UNIVERSITY OF BIRMINGHAM

# ACTIVE TARGETING TO HEPATOCYTES: HOW IMPORTANT IS OPTIMISING THE LIPOSOMES' PHYSICOCHEMICAL PROPERTIES AND SURFACE MODIFICATION FOR TARGETING THE HEPATOCYTES.

By

MAHA ABDULAZIZ ALSUNBUL

A thesis submitted to the University of Birmingham for the degree of
DOCTOR OF PHYLOSOPHY

Institute of clinical sciences
College of Medical and Dental Sciences
School of Pharmacy
University of Birmingham
August 2021

**Abstract**

The ability to selectively target drugs to their site of action is an increasingly important aspect in medicine. Using drug delivery systems and introducing active targeting strategies could maximise the accumulation of a drug to the desired site, while lowering its toxicity. Nanosized-drug carriers have been suggested to achieve this goal, and liposomes are one of the most studied nanocarriers. However, exploiting liposomes and other nanocarriers for delivery to the hepatocytes remains challenging, despite liver disease representing some of the most common diseases globally. In this work, different approaches have been taken to identify the best strategy for targeting the hepatocytes and to study the impact of composition on the physicochemical properties of liposomes.

First, a systematic review of the literature on hepatocyte targeting was conducted. Two databases (Medline and Embase) were searched for published primary research over the past 5 years. The initial search identified 6460 articles, out of which 142 were eligible for full-text screening and 25 were included in the review based on pre-defined inclusion/exclusion criteria. The impact of the liposomes size, PEGylation and active targeting on liver accumulation was probed using *in vitro* cellular uptake, and *in vivo* and clinical pharmacokinetics data. Overall, the results showed that a liposome size between 100-200 nm and using a ligand such as galactose, with a density no more than 20 mol% associated with higher cellular uptake and better pharmacokinetics profile. Adding PEG increases the circulation time but did not enhance the cellular uptake. However, using a dual modification of the liposome's surface with PEG and galactose appeared to be a promising strategy to target the hepatocytes.

Secondly, three neutral liposomal formulations (100-200 nm) made from phosphatidylcholine derivatives (DSPC, soybean (SPC) and hydrogenated soybean (HSPC)) and cholesterol, using thin film hydration method. Initially, the aim was to test the impact of composition on liposome accumulation in different liver cells, but due to the pandemic, the full aim was not achieved.

Still, the liposomes were evaluated in terms of size, zeta potential and membrane fluidity. Preliminary studies using SPC liposomes, suggested that a phospholipid: cholesterol ratio of 70:30 produced the best formulations; this ratio was then selected for all liposome formulations. Overall, liposomes size was below 200 nm with a narrow distribution index (PDI <0.3 a.u.). The zeta potential was within the range for neutral liposomes (-10 to 10 mV). Surface modification was performed using 2 or 5 mol% PEG (neutral, cationic, anionic). In general, PEGylation led to an increase in size, which was statistically significant for SPC liposomes (p-value<0.01). As expected, the surface charge was reduced compared to non-PEGylated vesicles. PEGylation efficiency varied between (22-85%), with DSPC liposomes achieving the highest efficiencies (85% when 2 mol% PEG was used). Initial PEG loading also affected PEGylation efficiency, with higher loadings achieved when 2 mol% PEG was used. Membrane permeability studies performed using calcein as a hydrophilic probe, showed temperature- and PEGylation-dependent patterns for SPC and HSPC liposomes (p-value<0.05). Finally, lyophilisation was performed on SPC liposomes using 150 mM sucrose using pre-and post-insertion method. In both cases, liposome size was maintained, but the post-insertion resulted in better homogeneity. Altogether, this study confirmed that composition must adjusted carefully when formulating liposomes, as this can affect the ease with which the surface can be modified and the release rate of an encapsulated compound.

**Dedication**

This thesis is dedicated to the best mother ever (Shaikhah), who retired from her job to support me during the whole of my scholarship period. I am speechless and cannot express my thanks towards you. I owe you.

To the soul of my lovely father (Abdulaziz), who believed in me, but passed away before the end of my Ph.D. I am almost there and I wish if you were with me.

To my small family, my great husband (Abdullah) and my beautiful son (Faisal) for being my positive energy during my Ph.D. Thank you for your love, understanding, and encouragement. I am grateful to have you in my life. I love you.

To my twin (Ghaidaa) and my lovely brothers (Saad and Abdullah) for their encouragements and inspiration. I am proud to have you in my life.

To my friends (Dena and Nourah) for being close and supportive all the time even when we are far away.

**Acknowledgment**

I would like to thank my supervise Dr. Marie-Christine Jones to give me the chance to undergo her supervision for my Ph.D. journey. I appreciate her unlimited support, caring, and understanding during all of these years.

Many thanks for my lab and office colleagues for their help and support. I would like to thank my friend Hanouf for her support for some software I used. A huge thank you for my friend Ruba for being close all the time and for her help, advice and for being the second reviewer for the systematic review work.

A huge appreciation for my lovely mother for being supportive during my whole scholarship period and for the soul of my father who believed in me, but passed away before the end of my Ph.D.

A huge appreciation for my great husband for the unlimited support, love, caring, understanding, and encouragement, and for my son for being my positive energy during this time.

This appreciation is also for all of my family members including my sister and my brother for their continuous encouragement and inspiration.

Many thanks for my government (Saudi Arabia) represented by higher education ministry and my academic institute (Princess Nourah bint Abdulrahman University) for their funding and supportive efforts to success my Ph.D. degree.

**Table of Contents**

## List of tables

## List of figures

**List of abbreviation**

| Abbreviation | Full term |
|---|---|
| ABC | Accelerated blood clearance |
| API | Active pharmaceutical ingredient |
| ASGP | Asiloglycoprotien |
| AUC | Area under the curve |
| Chol | Cholesterol |
| CL | Clearance |
| CU | Cellular uptake |
| 11-DAG-3-O-Gal | 3-Galactosidase-30-stearyl deoxyglycyrrhetinic acid |
| DiO | 3- ,3′-Dioctadecyloxacarbocyanine |
| DLS | Dynamic light scattering |
| DMPG | Dimyristoyl phosphatidylglycerol |
| DOPE | 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine |
| DSPC | Distearoylphosphatidylcholine |
| EDTA | Ethylenediaminetetraacetic acid |
| EE | Encapsulation efficiency |
| ELS | Electrophoretic Light Scattering |
| FDA | Food and drug administration |
| FI | Fluorescence intensity |
| Gal | Galactose |
| GalNAc | Galactose N-acetylgalactosamine |
| HC | Hepatocytes |
| HCC | Hepatocytes carcinoma |
| HFF | hydrodynamic flow focusing methods |
| HSPC | Hydrogenated Soybean phosphatidylcholine |
| ID | Injected dose |
| IA | Injected radioactivity |
| KC | Kupffer cells. |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LUV | Large unilamellar vessels |
| mGy/MBq | Organ absorbed dose |
| Mins | Minutes |
| Man-DLD-Chol | Mannose-diesterlauric diacid-cholesterol |
| MLV | Multi-lamellar vesicles |
| MPS | Mononuclear phagocytes system |
| NMR | Nuclear magnetic resonance |
| NP | Nanoparticles |
| PDI | Polydispersity index |
| PEG | Polyethylene glycol |
| PK | Pharmacokinetics |
| RCT | Randomised control trial |
| RES | Reticuloendothelial system |
| REV | Reverse phase evaporation |
| SAXS | small angle X-Ray scattering |
| SHM | staggered herringbone mixer |

| | |
|---|---|
| SPC | Soybean phosphatidylcholine |
| SR | Systematic review |
| SUVs | Small unilamellar vesicles. |
| Tc | Transition temperature |
| TES | N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid |
| THF | Thin film hydration |
| T1/2 | Half-life time. |
| Vd | Volume of distribution |
| ZP | Zeta potential |

**COVID-19 Statement**

Due to the spared of Corona virus (COVID-19) early 2020, there was a disruption of the research work. This starts with a university and lab closure on 18-3-2020 until further notification. During this time, there was an increase in the panic worldwide, and I decide to go back home and continue working from there. Labs continued being closed until August. In order compensate the incomplete lab work, me and my supervisor tried to find a way by finding a topic and a research question to start working on a systematic review. This is a new field for me and requires time and effort to do it on the right way. Many trial and error has been undertaken, until we agreed on a topic. Then, I started collecting the information and writing. I needed to change the research methodology as the borders in Saudi Arabia were still closed and travelling from or to Saudi Arabia is banded. By the end of the year, a new law was introduced to allow the student to go back to their studies after having a permission. I applied for a permission many times, but none of the attempts was accepted until the beginning of 2021.This means approximately one year of my lab work was not counted in this work, and working on a different field requires a knowledge, effort, and time. During this pandemic, I got pregnant, and I welcome my baby in March 2021. This is my first time to become a mother, and experiencing a motherhood for the first time, taking care of a baby, and managing your work is not easy. In addition, two weeks before my submission, me, my husband, and my four months old son tested positive for COVID-19 test. These all-cumulative factors influence my research work and leads to a huge disruption.

# Chapter 1: Literature review

## 1.1. Introduction

### 1.1.1. Liposomes (general overview)

Liposomes can be defined as small spherical vesicles and are obtained from the self-assembly of phospholipids in water (Akbarzadeh et al., 2013). Depending on the number of bilayers formed and the preparation method, the size of a liposome can vary from 30 nm to > 1 μm (Figure 1.1.).

(A) (B)

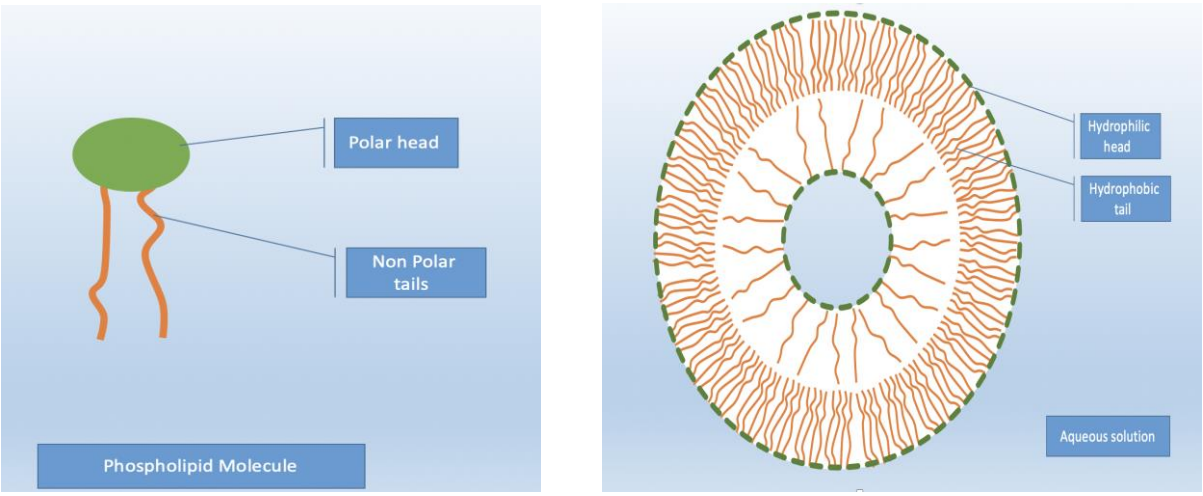
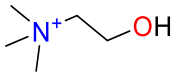
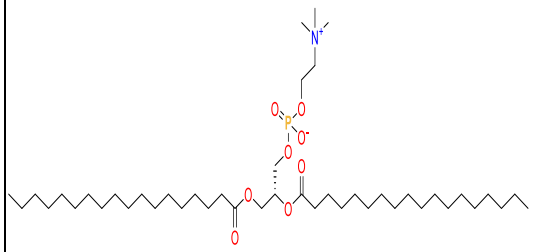
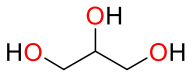
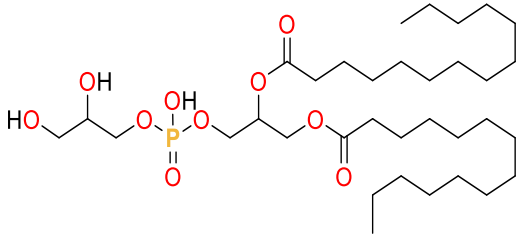


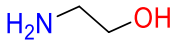
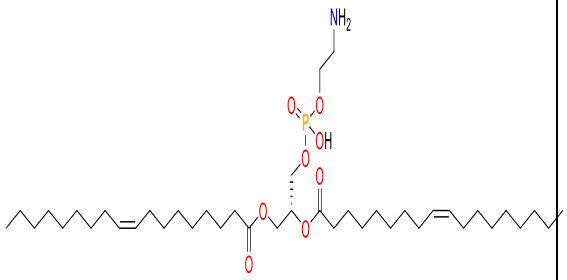

**Figure 1.1.Phospholipid (A) and liposome structure (B).**

### 1.1.2. Phospholipids composition and phase transition

As mentioned above, liposomes are formed from the self-assembly of phospholipids in water. Phospholipids, the main constituent of the cell membrane, are amphiphilic molecules where two alkyl chains are linked to a polar head via a phosphate group. The alkyl chains can have different lengths, although chains with 16 to 18 carbons (Racey et al., 1989) are the most common for liposome preparation. Both saturated and unsaturated chains can be used, which will affect liposome properties as discussed later. Depending on the nature of the polar head, phospholipids can have a neutral (zwitterion), positive or negative charge (Table 1.1.)

Table 1.1.Phospholipid classification based on the surface charge.

| Type | Source of surface Charge | Example | Properties | References |
|---|---|---|---|---|
| Neutral | Choline.  | Distearoylphosphatidylcholine (DSPC).  | • Do not interact with the cell significantly, leading to drug release extracellularly. | • (Bozzuto and Molinari, 2015).<br>• (Zhao et al., 2011). |
| Anionic | Glycerol, serine, inositol, and hydrogen.  | Dimyristoyl phosphatidylglycerol (DMPG).  | • Associated with some toxic effects e.g. pseudo-allergy when injected systematically.<br>• Used commonly with transdermal DDS due to their rapid penetration property. | • (Bozzuto and Molinari, 2015)<br>• (Campbell, 1983). |

| | | | | |
|---|---|---|---|---|
| Cationic | Phosphatidylethanolamine.<br>  | 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE).<br>  | • A carrier for marketed drug such as Doxorubicin, Paclitaxel and Oxaliplatin.<br>• Used commonly in gene therapy.<br>• Can cross BBB. | • (Bozzuto and Molinari, 2015).<br>• (Koynovaetal, 2008). |

Phospholipids can be derived either from natural or synthetic sources (Li et al., 2015). Natural phospholipids can be obtained either from vegetables (e.g., soybean, cotton seeds and sunflower), or from animal sources (e.g. lecithin from egg yolk) (Li et al., 2015; Singh, Gangadharappa and Mruthunjauya, 2017). Furthermore, various synthetic phospholipids have been manufactured for example1,2-Dimyristoyl-*sn*-glycero-3-phosphorylcholine (DMPC), 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) (van Hoogevest and Wendel, 2014). Because synthetic phospholipids require long and sophisticated production and purification processes, semi-synthetic phospholipids have been introduced (van Hoogevest and Wendel, 2014). There are several reported methods for producing semi-synthetic phospholipids throughout a modification of the tail, head or both of natural phospholipids (van Hoogevest and Wendel, 2014). Current commercial liposome formulations have been prepared from both natural and synthetic phospholipids; for example, soybean phosphatidylcholine (18 °C) and dimyristoyl phosphatidylcholine (DMPC) (14 C) were incorporated in liposomal amphotericin B and daunorubicin formulations respectively (Bulbake et al., 2017; Hamill, 2013; Warmack and Gubbins, 2010).

Along with phospholipids, cholesterol is commonly added to liposome formulations at molar ratios up to 50% of total lipids. Cholesterol is generally added to provide better control of the membrane rigidity (Perrie, 2013). Cholesterol prevents lipid aggregation at low temperature, and increase their stability at higher temperatures when cholesterol concentration is above 20% (Briuglia et al., 2015). The most commonly molar ratio used for liposomes preparation is 70: 30 (phospholipid: cholesterol) molar ratio (Briuglia et al., 2015). It is suggested to use at least 30% cholesterol with the formulation can be beneficial throughout reducing the leakage of the drug from liposomes, and decreasing the interaction between liposomes and plasma proteins (Perrie, 2013).

Lipid composition plays a crucial role in determining the bilayer's structure which ultimately influences liposome properties (Perrie, 2013). The transition temperature (Tc) is an important characteristic to consider when formulating liposomes (Ikeda et al., 2010). It is a unique feature for each phospholipid and is defined as the temperature at which the lipid closed pack structure convert into a random liquid crystalline phase (Figure 1.2.) (Anderson and Omri, 2004; Eze, 1991). The value of the Tc varies from –18 to 67 C and is affected by the properties of the alkyl chains; for instance, the increase chain length will increase Tc simultaneously, and this will lead to an increase the membrane rigidity, improving the stability and drug entrapment efficiency (Ikeda et al., 2010).

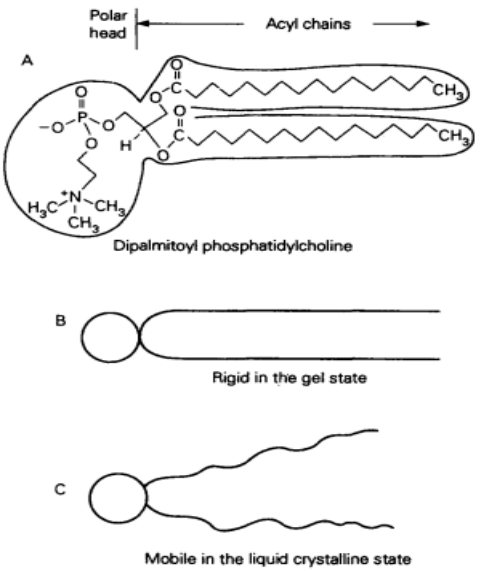



**Figure .1.2.Illustration of the influence of the Tc on the phospholipid state. A structure of the phospholipid (A), lipid structure in gel state for instance below Tc (B), The crystalline state of the phospholipid at Tc (C). Adopted from (EZE, 1991).**

### 1.1.3. Liposomes preparation

Four conventional methods have been reported for liposome preparations: thin film hydration, reverse phase evaporation (REV), solvent injection and detergent dialysis. Out of these, thin film hydration and REV are probably the most common. All these techniques involve four basic steps, beginning with dissolving the lipid (±cholesterol) in a volatile organic solvent (often methanol or chloroform) followed by solvent evaporation. The dried lipids are then hydrated

with water or an isotonic solution. The methods differ only in the way lipids are dried and rehydrated (Fendler and Romero, 2011; Laouini et al., 2012).

### 1.1.3.1.Thin film hydration

Thin film hydration method is considered the original method and still and simplest. This method was proposed by Bangham in the early 60$^{th}$ century (Laouini et al., 2012). A schematic diagram of this process is illustrated below (Figure 1.3).

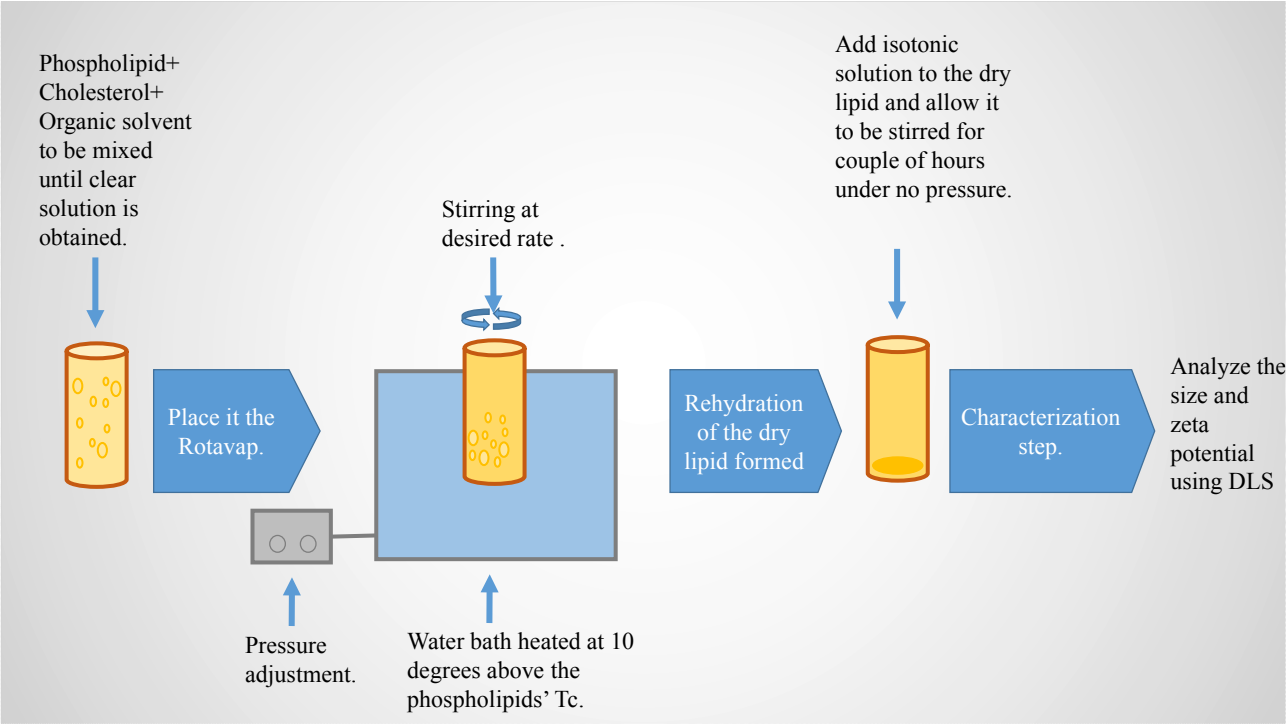



**Figure 1.3.Thin film hydration method.**

This method usually yields multilamellar liposomes (MLVs) which have large size and shape heterogeneity (Laouini et al., 2012). To reduce the size and gain a more uniform profile, two size reduction techniques were suggested: sonication and extrusion. Sonication can be done either with a probe or ultrasound bath and is used for small unilamellar vesicles (SUV) formation (Ong et al., 2016). Similarly, extrusion over polycarbonate filters produces SUV or LUV, depending on the membrane used (Berger et al., 2001). An example of a drug formulated by thin film hydration is ibuprofen (Mohammed et al., 2004). The thin film method has the

advantage of the ease of handling with the limitation of having low drug encapsulation efficiency (Laouini et al., 2012).

#### 1.1.3.2.Reverse phase evaporation (REV)

The REV method has the same framework of the previously mentioned method with the exception that the lipids are dispersed in an organic solvent which is mostly either diethyl or isopropyl ether emulsified within an aqueous buffer; both solvents are highly volatile and can be removed easily under the rotary evaporator (Andra et al., 2022; Cortesi, 1999). When the organic solvent is then removed from the system under reduced pressure, LUV liposomes would be formed. (Akbarzadeh et al., 2013; Laouini et al., 2012). This approach has the advantage of high encapsulation efficiency as vesicles have 30 times more aqueous volume to lipid ratio compared with the hydration method (Laouini et al., 2012). This is due to that, the mixture undergoes solvent removal under rotary evaporator, and leading to the conversion of the micelles to a viscous or gel which give this method an advantage of having large aqueous volume (Andra et al., 2022). In addition, the immiscibility between the water and organic solvents provides a high space between the water and lipid in which the drug can be encapsulated (Akbarzahed et al., 2013). Gomez et al., (2019) studied the effect of using different liposomal preparations (thin film hydration method, reverse phase evaporation method, and freezing and thawing method) on the %EE. In this study, different antibiotics namely (vancomycin hydrochloride, teicoplanin, and rifampin) were encapsulated in a DPPC liposome. The results showed the highest encapsulation efficiency was obtained when using REV method with an average of 90% compared to 50% and 33% for TFH and freeze and thawing method respectively (Gomez et al., 2019). Another study carried out by Shi and Qi (2021) to evaluate the encapsulation efficiency of different liposomes sizes prepared by REV method. The Adriamycin liposomes shared the same composition (PtdGro: PtdCho: Chol 1:4:5). The below table gives an overview of the main findings of the study (Table 1.2.).

**Table 1.2. The %EE for different liposome types and sizes using REV method.**

| Liposome type | Liposomes size (nm) | EE (%) |
|---|---|---|
| LUV | 200-1000 | 35-65 |
| MLV | 400-3500 | 5-15 |
| SUV | 20-50 | 0.5-1 |

### 1.1.3.3.Solvent injection and detergent dialysis

Solvent injection technique in which lipid dissolved either in ethanol or ether is injected in aqueous media to form liposomes. If using ethanol, as the solvent is miscible in water, the desired liposomes are gained directly upon ethanoic lipid injection. In comparison, ether is immiscible in water, and solvent removal is necessary in this case. This can be accomplished by heating the water above the boiling point of ether to allow its evaporation (Laouini et al., 2012). The solvent injection method generates SUV, with the advantage of a high entrapment level but a drawback of its complexity (Mohammed et al., 2004). Liposomal salidroside is an example of a drug formulated following this method (i Mohammed et al., 2004). Finally, the detergent dialysis method aimed to form homogenous unilamellar vesicles by mixing the lipid with a detergent followed by lipid removal by continuous dialysis (Kong et al., 2013). Although this method is easy and requires low cost materials, it showed some drawbacks including uncontrolled dialysis rate, the uncertainty of the obtained yield as the materials could be retained on the filter membrane, poor reproducibility and being time-consuming (Ollivon et al, 2000). Paclitaxel which is used to treat ovarian cancer was formulated using this technique (Crossaso et al, 2000).

As drug carriers, liposomes have advantages and drawbacks. On one hand, the attractive biological properties of these vectors include enhancement of drug stability and efficacy, selective targeting by the enhanced permeation and retention effect, ability for containment of both hydrophilic and hydrophobic active ingredients, commercial availability, biocompatibility, biodegradability, and lack of toxicity, (Hofheinz et al., 2005; Laouini et al., 2012). On the other hand, liposomes have a low water solubility profile, can be susceptible to

oxidation particularly ones with higher unsaturation degrees and probability of leakage of the loaded drug when formulated using for instance, thin film hydration method (Akbarzadeh et al., 2013; Sahoo and Labhasetwar, 2003). Nevertheless, the previously mentioned advantages and disadvantages can be balanced by carefully tailoring the physicochemical properties of the formulated liposome (Samad et al, 2007).

#### 1.1.3.4.Microfluidics for liposomes preparations in large scale

Although the previous reported methods can give a promising preliminary overview of the prepared liposomes, the produced batches are small and the needs forwards finding a robust methods for producing large quantities to meet the market needs is necessary. Microfluidics has been reported as a potential technique for achieving larger amount of liposomes with controlled size, PDI, and lamellarity (Carugo et al., 2016). The microfluidics system consists of three inputs (the central one is for the phospholipids, and the remaining two inputs are for the aqueous medium).

Liposome manufacture using microfluidics rely on mixing the organic solvent containing phospholipids with the aqueous phase which driven through the stream of the channels' groove. Afterwards, the mixing continues in the chamber and the lipid starts to assemble into liposomes (Shah et al., 2019). There are different methods for producing liposomes using microfluidic which are hydrodynamic flow focusing methods (HFF), micromixer, and emulsion templets (Zhang and Sun, 2021).

##### 1.1.3.4.1. Hydrodynamic flow focusing methods (HFF)

HFF was first proposed by Jahn et al., (2004). Herein, the organic solvent is mixed with the queues solvent until reaching the critical concentration. Then, the lipids self-assemble into liposomes spontaneously (Yu et al., 2009). This method has an advantage of producing batches with reproducible sizes. (Zhang and Sun, 2021). However, some limitations including lower encapsulation efficiency compared to the remote loading. In addition, limitations in the size and

the material to be generated. For example, partial removing of some solvents such as ethanol which by then can impact the stability. Furthermore, when aiming to formulate liposomes with a size smaller than 50 nm, the flow rate ratio should be 30 or higher (Zhang and Sun, 2021). An illustration of the microfluidic process is provided below (Figure 1.4.).

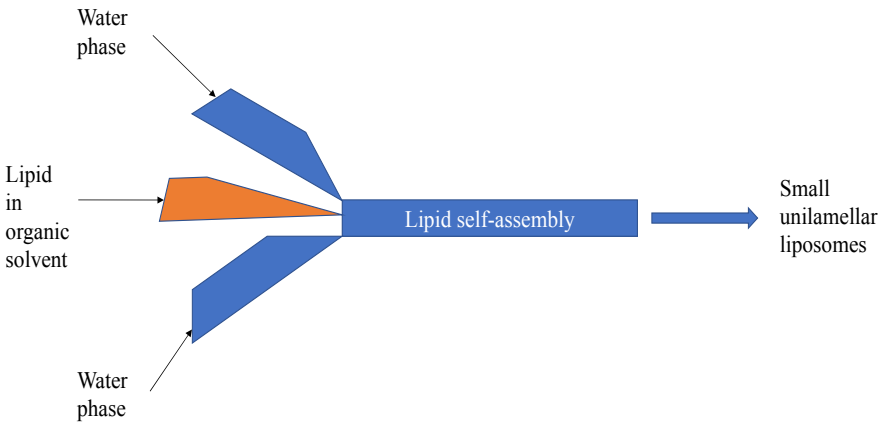



**Figure 1.4.An illustration diagram of Hydrodynamic flow focusing method process (Carugo et al., 2016).**

#### 1.1.3.4.2. Micromixer

Micromixer-based microfluidics are somewhat similar to HFF. It is a powerful method to produce small liposomes (<20 nm). The staggered herringbone mixer (SHM) used in this technique was designed by Stroock et al (2002), aiming to improve the liposomes scale-up process (Stroock et al., 2002). The mixing efficiency using SHM is improved due to the special zigzag-shaped champers involved in the system in which the materials passed and many mixing cycles are applied. An illustration of the of the process is provided below (Figure 1.5.).

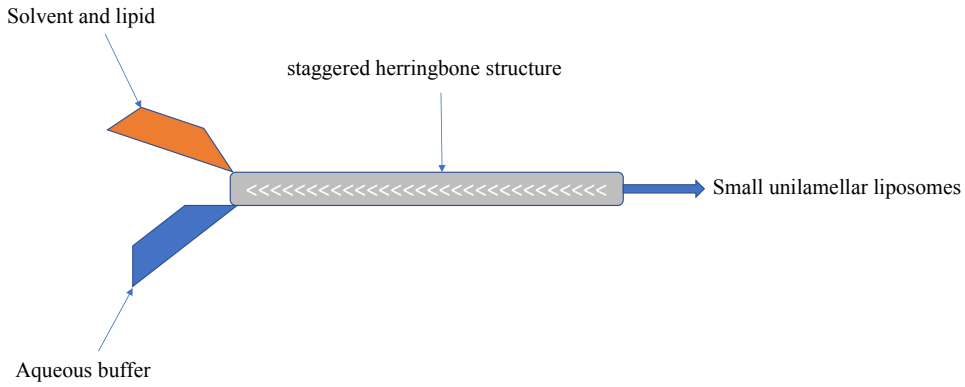



**Figure 1.5.An illustration diagram of the staggered herringbone mixer (SHM) (Carvalho et al., 2022).**

Both flow rate and number of cycles affect the quality of the product i.e., as increasing the flow rate and number of cycles associated with production of < 20 nm liposomes' size (Zhang and Sun, 2021). Although this technique requires a careful selection of the solvent, flow rate, number of cycles, lipid composition and concentration to ensure having the best results (Zhang and Sun, 2021), it provides a good encapsulation efficiency and they showed their excellence in the market for various marketed medications and vaccines including COVID-19 vaccine manufactured by Pfizer (Tenchov et al., 2021).

#### 1.1.3.4.3. Emulsion templates

In the emulsion template technique, a droplet with a defined size is mixed with immiscible liquids to form giant liposomes (>10 μm) (Zhang and Sun, 2021). This method developed by Weiss et al. (2018) aiming to encapsulate large biomaterials e.g. proteins and DNA (Weiss et al., 2018). It has the advantage of high encapsulation efficiency and ensuring the complete removal of the excess oil of the final product (Zhang and Sun, 2021). The drawback of this method is that it requires a special Pico injection device to inject the emulation inside the narrow microchannel which has a width of less than 2 μm (Zhang and Sun, 2021).

For all of the above microfluidics techniques, it is important to take care of the factors affecting the liposomes quality including the flow rate, lipid concentration, and the temperature

micromixer (Zhang and Sun, 2021). Zizzari et al., (2017) reported that lipid composition is an important factor in the determination of liposomes size. The researchers proved that there is a direct proportional relationship between the lipid concentration and the liposomes size i.e. as the concentration increases, the size increases too (Zizzari et al., 2017). In contrast, there is an inverse relationship between both flow rate and the applied temperature corresponding to the liposomes' size i.e. decreasing the flow rate associated with increasing the liposomes size (Zhang and Sun, 2021) and increasing the temperature associated with reducing the vesicles size (Sulkowsk et al., 2005).

### 1.1.4. Liposomes characterisation

Characterisation is required to assess the quality of prepared liposomes (Ruozi et al., 2011). As for most nanocarriers, this includes testing the main characteristics including size, zeta potential, lamellarity lipid analysis and stability. If used for drug loading, characterisation should include encapsulation efficiency, drug release (Laouini et al., 2012). As initially drug-free liposomes will be used in this project, this section will focus on physicochemical characterisation.

#### 1.1.4.1. Particle size analysis

Liposome size is an important factor that influences blood circulation half-life time and recognition by the mononuclear phagocytic system (MPS). MPS is a system which responsible for the clearance of foreign bodies and composed of monocytes and macrophages. RES can be found in blood, connective tissue, spleen, and bone marrow (Bozzuto and Molinari, 2015). The literature stated that small vesicles (< 200 nm) interact less with the RES compared to larger vesicles (Bozzuto and Molinari, 2015; Zalipsky et al., 1994). Several size analysis techniques are available for instance dynamic light scattering (DLS), field-flow fractionation (FFF), and atomic force microscopy (AFM) (Laouini et al., 2012). DLS is employed extensively for size measurements and works by measuring the fluctuation of the scattered light from the particles

which result from the collision between the suspended particles and the solvent over time (Edwards and Baeumner, 2006). The advantages of this technique include its ease of use and requiring small sample volumes (Edwards and Baeumner, 2006; Laouini et al., 2012). Disadvantages came from the lack of morphological analysis, and risk of gaining inaccurate readings if small quantities of high molecular weight compounds or impurities are present (Bozzuto and Molinari, 2015; Laouini et al., 2012).

#### 1.1.4.2. Zeta potential (surface charge)

Surface charge is measured as the zeta potential and is related to the stability of colloids in aqueous dispersions (Karmakar et al., 2012; Laouini et al., 2012). Charged particles tend not to aggregate as they repeal each other due to repulsive forces to maintain stability (Kanásová and Nesměrák, 2017). Zeta potential values that are greater than + 30 mV or less than -30 mV are needed to provide colloidal stability (Kanásová and Nesměrák, 2017; Laouini et al., 2012). However, a high positive charge can also be a problem because of toxicity and easy recognition by RES (Bozzuto and Molinari, 2015). Similarly, anionic liposomes can be cleared quickly because of the charge. Neutral liposomes may be stabilised by steric repulsion as will be discussed in the section on stealth liposomes. The zeta potential is measured by Electrophoretic Light Scattering (ELS). ELS is used to measure particle mobility in an electrical field; the movement of particles towards a specific electrode at a certain velocity determines the zeta potential (Laouini et al., 2012).

#### 1.1.4.3. Lamellarity

Lamellarity, i.e. a number of lipid bilayers, can be determined by spectrofluorimetry, NMR and small angle X-Ray scattering (SAXS) (Kanásová and Nesměrák, 2017; Mukherjee et al., 2004). Techniques involved in evaluating the number of bilayers are relatively simple (Edwards and Baeumner, 2006; Laouini et al., 2012). They rely on detecting the alterations of lipids'

fluorescent signals when reagents added and compare it with total signals without treatment (Edwards and Baeumner, 2006).

#### 1.1.4.4.Lipid analysis

Lipid analysis is used to quantify phospholipid content (Kanásová and Nesměrák, 2017; Laouini et al., 2012). Molybdate-containing reagents have been used extensively with some methods such as Bartlett, Ascorbic acid and Enzymatic assays for lipid analysis (Laouini et al., 2012). All of these methods are associated with the colorimetric detection of lipids after digestion, oxidation and/or hydrolysis. Also, chromatographic techniques, for instance, TLC and UV were used for separation and lipid composition evaluation (Kanásová and Nesměrák, 2017; Laouini et al., 2012).

#### 1.1.4.5.Colloidal stability

Stability is a vital principle in pharmaceutical manufacturing (Laouini et al., 2012). This includes monitoring liposomes stability physically, chemically and microbiologically (Laouini et al., 2012). From the physical perspective, this can be evaluated visually or through size measurements (Laouini et al., 2012; Mohammed et al., 2006). From a chemical point of view, phospholipids are susceptible to oxidation reactions which might alter the chemical stability and induce membrane permeability changes (Laouini et al., 2012). Microbiologically, as the majority of liposomes formulations are prepared to be injected parenterally, they should be sterilised and free from any pyrogens throughout following the ascetic techniques during the manufacturing process, and post-manufacturing throughout using a conventional stated method such as filtration and lyophilisation to produce stable liposomal products (Kanásová and Nesměrák, 2017; Laouini et al., 2012; Mohammed et al., 2006).

### 1.2. Liposomes bio-distribution and stealth liposomes

One of the major issues that liposomes face is the rapid capture by mononuclear phagocyte systems (MPS). MPS can be defined as a family of cells part of the immune system comprising

macrophages, monocytes, and dendritic cells (DCs) (Lavoie and Levy, 2017; Hume et al.;2019). Monocytes are the main components of MPS present in blood, macrophages are located in tissues (Lavoie and Levy, 2017). However, DCs holds the responsibility for releasing antigen-presenting cells. In general, MPS has many functions. This include digestion of the large particles such as bacteria and fungi, presenting the antigen stimulate the adaptive immunity, secreting some chemical i.e. cytokines to fight against any inflammation or infection, and it exhibits cytotoxic activity against tumour and old cells (Lavoie and Levy, 2017). This lead to a negative impact on the circulation time of liposomes (Immordino et al.; 2006). Removal by the MPS depends on size, surface stiffness, and surface prosperities. In general, larger and negatively charged liposomes are more susceptible to clearance than smaller and neutral ones (Lavoie and Levy, 2017). Researchers hypothesised that naked liposomes i.e. non-stealth ones are cleared more rapidly than PEGylated ones. Non-modified liposomes are usually cleared to the liver and spleen. On the other hand, PEGylated liposomes clearance depend on many factors as the following. Size plays a key role in stealth liposomes clearance i.e. Liposomes with larger sizes i.e. 250 nm cleared more rapidly from the bloodstream than smaller ones i.e. 70 nm. Researchers hypothesised that liposomes containing cholesterol are accumulated in the spleen due to the presence of specific opsonin in the spleen phagocytes. Large stealth liposomes bind to Kupffer cells and accumulate in the liver whereas smaller ones are commonly directed to the bone marrow (Owensiii and Peppa, 2006).

Liposomes as an example for our research has progressed from conventional liposomes to stealth liposomes aiming to provide longer circulation time and better targeting efficiency (Bozzuto and Molinari, 2015). This is affected by different parameters which will be explained. Generally, larger liposomes (400- 800 nm) are removed from blood circulation more rapidly than smaller ones (200 nm). Liposomes containing high amounts of cholesterol are removed more easily than liposomes containing a lower amount of cholesterol. Having a large liposome

with a high amount of cholesterol showed a synergetic effect for rapid removal by MPS (Ishida et al., 2002). Liposomes charge play a role in complement activation. Both neutral and positively charged liposomes retained more in the bloodstream, whereas negatively charged liposomes activate the immune system and removed more quickly due to their tendency to aggregate in the presence of serum proteins (Amoozgar and Yeo, 2012). Regarding lipid composition, unsaturated lipids activate the complement more potent than saturated ones. This is due to that unsaturated lipids are common with natural phospholipids and being less stiff than saturated ones (Monteiro et al., 2014).

Surface modified liposomes were used as a strategy to enhance the circulation time. This can be obtained by using some materials such as PEG to gain stealth properties. These long circulating liposomes are able to evade the immune system through the formation of a hydrophilic shell, which 1) mask the hydrophobic surface of the liposomes and prevent aggregation and 2) has been suggested to decrease protein adsorption and opsonin (Bozzuto and Molinari, 2015; Verhoef and Anchordoquy, 2013). Still, the efficiency of PEGylation depends on selecting a PEG chain with an adequate molecular weight and ensuring sufficient coverage of the liposome (Suk et al., 2016). First, for the PEG molecular weight, there is a direct proportional relationship between the PEG chain length and its molecular weight. The suggested range of the PEG weight is to be between 2 and 20 kDa to prevent the aggregation and the adsorption on the blood components (Miteva et al., 2015). Second, PEG density and configuration, previous literature suggested that using lower PEG density i.e. less than 5% can lead to liposomes aggregation overtime whereas using higher than 5% up to 10% can prevent liposomes' aggregation (Braeckmans et al., 2011). For the PEG configuration, it is suggested that obtaining a good configuration between brush and mushroom is desired to ensure a full coverage of the liposomes' surface (Parambath, 2018). The detailed explanation is provided in

below in the same section. The figure below shows the main pathways for complement activation (Figure 1.6.).

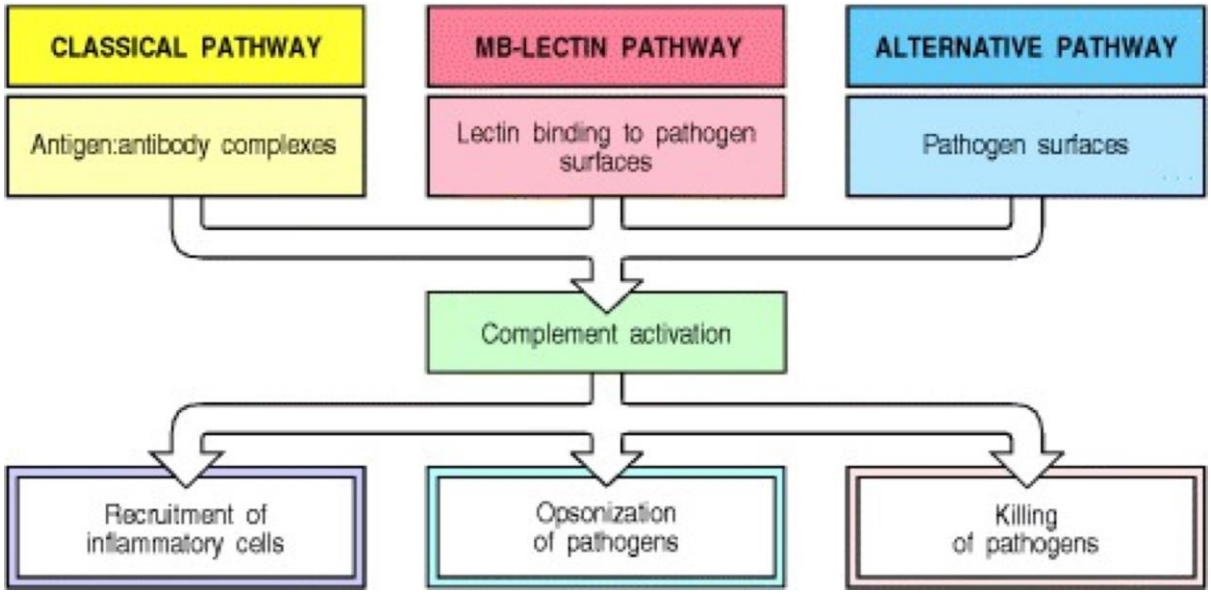



**Figure 1.6.The three main ways for complement activation. Retrieved from (Janeway et al., 2001).**

PEG has several advantages in pharmaceutical manufacturing e.g. biocompatibility, low toxicity and immunogenicity, and its good excretion prosperities (Bozzuto and Molinari, 2015). PEG has been used extensively due to its hydrophilic property, natural flexibility, allows PEG chains to extend freely in aqueous solution (Owensiii and Peppa, 2006). This property creates a repulsive force to balance the attractive force between the particles and opsonin (Owensiii and Peppa, 2006). The idea behind this approach is based on decorating the outer surface of liposomes with PEG to hide the liposomes from the recognition by the MPS (Nag and Awasthi, 2013). This would generally leads to the formation of a hydrophilic shell, increases the half-life time, and evading the capture by MPS (Salmaso and Caliceti, 2013). Table 1.3. summarises the main methods used to prepare stealth liposomes.

**Table 1.3. Summary of different materials used for stealth manufacturing (Amoozgar and Yeo, 2012; Barclay et al., 2019; Boddou et al., 2021; Franco and De Marco, 2020; Harijan and Singh, 2022; Nag and Awashti, 2013).**

| Material | Example | Advantages | Disadvantages |
|---|---|---|---|
| PEG lipo-polymers | There are three classes:<br>• PEG-Phospholipid: PEG-DSPE.<br>• 2-PEG-Non-phospholipid: Cholesteryl-PEG and PEG-Sphingomycine.<br>• 3-Hyerbranched PEG: PEG350 Derivatives. | • Increase the circulation time.<br>• Low toxicity.<br>• The gold standard for most formulations.<br>• There are approved PEGylated products for treatment such as Doxil. | • PEG prevents the liposomes' hydration leading to the leakage when inserted during manufacturing.<br>• Some PEGylated products can generate anti-PEG antibody which leads to ABC phenomena.<br>• Can induce immunogenic response depending on the configuration brush or mushroom. |
| Vinyl based lipo-polymers | Polyvinyl pyrrolidone (PVP) and poly (cryl amide) (PAA). | • Increase the circulation time.<br>• Reduce the accumulation in the liver.<br>• Non-toxic.<br>• High temperature resistant and pH stable. | • Non-biodegradable<br>• When used with a molecular weight above 25 KDa accumulates in the body.<br>• Needs further investigations in the liposomes research as it is not commonly used.<br>• Possibility of the presence of the solvent residue in the final product. |
| Poly(2-Oxazoline)-Based Lipopolymers | Poly(2-methyl-2-oxazoline) (PMOZ)- and poly(2-ethyl-2-oxazoline) (PEOZ)-linked DSPE . | • Increase the circulation time.<br>• Reduce the accumulation in the liver and spleen.<br>• Has a close behaviour to PEG-lipo-polymers.<br>• Good choice for low soluble and metabolically low-stable materials. | • Biological and stability studies are few and need further evaluation to be confirmed.<br>• Possibility of inducing microbial contamination.<br>• Upon storage, excessive hydration and reduced viscosity can occur. |
| Poly (Amino Acid)-Based Lipopolymers | polyglutamic acid (PGA), poly(hydroxyethyl-L-asparagine) (PHEA) and poly(hydroxyethyl-L-glutamine) (PHEG) | • Increase the circulation time.<br>• Reduce the accumulation in the live and spleen.<br>• Biodegradable. | • Increase the compliment activation which limits their use in surface engineering. |

| | | | |
|---|---|---|---|
| | | • Has been used in drug delivery application e.g. with Paclitaxel. | |
| Zwitterionic Lipopolymers | phosphobetaine, sulfobetaine or carboxybetaine moieties. | • Low fouling material.<br>• Good stability and more resistance against aggregation when using at 5 mol% and higher.<br>• Low compliment activation.<br>• Highly hydrophilic material which reduces the interaction with the extracellular components. | • Difficult to be synthesized as solvents needed for their preparation are not common. |
| Polysaccharides | Dextran and Chitosan. | • Biodegradable.<br>• Availability.<br>• Low immunogenicity.<br>• Increase the circulation time.<br>• Abundant functional groups to be a ligand for targeting. | • Expensive.<br>• High tendency towards complement activation.<br>• Low solubility in organic solvents.<br>• When formulating a sustained release formulation, polysaccharides-based drugs require a slow enzymatic degradation. |

From the above table, it is clear that the different stealth materials share some advantages including low toxicity and increasing circulation time. Different disadvantages for different types of stealth materials were reported including the difficulty of synthesis, lack of biological and stability studies, and the possibility of complement activation (Salmaso and Caliceti, 2013). PEG appeared to be the most extensive hydrophilic polymer used for surface modification. PEG has different types and molecular weights and can exist in mushroom and branched configurations (Immordin et al, 2006). PEG act as a steric stabilizer (Milla et al., 2012). It act by hiding the hydrophobic surface, thus, decreases the liposome recognition by the macrophages and extend their circulation half-life time (Milla et al., 2012).

PEG can be incorporated into liposomes in different ways. First, by adding the polymer during the liposomes preparation (pre-insertion method) either with the lipids in an organic solvent or during rehydration. This method has the advantage of simplicity, but some drawbacks were reported; for instance, pre-insertion makes the extrusion step more difficult. Also, modification of both the inner and outer surfaces could result in interference with the interior space, which is intended for drug loading (Nag and Awasthi, 2013). In contrast, the post-insertion method ensures that modification will take place on the outer surface only. For this method, PEG-phospholipid is added to pre-performed liposomes at a transition temperature above the Tc of the lipid. The PEG-phospholipid concentration should be higher than its critical micelles concentration (CMC) 10-20 μM (Milla et al., 2012; Sou et al., 2000). PEG-DSPE appeared to be the most common type of PEG used due to its biocompatibility, having biodegradable characteristic, and due to its amphiphilic property which allow it to be used with various molecules for different purposes (Che, et al., 2015). Moreover, the idea behind adding PEG-phospholipid at a temperature above Tc is to enhance the transition of the phospholipid from gel to the crystalline phase, to facilitate insertion of the PEG chains (Kastantin et al., 2009). An illustration of the two different methods used to insert PEG is provided below (Figure 1.7.).

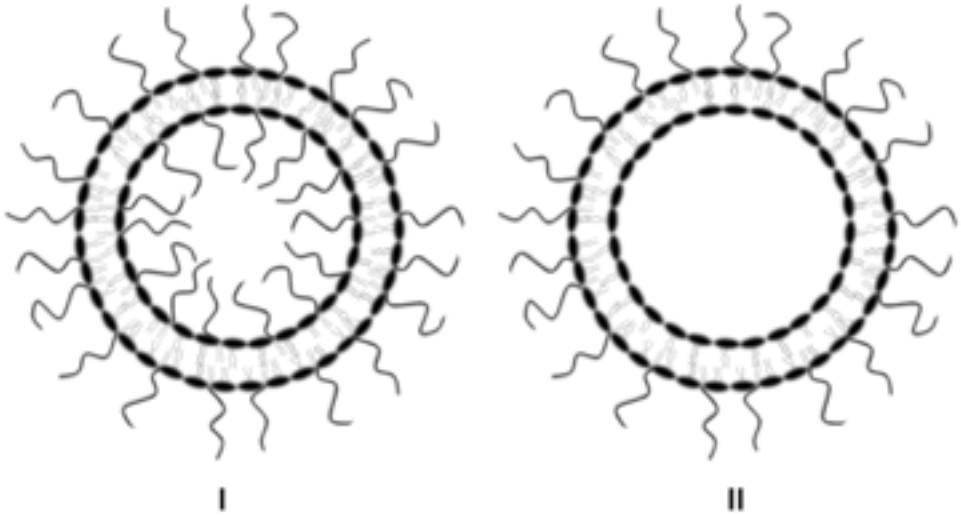



**Figure 1.7.The two methods of adding PEG to liposomes, pre-insertion methods (I), and post-insertion method (II). Retrieved from (Nag and Awasthi, 2013).**

In order to ensure the success of PEGylation, several factors must be set-up (Salmaso and Caliceti, 2013). This includes PEG molecular weight, PEG density, liposome rigidity, and polymer surface confirmation. PEGylation efficiency is proportional to its chain length; for instance, if longer chains are used, greater stability and longer circulation time can be achieved compared with shorter ones (Milla et al., 2012). Using low molecular weight PEG (less than 2000) (Owensiii and Peppa, 2006) can yield inhomogeneous coating and poor steric stability, thus liposomes being more susceptible to clearance and vice versa (Salmaso and Caliceti, 2013). Liposomes rigidity is an important factor in ensuring good incorporation of PEG into liposomes. Low rigidity and using phospholipids with low Tc enhance the leakage.

The polymer conformation can either be brush or mushroom. Brush confirmation is usually associated with higher PEG density (5 mol% or above), whereas the mushroom occurred with lower densities (Salmaso and Caliceti, 2013). In both cases, it is necessary to ensure using PEG with a molecular weight of 2000 and above to ensure its flexibility as shorter are less flexible.. Mushroom configuration is associated with low surface coverage or partial coverage leads to their recognition by MPS and clearance. On the other hand, a high surface coverage was obtained with brush configuration, but PEG's steric hindrance prosperities were decreased due

to reducing their mobility (Parambath, 2018). The optimal surface coverage is between mushroom and brush where sufficient coverage was guaranteed, with ensuring PEG flexibility (Owensiii and Peppa, 2006). Researchers suggested that using 5% of PEG-2000 and higher can be a good solution for providing good coverage besides to ensuring PEG flexibility (Owensiii and Peppa, 2006). The figure below is showing the difference between the two PEG confirmations (Figure 1.8.).

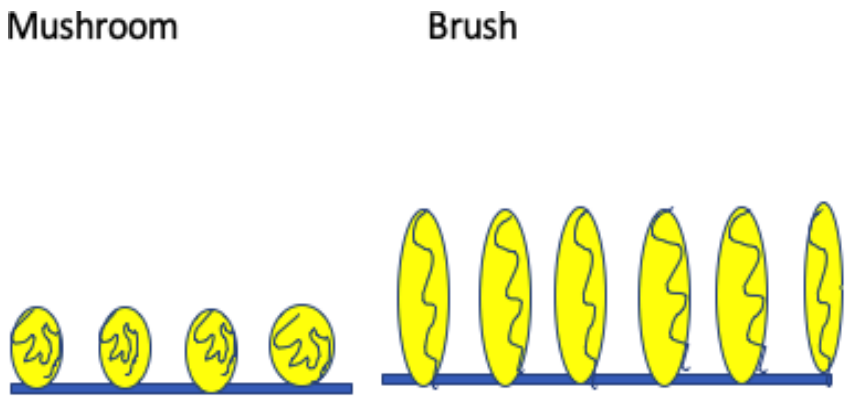



**Figure 1.8. PEG brush Vs mushroom configuration**

Several approved PEGylated liposomal formulations in the market showed their succeed are listed in the below (Table 1.4.).

**Table 1.4. List of the approved PEGylated formulations (Mohamed et al., 2020).**

| The approved drug | Clinical indication | Type of PEG used |
|---|---|---|
| ONPATTRO | Hereditary transthyretin amyloidosis | PEG (2000)-C-DMG |
| Onivyde | Pancreatic adenocarcinoma. | MPEG-2000: DSPE |
| Lipusu | Gastric, ovarian and lung cancer. | PEG2000 |
| Lipo-Dox | Breast and ovarian cancer. | PEG 2000-DSPE |
| Doxil | Kaposi's sarcoma. | PEG 2000-DSPE |

After administrating, these liposomes will undergo several pharmacokinetics processes inside the body. These stealth liposomes are designed to enhance their targeting. Thus, these are required to be formulated with care to avoid their clearance. PEGylated liposomes are mainly removed by phagocytosis through RES (Mohamed et al.; 2020).

There are several methods reported to quantify PEG. It can be quantified colorimetrically using spectrophotometer instrument i.e. UV-Vis, HPLC or Mass stereoscopy (Jones et al.; 2014), or by NMR to assess the grafting density (Lu et al.; 2019). The colorimetric assay is considered the simplest assay for PEG quantification. It is based on reacting the liposomes with Phospholipase C and deoxycholate to solubilize the phospholipid, then adding an equal amount of the same solubilizing solvent of the liposomes with ammonium ferrothiocynate. This mixture was incubated and shaked for 30 minutes. The upper layer was removed, and the bottom layer was measured using UV-Vis at $\lambda = 510$ nm (Jones et al.; 2014). The higher the concentration, the darker colour to be obtained.

### 1.3. Lyophilisation

Lyophilisation (Freeze-drying) is a common drying method and, for nanomedicines, offers a means to ensure long-term stability (Abdelwahed et al.; 2006). Nanoparticles in the aqueous form are susceptible to both physical instabilities, *e.g.* aggregation and fusion, and chemical instability including hydrolysis and the possibility of microorganism growth (Fonte et al.; 2016). Specifically, for liposomes, both chemical and microbiological instability can be derived from the tendency of the phospholipid to be hydrolysed in the aqueous solution. This was reported mainly with the short acyl chain lipids with an anionic polar head group *e.g.* DMPG (Fouladi et al., 2017). To avoid this, it is suggested to convert these formulations to a solid state, thus ensuring long-term stability with an easy reconstitution profile. Lyophilisation is one of the most commonly techniques used to achieve this goal. Up to date, twenty liposomal formulations approved for clinical use are lyophilised (Wang and Grainger, 2019). However,

there are some challenges represented by the presence of destabilisation factors as presented in (Figure 1.9.).

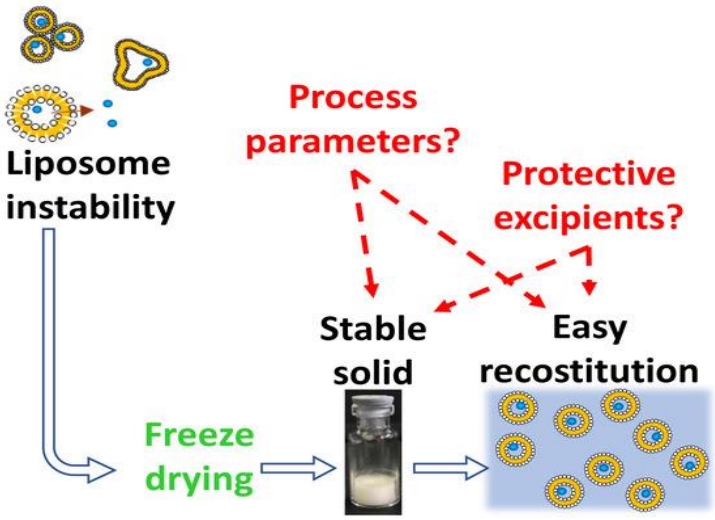



**Figure 1.9. Different liposomal instability and the benefits of lyophilisation. Retrieved from (Franzé et al., 2018).**

The freeze-drying process consists of three steps, starting with freezing the solution, then ice removal through sublimation (First drying), followed by desorption (Secondary drying) (Abdelwahed et al., 2006; Jensen et al.; 2002). During the freezing step, the solution is frozen, leading to the formation of ice crystals. The freezing step is crucial, as the lyophilisation stress has an effect. At higher temperatures, above the freezing temperature, the suspension separates into two phases, water, and bulk phase. While freezing at decreased temperature leads to a decrease in the spacing between the phospholipid and head group, leading to micelle formation and aggregation (Abdelwahed et al., 2006). This gives an indication of the importance of Tc and why paying an attention to this factor when selecting the material required for the freezing. The freezing rate can be rapid or slow. Rapid freezing speed usually leads to the formation of microscopic damage for liposomal membrane whereas slow freezing (blow 0.5 K\min) protects the formulation, ensuring the formation of a high-quality dried product (Arshinova et al.; 2012). The primary drying step involves lowering pressure and increasing the temperature to achieve the sublimation. Drying time differ depending on the formulation type and vial depth (Franzé

et al.; 2018). In secondary drying, the temperatures increased and water desorption for the residual moisture content occur (Franzé et al.; 20). The estimation of the drying time is computerised and differ from one formulation to another (Kodama et al.; 2014). The estimated average time for the secondary drying is 6 hours (Kodama et al.; 2014). The net result for these steps is preventing the hydrolysis of both liposomal phospholipids and the encapsulated drug and the formation of a stable product (Torchilin et al.; 2003 and Tsinontides et al.; 2004).

A good lyophilised liposome should show easy reconstitution characteristics, good dried powder formation, a low moisture content (1-2%), and a long stability profile (Arshinova et al., 2012; Williams and Polli, 1984). The quality of the final lyophilised product can be assessed using various methods. The table below shows the different techniques used to evaluate the quality of the lyophilised products (Table 1.5.).

**Table 1.5.Different techniques used for assessing the lyophilised product quality.**

| **Assessment Technique** | **Applications** | **Reference** |
|---|---|---|
| Atomic force microscope (AFM), Transmission electron microscopy (TEM), and Scanning electron microscopy (SEM). | To visualize the microstructure of the dried powder. | (Wahl et al., 2016) |
| X-Ray photoelectron spectroscopy (ESCA), and Differential scanning calorimetry (DSC). | For powder and changes in temperature analysis.<br>DSC is the equipment of choice for thermal analysis. | (Wahl et al., 2016) |
| Dynamic light scattering (DLS) | For size and zeta potential measurements | (Franzé et al., 2018). |
| Ultra-violet spectroscopy (UV) and High performance liquid chromatography (HPLC) | For drug content determinations | (Ghanbarzadeh et al., 2013). |
| Infra-red moisture balance | Residual moisture content determination. | (Khampakool et al., 2020). |

Each technique is intended for a specific use. To study the structure of the dried powder, atomic force microscopy (AFM) is suggested. AFM detects the structure through that, when the cantilever deflects, it interacts with the sample, the laser light will then produce, and the topography of the sample can be detected (Chang et al., 2012). In order to study the sample composition, DSC can be used. DSC work by detecting the thermodynamic of both blank and sample and record the difference in the enthalpy as a function of the temperature (Gill et al., 2010). Other techniques are also played a role for their use e.g., DLS for size measurements, HPLC for evaluating drug content, and infra-red moisture balance for moisture content determination.

Emami et al.; (2018) reported the main fundamental aspects for the obtaining good lyophilisation. This includes the control of the process parameter including cryoprotectant concentration, freezing rate and temperature, and drying time and temperature. They affect different stress types for example crystallization, dehydration, and interfacial stress. The resultant product can be assessed for their quality characteristics. Good lyophilised powder should be intact as a layer, have uniform colour, have a high strength to compensate the cracking. A summary of the main fundamental lyophilisation process and the advantages and limitation is provided in (Table 1.6.).

**Table 1.6. The main fundamentals of freeze-drying process. Retrieved from (Emami et al., 2018).**

| Process parameter | Stress | Advantages | Limitations | Powder characteristic |
|---|---|---|---|---|
| • Cryoprotectant concentration.<br>• Freezing temperature and rate.<br>• Drying temperature and time. | • Crystallisation.<br>• Dehydration stress.<br>• Interfacial stress (Ice-Liquid). | • Elevated temperatures are not required.<br>• Moisture content is controlled.<br>• Short reconstitution time.<br>• Homogenous dispersion.<br>• Suitable for easily oxidised material. | • Expensive set-up and maintenance cost.<br>• Long processing time.<br>• Complex process | • intact cake.<br>• Uniform colour.<br>• Consistency.<br>• High surface area.<br>• High strength to prevent cracking. |

Liposomes lyophilisation is a challenging process due to the possibility of bilayer destruction, aggregation, and leakage of water-soluble drugs (Fonte et al.; 2016). Therefore, some excipients called "cryoprotectants or lyoprotectans" were suggested to be used (Fonte et al.; 2016). They act as protective materials against the lyophilisation stress and preserve the liposomes' structure and physicochemical properties from damage by various mechanisms (Wolfe and Bryant, 1999). For instance, cryoprotectant enhances the vesicles withstand during the freezing step. In addition, they inhibit both aggregation and phase separation. Furthermore, they keep the encapsulated drug from leakage and improve the liposomes' rehydrating property (Chen et al.; 2010). An illustrative figure for the difference of the freeze-dried lipid with and without using sugars (Figure 1.10.).

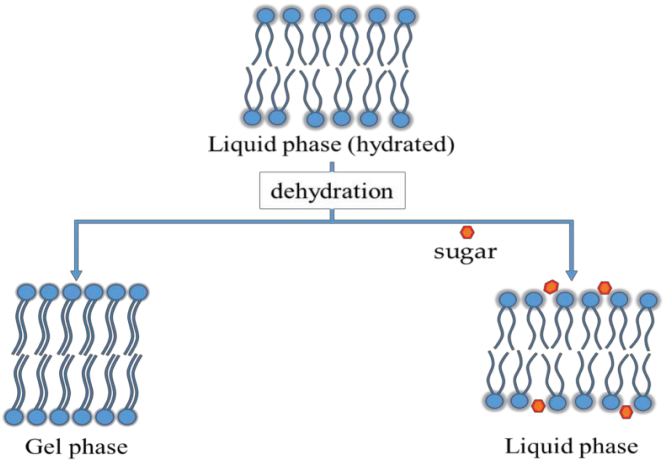



**Figure 1.10. Phospholipid bilayer after rehydration with and without sugar. Retrieved from (Franzé et al., 2018).**

Both lyoprotectant and cryoprotectant are used during lyophilisation and known for their low transition temperatures, thus guaranteeing the formulation's complete freezing (Chen et al.; 2010). Lyoprtoectant protects the formulation during the drying steps while cryoprotectant protects during freezing (Haeuser et al., 2020). The most typical cryoprotectants are sugars including monosaccharides (glucose, fructose, and galactose), disaccharides (sucrose, lactose, trehalose, and maltose), and trisaccharides (Raffinose) (Mohammady et al.,2020). Some compounds have an advantage over others due to their optimum Tc i.e., Trehalose (-110 °C), and Sucrose (-65 ° C) (Jovanovic et al.; 2006). This is due to that, if exceeded the Tc (the temperature required during the first drying stage), the glass temperature (Tg) which is (the temperature required to convert the sample from highly viscose to crystalline form) will increase, and a collapse can occur (Barresi et al., 2009) A table for the most common used cryoprotectant and lyoprotectant is provided below (Table 1.7.).

**Table 1.7. Main classes for cryoprotectant/lyoprotectant.**

| Cryoprotectant/lyoprotectant class | Examples | Explanation | Reference |
|---|---|---|---|
| Sugars (Lyoprotectant). | Monosaccharides (glucose, fructose, and galactose), disaccharides (sucrose, lactose, trehalose, and maltose) and tri-saccharides (Raffinose) | • Trehalose at 5% concentration is more efficient than other sugars due to lack of internal hydrogen bonding, low hygroscopicity, low chemical reactivity.<br>• For stabilizing the PS of chitosan NPs, glucose, sucrose, trehalose, mannitol as cryoprotectants at concentrations of 5%, 10%, 20%, and 50%.<br>• Trehalose at 5% concentration showed the best results. | 1-(Mohammady et al., 2020).<br>2-(Ma et al., 2017). |
| Polyols (Cryoprotectant) | Glycerol, mannitol, sorbitol, adonitol, and inositol. | • Less effective than sugars.<br>• Glycerol was used more than 50 years ago.<br>• It is less toxic than DMSO and PVP. | 1-(Mohammady et al., 2020).<br>2- (Umerska et al., 2018). |
| Polymers (Cryoprotectant) | Dextran, starch, PEG, and polyvinyl pyrrolidone. | • Have stabilising effect for the nanoparticles.<br>• Using 1% PEG-2000 showed a good stabilising effect compared with 3% of trehalose.<br>• PVP can be used as a cryoprotectant to avoid stress occurred during freezing step. | 1-(Mohammady et al., 2020).<br>2- (Umerska et al., 2018). |
| Oxides (Cryoprotectant) | DMSO | • Provide the maximum protection of the liposome's integrity during the freezing step. | 1-(Mohammady et al., 2020).<br>2-(Salazar et al.,2012). |

Cryoprotectant/lyoprotectant works by two mechanisms which are water replacement and matrix formation (Wang and Grainger, 2019; Arshinova et al.; 2012). Water replacement hypothesis was first stated by Crowe et al.; (1996) in which sugars interact with the head group in the phospholipid through the formation of hydrogen bonds, leading to Tc reduction of the lipid membrane in the solid state. Also, sugars reduce the interaction between the water and phospholipid and replacing the water (Chen et al.; 2010). Examples of cryoprotectants used with some of the approved liposomal formulation for clinical use as stated in (Table1. 8.).

**Table 1.8. A list of Some approved liposomal formulations as freeze-dried form. Retrieved from (Arshinova, et al., 2012).**

| Approved formulation | Liposome composition | Cryoprotectant used | Clinical indication |
|---|---|---|---|
| Ambisome (amphotericin B) | HSPC: Cholesterol | Sucrose | Systemic fungal infections |
| Myocet (doxorubicin) | EPC: Cholesterol | Lactose | Breast cancer metastases |
| Nyotran (nystatin) | DMPC: DMPG | Dextrose | Systemic fungal infections |
| Lipoferon (interferon-alpha 2b) | Lecithin: Cholesterol | Lactose | Acute hepatitis B, chronic hepatitis C. |
| Lipoflavone (quercetin) | Lecithin | Lactose | Wounds and post-operation wounds of cornea, keratitis. |
| Visudin (verteporphyrin) | DMPC: PG | Lactose | For photodynamic therapy in ophthalmology. |
| Lipodox (doxorubicin) | Lecithin | Lactose | Kaposi sarcoma, ovary cancer metastases. |

From the above table, lactose is the most abundant used cryoprotectant with the approved liposomal formulations. A series of studies proved that lactose and sucrose are the most suitable cryoprotectant (Mohammady et al., 2020). Sucrose is a highly soluble compound in water,

facilitate the sublimation step without affecting or changing the characteristics of the encapsulated drug. One the other hand, the use of lactose enabled an aqueous dispersion to be lyophilised without losing its integrity, homogeneity, and liposome size (Mohammady et al., 2020). However, some excipients are known to be used during freeze-drying for different purposes. A list of the common excipients used during freeze-drying and their role is provided below (Table 1.9.).

**Table 1.9.A list of the common excipient used during freeze-drying and their role. Retrieved from (Sadikoglu et al., 2006).**

| **Excipient** | **Examples** | **Role** |
|---|---|---|
| Bulking agent | Hydroxyethyl starch, trehalose, mannitol, lactose, and glycine. | Provide bulk to the formulation specially when using low volume of the product |
| Buffer stabilizer | Phosphate, tris HCl, citrate, and histidine, and sodium chloride. sucrose, lactose, and sodium chloride. | PH adjustment. Protect the formulation from drying stress. |
| Tonicity adjustment | Mannitol, sucrose, glycine, glycerol, and sodium chloride | Controlling the osmotic pressure and yielding isotonic solution. |
| Collapse temperature modifier. | Dextran, hydroxypropyl-β-cyclodextrin, PEG, and PVP. | To allow the formulation to withstand high drying temperatures. |

The type and concentration of the cryoptectant/lyoprotectant used, and the insertion method of the cryoprotectant/lyoprotectant (Pre- or post-insertion method) affect the liposomes size. One study was carried out to compare the difference of liposome' size after lyophilisation without any excipients and with a different type of sugars and assessing the optimal lipid: cryoprotectant ratio based on the size consistency before and after lyophilisation (Stark et al., 2010). The liposomes consist of lyso-stearyl-PG, POPC, or DSPC with molar ratios of 5:38:57 with 30 mg

of DSPE-PEG2000, prepared using thin film hydration method. Cryoprotectants were added post-manufacturing, then froze for 48 hours at -80 ◦C. The initial liposomal size was 100 nm. The size increased approximately 2-fold (260 nm) when lyophilised without any cryoprotectants. When using 1:5 (liposomes: cryoprotectants), particle size changed according to the cryoprotectant used as the following 175 nm, 160 nm,150 nm, and 160 nm when using glucose, lactose, trehalose, and mannitol respectively. However, size returns to 100 nm when using 1:10 ratio (Stark et al., 2010). For lactose and trehalose, better homogeneity obtained confirmed by the PDI (0.15 a.u.). In contrast, glucose, and mannitol, even if there was no obvious difference in terms of size, PDI was high (ca. 0.3 a.u.) (Stark et al., 2010).

Lyophilisation is affected by some factors including the type of phospholipid, cholesterol concentration, and surface modification of the liposomes, and liposomes size (Arshinova et al., 2012). First, phospholipid length and saturation degree affect the Tc (Hays et al., 2011). Cholesterol as a rigidity modifier has an impact on lyophilisation and formulation stability. Liposomes with no or low cholesterol concentration are more susceptible to dehydration over long storage time (Popova et al., 2000). Having an optimal concentration of cholesterol in the formulation increase the liposomes rigidity and enhance the withstand ability of the liposomes against the quick cooling (Franzé et al., 2018).

For surface-modified liposomes with PEG, using various PEG molecular weights are often (2000-5000 etc) for the protective effect. A study was performed aiming to assess the difference between PEG-2000 and PEG-5000 when used as a cryoprotectant depending on the diameter size and drug inclusion percentage. Liposomes were prepared from lecithin and cholesterol and were loaded with cardiolipin. Results obtained with PEG-5000 appeared to be more promising, confirmed by 155 ± 23 nm average size and 90% % encapsulation compared with 105 ± 30 nm average size and 78% when using PEG-2000 (Arshinova et al., 2012). For PEGylated

liposomes, sugars are the best choice for lyophilisation (Hinrichs et al., 2006). This was confirmed by using sucrose with PEGylated paclitaxel liposomes (Chen et al., 2010). Sucrose is added to prevent the stress during the drying stage (Guimarães et al., 2019), whereas PEG protect the integrity of the liposomes during the freezing step (Amin et al., 2004). In general, most of the approved liposomal formulations are in a lyophilised form. This would lead us to explore more on these approved formulations, advantages, disadvantages, and the challenges to overcome the reported toxicities.

## 1.4. Rationale of this work

There is a growing attention for the use of liposomal formulations in pharmaceutical field. There are currently many successful approved liposomal products in the market and in clinical development too. Examples of the successfully approved products include Doxil, Lipodox, Onpattro, Onivyde, Vyxeos, and DaunoXome. Notably, the approved formulations were mainly indicated for a wide range of non-liver cancers such as breast, ovarian, lung, colon and blood cancer. Interesting reports from post-marketing data reported hepatic-related adverse drug reactions associated with the use of these products. According to the adverse drug events data from the FDA and the Surveillance databases, there was a noticeable number of reports reporting hepatic toxicities that included liposomal formulation. Records form the FDA, reported the highest incidence rate for the hepatotoxicity was reported with (Ambisome), with an average of 333 out of 3515. The least reported hepatotoxicity incidences were Lipodox only one incidence (U.S. Food and Drug Administration, 2021). Likewise, data from the Surveillance database showed that, Doxil had 79 hepatotoxicity reports out of 3399. The least number of reported liver toxicity for the liposomal formulations was 4 out of 211 with Onivyde (EudraVigilance - European Medicines Agency, 2021).

Literatures suggested that, the induced hepatotoxicity can be due to the interaction of the liposomal formulation with the liver cells which leads to the toxicity (David and Hamilton,

2010). Different mechanisms are suggested according to the toxicity induced by each formulation. For example, Amphotericin B which used as an antifungal medication (Bozzuto and Molinari, 2015) lead to an elevation in the liver enzymes, serum aminotransferase. The exact mechanism is still unknown (David and Hamilton, 2010). However, the reported hyperbilirubinemia is expected to happen due to the inhibition of bilirubin transport mechanism (Hoofnagle et al., 2013). The hepatotoxicity with the anticancer drugs e.g., doxil and Vyxoses is rare (Hoofnagle et al., 2013). However, as the metabolism of the active compounds takes place in the liver. Upon their metabolism, some toxic and immunogenic intermediate are released might trigger the liver. For the reported elevations in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes can be lined with the activation of the reactive oxygen specious which leads to lipid peroxidation, and ultimately leads to elevation in liver enzymes (Kalender et al., 2005). Onyvide is metabolized in the liver by Cytochrome P450 (CYP 3A) and uridine diphosphate-glucuronosyltransferase (UGT1A1). The reported steatohepatitis might occur as a result of the formation of toxic metabolites upon their metabolism in the liver (Hoofnagle et al., 2013). It is difficult to decide if this hepatotoxicity is related to the use of these drugs directly as several factors including genetic differences, medical history, the use of other medication which can lead to drug-drug interaction has an influence on these findings. It worth the has a comprehensive analysis for each incidence rate and establish a causality relationship to determine if the hepatotoxicity was related to the use of liposomal formulations or not.

## 1.5. Liposomes as drug targeting system for the liver and associated challenges

Human liver cell can be classified into parenchymal cells (PCs) and Non-parenchymal cells. Depending on the medical conditions, different cells can be targeted. Hepatic stellate cells (HSC) are the targets for cirrhosis and fibrosis, whereas Kupffer cells and liver sinusoidal endothelial cells (LESC) are common targets for both acute and chronic inflammatory liver

conditions (Poelstra et al, 2012). Drug targeting can be defined as the ability of the drug to accumulate on the target site selectively and quantitatively. Two different methods were reported to enhance drug-carrier accumulation in specific liver cells (active and passive targeting). Active targeting can be accomplished through surface modification with specific ligands expressed on the surface of the target site. In terms of liver targeting, this can be done by exploiting differences in the expression of carbohydrate receptors for different liver cell types. However, targeting hepatocytes can be challenging (Iredale J, 2008; Poelstra et al., 2012). This is due to the indirect role of non-parenchymal cells mainly the Kupffer cells (KCs). Previous work reported that the injected liposomes tend to naturally accumulate in KCs after removal by the MPS. The non-parenchymal cells namely KCs enhance the immune system through digesting foreign substances including nanoparticles, while SCs aid in the extracellular matrix formation. LSECs play a role in transporting nutrients from the bloodstream to PCs (Poelstra, etal., 2012; Wu et al., 2008). According to the evidence, galactose and lactose are found mainly on the surface of hepatocytes, especially on Asiloglycoprotein receptors (Nosova et al., 2017; Sonoke et al., 2011), LSECs express mainly mannose receptors, thus; can be targeted by using mannose moieties (Kelly et al.,, 2011). However, mannose is a common strategy to target macrophages (Craparo et al., 2013; Zhang et al., 2017). Focusing on hepatocytes targeting, an active targeting strategy mainly to asiloglycoprotein receptor is recommended (Gorad et al., 2013). This is due to their high expression on the surface of the hepatocytes (Gorad et al., 2013). Active targeting success depends on different factors. This includes liposomes size, surface charge, and surface modification. Liposomes need to be small size (Below 200 nm) to pass the fenestrations, thus gain direct access to the hepatocytes. The ideal zeta potential range is between 0 and -10 mV. Positively charged liposomes interact with the negatively charged cell surfaces leading to unfavourable outcomes. On the other hand, negatively charged liposomes recognised quickly by the scavenger receptors that present on

Kupffer cells leading to a quick clearance. Liposomes PEGylation increases the liposomes hydrophilicity. Thus, vesicles' stability increases an longer circulation time can be obtained. However, It is important to optimize the targeting ligand concentration. Excessive using of the ligand could lead to their clearance by RES through the opsonin mechanism. By contrast, using low ligand density leads to reduction in cell specificity. ASGPR receptors appeared to be a promising targeting site for hepatocytes targeting. This is due to their abundant presence on the hepatocytes surface i.e. 500000 receptors/cells. ASGPR is a lectin receptor that is connected to terminal galactose and N-acetylgalactosamine residues. In order to improve the hepatocytes targeting, some ligands are suggested to be used. This includes the use of antibodies, and carbohydrates as targeting ligands (Witzigmann, 2016). These eventually present a few useful applications in targeting hepatic cells, which eventually can aid the development of sterically stabilized liposomes for hepatic drug delivery. For this purpose, the presented work aimed to evaluate the impact of the physicochemical properties and surface modification on liposomes membrane permeability. As we hypothesised that controlling physicochemical properties such as size and surface hydrophobicity could affect the liposomes' membrane permeability. Additionally, we aimed to prepare liposomes with a size range below 200 nm, modified with different PEG type and densities to mimic targeting the liposomes.

## 1.6. Aim and objectives of this work

### 1.6.1. Aim

To investigate the effect of liposomes' physicochemical properties e.g., liposomes size and surface modification with PEG and targeting ligand on targeting the hepatocytes.

### 1.6.2. Objectives

1- Systematic review of the literature for the factors affecting liposomes as a drug targeting system to the hepatocytes. The search was conducted using two databases (Medline and Embase) based on certain inclusion/exclusion criteria for the last five years published articles.

2-Based on both literature and systematic review data, different liposomal formulations were prepared in the lab with the below characteristics. The selection of these compositions was according to that their safety and for being components of approved liposomal formulations in the market.

2.1-Size and PDI: we were aiming to formulate liposomes with an average size of (150- 200 nm) and a narrow polydispersity index (less than 0.3 a.u.). In order to achieve the goal, different molar ratios and various pressure profiles were tested to choose the best two formulations. Size and PDI were evaluated using DLS.

2.2-Zeta potential: a stability marker for liposomes was tested using ELS. The expected average zeta potential values for the neutral liposomes between ( -10 mV and +10 mV).

2.3-Stability studies: The selected two formulations were kept in the refrigerator and their size, PDI, and zeta potential were evaluated monthly.

2.4-Membrane permeability: To evaluate liposomes' membrane permeability at different temperatures (25°C, 37°C, and Tc). Calcein used as a fluorescent probe. The data were adopted from UV-Spectrofluorophotometer.

2.5-PEGylation: adding 2 and 5 mol% of different PEG polymer (PEG2000-DSPE, PEG2000-DSPE-COOH, PEG2000-DSPE-NH2) to pre-prepared liposomes aiming to introduce stealth property which leads to stabilise the liposomes and provide longer circulation time for the prepared liposomes.

Due to the COVID-19 pandemic, it was not feasible to conduct the phase related to the cell work which aimed to evaluate the cellular up-take of the prepared formulations in the laboratory.

**References**


Abdelwahed, W., Degobert, G., Stainmesse, S. and Fessi, H., (2006). Freeze-drying of nanoparticles: formulation, process and storage considerations. *Advanced drug delivery reviews*, 58(15), pp.1688- 1713.

Abraham, S., Waterhouse, D., Mayer, L., Cullis, P., Madden, T. and Bally, M. (2005). The Liposomal Formulation of Doxorubicin. In: J. Abelson, ed., Methods in Enzymology. *Elsevier*, p.76.

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S.W., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M. and Nejati-Koshki, K., (2013). Liposome: classification, preparation, and applications. *Nanoscale research letters*, 8(1), p.102.

Amin, K., Dannenfelser, R. M., Zielinski, J., & Wang, B. (2004). Lyophilization of polyethylene glycol mixtures. *Journal of pharmaceutical sciences*, *93*(9), 2244-2249.

Andra, V., Pammi, S., Bhatraju, L. and Ruddaraju, L., (2022). Correction to: A Comprehensive Review on Novel Liposomal Methodologies, Commercial Formulations, Clinical Trials and Patents. *BioNanoScience*,.

Arshinova, O.Y., Sanarova, E.V., Lantsova, A.V. and Oborotova, N.A., (2012). Lyophilization of liposomal drug forms. *Pharmaceutical Chemistry Journal*, 46(4), pp.228-233.



Amin, K., Dannenfelser, R. M., Zielinski, J., & Wang, B. (2004). Lyophilization of polyethylene glycol mixtures. *Journal of pharmaceutical sciences*, *93*(9), 2244-2249.



Amoozgar, Z., & Yeo, Y. (2012). Recent advances in stealth coating of nanoparticle drug delivery systems. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, *4*(2), 219-233.

Berger, N., Sachse, A., Bender, J., Schubert, R. and Brandl, M., (2001). Filter extrusion of liposomes using different devices: comparison of liposome size, encapsulation efficiency, and process characteristics. *International journal of pharmaceutics*, 223(1-2), pp.55-68.

Barresi, A. A., Ghio, S., Fissore, D., & Pisano, R. (2009). Freeze drying of pharmaceutical excipients close to collapse temperature: Influence of the process conditions on process time and product quality. *Drying Technology*, *27*(6), 805-816.

Bozzuto, G. and Molinari, A. (2015). Liposomes as nanomedical devices. *International Journal of Nanomedicine,* p.975.

Briuglia, M., Rotella, C., McFarlane, A. and Lamprou, D. (2015). Influence of cholesterol on liposome stability and on in vitro drug release. *Drug Delivery and Translational Research*, 5(3), pp.231-242.

Bulbake, U., Doppalapudi, S., Kommineni, N. and Khan, W. (2017). Liposomal Formulations in Clinical Use: An Updated Review. *Pharmaceutics*, 9(2), p.12.

Campbell, P. I. (1983). Toxicity of some charged lipids used in liposome preparations. *Cytobios*, *37*(145), 21-26.

Carugo, D., Bottaro, E., Owen, J., Stride, E. and Nastruzzi, C., (2016). Liposome production by microfluidics: potential and limiting factors. *Scientific Reports*, 6(1).

Carvalho, B. G., Ceccato, B. T., Michelon, M., Han, S. W., & de la Torre, L. G. (2022). Advanced microfluidic technologies for lipid nano-microsystems from synthesis to biological application. *Pharmaceutics*, *14*(1), 141.

Chang, K. C., Chiang, Y. W., Yang, C. H., & Liou, J. W. (2012). Atomic force microscopy in biology and biomedicine. *Tzu Chi Medical Journal*, *24*(4), 162-169.

Chen, J., Ping, Q., Guo, J., Chu, X. and Song, M. (2006). Effect of Phospholipid Composition on Characterization of Liposomes Containing 9-Nitrocamptothecin. *Drug Development and Industrial Pharmacy*, 32(6), pp.719-726.

Chen, C., Han, D., Cai, C. and Tang, X., (2010). An overview of liposome lyophilization and its future potential. *Journal of controlled release*, 142(3), pp.299-311.

Chen, Y., Chen, J., Cheng, Y., Luo, L., Zheng, P., Tong, Y. and Li, Z., (2016). A lyophilized sterically stabilized liposome-containing docetaxel:in vitroandin vivoevaluation. *Journal of Liposome Research*, 27(1), pp.64-73.

Cócera, M., López, O., Coderch, L., Parra, J. and de la Maza, A. (2003). Permeability investigations of phospholipid liposomes by adding cholesterol. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 221(1-3), pp.9-17.

Cortesi,, R., (1999). Preparation of liposomes by reverse-phase evaporation using alternative organic solvents. *Journal of Microencapsulation*, 16(2), pp.251-256.

Craparo, E., Triolo, D., Pitarresi, G., Giammona, G. and Cavallaro, G. (2013). Galactosylated Micelles for a Ribavirin Prodrug Targeting to Hepatocytes. *Biomacromolecules,* 14(6), pp.1838-1849.

Crowe, L.M., Reid, D.S. and Crowe, J.H., (1996). Is trehalose special for preserving dry biomaterials?. *Biophysical journal,* 71(4), pp.2087-2093.

David, S., & Hamilton, J. P. (2010). Drug-induced liver injury. *US gastroenterology & hepatology review*, *6*, 73.

Edwards, K. and Baeumner, A. (2006). Analysis of liposomes. Talanta, 68(5), pp.1432-1441.

Emami, F., Vatanara, A., Park, E.J. and Na, D.H., (2018). Drying technologies for the stability and bioavailability of biopharmaceuticals. Pharmaceutics, 10(3), p.131.

Eze, M. (1991). Phase transitions in phospholipid bilayers: Lateral phase separations play vital roles in biomembranes. Biochemical Education, 19(4), pp.204-208.

Fendler, J.H. and Romero, A., (2011). Liposomes as drug carriers. Life Sciences, 20(7), pp.1109-1120.

Franco, P., & De Marco, I. (2020). The Use of Poly (N-vinyl pyrrolidone) in the Delivery of Drugs: A Review. *Polymers*, *12*(5), 1114.

Franzé, S., Selmin, F., Samaritani, E., Minghetti, P. and Cilurzo, F., (2018). Lyophilization of liposomal formulations: still necessary, still challenging. Pharmaceutics, 10(3), p.139.

Franzè, S., Selmin, F., Rocco, P., Colombo, G., Casiraghi, A. and Cilurzo, F., (2020). Preserving the Integrity of Liposomes Prepared by Ethanol Injection upon Freeze-Drying: Insights from Combined Molecular Dynamics Simulations and Experimental Data. Pharmaceutics, 12(6), p.530.

Fonte, P., Reis, S. and Sarmento, B., (2016). Facts and evidences on the lyophilization of polymeric nanoparticles for drug delivery. Journal of controlled release, 225, pp.75-86.

Fouladi, F., Steffen, K. J., & Mallik, S. (2017). Enzyme-responsive liposomes for the delivery of anticancer drugs. *Bioconjugate chemistry*, *28*(4), 857-868.

Gabizon, A., (2001). Pegylated Liposomal Doxorubicin: Metamorphosis of an Old Drug into a New Form of Chemotherapy. *Cancer Investigation*, 19(4), pp.424-436.

Ghanbarzadeh, S., Valizadeh, H., & Zakeri-Milani, P. (2013). The effects of lyophilization on the physico-chemical stability of sirolimus liposomes. *Advanced pharmaceutical bulletin*, *3*(1), 25.

Gill, P., Moghadam, T. T., & Ranjbar, B. (2010). Differential scanning calorimetry techniques: applications in biology and nanoscience. *Journal of biomolecular techniques: JBT*, *21*(4), 167.

Gonzalez Gomez, A., Syed, S., Marshall, K., & Hosseinidoust, Z. (2019). Liposomal nanovesicles for efficient encapsulation of staphylococcal antibiotics. *ACS omega*, *4*(6), 10866-10876.

Gorad, R., Mandlik, S. and Gujar, K. (2013). LIVER SPECIFIC DRUG TARGETING STRATEGIES: A REVIEW. *International Journal Of Pharmaceutical Sciences and Research,* 4(11), pp.4145-4157.

Guimarães, D., Noro, J., Silva, C., Cavaco-Paulo, A., & Nogueira, E. (2019). Protective effect of saccharides on freeze-dried liposomes encapsulating drugs. *Frontiers in bioengineering and biotechnology*, *7*, 424.

Harijan, M., & Singh, M. (2022). Zwitterionic polymers in drug delivery: A review. *Journal of Molecular Recognition*, *35*(1), e2944.

Hays, L.M., Crowe, J.H., Wolkers, W. and Rudenko, S., (2001). Factors affecting leakage of trapped solutes from phospholipid vesicles during thermotropic phase transitions. Cryobiology, 42(2), pp.88-102.

Hamill, R. (2013). Amphotericin B Formulations: A Comparative Review of Efficacy and Toxicity. Drugs, 73(9), pp.919-934.

Hofheinz, R., Gnad-Vogt, S., Beyer, U. and Hochhaus, A. (2005). Liposomal encapsulated anti-cancer drugs. Anti-Cancer Drugs, 16(7), pp.691-707.

Hinrichs, W.L.J., Sanders, N.N., De Smedt, S.C., Demeester, J. and Frijlink, H.W., (2005). Inulin is a promising cryo-and lyoprotectant for PEGylated lipoplexes. *Journal of controlled release*, 103(2), pp.465-479.

Hoofnagle, J. H. (2013). LiverTox: a website on drug-induced liver injury. In *Drug-Induced Liver Disease* (pp. 725-732). Academic Press.

Hume, D., Irvine, K. and Pridans, C., (2019). The Mononuclear Phagocyte System: The Relationship between Monocytes and Macrophages. Trends in Immunology, 40(2), pp.98-112.

Ikeda, A., Kawai, Y., Kikuchi, J. and Akiyama, M. (2010). Effect of phase transition temperature of liposomes on preparation of fullerene-encapsulated liposomes by the fullerene-exchange reaction. Chemical Communications, 46(16), p.2847.

Immordino, M., Dosio, F. and Cattel, L. (2006). Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. International Journal of Nanomedicine, 1(3), pp.297–315.

Ishida, T., Harashima, H. and Kiwada, H.,(2002). Liposome Clearance. *Bioscience Reports*, 22(2), pp.197-224.

Jensen, T., Halvorsen, S., Godal, H.C. and Skjønsberg, O.H., (2002). Influence of freeze-drying on the clotting properties of fibrinogen in plasma. Thrombosis research, 105(6), pp.499-502.

Jovanović, N., Bouchard, A., Hofland, G.W., Witkamp, G.J., Crommelin, D.J. and Jiskoot, W., (2006). Distinct effects of sucrose and trehalose on protein stability during supercritical fluid drying and freeze-drying. *European journal of pharmaceutical sciences,* 27(4), pp.336-345.

Jones, M. C., Jones, S. A., Riffo-Vasquez, Y., Spina, D., Hoffman, E., Morgan, A., & Dailey, L. A. (2014). Quantitative assessment of nanoparticle surface hydrophobicity and its influence on pulmonary biocompatibility. *Journal of Controlled Release*, 183, 94-104.

Kanásová, M. and Nesměrák, K. (2017). Systematic review of liposomes' characterization methods. Monatshefte für Chemie - Chemical Monthly, 148(9), pp.1581-1593.

Karmakar, S., (2012). Zeta Potential and Dynamic Light Scattering Measurements of Phospholipid Vesicles: A Model System for Biological Membrane.

Kastantin, M., Ananthanarayanan, B., Karmali, P., Ruoslahti, E. and Tirrell, M. (2009). Effect of the Lipid Chain Melting Transition on the Stability of DSPE-PEG(2000) Micelles. Langmuir, 25(13), pp.7279-7286.

Kelly, C., Jefferies, C. and Cryan, S. (2011). Targeted Liposomal Drug Delivery to Monocytes and Macrophages. Journal of Drug Delivery, 2011, pp.1-11.

Khampakool, A., Soisungwan, S., You, S., & Park, S. H. (2020). Infrared assisted freeze-drying (IRAFD) to produce shelf-stable insect food from Protaetia brevitarsis (white-spotted flower chafer) larva. *Food Science of Animal Resources*, *40*(5), 813.

Kodama, T., Takeuchi, M., Wakiyama, N. and Terada, K., (2014). Optimization of secondary drying condition for desired residual water content in a lyophilized product using a novel simulation program for pharmaceutical lyophilization. *International journal of pharmaceutics*, 469(1), pp.59-66.

Kong, T., Wu, J., Yeung, K.W.K., To, M.K.T., Shum, H.C., and Wang, L., (2013). Microfluidic fabrication of polymeric core-shell microspheres for controlled release applications. *Biomicrofluidics*, 7(4), p.044128.

Koster, K. L., Lei, Y. P., Anderson, M., Martin, S., & Bryant, G. (2000). Effects of vitrified and nonvitrified sugars on phosphatidylcholine fluid-to-gel phase transitions. *Biophysical Journal*, *78*(4), 1932-1946.

Koynova, R., Wang, L. and MacDonald, R. (2008). Cationic Phospholipids Forming Cubic Phases: Lipoplex Structure and Transfection Efficiency. Molecular Pharmaceutics, 5(5), pp.739-744.

Laouini, A., Jaafar-Maalej, C., Limayem-Blouza, I., Sfar, S., Charcosset, C. and Fessi, H., (2012). Preparation, characterization and applications of liposomes: state of the art. Journal of colloid Science and Biotechnology, 1(2), pp.147-168.

Lavoie, P.M. and Levy, O., 2017. Mononuclear phagocyte system. In Fetal and Neonatal Physiology (pp. 1208-1216). Elsevier.

Li, J., Wang, X., Zhang, T., Wang, C., Huang, Z., Luo, X. and Deng, Y. (2015). A review on phospholipids and their main applications in drug delivery systems. Asian Journal of Pharmaceutical Sciences, 10(2), pp.81-98.

Lu, J., Xue, Y., Shi, R., Kang, J., Zhao, C. Y., Zhang, N. N., ... & Liu, K. (2019). A non-sacrificial method for the quantification of poly (ethylene glycol) grafting density on gold nanoparticles for applications in nanomedicine. Chemical science, 10(7), 2067-2074.

Milla, P., Dosio, F. and Cattel, L. (2012). PEGylation of Proteins and Liposomes: a Powerful and Flexible Strategy to Improve the Drug Delivery. Current Drug Metabolism, 13(1), pp.105-119.

Mohamed, M., Alaaeldin, E., Hussein, A., & A Sarhan, H. (2020). Liposomes and PEGylated liposomes as drug delivery system. *Journal of advanced Biomedical and Pharmaceutical Sciences*, *3*(2), 80-88.

Mohammed, A., Bramwell, V., Coombes, A. and Perrie, Y. (2006). Lyophilisation and sterilisation of liposomal vaccines to produce stable and sterile products. Methods, 40(1), pp.30-38.

Mohammed, A., Weston, N., Coombes, A., Fitzgerald, M. and Perrie, Y. (2004). Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. International Journal of Pharmaceutics, 285(1-2), pp.23-34.

Mohammady, M., & Yousefi, G. (2020). Freeze-drying of pharmaceutical and nutraceutical nanoparticles: The effects of formulation and technique parameters on nanoparticles characteristics. *Journal of Pharmaceutical Sciences*, *109*(11), 3235-3247.

Monteiro, N., Martins, A., Reis, R. L., & Neves, N. M. (2014). Liposomes in tissue engineering and regenerative medicine. Journal of the Royal Society Interface, 11(101), 20140459.

Mukherjee, S., Raghuraman, H., Dasgupta, S. and Chattopadhyay, A. (2004). Organization and dynamics of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labeled lipids: a fluorescence approach. Chemistry and Physics of Lipids, 127(1), pp.91-101.

Nag, O. and Awasthi, V. (2013). Surface Engineering of Liposomes for Stealth Behavior. Pharmaceutics, 5(4), pp.542-569.

Nosova, A., Koloskova, O., Shilovskiy, I., Sebyakin, Y. and Khaitov, M. (2017). Lactose-based glycoconjugates with variable spacers for design of liver-targeted liposomes. Biomeditsinskaya Khimiya, 63(5), pp.467-471.

Ollivon, M., Lesieur, S., Grabielle-Madelmont, C. and Paternostre, M., (2000). Vesicle reconstitution from lipid–detergent mixed micelles. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1508(1), pp.34-50.

Owens III, D. E., & Peppas, N. A. (2006). Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. International journal of pharmaceutics, 307(1), 93-102.

Parambath, A., n.d. Engineering Of Biomaterials For Drug Delivery Systems.

Perrie, Y. (2013). Pharmaceutical nanotechnology and nanomedicines. In: M. Aulton and K. Taylor, ed., Aultons' pharmaceutics: the design and manufacture of medicines, 4th ed. Elsevier, pp.790-795.

Poelstra, K., Prakash, J. and Beljaars, L. (2012). Drug targeting to the diseased liver. Journal of Controlled Release, 161(2), pp.188-197.

Popova, A.V. and Hincha, D.K., (2007). Effects of cholesterol on dry bilayers: interactions between phosphatidylcholine unsaturation and glycolipid or free sugar. Biophysical journal, 93(4), pp.1204-1214.

Racey, T. J., Singer, M. A., Finegold, L., & Rochon, P. (1989). The influence of fatty acyl chain length and head group on the size of multilamellar vesicles. *Chemistry and physics of lipids*, *49*(4), 271-288.

Ruozi, B., Belletti, D., Tombesi, A., Tosi, G., Bondioli, L., Forni, F. and Maria Angela Vandelli (2011). AFM, ESEM, TEM, and CLSM in liposomal characterization: a comparative study. International Journal of Nanomedicine, p.557.

Sadikoglu, H., Ozdemir, M., & Seker, M. (2006). Freeze-drying of pharmaceutical products: Research and development needs. *Drying Technology*, *24*(7), 849-861.

Sahoo, S. and Labhasetwar, V. (2003). Nanotech approaches to drug delivery and imaging. Drug Discovery Today, 8(24), pp.1112-1120.

Salazar, J., Ghanem, A., Müller, R. H., & Möschwitzer, J. P. (2012). Nanocrystals: comparison of the size reduction effectiveness of a novel combinative method with conventional top-down approaches. *European Journal of Pharmaceutics and Biopharmaceutics*, *81*(1), 82-90.

Salmaso, S. and Caliceti, P., (2013). Stealth Properties to Improve Therapeutic Efficacy of Drug Nanocarriers. Journal of Drug Delivery, 2013, pp.1-19.

Samad, A., Sultana, Y. and Aqil, M. (2007). Liposomal Drug Delivery Systems: An Update Review. Current Drug Delivery, 4(4), pp.297-305.

Shi, N. Q., & Qi, X. R. (2021). Preparation of drug liposomes by reverse-phase evaporation. *Liposome-Based Drug Delivery Systems*, 37-46.

Shah, V., Nguyen, D., Patel, P., Cote, B., Al-Fatease, A., Pham, Y., Huynh, M., Woo, Y. and Alani, A., (2019). Liposomes produced by microfluidics and extrusion: A comparison for scale-up purposes. *Nanomedicine: Nanotechnology, Biology and Medicine*, 18, pp.146-156.

Singh, R., Gangadharappa, H. and Mruthunjaya, K. (2017). Phospholipids: Unique carriers for drug delivery systems. Journal of Drug Delivery Science and Technology, 39, pp.166-179.

Sonoke, S., Ueda, T., Fujiwara, K., Kuwabara, K. and Yano, J. (2011). Galactose-Modified Cationic Liposomes as a Liver-Targeting Delivery System for Small Interfering RNA. Biological & Pharmaceutical Bulletin, 34(8), pp.1338-1342.

Sou, K., Endo, T., Takeoka, S. and Tsuchida, E. (2000). Poly(ethylene glycol)-Modification of the Phospholipid Vesicles by Using the Spontaneous Incorporation of Poly(ethylene glycol)-Lipid into the Vesicles. Bioconjugate Chemistry, 11(3), pp.372-379.

Stark, B., Pabst, G. and Prassl, R., (2010). Long-term stability of sterically stabilized liposomes by freezing and freeze-drying: Effects of cryoprotectants on structure. European journal of pharmaceutical sciences, 41(3-4), pp.546-555.

Suk, J., Xu, Q., Kim, N., Hanes, J. and Ensign, L., (2016). PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Advanced Drug Delivery Reviews*, 99, pp.28-51.

Sulkowski, W., Pentak, D., Korus, W. and Sulkowska, A., (2005). Effect of temperature on liposome structures studied using EPR spectroscopy. *Spectroscopy*, 19(1), pp.37-42.

Torchilin, P.V., Torchilin, V.P., Torchilin, V. and Weissig, V. eds., (2003). Liposomes: a practical approach (No. 264). Oxford University Press.

Tenchov, R., Bird, R., Curtze, A. and Zhou, Q., (2021). Lipid Nanoparticles─From Liposomes to mRNA Vaccine Delivery, a Landscape of Research Diversity and Advancement. *ACS Nano*, 15(11), pp.16982-17015.

Tsinontides, S.C., Rajniak, P., Pham, D., Hunke, W.A., Placek, J. and Reynolds, S.D., (2004). Freeze drying—principles and practice for successful scale-up to manufacturing. International journal of pharmaceutics, 280(1-2), pp.1-16.

Umerska, A., Paluch, K. J., Santos-Martinez, M. J., Corrigan, O. I., Medina, C., & Tajber, L. (2018). Freeze drying of polyelectrolyte complex nanoparticles: Effect of nanoparticle composition and cryoprotectant selection. *International journal of pharmaceutics*, *552*(1-2), 27-38

Van Hoogevest, P., & Wendel, A. (2014). The use of natural and synthetic phospholipids as pharmaceutical excipients. *European journal of lipid science and technology*, *116*(9), 1088-1107.

Verhoef, J. J., & Anchordoquy, T. J. (2013). Questioning the use of PEGylation for drug delivery. *Drug delivery and translational research*, *3*(6), 499-503.

Wang, Y., & Grainger, D. W. (2019). Lyophilized liposome-based parenteral drug development: Reviewing complex product design strategies and current regulatory environments. *Advanced drug delivery reviews*, *151*, 56-71.

Yu, B., Lee, R. J., & Lee, L. J. (2009). Microfluidic methods for production of liposomes. Methods in enzymology, 465, 129-141.

Yu, S., Yuan, H., Chai, G., Peng, K., Zou, P., Li, X., Li, J., Zhou, F., Chan, H. and Zhou, Q., (2020). Optimization of inhalable liposomal powder formulations and evaluation of their in vitro drug delivery behaviour in Calu-3 human lung epithelial cells. International Journal of Pharmaceutics, 586, p.119570

Wahl, V., Khinast, J., & Paudel, A. (2016). Lyophilized protein powders: A review of analytical tools for root cause analysis of lot-to-lot variability. *TrAC Trends in Analytical Chemistry*, *82*, 468-491.

Warmack, S. and Gubbins, P. (2010). Liposomal Amphotericin B: A Review of its use in the Treatment of Invasive Fungal Infections. Clinical Medicine Reviews in Therapeutics, p.313.

Williams, N.A. and Polli, G.P., (1984). The lyophilization of pharmaceuticals: a literature review. PDA Journal of Pharmaceutical Science and Technology, 38(2), pp.48-60.

Witzigmann, D. (2016). Hepatocyte-specific drug delivery using active targeted nanomedicines-evaluation of targeting strategies in vitro and in vivo (Doctoral dissertation, University_of_Basel).

Wolfe, J. and Bryant, G., (1999). Freezing, drying, and/or vitrification of membrane–solute–water systems. Cryobiology, 39(2), pp.103-129.

Wu, J., Meng, Z., Jiang, M., Pei, R., Trippler, M., Broering, R., Bucchi, A., Sowa, J., Dittmer, U., Yang, D., Roggendorf, M., Gerken, G., Lu, M. and Schlaak, J. (2008). Hepatitis B virus suppresses toll-like receptor-mediated innate immune responses in murine parenchymal and nonparenchymal liver cells. Hepatology, 49(4), pp.1132-1140.

Zhang, G. and Sun, J., (2021). Lipid in Chips: A Brief Review of Liposomes Formation by Microfluidics. *International Journal of Nanomedicine*, Volume 16, pp.7391-7416.

Zhang, T., Zhou, S., Kang, L., Luo, X., Liu, Y., Song, Y., Liu, X. and Deng, Y. (2017). The effect of monosialylganglioside mix modifying the PEGylated liposomal epirubicin on the accelerated blood clearance phenomenon. Asian Journal of Pharmaceutical Sciences, 12(2), pp.134-142.

Zhao, W., Zhuang, S., & Qi, X. R. (2011). Comparative study of the in vitro and in vivo characteristics of cationic and neutral liposomes. *International journal of nanomedicine*, *6*, 3087.

Zalipsky, S., Brandeis, E., Newman, M. and Woodle, M. (1994). Long circulating, cationic liposomes containing amino-PEG-phosphatidylethanolamine. FEBS Letters, 353(1), pp.71-74.

Zizzari, A., Bianco, M., Carbone, L., Perrone, E., Amato, F., Maruccio, G., Rendina, F. and Arima, V., (2017). Continuous-Flow Production of Injectable Liposomes via a Microfluidic Approach. *Materials*, 10(12), p.1411.

# Chapter 2: Factors affecting liposomes as hepatocytes targeting system: A systematic review of the literature.

## 2.1. Introduction

The liver is a vital organ which contributes to metabolic functions, including the detoxification and clearance of endogenous and exogenous elements (Gorad et al., 2013). Four different cell types are present in the liver, hepatocytes/parenchymal cells (PCs) (up to 80% of liver mass), non-parenchymal cells represented by Kupffer cells (KCs), stellate cells (SC) and liver sinusoidal endothelial cells (LSECs) (Poelstra et al., 2012). Parenchymal cells play a role in the synthesis of important components (e.g., proteins, phospholipids, bile acid and glycoproteins) in carbohydrate metabolism, protein storage and detoxification of toxic substances. Non-parenchymal cells act as bodyguards for the liver (Poelstra et al., 2012). KCs enhance the immune system through digesting foreign substances including nanoparticles while SCs aid in the extracellular matrix formation. LSECs play a role in transporting nutrients from the blood stream to PCs (Poelstra et al., 2012). Depending on the medical conditions, different cells can be targeted. Hepatic stellate cells (HSC) are the targets for cirrhosis and fibrosis, whereas Kupffer cells and liver sinusoidal endothelial cells (LESCs) are common targets for both acute and chronic inflammation leading to liver fibrosis (Poelstra et al., 2012). There are two reported drug targeting methods: passive and active targeting (Iredale J, 2008). The theory behind passive targeting relay on the accumulation of the drug on the target site according to enhances permeability and retention (EPR) mechanism. This theory was firstly introduced by Matsmura and Meda (1986) based on certain observations. The first observation was the spontaneous accumulation of the drug in leaky tumours. The second one is the nanoparticles' retention due to the immune system compromise (Narum et al., 2020). The limitation of this method is that being non-specific in which healthy cells can be affected. The drug can also be recognised by the KCs and hence cleared more quickly from systematic circulation. In addition, the heterogeneity of tumour types can affect the dug distribution and might lead to drug resistance (Narum et al., 2020). Targeting the hepatocytes in the liver is challenging (Iredale J, 2008;

Poelstra et al., 2012) as the injected liposomes were found to naturally accumulate in KCs after being removed by the MPS. Approaches to enhance drug-carrier accumulation in specific liver cells will require active targeting through surface modification with cell-specific ligands; this can be done by exploiting differences in the expression of carbohydrate receptors for different liver cell types. For example, galactose and lactose are found mainly on the surface of hepatocytes (Nosova et al., 2017; Sonoke et al., 2011), LSECs express mainly mannose receptors (Kelly et al., 2011). Hepatocytes considered as key pro-pathological cells for hepatocellular carcinoma, some viral infections i.e., hepatitis B and C, and some genetic diseases such as Wilson's disease (Witzigmann, 2016). Research has moved forward to treat these conditions aiming to provide a high efficacy with lower toxicity via targeting the preferred site using nanoparticles. Liposomes are among the most commonly used nanoparticles and have been proved to show a good targeting profile with multiple approved formulations in the market. To target hepatocytes, it is recommended to follow the active targeting strategy by using a ligand i.e., carbohydrates to target the hepatocytes or certain receptors present on their surfaces (Nosova et al., 2017). Several factors have an impact on the success of liposomes targeting including liposomes size, zeta potential, and surface modification, and the cell type to be targeted. First, liposomes size suggested to be below 200 nm to escape from KCs to HCs. Zeta potential should be between 0 and -10 mV, as the positively charged nanoparticles interact with the cell membrane, and the negatively charged ones will quickly be recognised by the scavenger receptors on KC leading to their removal from the circulation (Witzigmann, 2016). Moving forward surface modification, PEG has a positive indirect impact on drug targeting throughout increasing the circulation time allowing longer time for targeting the preferred site. To enhance the success of drug targeting, the targeted site should be abundant and show an exclusive expression on the cell e.g., the hepatocytes occupy 80% of the liver morphology with an abundant ASGP-R expression (Witzigmann, 2016).

### 2.1.1Rationale of the review

Systematic reviews of the studies discussing liposomes as a drug delivery system for the hepatocytes can significantly contribute to developing therapeutic modalities in hepatic therapy.

### 2.1.2. Aim and objectives

To evaluate the impact liposome size and certain surface modifications (e.g., PEG and ligand type/density) on the cellular uptake and pharmacokinetics properties.

This evaluation will be based on data collected and extracted from Medline and Embase databases using certain keywords and inclusion/exclusion criteria. Descriptive narrative analysis and linear regression modelling to predict the factors play role in in hepatocytes targeting will be discussed.

## 2.2. Methodology

Systematic review (SR) can be defined as an evidence of the literature in which the primary research is identified, collected, and critically apprised based on a clearly stated research question and inclusion/exclusion criteria (Research guides, 2022). SR has the advantages of transparency and reproducibility (Research guides, 2022). Systematic review of literature in our project aimed to predict the optimum liposomes characteristics to target the hepatocytes. The search conducted according to the Cochrane and the Centre for Review and Dissemination (CRD) guidelines for systematic reviews. The Preferred Reporting Items for Systematic Reviews (PRISMA) were followed in reporting this review (PRISMA, 2021; Cochrane Handbook for Systematic Reviews of Interventions, 2021).

### 2.2.1. Literature search and eligibility criteria

A comprehensive literature search of two electronic databases: Medline and Embase was conducted on (December 2021) covering the last five years. The keywords were formulated using three main domains that reflect: (liposome), (hepatic cells and liver targeting), (cellular

uptake, pharmacokinetics, and liver accumulation) of the targeted liposomes. The keywords were combined using the "OR & AND" operators (Appendix A, Table 2.A.). See appendix B for the full searching strategy.

The focus was on both experimental studies and RCTs that addressed any liposomal preparation as a drug delivery system to the liver, including modified and non-modified preparations. The search was conducted on both laboratory-based experiments, including cellular and animal studies, and RCTs conducted on patients. We included any study investigating the effects of the liposomal formulation as a liver drug delivery system alone or tested against a control.

Studies reported outcomes that directly related to cellular assays; particularly the percentage of cellular uptake, and fluorescence intensity was included in the review. Studies reporting other cellular assays that did not reflect liposomal cellular uptake were excluded. Additionally, studies reporting any reflective liver targeting parameters such as pharmacokinetics either directly i.e., liver accumulation, drug distribution, drug concentration in the liver, or indirectly e.g., changes in tumour growth for either liposomal contain drugs or liposome alone were included. Only English language articles were included in this review. Also, abstracts only and unpublished records were excluded.

### 2.2.2. Data extraction and management

First, title and abstracts of eligible records were screened against the inclusion criteria. Records with irrelevant titles or abstracts were excluded. Eligible records were imported into a reference manager software (Mendeley). At this stage, duplicated records were checked and merged before proceeding to the next step (full-text screening). Full text for the records that was found to be for full text screening were retrieved. After that full text of potentially eligible records were assessed for the decision on inclusion or exclusion for the following step (data extraction). Cross-references were done by using Mendeley as a software manager. The included articles

were then assessed for their quality according to the type of the study and the scoring system based on the provided guideline.

The data were extracted using a pre-designed data extraction tool. The tool is provided in appendix C (Table 2.C.). The collected data includes the information of the study ID, year of publication, study type (in-vitro-in-vivo, RCT), the applied intervention, liposome composition, ligand/PEG and their concentration, and the reported outcome. Different outcomes were extracted including cellular uptake, PK outcomes (AUC, T1/2, accumulation, and clearance), drug concentration in the liver, and change in tumour growth for the used liposomes. Quantitative data related to cellular uptake and other bio-distribution and efficacy data were extracted to be used in the mathematic model. Data reported in graphs were visualised and extracted using OriginPro software version (9.8.5.201) and ImageJ 2021 version (1.8.0.172). The tool was piloted by re-extracting the data to check the tool sensitivity and to catch the required information.

Quantitative data that used in the modelling was cleaned by preparing the data for analysis by removing or modifying data that is incorrect, incomplete, irrelevant, duplicated, or improperly formatted (Sisense, 2021). The data cleaning was done in three steps. First, data standardisation was done by unifying the units, rounding numbers and labelling the variable names. Second, encoding string variables (variables that are alphabets in nature) into numbers so they could be translated and processed by STATA. Third, since we were applying regression, corresponding dummy variables were generated from original variables. Such variables enable testing subgroups within a single regression model. Example of a dummy variable, a list of three different liposomes formula can produce three dummies; each one represents a category of a liposome formula where its coded as (1) if the attribute is present and (0) if it is absent. The cleaning and analysis were conducted using STATA version 17.

### 2.2.3. Quality assessment of the included studies

Quality assessment can be defined as the extent of keeping the research aspects away from errors including implementation, inferential, and unsystematic errors (Natto and AlGhamdi, 2019). The aim of this step is to build a solid base judgment not only on the reported outcome, but to understand the strength of the applied methodology and how relevant the article to the research question (Sox et al., 2012). In this review, different study designs were assessed (in-vitro, In-vivo, and RCTs). Thus, different quality assessment tools were applied e.g., CASP was applied for the RCTs (Brice, 2022), Syrcle's tool was applied for in-vivo studies (Hooijmans et al., 2014) (Brice, 2022), and for in-vitro studies we adapted a tool designed by (Nasser et al., 2022). The tools used are provided in the appendix D (Table 2.1.D, 2.2.D, and 2.3.D.).

### 2.2.4. Data analysis and synthesis

Descriptive narrative analysis and linear regression modelling to predict the factors that play a role in liver targeting are provided. The qualitative narrative analysis aims to capture and summarise common themes related to liposomes size and surface modification using (PEG/ligand) that were addressed in the included studies. In addition, it discusses how the reported factors may affect the liposomes behaviour as targeting vectors for the liver cells by assessing the cellular uptake and the related PK and efficacy outcomes. Additionally, we applied a linear regression model to predict the effect of changing one or multi factors on the liposome's uptake by the hepatocytes and other outcome of interest.

### 2.2.5. Linear regression modelling

Regression modelling is a mathematical approach that is applied to test the relationship between the stated outcome corresponding to different variables (Alexopoulos, 2010). This type of analysis helps to understand and predict the factors that might play a role on the outcome (Alexopoulos, 2010). The model was used to predict the impact of liposomal size on the hepatic

cellular uptake, the drug accumulation, and tumour volume growth. We applied univariate linear regression analysis. STATA software version 17 was used to analyse quantitative data, and significance were set up at $p< 0.05$.

## 2.3.Results and discussion

### 2.3.1.Search results

The initial search yielded 142 records that were eligible for full-text screening. 25 studies were included in the final analysis. The full search process and the reasons for exclusion are demonstrated in Figure 2.1.

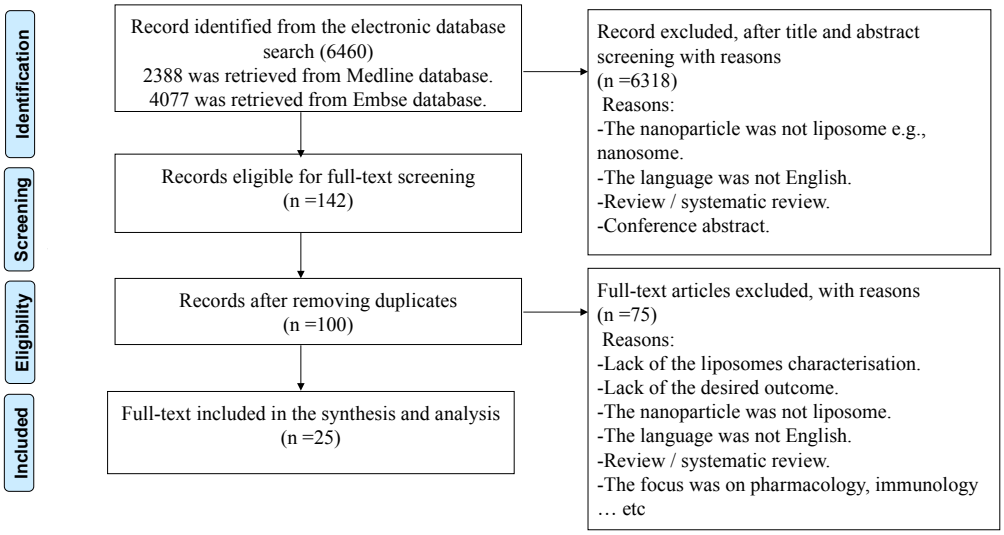



**Figure 2.1. PRISMA flow chart for search results.**

### 2.3.2. Quality assessment of the included studies

Three different tools used to assess the quality of the included studies according to the study type are provided in appendix D (Table 2.1.D, 2.2.D, and 2.3.D.). Studies reporting in-vitro outcomes were assessed against 7 domains (sample selection and experimental setup, reagents and cells, sample size, allocation to groups, allocation concealment, blinded assessment to the outcome, attrition). Sample selection and experimental setup, and reagents and cells were described by most of the studies. However, only one study described how the sample size was

selected (Yi et al., 2021). Studies reported in-vivo outcomes were assessed against five domains (selection, performance, detection, attrition, and reporting bias). Most of the studies showed a good quality when assessed for example they reported the methodology, stating the baseline, randomisation and blindness and other criteria. The reporting for the outcomes were good too. Only one study was included as an RCT study and assessed against four domains (randomisation, methodology, results, and applicability). The methodology was clearly stated but the participants were neither blinded nor randomised i.e., they did not mention anything about the randomisation and blindness in patient selection section. They reported the results and the side effects, but they did not report the CI-95%.

### 2.3.3.Results of the included studies

Characteristics of the included articles are listed in (Table 2.1.).

**Table 2.1. Linear regression analysis of cellular uptake of the formulated liposomes against liposome size.**

| Reference | Aim | Study design | Liposome preparation | Liposome size | PEG type and density | Ligand type and density | Reported outcome |
|---|---|---|---|---|---|---|---|
| (Chen et al., 2017) | Enhancing the liver targeting efficiency both in-vitro and in-vivo using a targeting ligand. | In-vitro and in-vivo. | 1-Un-modified liposomes (EPC/Chol 70/30).<br><br>2-Mannosylated liposomes<br><br>(EPC:Chol:Mannose) (70:25:5) | 1-124.37± 1.43 nm.<br><br>2-148.10 ± 0.95 nm | NA | Mannsose (10 mol%) | Cellular uptake and pharmacokinetics. |
| (Chen et al., 2020) | To improve the drug delivery to treat the HCC. | In-vitro | 1-Conventional liposomes: celastrol, SPC, cholesterol (1:15:3)<br><br>2-Galactosylated liposomes: celastrol, SPC, cholesterol, and gala-PEG-DSPE at a mass ratio of (1:15:3:15) | 1-143.2 ± 2.4 nm.<br><br>2-139.4 ± 2.7 nm. | NA | Gala-PEG2000-DSPE (15 mol%) | Cellular uptake. |
| (Cheng et al., 2018) | Preparing a combination therapy using liposomal formulation to treat HCC for overcoming the unsatisfactory clinical outcome of CDDP monotherapy. | In-vivo | DMPC: Cholesterol: DSPE-PEG2000 (75:25:5) | 294.6 ± 14.8 nm. | DSPE-PEG2000 (5 mol%) | NA | pharmacokinetics and tumour volume. |
| (Ding et al., 2019) | To improve the hepatocytes | In-vitro | SPC:Chol:ligand:HCPT (10:1:1:1). | 1-Mono-Galactose-Liposomes: 230.3 ± | NA | Mono, Di-and Biotin- | Cellular uptake. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | selective targeting. | | | 1.8 nm<br>2-Di-Galactose-Liposomes:<br>218.7 ± 3.4 nm.<br>3-Biotin-Galactose-Liposomes:<br><br>213.6 ± 2.9 nm. | | Galactose-stearate (1 mol%). | |
| (e Silva et al., 2018) | To improve the bio-distribution for liver targeting. | In-vivo | HSPC:EPG:CHOL:Carbohydrate:CHOL-DTPA (11.2:2.16:6.89:1.14:0.22) | 1-When using galactose:<br>131.2 ± 0.3 nm.<br>2-When using glucose:<br>132.9 ± 0.7 nm. | NA | Galactose and Glucose-cholesteryl (2 mol%) | Pharmacokinetics. |
| (Fatouh et al., 2021) | Enhance the drug delivery to hepatocytes. | In-vivo. | 1-Un-modified liposomes:<br>LDV, CHL and Lecithin (20:20:60)<br>2-Galactosylated-chitosan liposomes:<br>LDV, CHL, Lecithin, and galactosylated chitosan.<br>(20:20:42:18) | 1-For the un-modified liposomes:<br>179.45nm<br>2-For the modified liposomes:<br>For 0.3%:<br><br>218.20 nm | NA | Galactose-chitosan (0.3%). | Pharmacokinetics. |
| (Jiang et al., 2020) | To evaluate the anti-tumour efficacy of the prepared liposomes. | In-vitro and in-vivo | 1-Conventional liposomes:<br>N-14NCTDA/PC-98T/Chol (0.015/0.200/0.030)<br>2-Galactosylated liposomes:<br>N-14NCTDA/PC-98T/Chol/DSPE-PEG2000/DSPE-<br><br>PFG2000-GAL (0.015/0.200/0.030/0.010/0.011) | 1- 111.33 ± 4.12 nm.<br><br>2- 131.37 ± 3.89 nm | DSPE-PEG2000<br><br>(1 mol%). | DSPE-<br><br>PFG2000-GAL (1mol%). | Cellular uptake. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (Karimi et al., 2020) | To determine the bio-distribution of the prepared liposomes. | In-vivo | 1--Conventional liposomes: HSPC/DPPG/Chol/DSPE-mPEG2000 (50:10:35:5)<br>2-Pegylated liposomes:<br><br>HSPC/Chol/DSPE-mPEG2000 (55:40:5) | 1-129.4 ± 9.5 nm.<br><br>2-114.8 ± 7.9 nm. | DSPE-mPEG2000 (5 mol%). | NA | Pharmacokinetics. |
| (Karim et al., 2021) | To explore the protective effect of NH-loaded pectin-chitosan decorated liposomes (P-CH-NH-NL) in case of induced hepatic oxidative injury. | In-vitro | 1-SPC: Chol (6:1)<br>2-SPC: Chol: Chitosan (6:1:6)<br>3-SPC: Chol: Chitosan: Pectin (6:1:5:6) | 1-NH-Liposome 66.15 ± 0.99<br>2-Chitosan-NH-Liposome 244.97 ± 1.34 nm<br>3-Pectin-Chitosan-NH-Liposome 365.23 ± 2.66 | NA | Pectin (5 mol%) and Chitosan (6 mol%). | Cellular uptake. |
| (Li et al., 2019) | To improve the hepatoma treatment. | In-vivo | DSPE–PEG–cholic acid (3 mg/mL), phospholipid (40 mg/mL), cholesterol (8 mg/mL), and SLB (5 mg/mL) | 100 nm | DSPE–PEG2000–cholic acid (3 mol%) | NA | The tumour volume. |
| (Lian et al., 2021) | To inhibit the development of HCC | In-vivo | 1-Conventional liposomes: L-α-phosphatidylcholine, cholesterol (12:4)<br>2-Galactosylated liposomes: L-α-phosphatidylcholine, cholesterol, and DSPE- PEG-Gal (12:4:1) | 1- 137.12±4.59 nm.<br>2- 143.01 ±7.32 nm. | NA | DSPE-PEG2000-Gal (1 mol%) | The tumour volume. |
| (Liu et al., 2017) | Improving hepatocellular carcinoma targeted therapy. | In-vitro | 1-Un-modified liposomes: PC-98T/Chol(76/24)<br>2-Galactosylated liposomes: PC-98T/Chol/DSPE-$PEG_{2000}$/DSPE- $PEG_{2000}$-GAL (75/23/1/1) | 1-105.35 ± 3.79 nm<br>2-128.47 ± 1.09 nm | DSPE-$PEG_{2000}$ (1 mol%) | DSPE-$PEG_{2000}$-GAL (1 mol%) | Cellular uptake. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (Nie et al., 2019) | Preparing Hepatocyte selective targeting liposomes. | In-vivo | 1-Unmodified Liposomes: DSPC/CHS (1/1)<br>2-GalNAc-Liposomes: DSPC/CHS/ CHS-6-GalNAc (5/4.5/0.5)<br>3-Glasctosylated liposomes: DSPC/CHS/ CHS-6-Gal (5/4.5/0.5) | 1-76.27 ± 0.29 nm<br>2-80.06 ± 0.56 nm<br>3- 85.07 ± 0.69 nm | NA | GalNAc and Galactose-cholesteryl (1 mol%). | Pharmacokinetics |
| (Nunes et al., 2018) | To understand the effect of PEG inclusion on the targeting and liposomes' bio-distribution. | In-vivo | 1-Conventional liposomes: DOPE, CHEMS, and DSPE (5.7:3.8:0.5).<br>2-For the PEGylated liposomes, 5 mol% of the PEG was added. | 1- 212.3 ± 17.9 nm.<br>2-For DSPE-PEG2000 and PEG1000/5000, the size was 137.0 ± 2.1 nm and 146.6 ± 7.6 nm respectively. | DSPE-PEG2000 and PEG1000/5000 (5 mol%) | NA | Pharmacokinetics and drug accumulation in the liver. |
| (Pireddu et al., 2018) | To develop an effective targeted drug delivery system to teat HCC. | In-vitro. | 1-Un-modified liposomes: POPC: DDAB (78.94: 0.63 µmol)<br>2-Stealth liposomes: POPC: DDAB: DSPE-PEG2000 (78.94: 0.63: 2.4 µmol)<br>3-Lactoferin-Stealth-liposomes: POPC: DDAB: DSPE-PEG2000: Lactoferrin (78.94: 0.63: 2.4: 0.04 µmol) | 1- 98.1 ± 1.04 nm.<br>2- 94.6 ± 1.53 nm.<br>3- 126.5 ± 2.58 nm. | DSPE-PEG2000 (2.4 mol%) | Lactoferrin (0.3 mol%) | Cellular uptake. |
| (Sesarman et al., 2019) | To improve the bio-distribution of the liposome containing doxorubicin. | In-vivo. | 10:1:5 (SPC: Cholesterol: DSPE-PEG2000). | 1-For the Dox-liposomes: 172.7 ± 0.6 nm<br>2-For Dox-Curc-liposomes:171 ± 1.4 nm | DSPE-PEG2000 (5 mol%). | NA | Pharmacokinetics. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (Wang et al., 2019) | To improve the tumour targeting, bio-distribution, and pharmacokinetics of the Rhenium-188 (188Re) using a liposomal formulation. | RCT | HSPC, cholesterol, mPEG2000- DSPE (molar ratio 3:2:0.3) | 90 nm | mPEG2000-DSPE (0.3 mol%). | NA | Pharmacokinetics. |
| (Wang et al., 2020) | Optimising a liposomal formulation to target the hepatocytes. | In-vitro | 1-Blank liposomes: SPC: Chol: DSPE-PEG600 (4 mg: 1.5 mg: 1 mg) 2-Galactosylated liposomes: SPC: Chol: DSPE-PEG600-Galactose (4 mg: 1.5 mg: 1 mg) | 1-165.4 ± 3.5<br>2-161.9 ± 6.4 | DSPE-PEG600 (1 mol%) | DSPE-PEG600-Galactose (1 mol%) | Cellular uptake. |
| (Xu et al., 2017) | To improve the bio-distribution of the liposomes using a targeting ligand. | In-vivo. | 1-Conventional liposomes: SPC: Chol: DPPE (3:1:0.2) 2-Galactosylated liposomes: SPC: Chol: DPPE or Gal-DPPE (3:1:0.2:0.1) | 1- 103 nm. 2-121 nm. | NA | Gal-DPPE (1 mol%) | Pharmacokinetics. |
| (Yi et al., 2021) | To improve the bio-avilability and anti-tumour efficacy. | In-vitro | 1-Conventional liposomes: Uro-A (2 mg), Chol (6 mg), SPC (40 mg) 2-PEGylated liposomes: Uro-A (2 mg), Chol (6 mg), SPC (40 mg), and DSPE-mPEG2000 (20 mg) | 1- 102.0 ± 5.8 nm.<br>2- 122.8 ± 7.4 nm. | DSPE-mPEG2000 (2 mol%). | NA | Cellular uptake. |
| (Yin et al., 2018) | Improve the therapeutic effect for HCC | In-vitro and in-vivo. | Lipoid E80, DSPE-MPEG 2000, cholesterol, SF, and | 175 nm. | DSPE-PEG2000 (1mol%) | Ceremide (0.15 mol%) | Cellular uptake and the tumour volume. |

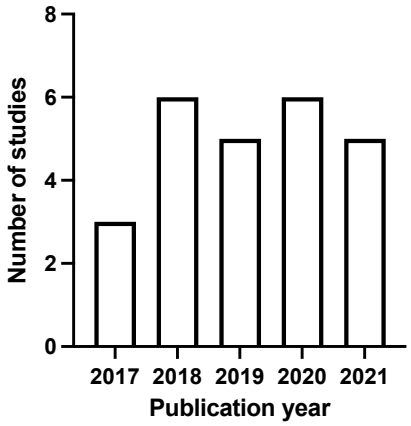
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | treatment. | | CE (4:1:0.3:0.3:0.15) | | | | |
| (Zhang et al., 2020) | To determine the difference in cellular uptake of different organs towards different sugar ligand used. | In-vivo | 0.907 mg SPC: 1.518mg cholesterol: 10 mg of DSPE-PEG750: 1mg of sugar ligand. | 1-Blank-Liposome: 165.4 ± 3.5 nm<br>2-Mannose-Liposomes: 155.9 ± 3.1 nm<br>3-Galactose-Liposomes: 161.9 ± 6.4 nm<br>4-Glucose-Liposome: 156.5 ± 5.4 nm | DSPE-PEG750 (4 mol%) | Different sugar used (Mannose, Galactose, Glucose-DSPE-PEG600) with a concentration of 2 mol%). | Pharmacokinetics. |
| (Zhang et al., 2021) | To study the targeting and the anti-tumour effect of the prepared liposomes against HCC. | In-vivo | 1-Conventional liposomes: Lupeol, HSPC, cholesterol at a molar ratio of 1:10:2.<br>2-Galactosylated liposomes: Lupeol, HSPC, cholesterol, and galactose-PEG-DSPE were dis- solved at a molar ratio of 1:10:2:2 | 1- 97.23 ± 0.6 nm.<br>2-130 ± 3 nm. | NA | Galactose-PEG2000-DSPE (2 mol%). | The tumour volume. |
| (Zhou et al., 2019) | Creating novel liver-targeting nanoliposomes. | In-vivo | 1-Conventional liposomes: Soybean phospholipids (SPC), cholesterol, and CTD were prepared at a molar ratio of 10:1:1<br>2-Galactosylated liposomes: SPC, cholesterol, CTD, and 11-DGA-3-O-Gal at a molar ratio of 10:1:1:1. | 1- 102 ± 3 nm.<br>2-107 ± 6 nm. | NA | 11-DGA-3-O-Gal (1 mol%). | Pharmacokinetics. |
| (Zhu et al., 2020) | Improving drug targeting to the liver. | In-vivo | 1-Conventional liposomes: SPC (4 mg/mL), cholesterol (0.4 mg/mL), and CTD (0.4 mg/mL) | 1- 144.03 ± 1.06 nm.<br>2- 95.78 ± 1.38 nm. | NA | Galactose-succinyl (4%). | Pharmacokinetics. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | 2-Galactosylated-succinyl-liposomes:<br>SPC (4 mg/mL), cholesterol (0.4 mg/mL), CTD (0.4 mg/mL), and 8-GA- Suc (0.4 mg/mL) | | | | |

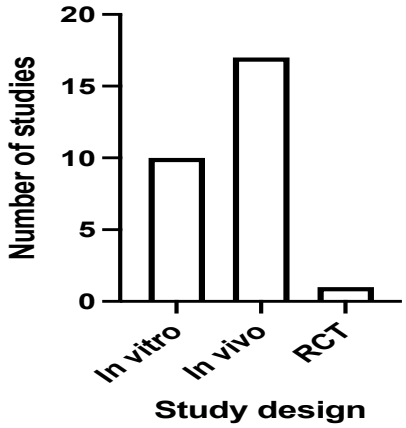
### 2.3.4. Characteristics of the selected papers

Based on the above table, a demographics bar charts are provided below to give a summary for the main characteristics of the included studies.

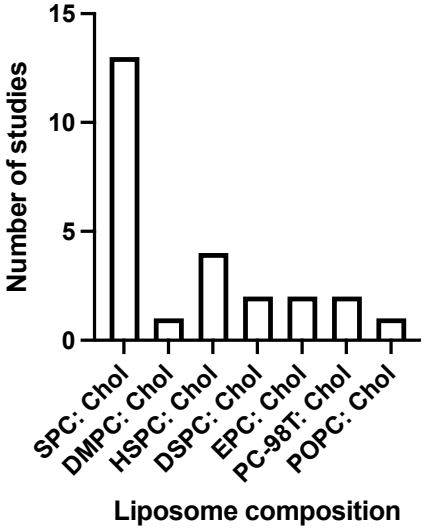
(A)



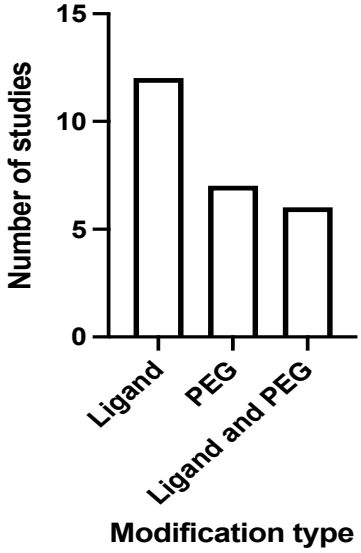

(B)



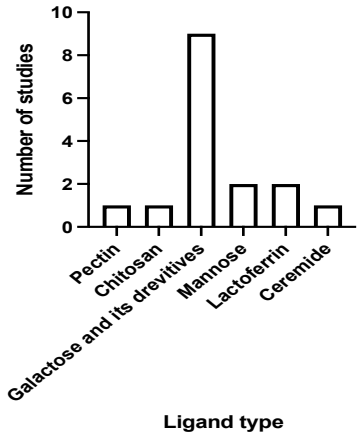

(C)



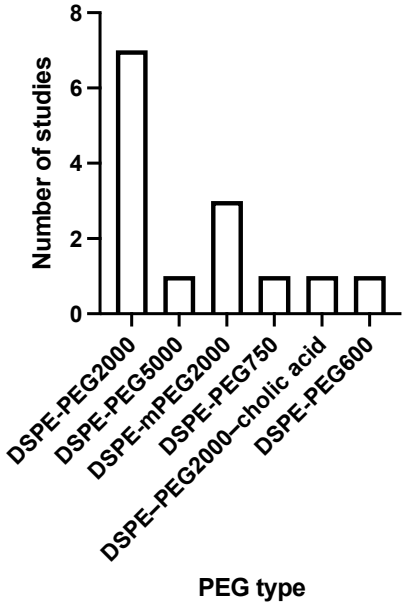

(D)



(E)




(F)




(G)

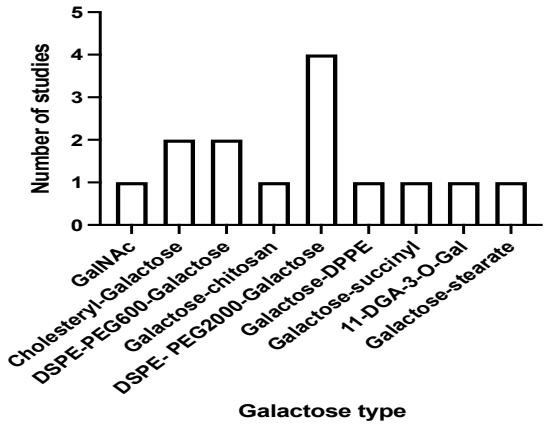



**Figure 2.2. Demographic representation for the number of publications of the included articles per year (A), study design (B), the most common liposomal composition (C), the modification type (D), ligand types (E), PEG types (F), and galactose-based ligand (G).**

Most of the included articles were published in 2018 and 2020 (six articles) and the least was in 2017 for (three articles) (Figure 2.2.A.). Most of the studies were conducted in-vivo (17 studies) (Figure 2.2.B.). SPC appeared to be the most extensively used phospholipid with a frequency of 13 out of 25 (Figure 2.2.C.). In term of liposomes modification, most of the studies used non-PEGylated liposomes (only 7 out of 25 were PEGylated liposomes). PEG-DSPE2000 was the most commonly PEG type used (Figure 2.2.F.). However, ligand was used in 12 studies (Figure 2.2.D.). Galactose and its derivatives are the most common ligands used in liver

targeting (Figure 2.2.E), in specific DSPE-PEG2000-Galactose was the most frequently used and reported in 4 studies (Figure 2.2.G.).

### 2.3.4. Liposome size and composition

In this review, we focused on the liposomes used for targeting the hepatocytes. All studies included the size as an independent variable. Most studies used small unilamellar liposomes with a diameter ranging from 66 to 365 nm. Dynamic light scattering was the most common method used to assess the liposomes' particle size, and liposomes were generally narrowly dispersed. SPC: Chol liposomes are the most used liposomal formulation with a frequency of 13 out of 25 selected papers. SPC has a Tc of 40 °C, which is close to the body temperature. This will ensure maintaining good drug retention in blood circulation (Alavizadeh et al., 2014). This is the reason why lipids with a high Tc are less commonly used i.e., (DSPC has a Tc of 55 °C) (Alavizadeh et al., 2014). These types of phospholipids require a higher temperature to liquefy them, and then releasing their content. Tc can be affected by the addition of cholesterol. As cholesterol is required to increase liposome rigidity, optimising the lipid to cholesterol ratio during the formulation process is vital (Bozzuto and Molinari, 2015). Using excessive amount of cholesterol leads to increase rigidity reducing drug release. However, adding a minute amount of the cholesterol leads to release the drug extracellularly (Bozzuto and Molinari, 2015).

#### 2.3.4.2. Surface modification

PEG type/concentration, and their impact on cellular uptake was reported in one study (Yi et al., 2021). The effect of PEGylation on the PK and bio-distribution was reported in 6 studies (Cheng et al., 2018), (Karimi et al., 2020), (Li et al., 2019), (Nunes et al., 2018), (Sesarman et al., 2019), (Wang et al., 2019). Most of the studies were non-PEGylated, and only quarter of them were PEGylated. Inserting PEG to the prepared liposomes showed an increase in liposomes' sizes. PEG has an advantage of escaping the MPS (Bozzuto and Molinari, 2015). In

addition, there are some available PEGylated liposomes in the marked e.g. Doxil and Onpattro (Anselmo and Mitragotri 2019; Bozzuto and Molinari, 2015).

Ligand type and concentration and the relationship with the cellular uptake were reported in 4 studies (Chen et al., 2017), (Chen etal., 2020), (Ding et al., 2020), (Karim et al., 2021). The impact of ligand on the liposomes' bio-distribution properties were reported in 10 studies (Chen et al., 2017), (e Silva et al., 2018), (Fatouh et al., 2021), (Lian et al., 2021), (Liu et al., 2017), (Nie et al., 2019), (Xu et al., 2017), (Zhang et al., 2021), (Zhou et al., 2019), (Zhu et al., 2020). Only 6 studies reported formulation had PEGylation and target modifiers. 5 of them reported their impact on the cellular uptake (Jiang et al., 2020), (Liu et al., 2017), (Pireddu et al., 2018), (Wang et al., 2020), (Yin et al., 2018). The effect of containment of both PEG and ligand in the formulation on the bio-distribution was reported in three studies (Jiang et al.,2020), (Yin et al., 2018) and (Zhang et al., 2020).

According to Figure 2.2.E., as the galactose used extensively as a targeting ligand in 9 studies. Thus, we will focus on this ligand in the review. Adding galactose changed the size slightly. From figure 2.2.G., DSPE-PEG2000-Galactose was the most common galactose type used and reported in 4 studies. The ligand was either prepared in the lab as in (Lian et al., 2021) study or purchased from different suppliers e.g., from Xian Ruixi Biological Technology Co., Ltd. (Xian, China) with (Chen et al., 2020) and (Zhang et al., 2021) studies, and from Shanghai A.V.T. pharmaceutical L.T.D. with (Jiang et al., 2020) study. Giorgi and Agusti (2014) suggested that, using PEG as an anchoring moiety yields longer circulation time and systematic bioavailability which can lead to enhancing the possibility of galactose receptor interaction. The ligand in all studies was added post-manufacturing. The literature proved that adding ligand using post-insertion method has the advantage of being fully decorated on the surface without occupying the inner space of the liposomes, unlike the pre-insertion method (Nag and Awasthi, 2013).

### 2.3.5. Data synthesis and narrative analysis

This part presents the qualitative analysis of the included studies. We will discuss the main findings and explain the obtained results from the literature. All reported liposome characteristics will undergo a comprehensive analysis relating these properties to their cellular uptake and PK profile.

#### 2.3.5.1. Cellular uptake

##### 2.3.5.1.1. PEGylation

The literature highlighted that liposomal cellular uptake is influenced by several factors, and one of the most important factors is particle size (Witzigmann, 2016). It is suggested that liposomes' size for targeting hepatocytes to be between 100 and 200 nm (Mishra et al., 2013; Shilpi, 2018). Liposomes with smaller sizes have a lower stability profile due to the increase in the surface tension. In addition, they have a high tendency for aggregation, fusion, and precipitation (Immordino et al., 2006). In order to stabilize the liposomal formulation, lyophilisation is one of the most common methods used (Abdelwahed et al., 2006). Lyophilisation aims to reduce the physical instability of the molecules (Fonte et al., 2016), and there are some available freeze-dried liposomal formulations in clinical use e.g. Lipodox (with a size of 20 nm), and Ambisome (with a size of 50 nm) (Bozzuto and Molinari 2015, 2015). Liposomes with a size range greater than 200 nm can induce non-specific recognition by MPS (Chu et al., 2016; Nozawa et al., 1986). In order to increase the liposomes circulation time, surface modification with PEG is recommended. The idea behind PEGylation is to enhance the hydrophilic characteristics and hidden the nanoparticles from the MPS (Bozzuto and Molinari 2015, 2015). However, it is important to control PEG concentration during liposomes manufacturing. Garbuzenko et al., (2005) studied the impact of different DSPE-PEG2000 densities (0-25%) on HSPC liposomes size. Results revealed that, particle size decreases as %PEG increases (Above 8 mol%). This can be explained as that, at higher temperatures (HSPC

has a Tc of 63 °C), liposomes mobility increases, reducing the bilayer thickness, and having a higher amount of PEG on the surface leads to micelles formation on the liposomes surface (Garbuzenko et al., 2005). Garbuzenko et al., (2005) suggested that using a maximum of 7 mol% of DSPE-PEG2000 ensures the biological stability of the prepared liposomes.

In addition, PEG chain length has an impact on cellular uptake. Pozziet al., (2014) investigated different PEG chain lengths (1000, 2000, and 5000 KDa) on the uptake by prostate cancer cells. Here, the prepared liposomes have an average size (100-160 nm) which is within the target size for targeting the hepatocytes However, PEGylation with (DSPE-PEG1000/2000) showed an improvement in the cellular uptake. In the contrary, PEGylation with (DSPE-PEG5000) reduces the cellular uptake to half. The explantion for the findings can be linked to that with longer PEG chain lengths, chain fold-up rather than stretched on the liposome's surface. In addition, long PEG chains interact via van der Waals attraction force and forming inter-chain hydrogen bonds, and ultimately leads to the formation of phase separate lamellar structure (Pozziet al., 2014). Using PEG as a steric stabilisation method appeared to be an efficient in term of reducing its interaction with MPS components but did not show an enhancement of the cellular uptake (Verhoef and Anchordoquy, 2013).

There is only one study which discussed the effect of PEGylation on the cellular uptake. The size for the prepared liposomes was 102 nm for the un-modified and 123 nm the PEGylated liposomes. In this study, they used DSPE-PEG2000 with a density of 2 mol%. The findings showed approximately the same cellular uptake on HepG2 cells (30 and 33%) for the conventional and the PEGylated liposomes respectively. Although it is known that PEG has an advantage of escaping the MPS and providing a longer circulation time, it did not show an improvement on the cellular uptake. Additional information on the impact of using PEG on the PK will be discussed later to help in building a conclusion.

#### 2.3.5.1.2. Ligand type and density

Ligand density slightly impacts liposomal size, as stated in previous studies (Uster et al., 1996; Li and Wang, 2017). However, this slight increase showed no effect on liposomal cellular uptake. Li et al., (2016) studied the effect of different octreotide ligands (0.5, 1, 2, 3, and 4 mol%) on PC liposomes used for targeting somatostatin receptors. All liposomal sizes were approximately 100 nm with no obvious differences among the stated ligand concentrations.

Several ligands were used as targeting modifiers such as sugars, polymers, and glycolipids (Allen et al.,1995). Based on the findings of the cellular uptake markers (%CU and FI) for the included studies, a positive effect of ligand insertion on liposomal cellular uptake was observed (Chen et al., 2017), (Chen et al., 2020), (Ding et al., 2020), (Karim et al., 2021). Notably, the most commonly ligand used was galactose and its derivatives. A study aiming to investigate the effect of adding 15 mol% of DSPE-PEG2000-Galactose as a targeting ligand was carried out by (Chen et al., 2020). The reported cellular uptake by HepG2 cells was 1.5 higher for the modified liposomes (50%) than conventional liposomes (30%). Another study that evaluated the impact of using galactose was carried out by Ding et al., (2019). The aim of the study was to prepare a liposomal formulation for treating hepatoma. Three hydroxycamptothecin liposomes were prepared and modified using different three modifiers (Mono-galactose, Di-galactose, and Biotin-galactose). The concentration of the ligand was 1 mol%. The uptake was the highest when using the biotin--galactose (50%), followed by di-galactose (45%), and the least was recorded with mono-galactose (35%). This finding comes in-line with (Chiang et al., 2013) study. The researcher formulated a doxorubicin liposomal formulation for treating colon cancer. The prepared liposomes were modified with biotin attached to a polymer (PEG). The main findings showed excellent accumulation in liver cells and a decrease in the distribution to the healthy cells. The idea behind adding the biotin with the galactose is to trigger two receptors, ASGP-R which excessively clustered on hepatocytes and biotin receptors which present on

cancer cells. In addition, Li et al., (2017) experimented to trigger the tumour cells by conjugating biotin to a rhodamine-triphenylphosphonium probe showed a good tumour targeting. A strong fluorescence confirmed this obtained when applied to the tumour cells (Hela cells) compared to approximately no or weak fluorescence when using epithelial kidney cells (HEK293) (Li et al., 2017). This finding supports the previous two findings in a considering biotin a promising ligand for treating hepatocellular carcinoma.

Mannose was also used as a targeting modifier to the hepatocytes (Chen et al., 2017). This liposomal formulation has an average size of 148 nm and contains 10% Mannose-diesterlauricdiacid-cholesterol (Man-DLD-Chol) as a targeting ligand which was prepared in the lab. The reported FI was 400 a.u., and it was not compared to the conventional liposomes. Thus, it is difficult to determine if this observation can be promising only based on the reading of the FI. However, comparing with a control is recommended to give a better understanding.

Karim et al., (2021) conducted an experiment to investigate the effect of using different ligands when preparing liposomal formulation on reducing the hepatic injury. Here, the researchers prepared three groups (un-modified, chitosan, chitosan-pectin liposomes). The different liposomes were tested on L02 cells and the reported %CU was 190%, 150%, and 125% for chitosan-pectin, chitosan, and the un-modified liposomes, respectively. The higher cellular uptake of the dual-modified liposomes can be linked to the formation of high methoxylated pectin, which has a good cell membrane adhesive characteristic (Jiang et al., 2017). In addition, previous literature suggested that using multiple ligands can provide better stability and controlled release properties for the prepared liposomes compared to using a single ligand (Liu et al., 2017).

#### 2.3.5.1.3. Dual use of PEG and ligand

The impact of modifying the liposomes' surface with PEG and ligand on the cellular uptake was investigated in five studies (Jiang et al., 2020), (Liu et al., 2017), (Pireddu et al., 2018), (Wang et al., 2020), (Yin et al., 2018).

Liu et al., (2017) investigated the effect of using 1 mol% PEG2000-DSPE-Gal to modify PC liposomes and tested the cellular uptake of the encapsulated norcantharimide using HepG2 cells. The formulation was compared to the un-modified liposomes. The reported size was (105 and 128 nm) for the conventional and modified liposomes respectively. The authors concluded that the galactose surface modified liposomes exhibited higher accumulation rates within the cells compared to the conventional ones, as confirmed by the fluorescence intensity of coumarin (800 and 6000 a.u.) respectively. Wang et al., (2020) carried out a study to evaluate the cellular uptake of HepG2 cells for different modified liposomes (DSPE-PEG600 Vs DSPE-PEG600-Galactose). The fluorescence intensity showed a significance difference ($p<0.05$) between galactose liposomes (600000 a.u.) compared to the PEGylated liposomes (300000 a.u.).

This agrees with (Naicker et al., 2014) who proved that the Galactose ligand can increase the interaction between the cell and liposomes leading to better hepatocytes targeting. Asiloglycoprotein receptor (ASGP-R) is abundant on the surface of hepatocytes (500,000/cell) (Yan et al., 2008), which possess a high binding affinity to the galactose moieties resulting in drug endocytosis, and hence better drug delivery. Similarly, 1mol% of DSPE-PEG2000 and DSPE-PFG2000-Galactose was found to improve hepatocyte uptake of norcantharimide (Jiang et al., 2020). The reported size was (111 and 131 nm) for the conventional and targeted liposomes respectively. According to the findings, the reported fluorescence intensity of the conventional compared to the galactosylated liposomes was (7405 and 41925) respectively.

Pireddue et al., (2018) studied the effect of POPC conventional, stealth, and stealth-lactoferrin-targeted liposomes which has a size range of (94, 98, and 127 nm) respectively. The findings

showed that for the conventional liposomes %CU was 12%, and 20% and 41% for conventional, PEGylated and PEGylated-lactoferrin modified stealth liposomes, respectively. This can explain that; targeted liposomes have a higher affinity to certain receptors on the hepatocytes surface i.e., ASGP-R which enhance their uptake compared to the un-modified/PEGylated liposomes (Mirsha et al., 2013).

Yin et al., (2018) investigated the effect of DSPE-mPEG2000 and ceramide on the carcinoma hepatocytes uptake compared to the free drug. Results proved that both free coumarin and encapsulated coumarin in PEG-ceramide liposomal formulation showed the ability to internalize the cells (FI was 20 vs 13) respectively. The liposomal formulation endocytosed slower than the free coumarin, and the changes in the FI profile can be due different mechanisms in cell entry. (Yin et al., 2018).

Overall findings gave an impression on the positive impact of using ligand, specifically galactose alone or with the PEG on the cellular uptake. However, when compared with using PEG alone, the reported %CU or FI is lower than ligand-modified liposomes. Additional evaluation of the PK will be discussed in the next section to help in reaching a good targeting strategy for the hepatocytes.

#### 2.3.5.2. The impact on pharmacokinetics, bio-distribution, and efficacy

##### 2.3.5.2.1. PEGylation

Drug bio-distribution and accumulation are among the key elements of drug pharmacokinetics which affected by different liposome characteristics, including liposome size, surface charge, and surface modification (Song et al.,2012). In this review, there are six studies reported the effect of PEGylation on the PK and bio-distribution (Cheng et al., 2018), (Karimi et al., 2020), (Li et al., 2019), (Nunes et al., 2018), (Sesarman et al., 2019), (Wang et al., 2019).

Cheng et al., (2018) reported the effect of using 5% PEG-DSPE on the efficacy by measuring the tumour volume compared to the solution formulation. The tumour volume was reduced to

the half (900 vs. 400 $mm^3$) for the liposomes and the solution form respectively. Although the liposomal formulation was compared to the solution formulation, it is worth including the findings for the PEGylated liposomes. The improvement of the drug efficacy can be due to adding the PEG through increasing the circulation time which enhances the targeting probability. Findings are in line with (Baumann et al., 2014), who stated that PEG insertion increase half-life, thus improving the bio-distribution profile and can enhance the chances for the targeting.

Karimi et al., (2020) prepared un-modified and 5 mol% of DSPE-mPEG2000 HSPC liposomes. The average size was 129 and 115 nm for the PEGylated and conventional liposomes, respectively. These vehicles were tested on mice bearing tumours and the drug concentration on the liver was 250 and 450 ng/g for the PEGylated and conventional liposomes. The tumour volume was evaluated too. The findings showed a significance reduction (p-value<0.05) when using PEGylated liposomes (500 $mm^3$) compared to when using blank liposomes (600 $mm^3$). Li et al., (2019) has explored the effect of adding 3 mol% DSPE-PEG-cholic acid to the doxorubicin/silybin liposomes. The drug concentration on the liver was 35,000 ng/g for the conventional liposomes and 80,000 ng/g for the un-modified and PEGylated liposomes, respectively. In addition, the tumour has been reduced from 700 $mm^3$ to 200 $mm^3$ for the saline group and the PEGylated liposomes, respectively. Allen and Cullis, 2004 (2014) suggested that the success of the bio-distribution and drug efficacy is linked to the drug release characteristics, which was the highest in the study for the PEGylated liposomes. In addition, using PEG prolongs the circulation time, enhances the tumour accumulation, and thus the drug exerts the pharmacological desire effect at the target site (Karimi et al., 2020).

Wang et al., (2019) conducted an RCT on 12 patients with different metastatic cancers. The study aimed to determine the effectiveness of using liposomes for the delivery of radioactive renium-188 to the tumour. The activity of the drug was determined by monitoring the

pharmacokinetics and bio-distribution of the encapsulated agent. HSPC liposomes (90 nm) were prepared and decorated with 3 mol% DSPE-mPEG2000 using the post-insertion method. The pharmacokinetics parameters (AUC, CL, and elimination half-life) were measured over 72 hours. The maximum concentration was higher in the plasma than in blood (0.1 and 0.2 %ID/ml). The reported AUC was 0.33 %ID/ml.h and 0.5 %ID/ml.h for the blood and plasma respectively. The elimination half-life was 37 h in blood while 52 h in the plasma. The drug was cleared faster from the plasma (494 ml/h) compared to the blood (794 ml/h). The results revealed that the maximum bio-distribution was observed in the liver (15 ± 5 %ID/Kg) followed by spleen (13 ± 8 %ID/Kg). The maximum absorbed dose in normal organs was reported in spleen (1.4 ± 8 mGy/MBq) followed by liver (0.92 ± 5 mGy/MBq). The data showed that the accumulation in the hepatic tumour lesions was higher compared to healthy hepatic tissue (1.1 mGy/MBq) which gives suggests some selectivity. Wang et al., (2019) hypothesised that the higher uptake by the liver and spleen can be linked to the presence of the HSPC in the liposomal formulation which can be detected by the MPS system. The injected dose was evaluated in term of its safety via testing urine and blood samples and no serious adverse drug reaction was reported. However, mild symptoms including chillness and palpitation occurred and resolved on the same day. This study does not have a control to compare with but this formulation reminds us with the first approved liposomal formulations in the market (Doxil) (Soundararajan et al., 2009). Doxil is used in the treatment of a variety of cancers (breast, ovarian, and Kaposi's sarcoma) (Bulbake et al., 2017). It consists of HSPC: Chol: DSPE-PEG2000 (56:39:5) with a size close to the liposomes used by Wang et al. (2019) in their study. Doxil has HSPC as a phospholipid which has a high Tc (63 °C) and cholesterol, thus, more drug retention was obtained as the drug dose not leak at a degree below the body temperature (Bulbake et al., 2017). The incorporation of 5 mol% of DSPE-PEG2000 enhanced the pharmacokinetics properties of Doxorubicin. The exact mechanism for its drug accumulation is unknown.

However, it is suggested that the insertion of PEG increase the circulation time which enhance the chances for the drug to be circulated in the leaky vessels of the tumour site and thus been uptake by the tumour (Gabizon et al., 2003). Gabizon et al., (1994) reported the results of a clinical trial compared the pharmacokinetics of Doxil and free doxorubicin conducted on 15 patients. The overall pharmacokinetics has improved. The clearance was reduced from 45 L/h to 0.1 L/h, Vd was reduced from 254 L to 45 L, and the accumulation on the tumour was 4 to 16 times greater than the free doxorubicin. This can explain the reduction in the side effect of cardiotoxicity as low drug availability on the circulation to reach the heart and more accumulation on the target site besides to using the PEG in the formulation which makes the drug available in the circulation to enhances the chance for its targeting to the tumour (Batist et al., 2007). According to the evaluated PK parameters for the encapsulated radioactive material of the included study (Wang et al., 2019), the formulation showed a potential for being a good targeting system for treating tumour diseases. Additional comparative analysis between the free and liposomal formulation and testing the formulation on larger number of patients is recommended to give a better understanding of the targeting profile.

In contrast, Sesarman et al., (2019) formulated PEGylated (5 mol%) doxorubicin-curcumin liposomes. The accumulation in the liver was comparable to the free doxorubicin. The results were the same for both formulations (400 ng/g). This finding came in line with Nunes et al., (2018) who investigated the effect of using PEG on liposome accumulation and bio-distribution. 5 mol% of two PEG chain lengths were used (DSPE-PEG2000 and DSPE-PEG5000) and compared to the un-modified liposomes. The reported size was 212, 147, and 137nm for the un-modified, DSPE-PEG5000, and DSPE-PEG2000 liposomes, respectively. The accumulation in the liver was 3%, 4%, and 7% for the un-modified, DSPE-PEG2000, and DSPE-PEG5000, respectively. Although PEG is known for its advantage in increasing the half-life time which can improve the pharmacokinetics properties, this was not the case with these

two studies. The author suggested that this might be arisen due to the low rigid membrane of the liposomes when using DOPE lipids can explain these findings. DOPE tends to form a hexagonal form when it is in an acidic medium (Farhood et al., 1995).

**2.3.5.2.2.Ligand type and density**

Surface-modified liposomes appeared to positively impact the body's response to the administered drug (Allen et al., 1995). This includes altering drug's half-life time, clearance (CL), and area under the curve (AUC), bio-distribution and drug accumulation on the target site. Evidence reported that the addition of different ligand types i.e., carbohydrates, glycolipids, or polymers, can affect pharmacokinetics in several ways (Chen et al., 2017), (e Silva et al., 2018), (Fatouh et al., 2021), (Lian et al., 2021), (Liu et al., 2017), (Nie et al., 2019), (Xu et al., 2017), (Zhang et al., 2021), (Zhou et al., 2019), (Zhu et al., 2020). A study exploring the effect of using DSPE-PEG2000-Galactose on the pharmacokinetics behaviour of norcantharidin as an anticancer drug using mice as an animal model was carried out by (Liu et al., 2017). The comparative analysis of the un-modified and galactosylated-PEGylated liposomes was as the following AUC (2.319 ±0.121 and 6.700 ± 2.964 mg.h/L), T1/2 (0.413 ± 0.238 and 1.347 ± 0.519 h), and CL (5.055 ± 0.271 and 1.882 ± 0.579 L/h/Kg) for conventional and modified liposomes respectively. The prolonged T1/2 with glycosylated liposomes can be explained as a result of the PEG inclusion (Li and Huang, 2010; Photos et al., 2003). PEG and Galactose worked synergically in this preparation i.e., PEG increased the half-life time and galactose enhance the targeting properties. PEG density in this study played a key role in improving the pharmacokinetics characteristics whereas the inclusion of galactose improving the PK profile by being cleared more quickly to the target site. It was used with a molar ratio of 2 mol%. Thus, PEG arranged in a mushroom configuration and guarantee a full coverage and protection, increasing both AUC and T1/2 (Li and Huang, 2010; Photos et al., 2003).

Other studies proposed the effect of adding the ligand on the tumour volume by Zhang et al., (2021) and Lian et al., (2021). Zhang et al., (2021) investigated the impact of modifying lupeol-loaded liposomes using 2% DSPE-PEG2000-Gal. Lupeol is used as an anticancer drug (Thomas et al., 2008). In this study, the prepared liposomes were tested for their anti-tumour efficacy using mice. The results showed that the tumour was reduced from 300 $mm^3$ to 200 $mm^3$ in which doxorubicin liposomes modified with 5 mol% DSPE-mPEG2000. Both studies used DSPE-PEG2000-galactose as a targeting ligand to improve the selectively and reduce systemic toxicity for targeting the liver through ASGP-R. In addition, linking the ligand to PEG can enhance the circulation time and stabilize the liposomal formulation (Allen and Cullis, 2013).

Fatouh et al., (2020) addressed the impact of galactosylated chitosan on the targeting of liposomes containing ledipasvir to the liver in rats. The reported AUC was (25 μg·h/mL and 89 μg·h/mL), T1/2 has doubled i.e. (18 and 32 h) for free drug and the modified liposomes, respectively. The relative uptake by hepatocytes of the targeted liposomes was 3.4 times greater than the free drug. The bio-distribution studies revealed that, drug concentration on the liver was three times higher for the liposomal formulation compared to the dispersion form (300 ng/g Vs 1000 ng/g). Theoretically, it is suggested that the free drug lacks the ligand which facilitates its localization on the target site. Ultimately, this will lead to its accumulation in the systematic circulation in relatively higher amounts than the target site. Although this study compared a free drug to a liposomal formulation, these results are considered as a promising approach for liver targeting.

Chen et al., (2017) conducted a study about the impact of mannose-diesterlauricdiacid-cholesterol (Man-DLD-Chol) on liposomes containing glycyrrhetinic acid for targeting liver cells using rabbit as an animal model. The aim is to us the ligand to enhance the liver targeting through the mannose receptor. Comparing the conventional and targeted liposome, AUC was (1053 ±65.44 μg·h/ mL, 906 ± 49 μg·h/ mL), the T1/2 was (2.51±0.44 h, 1.78 ± 0.05 h), and

the CL was (5.00 ± 0.30 L/Kg/h and 5.81 ± 0.30 L/Kg/h) respectively. The slight difference in the clearance of mannosylated liposomes suggested their recognition and binding to mannose receptors. Theoretically, mannose as a sugar can be used to target KCs. However, in this case as mannose receptors are expressed on the surface of Kupffer cells which might play a role in their clearance (López-Guisa et al., 2011). The concentration of the drug on the liver was significantly higher with the target modified liposomes (12000 ng/g) compared to (5000 ng/g) of the conventional liposomes.

Nie et al., (2019) compared the effects of different galactose residues (GalNAC-terminated glycolipid vs cholesteryl-galactose) (5 mol% was applied for all the types) as targeting doxorubicin liposomes mice. The accumulated drug on the liver was 18, 59, 48, and 75% for the unmodified, cholesteryl-1-Galactose, Cholesteryl-6-Galactose, and N-acetylgalactosamine (GalNAc), respectively. This might arise because of a difference in the sugar structure on the liposomes' surface. The researchers suggested a theory explaining the advantages GalNAc and cholesteryl-galactose in liver targeting according to their structure. (GalNAc and cholesteryl-galactose) have *β*-Gal but differ in the glycosidic linkage in positions 1 and 6. Cholesteryl-1-Galactose has D-glucitol leading to an increase in the number of hydrogen bonds with ASGP-R, thus better accumulation on hepatocytes. GalNAc exhibited higher drug targeting properties explained by the fact ASGP-R has a high affinity for GalNAc-terminated glycolipid over Gal-terminated glycolipid (Khorev et al. 2008; Kinberger et al. 2016). eSilva et al., (2018) investigated the effect of cholesteryl-galactose (7 mol%) vs. glucosamine modified liposomes on liver targeting using mice as an animal model. The bio-distribution in the liver for galactosylated liposomes was higher 1.7 times compared to glucose modified one which was used as a control in this experiment. This finding is expected as the liver lack the glucose receptor and on the other hand abundant galactose receptors are present on the hepatocytes surface (e Silva et al., 2018).

Xu et al., (2017) prepared SPC liposomal formulation modified with 1% Galactose-DPPE and studied their bio-distribution against un-modified liposomes using mice as an animal model. The average size was 103 nm and 121 nm for the un-modified and the modified liposomes respectively. The reported concentration on the liver was three times greater when using galactose as a ligand (300 Vs 900). This can be explained as ASGP-R has a strong affinity for galactose on its surface leading to better targeting and drug accumulation at the target of interest (Rigopoulou et al., 2012).

Arguably, the addition of a ligand appeared to behave differently at certain extend. Zhou et al., (2019) investigated the effect of 11-DAG-3-O-Gal insertion into liposomes containing Cantharidin. The AUC was (596 ±18 μg·h/mL, 509 ± 16 μg·h/ mL), the $t_{1/2}$ was (0.25±0.08 h, 0.18 ± 0.09 h), and the CL was (0.57 ± 01 L/Kg/h and 0.74 ± 0.13 L/Kg/h) for the conventional and modified liposomes respectively. This high clearance can be linked to their removal to the target site. This was confirmed by the concentration of the drug on the liver which 4 times greater for the modified liposomes (2000 ng/g) compared to the conventional liposomes (500 ng/g).

Liposomes containing Cantharidin was evaluated and compared to the modified form using rats (Zhu et al., 2020). The results for the conventional and modified liposomes showed a significant difference (P-value<0.05) among the studied pharmacokinetics parameter similar to (Zhou et al., 2019) study. These findings suggested that modified liposomes cleared quickly from the plasma as they distributed in a higher rate in the tissues as they can be recognized easily by the ASGP-R. AUC considered as a key factor in the determination of the drug concentration at the systematic circulation (drug bioavailability) (Scheff et al., 2011). The more available drug, the higher chance for cellular uptake, and higher targeting would have achieved when having a targeting ligand on the surface (El-Kareh and Secomb, 2003). AUC has a proportional relationship with T1/2 and an inverse relationship with the clearance rate (Riviere, 2011).

#### 2.3.5.2.3.Dual use of PEG and ligand

The impact of using both ligand and PEG on the bio-distribution was investigated in two studies (Yin et al., 2018) and (Zhang et al., 2020). Liposomes containing DSPE-PEG750 and different sugar moieties (galactose, glucose, and mannose linked to DSPE-PEG600) were visualised using DiO dye and evaluated using rats (Zhang et al., 2020). The results showed the maximum accumulation in the liver when using galactose (15,000 a.u.) compared to (2000 and 5000 a.u.) for glucose and mannose. The maximum accumulation of the galactosylated liposomes is due to the high expression of ASGP-R on the hepatocytes. Although there are some mannose receptors in the macrophages, it is presents more on the lung cells which was proved by the fluorescence intensity in this study (Zhang et al., 2020). Yin et al., (2018) carried out an experiment to investigate the impact of ceramide and DSPE-mPEG2000 on sorafenib liposomes for treating hepatoma using H-22 tumour bearing mice. The results proved that, there was a significance reduction in the tumour volume by third i.e., from 900 mm3 when using the un-modified liposomes to 300 mm3 when using dual modified liposomes (P-value<0.05). This is due to that ceramide provide a synergetic effect when combined with sorafenib as it is known for its tumour suppressing property (Yin et al., 2018). This was also proved by (Feng et al., 2014) who formulated a docetaxel liposomal formulation using ceramide as a ligand and observed a synergetic effect and obtain better tumour inhibition.

In order to get an overview on how different factors can impact the cellular uptake and the bio-distribution, we applied a linear regression model.

### 2.3.6. Factors affecting liver targeting (results of linear regression modelling)

Performing meta-analysis of our data was not feasible. This is due to that the heterogeneity was high ($I^2 = 100\%$). Instead, linear regression modelling was performed to investigate the impact of liposomes' size on the cellular uptake, drug accumulation represented by the drug concentration on the liver, and tumour volume was evaluated. Appendix E provides raw data for the STATA

output (Table 2.2.E-2.4. E). Data reflecting the effects of inserting the PEG and ligand into the liposomal formulations were limited (less than ten observations). Therefore, it was not feasible to include these variables in the analysis.

**Table 2.2. Linear regression analysis of cellular uptake of the formulated liposomes against liposome size.**

| Predictor variable | $N_o$ studies | Cellular uptake (%) | | |
|---|---|---|---|---|
| | | Coef | sig | CI-95% |
| Liposome Size | 13 | 0.529 | 0.016 ** | (0.12-0.936) |

Table notes: *** p<.01, ** p<.05, * p<.1

**Table 2.3. Linear regression analysis of cellular uptake of the formulated liposomes against liposome size.**

| Predictor variable | $N_o$ studies | Drug accumulation in the liver (ng\g) | | |
|---|---|---|---|---|
| | | Coef | sig | CI-95% |
| Liposome Size | 14 | 48.343 | 0.094 | (-9.5-106) |

Table notes: *** p<.01, ** p<.05, * p<.1

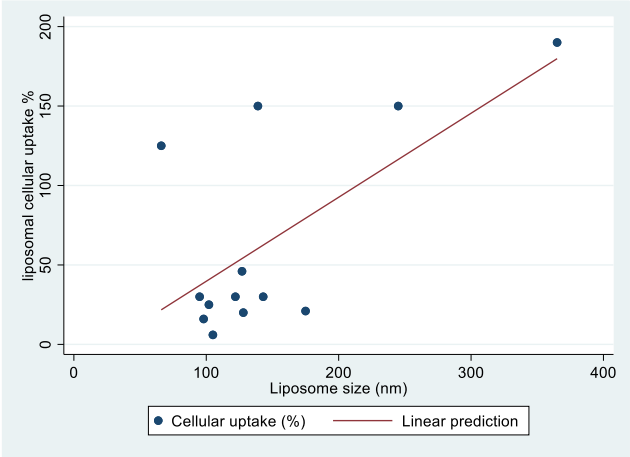
**Table 2.4. Linear regression analysis of cellular uptake of the formulated liposomes against liposome size.**

| Predictor variable | $N_o$ studies | Tumour volume (mm3) | | |
|---|---|---|---|---|
| | | Coef | sig | CI-95% |
| Liposome Size | 10 | -3.261 | 0.46 | (-13-6.5) |

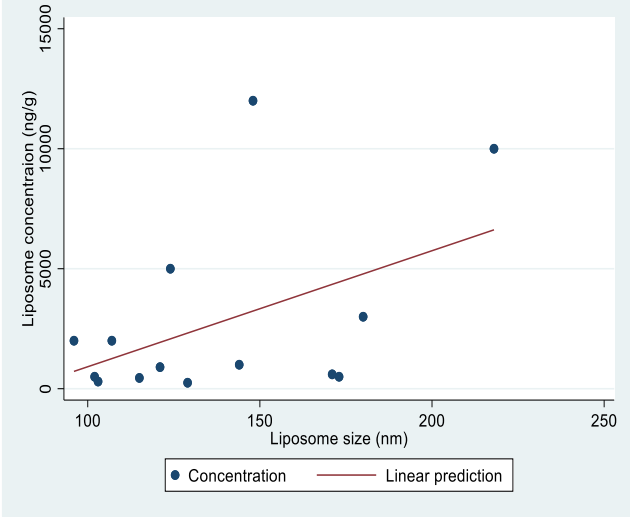
Table notes: *** p<.01, ** p<.05, * p<.1

Table 2.2. and Table 2.3 show the correlation between the liposomes size against cellular uptake and the drug concentration in the liver respectively. The positive correlation between liposomes' size and the cellular uptake was significant and confirmed by the p-value (0.016). Table 2.4. suggested that, there is an inverse relationship between the liposomes size and the tumour volume i.e., as the liposomes size increases the tumour growth reduced. However, this finding was not significant (p-value= 0.46).

(A)




(B)




(C)

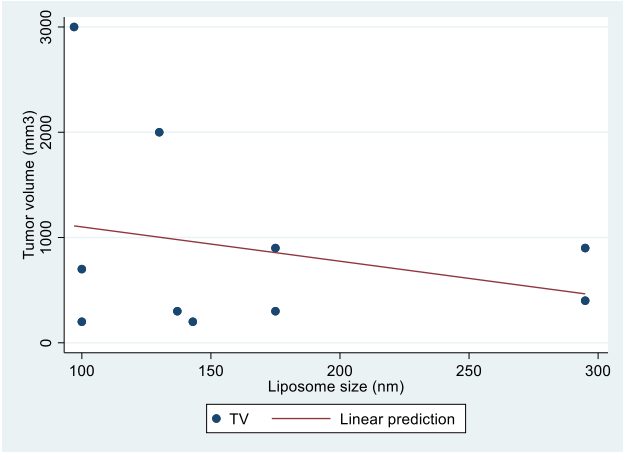



**Figure 2.3. Linear regression plot for liposome size vs cellular uptake (A), drug concentration in the liver (B), and tumour volume (C).**

Figure 2.3. demonstrates the above findings in the tables (2.2., 2.3., and 2.4.). This linear regression model investigated the effect of the liposome size on the cellular uptake, the drug accumulation, and the tumour volume. Most of the size range reported between 100-200 nm. Only few liposomes' sizes range were below or above 200 nm.

In term of cellular uptake, the size has a direct proportional relationship with the uptake i.e., as size increased, cellular uptake increase. However, it is important to emphasise that, the majority of liposomes has an average size between (100-200 nm), and only one reported liposomal formulation has an average size of ca. 350 nm (Karim et al., 2021). Liposomes' size is an important factor that impacts the cellular uptake and needs to be tailored carefully. Particle size over 200 nm is more susceptible to be capture by MPS (Elvevold et al., 2008), and smaller liposomes are more susceptible to instability and rapid elimination by the kidney (Elvevold et al., 2008). Park et al., (2016) investigated the impact of PLGA size on liver cellular uptake. NP size was 241 nm, and it was mostly taken up by Kupffer cells (89%), and only 4% was found in hepatocytes.

In order to avoid particle aggregation and MPS recognition, liposomes can be hidden through modulating their hydrophilicity (Kelf et al., 2010). Although we were unable to predict the impact of the PEG on the cellular uptake, it is good to consider and discuss this point based on the previous literatures as it is one of the most common methods used to ontain stealth properties and increase the liposomes' half-life time. Vasir and Labhasetwar (2008) compared the cellular uptake for un-modified and PEGylated PLGA. Findings showed that modified NPs had five-folds higher uptake compared to the un-modified ones. However, PEG chain length and concentration has an impact on cellular uptake. Pozziet al., (2014) suggested that the use of 1 and 2KDa PEG associated with uptake enhancement. However, the PEGylation with PEG-5K reduces the cellular uptake to half. Two theories can explain this phenomena. First, PEG with a longer chain length fold up on the surface rather than stretch. Second, long PEG chains interact

via short van der walls attraction force and forming inter-chain hydrogen bonds, and ultimately leads to chain enlargement. This will lead to phase-separated lamellar structure, which impacts their uptake (Kelf et al., 2010).

Carbohydrate modified liposomes can be used as a targeting ligand alone or combined with PEG (Lemarchand et al., 2004). Galactose appeared to be the ligand of choice for hepatocytes targeting according to the qualitative narrative analysis (Figure 2.2.E). This is due to their abundance expression as targeting moieties on ASGP-R which present selectively on the hepatocytes' surface (Witzigman 2017). The impact of using ligand on the cellular uptake was not feasible to be predicted using the linear regression modelling. However, we can give an overview on their effect according to the previous studies. Wang et al., (2020) reported the difference in the cellular uptake between un-modified and DSPE-PEG2000-Galactose liposomes. The reported florescence intensities were 35000 and 65000, respectively. Another study conducted by (Li et al., 2018), who studied the effect of different galactose concentrations on cellular uptake. Findings showed a concentration-dependent cellular uptake pattern and the receptor saturation occurred when using a ligand with a concentration higher than 20 mol%.

It is difficult to predict the behaviour of liposomes containing dual modification because of that, data was not enough to build a prediction. There were only five studies discussed the impact of using PEG and the targeting ligand for liver targeting (Jiang et al., 2020), (Liu et al., 2017), (Pireddu et al., 2018), (Wang et al., 2020), (Yin et al., 2018). Three out of five studies used galactose as a targeting ligand (Jiang et al., 2020), (Liu et al., 2017), (Wang et al., 2020). This is data is not enough to build a robust decision. However, some literature addressed their expectations earlier. Hstoshyar et al., (2016) suggested that decorating NPs surface with PEG and ligand would lead to an increase in the cellular uptake. This is due to that PEG will mask the NPs from the MPS recognition, and the ligand would facilitate their binding to the receptor and their uptake. It is important to take in consideration that; PEG chain length is an important

factor can impact the cellular uptake. Long chain lengths (2,3.5, 5, and 10KDa) has the advantage of presenting the ligand more clearly. However, long chains tend to form a mushroom configuration and thus hinder the targeting ligand in the PEG coat and reduce their targeting effectiveness (Stefanick et al., 2013). On the other hand, using shorter PEG lengths (350, 550, and 750 KDa) associated with more thermodynamic interaction between the ligand and the receptor (Stefanick et al., 2013). Stefanick et al., (2013) evaluated different PEG: ligand lengths using a constant 5% for PEG. Findings proved that using adjusted short PEG and peptide as a targeting ligand associate with the formation of linear configuration and better cellular uptake unlike longer chain lengths. This finding came in line with (Abstiens et al., 2018; Ding et al., 2014) study. Both studies studied the effect of NPs ligand density and linker length on cellular uptake. The surface of the polymeric nanoparticles was functionalised with different ligand (0-100%) concentrations connected to different PEG spacer lengths (2,3.5, and 5KDa). The maximum cellular uptake was observed with 100% functionalisation connected to a small PEG length confirmed by the fluorescence intensity. This can be explained as that with PEG 2KDa, stretching (brush) can be formed compared to the medium and high PEG chain, which fold up (mushroom configuration) and reduce their interaction with the cells rather than with smaller PEG chains where linear configurations can be obtained.

The liposomes' size has a direct proportional relationship on drug accumulation on the liver (Figure 2.3.B). This prediction agrees with Tang et al., (2014) study who investigated the optimal NP size to be used for anticancer therapy. In this experiment, different size ranges of PEG coated silica NPs containing camptothecin as an anticancer API were prepared (20, 50, and 200 nm). The formulated NPs were tested MCF-7 cells. The drug accumulation on the liver was the maximum with 50 nm size which is higher than 20 nm and 200 nm by 84% and 24% respectively. Similar findings were reported by (Tang et al., 2012). When compared with 200 nm, smaller sizes have better accumulation profile on the cancer cells could be due to that

smaller sizes are likely have more efficient probability to penetrate the cells by enhanced permeation and retention effect. Although 20 nm has smaller size compared to 50 nm, their lower accumulation on the cancer cells can be linked to their fast clearance from the circulation (Perrault et al., 2009).

Liposomes' size and efficacy on reducing tumour volume has an inverse relationship i.e. as the liposomes' size increases the efficacy for drug to reduce the tumour volume decrease (Figure 2.3.C). A study carried out by (Bao et al., 2016) aiming of determining the optimum size nanomedicine antitumor activity. The researchers prepared 10, 25, and 50 nm gold nanoparticles containing hydroxycamptothecin which is an anticancer API. The formulations were tested on MDA-MB-321 (breast carcinoma cells) of bearing mice. The anti-tumour activity represented by the changes in tumour growth was significantly different between the three groups (P-value<0.05). The maximum anti-tumour activity was for 10 nm (905±104 mm3), 50 nm followed by 25 nm (648±87 mm3) and the least was for 50 nm (529±79 mm3). This came in line with (Tang et al., 2014) which discussed in the previous point of the relationship between liposomes' size and the drug accumulation and proved that 50 nm was the best. This can be explained as much drug accumulated on the desired site, drug would exert its pharmacological activity at higher extent. Cabral et al., (2011) investigated the effective size in cancer treatment through preparing different PEGylated liposomes (30, 50, 70, and 100 nm). The anticancer activity of the formulated micelles was tested on pancreatic tumour using BxPC3 cells. The accumulation of the liposomes was the maximum with 30 nm which was two times higher than 50 nm, and four times higher than both 70 and 100 nm micelles. This finding suggests there is a size-dependent of the prepared liposomes on reducing tumour volume. However, it is important to explain that, although having small micelles are susceptible for quick clearance from the circulation, they are necessary to enhance the tumour penetration (Perrault et al., 2009). Thus, it is suggested to include the PEG on the surface of the NPs to

escape the quick clearance by the MPS and facilitating the tumour penetration of the smaller size NPs (Bozzuto and Molinari, 2015). On contrary, Bae et al., (2011) hypothesized that, increasing the liposomes size up to 200 nm can increase the tumour accumulation and retention with majority on liver and spleen. However, some factors can play role in determining the limit of accumulation including tumor model and the degree of fenestration in which the size needs to be smaller than the cut-off it (Bertrand et al., 2014).

In conclusion, for liver targeting, it is suggested to formulate liposomes with a size range between 100-200 nm. According to the narrative analysis and the linear regression model, liposomes' size with a range (100-200 nm) impact positively on the cellular uptake, the drug accumulation on the hepatocytes, and tumour volume reduction. Adding PEG appeared to increase the liposomes' circulation time. However, it did show a reduction in the cellular uptake. Using ligand specifically galactose improved the hepatocytes targeting via binding to ASGP-R. The most common concentration used for both PEG and ligand is 5 mol% and lower. A dual surface modification for the liposomes i.e., coating the surface with PEG and galactose appeared to be a promising strategy. By applying the suggested strategy, longer circulation time is provided, and the liposomes can escape the macrophages present in the liver as a function of the PEG while the galactose act as a targeting ligand to facilitate the liposomes' attachment with ASGP receptor present in the hepatocyte to give the desired outcome.

# Appendices

## Appendix A

**Table 2.A. Keywords for database search**

| Key | Operator |
|---|---|
| Domain 1: Liposome, Liposomes. | Joint by OR |
| Domain 2: Liver targeting, Hepatocytes. | Joint by OR |
| Domain 3: Cellular uptake, Pharmacokinetic, Pharmacokinetics, Liver accumulation. | Joint by OR |
| Results of domain 1,2, and 3. | Joint by AND |

## Appendix B

What is the best strategy for targeting hepatocytes?

Medline search strategy “Problem and intervention”

Accessed on 29/12/2021

1. Liposomes/ or Liposome*.mp.

2. Liver targeting.mp.

3. Hepatocytes.mp.

4. cellular uptake.mp.

5. Pharmacokinetic*.mp. or pharmacokinetics/.mp.

6. Liver accumulation.mp.

7. 1 OR 2 OR 3

8- 4 OR 5 OR 6

9- 7 AND 8

10. limit 9 to last 5 year

## Appendix C

**Table 2.C. data extraction tool for the included studies.**

| Reference | Liposome composition | Liposo me size | PEG type and density | Ligand type and density | Drug used and concentrat ion | Study design (In-vitro, In-vivo, RCT) | Cell line/Animal used, Number of patients | The reported outcome (%CU, FI, drug concentration, drug accumulation, change in tumour growth … etc) | Effective size |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| | | | | | | | | | |

## Appendix D

**Table 2.1.D. CASP tool for RCT study.**

| The domain | Description of the domain | Yes, No, Cannot tell |
|---|---|---|
| Domain A: Is the basic study design valid for a randomised controlled trial? | **Did the study address a clearly focused research question?**<br>*CONSIDER:*<br>• *Was the study designed to assess the outcomes of an intervention?*<br>• *Is the research question 'focused' in terms of:*<br>  • *Population studied*<br>  • *Intervention given*<br>  • *Comparator chosen*<br>*Outcomes measured?* | |
| | **Was the assignment of participants to interventions randomised?**<br>*CONSIDER:*<br>• *How was randomisation carried out? Was the method appropriate?*<br>• *Was randomisation sufficient to eliminate systematic bias?*<br>• *Was the allocation sequence concealed from investigators and participants?* | |
| | **Were all participants who entered the study accounted for at its conclusion?**<br>*CONSIDER:*<br>• *Were losses to follow-up and exclusions after randomisation accounted for?* | |

| | | |
|---|---|---|
| | • *Were participants analysed in the study groups to which they were randomised (intention-to-treat analysis)?*<br>• *Was the study stopped early? If so, what was the reason?* | |
| Domain B: Was the study methodologically sound? | • **Were the participants 'blind' to intervention they were given?**<br>• **Were the investigators 'blind' to the intervention they were giving to participants?**<br>• **Were the people assessing/analysing outcome/s 'blinded'?** | |
| | **Were the study groups similar at the start of the randomised controlled trial?**<br>*CONSIDER:*<br>• *Were the baseline characteristics of each study group (e.g. age, sex, socio-economic group) clearly set out?*<br><br>*Were there any differences between the study groups that could affect the outcome/s?* | |
| | **Apart from the experimental intervention, did each study group receive the same level of care (that is, were they treated equally)?**<br><br>*CONSIDER:*<br>• *Was there a clearly defined study protocol?*<br>• *If any additional interventions were given (e.g. tests or treatments), were they similar between the study groups?*<br><br>*Were the follow-up intervals the same for each study group?* | |
| Domain C: What are the results? | **Were the effects of intervention reported comprehensively?**<br><br>*CONSIDER:*<br>• *Was a power calculation undertaken?*<br>• *What outcomes were measured, and were they clearly specified?*<br>• *How were the results expressed? For binary outcomes, were relative and absolute effects reported?*<br>• *Were the results reported for each outcome in each study group at each follow-up interval?*<br>• *Was there any missing or incomplete data?*<br>• *Was there differential drop-out between the study groups that could affect the results?*<br>• *Were potential sources of bias identified?*<br>• *Which statistical tests were used?*<br>• *Were p values reported?* | |

| | | |
|---|---|---|
| | | |
| | **Was the precision of the estimate of the intervention or treatment effect reported?**<br><br>*CONSIDER:*<br>*Were confidence intervals (CIs) reported?* | |
| | **Do the benefits of the experimental intervention outweigh the harms and costs?**<br><br>*CONSIDER:*<br>• *What was the size of the intervention or treatment effect?*<br>• *Were harms or unintended effects reported for each study group?*<br><br>*Was a cost-effectiveness analysis undertaken? (Cost-effectiveness analysis allows a comparison to be made between different interventions used in the care of the same condition or problem.)* | |
| Domain D: Will the result help locally? | **Can the results be applied to your local population/in your context?**<br><br>*CONSIDER:*<br>• *Are the study participants similar to the people in your care?*<br>• *Would any differences between your population and the study participants alter the outcomes reported in the study?*<br>• *Are the outcomes important to your population?*<br>• *Are there any outcomes you would have wanted information on that have not been studied or reported?*<br><br>*Are there any limitations of the study that would affect your decision?* | |
| | **Would the experimental intervention provide greater value to the people in your care than any of the existing interventions?**<br><br>*CONSIDER:*<br>• *What resources are needed to introduce this intervention taking into account time, finances, and skills development or training needs?*<br>• *Are you able to disinvest resources in one or more existing interventions in order to be able to re-invest in the new intervention?* | |

**Table 2.2.D. Syrcle's tool for In-vivo study.**

| Type of bias | The domain | Description of the domain | Yes, No, Unclear |
|---|---|---|---|
| Selection bias | Sequence generation | Describe the methods used, if any, to generate the allocation sequence in sufficient detail to allow an assessment whether it should produce comparable groups. | |
| | Baseline characteristics | Describe all the possible prognostic factors or animal characteristics, if any, that are compared in order to judge whether or not intervention and control groups were similar at the start of the experiment. | |
| | Allocation concealment | Describe the method used to conceal the allocation sequence in sufficient detail to determine whether intervention allocations could have. | |
| Performance bias | Random housing | Describe all measures used, if any, to house the animals randomly within the animal room. | |
| | Blinding | Describe all measures used, if any, to blind trial caregivers and researchers from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective. | |
| Detection bias | Random outcome assessment | Describe whether or not animals were selected at random for outcome assessment, and which methods to select the animals, if any, were used | |
| | Blinding | Describe all measures used, if any, to blind outcome assessors from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective. | |
| Attrition bias | Incomplete | Describe the completeness of outcome data for each main outcome, including attrition and exclusions from the analysis. State whether attrition and | |

| | | | |
|---|---|---|---|
| | outcome data | exclusions were reported, the numbers in each intervention group (compared with total randomized animals), reasons for attrition or exclusions, and any re-inclusions in analyses for the review. | |
| Reporting bias | Selective outcome reporting | State how selective outcome reporting was examined and what was found. | |
| Other | Other sources of bias | State any important concerns about bias not covered by other domains in the tool. | |

**Table 2.3.D. Adapted tool for In-vitro study.**

| **Quality assessment tool for In-vitro studies** | | **Description** | **Yes,No, Unclear.** |
|---|---|---|---|
| Sample Selection & Experimental Setup | Do authors report a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates? (Yes, No, Unclear) | Technical replicates: a test performed on the same sample multiple times<br>Biological replicates: a test performed on biologically distinct samples representing an identical time point or treatment dose (Altogen Biosystems, 2017, Lazic et al., 2018) | |
| | Is the experimental unit clearly stated? (Yes, No) | Three types are defined by Lazic as biological unit of interest (BU), experimental unit (EU) and Observational unit (OU). BU is the entity about which interferences are made – the associated experiment tests a hypothesis, estimate a specific property that leads to a conclusion. The experimental unit (EU) is randomly and independently assigned to the test environment (the sample size N is equal to number of EUs). There are other factors that affect it – this can be a BU of interest, group of Bus, parts of a BU or a sequence observations on a BU. The ideal approach to genuine replication Is random and independent assignment. Moreover, treatment should be applied independently to each EU and EUs should not influence each other. If this cannot be met – a different unit can be used as EU – this can be one level up in the biological and | |

| | | | |
|---|---|---|---|
| | | technical hierarch. Finally observational unit is the entity which the measurement are taken. The latter might be different from EUs and Bus. The increase in OUs does not increase the sample size (Lazic et al., 2018). | |
| | Do authors report a statement of how many times the experiment shown was replicated in the laboratory? (Yes, No, Unclear) | "In cell culture experiments cells are often both the OUs and BUs of interest, but rarely the EU.<br>Suppose an aliquot of cells is thawed and the cell suspension is pipetted into different wells of a microtiter plate. Cells are randomised to wells, and then wells to treatments, so the first criterion is met. But treatments are applied simultaneously to all cells in a well, not independently to each cell, so the second criterion is not met. In addition, it is unreasonable to assume that cells in a well have no influence on each other; they form cell-to-cell connections, release signalling molecules, and compete for the same nutrients in the media. Hence, the third criterion is not met for using cells as the EU. Thus, a well, culture dish, or another plastic container is the appropriate EU for cell culture experiments." …. "The multiple wells on each day are then treated as subsamples and do not contribute to N (for example, by averaging values across wells in the same condition on each day)". | |
| | Is the sex of the cells sufficiently reported? (Yes, No, Unclear) | Sex is our experimental variable, there needs to be an adequate description of which data belongs to which sex (Shah et al., 2014). 10.1152/ajpcell.00281.2013 | |
| Reagents & Cells | Is the source of cell lines provided? (Yes, No, not applicable) | If it explains the source of the cell lines e.g Neonatal foreskin keratinocytes (NFSKs) select yes. If it does not, the answer is no. if the study does not provide cell lines, it will be not applicable (NPQIP, 2016). | |
| | Do the authors report whether the lines used have been authenticated recently (e.g., by STR profiling: within 1 year of use)? (Yes, No, not applicable.) | If the article has a sentence saying that cell authentication was done within a year from publication using Short Tandem Repeat (STR) profiling, then the answer is yes. If the statement is not available, the answer is no and if they do not report cell lines, it is not applicable(NPQIP, 2016). | |

| | | | |
|---|---|---|---|
| | | | |
| | Do the authors report whether the lines used have been tested for mycoplasma contamination recently (within 6 months of use)? (Yes, No, not applicable) | If there is a statement reporting the contamination, the answer is yes. If not, it is no. If no cell lines were used, the answer is no(NPQIP, 2016). | |
| | Do authors report a description allowing the reading to understand whether cells are primary cell cultures or continuous cell line? (Yes, No) | Primary cell cultures most closely represent the tissue of origin. When sub cultured they have a finite lifespan and are more prone to significant changes with increasing passage as they adapt to in vitro culture. This means the population doubling number should be carefully recorded. Continuous cell line, e.g. derived from a human cancer, can be passaged an infinite number of times (Public Health England) | |
| | Is the passage number and/or population doubling numbers adequately reported? (Yes, No) | *"The passage number of a cell culture is a record of the number of times the culture has been sub-cultured, i.e. harvested and reseeded into multiple 'daughter' cell culture flasks. The question about whether thawing cells represents a passage or not is one that is asked frequently. When cells are trypsinised for freezing and then thawed and reseeded, this represents one passage, albeit with time out in the freezer. As a passage is recognised as the transfer of the cells to another culture dish, the passage number should be increased on reseeding, but not on freezing."(Public Health England)* | |
| | Has every antibody used in this manuscript been profiled for use in the system under study, by either citation, catalogue number, clone number or validation profile? (Yes, No, Unclear) | ABB to add here from NPQIP study | |
| Sample Size | Does the manuscript describe how the sample size was chosen to ensure adequate power to detect a pre-specified effect size? (Yes, No, Unclear) | | |

| | | | |
|---|---|---|---|
| | - If Yes, Was the sample size calculation appropriate? (Yes, No, Unclear) | | |
| Allocation to groups | Was the methods of allocation samples to experimental groups appropriate?<br>(If it is randomised, the answer is yes, otherwise is no or unclear) | | |
| Allocation Concealment | Were the investigators blinded to the group allocation during the experiment? (Yes, No, Unclear) | | |
| Blinded Assessment of Outcome | Were the investigators blinded to the group allocation when assessing the outcome(s)? (Yes, No, Unclear) | | |
| Attrition | Does the manuscript describe if samples were excluded from the analysis? (Yes, No, Unclear) | | |
| | If yes, were exclusion criteria pre-defined? (Yes, No, Unclear) | | |

**Appendix E**

**Table 2.1.E. Linear regression analysis of the cellular uptake against liposomes size.**

| Cellular uptake | Coef. | St.Err. | t-value | p-value | [95% Conf | Interval] | Sig |
|---|---|---|---|---|---|---|---|
| Liposome size (nm) | 0.529 | .185 | 2.85 | .016 | .121 | .936 | ** |
| Constant | -13.125 | 30.628 | -0.43 | .677 | -80.537 | 54.288 | |
| Mean dependent var | | 64.538 | SD dependent var | | | 63.999 | |
| R-squared | | 0.425 | Number of obs | | | 13.000 | |
| F-test | | 8.144 | Prob > F | | | 0.016 | |
| Akaike crit. (AIC) | | 140.779 | Bayesian crit. (BIC) | | | 141.909 | |

*** p<.01, ** p<.05, * p<.1*

**Table 2.2.E. Linear regression analysis of the drug concentration in the liver against liposomes size.**

| Concentration | Coef. | St.Err. | t-value | p-value | [95% Conf | Interval] | Sig |
|---|---|---|---|---|---|---|---|
| Liposome size | 48.343 | 26.56 | 1.82 | .094 | -9.526 | 106.212 | * |
| Constant | -3917.864 | 3778.172 | -1.04 | .32 | -12149.794 | 4314.067 | |
| Mean dependent var | | 2750.000 | SD dependent var | | | 3753.460 | |
| R-squared | | 0.216 | Number of obs | | | 14.000 | |
| F-test | | 3.313 | Prob > F | | | 0.094 | |
| Akaike crit. (AIC) | | 269.732 | Bayesian crit. (BIC) | | | 271.010 | |

*** p<.01, ** p<.05, * p<.1*

**Table 2.3.E. Linear regression analysis of the tumour volume in the liver against liposomes size.**

| Tumour volume | Coef. | St.Err. | t-value | p-value | [95% Conf | Interval] | Sig |
|---|---|---|---|---|---|---|---|
| Liposome size | -3.261 | 4.226 | -0.77 | .463 | -13.006 | 6.484 | |
| Constant | 1427.07 | 756.962 | 1.89 | .096 | -318.488 | 3172.628 | * |
| Mean dependent var | | 890.000 | SD dependent var | | | 919.481 | |
| R-squared | | 0.069 | Number of obs | | | 10.000 | |
| F-test | | 0.595 | Prob > F | | | 0.463 | |
| Akaike crit. (AIC) | | 167.084 | Bayesian crit. (BIC) | | | 167.689 | |

*** p<.01, ** p<.05, * p<.1*

## References


Abdelwahed, W., Degobert, G., Stainmesse, S. and Fessi, H., (2006). Freeze-drying of nanoparticles: formulation, process and storage considerations. *Advanced drug delivery reviews*, 58(15), pp.1688- 1713.

Abstiens, K., Gregoritza, M., & Goepferich, A. M. (2018). Ligand density and linker length are critical factors for multivalent nanoparticle–Receptor interactions. *ACS applied materials & interfaces*, *11*(1), 1311-1320.

Alavizadeh, S. H., Badiee, A., Golmohammadzadeh, S., & Jaafari, M. R. (2014). The influence of phospholipid on the physicochemical properties and anti-tumour efficacy of liposomes encapsulating cisplatin in mice bearing C26 colon carcinoma. *International journal of pharmaceutics*, *473*(1-2), 326-333.

Alexopoulos, E. C. (2010). Introduction to multivariate regression analysis. *Hippokratia*, *14*(Suppl 1), 23.

Allen, T. M., Brandeis, E., Hansen, C. B., Kao, G. Y., & Zalipsky, S. (1995). A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1237*(2), 99-108.

Allen, T. M., & Cullis, P. R. (2004). Drug delivery systems: entering the mainstream. *Science*, *303*(5665), 1818-1822.

Anselmo AC, Mitragotri S. Nanoparticles in the clinic : an update. Bioeng Transl Med. 2019.

Bae, Y. H., & Park, K. (2011). Targeted drug delivery to tumours: myths, reality and possibility. *Journal of controlled release*, *153*(3), 198.

Bao, H., Zhang, Q., Xu, H., & Yan, Z. (2016). Effects of nanoparticle size on antitumor activity of 10-hydroxycamptothecin-conjugated gold nanoparticles: in vitro and in vivo studies. *International Journal of Nanomedicine*, *11*, 929.

Batist, G. (2007). Cardiac safety of liposomal anthracyclines. *Cardiovascular toxicology*, *7*(2), 72-74.

Baumann, A., Tuerck, D., Prabhu, S., Dickmann, L., & Sims, J. (2014). Pharmacokinetics, metabolism and distribution of PEGs and PEGylated proteins: quo vadis?. *Drug Discovery Today*, *19*(10), 1623-1631.

Bertrand, N., Wu, J., Xu, X., Kamaly, N., & Farokhzad, O. C. (2014). Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. *Advanced drug delivery reviews*, *66*, 2-25.

Bozzuto, G. and Molinari, A. (2015). Liposomes as nanomedical devices. *International Journal of Nanomedicine,* p.975.

Brice, R., 2022. *CASP CHECKLISTS - CASP - Critical Appraisal Skills Programme*. [online] CASP - Critical Appraisal Skills Programme. Available at: <https://casp-uk.net/casp-tools-checklists/> [Accessed 20 February 2022].

Bulbake, U., Doppalapudi, S., Kommineni, N., & Khan, W. (2017). Liposomal formulations in clinical use: an updated review. *Pharmaceutics*, *9*(2), 12.

Cabral, H., Matsumoto, Y., Mizuno, K., Chen, Q., Murakami, M., Kimura, M., ... & Kataoka, K. (2011). Accumulation of sub-100 nm polymeric micelles in poorly permeable tumours depends on size. *Nature nanotechnology*, *6*(12), 815-823.

Chen, J., Chen, Y., Cheng, Y., & Gao, Y. (2017). Glycyrrhetinic acid liposomes containing mannose-diester lauric diacid-cholesterol conjugate synthesized by lipase-catalytic acylation for liver-specific delivery. *Molecules*, *22*(10), 1598.

Chen, X., Hu, X., Hu, J., Qiu, Z., Yuan, M., & Zheng, G. (2020). Celastrol-loaded galactosylated liposomes effectively inhibit AKT/c-Met-triggered rapid hepatocarcinogenesis in mice. *Molecular Pharmaceutics*, *17*(3), 738-747.

Cheng, Y., Zhao, P., Wu, S., Yang, T., Chen, Y., Zhang, X., ... & Xiang, G. (2018). Cisplatin and curcumin co-loaded nano-liposomes for the treatment of hepatocellular carcinoma. *International journal of pharmaceutics*, *545*(1-2), 261-273

Chiang, Y. T., Cheng, Y. T., Lu, C. Y., Yen, Y. W., Yu, L. Y., Yu, K. S., ... & Lo, C. L. (2013). Polymer–liposome complexes with a functional hydrogen-bond cross-linker for preventing protein adsorption and improving tumor accumulation. *Chemistry of Materials*, *25*(21), 4364-4372.

Chu, C., Xu, P., Zhao, H., Chen, Q., Chen, D., & Hu, H. et al. (2016). Effect of surface ligand density on cytotoxicity and pharmacokinetic profile of docetaxel loaded liposomes. *Asian Journal Of Pharmaceutical Sciences*, *11*(5), 655-661. doi: 10.1016/j.ajps.2016.04.001

Ding, H., Sagar, V., Agudelo, M., Pilakka-Kanthikeel, S., Atluri, V. S. R., Raymond, A., ... & Nair, M. P. (2014). Enhanced blood–brain barrier transmigration using a novel transferrin embedded fluorescent magneto-liposome nanoformulation. *Nanotechnology*, *25*(5), 055101.

Ding, R., Li, Z., Wang, J., Zhu, X., Zhao, Z., & Wang, M. (2019). Design and synthesis of galactose-biotin lipid materials for liposomes to promote the hepatoma cell–targeting effect. *Journal of Pharmaceutical Sciences*, *108*(9), 3074-3081.

El-Kareh, A., & Secomb, T. (2003). A Mathematical Model for Cisplatin Cellular Pharmacodynamics. *Neoplasia*, *5*(2), 161-169. doi: 10.1016/s1476-5586(03)80008-8

Elvevold, K., Smedsrød, B., & Martinez, I. (2008). The liver sinusoidal endothelial cell: a cell type of controversial and confusing identity. *American Journal Of Physiology-Gastrointestinal And Liver Physiology*, *294*(2), G391-G400. doi: 10.1152/ajpgi.00167.2007

e Silva, A. T. M., Maia, A. L. C., de Oliveira Silva, J., de Barros, A. L. B., Soares, D. C. F., de Magalhães, M. T. Q., ... & Ramaldes, G. A. (2018). Synthesis of cholesterol-based neoglycoconjugates and their use in the preparation of liposomes for active liver targeting. *Carbohydrate research*, *465*, 52-57.

Farhood, H., Serbina, N., & Huang, L. (1995). The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1235*(2), 289-295.

Fatouh, A. M., Elshafeey, A. H., & Abdelbary, A. (2021). Galactosylated chitosan coated liposomes of ledipasvir for liver targeting: Chemical synthesis, Statistical optimization, In-vitro and In-vivo evaluation. *Journal of Pharmaceutical Sciences*, *110*(3), 1148-1159.

Feng, L. X., Li, M., Liu, Y. J., Yang, S. M., & Zhang, N. (2014). Synergistic enhancement of cancer therapy using a combination of ceramide and docetaxel. *International journal of molecular sciences*, *15*(3), 4201-4220.

Fonte, P., Reis, S. and Sarmento, B., (2016). Facts and evidences on the lyophilization of polymeric nanoparticles for drug delivery. Journal of controlled release, 225, pp.75-86.

Gabizon, A., Shmeeda, H., & Barenholz, Y. (2003). Pharmacokinetics of pegylated liposomal doxorubicin. *Clinical pharmacokinetics*, *42*(5), 419-436.

Gabizon, A., Catane, R., Uziely, B., Kaufman, B., Safra, T., Cohen, R., ... & Barenholz, Y. (1994). Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer research*, *54*(4), 987-992.

Garbuzenko, O., Barenholz, Y., & Priev, A. (2005). Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. *Chemistry and physics of lipids*, *135*(2), 117-129.

Giorgi, M. E., Agusti, R., & de Lederkremer, R. M. (2014). Carbohydrate PEGylation, an approach to improve pharmacological potency. *Beilstein journal of organic chemistry*, *10*(1), 1433-1444.

Gorad, R., Mandlik, S. and Gujar, K. (2013). LIVER SPECIFIC DRUG TARGETING STRATEGIES: A REVIEW. *International Journal Of Pharmaceutical Sciences and Research*, 4(11), pp.4145-4157.

Guides.temple.edu. 2022. *Research Guides: Systematic Reviews & Other Review Types: What is a Systematic Review?*. [online] Available at: <https://guides.temple.edu/c.php?g=78618&p=4178713> [Accessed 13 July 2022].

Hooijmans, C., Rovers, M., de Vries, R., Leenaars, M., Ritskes-Hoitinga, M. and Langendam, M., 2014. SYRCLE's risk of bias tool for animal studies. *BMC Medical Research Methodology*, 14(1).

Immordino, M., Dosio, F. and Cattel, L. (2006). Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. International Journal of Nanomedicine, 1(3), pp.297–315.

Iredale, J. (2008). Defining therapeutic targets for liver fibrosis: Exploiting the biology of inflammation and repair. *Pharmacological Research*, 58(2), pp.129-136.

Jiang, Y., Liu, X., Tan, X., Hou, Y., Sun, W., Gou, J., ... & Tang, X. (2020). In Vitro and In Vivo Evaluation of SP94 Modified Liposomes Loaded with N-14NCTDA, a Norcantharimide Derivative for Hepatocellular Carcinoma-Targeting. *AAPS PharmSciTech*, *21*(7), 1-11.

Karim, N., Shishir, M. R. I., Rashwan, A. K., Ke, H., & Chen, W. (2021). Suppression of palmitic acid-induced hepatic oxidative injury by neohesperidin-loaded pectin-chitosan decorated nanoliposomes. *International Journal of Biological Macromolecules*, *183*, 908-917.

Karimi, M., Gheybi, F., Zamani, P., Mashreghi, M., Golmohammadzadeh, S., Darban, S. A., ... & Jaafari, M. R. (2020). Preparation and characterization of stable nanoliposomal formulations of curcumin with high loading efficacy: In vitro and in vivo anti-tumor study. *International journal of pharmaceutics*, *580*, 119211.

Kelf, T. A., Sreenivasan, V. K. A., Sun, J., Kim, E. J., Goldys, E. M., & Zvyagin, A. V. (2010). Non-specific cellular uptake of surface-functionalized quantum dots. *Nanotechnology*, *21*(28), 285105.

Kelly, C., Jefferies, C. and Cryan, S. (2011). Targeted Liposomal Drug Delivery to Monocytes and Macrophages. *Journal of Drug Delivery*, 2011, pp.1-11.

Khorev, O., et al., 2008. Trivalent, Gal/GalNAc-containing ligands designed for the asialoglycoprotein receptor. Bioorganic and medicinal chemistry, 16(9), 5216–5231.

Kinberger, G.A., et al., 2016. Conjugation of mono and di-GalNAc sugars enhances the potency of antisense oligonucleotides via ASGR medi- ated delivery to hepatocytes. Bioorganic and medicinal chemistry let- ters, 26(15), 3690–3693.

Lemarchand, C., Gref, R., & Couvreur, P. (2004). Polysaccharide-decorated nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics*, *58*(2), 327-341.

Li, S. D., & Huang, L. (2010). Stealth nanoparticles: high density but sheddable PEG is a key for tumor targeting. *Journal of controlled release: official journal of the Controlled Release Society*, *145*(3), 178.

Li, Y., Yang, D., Wang, Y., Li, Z., & Zhu, C. (2019). Co-delivery doxorubicin and silybin for anti-hepatoma via enhanced oral hepatic-targeted efficiency. *International Journal of Nanomedicine*, *14*, 301.

Li, K., Hou, J. T., Yang, J., & Yu, X. Q. (2017). A tumor-specific and mitochondria-targeted fluorescent probe for real-time sensing of hypochlorite in living cells. *Chemical Communications*, *53*(40), 5539-5541.

Li, S., & Wang, X. P. (2017). In vitro and in vivo evaluation of novel NGR-modified liposomes containing brucine. *International journal of nanomedicine*, *12*, 5797.

Li, W. J., Lian, Y. W., Guan, Q. S., Li, N., Liang, W. J., Liu, W. X., ... & Luo, H. (2018). Liver-targeted delivery of liposome-encapsulated curcumol using galactosylated-stearate. *Experimental and therapeutic medicine*, *16*(2), 925-930.

Li, H., Yuan, D., Sun, M., & Ping, Q. (2016). Effect of ligand density and PEG modification on octreotide-targeted liposome via somatostatin receptor in vitro and in vivo. *Drug Delivery*, *23*(9), 3562-3572.

Lian, B., Wei, H., Pan, R., Sun, J., Zhang, B., Wu, J., ... & Tian, G. (2021). Galactose modified liposomes for effective co-delivery of doxorubicin and combretastatin A4. *International Journal of Nanomedicine*, *16*, 457.

Liu, X., Han, M., Xu, J., Geng, S., Zhang, Y., Ye, X., ... & Tang, X. (2017). Asialoglycoprotein receptor-targeted liposomes loaded with a norcantharimide derivative for hepatocyte-selective targeting. *International journal of pharmaceutics*, *520*(1-2), 98-110.

Liu, W., Tian, M., Kong, Y., Lu, J., Li, N., & Han, J. (2017). Multilayered vitamin C nanoliposomes by self-assembly of alginate and chitosan: Long-term stability and feasibility application in mandarin juice. *LWT*, *75*, 608-615.

López-Guisa, J., Cai, X., Collins, S., Yamaguchi, I., Okamura, D., & Bugge, T. et al. (2011). Mannose Receptor 2 Attenuates Renal Fibrosis. *Journal Of The American Society Of Nephrology*, *23*(2), 236-251. doi: 10.1681/asn.2011030310.

Mishra, S., Shimpi, N. G., & Sen, T. (2013). The effect of PEG encapsulated silver nanoparticles on the thermal and electrical property of sonochemically synthesized polyaniline/silver nanocomposite. *Journal of Polymer Research*, *20*(1), 1-10.

Nag, O. and Awasthi, V. (2013). Surface Engineering of Liposomes for Stealth Behavior.

Pharmaceutics, 5(4), pp.542-569.

Naicker, K., Ariatti, M., Singh, M., 2014. Colloids Surf. B: Biointerfaces 122, 482–490. Pan, Z., Niu, Y., Wang, Y., Tang, Y., Tang, X., Cai, C., 2015. Eur. J. Lipid Sci. Technol. 117, 55–64.

Narum, S. M., Le, T., Le, D. P., Lee, J. C., Donahue, N. D., Yang, W., & Wilhelm, S. (2020). Passive targeting in nanomedicine: fundamental concepts, body interactions, and clinical potential. In Nanoparticles for Biomedical Applications (pp. 37-53). Elsevier.

Nasser, M., Mackay, M., Tuohy, E., Bannach-Brown, A., Fogtman, A. and Gianni, M., 2022. *Quality assessment tools for in-vitro studies*. [online] OSF. Available at: <https://osf.io/wr748/> [Accessed 20 February 2022].

Natto, Z. S., & AlGhamdi, D. S. (2019). Quality assessment of systematic reviews and meta-analyses published in Saudi journals from 1997 to 2017. *Saudi medical journal*, *40*(5), 426.

Nie, H., Qiu, B., Yang, Q. X., Zhao, Y., Liu, X. M., Zhang, Y. T., ... & Zhang, S. Y. (2019). Effect of gal/GalNAc regioisomerism in galactosylated liposomes on asialoglycoprotein receptor-mediated hepatocyte-selective targeting in vivo. *Journal of liposome research*, 1-11.

Nosova, A., Koloskova, O., Shilovskiy, I., Sebyakin, Y. and Khaitov, M. (2017). Lactose-based glycoconjugates with variable spacers for design of liver-targeted liposomes. *Biomeditsinskaya Khimiya*, 63(5), pp.467-471.

Nozawa, Y., Banno, Y., Ohki, K., Morita, T., Yoshioka, S., & Sekiya, T. (1986). Interaction of Targeted Liposomes with Hepatocytes. In *Medical Application of Liposomes* (pp. 55-65). Karger Publishers.

Nunes, S. S., Fernandes, R. S., Cavalcante, C. H., da Costa César, I., Leite, E. A., Lopes, S. C. A., ... & de Barros, A. L. B. (2019). Influence of PEG coating on the biodistribution and tumor accumulation of pH-sensitive liposomes. *Drug delivery and translational research*, *9*(1), 123-130.

Park, J., Utsumi, T., Seo, Y., Deng, Y., Satoh, A., Saltzman, W., & Iwakiri, Y. (2016). Cellular distribution of injected PLGA-nanoparticles in the liver. *Nanomedicine: Nanotechnology, Biology And Medicine*, *12*(5), 1365-1374. doi: 10.1016/j.nano.2016.01.013

Perrault, S. D., Walkey, C., Jennings, T., Fischer, H. C., & Chan, W. C. (2009). Mediating tumor targeting efficiency of nanoparticles through design. *Nano letters*, *9*(5), 1909-1915.

Photos, P. J., Bacakova, L., Discher, B., Bates, F. S., & Discher, D. E. (2003). Polymer vesicles in vivo: correlations with PEG molecular weight. *Journal of Controlled Release*, *90*(3), 323-334.

Pireddu, R., Pibiri, M., Valenti, D., Sinico, C., Fadda, A. M., Simbula, G., & Lai, F. (2018). A novel lactoferrin-modified stealth liposome for hepatoma-delivery of triiodothyronine. *International journal of pharmaceutics*, *537*(1-2), 257-267.

Poelstra, K., Prakash, J. and Beljaars, L. (2012). Drug targeting to the diseased liver. *Journal of Controlled Release*, 161(2), pp.188-197.

Pozzi, D., Colapicchioni, V., Caracciolo, G., Piovesana, S., Capriotti, A. L., Palchetti, S., ... & Laganà, A. (2014). Effect of polyethyleneglycol (PEG) chain length on the bio–nano-interactions between PEGylated lipid nanoparticles and biological fluids: from nanostructure to uptake in cancer cells. *Nanoscale*, *6*(5), 2782-2792.

Prisma-statement.org. 2021. *PRISMA*. [online] Available at: <http://prisma-statement.org/prismastatement/flowdiagram.aspx> [Accessed 27 May 2021].

Rigopoulou, E. I., Roggenbuck, D., Smyk, D. S., Liaskos, C., Mytilinaiou, M. G., Feist, E., ... & Bogdanos, D. P. (2012). Asialoglycoprotein receptor (ASGPR) as target autoantigen in liver autoimmunity: lost and found. *Autoimmunity reviews*, *12*(2), 260-269.

Riviere, J. (2011). *Comparative pharmacokinetics*. Ames: Wiley-Blackwell.

Scheff, J., Almon, R., DuBois, D., Jusko, W., & Androulakis, I. (2011). Assessment of Pharmacologic Area Under the Curve When Baselines are Variable. *Pharmaceutical Research*, *28*(5), 1081-1089. doi: 10.1007/s11095-010-0363-8

Sesarman, A., Muntean, D., Abrudan, B., Tefas, L., Sylvester, B., Licarete, E., ... & Porfire, A. (2021). Improved pharmacokinetics and reduced side effects of doxorubicin therapy by liposomal co-encapsulation with curcumin. *Journal of Liposome Research*, *31*(1), 1-10.

Sisense. 2021. *What is Data Cleaning? | Sisense*. [online] Available at: <https://www.sisense.com/glossary/data-cleaning/> [Accessed 22 May 2021].

Song, G., Wu, H., Yoshino, K., & Zamboni, W. (2012). Factors affecting the pharmacokinetics and pharmacodynamics of liposomal drugs. *Journal Of Liposome Research*, *22*(3), 177-192. doi: 10.3109/08982104.2012.655285

Sonoke, S., Ueda, T., Fujiwara, K., Kuwabara, K. and Yano, J. (2011). Galactose-Modified Cationic Liposomes as a Liver-Targeting Delivery System for Small Interfering RNA. *Biological & Pharmaceutical Bulletin*, 34(8), pp.1338-1342.

Soundararajan, A., Bao, A., Phillips, W. T., Perez III, R., & Goins, B. A. (2009). [186Re] Liposomal doxorubicin (Doxil): in vitro stability, pharmacokinetics, imaging and biodistribution in a head and neck squamous cell carcinoma xenograft model. *Nuclear medicine and biology*, *36*(5), 515-524.

Sox, H. C., & Goodman, S. N. (2012). The methods of comparative effectiveness research. *Annual review of public health*, *33*, 425-445.

Stefanick, J. F., Ashley, J. D., Kiziltepe, T., & Bilgicer, B. (2013). A systematic analysis of peptide linker length and liposomal polyethylene glycol coating on cellular uptake of peptide-targeted liposomes. *ACS nano*, *7*(4), 2935-2947.
Tang, L., Fan, T. M., Borst, L. B., & Cheng, J. (2012). Synthesis and biological response of size-specific, monodisperse drug–silica nanoconjugates. *ACS nano*, *6*(5), 3954-3966.

Tang, L., Yang, X., Yin, Q., Cai, K., Wang, H., Chaudhury, I., ... & Cheng, J. (2014). Investigating the optimal size of anticancer nanomedicine. *Proceedings of the National Academy of Sciences*, *111*(43), 15344-15349.

Training.cochrane.org. 2021. *Cochrane Handbook for Systematic Reviews of Interventions*. [online] Available at: <https://training.cochrane.org/handbook> [Accessed 27 May 2021].

Uster PS, Allen TM, Daniel BE, et al. (1996). Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time. FEBS Lett. 386:243–6.

Vasir, J. K., & Labhasetwar, V. (2008). Quantification of the force of nanoparticle-cell membrane interactions and its influence on intracellular trafficking of nanoparticles. *Biomaterials*, *29*(31), 4244-4252.

Verhoef, J. J., & Anchordoquy, T. J. (2013). Questioning the use of PEGylation for drug delivery. *Drug delivery and translational research*, *3*(6), 499-503.

Wang, Q., Tardi, P., Sadowski, N., Xie, S., Heller, D., & Mayer, L. (2020). Pharmacokinetics, drug metabolism, and tissue distribution of CPX-351 in animals. *Nanomedicine: Nanotechnology, Biology and Medicine*, *30*, 102275.

Wang, S. J., Huang, W. S., Chuang, C. M., Chang, C. H., Lee, T. W., Ting, G., ... & Lan, K. L. (2019). A phase 0 study of the pharmacokinetics, biodistribution, and dosimetry of 188Re-liposome in patients with metastatic tumors. *EJNMMI research*, *9*(1), 1-13.

Witzigmann, D. (2016). Hepatocyte-specific drug delivery using active targeted nanomedicines-evaluation of targeting strategies in vitro and in vivo (Doctoral dissertation, University_of_Basel).

Xu, H., He, C., Liu, Y., Jiang, J., & Ma, T. (2017). Novel therapeutic modalities and drug delivery–erlotinib liposomes modified with galactosylated lipid: in vitro and in vivo investigations. *Artificial cells, nanomedicine, and biotechnology*, *46*(8), 1902-1907.

Yan, J. J., Liao, J. Z., Lin, J. S., & He, X. X. (2008). Active radar guides missile to its target: receptor-based targeted treatment of hepatocellular carcinoma by nanoparticulate systems. *Tumor Biology*, *36*(1), 55-67.

Yi, S., Zhang, C., Hu, J., Meng, Y., Chen, L., Yu, H., ... & Qiu, Z. (2021). Preparation, characterization, and in vitro pharmacodynamics and pharmacokinetics evaluation of PEGylated urolithin A liposomes. *Aaps Pharmscitech*, *22*(1), 1-12.

Yin, X., Xiao, Y., Han, L., Zhang, B., Wang, T., Su, Z., & Zhang, N. (2018). Ceramide-fabricated co-loaded liposomes for the synergistic treatment of hepatocellular carcinoma. *AAPS PharmSciTech*, *19*(5), 2133-2143

Zhang, X., Lin, C. C., Chan, W. K. N., Liu, K. L., Yang, Z. J., & Zhang, H. Q. (2017). Augmented anticancer effects of cantharidin with liposomal encapsulation: In vitro and in vivo evaluation. *Molecules*, *22*(7), 1052.

Zhang, X., Lin, C., Chan, W., Liu, K., Lu, A., Lin, G., ... & Yang, Z. (2019). Dual-functional liposomes with carbonic anhydrase IX antibody and BR2 peptide modification effectively improve intracellular delivery of cantharidin to treat orthotopic hepatocellular carcinoma mice. *Molecules*, *24*(18), 3332.

Zhou, L., Zou, M., Zhu, K., Ning, S., & Xia, X. (2019). Development of 11-dga-3-o-gal-modified cantharidin liposomes for treatment of hepatocellular Carcinoma. *Molecules*, *24*(17), 3080.

Zhu, K., Zhou, L., Zou, M., Ning, S., Liu, S., Zhou, Y., ... & Xia, X. (2020). 18-GA-Suc modified liposome loading cantharidin for augmenting hepatic specificity: preparation,

characterization, antitumor effects, and liver-targeting efficiency. *Journal of Pharmaceutical Sciences*, *109*(6), 2038-2047.

# Chapter 3: Liposomes preparation, characterisation, and surface modification.

## 3.1. Introduction

Liposomes are among the most commonly used carriers as drug delivery systems (Akbarzadeh et al., 2013; Allen and Cullis, 2013). They result from self-assembly of phospholipids in water (Akbarzadeh et al., 2013), with a size range between 30 nm and > 1 μm (Akbarzadeh et al., 2013). Different methods have been reported for the preparation of liposomes including the thin film hydration method, reverse phase evaporation (REV), solvent injection and detergent dialysis. The resulting vesicles need to undergo some characterisation to assess their quality (Ruozi et al., 2011). Typically, characterisation includes particle size analysis, polydispersity index (PDI), zeta potential (ZP), phospholipid quantification, and membrane permeability (Laouini et al., 2012). Dynamic Light Scattering (DLS) appeared to be the most common instrument used for size and PDI measurements (Edwards and Baeumner, 2006) while ZP, which represents the surface charge, can be measured using Electrophoretic Light Scattering (ELS). UV is one of the reported methods for phospholipid analysis (Kanásová and Nesměrák, 2017; Laouini et al., 2012). However, even if all of these properties are controlled well, there is a possibility for rapid capture of the liposomes by mononuclear phagocyte systems (MPS) (Lavoie and Levy, 2017; Hume et al., 2019). In order to avoid this phenomenon, stealth properties can be introduced to the liposomes either during or after vesicles production. The idea behind stealth manufacturing is to prolong the circulation time and to evade recognition by the immune system by modifying the surface properties of the liposomes (Lavoie and Levy, 2017).

Different materials can be used to provide stealth properties, but PEG is the most commonly used (Bozzuto and Molinari, 2015). This is due to its hydrophilic properties and flexibility (Owensiii and Peppas, 2006). PEGylated liposomes are also undergone the same quality attributes measurements for the un-modified liposomes. Quantifying the amount of PEG can be performed using colorimetric assay (Jones et al., 2014). Maintaining the physicochemical

stability of the prepared liposomes is challenging. This is due to that; they are formed in an aqueous solution (Fonte et al., 2016), and to overcome this problem, lyophilising/freeze-drying method is suggested (Abdelwahed et al., 2006). Several materials can convert the solution to solid particulates, and sugars i.e., sucrose and lactose are the most common in use (Abdelwahed et al., 2006).

In this project, we will prepare liposomal formulations using different neutral phospholipids (SPC, DSPC, and HSPC) using the thin film hydration method and assess their size, PDI, and zeta potential and phospholipid content. We aim to produce liposomes size below 200 nm with a good homogeneity (Ca. 0.3 a.u.) and a neutral ZP value (10 and -10 mV). These liposomes were kept in the fridge to assess their stability over time. Decorating liposomes' surface with different PEG types (PEG2000-DSPE, PEG2000-DSPE-COOH, PEG2000-DSPE-NH2) using two different molar ratios (2% and 5%) to obtain stealth characteristics was also performed. Their quality attributes and membrane fluidity were tested too at three different temperature profile (25°C, 37°C, and Tc). To investigate the impact of the temperature on the release kinetics profile, we established a linear regression model. Lyophilised liposomes were prepared using the pre- and post-insertion methods for the selected cryoprotectant (sucrose) to improve the liposomes shelf-life time. The size, PDI, and zeta potential were evaluated.

## 3.2. Experimental section

### 3.2.1. Material

Fat-free Soybean phospholipid with 70% Phosphatidylcholine (SPC), Hydrogenated phosphatidylcholine from soybean (HSPC), 1,2-Disteraroyl-s-glycerol- phosphatidylcholine (DSPC), and N-(Carbonyl-methoxypolyethylenglycol-2000) -1, 2-distearoyl-sn-glycero-3-phosphoethanolamine, MPEG-2000-DSPE (Na-salt) were purchased from Lipoid (Ludwigshafen, Germany). Cholesterol,) Calcein, Sephadex G-75 and Sepharose CL-4B, all

were purchased from Sigma Aldrich (Gillingham, UK). NaCl was obtained from Oxoid Ltd (Basingstoke, UK), Chloroform, TES, EDTA and sucrose were obtained from Fisher Chemicals (Loughborough, UK), Methanol from VWR Chemicals (Leicestershire, UK). All reagents were used without any further purification. De-ionised water which used in these preparations was dispensed from the Arium Mini station (enhanced with biofilters; Sartorious Company (Goettingen, Germany)). Phospholipase C, Ammonium thiocyanate, Triton-100X were obtained from Sigma Aldrich from different regions (Spain and U.K.). Ferric chloride anhydrous was obtained from Scientific Laboratory Suppliers (UK, Nottingham). DSPE-PEG (2000) and its derivatives both carboxylated and aminated were obtained from Avanti polar lipids (Alabama, USA).

### 3.3. Methodology

#### 3.3.1. Liposomes preparation

Five different molar ratios of Soybean phospholipid: cholesterol (SPC: CHOL; 90:10, 80:20, 70:30, 60:40 and 55:45) were used for screening purposes. Liposomes were prepared by the thin film hydration method as previously described (Varona et al., 2011). Briefly, soybean phospholipid and cholesterol were dissolved in 3 ml of chloroform and added to a 25 ml round bottom flask. The solvent was removed under vacuum at a temperature above the Tc using a rotary evaporator (Staufen, Germany) to allow the thin film to form. The latter was re-hydrated with 3 ml of a pre-heated normal saline solution and allowed to be mixed for 2h h under stirring (170 rpm). The suspension was then vigorously stirred and sonicated at 50 °C for an hour using ultrasonic bath (VWR, Leicestershire, UK). Samples were taken every 10 minutes to evaluate the impact of the sonication on liposomes size. Further investigation of the influence of vacuum pressure on average hydrodynamic size was carried out. Herein, different vacuum levels (100, 200, 300, 400, 500 and 600 mbar) were applied during solvent removal step of liposomes preparation. The best formulations were identified and taken

through further studies. All experiments were performed in triplicate. Liposomes preparation includes using for three neutral phospholipids SPC, DSPC, HSPC. All formulations were composed of phospholipid: cholesterol (70:30) and were characterized in terms of size and charge. All experiments were performed in triplicate.

### 3.3.2. Liposomes characterisation

#### 3.3.2.1. Particle size analysis

Particle sizes were determined on liposomes after 100-fold dilution with 0.9% NaCl. The average particle size and polydispersity index (PdI) of samples were evaluated using Malvern Zeta-sizer instrument (Zetasizer Nano ZSP model; Malvern, Worcestershire, UK). Analysis was made at a backward scattering angle of 173°, at 25, 37 and 50 Tc (50, 63, and 65 °C). Each measurement was repeated thrice.

#### 3.3.2.2. Surface charge determination

Electrophoretic Light Scattering (ELS) was used in this experiment to evaluate the zeta potential. Liposomes were diluted with 0.9% NaCl 1000 times, then transferred to a folded capillary cell. Measurements were taken at 25 °C. All experiments were performed in triplicate.

#### 3.3.2.3. Physicochemical stability

Liposomes (70:30 and 55:45 phospholipid: cholesterol) were kept in the refrigerator at 4°C. The mean particle size and zeta potential were determined every week for the first month, then tested monthly.

### 3.3.3. PEGylation of liposomes

Stealth liposomes were produced using the post-insertion method (Steenpaß et al., 2006). PEG-DSPE, PEG-DSPE-COOH, or PEG-DSPE-$NH_2$ (2 and 5 mol% *vs.* total lipid) were dissolved in 2 ml of 0.9% $NaCl_{aq}$. The PEG solution was then added to the pre-formed liposomes (700 ml of liposomes: 300 µL of PEG). Afterward, free PEG was removed by

eluting 300 µL of PEGylated liposomes on Sepharose CL-4B (75 Sepharose CL-4B: 25 buffer) (2.5 X 30 cm column). The purified PEGylated liposomes were evaluated for their size and zeta potential as described previously.

### 3.3.4. Phospholipid quantification

Liposomes were mixed with methanol (50 %v/v) for 30 minutes to allow complete dissolution of the liposomes (Cipolla et al., 2014). The same procedure as for PEG quantification was applied (See section 3.3.6). Measurements were taken at λ= 488 nm on a Shimadzu spectrophotometer (Model RF-5301PC) (Jimah, et al., 2017). The experiment was validated three times.

### 3.3.5. Yield measurements

0.5 ml of liposomes was dried in the oven for 24 hours. The solid was then measured and compared to the initial concentration to determine the yield. This experiment was performed in triplicate.

$$\%Yield = (\ Actual\ concentration/Theoritical\ concentration)X\ 100$$

### 3.3.6. PEG quantification

To quantify PEG, liposomes were digested at 37 °C in the presence of phospholipase C (0.001 g/ 1 ml) and of deoxycholate (4 M). 50 µL of the digested liposomes were withdrawn. Then, equal volume of 700 µl chloroform and 700 µL of the ferrocyantae solution was added. The samples were gently shaken for 30 minutes at room temperature. Afterwards, the upper layer was removed and the bottom layer which contain the extracted PEG in chloroform was assayed colorimetrically using UV-Spectrophotometer at λ= 510 nm as described previously (Jones et al., 2014; Nag et al, 1996).

### 3.3.7. Validation method for PEG and phospholipid quantification

The validation aims to test the linearity, reliability, and the precision for the quantification method used according to (ICH guidelines Q2 (R1), 2005). This includes an estimation of

the limit of detection (LOD), and Limit of quantification (LOQ) (Armbruster and Pry, 2008). LOD can be defined as lowest concentration of the sample can be detected by the instrument. LOQ is usually greater than LOD, and in which the redefined goals are met (Armbruster and Pry, 2008). Both predictors can be calculated using the following equations:

$$LOD = 3.3\ (SD\ of\ the\ intercept \backslash slope)$$

$$LOQ = 10\ (SD\ of\ the\ intercept \backslash slope)$$

### 3.3.8. Membrane permeability

A calcein stock solution (100 mM) was prepared in TES buffer (10mM, pH 7.4) and diluted to 50 ml with Buffer C (100 mM NaCl, 10 mM TES, 0.1 mM EDTA, pH 7.4). This solution was used to re-hydrate the lipid film during liposome preparation. Free calcein was removed by passing the sample on a Sephadex G-75 Column (2.5 X 30 cm). Calceino-liposomes were incubated at 25°C, 37°C and their Tc (50°C, 63°C, and 65°C) for 24 hours at which point the fluorescent signal was measured ($\lambda_{ex:\ 480\ nm\ and} = \lambda_{em:}$ 520 nm) (Düzgünes et al., 2010). Liposomes were lysed in Triton X-100 to obtain 100% calcein release (Düzgünes et al., 2010). All experiments were repeated at least three times on three distinct formulations.

### 3.3.9. Lyophilised liposomes

Pre-formed liposomes were mixed with a 150 mM sucrose solution (Kannan et al., 2014) at a volume ratio of 70:30. The formed mixture was then frozen at -80 °C for 24 hours then placed in the freeze-dryer for 48 hours. 1 mg of the resulting powder was reconstituted in 1 ml of 0.9% $NaCl_{aq}$. Measurements for the size, PDI, and zeta potential were taken at 25 °C, 37 °C, and their Tc three times.

## 3.4. Statistical analysis

Descriptive analysis for the collected data including mean± SD was calculated and drawn using GraphPad Prism (version 9.2.). The differences in the size, ZP, and the release profile

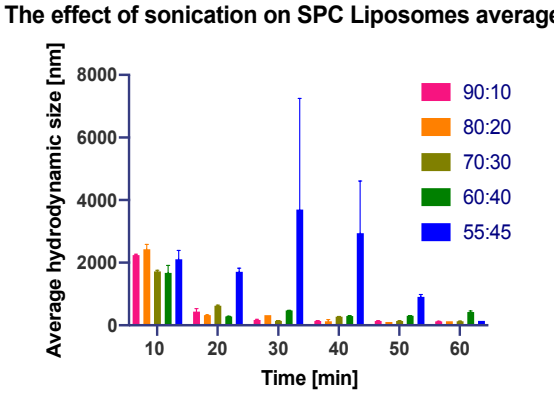
was tested between the three different formulations using ANOVA test. Linear regression analysis model to predict the effect of the temperature profile on the release behaviour was studies using STATA software (version 17).

## 3.5. Results and discussion

### 3.4.1. Effect of sonication on average size

The impact of sonication times was studied for different phospholipid: cholesterol ratios (Figure.3.1). In this experiment, we aim to determine the best formulation to be selected for further characterisations. The average size needs to be below 200 nm.

(A)




(B)

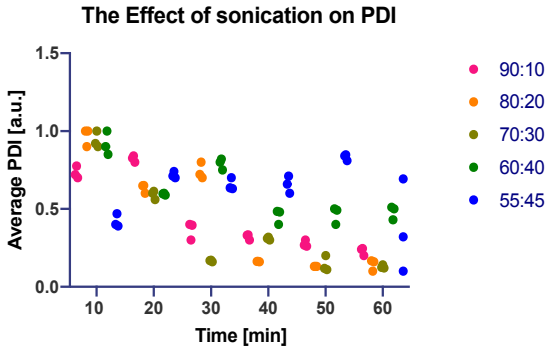



**Figure 3.1. The effect of sonication on size (A) and PDI (B) for liposomes prepared at different phospholipid: cholesterol ratios. Data represents the mean± SD of three individual experiments.**

Liposomes manufacturing started with the screening of five different molar ratios, 90:10, 80:20, 70:30, 60:40 and 55:45 (soybean phosphatidylcholine: cholesterol), taking into account that, the cholesterol fraction should not exceed 50 mol% (Perrie, 2013). Having high

cholesterol concentration is associated with increasing liposomes rigidity, reducing membrane permeability and drug retention inside the liposomes (Perrie, 2013). Here, liposomes were prepared by the thin film hydration followed by sonication. Sonication works by transforming the electrical energy into physical vibrations, which leads to sample size reduction (Nascentes et al., 2001). Bath sonicators has some advantages over the probe sonicator including simplicity and lower risk of the metal contamination and/or lipid deterioration (Hielscher ultrasound technology, 2021; Chung et al., 2014). For liposome size reduction, sonication times are usually around 30 to 60 min (Düzgünes et al., 2010) in order to get small unilamellar vesicles (SUVs). This is confirmed by the results here as 40 min of sonication was required to produce smaller vesicles (Figure 3.1. A.).

Most formulations experienced a reduction in size with increased sonication time; this was mostly independent of the phospholipid: cholesterol ratio. Ten mins of sonication led to the formation of large vesicles at all ratios (1700-2200 nm). Increasing sonication time to 20 mins, produced liposomes with a diameter ranging between 500 and 1100 nm. For most formulations, minimal size reduction was observed after 40 mins except with for SPC: Chol ratios of 60:40 and 55:45. A ratio of 55:45 needed longer time to reach the desired size (at 30 mins, the size was nearly 1500 nm, whereas the remaining ratios were below 500 nm. However, even with getting the desired size with sonication, extrusion is still recommended to ensure the mixture homogeneity (Ong et al., 2016). Ong et al., (2016) studied the impact of different size reduction techniques on the liposomes size and evaluated the poly dispersity index. The different techniques used were homogenization, sonication, ultra-sonication, freeze-thaw sonication, and extrusion. The size and PDI results for the control (purchased pro- SPC liposomes) were 322 nm and 0.4 a.u. respectively. Apart from the extrusion, the size range was 250 nm with PDI of Ca. (0.3-0.4 a.u.). The reported obvious difference was

with the extrusion in which the size reduced to 103 nm and the PDI reduced by the half of the control and reached 0.2 a.u.

The molar ratio of cholesterol had an important impact on size reduction by sonication. At the highest cholesterol ratio, at least 1h of sonication was required to reduce the size below 600 nm (Figure 3.1. A). Even at 40 mol% cholesterol, size reduction required longer sonication time. This might be related to the change in membrane fluidity at high cholesterol ratios (i.e., membrane rigidity increases as cholesterol concentration increases) (Bozzuto and Molinari, 2015). Literature also reported that, the changes in the Tc occur when using cholesterol with a concentration greater than 20 mol% (Kraske et al., 2001). Soybean phosphatidylcholine has a Tc of 30 °C but may still be in a relatively rigid state due to the cholesterol (Chen et al., 2006). Thus, it is suggested to increased up to 50°C in the presence of 30% of the cholesterol. Cholesterol acts by controlling the membrane permeability of the liposomes' membrane by alteration the packing form of the phospholipid molecules. However, using excessive amounts can increase membrane rigidity (Chen et al., 2006). Briuglia et al., (2015) tested different phosphatidylcholine: cholesterol ratios (80:20, 70:30: 60:40, and 50:50 mol%) after exposure to a 60 min sonication step. Findings showed that formulations with the highest cholesterol content (>30%) also had the highest size (i.e., > 300 nm). In contrast, similar sizes were obtained here and in Bozzuto's study for a 70:30 ratio (ca. 260 nm). Figure 3.1.B. illustrates the trends of PdI values for the tested ratios. For all formulations, PdI was shown to decrease with sonication times, except for 60:40 and 55:45 ratios. The highest PdI values after 1h sonication were 0.693 and 0.511 a.u. for ratios 55:45 and 60:40 respectively. The lowest PDI was obtained when using 70:30 ratio (0.124 a.u.).

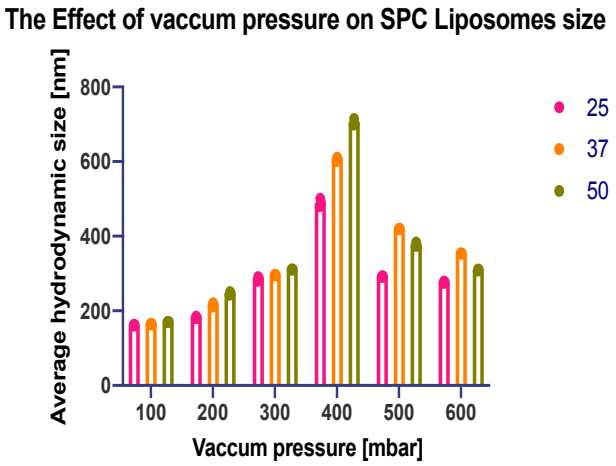
Based on these results, two ratios (70:30 and 55:45) were selected for stability studies. A molar ratio of 70:30 has been shown to produce the most stable preparations and is the ratio

used in Amphotericin B formulations (Briuglia et al., 2015; Jadhav et al., 2011). The second rate selected was 55:45 as it is also used in commercial liposomal formulations (Abraham et al., 2005). However, 70:30 was selected to undergo additional study of the impact of the vacuum pressure on the liposomes size, as it contains a desirable amount of cholesterol. Other ratios were excluded for the time being, but maybe evaluated in the future.

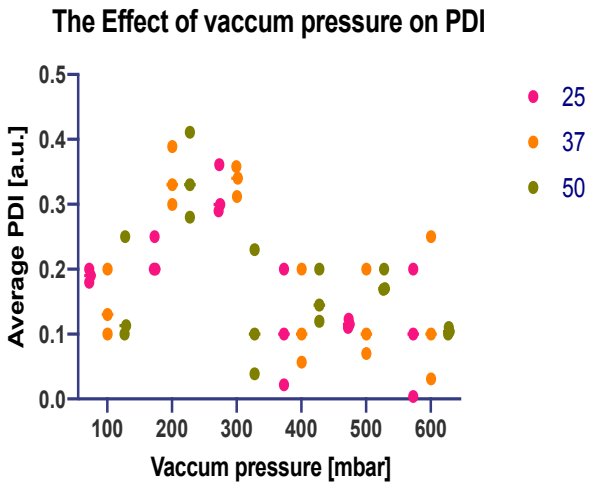
### 3.4.2. Impact of vacuum pressure on liposomes' formulation

This experiment is carried out to investigate the impact of the vacume pressure on the liposomes' physicochemical properties. The average size, PDI, and ZP for liposomes prepared at a 70:30 (SPC: Chol) were recorded at three different temperature presents in charts below. We aim to find out the pressure value that gives a liposomal size below 200 nm.

(A)



(B)




(C)

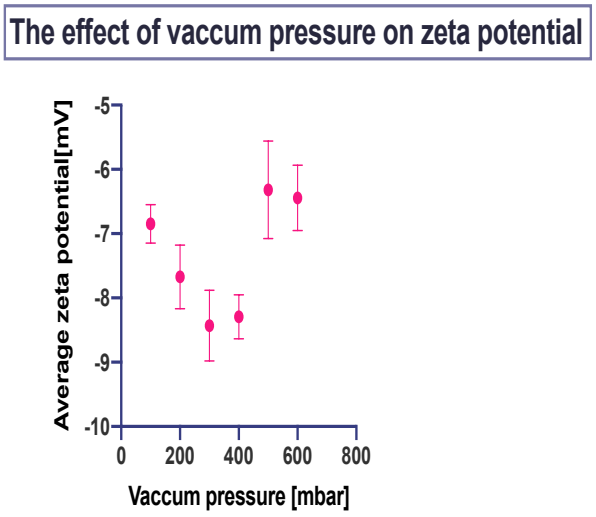



**Figure .3.2.The effect of applying different pressure on liposomes size (A),PDI (B), and Zeta potential (C) for 70:30 ratio. Data represents the mean± SD of three individual experiments.**

It has been reported that, vacuum pressure has an impact on liposomes size (Chung et al., 2014). Different vacuum systems have been used in both small- and large-scale production e.g., high pressure homogenisers and microfluidiser (Barnadas-Rodríguez and Sabés, 2001). As good liposomes characterisation is required to ensure the encapsulation efficiency and release profile, it is recommended to control the factors during manufacturing to obtain the desired outcome (Kulkarni et al., 1995).

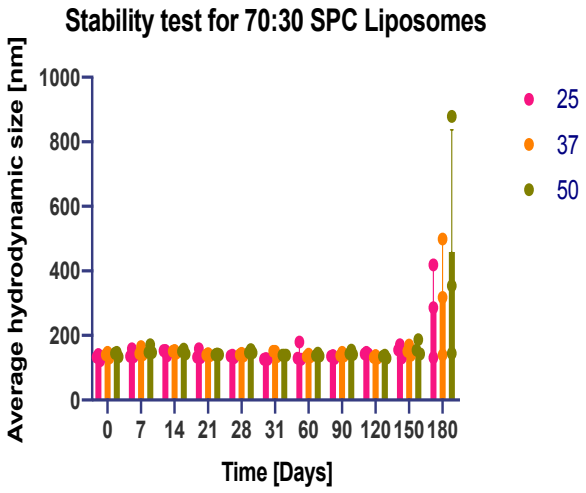
Here, liposomes size showed a consistent size enlargement at 100, 200, 300 and 400 mbar, with a proportional relationship with the temperature (i.e., size increase as the temperature increase) (Figure 3.2.A.). Particles sizes at 500 and 600 mbar decreased again and reached the minimum with an average size of 260 nm. In term of PDI, the highest value was attained when 400 mbar were applied at 50 °C. The lowest PDI values were obtained at a 100 mbar vacuum pressure. Zeta potential represents the surface charged, and the values ranged between -6 and -8.5 mbar at 600 mbar and 300 mbar respectively, which is in the expected range size for the neutral liposomes (Figure 3.2.C.). In a study from Chung et al., (2014) liposome size was reduced as vacuum pressure increased (264 nm at 500 mbar vs. 156 nm at 1000 mbar). This phenomenon is suggested to take place as a result of the impact of the pressure stress share force on liposomes uniformity (Barnadas-Rodríguez and Sabés, 2001). Based on these findings, we selected to use 100 mbar as a vacuum pressure rate of choice as it produced the smallest liposomes size. In the next section, a detailed characterisation for their quality attributes for the three-formulated neutral liposomal formulations will be discussed.

### 3.4.3. Liposomes characterisation and stability studies

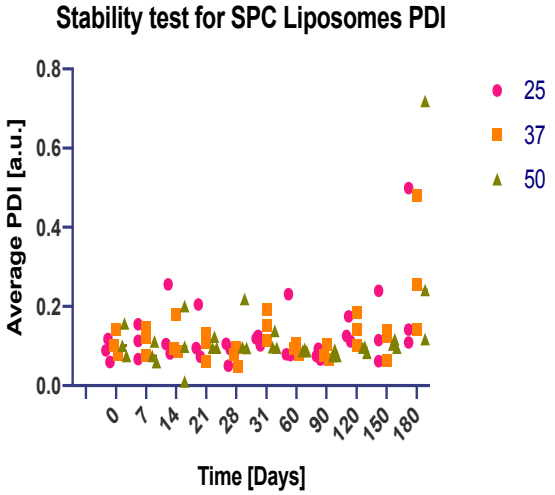
In this section, a full characterisation of liposomes size, PDI, and ZP over time was evaluated for the SPC, DSPC, and HSPC. These neutral liposomes were selected due to their low toxicity profile. We are aiming to produce homogenous liposomes with a size range below 200 nm, and acceptable zeta potential values (Between 10 and -10 mV).

### 3.4.3.1. Characterisation and stability studies for 70:30 and 55:45 SPC Liposomes

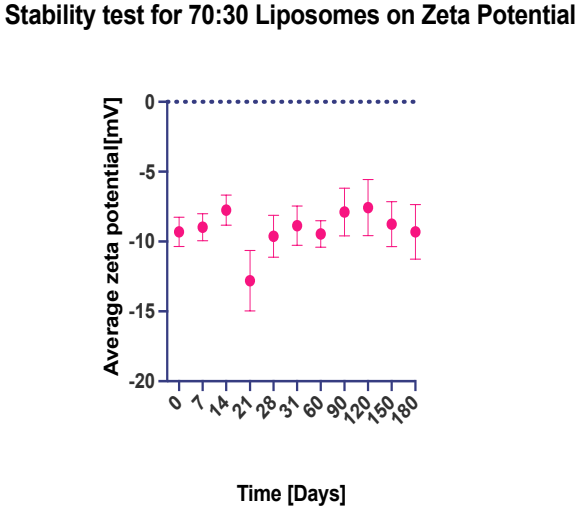
(A)




(B)




(C)

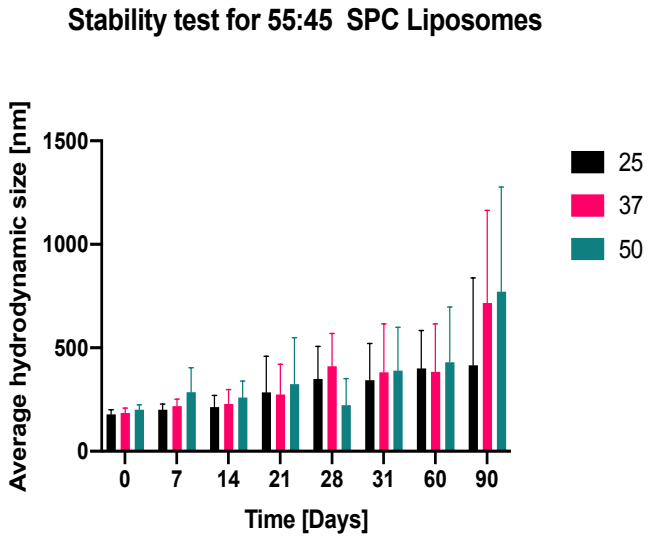



1.

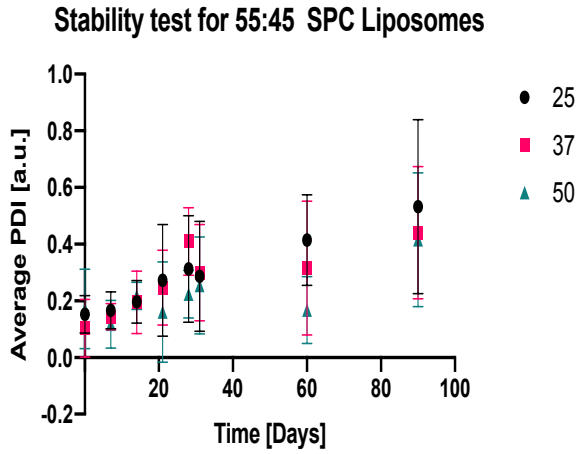
**Figure .3.3. Stability test for 70:30 over six months with information of the average size (A), PDI (B), and zeta potential (C). Data represents the mean± SD of three individual experiments.**

Figure 3.3 showed that, the 70:30 SPC liposomes showed a god stability when kept in the fridge for approximately five months. These formulations showed a good homogeneity with average size was within our goal (ca. 130 nm) (Figure 3.3.A), and an average zeta potential (Ca. -7 mV) (Figure 3.3.C).

(A)




(B)



(C)

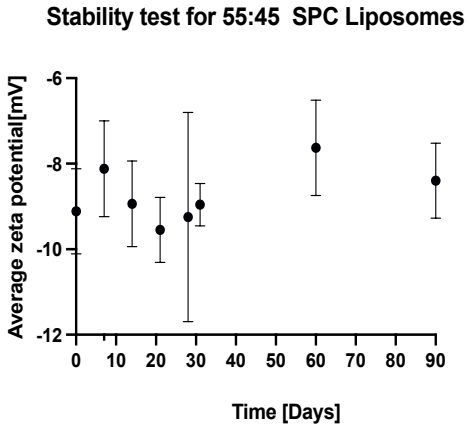
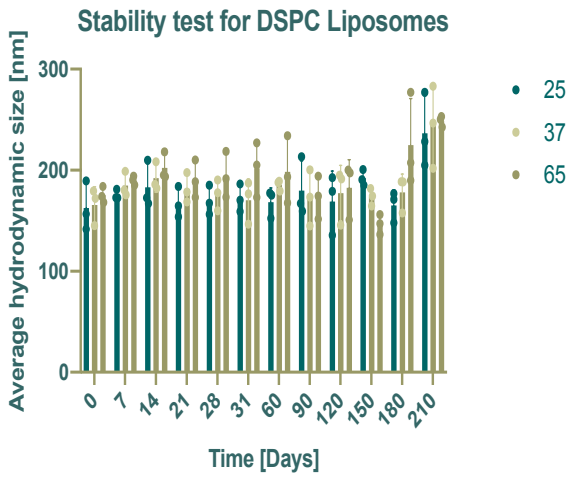



**Figure .3.4. Stability test for 55:45 over three months with information of the average size (A), PDI (B), and zeta potential (C). Data represents the mean± SD of three individual experiments.**

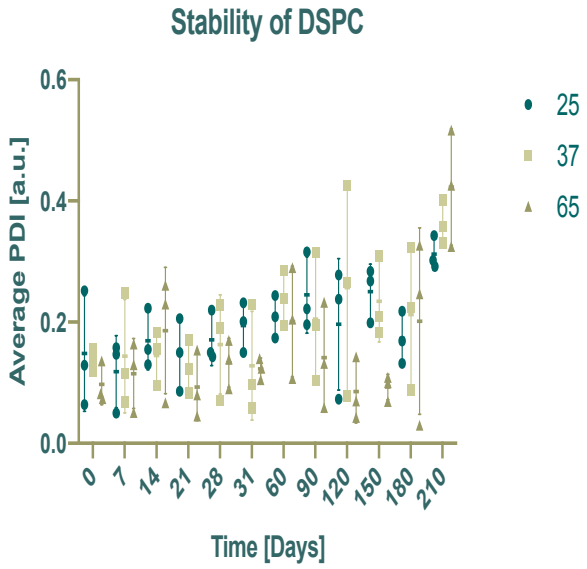
The physicochemical characteristics of 55:45 SPC liposomes is presented in (Figure 3.4). These formulations showed a lower stability profile (3 months) compared to (5 months) for 70:30 SPC liposomes. The average size start with (ca. 150 nm) for the first week and increased until reaching 800 nm by the third month (Figure 3.4.A). This formulation showed a greater heterogeneity with PDI values reached up to (0.5 a.u.). The zeta potential was similar to 70:30 formulation.

### 3.4.3.2. Characterisation and stability studies for 70:30 DSPC Liposomes

(A)




(B)




(C)

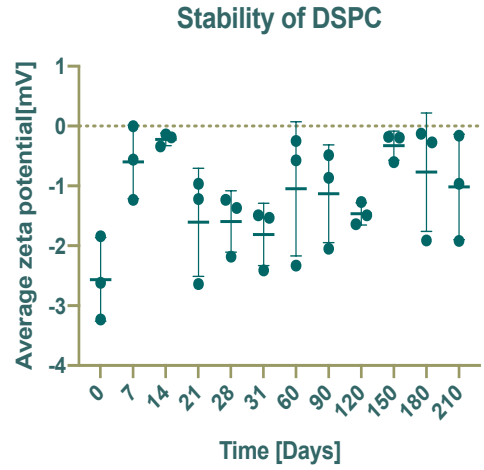
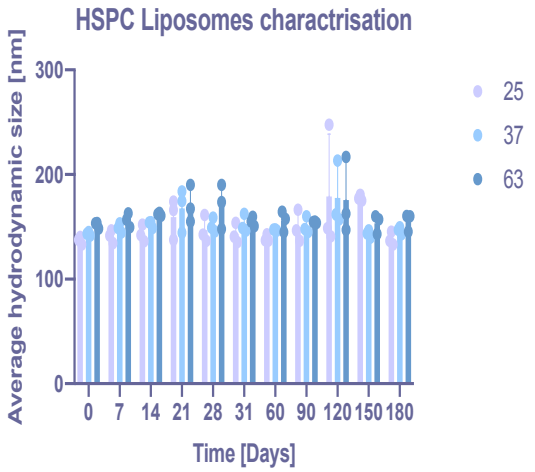



**Figure 3.5. Stability test for 70:30 DSPC liposomes over six months with information of the average size (A), PDI (B), and zeta potential (C). Data represents the mean± SD of three individual experiments.**

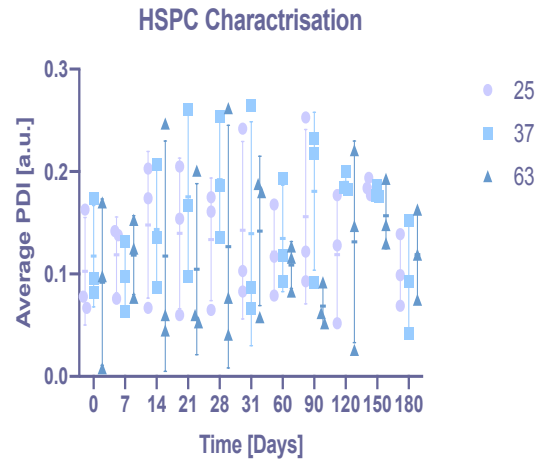
The DSPC liposomes showed a good stability for at least six months. The average size was greater than SPC liposomes (ca. 170 nm) (Figure 3.5.A). The PDI at 37°C was (ca. 0.2 a.u.) and the ZP was approximately -2 mV.

### 3.4.3.3. Characterisation and stability studies for 70:30 HSPC Liposomes

(A)




(B)




(C)

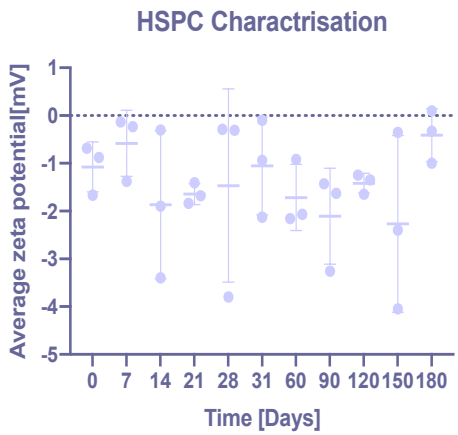


**Figure 3.6. Stability test for 70:30 HSPC liposomes over six months with information of the average size (A), PDI (B), and zeta potential (C). Data represents the mean± SD of three individual experiments.**

Figure 3.6. denote the stability and the physicochemical properties for the HSPC liposomes. This formulation was stable for six months. The average size was 150 nm, with an average PDI of 0.25 a.u. The ZP values ranged between -0.5 and -4 mV (Figure 3.6.C). All studies for liposomes characterisation were performed on liposomes prepared by sonication (60 min), followed by extrusion on 100 nm membrane. Looking at the behavior of SPC: Chol (70:30 and 55:45) liposomes, both formulations had sizes ca. 100 nm with a PdI ca. 0.15-0.25 a.u. Colloidal stability of both liposome formulations was tested by following particle diameter and zeta potential over time. All formulations were kept in the fridge, but the size was measured at three different temperatures (25, 37 and 50 °C or the expected Tc). Testing size at these temperatures expected to show size enlargement with increasing the temperature (Bozzuto and Molinari, 2015; Roy et al., 2016). This is due to the kinetic transformation of phospholipid packing from gel phase at room temperature to a liquid crystalline state above the transition temperature (Bozzuto and Molinari, 2015). Roy et al., (2016) evaluated the effect of temperature on different liposomal formulations (SPC, DPPC, and DPPG). The average size for SPC liposomes at 25 and 50 °C was 100 and 170 nm respectively. However, size at 37 °C was tested to mimic body temperature (Bozzuto and Molinari, 2015). On the one hand, 70:30 SPC: Chol liposomes appeared to be stable for the five months at the three temperature tested with size ranging between 120 to 160 nm (Figure 3.3.A.). On the other hand, 55:45 liposomes showed good stability over the first two months, but aggregated after three months to reach a size of 880 nm. These findings agreed with the literature, which proved that 70:30 is the most appropriate ratio to be used for liposome formulations. However, the reason is still unknown (Briuglia et al., 2015). Briuglia et al., (2015) investigated the stability at 25 and 50 °C, over one month. For 70:30, size was

approximately 260 nm (initial size was 250 nm) during this period for both temperatures. Size was higher with 50:50 to reach 300 nm, and this size was consistent over a month. However, different sonication times employed from our experiment (30 mins vs. 60 mins), and the shorter time of testing stability might play a role in these variations (1 month vs. 6 months).

DSPC and HSPC liposomes were prepared at a 70:30 phospholipid: chol ratio , and were stable for at least five months. Most of the liposomal formulations have an average shelf-life time of 18 months (Doxil) (Barenholz, 2012) and 48 months (Ambisome) (Crommelin et al., 2020). However, after reconstitution they tend to be stable for an average of 7 days (Jakoby et al., 2015). For the all formulations, size had enlarged when temperature increased (Bozzuto and Molinari, 2015; Roy et al., 2016). The size of SPC liposomes was as small as 120 nm at 25°C, which slightly increased at 37 °C to reach 135 nm, and the largest size obtained at Tc. Average DSPC (ca. 165 nm) and HSPC (ca. 150 nm) liposome sizes were greater than for SPC. PDI which is the homogeneity marker (Clayton et al., 2016) was good for all preparations and did not exceed 0.2 a.u. at the three different temperature profiles.

Zeta potential which is an indicator of the nanoparticles' surface charge (Smith et al., 2017) was assessed for the prepared formulations. For SPC, the zeta potential values, which were higher than the other two formulations, ranged from -12.9 to -7.9 mV (70:30 ratio), and from -7.7 to -9.8 mV (55:45 ratio). These findings agreed with a case study performed on the different type of phospholipids, and the obtained zeta potential values for neutral phospholipids were between -9 and -11 mV (Smith et al., 2017). Zeta potentials were similar for both DSPC and HSPC liposomes with values between -0.5 and -2 mV. Although the phospholipid used was neutral and expected to show values close to zero, this could result from water polarization of the hydrated layers, head group of a phospholipid, or might come from impurities (Karmakar, S, 2012). Statistical analysis showed that there is no significant

impact of the temperature among the different liposomal formulations size see Appendix A (Table A1). Although we used neutral liposomes differ in the lengths (18 for DSPC and 14 for HSPC and SPC), with a different Tcs, this can be expected. This agrees with (Funakoshi et al.; 2015) who studied the effect of different alkyl chain lengths on liposomes properties. The findings proved that, there was no significant difference in liposomes characteristics when using different phospholipids chain length which have different Tcs. In general, all prepared liposomal formulations consist of neutral phospholipids and differ only in their chemical structure. Neutral liposomes have an advantages of being a biologically inert with less toxic profile when compared to both anionic and cationic liposomes (Shen z. et al., 2018). SPC is unsaturated phospholipid with C-14 chain length, it require lower temperature (50 °C) which is 10 °C higher than their melting point (40 °C). The remaining two lipids (DSPC and HSPC ) have a Tc of (63 and 65°C) which is 10 °C higher than their Tc (53 and 55 °C ) respectively (Funakoshi et al., 2015). The latter lipids are saturated phospholipids with 16 and 18 chain lengths for HSPC and DSPC (Funakoshi et al., 2015). According to the approved liposomal formulations, DSPC was the most extensive lipid used with Onpattro, Vyxoes, Onivyde, and Lipodox (Anselmo et al., 2019; Blair, 2018; Pelzer et al., 2017; Smith et al., 2016). HSPC was used with Ambisome and Doxil (Bozzuto and Molinari 2015) while SPC used in formulating Lipusu (Ye et al., 2013). This is expected due to its high stability profile confirmed by the higher Tc required for their conversion from solid state to a liquid crystalline phase. Thus, drug retention inside the vesicles until administrated inside the body can be guaranteed (Anderson and Omri, 2004). Among different liposome preparations, a phospholipid: cholesterol ratio of 70:30 appeared to be the most commonly used for unknown reasons (Briuglia et al., 2015).

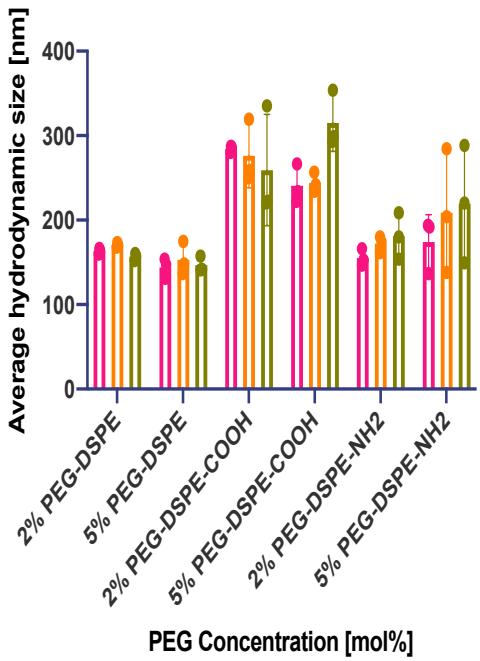
Although the prepared liposomes showed a great stability profile, further surface modification with PEG will be studied in the upcoming section aiming to obtain stealth properties.

### 3.4.4. Stealth liposomes with different PEG types

In this experiment, we insert different type and concentrations of PEG polymer into the pre-formed liposomes. We were aiming to formulate a drug carrier close to the ones approved in the clinical used which most of them are in a PEGylated form e.g., doxil. PEGylation leads to increase the hydrophilicity characteristics and ultimately increase the half-life time for the liposomes when injected inside the body (Lavoie and Levy, 2017).

#### 3.4.4.1. Characterisation and stability studies for SPC Liposomes

(A)



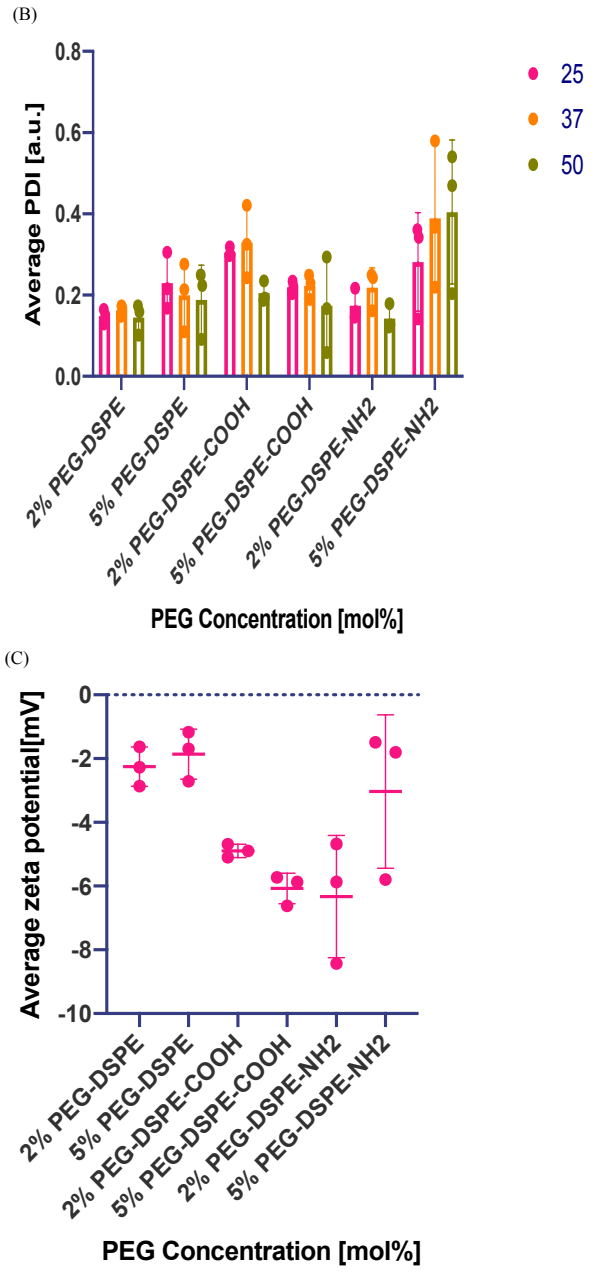


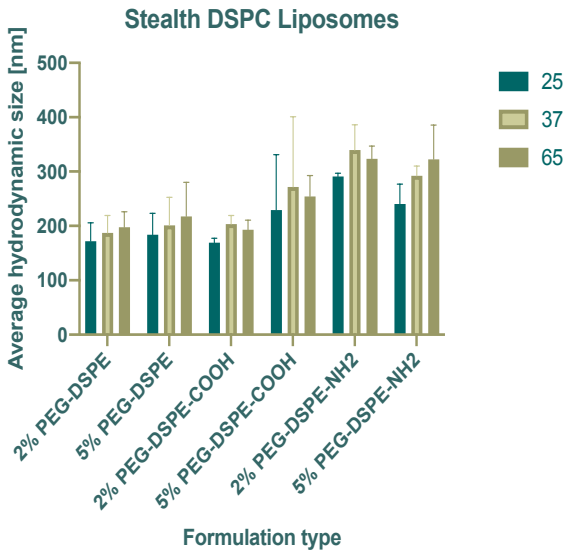

**Figure .3.7. Impact of inserting different PEG-2000 types using 2 and 5mol% on SPC Liposomes average size (A), PDI (B), and Zeta potential (C). Data represents the mean± SD of three individual experiments.**

Figure 3.7. showed the impact of PEG insertion on liposomes size, PDI, and ZP. Overall size remained below 200 nm except with DSPE-PEG2000-COOH, in which it reached (ca. 330 nm) at 5 mol%. One theory can explain this finding is that, when the PEG concentration is below above 4 mol%, the brush confirmation takes place. Here, it is expected to lead to a head group dehydration and a conjugation of the water with a PEG on the surface of the liposomes. This would lead to a formation of micelles and then size enlargements (Tirosh et

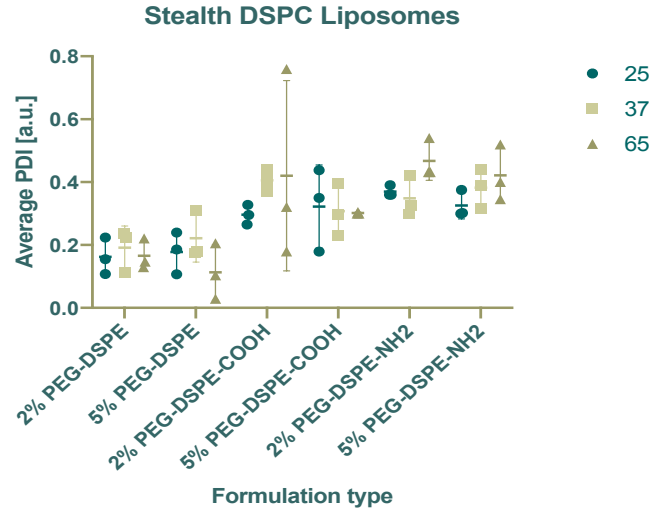
al., 1998). PDI was below 0.3 a.u. except with 5% DSPE-PEG2000-NH2 (Figure 3.7.B.). The maximum ZP value was -6 mV and the lowest was – 2mV.

### 3.4.4.2. Characterisation and stability studies for DSPC Liposomes

(A)




(B)



(C)

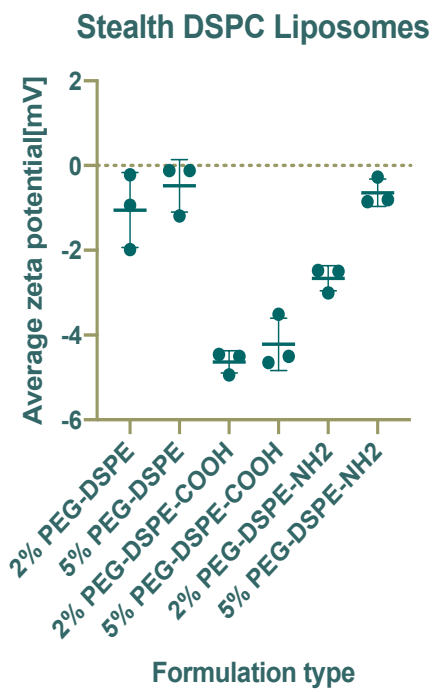


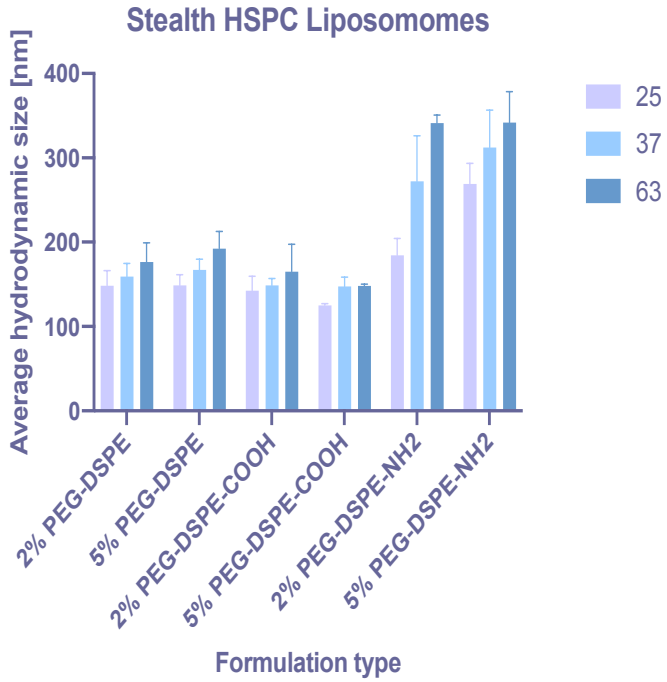

**Figure .3.8. Impact of inserting different PEG-2000 types using 2 and 5mol% on DSPC Liposomes average size (A), PDI (B), and Zeta potential (C). Data represents the mean± SD of three individual experiments.**

The modification with different types of PEG showed no impact on the targeted size (below 200 nm) except with 2 and 5 mol% DSPE-PEG2000-NH2 (330 nm) (Figure 3.8.A.). The average PDI with the functionalised PEG at 37 °C reached approximately 0.4 a.u. (Figure 3.8.B.). The ZP values ranged between -1 and -5 mV.

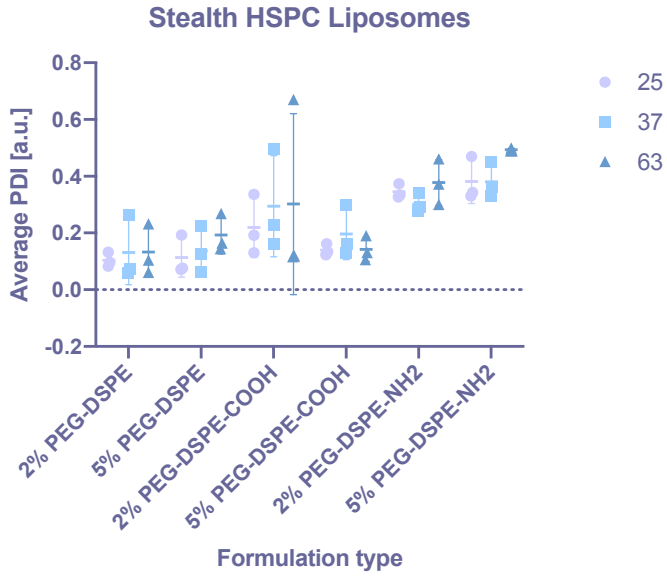
### 3.4.4.3. Characterisation and stability studies for HSPC Liposomes

HSPC liposomes' size did change when modified with both DSPE-PEG2000 and DSPE-PEG2000-COOH at both molar ratios (2 and 5 mol%). However, the size was doubled when modified with 2 and 5 mol% DSPE-PEF-NH2 (ca. 350 nm) (Figure 3.9.A.). The highest PDI value was observed with 2% DSPE-PEG2000-COOH (ca. 0.4 a.u.). The ZP showed an average of -2 mV (Figure 3.9.C.).

(A)


Stealth HSPC Liposomomes
Average hydrodynamic size [nm]
400
300
200
100
0
25
37
63
2% PEG-DSPE
5% PEG-DSPE
2% PEG-DSPE-COOH
5% PEG-DSPE-COOH
2% PEG-DSPE-NH2
5% PEG-DSPE-NH2
Formulation type

(B)


Stealth HSPC Liposomes
Average PDI [a.u.]
0.8
0.6
0.4
0.2
0.0
-0.2
25
37
63
2% PEG-DSPE
5% PEG-DSPE
2% PEG-DSPE-COOH
5% PEG-DSPE-COOH
2% PEG-DSPE-NH2
5% PEG-DSPE-NH2
Formulation type

(C)

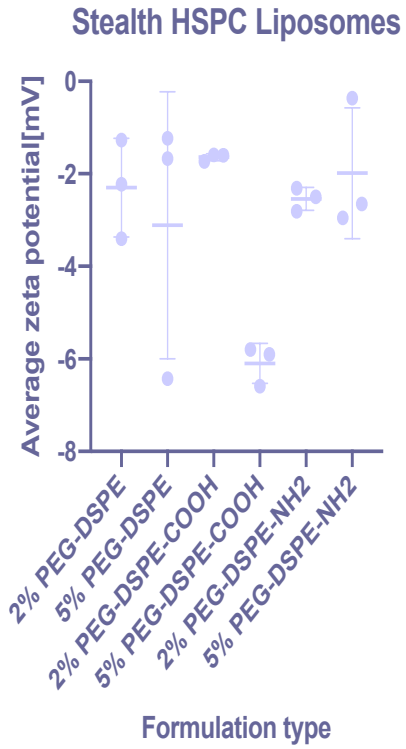



**Figure 3.9. Impact of inserting different PEG-2000 types using 2 and 5MOL% on HSPC liposomes average size (A), PDI (B), and zeta potential (C). Data represents the mean± SD of three individual experiments.**

Various liposomes preparations were produced with different surface modifications and have been tested for any size change at different temperature. Table 3.1. is provided below and shows the statistical analysis to determine the significance level of the changes in liposomes size under the influence of different temperatures.

**Table 3.1. Effects of temperature on the size of different liposomal formulations (SPC; DSPC; HSPC)**

| Modifications \ Liposome type | SPC liposome | | | |
|---|---|---|---|---|
| | changes in size at different temperature (nm) | | | p-value† |
| | 25° | 37 ° | 50 ° | |
| Conventional | 122 | 130 | 134 | 0.372 |
| 2% PEG2000-DSPE | 160 | 164 | 165 | |
| 5% PEG2000-DSPE | 153 | 154 | 136 | |
| 2% PEG2000-DSPE-COOH | 284 | 276 | 259 | |
| 5% PEG2000-DSPE-COOH | 241 | 244 | 315 | |
| 2% PEG2000-DSPE-NH2 | 150 | 170 | 190 | |
| 5% PEG2000-DSPE-NH2 | 174 | 209 | 219 | |
| **DSPC liposomes** | | | | |
| Conventional | 189 | 172 | 174 | 0.663 |
| 2% PEG2000-DSPE | 172 | 187 | 198 | |
| 5% PEG2000-DSPE | 184 | 201 | 218 | |
| 2% PEG2000-DSPE-COOH | 169 | 204 | 193 | |
| 5% PEG2000-DSPE-COOH | 229 | 271 | 254 | |
| 2% PEG2000-DSPE-NH2 | 291 | 340 | 324 | |
| 5% PEG2000-DSPE-NH2 | 230 | 290 | 321 | |
| **HSPC liposomes** | | | | |
| Conventional | 140 | 145 | 154 | 0.292 |
| 2% PEG2000-DSPE | 148 | 159 | 176 | |
| 5% PEG2000-DSPE | 149 | 176 | 192 | |
| 2% PEG2000-DSPE-COOH | 142 | 148 | 165 | |
| 5% PEG2000-DSPE-COOH | 125 | 147 | 148 | |
| 2% PEG2000-DSPE-NH2 | 148 | 152 | 176 | |
| 5% PEG2000-DSPE-NH2 | 149 | 176 | 192 | |

†Results of ANOVA tests for differences across different groups; *** *p<.01,* ** *p<.05,* * *p<.1*

**Table 3.2. Effect of Pegylation on the size of different liposomal formulations (SPC; DSPC; HSPC) at different temperature**

| Liposome type / Modifications | PEG-SPC liposome | | | |
|---|---|---|---|---|
| | change in size at different temperature (nm) | | | p-value† |
| | 25° | 37 ° | 50 ° | |
| Conventional | 122 | 130 | 134 | 0.011** |
| 2% PEG2000-DSPE | 160 | 164 | 165 | |
| 5% PEG2000-DSPE | 153 | 154 | 136 | |
| 2% PEG2000-DSPE-COOH | 284 | 276 | 259 | |
| 5% PEG2000-DSPE-COOH | 241 | 244 | 315 | |
| 2% PEG2000-DSPE-NH2 | 150 | 170 | 190 | |
| 5% PEG2000-DSPE-NH2 | 174 | 209 | 219 | |
| **PEG-DSPC liposomes** | | | | |
| Conventional | 189 | 172 | 174 | 0.474* |
| 2% PEG2000-DSPE | 172 | 187 | 198 | |
| 5% PEG2000-DSPE | 184 | 201 | 218 | |
| 2% PEG2000-DSPE-COOH | 169 | 204 | 193 | |
| 5% PEG2000-DSPE-COOH | 229 | 271 | 254 | |
| 2% PEG2000-DSPE-NH2 | 291 | 340 | 324 | |
| 5% PEG2000-DSPE-NH2 | 230 | 290 | 321 | |
| **PEG-HSPC liposomes** | | | | |
| Conventional | 140 | 145 | 154 | 0.038** |
| 2% PEG2000-DSPE | 148 | 159 | 176 | |
| 5% PEG2000-DSPE | 149 | 176 | 192 | |
| 2% PEG2000-DSPE-COOH | 142 | 148 | 165 | |
| 5% PEG2000-DSPE-COOH | 125 | 147 | 148 | |
| 2% PEG2000-DSPE-NH2 | 148 | 152 | 176 | |
| 5% PEG2000-DSPE-NH2 | 149 | 176 | 192 | |

†Results of ANOVA tests for differences across different groups; *** *p<.01,* ** *p<.05,* * *p<.1*

None of the liposomal formulations showed statistically significant differences in their sizes at 25 °C, 37 °C or Tcs (P-value >0.05) (Table 3.1). The calculated significance level for the

effect of PEG on the average liposomes' size is presented in (Table 3.2). Both SPC and HSPC formulas showed a significance difference in their sizes when modified with the PEG (P-value was 0.011 and 0.038) respectively.

There is a growing increase in the applications of neutral phospholipids in clinical industry, yet the use of PEGylated liposomes is still in its infancy. There are several liposomal formulations in clinical use is consist of neutral phospholipids. Some of them are PEGylated and other are not. The most commonly used neutral phospholipid is the DSPC which used with four liposomal formulations namely Onpattro, Vyxoses, Onivyde, and Lipodox (Anselmo and Mitragotri, 2019; Bozzuto and Molinari, 2015; Kaspers et al., 2013). Among these formulations, only Vyxosed is the non-PEGylated formulation. The DSPC takes its breakthroughs in the liposomes' formations due to its high stability, which was linked to the long acyl moiety. Previous work suggested that as the chain length increase, so does the Tc, increasing liposomes' stability (Andreson and Omri, 2004). Andreson and Omri (2004) carried out an experiment aiming to evaluate the effect of different liposome compositions on their stability characteristics and release profile. The researchers selected different phospholipids with different Tcs. The selected liposomes were DSPC which has a Tc above body temperature (55°C), DPPC which has a transition temperature close to body temperature (41.5°C), NS DMPC which has a temperature below the 37°C (23°C). The maximum encapsulation was achieved with DSPC. This may result from its rigid structure represented in the saturated long chain lengths. Although liposome stability is proffered to ensure the drug retention inside the vesicles, it is also important to confirm the encapsulated drug will be released and reach the target site. To ensure this liposome modifications with PEG were introduced. The insertion of PEG into the formulation increases the $T_{1/2}$ and thus allows more time for the contents to be released over time. The second most commonly used phospholipid was HSPC with two formulations. One of them is PEGylated (Doxil), and the

other one is non-PEGylated (Ambisome) (Bozzuto and Molinari, 2015). SPC liposomes reported as the main phospholipid modified with a PEG for the Lipusu formulation (Xu et al., 2013). Overall average size for these formulations was close to our findings (blow 200 nm) except the Lipusu which has a small size (ca. 400 nm).

According to the previous findings for the approved neutral liposomal formulations, most of them were PEGylated (5 out of 8). The introduction of PEG is a common method to avoid recognition of the liposomes by the immune system, including hepatic Kupffer cells which are part of the mononuclear phagocyte system (MPS) (Nag and Awasthi, 2013). Here, 2 or 5 mol% of PEG were introduced. The properties of the PEGylated liposomes mainly were the same as for non-PEGylated ones, apart from a small change in surface charge for SPC liposomes. PEG insertion did not seem to affect size at lower mol% when using DSPE-PEG2000. Interestingly, size decreased slightly at 5 mol% PEG-DSPE with SPC liposomes (Figure 3.7.A.). For all liposomal formulations, the size was dramatically increased when adding 2% and 5% PEG-DSPE-NH2. For example, for SPC liposomes it was (130 Vs 220 nm) and (150 Vs 300 nm) for DSPC and HSPC liposomes. Both SPC and DSPC liposomes size was also enlarged when inserting DSPE-PEG2000-COOH at 5mol%. However, size followed the same pattern for non-pegylated liposomes, in which size increased as temperature increased for the remaining formulations. Guzman-Villanueva et al., (2017) study which was conducted using PC: Chol 70:30 stated that the average size without modifications was 120 nm. When adding 5% PEG-DSPE-COOH, size increased to 132 nm, with a reduction of the ZP from -17 to -9 mV. Literature proved that the addition COOH group in an aqueous medium is related to increase the surface charge negativity due to their dissociation in the medium (Hanaor et al., 2012). On the other hand, the amine group tend to produce a positive charge on the surface when protonated (Oslolska and Wiśniewska, 2014). Although this was not the case with our findings, this might be related to some

reasons. It can be due to incomplete attachment of the PEG due to the rigidity or low fluidity obtained for the liposomes' membrane (Babchin et al., 1976). However, no obvious difference was recorded when using DSPE-PEG2000 with both concentrations. A study conducted by (Qi, Zhang et al., 2011) showed that adding a higher amount of DSPE-PEG2000 to liposomes is correlated to a reduction in both size and zeta potential values. This occurs because of the formed hydrophilic PEG shell around the liposomes. Thus, zeta potential value would be reduced by masking the surface charge (Zhang et al., 2011). Among the three formulations, only SPC liposomes showed a significant difference in average size when modified with PEG (P-value<0.01) (Table 3.2). Literature proved that there is no significant impact of PEG on the liposomes' average size when used below 8 mol% (Garbuzenko et al., 2005). The finding for SPC can be related to the changes in lipid bilayer packing form. SPC as an unsaturated phospholipid requires lower Tc than saturated lipids (Maherani et al., 2013). This can ensure getting better membrane fluidity and ensure the insertion of the PEG on the liposomes surface. However, further investigation by DSC to study the thermodynamic profile for SPC liposomes is required.

### 3.4.5. Phospholipid quantification and yield measurements

In this part, the amount of the phospholipids was evaluated using both colorimetric assay, and theoretical yield determination using evaporation method.

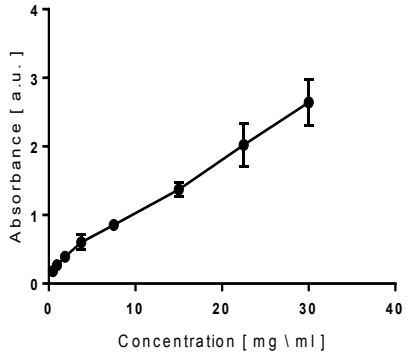
**Table 3.3. %Yield measurements for SPC, DSPC, and HSPC liposomes. Data represents the mean± SD of three individual experiments.**

| Formula | Initial concentration (g / ml) | Average theoretical concentration (g \ ml) | Average % yield |
|---|---|---|---|
| SPC | 0.02 | 0.013 | 66% |
| DSPC | 0.02 | 0.018 | 95% |
| HSPC | 0.02 | 0.016 | 85% |

Table 3.3. shows the average theoretical yield for the SPC, DSPC, and HSPC liposomes. The highest yield obtained with DSPC liposomes (95%) whereas the lowest was obtained with SPC liposomes (66%).

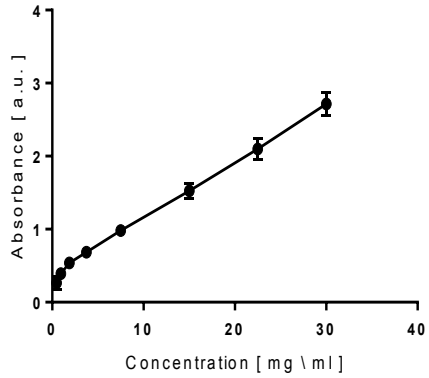
(A)

Y= 0.0804X + 0.2176
R2 = 0.9972




(B)

Y= 0.0795X + 0.3321
R2 = 0.9959



(C)

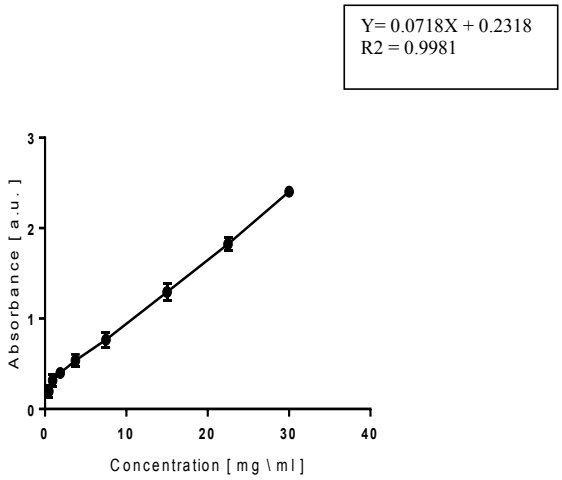



**Figure 3.10. Calibration curves for phospholipids quantification for SPC (A), DSPC (B), and HSPC (C). Data represents the mean± SD of three individual experiments.**

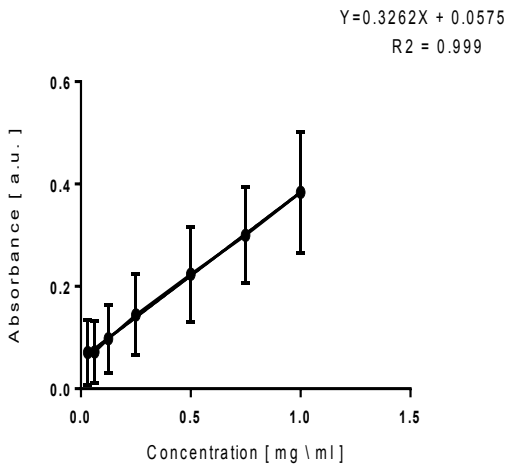
From the calibration curves, all liposomal formulations showed approximate full phospholipid incorporation confirmed by the calculated amount of the lipids in the liposomes. SPC liposomes has an average absorbance of 1.74. The mean calculated amount of phospholipid was 18.94 16 mg\ ml. DSPC liposomes have an average absorbance of 1.83. The mean calculated amount of phospholipid was 18.88 mg \ ml, and HSPC has an average absorbance of 1.6. The mean calculated amount of phospholipid was 19.01 mg \ml.

According to the obtained results from the actual yield, the highest yield was for DSPC> HSPC> SPC (95> 85> 66%). In general, all liposomes appeared to produce a great yield. However, some factors might lead to a reduction in the theoretical yield. This includes inaccurate measurements, insufficient drying time by which there are some droplets of the suspension did not solidify, and the presence of the impurities in the tested phospholipid (Paixão et al., 2020).

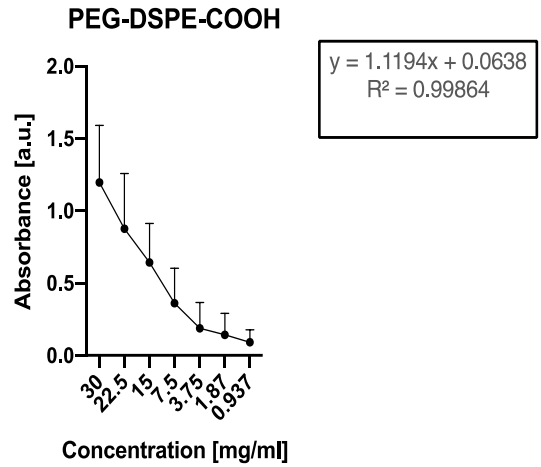
### 3.4.6. PEG Calibration curves and quantification

Standard calibration curves were performed three times for PEG quantification. The average standard curve is provided to calculate the amount of the PEG present in the prepared formulations according to the absorbance values obtained from the UV instrument.

(A)




(B)




(C)

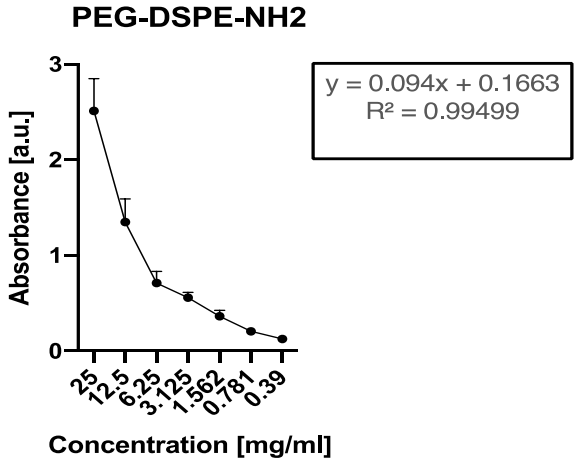



**Figure 3.11. Calibration curve for PEG-DSPE (A), PEG-DSPE-COOH (B), and PEG-DSPE-NH2 (C). Data represents the mean± SD of three individual experiments.**

Figure 3.11 Illustrates the calibration curves for different PEG types. All the curves showed a concentration dependant pattern corresponding to their absorbance values.

**Table 3.4. PEG absorbance values for liposomes containing different types of PEG and their %EE.**

| PEG mol% / %EE | SPC | | DSPC | | HSPC | |
|---|---|---|---|---|---|---|
| | **Absorbance** | **%EE** | **Absorbance** | **%EE** | **Absorbance** | **%EE** |
| 2% PEG-DSPE | 0.2 ± 0.029 | 76± 11 | 0.4± 0.063 | 67±11 | 0.2 ± 0.039 | 34±8 |
| 5% PEG-DSPE | 0.4± 0.027 | 52 ± 4 | .9 ± 0.004 | 67 ± 0.0 | 0.3± 0.042 | 22±3 |
| 2% PEG-DSPE-COOH | 0.2 ± 0.447 | 74 ±6.7 | 0.23 ± 0.47 | 85 ± 9.22 | 0.19 ± 0.3 | 39 ± 9 |
| 5% PEG-DSPE-COOH | 0.39 ± 0.62 | 40.4±6.3 | 0.45 ± 0.67 | 48 ± 7 | 0.3 ± 0.55 | 30 ± 5.4 |
| 2% PEG-DSPE-NH2 | 0.185 ± 0.4 | 73 ± 8.5 | 0.183 ± 0.45 | 65 ± 8 | 0.173 ± 0.4 | 26.3 ± 5 |
| 5% PEG-DSPE-NH2 | 0.2 ± 0.029 | 53 ± 4.3 | 0.19 ± 0.43 | 37 ± 6 | 0.187 ± 0.4 | 22 ± 3 |

† Results of N-ways ANOVA test; prop> F between liposomes (0.0001); prop> F between different modifications (0.0193).

Table 3.4 shows the absorbance values for different liposomal formulations modified with different PEG derivatives. The amount of inserted PEG on the liposomes' surface was determined as a function of %EE. HSPC liposomes showed the least amount of PEG on their surfaces. Results for both SPC and DSPC liposomes were comparable. However, the encapsulated amount on the liposomes' surface did not change dramatically between the two PEG densities i.e., the amount of PEG on the surface for SPC liposomes containing 2% DSPE-PEG2000 is 76% whereas the %EE for the modified SPC liposomes with 5 mol% was 52%, suggesting that the maximum PEG incorporation stands at ca. 2.5 mol% for liposomes of this size.

The amount of PEG on liposomes' surface differs slightly between the three formulations. However, these differences also reported for the same formulation when using 2 and 5 mol% PEG (Table 3.4). The encapsulation efficiency reached its maximum with DSPC liposomes

(85%) when PEGylating with 2% DSPE-PEG2000-COOH, and the minimum with HSPC when using 5% DSPE-PEG2000 and DSPE-PEG2000-NH2. In general, the incorporation of PEG on the liposomes' surface was increased as decreasing PEG concentration (Nicholas et al., 2000). Results for SPC was expected as this liposomal formulation has a low Tc. This means it is expected to easily liquefy the liposomes' surface and allow the insertion of the PEG. However, adding cholesterol to the liposomes can also alter the Tc, meaning that the membrane might not be fluid enough to allow PEG chains to be inserted as in case of HSPC (Redondo-Morata et al., 2012). Other factors can be considered for HSPC findings including that, as PEG was added using the post-insertion method, the liposomes' membrane might not fluid enough to incorporate all of the PEG quantity on its surface.

### 3.4.7. LOD and LOQ determination

In order to validate our quantification method, we calculate the LOD and LOQ. These two parameters determine the presence or absence of the sample to be quantified, and to increase the robustness and to confirm our estimations of the tested samples (Armbruster and Pry, 2008).

**Table 3.5. LOD and LOQ parameters and calculations for the quantified phospholipids.**

| | SPC | DSPC | HSPC |
|---|---|---|---|
| SD of the intercept | 0.025 | 0.03 | 0.018 |
| Slope | 0.08 | 0.08 | 0.07 |
| LOD | 1 mg/ml | 1.2 mg/ml | 0.8 mg/ml |
| LOQ | 3.125 mg/ml | 3.75 mg/ml | 2.6 mg/ml |

Table 3.5 showed that, the highest sensitivity for phospholipid detection was observed with HSPC liposomes (0.8). However, both SPC and DSPC showed a comparable detection level (ca. 1 mg/ml). All phospholipids showed a good quantification at 3 mg/ml which is within the range we have been used.

**Table 3.6. LOD and LOQ parameters and calculations for the quantified PEG.**

| | SPC | DSPC | HSPC |
|---|---|---|---|
| SD of the intercept | 0.018 | 0.01 | 0.03 |
| Slope | 0.0326 | 0.012 | 0.09 |
| LOD | 0.02 mg/ml | 0.01 mg/ml | 0.1 mg/ml |
| LOQ | 0.5 mg/ml | 0.1 mg/ml | 3.3 mg/ml |

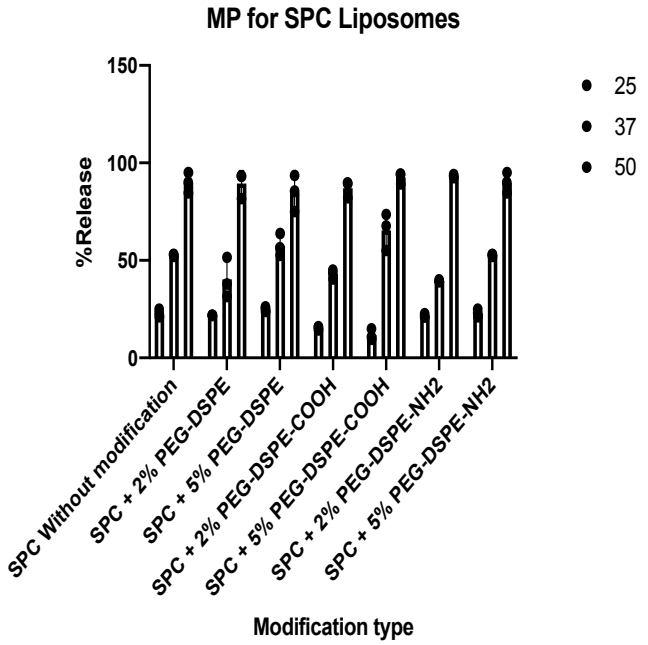
According to PEG quantification confirmation, the highest sensitivity was reported with SPC and DSPC (0.02 and 0.01 mg/ml) respectively. However, HSPC showed the least detection sensitivity (0.1 mg/ml) SPC and DSPC were quantified <0.5 mg/ml whereas HSPC showed the quantification profile at 3.3 mg/ml (Table 3.6).

These findings agree with PEG quantification data which showed the least detected amount of PEF on the surface was reported with HSPC. This can explain the high concentration required for their detection on the validation step. LOL and LOQ as functions are good to recognise the presence of the material to be tested. In order to investigate the membrane characteristics of the formulated liposomes, a membrane permeability experiment was obtained to assess the liposomes' surface characteristics which can affect the drug release kinetics.

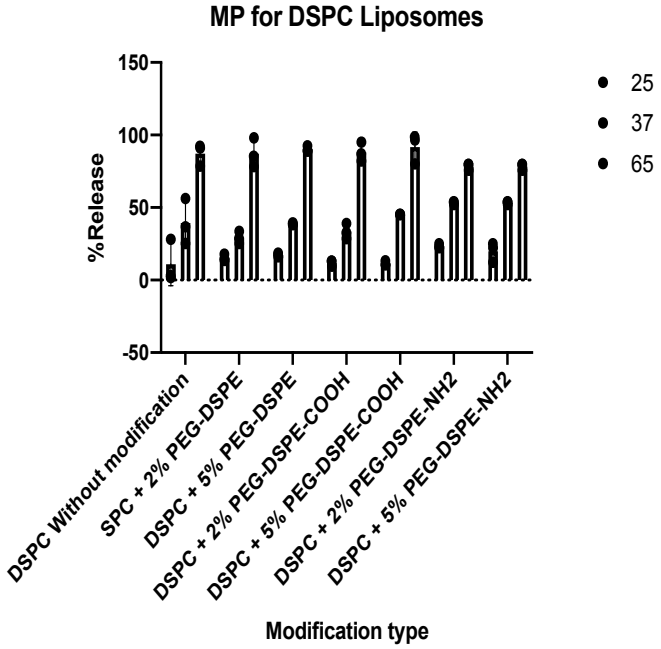
### 3.4.8. Membrane permeability

Calcein was used as a hydrophilic, fluorescent probe to investigate membrane permeability (Figure 12). The aim of this experiment is to evaluate the effect of the temperature on the membrane permeability profile among the liposomal formulations with different modifications. In addition, to identify the maximum calcien release at which temperature and with which formulation.

(A)


MP for SPC Liposomes
150
100
50
0
%Release
25
37
50
SPC Without modification
SPC + 2% PEG-DSPE
SPC + 5% PEG-DSPE
SPC + 2% PEG-DSPE-COOH
SPC + 5% PEG-DSPE-COOH
SPC + 2% PEG-DSPE-NH2
SPC + 5% PEG-DSPE-NH2
Modification type

(B)


MP for DSPC Liposomes
150
100
50
0
-50
%Release
25
37
65
DSPC Without modification
SPC + 2% PEG-DSPE
DSPC + 5% PEG-DSPE
DSPC + 2% PEG-DSPE-COOH
DSPC + 5% PEG-DSPE-COOH
DSPC + 2% PEG-DSPE-NH2
DSPC + 5% PEG-DSPE-NH2
Modification type

(C)

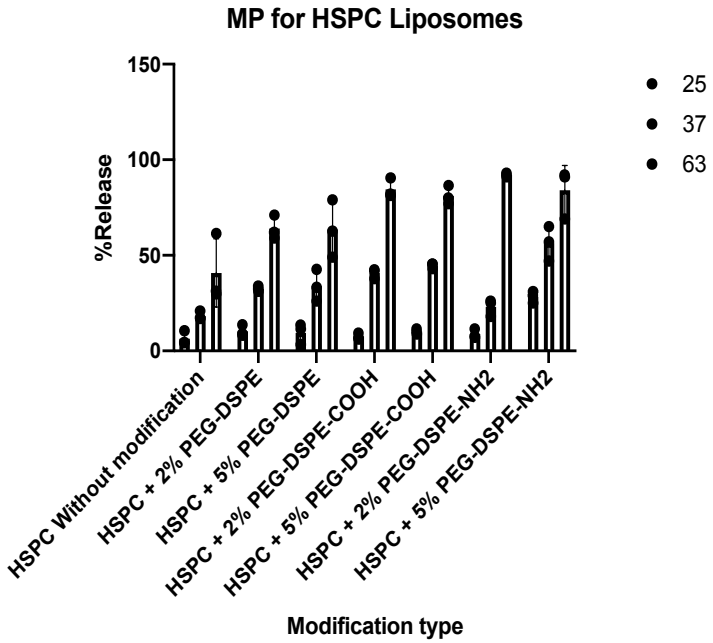



**Figure 3.12. Membrane permeability and %Release for SPC Liposomes (A), DSPC Liposomes (B), and HSPC Liposomes (C) with different modifications. Data represents the mean± SD of three individual experiments.**

Figure 3.12. represents the release profile for the three liposomes formulation without modification, and when modified with different types of PEG using 2 and 5 mol%. Overall results showed a direct proportional relationship between the temperature and the permeability. In another word, the membrane permeability increases as the temperature increase. The maximum permeability was observed with SPC liposomes without modification (ca. 95%) (Figure 3.12.A.), and the lowest was reported with the un-modified HSPC liposomes (Figure 3.12.C).

**Table 3.7. Effects of temperature on the membrane permeability of different liposomal formulations (SPC; DSPC; HSPC).**

| Liposome type / Modifications | SPC liposome | | | |
|---|---|---|---|---|
| | change in release at different temperature (%) | | | p-value† |
| | 25°C | 37°C | 50°C | |
| Conventional | 17.5 | 40.876 | 88.35 | 0.029** |
| 2% PEG2000-DSPE | 21.86 | 40.25 | 89.41 | |
| 5% PEG2000-DSPE | 25.06 | 46 | 84.73 | |
| 2% PEG2000-DSPE-COOH | 15 | 42.8 | 86.6 | |
| 5% PEG2000-DSPE-COOH | 11.75 | 65 | 92.52 | |
| 2% PEG2000-DSPE-NH2 | 21.6 | 39 | 92.88 | |
| 5% PEG2000-DSPE-NH2 | 23 | 53 | 90 | |
| **DSPC liposomes** | | | | |
| Conventional | 12.38 | 26 | 84.4 | 0.194 |
| 2% PEG2000-DSPE | 15.4 | 29.16 | 90.25 | |
| 5% PEG2000-DSPE | 17.38 | 38.67 | 90.29 | |
| 2% PEG2000-DSPE-COOH | 11.85 | 33.4 | 92 | |
| 5% PEG2000-DSPE-COOH | 11.44 | 45.24 | 92 | |
| 2% PEG2000-DSPE-NH2 | 23 | 53 | 78 | |
| 5% PEG2000-DSPE-NH2 | 25 | 53 | 78.5 | |
| **HSPC liposomes** | | | | |
| Conventional | 6.62 | 17.75 | 29.68 | 0.038** |
| 2% PEG2000-DSPE | 10.33 | 32.52 | 64.03 | |
| 5% PEG2000-DSPE | 9.41 | 0.34 | 0.64 | |
| 2% PEG2000-DSPE-COOH | 7.50 | 40.76 | 0.85 | |
| 5% PEG2000-DSPE-COOH | 10.30 | 45.00 | 0.81 | |
| 2% PEG2000-DSPE-NH2 | 8.80 | 23.00 | 0.92 | |
| 5% PEG2000-DSPE-NH2 | 28.30 | 56.30 | 0.84 | |

Table notes: †Results of ANOVA tests for differences across different groups; *** p<.01, ** p<.05, * p<.1

Table 3.7. shows that, the membrane permeability for SPC and HSPC liposomes are significantly affected when exposed to a different temperature profile (P-value<0.05).

**Table 3.8. Effect of Pegylation on the membrane permeability for different liposomal formulations (SPC; DSPC; HSPC) at different temperature.**

| Liposome type / Modifications | PEG-SPC liposome | | | |
|---|---|---|---|---|
| | change in release at different temperature (%) | | | p-value† |
| | 25° | 37 ° | 50 ° | |
| Conventional | 6.62 | 17.75 | 29.68 | 1 |
| 2% PEG2000-DSPE | 10.33 | 32.52 | 64.03 | |
| 5% PEG2000-DSPE | 9.41 | 0.34 | 0.64 | |
| 2% PEG2000-DSPE-COOH | 7.50 | 40.76 | 0.85 | |
| 5% PEG2000-DSPE-COOH | 10.30 | 45.00 | 0.81 | |
| 2% PEG2000-DSPE-NH2 | 8.80 | 23.00 | 0.92 | |
| 5% PEG2000-DSPE-NH2 | 28.30 | 56.30 | 0.84 | |
| **PEG-DSPC liposomes** | | | | |
| Conventional | 12.38 | 26 | 84.4 | 0.996 |
| 2% PEG2000-DSPE | 15.4 | 29.16 | 90.25 | |
| 5% PEG2000-DSPE | 17.38 | 38.67 | 90.29 | |
| 2% PEG2000-DSPE-COOH | 11.85 | 33.4 | 92 | |
| 5% PEG2000-DSPE-COOH | 11.44 | 45.24 | 92 | |
| 2% PEG2000-DSPE-NH2 | 23 | 53 | 78 | |
| 5% PEG2000-DSPE-NH2 | 25 | 53 | 78.5 | |
| **PEG-HSPC liposomes** | | | | |
| Conventional | 6.62 | 17.75 | 29.68 | 0.49 |
| 2% PEG2000-DSPE | 10.33 | 32.52 | 64.03 | |
| 5% PEG2000-DSPE | 9.41 | 0.34 | 0.64 | |
| 2% PEG2000-DSPE-COOH | 7.50 | 40.76 | 0.85 | |
| 5% PEG2000-DSPE-COOH | 10.30 | 45.00 | 0.81 | |
| 2% PEG2000-DSPE-NH2 | 8.80 | 23.00 | 0.92 | |
| 5% PEG2000-DSPE-NH2 | 28.30 | 56.30 | 0.84 | |

†Test for differences across different groups; *** $p<.01$, ** $p<.05$, * $p<.1$

The significance level of the impact of the PEGylation on the liposomal formulations is provided in (Table 3.8). PEGylation appeared to have no significance difference in the release kinetics for the formulated formulations proved by the P-vales<0.05.

Liposomes were tested for their membrane fluidity properties using calcein. Calcein is a hydrophilic probe with self-quenching characteristics (Maherani et al., 2013; Shimanouchi et al., 2009). The ability of calcein to self-quenching means that fluorescence will be low when concentrated inside the liposomes. At higher temperatures, the membrane permeability for the liposomes forwards the encapsulated calcein in the external medium increase which leads to an increase in fluorescence as the fluorescence is no longer quenched following dilution of the probe (Maherani et al., 2013). According to the obtained results, the maximum fluidity was recorded for all the formulations at their Tc, though none of the formulations achieved 100% calcein release. Factors known to have an impact on calcein release include size, lipid composition, lamillarity and temperature (Maherani et al., 2013; Shimanouchi et al., 2009). Here, composition and temperature seem to be the most important factors. Although the same cholesterol ratio was used in all formulations, the membrane permeability and availability of calcien in the external medium did not reach 100%, confirming that Tc was changed from the theoretical value, especially with HSPC liposomes. This can be due to that, the cholesterol incorporated fully in HSPC formulation and increase its rigidity. Thus, when adding the PEG post-insertion at Tc, it required higher temperature to fluidise the membrane and allow the PEG insertion.

For liposomes without any modification, the maximum permeability was recorded for Soybean phosphatidylcholine liposomes (88.35%) at 50 °C. Out of all the temperatures tested, the fluidity at 37°C is the most important as this shows the properties of the liposomes in the body. The results obtained confirm that liposomes with varying membrane fluidity have been made, with SPC being the most fluid and HSPC, the least. The SPC finding agreed with a study that revealed that maximum membrane permeability was achieved with soybean liposomes reaching 80.92% at their Tc (Chen et al., 2012). DSPC has a close fluidity profile as the value obtained for SPC liposomes (84.4%) at 65 °C (Figure 3.12.B.). Lu et al., (2012)

study stated that, the membrane permeability for the encapsulated probe from DSPC at 25°C was 16%, 22% at 37°C, and reached the maximum of 60% at 65 °C (Lu et al., 2012). At 25°C and 37°C, results were comparable. The 20% differences in release at the transition temperature could arise from using different phospholipid: cholesterol ratios i.e. they used 60:40 ratio instead of 70:30.This might arise as a results of increasing the membrane rigidity when using 60:40 in (Lu et al., 2012) study. Chen et al., (2012) carried out a study for testing the membrane permeability for different liposomal formulations and their behavior for the first 10 hours. Results showed that at 25°C, the membrane fluidity was 10.52%, 6.43% and 6.08% for Soybean, DSPC and HSPC respectively. These can be similar to the ones we had (17% for soybean, 12% for DSPC, and 6% for HSPC). Despite that, DSPC appeared to be the double value of the study; the difference between 10 hours and 24 hours can play a role (Lu et al., 2012). For HSPC, it was evident that the membrane permeability was low and did not exceed 7% after 10 hours of incubation in another study testing the influence of lipid composition of HSPC and DPPC on their membrane fluidity behavior (Chen et al., 2012).The low membrane permeability profile obtained at 25°C for all formulation was expected. This is due to their rigid gel configuration which did not allow the diffusion of the encapsulated calcein to the outside medium. With increasing the temperature, this gel phase converted to a liquid crystalline form with a less compact configuration. This can explain the findings for the liposomal formulations have a higher permeability upon increasing the temperature i.e. reach approximately 90% at their Tcs (Figure 3.12).

The introduction of the PEG-lipid was associated with a reduction in the membrane permeability at lower %mol except with HSPC liposomes. Silvander et al., (1998) carried out a study for assessing the liposomes fluidity for neutral liposomes (EPC and DSPC liposomes) containing different %mol of DSPE-PEG2000 using carboxyfluroscine probe. The main findings proved that, adding 5% DSPE-PEG2000 to the formulation improves the

permeability of the liposomes. However, adding 8 mol% of the polymer reduces the permeability significantly. The addition functionalized PEG was associated with an increase in permeability compared to liposomes without any modifications (Awad et al., 2019). However, the fluidity increases as the chain length of the added PEG decreases (Awad et al., 2019). Based on our findings from the lab, aminated PEG showed a positive correlation with the membrane permeability (i.e. the membrane permeability increased from 22% for plain liposomes to 57% for liposomes containing DSPE-PEG2000-NH2).

However, this was not the case with SPC liposomes. It is suggested as a smaller size (< 200 nm) which mainly associate with unilamillarity, the permeability pattern would be enhanced. This is due to that, the production of a unilamellar vesciels (ULVs) enclose a large aqueous core, and ideal for the encapsulation of a hydrophilic drugs (Bozzuto and Molinari 2015), which is in our study is Calcein. The membrane fluidity for SPC and HSPC liposomes showed a significant difference with both temperature and PEGylation (Table 3.8.). Both SPC and HSPC shares the same chain length and similar average size. Shimanouchi et al., (2009) confirmed that, calcein release increase as the liposomes diameter decrease. Another factor can also be considered which is the level of unsaturation. The presence of double bonds reduce the Tc of phospholipids and enhance the membrane permeability characteristics. SPC liposomes showed unsaturation prosperities with a low melting point and the smallest liposomes size, which excepted to show a good fluidity. HSPC has a smaller average size and shorter chain length than DSPC. This can explain it superiority over DSPC liposomes in term of membrane permeability prosperities. Previous literature stated that, the longer the chain length, the more stable system formed and the higher temperature is required for getting the desired outcome (Bouffioux et al., 2007).

### 3.4.9. Linear regression modelling for the impact of the temperature on release profile

This linear regression modelling was established to predict the effect of the temperature on the release kinetics.

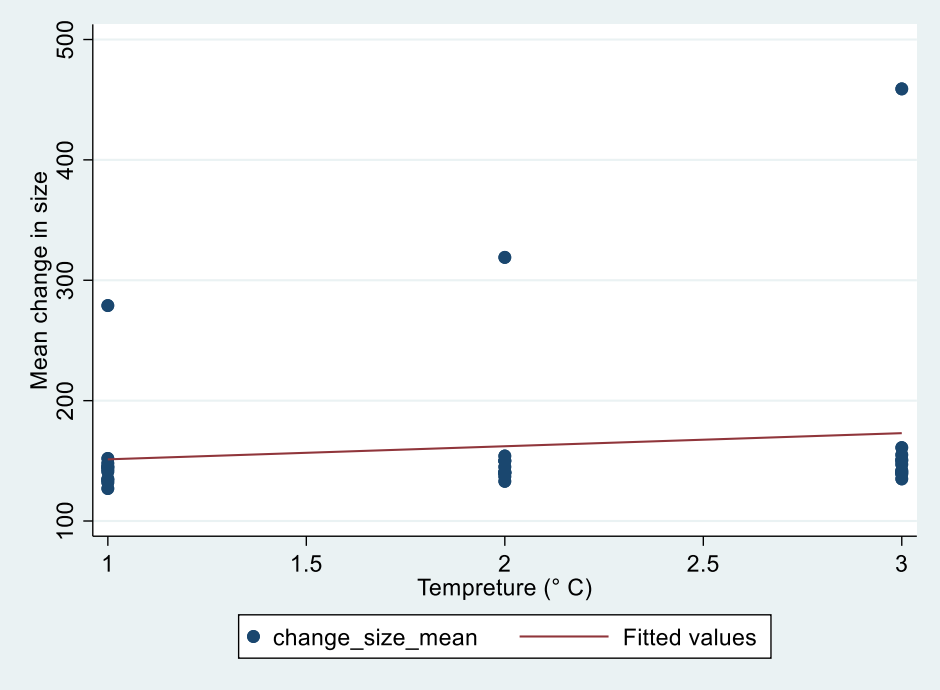
**Table 3.9. Linear regression analysis of the release behaviour for the formulated liposomes against different temperature profile.**

| Temperature °C | SPC | | | DSPC | | | HSPC | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Coef** | **P** | **CI 95%** | **Coef** | **P** | **CI 95%** | **Coef** | **P** | **CI 95%** |
| 25 (Ref) | - | - | - | - | - | - | - | - | - |
| 37 | 6.2 | 0.83 | (-37-80) | 5 | 0.58 | (-14-22) | 5.5 | 0.39 | (-7.3-18.3) |
| Tc[(a)] | 153 | 0.45 | (111-194) | 12.6 | 0.164 | (165-190) | 13.5 | 0.04** | (142-160) |

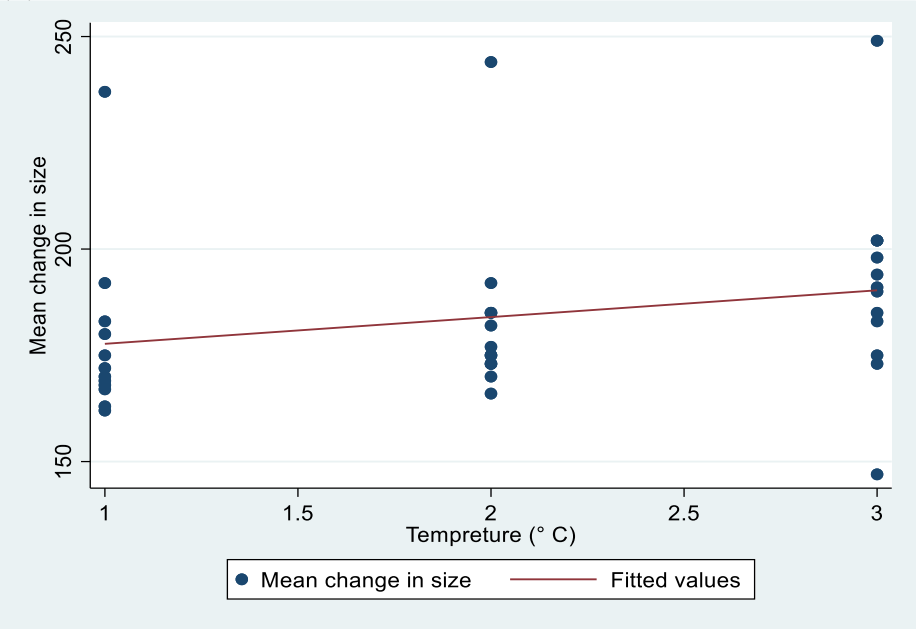
*Table keynotes: *** p<.01, ** p<.05, * p<.1; (a)* Tc = 50, 63 and 65 °C for SPC, DSPC and HSPC Liposomes

Table 3.9. stated the p-value for the three liposomal formulations to show which formulation showed a significance difference in the release behaviour at different temperature profiles. Only HSPC showed significance difference in its release (P-value<0.05).

(A)



(B)




(C)

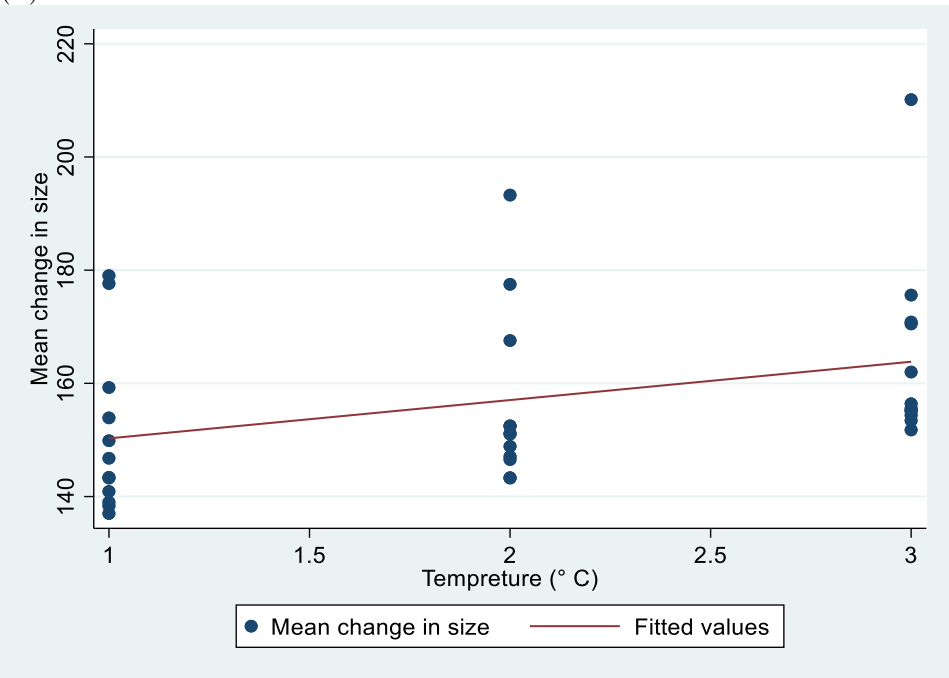



**Figure.3.13. Linear regression plots for the  against different temperature profile for the three formulated liposomes (A) SPC, (B) DSPC, and (C) HSPC Liposomes.**

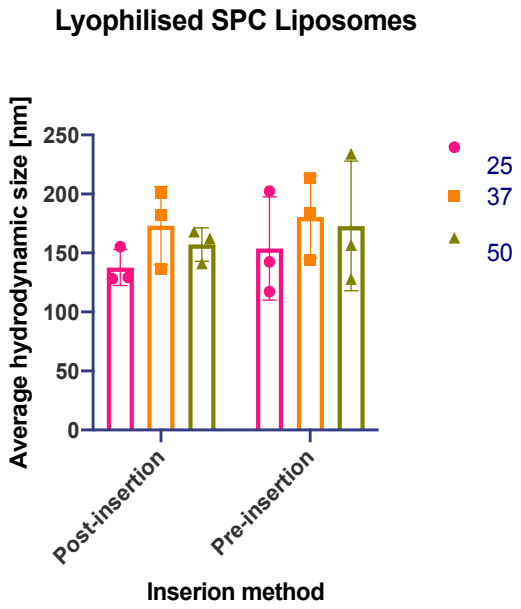
In order to investigate the impact of the temperature profile on the release kinetics, we established a linear regression model. Overall findings show a positive correlation for the release kinetics among the different stated temperatures. Table 3.9. showed that, only HSPC

liposomes showed a significant correlation for the temperature and the release profile (P-value<0.05) . This might arise as a result of its shorter chain length compared to DSPC, and having lower Tc which might be associated with a better release for the calcein. However, all the formulations showed a temperature-dependent pattern. All liposomal formulations expressed the maximum release at their Tcs when compared to 25 °C. For DSPC liposomes, as it is a saturated lipid with a long chain length (C-18), it requires higher energy to release the calcein (Maherani et al., 2013). Although we were expecting SPC to show a significant difference in the release with different temperature profile, as being an unsaturated lipid, having low Tc compared to DSPC and HSPC, which make it more liable to exert a good release profile (Shimanouchi et al., 2009). This might arise from that change in the packing form of the phospholipid due to the full inclusion of the cholesterol which increases its rigidity (Redondo-Morata et al., 2012). The reason, in this case, is unclear. Further research maybe required to address this outcome. After characterizing all the fundamental aspects of the liposomes, further modification for improving the shelf-life time of the liposomes was performed.

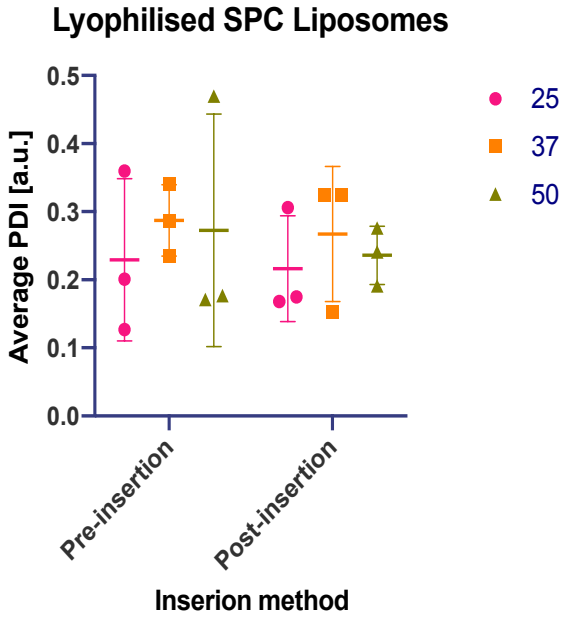
### 3.4.10. Lyophilised liposomes

The prepared liposomes were lyophilised using pre- and post-insertion methods of sucrose. The aim of this experiment is to reduce the liposomes instability and increase the shelf-life time. Also, we are trying to formulate a carrier close to the ones in the market.

(A)


Lyophilised SPC Liposomes
Average hydrodynamic size [nm]
250
200
150
100
50
0
Post-insertion
Pre-insertion
Inserion method
25
37
50

(B)


Lyophilised SPC Liposomes
Average PDI [a.u.]
0.5
0.4
0.3
0.2
0.1
0.0
Pre-insertion
Post-insertion
Inserion method
25
37
50

(C)

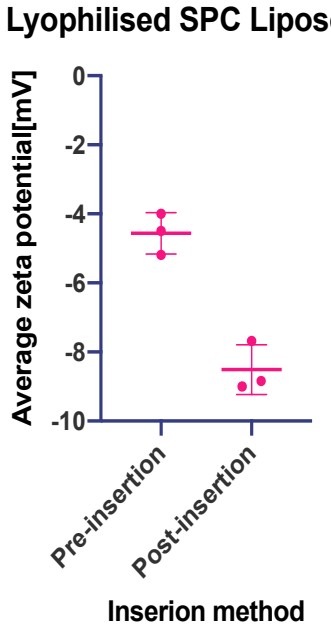



**Figure .3.14. Lyophilisation of SPC Liposomes using Pre- and Post insertion method, and their impact on average size (A), PDI (B), and Zeta Potential (C). Data represents the mean± SD of three individual experiments.**

Figure 3.14. shows the physicochemical properties for the lyophilized SPC liposomes. The sucrose as a lyoprotectant material was added using pre-and post-insertion method. The size with the pre-insertion method was slightly bigger (i.e., the size at 37 °C was 185 nm Vs 170 nm) for pre- and post-modification respectively (Figure 3.14.A.). Post-insertion method yields a greater homogeneity profile (Figure 3.14.B.). Both modifications produce a ZP values within the expected range for the neutral liposomes (Figure 3.14.C).

**Table 3.10. Anova test for the differences in size change between different lyophilisation methods at different temperature.**

| **Temperature** | **Changes in liposome size (nm)** | |
|---|---|---|
| | Pre-insertion | Post-insertion |
| **25** | 142 | 138 |
| **37** | 184 | 173 |
| **50** | 157 | 157 |

Table keynotes: † Results of ANOVA test; prop> F between different temperature (0.0204); prop> F between different insertion methods (0.2601).

Table 3.10 shows the output of the Anova results which applied to test the significance level for the impact of the insertion method on the liposomes size. The reported p-value was 0.02,

and thus, there is a significance difference in liposomes size when modified using pre- and post-insertion method.

Lyophilisation (Freeze-drying) is a process that was applied for liposomes aiming to enhance their stability (Alexopoulou, et al., 2006). The addition of some excipients e.g. sucrose, was recommended as a lyoprotectant (Kannan et al., 2014). Sucrose was added Pre and post-manufacturing. In both cases, the size was approximately the same (Figure 3.14.A.). However, the difference between the two groups was significant (0.02) (Table3.10). The post-insertion method yields better homogenous suspension than the post-manufacturing method (Figure 3.14.B.). Tavallaie (2016) studied the effect of sucrose on liposomes' size and PDI. The research findings showed that, the increase in both size and PDI occur as a result of vesicles' fusion and aggregation during the freezing step. During this stage, vesicles diffuse away from the ice crystals to the unfrozen medium and forms aggregation. Figure 14C showed that, the zeta potential value for post-insertion was double of the pre-insertion method (-4.5 and -9 mV). However, both values were within the suggested zeta potential for the neutral liposomes (10 and -10 mV) (Laouini et al., 2012). This suggested that, with the post-insertion method, most of the lyoprotectant was functionalized on the surface and masked the surface charge from recognition. On the contrary, when applying pre-insertion, some of the sucrose occupies some space inside the vesicles, and thus obtaining partial coverage on the liposomes' surface (Nag and Awasthai, 2013).

**References**

Abdelwahed, W., Degobert, G., Stainmesse, S. and Fessi, H., (2006). Freeze-drying of nanoparticles: formulation, process and storage considerations. Advanced drug delivery reviews, 58(15), pp.1688- 1713.

Abraham, S. A. *et al.* (2005) 'The liposomal formulation of doxorubicin', *Methods in Enzymology*. Academic Press Inc., 391(SPEC. ISS.), pp. 71–97. doi: 10.1016/S0076-6879(05)91004-5.

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S.W., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M. and Nejati-Koshki, K., (2013). Liposome: classification, preparation, and applications. Nanoscale research letters, 8(1), p.102.

Alexopoulou, E., Georgopoulos, A., Kagkadis, K. and Demetzos, C., (2006). Preparation and Characterization of Lyophilized Liposomes with Incorporated Quercetin. *Journal of Liposome Research*, 16(1), pp.17-25.

Allen, T. and Cullis, P. (2013). Liposomal drug delivery systems: From concept to clinical applications. Advanced Drug Delivery Reviews, 65(1), pp.36-48.

Anselmo AC, Mitragotri S. Nanoparticles in the clinic : an update. Bioeng Transl Med. (2019).

Armbruster, D. A., & Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. *The clinical biochemist reviews*, *29*(Suppl 1), S49.

Anderson, M., & Omri, A. (2004). The effect of different lipid components on the in vitro stability and release kinetics of liposome formulations. *Drug delivery*, *11*(1), 33-39.

Awad, N., Paul, V., Mahmoud, M., Al Sawaftah, N., Kawak, P., Al Sayah, M. and Husseini, G., (2019). Effect of Pegylation and Targeting Moieties on the Ultrasound-Mediated Drug Release from Liposomes. *ACS Biomaterials Science & Engineering*, 6(1), pp.48-57.

Babchin, A. J., Piliavin, M. A., & Levich, V. G. (1976). Rheoelectric effect in a polar liquid interphase layer. *Journal of Colloid and Interface Science*, *57*(1), 1-10.

Barnadas-Rodríguez, R., & Sabés, M. (2001). Factors involved in the production of liposomes with a high-pressure homogenizer. *International journal of pharmaceutics*, *213*(1-2), 175-186.

Barenholz, Y. C. (2012). Doxil®—the first FDA-approved nano-drug: lessons learned. *Journal of controlled release*, *160*(2), 117-134.

Blair, H. A. (2018). Daunorubicin/cytarabine liposome: a review in acute myeloid leukaemia. *Drugs*, *78*(18), 1903-1910.

Bouffioux, O., Berquand, A., Eeman, M., Paquot, M., Dufrêne, Y. F., Brasseur, R., & Deleu, M. (2007). Molecular organization of surfactin–phospholipid monolayers: Effect of phospholipid chain length and polar head. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1768*(7), 1758-1768.

Bozzuto, G. and Molinari, A. (2015) 'Liposomes as nanomedical devices', I*nternational Journal of Nanomedicine*. Dove Medical Press Ltd., pp. 975–999. doi: 10.2147/IJN.S68861.

Briuglia, M. L. et al. (2015) 'Influence of cholesterol on liposome stability and on in vitro drug release', *Drug Delivery and Translational Research*. Springer Verlag, 5(3), pp. 231–242. doi: 10.1007/s13346-015-0220-8.

Chen, J. et al. (2006) 'Effect of phospholipid composition on characterization of liposomes containing 9-nitrocamptothecin', *Drug Development and Industrial Pharmacy*, 32(6), pp. 719–726. doi: 10.1080/03639040500529077.

Chen, Yan, Hu, Gu, Chen, Gu, Chen and Chen (2012). Improved pharmacokinetics and reduced toxicity of brucine after encapsulation into stealth liposomes: role of phosphatidylcholine. *International Journal of Nanomedicine*, p.3567.

Chung, S. K., Shin, G. H., Jung, M. K., Hwang, I. C., & Park, H. J. (2014). Factors influencing the physicochemical characteristics of cationic polymer-coated liposomes prepared by high-pressure homogenization. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *454*, 8-15.

Cipolla, D., Wu, H., Gonda, I., Eastman, S., Redelmeier, T., & Chan, H. K. (2014). Modifying the release properties of liposomes toward personalized medicine. *Journal of pharmaceutical sciences*, *103*(6), 1851-1862.

Clayton, A. H., Kingsberg, S. A., & Goldstein, I. (2018). Evaluation and management of hypoactive sexual desire disorder. *Sexual medicine*, *6*(2), 59-74.

Crommelin, D. J., van Hoogevest, P., & Storm, G. (2020). The role of liposomes in clinical nanomedicine development. What now? Now what?. *Journal of Controlled Release*, *318*, 256-263.

Guzman-Villanueva, D., Migrino, R., Truran, S., Karamanova, N., Franco, D., Burciu, C., Senapati, S., Nedelkov, D., Hari, P. and Weissig, V., (2017). PEGylated-nanoliposomal clusterin for amyloidogenic light chain-induced endothelial dysfunction. *Journal of Liposome Research*, 28(2), pp.97-105.

Düzgüneş, N., Faneca, H. and Lima, M. C. (2010) 'Methods to monitor liposome fusion, permeability, and interaction with cells.', *Methods in molecular biology* (Clifton, N.J.), 606, pp. 209–232. doi: 10.1007/978-1-60761-447-0_16.

Edwards, K. and Baeumner, A. (2006). Analysis of liposomes. Talanta, 68(5), pp.1432-1441.

Fonte, P., Reis, S. and Sarmento, B., (2016). Facts and evidences on the lyophilization of polymeric nanoparticles for drug delivery. Journal of controlled release, 225, pp.75-86.

Funakoshi, Y., Iwao, Y., Noguchi, S., & Itai, S. (2015). Effect of alkyl chain length and unsaturation of the phospholipid on the physicochemical properties of lipid nanoparticles. *Chemical and Pharmaceutical Bulletin*, *63*(9), 731-736.

Garbuzenko, O., Barenholz, Y., & Priev, A. (2005). Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. *Chemistry and physics of lipids*, *135*(2), 117-129.

Hanaor, D., Michelazzi, M., Leonelli, C., & Sorrell, C. C. (2012). The effects of carboxylic acids on the aqueous dispersion and electrophoretic deposition of ZrO2. *Journal of the European Ceramic Society*, *32*(1), 235-244.

Hume, D., Irvine, K. and Pridans, C., (2019). The Mononuclear Phagocyte System: The Relationship between Monocytes and Macrophages. Trends in Immunology, 40(2), pp.98-112.

ICH Guidelines Q2 (R1), "Validation of analytical procedures: text and methodology," in *ICH Harmonized Tripartite Guide- lines*, 2005.

Jadhav, M. P. et al. (2011) 'Formulation and evaluation of long circulating liposomal Amphotericin B: A scinti-kinetic study using 99m Tc in BALB/C mice', I*ndian Journal of Pharmaceutical Sciences*. Medknow Publications, 73(1), pp. 57–64. doi: 10.4103/0250-474X.89757.

Jakoby, J., Beuschlein, F., Mentz, S., Hantel, C., & Süss, R. (2015). Liposomal doxorubicin for active targeting: surface modification of the nanocarrier evaluated in vitro and in vivo—challenges and prospects. *Oncotarget*, *6*(41), 43698.

Jimah, J. R., Schlesinger, P. H., & Tolia, N. H. (2017). Liposome disruption assay to examine lytic properties of biomolecules. *Bio-protocol*, *7*(15), e2433-e2433.

Jones, M.C., Jones, S.A., Riffo-Vasquez, Y., Spina, D., Hoffman, E., Morgan, A., Patel, A., Page, C., Forbes, B. and Dailey, L.A., (2014). Quantitative assessment of nanoparticle surface hydrophobicity and its influence on pulmonary biocompatibility. *Journal of Controlled Release*, 183, pp.94-104.

Kanásová, M. and Nesměrák, K. (2017). Systematic review of liposomes' characterization methods. Monatshefte für Chemie - Chemical Monthly, 148(9), pp.1581-1593.

Kannan, V., Balabathula, P., Thoma, L. and Wood, G., (2014). Effect of sucrose as a lyoprotectant on the integrity of paclitaxel-loaded liposomes during lyophilization. *Journal of Liposome Research*, 25(4), pp.270-278.

Karmakar, S. (2012) Zeta potential and dynamic light scattering measurements of phospholipids vesicles, researchgate.net.

Kaspers GJL, Zimmermann M, Reinhardt D, Gibson BES, Tamminga RYJ, Aleinikova O, et al. Improved outcome in pediatric relapsed acute myeloid leukemia: results of a randomized trial on liposomal daunorubicin by the international BFM Study Group. J Clin Oncol. (2013);31(5):599–607.

Kraske, W. V., & Mountcastle, D. B. (2001). Effects of cholesterol and temperature on the permeability of dimyristoylphosphatidylcholine bilayers near the chain melting phase transition. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1514(2), 159-164.

Kipps, E., Young, K., & Starling, N. (2017). Liposomal irinotecan in gemcitabine-refractory metastatic pancreatic cancer: efficacy, safety and place in therapy. *Therapeutic advances in medical oncology*, *9*(3), 159-170.

Kulkarni, S. B., Betageri, G. V., & Singh, M. (1995). Factors affecting microencapsulation of drugs in liposomes. *Journal of microencapsulation*, *12*(3), 229-246.

Lavoie, P.M. and Levy, O., 2017. Mononuclear phagocyte system. In Fetal and Neonatal Physiology (pp. 1208-1216). Elsevier.

Laouini, A., Jaafar-Maalej, C., Limayem-Blouza, I., Sfar, S., Charcosset, C. and Fessi, H.,( 2012). Preparation, characterization and applications of liposomes: state of the art. Journal of colloid Science and Biotechnology, 1(2), pp.147-168.

Lu, T. et al. (2012) 'Influence of polymer size, liposomal composition, surface charge, and temperature on the permeability of pH-sensitive liposomes containing lipid-anchored poly(2-ethylacrylic acid)', *International Journal of Nanomedicine*, 7, pp. 4917–4926.

Maherani, B., Arab-Tehrany, E., Kheirolomoom, A., Geny, D., & Linder, M. (2013). Calcein release behavior from liposomal bilayer; influence of physicochemical/mechanical/structural properties of lipids. *Biochimie*, *95*(11), 2018-2033.

Nag, O. K. and Awasthi, V. (2013) 'Surface engineering of liposomes for stealth behavior', Pharmaceutics. *Multidisciplinary Digital Publishing Institute (MDPI)*, pp. 542–569. doi: 10.3390/pharmaceutics5040542.

Nascentes, C. C. et al. (2001) 'Use of Ultrasonic Baths for Analytical Applications: A New Approach for Optimisation Conditions', *Journal of the Brazilian Chemical Society*. Sociedade Brasileira de Quimica, 12(1), pp. 57–63. doi: 10.1590/S0103-50532001000100008.

Nicholas, A., Scott, M., Kennedy, N. and Jones, M., (2000). Effect of grafted polyethylene glycol (PEG) on the size, encapsulation efficiency and permeability of vesicles. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1463(1), pp.167-178.

Ong, S., Chitneni, M., Lee, K., Ming, L. and Yuen, K. (2016). Evaluation of Extrusion Technique for Nanosizing Liposomes. Pharmaceutics, 8(4), p.36.

Ostolska, I., & Wiśniewska, M. (2014). Application of the zeta potential measurements to explanation of colloidal Cr 2 O 3 stability mechanism in the presence of the ionic polyamino acids. *Colloid and polymer science*, *292*(10), 2453-2464.

Owens III, D. E., & Peppas, N. A. (2006). Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. International journal of pharmaceutics, 307(1), 93-102.

Paixão, J. S., Casaroli, D., Battisti, R., Evangelista, A. W. P., Alves Júnior, J., & Mesquita, M. (2020). Characterizing Sugarcane Production Areas Using Actual Yield and Edaphoclimatic Condition Data for the State of Goiás, Brazil. *International Journal of Plant Production*, *14*(3).

Perrie, Y. (2013) 'Pharmaceutical nanotechnology and nanomedicines', in Aulton, M. and Taylor, K. (eds) *Aultons' pharmaceutics: the design and manufacture of medicines.* 4th edn. Elsevier, pp. 790–795.

Probe-Type Sonication vs. Ultrasonic Bath: An Efficiency Comparison. (2021). Retrieved 7 July 2021, from https://www.hielscher.com/probe-type-sonication-vs-ultrasonic-bath-an-efficiency-comparison.htm

Redondo-Morata, L., Giannotti, M. I. and Sanz, F. (2012) 'Influence of cholesterol on the phase transition of lipid bilayers: A temperature-controlled force spectroscopy study', Langmuir. *American Chemical Society*, 28(35), pp. 12851–12860. doi: 10.1021/la302620t.

Roy, B. et al. (2016) 'Influence of lipid composition, pH, and temperature on physicochemical properties of liposomes with curcumin as model drug', *Journal of Oleo Science*. Japan Oil Chemists Society, pp. 399–411. doi: 10.5650/jos.ess15229.

Ruozi, B., Belletti, D., Tombesi, A., Tosi, G., Bondioli, L., Forni, F. and Maria Angela Vandelli (2011). AFM, ESEM, TEM, and CLSM in liposomal characterization: a comparative study. International Journal of Nanomedicine, p.557.

Shen, Z., Ye, H., Kröger, M., & Li, Y. (2018). Aggregation of polyethylene glycol polymers suppresses receptor-mediated endocytosis of PEGylated liposomes. *Nanoscale*, *10*(9), 4545-4560.

Shimanouchi, T., Ishii, H., Yoshimoto, N., Umakoshi, H., & Kuboi, R. (2009). Calcein permeation across phosphatidylcholine bilayer membrane: Effects of membrane fluidity,

liposome size, and immobilization. *Colloids and Surfaces B: Biointerfaces*, *73*(1), 156-160.

Silvander, M., Johnsson, M. and Edwards, K., (1998). Effects of PEG-lipids on permeability of phosphatidylcholine/cholesterol liposomes in buffer and in human serum. *Chemistry and Physics of Lipids*, 97(1), pp.15-26.

Smith, J. A., Costales, A. B., Jaffari, M., Urbauer, D. L., Frumovitz, M., Kutac, C. K., ... & Coleman, R. L. (2016). Is it equivalent? Evaluation of the clinical activity of single agent Lipodox® compared to single agent Doxil® in ovarian cancer treatment. *Journal of Oncology Pharmacy Practice*, *22*(4), 599-604.

Smith, M. C., Crist, R. M., Clogston, J. D., & McNeil, S. E. (2017). Zeta potential: a case study of cationic, anionic, and neutral liposomes. *Analytical and bioanalytical chemistry*, *409*(24), 5779-5787.

Steenpaß, T., Lung, A., & Schubert, R. (2006). Tresylated PEG-sterols for coupling of proteins to preformed plain or PEGylated liposomes. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1758*(1), 20-28.

Tavallaie, M. (2016). Effect of Lyophilization on the Size and Polydispersity of Unilamellar and Multilamellar Liposomes. *Journal Of Nanotechnology And Materials Science*, *3*(2), 1-4. doi: 10.15436/2377-1372.16.1139

Tirosh, O., Barenholz, Y., Katzhendler, J., & Priev, A. (1998). Hydration of polyethylene glycol-grafted liposomes. *Biophysical journal*, *74*(3), 1371-1379.

U.S. Food and Drug Administration. (2021). Retrieved 3 August 2021, from https://www.fda.gov/

Varona, S., Martín, Á. and Cocero, M. J. (2011) 'Liposomal incorporation of lavandin essential oil by a thin-film hydration method and by particles from gas-saturated solutions', Industrial and Engineering Chemistry Research. *American Chemical Society*, 50(4), pp. 2088–2097. doi: 10.1021/ie102016r.

Xu X, Wang L, Xu H, Huang X, Qian Y, Xiang J. Clinical comparison between paclitaxel liposome (Lipusu) and Paclitaxil for treatment of patients with metastatic gastric cancer. *Asian Pac J Cancer Prev*. (2013);14(4):2591–4.

Xu, Q., Ensign, L. M., Boylan, N. J., Schön, A., Gong, X., Yang, J. C., ... & Hanes, J. (2015). Impact of surface polyethylene glycol (PEG) density on biodegradable

nanoparticle transport in mucus ex vivo and distribution in vivo. *ACS nano*, *9*(9), 9217-9227.

Ye, L., He, J., Hu, Z., Dong, Q., Wang, H., Fu, F., & Tian, J. (2013). Antitumor effect and toxicity of Lipusu in rat ovarian cancer xenografts. *Food and chemical toxicology*, *52*, 200-206.

Zhuang, Y., Ma, Y., Wang, C., Hai, L., Yan, C., Zhang, Y., ... & Cai, L. (2012). PEGylated cationic liposomes robustly augment vaccine-induced immune responses: Role of lymphatic trafficking and biodistribution. *Journal of Controlled Release*, *159*(1), 135-1

# Chapter 4: General conclusion and future work.

## 4.1. General discussion

### 4.1.1. The impact of the liposomes' size, ligand insertion and PEGylation on cellular uptake

Number of previous works highlighted the impact of different liposomal physicochemical properties, surface modification using PEG or targeting ligand on liposomes' cellular uptake, PK and targeting efficiency. Liposomes size appeared as one of the most important factors to target the hepatocytes (Witzigmann, 2016). The recommended size range for targeting the hepatocytes is between 100-200 nm (Mishra et al., 2013). This is the size range in which the nanoparticles can escape from the macrophages to the hepatocytes through the fenestration capillaries (Witzigmann, 2016). Small size liposomes have a low stability profile, and therefore show a great tendency for fusion, aggregation, and precipitation (Abdelwahed et al., 2006). There are several reported methods to solve the liposomes instability problems and one of the most common methods is lyophilisation (Fonte et al., 2016). However, liposomes with a size > 200 nm have been reported to be easily recognized by the MPS and hence cleared quickly from the circulation (Chu et al., 2016). The proposed linear regression model suggested a direct proportional relationship between liposomes size and the cellular uptake, and the suggested size range was between 100-200 nm. Decorating the liposomes' surface with PEG is recommended to increase the circulation time which can enhance the targeting probability (Chu et al., 2016). When using PEG, it is important to carefully control both the PEG concentration and its chain length. The evidence highlighted the importance of maintain the PEG concentration below 8mol% along a chain length of less than 2000 KDa to improve the cellular uptake (Pozziet et al., 2014). This can be explained by the brush configuration effect that can be induced by the long PEG chain as a result of chain folding-up rather than stretching on the surface of the liposome which can hinder their interaction with the targeted cells (Pozziet et al., 2014). Regarding using targeting ligand, it has been

reported that galactose is one of the most commonly used targeting ligand for the hepatocytes (Witzigman 2017). This can be linked to abundant expression of galactose moity on the hepatocytes surface specifically on ASPG-R (Witzigman 2017). Using galactose based ligand, allows liposomes to interact with the cell receptors, maximising the drug chances to enter the cell through receptor mediated endocytosis mechanism (Li et al., 2018). However, due to the limited number of studies discussing the impact of PEG and ligand on %CU, we were unable to establish the regression model.

Notably, it is important to control the ligand density. The reported findings from previous research suggested not to exceed 20 mol% as the receptor saturation would take place, leading to reduction in cellular uptake. This can give an idea on the recommended concentration to be used when formulating liposomal formulations for targeting the hepatocytes. However, these findings were based on limited number of articles and further studies are needed to establish the proper ligand concentration needed for hepatic cellular uptake.

As several factors, can affect the drug before it reaches the target site, it is important to understand how modulating the liposomes characteristics can impact the pharmacokinetics by studying the pharmacokinetics properties.

### 4.1.2. The impact of the liposomes' size, ligand insertion, and PEGylation on pharmacokinetics profile.

Liposome characteristics has reported to impact the PK parameters of the formulated liposomes including AUC, CL, T1/2, and accumulation (Song et al., 2012). Liposomes size has been considered as a main factor impacting the PK and liposomes efficiency (Chu et al., 2016). The linear regression showed a direct proportional relationship between liposomes' size and drug accumulation on the hepatocytes. On the other hand, the model showed an inverse relationship between liposomes' size and tumour volume. Previous literature

reported that PEG insertion was associated with an increase in the T1/2 (Baumann et al., 2014). However, there is possibility to reduce the liposomes' circulation when using PEG as a results of a reported ABC phenomenon (Nag and Awasthi, 2013). According to the findings of the systematic review conducted by (Li and Huang, 2010), ligand insertion appeared as a promising strategy to improve hepatic targeting. However, it is important to bear in mind that different sugars are used for targeting different liver cells. For example, mannose can be used for targeting KCs (Chen et al., 2017) while galactose can be used for targeting the hepatocytes (Liu et al., 2017). In addition, it is suggested to take care about both ligand and linker density. Additionally, the ligand density was suggested not to exceed 20 mol% (Li et al., 2018).

Number of studies addressed that the most used linker with the galactose was PEG. The PEG theory for PEG configuration is applied here (i.e., having PEG concentration below 5mol% shows a mushroom configuration and higher concentrations are responsible for brush formation). The mushroom configuration provides a full coverage of the liposomes surface, and thus, increasing the T1/2 and AUC for facilitating and increasing the chance to reach the target site (Li et al., 2018).

We acknowledge that there are some limitations in our work that might lead to inaccurate conclusion. However, according to our established model, it is suggested that using liposomes size between 100-200 nm associated with enhancing the cellular uptake and liver accumulation. This was confirmed by efficacy finding for reduction the tumour volume. Based on the narrative analysis, using PEG concentration below 5 mol%, and galactose with a density < 20 mol% as a dual system for targeting the hepatocytes would lead to desirable outcomes.

### 4.1.3. The impact of PEGylation on the liposomes' physicochemical properties and permeability profile.

A huge body of evidence suggesting that the use PEG would prevent liposomes recognition by the MPS (Nag and Awasthi, 2013). This work tested two different molar ratios (2 and 5 mol%) for different PEG types (DSPE-PEG2000, DSPE-PEG2000-COOH, and DSPE-PEG2000-NH2). The overall liposomes size results were mainly the same for the un-modified ones, and the size increased as the temperature increased. The ZP values did not affect significantly and appeared to be with the average of the un-modified liposomes. Although literatures suggested that, using functionalized PEG would lead to increase the negatively charge on the surface when using DSPE-PEG2000-COOH, this was not the case in our findings. This can be a result of the incomplete attachment of the PEG on the surface or insufficient protonation in the medium (Babchin et al., 1976). With all of these different modifications, only SPC liposomes showed a significance change in liposomes size (P-value<0.01). This might occur as a result of the unsaturated phospholipid nature of the SPC, which requires lower Tc to fluidize. As the membrane fluidity increases, the PEG insertion become more guaranteed and changes in the physicochemical properties can be obtained. However, further assessment with more advanced techniques such as DSC is required to confirm this finding.

Membrane permeability studies were conducted for the formulated liposomes to evaluate their fluidity behaviour. This was obtained by using Calcien as a hydrophilic probe (Maherani et al., 2013). The permeability profile for all liposomes showed a temperature dependent pattern in which as the temperature increase the permeability increase. The maximum fluidity achieved at the Tc for the different liposomal formulations. However, none of them showed 100% fluidity. Other authors suggested several factors can affect the membrane permeability including the liposomes composition, cholesterol content, and the

temperature (Maherani et al., 2013; Shimanouchi et al., 2009). Although the cholesterol concentration was constant within all the formulations, the observed findings can occur as a result of the changes in the theoretical Tc of the used phospholipids.

Our findings revealed that the insertion different PEG concentrations impacted the liposomes permeability profile. Adding the lower ratio (2 mol%) of DSPE-PEG2000 and its functionalized derivatives associated with lowering the fluidity. The liposome membrane permeability profile was reported to change significantly at the different temperature profiles and affected by PEG modification (P-value<0.05). Two factors might play a role for our findings. First, as the liposomes' size decreased, the release increased (Shimanouchi et al., 2009). Second, the level of unsaturation. SPC liposomes is composed of unsaturated saturated phospholipids with a low Tc, which require a low energy to be converted to a liquid crystalline form (Shimanouchi et al., 2009). Although HSPC is composed of a saturated phospholipid, it has a shorter chain length (14) compared to DSPC liposomes (18), which means less stability and the inducing membrane permeability changes can be easier than DSPC liposomes (Bouffioux et al., 2007).

The findings of our experiment were in line with our linear regression model, which showed a positive correlation between the temperature and the permeability behavior. However, only HSPC liposomes showed a significance differences in the release kinetics under the influence of different temperature profiles (P-value<0.05). Although we expected SPC to show a significance differences as it has a small diameter size, having unsaturated phospholipids which make an ideal model to show a good release characteristics, some factors might affect this findings. This includes a full encapsulation of cholesterol in the formulation which might lead to changes in their Tc. However, further studies are recommended to confirm the reason of this finding.

It is worth to test these formulated liposomal formulations on hepatocytes cell lines to assess their uptake characteristics in the presence of other factors i.e. proteins and enzymes, and to determine the best formulation for the clinical use.

### 4.2. General conclusion

This piece of work aims to investigate some aspects. First, to search the literature for the impact of the liposome's size, surface modification with PEG, and ligand insertion into liposomes on the hepatocytes targeting. Second, to understand how the liposomal physicochemical properties and surface modification can impact the membrane permeability profile. In order to answer these research questions, we followed different approaches.

In one hand, we performed a systematic search for two databases "Medline and Embase" for the last five years including certain inclusion and exclusion criteria (For the full search strategy refer to chapter 2, appendix B). According to the Prisma flowchart, only 25 out of 142 selected for the eligibility were included. This systematic review aimed to evaluate the potential factors that might play role and affect hepatocytes targeting. Both qualitative and quantitative analysis was conducted. Narrative analysis was conducted to describe the main theme for the aim qualitatively. Quantitatively, a linear regression model was established to evaluate the impact of the liposomes' size on the outcome (cellular uptake, drug accumulation, and tumour volume). From SR data, it is suggested to formulate liposomes with a particle size between 100-200 nm. In order to avoid the non-specific recognition by the MPS, PEGylation with no more than 5 mol% is suggested. However, introducing PEG reduces the cellular uptake. In order to facilitate the cellular uptake, the addition of a ligand is a good choice. The most common ligand used for liver targeting is the Galactose due to its abundant expression on ASGP-R on the hepatocytes' surface. However, ligand concentration suggested not to exceed 20 mol%. An illustrative chart for the systematic review findings is provided below (Figure 4.1.).

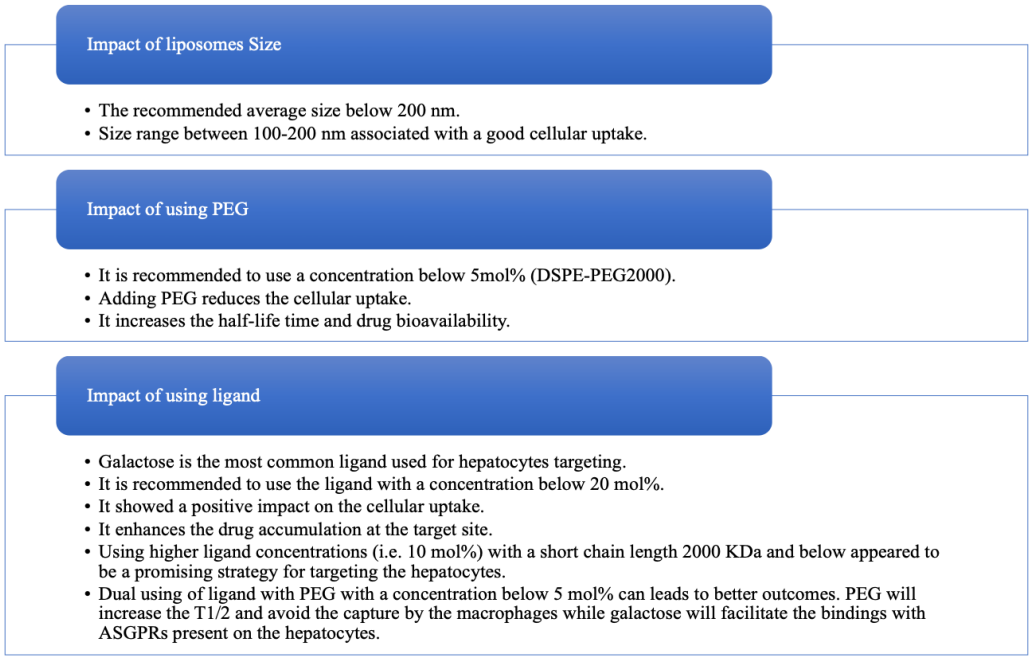



**Figure 4.1. Main findings from the systematic review search.**

On the other hand, a small industrial scale for liposome manufacturing took place in our lab. This work was carried out to prepare three liposomal formulations with similar sizes but different phospholipid compositions. All liposomes made up of neutral phospholipids. Neutral phospholipids have the advantage of being biologically inert with a low toxicity profile. The formulated liposomes undergo some tests of fundamental aspects including size, zeta potential and membrane permeability, followed by surface modification of the prepared liposomes with PEG polymer, and lyophilisation. Overall, stable liposomes with a neutral charge and narrow distribution were obtained from all phospholipids. In all formulations, size increased with temperature, likely due to a change in the arrangement of phospholipids in the bilayers as temperature increases above the Tc. The membrane permeability profile showed a temperature-dependent pattern with all prepared liposomes. Lyophilisation for the SPC liposomes was performed using sucrose.

In conclusion, all the formulations were stable for at least four months. They had a size smaller than 200 nm, good homogeneity, with acceptable zeta potential values. PEGylation

was implemented to increase the liposomes' stability and was successful for both 2 and 5 mol%. Better results were obtained with 5%, confirmed by small size, more uniform PDI, and lower zeta potential values. The encapsulation efficiency for the polymer within vesicles was not varied with the two %mol PEG when grafted to DSPC liposomes. However, entrapment efficiency was higher with 2% for Soybean and HSPC liposomes. Regarding membrane permeability, all three formulations showed a temperature-dependent pattern i.e., permeability increased as temperature increased. The maximum membrane permiability was achieved with soybean phospholipid, then DSPC, and finally HSPC. According to the established linear regression model which test the impact of the temperature on the membrane permeability characteristics, there is no significant difference among the tested formulations except with HSPC. SPC liposomes was undergone lyophilisation using pre- and post-insertion methods. In both cases, there was no obvious differences in size, PDI, and ZP profile. A summary of the lab work main experiments and findings is provided (Figure 4.2.).

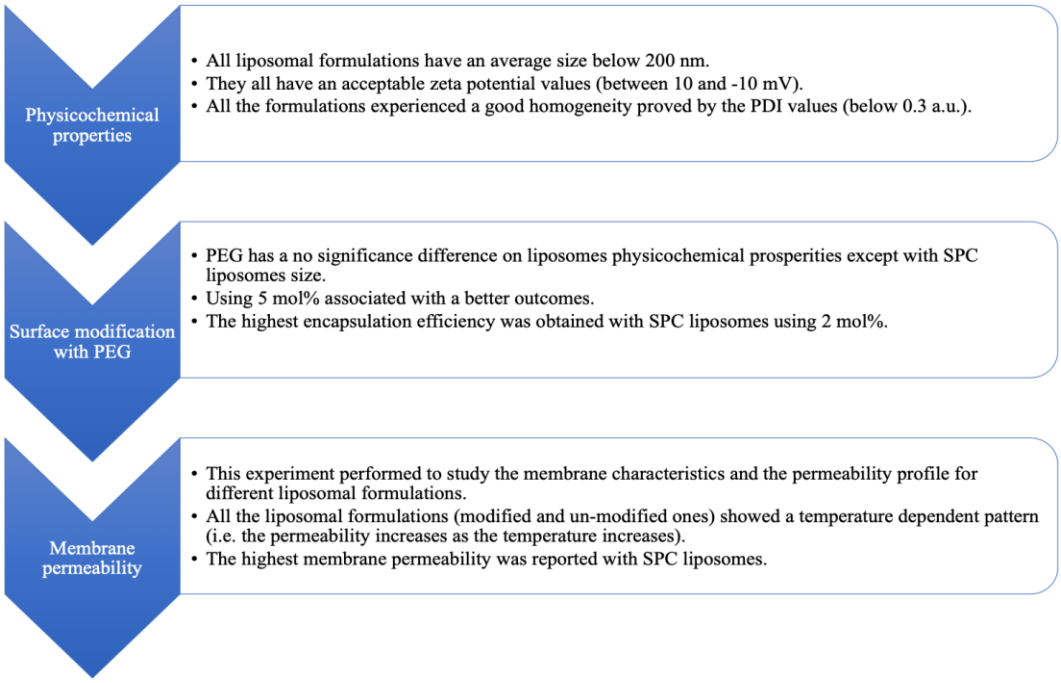



**Figure 4.2. The main conducted lab experiments and findings.**

### 4.3.Future work:

We acknowledge that our work has some limitations, and some future work suggestions are provided. For the systematic review findings, we included the most recent studies (for the last five years), which came back with a small number of the included studies based on our inclusion/exclusion criteria. It might worth to include the studies for 10 or 20 years to give a better understanding for the hepatocytes targeting. In addition, although we tried to standardized the data to the extend we could to, the heterogeneity was high which might affect the prediction. Second, none of the included studies measured the actual yield of the inserted PEG/ligand for liposomes modification. Third, most of the selected studies used mono-culture cell studies, in which other factors including proteins that might influence the uptake of the liposomes were isolated, which can give misleading prediction. In addition, the only one included RCT had a similar characteristic of Doxil and gives an overview on the liposomes PK, but the RCT was applied on a small group of patients (12 patients) and lack of blindness and randomisation. It is suggested to improve the quality of the study by applying a randomization and blindness and to look for the outcomes at bigger groups.

For the lab work results, further investigations for future work including using different liposomes compositions, lipid: chol ratios, surface modification with higher mol% of PEG to study their impact on liposomes characteristics. In addition, it is suggested to use phospholipids with different Tc values i.e., lipids with a Tc different than what we have selected to get better prediction on the impact of the temperature on membrane permeability. We could use the advantages of the systematic review findings and study the impact of dual surface modification using PEG and galactose at different concentrations to investigate their effect on liposomes physicochemical characteristics. As most of the approved liposomal formulations are in a lyophilised form, it is suggested to focus more on the lyophilisation for the prepared liposomes with different modifications.

Due to COVID-19 pandemic, we were unable to test our formulations on the cells. It worth testing these liposomal formulations in a hepatic cell line to study their cellular uptake for better understanding of their targeting efficiency.

**References**


Abdelwahed, W., Degobert, G., Stainmesse, S. and Fessi, H., (2006). Freeze-drying of nanoparticles: formulation, process and storage considerations. Advanced drug delivery reviews, 58(15), pp.1688- 1713.

Babchin, A. J., Piliavin, M. A., & Levich, V. G. (1976). Rheoelectric effect in a polar liquid interphase layer. *Journal of Colloid and Interface Science*, *57*(1), 1-10.

Baumann, A., Tuerck, D., Prabhu, S., Dickmann, L., & Sims, J. (2014). Pharmacokinetics, metabolism and distribution of PEGs and PEGylated proteins: quo vadis?. *Drug Discovery Today*, *19*(10), 1623-1631.

Bouffioux, O., Berquand, A., Eeman, M., Paquot, M., Dufrêne, Y. F., Brasseur, R., & Deleu, M. (2007). Molecular organization of surfactin–phospholipid monolayers: Effect of phospholipid chain length and polar head. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1768*(7), 1758-1768.

Bozzuto, G. and Molinari, A. (2015). Liposomes as nanomedical devices. International Journal of Nanomedicine, p.975.

Briuglia, M., Rotella, C., McFarlane, A. and Lamprou, D. (2015). Influence of cholesterol on liposome stability and on in vitro drug release. Drug Delivery and Translational Research, 5(3), pp.231-242.

Chen, J., Chen, Y., Cheng, Y., & Gao, Y. (2017). Glycyrrhetinic acid liposomes containing mannose-diester lauric diacid-cholesterol conjugate synthesized by lipase-catalytic acylation for liver-specific delivery. *Molecules*, *22*(10), 1598.

Chu, C., Xu, P., Zhao, H., Chen, Q., Chen, D., & Hu, H. et al. (2016). Effect of surface ligand density on cytotoxicity and pharmacokinetic profile of docetaxel loaded liposomes. *Asian Journal Of Pharmaceutical Sciences*, *11*(5), 655-661. doi: 10.1016/j.ajps.2016.04.001

David, S., & Hamilton, J. P. (2010). Drug-induced liver injury. *US gastroenterology & hepatology review*, *6*, 73.

Düzgüneş, N., Faneca, H. and de Lima, M.C.P., (2010). Methods to monitor liposome fusion, permeability, and interaction with cells. Liposomes: Methods and Protocols, Volume 2: Biological Membrane Models, pp.209-232.

Fonte, P., Reis, S. and Sarmento, B., (2016). Facts and evidences on the lyophilization of polymeric nanoparticles for drug delivery. Journal of controlled release, 225, pp.75-86.

Hoofnagle, J. H. (2013). LiverTox: a website on drug-induced liver injury. In *Drug-Induced Liver Disease* (pp. 725-732). Academic Press.

Laouini, A., Jaafar-Maalej, C., Limayem-Blouza, I., Sfar, S., Charcosset, C. and Fessi, H. ( 2012). Preparation, characterization and applications of liposomes: state of the art. Journal of colloid Science and Biotechnology, 1(2), pp.147-168.

Li, H., Yuan, D., Sun, M., & Ping, Q. (2016). Effect of ligand density and PEG modification on octreotide-targeted liposome via somatostatin receptor in vitro and in vivo. *Drug Delivery*, *23*(9), 3562-3572.

Li, S. D., & Huang, L. (2010). Stealth nanoparticles: high density but sheddable PEG is a key for tumor targeting. *Journal of controlled release: official journal of the Controlled Release Society*, *145*(3), 178.

Liu, X., Han, M., Xu, J., Geng, S., Zhang, Y., Ye, X., ... & Tang, X. (2017). Asialoglycoprotein receptor-targeted liposomes loaded with a norcantharimide derivative for hepatocyte-selective targeting. *International journal of pharmaceutics*, *520*(1-2), 98-110.

Maherani, B., Arab-Tehrany, E., Kheirolomoom, A., Geny, D. and Linder, M. (2013). Calcein release behavior from liposomal bilayer; influence of physicochemical/mechanical/structural properties of lipids. Biochimie, 95(11), pp.2018-2033.

Mishra, N., Yadav, N. P., Rai, V. K., Sinha, P., Yadav, K. S., Jain, S., & Arora, S. (2013). Efficient hepatic delivery of drugs: novel strategies and their significance. *BioMed research international*, *2013*.

Nag, O. and Awasthi, V. (2013). Surface Engineering of Liposomes for Stealth Behavior. Pharmaceutics, 5(4), pp.542-569.

Perrie, Y. (2013). Pharmaceutical nanotechnology and nanomedicines. In: M. Aulton and K. Taylor, ed., Aultons' pharmaceutics: the design and manufacture of medicines, 4th ed. Elsevier, pp.790-795.

Pozzi, D., Colapicchioni, V., Caracciolo, G., Piovesana, S., Capriotti, A. L., Palchetti, S., ... & Laganà, A. (2014). Effect of polyethyleneglycol (PEG) chain length on the bio–nano-interactions between PEGylated lipid nanoparticles and biological fluids: from nanostructure to uptake in cancer cells. *Nanoscale*, *6*(5), 2782-2792.

Shimanouchi, T., Ishii, H., Yoshimoto, N., Umakoshi, H. and Kuboi, R. (2009). Calcein permeation across phosphatidylcholine bilayer membrane: Effects of membrane fluidity, liposome size, and immobilization. Colloids and Surfaces B: Biointerfaces, 73(1), pp.156-160.

Song, G., Wu, H., Yoshino, K., & Zamboni, W. (2012). Factors affecting the pharmacokinetics and pharmacodynamics of liposomal drugs. *Journal Of Liposome Research*, *22*(3), 177-192. doi: 10.3109/08982104.2012.655285

Witzigmann, D. (2016). Hepatocyte-specific drug delivery using active targeted nanomedicines-evaluation of targeting strategies in vitro and in vivo (Doctoral dissertation, University_of_Basel).