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Ghenwa Nasr

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Etude de l'effet d'agents actifs et des nanovecteurs sur la perméabilité d'un produit hydrophile à travers des membranes liposomiales contenant différents taux de Cholestérol

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Résumé

La stabilité membranaire et particulièrement la perméabilité de la membrane des liposomes constitue un sujet de préoccupation dans la conception des formulations liposomales visant la délivrance de principes actifs. La composition membranaire des liposomes dicte les propriétés de la membrane : le taux de cholestérol (CHOL) joue alors un rôle primordial dans le maintien de la perméabilité membranaire. Ce travail de recherche vise à étudier la perméabilité membranaire de liposomes formés de dipalmitoylphosphatidylcholine (DPPC) et de différents taux de CHOL en présence d'agents actifs et de nanovecteurs. Des liposomes formés de DPPC et de CHOL et encapsulant un agent fluorescent hydrophile, la sulforhodamine B (SRB), sont préparés par la méthode d'évaporation en phase inverse suivie d'une extrusion. L'étude de perméabilité membranaire est effectuée en suivant le relargage de la SRB des liposomes au cours du temps et à 37 °C. Dans un premier temps, l'effet d'une série de 22 composés d'huiles essentielles (HE) sur la perméabilité membranaire des liposomes a été étudiée à un rapport molaire DPPC:HE 100:25. Les résultats ont démontré que la perméabilité membranaire dépend principalement de la structure de la molécule de HE, son hydrophobicité ainsi que le taux de CHOL dans la membrane. Il ressort également de cette étude que le CHOL réduit la perméabilité des composés de HE qui disparaît pour des taux supérieurs à 40 % CHOL. Dans un deuxième temps, l'effet des cyclodextrines (CDs) (3 CDs natives et 4 dérivés de la β -CD) sur la perméabilité membranaire des liposomes a été étudié. Les résultats ont révélé la dépendance de l'effet de ces CDs de plusieurs facteurs notamment : le taux de CHOL, la concentration de la CD et son type qui est déterminé par son affinité pour les composants lipidiques de la membrane ainsi que le temps d'incubation. Les membranes pauvres en CHOL étaient sensibles aux CDs qui présentent une affinité aux phospholipides (α -CD, la dérivée méthylée de la β -CD "CRYSMEB", la dérivée hydroxypropylée de la β -CD "HP- β -CD" et la dérivée sulfobutylée de la β -CD "SBE- β -CD") alors que les membranes riches en CHOL étaient sensibles aux CDs capables d'extraire le CHOL de la membrane (la β -CD et sa dérivée méthylée "RAMEB"). La dernière étude porte sur l'effet des complexes d'inclusion HP- β -CD/thymol et RAMEB/thymol sur la perméabilité des membranes liposomales formées de DPPC et CHOL au rapport molaire 100:25 et 100:100. Le complexe d'inclusion HP- β -CD/thymol ayant une constante de formation faible protège légèrement la membrane de l'effet du thymol et de la HP- β -CD à 25 % CHOL alors que le complexe RAMEB/thymol de constante de formation plus élevée exerce un effet protecteur de la membrane contre l'effet de la RAMEB et du thymol libre à

25 et 100 % CHOL, l'effet protecteur étant plus prononcé à 100 % CHOL. L'ensemble de ces résultats contribue à la compréhension des facteurs modulant la perméabilité membranaire et au développement des formulations liposomales stables pour l'encapsulation des molécules bioactives et des nanovecteurs tels que les CDs et les complexes d'inclusion CD/invité.

Mots-clés : cholestérol, composés d'huiles essentielles, complexes d'inclusion CD/invité, cyclodextrines, fluorescence, liposomes, perméabilité membranaire.

Abstract

The membrane stability, namely the permeability of the liposome membrane remains a matter of concern in the design of liposomal formulations aiming the delivery of active ingredients. The membrane composition of liposomes dictates the properties of the membrane: the cholesterol (CHOL) content plays a key role in maintaining the membrane permeability. Hence, our work aims to study the membrane permeability of liposomes composed of dipalmitoylphosphatidylcholine (DPPC) and different CHOL content in the presence of active agents and nanovectors. Liposomes made of DPPC and CHOL and encapsulating a hydrophilic fluorescent agent, the sulforhodamine B (SRB), are prepared by the reverse phase evaporation method followed by extrusion. The membrane permeability study is carried out by following the release of SRB from liposomes over time at 37 °C. First, the effect of a series of 22 essential oil (EO) components on the membrane permeability of liposomes was investigated at the DPPC:EO molar ratio 100:25. The results demonstrated that the membrane permeability mainly depends on the structure of the EO molecule, its hydrophobicity and the CHOL content in the membrane. We also found that CHOL reduces the effect of HE compounds, and this effect disappears beyond 40% CHOL. Secondly, the effect of cyclodextrins (CDs) (the 3 native CDs and 4 β -CD derivatives) on the membrane permeability of liposomes was studied. The obtained results revealed the dependence of the effect of these CDs on many factors including: the CHOL content, the concentration of the CD, the CD type which is determined by its affinity for the lipid membrane components as well as the time of incubation. CHOL poor membranes were sensitive to CDs showing a high affinity to phospholipids (α -CD, the methylated β -CD derivative "CRYSMEB", the hydroxypropylated β -CD derivative "HP- β -CD" and the sulfobutylated β -CD derivative SBE- β -CD) while CHOL rich membranes were sensitive to CDs that can extract CHOL from the membrane (β -CD and its methylated derivative "RAMEB"). Finally, we studied the effect of HP- β -CD / thymol and RAMEB / thymol inclusion complexes on the permeability of liposomal membranes composed of DPPC and CHOL at the molar ratio 100:25 and 100:100. Results demonstrated that HP- β -CD/thymol the inclusion complex having a low formation constant protects slightly the membrane from the effect of thymol and HP- β -CD at 25 % CHOL only while RAMEB/thymol inclusion complex having a high formation constant protects the membrane from the effect of RAMEB and thymol at 25 and 100 % CHOL, with a larger extent at 100 % CHOL. All these results contribute to the better understanding of the factors modulating the membrane

permeability and to the development of stable liposome formulations for the encapsulation of bioactive molecules and nanovectors such as CDs and CD / guest inclusion complexes.

Keywords: cholesterol, cyclodextrins, CD/drug inclusion complex, essential oil components, fluorescence, liposome, membrane permeability.

Liste des abréviations

CHOL: cholestérol

CD: cyclodextrines

CF: carboxyfluorescéine

CRYSMEB: β -cyclodextrine méthylée en position 2

DCL: drug-in-cyclodextrin-in-liposome

DLPG: Dilauroyl phosphatidylglycerol

DMPA : Dimyristoyl phosphatidic acid

DMPC: Dimyristoyl phosphatidylcholine

DMPE : Dimyristoyl phosphatidyl ethanolamine

DMPG : Dimyristoyl phosphatidylglycerol

DMPS : Dimyristoyl phosphatidylserine

DOPA : Dioleoyl phosphatidic acid

DOPC : Dioleoyl phosphatidylcholine

DOPE : Dioleoyl phosphatidylethanolamine

DOPG : Dioleoyl phosphatidylglycerol

DPPA : Dipalmitoyl phosphatidic acid

DPPC: dipalmitoylphosphatidylcholine

DPPE: Dipalmitoyl phosphatidyl ethanolamine

DPPG: Dipalmitoyl phosphatidylglycerol

DPPS : Dipalmitoyl phosphatidyl serine

DSPC: Distearoyl phosphatidylcholine

DSPE : Distearoyl phosphatidylethanolamine

DSPG : Distearoyl phosphatidylglycerol

HE: huile essentielle

HP- β -CD: hydroxypropyle- β -cyclodextrine

H-PC: hydrogenated phosphatidylcholine

Lo: liquid-ordered phase

L_d: liquid-disordered phase

LUVs: large unilamellar vesicles (vésicules larges unilamellaires)

PA: principe actif

PE: Phosphatidylethanolamine

PI: phosphatidylinositol

POPC: Palmitoyloleoyl phosphatidylcholine

POPG : Palmitoyloleoyl phosphatidylglycerol

PS: phosphatidylserine

RAMEB: the randomly methylated β -CD derivative

SBE- β -CD: sulfobutylether- β -CD

So: solid-ordered phase

SRB: sulforhodamine B

Introduction générale

Toutes les cellules vivantes sont entourées d'une membrane plasmique qui isole la cellule de son environnement. Toutefois, la membrane biologique n'est pas une barrière absolue car la cellule doit échanger continuellement avec l'extérieur de la matière. Il s'agit plutôt d'une barrière semi-perméable sélective qui permet le passage préférentiel des substances à travers la bicouche phospholipidique (Biochemistry of lipids, lipoproteins and membranes, 2015; Lodish, 2000). C'est ainsi que la perméabilité membranaire se définit comme étant la capacité d'un soluté ou d'une substance à franchir la barrière lipidique de la membrane (Frézard, 1999). Cette propriété se révèle d'une grande importance, puisqu'elle joue un rôle clé dans de nombreuses fonctions cellulaires vitales. De ce fait, le maintien de la perméabilité membranaire s'avère crucial.

Les liposomes sont des vésicules constituées de bicouches phospholipidiques renfermant un ou plusieurs compartiments aqueux. Communément utilisés en tant que modèles membranaires, les liposomes tentent de reproduire la structure et les propriétés de la membrane biologique. Ils servent à l'étude des propriétés membranaires telles que la fluidité, la stabilité et la perméabilité afin de mieux comprendre les mécanismes moléculaires se déroulant au niveau de la membrane (Eeman and Deleu, 2010; Sebaaly et al., 2019). De plus, les liposomes fournissent une base moléculaire pour l'étude de l'activité de certaines molécules ou agents bioactifs au niveau de la membrane permettant ainsi d'élucider leur mécanisme d'action (Eeman and Deleu, 2010). Effectivement, l'activité biologique exercée par de nombreuses molécules tels que les huiles essentielles (HE) est intimement liée à leur action au niveau de la membrane biologique (Bakkali et al., 2008). D'autre part, les liposomes ont été largement utilisés en tant que véhicules efficaces pour des médicaments hydrophiles et lipophiles (Choudhury, 2020). Cependant, la fuite des agents encapsulés constitue un problème majeur qui limite l'efficacité de ce système. C'est ainsi que la stabilité membranaire et particulièrement le contrôle de la perméabilité membranaire demeure un sujet de préoccupation des chercheurs depuis plusieurs décennies.

Les travaux menés sur la stabilité membranaire des liposomes se sont concentrés sur l'importance de la composition membranaire en tant que déterminant principal des propriétés physiques de la membrane liposomale. Dans ce contexte, le cholestérol (CHOL) est un constituant majeur de la membrane et il est connu comme un modulateur puissant de la perméabilité membranaire. En effet,

de nombreuses études ont démontré que le CHOL rend les membranes plus rigides et stables et moins perméables (Briuglia et al., 2015; Casciola et al., 2014; Cócera et al., 2003; de Meyer and Smit, 2009; Subczynski et al., 2017). De surcroît, le CHOL réduit la perméabilité membranaire de manière dose-dépendante (Kaddah et al., 2018). Par conséquent, le contenu membranaire en CHOL devrait être minutieusement choisi afin d'obtenir une formulation liposomale stable qui réduit et contrôle la libération des principes actifs encapsulés d'une part et qui présente une grande efficacité d'encapsulation d'autre part.

Ce travail de thèse a été réalisé dans le "Laboratoire de Molécules Bioactives" (Faculté des Sciences, Section II, Université Libanaise) et le "Laboratoire d'Automatique et de Génie des Procédés" (Université Claude Bernard Lyon 1). Il porte principalement sur l'étude de l'effet des agents actifs et des nanovecteurs sur la perméabilité membranaire des liposomes formés de dipalmitoyl phosphatidylcholine (DPPC) et différents taux de CHOL et encapsulant un agent fluorescent hydrophile, la sulforhodamine B (SRB). La perméabilité membranaire est évaluée par le suivi du relargage de la SRB des liposomes au cours du temps.

En premier lieu, nous avons choisi comme agents actifs les composés d'HE qui connaissent de nos jours un engouement sans précédent. En fait, ces composés présentent un large spectre d'activités biologiques et trouvent de nombreux débouchés dans les secteurs pharmaceutique, agroalimentaire et cosmétique (Bakkali et al., 2008). À cause de leur caractère hydrophobe, ces composés peuvent aussi interagir avec la membrane et altérer ses propriétés telle que la perméabilité membranaire (Nasr et al., 2020). Néanmoins, les HE souffrent d'une très faible solubilité dans l'eau ; ce sont des composés volatils, sensibles à la lumière, à la chaleur et à l'oxygène limitant alors leurs applications. Ces inconvénients peuvent être contournés par l'encapsulation (Sherry et al., 2013). Dans ce travail, nous allons étudier l'effet d'une série de 22 composés d'HE sur la perméabilité membranaire des liposomes formés de DPPC et différents taux de CHOL afin de choisir le rapport molaire DPPC:CHOL qui est adéquat pour l'encapsulation des composés de HE dans les liposomes. Cette étude permettra également d'extraire une relation structure activité entre les différentes molécules testées.

En second lieu, les cyclodextrines (CDs) ont été choisies en tant que nanovecteurs pour étudier leurs effets sur la perméabilité membranaire. Les CDs constituent une famille d'oligosaccharides

cycliques présentant la structure d'un cône tronqué. Ils possèdent une cavité interne hydrophobe et une surface externe hydrophile (Crini, 2014). Bien que les CDs aient démontré un effet perméabilisant sur la membrane (Hammoud et al., 2019), le taux de CHOL n'a pas été considéré dans les études déjà réalisées. Nous allons donc examiner dans notre travail si le CHOL module l'effet perméabilisant des CDs tenant compte de leur affinité pour les composants lipidiques membranaires.

Finalement, nous allons étudier l'effet des complexes d'inclusions CD/thymol sur la perméabilité des membranes liposomales pauvres et riches en CHOL dans le but de vérifier si la complexation de la CD pourrait modifier son comportement vis-à-vis de la membrane ainsi que l'effet de l'agent encapsulé. Ce travail contribuera également au développement du système mixte "drug-in-cyclodextrins-in-liposomes" (DCL).

Ce manuscrit est organisé en quatre parties incluant cinq chapitres présentés sous forme d'articles. La première partie « synthèse bibliographique » est composée de deux chapitres :

- Le premier chapitre présente un manuscrit qui décrit les techniques de préparation des liposomes et qui met l'accent sur leur utilisation en tant que modèles mimétiques des membranes.
- Le deuxième chapitre traite d'une revue bibliographique portant sur les techniques de fluorescence utilisées pour évaluer la perméabilité membranaire des liposomes ; elle rapporte également les différents agents perméabilisants de la membrane ainsi que les principaux facteurs modulant la perméabilité membranaire.

La deuxième partie est consacrée à l'objectif et la stratégie du travail.

La troisième partie « travail expérimental et résultats » comporte 3 chapitres (chapitres 3, 4 et 5) :

- Le chapitre 3 présente l'étude de l'effet d'une série de 22 composés d'HE sur la perméabilité membranaire des liposomes formés de DPPC et différents taux de CHOL.
- Le chapitre 4 aborde l'étude de l'effet d'une série de 7 CDs sur la perméabilité membranaire des liposomes contenant différents taux de CHOL.

- Le chapitre 5 est consacré à l'étude de l'effet des complexes d'inclusion CD/thymol sur la perméabilité membranaire de deux formulations liposomales formées de DPPC et de CHOL de ratio molaire 100:25 et 100:100.

Ce manuscrit s'achève par une quatrième partie « discussion générale et perspectives ».

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Partie 1 : Synthèse bibliographique

Chapitre 1 : Liposomes : techniques de préparation et applications.

Introduction

Ce chapitre sera présenté sous forme d'un manuscrit qui sera soumis en tant que chapitre d'ouvrage dans un livre scientifique. Il exposera la plupart des techniques de préparation des liposomes ainsi que les applications des liposomes dans les domaines biologiques et médicaux. L'accent sera également mis sur l'utilisation des liposomes en tant que modèle membranaire et l'importance de la stabilité des membranes liposomales lors de l'usage de ces vecteurs.

Les liposomes sont des vésicules lipidiques sphériques constituées essentiellement d'une ou de plusieurs bicouches de phospholipides délimitant deux compartiments aqueux ; l'un interne et l'autre externe. Ces structures microscopiques ont une taille qui varie de 20 nm à quelques dizaines de μm (Akbarzadeh et al., 2013; Laouini et al., 2012). La membrane des liposomes est généralement formée d'un ou de plusieurs types de phospholipides naturels ou synthétiques (Choudhury, 2020). Étant un modulateur de la fluidité et de la perméabilité membranaire (Kaddah et al., 2018; Subczynski et al., 2017), le CHOL est le plus souvent un composant des membranes liposomales.

Nombreuses sont les méthodes de préparation des liposomes développées et détaillées dans la littérature : chacune produit des vésicules ayant des caractéristiques morphologiques et physiques particulières. Malgré la diversité de ces méthodes, elles peuvent être regroupées en deux classes: les méthodes traditionnelles ou conventionnelles qui permettent la production des liposomes à petite échelle et les nouvelles méthodes ou les techniques innovantes visant la formation des liposomes à grande échelle ou à l'échelle industrielle (Has and Sunthar, 2019; Laouini et al., 2012). Ces dernières sont continuellement évoluées pour améliorer la qualité des liposomes produits et pour surmonter les problèmes rencontrés lors de l'utilisation des méthodes classiques.

La première partie du manuscrit présenté dans ce chapitre sera dédiée à la description des liposomes : leur composition, leur classification ainsi que les différentes méthodes utilisées pour la préparation de ces vésicules tout en montrant les avantages et les inconvénients de chacune.

Depuis leur découverte en 1965, les liposomes ont connu d'énormes progrès et ont fait l'objet d'un large éventail de champs d'applications. En fait, les liposomes ont été principalement employés comme modèle membranaire pour l'étude de la structure et des propriétés physico-chimiques et dynamiques des membranes biologiques (Bangham et al., 1965; Eeman and Deleu, 2010; Sebaaly et al., 2019). Bien que ce système ne reproduise pas la complexité des structures membranaires, les liposomes constituent de bons outils indispensables à la compréhension de plusieurs processus pouvant être liés aux bicouches lipidiques tels que la fusion, les mécanismes de transport et de diffusion à travers la membrane, etc. (Eeman and Deleu, 2010). Le mécanisme d'action de plusieurs agents bioactifs perturbant la stabilité de la membrane liposomale a été élucidé grâce à ce système membranaire (Nasr et al., 2020). Les liposomes ont suscité l'intérêt des chercheurs scientifiques en tant que nanovecteurs de choix pour des applications pharmaceutiques et cosmétiques (Choudhury, 2020; Daraee et al., 2016; Redziniak, 2003). Tout récemment, ils ont été utilisés pour véhiculer l'ARNm codant pour la protéine virale d'enveloppe S du gène SARS-CoV-2 dans le vaccin de Pfizer contre la Covid-19 ("Pfizer and BioNTech AVC). Effectivement, à part leur nature non-toxique, biodégradable et biocompatible, les liposomes se prêtent à l'encapsulation des molécules hydrophiles (dans le compartiment aqueux interne), lipophiles (dans la bicouche) et amphiphiles (à l'interface entre la cavité aqueuse et la bicouche), ce qui les rend aptes à la délivrance de nombreux principes actifs.

Néanmoins, la recherche d'une formulation liposomale stable constitue toujours un sujet de préoccupation étant donné que les molécules encapsulées doivent être retenues dans les liposomes jusqu'à atteindre leur site d'action. Cet aspect sera discuté dans la deuxième partie du manuscrit où l'utilisation des liposomes comme un modèle membranaire et un vecteur des molécules bioactives sera développée. L'influence de certains paramètres sur la stabilité membranaire des liposomes sera mise en relief. Enfin, l'application des liposomes dans trois domaines différents (vectorisation des médicaments, thérapie vaccinale et cosmétique) sera rapportée.

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An overview of the preparation methods and applications of liposomes. A focus on membrane stability.

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1. Introduction

For over half a century, liposomes have gained a great popularity in different application areas. These lipid-based vesicles have emerged as a versatile tool for many scientific disciplines including biology, biochemistry and medicine. First described by Bangham et al. in 1965 [1], liposomes are defined as synthetic spherical vesicles composed of one or several lipid bilayers surrounding an internal aqueous core with particle size ranging from 20 nm up to several micrometers (Figure 1) [2]. Thanks to their biphasic character, liposomes can entrap hydrophilic molecules in their aqueous core, hydrophobic molecules inside the membrane as well as amphiphilic molecules at the water-membrane interface [3]. In addition, the biocompatibility, biodegradability and low-immunogenicity of liposomes make them attractive candidates for drug delivery [4]. Currently, liposomes are being successfully applied as a potential delivery system (for drugs and other materials) in many fields including cancer therapy, immunotherapy, vaccine delivery, cosmetics, food technology, etc. For instance, many liposomal drug formulations loading a wide range of therapeutics already reached the market and many others are under the clinical trials [5], [6].

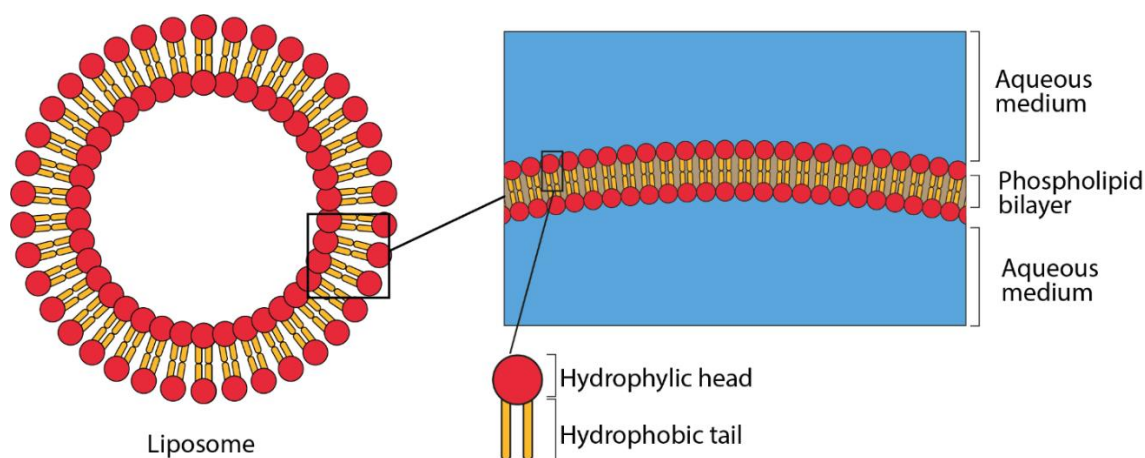


Figure 1 : Schematic representation of a liposome (left) and a phospholipid (right)

The control of the stability of liposome membranes is a matter of great concern in liposomal formulation design. In fact, the liposome functionality is strictly dependent on various parameters, most importantly the physicochemical properties of the membrane which, in turn, depend on the membrane composition of the liposomes. Hence, the composition of liposomal membrane can be

manipulated and optimized with respect to the desired applications and the drug properties in order to develop a stable formulation that provides controlled drug release.

Furthermore, liposomes have been largely used as a model of the biological membrane [7]. They helped to get a deep insight into the dynamic behavior of the membrane and to determine the key modulators affecting phospholipid bilayers stability and permeability [7], [8]. These model membranes were also useful to elucidate the mechanism of action of numerous bioactive agents [9]. Therefore, liposomes are regarded as an outstanding platform, accurately functionalized in order to fit the requirements for a large number of different applications.

In this chapter, we will first give a brief description of the liposomes composition and classification. Then, the methods of liposome preparation commonly used are presented with an emphasis on recent novel methods aiming large scale production of liposomes. The use of liposomes as a suitable model membrane to investigate the stability of the membrane is also reviewed. Finally, the application of liposomes in three different domains (drug delivery, vaccine therapy and cosmetics) will be discussed.

1.2. Composition and classification

Liposomes are lipid-based nanoparticles mainly constituted of phospholipids and other lipid species. Phospholipids are the backbones of liposomal formulations: they are amphiphilic molecules that possess a hydrophobic tail consisting of the hydrocarbon chain of two fatty acids and a hydrophilic headgroup containing polar organic entities such as choline, ethanolamine, serine, etc. attached to a phosphate group [2], [10]. Due to their amphiphilic nature, phospholipids are able to spontaneously self-assemble in an aqueous medium into lipid bilayers wherein the polar headgroups are oriented towards the inner and outer aqueous compartments and the hydrophobic tails are facing each other inside the bilayer. This self-assembly is stable and thermodynamically favored: indeed, it is well known that phospholipids reorganization in aqueous solutions is primarily driven by the hydrophobic effect which orients these amphiphilic entities in a position that minimizes the entropically unfavorable interactions between the aqueous medium and the hydrophobic chains of the molecules. This architecture is further strengthened by many intermolecular forces such as hydrogen bonds, Van der Waals forces and electrostatic interactions [4], [11]. Different categories of phospholipids could be employed for the preparation of liposomes, such as: naturally occurring phospholipids, modified phospholipids, semi synthetic

phospholipids, fully synthetic phospholipids, etc. They differ from each other by the length of the hydrocarbon chain of the fatty acids, the degree of unsaturation of the lipid chains and the charge of their polar head group. Some of the used phospholipids are shown in **Table 1**.

Table 1 Commonly used phospholipids in the preparation of liposomes and their properties.

Phospholipids	Abbreviation	Charge	T _c (°C) Main transition temperature	Saturated (S) / Unsaturated (US) acyl chain
Dimyristoyl phosphatidylcholine	DMPC	Neutral	24	S
Dipalmitoyl phosphatidylcholine	DPPC	Neutral	41	S
Distearoyl phosphatidylcholine	DSPC	Neutral	55	S
Dioleoyl phosphatidylcholine	DOPC	Neutral	-17	U
Palmitoyloleoyl phosphatidylcholine	POPC	Neutral	-2	U
Dilauroyl phosphatidylglycerol	DLPG	Negative	4	S
Dimyristoyl phosphatidylglycerol	DMPG	Negative	23	S
Dipalmitoyl phosphatidylglycerol	DPPG	Negative	41	S
Distearoyl phosphatidylglycerol	DSPG	Negative	55	S
Dioleoyl phosphatidylglycerol	DOPG	Negative	-18	U
Palmitoyloleoyl phosphatidylglycerol	POPG	Negative	-2	U
Phosphatidylethanolamine	PE	Neutral	48	U
Dimyristoyl phosphatidyl ethanolamine	DMPE	Neutral	50	S
Dipalmitoyl phosphatidyl ethanolamine	DPPE	Neutral	60	S
Distearoyl phosphatidylethanolamine	DSPE	Neutral	74	S
Dioleoyl phosphatidylethanolamine	DOPE	Neutral	-16	U

Dimyristoyl phosphatidic acid	DMPA	Negative	52	S
Dipalmitoyl phosphatidic acid	DPPA	Negative	65	S
Dioleoyl phosphatidic acid	DOPA	Negative	-4	U
Dimyristoyl phosphatidylserine	DMPS	Negative	35	S
Dipalmitoyl phosphatidyl serine	DPPS	Negative	48	S

Liposomes may also contain other lipid components, most commonly cholesterol which is the major sterol constituent of animal cell membranes. It can be included in liposomal formulations up to high cholesterol to lipid molar ratios [12]. The insertion of cholesterol into the membrane was shown to enhance the stability of liposomes. In addition, cholesterol can intercalate between phospholipid molecules and act as a fluidifying agent depending its concentration and on the temperature. It also alters the rotational freedom of the carbon molecule in the acyl chain and limits the transformation of trans to gauche conformation [13]. In some cases, liposomal membrane could include other lipid species (e.g. sphingolipid, cardiolipin, etc.) and even proteins in order to mimic the membrane composition of the desired cell type.

Liposomes are classified on the basis of different criteria: their method of preparation, their size and lamellarity (the number of phospholipids bilayers enclosing the aqueous compartment), their composition and applications, etc. (Figure 2) [4], [10], [14]. They are generally classified based on their size and lamellarity into two categories : MLV or multilamellar vesicles and UV or unilamellar vesicles. The former is made up of multiple concentric lipid bilayers separated from each other by aqueous layers similar to the onion structure (their diameter ranging from 500 nm to 3500 nm according to the number of lamellae) while the latter is made up of a single lipid bilayer enclosing an aqueous compartment. Unilamellar vesicles are in turn classified into three categories according to the size of the vesicle: SUV or small unilamellar vesicles (size range 20 - 100 nm), LUV or large unilamellar vesicles (size range 100 – 1000 nm) and GUV or giant unilamellar vesicles (size > 1000 nm) [15]–[17].

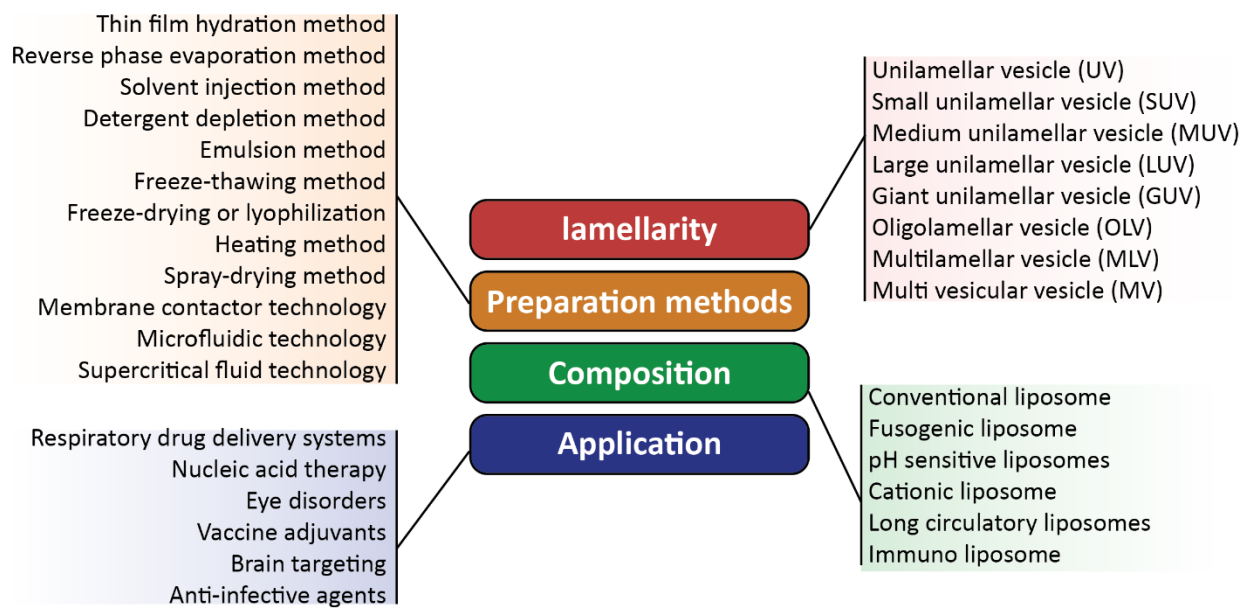


Figure 2 Classification of liposomes according to: their lamellarity, their preparation methods, their composition and applications.

2. Methods of preparation

Ever since their discovery in 1965, a large number of methods were developed for the preparation of liposomes both at the laboratory and the industrial scales. Each method produces vesicles with specific characteristics including size, size distribution, lamellarity, encapsulation efficiency, stability in a defined matrix, pharmacokinetic properties, target site, etc. Hence, the choice of the appropriate manufacturing method should consider various parameters: the physicochemical properties of the lipid ingredients used to form vesicles and those of the drug to be entrapped in liposomes, the toxicity of the loaded material, the optimal vesicle size and shelf-life for the desired application as well as the low-cost, simplicity, reproducibility and scale-up ability of liposomes production process.

The preparation methods are generally classified into 2 categories: the conventional methods and the novel methods. The former refers to the traditional methods used for the production of liposomal formulations at a small-scale while the latter comprises many procedures continuously innovated and improved to overcome the problems encountered when employing traditional methods. The following paragraphs are dedicated to the description of these methods with a special focus on their advantages and drawbacks.

2.1. The conventional methods

These traditional methods involve the dissolution of lipids in organic solvents and their dispersion in the aqueous phase. Depending on its chemical nature, the molecule to be encapsulated in liposomes is added either to the organic phase (in case of hydrophobic compounds) or to the aqueous medium (in case of hydrophilic compounds). There are mainly five classical methods for the preparation of liposomes: the thin film hydration method, the reverse phase evaporation method, the solvent injection method, the detergent depletion method and the emulsion method. They are briefly described below.

2.1.1. The thin film hydration method

Also known as “the Bangham method” [1], the thin film hydration method is the original technique used for liposomes preparation. It involves the solubilization of lipids in an organic phase followed by the removal of organic solvents usually by rotary evaporation at a reduced pressure to get a dry lipid film. The hydration of the film with an aqueous buffer will induce the self-assembly of phospholipids into liposomes. This final step is achieved under agitation at a temperature above the melting temperature of the phospholipids. The use of this technique is widespread due to its simplicity and easy handling. However, it yields a heterogeneous population of vesicles in terms of size and lamellarity. In fact, the thin film hydration method produces MLVs of several microns in size. These vesicles can be down-sized using mechanical techniques such as membrane extrusion cycles through polycarbonate filters forming LUVs or sonication to form SUVs [18]. Additional disadvantages of this method include low encapsulation efficiency particularly for hydrophilic drugs, difficulty in removing the organic solvents, non-homogeneity of vesicles and small-scale production [19]. A schematic representation of the thin film hydration method is shown in figure 3.

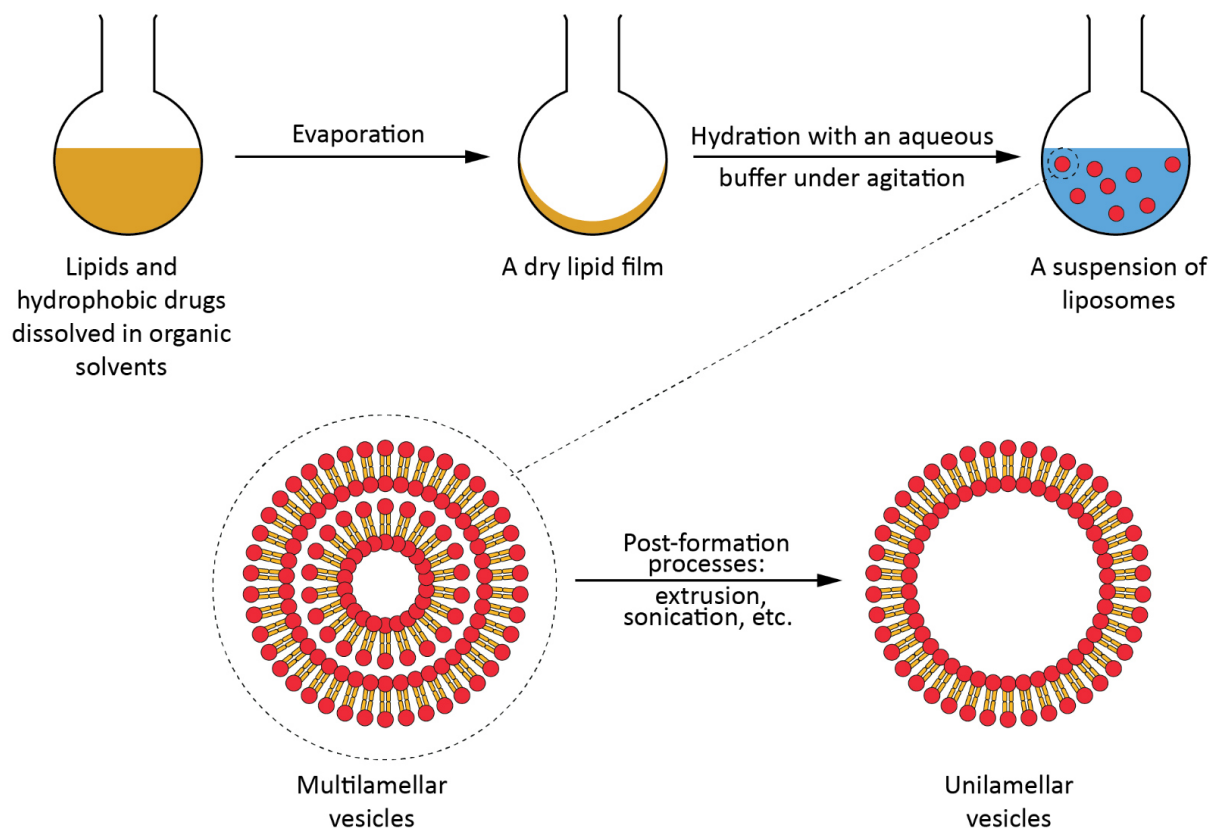


Figure 3 The thin film hydration method

2.1.2. The reverse phase evaporation method

First developed by Szoka and Papahadjopoulos in 1978 [20], the reverse phase evaporation method relies on the formation of inverted micelles also termed as water-in-oil (W/O) emulsions. It is performed by introducing an aqueous phase to a lipid solution made of lipids dissolved in organic solvents (generally diethyl ether and/or isopropyl ether). This will generate a two-phase system. A brief sonication of the mixture leads to the formation of inverted micelles. The slow removal of the organic solvents using rotary evaporation under reduced pressure initially forms a viscous gel which finally collapses to obtain a liposomal suspension (figure 4). Similar to the thin film hydration method, an extrusion step is required to obtain vesicles with the desired size [21]. The main advantage of this technique is the formation of liposomes with large internal aqueous core which allows the entrapment of large molecules (specially hydrophilic compounds) with high efficiency [20]. Nevertheless, the residual traces of the organic solvents in the final formulation pose a serious problem regarding the stability of the vesicles and their toxicity. Besides, the exposure of the molecule to be encapsulated within the vesicle to the organic solvents and to

sonication might damage its chemical structure which renders this method unsuitable for fragile compounds such as peptides [22].

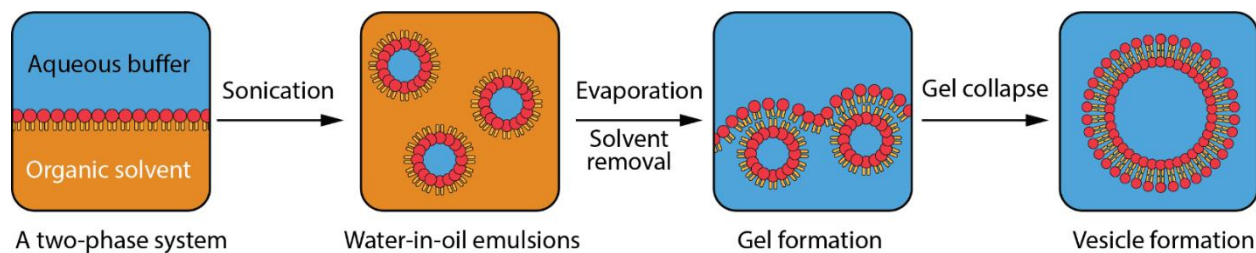


Figure 4 The reverse phase evaporation method

2.1.3. The solvent injection method

The preparation of liposomes using the injection method is described as follows: a lipid solution is injected into an aqueous phase resulting in the spontaneous formation of vesicles (figure 5). According to the organic solvent used for the dissolution of phospholipids, two methods are identified: the ethanol injection method and the ether injection method.

In the ethanol injection method, the lipid solution is rapidly injected through a fine needle into an excess of an aqueous phase promoting SUVs formation [23]. An additional step is needed to eliminate the ethanol from the liposomal suspension; it is commonly achieved by rotary evaporation. Despite the simplicity, rapidity and reproducibility of this technique, the presence of residual organic solvents is a major concern. Moreover, a heterogeneous liposomal suspension can be obtained with poorly-soluble lipids in ethanol [19]. In contrast, given the immiscibility of the ether in water, the lipid-ether solution is slowly injected into an aqueous solution warmed at a temperature above the boiling point of the ether. This allows the removal of the ether from the liposomal product (which is advantageous compared to the ethanol injection method) yielding a concentrated suspension of liposomes with high encapsulation efficiency [24]. Diethyl ether is the most commonly used solvent for lipid dispersion in this method. A mixture of ether and methanol could be also utilized. The ether injection method is recognized as a time-consuming technique since a slow injection rate is requisite.

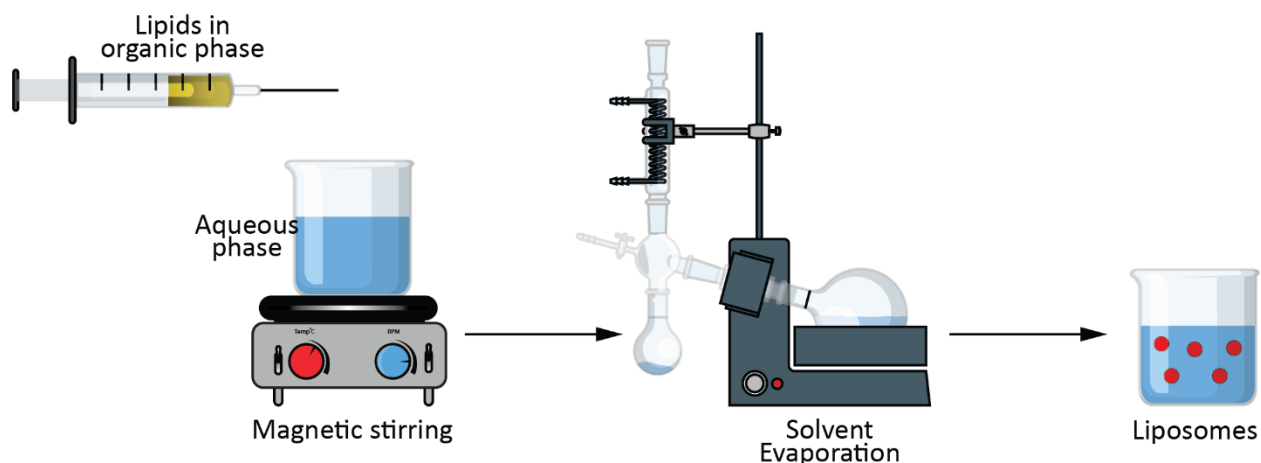


Figure 5 The solvent injection method

2.1.4. The detergent depletion method

The detergent depletion method relies on the formation of detergent-lipid micelles followed by the removal of the detergent enabling subsequently large vesicles (LUVs) to be formed. The detergent is usually removed from the micellar solution by dilution, dialysis, column chromatography, adsorption, etc. Although the dilution is the simplest method for detergent removal, the concentration of the final liposomal suspension is very low and a poor entrapment is reached for hydrophobic compounds when dilution is applied. The retention of traces of detergents within the liposomes is another drawback of this method. Besides, it is considered a very time consuming method [24].

2.1.5. The emulsion method

This method is based on the preparation of a double emulsion: a small volume of an aqueous media is first added to a large volume of an organic phase containing the phospholipids. This mixture will generate W/O emulsions consisting of tiny water droplets where the phospholipids align themselves with their polar headgroup towards water and their hydrophobic tails towards the organic solvent. This emulsion is next mixed with a large volume of aqueous solution so as to form water-in-oil-in-water W/O/W double emulsions. Upon the removal of the organic solvent from the double emulsion, unilamellar vesicles with large enclosing cavities are obtained [24].

Although regarded as straightforward techniques for small-scale preparations, the conventional methods are not convenient for an industrial scale due to many disadvantages. Indeed, these classical methods use large amounts of organic solvents which cause environmental harm and

health issues. Thus, the presence of residual toxic solvents in the final product is a major drawback given the high cost of handling and solvent removal process. Furthermore, the broad size distribution, the difficulty in achieving high entrapment in liposome formulations along with the exposure of the entrapped material to organic solvents, detergents, heat, sonication, etc. limited the use of these traditional techniques for the industrial production of liposomes. Another significant issue is that these methods are lengthy processes consisting of many steps and consuming a large amount of energy. To meet these shortcomings, new methods were developed for the production of efficient liposomal formulations that gather the best characteristics for drug delivery.

2.2. Novel methods

The on-going interest in liposomes as potential nanocarriers for a variety of drugs and bioactive agents has prompted the researchers to scale-up the production of liposomes and to develop new preparation methods that alleviate the issues associated with the use of conventional methods. Several techniques were invented including:

2.2.1. The freeze-thawing method

The freeze-thawing method is based on size transformation and fusion of preformed vesicles (figure 6). It is usually applied to increase the encapsulation capacity of MLVs by decreasing its lamellarity. A suspension of SUVs or MLVs prepared by the previously described conventional methods (usually the thin film hydration method) is subjected to repetitive cycles of freezing in liquid nitrogen followed by thawing in hot water ; the resulting suspension is then vortexed. LUVs are formed either by fusion of the starting SUVs or by rupture of the MLVs [2]. Multiple freeze-thawing cycles are generally performed in order to further improve the encapsulation efficiency of liposomes: by increasing the number of cycles, the size and the lamellarity of MLVs decrease while the liposome aqueous volume and the encapsulation efficiency of hydrophilic molecules increase [25], [26]. This method is widely used for the encapsulation of proteins [13].

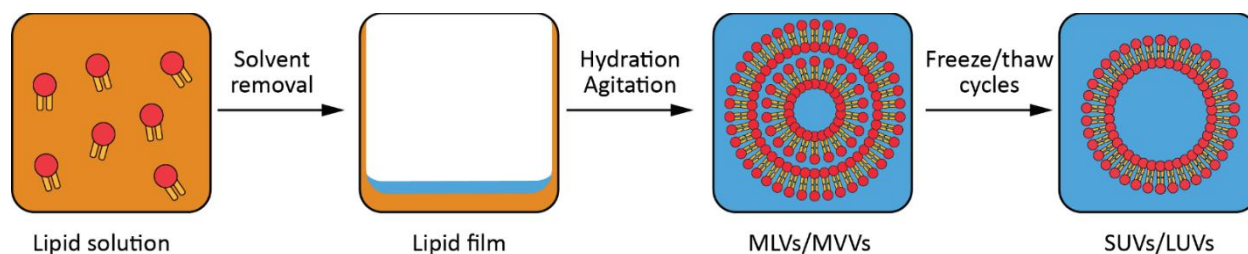


Figure 6 A scheme for liposomes preparation by the thin film hydration method followed by the freeze thawing process

2.2.2. Freeze-drying or lyophilization

This novel procedure was first developed by Li and Deng in 2004 to prepare LUVs of narrow size distribution [27]. It involves the formation of a monophasic solution of lipids and suitable water-soluble carriers which act as a lyoprotectant. A solution of sucrose dissolved in a tert-butyl alcohol/water cosolvent system is generally used to disperse the lipids. The monophasic solution is then lyophilized to remove the solvents. The freeze-drying process includes three steps: freezing at -40°C for 8h, a primary drying at -40°C for 48h and a secondary drying at 25°C for 10h. Upon rehydration, the lyophilized product spontaneously forms a homogeneous suspension of liposomes [27], [28]. This method allows the long-term storage of dried liposomes. It also prevents the hydrolysis of phospholipids and protects the vesicles from aggregation and fusion which usually occur in buffered suspension of liposomes [29]. High encapsulation efficiencies can be achieved with the freeze-drying process. Moreover, the freeze-drying method was used to encapsulate drugs in preformed liposomes; in this case, the process is denoted as “the dehydration-rehydration method” [30].

2.2.3. The heating method

The heating method has been described as a rapid process for the production of liposomes without using any hazardous chemical [31]. The liposomal ingredients are added to an aqueous phase containing glycerol (3% v/v) and the mixture is subjected to heat reaching temperatures up to 120°C [31]. Glycerol serves as an isotonic agent which enhances the stability of the liposomal preparation preventing vesicles aggregation and sedimentation. Since it is an acceptable and non-toxic agent already in use in many pharmaceutical products, there is no need to remove glycerol from the final suspension. Additionally, no sterilization step is required as high temperature is employed in this technique [18], [31]. The incorporation of drugs in liposomes could be achieved at different stages of the process : the drug could be added to the reaction medium containing the

liposome components and glycerol at the beginning of the heating, when the temperature has dropped below the transition temperature of the phospholipid, or to the liposomes after their formation. It depends on the sensitivity of the drug to temperature. The heating method proved to be suitable for the large-scale manufacture of safe and efficient liposomal formulations encapsulating nucleic acid drugs: the plasmid containing liposomes possessed long-term stability, high DNA entrapment capacity and high transfection efficiency [32].

2.2.4. Spray-drying method

Spray-drying is a simple technique widely used in the pharmaceutical industry for various purposes. Hence, a one-step spray-drying procedure was developed for the preparation of liposomes meeting both small and large-scale requirements. Basically, the lipids, the drug and a core carrier (carbohydrate carriers such as mannitol and lactose) are dissolved in convenient organic solvents (usually chloroform, ethanol, methanol) to get a homogenous solution and the mixture is then spray-dried generating dry powder called “proliposomes”. The hydration of proliposomes induces vesicles formation [14]. The spray-drying process is carried out at specific conditions; these include: inlet and outlet air temperature, feed flow rate, aspirator capacity and nozzle air pressure. These conditions are optimized depending on the drug and the lipids used for the preparation [22]. A simplified scheme of a spray dryer is represented in figure 7. A recent study attempted to prepare liposomes encapsulating lopinavir, an effective HIV protease inhibitor, using the spray-drying process. The resulting suspension displayed optimal characteristics including size, shape, encapsulation efficiency, etc. [33]. Similar studies developed liposomal formulations of spray-dried vesicles loaded with active ingredients demonstrating the potential of the spray drying technology to be a promising modality to treat different diseases including lung infections [34], lung cancer [35], etc.

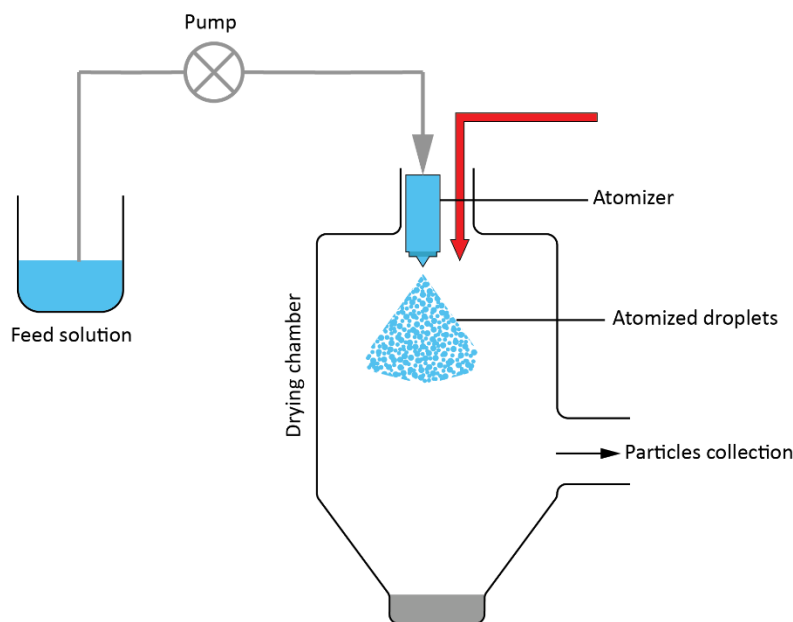


Figure 7 A schematic representation of a spray dryer used for the formation of dried particles such as proliposomes.

The spray drying process involves three stages: (1) Atomization, (2) Droplet-to-particle conversion and (3) Particle collection. For liposomes preparation, it is briefly described as follows: a feed solution (commonly a mixture of the lipid materials dissolved in organic solvents and mannitol) is pumped to an atomizer and transformed into a spray of droplets. The droplets are then subjected to a moisture vaporization in a drying chamber resulting in the formation of dry particles called “proliposomes”. Finally, the proliposomes powder are collected in a tank.

2.2.5. Membrane contactor technology

The membrane contactor technology is a novel approach for producing large-scale liposomal formulations in a single-step. It is based on the principle of ethanol injection method and uses a simple design unit where the organic phase (lipids and ethanol) permeates through the pores of a membrane into a tangential flow of an aqueous phase. As the aqueous solution flows perpendicular to the membrane and comes in contact with the organic phase, the ethanol is diluted, and lipids will instantly self-assemble to form vesicles which are transferred into a collector (figure 8). A final step consists of a rotary evaporation of the liposomes suspension to remove ethanol [36], [37]. Appropriate micromixing is highly important in this system because it directly influences the uniformity of liposomes. Further improvements were brought to this technique in order to control the liposomes size and to ensure a high encapsulation efficiency: several membrane devices were designed such as tubular SPG (Shirasu-porous-glass) membrane [37], hollow fibers Liqui-Cel® membranes [38] and nickel microengineered membranes [39]. Previous studies demonstrated the

suitability of this technology for large-scale production of liposomes encapsulating bioactive agents: for instance, Sebaaly et al. (2005) prepared clove essential oil and eugenol loaded liposomes using a membrane contactor and a pilot plant both equipped with a SPG membrane. A homogeneous suspension of nanosized MLVs with high encapsulation efficiency was produced [40]. This method was additionally applied for the large-scale preparation of clove essential oil-in-cyclodextrin-in-liposomes [41] and trans-anethole-in-cyclodextrin-in-liposomes [42].

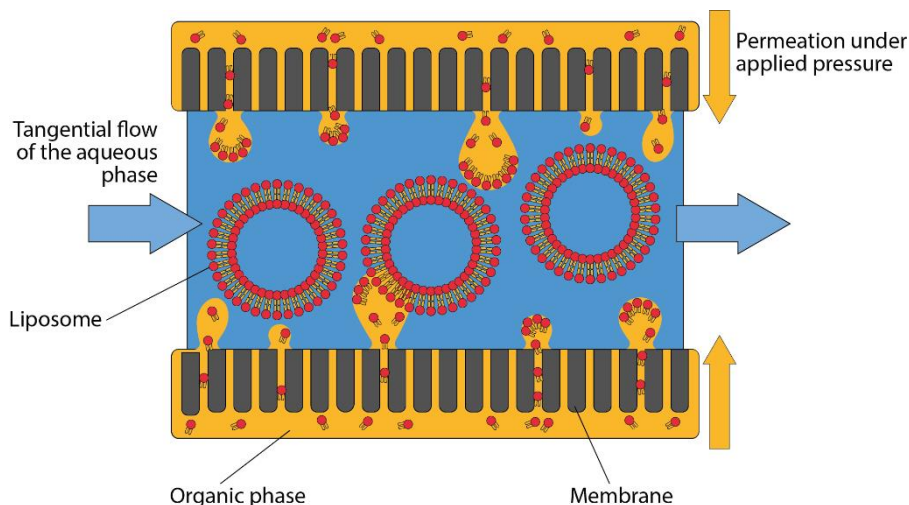


Figure 8 Schematic representation of a membrane contactor used for liposomes preparation

2.2.6. The microfluidic technology

The microfluidic technology is based on fluid flow into microchannels with cross-sectional dimensions in the range of 5-500 μm . During the past few decades, an extensive work has been done in this field and much effort has been involved in the production of liposomes using microfluidic tools. The originality of these methods over other novel approaches is manifested by liposomes formation in a confined microenvironment. Outstandingly, the microfluidic techniques provide accurate control of the physical properties of liposomes, particularly their size, size distribution and lamellarity. However, the scale-up ability of this technology remains a major limitation to their industrial application. Various microfluidic-based techniques have been developed for liposomes production; many reviews were recently published describing these methods and criticizing their potential in generating liposomes with the desirable characteristics [43]–[45]. On the basis of the size of the liposomes they produce, these techniques can be classified into 2 groups: the first includes the microfluidic methods for the formation of small and large vesicles while the second comprises those used for the production of giant vesicles. Some of these

microfluidic-based methods will be described in the following paragraphs. For more details, the reader is directed to references [43]–[45].

2.2.6.1. The microfluidic techniques generating small and large vesicles

Several microfluidic techniques were proposed to generate a monodisperse population of SUVs and LUVs of defined size. Among these, the “microfluidic hydrodynamic focusing” (MHF) method is the most applied. It was introduced by Jahn et al. in 2004 for the fast preparation of liposomes in a microfluidic platform [46]. The authors constructed a microfluidic channel comprising 3-inlet channels where the central channel is perpendicular to the oblique channels (**figure 9**).

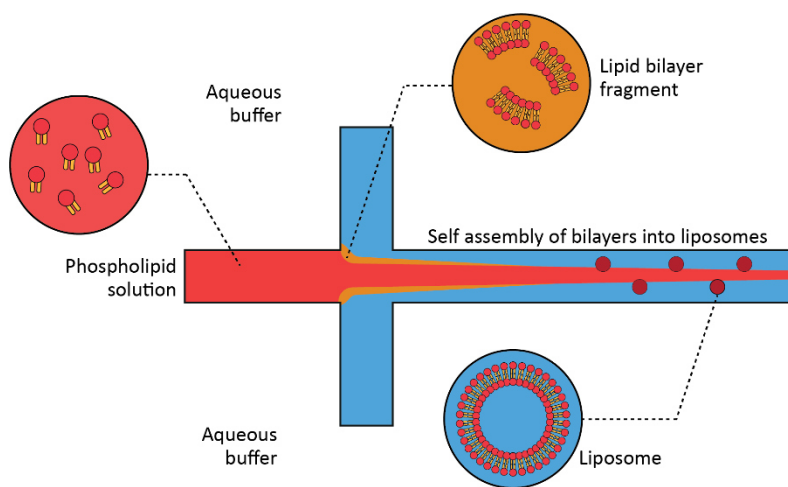


Figure 9 Liposome formation by micro-hydrodynamic focusing using a 3-inlet microchannel

Using this device, the method consists of a central flow of a lipid solution (containing phospholipids and ethanol) being intersected on either side by an aqueous solution. As a result, a diffusive mixing occurs between the two phases generating lipid vesicles having a size in the range of 100-300 nm [46]. The liposomes size is controlled by regulating the aqueous buffer-to-organic phase flow rate ratio in microchannels and the lipid concentration: a decrease in the vesicle size was reported following the increase of the flow rate ratio of the aqueous buffer-to-organic phase whereas larger vesicles were produced upon increasing the lipid concentration [47]. The microfluidic device was subjected to many modifications to improve the control of size and size distribution of the vesicles [36]. For instance, a five-inlet microfluidic device (**figure 10**) was developed to prepare liposomes containing oligonucleotides [48].

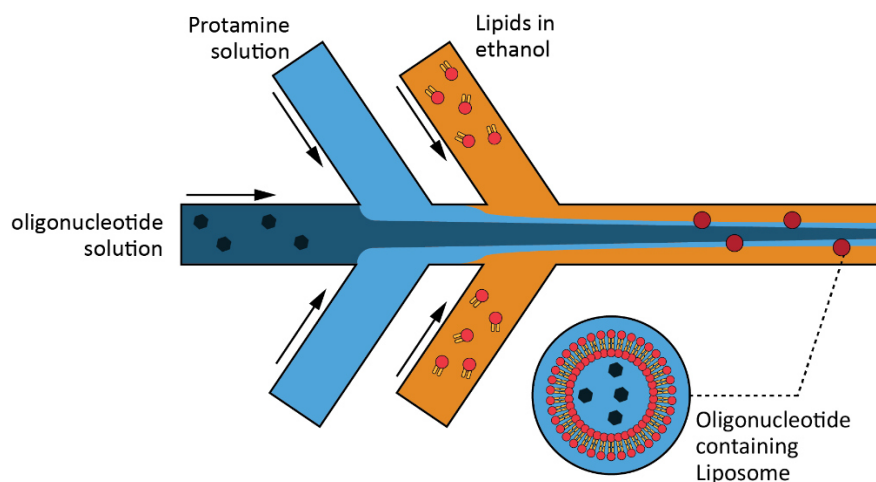


Figure 10 A five-inlet microchannel used for the production of oligonucleotide containing liposomes

Later on, the microfluidic “vertical flow focusing” (VFF) method was described by Hood and DeVoe in 2015 using planar microfluidic devices with different channel aspect-ratios (ratio of channel height to width), as an alternative to enhance the throughput of liposomes synthesis. The VFF platform enabled the formation of nano-sized liposomes (80-200 nm) with significant lower polydispersity index compared to the MHF method and with remarkably high production rates [49]. This technique was suggested for industrial applications.

In order to enhance the fluids mixing efficiency, microfluidic devices equipped with micromixer structures were developed giving rise to the “mixer-assisted microfluidics” techniques. The microfluidic channel consists of an array of “herringbone grooves” on one or more surfaces of the microchannel inducing turbulent mixing (or chaotic advection) within a continuous flow. This design is referred to as the “chaotic micromixer” or “staggered herringbone mixer” (SHM) [50]. The chaotic mixer is the most used microfluidic device for showing great liquid mixing performance and generating SUVs with diameters as small as 20 nm. Further studies reported the synthesis of doxorubicin loaded liposomes [51] and siRNA loaded liposomes [52] using SHM. **Figure 11** provides an illustration for the chaotic micromixer employed by Zhigaltsev et al. for the production of liposomes encapsulating doxorubicin [51].

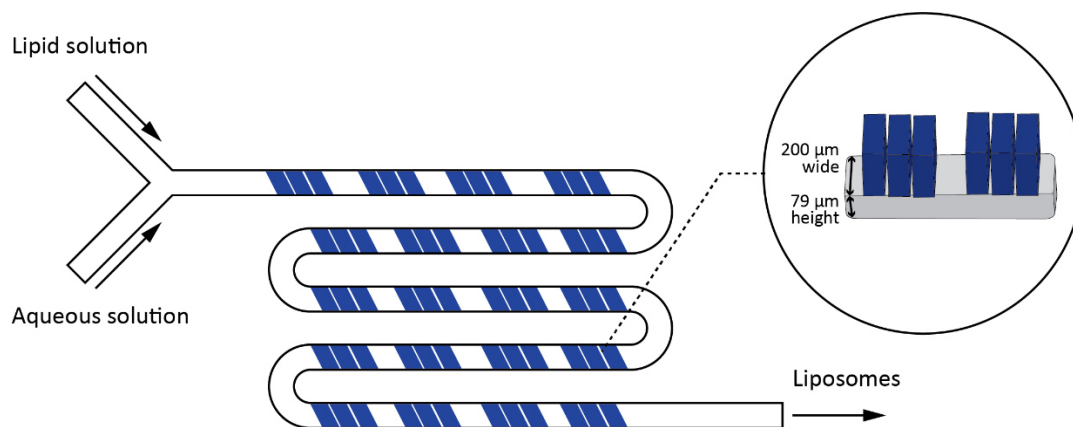


Figure 11: A microfluidic device equipped with a chaotic micromixer used for the synthesis of doxorubicin loaded liposomes

2.2.6.2. *The microfluidic techniques generating giant vesicles*

Owing to their capacity to form single or multiple emulsions with definite droplet size and composition, the microfluidic approaches were used to produce GUVs. Among many techniques, “droplet-based microfluidics” remains the most applied. Using a microfluidic device, Tan and coworkers generated an emulsion solution; it was then injected in ethanol-water solution where the phospholipid molecules constituting the droplets rearrange themselves forming subsequently the giant vesicles [53]. This method was suitable for the encapsulation of biological materials ranging from cells to nanosized proteins. In another study, a droplet emulsion technique was carried out in a Y-shaped microfluidic device (**figure 12**) in which the formation of W/O emulsion, the subsequent oil removal producing giant vesicles and the transfer of GUVs into an aqueous phase take place within the same implement. Known as the “droplet transfer method”, this technique yields giant liposomes of about 20 -70 μm with high entrapment efficiency [54].

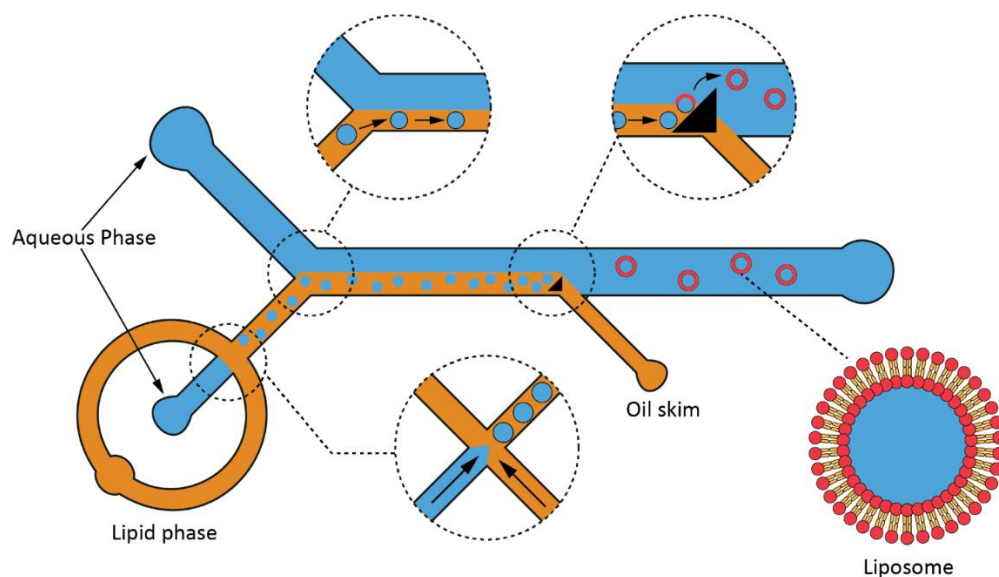


Figure 12 GUVs formation in a Y-shaped microfluidic device

In the following years, many attempts were made in order to better control droplet size and its distribution [36]. It was also possible to form monodisperse GUVs with high encapsulation efficiency by generating W/O/W double emulsions in a microfluidic device. These techniques were suitable for the encapsulation of both hydrophilic and hydrophobic drugs [55], [56]. One limitation of the double emulsion method is the presence of residual organic solvent in the final product altering thereby the stability of liposomes [47].

Furthermore, GUVs can be simultaneously produced and loaded with active ingredients using “a microfluidics method based on a pulsed microfluidic jet”. Initially, macro-sized water droplets are generated in a phospholipid-containing oil phase. When two water drops are brought in close proximity, a planar lipid bilayer is formed. Then, periodic pulses jet of an aqueous phase (buffer) are injected into the planar bilayer using a microdispenser resulting in giant vesicles (micrometer range) [57]. Since all the materials present in the aqueous phase are jetted across the planar bilayer, they will be directly encapsulated in the vesicles. Hence, this method enables a high encapsulation efficiency. Nevertheless, the jetting process induces high shear stress that can damage the biological materials [44]. The mechanism of formation of giant liposomes using pulsed microfluidic jetting is depicted in **figure 13**.

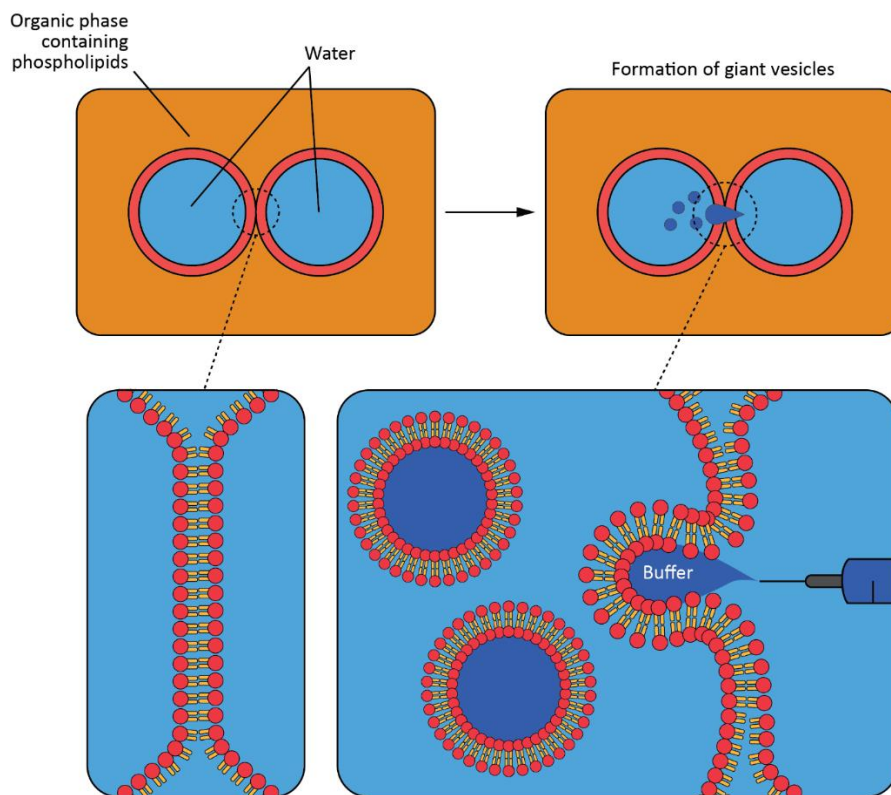


Figure 13 GUVs formation by pulsed microfluidic jetting

2.2.7. Supercritical fluid technology

For several years, supercritical fluid-based liposomes preparation has been one of the leading alternatives to conventional methods. Indeed, these methods provide one-step liposome preparation with high control over the physicochemical properties of the vesicles. The use of organic solvents is significantly reduced in these techniques and even sometimes totally suppressed [58]. Another advantage of this technology is the suitability of the manufacturing methods for large scale production of liposomes.

A supercritical state is defined as the state of a substance at which critical values of pressure and temperature are reached. In the supercritical region, no boundaries between the liquid and gaseous states are notable. In other words, when a compound is in the supercritical state, it has an intermediate behavior between that of a liquid and a gas: particularly, the supercritical fluid (SCF) is characterized by gas-like viscosity, liquid-like density, high diffusivity, high miscibility with organic solvents and thus, an enhanced solvent power. Numerous compounds are employed as supercritical fluids; among these, the carbon dioxide CO_2 is the most used SCF because it is an inert, colorless, odorless, non-toxic, non-flammable, inexpensive and recyclable gas and it is

classified as a safe solvent by FDA. Besides, CO₂ possesses low critical temperature ($T_c = 31.1^\circ\text{C}$) and pressure ($P_c = 73.8 \text{ bar}$) which are easily reachable [58], [59]. The phase diagram of CO₂ is shown in **figure 14**.

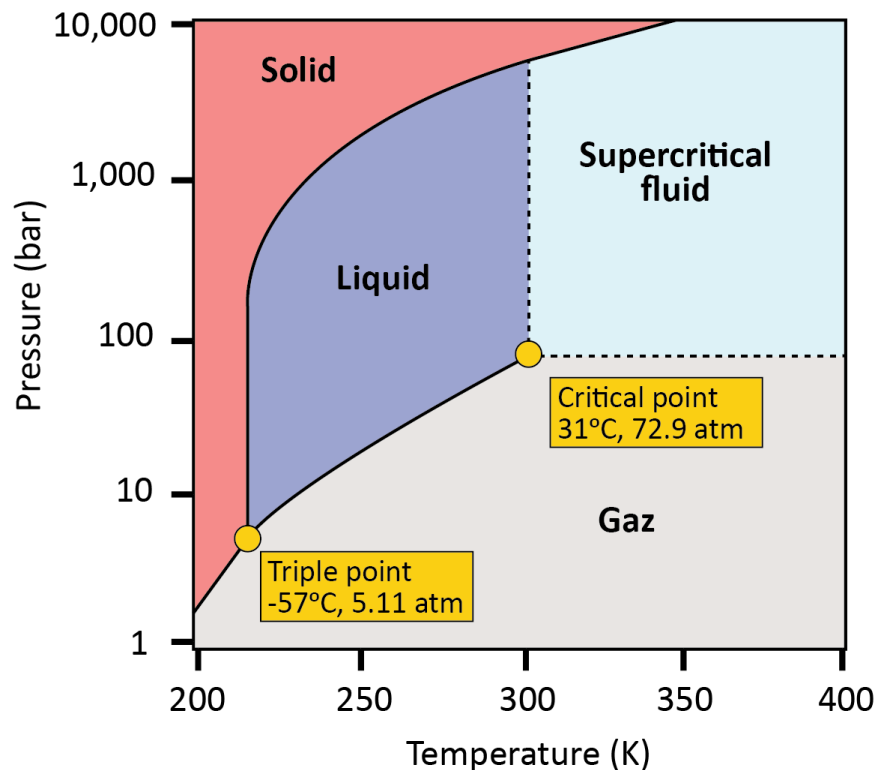


Figure 14 The temperature-pressure phase diagram of carbon dioxide

The use of SCF technology for liposomes preparation has gained much attention in recent decades. Many preparation processes were developed; these methods are classified with respect to the role exerted by the SCF on the lipids. In fact, the SCF can act as a solvent or a co-solvent to the lipids prior to their contact with the aqueous phase. It can also be used either as an antisolvent promoting vesicle formation after hydration or as a dispersing agent to finely disperse the lipids into the aqueous medium [58]. **Table 2** describes the various manufacturing processes developed for the preparation of liposomes using SCF. Their advantages and applications are also presented.

It is worth noting that an appropriate equipment and adaptive devices are being used for each method. For example, figure 15 illustrates the equipment setup designed for the rapid expansion of the supercritical solution process.

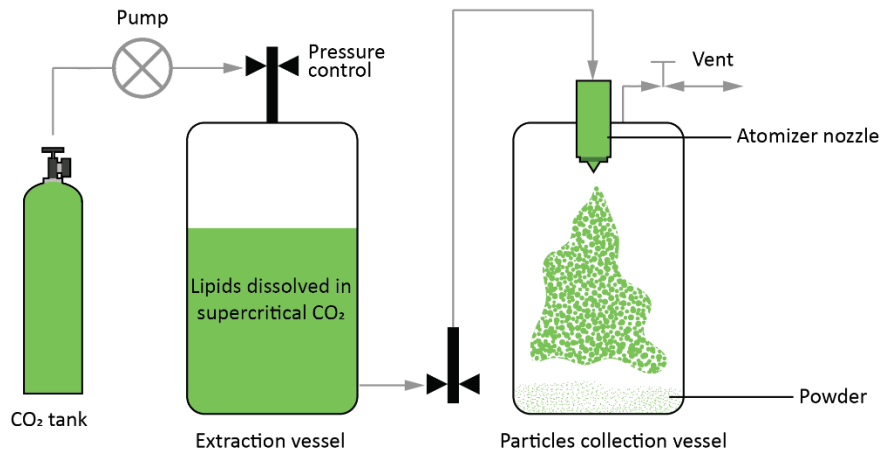


Figure 15 The supercritical fluid technology

Table 2 Supercritical fluid-based liposomes preparation methods and their characteristics

General procedure	Methods	Special features	Presence/absence of organic solvent	Hydration	Advantages and applications
SCF as a solvent					
<p>- Lipid materials are solubilized in the SCF in the absence or the presence of an organic solvent at a high pressure</p> <p>- The lipid supercritical solution is then hydrated with the aqueous phase either before or during the depressurization step</p> <p>- Once the depressurization is achieved, the SCF is eliminated yielding a liposome suspension</p>	Supercritical Reverse Phase Evaporation method (SCRPE)	The aqueous solution is slowly injected in the supercritical lipid solution under stirring	+	Before depressurization	<p>- Encapsulation of both hydrophilic (glucose and bovine serum albumin) and hydrophobic compounds (cholesterol) [60]</p> <p>- The liposomes generated from SCRPE process achieved higher encapsulation efficiencies and a better stability profile than the conventional thin film hydration (TFH) method [60]</p> <p>- The liposome size and morphology are tuned by adjusting the pressure and the amount of ethanol used in the process [61]</p>
	In-line mixing	The apparatus of the process contains an in-line mixer and a micro-pore cell through which the SCF/water emulsions will pass	+	Before depressurization	<p>- A better control of the size of the droplets (SCF/water emulsions) and the liposomes is provided with this technique</p> <p>- Preparation of nano-sized liposomes encapsulating a heat-sensitive compound [58]</p>
	Spraying method	The supercritical solution is sprayed into the	+	Before depressurization	Encapsulation of febrifugine (hydrophobic compound) [58]

		aqueous phase under pressure and the obtained mixture is depressurized			
	The injection method	The lipid supercritical solution is depressurized into the aqueous phase through a nozzle	+	During depressurization	<ul style="list-style-type: none"> - Control of liposome size and size distribution by modifying the nozzle diameter, the pressure and the type of cosolvent [58] - Small vesicles (less than 500 nm) with a unimodal size distribution are produced using this technique [58] - Encapsulation of cytochrome c and melatonin with an efficiency of 33.3% and 82.3% [58], [62] - Liposomes displayed a better physical stability compared to those produced by other methods [58]
	Rapid expansion of supercritical solution coupled with vacuum-driven cargo loading	The insertion of the aqueous phase with the supercritical solution into the expansion nozzle at an angle of 90°	-	During depressurization	<ul style="list-style-type: none"> - This technique is regarded as an eco-friendly process of liposome preparation without using toxic organic solvents [58] - LUVs and MVVs having a unimodal size distribution and a mean diameter ranging from 400 to 1200 nm are generated by this technique [63] - Co-encapsulation of hydrophilic and hydrophobic compounds [64]
	Rapid expansion of a supercritical solution through a T-piece	The supercritical solution is depressurized into the aqueous phase via a T-	+	During depressurization	<ul style="list-style-type: none"> - This method generates SUVs with a mean size in the range of 20-50nm and MLVs with a size up to 250 nm showing a wide size distribution [58] - Encapsulation of hydrophilic molecules such as FITC-dextran [65]

		piece connected to a capillary tube			- Low encapsulation efficiencies are obtained with this method compared to other conventional techniques [58], [65]
	Rapid expansion of a supercritical solution through a capillary tube	The supercritical solution is depressurized through a capillary tube in which an aqueous solution is pumped	+	During depressurization	- Formation of unilamellar liposomes with a wide size distribution ranging from 50 nm to 1 µm as well as oligolamellar vesicles consisting of 3 to 5 lipid bilayers - Encapsulation of both hydrophilic (ciprofloxacin A) and hydrophobic compounds (vitamin E) were achieved [66]
SCF as a cosolvent					
- Liposomal raw materials are dissolved in an organic solvent - The organic solution is then mixed with SCF to generate an expanded solution; this step is being achieved in specific conditions of pressure and temperature - The expanded solution is next hydrated either before or during the depressurization to produce the liposomal suspension	Supercritical assisted Liposome formation (SuperLip)	Water droplets are produced by atomization inside a high-pressure vessel, filled with a CO ₂ -expanded solution	+	Before depressurization	- Liposomes encapsulating hydrophilic compounds (such as BSA, olive pomace extract, etc.) with encapsulation efficiency values higher than those obtained with TFH method [67]–[69] - This method is suitable for the encapsulation of thermosensitive compounds such as proteins [68] - The exposure of the entrapped molecule to high pressure and/or high temperature during the process may cause its degradation and reduce its pharmacological effect. This was demonstrated with eugenol [69].
	Depressurization of an Expanded Solution into Aqueous Media (DESAM)	The CO ₂ -expanded solution is depressurized into the aqueous	+	During depressurization	- Unilamellar liposomes with a size ranging from 50 to 200 nm and showing high physical stability are produced by this method [70] - Encapsulation of hydrophilic and hydrophobic molecules

		media via a nozzle			- This process can reduce the residual organic solvent percentage in the final product to 2.2% [70], [71]
	Depressurization of an Expanded Liquid Organic Solution through a T-piece (DELOS-SUSP)	The expanded organic solution is depressurized into an aqueous solution within a T-mixer	+	During depressurization	This method produces liposomes with a degree of homogeneity similar to that obtained with TFH method [72]
SCF as an antisolvent					
<ul style="list-style-type: none"> - Lipids are solubilized in an organic solvent (such as ethanol) - The organic solution is then mixed with SCF allowing an antisolvent effect and thus proliposomes will form - Proliposomes are dispersed into an aqueous phase to produce liposomes 	Gas antisolvent method (GAS)	Hydration of proliposomes with 0.9% saline solution	+	Before or during depressurization	<ul style="list-style-type: none"> - Larger vesicles were obtained when the hydration was performed after the depressurization step [73] - Amphotericin B containing liposomes prepared by this technique showed nanosized particles with a narrow range of size distribution (between 0.5 and 15 µm) and a high entrapment efficiency [73]
	Supercritical AntiSolvent method (SAS)	The organic solution is sprayed through a capillary tube into a high-pressure vessel containing the SCF	+	After depressurization	<ul style="list-style-type: none"> - Small vesicles with a narrower size distribution are generated with SAS compared to those obtained with TFH [74] - A low encapsulation is achieved using this method [74]
	SAS with a T-piece	The SCF and the organic solution are brought together through a T-piece	+	After depressurization	Proliposomes were free from residual solvent thanks to a washing step performed during the preparation process [66]

	Continuous antisolvent process	Phospholipid hydration by introducing water into the high-pressure vessel before addition of SCF	+	Before depressurization	<ul style="list-style-type: none"> - Formation of liposomes with a unimodal size distribution (10 to 100 μm) [75] - Residual organic solvent is trapped into the aqueous phase which requires an additional process for solvent removal [75]
	Aerosol Solvent extraction system method (ASES)	The organic solution is sprayed into the SCF via a nozzle	+	After depressurization	<ul style="list-style-type: none"> - Low residues of organic solvent were found in the proliposomes suspension [76] - Encapsulation of various compound with high efficiency (at least 79%) - ASES was suitable for the preparation of PEGylated liposomes [77] - An <i>in vivo</i> study was successfully carried out using ASES-liposomes [78]
	Solution Enhanced Dispersion by Supercritical fluid (SEDS)	The lipid organic phase and the SCF are simultaneously introduced into a high-pressure vessel via a coaxial nozzle	+	After depressurization	This method was efficient for the preparation of vitamin D3 loaded liposomes with a high efficiency (nearly 100%) [79]
SCF as a dispersing agent					
<ul style="list-style-type: none"> - The SCF is mixed with the lipid materials and the aqueous phase before the depressurization - Lipids are finely dispersed during the depressurization step yielding either liposomes or proliposomes 	Depressurization of a mixture of SCF and an aqueous lipid suspension by the bottom of the high-pressure vessel	The depressurization is achieved from the bottom of the high-pressure vessel	-	Before depressurization	<ul style="list-style-type: none"> - Encapsulation of hydrophilic and hydrophobic compounds - The pressure, temperature and depressurization rate were found to influence the efficiency of encapsulation [80], [81]

					- Liposome size and size distribution are controlled by adjusting the depressurization rate [58]
	Improved Supercritical Reverse Phase Evaporation method (ISCRPE)	The depressurization is achieved from the top of the high-pressure vessel	-	Before depressurization	- Pressure, temperature and depressurization rate affect the size and size distribution of the liposomes [82]
	Particles from Gas Saturated Solution – drying process (PGSS-drying process)	An in-line mixer is used to mix the aqueous lipid suspension and SCF. The depressurization is achieved via a capillary tube into a tower where the temperature is well controlled in order to obtain a dry powder	-	After depressurization	- This method yields proliposomes which, upon hydration, are transformed into liposomes - Encapsulation of hydrophobic compounds including lavender essential oil [83] and β -carotene [84]

3. Liposomes as biomimetic model membrane

Liposomes are primarily used as model membranes since they are synthetic analogues to the biological membranes [85]. Actually, the phospholipids bilayer of liposomes defines a cell-like boundary which affords liposomes a functional scaffold tailored for the investigation of the fundamental cellular functions. As a model membrane system, liposomes unraveled the mechanism of many processes occurring at the membrane surface such as membrane fusion, molecular recognition, cell adhesion, [8], [85] etc.

The stability of the liposomal membrane is requisite for liposomes applications in different areas. These artificial membranes can mimic the biophysical properties of biological membranes : being a relatively unstable colloidal system, liposomes should achieve a good stability in order to improve their efficacy as a potent drug carrier. Membrane stability depends on the physicochemical properties of the membrane including membrane fluidity, permeability, elasticity, phase behavior, etc. These properties are majorly tuned by the lipid composition of the membrane [86]. In this context, liposomes were used as a model membrane to investigate the effect of membrane composition on its properties. It was demonstrated that increasing the length chain of the fatty acids of phospholipids results in tighter lipid packing and thereafter a stable formulation while increasing the degree of unsaturation of the hydrocarbon chain of phospholipids results in looser film packing and a leaky membrane [86]. Additionally, the inclusion of phospholipids inducing negative membrane curvature in the liposome formulation enhanced the permeability of liposomal membranes. Conversely, less permeable and more stable membranes are generated with phospholipids promoting positive membrane curvature [87]. Besides, cholesterol can markedly affect the physicochemical properties of the membrane. Effectively, the addition of cholesterol to the liposome membrane reduces its permeability and fluidity [88]. It also prevents the aggregation of liposomes through repulsive steric and electrostatic effects which confers a high stability to the liposomal formulation [89]. The effect of cholesterol on membrane characteristics was intensively studied and reviewed during the past two decades [88], [90]–[92].

Similarly, the encapsulation efficiencies of liposomes loaded with an active ingredient are influenced by the type of lipids constituting the liposomes. As a matter of fact, the choice of an appropriate combination of lipid components greatly affects the stability of the formulation. Hence, the adequacy of a liposome formulation as a carrier system for drugs should meet both

stability and high entrapment efficiency conditions. For instance, Azzi et al. found that, among three different formulations made of either unsaturated egg Lipoid E80, unsaturated soybean Lipoid S100 or saturated soybean Phospholipon 90H, E80-liposomes were suitable for the encapsulation of quercetin, either in its free form or its inclusion complex with sulfobutylether- β -cyclodextrin, with high efficiency [93]. The liposomes formulations were also stable after one year of storage at 4°C [93]. A large number of studies aiming to find the appropriate liposomal formulation for the encapsulation of bioactive agents can be found in the literature.

Furthermore, liposomes have been used to study the interaction of bioactive agents with the membrane. The membrane is the main target site of most drugs. Thus, it would be of paramount importance to evaluate the effect of the drug on membrane stability so as to better understand their mechanism of action. The investigations of drug-lipid interactions using liposomes as model membrane revealed the ability of some molecules to increase membrane fluidity: this was proven for progesterone derivatives [94], hydroxypropyl- β -cyclodextrin [95], and some essential oil components [96], [97]. Based on studies of liposomes leakage of self-quenching probes, the main permeabilizing agents were classified according to their process of membrane permeabilization in a recently published review [9]. These agents comprise: surfactants inducing membrane solubilization, cyclodextrins known as lipid membrane extracting agents, proteins and peptides promoting pore formation in the membrane, defect-forming agents, etc. [9]

On the other hand, liposomes can serve as in vitro model for drug permeability screening. It is employed for the prediction of the extent of oral absorption for new drug entities in early stages of drug discovery and development [98]. The original model is known as “phospholipid vesicle-based permeation assay” or PVPA; it was introduced in 2006 as an artificial liposome-based in vitro model representative for the intestinal epithelium and aiming to estimate passive drug permeability [99]. It consists of liposomes deposited into the pores of a filter support with a separate layer on the top of the support. The liposomes are then fused by solvent evaporation and a freeze–thaw cycle yielding a tight barrier comprised of a sandwich of phospholipid bilayers (figure 16). In this model, liposomes are composed of egg phosphatidylcholine naturally found in the intestinal epithelia and different biological barriers. In contrast to other artificial membrane models, no organic solvents are used in the PVPA which can accurately mimic the in vivo situation [100], [101]. This model is employed as a screening tool for passive permeability studies because it is

much less labor-intensive compared to cellular models [102]. A good correlation with in vivo data was reported for various drugs transported via passive diffusion [99].

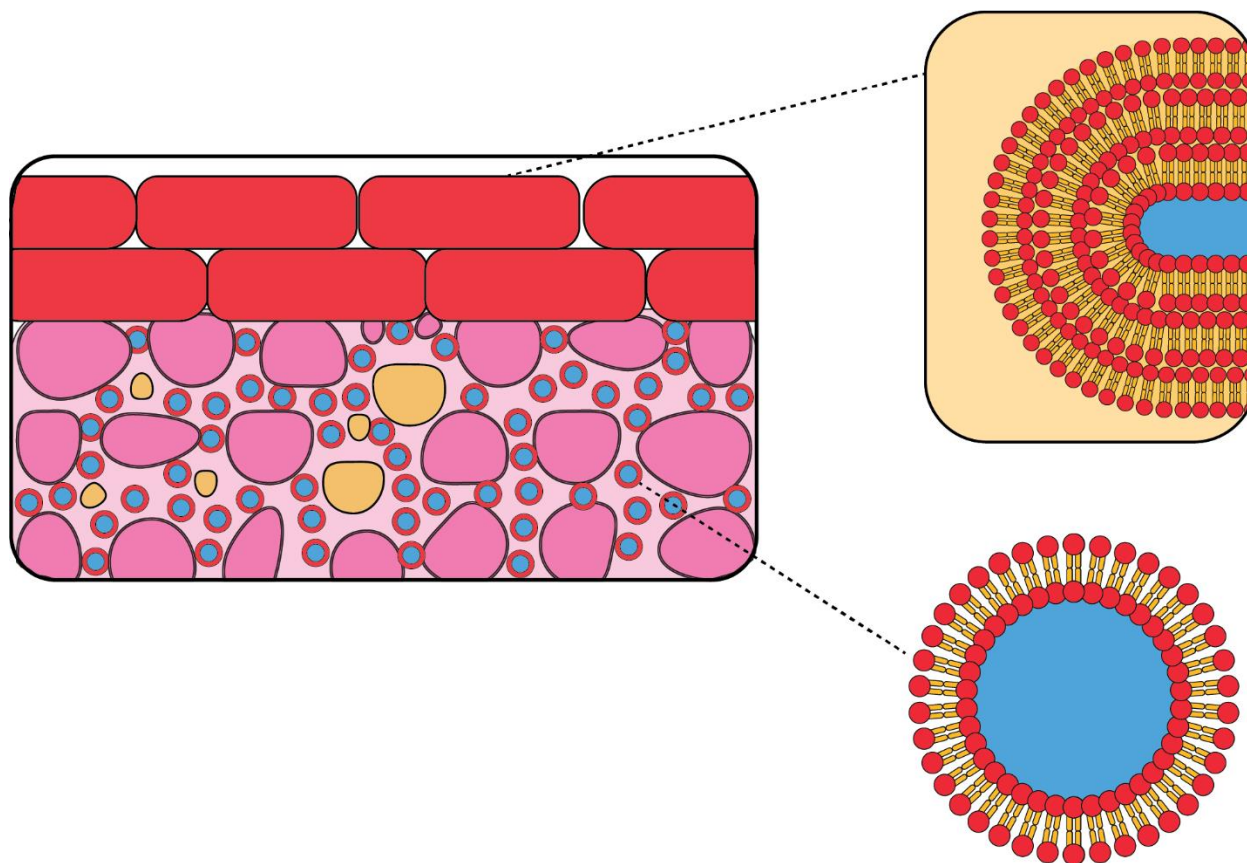


Figure 16 A schematic illustration of the PVPA barrier. It is generally inserted in Transwell (0.33 cm²) and Millicell well plates (0.6 cm²). The filter support is presented in pink while the liposomes are shown in red; the liposomes found within the pores are small and unilamellar whereas those placed on the top of the filter support are large and multilamellar.

Although the original PVPA is regarded as a general model for several biological absorption barriers, it was possible to improve this model by changing the lipid composition of liposomes in order to better mimic other specific barriers [102]. Different PVPA variants were developed: for example, a biomimetic PVPA model with a lipid composition closely resembling that of the intestinal epithelia was introduced by Naderkhani et al. (2014b) [101]. The manipulation of the composition of the original PVPA barrier was also performed to mimic the human stratum corneum composition giving rise to a new PVPA-variant model for screening skin transdermal absorption of drug candidates [103]. A novel approach for a Blood-Brain-Barrier (BBB) model based on phospholipid vesicles was as well designed for the prediction of BBB drug permeability [104]. Another variant of PVPA was recently described; it is the mucus-PVPA consisting of

liposomes with the same composition as the original PVPA model with a layer of mucus added to the top of the barrier [105]. These in vitro tools allow a reliable prediction of passive absorption through various biological barriers.

4. Applications of liposomes

Liposomal formulations possess characteristics that make them acknowledged as a promising strategy in bio-health domains. Their biocompatibility, ease of preparation, and ability to encapsulate a variety of molecules of different sizes and solubility profiles are essential for the development of novel approaches in drug delivery, vaccines, cosmetics, and other fields. In this part, the use of liposomes in the three abovementioned domains will be discussed.

4.1. Liposomes in drug delivery

Liposomes hold several advantages as drug carriers such as their ability to complex with both negatively and positively charged molecules, and their specificity and cell and tissue targeting. Their efficacy as drug carriers depends on their interaction with cells. In-vivo studies showed that liposomes can be internalized in cells via simple adsorption or subsequent endocytosis, and in some rare cases via fusion with the cell membrane [106].

Liposomes were used as drug carriers for anticancer therapy since the last century, and showed a reduction on side effects and activity improvement on the carried drug [107]. Since then, several anti-cancer liposomal formulations were commercialized [6]. Some of these formulations are cited in **Table 3**. Due to the success of liposomes in cancer therapy, more promising research continues to emerge. Recently, PEGylated liposomes were prepared via lipid thin-film hydration method as a carrier for both thymoquinone and docetaxel [108]. Docetaxel is known to be a key factor in standard chemotherapeutic regiment of breast cancer patients with a major drawback of high sensitivity due to high activation of Akt pathway. On the other hand, thymoquinone is known to inhibit and modulate several tumors progression signaling pathway such as the Akt pathway. By co-encapsulating these two molecules, it was found that a synergetic effect exists against MCF7 breast cancer cells proliferation with the ability to lower the dose of docetaxel needed [108]. Another study exploited liposomes in the emerging strategy of bacterial therapy for cancer treatment. Paclitaxel loaded in liposomes loaded in bacteria is prepared for inhalation treatment of

lung cancer. It was found that this strategy dramatically inhibits the proliferation of A549 lung cancer cells in-vitro, and on rat primary lung cancer [109].

Table 3 Commercially available liposomal drug formulations for cancer therapy

Commercial name	Drug	Manufacturer	Applications	Reference
Doxil®	Doxorubicin	Baxter Healthcare Corporation	Treatment of ovarian cancer, acquired immune deficiency syndrome (AIDS)-related Kaposi's Sarcoma, and multiple myeloma	[110], [111]
Exparel®	Bupivacaine	Pacira BioSciences, Inc.	Anesthetic	[112]
Marqibo®	Vincristine	Spectrum Pharmaceuticals, Inc.	Philadelphia chromosome negative acute lymphoblastic leukemia	[113]
Arikayce®	Amikacin	Insmed Incorporated	Mycobacterium avium complex lung disease	[114]
Lipodox®	Doxorubicin hydrochloride	Sun Pharmaceutical Industries Ltd.	Treatment of ovarian cancer, acquired immune deficiency syndrome (AIDS)-related Kaposi's Sarcoma, and multiple myeloma	[115]
Vyxeos®	Daunorubicin and cytarabine	Jazz Pharmaceuticals plc	Therapy-related acute myeloid leukemia	[116]

Liposomes are also widely used for the delivery of genetic materials. This is due to the ability of liposomes to carry large pieces of DNA (in a size of a chromosome) while offering a high degree of protection from degradative process. In fact, a recent study showed that liposomes made from DOTAP and DMPC lipids can carry 1.5 folds larger DNA quantity comparing to the recombinant adeno-associated virus vector which leads to the production of extended therapeutic levels of multiple transgene-encoded therapeutic human proteins. In addition, it was found that DNA-liposomal vectors were able to produce therapeutic levels of cytokine and monoclonal antibodies without integration into genomic DNA, all by overcoming the limitation of adaptive immune response caused by viral vectors [117].

The use of liposomes goes beyond cancer therapy and DNA delivery. During the last ten years, liposomes formulations were reported to be effective against central nervous system diseases [118], [119], lung diseases [120], [121], and microbial and fungal infections [122], [123]. In addition, liposomes were able to be administered topically [124], orally [125], intravenously [126] and by the intranasal route [127].

4.2. Liposomes in vaccines

Vaccine research is shifting towards replacing whole attenuated or killed pathogens with subunit vaccines composed of well characterized antigens such as recombinant proteins and peptides, offering better resolution, easier production, and more safety. On the other hand, the use of antigens is accompanied with a weaker immune response due to the inability of antigens to induce maturation of dendritic cells [128]. To enhance the immune response of such vaccines, adjuvants from different compounds are usually added to the vaccine formulations. In addition, a major roadblock in vaccine research is the delivery of the antigen. This is where liposomes fit into the picture.

Liposomes were used as vaccine adjuvants and protein carriers since the seventies when they were first proven to induce the immune response of associated antigens (**Table 4**) [129]. The versatility and plasticity of liposomes, same as their ability to encapsulate different antigens into their different compartments (**Figure 17**) open the possibility of tailoring liposomal vaccines for individual applications. In fact, several liposomal-based vaccines are already commercialized against several kinds of diseases. In addition, the rapid identification and purification of novel antigens, allows subunit vaccines to be used against not only infections, but also diseases such as autoimmunity and cancer.

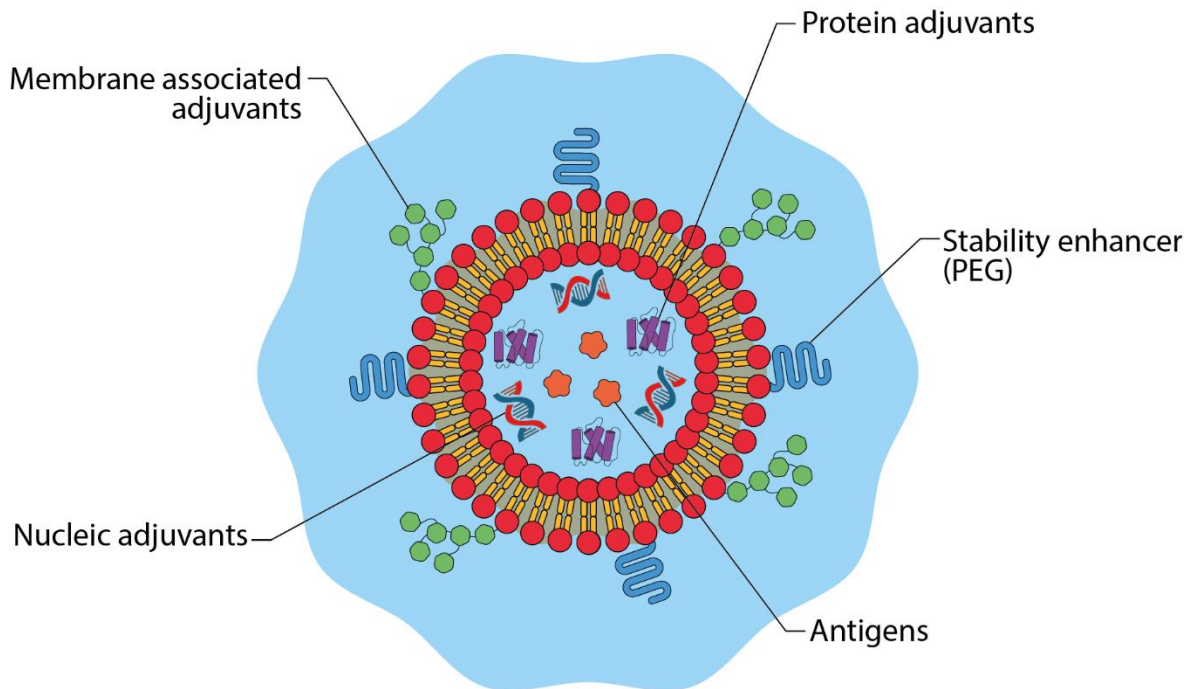


Figure 17 Different adjuvants and vaccine elements that can be incorporated within a liposomal system

Liposomes have repeatedly proved their ability to boost the immunogenicity of incorporated peptides in vaccine formulation. MUC1 peptide-loaded liposomes were able to induce humoral and cellular response in mice by stimulating a strong antigen-specific T cell responses and the production of MUC1-specific antibodies, while the administration of the free peptide did not induce such responses [130]. In addition, mannosylated liposomes were able to deliver antigens to dendritic cells providing an efficient solution to enhance immune responses of peptides and protein vaccines [131]. More recently, *Mycobacterium tuberculosis* vaccine formulation consisted of liposomal cationic adjuvant formulation and the multicomponent antigen H56 were modified by the inclusion of the Toll-like receptor agonist Pam₃Cys as immune modulatory adjuvant. The incorporation of Pam₃Cys led to the increase of macrophages intake and higher levels of TNF and several interleukins molecules in vitro and in vivo [132]. In another study, a novel immunomagnetic technology was used in the development of a whole parasite vaccine. Membranes from red blood cells parasitized with *P. falciparum* were used to construct liposomes later mannosylated for better targeting of antigen specific cells. This strategy was able to induce a potent strain- and species-transcending cell-mediated immune responses to parasite antigens with a low antibody response to human red cells [133].

Table 4 Commercially available liposome-based vaccine formulations

Name	Composition	Active ingredient	Disease	Reference
VaxiSome	ceramide carbamoyl-spermine and cholesterol helper lipid	Monovalent subunit/trivalent split virions	Influenza	[134]
IC 31®	Cationic peptide KLKL5KLK and the immunostimulatory oligodeoxynucleotide ODN1a	Recombinant subunit Ag85BTB10.4 protein	Tuberculosis	[135]
LDP	DOTAP lipid	E7 protein	HPV type 16	[136]
Escort™ Transreagen	DOTAP and DOPE	T.Gondii GRA7 antigen	Toxoplasma gondii	[137]
CLDC	DOTIM lipid and cholesterol helper lipid	HSV gD2	Herpes simplex virus	[138]
Pfizer-BioNTech COVID-19	ALC-3015, ALC-0159, DPSC, CHOL	modRNA encoding the spike protein of SARS-CoV-2	COVID-19	[139]

As mentioned previously, liposomes were also exploited for the development of cancer vaccines. Unlike infectious diseases vaccines, cancer vaccine cannot be serially injected over a long time in immune-replete children. Instead, it must reflect a rapid immunogenic activity in immunocompromised patients already introduced to cytotoxic chemotherapy. For this reason, several research groups have prioritized mRNA as a source of tumor specific antigen due to several factors such as the mechanisms of cells interactions with external mRNA, its direct effect on innate immunity, its ability to become a tumor specific vaccine for all patients, and its ease of production and storage [140]. RNA-lioplexes were able to target dendritic cells by activating a toll-like receptor 7 which leads to the production of type I interferon and the initiation of a robust anti-tumor activity [141]. Another study showed the usefulness of cationic liposomes as carriers of mRNA encoding ovalbumin, human papilloma virus E7, and tyrosinase related protein-2. These

particles were able to transfect dendritic cells and macrophages in spleen and liver, and induce a strong antigen-specific T cell response [142]. Other studies also report the use of peptide-loaded liposomes as vaccine formulations for breast cancer [143]. Recently, the global COVID-19 pandemic has led to the development and the validation of the first mRNA-based vaccines: the first to be registered was the Pfizer-BioNTech COVID-19 vaccine that has been authorized to prevent from the coronavirus disease. It is mainly constituted of a nucleoside-modified mRNA (modRNA) encoding the viral spike glycoprotein (S) of SARS-CoV-2 [139].

5. Conclusion

Since many decades, liposomes are used in a broad range of pharmaceutical applications because of their unique properties. After a brief description of the liposome's composition, the chapter reviews concisely the liposomes preparation methods with a special focus on their advantages and drawbacks. The preparation methods allowing industrial-scale production are pointed out since scaling-up is a major issue in the pharmaceutical industry.

The lipid composition of liposome formulation determines its stability and can markedly affect its in vivo performance and fate. Hence, liposomes are used as biomimetic model membranes for the investigation of the membrane properties under various conditions and the mechanistic behavior of active agents. These studies help in the optimization of an appropriate formulation for drug encapsulation.

Furthermore, the ability of liposomes to encapsulate a variety of molecules (hydrophilic, lipophilic, different sizes, etc.) offers an excellent opportunity to selective drug targeting especially for anticancer therapy and the delivery of genetic materials. Liposomal formulations are also employed as vaccine adjuvants and protein carriers and have been incorporated in skin gels and creams for either the delivery of cosmetic ingredients or fighting against skin aging. Liposomes formulation and characterization is an active research area. This paves the way to the emergence of new promising applications, especially in the biomedical field.

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Chapitre 2 : Évaluation de la perméabilité membranaire des liposomes par des techniques de fluorescence

Introduction

La membrane biologique est une barrière hydrophobe semi-perméable qui contrôle le passage des substances à travers la bicouche lipidique (*Biochemistry of lipids, lipoproteins and membranes*, 2015; Lodish, 2000). Ceci définit la perméabilité membranaire qui constitue une fonction vitale assurée par la membrane puisqu'elle intervient dans les échanges réalisés par la cellule avec son environnement. Le maintien de la perméabilité membranaire s'avère alors primordial.

La perméabilité membranaire peut être évaluée par une multitude de méthodes. Dans ce deuxième chapitre, nous allons nous concentrer sur celles qui permettent d'étudier la perméabilité des membranes liposomales. Ces méthodes peuvent être classées en deux catégories : i) les méthodes non-fluorescentes se basant sur la libération d'une molécule encapsulée à partir des liposomes et ii) les méthodes fluorescentes reposant sur le relargage de fluorophores à partir des liposomes (Deleu et al., 2014; Jain and Jain, 2016; Moreno-Bautista and Tam, 2011). Ces dernières sont largement utilisées tenant compte de la simplicité, la sensibilité et la précision des techniques de fluorescence. Dans la revue bibliographique suivante publiée dans « *International Journal of Pharmaceutics* », les différentes techniques seront décrites avec un accent particulier sur les techniques de fluorescence couramment employées pour l'évaluation de la perméabilité membranaire et les fluorophores de chacune. Parmi celles-ci, les techniques utilisant des agents fluorescents qui, à de hautes concentrations, provoquent un phénomène d'auto-extinction de la fluorescence demeurent la méthode de choix pour réaliser ce type d'études. Les fluorophores possédant cette propriété et habituellement utilisés sont : la calcéine, la carboxyfluorescéine (CF) et la sulforhodamine B (SRB). Un tableau récapitulatif montrant la structure de ces agents fluorescents et comparant leurs caractéristiques spectrales ainsi que leurs propriétés physico-chimiques est présenté dans la revue.

Les techniques de fluorescence ont bien servi à élucider le mécanisme d'action de nombreux agents actifs qui perméabilisent la membrane afin d'exercer leur activité biologique. En dépit de la pléthore d'études identifiant ce type de molécules, la classification des agents perméabilisants n'a pas été mise en vigueur dans la littérature. Dans cette revue, ces agents sont classés selon leur

mode de perméabilisation de la membrane. On distingue alors : les tensioactifs (ou les détergents) qui sont définis comme des agents solubilisant des membranes, les cyclodextrines (CDs) qui sont capables d'extraire les composants lipidiques membranaires, les protéines induisant la formation de pores au niveau de la membrane ainsi que d'autres molécules perturbant la stabilité membranaire et engendrant des défauts ou des lacunes dans la membrane. Pour chacune de ces quatre classes, le mécanisme d'action sera détaillé et un tableau regroupant l'ensemble des études de fluorescence qui montrent l'effet de ces molécules sur la perméabilité membranaire des liposomes de différentes compositions et structures sera également présenté et analysé. En fait, l'effet perméabilisant de ces différents agents s'est révélé dépendant de la composition lipidique de la membrane d'une part et des propriétés physico-chimiques et structurales des molécules d'autre part. Tous ces aspects seront discutés dans la revue.

Finalement, la perméabilité membranaire des liposomes dépend non seulement de la présence d'agents perméabilisants mais elle est aussi modulée par d'autres facteurs intrinsèques et extrinsèques à la membrane. En effet, en absence de tout agent perméabilisant, les propriétés de la membrane sont principalement régies par sa composition lipidique et ses caractéristiques structurales (facteurs intrinsèques) qui sont résumées dans un tableau. Des conditions expérimentales influençant la perméabilité comme le pH du milieu, la température, etc. (facteurs extrinsèques) seront de même discutées et analysées.

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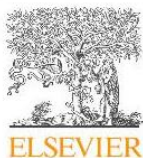
Liposomal membrane permeability assessment by fluorescence techniques : Main permeabilizing agents, applications and challenges

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Review

Liposomal membrane permeability assessment by fluorescence techniques: Main permeabilizing agents, applications and challenges

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Permeabilizing agent

ABSTRACT

Liposomes are lipid vesicles made of one or multiple lipid bilayers surrounding an internal aqueous core. They are broadly employed as models to study membrane structure and properties. Among these properties, liposome membrane permeability is crucial and widely assessed by fluorescence techniques. The first part of this review is devoted to describe the various techniques used for membrane permeability assessment. Attention is paid to fluorescence techniques based on vesicle leakage of self-quenching probes, dye/quencher pair or cation/ligand pair. Secondly, the membrane-active agents inducing membrane permeabilization is presented and details on their mechanisms of action are given. Emphasis is also laid on the intrinsic and extrinsic factors that can modulate the membrane permeability. Hence, a suitable liposomal membrane should be formulated according to the aim of the study and its application.

1. Introduction

Living cells are surrounded by a single or multiple membrane of particular structure and functions. As postulated by Singer and Nicolson in 1972, a biological membrane is regarded as a fluid matrix composed of a homogeneous lipid bilayer embedded with proteins and glycoproteins referring to the so called “fluid mosaic model” (Singer and Nicolson, 1972). Thenceforth, this model has been continuously developed and new definitions have emerged. Indeed, due to lipids polymorphism, some refinements were brought to the mosaic fluid model. It was demonstrated that biological membranes do not form a homogeneous fluid phase but, in contrast, membrane lipids form phase-separated domains, called “lipid rafts”, with both a specific composition

and particular dynamic properties (Brown and London, 1998; Simons and Ikonen, 1997).

The lipid bilayer sets a hydrophobic barrier responsible for the selective transport of molecules across the membrane. This property denotes the so-called “membrane permeability”. As a matter of fact, membrane permeability is defined as the measure of the flux or rate at which a solute would cross a lipid bilayer to get out into the aqueous compartment on the other side of the membrane (Frézard, 1999). It is a biological function of vital importance since transport phenomena occurring across biomembranes are involved in many cellular processes accounting for cell viability. The molecular transport mechanisms of substances across a biological membrane are classified into two major categories: passive transport and active transport. Passive transport is

Abbreviations: ANTS, aminonaphthalene trisulfonic acid; BUD, budesonide; CD, cyclodextrin; CF, carboxyfluorescein; Chol, cholesterol; CL, cardiolipin; Crysmeb, partially methylated crystallized- β -CD; Cuc E, cucurbitacin E; Dimeb, 2,6-Dimethyl- β -cyclodextrin; DMPC, dimyristoylphosphatidylcholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DPA, dipicolinic acid; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DPPG, dipalmitoylphosphatidylglycerol; DPX, p-xylene-bis-pyridinium bromide; DRV, dried-rehydrated vesicle; DSPC, distearoyl-*glycero*-PC; EDTA, ethylenediaminetetraacetic acid; Erol, ergosterol; Gm, gomesine peptide; GmL, linear analogue of Gm; GUV, giant unilamellar vesicle; H-PC, hydrogenated-PC; HP- β -CD, hydroxypropyl- β -cyclodextrin; HP- γ -CD, hydroxypropyl- γ -cyclodextrin; LPC, lysophosphatidylcholine; LUV, large unilamellar vesicle; MD, molecular dynamics simulations; Me- β -CD, methyl- β -cyclodextrin; MLV, multilamellar vesicle; MPER, membrane-proximal external region of the human immunodeficiency virus type 1 envelope glycoprotein 41 subunit; OSM, N-oleoyl-SM; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PFPs, pore forming proteins; PFTs, pore forming toxins; PG, phosphatidylglycerol; PI, phosphatidylinositol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; PR, prednisolone; PS, phosphatidylserine; PSM, palmitoyl sphingomyelin; Rameb, randomly methylated- β -cyclodextrin; SA, stearylamine; SBE- β -CD, sulfobutylether- β -cyclodextrin; SM, sphingomyelin; SPC, Soybean phosphatidylcholine; SRB, sulforhodamine B; Stol, stigmaterol; SUV, small unilamellar vesicle; Tb³⁺, Terbium III ion; Trimeb, 2,3,6-trimethyl- β -cyclodextrin; β -CD, β -cyclodextrin; γ -CD, γ -cyclodextrin

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the most common transport mechanism across cell membranes. It requires the interaction of the solute with lipid membrane components. To study these interactions, it would be crucial to choose proteins-free model membranes avoiding subsequently the interference of lipid-protein and solute-protein interactions. In this context, biomimetic model membranes, also called “artificial membranes”, are used as tools for studying membrane properties and structure. They are lipid-based synthetic membranes, commonly employed to investigate lipid-lipid and drug-lipid interactions as well as different processes occurring at the membrane surface (Deleu et al., 2014; Eeman and Deleu, 2010).

Liposomes are synthetic spherical vesicles composed of one or several lipid bilayers surrounding an internal aqueous core with particle size ranging from 20 nm up to several micrometers (Akbarzadeh et al., 2013). Their composition usually includes natural or synthetic phospholipids. It may also contain sterols (most commonly cholesterol) and other lipid species (e.g. sphingomyelin, cardiolipin, etc.). Liposomes are promising model membrane systems for several applications with a tremendous success in drug delivery. Indeed, several liposome-based drug formulations have been developed for therapeutic use and are available in the pharmaceutical market (Bulbake et al., 2017). For instance, Doxil®, DaunoXome®, Depocyt®, Myocet®, etc. have been approved for the treatment of various cancers while Abelcet®, Ambisome® and Amphotec® have been used for the management of fungal infections. Liposome-based products were also explored in other therapeutic areas including viral vaccines (Schwendener, 2014), pain management (Alam and Hartrick, 2005) and photodynamic therapy (Düzgüneş et al., 2018). Membrane permeability is a key parameter for improving the efficacy of liposomal formulations as a potent delivery system: in fact, the permeability of liposomes membrane has to be controlled in order to sustain the encapsulation of bioactive agents and to ensure their release at the target site. Besides, the effect of bioactive agents on liposomal membrane permeability usually serves to understand the mechanism of action of various natural and synthetic agents.

Fluorescence techniques proved to be very efficient to monitor the permeability of liposomal membranes and to demonstrate the permeabilizing effect of a large number of membrane-active agents. Countless studies have been addressing different aspects of membrane permeabilization in the absence and the presence of bioactive agents. Numerous factors, either intrinsic or extrinsic to the membrane system, were evaluated. In fact, these *in-vitro* experiments widely vary in lipid membrane composition, drug concentration and characteristics, buffer conditions, temperature, etc. The influence of these parameters on membrane permeability was highlighted.

Although a plethora of studies assessing liposomal membrane permeability by fluorescence techniques has been carried out during the last decades, there is, to the authors' knowledge, no reviews covering this topic. Therefore, this paper would be of great interest to the scientific community providing a comprehensive insight into the usefulness of these methods.

In this review, the different techniques used to evaluate liposomal membrane permeability are quickly overviewed and the fluorescence techniques based on the *in vitro* release of a fluorescent probe from liposomal vesicles are then presented. Secondly, the main permeabilizing agents are classified and their mechanistic behaviors are also described. Fluorescence studies investigating liposomal membrane permeability will be documented and analyzed to get a better understanding of the permeabilization process and to reveal the key modulators of the membrane permeability of liposomes in the absence and presence of membrane-active agents using conclusive tables. The choice of the fluorescence method and other related parameters (fluorophore, liposomes preparation method, vesicles type, etc.) is discussed throughout the review. Finally, the advantages of these techniques, their drawbacks and their potential to draw reliable conclusions are reported.

2. Non-fluorescent techniques used to assess liposomal membrane permeability

The *in vitro* release of an encapsulated agent from liposomes is generally conducted by various methods that could be mainly categorized into: “methods based on barriers diffusion” among which the dynamic dialysis method and the Franz cell diffusion are the most used, “methods based on mechanical forces” where the centrifugation method is commonly applied and “real time measurement methods”. Computational methods are also used for liposomal permeability assessment. All these methods will be briefly described in the next paragraphs.

2.1. Methods based on barriers diffusion

2.1.1. Dynamic dialysis

The dynamic dialysis method is described as follows: a solution comprising drug-loaded liposomes is placed inside a dialysis bag (donor compartment) (with a molecular weight cut-off larger than the size of the drug molecules), which is in turn introduced into a reservoir (receiver or acceptor compartment) of known buffer volume. Then the whole system is kept under agitation on a magnetic stirrer at a defined temperature allowing the release of the entrapped drug with time. Sampling is assayed at predetermined time intervals by withdrawing an aliquot of the solution of the receiver compartment. Finally, released drug is quantified (Fan et al., 2008; Panwar, 2010; Yang et al., 2007; Zhang et al., 2005; Zhou et al., 2018). The obtained data will help to construct the release kinetic profile and to determine the release constant.

However, the dynamic dialysis method has few disadvantages. First of all, the appearance of drug in the receiver compartment is the result of two consecutive diffusions: the drug is initially released from liposomes and then diffuses across the dialysis membrane to the bulk solution (receiver compartment). This dual diffusion barrier may lead to incorrect prediction of the drug release half-life since the rate constant obtained in these conditions may not indicate the rate of drug release from liposomes (Modi and Anderson, 2013; Zambito et al., 2012). Also, the required force for diffusion as well as the reversible binding of the released drug to liposomes membrane could also mislead the interpretation of the obtained data (Jain and Jain, 2016; Moreno-Bautista and Tam, 2011). Thus, careful consideration of these inconveniences has to be taken to validate the release rates obtained by the dynamic dialysis method. Interestingly, the use of a dialysis bag with low diffusional barrier and low affinity for the drug is recommended.

2.1.2. Franz cell diffusion

Similar to the dialysis method principle, the Franz cell diffusion system is additionally used to monitor the *in vitro* drug release from liposomal formulations designed for the delivery of their payload at specific sites of the body. The Franz cell apparatus consists of two chambers: the donor compartment (top chamber) and the receiver compartment (bottom chamber) separated by a membrane. The top chamber generally contains the compound of interest (drug) loaded in a variety of formulations (liquid solution, suspension, gel, cream, etc.) while the receptor medium is filled with an appropriate buffer. As for the membrane separating both compartments, it could be a synthetic membrane, a tissue construct or even a biological sample such as human skin. This assay has shown an excellent correlation between *in vitro* and *in vivo* studies (Franz, 1975). When testing drug release from liposomal formulations, drug-loaded liposomes are placed in the donor compartment and the diffusion of drug to the receiver compartment is followed by determining the amount of active ingredients that has permeated the membrane at each time point. This method is mostly applied as an *in vitro* tool for identifying the optimal liposomal formulation that allows the delivery of active agents through the skin (Hwang et al., 2007; Nava et al., 2011; Rukavina et al., 2018; Zhou

et al., 2014).

2.2. Methods based on mechanical forces

2.2.1. Centrifugation

Another popular technique used for drug release measurement is the centrifugation method, also known as the dispersion method. It involves the addition of the drug-loaded liposomes to an excess of the buffer solution in a glass vial. The system is placed in a shaking bath at a defined temperature. Then, at predetermined time intervals, aliquots of the solution are taken and centrifuged. The amount of drug in the supernatant or in the pellet is analyzed (Merodio et al., 2001; Shazly et al., 2008; Yingyuad et al., 2018). Nevertheless, exerting centrifugal forces on liposomal vesicles might promote the premature release of entrapped drug, leading subsequently to mistaken results since the amount of drug released in this case would be more than that released when no external forces are imposed on the liposomal samples (Moreno-Bautista and Tam, 2011).

Hence, despite their wide use for membrane permeability measurements, both the dialysis and the centrifugation methods lead to uncertainty regarding drug release data due to the problems related to diffusional barriers and the need to induce mechanical forces to separate released drug. More importantly, none of these techniques can directly monitor the real-time drug release. To meet these hurdles, an alternative technique has been developed: the drug-selective electrode system.

2.3. Real time measurement methods

2.3.1. Drug-selective electrode

This method relies on the drug electrochemical properties allowing to measure the concentration difference of the drug on both sides of the electrode (Paula et al., 1996). The main advantage of this technique includes their ability to measure drug concentrations at real time without requirement of released drug separation from nanocarriers such as liposomes (Mora et al., 2009; Xu et al., 2007).

2.4. Molecular dynamics simulations

Apart from classical methods, computational approaches known as Molecular Dynamics simulations (MD) are also used to investigate the interactions between permeants and the lipid membrane. As a matter of fact, experimental methods allow the measurement of permeability through lipid membrane indicating the stability of the membrane structure. However, they fail alone to describe the molecular interaction happening at the membrane surface. Therefore, computational modeling can complement experimental results giving more details on the mechanism of membrane transport (Shinoda, 2016). It is worth mentioning here that even though, in most MD studies, the entire

liposome isn't directly considered, the computational system is designed to accurately model the surface of the liposomal membrane and to act as such as a model of the liposome surface. A great number of papers used molecular simulations to model the membrane permeation of diverse molecules including water, neutral molecules, charged species, drug-like molecules, etc. (Awoonor-Williams and Rowley, 2016; Shinoda, 2016). Moreover, the effect of lipid composition on membrane permeability has been broadly studied computationally (Jedlovsky and Mezei, 2003; Khajeh and Modarress, 2014; Saito and Shinoda, 2011; Sugii et al., 2005). Due to their ability to predict drug permeability across biomembranes, MD simulations can contribute in the optimization of liposomal delivery systems (Xiang and Anderson, 2006).

3. Fluorescence techniques used for membrane permeability assessment

Fluorescence techniques are regarded as powerful analytical tools for studying membrane properties and notably, membrane permeability. They were broadly applied to this purpose during the last decades. These methods comprise fluorescence spectroscopy methods using either self-quenching probes, dye/quencher pairs or lanthanide cation/ligand pairs and confocal fluorescence microscopy methods using single or multiple fluorophores. Much attention has been paid to these straightforward techniques for their high sensitivity and precision. A detailed description of the principle of these methods in membrane permeability studies will be presented in the following paragraphs.

3.1. Vesicle leakage based on self-quenching probes

According to "fluorescence self-quenching method", the fluorescent agents are encapsulated within the liposomal aqueous compartment at self-quenching concentrations; the membrane permeability is evaluated by following their release from liposomes with time. An increase in the fluorescence signal is associated with an increase in the permeability of liposome membrane due to the release of the fluorescent probes from liposomes. The most commonly used self-quenching probes are: calcein, carboxyfluorescein and sulforhodamine B. The kinetic of dye release allows to determine the permeability of liposomal membrane.

Fluorescein and rhodamine fluorophores (Fig. 1) are xanthenes dyes. They have been broadly used in a variety of applications in biological, biochemical and medicinal research due to their outstanding spectral properties (Lakowicz, 2006). Many fluorescein and rhodamine derivatives have been known for their ability to self-quench; among these, calcein and carboxyfluorescein (CF) which are fluorescein derivatives as well as sulforhodamine B (SRB), a rhodamine derivative, are the most popular. Nonetheless, the mechanism underlying self-quenching phenomenon is not entirely understood nor demonstrated for these

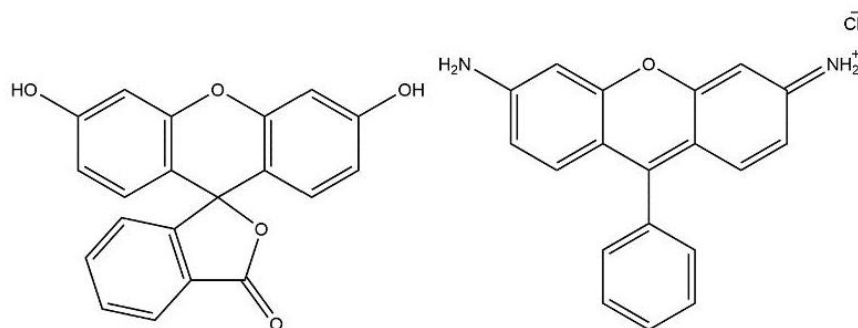
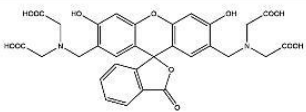
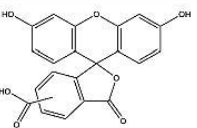
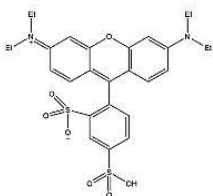
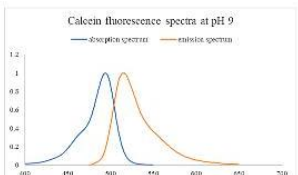
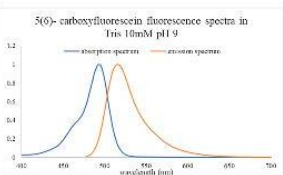
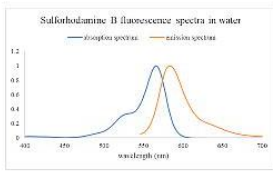


Fig. 1. the chemical structure of fluorescein (to the left) and rhodamine (to the right).

Table 1

The structure, physicochemical and spectral properties of three fluorophores commonly used as self-quenching probes: calcein, carboxyfluorescein and sulforhodamine B.

Fluorophore name	Calcein	Carboxyfluorescein	Sulforhodamine B
Chemical structure			
Molecular weight (g/mol)	622.539	376.32	558.664
LogP	1.56 (predicted) (chemspider)	2.66 (predicted) (chemspider)	2.2 (predicted) (pubchem)
Water solubility (g/L)	ND	ND	ND
Fluorescence emission spectrum			
Excitation wavelength	495 nm	495 nm	565 nm
Emission wavelength	515 nm	517 nm	586 nm
Quantum yield	High	High	High
Self-quenching concentration	Concentration range 50–100mM	Concentration range 25–100 mM	150 mM
pH effect on spectral properties	Increase of fluorescence with pH in the range 5.5–8.5 in the absence of metal ions and decrease of fluorescence in this range in the presence of metal ions (Furry, 1985)	Increase of CF fluorescence with pH in the pH range 5.5–8 (Massou et al., 2000)	Fluorescence intensity was independent on pH over the range 3–10 (Coppeta and Rogers, 1998)
Photostability	Stable over a pH range 7.5–8.5 (Furry, 1985)	Low photostability (Lakowicz, 2006)	High photostability (Hay, 1995; Lakowicz, 2006)

Abbreviations:

ND: not determined

dyes. For instance, a previous study reported that CF and SRB, when encapsulated inside the liposomes, underwent dimerization giving rise to non-fluorescent dimers. Also, self-quenching may result from a combination of dye dimerization, energy transfer to nonfluorescent dimers and collisional quenching interactions between fluorophore monomers (Chen and Knutson, 1988).

Although the popularity of these fluorescent probes and their widespread use stems from their ability to self-quench, there is, to our knowledge, no published data that precise the concentration above which self-quenching is achieved. Moreover, a careful examination of the studies using these self-quenching probes points out to the fact that different concentrations of the probes were used to reach auto-quenching. Based on these experiments, we could determine for each fluorophore a concentration range within which self-quenching occurs: a concentration range of 50–100 mM; 25–100 mM and a concentration of 150 mM for calcein, CF and SRB respectively. Table 1 summarizes the physicochemical and spectral properties of these fluorophores.

3.2. Vesicle leakage based on dye/quencher pair

To assess membrane permeability, vesicle leakage could be monitored by using a pair of two agents: a fluorophore and its quencher. The co-encapsulation of both agents in the same liposome core will generate a fluorescence quenching. The fluorophore release from vesicle and its subsequent dilution is measured by an increase in the fluorescence. The most common assay based upon this principle is: the ANTS/DPX (aminonaphthalene trisulfonic acid/ p-xylene-bis-pyridinium bromide)

assay where the fluorophore ANTS is quenched by DPX.

ANTS is a polyanionic dye, with maximum excitation and emission wavelengths of 356 nm and 516 nm, respectively. The fluorescence of this dye was shown to be effectively quenched by the cationic DPX through a collisional quenching mechanism. The ANTS/DPX assay was originally developed to track membrane fusion (Düzgüneş et al., 1985; Ellens et al., 1985). Then, it was used to monitor membrane permeabilization. For permeability assays, ANTS and DPX should be co-encapsulated into the same population of liposomes. Upon the leakage of both encapsulated agents and their subsequent dilution, ANTS fluorescence will increase because DPX quenching effect will be reduced.

3.3. Vesicle leakage based on lanthanide cation/ligand pair

This assay is less frequently used than the preceding ones for permeability studies. It relies on the co-encapsulation of a weakly fluorescent lanthanide cation and a fluorescence enhancer ligand in the same liposomal batch. This will produce high fluorescence intensity due to the chelation of the cation by the ligand. Consequently, the release of the cation/ligand pair from liposomes into the medium containing another chelator such as ethylenediaminetetraacetic acid (EDTA) will cause a fluorescence decrease due to the dissociation of the complex.

The Tb³⁺/DPA (Terbium III ion/dipicolinic acid) assay is commonly used in this case. Tb³⁺ is a trivalent lanthanide cation with a low intrinsic fluorescence in aqueous solutions. Upon chelation by ligands such as DPA, a highly fluorescent complex Tb³⁺/DPA will be formed (Sabbatini et al., 1993). The chelate has a maximum absorption at

270 nm and is detected at 490 nm or 545 nm. This assay was initially used to study the mechanism of membrane fusion (Wilschut et al., 1980). It can be also applied to assess membrane permeabilization by following the leakage of fluorescent probes from lipid vesicles (Gupta et al., 2013).

3.4. Time-lapse confocal fluorescence microscopy of single giant unilamellar vesicle (GUV)

This method involves the incubation of blank GUVs (devoid of any marker) with a solution containing a fluorescent marker and the testing agent. The influx of the fluorescent dye inside the vesicle is then monitored by time-lapse confocal microscopy. This technique offers valuable information regarding the permeabilization kinetics and the release mechanisms (Apellániz et al., 2010; Manzini et al., 2014; van Rooijen et al., 2010). It is also possible to test the stability of the permeabilization process by adding another fluorescent agent to the vesicles and taking additional pictures to see whether the permeabilization is temporary or not (Apellániz et al., 2010). A similar experimental strategy using the confocal fluorescence microscopy allows the real-time tracking of GUVs filled with water-soluble fluorophores of different molecular weights. This technique is generally used to determine whether membrane permeabilization occurs through pore formation mechanism or total membrane disruption.

The Alexa fluor dyes are a family of fluorescent probes introduced by the founders of “Molecular probes”. Originally synthesized by sulfonation of common fluorophores (rhodamine, fluorescein, coumarin, cyanine, etc.), they are frequently used to label cells and tissues and to monitor the permeability of liposomes in confocal fluorescence microscopy (“An Introduction to Alexa Dyes,” 2019). The excitation and emission spectra of Alexa Fluor dyes span the visible spectrum and beyond (extending to the infrared). Each member of this series is named according to its excitation maxima (for example, Alexa 350 has an excitation wavelength maximum of 350 nm, etc.) and possess unique characteristics (dye color, maximal absorption and emission wavelengths, molecular weight, etc.). The Alexa Fluor dyes are generally less pH-sensitive and more photostable than the original probes (Alexa Fluor® Dyes Spanning the Visible and Infrared Spectrum—Section 1.3). For permeability assays, blank vesicles are incubated with a fluorescent dye and the influx of the fluorophore is followed by fluorescence microscopy. Multiple of Alexa dyes (of different size) could be also used in the same experiment to determine the size of the pores that could be created during the permeabilizing process.

4. Membrane permeabilizing agents: Effect of the structure and membrane composition

Membrane permeability studies reported the effect of a large number of bioactive agents on liposomal membranes. Careful examinations of the published data reveal that the activity of these agents is attributed to their structural characteristics and their physicochemical parameters which rule their interaction with the lipid bilayer. Additionally, their effect on the membrane is modulated by intrinsic factors, most importantly the lipid membrane composition. The following paragraphs are dedicated to describe various membrane-active agents and to focus on the interaction between these agents and liposomal membranes, with respect to the membrane composition and the structure of the permeabilizing agent.

4.1. Surfactants

Surfactants are surface-active, water-soluble, amphiphilic compounds (Small, 1970). These molecules reduce the surface tension of water by forming a monolayer at the air-water interface orienting their polar group towards the water phase and their hydrophobic group outside the solution facing the air. These agents comprise synthetic

detergents, lysolipids, amphiphilic peptides, bile salts and other amphiphiles (Heerklotz, 2008).

Much work has been devoted to investigate surfactant-lipid interactions. Various changes in membrane properties were induced by these amphiphiles; most notably membrane permeabilization that results in significant leakage of aqueous solutes and disruption of membrane lamellar structure (Heerklotz, 2008). Hence, this mechanism is denoted membrane solubilization or membrane micellization. It was described by Helenius and Simons in 1975 as a three-stage model (Helenius and Simons, 1975) consisting of: (i) detergent-binding, (ii) lamellar-micellar phase transition and (iii) size decrease of mixed micelles leading to total membrane solubilization (Heerklotz, 2008; Lichtenberg, 1985).

Available literature data points out to the effect of a variety of surfactants on membrane permeability using fluorescence leakage assays. The permeabilizing effect of various surfactants including SDS (de la Maza and Parra, 1995), alkyl glucoside (de la Maza et al., 1998), dodecyl betaine (de la Maza et al., 1998), dodecyl maltoside (de la Maza and Parra, 1997), triton X100 and sodium cholate (Memoli, 1999) was investigated; the surfactant-induced dye leakage was proportional to the surfactant's concentration. However, few studies have addressed the influence of surfactant structure modification on permeabilization. Of note, alkyl glucoside surfactants (C_n-Glu) of varying alkyl chain length (C₆ to C₁₂-Glu) were demonstrated to promote liposomal membrane permeabilization; a better effect was observed with decreasing surfactant alkyl chain length (de la Maza et al., 1998).

4.2. Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides comprising (α-1-4)-linked α-D glucopyranose units. They are produced during the bacterial digestion of cellulose (Loftsson and Brewster, 2010). The most common CDs are α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), and γ-cyclodextrin (γ-CD) consisting of six, seven, and eight glucopyranose units, respectively. These native CDs can be methylated, hydroxypropylated, sulfomethylated, etc. giving rise to a great number of CD derivatives of different structures showing improved physicochemical properties in comparison to native CDs, especially a better aqueous solubility. CDs are cone-shaped molecules with the hydroxyl groups oriented to the cone exterior and the hydrocarbon skeleton and the glycosidic oxygen bridges lining the central cavity. Hence, this structure imparts a hydrophilic character for CD's outer surface and a hydrophobic character for its cavity (Jambhekar and Breen, 2016). As a result of this structure, CDs are able to home in their cavities hydrophobic guests forming thereby inclusion complexes “CD/guest”. CDs complexation has been the basis for their wide applications in pharmaceutical (Loftsson and Brewster, 1996; Varan et al., 2017), food and cosmetic fields (Bilensoy, 2011).

For the past three decades, interest has centered on the ability of CDs to induce changes in the physical properties of the membranes. As a matter of fact, the interaction of CDs with liposomal membranes was found to increase membrane fluidity and permeability. Also, the ability of CDs to extract membrane components including phospholipids, cholesterol and proteins was greatly reported. This effect is mainly related to CD's type and concentration as well as membrane composition. Furthermore, CDs can mediate lipid exchange between membranes and even possess cryoprotective properties during the freeze-drying of lipid vesicles and living cells. The CD interaction with synthetic and biological membranes is recently reviewed discussing the aforementioned properties (Hammoud et al., 2019).

Table 2 summarizes the studies related to CDs effect on membrane permeability using fluorescence quenching assays. These studies shed the light on CDs ability to permeabilize liposomal membrane and to trigger particular components of membrane depending on CDs structure and membrane composition. As shown in Table 2, methylated CDs seem to have the best permeabilizing effect, specially methylated β-CDs

Table 2
Effect of CDs on liposome membrane permeability using fluorescence self-quenching assays.

Lipid membrane composition	Preparation method	Entrapped fluorophore	Tested agent and operating temperature	Results	Reference
MLV, DRV and SUV composed of: - PC; PC/Chol (1:1) - H-PC; H-PC/Chol (1:1) - DSPC; DSPC/Chol (1:1) SUVs composed of: - SPC:SA (90:10) - SPC:Chol:SA (60:30:10)	Thin film hydration method (MLV) Thin film hydration followed by sonication (SUV) Hydration-rehydration method (DRV) Thin film hydration	Calcein (100 mM)	HP- β -CD, HP- γ -CD, Me- β -CD at 37 °C	- Me- β -CD induced the best permeabilizing effect - Calcein leakage from Chol containing membranes was higher than that from free Chol membranes - Calcein leakage from membranes composed of saturated phospholipids and treated with CDs was reduced compared to unsaturated PC membranes	(Hatzel et al., 2007)
SUVs composed of: - SPC:SA (90:10) - SPC:Chol:SA (60:30:10)	Thin film hydration	Calcein (concentration not mentioned)	β -CD, γ -CD, Dimeb, Trimeb, Rameb, Crysmeb, HP- β -CD, HP- γ -CD, SBE- β -CD at 37 °C	- Calcein release increased with CDs concentration - Calcein release was in the order: Dimeb > Rameb > Trimeb > γ -CD > β -CD = HP- β -CD = HP- γ -CD = SBE- β -CD = Crysmeb - Methylated β -CDs derivatives showed high affinity towards Chol; Crysmeb being the less effective	(Piel et al., 2007b)
LUVs composed of: - PC:SM:PI (4:4:3) - PC:SM:PI:Chol (4:4:3:5) molar ratios MLVs composed of: - POPC	Thin film hydration method followed by vortex / freeze/thaw cycles and extrusion	Calcein (73 mM)	HP- β -CD and BUD-HP- β -CD inclusion complex at 25 °C	- HP- β -CD and BUD-HP- β -CD both induced membrane permeabilization - Dye release was higher from Chol containing membranes	(dos Santos et al., 2017)
MLVs composed of: - POPC	Dehydration-rehydration method	CF (50 mM)	β -CD and β -Cb/fullerene (C60) complex at 37 °C	A better permeabilizing effect was observed for β -CD compared to the inclusion complex	(Gasbarri et al., 2010)
DRV composed of: - PC - PC/Chol (1:1)	Hydration-rehydration method	CF (100 mM)	PR, β -CD/PR complex or HP- β -CD/PR complex loaded in liposomes at 37 °C	- A higher CF release was observed from PC liposomes encapsulating β -CD/PR complexes compared to those encapsulating free PR and HP- β -CD/PR complexes - CF leakage decreased upon Chol addition	(Fatouros et al., 2001)

List of abbreviations:

MLV: multilamellar vesicle; SUV: small unilamellar vesicle; DRV: dried-rehydrated vesicle; PC: phosphatidyl-choline; Chol: cholesterol; H-PC: hydrogenated-PC; DSPC: distearoyl-glycero-PC; HP- β -CD: hydroxypropyl- β -cyclodextrin; HP- γ -CD: hydroxypropyl- γ -cyclodextrin; Me- β -CD: methyl- β -cyclodextrin; SPC: Soybean phosphatidylcholine; SA: stearylamine; β -CD: β -cyclodextrin; γ -CD: γ -cyclodextrin; Dimeb: 2,6-Dimethyl- β -cyclodextrin; Trimeb: 2,3,6-trimethyl- β -cyclodextrin; Rameb: randomly methylated- β -cyclodextrin; Crysmeb: partially methylated crystallized- β -CD, SBE- β -CD: Sulfobutylether- β -cyclodextrin; PI: L- α -phosphatidylinositol; BUD: budonide; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PR: prednisolone

derivatives. For instance, [Hatzl et al. 2007](#) tested the effect of two hydroxypropylated CDs (HP- β -CD and HP- γ -CD) and one methylated CD (Me- β -CD) on liposomal membranes made of phosphatidylcholine (PC), hydrogenated PC (H-PC) or distearoyl-glycero-PC (DSPC) with or without Chol and they found a significant permeabilizing effect for Me- β -CD. An increased dye release was particularly observed from rich Chol membranes; this finding indicates that methylation improves the affinity of β -CDs to Chol and enhances its extraction from membranes ([Hatzl et al., 2007](#)). This is true for many methylated β -CD derivatives with the exception of Crystmeb which showed a weak affinity towards Chol which is probably due to its low degree of substitution ([Piel et al., 2007a](#)). Phospholipid saturation appears to affect CD-membrane interaction since membranes made from saturated phospholipids were more resistant to the effect of CDs ([Hatzl et al., 2007](#)).

Besides these modifications, CDs are able to form inclusion complexes by incorporating in their internal cavity a hydrophobic guest. For some CDs, the complexation does not affect their permeabilizing activity: similar calcein release profiles were obtained from liposomes composed of PC, sphingomyelin (SM) and phosphatidylinositol (PI) with or without Chol and treated with either HP- β -CD or BUD:HP- β -CD, an inclusion complex of HP- β -CD and budesonide, a poorly soluble anti-inflammatory drug ([dos Santos et al., 2017](#)). Nonetheless, CDs complexation might hamper the permeabilizing activity of other CDs: a better permeabilizing effect was observed for free β -CD, compared to their inclusion complex with fullerene ([Gasbarri et al., 2010](#)). Furthermore, the stability of the combined delivery system “drug-in-cyclodextrin-in-liposome” was tested by studying the CF release from liposomes encapsulating prednisolone either in the free form or as an inclusion complex with: β -CD and HP- β -CD. The authors showed that liposomes incorporating β -CD:PR inclusion complexes were less stable than those incorporating HP- β -CD:PR and the plain drug PR ([Fatouros et al., 2001](#)).

4.3. Proteins and peptides

Many authors reported the ability of a variety of proteins and peptides to punch holes inside the membrane, altering thereby the membrane permeability barrier ([Gunn et al., 2016](#); [Pérez-Peinado et al., 2018](#); [Petit et al., 2016](#); [Yang et al., 2018](#)). These have been classified as pore-forming agents. By far the best known are the pore forming proteins (PFPs).

PFPs are produced by many organisms of different species, from bacteria to human. These proteins can exist in two different but stable states: a water-soluble and a transmembrane form. They are generally produced as soluble monomers which undergo a conformational change to insert into the membrane. At this level, they oligomerize in order to form water-filled pores or channels. This spontaneous conversion is an intrinsic property of these proteins and it is mediated by an interaction between the protein and a specific component (lipid, other protein or even carbohydrate) at the membrane surface ([Anderluh and Lakey, 2008](#); [Fradin et al., 2009](#)). A large subdivision of PFPs are toxins, referred to as pore-forming toxins (PFTs). They represent the largest class of bacterial protein toxins comprising important virulence factors ([Gonzalez et al., 2008](#)). PFPs are categorized into α -PFTs and β -PFTs accounting for their α -helical and β -sheet pore structure, respectively ([Gouaux, 1997](#)). The same classification exists for PFTs ([Iacovache et al., 2008](#)). Antimicrobial peptides (AMP) are PFP related compounds: they are cationic small peptides in which the lytic domain (accounting for the lysis of the bacterial membrane) covers the whole peptide structure. Akin to PFPs, they permeabilize lipid membrane through pore formation ([Ros and García-Sáez, 2015](#)).

4.3.1. Pore formation mechanisms

Pore formation mechanism by PFPs is a multi-step process involving consecutively: (i) membrane partitioning or binding of the protein to the membrane surface, (ii) insertion into the hydrophobic core of the

bilayer, (iii) oligomerization and finally (iv) pore formation ([Fradin et al., 2009](#); [Ros and García-Sáez, 2015](#)). This mechanism is responsible for cell damage and lysis ([Bhakdi, 1986](#); [Miao et al., 2011](#)). Two main modes of action have been reported here:

The transmembrane pore formation mechanism: various models were introduced to describe the pore structure including the barrel-stave ([Ehrenstein and Lecar, 1977](#)) and the toroidal pore ([Ludtke et al., 1996](#)) models. In a barrel-stave pore model, the peptide reaches the membrane either as monomers or oligomers, then inserts into the lipid core perpendicular to the plane of the bilayer where they self-associate to form bundles of transmembrane pores. A pore consists of a ring of adjacent protein domains, each protein domain being like the stave of the barrel. The pore size would increase following the recruitment of additional monomers. The barrel-stave pores are also referred to as “proteinaceous pores” or “protein-lined pore” since they are characterized by a well-defined protein structure from which lipids are excluded ([Brogden, 2005](#)).

In contrast, a toroidal pore is formed when the membrane undergoes a curvature into the shape of a torus, thereby allowing the contact between protein domains and phospholipids headgroups. Thus, this reorientation or lipid rearrangement will lead both the peptides and the lipids to bend into a toroidal structure defining the so-called “toroidal pore”, also sometimes named as “lipidic, lipid-lined or wormhole pore” ([Brogden, 2005](#); [Huang et al., 2010](#)). An example of a barrel-stave pore would be that induced by alamethicin, a peptide antibiotic ([Baumann and Mueller, 1974](#); [Qian et al., 2008](#)). Interestingly, most AMPs (adopting an α -helical structure) and α -PFPs are believed to form pores corresponding to the toroidal model: this was shown for magainins ([Matsuzaki et al., 1998](#); [1996](#)), protegrins ([Yamaguchi et al., 2002](#); [Yang et al., 2001](#)), melittin ([Matsuzaki et al., 1997](#)) as well as for colicin E1 ([Sobko et al., 2006](#)), Bcl-2 protein Bax ([Epanand et al., 2003](#)) and sticholysins ([Valcarcel et al., 2001](#)) (α -PFPs).

The carpet mechanism: it involves the ability of some membrane-active peptides to interact with model membranes causing the breakdown of the hydrophobic barrier through a detergent-like mechanism. Actually, such behavior was demonstrated for amphiphilic AMPs which affect membrane permeability by causing an extensive rupture of lipid bilayer. Unlike pore forming process, the carpet mechanism involves no peptide-peptide interactions ([Brogden, 2005](#)). Indeed, it is described as follows: peptides accumulate on the lipid bilayer surface and align in a parallel orientation (“in-plane orientation”) to the membrane surface. Then, the peptides associate with anionic phospholipids headgroups via electrostatic interactions, covering thereby the surface of the membrane in a carpet-like fashion. This association causes the displacement of lipids by peptides which disturbs membrane fluidity and stability. When a threshold peptide concentration is reached, the membrane integrity is breached: surface-associated peptides will break the bilayer into patches where lipids are aggregated and stabilized by the amphiphilic peptides ([Huang et al., 2010](#); [Li et al., 2012](#)). Hence, this detergent-like mechanism is associated with a dramatic membrane disruption which undoubtedly impairs membrane permeability. Cecropin ([Gazit et al., 1995](#)), δ -Endotoxin CytA ([Butko et al., 1996](#)), dermaseptin ([Pouny et al., 1992](#)), ovispirin ([Yamaguchi et al., 2001](#)) and many other AMPs were shown to act via the carpet mechanism.

The sinking-raft model: Other modes of action were further ascribed to AMPs such as peptide translocation across the lipid bilayer, referred to as “the sinking-raft” model. This model suggests the self-association of peptides (AMPs) side-by-side on the membrane surface and the aggregates will sink deeper into the membrane in a manner similar to a sinking raft. The translocation of the peptide aggregates from the outer to the inner leaflet of the bilayer creates a mass imbalance that disturbs the membrane; this is accompanied with the formation of transient cavities inside the membrane through which dye efflux occurs. This model has been proposed to describe the δ -lysin, Transportan 10 and PGLa induced membrane permeabilization ([Pokorny et al., 2002](#); [Pokorny and Almeida, 2004](#); [Ulmschneider, 2017](#); [Yandek et al., 2007](#)).

Table 3
The effect of proteins (PFs) and AMPs) structure and liposome membrane composition on their permeabilizing effect using fluorescence quenching methods.

Lipid membrane composition and Preparation method	Entrapped fluorophore and operating temperature	Tested cationic peptides and main characteristics	Results	Reference
LUVs composed of: POPC/POPG (75:25 and 50:50) molar ratios Thin film hydration method followed by extrusion	CF (50 mM) Temperature not mentioned	Gomesin (Gm) and a linear analogue of Gomesin (GmL) at various P/L molar ratios	<ul style="list-style-type: none"> Gm induced high CF leakage whereas GmL promoted a mild dye leakage of 25% POPG membranes Similar permeabilizing effect was found for Gm and GmL in the presence of 50% POPG Gm-induced leakage was proportional to P/L ratios reaching complete leakage at the highest P/L for both vesicle compositions. Both peptides exerted a permeabilizing effect on both vesicle types with the amidated peptide being more active than the acidic peptide CF leakage increased in a dose-dependent manner with both vesicle types The amidated peptide form induced 40-fold increase of dye leakage of PC vesicles Polybia-MP1 induced CF release from all vesicles with preference for anionic bilayers Dye leakage from all vesicle types was proportional to P/L ratios Cholesterol decreased the dye leakage rate from zwitterionic vesicles and increased the threshold peptide concentration CL reduced dye leakage in comparison with PG containing vesicles Both anionic lipids increased the threshold P/L ratio, with CL shifting more this value upwards. Both defensins displayed a membrane permeabilizing activity: HNP-2 induced an all-or-none leakage mechanism whereas rHNP-2 caused a graded mechanism of dye release HNP-2 broke the membrane permeability barrier via formation of stable and large pores All defensin peptides, except one NP-4, individually as well as the natural mixture of the 6 defensins exerted a permeabilizing effect on pure POPG vesicles via a slow graded leakage mechanism The presence of neutral lipids (POPC and POPG) abolished vesicle leakage All rabbit defensins, except NP-4 and NP-5, individually as well as the defensins natural mixture caused leakage of E. coli whole lipid extract vesicle content through large and transient membrane lesions Magainin promoted dye leakage via an all-or-none mechanism Higher dye leakage was noted from PG containing membranes in a dose dependent manner 	(Domingues et al., 2015)
LUVs composed of: - PC - PC/PG (70:30) Thin film hydration followed by extrusion	CF (25 mM) At 25 °C	Protonectarina-MP-NH ₂ and Protonectarina-MP-OH (the amidated and acidic forms of mastoparan) at different concentrations		(da Silva et al., 2014)
LUVs composed of: - PC - PC:Chol (80:20) - PC:PG (70:30 and 40:60) Hydration of lipid film dried under vacuum followed by extrusion	CF (25 mM) At 25 °C	Polybia-MP1 mastoparan cationic peptide at different P/L ratios		(dos Santos Cabrera et al., 2008)
LUVs composed of: POPG Thin film hydration followed by extrusion	ANTS/DPX (5 mM/ 25 mM) Temperature not mentioned	HNP-2 and rHNP-2 (the native dimeric cationic disulfide-stabilized peptide of human neutrophil defensins and its reduced form) at different concentrations		(Wimley et al., 1994)
LUVs composed of: - POPG - POPC/POPG (100:0; 75:25; 50:50; 25:75) molar ratios - POPE/POPG (35:65) molar ratio - E. coli whole lipid extracts Lipid film hydration followed by freeze-thaw cycles and extrusion	ANTS/DPX (9 mM/ 25 mM) Temperature not mentioned	NP-1, NP-2, NP-3A, NP-3B, NP-4 and NP-5 (cationic monomeric peptides of rabbit neutrophil defensins) tested individually and as the "natural" mixture		(Hristova et al., 1996)
LUVs composed of: - PC - PG Thin film hydration followed by freeze-thaw cycles and extrusion	CF (50 mM) or ANTS/DPX (5 mM/ 8 mM)	Magainin, a cationic AMP, at different P/L concentrations		(Hristova et al., 1997) (Gregory et al., 2009)
LUVs composed of: - PC - PC/PG (90:10; 80:20; 70:30; 50:50; 30:70) Thin film hydration followed by 5 cycles of freeze-thawing and extrusion	CF (50 mM)	BP100 cationic peptide (cecropin-melittin hybrid peptide) at different concentrations		(Manzini et al., 2014)

(continued on next page)

Table 3 (continued)

Lipid membrane composition and Preparation method	Entrapped fluorophore and operating temperature	Tested cationic peptides and main characteristics	Results	Reference
LUVs composed of: - DOPC - DOPC/Chol (60:40) - POPC - POPC/Chol (60:40) Thin film hydration followed by cycles of freeze-thawing and extrusion LUVs composed of: - POPC - POPC/PA (85:15 and 70:30) - POPC/POPG (90:10; 70:30 and 50:50) Freeze-thawing method followed by extrusion LUVs composed of: - POPC - POPC/SM (1:1) - POPC/POPG (1:1) molar ratio Hydration of lipid film followed by extrusion LUVs composed of: - CL/DOPC (1:2) - CL/DOPC/DOPE (1:1:1) - DOPG/DOPE/DOPC (1:1:1) Freeze-thawing method followed by extrusion LUVs composed of: - PC - PA - PC/LPC (80:20), (90:10) and (95:5) - PC/PS (75:25), (50:50) and (25:75) - PC/PA (50:50) - PC/PE (50:50), (75:25) and (90:10) - PC/PE/LPC (40:40:20) - PC/CL (90:10) Thin film hydration method followed by freeze-thaw cycles and extrusion LUVs composed of POPC/POPG (50:50), (70:30) and (80:20); Thin film hydration method followed by extrusion	CF (100 mM) At 25 °C	Melittin, at different concentrations	- Dye leakage increased with increasing melittin concentration for all systems - Dye leakage decreased for POPC/Chol only revealing the resistance of DOPC to Chol effect in the presence of melittin	(Wessman et al., 2008)
LUVs composed of: - POPC - POPC/PA (85:15 and 70:30) - POPC/POPG (90:10; 70:30 and 50:50) Freeze-thawing method followed by extrusion LUVs composed of: - POPC - POPC/SM (1:1) - POPC/POPG (1:1) molar ratio Hydration of lipid film followed by extrusion LUVs composed of: - CL/DOPC (1:2) - CL/DOPC/DOPE (1:1:1) - DOPG/DOPE/DOPC (1:1:1) Freeze-thawing method followed by extrusion LUVs composed of: - PC - PA - PC/LPC (80:20), (90:10) and (95:5) - PC/PS (75:25), (50:50) and (25:75) - PC/PA (50:50) - PC/PE (50:50), (75:25) and (90:10) - PC/PE/LPC (40:40:20) - PC/CL (90:10) Thin film hydration method followed by freeze-thaw cycles and extrusion LUVs composed of POPC/POPG (50:50), (70:30) and (80:20); Thin film hydration method followed by extrusion	Calcein (80 mM) Temperature not mentioned	Melittin	- Melittin induced calcein release from POPC vesicles via an all-or-none mechanism - Decreased dye leakage from anionic lipids containing membranes was observed in a dose depending manner.	(Benachir and Lafleur, 1995)
LUVs composed of: - POPC - POPC/PA (85:15 and 70:30) - POPC/POPG (90:10; 70:30 and 50:50) Freeze-thawing method followed by extrusion LUVs composed of: - POPC - POPC/SM (1:1) - POPC/POPG (1:1) molar ratio Hydration of lipid film followed by extrusion LUVs composed of: - CL/DOPC (1:2) - CL/DOPC/DOPE (1:1:1) - DOPG/DOPE/DOPC (1:1:1) Freeze-thawing method followed by extrusion LUVs composed of: - PC - PA - PC/LPC (80:20), (90:10) and (95:5) - PC/PS (75:25), (50:50) and (25:75) - PC/PA (50:50) - PC/PE (50:50), (75:25) and (90:10) - PC/PE/LPC (40:40:20) - PC/CL (90:10) Thin film hydration method followed by freeze-thaw cycles and extrusion LUVs composed of POPC/POPG (50:50), (70:30) and (80:20); Thin film hydration method followed by extrusion	ANTS/DPX (12.5 mM/45 mM) Temperature not mentioned	Melittin at different P/L molar ratios	- Melittin induced dye leakage preferentially from SM containing vesicles - Lower values of P/L ratios inducing release from SM vesicles compared to other vesicle types	(Gómara et al., 2003)
LUVs composed of: - POPC - POPC/PA (85:15 and 70:30) - POPC/POPG (90:10; 70:30 and 50:50) Freeze-thawing method followed by extrusion LUVs composed of: - POPC - POPC/SM (1:1) - POPC/POPG (1:1) molar ratio Hydration of lipid film followed by extrusion LUVs composed of: - CL/DOPC (1:2) - CL/DOPC/DOPE (1:1:1) - DOPG/DOPE/DOPC (1:1:1) Freeze-thawing method followed by extrusion LUVs composed of: - PC - PA - PC/LPC (80:20), (90:10) and (95:5) - PC/PS (75:25), (50:50) and (25:75) - PC/PA (50:50) - PC/PE (50:50), (75:25) and (90:10) - PC/PE/LPC (40:40:20) - PC/CL (90:10) Thin film hydration method followed by freeze-thaw cycles and extrusion LUVs composed of POPC/POPG (50:50), (70:30) and (80:20); Thin film hydration method followed by extrusion	ANTS/DPX (12.5 mM/45 mM) Temperature not mentioned	lBid peptide (20 nM)	- lBid exerted dye leakage from CL-containing liposomes - Dye leakage was increased in the presence of DOPE phospholipids	(Epanand, 2002)
LUVs composed of: - POPC - POPC/PA (85:15 and 70:30) - POPC/POPG (90:10; 70:30 and 50:50) Freeze-thawing method followed by extrusion LUVs composed of: - POPC - POPC/SM (1:1) - POPC/POPG (1:1) molar ratio Hydration of lipid film followed by extrusion LUVs composed of: - CL/DOPC (1:2) - CL/DOPC/DOPE (1:1:1) - DOPG/DOPE/DOPC (1:1:1) Freeze-thawing method followed by extrusion LUVs composed of: - PC - PA - PC/LPC (80:20), (90:10) and (95:5) - PC/PS (75:25), (50:50) and (25:75) - PC/PA (50:50) - PC/PE (50:50), (75:25) and (90:10) - PC/PE/LPC (40:40:20) - PC/CL (90:10) Thin film hydration method followed by freeze-thaw cycles and extrusion LUVs composed of POPC/POPG (50:50), (70:30) and (80:20); Thin film hydration method followed by extrusion	Calcein (80 mM) At room temperature	Bax ^{cs5} and Bid ^{cs6}	- Both peptides showed a permeabilizing effect with Bax ^{cs5} displaying a higher effect - Enhanced dye release was noted from vesicles containing negatively charged lipids in the presence of Bid ^{cs6} only - Enhanced dye leakage was observed from vesicles composed of positively curved phospholipids in the presence of Bax ^{cs5} only	(García-Sáez et al., 2005)
LUVs composed of: - POPC - POPC/PA (85:15 and 70:30) - POPC/POPG (90:10; 70:30 and 50:50) Freeze-thawing method followed by extrusion LUVs composed of: - POPC - POPC/SM (1:1) - POPC/POPG (1:1) molar ratio Hydration of lipid film followed by extrusion LUVs composed of: - CL/DOPC (1:2) - CL/DOPC/DOPE (1:1:1) - DOPG/DOPE/DOPC (1:1:1) Freeze-thawing method followed by extrusion LUVs composed of: - PC - PA - PC/LPC (80:20), (90:10) and (95:5) - PC/PS (75:25), (50:50) and (25:75) - PC/PA (50:50) - PC/PE (50:50), (75:25) and (90:10) - PC/PE/LPC (40:40:20) - PC/CL (90:10) Thin film hydration method followed by freeze-thaw cycles and extrusion LUVs composed of POPC/POPG (50:50), (70:30) and (80:20); Thin film hydration method followed by extrusion	CF (50 mM) or ANTS/DPX (5 mM/ 8 mM)	Cecropin A	- Cecropin A permeabilizes liposomal formulations via an all-or-none mechanism - Dye leakage increased upon increasing the ratio of anionic lipids (POPG) - Permeabilizing effect of both cationic peptides for all vesicle types following an all-or-none leakage process - Higher dye release was observed with increasing molar ratio of negatively charged phospholipids	(Gregory et al., 2008)
LUVs composed of: - POPC - POPC/PA (85:15 and 70:30) - POPC/POPG (90:10; 70:30 and 50:50) Freeze-thawing method followed by extrusion LUVs composed of: - POPC - POPC/SM (1:1) - POPC/POPG (1:1) molar ratio Hydration of lipid film followed by extrusion LUVs composed of: - CL/DOPC (1:2) - CL/DOPC/DOPE (1:1:1) - DOPG/DOPE/DOPC (1:1:1) Freeze-thawing method followed by extrusion LUVs composed of: - PC - PA - PC/LPC (80:20), (90:10) and (95:5) - PC/PS (75:25), (50:50) and (25:75) - PC/PA (50:50) - PC/PE (50:50), (75:25) and (90:10) - PC/PE/LPC (40:40:20) - PC/CL (90:10) Thin film hydration method followed by freeze-thaw cycles and extrusion LUVs composed of POPC/POPG (50:50), (70:30) and (80:20); Thin film hydration method followed by extrusion	Tb ³⁺ /DPA chelates	MAX1 and MAX35	- SiniI and SiniII exerted a permeabilizing effect even at low concentrations with preference to SM containing membrane - Increased dye release from PA (and other negatively charged lipids) containing membranes	(Gupta et al., 2013)
LUVs composed of: - pure PC, SM, PA - PC/SM (1:1) molar ratio - PA/SM (1:1 and 3:1) molar ratio Thin film hydration method followed by freeze-thaw cycles and extrusion LUVs composed of: - POPC/PSM (4:1) - POPC/dihydro-PSM (4:1) - pure OSM - pure dihydro-OSM	Calcein (80 mM) At room temperature	Sticholysin I and II peptides (SiniI and SiniII), basic cytolytins	- SiniI and SiniII exerted a permeabilizing effect, the former being more efficient than the latter - Reduced dye leakage from vesicles containing dihydro-PSM and dihydro-OSM - SiniI permeabilized LUVs by pore formation	(Valcarcel et al., 2001)
LUVs composed of: - pure PC, SM, PA - PC/SM (1:1) molar ratio - PA/SM (1:1 and 3:1) molar ratio Thin film hydration method followed by freeze-thaw cycles and extrusion LUVs composed of: - POPC/PSM (4:1) - POPC/dihydro-PSM (4:1) - pure OSM - pure dihydro-OSM	Calcein (100 mM) at 23 °C	Sticholysin I and II (SiniI and SiniII)	- SiniI and SiniII exerted a permeabilizing effect, the former being more efficient than the latter - Reduced dye leakage from vesicles containing dihydro-PSM and dihydro-OSM - SiniI permeabilized LUVs by pore formation	(García-Linares et al., 2016)(Alm et al., 2015)

(continued on next page)

Table 3 (continued)

Lipid membrane composition and Preparation method	Entrapped fluorophore and operating temperature	Tested cationic peptides and main characteristics	Results	Reference
<ul style="list-style-type: none"> - POPC/PSM/Chol (80:20:2.5; 80:20:5; 80:20:7.5 and 80:20:10) Thin film hydration followed by an extrusion LUVs composed of E coli phospholipidsHydration of lipid film followed by extrusion 	CF (50 mM) Temperature not mentioned Calcein (50 mM) Temperature not mentioned ANTS/DPX (12.5 mM/45 mM) and ANTS/DPX (5 mM/25 mM) Temperature not mentioned	Nisin	<ul style="list-style-type: none"> - Dye leakage increased in Chol containing membranes in a dose dependent manner - Nisin exerted a permeabilizing effect, the latter being pH-dependent - Dye leakage was low from anionic lipids containing vesicles - Both cationic peptides displayed a permeabilizing effect which is proportional to the peptide concentration - Dye leakage from CL containing membranes was low - Both peptides showed a permeabilizing effect; the induced-dye leakage followed an all-or-none leakage mechanism for PreTM-C and a graded mechanism for NpreTM - SM enhanced vesicle leakage compared to pure POPC vesicles - Increase of stable pore number in the presence of Chol in vesicles 	(Garcera et al., 1993) (Herrández-Villa et al., 2018) (Apellániz et al., 2009) (Apellániz et al., 2010)
<ul style="list-style-type: none"> LUVs composed of POPG/CL (100:0; 80:20 and 60:40)Thin film hydration followed by freeze-thaw cycles and extrusion LUVs composed of: <ul style="list-style-type: none"> - POPC - POPC:SM (1:1) molar ratio - POPC/Chol (4:1) molar ratio Thin film hydration followed by an extrusion 	CPreTM, a cationic peptide and NpreTM, an anionic peptide both derived from the MPEP sequence			

List of abbreviations:

POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; Gm: gomesin peptide; PG: phosphatidylglycerol; CL: cardiolipin; DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG: 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; PA: phosphatidic acid; LPC: lysophosphatidylcholine; PS: phosphatidylserine; PE: phosphatidylethanolamine; POPS: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; PSM: palmitoyl SM; OSM: N-oleoyl-SM; MPEP: membrane-proximal external region of the human immunodeficiency virus type 1 envelope glycoprotein 41 subunit

These mechanistic behaviors are not dependent on specific amino acid sequences or specific three-dimensional structures; they rather rely on the interfacial activity of peptides which refers to the ability of the peptide to bind to the membrane, partition into the membrane-water interface and induce changes in the packing and organization of lipids. Hence, the interfacial activity primarily depends on the physicochemical properties of the peptide which are tuned by its amino acid composition (Wimley, 2010).

4.3.2. All-or-none or graded dye leakage process

A great number of publications investigated the pore forming activity of PFPs and AMPs using entrapped fluorophore release from liposomes. Depending on the dye leakage process and the kinetic release profiles, the permeabilizing mechanisms of PFPs are classified as all-or-none or graded mechanism. Effectively, the dye release mechanism may be all-or-none, in which some vesicles lose all of their contents while others are intact; or it may be graded, in which all vesicles lose part of their contents. The former is usually accompanied by the formation of long-lived permeabilized state in a fraction of the vesicles while the latter is based on a transient permeabilization which disappears once equilibrium is reached (Apellániz et al., 2010). Literature data have shown that the barrel-stave model and the carpet model are consistent with an all-or-none leakage mechanism whereas the toroidal pore model and the sinking raft model are compatible with a graded dye efflux mechanism.

4.3.3. Peptide-membrane interactions, vesicle leakage studies

Many authors have undertaken the study of peptide-membrane interactions using liposomal membrane systems; focus was given on different parameters having a great impact on the membrane permeabilizing activity of these agents. Table 3 resumes the fluorescence studies highlighting the effect of peptides structure on liposomal membrane permeability. Also in this table, the effect of lipid membrane composition on the activity of these peptides is discussed.

Peptide structure and features: Several attempts were made to get a better understanding of the structure-function relationship of various membrane-active peptides. To start with, the peptide's secondary structure appears to play a major role in determining the lytic activity of AMPs. For instance, a recent work compared the membrane disruptive effect of Gomesine (Gm), an AMP, to that of its linear analogue "GmL". The native peptide Gm consists of 18 amino acids including 4 cysteine residues; the disulfide bonds confer a β -hairpin conformation to this peptide in aqueous solution. Conversely, GmL lacks the disulfide bonds which affects the peptide's secondary structure. The permeabilizing effect of GmL was found to be considerably lower than that of the native peptide Gm which induced a significant CF release from liposomal membranes. This finding underlines the implication of the peptide's secondary structure in AMPs-membrane interactions (Domingues et al., 2015). In addition, the amidation of the C-terminal carboxyl group improves the activity of cationic peptides (Huang et al., 2010). In fact, the C-terminal amidation stabilizes the secondary structure of the peptide with a high content of helical conformations allowing a deeper interaction with lipid membrane components. A study showed that the amidated form of a polycationic peptide (mastoparan peptide) was more efficient than the acidic form in permeabilizing liposomal membranes composed of PC or PC/PG (70:30) since a 40-fold increase of CF leakage was reported upon liposomes exposure to the amidated peptide (da Silva et al., 2014).

Wimley and coworkers, 1994 have previously studied the membrane permeabilizing effect of the dimeric human neutrophil defensin HNP-2: these cationic AMPs contain 3 disulfide bonds per molecule conferring a great structure rigidity to the peptide. The authors found that the reduction of the disulfide bonds (affecting the dimerization ability of rHNP-2) switches the leakage process from an all-or-none mechanism for the native defensin (HNP-2) to a graded mechanism for the reduced peptide (rHNP-2) suggesting that the dimeric state of

human defensins is required for the formation of large and long-lived pores (Wimley et al., 1994). Later on, Hristova et al., 1996 compared the membrane permeabilization mechanism of human defensins to that of rabbit defensins. The main difference between human and rabbit neutrophil defensin is that the former undergoes dimerization in aqueous solutions while the latter always exists in a monomeric state. The lack of aqueous dimerization of rabbit defensins was demonstrated to affect the peptide assembly at the membrane level and thereafter produce a graded leakage characterized by short-lived pores instead of an all-or-none mechanism (Hristova et al., 1996). Another study conducted by the same authors reported that a non-polar amino acid residue in position 21 of the defensin sequence is important for the peptide dimerization (Hristova et al., 1997).

Peptide size: The size of the peptide is believed to affect the membrane permeabilization process: a pore-forming lytic behavior for long sequence peptides and a carpet mechanism for short peptides. Indeed, short peptides cannot span the membrane to form pores; they rather permeabilize the membrane through a carpet mechanism. In contrast, a large peptide can easily form transmembrane pores inducing membrane permeabilization (Bowie et al., 2004; Chia et al., 2002).

Peptide/lipid ratio (P/L): Aside from structural characteristics, the peptide/lipid (P/L) ratio remains one of the main parameters affecting the leakage process. There are many instances where the peptide-induced leakage is proportional to this ratio (da Silva et al., 2014; Gregory et al., 2009; Manzini et al., 2014; Wessman et al., 2008). It was additionally reported that dye leakage starts to significantly occur and proportionally increase above a threshold value of P/L molar ratio. Below this value, leakage is very low and sometimes barely detectable. Total content release could be nearly reached at higher peptide concentrations. This behavior is consistent with a sequence of effects (as a response to an increasing peptide concentration) underscoring membrane permeabilization via a detergent-like mechanism. Surfactin (an antibacterial lipopeptide produced by *Bacillus subtilis*) (Heerklotz and Seelig, 2007) and Polybia-MP1 (an antimicrobial mastoparan peptide) (dos Santos Cabrera et al., 2008) were shown to permeabilize liposomal membranes this way. The examination of the peptide effect on lipid vesicles points to the dependence of the membrane permeabilization on the P/L ratio. In fact, AMPs are generally inactive at low P/L ratios ($P/L < 1:1000$). They become active and induce vesicle content leakage within the P/L ratio ranging from 1:500 to 1:50. True or ideal pore-forming peptides can permeabilize lipid vesicles at low P/L molar ratios (below 1:50). It is worth noting that at very high P/L ratios ($P/L < 1:50$), any membrane-active peptide could promote membrane disruption. Thus, the results showing an extensive vesicle leakage upon high peptide concentration treatment should be interpreted with caution for most of these are considered experimental artifacts (Wimley, 2010).

Phospholipid charge: Data collected in Table 2 clearly emphasizes the relationship between membrane lipid composition and the peptide's permeabilizing effect. Given their positive charge, AMPs are most commonly inactive on neutral membranes: no significant leakage was recorded for neutral vesicles treated with AMPs (dos Santos Cabrera et al., 2008; Hristova et al., 1996; Manzini et al., 2014). In contrast, anionic phospholipids or negatively charged lipids play a crucial role in peptide-lipid interactions and enhance the permeabilizing activity of several cationic peptides. Indeed, most of these peptides (displaying an antimicrobial activity against many bacterial and fungal species) possess a great affinity towards anionic lipids generally found in the outer bacterial membrane. Thus, cationic peptides reach the membrane surface and bind to anionic lipids which induce a strong negative membrane curvature allowing pore formation and membrane permeabilization to occur. Accordingly, significant dye release was observed in the presence of various cationic peptides from liposomal vesicles containing negatively charged phospholipids such as lysophospholipids, CL (cardiolipin), PA (phosphatidic acid), PS (phosphatidylserine), PG(phosphatidylglycerol), etc. (Epand, 2002; Fernandez et al., 2012;

García-Sáez et al., 2005; Gregory et al., 2008; Gupta et al., 2013; Manzini et al., 2014; Menestrina, 1988; Valcarcel et al., 2001). Notwithstanding, the opposite effect was observed with melittin (Benachir and Lafleur, 1995; Strömstedt et al., 2007) and nisin (Garcera et al., 1993) (cationic peptides) where the presence of negatively charged lipids considerably reduced fluorophore efflux from liposomes. CL appears to inhibit the permeabilizing activity of some cationic AMPs (Hernández-Villa et al., 2018). Similarly, Garcia-Saez et al., 2005 showed that the permeabilizing activity of Bax⁴⁵ (peptide fragment containing α -helix 5 of Bax) is enhanced with positively charged lipids containing membranes contrary to that of Bid⁶⁶ (peptide fragment containing α -helix 6 of Bid) (García-Sáez et al., 2005). Furthermore, the content of negatively charged phospholipids in liposomal membrane seems to have an impact of the activity of peptides. Despite its weak permeabilizing effect on membrane composed of 25% POPG, GmL, the linear analogue of Gm, retrieved its activity in the presence of 50% POPG membranes. This effect was similar to that of the native peptide against 50% POPG membranes. The authors explained that a high POPG density helped to restructure the linear peptide into a β -hairpin conformation that allowed the insertion of the peptide into the membrane and promoted vesicle leakage in a similar way to the native peptide (Domingues et al., 2015). Based on these results, one can conclude that the intrinsic lipid curvature can potentially modulate the poration activity and the permeabilization process of PFPs.

Cholesterol: With regards to Chol, this sterol is believed to generate a rigid membrane due to its ordering and condensing effect. Available data confirm this idea since peptide-induced dye efflux is generally decreased for Chol-containing membranes (dos Santos Cabrera et al., 2008; Wessman et al., 2008). However, in some cases, Chol could be beneficial for the leakage process: the presence of Chol in the liposome's membrane increased the number of stable pores induced by CpreTM and NpreTM peptides (Apellániz et al., 2010). Also relevant in this context is the effect of cholesterol on melittin activity. In fact, cholesterol doesn't prevent the permeabilizing activity of melittin. It rather imposes on melittin another mechanism of membrane permeabilization. It was suggested that in free cholesterol systems, melittin-induced leakage is due to pore formation whereas in cholesterol supplemented systems, melittin promotes complete membrane disruption (Wessman et al., 2008).

Sphingomyelin: SM could affect the permeabilizing effect of peptides: effectively, some PFTs promotes a significant dye leakage from SM containing vesicles (Gómara et al., 2003; Valcarcel et al., 2001). This is for example the case of NpreTM and PreTM-C peptides whose permeabilizing effect was enhanced in the presence of SM in the membrane; this latter reduced the amount of peptide required for the release of 50% of the entrapped fluorophore (Apellániz et al., 2009). Taken together, these findings imply a preferential interaction between the bioactive agent and membrane lipids, leading to membrane permeabilization.

Intermolecular hydrogen bonding: The intermolecular hydrogen bonding among lipid molecules seems to have a great influence on the membrane permeabilization process. This was proven for sticholysin peptides where the replacement of SM with dihydro-SM in liposomal membranes affected the sticholysin-induced membrane permeabilization. In fact, sticholysin binding to the lipid bilayer is known to be mediated by SM. The strong intramolecular hydrogen bonding in dihydro-SM inhibited the binding process and eventually prevent their ability to form pores and to permeabilize the membrane (García-Linares et al., 2016).

Biological membrane complexity: An additional remark could be made here: it concerns some active agents whose permeabilizing activity only occurs with a lipid membrane made from natural lipid extract of the host organisms. This was shown with *Limulus* pentraxin proteins (Harrington et al., 2008) and the rabbit neutrophil defensins (Hristova et al., 1997) inducing significant dye efflux from *E. coli* lipid extract vesicles only. One explanation for this could be that liposomal

formulations aren't always representative of all the interactions that may occur between the bioactive agent and the lipid bilayer. Working with a membrane composed only of two or three lipid species doesn't recall the exact composition of the biological membrane; therefore, this system lacking a broad range of lipid combinations would fail to show the many aspects of the complex drug-lipid interaction.

The membrane-disruptive effect of natural and designed proteins and peptides is still being investigated to date using various techniques including X-Ray diffraction, computational approaches (MD), fluorescence imaging, NMR, etc. These studies aim to establish a structure/activity relationship allowing a better understanding of the peptides' activity on membrane.

4.4. Other membrane-active agents

4.4.1. Polymers

Dendrimers are highly branched, globular macromolecules with a tree-like architecture. They comprise three structural components: (1) an inner core surrounded by (2) layers of repeatedly branched units emerging from the core and (3) terminal functional groups attached to the outer layer at the molecule surface. Each layer is called a generation (Abbasi et al., 2014). Dendrimers exert a striking effect on the lipid bilayer properties: effectively, the investigations of the interaction between PAMAM dendrimers (the most studied dendrimers) and model membranes evidenced their pore-forming capacity. The latter accounts for their permeabilizing effect and thereafter explains their induced-cell lysis (Kesharwani et al., 2014). The mechanism underlying membrane permeabilization by dendrimers involves the removal of lipids by dendrimers and the formation of dendrimers-filled lipid vesicles, leaving the membrane with pores through which leakage of intravesicular content will occur. This mechanistic model was first developed by Mecke and coworkers who additionally demonstrated that hole formation is sensitive to the structural and physicochemical properties of PAMAM dendrimers (Mecke et al., 2005). Another study investigated the effect of PAMAM dendrimer concentration and generation on their disruptive effect. An increasing calcein leakage was obtained with higher dendrimer's generation ($G2 < G4 < G6$). Dye leakage was also proportional to dendrimer's concentration (Parimi et al., 2010).

Other synthetic polymers are likely to induce pore formation causing thereafter membrane permeability alteration (Kepczynski et al., 2012). In fact, polycations are frequently used as transfection agents for the delivery of genetic materials into cells. It was shown that the transfection process is consistent with the formation of nuclear membrane pores (Sikor et al., 2010).

4.4.2. Small active molecules

This class includes a variety of small molecules of different chemical groups to which a permeabilizing effect was demonstrated. Two main mechanisms are attributed to these compounds: pore formation and defect formation. The latter refers to the ability of some membrane-active agents to disturb the lipid bilayer generating thereafter membrane defects or vacancies resulting in membrane leakiness and enhanced permeability. However, it should be noted that membrane defect formation isn't mainly due to a general mechanism; instead, it could be ascribed to different mechanistic behaviors. Examples are given below.

4.4.2.1. Pore-forming molecules. Saponins, naturally occurring plant triterpenoids or steroid glycosides, are amphiphilic compounds composed of a hydrophilic sugar moiety and a hydrophobic aglycone (generally triterpene or steroid) bound by a glycosidic linkage. They are known for their ability to interact with membrane lipids, specifically cholesterol and to alter membrane permeability through pore formation mechanism (Dourmashkin et al., 1962; Keukens et al., 1995; Nishikawa et al., 1984; Seeman, 1973). For instance, (Armah et al., 1999)

proposed a model to describe the pore formation made by avenacin A-1 saponin. According to this model, saponins will interact with Cholesterol-enriched membrane domains, aggregate and reorganize the membrane cholesterol into pores. As shown in table 4, a strong cholesterol dependence of terpenes-induced permeabilization was also demonstrated for α -hederin (Lorent et al., 2013), avicins (triterpenoid saponin) (Li et al., 2005) and digitonin (Sudji et al., 2015).

Other pore-forming agents include drugs with potential therapeutic effect. Amphotericin B, a polyene antibiotic used to fight fungal infections, promotes ion-permeable channels formation as a primary mechanism of killing fungal cells (Fujii et al., 1997; Holz, 1970; V. Coterio et al., 1998). Different models have been proposed to describe the channel formation mechanism: the sterol-independent channel formation (Hartsel et al., 1991), the sterol-dependent channel formation (Bonilla-Marín et al., 1991; De Kruijff et al., 1974) and the sequential channel formation mechanism (Cohen, 1992). Pore formation was also demonstrated for nystatin and etruscomycin (polyene antimicrobial drugs) (De Kruijff et al., 1974). Evenly, the anticancer drug tamoxifen is likely to interact with lipid membrane and to induce hole formation (Engelke et al., 2001).

4.4.2.2. Defect forming molecules. Some active agents cause membrane phase separation generating defects in the boundary regions of the membrane. In fact, short-chain alcohols can promote the formation of interdigitated structure membrane in which the terminal methyl of the phospholipid acyl chains will extend beyond the midplane of the bilayer and fully interpenetrate into the opposing monolayer. Accordingly, Komatsu and Okada, 1995 postulated that ethanol, in a defined concentration range, induces membrane phase separation of PC liposomes where both the normal bilayer structure and the interdigitated membrane structure coexist. This phase-separated membrane state is subjected to abrupt increase in membrane permeability proportionally to ethanol concentration and this could be explained as follows: the interdigitated structure is characterized by a thinner layer with more rigid hydrocarbon regions and closer packing than the normal bilayer structure. Hence, these differences appear to be responsible for the formation of membrane defects at the boundary regions of the phase-separated membranes (Komatsu and Okada, 1995). The same authors demonstrated that ethanol still enhance membrane permeability in the presence of cholesterol (Komatsu and Okada, 1997).

Another example of defect forming agent could be cucurbitacin E (Cuc E), an oxygenated triterpene, which is thought to destabilize the membrane altering membrane permeability of LUVs loaded with SRB (Habib et al., 2013). Likewise, the effect of cyclic hydrocarbons on the membrane integrity of liposomes made from E. coli lipid extracts revealed an increase in CF efflux (Sikkema et al., 1994).

Moreover, a series of essential oil components were previously investigated for their effect on the permeability of liposome membranes. A permeabilizing effect was reported for various compounds including menthol, thymol, carvacrol, etc. using vesicle leakage studies (Cristani et al., 2007; Trombetta et al., 2005). Their induced membrane damage may be also considered defect formation in the membrane. Indeed, due to their lipophilic character, essential oil components can partition into the membrane by hydrophobic interactions, causing membrane expansion and increased membrane fluidity and permeability. The disturbance of membrane stability provoked by these components is relevant to their antibacterial effect and explains to a large extent their mode of action on bacterial membranes (Bakkali et al., 2008). Previous studies evidenced that the permeabilizing effect of essential oil components is related to their physicochemical properties and to the lipid composition that affects the net surface charge of the membrane. In fact, Trombetta and coworkers investigated the damaging effect of three monoterpenes (thymol, (+) menthol and linalyl acetate) on the permeability of CF-loaded LUVs. Results showed that linalyl acetate, the less lipophilic component, was ineffective in permeabilizing vesicles indicating a correlation between the lipophilicity of the drug and its

Table 4
The effect of liposome's membrane composition on small active molecules permeabilizing effect using fluorescence quenching methods.

Lipid membrane composition	Preparation method	Entrapped fluorophore and operating temperature	Tested agent	Release results	Reference
Saponins					
LUVs composed of: - PC:SM:PI:Chol (4:4:3:0); 4:4:3:2.25 and 4:4:3:5.5) molar ratios - DMPC - DMPC/Chol (3:1) molar ratio	Hydration of the lipid film followed by extrusion	Calcein (72 mM) at 37 °C	α -hederin, δ -hederin and hederagenin saponins	- α -hederin showed a permeabilizing effect with chol-containing vesicles only - Calcein leakage increased with increasing Chol content - Dye release from the binary mixture followed this order: α -hederin > δ -hederin > hederagenin	(Lorent et al., 2013)
LUVs composed of: - DMPC/Chol - DMPC/Stol - DMPC/Erol - (3:1) molar ratio	Hydration of the lipid film followed by extrusion	Calcein at 25 °C	The saponin α -hederin	- The permeabilizing effect of α -hederin increased with saponin/sterol ratio - Same release kinetic profile regardless of the sterol type - Highest dye release rate was obtained for Chol-containing membrane	(Lorent et al., 2014)
LUVs composed of: - PC - PC/Chol (80:20) - PC/SM/Chol (40:40:20) - PC/CL	Thin film hydration followed by freeze-thaw cycles and an extrusion	Calcein (80 mM) at room temperature	Digitonin saponin at different concentrations	Digitonin permeabilized Chol-containing vesicles only, this effect increased with saponin concentration and Chol content	(Sudji et al., 2015)
Oxygenated triterpenes					
LUVs composed of: DPPC/DPPG/DPE/Cho (5/0.5/0.25/5) molar ratio	Reverse phase evaporation	SRB (150 mM) at 4 and 37 °C	Cuc E (encapsulated in liposomes)	- Faster SRB release from Cuc E-loaded liposomes at 37 °C (40% release after 8 incubation days) - Liposomes were stable at 4 °C with no effect of Cuc E on permeability	(Habib et al., 2013)
Essential oil components					
LUVs composed of: - PC - PC/PS (9:1) - PC/SA (9:1) - PG/CL (9:1) molar ratios	Hydration of a lipid film followed by extrusion	CF (20 mM) at room temperature	Linallyl acetate, (+) menthol and thymol, monoterpenes added individually to liposomes at various concentrations	- (+) menthol and thymol induced a concentration-dependent dye leakage from all vesicle types - Linallyl acetate was less damaging to the membrane than thymol and menthol - The order of CF leakage from PC vesicles was: Thymol > (+) menthol > linallyl acetate - Thymol-induced dye leakage was significantly higher from PC/SA vesicles than from other vesicles - Menthol caused a marked CF leakage from PC/PS and PG/CL vesicles compared to PC and PC/SA vesicles - Carvacrol and thymol showed a permeabilizing effect promoting a concentration-dependent CF efflux from all vesicle types - γ -terpinene and <i>p</i> -cymene had no significant effect CF leakage of vesicles - Dye leakage of PC vesicles was in the following order: thymol = carvacrol > γ -terpinene = <i>p</i> -cymene - Thymol induced a higher rate of CF efflux from PC/SA vesicles compared to other vesicle types - Carvacrol promoted a similar pattern of dye release from PC/SA and PC/PS vesicles. In both cases, dye leakage was lower than that from PC vesicles.	(Trombetta et al., 2005)
LUVs composed of: - PC - PC/SA (9:1) - PC/PS (9:1) molar ratio	Hydration of a lipid film followed by extrusion	CF (20 mM) at room temperature	Carvacrol, thymol, γ -terpinene and <i>p</i> -cymene, four monoterpenes added individually to the liposomes at various concentrations	- Carvacrol, thymol, γ -terpinene and <i>p</i> -cymene, four monoterpenes added individually to the liposomes at various concentrations	(Crisiani et al., 2007)

List of abbreviations:

PC: phosphatidylcholine; PI: phosphatidylinositol; Chol: cholesterol; DMPC: dimyristoylphosphatidylcholine; Chol: cholesterol; Erol: ergosterol; CL: cardiolipin; DPPC: dipalmitoylphosphatidylcholine; DPPG: dipalmitoylphosphatidylglycerol; SA: stearoylamine; PG: phosphatidylglycerol; SRB: sulforhodamine B; CF: carboxyfluorescein

ability to interact with lipid membrane components resulting in membrane destabilization. Additionally, (+) menthol elicited a significant dye release from negatively charged LUVs (PC/PS and PG/CL) while thymol promoted a remarkable leakage of positively charged vesicles (PC/SA). These findings are in agreement with the susceptibility of thymol and (+) menthol to kill gram + and gram - bacteria, respectively. Therefore, the permeabilizing effect of essential oil components is strongly influenced by the net surface charge of the membrane. Also, it is worthy to point out that a greater dye leakage was observed from vesicles made from phospholipids typical to the bacterial membranes such as PG and CL than from PC vesicles. This confirms again how the lipid composition can markedly impact the effect of essential oil component on membrane permeability (Trombetta et al., 2005). The influence of these factors was further studied by Cristani et al., 2007 who tested the effect of four monoterpenes of essential oil components (thymol, carvacrol, p-cymene and γ -terpinene) on LUVs permeability made of different lipid composition. The less hydrophobic components, p-cymene and γ -terpinene, were not effective in permeabilizing liposomal membranes whereas thymol was the most active on positively charged membranes. As for carvacrol, a greater dye leakage was evoked from pure PC vesicles over anionic and cationic vesicles (Cristani et al., 2007).

Before moving to another section, we should note that the permeabilizing agents described in this review are those studied by fluorescence and leakage assays. Other agents were also demonstrated to induce membrane permeabilization by other techniques but these are not within the scope of this paper.

5. Intrinsic factors affecting fluorescent probes release from liposomes

This section is devoted to discuss and analyze the data obtained from liposomal release fluorescence studies in order to draw conclusive ideas regarding the key modulators of membrane permeability. Two main factors affect dye release from liposomes: liposome membrane characteristics and experimental parameters. The former deals with lipid membrane composition and liposomes structural features (size, lamellarity, etc.) whereas the latter includes temperature and buffer

conditions (ionic strength, pH, etc.).

In the absence of bioactive agents, membrane permeability accounts for the physical state of the membrane. This latter is mainly dependent upon its lipid composition. The choice of lipid membrane constituents is very important for they provide the membrane with its barrier function. Therefore, blank liposomes data are collected in table 5 in an attempt to make a reasonable distinction between stable formulations and leaky ones on the basis of their lipid composition.

5.1. Cholesterol content

To start with, cholesterol exerts a deep modulatory effect on membrane physicochemical properties. Due to its condensing and ordering effect, cholesterol increases the stability of the liposomal membrane by reducing its permeability. This is consistent with leakage studies which reported a decrease in fluorophore leakage as a result of increasing cholesterol content in liposomal membranes (Cócera et al., 2003; Hatzi et al., 2007; Kaddah et al., 2018).

5.2. Membrane curvature

The effect of phospholipid species on blank liposomal membrane permeability was also investigated. Vesicles made up of only PC or DPPC are in general leaky (Cócera et al., 2003; Hatzi et al., 2007; Kaddah et al., 2018). Likewise, phospholipids inducing a negative membrane curvature such as DOPE usually produce, when included in liposomal formulations, more leaky vesicles. In contrast, phospholipids promoting a positive membrane curvature generate membrane with reduced permeability (Epanand, 2002). Hence, membrane curvature modulates membrane permeability. Furthermore, other lipid species such as SM (Ahumada et al., 2015) and CL (Firsov et al., 2015) had no effect on dye leakage and thereafter on the permeability of blank liposomes.

5.3. Vesicle type

Apart from membrane lipid composition, parameters related to the liposome structure, size, lamellarity, preparation method, etc. can affect

Table 5

The effect of membrane composition on membrane permeability of blank liposomes assessed by fluorescence leakage studies. For each study, the liposome composition, the fluorophore used and the main results are presented.

Liposome composition	Entrapped agent and operating temperature	Release study results	Reference
DPPC:Chol at molar ratios varying from 100:0 to 100:100	SRB (150 mM) at 37 °C	SRB release decreased with increasing Chol content in liposomal membranes	(Kaddah et al., 2018)
LUVs composed of DPPC/DPPG/DPPE/Chol (5/0.5/0.25/5) molar ratio	SRB (150 mM) at 4 and 37 °C	Stability of liposomal formulation at 4 °C (2% release after 6 days of incubation) versus 37 °C (17% release after 8 incubation days)	(Habib et al., 2013)
LUVs composed of DPPC and DPPC:SM (1:1) molar ratio	Calcein (80 mM) at 25 °C	The permeability of LUVs was not affected by the addition of SM	(Ahumada et al., 2015)
SUV, DRV and LUV composed of: - PC and PC/Chol (1:1) - H-PC and H-PC/Chol (1:1) - DSPC and DSPC/Chol (1:1)	Calcein (100 mM) at 37 °C	SUVs were found to be the most stable whereas MLV were leaky. Chol slowed down Cal release.	(Hatzi et al., 2007)
LUVs composed of: PC and PC/Chol (10:0; 9:1; 8:2; 7:3) molar ratios	CF (110 mM) Temperature not mentioned	Chol decreased CF release in a dose dependent manner	(Cócera et al., 2003)
LUVs composed of: - CL/DOPC (1:2) - CL/DOPC/DOPE (1:1:1) - DOPG/DOPC/DOPE (1:1:1)	ANTS/DPX (12.5 mM/45 mM) at 37 °C	Leakage percentage increases as follows: DOPG/DOPC/DOPE < CL/DOPC < CL/DOPC/DOPE. (effect of negative curvature)	(Epanand, 2002)
LUVs composed of PC/CL (80:20)	CF (100 mM) at room temperature	Negligible dye leakage	(Firsov et al., 2015)

List of abbreviations:

DPPC: dipalmitoylphosphatidylcholine; DPPE: dipalmitoylphosphatidylethanolamine; DPPG: dipalmitoylphosphatidylglycerol; Chol: cholesterol; SM: sphingomyelin; PC: phosphatidylcholine; H-PC: hydrogenated phosphatidylcholine; DSPC: distearoyl-glycero-phosphatidylcholine; CL: cardiolipin; DOPC: dioleoylphosphatidylcholine; DOPE: dioleoylphosphatidylethanolamine; DOPG: dioleoylphosphatidylglycerol; SRB: sulforhodamine B; CF: carboxyfluorescein

the drug release and thereafter the outcome of the study. In fact, many preparation methods were developed for the manufacturing of liposomes; the method to be used for liposomal release studies would be the one which presents a high encapsulation efficiency for the bioactive agent. Thus, thin film hydration and reverse evaporation phase are the most used for the preparation of liposomes for release studies. Liposome's lamellarity and size appear to have a great impact on the outcome of release studies. It was shown that small unilamellar vesicles (SUVs) are stable while multilamellar vesicles (MLVs) are very leaky vesicles (Hatzi et al., 2007). Effectively, SUVs are composed of a single lipid bilayer while MLV is composed of several concentric bilayers which complicates the use of MLVs in release studies.

5.4. Temperature

Growing evidence indicate that membrane permeability increases with temperature and it is drastically enhanced close to phase transitions (Papahadjopoulos et al., 1973). Instances where the leakage of solutes from liposomes significantly increases near the phase transition temperature were reported (Inoue, 1974; Oku et al., 1980). It was proposed that increasing the temperature near the gel/liquid-crystalline transition phase will induce a phase-separated state where leakage could occur due to the instability of the boundary regions of liposomal membrane and the discontinuity between the gel phase and fluid phase. Hence, this will lead to the formation of membrane defects or pores permitting the escape of solute out of the liposomes. It is worth mentioning that the permeability of a membrane composed of two or more lipid species is more complex since each lipid specie possesses a defined phase state depending on the temperature. This will lead to a heterogeneous membrane with different physical states.

Furthermore, liposomes have known a great popularity in the field of drug delivery. Various liposomal formulations were constructed among which the temperature-sensitive formulations, called also thermo-sensitive liposomes (TSL) were developed. Owing to the structural changes in the lipid membrane occurring at T_m and the highest permeability around this temperature, TSL will rapidly release the hydrophilic drug encapsulated inside their aqueous core at the T_m of the lipid bilayer. Therefore, many parameters should be controlled to ensure the stability and the ability of TSL formulations to release their cargos at a specific site of the body.

5.5. pH

Regarding buffer conditions, the investigations of the effect of bioactive agents on liposomal membrane permeability have revealed the pH-dependence of the permeabilizing activity of some agents. For instance, nisin and hemolysin display a pH-dependent activity on membrane permeability (Garcera et al., 1993; Menestrina, 1988). Additionally, the pore-forming activity of pentraxin proteins was pH-dependent, being highest at pH 5.5 (Harrington et al., 2008). Similarly, the effect of other peptides was found to change according to the ionic strength of the solution (Agafonov et al., 2003; Harrington et al., 2008; Manzini et al., 2014).

Taken all together, one can conclude that membrane permeability is modulated by the intrinsic properties of the liposomal system, the permeant or drug characteristics as well as experimental conditions.

6. Challenges

Fluorescence techniques are powerful tools frequently used in biochemistry, cell biology, molecular biology, biophysics, etc. Being sensitive, simple, rapid and reliable, these techniques were also applied to measure the stability of membrane under various conditions. Interestingly, fluorescence quenching assays were intensively used the past four decades to assess membrane permeability using liposomes as model membrane. They helped to elucidate the permeabilizing effect of

a large number of membrane-active agents, contributed to the mechanistic understanding of membrane permeabilization processes and provided a deep insight into the key modulators of membrane permeability.

Membrane permeability is modulated by intrinsic and extrinsic factors: the former relates to the membrane composition and the latter refers to the bioactive agent features and the experimental parameters. Hence, the composition of the liposomal formulation should be carefully prepared depending on the desired goal: investigation of the effect of an active agent on the membrane, determination of its mechanism of action or development of an appropriate liposomal system for drug delivery.

The agents promoting membrane permeabilization, presented in this review, belong to a variety of chemical classes. The literature data related to CD's effect on liposome membrane permeability proved that methylated derivatives of CDs and particularly those of β -CD are much more active than native CDs on Chol-enriched membranes. Almost all studies showed an increasing permeabilizing effect upon increasing CDs concentration. Also, phospholipid saturation increases the rigidity of membranes reducing thereafter the CDs-induced leakage of vesicles. These findings indicate that CDs effect on membrane permeability is mainly controlled by membrane composition. For instance, a methylated CD derivative exerts its permeabilizing effect on Chol containing membranes. Other CDs are active on poor or free Chol membranes. This reveals a preferential affinity between CDs and membrane components leading to a better understanding of the mechanism underlying the membrane permeabilization. The selection of HP- β -CD for the development of a combined system made of CD and liposome (DCL: drug-in-cyclodextrin-in-liposome) is being considered in many recent studies (Abada et al., 2019; Fernández-Romero et al., 2018; Gharib et al., 2019, 2018, 2017, 2016; Maestrelli et al., 2010).

As for PFPs, the published data underscores the importance of peptide's charge on their interaction with liposomal membrane. One can postulate that positively charged peptides possess a high affinity towards negatively charged membranes due to the electrostatic interactions occurring between positive amino acid residues and negative lipid components of the membrane and permitting the binding of the peptide to the membrane. This step is crucial in the membrane permeabilization process of PFPs. Furthermore, the secondary structure of the peptide is essential for the permeabilizing effect of PFPs to occur because the secondary structure exposes the amino acid residue in a position that favors peptide binding and insertion into the membrane. As stated earlier for CDs, membrane composition modulates here also the effect of peptides. For example, the presence of negatively charged membranes enhances the cationic peptide-induced permeabilization. Additionally, membrane composition can turn an inactive peptide into an active permeabilizing peptide.

Regardless the structure or the characteristics of the permeabilizing agent, unsaturated phospholipids generally form leaky membranes (Cócera et al., 2003). Also, loading the membrane with phospholipids inducing negative membrane curvature enhances the permeability (Epan, 2002). As for Chol content, it decreases liposomal permeability in a dose dependent manner due to the ordering and condensing effect of Chol on membranes (Kaddah et al., 2018). In addition, temperature can markedly affect the membrane permeability; the temperature effect depends on the phase transition of the phospholipids.

This implies that depending on the nature of the permeabilizing agent, the choice of the phospholipid type, the cholesterol content in the liposomal formulation and the operating temperature should be carefully considered for membrane permeability studies. One should note, that the choice of all these factors primarily depends on the aim and the subsequent applications of the study.

With regards to membrane fluidity, it is worth mentioning that numerous agents possessing a fluidifying effect were also found to exert a permeabilizing effect on the membrane. Nevertheless, to our knowledge, no correlation between membrane fluidity and membrane

permeability has been established whether in the absence or the presence of membrane-active agents.

7. Conclusion

Owing to their sensitivity and simplicity, liposomal membrane permeability has been intensively carried out using fluorescence techniques based on vesicle leakage of quenching probes. A vast literature survey was made in this review to describe the main permeabilizing agents and to summarize the key modulators of membrane permeability. We found that liposomal formulation should be carefully chosen depending on the agent's structure and characteristics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Partie II : Objectifs et stratégies de travail

1. Objectifs

Vue l'influence de la composition membranaire sur la stabilité de la membrane, l'étude de l'effet des composants lipidiques sur la perméabilité membranaire s'est avérée primordiale particulièrement dans le cadre de l'utilisation des liposomes en tant que systèmes d'encapsulation et de délivrance de principes actifs. Dans ce contexte, le CHOL demeure un composant lipidique principal qui affecte énormément la perméabilité et la stabilité membranaire. Néanmoins, à part son effet sur la membrane, le CHOL pourrait également moduler l'effet perméabilisant induit par certains agents bioactifs. Peu nombreuses sont les études portant sur ce sujet. Ce travail de thèse vise alors à combler cette lacune en étudiant l'effet du CHOL sur la perméabilité des membranes liposomales exposées à certains agents perméabilisants.

Deux types d'agents perméabilisants seront envisagés dans notre étude : des agents bioactifs tels que les composés d'HE et des nanovecteurs comme les cyclodextrines. Le choix de ces deux agents repose sur le fait que nous désirons évaluer d'une part l'effet d'un composé qui servirait de modèle d'un principe actif pouvant être encapsulé dans des vésicules lipidiques (c'est le cas des composés d'HE) et d'autre part l'effet d'un nanovecteur (tel que les cyclodextrines) qui est capable à la fois de véhiculer un principe actif et d'être lui-même encapsulé dans les liposomes. Dans ce dernier cas, l'effet des CDs libres et des complexes d'inclusion CD/invité sur la perméabilité membranaire seront étudiés. Cette étude complètera alors la thématique de recherche de notre laboratoire qui a évalué l'effet de plusieurs composants d'HE sur la fluidité membranaire (Gharib et al., 2017b, 2018) et qui vise l'optimisation de l'encapsulation de ces HE dans des liposomes ou bien dans des DCL (Gharib et al., 2019, 2017a, 2016a, 2016b; Hammoud et al., 2020, 2019; Sebaaly et al., 2016, 2015).

L'originalité de ces travaux expérimentaux réalisés réside dans l'utilisation de liposomes formés de DPPC et de différents taux de CHOL. En fait, varier le contenu membranaire en CHOL permettrait de souligner l'effet modulateur du CHOL non seulement sur la membrane mais aussi sur le comportement des agents perméabilisants testés sur la membrane.

2. Stratégies de travail

Pour réaliser ce travail, nous avons d'abord préparé des liposomes blancs formés de DPPC et de CHOL aux rapports molaires DPPC:CHOL (100:10; 100:25; 100:50; 100:75 et 100:100) par la méthode d'évaporation en phase inverse. Ces formulations sont caractérisées en termes de taille et composition lipidique. Cette première étude nous a permis de déterminer le rapport molaire DPPC:CHOL expérimental des différentes formulations préparées.

Ensuite, des liposomes formés de DPPC et CHOL aux mêmes rapports molaires et encapsulant la SRB ont été préparés, extrudés et purifiés par chromatographie de tamisage moléculaire. Ces liposomes sont utilisés pour l'étude de perméabilité membranaire qui consiste à suivre le relargage de la SRB des vésicules lipidiques avec le temps à 37°C après exposition des membranes liposomales aux agents actifs.

Dans un premier temps, nous avons exposé les membranes liposomales à une série de 22 composés d'HE de structures chimiques variées à un ratio molaire DPPC:HE (100:25). Ce ratio a été choisi en se référant à une étude récente réalisée dans notre équipe de recherche : celle-ci a montré que les composés d'HE exercent un effet fluidifiant de la membrane de liposomes formés de DPPC et cet effet croît avec la concentration de ces composés (Gharib et al., 2018). Les échantillons ont été incubés à 37°C et la perméabilité membranaire a été évaluée à 0h et 72h. Cette étude nous a permis d'abord de sélectionner les composés HE montrant un effet perméabilisant de la membrane à faible taux de CHOL (10 % CHOL), puis l'effet perméabilisant de ces composés a été étudié en présence de taux croissants de CHOL pour souligner l'influence de ce dernier sur l'effet perméabilisant des composés d'HE. Les résultats ont été analysés en considérant l'hydrophobicité de ces composés. De surcroît, une relation structure activité a été extraite de ce travail.

Dans un deuxième temps, les membranes liposomales ont été traitées par 3 CD natives et 4 dérivés de la β -CD aux ratios molaires CD:DPPC (1:1; 10:1 et 100:100) et incubés à 37 °C durant 24h. L'effet perméabilisant des CDs a été examiné à différents taux de CHOL et les résultats ont été analysés en fonction de la concentration des CDs, le taux de CHOL et l'affinité de la CD pour les composants lipidiques de la membrane.

Finalement, deux CDs ont été choisies pour former des complexes d'inclusion CD/thymol ; ce sont la RAMEB et la HP- β -CD. Le choix de ces deux CDs revient au comportement de chacune en

présence de taux croissant de CHOL dans la membrane : la RAMEB induit un effet perméabilisant qui s'accroît en présence du CHOL alors que la HP- β -CD exerce un effet qui s'affaiblit en présence du CHOL. Ainsi, des complexes d'inclusion CD/thymol ont été préparés en phase aqueuse puis lyophilisés. Des liposomes contenant 25 et 100 % CHOL ont été exposés aux complexes d'inclusion à un ratio molaire CD:DPPC 100:1 et l'étude de la perméabilité membranaire a été suivie durant 24 h. Les résultats ont permis de comparer l'effet des CDs libres, du thymol libre et du complexe d'inclusion CD/thymol.

La méthodologie suivie pour la réalisation de ce travail sont présentés dans les schémas I, II et III.

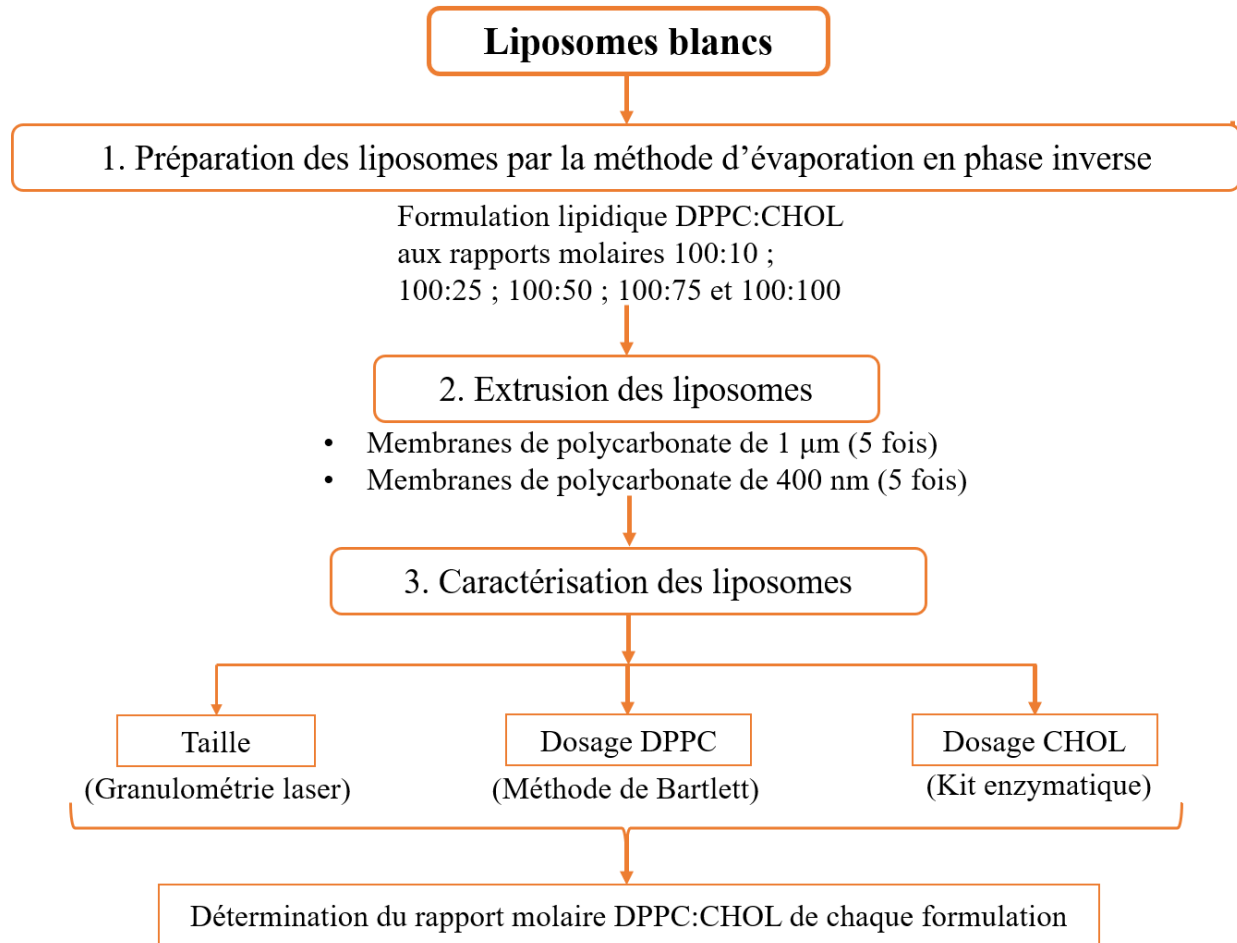


Schéma I : Méthodologie suivie pour la préparation et la caractérisation des liposomes blancs formés de DPPC et CHOL aux rapports molaires DPPC :CHOL (100:10 ; 100:25 ; 100:50 ; 100:75 et 100:100)

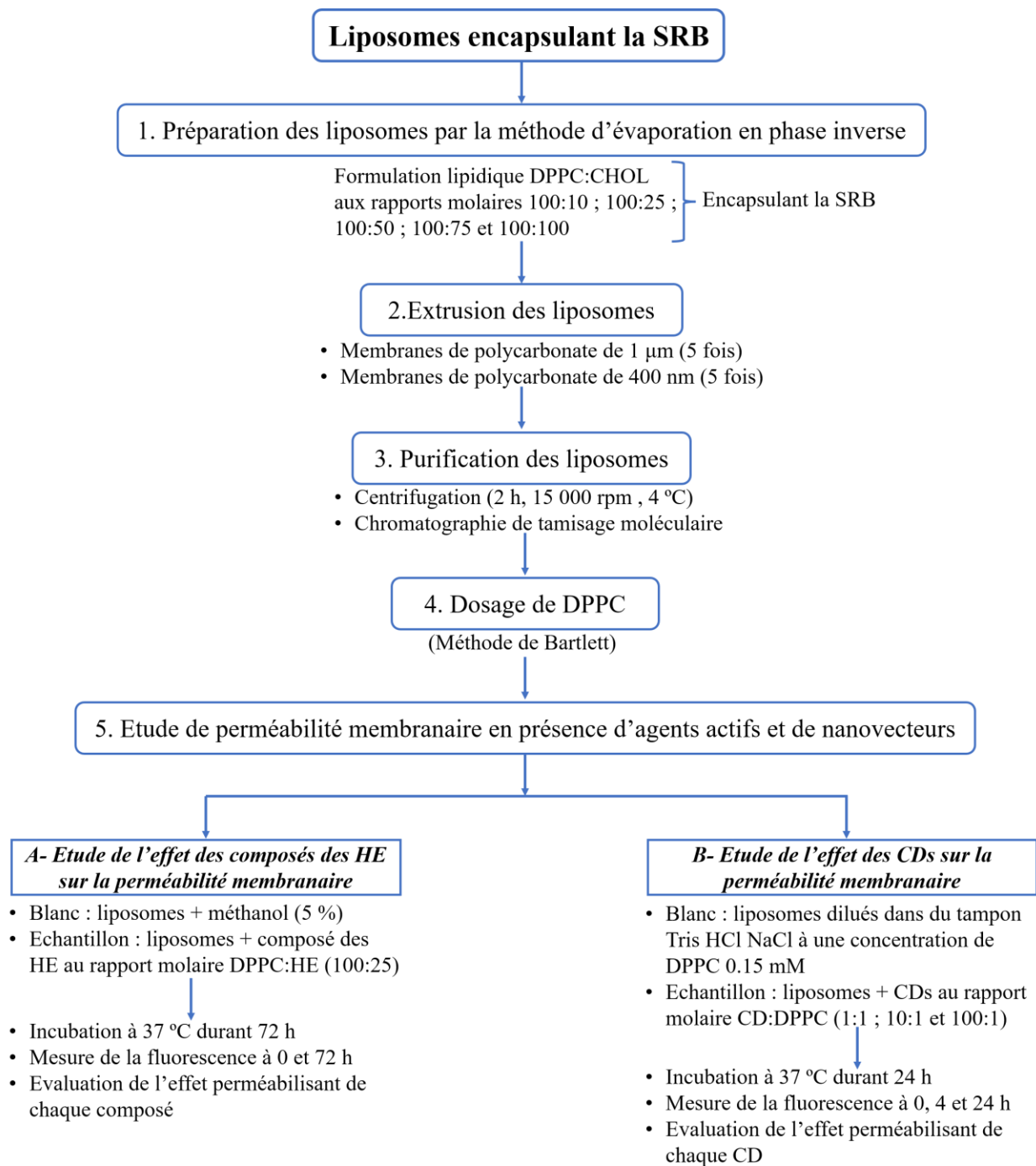


Schéma II : Méthodologie adoptée pour l'étude de l'effet d'agents actifs (les composés d'HE) et de nanovecteurs (CDs libres) sur la perméabilité membranaire des liposomes formés de DPPC et différents taux de CHOL.

L'effet des complexes d'inclusion CD/thymol sur la perméabilité membranaire des liposomes pauvres et riches en CHOL

RAMEB/thymol et HP-β-CD/thymol

1. Préparation des complexes d'inclusion CD/thymol en solution

- Un excès de thymol (15 mg) ajouté à une solution aqueuse de CD (HP-β-CD ou RAMEB) à une concentration 15 mM
- Le mélange est agité durant 24 h, à une vitesse 125 rpm, à 26 °C
- Filtration (filtre d'acetate de cellulose de taille 0.45 μm)
- Lyophilization du filtrat obtenu
- Dosage du thymol dans le complexe d'inclusion par HPLC

2. Etude de l'effet des complexes d'inclusion sur la perméabilité des membranes liposomales

- Blanc: liposomes (DPPC:CHOL 100:25 ou 100:100) dilué dans du tampon Tris HCl NaCl à une concentration de DPPC 0.15 mM
- Blanc thymol: liposomes + méthanol (5 %)
- Echantillons:
 - liposomes + CD (au rapport molaire CD:DPPC 100:1)
 - liposomes + complexes d'inclusion CD/thymol (au rapport molaire CD:DPPC 100:1)
 - liposomes + thymol (5mM)
- Incubation à 37 °C pendant 24 h
- Mesure de la fluorescence à 0, 2, 4, 6 et 24 h
- Evaluation de l'effet perméabilisant des complexes d'inclusion

Schéma III : Méthodologie suivie pour préparer les complexes d'inclusion solides RAMEB/thymol et HP-β-CD/thymol et pour étudier leur effet sur la perméabilité des membranes liposomales pauvres et riches en CHOL.

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Partie III : Travail expérimental et résultats

Chapitre 3 : Effet d'une série de composés d'huiles essentielles sur la perméabilité membranaire de liposomes formés de DPPC et de différents taux de cholestérol

Introduction

Dans ce chapitre, nous allons présenter une étude portant sur l'effet d'une série de 22 composés d'HE sur la perméabilité des membranes liposomales contenant différents taux de CHOL. Cette étude vise à évaluer l'effet du CHOL sur la perméabilité induite par les composés HE et permet de choisir pour chaque composé une formulation adéquate pour l'encapsulation de ces molécules dans les liposomes.

Les HE issues des plantes aromatiques sont des mélanges complexes formés de plusieurs dizaines, voire de plus d'une centaine de composés naturels, volatils, odorants et de faible poids moléculaire. Ces composés font partie des métabolites secondaires des plantes aromatiques (Burt, 2004). Les constituants d'HE possèdent des structures chimiques variées et sont réputés pour un large spectre d'activités biologiques : effectivement, de nombreuses HE ainsi que certains de ses composés ont démontré des propriétés antibactérienne, anti-inflammatoire, antioxydante, anticancéreuse et antidiabétique, etc. (Bakkali et al., 2008).

Cependant, les composés de HE sont instables. Outre leur faible solubilité aqueuse, ces constituants sont photosensibles et sont sujets à l'oxydation (Sherry et al., 2013). Plusieurs stratégies ont été développées pour contourner cette limitation ; l'encapsulation des composés de HE dans des liposomes s'est révélée efficace améliorant la stabilité de ces composés et conservant leur activité biologique (même dans certains cas leurs activités sont intensifiées) (Asbahani et al., 2015; Sherry et al., 2013). Les travaux menés sur l'encapsulation des HE ont utilisé des liposomes formés de phospholipides saturés (DPPC) ou insaturés (PC). Le CHOL faisait souvent partie de la composition membranaire (Azzi et al., 2018; Gortzi et al., 2007; Liolios et al., 2009; Moghimipour et al., 2012; Sebaaly et al., 2015b, 2015a). Toutefois, le contenu en CHOL dans la formulation destinée à encapsuler ces composés n'est pas encore optimisé. Bien que le CHOL réduit la perméabilité des membranes liposomales (Kaddah et al., 2018), un taux élevé de CHOL pourrait

aussi diminuer l'efficacité de l'encapsulation des composés d'HE (Jaafar-Maalej et al., 2010; Sherry et al., 2013). Par ailleurs, le mode d'action des constituants de HE dépend particulièrement de leur caractère hydrophobe qui leur permet de pénétrer dans la bicouche phospholipidique et perturber sa stabilité (Burt, 2004). Dans ce contexte, plusieurs composés de HE ont prouvé leur capacité de perméabiliser les membranes synthétiques et naturelles. Notons que la plupart des études évaluant l'effet de ces composés sur la membrane utilisent généralement les cellules bactériennes qui ne renferment pas de CHOL ou bien des liposomes sans CHOL ou contenant un taux faible de CHOL. Partant de ce qui précède, nous nous sommes intéressés à étudier l'effet d'une série de composés de HE sur la perméabilité des liposomes renfermant différents taux de CHOL.

Les 22 composés choisis appartiennent à plusieurs familles chimiques ; la plupart sont des composés terpéniques. Ils peuvent être groupés en deux classes : les composés phénoliques ayant dans leur structure un groupe phénol ou phényl et les composés non phénoliques ne possédant pas ce groupe. Chaque classe est à son tour subdivisée en sous-classes selon les caractéristiques structurales des composés de HE. À titre d'exemple, les composés phénoliques renferment des phénylpropènes et leurs dérivés (eugénol, isoeugénol, estragole, trans-anéthole, acétate d'eugényl), un monoterpène (thymol) et des composés phénoliques ayant une fonction éther (anisole et guaiacol). Quant aux composés non phénoliques, cette classe comprend des monoterpènes portant une fonction alcool (bornéol, géraniol, linalool, menthol), cétone (camphor), ester (acétate de bornyl et acétate de linalyl) ou aussi des monoterpènes hydrocarbonnés (camphène, p-cymène, α -phellandrène, α -terpinène et γ -terpinène). On trouve aussi parmi les composés non phénoliques un sesquiterpène hydrocarbonné (β -caryophyllène) et un sesquiterpène portant une fonction alcool (nérolidol). Les 22 composés de HE choisis pour ce travail se distinguent non seulement par leur structure, mais également par leur hydrophobicité et leur solubilité en milieu aqueux.

Pour réaliser cette étude, nous avons d'abord préparé des liposomes blancs (sans SRB) formés du mélange binaire DPPC:CHOL à différents ratios molaires (100:10; 100:25; 100:50; 100:75 et 100:100) par la méthode d'évaporation en phase inverse. Les liposomes ont ensuite subi une extrusion pour obtenir des vésicules de type LUVs et de taille moyenne environ de 400 nm. Ces différentes formulations ont été caractérisées en termes de taille (par granulométrie laser) et de

composition membranaire. Le taux d'incorporation du DPPC et du CHOL ainsi que le rendement d'encapsulation de chaque composant lipidique ont été déterminés pour chaque formulation. Ceci nous a permis de calculer expérimentalement le rapport molaire DPPC:CHOL relatif à chaque formulation.

Les résultats obtenus montrent que les liposomes préparés forment des suspensions homogènes et dont la taille augmente avec l'addition du CHOL à la membrane et ceci pour les formulations contenant 10 et 25 % CHOL. À des taux de CHOL membranaires supérieurs à 25 %, la taille des vésicules cesse d'augmenter : les liposomes formés de DPPC:CHOL de ratio molaire 100:50, 100:75 et 100:100 ont des tailles pratiquement les mêmes et elle sont voisines de la taille théorique prévue après l'extrusion (400 nm). Ceci est en concordance avec des résultats déjà obtenus dans notre laboratoire. En ce qui concerne la composition lipidique des différentes formulations, nous avons remarqué que l'addition du CHOL réduit le taux d'incorporation du DPPC dans le cas des membranes liposomales renfermant 10 et 25 % CHOL, sans avoir un effet pareil sur les autres formulations. De plus, l'ajout d'une quantité croissante de CHOL lors de la formation des liposomes semble réduire l'incorporation du CHOL dans la membrane et cet effet devient significatif d'autant plus que le taux de CHOL augmente. Ceci s'est traduit par une diminution progressive du taux d'incorporation du CHOL dans la membrane des liposomes affectant alors le contenu membranaire réel en CHOL. Par la suite, pour des taux élevés de CHOL, le ratio molaire DPPC:CHOL déterminé expérimentalement pour les formulations liposomales ne reflète pas le ratio théorique initialement utilisé pour la préparation des liposomes. Par exemple, la formulation théoriquement composée de DPPC:CHOL de ratio molaire 100:100 a effectivement un rapport de 100:55. Ainsi, les formulations préparées possèdent les rapports molaires DPPC:CHOL suivants : 100:11 ; 100:21 ; 100:40 ; 100:43 et 100:55.

Afin d'évaluer l'effet des composés de HE sur la perméabilité membranaire, des liposomes formés de DPPC et de CHOL aux mêmes rapports molaires et encapsulant la SRB ont été préparés par la même méthode. Après extrusion, les liposomes ont subi une centrifugation puis une séparation sur une colonne de gel Sephadex G25 dans le but de récupérer les liposomes encapsulant la SRB et se débarrasser des molécules libres de lipides et de SRB non encapsulées. Ainsi, les liposomes élués en premier sont utilisés pour l'étude de perméabilité après détermination de la concentration de DPPC dans les liposomes par la méthode de Bartlett. Cette étude a été effectuée en exposant les

membranes liposomales aux différentes molécules de HE selon un rapport molaire DPPC:HE de 100 :25. Puisque les solutions des composés de HE ont été préparées dans le méthanol, le volume de HE ajouté aux liposomes ne devrait pas dépasser 5 % du volume liposomal pour ne pas perturber la stabilité des liposomes. Pour chaque formulation, des liposomes traités par un volume de méthanol (5 %) ont été considérés comme blancs. Les liposomes blancs et les liposomes exposés aux composés de HE sont par la suite incubés à une température de 37 °C pendant 72 h. Des aliquotes ont été enfin prélevées des différents échantillons à t_0 et à 72 h et l'intensité de la fluorescence a été mesurée par spectroscopie de fluorescence permettant ainsi de calculer le pourcentage de libération de la SRB des vésicules reflétant ainsi la stabilité des liposomes exposés aux différents composés d'HE.

Dans un premier temps, nous avons testé l'effet des 22 molécules de HE sur la formulation formée de DPPC:CHOL de rapport molaire 100:10. Selon une étude récente, la membrane contenant un faible taux de CHOL n'est pas assez stable (Kaddah et al., 2018); elle sert donc à identifier les composés de HE exerçant un effet perméabilisant sur la membrane. Parmi les 22 molécules testées, 13 composés ont significativement augmenté la perméabilité membranaire des liposomes composés de 10 % CHOL; ceux-ci sont classés par ordre décroissant de leur pouvoir perméabilisant: nérolidol > eugénol > linalool > thymol > guaiacol > geraniol > α -phellandrène > trans-anéthole > α -terpinène > acétate de linalyl > menthol > γ -terpinène > acétate d'eugényl. Les autres molécules n'avaient pas d'effet sur la membrane supposant ainsi qu'un autre mécanisme d'action pourrait être attribué à leur activité antimicrobienne.

Ces résultats ont également permis d'établir une relation structure activité entre les différentes molécules étudiées : en effet, la majorité des molécules phénoliques et non phénoliques démontrant un effet perméabilisant étaient des composés terpéniques possédant un groupe hydroxyle dans leur structure. Le nérolidol, un sesquiterpène possédant un groupe OH, a présenté l'effet perméabilisant le plus significatif. Ceci est en accord avec de nombreuses études qui ont prouvé l'importance du groupe hydroxyle, en particulier le groupe phénol, et son implication dans l'activité de plusieurs composés de HE comme le thymol et l'eugénol. L'absence de la fonction alcool dans la structure des composés phénoliques oxygénés engendre un moindre effet sur la membrane ; tel était le cas du trans-anéthole et de l'anisole qui ont montré un effet perméabilisant plus faible en comparant leur effet respectivement avec celui de l'eugénol et du guaiacol. Dans cette catégorie aussi, les

composés non phénoliques hydrocarbonés n'ayant pas de groupement OH ont montré également un effet perméabilisant faible. De plus, la substitution du groupe hydroxyl par un autre groupement tel que l'acétate semble réduire ou bien masquer l'effet exercé par le composé : ceci a été observé avec l'acétate d'eugényl et l'acétate de linalyl. À part la présence du groupe hydroxyl, nous avons constaté que d'autres caractéristiques structurales peuvent influencer l'effet des composés de HE sur la perméabilité membranaire. À titre d'exemple, la présence d'un système d'électrons délocalisés pourrait être responsable de l'inactivité du composé phénolique l'isoeugénol par rapport à l'eugénol ainsi que l'inactivité du composé non phénolique le p-cymène par rapport à l' α -phellandrène. D'ailleurs, les molécules non phénoliques terpéniques linéaires (le nérolidol, le linalool, le géraniol) ayant une fonction alcool ont présenté un effet perméabilisant qui est plus important que le composé non phénolique cyclique, le menthol.

Dans un second temps, nous avons procédé à exposer les membranes liposomales des formulations DPPC:CHOL (100:25; 100:50; 100:75 et 100:100) aux 13 composés de HE choisis pour leur effet perméabilisant de la membrane formée de 10 % CHOL selon le même protocole adopté précédemment. Nous avons remarqué que le CHOL affecte considérablement la perméabilité induite par les composés de HE. En fait, parmi les 13 molécules ayant un effet sur la membrane formée de 10 % CHOL, 9 molécules seulement ont gardé un effet perméabilisant de la membrane renfermant 21 % CHOL. Ce nombre est davantage réduit à 5 composés (eugénol, thymol, guaiacol, linalool et menthol) avec les membranes composées de 40 % CHOL. Au-delà de ce pourcentage, aucune molécule n'a pu perméabiliser les liposomes.

Finalement, l'ensemble de ces résultats ont été analysés en considérant l'hydrophobicité des composés de HE étudiés. Ce paramètre s'est révélé crucial en discutant l'interaction ayant lieu entre ces composés et la membrane. Effectivement, si la molécule est très hydrophobe ($\log P > 4$), un effet perméabilisant est détecté à faible taux de CHOL seulement si la molécule possède un groupe hydroxyle. Ceci a été observé avec le nérolidol ($\log P$ 4.6) à 10 % CHOL. D'autre part, lorsque l'hydrophobicité de la molécule diminue, son effet va persister à des taux de CHOL plus élevé (40 % CHOL) si sa structure renferme un groupe OH. En absence de ce groupement, un effet perméabilisant très faible peut s'observer à 21 % CHOL pour quelques molécules (α -terpinène, γ -terpinène et trans-anéthole). Notons que le CHOL module l'état de phase de la membrane : les membranes renfermant 10 et 21 % CHOL existent à l'état solide ordonné – liquide ordonné noté

"So-Lo" alors que les autres formulations adoptent la phase liquide ordonné noté "Lo". La perméabilité membranaire dépend aussi de la phase de la membrane : elle est réduite à l'état So-Lo en présence des molécules d'HE alors qu'à l'état Lo un effet est uniquement observé pour quelques composés à 40 % CHOL.

En se basant sur l'ensemble des paramètres discutés, nous avons pu conclure qu'une formulation renfermant 40 % CHOL pourrait être adéquate aux molécules de HE très hydrophobes alors qu'une formulation contenant plus que 40 % CHOL serait recommandée aux molécules moins hydrophobes.

Ce chapitre sera présenté sous forme d'un article publié dans "Journal of Membrane Biology".

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Liposome Permeability to Essential Oil Components: A Focus on Cholesterol Content

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Liposome Permeability to Essential Oil Components: A Focus on Cholesterol Content

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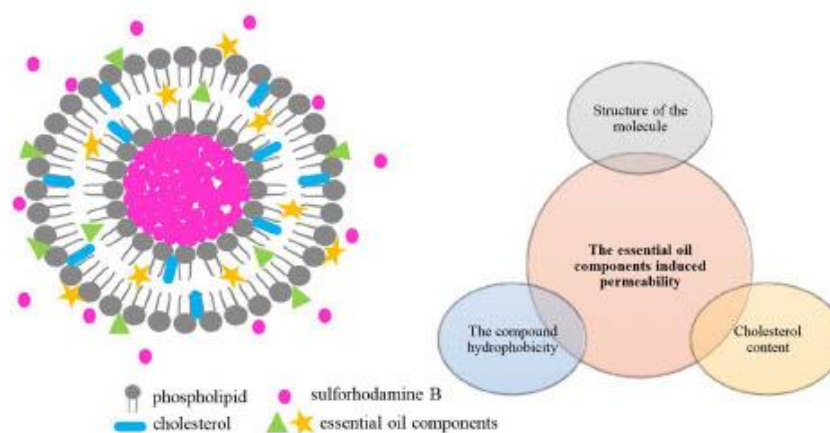
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Abstract

Encapsulation in liposomes has been an efficient strategy to improve the stability of sensitive bioactive compounds such as essential oils (EOs). However, the stability of liposomal formulations remains a key parameter controlling the delivery of encapsulated ingredients. Cholesterol (Chol) modulates the membrane properties conferring stability to the lipid bilayer. Thus, the Chol content in the liposome formulations encapsulating EO components should be carefully chosen. In this work, various liposome formulations differing by Chol content (DPPC:Chol 100:10; 100:25; 100:50; 100:75; 100:100) were exposed to a series of 22 EO components at DPPC/EO 100/25. The formulations were characterized for their final composition and their permeability to the hydrophilic fluorophore, sulforhodamine B (SRB), was monitored. Results showed that the Chol content experimentally determined for the various formulations (above 10% Chol) was below the theoretical weighed Chol. Among the tested components, 13 molecules displayed a significant permeabilizing effect on 10% Chol membranes. Most of these possess a hydroxyl group. The EO induced permeability was dependent on the Chol content which affects the membrane phase: their effect was reduced upon increasing Chol content keeping five EOs components effective at 40% Chol. The EO's effect was also linked to the hydrophobicity of the molecule. Hence, the DPPC:Chol ratio of the formulation is chosen considering the structure of the compound, its hydrophobicity and its effect on the permeability at different Chol content: a formulation comprising 40% Chol is suggested for highly hydrophobic molecules whereas a formulation with higher Chol content could be selected for less hydrophobic compounds.

Graphic Abstract



Keywords Liposomes · Membrane permeability · Essential oil components · Cholesterol content · Hydrophobicity · Encapsulation

Extended author information available on the last page of the article

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Abbreviations

DPPC	Dipalmitoylphosphatidylcholine
Chol	Cholesterol
SRB	Sulforhodamine B
EO	Essential oil
LUVs	Large unilamellar vesicles
PC	Phosphatidylcholine
LR	Loading ratio
IR	Incorporation ratio

Introduction

Aromatic and medicinal plants are rich in essential oils (EOs), a mixture of natural, volatile, fragrant compounds of low molecular weight, constituting the secondary metabolites of plants. These complex mixtures may comprise 20–100 different constituents belonging to a variety of chemical classes generally dominated by: terpenes, phenylpropanes and their oxygenated derivatives as well as other aromatic and aliphatic molecules (Hyldgaard et al. 2012; Asbahani et al. 2015). EOs are used in various sectors, mainly perfumery, cosmetics and food industry (Burt 2004; Bakkali et al. 2008). They cover a broad biological activity spectrum (Raut and Karuppaiyil 2014) including antibacterial (Nazzaro et al. 2013; Zhang et al. 2016), antifungal (Tian et al. 2012; Chen et al. 2013), antioxidant (Miguel 2010; Teixeira et al. 2013), anti-inflammatory (Hajhashemi et al. 2003; Miguel 2010), antiviral (Schnitzler et al. 2010; Astani et al. 2011) and insecticidal activities (Isman et al. 2008). Besides, some EOs (and some of their constituents) have displayed anticancer (Kaefer and Milner 2008; Jagannathan et al. 2011) and antidiabetic properties (Chung et al. 2010; Boukhris et al. 2012). Taken all together, EOs are currently used as an alternative natural cure.

Nevertheless, EOs are unstable compounds known for their poor water solubility and their high sensitivity to light, oxygen and temperature which render them prone to degradation (Majeed et al. 2015). Therefore, encapsulation would be a feasible solution to increase EOs stability, control their release and, hence, improve their efficiency (Sherry et al. 2013; Asbahani et al. 2015). During the past 2 decades, efforts have been made to determine the best formulation for the encapsulation of EO components in liposomes. In fact, the lipid composition of the liposomal membrane dictating the physicochemical properties of the lipid bilayer, affects considerably the stability of the vesicles. In addition, the liposomal formulation controls the release of encapsulated ingredients over time (Bozzuto and Molinari 2015). The available literature data shows that different liposomal formulations were demonstrated to improve the physicochemical stability of EO constituents and enhance their biological activities; the aforementioned are usually composed of either

saturated or unsaturated phospholipids in combination with cholesterol (Chol) (Gortzi et al. 2007; Liolios et al. 2009; Moghimipour et al. 2012; Sebaaly et al. 2015a,b; Lin et al. 2016; Cui et al. 2017; Azzi et al. 2018). Consequently, the liposome formulation should be tailored to meet the stability and release requirements of a suitable delivery system.

Liposomal membrane permeability is greatly influenced by the membrane lipid composition. Recently, we have investigated the effect of Chol on the membrane permeability of liposomes composed of various DPPC:Chol molar ratios and encapsulating a hydrophilic fluorescent dye, sulforhodamine B (SRB). The SRB release constants decreased upon increasing Chol content in the liposomal membranes proving thereby the ability of cholesterol to affect the membrane permeability in a dose dependent manner (Kaddah et al. 2018).

Compelling evidence supports the ability of EO components to increase the fluidity and permeability of liposomal membranes by interacting with the bilayer's phospholipids (Trombetta et al. 2005; Cristani et al. 2007; Gharib et al. 2017,2018). Indeed, some EO components are classified as permeabilizing agents inducing membrane permeabilization via defect formation (Nasr et al. 2020). This may lead to a low encapsulation efficiency due to the escape of the molecules out of the vesicles. To overcome this hurdle, a suitable liposomal formulation has to be found where the choice of the phospholipid to Chol ratio is actually a matter of importance. It is worth noting that much of the vast literature on the membrane permeabilizing effect of EO components deals with the antimicrobial effect of EOs where the bacterial cell membranes which are Chol free are used. Hence, in this work, we tested the effect of EO components on the permeability of liposomal membranes made of different DPPC:Chol. A series of 22 EO components was chosen for this study. The membrane permeability will be followed upon exposure of different liposome formulations to these components. This work will allow us to evaluate the effect of Chol on the membrane permeability that could be induced by these components and therefore deduce adequate formulations for EOs encapsulation. A structure activity relationship could be also extracted from the permeability study results.

Large unilamellar vesicles (LUVs) were first prepared from a mixture of DPPC and Chol, at different DPPC:Chol molar ratios (100:10; 100:25; 100:50; 100:75; 100:100) by the reverse phase evaporation method followed by extrusion. Liposomes were then characterized in terms of their size and their lipid composition (DPPC and Chol loading ratios and incorporation ratios) allowing the determination of the final liposome composition. Similarly, another set of LUVs loaded with SRB was prepared at the same DPPC:Chol molar ratios cited above. The se batches were exposed to EO components at

a molar ratio DPPC:EO of 100:25 and the SRB release from liposomes was followed during 72 h at 37 °C by fluorescence measurements.

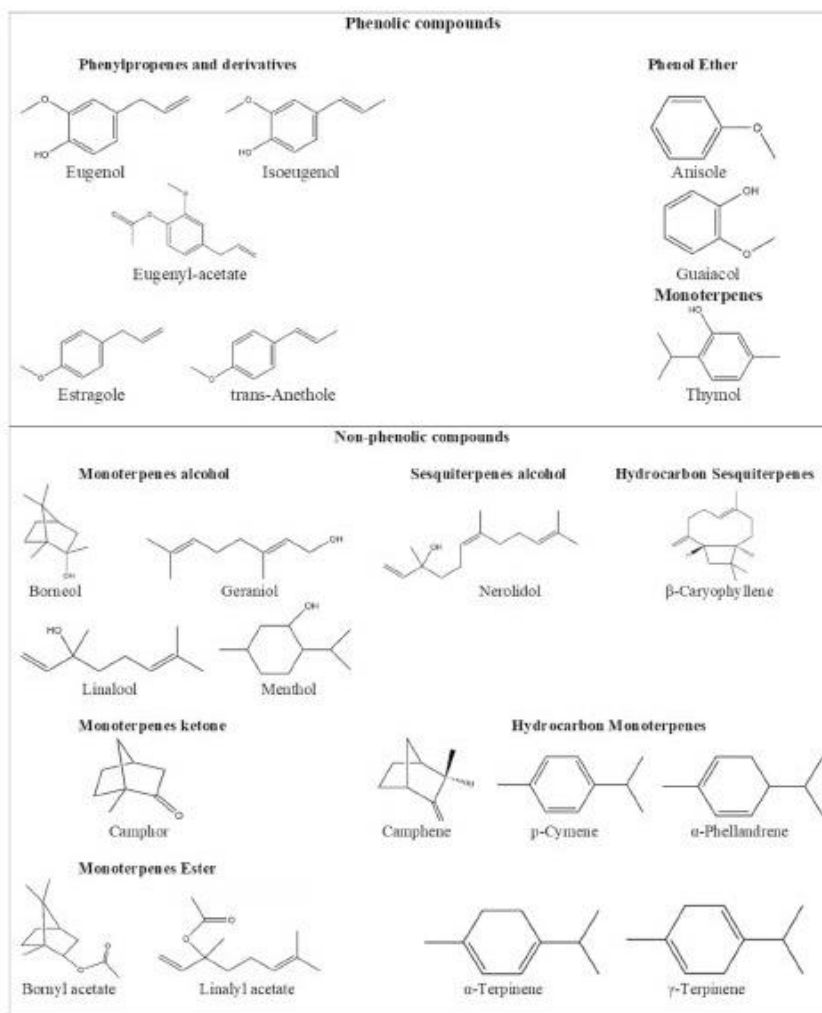
The chemical structures of the investigated molecules are shown in Fig. 1. The EO components series is divided into phenolic and non-phenolic compounds: the first group comprises phenylpropanoids, monoterpenes, and phenol ethers. The second group includes monoterpenes bearing different functional groups such as alcohol, ketone and ester. Sesquiterpenes and hydrocarbon monoterpenes are also present in this group. Table 1 in supplementary material presents the aqueous solubility, the log *P* values and the Henry constants of the tested molecules.

Materials and Methods

Chemicals

Dipalmitoylphosphatidylcholine (DPPC) ($\geq 99\%$) and trizma base (buffer reagent) were purchased from Sigma-Aldrich, Swiss. Triton X-100, sodium chloride (NaCl) and Sephadex G25 gel were purchased from Sigma-Aldrich, Belgium. Cholesterol (purity 99%), ammonium molybdate, hydrogen peroxide, potassium dihydrogenophosphate, sodium sulfite, sodium bisulfite, menthol, eugenol, chloroform and methanol were all purchased from Sigma-Aldrich, Germany. Linalool was purchased from Sigma-Aldrich, Switzerland. Trans-Anethole was purchased from Sigma-Aldrich, Spain. Geraniol and nerolidol were purchased

Fig. 1 The chemical structures of the 22 EO components tested in this study



from Sigma-Aldrich, USA. Sulforhodamine B and camphor were purchased from Sigma-Aldrich, China. Borneol, bornyl acetate, *p*-cymene, α -terpinene and γ -terpinene were purchased from Fluka Chemical, Switzerland. 4-amino-3-hydroxy-1-naphthalene sulfonic acid was purchased from Fluka, India. Anisole was purchased from Fluka, Germany. Guaiacol, thymol, estragole, isoeugenol were purchased from ACROS Organics, New Jersey, USA. The sulfuric acid was purchased from ACROS Organics, Belgium. The diethylether was purchased from VWR-Prolabo Chemicals, Belgium. β -Caryophyllene and eugenyl acetate were purchased from SAFC, USA. Linalyl acetate was purchased from SAFC, Spain. α -Phellandrene was purchased from SAFC, Germany. Camphene was purchased from MERCK, Schurhardt.

Preparation of Large Unilamellar Vesicles (LUVs) with and Without SRB Followed by Extrusion

LUVs were prepared by dissolving 25 μ mol of DPPC in 5 mL of a mixture of organic solvents: chloroform/diethyl ether/methanol (6/6/1—v/v/v) (Khreich et al. 2008). The Chol was added at different DPPC:Chol molar ratios (100:10, 100:25, 100:50, 100:75, 100:100) from a stock solution of Chol prepared in the same organic solvents mixture (25 mg/mL). The lipid solution was then sonicated (Sonicator Starsonic 35) for 1 min at 60 °C under nitrogen stream. 0.75 mL of Tris HCl buffer (0.1 M, pH 7.4) was added to the mixture, followed by a sonication for 6 min at 60 °C under nitrogen stream. The organic solvents were then removed by evaporation at 45 °C using a rotary vacuum evaporator (Heidolph, Germany). A white lipid film was obtained. In the last step, 1.5 mL of Tris HCl buffer (0.1 M, pH 7.4) was added and the mixture was sonicated again for 5 min at 60 °C under nitrogen stream. The obtained liposomal suspensions were stored in the refrigerator at 4 °C for further manipulation.

Before extrusion, the liposomes were incubated in a water bath for 35 min at 60 °C and then extruded through polycarbonate filters, using a mini extruder (Avanti polar lipids, Switzerland). Liposomes were forced to pass through polycarbonate membranes (Avanti polar lipids, Switzerland) of decreasing pore size (5 times through a 1 μ m membrane followed by 5 times through 0.4 μ m membrane). This step is important because it allows obtaining a homogenous mixture of LUV liposomes with a mean diameter theoretically very close to the pore size of the last membrane they were extruded through.

The same procedure was followed to prepare and extrude the SRB-loaded LUVs composed of DPPC:Chol molar ratios (100:10; 100:25; 100:50; 100:75 and 100:100). The only difference was that the SRB was added to the aqueous phase composed of Tris HCl (0.1 M, pH 7.4) buffer at a

concentration of 150 mM. After extrusion, Tris-HCl (0.1 M, pH 7.4) containing 150 mM NaCl was used to dilute the liposomes encapsulating SRB since it maintains the osmolarity of the medium and the integrity of the liposomes, preventing their burst and breakage.

Characterization of LUVs Prepared Without SRB

Size Analysis of Liposomes

The mean particle size of liposomes was determined by laser granulometry using a Partica LA-950V2 laser diffraction particle size distribution analyser (Horiba, Japan) that can detect sizes between 0.01 μ m and 3000 μ m. Two milliliter of each liposomal suspension obtained after extrusion was used for the size measurement.

Determination of Liposome Composition in the Final Liposomal Structure

Separation of Loaded and Unloaded Lipids by Centrifugation After a dilution of 5 times of the initial liposomal batches, aliquots of the LUVs of each formulation underwent an ultrafiltration on Vivaspin 500 tubes during 1 h. This step allows the separation of lipids (phospholipids and cholesterol molecules) incorporating the liposome structure from free lipid molecules present in the filtrate.

Determination of Lipids Incorporation Ratio and Loading Ratio in Liposomes **2.1.3.2.2.1. Phospholipids (DPPC)** According to the protocol previously described by (Habib et al. 2013), the total and free amounts of phospholipids (DPPC) were determined for each liposomal suspension using Bartlett method (Bartlett 1959). The total DPPC concentration was quantified for the various formulations after adequate dilutions of the initial liposome suspensions whereas the concentration of free DPPC was determined in the filtrates. The DPPC loading ratio (LR) was then calculated by the following equation:

$$LR = \frac{(m \text{ total DPPC} - m \text{ free DPPC})}{m \text{ theoretical weighed DPPC}} \times 100 \quad (1)$$

where: “*m* total DPPC” is the calculated mass of total DPPC found in the liposomal suspension, “*m* free DPPC” is the calculated mass of free DPPC that were not incorporated in the liposomal formulation and were present in the filtrates and “*m* theoretical DPPC” is the weighed mass of DPPC used for the preparation of liposomes.

The DPPC incorporation ratio (*IR*) was also calculated as follows:

$$IR = \frac{m \text{ total DPPC} - m \text{ free DPPC}}{m \text{ total DPPC}} \times 100 \quad (2)$$

where: “*m* total DPPC” is the calculated mass of total DPPC found in the liposomal suspension and “*m* free DPPC” is the calculated mass of free DPPC that were not incorporated in the liposomal formulation and were present in the filtrates.

Cholesterol Cholesterol dosage was performed after an enzymatic hydrolysis and an oxidation using a Chol kit (Cholesterol, spinreact, S.A.U., Spain). 1000 μ L of the standard kit were added to the 10 μ L of a solution containing Chol, and incubated for 10 min at room temperature. Then, the absorbance was read at a wavelength of 505 nm. Based on a calibrator concentration of 2 mg/mL, the concentration of Chol (mg/dL) was given by the following equation:

$$[\text{Chol}] = \frac{A \text{ sample} - A \text{ blank}}{A \text{ calibrator} - A \text{ blank}} \times 200 \quad (3)$$

To determine the total Chol concentration in each batch, 10 μ L of the liposomal suspension (after adequate dilutions) was sonicated for 20 min to ensure the degradation of the liposomes and the release of the total amount of cholesterol encapsulated in the membrane. As for free Chol, 10 μ L of the filtrates, obtained after centrifugation on vivaspin 500 tubes, was used for the dosage. The absorbance was measured using a spectrophotometer. Measurements were performed in triplicate.

The Chol loading ratio was calculated according to the following formula:

$$LR = \frac{(m \text{ total Chol} - m \text{ free Chol})}{m \text{ theoretical Chol}} \times 100 \quad (4)$$

where: “*m* total Chol” is the calculated mass of total Chol found in the liposomal suspension, “*m* free Chol” is the calculated mass of free Chol that were not incorporated in the liposomal formulation and were present in the filtrates and “*m* theoretical Chol” is the mass of Chol used for the preparation of liposomes.

The Chol incorporation ratio for each liposomal formulation was calculated as follows:

$$IR = \frac{m \text{ total Chol} - m \text{ free Chol}}{m \text{ total Chol}} \times 100 \quad (5)$$

where: “*m* total Chol” is the calculated mass of total Chol found in the liposomal suspension, “*m* free Chol” is the mass of free Chol that were not incorporated in the liposomal formulation and were present in the filtrates.

The results are expressed as the means of three independent experiments \pm SD.

Determination of the DPPC to Chol Molar Ratio for Each Formulation The DPPC and Chol incorporation ratios calculated for liposomal batches were used to determine the number of moles of DPPC and Chol incorporated into the liposomes of each formulation.

Then, the DPPC:Chol molar ratios were obtained after determining the percentage of Chol incorporated in the liposome formulation according to the following equation:

$$\text{Incorporated Cholesterol percentage} = \frac{n \text{ incorporated Chol}}{n \text{ incorporated DPPC}} \times 100 \quad (6)$$

With: “*n* incorporated Chol” and “*n* incorporated DPPC” corresponding to the number of moles of Chol and DPPC incorporated in the liposome formulation respectively.

Membrane Permeability Study

Purification of Liposomal Suspensions by Molecular Sieve Chromatography

The Sulforhodamine B (SRB)-loaded liposomes were subjected to a centrifugation for 2 h, at a speed of 15,000 rpm, at 4 °C. The pellets were suspended in 0.1 M Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. This preliminary step facilitates the purification of liposomes on the G25 column by eliminating the excess buffer and allows a non-delicate separation of non-encapsulated molecules (free SRB, phospholipids and cholesterol) that will remain in the supernatant.

Sulforhodamine B (SRB)-loaded liposomes were then separated from free SRB, phospholipids and cholesterol molecules by molecular sieve chromatography (using a Sephadex G25 column). The liposomal solution of each formulation was eluted with 0.1 M Tris-HCl buffer (pH 7.4) containing 150 mM NaCl.

Exposure of Liposomal Suspensions to EO Components

Liposomal suspensions of each preparation were diluted in a Tris HCl buffer containing 150 mM NaCl (0.1 M, pH 7.4) in order to obtain liposomal solutions with an optical density of 0.5 at 535 nm. The EOs components solutions prepared in methanol were then added to SRB-loaded liposomes at a molar ratio DPPC:EO of (100:25) and samples were incubated at 37 °C for 72 h. For each formulation, a liposomal solution treated with the same volume of methanol was used as a control and will be referred to as “blank liposomes”. The corresponding EO volume added to the liposomes did not exceed 5% of the

liposomal suspension volume. At this percentage, the methanol doesn't disrupt the liposomes stability (data not shown).

Membrane Permeability Study by Fluorescence Spectroscopy

Membrane permeability was monitored by means of fluorophore leakage from vesicles. In fact, the loading of the SRB (in the liposomes aqueous core) at a high concentration generates a fluorescence quenching phenomenon due to the formation of non-fluorescent aggregates (Nasr et al. 2020). Dye release from liposomes leads to its dilution and subsequently, the restitution of the fluorescence. Therefore, the effect of the different EOs components tested in our study on membrane permeability was followed by measuring the increase of the fluorescent signal resulting from the release of SRB from liposomes with time at 37 °C.

The SRB loaded liposomes suspensions of each formulation were exposed or not to EOs components and incubated in a water bath at 37 °C. Aliquots were removed from each sample at 0, 24, 48 and 72 h, diluted 50 times in 0.1 M Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and the fluorescence signal was measured immediately.

To eliminate an eventual interference of EO components in fluorescence measurements, EO components were dissolved in a Tris HCl NaCl (0.1 M, pH 7.4) buffer at the same concentration used for the liposomal permeability study.

Fluorescence measurements were performed using a spectrofluorometer (Hitachi F-7000 Spectrofluorometer) at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The emission spectrum was recorded in the range 540–700 nm. The permeability of liposomal membranes was evaluated by calculating the percentage of the fluorophore released from LUVs versus time, using the following equation:

$$\text{SRB release percentage} = \frac{(F_t - F_0)}{F_{\text{max}}} \times 100 \quad (7)$$

where F_t is the fluorescence intensity measured at time t , F_0 is the fluorescence intensity measured at time 0 and F_{max} is the maximum fluorescence indicating a complete release of SRB from vesicles and obtained in the presence of the nonionic detergent, Triton X-100 (1%) in a Tris HCl buffer (0.1 M; pH 7.4) containing 150 mM NaCl.

The effect of an EO component on the permeability of liposomal membrane of each formulation was determined as followed:

$$\text{Permeabilizing effect of an EO} = \frac{\text{SRB release \% from liposomes EOs}}{\text{SRB release \% of blank liposomes}} \quad (8)$$

where the "SRB release % from liposomes EOs" corresponds to the SRB release percentage of liposomes samples treated with EO components and the "SRB release % of blank liposomes" is the SRB release percentage of the control.

Statistical Analysis

To assess significant differences between values, statistical analysis was carried out using the Student's t test. A value of $P < 0.05$ was considered statistically significant. The P values compare the permeabilizing effect of EO component to the membrane permeability in the absence of these molecules (blank liposomes).

Results and Discussion

Characterization of LUVs Prepared Without SRB

Size Analysis

All the liposomal batches underwent an extrusion through polycarbonate membranes of 400 nm pore size, expecting thereafter a theoretical vesicle mean diameter equal to this size. Our results were in accordance with previously published data carried out in our laboratory where the mean size of DPPC/Chol formulations gradually increased following the addition of Chol to the membrane up to 30% Chol (Kaddah et al. 2018). No further increase in the vesicles size was observed at high Chol content (50% and 100% Chol membranes) since liposomal membranes had a mean diameter close to 400 nm regardless of increasing Chol content (data not shown).

Phospholipids and Cholesterol Quantification of LUVs

Total and free DPPC and Chol were determined for each formulation as described above. The incorporation ratios and the loading ratios were calculated according to Eqs. (1), (2), (3) and (4). The DPPC:Chol molar ratio determined experimentally in the final liposomal membranes are presented in Table 1.

The DPPC loading ratios were found to be around 75% for all liposomal batches except for 10% Chol membrane where a greater loading ratio value (85.86%) was obtained. These values show that the Chol content influences the incorporation of DPPC into the membrane: in fact, Chol reduces DPPC incorporation up to 25% Chol content (the LR of DPPC decreased from 85.86% for 10% Chol membranes to 77.22% for 25% Chol membranes). Above

Table 1 DPPC and Chol loading ratios and incorporation ratios for each liposomal formulation (DPPC:Chol molar ratios: 100:10; 100:25; 100:50; 100:75 and 100:100)

Theoretical DPPC:Chol molar ratio	DPPC		Chol		Experimentally determined DPPC:Chol ratio
	LR (%)	IR (%)	LR (%)	IR (%)	
100:10	85.86 ± 0.008	98.9 ± 0.007	100.00 ± 0.05	100.00 ± 0.41	100:11
100:25	77.22 ± 0.005	98.53 ± 0.06	65.98 ± 1.47	83.41 ± 0.89	100:21
100:50	76.2 ± 0.001	97.83 ± 0.004	60.43 ± 0.62	92.35 ± 0.92	100:40
100:75	75.36 ± 0.009	98.76 ± 0.004	43.58 ± 1.15	98.30 ± 1.25	100:43
100:100	77.6 ± 0.015	95.67 ± 0.002	42.55 ± 1.72	97.54 ± 0.20	100:55

The corresponding DPPC and Chol mole number as well as the experimentally determined DPPC:Chol molar ratio are also presented for each formulation. Values are expressed as the means of three measurements ± SD

this concentration, no influence of increasing Chol membrane content on the incorporation of DPPC is observed (the LR of DPPC ranged from 75 to 77% for all formulations). On the other hand, the DPPC and Chol incorporation ratios of all liposomal batches were high proving consequently the efficacy of the reverse evaporation phase method chosen for the preparation of liposomes. We noticed also that, at 10% Chol, all the Chol molecules were incorporated in the membrane (LR value was of 100%). With increasing Chol content, the loading ratios of Chol gradually decreased reaching 42.5% for the formulation of DPPC:Chol (100:100). Indeed, at low Chol content (up to 25%), the theoretical DPPC:Chol molar ratio is practically respected since the experimentally determined molar ratios were nearly the same as predicted. Conversely, at high Chol content (50% Chol and more), the experimentally determined DPPC:Chol molar ratios did not reflect anymore the theoretical ratios: at 50% Chol, the liposome membranes were found to contain 40% Chol. Similarly, DPPC membranes theoretically incorporating 75% and 100% Chol were actually composed of 43% and 55% Chol respectively. Our results are in agreement with (Ibarguren et al. 2010), who quantified the Chol content of liposome membranes made of different lipid binary mixtures before and after extrusion. In most cases, the extrusion resulted in liposomes with Chol contents lower than the theoretical ratios: particularly, the extruded DPPC/Chol membranes with Chol molar ratios ranging from 33 to 75% have shown Chol incorporation ratios below the predicted values. This effect was pronounced with increasing Chol content. A possible explanation for this is that the Chol molecules are kicked out of the membrane and remain stuck on the filters during the extrusion process. One can also note here that the effective composition of liposome formulations containing 50% and 75% Chol is nearly the same (40% and 43% Chol respectively) expecting no significant difference between these two formulations. Notwithstanding, the permeability study results revealed different behaviors of EO compounds on these membranes. Consequently,

this difference appears to be significant and could not be neglected.

Permeability Study: SRB Release from Liposomes

The liposomal membrane permeability was assessed by the commonly used fluorescence technique: vesicle leakage of self-quenching probes (Nasr et al. 2020). The LUVs encapsulating the fluorescent agent SRB were exposed to EOs components at a molar ratio DPPC:EO of 100:25. The choice of this ratio was based on a biophysical study conducted by (Gharib et al. 2018) where the effect of a series of EO monoterpenes compounds on the fluidity of DPPC membrane was investigated at various molar percentages of EO components to DPPC (from 0 to 25 mol%) and in the absence of Chol. The studied molecules exerted a fluidizing effect on DPPC membranes, this effect being enhanced in a dose dependent manner. Considering these results, the effect of Chol on the membrane permeability (Kaddah et al. 2018) and our aim to find adequate formulations for EOs encapsulation, we selected the highest ratio (25 mol%) expecting a high amount of the EO molecules retained in the liposome structures.

The permeability of LUVs was evaluated by measuring the fluorescence signal of liposomal suspensions at 37 °C between 0 and 72 h. It should be noted that the SRB release from liposomes composed of DPPC and various Chol content was time dependent and showed a biphasic release pattern (Kaddah et al. 2018). It includes an initial fast SRB release during the first 8–10 h, followed by a landing period (between 10 and 24 h) where no considerable dye leakage is observed. With respect to these results, we have chosen to carry out the permeability study in the presence of EOs components during 72 h to cover both phases of the SRB release kinetics.

Fluorescence of EOs Components

None of the studied EO molecules emit a fluorescence signal in the range of 500–700 nm and, therefore, the fluorescence

increase observed in the following experiments is due only to the leakage of SRB and thus the inhibition of fluorescence quenching rather than the molecules structure (Fig. 1, supplementary material).

SRB Release from Liposomal Formulations of Various Chol Content

According to (Kaddah et al. 2018), liposome membranes made of DPPC:Chol (100:10) was the less stable and the most permeable formulation. Its SRB release pattern was comparable to free Chol membranes. Therefore, this formulation was chosen to test the effect of a series of 22 EO components on membrane permeability. Table 2 represents the permeabilizing effect of EO components on DPPC:Chol

Table 2 The permeabilizing effect of EO components obtained with the DPPC:Chol (100:10) liposomes after 72 h of incubation at 37 °C

Sample (EO treated liposome)	Permeabilizing effect of EO component at 72 h
Blank liposomes	1.00
Phenolic compounds	
Eugenol	1.37 ± 0.02*
Thymol	1.30 ± 0.11*
Guaiacol	1.27 ± 0.06*
trans-Anethole	1.19 ± 0.02*
Eugenyl acetate	1.15 ± 0.01*
Anisole	1.04 ± 0.12
Estragole	0.99 ± 0.01
Isoeugenol	0.93 ± 0.02*
Non-phenolic compounds	
Nerolidol	1.43 ± 0.05*
Linalool	1.37 ± 0.04*
Geraniol	1.26 ± 0.08*
α-Phellandrene	1.23 ± 0.06*
α-Terpinene	1.19 ± 0.02*
Linalyl acetate	1.18 ± 0.05*
Menthol	1.17 ± 0.01*
γ-Terpinene	1.17 ± 0.06*
Bornyl acetate	1.05 ± 0.08
Camphor	1.03 ± 0.02
β-Caryophyllene	1.02 ± 0.04
Camphene	0.98 ± 0.05
Borneol	0.91 ± 0.01*
p-Cymene	0.86 ± 0.07*

Fluorescence measurements were carried out on a spectrofluorometer at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. Values are expressed as the means of three different measurements ± SD

* indicates a value of $P < 0.05$ statistically significant (the P values compare the permeabilizing effect of EO component to the membrane permeability in the absence of these molecules (blank liposomes))

(100:10) liposomes obtained after 72 h of incubation and calculated according to Eq. (7).

Based on the statistical significance t-test, among the 22 EO components tested, only 13 compounds increased the permeability of DPPC:Chol (100:10) liposomes after 72 h of incubation since their permeabilizing effects were significantly higher than 1 (release value of blank liposomes). In a descending order of permeabilizing effect, the 13 selected molecules are classified as follows: nerolidol > eugenol > linalool > thymol > guaiacol > geraniol > α-phellandrene > trans-anethole > α-terpinene > linalyl acetate > menthol > γ-terpinene > eugenyl acetate. At this DPPC/EO molar ratio, the remaining components (anisole, estragole, isoeugenol, bornyl acetate, camphor, β-caryophyllene, camphene, borneol and p-cymene) had no significant effect on the liposomal permeability suggesting that another mechanism of action may be accountable for their antimicrobial activities. Interestingly, we can notice that three EO components (isoeugenol: 0.93 ± 0.02 , borneol: 0.91 ± 0.005 and p-cymene: 0.91 ± 0.005) seem to exert a stabilizing effect on the liposome formulation since their SRB release values were significantly below that of the blank.

Our results showed that, among the 13 EO components previously mentioned, the majority were terpenic compounds bearing an alcohol function (phenolic compounds: eugenol, thymol and guaiacol and non-phenolic compounds: nerolidol, linalool, geraniol, menthol). In fact, numerous studies highlighted the importance of the hydroxyl group for the biological activity of several phenolic compounds exhibiting a permeabilizing effect (Ultee et al. 2002; Burt 2004). Among the most studied phenylpropanoids, eugenol was proved to permeabilize the bacterial cell membrane (Hyldgaard et al. 2012; Devi et al. 2013). Thymol, another OH-bearing compound, was demonstrated to interact with the cytoplasmic cell membrane and integrate into the membrane via its hydroxyl group at the polar head groups of the lipid bilayer leading to the loss of membrane integrity and to the increase in its passive permeability (Helander et al. 1998; Lambert et al. 2001; Walsh et al. 2003). Accordingly, in our study, eugenol and thymol showed a permeabilizing effect of 1.37 ± 0.02 and 1.30 ± 0.11 respectively. Moreover, DSC experiments conducted earlier in our laboratory on DPPC liposomes demonstrated that thymol and eugenol act as “substitutional impurities” replacing the lipid molecules in the bilayer (Gharib et al. 2017, 2018). Thymol also produced a phase separation indicating the presence of EO-rich and EO-poor regions inside the membrane (Cristani et al. 2007; Gharib et al. 2018). In addition, guaiacol which has common structural features with eugenol (both possess a hydroxyl and a methoxy groups) but lacks the prop-2-enyl side chain, was also able to permeabilize liposome membranes containing 10% Chol (1.27 ± 0.06). This suggests that the

prop-2-enyl chain may help the molecule to better permeabilize the membrane. On the other hand, the substitution of the hydroxyl group (case of eugenol) with an acetate group in eugenyl acetate reduced the effect of this latter on the membrane. Similarly, trans-anethole which has no hydroxyl group has shown a weak permeabilizing effect comparable to that of eugenyl acetate (1.19 ± 0.02 and 1.15 ± 0.01 for trans-anethole and eugenyl acetate respectively). This holds true when comparing the effect of guaiacol and anisole. Lacking the alcohol function, anisole did not show any effect on the membrane. This could be also the reason for which estragole does not affect the vesicle permeability. These instances further underline the importance of OH and its direct implication in the EO-membrane permeability.

Besides the importance of this OH group, our results revealed the presence of other structural features that could influence the membrane permeability. Isoeugenol, the isomer of eugenol, showed no permeabilizing effect on liposomes made from the binary mixture of DPPC:Chol (100:10); it rather displayed a stabilizing effect on the membrane (0.93 ± 0.02). Indeed, the double bond in the α , β positions and the methyl group in the γ position of isoeugenol's side chain create a conjugated system which is absent in the eugenol's structure. This structural feature appears to upset the permeabilizing effect of isoeugenol and to convert the eugenol induced permeability (1.37 ± 0.02) into a stabilizing effect with isoeugenol (0.93 ± 0.02). Based on their DSC studies, Gharib and coworkers (2017) reported that eugenol and isoeugenol both induce a membrane fluidifying effect and are inserted inside the membrane; yet, isoeugenol executes a deeper insertion compared to the parent compound, eugenol (Gharib et al. 2017). Therefore, we can suggest that the effect of EO components on lipid membrane permeability does not only account for the presence of a hydroxyl group. However, isoeugenol is known for its antibacterial activity against various bacterial strains (Zhang et al. 2017) and was also shown to induce the leakage of calcein-loaded LUVs made from *E. Coli* lipid extract (Hyldgaard et al. 2015). These discrepancies might be due to the presence of cholesterol in the present formulations comparing to other studies where cholesterol was absent.

Regarding the non-phenolic compounds, the importance of the hydroxyl group was also emphasized: a permeabilizing effect was obtained for OH-bearing molecules, with nerolidol (a linear sesquiterpene) exhibiting the highest effect (1.43 ± 0.05). Interestingly, the linear terpenes (nerolidol, linalool and geraniol) showed a better effect on 10% Chol membranes compared to the cyclic terpene, menthol, whose effect was similar to that of hydrocarbon monoterpenes (nerolidol: 1.43 ± 0.05 , linalool: 1.37 ± 0.04 and geraniol: 1.26 ± 0.08 versus menthol: 1.17 ± 0.01 , α -phellandrene: 1.23 ± 0.06 , α -terpinene: 1.19 ± 0.02 , γ -terpinene: 1.17 ± 0.06). Here also, the replacement of the

hydroxyl group of linalool with an acetate group reduced the effect of linalyl acetate (1.37 ± 0.04 and 1.18 ± 0.05 for linalool and linalyl acetate respectively). Although bearing an alcohol function, borneol (0.91 ± 0.005) was found to reduce the permeability of liposome membrane. We assume that the bulk structure of this bicyclic monoterpene could modulate its interaction with liposome membrane, and consequently its permeabilization effect. As regards the molecules lacking OH, the presence of a conjugated system in *p*-cymene reversed the permeabilizing effect of α -phellandrene and promoted a more stable membrane (0.86 ± 0.07 and 1.23 ± 0.06 for *p*-cymene and α -phellandrene respectively). Besides the presence of a conjugated system, the hydrophobicity of *p*-cymene ($\log P$ 4.02) can further explain its stabilizing effect for this molecule would be much retained inside the membrane. It is interesting to mention here that *p*-cymene was also classified as "substitutional impurity" of the membrane producing an enhancement of membrane fluidity (Cristani et al. 2007).

Overall, it could be concluded that the presence of a hydroxyl group, the presence of a conjugated system and the linearity of the molecule are important structural features modulating membrane permeability.

Based on the above preliminary results, liposomes of different Chol content were treated with only the EO compounds that showed a permeabilizing effect at 10% Chol (13 compounds). Figures 2 and 3 represent the permeabilizing effect of phenolic and non-phenolic EO components with all liposomal formulations (DPPC:Chol 100:10; 100:21; 100:40; 100:43 and 100:55 molar ratios) obtained after 72 h of incubation at 37 °C.

It is quite obvious how Chol drastically affected the membrane permeability induced by the tested molecules. As a matter of fact, upon increasing the Chol content in liposomes, the permeabilizing effect of these compounds started to fade and, in some cases, to disappear. Effectively, results show that, among the 13 EO components exerting a permeabilizing effect on liposomes containing 10% Chol, only 6 molecules bearing an OH group (eugenol, guaiacol, thymol, geraniol, linalool, menthol) along with the phenylpropene trans-anethole and two hydrocarbons monoterpenes (α and γ -terpinene) kept a permeabilizing effect on liposomes composed of 21% Chol. The effect of eugenol, guaiacol, thymol, linalool and menthol remained up to 40% Chol. As for liposomes composed of 43% and 55% Chol, none of the tested molecules showed a significant permeabilizing effect on the lipid bilayer. Although the slight difference in the experimentally determined Chol content between the 40% and 43% Chol membranes (50 and 75% Chol), the behavior of EO components towards these two formulations was not the same: our findings indicate that the permeabilizing effect of EO components can be observed up to 40% Chol membranes. Above this percentage, their effect

Fig. 2 The permeabilizing effect of phenolic EO components with the different liposomal formulations (DPPC:Chol 100:10; 100:21; 100:40; 100:43; 100:55) at 72 h (* indicates a value of $P < 0.05$ statistically significant. The P values compare the permeabilizing effect of EO component to the membrane permeability in the absence of these molecules (blank liposomes))

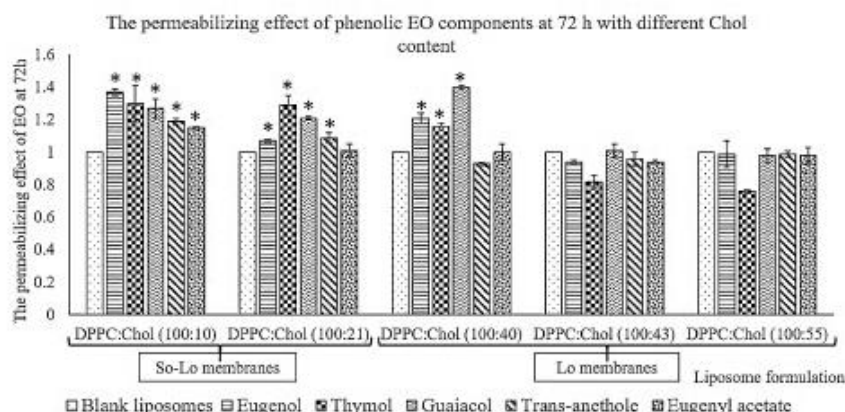
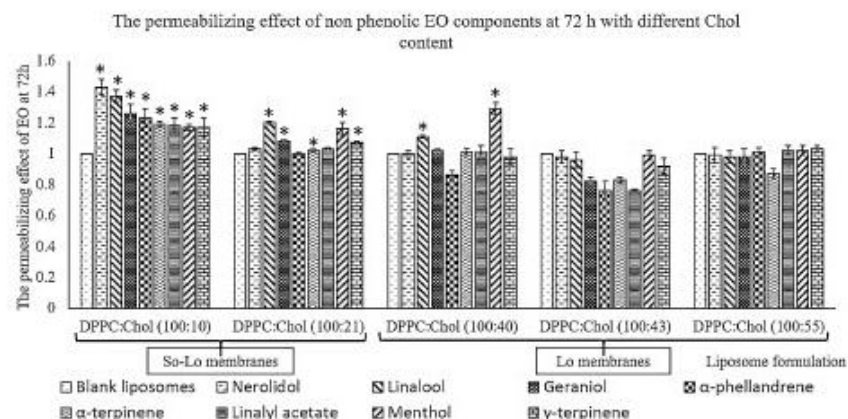


Fig. 3 The permeabilizing effect of non-phenolic EO components with the different liposomal formulations (DPPC:Chol 100:10; 100:21; 100:40; 100:43; 100:55) at 72 h (* indicates a value of $P < 0.05$ statistically significant. The P values compare the permeabilizing effect of EO component to the membrane permeability in the absence of these molecules (blank liposomes))



is inhibited. For instance, guaiacol and eugenol showed a permeabilizing effect of 1.40 ± 0.01 and 1.21 ± 0.03 on 40% Chol membranes respectively. Their effects were significantly reduced at 43% Chol membranes to reach 1.01 ± 0.04 and 0.94 ± 0.01 respectively. The same could be observed with linalool, menthol, thymol, etc.

The hydrophobicity of the compound is a key parameter that could affect the interaction of EO constituents with the membrane. Our results showed that when the EO component is highly hydrophobic ($\log P > 4$), two scenarios could take place depending on the structure of the molecule: in the presence of a hydroxyl group, a permeabilizing effect occurs only at low Chol content. This is the case of nerolidol, a hydrophobic compound ($\log P$ 4.6), which only permeabilized DPPC/Chol membranes made of 10% Chol. Conversely, when the hydrophobic molecule lacks a hydroxyl group, no permeabilizing effect is seen. For example, *p*-cymene ($\log P$ 4.02) and β -caryophyllene ($\log P$ 4.4), highly hydrophobic compounds with no alcohol function, did not enhance the permeability of any formulation.

Furthermore, it was previously stated that at high Chol content, Chol reduces the drug-membrane interaction because a competition might occur between EO compounds and Chol in the membrane (Sherry et al. 2013). For this reason, the permeabilizing effect of nerolidol might be inhibited at higher Chol content.

The decrease in the hydrophobicity of EO components possessing a OH group will increase the extent of membrane permeabilization at high Chol content: actually, the EO components bearing a hydroxyl group with $\log P$ below 4, had a permeabilizing effect visible up to 40% Chol. However, geraniol ($\log P$ 2.9) with similar hydrophobicity to linalool ($\log P$ 2.7) exerted a weaker permeabilizing effect only up to 21% Chol membranes (linalool: 1.37 ± 0.04 ; 1.20 ± 0.01 ; 1.11 ± 0.01 versus geraniol: 1.26 ± 0.06 ; 1.08 ± 0.01 ; 1.02 ± 0.01 at 10%, 21% and 40% Chol respectively). This could be probably explained by the differential position of the hydroxyl group in each compound: the OH group of geraniol is at the end side of the molecule which make the

structure less disturbing when it reaches the membrane comparing to geraniol.

Regarding EO components having no hydroxyl group and with a $\log P$ lower than 4, a very weak permeabilizing effect persisted for α -terpinene ($\log P$ 2.8), γ -terpinene ($\log P$ 2.8) and trans-anethole ($\log P$ 3.3) up to 21%. The remaining molecules in this category had no significant effect regardless the Chol content. All these results are summarized in Table 2, supplementary material.

On the other hand, lipid bilayers could exist in different states depending on many parameters; particularly, Chol concentrations and temperature (Vist and Davis 1990; McMullen and McElhaney 1995). In our study, membrane permeability was assessed by following SRB release from liposomes at 37 °C which corresponds to a temperature below the melting temperature of DPPC (41 °C). Therefore, membrane organization of the liposomes is controlled by Chol content. Based on the work of Sankaram and Thompson (1991), the liposomal formulations exist in two different membrane states. For DPPC membranes with Chol concentration ranging from 7 to 30 mol% and below T_m , the lipid bilayer is in the solid ordered—liquid ordered phase (So-Lo) in which both gel and liquid-ordered phases coexist. As for membranes with Chol concentrations above 30 mol%, lipid bilayers adopt the liquid-ordered phase (Lo) (Sankaram and Thompson 1991). Our results showed that in the (So-Lo) transition phase (membranes composed of 10% and 21% Chol effective ratios), the permeabilizing effect of EOs was pronounced at 10% and then decreased or disappeared by increasing cholesterol content (Table 3, supplementary material). In the Lo phase (for membranes effectively composed of 40%, 43% and 55% Chol), five EO components possessing a hydroxyl group kept their permeabilizing effect (menthol, eugenol, guaiacol, thymol and linalool) on 40% Chol membranes only. This underscores again the importance of OH group in the EO induced permeability and highlights as well the stability conferred by Chol to the membrane. In fact, the addition of increasing Chol concentrations implies the transition from a leaky to a more stable membrane leading to a decrease in the membrane permeability of liposomes as demonstrated by (Kaddah et al. 2018). Therefore, the introduction of Chol into the lipid bilayer results in a membrane that resists the disturbance and destabilization caused by bioactive agents as we obtained with the thymol, where the increase in Chol content results in a decrease of SRB release ratios from liposomes. (Table 3, supplementary material or Fig. 4a). Unexpectedly, for eugenol, guaiacol and menthol there was a re-increase in the permeabilizing effect in the Lo phase at 40% Chol content followed by a dramatical decrease at 43% where no permeabilizing effect was observed (Table 3, supplementary material or Fig. 4b). A plausible explanation regarding this observation could be related to the heterogeneity of the membrane at high Chol

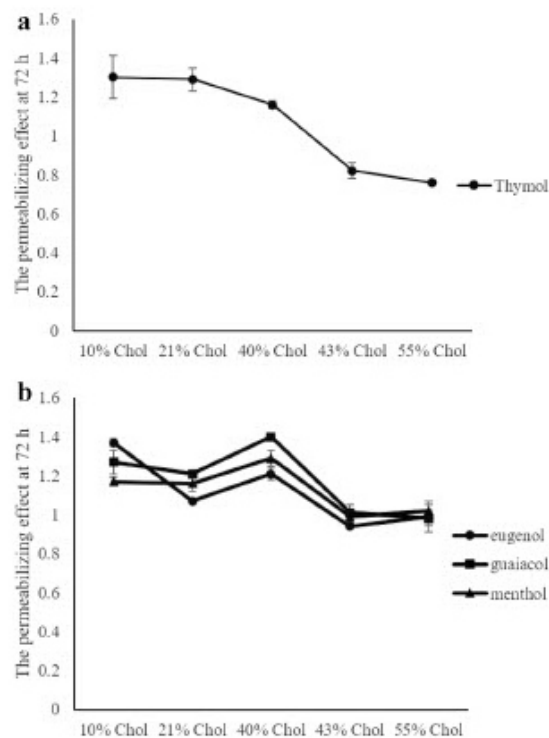


Fig. 4 The permeabilizing effect of EO components obtained at 72 h with various Chol content. Two patterns were obtained: a decreasing effect with increasing Chol content depicted in (a) for thymol, a re-increase in EO permeabilizing effect at 40% Chol shown in (b) for three components: eugenol, guaiacol and menthol

content. Effectively, the incorporation of large amount of Chol will give rise to lipid rafts, rich-Chol nanodomains, alongside with poor-Chol domains generating thereby an heterogeneous membrane (Simons and Sampaio 2011; Topozini et al. 2014; Javanainen et al. 2017). Hence, this membrane contains at the same time domains with high physical stability (rich-Chol domains) and domains with low physical stability (poor-Chol domains). The EO components will probably tend to permeabilize these unstable domains. This would account for the re-increase in the permeabilizing effect of some compounds at 40% or 43% Chol.

Taking in consideration all the discussed parameters, we can assume that the EOs effect on membrane permeability in the presence of Chol can be modulated by the structure of the molecule (presence of hydroxy group, position of the double bond, linearity of the molecule, length of the hydrocarbon chain), its hydrophobicity and the state of the membrane. Therefore, all these parameters can help us choose the best liposome formulation for the EOs encapsulation. As discussed above, the hydroxyl group in the EOs seems to be linked to the membrane permeability; however,

the hydrophobicity of the components should be taken into account when choosing the more stable encapsulation formulation. For instance, nerolidol, bearing an OH group and possessing a log P higher than 4, permeabilized the membrane up to 10% Chol. Nevertheless, due to its high hydrophobicity, a competition with the Chol may occur at high Chol content resulting in a low encapsulation efficiency as obtained for many hydrophobic drugs with liposomal formulations rich in Chol (Jaafar-Maalej et al. 2010; Varona et al. 2011; Sherry et al. 2013). Thus, a formulation moderately rich in Chol (not exceeding 40%) has to be chosen for the encapsulation of the compound. On the other side, linalool, bearing an OH group and possessing a low hydrophobicity permeabilized the membrane up to 40% Chol. Therefore, a formulation with a Chol content above 40% is recommended for a stable encapsulation. Consequently, a liposomal formulation loaded with either eugenol, thymol, guaiacol or menthol should be also composed of a Chol concentration higher than 40%. Considering the remaining non-phenolic compounds with no hydroxyl group and possessing a permeabilizing effect on the permeability of liposome membranes, we should note that these components were active at low Chol contents (10 and 21% Chol). These components must be encapsulated in a formulation moderately rich in Chol for most of these compounds are hydrophobic.

Though, we should keep in mind that the Chol content is not the only parameter that has to be controlled in the liposome formulation designed for EOs encapsulation. The lipid to EO component (either Chol:EO or phospholipid:EO) ratio is also of paramount importance since Chol and phospholipids can both influence the encapsulation of EO molecules in liposomes. As stated earlier for Chol at high content, it can reduce the encapsulation of hydrophobic EO components. Furthermore, Chol free formulations were previously evaluated for their efficiency in encapsulating EOs: it was shown that the choice of the phospholipid to EOs ratio should be taken into account since a large amount of lipophilic components would disturb the lipid bilayer, leading to membrane destabilization (Detoni et al. 2009; Yoshida et al. 2010; Khosravi-Darani et al. 2016; Dag and Oztop 2017).

Conclusions

In this work, we investigated the effect of a series of 22 EO components belonging to different classes (phenylpropenes, oxygenated monoterpenes and sesquiterpenes) on the permeability of DPPC liposome membranes with various Chol content (10; 21; 40; 43 and 55% Chol). Our results helped us to establish a correlation between the structure of a compound, its hydrophobicity, the membrane state and its effect on the membrane permeability.

EO constituents bearing a hydroxyl group have demonstrated the best permeabilizing effect; this effect being reduced with increasing Chol content in the So-Lo phase. Upon increasing Chol content, in the liquid-ordered phase, a re-increase in the permeabilizing effect of some EO components could be attributed to the heterogeneity of the membrane. The permeabilizing effect of EO components was visible up to 40% Chol only; none of the studied molecules displayed a significant effect at higher Chol content. Hydrophobicity of the compound played also a crucial role. Hydrophobic compounds having no hydroxyl group in their structure showed a low permeabilizing effect only in the So-Lo phase. No significant effect on the membrane was observed in the liquid-ordered phase. As for hydrophobic compounds possessing a hydroxyl group, when the hydrophobicity of EO component increases the Chol content up to which a permeabilizing effect is observed will decrease. Particularly in this category, the highly hydrophobic compound nerolidol displayed a significant permeabilizing effect at 10% Chol. This effect disappeared at higher content.

Accordingly, Cholesterol modulates not only the membrane permeability but also the interactions that may occur between the hydrophobic compounds and the membrane lipids. Therefore, increasing the Chol to phospholipid ratio to some extent wouldn't be useful for the encapsulation of highly hydrophobic compounds since Chol will reduce their encapsulation efficiency at high Chol concentrations. That's why, the DPPC:Chol ratio of the formulation is selected depending on the structure of the compound, its hydrophobicity and its consequent effect on the permeability at different membrane states. Based on the above considerations, we can conclude that a liposome formulation with Chol not exceeding 40% would be suitable for the encapsulation of highly hydrophobic molecules whereas a formulation comprising higher Chol content (above 40%) could be suggested for less hydrophobic compounds. Finally, the Chol to EO component ratio is also a crucial parameter that has to be carefully chosen to circumvent the problems associated with low encapsulation of EOs in liposomes. Further investigations should be undertaken in order to determine the appropriate molar ratio of the ternary mixture: DPPC, Chol and EO component resulting in a stable formulation for EO encapsulation.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00232-021-00180-3>.

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Supplementary Materials

Table 1 The hydrophobicity (log P), the aqueous solubility and the volatility (Henry constant) of the 22 EO components tested in our study

EO component	Log P	Aqueous solubility (mg/L)	Henry constant (mol /m ³ Pa)	Reference
Phenolic compounds				
Anisole	2.1	1600	2.3 x 10 ⁻³	HSDB 2015
trans-Anethole	3.2	408	0.14	HSDB 2015
Estragole	3.4	178	4.62 x 10 ⁻⁴	US EPA 2012
Eugenol	2	2460	5.1	HSDB 2015
Eugenyl acetate	2.1	ND	ND	
Guaiacol	1.3	18.7	5.2	(Hilal et al. 2008)
Isoeugenol	2.6	810	2.7	HSDB 2015
Thymol	3.3	900	2.8	(Hilal et al. 2008)
Non-phenolic compounds				
Borneol	2.7	738	2.2	(Sander 2015)
Bornyl acetate	3.3	23.2	9.52 x 10 ⁻⁵	US EPA 2012
Camphene	3.3	4.2	1 x 10 ⁻⁴	HSDB 2015
Camphor	2.2	1600	0.21	HSDB 2015
β-Caryophyllene	4.4	Less than 1	ND	
p-Cymene	4.02	23.4	9 x 10 ⁻⁴	HSDB 2015
Geraniol	2.9	100	0.17	HSDB 2015
Linalool	2.7	1590	0.69	(Hilal et al. 2008)
Linalyl acetate	3.3	8.2	0.58	HSDB 2015
Menthol	3	456	0.66	HSDB 2015
Nerolidol	4.6	36	ND	
α-Phellandrene	3.2	133	1.8 × 10 ⁻⁴	(Copolovici and Niinemets 2005)
α-Terpinene	2.8	5.9	2.8 × 10 ⁻⁴	(Copolovici and Niinemets 2005)
γ-Terpinene	2.8	8.6	2.8 × 10 ⁻⁴	(Copolovici and Niinemets 2005)

ND: not determined

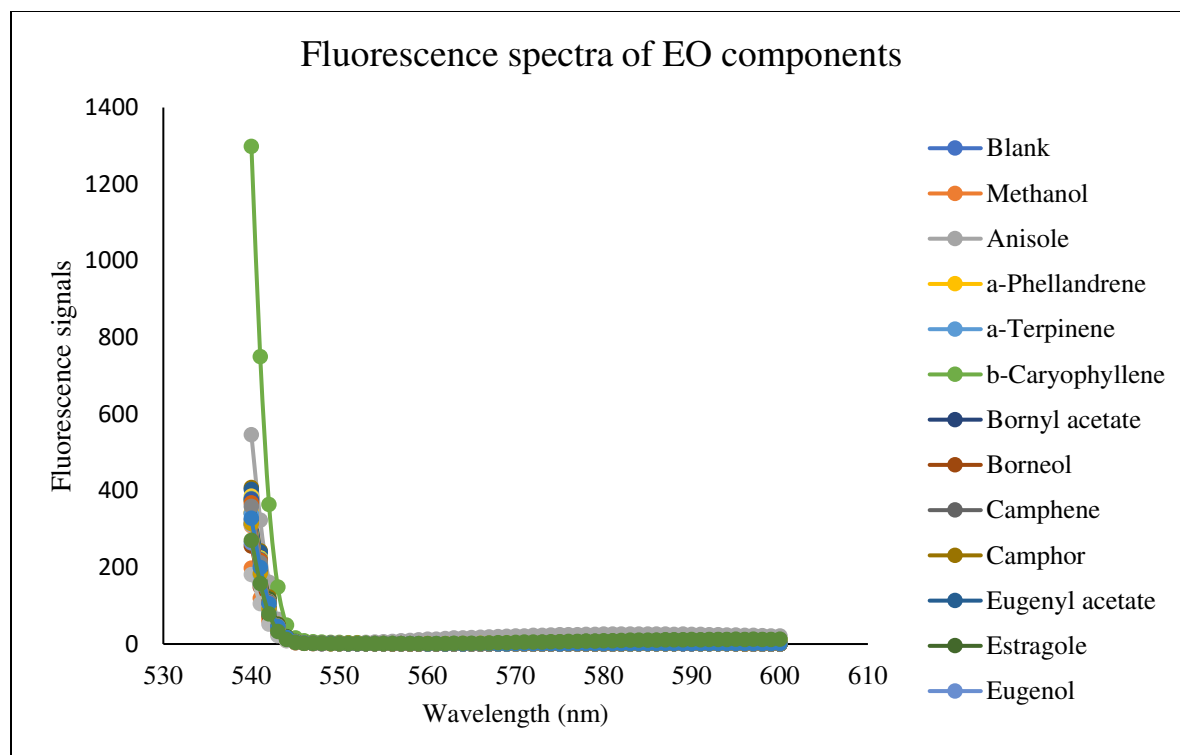


Fig. S1 The fluorescence spectra of the tested EO components in the range 540 – 600 nm.

Table 2 The hydrophobicity of EOs components and their corresponding Chol content membrane up to which a significant permeabilizing effect is observed (* indicates a value of $P < 0.05$ statistically significant)

Hydrophobicity	EO component	Log P	Chol content up to which a permeabilizing effect is observed	Permeabilizing effect of EO at the highest Chol content
Compounds bearing a hydroxyl group				
pH below 4	Guaiacol	1.3	40%	$1.40 \pm 0.01^*$
	Eugenol	2	40%	$1.21 \pm 0.03^*$
	Isoeugenol	2.6	----	-----
	Linalool	2.7	40%	$1.11 \pm 0.01^*$
	Borneol	2.7	----	----
	Geraniol	2.9	21%	$1.08 \pm 0.01^*$
	Menthol	3	40%	$1.25 \pm 0.01^*$
	Thymol	3.3	40%	$1.16 \pm 0.02^*$
pH above 4	Nerolidol	4.6	10%	$1.43 \pm 0.05^*$
Compounds with no hydroxyl group				
pH below 4	Eugenyl acetate	2.1	10%	$1.15 \pm 0.01^*$
	Anisole	2.1	----	-----

	Camphor	2.2	----	-----
	α -Terpinene	2.8	21%	1.09 \pm 0.03*
	γ -Terpinene	2.8	21%	1.07 \pm 0.01*
	α -Phellandrene	3.2	10%	1.23 \pm 0.06*
	Trans-anethole	3.3	21%	1.09 \pm 0.03*
	Bornyl acetate	3.3	----	-----
	Camphene	3.3	----	-----
	Linalyl acetate	3.3	10%	1.18 \pm 0.05*
	Estragole	3.4	----	-----
pH above 4	p-cymene	4.02	----	-----
	β -Caryophyllene	4.4	----	-----

Table 3 The permeabilizing effect of EO components obtained at 72h for the different formulations. (* indicates a value of P < 0.05 statistically significant)

Membrane phase	S ₀ – L ₀ transition phase		L ₀ phase		
	10% Chol	21% Chol	40% Chol	43% Chol	55% Chol
Permeabilizing effect of EO components at 72 h					
Nerolidol	1.43 \pm 0.05*	1.03 \pm 0.01	1.00 \pm 0.02	0.98 \pm 0.04	0.99 \pm 0.05
Eugenol	1.37 \pm 0.02*	1.07 \pm 0.01*	1.21 \pm 0.03*	0.94 \pm 0.01	0.99 \pm 0.08
Linalool	1.37 \pm 0.04*	1.20 \pm 0.01*	1.11 \pm 0.01*	0.96 \pm 0.05	0.98 \pm 0.04
Thymol	1.30 \pm 0.11*	1.29 \pm 0.06*	1.16 \pm 0.02*	0.82 \pm 0.04	0.76 \pm 0.01
Guaiacol	1.27 \pm 0.06*	1.21 \pm 0.01*	1.40 \pm 0.01*	1.01 \pm 0.04	0.98 \pm 0.04
Geraniol	1.26 \pm 0.06*	1.08 \pm 0.01*	1.02 \pm 0.01	0.82 \pm 0.03	0.98 \pm 0.05
α -phellandrene	1.23 \pm 0.06*	1.00 \pm 0.01	0.86 \pm 0.03	0.76 \pm 0.06	1.01 \pm 0.03
Trans-anethole	1.19 \pm 0.02*	1.09 \pm 0.03*	0.93 \pm 0.01	0.96 \pm 0.04	0.99 \pm 0.02
α -terpinene	1.19 \pm 0.02*	1.02 \pm 0.01	1.01 \pm 0.02	0.83 \pm 0.02	0.87 \pm 0.03
Linalyl acetate	1.18 \pm 0.05*	1.03 \pm 0.01	1.01 \pm 0.04	0.76 \pm 0.01	1.02 \pm 0.03
Menthol	1.17 \pm 0.02*	1.16 \pm 0.04*	1.29 \pm 0.04*	0.99 \pm 0.03	1.02 \pm 0.03
γ -terpinene	1.17 \pm 0.06*	1.07 \pm 0.01*	0.98 \pm 0.05	0.92 \pm 0.05	1.03 \pm 0.02
Eugenyl acetate	1.15 \pm 0.01*	1.01 \pm 0.04	1.00 \pm 0.05	0.94 \pm 0.01	0.98 \pm 0.05

Reference

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Chapitre 4 : Les cyclodextrines perméabilisent des membranes liposomales formés de DPPC : effet du contenu en CHOL, la concentration des cyclodextrines et leur type

Introduction

Les cyclodextrines (CDs) sont des oligosaccharides cycliques constitués d'unités de glucopyranose. Elles adoptent la forme d'un cône tronqué ayant une cavité interne hydrophobe et une surface externe hydrophile. Ceci leur confère la propriété de former des complexes d'inclusion (hôte/invité) avec des molécules lipophiles (Duchêne and Bochot, 2016). Les CDs les plus connues sont l' α -CD, la β -CD et la γ -CD ; elles sont appelées les CDs natives et sont composées de 6, 7 et 8 unités de glucose, respectivement. En raison de la faible solubilité aqueuse de la β -CD, des modifications chimiques peuvent avoir lieu sur les groupements hydroxyles de la β -CD engendrant par la suite plusieurs dérivés de la β -CD (Szente, 1999). Parmi ceux-ci, on distingue les dérivés hydroxypropylés : l'hydroxypropyle- β -CD (HP- β -CD); les dérivés méthylés : la RAMEB (méthylée de manière aléatoire sur l'intégralité des hydroxyles primaires ainsi que sur 7 à 9 hydroxyles secondaires) et la CRYSMEB (possédant un méthyle en position 2 de la β -CD) et les dérivés sulfobutylés : la sulfobutyléther- β -CD (SBE- β -CD) (Del Valle, 2004).

En dépit de leurs vertus en tant que nanovecteurs employés pour la délivrance des médicaments et pour l'amélioration des propriétés physico-chimiques des molécules instables, les CDs ont démontré une activité hémolytique sur les érythrocytes et plusieurs types cellulaires (Irie et al., 1982). Des études ont également rapporté l'altération des propriétés physiques de la membrane (fluidité et perméabilité) en présence des CDs. Ceci est principalement dû à la capacité des CDs d'extraire les composants lipidiques de la membrane (phospholipides et CHOL) (Hammoud et al., 2019). Cependant, le contenu membranaire en CHOL n'a pas été encore étudié lors de l'étude de l'interaction des CDs avec la membrane. Dans ce chapitre, nous allons présenter une étude de l'effet des CDs natives et de quatre dérivés de la β -CD sur la perméabilité membranaire de liposomes comprenant différents taux de CHOL.

Pour ce faire, des liposomes formés de DPPC et de CHOL aux rapports molaires DPPC:CHOL (100:10; 100:25; 100:50 et 100:100) et encapsulant la SRB ont été préparés par la méthode d'évaporation en phase inverse suivie d'une extrusion. Les liposomes sont séparés des molécules libres de lipides et de SRB après passage sur une colonne gel Sephadex G 25 et la concentration de DPPC dans les différentes formulations a été déterminée. Finalement, les liposomes ont été exposés aux différentes CDs à des rapports molaires CD:DPPC 1:1; 10:1 et 100:1. Pour chaque formulation, des liposomes non traités par les CDs ont été utilisés comme blancs. Les échantillons sont incubés à 37 °C et la perméabilité membranaire a été évaluée en suivant le relargage de la SRB des liposomes au cours de temps.

Les résultats obtenus ont révélé la dépendance de l'effet perméabilisant des CDs de 4 facteurs : le temps d'incubation, la concentration de la CD, le contenu membranaire en CHOL ainsi que le type de CDs.

En premier lieu, toutes les CDs ont exercé un effet perméabilisant instantané sur les différentes formulations liposomales. Parmi l'ensemble des CDs testées, la γ -CD a montré l'effet le plus faible. L'effet instantané des CDs augmente quelques heures après l'incubation (à 4 h) pour atteindre un plateau à 24 h.

En second lieu, l'effet des CDs sur la perméabilité membranaire dépendait de la concentration de la CD. En fait, à un ratio molaire CD:DPPC égal à 1:1, aucun effet n'a été observé avec les différentes CDs. Aux rapports molaires CD:DPPC 10:1 et 100:1, les CDs ont montré un effet perméabilisant dose-dépendant: ainsi, la membrane était le mieux perméabilisé par les CDs au ratio molaire le plus élevé.

Par ailleurs, il s'est avéré que la perméabilité induite par les CDs soit modulée par le CHOL. En effet, nous avons trouvé que le comportement des CDs vis-à-vis des membranes liposomales est lié au taux de CHOL qui affecte à son tour la stabilité membranaire : à 10 % CHOL, toutes les CDs ont présenté un effet instantané mais cet effet ne persiste pas et disparaît quelques heures plus tard. Au contraire, à des taux de CHOL supérieurs à 10 %, l'effet instantané exercé par les CDs sur la membrane est maintenu au cours du temps. La cause principale de ceci revient à la stabilité conférée à la membrane par le CHOL : en effet, l'instabilité de la membrane contenant un faible taux de CHOL (10 %) mène à l'établissement rapide de l'équilibre entre la cavité des CDs et la

membrane. Dans le cas des membranes renfermant un taux de CHOL supérieur à 10%, celles-ci sont plus stables et résiste alors à l'établissement de cet équilibre qui sera plus tardif.

De surcroît, nous avons constaté que le CHOL ne module pas l'effet des CDs en stabilisant uniquement la membrane, mais il le module dépendamment du type de la CD et de son affinité pour les composants lipidiques de la membrane. En présence d'une CD présentant une affinité élevée pour le DPPC, l'effet perméabilisant de la CD diminue progressivement avec l'ajout du CHOL aux rapports molaires CD:DPPC 10:1 et 100:1. Pour ce type de CDs, l'effet est le mieux observé avec les membranes contenant 25 % CHOL et qui sont alors relativement pauvres en CHOL. Ce résultat a été obtenu avec α -CD, HP- β -CD, CRYSMEB et SBE- β -CD. D'autre part, si la CD est connue pour son affinité élevée pour le CHOL, l'effet de la CD est réduit avec l'augmentation du taux de CHOL membranaire au ratio molaire CD:DPPC 10:1. Toutefois, au ratio molaire 100:1, un comportement remarquable a été observé avec ce type de CDs: dans ce cas, l'effet de la CD augmente significativement avec le taux de CHOL dans la membrane. Par conséquent, ces CDs montrent l'effet perméabilisant le plus prononcé avec les membranes riches en CHOL (100 % CHOL). Dans cette étude, la β -CD et la RAMEB font partie de cette catégorie. Quant à la γ -CD, celle-ci ne présente pas d'affinité ni pour le DPPC ni pour le CHOL vue sa large cavité. Ainsi, la γ -CD perméabilise légèrement la membrane et son effet est seulement visible à faible taux de CHOL (25 %).

Compte tenu de tout ce qui précède, nous pouvons assumer que les membranes pauvres en CHOL sont sensibles à l' α -CD, la γ -CD, la CRYSMEB, la HP- β -CD et la SBE- β -CD au ratio molaire CD:DPPC 10:1 alors que les membranes riches en CHOL sont sensibles à la β -CD et la RAMEB au ratio 100:1. Ces dernières pourraient extraire le CHOL des radeaux lipidiques de la membrane.

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Cyclodextrins permeabilize DPPC liposome membranes: effect of cholesterol content, cyclodextrin concentration and type

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Abstract

Cyclodextrins (CDs) are known for their ability to extract lipid components from synthetic and biological membranes and therefore to induce an increase of membrane permeability. However, the effect of cholesterol (CHOL) content in the membrane on the CD permeabilizing effect was not considered yet. Given that an increase in CHOL content reduces the membrane permeability, the aim of this work was to reveal how CHOL would modulate the CD effect on the membrane. Hence, liposomes made of dipalmitoyl phosphatidylcholine (DPPC) and various CHOL contents (DPPC:CHOL 100:10; 100:25; 100:50 and 100:100) encapsulating the hydrophilic fluorophore, sulforhodamine B (SRB), were prepared and exposed to the native CDs (α -CD, β -CD, γ -CD) and four β -CD derivatives: the randomly methylated- β -CD (RAMEB), the low methylated- β -CD (CRYSMEB), the hydroxypropyl- β -CD (HP- β -CD) and the sulfobutylether- β -CD (SBE- β -CD) at different CD:DPPC molar ratios (1:1; 10:1 and 100:1). The membrane permeability was monitored following the release of SRB with time. The results demonstrated the dependency of the CD effect on the membrane permeability on the CHOL content, the CD concentration, the CD type and time of incubation. A decrease in the CDs effect upon CHOL content increase was found with CDs displaying a preferential affinity towards phospholipids regardless CDs concentration whereas the effect of CDs was enhanced with CHOL content increase with CDs showing a better affinity towards CHOL membrane at high CDs concentration. Hence, CHOL poor membranes were more sensitive to α -CDs, γ -CD, CRYSMEB, HP- β -CD and SBE- β -CD at a CD:DPPC molar ratio above 10:1. In contrast, CHOL rich membranes were only sensitive to a high concentration of β -CD and RAMEB. These CDs can possibly extract CHOL from raft domains.

Keywords: cyclodextrins, liposome, membrane permeability, cholesterol content, vesicle leakage

Abbreviation: CD: cyclodextrins; DPPC: dipalmitoyl phosphatidylcholine; CHOL: cholesterol; SRB: sulforhodamine B; RAMEB: the randomly methylated- β -CD; CRYSMEB: the low methylated- β -CD; HP- β -CD: the hydroxypropyl- β -CD; SBE- β -CD: the sulfobutylether- β -CD; LUVs: large unilamellar vesicles; DCL: drug-in-cyclodextrin-in-liposomes; PI: phosphatidylinositol; PS: phosphatidylserine; Lo: liquid-ordered phase; H-PC: hydrogenated phosphatidylcholine

1. Introduction

Cyclodextrins (CDs) are a family of cyclic oligosaccharides made of glucopyranose units connected by $\alpha(1-4)$ glycosidic bonds. They possess a cone-shaped molecular structure with a hydrophobic internal cavity and a hydrophilic outer surface [1]. The natural common CDs are the native α -CD, β -CD and γ -CD consisting of 6, 7 and 8 D-glucopyranose units, respectively. Due to their limited water solubility (especially β -CD), the native CDs can undergo chemical or enzymatic modifications (such as alkylation, arylation, hydroxypropylation, amination, etherification, etc.) giving rise to synthetic CDs derivatives with greater water solubility [2]. Thanks to their unique structure, CDs can offer exclusive advantages by allowing the entrapment of lipophilic molecules inside their inner cavities. This inclusion improves the chemical stability and aqueous solubility of the guest molecule and results in the formation of a water soluble CD-guest complex [3]. Being recognized as non-toxic, biodegradable and sustainable carriers, CDs have attracted wide interest as potential carriers in different fields, mainly in drug delivery where they are used as pharmaceutical excipients to increase the drug permeability through biological membranes improving their bioavailability and their efficacy [2,4,5]. Furthermore, the CDs peculiarities helped to develop a combined system in which CD-guest complexes are encapsulated in the aqueous core of liposomes; it is generally known as “drug-in-cyclodextrin-in-liposomes” (DCL) [6].

Nevertheless, CDs are known to induce considerable damages in the membrane structure and composition. In fact, CDs can alter the biophysical properties of the membrane by increasing its fluidity and permeability [7]. They are even able to extract the lipid membrane components leading the membrane to lose its integrity[7]. This behavior was attributed to the hemolytic activity of CDs previously observed on erythrocytes and other cell membranes [8].

Numerous reports highlighting the CDs mediated lipid extraction demonstrated that some CDs displayed a higher affinity towards phospholipids such as α -CD for phosphatidylinositol (PI), phosphatidylserine (PS) and dipalmitoyl phosphatidylcholine (DPPC), [9,10] etc. while other CDs preferentially extracted cholesterol (CHOL) from membranes such as β -CD and its methylated derivatives [11,12]. Consequently, CDs are classified as permeabilizing agents for being able to promote the leakage of liposomal membranes [13]. Although a great number of papers demonstrated the membrane-damaging effect induced by several CDs, the CHOL content in the

membrane was not considered in the literature despite the remarkable effect of CHOL on the stability of the lipid bilayer. In fact, CHOL can greatly modulate the membrane permeability: a previous work showed that increasing CHOL content in the membrane results in a decrease in the membrane permeability in a dose dependent manner [14]. Additionally, the CHOL content was demonstrated to reduce and sometimes to inhibit the permeability of DPPC vesicles induced by bioactive agents [15,16]. Given the condensing and ordering effect that CHOL exerts on the membrane, the presence of CHOL in the lipid bilayer introduced a new phase to the membrane referred to as “the liquid-ordered” (Lo) alongside with the gel phase and the liquid-disordered phase [17].

Beside, CHOL is a major component of the so-called “lipid rafts” which are perceived as membrane domains rich in CHOL and sphingomyelin and involved in various cellular processes, (e.g. signaling transduction, proteins trafficking, etc.) [18]. However, many discrepancies could be found in the literature regarding the existence of lipid rafts in model membranes, especially CHOL-lipid binary mixtures. Studies of DPPC:CHOL bilayers have elucidated the formation of nanodomains enriched in CHOL within the membrane displaying a fluid-like structure as manifested in the Lo phase [19].

To the best of our knowledge, this is the first study investigating the effect of CDs on the permeability of DPPC liposome membrane of various CHOL content. The CD effect was examined at various CD:DPPC molar ratios. This work will provide a better understanding of the influence of CHOL content on the CD effect with regards to their affinity to lipid membrane components. It will also allow us to point out if the CDs induced lipid extraction is specific and may occur in “lipid rafts”.

Different CDs type were considered in this study; these include the native CDs: α -CD, β -CD, γ -CD and four β -CD derivatives: the randomly methylated- β -CD (RAMEB), the low methylated- β -CD (CRYSMEB), the hydroxypropyl- β -CD (HPBCD) and the sulfobutylether- β -CD (SBE- β -CD). A schematic representation of the chemical structure of the native CDs and their dimensions is depicted in figure 1. The general structure of the β -CD derivatives with the substituent and the degree of substitution of each derivative are represented in figure 2. Therefore, large unilamellar vesicles (LUVs) composed of DPPC and CHOL at various DPPC:CHOL molar ratios (100:10; 100:25; 100:50; 100:75 and 100:100) and encapsulating a fluorescent dye, the sulforhodamine B

(SRB), were prepared and treated with CDs at various CD:DPPC molar ratios (1:1; 10:1 and 100:1). Then, we followed the SRB release from liposomes over time at 37°C by fluorescence spectroscopy to measure the permeability of liposome formulations exposed to CDs.

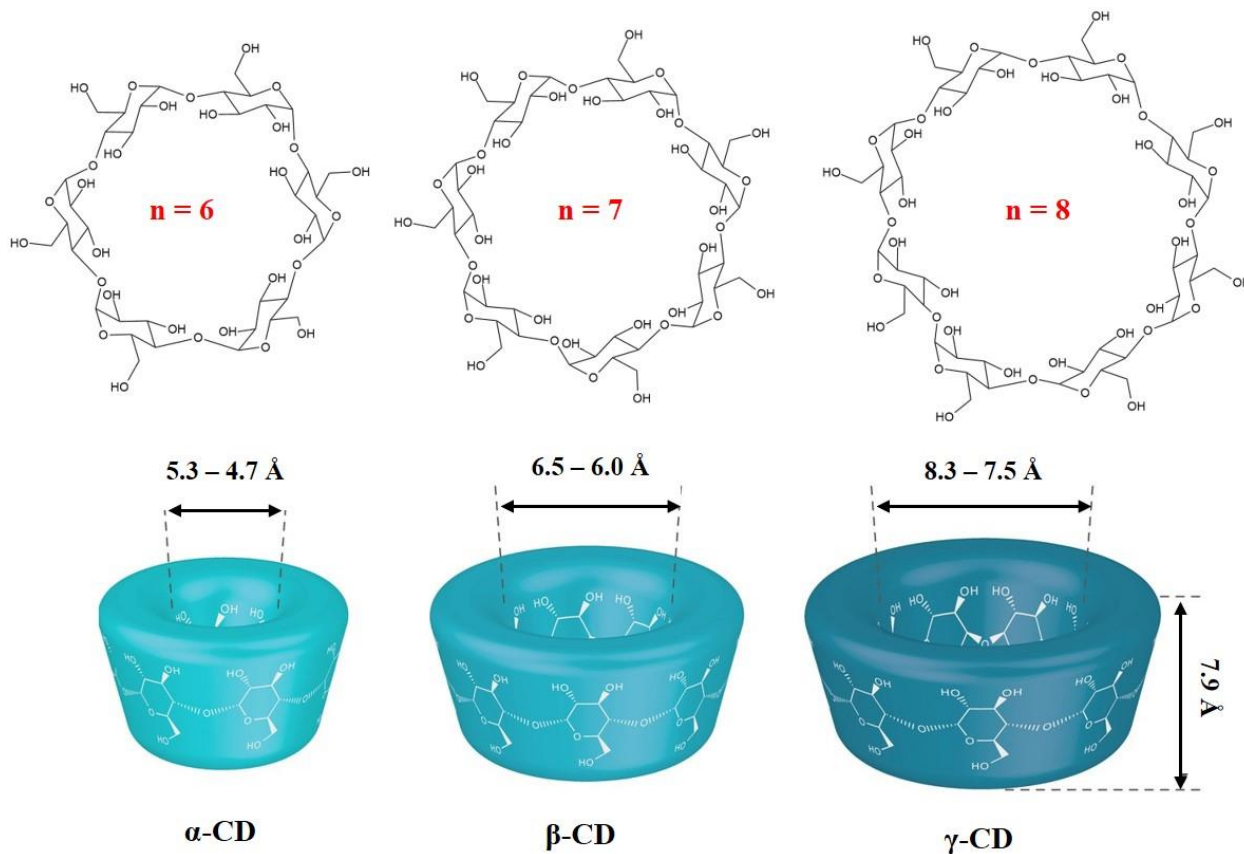


Figure 1: The chemical structure of the native CDs, their three-dimensional structure and their dimensions ($n = 6, 7$ and 8 glucopyranose units for α, β and γ -CD respectively)

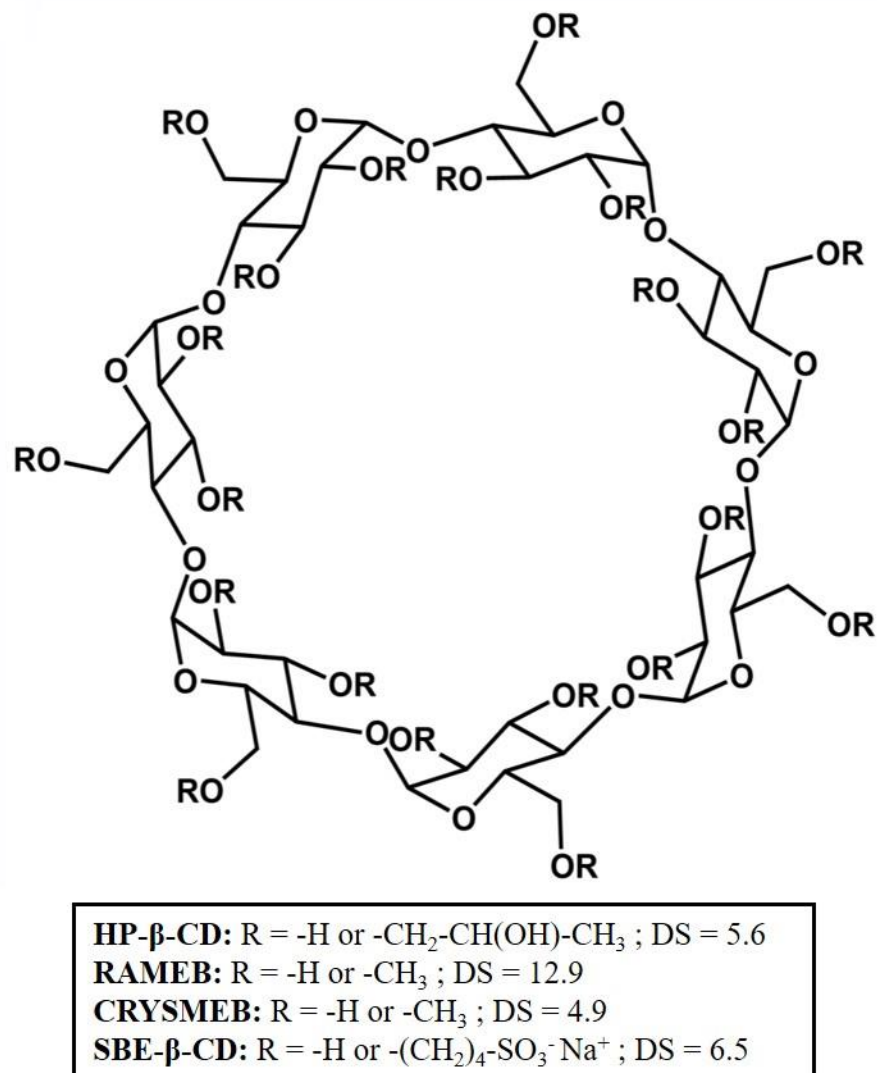


Figure 2: The general structure of the β -CD derivatives: substituents, degree of substitution and name of the corresponding β -CD derivatives.

2. Materials and methods

2.1. Materials

α -CD, β -CD, γ -CD and randomly methylated- β -CD (RAMEB, DS = 12.9), were provided by WackerChemie (Germany). Low methylated- β -CD (CRYSMEB, DS = 4.9) and hydroxypropyl- β -CD (HPBCD, DS = 5.6) were provided by Roquette Frères (Lestrem, France). Sulfobutylether- β -CD (SBE- β -CD, DS = 6.5) was provided by LIGAND Pharmaceuticals (San Diego, CA, USA). Dipalmitoylphosphatidylcholine (DPPC) ($\geq 99\%$) and trizma base (buffer reagent) were

purchased from Sigma-Aldrich, Switzerland. Triton X-100, sodium chloride (NaCl) and Sephadex G25 gel were purchased from Sigma-Aldrich, Belgium. Ammonium molybdate, hydrogen peroxide, potassium dihydrogenophosphate, sodium sulfite, sodium bisulfite, chloroform and methanol were purchased from Sigma-Aldrich, Germany. Cholesterol ($\geq 99\%$) and sulforhodamine B were purchased from Sigma-Aldrich, USA. 4-amino-3-hydroxyl-1-naphtalene sulfonic acid was purchased from Fluka, India. Sulfuric acid was purchased from ACROS Organics, Belgium and diethyl ether was purchased from VWR-Prolabo Chemicals, Belgium.

2.2. Liposomes preparation, extrusion and purification

The SRB-loaded liposomes were prepared, extruded and purified following the same method described by Nasr et al. [16]. Briefly, the lipid mixture of DPPC and CHOL at the different molar ratios (DPPC:CHOL 100:10, 100:25, 100:50 and 100:100) was dissolved in an organic phase made of: chloroform/diethylether/methanol (6/6/1 – v/v/v). After a short sonication, the aqueous phase made of SRB (150 mM) dissolved in Tris HCl buffer (0.1 M, pH = 7.4) was added to the lipid solution and the mixture was sonicated at 60°C under nitrogen stream. The removal of organic solvents was achieved by evaporation at 45°C using a rotary vacuum evaporator (Heidolph, Germany). Then, the aqueous phase (SRB containing buffer) was added to the dry film and the mixture was sonicated at 60°C under nitrogen stream to generate vesicles. The SRB-loaded liposomes were subjected to extrusion through polycarbonate filters membranes (Avanti Polar Lipids, Switzerland) of decreasing pore sizes resulting in a homogenous mixture of LUVs.

Finally, the purification of the SRB loaded LUVs to eliminate unencapsulated SRB and lipid molecules from liposomes was carried out via a centrifugation (2 hours, 15 000 rpm, 4 °C) and a molecular sieve chromatography (using a Sephadex G25 gel filtration column). A Tris HCl buffer (0.1 M, pH 7.4) containing 150 mM NaCl was used for elution and liposome storage.

2.3. Exposure of SRB-loaded liposomes to CDs

The concentration of DPPC was determined for each formulation according to Bartlett method, as previously described by Habib et al. [20]. Then, the liposomal suspensions were all diluted to obtain solutions with a DPPC concentration of 0.15 mM. At time of incubation, the CDs were individually added to the liposomes so that the concentration of CD in the final volume of CD treated liposomes is equal to 0.15 mM, 1.5 mM and 15 mM in respect to the CD:DPPC molar

ratios: 1:1, 10:1 and 100:1 respectively. The fluorescence signal was measured for each sample immediately after the exposure of LUVs to CDs and the samples were incubated at 37°C during 24 h. For each formulation, a solution containing only liposomes was used as the blank solution.

2.4. The membrane permeability study by fluorescence spectroscopy

The membrane permeability is commonly evaluated by following the leakage of self-quenching probes such as SRB from liposomes [13]. Indeed, a fluorescence auto-quenching effect is observed when SRB is encapsulated at a high concentration inside the liposomes. The recovery of the fluorescence signal is achieved upon the release of the dye from liposomes and its dilution in the external medium. Thus, the effect of CDs on the permeability of liposomal membranes was studied by measuring the fluorescence signal of liposomes treated with CDs and incubated at 37°C. An enhanced membrane permeability is detected when the fluorescence signal is increased demonstrating the permeabilizing effect of the tested agents.

As described in the previous section, the SRB loaded liposomes of each formulation treated or not with CDs were incubated in a water bath at 37°C. Aliquots were taken from each sample at 0, 4 and 24 h and the fluorescence signal was measured after a dilution of 100 times in the Tris HCl (0.1 M ; pH 7.4) buffer containing 150 mM NaCl.

The measurements were carried out on a spectrophotometer (Hitachi F-7000 Spectrofluorometer) at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The emission spectrum was recorded in the range 540-700 nm. The results of the permeability study were expressed as the percentage of the fluorophore released from LUVs obtained using the following equation:

$$\text{SRB release percentage} = \frac{(F_t - F_{0 \text{ blank}})}{F_{\text{max}}} \times 100 \quad (1)$$

Where F_t is the fluorescence intensity measured at time t for each sample, $F_{0 \text{ blank}}$ is the fluorescence intensity measured at time 0 for the blank liposomes of each formulation and F_{max} is the maximum fluorescence indicating a complete release of SRB from vesicles and obtained in the presence of the nonionic detergent, Triton X-100 (1%) in a Tris HCl buffer (0.1M ; pH 7.4) containing 150 mM NaCl.

The results are expressed as the means of three independent experiments \pm SD.

2.6. Statistical analysis

To assess significant differences between values, statistical analysis was carried out using the Student's t-test. A value of $p < 0.05$ was considered statistically significant. The p values compare the membrane permeability in the presence of CDs to that of blank liposomes.

3. Results and discussion

In this study, liposome membranes made of DPPC and various CHOL contents were prepared and extruded. The prepared formulations are usually classified as: CHOL poor membranes (10 and 25 % CHOL) and CHOL rich membranes (50 and 100 % CHOL) exhibiting particular behaviors depending on the CHOL content [21]. As a matter of fact, CHOL exerts a deep modulatory effect on the physical properties of the membrane [21]. By increasing the CHOL content in the membrane, the permeability of the lipid bilayer was shown to decrease gradually [14]. Additionally, an increase in CHOL content promotes the formation of microdomains called rafts that exist in the "Lo" (liquid ordered) phase [22]. The aim of this study was to outline the effect of the CDs on the membrane permeability of DPPC liposomes with different CHOL contents at various CD:DPPC molar ratios. For reasons of simplification, the liposome membranes made of DPPC:CHOL at the molar ratios 100:10; 100:25; 100:50 and 100:100 will be referred to as 10, 25, 50 and 100 % CHOL membranes respectively, as conventionally done in many papers.

The effect of the CDs on the permeability of liposome membranes was assessed by the commonly used self-quenching probe leakage method. Prior to the exposure of liposomal formulations to the studied CDs, the various suspensions were diluted in order to obtain liposome suspensions with a DPPC concentration of 0.15 mM. In fact, among the native CDs, β -CD was the less soluble in water (18.5 mg/mL or 16 mM) which urged us to conduct the comparative permeability study at a concentration of CD (15 mM) not exceeding the limit of solubility of β -CD at the highest CD:DPPC ratio of 100:1.

The liposome suspensions were individually treated with 0.15 mM, 1.5 mM and 15 mM of CDs (CD:DPPC molar ratios were of 1:1, 10:1 and 100:1, respectively). Following the exposure of liposomes to CDs, the samples were incubated at 37°C and the fluorescence signals were measured at time 0, 4 and 24h.

The obtained data are reported in tables 1, 2, 3 and 4 in which the vertical reading points out the effect of the CD concentration on the SRB release for a specific membrane composition while the horizontal reading of the table highlights the impact of CHOL content on the permeability of membranes at various intervals of time.

Table 1: The SRB release values obtained at t_0 , 4 h and 24 h for α -CD treated liposomes of different CHOL contents and different CD:DPPC molar ratios.

The SRB release value of blank liposomes was 0 % at t_0 for the different formulations.

Values are expressed as the means of three different measurements \pm SD. (* indicates a value of $p < 0.05$ statistically significant. The p values compare the membrane permeability in the presence of CD to that of the respective blank).

Time	DPPC:CHOL (100:10)			DPPC:CHOL (100:25)			DPPC:CHOL (100:50)			DPPC:CHOL (100:100)		
	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h
Blank	0	43.64 \pm 1.84	48.34 \pm 1.73	0	12.60 \pm 1.28	15.01 \pm 1.35	0	3.13 \pm 0.79	4.17 \pm 1.26	0	2.41 \pm 0.70	5.15 \pm 1.35
α -CD/DPPC 1:1	2.20 \pm 0.39*	38.20 \pm 0.97*	42.19 \pm 0.35*	2.16 \pm 0.42*	13.99 \pm 0.72	15.13 \pm 0.40	2.23 \pm 0.53*	2.31 \pm 0.30	3.14 \pm 0.13	1.06 \pm 0.12	2.46 \pm 0.86	5.20 \pm 0.34
α -CD/DPPC 10:1	13.08 \pm 0.38*	40.84 \pm 0.86*	42.76 \pm 1.20*	8.94 \pm 0.82*	19.67 \pm 0.17*	23.85 \pm 1.01*	4.20 \pm 0.39*	9.81 \pm 0.27*	14.48 \pm 0.43*	2.95 \pm 0.25*	4.27 \pm 0.73*	6.85 \pm 0.39*
α -CD/DPPC 100:1	15.00 \pm 0.95*	45.70 \pm 1.63	48.38 \pm 1.87	23.01 \pm 0.73*	40.16 \pm 1.85*	47.80 \pm 0.85*	18.50 \pm 1.23*	34.55 \pm 0.18*	44.48 \pm 1.45*	13.29 \pm 0.12*	30.50 \pm 0.54*	38.06 \pm 0.74*

Table 1 shows the SRB release values calculated for DPPC:CHOL blank liposomes and liposomes treated with α -CD at different CD:DPPC molar ratios. At t_0 , the SRB release values of blank liposomes were equal to zero for the different formulations according to equation number (1). As expected, the SRB efflux from blank liposomes was reduced with the increase of CHOL content in the membrane which is in accordance with previously published work in our team proving the decrease of membrane permeability [14].

Upon exposure to α -CD, an instant permeabilizing effect was observed with the different formulations. However, at a CD:DPPC molar ratio 1:1, the SRB leakage of liposomes was barely noted regardless CHOL content. The instantaneous effect of α -CD increased with CD concentration and became more significant at the CD:DPPC molar ratio 100:1. Also, this instant effect decreased gradually with increasing CHOL content.

As we can see in table 1, the instantaneous permeabilizing effect of α -CD on 10 % CHOL membranes disappeared at 4 and 24 h regardless the CD:DPPC molar ratio. The permeability of 10 % CHOL liposome membranes exposed to α -CD were almost similar to blank liposomes after t_0 . This may be due to the rapid equilibrium that could be established at 10 % CHOL between the membrane and the α -CD. In fact, α -CD can instantly interact with the liposome membrane constituents and the extraction of lipid molecules takes place resulting in membrane destabilization. This is illustrated by the rapid leakage of SRB loaded liposomes upon α -CDs exposure to membranes of different CHOL content. After this initial effect, α -CD would not influence the stability of the bilayer indicating that the membrane is re-organized and the equilibrium between the α -CD and the membrane is established. Our result for 10 % CHOL liposomes is in accordance with that of Hatzi et al. (2007) showing an instant calcein release from CHOL free liposomes (PC and hydrogenated phosphatidylcholine (H-PC) vesicles) exposed to CDs with no further leakage with time [11]. It is worthy to note that the 10 % CHOL membranes are less stable than the remaining formulations and evidenced the same SRB release pattern as CHOL free liposomes [14].

Taking our table horizontally, no permeabilizing effect was seen after t_0 with the CD:DPPC ratio 1:1 regardless CHOL content and the incubation time. At the CD:DPPC ratios 10:1 and 100:1, the permeability increased rapidly up to 4 h then slowed down to reach probably a plateau at 24h. The α -CD effect was first visible at 25% CHOL and decreased with increasing CHOL content. While

at the CD:DPPC ratio 10:1, the permeabilizing effect of α -CD decreased dramatically in CHOL rich membranes (50% et 100% CHOL), at the CD:DPPC 100:1, it decreased gradually from the highest SRB release at 25 % CHOL (47.80 % at 24 h) to reach 38.06% at 100 % CHOL.

Taking into account the ability of CDs to remove lipid membrane components, the obtained effect of α -CD could be mainly attributed to its preferential phospholipids extraction from the membrane [8]. Actually, a previous work reported that α -CD can extract DPPC through a special complex formation between the acyl chains of the phospholipid and the CD molecules where many α -CD units string along the nonpolar chains of DPPC forming a rotaxane-like ring [10]. DSC studies have also proved that α -CD forms an insoluble complex with DPPC [23]. Besides, α -CD has previously demonstrated the strongest destabilizing effect on the DPPC liposome membranes among the native CDs [10]. In our study, being more abundant in phospholipids than CHOL, the 25 % CHOL liposome membranes were more sensitive to α -CD compared to the remaining formulations (50 and 100 % CHOL) which explain the highest permeabilizing effect exerted by α -CD at this CHOL content. With increasing CHOL content (at 50 and 100 % CHOL), CHOL might be clustered into microdomains which may hinder the α -CD's effect. Additionally, it is important to mention that there was no significant difference between the effect of α -CD on 50 and 100 % CHOL membranes. This result is consistent with our previous study where we found that these formulations contained effectively 40 and 43 % CHOL, a closely similar membrane composition [16]. Based on the observed results, we can assume that α -CD is active mainly on CHOL poor membranes (25 % CHOL) at a CD:DPPC molar ratio above 10:1.

Table 2: The SRB release values obtained at time t_0 , 4 and 24 h for β -CD treated liposomes at different CHOL content and different CD:DPPC molar ratios.

The SRB release value of blank liposomes was 0 % at t_0 for the different formulations.

Values are expressed as the means of three different measurements \pm SD. (* indicates a value of $p < 0.05$ statistically significant. The p values compare the membrane permeability in the presence of CD to that of the respective blank.)

Time	DPPC:CHOL (100:10)			DPPC:CHOL (100:25)			DPPC:CHOL (100:50)			DPPC:CHOL (100:100)		
	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h
Blank	0	43.64 \pm 1.84	48.34 \pm 1.73	0	12.60 \pm 1.28	15.01 \pm 1.35	0	3.13 \pm 0.79	4.17 \pm 1.26	0	2.41 \pm 0.70	5.15 \pm 1.35
β -CD/DPPC 1:1	1.90 \pm 0.09*	39.45 \pm 1.03	44.05 \pm 0.16	1.40 \pm 0.25	14.24 \pm 0.37	14.60 \pm 0.59	2.50 \pm 0.32*	2.14 \pm 0.08	5.63 \pm 0.25	1.28 \pm 0.15	0.99 \pm 0.43	7.02 \pm 0.08
β -CD/DPPC 10:1	11.91 \pm 1.28*	35.79 \pm 0.81*	40.85 \pm 0.90*	6.32 \pm 0.20*	18.07 \pm 0.82*	21.45 \pm 0.94*	2.76 \pm 0.28*	2.53 \pm 0.01	7.93 \pm 0.20*	1.75 \pm 0.30*	2.16 \pm 0.01	4.60 \pm 0.24
β -CD/DPPC 100:1	17.10 \pm 0.83*	44.71 \pm 1.23	49.20 \pm 2.02	18.94 \pm 1.48*	38.29 \pm 1.89*	39.78 \pm 1.95*	23.30 \pm 0.63*	36.97 \pm 0.33*	45.85 \pm 1.54*	32.37 \pm 0.90*	52.3 \pm 1.00*	57.71 \pm 0.01*

As shown in table 2, the β -CD had no effect on the membrane permeability of liposomes at the CD:DPPC molar ratio 1:1 similar to α -CD. For the other concentrations of β -CD, it displayed an instant permeabilizing effect that increased with β -CD concentration. As obtained with α -CD, the 10 % CHOL formulation exposed to β -CD was comparable to the blank after 4 and 24 h of incubation, at the various CD/DPPC molar ratios. Furthermore, the effect of β -CD increased fast in the first 4 h reaching a plateau up to 24h of incubation for all other formulations. At the ratio CD:DPPC 10:1, β -CD exhibited a permeabilizing effect only visible at 25 % CHOL, as found with α -CDs. At the ratio 100:1, the permeabilizing effect of β -CD on 25% CHOL membranes increased compared to that obtained at the ratio 10:1 but was slightly below that induced by α -CD at the same ratio. However, contrary to α -CDs, the permeabilizing effect of β -CD was enhanced with CHOL content to reach 57.71 % of SRB release at 100% CHOL after 24 h of incubation. This value was the highest among the studied CDs which draws attention to the potency of β -CD in permeabilizing the membranes rich in CHOL. Indeed, β -CD can extract CHOL from the membranes [7] which explains the increase of its permeabilizing effect with CHOL addition at the CD:DPPC molar ratio 100:1 as more CHOL molecules are extracted from the membrane leading to the disruption of the membrane continuity and its subsequent leakage [13]. Though, this effect was not seen at the ratio 10:1 which is also considered a high β -CD concentration: this leads us to suppose that the CHOL extraction mediated by β -CD is probably achieved at a CD:DPPC molar ratio larger than 10:1. Here also, no major difference was found between 50 and 100 % CHOL formulations. Comparing the α -CD's induced permeability to that of β -CD, it is possible to state that CHOL poor membranes are sensitive to α -CD at the CD:DPPC molar ratios 10:1 and 100:1 whereas CHOL rich membranes are sensitive to β -CD at the highest ratio (100:1). Hence, β -CD is active on CHOL rich membranes (50 and 100% CHOL) where the rafts domains may be present. We suggest therefore that β -CD at high concentration would extract CHOL from raft domains.

Table 3: The SRB release values obtained at t_0 , 4 and 24 h for the β -CD derivatives treated liposomes of different CHOL content and at different CD/DPPC molar ratios.

The SRB release value of blank liposomes was 0 % at t_0 for the different formulations.

Values are expressed as the means of three different measurements \pm SD. (* indicates a value of $p < 0.05$ statistically significant. The p values compare the membrane permeability in the presence of CD to that of the respective blank).

Time	DPPC:CHOL (100:10)			DPPC:CHOL (100:25)			DPPC:CHOL (100:50)			DPPC:CHOL (100:100)		
	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h
Blank	0	43.64 \pm 1.84	48.34 \pm 1.73	0	12.60 \pm 1.28	15.01 \pm 1.35	0	3.13 \pm 0.79	4.17 \pm 1.26	0	2.41 \pm 0.70	5.15 \pm 1.35
RAMEB												
CD/DPPC 1:1	3.20 \pm 0.44*	39.22 \pm 0.53	45.49 \pm 0.19	2.46 \pm 0.11*	13.73 \pm 0.22	16.97 \pm 0.83	3.50 \pm 0.16*	2.09 \pm 0.09	6.38 \pm 0.30	1.06 \pm 0.27	0.97 \pm 0.18	6.34 \pm 0.11
CD/DPPC 10:1	13.32 \pm 0.27*	43.99 \pm 0.65	47.25 \pm 0.06	9.39 \pm 0.64*	19.32 \pm 0.42*	22.72 \pm 1.02*	4.78 \pm 0.09*	8.35 \pm 0.33*	11.04 \pm 0.27*	2.56 \pm 0.37*	1.90 \pm 0.21	3.47 \pm 0.28
CD/DPPC 100:1	28.70 \pm 0.48*	46.06 \pm 1.75	52.76 \pm 1.51*	23.80 \pm 1.77*	38.13 \pm 1.40*	45.24 \pm 1.87*	27.50 \pm 0.82*	43.51 \pm 0.80*	48.46 \pm 0.23*	40.74 \pm 0.46*	52.00 \pm 0.52*	57.07 \pm 0.24*
CRYSMEB												
CD/DPPC 1:1	1.11 \pm 0.53	41.81 \pm 0.16	42.24 \pm 0.28	2.57 \pm 0.17*	13.53 \pm 0.06	16.45 \pm 0.17	1.21 \pm 0.42	2.85 \pm 0.78	5.89 \pm 0.40	1.09 \pm 0.14	1.72 \pm 0.71	6.89 \pm 0.07
CD/DPPC 10:1	5.90 \pm 0.14*	38.18 \pm 0.06	40.21 \pm 0.27	6.09 \pm 0.38*	17.87 \pm 0.01	21.64 \pm 0.31*	1.28 \pm 0.10*	2.92 \pm 0.05	6.64 \pm 0.48*	1.85 \pm 0.72*	0.59 \pm 0.30	3.12 \pm 0.93
CD/DPPC 100:1	18.90 \pm 0.68*	47.07 \pm 1.72	47.55 \pm 0.98	18.41 \pm 1.05*	32.51 \pm 1.35*	36.54 \pm 0.81*	15.70 \pm 0.56*	16.68 \pm 1.70*	26.34 \pm 1.14*	12.92 \pm 0.82*	20.00 \pm 0.71*	20.88 \pm 0.69*
HP-β-CD												
CD/DPPC 1:1	1.36 \pm 0.43	38.30 \pm 0.67	41.45 \pm 0.71	1.12 \pm 0.37	14.78 \pm 0.29	14.28 \pm 1.14	0.98 \pm 0.08	1.66 \pm 0.16	5.77 \pm 0.21	1.26 \pm 0.19	1.64 \pm 0.36	9.56 \pm 0.39
CD/DPPC 10:1	6.86 \pm 0.68*	39.40 \pm 0.22	44.52 \pm 0.63	5.36 \pm 0.76*	15.17 \pm 0.10	20.40 \pm 0.89*	2.61 \pm 0.37*	3.64 \pm 0.29*	3.02 \pm 0.87*	2.18 \pm 0.30*	2.30 \pm 0.25	2.40 \pm 0.51

CD/DPPC 100:1	16.7 ± 0.48*	45.69 ± 2.39	49.35 ± 0.97	17.19 ± 1.04*	32.90 ± 0.76*	36.70 ± 0.24*	13.70 ± 0.50*	18.27 ± 0.27*	23.71 ± 0.91*	6.98 ± 0.25*	11.00 ± 0.64*	12.91 ± 1.65*
SBE-β-CD												
CD/DPPC 1:1	2.58 ± 0.29*	39.58 ± 0.15	42.52 ± 0.48	1.76 ± 0.10*	15.69 ± 0.58	14.08 ± 0.24	1.30 ± 0.45*	2.96 ± 0.42	6.27 ± 0.15	0.73 ± 0.18	1.33 ± 0.32	7.46 ± 1.16
CD/DPPC 10:1	7.11 ± 0.38*	41.52 ± 0.26	45.43 ± 0.41	5.94 ± 0.42*	16.27 ± 0.60	21.59 ± 1.12*	3.21 ± 0.75*	1.98 ± 0.71*	8.35 ± 0.27*	3.04 ± 0.44*	1.03 ± 0.08	3.71 ± 1.11
CD/DPPC 100:1	13.90 ± 0.53*	48.37 ± 1.73	52.70 ± 0.54	16.64 ± 1.62*	28.19 ± 1.06*	31.36 ± 0.74*	11.9 ± 0.18*	20.25 ± 0.70*	21.59 ± 0.80*	10.71 ± 0.07*	13.30 ± 0.87*	14.32 ± 0.21*

RAMEB, a methylated β -CD derivative, exhibited a behavior that was comparable to β -CD (table 3). As shown in table 3, RAMEB was able to instantly permeabilize the various liposome membranes at the CD:DPPC molar ratios 10:1 and 100:1 (with no significant effect at the ratio 1:1). This instantaneous effect increased with RAMEB concentration; it also decreased with CHOL content at the CD:DPPC molar ratio 10:1 but increased with the addition of CHOL at the ratio 100:1 as did happen with β -CD. However, the effect of RAMEB was more pronounced than that induced by β -CD at time of exposure. After 4 and 24 h of incubation, the effect of RAMEB persisted with the different formulations except for 10 % CHOL membranes, where similar release with blank liposomes was observed. Only a slight effect was visible at 10 % CHOL at a CD:DPPC molar ratio 100:1 but it appears to be negligible comparing to the effect of RAMEB at higher CHOL content. Above 10 % CHOL, the permeabilizing effect was reduced with CHOL increase at the CD:DPPC molar ratio 10:1 and was significantly enhanced upon CHOL addition at the ratio 100:1 at 4 and 24 h.

As explained earlier for β -CD, the main reason behind the behavior of RAMEB is its preferential affinity towards CHOL: in fact, many studies proved the ability of the methylated derivatives of β -CD such as RAMEB to promote vesicle leakage due to CHOL extraction from the membrane [11,12]. Besides, RAMEB was shown to induce CHOL efflux from biological membranes and to evoke cell damage [24]. This accounts for the significant increase in the membrane permeability upon RAMEB exposure at high CHOL content at the CD:DPPC molar ratio 100:1. Thus, RAMEB is active on CHOL rich membranes and can possibly extract CHOL from raft domains as previously demonstrated [7,24].

As for CRYSMEB, another methylated derivative of β -CD, its behavior was different from that of RAMEB. Although CRYSMEB exerted an instant permeabilizing effect on the different formulations at the CD:DPPC molar ratios 10:1 and 100:1, its effect remained lower than that induced by RAMEB. The effect of CRYSMEB observed at t_0 was increased with CD concentration and gradually decreased with increasing CHOL content at the CD:DPPC molar ratios 10:1 and 100:1. This instant effect did not persist during the incubation with 10 % CHOL liposome membranes as described with the previous CDs. Beyond 10 % CHOL, the effect of CRYSMEB persisted after 4 and 24 h of incubation and was reduced subsequent to the CHOL content increase at both CD:DPPC molar ratios 10:1 and 100:1. The highest effect was seen at 25 % CHOL with a

SRB release of 36.54 % at 24 h at the CD:DPPC ratio 100:1. The behavior of CRYSMEB at the CD:DPPC molar ratio 100:1 upon the increase of CHOL content was contradictory to those observed with RAMEB and β -CD whose effects were enhanced with CHOL increase at this ratio. Our result for CRYSMEB is in agreement with the work of Piel et al. who showed that CRYSMEB is less potent than RAMEB and other methylated CDs in promoting calcein leakage from liposomes comprising 30 % CHOL [12]. The authors stated that the low degree of substitution of CRYSMEB decreased its affinity to the lipid membrane components and resulted in a weaker disturbing effect compared to RAMEB and other methylated CDs. Consequently, CRYSMEB is active on CHOL poor membranes (25 % CHOL) at a CD:DPPC molar ratio above 10:1.

The non-methylated β -CD derivatives HP- β -CD and SBE- β -CD showed similar behaviors to CRYSMEB. As it can be seen in table 3, HP- β -CD and SBE- β -CD displayed an instant permeabilizing effect at the CD:DPPC molar ratios 10:1 and 100:1 that increased with CD concentration while at the ratio 1:1, no effect was observed with the different formulations. Increasing the CHOL content resulted in a decrease in the instant effect of the two β -CD derivatives. After t_0 , the effect of HP- β -CD and SBE- β -CD were no longer visible at 10 % CHOL and above 10 % CHOL, the increase in the membrane permeability remained up to 24 h. The observations noted at time of incubation were the same at 4 and 24 h: a decrease in the effect of HP- β -CD and SBE- β -CD upon increasing CHOL content with the highest effect obtained at 25 % CHOL for both CDs. Comparing to CRYSMEB, a weaker effect was observed with HP- β -CD and SBE- β -CD, showing the lowest SRB release at the CD:DPPC ratio 100:1 after 24h with 100 % CHOL liposomes (12.91 and 14.32 % of SRB leakage of liposomes treated with HP- β -CD and SBE- β -CD respectively versus 20.88 % in case of CRYSMEB treated liposomes).

According to a recent biophysical study, HP- β -CD demonstrated an increase in the fluidity of DPPC liposomes through the interaction of HP- β -CD with the polar head group region and the acyl chains of DPPC [25]. Although the complex between HP- β -CD and CHOL has been previously characterized [26], this complex seems to be unstable. Thus, a better interaction would occur between HP- β -CD and DPPC rather than with CHOL which explain the obtained results. As for SBE- β -CD, it was reported that charged CDs could not interact with CHOL molecules and form inclusion complexes due to charge repulsion [27]. This could explain the results obtained for these two CDs. Hence, HP- β -CD and SBE- β -CD are active on CHOL poor membranes.

The effects of β -CD derivatives obtained in this study present a good correlation with biological membranes studies: indeed, the data indicate that the methylated β -CD derivatives with high degree of substitution (RAMEB in our study) possess the strongest CHOL extraction capacity and can subsequently achieve the highest solubilization of CHOL [28] while the low substituted derivatives (CRYSMEB in our case) were less cytotoxic and maintained the integrity of endothelial cells assuming a lower affinity to CHOL membrane compared to the other derivatives [24]. Furthermore, the hydroxypropyl substituents are bulkier and less hydrophobic than the methyl groups resulting in a lower CHOL solubilizing capacity and a weaker hemolytic activity for HP- β -CD [29]. Besides, the ionic β -CD derivatives are less effective in promoting CHOL extraction given that the charge decreases the affinity of CDs towards CHOL [28].

Table 4: The SRB release values obtained at time t_0 , 4 and 24 h for γ -CD treated liposomes at different CHOL content and different CD:DPPC molar ratios.

The SRB release value of blank liposomes was 0 % at t_0 for the different formulations.

Values are expressed as the means of three different measurements \pm SD. (* indicates a value of $p < 0.05$ statistically significant. The p values compare the membrane permeability in the presence of CD to that of the respective blank.)

Time	DPPC:CHOL (100:10)			DPPC:CHOL (100:25)			DPPC:CHOL (100:50)			DPPC:CHOL (100:100)		
	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h
Blank	0	43.64 \pm 1.84	48.34 \pm 1.73	0	12.60 \pm 1.28	15.01 \pm 1.35	0	3.13 \pm 0.79	4.17 \pm 1.26	0	2.41 \pm 0.70	5.15 \pm 1.35
γ -CD/DPPC 1/1	0.60 \pm 0.11	43.49 \pm 0.47	49.66 \pm 0.81	0.74 \pm 0.30	14.81 \pm 0.12	17.07 \pm 0.29	0.15 \pm 0.05	1.50 \pm 0.10	3.25 \pm 0.66	0.13 \pm 0.08	1.00 \pm 0.11	6.04 \pm 0.13
γ -CD/DPPC 10/1	2.57 \pm 0.54*	32.95 \pm 0.88	40.04 \pm 0.59	1.04 \pm 0.81	9.69 \pm 0.34	14.38 \pm 0.70	0.8 \pm 0.12	1.5 \pm 0.65	3.26 \pm 0.73	0.45 \pm 0.23	0.39 \pm 0.20	3.66 \pm 0.90
γ -CD/DPPC 100/1	6.90 \pm 1.56*	39.29 \pm 1.03	40.20 \pm 1.84	2.63 \pm 1.01*	23.18 \pm 0.89*	24.00 \pm 0.81*	2.70 \pm 0.53*	6.04 \pm 0.64*	10.19 \pm 0.26*	1.76 \pm 0.76	3.80 \pm 0.23*	6.01 \pm 0.69*

With regards to the native γ -CD, non-significant SRB leakage was obtained at the CD:DDPC ratios 1:1 and also 10:1 at the different incubation times (table 4). The permeability of liposome membranes was even reduced in the presence of γ -CD at the CD:DPPC molar ratio 10:1. At the CD:DPPC ratio 100:1, no permeabilizing effect was observed at 10 % CHOL content as obtained and discussed previously with α - and β -CD. A slight permeabilizing effect was visible at 25 % CHOL content with a SRB release of 24 % after 24h of incubation. This value is the lowest obtained among the studied CDs at this CHOL content. We also observed that the γ -CD's effect almost disappeared in CHOL rich membranes.

These observations are in agreement with published data where γ -CD always exhibited low vesicle leakage [30]. Actually, γ -CD was found to be less lipid specific than the remaining native CDs (α -CD and β -CD) [7] which implies that the interaction of γ -CD with DPPC would be not favorable. Considering its large cavity in comparison to α and β -CD, γ -CD is not able to extract properly lipid membrane components. This result confirms that the mechanism of CDs induced permeability is mainly attributed to the lipid extraction mediated by CDs resulting in the formation of a complex between the CD and the lipid membrane components, as reviewed by Nasr et al., 2020 [13]. Based on our results, γ -CD was active on CHOL poor membranes. Yet, its effect remains very weak compared to the studied CDs.

Considering the above discussed results, we can assume the dependency of the CD effect on the membrane permeability on three main parameters: the CHOL content, the CD concentration and type or more precisely its affinity towards lipid membrane components. At the CD:DPPC molar ratio 1:1, the studied CDs had no effect on the membrane regardless the CHOL content. Their effect occurred above this ratio and was thereafter strongly modulated by the CHOL content depending on the CD's affinity or interaction with lipid membrane components. At 10 % CHOL, an instant effect was only induced by the CDs and it soon disappeared due to the rapid membrane re-organization. Beyond 10 % CHOL, the effect of CDs in the presence of CHOL was mainly dependent on their type: a decrease in the CDs effect is observed with CDs displaying a preferential interaction with phospholipids whereas an enhancement of the CDs effect is obtained with CDs showing a high affinity towards CHOL membrane.

On the other hand, these results can serve in the study of CDs as drug delivery system. Actually, we found that mainly α -CD was the most potent in permeabilizing the 25% CHOL membranes

which represents the average CHOL content in biological membrane. This indicates that one should keep in mind when using this CD as a drug vector its ability to destabilize the membrane. On the other side, γ -CD which had a very weak effect on the membrane might be used as a vector due to the inability of γ -CD to mediate lipid membrane extraction. Other parameters should be taken into account though; such as the size of the drug, the formation constant of the CD/drug inclusion complex, etc.

4. Conclusions

In this work, we investigated the effect of CDs on the membrane permeability of DPPC liposomes composed of different CHOL contents at different CD:DPPC molar ratios. The obtained data revealed the dependency of the CD's induced permeability on three main factors: the CHOL content, the CD concentration and the CD type interpreted by their ability to extract lipid membrane components. No effect was observed for the CD:DPPC molar ratio 1:1 on the membrane permeability for all the CDs. At the ratio 10:1, the CD effect was mainly visible at 25% CHOL content. At the highest CD:DPPC ratio (100:1), α -CD, CRYSMEB, HP- β -CD and SBE- β -CD (known to extract or to interact with membrane phospholipids) showed the strongest effect at 25 % CHOL, this effect was reduced with CHOL content increase in membranes. β -CD and RAMEB (known to extract CHOL) demonstrated the strongest effect at 100 % CHOL. Finally, γ -CD which is less lipid specific showed the weakest effect on the membrane that was only clearly visible at CHOL poor membranes (25% CHOL). Accordingly, CHOL poor membranes were more sensitive to α -CD, γ -CD, CRYSMEB, HP- β -CD and SBE- β -CD at the CD:DPPC molar ratios above 10:1 whereas CHOL rich membranes were sensitive to β -CD and RAMEB at the highest CD:DPPC molar ratio (100:1). Also, the CD's effect was time dependent since an instant permeabilizing effect was observed with the various formulations and this effect increased with time to reach a plateau after 24 h of incubation above 10 % CHOL.

These results contribute to the better understanding of the CD – membrane interaction and may be very useful in the choice of these CDs as a delivery system. Furthermore, these results may help in the development of the combined delivery system “drug-in-cyclodextrin-in-liposomes” (DCL) where CD/drug inclusion complexes are in contact with the membrane. The choice of CD in such system does not only depend on the drug affinity towards the CD cavity, but should also take into

consideration the affinity of the selected CD towards membrane lipids, the CD/phospholipid molar ratio and the CHOL content in the membrane.

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Chapitre 5 : Effet des complexes d'inclusion CD/invité sur la perméabilité membranaire des liposomes DPPC pauvres et riches en CHOL

Introduction

L'interaction des CDs avec la membrane a été largement étudiée ces dernières décennies ; en particulier, l'effet perméabilisant de différentes CDs a été démontré. Cet effet est principalement lié à la capacité des CDs d'extraire les lipides membranaires (phospholipides et ou CHOL) : en fait, certaines CDs (comme l' α -CD) présentent une affinité pour les phospholipides alors que d'autres (comme la β -CD et ses dérivés méthylés) présentent une meilleure affinité pour le CHOL et favorisent son extraction de la membrane. En revanche, il existe des CDs dont l'effet n'est pas spécifique des lipides (c'est le cas de la γ -CD). Nous venons également d'élucider l'effet du CHOL sur la perméabilité induite par les CDs et, suivant les résultats obtenus, nous avons classé les CDs en deux catégories : la première regroupe les CDs qui perméabilisent mieux les membranes pauvres en CHOL (ce sont l' α -CD, la CRYSMEB, la HP- β -CD et la SBE- β -CD) et la deuxième catégorie renferme les CDs qui induisent un effet perméabilisant significatif sur les membranes riches en CHOL (la β -CD et la RAMEB).

À part leur effet sur la membrane, les CDs sont employées en tant que vecteurs : en effet, ils sont capables de lier dans leur cavité interne hydrophobes, et de façon réversible via des interactions non-covalentes, diverses classes de molécules lipophiles (Liu and Guo, 2002). Il en résulte alors des complexes d'inclusion CD/invité où les propriétés physico-chimiques et/ou biologiques de la molécule encapsulée sont modifiées. De ce fait, les CDs améliorent considérablement la solubilité aqueuse des molécules incluses dans leur cavité, ainsi que leur biodisponibilité et leur stabilité ; elles permettent aussi d'assurer une libération prolongée et contrôlée des principes actifs (PAs) (Abarca et al., 2016; Bonnet et al., 2015; Nuchuchua et al., 2009). Plusieurs études ont également souligné la capacité des CDs à augmenter non seulement la solubilité du PA mais elle favorise aussi la pénétration de celui-ci à travers la membrane biologique, sous certaines conditions précises. La molécule invitée ou le PA doit être suffisamment lipophile pour traverser la bicouche lipidique comme il devrait être bien soluble dans la couche d'eau entourant la membrane (Muankaew and Loftsson, 2018). En vertu de ses propriétés d'inclusion, les CDs sont bénéfiques

pour le développement du système mixte “drug-in-cyclodextrins-in-liposomes” (DCL) qui repose sur l’encapsulation des complexes d’inclusion dans des liposomes combinant ainsi les avantages des deux systèmes d’encapsulation (Gharib et al., 2015).

De ce fait, la dernière partie de cette thèse sera consacrée à l’étude de l’effet de la formation de complexes d’inclusion CD/invité sur la perméabilité des membranes riches et pauvres en CHOL. Deux CDs ont été choisies : la HP- β -CD qui est active sur les membranes pauvres en CHOL et la RAMEB qui est active sur les membranes riches en CHOL. Le thymol a été choisi comme molécule invitée possédant une constante de formation (K_f) égale à 1448 M^{-1} pour le complexe d’inclusion HP- β -CD/thymol et 3337 M^{-1} pour le complexe RAMEB/thymol. L’effet de ces deux complexes sera évalué sur la perméabilité des membranes liposomales formées de DPPC:CHOL aux rapports molaires 100:25 et 100:100. L’objectif de ce travail est d’élucider les facteurs influençant la perméabilité induite par des complexes d’inclusion de stabilités différentes.

Pour ce faire, des liposomes formés de DPPC et CHOL au rapport molaire 100 :25 et 100 :100 et encapsulant la SRB ont été préparés. Les complexes d’inclusion HP- β -CD/thymol et RAMEB/thymol ont été préparés et lyophilisés. Ensuite, les suspensions liposomales ont été mélangées avec des CDs libres (15 mM) et des complexes d’inclusion CD/thymol à l’état solide. Le rapport CD:DPPC dans les deux cas est de 100:1. De même, des liposomes traités par le thymol (à une concentration égale à sa solubilité aqueuse) ont été testés. Le suivi du relargage de la SRB des liposomes blancs et des liposomes traités a été mené à $37 \text{ }^\circ\text{C}$ pendant 24 h.

Les résultats obtenus montrent que le thymol seul exposé aux liposomes 100 et 25 % CHOL à un ratio thymol:DPPC 33:1 a présenté un effet perméabilisant prononcé dans le deux cas. Cet effet perméabilisant est surtout dû à la haute concentration du thymol utilisée, qui est 132 fois supérieure à celle utilisée dans notre étude précédente où le thymol a pu perméabiliser les membranes uniquement entre 10 et 50 % CHOL.

Quant aux complexes d’inclusion CD/thymol, nous avons remarqué que les complexes d’inclusion RAMEB/thymol ont exercé un effet perméabilisant plus faible que la somme de l’effet du thymol libre et de la RAMEB libre ensemble. Un effet protecteur s’est alors produit à partir de t_0 et il est maintenu tout au long de la période d’incubation. Pourtant, cet effet est légèrement plus prononcé

à 100 % CHOL. Ce complexe semble alors protéger la membrane de l'effet du thymol et de la RAMEB libres.

En ce qui concerne le complexe d'inclusion HP- β -CD/thymol, deux comportements différents sont obtenus selon le type de membrane : à 100 % CHOL, le complexe induit un effet perméabilisant supérieur à celui obtenu en présence de la HP- β -CD seule. Vu que la HP- β -CD seule perméabilise faiblement ce type de membrane, on peut assumer que l'effet perméabilisant du complexe est dû aux molécules libres de thymol. Aucun effet protecteur n'a été observé au début de l'incubation. Un effet protecteur faible apparaît ultérieurement (après 4 h) révélant que l'équilibre entre les complexes d'inclusion et les molécules libres d'une part et la membrane de l'autre part s'est établi. À 25 % CHOL, la HP- β -CD seule perméabilise effectivement la membrane. Un effet protecteur a été observé avec les complexes d'inclusion HP- β -CD/thymol à partir de t_0 . Ainsi, l'inclusion protège dans ce cas la membrane contre l'effet de la CD.

En considérant les constantes de formation des deux complexes d'inclusion préparés, le complexe RAMEB/thymol est plus stable en solution que le complexe HP- β -CD/RAMEB. Ceci explique davantage l'effet protecteur prononcé exercé par le complexe d'inclusion RAMEB/thymol : le thymol est bien maintenu dans la cavité de la CD, ce qui protège alors la membrane de l'effet de la CD et du thymol. Ces résultats révèlent la dépendance de l'effet perméabilisant des complexes d'inclusion de leur stabilité d'une part et de la nature de la CD et son effet sur la membrane d'autre part.

Pour consolider nos résultats, il serait intéressant de choisir, avec les mêmes CDs, des PA ayant des valeurs différentes de constantes de formation (des complexes d'inclusion plus stable pour la HP- β -CD et moins stables pour la RAMEB) afin d'établir une relation entre la constante de formation du complexe d'inclusion et son effet sur la membrane. Ceci contribuerait à mieux comprendre le mécanisme d'action des complexes d'inclusion non encore élucidé.

Ce chapitre sera présenté sous forme d'un article en cours de préparation.

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CD/guest inclusion complex affects DPPC / cholesterol liposomes permeability depending on the CD type: application to thymol.

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Abstract

Cyclodextrins (CDs) are cone-shaped molecules with a hydrophilic outer surface and a hydrophobic central cavity. They are known as suitable carriers and solubilizing agents for lipophilic drugs improving their physicochemical properties including their stability. CDs are also known to increase the membrane permeability by extracting lipid components of the membrane (phospholipids and/or cholesterol (CHOL)). The aim of this work was to study the effect of two CDs (the hydroxypropyl- β -cyclodextrin (HP- β -CD) and the randomly methylated β -cyclodextrin (RAMEB)) in their free form and as CD/thymol inclusion complex on the membrane permeability. Therefore, liposomes composed of DPPC:CHOL at the molar ratios (100:25 and 100:100) and encapsulating the fluorescent probe, sulforhodamine B (SRB), were prepared by the reverse phase evaporation method. The CD/thymol inclusion complexes were prepared, freeze-dried. Liposomes were added to free CDs and CD/drug inclusion complexes at a CD:DPPC molar ratio 100:1. The membrane permeability was monitored by following the release of SRB over time at 37 °C. Our results showed that thymol at a concentration of 5 mM had a comparable strong permeabilizing effect on the both types of membranes. HP- β -CD/thymol inclusion complex with a low formation constant (K_f) had a very slight protective effect at equilibrium on the membrane permeability of 100 % CHOL liposomes. However, the same inclusion complex protected the membrane from the permeabilizing effect of HP- β -CD and thymol at 25% CHOL. On the other side, RAMEB/thymol inclusion complex with high K_f demonstrated a lower permeabilizing effect on the membrane, when compared to the sum of the effects of free RAMEB and free thymol at 25 and 100 % CHOL. Greater protection was obtained with 100% CHOL membranes. Hence, we can assume that when a guest molecule comes inside the cavity of the CD, the latter is no longer able to exert its effect on the membrane and so a weaker effect is more likely to occur. Thereby, our results showed that the protective effect of the inclusion complex depends on its stability, the CDs type and the membrane composition. Further studies are being conducted to establish a relationship between the inclusion complex formation constant and the membrane induced permeability.

Keywords: cyclodextrins, formation constant, inclusion complex, liposomes, membrane permeability, thymol

1. Introduction

Cyclodextrins (CDs) make up a family of cyclic oligosaccharides that are obtained from the enzymatic degradation of starch (Crini, 2014). Native CDs contain six (α -CD), seven (β -CD) or eight (γ -CD) D-glucopyranose units, linked by α (1-4) glycosidic bonds. They were subjected to chemical modifications (alkylation, hydroxypropylation, sulfonation, etc.) leading to various CD derivatives with improved water solubility compared to the native CD (Sharma and Baldi, 2016).

CDs are ring molecules featuring a cage-like structure with a hydrophobic cavity associated to a hydrophilic surface. As a result of their molecular shapes, CDs can form inclusion complexes with a wide range of hydrophobic molecules through noncovalent host-guest interactions. Such inclusion complexes improve the physicochemical properties of the host molecules. Indeed, numerous studies reported that CDs complexation enhanced the aqueous solubility and improved the physical stability and biological activity of poorly soluble agents such as essential oils and their individual components (Azzi et al., 2017; Kfoury et al., 2015, 2016, 2017b, 2018) and other phytochemical compounds including caffeic acid (Kfoury et al., 2019), nootkatone (Kfoury et al., 2017a), and others.

The effect of free CDs on the membranes was largely studied; they are known to increase the membrane permeability. This behavior is mainly attributed to their ability to extract lipid membrane components (Nasr et al., 2020). A recent work has investigated the effect of CDs on the membrane permeability of liposomes made of DPPC and different cholesterol (CHOL) contents. We previously demonstrated that at a high CD concentration (CD:DPPC molar ratio 100:1), the randomly methylated β -CD (RAMEB) displayed a permeabilizing effect that increased considerably with increasing CHOL content, whereas the hydroxypropyl β -CD (HP- β -CD) had an opposite behavior with a weak permeabilizing effect that decrease upon CHOL increase (Submitted manuscript in "Journal of steroids and molecular biochemistry"). Accordingly, we were interested to evaluate the effects of the both CDs encapsulating an active agent in their hydrophobic cavities. Thymol, an essential oil component, was selected to form CD/drug inclusion complexes with RAMEB and HP- β -CD. Actually, a study on the effect of a series of essential oil components on the permeability of liposome membranes was carried out using a mixture of DPPC and various CHOL content. Among the tested compounds, thymol exerted a permeabilizing effect

that decreased upon increasing CHOL content and had no effect on the stable formulation DPPC:CHOL (100:100) with a thymol:DPPC molar ratio of 25:100 (Nasr et al., 2021).

Although a great number of published articles deals with the effect of CDs on the membrane, the effect of CD/drug inclusion complex on the membrane is not entirely understood. In the aqueous media containing the CD/drug inclusion complex, the complex, the free CD, and the free drug coexist and interact with the medium components. The equilibriums depend on the stability of the complex and the interactions of the various species with the medium components.

Our study aims to investigate the effect of CD/thymol inclusion complexes on the permeability of liposome membranes made of DPPC:CHOL at the molar ratio 100:25 and 100:100. These two formulations were chosen with respect to the behavior of RAMEB and HP- β -CD on the membrane permeability stated earlier. Therefore, liposomes encapsulating the fluorescent dye, sulforhodamine B (SRB), were prepared by the reverse phase evaporation method followed by extrusion. Then, the CD/thymol inclusion complexes were prepared and freeze-dried. Finally, liposomes were added to free CDs and CD/drug inclusion complexes at a CD:DPPC molar ratio 100:1. The membrane permeability was assessed by the vesicle leakage of SRB during 24 h.

2. Materials and methods

2.1. Materials

The randomly methylated- β -CD (RAMEB, DS = 12.9) was provided from WackerChemie (Germany). The hydroxypropyl- β -CD (HPBCD, DS = 5.6) was provided by Roquette Frères (Lestrem, France). Dipalmitoylphosphatidylcholine (DPPC) ($\geq 99\%$) and trizma base (buffer reagent) were purchased from Sigma-Aldrich, Switzerland. Triton X-100, sodium chloride (NaCl) and Sephadex G25 gel were purchased from Sigma-Aldrich, Belgium. Ammonium molybdate, hydrogen peroxide, potassium dihydrogenophosphate, sodium sulfite, sodium bisulfite, chloroform and methanol were purchased from Sigma-Aldrich, Germany. Cholesterol ($\geq 99\%$) and sulforhodamine B were purchased from Sigma-Aldrich, USA. Thymol was provided by ACROS Organics, USA. The 4-amino-3-hydroxyl-1-naphtalene sulfonic acid was purchased from Fluka, India. Sulfuric acid was purchased from ACROS Organics, Belgium and diethyl ether was purchased from VWR-Prolabo Chemicals, Belgium.

2.2. Liposome preparation

Two liposome formulations were prepared: they were composed of DPPC:CHOL at molar ratios of 100:25 and 100:100.

The SRB-loaded liposomes were prepared by a reverse phase evaporation method following the same method described by (Nasr et al., 2021). Then, they underwent extrusion through polycarbonate filters membranes (Avanti Polar Lipids, Switzerland) of decreasing pore sizes resulting in a homogenous mixture of large unilamellar vesicles (LUVs). The liposome suspensions were centrifuged (2 hours, 15 000 rpm, 4 °C), and purified by a molecular sieve chromatography (using a Sephadex G25 gel filtration column). A Tris HCl buffer (0.1 M, pH 7.4) containing 150 mM NaCl was used for elution and liposome storage.

2.3. Preparation of solid RAMEB/thymol and HP- β -CD/thymol inclusion complexes for the permeability study

An excess of thymol (15 mg) was added to an aqueous solution of CDs (RAMEB and HP- β -CD) at a concentration of 15 mM. This concentration was chosen based on previous phase solubility studies where a concentration of 15 mM of CD allowed the highest complexation efficiency of thymol inside the cavity of the CDs (Hammoud et al., 2020; Kfoury et al., 2016).

Solutions were then stirred (125 rpm for 24 h at 26 °C). They were filtered through a 0.45 μ m pore size cellulose acetate filter to eliminate the undissolved thymol, frozen at - 80 °C and lyophilized using a freeze dryer (Martin Christ, Germany). The final dry powders were stored at 4 °C prior to analysis.

Determination of the thymol content in the solid complex

Aqueous solutions of HP- β -CD/thymol and RAMEB/thymol (5 mg/mL) were prepared from the obtained lyophilizates. Dilutions of 25 and 50 times were performed in ultrapure water for the CD/thymol inclusion complex solutions followed by sonication at 4 °C during 10 min. Finally, the amount of thymol in the complex was determined by HPLC as previously described by (Hammoud et al., 2019a).

Briefly, HPLC analysis was accomplished using an analytic column (C18: 5 cm × 4.6 mm (Agilent)) at a wavelength of 204 nm. Methanol and water mixture (70:30) was used as a mobile phase for all samples. The injection volume was 20 µl. The flow rate was set at 1 mL/min.

2.4. Study of the effect of CD/thymol inclusion complexes on the membrane permeability of liposomes

The membrane permeability study is monitored by following the release of SRB from liposomes at the same DPPC concentration. In fact, SRB is a self-quenching probe; it causes the quenching of the fluorescence when encapsulated at high concentration within the aqueous core of liposomes. The release of SRB from the vesicles results in an increase in the fluorescence and the inhibition of the auto-quenching effect due to the dilution of SRB outside the vesicles (Nasr et al., 2020).

2.4.1. Preparation of liposomal sample for the permeability study

Prior to the permeability study, the liposome suspensions obtained after purification was concentrated by centrifugation (15 000 rpm, 4 °C, 2h). The pellet was dissolved in a small volume (3mL) of the Tris HCl NaCl buffer. The resulting suspensions were assayed for DPPC concentration by Bartlett method (Bartlett, 1959) and used for the preparation of samples for the permeability study.

Aliquots of the concentrated suspensions of liposomes DPPC:Chol (100:25 and 100:100) were removed and diluted to prepare liposomal suspensions with a concentration of DPPC of 0.15 mM. The diluted liposome suspensions were added to the powders of CDs (11.25 mg and 10.2 mg of HP-β-CD and RAMEB respectively) and to those of CD/thymol inclusion complexes (12.55 mg and 11.24 mg of lyophilized HP-β-CD/thymol and RAMEB/thymol inclusion complex respectively). The concentration of CDs in the final volume was 15 mM, and the CD:DPPC molar ratio was of 100:1. A tube containing liposomes alone was used as a blank.

Additionally, in order to test the effect of free thymol alone on the permeability of liposomes, a solution of thymol prepared in methanol (15.2 mg/mL or 10 mM) was added to the diluted suspensions of liposomes in order to obtain a concentration of 5 mM corresponding to the aqueous solubility of thymol reported in published studies (Hammoud et al., 2020). The methanol did not

exceed 5% of the final sample volume. Liposomes treated with the same volume of methanol were used as a blank for these mixtures. The samples were prepared in triplicate.

All the mixtures were incubated at 37 °C for 48 h, and the effect of CDs alone, thymol, and CD/thymol inclusion complex on the membrane permeability were studied by monitoring the release of SRB from liposomes.

2.4.2. Fluorescence measurements by fluorescence spectroscopy

In our study, the fluorescence signals were measured at times 0, 2, 4, 6 and 24. Fluorescence measurements were performed using a spectrofluorimeter (Hitachi F-7000 spectrofluorimeter) at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The emission spectrum was recorded in the range 540-700 nm. The permeability of liposomal membranes was evaluated by calculating the percentage of fluorophore released as a function of time, using the following equation:

$$\text{Percentage of SRB release} = \frac{F_t(\text{sample}) - F_t(\text{blank})}{F_{\text{max}}} \times 100 \quad (\text{equation 3})$$

Where $F_t(\text{sample})$ stands for the fluorescence intensity of the sample at time t , $F_t(\text{blank})$ is the fluorescence intensity of the corresponding blank at time t and F_{max} is the maximum fluorescence intensity indicating the complete release of the encapsulated SRB from liposomes; it is determined following solubilization of the vesicles in Tris HCl NaCl buffer containing 1% of the nonionic detergent Triton X-100. It is worth mentioning that F_{max} values for each formulation are the same for blank and treated liposomes as we are working with the same DPPC concentration.

As for the blank, the SRB release values were calculated according to this equation:

$$\text{SRB release percentage} = \frac{F_t(\text{blank})}{F_{\text{max}}} \times 100 \quad (\text{equation 4})$$

2.5. Statistical analysis

The results of the kinetic study obtained by fluorescence spectroscopy were statistically analyzed by the Student test (t-test). The values are considered significant if $p < 0.05$. The p values compare the permeabilizing effect of the tested agent to the membrane permeability of blank liposomes.

3. Results and discussion

3.1. Characterization of solid CDs/thymol inclusion complexes

With respect to the phase solubility study conducted by (Hammoud et al., 2020; Kfoury et al., 2016), the concentration of 15 mM of CDs, achieving a better solubility for thymol was chosen to prepare the inclusion complexes for the release study. Thus, the RAMEB/thymol and HP- β -CD/thymol inclusion complexes were prepared, freeze-dried and characterized for their thymol content using HPLC. Eugenol was used as an internal standard. The retention times of eugenol and thymol were 3.55 ± 0.1 min and 5.4 ± 0.1 min, respectively.

Using the equation of the thymol calibration curve, the amount of thymol in the solid CD/thymol inclusion complexes was calculated; it was found to be 0.104 mg thymol / mg of HP- β -CD/thymol inclusion complex and 0.106 mg thymol / mg of RAMEB/thymol inclusion complex. These values are in agreement with that obtained with (Kfoury et al., 2016) corresponding to a high complexation efficiency of thymol with RAMEB and HP- β -CD.

3.2. The membrane permeability study: the SRB release kinetics from DPPC:CHOL liposomes treated with free CDs, thymol and CD/thymol inclusion complexes

For the permeability study, liposomes were added to free CDs and CD/thymol inclusion complexes in solid state (to avoid dilution of the liposomal solution) and samples were incubated at 37 °C during 24 h.

First, we studied the effect of the solid CD/thymol inclusion complexes on the permeability of DPPC:CHOL (100:100). Table 1 shows the SRB release values of the samples at different times.

Table 1: The SRB release values (%) at different times (0, 2, 4, 6 and 24 h) for DPPC:CHOL (100:100) liposomes treated with either free CDs, free thymol or CDs/thymol inclusion complex at the CD:DPPC molar ratio 100:1. Values are expressed as the means of three measurements \pm SD.

*indicates a value of $p < 0.05$ statistically significant (the p values compare the permeabilizing effect of the tested agent to the membrane permeability of blank liposomes).

Samples	SRB release % (0 h)	SRB release % (2 h)	SRB release % (4 h)	SRB release % (6 h)	SRB release % (24 h)
Blank	3.55 ± 0.46	3.87 ± 0.19	3.75 ± 0.26	4.14 ± 0.20	5.30 ± 0.35
Blank thymol (MeOH)	10.03 ± 0.78*	14.49 ± 0.58*	14.58 ± 0.98*	14.23 ± 0.31*	15.86 ± 0.22*
Thymol 5mM	39.50 ± 0.68*	40.65 ± 0.48*	43.23 ± 1.77*	44.23 ± 1.03*	45.20 ± 1.72*
HP-β-CD 15mM	8.14 ± 0.41*	17.68 ± 1.13*	18.93 ± 0.26*	18.87 ± 1.20*	20.23 ± 1.02*
HP-β-CD/Thymol	49.52 ± 1.92*	58.59 ± 1.31*	57.59 ± 1.41*	56.41 ± 0.95*	58.62 ± 0.98*
RAMEB 15mM	43.39 ± 0.66*	53.84 ± 1.65*	53.03 ± 1.65*	54.49 ± 0.73*	57.81 ± 1.56*
RAMEB/Thymol	38.34 ± 1.24*	59.06 ± 0.36*	59.79 ± 1.02*	60.86 ± 0.17*	65.54 ± 1.17*

As expected and with agreement with (Kaddah et al., 2018), our results showed that the liposomes made of DPPC:CHOL at a molar ratio 100:100 form a stable formulation with a SRB release around 5 % after 24 h of incubation. On the other hand, liposomes treated with 5% methanol (used as the blank for thymol treated liposomes) showed an increase of permeability compared to the blank (with the highest value 15.86 ± 0.22 at 24h). Remarkably, free thymol had a striking effect on membrane permeability with an instant SRB release value of 39.50 ± 0.68 %. SRB release % increased very slightly during the first hours of incubation to reach thereafter a plateau at 4h hours of incubation. In our previous work, thymol did not affect the permeability of DPPC:CHOL (100:100) liposomes (Nasr et al., 2021). Nevertheless, the amount of thymol used in our study was 132 times higher than that used in the previous work (with a thymol: DPPC molar ratio of 0.25:1 comparing to 33:1 in the present work) which explains the significant effect of thymol on the permeability. Actually, we have recently demonstrated that the presence of the hydroxyl group plays a key role in the permeabilizing effect promoted by thymol. At low concentration, this EO component induced the permeability of the membranes consisting of 10 to 50 % CHOL (Nasr et al., 2021). At higher concentration, thymol was able to permeabilize more stable membranes such as 100 % CHOL liposome membranes.

Regarding CDs, the results are in good agreement with a previous study (submitted manuscript). The both CDs at the used concentration (CD:DPPC molar ratio of 100:1) showed an instant permeabilizing effect that increased with time to reach a plateau after 4 h of incubation. At t_0 , HP- β -CD exhibited a weak permeabilizing effect on CHOL rich membranes (8.14 ± 0.41 % of SRB release at 0 h) compared to RAMEB which promoted the instant release of 43.39 ± 0.66 % of SRB from liposomes upon CD-membrane interaction. These values increased and reach a plateau after 2 hours of incubation (20.23 ± 1.02 % at 24 h for HP- β -CD treated liposomes and 57.81 ± 1.56 % at 24 h for RAMEB treated liposomes). Known for its ability to extract CHOL from the membrane (from raft and non-raft domains), RAMEB is able to better permeabilize CHOL rich membranes than HP- β -CD.

RAMEB/thymol inclusion complex showed a permeabilizing effect lower than free RAMEB. Effectively, RAMEB induced the instant leakage of 43.39 ± 0.66 % of SRB comparing to 38.34 ± 1.24 % for RAMEB/thymol complex. We assume that this reduction is mainly due to the occupation of the cavity of RAMEB by the thymol, leaving the CD less able to extract CHOL and permeabilize the membrane instantaneously. As for HP- β -CD/thymol inclusion complex, it displayed a significant permeabilizing effect on liposome membranes with an instant SRB release of 49.52 ± 1.92 %. Considering the weak instant effect of HP- β -CD on this formulation, the obtained result with the inclusion complex of HP- β -CD may be due to the release of thymol from the inclusion complex. However, the difference observed between the effect of HP- β -CD and its inclusion complex and RAMEB and its inclusion complex could be explained by their different formation constant values. According to (Kfoury et al. 2016), the formation constants of HP- β -CD/thymol and RAMEB/thymol inclusion complexes are 1488 and 3337 M^{-1} respectively. Taking into account these values, we can assume that RAMEB/thymol inclusion complex is more stable than HP- β -CD/thymol inclusion complex.

After t_0 , the SRB release values of CDs complexes increased slowly to reach a plateau after 2h of incubation. This may be due to the dissolution of the inclusion complex creating an equilibrium between free CD, free thymol molecules and CDs/thymol complex.

Permeability study on liposomes DPPC/CHOL 100/100

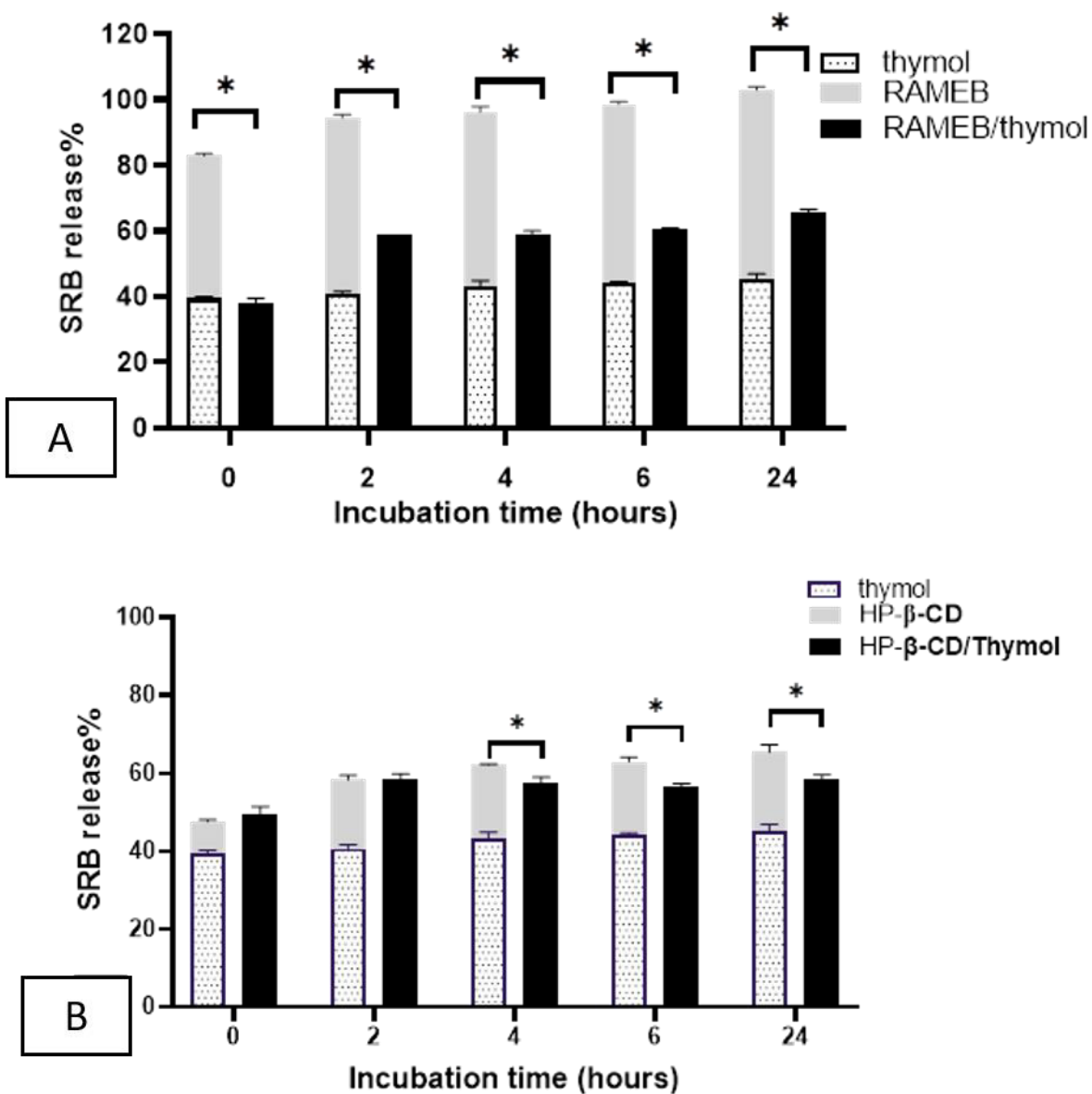


Figure 1: Histograms showing : (A) the stacked values of SRB release % from DPPC:CHOL (100:100) liposomes treated separately with thymol (5 mM) and RAMEB (15 mM) versus the SRB release value from liposomes treated with the RAMEB/thymol inclusion complex at different incubation time and (B) the stacked values of SRB release % from DPPC:CHOL (100:100) liposomes treated separately with thymol (5 mM) and HP-β-CD (15 mM) versus the SRB release

value from liposomes treated with the HPBCD/thymol inclusion complex at different incubation time.

(* indicates a value of $p < 0.05$ statistically significant. The p values compare the sum of the SRB release values of thymol and CDs to that of the corresponding complex)

Furthermore, we choose to analyze the effect of the inclusion complex on the permeability of the membrane by comparing the sum of the SRB release values from liposomes treated with free CD and free thymol with that of the CD/thymol inclusion complex at each incubation time. A protective effect is exerted by the complex formation when the release values of CD/thymol inclusion complex treated liposomes are lower than the sum of those of free CD and free thymol treated liposomes.

As observed in figure 1(A), a protective effect was instantly observed with RAMEB/thymol inclusion complex where the SRB release value from liposomes treated with RAMEB/thymol complex was 2 times lower than that of the sum of the SRB release percentage of both thymol and RAMEB treated liposomes. After t_0 , the protective effect was maintained over time. It decreased slowly to form a plateau after 2 h of incubation. As for HP- β -CD, no protective effect was observed at t_0 with HP- β -CD/thymol complexes (figure 1(B)). Afterwards, a slight significant protective effect starts to occur at 4h when the equilibrium is established.

Accordingly, we can state that the effect of CD/thymol inclusion complex is related to the stability of the complex in solution and to the CDs type and its effect on the membrane. Indeed, in the presence of HP- β -CD/thymol inclusion complex having a low formation constant and with HP- β -CD exerting a weak effect on the membrane permeability at high CHOL content, weak a slight protective effect appeared at equilibrium at 4 h of incubation. Conversely, in the presence of RAMEB/thymol inclusion complex having high formation constant and with RAMEB displaying a pronounced effect on the membrane rich in CHOL, a strong protective effect could be observed

Furthermore, to emphasize our results, we investigated the effect of the CDs/thymol inclusion complex on DPPC:CHOL (100:25) liposome membranes which also reflects the biological membrane. In fact, HP- β -CD was found to be active on CHOL poor membranes. The results are presented in table 2 and figure 2.

Table 2: The SRB release values (%) at different times (0, 2, 4, 6 and 24 h) for DPPC:CHOL (100:25) liposomes treated with either free CDs, free thymol or CDs/thymol inclusion complex at the CD:DPPC molar ratio 100:1. Values are expressed as the means of three measurements \pm SD.

*indicates a value of $p < 0.05$ statistically significant (the p values compare the permeabilizing effect of the tested agent to the membrane permeability of blank liposomes).

Sample	SRB release % (0 h)	SRB release % (2 h)	SRB release % (4 h)	SRB release % (6 h)	SRB release % (24 h)
Blank	14.90 \pm 0.94	18.65 \pm 0.68	19.11 \pm 0.43	19.79 \pm 0.59	19.74 \pm 0.83
MeOH	17.28 \pm 0.26	20.76 \pm 0.12	21.56 \pm 0.50	21.70 \pm 0.66	23.21 \pm 0.90
Thymol 5mM	36.13 \pm 0.43*	37.43 \pm 0.20*	40.61 \pm 1.43*	40.15 \pm 1.09*	40.62 \pm 0.62*
HP- β -CD 15mM	19.83 \pm 0.17*	27.63 \pm 1.76*	27.56 \pm 1.59*	27.55 \pm 0.97*	27.94 \pm 1.20*
HP- β -CD/Thymol	39.60 \pm 1.35*	43.85 \pm 0.44*	45.59 \pm 0.70*	43.73 \pm 1.46*	44.96 \pm 1.81*
RAMEB 15mM	27.87 \pm 1.77*	38.95 \pm 2.14*	40.75 \pm 0.44*	41.13 \pm 0.55*	41.17 \pm 1.86*
RAMEB/Thymol	42.64 \pm 1.24*	45.53 \pm 2.13*	50.76 \pm 0.43*	47.08 \pm 0.74*	47.13 \pm 0.50*

As demonstrated previously, CHOL poor membranes are more permeable and less stable than CHOL rich membranes: indeed, liposomes comprising 25 % CHOL were leakier than 100 % CHOL membranes (19.74 \pm 0.83 % of SRB release from 25 % CHOL membranes versus 5.30 \pm 0.35 % from 100 % CHOL liposomes within 24 h of incubation). Here also, the methanol affected the membrane permeability at the used percentage. We should note that the effect of methanol was more significant at 100 % CHOL despite the stability of this formulation. A possible reason for that might be the interaction between the OH group of CHOL and that of methanol which makes the 100 % CHOL membranes more sensitive to the effect of methanol than the 25 % CHOL membranes. The results obtained for free CDs were consistent with our previous work: the HP- β -CD exerted (at the CD:DPPC molar ratio 100:1) a significant permeabilizing effect at 25 % CHOL that was higher than that observed at 100 % CHOL with an instant SRB release of 19.83 \pm 0.17 % (compared to 8.14 \pm 0.41 at 100 % CHOL) that increased to reach a plateau after 2 h of incubation. Besides, the effect of RAMEB was weaker at 25 % CHOL: 27.87 \pm 1.77 % of SRB were released instantaneously reaching 41.17 \pm 1.86 % after 24 h of incubation. Unexpectedly, the effect of free thymol on 100 % CHOL membranes was comparable to that obtained with 25 % CHOL

membranes. This could be possibly due to the large concentration of thymol where the maximal effect on the membrane was reached.

Permeability study on liposomes DPPC/CHOL 100/25

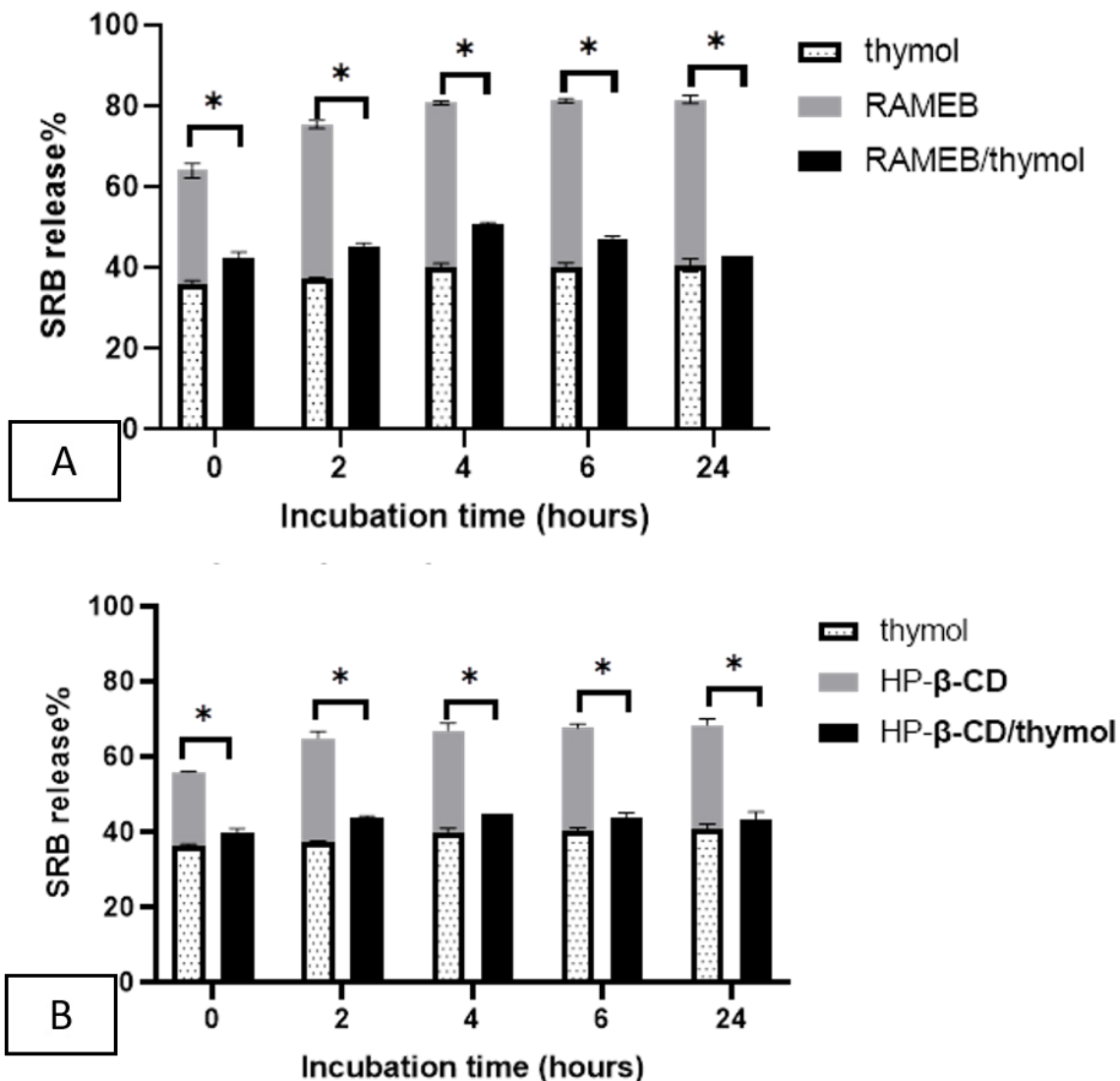


Figure 2: Histograms showing : (A) the stacked values of SRB release % from DPPC:CHOL (100:25) liposomes treated separately with thymol (5 mM) and RAMEB (15 mM) versus the SRB release value from liposomes treated with the RAMEB/thymol inclusion complex at different incubation time and (B) the stacked values of SRB release % from DPPC:CHOL (100:25) liposomes treated separately with thymol (5 mM) and HP-β-CD (15 mM) versus the SRB release

value from liposomes treated with the HPBCD/thymol inclusion complex at different incubation time.

(* indicates a value of $p < 0.05$ statistically significant. The p values compare the sum of the SRB release values of thymol and CDs to that of the corresponding complex)

With regards to the CD/thymol inclusion complex, as illustrated in figure 2(A), the RAMEB/thymol inclusion complex also exhibited a protective effect on 25 % CHOL membranes at t_0 that persisted throughout the incubation. However, this effect was weaker comparing with that observed at 100 % CHOL.

The HP- β -CD/thymol inclusion complex displayed again a significant permeabilizing effect with an instant release of 39.60 ± 1.35 % of SRB from liposomes (table 2). Interestingly, as it can be seen in figure 2(B), a protective effect was promoted by HP- β -CD/thymol inclusion complex at t_0 with 25 % CHOL membranes (the instant SRB release value from the complex was 1.5 times less than that obtained by summing the SRB values of free thymol and HP- β -CD). The protective effect was maintained with time. Here, the occupation of the HP- β -CD cavity by thymol reduced also the effect of free HP- β -CD on CHOL poor membranes.

Many authors have addressed the parameters influencing the effect of CDs and CD/guest inclusion complex on the stability of the membrane. For instance, the guest local concentration (at the membrane) appears to modulate the effect of CD/guest inclusion complex on the membrane: Angelini et al. reported that the dissociation of the β -CD/ibuprofen inclusion complex alters the integrity of POPC vesicles depending on the concentration of ibuprofen: at low local concentration, the intercalation of the released drug (from the complex) into the membrane would stabilize the bilayer against the effect of β -CD while a high local concentration of the drug may lead to an alteration of the membrane integrity due to the dual effect of ibuprofen and β -CD (Angelini et al., 2017). This might explain the considerable increase of membrane permeability of liposomes exposed to a high concentration of thymol, either as free molecules or as an inclusion complex. Other studies have additionally shown that the complexation of CDs can preserve the integrity of the membrane because the latter is protected from the effect of free CDs (Gasbarri et al., 2010), as we obtained in the case of RAMEB at 100 % CHOL and HP- β -CD at 25 % CHOL. CD/CHOL inclusion complexes are used for the preservation of the sperm cells integrity allowing the transfer of CHOL from the CDs cavity into the sperm plasma membrane (Hammoud et al.,

2019b). On the other side, CDs are frequently found in pharmaceutical products, owing to their ability to increase the solubility of poorly soluble drugs. However, its application in this field is not restricted to their solubilizing effect; CDs are also used as permeation enhancers since they promote the diffusion of the drug across the membrane (Muankaew and Loftsson, 2018). In this context, CDs inclusion complex will enhance the permeation of lipophilic drugs by increasing the permeability of the unstirred water layer surrounding the membrane and by decreasing the permeability of the membrane. This will allow the drug entrapped in the cavity of the CDs to be soluble in the unstirred water layer and to reach the membrane in which it can easily partition due to its hydrophobicity (Dahan and Miller, 2012; Muankaew and Loftsson, 2018). Hence, in this context our results can serve to better understand the effect of an inclusion complex on membrane permeability. This effect is thus dependent on the guest type and its concentration, the CDs effect on the membrane, the CDs concentration, the stability of the complex and the membrane composition.

4. Conclusion

In this work, we investigated the effect of the inclusion of thymol in the cavity of RAMEB and HP- β -CD on the permeability of liposomes comprising low and high CHOL content. Our results allowed us to correlate between the stability of the inclusion complex, the CDs affinity to lipid membrane and their effect on the membrane. Indeed, in the presence of CD/guest inclusion complexes with low formation constants, two scenarios could be presented; when the CD has a weak permeabilizing effect on the membrane, a slight protective effect could be seen at equilibrium. However, when the CD has a strong permeabilizing effect on the membrane, a more pronounced protective effect could be observed starting t_0 . On the other side, in the presence of CD/guest inclusion complexes with high formation constant, the inclusion will protect the membrane against the effect of the CD and the guest molecule. Thus, a high protective effect is observed instantaneously whereas the CDs had a low or high permeabilizing effect on the membrane. These findings seem to be valuable for the development of DCL as an efficient delivery system. Therefore, we suggest to carry out the membrane permeability study with different CD/guest inclusion complex possessing various formation constants in order to examine whether a relationship could be established between the formation constant of the inclusion complex and the effect on the membrane.

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Partie IV : Discussion Générale et Perspectives

Depuis plusieurs décennies, le développement de systèmes d'encapsulation et de délivrance des médicaments constitue un sujet de préoccupation de la communauté scientifique. Cette recherche vise l'édification d'un système de vectorisation qui garantit la protection des principes actifs d'une part et le contrôle de la délivrance de ces derniers à leur site d'action d'autre part. Plusieurs vecteurs ont été mis au point ; parmi ceux-ci, les liposomes demeurent le système membranaire le plus communément utilisé et qui doit sa popularité à sa simplicité, sa biodégradabilité et sa non toxicité comparant à d'autres systèmes (Akbarzadeh et al., 2013; Laouini et al., 2012). La composition des membranes liposomales jouent un rôle clé dans la stabilité de ce vecteur ; en particulier, le CHOL est un constituant lipidique capable de modifier les propriétés membranaires telles que la perméabilité membranaire (Kaddah et al., 2018a; Subczynski et al., 2017).

L'objectif de ce travail de thèse consiste à étudier la perméabilité membranaire de liposomes contenant différents taux de CHOL en présence d'agents actifs et de nanovecteurs. Des liposomes de types LUVs formés de DPPC et de CHOL à différents rapports molaires et encapsulant la SRB ont été choisis comme modèle membranaire propice pour l'étude de la perméabilité. Celle-ci a été évaluée en suivant au cours du temps le relargage de la SRB des liposomes blancs et des liposomes traités par les agents testés et incubés à 37 °C. Des composés d'HE de structures différentes ont été choisis comme agents actifs et les CDs comme nanovecteurs pour nos études.

Ce travail se divise en trois grandes parties :

- La première partie aborde l'effet du CHOL sur la perméabilité induite par les composés d'HE de différentes structures chimiques ;
- La seconde partie rapporte l'effet du CHOL sur l'effet perméabilisant des CDs libres présentant des affinités différentes vis-à-vis des composants membranaires ;
- La troisième partie étudie l'effet des complexes d'inclusion sur la perméabilité des membranes pauvres et riches en CHOL.

L'ensemble des résultats obtenus dans ce travail de thèse sont résumés dans le tableau 1 ci-dessous.

Tableau 1 : Tableau résumant l'effet des composés HE, des CDs et des complexes d'inclusion CD/thymol sur la perméabilité membranaire des liposomes formés de DPPC à différents taux de CHOL.

Liposomes au rapport molaire DPPC:CHOL	Les composés d'HE au rapport molaire DPPC:HE (100 :25)				Les CDs au rapport molaire CD:DPPC (100:1)								Complexes d'inclusion CD/thymol	
	Log P > 4 with OH	Log P < 4 with OH	Log P > 4 without OH	Log P < 4 without OH	Les CDs natives			Les dérivés de la β -CD				HP- β -CD / thymol	RAMEB / thymol	
					α -CD	β -CD	γ -CD	CRYSMEB	HP- β -CD	RAMEB	SBE- β -CD			
100:10	+ ^a	+ ^b	-	+ ^e	*	*	*	*	*	*	*	*		
100:25	-	+ ^c	-	+ ^f	+++	+	++	+++	+++	+	+++	+	+ ^g	++ ^g
100:50	-	+ ^d	-	-	++	++	+	++	++	++	++	++		
100 :75	-	-	-	-										
100:100	-	-	-	-	+	+++	+	+	+	+	+++	+	+++ ^h	+ ⁱ

Légende :

(+) : effet perméabilisant

(-) : absence d'effet perméabilisant

* : effet perméabilisant instantané qui disparaît plus tard

a : le nérolidol

b : eugénol, géraniol, guaiacol, linalool, menthol, thymol

c : eugénol, géraniol, guaiacol, linalool, menthol, thymol

d : eugénol, guaiacol, linalool, menthol, thymol

e : acétate d'eugényl, acétate de linalyl, trans-anéthole, α -phellandrene, α -terpinène, γ -terpinène

f : trans-anéthole, α -terpinène, γ -terpinène

g : effet protecteur à partir de t0

En premier lieu, nous avons caractérisé des liposomes blancs formés de DPPC et de différents taux de CHOL en termes de composition membranaire. Nous avons trouvé que le rapport molaire DPPC:CHOL déterminé expérimentalement ne correspond pas au rapport théorique surtout pour les formulations riches en CHOL : les formulations théoriquement formées de DPPC:CHOL (100:10; 100:25; 100:50; 100:75 et 100:100) sont effectivement formées de DPPC:CHOL (100:11; 100:20; 100:40; 100:43; 100:55). Ceci est principalement dû à l'étape d'extrusion qui chasse les molécules de CHOL des membranes liposomales, ce qui est en accord avec d'autres études (Ibarguren et al., 2010).

Ensuite, dans cette même étude, nous avons testé l'effet d'une série de 22 composés d'HE sur la perméabilité des membranes liposomales contenant différents taux de CHOL. En effet, il est bien connu que le CHOL module la fluidité et la perméabilité membranaire (Kaddah et al., 2018a); cependant, l'effet de ce composant membranaire sur la perméabilité qui pourrait être induite par des molécules bioactives telles que les composés d'HE n'a pas été encore envisagé. Tenant compte de la capacité de la plupart de ces composés hydrophobes à modifier la fluidité et la perméabilité membranaire en interagissant avec les composants de la membrane (la tête polaire des phospholipides par exemple) (Burt, 2004; Cristani et al., 2007; Gharib et al., 2018, 2017; Ultee et al., 2002), il nous a paru pertinent de vérifier si leur effet sur la membrane est influencé par la présence du CHOL. Ainsi, les liposomes sont exposés individuellement à ces différents composés de HE de structures variées selon un rapport molaire DPPC:HE de 100:25. L'étude de perméabilité montre que, parmi les 22 composés testés, 13 molécules possèdent un effet perméabilisant des membranes formées de 10 % CHOL (par ordre décroissant d'effet perméabilisant : nérolidol > eugénol > linalool > thymol > guaiacol > géraniol > α -phellandrène > trans-anéthole > α -terpinène > acétate de linalyl > menthol > γ -terpinène > acétate d'eugényl). Ces composés sont alors qualifiés d'agents perméabilisants de la membrane ; quant aux 9 molécules (anisole, estragole, isoeugénol, acétate de bornyl, camphor, β -caryophyllène, camphène, bornéol and *p*-cymène) qui ne montrent pas d'effet significatif sur la perméabilité membranaire, on suggère que leur activité antimicrobienne pourrait être dû à un autre mécanisme d'action. Une relation structure activité a été établie entre les différentes structures et nous avons constaté que la présence du groupe hydroxyle, la linéarité de la molécule ainsi que la présence d'un système conjugué constituent des facteurs structuraux modulant la perméabilité membranaire. En effet, la plupart des composés (phénoliques et non phénoliques) exerçant un effet significatif sur la perméabilité membranaire

présentent dans leur structure un groupement hydroxyle. Ceci est en accord avec plusieurs études qui ont souligné l'importance du groupe OH et son implication dans l'activité biologique des composés d'HE (Burt, 2004; Ultee et al., 2002). À titre d'exemple, il a été démontré que le thymol, un composé phénolique, s'intègre dans la membrane cytoplasmique bactérienne via l'interaction entre son groupe OH et les têtes polaires des phospholipides. Ceci conduit à la perte de l'intégrité de la membrane et à une augmentation de sa perméabilité (Helander et al., 1998; Lambert et al., 2001; Walsh et al., 2003). Des expériences de DSC menées précédemment dans notre équipe de recherche sur des membranes liposomales formées de DPPC ont également montré que l'eugénol et le thymol agissent comme des « impuretés de substitution » remplaçant les lipides de la bicouche (Gharib et al., 2017, 2018a). Le thymol a même produit une séparation de phase, ce qui indique la présence de régions pauvres et d'autres riches en thymol dans la membrane (Cristani et al., 2007; Gharib et al., 2018a). Ces données pourraient alors expliquer l'absence d'effet perméabilisant chez les composés d'HE ne possédant pas de groupe OH dans leur structure, comme l'estragole, le trans-anéthole et l'anisole. La substitution du groupement hydroxyle chez certains composés (eugénol et linalool) par un autre groupement tel que l'acétate peut soit réduire (cas de l'acétate d'eugényl), soit masquer (cas du l'acétate de linalyl) l'effet perméabilisant du composé. À part l'importance du groupement OH, d'autres facteurs structuraux semblent être impliqués dans l'interaction entre les composés d'HE et la membrane : nous avons remarqué que la présence d'un système conjugué dans la structure de la molécule peut inhiber l'effet de cette dernière sur la membrane (cas de l'isoeugénol, un isomère de l'eugénol et du p-cymène). De plus, les composés non-phénoliques linéaires (nérolidol, linalool, géranol) ont montré un effet perméabilisant plus important que le composé cyclique (le menthol) de ce groupe. Néanmoins, certains résultats obtenus ne sont pas en accord avec la littérature. En fait, l'isoeugénol présente une activité antibactérienne contre nombreuses souches bactériennes (Zhang et al., 2017) ; des études ont montré qu'il induit le relargage de la calcéine des LUVs formées d'extraits lipidiques d'*E. Coli* (Hyldgaard et al., 2015) et qu'il s'insère profondément dans la membrane (Gharib et al., 2017). Dans notre étude, l'isoeugénol n'affecte pas la perméabilité membranaire ; ceci pourrait s'expliquer par la présence du CHOL dans nos formulations liposomales en comparaison avec les autres études où le CHOL était absent. Concernant l'effet du contenu de CHOL, les résultats confirment que le CHOL réduit l'effet perméabilisant des molécules de HE étant donné que le nombre de composés capables de perméabiliser les membranes diminue avec l'augmentation du

taux de CHOL membranaire jusqu'à 40 %. Au-delà de ce pourcentage, aucune molécule n'a gardé son effet perméabilisant. Ceci s'explique par la stabilité conférée à la membrane en présence du CHOL ; ce qui rend la membrane moins sensible à l'action des molécules bioactives.

Cette étude fait partie d'un ensemble de travaux effectués dans notre équipe de recherche où l'effet des agents actifs tels que les corticostéroïdes (Kaddah et al., 2018b), les cucurbitacines (Habib et al., 2013) sur la membrane a été étudié en variant la composition membranaire des liposomes et particulièrement le taux de CHOL. Ainsi, vient s'ajouter ce travail avec les différents composés d'HE qui a permis d'établir une relation entre l'hydrophobicité de la molécule et son effet perméabilisant et ceci dépendamment de la présence du groupe OH dans sa structure. En fait, les constituants d'HE très hydrophobes et portant le groupe OH exercent un effet perméabilisant uniquement sur les membranes renfermant de faible taux de CHOL (10 % CHOL). La raison principale pour ceci serait la compétition qui peut avoir lieu entre les composés hydrophobes d'HE et le CHOL au niveau de la membrane (Sherry et al., 2013): plusieurs auteurs ont rapporté une diminution du pourcentage d'encapsulation des composés hydrophobes dans des vésicules lipidiques riches en CHOL (Jaafar-Maalej et al., 2010; Sherry et al., 2013; Varona et al., 2011). En absence du groupement OH, le composé hydrophobe ne pourrait pas perméabiliser la membrane. En passant aux molécules moins hydrophobes, nous avons remarqué que quand l'hydrophobicité de la molécule portant une fonction alcool diminue, l'effet sur la membrane persiste à des taux de CHOL plus élevés et disparaît à partir de 40 % CHOL. En revanche, si la molécule n'a pas de fonction alcool, certaines molécules d'HE pourraient légèrement perméabiliser les membranes formées de 21 % CHOL.

Toutes ces données pourraient servir pour l'encapsulation des composés HE dans des liposomes pour des applications dans des domaines divers, notamment dans l'industrie cosmétique, alimentaire, etc. (Asbahani et al., 2015; Bakkali et al., 2008; Burt, 2004). En se basant sur ce travail, le choix de la formulation liposomale visant l'encapsulation d'un composé de HE devrait tenir compte de : la structure de la molécule, son effet sur la perméabilité membranaire en présence du CHOL ainsi que son hydrophobicité.

Par ailleurs, l'effet des CDs libres sur la perméabilité membranaire des liposomes formés de DPPC et différents taux de CHOL a été élucidé. Les CDs sont des oligosaccharides cycliques constitués de plusieurs unités de glucopyranoses ; il s'agit de nanovecteurs largement utilisés pour solubiliser

et véhiculer les molécules hydrophobes dans différents domaines, principalement dans le domaine pharmaceutique et biomédical (Arima et al., 2017; Bilensoy, 2011). Les CDs communes sont les CDs natives : l' α -CD, la β -CD et la γ -CD formées de 6, 7 et 8 unités de glucopyranoses respectivement. Vu la limite de solubilité aqueuse des CDs natives et particulièrement celle de la β -CD, des dérivés de CDs ont été synthétisés par modification chimique des groupements polaires des CDs (Gharib et al., 2015; Szente, 1999). L'attention accordée aux CDs en tant que nanovecteurs revient à leur capacité de former des complexes d'inclusion avec des molécules hydrophobes améliorant ainsi la solubilité et la biodisponibilité des molécules invitées ou des PA. Elles favorisent également la pénétration de ces molécules à travers la membrane (Muankaew and Loftsson, 2018). D'autre part, les CDs peuvent perturber la stabilité de la membrane en assurant l'extraction des composants lipidiques membranaires tels que les phospholipides et/ou le CHOL (Hammoud et al., 2019). A titre d'exemple, l' α -CD peut extraire les phospholipides tels que la DPPC et le PI alors que la β -CD et ses dérivés méthylés interagissent avec le CHOL membranaire et favorise son extraction (Beseničar et al., 2008; Castagne et al., 2009; Hammoud et al., 2019; Kilsdonk et al., 1995). Par suite, les CDs sont classés en tant qu'agents perméabilisants de la membrane (Nasr et al., 2020).

Dans notre étude, nous avons investigué l'effet du taux de CHOL sur la perméabilité induite par les CDs. Les liposomes encapsulant la SRB ont été traités par les CDs selon des rapports molaires CD:DPPC égaux à : 1:1; 10:1 et 100:1. L'étude de perméabilité a démontré la dépendance de l'effet des CDs de différents facteurs: le temps d'incubation, le taux de CHOL au niveau de la membrane, la concentration des CDs ainsi que le type de CD interagissant avec les composants lipidiques de la membrane. Toutes les CDs ont présenté un effet perméabilisant instantané ; l'effet de la γ -CD étant le plus faible. Pourtant, cet effet n'est pas visible pour le rapport CD:DPPC 1:1. Il apparaît à partir du ratio 10:1 et augmente avec la concentration des CDs. Ceci est en accord avec de nombreuses études démontrant que l'effet des CDs sur la membrane est proportionnelle à sa concentration (Hammoud et al., 2019).

Le CHOL semble moduler l'effet des CDs selon l'affinité de chacune pour les constituants de la membrane. Effectivement, la présence du CHOL réduit l'effet perméabilisant des CDs possédant une affinité élevée pour les phospholipides tels que la DPPC et sont capables de l'extraire ; celles-ci comprennent l' α -CD, la HP- β -CD, la CRYSMEB et la SBE- β -CD. En effet, une étude a montré

que l' α -CD est capable d'extraire le DPPC et former un complexe de structure particulière où les chaînes acyles de la DPPC sont incorporées dans la cavité de la CD formant un anneau de rotaxane (Puskás and Csempez, 2007). Il a été aussi démontré que l' α -CD forme un complexe insoluble avec la DPPC (Nishijo and Mizuno, 1998). Pareillement pour la HP- β -CD, un dérivé hydroxypropylé de la β -CD, une étude de fluidité membranaire a révélé la capacité de cette CD à interagir avec les têtes polaires de la DPPC, engendrant un effet fluidifiant de la membrane (Gharib et al., 2018b). Bien que le complexe entre la HP- β -CD et le CHOL ait été caractérisé, ce complexe est instable (Williams III et al., 1998). Par conséquent, une meilleure interaction aurait lieu entre la HP- β -CD et la DPPC. Quant à la SBE- β -CD, il a été rapporté que les CD chargées n'interagissent pas avec les molécules de CHOL et par la suite ne forment pas des complexes d'inclusion avec celui-ci en raison de la répulsion de charge (Stella and Rajewski, 2020). La CRYSMEB, un dérivé méthylé de la β -CD, a exercé un effet comparable aux dérivés HP- β -CD et SBE- β -CD, mais cet effet était contradictoire avec celui du dérivé méthylé de la CD, la RAMEB. Nos résultats pour la CRYSMEB et la RAMEB sont conformes à ceux obtenus par Piel et al., 2007, qui ont postulé que le faible degré de substitution du CRYSMEB diminuait son affinité pour les composants lipidiques de la membrane; ce qui résulte en un effet perturbateur plus faible par rapport au RAMEB et aux autres CD méthylés (Piel et al., 2007). C'est ainsi que nous avons constaté que ces CDs sont actives sur les membranes pauvres en CHOL.

Au contraire, la présence du CHOL augmente considérablement l'effet perméabilisant des CDs ayant une affinité élevée pour le CHOL et pouvant l'extraire ; il s'agit dans notre cas de la β -CD et de la RAMEB. En fait, la capacité de la β -CD d'extraire le CHOL a été soulignée dans de nombreuses études (Hammoud et al., 2019; Nasr et al., 2020), ce qui explique l'augmentation de l'effet perméabilisant de cette CD à des taux élevés de CHOL. Cependant, cet effet n'a pas été observé au rapport 10:1 qui est également considéré comme une concentration élevée en β -CD : cela nous amène à supposer que l'extraction de CHOL médiée par la β -CD est probablement réalisée à un rapport molaire CD:DPPC supérieur à 10:1. De même, un effet comparable a été obtenu avec la RAMEB, un dérivé méthylé de la β -CD. Celle-ci est également capable d'extraire le CHOL des membranes synthétiques et biologiques, induisant par la suite la perméabilisation membranaire (Castagne et al., 2009; Hatzi et al., 2007; Piel et al., 2007).

En ce qui concerne la γ -CD, celle-ci ne présente pas d'affinité pour les composants lipidiques de la membrane (Hammoud et al., 2019; Nishijo et al., 2000); ceci se traduit par son effet perméabilisant très faible.

Ainsi, les CDs peuvent être regroupées en deux classes : les CDs actives sur les membranes pauvres en CHOL (l' α -CD, la CRYSMEB, la HP- β -CD, la SBE- β -CD) et les CDs actives sur les membranes riches en CHOL (la β -CD et la RAMEB).

Par ailleurs, les CDs sont des vecteurs puissants des molécules hydrophobes. Caractérisées par une structure de forme conique, les CDs possèdent une surface hydrophile et une cavité hydrophobe pouvant se lier à des molécules lipophiles et formant des complexes d'inclusion CD/invité (Gharib et al., 2015). Ces complexes d'inclusion présentent plusieurs avantages qui ont été détaillés dans les paragraphes précédents. Outre ces avantages, la capacité des CDs à former des complexes d'inclusion CD/invité a permis de développer un nouveau système d'encapsulation « DCL » qui consiste à encapsuler des complexes d'inclusion dans la cavité aqueuse des liposomes. Ce système d'encapsulation prometteur combine les avantages offerts par les liposomes et les CDs : l'encapsulation du PA dans la cavité des CDs réduit l'effet de ces derniers sur la membrane, augmente la stabilité des vésicules et améliore l'encapsulation des molécules hydrophobes dans les liposomes (Chen et al., 2014). De plus, les liposomes chargés de molécules libres et de complexes d'inclusion permettant l'encapsulation à la fois du médicament libre et du médicament complexé, présentent une amélioration significative de l'intensité et de la durée de l'effet thérapeutique du médicament (Gharib et al., 2015).

Pour toutes ces raisons, nous avons décidé de clôturer ce travail par une étude de l'effet des complexes d'inclusion CD/thymol sur la perméabilité membranaire des liposomes formés de DPPC et de CHOL au rapport molaire 100:25 et 100:100 couvrant ainsi les membranes pauvres et riches en CHOL. Après avoir exposé les liposomes aux CDs libres et complexés au thymol (à l'état de poudre), l'étude de perméabilité membranaire rapporte que la stabilité du complexe d'inclusion ainsi que l'effet de la CD sur la membrane selon le taux de CHOL influencent fortement l'effet des complexes d'inclusion sur la membrane. En effet, la HP- β -CD génère avec le thymol un complexe de constante de formation inférieure à celui formé avec la RAMEB ; ce dernier est alors plus stable à l'équilibre. Nos résultats montrent que l'inclusion du thymol dans la cavité de la RAMEB protège la membrane de l'effet de la CD libre et du thymol pris séparément à 25 et 100

% CHOL, mais plus significativement à 100 % CHOL puisque la RAMEB perméabilise principalement les membranes riches en CHOL. D'autre part, l'inclusion du thymol dans la cavité de la HP- β -CD protège légèrement la membrane de l'effet du thymol libre à 100 % CHOL et protège la membrane de l'effet de la HP- β -CD et du thymol à 25 % CHOL (étant donné que la HP- β -CD agit sur les membranes pauvres en CHOL). Tenant compte des valeurs de la constante de formation des complexes d'inclusion HP- β -CD/thymol et RAMEB/thymol, nous pourrions alors expliquer l'effet protecteur plus important exercé par le complexe RAMEB/thymol tout au long de la période d'incubation vu que le thymol est maintenu dans la cavité de la RAMEB plus que dans celle de la HP- β -CD. Ces résultats révèlent alors une corrélation entre la stabilité du complexe (déterminée par la valeur de sa constante de formation) et son effet sur la membrane.

En conclusion, l'ensemble de ces résultats souligne que la composition membranaire, en particulier le taux de CHOL, ainsi que les caractéristiques structurales et physico-chimiques des agents actifs tels que les composés d'HE sont les principaux facteurs modulant la perméabilité membranaire induite par ces agents. Par ailleurs, en ce qui concerne les CDs, la géométrie de la CDs (taille de la cavité), sa concentration, son affinité pour les composants lipidiques de la membrane et le taux de CHOL sont les facteurs principaux affectant la perméabilité induite par ces nanovecteurs. L'inclusion d'une molécule dans la cavité de la CD pourrait protéger la membrane de l'effet de la CD ou de la molécule et ceci dépend de la nature de la CD, son affinité pour les composants lipidiques de la membrane (et alors son effet sur la membrane) et de la constante de formation du complexe mis en jeu.

Ce travail de thèse s'inscrit dans la thématique et les travaux de recherche menés dans notre laboratoire. En fait, à part l'étude de l'effet des composés bioactifs sur les propriétés de la membrane et sa stabilité, notre équipe de recherche vise à encapsuler ces agents dans un système d'encapsulation adéquat qui garantit le maintien et la protection des principes actifs dans un système membranaire stable d'une part et le contrôle de la libération de ces molécules à leur site d'action d'autre part. Les résultats apportés par ce travail nous permettraient alors de pousser nos recherches vers l'optimisation des formulations liposomales encapsulant des composés HE dans un système binaire formé de DPPC et de CHOL. Le taux de CHOL sera choisi en se référant à nos résultats, dépendamment de la structure de la molécule, son hydrophobicité, son effet sur la membrane et l'effet modulateur du CHOL. Nous pourrions également déterminer la concentration

des composés hydrophobes de HE qui devrait être utilisée lors de l'encapsulation sans altérer la stabilité de la membrane.

De surcroît, nous nous intéressons à développer davantage le système d'encapsulation mixte « DCL » qui est déjà mis au point dans notre laboratoire. Ce travail pourrait alors être complété par une étude de l'effet des complexes d'inclusion CD/invité de différentes constantes de formation avec la HP- β -CD et la RAMEB ainsi que d'autres CDs afin d'établir une corrélation entre la constante de formation du complexe qui détermine sa stabilité et l'effet du complexe sur la membrane selon le taux de CHOL membranaire. Ceci nous permettrait de mieux comprendre le comportement des complexes d'inclusion dans le système mixte DCL et de faire le bon choix de la CD et de la molécule invitée pour former des complexes d'inclusion stables en vue de les encapsuler dans la cavité des liposomes sans perturber la stabilité de ce système. Ainsi, nous pourrions comparer l'efficacité des deux systèmes d'encapsulation (les liposomes conventionnels et le système DCL) en termes de libération de la molécule encapsulée.

De même, nous souhaitons examiner si une relation peut exister entre la perméabilité membranaire et la fluidité membranaire, sachant que de nombreuses molécules qui fluidifient la membrane sont capables aussi de la perméabiliser.

Toutes ces études contribueront à la compréhension des mécanismes ayant lieu au niveau de la membrane et modifiant sa stabilité et participeront à l'édification de systèmes d'encapsulation et de délivrance appropriés avec les liposomes.

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