Amount of drug delivered to NS, PFS, and PUS by nonliposomal and liposomal formulations.

Fluoresence microscopy

Fluorescence microscopy was performed to confirm the deposition potential of the developed liposomal formulation in pilosebaceous compartments. A fluorescence photomicrograph (Figure 8) revealed the qualitative targeting of selected liposomes as vesicular drug carriers and their contents by showing the extensive deposition of rhodamine 123 in pilosebaceous units by developed formulation. The results confirmed the better skin penetration and deposition potential of the developed liposomal (LF2-M) formulation.

Stability studies

The stability studies performed on the final liposomal formulation (LF_2-M) indicated that the formulations stored at 4 ± 1°C were more stable than those stored at 37 ± 1°C, as shown in Table 3. However, the vesicular size of liposomes was found to increase during storage, which was attributable to the fusion of vesicles. Increase in size was, however, negligible in the preparation stored at 4 ± 1°C, which indicated vesicle fusogenicity to be a temperature-dependent phenomenon. The study of effect of storage on vesicle fusogenicity revealed that the

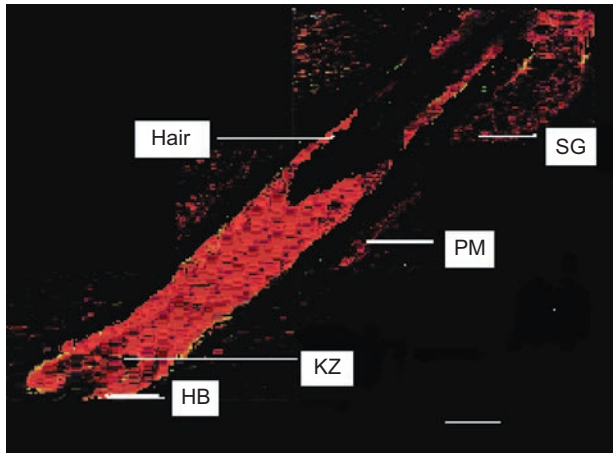

Figure 8. Fluorescent image of rat pilosebaceous compartment after application of rhodamine 123–loaded LF2-M formulation (X400). Scale bar = 10 μm. SG, sebaceous gland; PM, pilosebaceous muscles; KZ, keratogenous zone; HB, hair bulb.

Table 3. Effect of storage conditions and time on vesicle size, vesicle fusogenecity, and residual drug content of optimized liposomal preparation of minoxidil.

	Stability parameters					
	37 ± 1°C			4 ± 1°C		
Time points	Vesicle size (μm)	FG (no. × 10^3)	RDC (%)	Vesicle size (μm)	FG (no. × 10^3)	RDC (%)
Freshly prepared	3.83 ± 0.18	61	100	3.83 ± 0.18	61	100
After 10 days	4.06 ± 0.28	49	87 ± 1.2	3.97 ± 0.31	58	99 ± 1.4
After 20 days	4.34 ± 1.12	37	81 ± 1.8	4.12 ± 1.27	54	96 ± 1.5
After 30 days	4.93 ± 1.26	21	76 ± 1.4	4.36 ± 1.17	52	93 ± 1.9

FG, fusogenecity (i.e., number of vesicles per cubic millimeter (no. × 10^3)); RDC, residual drug content (%).

number of vesicles decreased considerably when formulations were kept at 37 ± 1°C, as compared to the identical formulation stored at 4 ± 1°C. The LF_2-M formulation was found to be stable, with a little decrease in number of vesicles after 30 days. A significant proportion of the drug (i.e., 20–25%) (assuming initial drug content to be 100%) was lost from the vesicular formulation in 30 days, when stored at 37 ± 1°C. However, the loss of drug from the formulation stored at 4 ± 1°C was relatively much lower (i.e., only 5–7%). Thus, the stability studies carried out at various temperatures indicated a relatively much better stability of the formulation at lower temperatures.

Conclusions

The current studies establish a methodology to study the quantitative targeting of different liposomal drug carriers into the pilosebaceous units. Results of the study indicate that optimal vesicle size is essential for the optimal targeting of MXD to pilosebaceous units. The better ability of the vesicular systems to facilitate the delivery of the therapeutic agents into hair follicles, as compared to non-vesicular systems, was also confirmed. Liposomes with a positive surface charge showed the highest efficiency of follicular penetration, as compared to the negatively charged and neutral liposomes. Nevertheless, the retention ability toward pilosebaceous units was the highest in the case of neutral liposomes. Overall, the results of the present investigations suggest that MXD-loaded neutral liposomes, having selective PU drug-targeting potential, may have profound therapeutic applications in improving hair growth and treating alopecia.

As we have gotten potential results to pursue our studies, the next step in continuation will be to prepare various dosage formulations of optimized liposomal systems and compare with conventional MXD solution to study the pharmacological effect of liposomal delivery of MXD on hair growth.

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