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The role of liposomes and their future perspective

Keywords: Liposomes, drug carrier system, penetration, surface modification

Abstract

Liposomes have been extensively studied and suggested as a vehicle for topical drug delivery systems in cosmetics and pharmacy. To clarify the effects of different modifications of liposomes (surface charge, sterical stabilisation, varying solvents, dependence on phase transition temperature) on the percutaneous absorption, we determined the penetration profiles of encapsulated components into the skin (6 hours application). For the measurements both a hydrophilic fluorescent compound [5(6)-Carboxyfluorescein = CF] and a lipophilic one [1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate = DiI] were encapsulated into the vesicles. Liposomal formulations were prepared by extruding the vesicles through polycarbonate membrane filters. *In vitro* penetration studies into human abdominal skin were performed by using Franz diffusion cells and a non-occlusive application. Confocal laser scanning microscopy (CLSM) was used to visualize the effect of the penetration ability of encapsulated dyes. After 6 hours, it was clear that liquid-state liposomes (negatively charged) showed a high fluorescence intensity in the deeper skin layers in contrast to the positive charged ones. A sterical stabilisation of the vesicles by PEG-coating prevented the liposomes from entering the skin. Also the solvent showed an important influence on the percutaneous absorption, good penetration efficacy could be obtained with either ethanol or pentylene glycol but no penetration into the deeper viable skin layer was observed with liposomes in a propylene glycol/water solution. Liposomes composed of DMPC (1,2-Dimyristoyl-glycero-3-phosphocholin) (phase transition at 24 °C, gel-state liposomes at RT but liquid at skin temperature) show only a slight penetration into the stratum corneum (SC). Several factors in the modification of liposomal formulations play an important role in improving or hindering the percutaneous absorption of the vesicles.

Introduction

A broad range of topical products with active ingredients is used in cosmetics as well as in pharmacy. The efficacy of many of these products has been enhanced by the encapsulation of the actives in liposomes for better penetration into the skin.

The skin's barrier function is accomplished, entirely and quite remarkably, by the outermost few microns of skin – the stratum corneum (SC), a compositionally and morphologically unique bio-membrane. This extremely thin (approx. hundredth of a mm), least permeable of skin layers is the ultimate stage in the epidermal differentiation process, forming a laminate of compressed keratin-filled corneocytes anchored in a crystalline state lipophilic matrix consisting of non polar lipids (ceramides, free fatty acids and cholesterol). The staggered corneocytes arrangement in a lipid continuum (similar to a brick and mortar assembly) is suggested to bestow a highly tortuous lipoidal diffusion pathway rendering the membrane 1000-times less permeable to water relative to most other biomembranes. The stratum corneum contributes over 80 % to the transport resistance of actives into the skin – it is hence very difficult to bring molecules greater than 200 – 350 Dt efficiently across the intact SC and the same task for molecules > 750 Dt is all but possible. (1), **(Fig. 1)**.

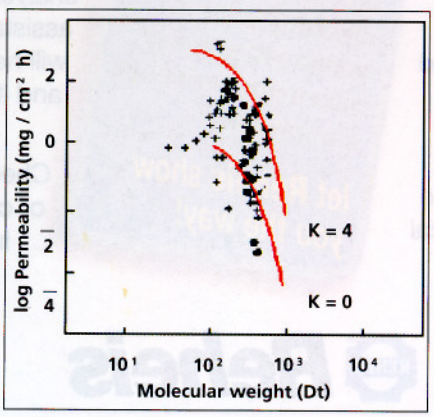

Fig. 1 Penetration of molecules through the skin

In the 80ies the first reports of the potential use of liposomes carrying active ingredients for topical applications were published (2-4) and in 1987 the first liposomal product entered the cosmetic market (Dior with Capture). Since then a continuous increase in the usage of liposomes could be noticed (US sales of advanced drug delivery systems, liposomes: 1996 = 117 millions of $US and 1997 = 298 millions of $US). Liposomes are microscopic small vesicles (hollow spheres), consisting of one or several lipid bilayers that surround a watery nucleus. The use of these phospholipid (PC, phosphatidyl-choline) vesicular systems to deliver active agents to the skin has recently received much attention: liposome encapsulation can overcome various problems commonly encountered in the delivery of biologicals such as toxicity, solubility, stability and bioavailability of the component to be delivered.

Very few papers were published, describing the modification of phospholipid vesicles to improve their ability to penetrate the skin: like charge modification (5) or the role of the size of liposomes (6) or the adding of edge-active molecules like bile salts (7).

The aim of this study was therefore to investigate the influence of modifying the vesicle structure or liposomal for-

mulation in order to find the best combination for topical drug delivery into the skin. The effects of differently charged liposomes on the skin penetration were determined – also the influence of sterical stabilisation of liposomes was studied as well as the usage of different solvents for the liposomal preparations. Confocal laser scanning microscopy (CLSM) was used to visualise the efficacy of penetration of fluorescently labelled vesicles.

Formulation	Composition	Size (nm)	Charactristics
PL 90 + DC	(9:1 mol/mol)	132	negatively charged liposomes: (- 47 mV)
PL 90 + SA	(9:1 mol/mol)	128	positively charged liposomes (+ 35 mV)
DMPC		125	phase transition (24 °C)
PL 90		134	phase transition (< 0 C°)
PL 90 / EtOH	(20 vol%)	138	
PL 90 / Propylene Glycol	(20 vol%)	165	
PL 90 / Pentylene Glycol	(20 vol%)	397	
PL 90 + PE-PEG	(9:1 mol/mol)	88	sterically stabilised liposomes

Table 1 Formulations of liposomes used for the penetration studies

Material and methods

Material

Phospholipon 90 (PL 90) was purchased from Nattermann, Germany:
Lipids like DMPC (1,2-Dimyristoyl-glycero-3-phosphocholine) and PE-PEG (1,2- Dipalmitoyl-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol-2000)] were from Avanti Polar Lipids, USA. Stearylamine (SA) and Dicetylphosphate (DC) were obtained from Sigma.
CF (5(6)-Carboxyfluorescein) and DiI (1,1'-Dioctadecyl-3,3,3',3'-tetramethyl-indo-carbocyanine perchlorate) were purchased from Sigma-Aldrich Chem. Co. USA. 1,2 pentandiol was from Symrise, Germany. All other chemicals like 1,2 propanediol were obtained from Fluka GmbH, CH. The water used was double distilled, deionized and filtered with a Milli-Q system. Mini extruder Liposofast was from Avestin, Canada. The cryotome was from Vogel Cryotome AS 620 (Anglia-Scientific, U.K.) and the CLSM-Unit (Zeiss-Axiovert 100 – BioRad MRC 1024).

Liposomes preparation and characterisation

Liposomes were prepared as follows: lipids (100 mg/ml) and DiI (50 µg/ml) were dissolved in ethanol or other polar solvents (propylene glycol, pentylene glycol). Then 0.1mM CF-solution was slowly added to this mixture and stirred. These spontaneously formed multilamellar vesicles (MLV) were pressed through a 200 nm pore size poly- carbonate membrane to get unilamellar liposomes with the help of Avestin extrusion device.
The diameter of vesicles and the zeta-potential were determined by a Zetamaster instrument (Malvern Instruments, UK).
Table 1 shows formulations of liposomes were used for the penetration studies.

The skin

Excised human skin from a male patient, who had undergone abdominal plastic surgery, was used. Immediately after excision the subcutaneous fatty tissue was removed using a scalpel. The skin was wrapped in aluminium foil and stored in polyethylene bags at -25 °C until use. Under these conditions the skin is stable with regard to the penetration of drugs as well as to the thickness of the stratum corneum over a time period of 3 and 6 months, respectively (8,9). For penetration experiments, skin disks of 25 mm in diameter were punched out, thawed, cleaned with cotton, which was soaked with Ringer solution, and transferred onto the Franz diffusion cell.
On the Franz diffusion cell (Gauer Glas, Püttlingen, Germany) the epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed by phosphate buffer saline pH 7.4. To achieve higher reproducibility the skin was pre-hydrated with the basolateral receptor medium for 30 minutes before applying the formulation. The Franz diffusion cells were put into a temperature controlled water bath (37 °C) to maintain the skin temperature constant for the whole period of the penetration experiment. The duration of the penetration studies was 6 hrs. Twenty µl of each liposomal preparation containing CF and DiI were non-occlusively applied per 2 cm² onto the skin surface.
For controlling purposes, a solution of CF and DiI in water / ethanol (8:2 w/w) was investigated in the same matter.

Pentration study

After incubation for a predetermined time period, the liposomal formulation was removed by wiping the skin with cotton and cross-sections (20 µm thickness) were made by the help of a cryotome. The fluorescence of both dyes in different skin layers were visualised by CLSM.

Results

Penetration of liposomes in dependence of phase transition of the vesicles

Conventionally used liposomes are prepared of soya PC (phosphatidylcholine) and are in a liquid state at

room temperature. Compared to that, DMPC has a phase transition temperature of 24 °C (RT) and should change into the liquid state at skin temperature (32 °C). But in contrast to PL 90 liposomes, the DMPC-vesicles showed only a penetration ability into the SC and much less fluorescence could be observed in deeper skin layers **(Fig. 2)**. Changing to liposomes prepared of hydrogenated C18-PC (phase transition >50 °C), former observations only showed fluorescense on the top of the skin (10, 11). These results indicate that there might be a relation between the depth of penetration and the phase transition temperature of liposomes. This could be of interest when achieving a specific drug targeting to special layers of the skin (i.e. immune stimulation of Langerhans cells).

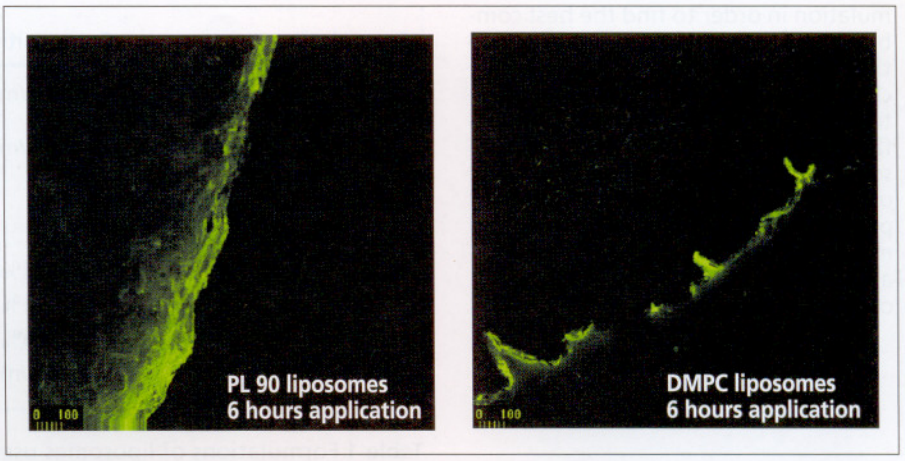

Fig. 2 Penetration of encapsulated CF into human skin

Penetration in dependence of charge of liposomes

The penetration of liposomes with a charged surface is controversially discussed. On the one hand, *Yu* (12) described a significantly increased penetration of triamcinolone acetonide into rat skin by negatively charged liposomes and on the other hand, *Montenegro* (13) reported an enhanced delivery of retinoic acid into the skin by positively charged vesicles.

Our findings clearly indicate that negatively charged liposomes carry the encapsulated fluorescence dyes to a much higher extent into the deeper skin layers. The CLSM images show nearly identical fluorescence pictures of the applied control solution (CF + DiI in water / ethanol 8:2) and the positively charged liposomes **(Fig. 3)**.

These results are in accordance with the observation made by *Ogiso* (14), who found that formulations with negatively charged liposomes generally showed an enhanced delivery of drugs through rat skin compared to positively charged ones. The positively charged liposomes accumulated in the upper layer of the SC which is probably due to the interaction of the charged vesicles with the negative charge of the intercellular lipids (like free fatty acids).

Penetration of liposomes in dependence of the solvent

It is postulated that the transepidermal water gradient results in fields enforcing a lipid flow into or through the intact skin surface provided that lipids are applied in the form of special vesicles (15). These vesicles have to be in a liquid state, so that they consist of a flexible membrane, which enables the liposomes to enter the skin. When such a kind of liposomal dispersion is occlusively applied onto the skin, the water gradient breaks down and no penetration can be observed (15). Evaporation of the solvent of liposomal preparation (water-suspension up to complex cosmetic formulations) is the time-defining factor for the penetration kinetics (16).

After 6 hours application the fluorescence images show a nearly uniform distribution of the lipophilic DiI in the skin down to a depth of 300 µm (viable skin) when an ethanolic liposomal dispersion was applied (high vapour pressure of ethanol causes a fast evaporation).

For both other solvents, which are also used in cosmetics to improve the moisture of the skin, an intensively red colouration of the SC could be observed. But there is a significant difference in the amount of DiI being found in the deeper skin layer. Also with the liposomes in the pentylene glycol solution we are able to detect fluorescence in the viable skin **(Fig. 4)**. This data is also of interest with respect to the size of liposomes. Liposomes prepared in a pentylene glycol / water so-

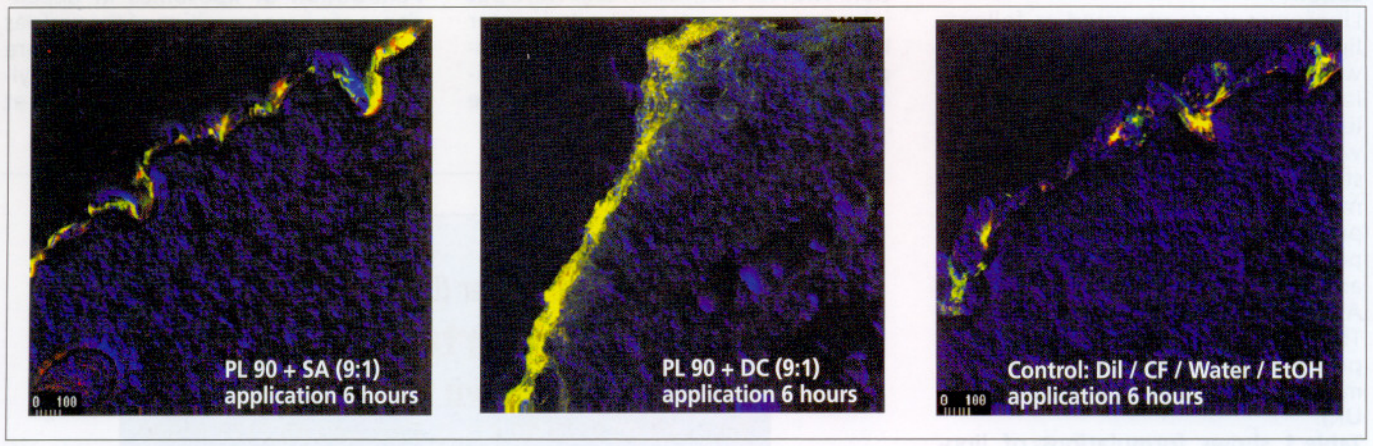

Fig. 3 Penetration of lipophilic DiI (red) and hydrophilic CF (green) into human skin (Overlapping fluorescence images)

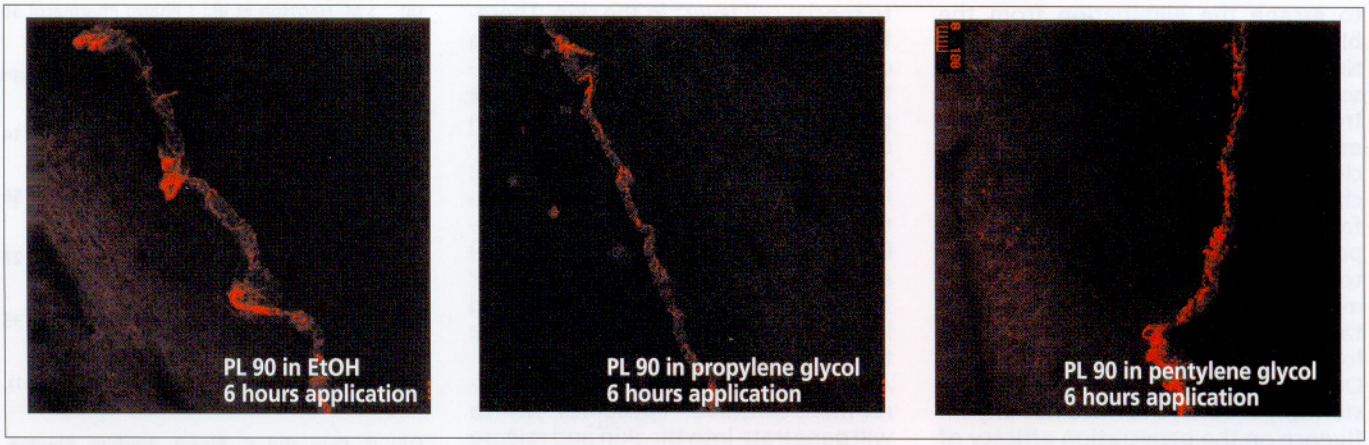

Fig. 4 Penetration of lipophilic DiI (red) into human skin

lution exhibited a vesicle size of 130 nm directly after extrusion through the polycarbonate filter (200 nm). But after storage of one day an increase in vesicle size up to 3-fold could be observed and then the size was kept at constant value over time. The penetration study was investigated with liposomes (PL 90/pentylene glycol) with a size of 400 nm and it seems that the percutaneous absorption is independent from vesicles diameter (up to 450 nm).

Penetration in dependence of sterical modification

In the recent years so-called sterically stabilised liposomes have achieved a large popularity owing to their ability

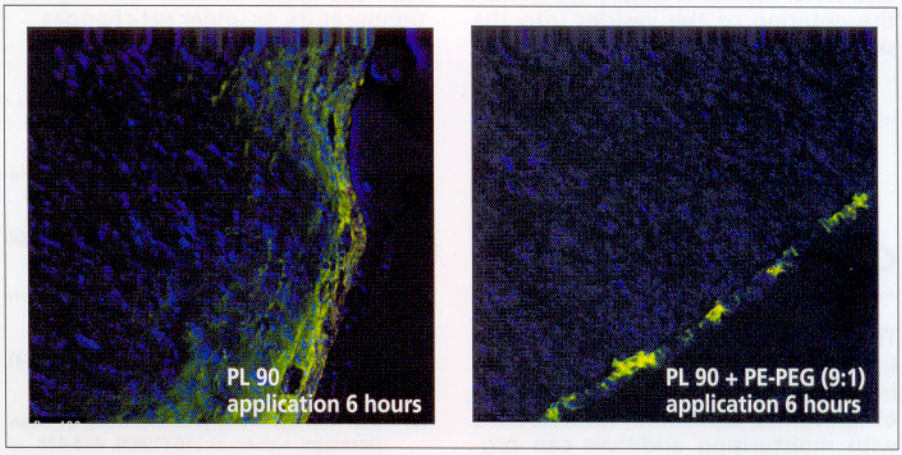

Fig. 5 Penetration of lipophilic DiI (red) and hydrophilic CF (green) into human skin (Overlapping fluorescence images)

to escape the elimination from the blood and their prolonged circulation time. These systemically applied liposomes are used in the pharmacy to treat fungal infections (AmBiosome™) or Kaposi Sarcoma (Doxil™). Such vesicles carry long polyoxyethylene (PEG) chains attached to the liposomal surface which provides the vesicles with a polar steric hindrance. The mobile interface suppresses any adsorption of macromolecules and prevents the uptake of liposomes into the RES (reticulo-endothelial-system) (17).
Our aim was to examine whether the sterically modified surface has any influence on the penetration efficacy of PL 90 liposomes. Our fluorescence pictures showed only some adsorption of these sterically stabilised liposomes on the surface of human skin, therefore also here the steric hindrance inhibit the entering of the vesicles into the skin **(Fig. 5)**.

Conclusion

These penetration studies gave an idea of the variety of factors which can influence the penetration properties of liposomes. Liposomal formulations with moisturising additives can prolongate or totally inhibit the percutaneous adsorption and each formulation has to be checked individually on its ability to penetrate into the skin.
Among other things, future perspectives will be a specific drug targeting to special cell layers in the skin. Therefore, it is of interest whether there is a correlation between the phase transition temperature of liposomes and the penetration profile of drugs (depth) in different skin layers. This study indicates that there could be a relation: liposomes in the liquid state can penetrate into the very deep skin layers – DMPC-liposomes (liquid to gel state) are more enriched in the SC and upper skin layers – meanwhile hydrogenated liposomes (gel state) are fixed on the top of the skin.
Also future perspectives will be environment-sensitive liposomes which will penetrate into the skin and release their encapsulated component on external stimulus like UV-radiation or heat. Some liposomes are under investigation which are able to show fusion with the upper skin at a well defined temperature or under pH-changes.

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