Review

Topical Liposome Targeting of Dyes, Melanins, Genes, and Proteins Selectively to Hair Follicles

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For therapeutic and cosmetic modification of hair, we have developed a hair-follicle-selective macromolecule and small molecule targeting system with topical application of phospholipid-choline-based liposomes. Liposome-entrapped melanins, proteins, genes, and small-molecules have been selectively targeted to the hair follicle and hair shafts of mice. Liposomal delivery of these molecules is time dependent. Negligible amounts of delivered molecules enter the dermis, epidermis, or bloodstream thereby demonstrating selective follicle delivery. Naked molecules are trapped in the stratum corneum and are unable to enter the follicle. The potential of the hair-follicle liposome delivery system for therapeutic use for hair disease as well as for cosmetics has been demonstrated in 3-dimensional histoculture of hair-growing skin and mouse in vivo models. Topical liposome selective delivery to hair follicles has demonstrated the ability to color hair with melanin, the delivery of the active lac-2 gene to hair matrix cells and delivery of proteins as well. Liposome-targeting of molecules to hair follicles has also been achieved in human scalp in histoculture. Liposomes thus have high potential in selective hair follicle targeting of large and small molecules, including genes, opening the field of gene therapy and other molecular therapy of the hair process to restore hair growth, physiologically restore or alter hair pigment, and to prevent or accelerate hair loss.

INTRODUCTION

There is an important need to find improved methods to directly influence hair growth, color, appearance, and removal. The existing approaches are expensive, labor-consuming and relatively short-lasting (carcinogenic dyes, color, and surgical transplantation), or relatively low-efficient (the use of different creams and lotions with biologically-active substances).

The use of biologically-active modulators of hair color and growth seems to be the more natural and attractive approach, especially at the stage when hair-follicle cells still exist but hair growth, for
unknown reasons, is affected. The relative low efficacy of this approach can possibly be explained by the inability of modulators to penetrate the cellular membrane of hair-follicle cells and to enter inside cells where their action is needed.

Liposomes, which are artificial phospholipid vesicles, have been successfully used for the delivery of various low- and high-molecular-weight water-soluble and oil-soluble compounds into different cell types (Gregoriadis, 1988; Schmidt, 1986; Lasic and Barenholz, 1995). We review here our development of the use of liposome-mediated targeting of molecules into hair follicle cells. This was initially made possible by us by first developing skin histoculture as a discovery system.

Long-term histoculture (Leighton, 1951; Hoffman, 1991) of whole human and mouse skin was developed by utilizing a collagen-sponge-gel-supported in vitro three-dimensional histoculture system, such that long-term effects of various agents can be studied with regard to toxicity, penetration and hair growth (Li, et al., 1991).

We have been able to culture both white (Li, et al., 1992a) and black mouse skin (Li, et al., 1992b) with hair growing for periods of at least 10–15 days respectively, and human scalp tissue with hair follicle cells proliferating for at least 40 days (Li, et al., 1992c). We have been able to determine cytotoxicity and viability with fluorescent dyes identifying dead and living cells, including cells in hair follicles, three-dimensionally in the histocultured skin in the living state, in particular with confocal microscopy (Li, et al., 1991).

A key aspect of the intact skin histocultured in our system is the presence of the major types of cells, including hair follicles, as well as growing hair.

The histoculture model for skin may be an effective replacement for animal systems and superior to the dispersed and reconstituted skin equivalent cell system used previously (Naughton, et al., 1989) for measurements of the effect of manufactured products, drugs, and pollutants on skin and hair growth.

The use of three-dimensional histoculture in conjunction with confocal microscopy opens the opportunity to follow fine details of product-delivering liposome interactions with hair-follicles at the cellular level. As a result, optimal liposome compositions can be established as well as the conditions for the delivery of liposomal contents into target cells.

We review here the specific affinity of product-delivering liposomes for hair follicles in histocultured skin as well as in vivo in mice.

LIPOSOME TARGETING OF DYES TO HAIR FOLLICLES

Pieces of shaved outbred white-haired-mouse or nude-mouse skin (appro., 2 × 5 × 2 mm) were harvested under a dissection microscope and then histocultured on collagen-gel supported sponge as described earlier (Li, et al., 1991). Liposome interaction with the skin was initiated after 24 hours of histoculture.

Liposomes were prepared by sonication of phosphatidylcholine (PC)(25 mg) emulsion in phosphate buffered saline (PBS) containing the fluorescent dye calcine (20 mg/ml). Liposomes were separated from the non-entrapped dye by gel-filtration on a Sepharose 4B column diluted with phosphate buffered saline (PBS). The amount of the entrapped dye was measured spectrophotometrically. Two types of PC were used: egg PC (EPC) and dipalmityloyl PC (DPPC). Due to their phase transition temperatures, liposomes made of DPPC are in a gel phase at 37°C while liposomes prepared from EPC are in a liquid-crystalline state.

Mouse skin histocultures were incubated for 20 minutes with liposomes or with a solution of “free” calcine dye at the same concentration as was in the liposome preparation. After the tissues were thoroughly washed, the specimens were analyzed with a Biorad MRC 600 laser confocal microscope with a BHS filter block, which excites the tissue at 488 nm and passes the light emitted at 520. These parameters are close to the excitation and emission maxima reported for calcine (Haugland, 1989). It is also important to note, that there is no autofluorescence of tissue when these emission and excitation wavelengths are used.
The histocultured skin incubated with free calcine solution exhibited relatively low fluorescence with no preferential staining of any particular skin structure. Liposome-entrapped dye in striking contrast to free dye becomes associated with hair follicles (Li et al. 1992d).

In order to understand whether liposomes are preferentially targeting hair follicles, we have used gel-phase liposomes, which are known for their stability in living systems. These liposomes also become associated preferentially with hair follicles both when liposomes were loaded with calcine or when the liposomes were fluorescently labeled with NBD-phosphatidylethanolamine (Li et al., 1992d).

Based on the results of the experiments, we concluded: 1) liposomes seem promising as a preferential delivery vehicle to hair follicles; 2) depending on the goal, different type of liposomes can be used to deliver entrapped substances into the follicle or to its surface; 3) confocal laser microscopy is a highly efficient instrument to study hair follicles in the histocultured skin specimens stained with fluorescent dyes; and 4) the histoculture-liposome system coupled with confocal scanning laser microscopy and other means of analysis such as measurement of hair growth itself (Li et al., 1992a, 1992c) could serve as an effective screen for agents which perturb the hair cycle such as hair-growth stimulators.

The liposome delivery of calcine to hair follicles in vivo has also been achieved (Li and Hoffman, 1997). When calcine entrapped in phosphatidylethanolamine (PC) liposomes is topically applied to mice, the calcine entered deeply into the hair follicle cells as well as the hair shafts within the follicle. In striking contrast, topically-applied naked calcine, which is a polar, negatively-charged compound, could not enter below the stratum corneum. Indeed the naked dye seemingly was trapped in the stratum corneum.

A time course of entry of liposome-entrapped calcine into the skin of the mice was carried out. By 24 hours, 22 ng/mm² of calcine was delivered when the calcine was entrapped in the liposomes. The amount of liposome-delivered calcine increased with time. In striking contrast less than 1.4 ng/mm² of naked calcine was delivered and this amount did not increase with time (Li and Hoffman, 1997).

Over the 24-hour period of the experiment, no detectable calcine entered the blood circulation when applied topically in liposomes. This is a very important result in that it shows a molecule, in this case calcine, can be delivered to the skin without entry to the systemic circulation thus indicating that safe follicle delivery of a product is possible (Li and Hoffman, 1997).

Our results were confirmed by Lieb et al.'s studies (1992) demonstrating that liposomal formulations are more efficient than any other vehicle tested for delivery of carboxyfluorescein through the follicular route into the pilosebaceous units in the hamster ear model.

LIPOSPHERE TARGETING OF MELANIN TO HAIR FOLLICLES

Liposome-mediated targeted delivery of melanin into hair follicles and the hair shaft itself in histocultured white-haired mouse skin has been carried out. Liposomes were prepared by sonication. 20 mg of egg phosphatidylethanolamine (PC) were rotary evaporated from a chloroform solution to form a thin film on the walls of a 5 ml round-bottomed flask for 1 hour. The dried thin film lipid was suspended in 0.5 ml phosphate buffered saline (PBS, pH 7.4) on a vortex mixer and then sonicated with a Branson probe-type sonicator fitted with a microtip at power level 3, for 8 minutes. Then 0.5 ml melanin solution (10 mg/ml) was added and the melanin was entrapped into the liposomes by sonication for an additional 4 minutes. Liposomes were separated from the non-entrapped melanin by gel-filtration with a Sepharose 4B column diluted with phosphate buffered saline (PBS).

Pieces of outbred white-haired-mouse skin derived from 1-2 week-old animals (approx. 2 x 5 x 2 mm) were harvested under a dissection microscope and then histocultured on collagen-gel supported sponges. Liposome interaction with the skin was initiated after 24 hours of histoculture.
Mouse skin histocultures were incubated for 12 hours with liposomes. A solution of “free” melanin, at the same concentration as was used in the liposome preparation, served as the control and was also incubated for 12 hours with the histocultured skin. The skin histocultures were counter-stained with (BCECF-AM). After the tissues were thoroughly washed, the specimens were analyzed with a Nikon fluorescence microscope equipped with a fluorescein cube. Microscopically the live tissues and cells fluoresced green such that melanin localized in the tissues could be clearly identified. All skin samples were then fixed with formalin and processed through dehydration, paraffinization, paraffin embedding and hematoxylin and eosin staining.

Under the fluorescence microscope, the majority of the melanin was localized around the hair follicles (Li et al., 1993a). The melanin could be both observed at the periphery of follicles and in the follicle cells themselves. As a control, the skin histocultures were also incubated with “free” melanin at the same concentration used in preparing the liposomes. No “free” melanin could be seen to be localized in the follicles.

In order to substantiate the results obtained with fluorescent microscopy, the skin histocultures were processed for histological studies. The liposome-entrapped melanin was seen to be transferred into the follicular cells. The liposome-entrapped melanin was also delivered into the hair shaft itself to form the band-like melanin-distribution pattern in the terminally-differentiated keratinocytes of the typical normal mouse pigmented hair shaft (Li et al., 1993a). In the control, in which the skin histocultures had been incubated with the “free” melanin, no “free” melanin could be observed either in hair shafts or the hair follicular cells.

Thus we have demonstrated that liposomes can specifically target an important molecule, in this case melanin, to hair follicles in histocultured mouse skin and even enter the hair shaft itself in a normal pattern. Our results demonstrated the great potential for liposome-targeting of melanin to hair follicles to modify the pigment of the hair follicle and hair shaft itself.

Liposome delivery of melanin to hair follicles in vivo was also carried out by topical application (Li and Hoffman, 1997). Liposome-entrapped melanin, topically applied to the mouse skin, entered the hair follicle as well as the hair shaft. Melanin could enter the follicle deeply and the hair shaft. Naked melanin topically applied to the mouse skin could not enter below the stratum corneum and indeed appeared to be trapped in the stratum corneum.

A time course study was done on liposome melanin delivery into the skin. By 16 hours, 19 μg/mm² of melanin was delivered to the mouse hair follicles, when the melanin was entrapped in the liposomes. In striking contrast less than 2 μg/mm² of naked melanin was delivered and this amount did not increase with time.

Thus liposomes can deliver melanin to the hair follicle in vivo. Histological preparations also demonstrated that the liposome-targeted melanin was delivered to the hair follicle cells and hair shafts. The liposome-delivered melanin acquired the exact pattern that occurs in naturally melanized mice.

LIPOsome TARGETING OF HIGH MOLECULAR WEIGHT DNA TO THE HAIR FOLLICLES OF HISTOCULTURED SKIN

A number of years ago we developed the technique of entrapping DNA in liposomes (Hoffman et al., 1978). We have utilized DNA liposomes to target high molecular weight DNA to the hair follicle itself as a model of gene therapy of the hair growth processes (Li et al., 1993a).

A one kb DNA fragment was isolated from a mouse genomic DNA library. 50 ng of DNA was labeled with [35S]dATP (DuPont) with the Random Primer DNA Labeling Kit (BioRad, Richmond, VA). The specific activity of the labeled DNA with [35S]dATP was 2.6 × 10⁸ cpm/μg.

Liposomes were prepared by freezing and thawing. 20 mg of egg phosphatidylethanolamine (PC) were rotary evaporated with a vacuum drier from a chloroform solution to form a thin film on the walls of a 5 ml round-bottomed flask for 1 hour. The
TABLE 1  Liposome Transfer of $^{35}$S]DNA to hair follicles of histocultured skin (Li, et al., 1993b)

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<th>Lipo-$^{35}$S]DNA</th>
<th>Naked-$^{35}$S]DNA</th>
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<tr>
<td>Percent of labeled follicles per 20 x field</td>
<td>37.50 (6/16)</td>
<td>2.41 (2/37)</td>
</tr>
<tr>
<td>Percent of labeled cells per follicle</td>
<td>51.06 (24/47)</td>
<td>9.30 (4/43)</td>
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dried thin film lipid was suspended in 0.5 ml phosphate buffered saline (PBS, pH 7.4) on a vortex mixer and then sonicated with a Branson probe-type sonicator fitted with a microtip at power level 3, for 8 minutes. 0.5 ml of $^{35}$S]dATP-labeled DNA solution was entrapped with the above suspension by extensive vortexing for 1 minute followed 3 times by freezing and thawing. Liposomes were separated from the non-entrapped $^{35}$S]dATP by gel-filtration on a Sepharose 4B column diluted with PBS. 50 $\mu$l of Calcein (10 mg/ml) was added into the above solution in order to mark the liposomes during the separation. The specific activity of entrapped DNA labeled with $^{35}$S]dATP was $2.5 \times 10^{10}$ cpm/$\mu$l measured by liquid scintillation counting.

Pieces of outbred white-haired mouse skin (approximately 2 x 5 x 2 mm) derived from 1-5 week-old animals were harvested under a dissection microscope and then histocultured on collagen-gel-supported sponges. Liposome interaction with the skin was initiated after 24 hours of histoculture. Mouse skin histocultures were then incubated subsequently for 44 hours with liposomes. A solution of naked-$^{35}$S]DNA at the same concentration was used in the liposome preparation to serve as the control and was also incubated with skin histocultures.

The skin histocultures were then fixed with formalin and processed through the standard procedure of dehydration, paraffin-embedding and sectioning. The slides then were prepared for autoradiography (Li, et al., 1991).

Histological autoradiography showed $^{35}$S]DNA-labeled hair and follicle cells in histocultured skin after the histocultured skin was incubated with the DNA liposomes for 44 hours. High radioactive labeling by the $^{35}$S]DNA in the cell membranes and cell cytoplasm as well as in the cell nucleus was seen. This suggests that the liposomes have delivered the DNA across the cell membrane and the DNA is eventually transported through the cytoplasm to the nucleus.

When the histocultured skin was treated with naked-$^{35}$S]DNA, there were only a few radioactively-labeled cells. For further comparison, we have calculated from the autoradiograms the percent of labeled follicles per 20 x field and the percent of labeled cells per follicle in the areas of maximum labeling. Both the percent of labeled follicles per 20 x microscope field and the percent of labeled cells per follicle in liposome-$^{35}$S]DNA-treated skin histocultures were significantly higher than in naked-$^{35}$S]DNA treated histocultures (Li, et al., 1993b).

Naked $^{35}$S]DNA was used as a control. Follicular $^{35}$S]DNA was analyzed by histological autoradiography as described in the text in the areas of maximum labeling.

LIPOsome TARGETING OF THE ACTIVE
lac-Z GENE TO HAIR FOLLICLES OF
HISTOCULTURED SKIN

The capability of liposomes to target DNA to hair follicles suggested that liposomes could target active genes to the hair follicles. We then demonstrated liposome targeting of the active bacterial lac-Z gene to hair follicles of histocultured mouse skin (Li and Hoffman, 1995a).

A recombinant retrovirus containing the lac-Z gene (pM-MuLV-SV-lac-Z) was obtained from Dr. Joshua R. Sanes (Washington University Medical Center, St. Louis, MO). The lac-Z plasmid was transfected into HB101 E. coli competent cells (Promega, Madison, WI) by standard methods. The purification of lac-Z plasmid DNA was carried out with the Promega Wizard™ Maxiprep DNA purification system.
A total of 20 mg of lipid in a ratio of 5:3:2 of phosphatidylcholine (PC), cholesterol (Chol); phosphatidylethanolamine (PE) were rotary evaporated for 1 h with a vacuum drier from a chloroform solution to form a thin film on the wall of a 5 ml round-bottomed flask. The dried thin film lipid was suspended in 2 ml phosphate buffered saline (PBS, pH 7.4) on a vortex mixer and then sonicated with a Branson probe-type sonicator fitted with a microtip at power level 3, for 8 minutes. Then 300 µg of the plasmid lac-Z DNA were entrapped in the above suspension by water bath sonication for 3 minutes followed by freezing and thawing three times.

Pieces of outbred white-haired-mouse skin (approximately 2 x 5 x 2 mm) derived from 2- to 3-week-old animals were harvested under a dissection microscope and then histocultured on collagen-gel-supported sponges. Liposome interaction with the skin was initiated after 24 h of histoculture. Mouse skin histocultures were then incubated with liposome-lac-Z for 4 d. A solution of naked-lac-Z DNA at the same concentration that was used in the liposome preparation served as the control and was also incubated with skin histocultures.

The histocultured skin samples were first fixed in 2% (vol/vol) formaldehyde-0.2% (vol/vol) and glutaraldehyde in PBS for 30 min at 4°C. Then the tissues were rinsed with PBS three times and incubated in the X-gal staining solution containing 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl₂ in PBS, at 37°C for 18 h. Skin tissues were directly observed under light microscope.

Histocultured skin samples were then evaluated using light microscopy at 125 x and 250 x magnification. The presence of the expressed lac-Z gene was only seen in the histocultures which received liposome-entrapped plasmid. The control treated with the naked lac-Z gene did not incorporate the active gene. Furthermore, liposome-delivered lac-Z was observed in the hair follicles and was not significantly observable in the tissues adjacent to the hair follicles.

The results showed that the liposome-targeted lac-Z gene was expressed in hair follicles and was not detectable in the other portions of the histocultured skin sample, indicating the hair-follicle selectivity of the liposome delivery method (Li and Hoffman, 1995b).

**TOPICAL LIPOsome TARGETED GENE DELIVERY TO THE HAIR FOLLICLE OF MICE**

Experiments were then carried out to target the lac-Z gene to the hair follicles in mice after topical application of the gene entrapped in liposomes (Li and Hoffman, 1995b). Preslaved 5- to 6-week-old BALB/c mice were used. The skin area for application of liposome-lac-Z was prehydrated with phosphate-buffered saline (PBS) for 10-30 minutes. Liposome-lac-Z formulation (50 µl) was placed directly on the skin with re-application after 1 hr. Untreated mice and mice treated with naked lac-Z DNA were used as controls. The skin was carefully cleaned by 70% isopropyl alcohol before harvest for X-gal staining 3 days after application of the liposome-lac-Z formulation.

The harvested skin samples were immediately put into a modified Eagle's medium (MEM) wash containing a combination of antibiotics at 4°C for 1 h and then fixed in 2% (vol/vol) formaldehyde-0.2% (vol/vol) and glutaraldehyde in PBS for 30 minutes at 4°C. Tissues were then rinsed with PBS three times and incubated in the X-gal staining solution containing 1 mg ml⁻¹ X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl₂ in PBS, at 37°C for 18 h. Skin tissues were processed for paraffin sectioning by standard histological procedures and photographed under light microscopy after counter-staining with 0.1% nuclear fast red.

After topical application of liposome-lac-Z, expression of the lac-Z gene, indicated by blue staining of the X-gal substrate, was in the hair-forming hair matrix cells in the hair follicle bulbs and in the bulge area below the opening of the sebaceous gland (Li and Hoffman, 1995b). The transfection frequency was high as many follicles were stained.
No other cells were transfected with lac-Z outside the follicle in the dermis or epidermis. Extensive lac-Z expression in the hair matrix cells was shown. Topical application of the naked lac-Z gene did not result in gene transfer, and no lac-Z staining was seen in follicles in animals not treated with liposome-lac-Z.

These results demonstrate that genes can be selectively targeted to the most important cells of the hair follicle by liposomes representing the most selective targeting of a gene observed thus far in vivo. This selectivity of gene targeting by topical liposome application suggests the feasibility of targeting hair matrix cells and possibly follicle stem cells to restore hair color perhaps by delivery and expression of the tyrosinase gene (Bouchard et al. 1989, Shibahara et al. 1988, Tanaka et al. 1990) and in the future, delivery of genes to restore hair growth. The highly selective nature of the topical liposome gene targeting lends itself to the development of practical and safe procedures.

Recently, Alexander and Akhurst (1995) used cationic DOTAP liposomes to topically deliver the lac-Z gene to mice. In telogen skin, they found expression predominantly in the hair follicle, confirming our observations in telogen mice. However, in anagen-skin, gene expression was found in the hair follicle as well as outside the hair follicle. This could be due to reduced selective targeting by cationic liposomes, the use of a chemical depoliarizing agent which permeabilized the stratum corneum, and/or a difference between anagen and telogen skin.

**TOPOCAL LIPOsome TARGETED PROTEIN DELIVERY TO THE HAIR FOLLICLES**

Recently, Yarosh et al. (1992, 1994) demonstrated that liposomes can deliver the DNA repair protein T4 endonuclease V to the skin where it stimulated the removal of UV-light induced cyclobutane pyrimidine dimers with concomitant reduction of squamous-cell carcinomas in mice. Yarosh demonstrated that hair follicles were targeted by the endonuclease-containing liposomes. Balsari et al. (1994) also reported that liposomes can deliver monoclonal antibodies into hair follicles of rats for protection against doxorubicin-induced alopecia. Yarosh (1994) also reported very little entry into the blood after topical liposome delivery of the T4 endonuclease, demonstrating the safety of the procedure.

**CONCLUSION**

A major question is how have the liposome-entrapped molecules reached the hair shafts? One hypothesis is that when liposomes are topically applied on the skin, the liposomes penetrate via a "lipid-rich channel" coating the hair follicles with subsequent entry into the hair follicle keratinocytes. We believe there are two ways of liposome-entrapped molecules reaching the hair shafts. One is that the liposomes first deliver the molecules into the hair matrix cells, then the molecules move to the hair shafts by differentiation and hair growth. Second is that the liposomes may directly penetrate into the hair shafts from the tip of the hair shafts.

In our most recent experiments (unpublished data), we have shown that liposomes can also deliver calcine into hair follicles in histocultured human scalp tissue without detectable calcine found in non-follicles sites. These data demonstrate that the liposome delivery to hair follicles is effective in human skin (L.L. and Hoffman, R.M., unpublished data).

Thus the liposome hair-follicle delivery system has great potential to test the effect of drugs and genes on the hair follicle and on the hair process for cure of hair diseases and cosmetics without significant systemic side effects.

**References**


