New Nanovesicles for Dermal Drug Delivery

Dissertation

Zur Erlangung des akademischen Grades

„doctor rerum naturalium“ (Dr. rer. nat.)

Vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena

von

Amaraporn Roopdee

Master of Pharmaceutics (M.S.)

Geboren am 26th of September, 1982 in Ubon Rachathani, Thailand

Reviewers

Reviewer 1: Univ.-Prof. Dr. Alfred Fahr (Emeritus)

Friedrich-Schiller-Universität Jena

Biologisch-Pharmazeutische Fakultät

Institut für Pharmazie, Lehrstuhl für Pharmazeutische Technologie

Lessingstraße 8

D-07743 Jena, Germany

Reviewer 2: Prof. Dr. Gerrit L. Scherphof (Emeritus)

University of Groningen

Groningen, Netherlands

Reviewer 3: Prof. Dr. Rolf Schubert

Albert-Ludwigs-Universität Freiburg

Lehrstuhl für Pharmazeutische Technologie und Biopharmazie

Hermann-Herder-Straße 9

D-79104 Freiburg i. Br., Germany

Day of the public defense: February 22, 2017

Contents

Abbreviation

CF	5(6)-Carboxyfluorescein
CG	Cetearyl glucoside
DSPG	1,2-Distearyl-sh-glycero-3-phospho-rac-glycerol
DSPS	1,2-Distearyl-sn-glycero-3-phosphoserine
EM	EMULSENSE®; Brassicyl isoleucinate esylate
LPC20	Phospholipon® R LPC 20 (partially hydrolysed rapeseed Lecithin containing lysophosphatidylcholine 20%)
NAT	NAT® 8539 (Soybean phosphatidylcholine (SPC) dissolved in ethanol containing: 77% phosphatidylcholine)
NMR	Nuclear magnetic resonance
PEG	Polyethyleneglycol
PG	Polyglycerol
PGMO	Polyglycerol monooleate
PL90G	Phospholipon® 90G; PL90G (phosphatidylcholine > 95%)
QA	quaternary ammonium compound with isopropanol (15%) (Behentrimonium Chloride 85%; VARISOFT BT 85 PELLETS®)
SO	Sucrose oleate (SURFHOPE® SE COSME)
TE	polyglycerol-3 methylglucose distearate (TEGO CARE 450®)
TP	Terpene mixture (limonene: citral: cineole = 1: 4.5: 4.5, v/v)
TW	Tween 80

Part 1

Introduction and the Aims of research

Introduction

Dermal drug delivery is of global interest in the pharmaceutical and cosmetic productions. Its advantages over other routes of administration (such as oral and intravenous routes) include 1) avoidance of the hepatic first pass effect, 2) decreased side effects of drugs and 3) improved patient compliance. Traditionally, conventional liposomes are chosen by the large majority of researches as the candidate of vesicle tool for enhancing the drug penetration. Even though the conventional liposomes could enhance dermal drug delivery to a certain degree, they however have some disadvantages *per se*, and can be subjected to further improvement. Recent studies have demonstrated the various vesicular formulations (such as deformable liposomes, niosomes) can improve the vesicular properties when compared to conventional liposomes.

Since the *type of phospholipids selected* for liposome preparation plays an important role for our investigation of emulsifier effect, the ideal liposomes which are deemed appropriate should consist of uncharged phospholipids, making them neutral liposomes and thus exhibit a noninterfering effect to added emulsifier. In addition, our ideal liposomes should have, at least, increased drug entrapment and high membrane elasticity of lipid bilayer for better penetration through deeper skin. While many strategies exist to enhance drug penetration, one of the most interesting which constitutes whole studies of this dissertation is the *surface modification on lipid bilayer*.

Notwithstanding the fact that prior studies have demonstrated the surface modifications using either nonionic surfactant (Tween® and Span®) or long chain polymeric surfactant incorporated into lipid bilayer could enhance the elasticity of bilayer and drug penetration of liposomes [1, 2], other interesting substances for improving the drug penetration capacity are yet to be thoroughly observed.

In the light of the above, we therefore tested a set of chosen emulsifiers in order to investigate their influence on drug permeation capability and membrane elasticity of liposomes. We divided these emulsifiers into four groups based on their characteristics: 1) charged phospholipids/surfactants, 2) sugar derivatives (carbohydrate-based surfactants), 3) polymeric surfactants, and 4) terpenes. Using

biological testing methods, i.e., *ex vivo* skin penetration and tape stripping on abdominal human skin obtained by plastic surgery, we are able to systematically categorize the enhancement capability of all tested liposomes. All in all, our studies would not only result in a valuable suggestion for further topical application, but also point out some liposomal formulations which have a high, promising potential for being a candidate for further advanced researches in this field.

Aims of this research

Even though, as mentioned earlier, dermal drug delivery has advantages over other routes of administration, it has also one major obstacle, the main barrier of the skin or the stratum corneum (SC), which is to be overcome as the aim of this research. Barrier features of SC limit drug transport therefore making it a less attractive mode for delivery of medications. In order to effectively across the skin, drug carriers must be able to pass not only through corneocytes but also in between them (via intercellular spaces). As a result of which, we have to modify our liposomes by incorporating them different emulsifiers (which are already divided into four groups), and then characterize the physicochemical properties and biological performance of those modified liposomes accordingly. At last stage, they will all be categorized based on their particular drug penetration capability.

Therefore, the aims of the whole study can be divided into *four* parts, albeit interconnected themes, as follows:

1) To compare the physicochemical properties and skin penetration study of carrier systems with five different phospholipid types.

Five phospholipids **(**PL90G, NAT8539, LPC20, SUN70P and SUN90P**)** were used for preparing liposomal formulations**.** Hydrophilic dye, carboxyfluorescein was entrapped in the aqueous core**.** All phospholipids are unsaturated, having one to two double bonds in the fatty acid tails**.** They are mainly composed of difference percentage of phosphatidylcholine **(**PC**)** and other residuals**.** Liposomes can be normally and in practical formed with PLs containing more than 80**%** PC**.**

2) ***To study the influence of the addition of a charge into liposomes on the physicochemical properties and skin penetration study of liposomes.***

One of the factors affecting to skin penetration is the surface charge of liposomes**.** Positive or negatively charged liposomes entrapping CF were prepared using PL90G, in combination with brassicyl isoleucinate esylate **(**EM**)**, quaternary ammonium **(**QA**)** or DSPG, DSPS**.** Liposomal formulations were characterized by dynamic laser light scattering for size distribution and polydispersity index **(**PDI**)**, and electrophoretic mobility for zeta potential determination**.** In order to obtain more information about skin penetration, Franz**'**s cell diffusion technique was employed**.**

3) ***To investigate the effect of surface modification of liposomes with hydrophilic polymer on the physicochemical properties and skin penetration study of liposomes.***

As the result of poor colloidal stability of liposomes, the incorporating hydrophilic polymers into liposomes can introduce a sterical barrier between adjacent bilayer membranes to prevent aggregation**.** In this study, two different groups**:** polyethylene glycol **(**PEG**)** series **(**PEG300, 600, 1000, and 2000**)** and polyglycerol monooleate **(**PGMO**)** series **(**PGMO4, 5, and 10**)** are incorporated into lipid bilayer of liposomes**.**

4) ***To study the effect of the surface modification of liposomes with sugar derivatives on the physicochemical properties and skin penetration of liposomes.***

The fourth and the last purpose of this study was to investigate the effect of sugar derivatives (sucrose oleate, SO; cetearyl glycoside, CG; polyglyceryl-3 methylglucose distearate, TE) incorporated in lipid bilayer of liposomes on the physicochemical properties and skin penetration efficacy of liposomes. These selected sugar derivatives are usually used as emulsifying agent for topical formulations.

References

1. Cevc, G., *et al*., *Ultraflexible vesicles, Transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1998. **1368**(2): p. 201-215.
2. Knudsen, N.O., *et al*., *Calcipotriol delivery into the skin with PEGylated liposomes.* Eur J Pharm Biopharm, 2012. **81**(3): p. 532-9.

Part 2

Background information

Background information

1. Structure and function of human skin

Skin is the largest organ of human body, accounting for approximately 16% of total body weight [1]. Its main vital function is to prevent loss of water of the body to the environment and protect the body from a variety of environmental insults as described in ***Figure 1*** [1, 2]. Furthermore, skin also has important immune and sensory functions, helps to regulate body temperature, and synthesizes vitamin D [3, 4]. The structure of skin and its barrier function have been extensively described in the literature [5].

1.1 Structure of human skin

Human skin is composed of three layers, the epidermis, dermis and subcutaneous fat as illustrated in ***Fig 1***.

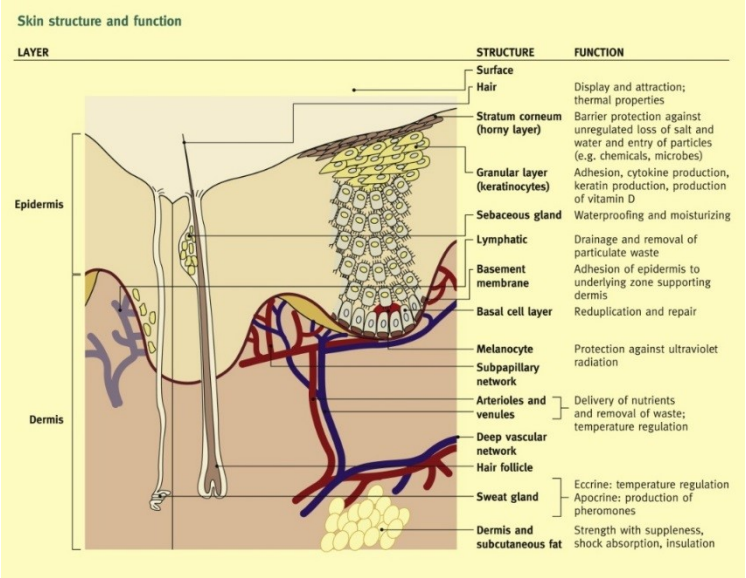


Figure 1 Structure and function of human skin (Source from Venus *et al*., 2011) [1].

1.1.1 Epidermis

Epidermis is divided into several layers, all of which can be categorized into non-viable epidermis and viable epidermis. The upper layer of the epidermis is the non-viable stratum corneum (SC), which constitutes the main barrier function of the

skin. Underneath the viable epidermis is located, which is classified into three separate layers; stratum granulosum, stratum spinosum and stratum basale just above the dermis proceeding upward through the prickle and the granular layers to the top layer, the SC. It ranges in thickness from 75 to 150 μm [6]. ***Figure 2*** is a diagram representing the major strata of the epidermis. The predominant cell type of the epidermis is the keratinocyte.

Keratinocytes synthesize keratin and many other proteins. Keratins are the major structure proteins of the SC. In addition, there are two other cell types in the epidermis, which also play a significant role: melanocytes (which involve in skin pigmentation) and Langerhans (which relate to antigen presentation and immune response) cells [3, 5].

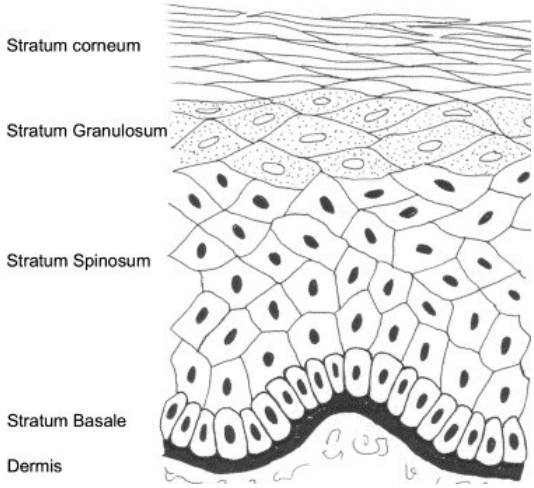


Figure 2 Diagram of the epidermis showing the main layers (Source from Wickett and Visscher, 2006) [3].

1.1.2 Dermis

Dermis has generally various in thickness, ranging from 0.6 mm on the eyelids to 3 mm on the back, palms and soles. It is composed of a tough and supportive cell matrix which contains collagen and fibrous proteins embedded in a semigel matrix (water, hyaluronic acid, ions and mucopolysaccharides) [3, 5].

There are two layers, a thin papillary layer and a thicker reticular layer, which comprise the dermis. The papillary layer contains thin loosely arranged

elastics collagen fibers and is an important layer for the nutrition of the epidermis. Thicker bundles of collagen run parallel to the skin surface in the deeper reticular layer, which extends from the base of the papillary layer to the subcutis tissue. The dermis is made up of fibroblasts, which produce collagen, elastin and structural proteoglycans, together with immunocompetent mast cells and macrophages. Up to 70% of the dermis are collagen fibers, giving it strength and toughness. Elastin maintains normal elasticity and flexibility while proteoglycans provide viscosity and hydration [3, 5].

1.1.3 Subcutaneous layer

The subcutaneous layer or hypodermis is the innermost and thickest layer of the skin. It is located underneath the dermis and composed of a type of cell specialized in accumulating and storing fats (adipocytes). These cells are grouped together in lobules separated by connective tissue. This layers acts as energy storage, protect against injury and participates in thermoregulation (since fat is a heat insulator). It can act as a depot for lipophilic compounds, which permeate the dermis and SC [3, 5].

1.2 Skin functions

a) ***Barrier function:*** Skin prevents both excessive water loss from the body and the ingress of xenobiotic [6].

b) ***UV protection:*** Melanocytes, located in the basal layer produce melanin has an important role in preventing damages in the skin caused by UV radiation. In the inner layers of the epidermis, melanin granules form a protective shield over the nuclei of the keratinocytes; in the outer layers, they are more evenly distributed. Melanin absorbs UV radiation, thus inhibiting the formation of free radicals and protecting deoxyribonucleic acid (DNA) damages [1, 7].

c) ***Thermoregulation:*** The skin plays an important role in maintaining a constant body temperature through changes in blood flow in the cutaneous vascular system and evaporation of sweat from the surface [7].

2. The barrier of human skin

2.1 Formation of the SC barrier

The stratum corneum (SC) is seen as a wall-like structure with protein bricks and lipid mortar [6, 8] as shown in ***Figure 3***. The SC corneocytes, together with their resistant cell envelopes and keratin microfibrils are considered to be the bricks, and the layers of lipids found between the cells are considered to be the mortar [3, 9].

2.2 The bricks and mortar model of the SC barrier

The lipid "mortar" is the main barrier preventing water passing through the SC [10]. Both the bricks and the mortar of the SC are produced by keratinocytes at the stratum granulosum (SG). Keratinocytes release the lipids of the mortar into the space between the cells as they are being transformed into corneocytes "bricks". A major difference between the current and the earliest version of the wall model is that the bricks are linked by desmosomes as illustrated in ***Figure 3***.

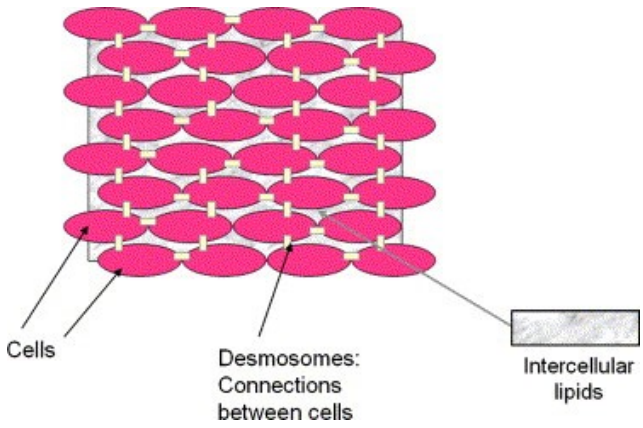


Figure 3 "Bricks and Mortar" model for human stratum corneum illustrating the corneocytes "Bricks", the intercellular lipid "Mortar", and the desmosomes connecting the corneocytes (Source from Wickett and Visscher, 2006) [3].

2.2.1 The bricks (corneocytes)

Keratinocytes move apically from basal layer to the skin surface in about 14 days, by a process called "desquamation" [11]. Epidermal differentiation begins with the migration of keratinocytes from the basal layer, and ends with the formation of the

cornified layer (SC). In epidermis differentiation, cell proliferation, differentiation and death occur sequentially, and each process is characterized by the expression of specific proteins. In normal epidermis, the proliferation rate in the basal layers is precisely balanced by desquamation of the cornified layer at skin surface. The epidermal homeostasis constantly rejuvenates the epidermis [12]. The corneocytes (terminally differentiated keratinocytes) are dead cells filled with keratin [13, 14].

The process of terminal keratinocyte differentiation gives rise to a cornified cell envelope forming beneath the plasma membrane. The cornified envelope provides a vital physical barrier to these tissues in mammals and consists of a 15 nm thick layer of highly cross-linked insoluble proteins. The assembly of the cornified envelope starts in the upper spinous layers of the epidermis. Involucrin is an early substrate for transglutaminase activity following by loricrin, cystatin and small proline-rich proteins (SPRs). Especially, loricrin and cystatin A are major constituents of the cornified envelope [15, 16].

2.2.2 The mortar

The lamellar bodies appear at the SG contain lipids, which are released into the intercellular space of the SC. These lipids are glucosyl ceramides, cholesterol, cholesterol esters, and long chain fatty acids. In the intercellular space, the glucosyl ceramides are converted to ceramides [17]. The lipids spontaneously organize into multiple layers between the SC cells [18].

The SC contains virtually no phospholipids [19]. The phospholipids from the keratinocytes in the viable layers are broken down by phospholipases [19] in the lower SC. This enzymatic process produces fatty acids, which are necessary for the development of functional SC barrier and may play a role in producing the acid pH of the SC. The SC has a surface pH of approximately 4 to 5.5. This so-called “acid mantle” of the skin [20], plays a role in preventing colonization of harmful bacteria [21].

2.2.3 SC desmosomes

Desmosomes are structures composed primarily of glycoproteins, joining corneocytes in the SC together. Since they are modified from those located in the epidermis by the addition of a protein called “corneodesmosine” [22], therefore they are sometimes referred to corneodesmosomes. In the deeper layer of SC, desmosomes are more and intact, but they are gradually digested by the time at the skin surface [3].

In summary, the specialized role ‘brick and mortar’ of the SC works together to produce a cover for the skin that is both flexible and superbly protective. If the SC is functioning properly, it will effectively defend the body against dehydration, external toxins, and bacterial assault as well as protecting the more fragile keratinocytes below from mechanical disruption [21, 23].

3. Permeation through the skin

3.1. Transappendageal route

There are various potential pathways for permeation through the SC. Here we consider firstly the “transappendageal route”. It comprises transportation via the sweat glands and along hair follicles with their associated sebaceous glands as shown in ***Figure 4*** [6, 24].

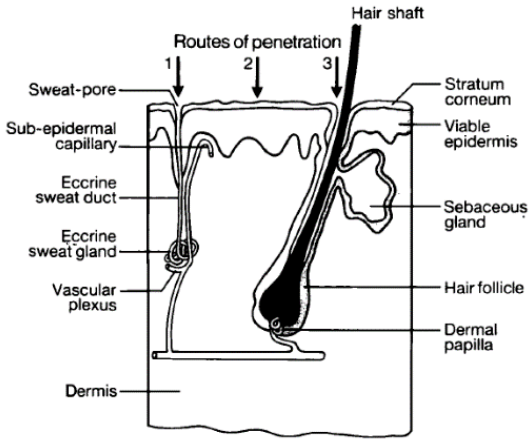


Figure 4 Possible routes for penetrant through the skin, 1: via the sweat glands; 2: across the continuous stratum corneum; 3: via the hair follicles with their associated sebaceous glands (Source from Barry, 2001) [25].

This route involves penetration through the hair follicles. Since the hair follicles constitute only 0.1% of the total area of human skin, this route is normally not considered to be significant for permeation. It is of some importance for targeting actives to the hair follicles [26], however.

3.2 Transepidermal pathway

The "transepidermal route" across the SC between the appendages comprises transportation via 1) the intercellular route through lipid layer or 2) transcellular through the cells themselves [24, 25, 27] as depicted in ***Figure 5***. The first pathway, the intercellular route, is a continuous and also tortuous way through the intercellular lipid domains. The intercellular spaces are filled with two layers. One of these layers contains lipids region (ceramides, sterols, sterol esters) and free fatty acids, which are organized in multilaminated sheets and other layer contains aqueous region.[28].

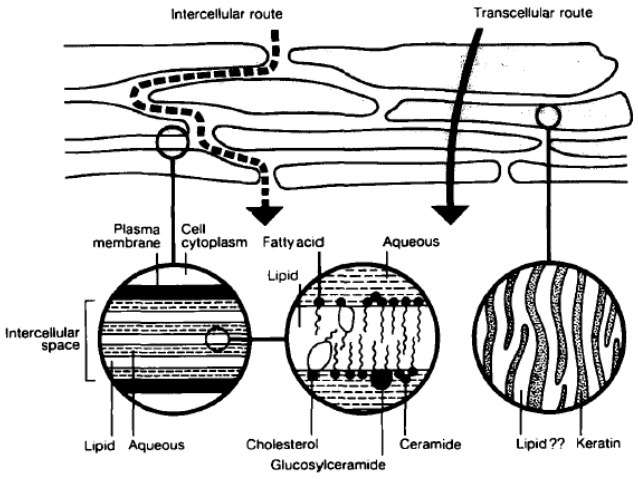


Figure 5 Suggested routes of transepidermal drug transport through human stratum corneum which has two micropathways; intercellular and transcellular routes (Source from Barry, 2001) [25].

However, the bilayers in the intercellular space in SC consist of straight closely packed and almost saturated hydrocarbon chains, resulting in impermeability. This route is widely accepted and regarded as the dominant pathway during steady-state permeation of compounds.[29]. The second pathway is the transcellular and requires diffusion through the keratinocytes and across the intercellular lipids. The intracellular spaces of corneocytes is highly dense and

virtually packed with structure protein [8]. All components of the intracellular spaces contribute to the barrier function of the SC.

Nevertheless, there possibly are deviations in this basic structure of the skin that can affect the percutaneous absorption of penetrants. Some of these deviations are due to species differences, regional skin site variations, skin age and skin condition variables [30].

4. Penetration of substances

Besides the structure of the barrier, other factors of the drug also play an important role for investigating percutaneous absorption. The important factors include the partition coefficient and the diffusion coefficient [6]. These factors depend on variables such as molecular weight, size, structure, pH and degree of ionization of the penetrants [6, 31-34]. In general, drug permeation across the skin requires drug substances with a molecular weight smaller than 500 Da and with an adequate partition between octanol and water (a log P value of 1-3) [35, 36]. The chemical structure of the penetrant is of importance in determining the permeation through human skin. The explanation and investigation of the percutaneous absorption of penetrant by one or the other factors are very difficult [24, 37]. In consequence, only general statements can be classified and based on homologous series of compounds, such as *n*-alkanols [24, 30].

As the barrier for percutaneous absorption is located in the SC for the majority of drugs, this study will be focused mainly on permeation through the SC which can be described using Fick's first law (as shown in ***Equation 1***).

$$J = \frac{D_{SC} \cdot C_{SC}}{L} \cdot \frac{C_V}{C_{S,V}} \qquad \textbf{Equation 1}$$

Where L is the diffusion pathway given by the thickness of the SC. The C_V is the concentration of drug dissolved in the vehicle and $C_{S,V}$ is the solubility of the drug in the vehicle.

This equation can predict the flux (J) across the SC based on solubility (C_{SC}) and diffusion (D_{SC}) of a substance [24, 38]. The influence of the SC thickness

involves the following: a) penetration of the drug from the vehicle into the SC, b) diffusion through the SC and c) penetration into the viable epidermis [24, 30, 39].

The role of concentration gradient in driving percutaneous absorption is considerable, where concentration in the outermost layer like SC is high followed by a decrease as we move to the deeper layer. This implicate the role of partition coefficient in the ability of the penetrant to reach the SC [40].

4.1. Chemical penetration enhancers

The use of chemical penetration enhancer in dermal/ transdermal drug delivery plays an important role for better enhancement of drug through skin barriers. There is a set of various chemical structures that have been reported as skin penetration enhancer, e.g. fatty acids, fatty alcohols, terpenes, pyrrolidones, and surfactants [41, 42]. The difference in chemical structures and physicochemical properties of these substances also affects their penetration enhancement capability [41]. Therefore, the correlation between the chemical structure and skin penetration enhancement activity has been studied and expressed in terms of structure-activity relationship (SAR) [41]. In our studies, we will examine the SAR of two groups of chemical penetration enhancers – *surfactants and terpenes* – and evaluate their skin penetration activities using *ex vivo* skin penetration method.

4.1.1. Surfactant

Surfactants are surface-active molecules and contain a *hydrophobic moiety*, usually alkyl chains or aryl moieties referred to as tail and a *hydrophilic head group* [43, 44]. The schematic illustration of a surfactant molecule is shown in ***Figure 6***. Surfactants are classified primarily by the characteristics of the headgroups. There are *four major categories of surfactants* based on the *charge of the headgroup* which are anionic, cationic, amphoteric, and nonionic. These are utilized as emulsifiers for the production of cream and cleansing purposes.

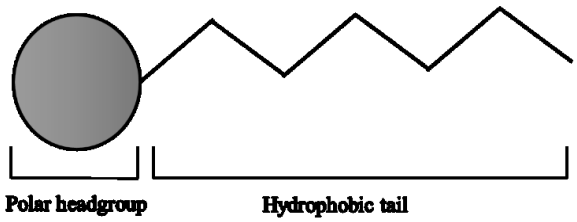


Figure 6 The basic molecular structure of a surfactant molecule

In the present, the use of surfactant has gradually increased in liposome technology in particular the incorporation of surfactant into lipid bilayer for enhancing the flexibility property of vesicle. Many surfactants, e.g. Tween80®, Span80®, and sodium cholate, have been reported that they could increase drug solubility and enhance drug penetration through the skin as compared to conventional liposomes [41]. The capability of each surfactant for improving flexibility of lipid bilayer and enhancing drug penetration depends on the chemical structure and physicochemical property [41], e.g. molecular weight and hydrophilicity-lipophilicity property. In addition, effect of headgroup size and fatty acid chain length of surfactant on the percutaneous penetration enhancement of drug has been discussed in several reports. Mishra *et al* (2007) investigated the effect of a series of Span® (40, 60 and 80) on the transdermal flux of propranolol hydrochloride through male albino rat skin using elastic liposomal formulations. The maximum permeation was observed with Span®80 [45]. Hashizaki *et al*. (2003) investigated the effect of PEG chain length of the PEG-lipid on the phase transition of liposomes and temperature dependency of CF permeability from liposomes [46].

Surfactant molecules are known for their ability to disturb the lipid bilayers and bind to proteins, causing changes in their structure, and have been shown to bind to the SC as well. After binding to the protein, surfactant causes swelling of the SC. Presumably, corneocytes were separated widely with small regions of cell to cell contact and their membranes appeared denuded of intercellular lamellar bilayers [47]. There is a good correlation between degree of swelling and the harshness of surfactant (individual or mixture). The effect of various charges on the SC has been studied in detail. The ionic surfactants interact with keratin in corneocytes and disorder lipid bilayer of SC. While non-ionic surfactants can

enhance penetration with two mechanisms: first, surfactants penetrate into the intercellular spaces of SC to increase fluidity of bilayer and second, they can interact and bind with keratin protein in corneocytes. Not only charge of surfactants affects to enhance drug permeation, but also lipophilicity of them. High lipophilic surfactants interact with lipid and open the lipid bilayers of SC and high hydrophilic surfactants expand the water channels between the lipid bilayers. The lipid mortar is the major barrier to permeability of the SC, and surfactants are known to increase the ability of exogenous compounds to penetrate the skin and to increase the rate of water loss through the skin, presumably because of their effects on the lipid SC [48].

The effect of surfactant; sodium dodecyl sulfate, on SC lipids in pig skin exposed in vitro at 46 °C showed that the surfactant could disrupt the barrier lipids, induce intercellular lamellar bilayer disruption, and produce corneocytes separation [47]. Furthermore, Warner et al. (1999) found that water alone also disrupt the SC barrier lipids with a slower onset and enhanced permeation, as the result causing the intercellular lamellar bilayer disruption [47]. Besides, Blank (1953) reported water as a key factor for maintaining the pliability of the SC and showed that well-hydrated SC is very flexible [49].

4.1.2. Terpenes

Terpenes – naturally found in essential oils – are a very safe and effective class of penetration enhancers, obtained from natural sources, the FDA classifies them as generally regarded as safe (GRAS) [50]. Terpenes are compounds consisting of isoprene (C5H8) units [41, 51]. They are classified according to the number of isoprene units as monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20) [41]. Terpenes have been widely evaluated as skin penetration enhancers for various drugs, e.g., 5-fluorouracil [52], indomethacin [53]. Both the mono- and sesquiterpenes are known to increase percutaneous absorption of compounds by increasing diffusivity of the drug in stratum corneum and/or by disruption of the intercellular lipid barrier [54].

5. Vesicles as skin delivery system

Vesicular systems such as liposomes, niosomes, transfersomes ethosomes, invasomes and elastic vesicles provide an alternative for improved skin drug delivery. In general, the walls of these vesicles consist of amphiphilic molecules in a bilayer conformation. Due to an increase hydration, these amphiphilic molecules can form spontaneously one (unilamellar vesicles) or more (multilamellar vesicles) concentric bilayers. Hydrophilic drugs can be entrapped into the internal aqueous compartment, whereas amphiphilic drugs and lipophilic drugs can be associated with the vesicle surface and lipid bilayer by hydrophobic and/or electrostatic interaction [47].

In last decades, most of the conventional liposomes were composed of PC, cholesterol and entrapped substances. When applied these liposomes to skin for topical drug delivery, they deposit in the upper layers of the SC or pilobaceous unit and are not penetrated into deeper skin [48]. Therefore, the new generation of liposomes has been developed for better drug penetration through the skin. In 1992, the first type of deformable liposomes, i.e. the 'Transfersomes®', was introduced by Cevc and his colleagues [42]. These vesicles are composed of PC and sodium deoxycholate as the "edge activator" in lipid bilayer. They claimed special feature that such vesicles could pass through the SC by squeezing through skin pores that are less than one-tenth the diameter of liposomes. (We will discuss in detail about the deformable liposomes in ***5.2.2 Deformable liposomes***.) A wide variety of lipids and surfactants can be used to prepare vesicles, which are commonly composed of phospholipids (liposomes) [49] or non-ionic surfactants (niosomes) [50]. Furthermore, Fahr and his colleagues developed another type of deformable liposomes, i.e. 'invasomes" composed of phosphatidylcholine, ethanol and mixture of terpenes (instead of other surfactants) as penetration enhancers [51]. Another kind of vesicle is ethosomes, which are composed of phospholipids and high content of ethanol (20–45%) [52]. The composition of the vesicles influences their physicochemical characteristics such as size, charge, thermodynamic phase, lamellarity and bilayer elasticity. These physicochemical characteristics have a significant effect on the behavior of the vesicles and hence on their effectiveness as

a drug delivery system. The rationale for using vesicles in dermal and transdermal drug delivery has many advantages [36, 53] as follows:

1. Vesicles act as drug carriers to deliver entrapped drug molecules into or across the skin;
2. act as penetration enhancers owing the penetration of the lipid components into the SC and subsequently the alteration of the intercellular lipid lamellae within this skin layer;
3. serve as a depot for sustained release of dermally active compounds; and
4. serve as a rate-limiting membrane barrier for the modulation of systemic absorption, hence providing a controlled transdermal delivery system.

5.1. Mechanisms of action of liposomes as skin drug delivery systems

Mechanisms have been suggested for liposomes acting as skin drug delivery systems as shown in ***Figure 6***.

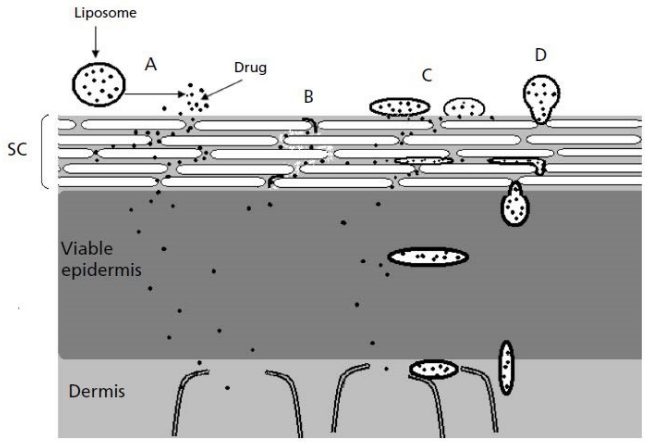


Figure 6 Possible mechanisms of action of liposomes as skin drug delivery systems. A: the free drug mechanism, B: the penetration enhancing process with liposomes components, C: vesicles adsorption and fusion with the SC, and D: intact vesicle penetration into skin (Source from El Maghraby *et al*., 2006) [54].

5.2. Liposomes as dermal drug delivery systems

Bangham and colleagues in 1965 were the first to introduce the liposomes as drug delivery vehicles [49]. Liposomes are spherical vesicles with concentric phospholipid bilayers, which they are hydrophilic on one side and lipophilic on the other side. When phospholipids – having one hydrophilic headgroup and double fatty acid chain i.e., PC – are placed in water and obtain sufficient energy, this results in the arrangement of lipids and spontaneous formation of bilayer vesicles [55] as described in ***Figure 7*** [56]. The amphipathic character of phospholipids allows entrapment of hydrophilic substances into the aqueous core and lipophilic ones in the lipid bilayer.

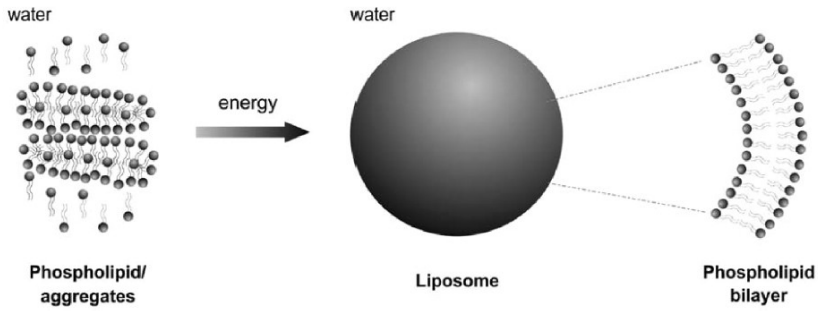


Figure 7 The simplified mechanism of liposomes formation (Source from Mozafari *et al*., 2008) [56].

These vesicles are preferred over other formulation because of their characteristics such as biocompatible, biodegradation, capacity of encapsulating both hydrophilic and lipophilic molecules, enhancement of solubility of hydrophilic and lipophilic drug, reducing the drug toxicity, increasing drug bioavailability and targeting delivery to skin appendages in addition to their potential for transdermal delivery [57-59]. However, the limitations of these vesicles are high cost of liposome production in large scale, instability and low entrapment of drug molecules into vesicles. Additionally, conventional liposomes remain confined to the upper layer of the SC and accumulate in the skin appendages, mainly owing to their large size and lack of flexibility [60]. ***Table 1*** [61, 62] described liposomes classification based on their structure with size and number of lipid bilayers.

Table 1 Vesicle types of liposomes with their size and number of lipid layers [62, 63].

Vesicle type	Abbreviation	Diameter size	No. of lipid bilayer
Multilamellar vesicle	MLV	500 nm - 10μm	-25
Small Unilamellar vesicle	SUV	20-100 nm	1
Large Unilamellar vesicle	LUV	More than 100 nm	1
Giant Unilamellar vesicle	GUV	10-1000 μm	1
Oligolamellar vesicle	OLV	0.1-1 μm	Approx. 5

The lack of stability and low drug entrapment (volume) of SUVs make them less suitable for drug delivery system. Magnitude of vesicle diameter is an important feature deciding penetration/ permeation of drugs through the topical route. Since too much increase of diameter may result in decrease of penetration through skin, the proper size of structure for this route of administration is therefore the LUVs having 50-300 nm Diameter size.[62].

Besides classifying by their structure, liposomes can also be classified based on their compositions. The first class of vesicles so called ‘conventional liposomes’ can be prepared with any types of phospholipids. Various types and purities of phospholipids are used in liposomal formulations [53]. Conventional liposomes in the past composed of mostly one or more phospholipids and cholesterol. In 1980, Mezei and Gulasekharam reported that liposomal triamcinolone acetonide (Multilamellar liposomes composed of DPPC and cholesterol) showed higher drug deposition in rabbit skin as compared with control lotion [64]. In order to overcome the limitation of low penetration of drug and drug deposition in superficial skin, drug carriers must pass through a very fine pores with an average diameter typically around ≤ 30 nm, under the proper transdermal gradient [65]. This approach has resulted in two novel designs of vesicular carriers, ethosomes and elastic vesicles (transfersomes). In 1997, ethosomes were introduced, which are composed of phospholipids, water and high amount of ethanol [66]. Indeed, ethanol cause the

carriers penetrate more easier into the deeper skin by fluidizing the lipid domain of SC [67]. Other vesicular system, Transfersomes, will be discussed in detail in topic ***5.2.2 (Deformable liposomes)***.

5.2.1 Choice of lipids

The choice of lipids for drug encapsulation into liposomes is dependent on the drug characteristics and intended applications. Liposomal composition determines the properties (including surface charge, rigidity, and steric interactions) and the *in vitro* and *in vivo* performance of liposomes. The specific properties of the liposomes are determined by the chemistry of the head and tail groups of the constituent lipids [68]. Liposomes can be prepared from a variety of neutral, cationic, and anionic phospholipids. Examples of these lipids include, PC [69] and phosphatidylethanolamines (PE) and phosphatidylserines (PS), phosphatidylglycerols (PG), phosphatidic acid (PA) and phosphatidylinositols (PI). At pH 7.4, PC has neutral charge, PE has positively charged while PS, PG, PA and PI have negatively charged [68]. Conventional liposomes can possess different lipid compositions; however, the most commonly used lipids are PC and cholesterol.

An important property of phospholipids is the phase transition temperature (T_m). T_m determines the phase behavior of the liposomal membranes which in turn affects characteristics of liposomes such as permeability, fusion, and aggregation [70]. The maximal release of drug is usually observed at temperatures below or close to the T_m of the lipid bilayer, where the bilayers transform from an ordered gel state into a more disordered liquid state [70].

There are several factors, which affect directly the phase transition temperature including hydrocarbon length, unsaturation, charge, and head group species. As the hydrocarbon length is increased, van der Waals interactions become stronger and require more energy to disrupt the ordered packing, thus the phase transition temperature increases. Introducing a double bond into the acyl group puts a kink in the chain which requires much lower temperatures to induce an ordered packing arrangement [71]. The addition of cholesterol causes an ordering of the disordered fluid phase and therefore increasing amounts of cholesterol eventually lead to an elimination of the phase transition. Consequently, liposomes containing

cholesterol are more adhesive, having high stability against proteins *in vivo* and against leakage of encapsulated materials.

One major problem related to the use of liposomes in topical products is their poor colloidal stability. One possibility to overcome such stability problems for liposomes is to coat them with hydrophilic polymers in order to establish a sterical barrier between adjacent bilayer membranes to prevent aggregation. The effect of PEGylating liposomes for topical application was slightly described in the literature, however. Only two relevant studies have reported the PEGylated liposomes application for topical drug delivery. First, PEGylated liposomes can increase the skin permeation of zidovudine [72]. Second, the D-vitamin analogue calcipotriol, pegylation of the saturated liposomes provided a significantly increased deposition of calcipotriol in the stratum corneum after topical application and an improved steric stabilization of liposomes [73].

A.) Phosphatidylcholine

Phosphatidylcholine (PC) is the most abundant phospholipid in animal and plants [74] and the key building block of membrane bilayers.

In addition to phosphatidylcholine, neutral lipid bilayers may be composed of sphingomyelin or alkyl ether lecithin analogues, which substitute entirely lecithin in the membranes. In sphingomyelin, the presence of the amide linkage and hydroxyl groups (in the region corresponding to the glycerol backbone of lecithin) gives rise to hydrogen bond interactions with its neighbors in the membrane [75].

B.) Charged phospholipids

It is well known that skin may act as a negatively charged membrane; it has been generally reported that the presence of charge on the vesicle surface affects the drug diffusion through the skin. One of interesting strategies enhancing the stability of liposomes and influencing drug penetration is the modification of surface charge of liposomes.

Previous studies have documented the modification by using charged phospholipids; coating liposomes with polymers and addition of charge induce

agents. Recently, Sinico *et al*. [76] have shown that negatively charged liposomes strongly improved newborn pig skin hydration and tretinoin retention as compared to both hydroalcoholic and oil solutions, though evidence of intact liposomes was not found. Gillet *et al*. [77] revealed that negatively charged liposomes significantly enhanced betamethasone penetration in the epidermis compared to positively charged and neutral liposomes, while negative non- liposomal dispersions were unable to enhance skin penetration. On the other hand, Manosroi *et al*. [78] demonstrated that positively charged liposomes produced a higher absorption amphotericin B through the SC than that negatively charged one. These results can be explained by the fact that the three possible forces affecting headgroup interaction of bilayer membranes can function, namely steric hindrance, hydrogen bonding and electrostatic charge [55, 64]. In summary, charge source (phospholipid or surfactant) for liposomes have not been tested separately resulting in a lack of explanation for enhanced bioactivity of charged liposomes (positively or negatively-charged) reported until now.

Furthermore, liposomes coating with charged synthetic or natural polymers have been attempted to enhance permeation. Hasanovic *et al*. [79] examined the influence of adding cationic polymers (chitosan or Eudragit EPO) on the stability and skin penetration of DPPC liposomes encapsulating acyclovir or minoxidil. They showed an increased stability by the addition of the two different cationic polymers and an increased skin permeation of drugs from coated liposomes. Several polymers were coated on surface of liposomes for enhancing the permeation properties of drugs, for example, chitosan [80], poly(vinyl alcohol) [81] and Eudragit® series [79]. Despite enhanced stability of charged liposomal formulation and drug penetration enhancement through the skin, their human toxicity evaluations reveal skin drying [82] and irritation [83]. Therefore, charging agents require a more careful consideration from the perspective of liposomes biocompatibility and components added during the preparation.

5.2.2 Deformable liposomes

In 1992, Cevc and Blume introduced the first generation of the highly deformable liposomes, elastic liposomes, referred to as Transfersomes® (IDEA AG, Munich, Germany) composed of PC and sodium cholate [53, 84]. Deformable liposomes mainly consist of phospholipids especially soybean PC [85, 86] that is an unsaturated phospholipid (PL) and surfactant (sodium cholate, sodium deoxycholate [87-90]) that acts as an 'edge activator' capable of destabilizing the lipid bilayer thus increasing its deformability. The reason for using unsaturated PLs for preparing deformable liposomes is that they form bilayer in liquid crystalline phase. Because of the kinks in the carbon chains at the cis double bonds of unsaturated fatty acids, the PL molecules do not fit closely together which in turn imparts certain flexibility to the bilayer membrane. Use of unsaturated PL and edge activator together are responsible for the highly deformable nature of Transfersomes.

In principle, deformable liposomes can accommodate any kind of molecules: very lipophilic substances are incorporated into lipid bilayer; water soluble compounds find shelter in the aqueous vesicles core; and amphiphatic molecules typically adsorb to the lipid-water interface. In case of large molecules, such as protein, these liposomes can encapsulate such a large macromolecule into vesicles using a combination of several interactions. Thus, the capacity of deformable liposomes to accommodate many types of molecules proves their utility for dermal drug delivery system.

Deformable liposomes can penetrate through SC in two ways. First, vesicles can act as drug carrier systems, whereby intact vesicles enter the SC carrying vesicle-bound drug molecules into the skin. Second, vesicles can act as penetration enhancers, whereby vesicle bilayers enter the SC and subsequently modify the intercellular lipid lamellae. These mechanisms facilitate penetration of free drug molecules into and across the SC [91, 92].

Cevc *et al*. (1995) reported that Transfersomes having an average diameter 500 nm are able to pass through the smaller polycarbonate membrane pores 5 times,

in spite of the large size difference between the vesicles and water characteristic dimensions in both systems as shown in ***Figure*** 8 (A) [85].

A) B)

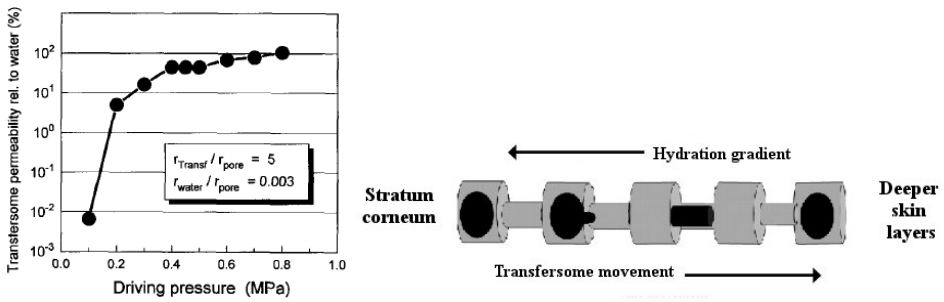


Figure 8 A) Relative permeation capability of the Transfersomes through the narrow pores under the influence of a trans-pore pressure difference (Source from Cevc *et al*., 1995) [66]. B) Hydration gradient (This gradient drives from high to low level) and deformability-driven movement of Transfersomes through small pores (Drawn from Cevc *et al*., 1996) [93].

Topically applied Transfersomes dehydrate on the skin surface by evaporation and thus an osmotic pressure difference is originated between the highly hydrated regions inside the skin and the dehydrated skin surface, which makes Transfersomes enter the SC moving into the deeper skin to avoid dehydration [94, 95]. Transfersomes require a hydration gradient to support skin penetration, that is, non-occlusive condition. Then the gradient operating from the dry skin surface towards waterlogged viable tissues drives Transfersomes through the SC (***Figure 7 (B)***) [93].

Using confocal laser scanning microscopy, Schatzlein and Cevc (1998) suggested two different penetration pathways in the SC. An intercluster route with low penetration resistance, where lateral inhomogeneity in the SC showed 'clusters' consisting of groups of 3 to 10 corneocytes. The other pathway was an intercorneocyte pathway that was more resistant to the penetration and was more abundant [96]. Within the intercellular lipid lamellae, these regions contain structural irregularities that can act as 'virtual channels' through which the deformable liposomes can penetrate because of their high flexibility owing to the edge activator molecules [96].

5.2.3 Surfactant as edge activator for deformable liposomes

Edge activator is often a single chain surfactant; its incorporation within the bilayer can destabilize the bilayers by increasing the radius of curvature of the bilayers which in turn also increases the deformability of the vesicles [97]. Ratio of an edge activator with the phospholipid is not fixed but usually varies with formulation requirements. Therefore, the concentration of each edge activator is systematically tested in order to search for suitable concentration which provides highest skin penetration while still maintaining adequate stability and drug loading.

6. Liposomes in dermatology

The advantage of the liposomal formulations over conventional dermatological formulations was particularly striking when the activity of local anesthetic agents was evaluated in cream, ointment or lotion forms versus liposomes. A liposomal containing 0.5% tetracaine exhibit more intensive activity (6-8 folds) than 1.0% tetracaine in commercial Pontocaine® cream [98, 99]. Similar results were obtained with liposomal formulations of another local anesthetic agent, e.g. lidocaine (Maxilene®) [100-102].

References

1. Venus, M., J. Waterman, and I. McNab, *Basic physiology of the skin.* Surgery (Oxford), 2011. **29**(10): p. 471-474.
2. Elias, P.M., *Epidermal barrier function: intercellular lamellar lipid structures, origin, composition and metabolism.* Journal of Controlled Release, 1991. **15**(3): p. 199-208.
3. Wickett, R.R. and M.O. Visscher, *Structure and function of the epidermal barrier.* American Journal of Infection Control, 2006. **34**(10, Supplement): p. S98-S110.
4. Lehmann, B., K. Querings, and J. Reichrath, *Vitamin D and skin: new aspects for dermatology.* Exp Dermatol, 2004. **13 Suppl 4**: p. 11-5.
5. Baroni, A., et al., *Structure and function of the epidermis related to barrier properties.* Clinics in Dermatology, 2012. **30**(3): p. 257-262.
6. Wiechers, J.W., *The barrier function of the skin in relation to percutaneous absorption of drugs.* Pharmaceutisch Weekblad, 1989. **11**(6): p. 185-198.
7. Farina, D.J., *CHAPTER 10 - Regulatory Aspects of Nasal and Pulmonary Spray Drug Products*, in *Handbook of Non-Invasive Drug Delivery Systems*, V.S. Kulkarni, Editor. 2010, William Andrew Publishing: Boston. p. 247-290.
8. Elias, P.M., *Epidermal lipids, barrier function, and desquamation.* J Invest Dermatol, 1983. **80 Suppl**: p. 44s-49s.
9. Elias, P.M., et al., *Percutaneous transport in relation to stratum corneum structure and lipid composition.* J Invest Dermatol, 1981. **76**(4): p. 297-301.
10. Grubauer, G., et al., *Lipid content and lipid type as determinants of the epidermal permeability barrier.* J Lipid Res, 1989. **30**(1): p. 89-96.
11. Jackson, S.M., et al., *Pathobiology of the stratum corneum.* West J Med, 1993. **158**(3): p. 279-85.
12. Candi, E., R. Schmidt, and G. Melino, *The cornified envelope: a model of cell death in the skin.* Nat Rev Mol Cell Biol, 2005. **6**(4): p. 328-40.
13. Norlen, L., I. Plasencia, and L. Bagatolli, *Stratum corneum lipid organization as observed by atomic force, confocal and two-photon excitation fluorescence microscopy.* Int J Cosmet Sci, 2008. **30**(6): p. 391-411.
14. Bouwstra, J.A. and M. Ponec, *The skin barrier in healthy and diseased state.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2006. **1758**(12): p. 2080-2095.
15. Jarnik, M., M.N. Simon, and A.C. Steven, *Cornified cell envelope assembly: a model based on electron microscopic determinations of thickness and projected density.* J Cell Sci, 1998. **111 (Pt 8)**: p. 1051-60.
16. Harding, C.R., et al., *The cornified cell envelope: an important marker of stratum corneum maturation in healthy and dry skin.* International Journal of Cosmetic Science, 2003. **25**(4): p. 157-167.
17. Holleran, W.M., et al., *Processing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function.* J Clin Invest, 1993. **91**(4): p. 1656-64.
18. Swartzendruber, D.C., et al., *Molecular models of the intercellular lipid lamellae in mammalian stratum corneum.* J Invest Dermatol, 1989. **92**(2): p. 251-7.
19. Fluhr, J.W., et al., *Generation of free fatty acids from phospholipids regulates stratum corneum acidification and integrity.* J Invest Dermatol, 2001. **117**(1): p. 44-51.
20. Schmid, M.H. and H.C. Korting, *The concept of the acid mantle of the skin: its relevance for the choice of skin cleansers.* Dermatology, 1995. **191**(4): p. 276-80.
21. Elias, P.M. and E.H. Choi, *Interactions among stratum corneum defensive functions.* Exp Dermatol, 2005. **14**(10): p. 719-26.
22. Jonca, N., et al., *Corneodesmosin, a component of epidermal corneocyte desmosomes, displays homophilic adhesive properties.* J Biol Chem, 2002. **277**(7): p. 5024-9.
23. Elias, P.M., *Stratum corneum defensive functions: an integrated view.* J Invest Dermatol, 2005. **125**(2): p. 183-200.
24. Scheuplein, R.J. and I.H. Blank, *Permeability of the skin.* Physiol Rev, 1971. **51**(4): p. 702-47.
25. Barry, B.W., *Novel mechanisms and devices to enable successful transdermal drug delivery.* European Journal of Pharmaceutical Sciences, 2001. **14**(2): p. 101-114.
26. Blume-Peytavi, U. and A. Vogt, *Human hair follicle: reservoir function and selective targeting.* Br J Dermatol, 2011. **165 Suppl 2**: p. 13-7.
27. El Maghraby, G.M., B.W. Barry, and A.C. Williams, *Liposomes and skin: from drug delivery to model membranes.* Eur J Pharm Sci, 2008. **34**(4-5): p. 203-22.
28. Smith, W.P., et al., *Effect of lipids on the aggregation and permeability of human stratum corneum.* J Invest Dermatol, 1982. **78**(1): p. 7-11.
29. Landmann, L., *The epidermal permeability barrier.* Anatomy and Embryology, 1988. **178**(1): p. 1-13.

30. Maibach, H., *Dermatological formulations: Percutaneous absorption. By Brian W. Barry. Marcel Dekker, 270 Madison Avenue, New York, NY 10016. 1983. 479 pp. 16 × 23.5 cm. Price $55.00 (2070 higher outside the US. and Canada).* Journal of Pharmaceutical Sciences, 1984. **73**(4): p. 573-573.
31. Cevc, G., *Lipid vesicles and other colloids as drug carriers on the skin.* Adv Drug Deliv Rev, 2004. **56**(5): p. 675-711.
32. Cevc, G. and G. Blume, *Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force.* Biochim Biophys Acta, 1992. **1104**(1): p. 226-32.
33. Asbill, C.S. and B.B. Michniak, *Percutaneous penetration enhancers: local versus transdermal activity.* Pharmaceutical Science & Technology Today, 2000. **3**(1): p. 36-41.
34. Idson, B., *Percutaneous absorption.* J Pharm Sci, 1975. **64**(6): p. 901-24.
35. Thong, H.Y., H. Zhai, and H.I. Maibach, *Percutaneous penetration enhancers: an overview.* Skin Pharmacol Physiol, 2007. **20**(6): p. 272-82.
36. Brown, M.B., et al., *Dermal and transdermal drug delivery systems: current and future prospects.* Drug Deliv, 2006. **13**(3): p. 175-87.
37. Flynn, G.L. and S.H. Yalkowsky, *Correlation and prediction of mass transport across membranes I: Influence of alkyl chain length on flux-determining properties of barrier and diffusant.* Journal of Pharmaceutical Sciences, 1972. **61**(6): p. 838-852.
38. Moser, K., et al., *Passive skin penetration enhancement and its quantification in vitro*. Eur J Pharm Biopharm, 2001. **52**(2): p. 103-12.
39. Moss, G.P., S.C. Wilkinson, and Y. Sun, *Mathematical modelling of percutaneous absorption.* Current Opinion in Colloid & Interface Science, 2012. **17**(3): p. 166-172.
40. Lademann, J., et al., *The tape stripping procedure – evaluation of some critical parameters.* European Journal of Pharmaceutics and Biopharmaceutics, 2009. **72**(2): p. 317-323.
41. Kanikkannan, N., R.J. Babu, and M. Singh, *Structure-Activity Relationship of chemical Penetration Enhancers*, in *Percutaneous Penetration Enhancers*, E.W. Smith and H.I. Maibach, Editors. 2006, Taylor & Francis Group: 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL 33487-2742. p. 17-33.
42. Thomas, N.S. and R. Panchagnula, *Combination strategies to enhance transdermal permeation of zidovudine (AZT).* Pharmazie, 2003. **58**(12): p. 895-8.
43. Chowhan, Z.T. and R. Pritchard, *Effect of surfactants on percutaneous absorption of naproxen I: comparisons of rabbit, rat, and human excised skin.* J Pharm Sci, 1978. **67**(9): p. 1272-4.
44. Ashton, P., et al., *Surfactant effects in percutaneous absorption I. Effects on the transdermal flux of methyl nicotinate.* International Journal of Pharmaceutics, 1992. **87**(1–3): p. 261-264.
45. Mishra, D., et al., *Elastic liposomes mediated transdermal delivery of an anti-hypertensive agent: propranolol hydrochloride.* J Pharm Sci, 2007. **96**(1): p. 145-55.
46. Hashizaki, K., et al., *Effects of Poly(ethylene glycol) (PEG) Chain Length of PEG-Lipid on the Permeability of Liposomal Bilayer Membranes.* Chemical and Pharmaceutical Bulletin, 2003. **51**(7): p. 815-820.
47. Kombath Ravindran Vinod, et al., *CRITICAL ISSUES RELATED TO TRANSFERSOMES – NOVEL VESICULAR SYSTEM.* Acta Scientiarum Polonorum, 2012. **11**(1): p. 16.
48. Barry, B.W., *Penetration enhancer classification*. Percutaneous penetration enhancers, ed. E. W.Smith and H. I.Maibach. 2006, 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL 33487-2742: Taylor & Francis Group, LLC.
49. Bangham, A.D., M.M. Standish, and J.C. Watkins, *Diffusion of univalent ions across the lamellae of swollen phospholipids.* Journal of Molecular Biology, 1965. **13**(1): p. 238-IN27.
50. Kazi, K.M., et al., *Niosome: A future of targeted drug delivery systems.* J Adv Pharm Technol Res, 2010. **1**(4): p. 374-80.
51. Dragicevic-Curic, N., et al., *Temoporfin-loaded invasomes: Development, characterization and in vitro skin penetration studies.* Journal of Controlled Release, 2008. **127**(1): p. 59-69.
52. Touitou, E., et al., *Ethosomes - novel vesicular carriers for enhanced delivery: characterization and skin penetration properties.* J Control Release, 2000. **65**(3): p. 403-18.
53. Sinico, C. and A.M. Fadda, *Vesicular carriers for dermal drug delivery.* Expert Opin Drug Deliv, 2009. **6**(8): p. 813-25.
54. El Maghraby, G.M., A.C. Williams, and B.W. Barry, *Can drug-bearing liposomes penetrate intact skin?* J Pharm Pharmacol, 2006. **58**(4): p. 415-29.
55. Li, J., et al., *A review on phospholipids and their main applications in drug delivery systems.* Asian Journal of Pharmaceutical Sciences, 2015. **10**(2): p. 81-98.
56. Mozafari, M.R., et al., *Nanoliposomes and their applications in food nanotechnology.* J Liposome Res, 2008. **18**(4): p. 309-27.
57. Vinod, K.R., et al., *Critical issues related to transfersomes - novel vesicular system.* Acta Sci Pol Technol Aliment, 2012. **11**(1): p. 67-82.
58. Mansoori, M., et al., *A review on liposome.* Internatioan Journal of Advanced Research in Pharmaceutical and Bio Sciences, 2012. **2**(4): p. 453-464.

59. El Maghraby, G.M. and A.C. Williams, *Vesicular systems for delivering conventional small organic molecules and larger macromolecules to and through human skin.* Expert Opin Drug Deliv, 2009. **6**(2): p. 149-63.
60. El Zaafarany, G.M., et al., *Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery.* Int J Pharm, 2010. **397**(1-2): p. 164-72.
61. de Leeuw, J., et al., *Liposomes in dermatology today.* J Eur Acad Dermatol Venereol, 2009. **23**(5): p. 505-16.
62. Hope, M.J. and C.N. Kitson, *Liposomes. A perspective for dermatologists.* Dermatol Clin, 1993. **11**(1): p. 143-54.
63. Khosravi-Darani, K., et al., *The role of high-resolution imaging in the evaluation of nanosystems for bioactive encapsulation and targeted nanotherapy.* Micron, 2007. **38**(8): p. 804-818.
64. Mezei, M. and V. Gulasekharam, *Liposomes--a selective drug delivery system for the topical route of administration. Lotion dosage form.* Life Sci, 1980. **26**(18): p. 1473-7.
65. Trotta, M., et al., *Elastic liposomes for skin delivery of dipotassium glycyrrhizinate.* International Journal of Pharmaceutics, 2002. **241**(2): p. 319-327.
66. Touitou, E., et al., *Ethosomes — novel vesicular carriers for enhanced delivery: characterization and skin penetration properties.* Journal of Controlled Release, 2000. **65**(3): p. 403-418.
67. Bahia, A.P., et al., *New insights into the mode of action of ultradeformable vesicles using calcein as hydrophilic fluorescent marker.* Eur J Pharm Sci, 2010. **39**(1-3): p. 90-6.
68. Dragicevic-Curic, N., et al., *Surface charged temoporfin-loaded flexible vesicles: in vitro skin penetration studies and stability.* Int J Pharm, 2010. **384**(1-2): p. 100-8.
69. Nii, T. and F. Ishii, *Properties of various phosphatidylcholines as emulsifiers or dispersing agents in microparticle preparations for drug carriers.* Colloids and Surfaces B: Biointerfaces, 2004. **39**(1–2): p. 57-63.
70. New, R.R.C., *Liposomes: a practical approach*. The practical approach, ed. D.D. Rickwood and D.B.D. Hames. 1990, Oxford University Press Inc., the United State: Oxford University Press. 301.
71. Nagle, J.F., *Lipid Bilayer Phase Transition: Density Measurement and Theory.* Proc Natl Acad Sci U S A, 1973. **70**(12): p. 2.
72. Jain, S., A.K. Tiwary, and N.K. Jain, *PEGylated elastic liposomal formulation for lymphatic targeting of zidovudine.* Curr Drug Deliv, 2008. **5**(4): p. 275-81.
73. Knudsen, N.Ø., et al., *Calcipotriol delivery into the skin with PEGylated liposomes.* European Journal of Pharmaceutics and Biopharmaceutics, 2012. **81**(3): p. 532-539.
74. Kontara, E.K., et al., *Comparison of native, lyso and hydrogenated soybean phosphatidylcholine as phospholipid source in the diet of postlarval Penaeus japonicus bate.* Arch Tierernahr, 1998. **51**(1): p. 1-19.
75. Weselake, J.L.H.R.J. *PHOSPHATIDYLCHOLINE AND RELATED LIPIDS*. STRUCTURE, OCCURRENCE, BIOCHEMISTRY AND ANALYSIS; Available from: http://lipidlibrary.aocs.org/Lipids/pc/index.htm.
76. Sinico, C., et al., *Liposomes as carriers for dermal delivery of tretinoin: in vitro evaluation of drug permeation and vesicle-skin interaction.* J Control Release, 2005. **103**(1): p. 123-36.
77. Gillet, A., et al., *Liposome surface charge influence on skin penetration behaviour.* Int J Pharm, 2011. **411**(1-2): p. 223-31.
78. Manosroi, A., L. Kongkaneramit, and J. Manosroi, *Stability and transdermal absorption of topical amphotericin B liposome formulations.* Int J Pharm, 2004. **270**(1-2): p. 279-86.
79. Hasanovic, A., et al., *Improvement in physicochemical parameters of DPPC liposomes and increase in skin permeation of aciclovir and minoxidil by the addition of cationic polymers.* Eur J Pharm Biopharm, 2010. **75**(2): p. 148-53.
80. Guo, J., et al., *Chitosan-coated liposomes: characterization and interaction with leuprolide.* International Journal of Pharmaceutics, 2003. **260**(2): p. 167-173.
81. Takeuchi, H., et al., *Polymer coating of liposomes with a modified polyvinyl alcohol and their systemic circulation and RES uptake in rats.* J Control Release, 2000. **68**(2): p. 195-205.
82. G., A.C.R.J.G.M.P.H., *Einfluss Verschiedener Liposomenzubereitungen auf die Hautfeuchtigkeit.* Parfümerie und Kosmetik, 1990. **71**: p. 324-325.
83. Parnham, M.J. and H. Wetzig, *Toxicity screening of liposomes.* Chemistry and Physics of Lipids, 1993. **64**(1–3): p. 263-274.
84. Cevc, G. and G. Blume, *Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1992. **1104**(1): p. 226-232.
85. Cevc, G., A. Schätzlein, and G. Blume, *Transdermal drug carriers: Basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides.* Journal of Controlled Release, 1995. **36**(1–2): p. 3-16.
86. Cevc, G., et al., *Ultraflexible vesicles, Transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1998. **1368**(2): p. 201-215.

87. El Maghraby, G.M.M., A.C. Williams, and B.W. Barry, *Skin delivery of oestradiol from lipid vesicles: importance of liposome structure.* International Journal of Pharmaceutics, 2000. **204**(1–2): p. 159-169.
88. Trotta, M., et al., *Deformable liposomes for dermal administration of methotrexate.* Int J Pharm, 2004. **270**(1-2): p. 119-25.
89. Cevc, G., *Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery.* Crit Rev Ther Drug Carrier Syst, 1996. **13**(3-4): p. 257-388.
90. Boinpally, R.R., et al., *Lecithin vesicles for topical delivery of diclofenac.* European Journal of Pharmaceutics and Biopharmaceutics, 2003. **56**(3): p. 389-392.
91. Honeywell-Nguyen, P.L., S. Arenja, and J. Bouwstra, *Skin Penetration and Mechanisms of Action in the Delivery of the D2-Agonist Rotigotine from Surfactant-Based Elastic Vesicle Formulations.* Pharmaceutical Research, 2003. **20**(10): p. 1619-1625.
92. Honeywell-Nguyen, P.L. and J.A. Bouwstra, *The in vitro transport of pergolide from surfactant-based elastic vesicles through human skin: a suggested mechanism of action.* Journal of Controlled Release, 2003. **86**(1): p. 145-156.
93. Cevc, G., et al., *The skin: a pathway for systemic treatment with patches and lipid-based agent carriers.* Advanced Drug Delivery Reviews, 1996. **18**(3): p. 349-378.
94. Cevc, G. and G. Blume, *New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers, Transfersomes.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2001. **1514**(2): p. 191-205.
95. Cevc, G., A. Schatzlein, and H. Richardsen, *Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements.* Biochim Biophys Acta, 2002. **1564**(1): p. 21-30.
96. Schatzlein, A. and G. Cevc, *Non-uniform cellular packing of the stratum corneum and permeability barrier function of intact skin: a high-resolution confocal laser scanning microscopy study using highly deformable vesicles (Transfersomes).* Br J Dermatol, 1998. **138**(4): p. 583-92.
97. Ogiso, T., et al., *Effect of positively and negatively charged liposomes on skin permeation of drugs*. J Drug Target, 2001. **9**(1): p. 49-59.
98. Mezei, M. and A. Gesztes, *Liposomal local anesthetic and analgesic products*. 1990, Google Patents.
99. Gesztes, A. and M. Mezei, *Topical Anesthesia of the Skin by Liposome-Encapsulated Tetracaine.* Anesthesia & Analgesia, 1988. **67**(11): p. 1079&hyhen;1081.
100. Poonai, N., et al., *A Comparison of Amethocaine and Liposomal Lidocaine Cream as a Pain Reliever Before Venipuncture in Children: A Randomized Control Trial.* Pediatric Emergency Care, 2012. **28**(2): p. 104-108 10.1097/PEC.0b013e3182442c3b.
101. Friedman, P.M., et al., *Comparative study of the efficacy of four topical anesthetics.* Dermatol Surg, 1999. **25**(12): p. 950-4.
102. Taddio, A., et al., *Liposomal lidocaine to improve procedural success rates and reduce procedural pain among children: a randomized controlled trial.* Canadian Medical Association Journal, 2005. **172**(13): p. 5.

Part 3

Materials and Methods

Materials

1. Materials for preparation of vesicles

PL90G (Phospholipon® 90G from soybean, unsaturated, phosphatidylcholine > 95%), DSPG (Distearoyl phosphatidylglycerol) and DSPS (Distearoyl-phosphatidylserine) were kindly supplied by Lipoid GmbH (Ludwigshafen, Germany). Phosphatidylcholine (PC), purified soybean phosphatidylcholine (SPC) dissolved in ethanol (NAT8539, SPC: ethanol 75:25, w/w) was purchased from Phospholipid GmbH (Cologne, Germany). SUN70P and SUN90P, unsaturated, phosphatidylcholine purified from sunflower were kindly provided by Berg and Schmidt (GmbH & Co.) (Hamburg, Germany).

Hydriol® OP.3 (Oleic acid-PEG300-ester), Hydriol® OP.6 (Oleic acid-PEG600-ester), Hydriol® OP.10 (Oleic acid-PEG1000-ester) and Hydriol® OP.20 (Oleic acid-PEG2000-ester) were gifts from Hydrior AG (Wettingen, Switzerland). Moreover, Hydriol® PGMO.4 (Polyglyceryl-4-oleate), Hydriol® PGMO.5 (Polyglyceryl-5-oleate), and Hydriol® PGMO.10 (Polyglyceryl-10-oleate) were gifts from Hydrior AG (Wettingen, Switzerland).

Emulsense® (Brassicyl Isoleucinate Esylate) was kindly provided by Inolex Chemical Company. TEGO® CARE 450 (Polyglyceryl-3 Methylglucose Distearate) and TEGO® CARE CG 90 (Cetearyl Glucoside) were supplied by Evonik Industries AG. Sucrose oleate was a gift from Mitsubishi Chemical Corporation.

D-limonene, citral, 1,8-cineole, Tween80 and cholesterol were purchased from Sigma – Aldrich.

2. Materials for permeation and penetration experiments

Phosphate buffer solution (PBS) was composed of disodium hydrogenphosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$) (Roth, Germany), potassium dihydrogenphosphate (KH_2PO_4) (Roth, Germany), sodium chloride (NaCl) (Merck, Germany), potassium chloride (KCl) (Merck, Germany) and distilled water.

Carboxyfluorescein (CF) was purchased from Sigma-Aldrich (Steinheim am Albuch, Germany). All other chemicals used in this study were of analytical grade and purchased from Merck (Darmstadt, Germany) and used as a hydrophilic fluorescent marker. The molecular formula is $C_{21}H_{12}O_7$ and molecular weight is 376.32 g/mol. The chemical structure of CF is shown in ***Figure 9***.

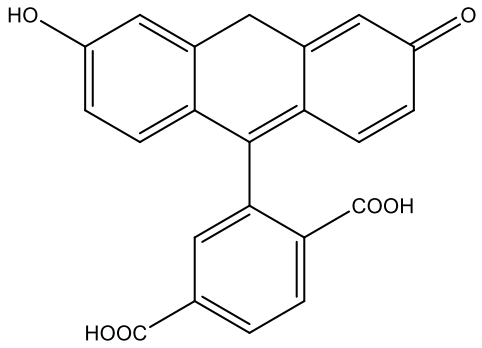


Figure 9 Chemical structure of carboxyfluorescein; 3′,6′-Dihydroxy-1-oxospiro [2-benzofuran.

Methods

1. Preparation of human skin models

Female human abdominal skin was kindly provided by Dr. Gruhl (Kassel, Germany) following plastic surgery. The subcutaneous fat tissue was removed from the skin using a scapel and surgical scissors as illustrated in ***Figure 10***. The full-thickness skin was obtained. Then, the skin was cleaned with PBS pH 7.4 and allowed to dry for about 20 min at room temperature. The prepared skin samples were wrapped in aluminium foil and stored at -20°C for later use. These samples have a shelf-life of 3 months for all experiments.

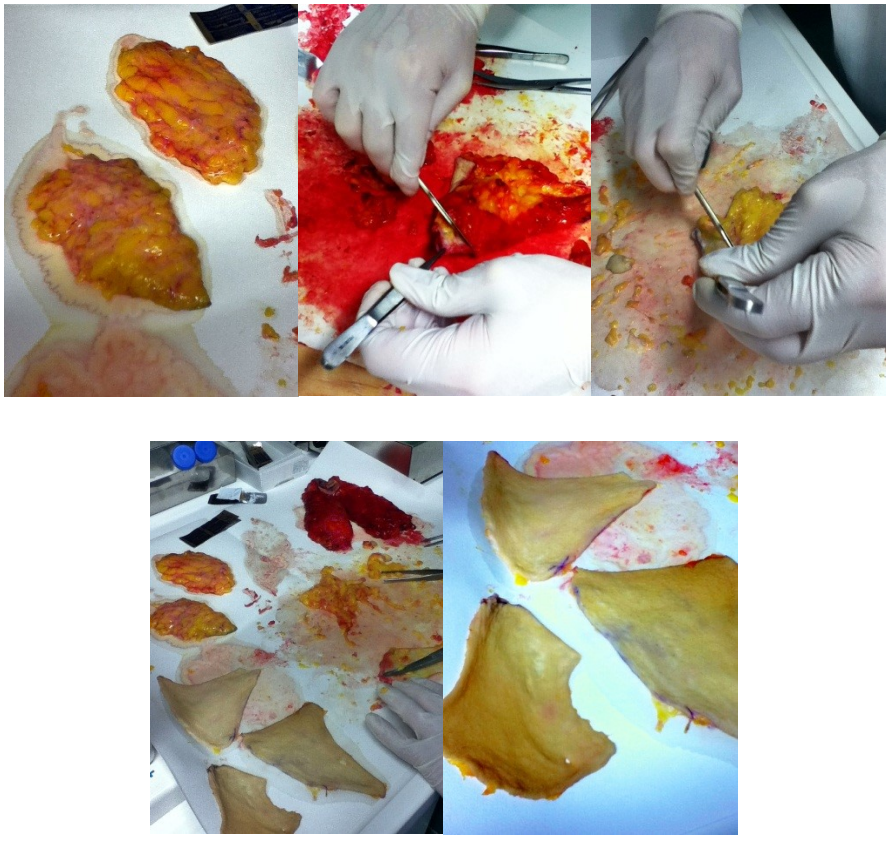


Figure 10 Removal the fat tissue from human abdominal skin

Before use, skin disks of 18 mm were punched out, cleaned with PBS (pH 7.4, 10mM) and thawed with the stratum corneum (SC) side up open to the atmosphere and the dermal side bathed with PBS for 2 h. Subsequently, transepidermal water loss (TEWL) (VapoMeter, Delfin Technology Ltd., Kuopio, Finland) of the skin disks were visually inspected to ensure that disks were free

form any surface irregularity such as holes. All investigations were conducted in full compliance with ethical principles, i.e. the studies were reviewed and approved by the Ethics Committee of the Friedrich-Schiller University Jena.

2. Preparation of liposomes

The liposomes containing PC and surface modifier were formulated and prepared essentially by a sonication method [101]. Briefly, the appropriate amounts of phospholipids (10%w/w in case of phospholipid liposomes) and surface modifiers (charged inducer agents, PEG-monooleate, PG- monooleate, sugar derivatives and penetration enhancers) in ratio of 9:1 (lipid: surface modifier in weight) were completely dissolved in 1 ml of ethanol/ PBS (total concentration of ethanol 16%w/w for all formulations), gently shaken for 2 min and then sonicated for 10 min in 10 cycles with a sonication ice-water bath. CF-containing liposomes were prepared in a similar way, 30mM CF was added instead of PBS (total concentration of CF 0.087%w/v for all formulations). Then these liposomal suspensions were extruded through polycarbonate membrane (diameter: 19 mm, pore diameter: 100 nm, Armatis, Schriesheim, Germany) 21 times with a Hamilton-Bonaduz extruder (GASTIGHT #1001, Bonaduz, Switzerland) to produce liposomes with homogenous sizes.

3. Liposomes characterizations

3.1 Particle size distribution and zeta potential measurement

The particle size, polydispersity index (PDI) and zeta potential of all lipid vesicles were determined by photon correlation spectroscopy (PCS, Zetasizer Nano series, Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK). Before size and zeta potential measurements, different liposome samples were diluted 20-times with filtered water prepared by using polycarbonate membrane (Minisart® syringe end filters 0.2 μm, Sartorious AG, Göttingen, Germany). Measurements were made at 25 ˚C with a fixed angle of 137°. Sizes quoted are the z-average mean for the liposomal hydrodynamic diameter.

The polydispersity index (PDI) was determined as a measure of homogeneity. Small values of PDI (< 0.3) indicate a homogenous population, while PDI > 0.3

indicate high heterogeneity [102]. For the measurement of zeta potential, the electrophoretic mobility of the dispersion was determined by laser Doppler velocimetry. The zeta potential values were calculated by the Smoluchowski approximation of Henry's equation. Calculation of zeta potential (mV) was done by the instrument from electrophoretic mobility.

3.2 Deformability study

The vesicle suspension was driven through a microporous filter by an external constant pressure of high pressure. The size of the vesicles was monitored by dynamic light scattering measurement before and after the filtration through a microporous filter with pore diameter 50 nm using a stainless steel pressure filter holder for 47 mm diameter filters, with 200 ml capacity barrel. Deformability value can be followed by the equation 2, which was developed by Bergh *et al*. (2001) [103].

$$D = J \times (r_v/r_p)^2 \qquad \textbf{\textit{Equation 2}}$$

Where J is the amount of vesicle suspension extruded (g) for 10 min, r_v the size of vesicles after extrusion (nm) and r_p the pore size of the barrier (nm). To measure J, the vesicles were extruded through a polycarbonate membrane (Nuclepore, Whatman Inc., MA, USA) with a pore diameter of 50 nm (r_p), at a pressure of 2.5 bar. After 10 min of extrusion, the extrudate was weighed (J), and the average vesicle diameter after extrusion (r_v) was measured by dynamic light scattering (DLS).

3.3 Percentage of Entrapment efficiency (%EE)

Free carboxyfluorescein was separated from entrapped CF using an ultracentrifugation method. The liposomal suspension (0.5 ml) was added to an Eppendorf tube (1 ml) and centrifuged at 4 °C at 14,000 ×g for 30 min. The non-entrapped CF in the filtrate was quantified the concentration by fluorescence spectrophotometry. Entrapment efficiency of CF was calculated indirectly from the amount of free CF, according to the following *equation 3*:

$$\text{Entrapment efficiency (\%)} = (1 - C_f/ C_t) \times 100 \qquad \textbf{\textit{Equation 3}}$$

with C_f as the amount of free CF and C_t as the total amount of CF.

3.4 Storage-stability studies

Samples were stored in tightly capped tubes at room temperature for 90 days. Physical stability studies of liposome formulations were carried out to investigate the particle size and percentage of CF remained in liposomes during storage. Samples from each were taken at time intervals of 0, 30, 60, and 90 days.

3.5 Skin permeability study

3.5.1 Ex vivo permeability of carboxyfluorescein

The skin samples were mounted in Franz diffusion cells under non-occlusive condition with the effective surface area of 0.5 cm^2 and a receptor volume of 5.7 ml. The dermal side of the skin was exposed to the receptor fluid and the SC remained in contact with the donor compartment left dry and open to the atmosphere. The temperature was maintained at 37 ± 0.1 °C, in order to maintain the skin surface at 32 °C, and the receptor solution was continuously stirred at 700 rpm with a small bar magnet placed inside the cell. Five µl of each liposomal formulation were applied onto the skin's surface and uniformly spread. Samples (1 ml) were taken from the receptor fluid at 3, 6, 12 and 24 h after application. At each point, the receptor cell content was replaced by 1 ml of fresh solution. The samples were diluted to proper concentration before measuring the amount of CF by spectrofluorometer. Three replicates were used for the study.

3.5.2 Ex vivo and in vivo tape-stripping of the stratum corneum

The *ex vivo* and *in vivo* skin penetration studies were carried out by the tape stripping technique in a modified form after Coderch *et al*. (1996) [104]. **Before this *ex vivo* measurement**, the excised human skin was stretched and mounted with pins on cork discs and covered with aluminium mask with a central hole of 8 mm in diameter. **In case of *in vivo* experiments**, two volunteers were tested on their own both (left and right) sides of forearm and were applied the tested formulations with a circle-shaped of 8 mm. The applied volume of liposomal suspension for each experiment was of 5 µl.

After 2 hours, the SC was successively removed by stripping with an adhesive tape (CristallKlarTesa®, Beiersdorf AG, Hamburg, Germany). Each tape was put onto the skin and a weight of 2 kg was placed on the tape for 10 s. Afterward the tape was rapidly removed with forceps and transferred into a glass vial of suitable size. Fifteen stripping procedures were performed consecutively. One ml of ethanol/ PBS pH7.4 (1:1, v/v) was added to each vial to extract the CF from the strip. Each vial was vortexed for 2 min and then sonicated for 2 min, after which they were kept overnight at room temperature under light protection. The strips were analysed for CF content by fluorescence spectrophotometry. The results were expressed as percentage of the applied CF dose penetrated into the skin.

3.5.3 Fluorescence assay of carboxyfluorescein

The concentration of CF was determined by fluorescence spectroscopy. Fluorescence detection was performed at an excitation of 490 nm and an emission of 520 nm in the case of CF. The method was validated for linearity, accuracy and precision. The linear range during the measurements for CF was from 0.1 to 4.0 µM ($r^2 = 0.99$). The software used was Optima, version 2.10, BMG Lab Tech.

3.6 Data analysis

All reported data are mean ± S.D. Statistical significance was interpreted by Student's t-test using Graph Pad Prism® Ver.6 software and considered to be significant at $P < 0.05$.

References

1 Muller RH, Keck CM: *Challenges and solutions for the delivery of biotech drugs--a review of drug nanocrystal technology and lipid nanoparticles*. Journal of biotechnology 2004;113:151-170.

2 Šentjurc M, Vrhovnik K, Kristl J: *Liposomes as a topical delivery system*: The role of size on transport studied by the epr imaging method. Journal of Controlled Release 1999;59:87-97.

3 van den Bergh BA, Wertz PW, Junginger HE, Bouwstra JA: *Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements*. Int J Pharm 2001;217:13-24.

4 Coderch L, Fonollosa J, De Pera M, Estelrich J, De La Maza A, Parra JL: *Influence of cholesterol on liposome fluidity by epr: Relationship with percutaneous absorption*. Journal of Controlled Release 2000;68:85-95.

Part 4

Results and Discussions

1. Phospholipids selection for preparing model liposomes

Phospholipids (amphipathic structure) are the main components for liposome production. Recently, various types of phospholipids have been used, each of which has advantages and disadvantages based on intended purpose of use. The purpose of this study was to examine the characteristics of phospholipids and select one model phospholipids which possesses preferable characteristics for further studies on surface modification of liposomes.

Vesicle size, charge, membrane elasticity, entrapment efficiency and drug penetration are factors that influence skin penetration of the liposomes [1-3]. Hence, these parameters were considered for selecting model liposomes for this dissertation. The particle size, especially small size, is a desirable physical property of vesicles, as it could enhance the skin penetration of drug as compared to large vesicles [4, 5]. Zeta potential – a key indicator of the magnitude of charge carried by the liposome – helps in gauging the stability of colloidal dispersions and drug penetration [6]. Ogiso *et al*. (2001) have previously shown that that drug penetration can be influenced by modifying the surface charge of liposomes [7]. To this effect, Gillet *et al*. (2011) observed that negatively charged liposomes exhibit the enhanced penetration of betamethasone when compared with neutral liposomes and positively charged liposomes [8], however, Manosroi *et al*. (2004) suggested that the positively charged liposomes might be the best formulation for amphotericin B, due to the highest flux in stratum corneum and higher stability than other formulations compared to negatively charged liposomes [9]. These effects indicate that the performance of the liposomes cannot be simply explained by charge but by several of the other factors such as payload and method of preparation of the liposomes.

In this study, we selected five phospholipids – namely PL90G, NAT8539, LPC20, SUN70P and SUN90P – to prepare liposomal formulations and investigate the physicochemical properties. Most natural phospholipids are mixed with the main component of the phospholipids being phosphatidylcholine (PC), lyso-PC and other residues (i.e., phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA)) [1]. The selected phospholipids were derived from plant source and vary in components as represented in ***Table 3***. Because of high

proportion of polyunsaturated fatty acid chains from plant source, these phospholipids are more flexible in lipid bilayer when compared to phospholipids extracted from egg yolk [1].

Table 3 Composition of different selected phospholipids [10].

Phospholipids	%PC	%Lyso-PC	%Others
PL90G	> 95	< 1	-
NAT8539	85	6	PI = 1.5 PE = 6 PA = 2
LPC20	32	50	PI =4 PE = 8 PA = 3.5
SUN70P	85.91	7.18	PI = 0.59 PE = 2.89 PA = -
SUN90P	40.07	16.54	PI = 2.42 PE = 2.22 PA = 18.81

PC (Phosphatidylcholine), Lyso PC (Lyso phosphatidylcholine), PI (Phosphatidylinositol), PE (Phosphatidylethanolamine), PA (Phosphatidic acid)

1.1. Selected physicochemical characteristics of liposomes

Physicochemical properties are a suitable indicator for the prediction of skin penetration efficiency of liposomal preparation. The evaluation of the physicochemical parameters of the liposomal formulations is a fundamental aspect to be investigated, due to their influence on the skin penetration behavior. The sizes, zeta potentials and deformability of the prepared liposomes are shown in ***Table 4***. The particle size analysis revealed that the size of all prepared liposomes was in the range of 33.33 ± 0.36 nm to 174.93 ± 2.41 nm, indicating that the most of them were in range of small unilamellar vesicles (SUVs) [1].

The other observation showed that the size of L3 was the largest among the tested vesicles. This might be due to the higher amount of lyso-PC in LPC20,

resulting in an increased particle size. The polydispersity index (PDI) of the prepared liposomes had values from 0.03 ± 0.05 (high homogeneity) to 0.37 ± 0.01 (lower and not in range acceptable homogeneity, since PDI < 0.3 [11]), indicating L5 had less homogenous population of vesicles as compared to the others. The zeta potential is related to the charge on the surface of the vesicle which influences both vesicular properties such as stability, as well as skin-vesicle interactions. In this study, L1, L4 and L5 possessed only a small negative surface charge and were therefore considered as neutral charge. Whereas L2 and L3 possessed a high negative surface charge (***Table 4***).

Table 4 The selected physical characterizations and liposomal compositions of non-entrapped phospholipid liposomes by vesicle size, PDI, zeta potential and deformability.

Rank	Vesicle properties		Zeta potential (mV) [a]	Deformability index (D) [a]
	Size (nm)[a]	PDI[a]		
1	174.93	0.37	-1.5	2.894
2	95.29	**0.28**	-2.55	**2.014**
3	69.48	0.22	**-5.75**	1.707
4	**65.37**	0.22	-40.16	1.26
5	33.33	0.03	-56.12	0.354

Code	L1	L2	L3	L4	L5
Composition	PL90G:EtOH =10:16	NAT8539:EtOH =10:16	LPC20:EtOH =10:16	SUN70P:EtOH =10:16	SUN90P:EtOH=10:16
	Chosen for further studies				

All prepared liposomal formulations were tested for their deformability by extrusion measurements. Results are expressed in terms of deformability index (D-value). This value is a unique parameter for elastic vesicles and represents the flexibility of vesicle membrane and stress-dependent adaptability. The minimum D value is '0' indicating that liposomal formulation are not able to pass through the polycarbonate membrane (a skin pore analogue) (amount of filtrate; $j = 0$) with a given pore size. On the other hand, the maximum D value has no limitation value,

depending on the capability of formulation passes through the membrane. The deformability of liposomes is also affected by the type of phospholipids used, which could be interpreted by the differences in their chemical structures. The D value of prepared liposomes decreased in the following order: *LPC20* > *PL90G* > *SUN90P* > *NAT8539* > *SUN70P*. The optimum deformability for the formulation was observed in LPC20 phospholipids (L3). This may be due to the high amount of lyso-PC (~ 50%w/w). Since the molecules of the type lyso-PC having a polar headgroup and single chain gives the molecule an overall conical shape, higher flexibility if incorporated in lipid vesicles compared with PC having two long chain of hydrophobic acyl hydrocarbon and one hydrophilic polar headgroup [1].

1.2. Entrapment efficiency

The physicochemical properties of CF entrapped liposomes are shown in ***Table 5***. The vesicle size distribution of all CF entrapped liposomal formulations has a diameter size in the range of 43.58 ± 0.56 – 175.77 ± 2.41 nm. Almost all formulations (L2 - L4) were marginally larger in vesicle size. These results may be the reason that CF molecules could increase the whole volume of vesicle core. Another property is entrapment efficiency. The entrapment efficiency (EE) was expressed as a function of the percentage of cumulative CF released (***Table 5***). The EE of CF ranged from 25.48 ± 7.88% to 35.10 ± 3.89% obtained from different liposomal formulations. The highest %EE was achieved by the PL90G liposomes (L1).

Table 5 Physicochemical properties of phospholipid liposomes entrapped with carboxyfluorescein (CF)

Vesicle size (nm)[a]	%Entrapment efficiency (%EE) [a]
175.77	**35.10**
82.21	30.77
70.21	28.18
44.6	27.12
43.58	25.48

1.3. Ex vivo skin permeation study

We investigated the delivery of CF into the *ex vivo* skin after topical application. Plots mean cumulative amount of CF released across abdominal fullskin from different liposomal formulations evaluated as a function of time are shown in ***Figure 12***. The cumulative amount of CF released at 24 h, the liposomal formulations evaluated in this study in the decreasing order of their ability to deliver the CF across fullskin was formulation *CF-L3* > *CF-L1* > *CF-L5* > *CF-L4* > *CF-L2*.

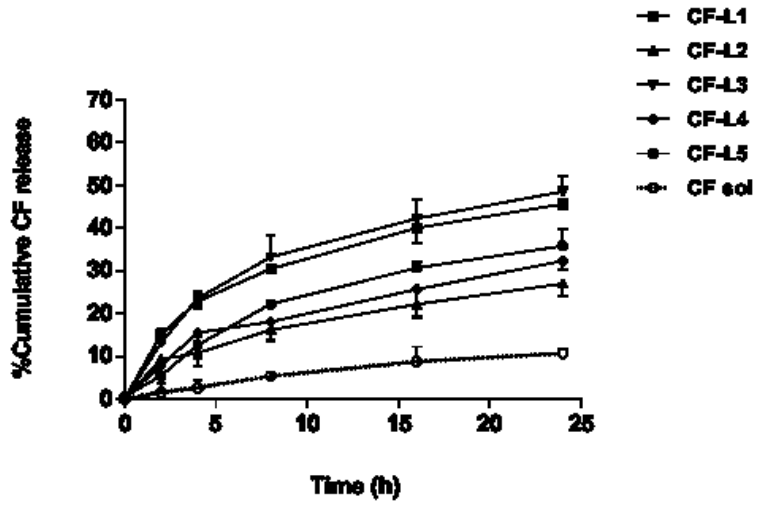


Figure 12 Skin permeation of CF after 24 h non-occlusive application of different CF formulations onto human abdominal skin (expressed as % cumulative of dose released ± S.D., *n*=3)

Comparison of the mean values using analysis of variance has revealed that they are significantly different ($P < 0.05$). Further, pairwise multiple comparison procedures (the Tukey's test) have revealed that the CF released across full thickness skin into receptor compartment was significantly lower ($P < 0.05$) than from all prepared liposomal formulations. There is an important to note that the type of phospholipids for liposomal preparation on the physicochemical properties (size, zeta potential, deformability) and penetration behavior of CF.

Even though, L3 shows the largest vesicles, it has the highest CF permeated through the fullskin. We speculate that the factors related to drug permeation enhancement not only include the phospholipid composition, but also particle size

and the interaction between lipid bilayer and deeper skin when penetrated through skin. However, these results disagree with Valenta and Janish (2003) who reported that small vesicles improved both deposition into and penetration through skin, with large vesicles improving deposition only [12]. NAT8539 and LPC20 liposomes exhibited high negative surface charge (***Table 4***) which eliminated them as the ideal candidates for the further study based on the minimum criterion decided – namely inability to clearly allocate the effects of surface modifications to the modifier or the native charge on the liposomes. On the other hand, SUN70P and SUN90P were observed to show low D values suggesting poor ability to penetrate to a great extent through the skin. We observed that among the liposomes synthesized PL90G exhibited an acceptable particle size, lowest negative zeta potential, high D value and high drug entrapment efficiency thus satisfying all criterion for our studies. Therefore, PL90G phospholipids was subsequently used for investigation of various surface modification treatments.

Apart from giving us the selected model liposomes for further experiments, this experiment illustrated the effects of lyso-PC upon some physicochemical properties of liposomes (size, zeta potential and deformability).

Table 7 Effect of LPC amount in PL90G phospholipid bilayer on characteristics of liposomes

% Lyso-PC [b]	Physicochemical properties		
	Particle size (nm)[a]	Zeta potential (mV)[a]	Deformability (D)[a]
3.2	67.21 ± 1.84	-10.18 ± 1.78	1.890 ± 3.22
8	89.77 ± 1.98	-36.85 ± 6.23	1.987 ± 2.85
16	120.65 ± 3.11	-48.12 ± 2.90	2.671 ± 1.89
32	169.09 ± 2.09	-57.88 ± 0.78	2.849 ± 2.44

[a] Average of three determinations ± standard deviation (SD)
[b] Lyso-PC; lyso phosphatidylcholine

With respect to component of phospholipids, PC is one of main component and the other one being is lyso-PC. Their utility in effectiveness of the formulations required further the investigations by incremental quantitative changes in concentration in the preparations. The effect of the amount of lyso-PC in PL90G phospholipid bilayer was expressed as shown in ***Table 7***. Interestingly, as we increased the concentration of lyso-PC into the vesicle bilayer, this resulted in increase in all the physicochemical parameters. We therefore concluded that lyso-PC alters the physicochemical characteristics of the liposomes. Lyso-PC molecule is generally known to result in micelle formation [1] but in our investigation we observed that lyso-PC did not result in micelle formation, but rather (only in the presence of phospholipids) formation of liposomes. This effect could be ascribed to interference of lyso-PC molecule in packing of the intercellular lipid bilayer which resulted in altered fluidization and polarity of the bilayer.

1.4. Conclusion

The conceptual framework of this dissertation is focused at understanding the effects of surface modification during the preparation method for liposomes on their performance for efficient skin penetration/permeation. Native charge of phospholipid can interfere with ascertaining the true effect of emulsifiers upon liposome performance. In order for emulsifier properties to be investigated, we consider the "neutral liposome" (as defined by Gillet *et al*. 2011) [13] as an ideal liposome candidate for our experiment. This allows us to understand the effects of such modifications (tested eventually) on liposome's characteristics and performance independent of the native charge possessed by the liposomes. Thus we propose that neutral liposomes are ideal candidates for estimation/ evaluation of effects of surface modifications. Membrane elasticity of liposomes is also one of the main parameters in addition to their size and surface charge discussed above. In order to investigate and compare the elasticity of our liposomes, all preparations were quantified and measured by their deformability index (D value) which enabled us to arrange our liposomes based on their membrane elasticity. A high value for this parameter represents high flexibility of the liposome membrane. Several studies have indicated a high deformability index can allow increased penetration efficiency for the claimed liposomes.

Based on these characteristics for our further studies we therefore define an ideal model liposome as

1) *preferable small size* (in range of small unilamellar vesicles)
2) *neutral charge*
3) *high deformability index and*
4) *high drug entrapment efficiency*

Type of phospholipids for preparing liposomal formulation can have an effect on the physicochemical properties directly through alteration of diameter size, deformability, entrapment efficiency and drug release. Considering the results from all above criteria, among five phospholipids, PL90G liposomes (L1) were selected for further experiment in this dissertation because of their acceptable small size, neutral charge, high elasticity of vesicle membrane and high drug entrapment efficiency.

References

1. New, R., *Liposomes: A practical approach*. 1990, The United State: Oxford University Press Inc.
2. Cevc, G., *Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery.* Crit Rev Ther Drug Carrier Syst, 1996. **13**(3-4): p. 257-388.
3. Cevc, G., A. Schätzlein, and G. Blume, *Transdermal drug carriers: Basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides.* Journal of Controlled Release, 1995. **36**(1–2): p. 3-16.
4. Verma, D.D., et al., *Particle size of liposomes influences dermal delivery of substances into skin.* Int J Pharm, 2003. **258**(1-2): p. 141-51.
5. Kohli, A.K. and H.O. Alpar, *Potential use of nanoparticles for transcutaneous vaccine delivery: effect of particle size and charge.* International Journal of Pharmaceutics, 2004. **275**(1–2): p. 13-17.
6. Sabin, J., et al., *Size and stability of liposomes: a possible role of hydration and osmotic forces.* Eur Phys J E Soft Matter, 2006. **20**(4): p. 401-8.
7. Ogiso, T., et al., *Effect of Positively and Negatively Charged Liposomes on Skin Permeation of Drugs.* Journal of Drug Targeting, 2001. **9**(1): p. 49-59.
8. Gillet, A., et al., *Liposome surface charge influence on skin penetration behaviour.* Int J Pharm, 2011. **411**(1-2): p. 223-31.
9. Manosroi, A., L. Kongkaneramit, and J. Manosroi, *Stability and transdermal absorption of topical amphotericin B liposome formulations.* Int J Pharm, 2004. **270**(1-2): p. 279-86.
10. GmbH, P., *Datasheet of Phospholipids*, P. GmbH, Editor. 2007: Nattermannallee 1 D-50829 Cologne.
11. Šentjurc, M., K. Vrhovnik, and J. Kristl, *Liposomes as a topical delivery system: the role of size on transport studied by the EPR imaging method.* Journal of Controlled Release, 1999. **59**(1): p. 87-97.
12. Valenta, C. and M. Janisch, *Permeation of cyproterone acetate through pig skin from different vehicles with phospholipids.* Int J Pharm, 2003. **258**(1-2): p. 133-9.
13. Gillet, A., et al., *Skin penetration behaviour of liposomes as a function of their composition.* Eur J Pharm Biopharm, 2011. **79**(1): p. 43-53.

2. Influence of surface charge on drug penetration performance of liposomes

For subsequent work of this dissertation we use PL90G phospholipid while emphasizing the importance of the neutral charge on the liposomes (phospholipid attributable). In order to estimate the actual impact of surface charge (independent of native charge) liposome selection stringency aids in pinpointing the actual charge related mechanism for effective drug penetration.

Liposomes used for measuring the influence of surface charge on skin penetration were generated using distearoyl phosphatidylglycerol (DSPG) and distearoyl phosphatidylserine (DSPS) – *negatively charged phospholipids*, coupled with two *cationic emulsifiers* – namely brassicyl isoleucinate esylate (EM) and quaternary ammonium (QA).

2.1. Selected physicochemical properties of liposomes

In our experiments, most of the charge inducer agents **(**with only exception of QA**)**, namely DSPG, DSPS and EM, **–** when incorporated into the liposomes **–** result in significant increase of the vesicle sizes as compared to *neutral* liposomes which have relatively smaller size **(***P* < 0**.**05**)** as shown in ***Table 9*****.** It was reported in some studies that this increase may be caused by an extraneous amount of these added inducers which increased the whole volume of lipid bilayer **[**1**].** Moreover, considering the PDI value, our results showed that incorporated *negative* charge decreased the PDI value of the liposomes in comparison with unmodified *neutral* liposomes. This decrease represents a narrower vesicle size distribution and improves the homogeneity of formulation. Conversely, incorporated *positive* charge increased the PDI value of the liposomes resulting in lower homogeneity of vesicle size which however remains in the range of acceptable PDI values (≤ 0.3) [2].

In term of zeta potential, we observed L1 to possess a slightly negative charge and with almost no surface charge making it comparable to *neutral* liposomes [1, 3]. L1_PS and L1_PG were observed to have a high *negative* charge on vesicle surface, while L1_EM and L1_QA possessed a high *positive* value surface charge

(***Table 9***). Moreover, the absolute values of the zeta potential of *negatively* – and *positively* – charged liposomes were in range above -/+ 30 mV for all charged liposomes, which indicates sufficient electrochemical stability for colloidal system [4].

Table 9 Physical characterizations of the different types of charged liposomes entrapping with 30mM CF in PBS **(**pH 7.4**) ***.

Vesicle properties		**Zeta potential (mV)**	**Deformability index (D) [a]**	**%Entrapment efficiency (%EE) [a]**
Size (nm)[a]	**PDI**			
167.1	**0.30**	**54.17**	**11.09**	**49.29**
160.9	**0.22**	**46.57**	**2.901**	**39.22**
112.2	**0.19**	**-7.45**	**2.322**	**35.10**
44.63	**0.15**	**-44.12**	**2.014**	**28.85**
30.21	**0.05**	**-55.28**	**1.674**	**27.00**

[a] Values represent mean ±SD **(**n = 3**)**

* Data including statistic in detail can be found in the *Appendix A (II)***.**

Code	***Neutral* liposomes**	***Negatively* charged liposomes**		***Positively* charged liposomes**	
Lipid bilayer composition (%w/w)	**L1**	**L1_PG**	**L1_PS**	**L1_EM**	**L1_QA**
	Only PL90G (10)	PL90G: DSPG (9:1)	PL90G: DSPS (9:1)	PL90G: EM (9:1)	PL90G: QA (9:1)

The essential characteristic of elastic/ flexible liposomes in comparison with classical liposomes and other type of colloidal systems is the *flexibility of vesicle membrane* – expressed in terms of deformability index (D) (***Table 9***) – under stress-dependent adaptability [5]. It should be noted, however, that L1 – despite being composed of only PC – showed in our results the slight deformability. This might be explained that PC molecules has resulted in more flexible in lipid bilayer; reason being their structure which contain also unsaturated fatty acid chain, i.e. oleic acid (C18:1) that, by itself, can act as penetration enhancer [6]. This finding is consistent with Williams and Barry (2012) [7].

This indicates that vesicle flexibility depends upon the additives, both in case of *negatively charged* phospholipids and *cationic* emulsifiers. D-values were higher for liposomes with DSPG, DSPS and EM than that of *neutral* liposomes (L1).

2.2. Entrapment efficiency

The assessment of capability to encapsulate for the liposomes was also investigated, in conjunction with CF (hydrophilic fluorescent dye) content in liposomal formulations. The entrapment efficiency (EE) was observed to be higher for both *negatively* charged liposomes (L1_PS and L1_PG) when compared to the *neutral* liposomes, while the *positively* charged liposomes (like L1_EM and L1_QA) showed lower relative drug entrapment. Being *cationic* emulsifiers, EM and QA markedly decreased the EE. Such behavior has been encountered in previous studies with one possible reasoning being that positive charged inducer agent induces a repulsion within the lipid bilayer possible [8] and another being interaction between positive charged inducer and lipid vesicles, thus destabilizing the packing characteristics and fluidizing properties of the lipid bilayer [9].

2.3. Ex vivo skin penetration study

We realize that a simple agreement of physicochemical properties and entrapment efficiency does not comprehensively explain effects upon actual human skin. Therefore, we undertook studies examining penetration of CF through skin of *negatively-, positively-* and *neutral* charged liposomes are applied. All liposomal formulations were directly applied to human abdominal skin mounted in a vertical Franz's diffusion cell. Penetration studies were carried out under non-occlusive conditions to allow the driving force provided by the osmotic gradient. The penetration profile is illustrated in ***Figure 13***. We used CF in the same concentration of CF entrapped in liposomes as a reference sample.

Upon 24 h incubation, small amount of CF (~10%) from CF solution was observed to permeate into the receptor compartment with most of fluorescent dye remaining on the skin. However, this was not the case for all liposomal formulations. The high penetration efficiency of charged liposomes compared to solution of drug corroborates previous finding [10]. The cumulative amount of CF permeated in the *positively-*, *negatively-*, and *neutral* charged liposomes were significantly higher than in the CF solution (~10%, $P < 0.05$), however we do not observe differences in cumulative amount of CF permeated between L1_PG, L1

and L1_QA (50.19, 45.61 and 44.11%, respectively). Such variations in CF release could be explained by variations in the headgroup of additive present in lipid bilayer effected by surface charge modifiers which has also been observed in previous studies [3, 11, 12].

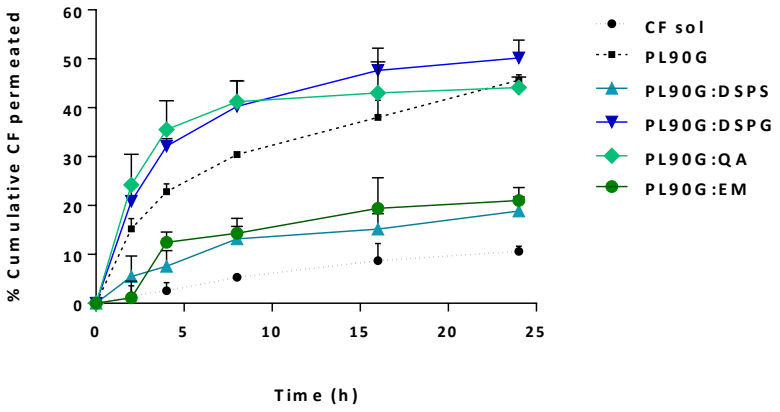


Figure 13 **%**Cumulative amount of CF permeated through full skin after 24 h**.** Applied with finite dose of 5 µl each liposomal formulation onto abdominal skin at 32°C under non-occlusive condition of as function of time **(**h**)**.

Despite L1_EM showing the highest deformability index and being more flexible (as compared to the other formulations), it surprisingly does not exhibit a corresponding high CF penetration into the receptor fluid. In agreement, L1_EM showed a reduction in cumulative CF permeated after 24 h. We suspect that the positive headgroup of EM in L1_EM and some protein molecule within the skin layers cause a lowered CF release. In summary, penetration of substances through skin does not depend merely on specific liposome characteristics (e.g., small particle size, more flexibility) but also on the chemical structure of emulsifier incorporated into the lipid bilayer. Of equal and crucial importance, our results concerning the *ex vivo* penetration interestingly showed that, even though the L1_PG (negatively charged) can enhance the CF penetration through skin better than neutral liposomes (L1), L1_PS – containing also a negative charge – in contrast showed even worse penetrability than neutral liposomes. This finding – which has never been reported by any other researches in the field – could be the result of *not only* the surface charge affecting the penetrability, *but also* the reaction between

other additives incorporated into lipid bilayer and the phospholipids, resulting in the change of conformation in lipid bilayer and the penetration enhancement.

2.4. Conclusion

Not only does the surface charge of the liposomes play a dominant role for the interaction between vesicles and skin, it also contributes substantially to the drug penetration through deeper skin layers. We report that among the charged liposomal formulations tested in our experiments, *only* the negatively-charged liposomes formulated by using DSPG can enhance the CF permeation through the skin. Nonetheless, apart from the aforementioned discussion, a comprehensive and complete understanding of the intricate mechanism of this penetration event is still missing. We thus suggest that the incorporation of charged components (positive or negative) into lipid bilayer for enhanced skin penetration as illustrated by previous works [3, 12-17] is more complex an event than previously anticipated. In order to understand this point more accurately and in deeper detail, further specific and advanced experiments (e.g. Nuclear Magnetic Resonance (NMR)) focusing on the interaction between PC molecules and charged inducer agents in the lipid bilayer are needed. Furthermore, deformability index investigated in our studies is also not able to explain high penetration efficiencies convincingly suggesting the importance of factors other than lipid proportions governing this phenomenon. More importantly, our studies show that surface charge ascribable to emulsifiers does not govern penetration efficiencies, rather, the head group property (in term of chemistry) along with characteristics of drug being delivered can be decisive in determining penetration efficiency. There exists *ipso facto* a discrepancy between two groups of experiments as to *how* the different surface charge of liposomes affects the drug penetration through the skin.

References

1. Fang, J.-Y., et al., *Enhancement of the transdermal delivery of catechins by liposomes incorporating anionic surfactants and ethanol.* International Journal of Pharmaceutics, 2006. **310**(1–2): p. 131-138.
2. Verma, D.D., et al., *Particle size of liposomes influences dermal delivery of substances into skin.* Int J Pharm, 2003. **258**(1-2): p. 141-51.
3. Dragicevic-Curic, N., et al., *Surface charged temoporfin-loaded flexible vesicles: In vitro skin penetration studies and stability.* International Journal of Pharmaceutics, 2010. **384**(1–2): p. 100-108.
4. Greenwood, R. and K. Kendall, *Selection of Suitable Dispersants for Aqueous Suspensions of Zirconia and Titania Powders using Acoustophoresis.* Journal of the European Ceramic Society, 1999. **19**(4): p. 479-488.
5. Mishra, D., et al., *Elastic liposomes mediated transdermal delivery of an anti-hypertensive agent: propranolol hydrochloride.* J Pharm Sci, 2007. **96**(1): p. 145-55.

6. Kanikkannan, N., R.J. Babu, and M. Singh, *Structure-Activity Relationship of chemical Penetration Enhancers*, in *Percutaneous Penetration Enhancers*, E.W. Smith and H.I. Maibach, Editors. 2006, Taylor & Francis Group: 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL 33487-2742. p. 17-33.
7. Williams, A.C. and B.W. Barry, *Penetration enhancers.* Advanced Drug Delivery Reviews, 2004. **56**(5): p. 603-618.
8. Fang, J.-Y., et al., *Effect of liposomes and niosomes on skin permeation of enoxacin.* International Journal of Pharmaceutics, 2001. **219**(1–2): p. 61-72.
9. El Zaafarany, G.M., et al., *Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery.* Int J Pharm, 2010. **397**(1-2): p. 164-72.
10. El Maghraby, G.M.M., A.C. Williams, and B.W. Barry, *Skin delivery of oestradiol from lipid vesicles: importance of liposome structure.* International Journal of Pharmaceutics, 2000. **204**(1–2): p. 159-169.
11. Gillet, A., et al., *Liposome surface charge influence on skin penetration behaviour.* Int J Pharm, 2011. **411**(1-2): p. 223-31.
12. Manosroi, A., L. Kongkaneramit, and J. Manosroi, *Stability and transdermal absorption of topical amphotericin B liposome formulations.* Int J Pharm, 2004. **270**(1-2): p. 279-86.
13. Katahira, N., et al., *Enhancement of Topical Delivery of a Lipophilic Drug from Charged Multilamellar Liposomes.* Journal of Drug Targeting, 1999. **6**(6): p. 405-414.
14. Montenegro, L., et al., *In vitro retinoic acid release and skin permeation from different liposome formulations.* International Journal of Pharmaceutics, 1996. **133**(1–2): p. 89-96.
15. Ogiso, T., et al., *Effect of Positively and Negatively Charged Liposomes on Skin Permeation of Drugs.* Journal of Drug Targeting, 2001. **9**(1): p. 49-59.
16. Sinico, C., et al., *Liposomes as carriers for dermal delivery of tretinoin: in vitro evaluation of drug permeation and vesicle-skin interaction.* J Control Release, 2005. **103**(1): p. 123-36.
17. Yoo, J., et al., *Skin penetration and retention of L-Ascorbic acid 2-phosphate using multilamellar vesicles.* Archives of Pharmacal Research, 2008. **31**(12): p. 1652-1658.

3 Surface modification using polymer coating of liposomes

Surface modifications of skin penetrable liposomes may have an effect on the enhancement of drug penetration through the skin. Here we evaluated one such method of enhancing drug delivery across skin by addition of polymer emulsifiers into the lipid bilayer.

Such polymer additions to the lipid bilayer are expected to enhance the colloidal stability of conventional liposomal formulation for topical administration and markedly alter the physicochemical properties, especially drug penetrability. To this end, we measured this enhancement of liposomal function using two *hydrophilic polymer* groups – namely *polyethyleneglycol (PEG)* mono-ester and *polyglycerol (PG)* mono-ester.

3.1. Polyethyleneglycol (PEG) mono-oleate group

Information about PEGylated liposome for topical application is obscure in hitherto published literature. To the best of our knowledge only two studies reported PEGylated liposomes to have enhanced penetration of low molecular weight drug into deeper skin layers [1, 2]. Traditionally, DSPE-PEG has been used for such modifications which possess two fatty acid chains thus producing much larger particle size as compared to single fatty acid chain with a consequent rise in production costs. PEGylated liposomes considered here are comprised of unsaturated phosphatidylcholine derived from soy bean which is the main component and PEG_x-mono-oleate (single chain surfactant), which provides the necessary physicochemical properties, especially deformability of these vesicles.

This study demonstrates the influence upon penetration characteristics of liposomes due to incorporation of surfactants like PEG300-, PEG600-, PEG1000-, and PEG2000-monooleate. (Note: All preparations consist of 9% phospholipids, 1% PEG-ester surfactant, 16% ethanol ad to 100% with CF solution or PBS by weight.)

3.1.1. Selected physicochemical properties of these liposomes

A particle size analysis revealed that the size of all investigated vesicles was in the range of 44 nm to 96 nm (***Table 10***), which indicated that these vesicles were of acceptable size. (In principle, a small size is a highly desirable size since it enhances penetration into the skin [3].) However, we noted that the mean particle size of PEGylated liposomes was greater than non-PEGylated liposomes due to an increase in bilayer surface hydrophilicity caused mainly by incorporation of PEG-esters. The polydispersity index (PDI) values of all investigated vesicles range from 0.09 to 0.30. Moreover, all PEGylated liposomal formulations had PDI values well below 0.20 as compared to non-PEGylated liposomes a phenomenon not reported in previous studies [4] which tested variations of PEG2000: DSPE ratio in the investigated liposomal preparations. These vesicles were of uniform size which as a factor was independent of the quantity of PEG2000-DSPE incorporated.

Table 10 Characterization of different liposomes (Data with standard deviation are presented in *Appendix A (III)*).

Rank	Vesicle properties		Zeta potential (mV)	Deformability index	% Entrapment efficiency(%EE)
	Size (nm)	PDI			
1	95.31	**0.30**	**-7.45**	3.67	41.09
2	89.24	0.20	-9.58	2.03	39.55
3	64.23	0.15	-11.77	**2.01**	37.67
4	60.14	0.14	-12.58	1.07	37.30
5	**44.60**	0.09	-12.63	0.93	**35.10**

Code	**L1**	**L1_PEG300**	**L1_PEG600**	**L1_PEG1000**	**L1_PEG2000**
Lipid bilayer composition (%w/w)	PL90G (10)	PL90G:PEG300 (9:1)	PL90G:PEG600 (9:1)	PL90G:PEG1000 (9:1)	PL90G:PEG2000 (9:1)

Both vesicular stability and skin-vesicle interactions are also functions of zeta potential, which describes the surface charge. Specifically, the absolute values of the zeta potential for *negatively* - and *positively* - charged liposomes should be in the range above -/+ 30 mV respectively for sustaining colloidal stability [5]. The

mean surface potential of PEGylated liposomes differs by -7.45 mV as compared to non-PEGylated vesicles and by -12.63 mV for PEG300-modified vesicles. This incorporation of PEG-monooleate into lipid bilayer though slightly affects the total surface charge of liposomes.

Further parameter, the capacity to pass through the skin barrier is dependent on the vesicle bilayer fluidity, reported as D value). ***Table 10*** shows a strong impact of PEG-monooleate addition on the deformability index, which in turn was related to the chain length of the polymer. *L1_PEG300* formulation exhibited a markedly higher deformability index followed by unmodified liposomes (*L1*) and *L1_PEG600*. Furthermore, low deformability was observed in long PEG-chain modified liposomes; *L1_PEG1000*and *L1_PEG2000*. A *short PEG-chain molecule (PEG300)* embedded into the bilayer can increase the fluidity of the vesicle membrane (addition of edge activators) than a *longer PEG-chain* due to increases steric hindrance and shows a lower penetration.

Increased drug entrapment efficiency in carrier vesicles can achieve a stronger therapeutic effect at the target site. As shown in ***Table 10***, there were no significant differences in the entrapment efficiency (EE) between *L1_PEG300* and *L1_PEG2000*. The highest EE of CF was determined in *L1_PEG600* with 41.09%, which was higher than that in non-PEGylated ones.

3.1.2. Stability studies

We also investigated the stability of liposomes in terms of mean particle size and entrapment efficiency after storage for 3 months at room temperature. The two PEGylated liposomal formulations composed of short chain PEG (*L1_PEG300* and *L1_PEG600*) maintained their vesicles sizes at low PDI value (PDI < 0.20) in contrast to the *L1_PEG1000* and *L1_PEG2000* liposomes (***Figure 14***(A)). However, in non-PEGylated liposomes, a strong increase in average particle size was observed which was accompanied by a high heterogeneity of the vesicles (PDI >> 0.30). This can be caused by aggregation, which results in a close proximity of vesicles surfaces and can result in fusion of liposome membrane, due to instability of the bilayer [6].

Figure 14(B) shows the liposomal stability in term of percentage of retained encapsulated CF which was only observed with *L1_PEG300*, while other formulations showed a decrease in CF entrapment over the time period of 3 months. We observe a marked increase of % CF leakage in *L1_PEG1000*, which could not be explained.

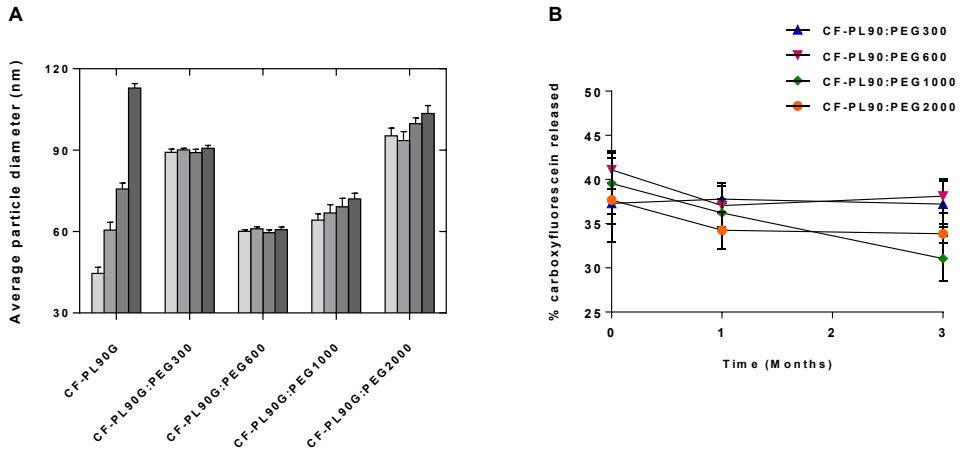


Figure 14 Colloidal stability of liposomes upon storage at room temperature. (A) The average size of the liposomes for 0 ( ), 1 ( ), 2 ( ), and 3 months ( ). (B) The percentage of carboxyfluorescein of liposomes over a period of 3 months.

3.1.3. Ex vivo skin permeation study

The permeation enhancing effect for hydrophilic substances could be higher importance than in case of lipophilic drugs, due to relatively lower permeation of hydrophilic molecules and hence needing more enhancement [7, 8]. Several studies suggest a possible permeation enhancing mechanism for surfactant-based elastic vesicles [9] and different kinds of deformable liposomes [10-15]. Therefore, we investigated whether the modification of the liposomal surface with different PEG molecules has an impact on the permeation of CF through the skin.

Figure 15 shows *ex vivo* skin permeation profiles conducted with different formulations of CF. Two references were used: a solution of CF and PL90G liposomes loaded with CF (CF-PL90G). Cumulative CF amount that permeated the skin in a time period of 24 h after non-occlusive application was highest for CF-PL90G:PEG300 liposomes (49.63 ± 1.26%), followed by the pure CF-PL90G liposomes (39.45 ± 2.01%), CF-PL90G:PEG600 liposomes (38.53 ± 1.91%) and at

the end CF-PL90G:PEG1000 liposomes (27.33 ± 1.88%) and CF-PL90G:PEG2000 liposomes (24.36 ± 1.34%), respectively.

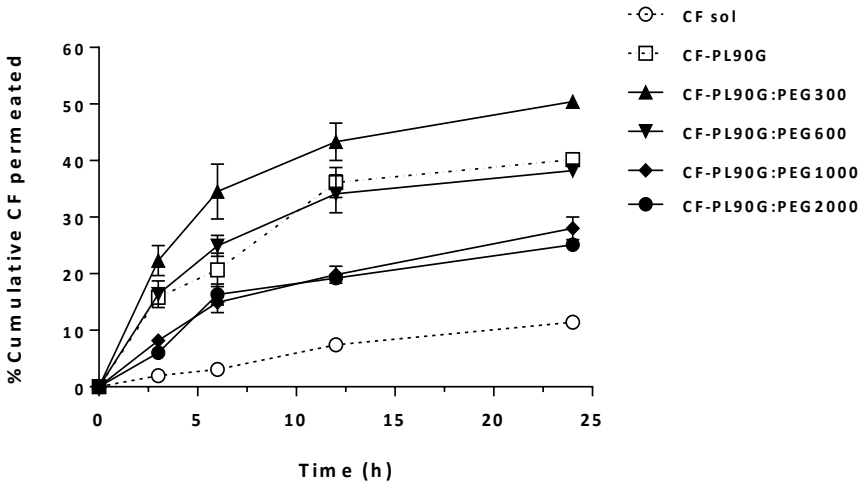


Figure 15 Percentage of carboxyfluorescein permeated from liposomes encapsulating carboxyfluorescein as a function of time. All values represent as mean ± SD (*n=3*).

3.1.4. Ex vivo and in vivo tape stripping study

Figure16 shows the amount of CF determined upon strips 1st, 5th, 10th and 15th. The other strips were not considered in order to maintain a feasible sample number (data not shown). The result showed that the amount of CF decreases with the number of strips taken from skin. As shown in ***Figure 16***, for the first strip, we observed a high difference in CF content between the CF solution and the PL90G:PEG300 liposomes. We see that PL90G:PEG300 liposomes was the best formulation when compared to the other formulations. Trebilcock *et al*. (1994) studied the correlation between *in vitro* and *in vivo* tape stripping method and observed the penetrant amount reduced gradually with increasing depth in both *in vivo* and *in vitro* situations, with enhanced rapidity for the first five strips [16]. The observation of this gradient was a result of the reduced structural integrity of the stratum corneum near the skin surface, hence more skin is removed in the first few strips. The amount of CF deposited in human skin strip 15th (see enlargement in ***Figure 16***) consistently high for *in vitro* skin penetration studies at 24h. (Note: A good correlation could be shown between the CF amount in strip 15 and % CF released in 24h.).

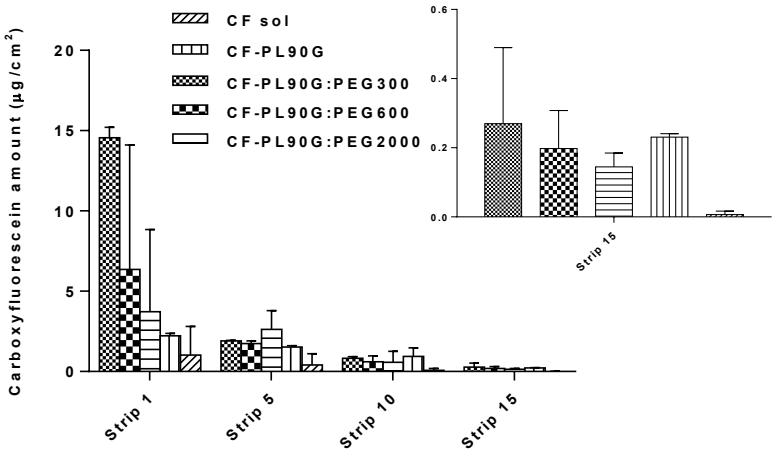


Figure 16 Amount of carboxyfluorescein as a function of the strip number (µg/cm^2). CF sol: solution of carboxyfluorescein in mixture solvent (ethanol: PBS, pH7.4) and CF-PL90G: PL90G liposomes loaded with carboxyfluorescein (without PEG surfactant) (n=3).

The biological variation of the human skin resulted in large standard deviations and therefore we incorporated replicates in the experimental design (*n*=3). The *ex vivo* penetration study used PEG surfactants incorporated into to measure influence on skin permeation. Moreover, the chain length (300 to 2000) of PEG molecules embedded in lipid bilayer influenced particle size, deformability, and showed a decreased CF permeation through skin. An increased permeation of CF from *PL90G:PEG300 liposomes*, compared with non-PEGylated liposomes, were in accordance with the study by Jain *et al*. [1], which reported an increased drug permeation across rat skin by PEGylated liposomes. In this investigation, the amount of CF permeated from *PL90G:PEG2000* liposomes was similar to CF-PL90G liposomes in both *in vitro* Franz diffusion study and *ex vivo* tape stripping study. There were no differences between the profiles measured by both studies. This might be resulting from decreased permeation of CF through the skin. Even though the mean diameter of PL90G:PEG2000 liposomes is about 95.31 nm (approximate to PL90G:PEG300 liposomes, 89.24 nm), PL90G:PEG2000 liposomes did not easily pass through the skin. This may be due to their ability to draw water into molecules of PEG chain, forming a water boundary around the vesicles. Furthermore, the skin permeation results were consistent with the

deformability studies (as shown in ***Table 10***), showing a low deformability value (D = 0.93) as compared with CF-PL90G liposomes (as control).

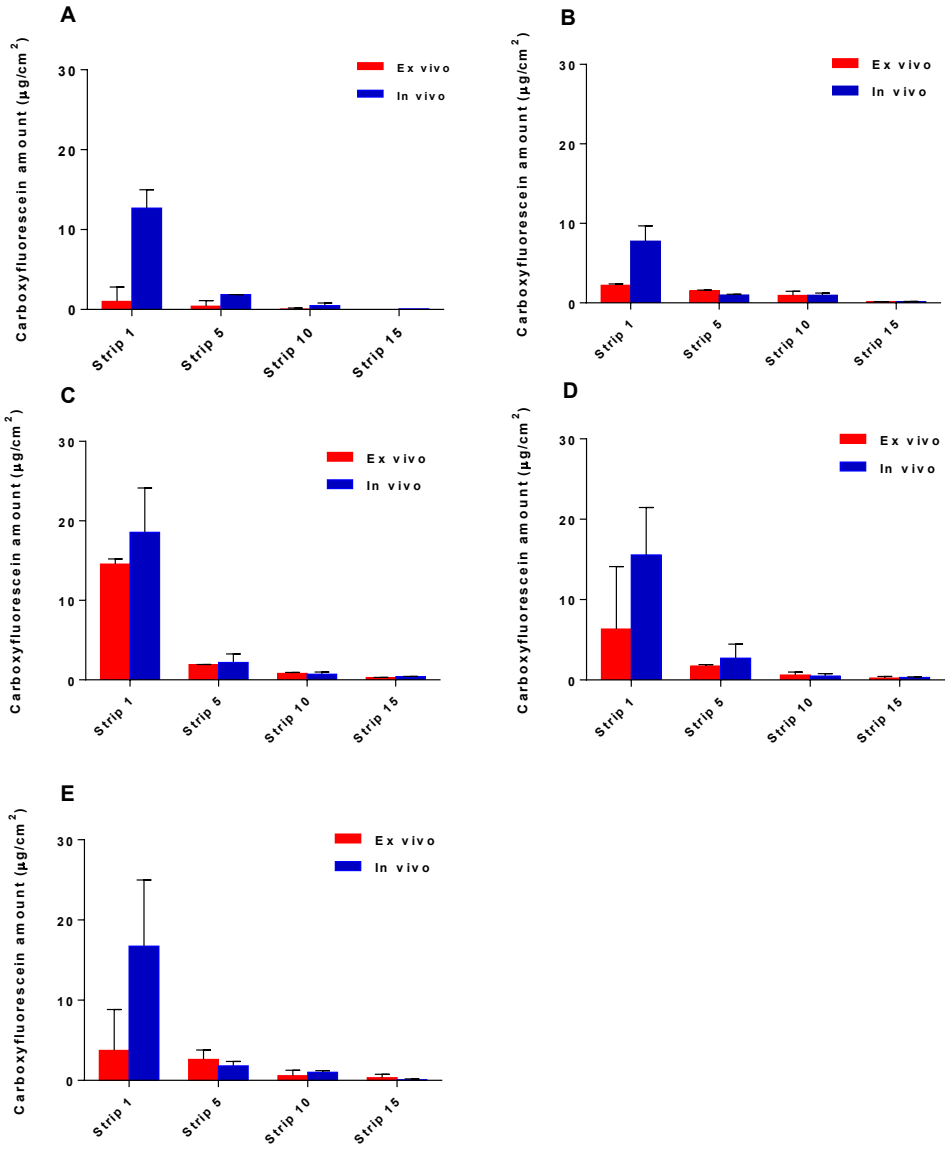


Figure 17 Tape stripping permeation study of carboxyfluorescein, *ex vivo – in vivo* correlation. A: Tape stripping study of *CF-solution*, B: Tape stripping study of *PL90G*, C: Tape stripping study of *PL90G:PEG300*, D: Tape stripping study of *PL90G:PEG600* and E: Tape stripping study of *PL90G:PEG2000*, respectively.

Skin tape stripping results obtained *ex vivo* and *in vivo* with CF in mixture solvent were also compared in ***Figure 17***. They showed a correlation of permeation between excised human skin (*ex vivo*) and human skin (*in vivo*) studies in these

liposomal formulations. Concerning the amount of CF permeated through the skin, *in vivo* tape stripping systematically showed a higher CF amount skin of penetration than *ex vivo* tape stripping study.

According to the results, an increase in polyethyleneglycol (PEG) chain length decreased the amount of CF permeated through human skin. These observations are in agreement with the results, obtained by deformability studies as shown in ***Table 10***. To verify the independence of deformability behavior from the molar ratio of phospholipid to PEG-ester is 1: 0.147 in case of PL90G:PEG300 liposomes and only 1: 0.038 in case of PL90G:PEG2000 liposomes, we examined variations in ratios of PL90G and PEG-esters for deformability.

Table 11 Effect of PEG concentration in liposomal formulations on physical properties of PEGylated liposomes.

Formulation Code	Molar ratio	Particle size (nm)	PDI	Deformability index (D)
PL90G:PEG300 [a]	1 : 0.038	85.65 ± 1.20	0.10 ± 0.09	1.95 ± 0.32
PL90G:PEG300 [a]	1 : 0.147	89.24 ± 1.21	0.14 ± 0.01	3.67 ± 0.03
PL90G:PEG2000[b]	1 : 0.038	95.31 ± 0.84	0.15 ± 0.11	0.93 ± 0.20
PL90G:PEG2000[b]	1 : 0.147	91.63 ± 0.44	0.20 ± 0.07	0.66 ± 0.18

Values represent mean ± S.D. (n = 3)
[a] PL90G liposomes composed of PEG300
[b] PL90G liposomes composed of PEG2000

As shown in ***Table 11***, an increase in the molar ratio of PEG-ester surfactant into phospholipid bilayer did not change the size of vesicles. Increasing the amount of the short PEG-chain ester into the vesicle bilayer rather led to an improvement in the deformability index which represents the penetration capability through the skin. This single chain emulsifier with only PEG 6 units may act as edge activator increasing the flexibility of the vesicles thus improving their ability to squeeze through artificial membranes with pore size ½ of vesicle size. Van den Bergh *et al*. [9] also described this phenomenon with an increased addition of PEG-8-laurate into vesicles formed by sucrose laurate with a concordant improvement in elasticity values achieved. In contrast to these findings, the PEG 2000 liposomes with the higher ratio in the polymer content exhibited less deformability. That can be due to

more pronounced steric hindrance at the particle surface increased by the immobility of the PEG chains.

Polymer-grafted lipid membranes (PEG2000-DSPE/ PEG5000-DSPE) showed a distinct molecular interaction at the lipid bilayer surface compared to non-modified ones [2]. The thickness of simple phospholipid/water interface is approximately $d_p \sim 1$ nm and rises to 5-10 nm when long-chain PEG-molecules were embedded in the membrane [10, 17]. A possible explanation for the differences in drug skin permeation of polymer-grafted liposomes is the difference in surface polarity combined with the steric hindrance on the particle surface. Cevc and Blume (1992) [10, 17] reported that transfersomes can penetrate through the skin owing to the transdermal osmotic gradients and hydration force. If the osmotic gradient is lowered by strong hydration on the vesicle surface due to the incorporation of long-chain PEG-esters, the permeation of PEG1000/ PEG2000 liposomes can also be diminished. There seems to be an exclusion size in the molecular weight of the PEG-esters: MW ~ 300 showed penetration enhancing effects and MW > 1000 suppressed the ability of penetration. An interesting factor might be the addition of selected PEG-ester being able to trigger the penetration depth as well as the kinetic of modified liposomes.

3.2. Polyglycerol (PG) mono-oleate group

Why we selected the group of polyglycerol (PG) emulsifiers for incorporating into lipid bilayer of liposomes? This study compares various polymer emulsifiers to find an alternative to PEG emulsifier embedding into lipid bilayer of liposomes for cosmetic production. However polymers – e.g., poly(glycerol-derived polymers [18], polyvinyl pyrollidone [19], and polyvinyl alcohol [20] – have been approved to be alternatives to PEG in preparing long circulating liposomes [21] for pharmaceutical purposes.

Therefore, the main purpose of this study was to modify the surface of liposomes using PG for improving hydrophilic fluorescent dye through skin using *ex vivo* skin penetration. The effect of observed differences of polyglyceryl chain length in lipid bilayer on physicochemical properties and skin permeation profiles were examined.

3.2.1. Physicochemical properties of liposomes

The current study demonstrated that it is possible to influence the penetration of liposomes by surfactants like PGMO4 (MW 500), PGMO5 (MW 575), and PGMO10 (MW 947). As shown in ***Table 12***, particle sizes of all investigated vesicles were in the range of 29 nm to 51 nm, indicating that vesicles were of a small size. This is a highly desirable attribute for the ability of penetration into the skin. Clearly, when increasing the polyglyceryl group from 4 to 10, a decrease in mean diameter of vesicles was detected.

The polydispersity index (PDI) of the investigated vesicles had values from 0.13 to 0.30. Moreover, all PGMO liposomal formulations were homogenously with low PDI values well below 0.30, as compared to conventional liposomes.

Table 12 Characterization of different liposomes (Data with standard deviation are presented in *Appendix A (III)*).

Rank	Vesicle properties		Zeta potential (mV) [e]	Deformability index (D)	%Entrapment efficiency(%EE) [e]
	Size (nm)[e]	PDI [e]			
1	50.32	0.30	-4.81	2.40	40.21
2	44.60	0.25	-7.45	2.01	38.44
3	35.07	0.18	-7.99	1.85	35.10
4	29.12	0.13	-14.1	1.70	32.48

Code	L1	L1_PGMO4	L1_ PGMO5	L1_ PGMO10
Lipid bilayer composition (%w/w)	Only PL90G (10)	PL90G:PGMO4 (9:1)	PL90G:PGMO5 (9:1)	PL90G:PGMO10 (9:1)

The zeta potential is related to the charge on the surface of the vesicle which may influence both vesicular properties such as stability, as well as skin-vesicle interactions. The result show that a high negative charge on L1_PGMO10 whereas L1_PGMO4 and L1_PGMO5 show decrease negative charge which also follows the trends of polyglyceryl group (***Table 12***).

Several reports in dermal drug delivery pointed out the capability of vesicles passing through the skin barrier is dependent of the vesicle bilayer fluidity. As shown in ***Table 12***, the addition of PG-mono-oleate had an effect on the deformability index depending on the chain length of the polymer. One formulation, CF-PL90G:PGMO4 exhibited a significantly higher deformability index, followed by CF-PL90G:PGMO5 and CF-PL90G:PGMO10, respectively. It might be explained that short PG chain molecule (PGMO4) embedded into the bilayer can increase the fluidity of the vesicle membrane (addition of edge activators), and as longer the PG-mono-oleate chain is as more steric hindrance will arise and lowers the penetration.

Drug entrapment efficiency in carrier vesicles can achieve a stronger therapeutic effect at the target site. As shown in ***Table 12***, there were no significant differences in the entrapment efficiency (EE) between the formulations.

3.2.2. Stability studies

After 3 months of storage at room temperature with light protection, the two PG-mono-oleate liposomal formulations composed of short chain PG (CF-PL90G:PGMO4 and CF-PL90G: PGMO5) still retained their vesicles sizes at low PDI value (PDI < 0.20) in contrast to the CF-PL90G:PGMO10 liposomes (as shown in ***Figure 18***).

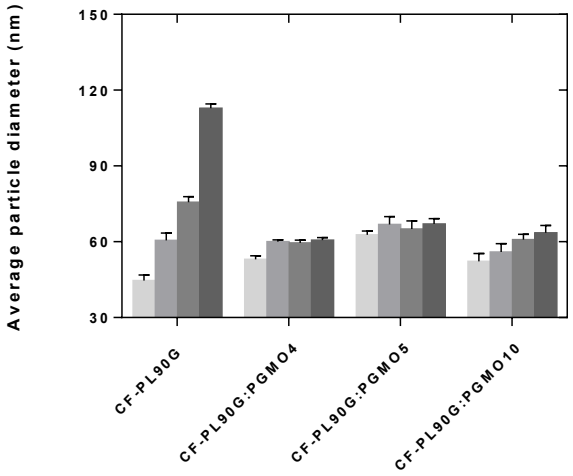


Figure 18 Colloidal stability of liposomes upon storage at room temperature. The average size of the liposomes for 0 (□), 1 (□), 2 (□), and 3 months (■).

CF-PL90:PGMO10 liposomes had slightly increasing in particle size after storage for 3 months. In part of CF-PL90G, a strong increase in average particle size was observed accompanied by high heterogeneity of the vesicles (PDI >> 0.30). This can be the result of aggregation, resulting in a close apposite of vesicles surface and fusion of liposome membrane, relating to instability bilayer [6].

3.2.3. Ex vivo skin permeation study

Penetration through skin for hydrophilic substances is subject to greater inhibition as compared to hydrophobic substances. Hence, this characteristic seems to be more important especially in case of lipophilic drugs, since permeation of hydrophilic molecules tends to be relatively lower and hence can be subjected to further enhancement [7, 8].

Several studies suggested a possible permeation enhancing mechanism for surfactant-based elastic vesicles [9] and different kinds of deformable liposomes [10-15]. Therefore, it was investigated whether the modification of the liposomal surface with different PG molecules has an impact on the permeation of CF through the skin.

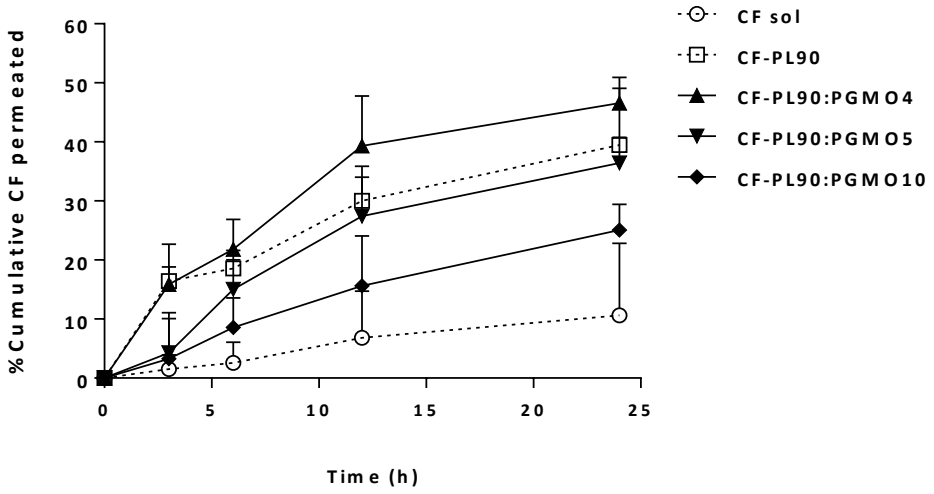


Figure 18 Percentage of carboxyfluorescein permeated from liposomes encapsulating carboxyfluorescein as a function of time. All values represent as mean ± SD (*n=3*).

Results obtained from *ex vivo* skin permeation profiles conducted with different formulations of CF are shown in ***Figure 18***. Two references were used:

solution of CF in ethanol: PBS (50: 50 w/w) (CF solution) and PL90 liposomes loaded with CF (CF-PL90G). Cumulative CF amount that permeated the skin in a time period of 24 h after non-occlusive application was highest for CF-PL90G: PGMO4 liposomes (46.57 ± 4.36%), followed by CF-PL90G: PGMO5 liposomes (36.43 ± 8.44%) and at the end CF-PL90G: PGMO10 liposomes (25.06 ± 6.84%) respectively.

3.3 Conclusion

We observe that incorporation of PEG ester surfactants like PEG300-oleate into lipid bilayer alters the liposome physicochemical properties. This is manifested as, larger particle diameter, low PDI values, increased deformability, even slightly higher entrapment efficiency and better physical stability over a time period of 3 months at room temperature. Moreover, *ex vivo* skin permeation studies revealed that *short chain length PEG* embedded in the lipid bilayer of vesicles enhances the transdermal activities of the hydrophilic fluorescent substance, CF. However, the quantity of CF penetrating into the skin is decreased by modification of liposomes with *long-chain PEG-esters* like PEG1000-oleate or PEG2000-oleate as compared to short chain PEG esters.. In conclusion, modifying liposomes with PEG-esters affect the physicochemical properties as well as the penetration depth of these vesicles which strongly depends on the chain length of the used PEG-esters.

The incorporation of PG-mono-oleate surfactant molecules with *short chain length*, especially PGMO4 surfactant, into lipid bilayer produces smaller and more stable liposomes. The elasticity of lipid bilayers appears to be of greater importance in the enhanced skin delivery of CF by PGMO liposomes, under non – occlusive conditions. Modifying liposomes with PG-mono-oleate not only affects the physicochemical properties but also the penetration depth of these vesicles which is strongly dependent on the chain length of the used PG-mono-oleate. In comparison, the results of PL90G: PGMO4 and PL90G: PG (L1_PG; in Chapter 2), they showed that PL90G: PGMO4 had a smaller size than PL90G: PG (160.9 nm) and a less negatively charged on liposomes surface. Not only were the observed elasticity of PL90G: PGMO4 and PL90G: PG similar, but also their CF permeation into the skin were also enhanced.

In case of PEG-esters and PG-esters when embedded into lipid bilayer of liposomes, resulted in short chain hydrophilic of PEG (PEG300) and PG (PGMO4) having good characteristics with subsequent enhancement of skin permeation.

References

1. Jain, S., A.K. Tiwary, and N.K. Jain, *PEGylated elastic liposomal formulation for lymphatic targeting of zidovudine.* Curr Drug Deliv, 2008. **5**(4): p. 275-81.
2. Knudsen, N.O., et al., *Calcipotriol delivery into the skin with PEGylated liposomes.* Eur J Pharm Biopharm, 2012. **81**(3): p. 532-9.
3. Katahira, N., et al., *Enhancement of Topical Delivery of a Lipophilic Drug from Charged Multilamellar Liposomes.* Journal of Drug Targeting, 1999. **6**(6): p. 405-414.
4. Sinico, C., et al., *Liposomes as carriers for dermal delivery of tretinoin: in vitro evaluation of drug permeation and vesicle-skin interaction.* J Control Release, 2005. **103**(1): p. 123-36.
5. Greenwood, R. and K. Kendall, *Selection of Suitable Dispersants for Aqueous Suspensions of Zirconia and Titania Powders using Acoustophoresis.* Journal of the European Ceramic Society, 1999. **19**(4): p. 479-488.
6. Maria Cristina, A., et al., *A Simplified Model for Liposome Aggregation and Fusion.* Journal of Liposome Research, 1993. **3**(3): p. 687-695.
7. Cevc, G., *Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery.* Crit Rev Ther Drug Carrier Syst, 1996. **13**(3-4): p. 257-388.
8. Williams, A.C. and B.W. Barry, *The enhancement index concept applied to terpene penetration enhancers for human skin and model lipophilic (oestradiol) and hydrophilic (5-fluorouracil) drugs.* International Journal of Pharmaceutics, 1991. **74**(2–3): p. 157-168.
9. van den Bergh, B.A., et al., *Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements.* Int J Pharm, 2001. **217**(1-2): p. 13-24.
10. Cevc, G. and G. Blume, *Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1992. **1104**(1): p. 226-232.
11. Dragicevic-Curic, N., et al., *Temoporfin-loaded invasomes: Development, characterization and in vitro skin penetration studies.* Journal of Controlled Release, 2008. **127**(1): p. 59-69.
12. El Maghraby, G.M., A.C. Williams, and B.W. Barry, *Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in-vitro.* J Pharm Pharmacol, 2001. **53**(8): p. 1069-77.
13. Subongkot, T., et al., *Visualization of ultradeformable liposomes penetration pathways and their skin interaction by confocal laser scanning microscopy.* Int J Pharm, 2013. **441**(1-2): p. 151-61.
14. Trotta, M., et al., *Deformable liposomes for dermal administration of methotrexate.* Int J Pharm, 2004. **270**(1-2): p. 119-25.
15. Verma, D.D., et al., *Liposomes increase skin penetration of entrapped and non-entrapped hydrophilic substances into human skin: a skin penetration and confocal laser scanning microscopy study.* European Journal of Pharmaceutics and Biopharmaceutics, 2003. **55**(3): p. 271-277.
16. Trebilcock, K.L., J.R. Heylings, and M.F. Wilks, *In vitro tape stripping as a model for in vivo skin stripping.* Toxicology in Vitro, 1994. **8**: p. 665-667.
17. Needham, D., T.J. McIntosh, and D.D. Lasic, *Repulsive interactions and mechanical stability of polymer-grafted lipid membranes.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1992. **1108**(1): p. 40-48.
18. Maruyama, K., et al., *Phosphatidyl polyglycerols prolong liposome circulation in vivo.* International Journal of Pharmaceutics, 1994. **111**(1): p. 103-107.
19. Torchilin, V.P., et al., *Amphiphilic poly-N-vinylpyrrolidones: synthesis, properties and liposome surface modification.* Biomaterials, 2001. **22**(22): p. 3035-44.
20. Takeuchi, H., et al., *Evaluation of circulation profiles of liposomes coated with hydrophilic polymers having different molecular weights in rats.* J Control Release, 2001. **75**(1-2): p. 83-91.
21. Abu Lila, A.S., et al., *Use of polyglycerol (PG), instead of polyethylene glycol (PEG), prevents induction of the accelerated blood clearance phenomenon against long-circulating liposomes upon repeated administration.* Int J Pharm, 2013. **456**(1): p. 235-42.

4. Sugar-derivatives: Emulsifier effects onto the lipid bilayer of liposomes for membrane fluidization improvement

Besides polymer esters, sugar derivatives can also behave as an emulsifier with capacities to enhance the skin penetration capability of conventional liposomes. As a result, we investigated the following sugar derivatives as a class of penetration enhancer – namely sucrose oleate (SO), cetearyl glycoside (CG), and polyglyceryl-3 methylglucose distearate (TE) – which behave as emulsifiers during formulation and are commonly used in the pharmaceutical industry. This class of molecules generally act by altering the ordered structure of the intercellular region of the stratum corneum [1-3] and allow the active ingredient to pass through stratum corneum to deeper skin layers. Furthermore, we observe that these three penetration enhancers show a hydrophilic-lipophilic balance (HLB) value in the range of 10-15 which suggest a balanced hydrophilic–lyophilic nature with possible controls on the size of the liposomes. These derivatives existed in liposomes in hydrophilic-lipophilic balance (HLB; ***Table 12***) or as mixed surfactant system so as to enhance ease of incorporation into lipid bilayer of liposomes.

4.1. Selected physicochemical characteristics of liposomes

As shown in ***Table 13***, all of the sugar derivatives – when incorporated into lipid bilayer of liposomes – resulted in a significant increase of the vesicle sizes as compared to conventional liposomes (L1) which had smaller size ($P < 0.05$). Such increase in size may be due to the incorporation of sugar ester molecules into the lipid bilayer and the disordering and destabilization of lipid bilayer [4, 5]. Especially after considering the increasing of vesicle size in the following order L1_SO > L1_TE > L1_CG we found an interesting observation that the increased size of modified liposomes varied inversely with the HLB value of sugar esters. However, this observation requires further prove for being capable of explaining the relation between the vesicle size and the HLB value. PDI values of all tested modified liposomes were in an acceptable range (PDI < 0.30) [6].

Table 12 Chemical Structure, HLB and overall shape value of sugar ester derivatives.

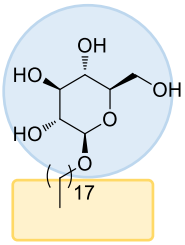
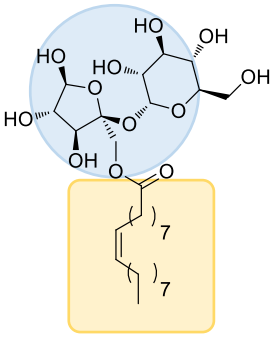
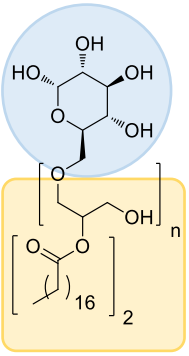
Chemical name	***Cetearyl Glucoside (CG)***	***Sucrose oleate (SO)***	***Polyglycerol-3-methylglucose distearate (TE)***
Polar headgroup ***Hydrophobic tail***			
HLB	11	15	12
Overall shape			

Table 13 Physical characterization of the different types of liposomes with sugar derivatives (Data with standard deviation are presented in *Appendix A (II)*).

Vesicle properties		**Zeta potential (mV)**	**Deformability index (D)**	**%Entrapment efficiency (%EE)**
Size (nm)	**PDI**			
119.3	0.29	5.87	3.297	58.44
72.98	0.25	**-7.45**	3.253	54.26
67.76	**0.19**	-13.57	3.192	49.95
44.60	0.17	-35.47	**2.014**	**35.10**

Code	**L1**	**L1_SO**	**L1_CG**	**L1_TE**
Lipid composition (%w/w)	Only PL90G (10)	PL90G:SO (9:1)	PL90G:CG (9:1)	PL90G:TE (9:1)

Zeta potentials of the liposomes indicate a slight positive charge on SO liposome whereas CG and TE show increased negative charge which also follows the trends of complexity caused by the sugar structure (***Table 13***). The liposomes despite affected packing structure were found to be significantly more flexible (in terms of D values from ***Table 13***, $P < 0.05$) compared to conventional liposomes without sugar derivatives. A possible explanation for such improved flexibility could be the disruption of ordered lipid bilayer structure by sugar derivatives incorporation.

4.2. Entrapment efficiency

Liposomes are often synthesized and selected for enhanced entrapment efficiency. Entrapment efficiency is not only dependent on bilayer permeability, stability and drug complexation in liposome but also upon component stoichiometry of the bilayer. The entrapment efficiency (***Table 13***) of all three modified liposomes were significant higher as compared to unmodified liposomes (L1) ($P < 0.05$). The sugar molecules in lipid bilayer could assist an increase of volume capacity of the inner core of liposomes also influencing entrapping hydrophilic compound.

4.3. Ex vivo drug penetration studies

Despite possessing favorable physicochemical characteristics, the true measure of liposome performance is its penetration into abdominal skin as investigated here using an *ex vivo* skin penetration study (as illustrated in ***Figure 20***).

The release of CF as a function of time was observed to be enhanced in all our formulation with SO and CG showed a 4 and 3.8 times increased enhancement respectively when compared to aqueous CF. However only L1_SO was observed to have and enhancement in excess of the conventional liposome into skin. This result may be due to unsaturated fatty acid chain (C18:1) and large polar headgroup of the SO molecule. The release rate decreased as follows: L1_SO > L1_CG > L1 > L1_TE. At first point 2 h, we observed that L1_SO and L1_CG had a higher release CF as compared to L1_TE.

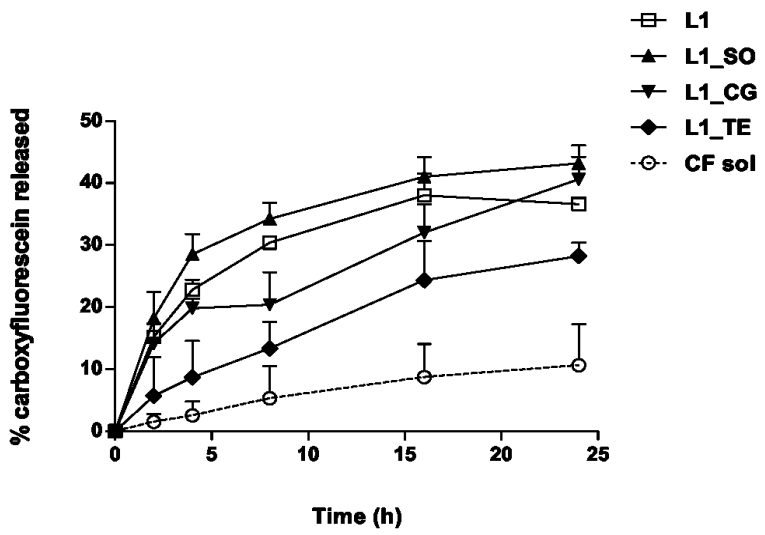


Figure 20 Skin permeation of CF after 24 h non-occlusive application of different CF formulations onto human abdominal skin (expressed as % cumulative of dose released ± S.D., n = 3)

Interestingly, we observe a sustained release over 4-6 h for L1_CG followed by a sudden release after 24 h. This could be explained by a sudden loss of membrane integrity due to small headgroup size of CG and saturated fatty acid chain. TE exhibited a prolonged release function into the receptor compartment which may be due to a large head group accompanied by two saturated fatty acid chain (C18:0) overall retaining a cylindrical shape, causing a denser lipid packing which could cause a controlled drug release.

4.4. Conclusion

Non-ionic surfactants have been clearly shown to influence percutaneous absorption rate of drugs i.e., lidocaine, ibuprofen [7-9]. Different functional groups, hydrocarbon chain length and degree and position of unsaturation can impact the efficacy of penetration enhancers. Sugar fatty acids – non-ionic surfactants – can alter membrane barrier properties temporarily [10-12]. We emphasize that incorporation of sugar esters into lipid bilayers can improve the flexibility of bilayer and enhance skin penetration profile, ultimately enhancing drug delivery into the skin. Sugar ester derivatives are due to their detergent-like structure able to influence liposome physicochemical properties in turn manipulating its penetration through human skin, specifically, we observe L1_SO to be the best formulation.

We suggest to consider the incorporation of sugar derivatives. The sugar ester structure should possess a large headgroup and long chain fatty acid for improving the membrane elasticity and enhancing the skin penetration of drug.

References

1. Williams, A.C. and B.W. Barry, *Penetration enhancers.* Adv Drug Deliv Rev, 2004. **56**(5): p. 603-18.
2. Williams, A.C. and B.W. Barry, *Penetration enhancers.* Advanced Drug Delivery Reviews, 2004. **56**(5): p. 603-618.
3. Moser, K., et al., *Passive skin penetration enhancement and its quantification in vitro.* Eur J Pharm Biopharm, 2001. **52**(2): p. 103-12.
4. Malmsten, M., *Liposomes*. Surfactants and Polymers in Drug Delivery, ed. J. Swarbrick. 2002, 270 Madison Avenue, New York, NY 10016: Marcel Dekker, Inc.
5. Badran, M., K. Shalaby, and A. Al-Omrani, *Influence of the Flexible Liposomes on the Skin Deposition of a Hydrophilic Model Drug, Carboxyfluorescein: Dependency on Their Composition.* The Scientific World Journal, 2012. **2012**: p. 9.
6. Verma, D.D., et al., *Particle size of liposomes influences dermal delivery of substances into skin.* Int J Pharm, 2003. **258**(1-2): p. 141-51.
7. Sarpotdar, P.P. and J.L. Zatz, *Evaluation of penetration enhancement of lidocaine by nonionic surfactants through hairless mouse skin in vitro.* J Pharm Sci, 1986. **75**(2): p. 176-81.
8. Cazares-Delgadillo, J., et al., *Skin permeation enhancement by sucrose esters: a pH-dependent phenomenon.* Int J Pharm, 2005. **297**(1-2): p. 204-12.
9. Bialik, W.W., K.A.; Brain, K.R.; Hadgraft, J., *Some factors affecting the in vitro penetration of ibuprofen through human skin.* International Journal of Pharmaceutics, 1993. **92**(1-3): p. 5.
10. Ganem-Quintanar, A., et al., *Ex vivo oral mucosal permeation of lidocaine hydrochloride with sucrose fatty acid esters as absorption enhancers.* International Journal of Pharmaceutics, 1998. **173**(1): p. 203-210.
11. Ayala-Bravo, H.A., et al., *Effects of sucrose oleate and sucrose laureate on in vivo human stratum corneum permeability.* Pharm Res, 2003. **20**(8): p. 1267-73.
12. Ahsan, F., et al., *Sucrose cocoate, a component of cosmetic preparations, enhances nasal and ocular peptide absorption.* International Journal of Pharmaceutics, 2003. **251**(1–2): p. 195-203.

Part 5

Summary/ Zusammenfassung

Summary

Liposomes are an important component of modern drug delivery strategies. There is increasing interest of late in using them as preferred drug carrier systems for topical application. In the past decades, conventional liposomes obtained by incorporation of phospholipids (e.g., hydrogenated soya phosphatidylcholine and dipalmitoylphosphatidylcholine; DPPC) and cholesterol in the lipid bilayer do not penetrate efficiently across the skin, confining them to the upper layers of the stratum corneum. In order to overcome the obstacle of drug penetration through the biological barrier, the next generations of deformable liposomes – namely Transfersomes®, ethosomes and invasomes – exhibit high skin penetration efficiency, greater control on drug delivery and improved drug solubility and stability, was developed by Cevc (1996), Touitou *et al*. (2000) and Dragicevic-curic *et al*. (2008) respectively [1-3]. The lipid bilayer of these deformable liposomes was composed of unsaturated phospholipids and penetration enhancer – e.g., sodium cholate, nonionic surfactants (Tween80®, Span80®, *etc.*), terpenes – which destabilizes the bilayer structure and generates the elasticity of lipid bilayer. Hitherto, among the characteristics of liposome, e.g. particle size, surface charge, flexibility of lipid bilayer, it was – and still is – not completely clear which characteristic or characteristics should be more intensively considered in order to improve the drug penetration enhancement, and what should be the ideal characteristic of surfactant (which serves as our interested group of penetration enhancer in this study). In the light of aforementioned characteristics, we anticipated that the surface modification of liposomes using different surfactants will yield the different penetration capability and therefore provide us a guideline as to which substances should be incorporated into the lipid bilayer in order to improve the penetration enhancement.

Thus, in order to investigate influences of emulsifiers we selected a model liposome with a native surface having no emulsifiers having a z-potential of – 7mV, which is representative for uncharged phospholipid bilayer membranes. Gillet *et al*. (2011) have observed that a net negative native surface charge in phospholipid liposomes exhibit high penetration efficiency [4], however, Manosroi *et al*. (2004) have suggested the same for positively charged liposomes [5]. This net native

charge of the liposomes due to the phospholipid composition interferes with attributing and measuring effects brought about by emulsifiers as they too can also alter surface charge. Membrane elasticity too can alter effectiveness of liposomes to perform upon skin. Here we measured the deformability index (D) for our liposomes and expect better skin penetration efficiency (due to enhanced flexibility) for liposome preparation possessing high D value. Hence, based on these characteristics an ideal model liposome for surface modifications should exhibit little or no surface charge, have a high deformability index and be of small size. We investigated five commercial phospholipids liposome models (PL90G, NAT8539, LPC20, SUN70P and SUN90P) and selected PL90G (> 95% unsaturated PC) as a candidate for surface modifications as it possessed lower negative charge compared to NAT8539 and LPC20, exhibited higher D values compared to SUN70P and SUN90P.

Charged phospholipids like distearoylphosphatidylglycerol (DSPG) and distearoylphosphatidylserine (DSPS) yielded liposomes with a negative charge whereas quaternary ammonium (QA) and brassicyl isoleucinate esylate (EM) incorporation resulted in liposomes with net positive charge. Slightly negative PL90G liposomes were used as control liposomes to compare these production iterations. Biological performance of these liposomes was tested using multiple *ex vivo* skin based assay methods. Tape stripping to human skin and collection of penetrating liposomes (*Franz*'s cell experiment) underneath the investigated skin revealed effective penetration of both positive and negative liposomes. Positively charged QA containing liposomes have higher penetration compared to EM containing liposomes and were similar to PL90G liposomes in this regard. In agreement with Gillet *et al*. (2011) finding [4], we also observed negative DSPG containing liposomes exhibited a high penetration efficiency when compared to DSPS and control PL90G liposomes. As we used neutral liposomal preparation for these surface changes we can report that this penetration enhancement can be only due to the use of DSPG (negatively charged headgroup modifier phospholipid) for better penetrability.

Since headgroup modifications were observed to exert effect on penetration characteristics we further hypothesized that the size of a modified hydrophilic

headgroup can affect performance of the liposomes. Polymeric surfactant molecules like Polyethyleneglycol (PEG) monooleate and Polyglycerol (PG) monooleate containing varying polymer chain length were used for production of liposomes. Compared to control PL90G liposomes these emulsifiers increased headgroup size producing liposomes with larger vesicle size. In agreement with previous study, we too observed that such headgroup size changes do enhance liposome shelf life at room temperature. Increase in polymeric chain length of headgroups caused a concurrent increase in vesicle size, however, this size increase was not able to enhance penetration efficiency of the liposomes. It must be noted that incorporation of emulsifier having smaller headgroup is able to enhance simultaneously both membrane elasticity and skin penetration properties, however, at higher headgroup size though there is no enhancement of skin penetration. The smallest polymer size in both PEG monooleate (PEG300) and PG monooleate (PGMO4) firstly enhanced deformability index values and % entrapment efficiency of the modified liposomes as compared to PL90G liposomes control and secondly, compared to their larger counterparts demonstrated higher penetration efficiency. Molecular weight (MW) of hydrophilic headgroup and emulsifier type plays a significant role in determining activity and in our case, we see that smallest PEG300 (MW ~300) headgroup is much more potent than PGMO4 (MW ~500) headgroup in penetrating skin and may be due to enhanced steric effects which directly affects the hydrophilic headgroup of the phospholipids enhancing liposome characteristics. We also reported here that incorporation of small polymeric headgroup (in our case, PEG300 and PGMO4) of liposomes are strongly associated with its ability to penetrate skin as revealed by *ex vivo* studies.

Headgroup modifications can also have profound effects on well-organized packing arrangements of lipid bilayer in liposomes. Membrane flexibility of vesicle is known to allow liposomes easier access to stratum corneum. Non-ionic surfactants like sugar derivatives can alter the liposome bilayer packing as a result affect the deformability index thus increasing liposome flexibility [6]. This study uses three sugar derivatives namely sucrose oleate, cetearyl glucoside and polyglyceryl-3 methylglucose distearate to assess changes in membrane packing. All three substances were observed to bring about skin penetration enhancement.

Sucrose oleate and cetearyl glucoside have very similar time dependent functions for skin penetration. These findings suggest that structural complexity of the headgroup molecule constrain the penetration enhancement effect. As compared, the complex molecule of polyglyceryl-3 methylglucose distearate was observed for less penetration through skin. Despite structural similarity between sucrose oleate and cetearyl glucoside (that is, both have one small hydrophilic head group and one hydrophobic tail), the former achieved in our experiment the highest penetration efficiency which could be – according to our construal – attributed to its differed conformation composing of C18:1 *unsaturated fatty acid chain* (in comparison with the latter composing of C18:0 *saturated fatty acid chain*), which makes the packing of lipid bilayer of sucrose oleate looser and more flexible.

Modification of liposomes by changes to the headgroup layer is a powerful tool for enhancing properties of the liposome itself. However, a potentially more powerful approach is to modify the skin's barrier resistance reversibly thus enabling the stratum corneum to be more susceptible to the liposomes. This is achieved through agents which can either disrupt the molecular arrangement of lipids enabling formation of penetration channel paths or use the existent pores on the stratum corneum to enable entry and drug delivery. Though a number of such compounds exist which can alter skin characteristics, in this study, we evaluated application of three, namely, highly lipophilic compounds like a terpene mixture, a non-ionic surfactant like tween 80 and phospholipids to impact skin barrier functions and enhance penetration. These compounds are broadly grouped as penetration enhancers [7, 8]. A number of liposome types were produced by us namely transfersomes (using tween80) [9], invasomes (using terpene mixtures) [3][7] and phospholipids liposomes and were compared with conventional liposomes (produced by emulsifying neutral lipids with cholesterol). Enhanced penetration properties are complex factors often depending on many aspects of the liposomes. We observe invasomes penetrate skin better than transfersomes whereas conventional liposomes are unable to penetrate skin effectively suggesting terpene mixture treated liposomes possess better membrane elasticity and penetration enhancement properties.

Concluding remarks

Neutral liposomes like PL90G are an ideal model liposome to evaluate effects of surface modifiers with potential to enhance liposome performance. This study also reports that surface charge of liposome, when modified by emulsifiers (especially negatively charged donors being used in our experiment: DSPG), can also enhance biological performance of the liposome compared to unmodified liposomes. Polymeric headgroup of PEG300 and PGMO4 (MW 300 and 500, respectively) enhanced liposomes can penetrate the stratum corneum efficiently, however, the performance gain is restricted to a certain size and does not apply across the entire possible size range. Sucrose oleate incorporated liposomes are observed to perform better than other sugar derivatives by synergistically modifying both the liposome bilayer characteristics and altering stratum corneum to enhance access. Similarly, penetration agents altering skin barrier characteristics too perform better than conventional liposomes. In the light of these findings we are also able to suggest a four level index based on CF permeation assays (***Figure 21***) based on the fact and discussion above.

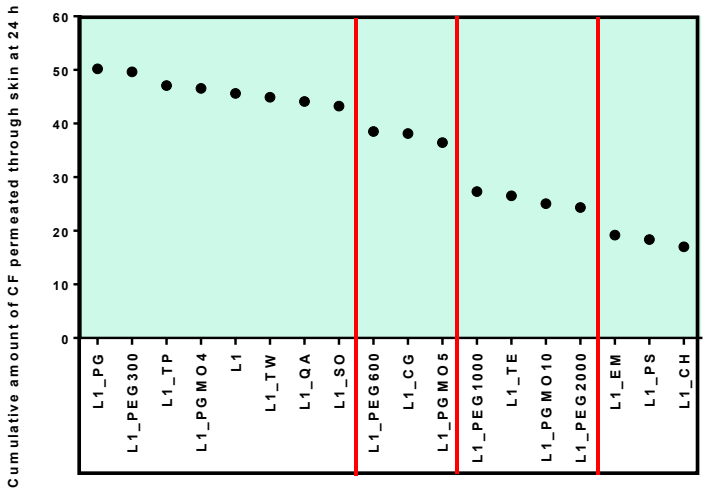


Figure 21 Comparison of cumulative amount CF permeated through the skin at 24h of all liposomal formulations based on PL90G-based liposomes (L1_PG: PL90G liposomes containing DSPG, L1_PEG300: PL90G liposomes containing PEG300-monooleate, L1_TP: PL90G liposomes containing terpene mixture, L1_PGMO4: PL90G liposomes containing PG4-monooleate, L1: PL90G liposomes without no additives, L1_TW: PL90G liposomes containing tween80, L1_QA: PL90G liposomes containing quarternary ammonium, L1_SO: PL90G liposomes containing sucrose oleate, L1_PEG600: PL90G liposomes containing PEG600-monooleate, L1_CG: PL90G liposomes containing cetearyl glucoside, L1_PGMO5: PL90G liposomes containing PG5-monooleate, L1_PEG1000: PL90G liposomes containing PEG1000-monooleate, L1_TE: PL90G liposomes containing polyglyceryl-3 methylglucose distearate, L1_PGMO10: PL90G liposomes containing PG10-monooleate, L1_PEG2000: PL90G liposomes containing PEG2000-monooleate, L1_EM: PL90G liposomes containing brassicyl isoleucinate esylate, and L1_PS: PL90G liposomes containing DSPS, L1_CH: PL90G liposomes containing cholesterol)

Hence, emulsifying agents are key components which improve native liposome attributes for skin penetration. Fundamentally, there are two important characteristics of preferable liposomes for penetration enhancement, 1). liposomal membrane should be flexible, allowing it to squeeze easily through skin and 2). consideration of emulsifier characteristic incorporating into vesicle membrane for producing elastic vesicle should be constructed with small hydrophilic headgroup and medium chain unsaturated fatty acid. These liposomes show potential promise in dermal drug delivery and commercial cosmetic applications. Future work in this area should be focused at two aspects namely increased characterization of studied liposomes in relation to drugs that require dermal delivery routes and examine combinations of emulsifiers to synergistically enhance liposome performance.

Zusammenfassung

Liposomen sind ein wichtiger Bestandteil moderner Strategien der Wirkstoffapplikation. Zuletzt entwickelte sich ein gesteigertes Interesse an der Anwendung als topisches Trägersystem. In der Vergangenheit waren konventionelle Liposomen, deren Bilayer aus Phospholipiden (Soja-Phosphatidylcholin, und Dipalmitoylphosphatidylcholin (DPPC)) und Cholesterol bestehen, nicht in der Lage, effizient durch die Haut zu penetrieren und verblieben in den oberen Schichten des Stratum corneums. Eine neue Generation von verformbaren Liposomen – Transfersomen®, Ethosomen und Invasomen – wurde von Cevc (1996), Touitou *et al*. (2000) bzw. Dragicevic-Curic *et al*. (2008) entwickelt [1-3], besitzt eine hohe Effizienz, in die Haut einzudringen, eine bessere Steuerung der Wirkstofffreisetzung sowie eine Erhöhung der Löslichkeit und Stabilität des Wirkstoffes und bietet auf diesem Weg eine Möglichkeit, diese biologische Barriere zu überwinden. Der Lipidbilayer dieser Vesikel bestand aus ungesättigten Phospholipiden und Penetrationsbeschleunigern – z.B. Natriumcholat, nichtionische Tenside (Tween 80®, Span80®, etc.), Terpene, welche die Membranstruktur destabilisieren und die Elastizität des Bilayers erzeugt. Bisher war und ist noch nicht vollständig bekannt, welche Eigenschaften – z.B. Partikelgröße, Oberflächenladung, Flexibilität des Lipidbilayers – näher untersucht werden sollten, um die Wirkstoffpenetration zu verbessern, und welche

Eigenschaften ein ideales Tensid (welches im Rahmen dieser Arbeit als untersuchte Gruppe der Penetrationsbeschleuniger diente) hierfür besitzen sollte. In Anbetracht der zur erwähnten Eigenschaften erwarteten wir, dass die Oberflächenmodifikation der Liposomen durch verschiedene Tenside unterschiedliche Penetrationsfähigkeiten bedingt und uns dadurch eine Richtlinie zur Verfügung steht, welche Substanzen in einen Bilayer eingearbeitet werden sollten, um die Penetration zu verbessern.

Um den Einfluss der Emulgatoren zu untersuchen, verwendeten wir daher nicht-modifizierte, ungeladene Liposomen mit einem ζ-Potential von -7 mV als Ausgangsmodell. Gillet *et al*. (2011) beobachteten, dass eine negative Oberflächenladung die Penetration begünstigt [4]. An anderer Stelle beschrieben Manosroi *et al*. (2004) den gleichen Effekt für positiv geladene Liposomen [5]. Dabei handelt es sich um die Nettoladung des Bilayers, die durch die Zusammensetzung der Membran (Phospholipide sowie andere Bestandteile, z.B. Tenside). Ein anderer Parameter, der die Penetration verbessern kann, ist die Membranelastizität. Wir bestimmten den Deformierbarkeitsindex (D) für die hergestellten Liposomen und vermuteten, dass hohe Werte für D aufgrund steigender Flexibilität mit verbesserter Penetration interpretiert werden können. Basierend auf diesen Merkmalen sollte eine liposomale Membran zur weiteren Modifikation folgende Eigenschaften aufweisen: geringe oder neutrale Oberflächenladung, einen hohen Deformierbarkeitsindex und eine geringe Größe. Wir untersuchten fünf Liposomenformulierungen, die aus kommerziellen Phospholipiden (PL90G, NAT8539, LPC20, SUN70P und SUN90P) aufgebaut waren und wählten PL90G (> 95% ungesättigtes PC) als Kandidat zur weiteren Oberflächenmodifikation aus, da es die geringer ausgeprägte negative Oberflächenladung besaß als NAT8539 und LPC20 und höhere D-Werte aufwies als SUN70P und SUN90P.

Geladene Phospholipide wie Distearoylphosphatidylglycerol (DSPG) und Distearoylphosphatidylserin (DSPS) bildeten Liposomen mit einer negativen Oberflächenladung, während der Einbau quartärer Amine (QA) und Brassicyl-Isoleucinat-Esylat (EM) eine positive Ladung zur Folge hatte. Leicht negativ geladene PL90G-Liposomen wurden als Kontrolle verwendet, um diese

Modifikationen zu charakterisieren. Die biologische Funktion dieser Liposomen wurde an verschiedenen *ex vivo* hautbasierten Untersuchungen getestet. Tape Stripping auf menschlicher Haut und die Bestimmung der penetrierenden Liposomen (*Franz*-Zell-Experimente) unter dieser Haut zeigte eine Penetration positiv *und* negativ geladener Liposomen. Positiv geladene QA-Liposomen zeigten dabei eine bessere Penetration als EM-haltige Liposomen und ähnliche Werte wie PL90G-beladene Liposomen. In Übereinstimmung mit den Ergebnissen von Gillet *et al*. (2011) [1], konnten wir ebenfalls beobachten, dass negativ geladene DSPG-Liposomen eine bessere Penetration aufwiesen als DSPS- und PL90G-Vesikel. Die Penetrationsverbesserung geht dabei ausschließlich auf den Einbau des an der Kopfgruppe ngeativ geladenen DSPGs zurück.

Da beobachtet werden konnte, dass Modifikationen der Kopfgruppe einen Einfluss auf das Penetrationsverhalten ausüben, vermuteten wir im Folgenden, dass die Größe der modifizierten hydrophilen Kopfgruppe dieses Verhalten ebenfalls verändern kann. Polymere Tenside wie Polyethylenglykol (PEG)-Monooleat und Polyglycerol (PG)–Monooleat mit unterschiedlichen Kettenlängen wurden verwendet, um Liposomen herzustellen. Verglichen mit den Kontrolllliposomen erhöhten diese Polymere die Größe der Kopfgruppe und der Vesikel. In Übereinstimmung mit vorherigen Untersuchungen konnten wir ebenfalls feststellen, dass diese Modifikationen die Lagerstabilität der Formulierungen bei Raumtemperatur verbesserte. Eine Verlängerung der Polymerketten resultierte in einer Zunahme der Größe, was allerdings keinen Einfluss auf das Penetrationsverhalten hatte. Es sollte erwähnt sein, dass der Einbau von Emulgatoren mit kleinen Kopfgruppen, gleichzeitig die Membranelastizität und Hautpenetration verbessern kann, wohingegen eine große Kopfgruppe keine Verbesserung der Hautpenetration zeigte. Die geringste Polymergröße in PEG-Monooleat (PEG300) und PG-Monooleat (PGMO4) konnten den D-Wert und die prozentuale Verkapselungseffizienz der modifizierten Liposomen im Vergleich zur Kontrolle erhöhen. Sie zeigten zusätzlich im Vergleich zu den höher polymerisierten Analogas ein verbessertes Penetrationsverhalten. Die Größe (Molmasse) der hydrophilen Kopfgruppe und der Emulgator haben einen signifikanten Einfluss auf das Verhalten der Formulierung; in unserem Fall konnten

wir feststellen, dass Liposomen, deren Oberfläche mit dem PEG300-Polymer (MW~300) modifiziert waren, eine deutlich bessere Hautpenetration besaßen als Vesikel, die mit dem PGMO4-Polymer (MW~500) modifiziert wurden. Ein Grund hierfür können verstärkte sterische Effekte sein, die einen direkten Einfluss auf die Kopfgruppen der Phospholipide ausüben können und dadurch die Eigenschaften der Liposomen verbessern können. Wir beobachteten ebenfalls, dass der Einbau einer polymeren Kopfgruppe und die daraus folgende Vergrößerung der Vesikel eng mit dem Penetrationsverhalten in *ex vivo*-Studien zusammenhängen.

Modifikationen der Kopfgruppen können des Weiteren zu tiefgreifenden Veränderungen in der Ordnungsstruktur des liposomalen Bilayers führen. Es ist bekannt, dass Liposomen durch eine entsprechende Flexibilität der Vesikelmembran einfacher in das Stratum corneum penetrieren. Ungeladene Tenside - wie z.B. Zuckerderivate - verändern die Packung des Bilayers und resultieren damit in einer Änderung des Deformabilitätsindexes, welche zu einer erhöhten Flexibilität beiträgt [6]. In der vorliegenden Arbeit wurden drei verschiedene Zuckerderivate zur Modifikation der Membranordnung verwendet, nämlich Saccharoseoleat, Cetearylglucosid und Polyglycerol- 3-Methylglucosedistearat. Alle drei Substanzen konnten in der Vergangenheit ihr Potential zur Verbesserung der Hautpenetration darlegen. Saccharoseoleat und Cetearylglucosid wiesen dabei sehr ähnliche Penetrationskinetiken in die Haut auf. Verglichen dazu, wurde für das komplexere Molekül Polyglycerol-3-Methylglucosedistearat eine geringere Hautpenetration beobachtet. Diese Resultate lassen vermuten, dass eine strukturelle Komplexität der Kopfgruppe die Erhöhung des Penetrationseffektes beschränkt. Trotz struktureller Ähnlichkeiten zwischen Saccharoseoleat und Cetearylglucosid (beide besitzen eine kleine hydrophile Kopfgruppe und einen hydrophoben Rest) erzielte erstgenanntes die höchste Penetrationseffizienz. Dieser Fakt lässt sich womöglich auf Unterschiede in der Konformation zurückführen; Saccharoseoleat besitzt eine ungesättigte C18:1 Fettsäurekette, wohingegen letztgenannte eine gesättigte C18:0 Fettsäurekette besitzt, wodurch der Lipidbilayer im Fall von Saccharoseoleat lockerer gepackt und dadurch flexibler ist.

Die Modifikation von Liposomen durch Veränderungen der Kopfgruppenschicht ist ein geeignetes Werkzeug, um die Eigenschaften der Vesikel zu verbessern. Nichtsdestotrotz stellt die Modifizierung des Hautbarrierenwiderstandes einen noch potenteren Ansatz dar, um das Stratum corneum empfänglicher für Liposomen zu machen. Erreicht wird dies durch Agentien, welche entweder die molekulare Ordnung der Hautlipide stören und damit Penetrationskanäle schaffen oder bereits vorhandene Poren im Stratum corneum nutzen um den Wirkstoffeintritt und -transport zu gewährleisten. Obwohl bereits einige dieser Substanzen zur Veränderung der Hautcharakteristiken beschrieben sind, wurden in der vorliegenden Arbeit drei weitere Vertreter untersucht. Dabei handelte es sich um stark lipophile Komponenten wie eine Terpenmischung, ein ungeladenes Tensid wie Tween 80 und Phospholipide, welche schlussendlich die Barrierefunktion der Haut beeinflussen und die Penetration verbessern sollten. Der Oberbegriff solcher Agentien lautet Penetrationsbeschleuniger [7, 8]. Verschiedene Liposomentypen wurden hergestellt, darunter Transfersomen (unter Nutzung von Tween 80) [9], Invasomen (unter Nutzung der Terpenmischung) [3] und Liposomen bestehend aus Phospholipiden. Diese wurden anschließend mit konventionellen Liposomen verglichen, welche durch Dispersion neutraler Lipide mit Cholesterol hergestellt wurden. Die Verbesserung von Penetrationseigenschaften ist ein komplexer Vorgang, der oftmals von vielen Aspekten der Liposomen abhängig ist. Es wurde beobachtet, dass Invasomen die Haut besser penetrieren als Transfersomen, wobei konventionelle Liposomen nicht in der Lage waren die Haut effektiv zu penetrieren. Daraus lässt sich schlussfolgern, dass Liposomen auf Terpenbasis bessere Membranelastizitäten und penetrationsverbessernde Eigenschaften besitzen.

Schlussfolgerungen

Neutrale Liposomen wie PL90G stellen ein ideales Modell dar, um die Effekte von Oberflächenmodifikationen hinsichtlich ihres Potentials zur Verbesserung des Liposomenverhaltens zu bewerten. In der vorliegenden Arbeit wurde ebenfalls festgestellt, dass die Oberflächenladung von Liposomen durch den Einbau von Emulgatoren (besonders das negativ geladene DSPG) dazu befähigt ist, die biologische Leistungsfähigkeit von Liposomen, verglichen mit unmodifizierten

Vesikeln, zu verbessern. Liposomen mit den polymeren Kopfgruppen PEG300 und PGMO4 können effizient ins Stratum corneum penetrieren, wobei die Verbesserung durch eine gewisse Größe begrenzt wird und sich nicht auf den gesamten Größenbereich übertragen lässt. Liposomen mit inkorporiertem Saccharoseoleat eignen sich besser als andere Zuckerderivate, da sie in synergistischer Art und Weise die liposomalen Bilayer-Charakteristiken (z.B. Membranoberfläche) modifizieren und das Stratum corneum für eine Penetrationsverbesserung verändern. Ebenso werden die Hautbarrierefunktionen durch Penetrationsagentien beeinflusst, womit ein Zusatznutzen gegenüber konventionellen Liposomen erkennbar ist. Im Zuge dieser Beobachtungen und Diskussion ist es möglich einen Vier-Stufen-Index auf Grundlage eines CF-Permeations-Assays (***Abbildung 22***) zu entwickeln.

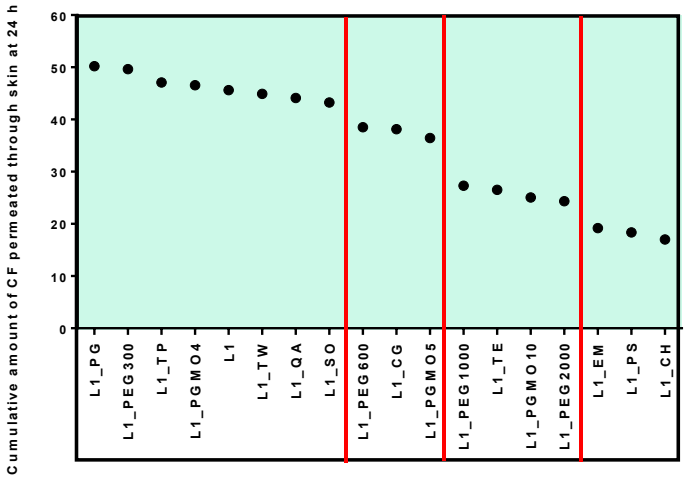


Abbildung 22 Vergleich der kumulativ permeierten Mengen an CF verschiedener PL90G-basierten Liposomen durch die Haut. (L1_PG: PL90G Liposomen mit DSPG, L1_PEG300: PL90G Liposomen mit PEG300-Monooleat, L1_TP: PL90G Liposomen mit Terpenmischungen, L1_PGMO4: PL90G Liposomen mit PG4-Monooleat, L1: PL90G Liposomen ohne Zusätze, L1_TW: PL90G Liposomen mit Tween80, L1_QA: PL90G Liposomen mit QA, L1_SO: PL90G Liposomen mit Saccharoseoleat, L1_PEG600: PL90G Liposomen mit PEG600-Monooleat, L1_CG: PL90G Liposomen mit Cetearylglucosid, L1_PGMO5: PL90G Liposomen mit PG5-Monooleat, L1_PEG1000: PL90G Liposomen mit PEG1000-Monooleat, L1_TE: PL90G Liposomen mit Polyglyceryl-3 Methylglucosedistearat, L1_PGMO10: PL90G Liposomen mit PG10-Monooleat, L1_PEG2000: PL90G Liposomen mit PEG2000-Monooleat, L1_EM: PL90G Liposomen mit Brassicylisoleucinatesylate, und L1_PS: PL90G Liposomen mit DSPS, L1_CH: PL90G Liposomen mit Cholesterol)

Folglich stellen Emulgatoren die Schlüsselkomponente zur Verbesserung von Liposomenattributen hinsichtlich der Hautpenetration dar. Grundsätzlich sind geeignete Liposomen zur Penetrationsverbesserung durch zwei wichtige Faktoren gekennzeichnet, 1.) die liposomale Membran sollte flexibel sein und somit einen

einfachen Durchgang durch die Haut gewährleisten und 2.) die Emulgatoren zum Einbau in die Vesikelmembran sollten aus kleinen hydrophilen Kopfgruppen und mittellangen ungesättigten Fettsäuren bestehen, um elastische Vesikel herstellen zu können. Solche Liposomen besitzen ein vielversprechendes Potential im Bereich des dermalen Arzneistofftransports und der kommerziellen Kosmetik. Weiterführende Arbeiten sollten sich verstärkt auf die Charakterisierung von liposomalen Formulierungen richten, deren Wirkstoff eine dermale Applikationsroute benötigt und des Weiteren die Kombination von Emulgatoren zur synergistischen Verbesserung der Liposomenpenetration untersuchen.

References

1. Cevc, G., *Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery.* Crit Rev Ther Drug Carrier Syst, 1996. **13**(3-4): p. 257-388.
2. Touitou, E., et al., *Ethosomes — novel vesicular carriers for enhanced delivery: characterization and skin penetration properties.* Journal of Controlled Release, 2000. **65**(3): p. 403-418.
3. Dragicevic-Curic, N., et al., *Temoporfin-loaded invasomes: Development, characterization and in vitro skin penetration studies.* Journal of Controlled Release, 2008. **127**(1): p. 59-69.
4. Gillet, A., et al., *Liposome surface charge influence on skin penetration behaviour.* Int J Pharm, 2011. **411**(1-2): p. 223-31.
5. Manosroi, A., L. Kongkaneramit, and J. Manosroi, *Stability and transdermal absorption of topical amphotericin B liposome formulations.* Int J Pharm, 2004. **270**(1-2): p. 279-86.
6. Ayala-Bravo, H.A., et al., *Effects of sucrose oleate and sucrose laureate on in vivo human stratum corneum permeability.* Pharm Res, 2003. **20**(8): p. 1267-73.
7. Williams, A.C. and B.W. Barry, *Penetration enhancers.* Advanced Drug Delivery Reviews, 2004. **56**(5): p. 603-618.
8. Thakur, R.A., Y. Wang, and B.B. Michniak, *Percutaneous penetration enhancers*. 2 ed. Essential Oils and Terpenes, ed. E.W. Smith and H.I. Maibach. 2006, 6000 Broken Spund Parkway NW, Suite 300, Boca Raton, FL 33487-2742: CRC Press, Taylor & Francis Group.
9. Cevc, G., et al., *Ultraflexible vesicles, Transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1998. **1368**(2): p. 201-215.

Appendix A: Data with standard deviation

I. Phospholipids selection for preparing model liposomes

Table 1 The composition and characterization of non-entrapped phospholipid liposomes by vesicle size, PDI, zeta potential and deformability.

Code	Composition	Vesicle size (nm)[a]	PDI[a]	Zeta potential (mV) [a]	Deformability index (D) [a]
L1	PL90G:EtOH=10:16	65.37 ± 2.13	0.28 ± 0.01	-5.75 ± 0.87	2.014 ± 0.21
L2	NAT8539:EtOH=10:16	69.48 ± 1.77	0.03 ± 0.05	-40.16 ± 0.74	1.260 ± 0.21
L3	LPC20:EtOH=10:16	174.93 ± 2.41	0.22 ± 0.12	-56.12 ± 1.24	2.894 ± 1.09
L4	SUN70P:EtOH=10:16	33.33 ± 0.36	0.22 ± 0.00	-2.55 ± 0.81	0.354 ± 2.88
L5	SUN90P:EtOH=10:16	95.29 ± 0.66	0.37 ± 0.01	-1.50 ± 0.32	1.707 ± 3.41

[a] Average of three determinations ± standard deviation (S.D.)

Table 2 Physicochemical properties of phospholipid liposomes entrapped with carboxyfluorescein (CF)

Code	Vesicle size (nm)[a]	%Entrapment efficiency (%EE) [a]
CF-L1	44.60 ± 2.25	35.10 ± 3.89
CF-L2	70.21 ± 1.77	28.18 ± 1.77
CF-L3	175.77 ± 2.41	30.77 ± 7.41
CF-L4	43.58 ± 0.56	25.48 ± 7.88
CF-L5	82.21 ± 0.74	27.12 ± 6.21

[a] Average of three determinations ± standard deviation (S.D.)

II. Influence of surface charge on drug penetration performance of liposomes

Table 1 Prepared liposomal formulations of charged liposomes

Formulation Code	PL90G	DSPG	DSPS	EM	QA
L1_PG[a]	9	1	-	-	-
L1_PS[b]	9	-	1	-	-
L1_EM[c]	9	-	-	1	-
L1_QA[d]	9	-	-	-	1

Add 16%w/w Ethanol and 74%w/w 30mM CF solution/ PBS in each formulation.

[a] L1_PG: PL90G liposomes containing distearoyl phosphatidylglycerol (DSPG)

[b] L1_PS: PL90G liposomes containing distearoyl phosphatidylserine (DSPS)

[c] L1_EM: PL90G liposomes containing brassicyl isoleucinate esylate (EM)

[d] L1_QA: PL90G liposomes containing quaternary ammonium (QA)

Table 2 Physical characterization of the different types of charged liposomes

(2a)

Formulation code	Particle size (nm)[a]	PDI [a]	Zeta potential (mV) [a]
L1	44.63 ± 2.25	0.19 ± 0.01	-7.45 ± 3.22
L1_PG	160.9 ± 1.22	0.15 ± 0.02	-55.28 ± 1.24
L1_PS	167.1 ± 0.88	0.05 ± 0.01	-44.12 ± 4.47
L1_EM	112.2 ± 1.43	0.30 ± 0.07	54.17 ± 2.11
L1_QA	30.21 ± 1.67	0.22 ± 0.03	46.57 ± 1.14

[a] Average of three determinations ± standard deviation (S.D.)

(2b)

Formulation code	Deformability index (D) [a]	%Entrapment efficiency (%EE) [a]
L1	2.014 ± 0.21	35.10 ± 3.89
L1_PG	2.322 ± 0.10	39.22 ± 8.01
L1_PS	2.901 ± 0.44	49.29 ± 7.02
L1_EM	11.09 ± 0.05	28.85 ± 3.19
L1_QA	1.674 ± 0.14	27.09 ± 7.09

[a] Average of three determinations ± standard deviation (S.D.)

III. Surface modification using polymer coating of liposomes

Table 1 Characterization of different liposomes

Formulation Code	Particle size (nm)	PDI	Zeta potential (mV)	Deformability index (D)	% Entrapment efficiency(%EE)
CF-PL90G[a]	44.60 ± 2.25	0.30 ± 0.01	-7.45 ± 3.22	2.01 ± 0.21	35.10 ± 3.89
CF-PL90G:PEG300[b]	89.24 ± 1.21	0.14 ± 0.01	-12.63 ± 1.54	3.67 ± 0.03	37.30 ± 2.35
CF-PL90G:PEG600[c]	60.14 ± 0.51	0.09 ± 0.00	-11.77 ± 3.01	2.03 ± 0.20	41.09 ± 2.18
CF-PL90G:PEG1000[d]	64.23 ± 0.36	0.20 ± 0.05	-12.58 ± 2.14	1.07 ± 0.12	39.55 ± 3.49
CF-PL90G:PEG2000[e]	95.31 ± 0.84	0.15 ± 0.11	-9.58 ± 1.26	0.93 ± 0.20	37.67 ± 4.72

Values represent mean ± S.D. ($n = 3$)

[a] Conventional liposomes containing CF

[b] PL90:PEG300 liposomes containing CF

[c] PL90:PEG600 liposomes containing CF

[d] PL90:PEG1000 liposomes containing CF

[e] PL90:PEG2000 liposomes containing CF

Table 2 Characterization of different liposomes

Formulation Code	Particle size (nm)[e]	PDI [e]	Zeta potential (mV) [e]	Deformability index (D) [e]	%Entrapment efficiency(%EE) [e]
CF-PL90[a]	44.60 ± 2.25	0.30 ± 0.01	-7.45 ± 3.22	2.01 ± 0.21	35.10 ± 3.89
CF-PL90:PGMO4[b]	50.32 ± 0.20	0.25 ± 0.10	-14.10 ± 3.64	2.40 ± 0.57	32.48 ± 4.22
CF-PL90:PGMO5[c]	35.07 ± 0.27	0.18 ± 0.01	-7.99 ± 2.83	1.85 ± 1.02	38.44 ± 1.19
CF-PL90:PGMO10[d]	29.12 ± 0.05	0.13 ± 0.01	-4.81 ± 0.37	1.70 ± 0.65	40.21 ± 5.22

Values represent mean ± S.D. ($n = 3$)

[a] Conventional liposomes containing CF

[b] PL90:PGMO4 liposomes containing CF

[c] PL90:PGMO5 liposomes containing CF

[d] PL90:PGMO10 liposomes containing CF

[e] Average of three determinations ± standard deviation (SD).

IV. Sugar-derivatives emulsifier effect into lipid bilayer of liposomes for membrane fluidization improvement

Table 1 Physical characterization of the different types of liposomes using sugar derivatives.

Formulation Code	Particle size (nm)[d]	PDI [d]	Zeta potential (mV) [d]	Deformability index (D) [d]	%Entrapment efficiency (%EE) [d]
L1	44.6 ± 2.25	0.19 ± 0.01	-7.45 ± 3.22	2.014 ± 0.21	35.10 ± 3.89
L1_SO [a]	67.76 ± 7.48	0.17 ± 0.03	+5.87 ± 1.74	3.297 ± 0.05	58.44 ± 6.18
L1_CG [b]	119.3 ± 3.63	0.25 ± 0.01	-13.57 ± 2.52	3.192 ± 0.02	54.26 ± 3.40
L1_TE [c]	72.98 ± 3.14	0.29 ± 0.02	-35.47 ± 1.77	3.253 ± 0.13	49.95 ± 3.04

Add 16% Ethanol and 74% 30mM CF solution in each formulation.

[a] L1_SO: PL90 liposomes containing sucrose oleate

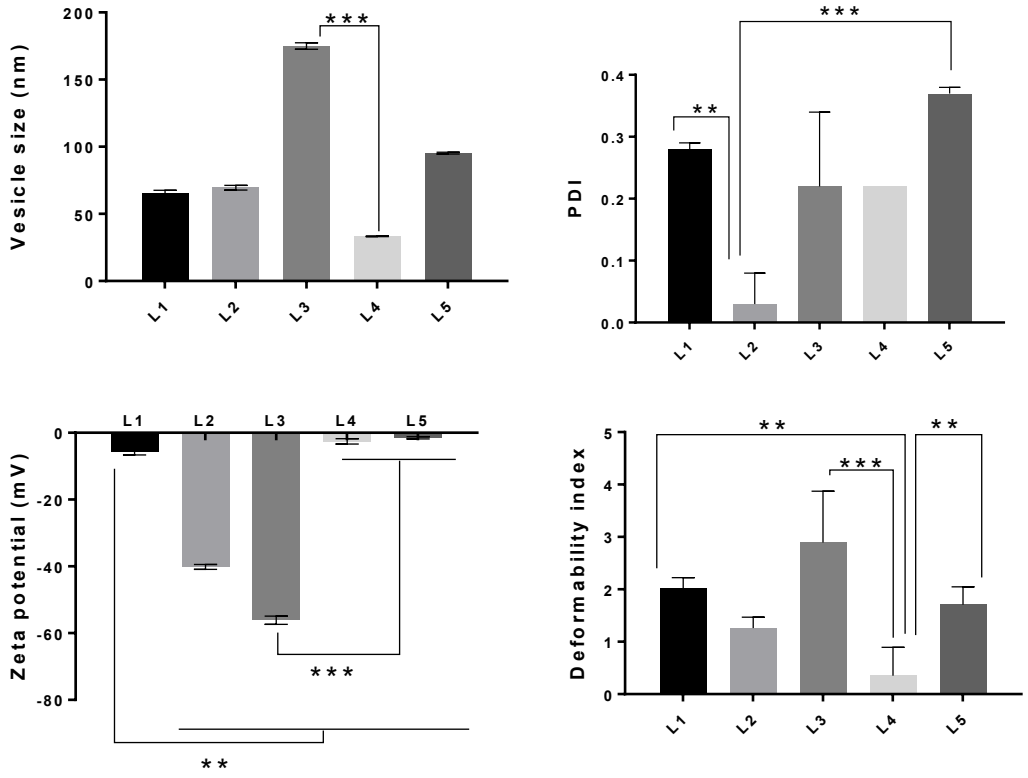
[b] L1_CG: PL90 liposomes containing cetearyl glucoside

[c] L1_TE: PL90 liposomes containing polyglyceryl-3 methylglucose distearate

[d] Average of three determinations ± standard deviation (S.D.)

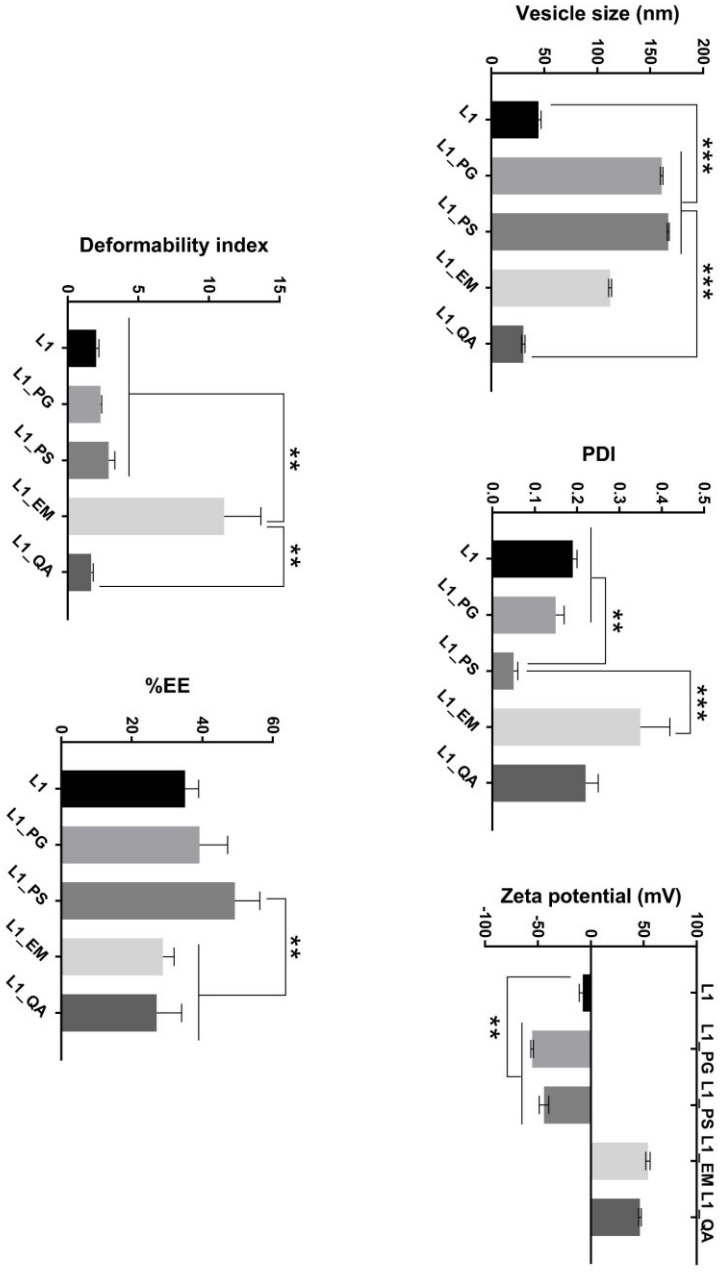
Appendix B: Statistical analysis

I. Phospholipids selection for preparing model liposomes



***, P < 0.05; **, P < 0.01; n.s., non-significant

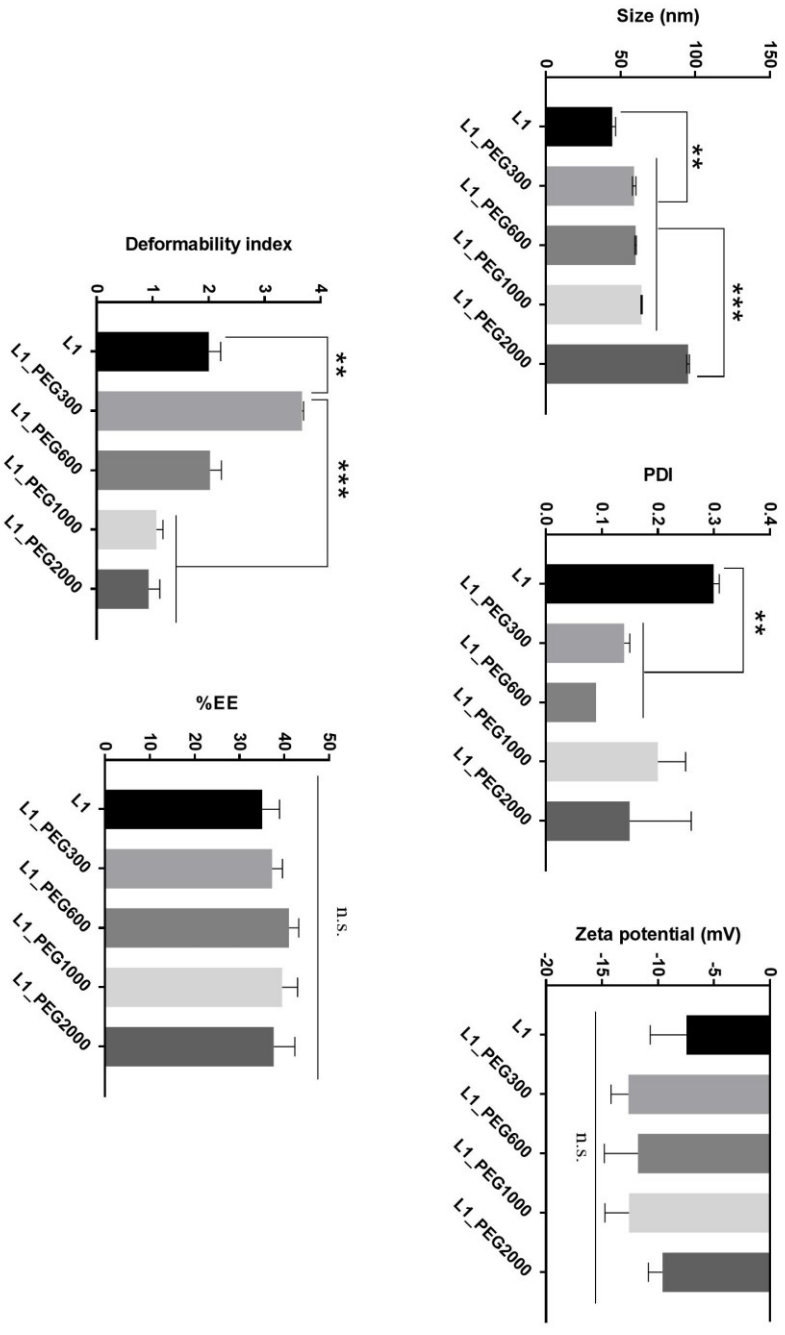
II. Influence of surface charge on drug penetration performance of liposomes



***, P < 0.05; **, P < 0.01; n.s., non-significant

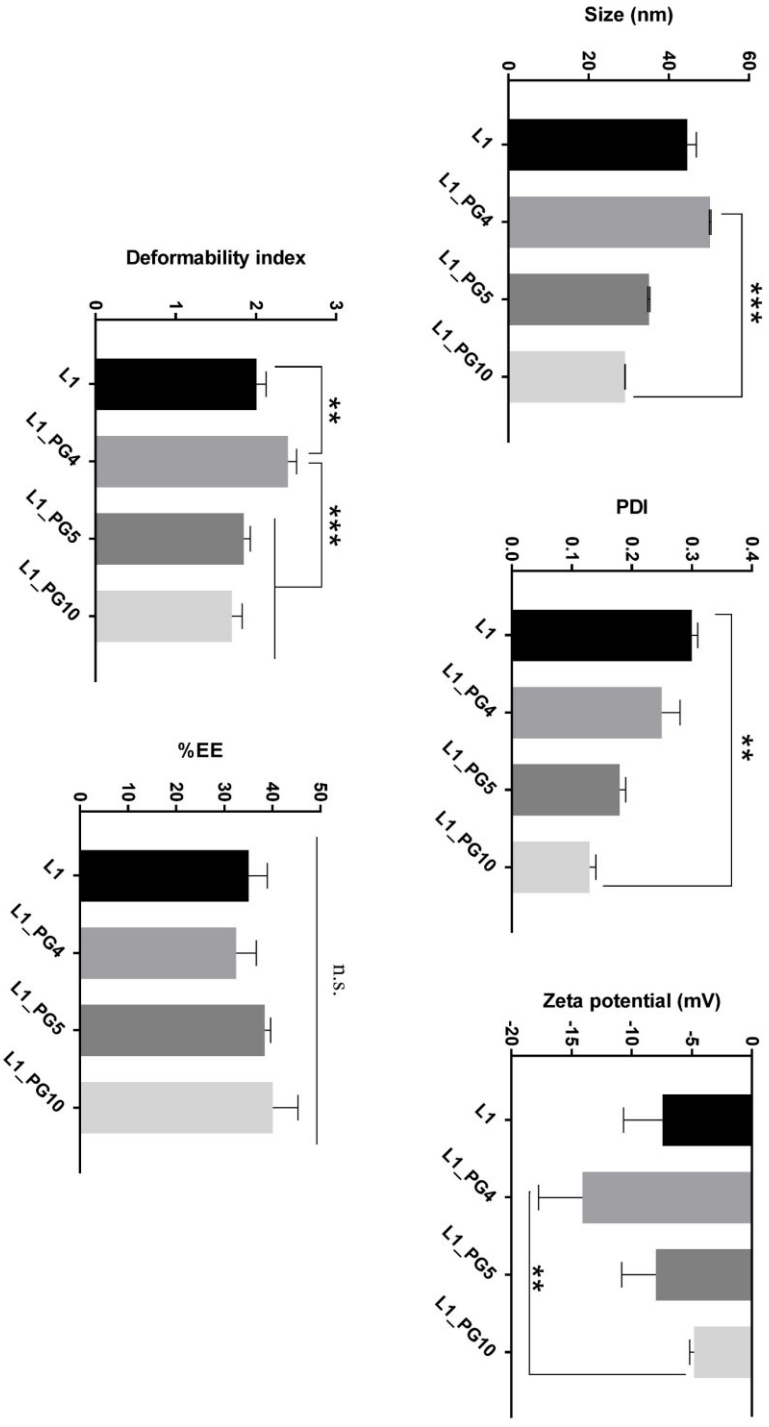
III. Surface modification using polymer coating of liposomes

1. PEG mono-oleate



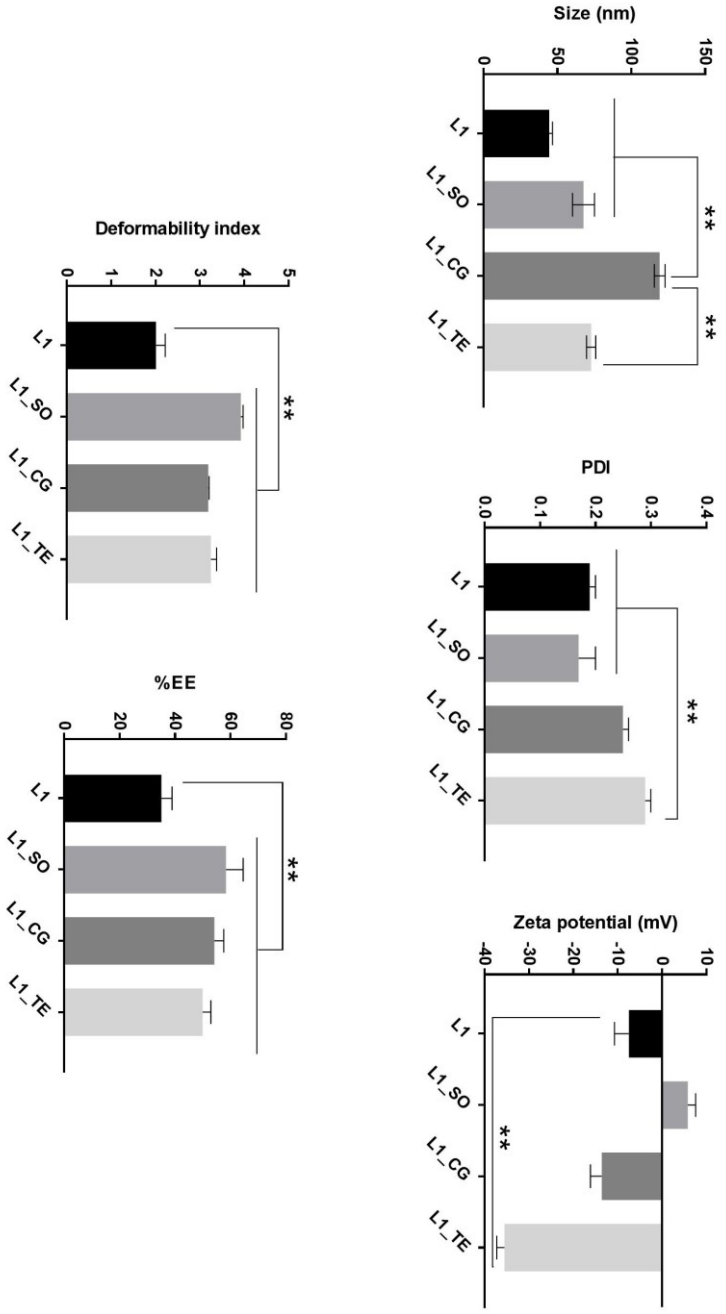
***, P < 0.05; **, P < 0.01; n.s., non-significant

2. PG mono-oleate



***, $P < 0.05$; **, $P < 0.01$; n.s., non-significant

IV. Sugar-derivatives emulsifier effect into lipid bilayer of liposomes for membrane fluidization improvement



***, P < 0.05; **, P < 0.01; n.s., non-significant

Acknowledgement

First and foremost, I would like to express my sincere gratitude to my main project and dissertation advisor, Prof. Dr. Alfred Fahr, for his moral supports, invaluable advices, academic guidance and especially for his patience with reviewing of my thesis. Secondly, I am also very grateful to my project and experimental supervisor, Dr. Gabriele Blume, for her valuable helps and advices during the experiment phase of the project. It is not exaggerated to say that without both of them this work cannot be done as it is.

I am also thankful to all companies mentioned in the material parts of this work for their supports of the high quality compounds and samples which are of significant importance for this project. Of equally importance, I would like to acknowledge my debt to the financial supports from the Deutscher Akademischer Austausch Dienst (DAAD) in the form of scholarship and from the Graduate Academy of FSU Jena in the form of grant. I would like to give my special thanks to Dr. Lutz Gruhl, Plastic Surgery Clinic Kassel for furnishing this project with human skins for the experiment.

In doing and finishing this project, I appreciate all personnel and faculty members for their grateful assistances in using the equipment at the Department of Pharmacy, Pharmaceutical Technology, and all involved technicians, especially Herr Alexander Mohn, Frau Ramona Brabetz, Frau Angela Herre and Dr. Jana Thamm, at the FSU Jena. With regard to general support and advice I am very thankful to all staffs of the Department of Pharmacy. For the translation of the summary part of this work from English into German I would like to express my big thanks to my PhD candidate colleagues, Herr Stephan Holzschuh and Herr Markus Rabenhold, for their support. Finally, I have received along the part of my project many helps and supports from a lot of people, Dr. Erica D'Aguanno, Dr. Kalpa Nagarsekar, Dr. Mukul Ashtikar, Dr. Keda Zhang, Dr. Kewei Yang, Dr. Ming Chen, Dr. Khaled Shalaby Ahmed, Dr. Christiane Decker, Dr. Markus Rabenhold, Dr. Stephan Holzschuh, Frau Kathrin Kaeß and PD. Dr. Ronny Rüger, Mr. (PhD student) Somak Chowdhury from the MPI for Biogeochemistry Jena and Mr. (PhD student) Thossaporn Saensawatt for their precious supports with regard to the dissertation, to name but a few.

Finally, I would like to use the space here to mention all my friends and in Jena and in Germany who always give me supports and especially their previous time in helping me when I faced some problems during my project period. Moreover, I would love to be grateful to my beloved parents, my lovely sisters and my love for their patience and all the supports (Even though my mother has still a big problem with severe disease, but she always cheers me up to succeed my dream.) and many thanks to all of my friends in Thailand for their love and encouragement throughout this study.

Statement

I am familiar with the Promotionsordnung of the Faculty of Biology and Pharmacy of the Friedrich-Schiller-Universität Jena. I produced all parts of the dissertation independently. I hereby declare that this thesis does not contain any material previously submitted for a degree or diploma at another university or any material previously written or published by any other person, except where due acknowledgement or references is made in the text. I also declare that I did not provide any direct or indirect financial remuneration to any third party in connection with the content of my dissertation.

Jena,_____ _____

Amaraporn Roopdee