Universidade do Minho
Escola de Engenharia

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Development of liposomal formulations for therapeutic applications

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Professor Doutor Artur Manuel Cavaco Paulo
e da
Doutora Eugénia Sofia da Costa Nogueira

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Desenvolvimento de formulações lipossomais para aplicações terapêuticas

RESUMO

Os lipossomas são considerados um sistema de entrega adequado para vários fármacos. O nosso grupo de investigação desenvolveu uma formulação lipossomal com metotrexato (MTX), a primeira linha de tratamento da artrite reumatoide (AR). Os lipossomas têm uma estratégia inovadora de direcionamento por folato, recorrendo a um péptido hidrofóbico, que tem como alvo o recetor de folato β presente na superfície de macrófagos ativados, células efetoras chave na AR. Estes lipossomas acumulam-se nas articulações de ratinhos com artrite, melhorando as pontuações clínicas comparativamente a ratinhos que recebem MTX livre. Estes resultados conduziram ao desenvolvimento do projeto europeu FOLSMART, com ênfase no desenvolvimento não-clínico e ensaios clínicos de fase I. O trabalho desenvolvido no meu doutoramento está inserido neste projeto, tendo como objetivo otimizar a produção de lipossomas com MTX e completar os ensaios não-clínicos em ratinhos com artrite, de modo a alcançar a implementação do ensaio clínico em humanos. Primeiramente, foi estabelecido um método apropriado para a quantificação de fármacos encapsulados nos lipossomas, explorando as vantagens da espectrometria de ressonância magnética nuclear (RMN). Após determinar a concentração de um fármaco hidrofóbico e outro hidrofílico recorrendo ao uso de um padrão interno, foi realizado um estudo comparativo com duas técnicas tradicionais para validar os resultados obtidos por RMN. O armazenamento da formulação lipossomal a longo prazo também foi estudado, através da incorporação de sacarose como crio e lioprotetor, de modo a preservar a integridade do lipossoma após o processo de liofilização. Lipossomas com o fármaco localizado na bicamada lipídica apresentaram perdas insignificantes de fármaco, preservando a sua atividade biológica após o processo de liofilização. De seguida, o trabalho focou-se no aumento de MTX encapsulado nos lipossomas, através do desenvolvimento e otimização de um novo método de produção, baseado nos princípios do método de injeção etanólica. Os lipossomas obtidos apresentam valores pequenos de tamanho e índice de polidispersividade (sem extrusão), alta encapsulação de MTX e um aumento do benefício clínico em ratinhos com artrite. No final, foram realizadas experiências em ratinhos com artrite de modo a completar os ensaios não-clínicos. O melhor rácio fármaco-lípido de lipossomas direcionados por folato encapsulando MTX foi determinado, bem como a dose necessária para se obter efeito terapêutico. Além disso, foi avaliado o padrão de biodistribuição e a via de injeção subcutânea. Em resumo, este trabalho apresenta resultados que permitem a implementação do ensaio clínico em humanos, demonstrando que lipossomas direcionados por folato constituem um sistema promissor de entrega de MTX para o tratamento de AR.

Palavras-chave: Artrite reumatoide; Direcionamento por folato; Lipossomas; Métodos de produção; Metotrexato.

Development of liposomal formulations for therapeutic applications

ABSTRACT

Liposomes are considered a suitable drug delivery system for several drugs. Our research group developed a liposomal formulation encapsulating methotrexate (MTX), the first-line therapy of rheumatoid arthritis (RA). The liposomes have an innovative strategy for folate-targeted delivery, using a hydrophobic peptide, that targets folate receptor β (FRβ) present at the surface of activated macrophages, key effector cells in RA. These liposomes showed a stronger accumulation at paws of arthritic mice, improving clinical scores compared to those receiving unformulated MTX. These results pave the way to develop the European project FOLSMART, emphases on the non-clinical development and phase I clinical trials. The work developed in my PhD is inserted in this project, aiming to optimize the production of liposomes encapsulating MTX and complete the nonclinical package in arthritic mice to further reach the implementation of the First-in-Human (FiH) clinical trial. Opening findings involved the establishment of an appropriate method for drug quantification inside liposomes, exploiting the advantages of ^{1}H Nuclear Magnetic Resonance (NMR) spectrometry. After determining the concentration of a hydrophobic and a hydrophilic drug using an internal standard, a comparative study was performed with two traditional techniques to validate the results obtained by ^{1}H NMR. A suitable approach to long-term storage of the liposomal formulation was also addressed, by incorporation of sucrose as cryo and lyoprotectant, to preserve its integrity after the freeze-drying process. Liposomes with a drug located in the lipid bilayer demonstrated negligible leakage preserving their biological activity after the freeze-drying process. Following efforts were dedicated on increasing MTX encapsulated inside liposomes through the development and optimization of a novel production method, based on the principles of the ethanol injection method. The liposomes obtained present small values of size and polydispersity index (without extrusion), a higher MTX encapsulation and increases the biological benefit in arthritic mice. At the end, a set of deeper experiments were performed in collagen-induced arthritis (CIA) mice model to complete the nonclinical package. The better drug-to-lipid ratio of folate-targeted liposomes encapsulating MTX was determined as well as the dosage needed to obtain a therapeutic effect in CIA mice. Furthermore, the biodistribution pattern was evaluated as well as the subcutaneous injection route. In summary, this PhD work present findings that allow the implementation of the FiH clinical trial, demonstrating that folate-targeted liposomes constitute a promising MTX delivery system for RA treatment.

Keywords: Folate-targeted; Liposomes; Methotrexate; Production methods; Rheumatoid arthritis.

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LIST OF SYMBOLS AND ABBREVIATIONS

AFM	Atomic force microscopy
AIDS	Acquired immunodeficiency syndrome
ASES	Aerosol solvent extraction systems
AUC	Area under the curve
CCD	Charge coupled device
$CDCl_3$	Deuterated chloroform
CH	Cholesterol
CIA	Collagen-induced arthritis
CII	Type II collagen
CIPA	Centre for Small Animal Imaging
CNRS	French National Centre for Scientific Research
CO_2	Carbon dioxide
COVID-19	Coronavirus disease 2019
Cryo–TEM	Cryogenic - Transmission electron microscopy
CT	Computed tomography
d	Doublet
D/L ratio	Drug-to-lipid ratio
D_2O	Deuterium oxide
dd	Doublet of doublets
DLS	Dynamic light scattering
DMARD	Disease-modifying anti-rheumatic drug
DOPC	1,2-dioleoyl-snglycero-3-phosphocholine
DOPE	1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
DOX	Doxorubicin
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DPPG	1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol
DSC	Differential scanning calorimetry
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
DSPE	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine
DSPG	1,2-Distearoyl-sn-glycero-3-phosphoglycerol

dt	Doublet of triplets
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
EPC	Egg phosphatidylcholine
EPR	Electron paramagnetic resonance
ER	Estrogen receptor
ESR	Electron spin resonance
FA	Folic acid, folate
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFF	Field-flow fractionation
FiH	First-in-Human
FITC	Fluorescein isothiocyanate
FL	Folate-targeted liposomes
FL–MTX	Folate-targeted liposomes encapsulating methotrexate
FR	Folate receptor
FTIR	Fourier transform infrared spectroscopy
GAS	Gas anti-solvent
GLP	Good laboratory practice
GMP	Good manufacturing practice
GUV	Giant unilamellar vesicle
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IL	Interleukin
^{111}In	Radioactive isotope of Indium
IP	Intraperitoneal
IV	Intravenous
L	Non-targeted liposomes
L–MTX	Non-targeted liposomes encapsulating methotrexate
LDE	Laser doppler electrophoresis
LUV	Large unilamellar vesicle

m	Multiplet
MDP	Methylene diphosphonate
MHz	Megahertz
MLV	Multilamellar vesicle
MPS	Mononuclear phagocytic system
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTX	Methotrexate
MVV	Multivesicular vesicle
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
NSAIDs	Nonsteroidal anti-inflammatory drugs
NTA	Nanoparticle tracking analysis
PA	Phosphatidic acid
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCS	Photon correlation spectroscopy
PCT	Patent Cooperation Treaty
PDI	Polydispersity index
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
ppm	Parts per million
PS	Phosphatidylserine
q	Quadruplet
RA	Rheumatoid arthritis
RES	Reticuloendothelial system
RESS	Rapid expansion of supercritical solutions
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RSD	Relative standard deviation
s	Singlet

SAS	Supercritical anti-solvent
SAXS	Small-angle X-ray scattering
SC	Subcutaneous
SCF	Supercritical fluid
SCRPE	Supercritical reverse-phase evaporation
SD	Standard deviation
SEC	Size exclusion chromatography
SP-D	Surfactant protein D
SPECT	Single photon emitted computed tomography
SUV	Small unilamellar vesicle
t	Triplet
TAM	Tamoxifen
Tc	Transition temperature
^{99m}Tc	Metastable nuclear isomer of technetium-99
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TGA	Thermogravimetric analysis
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
tt	Triplet of triplets
ULV	Unilamellar vesicle
UPLC	Ultra-performance liquid chromatography
UV-Vis	Ultraviolet-visible
XRD	X-ray diffraction
δ	Chemical shift (expressed in ppm)
J	Coupling constant (usually in frequency units, Hz)
°C	Degree Celsius
µL	Microlitre
µm	Micrometre
η	Yield
ζ-potential	Zeta potential

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PROLOGUE

Motivation and Thesis Outline

Motivation and Thesis Outline

Liposomes have gained extensive attention as drug delivery system due to properties such as increased drug stability, altered pharmacokinetics and target specific tissues. In this context, our research group previously developed a liposomal formulation for application in the treatment of rheumatoid arthritis (RA). The liposomes have an innovative strategy for folate-targeted delivery, using a bifunctional peptide, SP-DS3, serving as both targeting ligand linker and anchor. Folate (folic acid, FA) has high affinity for folate receptor β (FR)β overexpressed at the surface of activated macrophages, key effector cells in RA. These targeted-liposomes proved to significantly increase the clinical benefit and showed a complete prophylactic efficacy of methotrexate (MTX), the first-line therapy of RA, in an arthritic animal model. The effectiveness of the previous established targeted-liposomes in RA mouse models warranty the funding of European project H2020 FOLSMART, which focuses on the non-clinical development and phase I clinical trials.

The major goal of the PhD work was to optimize the production of liposomes encapsulating MTX and complete the nonclinical package in arthritic mice to further reach the implementation of the First-in-Human (FiH) clinical trial. Initial studies involved the establishment of a suitable method for drug quantification inside liposomes and an appropriate approach to their long-term storage. Following efforts were focused on increase the MTX encapsulated in liposomes through the development and optimization of a novel production method based on a well-known technique, the ethanol injection method. Finally, the behavior of the folate-targeted liposomes encapsulating MTX were evaluated by *in vivo* studies and their efficacy was compared using the RA mouse model.

In the quest to achieve the PhD objectives, this thesis is divided in seven chapters, in which four describe experimental work. The content of each chapter is summarized below.

The **Chapter I – General Introduction** is focused on a comprehensive overview on liposomes as the main subject of this thesis. Their physical, chemical and biological properties are addressed. A special focus is dedicated to the methods available for liposomal production, liposomal stability and their limitations. It was also reported their therapeutic application as drug delivery system.

Exploiting the advantages of ^{1}H Nuclear Magnetic Resonance (NMR) spectrometry, **Chapter II – Quantification of drugs encapsulated in liposomes by ^{1}H NMR** reports a method for quantification of drugs encapsulated in liposomes. Two different drugs were involved in this work, one hydrophilic (MTX disodium salt), and another hydrophobic (tamoxifen, TAM), as liposomes have the ability to encapsulated different types of drugs. A comparative study was also performed to validate the results obtained by ^{1}H NMR

method, using two traditional techniques: spectrophotometry and chromatography. This methodology presents some advantages such as great reproducibility, automation, and quantification without identical standard material. It was considered a suitable tool for the determination of drugs inside liposomes.

Chapter III – Protective effect of saccharides on freeze-dried liposomes encapsulating drugs describes a study involving the effect of five saccharides at different concentrations on preserving the stability and drug retention capacity after freeze-drying the liposomal formulation. The liposomes containing sucrose was considered, in a concentration dependent manner, the most suitable saccharide for the purpose and studied in terms of morphology, concentration, and anticancer drugs retention ability. This work involved two drugs encapsulated in the aqueous core, MTX (passive loading) and doxorubicin (DOX, active loading), and one drug located in the lipid bilayer, TAM. The inclusion of the cryo and lyoprotectant in the liposomal formulation can provide a protective effect to liposomes with drugs positioned in the lipid bilayer, as liposomes encapsulating TAM, maintaining their biological effect.

A novel strategy to increased encapsulation efficiency of MTX in liposomes through the new pre-concentration method was present in **Chapter IV – Increased encapsulation efficiency of methotrexate in liposomes for rheumatoid arthritis therapy**. Based on the principles of the ethanol injection method, the pre-concentration method achieves liposomes with adequate size distribution to further *in vivo* tests. The great potential of MTX to interact at the liposomal surface bilayer with the main phospholipid of the formulation appears to be a reason for the high encapsulation of MTX. The biological benefit of the liposomes produced by the novel method was proved in a mice model. This strategy shows to be a significant advance in MTX therapeutic applications.

In **Chapter V – Folate-targeted liposomes improve effect of methotrexate in collagen-induced arthritic mice**, a set of deeper experiments were performed to complete the nonclinical package in a collagen-induced arthritis (CIA) mice model. The establishment of the better drug-to-lipid ratio in folate-targeted liposomes encapsulating MTX, the dosage needed to obtain a therapeutic effect in CIA model and the biodistribution pattern of liposomes in the mice body were evaluated. To finish the nonclinical studies performed in CIA mice, subcutaneous injection of liposomes was tested since it is the more convenient and simpler route of administration for patient's self-administration.

The **Chapter VI – Liposomes encapsulating methotrexate: a powerful tool for rheumatoid arthritis therapy** is focused on the application of liposomes encapsulating MTX as a therapeutic strategy for rheumatoid arthritis therapy. The encapsulation of MTX in liposomes can be a promising approach to improve its pharmacological properties and reduce the main side effects. This review also addresses the

key aspects of MTX liposomal formulations, since their production, MTX quantification and storage conditions, highlighting the key contribution of the results obtained under my PhD in the state-of-art.

Finally, **Chapter VII – General discussion and Future perspectives** contains an integrative discussion focused on the results obtained through the developed work within the scope of this PhD thesis. Brief suggestions for future perspectives to complement the current research was presented here, describing the application of liposomes as an effective drug delivery system to the oncologic field.

CHAPTER I

General Introduction

CHAPTER I

General Introduction

Abstract

Liposomes are defined as spherical vesicles consisting of one or more concentric phospholipid bilayers enclosing an aqueous core. Being both nontoxic and biodegradable, liposomes represent one of the most innovative and powerful drug delivery system for a several therapeutic agents. They have improved the therapeutic effect via stabilizing therapeutic compounds, overcoming obstacles to cellular and tissue uptake and increasing biodistribution of drugs to target sites *in vivo*, while minimizing systemic toxicity. This review aims to offer an overview of liposomes, thought the exploration of the key fundamentals of liposomal formulations. The design of a great liposomal formulation includes a suitable composition, production method, functionalization and an effective targeting strategy, highlighting the active targeting. This targeting strategy includes the modification of the liposomes with a targeting ligand for enhanced delivery of the liposomal system. Several targeting ligands can be used for active targeting of liposomes being one of the most studied the folic acid due to its small size and ready availability. There is an abundant number of techniques for liposome production, categorized as conventional or novel methods. The chosen of an adequate technique is the primordial importance due different liposomal production methods can influence the final characteristics of liposomes. After production and before application it is required an extensive characterization of liposomes to assure their *in vitro* and *in vivo* performance. Here, several properties to characterize liposomes were explored, such as size, polydispersity index, zeta potential, shape, lamellarity, phase behavior, encapsulation efficiency, and *in vitro* drug release. Stability and some limitations of liposomes are also addressed. Finally, this review intends to explore the current market liposomes used as a drug delivery system in different therapeutic applications.

This chapter is based on the following scientific publication:

Diana Guimarães, Artur Cavaco-Paulo and Eugénia Nogueira. Design of liposomes as drug delivery system for therapeutic applications. Submitted.

I.1. Liposomes: an overview

Nanoscience can be described as the study of molecules and structures on the nanometer scale. The nanotechnology is the technology that utilizes the nanoscience in useful applications [1]. Thus, nanotechnology is the ability to convert the nanoscience theory to practical applications by observing, measuring, manipulating, assembling, controlling and manufacturing matter in a nanometer size range [2]. Novel developments in the field of nanotechnology are crucial to improve the drug delivery, thereby increasing their efficacy and decreasing their side effects. To develop new therapeutic applications, a wide selection of nanomaterials based on inorganic, organic, lipid, protein, glycan compounds and even on synthetic polymers have been employed [3]. Taking advantage of the lipids as nanomaterials was developed one of the most common and well-investigated nanodelivery system, called liposomes.

Liposomes were initial discovered in the 1960s by the British hematologist Dr. Alec D. Bangham and collaborators at the Babraham Institute, University of Cambridge, and the first report published in 1964 [4]. Liposomes are defined as a colloidal spherical structure formed by self-assembly of amphiphilic lipid molecules in solution, such as phospholipids [5]. Liposomal membrane can be composed of one or more lipid bilayers (lamellas) organized around an internal aqueous core, with the polar head groups oriented to the inner and outer aqueous phase [6]. This organized structure offers to liposomes the unique ability to encapsulate and deliver molecules with different solubility. Hydrophilic molecules in the internal aqueous core, hydrophobic molecules into the lipid bilayer and amphiphilic molecules at the water/lipid bilayer interface (Figure I.1) [7].

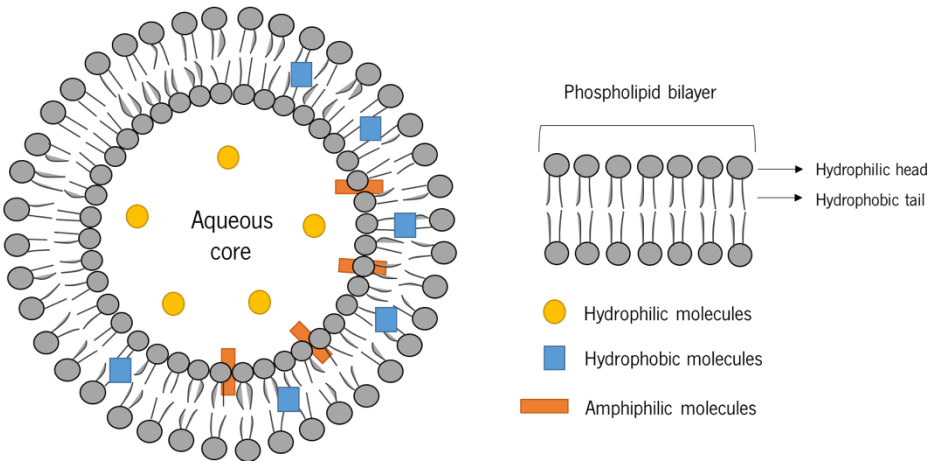


Figure I.1. Representation of the general structure of liposomes.

To date, liposomes have been investigated in several pharmaceutical research as drug delivery systems and continue to constitute an intense field of research [8]. Liposomes are considered a powerful drug delivery system due to their structural versatility as well as their biocompatibility, biodegradability, non-toxic and non-immunogenicity nature [9]. The amphiphilic character of phospholipids in solution mimic natural cell membranes, allowing excellent interactions between liposomes and mammalian cell membranes promoting an efficient cellular uptake [10]. Additional advantages of liposomes include their ability to carry large drug payloads, capacity for self-assembly and a wide range of physicochemical and biophysical properties that can be modified to control their biological characteristics [11].

Liposomes as a drug delivery system have improved therapies for a range of biomedical applications by stabilizing therapeutic compounds, overcoming obstacles to cellular and tissue uptake, and improving bio-distribution of compounds to target sites *in vivo* [12,13]. The drug encapsulated into liposome is protected against physiologically occurring events, such as enzymatic degradation, chemical and immunologic inactivation and fast plasma clearance, contributing to improve and extension of its action. Since the drug is inside the liposome occurs the minimization of its exposure of healthy tissue, thus reducing the undesirable side effects compared with the free drug form [8].

I.2. Design of liposomes

A suitable liposomal formulation can be achieved by choosing an adequate liposome composition, functionalization and even a targeting strategy, as developed deeper in the following sections. The selection of phospholipids, head group and chain length, as well as the ratio of liposomal components are crucial features to determine safety, stability, and efficiency of liposomes [14]. Moreover, the ability of liposomes as drug delivery system can be affected by the number and rigidity of lipid bilayers, size, surface charge, lipid organization and surface modification [5,15].

I.2.1. Liposome components and properties

The main component of liposomes are glycerophospholipids, which are amphiphilic lipids composed of a glycerol molecule bound to a phosphate group and to two fatty acid chains that may be saturated or unsaturated [16]. The phosphate group can be also bonded to another organic molecule [17,18]. According to this organic group, natural phospholipids are classified as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol

(PG) and phosphatidylserine (PS) [19]. Glycerophospholipids that are responsible to form liposomes can be divided in two different forms: natural and synthetic. The most natural phospholipids used to produce liposomes are PC and PE, that are abundant phosphatides in plants and animals [20]. The main sources of natural phospholipids are egg yolk or soya bean. Synthetic phospholipids are produced from natural lipids. Modification in head groups, aliphatic chains and alcohols of natural phospholipids creates a variety of synthetic phospholipids, that have proved to be more stable. Some examples of phospholipids in the synthetic form are 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-snglycero-3-phosphocholine (DOPC), 1,2-Distearoyl-sn-glycero-3-phosphoglycerol (DSPG), 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine, (DOPE) and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) [17].

In an aqueous environment, phospholipids have a strong ability to form membranes due to their amphipathic character. Therefore, liposomes are formed by hydrophilic interactions between polar head groups, van der Waals forces between hydrocarbon chains (keep the long hydrocarbon tails together) and hydrogen bonds with water molecules. Hydrophobic chains are repelled by water molecules and spontaneously occurs the self-assembly of liposomes in a closed bilayer [17,21]. Liposomes can be a combination of two or more phospholipids and consisted of single or multiple lipid bilayers. Depending on the head of the phospholipids, liposomes can acquire positive, negative, or neutral charges [22]. The final liposomal properties are influenced by the structure and characteristics of phospholipids. Liposomes can achieve different functionality with variations in head groups, aliphatic chains and in the saturation of fatty acids [23]. The stability of liposomes can be promoted using phospholipids with longer tails, and low degrees of tail unsaturation and ether linkages. Phospholipids with longer saturated hydrocarbon chains have higher ability to interact each other and to form rigidly ordered bilayer structures. Otherwise, phospholipids with shorter unsaturated hydrocarbon chains form liposomes with fluid and disordered bilayers [14,24].

In addition to phospholipids, there are more liposomal components that can enhance the stability of liposomes such as cholesterol (CH) and polyethylene glycol (PEG). These components can have pronounced effects on healthy tissues and cells, as well as activate or suppress the immune system [25]. The incorporation of CH to the lipid bilayer of liposomes can influence the bilayer fluidity and rigidity reducing their permeability and increased their *in vitro* and *in vivo* stability. CH, as a hydrophobic molecule, induces a dense packing of phospholipids and inhibits the interactions in the lipid chains by intercalating between them, promoting the stabilization of the liposomes membrane [8,26,27]. CH molecule accommodates itself among the phospholipids with its hydroxyl group close to the hydrophilic

region, and its aromatic rings parallel to the fatty acid chains into the lipid bilayer [18]. CH is crucial for the structural stability of liposomes, in their absence liposomes can interact with proteins (albumin, transferrin, macroglobulin and high-density lipoproteins). These interactions destabilize the structure of the liposomal membrane and consequently decreases their performance as drug delivery system [28–30]. The use of PEG molecule to coating liposomes can be a good approach to prolong blood circulation half-life from few minutes (conventional liposomes) to several hours (stealth liposomes) [18]. Indeed, one of the major drawbacks of conventional liposomes are their rapid clearance from the bloodstream and end up in organs and tissues in the reticuloendothelial system (RES) such as liver and spleen [31]. The improved surface properties of liposomes provide by PEG are associated to a camouflaged effect, mimicking water-like structures, providing a steric barrier that prevents the adsorption of proteins in liposome surface and avoiding their recognition by macrophages of the mononuclear phagocytic system (MPS) that otherwise leads to a rapid liposome clearance [14,17]. Besides the incorporation of CH and PEG on their composition, liposomes can be functionalized with specific ligands to improve their ability as drug delivery system, leads to a new category of liposomes called targeted liposomes. A more detailed description of the different types of liposomes will be reported below.

I.2.1.1. Phase transition temperature

Another important parameter that can affect the fluidity of the lipids within the bilayer is the transition temperature of phospholipids (Tc), which refers to the temperature at which phospholipids transit from gel to liquid crystalline phase [32]. The Tc depends on the length of the fatty acid chains, the degree of saturation of the hydrocarbon chains, the ionic strength of the suspension medium and the nature of the polar head group [33,34]. Lipid bilayers composed of phospholipids with long and saturated hydrocarbon chains should be rigid and less permeable, due the interactions between the chains are stronger, resulting in a higher Tc. Thus, hydrophobic interactions are stronger when the saturated hydrocarbon tails increase in length [35]. At a temperature lower than Tc, the phospholipids are in gel phase and presenting low fluidity and low permeability, individual molecules within the bilayer move gently. At a temperature higher than Tc, the phospholipids are in a liquid crystalline phase and having a high fluidity and usually relatively low permeability, individual molecules within the lipid bilayer move quickly. At a temperature around Tc, the liposome bilayer increases significantly the permeability due the presence of highly permeable interfacial regions between coexisting gel and liquid crystalline phase domains [18,36].

I.2.2. Liposome structure

According to their structure, liposomes are classified centered on the number of lipid bilayers (lamellae) and on the vesicle size (Figure I.2). Based on their lamellarity, liposomes can be classified as unilamellar (ULV, all size range), multilamellar (MLV, >500 nm) and multivesicular (MVV, >1000 nm) vesicles [35,37]. ULV can also be divided by their size into three categories, small unilamellar vesicles (SUVs, 20 – 100 nm), large unilamellar vesicles (LUVs, >100 nm) and giant unilamellar vesicles (GUVs, >1000 nm). ULVs are characterized by the presence of a single bilayer with more ability for the encapsulation of hydrophilic compounds. MLVs present two or more concentric lipid bilayers organized by an onion like structure, favorably for the encapsulation of lipophilic compounds. MVVs include several small non-concentric vesicles entrapped within a single lipid bilayer and are ideally suited for the encapsulation of large volume of hydrophilic material [37,38]. In addition to the vesicle size, the number of lamellae also affect the amount of certain compound to be encapsulated in liposomes [35,39].

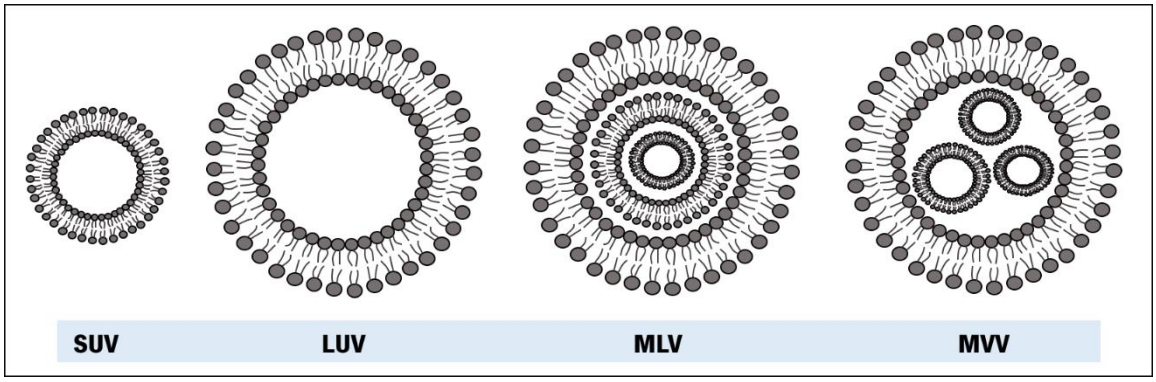


Figure I.2. Liposomal classification based on lamellarity and size. SUV (Small Unilamellar Vesicles), LUV (Large Unilamellar Vesicles), MLV (Multilamellar Vesicles) and MVV (Multi Vesicular Vesicles).

I.3. Methods for liposome production and drug loading

There are a great variety of techniques for liposome production, including the liposomal formulation methods itself and the size reduction methods. The different techniques can influence the final properties of liposomes, such as size, lamellarity, and encapsulation efficiency (EE) [40]. The methods to produce liposomal formulations can be categorized as conventional or novel. In the following section will be explored some of these methods.

I.3.1. Conventional methods

Despite the vast gamma of conventional methods applied in liposome preparation, the most common used are the thin film hydration, reverse phase evaporation, solvent injection, and detergent removal method [41,42]. These methods involve the following basic stages: (i) lipids dissolved in organic solvents, (ii) removal of organic solvent, (iii) purifying and isolation of liposomes and (iv) analysis of final liposomes [35].

I.3.1.1. Thin film hydration

The thin film hydration method, also known as the Bangham method, was the first described production process used in liposome technology [43]. In this simple method, lipids are initially dissolved in an organic solvent, generally chloroform, ether or methanol, and dried down to form a thin lipid film in a round-bottom flask by organic solvent evaporation. The obtained thin lipid film is hydrated using aqueous solvent and the liposomes are formed. Depending on hydration conditions, this method can create liposomes with different structural organization. A vigorous shaking at hydration process form MLVs with heterogeneous size, while a gentle hydration of the lipid film generates GUVs [44,45]. The main drawbacks of this method are the production of larger and heterogeneous liposomes, low entrapment ability, difficult to complete removal of organic solvent and to scale-up [41].

I.3.1.2. Reverse phase evaporation

One alternative method to prepare liposomes is the reverse phase evaporation. The initial procedure is the same of thin film hydration. Phospholipids are dissolved in an organic solvent to form a film and then the solvent are removed by evaporation. The film is re-dissolved in an organic solvent (typically, diethyl ether and/or isopropyl ether), followed by the addition of an aqueous phase, resulting in the formation of an oil-in-water emulsion [40]. The mixture is sonicated to produce inverted micelles, forming a homogeneous emulsion. The final evaporation of the organic solvent under reduced pressure form a viscous gel, that results subsequently into a liposomal suspension [35,38]. The advantage of this method is that permits a high EE [44,46]. The disadvantages include the exposure of the compounds to be encapsulated to sonication conditions and even the organic solvents [20]. This method is also described as time-consuming [41].

I.3.1.3. Solvent injection techniques

Liposomes can also be prepared by the solvent injection technique. This method involves the quick injection of the lipids dissolved in an organic solvent (ethanol or ether), into an aqueous medium, resulting in liposomes formation [47]. The ethanol injection method can be included in the category and will be the method used in this thesis. This method is usual in liposomes production due its simplicity, reproducibility, fast implementation, easy scale-up and not cause lipid degradation or oxidative alterations [48]. Ethanol has also the additional benefit to be an acceptable solvent for *in vivo* drug delivery applications, at lower concentration, according to the European pharmacopoeia [49]. Despite all the benefits, poor solubility of some lipids in ethanol, heterogeneity of liposomes when the agitation fail, very low EE of hydrophilic compounds and incomplete removal of ethanol from the liposomes, are the most concerns about this method [38,50]. Numerous parameters can be altered to control the particle size and EE obtained by ethanol injection method, such as lipid nature, lipid concentration in ethanol, drug to lipid ratio, diameter of injection orifice and injection rate [38,46].

I.3.1.4. Detergent removal

The detergent removal method is another known technique to produce liposomes. In this method, phospholipids are solubilized with detergents at critical micelle concentrations [45]. Upon detergent removal, by column chromatography or dialysis bag, and with an adequate aqueous medium, phospholipids molecules self-assemble into liposomes [35,40]. Numerous parameters can influence the size and homogeneity of the liposomes produced by this method, including initial ratio of phospholipids to detergents and rate of detergent elimination [38,46]. The drawbacks of detergent removal method can be the presence of impurities in the final liposomal formulation, possible interaction between the detergent and the encapsulated compound and the fact of this technique to be very time-consuming [41,51].

I.3.2. Size reduction techniques

Liposomes produced by the most of previous methods requires additional techniques to reduce their size, such as sonication, homogenization or extrusion [52]. There are two different sonication techniques that can be used to control the size of liposomes, a bath and a probe sonication [35]. The main disadvantages of sonication are the difficult to provide identical ultrasonic energy in a large volume of liposomal suspension (scale-up), possible risk of degradation on phospholipids and even on compound to be encapsulated, low EE and potential metal contamination from the probe tip [53,54]. In homogenization techniques, liposomes can be forced to pass within an orifice through under high pressure to reduce their size, resulting in a concept of high-velocity collision. Several techniques can be included in this category of size reduction, such as microfluidization, high-pressure homogenization, and shear force-induced homogenization processes [46]. Another technique of reducing the size of liposomes is the extrusion process. After their formation, the liposomes pass several times (extrusion cycles) through a membrane of defined pore size, normally a polycarbonate filter, to uniform size distribution [41,55]. This process requires much lower pressure and less volume of liposomal suspension compared with homogenizers [52].

I.3.3. Novel methods

The novel methods of liposome preparation are being investigated mainly to facilitate the scale-up for industrial production and to be applied to a wide range of phospholipids and drugs [40]. There are novel methods based on the modification or improvement of conventional methods, such as cross-flow injection (Wagner) method [46,56] and membrane contractor technology, both modified/improved of ethanol injection method [57,58]. The improved of detergent removal technique designs the cross-flow filtration method [59,60]. Furthermore, the use of supercritical fluid (SCF) methods has been explored in liposomes production. These methods use a supercritical fluid, such as carbon dioxide (CO_2), maintained under supercritical conditions (temperature and pressure). The SCF methods offers several advantages including a cheap and environmental harmless solvent, controlling of particle size, *in situ* sterilization and possibility of large-scale production [45,47]. The most used SCF methods are injection and decompression, rapid expansion of supercritical solutions (RESS), processes with supercritical CO_2 as an anti-solvent, gas anti-solvent (GAS), supercritical anti-solvent (SAS), aerosol solvent extraction systems (ASES) and supercritical reverse-phase evaporation (SCRPE) [42]. Recently, other methods can be also employed to produce liposomes, such as dual asymmetric centrifugation and microfluidics [41,61]. All

the novel methods referenced above have an extremely potential future in the therapeutic and pharmacological applications [59]. The main characteristics, (+) advantages and (–) disadvantages of the novel methods are outline in Table I.1, based in literature reviewing [38,40,42,61,62].

Table I.1. Main characteristics of the novel methods for liposome production.

Method	Main characteristics
Cross-flow injection (Wagner)	(+) Simple, scalable, continuous and sterile process. (–) Residual organic solvents can creates stability problems.
Membrane contractor	(+) Simple, rapid, scalable and continuous process; homogenous liposomes with higher EE for lipophilic drugs. (–) Less studied for hydrophilic drugs; high-cost material.
Cross-flow filtration	(+) Rapid, scalable, sterile process; homogeneous liposomes with high stability; facility to removal of detergent. (–) Understudy method.
Injection and decompression	(+) Sterile process; homogeneous liposomes by changing the nozzle diameter; narrow liposome size distribution; small organic solvent consumption. (–) Complex equipment with low yield; needs of high temperature and pressure; more adequate for hydrophobic drugs; nozzle can stay clogged.
RESS	(+) Simple fast and solvent-free process; liposomes with controllable size. (–) Low yield and EE.
GAS	(+) Suitable for a wide range of drugs; liposomes with variable size; and moderate stability; solvent-free and uncontaminated process. (–) Require organic solvent and needs gas and solvent separation; batch process.
SAS	(+) Simple, scalable; solvent-free and uncontaminated process; homogenous, small and stable liposomes; low use of organic solvent and moderate pressure and temperature. (–) Require organic solvents and needs gas and solvent separation; difficult to optimize conditions.
ASES	(+) Rapid, scalable and single step process; more adequate for dry liposomes; low organic residues. (–) Heterogeneous and large liposomes; uses a nozzle; understudy method.

SCRPE	(+) Simple, rapid and one-step process with scalable potential; no need for nozzles; reduced or no use of solvent; stable liposomes. (–) Understudy method; require high pressure; high-cost material.
Dual asymmetric centrifugation	(+) Simple, rapid and reproducible process; homogeneous and small liposomes; high EE for water soluble drugs. (–) Used only for small volumes; only laboratory-scale, not adequate for scale-up production, high pressure with agitation; understudy method.
Microfluidics	(+) Scalable process and used for biological samples; liposomes with controllable size. (–) Issues for thermolabile compounds; complex equipment; not adequate for scale-up production; difficult to clean after liposome production.

I.3.4. Drug loading methods

As mentioned before, liposomes are considered a good drug delivery system due their ability to encapsulate drugs with different characteristics [7]. The selection of an adequate method for drug encapsulation into liposomes depends of several factors such as EE, drug-to-lipid ratio, drug leakage and retention, sterility, facility of production and scale-up, cost efficiency and liposome stability [38,63]. Furthermore, the amount of encapsulated drug is related with the kind of drug, the composition of liposomes and the method used for the liposomal production [40]. There are two different processes to encapsulate drugs into liposomes, namely passive and active methods [35].

Passive loading method describes the procedure in which the drug is encapsulated during the liposome preparation. Hydrophilic drugs are dispersed in the aqueous phase (inside and outside of the liposomes), whereas hydrophobic drugs are located in the bilayer of the liposome [38]. In this procedure, immediately when they are being formed, liposomes can capture the aqueous volume containing the hydrophilic drug previous dissolved. Consequently, the concentration of the drug inside the aqueous core is similar to the aqueous volume enclosed by the liposomes. The EE of drugs encapsulated by passive loading changes due to numerous features, such as drug solubility, liposome size and charge, lipid concentration and production method [64]. The liposomal membrane is not permeable to ions and charged drugs. Otherwise, the uncharged drugs can diffuse through the lipid membrane, occurring drug leakage. Usually, this approach results in low EE, involving a large amount of non-encapsulated drug and

high drug leakage for the drugs permeable to liposomal bilayer [65]. However, hydrophilic drugs that have protonizable amine functions can be encapsulated into liposomes by active loading, improving their EE in comparison with passive loading [35].

The principle of active loading, also called remote loading, involves the creation of a transmembrane pH or ion gradient, that efficiently drives the drug through the lipid bilayer, leading to up to 100% loading in some drugs. This method is applied after the liposome formation. The gradient is created between the inside of the intact liposomes (already formed) and the outside of liposome, the aqueous medium, where the drug is solubilized. As uncharged drugs can diffuse across the lipid membrane, they become protonated, inhibiting their diffusion out of the liposome, enhancing their EE and retention inside liposome [65]. The ideal loading efficiency is achieved when the drug is an amphipathic weak base (pKa ≤ 11) or weak acid (pKa > 3) [66]. There are several approaches to performed active loading, such as ammonium sulfate transmembrane gradient for amphipathic weak bases, calcium acetate gradient for weakly acidic drugs, phosphate gradient method, ethylenediaminetetraacetic acid (EDTA) gradient method and ionophore loading method [65].

I.4. Characterization of liposomes

After production and before application, liposomes need to be extensively characterized for evaluation of their physical and chemical properties to guarantee their *in vitro* and *in vivo* performance [50]. The most investigated properties to characterize liposomes are size, size distribution (reported using the polydispersity index, PDI), surface charge (through zeta potential measurement), shape, lamellarity, phase behavior, EE, and *in vitro* drug release [40,45]. Table I.2 summarizes the main analytical techniques used for the assessment of liposomal characteristics.

Table I.2. Analytical techniques used for the evaluation of liposomal properties.

Properties	Analytical techniques
Size	Dynamic light scattering (DLS), Nanoparticle tracking analysis (NTA), Nuclear magnetic resonance (NMR), Field-flow fractionation (FFF), Size exclusion chromatography (SEC). Microscopy techniques: Transmission electron microscopy (TEM), Cryogenic-TEM (Cryo-TEM) and Atomic force microscopy (AFM).
Zeta potential	Laser Doppler electrophoresis (LDE) and Capillary electrophoresis.
Shape	Microscopy techniques: TEM, Cryo-TEM and AFM.
Lamellarity	Cryo-TEM, ^{31}P-NMR, Small-angle X-ray scattering (SAXS) and trapped volume determination techniques.
Phase behavior	Differential scanning calorimetry (DSC), Thermogravimetric analysis (TGA), fluorescence probe polarization, NMR, Electron paramagnetic resonance (EPR), Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD).
Encapsulation Efficiency	Ultraviolet-visible (UV–Vis) and fluorescence spectroscopy, enzyme or protein-based assays, High-performance liquid chromatography (HPLC), Ultra-performance liquid chromatography (UPLC), Liquid and Gas chromatography mass spectrometry, Electron spin resonance (ESR) and ^{1}H NMR.
Drug release	Spectrophotometry methods, HPLC and UPLC.

I.4.1. Size and Polydispersity index

The size and PDI of liposomes are the most relevant features in liposome characterization. It has known that the liposome size shown to be a crucial factor for inhalation and parental administrations [67] and to determine the circulation half-life of liposomes [68]. While small liposomes can circulate in the organism for long time, large liposomes are more quickly eliminated from the blood circulation [11]. For drug delivery, the desirable size of liposomes usually ranges between 50 and 200 nm [47]. The PDI value reveals in terms of size, the degree of sample heterogeneity, that can be monodisperse or polydisperse. PDI can be dimensionless and scaled such that values range from 0 to 1. A PDI value lower than 0.1 indicates a high homogeneity of the liposomal population, whereas high PDI value is associated with a very broad size distribution (heterogeneity) or even several liposomal populations in the sample [69]. The

calculation of PDI is based on the particle size, refractive index of the solvent, the measurement angle and the variance of the distribution [70].

The most used technique to measure these two features is DLS also known as photon correlation spectroscopy (PCS). DLS analyses the continuous motion of the dispersed particles in solution (Brownian motion), resulting in scattering of the incident light. The scattering of the light is correlated with the diffusion level of the liposomes in suspension (small particles diffused faster than the large particles). The evaluation of mean size is calculated based on the amount of light scattered. DLS is considered a simple, easy, fast and reliable method with the capacity to evaluate the liposome size in their native environment. Extensive range of measurement ability from a few nanometers to several micrometers is also applied [40,45]. However, this technique has some limitations involving the difficult to differentiate single particles from aggregates and the high sensitivity to detect low amount of impurities (contaminants) [71].

Recently, a size characterization tool called nanoparticle tracking analysis (NTA) was introduced to determine the size by measurement of the diffusion coefficient of particles in a sample [72]. DLS determine the diffusion coefficient of particles based on the reads of the intensity change of scattered light, whereas NTA find the diffusion coefficient by the movements of individual particles in successive optical video image. NTA can be a good approach to verify the size determined by DLS due they measure the same physical property. Therefore, the size measured by NTA should be similar to that observed in DLS technique [73,74]. The capacity of NTA to simultaneously measure size and particle scattering intensity, besides allowing to distinguish particles of different refractive index within the same sample solution, makes a direct estimation of particle concentration [75].

I.4.2. Zeta potential

The overall net charge of the particles is usually expressed as surface or zeta potential (ζ-potential) [52]. This feature of liposomes is considered an essential physical property in the control of the electrostatic interactions between the particles in suspension [76]. The net charge of liposomes is influenced by key parameters, such as lipid composition, the head group of lipids and associated ligands, differing from negative, neutral, or positive. The ζ-potential measurements are used to predict the stability of colloidal systems, such as liposomes in their surrounding medium. Usually, liposomes with low ζ-potential or uncharged have more probability to aggregate over time, because there will be no force to inhibit the liposomes flocculating. Otherwise, the liposomes in suspension with a large negative or positive

ζ-potential charge present repulsive forces in the medium that prevents the natural tendency to aggregation [67].

The measure of ζ-potential needs a laser to provide a light source to illuminate the liposomes within the sample. The laser beam passes through the middle of the sample cell used to the measurements at a specific angle [67]. Determination of the surface charge permits the evaluation of fluctuations in the scattered light intensity caused by the particle motion in the suspension due to the application of an electric field. The charge of liposomes is proportional to their mobility rate [40,77]. Posteriorly, the information is passed to a digital signal processor in a computer system and the value of ζ-potential is calculated by determining the electrophoretic mobility, i.e. a velocity of a particle in an electric field, and then applying one specific equation, called Henry equation [67,78]. LDE and capillary electrophoresis are the most known techniques used to measure the ζ-potential of liposomes through determination of their electrophoretic mobility [44,79].

I.4.3. Shape

The analysis of morphological characteristics, namely the shape, is vital for an adequate characterization of liposomes. The most select tool to ascertain the morphological features of liposomes is the microscopy [75]. The visualization of liposomes as individual particles by microscopy techniques provides a direct observation of their shape. Electron microscopy techniques such as TEM and cryo–TEM have been widely implemented for creating liposomal images [45]. TEM technique has some limitations at sample preparation level due the need to remove the native environment of liposomes. It is a time-consuming technique, thus is not flexible to being routine measurements. Moreover, this technique may induce alterations in liposomal shape, including possible vesicle shrinkage, swelling or artifacts formation in the created image [40,80]. To overcome these limitations, another possibility is the use of cryo–TEM. This approach keeps the liposomes close to their native state and minimize the shape distortion or shrinkage by involving the use of a flash freezing step with liquid nitrogen and then direct visualization of liposomes in a controlled environment. However, cryo–TEM usually works better with samples that have a lower nanometer range, because larger particles may be eliminated from the sample in the preparation step. The AFM technique appears for direct analysis of liposomes in their native environments without sample manipulation. It is considered a quick, powerful and non-invasive technique [67]. The main advantage of this technique over electron microscopy is the high resolution of the micrographs at three-dimensional level with resolution down to the nanometer and Angstrom scales [81].

I.4.4. Lamellarity

Lamellarity is also a characteristic that can have an impact on the further liposomal applications due their influence on the EE and drug release profile. Cryo–TEM is the most used method and provide useful information regarding liposome lamellarity such as their bilayer thickness and inter-bilayer distance [38]. Other methods to access the lamellarity are based on the visible or fluorescence signal variations of lipids marker upon the addition of certain reagents [45,67]. ^{31}P NMR approach has also been used to estimate the value of liposome lamellarity, particularly, the ratio of phospholipid amount in the outer to inner layers. The addiction of paramagnetic ions (Mn^{2+}, Co^{2+}, and Pr^{3+}) to the NMR sample preparation quenches the ^{31}P–NMR signal of the phospholipids. The interactions of the ions with the bilayer alter the NMR spectrum. Therefore, by comparison of both spectrum, before and after the incorporation of paramagnetic ions, it is possible to estimate the lamellarity [82]. SAXS and trapped volume determination are other techniques that also be used to estimate the lamellarity of liposomes [40,83].

I.4.5. Phase behavior

As mentioned above, the Tc represents an important feature that can affect the fluidity of the lipid bilayer. For drug delivery applications, phase behavior is highly considered due the fact that the lipid bilayer permeability to entrapped hydrophilic drugs increases with lipid membrane fluidity [84]. Several other liposomal properties including fusion, aggregation, stability and protein binding are also dependent on the phase behavior of a liposomal membrane [38]. Usually, the most common method used for study and determination of the Tc is the DSC. This thermal analysis technique is based on the evaluation of differences in heat flow, between a sample reference and a study sample. Both samples are subjected to a programmed heating, cooling or isothermal treatment using a meticulous control of the atmosphere, typically saturated with nitrogen gas [45]. The Tc can be also measure by other methodologies such as TGA, fluorescence probe polarization, NMR, EPR, FTIR and XRD [85,86]. To calculate the phase behavior of phospholipids in lipid bilayers can be also explored the molecular dynamics simulations [87].

I.4.6. Encapsulation efficiency

An optimal exploration of liposome characteristics may permit to develop liposomal formulations with ideal EE and allow the control of drug release. The liposome composition, the method of liposome production as well as the rigidity of the bilayer membrane can have a crucial impact on the EE of a certain

drug [38]. Load the proper amount of drug to achieve the therapeutic efficacy is the key in the field of medical applications [66]. EE is calculated as the percentage of the amount of drug inside liposomes (encapsulated drug), compared with the total amount of drug used in liposomal preparation (encapsulated and non-encapsulated drug). The immediate result of liposome preparation contains a mixture of encapsulated and non-encapsulated drug fractions. Thus, the first step to quantify the amount of drug within liposomes and consequently determined the EE is the separation of the free drug (non-encapsulated). Numerous techniques have been used for this purpose, including size exclusion chromatography based on the differences in size (liposome versus free drug), gravitation or centrifugation, dialysis membrane with an appropriate cut-off and ultracentrifugation [67]. The next step is the measurement of the amount of drug encapsulated into liposomes. There are two known ways to determine EE, namely the indirect and direct method. The indirect method focuses on assessing the non-encapsulated drug concentration in the eluted and subtract this concentration from the total drug concentration used in liposomal preparation. Otherwise, in the direct method the determination of EE can occur by direct disruption of liposomes with organic solvent and then the released material is quantified [88]. The conventional techniques used to estimate the concentration of drug encapsulated into liposomes depends mainly on their nature and include UV–Vis and fluorescence spectroscopy, enzyme or protein-based assays [67]. Moreover, the determination of the amount of drug can be obtained using more sophisticate equipment such as HPLC, UPLC, liquid chromatography and gas chromatography mass spectrometry (LC-MS and GC-MS, respectively) [89]. Additional techniques such as ESR and ^{1}H NMR has also been used to quantify the amount of drug [90,91].

I.4.7. *In vitro* drug release

The evaluation of the *in vitro* drug release profile can be performed using dialysis conditions. The selection of dialysis bag membrane should be in accordance with the drug specifications. It must be freely permeable to the drug and should not occur drug adsorption [67]. Liposomal sample is placed into the dialysis bag with specific molecular weight cut off, hermetically tied. The tubing membrane system is put into a simulated physiological fluid means release medium, usually a buffered saline at pH 7.4. The full system is kept at 37 °C to mimic an *in vivo* environment, and under continuous stirring. At defined time points, an aliquot of sample is taken and analyzed by the conventional methods used for drug quantification. The volume of samples needs to keep constant. Thus, an equal volume of fresh release medium is placed again in the system [40,45]. The data are used to establish the release profile by plotting the cumulative release percentage against the select time points. As extrapolation to *in vivo*

performance of liposomes as drug delivery system, the results obtained from the *in vitro* release study are widely considered in the development of liposomes for the controlled release of drugs [92].

I.5. Classification of liposomes

Liposomes compared to others colloidal delivery systems offers the advantage to alter their structural and physicochemical characteristics. Therefore, it is possible to modify liposomes behavior *in vivo* and targeting liposomes to a specific site in the organism. Liposomes can be classified based on their composition and functionalization. In addition to conventional, stealth and targeted liposomes, the recent improvement on the design of liposomes leads to a different types of liposomes such as immunoliposomes and stimuli responsive liposomes [6,93]. The differences between these categories of liposomes will be highlighted below.

I.5.1. Liposome composition and functionalization

Since their discovery, liposomes have been produced with different characteristics based on their composition and functionalization (Figure I.3). The first generation of liposomes to be used in therapeutic applications was the conventional liposomes [94–96]. These liposomes can be composed of neutral, cationic or anionic charged phospholipids, usually in combination with CH to promote the stabilization of the liposomal bilayer [11,17], as previous explained. However, this type of liposomes continues to be subjected to several difficulties, such as the instability in plasma which results in short blood circulation half-life. Liposomes are rapidly captured by RES and removed from the blood circulation [94]. The binding of opsonins, serum proteins, to the liposomes is the first signal for liposomes elimination. Opsonins, recognize the conventional liposomes as foreign particles, and consequently they are destroyed by phagocytes of the MPS [97].

To overcome the difficulties of conventional liposomes, a second generation of liposomes was developed, led to the creation of so-called stealth, long-circulating or PEGylated liposomes [98]. The stealth strategy involves mainly the possibility to coat the liposomal membrane surface with biocompatible hydrophilic polymer conjugates, such as PEG, chitosan, and others, increasing repulsive forces between liposomes and serum-components [99]. Therefore, results the reduction of immunogenicity and macrophage uptake, enhancing its blood circulation half-life and reducing the toxicity of encapsulated compound [100]. The methods to anchor the PEG in the liposome membrane involves the physical

adsorbing of the polymer onto the surface of the liposomes, the incorporation the PEG-lipid conjugate during liposome preparation, or thought the covalent attachment of reactive groups onto the surface of preformed liposomes [94]. However, an important restriction of stealth liposomes is their large body biodistribution. Thus, the encapsulated compound cannot be selectively delivered to specific target cells [101]. From this limitation, ligand-targeted liposomes were designed for targeted delivery of compounds at the desired tissues, promoting higher and more selective therapeutic activity [94]. In addition to surface modification of liposomes with PEG, targeted liposomes are also functionalized using glycoproteins, polysaccharides, or a ligand for specific receptors, such as antibodies, small molecules or peptides [97,101]. The ligand can target specific receptors which are overexpressed on the surfaces of the diseased cells, binding to them, resulting in a minimum off-target effects to healthy cells [102,103].

Following the principles of the previous strategy, it was considered the design of antibody-functionalized liposomes (immunoliposomes) and stimuli-responsive liposomes [6]. Immunoliposomes are formulated by chemically coupling of antibodies or their fragments to the liposomal surface, resulting in target antigens with an elevated degree of specificity [104]. In a stimuli sensitive liposomal system, the release of the drug occurs upon changes in some physicochemical or biochemical stimuli, such as pH, temperature, redox potential, enzymes and electrolyte concentrations, ultrasound, electric or magnetic fields [105,106]. The most common examples of stimuli-responsive liposomes are the pH-sensitive and temperature-sensitive liposomes [107,108]. Additionally to the delivery of drugs, liposomes can be used for other purposes, with simple modifications on their composition and charge [6]. A good example is the use of cationic liposomes in gene therapy as transfection vectors, to the delivery of genes. The encapsulation of genes into liposomes, permits the protection of nucleic acids against degradation during storage and in the systemic circulation [94].

More recently, multifunctional liposomes have been studied for their potential to perform a combination of multiple functions through surface modification techniques, resulting in liposomes with a wide range of functionalities [97]. In literature have been reported several examples of multifunctional liposomes. One example is the theragnostic liposomes, at the same liposome it is possible to have an imaging and therapeutic agent (diagnosis and treatment functions) [11,109]. Another example is the dual-targeting liposomes that involves liposomes having two different ligands [97].

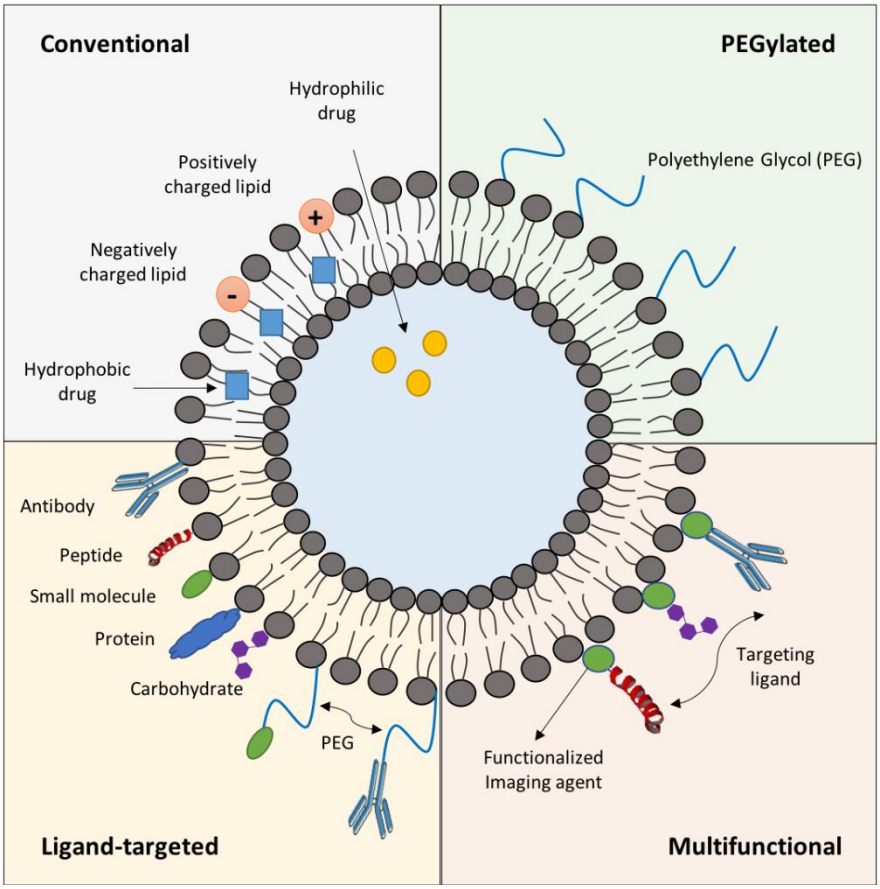


Figure I.3. Different types of liposomes used in therapeutic applications.

I.5.2. Targeting strategies of liposomes

Almost as intense, an area of research and development of the liposome formulation is their targeting strategies. The specific targeting is a primordial functional property of liposomes as drug delivery systems [110]. Thus, targeting of specific sites focuses on both the development of new diagnostic tools and improving the efficacies of therapeutic agents [111]. Currently, there are two main strategies by which targeting of liposomes can be broadly classified, namely passive and active targeting. Passive tissue targeting is mainly achieved through properties of cancer vasculature, and active tissue targeting through receptor-specific ligands on the liposome surface intended for cell binding (Figure I.4) [112].

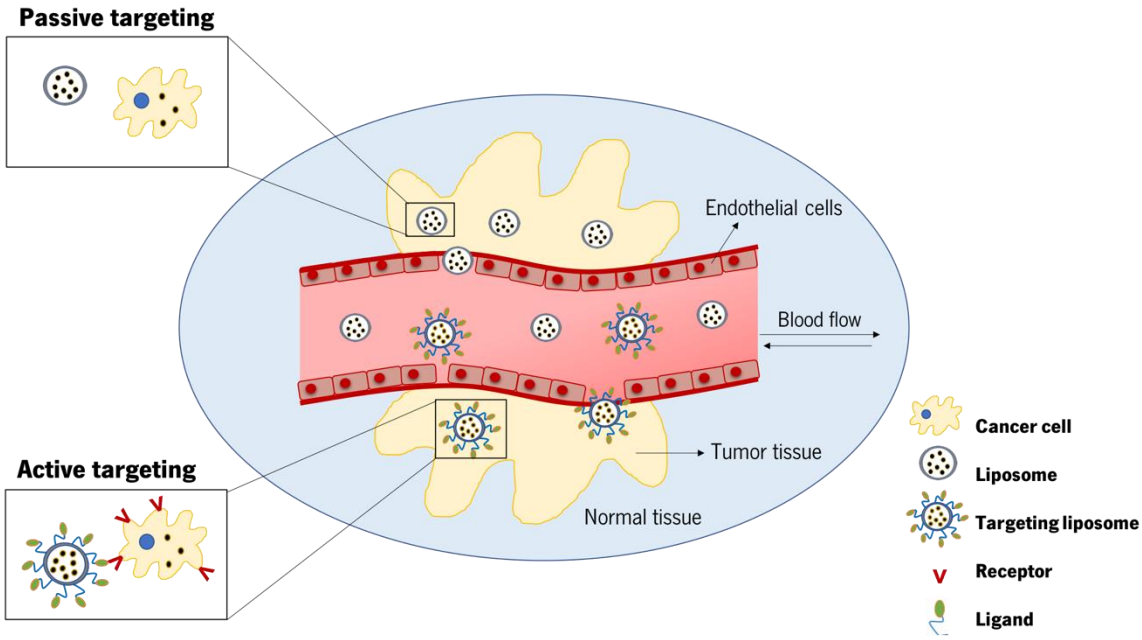


Figure I.4. Schematic illustration of passive and active targeting strategies of liposomes into a tumor for enhancing the therapeutic efficacy of drugs.

I.5.2.1. Passive targeting

Passive targeting approaches have been mainly applied in the oncology field due to pathophysiological features of cancers and their environment [3]. Passive targeting of liposomes to tissues or cells is performed by transport and delivery them into the tumor interstitium via leaky tumor vasculature through molecular drive within fluids [113]. In this way, non-targeted liposomes ranging from 10 to 500 nm in size can accumulate preferentially on the tumor and inflamed tissues via the enhanced permeability and retention (EPR) effect of the vasculature, because of abnormal leaky blood vessels and lack of functional lymphatics [114–116]. Passive targeting involves the needs to develop a liposomal formulation that can avoid their rapid elimination by organism defense mechanisms, such as phagocytic uptake or clearance by the cells of the MPS [52]. Thus, the preparation of stealth liposomes can be a good example to be used in passive targeting approaches due to surface modification of liposomes with PEG, that permits increase their circulation time [110]. This strategy also involves the use of typical features of liposomes, such as their charge, that can induce the specific targeting to the cancer cells. Another example is the cationic liposomes. This type of liposomes is found to bind the negatively charged phospholipid head groups, specially expressed on tumor endothelial cells by electrostatic interactions

[117]. The mechanism of targeting based only on the EPR effect is not enough to complete attenuate the side effects of cytotoxic drugs. The heterogeneity of EPR effect within tumors and their limitation to some solid tumors, can also affect the efficacy of drugs delivered by passive targeting [52,118]. Therefore, the development and searching of alternative targeting approaches with advanced functionalities such as active targeting have been explored [3].

I.5.2.2. Active targeting

In 1906, the visionary Paul Ehrlich introduced the concept of active targeting by describing a "magic bullet" needed to direct specific drug delivery within the body [112,119]. Since then, researchers worldwide have been searching for the "magic bullet" that would target selected cells with precision facilitating diagnosis and therapy [120]. Active targeting involves the attachment of a targeting ligand to the surface of liposomes for enhanced delivery of liposomal systems [97]. Numerous targeting ligands have been employed to active targeting, including antibodies, nucleic acids (aptamers), peptides and whole proteins (*e.g.*, transferrin) and small molecules such as vitamins (*e.g.*, folic acid) [3]. There are several aspects considered in the selection of target ligands, which include: relative degree of over-expression or selective expression on the target, target cell uptake of the ligand-targeted formulation, and degree of covering of the target molecule [121,122]. These ligands should also be selected to allow binding to the target cells while minimizing binding to healthy cells [101,112].

There are three main approaches available to functionalize liposomes. The first is binding the desired targeting ligand to a lipid prior to mixing them with other lipid components during liposome preparation. In second approach, immediately after preparation, liposomes are functionalized with the required targeting ligand [123]. Head group modified lipids with a PEG spacer functionalized at the end with amine, carboxylic acid, thiol or maleimide groups represent available options for this approach [124]. In another methodology, it was proposed the post-insertion of the functionalized lipid in preformed liposomes. This method is based on the spontaneous incorporation of functionalized lipids from the micellar phase into preformed and even drug-loaded liposomes. Derivatization of the targeting molecule happens in a separated step, as an approach to prevent the interference of activated lipids with other liposomal components such as those present in the buffer [125].

I.5.2.2.1. Folic acid

As pointed above, there are several targeting ligands that are used for active targeting of liposomes, being one of the most studied the folic acid (FA). FA, also known as vitamin B9, is a small molecule, stable over a broad range of temperatures and pH values, inexpensive, non-immunogenic, and it retains its ability to bind to the folate receptor (FR) after conjugation with drugs or diagnostic markers [126]. FRs include at least four isoforms, α, β, γ/γ'' and δ, exhibiting distinct patterns of tissue expression [127]. Functionalization of liposomes with FA offers some advantages for specialized drug delivery owing to its ease of conjugation to liposomes, its high affinity for FRs and the relatively low frequency of FRs in normal tissues as compared with their over-expression in activated macrophages (FRβ) and cancer cells (FRα) [128]. FRγ has been detected in certain normal and malign hematopoietic cells, while FRδ has been found to be expressed on regulatory T cells.

I.6. Limitations of liposomes

The stability of liposomes is a key consideration in drug delivery applications. Indeed, the therapeutic effect and safety of liposomes encapsulating drugs depend on their lifetime and their distribution within the body, and these features are directly related with their stability [129]. The stability is considered the main concern for liposome preparation, storage and further administrations steps [67]. The potential instability issues of liposomes are typically related to oxidation and/or hydrolysis of lipids, drug leakage, aggregates formation or even liposomal fusion [94]. Another challenge of liposomal formulations is the identification of a suitable large-scale production method and the needs to found an efficient sterilization technique for liposomes. These limitations of liposomal formulations will be addressed below.

I.6.1. Liposome stability

Liposomes itself are considered a thermodynamically unstable colloidal system, tending to aggregation over time [130]. A stable liposomal form preserves its physical integrity and does not negatively stimulus the chemical integrity of the encapsulated drug during its life [67]. The evaluation of liposomal stability includes the verification of some specific parameters such as (i) the chemical and physical stability, (ii) the conservation of their size and structure, (iii) the maintenance of encapsulated drug and (iv) the impact of biological fluids on the liposomal properties [17]. Thus, these parameters can

be interrelated. According to Food and Drug Administration (FDA), liposomes needs to be stable at least two years to be considered a liposomal drug product [38].

Chemical and physical stability are the main critical issues that influence the final performance of liposomes at biological level [20]. Normally, the evaluation of size and the visual inspection of liposomes appearance are two principal features to determine the liposomal physical stability. This event is related to the tendency to agglomeration or aggregation. Thus, fusion and breakage of liposomes on storage can also leads to drug leakage from liposomes [67]. Chemical stability can be considered the aptitude of liposomes to preserve the level of EE when changes in the medium can be occur, including pH alterations, electrolyte composition, oxidizing agents, and presence of surface active compounds [38]. The most important component in liposomes is the lipid. In its turn, lipids contain unsaturated fatty acids and can suffer oxidative reactions that can be stimulated by light, metal ions or temperature [17]. Chemical degradation may induce permeability changes within lipid membrane. Additionally, the interactions between the drugs and phospholipids can also interfere in liposomal chemical stability. The control of microbial stability of liposomal formulations is also important due the therapeutic formulations of liposomes are parenteral products and must be sterilized to remove the microbial contaminants from the final product [67].

I.6.1.1. Freeze-drying

Liposomal formulations can be stored in an aqueous solution or in a dry powder form [5]. To overcome the main instability issues of liposomes in an aqueous solution, their storage in a dry state can be an attractive way for long-term stability [131]. Among the feasible methodologies, freeze-drying, also known as lyophilization, remains the most studied and applied technique for this purpose. Freeze-drying consists on water removal from a frozen sample by sublimation and desorption under vacuum. However, the complexity of the process itself can compromise the liposomal membrane integrity from stresses caused by the freezing and drying steps. Thus, the choice of an ideal conditions to lyophilization is the main challenge to origin a final product with adequate characteristics, such as (i) elegant cake appearance with fast reconstitution time, (ii) suitable physicochemical characteristics after reconstitution, (iii) low water content and (iv) satisfactory long-term stability of final liposomal formulation [132]. The use of an appropriate excipient within the liposomal formulation, can maintain their size and avoid their drug leakage [133]. The excipients are included to protect the liposomes in the main steps of the freeze-drying

process, cryoprotectants assist in freezing stress and lyoprotectants contribute to drying stress. Table I.3 represents the most used excipients in freeze-drying of pharmaceutical products [132].

Table I.3. Examples of commonly used excipients in freeze-drying of pharmaceutical products.

Type of excipient	Main characteristics	Excipient
Bulking agents	Offers bulk to the formulations, in the case of very low concentration of the product to be freeze.	Trehalose, mannitol, lactose, hydroxyethyl starch and glycine.
Buffers	Regulate pH changes during freezing.	Phosphate, tris hydrochloride, citrate and histidine.
Stabilizers	Protect the liposomes during the lyophilization process, including freezing drying stresses.	Sucrose, lactose, glucose, trehalose, glycerol, mannitol, sorbitol, glycine, alanine, lysine, PEG and dextran.
Tonicity adjusters	Control the osmotic pressure and produce an isotonic solution	Sucrose, mannitol, glycine, glycerol and sodium chloride.
Collapse temperature modifiers	Obtain higher drying temperatures increasing the collapse temperature.	Hydroxypropyl-β-cyclodextrin, PEG and dextran.

The formulation features, including the liposomal composition, the nature of the drug as well as the type of excipient are the main responsible by the protective effect during lyophilization. Therefore, an exhaustive optimization of these features can be an appropriate way to improve the stability of the liposomes after lyophilization. The most used excipients are the sugars such as trehalose, sucrose and glucose. The sugars are considered ideal stabilizers to protect liposomal integrity during the lyophilization process. The stabilizer effect promoted by sugars depends on their nature and concentration. Thus, these parameters must be careful selected and optimized to guarantee an enhanced stabilizer effect of lyophilized liposomes (Figure I.5) [132].

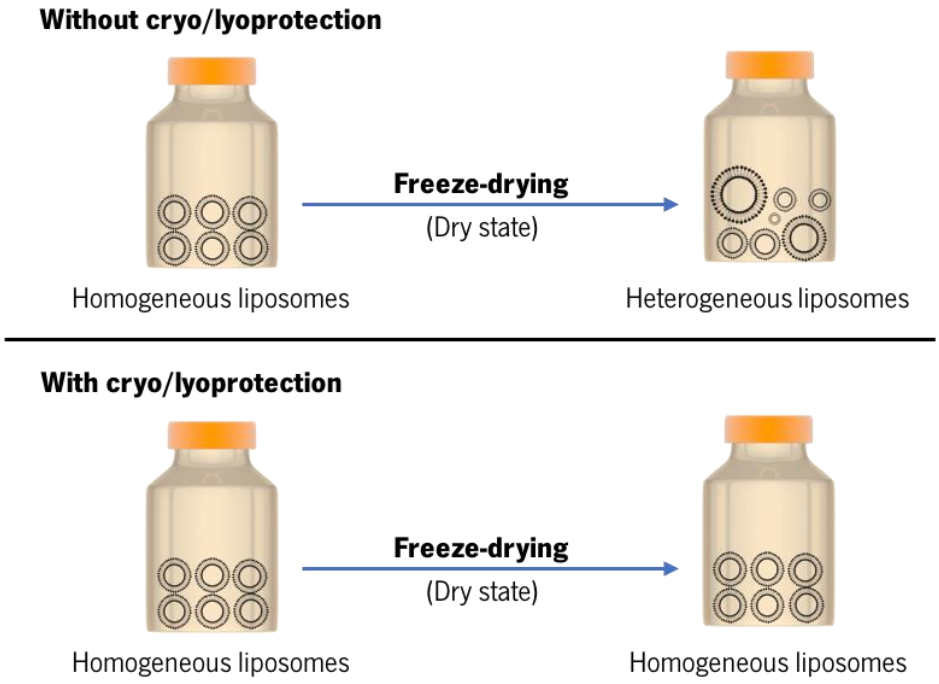


Figure I.5. Effect of cryo/lyoprotection on the size of liposomes after the freeze-drying process.

I.6.2. Scale-up and sterilization methods

The major limitation of liposomes application is the identification of a suitable method for large scale production as known as scale-up. To use liposomes as an acceptable pharmaceutical product, their production at large scale needs to be easily and economically feasible [67]. The slowed develop in scale-up process is associated to the time dispensed to resolve problems involving the quality and technological control. These problems included (i) accessibility of high-quality lipid raw materials, (ii) validated quality control analyses, (iii) unavailability of equipment, (iv) reliability and reproducibility batch to batch, (v) efficient and valid sterilization methods and (vi) long-term stability of produced liposomes. All these problems can be interrelated [98,134]. As discussed in section I.3, there are several methods available for production of liposomes at laboratory scale. However, only a few manufacturing techniques are available at industrial scale [46]. The production of liposomes involves an amount of unit operations which are not easy to transpose for commercial manufacturing [135]. The key issue for production of a successful liposomal formulation at industrial scale is the control and keep constant the characteristics of each batch maintaining the reproducibility of the method [136]. At laboratory scale, usually is easy to reach the reproducibility of the process, whereas at industrial scale the PDI of liposomes is difficult to control and the reproducibility of batch-to-batch is challenging to achieve [137]. The ethanol injection

method is considered the most interesting technique for this purpose due the reproducibility and fast implementation of this method [138,139].

Another limitation of liposomes is their sterilization that remains a challenging issue due the susceptibility of liposomes to physical and chemical degradation. Methods for liposomes sterilization should be a compromise between the inactivation of the microorganism's contamination and the degradation of liposomal product. The sterilization methods should not affect the physical and chemical characteristics of liposomal formulation and should be destructive for the microorganisms [134]. The most common technique to achieve sterilize small liposomes is the filtration using a sterile polycarbonate membrane with adequate pore size, normally 0.22 μm, under aseptic conditions [67]. This method has the advantage that is not destructive for small liposomes. Filtration is not appropriated for liposomes with high values of size (> 0.22 μm) and for large volume of liposomes due the possibility of filter clogging which compromises the final product. It should be noted that there are other methods for liposomes sterilization, for example, autoclaving, high pressure sterilization using nitrogen gas, utilization of saturated steam to sterilize pharmaceutical equipment, ethylene oxide treatment, UV sterilization, γ-irradiation and dense gas technique [134,135]. However, it is important to note that conditions required in these conventional sterilization techniques can be detrimental to the stability of the liposomal preparations [135].

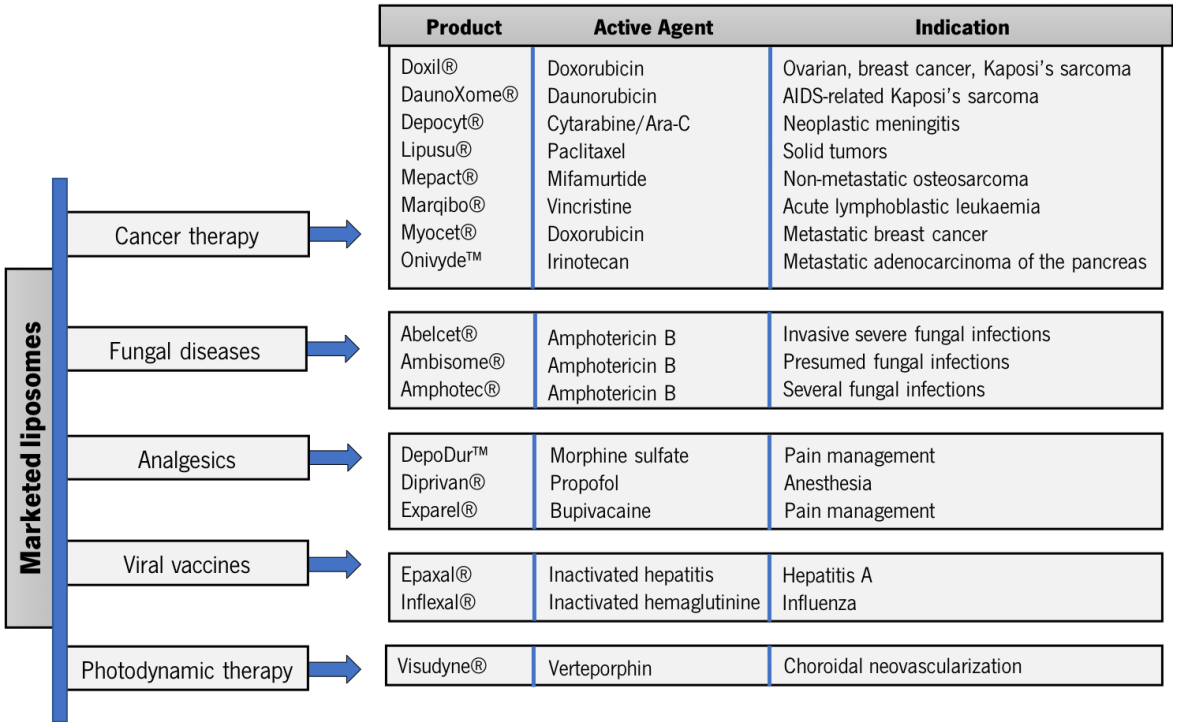
I.7. Therapeutic applications of liposomes

Liposomes have been revealing promising results as drug delivery system for numerous types of drugs. Thus, the intensive investigation of liposomes in medicine led the researches to develop different liposomal formulations for the controlling and management of a wide range of diseases besides an extensive variety of therapeutic applications. The encapsulation of drugs inside liposomes improve their therapeutic effect due the pharmacokinetics and pharmacodynamics alterations [140]. The modulation of the *in vivo* drug behavior and the reduction of the drug toxicity in the organism are the crucial features to design a suitable liposomal formulation. The use of liposomes in clinical applications focuses in the treatment and diagnosis of cancer. However, the potential of liposomes for therapeutic applications is not limited to cancer therapy. Liposomes are considered an extremely flexible platform and can be used in diverse field of research [33]. In this section will be explored the current market liposomes and the use of liposomes specifically in rheumatoid arthritis therapy.

I.7.1. Marketed liposomes

Liposomes have revealed significant therapeutic benefits in clinical applications. However, their applicability is limited due to the all stages of liposomal development and production process that comprises manufacturing methods, regulatory approval by the competent authorities and intellectual property [98]. Despite all the intensive research in the development of liposomal formulations to use in therapeutic application, in the moment, only a few liposomes have entered in the market as a commercialized liposomal product [141].

The first successful liposomal formulation, Doxil®, was introduced to the USA market in 1995 and it is the first liposomal product to obtain regulatory approval by FDA. Doxil®, or Caelyx® in Europe, is an intravenous injection product that contain doxorubicin (DOX) hydrochloride in their formulation. Doxil® is used to treat advanced ovarian cancer and acquired immunodeficiency syndrome (AIDS)-associated Kaposi's sarcoma, after the inefficiency of prior chemotherapy or intolerance therapies [140]. These liposomes proved to improve the pharmacokinetic properties of free DOX and minimize the life-threatening toxicities caused by the drug. Despite cancer therapy is the most studied area in terms of liposomal clinically approved products, liposomal products were also investigated for other diseases. Figure I.6 identifies the main therapeutic fields covered by liposomal formulations products [140]. The product name, active agent and pharmacological indications are also referenced. It can be prepared in different forms, liquid (suspension), solid (dry power) and semi-solid (gel or cream). The administration of liposomes in *vivo* can be topically or via parenteral route [67].



Product	Active Agent	Indication
Doxil®	Doxorubicin	Ovarian, breast cancer, Kaposi's sarcoma
DaunoXome®	Daunorubicin	AIDS-related Kaposi's sarcoma
Depocyt®	Cytarabine/Ara-C	Neoplastic meningitis
Lipusu®	Paclitaxel	Solid tumors
Mepact®	Mifamurtide	Non-metastatic osteosarcoma
Marqibo®	Vincristine	Acute lymphoblastic leukaemia
Myocet®	Doxorubicin	Metastatic breast cancer
Onivyde™	Irinotecan	Metastatic adenocarcinoma of the pancreas
Abelcet®	Amphotericin B	Invasive severe fungal infections
Ambisome®	Amphotericin B	Presumed fungal infections
Amphotec®	Amphotericin B	Several fungal infections
DepoDur™	Morphine sulfate	Pain management
Diprivan®	Propofol	Anesthesia
Exparel®	Bupivacaine	Pain management
Epaxal®	Inactivated hepatitis	Hepatitis A
Inflexal®	Inactivated hemaglutinine	Influenza
Visudyne®	Verteporphin	Choroidal neovascularization

Figure I.6. Main therapeutic fields covered by liposomal formulations products (adapted from Bulbake *et al.*, 2017 [142]).

It is important to highlight that most of liposomal products developed are nowadays under different pre-clinical studies and clinical trials. The translation of liposomes for clinical trials requires advanced models and methodologies. These models can predict the biosafety of liposomes inside the organism to enhanced their therapeutic applications [98].

I.8. Conclusion

Liposomes have been extensive attention as drug delivery system for numerous kinds of drugs. The direct application of liposomes in medicine encourages the researchers to create novel liposomes for treatments and diagnosis in a wide range of diseases as well as in a variety of therapeutic applications. In the context of liposomal therapy, the modulation of the *in vivo* drug behavior and the reduction of the drug toxicity in the organism are the crucial features to design a proper liposomal formulation. A suitable liposomal formulation product consists in three essential components, lipids to form a liposome,

molecules to functionalized them and a drug molecule that will be encapsulated. As we can see from this review, the development and improvement of liposomes are a complex challenge that involves the simultaneous optimization of several parameters to achieve a final liposomal formulation safe and effective. Although there are currently some liposomes approved on the market covering many health areas, it is possible to claim that there is still much to be done in the field of liposomal technology to overcome the limitations explored in this review. In summary, liposomes can contribute to treatments with key performance, hence it shall lead to a better clinical outcome, lower toxicity levels and fewer side effects.

CHAPTER II

Quantification of drugs encapsulated in liposomes by ^{1}H NMR

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Quantification of drugs encapsulated in liposomes by ^{1}H NMR

Abstract

Liposomes are one of the most important and extensively studied drug delivery system due to their ability to encapsulate different kinds of drugs. Exploiting the advantages of ^{1}H Nuclear Magnetic Resonance (NMR) spectrometry, we established a rapid and easy method for quantification of drugs encapsulated in liposomes. An internal standard, pyridine, was used for quantitative determination of drug concentration. Two different drugs were involved in this work, one hydrophilic, methotrexate disodium salt, and another hydrophobic, tamoxifen. The specificity and selectivity of the suggested method were evaluated by the absence of overlapping of at least one signal of each drug with pyridine in the NMR spectrum. The accuracy and precision of the method were assessed by adding a known amount of each drug to unloaded liposomes. Results obtained by quantitative NMR (qNMR) were validated and confirmed by comparing with two other traditional techniques, Ultraviolet-Visible (UV–Vis) spectrophotometry and High-Performance Liquid Chromatography (HPLC). It was found that the results were consistent with the ones obtained from our proposed qNMR method. Considering all the experiments conducted in this study, we deliberate that qNMR can be a suitable tool for the determination of drugs encapsulated in liposomes.

This chapter is based on the following scientific publication:

Diana Guimarães, Jennifer Noro, Ana Loureiro, Artur Cavaco-Paulo and Eugénia Nogueira. Quantification of drugs encapsulated in liposomes by ^{1}H NMR. *Colloids and Surfaces B: Biointerfaces.* 2019. 179: 414-420. doi:10.1016/j.colsurfb.2019.03.039.

II.1. Introduction

Liposomes have been considered the most suitable drug delivery system for a range of pharmaceutical and biomedical applications [143]. Due to their versatility, they can incorporate drugs with distinct solubilities. Hydrophobic drugs have affinity to the lipid bilayer and hydrophilic drugs are entrapped in their aqueous compartment. The delivery of drugs by liposomes enhances their therapeutic index and alters their biodistribution profile [142].

The immediate result of production of liposome encapsulating drugs is a mixture of encapsulated and free drug. After applying the separation process, several techniques are used for drug quantification, including spectrophotometry, fluorescence spectroscopy, enzyme-based methods, electrochemical techniques and chromatographic methods [7]. The assessment of the drug concentration encapsulated in liposomes by traditional methods such as Ultraviolet-Visible (UV–Vis) spectroscopy and High-Performance Liquid Chromatography (HPLC) can be a challenge for investigators. Although UV–Vis methodology allows a rapid and simple measurement of the drugs, some difficulties may appear. An inefficient drug quantification may occur due to possible interactions among components and when the maximum absorbance of the drug is close to the maximum absorbance of an eventual component of the formulation. Regarding the HPLC, the time of analysis is a principal concern. Before starting the experiments is necessary spend time with, for example, column equilibration [144], extensive preparation of samples and buffers, being also of high cost. To minimize some of these problems, Nuclear Magnetic Resonance (NMR) spectrometry can be a suitable solution.

NMR is a powerful technique for structure determination, that has also emerged as an important analytical tool in the biomedical and pharmaceutical field for quantitative determination of drugs in different matrices, providing high specificity and sensitivity [145–147]. Quantitative NMR (qNMR) have several advantages such as great reproducibility, automation, quantification without identical standard material, and total detection permitting an unbiased overview of the sample composition [148,149]. The principle of qNMR analysis reveals that integrated peak area of each ^{1}H NMR signal provided corresponds directly to the equal number of equivalent nuclei responsible for that signal. Therefore, adding an internal standard is possible to quantify the amount of test drug. The most significant conditions for an internal standard are its solubility and its chemical interaction with the drug to be quantified [150]. Numerous studies confirm that qNMR is a valid technique for pharmaceutical analysis [151,152].

In the scope of the current study, we outline a rapid and easy method based on ^{1}H NMR spectroscopy with an internal standard (pyridine) to determine the concentration of drugs encapsulated

in liposomes. The concentration of two different drugs was determined, one hydrophilic, methotrexate (MTX) disodium salt, and another hydrophobic, tamoxifen (TAM). A comparative study was performed based on the results obtained by ^{1}H NMR spectroscopy with two other techniques: UV–Vis spectrophotometry and HPLC–UV/Vis.

II.2. Materials and Methods

II.2.1. Materials

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE–mPEG) were achieved from Lipoid GmbH (Germany). Deuterium oxide (D_2O) and deuterated chloroform ($CDCl_3$) were obtained from Cortecnet (France). All the other chemicals using in this work were purchased from Sigma-Aldrich (USA), except the MTX that was acquired from Huzhou Zhanwang Pharmaceutical (China).

II.2.2. Liposome preparation

Liposomes composed of DOPE/Cholesterol/DSPE-mPEG (54:36:10, molar ratio) [153] were produced by ethanol injection method [154]. Firstly, an amount of DOPE, Cholesterol and DSPE-mPEG was dissolved in ethanol and secondly injected under vigorous magnetic stirring to phosphate-buffered saline (PBS, pH 7.4), at 70 °C. Encapsulation of drugs was done by their mixture during the formation of the liposomes. MTX disodium salt, as hydrophilic drug, was added in aqueous phase (PBS) and TAM, as hydrophobic drug, was included in organic phase (ethanol).

II.2.3. Physicochemical characterization of liposomes

The physicochemical characterization of liposomes was evaluated using dynamic light scattering (DLS) technique in terms of size distribution and ζ-potential. The analysis was determined at pH 7.4 ± 0.02 (PBS buffer) and at 25.0 °C, using a Malvern Zetasizer Nano ZS (Malvern Instruments) by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively. The values for viscosity and refractive index of dispersant were taken as 0.8616 cP and 1.332, respectively (values automatically calculated by the software, considering the components of PBS aqueous buffer). Each sample was measured in triplicate and results are presented as mean value ± standard deviation (SD).

II.2.4. Determination of drug concentration

The non-encapsulated drugs were removed from the liposomes after passage through a gel filtration chromatography column (GE Healthcare, UK), with 5 kDa cut-off (PD-10 Desalting Columns containing 8.3 mL of Sephadex™ G-25 Medium). After separation of the free drug from liposomal formulation, the concentration of each drug encapsulated in liposomes was determined using three different techniques: ^{1}H NMR, UV–Vis spectrophotometry and HPLC–UV/Vis.

II.2.4.1. ^{1}H NMR

^{1}H NMR experiments were performed using a Bruker Avance III Instrument, operating at 400 MHz. After freeze-drying of liposomes (remove water molecules to not interfere in NMR analysis), the ones contain MTX were dissolved in deuterium oxide and the ones with TAM dissolved in deuterated chloroform. For the quantitative analysis, a known amount of pyridine of high purity was used as internal standard. The calculations were assessed by comparing the integration of one peak of the drug with the integration of one peak of pyridine. NMR signal multiplicity is given as: s (singlet), d (doublet), t (triplet), q (quadruplet), dd (doublet of doublets), dt (doublet of triplets), tt (triplet of triplets) and m (multiplet).

II.2.4.2. UV–Vis spectrophotometry

Quantification of MTX and TAM was evaluated by measuring the absorbance at the maximum wavelength of each drug (MTX at 303 nm in PBS, and TAM at 280 nm in methanol). When necessary, dilutions of liposomes encapsulated drugs were performed, in order to be in the range of calibration curve. UV–Vis spectra of liposomes encapsulated drugs were recorded on spectrophotometer BioTek Synergy™ HT using a quartz microplate. The final drug concentration was determined based on the respectively calibration curve.

II.2.4.3. HPLC–UV/Vis detector

The HPLC–UV/Vis analysis were performed using an ultra HPLC Nexera, SHIMADZU (Kyoto, Japan). To both drugs, the separation was achieved using a KNAUER C18 column maintained at 35 °C. The mobile phases were filtered and degassed prior to use. To MTX, the mobile phase A was 0.1% trifluoroacetic acid (TFA) in ultra-pure H_2O and mobile phase B was 0.1% TFA in acetonitrile. The flow rate of mobile phase B consisted in a linear gradient from 20-40%, flowing at a rate of 1.2 mL/min. The

sample injection volume was 20 µL and MTX detected at 303 nm. Standard MTX solutions were prepared in a concentration range of 0.1 to 0.005 mg/mL, diluting the stock solution in PBS. To TAM, the mobile phase consisted of methanol / ammonium acetate buffer solution 0.5 M (75:25 v/v) at a flow rate of 1 mL/min, monitored at 280 nm. The injection volume was 40 µL. Standard TAM solutions were prepared in a concentration range of 0.5 to 0.015 mg/mL, diluting the stock solution in methanol.

II.2.5. Method validation

The accuracy of the method was determined by the standard addition method (recovery experiments), in which dispersions containing the liposomal formulation, were added to different amounts of drug standard solution to attain six different drug concentrations in a range of 0.1 to 5 mg/mL. After drug quantification by ^{1}H NMR, the obtained values were compared with theoretical values and reported as % recovery, following the equation:

$$\text{Recovery (\%)} = \frac{\text{Obtained value (mean value)}}{\text{Theoretical value}} \times 100$$

Equation II.1. Determination of the method accuracy.

The precision of the method was determined by replicate analysis of each calibration standards. The relative standard deviation (RSD) values were calculated from the ratios of the SD to the mean and expressed as percentage by the following equation:

$$\text{Relative Standard Deviation (\%)} = \frac{\text{Standard deviation (SD)}}{\text{Obtained value (mean value)}} \times 100$$

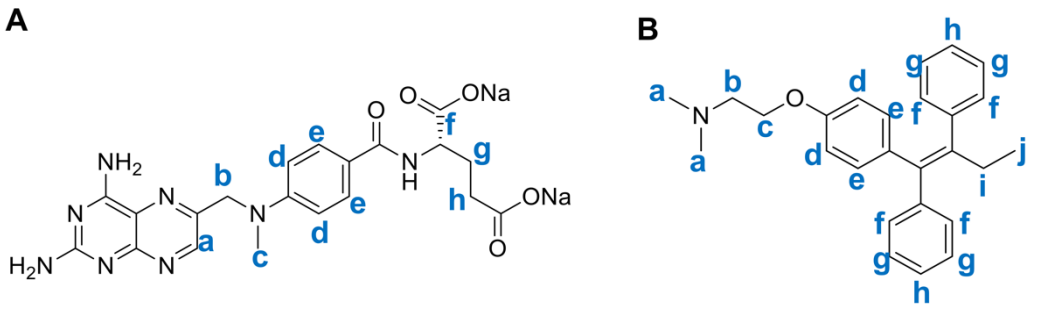
Equation II.2. Determination of the method precision.

II.3. Results and Discussion

II.3.1. Assignment of ^{1}H NMR signals

Prior to quantification of the drugs encapsulated in the liposome, the ^{1}H NMR analysis of the drugs structure was performed. Is possible to observe in Table II.1 the complete analysis of the ^{1}H NMR spectra of MTX and TAM. Both compounds have aliphatic protons that can be observed between δ_H 0.93 to 4.79 ppm and aromatic protons between δ_H 6.57 to 8.59 ppm.

Table II.1. ^{1}H NMR characterization of **(A)** MTX in D_2O and **(B)** TAM in $CDCl_3$.



	Protons (ppm)									
	a	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**
MTX (A) in D_2O	8.59 (s, 1H)	4.79 (s, 2H)	3.17 (s, 3H)	6.89 (d, J= 8.8 Hz, 2H)	7.72 (d, J= 9.2 Hz, 2H)	4.32 (dd, J= 8.8, 4.4 Hz, 1H)	1.29-2.08 (m, 1H), 2.12-2.21 (m, 1H)	2.29-2.34 (m, 2H)	—	—
TAM (B) in $CDCl_3$	2.29 (s, 6H)	2.65 (t, J= 6 Hz, 2H)	3.93 (t, J= 6 Hz, 2H)	6.57 (d, J= 8.8 Hz, 2H)	6.77 (d, J= 8.8 Hz, 2H)	7.09-7.20 (m, 5H), 7.24-7.29 (m, 4H), 7.35 (t, J= 8.4 Hz, 1H)			2.47 (q, J= 7.2 Hz, 2H)	0.93 (t, J= 7.2 Hz, 3H)

Our liposomes are constituted by a mixture of DOPE, Cholesterol and DSPE-mPEG which are based on aliphatic chains. The protons of these type of compounds are observed in the NMR spectra below δ_H 4.0 ppm (data not shown), leaving the aromatic area empty. Most constituents of liposomes found in the literature are based on the same compounds, or equivalents in terms of type of chemical character [155]. Based on our results, the signal of the liposome constituents does not interfere with the

aromatic signals of our tested drugs. Considering that a broad range of drugs possess in their constitutions aromatic rings, or allyl protons [156], the following methodology presented can be widely applied for drug quantification.

II.3.2. Quantification of drugs by ^{1}H NMR

The quantification of drugs by ^{1}H NMR is based on the addition of an internal standard. This standard is chosen centered on the tested drug structure, and its crucial for an accurate quantification. In this way, a suitable internal standard must consider certain criteria, (i) have signals (chemical shifts) that don't interfere with other signals, (ii) be accessible in pure form, (iii) have solubility in different NMR solvents, (iv) be easily measured, (v) nonreactive, (vi) nonvolatile, (vii) stable at long-term, and (viii) ideal molecular weight [157]. After analysis of the drug structure, the next step is the identification of the possible signals that can be used for quantification without interference of the internal standard (signal overlap) [158]. This statement means that we must guaranty that the signals of the drug and the internal standard are integrated separately [144]. The concentration of the drugs is calculated based on the integration of one peak of the internal standard compared with the integration of one peak of the drug. In the present study, pyridine was used as internal standard, since it supplies a well-separated signal without any interference with the tested drugs signal in the NMR spectra. Additionally, pyridine is soluble in both solvents used in this work, deuterium oxide and deuterated chloroform.

As can be observed in Figure II.1A and II.1B, pyridine is a compound that shows three distinct peaks, independently of the deuterated solvent used. In deuterium oxide, the first peak is observed at δ_H 7.49 ppm, corresponding to protons **2** (Figure II.1 in red), and should appear as a triplet. However, besides being possible to identify the triplet in the spectrum (J= 8 Hz), this signal unfolds being observed as a multiplet. The second peak, corresponding to proton **3,** appears at δ_H 7.91 ppm as a triplet of triplets (tt), with coupling constants of J= 8 Hz and J= 2 Hz. The protons in the *ortho* position of the nitrogen atom are observed at δ_H 8.56 ppm, corresponding to protons **1**, as a doublet of triplets (dt), with coupling constants of J= 4.4 Hz and J= 2 Hz. From these three peaks of pyridine, at least two of them are well separated from the peaks of the tested drugs, as can be observed in Figure II.1. The analysis of ^{1}H NMR spectra revealed that both aromatic protons **d** and **e** of MTX in D_2O and TAM in $CDCl_3$ were well separated from the other aromatic signals. For quantitative purposes, these signals were selected since they were not overlapped with any other signals, including the peaks of the internal standard.

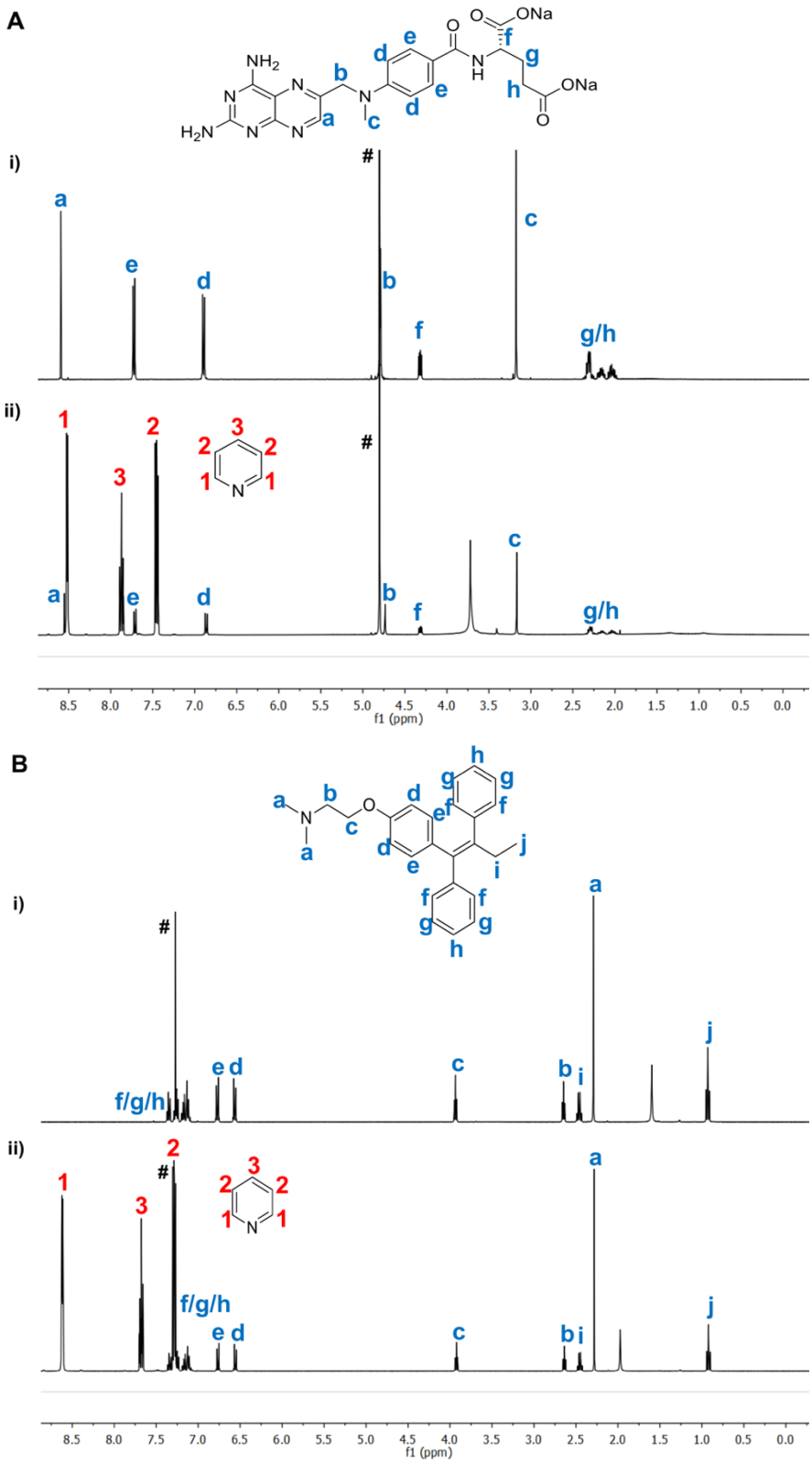


Figure II.1. ^{1}H NMR spectra of **(A)** i: MTX in D_2O and ii: MTX in D_2O with pyridine. **(B)** i: TAM in $CDCl_3$ and ii: TAM in $CDCl_3$ with pyridine. The blue letters represent the protons of each drug and the red numbers the protons of pyridine. # Peaks related to the solvent residual signal. Pyridine was used at the molar concentration of 0.124 M.

The liposomal formulation used in this study previously proved to be an efficient delivery system for the encapsulation and delivery of both hydrophilic and hydrophobic drugs [153]. The results support their use as therapeutic delivery systems as demonstrated by the biological effect of several drugs *in vitro* as well as *in vivo* [153,159]. Furthermore, pharmacokinetics studies demonstrated that to contrast to free MTX, the liposomes encapsulated MTX are selectively retained in plasma and are not subject to immediate filtering and absorption by the main organs [127]. As hydrophilic drug, MTX disodium salt is incorporated in the internal aqueous core of the liposome, and the hydrophobic TAM in the lipid bilayer [142]. The Table II.2 summarizes the physicochemical characterization of unloaded liposomes and liposomes encapsulating MTX and TAM. Since the extrusion process was not performed to promote the size reduction and homogeneity between samples, all liposomal formulations were in a higher size range, 120–150 nm. The zeta-potential of all liposomes close to zero underline the neutral charge of the DOPE-derived neutral liposomes [160]. The liposomal formulations used in this work were stable at least six months, without any significant size and PDI change and drug leakage (data not shown).

Table II.2. Physicochemical characterization of liposomes evaluated by DLS.

	Z-average (d.nm)	PDI	Zeta-potential (mV)
Liposomes	147.7 ± 2.631	0.084 ± 0.023	-0.591 ± 0.326
Liposomes + MTX	128.8 ± 1.833	0.091 ± 0.011	-0.675 ± 0.370
Liposomes + TAM	123.3 ± 0.849	0.097 ± 0.001	-2.930 ± 1.110

In the liposomal formulation, comparing the integration of a proton signal of the drug with pyridine, the concentration of the drugs can be easily calculated (Figure II.2). To remark, a shift of proton **3** occurred between analyses of both drugs since different deuterated solvents were used. Based on the ratio of pyridine/drug given by the integration of one peak of both compounds, is possible to determine the molar concentration, based on a known amount of internal standard used. Founded on these experiments, we consider qNMR an appropriate and fast technique to quantify drugs in the presence of liposomes.

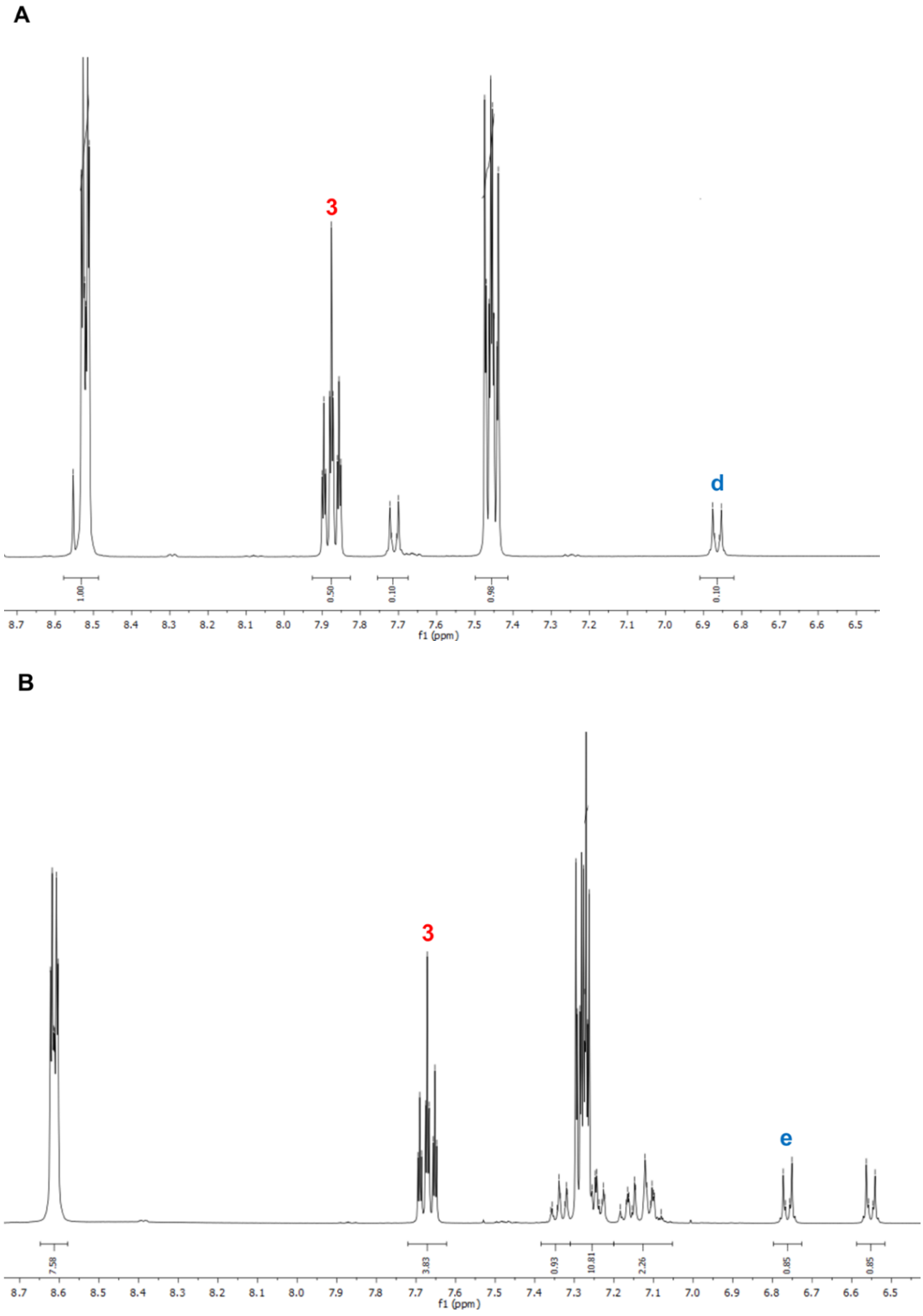


Figure II. 2. ^{1}H NMR spectra of liposomes encapsulating **(A)** MTX and **(B)** TAM. The blue letters, **d** and **e**, represent the protons used of each drug, respectively. The red number **3**, represent the proton used of pyridine for the quantification of both drugs. Pyridine was used at the molar concentration of 0.124 M.

II.3.3. ^{1}H NMR validation for quantification of drug encapsulated in liposomes

Accuracy and precision are the most important validation parameters. In this study, unloaded liposomes were added to the analytical drug solutions and the accuracy was investigated at six drug concentration levels, in the range of 0.1 to 5 mg/mL for both drugs. The mixture to be quantified is in the range of interest and the matrix composition like the test sample [150]. The accuracies were expressed as the closeness to the true value and are calculated as the percent recovery related to the theoretical values. Table II.3 and Table II.4 present the percentage of drug recovered relative to the theoretical values for MTX and TAM, respectively. The determined values were close to the true value, ranging the % recoveries from 99.4 to 101.9 for MTX and from 97.8 to 102.7 for TAM. These high values of the % drug recovered reflect the accuracy of the assay method. To remark that, the minimum theoretical value was taken as the lowest drug concentration (0.1 mg/mL), since that at lower concentrations the peaks intensity is very low for both drugs resulting in an inefficient quantification of the drugs.

Table II.3. Validation parameters calculated for MTX. Values represent the mean ± SD of 3 independent experiments. Differences were tested for statistical significance by two-way analysis of variance, being not significant.

Theoretical value (mg/mL)	Obtained value (mg/mL)	Recovery (%)	Relative Standard Deviation (%)
0.100	0.100 ± 0.001	100.9	0.25
0.250	0.250 ± 0.010	99.9	3.93
0.500	0.509 ± 0.013	101.9	2.73
1.000	0.994 ± 0.022	99.4	2.23
2.500	2.540 ± 0.049	101.6	1.94
5.000	5.004 ± 0.188	100.1	3.76

Table II.4. Validation parameters calculated for TAM. Values represent the mean ± SD of 3 independent experiments. Differences were tested for statistical significance by two-way analysis of variance, being not significant.

Theoretical value (mg/mL)	Obtained value (mg/mL)	Recovery (%)	Relative Standard Deviation (%)
0.100	0.100 ± 0.002	100.3	1.9
0.250	0.245 ± 0.004	97.8	1.48
0.500	0.502 ± 0.004	100.4	0.82
1.000	1.010 ± 0.018	101.1	1.86
2.500	2.486 ± 0.005	99.4	0.22
5.000	5.028 ± 0.195	100.6	3.87

The method precision was measured by the relative standard derivation (RSD) expressed as percentage over the concentration range of drug through validation. The RSD% values are presented in Table II.3 and Table II.4 for MTX and TAM, respectively. The RSD% of MTX ranged from 0.25 to 3.93 and of TAM ranged from 0.22 to 3.87. The low values of RSD% prove the precision of the NMR method for quantification of these drugs.

II.3.4. Comparison of ^{1}H NMR method with HPLC–UV/Vis and UV–Vis spectrophotometry

Several studies report the use of HPLC–UV/Vis and UV–Vis spectrophotometry for determination of MTX and TAM concentration [161–163]. The results obtained by qNMR were validate in comparison with these two techniques (Table II.5).

Table II.5. Comparison of different techniques for drugs quantification in liposomes. Values represent the mean ± SD of 2 independent experiments.

Technique	Standard	[MTX] mg/mL	[TAM] mg/mL
^{1}H NMR	Pyridine	3.495 ± 0.130	0.551 ± 0.001
UV–Vis	Calibration curve	3.507 ± 0.255	0.555 ± 0.019
HPLC–UV/Vis	Calibration curve	3.480 ± 0.113	0.556 ± 0.017

No significant differences were observed between the concentration values of each drug encapsulated in liposomes determined by the qNMR method and the other two techniques involved in the study. Taken together, the results indicated that the proposed qNMR method is effective for drug quantification as the HPLC–UV/Vis and UV–Vis spectrophotometry, despite the differences in the techniques' basic principle. HPLC–UV/Vis and UV–Vis spectrophotometry is based on light absorption, requiring a previous matrix effect evaluation to use the calibration curve approach. Otherwise, ^{1}H NMR signal provided corresponds directly to the equal number of equivalent nuclei responsible for that signal. The concentration of the drugs is then calculated based on the relation between the peak of the internal standard and the one of the drugs. Thus, the suggested qNMR is an accessible method and can be considered an alternative and reliable method for quantification of drugs encapsulated in liposomes.

II.4. Conclusion

The present study demonstrates that ^{1}H NMR should be used for quantification of drugs encapsulated in liposomes, independently of their hydrophilic or hydrophobic character. The internal standard used, pyridine, appears to be a versatile compound for quantification of drugs even encapsulated in nanoparticles such as liposomes. Comparing the assay results obtained by qNMR with other two different techniques, UV–Vis spectrophotometry and HPLC–UV/Vis, no significantly differences in drug concentration were observed. qNMR is an absolute quantification method that proves to be an excellent choice over previously described methods for quantification of drug concentration in liposomes. Furthermore, the determination of drug concentration by qNMR proves to be a precise and an accurate methodology. The implementation of this methodology for drug quantification in liposomes showed to be also inexpensive and fast, since is only necessary a simple step of sample preparation and a brief experiment time. Moreover, the present approach can be extended to other delivery systems. In summary, the present study offerings a simple, fast, reproducible, and relatively sensitive qNMR analysis method for quantitation of drugs encapsulated in liposomes.

CHAPTER III

Protective effect of saccharides on freeze-dried liposomes encapsulating drugs

CHAPTER III

Protective effect of saccharides on freeze-dried liposomes encapsulating drugs

Abstract

The production of freeze-dried liposomes encapsulating drugs is considered a key challenge since the drugs are prone to leakage. The aim of this work was to study the effect of different saccharides on preserving the stability and drug retention capacity of a previously developed liposomal formulation, when subjected to a freeze-drying process. The protective role of trehalose, lactose, glucose, mannitol and sucrose, known for their cryo/lyoprotective effect, was tested by addition of different concentrations to liposomes. Sucrose, in a concentration dependent manner (8:1 sugar:lipids mass ratio) proved to be a suitable cryo/lyoprotectant of these liposomes. Effectively, this saccharide prevents the fusion or/and aggregation of the liposomal formulation, protecting the integrity of the freeze-dried empty liposomes. The liposomal formulation containing sucrose was studied in terms of morphology, concentration and anticancer drugs retention ability. The study involved two drugs encapsulated in the aqueous core, methotrexate (MTX) and doxorubicin (DOX), and one drug located in the lipid bilayer, tamoxifen (TAM). After the freeze-drying process, liposomes with sucrose encapsulating drugs revealed high physical stability, maintaining their narrow and monodisperse character, however high leakage of the drugs encapsulated in the aqueous core was observed. Otherwise, no significant drug leakage was detected on liposomes containing the TAM, which maintained its biological activity after the freeze-drying process. These findings reveal that sucrose is a good candidate for the cryo/lyoprotection of liposomes with drugs located in the lipid bilayer.

This chapter is based on the following scientific publication:

Diana Guimarães, Jennifer Noro, Carla Silva, Artur Cavaco-Paulo and Eugénia Nogueira. Protective Effect of Saccharides on Freeze-Dried Liposomes Encapsulating Drugs. *Frontiers in Bioengineering and Biotechnology*. 2019. 7:424. doi: 10.3389/fbioe.2019.00424.

III.1. Introduction

Liposomes have received significant attention as drug delivery systems since they are composed of natural substances, making them nontoxic and biodegradable [164]. Drugs encapsulated within liposomes are protected from early inactivation, immediate dilution or degradation, suggesting these devices as good carriers to targeting sites [165]. Depending on their solubility, drugs can be encapsulated in the inner aqueous compartment (hydrophilic drugs), intercalated in the membrane bilayer structure or associated to the membrane surface (hydrophobic drugs) [166]. In addition, amphipathic acids or bases can be loaded in the inner aqueous core of liposomes [167,168]. The potential of liposomes for specific therapeutic applications continues to be a challenge due to their inherent physical and chemical instability for long-term storage. Some of the problems include hydrolysis or oxidation of phospholipids, liposome aggregation or/and fusion, and increased bilayer permeability resulting in drug leakage [39,169,170].

A usual approach to overcome these problems is the production of a dry liposomal product. Freeze-drying (i.e., lyophilization) is the method commonly used to improve the long-term stability of dry powder liposomes with low content of residual water [171,172]. However, freeze-drying itself may result in formulation physical changes, related with the increase of the size of liposomes, resulting from the fusion of the phospholipid membranes that can occur during the freezing, drying, or rehydration. The loss of the encapsulated drug is also a very common drawback related with this process [173]. Therefore, a meticulous optimization and selection of the components of the formulation is crucial to achieve a long-term stability of liposome based drugs [171].

The inclusion of cryo and lyoprotectants in the liposomal formulation has been undertaken to improve the functional properties and stability of the products after freezing and drying, respectively. Since they show the ability to act as the integrity membrane protectants, carbohydrates, more specific the saccharides, are the preferable cryo/lyoprotectants used during dehydration/rehydration of liposomes [174,175]. To achieve a highly stable liposomal formulation some aspects must be optimized, namely the type and concentration of saccharide. Some theories have been proposed to explain the stabilization mechanisms beyond the use of cryo/lyoprotectants during freeze-drying [176,177], however deeper studies must be undertaken to understand their protective effect on the freeze-dried liposomes.

We have previously described a liposomal formulation that prove to be an efficient system for the encapsulation and delivery of both hydrophobic and hydrophilic drugs [153]. The results support their use as therapeutic delivery systems as demonstrated by the biological effect of several drugs *in vitro* as well as *in vivo* [153,159]. The aim of this work was to optimize this liposomal formulation to preserve its

initial characteristics upon hydration of freeze-dried powder. For this, we introduced in the liposome suspension different concentrations of saccharides, namely trehalose, lactose, glucose, mannitol and sucrose, and freeze-dried the final formulation. We foresee that these new formulations, similarly to the raw formulation, will give rise to stable and homogeneous suspensions with a minimum of drug leakage. The physicochemical properties (like size, morphology and concentration) of the optimized liposomal formulations were investigated. Furthermore, the influence of the overall freeze-drying process on the leakage of three different anticancer drugs was explored and the biological activity of the most promising liposomal formulation was determined.

III.2. Materials and Methods

III.2.1. Materials

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE–MPEG) were obtained from Lipoid GmbH (Germany). Deuterium oxide and deuterated chloroform were purchased from Cortecnet. Cholesterol (CH), methotrexate (MTX), doxorubicin hydrochloride salt (DOX), tamoxifen (TAM), pyridine as well as the saccharides (trehalose, lactose, glucose, mannitol and sucrose) were received from Sigma-Aldrich (USA). All culture media and supplements were also purchased from Sigma-Aldrich (USA).

III.2.2. Liposome preparation

The production of liposomes encapsulating MTX and TAM was performed by passive loading of drugs. Liposomes composed of DOPE/CH/DSPE-mPEG (54:36:10, molar ratio) [153] were produced by ethanol injection method [154]. Briefly, an amount of DOPE, CH and DSPE-mPEG was dissolved in ethanol. The organic phase was injected under vigorous magnetic stirring to aqueous phase, phosphate-buffered saline (PBS) buffer (pH 7.4), at 70 °C. When indicated, sucrose was dissolved in PBS buffer at 8 g/g of dry lipids, to be present in both sides of liposomes. The vesicles were then extruded (extruder supplied by Lipex Biomembranes Inc., Vancouver, Canada) several times through polycarbonate filters of 200 nm and after 100 nm pore size (Nucleopore) to form unilamellar vesicles. Encapsulation of drugs was done by their mixture during the liposomes preparation, MTX disodium salt (7 mg/mL), as hydrophilic drug was included in aqueous phase (PBS buffer) and TAM (1 mg/mL) as hydrophobic drug was added in organic phase (ethanol). MTX disodium salt was prepared adding two NaOH molar equivalent to a PBS

buffer containing commercial MTX. After completely solubilization of MTX, the pH was adjusted to pH 7.4. The production of liposomes encapsulating DOX was obtained by active loading. Empty liposomes were prepared as described above, by ethanol injection method, using 120 mM ammonium sulfate buffer (pH 8.5) as aqueous phase, instead of PBS buffer. After extrusion, the buffer was exchanged in a Sephadex PD-10 desalting column (GE Healthcare, UK), equilibrated with 25 mM Tris Base sucrose (10%, w/v, pH 9.0). Remote loading of DOX (2.5 mg/mL) was carried out through ammonium sulfate gradient approach, upon incubation with liposomes for 1.5 hour at 60 °C [168].

III.2.3. Determination of drug encapsulation and leakage

The non-encapsulated drugs were removed from the liposomes after passage through a gel filtration chromatography column (GE Healthcare, UK), with 5 kDa cut-off (PD-10 Desalting columns containing 8.3 mL of Sephadex™ G-25 Medium) and eluted with PBS buffer for all liposomes. The concentration of MTX and TAM encapsulated was measured by proton nuclear magnetic resonance (^{1}H NMR) using a Bruker Avance III Instrument, operating at 400 MHz [178]. Powder liposomes containing drug were dissolved in deuterium oxide (for MTX) or deuterated chloroform (for TAM) to determine the amount of drug in the liposomal formulation. Pyridine was used as internal standard. Quantification of DOX was evaluated by UV–Vis spectrophotometry measuring the absorbance at 490 nm. UV–Vis spectra of liposomes encapsulated DOX were recorded on spectrophotometer BioTek Synergy™ HT using a plastic microplate. The final DOX concentration was determined based on the respective calibration curve.

The drug leakage was determined as follows:

$$\% \text{ Leakage} = 100 - \frac{[\text{Drug}] \text{ encapsulated after freeze-drying}}{[\text{Drug}] \text{ encapsulated before freeze-drying}} \times 100$$

Equation III.1. Determination of the drug leakage.

III.2.4. Determination of size distribution

The physicochemical characterization of liposomes was evaluated in terms of size and polydispersity index (PDI) using the dynamic light scattering technique (DLS) (at least three replicates for each formulation). The analysis was determined at pH 7.4 (PBS buffer) and at 25.0 °C, using a Malvern

Zetasizer Nano ZS (Malvern Instruments) by photon correlation spectroscopy (PCS). The viscosity and refractive index values used were 0.8616 cP and 1.332, respectively.

III.2.5. Freeze-drying and liposomes hydration

The saccharides used in this work (trehalose, lactose, glucose, mannitol and sucrose) were dissolved in PBS buffer at different concentrations of 2, 4, 6 and 8 g/g of dry lipids. Liposomal suspensions were diluted in an equal volume of each saccharide buffered solution in 50 mL tubes, at 10% of fill volume. As control, liposomal suspension was diluted in equal volume of PBS buffer. A very low freezing temperature seems to avoid damage of nanoparticles and improve the lyoprotective effect, as demonstrated in previous studies using nanoparticles with saccharides [179,180]. Considering these assumptions, all the liposomal suspensions were stored for 6 hours at -80 °C in a deep freezer and then freeze-dried. When indicated, liposomes were stored in a Corning® CoolCell™, in order to achieve a slow rate of freezing, ≈ -1 °C/min. The freeze-drying process was performed involved only the primary drying using the Labconco FreeZone 2.5 freeze-dryer (Labconco, Kansas City, USA), for 24 hours at -50 °C in a chamber with 6 Pa (conditions set by the equipment). The reconstitution of freeze-dried liposomes to their original volume was made at room temperature with PBS using a vortex mixer. The samples were equilibrated for 1 hour and subjected to further tests.

III.2.6. Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) measurements were performed using a NanoSight NS500 instrument (Salisbury, UK) equipped with a charge coupled device (CCD) camera that allows the visualization and tracking Brownian motion of laser-illuminated particles in suspension. The measurements were made at room temperature and each video sequence was captured over 60 seconds with manual shutter and gain adjustments. The samples were diluted with water and then injected into the system (at least three replicates for each formulation).

III.2.7. Thermogravimetric analysis

Thermogravimetric analysis (TGA) was performed in a TGA 4000 (Perkin Elmer, Waltham, MA, US) using an alumina crucible with sample weights of approximately 25 mg. The temperature calibration was established by Curie temperatures of reference materials: alumel, nickel and perkalloy at the analysis

scanning rate. The measurements were performed from 25 to 800 °C at 10 °C/min under a nitrogen atmosphere (flow rate: 20 mL/min). The weight loss percentage and its derivative were represented as function of temperature. Derivative weight percentages were used to measure the weight loss as function of temperature (establish the start and end of each degradation step) and compare the peak temperatures. The weight loss was used to calculate the total amount of residual water.

III.2.8. Microscopy imaging analysis

The morphology of liposomes was evaluated by scanning transmission electron microscopy (STEM). The initial liposomes suspensions and after freeze-drying with sucrose (8:1 mass ratio) were dropped in Copper grids with carbon film 400 meshes, 3 mm diameter. The shape and morphology of nanoparticles were observed using a NOVA Nano SEM 200 FEI system.

III.2.9. Cell culture conditions

The human breast adenocarcinoma cell line MCF-7 (ATCC® HTB-22™) was obtained from the American Type Culture Collection. Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2.0 g/L sodium bicarbonate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% (v/v) of fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin solution and 0.01 mg/mL insulin. Cells were grown in T75 flasks (SPL Life sciences, Korea) and maintained at 37 °C in a humidified atmosphere of 5% CO_2. MCF-7 cells were routinely sub-cultured two times a week.

III.2.10. Metabolic activity assay

Metabolic activity was studied using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. A ready-for-use CellTiter 96® Aqueous One solution of MTS (Promega, Madison, USA) was used according to the protocol suggested by the manufacturer. Cells were seeded at a density of $1.5x10^4$ cells per 100 µL/well on 96-well TCPS plates (TPP, Switzerland) in the day before the experiment to promote cell adhesion. The cells were incubated for 48 hours with then exposed to different liposome and drug concentrations (three replicates for each condition). After this time, the culture medium was refreshed, and 20 µL of CellTiter 96® Aqueous One solution was added to each well. After 4 hours of incubation at 37 °C, the absorbance at 490 nm was measured using a

microplate reader (Synergy Mx Multi-Mode Reader, BioTek, USA). Metabolic activity was expressed as a percentage relative to the negative control (untreated control cells).

III.2.11. Statistical analysis

Statistical analyses were performed with GraphPad Prism software (version 5.0). Differences were tested for statistical significance by a one-way Analysis of variance (ANOVA).

III.3. Results and Discussion

III.3.1. Influence of saccharides on liposomes' size distribution

The preservation of the physical integrity of liposomes during freeze-drying process is the primordial importance and can be achieved by the inclusion of saccharides at the final formulations. Since the stabilization effect promoted by cryo/lyoprotectants is concentration-dependent [181], we compared the protective effect of five saccharides at different concentrations on the final formulation. The fusion or aggregation of liposomes during the freeze-drying process was monitored by measuring the size distribution of liposomes after freeze-drying-rehydration cycle and compared with the results of freshly prepared liposomes. The values of size and PDI of liposomes before and after freeze-drying process are shown in Figure III.1A and III.1B, respectively.

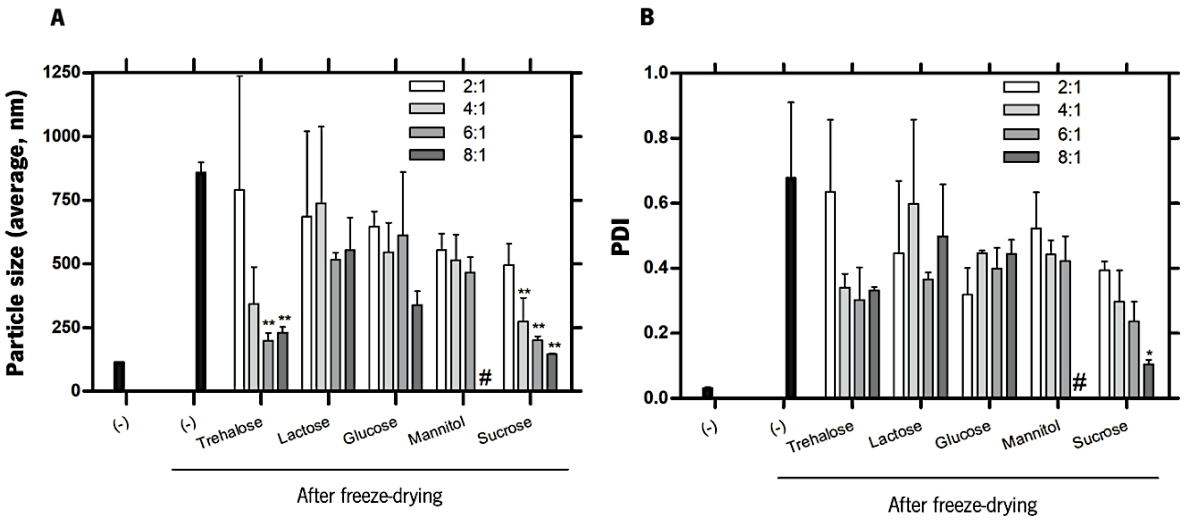


Figure III. 1. Physicochemical characteristics of liposomes evaluated by DLS: **(A)** Size (Z-average) and **(B)** PDI of liposomes with (w:w, sugar:lipids) and without (-) saccharides, before and after freeze-drying. # Values not determined due to non-homogenous dispersion obtained. Values represent the mean + SD of 2 independent experiments. Significant differences between liposomes without and with saccharides were detected as shown by an * ($P<0.05$) and ** ($P<0.005$).

The size and PDI of liposomes without saccharides in their composition increase significantly after the freeze-drying process (from 113.8±0.99 nm to 859.0±56.14 nm and from 0.03±0 to 0.57±0.49). One can also observe that the protective effect of the saccharides is concentration-dependent. Inadequate concentration of sugar can lead to incomplete coating of the glassy matrix around nanoparticles promoting aggregation [182]. Smaller particle sizes were obtained by using trehalose and sucrose at higher concentrations, however, only sucrose at 8:1 (sugar:lipids) mass ratio allows to achieve a homogeneous suspension, with PDI around 0.1. Higher sucrose molar ratios were also tested to decrease the size and polydispersity and meet the initial size of the liposomal formulation without cryo/lyoprotectants (Figure III.2). However, we found that higher concentrations of sucrose lead to similar liposomes' size and polydispersity as obtained when using the 8:1 mass ratio.

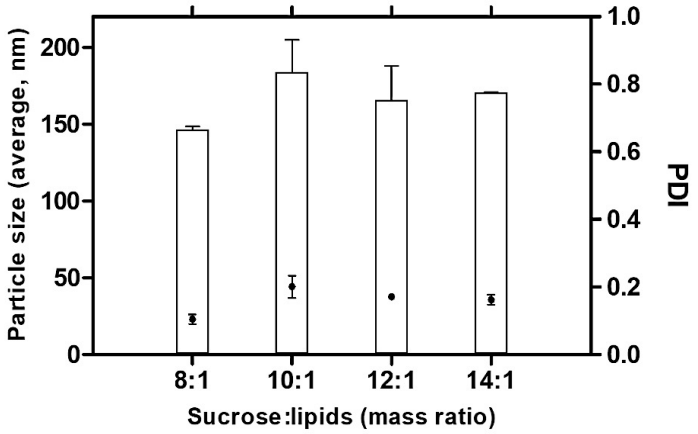


Figure III. 2. Influence of high concentration of sucrose in freeze-drying of liposomes. Size (average, nm) and PDI of liposomes in the presence of different sucrose concentrations, after freeze-drying. Values represent the mean + SD of 2 independent experiments.

A few theories have been proposed to explain the mechanism beyond the stabilizing action of cryo/lyoprotectants during the freeze-drying process. There is no generally accepted theory, being that these compounds exert their action via one or more of the following mechanisms. The water replacement theory attributes the stabilization effect of protectors to their ability to replace the bound water around the bilayers through specific interactions with the polar region of the lipid head group at low hydrations. In the vitrification theory, a highly viscous matrix is formed around the liposome which reduces the mobility during the freeze-drying process [131,183]. Kosmotropic effects, the less common theory, establishes that cryoprotectants interact with water and disrupt their normal structure. The damage during freeze-drying is prevented due to the reduction of water content at membrane interface [184]. Despite of the theories, deeper studies must be undertaken to understand their protective effect on the freeze-dried liposomes. However, saccharides such as sucrose or trehalose are described to be very effective lyoprotectants, as they show a very high viscosity, a low molecular mobility after drying and form an amorphous, glassy matrix [185], which corroborates our findings.

III.3.2. Residual water content

The residual water content of formulations can be one of the most important factors affecting the stability of freeze-dried products. It has been demonstrated that high levels of residual water content lead to a unexpected dissolution of the freeze-dried samples immediately after freeze-drying, or to a poor long-

term storage stability of nanoparticles [186]. After freeze-drying process, the liposomal formulation must have a water content less than 2% [175]. The total amount of residual water in liposomes after freeze-drying was herein determined by TGA. All freeze-dried liposomes contained less than 1.5% of residual water (Figure III.3). This very low water content proves the efficiency of primary drying, avoiding an additional secondary drying step.

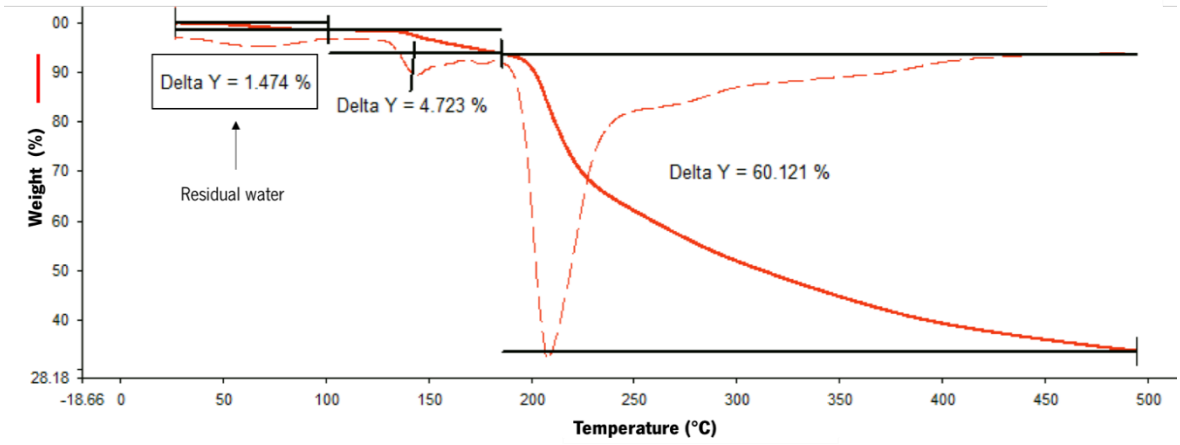


Figure III. 3. TGA of liposomes containing sucrose (8:1) after the freeze-drying process.

III.3.3. Influence of the freeze-drying process on the liposome's concentration

Fusion or aggregation of liposomes during freeze-drying process can be monitored by determination of the liposome's concentration. To determine if the freeze-dried liposomes maintain the same concentration as of the initial liposomes, the NTA was performed. The ability of NTA to simultaneously measure size and particle scattering intensity, makes possible the direct estimation of the particle's concentration. Furthermore, its ability to determine the size distribution of particles until 2 μm in diameter (according to the manufacturer, Malvern), allow us to evaluate liposomes in a micrometer range. A lower concentration of particles was observed for freeze-dried liposomes without cryo/lyoprotectants (Table III.1). These results are in good agreement with the results obtained by DLS, which revealed higher particle sizes, probably due to fusion or aggregation phenomena. After freeze-drying the concentration of liposomes containing sucrose remained similar to the initial formulation concentration, highlighting the protective role of the cryo/lyoprotectant.

Table III.1. Influence of freeze-drying on liposomes concentration, determined by NTA. Values represent the mean of ± SD of 3 independent experiments.

Sample	Freeze-drying	Concentration (E^{12} particles/mL)
Liposomes	-	20.1 ± 3.1
	+	10.3 ± 2.1
Liposomes + sucrose	-	23.3 ± 7.8
	+	21.3 ± 3.9

III.3.4. Morphology of liposomes after freeze-drying

The morphology of particles is of outmost importance to identify possible fusion and/or aggregation phenomena. Liposome suspensions, before and after freeze-drying, were observed by STEM. The profile of the initial liposomal formulation (Figure III.4A) and of the freeze-dried liposomes containing sucrose (8:1 sugar:lipids, mass ratio) as cryo/lyoprotectant (Figure III.4B), revealed few fused or aggregated particles, being the liposomes in the form of individual vesicles. Furthermore, the liposomal formulation presents a homogeneous population with spheroid and regular shape. The images show that freeze-dried liposomes containing sucrose were stable revealing no significant increase of the particle size and maintaining their spherical shape. This morphology can offer potential for controlled release and protection of incorporated drugs, as they provide minimum contact with the aqueous environment favoring a longest diffusion pathway. The result is in agreement with the DLS data showing a very similar particle size distribution. Significant changes in the lipid aggregate structure and size were noted upon rehydration of freeze-dried liposomes without the protective effect of sucrose (Figure III.4C). Liposomes exhibit fused or aggregated vesicles, presenting some cylinder-like shape particles. In addition, a distinct population of significantly larger liposomes was detected in the samples. These observations are also supported by DLS data.

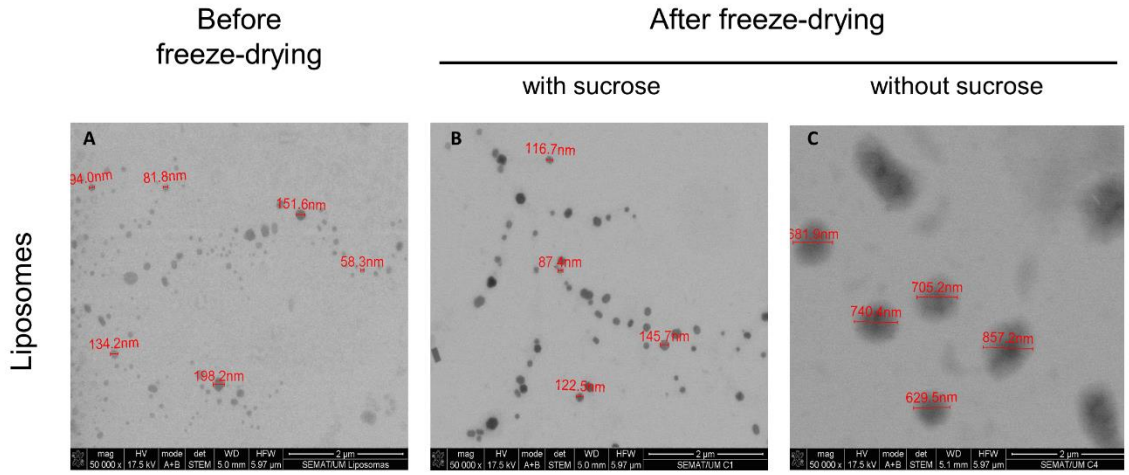


Figure III. 4. Morphology profile of liposomes. Representative STEM images of liposomal formulation **(A)** before freeze-drying, **(B)** with sucrose (8:1 mass ratio), after freeze-drying and **(C)** without sucrose, after freeze-drying. The scale bar in the figures represents 2 μm.

III.3.5. Influence of the freeze-drying on drug leakage from liposomes

Freeze-drying is an approach to dry liposomal formulations well-established by the pharmaceutical industry to improve the long-term storage stability of drugs [184]. However, the stresses imposed during the freeze-drying process might lead to the leakage of the encapsulated drugs [176]. The drug leakage depends on the liposome composition and on the nature of drug [64]. Thus, it is imperative to study the freeze-drying process on drug leakages and evaluate their influence on the final liposomal formulation behavior. Leakage of three anticancer drugs was evaluated only in liposomes with sucrose (8:1 sugar:lipids, mass ratio), since liposomes without cryo/lyoprotectant have a high values of size and PDI. MTX, a hydrophilic drug in a disodium salt form, was encapsulated into the aqueous core of liposomes and their behavior after the freeze-drying process was investigated. The results showed that despite the presence of sucrose, the leakage of MTX was very high after freeze-drying process (61.1%, Table II.2). From the data obtained (Table II.2), higher leakage levels remained even after using a slow rate of freezing (≈ -1 °C/min) and the distribution of the cryo/lyoprotetor in both sides of liposomes (outer and inner part). We also evaluated the behavior of different drug:lipid molar ratio, however high leakages levels were also observed (data not shown). It is noteworthy that buffer pH changes might influence drug leakage. For this reason, the pH of liposomes encapsulating MTX (in PBS buffer) was measured and did not showed any alteration after freezing and drying processes.

Table III.2. Influence of sucrose distribution and freezing rate on liposomes encapsulating MTX. Values represent the mean of ± SD of 2 independent experiments.

Freezing rate	Distribution	Z-average (nm)	PDI	Leakage (%)
Quick	One side	166.5 ± 4.1	0.165 ± 0.053	61.1
(non-fixed)	Two size	591.8 ± 13.8	0.272 ± 0.044	#
Slow	One size	181.8 ± 2.8	0.209 ± 0.015	69.0
(fixed: ≈ -1 °C/min)	Two size	433.0 ± 25.3	0.303 ± 0.158	#

Leakage not determined due to high values of Z-average and PDI.

The influence of the preparation method of liposomes was also evaluated using the remote loading. However, this approach can only be applied to weak amphipathic acids or bases, which MTX molecule does not belong to [187]. Liposomes encapsulating DOX, prepared by remote loading, was then used in this study, however, high leakage level is also observed (24.5%, Table III.3). These results indicate that the leakage is not directly governed by the loading method, but possibly by the location of drug in liposomes. This behavior might be justified by the presence of unsaturated phospholipid DOPE [17] in the liposomal composition. However, its presence in the optimal liposomal formulation is crucial to obtain pH-sensitive devices which, in therapeutic applications, facilitate the release of drugs into the cell cytoplasm [188]. The drug leakage of drugs encapsulated in the aqueous core may be also enhanced by eventual bilayer defects. These effects can cause a change in the pharmacokinetic profile of the encapsulated drug and lead to reduce the reproducibility of the therapeutic effect [64].

To study the behavior of a drug located in the lipid bilayer, TAM, was also encapsulated into liposomes. The results obtained indicate that freeze-dried liposomes containing sucrose with TAM encapsulated have lower drug leakage (4.0%, Table III.3). As performed for liposome encapsulating MTX, the pH of formulations containing TAM was also evaluated and no alterations were observed after freezing and drying processes. The pattern of size distribution is similar to the empty liposomes, showing small increase of size and PDI. These liposomes are stable for at least 3 months after rehydration (data not shown).

Table III.3. Characterization of liposomes before freeze-drying and after freeze-drying with addiction of sucrose. Values represent the mean ± SD of 3 independent experiments.

			Encapsulated Drug		
		Empty (-)	MTX	DOX	TAM
Before freeze-drying	Z-average (d.nm)	125.5 ± 4.3	126.5 ± 3.5	134.1 ± 0.5	107.8 ± 1.4
	PDI	0.068 ± 0.036	0.078 ± 0.008	0.111 ± 0.018	0.036 ± 0.003
	Encapsulation Efficiency (%)	-	2.6 ± 0.1	65.3 ± 1.4	93.9 ± 6.1
	Drug : lipid (molar ratio)	-	≈ 1:11	≈ 1:11	≈ 1:11
After freeze-drying	Z-average (d.nm)	154.3 ± 1.9	166.5 ± 4.1	169.1 ± 3.4	148.5 ± 4.4
	PDI	0.135 ± 0.009	0.165 ± 0.053	0.200 ± 0.012	0.130 ± 0.020
	Leakage (%)	-	61.1 ± 4.0	24.5 ± 0.3	4.0 ± 3.0

III.3.6. Influence of freeze-drying on the biological activity of liposomes encapsulating tamoxifen

It is of prime importance that a therapeutic drug preserves its functionality and biological activity, even after the freeze-drying process. In this way, liposomes encapsulating TAM are expected to present the same biological activity profile after the freeze-drying process. TAM, as a selective estrogen receptor (ER) modulator, is indicated for the treatment of breast cancer ER-positive [189]. As an hydrophobic drug, TAM may induce oxidative stress due to the accumulation in phospholipid bilayers of membranes resulting in cell death at highest concentrations [190]. The effect of freeze-drying process on TAM biological activity was evaluated *in vitro* using MCF-7 cell line, an ER-positive breast cancer cell line [191]. It was assessed the metabolic activity of cells after the incubation with different concentrations of TAM encapsulated into liposomes, before and after the freeze-drying process. Based on the collected data at 48 hours, liposomes encapsulating TAM after freeze-drying process did not exhibit significant loss of biological activity

compared to liposomes before freeze-drying (Figure III.5). These results confirm that the freeze-drying process does not affect the biological activity of the drug.

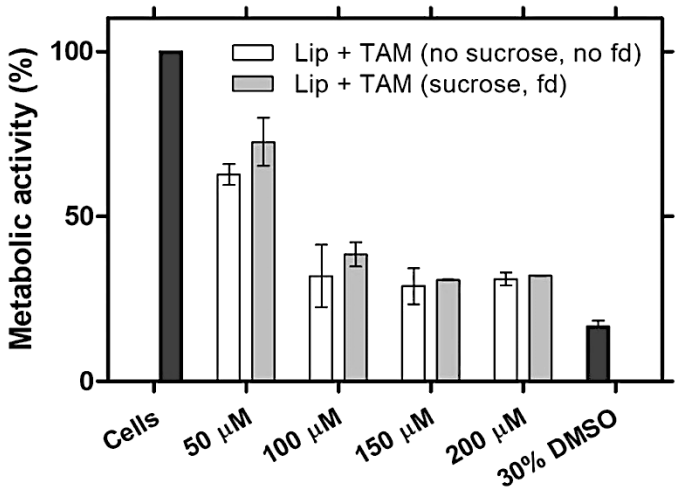


Figure III. 5. Biologic activity of liposomes encapsulating TAM after freeze-drying process. MCF-7 cell line metabolic activity after 48 hours of incubation with the initial liposomal formulations (without sucrose) and after freeze-drying (fd) with sucrose (8:1 sugar:lipids, mass ratio), at different concentrations of TAM. Values represent the mean ± SD of 2 independent experiments.

III.4. Conclusion

We have successfully optimized a liposomal formulation by incorporation of a sugar, sucrose, which preserved its integrity after the freeze-drying process. From all the saccharides tested, only sucrose at 8:1 mass ratio presented ability to protect the dry liposomal formulation. The size distribution, morphology analysis and concentration of the final formulations indicate that liposomes are not subject to fusion or/and aggregation. However, leakage of drugs encapsulated in the aqueous core, MTX and DOX, after freeze-drying process is observed, even though the different preparation method used (passive and active loading, respectively). Otherwise, liposomes with a drug located in the lipid bilayer, TAM, demonstrated negligible leakage preserving their biological activity after the freeze-drying process. In this way, leakage seems to be dependent of location of the drug in liposomes. Taken together, the results indicated that sucrose protects the physical and biological integrity of the liposomal formulations encapsulating TAM when exposed to freeze-drying process being a promising approach for storage.

CHAPTER IV

Increased encapsulation efficiency of methotrexate in liposomes for rheumatoid arthritis therapy

CHAPTER IV

Increased encapsulation efficiency of methotrexate in liposomes for rheumatoid arthritis therapy

Abstract

Methotrexate (MTX) is a common drug used to treat rheumatoid arthritis. Due to the excessive side effects, encapsulation of MTX in liposomes is considered an effective delivery system, reducing drug toxicity, while maintaining its efficacy. The ethanol injection method is an interesting technique for liposomes production, due to its simplicity, fast implementation, and reproducibility. However, this method occasionally requires the extrusion process, to obtain suitable size distribution, and achieve a low level of MTX encapsulation. Here, we develop a novel pre-concentration method, based on the principles of the ethanol injection, using an initial aqueous volume of 20% and 1:1 ratio of organic:aqueous phase (v/v). The liposomes obtained present small values of size and polydispersity index, without the extrusion process, and a higher MTX encapsulation (efficiency higher than 30%), suitable characteristics for *in vivo* application. The great potential of MTX to interact at the surface of the lipid bilayer was shown by Nuclear Magnetic Resonance (NMR) studies, revealing mutual interactions between the drug and the main phospholipid via hydrogen bonding. *In vivo* experiments reveal that liposomes encapsulating MTX significantly increase the biological benefit in arthritic mice. This approach shows a significant advance in MTX therapeutic applications.

This chapter is based on the following scientific publication:

Diana Guimarães, Jennifer Noro, Ana Loureiro, Franck Lager, Gilles Renault, Artur Cavaco-Paulo and Eugénia Nogueira. Increased Encapsulation Efficiency of Methotrexate in Liposomes for Rheumatoid Arthritis Therapy. *Biomedicines*. 2020. 8:630. doi: 10.3390/biomedicines8120630.

IV.1. Introduction

Methotrexate (MTX) is an effective drug used to treat autoimmune and inflammatory diseases such as rheumatoid arthritis, Crohn's disease, multiple sclerosis, and psoriasis [192,193]. However, free MTX has some limitations that restrict its use such as its poor bioavailability, low specificity, drug resistance, and dose-dependent side effects [194]. Innovative strategies have been investigated to increase the therapeutic effect of drugs [35,195,196]. Because of their lipid composition and structural similarity to cellular membranes, liposomes are considered the most used drug delivery system for the intracellular delivery of drugs [197]. Furthermore, they can encapsulate both hydrophilic and hydrophobic drugs [198]. A recent study of our research group reported a liposomal formulation encapsulating MTX that was shown to be a good therapeutic delivery system as demonstrated by its biological effect *in vitro* and *in vivo* [153,159].

The production of liposomes involved multiple steps in a complex and precise process that has a critical impact on the final liposome characteristics, such as size, stability and functionality of the finished liposomes [199,200]. The more adequate method for liposome production and drug encapsulation also depends on the physicochemical characteristics of the drugs to be encapsulated [201]. The ethanol injection method is commonly used to produce liposomes, due to its simplicity, ease of scale-up and safe production technique. Liposomes obtained by ethanol injection method were spontaneously formed when the organic phase containing the dissolved lipids was rapidly injected into an aqueous phase by agitation [202]. This method does not induce oxidative and degradation modifications in most encapsulated drugs or in the lipid components [41,203]. However, to reduce liposome size and form unilamellar vesicles, extrusion should be applied [204].

The Encapsulation Efficiency (EE) of the drug is determined by the properties of the liposomes, including their aqueous volume or membrane rigidity. Furthermore, the encapsulation is affected by hydrophilic or hydrophobic properties of drugs and their capacity to interact with the membrane bilayer [205–207]. There are two different approaches for the encapsulation of drugs into liposomes. In the active method, usually remote loading, the drug is encapsulated in liposomes after their preparation, through transmembrane gradient method, with an EE of around 100%. Since MTX molecule is not considered a weak amphipathic acid or base, the remote loading method cannot be used [187]. MTX is encapsulated into liposomes by a passive method, that corresponds to the encapsulation of drugs during the liposome formation or in a phase of preparation when the liposomal structure is extremely fluid [208]. The passive encapsulation of hydrophilic drugs depends on the capacity of liposomes to trap the aqueous

phase containing the drug. This methodology results in lower EE, since the drug retention is limited to the size of the aqueous compartment in liposomes and drug solubility [35].

The purpose of our study was to increase the encapsulation of MTX in liposomes, through the development of a novel production method based on the principles of the ethanol injection. The use of a reduced initial aqueous volume (pre-concentration) and optimization of organic:aqueous ratio proves to be essential to obtain a suitable size distribution and higher drug EE. The molecular interactions occurring between the MTX and the main phospholipid present in the liposomal bilayer were evaluated through nuclear magnetic resonance (NMR) studies. The biological benefit of the liposomes produced by the novel method was proved in a mice model of arthritis.

IV.2. Materials and methods

IV.2.1. Materials

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), egg phosphatidylcholine (EPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE–mPEG) were obtained from Lipoid GmbH (Germany). Deuterium oxide (D_2O) and deuterated dimethyl sulfoxide (DMSO-d_6) were acquired from Cortecnet (France). All the other chemicals involved in this work were purchased from Sigma-Aldrich (USA), except the MTX that was acquired from Huzhou Zhanwang Pharmaceutical (China), cholesterol from Anhui Chem-Bright Bioengineering Co Ltd (China), bovine collagen, type II purchased from Chondrex, Morwell Diagnostics (Switzerland) and complete Freund's adjuvant used in mice experiments from Fisher Scientific (France). All compounds were used without further purification.

IV.2.2. Liposome production by ethanol injection method

Liposomes composed of DOPE or EPC/Cholesterol/DSPE-mPEG [153] were produced by the ethanol injection method. Lipids were weighted at the initial molar ratio of 54:36:10, respectively. MTX disodium salt (soluble in aqueous buffer) was prepared by adding two sodium hydroxide (NaOH) molar equivalents to phosphate buffered saline (PBS) buffer containing commercial MTX. After the complete solubilization of MTX, the pH was adjusted to pH 7.4.

IV.2.2.1. Conventional method

For the production of 10 mL of liposomes, DOPE, Cholesterol and DSPE-mPEG were dissolved in 2 mL of ethanol. The organic phase was added through gravity, using a 20 gauge needle coupled to a plastic syringe, under magnetic stirring (500 rpm) to 10 mL of PBS (pH 7.4), at 70 °C. MTX disodium salt, as a hydrophilic drug, was added in the aqueous phase (PBS), to a final concentration of 20 mg/mL. The liposomes were then extruded (extruder supplied by Lipex Biomembranes Inc., Vancouver, Canada) several times through polycarbonate filters of 200 nm and after 100 nm pore size (Nucleopore) to form unilamellar liposomes.

IV.2.2.2. Pre-concentration method

The lipid components were dissolved in different volumes of ethanol (1, 2 and 4 mL), to further obtain different initial ratio of organic:aqueous phase (v/v) in liposomes, 1:2, 1:1 and 2:1, respectively. The organic phase was added through gravity, using a 20 gauge needle coupled to a plastic syringe under vigorous magnetic stirring (500 rpm) to 2 mL of PBS (pH 7.4) containing 100 mg/mL of MTX, at 70 °C. After ethanol evaporation, the remaining 8 mL of PBS was added to achieve the final volume (10 mL) and MTX concentration (20 mg/mL). The liposome suspension was then kept under stirring for 15 minutes at room temperature. When necessary (size ≥ 150 nm and polydispersity index (PDI) > 0.1), liposomes were extruded several times through polycarbonate filters of 200 nm and after 100 nm pore size (Nucleopore). When indicated, liposomes were prepared with D_2O instead PBS buffer.

IV.2.3. Determination of MTX concentration

The non-encapsulated MTX was removed from the liposomes after passage through a gel filtration chromatography column (GE Healthcare, UK), with 5 kDa cut-off (PD-10 Desalting Columns containing 8.3 mL of Sephadex™ G-25 Medium). After separation, the MTX concentration was determined by measuring the absorbance at 303 nm, the maximum wavelength of MTX in PBS, a method previously validated [178]. Briefly, ultraviolet-visible spectra of liposomes encapsulated MTX were recorded on spectrophotometer BioTek Synergy™ HT using a quartz microplate. The absorbance at 303 nm in empty liposome was used as blank. The final MTX concentration was determined based on the respective calibration curve. The EE is defined by the concentration of the encapsulated MTX detected in the final liposomal formulation over the initial MTX concentration used to make the liposomal formulation.

EE (%) was calculated using the following equation:

$$EE\ (\%) = \frac{[MTX]\ \text{encapsulated in liposomes}}{[MTX]\ \text{initial used in liposomes preparation}} \times 100$$

Equation IV.1. Determination of the encapsulation efficiency of MTX.

IV.2.4. Physicochemical characterization of liposomes

The physicochemical characterization of liposomes was evaluated in terms of size distribution using a dynamic light scattering technique. The analysis was determined at pH 7.4 (PBS buffer) and at 25.0 °C, using a Malvern Zetasizer Nano ZS (Malvern Instruments) by photon correlation spectroscopy. The viscosity and refractive index of dispersant were 0.8616 cP and 1.332, respectively. Each sample was measured in triplicate and the results are presented as mean value ± standard deviation (SD). The stability of liposomes over time was evaluated at 4 °C for 12 weeks, by monitoring changes in liposome size and PDI.

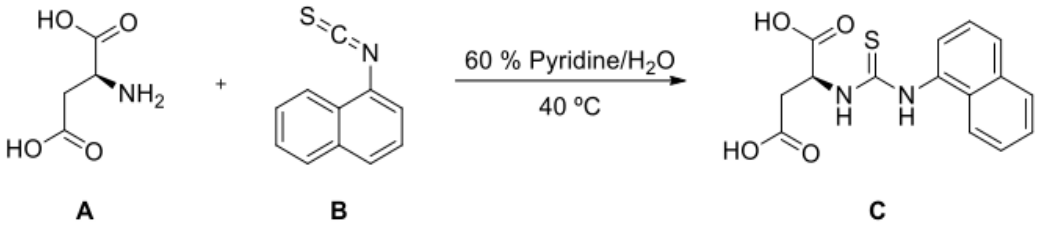
IV.2.5. Evaluation of compounds interactions by ^{1}H NMR

^{1}H NMR experiments were performed using a Bruker Avance III Instrument, operating at 400 MHz. To evaluate the distribution of MTX through the liposome's bilayer, the liposomes were formulated directly in D_2O. The procedure was followed as mentioned in section IV.2.2.2 but involved replacing the aqueous phase PBS buffer with D_2O. To evaluate the lipid-compound interactions, DOPE or EPC was mixed in DMSO-d_6 with an equimolar ratio of MTX or N-protected aspartic acid ((S)-2-(3-(naphthalen-1-yl)thioureido)succinic acid).

IV.2.6. Synthesis of (S)-2-(3-(naphthalen-1-yl)thioureido)succinic acid

To a round bottom flask with L-aspartic acid (**A,** 0.13 g, 0.98 mmol) and 1-naphthyl isothiocyanate (**B,** 0.18 mg, 0.98 mmol), 2 mL of pyridine were added (60% in water). The suspension was placed in an oil bath at 40 °C and kept stirred for 24 hours. The solvent was then removed in a rotary evaporator. N-amino protected L-aspartic acid (**C**) was obtained as a pure white solid after recrystallization with ethyl acetate (0.12 g, 0.38 mmol, η= 39%). ESI (m/z= 329.18). NMR (DMSO-d_6) δ_H: 2.42 (dd, J= 15.6, 2.8

Hz, 1H); 2.72 (dd, J= 16.0, 11.2 Hz, 1H); 3.78 (dd, J= 11.2, 2.4 Hz, 1H); 7.49-7.62 (m, 4H); 7.84 (d, J= 8.0 Hz, 1H); 7.96 (d, J= 7.6 Hz, 1H); 8.01 (d, J= 8.4 Hz, 1H); 9.85 (s, 1H) ppm (Scheme IV.1).



Scheme IV.1. Reactional scheme for the synthesis of (S)-2-(3-(naphthalen-1-yl)thioureido)succinic acid.

IV.2.7. Collagen-induced arthritis

Six-week-old male DBA/1 mice, which are susceptible to collagen-induced arthritis (CIA), were purchased from Janvier Laboratory (Le Genest-St-Isle, France). Mice were housed in groups of 6 per cage. Arthritis was induced with type II bovine collagen (CII). Male DBA/1 mice were injected subcutaneously at the base of the tail with 10 mg of CII emulsified in complete Freund's adjuvant. On day 21, mice were boosted with a subcutaneous injection of CII in incomplete Freund's adjuvant. In this model, arthritis develops 20 to 30 days after the first collagen injection [209]. Mice were monitored for evidence of arthritis in 4 paws using a blind procedure by a trained operator for arthritis scoring (20 years of experience). The severity of arthritis was evaluated using a clinical scoring front (4 fingers average, tarsus) and hind paws joints (5 fingers average, tarsus and ankle). Each joint was given a score ranging from 0 to 4 (0: normal joint, 1: erythema, 2: swelling, 3: deformity, 4: ankylosis) and summed, leading to a mouse individual score ranging from 0 to 40. As this model is strongly cage-dependent, groups were carefully randomized among mice cages. Treatments started on day 14, i.e., one week before the immune boost on day 21, and were continued throughout the testing period. All animals received the same injection volume for different treatments, intraperitoneally (IP), twice a week, free MTX at 7 mg/kg and liposomes encapsulating MTX at 2 mg/kg (according to preliminary study) Mice were scored on the same day. This study was approved by the local animal experimentation ethics committee (CEEA34, Comité d'ethique en experimentation animal de l'Université Paris Descartes) under agreement no. APAFIS#9696-2017031016246908 (first of December 2017). All experiments were carried out in accordance with National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Average severity scores per group and average weight variations per group were analyzed. The area under the curve (AUC) of severity curves was calculated in Excel. A therapeutic index was calculated on day 36 using the following equation:

$$\text{Therapeutic Index (\%)} = \frac{(AUC_v - AUC_p)}{AUC_v}$$

Equation IV.2. Determination of the therapeutic index.

Where AUC_p stands for area under the curve of the given product tested and AUC_v stands for the area under the curve of the vehicle group (PBS). This index somewhat reflects somewhat the percentage of the reduction of the severity of arthritis.

IV.2.8. Statistical analysis

A non-parametric one-way analysis of variance Kruskal-Wallis test on day 36 was used to test for significant differences between groups. The Dunn post hoc test without correction was then used to identify differences between all groups. All analyses were performed using R software and the "dunn.test" library for Dunn post hoc tests.

IV.3. Results and Discussion

IV.3.1. Liposome-encapsulated MTX prepared by the conventional ethanol injection method

Currently, MTX is considered the first line medication for rheumatoid arthritis patients [210]. Due to the excessive side effects, the encapsulation of MTX in liposomes can be an effective delivery system, reducing drug toxicity, while maintaining its efficacy. Ethanol injection is a common method to produce liposomes. It is known to be a simple, safe, reproducible, rapid, and easy to scale-up technique. However, this method occasionally requires the extrusion process, to obtain suitable size distribution, and achieve low level of MTX encapsulation. The size, PDI and EE of liposomes produced by ethanol injection method can depend on several conditions, such as lipid solution injection rate, temperature, homogenization

intensity, lipid concentration, and composition [138]. It is known that the size of liposomes can be controlled by the ratio of ethanol to aqueous phase [203,211].

The liposomal formulation used in this study (DOPE:Cholesterol:DSPE-mPEG, 54:36:10 molar ratio) was previously proved to be a good therapeutic delivery system of MTX, as demonstrated by the biological effect *in vitro* as well *in vivo* [153,159]. Liposomes-encapsulated MTX produced by the conventional ethanol injection method (Liposome A) presents a high value of size and PDI (Table IV.1). These values are considered not suitable for further in vivo applications (size < 150 nm and PDI ≤ 0.1), as the extrusion process is crucial to achieve liposomes with an appropriate size distribution. Furthermore, the low EE (%) of MTX decreases after the extrusion process (from 1.33 ± 0.19 to 0.88 ± 0.16). These results are in agreement with the literature, where the encapsulation of most drugs by the passive loading results in an EE around 1%, causing a large amount of drug waste [212]. Hydrophilic drugs as MTX normally have a poor EE due to rapid migration and consequently loss of drug into aqueous phase [213]. Indeed, due to the larger volume of the aqueous phase in the outside environment of the liposomes, compared to the limited aqueous volume inside the liposomes, the encapsulation of MTX results in low efficiency [32].

Table IV.1. Characterization of liposomes produced by conventional and pre-concentration ethanol injection method. Values represent the mean ± SD of 2 independent experiments.

Ethanol injection method	Liposomes*	Initial ratio Organic:Aqueous phase (v/v)	Ethanol (%)#	Extruder	Z-average (d.nm)	PDI	[MTX] (mg/mL)	Encapsulation Efficiency (%)
Conventional	A	1:5	20	-	160.60 ± 1.84	0.300 ± 0.01	0.26 ± 0.04	1.32 ± 0.19
				+	110.20 ± 1.84	0.083 ± 0.04	0.17 ± 0.03	0.88 ± 0.16
Pre -concentration	B	1:2	10	-	187.9 ± 14.14	0.272 ± 0.01	2.92 ± 0.60	14.60 ± 3.01
				+	122.55 ± 11.24	0.079 ± 0.02	0.75 ± 0.04	3.75 ± 0.22
	C	1:1	20	-	128.76 ± 7.78	0.107 ± 0.02	4.58 ± 0.03	22.90 ± 0.17
	D	2:1	40	-	257.7 ± 15.98	0.201 ± 0.02	6.47 ± 0.94	32.33 ± 4.70
				+	129.35 ± 2.33	0.060 ± 0.02	1.10 ± 0.43	5.52 ± 2.17
	E	1:1	20	-	201.1 ± 7.47	0.123 ± 0.03	2.37 ± 0.04	11.85 ± 0.23

* Liposomes A, B, C and D: DOPE-based liposomes. Liposomes E: EPC-based liposomes.

Relation between the initial Ethanol volume used to dissolve lipids and the final liposomal volume (considering 10 mL, after Ethanol evaporation).

IV.3.2. Liposomes encapsulating MTX prepared by the pre-concentration ethanol injection method

Based on the principles of the ethanol injection method (conventional method), we developed a novel strategy (pre-concentration method) to increase EE of MTX in liposomes and to obtain small values of size and PDI. The influence of three different initial ratios of organic:aqueous phase (v/v), 1:2, 1:1 and 2:1 (Liposomes B, C and D, respectively), was evaluated, initially using 20% of aqueous volume (pre-concentration) and adding the remaining 80% at the end of the process. The results showed that this method promotes an increase in EE (%) for all liposomes (Table IV.1). However, high values of size and PDI are obtained when ratios 1:2 and 2:1 (Liposomes B and D, respectively) are used. The extrusion of these liposomal formulations is imperative to achieve suitable size distribution but leads to a decrease in methotrexate concentration. Only the ratio 1:1 (organic:aqueous phase, v/v) achieved suitable values of size (128.76 ± 7.78 nm), PDI (0.107 ± 0.02) and high EE level (22.90 ± 0.17). In this way, Liposomes C has similar physicochemical characteristics to the liposomes obtained by the conventional ethanol injection method after extrusion with the advantage of a higher MTX encapsulation. The changes in ratio of organic:aqueous phase determines the initial lipid and MTX concentration in the suspension and consequently influence the final physicochemical characteristics of liposomes. Higher EE can be achieved, up to 40%, using a lowest initial MTX concentration (data not shown). To remark, liposomes C were shown to be very stable through time, maintaining its size (127.60 ± 9.95 nm) and PDI (0.102 ± 0.02) without significant drug leakage, for at least 12 weeks.

In both methods, an organic phase, composed of lipids dissolved in an ethanolic solution, is rapidly injected into an aqueous phase with vigorous magnetic agitation, promoting liposome formation [202]. The main difference is in the initial aqueous volume and the ratio of organic:aqueous phase (Figure IV.1). Through this pre-concentration ethanol injection method, we reduce the aqueous volume outside the liposomes, promoting the interaction between MTX and lipids. Figure IV.1 provides an overview of liposomes production by both methods.

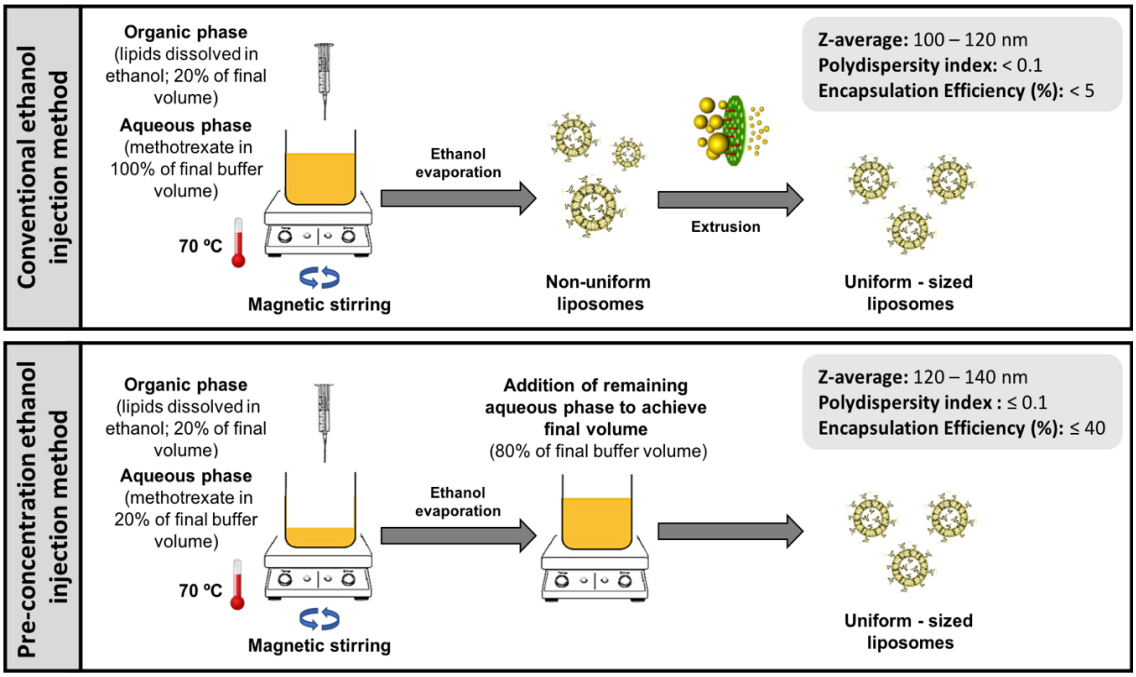


Figure IV.1. Schematic illustration of main differences between conventional and pre-concentration ethanol injection method.

IV.3.3. ^{1}H NMR of liposome encapsulated MTX

In order to study the effect of the extrusion process on the decrease in the EE, the ^{1}H NMR approach was applied. Liposome-encapsulated MTX was directly prepared in deuterium oxide, as described in section IV.2.2.2, and present similar physicochemical characteristics to those prepared with PBS buffer (data not shown). It should be noted that the NMR experiment was performed immediately after the removal of the non-encapsulated (free) MTX from the liposomes. In Figure IV.2 is depicted the ^{1}H NMR of the aromatic area of the encapsulated MTX, (A) before and (B) after extrusion. The singlet signal corresponds to the proton of the pteridine ring, and both doublets to the phenyl ring. It is possible to observed that MTX is distributed between two different environments, one in which the signal is well defined (dash line), and the other where the signals appear as broad singlets (solid line).

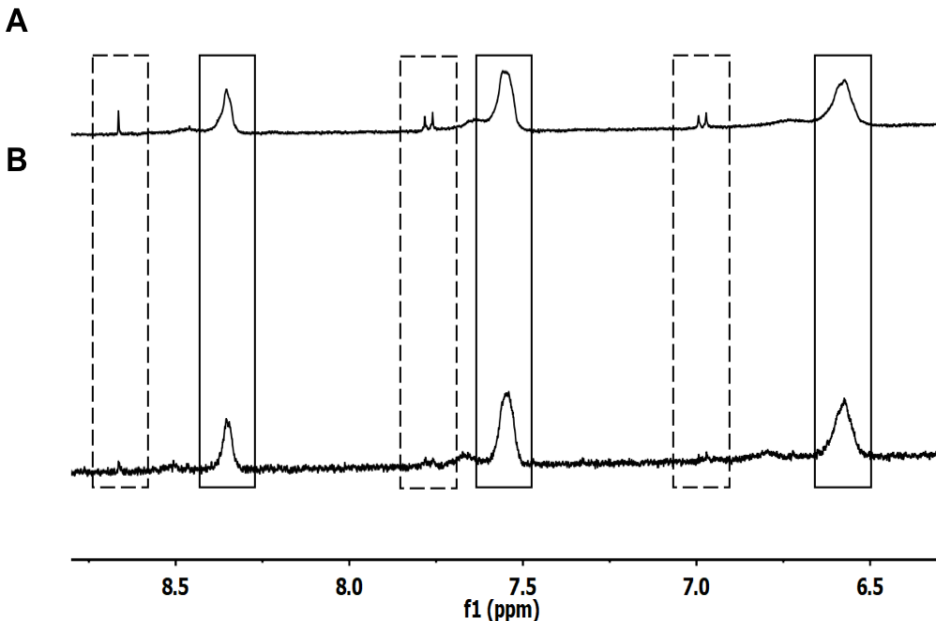


Figure IV.2. ^{1}H NMR spectra in D_2O of **(A)** liposome-encapsulated MTX and **(B)** liposome-encapsulated MTX after the extrusion process.

Given the two different environments observed for the MTX peaks, we may presume that the drug present near the surface of the liposome is represented by the well-resolved signals, since it should be more easily detected by the NMR apparatus. The drug present in the inner parts of the liposomes appears as broad singlets, given the presence of the lipid's bilayer. The presence of lipids in NMR spectra is commonly associated with the loss of resolution of the proton peaks of the existent solution [214]. By the peak's integration, it is possible to observe that 20% of the MTX is at the surface, while 80% is inside the liposomes. After extrusion, a pronounced decreased in the signal intensity is also observed, especially in the signals corresponding to the MTX at the liposome's surface (dashed line). The pressure applied during this procedure may cause higher release of this MTX, leading to lower EE.

IV.3.4. Role of the lipid in the encapsulation of MTX

The main lipids used to prepare liposomes are phospholipids. Their amphiphilic nature determines their precise distribution in the liposomal membrane. The carbon chains of phospholipids are considered hydrophobic regions and are aligned inside the lipid membrane. The polar heads of phospholipids are hydrophilic and are positioned to the inner and outer parts of the bilayer [215]. It was

known that the lipids used for the liposome formation greatly affected the EE [216]. Therefore, we compare liposomes produced with two different main lipids, DOPE and EPC, in order to evaluate their effect on MTX encapsulation.

From the results (Table IV.1), we may observe that the lipid used induced differences in the liposome size and EE. Both lipids have similar aliphatic chains, however, they differ in their hydrophilic head. EPC is composed by a quaternary amine, while DOPE is composed by a primary amine. Moreover, the phosphate group of EPC is ethylated, while in DOPE it remains negatively charged since it is not alkylated. These structural changes may induce a bulkier hydrophilic head group of EPC than DOPE, possibly leading to differences in packing during lipid bilayer formation, and consequently inducing discrepancies in the size achieved. The encapsulated drug can also affect the size of the formulation, given that the types of interaction that a drug can perform with a lipid membrane can modulate the proprieties of the lipidic membrane [217].

Regarding the differences detected in the EE (%) for liposomes composed of DOPE and EPC (22.90 ± 0.17, 11.85 ± 0.23, respectively), it is possible to observe that the drug could interact strongly with DOPE, leading to its higher retention in the formulation. Given the hydrophilic head of both lipids, EPC and DOPE, we proposed the possibilities of non-covalent interactions, as depicted in Figure IV.3. Nonetheless, we may not disregard other interactions that can occur between the two molecules, given the different chemical groups present.

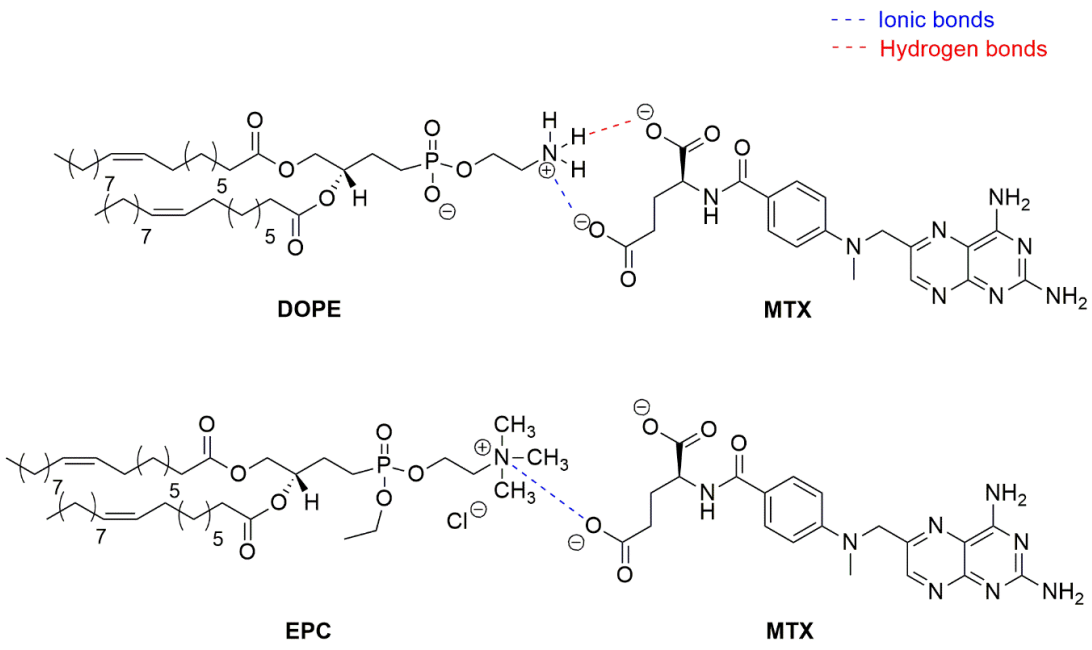


Figure IV.3. Possible non-covalent interaction between DOPE-MTX and EPC-MTX.

Considering the differences between the amine groups of both lipids, different possibilities of interactions are expected. Since DOPE is constituted by a primary amine, more possible interactions can occur (ionic and hydrogen bond). In the case of EPC only, an electrostatic interaction can be established. To observe the interactions between the lipids with MTX, we used ^{1}H NMR studies.

^{1}H NMR spectra of the aromatic area show that the addition of EPC to MTX (Figure IV.4B) does not lead to any changes in the chemical shift of all represented peaks. Meanwhile, with DOPE (Figure IV.4C), a chemical shift of the amide proton is observed from δ_H 8.07 to 7.88 ppm. The same behavior is observed for all peaks located in the glutamic moiety of MTX (data not shown). These findings suggest that an interaction between MTX and DOPE occurs, while the same interaction, do not happen when EPC is used. This non-covalent bond could explain the increase in the EE when DOPE is used as main lipid source, comparing with EPC.

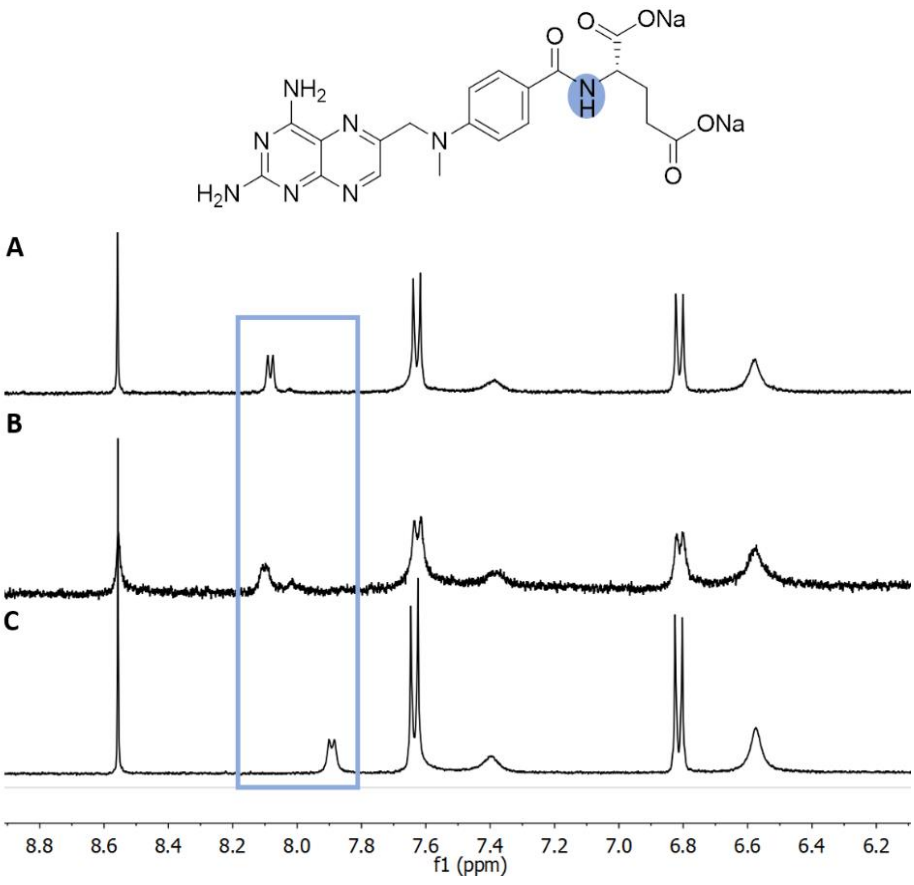


Figure IV.4. ^{1}H NMR spectra in DMSO-d_6 of **(A)** MTX, **(B)** MTX with an equimolar amount of EPC and **(C)** MTX with an equimolar amount of DOPE. Blue line represents the proton of the NH of the amide.

IV.3.5. Interaction of DOPE with *N*-protected L-aspartic acid

In order to verify that the lipid-MTX interaction is based on the terminal amino-acid moiety of MTX, we used ^{1}H NMR studies between DOPE and L-aspartic acid. The amino-acid was *N*-protected with an aromatic group (naphthyl) to mimic the terminal structure of MTX (compound C in Scheme IV.1). Figure IV.5 shows he ^{1}H NMR spectra of the protected amino-acid (Figure IV.5A) and the protected amino-acid with an equimolar amount of DOPE (Figure IV.5B). It is possible to observe that in presence of the lipid, the aliphatic signals of the aspartic acid lose its resolution and/or suffers a change in their chemical shift. The same behavior was observed previously for MTX with DOPE, but not for MTX with EPC (Figure IV.4). Based on these results, we hypothesized that the interaction drug-lipid occurs via hydrogen bond and appears to be the justification for the increased of EE in liposomes produced by the pre-concentration method. Although this interaction is the most plausible to happen, we cannot exclude the possibility of other interactions can occur between the key molecules.

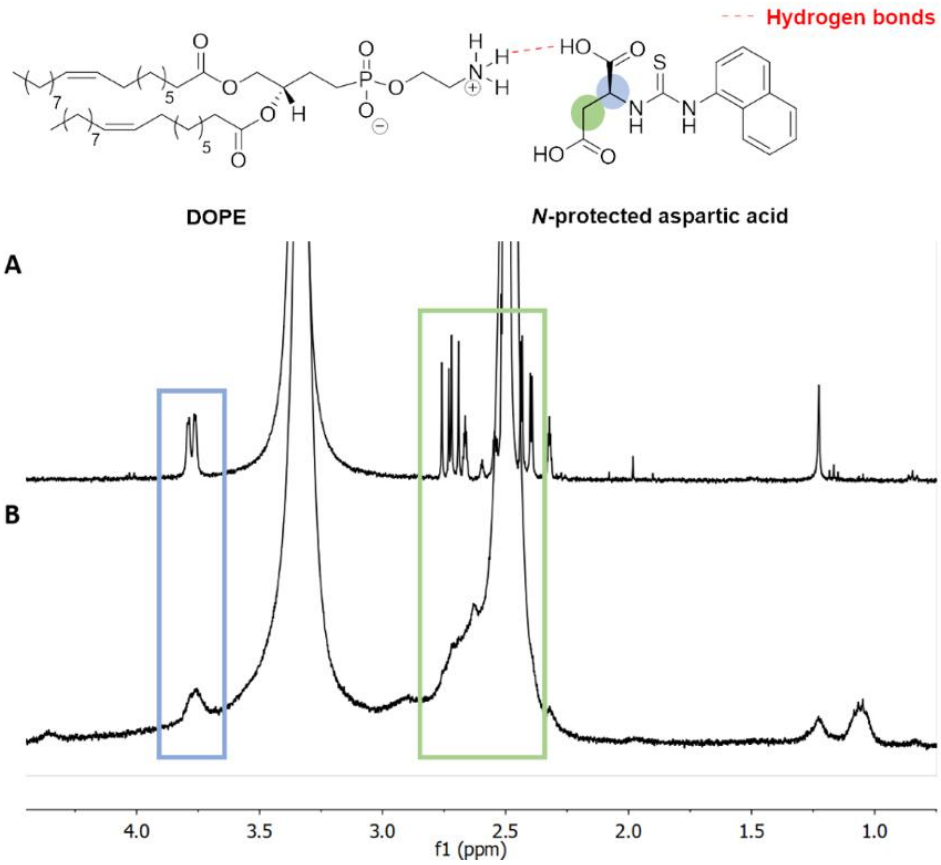


Figure IV.5. ^{1}H NMR spectra in DMSO-d_6 of **(A)** *N*-protected L-Aspartic acid and **(B)** DOPE with N-protected aspartic acid, in an equimolar ratio. Blue line represents the proton of the methylene (CH) group. Green line represents the protons from CH_2 group; both of the aspartic acid segment.

IV.3.6. Clinical effect of liposome-encapsulated MTX

The influence of the preparation method in the biological effect was evaluated *in vivo* using a mouse model of arthritis (collagen-induced arthritis in DBA/1 mice strain). Liposome-encapsulated MTX was administered twice a week IP in arthritic mice, before disease onset. Liposomal formulations produced by both methods were analyzed in two independent experiments, with a control of free MTX being used for each one. The results showed that the injection of MTX in a soluble form has an impact on the prevention of arthritis development (Figure IV.6). However, the encapsulation of MTX in liposomes improved the prophylactic efficacy. Comparing the liposomes produced by both methods, we can observe that liposomes produced by the pre-concentration method demonstrated a similar biological benefit to the conventional method. The results presented here show that the liposome-encapsulated MTX produced by this novel method maintains the prophylactic effect in the development of arthritis and can be a good alternative to the conventional production method.

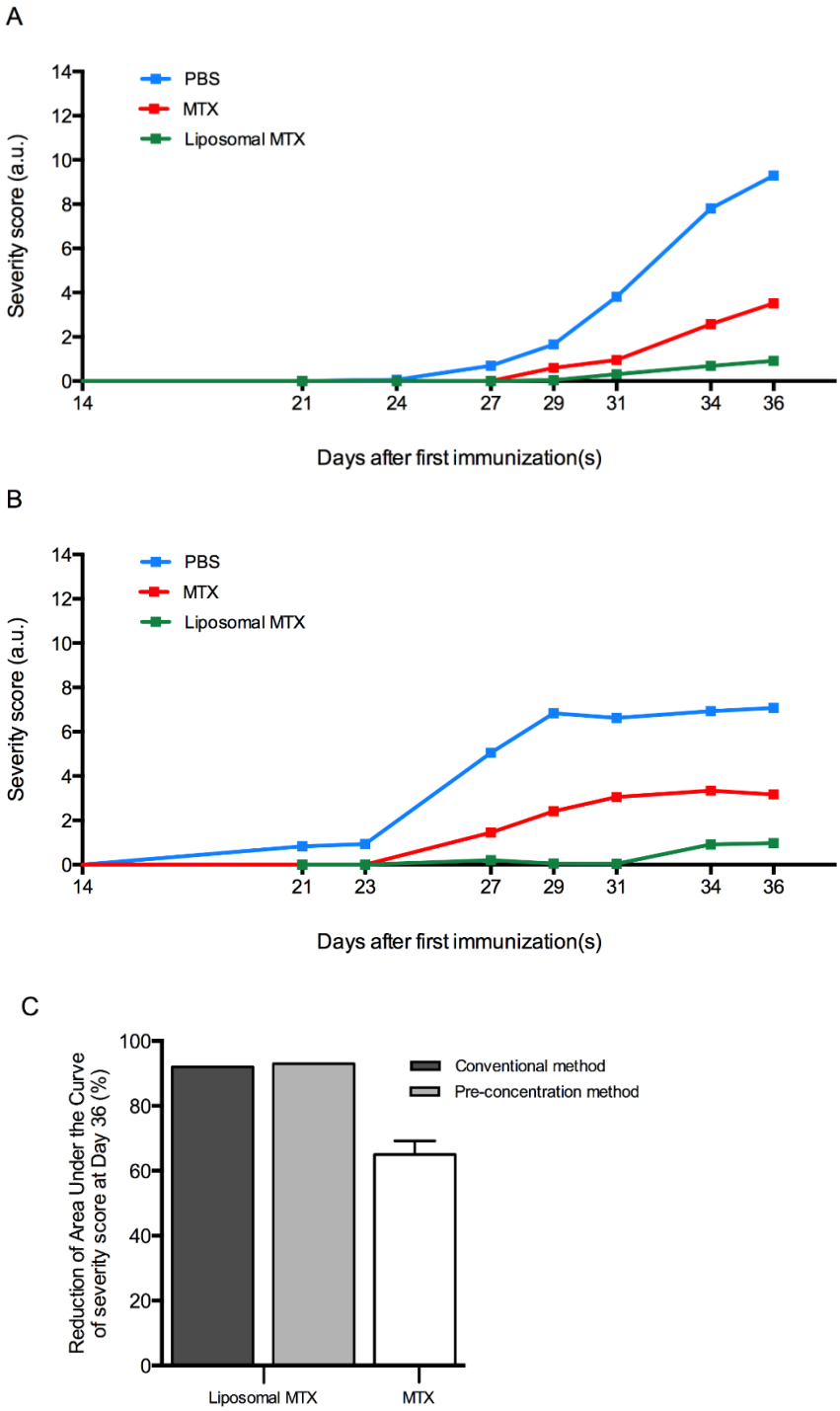


Figure IV. 6. Biological effect of MTX and liposome-encapsulated MTX in a mouse model of arthritis. Severity scores from two independent experiments with liposome-encapsulated MTX produced by **(A)** conventional method, liposome A, and **(B)** pre-concentration method, liposome C. Vehicle (PBS) in blue, 7 mg/kg MTX (red) and 2 mg/kg MTX into liposome (green). **(C)** Therapeutic index of liposomes encapsulating MTX (2 mg/kg) and the control of free MTX (7 mg/kg), on day 36. PBS as control reached the value of 0%.

IV.4. Conclusion

In this study, we have successfully developed a novel pre-concentration ethanol injection method to obtain higher MTX encapsulation in liposomes, using an initial aqueous volume of 20% and 1:1 organic:aqueous ratio (v/v). The optimized method represents an appropriate alternative to the conventional ethanol injection method, avoiding the extrusion process for size reduction and enabling a greater increase in the EE and concentration of MTX. Based on our findings, we can suppose that the specific MTX-DOPE interaction occurs via hydrogen bonds, increasing the EE. These results suggest that the interaction of the highly-charged drugs with liposomal membranes can be the drive for increased encapsulation in a novel liposome production method. Furthermore, liposome-encapsulated MTX produced by the novel method, as well as the conventional method, significantly increases the biological benefit in an arthritis animal model. In this way, this approach shows to be a significant advance in rheumatoid arthritis therapy using the liposomal encapsulation of MTX.

CHAPTER V

Folate-targeted liposomes improve effect of methotrexate in collagen-induced arthritic mice

CHAPTER V

Folate-targeted liposomes improve effect of methotrexate in collagen-induced arthritic mice

Abstract

Methotrexate (MTX) is the first line therapy for the treatment of rheumatoid arthritis (RA), however, its use may be limited by side effects notably post injection malaise. When patients are intolerant or become unresponsive, expensive biological agents must be considered. A previously developed folate-targeted liposomal formulation of MTX (FL–MTX) proved to accumulate specifically in arthritic paws and showed a complete prophylactic efficacy in collagen-induced arthritis (CIA) mouse model. In order to reach the implementation of the First-in-Human (FiH) clinical trial, we optimised the drug-to-lipid ratio (0.15) and establish the best dose to achieve therapeutic efficacy (2 mg/kg twice a week). These improved liposomes accumulate in inflamed joints, in proportion to the swelling degree of the paw and bone remodelling activity. FL–MTX showed to induce changes in the pharmacokinetics of MTX by reducing the rate of hepatic and renal excretion. Finally, the administration of FL–MTX by subcutaneous injection proved to be as effective as intraperitoneal injection. FL–MTX (2 mg/kg) have equivalent or even more efficiency than MTX (35 mg/kg) (same route and schedule) in reducing incidence and swelling in CIA model. These results suggest that FL–MTX is a more potent nanotherapeutic formulation than free MTX anchor treatment. Its potential benefits for patients may include reduced frequency of treatment and lower overall doses for a given response.

This chapter is based on the following scientific publication:

Diana Guimarães, Franck Lager, Gilles Renault, Jamil Guezguez, Michael Burnet, Joana Cunha, Artur Cavaco-Paulo and Eugénia Nogueira. Folate-targeted liposomes improve effect of methotrexate in collagen-induced arthritic mice. To be submitted.

V.1. Introduction

Rheumatoid arthritis (RA) is the most common form of chronic inflammatory arthritis, characterised by inflammation of the joints, resulting in synovial hyperplasia by infiltration of activated immune cells leading to cartilage and bone destruction [218]. RA is a common cause of disability. Mortality rates in RA patients (1.28-2.98%) are higher than in the general population [219]. Life expectancy is shortened by up to 3 to 5 years, especially in patients that develop treatment-related adverse effects including infections, tumors and gastrointestinal toxicity from drugs used in RA therapy [219,220]. Furthermore, RA patients have a higher risk of suffering from acute cardiovascular events, such as myocardial infarction, compared with the general population [221].

Methotrexate (MTX) is the anchor drug in first-line therapy indicated for the treatment of RA [222–224]. However, a careful monitoring of the patients is required to adjust dose and respond to treatment related effects. Minor toxic effects, such as stomatitis, malaise, nausea, diarrhoea, headaches and mild alopecia, are common but positively respond to folic acid (FA, folate) supplementation [223,225]. Other and more serious effects include gastrointestinal or bone marrow toxicity, pneumonitis, hepatotoxicity and cirrhosis, while major toxic effects include hepatic, pulmonary, renal and bone marrow abnormalities [224] require attentive medical supervision. Clinical reports refer that 10-30% of the patients taking MTX need to discontinue this therapy within 1-2 years of initiation [226–228]. If patients show moderate or high disease activity after 3-6 months of therapy despite dose optimization, another DMARD (disease-modifying anti-rheumatic drug; *e.g.* leflunomide, sulfasalazine, hydroxychloroquine, azathioprine) a biologic agent should be added or substituted into the therapeutic scheme [229]. Despite their clinical effectiveness, the use of biological agents have seen their implementation surrounded by health economics discussions due to questionable cost-effectiveness, since the cost of these therapies are from 20 to 200 times more expensive compared to traditional DMARDs [218,230].

RA therapies, while intended to reduce joint inflammation, act systemically leading to undesirable effects that increase the risk of adverse events. Therefore, there is a need for improved measures of disease control, as well as methods to better target therapies to act selectively on the tissues affected [231]. A large number of recent studies demonstrate that activated macrophages constitute the key effector cells in RA, reporting a direct correlation between the level of macrophage activity and the observed joint inflammation, articular pain and bone erosion [232]. Activated macrophages express an isoform of the receptor for the vitamin folic acid, the folate receptor (FR)β. Since only few cell types express this receptor, FRβ -macrophages accumulating in arthritic joints can be targeted by the use of

folate-linked agents for both imaging and therapeutic applications [233]. In this way, folate-targeted therapies selectively attack the pathogenic cell type at the sites of inflammation, leaving the healthy macrophages unharmed. Furthermore, since no other population of white blood cells appears to express a functional FRβ, the level of direct cellular toxicity associated with folate-targeted therapy seems to be very low [234].

Liposomes have gained extensive attention as carriers for a wide range of drugs. As they are composed of substances naturally occurring in biological membranes, liposomes are both nontoxic and biodegradable [235]. Biologically active materials encapsulated within liposomes are protected to a varying extent from immediate dilution or degradation, which making them good drug delivery systems for the transport of bioactive compounds to pathologically affected organs [93,236]. We previously reported the encapsulation of MTX in a new liposomal formulation (FL–MTX) using a hydrophobic fragment of the surfactant protein D conjugated to a spacer and folic acid (FA, folate) to enhance tolerance and efficacy. Our delivery system proved to be more efficient than classic systems where the FA is linked to liposomes by polyethylene glycol (PEG) [153]. We tested the specificity of these new liposomes in collagen-induced arthritic (CIA) mice, one of the most relevant animal models of RA, used for a decade in nonclinical studies to assess the efficacy of new molecules [237]. These liposomes strongly accumulated in the arthritic joints and the analysis of the cell populations retrieved from these joints revealed that macrophages expressing high levels of FRβ are more prone to uptake folate-targeted than the non-targeted liposomes. Prophylactic treatment of CIA mice with FL–MTX showed high efficacy in reducing arthritis severity, while free soluble form of MTX showed limited effect [159].

In support of the first-in-human (FiH) clinical trials with FL–MTX, a set of deeper studies were performed to complete the nonclinical package in CIA mice. We determined the better drug-to-lipid ratio of FL–MTX and dosage needed to obtain a therapeutic effect in CIA mice. Furthermore, using imaging approaches, the biodistribution pattern of labelled liposomes was evaluated in non-targeted organs and in the intended target – the arthritic paws. Finally, subcutaneous (SC) injection route efficacy was evaluated to meet the actual injection route foreseen for clinical applications.

V.2. Materials and Methods

V.2.1. Materials

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE–mPEG) were obtained from Lipoid GmbH (Germany) and diethylene-triamine-pentaacetate (DTPA) was obtained from Avanti (USA). Folate-peptide was synthetized by CSBio (USA). All the other chemicals involved in this work were purchased from Sigma-Aldrich (USA), except MTX that was acquired from Huzhou Zhanwang Pharmaceutical (China), cholesterol from Anhui Chem-Bright Bioengineering Co Ltd (China), bovine collagen type II (CII) purchased from Chondrex, Morwell Diagnostics (Switzerland) and complete Freund's adjuvant used in in vivo experiments from Fisher Scientific (France). All compounds were used without further purification.

V.2.2. Liposome preparation

Liposomes composed of DOPE/Cholesterol/DSPE-mPEG (54:36:10, molar ratio) [153] were produced by a pre-concentration ethanol injection method [238]. Briefly, the lipid components were dissolved in ethanol, to obtain a 1:1 initial ratio of organic:aqueous phase (v/v). The organic phase was added through gravity, using a 20 gauge needle coupled to a plastic syringe, to aqueous phase containing the drug MTX and folate-peptide (0.75%) dissolved in phosphate buffered saline (PBS) buffer, under vigorous magnetic stirring, at 70 °C. After ethanol evaporation, the liposomal suspension was diluted five times with PBS buffer. The non-encapsulated MTX and residual ethanol were removed from the liposomes after passage through a gel filtration chromatography column (GE Healthcare, UK), with 5 kDa cut-off (PD-10 Desalting Columns containing 8.3 mL of Sephadex™ G-25 Medium). After separation, the concentration of the encapsulated MTX was determined by measuring the absorbance at 303 nm, the maximum wavelength of MTX in PBS. The data were recorded on spectrophotometer BioTek Synergy™ HT using a quartz microplate.

V.2.3. Determination of size distribution

The determination of liposome size distribution was performed using dynamic light scattering technique. The analysis was conducted at pH 7.4 (PBS buffer) and at 25.0 °C, using a Malvern Zetasizer Nano ZS (Malvern Instruments) by photon correlation spectroscopy. The viscosity and refractive index of

dispersant were 0.8616 cP and 1.332, respectively. Each sample was measured in triplicate and results are presented as mean value ± standard deviation (SD).

V.2.4. Mice breeding

All animal experiments were conducted following the European and French regulations and were approved by the local Ethics Committee (CEEA 34, Paris Descartes University) and registered by the French ministry of research under reference #9696. CIA model is associated with severe swelling of the paws and pain was evaluated with a specific pain evaluation grid twice a week. This composite score included behavioural parameters, clinical score and weight loss. Increasing pain scores lead to sacrifice of the animals before signs exceeded the ethical threshold. Sanitary status from the animal facility in which were housed the animals was conducted every three months in a separate control cage. Six-week-old male DBA/1 mice, which are susceptible to CIA, were purchased from Janvier (Le Genest-St-Isle, France). Upon reception, animals were divided in groups of 6 individuals and placed in individually ventilated disposable cages (IVC Mouse Rack system, Innovive France). The cages (Disposable Cages 101) were provided with standard litter (corn cob) and pre-filled water bottles. Mice received standard diet food (SAFE A03 SP-10, batch U8994G10R00000). All these supplies were sterilized by suppliers.

V.2.5. Collagen-induced arthritis protocol

Arthritis was induced with native bovine collagen type II (CII) (Chondrex, Morwell Diagnostics, Zurich, Switzerland). Male DBA/1 mice were injected subcutaneously at the base of the tail with 10 mg of CII emulsified in complete Freund's adjuvant (Fisher Scientific). On day 21, mice were boosted with a subcutaneous injection above the tail of CII in incomplete Freund's adjuvant (Fisher Scientific). In this model, arthritis usually develops 20–30 days after the first collagen injection [209].

V.2.6. Clinical score evaluation

Mice were monitored for evidence of arthritis in their four paws using a blind procedure by a trained operator (FLA, senior technician 20 years of experience). For each mouse, the clinical severity of arthritis was scored (0, normal; 1, erythema; 2, swelling; 3, deformity; and 4, ankylosis) in 10 joints or group of joints: three joints of the two hind legs (toes, tarsus and ankle). The maximum score reached for each of the 10 joints was 4, so the maximum score of clinical arthritis reached for a single mouse on a

given day was 40. Animals were scored 2 to 3 times per week in the relevant period. The mean arthritic score on each clinical observation day was calculated in each group of treatment. Average severity scores per group and average weight variations per group were analysed.

V.2.7. Experimental design

The experimental design for CIA mice model was the following: treatment groups of 12 animals distributed over all cages (6 animals/cage) to avoid cage effects. Incidence of arthritis was calculated as the percentage of mice of one given group in which total clinical score was superior to 3. There was always both a naïve and vehicle group.

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V.2.8. Mouse collagen-induced arthritis treatment scheme

All animals received the same injection volume for treatment, intraperitoneally (IP), twice a week (unless otherwise stated). The injected doses for MTX encapsulated in folate-targeted liposomes (FL–MTX) or non-targeted liposomes (L–MTX) are expressed in MTX-equivalent dose. Empty liposomes (folate-targeted – FL, or non-targeted – L) were injected at the same lipid concentration as in FL–MTX and L–MTX. The negative treatment control group was PBS buffer. The positive control group was 7 mg/kg or 35 mg/kg MTX twice a week. Varying doses (1, 2 or 4 mg/kg MTX) and frequencies of injection (once or twice a week) of FL–MTX were used and are referred in the results section. Treatments started on day 14, *i.e.,* one week before the immune boost on day 21, and were continued throughout the testing period, unless stated otherwise.

V.2.9. Nuclear medicine imaging

This study was performed by CIPA-Orleans, a public owned (CNRS) pre-clinical imaging facility specialized in the evaluation of new therapies *in vivo*, with a specialization in nuclear medicine imaging for the assessment of biodistribution. The study followed radiolabelled liposomes using Single Photon Emitted Computed Tomography (SPECT) imaging *in vivo*, non-invasively, in the same animals over 4 days after injection. Liposomes were labelled with ^{111}Indium (^{111}In) and, consequently, the liposomes had to be modified to insert a linker for this purpose. The lipid DTPA, a chelating agent widely used in nuclear medicine to prepare radiolabelled pharmaceutical agents, was used at 1.5%, ensuring a very effective and specific binding of ^{111}In. A first trial was conducted to ensure that labelling of liposomes incorporating

^{111}In was indeed possible with sufficient ^{111}In label yield and specificity. Four mice were injected with L-MTX and 4 others with FL-MTX. A SPECT/CT imaging system (Mediso) was used for imaging at the following time points: 30 minutes, 24, 48 and 72 hours post injection. At 72 hours post injection an additional imaging modality was performed with the application of Methylene Di Phosphonate (MDP) labelled with ^{99m}Technetium (^{99m}Tc), allowing dual imaging of liposome and bone metabolism. Separation of ^{111}In and ^{99m}Tc activity was done by their signature rate of decay. *Ex vivo* CT scan of rear paws was performed to measure paw swelling at the end of experiment, in order to correlate paw thickness to liposome accumulation. These higher resolution acquisitions were performed using a Bruker Skyscan 1278 on fixed paws.

V.2.10. *In vivo* quantification of MTX concentration

To measure the concentration of MTX in most organs, some mice not subjected to arthritis were injected 24 or 48 hours before sacrifice. Organs were then dissected free and snap frozen in liquid nitrogen, then stored at -80 °C and later analysed. The concentration of MTX in the samples was measured by HPLC-MS/MS. The HPLC-MS/MS system comprises an Agilent Technologies 1260 Infinity liquid chromatography equipped with a binary pump and a column oven together with an auto-sampler. It is linked to the AB SCIEX triple-quadrupole mass spectrometry (MS) instrument with an electrospray ionization (ESI) interface. The system is controlled using Analyst Software 1.6.2. from Applied Biosystems Inc. in an associated computer. A Raptor Biphenyl (2.7 µm, 50 x 2 mm) chromatography column from Restek was used for separation.

Prior to measurement plasma and tissue samples were extracted using acetonitrile (6x volumes with sample weight in mg converted to µL 1:1) containing internal standard (terbuthylazine) (*e.g.* 10 µL sample + 60 µL acetonitrile). For plasma, the mix was allowed to precipitate on ice for 15 minutes and then centrifuged (14,000 g for 5 minutes) and the supernatant was placed into HPLC glass vials, suitable for the auto-sampler use. For tissue, samples were homogenised in an aqueous buffer containing protease K in one volume of buffer. The mix was incubated in an ultrasound bath for 5-10 minutes. Subsequently, six volumes of acetonitrile were added, and the samples were once again incubated in an ultrasound bath for 5-10 minutes. The mixture was centrifuged, and the supernatant was placed into HPLC glass vials, suitable for the auto-sampler.

A standard curve or calibration curve comprising 10 concentrations of MTX (in between 5 nM to 100 µM) was prepared in water. The calibration curve was extracted with 6 volumes of acetonitrile

including internal standard. The standard curve was measured twice throughout each run, at the beginning and at the end of each sample set and was freshly prepared for each measuring set. Linear regression analysis of the log peak area versus log theoretical concentration of MTX was used to obtain the apparent sample concentrations which were then corrected for dilution if appropriate. The parent ion for MTX was m/z H^+ 455.2 and the fragment measured was m/z H^+ 308.3.

V.2.11. Statistical Methods

Pathology development and incidence may provide non-normal distribution of severity scores in the study. A Shapiro test performed on severity scores always shows a significant value confirming this observation. Hence, we used a non-parametric one-way analysis of variance Kruskal-Wallis test at a given time point to test for significant differences in severity score response to treatment between groups. The Dunn post-hoc test without correction was then used to identify differences between all groups. All analyses were performed using R software and the "dunn.test" library for Dunn post-hoc tests. A linear model was used for nuclear imaging analysis of activity in paws.

V.3. Results and Discussion

V.3.1. Study of the drug-to-lipid ratio

The development of a liposomal product is quite a complex process as many critical parameters should to be investigated during the preparation process. The drug-to-lipid ratio (D/L ratio) is a critical process parameter that represents the capacity of the liposome to accommodate the drug. Thus, this parameter can influence the therapeutic efficacy of the liposomal product, expressing the actual dose of the drug being administrated [239]. Here we investigate the influence of D/L ratio on toxicity and therapeutic efficacy of FL–MTX after intraperitoneal (IP) administration in CIA mouse model. The versatility of the liposomal production method used in this study (a pre-concentrated ethanol injection method) [238] allows the MTX-to-lipid ratio to be greatly varied, with distinct values readily obtained. Three liposomal formulations were prepared with D/L ratio of 0.10, 0.15 and 0.20 and the therapeutic effect evaluated at a dose of MTX of 2 mg/kg. The liposomal formulations present small values of size and polydispersity index (PDI) (Table V.1).

Table V.1. Characterization of liposomes used in this work.

Liposomal formulation	Labelling	Drug-to-lipid ratio	Z-average (d.nm)	PDI
FL-MTX	-	≈0.10	130.6 ± 1.097	0.097 ± 0.041
	-	≈0.15	138.0 ± 0.351	0.092 ± 0.022
	-	≈0.20	121.2 ± 0.473	0.086 ± 0.009
L-MTX	-	≈0.15	129.7 ± 0.115	0.075 ± 0.010
FL	-	-	117.3 ± 1.917	0.162 ± 0.004
L	-	-	143.2 ± 0.757	0.057 ± 0.026
◊ FL-MTX	DTPA-^{111}In	≈0.15	100.1 ± 1.294	0.148 ± 0.005
◊ L-MTX	DTPA-^{111}In	≈0.15	99.71 ± 2.157	0.134 ± 0.016

◊ Radiolabelled liposomes

Results show that the most favourable D/L ratio is 0.15, demonstrating a negligible weight loss and a better therapeutic effect, compared with the other D/L ratios (Figure V.1). A compromise on the initial amount of lipids must be reached so that it is enough to achieve the target cells and cause the therapeutic effect, but not too high to cause toxicity. Indeed, considering that the amount of lipids allowed to be administered to a patient per day is limited and that the D/L ratio determines the final drug concentration in the product, this ratio determines the maximum dose of drug that can be administered and thus, the amount of liposomes that are going to be administrated per treatment [239]. In this sense, because of the elevated costs, high lipid concentrations may reduce the cost effectiveness of large-scale manufacturing.

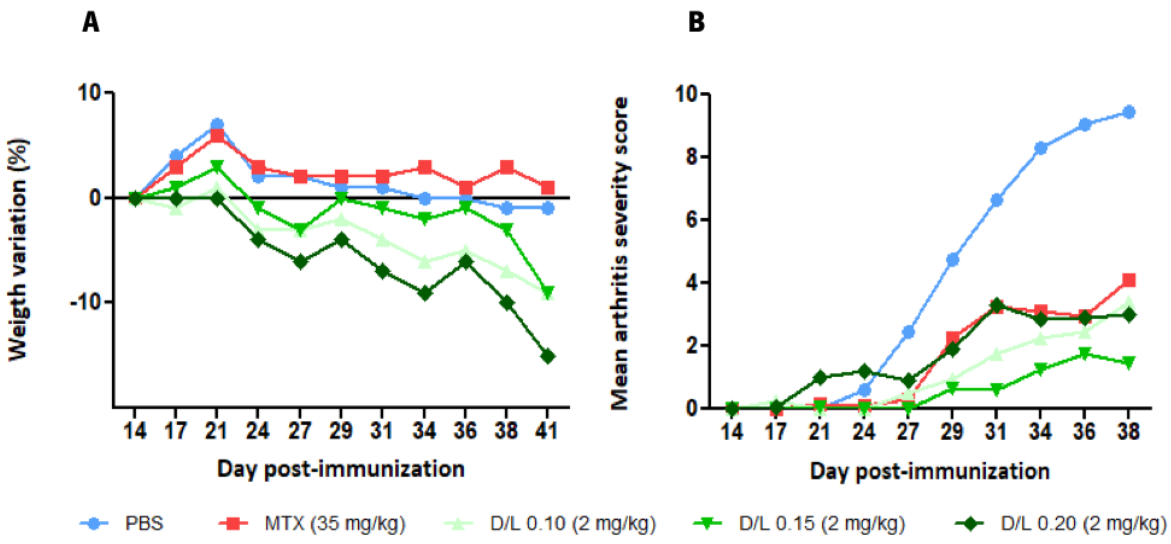

Figure V.1. Influence of D/L ratio on weight loss and therapeutic efficacy of FL–MTX in CIA mice. **(A)** Average weight variation after treatment. **(B)** Average clinical score per groups as a function of time. All treatments were injected at 2 mg/kg twice a week (n=12).

V.3.2. Effective dose in the murine collagen-induced arthritis model

In order to evaluate the most effective dose to prevent arthritis development, CIA mice were injected with several liposomal formulations at an MTX dose of 1, 2 or 4 mg/kg, twice a week. Results showed that the 4 mg/kg dose of FL–MTX rapidly induced weight loss in the animals (Figure V.2A), leading us to switch, during the experiment, from a twice to a once-weekly injection. This severe weight loss is most probably due to reduced food consumption. However, the dose of 4 mg/kg injected once week did not prove to be as effective as 2 mg/kg given twice a week (Figure V.2B). A 1 mg/kg dose injected twice a week had a mild efficacy. Recently, Chen *et al.* used a liposomal formulation to deliver MTX for the treatment of arthritis in C57BL/6 CIA mice [240]. A similar decrease of arthritic score was seen after IV injection of MTX 1 mg/kg once every two days for a total of five injections. Liposomes encapsulating MTX were also used in Lewis CIA rats, and their treatment was achieved after daily IV injection of MTX 2.5 mg/kg for 4 days [241]. Non-targeted liposomes (L–MTX) at a dose of 2 mg/kg given twice a week also showed a good efficacy, similar to FL–MTX. While folate targeting in FL–MTX has a better impact on the reduction of the clinical signs in CIA mice, though it is not possible to demonstrate this effect with appropriate statistics. L–MTX most often showed a slightly lower efficacy than FL–MTX at the same dose. The difficulty of obtaining a statistical difference is mostly due to the non-normal

distribution of clinical scores preventing from using multiparametric statistical tests and to the varying arthritis incidence of CIA mice.

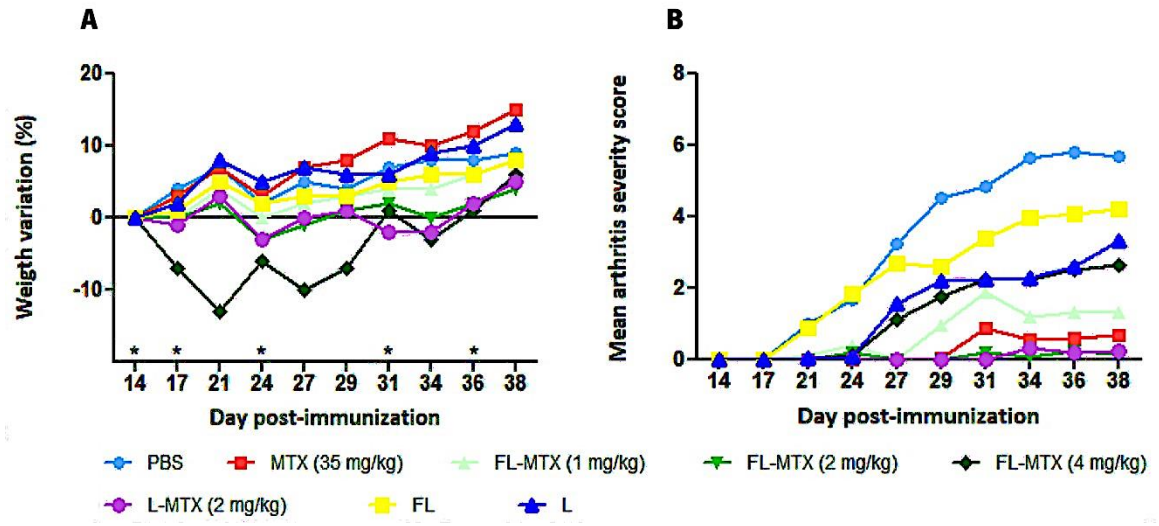

Figure V.2. Influence of MTX dose and folate targeting in CIA mice. Toxicity and efficacy were assessed after twice a weekly injection of free soluble form of MTX (MTX) and encapsulated MTX in folate-targeted (FL–MTX) or non-targeted (L-MTX) liposomes. Empty targeted or non-targeted liposomes (FL or L) and PBS were used as controls. **(A)** Average weight variation after injection of treatments. Each graph shows the average weight variation after day 14 (time of the first injection of treatments) among groups of mice treated (n=12). In the 4 mg/kg dose the frequency was reduced to once weekly (* assigned in the graphs). **(B)** Mean arthritis severity (n=12).

A clinical follow-up was performed for a few days after the experiment, leaving the animals without any treatment. This relapse trial clearly showed that mice treated with FL–MTX, had increased arthritis scores after the treatment was stopped (Figure V.3). These data suggest that disease is responding to therapy, however, that a population of pathological autoreactive T-cells are present (due to immunization) and that these are capable of inducing arthritis in the absence of treatment.

Indeed, CIA mice typical treatment scheme starts at day 14, whereas the immunization, to be completely effective, needs an additional injection a week after. One could think that the low incidence of pathology in FL–MTX-treated group could be the consequence of an inhibited immunization because of this injection timing. However, the onset of arthritis a few days after the end of treatment, even weeks after

immunization, clearly confirms that the induction of arthritis is effective, but its consequences are controlled by treatment. Comparatively, the incidence of arthritis on the relapse trail was much lower in PBS group, suggesting that if these mice were poorly or not arthritic all along the trial, this was because they did not mount a good immune response.

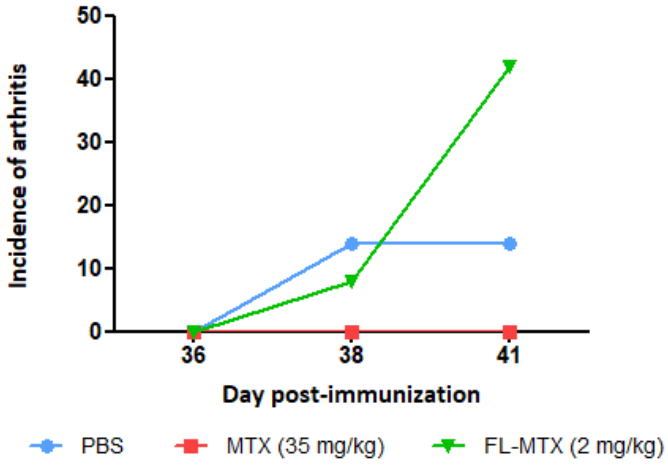

Figure V.3. Relapse of CIA arthritis upon arrest of treatment. Incidence of pathology is expressed in percentage of CIA mice of each group that had no clinical signs of arthritis at day 34 (last treatment injection).

V.3.3. Targeting of inflamed paws

We previously shown that FL strongly accumulated in the joints of the arthritic mice, using whole body mouse fluorescence imaging [159]. Since *in vivo* fluorescence is not quantitative, we assessed liposomes pharmacokinetics by nuclear imaging (SPECT-CT) throught the inclusion of 1.5% of DTPA allowing radiolabelling with ^{111}In [242]. After initial validation of the imaging protocol, showing no uptake of ^{111}In-labelled liposomes in healthy joints (data not shown), the pharmacokinetics in CIA mice was followed for 72 hours. Results showed accumulation of FL–MTX in inflamed joints within 30 minutes post injection (Figure V.4) and were still present 72 hours after injection, which confirmed a prolonged targeting of the inflamed joints. However, no significant diference is observed in the accumulation of liposomes with or without folate targeting. In contrast, the FL–MTX appear to enter the spleen and liver more rapidly and label both organs differently. The L–MTX signal increases in spleen more slowly. FL–MTX also appears to concentrate to a greater extent in proximal lymph nodes in affected paws.

Liposomes accumulation can be linked to various mechanisms. Neoangiogenesis is well known in inflammatory pannus both in patients and CIA mice, leading to a higher vascular density [243,244], which provides better distribution of the liposomes in the arthritic joints. Furthermore, vessels in arthritic joints are more permeable allowing higher rates of extravasation of liposomes and accumulation in the joint [245–247]. Finnaly, numerous immune cells present in the inflamed joints can phagocyte the liposomes, leading to an enhanced uptake in the joint [127,247]. Identification of which of these mechanisms is involved in the high accumulation of liposomes into the arthritic joints remains to be studied.

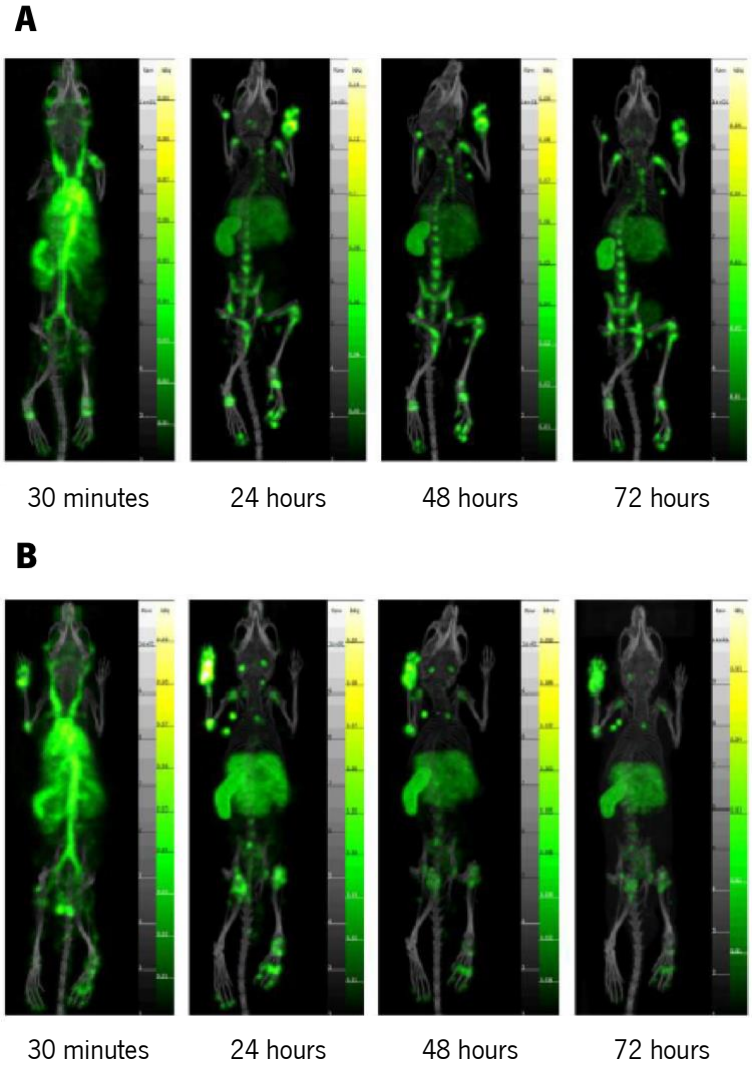

Figure V.4. Pharmacokinetics of ^{111}In-labelled liposomes encapsulating MTX assessed by nuclear imaging (SPECT-CT). **(A)** Non-targeted liposomes (L–MTX); **(B)** Folate-targeted liposomes (FL–MTX). Please note at 30 minutes the early targeting of inflamed joints and the strong signal from vessels. Targeting in inflamed joints remains very stable over time in inflamed joints.

The association between degree paw swelling and the accumulation of liposomes was assesed. The percentagem of swelling of the arthritic pawswas calculated using as reference the healthy front and rear paws as references. Then, liposomes accumulation was plotted against paw swelling for both FL–MTX and L–MTX (Figure V.5A). A strong accumulation of liposomes can be measured as the paws swell, which confirms the specificity of liposomes to arthritic tissue as non-arthritic paws do not retain the liposomes. Again, it is not possible to observe the influence of folate targeting on the accumulation of liposomes in the paws.

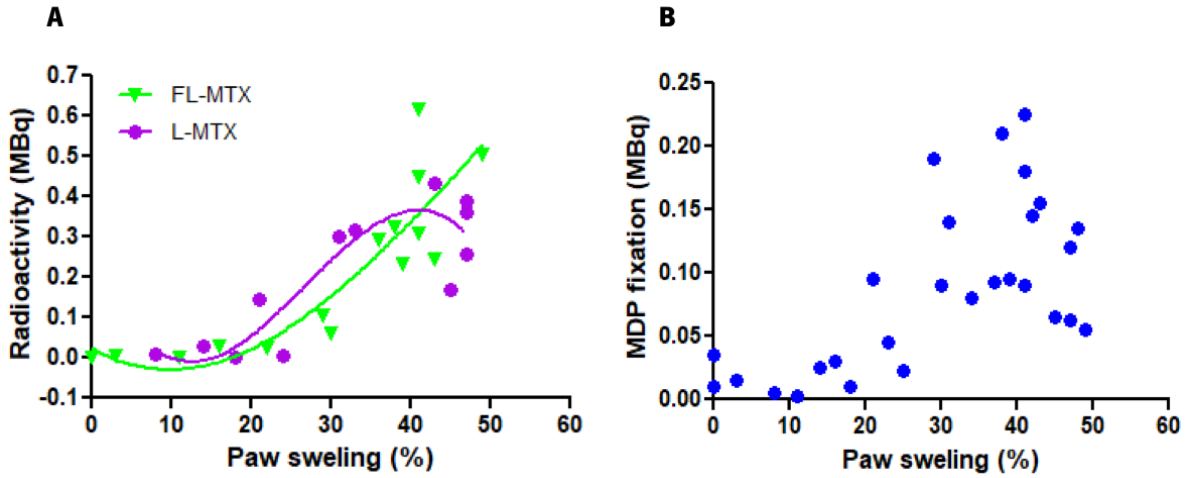

Figure V.5. Specificity of liposomes encapsulating MTX to arthritic joints. **(A)** Liposomes accumulation as a function of paw swelling. The accumulatio of liposomes directly correlates with the percentaage of swelling. **(B)** MDP activity as a function of paw swelling, strongly indicating bone erosion activity.

In order to evaluate the bone remodeling activity, ^{99m}Tc MDP, a radiotracer used in nuclear medicine for bone scans [248] was injected in these animals (Figure V.6). Bone remodeling activity partly matched with the paw swelling index, reflecting the occurrence of mechanisms related to bone erosion (Figure V.5B). Uptake of FL-MTX-DTPA-^{111}In to spleen and liver is also apparent and is consistent with the known distribution patterns of liposomes. Residual signal is also apparent at the injection site. CIA is normally not scored in the knee or shoulder joints, however signal is also apparent at these sites for both MDP and FL-MTX. These observations may indicate that arthritic reactions are more widespread than normally considered. FL-MTX also appears to be associated with the Popliteal and sub-mandibular lymph nodes as well as hip joints. MDP in contrast clears to the bladder.

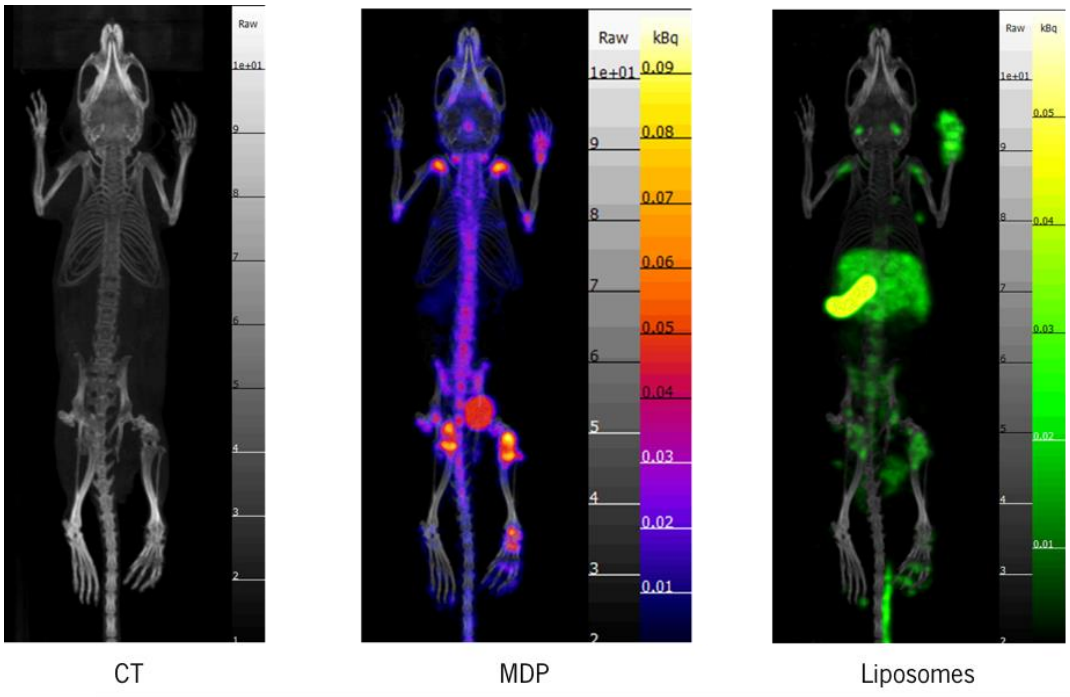

Figure V.6. Example of bone metabolism imaging and Liposome distribution in an arthritic mouse with severe monoliteral arthritis in the left fore and hind paws (see tarsal deformation). The left image shows the CT scanner of the animal, the middle one, the combination of Scanner and MDP-^{99m}Tc activity and the right one, combination of CT-scanner and FL-MTX-DTPA-^{111}In, 72 hours post injection. MDP reveals bone remodeling activity that correlates with accumulated FL–MTX liposome in joints. Please note that non-arthritic joints show no or very limited activity in both MDP and FL–MTX distribution.

V.3.4. MTX distribution in non-targeted tissues

In vivo biodistribution of different liposomal formulations after IP administration was evaluated by quantifying the amounts of MTX in serum and in several non-target organs. In naïve mice, biodistribution reveals that the absence of folate targeting leads to increased MTX delivery at 24 hours in bile, kidneys, spleen and possibly in gut (Figure V.7).

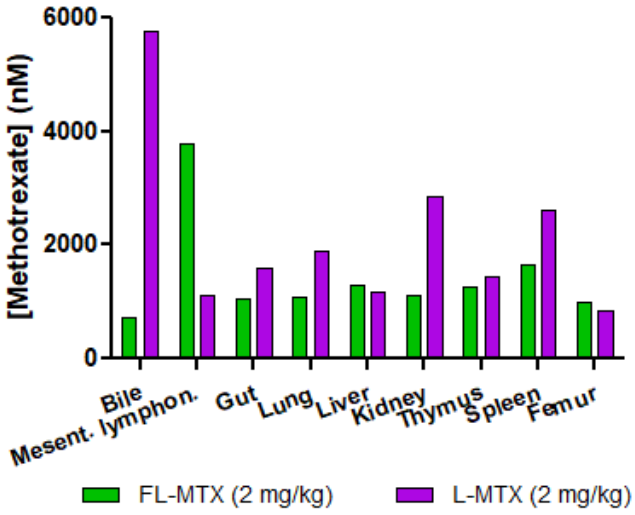

Figure V.7. Biodistribution of folate-targeted liposomal MTX (FL–MTX) compared to non-targeted liposomal MTX (L–MTX), in naïve mice. MTX concentration in non-target tissues 24 hours post IP injection (n=5).

In CIA mice, results shown that the encapsulated MTX remains in serum from 24 to 48 hours. The extended presence of MTX in serum is most probably indicative of intact liposomes circulating rather than free MTX, which is normally rapidly cleared [249]. Tissue concentration of MTX is also higher when the drug is encapsulated in liposomes as opposed to the free soluble form of MTX, even though the dose used is much lower (2 mg/kg in liposomes as opposed to 35 mg/kg in free MTX). There is a close dose-dependency between the quantity of liposomes injected and the measured MTX concentration in serum and various non-target organs (liver, spleen, kidneys, lungs) (Figure V.8). The dose delivered has an impact on liposomal treatment efficacy in CIA mice as well as on possible adverse effects.

Indeed, one other aspect of liposome targeting concerns non-target organs. At 24 hours post injection there is a clear impact of liposome targeting on MTX concentration. MTX concentration is higher in the liver and kidneys for L–MTX (Figure V.8B), whereas no differences could be observed in the serum between L–MTX and FL–MTX (2 mg/kg) (Figure V.8A). These results indicate that folate-targeting of liposomes induces pharmacokinetic changes by reducing the rate of drug excretion in hepatic and renal tissues. Indeed, liver and kidneys are the major organs responsible for nanoparticles excretion [250–252].

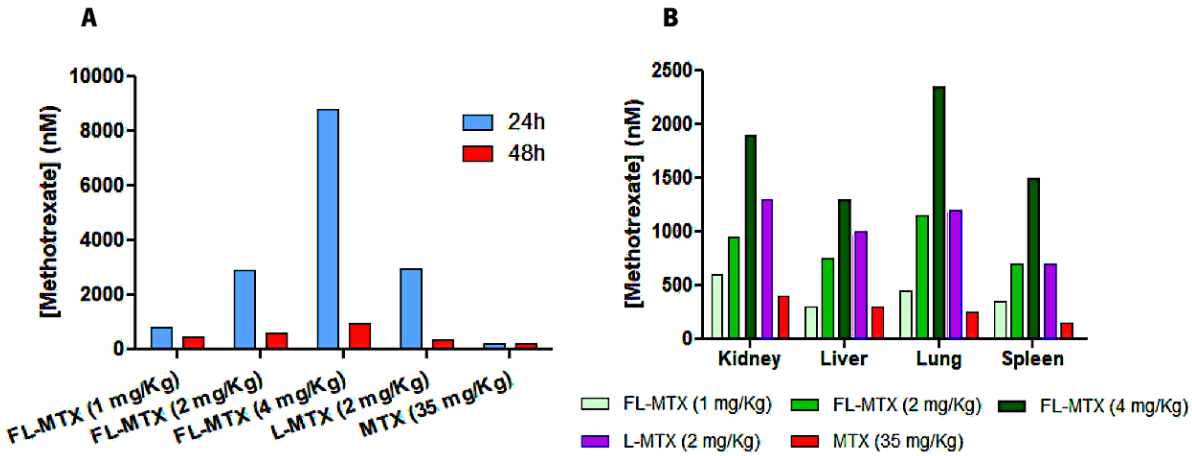

Figure V.8. Distribution of folate-targeted liposomal MTX (FL–MTX) in a dose-dependent manner compared to non-targeted liposomal MTX (L–MTX) and free MTX (MTX). **(A)** Serum MTX concentration at 24 and 48 hours post IP injection. **(B)** MTX concentration in non-target tissues 24 hours post IP injection.

V.3.5. Subcutaneous injection route

Although the parenteral administration in animal models is commonly IP (frequently the intravenous, IV, route is not feasible for technical reasons) [253], this route is unlikely to be of much use in human therapy. For liposomes in particular, the subcutaneous (SC) route of administration is preferable, not only because it is a simpler route for patient's self-administration, but also because it might serve as a depot for the sustained drug release *in vivo* [254]. With the behaviour of FL–MTX well established in CIA model when administered by IP injection, we wanted to assess the efficacy and toxicity of our liposomal MTX after SC administration. To determine the target dose to inject in animals, the concentration of MTX in serum was determined after 24 and 48 hours post IP, IV and SC injection. The results show that, comparatively with IP route, MTX serum concentration was lower via the SC route (1.5 $\log_{10}$ lower, Figure V.9A), which led us initially to increase the injected dose. However, severe weight losses in CIA mice were registered after the SC injection of FL–MTX at 8 and 16 mg/kg (data not shown). By contrast, soluble MTX showed no toxicity effect even at very high concentration (16 mg/kg). Not surprisingly, MTX serum concentration is not a good indicator of MTX bioavailability in mice injected with the drug encapsulated into liposomes. Indeed, Allen and colleagues reported that liposomes levels were significantly higher in the draining lymph nodes after SC administration and concentrations in other tissues were proportionately reduced relative to the IV and IP injections [254]. Furthermore, another study

found that the blood concentration of a pegylated liposomal formulation 24 hours after SC injection is much lower (aprox. 30 times) than after IV injection [255]. Administering the same 2 mg/kg dose by SC injection proved to be as effective as the IP injection in CIA mice. FL–MTX and L–MTX showed similar efficacy and performed better than free MTX which was given in a 3.5 times higher dose (Figure V.9B).

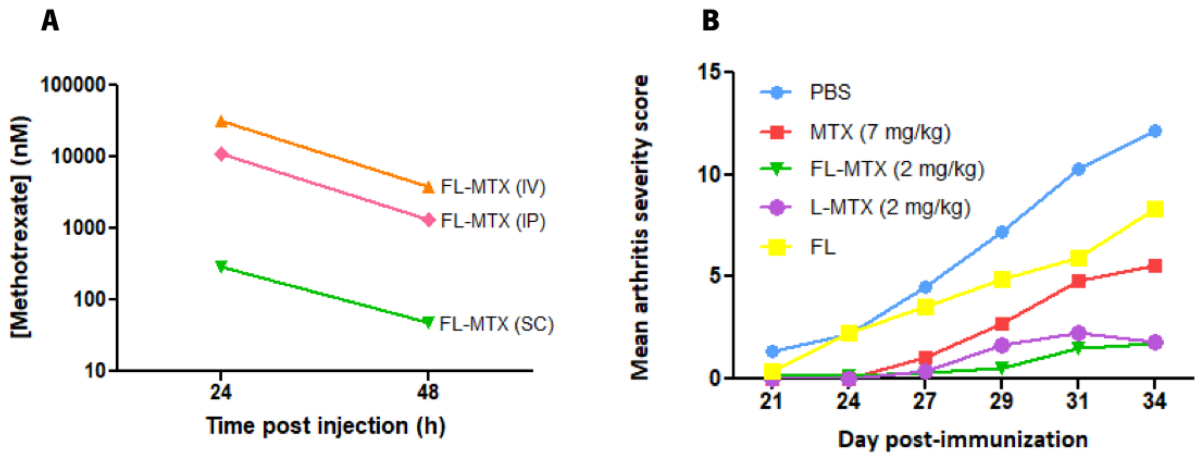

Figure V.9. Influence of SC route of administration in CIA mice. **(A)** MTX serum concentration after 2 mg/kg administration of FL–MTX by in IP, SC or IV injection. **(B)** Efficacy of 2 mg/kg of liposomal MTX (FL–MTX and L–MTX) given by SC injection. Free MTX was administered at a 7 mg/kg dose SC and PBS was also injected SC (n=12).

V.4. Conclusion

In the present work we performed several studies in CIA mouse model in order to optimize and characterize the new folate-targeted liposomal formulation encapsulating MTX (FL–MTX). The better D/L ratio was found (0.15), showing a negligible weight loss in mice. Furthermore, results showed that the optimal dose to achieve therapeutic efficacy was the dosage of 2 mg/kg twice a weekly, in comparasion with 1 mg/kg twice weekly (not effective) and 4 mg/kg once a week (not tolerated). Nuclear imaging revealed that the liposomes accumulate in inflamed joints in proportion to the paw swelling and bone remodeling activity. The pharmacokinetics of MTX is modified by its encapsulation in the liposomes, largely increasing the circulation time as MTX was quantified until 48 hours in the serum. Folate-targetting impacts on MTX distribution to non-target organs: a higher concentration was found in the liver and kidneys, whereas no differences could be detected in the serum between targeted and non-targeted liposomes. To finalise the set of experiments performed in CIA mice, the efficacy of FL–MTX treatment

for arthritis was assessed by administering it by SC injection, a route much simpler for patient's self-administration. The same dose was equally effective by both the SC and IP routes.

In conclusion, the new liposomal formulation FL–MTX provides new pharmacological properties to MTX, in particular, a far lower dose of MTX is required for a given reduction in arthritic score. The present findings demonstrate that folate-targeted liposomes constitute a promising MTX delivery system for RA treatment. This approach will potentially allow a reduction of the dose used clinically, which inherently will improve the tolerance of RA patients to MTX adverse effects that commonly requires the adding or changing to more expensive biological DMARDs.

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CHAPTER VI

Liposomes encapsulating methotrexate: a powerful tool for rheumatoid arthritis therapy

CHAPTER VI

Liposomes encapsulating methotrexate: a powerful tool for rheumatoid arthritis therapy

Abstract

Rheumatoid arthritis (RA) is an inflammatory disorder that affect almost 1% of the world population, considered a painful and incapacity disease. RA primarily affects the synovial membrane, leading to severe destruction of joints, deformity, and disability. The precise etiology of RA remains unknown but is clear the role of macrophages in RA pathogenesis, due to increased presence at the cartilage pannus junction and in the inflamed synovial membrane. The available RA therapy is still unsatisfactory and only intends to relief the symptomatic pain. Methotrexate (MTX), the first line treatment of RA, may reduce the inflammation level, pain, preventing joint erosion and functional damage. However, MTX can cause a long list of adverse effects in patients. In this context, researchers have the ambition to develop a safe and more efficiently system that can selectively the drug to the inflamed tissues without affecting the healthy cells. Liposomes are one of the most common and well-investigated drug delivery systems due to their suitable characteristics such as nontoxic, biodegradable, versatility, among others. MTX encapsulation into liposomes can be a promising approach to improve its pharmacological properties and reduce the main side effects. This review highlights the application of liposomes encapsulating MTX as therapeutic strategy in RA. Furthermore, key aspects of this formulation will be addressed, since their production, MTX quantification and storage conditions.

This chapter is based on the following scientific publication:

Diana Guimarães, Artur Cavaco-Paulo and Eugénia Nogueira. Liposomes encapsulating methotrexate: a powerful tool for rheumatoid arthritis therapy. To be submitted.

VI.1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that affect almost 1% of the adult population worldwide. RA is three folds more prone to developing in women than in men. The disease can develop in persons of any age, however, the high incidence is in the middle age, around 55 years [256,257]. RA is considered an inflammatory disease characterized by the chronic joint inflammation, acute synovitis, progressive erosion of articular cartilage, and bone destruction leading to a severe disability and premature mortality [258]. The synovial tissue is the initial target of the inflammatory process [259]. Posteriorly, RA patients can developed systemic complications including cardiovascular and vasculitis, skeletal, pulmonary and psychological disorders [260]. The precise etiology of RA is unknown, but it is evident that activated macrophages play a crucial role in the pathogenesis of RA, due to their higher presence in the inflamed synovial membrane and at the cartilage pannus junction, their activation status and their successful response to anti-rheumatic therapy [127].

The key objective in RA treatment is to minimize the symptoms, including inflammation and pain as well as to prevention the destruction and deformity of joint and maximize their function [261]. The drugs commonly used in RA therapy can be categorized as nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modifying anti-rheumatic drugs (DMARDs) and biologic agents. The most important and useful drug for RA clinical treatment is a type of DMARD, namely methotrexate (MTX). However, the administration of MTX in many patients along the time reveals reduction of efficacy and even toxicity [262,263]. In order to overcome this shortcoming, the pharmacological treatment of RA in the last years has been characterized by a firm evolution of new approaches and therapeutics agents [264].

An appropriate and effective approach for the RA therapy include the delivery of the antirheumatic drugs to the inflamed tissues using passive or active targeting strategies and a rapid drug release from nanosystems by a specific stimuli-responsive mechanism to reach successful therapeutic concentration into the inflammatory sites [240]. In this way, it is important the development of formulations safer with clear advantages for RA patients to decreasing the side effects and improved cost-benefit ratio [127,265]. In virtue of their suitable and versatile characteristics, such as the ability to encapsulate a wide range of therapeutic agents, liposomes have been studied in RA therapy. The use of liposomes for effective management of RA increase the therapeutic index of the antirheumatic drugs [259]. Furthermore, liposomes can be modified with targeting ligands for active targeting purposes [266]. As activated macrophages significantly influence the pathogenesis of the disease, the choose of this specialized cell to targeting is a great option for the development of RA therapies. Since folate receptor (FR) β are highly

over-expressed in activated macrophages but are limited in normal tissues, the production of liposomes with folate (folic acid, FA) as a targeting ligand represents a powerful approach for RA therapy.

This review provides an overview of the pathogenesis of RA and the current RA treatment, highlighting the MTX, that is considered the first line therapy in this disease. Liposomal formulations encapsulating MTX studied for the direct application in RA therapy are addressed. Since they represent a powerful tool for RA management, key aspects will be addressed, such as their production methods, MTX quantification and even storage conditions.

VI.2. Pathogenesis of rheumatoid arthritis

The pathogenesis of RA is still unclear. Although autoimmune phenomena and inflammatory processes appears to be the more dominant abnormalities [260]. As a prototype of an autoimmune disease, the RA pathogenesis is linked with the dysregulation of the immune system and could be divided into distinct stages: (i) triggering, (ii) maturation, (iii) targeting, and (iv) fulminant stage, that can occur sequentially or simultaneously [267]. During the first stage, the autoimmunity develops in healthy individuals appearing to be activated by environmental challenges acting as a triggering factor. Despite these individuals have not yet reveled any clinical manifestation, they have genetic susceptibility to the disease. The second stage include the clinical onset for the RA diagnosis. A specific inflammatory reaction occurs at joints leading to an early sign of the RA disease [268]. Third, a chronic inflammatory process develops, joint swelling is the reflection of synovial membrane inflammation, following immune activation [267]. At this stage, the patients have fulfilled the classification criteria of RA, becoming the focus of intense research and therapeutic approaches [268,269]. The last stage of RA is characterized by a cartilage damage, bone erosion and systemic consequences [267].

The precise RA etiology remains elusive but could be related to genetic factors, which show a key responsibility in initiation of the disease [270]. These genetic factors including specific genes, epigenetics and post-translational modifications of proteins, or due to environmental factors such as gut, smoking, pathogen infection, silicon and air pollution [270–273]. However, in combination with environmental factors play an important role in RA susceptibility because can trigger in a healthy person the formation of autoantibodies which can lead to joint problems without swelling [274]. Posteriorly, the complex interaction of these combined factors, RA autoantibodies, immune cells and immune organs begin to activate, causing infiltration of cells in the joints [275]. Synovitis, the inflammation of synovium (the lining

tissue of the joint) is caused by the influx and/or local activation of mononuclear cells and by angiogenesis, the formation of new blood vessels [276]. The infiltration of synovium by different types of immune cells, including macrophages, B cells, T cells, plasma cells, dendritic cells and neutrophils, disturbs the stock of oxygen and nutrients to the joints by the synovium, promoting the inflammatory tissue destructive lesions [218]. These issues lead to the characteristics of hypoxia (defined as low oxygen partial pressure) and acidity in RA inflammation joints [277]. The hypoxia itself can induce inflammation, apoptosis, angiogenesis, oxidative damage, cartilage erosion and irregular energy metabolism [260]. Acidity in joints can occur in the beginning of disease and can be progressive with the accumulation of acids in the connective and fatty tissues [278]. Figure VI.1 represents the schematic illustration between a normal joint and its main changes in the RA.

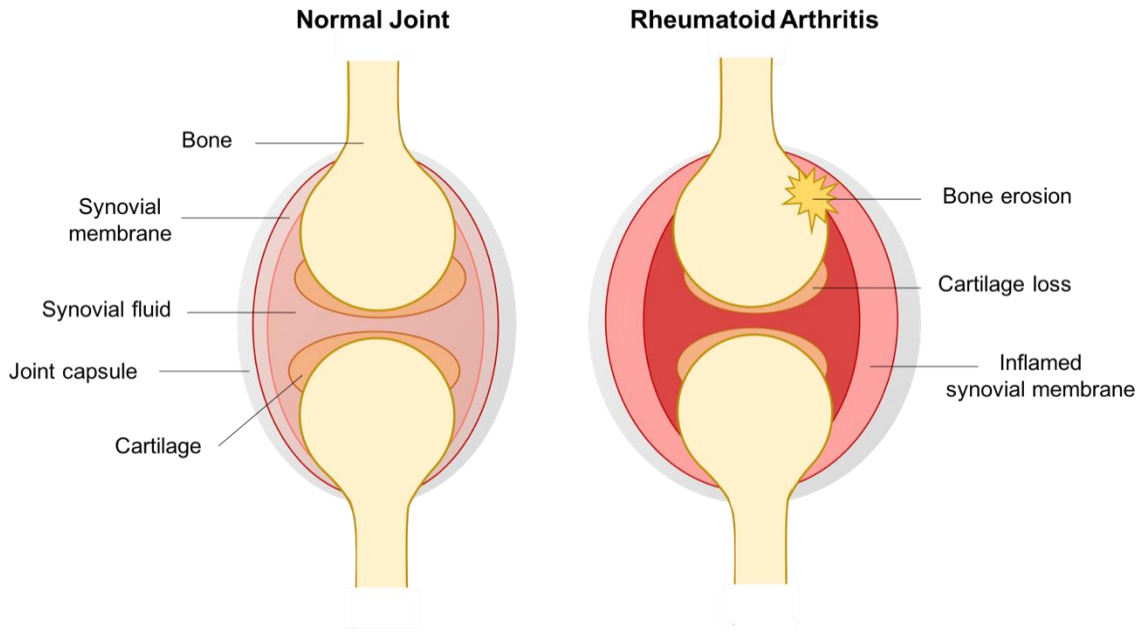

Figure VI. 1. Schematic illustration of a normal joint and a joint with RA.

Not excluding the important role of all the components involved in the RA pathogenesis, activated macrophages are key effector cells. They can contribute to the modulation of the immune response leading to autoimmunity. The autoimmunity reflects an imbalance between regulatory and effectors mechanisms, such as the defective elimination or the control of innate and adaptive responses, and the activation of cells with different subsets and phenotypes [279]. Macrophages are considered effector cells in the pathogenesis of RA, due to their higher incidence in the inflamed synovial membrane and at the cartilage pannus junction, posteriorly their activation status and the successful response to antirheumatic

therapy [280]. It is known that macrophages have the ability to exhibiting different and even opposing phenotypes, depending on their surrounding microenvironment [281]. There are two main phenotypes of macrophages activation status, namely M1 (classical, inflammatory) and M2 (alternative, anti-inflammatory) [282]. The M1 macrophages promote tissue inflammation and have high microbial activity, immune-stimulatory functions and tumor cytotoxicity. While the M2 macrophages are involved in the resolution of inflammation, wound repair and tumor promotion [281]. Activated macrophages produced several pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and transforming growth factor-β (TGF-β), chemokines, prostaglandins, metalloproteinases and reactive oxygen species (ROS), being hallmarks of RA disease [247,283].

VI.3. Treatment of rheumatoid arthritis

The RA therapy has evolved in the last years, allowing several patients to reach a remission phase or low disease activity, thus refining their life quality and limiting the late RA complications. An early diagnosis and treatment are prone to control the inflammation and limit the further tissue damage [269]. RA therapies can be cataloged into four classes: nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modifying anti-rheumatic drugs (DMARDs) and biological agents [284]. Most of these therapies need high doses for efficacy and are related with poor bioavailability, high clearance rate and inability to target inflamed joints (non-specificity), resulting in therapeutic effects weakness and increase of side effects [284,285]. NSAIDs are the most common drugs used to the management of RA due their involvement in antipyretic, analgesic and anti-inflammatory actions [286]. The administration of NSAIDs has several limitations because of their risk of gastrointestinal side effects, including perforation, obstruction and gastrointestinal hemorrhage [287] The adverse effects caused by NSAIDs also involve acute kidney ischemia, changes in blood pressure and increased bleeding [288]. Glucocorticoids are steroidal hormones with anti-inflammatory and immunosuppressive effects, which can be used single or in combination with DMARDs to moderate the advancement of RA symptoms. Prolonged use of glucocorticoids produce numerous side effects such as increased risks of cardiovascular diseases, osteoporosis, infections, hypertension, weight gain, fluid retention, and impaired glucose metabolism [289]. DMARDs are a class of drugs used for RA therapy that is effective in slowing down of the disease progression. These class of drugs do not have a similar action mechanism and the side effects are different depending on the drug used [290]. DMARDs administration by the intravenous or oral route induce toxicity to the immune system [291]. MTX, an antimetabolite-folate antagonist of DMARDs family,

has gained several attention among the rheumatologists, being considered the gold standard for the treatment of RA due to its outstanding effectiveness [292]. Biological agents are an emerging class in RA therapies. To date, mainly in adult with RA, some biologic agents targeting different mediators have been investigated in clinical trials [293]. In addition of high costs, one of the major drawbacks of biological agents is the occurrence of fungal and bacterial infections such as tuberculosis. It happens because this class of RA therapy suppress the immune response in patients and consequently the immune system fail [294].

Currently, there are an extensive variety of potential therapeutic agents for RA therapy, however, the response of these kinds of drugs by patients is between 50 and 70%. This can occurs due to the heterogeneous character of RA, the stage of the disease, and the presence of anti-drug antibodies [274]. Progress in knowledge about cellular targeting and molecular mediators of the inflammatory disease such as the mechanisms of RA leads to the development of new therapies have changed the overview of the scientific community about RA. The novel RA therapies should be easy to administer, deliver the drug in a controlled way, and preserve the desired drug concentration reducing the adverse effects [292]. A delivery system that targeting the drug specifically to the synovial cavity is observed to be more efficiently compared the drugs delivered systemically [295]. Nevertheless, most of the current RA therapies do not exhibit joint specificity [296].

VI.4. Role of methotrexate in rheumatoid arthritis therapy

In 1988, MTX has been approved by Food and Drug Administration (FDA) as the specific drug for the treatment of RA [297]. It is the most common used DMARD, being considered the first line therapy for patients with RA [210]. MTX can be used in monotherapy or in combination treatment with others DMARDs and biological agents. DMARD combinations are frequently considered the second line therapy when the MTX monotherapy unsuccessful [298].

There are multiple hypotheses to explain the complex mechanism of MTX efficacy in RA that contributes to their anti-inflammatory action. These mechanisms include (i) folate antagonism by the inhibition of purine and pyrimidine synthesis; (ii) adenosine signaling; (iii) ROS production, as a result of the increase in apoptosis of transformed T cells; (iv) decrease in adhesion molecules by decreasing chemotaxis and adhesion of inflammatory cells; (v) alteration of cytokine profiles inhibiting the production of pro-inflammatory cytokines and (vi) inhibition of polyamine synthesis [222]. Adenosine signaling is considered the most widely accepted approach for the explanation of the MTX action mechanism in RA.

MTX promote the over-expression of adenosine receptors on immune cells. Thus, the connection of adenosine with its extracellular receptors an intracellular cascade is activated promoting an overall anti-inflammatory state [210].

VI.4.1. Clinical pharmacokinetics of methotrexate

The research of the clinical pharmacokinetics of MTX can understanding the toxicity distribution and determine the adequate dose of the drug to be administered in virtue of optimize the therapeutic results [299]. Depending on the type and severity of disease, MTX can be administered in different doses. In the RA treatment and other rheumatic diseases, the dose of MTX administered in patients is relatively low [300]. MTX can be managed from the initial weekly dose of 7.5 – 10 mg to a weekly dose of about 30 mg, depending on the clinical response of the patient [301]. There are distinctive routes to administered MTX to patients, orally, subcutaneously or intramuscularly [302]. Thus, the bioavailability of MTX can be influenced by the route of MTX administration [303]. Usually, the bioavailability of MTX is measured in biological fluids by high performance liquid chromatography or fluorescence polarization immunoassay [304]. The pharmacokinetic properties of MTX can be unsatisfactory resulting in an inadequate clinical response. MTX bioavailability in different individuals was reported between 30% to 90% [305].

Regarding the distribution, approximately 46% of MTX binds to human serum albumin in plasma [303]. In this way, MTX is transported into the cells by two different processes: at low serum concentrations, MTX mainly enters through energy-dependent folate-receptor mediated, and at high serum concentrations enters cells through passive diffusion [306]. MTX can be metabolized into different metabolites, in the intestine, less than 5% is metabolized to 4-amino4-deoxy-N10-methylpteroic acid and in the liver, approximately 10% of MTX is metabolized to 7-hydroxy-methotrexate (7-OH-MTX). However, the principal metabolic pathway regarding the efficacy of MTX intracellular is the conversion to its polyglutamate form [307]. MTX is rapidly eliminated from the organism through the renal route. Around 80% of MTX (complete form) is excreted, and approximately 3% is excreted in its metabolite form, 7-OH-MTX [308]. The half-life of MTX is 8–15 hours and its derivates have a similar half-life, 7-OH-MTX of 10.2 hour and 4-amino4-deoxy-N10-methylpteroic acid of 9 hours [303].

VI.4.2. Adverse effects of methotrexate

MTX is considered the first line therapy in RA due its highly favorable cost effectiveness and efficacy/toxicity ratios. However, the drug toxicity is still a concern [309]. In some cases, MTX therapy needs to be discontinued due it unsatisfactory pharmacokinetic properties, resulting in insufficient clinical response and it high degree of toxicity creating an extensive list of adverse effects [266]. The more common side effects of MTX are gastrointestinal toxicities including nausea, stomatitis, vomiting, diarrhea and abdominal distress. Additionally, anemia, neutropenia, pulmonary fibrosis, dermatitis, bone marrow depression, mucositis, bruising, hepatitis are also reported [310]. Renal insufficiency can occurs and is caused by the accumulation of MTX or its metabolites in renal tubules [311]. MTX can also produce headache and drowsiness, itching, skin rash, dizziness, and hair loss [312].

The wide spectrum of adverse effects and the elevated frequency of occurrence led the researchers to explore the predictions to reduce it toxicity, while preserving at the same time the therapeutic efficacy of MTX [208]. Nanotechnology appears here as a promising approach for the treatment of several diseases by active and passive targeting. The effectiveness of the treatment is related with the capacity of a drug delivery system to target a specific cell population. In this way affecting the biological functions of ailing tissues and leaving minimal damage to healthy cells [313]. There are a lot of innovative delivery systems developed to improve the drawbacks of MTX therapy ranged from liposomes, microspheres, solid lipid nanoparticles, polymeric nanoparticles, dendrimers, polymeric micelles, carbon nanotubes, magnetic nanoparticles, and gold nanoparticles [266]. These delivery systems are establishing to targeting the drug to the inflamed site, reducing the amount of drug and adverse effect [314]. In this review, the focus are the liposomes encapsulating MTX to direct application in RA therapy.

VI.5. Liposomes encapsulating methotrexate to rheumatoid arthritis therapy

Despite all efforts by researchers to establish one treatment for RA, the effective cure is yet to be founded. The most current RA therapies do not have joint major specificity. Thus, the main challenge is to delivery efficient drug concentration to the affected area by the development of approaches that specifically target the drugs to the inflamed joints [315], such as liposomes. Liposomes are described as a colloidal spherical structure formed by self-assembly of phospholipids molecules in solution [5]. They have the ability to encapsulate and delivery drugs with different solubility. Hydrophilic drugs in the internal

aqueous core, hydrophobic drugs into the lipid bilayer and amphiphilic drugs at the water/lipid bilayer interface [7]. Due to their structural versatility as well as their biocompatibility, biodegradability, non-toxic and non-immunogenicity nature, liposomes are considered a powerful delivery system [9]. Liposomes as a drug delivery system have enhanced the treatment of diverse biomedical applications by stabilizing therapeutic drugs, overcoming obstacles to cellular and tissue uptake, and improving bio-distribution of drugs to target sites *in vivo* [12,13]. The drug inside liposome is protected against physiologically occurring events, such as chemical and immunologic inactivation, enzymatic degradation, and fast plasma clearance, leading to increase and extension of its action. Since the drug is encapsulated into liposome occurs the minimization of its exposure of healthy cells, thus reducing the adverse side effects compared with the free drug form [8].

The application of liposomes as drug delivery system in RA therapy can be a good strategy due its ability to use as delivery and targeting for the administration of the drugs at lower doses, thus reducing the toxicity of the drug [296]. Liposomes have proven to retaining the drug in the synovial cavity by benefit of their size and chemical composition [316]. There are diverse types of liposomes with different characteristics that depends of the liposomal components and even the production method. Small liposomes are suitable for passive targeting, large liposomes show enhanced retention and PEGylated liposomes also improve the liposomal circulation time by reducing the uptake by the liver and spleen [317].

MTX as an effective drug for RA therapy is currently formulated in several drug delivery systems [292]. As MTX is considered a low-permeability drug, it is predictable that it pharmacokinetics properties could be enhanced by it encapsulation in liposomes [310]. In this way, numerous studies in literature pronounced the liposomes as a gold drug delivery system to overcome some of the limitations caused by free MTX in RA treatment. The main goals of the use of liposomes to encapsulate MTX are the improvement of pharmacokinetics and drug efficacy, circulation time in blood, controlled release and therapeutic index and overcome drug resistance [318]. Figure VI.2 intends to show an example of liposomes encapsulating MTX in RA therapy, administrated by the intra-articular route.

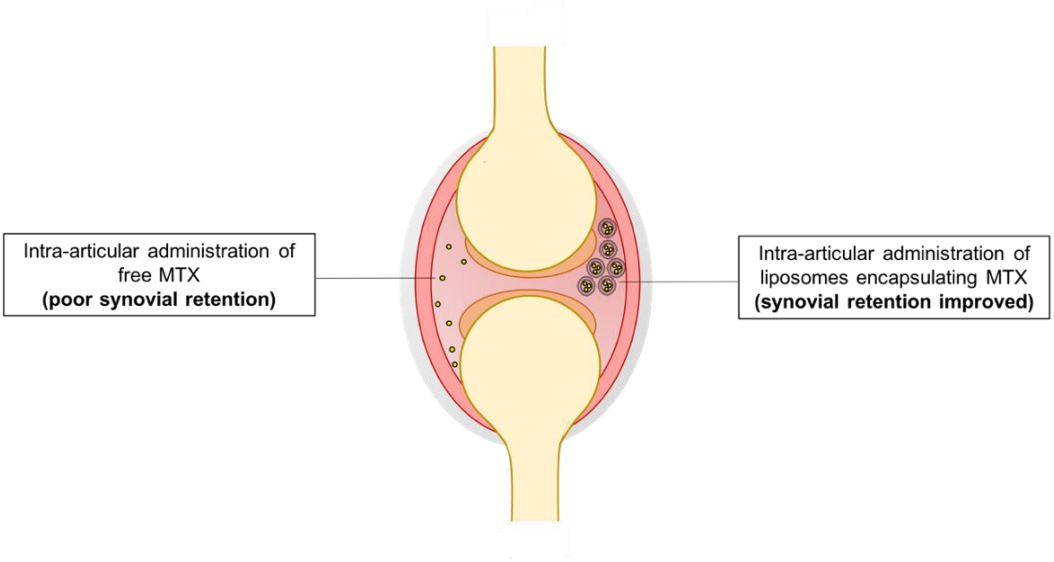

Figure VI. 2. Basis for the use of liposomes encapsulating MTX in RA therapy, via intra-articular route.

Over the last years, several authors reported the impact of liposomes containing MTX as a possible approach to use in RA therapy. Foong and Green showed that one liposomal formulation with MTX have great potential to suppress the development of arthritis. Results revealed that in antigen-induced arthritic rabbits, the joint swelling and rise in temperature were suppressed. Decrease in synovial hyperplasia, cellular infiltration, and cartilage erosion was also observed [319].

William *et al.* developed liposomes encapsulating MTX, comparing its efficacy with the free MTX. From the obtained results it can be showed that the liposomes have better results than the free MTX, revealing a significant effect on established arthritis [320]. In another study reported by this author it is demonstrated that multilamellar liposomes containing MTX have a significant anti-inflammatory effect compared to free MTX in Lewis rats with antigen-induced arthritis. These liposomes proved to inhibit the cellular infiltration associated with arthritis [321]. William *et al.* also reported conventional and long-circulation liposomes as carriers for the MTX delivery. Their data indicated that both formulations have potential for development into therapeutic modalities for the treatment of inflammatory joint [322]. Posteriorly, they observed that a single intra-articular injection of liposomes with MTX show to significantly reduced knee swelling as compared to free MTX in antigen-induced arthritis in rats. The treatment also inhibited the progression of antigen-induced arthritis [323].

Prabhu *et al.* found that liposomes incorporated MTX, administered by intravenous route, selectively targeting the arthritic lesions, reducing the toxicity to other organs. Results showed a pronounced reduction in edema volume in the rat group with the administration of stealth liposomes and

chitosan-coated conventional liposomes comparing to the control and standard (free MTX) group of rats [312]. A study reported by Bârcă *et al.* evaluated the toxicity of liposomes encapsulated MTX in comparison with a solution of MTX injectable in a murine model of arthritis. Results of the haematological and biochemical tests showed the reduction of toxicity in treatment with liposomes compared to MTX injectable treatment [324]. A study described by Gottschalk *et al.* demonstrated that MTX encapsulated into cationic liposomes have several advantages in contrast to generic and free MTX, reveling higher efficacy in anti-inflammatory and anti-angiogenic abilities [325]. Chen *et al.* designed a multifunctional liposomal formulation containing MTX. These PEGylated liposomes showed prolonged blood circulation time, enhanced accumulation of MTX in inflamed joints of CIA mice model, reinforced therapeutic efficacy and minimal toxicity toward major organs [240].

VI.5.1. Targeting the folate receptor

As noted above, macrophages play a critical role in inflammation development by promoting the interaction with the inflammatory microenvironment [326]. Numerous studies revealed that the inflamed joints of RA accumulate a subpopulation of macrophages expressing a receptor for the folate [233]. It was also observed that in RA, the changes in the number of synovial macrophages and the expression of inflammatory products reflect the therapeutic efficacy [327]. Tacking this into account, the potential of activated macrophages as a novel therapeutic target in autoimmune disease become a focus of considerable interest in the field of RA therapy.

Only a few cell types express the FRβ, so FA-targeted therapies can selectively attack the pathologic cell population, leaving the non-activated macrophages unharmed. Furthermore, since no other population of leucocytes appears to express a functional FRβ, the level of toxicity associated with FA-targeted therapy appears to be very low. Thus, in the search of joint specificity, the application of FR-targeting liposomes could be a noble selective approach for the diagnosis and therapeutic treatment of RA being a activated macrophage-mediated inflammatory disease [328]. FR-targeting liposomes with FA, covalently attached via a PEG linker to a phospholipid or CH anchor and incorporated into the bilayer during liposome preparation, were previously reported [329–331]. Lee and Low were the first to report the synthesis of FA conjugated liposomes [332]. They have demonstrated that a lengthy spacer, based on PEG, was required between FA and the lipid anchor to enable effective FR-mediated tumor cell targeting of the liposomes [333]. Two lipophilic derivatives have been synthesized for liposome targeting: FA-PEG-DSPE and FA-PEG-CH. Although these FA conjugates have been shown to effectively target FR-expressing tumor cells, there are concerns over the two negative charges carried by FA-PEG-DSPE and

the use of a carbamate linker in FA-PEG-CH, which has limited hydrolytic stability. Furthermore, there are concerns over the tendency for the FA moiety to self-aggregate on the surface of the liposomes, resulting in reduced FR targeting efficiency [334].

Taking this into account, Nogueira *et al*. developed an innovative strategy for targeted liposomal delivery that uses a hydrophobic fragment of surfactant protein D (SP-D) conjugated to a linker and FA, SP-DS3 peptide [153]. The peptide conjugate inserts deeply into the lipid bilayer without affecting liposomal integrity, being highly stable and specific. This delivery system was proven to be more efficient (2-fold) in FR overexpressing cells than classic systems where the FA moiety is linked to liposomes by PEG [153]. FA-targeting liposomes can be used to treat inflammatory disorders such as RA, targeting the specific population of activated macrophages [128]. Later, Nogueira *et al*. prove the ability of this liposomal formulation encapsulating MTX in an arthritic mice model, before disease onset. These liposomes encapsulating MTX are bioavailable *in vivo* resulting in a circulating steady-state low concentration in the body for longer periods. *In vivo* results showed that arthritic mice receiving MTX loaded liposomes have a stronger accumulation of the liposome at inflammation sites with improved clinical scores compared to mice receiving unformulated MTX drug. A complete prophylactic efficacy was observed in treated mice, since they did not show any clinical signs of arthritis. Taken together, these results demonstrate that the use of liposomes with the novel bifunctional SP-DS3 peptide linker for FA-mediated delivery represent a powerful drug delivery system for the treatment of collagen-induced arthritis (CIA) in mice [159].

Exploiting the potential of the liposomal formulation previous described by Nogueira *et al*., a set of deeper studies were recently performed using the liposomes encapsulating MTX, to complete the nonclinical package in CIA mice and to reach the implementation of the First-in-Human (FiH) clinical trial (unpublished data). Firstly, the establishment of the better drug-to-lipid ratio (D/L ratio) and the best dose to achieve therapeutic efficacy were evaluated. Results revealed that 0.15 is the better D/L ratio, exhibiting a more effective therapeutic effect. The best dose to achieve therapeutic efficacy was observed using the MTX dosage of 2 mg/kg twice a week (Figure VI.3) (unpublished data).

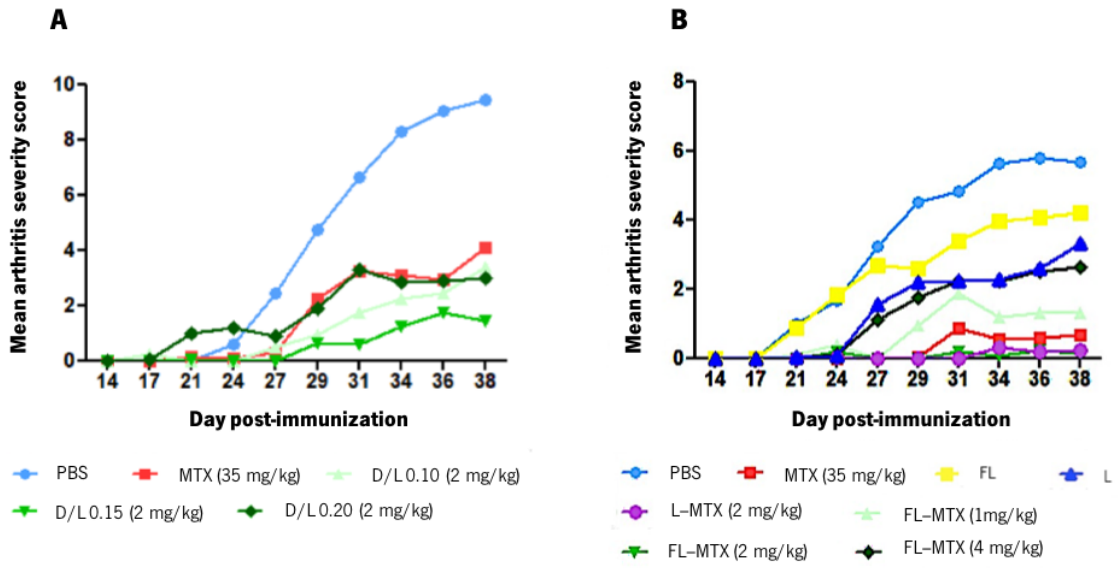

Figure VI. 3. Effect of the better D/L ratio and the best dose to achieve therapeutic efficacy of FL–MTX in CIA mice. **(A)** Average clinical score per groups as a function of time regarding the influence of D/L ratio. All treatments were injected at 2 mg/kg twice a week. **(B)** Mean arthritis severity among groups by the influence of MTX dose. Efficacy were assessed after twice a week injection of free soluble form of MTX and encapsulated MTX in folate targeted (FL–MTX) or non-targeted (L–MTX) liposomes. Empty targeted or non-targeted liposomes (FL or L) and phosphate buffered saline (PBS) were used as controls.

Furthermore, the biodistribution pattern of liposomes was evaluated by nuclear imaging in non-targeted organs and in the intended target – the arthritic paws. Results showed accumulation of FL–MTX in inflamed joints as soon as 30 minutes post injection (Figure VI.4) and remaining stable over time up to 72h after injection. FA-targeting of liposomes showed to induce changes in the pharmacokinetics of MTX, resulting in a large increase of the MTX circulation time (unpublished data).

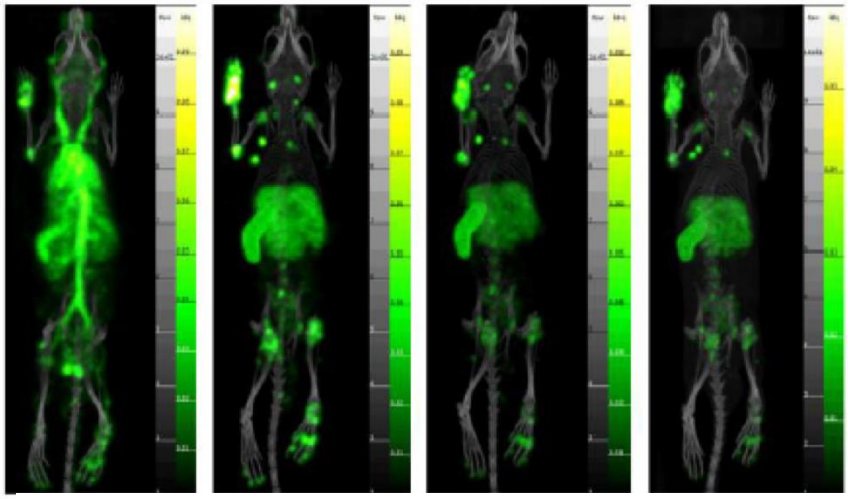

Figure VI. 4. Pharmacokinetics of liposomes encapsulating MTX assessed by nuclear imaging of FL–MTX. Please note at 30 minutes the early targeting of inflamed joints and the strong signal from vessels. Targeting in inflamed joints remains very stable over time in inflamed joints.

As observed from the examples above, the development of liposomes encapsulating MTX can be the future for the design of different and novel therapies to chronic inflammatory diseases such as RA. Liposomes can allow a decrease of the MTX dosage which inherently can improve the tolerance of RA patients to MTX side effects that commonly requires the adding or changing to more expensive biological DMARDs.

VI.5.2. Preparation of liposomes encapsulating methotrexate

The currently methods to prepare liposomes encapsulating MTX include mainly the lipid film hydration method [159,312] and the ethanol injection method [238]. To reduce the liposomal size and to obtain uniform liposomes, usually extrusion process or sonication should be applied [187,312]. As liposomes have the ability to incorporate drugs with different solubility, MTX can be encapsulated into liposomes as the hydrophobic form and its disodium salt form, the hydrophilic MTX. MTX encapsulation into liposomes has been succeeded by passive loading method, which encapsulates the drug during liposome formation or in a phase of production when the liposomal structure is extremely fluid [208]. However, the passive encapsulation of the hydrophilic drugs as hydrophilic MTX results in lower EE, since

the MTX retention is controlled by their solubility and the size of the aqueous internal core of liposomes [35].

The preparation of liposomes encapsulating MTX to RA or even other diseases has been reported by numerous authors. Prabhu *et al*. produced PEGylated liposomes of MTX by thin-film hydration method. Briefly, lipids were dissolved in a reduced amount of chloroform:methanol mixture (2:1) in a round bottom flask. Then, the flask was rotated at 60 rpm while immersed in a water bath to obtain a thin dry lipid film. The dry lipid film was hydrated accomplished by adding the MTX in PBS buffer forming the multilamellar vesicle suspension. These vesicles were subjected to ultra-probe sonication producing small unilamellar vesicle. After sonication, to produce a homogenous suspension, they used a 0.22 μm syringe filter. Liposomes obtained present an average size of 210 nm, with a polydispersity index in the range of 0.2-0.3 and an encapsulation efficiency of 23% - 31% [312].

Hu *et al*. proposed the production of PEGylated liposomes encapsulating MTX by another liposomal method, the ethanol injection. The liposomal components were dissolved in absolute ethanol (4.8 mL) and then mixed with 15.2 mL MTX solution in saline solution at 60 °C. Posteriorly, the formed liposomes were extruded through sequential filters to reduce liposomal size and obtain uniform liposomes. Depending of the main phospholipid used, liposomes present an average size ≤110 nm, a polydispersity index < 0.9 and a maximum encapsulation efficiency of 6.4% [335].

Recently, our group purposed a novel passive loading method to increase the encapsulation of MTX in PEGylated liposomes. Based on the principles of the ethanol injection method was developed a pre-concentration method that allows achieves liposomes with adequate physico-chemical characteristics to *in vitro* and *in vivo* tests, without using the extrusion process [238]. Results showed that the adjustments in the ratio of organic:aqueous phase permitted the control of the initial lipid and MTX concentration in the suspension with considerable impact in the final physicochemical characteristics of liposomes. The pre-concentration method requests for the use of an initial organic:aqueous ratio of 1:1 (v/v) and 20% of the aqueous volume, being the remaining 80% added after ethanol evaporation (Figure VI.5).

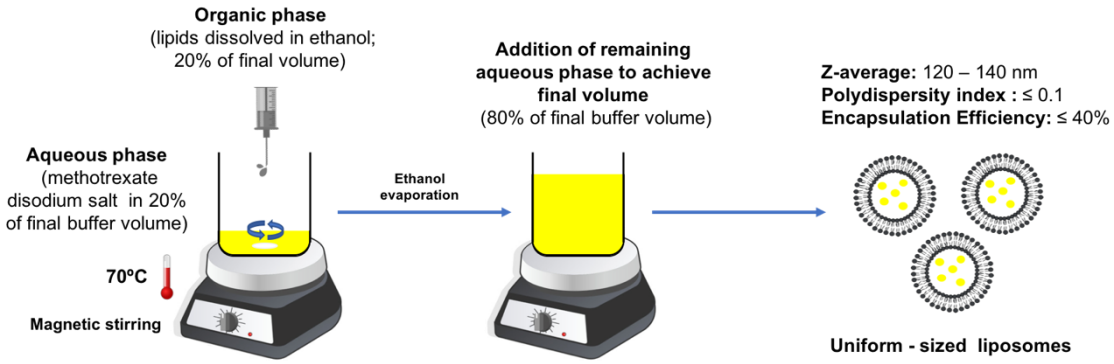

Figure VI.5. Schematic illustration of the pre-concentration method to prepare liposomes encapsulating MTX.

The initial reduction of the aqueous volume outside the liposomes may promote the interaction between MTX and lipids, highlighting the main lipid of the liposomal formulation, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Thus, the great interaction of MTX at the liposomal surface bilayer with DOPE appears to be the cause for the good results obtained in this study. Furthermore, it was possible to prove the biological benefit of the liposomes produced by this novel method in a mice model [238]. The developed approach shows to be a significant advance in MTX therapeutic applications.

VI.5.3. Methods for liposomal methotrexate quantification

The precise quantification of the drugs encapsulated in liposomes is an important step for the application of liposomes as a drug delivery system [336]. The immediate result of the preparation of liposomes encapsulated drugs is a mixture of encapsulated and free drug. Thus, it is necessary to achieve the separation of free drug that can be carried out by size exclusion chromatography, ultracentrifugation, extensive dialysis, ultrafiltration, gel filtration or centrifugal ultrafiltration [337]. Specifically, to separate free MTX from nanoparticles encapsulated MTX can be used for example a gel filtration chromatography column [159,178], ultrafiltration [335] and ultracentrifugation [240].

After the process of free drug separation, MTX quantification can be performed using standard analytical techniques, being the most common methods the ultraviolet-visible (UV–Vis) spectroscopy [338] and high performance liquid chromatography (HPLC) [240,265,339]. Hence, several researchers reported the separation and quantification methods of MTX encapsulated in liposomes and nanoparticles.

Chen *et al.* separated free MTX from liposomes by ultracentrifugation. The supernatant with free MTX was collected and the MTX quantification determined by HPLC system using a UV detector at 302 nm [240]. Prabhu *et al.* used centrifugation approaches to separated free MTX from liposomes and to achieve a liposomal pellet. A solution of sodium hydroxide was added to the liposomal pellet and after that, methanol was added to this suspension to completely lysed the liposomes and release the encapsulated MTX. This solution was diluted in methanol and the absorbance was determined using a UV-Vis spectrophotometer at a wavelength of 303 nm [312].

Despite the benefits of the methods reported above, occasionally some issues can occur in drug quantification inside nanoparticles which can create doubt if the quantification is correct. An inefficient drug quantification may occur due to possible interactions among components and sometimes a large dilution factor can be used to be possible the analyte signal in the equipment. Additionally, both methods assume that the drug absorption is in the UV–Vis range. These factors reveals uncertainty in drug quantification and decrease the precision and the accuracy of the determined drug content [336].

Tacking this into account, to overcome the possibility of the occurrence of these problems, our group developed a novel method to quantify drugs encapsulated in liposomes, exploiting the advantages of ^{1}H Nuclear Magnetic Resonance (NMR) spectrometry and using pyridine as an internal standard [178]. Among others, MTX was chosen as drug model. A comparative study was performed to validate the results obtained by ^{1}H NMR method, using the traditional techniques, already mentioned, UV–Vis spectroscopy and HPLC. Results showed that no significantly differences in MTX concentration were observed [178]. The procedure offers some advantages including great reproducibility, automation, and quantification without identical standard material. NMR proves to be an absolute quantification method and an excellent choice for quantification of MTX concentration in liposomes.

VI.5.4. Stability of liposomes encapsulating methotrexate

The use of liposomes for therapeutic applications continues to be a challenge due to their inherent physical and chemical instability for long-term storage [340]. Thus, the liposomal instability remains one of the main translational drawbacks for the development of liposomal formulations for therapeutic applications. Some of these problems includes the phospholipids oxidation, liposome aggregation or fusion, lack of re-dispersibility, and drug leakage [341].

Among the others possible methods, freeze-drying (i.e., lyophilization) appears as a usual approach to overcome these issues and ensure the long-term stability of liposomes. However, the freeze-

drying process itself is documented to can change the physical structure of liposomes, resulting from their fusion or aggregation. The encapsulated drugs can also release from the liposomes, leading to variability in formulation stability and reconstitution reliability [173,342]. To improve the functional properties and stability of the liposomes after the freeze-drying process, the inclusion of cryo and lyoprotectants in the liposomal formulation can be a good approach [343]. Therefore, numerous stabilizers are approved by FDA for pharmaceutical use, even in marketed drugs [344,345]. Since they have the ability to act as a protectant of the membrane integrity, carbohydrates, in particular the saccharides, are the desirable cryo/lyoprotectants used during dehydration/rehydration cycles of liposomal formulations [175,346].

To our knowledge, a few studies address the freeze-drying process in liposomes encapsulating MTX, reflecting the challenge of the scope. To achieve a highly stable liposomal formulation encapsulating drugs, specific aspects must be optimized, such as the type and concentration of saccharide. Sarbolouki and Toliat reported the use of different amounts of cryoprotectants to stabilize the liposomes. Results showed that a mixture of trehalose and PEG 20000 results in an MTX retention of about 70% after freeze-drying. They also performed stability tests of the freeze-drying liposomes at different temperatures and demonstrated that liposomes were stable even at 37 °C, highlighting their potential use in commercial applications [347].

Our group studied the protective role of five saccharides at different concentrations on the stability and drug retention capacity of the previously developed liposomal formulation encapsulating MTX [153], when subjected to a freeze-drying process. Sucrose, in a concentration dependent manner (8:1 sugar:lipids mass ratio) showed to be an appropriate cryo/lyoprotectant to protecting the integrity of the freeze-dried liposomes encapsulating MTX. However, it was revealed that in spite of these liposomes have high physical stability, maintaining their narrow and monodisperse character, higher MTX leakages were observed (≈60%) [340]. The stability of the liposomes without freeze-drying process was evaluated over time at 4 °C for 12 weeks, showing to be very stable without changes in size and polydispersity index. During this time, the MTX leakage remain insignificant. We believe that the long-term storage of this liposomal formulation in its liquid form brings more advantages compared to developed freeze-drying liposomes with MTX. Nonetheless, this procedure of freeze-drying has worked for other kinds of drugs, including the tamoxifen, a hydrophobic drug. Drug leakages may seems to be dependent of the drug location in liposomes [340]. It is already known that the drug leakage during the freeze-drying, behind the choice of an appropriate cryo/lyoprotectants, is essentially conditioned by the formulation factors, including the liposome composition, the nature of the drug [64].

VI.6. Conclusion

In the last years, the progress in knowledge about RA mechanisms and the development of new therapies have changed the overview of scientific community about RA. Despite all efforts, to implement new therapeutic applications for RA, the side-effects continue. To found and develop a new drug to RA therapy is a work extremely meticulous and can take many years. Therefore, the reuse of drugs already known will be the easier mission. MTX is the drug more used and the first choice for the treatment of RA by reduction of the inflammation level and prevent joint erosion. However, MTX intolerance and other adverse reactions can occur, leading to the finished of the MTX treatment. Thus, the key aim of RA therapy can go through the development of liposomal formulations with more advantages for RA patients, thought the decreased of side effects, improved of cost-benefit ratio and increased of life quality. Liposomes as a drug delivery system overcome some of the drawbacks associated with the conventional dosage form by the delivery of rheumatic drugs directly to the inflamed tissues. The current review focused on liposomes encapsulating MTX as a potential strategy for RA therapy. Several studies reveal the MTX into liposomes as an innovative therapeutic approach due their ability to accumulate at the select sites, vigorous biological response, increased blood circulation time and an overall safety. In summary, liposomes represent a powerful system for MTX delivery, opening new and reliable opportunities for RA therapy.

CHAPTER VII

General discussion and Future perspectives

VII.1. General discussion

Liposomes have been extensive attention as drug delivery system for numerous kinds of drugs. The direct application of liposomes in medicine encourages the researchers to create novel liposomes for treatments in a wide range of diseases and in a variety of therapeutic applications. The modulation of the *in vivo* drug behavior and the reduction of the drug toxicity in the organism are the crucial features to design a suitable liposomal formulation. The development and improvement of liposomes are a complex challenge that involves the simultaneous optimization of several characteristics to achieve a final liposomal formulation safe and effective. Taking this into account, this PhD work emphasis on liposomal characteristics (reviewed in chapter I) such as the production method, drug quantification technique, liposome storage and even the biological efficacy of liposomes *in vivo*.

The present PhD work was based on the potential of a liposomal formulation encapsulating methotrexate (MTX) previous developed in our research group, for the treatment of rheumatoid arthritis (RA). In the last years, the progress in knowledge about RA mechanisms and the development of new therapies have changed the overview of scientific community about RA. Despite all efforts, to implement new therapeutic applications for RA, the side-effects continue. Currently, it is important the development of liposomal formulations with more advantages for RA patients, thought the decreased of side effects, improved of cost-benefit ratio and increased of life quality.

In 1988, MTX has been approved by FDA as the specific drug for the treatment of RA [297]. However, in some cases, MTX therapy needs to be discontinued due it unsatisfactory pharmacokinetic properties, resulting in insufficient clinical response and it high degree of toxicity creating an extensive list of adverse effects [266]. The development of liposomes encapsulating MTX can be the future for the design of different and novel therapies to RA. With this in mind, our research group has been focused on the development of liposomes encapsulating MTX for application in the treatment of RA. The developed liposomes have an innovative strategy for targeting the folate receptor (FR)β, overexpressed in activated macrophages, key effector cells in RA. The liposomes use a hydrophobic peptide conjugated to folate (folic acid, FA), the SP-DS3 peptide, a targeting ligand that show high ability to bind the FR. The peptide conjugate inserts deeply into the lipid bilayer without affecting liposomal integrity, being highly stable and specific. This delivery system was proven to be more efficient (2-fold) in FR overexpressing cells than classic systems where the FA moiety is linked to liposomes by polyethylene glycol (PEG) [153]. These liposomes as have the FA at the surface specifically target FRα-cancer cells [348] and FRβ-activated macrophages [234,349]. These liposomes encapsulating MTX are bioavailable *in vivo* resulting in a

circulating steady-state low concentration in the body for longer periods. *In vivo* results showed that arthritic mice receiving MTX loaded liposomes have a stronger accumulation of the liposome at inflammation sites with improved clinical scores compared to mice receiving unformulated MTX drug [159]. Taken together, these results demonstrate that the use of liposomes with the novel bifunctional SP-DS3 peptide linker for FA-mediated delivery represent a powerful drug delivery system for the treatment of collagen-induced arthritis (CIA) in mice. Furthermore, it opens new opportunities for the treatment of human diseases, including chronic inflammatory diseases such as rheumatoid arthritis (RA) and cancer [153,159].

These results pave the way for further clinical designs which encouraged our research group to develop the European project H2020 FOLSMART (NMP-06-2015-683356, 2015-2020). This project aimed the good manufacturing practice (GMP) production and *in vivo* validation of the new liposomal formulation, as well as further application on the non-clinical development and phase I clinical trials. The work developed in my PhD is inserted in the FOLSMART research plan, seeking to reply to all the demanding requirements of this complex European project.

A liposomal formulation product consists in three crucial components, lipids to form a liposome, molecules to functionalized them and a drug molecule that will be encapsulated. An efficient quantification of the drug molecule encapsulated in liposomes is one of the most important features in the field of liposomes characterization. To investigate the efficiency of encapsulation, the free drug should be first removed from the initial liposomal suspension, applying a suitable technique of separation. The process of drug quantification continues with the use of a method to estimate the drug concentration. Traditional techniques include spectrophotometry, fluorescence spectroscopy, and chromatographic methods as high-performance liquid chromatography, ultra-performance liquid chromatography, among others. The selection of an adequate and specific method depends on the characteristics of the liposomal components and the encapsulated drug. Chapter II reports a rapid and easy method for quantification of drugs encapsulated in liposomes, exploiting the advantages of ^{1}H Nuclear Magnetic Resonance (NMR) spectrometry. The pyridine was used as an internal standard to determine the concentration of two different drugs, one hydrophilic, MTX disodium salt, and another hydrophobic, tamoxifen (TAM), encapsulated in the developed non-targeted liposomal formulation. The developed quantification method proves to be a proper tool, independently of the drug properties. A comparative study was also performed to validate the results obtained by ^{1}H NMR method, using two traditional techniques, spectrophotometry and chromatography. Results demonstrated that values obtained with these techniques were consistent with the ones obtained from our proposed quantitative NMR method, and not significantly differences in

drug concentration were observed. This methodology presents some advantages such as great reproducibility, automation, and quantification without identical standard material. The implementation of this method for quantification of drugs encapsulated in liposomes can also be extended to other delivery systems.

It is already known that liposomes as drug delivery system have massive benefits, but their correct and efficient long-term storage continues to be an issue, due to their inherent physical and chemical instability. Freeze-drying (i.e., lyophilization) appears as a promising approach to overcome these problems and ensure the long-term stability of liposomes. However, this approach itself can leads to physical changes in liposomes, including aggregation and drug leakage. The inclusion of a cryo and lyoprotectant in the liposomal formulation can provide a protective effect to liposomes. Several chemical compounds are considered effective cryo and lyoprotectants. The most used are carbohydrates, more specific the saccharides, since they can act as integrity membrane protectants, during dehydration/rehydration of liposomes in freeze-drying process. Chapter III mentions a study that involve the effect of five saccharides (trehalose, lactose, glucose, mannitol and sucrose) at different concentrations on preserving the stability in the developed non-targeted liposomal formulation, when subjected to a freeze-drying process. From all the saccharides tested, only sucrose, in a concentration dependent manner (8:1 sugar:lipids mass ratio) presented ability to protect the empty liposomes of fusion or/and aggregation. Therefore, liposomal formulation containing sucrose at 8:1 mass ratio, was studied in terms of morphology, concentration and drugs retention ability.

Depending on their solubility, drugs can be encapsulated into different compartments of the liposomal structure. Hydrophilic drugs in the aqueous core and hydrophobic drugs within the liposomal bilayer. This work involved three different drugs. MTX disodium salt and doxorubicin (DOX), drugs encapsulated in the aqueous core, and one drug located in the liposomal bilayer, TAM. Results showed that after the freeze-drying process, liposomes with sucrose encapsulating drugs revealed high physical stability, maintaining their narrow and monodisperse character. However, leakage of the drugs encapsulated in the aqueous core, MTX and DOX, was observed, independently of the liposomal preparation method used (passive and active loading, respectively). Otherwise, no significant drug leakage was detected on liposomes containing a drug located in the lipid bilayer, TAM, preserving its biological activity after the freeze-drying process. These findings reveal that sucrose seems to be a good candidate for the cryo/lyoprotection of the developed liposomal formulation with drugs located in the lipid bilayer.

The widespread use of liposomes for therapeutic applications created the need to develop different preparation methods which should be reproducible, efficient, fast and even simple to scale-up. There are different methods of liposomes preparation with numerous variants. The most common production method is the lipid thin-film hydration method. However, this method has concerns about the use of chlorinated solvents. Lipid film method also tend to be unsuitable for large scale production, that is a requirement for FOLSMART project. Therefore, the ethanol injection method was chosen as an alternative safer and up-scaling to produce liposomes encapsulating MTX for further *in vivo* studies. In the ethanol injection method, a part of the aqueous solution incorporating the MTX (hydrophilic drug in disodium salt form) is passively encapsulated inside the formed liposomes. That corresponds to encapsulation of drugs during the liposome formation. Although the several advantages of this method, only a very small percentage of the drug can be encapsulated, resulting in a low level of MTX encapsulation. Indeed, a high amount of drug is wasted and this could be an issue in a future scale-up process. Chapter IV presents a novel strategy (pre-concentration method) to encapsulate MTX into liposomes based on the principles of the ethanol injection method (conventional method) to increase encapsulation efficiency (EE) of MTX in liposomes and to obtain small values of size and PDI.

During both processes, an organic phase, constituted by the lipids dissolved in ethanol, is rapidly injected into an aqueous phase containing the MTX, occurring the liposome formation. The modifications in the ratio of organic:aqueous phase regulates the initial lipid and MTX concentration in the suspension and consequently influence the final physicochemical features of liposomes. Therefore, comparing both methods, the main difference is the initial aqueous volume and the ratio of organic:aqueous phase. In the conventional method a total aqueous volume is used since the beginning of the process, as well 1:5 organic:aqueous ratio (v/v), reaching a very low encapsulation of MTX. The pre-concentration method calls for the use of an initial 1:1 organic:aqueous ratio (v/v) and 20% of the aqueous volume, being the remaining 80% added after ethanol evaporation. This modification allows the production of liposomes with suitable size distribution and higher MTX encapsulation. We suppose that the initial reduction of the aqueous volume outside the liposomes promote the interaction between MTX and lipids, highlighting the main lipid, DOPE.

The great potential of MTX to interact at the liposomal surface bilayer was shown by NMR studies. This experiment explores the mutual interactions between MTX and the main phospholipid via hydrogen bonding. Liposomes produced with two independent lipid source, DOPE and EPC, were tested. The results suggest that an interaction between MTX and DOPE occurs, while the same interaction do not happen when is used EPC. The non-covalent bond observed could explain the increase in the EE when DOPE is

used as main phospholipid, comparing with EPC. NMR studies were also used to verify that the DOPE-MTX interaction is based on the terminal amino acid moiety of MTX, using L-aspartic acid. The amino acid was N-protected with an aromatic group (naphthyl) to mimic the terminal structure of MTX. The interaction between DOPE and *N*-protected L-aspartic acid presume that the interaction drug-lipid is based on the terminal amino-acid moiety of MTX and occurs via hydrogen bond, increasing the EE of MTX. The biological benefit of the liposomes encapsulating MTX produced by the novel method was proved in a mice model of arthritis, in a similar way to those produced by the conventional method.

Justified by the results described, a patent was submitted to the Portuguese Patent Office on 07 May 2019 (PT-20191000025346), which was extended as Patent Cooperation Treaty (PCT) patents (PCT Extension Application Number: PCT/IB2020/054346). On 12 November 2020 the international patent was published, under the international publication number WO/2020/225769. The present disclosure relates to a method for production of liposomes to obtain high encapsulation efficiency of encapsulated agents, including MTX.

Following up the needs of the FOLSMART project to perform the pre-clinical development and phase I clinical trials, was necessary to scale-up the production of liposomes in order to increase the volume of the produced liposomal formulation. A pilot line was designed and build-up in order to reply to all the demanding requirements of GMP manufacturing. Scale-up studies were carried out using the pilot unit to produce liposomes with the same final characteristics of liposomes produced in small-scale.

The FOLSMART European project propose to improve the treatment in RA therapy, intending to perform the translation of the laboratory-based medication into Phase I clinical trials. The project involves the evaluation of the folate-targeted liposomal formulation in animal models, preclinical toxicology tests and initial trials in humans. Chapter V reports a set of deeper experiments to complete the nonclinical package in CIA mice, performed at INSERM (*Institut National de la Santé et de la Recherche Médicale,* Paris, France). The establishment of the better drug-to-lipid (D/L) ratio in liposomes encapsulating MTX and the dosage needed to obtain a therapeutic effect in CIA mice were evaluated. Results showed that 0.15 is the better D/L ratio, exhibiting a negligible weight loss in mice and the more effective therapeutic effect. The best dose to achieve therapeutic efficacy was observed using the MTX dosage of 2 mg/kg twice a week. Furthermore, the biodistribution pattern of liposomes was evaluated by nuclear imaging in non-targeted organs and in the intended target – the arthritic paws. This experiment reveals that liposomes tends to accumulate in inflamed joints and it is dependent of the paw swelling and bone remodeling activity. Folate-targeted liposomes showed to induce modifications in pharmacokinetics of

MTX, resulting in a large increase of the MTX circulation time. Regarding, the impact of the FA-targeting on MTX distribution to non-target organs, a higher concentration was found in the liver and kidneys, whereas no differences could be detected in the serum between non-targeted and targeted liposomes. To finished the nonclinical studies performed in CIA mice, subcutaneous injection of liposomes was tested and validated, since it is the more convenient and simpler route of administration for patient's self-administration.

The non-clinical Good Laboratory Practice (GLP) studies (safety pharmacology and toxicology) were conducted by the contract research organization Aptuit (Verona, Italy). In first instance, dose range finding studies were carried out in the Sprague Dawley rat and the Beagle dog, with a liposomal formulation produced by us, in the developed pilot unit located at our research laboratory. Subsequently, major toxicological studies were carried out under GLP conditions with the objective to evaluate toxicity and toxicokinetics of the liposomal formulation in the same two species. These liposomes were produced by the pharmaceutical company Bluepharma (Coimbra, Portugal) with the pilot unit developed by us, following the GMP guidelines. The phase I clinical trials will determine the tolerability and safety in humans and identify the doses to be studied in the next phases of the clinical study. In this moment, due to force major derived from the COVID-19 pandemic, the phase I clinical trials are on standby. The trial will be led when possible by Blueclinical (Porto, Portugal), a private and independent clinical research organization devoted to the conduct and management of clinical studies.

The findings reported in this PhD work intends to give an optimistic contribution in the life quality of RA patients towards the optimization of production and physicochemical and biological characterization of a promising MTX delivery system for RA treatment (reviewed in chapter VI). FA-liposomes encapsulating MTX compared to free MTX treatment, can be used as a successful delivery system with increased benefits for patients with RA, including the reduction of the MTX dosage and reduction of the main side effects. Furthermore, the liposomes reported here can be a good drug delivery platform for FR-expressing diseases, highlighting the oncologic field.

VII.2. Future perspectives

The results presented in this thesis provides a clear overview about the importance of a suitable drug delivery system in therapeutic applications. The success of previous results on the application of the optimized FA-targeted liposomal formulation in RA therapy open news opportunities as the possibility of its application for other therapeutic areas. Based on the great potential for active targeting via FR, it is intended the future application of this functionalized liposomal formulation on cancer therapy. Taking this into account, we performed some preliminary experiments in view of the direct application of these liposomes with FA-peptide to cancer therapy. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females [350]. In this way, MCF-7 cells, a breast cancer FR-positive cell line, was used to assess the *in vitro* uptake specificity of folate-targeted (FL) liposomes integrating SP-DS3 peptide. Liposomal formulations were prepared with a fluorescent labelled, the fluorescein isothiocyanate (FITC), to be detected in the flow cytometer. Based on collected data (Figure VII.1), it is possible to observe that FR-expressing MCF-7 cells internalized more FL liposomes compared to liposomes non-targeted. This may occur due the fact of FL liposomes has the FA on their surface, and it is known that the FA specific binds to FRs, promoting the higher internalization of FL liposomes by MCF-7 cells as a FR-expressing cell line. These results were corroborated by a competitive study, using a high-affinity FR substrate via supplementation the medium with FA. When the FR was preliminarily blocked with the FA, no significant differences of the fluorescence intensity signal were observed in cell internalization with non-targeted liposomes. However, it is possible to detect a decrease in the signal of fluorescence intensity with FL liposomes, because the supplementation of medium with FA leads to a previous saturation of FRs in FR-expressing MCF-7 cells and consequently less FL liposomes were internalized.

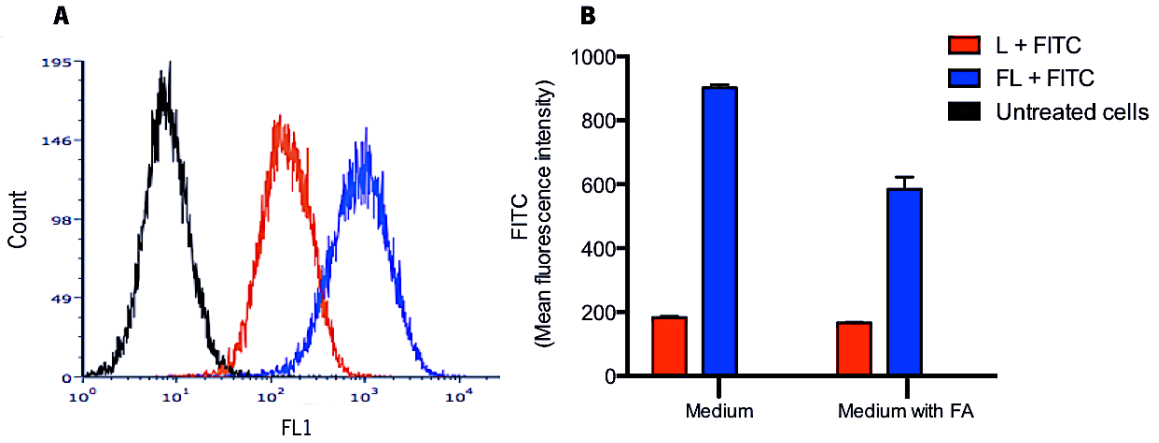

Figure VII. 1. Cellular uptake and fluorescence quantitative analysis of FITC labelling liposomal formulations in MCF-7 cells. **(A)** Flow cytometry profile. **(B)** Fluorescence quantitative analysis by incubation of liposomal formulation with or without the supplementation of FA in the medium at 37 °C for 30 minutes. Values are the mean + SD of 2 independent experiments.

The efficacy of the liposomal formulation was assessed using DOX, an anti-cancer chemotherapy drug routinely used in the treatment of several cancers, including breast [351]. To evaluate the cytotoxicity of liposomal formulations encapsulating DOX in MCF-7 cells the MTS assay was used. This assay corresponds to a colorimetric sensitive quantification of viable cells in proliferation. The viability of MCF-7 cells after 48 hours of incubation with liposomal formulations containing different concentrations of DOX was determined. Liposomal formulations empty did not induce toxicity at the wide range of concentrations tested [153]. Results showed that free DOX and encapsulated in liposomal formulations significantly reduced the viability of MCF-7 cells (Figure VII.2) in a similar form.

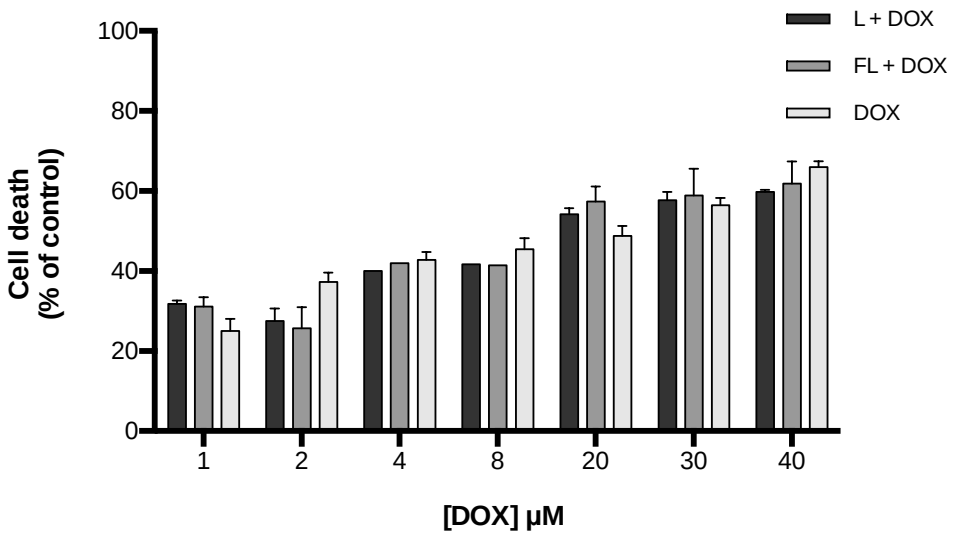

Figure VII. 2. Cell death of breast cancer cell line, MCF-7 cells after 48 hours of incubation with liposomal formulations at different DOX concentrations, compared with untreated cells (negative control), determined by the MTS assay. Values are the mean + SD of 2 independent experiments.

Recent developments in the field of drug delivery emphasize the development of a creative systems that simultaneously shows more than one useful targeting in the same system. Functionalized liposomes with two or more different ligands at the liposomal surface appear to be a promising approach for cancer therapy, taking advantage of the tumor aggressiveness. The design of novel targeted anticancer strategies must consider not only the tumor aggressiveness but also the cross-talk between cancer cells and other cells from the tumor microenvironment, such as endothelial cells. Therefore, targeting angiogenesis can improve the clinical efficacy, as tumor growth and metastases formation are angiogenesis-dependent [352]. The F3 peptide is a specific ligand for nucleolin, a shuttle protein that traffics between cell membrane and nucleus. Nucleolin is over-expressed on the surface of cancer cells and endothelial cells of tumor angiogenic blood vessels, but only exist in the nucleus among normal cells [353], offering the possibility to make a dual-targeting strategy toward the tumor microenvironment. As the developed FA-liposomes represent a good platform for cancer therapy, to further experiments it is planned the development of a dual-targeting system, adding the F3 peptide to the previous liposomal system. The study aims at assessing the therapeutic impact of targeting two different populations within the tumor microenvironment: cancer cells, through FA and F3 peptide ligands, and endothelial cells from angiogenic blood vessels, through F3 peptide ligand. FA/F3-targeted liposomes can also open new clinical

avenues for diagnosis in both diseases, as well as in other human diseases where their targeting receptors are also overexpressed.

The liposomes studied in this PhD thesis intend to provide a highly engineered liposomal formulation with much more health benefits to patients undergoing painful or incapacitate diseases. These liposomes can contribute to treatments with key performance, hence it shall lead to a better clinical outcome, lower toxicity levels and fewer side effects.

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