Liste des abréviations

ACN	Acétonitrile
BM ou BMS	Bétaméthasone
BMD	Bétaméthasone dipropionate
CD	Cyclodextrine
CLSM	« Confocal laser scanning microscopy » (microscopie confocale à balayage laser)
Crysmeb	β cyclodextrine méthylée
DCP	Dicétylphosphate
DEC	« Disposable extractive cartridge » (cartouche d'extraction jetable)
Déoxy Na	Déoxycholate sodique
DMPA	« 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt) » (acide dimyristoyl phosphatidique)
DMPC	Dimyristoylphosphatidylcholine
DRV	« Dehydration-rehydration vesicle » (vésicule formée par déshydratation-réhydratation)
EE	« Encapsulation efficiency » (pourcentage d'encapsulation)
EPR	« Electron paramagnetic resonance » (résonance paramagnétique électronique)
ESR	« Electron spin resonance » (résonance de spin électronique)
FRV	« freeze-dried rehydration vesicle » (vésicule formée par lyophilisation-réhydratation)
HPγCD	Hydroxypropyl – γ- cyclodextrine
HPLC	« High performance liquid chromatography » (chromatographie liquide haute performance)
LUV	« Large unilamellar vesicle » (grande vésicule unilamellaire)
MLV	« Multilamellar vesicle » (vésicule multilamellaire)

NBD-PC	1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-glycero-3-phosphocholine
OCDE	Organisation pour la Coopération et le Développement Économiques
P90	Phosphatidylcholine (Phospholipon® 90)
P90H	Phosphatidylcholine hydrogénée (Phospholipon® 90H)
PBS	« Phosphate buffer saline »
PCS	« Photon correlation spectroscopy » (spectroscopie à corrélation de photons)
SA	Stéarylamine
SPE	« Solid phase extraction » (extraction sur phase solide)
SUV	« small unilamellar vesicle » (petite vésicule unilamellaire)
TEM	« Transmission electron microscopy » (microscopie à transmission d'électrons)
T_m	Température de transition de phase
UV	Ultraviolet

INTRODUCTION

I. La barrière cutanée

La peau constitue un organe complexe. Ce chapitre donne un aperçu de la structure des principaux compartiments de la peau et des annexes cutanées. Le *stratum corneum* étant considéré comme la principale barrière cutanée, il sera décrit plus en détails. Ce chapitre comprend également la description des modèles de peaux utilisés en recherche.

I.1 La structure de la peau

La peau constitue le plus grand organe du corps humain avec une surface d'1,8 m^2 représentant approximativement 7 kg pour une personne de 65kg, soit plus de 10% de la masse corporelle [1]. Grâce à ses propriétés de barrière entre l'organisme et l'environnement, les fonctions de la peau sont d'une part, une protection contre les agressions de l'extérieur et d'autre part, un maintien de l'homéostasie. Les agressions extérieures comprennent par exemple les produits chimiques, les bactéries, les allergènes, les champignons, les radiations mais également les changements de températures et d'humidité. La peau contribue à l'homéostasie du corps grâce notamment à la thermorégulation et à la sécrétion de sébum et de sueur. La peau est également le siège du sens du toucher [2, 3]. Les principales couches cutanées comprennent de l'extérieur vers l'intérieur, l'épiderme constitué du *stratum corneum* et de l'épiderme vivant, le derme et l'hypoderme, auxquelles s'ajoutent les annexes cutanées dont les follicules pileux, les glandes sudoripares, les glandes sébacées et les ongles (Figure 1).

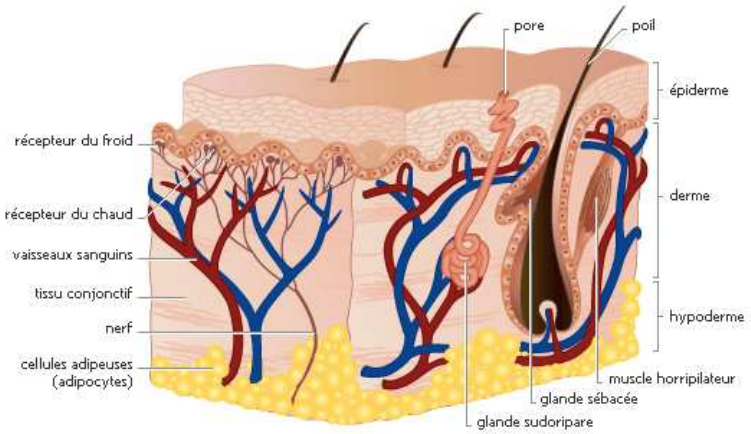

Figure 1 : Structure de la peau.

I.1.1 L'épiderme

L'épiderme comprend d'une part l'épiderme vivant constitué de plusieurs couches de cellules, les kératinocytes et d'autre part la couche cornée ou *stratum corneum* constituée de cellules mortes, les cornéocytes (Figure 2).

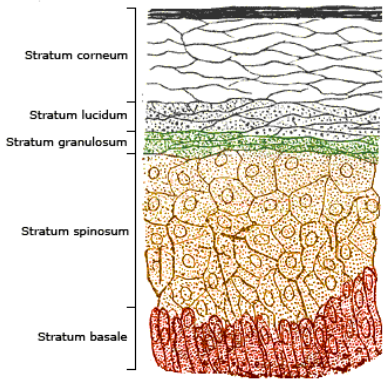

Figure 2 : Organisation de l'épiderme.

I.1.1.1 Le *stratum corneum*

Le *stratum corneum* ou couche cornée constitue la couche la plus externe de la peau. Dans la plupart des régions cutanées son épaisseur est d'environ 10 à 20 µm, bien qu'elle puisse varier de quelques micromètres à quelques millimètres. Le *stratum corneum* contient seulement 15% d'eau et est constitué de deux composants structurels : les cornéocytes et les lipides intercellulaires [4]. Le « brick and mortar model » a été proposé comme modèle d'architecture du *stratum corneum*, les briques représentant les cornéocytes riches en kératine et le ciment représentant la matrice riche en lipides [5] (Figure 3).

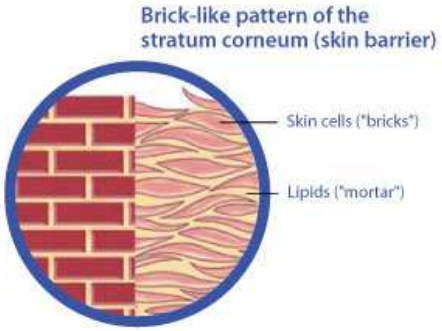

Figure 3 : Le modèle « brick and mortar ».

La fonction de barrière de la peau est facilitée par la desquamation continue de la couche cornée avec un turn over total en 2 à 3 semaines [3].

Les cornéocytes

Les cornéocytes sont des cellules aplaties de forme polyédrique, non nucléées, d'environ 40 µm de diamètre et 0,5 µm d'épaisseur [1]. Le *stratum corneum* est constitué de 15 à 20 couches de cornéocytes. Ces cellules sont originaires de l'épiderme vivant et subissent plusieurs

changements morphologiques avant la desquamation. Leurs composants cellulaires (comme les mitochondries et le noyau) et leur cytoplasme disparaissent durant le processus de cornéification. Cette disparition est accompagnée d'un remodelage des protéines constitutives restantes pour former les cornéocytes. La majorité des protéines intracellulaires sont composées de filaments de kératine. La cohésion du *stratum corneum* est assurée par les cornéodesmosomes , structures protéiques reliant les cornéocytes [1] (Figure 4).

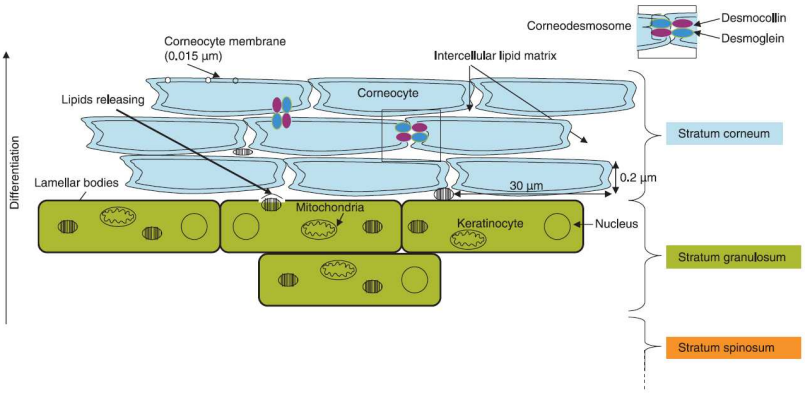

Figure 4 : Structure du *stratum corneum* et du *stratum granulosum* [4].

La matrice lipidique

La matrice lipidique, entre les cornéocytes, représente 10 à 15% du poids sec du *stratum corneum*. Elle est composée de 50% de céramides, 10% d'acides gras, 25% de cholestérol et 15% de dérivés du cholestérol et de glucosylcéramide. La majorité de ces lipides sont synthétisés par les kératinocytes dans la partie supérieure du *stratum spinosum* et dans le *stratum granulosum* (Figure 4). Ces lipides sont arrangés en structures lamellaires, très stables et constituant une barrière efficace contre toute pénétration de l'extérieur [4]. Le groupe de J. Bouwstra a largement étudié la structure des régions lipidiques lamellaires du *stratum corneum*. Ce groupe a montré que la matrice lipidique est organisée en lamelles parallèles à la surface des cornéocytes (Figure 5).

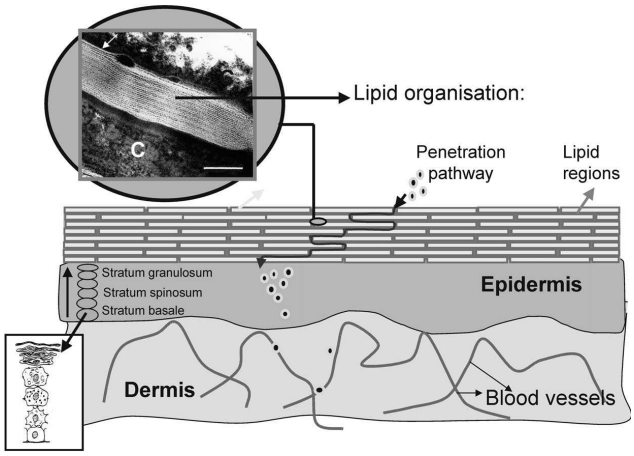

Figure 5 : Organisation lamellaire du *stratum corneum* [6].

Deux phases lamellaires coexistent dans le *stratum corneum*. Une phase possède une périodicité d'environ 6,4 nm, et l'autre une périodicité d'environ 13,4 nm [7]. Bien que la majorité des lipides soient présents dans un état cristallin appelé arrangement orthorhombique, quelques lipides sont occasionnellement présents dans un état gel appelé arrangement hexagonal dans la couche la plus superficielle de la couche cornée. Cette nature cristalline et la présence d'une phase lamellaire de 13 nm sont considérées comme les facteurs critiques pour la fonction de barrière de la peau. Un modèle moléculaire a été proposé pour décrire cette organisation, appelé « le modèle sandwich » (Figure 6) [8]. Récemment, Groen *et al*. ont cependant suggéré que l'organisation lamellaire était plus importante que l'arrangement orthorhombique pour les propriétés de barrière du *stratum corneum*. Ils ont observé que la perméabilité de l'acide benzoique n'était pas influencée par une transition de l'arrangement orthorhombique vers l'arrangement hexagonal. Par contre, en substituant les longues chaînes d'acide gras libres par des chaînes courtes, une augmentation de la perméabilité a été observée à cause de changements drastiques dans la structure lamellaire [9].

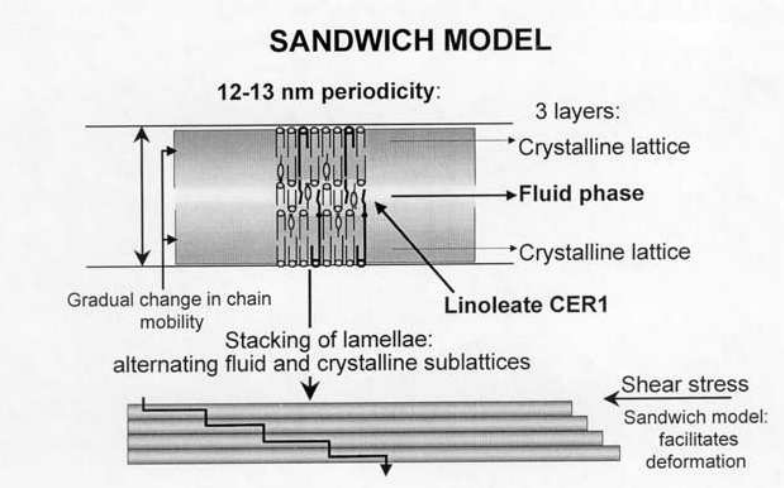

Figure 6 : Le modèle sandwich proposé par Bouwstra *et al*.[7].

I.1.1.2 L'épiderme vivant

Directement sous le *stratum corneum* se trouve l'épiderme vivant. On y distingue plusieurs couches de kératinocytes d'après leur degré de différenciation (aussi appelée kératinisation). L'origine des cellules de l'épiderme se trouve dans la membrane basale entre le derme et l'épiderme vivant. Se succèdent alors la couche basale ou *stratum basale*, la couche spinocellulaire ou *stratum spinosum*, la couche granuleuse ou *stratum granulosum*, et enfin la couche claire ou *stratum lucidum* (cette dernière est présente uniquement à la plante des pieds et la face palmaire des mains) (Figure 2). D'autres cellules sont également présentes comme les mélanocytes (impliqués dans la pigmentation), les cellules de Langerhans (impliquées dans l'immunité) et les cellules de Merkel (récepteurs du toucher) [3].

Couche basale

La couche basale contient des cellules de forme cylindrique ou cubique fonctionnant comme des cellules souches avec la capacité de se diviser pour produire de nouvelles cellules. Les cellules de cette couche sont

attachées à la membrane basale par des hémidesmosomes présents sur leur face ventrale et par des jonctions adhérentes.

Couche spinocellulaire

De forme polyédrique dans les couches profondes, les cellules de la couche spinocellulaire s'aplatissent progressivement vers les régions plus superficielles. Les cellules sont connectées entre elles et à la couche basale par des desmosomes. Des tonofibrilles (filaments de cytokératine) pénètrent dans les prolongements cytoplasmiques des kératinocytes et constituent le cytosquelette, assurant une certaine rigidité. Les mitoses cessent et la phase de maturation terminale est initiée. Dans les couches supérieures, des granules font leur apparition dans le cytosol, marquant la transition avec la couche granuleuse [3].

Couche granuleuse

De plus en plus aplaties, les cellules de la couche granuleuse voient progressivement disparaître leurs prolongements cytoplasmiques. Elles contiennent un grand nombre de granules intracytoplasmiques. On distingue deux types de granules. Les granules intracellulaires de kératohyaline et les kératinosomes. Les kératinosomes sont entourés d'une membrane d'origine golgienne et contiennent de nombreuses lamelles orientées parallèlement à leur surface. Ces granules, libérées par exocytose, sont supposées être les précurseurs des lamelles intercellulaires du *stratum corneum*. Des enzymes hydrolytiques y sont également associées et participent au processus de desquamation en déstructurant les desmosomes [3].

Couche claire

Cette couche est visible uniquement où la peau est épaisse. Elle est constituée de cellules où le noyau et la majorité des organites cytoplasmiques ont dégénéré.

I.1.2 Le derme

Le derme permet non seulement d'apporter les nutriments, l'immunité et d'autres supports à l'épiderme mais aussi d'avoir un rôle dans la régulation de la température, de la pression et de la douleur. Le derme a une épaisseur de 0,1 à 0,5 cm. C'est un tissu conjonctif composé de fibres de collagène (70%) et de fibres élastiques dans une matrice extracellulaire riche en protéoglycanes et glycoprotéines. Il est le siège d'un important réseau vasculaire et nerveux. Les principales cellules présentes sont les fibroblastes qui produisent le tissu conjonctif, et les mastocytes qui sont impliqués dans l'immunité et les réponses inflammatoires. Des cellules dendritiques et des globules blancs sont également présents [3]. On distingue plusieurs régions dans le derme en fonction de l'organisation des fibres conjonctives.

Le derme papillaire

Il s'enchevêtre avec l'épiderme par des papilles. Il est constitué d'un tissu conjonctif lâche dans lequel on observe des fibres de collagène qui ne sont guère groupées en faisceaux et des fibres élastiques, toutes étant orientées perpendiculairement à la jonction dermo-épidermique. La membrane basale est ancrée au derme par des fibrilles de collagène.

Le derme réticulaire

Il est très riche en fibres et constitué de faisceaux épais de fibres de collagène formant des travées onduleuses disposées parallèlement à la surface cutanée. Cette richesse en fibres lui confère une grande résistance mais a pour conséquence de le rendre moins souple que le derme papillaire et l'hypoderme.

Le derme profond

Il est constitué d'épais faisceaux de collagène se continuant dans l'hypoderme.

I.1.3 L'hypoderme

L'hypoderme est la couche la plus profonde de la peau. Il est constitué d'adipocytes arrangés en lobules et est relié au derme par des fibres de collagène et d'élastine, formant un tissu conjonctif lâche. L'hypoderme a un rôle d'isolant thermique, de protection contre les chocs et de réservoir énergétique [3].

I.1.4 Les annexes cutanées

On distingue quatre annexes cutanées : les glandes sudoripares eccrines, les glandes sudoripares apocrines, les follicules pileux associés aux glandes sébacées et les ongles. Bien que représentant seulement 0,1% de la surface cutanée, leur rôle comme voie potentielle de pénétration cutanée ne peut pas être négligé.

I.1.4.1 Les glandes sudoripares

Il existe deux types de glandes sudoripares : les glandes eccrines et les apocrines. Ce sont les plus nombreuses des annexes cutanées, distribuées sur tout le corps à une densité d'environ 400 glandes par cm^2. Elles sont responsables de la sécrétion de la sueur lorsque la température augmente [1].

Glandes eccrines

Les glandes eccrines sont de loin les plus nombreuses et se trouvent dans tout le revêtement cutané. Elles comprennent deux parties : une portion sécrétoire constituée d'un tube unique pelotonné localisé dans la partie profonde du derme et d'un tube excréteur (=canal sudoripare) qui achemine la sueur vers la jonction dermo-épidermique d'où elle gagne la surface.

Glandes apocrines

Elles sont beaucoup moins nombreuses et ne se rencontrent que dans certains endroits dont les plis inguinaux, les creux axillaires, les régions mammaires et périanales. Elles déversent leur contenu dans la gaine du poil.

I.1.4.2 Les follicules pileux

La structure générale d'un follicule pileux est représentée dans la Figure 7. Il comprend le bulbe pilaire et le poil entouré d'une gaine épithéliale interne et externe. Le muscle arrecteur pilaire et une glande sébacée y sont associés. Le bulbe papillaire contient la matrice du poil où des cellules se divisent et se différencient en se chargeant en eukératine dure pour former le poil.

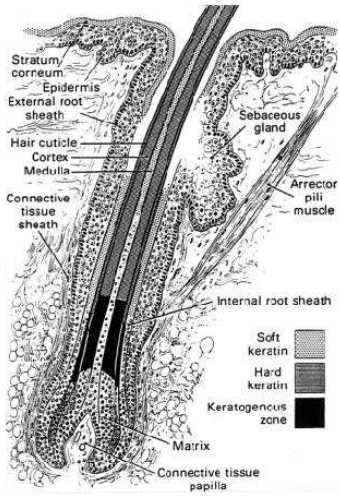

Figure 7 : Représentation d'un follicule pileux.

Les glandes sébacées sont constituées d'alvéoles piriformes. Les produits de sécrétions sont directement déversés dans la gaine du poil par un court conduit excréteur. Le sébum est composé de triglycérides, d'acides gras ainsi que de squalènes et de cires en plus faibles quantités. Bien que l'activité de ces glandes puisse avoir un impact significatif sur la

composition des lipides du *stratum corneum*, il est généralement accepté que le sébum ne participe pas à la fonction de barrière de la peau [1].

I.2 Voies de pénétration cutanée

Pour toute molécule appliquée sur la peau, on distingue deux voies de pénétration, la voie via les annexes cutanées et la voie transépidermale.
Les annexes cutanées ont d'abord été considérées comme insignifiantes comme voie de pénétration car elles ne représentent qu'approximativement 0,1% de la surface cutanée. Cependant, des études récentes suggèrent que la voie folliculaire peut être spécialement importante pour les molécules hydrophiles et de haut poids moléculaire, de même que pour les formes d'administration vésiculaires [10].
La voie transépidermale contient elle-même deux voies de pénétration (Figure 8). La pénétration transcellulaire à travers les cornéocytes et la matrice lipidique et la voie intercellulaire à travers les domaines lipidiques entre les cornéocytes. Il est généralement admis que la voie intercellulaire est la principale voie de pénétration pour la majorité des substances [11, 12].

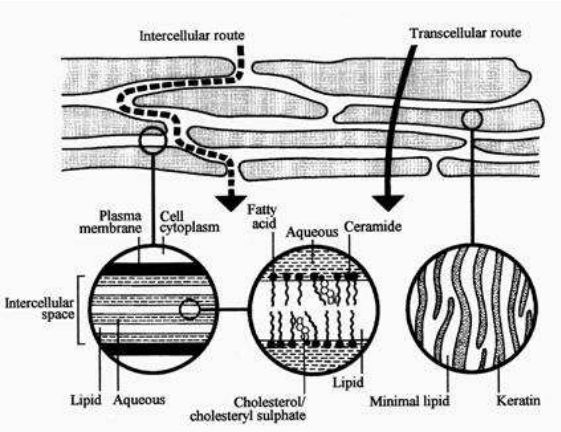

Figure 8 : Les voies de pénétration transcellulaire et intercellulaire au niveau du *stratum corneum* [11].

I.3 Modèles de peaux utilisés en recherche

La pénétration d'une substance dans et/ou à travers la peau s'évalue le plus souvent *ex vivo* au moyen de cellules de diffusion, appelées cellules de Franz. De nombreuses recommandations ont été publiées pour l'utilisation de ces cellules de diffusion, dont certaines ont été sélectionnées comme directives. La plus connue est la directive 428 de l'Organisation pour la Coopération et le Développement Économiques (OCDE ou guideline 428 OECD) [13, 14].
Une cellule de diffusion est constituée d'un compartiment donneur et d'un compartiment receveur entre lesquels est placée la peau. La préparation à étudier est appliquée sur la surface externe de la peau, dans le compartiment donneur. Le compartiment receveur contient un milieu, qui est maintenu à 37°C et sous agitation constante (Figure 9).

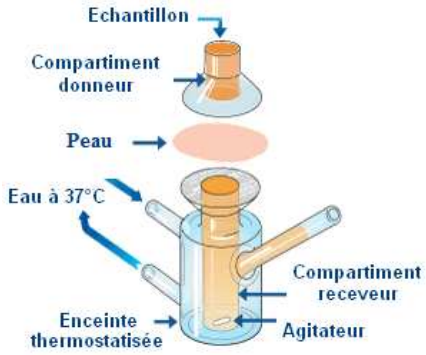

Figure 9 : Représentation d'une cellule de diffusion de Franz.

I.3.1 Corrélation *in vivo-ex vivo*

Franz a entrepris les premières validations de ce modèle d'étude *ex vivo* de l'absorption cutanée. Il a comparé la perméabilité de 12 molécules modèles à travers la peau humaine abdominale congelée avec des résultats obtenus précédemment *in vivo* par d'autres chercheurs. Il pu

démontrer une excellente correspondance qualitative entre les deux méthodes, malgré le fait que les données *in vivo* aient été obtenues par différentes sources [15]. Shaw *et al*. ont montré une excellente corrélation entre les flux de pénétration sur cellules de Franz et *in vivo* de 18 molécules, les deux études étant réalisées cette fois-ci par le même groupe (Figure 10) [16].

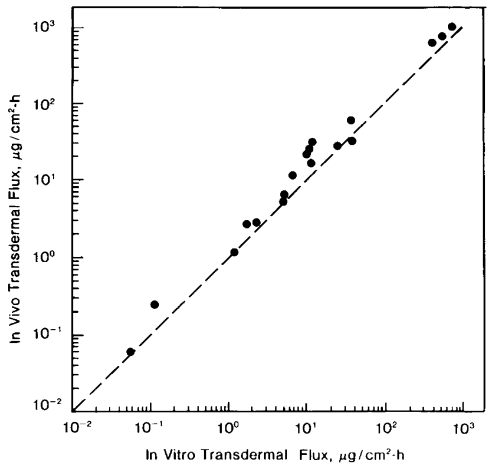

Figure 10 : Corrélation entre les flux transdermiques obtenus *in vivo* et au moyen des cellules de Franz, chaque point représentant un principe actif. La ligne en pointillés indique la corrélation absolue [16].

I.3.2 Corrélation peau humaine-peau animale

Comme la plupart des études tendent à prédire la pénétration d'une substance chez l'homme, la peau d'origine humaine devrait être préférentiellement utilisée. La peau d'origine humaine provient de peaux abdominales ou mammaires prélevées lors d'actes chirurgicaux. Malgré leurs avantages, les peaux d'origine humaine sont rarement utilisées à cause notamment de la difficulté d'approvisionnement et des charges administratives et éthiques. C'est pourquoi, la peau d'origine animale est souvent préférée. Les espèces animales les plus souvent utilisées dans les études *ex vivo* sont des cochons, des cochons d'Inde, des rats, des souris

et des lapins. Nous avons utilisé la peau d'oreilles de cochons. En effet de nombreuses publications ont montré une bonne corrélation entre les résultats obtenus sur peaux humaines et sur peaux d'oreilles de cochons [17-20].

I.3.3 Corrélation peau fraîche-peau congelée

Bien que la directive OECD 428 préconise l'utilisation de peaux humaines vivantes pour les tests de pénétration, des peaux qui ont été conservées congelées, peuvent également être utilisées [13]. Franz n'a pas observé de différence de perméabilité entre de la peau fraîche et de la peau humaine conservée 3 mois sous forme congelée [15]. Schreiber *et al*. ont montré une bonne corrélation entre la pénétration de la caféine et de la testostérone dans des peaux fraîches ou congelées [21].

I.3.4 Membranes synthétiques

Différentes membranes synthétiques sont également utilisées à la place de peaux [22, 23]. Dans nos premières études *in vitro*, nous avons utilisé des membranes en polycarbonate. Cependant, comme il est décrit dans la partie expérimentale, les résultats obtenus avec ces membranes ne se sont pas confirmés lors de l'utilisation de la peau d'oreilles de cochons.

I.3.5 Epidermes reconstitués

Des peaux reconstituées à partir de cultures de kératinocytes sont également utilisées pour pallier la difficulté d'approvisionnement des peaux humaines. Des épidermes reconstitués sont disponibles dans le commerce comme modèles de peaux tels qu'EpiDerm™, EPISKIN® et SkinEthic®. Netzlaff *et al*. ont montré des similitudes raisonnables entre la peau humaine et ces trois modèles en termes de morphologie, de composition en lipides et de marqueurs biochimiques. Ces modèles sont considérés comme des outils utiles pour tester la phototoxicité, la

corrosivité et le pouvoir irritant de substances. Des premières études ont montré leur compatibilité pour des études de transport de principes actifs. Cependant, la fonction de barrière de ces systèmes apparaît comme étant moins développée que celle de la peau humaine. Ces auteurs concluent que des adaptations de ces modèles restent un important défi pour l'avenir [24].

II Les liposomes en application cutanée

II.1 Structure et composition

Les liposomes sont définis comme des vecteurs vésiculaires formés d'une ou plusieurs paroi(s) composée(s) de molécules amphiphiles formant des bicouches lipidiques et entourant une cavité aqueuse. Les liposomes ont été découverts par Bangham en 1965 [25], mais c'est Mezei, qui le premier a suggéré que les liposomes pouvaient se révéler utiles comme systèmes de transport médicamenteux pour le traitement des maladies cutanées [26, 27].

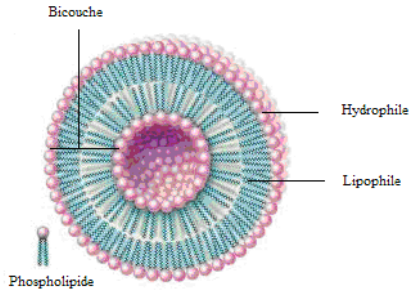

Figure 11 : Structure d'un liposome unilamellaire.

Les liposomes sont constitués à partir de différents phospholipides avec ou sans certains additifs. Les phospholipides les plus utilisés peuvent être d'origine naturelle (phosphatidylcholine d'œuf ou de soja) ou synthétique (par exemple la dimyristoylphosphatidylcholine, la dipalmitoylphosphatidylcholine). La phosphatidylcholine naturelle est constituée d'une partie hydrophobe, formée par une double chaîne hydrocarbonée, et d'une partie hydrophile, formée par la choline et un groupement phosphate, reliées entre elles par une molécule de glycérol. La phosphatidylcholine de soja est un mélange de plusieurs phospholipides

caractérisés par des chaînes hydrocarbonées possédant un nombre de carbones et de doubles liaisons variables. La Figure 12 représente le phospholipide majoritaire de la phosphatidylcholine de soja, avec une chaîne hydrocarbonée de 18 atomes de carbone et deux doubles liaisons ainsi qu'une chaîne de 16 atomes de carbone.

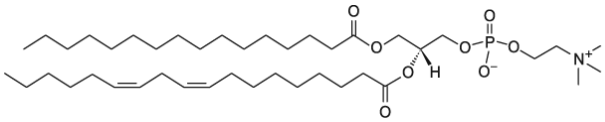

Figure 12 : Représentation du phospholipide majoritaire de la phosphatidylcholine naturelle.

Le cholestérol peut être ajouté au niveau des bicouches lipidiques des liposomes pour améliorer leurs caractéristiques, notamment en augmentant la microviscosité, en réduisant la perméabilité aux molécules hydrophiles, en stabilisant la membrane et en augmentant la rigidité des vésicules [28-30].
Au contraire, l'addition d'un "edge activator" déstabilise les bicouches lipidiques en donnant des liposomes dits déformables ou Transfersomes® [31]. Les « edge activators » le plus souvent utilisés pour former les liposomes déformables sont des tensioactifs comme le déoxycholate de sodium, les polysorbates ou les esters de sorbitane [32, 33].
Des composés lipidiques portant une charge peuvent également être ajoutés afin de conférer une polarité à la bicouche lipidique, comme la stéarylamine (SA) qui est un lipide portant une charge positive ou l'acide dimyristoylphosphatidique (DMPA) qui est un phospholipide portant sous sa forme de sel sodique, une charge négative.

Les liposomes sont capables d'encapsuler des principes actifs lipophiles au niveau de leur bicouche lipidique ainsi que des principes actifs hydrophiles au niveau de leur cavité aqueuse.
Une approche intéressante consiste à encapsuler un principe actif lipophile au niveau de la cavité aqueuse des liposomes grâce à l'utilisation de

complexes d'inclusion avec les cyclodextrines. Ce concept a abouti à un nouveau type de système appelé « drug-in-cyclodextrin-in-liposome » [34-37]. Les cyclodextrines sont des oligosaccharides cycliques produits par dégradation enzymatique de l'amidon. Elles sont composées d'un nombre variable d'unités α-D-glucopyranose en configuration chaise, reliées entre-elles par des liaisons α (1,4). On distingue les α-, β- et γ-cyclodextrines lorsqu'elles sont composées respectivement de 6, 7 ou 8 unités.

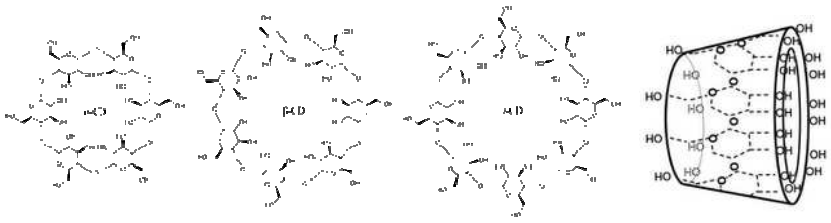

Figure 13 : Structure des α-, β- et γ-cyclodextrines et leur représentation sous forme de cône tronqué.

La configuration des cyclodextrines en trois dimensions apparaît sous la forme d'un cône tronqué hydrophile à l'extérieur grâce aux groupements hydroxyles et plus hydrophobe à l'intérieur. Grâce à leur structure particulière, les cyclodextrines sont capables d'augmenter la solubilité aqueuse de composés en les incluant dans leur cavité hydrophobe pour former des complexes d'inclusion [38].

II.2 Classification

Les liposomes sont classés en trois catégories en fonction de leur taille et du nombre de bicouches. On distingue les:

- « small unilamellar vesicles SUV » ou petites vésicules unilamellaires de 20 à 200 nm de diamètre ;
- « large unilamellar vesicles LUV » ou grandes vésicules unilamellaires d'un diamètre de 200 nm à 1 µm ;

- « giant unilamellar vesicles GUL » ou géantes vésicules unilamellaires d'un diamètre supérieur à 1 µm ;
- « multilamellar vesicles MLV » ou vésicules multilamellaires d'un diamètre supérieur à 500 nm [39, 40].

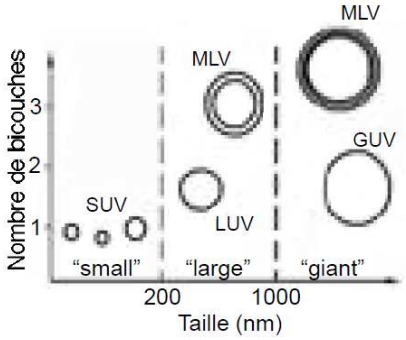

Figure 14 : Classification des liposomes selon leur nombre de bicouches et leur taille [40].

II.3 Préparation

Les liposomes peuvent être préparés de différentes manières.
La méthode la plus utilisée pour former les liposomes et développée par Bangham, est la méthode dite d'hydratation du film lipidique. Les différentes étapes de cette préparation sont décrites dans la Figure 15. En pratique, les lipides sont dissous dans un solvant organique comme l'éthanol absolu, le méthanol ou le chloroforme dans un ballon rond. La solution est alors évaporée à l'aide d'un évaporateur rotatif sous vide. Le film lipidique ainsi obtenu sur la paroi du ballon est hydraté par une solution aqueuse. La dispersion des lipides est facilitée par l'utilisation du vortex et de billes de verre. Des vésicules multilamellaires se forment alors spontanément. Différentes méthodes telles que la sonication ou l'extrusion peuvent ensuite être utilisées pour obtenir des liposomes unilamellaires de la taille souhaitée. L'extrusion consiste à forcer le passage de la suspension de liposomes à travers des membranes de

polycarbonate de taille de pores bien définies grâce à une pression d'azote.

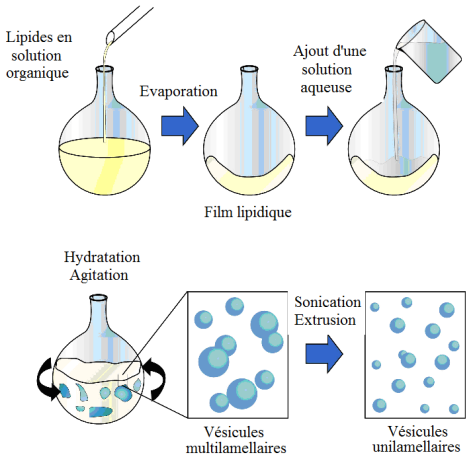

Figure 15 : Préparation des liposomes par la méthode d'hydratation du film lipidique.

Les liposomes peuvent également être préparés par évaporation en phase inverse [41]. Les premières étapes de cette préparation sont identiques à la méthode d'hydratation du film lipidique. Cependant, dans ce cas ci, le film lipidique est solubilisé par un solvant non miscible à l'eau comme l'éther. La phase aqueuse est alors ajoutée pour former une émulsion E/H (eau dans huile). Ensuite, l'évaporation du solvant organique conduit à la formation des vésicules.

Une troisième méthode est la méthode d'injection de solvant. L'injection d'une solution éthanolique (ou éthérée) de phospholipides dans une solution aqueuse conduit à la formation spontanée de liposomes.

D'après les propriétés physico-chimiques de la substance active destinée à être encapsulée dans les liposomes, celle-ci peut être ajoutée soit dans la phase lipidique, soit dans la phase aqueuse durant la préparation.

La préparation des liposomes est également possible par la technologie des fluides supercritiques [42].

La substance active peut aussi être incorporée dans des liposomes préformés. Différentes techniques peuvent être utilisées comme la lyophilisation – réhydratation ou l'utilisation de gradient de pH.

II.4 Mécanismes de pénétration cutanée

Dans deux revues récentes, El Maghraby *et al*. ont décrit cinq mécanismes possibles de pénétration cutanée des liposomes [11, 43]. Ces mécanismes sont décrits ci-dessous et correspondent aux points A à E dans la Figure 16.

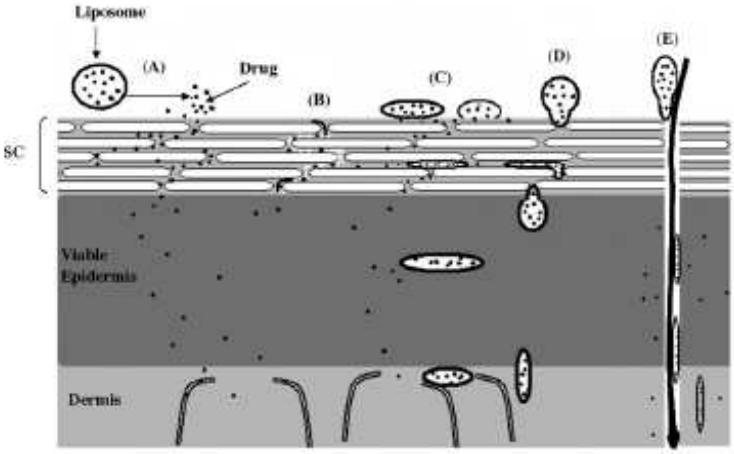

Figure 16 : Mécanismes de pénétration des liposomes au niveau cutané [11].

- Le mécanisme A correspond à la libération de la substance active seule, qui sort des liposomes pour pénétrer indépendamment des vésicules dans la peau.

- Pour le mécanisme B, les composants des vésicules engendrent une modification de l'ultrastructure de la matrice lipidique du *stratum corneum*. Les composants des liposomes jouent un rôle de promoteur d'absorption en diminuant l'imperméabilité du *stratum corneum*, favorisant ainsi la pénétration de la molécule encapsulée. Ce mécanisme a été confirmé par certains auteurs [44, 45] mais infirmé par d'autres [46, 47].

- Pour le mécanisme C, les liposomes pourraient s'adsorber sur la surface du *stratum corneum* avec pour conséquence un transfert direct de la molécule encapsulée du liposome vers la peau. Les vésicules pourraient également fusionner et se mélanger à la matrice lipidique du *stratum coneum*, augmentant ainsi la répartition de la molécule dans la peau [48].

- Le mécanisme D correspond à la pénétration des liposomes sous forme intacte. Bien qu'il soit maintenant généralement accepté que les liposomes conventionnels ne pénètrent pas sous forme intacte dans la peau, ce mécanisme de pénétration a été décrit par Cevc *et al*. pour les liposomes déformables ou Transfersomes® [31, 49-51]. Grâce à la présence d'un surfactant au niveau de leur bicouche, ces vésicules montrent des propriétés élastiques, qui leur permettraient de se déformer pour passer entre les cellules du *stratum corneum* et ainsi arriver sous forme intacte au niveau de l'épiderme. Cette pénétration serait rendue possible par la présence d'un « gradient transépidermique naturel en eau » (Figure 17). L'hydrophilie des phospholipides les pousserait à éviter les environnements secs. En conséquence, les liposomes vont essayer de suivre le gradient d'hydratation en se déplaçant vers les couches plus profondes et plus hydratées de la peau.

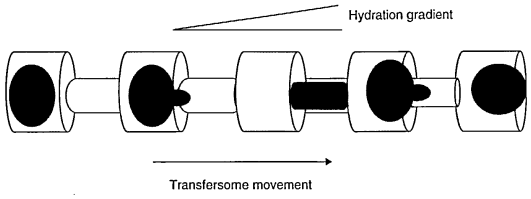

Figure 17 : Schéma représentant le passage de liposomes déformables au travers de petits pores grâce au gradient d'hydratation, adapté de [43].

- Enfin, le mécanisme E correspond à une pénétration des liposomes via les annexes cutanées. La pénétration des liposomes au niveau des follicules pileux a été montrée et mise à profit pour cibler ces annexes cutanées [10]. Malheureusement, il n'y a pas d'évidence d'une pénétration à travers ces follicules [11].

La revue récente de Benson souligne le manque de compréhension du mécanisme de pénétration cutanée des liposomes. Leur capacité à transporter un principe actif dans et à travers la peau a été étudiée mais reste un sujet controversé [52].

Après l'application cutanée de liposomes déformables, des changements morphologiques ont été identifiés dans le *stratum corneum* et des vésicules intactes ont été visualisées dans les régions lipidiques lamellaires du *stratum corneum* mais aucune vésicule intacte n'a pu être visualisée dans les couches profondes de la peau [53]. Bouwstra *et al*. ont montré que la quantité d'eau présente dans les couches profondes du *stratum corneum* près de l'épiderme est plus faible que dans la région centrale du *stratum corneum*. Sur la base de ce gradient en eau, ils ont donc suggéré que les liposomes déformables ne pénètrent pas au delà du *stratum corneum* [8]. Cependant, de nombreuses études montrent que les liposomes déformables entraînent une augmentation de la pénétration cutanée ou transcutanée [32, 54-58].

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III Paramètres influençant la pénétration cutanée des liposomes

Ce chapitre a fait l'objet d'une publication sous forme d'un article de revue dans le Journal of Drug Delivery Science and Technology.

LIPOSOMES AND PARAMETERS AFFECTING THEIR SKIN PENETRATION BEHAVIOUR

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Journal of Drug Delivery Science and Technology, sous presse

III. 1 Article

Abstract

It is known that classical and more recently deformable liposomes have shown their ability to increase dermal and transdermal drug delivery. However their penetration mechanism remains controversial and not understood at this time. Different vesicles compositions, preparation methods and drugs have been used, giving vesicles with diverse characteristics. This fact in addition to the experimental conditions probably account for the lack of understanding their mode of action. This review focuses on the penetration behaviour of liposomes as a function of their composition. Parameters influencing the mode of action of liposomes are discussed, like the preparation method, lipid composition, entrapment efficiency, addition of an edge activator or ethanol, size and lamellarity. It also reviews parameters of the experimental set-up (usually Franz type diffusion cells are used for *in vitro* skin absorption studies) which could affect results of diffusion, like the skin used, receptor fluid composition, occlusive or non occlusive mode, washing step. The purpose is to highlight parameters which are beneficial or detrimental to skin penetration with the goal to understand the penetration mechanism of liposomes.

Keywords
Liposome, vesicle, skin penetration, Franz diffusion cell, dermal drug delivery, transdermal drug delivery.

1. Introduction

Transdermal drug delivery has many potential advantages over other routes of administration. It allows the avoidance of gastrointestinal tract problems and hepatic first-pass effects, and improvement in patient compliance [1]. However, the barrier nature of the skin inhibits the penetration of most drugs. Researchers are developing various strategies to overcome this barrier. These include physical permeation enhancement techniques like iontophoresis by driving electrically molecules into and through the skin [2-4], electroporation by application of high-voltage pulses to the skin [5, 6] and sonophoresis by application of ultrasound [7]. Passive penetration enhancement techniques include for example use of supersaturated solutions [8, 9], penetration enhancers [10, 11] or microemulsions [12]. Combination of these strategies is also studied [3, 13].

Vesicular systems provide an alternative to improve drug delivery to and through the skin. Classical and more recently deformable liposomes have shown their ability to increase dermal and transdermal drug delivery. However their penetration mechanism remains controversial and not understood at this time. Different drug, vesicles composition and method of preparation have been employed, resulting in vesicles having diverse characteristics with respect to size, lamellarity, charge, membrane fluidity, permeability, elasticity and drug entrapment efficiency. This fact in addition to the skin model used (animal or human, *in vivo* or *in vitro*) probably account for the lack of understanding the mode of action of vesicles [14].

This review focuses on the penetration behaviour of liposomes as a function of their composition. The purpose is to highlight the parameters which are beneficial or detrimental on skin penetration with the goal to understand the penetration mechanism of liposomes. It will also reviews parameters of the experimental set-up (usually Franz type diffusion cells are used for *in vitro* skin absorption studies) which could affect results of

diffusion, like the skin used, receptor fluid composition, occlusive or non occlusive mode, washing step.

2. Skin structure

The skin is the largest organ of the body and forms an effective barrier between the organism and the environment. Its functions include protecting the body from injury and pathogens, maintaining fluid retention and controlling skin temperature [15]. The major skin layers, from inside to outside, comprise the subcutaneous hypodermis, the dermis of connective tissue and the stratified avascular, cellular epidermis [16]. The most abundant cell of the epidermis is the keratinocyte; cellular contacts are formed by desmosomes. Four epidermal layers are detectable by cellular differentiation: the *stratum basale* which is separated from the dermis by a basal membrane to which keratinocytes make contact by hemidesmosomes, the *stratum spinosum*, the *stratum granulosum* and the most superficial *stratum corneum* [17]. The *stratum corneum* is considered as the rate limiting barrier in penetration of most molecules. The *stratum corneum* consists of 15-20 layers of flat apoptotic keratinocytes, the so-called corneocytes. The thickness of the dry horny layer amounts to about 10-15 µm [16, 17].

The architecture of the *stratum corneum* has been described as the so-called bricks and mortar model. The bricks are the keratin rich corneocytes and the mortar is the lipid rich matrix [18]. The major lipid classes in this *stratum corneum* are ceramides (between 16-33 carbons), cholesterol and free fatty acids (chain lengths are C22 and C24) [19]. Bouwstra *et al*. considered that the organization in the lipid domains is very important for the skin barrier function. They showed that, in *stratum corneum*, crystalline phases are predominantly present, but most probably a subpopulation of lipids forms a liquid phase. Both the crystalline nature and the presence of a 13 nm lamellar phase are considered to be crucial for the skin barrier function. Based on these findings, they proposed a molecular model for the organization of the 13 nm lamellar phase,

referred to as "the sandwich model", in which crystalline and liquid domains coexist [20].

For any molecules applied to the skin, two main diffusional routes have been identified; the transappendageal and transepidermal pathways.

Initially, skin appendages were not considered to be significant transdermal penetration routes, as evidence suggested that they accounted for only approximately 0.1% of the skin area. However, recent studies suggest that the follicular penetration route may be especially relevant for hydrophilic and high molecular weight molecules, as well as by particle-based drug delivery systems [21].

The transepidermal pathway contains two micropathways. The transcellular pathway through the corneocytes and the lipid matrix, and the intercellular pathway through the lipids domains between the corneocytes. It is generally accepted that the intercellular pathway is regarded as the main route of permeation of most drug [16, 22].

3. Liposomes

Liposomes are built by amphiphilic molecules such as phospholipids which form bilayers containing water in their cavity and dispersed in an aqueous medium. The polar head groups of the phospholipids form the interface with the aqueous media. There are divided according to their size into three categories; the small unilamellar vesicles (SUV) have dimensions of 20 up to 100 nm, large unilamellar vesicles (LUV) are larger than 100 nm, and multilamellar vesicles (MLV) have dimensions exceeding 500nm [17]. Lipophilic agents are incorporated into the bilayers while hydrophilic agents are encapsulated in their aqueous compartment. Liposomes can also contain some additives like cholesterol. Cholesterol may be included to improve bilayers characteristics; increasing microviscosity of the bilayer, reducing permeability of the membrane to water soluble molecules, stabilizing the membrane and increasing rigidity of the vesicles [23-25]. In contrary, addition of a so-called edge activator destabilizes lipid bilayers giving deformable liposomes (see section 5.2.3).

4. Mechanisms of skin penetration

In recent reviews, El Maghraby *et al*. described five skin penetration mechanisms of liposomes [1, 16];

- The free drug mechanism where the drug exits from the vesicles and then permeates the skin independently;
- The penetration enhancing mechanism where vesicles components may enter the skin as monomers disrupting the packing characteristics of the *stratum corneum* lipid bilayers, thus enhancing drug permeation;
- Vesicles adsorption to and/or fusion with the *stratum corneum*;
- The intact vesicular skin penetration mechanism;
- The transappendageal penetration.

The review of Benson highlights the lack of understanding the penetration mechanism of vesicles. Their method of drug transporting into and through the skin has been investigated but remains an area of contention [26]. It is now generally accepted that conventional vesicles do not penetrate intact skin. Since description by Cevc of a penetration of intact elastic liposomes through the skin by the help of the naturally transepidermal water gradient, no proof of intact vesicular system in deeper layers of the skin has been shown. Bouwstra showed that the water content in the lowest *stratum corneum* layers close to the epidermis is much lower than in the central region of the *stratum corneum*. Thus, they suggested that, as a result of the osmotic gradient, vesicles will not penetrate beyond the level of the lower *stratum corneum* [19]. Many studies however showed an enhanced dermal or transdermal delivery by deformable vesicles [27-32].

5. Parameters influencing the skin penetration of liposomes

5.1 Liposome preparation

5.1.1 Method of preparation

Various types of liposomes (MLV, SUV, LUV, FRV (freeze-dried rehydration liposomes)) can be prepared by different methods. In the classical procedure a thin lipid film is deposited on the walls of a round-bottomed flask and shaken in excess of aqueous phase, resulting in MLV. In order to produce smaller and less lamellar liposomes, additional energy has to be dissipated into the system, like sonication or extrusion [33].

Weiner *et al*. evaluated the topical delivery of liposomally encapsulated interferon in the cutaneous herpes simplex virus guinea pig model. Comparing multilamellar (MLV) and large unilamellar liposomes (LUV) obtained by the classical film evaporation technique followed by extrusion in the case of LUV, with liposomes prepared by the dehydration/rehydration methods (DRV), DRV liposomes were superior to controls with respect to reduction of lesion scores. These authors explained that the dehydration and subsequent rehydration of the liposomes facilitate partitioning of the interferon into the liposomal bilayers, where the drug is positioned for transfer into the lipid compartment of the *stratum corneum*. Liposomes did not appear to function as permeation enhancers but seemed to provide the needed physicochemical environment for transfer of interferon into the skin [34]. The same group showed, in *in vitro* diffusion studies, that topical application of "skin lipid" liposomes prepared by the dehydration-rehydration method was twice as effective as were liposomes prepared by the reverse-phase evaporation method with respect to their ability to deposit interferon into the skin strata where the basal cell layers reside [35]. More recently, Maestrelli *et al*. investigated the effect of preparation

technique on the properties of liposomes encapsulating ketoprofen-cyclodextrin complexes. Liposomes were prepared by thin layer evaporation, freezing and thawing, extrusion through microporus membrane and reverse phase evaporation obtaining respectively multilamellar vesicles ((MLV), frozen and thawed MLV (FATMLV), small uni-lamellar vesicles (SUV) and large uni-lamellar vesicles (LUV). The drug permeation rate across cellulose nitrate membrane impregnated with lauryl alcohol as lipid phase depended on the vesicles characteristics and varied in the order SUV>MLV=FATMLV>LUV [36]. Authors performed previous comparative studies by using excised rat skin or such artificial lipophilic membranes to demonstrate the good correlation between permeation data obtained with the two methods [37].

5.1.2 Size and lamellarity

Mura *et al*. showed that the reduced dimensions of the vesicles did not have a significant effect on either the permeation across rat skin or the *in vivo* drug therapeutic effectiveness from benzocaine loaded liposomes [38]. Sinico *et al*. studied liposomes as carriers for the dermal delivery of tretinoin and showed that vesicles size and lamellarity did not affect tretinoin delivery through the pig skin [39]. Kim *et al*. investigated the penetration enhancing effect of phosphatidylcholine on caffeine. They showed that the amount of absorbed caffeine was nearly independent of the encapsulation efficiency and the vesicle size [40]. On the contrary, Verma *et al*. showed that the penetration of carboxyfluorescein was inversely related to the size of the liposomes [41]. The assumption that a decrease in the particle size of the liposomes would result in an increase in the amount of drug found in the deeper skin strata supports the concept of intact vesicular penetration as one of the possible mechanisms for improved skin accumulation [22]. Regarding to the contradictory results in the literature, no general conclusion can be formulated concerning the liposomes size influence on the intact vesicular penetration.

Yu *et al*. compared MLV and SUV within the same vesicle charge. Negative SUV gave higher penetrated amount and permeability of triamcinolone, and neutral SUV revealed lower skin retention than the corresponding MLV [42].

5.2 Membrane composition

5.2.1 Lipid composition

As explained in section 3, liposomes are lipid vesicles made of phospholipids with or without some additives like cholesterol.
Weiner's group investigated the impact of liposome lipid composition and showed that liposomes made of *stratum corneum* lipids appear more efficacious than the phospholipid-based liposomes [34, 35]. Skin lipid liposomes were also studied by Fresta to improve corticosteroid dermal delivery. Skin-lipid liposomes showed 1.3 times higher blanching effect than phospholipid based formulation. Skin-lipid liposomes also produced a reduction in drug levels in blood and urine, reducing possible side effects [43]. Yu *et al*. observed that liposomes consisting of skin lipid composition more significantly enhanced the skin permeation of triamcinolone than other liposomes [42]. In a recent paper, Puglia *et al*. evaluated the percutaneous absorption of naproxen from *stratum corneum* lipids liposomes and phosphatidylcholine/cholesterol liposomes. Results of *in vitro* study showed that naproxen formulated in *stratum corneum* lipids liposomes was characterized by a decreased permeation through the skin that could be related to a high reservoir capacity of the *stratum corneum* when the drug is applied in a formulation that has a similar lipid composition to this skin layer [44].
The hydrated liposome lipid membrane can appear in at least two different thermodynamic states, the so-called gel phase, in which the lipid molecules are packed in a quasi-crystalline two dimensional lattice, and the liquid-crystalline state, in which the lipids retain their lamellar arrangement but are now able to diffuse rapidly in the plane of the lipid

bilayer and also display fast rotational diffusion and trans-gauche isomerisation of the fatty acyl chains. The transition between the two states is reversible and is normally induced by temperature at the transition temperature T_m [45]. Bouwstra's group demonstrated that the thermodynamic state of the bilayer of the vesicles plays a crucial role in the effect of vesicles transport rate across skin *in vitro*. From confocal laser scanning microscopy images, it was clear that the label applied in micelles and gel-state liposomes did not penetrate as deep into the skin as the label applied in liquid-state vesicles. [19, 46]. Pérez-Cullell *et al*. also studied the influence of the fluidity of liposome compositions on percutaneous absorption. The skin penetration of sodium fluorescein was higher from fluid liposomes (phosphatidylcholine) than from rigid liposomes (hydrogenated phosphatidylcholine), but it was independent of the content of cholesterol. Authors concluded that the liquid-crystalline state of the lipids was the main aspect involved in the fluidity of the liposome bilayer itself as well as in the interaction with the lipids of the *stratum corneum* [47]. However, Sinico *et al*. have obtained higher drug diffusion when tretinoin was delivered in vesicles made from phospholipids with the highest transition temperature T_m (hydrogenated soy phosphatidylcholine P90H T_m = 52°C) than in soy phosphatidylcholine vesicles (P90, T_m<2°C). They explained these results as the consequence of the presence in the liposomal bilayers of both cholesterol and the amphipatic drug tretinoin, which was present in high molar ratio. These both compounds could affect the T_m of soy hydrogenated phosphatidylcholine in such a way that the fluidity of the P90H liposomal membrane increased at the temperature of the experiments [39].

5.2.2 Influence of the charge

Dimyristoyl phosphatidic acid (DMPA) or dicetylphosphate (DCP) are generally used as negative charge components while stearylamine or dioleoyltrimethylammonium propane chloride salt (DOTAP) are used to produce positively charged liposomes.

The lipid layer in the *stratum corneum* contains high ratio of negatively charged lipids and it is well known that the skin may act as a negatively charged membrane [39, 48]. It has been reported that the presence of a charge on the vesicle surface may affect the drug diffusion through the skin. Negatively charged vesicles generally give a higher flux than positively charge counterparts, which in turn can improve drug accumulation in the superficial skin strata [39]. The most efficient composition tested by Carrer *et al*. contained the highest proportion of charged edge activators: they suggested that the presence of negative charges in the membrane may allow for a better efficiency of penetration [49]. Manosroi *et al*. studied the transdermal absorption of amphotericine B liposomes. The absorption of amphotericine B entrapped in charged liposomes was higher than that in the uncharged. The positive liposomes appeared to exhibit higher absorption through the *stratum corneum* than the negative charged liposomes. This was explained by the fact that the cell surface of the skin bears a net negative charge. However, negative liposomes exhibited higher absorption through the viable epidermis and dermis than the positively charged liposomes [50]. Ogiso *et al*. showed that the percutaneous absorption of betahistine from a gel formulation containing negatively charged liposomes of betahistine was much higher than that from the formulation with positively charged ones. Histological studies confirmed their observation [51]. Katahira *et al*. found that negative dicetylphosphate liposomes provided better rhodamine B retention in the skin with lower skin permeability compared with positive and neutral multilamellar liposomes [52]. On the other hand, Sinico at al. showed that positively charged vesicles provided a tretinoin permeation similar or even statistically higher than negatively charged liposomes [39]. Hasanovic *et al*. studied the influence of adding cationic polymers (chitosan or Eudragit EPO) on the stability and skin penetration of DPPC liposomes encapsulating aciclovir 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. This increase in permeation was explained as a tendency of positively charged

liposomes to interact more strongly with the skin surface or as an interaction of the polymers with skin lipids, the polymers going more deeply disrupting the tight junctions in lower epidermis layers [53]. In our lab, we studied the skin penetration of charged phosphatidylcholine liposomes. We showed that negatively charged liposomes significantly enhanced betamethasone absorption in the epidermis compared to positively charged and neutral liposomes. This was observed with DMPA and DCP negative liposomes encapsulating betamethasone and also with negatively charged liposomes bearing betamethasone dipropionate (results to be published).

5.2.3 Addition of an edge activator

Recently, it became evident that, in most cases, classical liposomes are of little or no value as carriers for transdermal drug delivery, as they do not seem to penetrate skin deeply, but rather remain confined to the upper layers of the *stratum corneum* [24]. In order to target deeper underlying skin tissue, intensive research led to the introduction and development of a new class of lipid vesicles, the highly deformable (elastic or ultraflexible) liposomes, which were named Transfersomes® [54]. Several studies have reported that deformable liposomes are able to improve *in vitro* skin delivery of various drugs [27] and to penetrate intact skin, *in vivo*, while transferring therapeutic amounts of drugs [55]. According to Cevc and Blume, the improved drug delivery by deformable liposomes is due to the driving force provided by the osmotic gradient between the outer and inner layers of the *stratum corneum* [54]. The important difference between deformable liposomes and traditional liposomes is the high and stress-dependent adaptability of such deformable vesicles, which enables them to squeeze between the cells in the *stratum corneum*, despite the large average vesicle size [56]. Thus, they can pass through the intact skin spontaneously, under the influence of the naturally occurring, *in vivo* transcutaneous hydration gradient [57].

These vesicles consist of phospholipids and an edge activator. An edge activator is often a single chain surfactant, with a high radius of curvature, which destabilises lipid bilayers of the vesicles and increases their deformability [24]. Edge activators are for example, sodium cholate, sodium deoxycholate, Span®, Tween® and dipotassium glycyrrhizinate [26, 27]. In a study on estradiol skin delivery, El Maghraby *et al*. showed an improved skin delivery from ultradeformable liposomes. Moreover, Span 80® and Tween 80® were found to be equivalent to sodium cholate as edge activators [58]. Ita *et al*. studied the dermal delivery of selected hydrophilic drugs from elastic liposomes containing different surfactants. They obtained a higher flux value for liposomes containing sodium cholate compared with sodium deoxycholate. For the liposomes containing sorbitan monoesters (Span 20®, Span 40®, Span 60® and Span 80®), there was no clearly defined trend between alkyl chain length and flux values [59]. Jain *et al*. studied the transdermal delivery of dexamethasone by ultradeformable liposomes. The formulation optimizing study showed that specific types and concentrations of surfactant were required for providing the maximum deformability to vesicle membrane. They showed that Span 80® was more effective as compared with sodium deoxycholate and Tween 80® as an edge activator [60]. The same group studied elastic liposomes for transdermal delivery of an anti-jet lag agent. Among the three surfactants utilized namely, Span 80®, sodium cholate and sodium dodecylsulphate, formulation bearing Span 80® at an optimum lipid: surfactant ratio of 85:15% w/w proved also to be the best in all parameters studied [61].

In a first set of experiments, we studied the influence of adding sodium deoxycholate on the *in vitro* penetration of betamethasone-cyclodextrin inclusion complexes encapsulated in phosphatidylcholine liposomes through polycarbonate membrane (pore size 50 nm). These deformable liposomes increased the diffusion of betamethasone 1.3 times compared to classical liposomes [62]. In a second set of experiments, we studied the influence of adding sodium deoxycholate on the *ex vivo* penetration of betamethasone encapsulated in phosphatidylcholine liposomes through

pig skin. Despite an increased deformability determined by extrusion measurements, we could not show any enhancement compared to classical phosphatidylcholine liposomes (results to be published). We also tried to enhance the penetration of the same drug by adding Tween 80® or Span 80®, but failed to prove any significant enhancement (results not published).

5.2.4 Influence of ethanol

Another approach to improve skin penetration is an increase in vesicles flexibility due to the addition of ethanol; these liposomes are called ethosomes [17]. Touitou *et al*. first described the ethosomal system, which is composed of phospholipid, ethanol and water. They showed that ethosomal systems were much more efficient at delivering a fluorescent probe, rhodamine red, to the skin in terms of quantity and depth, than either liposomes or hydroalcoholic solution. The permeation enhancement from ethosomes observed in their work was much greater than would be expected from ethanol alone, suggesting some kind of synergistic mechanism between ethanol, vesicles and skin lipids. They illustrated a hypothetical model of how ethosomes may enhance penetration of drugs through the *stratum corneum* lipids. First, ethanol disturbs the organization of the *stratum corneum* lipid bilayer and enhances its lipid fluidity. The flexible ethosome vesicles can then penetrate the disturbed *stratum corneum* bilayers and even forge a pathway through the skin by virtue of their particulate nature. The release of drug in the deep layers of the skin and its transdermal absorption could then be the result of fusion of ethosomes with skin lipids and drug release at various points along the penetration pathway [63]. The same group demonstrated the superiority of ethosomes for delivering erythromycine [64, 65], testosterone [66], bacitracin [67], trihexyphenidyl HCl [68], cannabidiol [69] and buspirone [70]. Recently, Dubey *et al*. studied the transdermal delivery of an anti-HIV agent via ethanolic liposomes. Permeation studies of indinavir across human cadaver skin resulted in enhanced transdermal flux from ethanolic

liposomes that was significantly ($P < 0.05$) greater than that with ethanolic drug solution, conventional liposomes, or plain drug solution [71]. This group also showed an enhanced delivery by ethosomes of melatonin [72] and methotrexate [73]. Fang *et al*. concluded that application of ethosomal carriers with 5-aminolevulinic acid (ALA) in hyperproliferative murine skin could improve the penetration of ALA and the formation of protoporphyrin IX and significantly reduce TNF-α in this disordered skin compared to that after application of an ALA aqueous solution [74]. Rao *et al*. evaluated the *in vitro* percutaneous permeation and skin accumulation of finasteride using vesicular ethosomal carriers. The finasteride transdermal fluxes from ethosomes containing formulation (1.34 +/- 0.11 μg/cm^2/h) were 7.4, 3.2 and 2.6 times higher than that of finasteride from aqueous solution, conventional liposomes and hydroethanolic solution respectively ($P < 0.01$) [75]. All these studies demonstrated that ethosomes seem to be promising vesicular carriers for enhancing percutaneous absorption of several drugs.

5.3 Drug encapsulation

5.3.1 Entrapment efficiency

Mura *et al*. showed a possible correlation between liposome entrapment efficiency (EE) and benzocaine delivery through rat skin. In fact, the benzocaine permeability coefficient values from SUV (EE% 28.8) and MLV (EE% 29.7) were similar, independent of whatever the entrapped drug is in the aqueous (SUV) or in the lipophilic (MLV) liposomal phase, and of their different dimensions and structure (uni-lamellar or multi-lamellar bilayer) [38]. As mentioned above, Kim *et al*. showed that the amount of absorbed caffeine was nearly independent of the encapsulation efficiency and the vesicle size [40].

5.3.2 Properties of the drug encapsulated

With regard to liposome interaction, El Maghraby *et al*. classified drugs into three categories; the strongly hydrophilic ones, which are localized in the aqueous domains of liposomes and can exert some interaction with the head groups if they have a very high polar surface area, the less hydrophilic, more balanced molecules that adsorb at the water–lipid bilayer interface with some degree of penetration into the bilayer, and the strongly lipophilic drugs, which are located in the bilayer itself [76]. For hydrophilic drugs, the penetration enhancing effect seems to play a more important role in the enhanced skin delivery than in the case of lipophilic drugs (as for many penetration enhancers), since permeation of hydrophilic molecules tends to be relatively slower and hence more enhanceable [24].
We showed that properties of encapsulated drug are of high importance for skin penetration behaviour. We compared the skin penetration of negative liposomes encapsulating either betamethasone (log *P* = 2.06) or betamethasone dipropionate (log *P* = 3.66) with their respective ethanolic solution. The entrapment into liposomes enhanced the epidermis absorption more than 9 times for betamethasone while the entrapment of betamethasone dipropionate enhanced it only 1.6 times (results to be published).

5.4 Experimental set-up

Franz diffusion cell is the most common *in vitro* method to study skin absorption of chemicals. There have been numerous recommendations, and some of them have been selected as guidelines. The most widely known is the OECD guideline 428 [77, 78]. Regarding these numerous recommendations, standardisation problems occur. The lack of a standardized method recommended by the European Pharmacopea, gives rise to a variety of diffusion cells and different experimental protocols influencing the outcome and final conclusions.

As explained in the OECD guideline 428, the diffusion cell consists of a donor chamber and a receptor chamber between which the skin is positioned. The test substance is applied to the surface of the skin sample. Skin from human or animal sources can be used. Either epidermal membranes or split thickness skin prepared with a dermatome, or full thickness skin are acceptable. The receptor fluid should provide adequate solubility of the tested chemical so that it does not act as a barrier to absorption. A good mixing of the receptor solution in contact with the underside of the skin must be provided. The diffusion chamber and the skin should be maintained at a constant temperature close to the normal skin temperature of 32 ± 1°C. Static and flow-through cells are both acceptable.

Several data can be obtained from this method. The receptor fluid can be sampled at time points throughout the experiment and diffused drug assayed. An absorption profile with time is so obtained. The skin can be fractioned into *stratum corneum*, epidermis and dermis or by the help of a cryo-microtome into surface parallel sections in order to obtain the distribution of the tested substance into the skin. When infinite dose conditions of exposure are used the data may permit the calculation of permeability constant (Kp).

After the experiment using diffusion cells, the drug accumulation into the *stratum corneum* can be measured by the tape stripping method. This method allowed the removal of the *stratum corneum* layer by layer using adhesive tape. Many different tape stripping procedures have been employed over the last years using different methods for applying pressure on the tapes. This pressure can be applied with weight or with a roller. In order to obtain the drug distribution profile as a function of *stratum corneum* depth, the amount of *stratum corneum* on each tape strip has to be determined. Infrared densitometry is a suitable method for *stratum corneum* depth determination [79, 80]. Lindemann *et al*. compared three techniques; the pseudo-absorption of the corneocytes in the visible range (430 nm) correlated well with the protein absorption in the UV range (278 nm) and the absorption at 652 nm obtained after

staining of the *stratum corneum* proteins with Trypan blue [81]. Other techniques are available for the determination of *stratum corneum* amount on tape strips, an overview of these methods is given by Hahn *et al*. [79].
Franz diffusion cells are mainly used to compare the diffusion of a new developed formulation with an existing formulation serving as reference in order to show an improvement in delivery. Several parameters can influence the penetration of the tested formulation. They are developed below.

5.4.1 Occlusive *versus* non occlusive

The OECD guideline 428 recommends leaving donor chamber unoccluded during exposure to a finite dose of a test preparation. However, for infinite applications and certain scenarios for finite doses, the donor chambers may be occluded [77]. When studying skin penetration of deformable liposomes, diffusion studies are generally carried out in non occlusive conditions to allow the driving force provided by the osmotic gradient described by Cevc as the main driving force [57].
El Maghraby *et al*. showed that after open application, both ultradeformable and traditional liposomes improved estradiol skin delivery, with the ultradeformable liposomes being superior. Occlusion reduced the delivering efficiency of both vesicle types, supporting the theory that a hydration gradient provides the driving force [82]. Kim *et al*. adapted the delivery of hydrocortisone from liposomal suspensions to the hairless mouse skin following topical application under non-occlusive and occlusive conditions. They concluded that both excessive dehydration of liposomes (under non-occlusive condition) and excessive hydration of dosed skin (under occlusion) should be avoided in liposome topical application for efficient delivery of hydrocortisone for a prolonged period of time [83]. Van Kuijk-Meuwissen *et al*. investigated the interactions between liposomes and human skin *in vitro* by confocal laser scanning microscopy and showed that the label applied non-occlusively in flexible

liposomes penetrated deeper into the skin than after occlusive application [46].

5.4.2 Receptor fluid composition

The OECD guideline recommends the use of a physiologically conductive receptor fluid but others may also be used where justified. The selection of the receptor fluid must be compatible with the skin preparation and must meet the solubility requirements of the test substance [77].
An overview of receptor media used in transdermal penetration studies is given by Baert *et al*. Receptor media included water, phosphate buffer, normal saline, lactic acid solution, Hepes buffer, Hanks balanced salt solution, citrate-phosphate and acetate buffer. Solubility increasing additives may be used including surfactants (Tween 20®, Brij 98®), propylene glycol, polyethylene glycol, methanol, isopropanol, ethanol, cyclodextrins and bovine serum albumine [78]. When using human or animal skin, interactions of the receptor medium with the contacting lower skin layers can modify these layers (precipitation of proteins or dissolution of specific endogenous skin compounds) and consequently, the apparent diffusion characteristics of the skin for the tested compound can change [78].
Henning *et al*. showed that the thickness of the unstirred water layers has an enormous effect on the permeability coefficient and may lead to underestimation of the permeability by more than 90%. This unstirred water layer should be reduced by either thorough stirring or, preferably, by using additives such as bovine serum albumin [84]. Henning *et al*. also showed that the difference in permeability due to the temperature is less pronounced and probably negligible when using skin from different donors or comparing results from different laboratories [84].
When longer times of experiment are needed, sodium azide can be added in the receptor fluid as preservative. Hawkins and Reifenrath showed that the percutaneous penetration of benzopyrene on human and pig skin was unaffected by the presence of sodium azide in the tissue culture media;

however, with mouse skin, penetration was lower when sodium azide was present [85].

5.4.3 Skin used

As most studies are intended to predict skin absorption in man, human skin should be used in first instance. Human skin generally comes from medical or plastic surgery of human breast or abdomen. Despite their advantages, human skin samples are rarely used due to limited resources, high logistic effort and regulatory issues. Therefore porcine (ear) skin, bovine (udder) skin or rat skin is usually employed as an alternative [84]. The skin can be used as full-thickness or dermatomed. Trypsin-isolated *stratum corneum*, heat-separated epidermis or dermis is also used.
Schmook *et al*. concluded from their comparison between rat skin, domestic pigs skin, Göttingen minipigs and human abdominal cadaver, that pig skin was the most suitable model for human skin [86]. Comparing transepidermal water loss and impedance, Sekkat *et al*. also support the validity of pig ear skin as a model for the human membrane [87]. Dick and Scott compared the *in vitro* permeability of pig ear skin with human (abdominal) skin and rat (dorsal) skin using both hydrophilic and lipophilic penetrants. Pig skin was found to have a closer permeability character to human skin than rat skin, particularly for lipophilic penetrants [88]. From a recent review, Barbero and Frash concluded that both pig and guinea pig are good models for human skin permeability and have less variability than the human skin model [89]. Some authors used artificial membranes instead of skin [36]. Such artificial membranes should be used with care and correlation with skin permeation data should be confirmed before their use [37]. We observed a contradictory response when using polycarbonate membrane instead of pig skin (see section 5.2.3).

5.4.4 Washing step

Wiedersberg *et al*. investigated the influence of the skin washing procedure on the dermatopharmacokinetics of betamethasone valerate. They showed that removing excess formulation more aggressively with isopropylalcohol compared with cleaning with a dry paper towel resulted in a lower apparent uptake of drug into the *stratum corneum*. The brief exposure of the skin to isopropylalcohol served only to remove sequestered formulation more efficiently from furrows and did not artefactually drive drug into the *stratum corneum* [90].

6. Conclusion

This review gives an overview of the parameters affecting the skin penetration behaviour of liposomes. Despite the debate over the exact mechanism of skin penetration of liposomes, the evidence of enhanced penetration is impressive. Modulations in composition and structure of liposomes result in vesicles with different penetration properties allowing dermal, follicular or even transdermal delivery. This potential to optimise the permeability of a range of therapeutic molecules will lead to development in the future. As conclusion we would have wanted to highlight a perfect liposome composition for dermal and/or transdermal delivery. It seems evident that for higher and deeper penetration, this utopian formulation has to show flexible properties of its lipid bilayer by addition of surfactant or ethanol or better a combination of both. The incorporation of a negative charge would enhance the penetration even more. Regarding the lack of standardized method for the evaluation of the effectiveness of skin penetration, inter laboratory differences will still appear, giving rise to different results and conclusions.

7. Acknowledgements

A. Gillet is a PhD student supported by the FNRS, Brussels, Belgium.

8. References

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OBJECTIFS ET PLAN DE LA THESE

Au cours des dernières années, de nouvelles techniques ont été explorées afin d'augmenter l'absorption de substances médicamenteuses dans et à travers la peau. Ces techniques peuvent être, par exemple, actives comme l'électroporation, l'iontophorèse et la sonophorèse ou passives comme l'utilisation de promoteurs d'absorption. Les systèmes vésiculaires, comme les liposomes, représentent une alternative intéressante pour améliorer l'administration cutanée de médicaments. Les liposomes sont des vésicules sphériques composées d'une ou plusieurs bicouches lipidiques entourant une cavité aqueuse. Ils sont capables d'encapsuler des molécules hydrophiles dans leur cavité aqueuse, ainsi que de retenir des molécules hydrophobes dans leur bicouche lipidique.

La première partie de la thèse s'intéressera au développement d'un nouveau système d'administration cutanée combinant les avantages des complexes d'inclusion dans les cyclodextrines et ceux des liposomes déformables. En effet, il a été proposé de piéger les substances actives lipophiles dans la cavité aqueuse des liposomes sous la forme de complexes d'inclusion dans des cyclodextrines, aboutissant à un nouveau concept appelé : « drugs-in-cyclodextrin-in-liposome». D'autre part, des études récentes ont abouti à la conception de nouveaux transporteurs vésiculaires, les liposomes déformables appelés aussi « Transfersome®». Ce sont des vésicules élastiques, qui pénétreraient intactes dans la peau permettant d'y atteindre des concentrations efficaces en principe actif.
Ces nouveaux systèmes seront mis au point et caractérisés. La bétaméthasone sera utilisée comme molécule modèle. Les premières études de diffusion seront réalisées *in vitro*, à travers des membranes synthétiques au moyen des cellules de diffusion de Franz.

Afin de se rapprocher des conditions *in vivo*, des études de pénétration des liposomes à travers des peaux d'oreilles de cochons seront mises au point. Ces méthodes *ex vivo* seront validées avant utilisation.

La seconde partie de la thèse concernera l'étude de la pénétration cutanée d'autres systèmes vésiculaires, afin de permettre d'orienter les recherches vers des études permettant de mieux comprendre les mécanismes de pénétration cutanée des liposomes. En effet, il existe encore dans la littérature des divergences quant aux mécanismes permettant d'expliquer la pénétration cutanée des systèmes vésiculaires.

L'influence de différents paramètres sur la pénétration cutanée sera alors envisagée. Nous étudierons l'influence de l'ajout de différents additifs au niveau de la bicouche des liposomes comme des tensio-actifs ou des substances anioniques ou cationiques. La molécule modèle sera encapsulée soit comme précédemment à l'aide de cyclodextrines au niveau de la cavité aqueuse des liposomes soit seule au niveau de la bicouche lipidique, afin d'évaluer l'influence du mode d'encapsulation. L'influence des propriétés de la molécule modèle sera évaluée par le remplacement de la bétaméthasone par son ester plus lipophile, le dipropionate de bétaméthasone.

Enfin, l'efficacité de systèmes ayant donné des résultats prometteurs *ex vivo* sera évaluée *in vivo* sur des volontaires grâce à l'évaluation du pouvoir vasoconstricteur des corticostéroïdes au niveau de la peau.

RESULTATS

I. Chapitre 1

DEVELOPMENT OF A NEW TOPICAL SYSTEM: DRUG-IN-CYCLODEXTRIN-IN-DEFORMABLE LIPOSOME

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Ce chapitre est publié dans International Journal of Pharmaceutics, 380, (2009), 174–180

I.1 Résumé

Développement d'un nouveau système d'administration cutanée : « Drug – in – cyclodextrin - in deformable liposome ».

I.1.1 Introduction

Les liposomes sont des systèmes de transport capables à la fois d'encapsuler des molécules hydrophiles dans leur cavité aqueuse, ainsi que de retenir des molécules hydrophobes dans leur bicouche lipidique. Cependant, la rétention des molécules hydrophobes dans la bicouche lipidique pourrait interférer avec la formation et la stabilité de cette bicouche. Comme les cyclodextrines permettent d'augmenter la solubilité aqueuse des molécules lipophiles, il a été proposé d'encapsuler ces molécules dans la cavité aqueuse des liposomes sous la forme de complexes d'inclusion dans des cyclodextrines. D'autre part, des études récentes ont abouti à la conception de nouveaux transporteurs vésiculaires, les liposomes déformables, qui grâce à l'ajout d'un « edge activator » pénétreraient intacts dans la peau y atteignant des concentrations efficaces en principe actif.
Le but de ce travail est de développer un nouveau système d'administration cutanée combinant les avantages des complexes d'inclusion dans les cyclodextrines et ceux des liposomes déformables. Ces nouvelles vésicules sont caractérisées par leur diamètre, leur morphologie, leur pourcentage d'encapsulation, leur déformabilité et leur stabilité en termes de libération du matériel encapsulé. L'efficacité de ces liposomes est évaluée *in vitro* par des mesures de diffusion au travers de membranes en polycarbonate dans des cellules de diffusion de Franz.

I.1.2 Résumé des résultats

Les liposomes déformables contenant du déoxycholate sodique comme « edge activator » et encapsulant des complexes d'inclusion bétaméthasone-HPγCD ou bétaméthasone-Crysmeb sont comparés aux liposomes non déformables correspondants, c'est-à-dire ne renfermant pas de déoxycholate sodique.

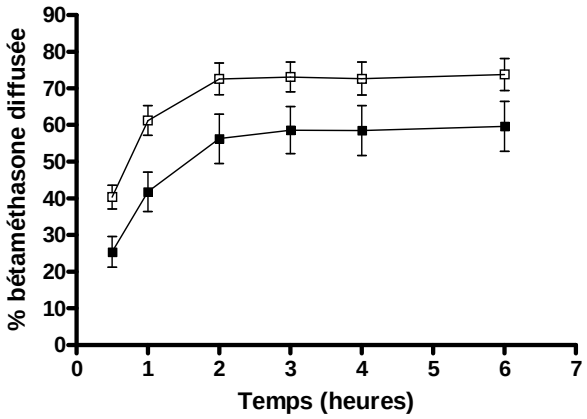
I.1.2.1 Caractérisation

La mesure du diamètre des liposomes et l'évaluation de leur morphologie ont montré la présence de vésicules unilamellaires d'une taille d'environ 200 nm. Deux types de lipides ont été testés : la phosphatidylcholine (PC) et le dimyristoyl-phosphatidylcholine (DMPC). Des liposomes déformables à base de DMPC n'ont pas pu être formulés. Ce lipide a donc été rapidement abandonné et seuls des liposomes à base de PC seront formulés. L'ajout de déoxycholate de sodium permet d'augmenter le pourcentage d'encapsulation de la bétaméthasone de 1,63 ± 0,09% à 2,11 ± 0,17% pour les liposomes encapsulant les complexes bétaméthasone-HPγCD (c'est à dire une augmentation d'environ 30%) et de 1,31 ± 0,01% à 2,29 ± 0,21% pour les liposomes encapsulant les complexes bétaméthasone-Crysmeb (c'est à dire une augmentation d'environ 75%) ($p < 0,05$). La déformabilité des liposomes a été étudiée au moyen de la mesure de viscosité des membranes lipidiques par la technique de résonance paramagnétique électronique (RPE). Les résultats ont pu montrer que la microviscosité des liposomes classiques vides est plus élevée que celle des liposomes déformables. Ces nouvelles vésicules montrent une bonne stabilité, en termes de rétention de la bétaméthasone encapsulée, durant un mois lorsqu'elles sont conservées à 4°C.

I.1.2.2 Etude de diffusion

Les études de diffusion sur cellules de Franz ont montré que les liposomes déformables permettent d'augmenter le pourcentage de diffusion de la bétaméthasone de 59,63 ± 7,03% à 73,79 ± 4,61%, quand la HPγCD était utilisée (c'est-à-dire une augmentation d'environ 25%) ($p < 0,001$) (Figure 1.1 A). Quand la Crysmeb était utilisée, la présence du déoxycholate de sodium permet d'augmenter la diffusion de la bétaméthasone de 51,64 ± 4,25% à 67,92 ± 0,26% (c'est-à-dire une augmentation d'environ 30%)($p < 0,001$) (Figure 1.1 B).

A



B

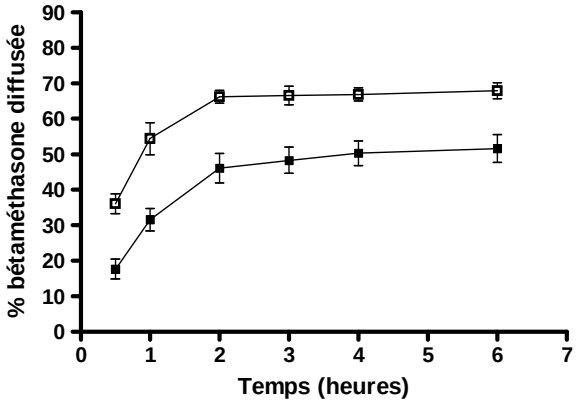


Figure 1.1: Comparaison des pourcentages de diffusion de la bétaméthasone à partir de liposomes classiques contenant les complexes d'inclusion bétaméthasone-cyclodextrines (■) et à partir des liposomes déformables contenant les complexes d'inclusion bétaméthasone-cyclodextrines (□). (A) HPγCD; (B) Crysmeb (n=3).

I.1.3 Conclusion

Cette étude a montré la faisabilité d'un système d'administration cutanée appelé drug-in-cyclodextrin-in deformable liposome. L'ajout de déoxycholate sodique a permis d'augmenter le pourcentage d'encapsulation de la bétaméthasone jusqu'à un facteur 1,8 et la déformabilité des liposomes. Ces nouvelles vésicules ont montré une bonne stabilité lorsqu'elles sont conservées à 4°C. L'ajout du déoxycholate de sodium a augmenté la diffusion à travers les membranes en polycarbonate de porosité de 50 nm jusqu'à un facteur 1,3 par rapport aux liposomes non déformables. Ces résultats prometteurs devront néanmoins être vérifiés *ex vivo* sur des peaux d'oreilles de cochons avant de pouvoir conclure à une efficacité supérieure de ces nouveaux systèmes.

I.2 Article

Abstract

A new delivery system for cutaneous administration combining the advantages of cyclodextrin inclusion complexes and those of deformable liposomes was developed, leading to a new concept: drug-in-cyclodextrin-in-deformable liposomes. Deformable liposomes made of soybean phosphatidylcholine (PC) or dimyristoylphosphatidylcholine (DMPC) and sodium deoxycholate as edge activator were compared to classical non deformable liposomes. Liposomes were prepared by the film evaporation method. Betamethasone, chosen as the model drug, was encapsulated in the aqueous cavity of liposomes by the use of cyclodextrins. Cyclodextrins allow an increase in the aqueous solubility of betamethasone and thus, the encapsulation efficiency in liposome vesicles. Liposome size, deformability and encapsulation efficiency were calculated. The best results were obtained with deformable liposomes made of PC in comparison with DMPC. The stability of PC vesicles was evaluated by measuring the leakage of encapsulated calcein on the one hand and the leakage of encapsulated betamethasone on the other hand. *In vitro* diffusion studies were carried out on Franz type diffusion cells through polycarbonate membranes. In comparison with non deformable liposomes, these new vesicles showed improved encapsulation efficiency, good stability and higher *in vitro* diffusion percentages of encapsulated drug. They are therefore promising for future use in *ex vivo* and *in vivo* experiments.

Keywords

Deformable Liposome; Cyclodextrin; Betamethasone; Franz cell

1. INTRODUCTION

A major obstacle to cutaneous drug delivery is the permeation characteristics of the *stratum corneum*, which limits drug transport, making this route of administration frequently insufficient for medical use. During the past few decades there has been wide interest in exploring new techniques for increasing drug absorption through the skin [1, 2]. Topical delivery of drug by liposomes has aroused a considerable interest.

Recently, it became evident that, in most cases, classical liposomes are of little or no value as carriers for transdermal drug delivery as they do not penetrate skin deeply, but rather remain confined to upper layers of the *stratum corneum* [3]. In order to target deeper underlying skin tissue, intensive research led to the introduction and development of a new class of lipid vesicles, the highly deformable (elastic or ultraflexible) liposomes, which have been called Transfersomes® [4]. Several studies have reported that deformable liposomes are able to improve *in vitro* skin delivery of various drugs [5] and to penetrate intact skin, *in vivo*, transferring therapeutic amounts of drugs [6]. According to Cevc and Blume, the improved drug delivery by deformable liposomes is due to the driving force provided by the osmotic gradient between the outer and inner layers of the *stratum corneum* [4]. The important difference between deformable liposomes and traditional liposomes is the high and stress-dependent adaptability of such deformable vesicles, which enables them alone to squeeze between the cells in the *stratum corneum*, despite the large average vesicle size [7]. Thus, they can pass through the intact skin spontaneously, under the influence of the naturally occurring, *in vivo* transcutaneous hydration gradient [8]. These vesicles consist of phospholipids and an edge activator. An edge activator is often a single chain surfactant, with a high radius of curvature, which destabilizes lipid bilayers of the vesicles and increases their deformability [3].

Liposomes are able to encapsulate hydrophilic drugs in their aqueous compartment, while lipophilic drugs are encapsulated in their lipid bilayer. However the accommodation of lipophilic compounds in the lipid phase can be problematic as some drugs can interfere with bilayer formation and stability. This accommodation is often limited in terms of drug to lipid mass ratio [9, 10]. In the case of the encapsulation of betamethasone into liposomes, the use of cyclodextrins was shown to increase the drug to lipid mass ratio [11]. Entrapping water-soluble drug-cyclodextrin inclusion complexes in the aqueous compartment of liposomes has been proposed in order to avoid such drawbacks [9, 10]. Cyclodextrins are cyclic (β-1,4)-linked oligosaccharides of d-glucopyranose containing a relatively hydrophobic central cavity and a hydrophilic outer surface. Cyclodextrins are able to form inclusion complexes with poorly water-soluble drugs. In a previous piece of research, we studied the effect of cyclodextrins on the encapsulation efficiency and release kinetics of betamethasone from liposomes. We showed the feasibility and the advantages of the betamethasone-in-cyclodextrin-in-liposome formulation [11]. Hydroxypropylated γ cyclodextrin (HPγCD) and Crysmeb®, a methylated β cyclodextrin, increased the aqueous solubility of betamethasone at the highest level when compared to other cyclodextrins. The affinity constants ($K_{1\text{-}1}$) of betamethasone for HPγCD and Crysmeb® were 12,606 and 10,011 M^{-1}, respectively. These two cyclodextrins were shown to be particularly effective for obtaining high betamethasone concentrations and were consequently selected for this study. Piel *et al*. [11] also demonstrated that these cyclodextrins had a higher affinity for betamethasone than for membrane lipids, allowing the entrapment of highly concentrated betamethasone inclusion complex solutions without or with minimal interaction with lipid components of the liposome membrane.

The purpose of this study is to develop a new delivery system for cutaneous administration combining the advantages of drug-cyclodextrin inclusion complexes and those of deformable liposomes, leading to a new concept: drug-in-cyclodextrin-in-deformable liposomes, first developed by

Jain S.K. *et al*. [12]. This present study deals with the characterization and the *in vitro* diffusion properties of these vesicles.
Betamethasone was chosen as the model drug, because of its hydrophobic nature and its well known ability to form inclusion complexes with various cyclodextrins [13].

2. MATERIALS AND METHODS

2.1 Materials

Soybean phosphatidylcholine (PC) was purchased from Lipoïd (Ludwigshafen, Germany). 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) came from Avanti®Polar Lipids (Pelham, AL, USA). Hydroxypropylated γ cyclodextrin (HPγCD, D.S. 0.7, 3.41% H_2O) was obtained from Wacker-Chemie GmbH (Munich, Germany) and Kleptose® Crysmeb (Crysmeb, D.S 0.5, 4.29% H_2O) from Roquette Frères (Lestrem, France). Sodium deoxycholate and calcein were purchased from Sigma Aldrich (Bornem, Belgium). Betamethasone (E.P.) was purchased from Medeva (Braine L'Alleud, Belgium). All other reagents and solvents were of analytical grade.
All experiments were performed using a 0.22 µm-filtered 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) buffer (Sigma Aldrich, Bornem, Belgique), containing 145 mM NaCl and adjusted to pH 7.4 with 0.1 N NaOH solution.

2.2 Formation of inclusion complexes

Cyclodextrins were dissolved in Hepes buffer pH 7.4 in order to obtain a 10 mM HPγCD or Crysmeb solution. Betamethasone was added in excess to the cyclodextrin solution. Water-soluble inclusion complexes were formed after stirring the mixture for 48 h at 25 °C. The milky solutions

were then filtered through a 0.22 µm filter. Betamethasone concentrations obtained for HPγCD and Crysmeb inclusion complexes were 2.02 mg/mL and 2.16 mg/mL respectively.

2.3 Liposome preparation

Classical liposomes were made of PC or DMPC with (70:30 mol%) or without cholesterol, while PC or DMPC and sodium deoxycholate at 13% m/m as "edge activator" were used for deformable liposomes. Liposomes were prepared by hydration of lipid films. In practice, the required amount of lipids was dissolved in ethanol in a round-bottomed flask, using a rotary evaporator and was dried under vacuum. The resulting lipid film was hydrated using 3 mL of the corresponding betamethasone-cyclodextrin complex solution. Suspensions were then extruded through Nucleopore® polycarbonate membranes of successive 0.4 and 0.2 µm pore diameters (Whatman, Maidstone, UK). Free betamethasone complexes were separated from liposome-encapsulated betamethasone complexes by three successive ultracentrifugations at 35,000 rpm. The first cycle lasted 3 h followed by two cycles of 1 h 30 at 4 °C. The supernatant was removed and the pellet was resuspended in Hepes buffer. Betamethasone and PC were assayed in purified liposomes. The same preparation method was used to make liposomes encapsulating calcein in order to study liposome stability. A 3 mL calcein solution was used to hydrate the lipid film. In order to detect a leakage-induced effect, the self-quenching fluorescent dye calcein was entrapped in these vesicles at a concentration of 40 mM. At this concentration, calcein shows minimal fluorescence, owing to the formation of ground state dimers. Any fluorescence measured will be due to calcein leakage and dilution in the exterior aqueous media. After extrusion, external calcein was removed by a first ultracentrifugation at 35,000 rpm for 3 h at 4 °C followed by four successive ultracentrifugations at 35,000 rpm for 45 min at 4 °C.

2.4 Liposome characterisation

2.4.1 Measurement of liposome size

Liposome dispersions were sized by photon correlation spectroscopy (PCS) (HPPS, Malvern Instruments). Measurements were made at 25 °C with a fixed angle of 90° and the results were expressed as the average liposomal hydrodynamic diameter (nm).

2.4.2 Freeze-fracture electron microscopy

Freeze-fracture replicas of liposome suspensions were examined in transmission electron microscopy. A drop of liposome suspension, containing 20% glycerol as a cryoprotectant, was deposited in a small gold cup and rapidly frozen in liquid nitrogen. Fracturing, freeze etching and shadowing with Pt-C were performed at -100 °C in shadowing equipment (Balzers® BAF-400) fitted with a freeze-fracture and etching unit. The replicas were examined with a JEOL (JEM-100SX) transmission electron microscope, operating at 80kV accelerating voltage.

2.4.3 Encapsulation efficiency

Encapsulation efficiency was calculated by two different ways. The first encapsulation efficiency ($EE_{B/l}$) corresponds to the drug to lipid mass ratio or the amount of betamethasone in purified liposomes (C_B) compared to the total lipid concentration (C_l) in purified liposomes:

$$EE_{B/l}\ (\%) = (C_B / C_l) \times 100$$

The second encapsulation efficiency ($EE_{b/bt}$) was the yield obtained. It corresponds to the concentration of betamethasone encapsulated in liposomes (C_B) compared to the total drug concentration first introduced

(C_{Bt}). This $EE_{b/bt}$ was corrected to the concentration of lipids in order to take into account the loss of liposomes during the liposomes preparation:

$$EE_{B/Bt}(\%) = (C_B / C_l) / (C_{Bt} / C_{lt}) \times 100$$

C_l is the lipid concentration in purified liposomes and C_{lt} is the lipid concentration first introduced.

2.4.3.1 Betamethasone determination

Betamethasone was determined using a validated HPLC method. HPLC was performed using a system consisting of a LaChrom® Merck Hitashi system L-7100 pump, an L-4000 UV detector, a L-7200 autosampler and a D-2500 chromato integrator. Twenty microliter samples were injected onto a LiChroCART® column (250 mm × 4 mm i.d.) prepared with an octadecylsilane (C18) phase Superspher® (Merck) and maintained at 30 °C. The mobile phase consisted of a 50:50 (v:v) mixture of HPLC grade acetonitrile and water. The flow rate was 0.8 mL/min. Betamethasone was detected at 240 nm.

2.4.3.2 Quantification of lipids

Total lipid concentrations were calculated by measuring PC or DMPC by an enzymatic method (LabAssay™ Phospholipid, Wako, Osaka, Japan). The principle of this enzymatic assay consists of the cleavage of PC in choline by phospholipase D and the oxidation of choline into betaine with the simultaneous production of hydrogen peroxide. The hydrogen peroxide, which is produced quantitatively, couples 4-Aminoantipyrine and *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS). Peroxidation results in the generation of a coloured compound quantified by spectrophotometry at 600 nm (spectrophotometer Perkin-Elmer Lambda 11).

2.4.4 Deformability

Electron spin resonance (ESR) is currently used to investigate the microenvironment in membrane liposome. The relative anisotropy observed in an ESR spectrum of a nitroxide spin probe is directly related to the rotational mobility of the probe, a term that can be correlated with the probe's microviscosity. Here, the microviscosity is defined as homogenous solution viscosity, which results in the same spectrum as that recorded in the microenvironment. Standard curves of microviscosity values have been established by calibration of the ESR spectra of three n-doxyl stearic acids (n-DSA: n = 5, 12, 16) probes in glycerol-ethanol mixtures of known viscosities [14]. These curves allow the quantifying of the effective microviscosity at different depths inside liposomes by measuring the order parameter (*S*) and the correlation time (τ_c) on n-DSA ESR spectra. *S* and τ_c were calculated according to the method of McConnel [15].
In this study, we used a 5-doxyl stearic acid (5-DSA) probe to measure the microviscosity of the liposomes. All ESR measurements were performed at 9.5 GHz using a Brucker ESR 300E spectrometer (Brucker, Germany) equipped with a variable temperature controller accessory and operating at a centre field strength of 3360 G with 120 G as the scan range. The measurements were made at 32 °C. Each measurement was repeated at least four times and the microviscosity standard deviation was calculated to be 2%.

2.4.5 Liposome permeability

The membrane integrity of liposomes at three temperatures (4 °C, 25 °C, and 37 °C) was evaluated by measuring the leakage of encapsulated calcein on the one hand and the leakage of encapsulated betamethasone-cyclodextrin inclusion complexes on the other hand. The leakage of

liposomal content into the medium is indicative of changes in the membrane permeability.

2.4.5.1 Calcein leakage

In practice, 100 µL liposome suspension were added to 100 µL Hepes buffered solution or 100 µL of a 2% Triton X-100 solution, for complete liposome destruction, in a 96-well plate. Calcein release from liposomes was measured fluorometrically (SpectraMax Gemini XS); excitation and emission wavelengths were 490 and 520 nm, respectively. The amount of calcein released was calculated by the following equation:

$$\% \text{ calcein released} = I \ / \ I_T \times 100$$

Where I is the fluorescence intensity at 520 nm and I_T is the fluorescence intensity at 520 nm after complete destruction of the liposomes by Triton X-100.

2.4.5.2 Betamethasone release

In practice, every day, a sample stored at each temperature was centrifuged at 35,000 rpm for 45 min at 4 °C. The supernatant was then assayed to determine the released betamethasone. The amount of betamethasone released was calculated by the following equation:

$$\% \text{ betamethasone released} = C_{Bt} / C_{B0} \times 100$$

Where, C_{Bt} is the concentration of betamethasone in the supernatant after t days of storage and C_{B0} is the concentration of betamethasone encapsulated in liposomes at day 0.

2.5 *In vitro* diffusion studies

Diffusion studies were carried out using Franz type glass diffusion cells. These consist of two compartments with a polycarbonate membrane (pore size: 50 nm) clamped between the donor and receiver chambers. The cell body was filled with 7.5 mL of a receptor phase consisting of Hepes buffer solution pH 7.4 which was constantly stirred with a small magnetic bar and thermostated at 37°C throughout the experiments. 350 µL of liposome suspension adjusted to 150 µg/ml betamethasone concentration, were placed in the donor chamber onto the polycarbonate membrane, in non occlusive conditions. The diffusion area was 1.767 cm^2. Samples of receptor phase (< 0.5 mL) were withdrawn after predetermined time intervals (0.5, 1, 2, 3, 4, and 6 h) and the betamethasone concentration was measured by HPLC. Each sample removed was replaced by an equal volume of fresh receptor phase. The calculated betamethasone was plotted as a function of time. All the permeation studies obtained were determined in triplicate in three independent experiments and the mean values ± standard deviation were calculated.

3. RESULTS AND DISCUSSION

3.1 Liposome characterisation

Deformable liposomes containing betamethasone-HPγCD or betamethasone-Crysmeb inclusion complexes with sodium deoxycholate as the edge activator were always compared to the corresponding formulation of non deformable liposomes. Two lipid compositions were tested: PC or DMPC. PCS and freeze-fracture electron microscopy were performed for size analysis and morphology determination. As shown in Table 1, liposomes are characterized by mean hydrodynamic diameters between 206 nm (± 2.5) and 280 nm (± 26.1). The polydispersity indexes

(not shown) were always lower than 0.2, indicating that liposomes were homogeneous in size, except for deformable liposomes made of DMPC, whatever the cyclodextrin used. In the latter case, different populations with sizes ranging from a few nm up to more than 500 nm were observed.

Table 1: Size ± S.D. (nm) and encapsulation efficiencies ($EE_{B/I}$) ± S.D. (%) and ($EE_{B/Bt}$) ± S.D. (%) of liposomes containing betamethasone (BMS)-cyclodextrin complexes as a function of their composition (n = number of batches, ND = not determined).

Composition	Size ± S.D. (nm)	n	$EE_{B/I}$ ± S.D. (%)	n	$EE_{B/Bt}$ ± S.D. (%)	n
DMPC – BMS-HPγCD	280 ± 26.1	4	0.98 ± 0.14	4	24.1 ± 3.4	4
DMPC – BMS-HPγCD – deoxycholate Na	ND		0.89 ± 0.13	4	19.2 ± 2.9	4
DMPC – BMS-Crysmeb	265 ± 27.6	3	0.74 ± 0.04	3	17.2 ± 1.0	3
DMPC – BMS-Crysmeb– deoxycholate Na	ND		0.73 ± 0.03	3	14.5 ± 0.8	3
PC – BMS-HPγCD	269 ± 7.9	3	1.63 ± 0.09	7	40.6 ± 2.1	7
PC – BMS-HPγCD – deoxycholate Na	209 ± 4.9	3	2.11 ± 0.17	7	45.3 ± 3.7	7
PC – BMS-Crysmeb	261 ± 6.7	3	1.31 ± 0.01	3	30.4 ± 0.1	3
PC – BMS-Crysmeb – deoxycholate Na	206 ± 2.5	3	2.29 ± 0.21	3	46.0 ± 4.3	3

These observations were confirmed by freeze fracture electron microscopy and could not be explained at the present time. This phenomenon was not observed either for classical liposomes made of DMPC or for deformable liposomes composed of PC. These deformable PC liposomes showed a significantly smaller size than that of the corresponding non deformable liposome, whatever the cyclodextrin used (206 and 209 nm for PC deformable liposomes with Crysmeb or HPγCD betamethasone complexes *versus* 261 and 269 nm for PC non deformable liposomes with Crysmeb or HPγCD betamethasone complexes; $p < 0.05$). This reduction of the particle size for deformable PC liposomes may be ascribed to increased

flexibility and reduced surface tension of the vesicles due to the presence of sodium deoxycholate, as observed by Chen *et al*. [16]. Betamethasone in cyclodextrin in deformable PC liposomes showed very good size reproducibility from batch to batch. The choice of cyclodextrins, HPγCD or Crysmeb, did not influence the size of PC deformable liposomes containing inclusion complexes. To confirm the results by an imaging method, a freeze fracture technique with subsequent transmission electron microscopy was used. This technique was only applied on liposomes containing betamethasone-HPγCD inclusion complexes. Pictures obtained are shown in Figure 1. The bar represents 200 nm. Figures 1 (A) and (C) represent classical liposomes made of PC and DMPC respectively. These two pictures show the presence of ± 200 nm small unilamellar vesicles of homogeneous size. Deformable liposomes made of PC are shown in Figure 1 (B). We can observe that these vesicles showed a more flattened shape in comparison with the shape of the corresponding classical liposomes, maybe due to their elastic properties. Figure 1 (D) shows deformable liposomes made of DMPC. This picture shows the presence of big vesicles with a size greater than 1 µm. The results from PCS and freeze-fracture electron microscopy measurements were in good agreement.

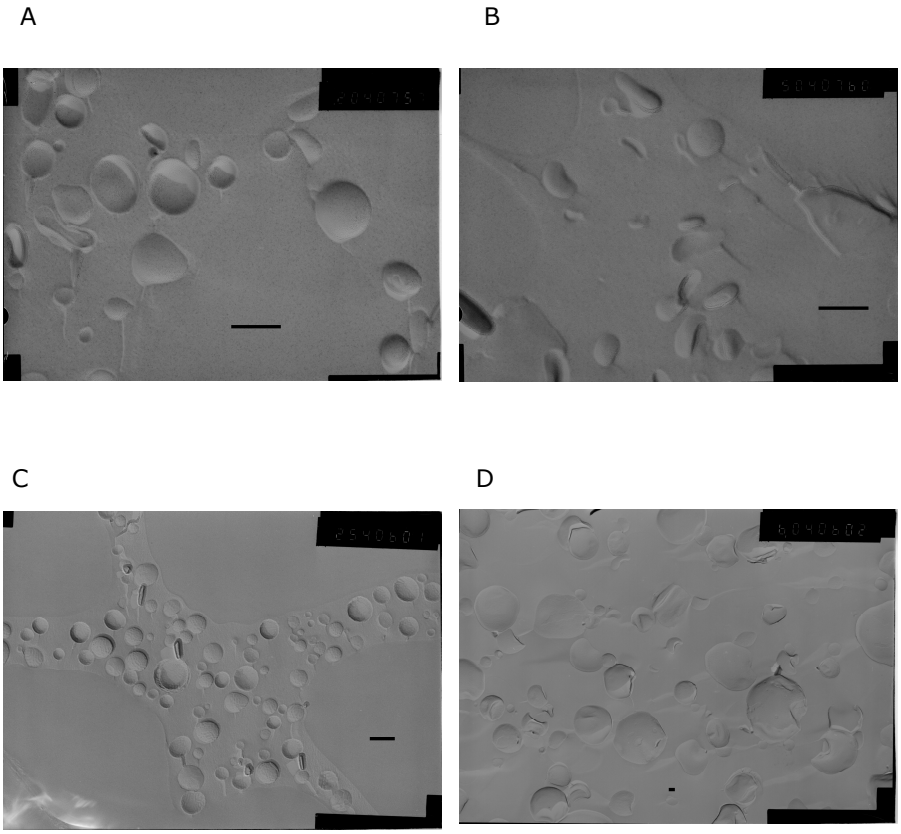


Figure 1: Transmission electron micrographs of freeze-fractured liposomes containing betamethasone - HPγCD inclusion complexes. Classical PC liposomes (A), deformable PC liposomes (B), classical DMPC liposomes (C), and deformable DMPC liposomes (D). The bar represents 200nm.

Betamethasone encapsulation efficiencies are reported in Table 1. It must be noted that encapsulation efficiency $EE_{B/l}$ represents the drug to lipid ratio, which explains the relatively low values obtained in comparison with the $EE_{B/Bt}$ expressing the encapsulation efficiency as a function of the total drug concentration. The addition of sodium deoxycholate significantly enhanced the $EE_{B/l}$ from 1.63% (± 0.09) to 2.11% (± 0.17) for PC liposomes containing betamethasone-HPγCD complexes, and from 1.31% (± 0.01) to 2.29% (± 0.21) for PC liposomes containing betamethasone-Crysmeb complexes ($p < 0.05$). Concerning the calculation of the yield,

the addition of sodium deoxycholate significantly enhanced the $EE_{B/Bt}$ from 40.6 ± 2.1 to 45.3 ± 3.7 for PC liposomes containing betamethasone-HPγCD complexes, and from 30.4 ± 0.1 to 46.0 ± 4.3 for PC liposomes containing betamethasone-Crysmeb complexes ($p < 0.05$). This increase in encapsulation efficiency could be explained by the presence of sodium deoxycholate in the bilayer, which could be supposed to be able to "solubilise" and "hold" the free betamethasone (betamethasone-cyclodextrin complex is in equilibrium with free betamethasone and cyclodextrin) in the lipid bilayer and therefore enhance the encapsulation efficiency for the deformable liposomes, as observed by Chen *et al*. [16] with fenofibrate. Concerning DMPC liposomes, the encapsulation efficiency was always lower than that of corresponding PC liposomes. In addition, no difference was observed between deformable DMPC liposomes and DMPC liposomes without sodium deoxycholate, whatever the cyclodextrin used ($p > 0.05$). The results obtained for size and encapsulation efficiency led us to abandon the formulation of liposomes made of DMPC.

The deformability was evaluated by measuring the microviscosity of the liposome membrane by electron spin resonance spectroscopy. These experiments were only carried out on PC liposomes. ESR spectra were collected for the 5-DSA probe. The *S* parameter was measured at 32 °C, to evaluate the microviscosity of vesicles at skin temperature. Results are summarised in Table 2.

Table 2: Microviscosity as a function of the liposome composition (n > 4; SD < 2%)

Composition	Microviscosity
PC – Cholesterol	226.0 mPa.s
PC	175.9 mPa.s
PC – Deoxycholate Na	169.2 mPa.s
PC – Deoxycholate Na – BMS-Crysmeb	166.2 mPa.s
PC – Deoxycholate Na – BMS-HPγCD	166.2 mPa.s

Measurements showed that the microviscosity of empty PC liposomes is higher in the presence of cholesterol (70:30 mol%) and in the absence of cholesterol, respectively 226 mPa.s and 175.9 mPa.s, than the PC deformable ones (169.2 mPa.s). In fact, cholesterol is known to increase the stability and thus, the rigidity of liposome membranes. This explains the difference observed for classical liposomes with or without cholesterol (226 mPa.s *versus* 176 mPa.s). The addition of the edge activator allowed a decrease in the microviscosity of the membrane to around 169 mPa.s. This decrease in membrane microviscosity may reflect a deformation increase in these vesicles. Moreover, in comparison with the non deformable liposomes, the incorporation of betamethasone-cyclodextrin complexes allowed a reduction of the microviscosity to 166 mPa.s, whatever the cyclodextrin used. The difference in microviscosity between empty deformable liposomes and deformable liposomes containing the inclusion complexes is not significant. Thus, the addition of inclusion complexes did not significantly influence the viscosity of the membrane of the deformable liposomes. EPR spectroscopy seemed to show a tendency towards higher deformability of the new deformable vesicles.

The stability of PC liposomes was first evaluated by measuring the leakage of encapsulated calcein. When calcein was able to leak out from the vesicles into the surrounding buffer, the fluorescence increased dramatically, due to the relief from the self-quenching that occurs. Control wells contained the detergent Triton X-100® to rupture the vesicles and cause complete calcein release. This allowed us to determine maximum fluorescence for the proper normalization of results. The leakage of calcein from the interior of liposomes was measured every day for 8 days. The results are shown in Figures 2 (A) and (B).

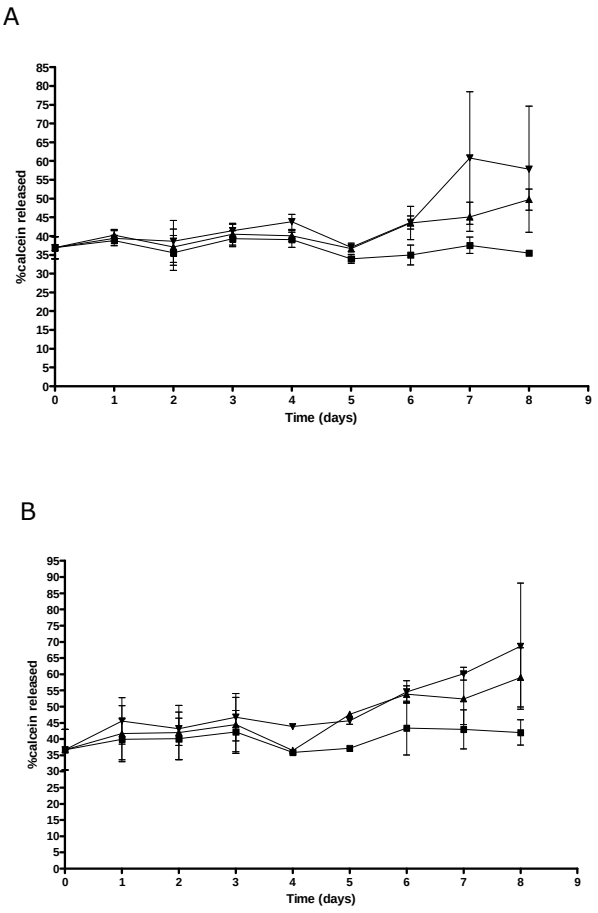


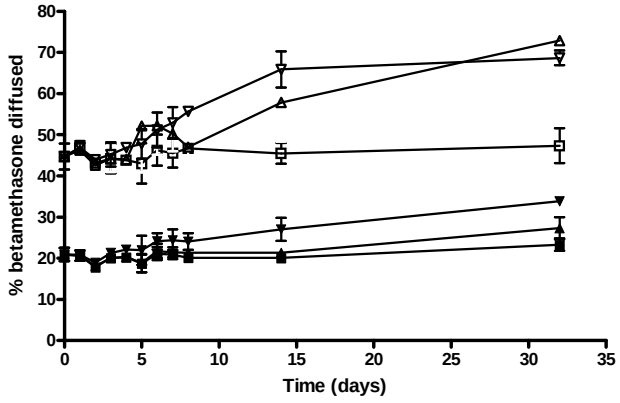
Figure 2: Liposome membrane permeability, expressed as a percentage of calcein released from PC liposomes as a function of time. (A) Classical liposomes and (B) deformable liposomes are stored at three different temperatures: 4 °C (■); 25 °C (▲); and 37 °C (▼) (n=3).

No significant increase in calcein leakage was observed when liposomes were stored at 4 °C, under nitrogen ($p > 0.05$). However, Figure 2 (B) shows a rise in calcein leakage with temperature, up to around 32% in comparison with day 0, after 8 days of storage at 37 °C for deformable liposomes. Loss of calcein from the vesicles stored at elevated temperatures may be attributed to the effect of temperature on the gel to liquid transition of lipid bilayers together with possible chemical

degradation of the phospholipids, leading to defects in the membrane packing, as observed by Dubey *et al*. [17].
After one month of storage at 4 °C, the increase in the percentage of calcein released compared to day 0 was 9.43% (± 5.38) and 23.75% (± 4.36) for classical and deformable liposomes respectively (results not shown). It must be noted that the basal fluorescence, at day 0, was around 37% for both classical and deformable PC liposomes.

In a second experiment, the stability of PC liposomes containing betamethasone-cyclodextrin inclusion complexes was evaluated by measuring the leakage of encapsulated betamethasone. Figures 3 (A) and (B) show the results obtained for liposomes containing betamethasone-HPγCD and betamethasone-Crysmeb inclusion complexes respectively.

A



B

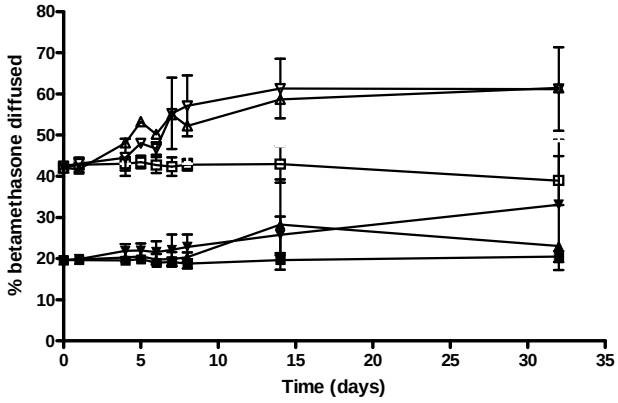


Figure 3: Liposome membrane permeability, expressed as a percentage of betamethasone released from PC liposomes as a function of time. (A) Liposomes containing betamethasone-HPγCD inclusion complexes, and (B) Liposomes containing betamethasone-Crysmeb inclusion complexes. Classical (dark symbol) and deformable (open symbol) liposomes are stored at three different temperatures: 4 °C (■,□); 25 °C (▲,Δ); and 37 °C (▼, ▽) (n=3).

We can observe that, whatever the cyclodextrin used, PC classical and deformable liposomes were stable for one month at 4 °C ($p > 0.05$). As observed for the release of calcein, the leakage of betamethasone increased with an increased temperature. However this increase occurred more slowly than was observed with calcein. The higher release of calcein compared to betamethasone could be explained by the concentration gradient that appears through the lipid bilayer. Betamethasone-cyclodextrin inclusion complexes are in equilibrium with free betamethasone and cyclodextrin. Because only free betamethasone can diffuse through the liposome membrane, the gradient is smaller in the case of betamethasone than for calcein, which is not complexed with cyclodextrin. According to Fick's law, higher is the gradient of concentration higher is the flux through the membrane. The percentage of betamethasone diffused at day 0 was about 20% for classical liposome

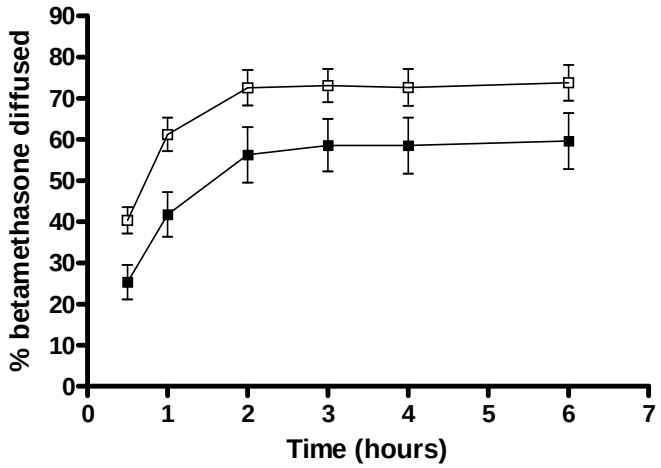
and more than 40% for deformable ones, whatever the cyclodextrin used. This high level of difference was not observed for liposomes encapsulating calcein. The ultracentrifugation cycle, needed before each measurement in the betamethasone release study, could explain this difference. The loss of entrapped drug was probably due to the deformation phenomenon of lipid membrane that occurs during the ultracentrifugation process, as observed by Lopez-Pinto *et al*. [18]. Deformable liposomes are certainly more sensitive to ultracentrifugation than classical liposomes because of the presence of sodium deoxycholate, which destabilizes the membrane and makes it more permeable.

3.2 *In vitro* diffusion studies

Franz type diffusion cells were used to evaluate the *in vitro* diffusion of betamethasone from liposomes. Test conditions were chosen in order to respect sink conditions in the receiver compartment. Saturation concentration of betamethasone in Hepes buffer was evaluated at 65.2 µg/mL, corresponding to around 10 times the maximum concentration of betamethasone that could be found in the receiver compartment. All samples were adjusted at a final betamethasone concentration of 150 µg/ml.

The polycarbonate membrane used had a pore size of 50 nm, a smaller size than the mean diameter of liposomes. The diffusion studies were carried out in non occlusive conditions to allow the driving force provided by the osmotic gradient. Figures 4 (A) and (B) show the cumulative percentage of betamethasone released from classical and deformable liposomes containing betamethasone-HPγCD and betamethasone-Crysmeb inclusion complexes respectively, during 6 hours of diffusion.

A



B

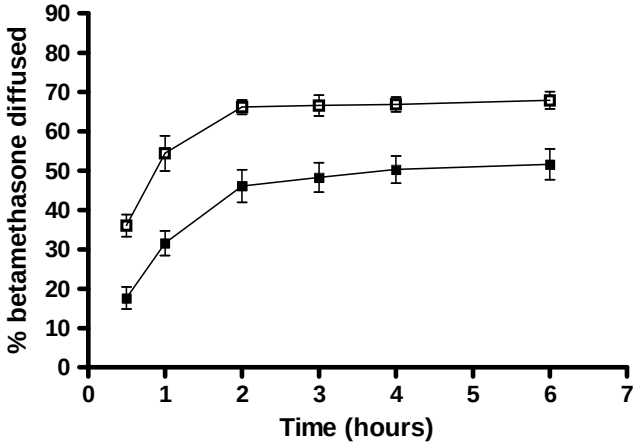


Figure 4: Comparison of betamethasone release kinetics from (■) classical PC liposomes containing betamethasone-cyclodextrin inclusion complexes and from (□) deformable PC liposomes containing betamethasone-cyclodextrin inclusion complexes. (A) HPγCD; (B) Crysmeb (n=3).

The presence of sodium deoxycholate enhanced the percentage of betamethasone diffused from 59.63% (± 7.03) to 73.79% (± 4.61), when HPγCD was used ($p < 0.001$) (Figure 4 (A)). When Crysmeb was used, the presence of sodium deoxycholate enhanced the percentage of betamethasone diffused from 51.64% (±4.25) to 67.92% (±0.26) ($p < 0.001$) (Figure 4 (B)). The type of cyclodextrin did not influence significantly the percentage of betamethasone diffused (for classical liposome, $p = 0.1676$ and for deformable liposomes, $p = 0.0925$). The release profiles exhibited a plateau effect after about 3 hours diffusion. This plateau was due to donor depletion, which occurs where the proportion of permeant entering the membrane is large, relative to the amount applied (finite dose technique). The percentage of betamethasone diffused did not rise up to 100%, even after 24 hours of diffusion (results not shown) and even if sink conditions were respected. This observation could be explained by the fact that the liposomal formulation did not completely dry up after the diffusion time; a part of sample remaining in the donor chamber. The amount of phospholipids that had permeated through the membrane was determined in the receptor phase by the enzymatic assay. At the end of the experiments, 4 mL of receptor phase were lyophilized and dispersed in 200 µL water in order to concentrate this receptor phase. Even then, no phospholipids were found in the receiver compartment. The first explanation was that the membrane used is hydrophilic and may not be appropriate for studying the diffusion of liposomes, but only that of the aqueous betamethasone-cyclodextrin complexes. However, this fact was also observed by Sinico *et al*. [19]. They studied the diffusion of liposomes encapsulating tretinoin through a silicone membrane, which is a polymeric hydrophobic membrane, and they did not find any phospholipids in the receiver compartment either; even though about 16% of tretinoin permeated after 24 hours from their liposomal formulation. The improvement of betamethasone release from deformable liposomes could be explained by a higher bilayer permeability because of the presence of sodium deoxycholate. We could not show a diffusion of intact deformable liposomes through the polycarbonate

membrane. *Ex vivo* diffusion studies using pig skin would help us to confirm these results and to understand the diffusion mechanism.

4. CONCLUSION

This study proved the feasibility of a new topical delivery system: drug-in-cyclodextrin-in deformable liposomes and showed its promising characteristics for future experiments using pig ear skin and human skin. The use of sodium deoxycholate as the edge activator increased up to 1.8 times the encapsulation efficiency and up to 1.3 times the percentage of betamethasone diffused in comparison with classical liposomes. These new vesicles showed higher deformability and a good stability, when stored at 4 °C.
Ex vivo diffusion studies using pig skin and *in vivo* studies would help us to confirm these *in vitro* results.

5. ACKNOWLEDGEMENTS

A. Gillet is a PhD student supported by the FNRS, Brussels, Belgium.

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II. Chapitre 2

SKIN PENETRATION BEHAVIOUR OF LIPOSOMES AS A FUNCTION OF THEIR COMPOSITION

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Ce chapitre a été accepté dans European Journal of Pharmaceutics and Biopharmaceutics

II.1 Résumé

Evaluation de la pénétration cutanée des liposomes en fonction de leur composition

II.1.1 Introduction

Les liposomes déformables ont été développés et testés comme systèmes d'administration cutanée et transcutanée. Il n'existe pas encore de consensus concernant les mécanismes de pénétration de ces liposomes. Ce travail décrit des études de diffusion *ex vivo* comparant les mécanismes de pénétration des liposomes classiques et déformables. Les liposomes classiques et déformables encapsulant la bétaméthasone dans leur cavité aqueuse par l'utilisation d'HPγCD (CD) (liposomes classiques BMS-CD et liposomes déformables BMS-CD) sont comparés à des liposomes encapsulant la bétaméthasone au niveau de leur bicouche lipidique (liposomes classiques BMS et liposomes déformables BMS). Ces liposomes sont caractérisés par leur diamètre, leur pourcentage d'encapsulation, leur stabilité (en termes de variation de diamètre et de libération du matériel encapsulé) et leur déformabilité. Les études de pénétration *ex vivo* sont réalisées à l'aide des cellules de diffusion de Franz et au travers de peaux d'oreilles de cochons. La pénétration de la bétaméthasone dans le *stratum corneum* est évaluée par la méthode du stripping. Les méthodes analytiques de dosage sont mises au point et validées (voir annexe 1). La microscopie confocale est utilisée pour visualiser la pénétration de liposomes rendus fluorescents.

II.1.2 Résumé des résultats

II.1.2.1 Caractérisation

Les liposomes dits « classiques » sont constitués de PC tandis que les liposomes déformables contiennent en plus du déoxycholate sodique comme « edge activator ».
L'encapsulation de la bétaméthasone au niveau de la bicouche lipidique permet d'augmenter le pourcentage d'encapsulation par rapport à une encapsulation à l'aide de cyclodextrines de 39,1 ± 1,9% à 97,8 ± 5,4% pour les liposomes classiques et de 42,5 ± 2,0% à 74,6 ± 2,5% pour les liposomes déformables. Par contre, l'encapsulation des complexes bétaméthasone-cyclodextrine au niveau du compartiment aqueux des liposomes augmente la rétention de la bétaméthasone au sein des vésicules. La mesure de la variation de diamètre au cours du temps montre une bonne stabilité des liposomes lorsqu'ils sont conservés à 4°C pendant un mois. La mesure de la déformabilité des liposomes par extrusion confirme la plus grande élasticité des liposomes déformables.

II.1.2.1 Etude de pénétration cutanée

Les résultats de pénétration cutanée sont repris dans la Figure 2.1.

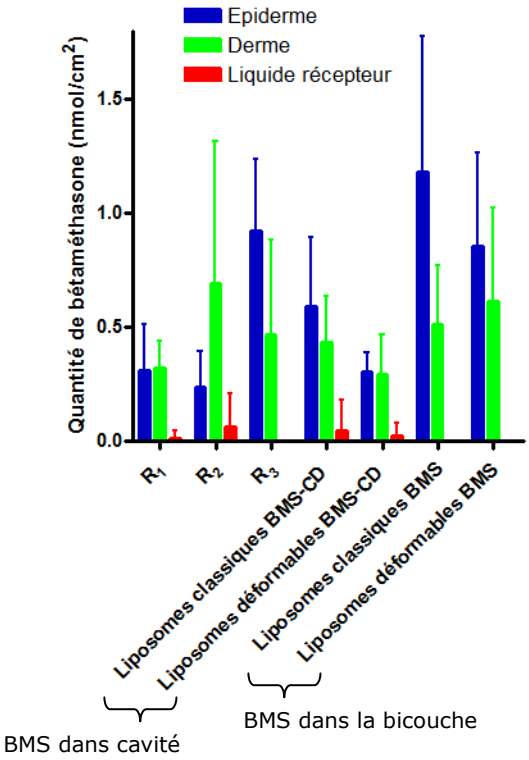


Figure 2.1 : Quantité de bétaméthasone accumulée au niveau de l'épiderme, du derme et du liquide récepteur des cellules de Franz (nmol/cm^2) avec R_1 = solution de bétaméthasone dans l'éthanol, R_2 = solution de complexes bétaméthasone-cyclodextrines et R_3 = dispersion de PC et bétaméthasone dans le tampon Hepes (n = 9).

L'encapsulation à l'aide des complexes avec l'HPγCD réduit la quantité de bétaméthasone au niveau de l'épiderme de 1,18 ± 0,59 nmol/cm^2 à 0,59 ± 0,3 nmol/cm^2 dans le cas des liposomes classiques et de 0,86 ± 0,41 nmol/cm^2 à 0,31 ± 0,09 nmol/cm^2 pour les liposomes déformables ($p < 0,05$).

Comme le montre la Figure 2.1, les liposomes déformables ne permettent pas d'augmenter la pénétration de la bétaméthasone par rapport aux liposomes classiques.

La rétention moindre des liposomes encapsulant la bétaméthasone au niveau de leur bicouche et la meilleure pénétration de ces systèmes suggèrent que la bétaméthasone est relarguée des liposomes et pénètre

ensuite sous forme libre dans la peau. De plus, il n'y a pas de différence significative entre la pénétration à partir d'une dispersion non liposomale de PC et de bétaméthasone (R_3) et à partir des liposomes classiques BMS, suggérant que la formulation sous forme de vésicules ne serait pas nécessaire. La PC agirait comme promoteur d'absorption puisque la dispersion non liposomale donne de meilleurs résultats de pénétration que les solutions de bétaméthasone dans l'éthanol (R_1) ou que la solution de complexes (R_2).
Les résultats de microscopie confocale semblent en accord avec les quantités de bétaméthasone accumulées au niveau de la peau et du liquide récepteur. La calcéine, hydrosoluble et utilisée à la place des complexes d'inclusion de la bétaméthasone dans la cavité aqueuse des liposomes, ne pénètre pas dans l'épiderme alors que la rhodamine B, plus lipophile et simulant l'encapsulation de la bétaméthasone seule au niveau de la bicouche lipidique, pénètre plus profondément dans l'épiderme. La rhodamine B suit la pénétration de la PC fluorescente (NBD-PC). En outre, les images de microscopie confocale montrent une pénétration des liposomes au niveau des follicules pileux.

II.1.3 Conclusion

Cette étude a montré que la bétaméthasone ne semble pas pénétrer dans la peau sous forme encapsulée dans les liposomes et que la PC agirait comme promoteur d'absorption. La bétaméthasone serait relarguée des liposomes et pénétrerait ensuite sous forme libre dans la peau. Dans cette étude, les liposomes déformables ne permettent pas d'augmenter la pénétration de la bétaméthasone. L'utilisation des complexes d'inclusion bétaméthasone-cyclodextrines augmente la stabilité des liposomes mais n'améliore pas la pénétration cutanée.

II.2 Article

Abstract

Deformable liposomes have been developed and evaluated as a novel topical and transdermal delivery system. Their mechanism of drug transport into and through the skin has been investigated but remains a much debated question. The present study concerns *ex vivo* diffusion experiments using pig ear skin in order to explain the penetration mechanism of classical and deformable liposomes. Classical and deformable vesicles containing betamethasone in the aqueous compartment through the use of cyclodextrin inclusion complexes were compared to vesicles encapsulating betamethasone in their lipid bilayer. Deformable liposomes contained sodium deoxycholate as the edge activator. Liposomes were characterised by their diameter, encapsulation efficiency, deformability, stability (in terms of change in diameter) and release of encapsulated drug. *Ex-vivo* diffusion studies using Franz diffusion cells were performed. Confocal microscopy was performed to visualise the penetration of fluorescently labelled liposomes into the skin. This study showed that liposomes do not stay intact when they penetrate the deepest layers of the skin. Betamethasone is released from the vesicles after which free drug molecules can diffuse through the *stratum corneum* and partition into the viable skin tissue.

Keywords

Liposome; cyclodextrin; betamethasone; Franz cells; pig skin

1. INTRODUCTION

A major obstacle to cutaneous drug delivery is the permeation characteristics of the *stratum corneum,* which limits drug transport, making this route of administration frequently insufficient for medical use. During the past few decades, there has been a wide interest in exploring new techniques for increasing drug absorption through the skin [1, 2]. Topical delivery of drug by liposomes has aroused considerable interest.

Recently, it became evident that, in most cases, classical liposomes are of little or no value as carriers for transdermal drug delivery, as they do not penetrate skin deeply, but rather remain confined to the upper layers of the *stratum corneum* [3]. In order to target deeper underlying skin tissue, intensive research led to the introduction and development of a new class of lipid vesicle, the highly deformable (elastic or ultraflexible) liposomes, which were named Transfersomes® [4]. Several studies have reported that deformable liposomes are able to improve *in vitro* skin delivery of various drugs [5] and to stay intact when they penetrate the skin, *in vivo*, transferring therapeutic amounts of drugs [6]. According to Cevc and Blume, the improved drug delivery by deformable liposomes is due to the driving force provided by the osmotic gradient between the outer and inner layers of the *stratum corneum* [4]. The important difference between deformable liposomes and traditional liposomes is the high and stress-dependent adaptability of such deformable vesicles, which enables them to squeeze between the cells in the *stratum corneum*, despite the large average vesicle size [7]. Thus, they can pass through the intact skin spontaneously, under the influence of the naturally occurring, *in vivo* transcutaneous hydration gradient [8]. These vesicles consist of phospholipids and an edge activator. An edge activator is often a single chain surfactant, with a high radius of curvature, which destabilises the lipid bilayers of the vesicles and increases their deformability [3].

Following topical application, structural changes in the *stratum corneum* have been identified and intact vesicles have been visualised within the

stratum corneum lipid lamellar regions, but no intact vesicles have been identified in the deepest viable tissues [9]. There is still considerable debate as to whether deformable vesicles behave as a true carrier system by penetrating the skin intact or whether they act as a permeation enhancer [10].

Depending on the composition and method of preparation, vesicles can vary with respect to size, lamellarity, charge, membrane fluidity or elasticity and drug entrapment. This variability, in addition to the skin model used (man or animal, *in vitro* or *in vivo*), probably accounts for the lack of understanding of the penetration mechanism of vesicles. In this context, systematic physicochemical and pharmacokinetic studies are still needed to define the mode of action of these vesicles [10, 11].

The present study concerns *ex vivo* diffusion experiments using pig ear skin in order to compare the penetration mechanism of classical and deformable liposomes. Betamethasone was chosen as a model drug only for *ex-vivo* studies.

When incorporated into phospholipid films or bilayers, betamethasone, because of its hydrophobic nature, would be expected to be associated with the hydrocarbon chain region of lipid molecules. Betamethasone is also known to form inclusion complexes with various cyclodextrins. Cyclodextrins are cyclic (β-1,4)-linked oligosaccharides of d-glucopyranose containing a relatively hydrophobic central cavity and a hydrophilic outer surface. Cyclodextrins are able to form inclusion complexes with poorly water-soluble drugs. This inclusion allows the increase of the "hydrophilic" character of betamethasone and for the preparation of aqueous solutions. This cyclodextrin inclusion allows the incorporation of betamethasone-cyclodextrin complexes in the aqueous cavity of liposomes instead of in the lipid bilayer compartment. In a previous work, we studied the effect of cyclodextrins on the encapsulation efficiency and released kinetics of betamethasone from liposomes. We showed the feasibility and the advantages of the betamethasone-in-cyclodextrin-in-liposome formulation [12]. The influence of drug incorporation in liposomes on skin penetration is studied by comparing either liposomes encapsulating betamethasone

into their lipid bilayer or liposomes encapsulating betamethasone into their aqueous cavity by using betamethasone-cyclodextrin complexes.
Classical and deformable vesicles containing betamethasone (BMS) in the aqueous compartment through the use of cyclodextrin inclusion complexes (PC-BMS-HPγCD and PC-Na Deoxy-BMS-HPγCD) were compared to vesicles encapsulating betamethasone in their lipid bilayer (PC-BMS and PC-Na Deoxy-BMS). Confocal microscopy was used in order to visualize the penetration of fluorescently labelled liposomes.
The aim of this study is to compare physicochemical characteristics and the skin penetration of classical and deformable liposomes encapsulating a hydrophobic drug in the lipid bilayer or in the aqueous cavity by the use of cyclodextrins.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Betamethasone (E.P.) was purchased from Medeva (Braine L'Alleud, Belgium). Soybean phosphatidylcholine (PC) was purchased from Lipoïd (Ludwigshafen, Germany). 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphocholine (NBD-PC) was purchased from Avanti Polar lipids (Alabaster, AL, USA). Hydroxypropylated-γ-cyclodextrin (HPγCD, D.S. 0.7, 3.41% H_2O) was obtained from Wacker-Chemie GmbH (Munich, Germany). Sodium deoxycholate and calcein were purchased from Sigma Aldrich (Bornem, Belgium). Rhodamine B and acetonitrile were obtained from Merck (Darmstadt, Germany). Pure water was generated from the Milli-Q system (Millipore, Bredford, MA, USA). All experiments were performed using a 0.22 µm-filtered 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) buffer (Sigma Aldrich), containing 145 mM NaCl and adjusted to pH 7.4 with 0.1 M NaOH solution. All other reagents and solvents were of analytical grade.

Pig ears (Race: Piétrain, age: 2-months-old) were purchased from the Faculty of Veterinary Medicine at the University of Liège, Belgium.

2.2 Preparation of inclusion complexes

Cyclodextrins were dissolved in Hepes buffer pH 7.4 in order to obtain a 10 mM HPγCD solution. Betamethasone was added in excess to the cyclodextrin solution. Water-soluble inclusion complexes were formed after stirring the mixture for 48 h at 25 °C. The milky solutions were then filtered through a 0.22 µm filter. The betamethasone concentration obtained for HPγCD inclusion complexes was 2.02 mg/mL. Solubility of betamethasone in Hepes buffer without cyclodextrin was evaluated to 65.2 µg/mL.

2.3 Liposome preparation

2.3.1 Entrapment of inclusion complexes into liposomes

Classical liposomes were made from PC, while PC and sodium deoxycholate (Na Deoxy) (87:13; m/m) as the "edge activator" were used for deformable liposomes. Liposomes were prepared by the hydration of lipid films. In practice, the required amount of lipids was dissolved in ethanol in a round-bottomed flask. The solution was then dried under vacuum using a rotary evaporator. The resulting lipid film was hydrated using 3 mL of betamethasone-HPγCD complex solution. Suspensions were then extruded three times through Nucleopore® polycarbonate membranes of successive 0.4 and 0.2 µm pore diameters (Whatman, Maidstone, UK). Free betamethasone complexes were separated from liposome-encapsulated betamethasone complexes by three successive ultracentrifugations at 165,052 × *g* (35,000 rpm). The first cycle lasted 3 h followed by two cycles of 1 h 30 at 4 °C. The supernatant was removed and the pellet was resuspended in Hepes buffer. Liposome diameter was

measured after this preparation. Betamethasone and PC were assayed in purified liposomes.

2.3.2 Entrapment of betamethasone into liposomal bilayers

Liposomes were prepared as described in 2.3.1 by hydration of lipid films. However, in this case, 3 mL of betamethasone solution (2 mg/mL in absolute ethanol) was added at the same time as the lipids before evaporation. The resulting lipid film was hydrated using 3 mL of Hepes buffer. Suspensions were then extruded and free betamethasone removed by ultracentrifugation, as described above.

2.4 Liposome characterisation

2.4.1 Measurement of liposome diameter

Liposome dispersions were sized by photon correlation spectroscopy (PCS) (HPPS, Malvern Instruments Ltd, Worcestershire, UK). Measurements were made at 25 °C with a fixed angle of 90° and the results were expressed as an average liposomal hydrodynamic diameter (nm).

2.4.2 Encapsulation efficiency

The encapsulation efficiency ($EE_{B/Bt}$) corresponds to the concentration of betamethasone encapsulated in liposomes (C_B) compared to the total drug concentration first introduced (C_{Bt}). This $EE_{B/Bt}$ was corrected to the concentration of lipids in order to take into account the loss of liposomes during their preparation:

$$EE_{B/Bt}(\%) = (C_B / C_L) / (C_{Bt} / C_{Lt}) \times 100$$

C_L is the lipid concentration in purified liposomes and C_{Lt} is the lipid concentration first introduced.

2.4.2.1 Betamethasone chromatographic determination

The HPLC used was a LaChrom Merck-Hitachi (Darmstadt, Germany) consisting of an L-7100 pump, an L-7200 autosampler, an L-7400 UV detector, an L-7350 column oven and a D-7000 interface. The system was controlled by "D-7000 HPLC System Manager" software. The analytical column was a LiChroCART (250 x 4 mm, i.d.) packed with Superspher 100 RP-18 (particle size: 5µm) and preceded by a guard column LiChroCART (4 x 4 mm, i.d.) packed with LiChrospher 100 RP-18 (particle size: 5 µm). Isocratic separation was performed at a temperature of 35 °C using a mobile phase consisting of a mixture of acetonitrile and water (50/50, v/v). The flow rate settled at 0.8 mL/min, and the sample injection volume was 20 µL. Betamethasone was monitored at 240 nm.

2.4.2.2 Quantification of lipids

Total lipid concentrations were calculated by measuring PC through an enzymatic method (LabAssay™ Phospholipid, Wako, Osaka, Japan). The principle of this enzymatic assay consists of the cleavage of PC in choline by phospholipase D and the oxidation of choline into betaine with the simultaneous production of hydrogen peroxide. The hydrogen peroxide, which is produced quantitatively, couples 4-Aminoantipyrine and *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS). Peroxidation results in the generation of a coloured compound quantified by spectrophotometry at 600 nm (spectrophotometer Perkin-Elmer Lambda 11).

2.4.3 Deformability

Comparative measurements of elasticity of the bilayers of the different liposome formulations were carried out by extrusion measurements [13]. Briefly, the vesicles were extruded through a polycarbonate membrane

with a pore size of 50 nm (Nucleopore, The Netherlands) at a constant pressure of 5 bars. The elasticity was expressed in terms of a deformability index D, which is proportional to $j(r_v/r_p)^2$, where j is the weight of the suspension, which was extruded in 5 min through the polycarbonate membrane, r_v the diameter of the vesicles after extrusion and r_p the pore size of the membrane. Liposome suspensions were diluted at the same lipid concentration. The loss of phosphatidylcholine during the extrusion was calculated. A corrected deformability index D_{cor} to the maximum deformability index for each formulation was calculated too.

$$D_{cor} = [(j(r_v/r_p)^2) / (j_0(r_{v0}/r_p)^2)] \times 100$$

Where r_{v0} is the liposomes diameter before extrusion and j_0 is the weight of suspension used for extrusion.

2.4.4 Leakage after ultracentrifugation

Liposome resistance to ultracentrifugation was evaluated by measuring the leakage of the encapsulated drug. In practice, a sample was centrifuged at 35,000 rpm for 1 h 30 at 4 °C. The supernatant was then assayed to determine the released betamethasone. The amount of betamethasone released was calculated by the following equation:

$$\% \text{ betamethasone released } = C_{Bsup} / C_B \times 100$$

where, C_{Bsup} is the concentration of betamethasone in the supernatant and C_B is the concentration of betamethasone encapsulated in liposomes.

2.4.5 Liposome stability

The stability of liposomes was evaluated by measuring the particle mean diameter and polydispersity indexes by PCS after one month of storage at 4°C.

2.4.6 Release of the encapsulated drug

The release of the encapsulated betamethasone was evaluated at three temperatures (4 °C, 25 °C, and 37 °C) for a period of one month. In practice, every week, a sample stored at each temperature was centrifuged at 35,000 rpm for 1 h 30 at 4 °C. The supernatant was then assayed to determine the released betamethasone. The amount of betamethasone released was calculated by the following equation:

$$\% \text{ betamethasone released}_t = [(C_{Bsup\ t} / C_{Bt}) / (C_{Bsup\ t0} / C_{B\ t0})] \times 100$$

where, C_{Bsup} is the concentration of betamethasone in the supernatant at the day of preparation ($C_{Bsup\ t0}$) or after t days of storage($C_{Bsup\ t}$) and C_B is the concentration of betamethasone encapsulated in liposomes at the day of preparation ($C_{B\ t0}$) or after t days (C_{Bt}) [14]. Results are calculated in order to remove the leakage due to ultracentrifugation (t_0), this leakage being reproducible.

2.5 *Ex vivo* diffusion study

2.5.1 Skin preparation

Full-thickness skin was removed from the dorsal side of the freshly excised pig ear, stored at -20 °C and used within 6 months. On the day of the experiment, punches were cut out and hairs cut with scissors.

2.5.2 Permeation experiments

Diffusion studies were carried out using Franz type glass diffusion cells. These cells consist of two compartments with the skin clamped between the donor and receiver chambers, dermal side down. The cell body was filled with 7.5 mL of a receptor phase consisting of Hepes buffer solution

pH 7.4 containing 0.01% NaN_3 as the preservative. Test conditions were chosen in order to respect sink conditions in the receiver compartment. Solubility of betamethasone in the Hepes buffer was evaluated to 65.2 µg/mL, corresponding to around 10 times the maximum concentration of betamethasone that can be found in the receiver compartment. The receptor medium was constantly stirred with a small magnetic bar and thermostated at 37 °C throughout the experiments. 350 µL of liposome suspension at 150 µg/ml betamethasone concentration, were placed in the donor chamber onto the *stratum corneum* of the skin, in non occlusive conditions. The diffusion area was 1.767 cm^2. At the end of the experiment (24 h), the receptor phases were removed and the diffusion cells were dismantled. The skin surface was washed with 3 mL Hepes buffer on each side to remove the residual donor sample and was thawed. The surface of the skin exposed to the donor compartment was punched out. The *stratum corneum* was removed by the stripping method with 15 successive strips of Corneofix® tape (CKelectronic, Germany). Only the first, fifth, tenth and fifteenth strips were kept and analysed for betamethasone content. The piece of skin was then separated into the epidermis and dermis by pressing the skin surface against a hot plate (65 °C) for 90 seconds and peeling off the epidermis. The four strips, the epidermis and dermis cut into small pieces were each soaked separately in a flask with 4 mL of Hepes for 24 h. Samples were then shaken for 30 minutes in an ultrasound bath, in order to extract the entire drug accumulated in the skin pieces. Each formulation was tested in triplicate and each batch was tested on three Franz cells (n = 9).

2.5.3 Betamethasone determination

2.5.3.1 Solid phase extraction (SPE) prior to chromatographic analysis

SPE was needed to clean up the samples before HPLC injection. The extraction procedure was carried out on Isolute® C18, 50 mg, 1 mL (Biotage, Uppsala, Sweden) disposable extractive cartridges (DEC). After

conditioning with 1 mL acetonitrile and 1 mL Hepes buffer, 1 mL sample was loaded onto the DEC and then washed with 1 mL water. Betamethasone was eluted with 500 µL acetonitrile and 500 µL water was added before the HPLC injection. The chromatographic conditions were described in 2.4.2.1.

2.5.3.2 SPE-HPLC-UV method validation

For the validation, two types of standards were prepared. The first were calibration standards, prepared in the mobile phase at seven concentration levels ranging from 20 to 10,000 ng/mL. The others were validation standards, prepared at seven concentration levels ranging from 20 to 10,000 ng/mL. Validation standards were made in the skin extract in the Hepes buffer so as to mimic real samples obtained after permeation experiments. The validation was performed in 3 series. For each series, all the calibration standards were analysed in duplicate, while each validation standard was analysed in triplicate.

The validation was based on an accuracy profile approach [15]. For betamethasone determination in the pig ear skin, the acceptance limits were set at 30% from 24.2 to 60 ng/mL and 15% from 60 to 10,000 ng/mL respectively, and the risk level was fixed at 10%. For betamethasone determination from the tape stripping method, the acceptance limits were set at 10% from 20.02 to 10,000 ng/mL and the risk level was fixed at 5% [16, 17]. The most appropriate calibration model was a weighted ($1/x^2$) linear regression. The e-noval software v3.0 (Arlenda, Liège, Belgium) was used to compute the validation results as well as to obtain the accuracy profiles.

2.6 Confocal laser scanning microscopy (CLSM) study

2.6.1 Liposome preparation

Liposomes were prepared and characterised as described in 2.3 and 2.4 with some modifications. Liposomes were made fluorescent in two ways. First, the aqueous cavity was made fluorescent by the incorporation of 16 mM calcein solution in order to avoid the self quenching phenomenon that occurs at higher concentration. Calcein (log *P* = -5.02) is a hydrophilic dye, which was expected to be encapsulated in the aqueous compartment of liposomes like the betamethasone-cyclodextrin inclusion complexes [18]. Secondly, rhodamine B (log *P* = 1.95) (0.01 % m/m) was incorporated into the lipid bilayer in order to mimic the encapsulation of betamethasone (log *P* = 1.94) [19]. In each case, the lipid bilayer became fluorescent through the incorporation of NBD-PC (1.33% m/m). After extrusion, non-encapsulated calcein, rhodamine B or NBD-PC were separated from liposomes by successive ultracentrifugations at 35,000 rpm.
It must be noted that calcein and NBD-PC are fluorescent in the same range of wavelengths, so they could not be encapsulated in the same formulation; two formulations were needed.

2.6.2 Confocal laser scanning microscopy

Diffusion studies were carried out as described in section 2.5.2. After 24 h, the remaining liposome formulation was washed and the diffusion area punched out. The diffusion area was then incorporated into an OCT compound (Tissue-Tek®, Sakura, The Netherlands) and frozen at -20 °C. The frozen skin was then sectioned with a cryostat into 7 µm slices. These tissues were counterstained with TOTO-3 iodide dye (Molecular Probes, Leiden, The Netherlands). The penetration of the fluorescent probes was assessed by confocal laser scanning microscopy (Leica TCS

SP2, Heidelberg GmBH, Germany). All optical sections were recorded with the same settings. Calcein and NBD-PC were excited with the 488 nm laser line from an argon laser and the fluorescent emission signals are represented by a green colour. Rhodamine B was excited with the 568 nm line from a Kr laser and the fluorescent emission signals are represented by a red colour. TOTO-3-stained cell nuclei were excited with the 633 nm line from a He/Ne laser and are shown in a blue colour. Images were acquired using a 40X objective lens immersed in oil.

2.7 Statistical analysis

The significance of the differences between different formulations was tested using the Student t-test (Graph Pad Prism Version 4). The differences are considered statistically significant when $p < 0.05$. Correlation was evaluated by the Pearson correlation test (Graph Pad Prism, Version 4). Correlation significance is considered when $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Liposome characterisation

Classical and deformable liposomes containing betamethasone-HPγCD inclusion complexes were compared to the corresponding formulation of liposomes with betamethasone included in their lipid bilayer. PCS was performed for diameter analysis. As shown in Table 1, liposomes are characterised by a mean hydrodynamic diameter of between 128 ± 6 nm and 178 ± 12 nm. The polydispersity indexes (not shown) were always lower than 0.2, indicating that the liposomes were homogeneous in size.

Table 1: Diameter ± S.D. (nm), encapsulation efficiency ($EE_{B/Bt}$) ± S.D. (%), deformability index D ± S.D., corrected deformability index D_{cor} ± S.D. and leakage of betamethasone after ultracentrifugation ± S.D. (%) of classical or deformable liposomes containing betamethasone-cyclodextrin complexes (BMS-HPγCD) or betamethasone (BMS) (n = 3).

Composition	Diameter (nm)	$EE_{B/Bt}$(%)	Deforma-bility index D	Corrected deformability index D_{cor}	Leakage after ultracentrifu-gation
PC – BMS-HPγCD	169 ± 4	39.1 ± 1.9	3.4 ± 0.3	11.3 ± 1.2	20.9 ± 1.6
PC – Na deoxycholate – BMS-HPγCD	140 ± 2	42.5 ± 2.0	8.2 ± 0.6	30.6 ± 1.6	44.8 ± 3.2
PC – BMS	178 ± 12	97.8 ± 5.4	5.2 ± 1.0	17.1 ± 2.7	76.2 ± 1.6
PC – Na deoxycholate – BMS	128 ± 6	74.6 ± 2.5	8.0 ± 0.4	32.5 ± 2.4	82.7 ± 4.1

Deformable liposomes were significantly smaller in size than the corresponding non deformable liposomes (140 and 128 nm for deformable liposomes encapsulating betamethasone-HPγCD complexes or betamethasone respectively, *versus* 169 and 178 nm for classical liposomes encapsulating betamethasone-HPγCD complexes or betamethasone respectively; $p < 0.05$). This reduction of the particle diameter for deformable liposomes may be ascribed to increased flexibility and reduced surface tension of the vesicles due to the presence of sodium deoxycholate, as observed by Chen *et al*. [20].

Betamethasone encapsulation efficiency is reported in Table 1. Encapsulation of betamethasone into the lipid bilayer significantly enhances the encapsulation efficiency from 39.1 ± 1.9% to 97.8 ± 5.4% for classical liposomes and from 42.5 ± 2.0% to 74.6 ± 2.5% for deformable liposomes. The addition of sodium deoxycholate significantly decreased the encapsulation efficiency from 97.8 ± 5.4% to 74.6 ± 2.5% for liposomes containing betamethasone in their lipid bilayer ($p < 0.05$). This can be explained by a competition phenomenon between betamethasone and sodium deoxycholate in the lipid bilayer. The absence

of drug crystals in the case of liposomes encapsulating betamethasone in their lipid bilayer was confirmed by the transmission electron microscopy (TEM) of freeze-fracture replica. Thus, betamethasone is rightly associated to the lipid bilayer.

Prepared formulations were subjected to the deformability test by extrusion measurements. Results are expressed in terms of deformability index D (Table 1). As expected, deformability is higher when liposomes contain sodium deoxycholate (D = 8.2 ± 0.6 when betamethasone is encapsulated by inclusion complexes and 8.0 ± 0.4 when encapsulated alone in the lipid bilayer) than that of classical liposomes (D = 3.4 ± 0.3 and 5.2 ± 1.0, respectively) ($p < 0.05$). The deformability index varies between a minimum value and a maximum value. The minimum value is 0 for all formulations, as no suspension passes through the polycarbonate membrane (j = 0). The maximum value depends on the formulation as liposome diameter varies with the formulation. Thus, a corrected deformability index to the maximum value was calculated (D_{cor} in Table 1). However, this correction does not change the above conclusions. The percentage of phosphatidylcholine recovered after the extrusion was calculated to ensure that the concentration of liposomes remains constant. For deformable liposomes, the percentage of phosphatidylcholine recovered is 89.5 ± 12.9% when betamethasone is encapsulated into the aqueous compartment and 84.1 ± 4.2% when betamethasone is encapsulated into the lipid bilayer. However, for classical liposomes, the percentage of phosphatidylcholine recovered is only 11.9 ± 1.7% when betamethasone is encapsulated into the aqueous compartment and 3.5 ± 2.8% when encapsulated into the lipid bilayer. In the case of classical liposomes, only low amounts of liposomes were extruded through the 50 nm pore size membrane. This observation confirms that classical liposomes are more rigid and less deformable than those containing the edge activator.

This observation is also confirmed by the leakage of betamethasone after ultracentrifugation (Table 1). The resistance of liposomes to ultracentrifugation was evaluated. The percentage of betamethasone released is 20.9 ± 1.6% for classical liposomes encapsulating betamethasone-cyclodextrin inclusion complexes *versus* 44.7 ± 3.1% for deformable ones. The loss of the entrapped drug is probably due to the deformation of lipid membrane that occurs during the ultracentrifugation process, as observed by Lopez-Pinto *et al*. [21]. Deformable liposomes are certainly more sensitive to ultracentrifugation than classical liposomes because of the presence of sodium deoxycholate, which destabilises the membrane and makes it more permeable. The percentage of betamethasone released is 76.5 ± 2.1% for classical liposomes containing betamethasone in the lipid bilayer and 82.7± 4.1% for deformable ones. These results show that the use of cyclodextrins improves the retention of betamethasone inside the liposome cavity. This high drug release was also observed by Bhardwaj *et al*. with DMPC liposomes encapsulating dexamethasone in their lipid bilayer. They observed that dexamethasone release from extruded liposomes was fast and that most of the content was released within 48h at 37°C. Based on differential scanning calorimetry, they showed that dexamethasone destabilized the liposome membranes as indicated by a decrease in enthalpy and an increase in the peak width of the main transition [22].
The results show that the incorporation of sodium deoxycholate and/or betamethasone in the lipid bilayer enhances the permeability of the membrane.

The stability of liposomes was evaluated by measuring their diameter after one month of storage at 4°C. Results are shown in Table 2. No significant change in diameter is observed, indicating the stability of the formulations. Polydispersity indexes (not shown) remain under 0.2.

Table 2: Diameter ± S.D. (nm) at the day of preparation (T_0) and after a minimum of one month of storage at 4°C for classical or deformable liposomes containing betamethasone-cyclodextrin complexes (BMS-HPγCD) or betamethasone (BMS) (n = 3).

Composition	Diameter T_0 (nm)	Diameter after one month 4°C (nm)
PC – BMS-HPγCD	169 ± 4	169 ± 1
PC – Na deoxycholate – BMS-HPγCD	140 ± 2	141 ± 1
PC – BMS	178 ± 12	172 ± 5
PC – Na deoxycholate – BMS	128 ± 6	128 ± 6

The release of encapsulated betamethasone was evaluated as a function of time. Fig. 1 shows the percentage of betamethasone released from classical and deformable liposomes encapsulating betamethasone-HPγCD complexes for a period of one month.

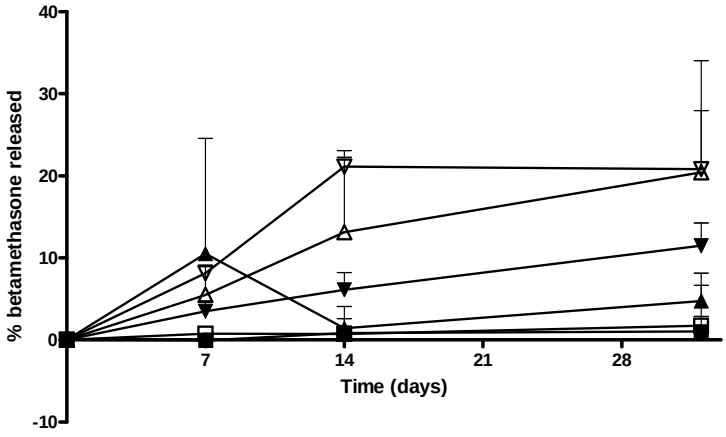


Fig. 1: Percentage of betamethasone released from liposomes encapsulating betamethasone-HPγCD complexes as a function of time. Classical (dark symbol) and deformable (open symbol) liposomes are stored at three different temperatures: 4 °C (■,□); 25 °C (▲,Δ); and 37 °C (▼, ▽) (n=3).

The release of the encapsulated drug does not increase significantly after one month when liposomes are stored at 4°C or 25 °C ($p > 0.05$). However, when liposomes are stored at 37°C, the release of betamethasone increases ($p < 0.05$). The difference in drug release between classical and deformable liposomes encapsulating betamethasone-cyclodextrin complexes after one month of storage at each temperature is not significant.

Fig. 2 shows the percentage of betamethasone released from classical and deformable liposomes encapsulating betamethasone in their lipid bilayer as a function of time.

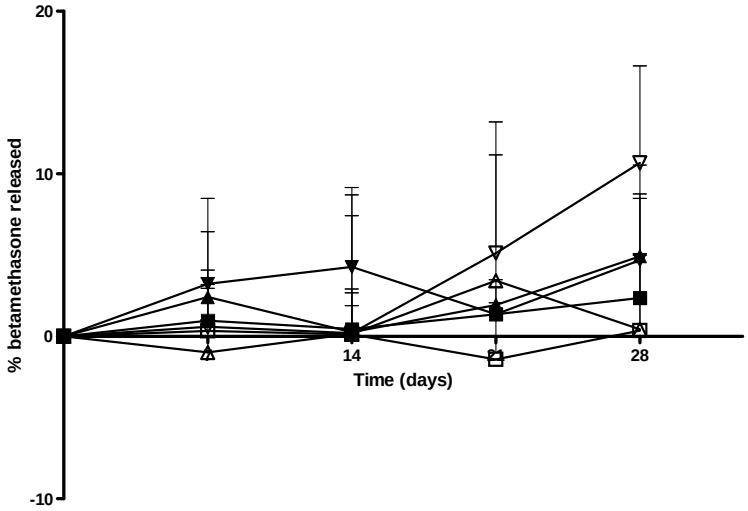


Fig. 2: Percentage of betamethasone released from liposomes encapsulating betamethasone in the lipid bilayer as a function of time. Classical (dark symbol) and deformable (open symbol) liposomes are stored at three different temperatures: 4 °C (■,□); 25 °C (▲,Δ); and 37 °C (▼, ▽) (n=3).

The release of the encapsulated drug does not increase significantly after one month when liposomes are stored at 4°C or 25 °C ($p > 0.05$). The release of betamethasone increases when liposomes are stored one month at 37°C. However, due to high standard deviations, results are not significantly different ($p > 0.05$). The difference in drug release between

classical and deformable liposomes encapsulating betamethasone alone after one month of storage at each temperature is not significant.

These results on characterisation show that the liposome diameter decreases when sodium deoxycholate is added. The encapsulation efficiency is higher for liposomes encapsulating betamethasone in their lipid bilayer compared with liposomes encapsulating betamethasone-cyclodextrin complexes. Concerning the deformability study, the addition of the edge activator increases the deformability of the vesicles. The use of betamethasone-cyclodextrines complexes enhances the drug retention in liposomes. Liposome diameter did not change after one month at 4°C. The release of the encapsulated drug increases with temperature.

3.2 *Ex vivo* penetration study

Franz type diffusion cells were used to evaluate the *ex vivo* penetration of betamethasone encapsulated in liposomes in pig ear skin. Despite their advantages, human skin samples are rarely used due to limited resources, high logistic effort and regulatory issues. Therefore, porcine (ear) skin, bovine (udder) skin or rat skin is usually employed as an alternative [23]. Pig skin is considered as a good model for human skin [24-27]. All samples were adjusted at a final betamethasone concentration of 150 µg/mL. Penetration studies were carried out in non occlusive conditions to allow the driving force provided by the osmotic gradient. Three reference samples were used for comparison: a solution of betamethasone in absolute ethanol (R_1), an aqueous solution of betamethasone-HPγCD inclusion complexes (R_2) and a dispersion of phosphatidylcholine and betamethasone in Hepes buffer (R_3).

3.2.1 Penetration in the *stratum corneum*

Fig. 3 shows the amount of betamethasone determined on strips one, five, ten and fifteen. The other strips were discarded to reduce the number of

samples. Histological studies confirmed that all the *stratum corneum* is removed after 15 strips.

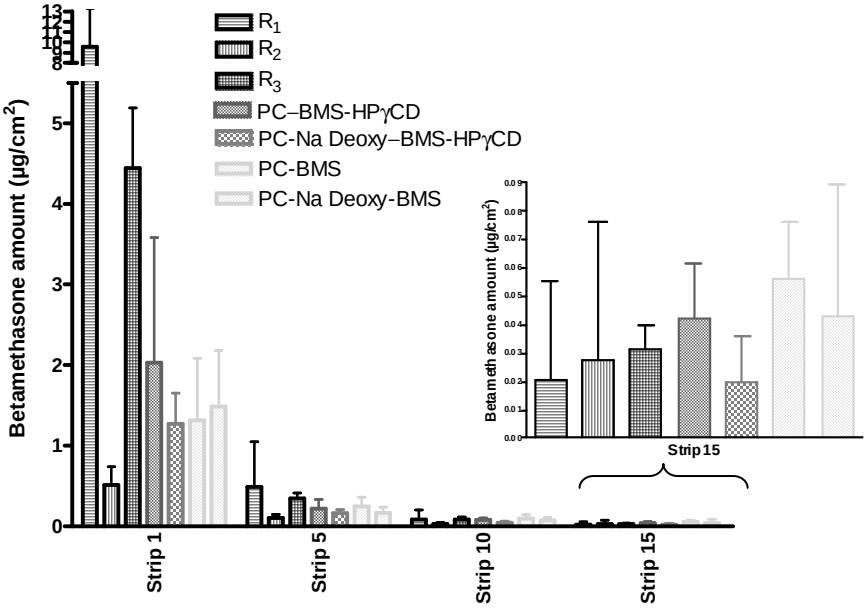


Fig. 3: Amount of betamethasone as a function of the strip number (µg/cm^2). R_1: solution of betamethasone in absolute ethanol, R_2: aqueous solution of betamethasone-HPγCD inclusion complexes and R_3: dispersion of phosphatidylcholine and betamethasone in Hepes buffer (n = 9).

We can observe that the amount of betamethasone decreases with the number of strips. As shown in Fig. 3, for the first strip, we observe a high difference in betamethasone content between the betamethasone ethanolic solution (R_1) and the aqueous solution containing the betamethasone-HPγCD inclusion complexes (R_2). The reason for this difference is the effectiveness of skin washing by Hepes, which can more easily remove betamethasone from the aqueous solution than from the ethanolic solution. Betamethasone included in cyclodextrin is water soluble and is easily removed by the washing procedure. Ethanolic solution, when

evaporated, leaves betamethasone not soluble in water and thus not easily washed.

3.2.2 Penetration in the epidermis, dermis and receptor medium.

The amount of betamethasone accumulated in the epidermis, dermis and in the receptor medium of the Franz cells is shown in Fig. 4.

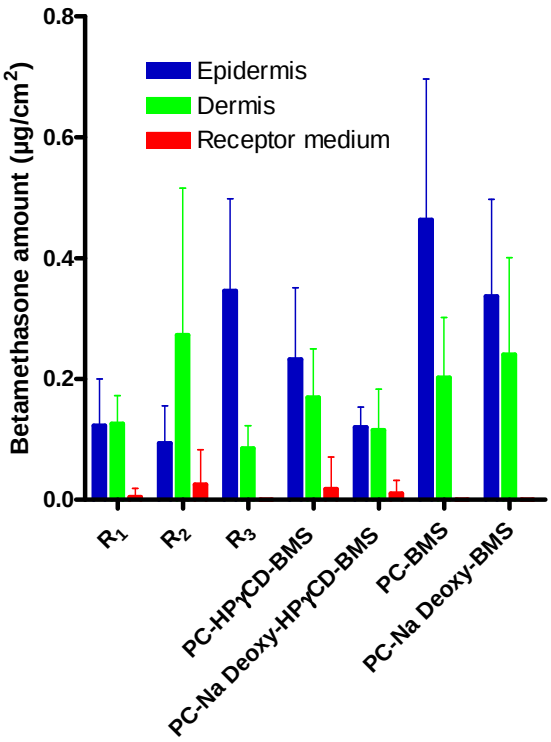


Fig. 4: Amount of betamethasone in the epidermis, dermis and receptor medium of Franz cells (µg/cm^2). R_1: solution of betamethasone in absolute ethanol, R_2: aqueous solution of betamethasone-HPγCD inclusion complexes and R_3: dispersion of phosphatidylcholine and betamethasone in Hepes buffer (n = 9).

Concerning the references, we observed that the dispersion of betamethasone and phosphatidylcholine in Hepes (R_3) penetrates the epidermis better than the ethanolic and the cyclodextrin complex solution (R_1 and R_2, respectively) ($p < 0.001$). The aqueous solution of betamethasone-HPγCD inclusion complexes (R_2) seems to penetrate the dermis better than the ethanolic solution (R_1) and the dispersion (R_3) but the standard deviation is too high and the difference is not significant. The better epidermis penetration of betamethasone from the dispersion (R_3), compared with other references, confirmed that phosphatidylcholine acts as a penetration enhancer.

Concerning liposome formulations, liposomes encapsulating betamethasone-cyclodextrin complexes systematically show a lesser cutaneous penetration than the corresponding formulation containing betamethasone alone. Cyclodextrin complexes reduce the amount of betamethasone in the epidermis from 0.46 ± 0.23 μg/cm^2 to 0.23 ± 0.12 μg/cm^2 for classical liposomes and from 0.34 ± 0.16 μg/cm^2 to 0.12 ± 0.03 μg/cm^2 for deformable liposomes ($p < 0.05$). The reservoir effect of liposomes containing cyclodextrins contributes to the reduced penetration. Regarding the high release of betamethasone from liposomes containing the drug in their lipid bilayer (Table 1) and the better epidermis penetration of this formulation, betamethasone would not penetrate the skin under an encapsulated form. Betamethasone is released from vesicles after which the free drug can penetrate through the *stratum corneum* and partition into the viable skin tissue. Maestrelli *et al*. also observed that ketoprofen permeated faster from liposomes containing plain drugs than from those containing drug-cyclodextrin complexes. This finding was attributed to the different preparation method of the liposomes. The drug alone, incorporated into the phospholipidic membrane bilayer, can be released rapidly, whereas the complexed drug, entrapped in the internal aqueous core, permeated more slowly, since it had to overcome the lipidic barrier of the vesicle membrane [28]. These results are in agreement with those observed in the present study.

We have also observed that sodium deoxycholate systematically show a lesser epidermis penetration than the corresponding formulation containing only PC. Sodium deoxycholate reduces the amount of betamethasone in the epidermis from 0.23 ± 0.12 µg/cm^2 to 0.12 ± 0.03 µg/cm^2 for liposomes encapsulating betamethasone-cyclodextrin complexes ($p < 0.05$) and from 0.46 ± 0.23 µg/cm^2 to 0.34 ± 0.16 µg/cm^2 for liposomes encapsulating betamethasone alone (however, $p > 0.05$).

No significant difference was found in the betamethasone content of the dermis between the different formulations. However, deformable liposomes encapsulating betamethasone in their lipid bilayer accumulate better in the dermis in comparison with deformable liposomes encapsulating betamethasone-cyclodextrin complexes ($p < 0.05$).

Only very small amounts of betamethasone were found in the receptor medium of Franz diffusion cells in some cases. This is certainly due to the use of full thickness skin instead of dermatomed skin or a heat separated epidermis. Thus, we were not able to observe any transdermal delivery of betamethasone.

As a conclusion of *ex-vivo* penetration study, we can observe that encapsulation of betamethasone-cyclodextrin complexes systematically decreases the betamethasone cutaneous penetration compared to the formulation containing betamethasone into lipid bilayers. We can also observe that surprisingly, deformable liposomes do not improve the penetration of betamethasone in the skin compared to classical liposomes. This observation cannot be explained at the present time. *Ex-vivo* studies showed that the best formulation seems to be liposomes encapsulating betamethasone in the lipid bilayer.

It must be noted that the difference of accumulation in the epidermis between the dispersion of phosphatidylcholine and betamethasone (R_3) and the corresponding (PC-BMS) liposomes was not significant, indicating that the incorporation of betamethasone in liposomes would not be an advantage.

3.2.3 Correlation between *stratum corneum* and epidemis penetration

Fig. 5 shows the relation between the betamethasone content in strip 15 (see enlargement in Fig. 3) and the amount of betamethasone in the epidermis.

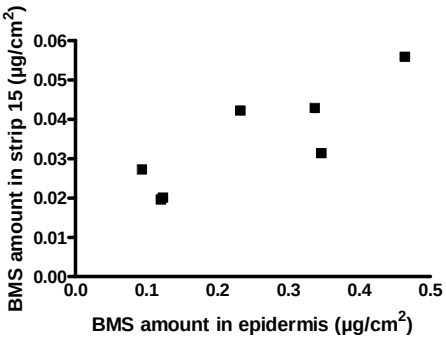


Fig. 5: Correlation between the amount of betamethasone (BMS) determined in strip 15 (µg/cm^2) and the amount of betamethasone in the epidermis (µg/cm^2).

A good correlation could be shown between the betamethasone amount in strip 15 and the betamethasone amount determined in the epidermis (Pearson test, $p < 0.05$, $r^2 = 0.8436$). The betamethasone amount in the last strip, close to the viable epidermis is reflective of the amount penetrated more deeply in the epidermis.

3.3 Confocal microscope observations

In order to visualize the skin delivery of the different formulations tested, classical liposomes are made fluorescent in two ways. In the first experiment, the aqueous cavity of classical liposomes is made fluorescent by the inclusion of calcein, a hydrophilic dye (log *P* = -5.02) supposedly encapsulated in the same compartment as the betamethasone-cyclodextrin inclusion complexes (Fig. 6A). In the second experiment, rhodamine B (log *P* = 1.95) was encapsulated into the lipid bilayers of

classical liposomes (Fig. 6C). This lipophilic dye is supposedly encapsulated into the lipid bilayer like free betamethasone (log *P* = 1.94). In the two cases, the lipid bilayer is made fluorescent by the incorporation of NBD-PC (Fig 6B and C).

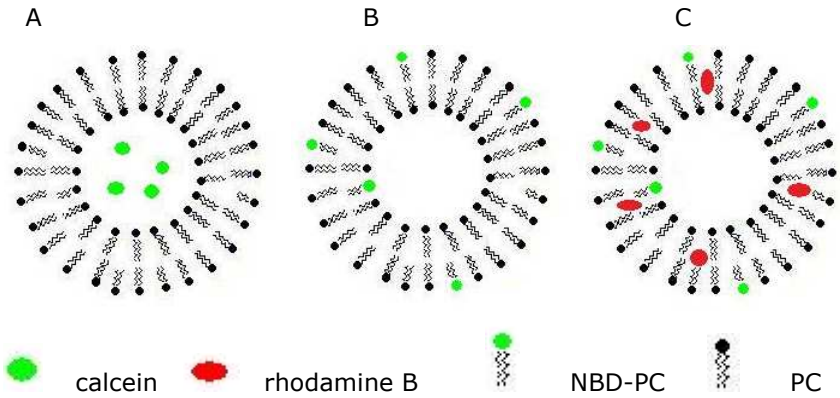


Fig. 6A, B and C: Schematic representation of liposomes encapsulating calcein in their inner cavity (A), liposomes encapsulating NBD-PC in their lipid bilayer (B) and liposomes encapsulating rhodamine B and NBD-PC in their lipid bilayer (C).

It must be noted that calcein and NBD-PC are fluorescent in the same range of wavelengths, so they could not be encapsulated in the same formulation but two formulations were needed (Fig 6A and B). Skin penetration of fluorescently labelled deformable liposomes was also realized. Deformable liposomes showed the same characteristics as classical liposomes with no visible difference. Thus, these results are not shown.

3.3.1 Skin autofluorescence

TOTO-3 iodide dye treated skins show an autofluorescence of the *stratum corneum* when excited with the 488 nm laser line (Fig. 7, part 1 in green colour) and a weak autofluorescence when excited with the 568 nm laser line (Fig. 7, part 2 in red colour). All measurements are made in

sequential mode, so the autofluorescence observed is due to the skin and not to an overlapping of the TOTO-3 iodide staining (Fig. 7, part 3 in blue colour).

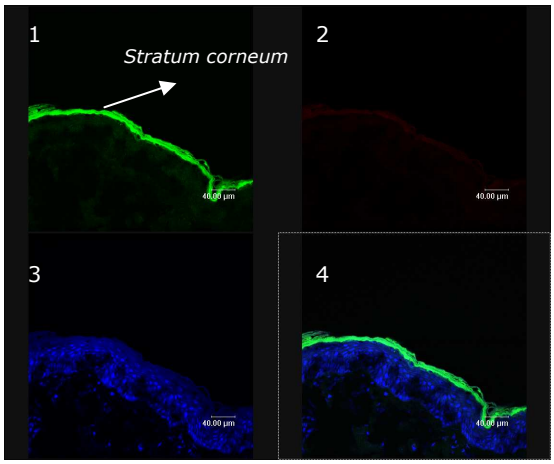


Fig. 7: CLSM images of skin autofluorescence (skin treated with TOTO-3 alone) divided into four parts with 1: green autofluorescence, 2: red autofluorescence, 3: fluorescence of cell nuclei and 4: overlay of 1, 2 and 3. Scale bar represents 40 µm.

3.3.2 Experiments with calcein

The penetration of an aqueous solution of calcein in Hepes buffer was evaluated and the results are shown in Fig. 8A and B. We can observe that calcein does not penetrate the epidermis. In addition, there is no accumulation in the hair follicles (Fig. 8B).

A B

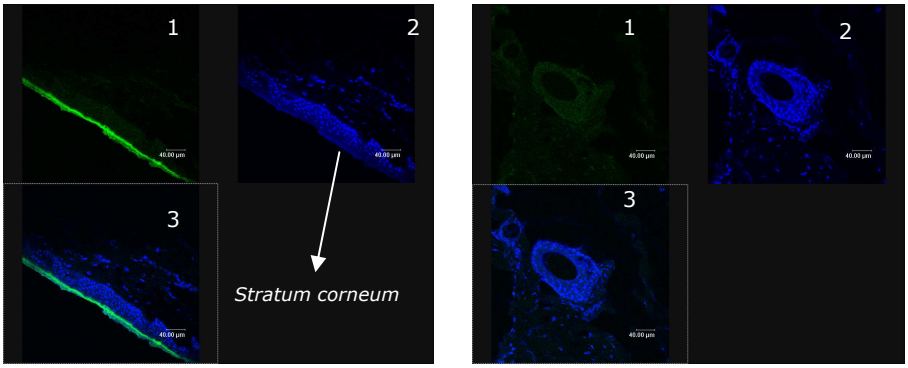
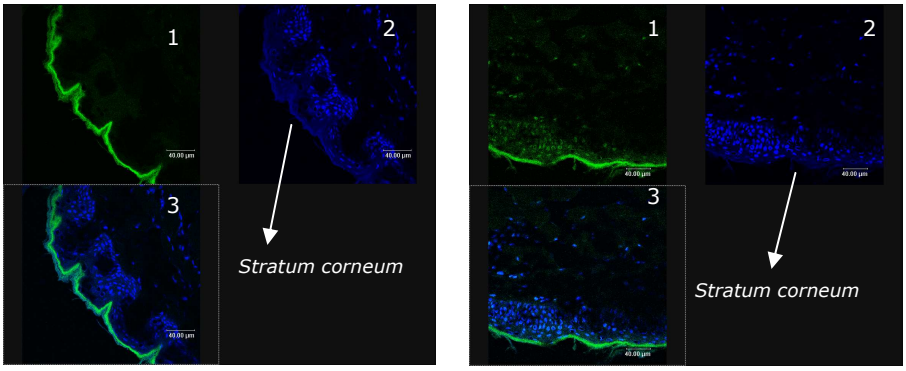


Fig. 8A and B: CLSM images of the penetration of a solution of calcein in Hepes buffer, each image divided into three parts with 1: fluorescence of calcein, 2: fluorescence of cell nuclei and 3: overlay of 1 and 2. Scale bar represents 40 µm.

Fig. 9A, B, C and D shows the first experiments with calcein and NBD-PC.

A B



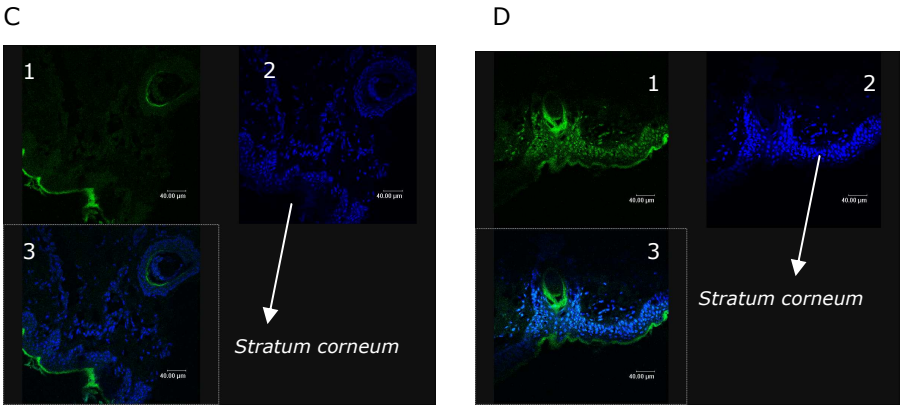


Fig. 9 A, B C and D: CLSM images, each image divided into three parts with 1: fluorescence of calcein or NBD-PC, 2: fluorescence of cell nuclei and 3: overlay of 1 and 2. A and C: classical liposomes encapsulating calcein; B and D: classical liposomes encapsulating NBD-PC. Scale bar represents 40 µm.

Fig. 9A shows the penetration of classical liposomes encapsulating calcein. Fig. 9B shows the penetration of classical liposomes containing NBD-PC as a marker of the lipid constituting the membrane. Each confocal image is divided into three parts in which 1 corresponds to the fluorescence of calcein (Fig. 9A) or NBD-PC (Fig. 9B), 2 corresponds to the fluorescence of cell nuclei and 3 is the overlay of image 1 and image 2. Fig. 9A shows that only a very low amount of the components of the liposome inner cavity (calcein) penetrates the epidermis. On the contrary, Fig. 9B shows that NBD-PC penetrates the epidermis and dermis deeply, which means that membrane components may penetrate more deeply. We can observe that calcein and NBD-PC penetrate the hair follicles of pig ear skin (Fig. 9C and D, respectively). These results confirm a penetration mechanism of liposomes already described in the literature that is a penetration of the hair follicles [29, 30]. From these confocal images, we can conclude that calcein in solution or in the inner cavity of liposomes does not penetrate the epidermis while NBD-PC penetrates the epidermis more deeply.

3.3.3 Experiments with rhodamine B

Fig. 10 shows the penetration of an aqueous solution of rhodamine B and NBD-PC. We can observe that rhodamine B accumulates in the *stratum corneum* and that a small amount penetrates the epidermis (Fig. 10A). Unlike the calcein solution, rhodamine B in the Hepes solution does penetrate the hair follicles (Fig. 10B).

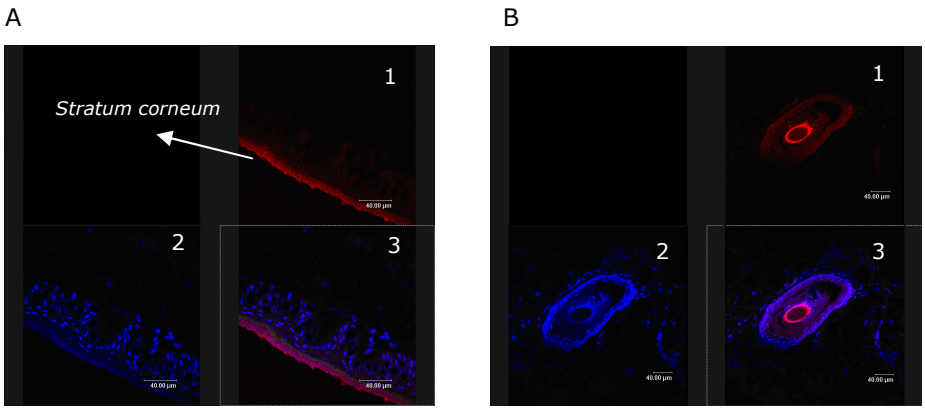


Fig. 10: CLSM images of the penetration of a solution of rhodamine B in Hepes buffer. The confocal image is divided into three parts with 1: fluorescence of rhodamine B, 2: fluorescence of cell nuclei and 3: overlay of 1 and 2. Scale bar represents 40 µm.

Fig. 11 shows the second experiments with liposomes encapsulating rhodamine B. Confocal images are divided into four parts in which 1 corresponds to the fluorescence of NBD-PC, 2 corresponds to the fluorescence of rhodamine B, 3 corresponds to the fluorescence of cell nuclei and 4 is the overlay of image 1, 2 and 3.

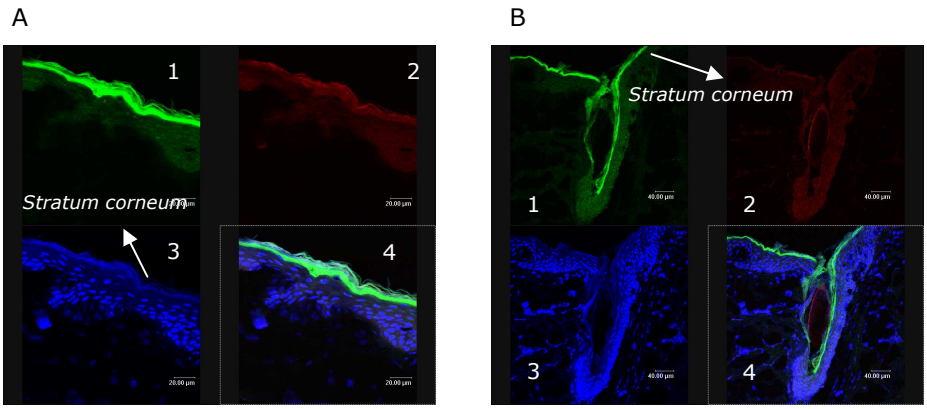


Fig. 11: CLSM images of the penetration of classical liposomes encapsulating rhodamine B and NBD-PC. The confocal image is divided into four parts with 1: fluorescence of NBD-PC, 2: fluorescence of rhodamine B, 3: fluorescence of cell nuclei and 4: overlay of 1, 2 and 3. Scale bar represents 20 µm (A) or 40 µm (B).

Rhodamine B penetrates the epidermis and the dermis deeply as well as NBD-PC and follows the penetration of NBD-PC (Fig 11A). Compared to the penetration of the rhodamine B aqueous solution, the encapsulation of rhodamine B int the liposome bilayer seems to enhance its penetration. Rhodamine B encapsulated in liposomes also penetrates the hair follicles (Fig. 11B).

The confocal microscope observations are in agreement with the analytical results. Calcein encapsulated in liposomes, which mimics the encapsulation of the betamethasone-cyclodextrin complexes, does not penetrate the epidermis, while rhodamine B encapsulated in liposomes, which mimics the encapsulation of betamethasone alone in the lipid bilayer, penetrates the epidermis.

The results also suggest that liposomes would not remain intact when penetrating the skin. Calcein and the aqueous inner cavity content are released and remain in the upper layers of the skin, in the *stratum*

corneum. When betamethasone is encapsulated as a cyclodextrin complex, betamethasone will remain in the upper layers of the skin. These results are in agreement with those of Bahia *et al*. [9]. They studied the penetration of deformable liposomes encapsulating calcein through full thickness hairless mouse skin. They concluded that the high membrane permeability to calcein of deformable vesicles and the non-encapsulated state of calcein after *in vitro* skin permeation are contradictory to a passage of calcein through the *stratum corneum* under the encapsulated form.

However, rhodamine B encapsulated in liposomes and NBD-PC penetrate the epidermis and the dermis deeply. Thus, when betamethasone is encapsulated in lipid bilayers, a deeper penetration may be obtained. Rhodamine B penetrates the epidermis better when encapsulated in liposomes than in the Hepes solution. Thus, PC could act as a penetration enhancer.

An accumulation of fluorescent dyes was observed in the hair follicles, confirming penetration of these cutaneous appendages. However, we could not confirm a diffusion of the fluorescent dye from these hair follicles.

4. CONCLUSION

This study shows that classical and deformable liposomes do not remain intact when penetrating the deepest layers of the skin and that phosphatidylcholine acts as a penetration enhancer. Betamethasone is released from the vesicles after which free drug molecules can diffuse through the *stratum corneum* and partition into the viable skin tissue. Within the framework of our study, deformable liposomes were not able to enhance the penetration of betamethasone compared to classical liposomes. The use of drug-cyclodextrin inclusion complexes enhanced the stability of the formulation but did not improve the penetration of betamethasone.

5. ACKNOWLEDGEMENTS

We are very thankful to the Giga Cell Imaging and Flow Cytometry Platform for their technical help in the confocal study. A. Gillet is a PhD student supported by the FNRS, Brussels, Belgium.

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III. Chapitre 3

LIPOSOME SURFACE CHARGE INFLUENCE ON SKIN PENETRATION BEHAVIOUR

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Ce chapitre a été soumis dans International Journal of Pharmaceutics

III.1 Résumé

Etude de l'influence de la charge des liposomes sur la pénétration cutanée

III.1.1 Introduction

Il a été décrit dans la littérature que la charge de surface des systèmes vésiculaires pouvait influencer la pénétration cutanée. La peau agirait comme une membrane chargée négativement puisque la matrice lipidique du *stratum corneum* contient une grande proportion de lipides portant une charge négative. Les vésicules chargées négativement donneraient des flux supérieurs à ceux des vésicules chargées positivement, qui en contre partie amélioreraient l'accumulation du principe actif dans les couches superficielles de la peau. Cependant, les résultats publiés sont contradictoires.

Dans ce travail, nous avons voulu étudier l'effet de l'ajout d'une charge au niveau de la bicouche des liposomes sur leur efficacité de pénétration cutanée. Le but est d'augmenter la pénétration de deux molécules modèles, la bétaméthasone et le dipropionate de bétaméthasone. L'ester de bétaméthasone a été comparé à la bétaméthasone base afin d'évaluer l'influence des propriétés de la molécule encapsulée sur la pénétration cutanée. La pénétration de ces vésicules est étudiée *ex vivo* à travers des peaux d'oreilles de cochons au moyen des cellules de Franz. La méthode du stripping est utilisée pour mesurer la pénétration du corticostéroïde dans le *stratum corneum*. Les méthodes analytiques de dosage du dipropionate de bétaméthasone sont mises au point et validées (voir annexe 1). La pénétration de liposomes rendus fluorescents est visualisée grâce à la microscopie confocale.

III.1.2 Résumé des résultats

Les liposomes neutres sont constitués de PC. Les liposomes chargés négativement contiennent en plus soit du DMPA soit du dicétylphosphate (DCP) et les liposomes chargés positivement contiennent en plus de la stéarylamine (SA). Ces vésicules ont été caractérisées par leur diamètre, leur morphologie, leur pourcentage d'encapsulation, leur stabilité en termes de sédimentation et de variation du diamètre au cours du temps et leur potentiel Zeta. La pénétration cutanée de ces systèmes a été comparée à la pénétration de dispersions non liposomales. Les résultats de pénétration cutanée sont repris dans la Figure 3.1.

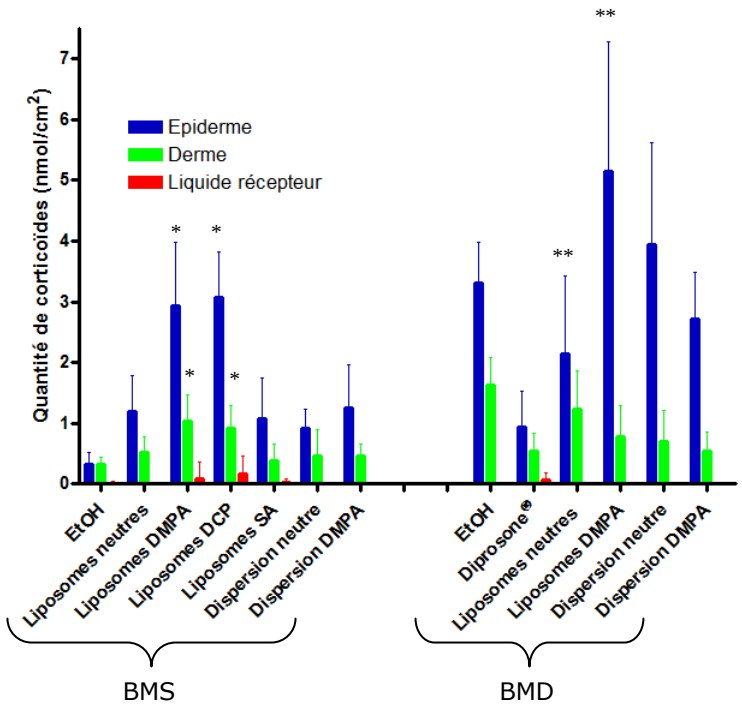


Figure 3.1 : Quantité de bétaméthasone (BMS, partie gauche) et du dipropionate de bétaméthasone (BMD, partie droite) au niveau de l'épiderme, du derme et du liquide récepteur des cellules de Franz en fonction des différentes formulations (nmol/cm^2) (* $p < 0,05$ par rapport aux autres BMS formulations et ** $p < 0,05$ par rapport à la lotion Diprosone®) (n = 9).

L'utilisation des liposomes chargés négativement (que ce soit au moyen de DMPA ou de DCP) permet d'augmenter d'un facteur supérieur à 9 la quantité de bétaméthasone au niveau de l'épiderme par rapport à la solution éthanolique, d'un facteur 2,5 par rapport aux liposomes neutres et d'un facteur 2,7 par rapport aux liposomes positifs ($p < 0,05$).
Dans le cas du dipropionate de bétaméthasone, l'utilisation des liposomes chargés négativement permet d'augmenter d'un facteur 1,6 la quantité de l'ester au niveau de l'épiderme par rapport à la solution éthanolique, d'un facteur 2,4 par rapport aux liposomes neutres et d'un facteur 5,5 par rapport à la spécialité Diprosone® lotion (solution hydro-alcoolique à 0,064% de bétaméthasone dipropionate) ($p < 0,05$).
La formulation sous forme de vésicules semble importante dans le cas des liposomes chargés négativement puisqu'une meilleure pénétration de la bétaméthasone est observée pour les liposomes par rapport à la dispersion non liposomale.
La méthode du stripping a permis de déterminer la quantité de corticoïdes ayant pénétré au niveau du *stratum corneum*. Celle-ci diminue lorsque le nombre de strips appliqués augmente. Une bonne corrélation a pu être montrée entre la quantité de bétaméthasone prélevée au niveau du dernier strip et la quantité de bétaméthasone qui a pénétré plus profondément dans l'épiderme et le derme.
La microscopie confocale semble confirmer les résultats de dosage.

III.1.3 Conclusion

Cette étude a montré le potentiel des liposomes chargés négativement, contenant soit du DMPA soit du DCP, à augmenter la pénétration cutanée de la bétaméthasone et du dipropionate de bétaméthasone. La microscopie confocale confirme ces observations.

III.2 Article

Abstract

Vesicular systems have shown their ability to increase dermal and transdermal drug delivery. Their mechanism of drug transport into and through the skin has been investigated but remains a much debated question. Several researchers have outlined that drug penetration can be influenced by modifying the surface charge of liposomes. In the present work we study the influence of particle surface charge on skin penetration. The final purpose is the development of a carrier system which is able to enhance the skin delivery of two model drugs, betamethasone and betamethasone dipropionate. Liposomes were characterised by their size, morphology, zeta potential, encapsulation efficiency and stability. *Ex-vivo* diffusion studies using Franz diffusion cells were performed. Confocal microscopy was used to visualise the penetration of fluorescently labelled liposomes into the skin. This study showed the potential of negatively charged liposomes to enhance the skin penetration of betamethasone and betamethasone dipropionate.

Keywords

Liposomes, charge, skin penetration, betamethasone, Franz cells.

1. INTRODUCTION

Transdermal drug delivery has many potential advantages over other routes of administration. It allows the avoidance of gastrointestinal tract problems and hepatic first-pass effects, and improvement in patient compliance [1]. However, a major obstacle to cutaneous drug delivery is the permeation characteristics of the *stratum corneum*, which limits drug transport, making this route of administration frequently insufficient for medical use. During the past few decades, there has been wide interest in exploring new techniques for increasing drug absorption through the skin. These include physical permeation enhancement techniques like iontophoresis by electrically driving molecules into and through the skin [2-4], electroporation by application of high-voltage pulses to the skin [5, 6] and sonophoresis by application of ultrasound [7]. Passive penetration enhancement techniques include, for example, use of supersaturated solutions [8, 9], penetration enhancers [10, 11] or microemulsions [12]. A combination of these strategies is also studied [3, 13].

Vesicular systems provide an alternative to improve drug delivery into and through the skin. Classical and more recently deformable liposomes have shown their ability to increase dermal and transdermal drug delivery.

Several researchers have outlined that drug penetration can be influenced by modifying the surface charge of liposomes. The lipid layer in the *stratum corneum* contains a high ratio of negatively charged lipids and it is well known that the skin may act as a negatively charged membrane [14, 15]. It has been reported that the presence of charges at the vesicle surface may affect the transcutaneous diffusion of drugs. Negatively charged vesicles generally give a higher flux than positively charge counterparts, which in turn can improve drug accumulation in the superficial skin strata [14]. However, results in the literature are contradictory.

The most efficient composition tested by Carrer *et al*. [16] contained the highest proportion of charged edge activators and the authors suggested

that the presence of a negative charge in the membrane may allow for a better efficiency of penetration. Manosroi *et al*. [17] showed that the transdermal absorption of amphotericine B was higher when entrapped in charged liposomes than in non-charged ones and that positive liposomes produced a higher absorption through the *stratum corneum* than the negatively charged ones. This result was explained by the fact that the skin surface bears a net negative charge. However, negative liposomes exhibited higher absorption through the viable epidermis and dermis than the positively charged liposomes. Ogiso *et al*. [18] showed that the percutaneous absorption of betahistine from a gel formulation containing negatively charged liposomes was much higher than that in the formulation with positively charged liposomes. Histological studies confirmed their observation. In contrast, Katahira *et al*. [19] found that negative dicetylphosphate liposomes provided better rhodamine B retention in the skin with lower skin permeability compared with positive and neutral multilamellar liposomes. Sinico at al. [14] also showed that negatively charged vesicles provided higher skin accumulation values of entrapped tretinoin with lower skin permeation in comparison with positively charged vesicles. Hasanovic *et al*. [20] studied the influence of adding cationic polymers (chitosan or Eudragit EPO) on the stability and skin penetration of DPPC liposomes encapsulating aciclovir 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. This permeation increase was explained as a tendency of positively charged liposomes to interact stronger with the skin surface or as an interaction of the polymers with skin lipids, the polymers going deeper and disrupting the tight junctions in lower epidermis layers.

In a previous paper we studied the influence of betamethasone encapsulation in liposomes. The drug was encapsulated either alone into the lipid bilayer or in the aqueous compartment of liposomes by the help of betamethasone-cyclodextrin complexes. We showed that the encapsulation into the lipid bilayer significantly enhanced the accumulation of betamethasone in the epidermis of pig ear skin (results to

be published). In the present work we would like to study the influence of the addition of a charge on the skin penetration. The aim of this study is to develop a carrier system able to enhance the skin delivery of a model drug, betamethasone. This corticoid is too hydrophilic for a good skin penetration behaviour by itself. We studied the influence of adding a charge into the lipid bilayers on the penetration efficiency of encapsulated betamethasone. The ester of betamethasone, betamethasone dipropionate, was also used in order to evaluate the properties of the drug encapsulated on the skin penetration behaviour. The penetration enhancing ability of these charged vesicles was tested *ex vivo* using pig ear skin as the model membrane. Confocal microscopy was made to visualize the penetration of fluorescently labelled liposomes.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Betamethasone (E.P.) was purchased from Medeva (Braine L'Alleud, Belgium), betamethasone dipropionate (E.P.) from Abc Chemicals (Wauthier-Braine, Belgium), Soybean phosphatidylcholine (PC) from Lipoïd (Ludwigshafen, Germany), 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt) (DMPA) and 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecan-sn-glycero-3-phosphocholine (NBD-PC) from Avanti Polar lipids (Alabaster, AL, USA), dicetylphosphate (DCP) from Santa Cruz Biotechnology (Santa Cruz, USA), hydroxypropylated-γ-cyclodextrin (HPγCD, D.S. 0.7, 3.41% H_2O) was obtained from Wacker-Chemie GmbH (Munich, Germany), stearylamine from Sigma Aldrich (Bornem, Belgium), and Rhodamine B and acetonitrile from Merck (Darmstadt, Germany). Pure water was generated from the Milli-Q system (Millipore, Bredford, MA, USA). All experiments were performed using a 0.22 µm-filtered 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) buffer (Sigma Aldrich), containing 145 mM NaCl and

adjusted to pH 7.4 with 0.1 M NaOH solution. All other reagents and solvents were of analytical grade.

Pig ears came from two sources. For the first experiments with betamethasone, pig ears (Race: Piétrain, age: 2-months-old) were obtained from the Faculty of Veterinary Medicine at the University of Liège, Belgium. Because of a lack of supply, experiments with betamethasone dipropionate were made with pig ears (Race: Landras, age: 6-month-old) obtained from the local slaughterhouse prior to scald.

2.2 Liposome preparation

Non charged liposomes were made of PC. Negatively charged liposomes contained in addition DMPA (12.5 % m/m) or DCP (5.9 % m/m), while positively charged liposomes contained SA (3.8 % m/m). Liposomes were prepared by hydration of lipid films. In practice, the required amounts of lipids and drug (6 mg of betamethasone or 7.7 mg of betamethasone dipropionate) were dissolved in a 2:1 chloroform/ethanol mixture in a round-bottomed flask. The solution was then dried under vacuum using a rotary evaporator. The resulting lipid film was hydrated using 3 mL of Hepes buffer. Suspensions were then extruded three times through Nucleopore® polycarbonate membranes of successive 0.4 and 0.2 µm pore diameters (Whatman, Maidstone, UK). Free drug was separated from liposome-encapsulated drug by three successive ultracentrifugations at 165,052 × *g* (35,000 rpm). The first cycle lasted 3 h followed by two cycles of 1 h 30 at 4 °C. The supernatant was removed and the pellet was re-suspended in Hepes buffer. Betamethasone or betamethasone dipropionate and PC were assayed in purified liposomes.

2.3 Non liposomal dispersion preparation

Dispersions were made by mixing the required amount of PC with or without DMPA (12.5%) in Hepes buffer containing betamethasone or betamethasone dipropionate without further preparation.

2.4 Liposome characterisation

2.4.1 Measurement of liposome diameter and Zeta potential

Liposome suspensions were sized by photon correlation spectroscopy (PCS) (HPPS, Malvern Instruments Ltd, Worcestershire, UK). Measurements were made at 25 °C with a fixed angle of 90° and results were expressed as the average liposomal hydrodynamic diameter (nm). The surface charge of the particles was determined using a Zetasizer® 2000 (Malvern Instruments Ldt, Worcestershire, UK).

2.4.2 Freeze-fracture electron microscopy

Freeze-fracture replicas of liposome suspensions were examined by transmission electron microscopy (TEM). A drop of liposome suspension, with 20% glycerol added as freeze-protectant, was deposited in a small gold cup and rapidly frozen in liquid nitrogen. Fracturing, freeze etching and shadowing with Pt-C were performed at -100 °C in a shadowing equipment (Balzers® BAF-400) fitted with a freeze-fracture and etching unit. The replicas were examined in a JEOL (JEM-100SX) transmission electron microscope, operating at 80kV accelerating voltage.

2.4.3 Encapsulation efficiency

Encapsulation efficiency was calculated in two ways. The first encapsulation efficiency ($EE_{D/L}$) corresponds to the drug to lipid mass ratio or the amount of drug in purified liposomes (C_D) compared to the total lipid concentration (C_L) in purified liposomes:

$$EE_{D/I}\ (\%) = (C_D / C_L) \times 100$$

The second encapsulation efficiency ($EE_{D/Dt}$) was the yield obtained. This corresponds to the concentration of drug encapsulated in liposomes (C_D) compared to the total drug concentration first introduced (C_{Dt}). This $EE_{D/Dt}$ was corrected to the concentration of lipids in order to take into account the loss of liposomes during their preparation:

$$EE_{B/Bt}(\%) = (C_D / C_L) / (C_{Dt} / C_{Lt}) \times 100$$

C_L is the lipid concentration in purified liposomes and C_{Lt} is the lipid concentration first introduced.

2.4.3.1 Drug chromatographic determination

The HPLC used was a LaChrom Merck-Hitachi (Darmstadt, Germany) consisting of an L-7100 pump, an L-7200 autosampler, an L-7400 UV detector, an L-7350 column oven and a D-7000 interface. The system was controlled by "D-7000 HPLC System Manager" software. The analytical column was a LiChroCART (250 x 4 mm, i.d.) packed with Superspher 100 RP-18 (particle size: 5µm) and preceded by a guard column LiChroCART (4 x 4 mm, i.d.) packed with LiChrospher 100 RP-18 (particle size: 5 µm). Isocratic separation was performed at a temperature of 35 °C using a mobile phase consisting of a mixture of acetonitrile and water (50:50, V/V) for betamethasone and (55/45, V/V) for betamethasone dipropionate. The flow rate was settled at 0.8 mL/min for betamethasone and at 1 mL/min for the ester, and the sample injection volume was 20 µL. Betamethasone was monitored at 240 nm while betamethasone dipropionate was monitored at 254 nm.

2.4.3.2 Quantification of lipids

Total lipid concentrations were calculated by measuring PC through an enzymatic method (LabAssay™ Phospholipid, Wako, Osaka, Japan). The principle of this enzymatic assay consists in the cleavage of PC in choline

by phospholipase D followed by the oxidation of choline into betaine with the simultaneous production of hydrogen peroxide. The hydrogen peroxide, which is produced quantitatively, couples 4-Aminoantipyrine and *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS). Peroxidation results in the generation of a coloured compound quantified by spectrophotometry at 600 nm (spectrophotometer Perkin-Elmer Lambda 11).

2.4.4 Liposome stability

The stability of liposomes was evaluated by measuring the particle mean diameter and polydispersity indexes by photon correlation spectroscopy after one month of storage at 4°C. The stability of the different suspensions was also evaluated visually.

2.5 *Ex vivo* diffusion study

2.5.1 Skin preparation

Full-thickness skin was removed from the dorsal side of freshly excised pig ear, stored at -20 °C and used within 6 months. On the day of the experiment, punches were cut out and hairs cut with scissors.

2.5.2 Permeation experiments

Diffusion studies were carried out using Franz type glass diffusion cells. These cells consist of two compartments with the skin clamped between the donor and receiver chambers, dermal side down. The cell body was filled with 7.5 mL of a receptor phase consisting of Hepes buffer solution pH 7.4 containing 0.01% NaN_3 as the preservative. Test conditions were chosen in order to respect sink conditions in the receiver compartment. The solubility of betamethasone in Hepes buffer was evaluated at 65 µg/mL. This corresponds to approximately 10 times the maximum

concentration of betamethasone that can be found in the receiver compartment. To assure sink conditions in the case of betamethasone ester, HPγCD 5mM was added in the receptor medium giving a solubility of 54 µg/mL. The receptor fluid was constantly stirred with a small magnetic bar and thermostated at 37 °C throughout the experiments. 350 µL of liposome suspension adjusted to 150 µg/mL betamethasone or 193 µg/mL betamethasone dipropionate concentration, were placed in the donor chamber onto the *stratum corneum* of the skin, in non occlusive conditions. The diffusion area was 1.767 cm^2. At the end of the experiment (24 h), the receptor phases were removed and the diffusion cells were dismantled. The skin surface was washed with 3 mL Hepes buffer on each side to remove the residual donor sample and was thawed. The surface of the skin exposed to the donor compartment was punched out. The *stratum corneum* was removed by the stripping method using 15 strips of Corneofix® tape (CKelectronic, Germany) successively. Only the first, fifth, tenth and fifteenth strips were kept and analysed for drug content. The piece of skin was then separated into the epidermis and dermis by pressing the skin surface against a hot plate (65 °C) for 90 seconds and peeling off the epidermis. The four strips, the epidermis and dermis cut into small pieces, were each soaked separately in a flask with 4 mL of Hepes (containing 5mM HPγCD in the case of betamethasone dipropionate) for 24 h. Samples were then shaken for 30 minutes in an ultrasonic bath, in order to extract the entire drug accumulated in the skin pieces.

2.5.3 Drug determination

2.5.3.1 Solid phase extraction (SPE) prior to chromatographic analysis

SPE was needed to clean up the samples before HPLC injection. The extraction procedure was carried out on Isolute® C18, 50 mg, 1 mL (Biotage, Uppsala, Sweden) disposable extractive cartridges (DEC). After conditioning with 1 mL acetonitrile and 1 mL Hepes buffer, 1 mL sample

was loaded onto the DEC and then washed with 1 mL water. Betamethasone or betamethasone dipropionate was eluted with 500 µL acetonitrile and 500 µL water was added before HPLC injection. The chromatographic conditions were described in 2.4.2.1.

2.5.3.2 SPE-HPLC-UV method validation

For the validation, two types of standards were prepared. The first were calibration standards, prepared in the mobile phase at seven concentration levels ranging from 20 to 10,000 ng/mL. The others were validation standards, prepared at seven concentration levels ranging from 20 to 10,000 ng/mL. Validation standards were made in skin extract in Hepes buffer so as to mimic real samples obtained after permeation experiments. The validation was performed in 3 series. For each series, all the calibration standards were analysed in duplicate, while each validation standard was analysed in triplicate.

The validation was based on an accuracy profile approach [21]. For betamethasone determination in pig ear skin, the acceptance limits were set at 30% from 24.2 to 60 ng/mL and 15% from 60 to 10,000 ng/mL, respectively and the risk level was fixed at 10%. For betamethasone determination from the tape stripping method, the acceptance limits were set at 10% from 20.02 to 10,000 ng/mL and the risk level was fixed at 5% [22, 23]. The most appropriate calibration model was a weighted ($1/x^2$) linear regression. For betamethasone dipropionate determination in pig ear skin, the acceptance limits were set at 30% from 40.92 to 100 ng/mL and 15% from 100 to 10,000 ng/mL, respectively and the risk level was fixed at 10%. For betamethasone dipropionate determination from the tape stripping method, the acceptance limits were set at 30% from 20.23 to 100 ng/mL and 10% from 100 to 10,115 ng/mL, respectively and the risk level was fixed at 10%. The most appropriate calibration model was a linear regression. The e-noval software v3.0 (Arlenda, Liège, Belgium) was used to compute the validation results as well as to obtain the accuracy profiles.

2.6 Confocal laser scanning microscopy study

2.6.1 Liposome preparation

Liposomes were prepared and characterised as described in 2.2 and 2.4 with some modifications. Liposomes were made fluorescent in two ways. First, rhodamine B (log *P* = 1.95) (0.01 % m/m) was incorporated into the lipid bilayer in order to mimic the encapsulation of betamethasone (log *P* = 1.94) [24]. Secondly, the lipid bilayer was made fluorescent by incorporation of NBD-PC (1.33% m/m). After extrusion, non-encapsulated rhodamine B and NBD-PC were separated from liposome-encapsulated rhodamine B and NBD-PC by successive ultracentrifugations at 35,000 rpm.

2.6.2 Confocal laser scanning microscopy

Diffusion studies were carried out as described in section 2.5.2. After 24 h, the remaining liposome formulation was washed and the diffusion area punched out. The diffusion area was then incorporated into OCT compound (Tissue-Tek®, Sakura, The Netherlands) and frozen at -20 °C. The frozen skin was then sectioned with a cryostat into 7 µm slices. These tissues were counterstained with TOTO-3 iodide dye (Molecular Probes, Leiden, The Netherlands). The penetration of the fluorescent probes was assessed by confocal laser scanning microscopy (Leica TCS SP2, Heidelberg GmBH, Germany), using sequential acquisition. NBD-PC was excited with the 488 nm laser line from an argon laser and the fluorescent emission signals are represented by a green colour. Rhodamine B was excited with the 568 nm line from a Kr laser and the fluorescent emission signals are represented by a red colour. TOTO-3-stained cell nuclei were excited with the 633 nm line from a He/Ne laser and are shown by a blue colour. Images were acquired using a 40X objective lens immersed in oil.

2.7 Statistical analysis

The significance of the differences between formulations was tested using the Student t-test (Graph Pad Prism, Version 4). The differences are considered statistically significant when $p<0.05$. Correlation was evaluated by the Pearson correlation test (Graph Pad Prism, Version 4). Correlation significance is considered when $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Liposome characterisation

Classical, non charged liposomes contained PC as phospholipids. In addition, positive liposomes contained stearylamine (SA), while negatively charged liposomes contained DMPA or DCP. Two model drugs were encapsulated, betamethasone and betamethasone dipropionate in order to evaluate the influence of the properties of the drug encapsulated on the efficiency of skin penetration. Non liposomal dispersions of PC and betamethasone or betamethasone dipropionate in Hepes with or without DMPA were also studied and compared with the liposome formulations. PCS and freeze-fracture electron microscopy were performed for diameter analysis and morphology characterisation. As shown in Table 1, liposomes are characterized by mean hydrodynamic diameters between 148 nm (± 21) and 178 nm (± 12). Polydispersity indexes (not shown) were always lower than 0.2, indicating that liposomes were homogeneous in size.

Table 1: Diameter ± S.D. (nm), Zeta potential ± S.D. (mV) and encapsulation efficiencies ($EE_{B/L}$) ± S.D. (%) and ($EE_{B/Bt}$) ± S.D. (%) of liposomes containing betamethasone (BM) or betamethasone dipropionate (BMD) (n = 3).

Composition	diameter (nm)	Zeta potential (mV)	$EE_{B/L}$ (%)	$EE_{B/Bt}$(%)
PC – BM	178 ± 12	-1.6 ± 2.8	3.96 ± 0.27	97.8 ± 5.4
PC – SA – BM	141 ± 6	+13.2 ± 2.2	3.97 ± 0.03	92.2 ± 1.2
PC – DMPA – BM	153 ± 2	-26.6 ± 3.5	4.52 ± 0.08	96.9 ± 1.7
PC – DCP – BM	153 ± 3	-19.9 ± 4.5	3.94 ± 0.11	90.1 ± 2.8
PC – BMD	171 ± 3	-3.6 ± 0.9	5.11 ± 0.05	96.0 ± 1.0
PC – DMPA – BMD	155 ± 7	-27.8 ± 5.3	5.70 ± 0.22	94.7 ± 3.8

Whatever the drug encapsulated, positively and negatively charged liposomes show significantly smaller sizes than non charged classical liposomes ($p < 0.05$). This fact was also observed by Namdeo *et al*. [25] where the incorporation of DCP in niosomes reduced the mean size. They explained that the presence of a charge in the bilayer due to DCP increases its tendency to become curved, thereby reducing the size of the vesicles. Liposomes show very good size reproducibility from batch to batch.

These results are in good agreement with the TEM-imaging of the freeze fracture replica as shown in Fig. 1. Indeed, the picture of PC liposomes encapsulating betamethasone (Fig. 1A) and those of negatively charged DMPA liposomes encapsulating either betamethasone (Fig. 1B) or betamethasone dipropionate (Fig. 1C) show very similar unilamellar vesicles with a homogeneous size of about ± 200 nm.

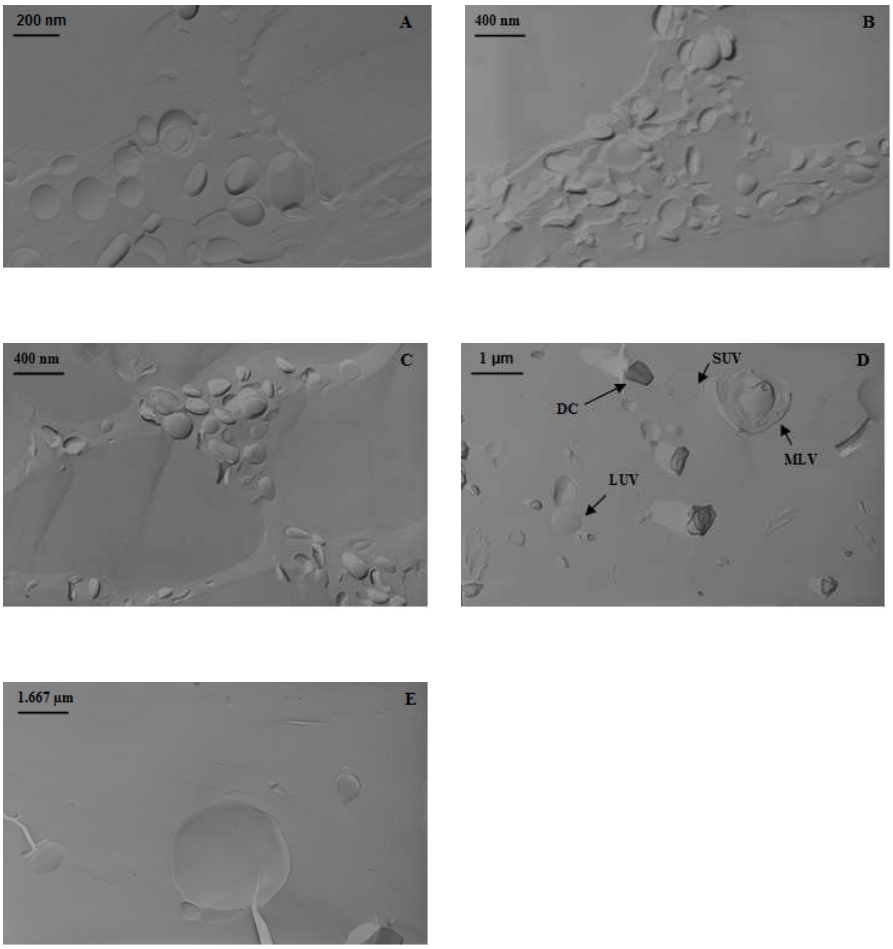


Fig.1: Transmission electron micrographs of freeze-fracture replica of liposomes and non liposomal dispersions. Classical PC liposomes encapsulating betamethasone (A), negatively charged PC-DMPA liposomes encapsulating betamethasone (B), negatively charged PC-DMPA liposomes encapsulating betamethasone dipropionate (C), non liposomal dispersion PC-betamethasone (D with SUV = small unilamellar vesicle, LUV = large unilamellar vesicle, MLV = multilamellar vesicle and DC = drug crystal), and non liposomal dispersion PC-DMPA-betamethasone (E).

In contrast, non liposomal dispersions of PC and betamethasone (Fig. 1D) or of PC, DMPA and betamethasone (Fig. 1E) in Hepes buffer appear as polymorphic vesicles in shape and size probably resulting from the self-assembling in suspension. The vesicle sizes range from a few nm up to one µm. In Fig. 1D, we observe the presence of different types of vesicles such as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and also multilamellar vesicles (MLV). The term "non liposomal dispersion" is therefore conflicting, as vesicles are formed in these dispersions. However, we maintain this term in order to easily differentiate it from the liposome formulations which consist of small unilamellar vesicles of the same diameter. In non liposomal dispersions, drug crystals (DC) are also found outside the vesicles (Fig. 1D).

Regarding Zeta potential values, PC liposomes possess a small negative charge and were therefore considered as neutral [26]. The amount of stearylamine incorporated into liposomes was selected according to Piel *et al* [27], giving positively charged vesicles, while the amount of DMPA was selected according to Yoo *et al*. [15], giving negatively charged liposomes. DCP containing vesicles were used to confirm the effect of a negative charge on skin penetration but the same molar ratio as used for DMPA could not be incorporated. The maximum amount of DCP that could be incorporated into the lipid bilayer gives thus a smaller negative charge than obtained with DMPA.

Drug encapsulation efficiencies are reported in Table 1. It must be noted that encapsulation efficiency $EE_{D/l}$ represents the drug to lipid ratio, which explains the relatively low values obtained in comparison with the $EE_{D/Dt}$ expressing the encapsulation efficiency as a function of the total drug concentration. The addition of DMPA significantly enhances the $EE_{D/l}$ from 3.96% (± 0.27) to 4.52% (± 0.08) for liposomes containing betamethasone, and from 5.11% (± 0.05) to 5.70% (± 0.22) for liposomes containing betamethasone dipropionate ($p < 0.05$). The higher efficiencies (expressed in mass) obtained for betamethasone dipropionate

in comparison with betamethasone are explained by the higher amounts used to hydrate the lipid film, in order to maintain the same molar ratio (6 mg of betamethasone corresponding to 7.7 mg of betamethasone dipropionate). Concerning the calculation of the yield, differences are less marked but allow an encapsulation over 90%.

Stability of formulations was first evaluated by visual observations. In Fig. 2, tube 1 corresponds to classical PC liposomes, tube 2 to negatively charged DMPA liposomes, tube 3 to the non liposomal dispersion of PC in Hepes and tube 4 to the non liposomal dispersion of PC and DMPA in Hepes. Pictures are taken at time 0 (A), after 2 hours (B), 6 hours (C) and 24 hours (D).

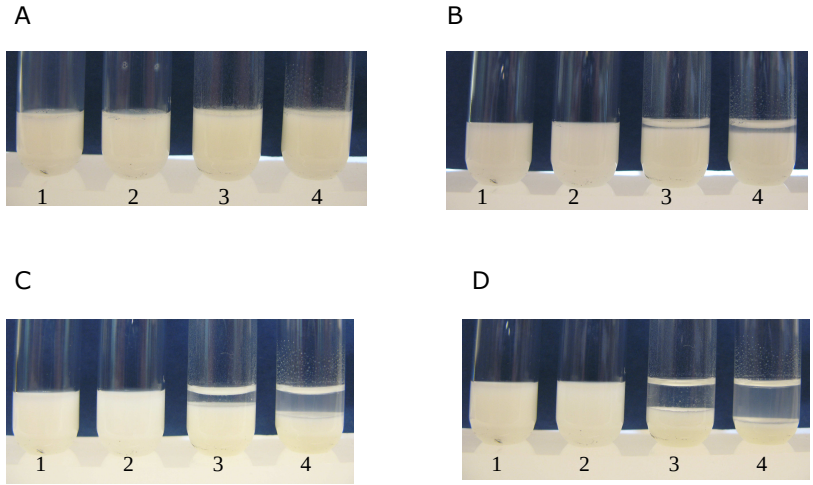


Fig. 2: Visual observations of the non liposomal dispersion sedimentation (tube 3: dispersion PC and betamethasone; tube 4: dispersion PC-DMPA and betamethasone) compared with the stable liposome suspensions (tube 1: neutral PC liposomes; tube 2: negative DMPA-PC liposomes) with time (A= T_{0h}; B=T_{2h}; C= T_{4h}; D=T_{24h}).

We can observe that neutral (1) and charged liposomes (2) appear in white suspensions without sedimentation, indicating that the suspensions are physically stable due to the presence of small and uniform distributed vesicles obtained after the extrusion process. The non liposomal dispersions (3 and 4), however, show high sedimentation levels with time. The stability of liposomes was also evaluated by measuring their diameter after one month of storage at 4°C. Results are shown in Table 2. No significant change in diameter is observed indicating the stability of the formulations. Polydispersity indexes (not shown) remain under 0.2.

Table 2: Diameter ± S.D. (nm) at the day of preparation (T_0) and after a minimum of one month of storage at 4°C for liposomes containing betamethasone (BM) or betamethasone dipropionate (BMD) (n=3).

Composition	Diameter T_0 (nm)	Diameter after one month 4°C(nm)
PC – BM	178 ± 12	172 ± 5
PC – SA – BM	141 ± 6	141 ± 5
PC – DMPA – BM	153 ± 2	155 ± 4
PC – DCP – BM	153 ± 3	151 ± 4
PC – BMD	171 ± 3	171 ± 1
PC – DMPA – BMD	155 ± 7	157 ± 5

3.2 *Ex vivo* diffusion studies

Franz type diffusion cells were used to evaluate the *ex vivo* diffusion of betamethasone or betamethasone dipropionate from liposomes in pig ear skin. Three reference samples were used: a solution of drug in absolute ethanol and the commercially available lotion Diprosone® (containing 0.05% of corticoid expressed in betamethasone).

3.2.1 Penetration into the *stratum corneum*

Fig. 3A shows the amount of betamethasone determined on strips one, five, ten and fifteen. The other strips were discarded to reduce the number of samples. We can observe that the amount of drug decreases with the number of strips.

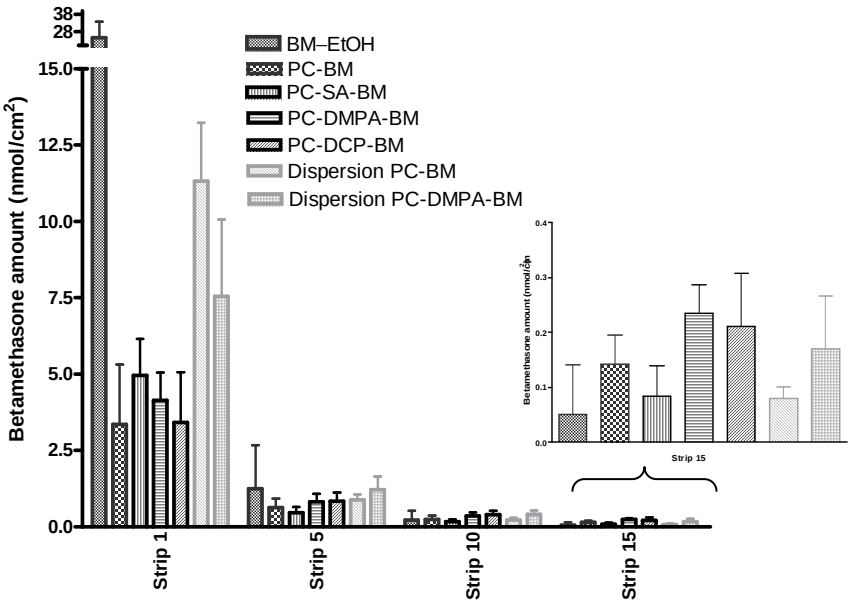


Fig. 3A: Amount of betamethasone (BM) as a function of the strip number (n = 9).

As shown in Fig. 3A, for the first strip we observe a high betamethasone content for the betamethasone ethanolic solution. The reason is the effectiveness of skin washing by Hepes, which can more easily remove betamethasone from aqueous medium than from the ethanolic solution. A higher amount of the drug is also determined on the first strip in the case of non liposomal dispersions in comparison with liposomes. It can be

explained by sedimentation of the drug on the skin surface due to the instability of the non liposomal dispersions. Fig. 3B shows the amount of betamethasone dipropionate determined on strips one, five, ten and fifteen. We also observe that the concentration of the drug decreases with the number of strips. As shown in Fig. 3B, for the first strip we observe a high betamethasone content from the betamethasone ethanolic solution and from Diprosone®.

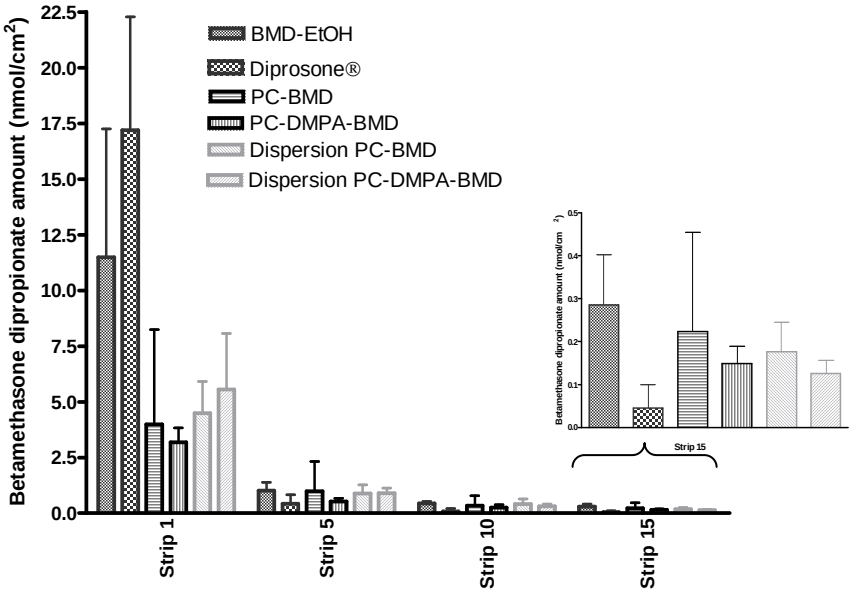


Fig. 3B: Amount of betamethasone dipropionate (BMD) as a function of the strip number (n = 9).

3.2.2 Penetration into the epidermis, dermis and receptor medium

Fig. 4 shows the amount of betamethasone (BM; left part of the graph) and betamethasone dipropionate (BMD; right part) in the epidermis, dermis and receptor medium of Franz type diffusion cells.

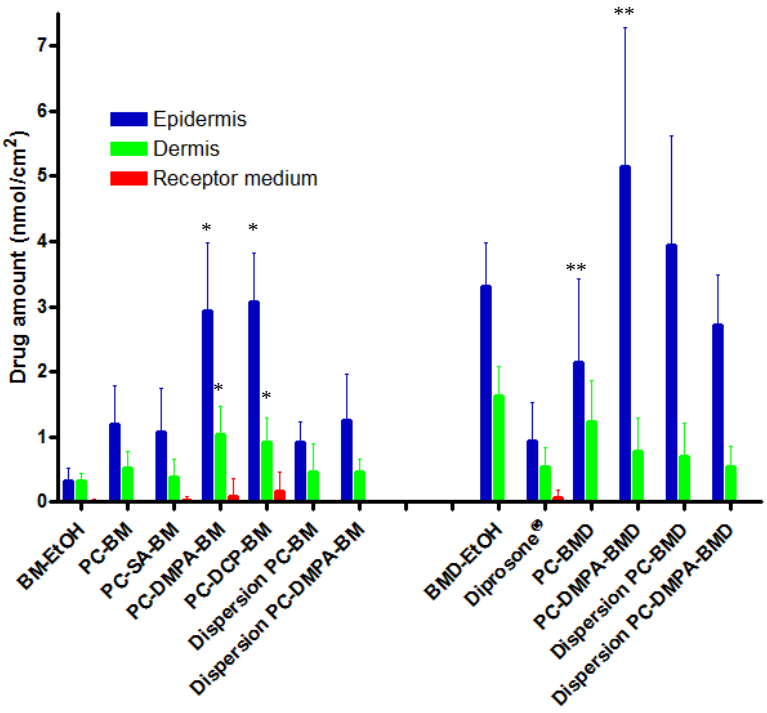


Fig. 4: Amount of betamethasone (BM, left part of the graph) or betamethasone dipropionate (BMD, right part of the graph) in the epidermis, dermis and receptor medium of Franz cells (n = 9) (* $p < 0.05$ compared with other BM formulations; ** $p < 0.05$ compared with Diprosone® lotion).

Regarding the betamethasone amount in the epidermis (left part in Fig. 4), the encapsulation in liposomes significantly enhances the penetration of betamethasone compared with the ethanolic solution ($p < 0.05$). No significant difference in penetration is observed between neutral liposomes (PC-BM), positive liposomes (PC-SA-BM), and the non liposomal dispersions (Dispersion PC-BM, Dispersion PC-DMPA-BM) ($p > 0.05$). However, negatively charged PC-DMPA-BM liposomes enhance the penetration of betamethasone 9.3 times compared with the ethanolic solution, 2.5 times compared with neutral liposomes and 2.7 times compared with positively charged vesicles. In order to understand if this increase is due to the incorporation of DMPA or to the presence of a negative charge, DMPA was replaced by DCP, another negative compound.

As shown in Fig. 4, the incorporation of DCP increases the penetration of betamethasone at the same level as DMPA ($p > 0.05$), indicating that the presence of a negative charge in the lipid bilayer of liposomes is enough to enhance the penetration of the encapsulated drug. For neutral liposomes, there is no significant difference between liposomes and the non liposomal dispersion, indicating that the vesicle formulation is not necessary ($p > 0.05$). However, in the case of negatively charged liposomes, the vesicle formulation is of high importance for the enhanced penetration. These differences between charged and uncharged liposomes and non liposomal dispersions could not be explained at this time and further investigations are needed. Regarding the betamethasone accumulation in the dermis, the penetration of betamethasone is also higher for negative liposomes compared with the other formulations ($p < 0.05$).

The right part of Fig. 4 shows the penetration of betamethasone dipropionate from the different formulations. Differences with betamethasone are obvious. Regarding the accumulation in the epidermis, betamethasone dipropionate ethanolic solution penetrates well. The more lipophilic properties of the ester of betamethasone (log $P = 4.07$) compared with betamethasone (log $P = 1.94$) could explain this difference in the penetration behaviour [24]. Neutral liposomes (PC-BMD) and negative liposomes (PC-DMPA-BMD) enhance the penetration in comparison with the commercially available lotion Diprosone® ($p < 0.05$). Negatively charged liposomes enhance the penetration of betamethasone dipropionate 1.6 times compared with the ethanolic solution, and 2.4 times compared with neutral liposomes. Negative liposomes also penetrate the epidermis better than the dispersion PC-DMPA-BMD ($p < 0.05$). However, the enhanced epidermis accumulation of the negative liposomes compared with the dispersion PC-BMD is not significant ($p > 0.05$).

Properties of the encapsulated drug seem to be of high importance for skin penetration behaviour. The entrapment in negatively charged liposomes enhances the epidermis absorption more than 9 times for

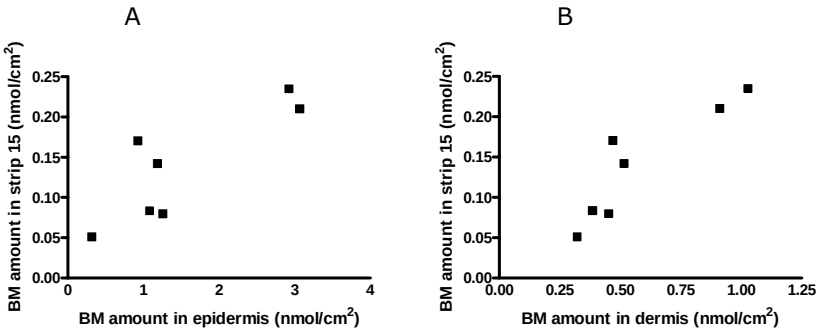
betamethasone while the entrapment of betamethasone dipropionate enhances it only 1.6 times compared with their respective ethanolic solution. The penetration of a more lipophilic drug, with a high intrinsic penetration in ethanolic solution such as betamethasone dipropionate, is less enhanceable when incorporated into liposomes. The encapsulation in liposomes is therefore more effective to improve the penetration of a drug with poor intrinsic penetration such as betamethasone.

Only very small amounts of the drug are found in the receptor medium of Franz diffusion cells in some cases. This is certainly due to the use of full thickness skin instead of dermatomed skin or heat separated epidermis. Thus, we were not able to observe any transdermal delivery of corticoids.

As a conclusion of *ex-vivo* diffusion study, we can observe that the encapsulation of betamethasone and its ester in negatively charged liposomes significantly enhances skin penetration. These results are in agreement with those of Sinico *et al*. [14] where the use of negatively charged DCP liposomes increased skin accumulation of entrapped tretinoin in comparison with positively charged SA liposomes.

3.2.3 Correlation between *stratum corneum* and viable skin penetration

Fig. 5A, B and C shows the relation between the betamethasone content in strip 15 (see enlargement in Fig. 3A) and the amount of betamethasone in the epidermis, dermis and total skin (epidermis and dermis), respectively.



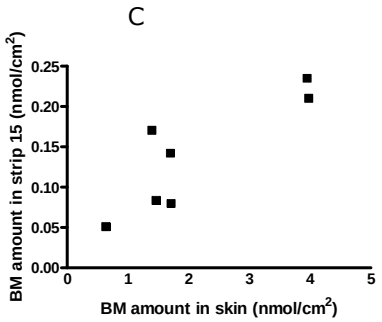


Fig. 5: Correlation between the amount of betamethasone (BM) determined in strip 15 (nmol/cm^2) and the amount of betamethasone in the epidermis (A), dermis (B) and total skin (epidermis + dermis) (C) (nmol/cm^2).

A good correlation could be shown between the betamethasone content in strip 15 and the betamethasone amount determined in the epidermis (Fig. 5A, Pearson test, $p < 0.05$, $r^2 = 0.8436$), in the dermis (Fig. 5B, Person test, $p < 0.05$, $r^2 = 0.9035$) and in the total skin (Fig. 5C, Pearson test, $p < 0.05$, $r^2 = 0.8603$). The betamethasone amount in the last strip, next to the viable epidermis is reflective of the amount in the epidermis and dermis.

Fig. 6A, B and C shows the relation between betamethasone dipropionate content in strip 15 (see enlargement in Fig. 3B) and the amount of betamethasone dipropionate in the epidermis, dermis and total skin (epidermis and dermis), respectively.

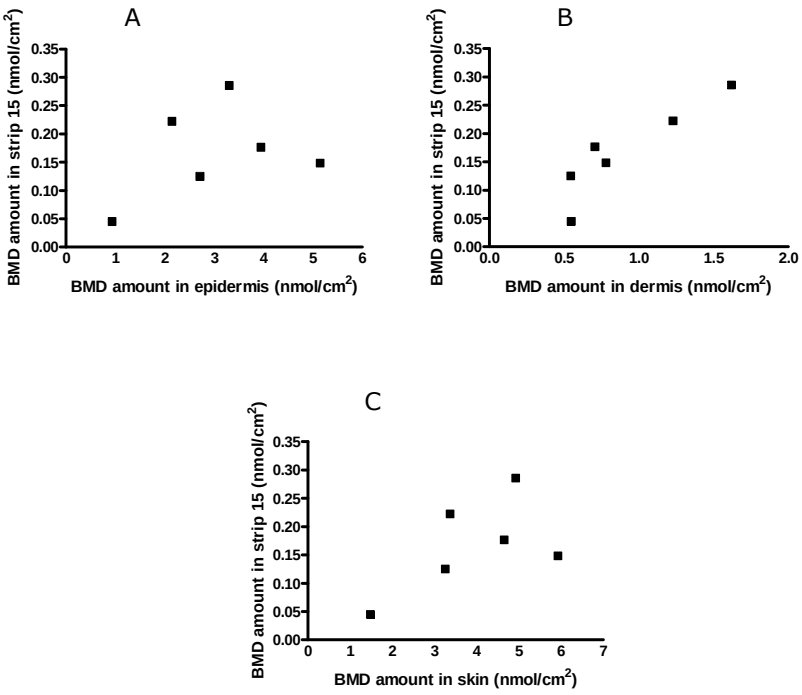


Fig. 6: Correlation between the amount of betamethasone dipropionate (BMD) determined in strip 15 (nmol/cm^2) and the amount of betamethasone dipropionate in the epidermis (A), dermis (B) and total skin (epidermis + dermis) (C) (nmol/cm^2).

Regrettably, the only significant correlation for betamethasone dipropionate is between the drug content in strip 15 and in the dermis (Fig. 6B, Pearson test, $p < 0.05$, $r^2 = 0.9086$).

3.3 Confocal microscope observations

In order to visualize the skin delivery of the different formulations tested, liposomes are made fluorescent by two ways. Rhodamine B is encapsulated in the lipid bilayers of neutral and negatively charged liposomes, this lipophilic dye being supposed to mimic the encapsulation of betamethasone (similar log *P* values). Otherwise, the lipid bilayer is

also made fluorescent by the incorporation of NBD-PC. This double labelling allows visualising the penetration of the lipid bilayer materials and the encapsulated drug simultaneously.

TOTO-3 iodide dye treated skins show an autofluorescence of the *stratum corneum* when excited with the 488 nm laser line (Fig. 7, part 1 in green colour) and a weak autofluorescence when excited with the 568 nm laser line (Fig. 7, part 2 in red colour). All measurements are made in sequential mode, so that the autofluorescence observed is due to the skin and not to an overlapping of the TOTO-3 iodide staining.

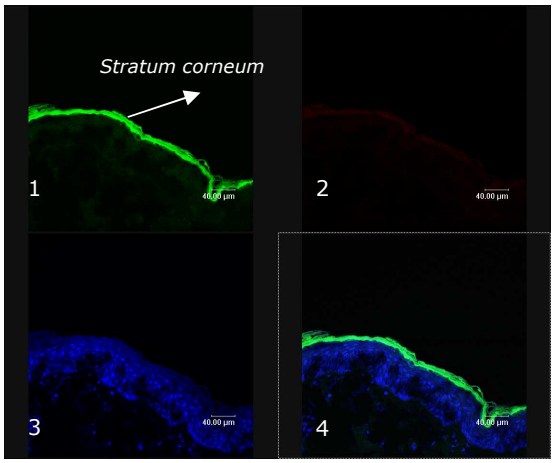


Fig. 7: CLSM images of skin autofluorescence (skin treated with TOTO-3 alone) divided into four parts with 1: green autofluorescence, 2: red autofluorescence, 3: fluorescence of cell nuclei; 4: overlay of 1, 2 and 3. Scale bar represents 40 µm.

As a control, the Fig. 8 shows the weak penetration in the epidermis of an aqueous rhodamine B solution in Hepes buffer.

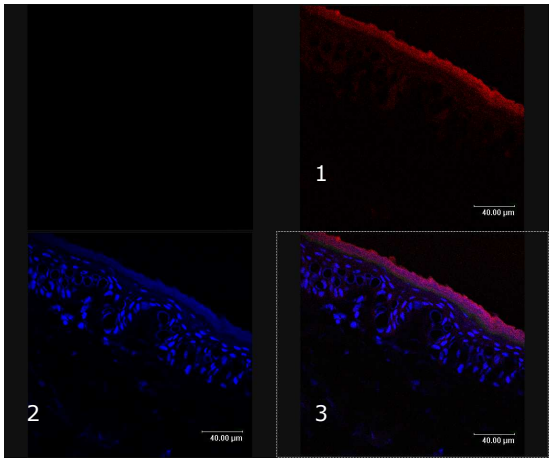


Fig. 8: CLSM images of the penetration of a solution of rhodamine B in Hepes buffer. The confocal image is divided into three parts with 1: the fluorescence rhodamine B, 2: the fluorescence of cell nuclei and 3: overlay of image 1 and image 2. Scale bar represents 40 µm.

Fig. 9A and B reveals the penetration of neutral liposomes and negatively charged PC-DMPA liposomes, respectively. These images are divided into four parts in which 1 corresponds to the fluorescence of NBD-PC, 2 corresponds to the fluorescence of rhodamine B, 3 corresponds to the fluorescence of cell nuclei and 4 is the overlay of the three first images.

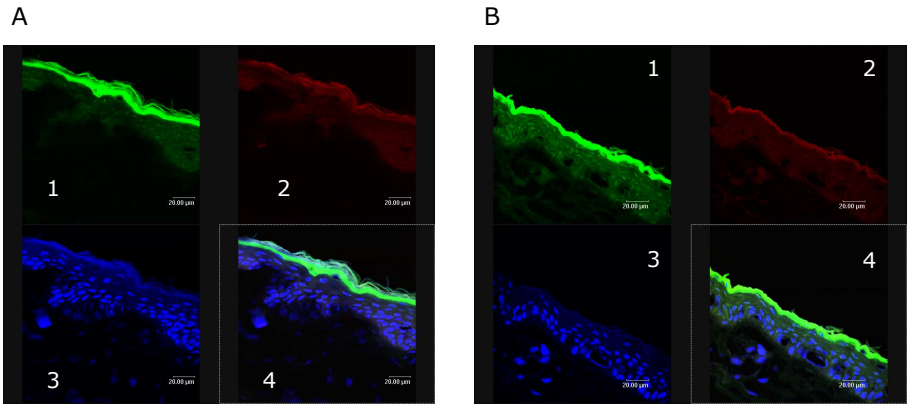


Fig. 9: CLSM images of the penetration of neutral liposomes encapsulating Rhodamine B and NBD-PC (A) and negatively charged liposomes encapsulating Rhodamine B and NBD-PC (B). Each confocal image is divided into four parts with 1: fluorescence due to NBD-PC, 2: the fluorescence due to rhodamine B, 3: the fluorescence of cell nuclei and 4: overlay of image 1, image 2 and image 3. Scale bar represents 20 µm.

Rhodamine B seems to penetrate the epidermis deeply as well as NBD-PC and to follow the penetration of NBD-PC. Compared with the solution of rhodamine B in Hepes (Fig. 8), the encapsulation into neutral or negatively charged liposomes clearly enhances the penetration of rhodamine B (Fig. 9A and B). Similar results were obtained by Mura *et al* [28], where the encapsulation of rhodamine 6G in multilamellar liposomes increased its skin penetration in comparison with a rhodamine 6G solution. However, it must be noted that rhodamine 6G (log *P* = 4.02) is more lipophilic than rhodamine B [29]. In addition, compared with neutral liposomes (Fig. 9A), negatively charged liposomes appear to enhance the NBD-PC and rhodamine B fluorescence in the epidermis (Fig. 9B).

The present observations obtained by confocal microscopy show that negatively charged vesicles do enhance the penetration of rhodamine B in the epidermis. They are in good agreement with the analytical results with betamethasone. In addition, owing that rhodamine B is supposed to mimic an encapsulated drug into liposomes and since it is strongly associated to

the lipid bilayer, the results suggest that negative charges at the surface of liposomes enhance the penetration of the vesicles together with their encapsulated drug. However, as only the fluorescent dyes are visible, this statement needs to be confirmed. Indeed, the liposomes physical integrity during the penetration remains to be demonstrated.

4. CONCLUSION

In this study, we show that negatively charged liposomes significantly enhance betamethasone penetration in the epidermis compared to positively charged and neutral liposomes. This is observed with DMPA and DCP negative liposomes encapsulating betamethasone or betamethasone dipropionate. Negative non liposomal dispersions of PC, DMPA and betamethasone are unable to enhance skin penetration at the same level. Observations in confocal microscopy study seem to confirm the potential of the negatively charged vesicles. Further studies will be made in order to understand the mechanisms by which negatively charged liposomes enhance penetration. TEM observation of freeze fracture replica of skin samples will probably help us to see any effect on the *stratum corneum* ultra-structure and to see if intact vesicles are present.

5. ACKNOWLEDGMENTS

We are very thankful to the Giga Cell Imaging and Flow Cytometry Platform for their technical help in the confocal study. A. Gillet is a PhD student supported by the FNRS, Brussels, Belgium.

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IV. Chapitre 4

NEGATIVELY CHARGED LIPOSOMES ENCAPSULATING A CORTICOSTEROID DRUG SUBSTANCE: ASSESSMENT OF *IN VIVO* TOPICAL ACTIVITY

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Etude préliminaire

IV.1 Résumé

Evaluation de l'activité topique de corticostéroïdes encapsulés dans des liposomes chargés négativement.

IV.1.1 Introduction

Dans le chapitre précédent, nous avons montré que l'utilisation de liposomes chargés négativement permettait d'augmenter d'un facteur 6 la quantité de bétaméthasone au niveau de la peau (épiderme et derme) par rapport à une solution éthanolique de bétaméthasone. En ce qui concerne le dipropionate de bétaméthasone, l'encapsulation dans les liposomes négatifs a augmenté d'un facteur 4 la quantité d'ester dans la peau par rapport à la spécialité Diprosone® lotion (solution hydro-alcoolique renfermant 0,064% de bétaméthasone dipropionate).
Dans cette étude préliminaire, l'efficacité de ces vésicules sera évaluée *in vivo* sur des volontaires grâce au test de blanchiment cutané. Le blanchiment de la peau est provoqué par une vasoconstriction de la micro-vascularisation au niveau de la peau et peut être mesuré grâce à un chromamètre. Cette vasoconstriction est en corrélation avec l'efficacité clinique des corticostéroïdes.

IV.1.2 Résumé des résultats

Les résultats de blanchiment cutané sont exprimés par la valeur Δa [la chromaticité a évalue le spectre du rouge (valeur +100) au vert (valeur -100)] (recommandé par la FDA). Plus cette valeur est négative plus le blanchiment observé est intense. La Figure 4.1 reprend les résultats obtenus lorsque seuls les « bons répondeurs » sont pris en compte. Les « bons répondeurs » sont définis dans cette étude comme les volontaires montrant une valeur ΔL positive (L est la luminance dont l'échelle s'étend

de la valeur 0 pour le noir jusqu'à 100 pour le blanc) et une valeur Δ*a* négative pour chaque site traité.

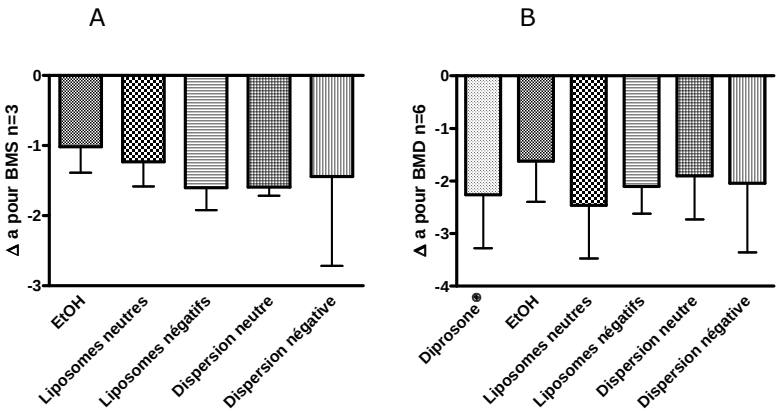

Figure 4.1: Valeurs de Δ*a* en fonction des différentes formulations contenant soit la bétaméthasone BMS (n = 3) (A) soit le dipropionate de bétaméthasone BMD (n = 6) (B).

Lorsque seuls les « bons répondeurs » sont considérés, les liposomes chargés négativement semblent augmenter l'efficacité de la bétaméthasone par rapport aux liposomes neutres et au gel éthanolique de bétaméthasone bien que ces résultats ne soient pas significativement différents. En effet, la puissance du test statistique est trop faible vu le petit nombre de « bons répondeurs ». Cependant, une certaine tendance peut être observée.

Les résultats obtenus précédement *ex vivo* montraient que les dipersions non liposomales ne permettaient pas d'augmenter la pénétration cutanée de la bétaméthasone au même niveau que les liposomes chargés négativement. Cette observation est expliquée par la sédimentation de la bétaméthasone sur la surface cutanée due à l'instabilité des dispersions non liposomales. La bonne réponse à la bétaméthasone dans les dispersions non liposomales *in vivo* peut s'expliquer par une diminution de cette sédimentation grâce à la viscosité supérieure des gels par rapport aux dispersions dans le tampon Hepes seul.

Pour les formulations contenant le dipropionate de bétaméthasone, il est difficile de dégager une tendance.
L'utilisation des liposomes chargés négativement semble plus intéressante dans le cas d'une molécule ne possédant pas une bonne pénétration cutanée intrinsèque comme c'est le cas pour la bétaméthasone.
Le caractère éventuellement saturable de la réponse aux corticostéroïdes pourrait expliquer le manque de différenciation entre les formulations.

IV.1.3 Conclusion

Bien que la puissance du test statistique soit trop faible vu le petit nombre de « bons répondeurs », cette étude préliminaire semble montrer une certaine tendance. Les liposomes chargés négativement semblent améliorer l'efficacité de la bétaméthasone. Une étude sur un plus grand nombre de « bons répondeurs » est cependant nécessaire afin de confirmer cette hypothèse.

IV.2 Preliminary study

1. INTRODUCTION

The *in vivo* clinical effectiveness of a topically applied glucocorticoid depends on its bioavailability within the skin at the site of action. The target cells are the keratinocytes and fibroblasts within the viable epidermis and dermis, where the glucocorticoid receptors are located. The clinical effect of steroids will be determined by the cellular uptake, the residence time as well as their affinity for the glucocorticoid receptor. Within the cytoplasm, the steroid binds to the glucocorticoid receptor, forming a complex which is rapidly transported to the nucleus. Then the glucocorticoid-receptor complex inside the nucleus binds to a region of DNA called the glucocorticoid responsive element (GRE) to either stimulate or inhibit transcription and thereby regulate the inflammatory process [1] (Figure 4.2).

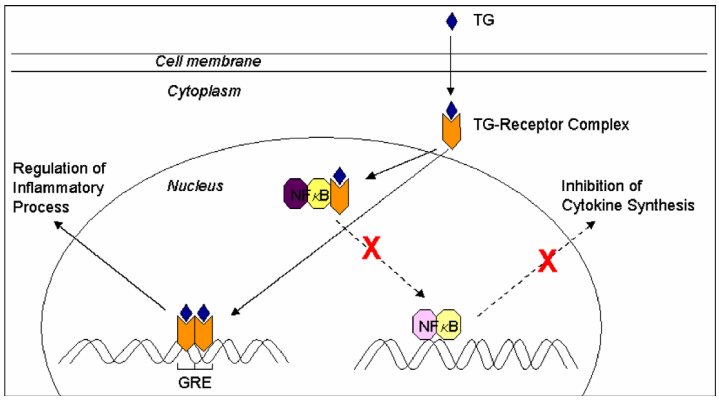

Figure 4.2: Schematic representation of the mechanism of action of topical glucocorticoids (TG). Nuclear factor-jB (NF-jB); Glucocorticoid response element (GRE) [1].

Methods available for testing the effectiveness of topical glucocorticoids include the tape stripping, microdialysis and the vasoconstrictor assay.

The tape stripping method allows the measurement of the drug accumulation in the *stratum corneum*, which is removed layer by layer using adhesive tape. In order to obtain the drug distribution profile as a function of stratum corneum depth, the amount of *stratum corneum* has to be determined on each tape strip. Infrared densitometry is a suitable method for *stratum corneum* depth determination [2, 3]. Lindemann *et al*. compared three techniques; the pseudo-absorption of the corneocytes in the visible range (430 nm) correlated well with the protein absorption in the UV range (278 nm) and the absorption at 652 nm obtained after staining the *stratum corneum* proteins with Trypan blue [4]. Other techniques are available to determine the amount of *stratum corneum* on tape strips. An overview of these methods was published by Hahn *et al*. [2].

Microdialysis involves placing an ultra thin hollow fibre, called a probe, in the dermis. The probe is semi-permeable and perfused with a sterile buffer at a slow rate using a microdialysis pump. The probe serves as an artificial vessel, allowing the exchange of small diffusible molecules from the extracellular fluid to the probe and vice versa. Thus, this method can provide concentration-time profiles [5] (Figure 4.3).

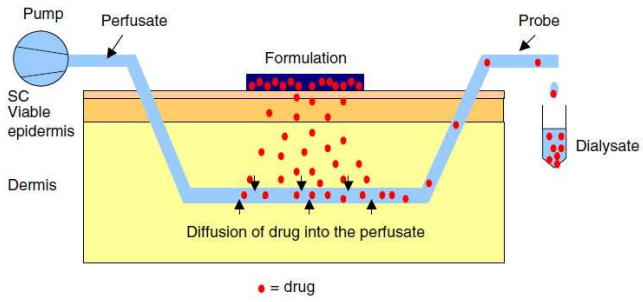

Figure 4.3: Schematic representation of the microdialysis principle. The probe is inserted into the dermis and is perfused at a flow rate controlled by the pump. The drug from the applied formulation permeates the SC and viable epidermis and eventually diffuses passively into the lumen of the membrane. The dialysate is sampled at fixed time intervals [1].

The vasoconstrictor assay is the only method approved by the FDA for topical glucocorticoid bioequivalence testing [6]. The skin blanching or

whitening is due to the ability of topical glucocorticoids to produce a vasoconstriction of the microvasculature of the skin [1]. This assay was first described by McKenzie and Stoughton in 1962 [7] . Vasoconstriction is a pharmacological activity, which correlates well with clinical activity [8]. Although the skin blanching was first evaluated subjectively by visual scoring, the FDA recommends the use of the Minolta chromameter as an objective instrumental method. Chromameters are compact portable instruments used for the assessment of surface colour based on the tristimulus analysis of a reflected xenon light pulse. The colour is measured in terms of three indices: the *L*-scale (light/dark; 100/0) and the two chromaticity co-ordinates, which are the *a*-scale (red/green; +60/-60) and the *b*-scale (yellow/blue; +60/-60) [9, 10]. These three values can be used to define a point in three-dimensional space that characterizes a colour in absolute terms. The magnitude of the difference between two colours, as perceived by the human eye, is proportional to the distance between two points defining those colours in a three-dimensional space. The difference between two colours, the Euclidean distance ED, is defined by the following equation [11]:

$$ED = \sqrt{(\Delta a)^2 + (\Delta b)^2 + (\Delta L)^2}$$

In a previous study, we developed negatively charged liposomes encapsulating either betamethasone or betamethasone dipropionate. These liposomes were compared to neutral liposomes and to non liposomal dispersions. Skin penetration studies were performed *ex vivo* on pig ear skin by using Franz diffusion cells. Figure 4.4 shows the amount of betamethasone and betamethasone dipropionate determined in the skin for the different formulations. As the glucocorticoid receptors are located in the keratinocytes and fibroblasts within the viable epidermis and dermis, Figure 4.4 represents the cumulated amounts of drug determined in the epidermis and the dermis.

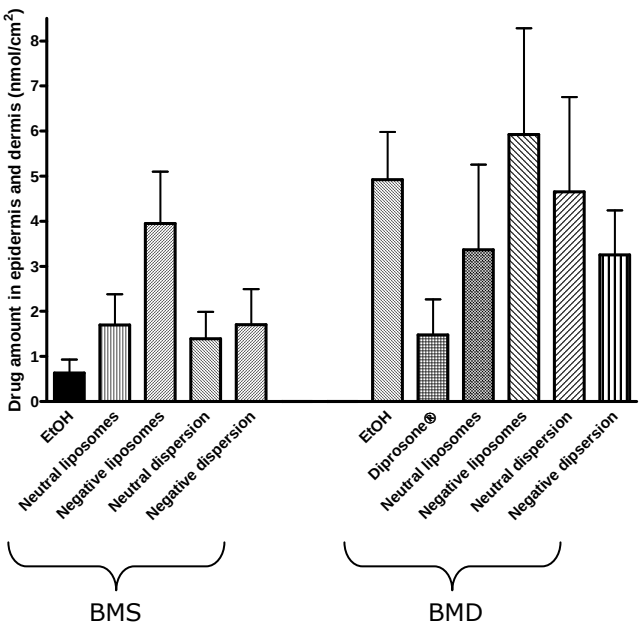

Figure 4.4: Drug amounts in the total viable skin (epidermis and dermis) as determined *ex vivo* with Franz diffusion cells in pig ear skin (nmol/cm^2) (n = 9). Betamethasone (BMS) left and betamethasone dipropionate (BMD) right.

Figure 4.4 shows that the use of negatively charged liposomes increased the amount of betamethasone in the skin 6 times compared with an ethanolic solution, and the amount of betamethasone dipropionate 4 times compared with Diprosone® lotion, a marketed product containing 0.064% betamethasone dipropionante in a hydro-alcoholic solution.

In the present study, the effectiveness of these negatively charged liposomes will be evaluated *in vivo* by the vasoconstrictor test using the Minolta Chroma Meter CR-400®.

2. MATERIALS AND MATHODS

2.1 Chemicals and reagents

Betamethasone (E.P.) was purchased from Medeva (Braine L'Alleud, Belgium). Betamethasone dipropionate (E.P.) was purchased from ABC Chemicals (Wauthier-Braine, Belgium). Soybean phosphatidylcholine (PC) was purchased from Lipoïd (Ludwigshafen, Germany). 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt) (DMPA) came from Avanti Polar lipids (Alabaster, AL, USA). Pure water was generated from the Milli-Q system (Millipore, Bredford, MA, USA). All experiments were performed using a 0.22 µm-filtered 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) buffer (Sigma Aldrich), containing 145 mM NaCl and adjusted to pH 7.4 with 0.1 M NaOH solution. Hydroxypropylcellulose and trometamol came from Fagron (Waregem, Belgium). Carbomer 980 was purchased from Pharminnova (Waregem, Belgium). Diprosone® lotion is a hydro-alcoholic lotion containing 0.064% betamethasone dipropionate, carbomer, NaOH, isopropyl alcohol and water.

2.2 Gel preparation

Neutral liposomes and negatively charged DMPA liposomes and their respective non liposomal dispersions containing either betamethasone or betamethasone dipropionate were prepared and characterised as previously described (section 2.2, Chapter 3). Liposomes and non liposomal dispersions were formulated in carbomer gels. Appropriate and equal amounts of carbomer 980 and trometamol were ground in a mortar to obtain a homogenous mixture. Then liposome suspensions and a Hepes buffer where added to obtain a final betamethasone and betamethasone dipropionate concentration of 583 µg/g and 750 µg/g (corresponding to 583 µg/g betamethasone) respectively, and a final carbomer concentration of 1.5% (m/m). In the *ex vivo* study on Franz diffusion

cells, drug in ethanolic solutions were used as references. In order to formulate a 100% ethanolic gel, hydroxypropylcellulose gels (7% m/m) with 100% ethanol and containing a free drug were used. Each formulation contained 0.058% betamethasone or 0.075% betamethasone dipropionate (same molar ratio). Tested formulations are:

For betamethasone:

- hydroxypropylcellulose gel containing a 0.058 % free drug (BMS-EtOH)
- neutral liposomes in carbomer gel
- negative liposomes in carbomer gel
- neutral non liposomal dispersion in carbomer gel
- negative non liposomal dispersion in carbomer gel

For betamethasone dipropionate:

- Diprosone® lotion (containing a 0.064% free drug)
- hydroxypropylcellulose gel containing a 0.075 % free drug (BMD-EtOH)
- neutral liposomes in carbomer gel
- negative liposomes in carbomer gel
- neutral non liposomal dispersion in carbomer gel
- negative non liposomal dispersion in carbomer gel

2.3 Volunteers

Twenty healthy volunteers participated in the study (ten men and ten women, between 23 and 45 years old) and were divided into two groups; ten volunteers (five men and five women) received the formulations containing betamethasone and ten volunteers received the formulations containing betamethasone dipropionate. The study was approved by the ethics committee of the University of Liège (see annexe 2). The volunteers provided written informed consent to participate and all of them considered themselves healthy and not to have skin diseases or contact allergy. They were asked not to use skin products on their forearms on the day of the study.

2.4 Method of application of the formulations

The volunteers stayed five minutes with their forearms positioned horizontally before baseline measurements (zero time) were taken. Application areas were delimited on the forearms with auto adhesive ring tapes (1.67 cm diameter) and placed at least 2 cm apart, avoiding nevus and visible blood vessels. One site was used for control, whilst the other sites were treated randomly with the formulations. 90 mg of the different gels were applied with pre-filled syringes without needles and covered with non occlusive bandages. The preparations were left in contact with the skin for five hours before removal. After this time, the protective covers and adhesive labels were removed. The excess formulation was removed by cleaning the sites with gauze impregnated with distilled water. The skin sites were then allowed to equilibrate horizontally for five minutes before measurements were taken.

2.5 Chromameter assessment of blanching response

The blanching response was evaluated with a Minolta Chroma Meter CR-400® (Konica Minolta Sensing, Inc., Osaka, Japan) calibrated with the white plate immediately before use. Baseline readings (zero time) were taken from all sites (including the untreated control site) prior to the application of the formulations. After their removal, blanching responses were assessed at all application and control sites. Each reading was taken in duplicate and the mean value was calculated. The recorded *L*, *a* and *b*-values for each site were corrected by subtracting the baseline (time zero) values to obtain baseline-corrected values. In addition, the untreated control site value (also baseline-corrected) was further subtracted from the baseline value, to yield Δ*L*, Δ*a* and Δ*b* values:

$$\Delta\text{value} = (M_{t=5h} - M_{t=0}) - (U_{t=5h} - U_{t=0})$$

Where M is the *L*, *a* and *b* value at the medicated site, U is the corresponding value for the untreated control site and t is the observation time (0= baseline readings, 5h= reading time). This double correction procedure is only suggested for the *a*-scale in the FDA Guidance as the *L* and *b*-scale data are not advocated for use in the Guidance.

2.6 Statistical analysis

The significance of the differences between different formulations was tested using paired Student t-test (Graph Pad Prism Version 4). The differences are considered as statistically significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

The effectiveness of negatively charged liposomes containing either betamethasone or betamethasone dipropionate formulated in carbomer gel was evaluated by the vasoconstrictor assay and compared with neutral liposomes and their non liposomal dispersions. Three formulations were used as references: two hydroxypropylcellulose-based gels containing free betamethasone or free betamethasone dipropionate and Diprosone® lotion.
Visual observation of the skin blanching is shown in Figure 4.5:

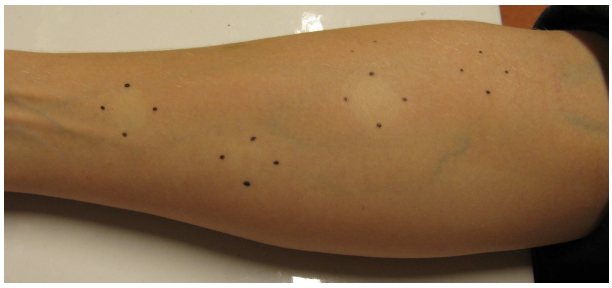

Figure 4.5: A typical skin blanching response.

The FDA Guidance suggests the use of only the *a*-scale value in quantifying the blanching response. As recommended by several authors, the Euclidean distance (ED) was also calculated [9, 11].

Figure 4.6 A and B represents the *a*-scale values for betamethasone and betamethasone dipropionate containing formulations, respectively. As the *a*-scale values decrease with increasing skin blanching, Δ*a* are negative.

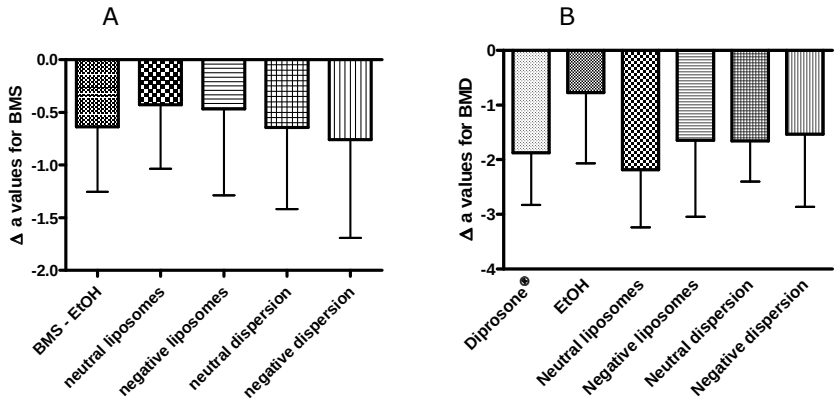

Figure 4.6: Δ*a* values for the different formulations containing betamethasone (A) or betamethasone dipropionate (B) (n = 10).

When including all the volunteers who participated in the study (Figure 4.6 A and B), standard deviations are quite high and therefore we cannot observe any enhanced effect of a formulation over the others. The FDA recommends including only "good responders" in the skin blanching assay. A good responder is defined as "a subject who shows a response to a single dose duration of the reference listed drug under the same occlusion or nonocclusion conditions used in the pilot and pivotal studies. A responder shows a visual reading of at least one unit" [6] . When including only the good responders (defined in this study as having a positive Δ*L* value and a negative Δ*a* value for each treated site), we obtain Figure 4.7 A and B for betamethasone and betamethasone dipropionate containing formulations, respectively.

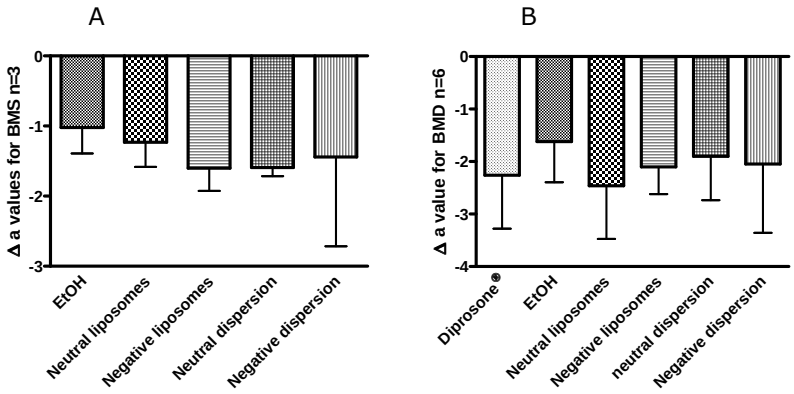

Figure 4.7: Δ a values for the different formulations containing betamethasone (A) (n = 3) or betamethasone dipropionate (B) (n = 6).

The difference in the number of good responders between formulations containing betamethasone (n = 3) and betamethasone dipropionate (n = 6) is explained by the fact that betamethasone is considered as a less potent glucocorticoid than its ester (classified as potent in the British National Formulary) [1].

When including only the good responders, negatively charged liposomes seem to enhance the pharmacodynamic response of betamethasone in comparison with the neutral liposomes and the ethanolic gel. Unfortunately, differences are not significant. This can be explained by the small number of "good responders". Unlike *ex vivo* results, the non liposomal dispersions give the same response as negative liposomes. In a previous work, we showed that non liposomal dispersions are not physically stable; high sedimentation was visible with time. The gel formulation would decrease this sedimentation at the skin surface and could therefore enhance the amount of the drug available for skin penetration.

Regarding betamethasone dipropionate, it is difficult to highlight a tendency.

Figure 4.8 A and B represents the ED values for betamethasone and betamethasone dipropionate containing formulations, respectively.

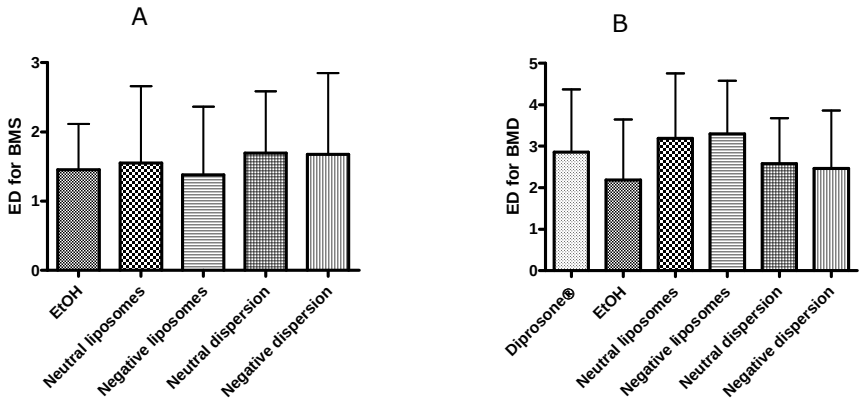

Figure 4.8: ED values for formulations containing betamethasone (A) and betamethasone dipropionate (B) (n = 10).

When including only the "good responders", we obtain Figure 4.9 A and B.

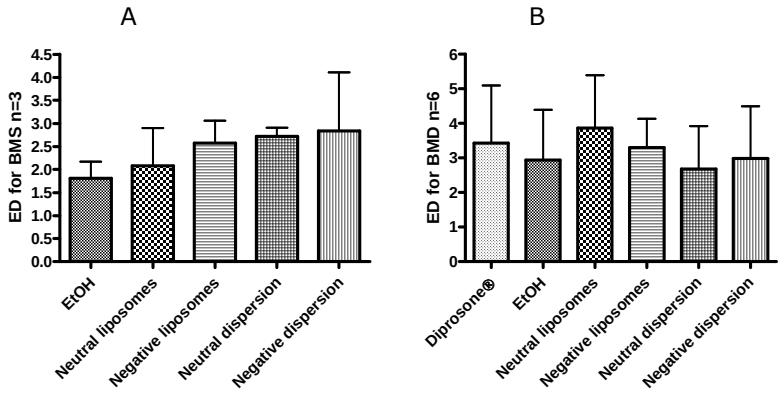

Figure 4.9: ED values for different formulations containing betamethasone (A) (n = 3) or betamethasone dipropionate (B) (n = 6).

Data follow the same tendency when comparing the Δ*a* values with the ED values.

Wiedersberg *et al*. compared the vasoconstrictor assay (= pharmacodynamic response) with the tape stripping method (= dermatopharmacokinetic technique) on topical applied betamethasone

valerate. They showed that the pharmacodynamic response did not increase from 1 mg/ml to 10 mg/ml and was, therefore, saturable, while the tape stripping method distinguished unequivocally between the different formulations and different concentrations [12]. We applied a concentration of 0.58 mg/g for betamethasone and 0.75 mg/g for betamethasone dipropionate; corresponding to a concentration of 0.71 mg/g for betamethasone valerate (same molar ratio). Although the concentrations used in this study were smaller than 1mg/mL, a saturation effect could not be excluded. Therefore, it could be worthwhile to use smaller concentrations.

Pershing *et al*. also compared the skin blanching assay with the tape stripping method. The tape stripping method distinguished between three triamcinolone acetonide cream concentrations (0.025%, 0.1% and 0.5%). The *stratum corneum* concentration achieved with the 0.1% cream was statistically greater than the drug content produced with the 0.025% cream and the 0.5% cream was statistically higher than both the 0.1% and 0.025% creams. However, when studying the skin blanching response, there was statistically no difference between the 0.025% and 0.1 % creams [13]. In their study, the skin blanching assay failed to show any significant difference between the lowest concentrations tested.

Franz *et al*. evaluated the bioavailability of a new topical formulation as a betamethasone valerate foam. By using human cadaver skin and Franz diffusion cells, the rate of betamethasone valerate absorption from the foam was found to be nearly 300% higher than that from the lotion. In addition, the foam product was found to be 50% more effective than the lotion in the treatment of scalp psoriasis. However, the vasoconstrictor assay was unable to demonstrate the greater potency of the foam product [14, 15].

The skin blanching assay is recommended by the FDA to determine the bioequivalence of topical formulations containing a corticoid. Regarding the saturable nature of the blanching response and the results obtained in the *ex vivo* experiments, the use of the vasoconstrictor assay seems

questionable. In the present study, we could conclude that the different formulations tested are bioequivalent, even if *ex vivo* results showed significant differences between negatively charged liposomes encapsulating betamethasone (a 9-fold increase) and the betamethasone ethanolic solution.

4. CONCLUSION AND PERSPECTIVE

Although the results were not significantly different, this preliminary study seems to show that the use of negatively charged liposomes could improve the *in vivo* efficacy of betamethasone. The use of liposomes seems to be more effective in improving the penetration of a drug with poor intrinsic skin penetration like betamethasone compared with a more lipophilic drug with higher intrinsic penetration like betamethasone dipropionate.

Further studies with a higher number of "good responders" are needed to confirm the potential of negatively charged liposomes encapsulating betamethasone.

5. REFERENCES

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DISCUSSION

La première partie de la discussion sera consacrée à la caractérisation des liposomes testés sur le plan de la taille, de la morphologie, du pourcentage d'encapsulation, de la stabilité, de la déformabilité et de la charge. La seconde partie discutera de l'influence de différents paramètres sur l'efficacité de la pénétration cutanée mesurée d'abord *in vitro*, ensuite *ex vivo* et enfin *in vivo*.

1 Caractérisation des liposomes

a. Diamètre – morphologie

Le diamètre des liposomes a été déterminé par spectroscopie à corrélation de photon (PCS) et leur morphologie à été évaluée par microscopie électronique après cryofracture. Ces deux méthodes ont donné des résultats semblables.

Le diamètre des liposomes réalisés est proche de 200 nm. Les liposomes contenant du DMPC en association avec le déoxycholate sodique montraient des tailles extrêmement variables allant de quelques nm à plus de 500nm, ce qui nous a amené à abandonner cette formulation. Nous avons également observé que les liposomes constitués de PC seule avaient une taille plus grande comparée à celle des liposomes contenant soit un tensio-actif soit une charge. La réduction de taille observée pour les liposomes contenant un tensio-actif peut être attribuée à une augmentation de la flexibilité et à une réduction de la tension de surface des vésicules, comme observé et expliqué par Chen *et al* [1]. La réduction de taille due à l'ajout d'une charge a également été observée par Namdeo *et al*., dans le cas de niosomes dans lesquels le DCP a été incorporé. Ils ont expliqué que la présence d'une charge au niveau de la bicouche augmentait le degré de courbure et de ce fait réduisait la taille des vésicules [2].

L'étude de la morphologie des liposomes a montré la présence de petites vésicules unilamellaires de taille homogène (SUV ou small unilamellar vesicles).

b. Pourcentage d'encapsulation

Le pourcentage d'encapsulation des liposomes est déterminé après élimination du principe actif non encapsulé par ultracentrifugation.
L'incorporation du principe actif au niveau de la bicouche des liposomes au lieu de la cavité aqueuse à l'aide des cyclodextrines a permis d'augmenter le pourcentage d'encapsulation de 35-55% à 80-97% selon les différentes compositions. Ce fait à également été observé par Maestrelli *et al*., puisque l'incorporation du kétoprofen au niveau de la bicouche des liposomes augmentait le pourcentage d'encapsulation de 26,8 ± 1,6% à 56,0 ± 3,2% par rapport à une encapsulation à l'aide de complexes d'inclusion kétoprofen - β -cyclodextrine [3].

Dans le cas d'une insertion de la bétaméthasone au niveau de la bicouche, l'absence de cristaux a été confirmée par microscopie électronique. L'absence de cristaux a également été vérifiée par dosage de la bétaméthasone avant et après filtration, montrant la bonne dispersion de la bétaméthasone au niveau de la bicouche lipidique.

Nous avons réalisé des premières études de microscopie électronique dans le but de caractériser les liposomes en identifiant la position de la bétaméthasone au sein des liposomes. Les liposomes encapsulant différents marqueurs (comme la biocytine) ont été réalisés et fixés au tétroxyde d'osmium (OsO_4). Ce colorant est utilisé en microscopie électronique pour donner du contraste à l'image. C'est un oxydant qui va permettre de marquer préférentiellement les lipides insaturés. Cette réduction se produit au niveau des doubles liaisons carbone-carbone des lipides insaturés. L'OsO_4 permet également de fixer certaines protéines au niveau de leurs acides aminés porteurs de groupements sulfhydriles. C'est

cette propriété qui a été mise à profit pour marquer la biocytine encapsulée dans des liposomes. Les résultats obtenus avec la biocytine (témoin positif) ont été comparés avec ceux obtenus avec la bétaméthasone encapsulée dans les liposomes. Cette comparaison a permis de confirmer que la bétaméthasone encapsulée seule est bien associée à la bicouche lipidique.

La Figure 2 reprend les différentes étapes de préparation des échantillons.

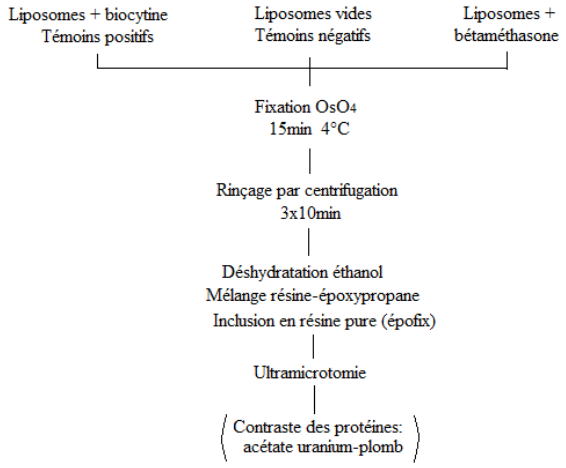

Figure 2 : Les différentes étapes de la préparation des échantillons

Le marquage des liposomes à l'OsO_4 a permis de confirmer que la bétaméthasone est bien située au niveau de la bicouche lipide lorsqu'elle est encapsulée seule. Cette visualisation est nettement plus marquée sur les coupes contrastées à l'acétate d'uranium/plomb comme le montre les Figures 3 et 4.

La Figure 3 montre les résultats obtenus sur des coupes non contrastées. Il est difficile de voir une différence entre les liposomes blancs (Figure 3B) et les liposomes encapsulant la bétaméthasone seule (Figure 3C).

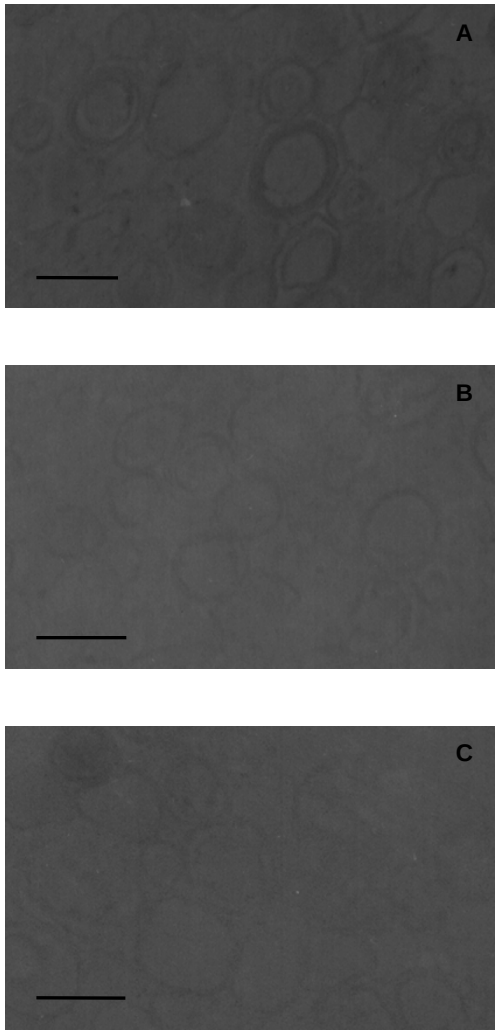

Figure 3 : Images de microscopie électronique (TEM) de liposomes marqués au OsO_4. A : témoin positif, liposomes encapsulant de la biocytine, B : témoin négatif, liposomes blancs et C : liposomes encapsulant la bétaméthasone seule (échelle = 200nm).

La Figure 4 montre les photos obtenues sur des coupes contrastées. Les différences sont beaucoup plus marquées.

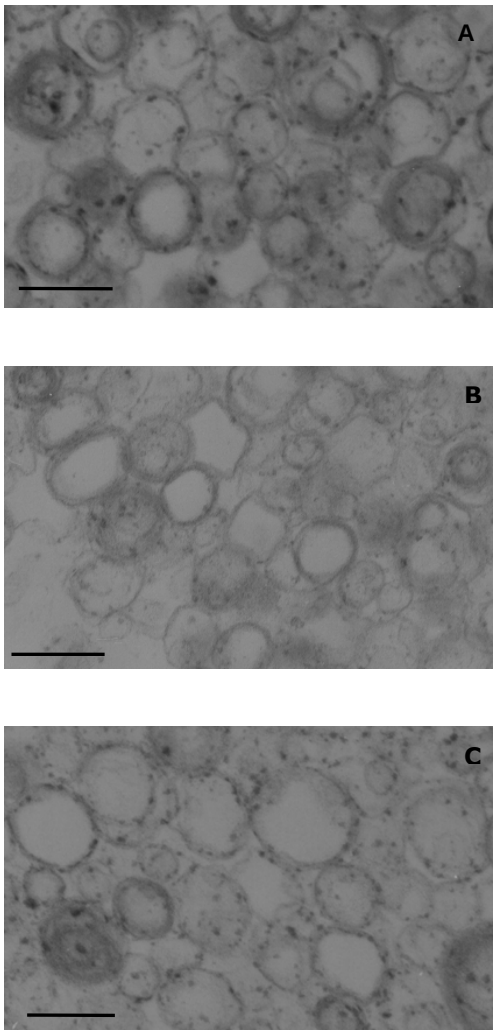

Figure 4 : Images de microscopie électronique (TEM) de liposomes marqués au OsO_4 et contrastée à l'acétate d'uranium/plomb. A : témoin positif, liposomes encapsulant de la biocytine, B : témoin négatif, liposomes blancs et C : liposomes encapsulant la bétaméthasone seule (échelle = 200nm).

Cette étude a permis de confirmer que la bétaméthasone est bien associée à la bicouche lipidique lorsqu'elle est incorporée seule dans les liposomes.

c. Rétention de la bétaméthasone dans les liposomes

Le pouvoir de rétention de la bétaméthasone dans les liposomes a été évalué par ultracentrifugation. L'encapsulation de la bétaméthasone dans la cavité des liposomes à l'aide des complexes d'inclusion avec les cyclodextrines a permis de diminuer la fuite de la bétaméthasone de 76,2 ± 1,6% à 20,9 ± 1,6% dans le cas des liposomes classiques et de 82,7 ± 4,1% à 44,8 ± 3,2% dans le cas des liposomes déformables. Ces résultats montrent que l'ajout de bétaméthasone et/ou de déoxycholate sodique au niveau de la bicouche lipidique diminue la rétention de la bétaméthasone dans les liposomes.

d. Stabilité

La stabilité des liposomes a été évaluée de différentes manières. D'abord visuellement par l'absence de sédimentation puis par la mesure de la variation du diamètre et enfin par la mesure de la perte de matériel encapsulé (le matériel étant soit la calcéine, soit la bétaméthasone seule soit les complexes bétaméthasone-cyclodextrines). Ces différentes techniques ont permis de montrer que les liposomes sont stables lorsque ceux-ci sont conservés pendant 1 mois à 4°C sous azote. La température de conservation est l'élément majeur de la déstabilisation des liposomes. Une conservation à 37°C a entraîné une dégradation des vésicules en termes de diamètre et de fuite du matériel encapsulé après un mois de conservation.

e. Déformabilité

Outre le déoxycholate sodique, deux autres tensio-actifs ont été utilisés. Il s'agit du polysorbate 80 et du sorbitane monooléate (résultats non publiés).

La déformabilité de ces liposomes a d'abord été étudiée par la mesure de viscosité des membranes lipidiques par la technique de résonance paramagnétique électronique (RPE). Le laboratoire du Prof. M. Hoebeke, Spectroscopie Biomédicale (Faculté des Sciences, ULg) a établi les équations permettant de faire le lien entre les paramètres obtenus à partir des spectres RPE et la microviscosité. Les résultats ont pu montrer que la microviscosité des liposomes classiques vides est plus élevée que celle des liposomes déformables. Dans la littérature, un autre test est souvent réalisé pour mesurer la déformabilité des vésicules. Il s'agit de mesurer un coefficient de déformabilité D par extrusion. Cette technique consiste à extruder la suspension de liposomes à travers une membrane de polycarbonate de porosité connue sous une pression faible et constante en azote. Nous avons appliqué la formule suivante :

$$D = j*(r_v/r_p)^2$$

où j correspond au poids de la suspension extrudée, r_v correspond au diamètre des liposomes après extrusion et r_p correspond à la porosité de la membrane de polycarbonate [4]. Les conditions d'extrusion comme la pression en azote et la porosité de la membrane ont été adaptées [5, 6]. L'index de déformabilité D est d'autant plus élevé que la déformabilité est grande. La mesure par extrusion a permis de confirmer la plus grande déformabilité des liposomes contenant soit le déoxycholate sodique (D = 8,0 ± 0,4), soit le polysorbate 80 (D = 7,5 ± 0,2) par rapport aux liposomes classiques (D = 5,2 ± 1,0). De plus, la mesure de la quantité de PC présente avant et après extrusion a montré que dans le cas de liposomes classiques, seuls 3,5% (± 2,8) de PC étaient récupérés après extrusion en comparaison à 84,1 % (± 4,2) et 94,4% (± 2,3) pour les liposomes déformables contenant soit du déoxycholate sodique soit du polysorbate 80, respectivement. Cependant, les résultats diffèrent en ce qui concerne la formulation contenant le sorbitane monooléate. Dans ce cas, un faible index de déformabilité D est obtenu (D = 2,8 ± 0,2). Cela pourrait être expliqué par la valeur de la HLB (balance

hydrophilie /lipophilie) des tensio-actifs. Un tensio-actif présentant une HLB < 7 est plutôt lipophile tandis qu'un tensio-actif dont la HLB > 7 est plutôt hydrophile. La valeur de HLB du sorbitane monooléate est de 4,5 alors qu'elle est de 14,8 pour le polysorbate 80 et de 16 pour le déoxycholate sodique. Les différences observées s'expliquent par des interactions différentes au niveau de la bicouche lipidique.

La déformabilité des liposomes chargés et ne contenant pas de tensio-actifs a également été évaluée. Les liposomes encapsulant la bétaméthasone au niveau de leur bicouche et chargés positivement ou négativement ont montré un faible index de déformabilité de D = 2,0 ± 0,2 et de D = 2,3 ± 0,1, respectivement (résultats non publiés). Ces vésicules chargées ne peuvent donc pas être considérées comme déormables.

L'index de déformabilité minimum est de 0 pour toutes les formulations. L'index de déformabilité maximum varie en fonction des formulations puisque le diamètre des liposomes est différent pour chaque formulation. C'est pourquoi, un index de déformabilité corrigé (D_{cor}) par rapport à l'index maximum a été calculé. Cette correction ne change cependant pas les conclusions.

f. Charge

L'ajout d'une charge au niveau de la bicouche des liposomes a été décrit dans la littérature comme pouvant influencer la pénétration cutanée (voir discussion au point 2.b.i.5). C'est pourquoi des liposomes chargés ont été formulés. La charge de surface des vésicules a été mesurée par Zetasizer® 2000 (Malvern Instruments Ldt, Worcestershire, UK). La charge légèrement négative des liposomes constitués uniquement de PC (-1,6 ± 2,8 mV) a été considérée comme neutre [7]. La quantité de SA incorporée dans la bicouche des liposomes positifs a été sélectionnée d'après Piel *et al*. [8], pour donner des vésicules chargées positivement. La quantité de DMPA a été sélectionnée d'après Yoo *et al*. [9], pour donner des vésicules chargées négativement. Le DCP a été utilisé pour confirmer les résultats

prometteurs obtenus avec le DMPA. L'ajout de SA a permis d'obtenir des vésicules chargées positivement (+13,2 ± 2,2 mV) tandis que l'ajout de DMPA et de DCP a permis d'obtenir des vésicules chargées négativement (-26,6 ± 3,5 mV et -19,9 ± 4,5 mV, respectivement). La même proportion de DCP que celle utilisée pour le DMPA n'a pas pu être entièrement incorporée dans les liposomes, c'est pourquoi la quantité maximale incorporable a été utilisée, donnant une charge négative légèrement plus faible.

2. Etudes d'efficacité des liposomes

a. In vitro

Nous avons tout d'abord étudié la diffusion de la bétaméthasone encapsulée à l'aide des cyclodextrines à partir des différentes formulations de liposomes au moyen de cellules de diffusion de Franz, à travers des membranes de polycarbonate de porosité de 50 nm. La taille des pores des membranes utilisées étant inférieure au diamètre moyen des liposomes (~ 200 nm), les liposomes déformables devraient se déformer pour pouvoir passer à travers ces pores. Nous avons ainsi pu montrer que la présence de déoxycholate sodique (liposomes déformables) permettait d'augmenter le pourcentage de diffusion de la bétaméthasone par rapport à des liposomes classiques. Ces premiers résultats étaient très encourageants. Cependant, des études de pénétration *ex vivo* sur peaux s'imposaient avant de pouvoir conclure à une supériorité de ces systèmes.

Avec le recul, ces premiers résultats peuvent être interprétés différement. Les liposomes classiques encapsulant les complexes bétaméthasone-cyclodextrine montrent une fuite de la bétaméthasone de 20,9 ± 1,6% au T_0 et les liposomes déformables encapsulant les mêmes complexes montrent une fuite de 44,8 ± 3,2% au T_0 (voir Discussion 1.c). L'augmentation du pourcentage de diffusion observée aux cellules de

Franz pour les liposomes déformables serait due au relarguage plus élevé de la bétaméthasone des liposomes déformables au T_0 plutôt qu'à une vitesse de diffusion supérieure.

b. Ex vivo

i. Cellules de Franz

Les résultats des premières études de pénétration sur peaux d'oreilles de cochons n'ont pas permis de confirmer les résultats obtenus précédemment *in vitro*. Sur les peaux, les liposomes classiques encapsulant les complexes bétaméthasone-cyclodextrines permettaient une meilleure accumulation de la betamethasone au niveau de l'épiderme par rapport aux liposomes déformables ($p < 0,05$). L'ajout d'une charge au niveau de la bicouche lipidique de ces liposomes a alors été envisagé. Les résultats observés pour les liposomes chargés, tant positivement que négativement, étaient prometteurs puisque ces vésicules permettent d'augmenter significativement l'accumulation de la bétaméthasone au niveau de l'épiderme ($p < 0,05$) (résultats non publiés). Des liposomes encapsulant la bétaméthasone seule au niveau de leur bicouche ont aussi été testés. Ces vésicules présentaient des résultats très prometteurs.
La formulation des liposomes s'est donc avérée très importante pour l'efficacité de la pénétration cutanée. Nos recherches se sont orientées vers des études permettant de mieux comprendre les mécanismes de pénétration cutanée de systèmes vésiculaires sans se limiter à l'utilisation des cyclodextrines et des liposomes déformables.
L'influence de différents paramètres sur l'efficacité de pénétration cutanée a alors été étudiée. Les différents paramètres testés sont repris ci-dessous.

1) Influence du diamètre des liposomes

Il n'y a pas de corrélation entre la taille des liposomes et l'efficacité de la pénétration cutanée (Figure 1, test de Pearson, $p > 0,05$, $r^2 = -0,09172$). Par exemple, les liposomes positifs encapsulant la bétaméthasone montrent une taille significativement inférieure aux liposomes neutres (141 ± 6 nm et 178 ± 12 nm, respectivement) tout en permettant une accumulation semblable de la bétaméthasone au niveau de l'épiderme.

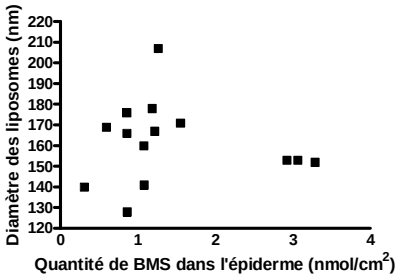

Figure 1 : Corrélation entre le diamètre des liposomes et la quantité de bétaméthasone (BMS) dans l'épiderme.

Au niveau de la littérature, les résultats sont contradictoires en ce qui concerne l'influence de la taille des liposomes sur l'efficacité de la pénétration cutanée. Mura *et al*. ont montré qu'une réduction de la taille des vésicules n'a pas d'effet significatif ni sur la pénétration à travers la peau de rat ni sur l'efficacité *in vivo* de liposomes encapsulant de la benzocaïne [10]. Sinico *et al*. ont étudié les liposomes comme transporteurs cutanés de la trétinoine et ont montré que la taille des liposomes n'affectait pas la pénétration de la trétinoine à travers la peau de cochon [11]. Kim *et al*. ont également montré que l'absorption de caféine était indépendante de la taille des vésicules [12]. Par contre, Verma *et al*. ont montré que la pénétration de carboxyfluorescéine était inversement corrélée à la taille des liposomes [13]. La supposition qu'une réduction de la taille conduit à une augmentation de la pénétration cutanée est en accord avec le concept d'une pénétration des liposomes sous forme intacte comme mécanisme de pénétration [14]. Comme dans

notre cas le diamètre des liposomes ne semble pas influencer la pénétration cutanée, nous ne pouvons pas être en accord avec ce mécanisme de pénétration.

2) Influence du pourcentage d'encapsulation

Les Figures 2 A et B montrent la corrélation entre la quantité de bétaméthasone ayant pénétré au niveau de l'épiderme et les pourcentages d'encapsulation de la bétaméthasone $EE_{B/Bt}$ et $EE_{B/L}$, respectivement. Quel que soit le moyen de calculer le pourcentage d'encapsulation, une corrélation a pu être mise en évidence (Figure 2A: test de Pearson, $p < 0,05$, $r^2 = 0,5540$ et Figure 2B : test de Pearson, $p < 0,05$, $r^2 = 0,6277$).

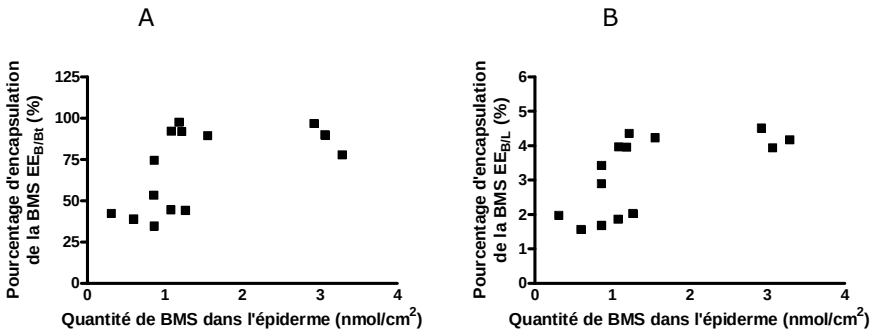

Figure 2: Corrélation entre les pourcentages d'encapsulation de la bétaméthasone $EE_{B/Bt}$ (A) et $EE_{B/L}$ (B) et la quantité de bétaméthasone (BMS) dans l'épiderme.

Au niveau de la littérature, les résultats sont également contradictoires en ce qui concerne l'influence du pourcentage d'encapsulation des liposomes sur l'efficacité de la pénétration cutanée. Mura *et al*. ont montré une possible corrélation entre le pourcentage d'encapsulation et la pénétration de la benzocaine à travers la peau de rat. Les coefficients de perméabilité de la benzocaine étaient identiques, indépendamment du fait que l'encapsulation soit au niveau de la cavité aqueuse ou au niveau de la

bicouche lipidique, des dimensions et de la structure des vésicules. Le seul élément commun à ces formulations était le pourcentage d'encapsulation (environ 29%) [10]. Par contre, Kim *et al*. ont montré que la quantité de caféine absorbée était indépendante du pourcentage d'encapsulation [12].

3) Influence du mode d'encapsulation

La bétaméthasone a été encapsulée soit dans la cavité aqueuse des liposomes, à l'aide de cyclodextrines, soit seule au niveau de la bicouche. Bien que montrant une moins bonne stabilité en termes de rétention de la molécule encapsulée, les liposomes encapsulant la bétaméthasone seule ont permis d'augmenter significativement la pénétration au niveau de l'épiderme par rapport aux liposomes encapsulant les complexes bétaméthasone-cyclodextrines. La pénétration d'une dispersion non liposomale de PC et bétaméthasone n'a pas montré de différence par rapport aux liposomes. La formulation sous forme de vésicules ne semblait pas nécessaire dans ce cas. Par contre, la dispersion pénétrait mieux au niveau de l'épiderme que la solution éthanolique et la solution de complexes d'inclusion ($p < 0,001$), indiquant que la PC agirait comme promoteur d'absorption.

Maestrelli *et al*. ont étudié l'encapsulation du kétotifène à l'aide de cyclodextrines dans des liposomes. Ils ont montré que les cyclodextrines permettaient d'augmenter la solubilité du kétotifen dans la cavité aqueuse des liposomes et conduisaient à une encapsulation plus stable par rapport à une encapsulation au niveau de la bicouche lipidique. Par contre, l'encapsulation des complexes kétotifen-cyclodextrines n'a pas permis d'augmenter la perméabilité par rapport aux solutions [3]. Par contre, Jain *et al*. ont montré que l'encapsulation de méloxicam à l'aide des cyclodextrines dans des liposomes permettait d'augmenter la perméabilité cutanée par rapport à des liposomes encapsulant le principe actif seul dans leur cavité aqueuse et par rapport à une solution de méloxicam [15].

4) Influence de l'ajout d'un tensio-actif

Les liposomes déformables sont constitués de phospholipides et d'un « edge activator ». L'edge activator est le plus souvent un tensio-actif qui va déstabiliser la bicouche lipidique et ainsi augmenter la déformabilité des liposomes [16]. Dans les deux types de liposomes (avec et sans cyclodextrines), l'ajout du déoxycholate sodique n'a pas permis d'augmenter la pénétration cutanée de la bétaméthasone. Comme la majorité des publications montrent la supériorité des liposomes déformables, nous avons testé d'autres tensio-actifs à la place du déoxycholate sodique [17, 18]. Nous avons ainsi réalisé des liposomes déformables contenant soit du polysorbate 80 soit du sorbitane monooléate afin de tenter d'augmenter la pénétration [19, 20]. Malheureusement la faible augmentation observée n'était pas significative. Les liposomes déformables ne permettaient donc pas d'augmenter la pénétration de la molécule encapsulée (résultats non publiés). D'autres temps de contact aux cellules de Franz ont également été envisagés. Il a été suggéré de réduire le temps initial de 24 heures afin de montrer une supériorité des liposomes déformables, qui pourraient permettre une pénétration plus rapide de la bétaméthasone. Des durées de 6 et 4 heures ont été testées mais cette réduction de temps de contact n'a pas permis de montrer un avantage des liposomes déformables par rapport aux liposomes classiques (résultats non publiés).

Il n'y a pas de corrélation entre l'index de déformabilité D et la pénétration de la bétaméthasone (Figure 3: test de Pearson, $p > 0,05$, $r^2 = -0,4359$).

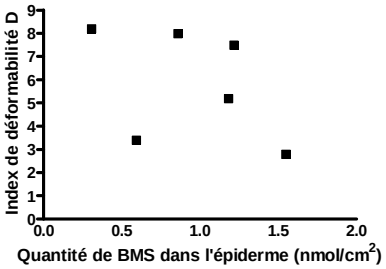

Figure 3: Corrélation entre l'index de déformabilité D et la quantité de bétaméthasone (BMS) dans l'épiderme.

5) Influence de l'ajout d'une charge

Dans le cas des formulations de liposomes contenant les complexes d'inclusion bétaméthasone-cyclodextrines, l'ajout d'une charge, tant positive que négative, au niveau de leur bicouche lipidique a permis d'augmenter significativement la pénétration de la bétaméthasone au niveau de l'épiderme (résultats non publiés). Dans le cas des liposomes encapsulant la bétaméthasone seule, l'ajout d'une charge négative (DMPA ou DCP) a permis d'augmenter significativement la pénétration de la bétaméthasone. Cette augmentation ne se confirme pas lorsqu'on étudie la pénétration d'une dispersion non liposomale de PC, DMPA et bétaméthasone, montrant que la formulation sous forme de vésicules est importante.

La matrice lipidique au niveau du *stratum corneum* contient une grande proportion de lipides chargés négativement et il est connu que la peau pourrait agir comme une membrane chargée négativement [9, 11]. Il a été décrit que la présence d'une charge sur la surface des vésicules pouvait influencer la diffusion d'un principe actif à travers la peau. Les vésicules chargées négativement donneraient des flux supérieurs aux vésicules chargées positivement, qui en contre partie amélioreraient l'accumulation du principe actif dans les couches superficielles de la peau [11]. Mais les résultats publiés sont contradictoires.

Comme la meilleure formulation testée par Carrer *et al*. contenait la plus grande proportion d' « edge activators » chargés, ils ont suggéré que la présence de charges négatives dans la membrane permettait la meilleure efficacité de pénétration [21]. Manosroi *et al*. ont étudié l'absorption transdermique de liposomes contenant de l'amphotéricine B. L'absorption d'amphotéricine B encapsulée dans des liposomes chargés était supérieure à celle de liposomes non chargés. Les liposomes positifs ont montré une meilleure absorption à travers le *stratum corneum* par rapport aux liposomes négatifs. Cependant, les liposomes chargés négativement ont présenté une meilleure absorption à travers l'épiderme vivant et le derme que les liposomes positifs [22]. Ogiso *et al*. ont montré que l'absorption percutanée de bétahistine à partir d'un gel contenant des liposomes chargés négativement était bien meilleure qu'à partir de la formulation contenant les liposomes positifs [23]. Katahira *et al*. ont montré que les liposomes négatifs contenant du DCP donnaient une meilleure rétention cutanée de rhodamine B accompagnée d'une diminution de la perméabilité par comparaison aux liposomes positifs et neutres multilamellaires [24]. D'un autre côté, Sinico *et al*. ont montré que les vésicules positives permettaient une perméation de la trétinoine similaire voire supérieure aux liposomes chargés négativement [11]. Hasanovic *et al*. ont étudié l'influence de l'ajout de polymères cationiques (chitosan ou Eudragit EPO) sur la stabilité et la pénétration cutanée de liposomes encapsulant soit de l'aciclovir soit du minoxidil. Ils ont montré une augmentation de la stabilité et de la pénétration cutanée grâce à cet ajout. Cette augmentation de pénétration a été expliquée par une tendance des liposomes cationiques à interagir plus fortement avec la surface de la peau ou à une interaction des polymères avec les lipides cutanés, les polymères pénétrant plus profondément en rompant les « tight junctions » dans les couches plus basses de l'épiderme [25].

6) Influence des propriétés de la molécule encapsulée

La bétaméthasone (log *P* entre 1.94 et 2.08 d'après les sources) a été remplacée par son ester plus lipophile, le dipropionate de bétaméthasone (log *P* entre 3.85 et 4.07) [26, 27]. L'utilisation de l'ester nous a permis de montrer que les propriétés de la molécule encapsulée ont une grande importance sur la pénétration cutanée. Si l'on compare la pénétration des liposomes négatifs encapsulant soit la bétaméthasone soit l'ester à celle de leur solution éthanolique respective, l'encapsulation dans les liposomes permet d'augmenter d'un facteur supérieur à 9 la pénétration dans l'épiderme dans le cas de la bétaméthasone alors que l'encapsulation de l'ester l'augmente seulement d'un facteur 1,6. Les liposomes neutres et négatifs encapsulant le dipropionate de bétaméthasone ont cependant permis d'augmenter la pénétration par rapport à la spécialité Diprosone® lotion ($p < 0,05$). Par contre, il n'y a pas de différence entre les liposomes négatifs et la dispersion non liposomale de PC et de dipropionate de bétaméthasone. L'encapsulation sous forme de liposomes serait moins avantageuse dans le cas d'une molécule plus lipophile montrant déjà une bonne pénétration cutanée. Elsayed *et al*. expliquent que pour les molécules hydrophiles, le mécanisme promoteur d'absorption semble jouer un rôle plus important pour l'augmentation de la pénétration cutanée que dans le cas de molécules plus lipophiles. La pénétration cutanée des molécules plus hydrophiles est relativement plus faible et donc pourrait être plus facilement augmentée [16].

ii. Microscopie confocale

La microscopie confocale a été réalisée afin de visualiser la pénétration de liposomes rendus fluorescents. Nous avons pu montrer qu'un marqueur hydrophile (la calcéine, log *P* = -5,02) encapsulé dans la cavité aqueuse des liposomes ne pénétrait pas ou très peu dans l'épiderme en

comparaison avec un marqueur plus lipophile (la rhodamine B, log *P* = 1,95) encapsulé au niveau de la bicouche des liposomes qui pénétrait plus profondément dans l'épiderme et le derme. L'utilisation de la calcéine permet de mimer l'encapsulation des complexes d'inclusion bétaméthasone-cyclodextrines tandis que l'utilisation de la rhodamine B (log *P* proche de celui de la bétaméthasone qui possède un log *P* entre 1,94 et 2,08) permet de mimer l'encapsulation de la bétaméthasone seule au niveau de la bicouche lipidique. Ces résultats sont en accord avec ceux obtenus par dosage de la bétaméthasone au niveau des différentes couches de la peau. L'encapsulation de la bétaméthasone au niveau de la bicouche des liposomes (*cf*. rhodamine B) permettait d'obtenir une meilleure pénétration par rapport à une encapsulation de la bétaméthasone dans la cavité aqueuse des liposomes grâce à l'utilisation des cyclodextrines (*cf*. calcéine).

La microscopie confocale a également permis de mettre en évidence une pénétration des liposomes au niveau des follicules pileux.

Le désavantage de cette méthode est que seules les substances fluorescentes peuvent être étudiées. Il est donc difficile de pouvoir conclure si le marqueur fluorescent (calcéine ou rhodamine B) reste encapsulé ou non dans les liposomes en pénétrant dans l'épiderme. La bicouche des liposomes à été rendue fluorescente par l'ajout de PC fluorescente (NBD-PC). Lorsque l'on considère les liposomes encapsulant la calcéine, comme celle-ci ne pénètre pas ou peu dans l'épiderme alors que la PC fluorescente bien, il semblerait qu'il n'y ait pas de pénétration sous forme encapsulée. Par contre, la rhodamine B suit la pénétration de la PC fluorescente. Cependant, une co-localisation de la rhodamine B et de la PC fluorescente n'a pas pu être démontrée.

Cette méthode ne donne pas non plus d'information sur la quantité absolue de substance dans la peau, mais plutôt une évaluation de la distribution de la molécule utilisée dans les différents compartiments de la peau (épiderme, derme, follicules pileux).

c. In vivo

L'efficacité de gels contenant des liposomes chargés négativement et encapsulant soit la bétaméthasone soit l'ester de bétaméthasone a été évaluée *in vivo* sur des volontaires par le test du blanchiment cutané. Bien que les résultats ne montrent pas de différence significative, cette étude préliminaire semble montrer que les liposomes chargés négativement permettent d'améliorer l'efficacité de la bétaméthasone. Cependant, une étude sur un plus grand nombre de volontaires sera nécessaire pour confirmer cette observation. En ce qui concerne le dipropionate de bétaméthasone, il est difficile de dégager une tendance.

Ces résultats semblent confirmer les résultats obtenus *ex vivo* par dosage du corticostéroïde au moyen des cellules de Franz. L'utilisation des liposomes négatifs semble plus intéressante dans le cas d'une molécule moins lipophile et ne possédant pas une bonne pénétration cutanée intrinsèque comme c'est le cas pour la bétaméthasone par rapport à une molécule plus lipophile et possédant une meilleure pénétration cutanée intrinsèque comme c'est le cas pour le dipropionate de bétaméthasone. La faible augmentation observée *ex vivo* pour l'ester ne semble pas être suffisante pour observer un effet *in vivo*.

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CONCLUSIONS ET PERSPECTIVES

Ce travail a permis de mettre en évidence divers facteurs influençant la pénétration cutanée d'une molécule modèle, la bétaméthasone, encapsulée dans des liposomes. Nous avons ainsi pu mettre en évidence l'influence du mode d'encapsulation de la molécule modèle, l'influence des propriétés de celle-ci, l'influence de l'ajout de différents composants au niveau de la bicouche lipidique des vésicules notamment des tensio-actifs et des charges ainsi que l'influence des propriétés physiques des liposomes (comme le diamètre, la stabilité et le pourcentage d'encapsulation).

Le graphique ci-dessous résume les résultats obtenus avec les différentes formulations testées.

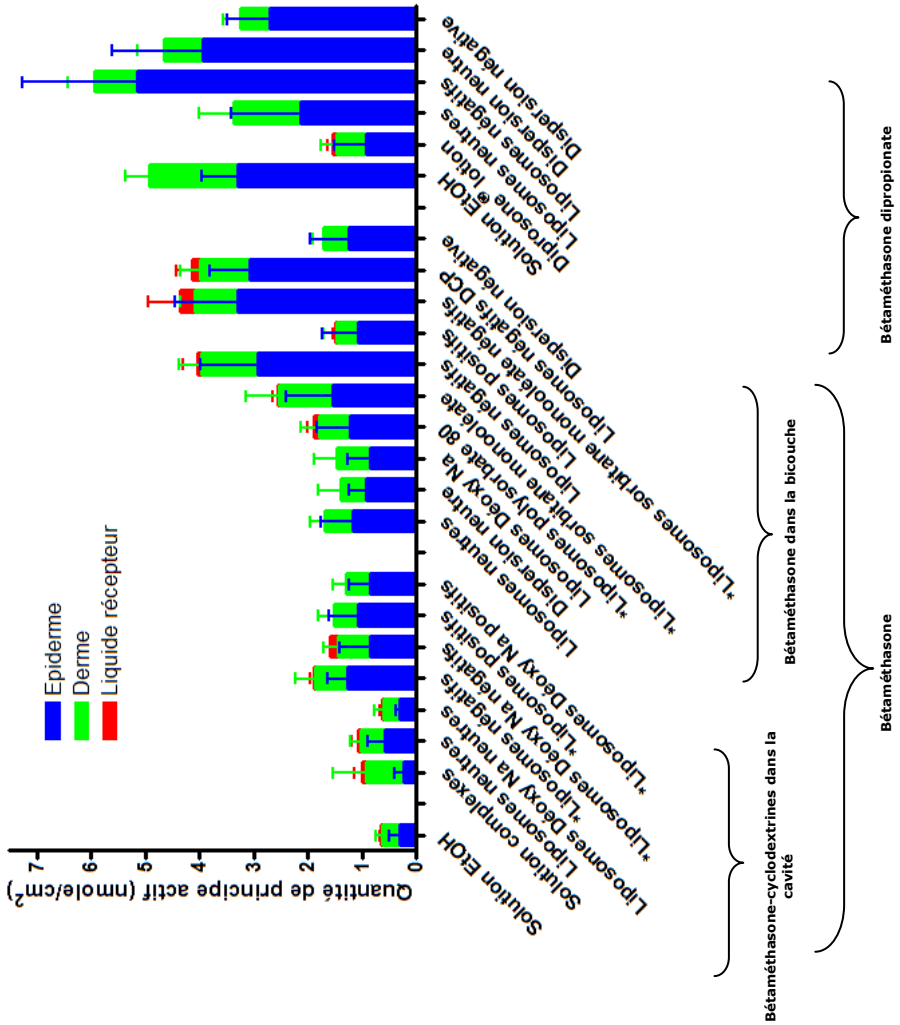

Figure 1 : Quantité de bétaméthasone ou bétaméthasone dipropionate (nmol/cm^2 ± S.D.) dosée au niveau des différentes couches de la peau dont l'épiderme et le derme ainsi qu'au niveau du milieu récepteur des cellules de diffusion de Franz (n = 9) (* résultats non publiés).

Un tableau récapitulatif des différentes formulations testées est repris ci-dessous. Ce tableau classe les formulations d'après leur potentiel à augmenter la pénétration cutanée de la bétaméthasone en trois catégories, depuis le potentiel le plus faible au plus élevé, par rapport à la solution éthanolique de bétaméthasone. Les meilleures formulations contiennent une charge négative, apportée par le DMPA ou le DCP, et encapsulent la bétaméthasone au niveau de leur bicouche lipidique.

Table 1 : Classification des différentes formulations testées d'après leur potentiel à augmenter la pénétration cutanée de la bétaméthasone par rapport à une solution éthanolique de bétaméthasone.

Potentiel	Composition
Faible (facteur <1 à 2)	PC-HPγCD-BMS
	PC-déocycholate Na-HPγCD-BMS
	solution HPγCD-BMS
Moyen (facteur 3 à 5)	PC-DMPA-HPγCD-BMS
	PC-DMPA-déoxycholate Na-HPγCD-BMS
	PC-SA-HPγCD-BMS
	PC-SA-déoxycholate Na-HPγCD-BMS
	PC-BMS
	PC-déoxycholate Na-BMS
	PC-polysorbate 80-BMS
	PC- sorbitane monooléate-BMS
	Dispersion PC-BMS
	Dispersion PC-DMPA-BMS
Elevé (facteur 9 à 11)	PC-DMPA-BMS
	PC- sorbitane monooléate -DMPA-BMS
	PC-DCP-BMS

La microscopie confocale a permis de confirmer que l'encapsulation dans les liposomes chargés négativement permettait d'augmenter la pénétration dans l'épiderme d'un marqueur (rhodamine B) possédant un coéfficient de partage (log *P*) similaire à celui de la bétaméthasone.

L'utilisation du dipropionate de bétaméthasone a permis de mettre en évidence l'influence des propriétés de la molécule encapsulée dans les liposomes sur la pénétration cutanée. L'encapsulation d'une molécule lipophile comme le dipropionate de bétaméthasone, qui possède une bonne pénétration cutanée en solution éthanolique est moins avantageuse en comparaison à l'encapsulation d'une molécule moins lipophile comme la bétaméthasone, qui possède une faible pénétration cutanée intrinsèque. La bétaméthasone est donc une bonne molécule modèle qui nécessite une approche galénique afin d'améliorer sa pénétration cutanée.

En conclusion de la revue, nous avions définit une formulation utopique de liposomes pour améliorer la pénétration cutanée. Ces liposomes devaient montrer des propriétés flexibles de leur bicouche soit par l'addition d'un « edge activator » soit d'éthanol. L'incorporation d'une charge négative devait encore améliorer la pénétration cutanée. En conclusion de nos résultats, les meilleures formulations contiennent une charge négative, apportée par le DMPA ou le DCP, et encapsulent la bétaméthasone au niveau de leur bicouche lipidique. L'ajout d'un « edge activator » ne s'est pas révélé bénéfique.

En ce qui concerne les mécanismes de pénétration des liposomes, les premières études ont montré qu'ils ne pénètreraient pas sous forme intacte dans la peau. L'encapsulation de la bétaméthasone au niveau de la bicouche lipidique diminue sa rétention dans les liposomes tout en augmentant sa pénétration cutanée par rapport à une encapsulation plus stable dans la cavité. Par conséquent, la bétaméthasone serait relarguée des liposomes et pénétrerait ensuite seule dans les couches profondes de l'épiderme. La microscopie confocale confirme cette hypothèse. En effet,

un marqueur hydrophile (calcéine) dans la cavité des liposomes ne pénètre pas dans l'épiderme alors que le matériel lipidique (NBD-PC) y pénètre. De plus, les liposomes encapsulant la bétaméthasone au niveau de leur bicouche donne les mêmes résultats en termes de pénétration cutanée qu'une dispersion non liposomale de PC et bétaméthasone montrant que la formulation sous forme de vésicules ne serait pas un avantage.

La PC semble jouer le rôle de promoteur d'absorption. En effet, la dispersion non liposomale de PC et bétaméthasone permet une meilleure pénétration de la bétaméthasone par rapport à la solution éthanolique ou à la solution de complexes avec les cyclodextrines.

Cependant, dans les dernières études, les liposomes chargés négativement ont montré une meilleure pénétration cutanée de la bétaméthasone par rapport à la dispersion non liposomale correspondante. Dans ce cas-ci, la formulation sous forme de vésicules est donc très importante. Cette observation n'a pas encore pu être expliquée.

Ce travail a soulevé plusieurs questions concernant les mécanismes de pénétration cutanée des liposomes et les interactions occasionnées lors de leur passage. A l'avenir, nous voudrions déterminer les paramètres qui sont bénéfiques pour une augmentation de la pénétration. Nous avons montré qu'une charge négative incorporée dans la bicouche lipidique augmente la pénétration cutanée de la molécule encapsulée. Les perspectives de ce travail seront de comprendre pourquoi une charge négative est favorable. Une charge négative permettrait-elle d'interagir au niveau de la matrice lipidique du *stratum corneum* pour favoriser le passage ?

Il sera d'abord intéressant d'étudier d'une part les propriétés de barrière de la peau afin d'évaluer les paramètres qui sont favorables à la pénétration et d'autre part les vecteurs eux-mêmes. Ensuite, les interactions entre la peau et les vecteurs pourront être étudiées.

Plusieurs explications concernant ces mécanismes ont été apportées dans la littérature. Malheureusement, celles-ci sont divergentes et souvent insatisfaisantes pour expliquer nos résultats. La peau agirait comme une membrane chargée négativement puisque la matrice lipidique du *stratum corneum* contient une grande proportion de lipides portant une charge négative. Les vésicules chargées négativement donneraient des flux supérieurs à ceux des vésicules chargées positivement, qui en contre partie amélioreraient l'accumulation du principe actif dans les couches superficielles de la peau [1]. Cependant, des résultats contradictoires ont été décrits.

Des études ont été réalisées par le laboratoire du Prof. J. Bouwstra (Université de Leiden, Leiden, Pays-Bas) pour tenter de comprendre la pénétration des liposomes déformables. Grâce notamment à la microscopie électronique, Van den Bergh *et al*. ont montré que les liposomes déformables affectaient les régions intercellulaires lamellaires du *stratum corneum* ; leur bicouches lipidiques y formant des petits amas. La microscopie sous excitation à deux photons a permis de montrer que ces vésicules pourraient rompre le caractère compact de ces régions et créer des voies de pénétration définies comme des canaux filiformes [2]. Honeywell-Nguyen *et al*. ont confirmé la présence de ces voies de pénétration et ont pu y observer la présence de vésicules intactes jusqu'au 9ème strip [3]. Une autre équipe s'est également intéressée à la microscopie électronique pour tenter d'expliquer les interactions vésicules-peau. Ils ont montré que les liposomes modifiaient l'ultrastructure des régions intra et intercellulaires de la couche cornée. Les cornéocytes apparaissent gonflés et moins denses aux électrons, les espaces intercellulaires sont plus larges, onduleux et plus irréguliers que la peau traitée au tampon PBS. [1].

Différentes techniques sont disponibles pour étudier les propriétés de barrière de la peau, les propriétés des vecteurs contenant différents marqueurs et les interactions entre ces vecteurs et la peau. Certaines de ces techniques ont déjà été utilisées et pourront être poursuivies.

Des techniques de dosage des marqueurs incorporés dans les vecteurs ont déjà été utilisées lors de la mesure de la quantité de bétaméthasone pénétrée dans les différentes couches de la peau.

Des techniques de visualisation comme la microscopie confocale ou la microscopie électronique sont également disponibles. Dans les études de microscopie confocale, nous avions montré que la rhodamine B encapsulée dans des liposomes chargés négativement ainsi que le matériel lipidique pénètraient dans l'épiderme. L'intégrité physique des liposomes devait, cependant, encore être confirmée. La microscopie électronique pourra montrer si des vésicules intactes peuvent être visualisées dans la peau.
La charge négative des liposomes contenant du DMPA pourrait être visualisée grâce à l'ajout d'un polycation au fixateur comme le rouge ruthénium ou le bleu alcian. Le polycation pourrait immobiliser les charges et la densité électronique du ruthénium et du cuivre serait visualisée en microscopie [4].
Des changements structuraux entre les kératinocytes pourraient être visualisés en STEM (microscopie à balayage électronique).

Des techniques spectroscopiques pourront également être utilisées comme la spectroscopie de réflexion totale atténuée infra-rouge à transformée de fourier (ATR-FTIR) ou la spectroscopie Raman.
La spectroscopie FTIR est basée sur l'absorption d'un rayonnement infrarouge par l'échantillon analysé. Elle permet via la détection des vibrations caractéristiques des liaisons chimiques, d'effectuer l'analyse des fonctions chimiques présentes dans l'échantillon. Cette technique a été utilisée pour étudier les fonctions de barrière de la peau. Elle a permis de mesurer la quantité d'eau présente dans la couche cornée [5], d'étudier les actions de promoteurs d'absorption[6] et d'évaluer la pénétration cutanée de molécules modèles [7].
La spectroscopie Raman est une technique d'analyse non destructive, basée sur la détection des photons diffusés inélastiquement suite à

l'interaction de l'échantillon avec un faisceau de lumière monochromatique. La différence de fréquence entre photon excitateur et photon diffusé renseigne sur la nature chimique de la molécule à l'origine de la diffusion. Cette méthode à été mise à profit pour déterminer la concentration en eau dans le *stratum corneum in vivo* [8] et pour évaluer l'efficacité de promoteurs d'absorption sur la pénétration cutanée *in vivo* de trans-rétinol [9]. La spectroscopie Raman présente l'avantage par rapport à la spectroscopie IR de pouvoir analyser la peau jusqu'à plusieurs centaines de micromètres en-dessous de la surface. En effet, en raison de l'absorption importante des rayons IR moyen et lointain par les molécules d'eau, la pénétration de ces rayons est limitée à quelques micromètres de profondeur [8].

Ces différentes techniques montrent qu'il sera nécessaire d'établir des correspondances entre les méthodes utilisées afin de pouvoir comparer les résultats notamment en utilisant des marqueurs avec des propriétés physico-chimiques proches. C'est par exemple le cas lorsque la bétaméthasone est remplacée par la rhodamine B (log *P* similaires) lors des expériences de microscopie confocale.

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RESUMES

Les systèmes vésiculaires, comme les liposomes, représentent une approche intéressante pour améliorer l'administration cutanée de médicaments. Les liposomes sont des vésicules sphériques composées d'une ou plusieurs bicouches lipidiques entourant une cavité aqueuse. Ils sont capables d'encapsuler des molécules hydrophiles dans leur cavité aqueuse, ainsi que de retenir des molécules hydrophobes dans leur bicouche lipidique.

Dans la première partie de la thèse, nous nous sommes intéressés au développement d'un nouveau système d'administration cutanée combinant les avantages des complexes d'inclusion dans les cyclodextrines et ceux des liposomes déformables, aboutissant à un nouveau concept appelé : « drugs-in-cyclodextrin-in-deformable liposome». La bétaméthasone a été utilisée comme molécule modèle. Ces nouveaux systèmes ont été mis au point et caractérisés avec succès. Les premières études de diffusion réalisées *in vitro*, à travers des membranes synthétiques et au moyen des cellules de diffusion de Franz, étaient prometteuses.

Afin de se rapprocher des conditions *in vivo*, des études de pénétration des liposomes ont alors été mises au point, *ex-vivo*, au moyen de cellules de diffusion et à travers des peaux d'oreilles de cochons. La méthode du « tape stripping » a permis de déterminer la quantité de bétaméthasone dans le *stratum corneum*. La quantité de substance active dans l'épiderme, le derme et le mileu récepteur des cellules de Franz a également été déterminée. Ces méthodes ont été validées avec succés.

Malheureusement, les études sur peaux d'oreilles de cochons n'ont pas permis de confirmer nos premières conclusions.

Les recherches ont alors été orientées vers des études permettant de mieux comprendre les mécanismes de pénétration cutanée des liposomes.

Dans la seconde partie de la thèse, l'influence de différents paramètres sur la pénétration cutanée a été étudiée.

La comparaison de la pénétration cutanée de deux systèmes liposomiaux différents a mis en évidence l'importance de la formulation des liposomes sur leur efficacité. Dans le premier, la bétaméthasone est encapsulée comme précédemment à l'aide de cyclodextrines dans la cavité aqueuse des liposomes déformables. Dans le second système, la bétaméthasone est encapsulée seule au niveau de la bicouche lipidique des liposomes. Ce second système a montré une plus grande perméabilité de la molécule modèle encapsulée. Malgré cette plus faible rétention, le deuxième système a permis d'augmenter la pénétration de la molécule modèle. La plus grande perméabilité et la meilleure pénétration du deuxième système permettent de conclure que la bétaméthasone pénétrerait sous une forme non encapsulée dans les liposomes. De plus, aucune différence n'a été observée entre la pénétration cutanée des liposomes encapsulant la bétaméthasone dans leur bicouche et une dispersion non liposomale.

Plusieurs tensio-actifs ont également été incorporés au niveau de la bicouche des liposomes afin d'obtenir des liposomes déformables. Malheureusement, la faible augmentation de pénétration observée par rapport aux liposomes classiques non déformables n'est pas significative.

L'ajout de lipides chargés au niveau de la bicouche lipidique de liposomes encapsulant soit la bétaméthasone soit le dipropionate de bétaméthasone a été investigué. L'addition de lipides chargés négativement a permis d'augmenter la pénétration de la molécule encapsulée. L'utilisation de liposomes chargés négativement a permis d'augmenter la quantité de bétaméthasone au niveau de l'épiderme d'un facteur 9,3 par rapport à une solution éthanolique de bétaméthasone. Cette augmentation n'a pas été observée avec la dispersion non liposomale correspondante, indiquant que la formulation sous forme de vésicules est très importante. En ce qui concerne le dipropionate de bétaméthasone, l'encapsulation dans les

liposomes chargés négativement a augmenté la quantité d'ester dans l'épiderme d'un facteur 5,5 par rapport à la spécialité Diprosone® lotion et seulement d'un facteur 1,6 par rapport à la solution éthanolique.
L'encapsulation dans les liposomes chargés négativement semble donc moins avantageuse dans le cas d'une molécule plus lipophile, comme le dipropionate de bétaméthasone et qui possède déjà une bonne pénétration cutanée intrinsèque par rapport à une molécule plus hydrophile comme la bétamethasone.

La pénétration de liposomes rendus fluorescents a été visualisée grâce à la microscopie confocale. Un marqueur lipophile, la rhodamine B, encapsulé dans la bicouche comme la bétaméthasone, pénètre bien dans la peau de la même façon que la phosphatidylcholine fluorescente. Par contre, un marqueur hydrophile, la calcéine, encapsulé dans la cavité des liposomes comme les complexes bétaméthasone-cyclodextrines, ne pénètre pas dans l'épiderme. Ces observations confirment les résultats de dosage *ex vivo* au moyen des cellules de Franz. De plus, la pénétration dans la peau de la rhodamine B semble être plus importante lorsqu'elle est encapsulée dans les liposomes chargés négativement par rapport aux liposomes non chargés.

Enfin, l'efficacité des liposomes négatifs a été évaluée *in vivo* sur des volontaires grâce à l'évaluation du pouvoir de blanchiement des corticostéroïdes. Le gel de carbomère contenant les liposomes chargés négativement semble augmenter la réponse de la bétaméthasone par rapport au gel éthanolique d'hydroxypropylcellulose. Cependant, les résultats ne sont pas significatifs. Une explication pourrait être le faible nombre de volontaires.
Par contre, dans le cas des liposomes contenant le dipropionate de bétaméthasone, il a été plus difficile de dégager une tendance.
Ces résultats semblent confirmer que l'utilisation des liposomes chargés négativement est plus intéressante dans le cas d'une molécule moins

lipophile comme la bétamethasone par rapport à une molécule plus lipophile comme le dipropionate de bétaméthasone.
Ces résultats nécessitent cependant d'être confirmés sur un plus grand nombre de volontaires.

Vesicular delivery systems, like liposomes, represent an attractive approach to improving skin delivery of drugs. Liposomes are composed of one or several lipid bilayers surrounding an aqueous compartment. They are able to encapsulate hydrophilic compounds into their inner compartment and lipophilic compounds into their lipid bilayers.

In the first part of this thesis, we developed a new dermal delivery system combining the advantages of cyclodextrin inclusion complexes and those of deformable liposomes. This system was called "drugs-in-cyclodextrin-in-deformable liposome". Betamethasone was chosen as the model drug. These systems were successfully developed and characterised. *In vitro* diffusion studies using Franz diffusion cells and polycarbonate membranes gave promising results.

In order to better mimic the *in vivo* conditions, *ex vivo* penetration studies using pig ear skin and Franz diffusion cells were developed. The tape stripping method was used to determine the amount of drug in the *stratum corneum*. The amount of drug in the epidermis, dermis and receptor medium was also assayed. These methods were successfully validated.

Unfortunately, *ex vivo* penetration studies of the developed skin delivery system could not confirm the promising *in vitro* results.

Our research was then directed towards studies allowing a better understanding of liposome skin penetration mechanisms. In the second part of this thesis, the influence of several parameters on the skin penetration behaviour of liposomes was investigated.

The formulation importance on skin penetration efficiency was highlighted by the comparison of two liposomal systems. In the first system,

betamethasone is encapsulated by the help of cyclodextrin inclusion complexes in the aqueous compartment. In the second system, betamethasone is encapsulated alone in the lipid bilayer. The second system shows high membrane permeability of the encapsulated betamethasone. Despite this decreased drug retention, the second system enhances the skin penetration of betamethasone. This high membrane permeability and the better penetration of the second formulation support the mechanism of penetration as a free drug substance. In addition, no difference in penetration is observed between liposomes containing betamethasone alone and a non liposomal dispersion.

Several surfactants or "edge activators" were incorporated in the lipid bilayer of liposomes in order to obtain deformable liposomes. Unfortunately, these deformable liposomes are unable to enhance skin penetration compared to classical non deformable liposomes.

The addition of charged lipids in the lipid bilayer of liposomes encapsulating either betamethasone or betamethasone dipropionate was investigated. The addition of negatively charged lipids enhances the penetration of the encapsulated drug. The use of negatively charged liposomes enhanced the epidermis accumulation of betamethasone 9.3 times compared with the ethanolic solution. This improvement is not observed with the corresponding non liposomal dispersion, indicating that the vesicle form is of high importance. In the case of the ester, the use of negatively charged liposomes enhances the epidermis accumulation 5.5 times compared with the Diprosone® lotion and only 1.6 times compared with the ethanolic solution. The penetration of a more lipophilic drug, with a high intrinsic penetration, is less enhanceable when incorporated in liposomes.

Confocal microscopy was performed to visualise skin penetration of fluorescently labelled liposomes. The lipophilic dye rhodamine B encapsulated in the lipid bilayer as betamethasone penetrates the

epidermis at the same level as NBD-phosphatidylcholine. On the other hand, the hydrophilic dye calcein encapsulated in the aqueous compartment as betamethasone-cyclodextrin complexes does not penetrate the epidermis. These observations seem to confirm the *ex vivo* results. In addition, the skin penetration of rhodamine B seems to be improved by the incorporation in negatively charged liposomes compared with classical ones.

Finally, the efficiency of the negatively charged vesicles was evaluated *in vivo* on volunteers by the skin blanching assay. Carbomer gel containing the negatively charged vesicles seems to enhance the blanching response of encapsulated betamethasone in comparison with the ethanolic hydroxypropylcellulose gel. However, results are not significantly different. This could be explained by the small number of volunteers. On the other hand, in the case of formulations containing betamethasone dipropionate, no tendency could be highlighted. These results seem to confirm that the use of negatively charged liposomes is more attractive for improving the efficiency of a lesser lipophilic drug, betamethasone, in comparison with the more lipophilic ester, betamethasone dipropionate. These results need to be confirmed using a higher number of volunteers.

ANNEXES

1. Validation des méthodes analytiques

1.1 Bétaméthasone

Le dosage de la bétaméthasone extraite des peaux a été validé avec succès. Le risque α est de 10%. Les limites d'acceptation ont été placées à 15% de 10000,0 ng/ml à 60,0 ng/ml puis à 30% de 60,0 ng/ml à 24,2ng/ml. Le modèle le plus approprié est une régression linéaire pondérée en $1/x^2$ (Figure 1)

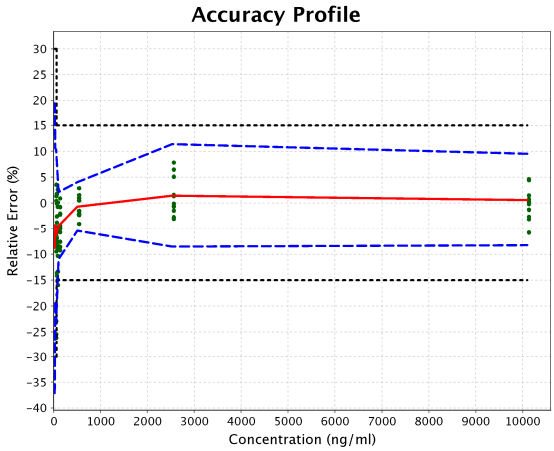


Figure 1 : Profil d'exactitude de la bétaméthasone dans les extraits de peaux d'oreilles de cochon en appliquant le modèle de la régression linéaire pondérée en $1/x^2$.

Le dosage de la bétaméthasone extraite à l'aide des strips a été pré-validé (une série) avec succès. Le risque α est de 5%. Les limites d'acceptation ont été placées à 10% de 10010,0 ng/ml à 20,02ng/ml. Le modèle le plus approprié est une régression linéaire pondérée en $1/x^2$ (Figure 2).

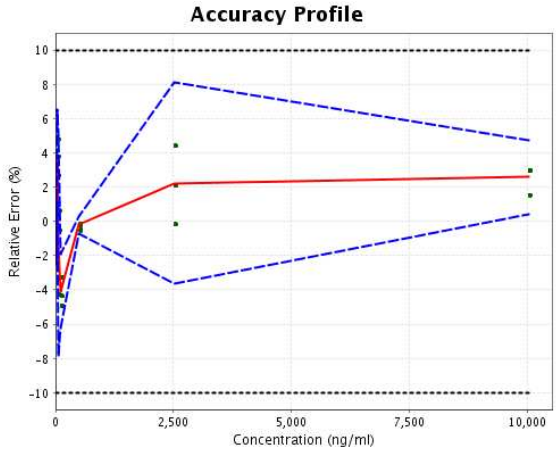


Figure 2 : Profil d'exactitude de la bétaméthasone extraite à l'aide des strips en appliquant le modèle de la régression linéaire pondérée en $1/x^2$.

1.2 Dipropionate de bétaméthasone

Le dosage du dipropionate de BMS extrait des peaux a été validé avec succès. Le risque α est de 10%. Les limites d'acceptation ont été placées à 15% de 10000,0 ng/ml à 100,0 ng/ml puis à 30% de 100,0 ng/ml à 40,92 ng/ml. Le modèle le plus approprié est une régression linéaire.

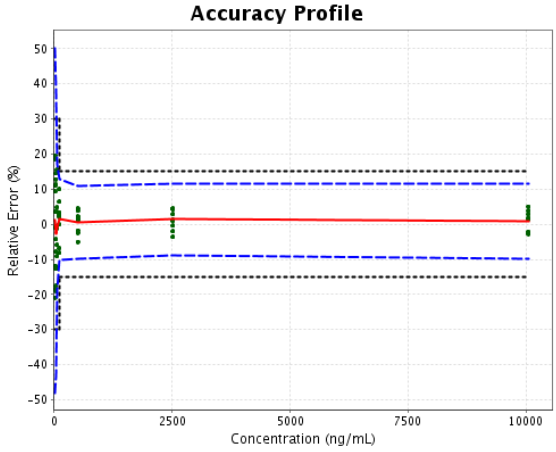


Figure 3 : Profil d'exactitude du dipropionate de bétaméthasone dans les extraits de peaux d'oreilles de cochon en appliquant le modèle de la régression linéaire.

Le dosage du dipropionate de bétaméthasone extrait à l'aide des strips a été pré-validé (une série) avec succès. Le risque α est de 10%. Les limites d'acceptation ont été placées à 10% de 10115,0 ng/ml à 100,0 ng/ml puis à 30% de 100,0 ng/ml à 20,23 ng/ml. Le modèle le plus approprié est une régression linéaire (Figure 4).

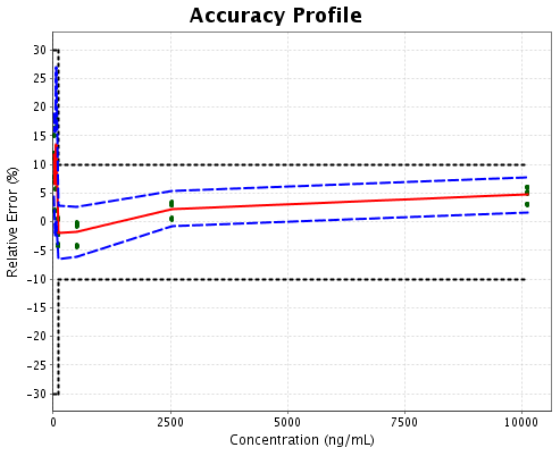


Figure 2 : Profil d'exactitude du dipropionate de bétaméthasone extrait à l'aide des strips en appliquant le modèle de la régression.

2. Dossier Comité d'Ethique Hospitalo-Facultaire Universitaire de Liège

2.1 Lettre d'acceptation

Comité d'Ethique Hospitalo-Facultaire Universitaire de Liège (707)

Université de Liège

CHU de Liège

Sart Tilman, le 12 octobre 2010

Monsieur le **Prof. G. PIERARD**
Madame le **Prof. B. EVRARD**
Mesdames les **Drs. G PIEL et A GILLET**
DERMATOPATHOLOGIE
ET TECHNOLOGIE PHARMACEUTIQUE
CHU B35

Concerne: Votre demande d'avis au Comité d'Ethique
Nr belge : B70720109043; Notre réf: 2010/145

Cher Collègue,

J'ai le plaisir de vous informer que le Comité d'Ethique a donné une réponse favorable à votre demande d'avis intitulée :

"Evaluation de l'efficacité de gels dermatologiques à base de liposomes contenant un corticoïde. "

Vous trouverez, sous ce pli, le formulaire de réponse reprenant, en français et en anglais, les différents éléments examinés et approuvés et la composition du Comité d'Ethique.

Je vous prie d'agréer, Cher Collègue, l'expression de mes sentiments les meilleurs,

Prof. G. RORIVE
Vice-Président du Comité d'Ethique

Note: l'original de la réponse est envoyé au Chef de Service, une copie à l'Expérimentateur principal.

Copie à la **Direction de l'AFMPS**

2.2 Protocole complet d'expérimentation

Titre du projet:

Evaluation de l'efficacité de gels dermatologiques à base de liposomes contenant un corticoïde.

Objectif :

Comparer l'effet de blanchiment ainsi que la profondeur de pénétration dans le *stratum corneum* de la bétaméthasone ou du dipropionate de bétaméthasone lorsque ceux-ci sont encapsulés dans des liposomes classiques à l'efficacité obtenue lorsqu'ils sont encapsulés dans des liposomes contenant soit un tensio-actif (liposomes dits déformables) soit une charge (liposomes chargés). La bétaméthasone sera encapsulée soit seule soit à l'aide de cyclodextrines, le dipropionate de bétaméthasone sera encapsulé seul.

Les liposomes chargés ou contenant un tensio-actif sont des vésicules, qui pénétreraient dans la peau permettant d'y atteindre des concentrations efficaces en principe actif. En pratique nous devrions montrer que ces liposomes permettront d'obtenir un blanchiment plus important ainsi qu'une pénétration plus profonde que des liposomes classiques.

Cette étude constitue la dernière partie avant d'aboutir au dépôt d'une thèse en sciences biomédicales et pharmaceutiques.

Préparations testées :

Gels topiques contenant :

- Des liposomes classiques encapsulant la bétaméthasone soit seule soit à l'aide de cyclodextrines.
- Des liposomes soit déformables soit chargés encapsulant la bétaméthasone soit seule soit à l'aide de cyclodextrines ou du dipropionate de bétaméthasone.
- Des complexes d'inclusion bétaméthasone-cyclodextrines.

- De la bétaméthasone ou du dipropionate de bétaméthasone seul.

Des seringues à tuberculine sans aiguille (pour usage topique) seront remplies avec les différentes préparations et identifiées de façon à maintenir le double aveugle. Un étalonnage gravimétrique permettra de déterminer la quantité de gel dispensée par graduation.

Sujets :

Les sujets seront inclus sur base des critères suivants :

(1) âge compris entre 18 et 65 ans ;
(2) aucun traitement médicamenteux en cours pendant l'étude, à l'exception des traitements contraceptifs ;
(3) aucune administration topique de corticostéroïdes sur les bras dans le mois précédent l'étude ;
(4) aucune lésion visible de la peau sur les avant-bras ;
(5) pas d'allergie connue aux corticostéroïdes ou au sparadrap ;
(6) pas de dépendance nicotinique ni d'antécédent d'hypertension artérielle sévère.

Procédure :

Une étude en double aveugle sera menée sur des sujets volontaires caucasiens. Pendant toute la durée de l'étude, il sera demandé aux sujets de ne pas pratiquer d'efforts intensifs, de ne pas se doucher ou se laver, ni d'appliquer une quelconque préparation sur les bras.

Préalablement à l'étude à proprement parler, un pré-test sera réalisé afin de s'assurer que chaque sujet répond aux corticostéroïdes. Une dose de Diprosone® crème (Diprosone® crème, Schering-Plough Labo n.v., Heist-op-den-Berg, Belgique) sera appliquée sur la partie supérieure du bras, au niveau du biceps. Le blanchiment sera visuellement apprécié après 5 heures d'application dans des conditions non occlusives (compresses Melolin®- Smith & Nephew Medical Limited, Hull, Grande-Bretagne).

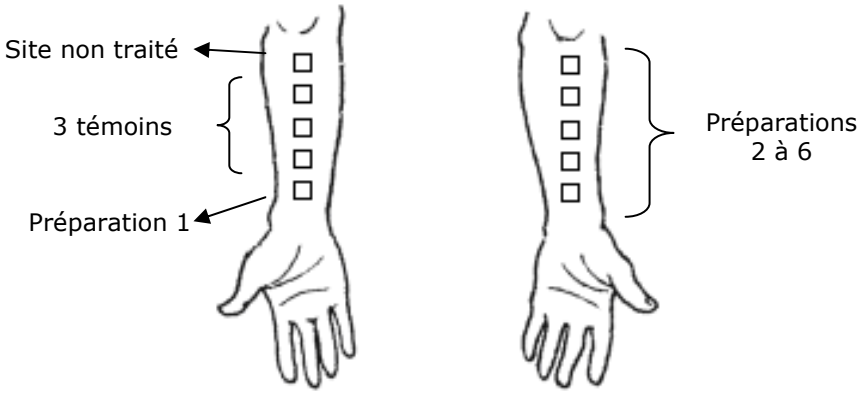
L'étude inclura une dizaine de volontaires. Le premier jour, la procédure du test sera expliquée aux volontaires et l'investigateur vérifiera l'absence de lésions cutanées sur les avant-bras. Cinq carrés de 2 cm de côté ($4cm^2$) seront dessinés avec précision à l'aide d'un crayon dermographique sur la face antérieure des avant-bras (voir schéma). La position des sites sera déterminée de telle façon que les centres de deux carrés adjacents soient distants d'au moins 4 cm et que l'ensemble des carrés soit confiné sur la surface de la face antérieure de l'avant-bras délimitées depuis 3 cm sous la fosse antécubitale et 3 cm au dessus du poignet. Les zones velues, les naevi et les vaisseaux sanguins visibles seront évités. Après repos de l'avant-bras en position horizontale pendant 5 minutes, les mesures chromamétriques seront réalisées (chromamètre Visi-Chroma VC-100®). Chaque zone sera mesurée de façon indépendante à 4 reprises et la valeur moyenne sera calculée. Les volontaires recevront 9 seringues numérotées et un schéma d'application topique. Un site devra rester non traité, trois sites recevront les préparations témoins (bétaméthasone seule (1) ou encapsulée dans des cyclodextrines (2), dipropionate de bétaméthasone seul (3), sans liposomes), chaque autre site recevra une préparation de liposomes encapsulant de la bétaméthasone soit seule soit à l'aide de cyclodextrines ou du dipropionate de bétaméthasone. Au jour 2, les sujets appliqueront les préparations au centre des zones, en respectant le schéma personnel d'application délivré. Il sera demandé aux volontaires de masser délicatement les zones traitées pendant 30 secondes, en respectant les contours dessinés. Les zones seront ensuite recouvertes par des compresses non occlusives maintenues sur leurs côtés à l'aide d'adhésifs hypoallergéniques. 5 heures plus tard, les compresses seront retirés et les éventuels excédents de préparations enlevés sans frotter à l'aide de coton-tiges secs. Après repos des bras pendant 5 minutes en position horizontale, les paramètres de couleurs de la peau seront mesurés comme précédemment décrit à l'aide du chromamètre. Immédiatement après ces mesures, les contours de la zone blanchie seront appréciés visuellement

par l'investigateur et délimitées au feutre indélébile noir. A l'aide d'un calque, ces traits seront transférés sur un support papier.
La profondeur de pénétration de la bétaméthasone ou du dipropionate de bétaméthasone sera ensuite évaluée par la méthode du stripping. Cette méthode consiste à décoller successivement de fines couches de cellules du *stratum corneum* au moyen d'un papier adhésif, appelé strip. La quantité exacte de corticoïde retenue sur chaque strip pourra être dosée par une méthode chromatographique. Une quinzaine de strips est généralement nécessaire pour retirer la couche cornée. Une mesure de la perte transépithéliale en eau permettra de confirmer l'enlèvement complet de cette couche cornée.

Préparations appliquées :

(1) Un gel contenant de la bétaméthasone seule.
(2) Un gel contenant des complexes bétaméthasone-cyclodextrines.
(3) Un gel contenant du dipropionate de bétaméthasone seul.
(4) Un gel contenant de la bétaméthasone encapsulée dans des liposomes classiques.
(5) Un gel contenant des complexes bétaméthasone-cyclodextrines encapsulés dans des liposomes classiques.
(6) Un gel contenant de la bétaméthasone encapsulée dans des liposomes déformables.
(7) Un gel contenant de la bétaméthasone encapsulée dans des liposomes chargés.
(8) Un gel contenant du dipropionate de bétaméthasone encapsulé dans des liposomes chargés.
(9) Un gel contenant des complexes bétaméthasone-cyclodextrines encapsulés dans des liposomes chargés.

Schéma d'application :



Les liposomes classiques sont constitués de phosphatidylcholine de soja. Les liposomes déformables contiennent de la phosphatidylcholine de soja et du Span 80®. Les liposomes chargés contiennent de la phosphatidylcholine de soja, de l'acide dimyristoyl phosphatidique et du dicétyl phosphate.
Les préparations sont gélifiées au moyen de carbomère (Carbopol NF® 80), et de trométamol.
Toutes les préparations contiendront de la bétaméthasone ou du dipropionate de bétaméthasone à une concentration de 150 µg/g ou 193µg/g respectivement.

Risques potentiels :
Les risques potentiels sont extrêmement faibles. Appliqués séparément les cyclodextrines, les composants des liposomes (phosphatidylcholine de soja, Span 80®, acide dimirystoyl phosphatidique et dicétyl phosphate) ainsi que la bétaméthasone ou le dipropionate de bétaméthasone ne provoquent pas d'irritation et sont généralement bien tolérés. Comme toute substance appliquée sur la peau, de rares cas d'allergies sont possibles. La méthode du stripping est pratiquement indolore vu quelle n'enlève que les cellules mortes de la couche superficielle de l'épiderme.

2.3 Formulaire d'information aux volontaires

Titre du projet:
Evaluation de l'efficacité de gels dermatologiques à base de liposomes contenant un corticoïde.

Objectif:
Comparer l'effet de blanchiment ainsi que la profondeur de pénétration dans le *stratum corneum* de la bétaméthasone ou du dipropionate de bétaméthasone lorsque ceux-ci sont encapsulés dans des liposomes classiques à l'efficacité obtenue lorsqu'ils sont encapsulés dans des liposomes contenant soit un tensio-actif (liposomes dits déformables) soit une charge (liposomes chargés). La bétaméthasone sera encapsulée soit seule soit à l'aide de cyclodextrines, le dipropionate de bétaméthasone sera encapsulé seul.
Les liposomes chargés ou contenant un tensio-actif sont des vésicules, qui pénétreraient dans la peau permettant d'y atteindre des concentrations efficaces en principe actif. En pratique nous devrions montrer que ces liposomes permettront d'obtenir un blanchiment plus important ainsi qu'une pénétration plus profonde que des liposomes classiques.

Modalités :
9 préparations à base de bétaméthasone ou de dipropionate de bétaméthasone seront appliquées et laissées en contact avec la peau pendant 5 heures. Une évaluation du pouvoir blanchissant se visualisera par une différence de coloration de la peau. Une mesure colorimétrique se fera directement sur la peau à l'aide d'un chromamètre. Une évaluation de la profondeur de pénétration se fera par la méthode du stripping. Cette méthode consiste à décoller successivement de fines couches de cellules du *stratum corneum* au moyen d'un papier adhésif, appelé strip. Une quinzaine de strips est généralement nécessaire pour retirer la couche

cornée. Une mesure de la perte transépithéliale en eau permettra de confirmer l'enlèvement complet de cette couche cornée.
Tous les frais inhérents à l'étude sont pris en charge par l'expérimentateur.

Risques potentiels :
Les risques potentiels sont extrêmement faibles. Appliqués séparément les cyclodextrines, les composants des liposomes (phosphatidylcholine de soja, Span 80®, acide dimyristoyl phosphatidique et dicétyl phosphate) ainsi que la bétaméthasone ou le dipropionate de bétaméthasone ne provoquent pas d'irritation et sont généralement bien tolérés. Comme toute substance appliquée sur la peau, de rares cas d'allergies sont possibles. La méthode du stripping est pratiquement indolore vu quelle n'enlève que les cellules mortes de la couche superficielle de l'épiderme.

Participation :
La participation est volontaire et peut être suspendue à tout instant. La participation n'est pas rémunérée.
Les expérimentateurs assureront l'anonymisation des données. Les résultats de cette étude resteront confidentiels.

Assurance :
Le risque résultant de cette expérimentation est couvert conformément à l'article 29 de la loi belge du 7 mai 2004 relative aux expérimentations sur la personne humaine qui impose au promoteur d'assumer, même sans faute, la responsabilité du dommage causé au participant et aux ayants-droit, dommage lié de manière directe ou indirecte à l'expérimentation. Le promoteur à contracté une assurance couvrant cette responsabilité.

L'étude a été examinée et approuvée par le Comité d'éthique.

Le volontaire conserve une copie de ce document