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Encapsulation and Targeting of Biofunctional Molecules in Nanoliposomes: Study of Physico-Chemical Properties and Mechanisms of Transfer through Liposome Membrane

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Université de Lorraine

École Nationale Supérieure d'Agronomie et des Industries Alimentaires

Ecole Doctorale Sciences et Ingénierie des Ressources, Procédés, Produits, Environnement

(RP2E)

Laboratoire d'Ingénierie des Biomolécules (LIBio)

Spécialité : Procédés Biotechnologiques et Alimentaires

THESE

Présenté devant L'Université de Lorraine

Pour obtenir le grade de Docteur de l'Université de Lorraine

par

Mme Behnoush MAHERANI

Encapsulation et vectorisation de molécules biofonctionnelles par des nanoliposomes : Etude des propriétés physico-chimiques et des mécanismes de transfert à travers la membrane liposomale

Encapsulation and Targeting of Biofunctional Molecules in Nanoliposomes: Study of Physico-Chemical Properties and Mechanisms of Transfer through Liposome Membrane

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Ma petite puce, Qu'elle m'a donnée l'amour et la vie, celle qui m'a fait oublier la fatigue et les contrariétés, celle qui m'a supportée et comprise tout au long du chemin. La plus adorable des petites filles.

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La Terre des Aryens

Le berceau des plus anciennes civilisations et cultures.

Le symbole de l'épanouissement de la Littérature, la Philosophie, la Médecine, l'Astronomie, les Mathématiques et l'Art.

A l'origine de la première Charte des droits de l'Homme

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The life is the unique artistic scene
Every one sings your song and leaves the scene
The scene remains forever
It's the best, the song which people remember forever

“Jaleh Esfahani - Iran “

GLOSSAIRE

AFM	Atomic Force Microscopy
BBB	Blood Brain Barrier
BLM	Bilayer Lipid Membrane
CADs	Cationic Amphiphilic Drugs
CLSM or LSCM	Confocal Laser Scanning Microscopy
C_s^{-1}	Compressibility Modulus
D₂O	Deuterium Oxide
DAC	Dual Asymmetric Centrifugation
DCP	Di – Cethyl Phosphate
DHA	Docosahexaenoic Acid
DLS	Dynamic Light Scattering
D_m	Membrane Diffusion Coefficient
DOPC	1,2-Dioleoyl- <i>sn</i> -Glycero-3-Phosphocholine
DOTAP	Dioleoyl Trimethyl Ammonium Propane
DPPC	1,2-Dipalmitoyl- <i>sn</i> -Glycero-3-Phosphocholine
DPPG	Dipalmitoylphosphatidyl Glycerol
DPPE	Dipalmitoylphosphatidyl Serine
DSC	Differential Scanning Calorimetry
DE	Equilibrium Dialysis
ELSD	Evaporative Light Scattering Detectors
EPA	Eicosapentaenoic Acid
EPR	Electron Spin Resonance
HB	H-bonding
HBD	Hydrogen Bond Donors
HLB	Hydrophilic- Lipophilic Balance
HOMO	Highest Occupied Molecular Orbital
HPH	High-Pressure Homogenization method
HPLC	High-Performance Liquid Chromatography
HSDSC	DSC and High Sensitivity DSC
IC-AFM	Intermittent Contact Mode
ISCRPE	Improved Supercritical Reverse Phase Evaporation
K_A	Area Modulus
K_c	Bending Elasticity
K_p	Liposome/water Partition Coefficient
LC	Liquid-Condensed
LCFA	Long Chain Fatty Acid
LDE	Laser Doppler Electrophoresis
LE	Liquid-expanded
Log <i>D</i>	Distribution Coefficient
Log <i>P</i>	Partition Coefficient
LPO	Lipid Peroxidation
LUMO	Lowest Unoccupied Molecular Orbital

LUVs	Large Unilamellar Vesicles
MDOE	Mixture Design of Experiments
MDT	Magnetic Drug Targeting
MG	Malachite Green
MLV	Multilamellar Vesicles
MS	Mass Spectrometric
MVV	Multivesicular Vesicle
MW	Molecular Weight
NEFA	Non-Esterified Fatty Acids
NMR	Nuclear Magnetic Resonance
P₀	Permeability Coefficient
PBS	Phosphate Buffered Saline
PCS	Photon Correlation Spectroscopy
PDI	Polydispersity Index
PEG	Poly Ethylene Glycol
Pgp	P-glycoprotein
PLA	Poly Lactic Acid
PM-IRRAS	Polarization Modulation Infrared Reflection-Absorption Spectrometry
POPC	1-Palmitoyl-2-Oleoyl- <i>sn</i> -Glycero-3-Phosphocholine
P_s	Permeability
PSA	Polar Surface Area
PUFA	Polyunsaturated Fatty Acid
QSAR	Quantitative Structure–Activity Relationships
RES	Reticuloendothelial System
RMS	Root-Mean-Square
S/N	Signal-to-Noise
SA	Stearyl Amine
SAXS	Small Angle X-ray Scattering
SCRPE	Supercritical Reverse Phase Evaporation
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SHG	Second Harmonic Generation
SPR	Surface Plasmon Resonance
STED	Stimulated Emission Depletion Microscopy
SUV	Small Unilamellar Vesicles
T_c	Phase Transition Temperature
TEM	Transmission Electron Microscopy
TMA-DPH	1-(4-Trimethylammonium-Phenyl)-6-Phenyl-1,3,5-Hexatriene
TMR	Tetramethylrosamine
ULV	Unilamellar Vesicles
VPGs	Vesicular Phospholipid Gels

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- 1- **Maherani, B.**, Arab-Tehrany, E and Linder. M; **2011**, Mechanism of bioactive transfer through liposomal bilayers, *Current Drug Targets*, Apr 1; 12(4): 531-45.
- 2- **Maherani, B.**, Arab-Tehrany, E., Gaiani, C. and Linder, M; **2011**, Liposomes: A Review of Manufacturing Techniques and Targeting Strategies. *Current Nanoscience*, 7(3): 436-452.
- 3- **Maherani, B.**, Arab-Tehrany, E., and Linder, M.; **2011**, Optimization and Characterization of Liposome Formulation By Mixture Design, *Analyst*, 137: 773- 786.
- 4- **Maherani, B.**, Arab-Tehrany, E., Kheiriloom, A., Cleymand, F., Linder, M.; **2012**, Influence of lipid composition on physicochemical properties of nanoliposomes encapsulating natural dipeptide antioxidant L-carnosine, *Food chemistry*, 134 (2): 632-640.

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- 1- **Maherani, B.**, Arab-Tehrany, E, Kheiriloom, A., Korchowicz, B. Rogalska, E., and Linder. M; **2012**, Investigation of molecular interaction between calcein and lipid model membranes by Raman spectroscopy, Langmuir balance study and Differential scanning calorimetry, *BBA- Biomembrane*.
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- 1- Arab-Tehrany, E., Baravian, Ch., **Maherani, B.** Belhaj, N., Wang, X. and Kahn, C. J.F., Linder, M.; **2012**, Elaboration and characterization of nanoliposome made of soya; rapeseed and salmon lecithins: Application to cell culture, *Colloids and Surfaces B: Biointerfaces*, 95: 75-81.
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- **Maherani, B.** Arab-Tehrany, E., and Linder, M., **2012**, “Atomic force microscopy; a tool for investigation the effect of lipid composition on nanoliposomes characterizatio,” 103rd AOCS Annual Meeting & Expo. May 1-4, Long beach, California, USA (2012).
- **Maherani, B.**, Arab-Tehrany, E., and Linder, M., **2011**, “Characterization of Carnosine-encapsulated liposome as natural antioxidant “. 102nd AOCS Annual Meeting & Expo. May 1-4. 2011, Cincinnati, Ohio, USA.
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I. Introduction et objectifs de l'étude

Introduction

Les liposomes font l'objet de nombreuses investigations en matière de recherche dans les domaines pharmacologique, médical, mais aussi dans certaines applications biologiques et alimentaires en raison de leurs propriétés de vectorisation de biomolécules vers des cibles cellulaires d'intérêt, ou de relargage de principes actifs dans des matrices alimentaires. Les liposomes ont été synthétisés pour la première fois en Angleterre par Alec D. Bangham, qui a trouvé que des phospholipides en présence d'eau formaient des vésicules, en raison de leurs propriétés amphipathiques.

Les liposomes sont des structures sphériques composés de bicouches lipidiques encapsulant une partie de la phase aqueuse environnante. Principalement constitués de molécules amphiphiles, les phospholipides confèrent aux liposomes des propriétés d'auto-assemblage en milieux aqueux faisant de ces systèmes d'excellents vecteurs dans les domaines alimentaire, nutraceutique, cosmétique et pharmaceutique.

Les liposomes sont identifiés en fonction de leur taille, du nombre de multicouches et de leur méthode de préparation. On les dénomme vésicule unilamellaire (ULV pour Unilamellar Vesicles), de moins de 100 nm (Small Unilamellar Vesicles) et de plus de 100 nm (Large Unilamellar Vesicles). Structurés en multicouches lipidiques, ils se dénomment vésicules multilamellaires (MLV ou Multilamellar Vesicle), ou Multivesicular vesicle (MVV) lorsque plusieurs vésicules non concentriques sont encapsulées par une seule bicouche lipidique.

Les liposomes permettent de vectoriser et de relarguer simultanément et de façon progressive des biomolécules d'intérêt, de polarités différentes, vers des cibles cellulaires. Cet adressage spécifique est une propriété recherchée de ces liposomes, tout comme le relargage progressif, permettant de maîtriser la concentration du principe actif. Ceci ouvre de plus larges voies d'application, un dosage optimal évitant une surconcentration et limite le coût de la molécule à vectoriser.

Le relargage d'un principe actif sur une cible dépend de nombreux facteurs :

- la biomolécule doit être administrée en tenant compte de la concentration, du temps d'administration et de la cinétique de diffusion à la concentration thérapeutique.

- le principe actif doit rester chimiquement et physiquement stable dans sa formulation pendant un temps prédéfini.

- le choix de la méthode de libération doit être adapté à la nature de la molécule.

La quantité de principe actif délivrée à travers la bicouche lipidique dépend de la structure et de l'arrangement moléculaire des lipides. Le coefficient de partition de la biomolécule est aussi lié à la taille du liposome et aux contraintes engendrées par les structures vésiculaires.

La formulation du système de vectorisation permettra un relargage contrôlé de la molécule encapsulée en fonction de la perméabilité de la membrane, qui conditionne la pharmacocinétique du principe actif. Sur le plan moléculaire, le passage de petites molécules à travers la bicouche lipidique dépend de nombreux paramètres comme la perméabilité membranaire, les propriétés physicochimiques du principe actif et de la force des interactions du système membrane-ligand.

En dépit de l'importance du sujet, peu d'information sont actuellement disponibles sur le transfert de principes actifs à travers la membrane liposomale.

L'objectif de ce travail est d'étudier les interactions entre une molécule hydrophile et son vecteur par différentes méthodes physicochimiques utilisant la spectroscopie Raman, l'analyse thermique différentielle et les mesures de pressions interfaciales à l'aide d'une balance de Langmuir. L'étude du comportement de cette biomolécule s'effectuera sous la forme encapsulée dans une bicouche liposomale afin d'étudier les mécanismes de transfert à travers la membrane.

Ce travail se divisera en plusieurs parties prenant en compte la formulation du vecteur, l'étude de ses propriétés physicochimiques, les interactions avec le principe actif, ainsi que l'étude de transfert.

1 - Optimisation et caractérisation de la formulation liposomale:

L'optimisation de la formulation de la bicouche lipidique du liposome a été réalisée à partir d'un plan de mélanges. Une matrice de Scheffé a été générée à partir du logiciel NEMROD permettant de modéliser la formulation en phospholipides en un minimum d'essais. La calcéine, molécule hydrophile, a été choisie comme marqueur pour étudier les interactions liposome-principe actif et déterminer l'efficacité d'encapsulation, notamment par les techniques de microscopie confocale.

Dix mélanges de liposomes ont été générés par une matrice simplexe centroïde de Scheffé après hydratation d'un monofilm et procédé d'extrusion.

Les liposomes obtenus ont été ensuite caractérisés en termes de taille, potentiel zéta, température de transition de phase, fluidité, perméabilité et efficacité d'encapsulation.

Les résultats du plan de mélanges ont permis de trouver les conditions optimales permettant d'améliorer les propriétés physicochimiques préalablement citées.

2 - Influence de la composition lipidique sur les propriétés physicochimiques de nanoliposomes vectorisant un dipeptide antioxydant (L-carnosine) :

Le dipeptide L-carnosine a largement été étudié en raison de ses propriétés antioxydantes naturelles dans le domaine agro-alimentaire. L'encapsulation d'antioxydant sous forme liposomale représente une alternative à l'application directe de ce type de composés dans les aliments.

Dans ce travail, les différents phospholipides formulés (DOPC, POPC et DPPC) ont été caractérisés avant incorporation de la L-carnosine. Trois formulations liposomales ont été étudiées en termes d'efficacité d'encapsulation de ce principe actif par une approche précise de RMN protonique sans avoir à déstructurer physiquement les nanoliposomes formulés pour évaluer la quantité de L-carnosine encapsulée. La morphologie des systèmes de nanoliposomes unilamellaires de compositions différentes, a d'autre part été étudiée par microscopie à force atomique.

3- Etude des interactions moléculaires de la calcéine avec la bicouche liposomale :

Sur le plan moléculaire, les principes actifs capables de s'intégrer dans la bicouche lipidique peuvent entraîner des perturbations au niveau de la forme, de la taille et de la structure des liposomes. La localisation de la molécule dans la bicouche lipidique soulève un point d'intérêt en termes d'interactions avec son vecteur. En effet, les changements thermodynamiques observés au niveau de la membrane lipidique, consécutifs à l'intégration de la biomolécule, peuvent provoquer des perturbations conformationnelles. Ces changements ont été pris en compte dans la formulation lipidique permettant une libération progressive du principe actif. Une meilleure compréhension des interactions membrane –ligand conduirait à une meilleure maîtrise de leur libération dans les systèmes biologiques. Nous avons étudié les effets moléculaires de la calcéine sur les lipides membranaires composé de DOPC, POPC et DPPC, purs ou en mélanges. Des investigations par ATD, spectroscopie Raman et pressions de surface, ont permis d'optimiser le modèle de libération progressive de cette molécule. Les effets de l'intégration de la calcéine au niveau de la membrane ont pu être mis en évidence par analyse thermique différentielle. Les mesures de tension de surface à l'aide d'une balance de Langmuir ont permis de caractériser les variations de surface que la calcéine occupe au niveau d'une monocouche lipidique. Ces interactions ont été étudiées sur le système modèle composé de DOPC, POPC et DPPC purs, puis en mélanges. Ceci a été réalisé en mesurant les pressions de surface après compression de la monocouche, par microscopie à angle de Brewster et spectroscopie d'adsorption – réflexion en mode polarisé (PM IRRAS).

4 - Transfert du calcéine par la bicouche liposomale; Estimation du coefficient de partition de la calcéine dans un système de bicouche lipidique liposomal :

L'objectif a été de déterminer les propriétés de perméation dans un système liposomal pour élaborer un modèle de libération contrôlée d'un principe actif. La perméabilité de la calcéine au travers d'une membrane de liposome a été évaluée préalablement sur la base d'un système cinétique de premier ordre. Des liposomes élaborés à partir de phospholipides neutres ont été utilisés pour éliminer les contributions d'interactions électrostatiques entre les lipides membranaires et la calcéine chargée négativement. L'environnement ionique et la température ont été pris en considération sur la libération de cette molécule. L'optimisation par planification expérimentale a permis d'élaborer une formulation liposomale qui a été testée en conditions drastiques de pH (stomacal et intestinal), pour mesurer les coefficients de diffusion et de partition de la calcéine.

5 – Observation et Investigation de mécanisme de transfert du calcéine

Le but principal de cette étude était de déterminer les mécanismes de transfert du bioactif (comme calcéine) à travers les bicouches liposomales. Plusieurs approches ont été employées afin d'obtenir plus d'informations sur le transfert de calcéine par bicouche des liposomes. Nous avons suivi cette étude en utilisant des techniques différentes, y compris; génération de seconde harmonique (SHG), stimulé microscopie épuisement des émissions (STED). Nous avons également utilisé la microscopie électronique de transition (TEM), et la microscopie à force atomique (AFM) qui sont largement utilisés pour l'étude de la translocation à travers les bicouches bioactif.

Introduction

Liposomes were first made synthetically in England in 1961 by Alec D. Bangham, who found that phospholipids combined with water form a sphere because one end of each molecule is water soluble, while the opposite end is water insoluble. Liposomes are spherical, closed structures, composed of curved lipid bilayers, which enclose part of the surrounding solvent into their interior. The main constituents of liposomes are phospholipids, which are amphiphilic molecules containing water soluble, hydrophilic head section and a lipid-soluble, hydrophobic tail section. This property of phospholipids gives liposomes unique characteristics, such as self-sealing in aqueous media and makes them an ideal carrier system with applications in different fields including food, cosmetic, agriculture and pharmaceuticals.

Liposomes are classified based on vesicle size, number of lamella and preparation method, e.g. unilamellar vesicles (ULV), small unilamellar vesicles (SUV, less than 100nm) and large unilamellar vesicles (LUV, larger than 100nm). A multilamellar vesicle (MLV) is a liposome composed of a number of concentric lipidic bilayers. A vesicle composed of several non-concentric vesicles encapsulated within a single bilayer is known as a multivesicular vesicle (MVV).

In order to exert a bioactive agent's intended effect, it needs to be in physical contact with its physiological target. A possible approach to facilitate material transport into cells or target sites is the application of liposome. A significant advantage of liposome is that it can incorporate and release two materials with different solubilities simultaneously. Furthermore, targetability is another extremely useful characteristic of liposome. These particular properties make liposome to be useful in many applications due to its ability to increase the effectiveness of the encapsulated active agents and optimizing their dosage.

Targetability is an important attribute of the lipid vesicles. Targeting bioactive agents is necessary to obtain adequate concentration of bioactive at the target site for their optimum efficacy. Targeted release increases the effectiveness of bioactive, broadens their application range and ensures optimal dosage, thereby improving the cost-effectiveness of the product.

The goal of bioactive delivery system is also to administer a drug at a therapeutic concentration to a particular site of action for a specified period of time. The design of the final product for drug delivery depends upon different parameters. The drug must be administered by considering to some factors which effects on therapeutic action of the drug. These parameters include the site of action, the concentration of the drug at the time of administration, the period of time that drug must remain at a therapeutic concentration, and the initial release rate of the drug for controlled release systems. The drug must remain

physically and chemically stable in the formulation for a defined time. Finally, the choice of delivery method must indicate the effective administration route for the drug.

Additionally, the amount of bioactive penetration through lipid bilayers depends on bioactive structure and the molecular packing of the lipids. The partition coefficient of bioactive also depends on vesicle size and relates to differences in the curvature and the area compressibility of different vesicle structures.

A main process in bioactive delivery and targeting using liposome technology is the mechanism of material transfer through the liposomal lipid bilayer. The release of efficacious dose of liposome-entrapped bioactive depends on the permeability of the liposomal formulation with respect of the entrapped bioactive.

It is well known that bioactive agents have to pass several membrane barriers for exerting their suitable effects. These barriers affect on their pharmacokinetic and nutraceutical behavior and their capability to access the target site.

From a molecular point of view, transport of small molecules across lipid bilayers is a fundamental and functional process. The release of efficacious dose of bioactive-entrapped in liposome depends on different parameters such as liposome permeability, bioactive structural properties and strength of liposome / bioactive interaction.

Despite the importance of this subject, there is not sufficient and noticeable information concerning bioactive transfer through liposomal bilayer. For this reason, we tried to investigate hydrophilic bioactive agents' interaction with liposome by Raman Spectroscopy, Langmuir Balance and Differential Scanning Calorimetry.

Also, we studied the bioactive behavior which able to insert or entrapped into liposomal bilayer and their possible mechanism of transfer through liposomal bilayer.

Objects of study:

In our research study, we present 5 parts:

1- Optimization and characterization of liposome formulation:

We applied the mixture design technique to generate the optimal mixture of liposome formulation by using the different lipids in type and percentage (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) in liposome composition.

Mixture Design of Experiments (MDOE) is a technique that used to determine the optimum combination of chemical constituents that deliver a desired response by using a minimum number of mixture runs. Calcein was chosen as hydrophilic marker which has been widely

used as a model for drug/liposome interactions and determining the encapsulation efficiency. It is also easily detected by Confocal Microscopy Techniques.

Ten lipid mixtures were generated by the Simplex Centroid Design technique and liposomes were prepared by thin hydration method and extrusion method.

Then, liposomes were characterized with respect to size, zeta potential, phase transition temperature, fluidity, permeability and efficiency in loading calcein using a Nano Zetasizer and Differential Scanning Calorimeter, Spectrofluorimeter, Fluorescence Spectrophotometer, respectively.

Results of this mixture design were then applied to find the optimal point of experience to evaluate the possibility of improving the encapsulation efficiency, size, transition temperature and zeta potential of liposomes which prepared by extrusion method and different compositions.

2- Influence of lipid composition on physicochemical properties of nanoliposomes encapsulating natural dipeptide antioxidant L-carnosine.

Natural dipeptide antioxidants (L-carnosine) are receiving increasing attention because of their noticeable potential as biopreservatives in food recent technology.

Encapsulation of antioxidants by nanoliposomes could represent an ameliorative approach to overcome the problems related to the direct application of these antioxidant peptides in food.

In this study, nanoliposomes prepared from different lipids (DOPC, POPC and DPPC) by thin film hydration method, were assessed by considering their size, zeta potential, phase transition temperature and fluidity. One important parameter of interest in this article was to compare the encapsulation efficacy of L-carnosine in three different nanoliposomes using a rapid and precise approach ¹H-NMR without the need for physical separation of entrapped and non-entrapped L-carnosine. Furthermore, the morphology of small unilamellar nanoliposomes with different compositions on mica surface was investigated using Atomic force microscopy.

3- Investigation of calcein molecular interaction with liposomal bilayer

From a molecular point of view, bioactive substances able to insert or entrapped into liposomal bilayer can alter the shape, size distribution and chemical properties of liposome. Additionally, the localization of bioactive substances within the bilayer is also a question of great importance in order to determine the interaction with liposomes. Interactions of bioactive with model lipid bilayers could provoke changes on their thermotropic behavior as well as their conformation properties. These effects were taking into account in the design of

liposomal formulations as controlled release drug delivery systems. Also, by understanding the signaling and interaction between the bioactive and liposomes, it would be possible to mimic biological systems.

We studied the molecular effect of calcein on lipid model membrane composed of DOPC, POPC and DPPC and their mixture by using DSC, Raman spectroscopy and Langmuir balance in order to contribute to the knowledge of designing and optimizing model drug delivery systems. DSC measures thermal changes on the lipid bilayers that are caused by calcein. Raman spectroscopy is used to investigate the location of the bioactive compound in the lipid bilayers. Additionally, measurements on monolayers were performed by Langmuir balance in order to get information on the area occupied by the calcein on the surface of the monolayer. In this study, interactions between model membranes and calcein as a model of polar drug were investigated by comparing the behavior of pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and their mixture. The monolayers were studied using surface pressure and potential measurements, Brewster angle microscopy (BAM) and polarization modulation infrared reflection-absorption spectrometry (PM-IRRAS).

4- Calcein transfer through liposomal bilayer; Estimation of its diffusion and partition coefficient

The aim of this study was to determine the basic characteristics of calcein permeation from liposomal bilayer to simulate a model of polar drug - delivery system.

In this study, the permeability of calcein across some liposome membranes was first evaluated on the basis of the first-order kinetics. The neutral phospholipid was used to rule out the contribution of the electrostatic interaction of lipid membranes and the negatively charged calcein. Second, the pH effect of medium as well as the temperature effect on calcein release was investigated.

We also prepared the liposomes according to optimal point estimated by mixture design to design the drug carrier model. By considering the liposomal bilayer properties such as fluidity and permeability, we applied pH simulating stomach and intestine conditions to measure the diffusion and partition coefficient of calcein.

5- Observation and Investigation of calcein Transfer mechanism

The main purpose of this study was to determine the mechanisms of bioactive (such as calcein) transfer through liposomal bilayers. Several approaches were employed in order to obtain more information about calcein transfer through liposomes bilayer. We followed this study by using different techniques such as fluorescence-labeled markers in combination with various microscopic techniques including; Second Harmonic Generation (SHG), Stimulated Emission Depletion Microscopy (STED). We also used Transition Electron Microscopy (TEM), and Atomic Force Microscopy (AFM) which are widely used for investigation of bioactive translocation through the bilayers.

II. Synthèse bibliographique

I. Introduction

Les liposomes sont des particules sphériques composées de bicouches lipidiques refermées sur elles mêmes. Initialement, ces liposomes étaient utilisés comme modèles membranaires pour étudier leurs propriétés mécaniques et les modifications engendrées par les réactions biochimiques au cours de leur dégradation. Ces assemblages lipidiques trouvent de nombreuses applications dans le domaine cosmétique, les traitements anti-cancer, la thérapie génique, la vaccination et l'alimentation. Les caractères non toxique et biocompatible de ces vecteurs en font des systèmes intéressants pour des applications in vivo (Gregoriadis, 1976). L'objectif recherché consiste à encapsuler un principe actif hydrosoluble, liposoluble ou amphiphiles, de le vectoriser et de maîtriser sa libération de façon contrôlée, en fonction des conditions environnementales (Khosravi-Darani et al., 2007 ; Mozafari et al., 2008).

De nombreux travaux sur les liposomes ont permis de suivre les cinétiques de libération de molécules possédant des propriétés différentes en termes de solubilité et d'hydrophobicité comme l'alpha-tocophérol liposoluble ou le glutathion hydrosoluble (Mozafari et Mortazavi, 2005).

L'utilisation « maîtrisée » des liposomes devrait permettre d'atteindre des cibles cellulaires afin de délivrer un principe actif à la concentration optimale. Cependant, la stabilité de ces structures dépend de la nature de la molécule vectorisée, de la composition lipide, mais aussi des interactions entre le principe actif et la bicouche lipidique qui affectera son transfert. Il est de ce fait, important d'étudier en détails les propriétés physicochimiques de ces systèmes.

Au travers d'une synthèse bibliographique structurée en trois parties, nous allons faire un état de l'art sur les différentes techniques d'élaboration des liposomes, de leurs propriétés physicochimiques, avant de se focaliser sur le transfert de biomolécules fonctionnelles vectorisées.

II- Notions sur le liposome

2.1-Définition

Les liposomes sont des systèmes d'encapsulation les plus utilisés à des fins de vectorisation. Ceux sont des structures sphériques fermées, caractérisées par la courbure des bicouches lipidiques entourant une partie du solvant environnant et principalement composés de phospholipides, mais pouvant contenir du cholestérol ou d'autres composés. Leur taille est environ 70 fois plus petite qu'un globule rouge, de l'ordre de quelques dizaines à quelques milliers de nanomètres de diamètre. Le liposome peut être composé d'une ou plusieurs membranes concentriques d'une épaisseur de 4 nm (Torchilin, 2006; Augustin et Hemar, 2009; Mozafari *et al.*, 2008).

Les phospholipides sont un exemple de lipides complexes bien connus, ils sont rencontrés principalement dans les membranes cellulaires (bicouches lipidiques), ils sont aussi présents dans les globules gras du lait et dans le jaune d'œuf (lécithines).

La structure du phospholipide se caractérise par la présence sur le glycérol de deux acides gras et d'un groupement phosphate auquel est lié une autre molécule ; un composé azoté plus ou moins chargé ou neutre (sérine, choline, inositol, etc).

Les phospholipides sont des molécules amphiphiles présentant une structure caractéristique, montrant une tête polaire (partie hydrophile) et une queue apolaire (partie hydrophobe). Cette structure joue un rôle important dans la stabilisation de la matière grasse dans la phase aqueuse.

La conformation la plus stable des phospholipides est de se mettre en bicouches, elle est basée sur le fait que lorsque de tels composés sont mis en présence d'un excès de solution aqueuse, ils s'organisent de manière à minimiser les interactions entre leurs chaînes hydrocarbonées et l'eau. Les têtes polaires se regroupent entre elles face à la phase aqueuse de part et d'autre de la bicouche formée et les queues apolaires, hydrophobes se mettent au centre de la bicouche, inaccessibles à l'eau. L'effet hydrophobe constitue la force principale dirigeant la formation des bicouches lipidiques (Jesorka et Orwar, 2008).

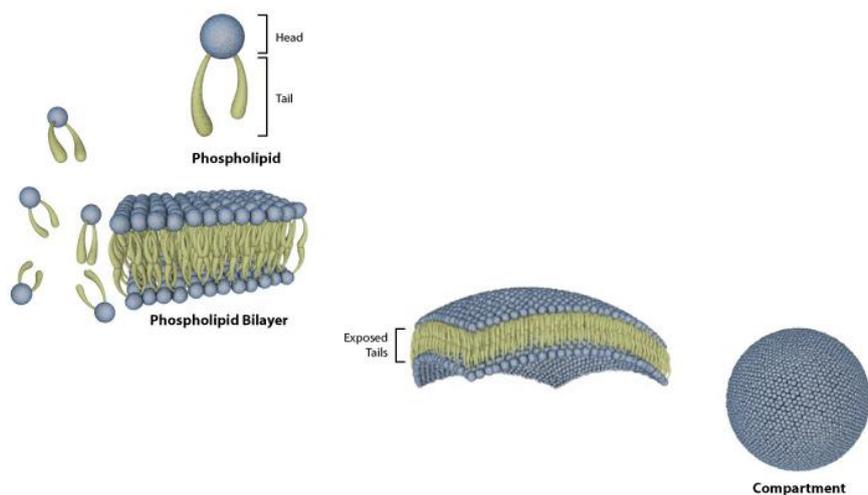


Figure 1. Mécanisme simplifié de formation de liposomes.

Un apport d'énergie à ces structures phospholipidiques va leur permettre de se refermer sur elles mêmes formant une vésicule (liposome). Pendant ce processus, le piégeage des solutés présents dans le milieu aqueux survient (Mozafari *et al.*, 2008). Les phospholipides naturels forment spontanément des liposomes en milieu aqueux (Lorin *et al.*, 2004).

2.2-Classification

Les liposomes sont classés selon leurs tailles, le nombre de bicouches et la méthode entreprise pour leur préparation (Mozafari et Mortazavi, 2005). On distingue des liposomes unilamellaires répartis en trois catégories selon l'importance de leur taille : les liposomes de grande taille (LUV) allant de 80 nm à 1 µm de diamètre, des liposomes unilamellaires géants (GUV) d'une taille supérieure à 1 µm, et des liposomes nanométriques SUV de petite taille mesurée entre 20 et 80 nm. On arrive aussi à observer des liposomes multilamellaires (MLV) dont la taille est supérieure à 400 nm et des liposomes à plusieurs vésicules non concentriques encapsulées dans de grandes vésicules uniques appelées vésicules multi-vésiculaires (MVV) d'une taille avoisinant 1 µm en diamètre (Lorin *et al.*, 2004).

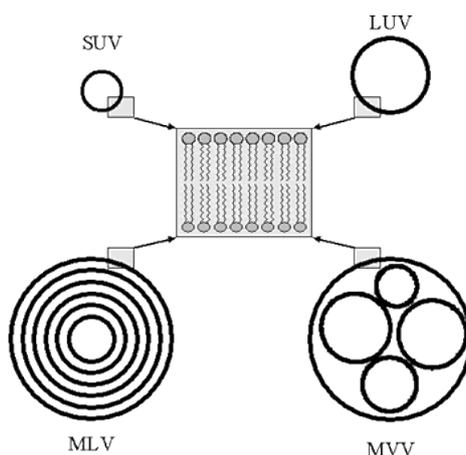


Figure 2. Schématisation de liposomes de taille différente et le nombre de lamelles. SUV: petites vésicules unilamellaires; LUV: De grandes vésicules unilamellaires; MLV: vésicules multilamellaires; MVV: vésicules multivésiculaires.

Les premières formulations ont donné naissance à des liposomes de première génération. Ils présentent une surface non modifiée et sont rapidement et efficacement retirés de la circulation sanguine, ils sont captés par les macrophages du foie ; les cellules de Kupffer. Le système hépatique est la cible principale de ces vecteurs. Ils vont ainsi être particulièrement efficaces pour délivrer des médicaments au niveau de cet organe.

Les liposomes de deuxième génération sont appelés, liposomes furtifs. L'émergence de ce type de particules a pu répondre à une longue recherche, essayant de développer un système support pouvant éviter la phagocytose et ainsi circuler plus longtemps dans le sang. Le liposome furtif est élaboré en couvrant la surface du support avec des chaînes hydrophilestels que le polyéthylène glycol (PEG) (Lasic, 1993 ; Gref *et al.*, 1994). Ils ne seront pas captés par le foie comme le sont les vecteurs de première génération et vont ainsi pouvoir atteindre l'organe malade et y amener de façon sélective le principe actif d'un médicament. La troisième génération portera en plus des molécules de surface permettant de fuir les macrophages celles du ciblage, et c'est de cette façon que les immunoliposomes ont été conçus afin de mieux cibler les agents bioactifs à l'intérieur du corps humain (Mozafari et Mortazavi, 2005).

Le nanoliposome est un nouveau concept tandis que les liposomes ont une histoire de plusieurs décennies. Grâce à leur taille nanométrique, ils permettent une circulation dans le système sanguin sans être reconnus par le macrophage. Ils présentent une facilité remarquable de pénétration dans le tissu à travers les capillaires et les membranes plasmiques, leur permettant une absorption facile par les cellules, et un ciblage contrôlé en plus de l'augmentation de leur biodisponibilité (Mozafari *et al.*, 2008).

2.3-Méthodes de préparation des liposomes

L'objectif visé est d'arriver à un assemblage de bicouches membranaires afin d'obtenir des vésicules ayant la taille, la structure et l'élasticité désirées avec une répartition homogène, ainsi qu'une polydispersité et une efficacité d'encapsulation considérables (Mozafari et Mortazavi, 2005). Le bon choix de la méthode de préparation dépend des propriétés physico-chimiques, des caractéristiques du matériau à piéger et celles des ingrédients du liposome, ainsi que la nature du milieu dans lequel les vésicules lipidiques sont dispersées. La concentration efficace de la substance à piéger et sa potentielle toxicité, les processus supplémentaires impliqués lors de l'application et le transfert des vésicules, la taille optimale, la polydispersité sont des paramètres importants à prendre en compte pour choisir une méthode de préparation adéquate (Gomez-Hens et Fernandez-Romero, 2006).

A- Méthode de Bangham

Bangham est la première personne à avoir fabriqué des liposomes en 1965. Cette méthode est très simple à réaliser, elle consiste à évaporer le solvant organique dans lequel sont dissous les lipides, puis à les remettre en suspension dans un solvant aqueux. Cette opération doit se dérouler dans des conditions de température dépendant de la nature du (des) lipide(s) choisi(s) (Bangham *et al.*, 1965).

Dans un milieu aqueux, le film lipidique s'hydrate et les phospholipides s'associent de manière à ne pas exposer leurs chaînes acyles au solvant, il en résulte la formation de bicouches, qui se referment en emprisonnant du solvant. Des bicouches peuvent enfermer d'autres bicouches de plus petite taille, ainsi lors de cette préparation, des liposomes multilamellaires se constituent en bicouches lipidiques concentriques et séparées les unes des autres, par des couches d'eau (Bangham *et al.*, 1965).

B- Méthode d'évaporation de la phase inverse

Le processus d'évaporation en phase inverse a été décrit par Szoka et Papahadjopoulos en 1978. La technique est réalisée par solubilisation des lipides dans un solvant organique, en ajoutant des aliquotes de la phase aqueuse. Le mélange est ensuite soniqué pour produire des micelles inverses. Le solvant organique est éliminé à l'aide d'un évaporateur rotatif. Il se forme alors un gel visqueux. Cette méthode présente l'inconvénient de mettre en contact le principe actif avec un solvant organique avant le procédé d'encapsulation.

C- Méthode de chauffage

Une méthode développée par Mozafari implique l'hydratation des composants phospholipidiques dans une solution aqueuse contenant du glycérol à 3% du volume total pendant une heure, suivie d'un chauffage à une température allant de 60 °C à 120 °C et d'une agitation à 1000 tours par minute (Mortazavi *et al.*, 2007). Le glycérol est le solvant utilisé car il est soluble dans l'eau et physiologiquement acceptable. Cet agent isotonique permet d'augmenter la stabilité des vésicules lipidiques due à son effet anticoagulant et empêchant de ce fait la sédimentation (Mozafari *et al.*, 2002). Cette méthode économique permet d'élaborer des transporteurs bioactifs, y compris des liposomes et nanoliposomes, avec une monodispersité et une stabilité supérieure, en utilisant un protocole simple. Une autre caractéristique importante est qu'elle peut être adaptée à petite et à grande échelle (Colas *et al.*, 2007). Aucune dégradation des ingrédients lipidiques n'a été signalée pour les liposomes fabriqués par ce procédé de chauffage (Mozafari *et al.*, 2002). Une version encore améliorée de la méthode de chauffage, appelée méthode Mozafari, a récemment été employée pour l'encapsulation et le ciblage d'antimicrobiens alimentaires tels que la nisine (Colas *et al.*, 2007). Cette méthode permet une fabrication à grande échelle des systèmes support en une seule étape, sans la nécessité de préhydratation des ingrédients formant la bicouche et sans employer de solvants toxiques ou de détergents.

D- Méthodes à haute énergie basées sur le phénomène de cisaillement

Deux technologies différentes permettent de fournir un cisaillement suffisamment puissant pour former des nanoliposomes : les homogénéiseurs haute pression et les sondes à ultrasons (Goutayer, 2008). Le principe de fonctionnement des homogénéiseurs haute pression est le suivant : la phase dispersée et la phase continue sont pré-mélangées de manière à former une émulsion grossière. Cette solution est ensuite introduite à haute pression (jusqu'à 150 MPa) dans une chambre d'interaction, dont la géométrie est étudiée pour générer un intense cisaillement capable de réduire fortement la taille des globules. Grâce au réarrangement de la structure qui se fait sous l'effet de la cavitation et /ou des phénomènes de cisaillement, on arrive à obtenir des vésicules liposomales (Sonneville-Aubrun *et al.*, 2004 ; Yilmaz et Borchert, 2006 ; Singh et Vingkar, 2008 ; Tadros *et al.*, 2004 ; Saupe *et al.*, 2006). Les propriétés des liposomes préparés par homogénéisation à haute pression dépendent de la pression et du nombre de cycles que l'échantillon subit (Otake *et al.*, 2006 ; Barnadas-Rodríguez et Sabés, 2001). Cette technique présente l'inconvénient de pouvoir traiter que de grands volumes de solutions, mais particulièrement utile pour la production de très petits

liposomes destinés à une application par voie intraveineuse (Mukherjee *et al.*, 2007). Le processus d'obtention de liposomes par sonication est rappelé ci-après. Un générateur convertit le courant électrique discontinu (50/60 Hz) en une énergie électrique à haute fréquence (20kHz). Ce courant est transmis vers un transducteur où il est changé en vibrations mécaniques longitudinales grâce à un cristal piézo-électrique. Ces vibrations sont ensuite amplifiées par la sonde et transmises au liquide sous la forme d'ondes ultrasoniques consistant en une succession de compressions et de dépressions. Ces variations de pression engendrent la formation de bulles microscopiques (d'air ou de vapeur) appelées cavités. Ces cavités se dilatent durant les phases de dépression et implosent violemment durant les phases de compression. L'effondrement des bulles provoque alors localement un puissant cisaillement s'accompagnant d'une élévation de la température (Goutayer, 2008).

Bien que ce phénomène, appelé cavitation, dure seulement quelques millisecondes et que l'énergie libérée par chaque bulle soit faible, sa fréquence fait que l'énergie cumulée générée par toutes les bulles de cavitation est très élevée. Il en résulte une intense agitation à l'échelle de l'échantillon, et donc la dispersion de la phase huile dans la phase aqueuse sous forme de liposomes de faibles diamètres (Goutayer, 2008).

L'intensité du phénomène de cavitation varie fortement avec les propriétés du milieu, notamment la tension de vapeur, la viscosité, la densité, et toutes les propriétés liées à la quantité de molécules ou d'ions en solution (Jafari *et al.*, 2006 ; Jafari *et al.*, 2007). L'énergie requise pour former une bulle de cavitation est proportionnelle à la tension de surface et à la pression de vapeur. Ainsi, plus la tension de surface est importante, plus l'énergie nécessaire pour produire les cavités est importante. Cependant l'énergie de l'onde de choc libérée est plus importante lorsque les bulles s'effondrent. De la même manière, plus la viscosité de la solution est importante, plus la puissance nécessaire pour former les bulles de cavitation est grande, mais en contrepartie l'énergie libérée par l'effondrement de ces bulles est également plus intense (Abismail *et al.*, 1999).

La sonication possède des avantages non négligeables, en tant que technique à haute énergie, elle permet d'avoir une grande liberté sur la formulation, et à l'inverse de l'homogénéisation à haute pression, elle permet de travailler sur des plus petits volumes et avec des viscosités plus importantes (Goutayer, 2008).

III- Caractérisation du liposome

Après la production, la caractérisation des liposomes est nécessaire pour qualifier, quantifier et approuver la capacité des liposomes pour une application bien précise. Cette connaissance

permet de développer des formulations ayant des rendements optimaux de piégeage et de libération contrôlée. Les méthodes de caractérisation doivent être exactes et rapides.

3.1- Propriétés physico-chimiques des liposomes

Elles représentent un ensemble de paramètres physico-chimiques lors de la mise en œuvre des procédés de préparation des liposomes. La variation de ces paramètres va favoriser une utilisation ultérieure d'un type de vecteur lipidique en dépit d'un autre.

a. Taille et forme des liposomes

La taille moyenne et la distribution de taille sont des paramètres qui doivent être modulés en fonction de l'application ultérieure du système liposomal (Meerovich *et al.*, 2008).

Il a été démontré que la taille moyenne des liposomes est influencée par la composition lipidique et la méthode de préparation. Les techniques de mesure utilisées relèvent de la nanotechnologie. La taille moyenne d'une dispersion aqueuse de liposomes peut être mesurée en utilisant la diffusion dynamique de la lumière (DLS) fonctionnant avec la détection hétérodyne (Otake *et al.*, 2001 ; Castor et Chu, 1998). Elle permet d'indiquer la taille mais ne donne pas de précision sur la forme et la structure liposomales.

En revanche, les techniques de microscopie électronique permettent des observations possibles de la forme des liposomes, et la présence de toute fusion ou agrégation (Reimer, 1998). Elles fournissent également des informations sur l'épaisseur de la bicouche lipidique et la distance inter-lamellaire. Ces techniques sont faites à base de transmission d'électrons, l'exemple du microscope électronique à balayage. La microscopie à force atomique (AFM) est de très-haute résolution. Elle permet de créer en trois dimensions des micrographies avec une résolution jusqu'à l'échelle du nanomètre et de l'Angstrom (Spyratou *et al.*, 2009). En raison de ses performances, l'AFM est une technique d'imagerie directe parfaitement adaptée aux nanoparticules, elle permet une caractérisation morphologique et nous renseigne sur la stabilité et le processus dynamique des nanocapsules lipidiques (Luykx *et al.*, 2008 ; Edwards et Baeumner, 2006).

b. Mobilité électrophorétique des liposomes

La charge à la surface des liposomes varie. Elle pourrait être neutre en employant des phospholipides, comme la phosphatidylcholine ou la phosphatidyléthanolamine, négative avec des phospholipides acides tels que la phosphatidylsérine, le phosphatidylglycérol, l'acide phosphatidique ou le diacétylphosphate. Des charges positives peuvent être générées par l'utilisation des lipides tels que le propane dioléoyl triméthyl ammonium (DOTAP) ou le

stéarylamine (SA) dans des gammes de pH physiologique. La charge liposomale est une caractéristique importante qui détermine la stabilité et l'efficacité d'encapsulation des liposomes. L'attraction électrostatique entre les bioactifs chargés et les liposomes est un moyen d'accroître l'efficacité d'encapsulation (Nagahiro *et al.*, 2000 ; Filion et Phillips, 1997). La densité de charge des surfaces liposomales et l'affinité de liaison des différents ions à des vésicules lipidiques peuvent être déterminées par mesure d'un paramètre appelé, potentiel zeta. La mobilité électro-phorétique est une fonction de la charge de surface de la vésicule lipidique. Elle n'est pas mesurable directement, mais peut être calculée à l'aide des modèles théoriques et une électrophorèse détermine de manière expérimentale la mobilité ou la dynamique électrophorétique (Gregoriadis, 2007). La plus grande mobilité électrophorétique entraîne une suspension liposomale stable car les vésicules chargées se repoussent les unes les autres et surmontent la tendance naturelle à s'agréger. En général, les vésicules lipidiques précipitent pendant le stockage. Accroître la répulsion inter-particulaire, soit de façon électrostatique ou stérique, peut améliorer leur stabilité (Grabielle-Madelmont *et al.*, 2003). La charge de surface des liposomes peut influencer le temps de leur circulation dans le système sanguin et les valeurs de la mobilité électrophorétique sont influencées par la composition lipidique des liposomes (Keller, 2001). La lumière de diffusion doppler peut être utilisée pour mesurer la mobilité électrophorétique des liposomes (Filion et Phillips, 1997).

c. Température de transition

Les molécules amphipathiques telles que les phospholipides peuvent subir une transition de phase thermotrope à des températures beaucoup plus basses que leur point de fusion. Les liposomes à base de phospholipides purs ne peuvent pas se former à des températures inférieures à cette température de transition. A cette température, la bicouche perd son organisation ordonnée et augmentant sa fluidité (Mozafari *et al.*, 2008). Cette valeur de température dépend de plusieurs paramètres, comme la polarité du groupement de tête plus ou moins chargé, la longueur de la chaîne acyle, la présence de méthyle sur la chaîne hydrocarbonée, le degré de saturation des chaînes, ainsi que la nature de la force ionique du milieu de suspension (Mozafari *et al.*, 2008).

La température de transition augmente avec le nombre d'interactions entre les lipides, notamment les interactions hydrophobes (Seydel *et al.*, 1981). Le nombre d'interactions hydrophobes augmentent avec la longueur des chaînes acyles. La température de transition augmente proportionnellement avec la longueur des chaînes acyles. Par contre, les insaturations *cis* défavorisent les interactions entre chaînes. La température de transition

diminue fortement avec le nombre d'insaturations *cis*. La température de transition dépend ainsi de la longueur et du degré d'insaturation des chaînes d'acides gras constituant les lipides de la membrane. Il y a donc une relation linéaire entre la température de fusion des acides gras d'un lipide et la température de transition d'une membrane composée de ce lipide (Chaudhury et Ohki, 1981).

La température de transition décroît lors de la diminution de la longueur de la chaîne, par l'augmentation du degré d'insaturation des chaînes acyles, ainsi que par la présence des chaînes ramifiées introduisant la notion de groupements « encombrants ». Tout hydrocarbure dont l'insaturation est configurée en *cis* a une plus faible température de transition (TC) que ceux qui sont trans-insaturés (Taylor et Morris, 1995).

Cette exigence de température est réduite dans une certaine mesure mais pas éliminée par ajout de cholestérol (Leserman *et al.*, 1994). Dans certains cas, il est recommandé de préparer des liposomes à des températures bien au-dessus de la (TC) des vésicules. Par exemple, dans le cas des vésicules contenant du dipalmitoylphosphatidylcholine (DPPC, TC = 41 ° C), elle se fait à 51°C, dix degrés de plus que la (TC), afin de s'assurer que tous les phospholipides soient dissous dans le milieu de suspension homogène et ayant une flexibilité suffisante, nécessaire pour s'aligner dans la structure des vésicules lipidiques (Mozafari et Mortazavi, 2005).

La détermination de la température de transition s'avère donc très importante. Obtenir une faible température de transition de phase est avantageux pour les liposomes utilisés comme vecteurs de médicaments. En effet, les agents actifs ont une libération plus longue par rapport à ceux encapsulés dans des liposomes ayant une TC plus élevée (Betz *et al.*, 2005). La calorimétrie différentielle à balayage (DSC) a été largement utilisée pour la détermination des températures de transition des phospholipides (Sot, 2005 ; Saroglou, 2006).

d. Lamellarité

La lamellarité et la taille des vésicules lipidiques sont généralement les caractéristiques les plus importantes. La lamellarité est le nombre de bicouches lipidiques entourant l'espace aqueux interne des vésicules lipidiques. Les vésicules sont observées par différentes techniques analytiques, comme par exemple, la microscopie électronique (Johnson., 1971). L'observation microscopique directe donne des informations sur la taille, l'homogénéité de l'échantillon et la lamellarité des liposomes (Mozafari *et al.*, 2006). La lamellarité d'une préparation de liposomes peut être également déterminée en utilisant la résonance magnétique nucléaire (RMN) (Hope *et al.*, 1985 ; Yamauchi *et al.*, 2007). Ruozi et ses collaborateurs

(2007) ont utilisé la résonance magnétique nucléaire (RMN) et la résonance paramagnétique électronique (RPE) pour étudier la lamellarité, la perméabilité de la bicouche et l'influence de la taille des particules sur le transport de molécules bioactives à travers des liposomes (Ruozi *et al.*, 2007).

3.1- Propriétés mécaniques des liposomes

La littérature scientifique est abondante dans le domaine du développement, de la caractérisation et de la validation des suspensions de liposomes. Toutefois, la stabilisation de la mobilité de surface des liposomes est pour l'instant moins étudiée. Elle pourrait trouver des applications dans les domaines pharmaceutique et médical (Ogiso *et al.*, 1996 ; Shimanouchi *et al.*, 2009).

Diverses techniques ont été utilisées pour incorporer les vésicules liposomales dans différents substrats tels que le collagène (Liebau *et al.*, 1998 ; Luthgens *et al.*, 2003) et le chitosane (Weiner *et al.*, 1985 ; Trafny *et al.*, 1996). La rigidité de la bicouche reflète l'ordre et la dynamique des chaînes alkyles des phospholipides dans la bicouche (Letchford *et al.*, 2007), ce qui fait de ce paramètre l'un des plus importants qui affecte l'efficacité de délivrance des molécules bioactives, évaluée par la stabilité des particules et le profil de libération.

3.2.- Stabilité colloïdale

C'est l'un des facteurs les plus importants autour duquel sont fondées des relations interdépendantes entre la variante physique, chimique et biologique. La stabilité physique fait entrer la notion de courbure de la bicouche et la rigidité de la membrane. L'apport d'énergie lors de la formation de liposomes par sonication, homogénéisation ou chauffage est nécessaire à l'allongement des molécules de lipides, puis la courbure se fait de façon spontanée par la suite. Le degré de courbure membranaire est fonction du type de lipides, ainsi que de la présence ou de l'absence de stérols dans la formulation (Mozafari et Mortazavi, 2005).

Des membranes plus rigides avec des points de fusion plus élevés et des courbures à proximité de leur courbure naturelle seraient plus stables contre les troubles tels que l'augmentation de température, du cisaillement, des vibrations et du gel-dégel (Garti, 2008). La stabilité chimique se réfère à la capacité des liposomes à maintenir le niveau d'efficacité d'encapsulation face à des changements environnementaux (pH, composition de l'électrolyte, présence d'agents oxydants et de tensio-actifs comme les surfactants, le cholestérol et les sels biliaires) (Couvreur *et al.*, 1979).

La dégradation chimique de la bicouche liposomale réduit de façon importante la stabilité biologique et physique des liposomes. La réduction de la stabilité physique, due à l'agrégation ou à la libération précoce du matériel encapsulé, réduit l'utilité des liposomes. La variante biologique quant à elle, se réfère au processus de libération du principe actif dans son milieu biologique en traversant la membrane du liposome (Couvreur *et al.*, 1979). La stabilité colloïdale ci-mentionnée se traduit aussi par la capacité des liposomes à maintenir leur taille sous différentes conditions de stockage (Acosta., 2008). Le stockage à long terme dans des conditions spécifiques, par exemple à une exposition à la lumière et à haute température, affecte la stabilité chimique et physique des liposomes (De Luca *et al.*, 2006). Certains substrats comme le cholestérol et les antioxydants offrent une protection contre la dégradation des liposomes (Smith, 1991). Concernant le stockage à long terme, deux aspects d'instabilité peuvent être considérés ; l'instabilité chimique qui reflète la dégradation des composants de liposomes par l'hydrolyse et / ou l'oxydation et l'instabilité physique où la structure des liposomes peut être affectée, par agrégation ou par une fusion de la bicouche (Brandl et Massing, 2007). La chromatographie liquide à haute performance (HPLC) a été introduite pour évaluer la stabilité des liposomes (Zuidam *et al.*, 1993 ; Zuidam *et al.*, 2003). Les détecteurs d'évaporation par diffusion de lumière (ELSD) deviennent des détecteurs de choix pour quantifier, les matériaux insensibles aux UV tels que la plupart des phospholipides (Sas *et al.*, 1999). La chromatographie en phase inverse liquide à haute performance associée à un spectromètre de masse à ionisation permet de quantifier les lipides, et d'attribuer également la localisation de la chaîne acyle sur les molécules de phospholipides (Vernooij *et al.*, 1998). La microscopie électronique à transmission a aussi été utilisée afin d'observer la stabilité des vésicules liposomales et leur tendance globale (Tchoreloff *et al.*, 1991). La résonance électronique paramagnétique (REP) sert à déterminer en plus de la fluidité, les modifications de la structure des bicouches lipidiques des liposomes (Coderch *et al.*, 2000).

IV-Techniques d'encapsulation

La sélection d'un protocole d'encapsulation est largement liée aux paramètres, tels que l'efficacité d'encapsulation, le degré d'hydrophobicité du principe actif, la stérilité du milieu, la facilité de préparation et l'échelle de sa mise en place. La compatibilité avec les organismes de réglementation, l'efficacité des coûts, ainsi que la stabilité des liposomes et des biomolécules jouent aussi des rôles importants dans cette sélection (Barenholz *et al.*, 1994). Deux façons différentes d'encapsulation des composés bioactifs peuvent être distinguées : le piégeage réalisé au cours du processus de formation des vésicules appelé encapsulation

passive et le chargement dans des vésicules lipidiques sous la forme d'un piégeage dit actif (Mozafari et Mortazavi, 2005).

4.1-Techniques de piégeage passif

Les techniques de piégeage passives reposent sur la capacité des liposomes à capturer un certain volume aqueux y compris les solutés dissouts, pendant la formation des vésicules (Mayer *et al.*, 1994 ; Gregoriadis, 2007). Pour les composés hydrosolubles qui n'interagissent pas avec la bicouche, l'efficacité d'encapsulation est proportionnelle au volume aqueux enfermé par les vésicules, qui dépend lui-même de la concentration des phospholipides, de la dispersion, de la lamellarité et de la morphologie des vésicules. Pour les moins hydrosolubles qui interagissent avec la bicouche, le paramètre d'encapsulation dépendra plus de la concentration des phospholipides et de leur sélection que sur des paramètres morphologiques (Lasic, 1996). Pendant ce processus, les molécules hydrophiles se trouveront dans la phase aqueuse interne du liposome tandis que les molécules hydrophobes (liposolubles) seront situées dans la bicouche (phase lipidique) du liposome. Les molécules amphiphiles seront placées de telle façon que la partie liposoluble sera intégrée entre les chaînes lipidiques alors que leur partie hydrosoluble sera située dans la phase aqueuse (Mozafari et Mortazavi, 2005).

4.2-Techniques de piégeage actif

En principe, la technique de piégeage actif est constituée de l'assemblage de liposomes vides avec une solution concentrée de l'agent bioactif jusqu'à ce que ce dernier traverse par diffusion (Brandl et Massing, 2007). Cette méthode présente certains avantages, car les bicouches des vésicules sont suffisamment perméables pour permettre la diffusion des molécules bioactives dans les liposomes dans un délai raisonnable. Cette pénétration se fait suivant un gradient de concentration jusqu'à ce qu'un équilibre moyen entre l'intérieur et l'extérieur des vésicules soit obtenu (Brgles *et al.*, 2008).

Le taux de molécules hydrophobes que peut contenir un liposome dépend de la restriction de la bicouche lipidique et par conséquent, les formulations de liposomes pour cette classe d'agents bioactifs sont en continuel développement d'un agent à un autre. Les particules hydrosolubles interagissent avec les groupements de tête polaires des phospholipides et sont séquestrées par les liposomes, mais les agents amphiphiles sont souvent difficiles à retenir au sein des liposomes en raison de leur infiltration dans les bicouches lipidiques (Mayer *et al.*, 1986). Cette technique est limitée à une petite gamme de molécules bioactives qui se comportent comme des bases amphipathiques faibles ou des acides.

4.3- Efficacité d'encapsulation

L'efficacité d'encapsulation va dépendre des propriétés liées à la bicouche du liposome d'un côté et celles des molécules à encapsuler de l'autre côté. Les biomolécules actives peuvent interagir avec les liposomes sous différentes formes en fonction de leurs propriétés, telles que la solubilité et la polarité. Ils peuvent être piégés dans la bicouche lipidique, intercalés dans les groupements de têtes polaires, adsorbés sur la surface de la membrane, ancrés dans une queue hydrophobe ou encapsulés dans la phase aqueuse du compartiment interne (Grabielle-Madumont *et al.*, 2003). Une réalisation majeure dans l'application médicale des liposomes est la possibilité de vectoriser une quantité suffisante de principe actif nécessaire à l'efficacité thérapeutique. Cette efficacité d'encapsulation est évaluée par l'équation suivante (Laridi *et al.*, 2003).

$$\% \text{ EE} = (\text{Biomolécules encapsulées}) / \text{Biomolécules total} * 100$$

La connaissance des caractéristiques des liposomes est nécessaire pour développer des formulations de liposomes qui ont des rendements optimaux de piégeage et permettent une libération contrôlée des substances bioactives. La composition lipidique et la méthode de préparation peuvent influencer l'efficacité d'encapsulation (Lasic, 1993).

4.4-Effet des propriétés du liposome sur l'efficacité d'encapsulation

La perméabilité est étroitement reliée à la composition de la membrane liposomale (Komatsu et Chong., 1998). Des études de perméabilité des systèmes bicouches modèles indiquent que la région de la chaîne acyle adjacent le groupement de tête est, le site susceptible d'offrir la plus grande résistance à la perméation de l'eau et des solutés (Xiang *et al.*, 1992 ; Xiang, 1993). La perméabilité de la membrane diminue par l'utilisation de l'acide acétique comme acide gras lors de la formulation des liposomes conférant une longueur plus courte des chaînes de phospholipides (Xiang et Anderson, 1997). D'autres recherches ont confirmé que la perméabilité des bicouches lipidiques dépend fortement du degré de tassement des chaînes lipidiques dans la membrane (Xiang et Anderson, 1998).

L'analyse des coefficients de perméabilité de liposomes, à composition variable a montré que la présence de doubles liaisons au sein de la chaîne acyle conduit à une diminution de la densité, qui à son tour perturberait la barrière à la perméation (Shimanouchi *et al.*, 2009). Il a aussi été constaté, que le taux de cholestérol réduit aussi la perméabilité de la bicouche (Papahadjopoulos *et al.*, 1971). La taille des liposomes est un facteur déterminant de la perméabilité des vésicules unilamellaires. Une diminution uniforme du coefficient de perméabilité PS a été observée en augmentant la taille des liposomes. Il est généralement

connu que les liposomes de plus de 200 nm de diamètre ont tendance à avoir une structure multilamellaire. L'augmentation de la lamellarité pourrait se traduire par la résistance à la perméation, donc la valeur PS devrait diminuer sensiblement (Shimanouchi *et al.*, 2009).

V. Conclusion

L'utilisation des nanoliposomes à des fins de transfert et de ciblage des molécules biofonctionnelles, est une approche très intéressante, dans la mesure où l'on arrive à contrôler même la libération des principes actifs, en prolongeant ou encore en retardant leur relargage pour une meilleure efficacité et pouvoir ainsi déclencher l'effet thérapeutique désiré au moment et à l'endroit voulu. Il s'est avéré que la structure des nanoliposomes présente un effet très important sur l'efficacité d'encapsulation et le maintien de la stabilité de ces particules colloïdales tout au long de la durée du transfert. Le choix de la composition phospholipidique et la taille de ces particules est une étape pertinente dans la formulation à des fins de transfert. Plusieurs formulations liposomales se sont développées afin de remédier à ses limites structurales en apportant des propriétés de ciblage et de stabilité nouvelles.

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Liposomes: A Review of Manufacturing Techniques and Targeting Strategies

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Abstract: Today, liposomes are among the most applied technologies for the encapsulation and delivery of bioactive agents and many different compounds in biological, pharmaceutical, medical and nutritional research. In this review, classification of liposomal vesicles, methods of their preparation and encapsulation, as well as their applications in food, cosmetics and pharmaceutical industries are reviewed. In addition, the main analytical approaches used to study liposome characteristics such as size, transition temperature, surface charge, fluidity, lamellarity, stability and encapsulation efficiency are presented. In the final part of the article, mechanisms of liposome targeting are discussed.

Keywords: Liposome composition, liposome production, oxidative stabilization, encapsulation efficiency, liposome targeting, release mechanisms.

INTRODUCTION

Microencapsulation is the packaging of small particles (known as active agents) within an encapsulating system (known as a capsule or shell). The contents of the capsule are isolated from the environmental stress that surrounds the capsule and can be released in a targeted site in a suitable time. There are several types of encapsulation systems, each made using different material such as polymers, surfactants, lipids or phospholipids. One of the most applied encapsulation systems is liposome, which is mainly composed of phospholipid molecules [1, 2].

Liposomes were first made synthetically in England in 1961 by Alec D. Bangham [3], who found that phospholipids combined with water form a sphere because one end of each molecule is water soluble, while the opposite end is water insoluble.

Liposomes are spherical, closed structures, composed of curved lipid bilayers, which enclose part of the surrounding solvent into their interior. The size of a liposome ranges from 20 nm up to several micrometers and they may be composed of one or several concentric or nonconcentric membranes, each with a thickness of about 4 nm [4].

The main constituents of liposomes are phospholipids, which are amphiphilic molecules containing water soluble, hydrophilic head section and a lipid-soluble, hydrophobic tail section. This property of phospholipids give liposomes unique characteristics, such as self-sealing, in aqueous media and makes them an ideal carrier system with applications in different fields including food, cosmetic, agriculture and pharmaceuticals [3].

A significant advantage of liposome is that it can incorporate and release two materials with different solubilities simultaneously. One example for which is the incorporation of two antioxidant agents namely alpha-tocopherol (a lipid-soluble molecule) and glutathione (a water-soluble molecule) in the same lipid vesicle [5].

CLASSIFICATION OF LIPOSOMAL VESICLES

Liposomes are classified based on vesicle size, number of lamella and preparation method [5]. Liposomes that only contain a single bilayer membrane are called unilamellar vesicles (ULV).

Unilamellar vesicles can be divided into small unilamellar vesicles (SUV, less than 100nm) and large unilamellar vesicles (LUV, larger than 100nm) [6].

A multilamellar vesicle (MLV) is a liposome composed of a number of concentric lipidic bilayers. A vesicle composed of several non-concentric vesicles encapsulated within a single bilayer is known as a multivesicular vesicle (MVV).

Some of the carrier systems developed based on liposome technology are briefly introduced in the following sections [5]:

LIPOSOME-BASED CARRIER SYSTEM

- Immunoliposomes

One class of lipid vesicles designed for active targeting of the bioactive agents inside the body is known as immunoliposome [5]. This type of liposome is explained in more details in the following sections.

- Virosomes

Virosome or artificial viruses are another type of liposomal system used in active targeting and contain reconstituted viral proteins in their structure [7].

- Stealth Liposomes

During many years, researchers tried to develop carrier systems which can avoid phagocytosis and thus circulate longer in the blood. As a result of these studies, a kind of liposome so-called "Stealth particles" has emerged. Stealth liposome is made by covering the carrier surface with hydrophilic chains such as poly ethylene glycol (PEG) [8, 9].

- Transferosomes and Transdermal Bioactive Delivery

For transdermal delivery of bioactive agents, the carrier system needs to possess required deformability to be able to pass the skin layers. For this aim, transferosome has been introduced.

Transferosomes consist of phospholipids, cholesterol and additional surfactant molecules such as sodium cholate. Transferosomes are ultra deformable and squeeze through pores less than one-tenth of their diameter [5, 10].

- Archaeosomes

Archaeosomes can be defined as liposomes made from one or more of the polar ether lipids extracted from the domain *Archaea* (Archaeobacteria) [11]. Compared with liposomes, archaeosomes

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are relatively more thermostable, more resistant to oxidation and chemical and enzymatic hydrolysis. Considering that some liposome formulations have been found to retain their structural integrity with high encapsulation efficiency after treatments at sterilisation temperatures, the fact that archaeosomes are even more thermostable makes them ideal candidates to protect antioxidants during food processing [12, 13].

- Vesicular Phospholipid Gels

Vesicular phospholipid gels (VPGs) are highly concentrated phospholipid dispersions of semisolid consistency and vesicular morphology. VPGs can be prepared by high-pressure homogenization of high concentrations of phospholipid molecules [14]. VPGs can be useful as parenteral depot formulations. They are also useful as intermediates for liposome dispersions, especially those with drugs with high leakage rates and poor storage stabilities [5].

- Cochleates

Cochleates are small-sized and stable lipid-based carriers comprised mainly of a negatively charged lipid (e.g. phosphatidylserine) and a divalent cation such as calcium. They have a cigar-shaped multilayered structure [15, 16]. Hydrophobic, amphiphilic, negatively or positively charged molecules can be delivered by cochleates. The unique structure and properties of nanocochleates make them ideal candidates for oral and systemic delivery of antioxidants and other sensitive moieties [12].

- Nanoliposome

The nanometric versions of liposomes are known as nanoliposomes [5]. Nanosystems have many advantages over the micro systems such as longer circulation time in the blood stream without being recognized by macrophages, ease of penetration into tissues through capillaries and biological membranes, ability to be taken up by cells easily, demonstrating high therapeutic activity at the target site, sustaining the effect at the desired area over a period of days or even weeks, improve controlled release and precision targeting of the entrapped compounds to a greater extent [17]. Nanoliposomes are able to amplify the performance of bioactive agents by improving their solubility and bioavailability, *in vitro* and *in vivo* stability, as well as preventing their unwanted interactions with other molecules [18].

Nanoliposomes are employed for encapsulation and delivery of the antioxidants and they also can incorporate and deliver both vitamin E and ascorbic acid to a site of oxidation in the food system [12, 18].

APPLICATIONS OF LIPOSOMES

In order for a bioactive agent to exert its intended effect, it needs to be in physical contact with its physiological target. A possible approach to facilitate material transport into cells or target sites is the application of liposome. A significant advantage of liposome is that it can incorporate and release two materials with different solubilities simultaneously [19, 20]. Furthermore, targetability is another extremely useful characteristic of liposome. These particular properties make liposome to be useful in many applications due to its ability to increase the effectiveness of the encapsulated active agents and optimizing their dosage [2].

The unique properties of liposomes have triggered numerous applications in various fields of science and technology, from basic studies to gene and drug delivery [21]. Some of the main applications of liposomes are described below:

- Liposome Applications in the Pharmaceutical and Medical Research

Today liposomes are an important part of biological, pharmaceutical, and medical research. Liposomes not only serve as unique model membranes and nucleic acid delivery vehicles, but they also

have been reported to be used as delivery systems of enzymes, various drugs, hormones and blood factors [22, 23].

Particular areas in which liposomes display therapeutic potentials are drug delivery, cancer treatment and gene therapy. They act as carriers for anticancer agents, anti-fungal drugs, antibacterials, anti-virals and certain anti-parasitics. Some of the other applications of liposomes include encapsulation of contrast agents for use in diagnostic X-ray and NMR imaging [24-27].

Because liposomes are one of the most effective carriers for the delivery of many different types of bioactive agents into cells, the applications of liposome-based formulations and products are extremely wide. Some of the new developments in this respect include applications in tumor targeting, gene and antisense therapy, DNA vaccination, immunomodulation, lung therapeutics, and cyclodextrin-controlled drug release *in situ* [28-30]. In immunology, antigens encapsulated in liposomes are used to generate antibodies, to mediate active and passive immunization and for many other applications (For a review see, [31]). The first report on the application of liposomes as immunological adjuvant was made 20 years ago by Allison and Gregoriadis [32] and since then, numerous studies showing the adjuvant action of liposomes have been published. Applications of liposomes as adjuvant include hepatitis B-derived polypeptides, subunit antigens from the influenza virus, adenovirus type 5 hexon, allergens, and polysaccharide-protein conjugates, to name a few. Several laboratories have studied liposomes made with detergent-extracted envelope glycoproteins from HIV-1, and synthetic peptide carrying a CTL epitope from the simian immunodeficiency virus gag protein [31]. Liposomes also can be taken by cells through different mechanisms such as endocytosis, phagocytosis, fusion and others [31].

More recently, liposomes have also been applied as contrast agents for molecular imaging [33].

- Applications of Liposomes in Food Science

Liposomes and nano-liposomes are used for improving and/or developing new taste, controlling the release of flavor, improving the food color and altering the texture of food components. They are also able to increase the absorption and bioavailability of nutraceutical and health supplements and develop food antimicrobials. In addition, lipid vesicles can be used to construct new food packaging materials with improved barrier and antimicrobial properties as well as some kinds of nanosensors for traceability and monitoring the condition of food during transport and storage [34].

The range of applications for micro- and nanoliposome in the food industry has been increasing because of the many advantages that liposome provide by protecting the active agent from the enzymatic and chemical changes, as well as temperature and ionic strength variations [2]. In addition, liposomes can be effective carriers for nutritionally valuable ingredients [35].

In the following sections, some applications of the liposomes in the food industry are explained:

One of the first reported liposome applications in food products was in cheese manufacture [36], in order to decrease the time and cost of the cheese ripening by adding the proteinases encapsulated in liposome to cheese mixes. Researchers have shown that encapsulated enzymes in liposome improve the stability and activity of enzymes and control their release time and improve the flavor of cheese and reduce the cost of production [37-39].

Studies have shown that liposome-entrapped proteinases reduce the firmness of cheddar cheeses but increase their elasticity and improve their flavor and liposome-entrapped lipase increases cheddar cheese cohesiveness and elasticity, but reduces the cheese firmness [40, 41]. They encapsulated negatively charged bacterial and fungal proteinase and lipases separately in extremely positively charged liposome.

Lee *et al.* encapsulated the enzyme bromelain (used as a meat tenderizer) in liposomes and they also found that the stability and bioavailability of enzyme significantly increase [42].

Matsuzaki *et al.* and Rao *et al.* [43, 44] separately used liposome-entrapped β -galactosidase in order to aid the digestion of dairy foods by the lactose intolerance. They found out that liposomes can stabilize the enzyme during the storage.

Vitamins are also encapsulated in liposomes to enhance their retention. Tesoriere *et al.* encapsulated vitamin systems including vitamins A and E, and β -carotenes in liposome and they resulted that liposome able to extend the half-life of the entrapped antioxidants and facilitates their intracellular uptake [45]. Studies also showed that better bioavailability of the hydrophobic Coenzyme Q₁₀ obtained with encapsulation in a liposomal system compared with entrapment in a gelatin capsule [13].

Liposomes have also been used to increase the nutritional quality of dairy products by entrapping the vitamin D in cream and cheese [46]. These researches indicate that liposome can protect vitamins from degradation.

Ascorbic acid incorporated in liposomes exhibited a half-life of 100 days compared with a pure solution of ascorbic acid with a half-life of 18 days at 4 °C [47].

Another useful application is encapsulation of food preservatives in liposomes in order to control cheese spoilages. Lysozyme, a natural preservative derived from egg is used in food systems as a replacement for nitrite [38]. Liposome-entrapped lysozyme has the potential to prevent cheese spoilage.

Antimicrobial peptides have been extensively examined as potential biopreservatives in food technology. However, their stability in food because of proteolytic degradation and the potential interaction of the antimicrobial peptide with food components, might terminate to decrease their antimicrobial activity. The entrapment of bacteriocins into liposomes represents an alternative to overcome this problem [48].

Application of liposome - entrapped bactericide in dairy science and industry has also shown great potential [49]. Another group of researchers encapsulated the antimicrobials pediocin [50] and nisin Z [51-54] separately in liposomes and found that liposome-entrapped antimicrobials can reduce or eliminate undesirable interactions in foods and also have long-term preservative effects and can be a useful replacement for the synthetic preservatives that have undesirable side effects [55].

Another useful application of liposome is fortification of foods with ferrous sulfate and vitamin C [56], using haem liposome as a source of iron [57]. They resulted, by addition of haem liposomes to wheat flour, the fat content of flours increased and it also had a positive effect on the stability and rheological properties of the dough. Additionally, loaf volume and crumb uniformity was improved [57].

Encapsulation of enzymes in liposomes [58, 59], in order to stabilize the enzymes against food manufacture processes and preserving them for a long-time and maintain their useful effects in foods, is another application of liposome technology in food production industry.

Liposomes and nanoliposomes have also been employed for encapsulation and delivery of the antioxidant glutathione (GSH) [60-63]. The influence of α -tocopherol incorporation into liposomes and its effect on physical and chemical stability of biomembranes was studied by Nacka *et al.* [64]. Another liposomal antioxidant system containing ascorbic acid and α -tocopherol has been reported by Kirby [65]. In a more recent study, Mozafari *et al.* [66] incorporated two antioxidant agents, α -tocopherol and glutathione, in the same liposome composition, creating a bifunctional carrier system.

All the researchers reported thus far have shown that liposome entrapment of the ascorbic acid and α -tocopherol can prevent the

degradation of these active agents and maximize their antioxidant effects [12].

Liposomes are also used to deliver minerals such as Ca⁺² and Mg⁺² into foods [13].

The benefits obtained through the application of liposome in food are, acting such as an effective controlled-release system which protects the active ingredients throughout the processes and ability to releases the ingredients where and when required [67].

- Applications of Liposomes in Cosmetics

Liposome applications in skin treatment are based on the similarity of the bilayer structure of liposome to that of the natural membranes, so depending on the lipid composition of liposome, they can alter cell membrane fluidity and deliver active drugs to the target site. Different forms of liposome preparations such as solution, creams, gels and ointments can deliver compounds across the stratum corneum [68, 69].

Liposomes based on a natural marine lipid containing a high polyunsaturated fatty acid (PUFA) ratio such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were recently introduced as Marionosomes[®] for the prevention and treatment of skin diseases [70]. These lipids exhibit anti-inflammatory properties *in vitro* and have variety of benefits regarding inflammatory skin disorders and metabolized by skin epidermal enzymes into anti-inflammatory and antiproliferative metabolites [71].

These types of liposomes provide valuable raw material for the regeneration, humidifying and softening of skin [70].

Liposomes can repair and accelerate the removal of pyrimidine dimers after skin exposure to the ultraviolet radiation. Specific antioxidants in the liposome reduce the rate of formation of secondary ultraviolet-induced damages [72].

Anti-inflammatory agents, immunostimulants, and enhancers of molecular and cellular detoxification within liposomes could avoid age spots, dark circles, wrinkles, and other clinical aspects of skin aging [72].

Nutricosmetics are an emerging class of health and beauty aid products that combine the herbs and liposomes to maintain and enhance human beauty because of their beneficial properties, such as sunscreen, anti-aging, moisturizing, antioxidant, anticellulite, and antimicrobial effects [73].

LIPOSOME COMPOSITION

Calvagno *et al.* [74] showed that mean size, polydispersity index, zeta potential, loading capacity, drug release, antitumoral activity and intracellular uptake of the encapsulated drugs were influenced by liposome lipid composition and preparation methods.

Chemical components of liposomes are lipid and/or phospholipid molecules with various head groups. These components can be subjected to certain chemical manipulations, for instance to prepare liposomes with optimized physico-chemical characteristics. Different lipid compositions could modulate both technological and biopharmaceutical parameters of colloidal vesicles thus influencing the application of liposomes as carrier systems [74].

- Types of Phospholipids

Liposome features are strictly related to chemical properties of the phospholipids used for their preparation. In fact, lipids can modify biodistribution, surface charge, permeability, and release and clearance of liposomal formulations [75]. Type of the phospholipid ingredients also influences the encapsulation efficiency, toxicity and stability of liposome [17].

The hydrophilic-lipophilic balance (HLB) is a good indicator of the vesicle forming ability of any surfactant. Single-chain surfactants were found to be compatible with vesicle formation but they

possess less encapsulation efficiency in the presence of cholesterol [76, 77]. The most conventional groups of surfactant molecules are phospholipids and possess higher encapsulation efficiency. Phospholipids are amphipathic (amphiphilic) molecules, being both hydrophilic and hydrophobic. The head group of a phospholipid is hydrophilic and its fatty acid tail (acyl chain) is hydrophobic [5].

- Sterols

Sterols are important components of cell membranes and their presence in membranes causes significant changes with regard to bilayer stability, fluidity and permeability. There are some additives incorporated to liposome structure to enhance vesicular stability. Some of these additives (or moieties) improve the stability of liposomes by providing steric hindrance. In some other instances charged molecules are used to enhance the stability of the lipid vesicles by providing electrostatic repulsion [17]. One of the most applied molecules used to increase liposome stability is cholesterol. It is known that cholesterol is able to modulate the fluidity of lipid bilayers. It is usually used in most formulations to stabilize the system against the formation of aggregates by repulsive steric or electrostatic effects and modulate the fluidity of lipid bilayers [17]. Adding cholesterol to liposome reduce the permeability of the liposomal membrane to solutes. The amount of cholesterol to be used in the liposomal formulation mainly depends on the liposome application area [5]. Cholesterol has been shown to modify the order and mobility of the phospholipids in the bilayer and hence affects the bilayer fluidity [78, 79].

- Other Additives

The presence of polyethylene glycol on the surface of liposomes provides long circulating properties, improved stability, protecting the encapsulated drug against metabolic degradation /

inactivation and increased intracellular uptake of the vesicles [80].

In some liposome formulations, charged phospholipids such as dicetylphosphate (DCP) and stearyl amine (SA) have been used to produce charged vesicles.

Adding sphingomyelin exhibited reduction in water permeability and increasing the proton permeability of some kind of liposomes [81].

EFFECT OF THE BIOACTIVES NATURE ON LIPOSOME FORMATION

One of the most important factors is the influence of the nature of the encapsulated drug on vesicle formation [17]. The electrostatic attraction between charged bioactives and liposomes is a mean to increase the entrapment efficiency. Zucker *et al.* [82] found that high positive charge (>1) of the drug leads to high encapsulation efficiency and high charge increases drug's partitioning to the aqueous phase and its interaction with the counteranions inside the liposomes.

The chemical and physical structure of various drugs or active molecules can also have a profound effect on the burst effect in controlled release systems [83]. Fig. (1), represents different types of bioactives entrapped in a unilamellar liposome

METHODS OF LIPOSOME PREPARATION

There are a wide variety of conventional techniques that can be used to produce liposomal formulations. All methods for producing liposomes require lipids to be combined by some means with an aqueous phase [84, 85].

In the following sections, some of the most applied conventional methods of liposome production are described.

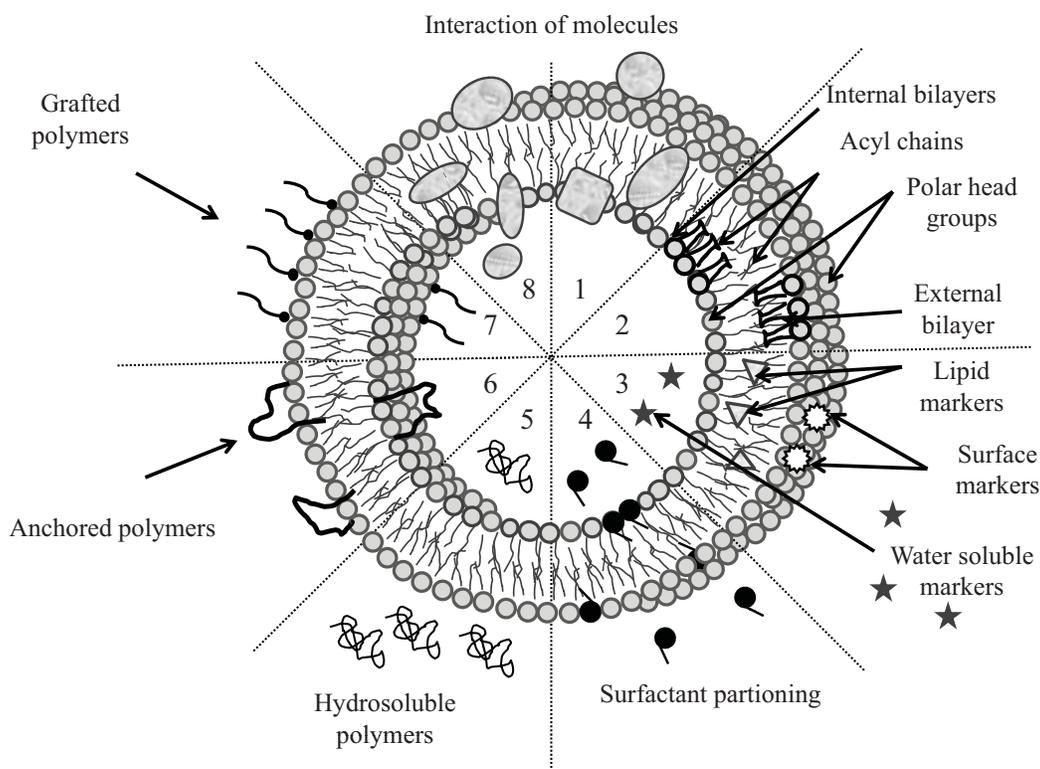


Fig. (1). Schematic representation of unilamellar liposomes (1) and positions of loaded substances (2– 8) / Hydrophobic markers are essentially located within the acyl chains of the bilayer; amphipathic labels are inserted in the polar head groups and water-soluble ones in the internal and external aqueous medium (2)/ Surfactant molecules, which are amphiphilic, partition between the liposome bilayer and the aqueous medium (3)/ Hydrosoluble polymers of various average molecular mass can be entrapped (4)/ Stealth liposomes may be formed with anchored (5) and covalently attached (6) polymers. Interaction sites of various molecules such as drugs, proteins or biological macromolecules (7, 8): water-soluble molecules can be loaded into the liposome interior or bound to the bilayer surface; amphiphilic molecules orient into bilayers; transmembrane proteins span the lipid bilayer with sites exposed to the aqueous phase. Source: Adapted from [121].

- Bangham Method

The Bangham method is one of the first methods for liposome formation and is widely used [86, 87]. The process involves the dissolution of lipids in an organic phase, removal of the organic solvent, usually via evaporation, to form a lipid film. The solvent removal stage is time-consuming. The final step is the dispersion or hydration of the lipid film with an aqueous media, carried out in conjunction with agitation to separate the swelling lamellae from the vessel surface and form sealed spherical structure. Bangham method often produces liposomes with several microns in size and consequently MLVs which limit their consumption due to low entrapment efficiency specially for water soluble active agents, difficulty in removing organic solvent and small scale production [84].

- Detergent Depletion Method

The detergent depletion method is a mild process for the production of a wide variety of vesicle types and highly homogeneous liposomes. The method is based on the formation of detergent-lipid micelles, followed by the removal of the detergent to form liposomes [88]. The disadvantages of this method are that the final concentration of liposomes in the solution is low and entrapment of any hydrophobic compound is also low. The detergent also remains in the formulation. The size and homogeneity of liposomes produced using detergent depletion are based on the rate at which the detergent is removed and the initial ratio of detergent to phospholipid. The method is very time consuming and the process of removing the detergent may also remove any other small hydrophilic compound [84, 89].

- Injection Methods

The ethanol injection method was first described in 1973 by Batzri and Korn [90]. The ethanol and ether injection methods involve the dissolution of the lipid into an organic phase, followed by the injection of the lipid solution into aqueous media, forming liposomes. The ethanol injection method is a simple method, but some lipids are poorly soluble in ethanol and heterogeneous liposomes are formed if adequate mixing is not achieved. The ether injection method differs from the ethanol injection method since the ether is immiscible with the aqueous phase, which is also heated so that the solvent is removed from the liposomal product [91, 92]. Jaafar-Maalej and colleagues [93] have used this method for encapsulation the hydrophobic and hydrophilic drugs and they found that the higher encapsulation efficiencies were about 100% for the hydrophobic drug and about 16% for the hydrophilic one and they also found small multilamellar vesicles, with sizes ranging from about 80 to 170nm.

An advantage of the ether injection method compared to the ethanol injection method is to form a concentrated liposomal product with high entrapment efficiencies. The ethanol injection method is rapid, simple and reproducible for production of a ready-to-use liposome suspension. The particle size of liposomes produced by this method is a function of lipid nature and concentration, the drug to lipid ratio and the organic solvent and aqueous phase composition. The inkjet method is a modern variation of the ethanol injection method and was developed by Hauschild *et al.* [94] for liposome formation with excellent control on particle size and high potential for scaling up.

- Reverse Phase Evaporation Method

The reverse-phase evaporation process was first described by Szoka and Papahadjopoulos [95]. The technique is carried out by dissolving the lipids in an organic solvent, adding a small volume of aqueous phase, then sonicating the solution to produce inverted micelles. The organic solvent is removed using a rotary evaporator and a viscous gel forms. A disadvantage of this method is that the

compound to be encapsulated within the vesicles is in contact with an organic solvent, therefore the process is not suitable for fragile molecules such as peptides [84].

- Microfluidic Channel Method

Jahn *et al.* [96] developed a microfluidic method for controlled liposome formation. The process involves a stream of lipid dissolved in alcohol passing between two aqueous streams in a microfluidic channel, with mixing occurring at the liquid interfaces and thus liposomes forming. The laminar flow in the channels enables controlling the size and size distribution of the liposomes. Liposome self assembly by this microfluidic method can be used for drug encapsulation immediately prior to use [96].

- Heating Method

The heating method developed by Mozafari [97] to produce liposomes involves hydration of the phospholipid components in an aqueous solution containing 3% (vol) glycerol and increasing the temperature to 60°C or 120°C, depending on the absence or presence of cholesterol, respectively. Glycerol is utilized since it is a water soluble and physiologically acceptable chemical with the ability to act as an isotonic agent and increase the stability of lipid vesicles due to preventing coagulation and sedimentation. No degradation of the lipid ingredients was reported for liposomes fabricated by the heating method [98]. Also there is no need for sterilization once high temperature (i.e. 120°C) is used in this technique [5, 99]. A further improved version of the heating method, called the Mozafari method, has recently been employed for the encapsulation and targeted delivery of the food-grade antimicrobial nisin [39]. The Mozafari method allows large-scale manufacture of the carrier systems in one step without the need for the prehydration of the ingredient material, and without employing toxic solvents or detergents [39].

- Dense Gas Techniques

The term dense gas is a general expression used to refer to a substance in the region surrounding the critical point. Dense gases possess solvent characteristic similar to that of liquids along with mass transport properties similar to those of gases. The unique properties of dense gases have been exploited to replace many organic solvents and enable improved processing techniques, in particular separation, purification and size reduction processes. The most widely used dense gas is carbon dioxide since it is non-flammable, non-toxic, non-corrosive, inexpensive, environmentally acceptable and has easily accessible critical parameters (31.1°C and 73.8 bar). The solvent can be easily recovered after processing by simply returning to atmospheric pressure. Dense gas processing can provide sterile operating conditions and one-step production that can alleviate the current liposome sterilization issues [100, 101].

- Supercritical Fluid Injection and Decompression Method

The first dense gas techniques for the formation of liposomes, referred to as the injection and the decompression methods, were described by Castor and Chu in 1998 [102]. In the injection method, a mixture of lipid, organic solvent and compressed gas is injected through a nozzle into an aqueous solution. Alternatively, the decompression method involves a mixture of lipid, organic co-solvent, compressed gas and aqueous solution being decompressed through a nozzle to form liposomes. In the injection method the compressed phase is sprayed into water, whereas in the decompression method, the aqueous phase is incorporated into the compressed phase, which is sprayed into air. The injection and decompression processes are capable of producing sterile and pharmaceutical grade liposomes of a pre-determined size with narrow particle size distributions [103].

- Supercritical Fluid Method

Frederiksen *et al.* [104] described the supercritical liposome method in 1994, which is similar to the injection method developed by Castor and Chu [102] and produced small unilamellar vesicles (SUVs) with particle sizes between 20 and 50 nm [100, 104]. The process involves the dissolution of lipid and cholesterol into supercritical carbon dioxide. The solution is then rapidly expanded into an aqueous phase containing the hydrophilic compound to be entrapped. The encapsulation efficiency was lower than that achieved using conventional liposome formation techniques [105].

- Supercritical Reverse Phase Evaporation Process

Supercritical Reverse Phase Evaporation (SCRPE) method was developed by Otake *et al.* [101]. The lipid, organic co-solvent and compressed gas are combined in a stirred variable volume cell at a temperature above the lipid phase transition temperature. An aqueous solution is then slowly introduced to the cell. The pressure is reduced by the release of the compressed gas and liposomes are formed. The principle of the SCRPE method is similar to the decompression method. The trapping efficiencies achieved were extremely low [104, 105]. Otake *et al.* [106] recently developed a new method known as the improved supercritical reverse phase evaporation (ISCRPE) technique to avoid the use of organic solvents in liposome formation and enhance the stability and the drug loading efficiency of the vesicles.

- High-pressure Homogenization Method (HPH)

High-pressure homogenizers are used for the preparation of liposomes and lipid dispersions because of their vesicle disruption capability. The sample is injected at high and constant pressure in a specially designed part of the homogenizer where rearrangement of liposome structure takes place due to turbulence, cavitations and / or shear phenomena. Properties of liposomes prepared by high-pressure homogenization depend on the pressure and number of times that the sample is processed (number of cycles) [107, 108]. HPH is especially useful for the production of very small liposomes as they are especially suitable for intravenous applications [84].

- Dual Asymmetric Centrifugation (DAC)

Dual asymmetric centrifugation (DAC) is a special kind of centrifugation in which as usual a vial is turned around the main rotation axis with a defined distance and speed. The main difference of DAC to normal centrifugation is that the vial is turned around its own center (vertical axis) during the normal centrifugation process. The energy that transfers to the sample, in the form of mechanical turbulence and cavitations produces nanoliposomes with 60nm in size and homogenous size distribution. DAC has high trapping efficiency but this method is especially useful for producing batch sizes of about a gram or even less [109].

Table 1 shows the advantages and disadvantages of the conventional methods of liposome production.

TECHNIQUES FOR ENCAPSULATING BIOACTIVE AGENTS

Selection of an encapsulation protocol is largely related to parameters such as encapsulation efficiency, drug/lipid ratio, drug retention, sterility, ease of preparation and scale up, compatibility with regulatory agencies, cost efficiency, as well as liposome and drug stability [27]. Two different ways for the encapsulation of bioactive compounds in liposomes can be distinguished: (i) bioactive entrapment during the vesicle formation process (passive encapsulation) and (ii) loading the bioactive into intact vesicles (active loading) [5].

- Passive Trapping Techniques

The passive entrapment techniques rely on the ability of liposomes to capture a certain aqueous volume (including dissolved

solutes) during vesicle formation [27, 110, 111]. For water soluble compounds which do not interact with the bilayer, the encapsulation efficiency after passive encapsulation is proportional to the aqueous volume enclosed by the vesicles, which itself depends on the phospholipid concentration of the dispersion and the lamellarity and morphology of the vesicles. As with the less water-soluble drugs which interact with the bilayer, both encapsulation parameters will depend more on the phospholipid concentration and selection than on morphological parameters [31]. During this process water-soluble (hydrophilic) molecules will be encapsulated inside the aqueous phase of the liposome while lipid-soluble (hydrophobic) agents will be located in the bilayer (lipidic phase) of the liposome. Amphiphilic molecules will be located in such way that the lipid-soluble part will be embedded between the liposomal lipids while their water-soluble part will be located in the liposomal aqueous phase [5].

- Active Trapping Techniques

In principle, active trapping technique consists of the blending of "empty" liposomes with concentrated drug solution and thereafter incubation until the drug is equally distributed by diffusion [112]. This method has some advantages, because vesicle bilayers are sufficiently permeable for drugs to allow diffusion into the liposomes within reasonable time. The drug permeates through the lipid bilayers into the vesicles following the concentration gradient until equilibrium between the interior of the vesicles and the surrounding medium is achieved [113]. The amount of hydrophobic drug that can enter in a liposome actually depends on packing restrictions in the lipid bilayer and, as a result, liposome formulations for this class of drugs change sensationally from one agent to others. Water-soluble drugs interact with the polar head groups of phospholipids and are sequestered by the liposomes but amphiphilic agents are often difficult to retain inside liposomes as they can rapidly permeate through lipid bilayers [27]. The active loading technique, however, is restricted to a small range of drugs that behave as weak amphipathic bases or acids and can permeate bilayers in the uncharged, but not in the charged, state. Active loading has certain advantages, because the active ingredient is not yet present during the preparation of the liposomes, hence the safety precautions that have to be taken when toxic drugs are handled may be minimized [114].

LIPOSOME CHARACTERIZATION

After production, characterization of liposome is required to qualify, quantify and approve the liposome capability for special application. Methods of characterization have to be exact and rapid.

-Size and Size Distribution

Calvagno *et al.* [74] have demonstrated that the mean size of liposomes was influenced by both the lipid composition and the preparation method. They found that largest mean size and polydispersity values were obtained for liposomes which were composed of oleic acid and dipalmitoylphosphatidylserine (DPPS), respectively. Oleic acid is also able to increase the liposome bilayer fluidity. Mean size and size distribution are parameters that have to be modulated as a function of the proposed application for a certain liposomal system [115].

The mean size of an aqueous dispersion of liposomes can be measured by using dynamic light scattering (DLS) operating with heterodyne detection [107, 108].

Electron Microscopy techniques (e.g. Transmission Electron Microscopy, Scanning Electron Microscopy) are also used as direct imaging techniques for liposomes, enabling not only quantitative analysis (the number of particles) but also qualitative information on the size and shape of liposomes. DLS provides information about the size but not about the shape of the lipid vesicles. In contrast, electron microscopic techniques [116] make direct observa-

Table 1. Advantages and Disadvantages of Conventional Methods of Liposome Production

Methods	Advantages	Disadvantages	Application
Bangham	Simple process	Contains the organic solvent, with agitation, large vesicles without controlling on particle size, time consuming, sterilization issue [86, 87]	Diffusion of univalent ions across the liposome by Bangham <i>et al.</i> [86]
Ethanol/ether injection	Simple process	Organic solvent residue, readily to nozzle blockage in ether system, time consuming, sterilization issue [91,92]	Preparation of Azithromycin liposomes by Wang and Zhu [212]
Reverse phase evaporation	Simple design, suitable encapsulation efficiency	Not suitable for encapsulation of fragile molecule due to large quantity of organic solvent use, time consuming, sterilization issue [95]	Amphotericin B liposomes preparation by Rojanapanthu <i>et al.</i> [213]
Microfluidic channel	Control of particle size, production of vesicles with diameter up to 29 nm	Not suitable for bulk production, organic solvent use, with agitation [96]	Encapsulation of ferrous sulfate in liposome by Kosaraju <i>et al.</i> [56]
Detergent depletion	Simple design, homogenous product, control of particle size	Contains of organic solvent, detergent residue, time consuming, poor entrapment efficiency, low yield, need to sterilization [88]	Liposome preparation by Winterhalter and Lasic [214]
Supercritical fluid injection and decompression	Control of particle size, possible in situ sterilization, low organic solvent consumption	High cost, low yield, high pressure up to 350 bar used [102,103]	liposome dispersion containing an active agent by Anton <i>et al.</i> [100]
Improved/ supercritical reverse phase evaporation	No need for using nozzles, one-step production, low organic solvent consumption, rapid process, scale-up potential, enhance stability	High cost, high pressure up to 200 bar used [106]	Preparation liposome with different phospholipids by Sakai <i>et al.</i> [212]
Dense Gas Techniques	Possible in situ sterilization, producing stabilize and homogenous liposome, low organic solvent consumption	Need to multiple stages to achieve the final size of liposome, high pressure up to 200-300 bar, readily block nozzles [84]	Processing Pharmaceutical Compounds by Foster <i>et al.</i> [216]
Dual asymmetric centrifugation	Simple method, homogenous liposome production with 60 nm size, high trapping efficiency	Not suitable for bulk production, high pressure, with agitation [109]	Preparation of vesicular phospholipids which encapsulate calcein by Massing <i>et al.</i> [109]
High-pressure Homogenization method	Able to produce liposome with diameter up 100 nm, simple design, suitable for bulk production	High pressure, sterilization issue, not homogenous liposome production, organic solvent residue [107, 217]	PEG-modified CPT-11 liposomes by Li <i>et al.</i> [218]
Heating method	Simple design, organic solvent free, without need to sterilization, scale-up possible	Use of high temperature [97, 99]	Preparation of liposomal gene therapy vectors by Mozafari <i>et al.</i> [97]

tion possible, so the shape of liposomes and presence of any fusion or aggregation can be observed. Electron microscopic techniques also provide information on the bilayer thickness and interbilayer distance of liposome.

Atomic Force Microscopy (AFM) is very-high-resolution type of scanning probe microscopy that can create three-dimensional micrographs with resolution down to the nanometer and Angstrom scales [117]. AFM is also ideally suited to characterizing nanoparticles, a more recently developed microscopic technique that can be used for the structural characterization of liposomes and has also been utilized to study the morphology, size, stability, and dynamic processes of lipid nanocapsules [118, 119]. AFM allows biomolecules to be imaged not only under physiological conditions but also during biological processes.

Gel permeation chromatography was performed to compare the elution characteristics, size distribution and homogeneity of the liposomes.

Chromatography is a kind of separation method which separates molecules in solution according to their size. With organic mobile phases, the technique is known as gel-permeation chromatography and with aqueous mobile phases, the term gel-filtration chromatography has been used. The size separation takes place by repeated exchange of the solute molecules between the solvent of the mobile phase and the same solvent in the immovable liquid phase (stationary phase) within the pores of the column-packing material. The pore size range of the packing material in the column determines the molecular size range and separation can occur [5, 120].

Size Exclusion Chromatography (SEC) separates liposomes on the basis of size and makes it possible to estimate the molecular mass of a compound. Liposomal delivery systems can be indicated during SEC elution by separating and collecting fractions and analyzing them by *photon correlation spectroscopy (PCS)*. By additional analyses, e.g., with an enzymatic phosphatidylcholine assay,

the lipid content of the liposomes can be determined in details [121].

-Transition Temperature (T_C)

Amphipathic molecules such as phospholipids have an important characteristic, they can undergo a thermotropic phase transition at temperatures much lower than their melting point. The temperature of transition depends on the nature of the hydrocarbon chains (acyl chain length, structure and degree of unsaturation of the hydrocarbon chains, presence of a methyl branch on the hydrocarbon chain), the polar region of the molecule and nature and ionic strength of the suspension medium (the amount of water molecules and any solutes dissolved in the water) [2, 122]. T_C is lowered by decreased chain length, by the degree of unsaturation of the acyl chains, as well as presence of branched chains and bulky side groups. Any hydrocarbon with cis-unsaturated tail will have a lower T_C than those which are trans-unsaturated [122].

Phase transitions and fluidity of phospholipid membranes are important in the manufacture and application of liposome. The phase behavior of a liposomal membrane determines some properties such as permeability, fusion, aggregation and protein binding. All of these properties affect the stability of liposomes and their applications [5].

Transition temperature has important effects on the liposome properties. For example having low phase transition temperature is advantageous for liposomes as drug carrier systems due to the fact that actives stored in liposomes with high phase transition temperature are generally released slower than those encapsulated in liposomes with lower phase transition temperature. Consequently, determining the transition temperature of liposome is very important [69].

Differential scanning calorimetry (DSC) has been used extensively for the determination of transition temperatures of phospholipids [123, 124].

-Surface Charge

The surface charge of liposomes can be varied, they could be neutral (by employing phospholipids such as phosphatidylcholine, or phosphatidylethanolamine), negative (with acidic phospholipids such as phosphatidylserine, phosphatidylglycerol, phosphatidic acid or dicetylphosphate) or positive (by the use of lipids such as dioleoyl trimethyl ammonium propane (DOTAP) or stearylamine) in physiological pH ranges. Liposomal charge is an important characteristic that determines liposome stability and encapsulation efficiency. The electrostatic attraction between charged bioactives and liposomes is a mean to increase entrapment efficiency [125, 126]. The charge density of liposomal surfaces and the binding affinity of various ions to the lipid vesicles can be determined by measuring a parameter called *zeta potential*.

-Zeta Potential

Zeta potential is a function of the surface charge of the lipid vesicle, any adsorbed layer at the interface and the nature and composition of the medium in which liposome is suspended. Zeta potential is not measurable directly but it can be calculated using theoretical models and an experimentally-determined electrophoretic mobility or dynamic electrophoretic mobility [113].

The greater zeta potential causes the liposomal suspension to be stable because the charged vesicles repel each other and thus overcome the natural tendency to aggregate. The lipid vesicles will aggregate, fuse, fluctuate and precipitate during storage. Increasing inter-particle repulsion, either electrostatic or steric, can enhance their stability [35]. The surface charge of liposomes can influence blood circulation time. Zeta potential values are influenced by lipid composition of liposomes [127].

Filion and Phillips [126] used Doppler *electrophoretic light scattering* for zeta potential measurement of liposomes formulated with cationic lipids.

Laser doppler electrophoresis (LDE) and *Zetasizer* [113, 124] measure the zeta potential by applying an electric field across the dispersion of liposomes.

- Fluidity

Bilayer fluidity reflects the order and dynamics of phospholipid alkyl chains in the bilayer. The influence of liposome composition on bilayer fluidity and its effect on the liposome application were investigated by Coderch *et al.* [79]. The presence of cholesterol in the membrane structure weakens Vander Waals-interactions between hydrocarbon chains of fatty acids and prevents liposome crystallization [128] and effect on bilayer fluidity. Researchers demonstrated that the incorporation of some fluid lipids into the bilayer of liposomes could interfere with the barrier function and thus lowering its phase transition temperature (T_C) and increasing its fluidity [74, 129].

The release of the entrapped drug from the liposome depends on the number of bilayers, and the bilayer permeability and fluidity of the bilayer [74, 130].

Electron paramagnetic resonance (EPR), H NMR spectroscopy and depolarization of fluorescence methods are generally used to study liposome fluidity [128].

EPR is a useful technique for determining fluidity and the structural changes of the lipid bilayers of liposomes [79].

- Lamellarity Determination

Among the most important characteristics of lipid vesicles are generally their lamellarity and size. Lamellarity is the number of lipid bilayers surrounding the inner aqueous space of the lipid vesicles. Vesicles are observed in the intermediate protonation range, as independently confirmed by various analytical techniques, for instance, electron microscopy [131].

Direct microscopical observation gives information about size, homogeneity of the sample and lamellarity of liposomes [17].

Lamellarity of a liposome preparation can be also determined by using ^{31}P -*nuclear magnetic resonance (NMR)* to monitor the phospholipid phosphorus signal intensity at the outer monolayer compared to the total signal [132-134].

Ruozi *et al.* used *Nuclear magnetic resonance (NMR)* and the *Electron paramagnetic resonance (EPR)* to investigate the lamellarity, the permeability of the bilayer and the influence of particle size on the liposomal transport of bioactive molecules [135].

- Encapsulation Efficiency / Entrapment Efficiency

Bioactives can interact with liposomes in several different styles depending on their special properties such as solubility and polarity. They can be entrapped in the lipid bilayer phase, intercalated in the polar head groups, adsorbed on the membrane surface, anchored by a hydrophobic tail or encapsulated in the inner aqueous compartment [121].

A major achievement in the medical application of liposomes is the ability to load sufficient amount of drug needed to achieve therapeutic efficacy [82]. Liposome - encapsulated compound refers to a compound which is sequestered, at least in part, in the internal compartment of liposome or within the liposomal membrane [136].

Knowledge of liposome characteristics is required to develop liposome formulations that have optimal entrapment efficiencies and allow the controlled release of bioactives. Lipid composition and preparation method can influence the entrapping efficiency of liposome formulations. Addition of cholesterol significantly alters the entrapment efficiency [17].

The encapsulation efficiency of liposome depends on the rigidity of the bilayer membrane. Laridi *et al.* [137] encapsulated nisin Z in liposome and found that the encapsulation efficiency of 'soft' liposomes (having melting temperatures near 25°C) was about to 10%, whereas a 'hard' liposome (melting temperature of 65°C) had an encapsulation efficiency of 35%. They also found that by addition of fluidizing agents such as cholesterol, the encapsulation efficiency of these systems reduced [137].

Water-soluble labeled bioactive molecules (e.g. radioactive or fluorescently labeled) are used to determine encapsulation efficiency, release profile and leakage kinetics of liposomes [138].

Most of the reported experimental methods to determine liposomal encapsulation efficiency, require removal of the free (unencapsulated) bioactives from liposome encapsulated bioactives by *column chromatography* [139], size exclusion chromatography (SEC) [55], ultracentrifugation [54], equilibrium dialysis (ED)[140], ultrafiltration [141], before quantification of the entrapped material by analytical techniques such as UV/VIS Spectrometry, HPLC, Spectrofluorimetry [140, 142, 143]. Oku *et al.* [144] determined liposomal encapsulation efficiency using the fluorescent dye calcein based on fluorescence quenching of the untrapped calcein by addition of cobalt cation. This method, in contrast to the common entrapment efficiency evaluation methods, does not require application of any separation technique.

Entrapment efficiency of liposomes was determined fluorometrically by using a *fluorescence spectrophotometer* (fluorescence quenching of calcein) by Were *et al.* [55].

Electron spin resonance (ESR) spectroscopy has also been used to determine the liposomal encapsulation efficiency whereby the addition of a paramagnetic agent such as ferricyanide results in significant broadening of the external spin-labeled marker [145]. Advantage has also been taken of the difference in diffusion coefficients between an entrapped and free marker substance such as sucrose using diffusion-ordered 2D NMR spectroscopy to assess entrapment efficiency [146].

Zhang *et al.* [139] presented a rapid and simple experimental approach using ¹H NMR in conjunction with a pH-sensitive marker compound (homocarnosine) to determine the liposomal encapsulation efficiency without the need to physically separate free from encapsulated marker.

-Liposome Stability

Liposome stability is one the most important factors in liposome applications and depends on a number of factors such as size and chemical composition of the vesicles. Liposomal drug products have to be stable for over two years at a minimum according to the regulatory principles of FDA [112].

Liposome is a relatively unstable colloidal system. Liposome stability can be divided into physical, chemical, colloidal and biological stability. All of these three aspects are interrelated.

The physical stability depends on the natural curvature of the lipid mixture (equilibrium curvature of the liposome), and the rigidity of the bilayer. More rigid membranes (with higher melting points) with curvatures near to their natural curvature would be more stable against disorders such as temperature increase, shear, vibration, freeze-thawing cycles [13].

Chemical stability refers to the ability of liposome to maintain the level of encapsulation efficiency with changes in solution chemistry such as pH, electrolyte composition, oxidizing agents, and presence of surface active compounds (e.g. surfactants, cholesterol, bile salts).

Colloidal stability mentioned by ability of the liposomes to maintain their size under various storage conditions [147].

Chemical degradation reduces the biological and physical stability of liposomes. Reduction of physical stability due to aggrega-

tion or drug leakage reduces liposome utility. The major chemical reactions are acyl ester bond hydrolysis and oxidative damage to polyunsaturated acyl chains, cholesterol, and (primary) amino groups. As for physical stability, the most important parameters in quality control and characterization of liposomal formulations are liposome size distribution and liposome physical integrity [21, 112].

Vesicles are stabilized based upon formation of different forces: Vander Waals forces among the phospholipids, repulsive forces among charged groups of phospholipid molecules, repulsive forces of the head groups of phospholipids and also short-acting repulsive forces.

Electrostatic repulsive forces are formed among vesicles upon addition of charged ingredients to the double layer, enhancing the stability of the system [17].

To overcome instability problems, liposomes may be freeze-dried. However, freezing may cause phase-transition changes, osmotic stress, and the expansion of bilayers due to ice formation [148]. This, in turn, may lead to bilayer disruption, fusion, and vesicle aggregation, resulting in loss of entrapped material and changes in liposome size distribution. Such effects can be minimized by the inclusion of cryoprotectants (e.g., disaccharide sugars) within the liposome formulations [149, 150]. As an alternative to freeze-drying, proliposome approach to liposome formation have been described as a mean of enhancing stability [151]. Proliposomes may be of two types: particulate-based proliposomes comprise soluble, free-flowing carrier particles coated with phospholipids [152], whilst alcohol-based proliposomes comprise a concentrated alcoholic solution of phospholipids [153]. Both these types of proliposomes generate liposomes on addition of an appropriate aqueous phase.

A) Degradative Damage upon Long-Term Storage

The long-term storage under specified conditions (e.g. temperature, light) affects the chemical and physical stability of liposomes. Some materials such as cholesterol and antioxidants provide protection against liposome degradation. Cholesterol in lipid bilayers has a role as an antioxidant in biological membranes [154]. With respect to long-term storage, two stability aspects have been considered for systems containing liposomes: (i) the liposome components may be degraded by hydrolysis and/or oxidation, and (ii) the physical structure of the liposomes may be affected, by aggregation, fusion or changes within the bilayer [112].

- *High-performance liquid chromatography (HPLC)* equipped with special column material with superior separation properties was introduced for the evaluation of liposome stability [155, 156]. On the detector side, several technologies such as Evaporative Light Scattering Detectors (ELSD) entered the field providing quantification approaches for liposome analysis.

- *Evaporative Light Scattering Detectors (ELSD)* is becoming the detector of choice to quantitate, UV-insensitive material such as most phospholipids. Moreover, the response of a UV detector is sensitive toward oxidative changes in phospholipids. ELSDs can be used to monitor phospholipid stability during liposome formulation studies [157].

- *Mass spectrometric (MS)* analysis in combination with HPLC enables quantification of different phospholipids in the sample. *Reverse-Phase High-Performance liquid Chromatography _ Electrospray ionization-Mass spectrometer* setup were able to quantify the lipids, also to assign acyl chain positions on the phospholipid molecule [158].

- Lasic and Papahadjopoulos used radiolabeled liposomes to evaluate and determine formulation parameters that would preserve physical stability under physiological conditions in circulation [31].

- Tchoreloff *et al.* used *Transmission electron microscopy* to observe the stability of liposome vesicles and their tendency to aggregate [159].

B) Lipid Peroxidation

Lipid oxidation, also called lipid peroxidation (LPO) or lipid auto-oxidation, is mediated by free radicals, leads to the formation of a broad spectrum of intermediates and products, and has long been a problem in the preparation and preservation of the lipid-based formulations [160].

In liposomal formulations, the two main types of lipid components, phospholipids and cholesterol, are susceptible to peroxidation reactions [161]. Oxidation of phospholipids takes place in their unsaturated, mainly polyunsaturated fatty acid (PUFA) chains [162].

Acyl chain and cholesterol peroxidation are interrelated. Cholesterol seems to inhibit phospholipid acyl chain peroxidation in the lipid bilayer, and the PUFA level influences cholesterol peroxidation, with higher PUFA levels decreasing cholesterol peroxidation [163, 164].

Normally, oxidation is hardly a problem in practise, but it can be minimized by using an inert atmosphere (e.g., nitrogen), metal-complexing agents (e.g., EDTA) and antioxidants (e.g., α -tocopherol) [31].

Several techniques are available for measuring and quantitating the rate of LPO in membranes. Quantitative determination of individual fatty acids (using GC) or cholesterol (using HPLC) enables an accurate follow-up of liposome lipid degradation [165].

- Variations in liposome size and polydispersity index are indicative of liposome stability. Liposome size, homogeneity, and stability can be determined by *laser light scattering*, by *gel filtration columns* and *freeze fractures Electronic Microscopy* [113].

- Oxidative Stabilization

Preventive and protective procedures are generally performed to minimize oxidative damage. Preventive procedures include the efficient chelating of ions of transition metals such as Fe^{+2} and Cu^{+2} and protection from light and from exposure to air [162].

These preventive measures are not sufficient to prevent LPO completely. Therefore, in many cases there is a need to use additional preventative methods. A common strategy of protection against LPO employs reducing agents (conventional antioxidants) that act as preventive and chain-breaking antioxidants (e.g. α -tocopherol) (Lichtenberg and Barenholz, 1988). The stabilizing effect of the various carbohydrates such as Trehalose, have also been reported [150]. In fact, a wide spreading application for Trehalose has been reported, ranging from stabilization and preservation of vaccines and liposomes to hypothermic storage of human organs [149]. Trehalose is very effective at preventing of fusion between liposomes during drying and it is able to inhibit leakage of water-soluble marker from unilamellar liposomes during freeze-drying [149, 150].

Trehalose is prominently listed as an ingredient in cosmetics [166]. Trehalose has been shown by several groups to suppress free-radical damage [167], protect against damage from anoxia [168, 169], inhibit dental caries [170], enhance ethanol stabilize and the flavors in foods [171].

C) Liposome Hydrolysis

In aqueous liposome dispersion, the liposomal phospholipids can be hydrolyzed to free fatty acids and 2-acyl and 1-acyl lysophospholipids [172, 173]. Further hydrolysis of both of the above-mentioned lysophospholipids results in glycerophospho compounds. The hydrolysis of liposomal phospholipids is catalyzed by protons and hydroxyl ions and the hydrolysis rate reaches a minimum at pH 6.5 [174]. The effect of temperature on the hydrolysis rate of phospholipids can be adequately described by an Arrhenius

equation, if no phase transitions occur in the experimental temperature range [172, 175].

- The phospholipids hydrolysis determined by quantification of Non-Esterified Fatty Acids (NEFA) by using a *Biochromatic ELISA* reader [176].

The most conventional methods for characterization assays of liposome formulation are represented in Table 2.

LIPOSOME TARGETING MECHANISMS

One of the challenges in nanotherapy is to reduce or completely eliminate side-effects. If bioactive agents act solely on their chosen target to produce the desired effect without causing unwanted effects on other systems, their usefulness will be enhanced significantly [177]. One of the most important aspects of nanomedicine and nanotherapy is the targeting of therapeutic agents to the desired organs and tissues using special nanocarrier systems. Because of their unique properties, nanosystems enhance the performance of medicines by improving their solubility and bioavailability, increasing their stability and establishing high concentrations of bioactives in target cells and cellular compartments in order to gain therapeutic efficiency [17].

The use of liposomes for targeted drug delivery offers several advantages over direct conjugation of a targeting ligand to the therapeutic agent. First, the availability of functional groups for direct ligand conjugation to a drug molecule may be limited, rendering the conjugation chemistry problematic. Second, biological activity can be compromised, requiring the additional construction of a cleavable linker to enable drug release following endocytosis. Moreover, multiple drug molecules can be delivered upon internalization in a single liposome, whereas only single drug molecules are generally delivered following the uptake of directly conjugated agents. Thus, targeted liposomal formulations may be preferred over directly conjugated therapeutic agents [178].

Targeted delivery can be achieved by either active or passive targeting.

A) Active Targeting

Active targeting, involves the directed movement of the lipid vesicle to the given organ, tissue or cell before release of bioactive agent occurs. This can be achieved via appropriately engineered modifications to the liposomal structure. For active targeting of liposomes, thermo-labile, pH-sensitive, photo-sensitive and antibody coated vesicles have been designed [179, 180].

- Active targeting can also be achieved by utilizing external stimuli such as ultrasound, magnetic or laser field and light [181]. Experiments have shown that ultrasound focused on tumor tissue causes the release of drugs from polymeric micelles only at the tumor site [182]. This method has some advantages as well as some disadvantages. The beneficial effects, with respect to drug and gene delivery, include the loosening of cell-to-cell junctions, the permeabilization of cell membranes, the stimulation of stress response (or other) pathways in cells, the release of drugs and genes from various carriers, the deposition of heat and the activation of some chemicals by free radicals. The detrimental effects include unwanted cell injury / death and degradation of the drugs and polynucleotides. Thus one of the challenges to ultrasonic drug and gene delivery is to find the correct balance of ultrasonic parameters that maximize helpful and minimize harmful effects in order to create a functional therapeutic approach [182, 183].

- Magnetic drug targeting (MDT) uses paramagnetic particles as drug carriers, for accumulating them in target tissues by using strong local magnetic fields, and has been used in the treatment of cancer patients with some success [184]. Zheng *et al.* reported that the magnetic drug targeting was applicable in gene delivery [185].

Magnetic particles have been widely used in various aspects in biotechnology and biomedicine fields, such as biosensor, MRI

Table 2. Characterization Assays for Liposomal Formulations

Basic Assays	Methodology
pH	pH meter
Osmolarity	Osmometer
Lipid concentration	Lipid phosphorus content, HPLC, Enzymatic assays, HPTLC
Lipid composition	TLC, HPLC
Cholesterol concentration	HPLC, HPTLC, Enzymatic assays
Lipid hydrocarbon chains composition	GC
Residual organic solvent	GC
Heavy metals	NMR
Chemical stability	
Lipid hydrolysis	HPLC, HPTLC
Lipid hydrocarbon chain oxidation	Lipid peroxides, Conjugated dienes, Fatty acid composition (GC), TBA method
Free fatty acid concentration	HPLC
Cholesterol oxidation	HPLC, TLC
Physical stability	
Appearance, color, clarity	Pharmacopoeia methods
Vesicle size distribution, submicron range	DLS, SLS, Electron Microscopy, HPSEC, Turbidity
Micron range	Light obscuring method, Light microscopy, Laser diffraction, SLS, Coulter counter
Lamellarity	Electron microscopy
Zeta potential	Electrophoretic mobility, Zetasizer
Thermotropic phase behaviour	DSC, NMR, FTIR, Fluorescence spectroscopy, Raman spectroscopy
Phase separation	DSC, NMR, FTIR, Fluorescence spectroscopy, Raman spectroscopy, ESR, turbidity, AFM
Microbiological assay	
Sterility	Pharmacopoeia methods
Endotoxin	Pharmacopoeia methods (LAL)

Abbreviations: HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer liquid chromatography; TLC, thin-layer chromatography; GC, gas chromatography; NMR, nuclear magnetic resonance; TBA, thiobarbituric acid; DLS, dynamic light scattering; SLS, static light scattering; HPSEC, high-performance size-exclusion chromatography; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy; ESR, electron paramagnetic spin relaxation spectroscopy; AFM, atomic force microscopy; LAL, limulus amoebocyte lysate assay. Source: Adapted from [111].

contrast agent, cell separation, immunoassay, targeted drug delivery, and hyperthermia and they have a long and controlled sustained release profile by changing the number of polymeric lipid layer [186].

- Asmatulu *et al.* synthesized biodegradable magnetic nanosystems for the purpose of magnetic targeted drug delivery [187]. However, the amount of drug that magnetite nanosystems can carry is limited, and stability of the dispersion with respect of precipitation and oxidation need to be improved. Coating with polymers is a good route to solve these problems, for example, recently the results have shown that different problems are associated with drug delivery across the blood brain barrier (BBB) will be solved by using polymeric/solid lipid nanoparticles [188, 189].

A potential benefit of using magnetic nanosystems is the use of localized magnetic field gradients to attract the particles to a chosen site and the possibility to hold them until the therapy is complete [190].

- In order to release the liposomal content at the target site, photosensitivity is another property that has been introduced into liposomes. Photosensitive liposomes have been produced by conjugation of retinoids, photopolymerizable or photolabile lipids and lipoidal derivatives of retinoic acid into bilayer membranes [180]. The photoactive agents in the lipid bilayer were used for destabilizing the liposome membrane and causing the release of contents in the aqueous compartment due to exposure to UV-A, UV-B or gamma [191, 192].

- The pH-sensitive carriers destabilize endosomal membrane under the low pH inside the endosome / lysosome compartment and liberate the entrapped bioactive into the cytoplasm and the liposomes subsequently fuse with the endosomal membranes resulting in release of their contents into the cytosol [193].

- Recently, a number of methods have been developed for the modification of the liposome surface using thermally responsive polymers to enhance the temperature-sensitivity of the vesicles.

This mechanism can be used for increasing the release of anti-cancer drugs at external hyperthermic temperatures of tumors [194, 195]. Temperature-sensitive liposome is a kind of targeted delivery system, which releases its contents in response to environmental temperature. Liposomes that exhibit a response at few degrees above physiological temperature are considered to be especially useful for site-specific targeted delivery of drugs in the body because such liposomes can release drugs specifically at a target area where heat is applied [196].

Li *et al.* [197] hypothesized that: (i) The sol-gel transition temperature of thermosensitive targeted drug delivery system should be below the body temperature, and its gel-sol transition temperature should be greater than physiological body temperature, (ii) The thermosensitive drug delivery system has none or a little release in the body temperature, while it possess large accumulative release at higher temperatures of the tumor site, about (40–44°C), (iii) In order to be effective, the thermosensitivity of drug delivery systems should be among a narrow temperature range. Temperature-sensitive drug delivery system should have a reversible structural transition from a closed form to an open form as a result of changes the external temperature giving on-off switches for active drug delivery.

- Active targeting of a therapeutic agent can be obtained by conjugating the therapeutic agent or the carrier system to a tissue or cell-specific ligand. Conjugation of targeting ligands to bioactives or carriers is the most conventional method of site-specific drug delivery. For this purpose, various techniques have been devised, including covalent and non-covalent conjugation [198]. Direct coupling of bioactives to a targeting ligand restricts the coupling capacity to a few bioactive molecules. In contrast, coupling of nanocarriers to ligands allows import of thousands of bioactive molecules by means of one receptor targeted ligand [199, 200]. The coupling reactions must not affect the biological activity of ligand and should not adversely affect the structure of nanocarrier systems. Moreover, such coupling reactions must be optimized so that binding of ligands takes place in a homogeneous manner on the surface of the nanocarrier system. The identity and characteristics of the targeting moiety are important for circulation time, cellular uptake, affinity and extravasation [201]. Virosomes [202, 203], or artificial virus particles, are one type of liposomes that contain reconstituted viral proteins in their structure. Due to the presence of the specialized viral proteins on the surface of virosomes, they can be used in active targeting [177, 204].

- Another class of lipid vesicles designed for active targeting of their encapsulated / entrapped material inside the body is known as immunoliposome. The immunoliposomes possess moieties such as antibodies, carbohydrates and hormones on the outer surface of their membrane [205, 206]. Various ligands can be attached to the outer surface of the lipid vesicles by either insertion into the membrane, adsorption to the surface, via biotin - avidin pair or through the most preferable method covalent binding [161]. These ligands attached to the immunoliposome have a complementary binding site on the target cell.

B) Passive Targeting

In this method the bioactive-carrier complex reaches its destination by following the physio-anatomical conditions of the body. Site-specific delivery is achieved based on the physicochemical properties of bioactive carrier complexes and does not require utilization of any targeting strategy. The clearance kinetics and *in vivo* biodistribution of carrier systems depend on the physicochemical factors like size, charge and hydrophobicity and can be manipulated to enable passive targeting [207].

Liposomes with a mean diameter of 100-nm, for example, can selectively extravasate in tissues characterized by leaky vasculature such as solid tumors [208], while liposomes with larger diameters (≥ 1 micrometer) are taken up by the reticuloendothelial system

(RES) in a passive manner. Stealth carriers are a kind of liposomes, which can be made by covering the surface of the lipid vesicles with hydrophilic chains to prevent opsonization [209]. This will provide liposomes with long circulation time and less elimination from the blood and, as a result, higher drug uptake. The vesicles will migrate and accumulate in the tumorous or infected area and as the stealth liposomes become degraded, they will release their drugs into the surrounding area [210, 211]. This is an example of passive targeting because the stealth liposomes migrate and treat the injured area passively, without employing any active targeting strategy.

SUMMARY

Due to their unique properties, including low cytotoxicity, good biocompatibility and biodegradability, liposomes possess wide applications in different fields including gene and drug delivery, food and nutrition industries and cosmetics. Most of the liposome producers are interested in using a low cost and simple process without contamination of the product. Many processes are under development to produce sterile and homogenous liposome with low cost and high stability and eliminate organic solvent usage. The method of preparation has significant effects on liposome properties such as size, shape and lamellarity, which, in turn, determine the encapsulation and entrapment efficiency. Several techniques are available for assessing physical and chemical properties of liposomes. However some of them are time consuming and involve high operation costs.

One of the most important aspects of nanomedicine and nanotherapy is the targeting of therapeutic agents to the desired organs and tissues using special nanocarrier systems of different natures to cure human diseases. Recently, a number of methods have been developed in order to modify the liposome structure and improve their stability as well as establishing high concentrations of bioactives in the target cells and cellular compartments in order to gain maximum therapeutic efficiency. Active targeting can be achieved via appropriately engineered modifications to the liposomal structure. For active targeting of liposomes, thermo-labile, pH-sensitive, photo-sensitive and antibody coated vesicles, have been designed. By passive targeting, the bioactive-carrier complex reaches its destination based on the physicochemical properties of bioactive carrier complexes and does not require utilization of any targeting strategy.

ABBREVIATIONS

AFM	=	Atomic Force Microscopy
BBB	=	Blood brain barrier
DAC	=	Dual asymmetric centrifugation
DCP	=	Dicethylphosphate
DHA	=	Docosahexaenoic acid
DLS	=	Dynamic light scattering
DOTAP	=	Dioleoyl trimethyl ammonium propane
DPSP	=	Dipalmitoylphosphatidylserine
DSC	=	Differential scanning calorimetry
ED	=	Equilibrium dialysis
ELSD	=	Evaporative Light Scattering Detectors
EPA	=	Eicosapentaenoic acid
EPR	=	Electron Paramagnetic Resonance
ESR	=	Electron spin resonance
HLB	=	hydrophilic- lipophilic balance
HPH	=	High-pressure Homogenization
HPLC	=	High-performance liquid chromatography
ISCRPE	=	Improved supercritical reverse phase evaporation

LDE	=	Laser doppler electrophoresis
LPO	=	Lipid peroxidation
MDT	=	Magnetic drug targeting
MLV	=	Multilamellar vesicle
MS	=	Mass spectrometric
MVV	=	Multivesicular vesicle
NEFA	=	Non-Esterified Fatty Acids
NMR	=	Nuclear magnetic resonance
PCS	=	Photon correlation spectroscopy
PEG	=	Poly ethylene glycol
PUFA	=	Polyunsaturated fatty acid
RES	=	Reticuloendothelial System
SA	=	Stearyl amine
SCRPE	=	Supercritical Reverse Phase Evaporation
SEC	=	Size Exclusion Chromatography
SUV	=	Small unilamellar vesicles
Tc	=	Transition temperature
ULV	=	Uunilamellar vesicles.
VPGs	=	Vesicular phospholipid gels

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Mechanism of Bioactive Transfer through Liposomal Bilayers

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Abstract: Today, liposomes are one of the most effective carrier systems employed in biological, pharmaceutical, medical and nutritional research. In order to optimize a liposomal formulation for the encapsulation, delivery and release of the entrapped material, it is necessary to study material passage through the lipidic and aqueous phases of the lipid vesicle. Towards this end, this article aims to review the mechanisms of bioactive transfer between different layers of a liposome and it also discusses the bioactive release mechanism. Several methods of detection and observation of bioactive transfer in liposomal systems are presented.

Keywords: Liposomal bilayer, liposome observation, mechanism of transfer, membrane permeability, release mechanism.

1. INTRODUCTION

Currently liposomes are an important part of biological, pharmaceutical, medical and nutritional research. This is because, liposomes are one of the most effective carriers for the introduction of many different types of bioactive agents into target cells and to different parts of non-living systems such as food material [1].

Targetability is an important attribute of the lipid vesicles. Targeting bioactive agents is necessary to obtain adequate concentration of bioactives at the target site for their optimum efficacy. Targeted release increases the effectiveness of bioactives, broadens their application range and ensures optimal dosage, thereby improving the cost-effectiveness of the product [2].

The transport of small molecules across lipid bilayers is a fundamental biological process. Currently, there are two proposed models for drug translocation across membranes, i.e. active and passive translocation [3]. Transport of molecules across the lipid bilayers consists of a multistep process which is comprised of the following steps: adsorption, dehydration, diffusion, rehydration and desorption [4].

This article explicates the conventional techniques of liposome targeting release and discusses the mechanism of bioactive transfer from liposome bilayers and the techniques of bioactive location and observation.

2. RELEASE MECHANISMS

The goal of drug delivery system is to administer a drug at a therapeutic concentration to a particular site of action for a specified period of time. The design of the final product for drug delivery depends upon different parameters. 1) The drug must be administered by considering some factors which affect therapeutic action of the drug. These parameters

include the site of action, the concentration of the drug at the time of administration, the period of time that drug must remain at a therapeutic concentration, and the initial release rate of the drug for controlled release systems. 2) The drug must remain physically and chemically stable in the formulation for a defined time. 3) The choice of delivery method must indicate the effective administration route for the drug [5].

In recent years, the study of controlled release of drugs and other bioactive agents from carrier systems has attracted many researchers from around the world [6]. As mentioned above, liposomes have been extensively used as a carrier for both hydrophobic and hydrophilic bioactives. In order to achieve specific targeting; liposomes should release their contents in special sites with effective doses. In the following sections, the mechanisms of bioactive release from carrier systems are described:

2.1. Bioactive Release Mechanisms

Three types of release mechanisms for non-immediate-release systems have been defined: delayed release, prolonged release, and controlled release.

Delayed release system allows multiple doses to be incorporated into a single dosage form and reducing the problem of frequent dosing. The prolonged-release system extends the release of the drug, for example, by controlling the dissolution rate of the drug compared to an immediate release system. The controlled-release systems are able to maintain the drug release in a constant rate throughout during the desired time [5].

Currently, many efforts in the field of drug delivery have been performed for developing the targeted delivery systems in which the drug is only active in the target site and formulating the sustained release systems in which the drug is released over a period of time in a controlled manner [7].

The controlled release technologies have advanced during the last four decades. As a result, hundreds of commercial products have been presented based on the controlled drug delivery technologies such as Phenylpropanolamine hydrochloride and Doxorubicin [8-10]. Controlled

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release formulations can be used to reduce the amount of drug necessary to cause the same therapeutic effect in patients. The convenience of fewer and more effective doses also increases patient compliance [11].

Selection of a controlled drug delivery technology suitable for each drug depends on many factors, including physicochemical properties of the drug, duration of release and the release profiles [8].

The inability to control drug release rates during the circulation in body is perhaps the most significant limitation of some of the present available liposomes. Ideally, they must be able to completely eliminate drug leakage during the circulation and then to increase the release rate at the target site to an acceptable concentration; which provides the optimal effect for the specific drug [12].

Different types of drug delivery systems can be designed to release the bioactive agent by a variety of mechanisms. According to these mechanisms, Langer [13] categorized these systems into diffusion-controlled, chemically controlled, swelling-controlled and magnetically controlled devices.

2.1.1. Diffusion-Controlled Drug Release

Diffusion-controlled drug release model can happen in two different ways, reservoir and matrix method. In the reservoir methods, the drug reservoir is covered with a thin polymer layer like Poly lactic acid (PLA) which acts as a rate-controlling for membrane and vary considerably with respect to their physical properties (crystallinity, glass transition temperatures, water uptake, etc.) [14].

At the steady state, the drug release rate remains constant to result in a zero-order (constant) release. In the matrix methods, a drug is usually dispersed inside the polymer matrix, and is released without any rate-controlling barrier layer. During the migration of drug molecules from the surface to longer distances, the drug release rate decreases over time and resulting in non-zero-order release. In recently developed controlled drug delivery technologies, it was identified that the zero-order release would be more desirable than other methods of drug release. In these systems of polymeric matrices, including both the membranes of reservoir devices and the bulk porous structure of matrices, drug diffusion is the rate-limiting step [8,15]. Narasimhan and Langer [16] proposed that, in the systems of polymeric matrices the burst effect is controlled by the drug solubility and diffusion coefficient in the release medium.

In many of the controlled release formulations, immediately after placement in the release medium, an initial large volume of drug is released before the release rate reaches a stable profile. This phenomenon is typically referred to as "burst release" [8].

In addition to biological and medical science, food companies also are very interested in the development of burst release systems in the foods. Coatings are desired to protect flavors and aromas during processing and storage, but they must release their contents rapidly when the product is consumed [6].

2.1.2. Dissolution/Degradation-Controlled Drug Release

Dissolution/degradation-controlled drug release is based on the dissolution or degradation of a polymer membrane

encapsulating the drug or a drug-containing polymer matrix itself. In this case, the biodegradable polymers can be effectively used because water-soluble polymers dissolve almost quickly than biodegradable polymers such as poly (glycolic acid), poly (lactic acid), so they may not be a good option for controlled release during the weeks and months [8, 17].

2.1.3. Ion Exchange-Based Drug Release

The Ion exchange method can be used very effectively for controlled release of ionized drugs which bind to the drug delivery systems by electrostatic interactions. The ions having the same charge as the drug, replace the drug molecules for release. This method is exactly applicable for delivery of ionized drugs including DNA molecules [8].

2.1.4. Swelling-Controlled Systems

In the swelling-controlled systems, absorption of solvent (water) leads to polymer expansion (a matrix with a constant releasing area, which is the main control element of the drug release) and thus different release kinetics, where convective transport of water is combined with Fickian diffusion to determine the overall release profile [15]. The release rate is determined by the rate of diffusion of fluid in the polymer and its macromolecular relaxation [18].

More possible mechanisms for controlled release systems including:

2.1.5. Osmosis-Based Controlled Release

In this way, the drug is released at zero-order release because of the osmotic pressure inside the capsule. The drug molecules are pumped out into the environment with convection, not just by diffusion, this type of drug delivery systems may be a good option where drug release by convection is considered [19].

2.1.6. Prodrug Delivery

Prodrug delivery is a novel strategy to improve specificity and therapeutic efficacy with lower cytotoxicity and it acts based on chemical (e.g., hydrolysis) or enzymatic degradation in the body [20]. The drug release kinetics is likely to be affected by certain parameters, such as pH and the enzyme concentration, which cannot be controlled by the system itself [8].

Prodrugs are pharmacological molecules that are administered in an inactive or significantly less active form. Once administered, the prodrug is metabolized *in vivo* into an active form. Thus, prodrugs are molecules that must undergo biotransformation prior to exhibiting their therapeutic effects, for instance Isoniazid [21]. Additionally, the use of a prodrug strategy increases the selectivity of the drug for its intended target [22].

2.1.7. Thermosensitive Hydrogels

Thermosensitive hydrogels are a kind of nanoparticles that have been widely investigated for controlled delivery based on their phase transition [23]. It is well known that membrane permeability reaches a maximum around a lipid's gel-to-liquid crystalline phase, L_{α} , at transition temperature, T_C (i.e., the temperature at which the lipid's acyl chains melt). This increased permeability has previously been attributed to either or both of the following possibilities: (1)

defects caused by mismatched gel and L_{α} phase hydrocarbon chain domains; (2) strong density and thermal fluctuations resulting in increased lateral membrane compressibility, which lowers the energy barrier for molecules to pass through the membrane, or to create defects [24]. This sharp increase in membrane permeability at T_C provides the possibility of controlling the release of the liposome contents.

2.1.8. Other Controlled Release Technologies

Other liposome release mechanisms include pH-sensitive liposomes, magnetic and microwave induced, and laser-triggered release [11, 25].

- Magnetic drug targeting (MDT) uses paramagnetic particles as drug carriers, for accumulating them in target tissues by using strong local magnetic fields, and has been used in the treatment of cancer patients with some success [25, 26]. Zheng and colleagues reported that the magnetic drug targeting was applicable in gene delivery [27].
- Photosensitivity is another property that has been introduced into liposomes. Photosensitive liposomes have been produced by conjunction of retinoids, photopolymerizable or photolabile lipids and lipoidal derivatives of retinoic acid into bilayer membranes [28]. The photoactive agents in the lipid bilayer were used for destabilizing the liposome membrane and causing the release of contents in the aqueous compartment due to exposure to UV-A, UV-B or gamma [29].
- The pH-sensitive carriers destabilize endosomal membrane under the low pH inside the endosome / lysosome compartment and liberate the entrapped bioactive into the cytoplasm and the liposomes subsequently fuse with the endosomal membranes resulting in release of their contents into the cytosol [30].
- Recently, a number of methods have been developed for the modification of the liposome surface using thermally responsive polymers to enhance the temperature-sensitivity of the vesicles. This mechanism can be used for increasing the release of anti-cancer drugs at external hyperthermic temperatures of tumors [31]. Temperature sensitive liposomes leak more readily above the phase transition temperature of their membrane lipids. These liposomes are designed to be stable up to 37° C but will break down as they pass through an area of the body where the temperature is above 40° C such as the interior of a tumor. Thermosensitive liposomes represent one of the advanced delivery methods for anticancer agents [32].

2.1.9. Physical and Chemical Mechanisms for Controlled Release Systems

In another category of drug delivery systems, the controlled release mechanisms can be classified into physical and chemical mechanisms. The physical mechanisms include diffusion of drug molecules through the vesicle membranes, dissolution or degradation of polymer matrix (in case of

polymeric carriers), or disintegration of the liposomal vesicles. One of the main advantage of using physical mechanisms is that the drug release kinetics can be controlled by the drug delivery system itself. Each drug delivery system has predetermined drug release kinetics that can be adjusted by varying simple parameters, such as thickness of the carrier membrane, type of a carrier used and surface area [8]. In chemical mechanisms, the covalent bonds which connect drug molecules to a delivery system break by either chemical or enzymatic degradation. The main disadvantage of these mechanisms is that drug molecules have to be chemically modified for connecting to the delivery system and these result in new chemical qualities which are called prodrugs. For this reason, the physical mechanisms have been used widely. They are used simply and highly effective in controlling the drug release kinetics [8].

2.2. Effective Parameters on Release Mechanism

The physicochemical properties of the target molecule can significantly impact the rate of release [33].

The drugs' physicochemical properties are critical subject in the design of the delivery systems. Solubility, stability, and pH of drugs can effectively change the mechanism of drug delivery in controlled delivery systems [5].

Experimental data acquired by using ^{31}P -NMR analysis and trap volume measurements, designated that the number of lipid bilayers in liposomes augmented by increasing the particle size. Increasing the lipid bilayers motivates a more effective barrier and consequently slowing the release of drugs [34]. Nagayasu *et al.* reported that the inflection of small unilamellar vesicles (SUVs) compared with large unilamellar vesicles, is greater and conjunction between the lipids in the membranes of large unilamellar vesicles is more stable compared with small unilamellar vesicles. In addition, they also found that the rate of release from liposomal formulations is drug-dependent [35].

Zhang *et al.* [36] concluded that the release of the amphiphilic drugs (such as 5-carboxyfluorescein) from the unilamellar liposomes was greater than multilamellar liposomes with similar size. The curvature of small unilamellar liposomes is greater and consequently the packing between the lipids because of acyl chain orders in these membranes, is weaker. Xing and Anderson [37] proposed that chain ordering in lipid bilayers, which can be characterized by both segmental order parameters and bilayer surface density, is a major determinative of molecular transport across lipid bilayers. Additionally, by increasing chain ordering within lipid bilayers, solute partitioning into bilayers substantially reduces [37].

Significant difference in the release profiles of drugs were determined by Calvagno *et al.* [38] by the presence of two factors, i) the strength of the drug-liposomal lipid interaction, i.e. the stronger the interaction causes the less of desorption and so the burst effect, ii) the fluidity of the bilayer, i.e. by increasing the fluidity of the bilayer, the drug leakage to outer liposomal aqueous compartments is rapidly increased.

2.2.1. Coating Strategies for Prevention of Bioactive Delivery Systems

The challenge in bioactive targeting is not only the targeting of bioactive to a specific site but also retaining it for optimum duration to elicit the desired action [39], because, they are very rapidly taken up by the reticulo-endothelial system (RES), which limits the blood circulation time of liposomes and can release the entrapped drug into the blood stream. 1) One of the most conventional methods that mentioned before, is the use of polymers, particularly hydrophilic polyethylene glycol (PEG), to modify liposomes. 2) Another way of protecting the liposomes from the immune system is to encapsulate the drug-containing liposomes in a polymer matrix in the form of a microcapsule, 3) The third method of obtaining stabilized liposomes is to use the archaeobacterial membrane lipids or their analogs as the lipid component of the liposomes. Archaeobacteria and some gram-positive bacteria compared with liposomes, are relatively more thermostable, more resistant to oxidation and chemical and enzymatic hydrolysis and extreme changing of pH (2 to 10) [40-42].

3. BIOACTIVE TRANSLOCATION ACROSS BILAYER MEMBRANES

Currently there are two proposed models for drug translocation across cellular membranes, i.e. active and passive translocation.

Passive translocation is dominated by diffusion across and out of the membrane, without the consumption of cellular energy. This process (i) is without carrier proteins, (ii) is unsaturable, and (iii) is the main mechanism by which considerable quantity of drugs diffuse through lipid membranes into the body. Generally, passive translocation exhibits low structural specificity and is due to a concentration gradient across the membrane.

In active drug translocation, energy is consumed by the cell in processes such as endocytosis, carrier-mediated transport or the use of especially efflux receptors. Active translocation is especially important for large drug molecules, whereas the endocytosis and exocytosis processes are applicable to the movement of even larger systems such as vitamin B₁₂ [43-45].

Baciu *et al.* [43] determined third category of translocation mechanism for cationic amphiphilic drugs (CADs), i.e. haloperidol and spiperone, by degradation of the model membrane formed by a single, well-characterized phospholipid with the CAD in a physiological buffer.

They observed different liquid crystalline phase of membrane by using the small-angle X-ray scattering (SAXS) techniques.

After a period of three weeks, they could see different liquid crystalline phase, in both spiperone and haloperidol systems by using the time-dependent two-dimensional X-ray diffraction pattern. This indicates that there is a continuous action of the CAD upon the phospholipid bilayer. In contrast, the changing of lipid bilayer is not evident in the control system without CADs [43].

For further investigations of CAD–membrane interactions, Baciu *et al.* [43] used the solid-state ³¹P NMR spectroscopy of anisotropic and the residual chemical shift anisotropic tensors (powder patterns) from the ³¹P nucleus in the phospholipids indicated the presence and the nature of different liquid crystalline phases. The results achieved by the ³¹P NMR spectra of the systems during the three weeks were also confirmed by the SAXS data [43]. A solid-state ¹³C MAS NMR experiment gives high-resolution information about the framework and the structure of the phospholipid bilayer.

The chemical shifts of peaks are indicative of the presence of the carbonyl group of oleic acid and ester moiety in the phospholipid. The intensity of the oleic acid signal increased with time and that of the phospholipid decreased with time [45].

Baciu *et al.* [43] prepared the fluorescent – labeled CAD and directly visualized the membrane degradation process by fluorescence and transmission electron microscopy. The fluorescent – labeled CAD binds rapidly to the membrane interface and visibly distort the membrane. The formation of the membrane fragments is a result of drug-induced ester hydrolysis. The degradation continues until they are completely disintegrated and acyl chain formed [43].

Baciu *et al.* concluded that CADs will bind rapidly to membrane surfaces with selection of some mechanical state of the membrane. Once at the membrane–water interface, the CAD rapidly catalyses chain hydrolysis, although, the rates depend on the state of membrane stress and the chemistry of the CAD itself. It has been shown that CADs will have different reaction rates for membranes with different curvature elastic stresses [43].

3.1. Permeability of Bilayer Membranes

It is well known that drugs have to pass several membrane barriers for exerting their pharmacotherapeutic effects. These barriers affect the pharmacokinetic behavior of drugs and their capability to access the target site. In addition, partitioning always occurs because of drugs' binding to membrane receptors and transporters, so prediction of drug–membrane permeability is important for optimum efficacy [46].

The transport of small molecules across lipid bilayers is a fundamental biological process. Most of the biologically important transport of ions and bulky molecules with very low permeability across the lipid membrane occurs via proteins. Small, uncharged molecules (*e.g.* water and glycerol), however, permeate across the lipid component of the membrane at an appreciable rate [3].

Shimanouchi *et al.* [47] proposed that the main possible mechanism for permeation of water-soluble probe across the simple phospholipid membrane could be diffusion through the hydrocarbon portion of the membrane. It must overcome the barrier of the hydrophilic part of the membrane.

In the following section, the theories and models for explaining the permeability of membrane are discussed:

3.1.1. Theories and Models for Explaining the Permeability of Liposome

Theory 1

Cherny *et al.* [48] explained the transport of the amphiphilic drugs through the bilayer lipid membrane (BLM) by a model that contains 5 steps. The process includes: 1) Adsorption of RH^+ (amphiphilic drug) on the BLM surface, 2) Formation of the neutral form of the drug [$RH^+ \rightarrow RH + H^+$]; 3) Transport of the neutral form of the drug through the BLM; 4) Formation of the charged form of the RH on the other side of the BLM [$RH^+ \rightarrow RH + H^+$]; 5) Desorption of the charged form of the drug from BLM.

The Inner Field Compensation (IFC) method measures the difference between the boundary potential on each side of BLM [48].

The membrane permeability of some molecules such as certain peptides could be proportional to either the partition coefficient into the BLM or to the diffusion coefficient across the BLM [49].

Theory 2

The most generally accepted model to describe the permeation of small neutral permeants across lipid bilayer membranes is the solubility-diffusion model. Solubility-diffusion theory depicts the bilayer membrane as a thin, homogeneous slab of bulk organic material into which the permeant must partition and diffuse across [50].

Most results have relied on the “size / lipophilicity rule”, which originates from the solution-diffusion model for bilayer transport [51]. According to this model (Eq. 1), the permeability coefficient, P_m , of a drug through membranes is directly proportional to its water-lipid partition coefficient, K_m and the membrane diffusion coefficient, D_m of the solute and inversely proportional to the membrane thickness ($L \sim 30$ Å for the hydrocarbon domain of the bilayers). Although K_m is the major source of variation to drug permeability, passive drug diffusion through cell membranes also depends on D_m [52].

$$P_m = \frac{D_m \cdot K_m}{L} \quad \text{Eq. (1)}$$

Furthermore, D_m significantly depends on the molecular size or molecular weight of the drug. As molecular weight increases, D_m dramatically decreases such that very few of the commercially available drugs have high molecular weight [52].

The lipid solubility of a drug in systems such as the liposome/water partition coefficient (K_p), remains a primary characteristic accounting for quantitative structure/activity relationship studies (QSAR). The value of K_p determines the drug's distribution between the aqueous and lipid phases, and thereby the extent of penetration into the membrane and/or interactions with phospholipids or other membrane components [53, 54].

Theory 3

The transport dynamics of the hydrophobic ion through the liposome bilayers can be explained by the free - volume

theory [55]. Xiang and Anderson [55] proposed that a free volume is elongated in the highly ordered chain region so that is a critical variable in determining molecular diffusion and has been considered to have an important role in determining molecular permeability across biological membranes. In addition, free volume has been shown to be a critical variable in determining molecular diffusion and chemical potential in simple fluids and polymers and has been investigated to play an important role in determining molecular permeability across biological membranes [55].

In the original diffusion theory, translational diffusion of a permeant occurs when statistical redistribution of free volume opens up a void of a critical size in the immediate vicinity of the permeant [56]. The concept of free volume has also been utilized to describe the effects of molecular size and shape and medium density on partitioning of solutes into inter phases as well as between simple liquids (for a more detailed discussion of free - volume distributions in lipid bilayers, see [56, 57]).

Theory 4

Joguparthi *et al.* [58] determined that the partition coefficient is independent of drug and lipid concentration. They suggested that the simplest method and model is the bulk-solubility diffusion model which assumes that the membrane is homogenous and isotropic. The permeability coefficient derived from this model is according to Eq. (2):

$$P_0 = \frac{PC_{m/w} D_m}{h_m} \quad \text{Eq. (2)}$$

Where, P_0 is the permeability coefficient, $PC_{m/w}$ is the membrane/water partition coefficient, h_m is the thickness of the membrane and D_m is the diffusion coefficient [58].

3.1.2. Partition Coefficient Determination

Methods for the determination of the membrane/water partition coefficient of a solute can be categorized under three methods: (i) One involves liposome separation from the aqueous phase (dialysis, centrifugation or filtration) and after that determination of the amount of solute in each phase; (ii) Another method measures changes in solution properties upon incorporation in the membrane (acid/base, stability, solubility or spectroscopic properties - UV-Vis, IR, NMR, fluorescence); and (iii) The third method uses a probe inserted in the lipid bilayer whose spectroscopic characteristics change with the proximity of the solute [53].

Gel filtration and ultra filtration methods have been used to study the bilayer permeability of various compounds, but recently a dynamic dialysis method to study membrane permeability of hydrophobic solutes has been developed and validated [58].

3.1.3. Influences of Liposome Composition and Structure on Permeability

The Archaeobacterial lipid membranes have been shown to exhibit low permeability to protons and 5, 6-carboxy-fluorescein, it has been shown that the liposomes made from caldarchaeol lipids have remarkable thermal and mechanical stability [59]. The low proton permeability was attributed to tight and rigid packing of the lipids in these membranes [60].

Permeability studies of model bilayer systems indicate that the region of the acyl chain adjacent to the head group is the site likely to offer the most resistance for water and solute permeation [57, 61].

Shimanouchi *et al.* [47] analyzed the permeability coefficient (P_s) values of several liposomes with different compositions and showed that the fluidity of the hydrocarbon portion of the membrane is very important. The double bonds within the acyl chain resulted in a decrease in the packing density, which in turn perturbed the barrier to permeation so, the liposome entering the liquid-crystalline phase at temperature above the phase-transition, shows P_s value larger than other. The permeation appeared to be controlled by the membrane fluidity [62]. In general, the permeability of the layer increases by increasing the temperature [63].

Gazzara *et al.* [64] found that the penetration of the drugs depends on their structure and on the molecular packing of the lipids. The dependence of the partition coefficient on vesicle size related to differences in the curvature and the area compressibility of different vesicle structures.

Other researches confirmed that permeability of biological membranes and model lipid bilayers depends strongly on the degree of packing of lipid chains in the membrane and the size of the permeating solute. Membranes that are highly ordered show very low permeability and exhibit a steep dependence on size of the solute [37, 65].

Papahadjopoulos *et al.* [66] found that cholesterol retard the rate of transport of some molecules across the liposome bilayer because of decreasing the permeability of the bilayer. Szabo [67] proposed that cholesterol changes membrane permeability by two well-known mechanisms, i) by changing the electric potential difference across the interface of the membrane and the surrounding medium (in case of liposomes the suspension medium), thereby changing the partition of charged species across the membrane; and ii) by changing the fluidity of the membrane, thereby changing the rate of ionic transfer and the partition of the solute across the membrane. The first mechanism is expected to act in the same way on the permeability of charged species of widely different molecular structure. In contrast, the second mechanism is expected to act differently on the permeability of different ionic species depending on the structural details of the membrane and that of the ion.

Some molecules such as water, fluorescence probe, acetic acid, Na^+ -ionophore complex and Ca^{2+} were also found to cross membranes at a slower rate when cholesterol is incorporated into the membranes [68].

Srivastava and Eisenthal [69] proposed that the impermeability of the dipalmitoylphosphatidyl glycerol (DPPG) liposome bilayer to some molecules like malachite green (MG), versus the permeability of the dioleoylphosphatidyl glycerol (DOPG) liposome is attributed to the different structural states of the DOPG and DPPG liposomes. At room temperature, the DOPG liposome is in a liquid crystalline state whereas the DPPG liposome is in the gel state. In the gel state, the phospholipid acyl chains are packed in a highly ordered arrangement with only restricted motions of the chain. In the liquid crystalline state, the bilayer is more fluid because the chains are more disordered and are free to undergo fast rotational motions unlike the gel state. This

difference in the packing of the acyl chains in DOPG and DPPG, is the origin of the permeability of MG across the DOPG bilayer and its impermeability across the DPPG bilayer.

The steep decrease in P_m with temperature in the gel phase may originate from a change in the molecular packing of the acyl chains from near hexagonal to orthorhombic or monoclinic [55].

Xiang and Anderson [55] determined that the membrane permeability to acetic acid decreased with decreasing phospholipid chain length and correlated with the sensitivity of chain ordering to temperature, as chain length varied.

The ability of drugs to diffuse across membranes is more influenced by the ionizability of the drug in the surrounding medium. However, relatively few kinds of endogenous molecules cross membranes by lipid diffusion [55].

3.1.4. Influences of Liposome Size on Permeability

Shimanouchi *et al.* [47] showed that the liposome size is a factor determining the permeability of the unilamellar vesicles. They observed a uniform decrease in the P_s by increasing the liposome size. It is generally known that liposomes larger than 200 nm in diameter tend to have a multilamellar structure and since increasing the lamellarity could result in permeation resistance, the P_s value is expected to decrease significantly.

3.1.5. Influences of pH on Permeability

A large part of drugs are weak acids or weak bases which exist in either charged (ionized) or uncharged (nonionized) forms. The ratio of the charged to uncharged form depends on the drug's pKa, and the pH of the environment. Since, diffusion across a lipid bilayer requires that a drug be lipid-soluble, the ionized form of a drug cannot cross membranes. Thus, weak acids that are nonprotonated and weak bases that are protonated cannot diffuse across membranes. At a pH that is equal to a drug's pKa, equal amounts of the protonated and nonprotonated forms are present. Assuming, the pH is the same on both sides of a given membrane, the drug will be at equilibrium across the membrane. If the pH is less than the pKa (such that there are excess protons available), the protonated form of a drug predominates. Thus, weak acids exposed to a low pH environment are favored to diffuse across membranes, while, weak bases are not. The opposite is true at a higher pH [44].

3.1.6. Influences of Drug Properties on Permeability

Drug – liposome interaction depends not only on the partition coefficient of the drug but also on its functional groups such as hydrogen bonding sites and its polar surface area (PSA). PSA is the sum of surfaces of polar atoms in a molecule that reflects the physicochemical characters of all drugs [70].

Increasing the polar surface area reduces the power of interaction with the lipid bilayer and raises the tendency for surface interaction with the head group. Considering the interactions of drugs with liposomes, they can be grouped into three categories; i) the very strong hydrophilic drugs, which are localized in the aqueous medium of liposomes and if they have a very high PSA, can display some interaction

with the head groups, ii) the less hydrophilic drugs, more balanced molecules that adsorb at the water–lipid bilayer interface with some degree of penetration into the bilayer, and iii) the strong lipophilic drugs, which are located in the bilayer itself [71].

Another parameter to predict passive diffusion of particles through biological membranes is lipophilicity which is expressed as the logarithm of the partition coefficient between an organic solvent and an aqueous phase ($\log P$) which is widely used to indicate membrane affinity. Especially for neutral compounds, the $\log P$ is a good descriptor of lipophilicity. Indeed, the ability of ionized particles to partition in biological membranes has been reported to depend on complex mechanisms. In addition, molecular volume and shape of the molecules are also relevant for penetration through membranes [46, 72, 73].

Simard *et al.* [74] utilized stopped-flow fluorescence (millisecond time resolution) to monitor different aspects of Long Chain Fatty Acid (LCFA) binding to phospholipid vesicles. They determined a very rapid binding and of FA to the lipid membrane of phospholipids vesicles by changing in fluorescence intensity and they also found that lipid membrane is permeable to LCFA.

They measured the transmembrane movement of LCFA by controlling the pH inside the phospholipid vesicles. They showed that 50% of the LCFA in the outer part of the membrane becomes ionized on binding and also the energy barrier for translocation of the uncharged LCFA is lower than that of the charged groups [74].

A low permeability of phospholipids vesicles to LCFA could be a result of a low affinity of LCFA for a membrane or slow kinetics of adsorption, translocation, and possibly dissociation [75].

The differences in the magnitude of fluorescence changes can be explained by the physical–chemical properties of the different fatty acids, as where by decreasing acyl chain length, the partition coefficient (K_p) decreases and by increasing acyl chain length more than 12 carbons, affinity for LCFA increases [75].

Several antimicrobial peptides have been discovered in both animals and plants which are good candidates for novel antibiotics. Different mechanisms have been proposed for membrane permeabilization induced by these peptides [76]. The peptide forms an amphipathic helix in lipid bilayers, which effectively lies parallel to the membrane surface and causes a positive curvature strain on the membrane and consequently forming a membrane-spanning pore, which allows not only ion transport but also rapid flip-flop of the membrane lipids [76, 77].

In another mechanism, for transporting the molecular peptides; the peptide must move from the interface into the hydrophobic membrane interior. This transfer has been suggested to be much dependent on hydrophobic properties of the peptide. The rate of peptide flux across membrane is dependent on the lipophilicity of the peptides, its conformational structure and molecular size [49].

For passive transport across lipid bilayers, rigid molecules must be reasonably small, and their hydrophilic / lipophilic properties must be within narrow ranges [78].

3.2. Drug Transfer by P-Glycoprotein

Lu *et al.* [79] have shown that transport of Tetramethylrosamine (TMR) in liposome increases as the temperature is increased and reaches a maximum at the phase transition or melting temperature of the bilayer.

They used the P-glycoprotein (Pgp) in proteoliposome for transferring drugs and concluded that drugs must partition into the membrane before arriving to the transport site on Pgp, because the rate of transport by Pgp will depend directly on the drug concentration within the bilayer, on the other hand, it is related to the degree of membrane partitioning. It seems likely that the rate of drug transport depends obviously on the lipid/water partition coefficient, P_{lip} . The temperature dependence of P_{lip} may thus be reflected directly in the temperature dependence of drug transport. The value of P_{lip} showed a maximum at T_C , decreased consequently at temperatures higher than T_C , and remained constant, or decreased slightly at temperatures lower than T_C [79].

Kheirilomoom and Ferrara [80] studied the transport of imaging probes attached to cholesterol across the bilayer of a liposomal formulation. They used a simple liposomal formulation with poly ethylene glycol (PEG) conjugated lipids and determined the internalization efficiencies of two fluorescent cholesterol analogues - the cholesterol molecule carried two molecules fluorophore on the head and the other on the tail. Confocal microscopy was used in order to determine the intracellular location of fluorescent probes after incubation and the internalization of cholesterol was quantified by using the FACScan flow cytometer and the CELLQuest software system. They found that transport of cholesterol was approximately constant during the time and in the absence of PEG within the liposome, the transfer rate of cholesterol decreased [80]. Reaven *et al.* [81] have also observed that the quantified fluorescence intensity of labeled cells increased with liposome concentration, showing a tendency to saturation at higher liposome concentrations, by increasing the time of incubation, the number of lipid goblets had increased, perinuclear regions became more highlighted, and the cell background was more intense. Kheirilomoom and Ferrara also reported that the internalization proceeds much slower at 4 °C compared to 37 °C and proposed this change was a result of changes in the rigidity of the lipid bilayer, its functionality, and/or the reduced level of cellular energy. They also observed that when PEG lipids were included in the liposome formulation, because of stabilization of the membrane against vesicle–vesicle fusion, it inhibits the formation of multi-lamellar liposomes [81]. To clarify the mechanism by which cholesterol is transferred from liposomes, cells were pretreated with three inhibitors of cellular function: Filipin, Nocodazole and Chloroquine diphosphate. Chloroquine diphosphate blocks lysosomal functions, Nocodazole interferes with the microtubule assembly, inhibiting the clathrin-mediated pathway and disrupting golgi membranes [82–84]. Filipin was used to specifically block caveolae-mediated uptake without affecting the function of coated pits and they observed that using the Filipin caused 70% inhibition of cholesterol internalization in treated cells, suggesting that cholesterol internalization follows a caveolae-mediated pathway [85].

3.3. Molecular Umbrella – Assisted Transport

One of the most significant challenges for medicinal chemists is to find methods of promoting the passive transport of polar, biologically active agents across lipid membranes. This challenge continues not only for large - and intermediate - sized molecules (e.g., DNA and antisense oligonucleotides), but also for relatively small molecules (e.g., peptides) [86].

Janout *et al.* [87] have introduced a new class of surfactants termed “molecular umbrellas” that is based on molecules that mimic the structure and function of umbrella, i.e., molecules that can cover an attached agent and shield it from an incompatible environment. Molecular umbrellas (i.e.,

molecules composed of two or more amphiphilic units that attached to a central scaffold) have been designed to enhance the permeability of polar molecules across lipid bilayers [87]. They constructed a molecular umbrella consisting of amphiphilic molecules that maintain a hydrophobic as well as a hydrophilic face. Principally, two or more amphiphiles (umbrella walls) are coupled to a suitable scaffold either before or after a desired agent is attached to a central location. They have shown that molecular umbrellas display “molecular amphotorphism”, that means, they are able to form a shielded or exposed conformation in hydrophobic and hydrophilic mediums, respectively [88]. Fig. (1) shows some types of molecular umbrella.

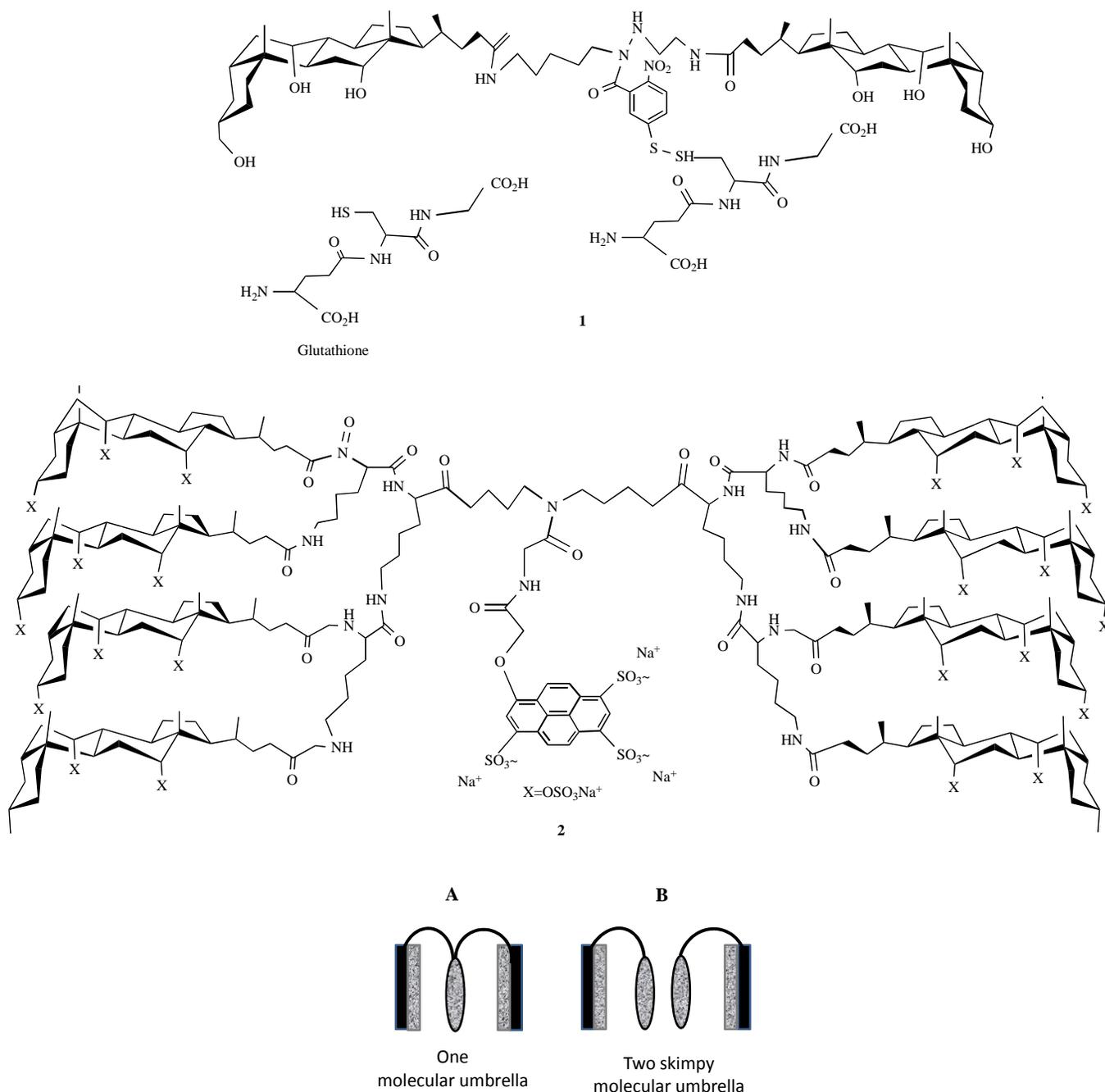


Fig. (1). Schematic illustration of, 1. Di-walled and 2. Tetra-walled molecular Umbrella, (Reproduced with permission from [4, 89]).

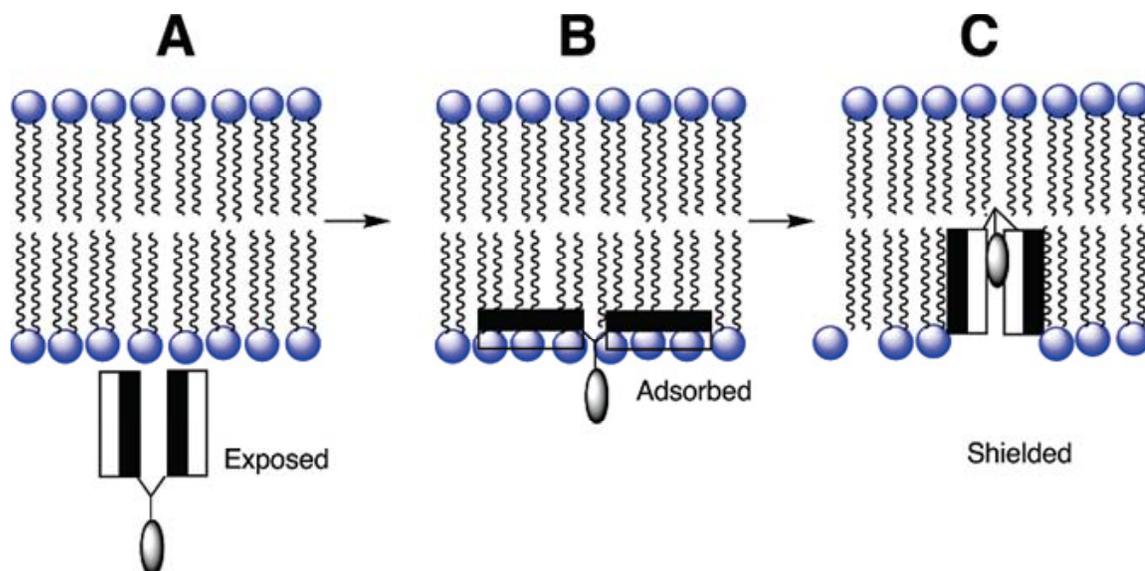


Fig. (2). Mechanism of transfer of molecular umbrella across lipid bilayer, (Reproduced with permission from [93]).

In suitable environmental conditions, the amphiphilic part of each wall connects with the hydrophobic or hydrophilic parts of the agent to produce a “shielded” construction. For hydrophobic agents, “immersion” in water causes to make a shielded construction such that intramolecular hydrophobic interactions are maximized and the external face of each wall is hydrated. When immersed in a hydrocarbon solvent, the umbrella desires to make a fully exposed construction where solvation and intramolecular dipole-dipole and hydrogen bonding interactions can be optimized. For those umbrellas that carry a hydrophilic agent, these same conditions are expected to produce shielded and fully exposed constructions in hydrocarbon and aqueous mediums, respectively, i.e., the opposite conformational preferences [87, 89]. (Fig. 2) shows the mechanism of transfer of molecular umbrella.

Molecular umbrellas can serve as novel vehicles for transporting polar drugs across biological membranes. Specifically, the molecule first approaches a lipid bilayer in a fully exposed conformation (structure A, Fig. 2). Hydrophobic interactions with the membrane interior then lead to an adsorbed state in which the hydrophilic faces are in contact with the polar head group region and the hydrophobic faces are in intimate contact with the hydrocarbon region of the lipid bilayer (structure B, Fig. 2). Subsequent absorption into the interior of the membrane, being driven by hydrophobic forces, then affords (structure C, Fig. 2). Translocation to the adjoining leaflet, 180° rotation, and reversal of steps B and A (not shown) then release the conjugate from the other side of the membrane [90, 91]. Umbrella molecule should facilitate the transport of an attached agent by shielding its hydrophilicity from the hydrophobic part of a membrane [92].

Janout *et al.* [89] reported that bilayer membrane permeation is rate limiting and the umbrella molecules act as the transport-active species and also some kinds of umbrella molecules with hydrophobic/hydrophilic balance have greater membrane permeability. They found that the ability of this conjugate to cross these bilayers depends more on

their facial amphiphilicity than on their hydrophobic/ hydrophilic balance [90]. Their studies have shown that molecular umbrellas can transport small hydrophilic peptides (polar molecules) such as glutathione (GSH) across phospholipid bilayers [86].

It is also possible to conjugate thiolated forms of AMP and ATP to molecular umbrellas and transport these nucleosides across liposomal membranes based on the proposed mechanism [90, 93].

Furthermore, Janout and Regen [94] have shown that the molecular umbrella moiety functions like a “needle” for creating a pathway for the much larger molecules like oligonucleotides (the “thread”) to cross the liposomal membrane (Fig. 3).

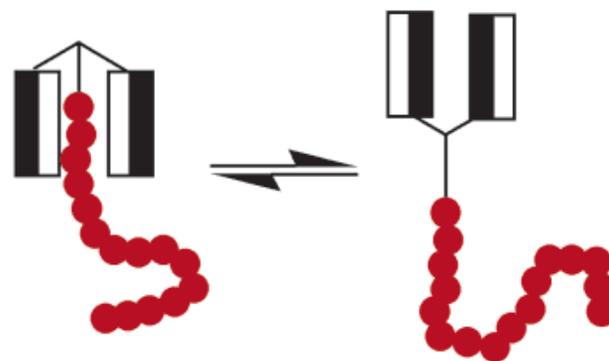


Fig. (3). Schematic representation of a molecular umbrella in a shielded conformation covering a small section of an attached polar agent (left), and the same umbrella in a fully exposed conformation (right). Here, the shaded and unshaded rectangles show hydrophobic and hydrophilic parts, respectively; the polar agent appears as a red beaded chain, (Reproduced with permission from [94]).

The other possible hypothesis is, the molecular umbrella acting like a leader (Fig. 4), the conjugate first penetrates into membrane to produce a shielded construction (A). Subsequent opening of the umbrella across the bilayer, and simultaneous folding of the agent e.g. oligonucleotide into a

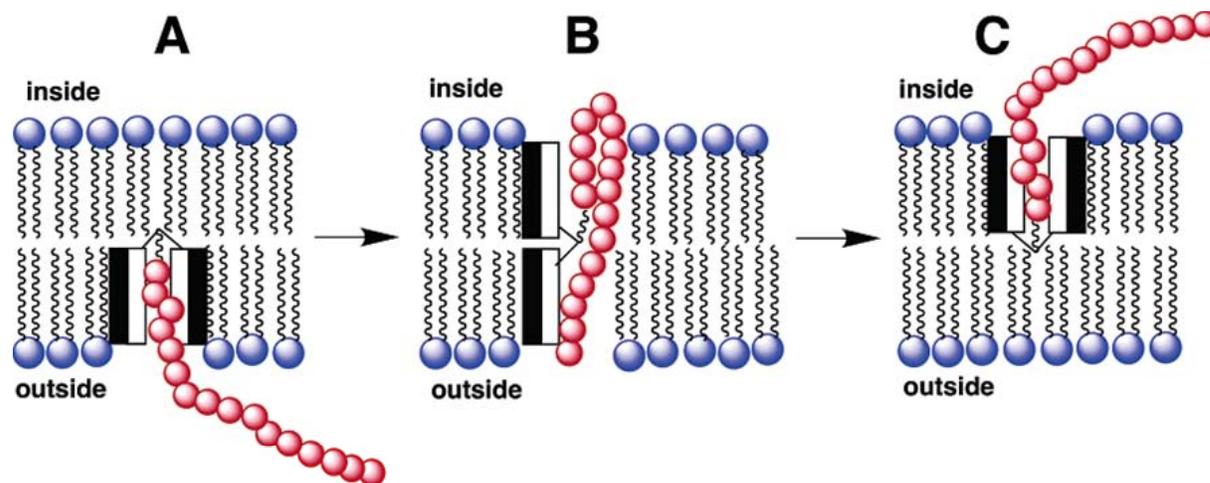


Fig. (4). Schematic representation of transfer of the Molecular Umbrella - oligonucleotide conjugate into the lipid bilayer, (*Reproduced with permission from [91]*).

partial “U”, could leave most of the membrane and proceed to the hydrophilic parts of the umbrella (**B**), allowing for translocation to other side of membrane (**C**). Release into the vicinal aqueous phase would happen by a simple desorption [91].

Furthermore, dynamic light scattering measurements showed no significant change in particle size and size distribution of the target liposomal membrane after exposure to the molecular umbrella - oligonucleotide conjugates and throughout the course of the transport experiments [91].

From these results, it is clear that a molecular umbrella’s ability to transport an oligonucleotide across a lipid membrane is strongly dependent on the number of facial amphiphiles present, but only moderately dependent on the change from three to two hydroxyl groups per sterol and also indicates that the rate of the translocation process, itself, is more important than membrane partitioning for overall membrane transport [91]. The greater transport rates are associated with the large molecular umbrella that is probably a consequence of their greater capacity to shield the attached oligonucleotide [91]. The observed results indicate that the rate of transport and splitting is controlled by permeation and not by chemical reaction; partitioning measurements also indicate that permeation is dominated by translocation [91]. The results also confirmed that molecular umbrellas that have more walls could exhibit higher transport rates due to a greater shielding capacity [91].

Mehiri *et al.* [4] reported that transport rates of the molecular umbrella were found to be in contrast to classic solution-diffusion theory, transport rates increase with increasing umbrella size. Additionally, they hypothesized that rate constants for the translocation step of molecules across the bilayer indicated that the less lipophilic umbrella crosses lipid bilayers more readily.

Mehiri *et al.* [4] also reported that transport of molecules across the lipid bilayers consists of a multistep process including adsorption, dehydration, diffusion, rehydration, and desorption. Depending on the nature of the molecule to be transported, as reflected by the number, type, and distribution of polar groups, its shape, and its hydrophilic/lipophilic balance (HLB), any one of these steps can become rate-limiting [4].

Fig. (5) summarize the process of transfer of bioactive agent from the bilayer.

In most of these studies, liposomes are used as cellular models to study the permeability of the cell membrane to drugs and other bioactives.

4. OBSERVATIONS AND LOCALIZATION OF BIO-ACTIVE AGENTS

Xing *et al.* [61] used differential scanning calorimetry, x-ray diffraction, and ^2H -NMR measurements in order to indicate the location of solvent trapped in the bilayer.

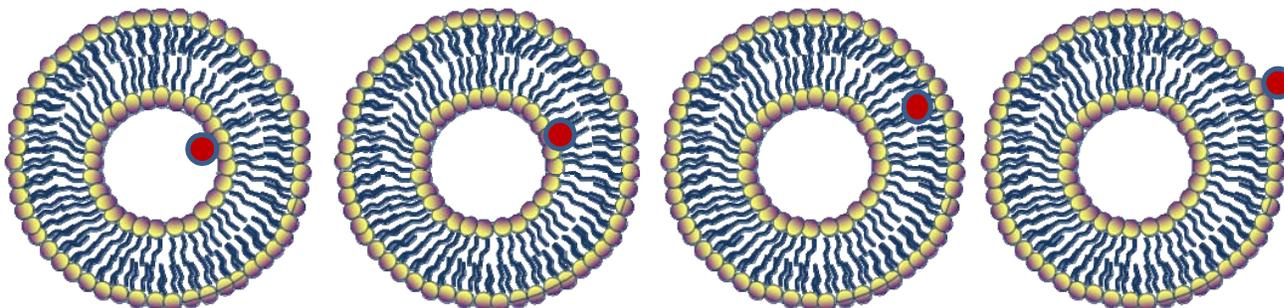


Fig. (5). Schematic of the process of bioactive agent transfer from the bilayer.

4.1. Radioactive or Fluorescence-Labeled Bioactives

Radioactive or fluorescence-labeled markers and drugs entrapped in liposomes in combination with various electron and (laser) light microscopic techniques are widely used to investigate the translocation of bioactives across the bilayer. These models with other techniques (i.e. Electron Spin Resonance, EPR), give some information about the interaction and fate of vesicles [95].

Breukink *et al.* [96] used radioactive labeled nisin to study material translocation in model membrane systems.

Inorganic ions are highly suitable markers for monitoring and investigating the release of the inner content of liposomes. Silberstein *et al.* [97] used Ion Selective Electrodes (ISEs) to detect the rate of ion release from liposomes.

4.2. Confocal laser Scanning Microscopy (CLSM or LSCM)

CLSM is a technique for obtaining high-resolution optical images with depth selectivity. The key feature of confocal microscopy is its ability to catch in-focus images from selected depths, a process known as optical sectioning. Images are captured point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically-complex objects. By this method, interior structures of samples can be imaged. For interior imaging, the quality of the image is greatly enhanced over simple microscopy because image information from multiple depths in the specimen is not superimposed [98, 99]. Confocal microscopy can also be used to locate the magnetic and fluorescent particles in the aqueous core of the liposomes. This makes it possible to measure membrane permeability and also make it easier to visualize liposome deformations [100].

4.3. Fluorescence Stopped-Flow Technique Method

Stopped-flow is a kind of technique used to study the kinetics of reactions in solution. In the simplest form of the technique, two reactant solutions are rapidly and forcedly mixed in a chamber, and then through an observation cell. Sometimes, the flow is suddenly stopped, and the reaction monitored by using a suitable spectroscopic probe, such as absorbance, fluorescence. The change in the spectroscopic signal as a function of time is recorded [74, 101].

Przybyło *et al.* [102] used the fluorescence stopped-flow technique for evaluating the transfer of a compound through a lipid bilayer. The liposome suspension is driven out of equilibrium in the stopped-flow apparatus chamber while mixed with the isotonic solution of the compound. Due to concentration difference across the lipid bilayer, the flux of the compound is created and detected by the variation in the fluorescence intensity of a fluorophore which is covalently attached to a lipid at both sides of the lipid bilayer.

4.4. Second Harmonic Generation (SHG) Method

Second harmonic generation (SHG) is a surface-specific and second-order spectroscopic technique that involves the transformation of common light at a fundamental frequency (ω) to light at twice frequency (2ω) via a nonlinear interaction with the environmental [103].

The optical second harmonic generation (SHG) is an in-situ surface-specific technique which enables the real-time observation of the molecular adsorption and transport without disturbing the liposomal system [104]. The orientation of molecules adsorbed on the outer and inner surfaces should be opposite to each other due to the bilayer symmetry. Therefore, the SH electric field from the liposomes of several hundred nanometers size is proportional to the difference of the number of adsorbents on the outer and inner surfaces [69]. In this system, the common light irradiates the solution of liposomes and consequently the SH signal is generated and detected by a single photon-counting system [69].

Kim and Kim [104] measured all of the adsorption, desorption, outside-to-inside transport and inside - to - outside transport phenomena in a liposomal system by using the SHG.

They used triphenyl cationic dye, Malachite Green (MG) as a model system for an organic hydrophobic ion and unilamellar liposomes for observing the transport dynamics. Before mixing MG and liposomes, the SHG field from the MG solution was negligible, because MG molecules were randomly oriented in water [104].

With an injection of the liposome solution into the MG solution at time zero, the SHG square suddenly increased to a high value within 1s and then slowly decreased to the final equilibrium value. The initial rapid increase in the SHG square is due to the rapid adsorption of MG onto the outer surface of the liposome, and the next slow decrease of the SHG square can be explained by the transport of MG across the lipid bilayer and subsequently adsorption on the inner surface of the liposome [68, 105]. The transport rate, k , can be described as the inverse of the exponential decay time constant obtained by fitting the time profile of the SHG field to a single exponential decay function. They observed that adsorption amount of MG on the outer surface of liposomes did not depend on changing the temperature, whereas the transport rate significantly increased with increasing temperature [105].

4.5. High Sensitivity Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) provides accurate information on the temperature dependence of the excess specific heat over a wide temperature range. This allows the determination of thermodynamic parameters for well-defined molecular systems [106, 107].

Lo and Rahman [101] used both DSC and high sensitivity DSC (HSDSC) to investigate the location of proteins in liposomes. HSDSC has several advantages over traditional DSC. The resolution of the instrument is greater, the thermopile is more sensitive than traditional DSC instruments and it is suitable for liquids and samples with distinctive volume, around 1ml. It was suggested that hydrophobic proteins had an enormous effect on the phase transition behavior of liposomes by adjoining into the bilayer structure, whereas hydrophilic proteins did not interact with the bilayer extensively, which altered the phase transition behavior of the liposomes [108].

El Maghraby *et al.* [71] also used the high sensitivity differential scanning calorimetry (HSDSC) to probe the

interactions of drugs with liposomes in a further attempt to specify the position of drugs in liposomes.

4.6. Transition Electron Microscopy (TEM)

TEM is a kind of nano-scale electron imaging technique with 0.2 nm resolution. This technique transmits a beam of electrons through a sample and consequently an image is formed. TEM provides information about the location of the bioactive with respect to the carrier (e.g., internalized or attached to the surface) [109, 110].

4.7. Scanning Electron Microscopy (SEM)

SEM is a type of electron microscopy techniques that is able to produce high-resolution three-dimensional images of liposome surface. By using this system closely spaced features of sample with larger depth of focus can be easily examined [111].

4.8. Atomic Force Microscopy (AFM)

AFM is very-high-resolution type of scanning probe microscopy that can create three-dimensional micrographs with resolution down to the nanometer and Angstrom scales (with resolution of 0.1 nm) which can be used for the structural characterization of liposomes and directly evaluating and imaging liposomal delivery systems. This technique provides information about the surface, as well as the morphological and technological properties of liposome. AFM allows biomolecules to be imaged not only under physiological conditions but also during biological processes [110, 112-114].

4.9. Surface Plasmon Resonance (SPR)

Branden *et al.* [115] presented a novel approach enabling direct measurements of bimolecular transfer across lipid bilayer membranes by using a biosensor technique, called surface plasmon resonance (SPR).

SPR-based instruments uses an optical method to measure the refractive index near (within ~300 nm) a sensor surface [116].

This technique utilizes the rule that biorecognition reactions sufficiently close to a metal surface stimulate a change in interfacial index which leads to altered conditions for surface Plasmon excitation. Surface-based techniques, however, are still not commonly applied for studying molecular transfer across the liposomal membrane (For a review see [115]).

All approaches, except SHG method, developed for the membrane permeability evaluation are based on direct measurements of the amount of a compound passing through the membrane. Such an experimental design requires a model system which consists of two macroscopically well-defined segments separated by a membrane and the quantity of the material passing through the membranes are determined at various time points during the transport process [102].

SUMMARY

The benefits of liposome in drug research critically depend on manner and mechanism of release of bioactive

agent. Furthermore, in order to achieve specific targeting, they should release their contents in special sites with effective doses. Liposome targeting mechanism reduces or completely eliminates side effects of drugs.

Determination of a controlled drug delivery technology suitable for each drug depends on many factors, including physicochemical properties of the drug, duration of release and the release profiles.

A main process in drug delivery and targeting using liposome technology is the mechanism of material transfer through the liposomal lipid bilayer. The release of efficacious dose of liposome-entrapped drug depends on the permeability of the liposomal formulation with respect of the entrapped drug.

The studies have been shown that it is not necessarily sufficient to develop drug carriers that accumulate at the disease site to high levels but also it must engineer appropriate drug release rates with controlling the properties of liposomal bilayer such as permeability, size.

The amount of drug penetration through lipid bilayers depends on drug structure and on the molecular packing of the lipids. The partition coefficient of drugs also depends on vesicle size and relates to differences in the curvature and the area compressibility of different vesicle structures.

Radioactive or fluorescence-labeled markers and drugs entrapped in liposomes in combination with various microscopic techniques; e.g. CLSM, TEM, AFM, are widely used for the investigation of translocation of bioactives across the bilayers.

Among the direct methods for measuring the translocation of bioactives, SHG, enables the real-time observation of the molecular adsorption and transport by measuring a SH signal which is detected by a single photon-counting system.

More studies are needed to determine the mechanisms of bioactive transfer between the layers of liposomes in order to achieve optimized liposomal systems for efficient encapsulation and release of bioactive agents.

ABBREVIATIONS

AFM	=	Atomic force microscopy
BLM	=	Bilayer lipid membrane
CADs	=	Cationic amphiphilic drugs
CLSM or LSCM	=	Confocal laser scanning microscopy
DSC	=	Differential scanning calorimetry
DOPG	=	Dioleoylphosphatidyl glycerol
DPPG	=	Dipalmitoylphosphatidyl glycerol
HSDSC	=	DSC and high sensitivity DSC
EPR	=	Electron spin resonance
LCFA	=	Long chain fatty acid
MDT	=	Magnetic drug targeting
MG	=	Malachite green
Pgp	=	P-glycoprotein

PSA	=	Polar surface area
PEG	=	Poly ethylene glycol
PLA	=	Poly lactic acid
RES	=	Reticuloendothelial system
SEM	=	Scanning electron microscopy
SHG	=	Second harmonic generation
SAXS	=	Small-angle X-ray scattering
QSAR	=	Quantitative structure/activity relationship
SPR	=	Surface plasmon resonance
TMR	=	Tetramethylrosamine
TEM	=	Transition electron microscopy

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III. Résultats & Discussion

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Chapitre III.I: Caractérisation physico-chimique des liposomes

Objectives

Knowledge of liposome characteristics is required to develop liposome formulations that have optimal entrapment efficiencies and allow the controlled release of bioactive agents. Optimization of liposome formulation can also made it possible to deliver bioactive agents directly to target site, hence reducing systemic side effects. This is an interesting approach to correlate different properties of liposome and bioactive drug properties.

The liposomes characterized with respect to size, phase transition temperature, zeta potential, lamellarity, fluidity and efficiency in loading calcein by using different techniques. Calcein was chosen as a model of hydrophilic active agnet for liposome characterization. L-carnoisne was also introduced as a hydrophilic antioxidant to present more techniques for liposome characterization. Furthermore, antioxidants encapsulation used to represent an ameliorative approach to overcome the problems related to the direct application of these antioxidant peptides in food systems.

Optimisation et caractérisation de liposomes par plan de mélanges

Résumé

Cette étude présente l'optimisation de formulations lipidiques de liposome à l'aide de plan de mélanges. Les formulations de dix mélanges de phospholipides purs (DOPC, POPC et DPPC) ont été générées à l'aide d'une matrice simplex-centroïde de Scheffé. Les liposomes, préparés par extrusion, ont été caractérisés sur le plan physicochimique avec et sans encapsulation de calcéine en terme de taille, température de transition de phase, potentiel zéta, lamellarité, fluidité et efficacité d'encapsulation du principe actif. Les effets et les interactions entre les constituants du mélange ont permis de générer un modèle mathématique afin d'optimiser la composition de la formulation lipidique par la méthode des surfaces de réponse. La formulation en phospholipides de la membrane du liposome (DOPC: 46%, POPC: 12% et DPPC: 42%) permet d'obtenir un diamètre moyen de 127,5 nm, une température de transition de phase de 11,43 C, un potentiel zéta de 7,24 mV, une valeur de fluidité de 2,87 exprimée en (1/P)TMA–DPH, ainsi qu'une valeur d'efficacité d'encapsulation de 20,24%. Les résultats expérimentaux générés à partir de cette formulation sont en accord avec la prédiction du modèle mathématique.

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PAPER

Optimization and characterization of liposome formulation by mixture design

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This study presents the application of the mixture design technique to develop an optimal liposome formulation by using the different lipids in type and percentage (DOPC, POPC and DPPC) in liposome composition. Ten lipid mixtures were generated by the *simplex-centroid* design technique and liposomes were prepared by the extrusion method. Liposomes were characterized with respect to size, phase transition temperature, ζ -potential, lamellarity, fluidity and efficiency in loading calcein. The results were then applied to estimate the coefficients of mixture design model and to find the optimal lipid composition with improved entrapment efficiency, size, transition temperature, fluidity and ζ -potential of liposomes. The response optimization of experiments was the liposome formulation with DOPC: 46%, POPC: 12% and DPPC: 42%. The optimal liposome formulation had an average diameter of 127.5 nm, a phase-transition temperature of 11.43 °C, a ζ -potential of -7.24 mV, fluidity $(1/P)_{\text{TMA-DPH}}^{-1}$ value of 2.87 and an encapsulation efficiency of 20.24%. The experimental results of characterization of optimal liposome formulation were in good agreement with those predicted by the mixture design technique.

1. Introduction

Currently, liposomes are an important part of biological, pharmaceutical, medical and nutritional research, as they are considered to be among the most effective carriers for the introduction of various types of bioactive agents into target cells and to different parts of non-living systems such as food materials.^{1,2} Liposomes are also some of the best drug delivery systems for low molecular weight drugs, imaging agents, peptides, proteins, and nucleic acids.^{3,4}

Encapsulation of bioactive agents in biodegradable and biocompatible liposomes has been considered a safe way of delivering effective bioactive substances. On the other hand, the effective delivery of bioactive agents necessitates protection from the enzymatic and chemical changes, as well as temperature and ionic strength variations in different environments, implying that

liposome composition could be a determining factor in the fate of liposomes.¹

Extrusion of hydrated lipid films is a common method for liposomes production on a laboratory scale and there are numerous reports on liposome preparation with this technique.⁵ Apart from the wide choice of different extrusion devices used for liposome preparation, extrusion parameters including filter pore sizes, number of passages and use of freeze–thaw cycles prior to extrusion could be varied. Another type of variation included the differences in employed lipid compositions and lipid concentrations (from <10 mg ml⁻¹ to >150 mg ml⁻¹).⁵

Liposomes are one of the most effective carriers, whose *in vivo* behavior is altered by their various physicochemical properties.^{6,7} Particle size is one such property that is well known to have an influence on removing liposomes from the circulation by the reticuloendothelial system (RES), which limits the blood circulation time of liposomes and can release the entrapped drug into the blood stream.⁸ Additionally, it is well known that liposomes larger than 200 nm in diameter tend to have a multilamellar structure and since increasing the lamellarity could result in permeation resistance consequently the Partition Coefficient (P_s) value significantly decreases.³

Liposomal structure also can significantly influence the stability and the drug release from liposomes.⁹ Controlling the above factors is important when using liposomes as drug carrier systems.^{10,11}

In addition, entrapment efficiency of liposomes is an important factor in their practical use. A major achievement in the medical application of liposomes is the ability to load sufficient

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amounts of drugs needed to achieve therapeutic efficacy. Knowledge about liposome characteristics is required to develop liposome formulations that have optimal entrapment efficiencies and allow the controlled release of bioactive agents. Entrapment efficiency depends on the phospholipid concentration of the dispersion and the lamellarity and morphology of the liposomes. The lipid composition and preparation method can also influence the entrapping efficiency of liposome formulations.^{12,13}

Phase transition temperature (T_c) exerts significant effects on the liposome properties. T_c and fluidity of phospholipid membranes are important in the manufacture and application of liposomes, *e.g.*, having low phase transition temperature is advantageous for liposomes as drug carrier systems due to the fact that bioactive drugs which are encapsulated in liposomes with high phase transition temperature are generally released slower than those encapsulated in liposomes with lower phase transition temperature.^{14,15}

Furthermore, the fluidity of lipid membrane also reflects the order and dynamics of phospholipid alkyl chains in the bilayer and liposome permeation appeared to be controlled by the membrane fluidity.¹⁶

The significant difference in the release profiles of drugs also depends on the fluidity of the bilayer, *i.e.* by increasing the fluidity of the bilayer, the drug leakage to outer liposomal aqueous compartments is rapidly increased. Additionally, the release of the entrapped drug from liposomes also depends on the number of bilayers and obviously permeability of the bilayer.¹⁷

There is plenty of data available on liposomes but it is unclear whether the observed differences in the liposome characteristics are due to the use of different extrusion devices, different process parameters or differences in the employed analytical techniques. In this work, we developed a model of mixture design using 10 different formulations and compared the effect of composition on the liposomal properties such as size, entrapment efficiency, ζ -potential, transition temperature, lamellarity and fluidity in order to find the optimal liposome formulation.

2. Materials and methods

2.1. Materials

Phospholipids used in the study were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), all purchased from Avanti Polar Lipids (Alabaster, AL, USA). 3,3-Bis[*N,N*-bis(carboxymethyl)-amino-methyl] fluorescein (calcein) and 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) were acquired from Invitrogen (France); sodium hydroxide, hydrogen chloride and Phosphate Buffered Saline (PBS) and Triton® X-100 were from Sigma-Aldrich (France). All other reagents of analytical grade were obtained from Sigma-Aldrich (France).

2.2. Mixture Design of Experiments

Mixture Design of Experiments (MDOE) is a technique used to determine the optimum combination of chemical constituents that deliver a desired response by using a minimum number of mixture runs.¹⁸

The mixture design was developed to characterize liposomes having various ratios of lipids in their formulation. Obtained results were then applied as a model to find the optimal point of experiment for evaluating the possibility of improving the entrapment efficiency, size, phase transition temperature (T_c), fluidity and ζ -potential of liposomes prepared by the extrusion method and of different compositions.

The mixture design was generated by NEMROD® software (New Efficient Methodology for Research using Optimal Design) (Mathieu and Phan-Tan-Luu 2002).

The fact of this model that the proportions must add up to one is the key attribute of mixture designs. Specifically, the settings for various factors must satisfy: $x_i \geq 0$, for all i , $\sum_i x_i = 1$.

The design region for mixture proportions is a simplex, a regularly sided figure of dimension $k - 1$ with k vertices (usually embedded into a k dimensional space). For example, with two factors, the simplex is the line segment from (0,1) to (1,0). With three factors, the simplex would have vertices at (1,0,0), (0,1,0), and (0,0,1). There is a corresponding simplex coordinate system.¹⁸

We can now consider models for mixture experiments. The usual first order model is:

$$E(y) = \beta_0 + \sum \beta_i x_i \quad (1)$$

However, since $\sum x_i = 1$ for a mixture model, the β_i 's will not be uniquely determined. We could choose to eliminate one of the x_i 's, but a better approach was suggested by Scheffé. In the equation above multiply β_0 by $1 = \sum x_i$ to get:

$$E(y) = \sum (\beta_0 + \beta_i) x_i \quad (2)$$

Relabeling the β_i 's, we get the following canonical forms. Linear: $E(y) = \sum \beta_i x_i$ and special cubic:

$$y = \sum_{i=1}^n \beta_i x_i + \sum_{i < j} \sum \beta_{ij} x_j + \sum_{i < j < k} \sum \beta_{ijk} x_i x_j x_k \quad (3)$$

In order to estimate the Scheffé coefficients, we designed a plan of 10 experiments representing different liposome compositions. Table 1 shows the plan of mixture design of different liposomes.

Data from the liposome characterizations were used to calculate the special cubic equation for three components (eqn (3) and (4)).

Table 1 Mixture design plan of ten liposomal formulations with different compositions ($n = 5$)^a

No. exp.	Name	DOPC	DPPC	POPC
1	b_1	1.0000	0.0000	0.0000
2	b_2	0.0000	1.0000	0.0000
3	b_3	0.0000	0.0000	1.0000
4	b_{12}	0.5000	0.5000	0.0000
5	b_{13}	0.5000	0.0000	0.5000
6	b_{23}	0.0000	0.5000	0.5000
7	b_{123}	0.3333	0.3333	0.3333
8	b_{123}	0.6667	0.1667	0.1667
9	b_{123}	0.1667	0.6667	0.1667
10	b_{123}	0.1667	0.1667	0.6667

^a The mixture results were then analyzed by NEMROD® software with a cubic model.

$$y = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (4)$$

where y is the studied response (size, transition temperature, ζ -potential, fluidity and entrapment efficiency), β_{ijk} are regression parameters and x_1 , x_2 and x_3 represent level constituents of three phospholipid types.

2.3. Liposome preparation

Large unilamellar vesicles (LUVs) were prepared as described elsewhere.⁴ In brief, phospholipids were dissolved in a chloroform solution. The organic solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under vacuum overnight, was hydrated with calcein suspension to obtain multilamellar vesicles. Calcein was dissolved in Phosphate Buffered Saline (pH 7.4, 1 M NaOH, 100 mM HCl) to obtain a final calcein concentration of 10 mM before use. The suspension was subjected to sonication (20 seconds; 1 s on and 1 s off). Sonication was done at 25 °C in a sonicator bath (Sonicator Vibra cell 75115, 500 watt, Bioblock Scientific Co.), and a nominal frequency of 40 kHz at 25% of full power and then extruded through a polycarbonate filter (100 nm pore size filter, 11 times) above the phase transition temperature of the vesicles by using an Avanti Mini extruder (Avanti Polar Lipids, Alabaster, USA).^{19,20} The lipid concentration in the final vesicle suspension was determined with an enzymatic assay kit (Test Wako-C) from Wako Pure Chemical Co. Ltd. (Osaka, Japan).

2.4. Particle size determination

The mean diameter and particle size distribution of vesicles were determined using dynamic light scattering (DLS) technique employing a Zetasizer Nano ZS (Malvern Instruments Ltd, UK). The software used was DTS Nano, version 6.12 supplied by the manufacturer (Malvern Instruments Ltd, UK). The samples were diluted (1 : 100) and all measurements were carried out at 25 °C, by considering a medium viscosity of 1.020 and medium refractive index of 1.335. Results are presented as an average diameter of the liposome suspension (z -average) with the polydispersity index (PDI).

2.5. ζ -Potential determination

ζ -Potential is a function of surface charge of the lipid vesicle, any adsorbed layer at the interface, and the nature and composition of the medium in which liposomes are suspended. ζ -Potential is not measurable directly but it can be calculated using theoretical models and an experimentally determined electrophoretic mobility or dynamic electrophoretic mobility.²¹

The ζ -potential of liposomes was determined using a Zetasizer Nano ZS. The sample was put in a standard capillary electrophoresis cell equipped with gold electrodes. The liposome suspensions were diluted (1 : 50) to avoid multiple scattering effects and then directly placed into the module; all measurements were carried out at 25 °C and results are presented as an average ζ -potential of the liposome suspension (z -average).

2.6. Entrapment efficiency determination

Calcein was chosen as a hydrophilic marker. It is widely used to determine entrapment efficiency. The entrapment efficiency was calculated according to the following method as previously reported.²²

$$\% \text{ Entrapment efficiency} = \left(\frac{C_{\text{total}} - C_{\text{out}}}{C_{\text{total}}} \right) \times 100 \quad (5)$$

C_{total} : 20 μl of liposome suspension (with concentration of 20 mg ml^{-1}) were diluted 50-fold with buffer solution (PBS) and liposomes were disrupted with 20 μl of Triton® X-100 (1%) to release the encapsulated calcein. The fluorescent intensity of calcein was measured for the sample with a spectrofluorimeter SAFAS (FLX-Xenius, Monaco) at 490 nm excitation and 520 nm emission.

C_{out} : liposome suspension was eluted through a Sephadex-G75 column (10 mm \times 200 mm) which was thoroughly pre-equilibrated with PBS to remove the non-encapsulated calcein. The fluorescence intensity of calcein was measured for the sample according to the procedure described above.^{22,23}

2.7. Phase transition temperature determination

Liposome suspension was analyzed with differential scanning calorimetry. Calorimetric scans from -30 to 90 °C were performed on Netzsch 204 F1 (Netzsch-Gerätebau GmbH, Germany) at a scanning rate of 5 °C/min. The software used was Proteus Analysis, version 4.8.5 supplied by the manufacturer (Netzsch-Gerätebau GmbH, Germany).²⁴

2.8. Membrane fluidity

The liposome membrane fluidity was determined by fluorescence polarization (P) by measuring the fluorescent intensity of TMA-DPH, according to the conventional method.¹⁷ The solution of TMA-DPH (in ethanol) was added to the liposome suspension to maintain the lipid/probe molar ratio of 250 ($[\text{TMA-DPH}]_{\text{final}} = 4 \mu\text{M}$). The mixture was then incubated for at least 1 h at room temperature with gentle stirring. The fluorescence probe was vertically oriented in the lipid bilayer. The amount of probe remaining in the external aqueous medium was negligible due to high lipophilicity coefficient, and the non-incorporated TMA-DPH was non-fluorescent due to aggregation. The fluorescence intensity of samples was measured with a Perkin-Elmer LS 55B spectrofluorimeter equipped with fluorescence polarizers (Perkin-Elmer, Waltham, USA). Samples were excited at 360 nm, and emission was registered at 430 nm under constant stirring at a temperature of 25 °C (PTP-1 temperature controller). The P value of TMA-DPH was calculated from the following equation:

$$P = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \quad (6)$$

where I_{\parallel} is the intensity of fluorescence parallel to excitation plane, I_{\perp} is the intensity of fluorescence perpendicular to excitation plane, and G is the factor that accounts for transmission efficiency. The reciprocal value of polarization ($1/P$) was defined as membrane fluidity.

2.9. Lamellarity determination

The average number of bilayers is another important factor which determines the relationship between vesicle size and encapsulated volume. Lamellarity of liposomes was determined from Small Angle X-ray Scattering (SAXS). Measurements were performed with SAXSess (Anton Paar KG, Graz, Austria) equipped with a sealed tube (Cu anode $\lambda = 1.542 \text{ \AA}$). The samples were analyzed in a quartz capillary (with an outer diameter of 1 mm, at 20 °C). The 2D scattering pattern was detected by a CCD camera featured with a 2084×2084 pixel array and $24 \mu\text{m} \times 24 \mu\text{m}$ pixel size. Via SAXSQuant software (Anton Paar), the 2D image was integrated into the 1D scattering curves as a function of the magnitude of the scattering vector (q). Data were obtained in the q range of $0.11\text{--}6 \text{ nm}^{-1}$ and corrected for the solvent (PBS)-filled capillary scattering. Since the beam incident is linear, desmearing treatment allows correcting the spectra.^{25,26}

2.10. Transmission electron microscopy (TEM)

The microstructure of nanovesicles was investigated by TEM with a negative staining method. For examination, the liposome samples were diluted 10-fold with PBS to reduce the concentration of the vesicles. Equal volumes of the diluted sample and an ammonium molybdate solution (2%) were combined and left for 3 min at room temperature. A drop of this solution was placed on a Formvar-carbon coated copper grid (200 mesh and 3 mm diameter HF 36) for 5 min. The excess of the liquid was removed by using the filter papers. After drying the grid at room temperature for 5 min, micrographs were made using a Philips CM20 transmission electron microscope operating at 200 kV. Micrographs were recorded using an Olympus TEM CCD camera.²⁷

2.11. Statistical analysis

A *simplex-centroid* design was applied to model the kinetics of liposome formulation with three different types of phospholipids.

The results of the mixture design were interpreted by NEM-ROD® software with a cubic model. The presented results are the averages of five complete and independent experiments. Data

were reported as mean \pm SD. One-way ANOVA was employed to identify differences in means, using *SPSS software* (SPSS for Windows, Rel.10.0.5. 1999; SPSS Inc., Chicago, IL). Statistical significance was declared at $P < 0.05$.

3. Results and discussion

3.1. Influence of composition on liposome size and ζ -potential

3.1.1. Size. Determination of vesicle size distribution is a fundamental quality control assay due to the following reasons: (i) size distribution of bioactive carriers is an important parameter with respect to the physical properties and stability; (ii) size distribution defines plasma biodistribution and stability of nanocarriers and also their behavior in plasma and blood; (iii) nanocarrier size is a major factor in their permeation through tumor tissue and other organs;²⁸ and (iv) in pulmonary applications, the deposition region of bioactive carriers depends mainly on density, shape, and size of the particles.^{29,30}

Mean size, size distribution and ζ -potential values are physicochemical parameters that have to be modulated as a function of the proposed application for a certain liposomal system.¹⁶

There are different techniques for liposome preparation which are able to produce vesicles of different sizes ranging from 20 nm to several microns in diameter and composed of one or more bilayers. The extrusion method is one of the most commonly used procedures for reducing liposome size and produce unilamellar vesicles on the research scale. The advantage of the extrusion technique is its applicability to a variety of lipid species to make a homogeneous-sized colloidal suspension.¹⁴

The greatest mean size and polydispersity values were obtained for liposomes composed of DOPC which contains unsaturated phospholipids (Fig. 1). Liposome size increases with incorporation of unsaturated fatty acids on phospholipid chains such as oleic acid in the liposomal formulation, due to augmentation of liposome membrane fluidity. Our results are in good agreement with the results of Calvagno *et al.* (2007).¹⁶ Ferreri *et al.* also investigated that the type of fatty acid in lipid membrane influenced vesicle dimensions, as the vesicle diameter decreases along the series *cis*-unsaturated FA > *trans* > saturated FA.³¹

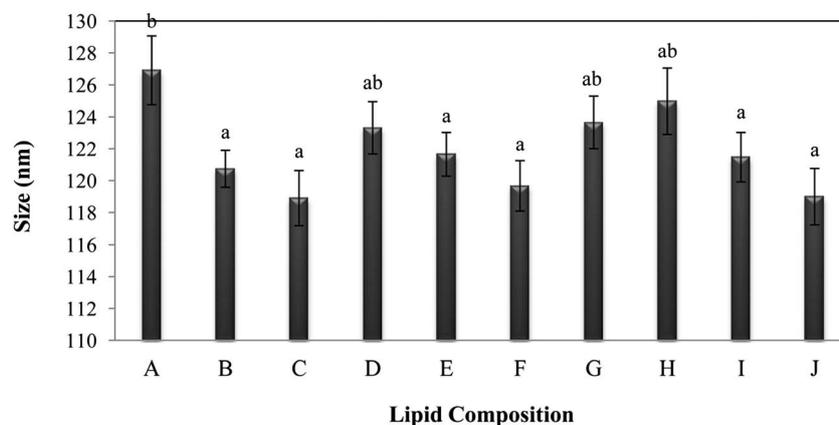


Fig. 1 The effect of the lipid composition on liposome size. (A) b_1 , (B) b_2 , (C) b_3 , (D) b_{12} , (E) b_{13} , (F) b_{23} , (G) b_{123} , (H) b_{123} , (I) b_{123} , and (J) b_{123} . The vertical bars represent the standard deviation ($n = 5$) of each data point. Different letters represent significant differences of lipid composition ($P < 0.05$).

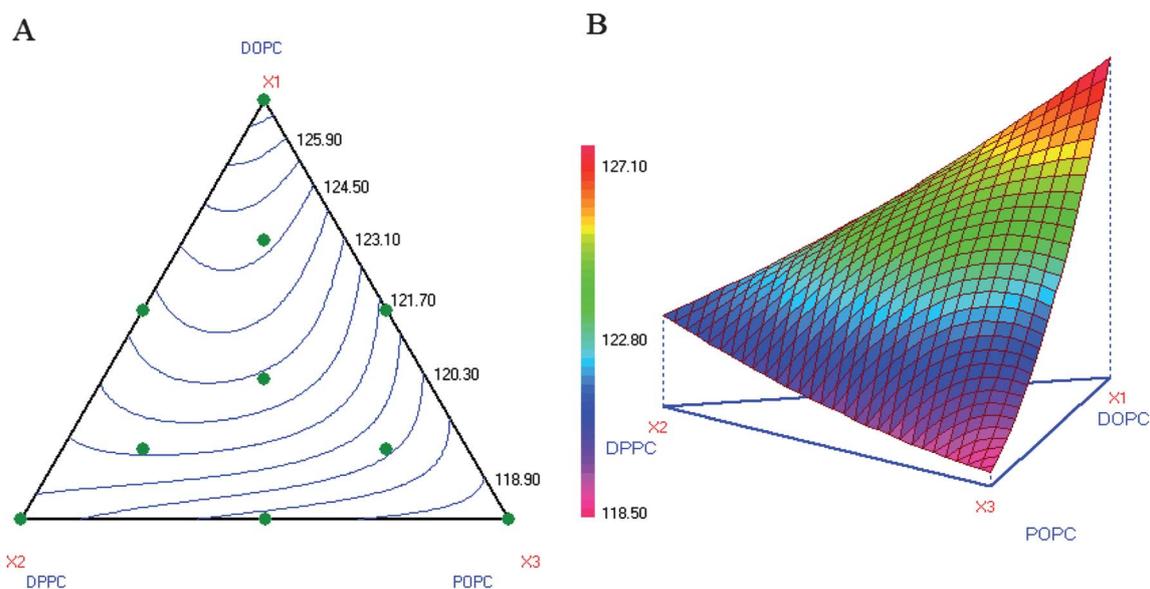


Fig. 2 Variation of size response as a function of lipid composition.

The basic principle for the formation of liposomes, regardless of the preparation methodology, is the hydrophilic/hydrophobic interactions between lipid–lipid and lipid–water molecules. The input of energy (*e.g.* in the form of sonication, homogenization, shaking, heating, *etc.*) results in the arrangement of the lipid molecules, in the form of lipid bilayer vesicles, to achieve a thermodynamic equilibrium in the aqueous phase.³² It is recommended that liposome preparation is carried out at temperatures above T_c of the vesicles,²⁰ to make sure that all the phospholipids are dissolved in the suspension medium homogeneously and have sufficient flexibility to align themselves in the structure of lipid vesicles.³³ At elevated temperatures, lipids undergo a phase transition that alters their permeability. This is known as gel to liquid crystalline transition temperature, T_c , at which the lipid bilayer loses much of its ordered packing and therefore its fluidity increases. It was perceived that the most important compositional factor in liposome extrusion is T_c of the liposome membrane.²⁰ An understanding of phase transitions and fluidity of phospholipid membranes is essential both in the manufacture and exploitation of liposomes.³⁴

In the case of vesicles containing DPPC, $T_c = 40$ °C, it has been suggested that the liposome preparation procedure be

carried out at 10 °C higher than the T_c , at 50 °C. This phenomenon promotes disordering in the lipidic bilayer and possibly increases the liposome size.³⁵ Temperatures higher than T_c are probably able to increase liposome size. The T_c temperature of liposome preparation which is related to the transition temperature of each phospholipid is also a factor affecting the liposome size. In comparison with different liposome formulations, liposomes composed of completely saturated lipids such as DPPC also have large size (Fig. 1 and 2). Our results are in good agreement with the results of Nii *et al.* (2003).³⁶ The thermal reduction of the bending rigidity of the bilayers leads to increase of liposome size.

It is worth noting that all size determination experiments were performed at 25 °C, as liposomes composed of DPPC are in gel-ordered form but other liposomes are in liquid-disordered forms; thus during size determination, we observed a small decrease and increase in the size depending on the type of fatty acid chains branched on phospholipids.

Briefly, findings have shown that conditions of liposome preparation including preparation temperature in relation to the T_c of each sample, the number of passages through polycarbonate filter and lipid composition affect liposome size.

Table 2 Particle size, polydispersity index (PDI) and ζ -potential of calcein-loaded nanoliposomes

Liposome composition	Molar ratio	Average diameter ^{ab} /nm	PDI ^{ab}	ζ -potential ^{ab} /mV
DOPC	1	126.92 ± 2.15 ^b	0.10 ± 0.03	−7.75 ± 2.36 ^a
DPPC	1	120.75 ± 1.16 ^a	0.09 ± 0.02	−6.57 ± 2.21 ^a
POPC	1	118.92 ± 1.72 ^a	0.10 ± 0.02	−7.11 ± 1.98 ^a
DOPC + DOPC	1 : 1	123.32 ± 1.64 ^{ab}	0.10 ± 0.03	−7.09 ± 1.90 ^a
DOPC + POPC	1 : 1	121.66 ± 1.37 ^a	0.09 ± 0.02	−7.45 ± 1.89 ^a
POPC + DPPC	1 : 1	119.68 ± 1.58 ^a	0.10 ± 0.02	−6.89 ± 1.87 ^a
DOPC + DPPC + POPC	1 : 1 : 1	123.66 ± 1.65 ^{ab}	0.10 ± 0.03	−7.33 ± 2.22 ^a
DOPC + POPC + DPPC	3 : 1 : 1	124.98 ± 2.07 ^{ab}	0.10 ± 0.03	−7.45 ± 2.18 ^a
DOPC + DPPC + POPC	1 : 3 : 1	121.48 ± 1.55 ^a	0.09 ± 0.02	−6.98 ± 2.13 ^a
DOPC + DPPC + POPC	1 : 1 : 3	119.10 ± 1.76 ^a	0.09 ± 0.02	−7.23 ± 2.20 ^a

^a Data were expressed as mean ± SD ($n = 5$). Data were adjusted to that at 25 °C. ^b The mean diameter and particle size distribution of vesicles were determined using the DLS technique. Different letters represent significant differences of lipid composition ($P < 0.05$).

Table 3 Difference in ζ -potential between liposomes with and without encapsulated calcein

Liposome composition	With encapsulated calcein/mV	Without encapsulated calcein/mV
DPPC	-7.0 ± 1.7	1.0 ± 0.3
POPC	-7.5 ± 1.3	-1.1 ± 0.5
DOPC	-7.4 ± 1.5	-1.6 ± 0.5
POPC + DOPC + DPPC	-7.9 ± 1.4	-2.0 ± 0.6

Table 4 Estimation of model coefficients (eqn (4))^a

Name	Model coefficient				
	Size	ζ -potential	Transition temp.	Entrapment efficiency	Fluidity
b_1	127.102***	-7.733***	-18.515***	23.298***	3.335***
b_2	120.719***	-6.583***	39.222***	18.985***	2.455***
b_3	118.532***	-7.109***	0.549**	17.461***	2.774***
b_{12}	-1.757	0.288	27.414	-5.993	0.139
b_{13}	-5.450	-0.045	-15.132	-9.162	0.297
b_{23}	-1.457	-0.225	4.341	-3.107	0.177
b_{123}^b	48.529	-4.776	-53.047	54.762	-3.568
R^2	0.95	0.99	0.99	0.93	0.95

^a Confidence levels: **, 99%, ***, 99.9%. ^b Liposome composition of 33% DOPC + 33% POPC + 33% DPPC.

In Table 2 mean size, ζ -potential and polydispersity index (PDI) of the vesicles prepared in this study assessed by the DLS method are shown.

Fig. 1 presents the effect of lipid composition on liposome size. There is a significant difference between the size of liposomes composed of DOPC and those composed of DPPC and other liposome formulations. No significant difference was observed in sizes of liposomes composed of two or three types of phospholipids ($P < 0.05$) (Table 1).

Different letters on vertical bars represent significant differences of lipid composition ($P < 0.05$); liposomes composed of POPC and DOPC have the smallest and the largest liposome size, respectively as shown by (a) and (b) letters. It was shown that there is a significant difference between these liposome formulations in 99.9% confidence level. Liposomes composed of DPPC or a combination of two or three liposomes have an intermediate liposome size, presented by (ab) which shows no significant difference in 99.9% confidence level.

To verify the influence of calcein on mean size and polydispersity index of the liposome, light scattering analysis was also carried out in the presence and absence of calcein and we found that calcein had no effect on the liposome size.

Comparison of parallel curves around the DOPC point in the topographic chart (Fig. 2A) also confirms that by increasing the concentration of phospholipids containing the oleic acid in their formulation, liposome size increases. Additionally augmentation of temperature for liposome preparation influences the liposome size. The curvature and color change show a noticeable effect of lipid composition on liposome size. These results are verified by considering the 2D graph (Fig. 2B).

3.1.2. ζ -Potential. Knowledge about the ζ -potential of a liposome preparation can help to predict the fate of the

liposomes *in vivo*. Liposomal charge is an important characteristic determining liposome stability and entrapment efficiency. The electrostatic attraction between charged bioactive agents and liposomes is a means to increase entrapment efficiency. Any subsequent modification of the liposome surface can also be monitored by measurement of the ζ -potential.^{21,37}

The greater absolute values of ζ -potential cause the liposomal suspension to be stable due to repulsion of charged vesicles, thus overcoming the natural tendency to aggregate. ζ -Potential values are also influenced by lipid composition of liposomes (Table 2). The ζ -potential of a liposome is the overall charge that the particle acquires in a particular medium. The surface charge of liposomes can be varied; they could be neutral (by employing phosphatidylcholine), negative (with acidic phospholipids such as phosphatidylserine) or positive (by dioleoyl trimethylammonium propane (DOTAP) or stearylamine) in physiological pH ranges.³⁸

It was found that the neutral liposomes such as phosphatidylcholine prepared with calcein solution are slightly negatively charged due to charge of calcein that surrounded and encapsulated in liposomes.

To verify the influence of the calcein on liposome ζ -potential, light scattering analysis was also carried out in the presence and absence of calcein in liposome suspension and we found that calcein solution is negatively charged and ζ -potential of solution fully depends on calcein concentration, *e.g.* ζ -potential of calcein solution with concentration of 10 mM and 30 mM is -6 mV and -22 mV, respectively (Table 3). ζ -Potential results of liposome suspension before and after separation on gel chromatography also confirm the effect of calcein solution on ζ -potential of liposome suspension, as ζ -potential of calcein-encapsulated liposomes composed of POPC before and after separation on gel chromatography was -7.6 mV and -6.2 mV, respectively. By dilution of samples, calcein desires to egress from the liposomal bilayer and it considerably influences the ζ -potential value. It was also mentioned that after separation, the encapsulated materials can leak out at storage conditions higher than their T_c .

This phenomenon was clearly observed in liposomes composed of unsaturated lipids.

Table 4 summarizes the model coefficients for each response. As mentioned above, b_1 , b_2 and b_3 represent three types of phospholipids and the coefficients show that lipid composition (DOPC, POPC, and DPPC) influenced the size and ζ -potential of liposomes. Their impacts are significant at the 99.9% confidence level. The results confirmed that lipid composition has influence on the liposome size and ζ -potential.

The coefficients of model for combination of lipids are not significant. The negative coefficients have shown that combination of two or three lipids has an inverse effect on some factors. The combination of DOPC and DPPC, had an increasing and reducing factor on liposome size, respectively, due to the negative effect of their combined, b_{12} , on liposome size. The obtained results of size in Table 2 also confirmed this impact.

3.2. Influence of composition on liposome entrapment efficiency

Knowledge about liposome characteristics is required to develop liposome formulations that have optimal entrapment efficiencies to achieve therapeutic efficacy and allow the controlled release of bioactive agents.³⁹

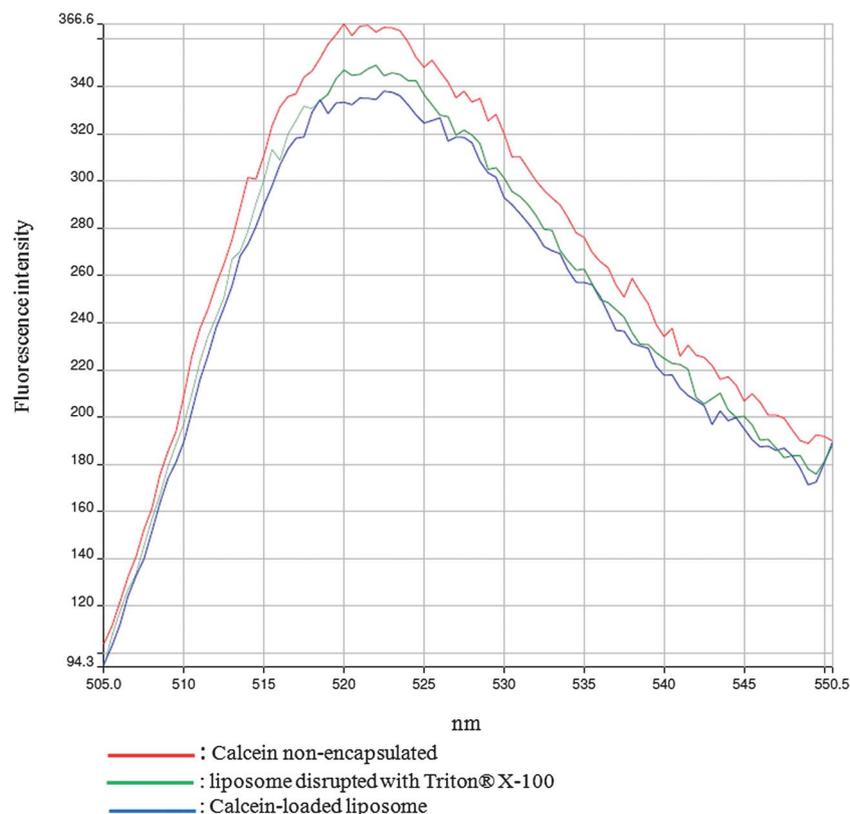


Fig. 3 The fluorescence spectra of calcein non-encapsulated (red line), liposome disrupted with Triton® X-100 (green line) and calcein-loaded liposome (blue line).

Bioactive agents can interact with liposomes in several different ways depending on their special properties such as solubility and polarity. They can be entrapped in the lipid bilayer phase, intercalated in the polar head groups, adsorbed on the membrane surface, anchored by a hydrophobic tail or encapsulated in the inner aqueous compartment. A major achievement in the medical application of liposomes is the ability to load sufficient amount of drug needed to achieve therapeutic efficacy.^{13,39} The electrostatic attraction between charged bioactive agents and liposomes is a means to increase the entrapment efficiency.

The gel filtration serves to separate liposome-encapsulated materials from those that remain in the suspension medium. Therefore, they can also be used to monitor the storage stability of liposomes in terms of leakage or the effect of various disruptive conditions on the retention of encapsulants. Retention and leakage of the encapsulated material depend on the type of the vesicles, their lipid composition and T_c . It has been reported that small unilamellar vesicles (SUV) and multilamellar vesicles (MLV) are less sensitive than large unilamellar liposomes (LUV) to temperature-induced leakage.⁴⁰

In this study calcein was used as a model of hydrophilic drug because of its stability and fluorescence properties, to which lipid membranes were essentially impermeable. It registers the fraction of the aqueous entrapped phase and hence provides a good measure of liposome encapsulation. Fig. 3 shows the fluorescence spectra of liposome of optimal point formulation before and after disruption with Triton® X-100 and calcein non-entrapped in liposome.

To evaluate the effect of lipid concentration on entrapment efficiency of vesicles, different lipid concentrations of POPC (10, 20, and 40 mg ml⁻¹) were prepared with the same size (117 ± 2 nm) and the entrapment efficiency was determined. A significant correlation between lipid concentration and entrapment efficiency was observed, as by increment in lipid concentration, entrapment efficiency also increased (Fig. 4). Schneider *et al.* (1995)⁴¹ also demonstrated the dependence of entrapment efficiency of extruded liposomes on lipid concentration.

It was observed that the liposome composition influenced the entrapment efficiency of vesicles. Fig. 5 shows the relationship between the liposome composition and entrapment efficiency. It was found that the liposome composition has probably indirect effects on entrapment efficiency of vesicles by changing the size and lamellarity of vesicles.

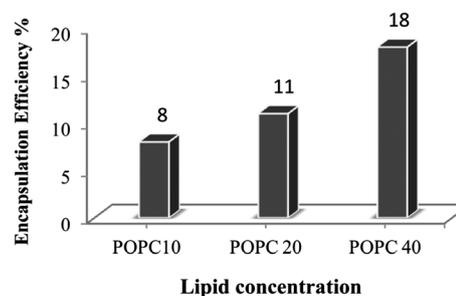


Fig. 4 The effect of lipid concentration (POPC: 10, 20, 40 mg ml⁻¹) on encapsulation efficiency.

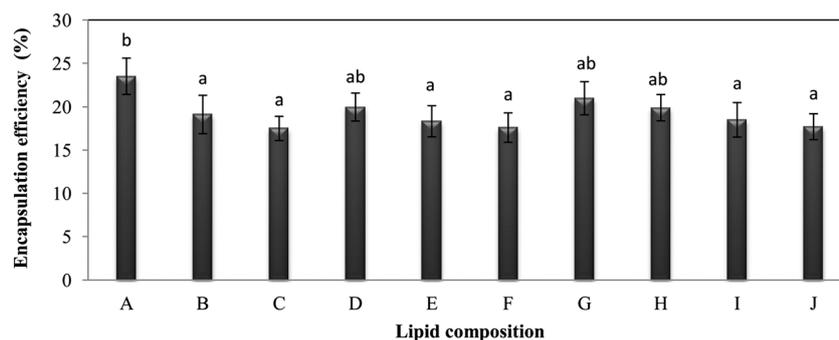


Fig. 5 The effect of lipid composition on encapsulation efficiency. (A) b_1 , (B) b_2 , (C) b_3 , (D) b_{12} , (E) b_{13} , (F) b_{23} , (G) b_{123} , (H) b_{123} , (I) b_{123} , and (J) b_{123} . The vertical bars represent the standard deviation ($n = 5$) of each data point. Different letters represent significant differences of lipid composition ($P < 0.05$).

A significant difference was observed in encapsulation efficiency between liposomes composed of DOPC and other liposomes ($P < 0.05$). Considering the size of liposome, the maximum encapsulation efficiency was observed in liposomes composed of DOPC and DPPC, respectively.

As demonstrated in topographic chart and 2D graph (Fig. 6), increasing the density of parallel curves around the DOPC point indicates that liposomes with soft membranes (with unsaturated lipid) have larger size and consequently have greater entrapment efficiency. The density of parallel curves around the DOPC point confirms more encapsulation efficiency in comparison with DPPC and POPC.

By comparing entrapment efficiencies of liposomes with soft membranes such as DOPC with the ones having rigid membranes composed of saturated lipids, it can be concluded that the entrapment efficiency of liposomes also depends on the rigidity of the bilayer membrane. Additionally, by increasing the temperature higher than the transition temperature of liposomes during their preparation and storage, especially for liposomes composed of saturated lipids, the permeability and fluidity increase, and partly low entrapment efficiency was found

probably due to leakage of calcein during the preparation of liposomes.⁴¹ Nii *et al.* (2003)³⁶ also concluded that high preparation temperature resulted in lower entrapment efficiencies suggesting that the leakage of encapsulated calcein was facilitated at high temperature prepared by the microentrapment vesicle (MCV) method.

It is also recommended to keep the final product at temperatures above T_c under an inert atmosphere such as nitrogen or argon for 1 h to allow the sample to anneal and stabilize but for long storage keep at temperatures below T_c under an inert atmosphere.²⁰ It was observed that after separation, the encapsulated materials can leak out at storage conditions higher than their T_c .

Our findings have shown that liposome size and entrapment efficiency are considerably correlated, as by increasing liposome size, entrapment efficiency is also increased in liposomes with preserved unilamellarity (Fig. 7). Further studies have shown that by increasing the lamellarity and decreasing the space between the layers in multilamellar vesicles, entrapment efficiency is decreased so there is an inverse relation between entrapment efficiency and vesicle diameter.⁴³ Fig. 7 shows the

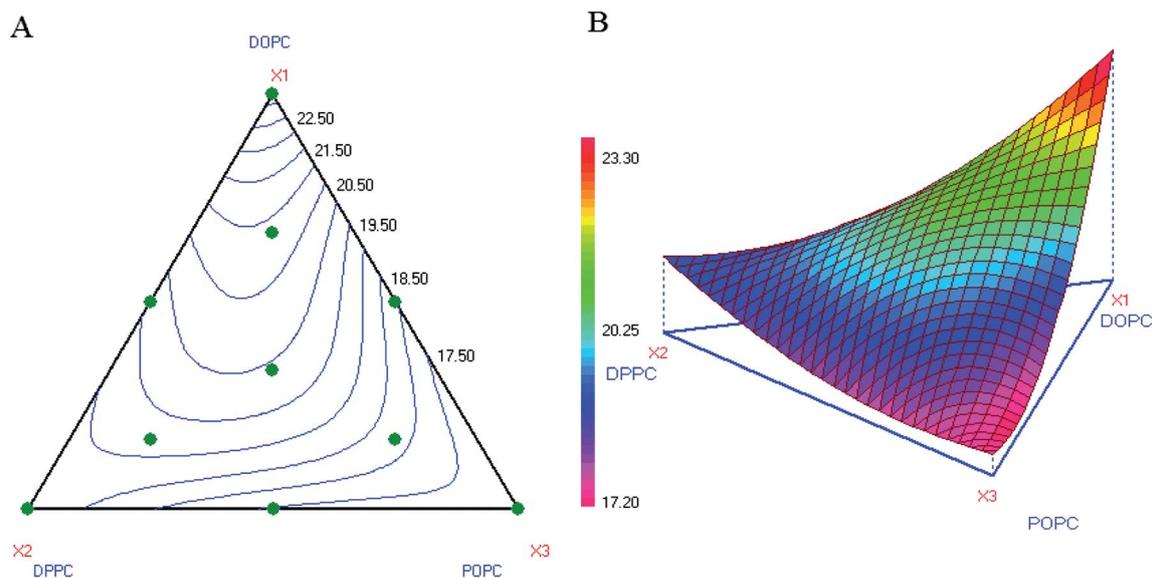


Fig. 6 Variation of entrapment efficiency as a function of lipid composition.

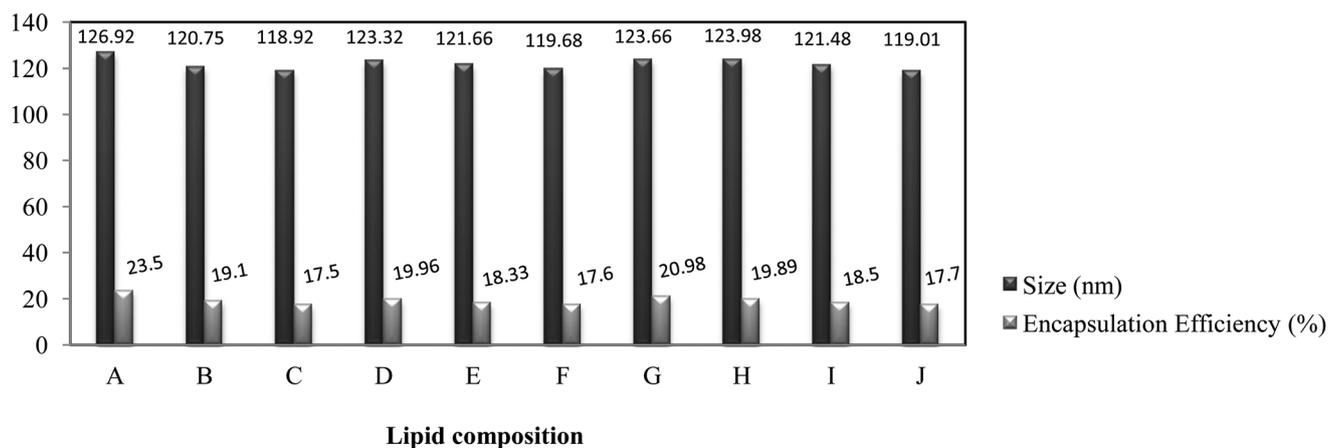


Fig. 7 The effect of liposome composition on liposome size and encapsulation efficiency. (A) b_1 , (B) b_2 , (C) b_3 , (D) b_{12} , (E) b_{13} , (F) b_{23} , (G) b_{123} , (H) b_{123} , (I) b_{123} , and (J) b_{123} . The vertical bars represent the standard deviation ($n = 5$) of each data point.

effect of liposome composition on liposome size and entrapment efficiency.

Our findings are in good agreement with the results of Mozafari.¹² It was observed that the lipid composition and the preparation method influence entrapping efficiency of liposome formulations. The coefficients estimated for entrapment efficiency (Table 4) as a response of the model could describe and predict the effect of lipid composition (DOPC, POPC, and DPPC) on entrapment efficiency of liposome. The results were significant with 99.9% confidence level.

3.3. Influence of lipid composition on phase transition temperature of liposomes

For first-order phase transitions, such as the bilayer gel to liquid crystalline transition, T_c can be directly obtained by the DSC thermogram as the maximum of the excess heat capacity function. The transition temperature of liposome depends on the nature of the hydrocarbon chains (acyl chain length, structure and degree of unsaturation of the hydrocarbon chains and presence of a methyl branch), the polar region of the molecule, nature and ionic strength of the suspension medium (the amount of water molecules and any solutes dissolved in the water).^{1,24}

Table 5 Transition temperature of liposomes with different compositions

Liposome composition	Molar ratio	Transition temperature ^a /°C
DOPC	1	-18.9 ± 1.22^a
DPPC	1	39.8 ± 1.91^j
POPC	1	0.3 ± 0.73^d
DOPC + DPPC	1 : 1	17.4 ± 1.82^e
DOPC + POPC	1 : 1	-13.4 ± 2.32^b
POPC + DPPC	1 : 1	21.3 ± 1.54^h
DOPC + DPPC + POPC	1 : 1 : 1	6.8 ± 1.92^f
DOPC + POPC + DPPC	3 : 1 : 1	-4.0 ± 1.46^c
DOPC + DPPC + POPC	1 : 3 : 1	23.6 ± 2.13^i
DOPC + DPPC + POPC	1 : 1 : 3	3.2 ± 1.11^e

^a Data were expressed as mean \pm SD ($n = 5$). Different letters represent significant differences of lipid composition ($P < 0.05$).

The greatest ($\sim 39^\circ\text{C}$) and smallest ($\sim -18.9^\circ\text{C}$) T_c were obtained for liposomes composed of saturated and unsaturated lipids, respectively.

Results show that T_c is lowered by decreasing the chain length, the degree of unsaturation of the acyl chains, as well as the presence of branched chains and bulky side groups (Table 5).

Furthermore, T_c depends critically on the position of the *cis*-double bond, and any hydrocarbon with *cis*-unsaturated tail has a lower T_c than those which are *trans*-unsaturated.²⁴

The liposomal thermotropic characteristics are influenced both by the chemical composition of liposomes and their physical state (especially vesicle size, curvature, and number of lamellae).⁴⁴ The thermotropic behavior also provides information on the homogeneity and the lateral organization of the lipid bilayer. Koynova and Caffrey (1998)⁴⁵ also found that for unilamellar vesicles composed of 16 : 0/16 : 0 PC, the phase transition temperature parameters depend on vesicle size but for larger vesicles (diameters > 70 nm) they are essentially independent of size.

It was observed that if the lipid molecules of the bilayer were perfectly ordered, the transition would be isothermal, as the transition would involve all molecules at the same temperature. However, imperfect ordering of lipid molecules due to confining scan rates broadens the observed transitions. The packing of molecules within the bilayer can be disrupted by the presence of an impurity, by low miscibility of components or by phase separation; the reduced lipid–lipid interaction will be reflected by the thermal phase transition profile due to interference with cooperative movements occurring during phase transition. Barenholz and Amselem (1993)³⁰ also mentioned that perturbation of a lipid bilayer can cause a decrease in T_c due to increase in the available space between hydrophobic chains. Fig. 8 shows the effect of lipid composition on T_c .

3.4. Influence of liposome composition on its fluidity

Fluidity of phospholipid membranes is an effective factor in manufacturing and application of liposome. Calvagno *et al.* (2007),¹⁶ determined a significant difference in the release profiles of drugs by the presence of two factors: (i) the strength of the

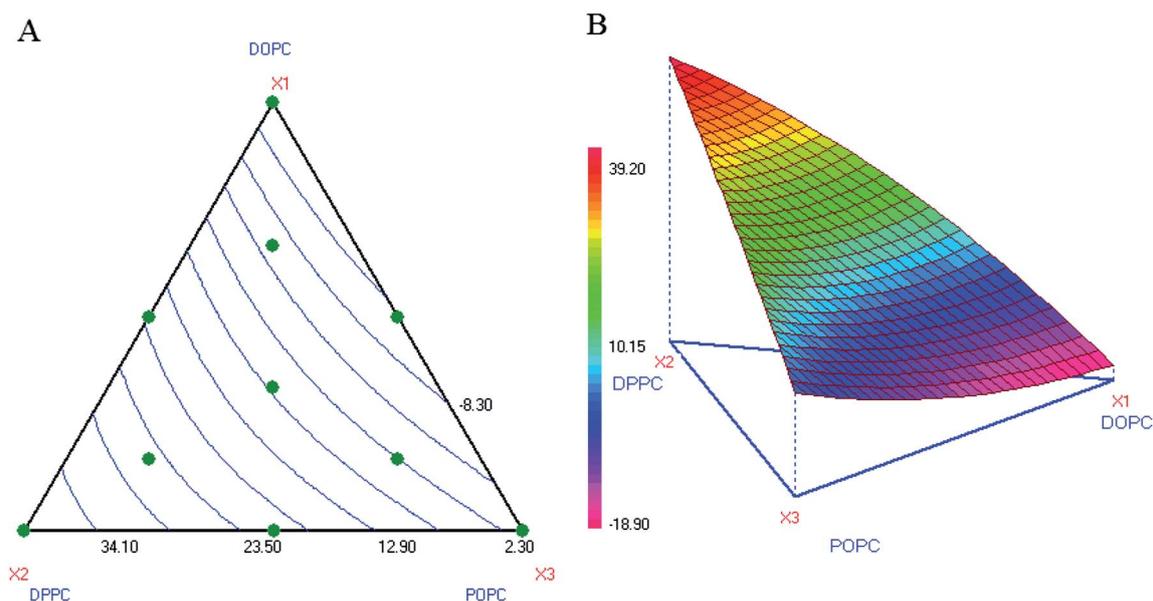


Fig. 8 Variation of transition temperature as a function of lipid composition.

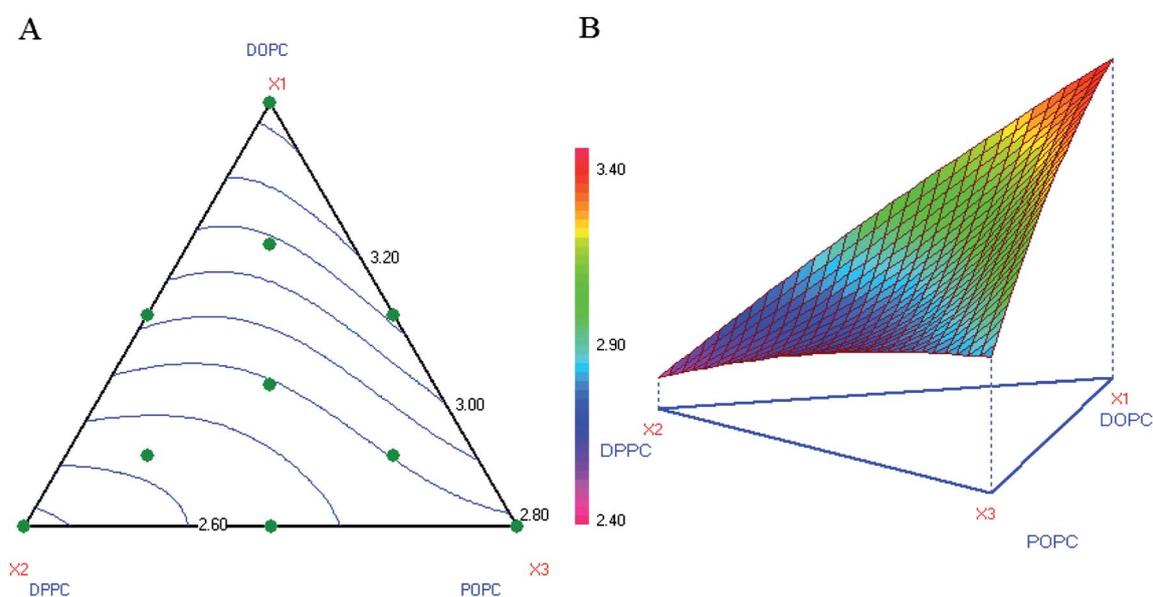


Fig. 9 Variation of liposomes fluidity as a function of lipid composition.

drug–liposomal lipid interaction and (ii) the fluidity of the bilayer, *i.e.* by increasing the fluidity of the bilayer, the drug leakage to outer liposomal aqueous compartments was rapidly increased. Bilayer fluidity reflects the order and dynamics of phospholipid alkyl chains in the bilayer (Fig. 9).⁴⁶

The comparison of fluidity values of DOPC (two C=C bonds), POPC (one C=C bond), and DPPC (zero C=C bond) shows the noticeable role of double bonds in lipids (Table 6). Liposomes formed with saturated or unsaturated phospholipids show different fluidity values of membranes as liposomes composed of unsaturated and saturated lipids have greater and lower fluidity values, respectively. The double bonds within the acyl chain resulted in a decrease in the packing density and chain

ordering in lipid bilayers and consequently an increase in the liposomes fluidity. As order and subdynamics of phospholipid alkyl chains in the bilayer depend on their composition, fluidity values depend on liposome composition.⁴⁷

Our results have also indicated that incorporation of some fluid lipids into a liposome bilayer could interfere with the barrier function and thus lower its T_c and increase its fluidity. These data correspond well with the previous findings.¹⁷

A significant difference in fluidity values of liposomes completely or partially composed of DOPC with liposomes composed of DPPC and POPC was observed. No significant difference was observed in the fluidity of liposomes that were a hybrid of three types of phospholipids ($P < 0.05$) (Table 6).

Table 6 Relation between transition temperature and fluidity of liposome

Lipid	T_c^a (°C)	$(1/P)_{TMA-DPH}^{(-)}$ ^b
b_1	-18.9 ± 1.2	3.35 ± 0.4^c
b_2	39.8 ± 1.9	2.44 ± 0.3^a
b_3	0.3 ± 0.7	2.81 ± 0.2^{ab}
b_{12}	17.4 ± 1.8	2.88 ± 0.3^{ab}
b_{13}	-13.4 ± 2.3	3.13 ± 0.2^c
b_{23}	21.3 ± 1.5	2.68 ± 0.2^{ab}
b_{123}	6.8 ± 1.9	2.75 ± 0.3^{ab}
b_{123}	-4.0 ± 1.4	3.2 ± 0.2^c
b_{123}	23.6 ± 2.1	2.69 ± 0.3^{ab}
b_{123}	3.2 ± 1.1	2.71 ± 0.2^{ab}

^a T_c means the gel-to-liquid crystalline transition temperature. Data were expressed as mean \pm SD ($n = 5$). ^b All experiments were carried out at 25 °C. Different letters represent significant differences of lipid composition ($P < 0.05$).

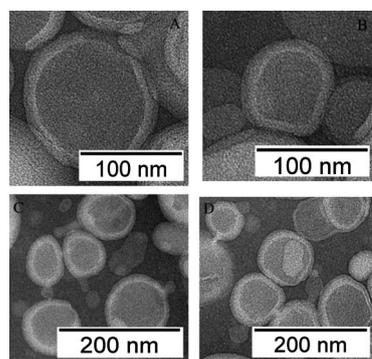


Fig. 10 Transmission electron micrographs of liposomes prepared by the extrusion technique. (A) liposomes composed of DOPC; (B) liposomes composed of DPPC; (C) liposomes composed of POPC; (D) liposomes composed of three lipids (DOPC, POPC, and DPPC).

All experiments were performed at 25 °C. In this condition, most of the liposomes were in the liquid-disordered forms (above T_c), thus showing the maximal fluidity values. Our findings also

confirmed that liposome fluidity depends on temperature, as by increasing the temperature, bilayer fluidity values also increase, e.g. liposomes composed of DPPC have fluidity value of 2.44 $(1/P)_{TMA-DPH}$ and 2.85 $(1/P)_{TMA-DPH}$ at 25 °C and 45 °C, respectively.

3.5. Transmission electron microscopy (TEM)

The physical and chemical properties of liposomes rely on their surface and structures. Transmission electron microscopy (TEM) is a powerful and unique technique for structure characterization. It provides information on the size distribution and shape of nanocarrier systems.²⁹ In addition to the configuration of liposome, electron microscopy can also provide information on the interaction between liposomes (e.g., in the form of aggregation or fusion) and their stability.⁴⁵

Vesicles in the nanometre size range (after extrusion through 100 nm pore size filters) were observed by transmission electron microscope. The extrusion step was performed in order to produce nanometric unilamellar vesicles with a homogeneous size distribution.

TEM images indicate that the extruded vesicles are in the form of large unilamellar vesicles and multilamellar vesicles. The bilayer nature of the vesicles is clearly visible in these micrographs confirming that the prepared lipid vesicles are liposomes (defined as closed continuous bilayer structures) (Fig. 10).

3.6. SAXS measurements

SAXS is suitable for structural analysis of materials. It enables determination of the structure of objects such as the solutions of biological macromolecules and liposomes. The scattering pattern of SAXS contains information about the structure, shape, and size of macromolecules and the surface to volume ratio of particles. The scattering function can be described as the product of a structure factor and a form factor. The structure factor is related to the ordering of the bilayers and is responsible for the typical equidistant peaks, but it also contains information about the bilayer flexibility and the number of coherently scattering

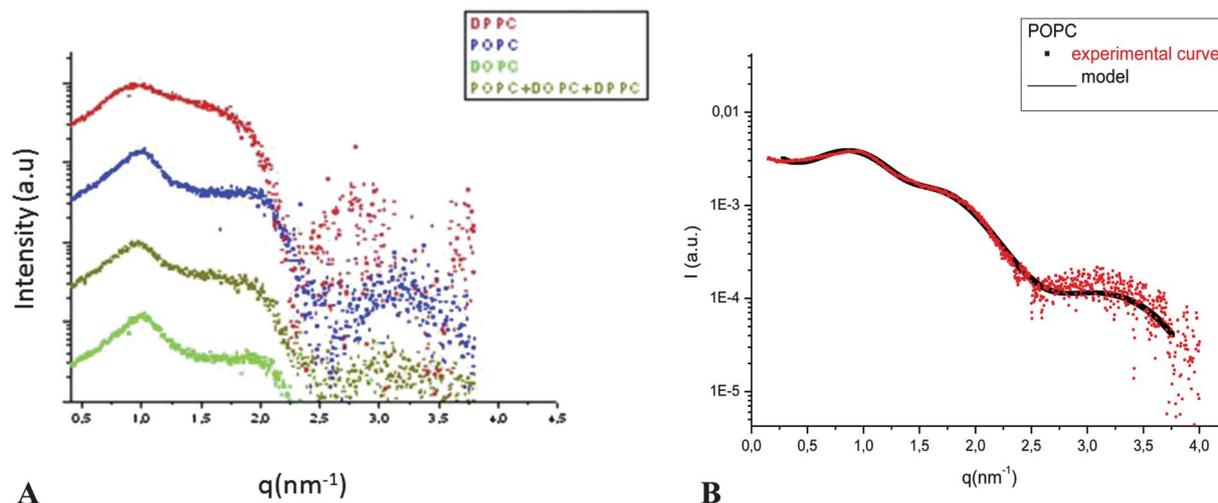


Fig. 11 (A) SAXS curves of some of the liposome dispersions (DOPC, POPC, DPPC, and a mixture of three lipids) and (B) scattering curve (line) and best fit of POPC (symbol).

Table 7 The compression of the mixture results and the responses gave by mixture design ($n = 5$)

Factors	Size ^a /nm	Transition temperature ^a /°C	Entrapment efficiency ^a (%)	ζ-Potential ^a /mV	Fluidity (1/ <i>P</i>) _{TMA-DPH} ^{(c)b}
Mixture design response	127.5 ^a	11.43 ^a	20.24 ^a	-7.24 ^a	2.87 ^a
Experimental result	125.9 ± 1.8 ^a	10.9 ± 1.5 ^a	19.1 ± 2.1 ^a	-7.15 ± 1.4 ^a	2.56 ± 0.8 ^a

^a Data were expressed as mean ± SD ($n = 5$). Different letters within each column represent significant differences ($P < 0.05$).

bilayers. The method is accurate, nondestructive, and usually requires only a minimum of sample preparation.⁴⁸

Lamellarity also is a noticeable property in liposomal structure having a significant effect on liposomal application, since an increase in lamellarity could result in permeation resistance, and release of the amphiphilic drugs from unilamellar liposomes is greater than from multilamellar liposomes with similar size.

In order to estimate the average number of bilayers in the vesicles, small-angle X-ray scattering experiments for 10 liposome suspensions were recorded.

All the SAXS patterns of the liposome suspensions have the same profile (Fig. 11). Several equidistant broad peaks are also observed, suggesting a multilamellar stack of bilayers.⁴⁹

Scattering intensity $I(q)$ can be represented as the product of the form factor $P(q)$ and the structure factor $S(q)$, where q is the length of the scattering vector, given by eqn (7).

$$q = (4\pi/\lambda)\sin \theta \quad (7)$$

where λ and θ are wavelength and scattering angle, respectively. The arrangement of multi-lamellar bilayers is represented by $S(q)$ and $P(q)$ corresponding to the bilayer structure (electron density profile).

The data were analyzed with the generalized indirect Fourier transformation method elaborated by Frühwirth *et al.* (2004).⁴⁹ As the structure extends further in two dimensions compared to its thickness, the form factor can be described as the separated product of the factor $\sim 1/q^2$ and the thickness scattering function, and the indirect Fourier transform is calculated for the thickness of a flat structure.⁵⁰ The structure factor is calculated for a lamellar structure according to the modified Caillé theory.⁵¹ This model is defined by three parameters: the number of coherently scattering bilayers in the stack, which corresponds to the lamellarity of liposomes, the spacing of bilayers and the Caillé parameter which can be related to the flexibility of the bilayers.

For Fourier transformation studies, the scattering curves were used in the q range from 0.2 to 3.7 nm⁻¹. The best fit obtained with this method for the liposomes prepared with POPC is shown in Fig. 11.

It was found that liposomes formed well-ordered bilayers with a lamellar spacing of $d = 5\text{--}6$ nm at 20 °C. Liposomes composed of DPPC had the lamellar spacing around 5 nm and others had the lamellar spacing of about 6 nm. The difference can be due to the type of fatty acid chains branched on phospholipids and T_c of phospholipid, as at 20 °C, liposomes composed of DPPC are in gel-ordered form but other liposomes are in liquid-disordered forms. Most of the liposomes were large unilamellar vesicles but in comparison with TEM images, a negligible amount of large multilamellar vesicles was also observed.

3.7. Optimization of liposome formulation with the mixture design

Mixture Design of Experiments (MDOE) was used as a model to determine the optimum combination of different lipids for delivering a desired response using a minimum number of experimental runs.

Mixture design proposed a liposome formulation with an appropriate size for achieving the maximum possible entrapment efficiency and phase transition temperature of ≤ 20 °C for preparation of liposomes with minimal leakage of bioactive agents, with appropriate fluidity for bioactive releasing under considered conditions.

The optimal point of liposome formulation estimated by mixture design is 46% DOPC, 12% POPC and 42% DPPC.

We prepared the liposomes according to the optimal point estimated by the model of mixture design and characterized the liposomes and compared the experimental results with the responses obtained using the mixture design model. Table 7 presents the mixture design responses and experimental results.

There is no significant difference between the experimental results and mixture design responses. This model is an easy and straightforward approach for optimization of lipid mixtures of drug-loaded liposomes. Any deviation of this point will create a mixture composition that will change the results.

These mixed systems are interesting not only for their potential therapeutic applications, but also as models for investigating the structure and the dynamics of complex lipid membranes and other assemblies.

Currently, several types of liposomes with numerous variations in lipid composition are used in drug delivery. The range of applications for micro- and nanoliposome in the food industry has been also increased because of the many advantages that liposomes provide by protecting the active agent from the enzymatic and chemical changes, as well as temperature and ionic strength variations.⁴⁶ Model of Mixture Design of Experiments could be an impressive approach which enables theoretical understanding of optimal liposome formulation including construction of liposomes with improved stability, favorable size, expected encapsulation efficiency and controlled interaction properties, *e.g.*, smaller size increases blood circulation times, increases the volume of biodistribution and allows extravasation through blood vessels, while, on the other hand, reducing the amount of encapsulated contents per mass of lipid. Therefore, for various applications, the optimal size may vary depending on the target site.

For example, transdermal drug delivery is also a user-friendly delivery method for therapeutics. A liposome formulation composed of different lipids with appropriate size for skin

permeation of drugs with high molecular weight and poor water solubility can be developed by this model.

4. Conclusion

The possibility of using liposomes as a carrier system for different applications depends on their physicochemical properties; lipid composition and preparation method of vesicles strongly influence vesicle behavior in biological systems.

The reported results show that mean size, polydispersity index, ζ -potential, phase transition temperature, entrapment efficiency and fluidity were influenced by liposome lipid composition and preparation method. The greatest mean size and polydispersity values were obtained for liposomes composed of DOPC which contains unsaturated phospholipids, and DPPC, composed of saturated phospholipids prepared at high temperature, respectively.

The size of the liposome and the entrapment efficiency are considerably correlated, as by increasing liposome size, entrapment efficiency also increases in liposomes with preserved unilamellarity. Therefore, in giant liposomes, entrapment efficiency of hydrophilic bioactive agents is decreased. In multilamellar vesicles, by increasing the lamellarity and decreasing the space between the layers, entrapment efficiency is decreased, so there is an inverse relation between entrapment efficiency and vesicle diameter. By considering the liposome size and liposomal bilayer properties such as fluidity and T_c , maximum encapsulation efficiency of calcein was found in liposomes composed of DOPC and DPPC, respectively.

Generally, particle size and ζ -potential are the two most important properties that determine the fate of liposomes. Knowledge of the ζ -potential is also useful in controlling the aggregation, fusion and precipitation of liposomes, which are important factors affecting the stability of liposomal formulations.

The results confirmed the relationship between ζ -potential, liposome composition and bioactive agents' properties, as by increasing the calcein concentration, ζ -potential of liposome suspensions also increase. The greater absolute value of ζ -potential causes the liposomal suspension to be stable because the charged vesicles repel each other and thus overcome the natural tendency to aggregate.

It was observed that the transition temperature of liposomes depends on the nature of the hydrocarbon chains, the polar region of the molecule, nature and ionic strength of the suspension medium. The greater and lower T_c and fluidity values were observed for liposomes composed of saturated and unsaturated lipids, respectively.

Bilayer fluidity also reflects the order and dynamics of phospholipid alkyl chains in the bilayer and is mainly dependent on its composition as liposomes composed of unsaturated lipids have more fluidity.

TEM images indicate that the extruded vesicles are in the form of large unilamellar vesicles and partially multilamellar vesicles. Additionally, all the SAXS patterns of the liposome suspensions have the same profile, with spacing bilayer about 5–6 nm.

Furthermore, we used the mixture design as a model for determining the optimum combination of different lipids for delivering a desired response by using a minimum number of

mixture runs. These mixed systems are interesting as models for investigating the structure and the dynamics of complex lipid membranes and other assemblies. Optimization of liposome formulation can make it possible to deliver bioactive agents directly to target sites, hence reducing systemic side effects. The use of this model is an interesting approach to correlate different properties of liposomes and bioactive drug properties (hydrophobicity or hydrophilicity). From this point of view it can be an interesting model study that has relevance for biological applications.

Abbreviations

DLS	dynamic light scattering
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
LUVs	large unilamellar vesicles
MDOE	Mixture Design of Experiments
PBS	phosphate buffered saline
PDI	polydispersity index
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
RES	reticuloendothelial system
SAXS	small angle X-ray scattering
SUV	small unilamellar vesicles
T_c	phase transition temperature
TEM	transmission electron microscopy
MLV	multilamellar vesicles
MCV	microentrapment vesicle

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Conclusion

Les caractéristiques et les domaines d'application des liposomes, comme système vecteur de biomolécules, dépendent fortement des propriétés physicochimiques (taille, indice de polydispersité, ζ -potentiel, température de transition de phase, efficacité d'encapsulation, fluidité), de la composition lipidique et du mode de préparation des vésicules. Les liposomes les plus volumineux ont été obtenus avec une formulation riche en DOPC, principalement composée de phospholipides insaturés. L'indice de polydispersité maximal a été obtenu avec une formulation riche en phospholipides riches en acides gras saturés (DPPC), préparés à haute température. La taille des liposomes et l'efficacité d'encapsulation sont fortement corrélées. En effet, l'augmentation de taille d'un liposome accroît cette propriété en préservant l'uni-lamellarité de la membrane. Cependant, dans le cas de liposomes géants, cette efficacité d'encapsulation de biomolécule hydrophile diminue. Dans les vésicules multi-lamellaires, on observe une diminution inversement proportionnelle à la taille du liposome qui s'explique par la diminution de l'espace entre les différentes couches. En tenant compte des caractéristiques physicochimiques préalablement détaillées de la bicouche lipidique (taille, fluidité, T_c), l'efficacité d'encapsulation maximale de la calcéine est obtenue pour des membranes composées de DOPC et de DPPC. La stabilité des formulations liposomales dépend des phénomènes d'agrégation, de fusion et de précipitation, pouvant subvenir. Les charges des lipides membranaires permettent de contrer la tendance naturelle des vésicules à s'agréger. Cette propriété, mesurée par la valeur du potentiel zéta, augmente lors de l'encapsulation de la calcéine dans des liposomes. La température de transition de phase des liposomes dépend de la nature des chaînes hydrocarbonées, de la région polaire de la molécule et de la nature et la force ionique du milieu. Les valeurs maximales de T_c et de fluidité sont respectivement observées pour des formulations riches en lipides saturés et insaturés. En effet, le nombre d'insaturations augmente la mobilité et la fluidité des chaînes d'acides gras estérifiées sur les phospholipides de la bicouche membranaire du liposome.

Les images obtenues par microscopie électronique à transmission montrent que les vésicules extrudées ont une structure uni-lamellaire et partiellement multi-lamellaire, avec une distance entre les bicouches de 5 à 6 nm, mesurée par SAXS. Nous avons optimisé la formulation des différents lipides constituant les liposomes pour obtenir les caractéristiques d'un système en un minimum d'essais, dans le but d'étudier la structure, l'assemblage et la dynamique des membranes lipidiques liposomales. L'optimisation de la formulation des lipides de ce type de vecteur permet de délivrer des molécules bioactives vers des tissus ciblés, en réduisant les

III. Résultats & Discussion
Chapitre III.I: Caractérisation physico-chimique des liposomes

effets systémiques secondaires. L'utilisation de ce type de modèle permet d'accroître les connaissances des interactions entre les différentes propriétés du liposome et l'hydrophilie de la molécule à vectoriser, en fonction des applications.

Influence de la composition lipidique sur les propriétés physicochimiques de nanoliposomes encapsulant un peptide naturel antioxydant (L-carnosine)

Résumé

Les peptides comme la L-carnosine sont de plus en plus étudiés en raison de leur propriété antioxydante recherchée dans le domaine alimentaire. L'encapsulation de ces biomolécules par des nanoliposomes présente une alternative aux problèmes posés par une incorporation directe des antioxydants dans les aliments.

Dans cette étude, les nanoliposomes préparés à partir de différentes sources lipidiques (DOPC, POPC et DPPC) par la méthode d'hydratation du film phospholipidique, ont été caractérisés par leur taille, leur potentiel zéta, la température de transition de phase et la fluidité. L'efficacité d'encapsulation de la L-carnosine dans trois systèmes liposomales de composition différente a été comparée par une méthode précise et rapide (RMN 1H), sans séparation de la fraction de L-carnosine piégée de celle non encapsulée. De plus, la morphologie des différents nanoliposomes unilamellaires obtenus, a été caractérisée par microscopie à force atomique.

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Influence of lipid composition on physicochemical properties of nanoliposomes encapsulating natural dipeptide antioxidant L-carnosine

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ABSTRACT

Natural dipeptide antioxidants (L-carnosine) are receiving increasing attention because of their noticeable potential as biopreservatives in food recent technology. Encapsulation of antioxidants by nanoliposomes could represent an ameliorative approach to overcome the problems related to the direct application of these antioxidant peptides in food.

In this study, nanoliposomes prepared from different lipids (DOPC, POPC and DPPC) by thin film hydration method, were assessed by considering their size, ζ -potential, phase transition temperature and fluidity. One important parameter of interest in this article was to compare the encapsulation efficacy of L-carnosine in three different nanoliposomes using a rapid and precise approach ¹H NMR without the need for physical separation of entrapped and non-entrapped L-carnosine. Furthermore, the morphology of small unilamellar nanoliposomes with different compositions on mica surface was investigated using Atomic Force Microscopy.

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1. Introduction

Antioxidant peptides have been extensively examined as potential biopreservatives in food recent technology. However, stability issues like proteolytic degradation and the potential interaction of peptide with food components might result in a decrease in their activity (Da Silva Malheiros, Daroit, & Brandelli, 2010).

Traditionally, antimicrobials or antioxidants agents are mixed into initial food formulations, or alternatively foods are immersed into solutions containing these additives. Limitations of these approaches are: loss of protection effect once the active compounds are consumed in complex reactions of food system and lack of selectivity to target the food surface where most microbial and

Abbreviations: AFM, Atomic Force Microscopy; DLS, Dynamic Light Scattering; D₂O, deuterium oxide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; IC-AFM, intermittent contact mode; LUVs, large unilamellar vesicles; PDI, polydispersity index; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; RMS, root-mean-square; SAXS, Small Angle X-ray Scattering; S/N, signal-to-noise; SUV, small unilamellar vesicles; T_c, phase transition temperature; TEM, Transmission Electron Microscopy; TMA-DPH, 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene; MLV, Multilamellar Vesicles; LUVs, Large Unilamellar Vesicles.

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oxidative spoilage reactions occur intensively (Gemili, Yemencioğlu, & Altinkaya, 2010).

The entrapment of antioxidants into nanoliposomes might represent an alternative to overcome the problems related to the direct application of these antioxidant peptides in food (Maherani, Arab-Tehrany, Mozafari, Gaiani, & Linder, 2011). Encapsulation of antioxidants in nanoliposomes not only offers a potential solution to protect antioxidants but also enhance their efficacy and stability in food applications. Another advantage of liposomal delivery systems is the ability to release components on demand (Were, Bruce, Davidson, & Weiss, 2003).

As mentioned above, the great advantage of nanoliposomes over other encapsulation technologies (spray-drying, extrusion, fluidised beds) is their stability (Desai & Park, 2005). Liposome entrapment has been shown to stabilise the encapsulated materials against a range of environmental and chemical changes, including enzymatic and chemical modification, as well as buffering against extreme pH and temperature (Mozafari et al., 2008; Taylor, Davidson, Bruce, & Weiss, 2005).

The ability of nanoliposomes to trap water-soluble substances has been employed in various pharmaceutical and cosmetic applications to protect and control the release of active compounds. Thus, the entrapment of antioxidants into nanoliposomes might represent an alternative to overcome some problems related to

the direct application of bacteriocins in food, such as proteolytic degradation or interaction with food components (Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008).

Another significant advantage of liposome is that it can incorporate and simultaneously release two materials with different solubilities. One example for which is the incorporation of two antioxidant agents namely alpha-tocopherol (a lipid-soluble molecule) and glutathione (a water-soluble molecule) in the same lipid vesicle (Mozafari & Mortazavi, 2005).

Encapsulation of antioxidants into nanoliposomes is mainly reported to be achieved by thin film hydration method, and phosphatidylcholine is the most common phospholipid employed in liposome manufacture (Da Silva Malheiros et al., 2010).

As nanoliposomes could be recently prepared from natural components, regulatory limits of their application in food systems are potentially reduced or eliminated, and new formulations based on natural components could be quickly introduced (Da Silva Malheiros et al., 2010).

The dipeptide carnosine (β-alanyl-L-histidine) is widely distributed in mammalian tissues. It is synthesised by carnosine synthetase from its component amino acids and degraded by carnosinase (Holliday & McFarland, 1996). There have been many theories about its biological function such as peroxy radical trapping activity. Furthermore, carnosine inhibited the oxidative hydroxylation of deoxyguanosine induced by ascorbic acid and copper ions. Other roles of carnosine, such as chelation of metal ions, quenching of singlet oxygen, and binding of hydroperoxides, pH buffering and regulation of enzyme activity are also discussed (Babizhayev, 2006; Kohen, Yamamoto, Cundy, & Ames, 1988).

Furthermore, it has been shown that ageing and neurodegenerative conditions are often associated with proteasome dysfunction, possibly mediated by zinc and/or copper ions. The nasal administration of carnosine has been suggested as a possible way for repression of zinc/copper-mediated proteasome inhibition and consequent neurodegeneration (Hippkiss, 2005).

L-Carnosine synthesized in muscles may also be able to control the blood glucose level through the regulation of the autonomic nervous system (Nagai et al., 2003).

In this work, we compared three different formulations and effect of composition on L-carnosine encapsulated liposome properties such as size, ζ-potential, phase transition temperature, lamellarity and fluidity. We also determined L-carnosine encapsulation efficiency by using a rapid approach ¹H NMR without the need for physical separation of entrapped and non-entrapped L-carnosine. Furthermore to complete the liposome characterisation, the tapping mode Atomic Force Microscopy approach was used.

2. Materials and methods

2.1. Materials

Phospholipids used were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), all of which were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-(4-Trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was purchased from Invitrogen (Paris-France). L-Carnosine and phosphate buffer salts, monobasic sodium phosphate and dibasic sodium phosphate, were purchased from Sigma-Aldrich (Paris, France). All other reagents of analytical grade were purchased from Sigma-Aldrich (Paris, France).

2.2. Liposome preparation

Large unilamellar vesicles (LUVs) were prepared as described elsewhere (Lichtenberg & Barenholz, 1998). In brief, phospholipids

were dissolved in a chloroform solution. The organic solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under vacuum overnight, was hydrated with L-carnosine solution to obtain multilamellar vesicles. Isotonic phosphate buffer solutions with pH values 7.4 were prepared using monobasic sodium phosphate and dibasic sodium phosphate in deuterium oxide solution. L-Carnosine was dissolved in phosphate deuterium oxide (D₂O) buffer solution (pH 7.4); to get a final L-carnosine concentration of 50 mM before use. The suspension was subjected to five cycles of freezing (−80 °C) and thawing (at temperature above the phase transition temperature) to obtain multilamellar vesicles (MLVs) and then extruded through a polycarbonate filter (100-nm pore size filter, 11 times) above the phase transition temperature of the vesicles by using an Avanti-mini extruder (Avanti Polar Lipids, Alabaster, USA) (MacDonald et al., 1991; Mozafari, 2010). The lipid concentration in the final vesicle suspension was determined with an enzymatic assay kit (Test Wako-C) from Wako Pure Chemical Co. Ltd. (Osaka, Japan).

2.3. Particle size and ζ-potential determination

When designing liposome-based bioactive carrier systems, a reliable and reproducible analysis of their size and size distribution is important. Determination of vesicle size distribution is a fundamental quality control assay.

The mean diameter, particle size distribution and ζ-potential of vesicles were determined upon empirical dilution of the samples using dynamic light scattering (DLS) technique employing a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Viscosity and concentration of liposome suspension are most important parameters for DLS. Viscosity directly affects the Brownian motion of nano-particles and thus the calculated liposome size result, so sample should be diluted to an appropriate concentration.

The software used is DTS Nano, version 6.12 supplied by the manufacturer (Malvern Instruments Ltd., UK). To avoid multiple scattering effects, liposome suspensions were diluted (1:25), and then the sample was put into a standard capillary electrophoresis cell equipped with gold electrodes. All measurements were carried out at 25 °C by considering a medium viscosity of 1.020 and medium refractive index of 1.333. Results are presented as an average diameter of the liposome suspension (z-average) with the polydispersity index (PDI) and ζ-potential (z-average) of the liposome suspension (Gregoriadis, 2007).

2.4. Encapsulation efficiency determination by NMR spectroscopy

The encapsulation efficiency, which is a measure of the percentage of the total compound entrapped within the liposome, is an important parameter in liposomal characterisation. Most of the reported experimental methods in the literature to determine liposomal encapsulation efficiency require removal of the free (unencapsulated) bioactive component using column chromatography, centrifugation, or dialysis before measurement (Perkins, Minchey, Ahl, & Janoff, 1993). These methods may lead to vesicle leakage or separation may not always be complete. Thus, quantitative methods not requiring physical separation would be desirable for liposome characterisation (Perkins et al., 1993).

In the present study, a rapid and simple experimental approach is reported using ¹H NMR to determine the liposomal encapsulation efficiency without the need to physically separate of encapsulated bioactive components from free ones.

The osmotic balance between the encapsulated and external media is very important. The NMR sample (600 μl) was prepared in a 5 mm NMR tube without dilution of the sample. The phospholipid concentration of the final liposomal dispersions was about 40 mg/ml, and the total L-carnosine concentration of the final

liposomal dispersions was 50 mM. The vesicles were lysed completely by addition of 20 μ l of Triton X-100 (1%) to the NMR sample tube for release the encapsulated L-carnosine (Zhang, Patel, De Graaf, & Behar, 2004). The pH values of the liposome suspension were measured directly in the NMR tube using a pH-microelectrode.

The NMR experiments were performed at a magnetic field strength of 14.1 T (AVIII-600 spectrometer, Bruker Biospin GmbH, Karlsruhe, Germany) and at 25 °C (298 K). Proton NMR spectra were acquired with the following acquisition parameters: number of scans: 16, number of points: 65536, relaxation delay time: 15 s, 90° pulse length: 12.5 μ s. Standard precautions for quantitative experiments were taken (e.g. fully-relaxed, same experimental parameters for each experiment such as pulse length, receiver gain). The sensitivity of the NMR measurement to detect the entrapped carnosine was estimated by calculating the root-mean-square (RMS) signal-to-noise (S/N) ratio defined as $2.5 \times S/N_{pp}$, where S is the peak height of the entrapped species, N_{pp} is the peak to peak noise, and 2.5 is a factor for converting peak-to-peak to RMS noise. For a typical NMR experiment in which the entrapped carnosine concentration was 50 mM, the average S/N_{RMS} was about 396:1 after 16 scans.

Data were acquired and processed using Bruker Topspin Software, supplied by the manufacturer.

2.5. Membrane fluidity

Liposomal membrane fluidity was determined as fluorescence polarisation (P) by measuring the fluorescent intensity of 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), according to the conventional method. The fluorescence probes were oriented into the lipid bilayer by the following method. The solution of TMA-DPH (in ethanol) was added to the liposome suspension to maintain the lipid/probe molar ratio at 250 ([TMA-DPH]_{final} = 4 μ M). The mixture was then incubated for at least 1 h at room temperature with gentle stirring. The fluorescence probe was vertically oriented in the lipid bilayer. The amount of probe remaining in the external aqueous medium was negligible due to high lipophilicity coefficient, and the non-incorporated TMA-DPH was non-fluorescent due to aggregation. The fluorescence intensity of samples was measured with a Perkin-Elmer LS 55B spectrofluorimeter equipped with fluorescence polarisers (Perkin-Elmer, Waltham, USA). Samples were excited at 360 nm, and emission was registered at 430 nm under constant stirring at the temperature of 25 °C (PTP-1 temperature controller). The P value of TMA-DPH was calculated from the following Eq. (1):

$$P = \frac{I_{||} - G \cdot I_{\perp}}{I_{||} + 2G \cdot I_{\perp}} \quad (1)$$

where $I_{||}$ is the intensity of fluorescence parallel to excitation plane, I_{\perp} is the intensity of fluorescence perpendicular to excitation plane and G -factor that accounts for transmission efficiency. The reciprocal value of polarisation ($1/P$) was defined as membrane fluidity (Shimanouchi, Ishii, Yoshimoto, Umakoshi, & Kuboi, 2009).

2.6. Phase transition temperature (TC)

Liposome suspension was analysed with Differential Scanning Calorimetry (DSC Netzsch, 204 F1 – Gerätebau GmbH, Germany). Calorimetric scans from –30 to 90 °C were performed on DSC Netzsch at a scanning rate of 5 °C per minute. The software used is Proteus Analysis, version 4.8.5 supplied by the manufacturer (Netzsch-Gerätebau GmbH, Germany) (Taylor & Morris, 1995).

2.7. Lamellarity determination

The average number of bilayers is another important factor which determines the relation between vesicle size and encapsulated volume. Lamellarity of nanoliposomes was determined from Small Angle X-ray Scattering (SAXS). The measurements were performed with SAXSess, (Anton Paar KG, Graz, Austria) equipped with a sealed tube (Cu anode $\lambda = 1.542$ Å). The samples were measured in a quartz capillary (with an outer diameter of 1 mm). The 2D scattering pattern was detected by a CCD camera featured a 2084 \times 2084 pixel array with 24 μ m \times 24 μ m pixel size. via SAXS-Quant software (Anton Paar), the 2D image was integrated into the 1D scattering curves as a function of the magnitude of the scattering vector (q). Data were obtained in the q range of 0.11–6 nm⁻¹ and corrected for the solvent (phosphate buffer solution)-filled capillary scattering. Since the beam incident is linear, desmearing treatment allows correcting the spectra. The experiments were performed at 20 °C (Bunjes & Unruh, 2007; Jousma, Talsma, & Spies, 1987).

2.8. Transmission Electron Microscopy (TEM)

The microstructure of nanovesicles was investigated by TEM with a negative staining method. To do this, the nanoliposome samples were diluted 10-fold with de-ionised water to reduce the concentration of the vesicles. Equal volumes of the diluted sample and an ammonium molybdate solution (2%) were combined and left for 3 min at room temperature. A drop of this solution was placed on a Formvar-carbon coated copper grid (200 mesh, 3 mm diameter HF 36) for 5 min. The excess of liquid was drawn off by using the filter papers. After drying the grid at room temperature for 5 min, micrographs were made using a Philips CM20 Transmission Electron Microscope operating at 200 kV. Micrographs were recorded using an Olympus TEM CCD camera (Colas et al., 2007).

2.9. Atomic Force Microscopy

Atomic Force Microscopy (AFM) has been widely used to study the structure and morphology of nanoliposomes at nanoscale resolution. Most prominently, tapping mode AFM has been applied, providing both, height and phase contrast of nanoliposomes (Krapf, Dezi, Reichstein, Köhler, & Oellerich, 2011). This approach completes the morphological, dimensional and surface investigation of samples applied in the pharmaceutical and medical fields, strengthening the versatility of AFM (Ruozi et al., 2009).

All the topographical imaging was performed using a Dimension 3100 equipped with a Nanoscope V electronic from Veeco manufacturer (Digital Instruments, Veeco, California, USA).

The images were recorded at ambient conditions (25 °C and 50% RH) in tapping mode (IC-AFM). Tap150 tapping mode cantilevers (Veeco model No MPP-12,100 tip diameter \approx 15 nm with a typical spring constant of about 2 N/m and a resonance frequency around 165 kHz was used for scanning. The scan rate was adjusted in the range of 0.5 Hz over a selected area in dimension of 1.2 \times 1.2 μ m.

Tapping force was adjusted by the ratio of the engaged or set-point amplitude (A_{sp}) and free-air amplitude (A_0).

A drop (50 μ l) of diluted liposome suspension (1:25) was deposited on freshly cleaved mica substrates, to avoid dust contamination, incubated in desiccators (over 1 h at room temperature). The excess vesicles were removed by flushing the mica with pure water. The sample was then dried and visualised under AFM.

Acquired images were processed and analysed by using a program supplied by the manufacturer. The height and diameter of nanoliposomes were measured from the profile section of AFM line scans analysing the height images.

2.10. Statistical analysis

The presented results are the averages of five complete and independent experiments. Data were reported as mean \pm SD. One-way ANOVA was employed to identify differences in means, using SPSS software (SPSS for Windows, Rel. 10.0.5, 1999; SPSS Inc., Chicago, IL). Statistical significance was declared at $P < 0.05$.

3. Results and discussion

3.1. Size distribution and ζ -potential determination

Liposome size influences the *in vitro* characteristics of nanocarriers such as drug loading capacity, aggregation and sedimentation. It is also generally acknowledged that the pharmacokinetic behaviour and biodistribution such as blood circulation time, endocytosis, fusion, adsorption on target cell membrane and contact release of the carrier is strongly size-dependent (Hupfeld, Holsaeter, Skar, Frantzen, & Brandl, 2006).

The greatest and smallest mean size and polydispersity index were obtained for nanoliposomes composed of DOPC and DPPC, which contain unsaturated and saturated phospholipids, respectively.

In general, in addition to the effect of extrusion device used for liposome preparation, the process parameters such as extrusion pressure employed, sample flow rate, filter pore sizes, number of passages and inclusion of freeze–thaw cycles as part of the production protocol, also influence liposome size. By increasing the number of passages, liposome size is decreased (Lesieur, Grabielle-Madellmont, Paternostre, & Ollivon, 1991). Size reduction mechanism was suggested to include the rupturing of vesicles and spontaneous rearrangement after membrane passage resulting in the formation of smaller and less lamellar nanoliposomes. It was also observed that higher flow rate and pressure employed in liposome extrusion result in smaller nanoliposomes (Lesieur et al., 1991). During freezing process ice crystals are formed and disrupt the bilayers. Disrupted bilayers reassemble to form newer vesicles and decreasing the liposome size and number of lamellarity (Agashe, Lagisetty, Awasthi, & Awasthi, 2010).

In addition, our findings have shown the most effective factors are employed lipid compositions and lipid concentrations. Extrusion of multilamellar liposome suspension composed of soft membranes such as DOPC represents liposomes with an average size around 125 nm while liposomes composed of rigid membranes such as DPPC represents particles in average size around 116 nm (Table 1). This can be attributed to noticeable difference in liposomal membrane fluidity and their elasticity deformation during passage through pores of polycarbonate filters. In comparison to liposomes composed of DPPC, DOPC and POPC nanoliposomes have permeable membranes with larger fluidity values. These phenomena cause to form large unilamellar liposomes, our finding is in good agreement with the results presented by Calvagno et al. (Calvagno et al., 2007; Nii, Takamura, Mohri, & Ishii, 2003).

ζ -potential results of liposome suspension are represented in Table 1. Liposomal charge is another important characteristic

determining liposome stability and entrapment efficiency which is a function of lipid charge or any adsorbed layer at the interface, and the nature and composition of the medium in which liposome is suspended (Maherani et al., 2011).

The lipids employed, phosphatidylcholine, are neutral lipids in physiological pH ranges, while the DLS results showed weak negatively charged liposome suspensions.

To verify the influence of the L-carnosine dissolved in phosphate buffer solution on liposome ζ -potential, light scattering analysis was carried out in the presence and absence of L-carnosine in liposome suspension. Our findings showed that L-carnosine dissolved in phosphate buffer solution is a negatively charged solution and ζ -potential of solution also depends on its concentration, e.g. ζ -potential of L-carnosine solution at concentrations of (20 mM) and (50 mM) are -10 and -25 mV, respectively.

Also it was found that neutral nanoliposomes loading L-carnosine dissolved in phosphate buffer solution were slightly negatively charged due to charge of L-carnosine that surrounded and encapsulated in nanoliposomes.

3.2. Assessment of encapsulation efficiency by NMR spectroscopy

One of the critical parameters that has to be optimised in the development of a delivery system is the encapsulation efficiency.

Encapsulating a sufficient amount of therapeutic agent is one of the most desirable properties for liposomal use (Lohse, Bolinger, & Stamou, 2008).

The liposomal encapsulation efficiency is defined as the ratio of the encapsulated to the total (encapsulated + non-encapsulated) carnosine.

NMR spectroscopy has become the standard method for determination of bioactive agents loading in liposome. Its application for this purpose is based on the detection of an endogenous acid or base having a resonant frequency (chemical shift) sensitive to the molecule's degree of protonation (Gasparovic et al., 1998).

Carnosine possesses two well-resolved, pH-sensitive imidazole ring proton resonances (H_a and H_b) around the physiological pH range (7.4). Due to the close proximity of the amide proton resonance (8.10 ppm) with the downfield imidazole ring proton (H_a) resonance, all of the liposomal encapsulation efficiency measurements were made by integration of the upfield singlet imidazole ring proton (H_b) resonances for the encapsulated and non-encapsulated carnosine (Fig. 1).

The imidazole ring proton (H_b) of carnosine is sensitive to pH change. As shown in Fig. 2, the larger peak shifted downfield from 6.98 to 7.13 ppm, when the pH value of the liposome suspension (external medium) was changed from 8.0 to 6.8, indicating that the larger peak corresponds to carnosine in the external medium. Thus, the smaller and broader proton resonance (7.03 ppm), which was unaffected by the pH change of the external medium, was attributed to the carnosine encapsulated in the phospholipid vesicle.

It was found that the pH value of the internal medium (encapsulated carnosine) was fixed around the physiological pH value (7.4), and the proton resonances of encapsulated and non-encapsulated carnosine imidazole ring were resolved completely.

Table 1

Particle size, polydispersity index (PDI) and ζ -potential of L-carnosine-loaded nanoliposomes.

Liposome composition	Average size (nm) ^a	PDI ^a	ζ -potential (mv) ^a
DOPC	122.0 \pm 1.6 ^a	0.10 \pm 0.03 ^a	-7.43 ± 1.6^a
POPC	118.4 \pm 1.8 ^{ab}	0.09 \pm 0.04 ^a	-7.19 ± 1.3^a
DPPC	116.2 \pm 1.2 ^{ab}	0.09 \pm 0.02 ^b	-6.98 ± 1.6^a

^a Data were expressed as mean \pm SD ($n = 5$). Data were adjusted to that at 25 °C. Different letters represent significant differences of lipid composition ($P < 0.05$). The mean diameter, particle size distribution and ζ -potential of vesicles were determined using dynamic light scattering technique.

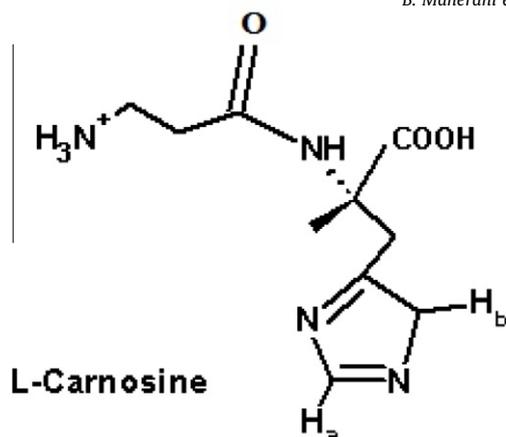


Fig. 1. Schematic of L-carnosine.

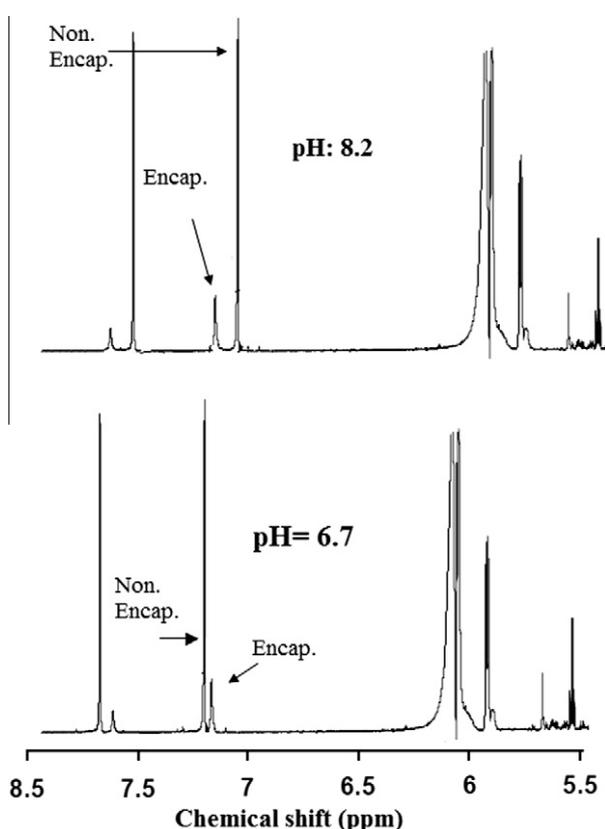


Fig. 2. Dependence of L-carnosine imidazole ring proton resonance (H_b) vs. the pH value of the external media.

Thus, the liposomal encapsulation efficiencies could be determined directly by integration of the resolved proton resonances corresponding to the encapsulated (internal) and non-encapsulated (external) imidazole ring protons.

Furthermore, addition of Triton X-100, to rupture the vesicle membranes, resulted in complete leakage of their contents and was shown as one large peak.

The functional properties of liposomal carriers to encapsulate antioxidants depend on the interaction of antioxidant with both liposome membrane and target site. It has been shown that addition of negatively charged polypeptides antioxidants to liposome composed of cationic phospholipid [dioleoyl trimethyl ammonium propane (DOTAP) or stearylamine] may result in association due to attractive electrostatic interactions, whereas addition to neutral

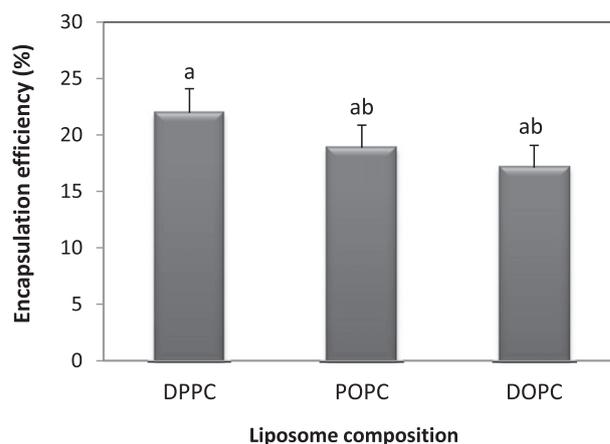


Fig. 3. represents the results of L-carnosine encapsulation efficiency. Data were expressed as mean \pm SD ($n = 5$). Different letters represent significant differences of lipid composition ($P < 0.05$).

nanoliposomes may result in association due to hydrophobic interactions (Were et al., 2003).

Furthermore, factors affecting the encapsulation efficiency in nanoliposomes are different and come from the properties of both nanoliposomes and encapsulated bioactive agent. For example, the encapsulation efficiency is affected by hydrophilic or lipophilic properties of the bioactive agent and its tendency to interact with the membrane bilayer. As concerning liposome properties; aqueous volume, membrane rigidity, surface area and preparation methods are reported to have influenced the encapsulation efficiency (Nii & Ishii, 2005).

L-Carnosine loading efficiency in liposome appears to decrease in the order of DPPC > POPC > DOPC, by value of 21.96%, 18.86% and 17.12%, respectively. The encapsulation efficiency tended to increase as the degree of saturation of the lipid used for the liposome membrane increased (Fig. 3). Membrane fluidity was one of the major factors affecting the encapsulation efficiency of the bioactive agents. Unsaturated phospholipids increased the fluidity of the membrane, which were expected to facilitate the leakage of trapped drugs. Orientation of lipids within the bilayer also might affect packing of nanoliposomes and rigidity. Our results are in good agreement with results obtained by Nii and Ishii (Kulkarni, Betageri, & Singh, 1995; Nii & Ishii, 2005).

3.3. T_c and fluidity values determination

3.3.1. T_c

For first-order phase transitions, such as the bilayer gel to liquid crystalline transition, T_c can be directly obtained by a DSC thermogram as the maximum of the excess heat capacity function (Taylor & Morris, 1995).

Results of DSC have shown that T_c is lowered by decreased chain length and degree of unsaturation of the acyl chains (Table 2).

Additionally, phase transition temperature of liposomes depends not only on the nature of hydrocarbon chains but also on the polar region of the molecule, nature and ionic strength of the suspension medium and the position of the *cis*-double bond (Taylor & Morris, 1995).

3.3.1.1. Membrane fluidity value. Bilayer fluidity reflects the order and dynamics of phospholipid alkyl chains in the bilayer. The release of the entrapped bioactive agents from nanoliposomes depends on the number of bilayers and permeability and fluidity of the bilayer (Calvagno et al., 2007).

Table 2
Relation between transition temperature and fluidity of liposome.

Lipid	T_c^a (°C)	$(1/P)_{TMA-DPH}^{(-)b}$
DOPC	-18.9 ± 1.2^a	3.35 ± 0.4^a
POPC	0.3 ± 0.7^b	2.81 ± 0.2^{ab}
DPPC	39.8 ± 1.9^c	2.44 ± 0.3^{ab}

^a T_c means the gel-to-liquid crystalline transition temperature. Data were expressed as mean \pm SD ($n = 5$). Different letters represent significant differences ($P < 0.05$).

^b All experiments were carried out at 25 °C.

The comparison of fluidity values of DOPC (two C=C bonds), POPC (one C=C bond), and DPPC (zero C=C bond) shows the noticeable role of double bonds and degree of saturation in lipids (Table 2). Fluidity values of nanoliposomes appear to increase in the order of DPPC < POPC < DOPC. The presence of double bonds within the acyl chain resulted in a decrease in the packing density and chain ordering in lipid bilayers and consequently increasing the nanoliposome fluidity.

It was found that the liposome fluidity depends on temperature. By increasing temperature, bilayer fluidity values also increase, e.g. liposome composed of DPPC, has fluidity value of 2.44 $(1/P)_{TMA-DPH}$ and 2.85 $(1/P)_{TMA-DPH}$ at 25 and 45 °C, respectively.

The results have also indicated that incorporation of some fluid lipids into liposome bilayer could interfere with the barrier function and thus lower its T_c and increase its fluidity. These data correspond well with the previous findings (Shimanouchi et al., 2009).

3.4. Lamellarity analysis and TEM observation

SAXS plays a prominent role in the characterisation of nanoliposomes. The method is accurate, nondestructive, and usually requires only a small volume of sample preparation (Luykx, Peters, Van Ruth, & Bouwmeester, 2008).

In order to estimate the average number of bilayers in the vesicles, small-angle X-ray scattering experiments for three types of liposome suspensions were recorded. All the SAXS patterns of the liposome suspensions demonstrated the same profile (Fig. 4).

Scattering intensity $I(q)$ can be represented as the product of the form factor $P(q)$ and the structure factor $S(q)$, where q is the length of the scattering vector, given by Eq. (2).

$$q = (4\pi \sin \theta) / \lambda \quad (2)$$

where λ and θ are wavelength and scattering angle, respectively. The arrangement of multi-lamellar bilayers is represented by $S(q)$

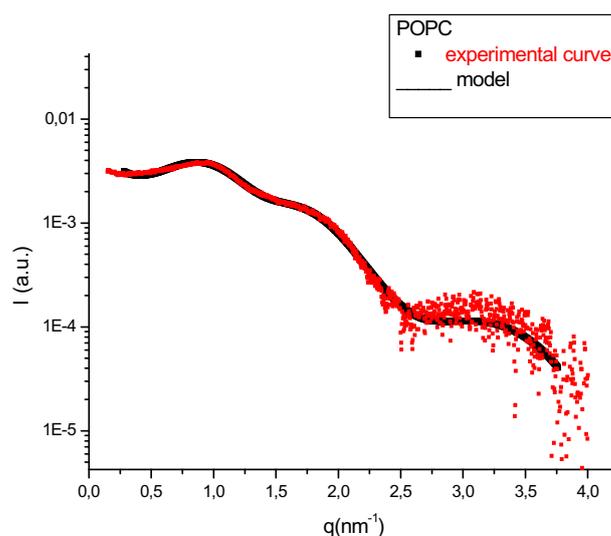


Fig. 4. SAXS-curve and best fit of POPC (symbol).

and $P(q)$ corresponding to the bilayer structure (electron density profile).

The data were analysed with the generalised indirect Fourier transformation method elaborated by Fruhwirth, Fritz, Freiburger, and Glatter (2004).

The best fit obtained with this method for the nanoliposomes prepared with POPC is shown in Fig. 4. It was determined that nanoliposomes formed well-ordered bilayers with a lamellar spacing of $d = 5\text{--}6$ nm at 20 °C.

Furthermore, the obtained TEM images confirmed that extruded vesicles are in the form of large unilamellar vesicles. The bilayer nature of the vesicles is clearly visible in these micrographs confirming that the prepared lipid vesicles are liposome (defined as closed continuous bilayer structures) (Fig. 5).

3.5. AFM studies

Atomic Force Microscopy (AFM) was successfully applied to evaluate the morphological and technological properties of nanoliposomes. To investigate soft samples such as nanoliposomes, two types of operating dynamical modes are currently used; intermittent contact mode (IC-AFM), tapping mode, and contact mode (Liang, Mao, & Ng, 2004a). To evaluate the surface properties and

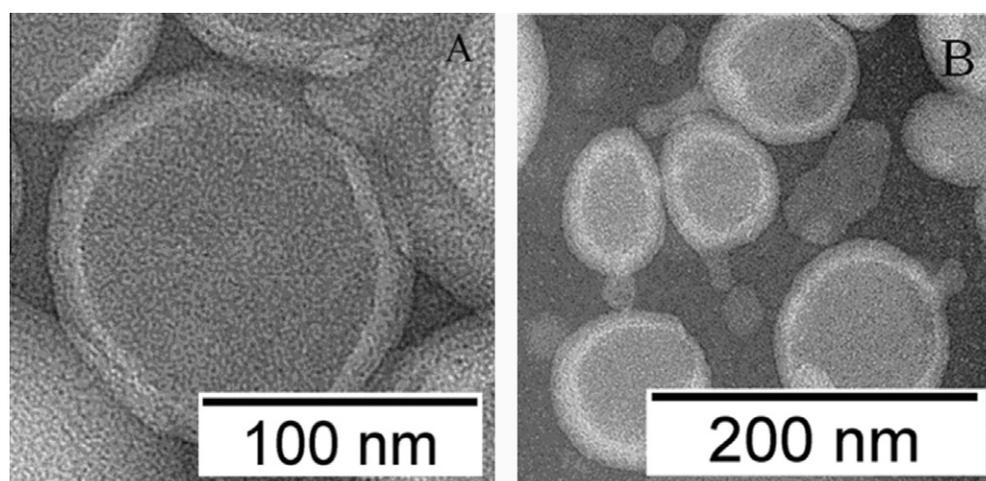


Fig. 5. Transmission electron micrographs of L-carnosine-encapsulated nanoliposomes prepared by the extrusion technique.

Table 3
AFM and DLS data of nanoliposomes.

Liposome composition	Average size (nm) ^a	
	DLS measurements	AFM measurements
DOPC	122.0 ± 1.6 ^a	154 ± 11.1 ^a
POPC	118.4 ± 1.8 ^{ab}	137 ± 9.2 ^{ab}
DPPC	116.2 ± 1.2 ^{ab}	129 ± 8.9 ^{bc}

^a Data were expressed as mean ± SD ($n = 5$). Data were adjusted to that at 25 °C. Different letters represent significant differences of lipid composition ($P < 0.05$).

to complete the characterisation, the tapping mode AFM approach was commonly used (Ruozi et al., 2009).

In this context, soft IC-AFM was used to directly image and measure the size and height of adsorbed liposomes onto a surface (mica).

The nanoliposomes size (diameter) was compared with the height measured by sectional cross sections obtained by IC-AFM on different areas of specimen and the DLS data.

By operating in soft tapping mode, liposome diameters remain comparable to the heights of the vesicles and in a spherical defined shape within the first minutes after sample preparation on mica. The AFM data were almost in agreement with DLS ones but we observed a little difference between the results (Table 3). For instance, AFM size measurements of liposome composed with DOPC revealed an average diameter of 154 ± 2 nm which is larger than the value of 122.0 ± 1.6 nm for the same vesicles obtained through the DLS measurements.

It is worth noting that most of IC-AFM studies on liposomes, reported noticeable differences between these two techniques (Colas et al., 2007; Liang, Mao, & Ng, 2004b). As Colas et al., have obtained an average diameter of 321 ± 9.6 nm with AFM analyses cross sectional analysis which is larger than the value of 195 ± 2.8 nm for

the same vesicles obtained through the light scattering measurement technique (Colas et al., 2007).

This can be due to difference in liposome composition and typical forces used for AFM studies. Typical forces of 2 nN used in our AFM studies provided a very soft tapping mode compared to ≈ 20 nN typical forces used in the past studies (Colas et al., 2007; Liang et al., 2004b).

It also should be considered that in the AFM analysis, nanoliposomes are adsorbed on solid surface while the DLS technique evaluates nanoliposomes in suspension. The interaction between the sample and the substrate, as well as the continuous movement of the tip, can also be able to induce a liposome deformation and consequently difference between the results of two techniques (Nakano, Tozuka, Yamamoto, Kawashima, & Takeuchi, 2008; Ruozi, Tosi, Leo, & Vandelli, 2007).

However, by considering different images of liposomes, no significant changes in liposome shape were observed. It suggests that liposome deformation mainly could be attributed to the specimen/substrate interaction.

A few minutes after preparation, morphological and dimensional characteristics of the vesicles begin to change. Nanoliposomes showed a progressive tendency to turn into an asymmetrical and flattened structure (Fig. 6A and B).

It is well-known that adsorption of liposome on solid substrate generally modifies its shape. Furthermore, evaporation of medium suspending liposome causes the nanoliposomes to collapse. Nanoliposomes with different compositions have a different tendency to change their structure after mica adsorption: the elastic properties of the lipidic composition influence the interaction with the tip (Ruozi et al., 2007).

In order to correctly evaluate the shape and to measure the size of the adsorbed vesicles, it is necessary to consider different factors such as the type of liposomes, the time of analysis, method of

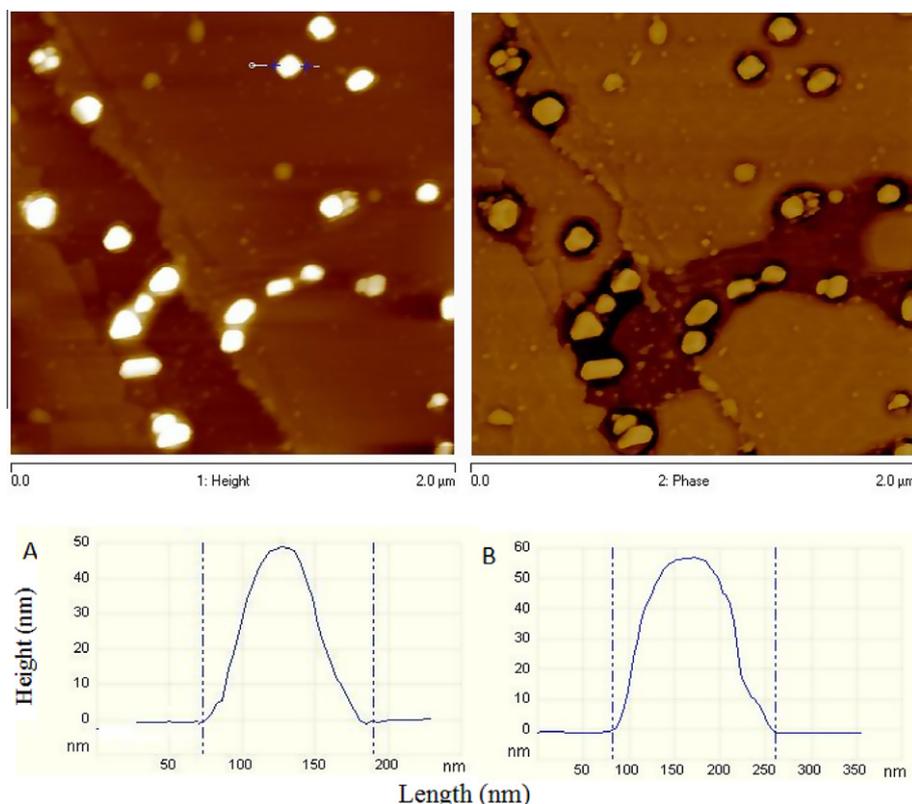


Fig. 6. AFM images (height and phase) of l-carnosine encapsulated DPPC liposome 30 min after sample preparation. Profiles of liposome (DPPC); (A and B) Immediately and 30 min after sample preparation, respectively.

sample preparation for AFM analysis as well as the vertical and lateral forces applied by the tip on the liposomes during the scanning procedure (Ruozi et al., 2007). Other findings have been also shown that by using tapping mode and operating in aqueous solution, nanoliposomes absorbed on to the mica surface can be less changed. As, tapping mode is less likely to damage the sample than the contact mode because of elimination of the lateral forces (friction or drag) between the sample and the tip and also because liposomes tend to flatten during drying. The drying process is also due to liposome deformation (Krapf et al., 2011; Ruozi et al., 2009).

Fig. 6 presents profiles of DPPC liposomes containing L-carnosine. The height image of samples showed separated and defined vesicles. The diameter of vesicles corresponds to the width of the peak at the base of graphic. It was observed that the average height and width of individual surface – bound nanoliposomes were changed after deposition of nanoliposomes on mica surface. Nanoliposomes with hard membrane such as DPPC showed the highest value after analysing height images. The average height of individual surface – bound DPPC liposome was 48 ± 2 nm (Fig. 6A). This phenomenon could be probably due to high phase transition temperature and low fluidity of this membrane. As, liposome composed of DPPC with highest transition temperature exists as gel state at room temperature, showed highest value. Our findings are in good agreement with the results of Nakano et al. (2008). They also confirmed that liposomes with soft membranes composed of unsaturated lipids and high fluidity values tended to collapse by interaction with the mica surface. Additionally, liposomes composed with the phospholipid having the highest phase transition temperature showed the highest value in IC-AFM (Nakano et al., 2008; Ruozi et al., 2009).

The IC-AFM analysis both height mode and phase mode are commonly used to describe the morphology of our liposomes and to confirm the data of TEM. Moreover, it is well-known that the phase images in IC-AFM are affected by the tip-sample adhesion, the elasticity of materials and the viscosity of surface which can be related, in this case, to the composition and hydrophilicity/hydrophobicity properties of liposomes surfaces (Dong & Yu, 2003; Ruozi et al., 2009).

In fact in most cases, softer materials lead to a larger contact area and to a longer duration tip-sample contact than on a hard material. Some researchers introduced the surface properties of samples by using Young's modulus. They described that larger, brighter positive phase in phase image represents stiffer surface with a higher elastic modulus but dark and negative phase represents softer surface (Leclère et al., 1996; Schön et al., 2011).

By considering the phase image, it was concluded that the dark phase corresponds to the liposome membrane. Since liposomes comprised of a dark frame have higher viscous/attractive forces between tip and sample and also softer surface. It was also observed that the clear/brighter part corresponds to the L-carnosine encapsulated in liposome (Fig. 6). Our findings were verified by estimating the size of these phases. It was found that IC-AFM findings are in good agreement with the results of SAXS and TEM.

It was estimated that the lamellar spacing of nanoliposomes by a value of ≈ 8 –9 nm which is comparable with the results of SAXS.

These findings will be useful for completing the morphological investigation and surface properties evaluation of different liposomes applied in the pharmaceutical, biological and medical fields as well as food systems.

4. Conclusion

In this study, three different phospholipids used for preparation of L-carnosine loaded liposome. The greatest and smallest mean size and polydispersity index were obtained for nanoliposomes

composed of DOPC and DPPC. This can be attributed to noticeable difference in liposome membrane fluidity and their elasticity deformation.

It was determined that encapsulation efficiency tended to increase by augmentation of the saturation degree of the lipids use in liposome membrane. Membrane fluidity was also one of the major factors affecting the encapsulation efficiency of the bioactive agents.

It was observed that the presence of double bonds within acyl chain, packing density and chain ordering in lipid bilayers influence T_c and fluidity value of nanoliposomes.

Additionally, the tapping mode AFM approach was used to describe the variation of the phase during the cantilever oscillation. As, liposomes comprised of a dark frame, have shown higher viscosity values with softer surface and brighter phase with stiffer surface, having a higher elastic modulus.

Despite the wide range of encapsulated products that have been successfully manufactured and developed in pharmaceutical, biological and cosmetics industries, nanoencapsulation is found comparatively much smaller in food industry. Evaluation and optimisation of liposome composition can make it possible to deliver bioactive agents directly to the target site and reducing systemic side effects. From this point of view, handling liposome composition and evaluating its stability, can be an interesting approach to optimise liposome formulation. The AFM findings could be useful for completing the morphological, technological and evaluation of surface properties of different liposomes applied in the pharmaceutical, biological and food systems.

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Conclusion

Dans cette étude, trois phospholipides différents ont été utilisés pour encapsuler de la L-carnosine dans des liposomes. La taille optimale moyenne et l'indice de polydispersité minimal ont été obtenus pour des liposomes composés de DOPC et DPPC. Cela peut être attribué à la fluidité et à la déformation élastique différente de la membrane du liposome en fonction de la composition lipidique. Il a été montré que l'efficacité d'encapsulation tend à croître avec l'augmentation du degré de saturation des lipides utilisés dans la formulation de la membrane des liposomes. La fluidité de la membrane est un des facteurs importants qui affectent l'efficacité d'encapsulation de molécules bioactives.

La présence de doubles liaisons, augmentant la densité et l'arrangement des chaînes acylées de la membrane lipidique, influence la température de transition de phase (T_c) et les valeurs de fluidité des nanoliposomes. De plus, l'approche par Microscopie à Force Atomique permet de révéler la morphologie de surface des liposomes durant l'oscillation du bras de levier. En mode Tapping, les variations d'amplitude d'oscillation en contact avec la surface de la membrane du liposome (image foncée) ont montré des valeurs de viscosité plus élevée avec une surface

Malgré le large éventail de produits encapsulés développés dans les industries pharmaceutiques, et cosmétiques, les produits nano-encapsulés trouvent des applications plus restreintes dans le secteur alimentaire. La formulation et l'optimisation de la composition de ces vecteurs permet de délivrer des molécules d'intérêt vers des tissus ciblés tout en limitant les effets secondaires systémiques. Il est de ce fait important d'évaluer la stabilité des liposomes en fonction de leur composition lipidique pour optimiser la formulation. Les résultats obtenus par Microscopie à force Atomique permettent d'évaluer les propriétés et caractériser la morphologie de surface de liposomes utilisés dans les domaines pharmaceutique, biologique et alimentaire.

III. Résultats & Discussions

Chapitre III.II: Interaction moléculaire de la calcéine avec les membranes lipidiques

Objectives

The goal of bioactive delivery system is also to administer a drug at a therapeutic concentration to a particular site of action for a specified period of time. The design of the final product for drug delivery depends upon different parameters. The drug must be administered by considering to some factors which effects on therapeutic action of the drug such as the initial release rate of the drug for controlled release systems. The drug must also remain physically and chemically stable in the formulation for a defined time. So, one of the most noticeable factors in release profiles is the strength of the drug-carrier interaction. To adjust the pharmacokinetic / nutraceutical properties of molecule agents, it is necessary to optimize the drug-carrier interaction.

To get a better understanding of calcein / lipid membrane, the thermodynamic changes caused by calcein and its location in lipid bilayers were determined was studied by using DSC, Raman spectroscopy and Langmuir film balance.

***Etude sur l'interaction moléculaire entre calcéine et des membranes lipidiques
modèles par spectroscopie Raman, Balance de Langmuir et
la calorimétrie différentielle à balayage***

Résumé :

Les liposomes sont couramment utilisés comme vecteur permettant une libération contrôlée de principes actifs. Les propriétés pharmacocinétiques et pharmacodynamiques dépendent fortement des forces d'interaction entre la biomolécule et le liposome. Afin d'approfondir les connaissances sur la nature de ces interactions, des liposomes unilamellaires ont été préparés en utilisant différentes formulations de phospholipides (1,2-dioléoyl-sn-glycéro-3-phosphocholine, 1-palmitoyl-2-oléoyl-sn-glycéro-3-phosphocholine, 1,2-palmitoyl-sn-glycéro-3-phosphocholine). La calcéine a été choisie comme molécule polaire à vectoriser. Les modifications thermodynamiques induites par la présence de ce marqueur dans la bicouche lipidique ont été étudiées par analyse thermique différentielle et spectroscopie Raman. Les résultats obtenus montrent que cette molécule hydrophobe modifie les propriétés thermotropes des lipides membranaires, en modifiant la température de transition de phase (T_c). Les variations d'intensité des signaux Raman nous renseignent sur les interactions entre la calcéine et les groupements cholines des phospholipides. La molécule peut perturber l'intégrité de la membrane en s'intercalant entre les chaînes acylées et ces résultats sont d'autant plus marqués pour des liposomes composés de lipides insaturés. Une étude de pression de surface a permis de caractériser l'influence de la calcéine sur une monocouche phospholipidique à l'aide d'une balance de Langmuir. Les isothermes de compression ont été couplées à la spectroscopie infrarouge et la microscopie à angle de Brewster. Les résultats obtenus indiquent que la calcéine a un effet plus marqué avec les systèmes lipidiques purs qu'en présence d'un mélange de phospholipides.

**Investigation of molecular interaction between calcein and lipid model membranes
by Raman spectroscopy, Langmuir balance study and Differential scanning calorimetry**

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Abstract

Liposomes are commonly used as a carrier in controlled release drug delivery systems. Controlled release formulations can be used to reduce the amount of drug necessary to cause the same therapeutic effect in patients. One of the most noticeable factors in release profiles is the strength of the drug-carrier interaction. To adjust the pharmacokinetic and pharmacodynamic properties of therapeutic agents, it is necessary to optimize the drug-carrier interaction.

To get a better understanding of this interaction, large unilamellar liposomes containing calcein were prepared using 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-choline and 1,2-palmitoyl-sn-glycero-3-phosphocholine, and a mixture of them; calcein was chosen as a model polar molecule.

The thermodynamic changes caused by calcein and its location in lipid bilayers were determined by Differential Scanning Calorimetry and Raman spectroscopy.

The results reveal that calcein influences the thermotropic properties of the lipid membrane, causing abolition of the pretransition and a negligible decrease or increase in the phase transition temperature. The change in intensity of the Raman peaks represents the interaction of calcein with choline head groups. The results indicated that calcein maybe could intercalate into the acyl chains and disturb the chain order. Indeed, this augmentation is more visible in liposomes composed of unsaturated lipids.

Moreover, the impact of calcein on phosphoglyceride Langmuir layers spread at the air-water interface was studied using surface pressure-area and surface potential-area isotherms, as well as polarization-modulation infrared reflection-absorption spectroscopy and Brewster angle microscopy. The results obtained indicate that, while calcein has a meaningful effect on the systems prepared with pure lipids.

Keywords: hydrophilic drugs, mechanical properties, compressibility modulus, methylene scissoring, ,thermodynamic changes.

Introduction

Currently, liposomes and nanoliposomes are being used successfully as models of biomembranes and also as delivery and controlled release systems for drugs, diagnostics, nutraceuticals, minerals, food material and cosmetics [1]. Due to the extra-ordinary success of liposome technology in many fields, both in research and industry, several liposome-derived systems have been developed in recent years [1].

Liposomes can increase the therapeutic effectiveness of the encapsulated drugs and decrease their toxicity. They are widely accepted as drug delivery systems. Particularly, nanoliposomes are considered as promising carriers, especially in the case of bioactive agents, cosmetics and nutraceuticals [2].

The goal of a drug delivery system is to administer a drug at a therapeutic concentration to a particular site of action for a specified period of time. The design of the final product for drug delivery depends on different parameters: a) The drug must be administered by considering factors which affect the therapeutic action of the drug. These parameters include the site of action, the concentration of the drug at the time of administration, the period of time that the drug must remain at a therapeutic concentration, and the initial release rate of the drug for controlled release systems, b) The drug must remain physically and chemically stable in the formulation for a defined period of time, c) The choice of delivery method must indicate the effective administration route for the drug [3]. The properties of the liposomes vary substantially with composition, size, surface charge and preparation method. It is obvious that the design and development of drug carriers is a difficult issue because they have to behave as biocolloidal systems after being administered [4].

Physicochemical properties of drugs are also a critical subject in the design of the delivery systems [3]. The bioactive agents are sequestered within the aqueous interior compartment of a liposome, either partly or fully trapped between the fatty acyl chains of a liposome [5].

Calvagno *et al.* (2007) have determined that one of the noticeable factors in the release profiles of drugs is the strength of the drug-liposomal lipid interaction, i.e. a strong interaction causes less desorption, thus causing the burst effect [6].

From a molecular point of view, bioactive substances able to insert themselves or become entrapped in the liposomal bilayer can alter the shape, size distribution and chemical properties of a liposome [7].

Additionally, the localization of bioactive substances within the bilayer is also a question of great importance for determining their interaction with liposomes [7].

Interactions of bioactive compounds with model lipid bilayers could provoke changes in their thermotropic behaviour as well as in their conformation properties. These effects were taken into account in the design of liposomal formulations as drug controlled release delivery systems. Also, by understanding the signalling and interaction between the bioactive substances and liposomes, it is possible to mimic biological systems [4],[8].

To this purpose, liposomes prepared in the presence of bioactive substrates sitting closer to the polar head, or even inserted into the hydrophobic moieties of the bilayer, were studied by DSC and Raman spectroscopy to specify the location of the bioactive compound in the lipid bilayer [8].

Differential scanning calorimetry (DSC) has been proved as a valuable tool for studying the interaction of bioactive compounds with model lipid bilayers. It has been considered as a sensitive tool in the exploration of the thermodynamic lipid phase transition. DSC measures the thermal changes on the lipid bilayers and has been extensively used in studying the molecular interactions of bioactive compounds with model lipid bilayers [9],[10].

Raman spectroscopy can also be used as a tool to examine localized interactions, by studying the behaviour of the vibrational stretching modes of each group in the lipid membrane [7].

Furthermore, measurements on monolayers by means of a Langmuir balance have been performed in order to obtain the necessary information on the area occupied by the bioactive compounds on the surface of the monolayer and, hence, on their conformation. This study was conducted to determine if the dissolved bioactive compound interacts with lipid monolayers or whether it penetrates into the liposomal bilayer [11],[12].

More information is available for small, lipophilic substances which are able to penetrate deeply into the hydrophobic bilayer [4],[7],[8]. However, there are no noteworthy studies about the hydrophilic substances which might interact with polar head group of the bilayer membrane.

In this work, we studied the molecular effect of calcein on a lipid model membrane composed of DOPC, POPC and DPPC and mixtures of them by using DSC, Raman spectroscopy and Langmuir balance in order to contribute to the knowledge of designing and optimizing model drug delivery systems. DSC was used to measure the thermal changes in the lipid bilayers that were caused by calcein. Raman spectroscopy was used to investigate the location of the bioactive compound in the lipid bilayers. Additionally, measurements on monolayers were performed by a Langmuir balance in order to get information on the area occupied by the calcein on the surface of the monolayer. In this study, interactions between model membranes

and calcein as a model of polar drug were investigated by comparing the behaviour of pure 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and mixtures of them. The monolayers were studied using surface pressure and potential measurements, Brewster angle microscopy (BAM) and polarization modulation infrared reflection-absorption spectrometry (PM-IRRAS).

2- Materials and Methods

2.1. Materials

The phospholipids used in this study were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), all purchased from Avanti Polar Lipids (Alabaster, AL, USA). 3,3-bis[N,N- bis(carboxymethyl)- aminomethyl] fluorescein (calcein) was acquired from Invitrogen (France), Sodium hydroxide, Hydrogen chloride were from Sigma–Aldrich (France). Pure water (Millipore Milli-Q system, 18 M Ω cm), surface tension (γ) of 72.8 mN/m at 20 °C, was used for preparing the calcein solution. All other reagents of analytical grade were obtained from Sigma–Aldrich (France).

2.2. Liposome Preparation

Large unilamellar vesicles (LUVs) were prepared as follows. In brief, phospholipids were dissolved in a chloroform solution. The organic solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under vacuum overnight, was hydrated with calcein solution to obtain multilamellar vesicles. Calcein was dissolved in pure water (pH adjusted to 7.4 by 1 M NaOH, 100 mM HCl) to obtain a final calcein concentration of 10 mM. The calcein concentration of 10 mM was kept constant throughout the study unless otherwise stated. The suspension was subjected to 5 cycles of freezing and thawing to obtain Multilamellar Vesicles (MLVs) and then extruded through a polycarbonate filter (100-nm pore size filter, 11 times) at a temperature above that for the phase transition of the vesicles by using an Avanti-mini extruder (Avanti Polar Lipids, Alabaster, USA) [13] [14]. Liposome suspension for Raman spectroscopy analysis was eluted through a Sephadex-G75 column (10 mm \times 200 mm) which was thoroughly pre-equilibrated with PBS to remove the non-encapsulated calcein. The lipid concentration in the final vesicle suspension was determined

with an enzymatic assay kit (Test Wako-C) from Wako Pure Chemical Co. Ltd. (Osaka, Japan).

2.3. Raman Spectroscopy

High-frequency Raman spectra were recorded with a Thermo Scientific, Smart DXR Fourier transform spectrometer. The spectra were analyzed by *OMNIC software (version 7.0)* provided by *Thermo Fisher Scientific*. A diode laser at 780 nm was used as an excitation source and the spectra were obtained at 4 cm^{-1} resolution from 3400 to 50 cm^{-1} with interval 5 cm^{-1} . The laser power was constant at 10 mW during the experiments. Quantities of 10–12 mg (total weight) were used for the Raman Spectroscopy. All the experiments were held at $20 \text{ }^\circ\text{C}$.

2.4. Compression Isotherms and Brewster Angle Microscopy

The surface pressure and electric surface potential measurements were carried out with a KSV 5000 Langmuir balance (KSV Instruments Ltd, Helsinki, Finland). A Teflon trough [58 cm (length, l) \times 15 cm (width, w) \times 1 cm (depth, d)] with two hydrophilic Delrin barriers (symmetric compression) was used in compression isotherm experiments. The system was equipped with an electrobalance and a platinum willhelmy plate (perimeter 3.94 cm) as surface pressure sensor. Surface potential was measured using a KSV Spot 1 with a vibrating plate electrode and a steel counter electrode immersed in the subphase. Temperature was kept constant at $20 \text{ }^\circ\text{C}$. Aqueous subphases for monolayer experiments were prepared with MilliQ water, which had a surface tension of 72.8 mN m^{-1} at $20 \text{ }^\circ\text{C}$, pH 5.6. Concentration of calcein in the subphases was 10 mgL^{-1} . Monolayers were spread from calibrated phospholipid solutions (concentration around 0.5 mg mL^{-1}) in pure chloroform using a microsyringe (Hamilton Co., USA). All solvents used for cleaning the trough and the barriers were of analytical grade.

The compression isotherms allowed calculating the compressibility modulus (C_S^{-1} ; $C_S^{-1} = -A(\partial\Pi/\partial A)_T$) [15]. The collapse parameters ΔV_{coll} , Π_{coll} , and A_{coll} , as well as the parameters corresponding to surface pressure of 15 and 30 mN m^{-1} , were determined directly from the compression isotherms.

The morphology of the films was visualized using a computer-interfaced KSV 2000 Langmuir balance combined with a Brewster angle microscope (KSV Optrel BAM 300,

Helsinki, Finland). The Teflon trough dimensions were 58 cm (l) × 6.5 cm (w) × 1 cm (d); other experimental conditions were as described above.

2.5. Polarization-Modulation Infrared Reflection-Absorption Spectroscopy (PM-IRRAS)

The PM-IRRAS spectra of phospholipid monolayers spread on pure water or on aqueous solutions of calcein were registered at 20 °C. The Teflon trough dimensions were 36.5 cm (l) × 7.5 cm (w) × 0.5 cm (d); other experimental conditions were as described in the preceding paragraph. The PM-IRRAS measurements were performed using a KSV PMI 550 instrument (KSV Instruments Ltd, Helsinki, Finland) [16].

2.6. Differential scanning calorimetry

Samples containing calcein-loaded liposome were prepared in 40 µl crucibles hermetically sealed with lid. All samples were scanned two or three times (from - 30 to 60 °C) until identical thermograms were obtained using a TA Instrument Q200 DSC, coupled with a refrigerated cooling system 90 with a scanning rate of 5 °C/min. An empty crucible was used as a reference sample and the temperature scale of the calorimeter was calibrated using indium (melting temperature, $T_m = 156.6$ °C). A quantity of ≈ 10 mg (total weight) was used for the DSC measurements.

3. Results and Discussion

3.1. Raman spectroscopy

When small molecules are encapsulated or loaded into an aqueous lipid bilayer system, penetration or interaction of these molecules into the lipid bilayer may be expected, resulting in conformational changes (*trans/gauche*) of the alkyl chains and in changes of the spectral range characterizing the polar head group[17].

Raman spectroscopy is a spectroscopic technique used to study vibrational, rotational, and other low-frequency modes in a system [18]. In general, three spectral regions are often used to describe structural changes of lipids: a) The spectral changes in the hydrocarbon chain C-H stretching mode region, 2800–3100 cm^{-1} , present information about the intrachain interactions between the alkyl chains of the phospholipids and thus about their conformation. By determining the symmetrical and asymmetrical vibrational stretching mode of methylene groups at 2850 cm^{-1} , and 2880 cm^{-1} and calculating the disorder/order parameter, the chain

order in the bilayer can be determined. It also shows the peak at 2935 cm^{-1} , which is attributed to the symmetrical vibration resulting from the C-H bond stretching in the final methyl group of the alkyl chain [19],[20].

b) The C-C stretching mode region, spectral region $1000\text{--}1200\text{ cm}^{-1}$, reflects intramolecular *trans/ gauche* conformational changes within the alkyl chains of the phospholipids. The bands at 1063 cm^{-1} and 1128 cm^{-1} are assigned to the *trans* conformation of C-C bonds while that at 1095 cm^{-1} has been attributed to the *gauche* one [17]. The population ratio of *trans/ gauche* conformation changes were noticeable during the phase transitions. This change can be monitored by calculating the intensity ratios of the *trans* and *gauche* bands (I_{1063}/I_{1095} and I_{1128}/I_{1095}) of the skeletal optical modes [18, 21, 22]. They provide information about the acyl chain conformational states. The methylene scissoring mode in the $1400\text{--}1500\text{ cm}^{-1}$ spectral region has also been recently used to indicate changes in the lipid-chain lateral packing characteristics of DPPG (1,2-dipalmitoyl-phosphatidylglycerol) [23], [24].

The Raman intensity ratios $I_{2850/2880}$ and $I_{1095/1128}\text{ cm}^{-1}$ reflect the degree of the fluidity of the lipid bilayer and the peak height intensity ratios of the bands at 2935 and 2880 cm^{-1} , or 2850 and 2880 cm^{-1} represent the interchain and intrachain order-disorder processes in the bilayer alkyl chains during phase transition temperature [25].

c) Spectral region $700\text{--}800\text{ cm}^{-1}$ with the peak at 716 cm^{-1} , represents the stretching vibration of the C-N bond of the choline group [4, 8].

To follow the structural changes of liposomes in the presence of calcein, the vibrational bands of acyl chains as well as of the head group were analyzed.

The following vibrational modes of Raman spectroscopy were selected to characterize the structural changes of liposomes: (i) one band from the head group assigned to the C-N stretching mode at 716 cm^{-1} , (ii) the second band is one of the skeletal optical modes at 1128 cm^{-1} assigned to the *trans* conformation state and another at 1095 cm^{-1} assigned to the *gauche* conformation state (iii) the methylene scissoring mode at 1440 cm^{-1} , and (iv) the symmetrical and asymmetrical methylene stretching bands at 2844 cm^{-1} and 2880 cm^{-1} [8, 18, 19], [20].

Calcein is a water soluble, fluorescent and self-quenching probe that is *widely used* in studies of cell viability and mitochondrial function by microscopy fluorescence imaging. It was first prepared by Diehl and Ellingboe through the interaction of fluorescein, formaldehyde, and iminodiacetic acid [26]. Its structure was determined by Wallach *et al.* [27] and is shown in

Figure 1. It can be considered chemically as a combination of fluorescein and ethylene diaminetetracetic acid and shares the properties of both of these compounds.

Considering the interactions of drugs with liposomes, they can be grouped into three categories; i) the very strong hydrophilic drugs, which are localized in the aqueous medium of liposomes and if they have a very high PSA, can display some interaction with the head groups, ii) the less hydrophilic drugs, more balanced molecules that adsorb at the water–lipid bilayer interface with some degree of penetration into the bilayer, and iii) the strongly lipophilic drugs, which locate in the bilayer itself [28]. Calcein was chosen as hydrophilic marker which notably used as a model for interactions drug / liposome [29].

In this study, calcein was used as a model of hydrophilic drug because of its stability and fluorescence properties, to which lipid membranes were essentially impermeable. As, a large part of drugs are weak acids or weak bases, the calcein selected as a model of hydrophilic drug which is also negatively charged between pH 5.4 and 9.2 and shows about 3.5 negative charges per molecule at pH 7.4. As, the zeta potential of the calcein-encapsulated liposome suspension fully depends on the calcein concentration. However, the light scattering analysis of the liposome suspension in the presence and absence of calcein showed that calcein had no effect on liposome size [30].

Calcein has hydrophobic regions as well as highly charged regions which will interact differently with the lipid polar head groups and the alkyl side chains. The pure calcein spectrum exhibits two characteristic areas from which useful information can be derived about the interaction of the molecule with lipid bilayers.

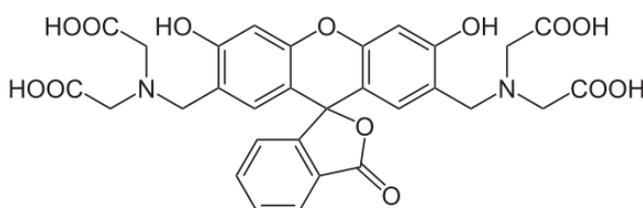


Figure 1. Schematic of Calcein.

Some common Raman signals were observed for the calcein solution, such as the C=C ring mode around spectral region 1620-1670 cm^{-1} . The carboxylate moieties, typical C=O stretching features were clearly detected at about 1650–1700 cm^{-1} .

All experiments were realized at 20 °C in order to assess the change of the interaction between the different liposome and calcein.

The peak height intensity ratios $I_{2935/2880}$, $I_{2850/2880}$ and $I_{1090/1128}$ provided us with information about the conformation of the alkyl chain of different lipids in the presence of calcein [31].

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) is an unsaturated phospholipid with equivalent *sn*-1 and *sn*-2 acyl chain structures and a phase transition temperature of ~ -17.5 °C. The presence of double bonds leads to a curvature of the carbon chain that interferes with the ability to form highly ordered compact structures. Within the bilayer, the packing of the lipids affects the membrane mechanical properties, including their resistance to stretching and bending [31, 32].

The Raman spectra analyzing calcein - encapsulated liposomes composed of DOPC, showed no significant difference in the peak positions related to stretching vibration of $\nu_{\text{C-C}}$, $\nu_{\text{C-H}}$ and $\nu_{\text{C-N}}$ bonds in liposomes containing calcein in comparison to DOPC liposomes without calcein (Figure 2).

The peak at 2935 cm^{-1} exhibited no increase in peak wavenumber but an increase in peak height intensity which is attributed to the increasing the symmetrical stretching vibration of the C-H bond of the final methyl group of the alkyl chain [21].

The change of the disorder/order parameter calculated from the methylene stretching bands ($I_{2935/2880}$) also exhibited a slight increase in the intensity ratio, suggesting an augmentation of disorder degree in the alkyl chains. The *disordered* conformation is more dominant in a calcein-encapsulated liposome composed of DOPC than in a liposome without calcein (Table 1).

Both changes of the peak positions and the intensity ratio of *trans* bands to *gauche* well describe the conformational state of the alkyl chains and the *trans/gauche* conformation population of the phospholipids [33].

Table 1. Interaction of calcein with lipid membranes.

Sample	$I_{1095/1128}$	$I_{2850/2880}$	$I_{2935/2880}$
DOPC	0.92	0.98	1.07
DOPC with calcein	0.95	0.90	1.15
POPC	0.99	0.94	1.22
POPC with calcein	1.01	0.91	1.41
DPPC	0.98	0.91	1.22
DPPC with calcein	0.97	0.89	1.26
Mixed lipids	1.00	0.93	0.80
Mixed lipids with calcein	1.02	0.93	1.10

The height intensity ratios $I_{1095/1128}$, $I_{2850/2880}$, $I_{2935/2880}$ corresponds to proportion between disorder and order that exists in the conformation of the alkyl chain, their bending degree and interactions between the alkyl chains of lipid bilayers at 25 °C, respectively.

The stretching vibrations of the ν_{C-C} bonds of the alkyl chains in DOPC show no increase in the peak wavenumber, but do show an increase in peak height intensity in the presence of calcein. Moreover, the height intensity ratio of alkyl chain stretching bands ($I_{1095/1128}$) showed a slight increase, suggesting an augmentation of the disorder between the alkyl chains. Furthermore, it was shown that *gauche* conformation in disordered chains is more dominant in calcein-encapsulated liposomes composed of DOPC than in liposomes without calcein [20].

Additionally, the Raman spectra of the stretching vibration of ν_{C-N} bond showed an increase in peak height intensity in presence of calcein. The peak height intensity increasing at 715 cm^{-1} indicated significant interaction between the choline head group and calcein (Figure 2).

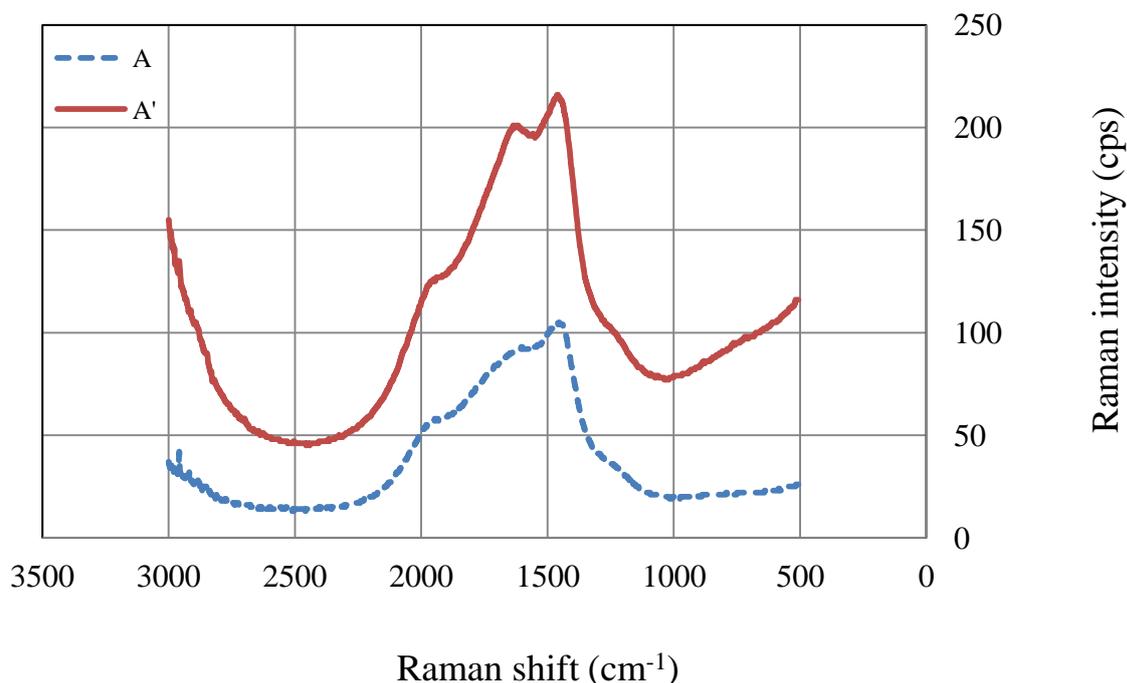


Figure 2. Raman spectra of the area 100 – 3500 cm^{-1} , including the C-N stretching mode of the choline group at 700 - 800 cm^{-1} , C-C bonds of the alkyl chains at 1000-1200 cm^{-1} , C-H stretching mode of the methylene group at 2800 – 3000 cm^{-1} and 25 °C, of A: DOPC without calcein and A': DOPC with calcein.

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) is a demi-unsaturated phospholipid with inequivalent *sn*-1 and *sn*-2 acyl chains and a phase transition temperature of ~ 0.5 °C.

Differences in the length and saturation of the fatty acid tails are important because they influence the ability of phospholipid molecules to pack against one another, thereby affecting the fluidity of the membrane [32].

Raman analysis was performed on POPC liposomes with or without encapsulated calcein. The Raman spectra, in the region of the $\nu_{\text{C-C}}$ and $\nu_{\text{C-H}}$ stretching modes exhibited no significant change in the peak positions of all observed bands in the POPC liposomes with calcein.

The change of the disorder/order parameter calculated from the methylene stretching bands ($I_{2935/2880}$) exhibited a slight increase of disorder degree in the alkyl chains in the presence of calcein.

Furthermore, the Raman spectra of the stretching vibration of the ν_{C-N} bond showed an increase in peak height intensity with no changes in the peak wavenumber in the presence of calcein. The peak height intensity increasing at 715 cm^{-1} induced the interaction between the choline head group and calcein (Figure 3).

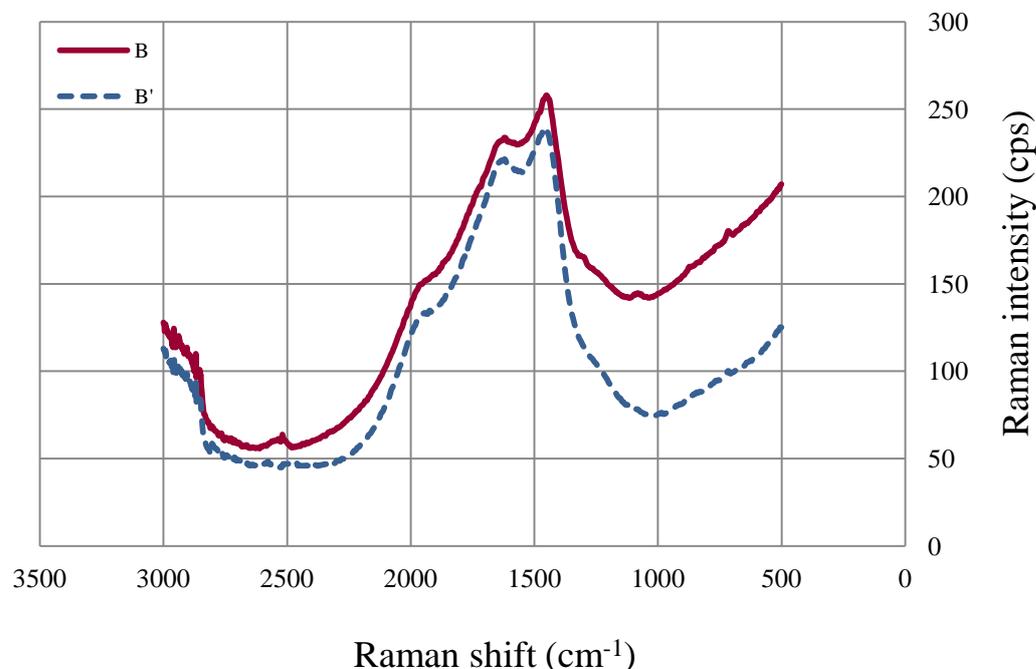


Figure 3. Raman spectra of the area $100 - 3500\text{ cm}^{-1}$, including the C-N stretching mode of the choline group at $700 - 800\text{ cm}^{-1}$, C-C bonds of the alkyl chains at $1000-1200\text{ cm}^{-1}$, C-H stretching mode of the methylene group at $2800 - 3000\text{ cm}^{-1}$ and $25\text{ }^{\circ}\text{C}$, of B: POPC without calcein and B': POPC with calcein.

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) is a saturated phospholipid with equivalent *sn*-1 and *sn*-2 acyl chain structures and a phase transition temperature of $\sim 40\text{ }^{\circ}\text{C}$.

The pure DPPC spectrum exhibits three characteristic areas from which useful information can be derived about the conformation of the molecule. The height intensity ratio $I_{2935/2880}$ indicated the interactions between the alkyl chains, and their conformation.

The peak at 2935 cm^{-1} exhibited no increase in peak wavenumber but an increase in peak height intensity, which is attributed to the increased symmetrical stretching vibration of the C-H bond of the final methyl group of the alkyl chain [21].

The Raman spectra of the lipid bilayer composed of DPPC in the presence of calcein exhibited no significant change in the peak positions of the ν_{C-C} and ν_{C-H} stretching mode regions. The change in the disorder/order parameter calculated from the ν_{C-H} stretching mode regions of the methylene groups of the alkyl chains ($I_{2935/2880}$) exhibited a slight increase in

intensity ratio. It was shown that the change of the disorder/order parameter calculated from the methylene stretching bands noticeably exhibited a significant increase of the disorder degree in acyl chains. Our results are in good agreement with the results presented by Gardikis *et al*, [8]. They explained that the conformational properties of the alkyl chains have changed due to the incorporation of PAMAM G4 and G3,5 dendrimer into DPPC lipid membrane.

The stretching vibrations of the ν_{C-C} bonds of the alkyl chains in DPPC show no increase in wavenumber and peak height intensity in the presence of calcein. Although, the height intensity ratio of the alkyl chain stretching bands ($I_{1095/1130}$) did not change significantly, the *trans* conformation in the acyl chains was found to be more dominant in calcein-encapsulated liposomes composed of DPPC. By considering the temperature of the experiments performed (20°C below the transition temperature of DPPC), the conformational properties of DPPC liposome were predictable.

The Raman spectra, in the region of the ν_{C-N} stretching mode, showed no wavenumber change in the presence of calcein, but a significant increase in band height intensity was observed. The intensity changes of the peak at 715 cm^{-1} showed the interaction between the choline head group and calcein (Figure 4).

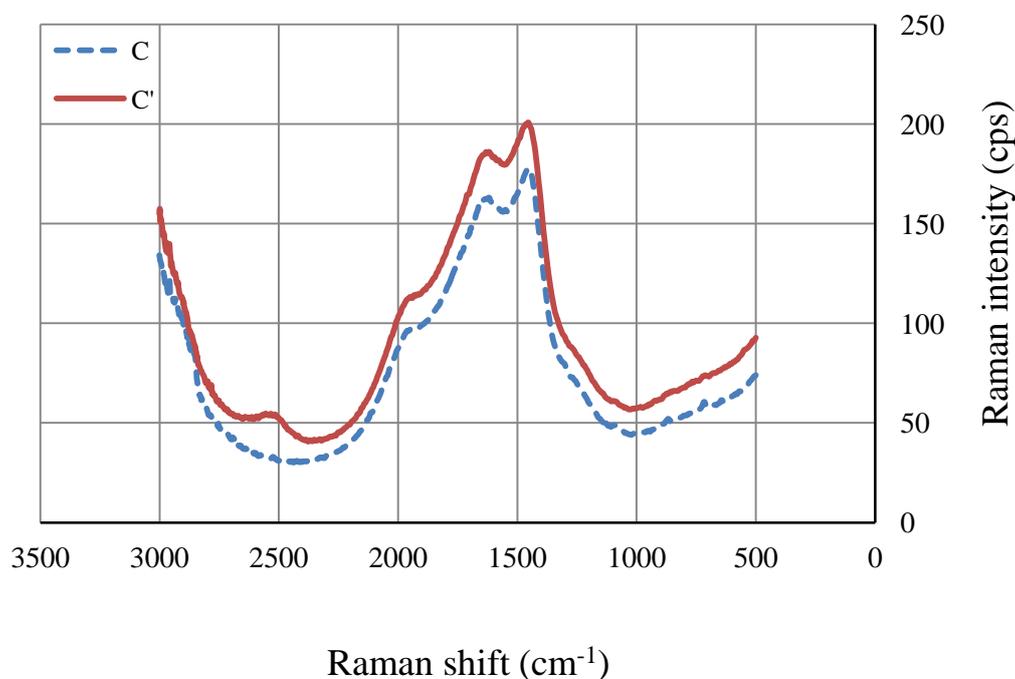


Figure 4. Raman spectra of the area $100 - 3500\text{ cm}^{-1}$, including the C-N stretching mode of the choline group at $700 - 800\text{ cm}^{-1}$, C-C bonds of the alkyl chains at $1000-1200\text{ cm}^{-1}$, C-H stretching mode of methylene group at $2800 - 3000\text{ cm}^{-1}$ and $25\text{ }^{\circ}\text{C}$, of C: DPPC without calcein and C': DPPC with calcein.

The mixed lipid formulation (46% DOPC, 12% POPC and 42% DPPC) optimized with the Mixture design model explained in our previous paper was also analyzed (phase transition temperature of ~ 10 °C) [30].

Mixture Design of Experiments (MDOE) is a technique used to determine the optimum combination of chemical constituents that deliver a desired response by using a minimum number of mixture runs [34].

The Raman spectra of the lipid bilayer composed of mixed lipids in the presence of calcein exhibited no significant change in the peak positions of the ν_{C-C} , ν_{C-H} and ν_{C-N} stretching mode regions. The change of the disorder/order parameter calculated from ν_{C-H} stretching mode regions of the methylene groups of alkyl chains ($I_{2935/2880}$) showed a slight increase in intensity ratio. It was shown that the change of the disorder/order parameter calculated from the methylene stretching bands noticeably exhibited a significant increase in degree of disorder in the acyl chains.

The stretching vibrations of the ν_{C-C} bonds of the alkyl chains in mixed lipids showed only a minor increase in peak height intensity in the presence of calcein. Although, the height intensity ratio of the alkyl chain stretching bands ($I_{1095/1130}$) showed a slight increase, it suggests that the *gauche* conformation in disordered chains is more dominant in calcein-encapsulated liposomes composed of mixed lipids than in liposomes without calcein.

The Raman spectra, in the region of the ν_{C-N} stretching mode, showed a significant increase in height intensity. This change in the peak at 715 cm^{-1} represents a significant interaction between the choline head group of the acyl chain and calcein (Figure 5).

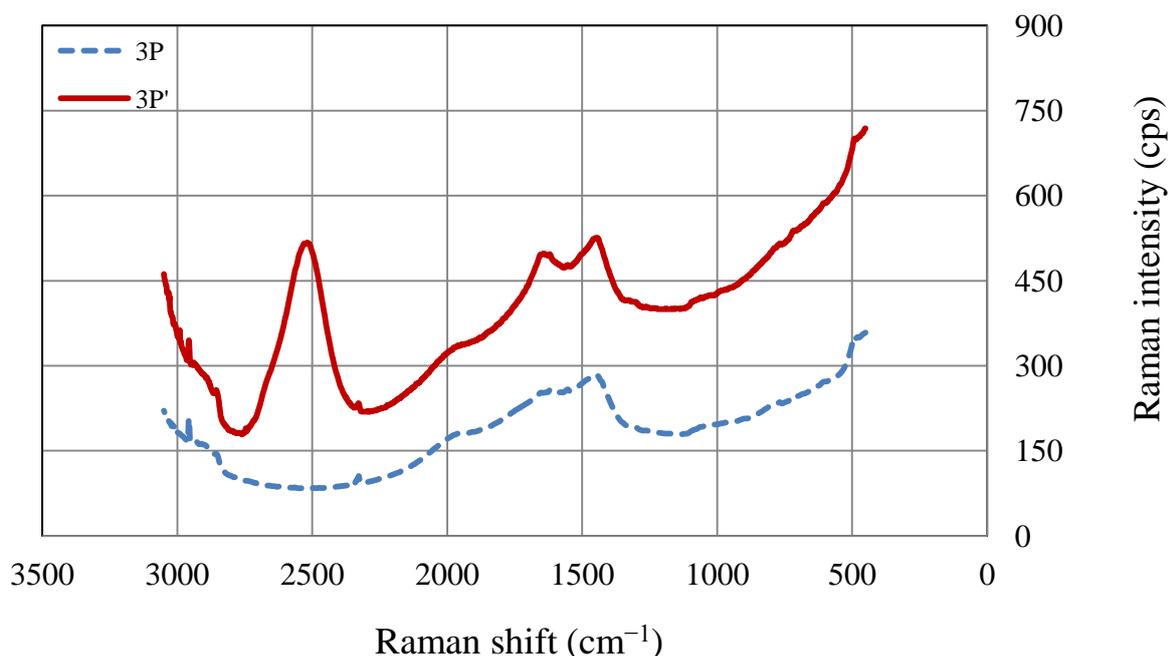


Figure 5. Raman spectra of the area 100 – 3500 cm^{-1} , including the C-N stretching mode of the choline group at 700 - 800 cm^{-1} , C-C bonds of the alkyl chains at 1000-1200 cm^{-1} , C-H stretching mode of the methylene group at 2800 – 3000 cm^{-1} and 25 °C, of C: Mixed lipids without calcein and C': Mixed lipids with calcein.

We observed an increase of the perturbation in the carbon-chain and the terminal methyl group of all liposomes in the presence of calcein. Therefore, we conclude that calcein shows different molecular effects on liposomal bilayers and this phenomenon is based on differences in the lipid structure and their properties. The obtained results showed that the fluidity of the hydrocarbon portion of the membrane is very important. The double bonds within the acyl chain resulted in a decrease in the packing density, which in turn perturbed the barrier to calcein permeation. The double bond in the normal *cis* configuration which is asymmetric leads to a kink of the hydrocarbon chain. Consequently, the unsaturated lipids are packed together loosely.

The packing of lipids within the bilayer affects its mechanical properties, including its resistance to stretching and bending. For example, the length and degree of saturation of the lipid acyl chains determine the thickness and ordering of the hydrophobic region of the membrane [31].

Thus, at moderate temperatures, the terminal region of the longer *sn-1* acyl chain would be expected to display some motional disorder in order to occupy the free space generated below the terminal methyl group of the contiguous shorter *sn-2* chain. As the overall phospholipid

chain lengths become shorter, the terminal methyl regions, with their inherent disruptive nature, then proportionately contribute more to the overall disordering characteristics of the bilayer matrix [35, 36].

Mason *et al.*, [37] proposed that the inequivalent *sn-1* and *sn-2* acyl chain configurations for the terminal methyl groups lead to perturbations in both the intramolecular conformational statistics of the acyl chains and the intermolecular interactions existing between chains. They also mentioned that the perturbation parameter is inversely related to the acyl chain length. With an increasing degree of unsaturation, the packing-free volume within the hydrophobic region of the bilayer exhibited a progressive increase [31, 38].

As, we expected the molecular effect of calcein on liposomes composed of unsaturated lipids (in gel-form at 20 °C) is more dominant than liposomes composed of saturated lipids. Indeed, this effect is more noticeable in mixed lipids. The inequivalent chain configurations of diacylphosphatidylcholines lead to distortions at the methyl termini, which perturb the gel-phase packing properties of the remaining hydrocarbon chain segments within the bilayer assembly (Figure 6)[31].

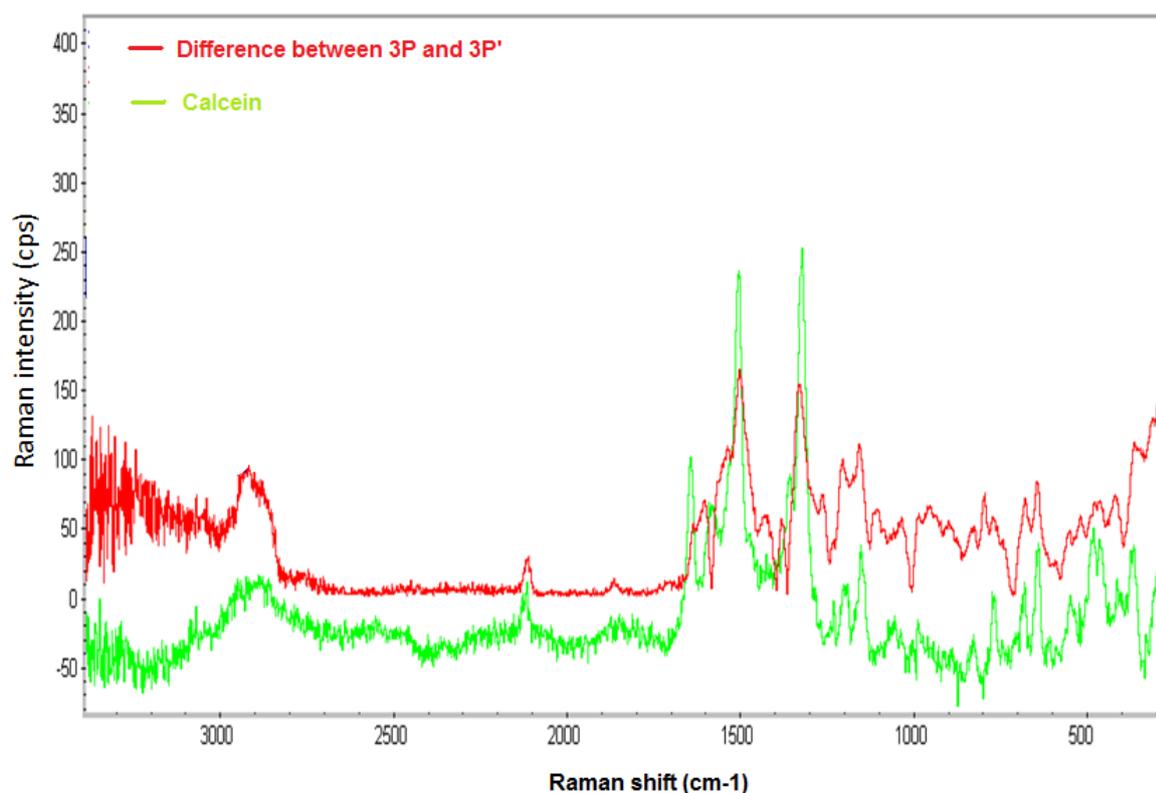


Figure 6. Raman spectra of the area 100 – 3500 cm^{-1} , including the C-N stretching mode of the choline group at 700 - 800 cm^{-1} , C-C bonds of the alkyl chains at 1000-1200 cm^{-1} , C-H stretching mode of the methylene group at 2800 – 3000 cm^{-1} and 25 °C, difference between mixed lipids without and with calcein and calcein.

Furthermore, the Raman shifts of the methylene scissoring ($1435 - 1445 \text{ cm}^{-1}$) band of different lipids may be assigned to the mesophases at different temperatures. For example, for liposomes composed of DOPC, POPC and mixed lipids at 20°C , no lattice order was observed. This lack of order can be identified by the scissoring band being shifted to 1443 cm^{-1} . Whereas for liposomes composed of DPPC, the $L_{\beta'}$ phase with a hybrid hexagonal lattice was observed, which is characterized by a split methylene scissoring band at 1437 cm^{-1} [21]. It is worth mentioning that the Raman shift of the methylene scissoring band of different lipids showed a noticeable increase in peak height intensity in the presence of calcein. Indeed, this augmentation is more visible in liposomes composed of unsaturated lipid.

3.2. Langmuir monolayer studies

The bilayer properties of the liposomal carrier can significantly alter its interaction with cell membranes and also considerably modify the pharmacokinetics, pharmacodynamic properties and the efficiency of the drug delivery systems. Lipid monolayers are an appropriate membrane target model for studying liposomal drug carriers [39].

Hernandez-Borrell *et al.*[39] have shown that a simple lipid monolayer can be used to experimentally simulate the observed interactions between drug-loaded lipid vesicles. Such interactions can be investigated by spreading a lipid over a subphase containing drug or lipid/drug mixture over a pure subphase, such as water, to form a monolayer in a Langmuir trough. The monolayer is then compressed, and the surface pressure (π) – molecular area (A) isotherms of the pure lipid spreading over a subphase with/without a drug are compared [39]. The Langmuir technique allows for preparing model lipid membranes by spreading phospholipid monolayers at the air/water interface and offers a unique way of investigating the interactions between the membranes and different molecules [11].

- Compression isotherms

The response of the monolayer to applied external forces indicates its elastic and mechanical properties. Since the lipid structure is not rigid, an applied tension or compression will change the area per molecule in monolayer. The π -A and ΔV -A isotherms obtained upon compression of the formed films are shown in Figure 7.

The isotherms for DOPC, POPC and the mixed lipids spread on pure water as a subphase demonstrate the phase behaviour characteristic of a liquid phase, as a consequence of the lipid composition, representing molecules containing unsaturated fatty acids. The presence of

unsaturated chains in the molecules prevents them from close-packing at the interface. The initial increase in the surface pressure upon film compression for DOPC, POPC and mixed lipids occurred at about 118, 105 and 103 Å²/molecule, respectively, and the pressure rose steadily as the molecular area decreased. The DOPC and POPC monolayers can be compressed to nearly 46 and 47 mN/m, respectively, before collapsing (Figure 7). The collapse pressures of the DOPC and POPC monolayers on pure water were also 42.1, 42.9 mN/m, respectively. The isotherm of the mixed lipids monolayer appeared between the isotherms of pure lipids. The mixed monolayer was more condensed than that with pure DOPC, POPC and attained the maximum value of compressibility modulus, 39.2 mN/m and it could have been compressed to nearly 44 mN/m before collapsing.

The expanded monolayer of the DOPC and mixed lipid films becomes more unstable in the presence of calcein compared to pure water.

The isotherm of the pure DPPC monolayer is similar to that reported in the literature [40, 41]. A phase behaviour of solid-condensed states was observed [42].

At 20 °C on pure water, the isotherms of DPPC showed a break characteristic of fluid-solid phase transitions, while other films were fully expanded at all surface pressures. The initial increase in the surface pressure upon film compression for the DPPC monolayer occurred at about 90 Å²/molecule and the pressure rose steadily as the molecular area decreased. The DPPC monolayer could have been compressed to nearly 58 mN/m before collapsing (Figure 7-A), its collapse pressure on pure water is 53.5 mN/m.

In the presence of calcein, a small shift of the π -A isotherms to higher molecular areas was observed in all types of monolayers. This shift was most important in the liquid-expanded (LE) and liquid-condensed (LC) phase transition region and included an instability in the monolayer packing (Figure 7-A). Indeed, the DPPC monolayer formed over the subphase containing calcein was less compressible compared to those spread on pure water, as indicated by the values of the compressibility modulus (Table 2). Corvis *et al.* (2006)[43] also observed this phenomenon in DPPC films spread on a subphase containing Griseofulvin (used as an oral antibiotic).

In general, a decreased molecular area could be a manifestation of the subphase electrostatic effect, which would reduce the electrostatic interactions between the locally charged groups in lipid molecules. A decrease in repulsive electrostatic interactions favors tighter close-packing between the lipids at the interface, or a reduction in the average molecular area, which allows

the collapse pressure to increase. Increasing the interaction area eventually destabilizes monolayer packing [44, 45].

Zhao *et al.* (2004)[40] also observed that increasing the mole percentage of paclitaxel caused the isotherms to shift to smaller areas per molecule in the mixed paclitaxel/ DPPC monolayer. Additionally, Corvis *et al.* (2006)[43] explained that DPPC monolayers spread on the Griseofulvin solution subphase shift to higher molecular areas at low surface pressures compared to those of the pure water subphase. This observation suggests that, at low surface pressures, Griseofulvin penetrates into the phospholipid film from the subphase.

Table 2 . Isotherm parameters at the collapse point.

	A_{coll} (\AA^2)	Π_{coll} (mN m^{-1})	ΔV_{coll} (V)	C_s^{-1} (mN m^{-1})
DPPC on pure water	40	53.5	0.58	257.2
DPPC on 10 mg L ⁻¹ calcein	42	50.1	0.53	288.0
POPC on pure water	54	42.9	0.47	119.0
POPC on 10 mg L ⁻¹ calcein	58	42.3	0.40	120.9
DOPC on pure water	60	42.1	0.43	116.2
DOPC on 10 mg L ⁻¹ calcein	62	41.4	0.39	107.1
Mix PC on pure water	51	39.2	0.44	100.7
Mix PC on 10 mg L ⁻¹ calcein	53	38.9	0.40	99.0

Moreover, the decrease in the π_{coll} values (Table 2) of the monolayers in the presence of calcein reflects a lower stability in the films. In general, both changes in the monolayer properties, i.e., liquefaction and destabilization of the phospholipid films, are induced by the calcein present in the subphase.

As shown in Figure 7-B, the influence of the calcein on the surface properties of the lipid monolayers can be also observed using ΔV -A measurements. The results indicate that there is a slight reorientation of the molecules upon film compression. These findings are in accordance with the surface pressure isotherm results and indicate more liquefaction of the DOPC and mixed lipids films in the presence of the calcein. On the other hand, the surface potential of these phospholipid monolayers is lower for the films spread on the calcein solution subphase compared to the films spread on pure water (Figure 7-B, Tables 2).

Concerning surface potential isotherms, a slight shift to larger molecular areas of the liquid-expanded (LE) and liquid-condensed (LC) phase regions can be observed. Nevertheless, the presence of calcein in the uncompressed phase monolayer is more easily detected by the surface potential shift.

It should be noted that, in the case of ΔV - A dependencies, the isotherms obtained from DOPC, POPC and mixed lipid monolayers spread on pure water and calcein solution subphases are almost superposed in the most condensed phase regions.

The adsorbed calcein significantly shifts the π - A and ΔV - A isotherms to larger molecular areas, showing that calcein stays strongly adsorbed to the monolayer, even at the most condensed state, and that it may penetrate into lipid molecules [46].

On the other hand, the decrease in the surface potential values (Table 2) indicates a less vertical orientation of the lipid forming the film over the calcein solution, relative to the water surface. It is also reasonable to suppose that polar interactions of the hydrophilic moiety of calcein with the polar head group of lipids allow the calcein to take anchor in the monolayer.

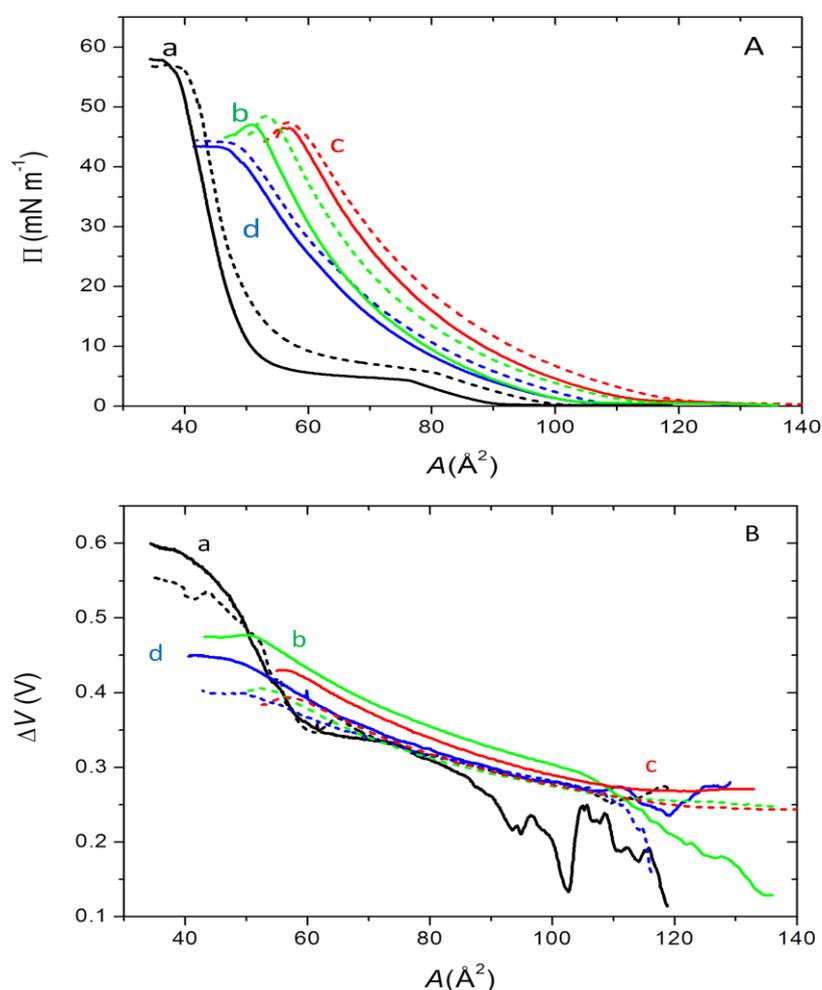


Figure 7. (A) Surface pressure – area, (B) Surface potential – area isotherms of DPPC (a), POPC (b), DOPC (c) and mixed PC (DPPC/DOPC/POPC = 4:4:2; d) monolayers spread on pure water (solid lines) and on calcein 10 mg L^{-1} solution (dashed line), $T = 20 \text{ }^\circ\text{C}$.

Most of the liquid-expanded monolayers of the lipids (Table 2) would collapse at molecular areas between 40 and 62 \AA^2 and surface pressures between 38.9 and 53.5 mN/m . This means,

the effects of chain length and unsaturation are greater on the collapse area, A_{coll} , than on the collapse pressure, Π_{coll} , which lies within a narrow range.

The obtained results shown the areas per lipid molecule of monounsaturated lipids are larger than those of the corresponding saturated. The unsaturated lipids are packed together loosely and consequently, A_{coll} of monolayer composed of them increases (Table 2). This confirmed that the active molecules insertion in lipid layers depends strongly on its structure and the degree of packing of lipid chains [29].

Moreover, the decrease of π_{coll} values of monolayers in the presence of double bond was observed which reflects a lower stability of the films by increasing the degree of unsaturation. Additionally, the decrease of the surface potential values of monolayers composed unsaturation lipids indicates a less vertical orientation of the lipid forming the film and their more penetrable to calcein.

The surface compressibility modulus (C_s^{-1}) of the monolayer as a function of lateral packing pressure is calculated from surface pressure and area per molecule data according to the following equation; [44, 47].

$$C_s^{-1} = -A \left(\frac{d\pi}{dA} \right)_T$$

Where A is the area per molecule and $\left(\frac{d\pi}{dA} \right)_T$ is the surface pressure change with area under isothermal conditions. High C_s^{-1} values suggest a closely-packed film that requires a small area change to attain lower surface tensions (higher surface pressures). The collapse parameters, ΔV_{coll} , π_{coll} , and A_{coll} , corresponding to the highest packing of molecules in the monolayer, were also determined directly from the compression isotherms. Compressibility is a measurement of the elastic packing interactions of the monolayer [11, 40].

The incorporation of calcein into the DPPC monolayer increases the compressibility coefficient and in the case of a POPC monolayer, the presence of calcein induces a slight increase in the compressibility coefficient.

A higher C_s^{-1} value indicates that the films are in less compressible solid phase form. From the results presented in Table 2, it can be concluded that calcein has a condensing effect on DPPC monolayers and makes the monolayer structure somewhat harder and more solid-like. The obtained results are in good agreement with the results obtained by Korchowiec *et al.* [48] who observed that the C_s^{-1} values of lipid monolayers increased in the presence of the salts, which suggests a condensing effect, however, Zhao *et al.* (2006) [40] also explained that

the incorporation of Paclitaxel (used as an antineoplastic drug) in the DPPC monolayer was the reason for its increased compressibility.

This effect might have to do with this hypothesis that calcein maybe affects the packing of the acyl chain domain of DPPC, as made evident by DSC analysis and discussed later. Because the lipid tails are near the aqueous surface, calcein is in closer proximity with the polar head groups of the lipids and can experience repulsive forces. This phenomenon could explain the observed surface pressure increment that indicates increased pressure or an area expansion effect.

However, as can be seen in Table 2, the DPPC and POPC monolayers in the presence of calcein are more compressible than those spread on pure water.

Interestingly, there is an opposite effect: more compressibility and expanded film is observed with DOPC and mixed lipid films. The incorporation of calcein into the DOPC monolayer decreases the compressibility coefficient. A lower C_s^{-1} value indicates the films are in a more compressible liquid-expanded form (Table 2). This phenomenon can be observed in the mixed lipids monolayer by a slight decrease in the compressibility coefficient, from 100.7 to 99 mN/m. It can be concluded that calcein has an expanding effect on DOPC and probably on mixed lipid monolayers. This phenomenon makes the monolayer structure slightly softer and more compressible in comparison to DOPC and mixed monolayers spread on pure water. The results of DSC also confirmed a slight decrease in the phase transition temperatures of DOPC and mixed lipid bilayers due to calcein encapsulation (Table 4).

Table 4, Main transition of lipid bilayers with and without calcein encapsulated.

Sample	T_c^a (°C)		ΔH (J/g)	
	With calcein	Without calcein	With calcein	Without calcein
DOPC	-17.80 ± 0.44	-17.15 ± 0.31	195.50 ± 0.51	185.65 ± 0.7
POPC	1.01 ± 0.37	0.78 ± 0.24	291.15 ± 0.46	285.9 ± 0.42
DPPC	41.77 ± 0.28	41.83 ± 0.31	329.16 ± 0.62	315.86 ± 0.56
Mixed of lipids ^b	6.30 ± 0.42	6.72 ± 0.34	310.32 ± 0.22	301.34 ± 0.32

T_c : temperature at which heat capacity (ΔC_p) at constant pressure, is maximum;
 ΔH : transition enthalpy normalized per mol of DPPC.

^a Mean of four runs of each experiment.

^b mixed lipids: 46% DOPC + 42 % DPPC + 12 % POPC.

In comparison to membrane mechanical properties of lipid films made from PC_S, our obtained results shown the surface compressibility modulus (C_s^{-1}) increases in monolayer composed of saturation phospholipids which is an indicator for rigid monolayer.

Our findings confirmed that the structural changes caused by the double bond, reducing the molecular packing of the lipids and increasing the area per molecule, lead to decreasing the barrier property of the films.

Furthermore, the obtained results showed that the penetration of the calcein in lipid films depends on their structure and on the molecular packing of the lipids and as well as the area compressibility of different lipid structures.

- *Brewster angle micrographs (BAM)*

The BAM images show that calcein modifies the morphology of the monolayers. The BAM micrographs indicate that in the presence of calcein in the subphase, the domains characteristic for the LE-LC phase transition in all lipid films coalesce at slightly higher surface pressures compared to those spread on a pure water subphase. This result is in accordance with the shift of the phase transition to the higher surface pressures seen in the compression isotherms. In accordance with the π - A isotherms results, the BAM images show that the LE-LC phase transition is shifted to higher surface pressures in the presence of the calcein compared to the pure water in DPPC films (Figure 8).

Indeed, the bright condensed phase domains are observed at 5.0 mNm⁻¹ in the DPPC film spread on pure water (Figure 8A), while no bright spots could be observed in the DPPC film at this surface pressure over subphase containing calcein (Figure 8E). While the condensed phase domains coalesce at 6.0 mNm⁻¹ in the case of the DPPC film spread on pure water (Figure 8C), in the DPPC film over subphase containing calcein; the domains are, on the contrary, clearly seen (Figure 8G) and the condensed phase domains coalesce at 8.0 mNm⁻¹ (Figure 8H).

When the monolayer is in the solid-condensed state, the phospholipids are probably quasi-perpendicular with respect to the water surface and, consequently, some transition moments should be preferentially oriented parallel or perpendicular to the surface.

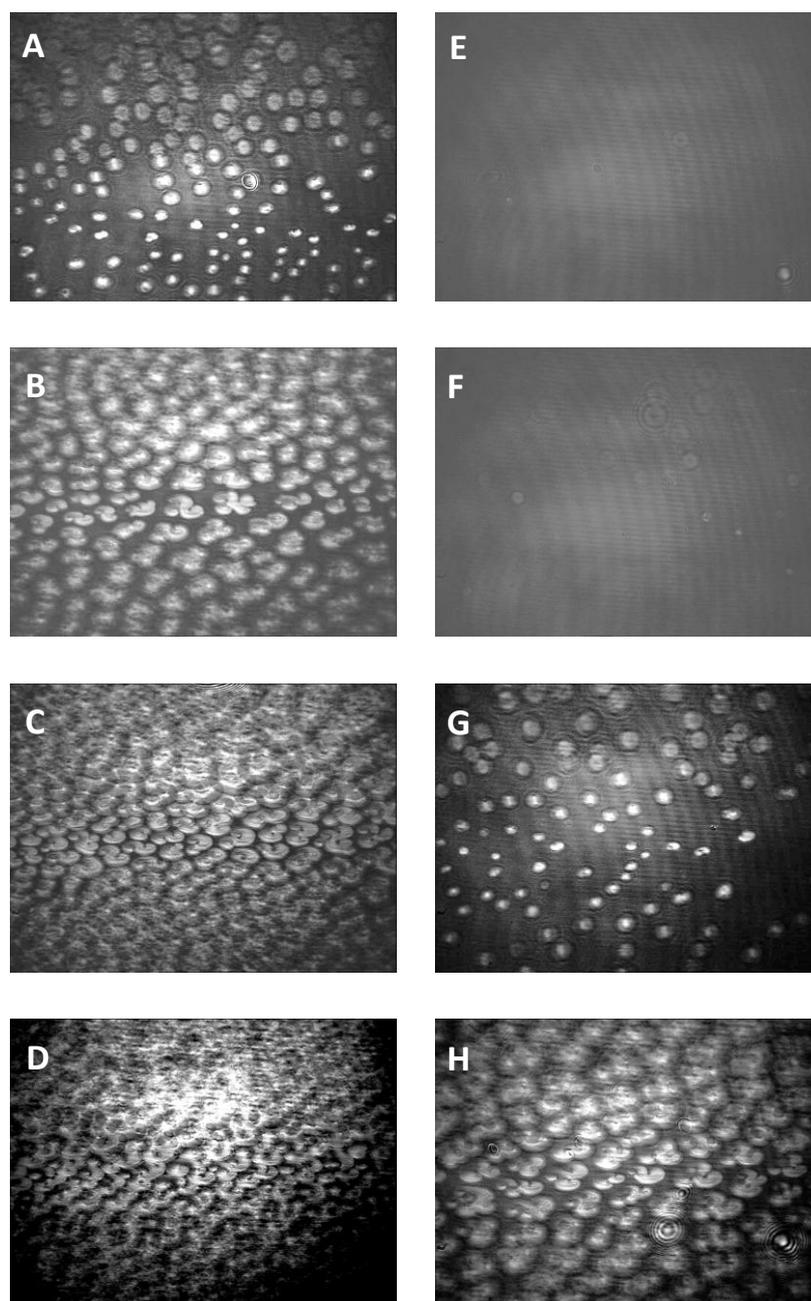


Figure 8. Brewster angle micrographs of DPPC monolayers spread on pure water (A-D) and calcein solution (E-H). The micrographs were taken at $\Pi = 5 \text{ mN m}^{-1}$ (A, E), $\Pi = 5.5 \text{ mN m}^{-1}$ (B, F), $\Pi = 6 \text{ mN m}^{-1}$ (C, G), and 8 mN m^{-1} (D, H). Scale: the width of the snapshots corresponds to $400 \mu\text{m}$.

3.3. PM-IRRAS studies

In order to better understand the interactions between calcein and the phospholipid membranes, PM-IRRAS experiments were performed. The lipid perturbations induced by incorporating calcein into the monolayers can be investigated by analysing the lipid symmetric and antisymmetric methylene group stretching vibrations [$\nu_s(\text{CH}_2)$ and $\nu_{as}(\text{CH}_2)$] (around 2920 and 2850 cm^{-1} , respectively) and the ester carbonyl stretching band (C=O) (around 1730 cm^{-1}). The CH_2 stretching mode region between 2850 and 3000 cm^{-1} in the infrared spectra of the phospholipid is particularly useful because the frequency and width of the methylene bands are sensitive to the conformation of phospholipid acyl chains [49, 50].

- CH_2 Stretching Region

The CH_2 stretching region is located between 2800 and 3000 cm^{-1} ; the two dominating peaks are observed in the phospholipid spectra at around 2920 and 2850 cm^{-1} , which correspond to the antisymmetric and symmetric methylene group stretching vibrations, respectively. The frequencies of these bands are sensitive to the conformation of the phospholipid acyl chains and thus provide valuable information on the orientation of the lipid acyl chains in the membrane.

In general, the downward shift of the $\nu_{as}(\text{CH}_2)$ and $\nu_s(\text{CH}_2)$ frequencies from 2920 and 2850 cm^{-1} indicates a higher chain ordering in the film, i.e., an ordered all-trans conformation of the chains, while their upward shift suggests chain disordering with an increase in the number of gauche conformers in the chain (Table 3) [50, 51].

In the pure lipid monolayers, the $\nu_{as}(\text{CH}_2)$ and $\nu_s(\text{CH}_2)$ bands appear at wavenumbers slightly higher than 2920 and 2850 cm^{-1} , respectively (Table 3). Those peak positions are typical for liquid-expanded films with disordered acyl chains, which is in agreement with the compression isotherm analysis (Table 2). In the case of monolayers formed in the presence of calcein, the compression isotherm analysis clearly shows that calcein interacts with monolayers on the subphase and changes the monolayer ordering (Table 2, Figure 5).

The conformational changes of phospholipid chains presented by the $\nu_{as}(\text{CH}_2)$ and $\nu_s(\text{CH}_2)$ band wavenumbers, showed a shift to higher values compared to the monolayer films spread on pure water. This effect suggests that the interaction of calcein with the monolayer favors phospholipid chain disordering, i.e., the number of gauche conformers in the chains increases [52].

Table 3. PM-IRRAS: methylene and carbonyl stretching vibrations of phospholipids in the absence and in the presence of calcein ($\Pi = 30 \text{ mN m}^{-1}$)

Monolayers/subphases	$\nu_{\text{as}}(\text{CH}_2)$ (cm^{-1})	$\nu_{\text{s}}(\text{CH}_2)$ (cm^{-1})	$\nu(\text{C}=\text{O})$ (cm^{-1})	$\nu_{\text{as}}(\text{PO}_2^-)$ (cm^{-1})
DPPC / pure water	2921	2842	1727	1247
DPPC / calcein	2923	2848	1734	1238
POPC / pure water	2930, 2911	2856	1734	1248
POPC / calcein	2931, 2918	2859	1738	1245
DOPC / pure water	2928	2847	1730	1248
DOPC / calcein	2929	2847	1728	1240
Mixed PC / pure water	2938, 2920	2850	1745	1247
Mixed PC / calcein	2940, 2922	2855	1739	1244

- Carbonyl Stretching Region

The $\nu(\text{C}=\text{O})$ stretching band observed at around 1730 cm^{-1} is often used to investigate the interfacial region of phospholipids. However, the ester carbonyl $\text{C}=\text{O}$ stretching band is quite complex because it appears to be the summation of two overlapping bands. The frequency of this band depends on the hydration of the ester group and thus provides useful information concerning changes in this hydration [16, 53] since, in a given phospholipid, the low- and high-frequency bands correspond, respectively, to hydrated and dry carbonyl groups.

The PM-IRRAS spectra of DPPC, POPC, DOPC and mixed lipids monolayers spread on pure water display stretching $\nu(\text{C}=\text{O})$ bands at around 1727, 1734, and 1730 and 1745 cm^{-1} , respectively.

However, a negligible increase in the higher wavenumber on the $\nu(\text{C}=\text{O})$ band of DOPC can be observed in presence of calcein. A clearly visible shift of this band to higher wavenumbers occurs in the case of DPPC and POPC. This augmentation indicated dehydration of carbonyl moieties and a decrease of the number of hydrogen bonded carbonyl groups. This also showed that the hydrogen bond network would be less ordered in the more lipid films spread on calcein solution [54, 55].

A slight shift of the $\nu(\text{C}=\text{O})$ band of mixed lipids to lower wavenumbers can be observed, which demonstrates hydration of carbonyl moieties and an increase in the number of hydrogen bonded carbonyl groups [49, 51].

Additionally, the shift of the stretching $\nu(\text{PO}_2^-)$ band of all monolayers spread on the calcein solution to lower wavenumbers indicates that a lower number of phosphate groups are hydrogen bonded in these films compared to film spread on pure water. This augmentation shows that the hydrogen bond network would be less ordered in the more lipid films spread on calcein solution.

All PM-IRRAS results are in accordance with those obtained from the compression experiments and indicate interaction between calcein and phospholipid molecules. However, the interaction with different lipid monolayer differs.

3.4. Differential scanning calorimetry

Lipid bilayers undergo phase transition with an increase in temperature. However, DPPC in an aqueous environment exists in two totally different mesomorphic phases known as L_α and L_β' . The L_β' crystalline form of DPPC corresponds to the gel phase while the L_α crystalline form corresponds to the liquid crystalline phase. The transition from the gel phase to the liquid-crystalline phase can be done by increasing the temperature [55].

The exact knowledge and control of the thermodynamic properties of the lipid bilayers [ΔH (transition enthalpy), T_m (transition temperature), $T_{1/2}$ (temperature at which the transition is half completed), $\Delta T_{1/2}$ (cooperatively of the bilayer)], is of importance in the development of lipidic controlled release systems (i.e. liposomes) because the encapsulation, stability and release of biologically active molecules depend on them [4].

In this study, DSC was applied to assess the thermodynamic changes in the lipid bilayers caused by incorporating calcein and thus further understanding how the calcein interacts with lipid bilayers.

The thermogram of pure DPPC (Figure 9) shows two characteristic peaks (a): at 37.46 and 41.83 °C. The first peak presents a low enthalpy transition (pretransition) attributed to the mobility of the choline polar head of DPPC, while the sharp enthalpy main transition is attributed to the mobility of the alkyl chains.

Fully hydrated DPPC bilayers incorporating calcein indicated no significant changes in transition values but it showed thermograms consisting of broad enthalpy transitions,

abolition of the pretransition and a negligible increase of the enthalpy change of the gel to liquid–crystalline phase transition of DPPC bilayers.

According to a previous study, the molecules localized within the hydrophobic interior of phospholipid bilayers cause a decrease in the enthalpy of the transition, while those localized at the interfacial region of the phospholipids increase the enthalpy of the transition [56].

DSC analysis showed that with the incorporation of calcein at 10mM, the DPPC pretransition weakened since the increase in the calcein concentration is undetectable (10, 15, 20, 30 mM). The pretransition arises from the transformation from a $L_{\beta'}$ bilayer structure to the $P_{\beta'}$ conformation. Because the pretransition is highly sensitive to the presence of other molecules in the polar region of the phospholipids, the weakening of the pretransition cannot be ascribed to any specific molecular changes. It can be observed that there was no significant change in the peak melting temperature of the DPPC bilayers, which demonstrates that calcein was localized in the outer hydrophobic cooperative zone of the bilayer (Figure 9).

Our results are in good agreement with the results of Zhao *et al.* [4], which show that paclitaxel caused a broadening of the main phase transition.

The results of other lipid bilayers (DOPC, POPC and mixed lipids) have also shown a slight shift in the transition values. This observation confirmed that calcein is being able to interact with the choline polar-head group of the lipids but probability it could insert itself into the hydrophobic bilayers.

As T_c shows the main transition is attributed to the mobility of the alkyl chains, Table 4, presents the main transition of the four lipid bilayers studied, with and without calcein encapsulated.

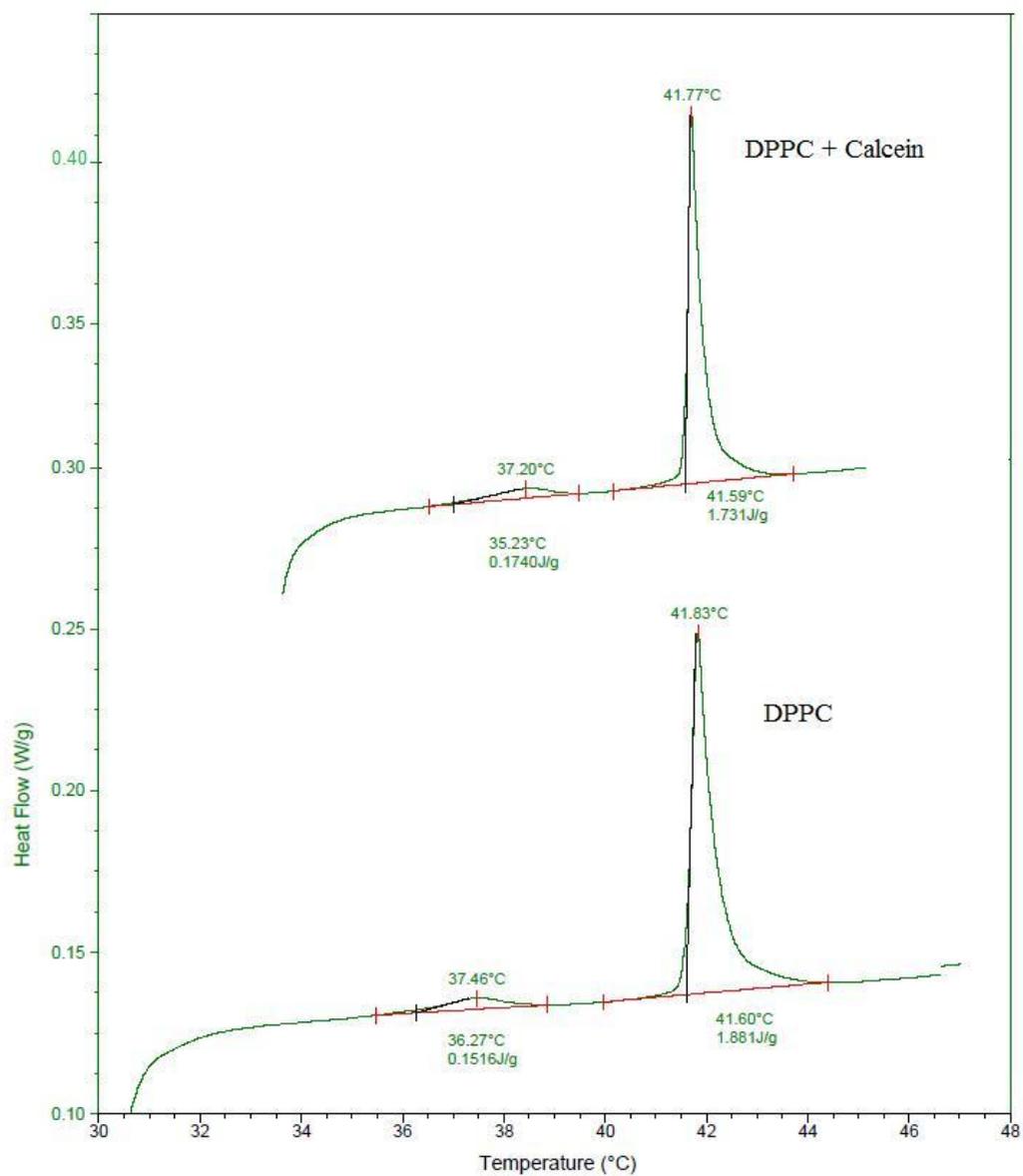


Figure 9. DSC thermograms of fully hydrated DPPC lipid bilayers with and without calcein.

4- Conclusion

Interactions of additives with model lipid bilayers could provoke changes in their thermotropic behaviour as well as their conformation properties. These effects were taken into account in the design of liposomal formulations as controlled release drug delivery systems. Also, by understanding the signalling and interaction between the bioactive compounds and liposomes, it would be possible to mimic biological systems.

We address the composition of lipids and the structure of the cell membrane and comment on the physical forces present in the membrane which may impact polar drug interactions.

Phospholipids are differentiated from each other by their head groups, chain length, and degree of chain unsaturation. From a pharmaceutical viewpoint, it would be essential to investigate the molecular interactions between model polar drug and lipids of various head group types, chain lengths, and degrees of chain unsaturation.

The inequivalent chain configurations of diacylphosphatidylcholines lead to distortions at the methyl termini which perturb the gel-phase packing properties of the remaining hydrocarbon chain segments within the bilayer assembly.

The Raman spectra analysis of calcein - encapsulated liposomes composed of DOPC, mixed lipids, POPC and DPPC showed that the stretching vibration of the ν_{C-C} , ν_{C-H} and ν_{C-N} bonds exhibited no significant change in the peak positions in samples containing calcein in comparison to liposomes without calcein.

The change of the disorder/order parameter calculated from the methylene stretching bands ($I_{2935/2880}$) and alkyl chain stretching bands ($I_{1095/1130}$) of all liposomes in the presence of calcein exhibited a slight increase in the intensity ratio, which mentioned an augmentation of a disorder degree in the alkyl chains. It was observed that the *gauche* conformation was more dominant in calcein-encapsulated liposomes than in liposomes without calcein.

Furthermore, the Raman spectra of the stretching vibration of ν_{C-N} bond of all liposomes in the presence of calcein showed an increase in peak height intensity in the presence of calcein. The increase in peak height intensity at 715 cm^{-1} indicates significant interaction between the choline head group and calcein.

In the presence of calcein, a small shift of the π -A isotherms to higher molecular areas is observed in all types of monolayers. A decrease in the molecular area could be a manifestation of the subphase electrostatic effect which would reduce the electrostatic interactions in acyl chains of lipids. Moreover, the decrease of π_{coll} values in the monolayers in the presence of calcein reflects a lower stability of the films. The compression isotherm

analysis also clearly showed that calcein interacts with monolayers from the subphase and changes the monolayer ordering.

Additionally, the shift of the stretching $\nu(\text{PO}_2^-)$ band of all monolayers spread on calcein solution to lower wavenumbers indicates that a lower number of phosphate groups are hydrogen bonded in these films compared to those spread on pure water.

The obtained results indicated that calcein could be able to intercalate into the acyl chains and disturb the chain order. The chain disorder strongly increases in the presence of unsaturated lipids in the liposome structure.

Currently, several types of liposomes with numerous variations in lipid composition are used in drug delivery. Bioactive interaction with the liposomal bilayer could be an impressive approach to enable a theoretical understanding of controlling interaction properties.

Additionally, the bilayer properties of the liposomal carrier can significantly alter its interactions with the biological environment, including the cell membranes and, as a result, modify significantly the pharmacokinetics and efficacy of the drug delivery system.

The techniques used could be a valuable approach to correlate the different properties of liposome and bioactive properties (hydrophobic or hydrophilic); e.g. Lipid monolayers are also suitable as membrane target models for studying liposomal drug carriers.

Furthermore, these observations could be useful for developing efficient liposomal systems in delivering polar and non-polar drugs, nutraceutical and antibacterial agents in different fields.

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Conclusion

Les interactions entre les substances actives à vectoriser et la membrane lipidique du liposome influencent fortement les propriétés thermiques et la structure du complexe ainsi formé. Ces effets ont été pris en compte dans la formulation liposomale pour une meilleure maîtrise de la libération de la biomolécule active. Une meilleure compréhension du mode de signalisation des liposomes et de la composition en acides gras des bicouches lipidiques permettrait une meilleure intégration de ces vecteurs au niveau des membranes biologiques.

Les phospholipides présentent des propriétés différentes en fonction de la nature de la tête polaire, la longueur des chaînes d'acide gras et le nombre d'insaturations. Cette diversité de composition des chaînes acyles peut entraîner des perturbations et des modifications de structure au niveau de l'intégrité de la membrane liposomale.

Les analyses des spectres Raman d'un système liposomal composé de DOPC, POPC et DPPC montrent peu de variation significative des vibrations d'élongation ν_{C-C} , ν_{C-H} en présence de calcéine encapsulée, en comparaison avec le vecteur seul. Une légère augmentation de l'intensité du ratio des bandes méthyles ($I_{2935/2880}$) et alkyles ($I_{1095/1130}$) des liposomes en présence de calcéine indique une augmentation du degré de désordre au niveau des chaînes alkyles. Une prépondérance de la conformation gauche a été observée dans le système calcéine-liposome par rapport au vecteur seul.

L'intensité des spectres Raman, relatifs aux vibrations d'élongation de la liaison ν_{C-N} augmente en présence de la calcéine encapsulée, notamment à 715 cm^{-1} indiquant une interaction significative entre le groupement choline et la calcéine.

En présence de calcéine, un léger décalage des isothermes π -A vers des aires moléculaires plus importantes est observé pour toutes les monocouches. Une diminution de l'aire moléculaire pourrait être attribuée à une diminution des effets électrostatiques entre les chaînes acylées. De plus, une diminution des pressions de la valeur de π_{coll} des monocouches en présence de calcéine reflète une diminution de la stabilité du film moléculaire.

L'analyse des isothermes de compression du film moléculaire en présence de calcéine montre une diminution des pressions de surface qui se traduit par une déstabilisation de l'arrangement moléculaire des chaînes acylées.

En effet, la calcéine peut s'insérer entre les chaînes d'acyles et modifier l'intégrité de la membrane. Ce phénomène est d'autant plus marqué en présence d'acides gras polyinsaturés issus de la formulation liposomale. Différentes formulations lipidiques ont été testées pour étudier les interactions entre la biomolécule et son vecteur, en tenant compte des paramètres

environnementaux. La maîtrise de la formulation et des propriétés physicochimiques de ces systèmes vecteurs permet de contrôler le relargage de molécules d'intérêt (médicament, molécule antibactérienne) en fonction de la nature des applications visées.

III. Résultats & Discussion

***Chapitre III.III: Mécanisme de transfert de molécules hydrophiles dans
une bicouche liposomale***

Objectives

Targetability is an important attribute of the lipid vesicles. Targeting bioactive agents is necessary to obtain adequate concentration of bioactive at the target site for their optimum efficacy. Targeted release increases the effectiveness of bioactive, broadens their application range and ensures optimal dosage, thereby improving the cost-effectiveness of the product.

A main process in bioactive delivery and targeting using liposome technology is the mechanism of material transfer through the liposomal lipid bilayer. The release of efficacious dose of liposome-entrapped bioactive depends on the permeability of the liposomal formulation with respect of the entrapped bioactive.

It is well known that bioactive agents have to pass several membrane barriers for exerting their suitable effects. These barriers affect on their pharmacokinetic and nutraceutical behavior and their capability to access the target site.

We used calcein as a model of hydrophilic drug to study molecular transfer through liposomal bilayer. The permeability of calcein across liposomal membranes also evaluated based on the assumption of the first-order kinetics by spectrofluorometer. Furthermore, the lipid composition effect on calcein release was investigated.

***Comportement de libération de la calcéine,
Estimation des paramètres de libération temporelle dans des bicouches
liposomales de différentes compositions***

Résumé

L'objectif d'un système de libération d'agents médicamenteux est d'administrer un médicament à une concentration thérapeutique au niveau d'un site d'action spécifique pour une durée déterminée. Le mode d'administration du médicament dépend de plusieurs paramètres. L'un des plus notables est le profil de libération du médicament qui détermine le site d'action spécifique, la concentration du médicament au moment de son administration et la période durant laquelle il doit rester à une concentration thérapeutique.

Afin de mieux comprendre les processus liés à la libération de médicaments, des liposomes unilamellaires de grande taille contenant de la calcéine ont été préparés à partir de 1,2-dioléoyl-sn-glycéro-3-phosphocholine, de 1-palmitoyl-2-oléoyl-sn-glycéro-3-phosphocholine, de 1,2-palmitoyl-sn-glycéro-3-phosphocholine ou d'un mélange de chacun d'eux; la calcéine ayant été choisie comme modèle de médicament hydrophile. Au cours de cette étude, la perméabilité membranaire des liposomes vis-à-vis de la calcéine a d'abord été mesurée sur la base d'une cinétique d'ordre 1 par spectrofluorimétrie. Dans un second temps, la composition/fluidité des liposomes ainsi que les effets de la température d'incubation/pH ont été évalués.

De plus, les conditions de digestion régnant dans le tractus gastro-intestinal humain ont été reconstituées afin de simuler la digestion gastro-duodénale et de suivre la libération de la calcéine au cours du processus de digestion. Le modèle de digestion *in vitro* "pH stat" a été utilisé afin d'examiner systématiquement l'influence du pH/enzyme sur la digestion des liposomes phospholipidiques au cours de la simulation de la digestion gastro-duodénale.

Les résultats ont montré que la calcéine perméabilise les membranes liposomales sans entraîner de rupture membranaire. Le taux de libération de la calcéine à partir des liposomes dépend du nombre et de la fluidité des bicouches membranaires et de leurs propriétés physicomécaniques telles que perméabilité et flexion élastique. Les propriétés chimio-structurales des médicaments telles que coefficient de partition (Log P), liaisons hydrogènes

(H-bonding), polarité de surface (PSA) sont aussi des paramètres déterminants dans le comportement de libération du médicament.

Enfin, dans le but d'avoir une meilleure connaissance des phénomènes de translocation de la calcéine à travers les bicouches membranaires, des observations par microscopie de déplétion par émission stimulée (« stimulated emission depletion –STED- microscopy ») ont été effectuées.

Calcein release behavior;
Parameter estimation of the release time course in liposomal bilayers
composed of different lipid compositions

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Abstract

The goal of drug delivery system is to administer a drug at a therapeutic concentration to a site of interest for a specified period of time. The design of the drug delivery depends upon different parameters. One of the most important factors in design of the drug delivery is drug-release profile which navigates the site of interest, the concentration of the drug at the time of administration, the period of time that the drug must remain at a therapeutic concentration.

To get a better understanding of drug release, large unilamellar liposomes containing calcein were prepared using 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-choline and 1,2-palmitoyl-sn-glycero-3-phosphocholine, and a mixture of them; calcein was chosen as a model of hydrophilic drug. In this study, the permeability of calcein across liposomal membranes was first evaluated based on the assumption of the first-order kinetics by spectrofluorometer. Second, the composition/fluidity effect of liposome as well as the incubation temperature/pH effect was investigated.

Furthermore, we simulated the digestion condition in the gastrointestinal tract in humans, to mimic human gastro-duodenal digestion by monitoring calcein release during the course of the digestion process. In vitro digestion model “pH stat” was used to systematically examine the influence of pH/enzyme on phospholipid liposomes digestion under simulated gastro-duodenal digestion.

The results revealed that calcein permeates through liposomal membrane without membrane disruption. The release rate of calcein from the liposomes depends on the number and fluidity of bilayers and its mechanical/physical properties such as permeability, bending elasticity. Chemo-structural properties of drugs such as partition coefficient ($\text{Log } P$), H-bonding, polar surface area (PSA) are also determinative parameters in release behavior.

Finally, we used stimulated emission depletion (STED) microscopy to monitor calcein translocation through the bilayers,

Keywords: polar drugs, diffusion, hydrophobic thickness, area modulus, photo-bleaching.

1-Introduction

Currently, many efforts in the field of drug delivery have been made to develop the targeted delivery systems in which the drug is only active in the target site and to formulate the sustained release systems in which the drug is released over a period of time in a controlled manner [1].

The goal of drug delivery system is also to administer a drug at a therapeutic concentration to a particular site of action for a specified period of time. The design of the final product for drug delivery depends upon several parameters. a) The drug must be administered by considering some factors which affect therapeutic action of the drug. These parameters include the site of action, the concentration of the drug at the time of administration, the period of time that drug must remain at a therapeutic concentration, and the initial release rate of the drug for controlled release systems. b) The drug must remain physically and chemically stable in the formulation for a defined time. c) The choice of delivery method must indicate the effective administration route for the drug [1].

In the last decade, phospholipid liposomes have become increasingly popular as vehicles for systemic delivery of drugs, enzymes and genetic material. Due to the widespread use of sterically stabilized liposomes, the inherently short circulation times of conventional liposomes do no longer constitute a problem [2].

In recent years, the study of controlled release of drugs and other bioactive agents from carrier systems has attracted many researchers from around the world. When seeking to develop efficient liposomes for drug delivery, a low leakage of the encapsulated substance is often a prerequisite in order to minimize unwanted side effects while the liposomes are in circulation. However, once the liposomes have reached their destination, e.g. the interior of a target cell, the cargo needs, for most applications, to be quickly and efficiently released. The need for a quick release arises from the fact that (1) the active substance is normally prevented from exercising its therapeutic effect while still being enclosed in the lipid carrier and (2) liposomes internalized via the endocytotic pathway often end up in lysosomes where the action of degrading enzymes may decrease, or even destroy, the biological activity of the delivered substance [3].

Indeed, liposome properties vary substantially with composition, size, surface charge and preparation method. It is obvious that the design and development of drug carriers is a difficult issue because they have to behave as biocolloidal systems after administration. Physicochemical properties of drugs are also a critical subject in the design of the delivery

systems [4, 5]. As drug solubility and drug dissolution are of crucial importance for orally administered drugs, to achieve the desirable systemic exposure after oral dosing, the active drug substance should be in solution in the aqueous environment of the intestine in order to cross the luminal wall. Additionally, the amount of bioactive penetration through lipid bilayers depends on bioactive structure and the molecular packing of the lipids. The partition coefficient of bioactive also depends on vesicle size and relates to differences in the curvature and the area compressibility of different vesicle structures [1].

A main process in bioactive delivery and targeting using liposome technology is the mechanism of material transfer through the liposomal lipid bilayer. The release of efficacious dose of liposome-entrapped bioactive depends on the permeability of the liposomal formulation with respect to the entrapped bioactive [1].

Several approaches are employed in order to obtain liposomes that are non-leaky during circulation and yet capable of rapidly releasing their contents upon reaching their organ, tissue, or cell of destination by a variety of mechanisms. The rationale behind the approaches varies but they have generally one thing in common; the liposomes are made from lipid components, or lipid mixtures, that in response to a given change in conditions develop a propensity to form leaky or non-lamellar structures. Recently, a number of methods have been developed for the modification of the liposome surface to enhance the temperature / light / pH sensitivity of the vesicles which release their loading in response to external stimuli [6].

Diffusion-controlled drug release model which controls the drug release by the drug solubility and diffusion coefficient in the release medium is an alternative approach. At the steady state process, the drug release rate remains constant to result in a zero-order (constant) release. In this system, a drug is usually dispersed inside liposomal bilayer, and is released without any rate-controlling barrier layer. During the migration of drug molecules from the surface to longer distances, the drug release rate decreases over time and results in a non-zero-order release. In recently developed controlled drug delivery technologies, it was identified that the zero-order release would be more desirable than other methods of drug release. In these systems, structural properties of carriers, drug solubility and diffusion coefficient in the release medium are the rate-limiting steps. The release of the entrapped drug from the liposome also depends on the number and fluidity of bilayers and its permeability [7-9]. However, a common mechanism for permeation of entrapped molecules across the phospholipid bilayer membrane has not been thoroughly discussed.

In this study, we used calcein as hydrophilic marker which has been notably used as a model for interactions drug / liposome and determining the encapsulation efficiency. Calcein is a water soluble and a fluorescent and self-quenching probe that is *widely used* in studies of cell viability and mitochondrial function by microscopy fluorescence imaging [10]. Calcein-release phenomena have been utilized as an effective index to characterize the membrane properties of (model) biomembranes and to evaluate their stability in a variety of conditions. In addition, the calcein-release phenomena have been investigated with regard to design of a drug-delivery system.

The purpose of this study was to determine the basic characteristics of calcein permeation as a model of polar- hydrophilic drug from the liposome to apply them in design of a drug-delivery system. In this study, the permeability of calcein across some liposome membranes was first evaluated on the basis of the first-order kinetics. The neutral phospholipids liposomes were used to rule out the contribution of the electrostatic interaction of lipid membranes and the negatively charged calcein. Second, the composition/fluidity effect of liposome as well as the temperature effect was investigated.

Furthermore, we have used an *in vitro* model designed to mimic closely human gastro-duodenal digestion to monitor calcein release during the course of the digestion process. Simulation of gastrointestinal conditions is essential to adequately predict the *in vivo* behavior of water soluble drugs. We simulated the digestion process in the gastrointestinal tract in humans, in a simplified manner by applying physiologically based conditions. *In vitro* digestion model “pH stat” was used to systematically examine the influence of pH/enzyme on phospholipid liposomes digestion under simulated gastro-duodenal digestion.

Finally, in an attempt to obtain a better understanding of calcein translocation through the bilayers, we used stimulated emission depletion (STED) microscopy in this study. Unlike conventional microscopy, STED overcomes the normal diffraction limit and allows us to investigate calcein distribution within the confined medium.

2- Materials and Methods

2.1. Materials

Phospholipids used in this study were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), all purchased from Avanti Polar Lipids (Alabaster, AL, USA). 3,3-bis[N,N-bis(carboxymethyl)-aminomethyl] fluorescein (calcein) 1-(4-

trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) were acquired from Invitrogen (Paris, France). Rhodamine B1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Rhodamine-DHPE) was purchased from Interchim (Paris, France). Enzymes were purchased from Sigma (Paris, France) as freeze-dried powders: pepsin from porcine gastric mucosa (Sactivity: 3300 U/mg of protein), porcine pancreatic trypsin (activity: 13,800 U/mg of protein), bovine pancreatic α -chymotrypsin (Sactivity 40 U/mg of protein), bile salts (Bile extract porcine) and Bowman-Birk trypsin-chymotrypsin inhibitor. Phosphate buffer salts, monobasic sodium phosphate and dibasic sodium phosphate, were purchased from Sigma–Aldrich (Paris, France). All other reagents were of analytical grade.

2.2. Liposome Preparation

Large unilamellar vesicles (LUVs) were prepared as described elsewhere [11]. In brief, phospholipids were dissolved in chloroform solution. The organic solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under vacuum overnight, was hydrated with calcein solution to obtain multilamellar vesicles. Calcein was dissolved in pure water and pH was adjusted to 7.4 to obtain a final calcein concentration of 20 mM. Calcein concentration of 20 mM was used throughout the study unless otherwise stated. The suspension was subjected to 5 cycles of freezing and thawing to obtain Multilamellar Vesicles (MLVs) and then extruded through a polycarbonate filter (100-nm pore size filter, 11 times) above the phase transition temperature of the vesicles by using an Avanti-mini extruder (Avanti Polar Lipids, Alabaster, USA)[12][13]. Liposome suspension was eluted through a Sephadex-G75 column (10 mm \times 200 mm) which was thoroughly pre-equilibrated with phosphate buffered salts solution, to remove the non-encapsulated calcein. The lipid concentration in the final vesicle suspension was determined with an enzymatic assay kit (Test Wako-C) from Wako Pure Chemical Co. Ltd. (Osaka, Japan).

2.3. Particle size and ζ - potential determination

When designing liposome-based bioactive carrier systems, a reliable and reproducible analysis of their size and size distribution is important. Determination of vesicle size distribution is a fundamental quality control assay.

The mean diameter, particle size distribution and ζ - potential of vesicles were determined upon empirical dilution of the samples using dynamic light scattering (DLS) technique

employing a Zetasizer Nano ZS (Malvern instruments Ltd, UK). Viscosity and concentration of liposome suspension are important parameters for DLS. Viscosity directly affects the Brownian motion of nano-particles and thus the calculated liposome size result, so sample should be diluted to an appropriate concentration. The software used is DTS Nano, version 6.12 supplied by the manufacturer (Malvern instruments Ltd, UK). To avoid multiple scattering effects, liposome suspensions were diluted (1:25), and then sample was put in a standard capillary electrophoresis cell equipped with gold electrodes. All measurements were carried out at 25°C by considering a medium viscosity of 1.020 and medium refractive index of 1.333. Results are presented as an average diameter of the liposome suspension (z-average) with the polydispersity index (PDI) and ζ - potential (z- average) of the liposome suspension [14].

2.4. Phase Transition temperature determination

Liposome suspension was analyzed with differential scanning calorimetry. Calorimetric scans from - 30 to 90 °C were performed on Netzsch 204 F1 (Netzsch-Gerätebau GmbH, Germany) at a scanning rate of 5 °C per minute. The software used was Proteus Analysis, version 4.8.5 supplied by the manufacturer (Netzsch-Gerätebau GmbH, Germany).

2.5. Membrane fluidity

Liposomal membrane fluidity was determined as fluorescence polarization (P) by measuring the fluorescent intensity of 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene(TMA-DPH), according to the conventional method. The fluorescence probes were oriented into the lipid bilayer by the following method. The solution of TMA-DPH (in ethanol) was added to the liposome suspension to maintain the lipid/probe molar ratio at 250 ($[TMA-DPH]_{final} = 4 \mu M$). The mixture was then incubated for at least 1 h at room temperature with gentle stirring. The fluorescence probe was vertically oriented in the lipid bilayer. The amount of probe remaining in

the external aqueous medium was negligible due to high lipophilicity coefficient, and the non-incorporated TMA-DPH was non-fluorescent due to aggregation. The fluorescence intensity (FI) of samples was measured with a Perkin-Elmer LS 55B Spectrofluorometer equipped with fluorescence polarizers (Perkin-Elmer, Waltham, USA). Samples were excited at 360 nm, and emission was registered at 430 nm under constant stirring at the temperature of 25 °C (PTP-1

temperature controller). The P value of TMA-DPH was calculated from the following equation (1):

$$P = \frac{I_{\parallel} - G \cdot I_{\perp}}{I_{\parallel} + 2G \cdot I_{\perp}} \quad (1)$$

Where I_{\parallel} is the intensity of fluorescence parallel to excitation plane, I_{\perp} is the intensity of fluorescence perpendicular to excitation plane and G -factor that accounts for transmission efficiency. The reciprocal value of polarization ($1/P$) was defined as membrane fluidity [15].

2.6- pH- Dependent calcein fluorescence intensity

The calcein – encapsulated liposome suspensions after separation by gel sephadex G-75, were diluted (1:20) in pre-adjusted phosphate buffer salts solutions (pH ranging from 2.4 – 10.4). The change of fluorescence intensity of samples was monitored at 525 nm with excitation at 494 nm. The percent release of calcein was determined as follows:

$$\% \text{ release} = \frac{F_t - F_i}{F_f - F_i} \times 100 \quad (2)$$

Where F_t and F_i are the intensity of fluorescence at a given pH and at pH 7.4, respectively. F_f is the total fluorescence after adding triton X-100 (3% v/v). Since the intensity of the fluorescence strongly depends on pH, F_t was corrected by using calibration curves [16].

2.7- Calcein-release measurement

Calcein release from the liposome membrane was evaluated according to a previous report [17]. Liposomes containing calcein (20 mM) were prepared in a manner similar to that described above. Untrapped calcein molecules were removed from the calcein containing vesicles by Sephadex-G75 column (10 mm × 200 mm) which was thoroughly pre-equilibrated with phosphate buffered salts solution.

The fluorescence intensity of the liposomes containing entrapped calcein was measured and found to show low fluorescence due to its self-quenching. The calcein-release experiments were initiated by mixing a liposome suspension with a buffer solution to yield 0.1mM lipid to monitor the calcein release. The change in fluorescence intensity due to calcein release from the vesicles was monitored with Spectrofluorometer SAFAS (FLX –Xenius, Monaco) at 490

nm excitation and 520 nm emission, respectively. The amount of calcein released after time t was calculated according to Equation (3):

$$Rf(\%) = 100(I_t - I_0)/(I_{max} - I_0) \quad (3)$$

where Rf is the fraction of calcein released, I_0 , I_t and I_{max} are the fluorescence intensities measured at the beginning of the experiment, at time t , and after the addition of 3% triton X-100, respectively [17, 18]. Some of molecular descriptors of calcein and PCs were calculated by ChemSpider calculator using SMILES notations or chemical structure inputs.

2.8- Simulated gastric digestion

The digestion model was simulated on *in vitro* model presented in previous studies [19, 20]. Briefly, sample was added in simulated gastric buffer (0.15 M NaCl, pH 2.5, 1 mg/ml) and pH was adjusted to 2.5 with 0.5 M HCl. After incubation at 37 °C for 10 min, pepsin (gastric buffer, pH 2.5, 5 mg/ml) was added (100 μ l), to give 165 U of pepsin per mg of protein in the final digestion mix containing 10 mg of protein and 0.5 mg of pepsin (pepsin: protein ratio of 1:20, w:w).

Samples were placed in an orbital shaking incubator (170 rpm, 37 °C) and aliquots (100 μ l) withdrawn after 0.5, 1, 2, 5, 10, 15, 20, 30, 40, 50 and 60 min. Digestions were stopped by raising the pH to 7.5 by addition of 0.5 M ammonium bicarbonate (20 μ l), to irreversibly inactivate pepsin. All assays were performed in triplicate [21].

2.9- Simulated duodenal digestion

Digests resulting from 60 min of *in vitro* gastric digestion (5 ml) were used as the starting material. The pH was adjusted to 6.5 with 0.1 M NaOH and solutions of bile salts, Bis-Tris, and enzymes were added to give the following: 4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM Bis-Tris buffer pH 6.5, 0.4 U per mg of test protein bovine α -chymotrypsin, 34.5 U per mg of test protein porcine trypsin. This gave enzyme: test protein ratios of 1:400 (w:w) for trypsin and 1:100 (w:w) for chymotrypsin (5 mg of protein incubated with 2.5 μ g of trypsin and 0.05 mg of chymotrypsin). Digestions were performed in a shaking incubator (170 rpm, 37 °C) for 30 min and aliquots (100 μ l) taken at 0.5, 1, 2, 5, 10, 15, 20, 25 and 30 min for further analysis. Reactions were stopped by adding a solution of Bowman-Birk trypsin-chymotrypsin inhibitor dissolved in 150 mM NaCl (pH 6.5) from soybean (Sigma) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix.

2.10- Introduced stimulated emission depletion (STED) microscopy study

Stimulated emission depletion (STED) microscopy is a recently developed optical technique that breaks the classic resolution limit of light microscopy. STED microscopy is a fluorescence microscopy technique that uses the non-linear de-excitation of fluorescent dyes to overcome the resolution limit imposed by diffraction with standard confocal laser scanning microscopes and conventional far-field optical microscopes. Therefore, it is also described as a form of super-resolution microscopy. It offers the ability to shed light on cell biological processes inside neurons and synapses that have been out of reach for confocal and two-photon microscopy, whose spatial resolution is limited by the diffraction of light. The resolution of such a microscope is limited to the spot size to which the excitation spot can be focused. This size depends on system parameters, but is limited by approximately half the wavelength of the light used. Nearby structures in the focal plane with a distance smaller than about 200 nm cannot be resolved [22-24].

With STED continuous wave (CW) technique, it is possible to study structures with a resolving distance lower than 90 nm. The phase plate, a doughnut shaped area is generated in the focal plane. With the illumination of two superimposed laser (one for excitation and one for depletion) the emitting molecules within this area are forced back to the ground state at the same wavelength than the depletion laser, which is eliminated by a notch filter. The resulting fluorescence signal comes from an area that is smaller than the diffraction limit. The images were recorded at ambient conditions (23°C and 50% RH) with a 100x STED objective (PL APO, 100x, Oil, 1.4-NA, Leica Microsystems).

An auto alignment was processed just before the experiment, to make sure that depletion doughnut and laser beam are correctly aligned.

Our setup used two synchronized trains of laser excitation: a 488 nm laser (6% power of 100 mW Argon laser) was used for calcein excitation and a 592 nm laser (coherent, 100% power of 1.5W VFL Depletion laser) was used for stimulated emission depletion (STED). A notch filter was then used to avoid detection of stimulated emission and calcein fluorescence signal was detected on a photomultiplier tube in the 500 nm to 550 nm spectral window.

For STED experiment, the studied liposome suspension was eluted through a Sephadex-G75 column (10 mm × 200 mm) which was thoroughly pre-equilibrated with phosphate buffer salts solution to remove the non-encapsulated calcein.

For STED studied, fluorescent-labeled liposomes were similarly prepared as the method described in previous studies [25, 26]. Briefly, the phospholipids, such as 1mM POPC were dissolved with Rh-PE (1 mol% lipid) in chloroform in round bottom-flask before evaporation. More, fluorescent-labeled liposomes preparation was continued as described above. Finally, liposome suspension was eluted through a Sephadex-G75 column (10 mm × 200 mm) which was thoroughly pre-equilibrated with phosphate buffered salts solution to remove the non-encapsulated calcein.

A drop (100 µl) of diluted liposome suspension (1:10) was deposited in the middle of clean glass microscope slide and after the cover slip was placed gently over the drop at an angle, with one edge touching the slide first. The sample was spread out between the two pieces of glass without applying pressure. The excess samples were removed by soft facial tissue. Immediately, the sample was observed by microscope.

2.11- Statistical analysis

The presented results are the averages of three complete and independent experiments. Data were reported as mean ± SD. One-way ANOVA was employed to identify differences in means, using *SPSS software* (*SPSS for Windows, Rel.10.0.5. 1999; SPSS Inc., Chicago, IL*). Statistical significance was declared at $P < 0.05$.

3. Results and discussion

3.1. pH-Dependent Calcein fluorescence intensity

The calibration curves of calcein fluorescence were obtained at various pH values, where the degree of release was observed. The slope of each curve decreased with decreasing pH, which means that the intensity of fluorescence is suppressed at acidic pH values. With these curves, the fluorescence intensities were corrected (Figure 1). The fluorescence intensity (FI) at the various pH values should be converted to the intensity at pH 7.4, since the liposomes were prepared at pH 7.4 but the release experiment was performed at various pH values different from pH 7.4.

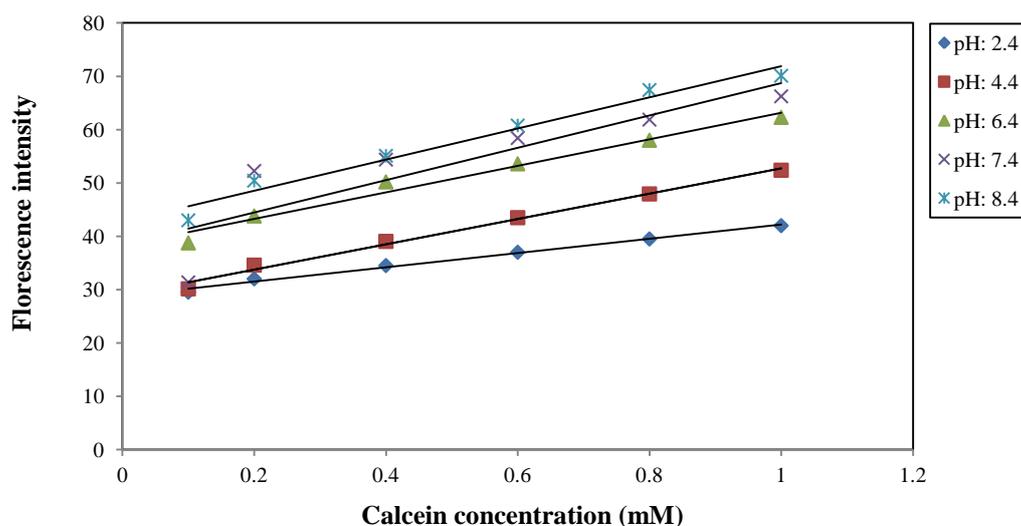


Figure 1. Calibration curve of calcein fluorescence at various pH values (2.4 – 8.4).

3.2- Calcein release behavior study

The benefits of liposome in drug research critically depend on manner and mechanism of release of bioactive agent. Furthermore, in order to achieve specific targeting, they should release their contents in special sites with effective doses. Selection of a controlled drug delivery technology suitable for each drug depends on many factors, including physicochemical properties of the drug, duration of release and the release profiles [1].

Currently there are two different models proposed for drug translocation across membranes, i.e. active and passive translocation.

Passive translocation is dominated by diffusion across and out of the membrane, without the consumption of extra energy. This process is without attendant carrier and maybe is the main mechanism by which considerable quantity of drugs diffuse through lipid membranes into the body. Generally, passive translocation exhibits low structural specificity and is due to a concentration gradient across the membrane [1].

We first characterized the amount of released calcein with regard to the size, fluidity, permeability of liposomes as well as temperature and pH of medium.

In this study, we observed that calcein released according to a zero-order release mechanism because of the osmotic pressure inside the liposome. The calcein molecules migrate into the environment through the vesicle membranes with physical mechanisms include diffusion. One of the main advantages of physical mechanisms is that the drug release kinetics can be controlled by the drug delivery system itself. Each drug delivery system has predetermined drug release kinetics that can be adjusted by varying simple parameters, such as thickness of

the carrier membrane, type of carrier used and surface area. Figure 2, shows a typical example of the time-course of the RF values of calcein from liposomes that were $\approx 117 \pm 2.1$ nm diameters.

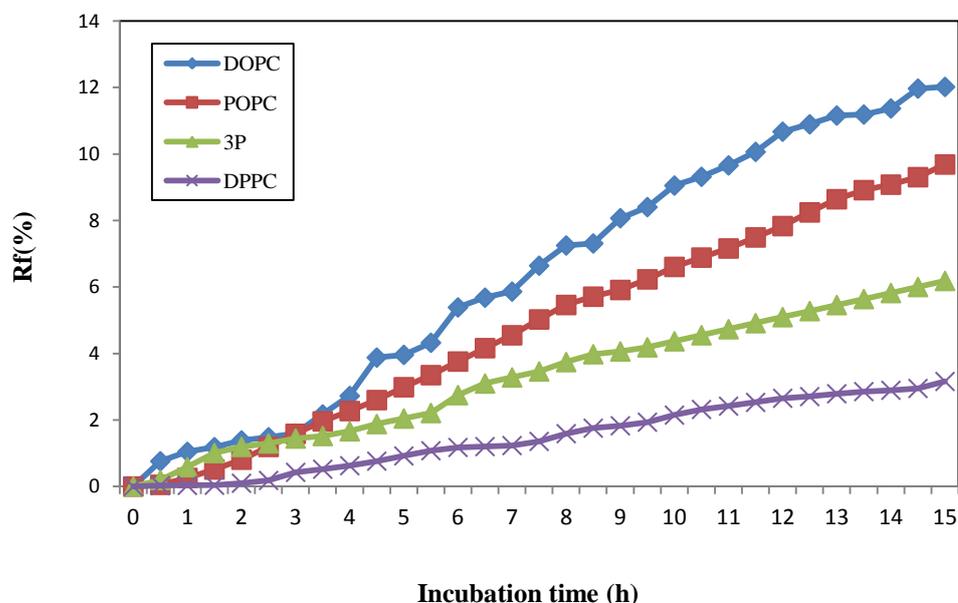


Figure 2. Time-course of calcein release from liposome with different compositions. Liposomes with 117 ± 2.1 nm diameter was prepared with a freeze/thaw method and extrusion method. The temperature was 25 °C. The data with standard error were obtained from at least three independent experiments. 3P represents liposome formulation mixed of lipids.

3.3- Theories and Models for explaining the liposome permeability to calcein

The most generally accepted model to describe the permeation of small neutral permeant across lipid bilayer membranes is the solubility-diffusion model. Solubility-diffusion theory depicts the bilayer membrane as a thin, homogeneous slab of bulk organic material into which the permeant must partition and diffuse across [27].

In the following section, the theories and models for explaining the permeability of membrane are discussed:

- Theory 1:

Whereas neutral vesicles have surface potential is zero, calcein solution has negatively surface potential. Apart from its fluorescent properties, the calcein molecule is a polyanion. An acid-base titration of calcein gives about 3.5 negative charges per molecule at pH 7.4. Thus, it has the effect of significantly increasing ionic strength with respect to its osmotic properties.

Therefore, calcein causes a great difference in osmolarity values of internal and external of liposome.

In fact, internal surface potential can influence the external one, because the distance between the two leaflets is very small (about 4 nm). Where the differences between internal and external ionic strength are very great, it is considered as a noticeable effect and an internal stimuli for calcein release. This phenomenon is due to a time-dependent random process for calcein release.

By considering the Time –course of calcein release behavior, we analyzed the permeability coefficient (P_s) values of four lipid membranes with different compositions (Table 1), based on the kinetics analysis of calcein fluorescence according to the Equation (4):

$$RF_{(t)} = RF_{max} (1 - \exp(-kt)) \quad (4)$$

The apparent rate constant k was obtained and the resulting permeability coefficient to calcein was calculated by using the Equation (5):

$$P_s = (r/3)k \quad (r: \text{the radius of liposome}) \quad (5)$$

Table1. Summary of properties of liposomes made from various phospholipids and their permeability to calcein

Lipid	T_c (°C)	K	$(1/P)_{TMA-DPH}^{(-)a}$	P_s (cm/s) ^a
DOPC	-18.9 ± 1.2	1.255×10^{-5}	3.35 ± 0.4	$2.64 \times 10^{-11} \pm 0.3$ cm/s
POPC	0.3 ± 0.70	7.673×10^{-6}	2.81 ± 0.2	$1.49 \times 10^{-11} \pm 0.2$ cm/s
Mixture of lipids	10.9 ± 1.5	7.53×10^{-6}	2.56 ± 0.8	$1.40 \times 10^{-11} \pm 0.15$ cm/s
DPPC	39.8 ± 1.9	2.784×10^{-6}	2.44 ± 0.3	$0.545 \times 10^{-11} \pm 0.18$ cm/s

^a All experiments were carried out at 25 °C.

-Theory 2:

Most results have relied on the “size / lipophilicity rule”, which originates from the solution-diffusion model for bilayer transport [28]. According to this model (Equation 6), the permeability coefficient, P_m , of a drug through membranes is directly proportional to its water-lipid partition coefficient, K_m and the membrane diffusion coefficient, D_m of the solute and inversely proportional to the membrane thickness ($L \sim 30$ Å for the hydrocarbon domain of the bilayers). Although K_m is the major source of variation to drug permeability, passive drug diffusion through membranes also depends on D_m [29]:

$$P_m = \frac{D_m \cdot K_m}{L} \quad (6)$$

Furthermore, D_m significantly depends on the molecular size or molecular weight of the drug. As molecular weight increases, D_m dramatically decreases such that very few of the commercially available drugs have high molecular weight [29].

The lipid solubility of a drug in systems such as the liposome/water partition coefficient (K_p), remains a primary characteristic accounting for quantitative structure/activity relationship studies (QSAR). The value of K_p determines the drug's distribution between the aqueous and lipid phases, and thereby the extent of penetration into the membrane and/or interactions with phospholipids or other membrane components [30, 31].

- *Theory 3:*

Joguparthi *et al.* [32] determined that the partition coefficient is independent of drug and lipid concentration. They suggested that the simplest method and model is the bulk-solubility diffusion model which assumes that the membrane is homogenous and isotropic. The permeability coefficient derived from this model is according to Equation 7:

$$P_0 = \frac{PC_{m/w}D_m}{h_m} \quad (7)$$

Where P_0 is the permeability coefficient, $PC_{m/w}$ is the membrane/water partition coefficient, h_m is the thickness of the membrane and D_m is the diffusion coefficient [58].

3.3. Effective Parameters on calcein-release behavior

It is worthy to know how drugs pass several membrane barriers for exerting their pharmacotherapeutic effects. These barriers affect the pharmacokinetic behavior of drugs and their capability to access the target site. In addition, partitioning always occurs because of drugs' binding to membrane receptors and transporters, so prediction of drug-membrane permeability is important for optimum efficacy [33].

- *Effect of liposome size and lamellarity*

According to the obtained results, the liposome size is an effective factor on calcein release. By increasing liposome size, permeability increases in liposomes with preserved unilamellarity. Whereas in large liposomes, permeability of hydrophilic bioactive molecules

decreases. It is generally known that liposomes larger than 200 nm in diameter tend to have a multilamellar structure and since increasing the lamellarity could result in permeation resistance, the P_s value is expected to decrease significantly. The release of amphiphilic drugs from unilamellar liposomes is greater than from multilamellar liposomes with similar size. We also concluded that the curvature of small unilamellar liposomes is greater and consequently the packing between the lipids because of acyl chain orders in these membranes, is weaker. Shimanouchi *et al.* [15] also demonstrated that the liposome size is a factor determining the permeability of the unilamellar vesicles. They observed a uniform decrease in the P_s by increasing the liposome size.

Furthermore, Zhang *et al.* [34] also concluded that the release of the amphiphilic drugs (such as 5-carboxyfluorescein) from the unilamellar liposomes was greater than from multilamellar liposomes with similar size.

Recently, some experimental data acquired by ^{31}P -NMR analysis and trap volume measurements designated that increasing the number of lipid bilayers in liposomes increases the particle size, forms more effective barriers and consequently slows the release of drugs [35].

Olbrich *et al.* [36] also found that the membrane rigidity calculated using micropipette aspiration for multilamellar vesicles (MLV) is ~ 3 times larger than unilamellar vesicles (ULV).

- Influences of liposome composition and structure on permeability

The properties of the liposomes vary substantially with composition, size, surface charge and preparation method. It is obvious that the design and development of drug carriers is a difficult issue because they have to behave as biocolloidal systems after being administered.

We address the composition of lipids and their structure and comment on the physico-chemical forces present in the membrane which may impact calcein release.

- *Membrane fluidity*

The obtained results showed that the fluidity of the hydrocarbon portion of the membrane is very important. The double bonds within the acyl chain resulted in a decrease in the packing density, which in turn perturbed the barrier to calcein permeation. Therefore, the liposomal membranes in the liquid-crystalline phase formed at temperatures above the phase-transition, show P_s values larger than those in gel-crystalline phase (Table 1). We calculated the

parameters of calcein release by considering the first-order kinetic and we found our results in good agreement with experimental and theoretical results presented by Shimanouchi *et al.* [15].

We also observed that the rate of calcein release increases with increasing the number of double bonds in liposomal bilayer as well as with increasing the fluidity values of liposome membrane. Calcein release rate from liposome appears to decrease in the order of DOPC > POPC > Mixture of lipids > DPPC.

The double bond in the normal *cis* configuration which is asymmetric, leads to a kink of the hydrocarbon chain. Consequently, the unsaturated lipids are packed together loosely, or crystallize less readily compared to the straight chain saturated lipids. Recent studies by x-ray scattering have found that the areas per lipid molecule of monounsaturated lipids are larger than those of the corresponding saturated lipids with the same chain length [37].

- *Phase transition temperature of bilayer*

Furthermore, transition temperature of liposome is another determinative factor in drug delivery application which depends on the nature of the hydrocarbon chains (acyl chain length, structure and degree of unsaturation of the hydrocarbon chains and presence of a methyl branch) (Table 1). The effect of the double bond on the temperature of the gel-to-liquid-crystal phase transition was greatest when it was situated in the middle of the chain. The main transition temperature (T_c) of saturated phospholipid bilayers is known to be proportional to the length of the alkyl chains, as by increasing the length of acyl chain, T_c increases. This dependence originates from the balance between the interaction energy of the head groups, which is constant, and the total interaction between the chains, which decreases with alkyl chain length [37]. Furthermore the change of hydrophobic thickness also has been linked to such properties as ion permeability[38].

- *Membrane mechanical properties*

In comparison to membrane mechanical properties of vesicles made from PC_s, our obtained results showed that the surface compressibility modulus (C_s^{-1}) increases in monolayers composed of saturated phospholipids which is an indicator for rigid monolayers (Data not yet published).

Yi *et al.* [37] confirmed that, contrary to the strong effect on the main transition temperature, the monounsaturations has a limited influence on the bending elasticity of lipid bilayers. In

addition, when the area modulus (K_A) varies little with chain unsaturation or length, the elastic ratios $(K_c/K_A)^{1/2}$ of saturated and monounsaturated phospholipids bilayers varies linearly with lipid hydrophobic thickness d which agrees well with the theory of ideal fluid membranes. The bending elasticity is an important mechanical property that governs the thermal fluctuations of bilayers, gives rise to undulation forces and predetermines the contact time of the membranes with solid substrates and other objects [39].

The obtained results have shown that the structural changes caused by the double bond such as reducing the hydrophobic thickness and increasing the area per molecule, lead to decreasing of the bending elasticity. Since the bending elasticity is related to the undulation of the membrane tiny structural changes of phospholipid conformations only give rise to small changes in the dynamics of biomembranes. The recently obtained results showed that the bending elasticity of the monounsaturated bilayer in the liquid crystalline phase is slightly smaller than that of the saturated one. However, the monounsaturation has a strong effect on the transition temperature, which leads to a reduction of the transition temperatures of membranes without changing the mechanical properties of total lipid bilayers. It seems that the variations in bending elasticity (K_c) in different phospholipids bilayers are due to the differences in bilayer hydrophobic thickness [37, 40]. For fluid membranes K_c is controlled by two independent parameters K_A and d . On the other hand K_A is independent of the hydrophobic thickness d and depends strongly on the interfacial structures.

As, we mentioned above, the presence of the double bond due to reduction of the hydrophobic thickness and increasing the area per molecule. Therefore, bending elasticity $K_c \sim 0.41 \pm 0.01$ (10^{-19} J) of mono-saturated 18:1 PC bilayer with hydrophobic thickness $d \sim 2.70 \pm 0.1$ nm and area modulus $K_A \sim 0.236$ (Nm^{-1}) are smaller in comparison to $K_c \sim 0.62 \pm 0.01$ (10^{-19} J) of saturated 18:0 PC bilayer with $d \sim 2.95 \pm 0.1$ nm and $K_A \sim 0.235$ (Nm^{-1}) (Table 2) [37].

These data confirmed that the rate of calcein release increases with increasing the number of double bonds.

Table 2. Bending elasticity K_c , area modulus K_A and bilayer hydrophobic thickness d of saturated and monounsaturated bilayers (table was adapted from the [37]).

Lipid	K_C (10^{-19} J)	K_A (Nm^{-1})	d (nm)
16:0 PC	0.44 ± 0.01	0.234	2.60 ± 0.1
16:1 PC	0.38 ± 0.02	0.250	2.35 ± 0.1
18:0 PC	0.62 ± 0.01	0.235	2.95 ± 0.1
18:1 PC	0.41 ± 0.02	0.236	2.70 ± 0.1

Ipsen *et al.* concluded that the orientational order parameter and the hydrophobic thickness are linearly related. The maximum possible value of the hydrophobic thickness is associated with molecules in the all-trans conformation of acyl chains oriented parallel to the bilayer normal [41].

Rawicz *et al.* [42] also used micropipette approach to quantitate mechanical stretch properties of bilayers. They observed that the direct stretch moduli varied little with either chain unsaturation or length. On the other hand, the bending moduli of saturated/monounsaturated chain PCs increased progressively with chain lengths. Furthermore, the bending rigidity increased in a steady and a progressive manner with the number of carbons for saturated / monounsaturated chain bilayers (Table 2) [42].

Lipids with a high degree of unsaturation in their hydrocarbon tails have been found to reduce the bending rigidity of a vesicle by a factor of two compared to their saturated counterparts, while leaving the membrane's area stretch modulus approximately unchanged [43]. The balance Langmuir studies have been shown that monolayers of lipids with asymmetric chains are also less rigid than those composed of lipids with equal length tails [44]. Feng *et al.* [45] also found that the interfacial elastic modulus increases with the number of double bonds. It means the bilayer composed of unsaturated lipids tend to be deformed elastically in the passing of calcein. This deformation elastically increases the rate of calcein passage in comparison to rigid bilayer with lower elastic modulus.

Furthermore, the saturated lipid membranes have been exhibited low permeability to calcein. This means liposomes made from saturated lipids have remarkable thermal and mechanical stability. The low calcein permeability was attributed to tight and rigid packing of the lipids in these membranes. Permeability studies of model bilayer systems indicate that the region of the acyl chain adjacent to the head group is the site likely to offer the most resistance for water and solute permeation [46, 47].

We observed that in the case of hydrophilic drugs, liposome-membrane rigidity is the most important parameter that determines the release rate of the drug from liposomal bilayer. The permeation appeared to be controlled by the membrane fluidity. Our experimental results in good agreement with the results of Mourtas *et al.* [18], [48].

Xing and Anderson [49] proposed that chain ordering in lipid bilayers, which can be characterized by both segmental order parameters and bilayer surface density, is also another determinative parameter of molecular transport across lipid bilayers. Additionally, by

increasing chain ordering within lipid bilayers, solute partitioning into bilayers substantially reduces.

Calvagno *et al.* [50] also reported that the significant difference in the release profiles of drugs is caused by presence of two factors, i) the strength of the drug-liposomal lipid interaction, i.e. the stronger the interaction causes less desorption and increases the burst effect, ii) the fluidity of the bilayer, i.e. by increasing the fluidity of the bilayer, the drug leakage to outer liposomal aqueous compartments is rapidly increased.

- *Membrane structure*

More, Gazzara *et al.* [51] found that the penetration of the drugs depends on their structure and on the molecular packing of the lipids. The dependence of the partition coefficient on vesicle size related to differences in the curvature and the area compressibility of different vesicle structures.

Other studies confirmed that permeability of biological membranes and model lipid bilayers depend strongly on the degree of packing of lipid chains in the membrane and the size of the permeating solute. Membranes that are highly ordered show very low permeability and exhibit a steep dependence on size of the solute [34, 52].

Our findings also showed membrane permeability to calcein decreased with decreasing phospholipid chain length and correlated with the sensitivity of chain ordering and their rigidity. These findings are in good agreement with the results of Xiang and Anderson [53] which presented on membrane permeability to acetic acid.

Additionally, Nagayasu *et al.* reported that the inflection of small unilamellar vesicles (SUVs) compared with large unilamellar vesicles, is greater and conjunction between the lipids in the membranes of large unilamellar vesicles is more stable compared with small unilamellar vesicles.

In addition, they also found that the rate of release from liposomal formulations is drug-dependent [54].

Shimanouchi *et al.* [15] proposed that the main possible mechanism for permeating of water-soluble probe to across the simple phospholipid membrane could be diffusion through the hydrocarbon portion of the membrane. The drug must overcome the barrier of the hydrophilic part of the membrane.

The physicochemical properties of the target molecule can significantly impact the rate of release. The physicochemical properties of drugs are critical subject in the design of the

delivery systems. Solubility, stability, and pH of drugs can effectively change the mechanism of drug delivery in controlled delivery system.

- Effect of temperature

It is well known that membrane permeability reaches a maximum around a lipid's gel-to-liquid crystalline ($L\alpha$) phase transition temperature, T_c (i.e., the temperature at which the lipid's acyl chains melt). This increased permeability has previously been attributed to either or both of the following possibilities: (1) defects caused by mismatched gel and $L\alpha$, phase hydrocarbon chain domains; (2) strong density and thermal fluctuations resulting in increased lateral membrane compressibility, which lowers the energy barrier for molecules to pass through the membrane, or to create defects [55]. This sharp increase in membrane permeability at T_c provides the possibility of controlling the release of the liposome contents. The effect of heating temperature on the permeability of calcein was investigated at two temperatures of 25 and 50 °C. The P_s value for DPPC-liposome increased to 1.18×10^{-11} cm/s at 50 °C, above the phase transition temperature of DPPC ($39.8 \pm 1.9^\circ\text{C}$) as shown in **Figure 3**. This incremental trend in permeability for DPPC liposomes is related to the fact that DPPC-liposome reaches the liquid-crystalline phase at temperatures more than 41°C . In contrast, at 25 °C below T_c DPPC liposomes showed significantly smaller P_s value of 0.545×10^{-11} cm/s since liposomal membrane at the gel phase becomes semi-impermeable to calcein.

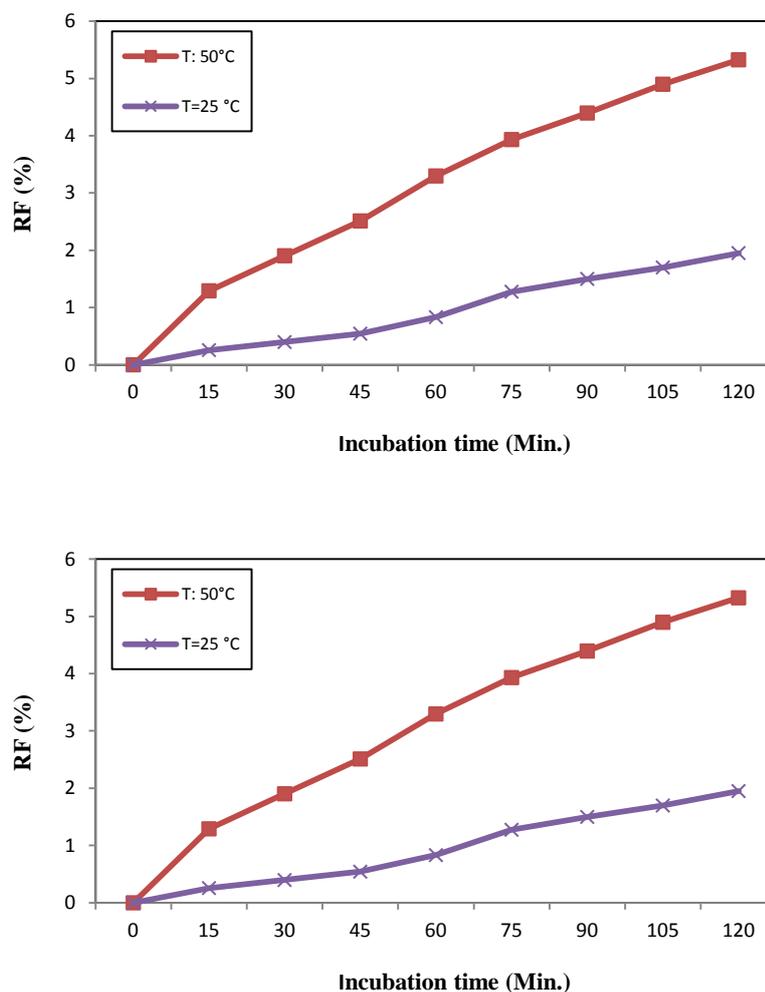


Figure 3. Temperature dependency of permeability to calcein of DPPC-liposome. The diameter of the liposomes were ~ 117 nm. The data with standard error were obtained from at least three independent experiments.

- Influences of drug properties

Physicochemical properties of drugs are also a critical subject in the design of the delivery systems [1]. Calvagno *et al.* (2007) have determined that one of the noticeable factors in the release profiles of drugs is the strength of the drug-liposomal lipid interaction, i.e. the strong interaction causes less desorption and so the burst effect [50].

Drug – liposome interaction depends not only on the partition coefficient of the drug but also on its functional groups such as hydrogen bonding sites and its polar surface area (PSA). PSA is the sum of surfaces of polar atoms in a molecule that reflects the physicochemical characters of all drugs [56].

PSA is a very useful parameter for prediction of drug transport properties. It is defined as a sum of surfaces of polar atoms (usually oxygens, nitrogens and attached hydrogens) in a molecule [57].

Increasing the polar surface area reduces the power of interaction with the lipid bilayer and raises the tendency for surface interaction with the head group. Especially for neutral compounds, the partition coefficient, $\log P$ is a good descriptor of lipophilicity. Considering the interactions of drugs with liposomes, they can be grouped into three categories; i) the very strong hydrophilic drugs, which are localized in the aqueous medium of liposomes and if they have a very high PSA, can display some interaction with the head groups, ii) the less hydrophilic drugs, more balanced molecules that adsorb at the water–lipid bilayer interface with some degree of penetration into the bilayer, and iii) the strongly lipophilic drugs, which locate in the bilayer itself [58]. Molecules with a polar surface area of greater than 140 angstroms squared tend to be poor at permeating cell membranes.

Calcein is a hydrophilic dye, which was expected to be encapsulated in the aqueous compartment of liposomes

Since, calcein with PSA ~ 231.67 higher than acyl chains shows strong hydrophilic property, can display some interaction with the choline head groups of phospholipids without any interaction with the lipid bilayers. This means the calcein diffusion could be happened without any interaction with lipid bilayers.

Another parameter to predict the diffusion of particles through biological membranes is lipophilicity which is expressed as the logarithm of the partition coefficient between an organic solvent and an aqueous phase ($\log P$) and is widely used to indicate membrane affinity. $\log P$ is used in quantitative structure–activity relationships (QSAR) studies and rational drug design as a measure of molecular hydrophobicity. Hydrophobicity affects drug absorption, bioavailability, hydrophobic drug–receptor interactions, metabolism of molecules, as well as their toxicity. $\log P$ has become also a key parameter in studies of the environmental fate of chemicals [59].

Calcein with a $\log P$ value about -4.02 has hydrophilic properties, and therefore, cannot be entrapped in liposome membrane. Calcein with low $\log P$ value compared to lipids with $\log P \sim 8-9$ exhibits a strong hydrophilicity with little or no affinity to lipid bilayers. Absence of membrane affinity justified the low permeability of membrane to calcein and consequently this proposed a slow calcein passage through membranes without any internal interaction with lipids.

Table 3. The chemostructural properties of lipids and calcein

Parameter	DOPC	POPC	DPPC	Calcein
LogP	9.17	8.64	8.11	-4.04
Molecular polarizability	89.27	85.83	82.39	58.20
Polar surface area (PSA)	111.19	111.19	111.19	231.67
Molecular surface area (A ²)	1469.49	1435.66	1406.97	794.81
pi energy	33.89	31.89	29.89	81.47
LUMO	0.16	0.16	0.15	6.24
HOMO	- 0.45	- 0.45	-0.45	6.04

A low permeability of phospholipids vesicles to calcein could be a result of a low affinity of calcein for a membrane or slow kinetics of adsorption, translocation, and possibly dissociation [60].

Comparing different lipids, the differences in the calcein release behavior could be explained by the physical–chemical properties of the different fatty acids of phospholipids, as where by decreasing acyl chain length, Log *P* decreases and by increasing acyl chain length in phospholipids calcein – membrane affinity decreases [60]. In contrast to a significant difference observed for Log *P* values of liposomes composed of POPC and those of mixed lipids, there is a little difference in Log *P* values of liposomes composed of DOPC compared to those of DPPC. However, by considering the obtained results showing a higher rate of calcein release in DOPC compared to DPPC, it seems this difference is not significant. Furthermore, Distribution coefficient, Log *D*, is defined as the water/ octanol partition coefficient (log *P*) at a distinct pH value, pH 7.4 in this study. Fichert *et al.* concluded that compounds with log *D* values higher than 0 and less than 3 falls in the highly permeable class. Most of the compounds with log *D* values below 0 are moderately permeable [61]. Calcein with Log *D* ~ -3.29 is categorized in low permeable class.

Flaten *et al.* also expressed that compounds with experimentally determined log *D* values between -0.5 and 4.5 are likely to be well absorbed, 55% of the drugs would be correctly classified [62].

H-bonding is obtained by counting the number of potential H-bonds (H – bond donors, H-bond acceptors) on electronegative atoms (N, O, S) and the number of all atoms capable of H-bonding (HB) [63]. More recent papers found linear correlations between permeability and solubility as well as lipophilicity and hydrogen bonding descriptors. Moreover, the results indicated that lower number of hydrogen bond donors (HBD) is associated with higher permeability.

III. Résultats & Discussion

Chapitre III.III: Mécanisme de transfert de molécules hydrophiles

Calcein shows about 3.5 negative charges per molecule at pH 7.4 and has electron bonding energy more than lipids which enables it to act as a hydrogen bond donor and constitute van der Waals bonding. In contrast, the PCs are neutral molecules without any hydrogen bond donor-acceptor with low electron bonding energy suggesting lack of any calcein- acyl chain interaction in membranes (Figure 4). In other side, having low electron bonding energy for lipids is an effective parameter in facilitating the calcein passage through lipid membranes, as calcein with a HBD ~ 6 indicated lower permeability.

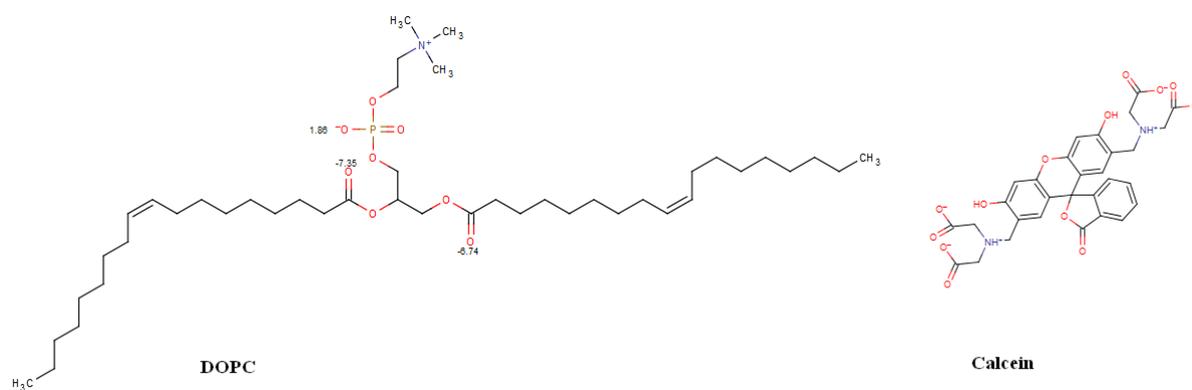


Figure 4. Molecular structure of DOPC and calcein.

Pi energy of molecules also represents a value to determination of energies of molecular orbitals of pi electrons in conjugated hydrocarbon systems which determines the general properties of the molecules and predicts how many energy levels exist for a given molecule, which levels are degenerate. This value explains the frontier orbitals, and in particular the effects of the Highest Occupied Molecular Orbital (HOMO) and the Lowest Unoccupied Molecular Orbital (LUMO) on reaction mechanisms [64, 65]. These values used to realize the possible reactivity which maybe be happened in molecules. As, according to this theory, the occupied orbitals of one molecule and the unoccupied orbitals of the other (especially the HOMO and LUMO) interact with each other causing attraction. Since the low values of HOMO and LUMO of lipids in comparison to values of calcein, well confirms the lack of calcein – acyl chain membrane interaction.

Furthermore, molecular size of compounds is another parameter which might be taken into account for prediction of the membrane permeability to molecules. Whereas, Van de Waterbeemd and Camenisch [63] concluded that permeability is not simply correlated to the

molecular weight (MW) of the compounds. As, the Van der Waals volume V and the surface area S shows the best correlation with Mw, similarity poor correlation are observed with these size descriptions.

- Influences of pH on permeability

A large part of drugs are weak acids or weak bases which exist in either charged (ionized) or uncharged (nonionized) forms. The ratio of the charged to uncharged form depends on the pKa of drug, and the pH of the environment. Since, diffusion across a lipid bilayer requires that a drug be lipid-soluble, the ionized form of a drug cannot cross membranes. Thus, weak acids that are nonprotonated and weak bases that are protonated cannot diffuse across membranes. At a pH that is equal to a drug's pKa, equal amounts of the protonated and nonprotonated forms are present. Assuming that the pH is the same on both sides of a given membrane, the drug will be at equilibrium across the membrane. If the pH is less than the pKa (such that there are excess protons available), the protonated form of a drug predominates. Thus, weak acids exposed to a low pH environment are favored to diffuse across membranes, while, weak bases are not. The opposite is true at a higher pH [66]. The ability of drugs to diffuse across membranes is more influenced by the ionizability of the drug in the surrounding medium [53].

3.4- In vitro gastro-duodenal digestion

Finally, to obtain a better understanding of calcein translocation through the bilayer in gastro – duodenal digestion, mixture of lipids –liposome contacting calcein was assessed.

During gastric digestion, fluorescence intensity of calcein increased over time indicating the calcein release (Figure 5 -A). The release was rapidly starts, during 5 min after incubation in digestion condition. The liposome remained stable for 2 h gastric digestion, around the time taken for gastric digestion *in vivo*.

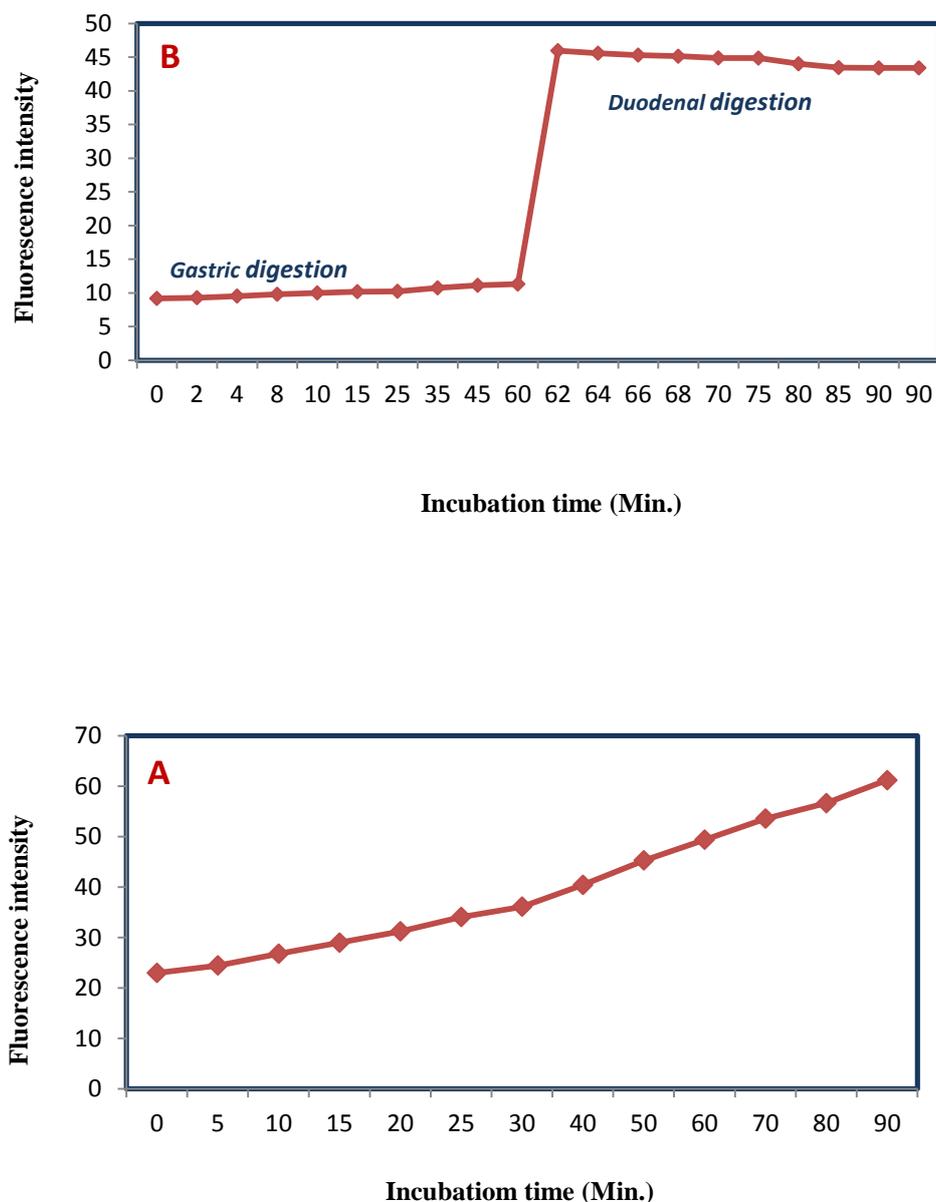


Figure 5. Calcein release in gastric digestion during 1.5h (A), in duodenal digestion, 30 Min (B). The liposome with 117 ± 2.5 nm diameter was prepared with a freeze/thaw method and extrusion method. The temperature was 37 °C. The data with standard error were obtained from at least three independent experiments.

Gastric digests were then subjected to a stimulated duodenal digestion, which resulted in a very rapid degradation of liposomes remained intact following gastric digestion (Figure 5-B). Digestion was continued for 30 min, as this is approximately the transit time down the small intestine to the site of the first Peyer's patch. All encapsulated calcein was released very

rapidly after bile salts addition. We observed a jump in FI of calcein in duodenal digestion. This sudden increase was due to liposome disruption and burst release of calcein. It is also worth to note that the FI of calcein in duodenal condition (pH: 6.5) shows the real value of FI in comparison to gastric condition (pH: 2.5) and the pH suppression of FI value has not been seen in pH range of 6.5 to 8. Calcein FI is independent of pH in the range of 6.5 and 12 but decreases with pH below pH 4.5 (Figure 6).

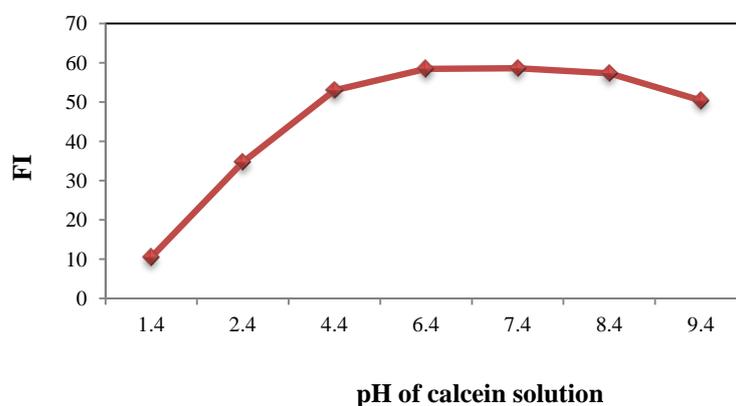


Figure 6. pH dependency of calcein fluorescence intensity.

Bile salts are bile acids compounded with a cation, usually sodium. In digestion condition, the salts of taurocholic acid and glycocholic acid (derivatives of cholic acid) represent approximately eighty percent of all bile salts. The main function of bile acid is to facilitate the formation of micelles, which promotes processing of dietary fat. Bile salts are secreted by the liver, and have a hydrophobic and a hydrophilic side. These will attach to fat globules, emulsifying them, and causing them to form micelles (Figure 7).

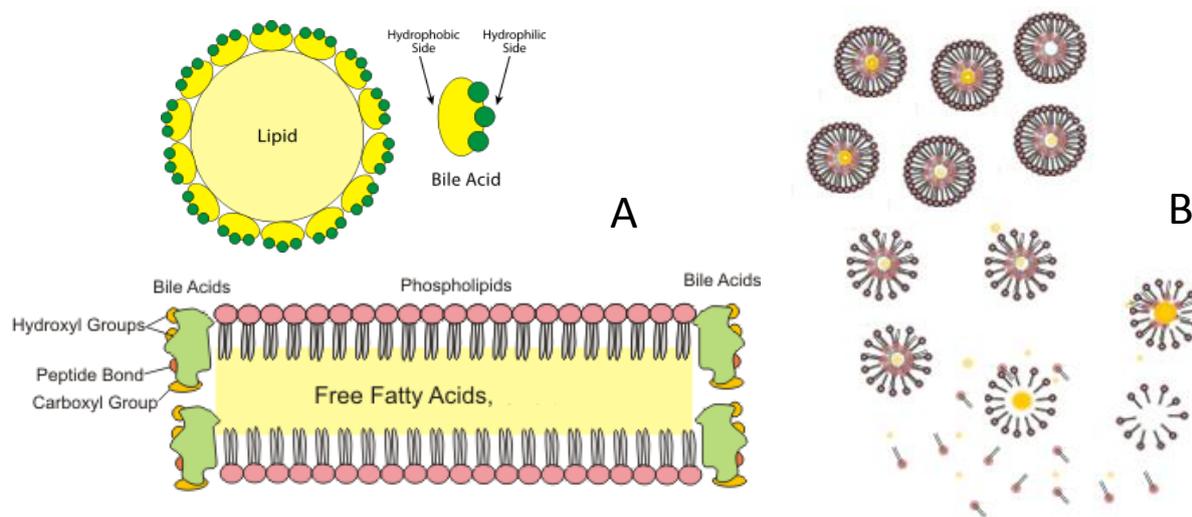


Figure 7. Bile salts action on lipid particles (liposome).

Obtained results have shown the mixed-lipid liposome is stable during gastric digestion and the calcein release about 3 % occurred in this step, however they are not stable in duodenal digestion. It means liposome composed of natural phospholipids without any protecting cover could be able to use as oral drug delivery systems for treatment of gastrointestinal diseases. As this way may facilitates the polar drug absorption by small intestine epithelium and also protects them from degradation by oral degradation and gastric digestion. Indeed, liposomes with modified surfaces could be developed by using several molecules to prolong the drug circulation and stability in gastrointestinal sections.

Today's, the challenge in bioactive targeting is not only the targeting of bioactive to a specific site but also retaining it for optimum duration to elicit the desired action. Recently, a number of methods have been developed for the modification of the liposome for increasing the release of drugs at desired site of action [1]. One of the most conventional methods is the use of polymers, particularly hydrophilic polyethylene glycol (PEG), to modify liposomes. Another way of protecting the liposomes is to encapsulate the drug-containing liposomes in a polymer matrix in the form of a microcapsule and finally, the third method of obtaining stabilized liposomes is to use the archaeobacterial membrane lipids or their analogs as the lipid components of the liposomes. Archaeobacteria and some gram-positive bacteria compared with liposomes, are relatively more thermostable, more resistant to oxidation, chemical, and enzymatic hydrolysis and to extreme changes of pH (2 to 10) [67, 68].

3.5- STED studies

To get better understanding of calcein release through liposomal bilayer, STED continuous wave (CW) was used. The images have been acquired by sequential scanning the different channels, using a 6x numerical zoom (25.83nm pixel size). The accumulation of liposomes was estimated by determining the fluorescence of Rhodamine (red channel) in the 550–650 nm spectral windows by exciting at 561nm. The fluorescence of Calcein (green channel) has been acquired using photomultiplier tube (PMT) in the 500–550 nm spectral window by exciting at 488 nm, 4 lines averaging, 1400 Hz scanning and 1 Airy pinhole size. In the STED mode, the 592 nm CW laser beam is superimposed at a typical power of 200–300 mW at the sample.

Resolution difference between confocal and STED is considerable. STED has an average resolution of below 100 nm while confocal resolved around 300 nm.

Normally, the best STED results are obtained from bright samples with high contrast (i.e. high signal-to-noise ratios). A good rule of thumb is that if the signal is dim or easily photobleachable under a traditional confocal system, it will not be suitable for STED imaging. So some optimization of existing labeling procedures might be necessary for each new specimen.

Other important criteria that one should consider when imaging in the 100 nm range relate to the size of the label itself and the density of the labeling probes. A multi-step indirect IF labeling approach allows signal enhancement but will add size to the structure of interest. On the other hand, a direct IF labeling approach will keep the fluorophore closer to the structure of interest but will result in lower signal intensity.

The main key in super-resolution imaging using STED is the non-linear dependence of the depleted population of the excited electrons on STED laser beam intensity. Simply speaking, if the STED laser intensity is more than a certain threshold, all the spontaneous emission is dominated by the stimulated emission. This leads to complete suppression of the fluorescence or what is known as *depletion*. Hence, there are two determining factors in the resolution of STED microscopy: STED laser intensity and saturation intensity of the fluorophore (Figure 8C). The latter is a function of the STED laser wavelength, pulse width, as well as intrinsic characteristics of the fluorophore such as lifetime and kinetics of the population of ground and excited states [69].

Finally, super-resolution (SR) imaging requires that the density of the labeling probes be sufficient to meet the so-called Nyquist sampling criterion (Figure 8).

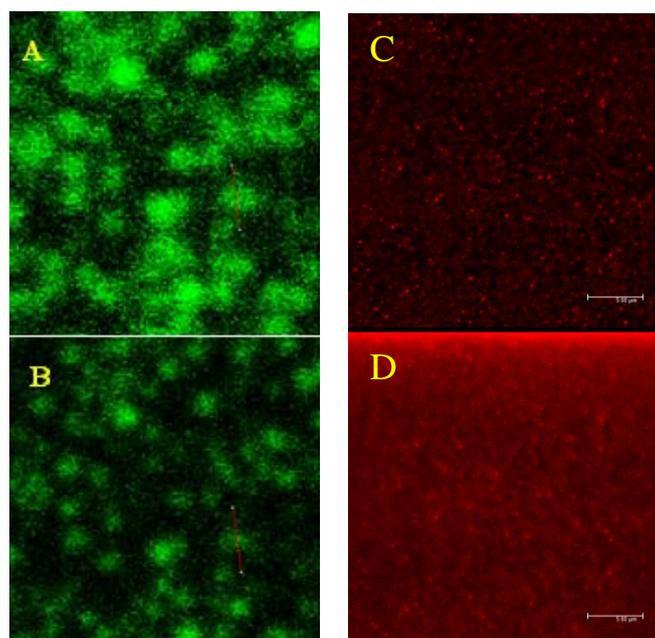
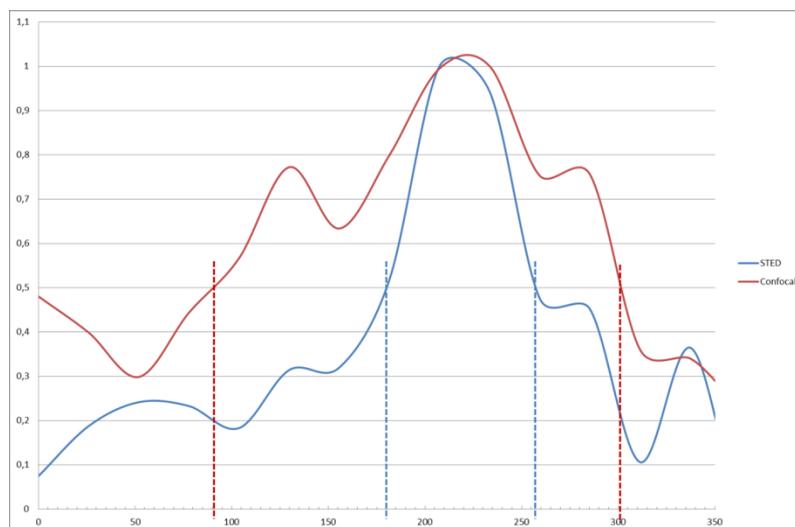


Figure 8. Resolution comparison of images of calcein- encapsulated liposome by Confocal microscopy (A) and STED (B). Liposome labeled with Rhodamine observed with Confocal microscopy (C) and STED (D).

To better visualize membrane bilayers of vesicles, Rhodamine-DHPE labeled- liposome were prepared (Figure 9). Calcein- encapsulated liposome was obviously observed in blue points

which indicate the self-quenching concatenation of calcein in liposome. As, the green points in surrounding area of liposome (exterior of liposome) showed calcein dilution during the gradual release.

The STED images showed the vesicles in the form of large unilamellar vesicles encapsulating calcein. This confirmed the lack of liposome disruption by calcein release. Moreover, if calcein disrupts vesicles during egression, a suddenly jump in fluorescence intensity values were detected by spectrofluorometer.

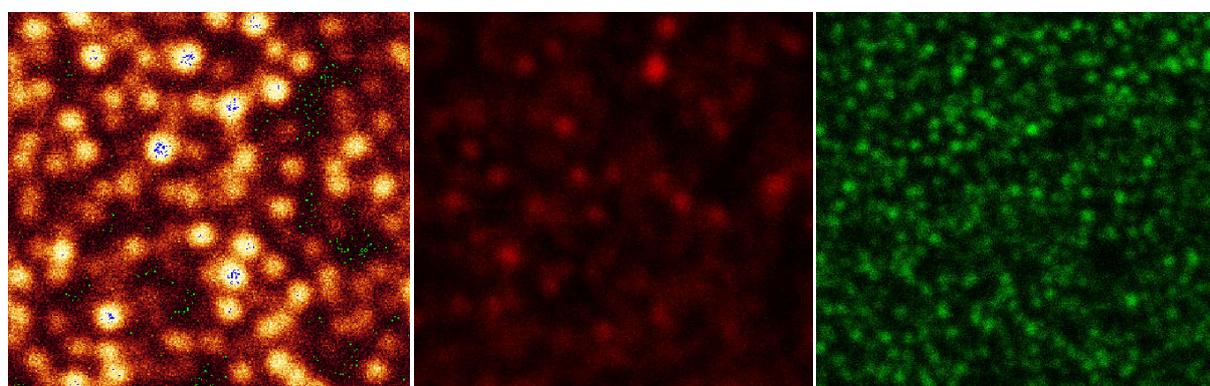


Figure 9. Rhodamine – labeled POPC encapsulating calcein in STED multiphoton (red liposome with green calcein), Rhodamin labeled-POPC (red channel), calcien-encapsulated liposome (green channel).

The obtained images of STED during release incubation's time (5 h), obviously showed a gradual increase in fluoresce intensity of calcein in surrounding medium. It confirmed that calcein permeates slowly through liposomal membrane by diffusion without liposome disruption (Figure 10).

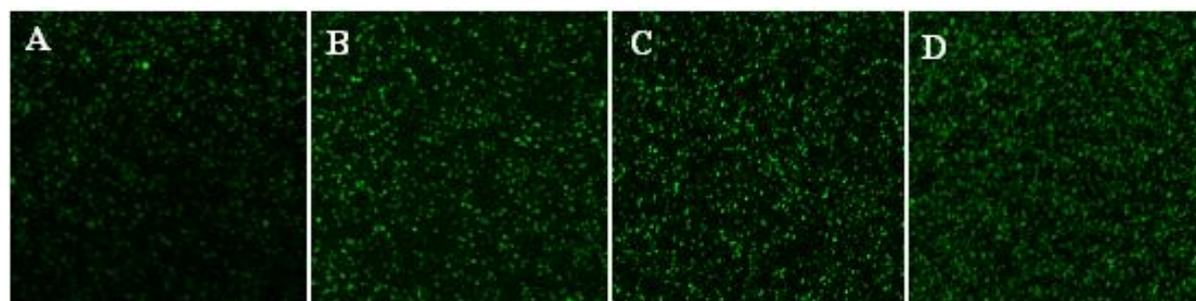


Figure 10. Calcein release form DOPC liposome during incubation at 25°C (5h).

Furthermore, the effect of heating temperature on the permeability of calcein was observed with STED in the temperature 25 and 50 °C. The FI induced by calcein release from DPPC-liposome increased with increasing temperature in the same period of incubation. This dramatic increasing of FI should be originated from an increased permeability of DPPC-liposome induced by temperature rather than its phase transition temperature (Figure 11).

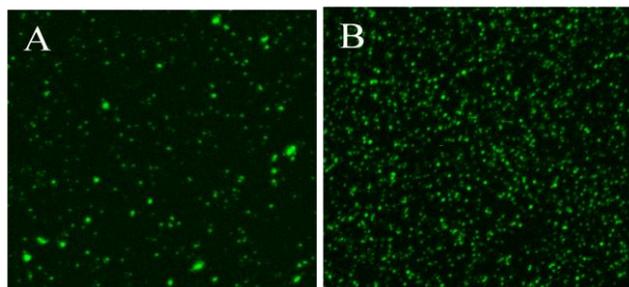


Figure 11. Effect of temperature on calcein release of DPPC liposome, A and B presents the FI of calcein release from DPPC-liposome incubated in 25°C and 50°C, respectively.

The images acquired in acidic and basic pH values confirmed that FI of calcein depends on environmental pH condition, as images in basic pH are bright whereas the FI of calcein is quenched at acidic pH values, producing a dark image.

Comparison between the liposome size determined by STED and with DLS technique showed a negligible difference which might be due to the dilution factors of 10 and 25 for STED and DLS measurements, respectively.. Since, dilution of liposomal suspension may cause a difference in osmolarity between interior and exterior of liposome, which in turn may result in enlargement of liposome size, as liposome size measured by STED are about 109 -110 nm and those measured by DLS technique are about 116 - 117 nm.

4-Conclusion

Indeed, liposomes properties vary substantially with composition, size, surface charge and preparation method. It is obvious that the design and development of drug carriers is a difficult issue because they have to behave as biocolloidal systems after administration. Physicochemical properties of drugs are also a critical subject in the design of the delivery systems.

Diffusion-controlled drug release model which controls the drug release by physical mechanisms such as drug solubility and diffusion coefficient in the release medium is an ameliorate approach in controlled drug release. For this reason, the physical mechanisms have

been used widely. They are used simply and highly effective in controlling the drug release kinetics.

The results confirmed that calcein released according to zero-order release mechanism. Physicochemical properties of liposome are effective factors in calcein release. As by increasing number of lamellarity and liposome size more than 200 nm permeability decreases. Membrane fluidity and T_c related to liposome composition has direct effect on release, as membrane composed unsaturation lipids with more fluidity values and lower T_c , shows more permeability to calcein.

Furthermore, the results have shown the structural changes caused by the double bond, reducing the hydrophobic thickness and increasing the area per molecule, lead to decreasing of the bending elasticity and increasing membrane permeability to calcein.

Physicochemical properties of drugs are also a critical subject in the design of the delivery systems. As partition coefficient of the drug and hydrogen bonding sites as well as its polar surface area (PSA) influence in calcein – liposome interaction. Since the calcein is a negatively charge hydrophilic compound, it could be adsorb with choline head groups of phospholipids. However, no interaction between calcein and hydrophobic section of membrane was determined but the results showed that calcein could permeate in membrane by the force of osmotic pressure between interior and exterior of membrane and diffuses through it. The molecular size of compounds is another parameter which maybe takes account for predicting the membrane permeability to molecules. As, for passive transport across lipid bilayers, rigid molecules must be reasonably small, and their hydrophilic / lipophilic properties must be within narrow ranges. Indeed, the ability of ionized particles to partition in biological membranes has been observed to depend on complex mechanisms. In addition, molecular volume and shape of the molecules are also relevant for penetration through membranes.

During gastro- duodenal digestion, calcein release from mixture of lipids –liposome happened, which was appeared as increasing the fluorescence intensity of calcein. The calcein release was rapidly starts, during 5 min after incubation in digestion condition. Gastric digesta were subjected to a stimulated duodenal digestion, resulted in a very rapid degradation of liposomes and a jump in FI of calcein in duodenum digestion.

The obtained images of STED confirmed the effect of temperature on membrane permeability to calcein and it showed the suppression of FI by acidic pHs, as the taken images in acidic pH are black because of weakened emission.

Indeed, this Calcein-release model could be utilized as an effective index to characterize the membrane properties of (model) biomembranes and to evaluate their stability in a variety of conditions. In addition, the calcein-release phenomena have been investigated with regard to design of a drug-delivery system.

Also, STED is a super-resolution technique which breaks the classic resolution limit of light microscopy. It offers the ability to shed light on cell biological processes inside neurons and synapses that have been out of reach for confocal and two-photon microscopy, whose spatial resolution is limited by the diffraction of light. STED is able to be used as a useful tool to investigate drug-membranes interaction and follow the fate of liposome in vivo systems.

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Conclusion

Les propriétés des liposomes varient considérablement en fonction de leur composition, de leur taille, de leur charge de surface et de leur méthode de préparation. Les difficultés de conception et de développement de vecteurs médicamenteux résident dans le fait que ces derniers doivent se comporter comme des systèmes biocolloïdaux après leur administration. Les propriétés physicochimiques des médicaments constituent également un point critique dans la conception des systèmes de libération des agents actifs.

Le modèle de libération de médicaments par diffusion contrôlée, qui contrôle la libération du médicament par des mécanismes physiques tels que sa solubilité et son coefficient de diffusion dans le milieu de libération, constitue une approche améliorée dans la libération contrôlée de médicaments. Pour cette raison, les mécanismes physiques ont été largement utilisés. Ils sont facilement mis en œuvre et hautement efficaces quant au contrôle de la cinétique de libération du médicament.

Les résultats ont confirmé la libération de la calcéine selon un mécanisme d'ordre zéro. La libération de la calcéine est intrinsèquement liée aux propriétés physicochimiques des liposomes. Ainsi, en augmentant le nombre de lamelle et la taille des liposomes de plus de 200 nm la perméabilité diminue. La fluidité membranaire et T_c lié à la composition des liposomes agissent directement sur la libération dans la mesure où les membranes composées de lipides insaturés avec une fluidité plus élevée et des valeurs de T_c plus faibles montrent davantage de perméabilité à la calcéine.

De plus, les résultats ont montré que les changements structuraux causés par les doubles liaisons, réduisant l'épaisseur de la couche hydrophobe et augmentant la surface par molécule, conduisaient à une diminution de la flexion élastique et augmentaient la perméabilité membranaire à la calcéine.

Les propriétés physicochimiques des médicaments sont aussi d'une grande importance dans la conception de systèmes de libération. Les coefficients de partage du médicament, leurs sites de liaisons hydrogène ainsi que leur polarité de surface (PSA) influencent les interactions calcéine-liposomes. En considérant que la calcéine est un composé négativement chargé, il pourrait se trouver adsorbé aux groupements de tête choline des phospholipides. Cependant, toutes les interactions entre la calcéine et la section hydrophobe de la membrane ont été déterminées mais les résultats ont montré que la calcéine pouvait perméabiliser la membrane par la force de pression osmotique entre l'intérieur et l'extérieur de la membrane et diffuser à travers elle. La dimension moléculaire des composés est un autre paramètre qui pourrait être

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Chapitre III.III: Mécanisme de transfert de molécules hydrophiles

pris en considération pour prédire la perméabilité membranaire des molécules. Pour les transports passifs à travers les bicouches lipidiques, les molécules rigides doivent être relativement petites et leurs propriétés hydrophiles/lipophiles doivent être dans des intervalles étroits. En effet, il a été montré que le partage des particules ionisées de part et d'autre des membranes biologiques dépend de mécanismes complexes. De plus, le volume et la forme des molécules sont aussi déterminants pour le passage à travers les membranes.

Au cours de la digestion gastro-duodénale, la libération de calcéine a pu être observée à partir de mélange lipides-liposomes, indiquée par l'augmentation de l'intensité de fluorescence de la calcéine. La libération de calcéine a débuté rapidement, durant 5 min après incubation dans les conditions de digestion. Le digestat gastrique a été soumis à une simulation de digestion duodénale, conduisant à une très rapide dégradation des liposomes et un « saut » de FI de calcéine au cours de la digestion duodénale.

Les images obtenues à partir de microscopie STED ont confirmé l'effet de la température sur la perméabilité membranaire à la calcéine et témoignèrent de la suppression de FI aux pH acides, les images obtenues aux pH acides étant noires du fait des faibles émissions.

En effet, ce modèle de libération de la calcéine pourrait être utilisé comme indice dans la caractérisation des propriétés membranaires de biomembranes (modèles) et dans l'évaluation de leur stabilité dans des conditions variées. De plus, les phénomènes de libération de calcéine ont été évalués dans le cadre de la conception d'un système de libération de médicament.

Aussi, la microscopie STED est une technique à haute résolution qui dépasse la limite de résolution classique de microscopie à luminescence. Elle offre la possibilité de focaliser l'attention sur des processus biologiques au niveau cellulaire, à l'intérieur des neurones et aux interfaces synaptiques, ce qui est impossible en microscopie confocale et à deux-photons dont la résolution spatiale est limitée par la diffraction de la lumière. STED peut également être utilisée efficacement en tant qu'outil dans l'évaluation des interactions médicaments-membrane et le suivi du devenir des liposomes dans les systèmes *in vivo*.

IV. Conclusion & Perspectives

Conclusion

Le vecteur lipidique représenté par le système liposomal est un modèle largement étudié en tant que membrane lipidique permettant de simuler le comportement des membranes des biologiques. L'utilisation des liposomes dans les domaines alimentaire, nutraceutique, pharmaceutique et médical, est largement développée afin de vectoriser et relarguer des molécules d'intérêt vers des zones cibles localisées. Formés de phospholipides agencés en bicouches ou sous forme de monocouches multilamellaires concentriques ou non concentriques, les liposomes peuvent contenir aussi des protéines dans leur structure.

Ce sont des vecteurs de biomolécules très efficaces, dont le comportement *in vivo* dépend fortement des conditions environnementales affectant leurs propriétés physicochimiques. Les liposomes possèdent des propriétés remarquables comme la possibilité d'encapsuler simultanément des molécules présentant des solubilités différentes, un ciblage spécifique et un relargage progressif de molécules d'intérêt à de faibles concentrations. Une connaissance approfondie de la structure de ces vecteurs et des interactions avec la molécule encapsulée est nécessaire afin d'optimiser l'efficacité d'encapsulation. La caractérisation physico-chimique est indispensable afin d'optimiser la formulation des phospholipides structurant la bicouche lipidique, l'efficacité d'encapsulation et la libération maîtrisée des molécules d'intérêt. La taille des liposomes est un facteur déterminant dans le processus de libération du principe actif et de leur élimination de la circulation sanguine par le système réticuloendothélial pour des applications médicales. Il a été montré que la nature des acides gras estérifiés sous la forme phospholipidique et le mode de préparation jouaient un rôle déterminant sur la structure et la polydispersité des liposomes. L'utilisation d'acides gras polyinsaturés entraîne généralement une augmentation de la taille.

La connaissance des propriétés physicochimiques des molécules d'intérêt est essentielle afin de mieux comprendre leur mode d'intégration dans la bicouche lipidique, adsorbées à la surface ou piégées à l'intérieur du liposome, en fonction de leur solubilité et de leur polarité.

La formulation de la composition lipidique des liposomes permet de jouer sur la libération progressive des molécules d'intérêt encapsulées en fonction des conditions environnementales. Il est ainsi nécessaire de connaître la température de transition de phase (T_c) tout comme la fluidité membranaire afin d'optimiser au mieux la formulation lipidique de ces liposomes.

Le transfert des biomolécules à travers les membranes représente un obstacle à la libération progressive des principes actifs vers leurs cibles. Il est donc important de comprendre les phénomènes de perméabilité membranaire afin de prédire une efficacité optimale. L'objectif

étant ici de limiter l'apport en principes actifs avec une concentration optimale. Il est de ce fait nécessaire d'étudier les interactions ligand-liposome. Sur le plan moléculaire, les interactions et l'insertion de la molécule dans la bicouche entraînera des modifications chimiques, conformationnelles et structurales des lipides du liposome. L'utilisation de l'analyse thermique différentielle et de la spectroscopie Raman a permis d'étudier les interactions ligand-liposomes mais aussi les interactions plus localisées, par l'étude du comportement des vibrations de chacun des groupes. L'analyse des spectres Raman du système calcéine-liposome montre que la vibration d'élongation des liaisons ν_{C-C} , ν_{C-H} et ν_{C-N} ne présentait pas d'évolution significative en présence ou en absence de calcéine. A partir des ratios d'intensité des bandes d'étirement concernant les groupements méthylène ($I_{2935/2880}$) et des bandes caractéristiques des chaînes alkyle ($I_{11095/1130}$), il a été montré que la calcéine induisait une légère modification de l'intégrité de la membrane avec une conformation gauche dominante, par rapport au liposome sans calcéine.

De plus, les spectres Raman de la vibration d'élongation de la liaison ν_{C-N} des liposomes en présence de calcéine montrent une augmentation de l'intensité des pics. L'augmentation de l'intensité à 715 cm^{-1} indique une interaction significative entre le groupe choline des phospholipides et la calcéine.

Les mesures de tension de surface appliquées à la surface de monofilms de Langmuir ont permis d'obtenir des informations sur la conformation et l'insertion de composés bioactifs.

En présence de calcéine, un léger décalage des isothermes π -A est observé pour tous les types de monocouches.

L'efficacité d'un système liposomal dépend de la libération progressive de la biomolécule encapsulée sur une période déterminée, à la concentration optimale. Le modèle de molécule d'intérêt utilisé dans cette étude montre que la calcéine s'intègre parfaitement à la membrane. La libération progressive de cette molécule dépend de la fluidité des membranes lipidiques et de leurs propriétés mécaniques et physiques comme la perméabilité et l'élasticité. Les propriétés physico-chimiques des molécules encapsulées tel que le coefficient de partage ($\log P$) et la polarité de surface sont également des paramètres importants dans le comportement du système. Les résultats ont confirmé que le mode de libération de la calcéine est une cinétique d'ordre zéro. La lamellarité et la taille des liposomes de plus de 200 nm diminuent la perméabilité des membranes. La fluidité augmentée par la présence de lipides insaturés des phospholipides de la bicouche lipidique et la composition en acides gras ont un effet direct sur la libération de la molécule. En effet, la présence de double-liaisons induit des modifications

structurales, notamment en augmentant l'aire par molécule limitant l'élasticité de flexion et augmentant la perméabilité de la calcéine à travers la membrane.

Cependant, aucune interaction entre la calcéine et la partie hydrophobe de la membrane a été mise en évidence. Les résultats ont montré que la calcéine pourrait s'intégrer et diffuser à travers la bicouche par la différence de pression osmotique existante entre l'intérieur et l'extérieur de la membrane. Ce modèle de libération de la calcéine pourrait être utilisé pour caractériser les propriétés de biomembranes et évaluer leur stabilité en fonction des conditions expérimentales de température / pH / traitement enzymatique.

L'étude de l'encapsulation et du transfert de principes actifs (calcéine, carnosine) dans un système liposomale, a permis d'obtenir de nombreuses informations nécessaires à la compréhension des mécanismes permettant d'optimiser l'encapsulation, le transfert et la libération progressive des biomolécules vectorisées. La compréhension des interactions entre ces molécules hydrophiles et la bicouche lipidique a été rendu possible par l'utilisation de techniques avancées comme la spectroscopie Raman, l'analyse thermique différentielle et les études de tension superficielle à l'aide de monofilms de Langmuir.

Il serait intéressant de poursuivre ces investigations par la mesure des interactions en utilisant la calorimétrie isotherme à titration. Cette technique permettrait de mesurer les interactions entre la macromolécule et son ligand, les isothermes de liaison et certains paramètres thermodynamiques d'interaction. Dans le présent travail, les liposomes ont été formulés à partir de phospholipides purs, où la longueur de chaîne et le nombre d'insaturations des acides gras ont été modifiés afin d'optimiser la fluidité membranaire. Il serait intéressant d'élaborer de nouveaux vecteurs à partir de lécithines végétale et marine, contenant des acides gras indispensables, à savoir les acides linoléique (18:2 n-6) et α -linoléique (18:3 n-3), ou les chefs de file des acides gras polyinsaturés à longue chaîne comme l'acide docosahexaénoïque (C22:6 n-3 ; DHA) et l'acide éicosapentaénoïque (C20:5 n-3 ; EPA). Les implications des AGPI-LC de la série n-3 les plus significatives se retrouvent au niveau des besoins et du développement du fœtus et du nourrisson, la prévention des maladies cardio-vasculaires, la vision, certaines réponses immunitaires et maladies inflammatoires, un rôle inhibiteur dans la genèse et la progression de certains cancers, pour ne citer que ceux-ci.

Le mode de préparation des liposomes permet d'obtenir des vecteurs présentant des propriétés physico-chimiques différentes. Outre le modèle unilamellaire formulé tout au long de ce travail, il est envisagé d'étudier les liposomes multicouches permettant de développer un modèle de diffusion et de transfert pharmacocinétique de principes actifs aussi bien hydrophiles qu'hydrophobes.

Les molécules hydrophobes peuvent plus facilement s'intégrer aux membranes phospholipidiques et interagir avec les chaînes acyles induisant un comportement différent au niveau des mécanismes de libération progressive des principes actifs recherchée dans les domaines de la nutraceutique, de la pharmacologie, du ciblage thérapeutique ou de la cosmétique.

Perspectives

Malgré l'importance de ce sujet, peu de travaux portent sur le transfert de molécules bioactives à travers une bicouche liposomale. Pour cette raison, nous avons décidé d'étudier le comportement de biomolécules et les mécanismes de transfert au travers d'une membrane modèle.

- ✓ Nous avons étudié au cours de ce travail les interactions principe actif – membrane à l'aide de la spectroscopie Raman, de la balance de Langmuir et de l'analyse thermique différentielle.
 - L'objectif serait de poursuivre les investigations par calorimétrie isotherme à titration pour mesurer et approfondir nos connaissances sur les interactions moléculaires.

- ✓ En raison de la complexité du sujet de recherche, nous nous sommes focalisés sur le transfert de molécules actives dans un liposome composé de lipides purs.
 - Nous envisageons de poursuivre ces recherches en formulant des liposomes à partir de lipides polaires complexes issus de lécithine marine, riche en acides gras polyinsaturés à longue chaîne.

- ✓ Dans ce travail, nous avons élaboré et étudié des liposomes unilamellaires. Il serait intéressant d'étudier les mécanismes de transfert pour des liposomes multicouches.

- ✓ Le transfert de molécules hydrophobes localisées dans la membrane liposomale ou partiellement insérées constituerait une perspective de recherche pour maîtriser les phénomènes de relargage contrôlé.

Conclusion

Liposomes (also known as bilayer lipid vesicles) are ideal models of cells and biomembranes. Their resemblance to biological membranes makes them an ideal system, not only for the study of contemporary biomembranes, but also in studies investigating the emergence, functioning and evolution of primitive cell membranes. Furthermore, they are being used by the food, cosmetic, agricultural and pharmaceutical industries as carrier systems for the protection and delivery of different material including drugs, nutraceuticals, pesticides and genetic material. Liposomes are composed of one or more concentric or nonconcentric lipid and/or phospholipid bilayers and can contain other molecules such as proteins in their structure.

Liposomes are one of the most effective carriers, whose *in vivo* behavior is altered by their various physicochemical properties. A significant advantage of liposome is that it can incorporate and release two materials with different solubilities simultaneously. Furthermore, targetability is another extremely useful characteristic of liposome. These particular properties make liposome to be useful in many applications due to its ability to increase the effectiveness of the encapsulated active agents and optimizing their dosage.

Knowledge of liposome characteristics is required to develop liposome formulations that have optimal entrapment efficiencies and allow the controlled release of bioactives. Liposome characterization is required to qualify, quantify and approve the liposome capability for special application.

Particle size is one such property that is well known to have an influence in removing liposomes from the circulation by the reticuloendothelial system (RES), which limits the blood circulation time of liposomes and can release the entrapped drug into the blood stream. We found that the mean size of liposomes was influenced by both the lipid composition and the preparation method. As, largest mean size and polydispersity values were obtained for liposomes which were composed unstaured lipids.

Bioactives can interact with liposomes in several different styles depending on their special properties such as solubility and polarity. They can be entrapped in the lipid bilayer phase, intercalated in the polar head groups, adsorbed on the membrane surface, anchored by a hydrophobic tail or encapsulated in the inner aqueous compartment. Lipid composition and preparation method can influence the entrapping efficiency of liposome formulations.

Indeed, the goal of drug delivery system is to administer a drug at a therapeutic concentration to a particular site of action for a specified period of time. The design of the final product for drug delivery depends upon different parameters. As, phase transition temperature (T_c) exerts

significant effects on the liposome properties. T_c and fluidity of phospholipid membranes are effective parameters in the manufacture and application of liposome, were influenced by lipid composition.

It is well known that drugs have to pass several membrane barriers for exerting their pharmacotherapeutic effects. These barriers affect the pharmacokinetic behavior of drugs and their capability to access the target site. In addition, partitioning always occurs because of drugs' binding to membrane receptors and transporters, so, prediction of drug-membrane permeability is important for optimum efficacy. The transport of small molecules across lipid bilayers is a fundamental biological process. Controlled release formulations can be used to reduce the amount of drug necessary to cause the same therapeutic effect in patients. One of the most noticeable factors in release profiles is the strength of the drug-carrier interaction. To adjust the pharmacokinetic and pharmacodynamic properties of therapeutic agents, it is necessary to optimize the drug-carrier interaction.

From a molecular point of view, bioactive substances able to insert themselves or become entrapped in the liposomal bilayer can alter the shape, size distribution and chemical properties of a liposome.

Additionally, the localization of bioactive substances within the bilayer is also a question of great importance for determining their interaction with liposomes.

Interactions of bioactive compounds with model lipid bilayers could provoke changes in their thermotropic behavior as well as in their conformation properties. These effects were taken into account in the design of liposomal formulations as drug controlled release delivery systems.

Differential scanning calorimetry (DSC) has been proved as a valuable tool for studying the interaction of bioactive compounds with model lipid bilayers. Raman spectroscopy can also be used as a tool to examine localized interactions, by studying the behavior of the vibrational stretching modes of each group in the lipid membrane.

The Raman spectra analysis of calcein - encapsulated liposomes showed that the stretching vibration of the ν_{C-C} , ν_{C-H} and ν_{C-N} bonds exhibited no significant change in the peak positions in samples containing calcein in comparison to liposomes without calcein.

The change of the disorder/order parameter calculated from the methylene stretching bands ($I_{2935/2880}$) and alkyl chain stretching bands ($I_{1095/1130}$) of all liposomes in the presence of calcein exhibited a slight increase in the intensity ratio, which mentioned an augmentation of a disorder degree in the alkyl chains. It was observed that the *gauche* conformation was more dominant in calcein-encapsulated liposomes than in liposomes without calcein.

Furthermore, the Raman spectra of the stretching vibration of ν_{C-N} bond of all liposomes in the presence of calcein showed an increase in peak height intensity in the presence of calcein. The increase in peak height intensity at 715 cm^{-1} indicates significant interaction between the choline head group and calcein.

Furthermore, measurements on monolayers by means of a Langmuir film balance have presented the necessary information on the area occupied by the bioactive compounds on the surface of the monolayer and, hence, on their conformation.

In the presence of calcein, a small shift of the π -A isotherms to higher molecular areas is observed in all types of monolayers. A decrease in the molecular area could be a manifestation of the subphase electrostatic effect which would reduce the electrostatic interactions in acyl chains of lipids.

As, we mentioned the design of the drug delivery depends upon different parameters. One of the most noticeable factors in design of the drug delivery is drug- release profile which determines the site of action, the concentration of the drug at the time of administration, the period of time that the drug must remain at a therapeutic concentration.

The results revealed that calcein permeates in liposomal membrane without membrane disruption. The release rate of calcein from the liposomes depends on the number and fluidity of bilayers and its mechanical/physical properties such as permeability, bending elasticity. Structural properties of drugs like as partition coefficient ($\log P$), H-bonding, surface polar (PSA) are also determinative parameter in release behavior.

The results confirmed that calcein released according to zero-order release mechanism. Physicochemical properties of liposome are effective factors in calcein release. As by increasing number of lamellarity and liposome size more than 200 nm permeability decreases. Membrane fluidity and T_c related to liposome composition has direct effect on release, as membrane composed of unsaturated lipids with more fluidity values and lower T_c , shows more permeability to calcein.

Furthermore, the results have shown the structural changes caused by the double bond, reducing the hydrophobic thickness and increasing the area per molecule, lead to decreasing of the bending elasticity and increasing membrane permeability to calcein. Physicochemical properties of drugs are also a critical subject in the design of the delivery systems. Partition coefficient of the drug and hydrogen bonding sites as well as its polar surface area (PSA) influence in calcein – liposome interaction.

However, no interaction between calcein and hydrophobic section of membrane was determined, but the results showed that calcein could permeate in membrane by the force of osmotic pressure between interior and exterior of membrane and diffuses through it.

Indeed, this calcein-release model could be utilized as an effective index to characterize the membrane properties of (model) biomembranes and to evaluate their stability in a variety of conditions of temperature / pH / enzymatic treatment. In addition, the calcein-release phenomena have been investigated with regard to design of a drug-delivery system.

Perspectives

Despite the importance of this subject, there is not sufficient and noticeable information concerning bioactive transfer through liposomal bilayer. For this reason, we decided to study the bioactive substances behavior which able to insert or entrapped into liposomal bilayer and their possible mechanism of transfer in liposome.

- ✓ We tried to investigate hydrophilic bioactive agents' interaction with liposome by Raman Spectroscopy, Langmuir Balance and DSC.
 - We can also use Isothermal Titration Calorimetry (ITC) for measuring biomolecular interactions. ITC simultaneously determines all binding parameters (n , K , ΔH and ΔS) in a single experiment – *information that cannot be obtained from any other method.*
- ✓ In the first step due to the complexity of the research subject, we focused on transfer of active molecules from the liposome composed of pure lipids.
 - We can also consider elaborating the liposomes from the lecithin composed of various phospholipids such as marine and vegetable lecithins which contain essential fatty acids such as α -linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These fatty acids are considered for the efficient functioning of the brain and the body at a cellular level. They have also noticeable role in healthy development of the brain and vision in young children. Additionally, they also prevent heart disease and the complications of heart attack. For this purpose, using these natural lecithins, facilitate their application in pharmaceutical, medical and nutraceutical aspects.
- ✓ Furthermore, in our study, we formed the large unilamellar liposome; we can also prepare the multilayer liposomes in order to study the molecular transfer.
 - It will be interesting to determine mechanism of drug transfer from several membrane barriers for exerting their suitable effects. These barriers influence on pharmacokinetic and nutraceutical behavior and capability of drugs to access the target sites.
- ✓ Additionally, it is really interesting to study the hydrophobic molecules transfer. These molecules may be entrapped in hydrophobic part of lipid membrane or partially inserted in lipid membrane and interact with acyl chain. The molecules / membrane interaction acts as barrier. These barriers affect on their pharmacokinetic and nutraceutical behavior and their

capability to access the target site. These effects were taken into account in the design of liposomal formulations as controlled release drug delivery systems. Also, by understanding the signalling and interaction between the bioactive compounds and liposomes, it would be possible to mimic biological systems.

Résumé

Sur le plan moléculaire, le transport de petites molécules à travers les bicouches lipidiques est un processus fondamental. Le relargage d'une molécule bioactive encapsulée dans un liposome dépend de différents paramètres tels que la perméabilité membranaire, les propriétés structurales, ainsi que les forces d'interaction entre le liposome et la biomolécule.

L'objectif de ce travail consiste à étudier les différents mécanismes de transfert de molécules hydrophiles à travers la bicouche liposomale. La calcéine a été choisie comme molécule hydrophile modèle pour simuler la vectorisation de principes actifs.

Dans une première étape, nous avons optimisé la formulation des liposomes en considérant ses propriétés physico-chimiques (taille, efficacité d'encapsulation, fluidité et etc.) par différentes méthodes (DSC, TEM, SAXS, DLS, RMN et spectrofluorométrie). Les résultats obtenus montrent que la taille moyenne, le potentiel zêta, T_c , l'efficacité d'encapsulation et la fluidité, sont influencés par la composition lipidique des liposomes. Les interactions entre les molécules hydrophiles encapsulées et le vecteur ont été étudiées par spectroscopie Raman, balance de Langmuir et analyse thermique différentielle.

Les résultats obtenus montrent que la calcéine est capable d'interagir avec le groupement polaire de la phosphatidylcholine, en s'intercalant entre les chaînes acyles et modifiant de ce fait l'organisation de la membrane. La perméabilité des membranes à la calcéine a d'abord été évaluée sur la base d'une cinétique du premier ordre par spectrofluorimétrie. L'effet de la composition en lipides sur la fluidité membranaire a été étudié en fonction des conditions environnementales.

Un modèle simulant les conditions de la digestion a été élaboré pour estimer la vitesse de libération du calcéine à travers la bicouche liposomale, son coefficient de partage, en utilisant l'AFM et la méthode STED. Les résultats obtenus ont confirmé que la calcéine diffuse lentement à travers la membrane liposomale sans pour autant déstructurer le liposome.

Mots clés : Nanoliposome, libération, l'interaction moléculaire, transfert, caractérisation physico-chimique

Summary

From a molecular point of view, transport of small molecules across lipid bilayers is a fundamental and functional process. The release of efficacious dose of bioactive-entrapped in liposome depends on different parameters such as liposome permeability, bioactive structural properties and strength of liposome / bioactive interaction.

The aim of this study was investigation the possible mechanisms of hydrophilic molecules transfer through liposomal bilayer. Calcein was chosen as model of hydrophilic drugs. In the first step, we optimized liposome formulation by considering its physicochemical properties (size, encapsulation efficiency, fluidity and etc.) by different methods such as DSC, TEM, SAXS, DLS, NMR and Spectrofluorentere. The reported results show that mean size, zeta potential, T_c , entrapment efficiency and fluidity were influenced by liposome lipid composition. Then, we tried to investigate hydrophilic bioactive agents' interaction with liposome by Raman Spectroscopy, Langmuir Balance and Differential Scanning Calorimetry. The obtained results indicated that calcein is being able to interact with the choline polar-head group of the lipids but probability it could intercalate into the acyl chains and disturb the chain order.

Finally, the permeability of calcein across some liposome membranes was first evaluated on the basis of the first-order kinetics by spectrofluorometer. Second, the composition/fluidity effect of liposome as well as the incubation temperature/pH effect was investigated. Furthermore, a model simulating the conditions of digestion was developed to estimate the partition coefficient and to determine the mechanism transfer through liposomal bilayer by using AFM and STED methods. The results confirmed that calcein permeates slowly through liposomal membrane by diffusion without liposome disruption.

Keywords: Nanoliposome, release, molecular interaction, transfer, physicochemical characterization.