Development of liposomal nanoencapsulation of lumefantrine with phosphatidylcholine/Tweens system

This thesis is submitted in partial fulfilment for the requirements for the degree of Master of Pharmacy

By

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Abstract

This study examines the potential of lumefantrine-loaded liposome delivery in vitro by employing phospholipid in the combination of two polysorbates. Liposomes were prepared using the thin-film hydration method, and the size was then downsized using sonicator and homogenizer to achieve a small uni-lammelar vesicles (SUVs). Formulations of phosphatidylcholine (PC), phosphatidylcholine/tween 20 (PC/T20) and phosphatidylcholine/tween 80 (PC/T80) in different mole ratios, were used in each liposomal formulation.

Two different homogenizing rates (10000rpm and 20000rpm) were utilized in down-sizing liposomal formulations. The size of the liposomes was further reduced using a 0.2μm filter into a nanosized scale. The physiochemical characteristics of liposomal formulations on their, size distribution, zeta potential, pH, and morphology were determined.

PC/T80-liposomes entrapped 53.2- 71.5 % of the available LUM, and 53-68.7% and 40.2- 49.6% entrapped by liposomes made from PC/T20 and only PC liposomal formulations, respectively. The encapsulation efficiency of liposomes was dependent on the lipid bilayer properties and surfactant type and concentrations.

In vitro studies exhibited that LUM release was higher in PC/ T80 liposomes than in the PC/T20 or surfactant-free liposome formulations. The drug release was dependent on the lipid and surfactant concentration. Thus, T80-liposomes had a higher LUM release of approximately 31.5 - 42.7%. Similarly, drug release in T20-based formulations revealed to be 30.7-38.6% in 24 hours. However, drug release in surfactant-free liposomes proved to be slow (approximately 27%). The characteristics of different liposome formulations were essential in understanding their drug delivery mechanism.

The impact of lyophilization without cryoprotectant on the stability of liposomes was also determined by comparing the mean vesicle size, PDI, and encapsulation efficiency before and after freeze-drying. The process of lyophilization resulted in particle size increment and a significant decrease in drug entrapment in all formulations.

***Keywords*:** Liposomes; Malaria; Lumefantrine; Encapsulation efficiency; Drug-release; Lyophilization

List of Abbreviations

ACN	Acetonitrile
ARM	Artemether
DLS	Dynamic Light Scattering
DL	Drug Loading
EE	Encapsulation efficiency
HPLC	High-performance liquid chromatography
HPH	High pressure homogenization
HLB	Hydrophilic-Lipophilic Balance
LC	Loading capacity
LET	Liposomal encapsulation technology
LPH	Low pressure homogenization
LUM	Lumefantrine
LUV	Large unilamellar vesicle
MLV	Multi lamellar vesicles
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDI	Polydispersity Index
PLs	Phospholipids
PNs	Phospholipid nanoparticles
RES	Reticuloendothelial system
SEC	Size exclusion chromatography
SUV	Small unilamellar vesicle
T20	Tween-20
T80	Tween-80
Tc	phase transition temperature
TEM	Transmission electron microscopy
WHO	World Health Organization
ZP	Zeta potential

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1 Introduction

The absorption mechanism and the nature of the drug are the fundamental factors that determine the appropriate delivery systems for achieving the highest bioavailability and effectiveness [1]. Therefore, the solubility of the drug is likely to influence the absorption profile and bioavailability after oral administration. For instance, orally given drug molecules must first undergo dissolution and absorption in gastrointestinal (GI) fluid to reach the systemic circulation[1]. However, these processes are challenging factors for drug molecules with hydrophobic properties. Because when an active ingredient is taken orally, it must first dissolve in GI fluids before permeating the gastrointestinal tract (GIT) membranes. Drug molecules such as Lumefantrine (LUM) are examples of highly lipophilic ($logP = 9.19$) molecules with low aqueous solubility[2].Thus, LUMs poor solubility nature leads to incomplete absorption and low bioavailability and is consequently poor for acute malaria infections. These also outcomes to treatment failure, which is again associated with increased morbidity and development of resistance [3]. Therefore, the oral bioavailability of LUM is more certainly gained by co-administration with a fatty meal intake. However, it is still challenging as malaria patients have nausea and vomiting indications commonly.

To overcome these difficulties finding suitable formulation, such as encapsulation of drug molecules in nanocarriers for the parenteral delivery system, is needed. Thus, the active substance was administered directly in a blood vessel and avoided the first-pass metabolism. Compared with oral administration, the parenteral route exhibits several advantages, such as first-pass metabolism avoidance leading to better bioavailability, improving patient compliance (For example malaria patients with high vomiting tendency), and controlling the dosage. Additionally, the route benefits patients who cannot take the drug orally and require rapid onset of action[4, 5].

Recently, parenteral drug delivery systems using liposomal encapsulation technology(LET) are promising in pharmaceutical applications[6]. LET is a method of designing sub-microscopic liposomes, which encapsulates various pharmaceutical agents. This method provides efficient drug

loading, decreasing systemic toxicity associated with the drug and improving its unfavorable pharmacokinetics [7, 8]

1.1 Liposomes

Liposomes are lipid-based vesicular structures consisting of one or more hydrated lipid bilayers that form spontaneously when phospholipids are dispersed in an aqueous medium [9]. Liposomes consist of an aqueous core surrounded by a lipid bilayer, like cell membrane, separating the inner aqueous core from the bulk outside. They were first discovered and described by Bangham and his co-workers in 1961. The name liposomes were derived from two Greek words, “lipos” meaning fat and “soma,” meaning body[10].

Liposomes are microscopic spherical vesicles with particle sizes varying from 15 nm to several micrometers. They have either consistent of either single or multi-lipid bilayers enclosing aqueous units, where the polar head groups are oriented toward the interior and exterior aqueous phases. [9].

1.1.1 Composition of liposomes

Liposomes are composed of physiologically acceptable natural or synthetic phospholipids found in lipid bilayer membranes of human cells. Additionally, other ingredients such as cholesterol and surfactants are also added in formulation, and these can affect liposomes behavior and afford the desired encapsulation or delivery profiles[10].

1.1.1.1 Phospholipids

Phospholipids (PLs) are lipid molecules that occur naturally in all living organisms as a main component of the cell membrane. The unique characteristics of Phospholipids are their excellent biocompatibility and amphiphilicity. These unique properties make PLs suitable for critical pharmaceutical excipients and have a wide range of applications in drug delivery systems[11]. PLs are widely used in the formulation of poorly water-soluble drugs for oral and primarily parenteral administration. Also, they play a crucial role in the physiological dissolution mechanisms after oral and parenteral administration of hydrophobic drugs[12].

1.1.1.2 Phospholipid Structure

PLs are amphiphilic molecules containing both hydrophilic phosphate head groups and two long-chain hydrophobic fatty acids. The hydrophobic tail of the PLs is nonpolar, composed of hydrogen and carbon. Because of that fatty acids can easily interact with other nonpolar groups. While the polar head group is negatively charged, it consists of a phosphorus molecule attached with four oxygen molecules.The hydrophilic and hydrophobic chains link via the third molecule, either glycerol or sphingomyelin as the backbone [11].The alcohol group present in glycerol is attached to the phosphate molecule, which is linked to a small molecule containing an alcohol group, such as choline, inositol, glycerol and serine [12]. The molecular structure of a phospholipid is provided in Figure 1.1.

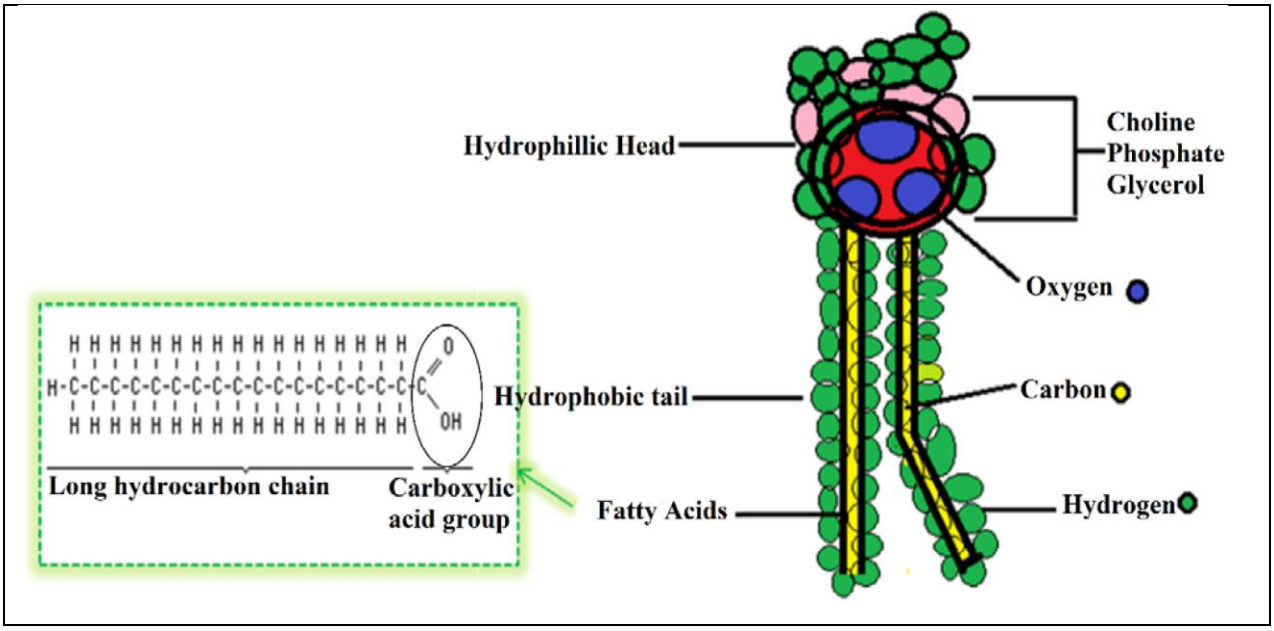


Figure 1.1: *Illustrates the general structure and constituents of phospholipids (in this example: phosphatidylcholine [13]*

Due to their amphipathic nature, phospholipids are well-suited to form a membrane bilayer. In water or aqueous solution, lipid bilayer forms when PLs arrange themselves next to each other that the hydrophilic head group faces to the aqueous fluid of both the bilayer's outer sides and forms

electrostatic interactions with the water molecule. On the other hand, the hydrophobic tail faces each other to the bilayer's interior, away from the water phase [14]. A fatty acid with single tails forms a small, single-layered sphere called a micelle, while PLs with larger tails form a hollow droplet bilayer, liposomes[10]. Figure 1.2 below shows structure of liposome and micelle *[15]*.

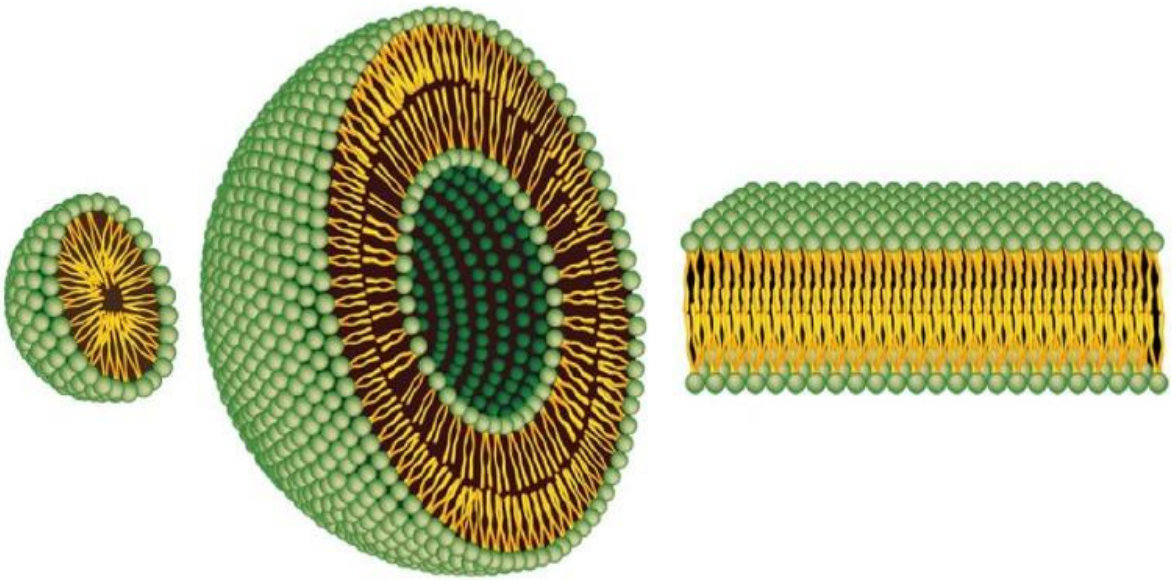


Figure 1.2: *Illustration of the steric construction of a micelle (left), a liposome (center), and a lipid bilayer (right). Whereas liposomes are made of a lipid bilayer, micelles are constructed of one lipid layer in which the non-polar part bends inward and the polar heads interact with the environment*[15] .

Based on the nature of sources, phospholipids are categorized into natural phospholipids and synthetic phospholipids. Natural phospholipid excipients are obtained from natural sources like, e.g., soybeans, sunflower seeds, milk, egg yolk, etc., while synthetic phospholipids are manufactured using organic chemical and enzymatic synthesis. Soya phosphatidylcholine (SPC), phosphatidylethanolamine (PE) are examples of natural PLs. and Synthetic PLs include dimyristoylphosphatidylcholine (DMPC) and dipalmitoyl phosphatidylcholine (DPPC)[16, 17].

Table 1.1: List of commonly used phospholipid for liposomes formulations [16]

Name	Abbreviation	Net charge in pH 7
Phosphatidylcholines	PC	Zwitterionic
Phosphatidylethanolamines	PE	Zwitterionic
Phosphatidyl Serines	PS	Negative
Phosphatidylglycerol	PG	Negative
Lysyl phosphatidylglycerol	LPG	Positive

Phosphatidylcholines (PCs) obtained from both natural and synthetic sources are commonly used in different formulations. Under physiological conditions (pH values of about 7), PCs are zwitterionic, a positive charge on the choline and a negative charge on the phosphate[16].
Figure 1.3 shows the structure of phosphatidylcholines.

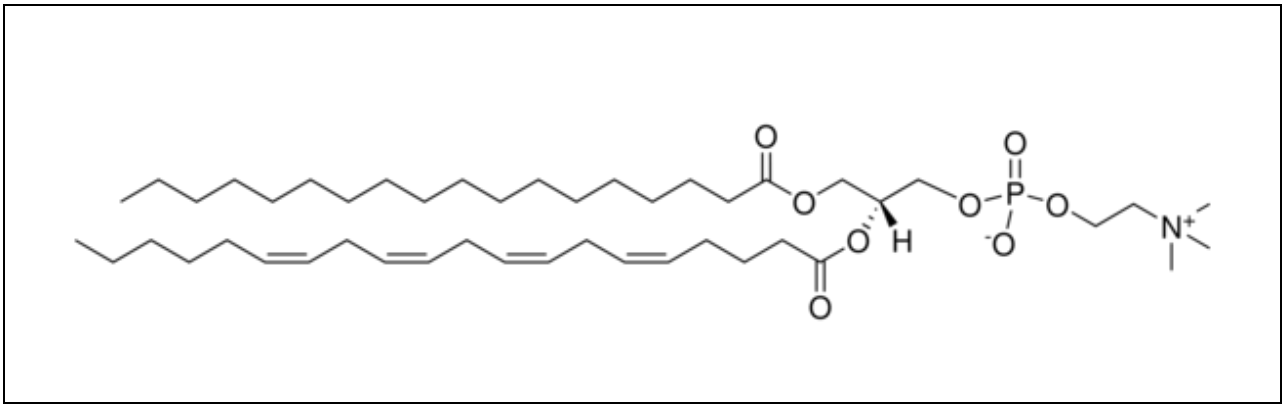


Figure 1.3: *Chemical structure of a Phosphatidylcholines molecule; with a chemical structure of* $C_{46}H_{84}NO_8P$*.[18]*

1.1.1.3 Phase transition temperature of lipid (Tc)

All phospholipids have a particular temperature at which they undergo a phase transition from a solid (gel) to a liquid form known as phase transition temperature (Tc). When PLs are at a

temperature above Tc, their hydrocarbon chains (tail) are randomly oriented and form liposome bilayer vesicles. On the opposite, when PLs are below their Tc, the hydrocarbon tail is in a gel state and remains fully extended and closely packed, hence they are not suitable for liposome formulation [11]. Tc of PLs is directly affected by several factors such as charge in the headgroup, the length of hydrocarbon chain and degree of saturation. The longer the hydrocarbon chain, the higher the Tc is [19].Phospholipid Tc has a significant impact on liposome structure and permeability as it regulates the movement of a target molecule in the lipid bilayer [20].

1.1.2 Surfactants

Polysorbates (PSs) with commercial name (Tween®) are amphipathic nonionic surfactant molecules made of fatty acid esters of polyoxyethylene sorbitan [21]. PS have a characteristic of good biocompatibility, stabilizing, emulsifying, and wetting properties. These unique properties result in increasing colloidal stability, making PSs the most suitable excipient in pharmaceutical products.

Tween 80 and Tween 20 are polysorbate surfactants with a fatty acid ester moiety and a long polyoxyethylene chain. Both have a common backbone, but they differ in the hydrocarbon chain structures as illustrated in Figure 1.4 . Tween 80 has oleic acid as primary fatty acid, while Tween 20 has lauric acid[21]. Their structures are shown in Figure 4. Tween 20 has the shortest length of the fatty acid and the highest HLB value (16.7) in comparison to Tween 80 with HBL value of 15[22]. Both Tweens are water-soluble, and they can form micelles in water [12]. The micellization process typically occurs when the surfactant concentration increases, and consequently become saturated on the water -air interface, the monomers in solution associate and form micelles [21]. Due to the longer fatty acid chain Tween 80 is more surface active with a lower critical micellization concentration (CMC) than tween 20 [23].

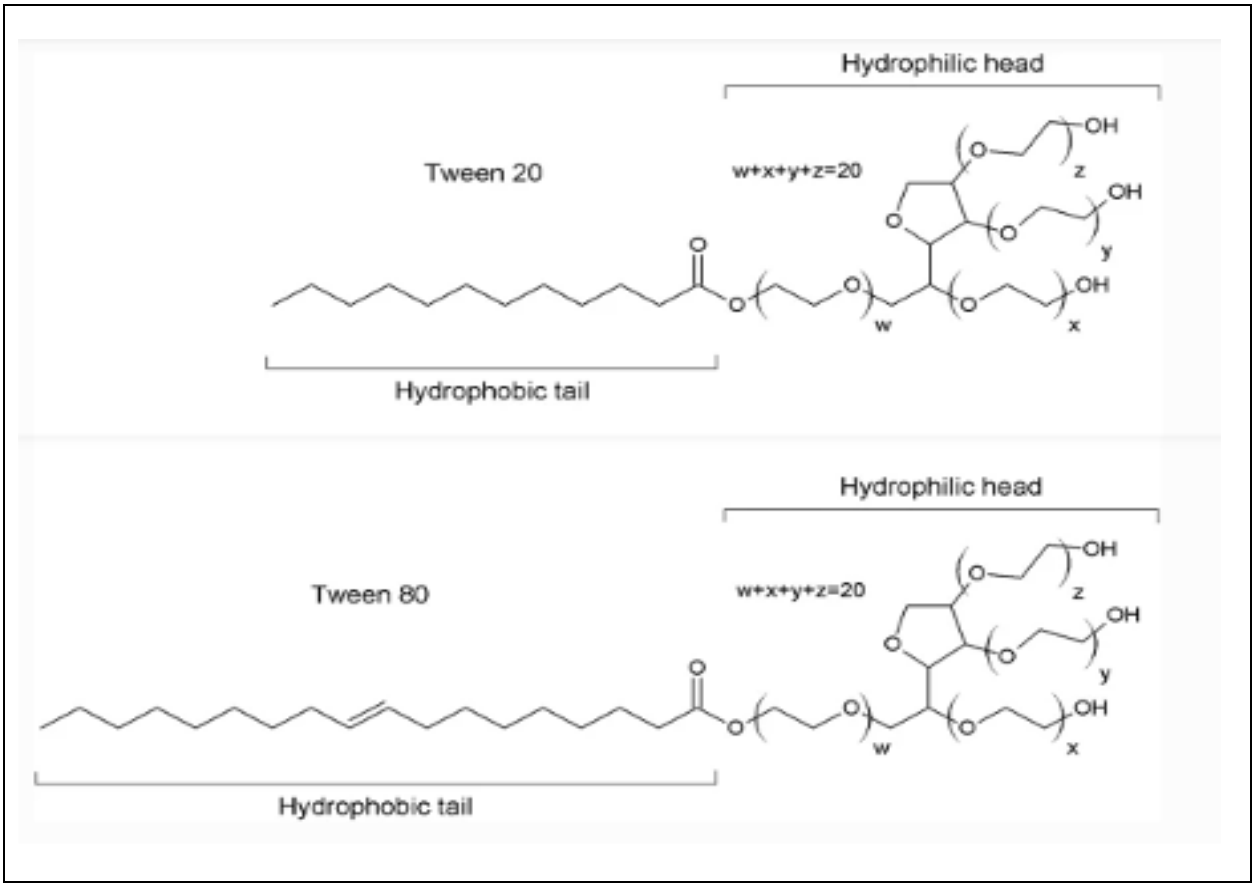


Figure 1.4: *Chemical structure of Tween 20 and Tween 80 [15]. Tween 20 has a molecular weight of 1228 g/ml, assuming 20 ethylene oxide units, one sorbitol, and one shortest length lauric acid as the basic fatty acid. Tween 80 (right), polyethylene sorbitol ester has MW 1310 g/ml , 1 sorbitol and one long chain oleic acid as the primary fatty acid.*

Currently Tween-20 and Tween 80 are the most commonly used surfactants in the parenteral biopharmaceutical formulations [24] and both are effective functionally in preventing surface adsorption and as a stabilizers against aggregations [23]. Additionally , Tweens are excellent solubilizers, and solubility of drugs increases with tween concentrations [22]

1.2 Liposome classification

There are various classes of liposomes, and these can be distinguished depending on the number of bilayers forming the vesicle, particle of size, and the method of their preparation. Liposome lamellarity refers to the number of lipid bilayers surrounding the inner aqueous space[25].

Based on vesicle lamellarity, liposomes are categorized into two categories: multilamellar vesicles (MLV) and unilamellar vesicles (ULV). Further, depending on the particle diameter ULV is described as small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) [8] as shown in Figure 1.5. The ULVs or MLVs formulations depends on the synthesis and post-formation processing methods used for their preparation [15]

1.2.1 Multilamellar vesicles (MLV)

MLV are liposome vesicles consist of several lipid bilayers and their diameter size range from 500 nm to several micrometers [17], depending on the preparation method. MLV forms spontaneously when an excess volume of buffer is added to hydrate lipid film. Due to their multi lamellarity, they are more suited to the incorporation of hydrophobic molecules compared to hydrophilic substances[26].

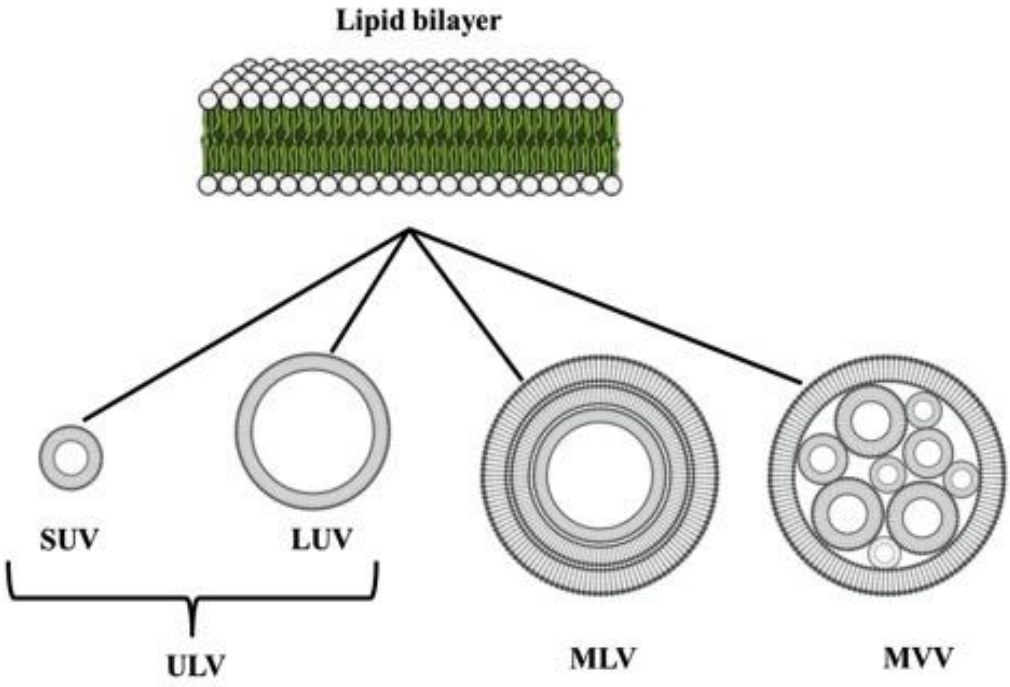


Figure 1.5: *Illustration for liposome classification based on vesicle size and lamellarity of lipid bilayer: Small unilamellar vesicles(SUV),large unilamellar vesicles (LUV), multilamellar vesicles (MLV) and multivesicular vesicle (MVV)[27]*

1.2.2 Large unilamellar vesicles (LUV)

LUVs are liposome vesicles bounded by a single lipid's bilayer with size range >100 nm in diameters. LUVs liposomes are prepared by several techniques such as freeze-thaw cycling, dehydration followed by rehydration[17]. These method enables the preparation of large unilamellar vesicle which gives the ability to carry on a large volume of solution. Thus, larger quantities of drug encapsulate in smaller amount of lipid. Due to their large capture volume LUVs are able to encapsulate higher amount of hydrophilic drug molecules than SUVs [26].

1.2.3 Small unilamellar vesicles (SUV):

SUV are liposome vesicles surrounded by a single lipid bilayer with a particle size range of 20–100 nm in diameter. They are prepared by mixing PL dispersions in water using sonication extrusion through filters and high-pressure techniques[17]. This method enables size reduction and homogeneous SUVs production, which are more suited for *i.v* administration with a prolonged circulation time than MLVs. However, as the vesicle size is small in volume, consequently, the entrapped amount is much lower than MLVs[28].

1.3 Liposome lamellarity effect in drug encapsulation

The number of lipid bilayers (lamellarity) of liposomes has greatly influenced encapsulation efficiency and the release kinetics of the entrapped drug molecules [29]. Since UVLs are single bilayer vesicles, they enclose a large aqueous core, making UVLs preferable for the entrapment of drugs with hydrophilic character. On the other hand, MVLs with multiple lipid bilayers are more suited for the encapsulation of lipophilic drug molecules. Generally, MLVs have multiple lamellar bilayers and are larger in diameter, thus having a larger drug encapsulation volume than ULVs. As a result, MVLs have slow drug release rate than single-layered UVLs[15]. The vesicle lamellarity depends on the level of mechanical stress during the dispersion process. Techniques such as high-pressure homogenization are used for formation of small and uni-lamellar vesicles[25].

1.4 Liposomes as a type of drug carrier

Since the first liposomal drug (Doxil®) approval, the application of liposomes as a drug carrier has become one of the most studied and widely used delivery systems for pharmaceutical

applications[30]. The unique feature of liposomes as a drug carrier is their biocompatibility, biodegradability, low toxicity, and ability to encapsulate drugs with different lipophilicity[8]. Liposomes can encapsulate both hydrophobic and hydrophilic compounds where hydrophobic drugs place themselves inside the lipid bilayer of the liposome and hydrophilic drugs located entirely in the aqueous compartment inside the core or at the bilayer interface as illustrated in Figure 1.6. Consequently, water-insoluble drugs may solubilize in the liposome and provide stable aqueous formulations[31].

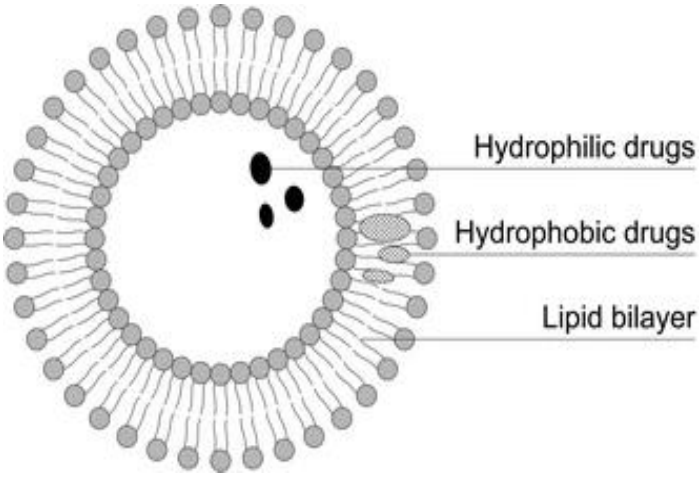


Figure 1.6: *Structure of unilamellar liposome; which illustrates that hydrophilic drug molecules (dark black) incorporate at the aqueous core while hydrophobic drugs (gray colored) encapsulate at the lipid bilayer [32]*

Furthermore, liposomes have been used to improve the drug's protection from degradation, to improve unfavorable pharmacokinetics of the drug by modifying drug absorption, reduce side effects or toxicity, and exhibit great benefit as direct drug delivery [28, 31]

1.5 Liposomes for Parenteral drug administration

Intravenous (*i.v*.) drug delivery is the most preferred administration route for low absorption lipophilic drugs. The purpose of a liposomal carrier in *i.v*. administration is to circulate the drug in the bloodstream and reach desired target organ or tissue[28]. However, formulations of parenteral drug delivery systems are a critical and challenging task as drug particle size approved for *i.v* administration should be small to avoid capillary blockage[33].Additionally, due to their biological component, liposomes may also excrete from blood circulation within hours after administration.

Therefore the drug circulation in the blood and pharmacokinetics activities are primarily dependent on liposome vesicle size and surface charges[34].

1.5.1 The role of liposome vesicle size

Larger particles with diameters range above 200nm are rapidly cleared by cells reticuloendothelial system (RES) in liver and spleen, thus results rapid disappearance of particles from blood circulation before reaching the desired sites. While particle with size range under 50 nm results for better prolonged biodistribution and to bypass the liver hepatocytes[35]. However, the very small sized liposomes have a limitation as they possess higher circulation time. Therefore, better retention after IV injection is offered by liposome vesicle size with a range of 70- 150nm in diameters. Consequently, these intermediate size vesicles can have a chance to escape the RES resulting in prolonged blood circulation and reach the target site[28].

Overall, the size range determines the biodistribution and the mononuclear phagocytic system's (MPS) clearance of liposome particles. As the particle sizes below 50nm and above 300nm, the higher clearance tendency will be achieved by renal filter and MPS consecutively. To overcome rapid clearance problems additives such as PEG and Polysorbate 80 are broadly applied as a coating material used to enhance nanoparticles' circulation time[36].

1.5.2 The role of liposome surface charge

Generally, liposomes obtain their surface charge from the PLs and additives used on formulations. Thus, the surface charge exhibits its influence on pharmacokinetics and MPS uptake. The surface charge of nanoparticles can be determined by means of Zeta potential (ZP). Reports show that negatively charged liposomes have higher MPS uptake, and toxic effects, such as pseudo allergy [15] and positively charged nanoparticles >10 mV generate a higher immune response. On the opposite, neutrally charged particles with ZP (+/- 10mV) have been associated with the lowest MPS uptake and prolonged circulation time[35].

1.6 Storage stability of liposomal formulations

Stability is the primary consideration during the formulation of drug products[37].The drug's therapeutic activity is determined by the stability of the formulations from the production steps to storage and delivery. Physical, chemical, and biological stabilities are basic parameters applied to evaluate the stability study of dosage formulations, consequently determining the shelf-life of dosage forms[28].

Liposome formulations are a relatively unstable colloidal system [25]. The stability and formation of the different structures are governed by their thermodynamic properties. Physical instability is manifested in vesicle aggregation and fusion, associated with changes in vesicle size and loss of entrapped hydrophilic materials. Whereas chemical stability primarily indicates liposomes breakdown and alters drug-release related with hydrolysis and oxidation of lipid[25].

SUVs have much more tendency to fusion when compared to large liposomes due to the presence of high surface energy. This can occur specifically at the transition temperature of the membrane [31]. Hence to minimize particle agglomeration and the possibility of Ostwald ripening, a stabilizer such as polysorbate is useful for the preparation of the negatively charged liposomes. These charged liposomes give higher stability as charged particles repel each other and reduce aggregation tendencies[23]. The liposomal formulation in dispersion should store at a much lower temperature, a range of 4-8℃, than the lipids' transition temperature[31].

PLs are chemically unsaturated fatty acids prone to oxidation and hydrolysis, which may alter the shelf life of a product[37]. These instability problems can be solved by storing the liposomal formulation in a dry state by the so-called freeze-drying (lyophilization) process. The primary concept of using this method is the belief that water removal prevents hydrolysis and maintains the physical and chemical stability of liposomes for an extended period[25] Saccharides such as sucrose and lactose are applied to protect the liposome membranes against possible fracture and rupture that might induce a change in size distribution and a loss of the encapsulated material[31].

1.7 Choice of compounds

PLs are the most abundant lipid membrane. Differences in the length and saturation of the fatty acid tails are essential because they determine the efficacy of PLs to pack toward one another, thereby altering the fluidity of the bilayer. Due to their amphipathic nature, PLs molecules are spontaneously aggregate to hide their fatty acid tails in the depths and reveal their hydrophilic heads to the water. Consequently, PLs can form spherical micelles with the fatty acid tails inward or form bilayers, with the hydrophobic tails sandwiched between the hydrophilic head groups[14].

PLs are components of the liposomal membrane; hence, they can directly influence liposome particle size [38]. However, the liposome bilayer is not composed exclusively of phospholipids. It often also contains other excipients, including cholesterol and surfactants. Surfactants such as T20 and T80 are also commonly used excipients in the pharmaceutical industry of nanocarrier productions due to their ability to solubilize poorly soluble drugs and improve the delivery system's flexibility and aid drug delivery across biological membranes such as the blood-brain barrier (BBB)[39, 40]. Surfactants at low concentrations were taken up into the liposome bilayer without breaking up the vesicle [38]. Studies suggest that the surfactant presence in lipid-based vesicle systems has a noticeable effect on improving drug loading, drug release, stability, and other physiochemical properties [39].

However, as surfactant concentration increases, the proportion of surfactant molecules in the membrane increases until a critical value. In contrast, over this threshold value, the permeability of the vesicle's membrane might increase due to the arrangement of surfactant molecules within the lipid bilayer structure, which could introduce holes within the membrane and increase its fluidity. This might induce the vesicle bilayer's rupture and reduce the drug entrapment efficiency and liposome physical instability[39].

1.8 Malaria infection

Malaria is a serious and sometimes fatal parasitic infection that is transmitted into the human body through the bites of infected female anopheles' mosquitoes. In humans, malaria is caused by five protozoa (single-celled) plasmodium species. The Plasmodium strains that infect humans are Plasmodium falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi. Among these P. Falciparum parasites are responsible for the majority of malaria cases and almost all malaria-related deaths [41].

Malaria is a global public health concern with high morbidity and mortality rate in children and adults. It is endemic to varying extents in 106 countries of the world's tropical and subtropical regions[42]**.** According to the latest WHO estimates, released in November 2020, there were an estimated 229 million clinical malaria cases in 2019 from that the estimated number of malaria deaths attained be at 409 000 among which 67% (274 000) of all malaria deaths were covered mainly by children under five years age. Africa as a continent accounted for a high share of the global malaria burden, 94% of malaria cases and deaths [42].

Patients diagnosed with malaria infection usually feel very sick with high fever, chill, sweat nausea, vomiting et**c** [43]. Additionally, patients with severe malaria which mainly caused due to the falciparum strain of the plasmodium parasite are also other complications such as Cerebral malaria, anaemia, and splenomegaly (enlargement of the spleen) [44]. Cerebral malaria is the most severe neurological complication of infection and happens when the infected red blood cells block the small blood vessels leading to the brain necrosis and swelling of the brain which lead to permanent brain damage, seizures, and coma. The plasmodium parasites infect and multiply inside the red blood cells (RBC) and if it is not treated it may eventually cause rupture of the infected cells where it leads to severe anaemia[44].

1.8.1 The Plasmodium Life cycle

The malaria parasite life cycle is very complex, and it requires two hosts including the humans (asexual) and the mosquitoes (sexual) [45]. The Plasmodium life cycle (Figure 1.7) is summarized in six stages as below [44, 46].

Stage I

During a blood meal, a malaria-infected female anopheles mosquito inoculates sporozoites into the human body and these enter the parenchymal cells of the liver where they multiply.

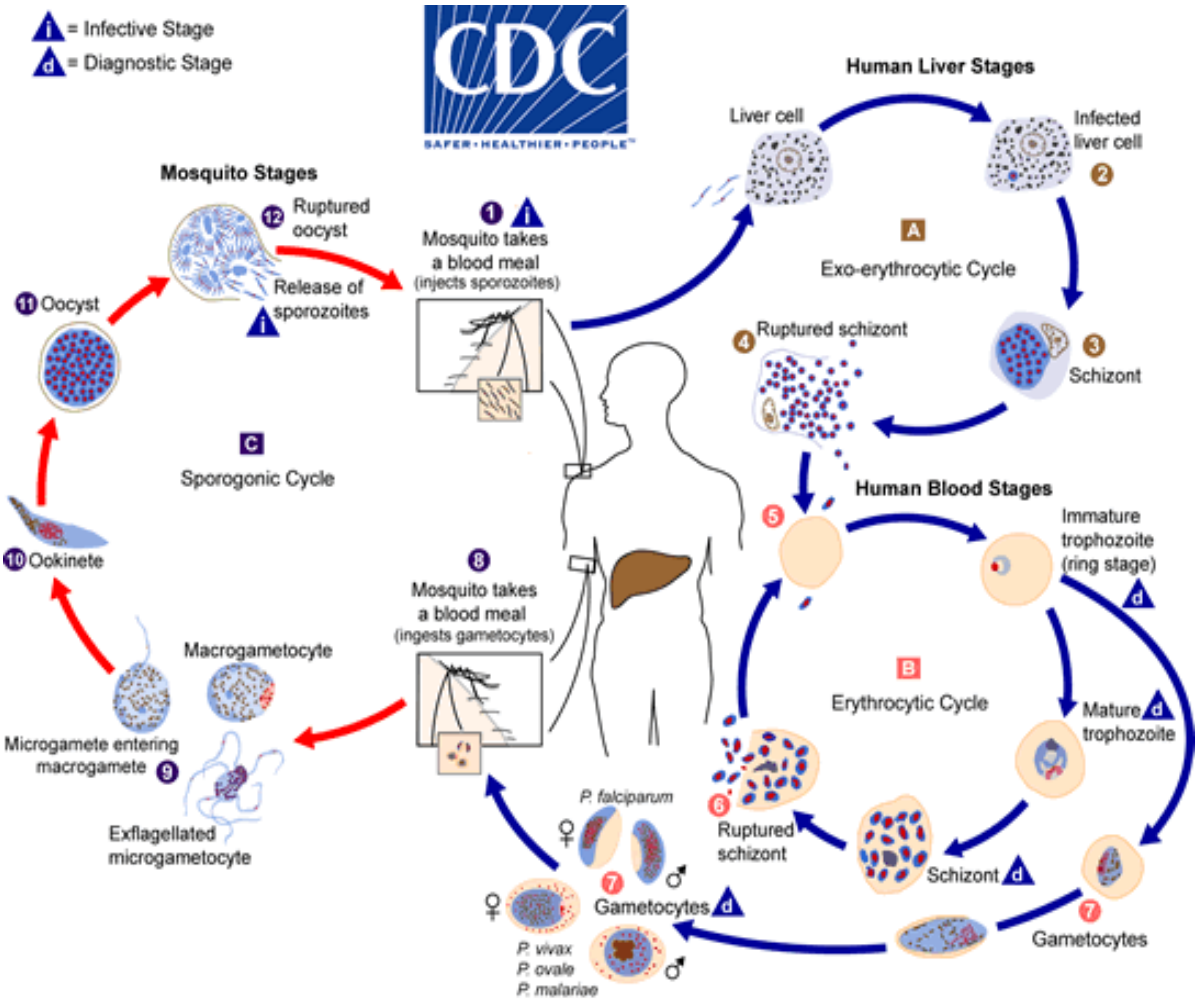


Figure 1.7*: Illustration of the life cycle of plasmodium parasite. Sporogonic cycle (sexual phase) multiplication in Anopheles mosquitoes and the Schizogony cycle multiplication in humans at the liver stage and in blood-stage [47].*

Stage II (Human Liver Stage)

At this stage, these sporozoites enter hepatocyte cells gets mature into schizonts which then ruptured, and release many invasive structures called merozoites. In the exoerythrocytic liver-stage

P. vivax and P. ovale a dormant stage [hypnozoites] can persist in the liver (if untreated) and cause relapses by invading the bloodstream for weeks, or even years after the primary infection.

Stage III (Human Blood Cell Stage)

After the initial replication in the liver merozoite further invades red blood cells (RBC) where they undergo asexual reproduction known as schizogony. Merozoites infect RBC and mature into schizonts, which rupture releasing merozoites re-enter the blood cells and begin a cycle of invasion of red blood cells and can result in thousands of parasite-infected cells in the host bloodstream. The blood-stage parasites are responsible for the clinical symptom and complications associated with malaria disease. In this stage, some merozoites can leave the cycle of asexual multiplication and form to the gametocytes.

Stage IV (Sexual Stage)

In this stage, the gametocytes differentiate into sexual erythrocytic stages as male (microgametocytes) and female (microgametocytes) and circulate in the bloodstream.

Stage V (Early and Intermediate Mosquito Stage)

During a blood meal, the female Anopheles mosquito ingests the gametocytes, and, on her stomach, the microgametocytes fertilize the microgametocytes resulting in zygotes production. Further, the zygote develops into motile Ookinetes and they invade the midgut wall of the mosquito and develop into oocysts.

STAGE VI (Late Mosquito Stage)

At last, the oocyst grows into several sporozoites through sexual multiplication and makes its way into the mosquito's salivary gland and is ready for another new human host that restarts the malaria life cycle.

The blood stage parasites are responsible for the clinical symptom and pathology associated with malaria disease. While in the exo-erythrocytic liver-stage does not produce any clinical symptoms. However parasites such as P. vivax and P. ovale can also persist as a dormant stage [hypnozoites] in the liver (if untreated) and cause relapses by invading the bloodstream in weeks, or even years after the primary infection [46].

1.8.2 Antimalarial drug

Plasmodium falciparum parasite is responsible for most of the mortality and morbidity associated with malaria infection. However malaria infection is curable and can be prevented with antimalaria drugs[48]. Most malarial drugs are schizonticidal blood drugs that target the asexual erythrocytic stage and some few schizonticidal tissue drugs targets the hypnozoites (liver stage) caused by P.Ovale and P.vivax infections [49].

Based on the mechanism action currently available antimalarial drugs were broadly categorized into three classes as summarized below. The drug in each class shares a common pharmacophore [45, 50, 51].

1. **The Aryl amino alcohol compounds*:*** include drugs such as quinine, quinidine, halofantrine, lumefantrine, chloroquine, amodiaquine, mefloquine, cycloquine, etc.
   *Mode of action**:*** *The quinoline and related drugs enters into RBC and inhibit heme, crystallization, thereby the accumulation of cytotoxic heme in vacuoles leads in parasites death.*

2. **The Antifolate compounds*:*** include proguanil, pyrimethamine, trimethoprim, etc.
   *Mode of action**:** Antifolate drugs act by inhibiting enzymes for synthesis of folate cofactors, thereby affecting DNA synthesis.*

3. ***The Artemisinin compounds****:* including artemisinin, dihydroartemisinin, artesunate, artemether, etc.
   Mode of action: Artemisinin and its derivate acts on food vacuoles of the parasite there, they bind with FE(II)-heme and generates free radicals, leading to killing of the parasites.

Oral chloroquine (CQ) and quinine were the most widely used treatments in the fight against uncomplicated Plasmodium infections. However, due to the emergence and spread of CQ and SP resistant parasites complicate the treatments of P. falciparum[49].

To counter the emergence of the malarial drug's resistance problem, WHO is promoting the use of antimalarial combination therapy. According to WHO the antimalarial combination therapy defines as "*the simultaneous use of two or more blood schizonticide medicines with an independent*

mode of action and thus different biochemical targets in the parasite"[48]. Consequently, Artemisinin Combination Therapies (ACTs) were introduced as a frontline treatment against uncomplicated P. falciparum malaria infection. The WHO currently approved five different ACTs listed in (Table 1.2). These antimalarial drug combinations' primary objective is to prolong Artemisinin's half-life and delay the spread of resistance. Oral artemisinin and its derivatives have a rapid onset of action, and they are eliminating rapidly (T1/2 0.5–1.4 h) from the human body [41, 48].

Table 1.2: List of WHO recommended ACTs drugs for the treatment of uncomplicated falciparum malaria ;*All combinations are in tablet form. [48, 52]*

Artemisinin derivative	**Co-Partner drug(s)**	**Formulation** **Abbreviation**	**Dosage**
Artemether	Lumefantrine	Co-formulated (AL*)*	*AL (20/120mg)*
Artesunate	Amodiaquine	Co-formulated (*AS+AQ)*	*AS+AQ (25/67,5mg,50/135mg, 100/270mg)*
Artesunate	Mefloquine	Co-blistered (*AS+MQ)*	*AS+MQ (50+250) mg*
Artesunate	Sulfadoxine /pyrimethamine	Co-blistered (AS+*SP)*	*AS+SP (50+500+25) mg*
Dihydroartemisinin	Piperaquine	Co-formulated (*DHA +PPQ)*	*DHA +PPQ (40/320mg)*

1.8.3 Lumefantrine

Lumefantrine (LUM), also known as benflumetol, is an aryl amino alcohol compound a member of fluorenes derivatives[53].LUM is used as an antimalarial drug combined with artemether mainly

for the treatment of multidrug-resistant and cerebral malaria infection [54]. Artemether-lumefantrine (AL) is an oral fixed-dose combination recently given in a standard tablet form containing 20 mg artemether and 120 mg lumefantrine, and 40 mg artemether and 240 mg lumefantrine[48].

Lumefantrine is a blood schizonticide, effective against chloroquine resistant P. falciparum strains. Currently LUM is only available in combination with Artemisinin with a marked name Coartem® [52]. Both compounds have potent antimalarial activity and they corporate in antimalarial clearing effects. Since Artemether has a rapid onset of action and it is rapidly eliminated from the body, thereby provides rapid symptomatic relief by reducing the number of malarial parasites. While Lumefantrine has a much longer half-life and is believed to clear residual parasites[55].

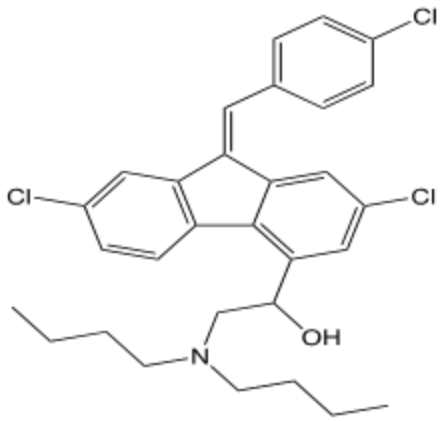


Figure 1.8*: Chemical structure of lumefantrine* ($C_{30}H_{32}C_{13}NO$)

Lumefantrine has highly lipophilic and weakly basic character. It is water insoluble, slightly dissolve in acetonitrile, methanol and completely dissolves in solvent such as chloroform, and dichloromethane. To improve the absorption limitations LUM is very dependent on coadministration with fatty foods [56].

Mode of action: The antimalarial mechanism of action of lumefantrine and its derivatives remains unclear. However, several theories suggest that lumefantrine inhibits the formation of β-hematin by forming a complex with hemin and hinders nucleic acid and protein synthesis. Lumefantrine has a much longer half-life and is believed to clear residual parasites[57].

1.9 Aim of the study

This study aimed to develop a liposomal formulation entrapping Lumefantrine for the use of parenteral administration. The plan can further divide in:

- To develop unloaded liposomal surfactant-based formulations using (Tween 20 and Tween 80).
- Formulate loaded liposomes using Tween 20 and Tween 80 containing lumefantrine and compare the encapsulation efficiency of both formulations.
- To determine both physical and chemical properties of unloaded and loaded liposomes using analytical instruments
- To perform *in vitro* lumefantrine release from the loaded liposomal formulations.

2 Materials and Methods

2.1 Materials

Table 2.1 Chemicals list used in the preparation of liposomes

Chemical	**Identification code**	**supplier**
Acetonitrile For HPLC-GC, ≥99.8% (GC)	Lot nr:STBJ9562	Sigma-Aldrich, Norway
Chloroform, 99.0-99.45% (GC)	Lot nr:STBH7859	Sigma-Aldrich, Norway
Ethanol	Lot nr:20D074007	VWRCHEMICHALS, France
Methanol for HPLC ≥99.9%	Lot nr:STBJ9184	Sigma-Aldrich, Norway
Phosphate Buffered Saline (PBS) Tablet	Lot nr:SLCF3040	Sigma-Aldrich, Norway
Propylene glycol, ≥ 99.5% FCC, FG	Lot nr:SHBM3251	Sigma-Aldrich, Norway
Sephadex® G-50 fine	Lot nr: SLBZ6430	Sigma-Aldrich, Norway
Trifluoroacetic acid (TFA), Reagent Plus 99%	Lot nr:STBJ0634	Sigma-Aldrich, Norway
Uranyl acetate reagent ACS	Lot nr:180404-01	Electron Microscopy Sciences. England

Table 2.2: Equipment's list used in the preparation of liposomes

Equipment	**Type /Supplier /country**
Bath Sonicator	Bandelin Electronic™ Soronex Super RK 102H Ultrasonic Bath. Germany
Buchi vacuum controller	Buchi B-721, Switherland
Column chromatography	Econo-Column(r) Chromatography Columns, product 1x20cm glass, Bio Rad Laboratories, inc , Catalog# 7371022
Copper grid	Carbon-coated Cu,3mm SPI 200MESH , Sigma-Aldrich Norway
Cuvette (with electrode for ZP)	Cuvette, polystyrol/polystyrene(10x10x45mm), Germany
Cuvette (disposable)	Cuvette, polystyrol/polystyrene(10x10x45mm), Germany
Dialysis bag	Spectra /Por® Dialysis membrane ;Biotech CE Tubing MWCO:20kd
Eppendorf Tubes®	Eppendorf AG, Hamburg, Germany
Homogenizer	IKA® T25. digital Ultra-Turrax rpm x1000 Model: T25D Laboratory Equipment
Filter	Acrodisc. 13mm with 0.2um Nylon Lot: FG0399
pH meter	914 pH/Conductometer, Metrohm Ltd, Switzerland
Pipette 0.5-5ml	Thermo Fisher Scientific Finn pipette 0.5-5ml. Made in Finland
Pipette 0.5-10ml	Eppendorf Tubes® 0.5-10 ml, Hamburg, Germany
Shaking water bath	Stuart SBS40, Thermo Fisher Scientific
Syringe 2ml	Malvern BBRAUN INJECT® Luer Solo ,Germany

Syringe 5ml	Malvern BBRAUN INJECT® Luer Solo ,Germany
TEM	JEM-1230TEM, JEOL Ltd., Japan
UHPLC	1260 Infinity II , Agilent Technologies Inc, USA
Vortex -Genie 2	Vortex -Genie 2
Vial Clear Conv Pk9mm	Supleco solutions within™ pack of 100. Lot:112546, USA
Weight	XA204, DeltaRange®, METTLER TOLEDO
Zetasizer	Zetasizer Nano series (Nano-ZS) Malvern instruments, Model: ZEN3600
HPLC column	ZORBAX Eclipse Plus C18 ,2.1x50mm 1.8-micron, Agilent Technologies Inc, USA

Table 2.3 Materials for liposome formulations

Material	Abbreviation	Identification code	Supplier /country
Lumefantrine powder Cat# HY-B0803/cs-5130	LUM	Lot#18510	MedchemExpress (MCE), USA
green PC Phosphatidylcholine 94%	PC	Product Code 2400421	Shanghai eca healthcare inc. China
Tween 20	T20	Lot#BCCC8540	Sigma-Aldrich , Norway
Tween 80	T80	Lot#BCCC7827	Sigma-Aldrich , Norway

2.2 Methods

2.2.1 Liposome preparation

There are several methods well used for liposome formulations. In this study, uni-lamellar structure vesicles were produced using the thin-film hydration method[58] for all liposome formulations regardless of their composition. Followed by using a bath sonicator, small unilamellar vesicles (SUV) were produced. Since the liposomal size is a crucial parameter for intravenous injections[59] we further employed a high-pressure homogenization technique and extrusion through a 0.2-micrometer filter to produce a reliable vesicle size. A cold homogenization method of 10000rpm and 20000rpm rate was employed. We prepare empty liposomes first; furthermore, we select a reliable size formulation for further drug loading.

2.2.1.1 Film hydration

2.2.1.1.1 Empty liposome preparation

In brief, 5 mg Phosphatidylcholine (PC) was weighed into a round-bottomed flask and dissolved by organic solvent chloroform to make a 200mg/ml final stock solution. Stock solutions of surfactants with a concentration of 200mg/ml were also prepared. Based on (Table 2.4A) we mixed the desired amount, either lipid alone or in different molar ratios with Tween. Further, the organic solvent was evaporated for two hours using a vacuum evaporator until it completely dried and a thin lipid film was formed on the bottom of the flask. The film was hydrated with phosphate buffered saline (PBS) and vortexed (for 2 min) until a complete detachment of the lipids from the flask wall. The sample was placed in the fridge at 4 °C overnight before further experiments were performed.

Table 2.4A : Composition of empty liposomal formulation batches

Batch A and B Liposome formulations in 95% PBS			
Formulation nr.	Phosphatidylcholine (PC) %	Tween %	Tween/PC molar ratio
1	100	0	0
2	95	5	0.03
3	90	10	0.07
4	85	15	0.11
5	80	20	0.15
6	70	25	0.26
Batch C and D Liposome formulations in 98%PBS			
Formulation nr.	Phosphatidylcholine (PC) %	Tween %	Tween/PC molar ratio
7	100	0	0
8	90	10	0.07
9	80	20	0.15
10	70	30	0.26
11	50	50	0.60
12	60	40	0.40
13	40	60	0.90
14	30	70	1.40

Based on table 2.4A total of 52 blank liposome formulations composed of only PC, PC/T20, and PC/T80 were prepared in water phase 95% and 98% and downsized using a high-pressure

homogenizer at a medium speed 10000 rpm and a high speed of 20000 rpm each. All formulations are categorized in four major batches where batches A and B are formulations prepared in 95% PBS while C and D prepared in 98% PBS as listed in table 2.4B below.

Table 2.4B: Batch code for empty liposome formulations

	Baches in 95% PBS		Baches in 98% PBS	
Components	**Batch A:**	**Batch B:**	**Batch C:**	**Batch D:**
Only PC	PC95M1	PC95H1	PC98M7	PC98H7
PC/T20	PC95T20M2-6	PC95T20H2-6	PC98T20M8-14	PC98T20H8-14
PC/T80	PC95T80M2-6	PC95T80H2-6	PC98T80M8-14	PC98T20H8-14

** Phosphatidylcholine (PC), Tween20 (T20), Tween80 (T80), 95 and 98 for PBS %, M for medium rate (10000rpm) and H for high rates 20000rpm and the numbers (1-14) indicates formulations numbers listed in Table 2.4A.*

2.2.1.1.2 Lumefantrine loaded liposomes preparation

The liposomal entrapment of LUM was obtained by the passive loading method, where the drug was added with the other liposome components during liposome formulation [60]. Stock solution 10mg/ml was prepared by dissolving lumefantrine in chloroform. The desired amount of lipid, surfactant, and lumefantrine components were mixed in a flask, and then the same procedure as for the empty liposome preparation was further performed.

The lumefantrine encapsulation was prepared based on the composition in Table 2.5A. Formulations from blank liposomes in batch D (PC98H7, PC98T20H8-11 and PC98T80H8-11) were selected for further studying, drug encapsulation. Each formulation further will load the drug molecule in PC: drug ratio of (1:10, 1:20, and 1:30). In total, 27 formulations were prepared as shown in Table 2.5A, and for simplicity, a formulation code is given as listed in Table 2.5B.

Table 2.5A: *Composition of drug loaded liposomal formulation batches*

Formulation number	**PC:Tween composition %**	**Tween / PC molar ratio**	**LUM: PC Mass (mg) ratio**	**LUM concentration (mg/ml)**
7	100:0	*Only PC*	*1:10* *1:20* *1:30*	*2.04* *1.02* *0.67*
8	90:10	*0.07*	*1:10* *1:20* *1:20*	*1.84* *0.92* *0.61*
9	80:20	0.15	*1:10* *1:20* *1:30*	*1.63* *0.82* *0.54*
10	70:30	*0.26*	*1:10* *1:20* *1:30*	*1.43* *0.71* *0.47*
11	50:50	0.60	*1:10* *1:20* *1:30*	*1.02* *0.51* *0.34*

Table 5.2B: Batch code for drug loaded liposome formulations

Components	**1:10**	**1:20**	**1:30**
Only PC	PCHL7	PCHL7(a)	PCHL7(b)
PC/T20	T20HL8-11	T20HL8-11(a)	T20M8-11(b)
PC/T80	T80HL8-11	T80HL8-11(a)	T80HL8-11(b)

** Phosphatidylcholine (PC). Tween 20 (T20), Tween 80(T80), High homogenization 20000rpm (H), Lumefantrine (L) , the numbers (7-11) indicates formulation numbers and alphabets a and b indicates for formulations with drug /lipid ratio (1:20) and (1:30) respectively.*

2.2.1.2 Reduction of particle size and lamellarity

After hydration of the dry lipids using PBS, it is assumed that the liposomes are present in multilamellar vesicles (MLVs)[17]. Further reducing of vesicle size and lamellarity is procured by manufacturing the liposome suspension with several processing techniques. The most commonly used approaches to minimize MLVs to SUVs includes sonication, filter extrusion, and high-pressure homogenization[28].

2.2.1.2.1 Sonication

Sonication disrupts MLV suspensions by using sonic energy to produce SUVs liposomes[31]. This work is conducted using a bath sonicator. The sample filled vials were locked and placed in the bath where temperature is adjusted at 60 °C which is 5°C above PC phase transition temperature. All formulations were sonicated for 10 minutes.

2.2.1.2.2 High-pressure homogenization

Using high-pressure homogenizers, the liposome vesicle size is readily reduced by its passage through a narrow gap or micro-channel without inducing degradation of the lipids[61].
Since liposomes are prone to degradation in high temperatures, the cold homogenization technique has been utilized in all preparations. The formulations were kept in a glass vessel on dry ice and were homogenized using two different sharing rates (10000Rpm and 20000 Rpm) for 10 minutes.

2.2.1.2.3 Filter extrusion

After homogenization, liposome dispersions were filtered through a 0.2 μm pore size filter using a 5ml syringe. This was carried out in order to downsize phospholipid vesicles. This technique is utilized for producing a desired homogeneous liposomes vesicle size is in the range of 50-200nm.

2.2.2 Liposome characterization

The most important parameters of liposome include visual appearance, size distribution, concentration, lamellarity, and stability [17].

2.2.2.1 Particle Size and particle size distribution determination

The particle size, particle size distribution and polydispersity index (PDI) of liposomes were analysed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano. The standard

operational principle of DLS is based on Brownian motion of dispersed particles. According to Brownian motion, when small particles are dispersed in a liquid, they undergo random thermal motion in all directions. Consequently, this thermal motion leads to a continuous collision with solvent molecules and causes a certain amount of energy transfer, which induces particle movement. The energy transfer is constant and therefore has a more significant effect on smaller particles. As a result, smaller particles diffuse at higher speeds than larger particles[10].

The DLS measurements were performed at 25 and scattering angle 173°C. Using too high a concentration sample can cause multi-scattering, where particles interact and lose intensity [10]. To exclude the multi-scattering problem, the liposome sample was diluted with PBS in a ratio of 1:100. The diluted sample was transferred into a disposable cuvette using a 5 ml syringe for size determination. Then the z-average diameter and PDI were measured using DLS in triplicate, and the results are given as an average.

2.2.2.2 Zeta Potential determination

Zeta potential (ZP) is a parameter usually used for the determination of colloidal stability where it is carried out on the Malvern Zetasizer nano. The ZP analysis of liposome was performed by filling a 0.8 ml diluted sample into the non-disposable cuvette. The liposome samples were diluted in 1:100 with PBS, and the ZP was measured for three cycles at 25 ℃. After every analysis, the non-disposable cuvette was then cleaned with distilled water using a 2 mL syringe.

2.2.3 Morphology determination

The size and morphology of lumefantrine encapsulated liposomes were studied by transmission electron microscopy (TEM). The analyses were prepared by placing 5µL of the liposome sample to a carbon-coated copper grid (200 mesh) and left for 1 min to allow adhesion between the sample and the carbon substrate. Then, the excess sample was removed by immersing the grid in a drop of water for 30 sec. followed by adding a drop of 2% uranyl acetate solution and leaving it for 30 seconds until it gets stained. Lastly, the excess uranyl solution was removed by immersing the grid in a drop of water for 30 sec and air-dried completely. The morphology of the liposome were examined using a JEM-1230TEM apparatus (JEOL, Japan). The images were observed using a microscope at an accelerating voltage of 80 kV.

2.2.4 Chromatography

2.2.4.1 Gel Filtration Chromatography

The drug-loaded liposomal formulations were fractionated by gel filtration chromatography method known as size exclusion chromatography (SEC). The primary separation principle is that the molecular weight difference between liposomes and non-trapped free drugs will lead to different retention times. Where, during separation process, low molecular weight (free drugs) can easily enter to the pores and spend a long time in the pores before they eluate. In contrast, larger particles (liposomes) do not enter the pores results in quick elution [34].

Briefly, a glass column of approximately 23 cm height was packed with a Sephadex G-50. First the dry powder Sephadex were prepare by dissolving in PBS buffer in a mass ratio (1: 9) and allowed to swell overnight at 4°C. After swelling, the Sephadex solution is filled into a column up to about 2 cm from the top. After every eluting, the column is washed first with 2 ml 70% ethanol and then using 5ml PBS to reuse the stationary phase. The Sephadex column was kept at 4°C in 20% ethanol to ensure no growth of microorganism.

2.2.4.2 Ultra-High-Performance Liquid Chromatography (UHPLC)

Ultra-high-pressure liquid chromatography (UHPLC) is a chromatographic method employed to separate, distinguish, and quantify compounds. The principle back to this method is that the mixture of particles move flow the mobile phase and to be separated through a column filled with a packing material known as the stationary phase. The analyte, which was separated from the mixture were further to be analysed with different types of detectors at different retention times. The amount of Lumefantrine entrapped in liposome was quantified using an UHPLC method equipped with a UV detector. In this work the UHPLC analyses were carried out based on the method reported by *Nogueira et al.* and *Iqbal et al*. with some modification [58, 59].

Briefly the used UHPLC-method were listed as follow:

- Mobile phase

    A. Trifluoroacetic acid (TFA) 0.05%
    B. Acetonitrile

C. mixture of Methanol and water (70:30) %
D. Acetonitrile

- Column: ZORBAX Eclipse Plus C18 (2.1 x 50 mm, 1.8 -Micron)
- Flow rate: 0.4mL/min
- Max column pressure limit: 1100bar
- Run time: 15 min
- Column temperature: 30°C

2.2.5 Standard calibration curve

Lumefantrine are dissolved in acetonitrile in a stock solution 2mg/ml. further the stock solution was diluted with the mobile phase (acetonitrile) to prepare standard solutions of 0.3,2.5, 5, 12.5, 25 and 50 µg/ml and the absorbance were detected at 355nm. The standard calibration curve was plotted in drug concentration (x-axis) versus peak area (y-axis).

2.2.6 Encapsulation efficiency and Drug loading capacity determination

The encapsulation efficiency (EE) determination was conducted by separating the un-entrapped drug from liposomal formulation using gel filtration chromatography. Briefly, liposome dispersion (300 µL) were loaded to the Sephadex G-50 column and using PBS as eluent, and total 20 fractions of each fraction around 750 microliters eluate were collected. The collected eluates were analysed using UHPLC, and the concentration of the UV detector at wavelength of 350 nm. The entrapped amount of lumefantrine in liposomes was calculated by substituting the obtained peak area value in the calibration curve. This amount was further divided by the total amount of lumefantrine in the liposomes to calculate the encapsulation efficiency (EE%). The EE% and DL% of Lumefantrine were determined using the following Equations 1 and Equation 2.

Encapsulation efficiency (EE%) = $\frac{Amount\ of\ incorporated\ drug}{Total\ amount\ of\ added\ drug} * 100\%$ Equation:1

Drug Loading capacity (DL%) = $\frac{Amount\ of\ incorporated\ drug}{Total\ amount\ of\ added\ Phospholipid} * 100\%$ Equation :2

2.2.7 Drug release determination

The drug release from liposomes was analysed using the dialysis method. Dialysis bag (molecular weight cut-off: 20 000 kD) were used. To remove the preservative and soften the dialysis bag, it was soaked before use in distilled water overnight.

A sample of liposomal dispersion equivalent to 2 mg liposomal incorporated lumefantrine was placed in the dialysis bag. The bag was tied at both ends and tested for leakage. The system was suspended in a release medium (25 mL, at 37±0.2°C), and rotated at a rotational speed 120 rpm. For control groups, 1 mg of Lumefantrine drug dissolved in acetonitrile were placed in a dialysis bag. The drug release analyses were carried out based on the method reported *Panwar, P., et al* with some modifications[62]. In this work the bags were suspended in glass beakers filled a release medium containing a mixture of glycol (7%), methanol 35% and 58% PBS buffer.

At various time intervals (0.5, 1, 2, 3,4,6,8,10 and 24, hours)1.5 ml aliquot from the release medium were withdrawn for the UHPLC analysis. The same amount of fresh release medium at the equivalent temperature was added to the release medium immediately to maintain the constant volume. The dialysis tubing length was kept constants for all methods to ensure that the dialysis's surface area remained constant. Further, based on the results from UHPLC, the drug release (%) is calculated using the following equation[63].

$$\textbf{Drug release (\%)} = \frac{Amount\ of\ \ Drug\ in\ releasing\ medium}{Total\ amount\ of\ Drug} * 100\% \qquad \text{Equation: 3}$$

2.2.8 Liposome Stability Determination

2.2.8.1 Physical stability

The stability analysis for both empty and drug-loaded liposomes was carried out over a period. After preparing the liposomal formulations, each were kept at 4°C refrigerate to assess stability. The physicochemical stability of the liposomal formulations was determined by physical appearance, pH, particle size, ZP, and drug content in both accelerated and after freeze drying conditions.

2.2.8.2 lyophilization process

Preparation: Freshly prepared LUM-loaded liposomal formulations were prepared. 1.5 ml of each sample were freeze by liquid nitrogen and put in freeze-dryer. The liposome suspension was then dried at −81°C.

Reconstitution of the freeze-dried liposomes:The lyophilized dry products were rehydrated with distilled water to their original volume. Subsequently stability of the formulation was evaluated by analysing various parameters such as particle size, ZP, EE of the liposomes. These results were compared with the results obtained before freeze drying to evaluate the changes and stability of the formulation.

2.2.9 Phosphate-buffered saline (PBS) preparation

As recommended by the distributor Sigma-Aldrich[64],we prepare the PBS buffer solution by dissolving 1 PBS tablets in 200ml of distilled water to make a solution of 0.1M. pH is examined using a pH-meter and shows 7.443. In this work distilled water is used for all preparations.

3 Results and discussion

Liposomal formulations' suitability and acceptance for parenteral drug delivery mainly depend on the vesicle size and PDI. However, controlling and validating these parameters remains a key concern for practical clinical applications [59]. This pre-liposomal formation is intended to find out an appropriate method for manufacturing small-sized liposomes. Liposomes, mainly production of SUVs liposome, was the main goal as the first step in this work. Therefore, most of the work at the beginning was focused on finding reliable particle sizes. Hence, the study was first conducted on preparing unloaded liposomes using PC alone and in combination with surfactants Tween 20 and Tween 80. In addition to that, formulations with the smallest mean particle size were selected for further experiments. This was because vesicle size and size distributions have a significant impact on determining biodistribution and the mononuclear phagocytic system's (MPS) clearance of liposome particles [33].

Liposomal formulations were prepared based on the thin film method with additional downsizing techniques. Hence, besides techniques such as sonication and filter extraction, high-pressure homogenization at two different speeds (10000rpm and 20000) has also been employed further to reduce the vesicles and control size and PDI. In total 52 unloaded liposome formulations composed of only PC, PC/T20, and PC/T80 were prepared in water phase 95% and 98% and further 9 unloaded formulations were selected for drug loading and total 27 lumefantrine loaded liposome formulation at drug:lipid (wt/wt) ratio (1:10, 1:20 and 1:30) were prepared.

3.1 Physicochemical characterization of blank liposomes

Particle size, PDI, and ZP of empty liposomes were analysed using the DLS instrument, and the results were illustrated in figures (3.1, 3,2, and 3,3), respectively. The result revealed that our liposome size and PDI value generally showed a reduction in all batches regardless of water phases when using a 20000rpm homogenizer. In contrast, the ZP measure revealed an increase towards zero with an increase in homogenization speed. These changes in physicochemical parameters could relate to homogenization rate, phospholipid, and surfactant concentration.

3.1.1 Influence of homogenization speed on (size, PDI, and ZP)

***Effect on particle size**:*

Particle size is a critical attribute in lipid-based liposomes, which influences the stability, degree of encapsulation efficiency, drug release profile, biodistribution, and cellular uptake of the formulations [59]. Various factors may affect the particle size of liposomal formulations. In our case, the preparation methods had more notable influences on the mean vesicle size. Figure 3.1 clearly shows that the shearing rate affected the difference in liposome size produced with the two different homogenization speeds. Even though both methods apply an equal duration of time (10 minutes), the size produced with 20000rpm was significantly smaller than formulations homogenized by 10000rpm. This proves that the shearing speed had a significant impact on the size reduction. The result was expected since the size and size distribution truly depends on the number of times that vesicles pass through the interaction chamber [60].

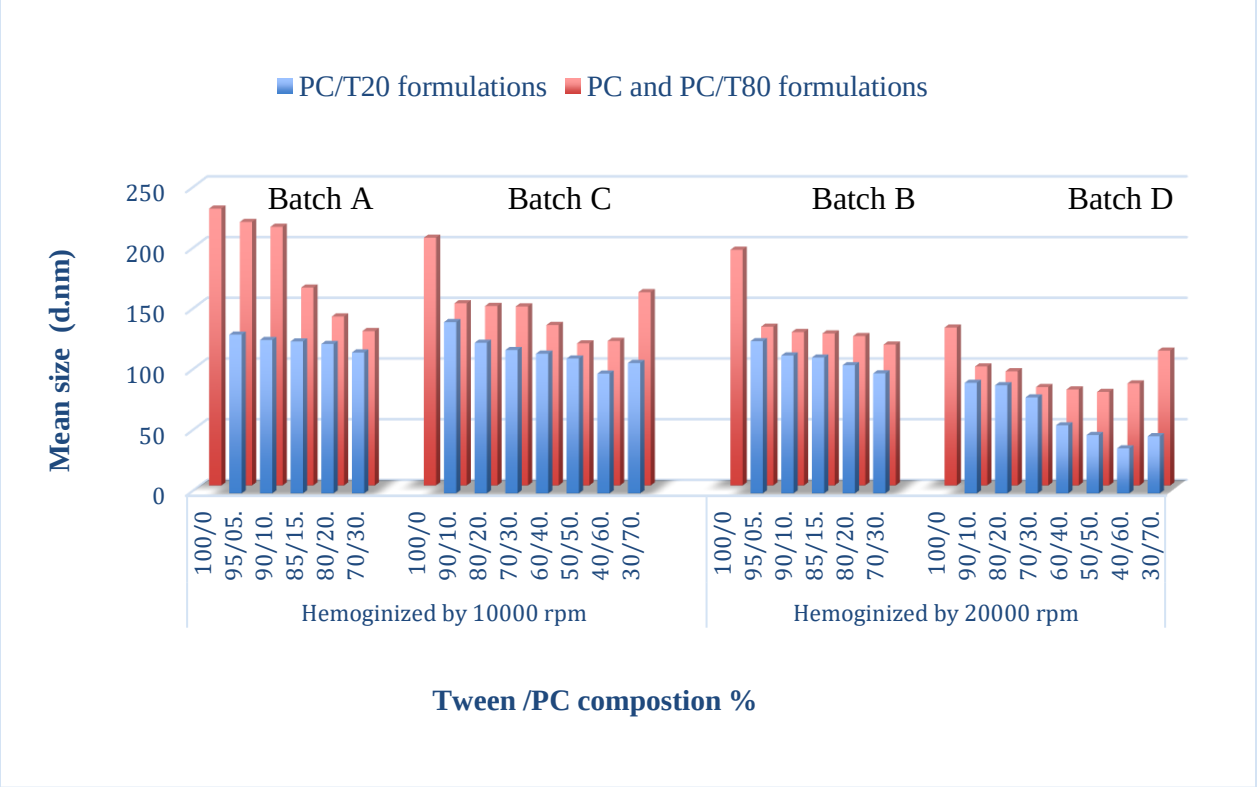


Figure 3.1: *The mean particle size of empty liposomal formulations. Formulations based on only PC and PC/T80 (red colour) and Blue coloured bars for formulations based on PC/T20.Batch A and B liposome formulations prepared respectively in 95% PBS and 98% PBS homogenised using*

LPH (10000rpm). Similarly, batches C and D prepared in 95% PBS and 98%PBS homogenised using HPH (20000rpm) (n=3).

Effect on the polydispersity index (PDI):

The polydispersity index (PDI) is another significant parameter in lipid-based nanocarriers that describes the width or spread of the particle size distribution. PDI values below 0.7 are considered as a suitable measurement [65], indicating that the sample has narrow particle size distribution. The lower PDI measure indicates the homogeneity of a sample based on the size of the dispersion.

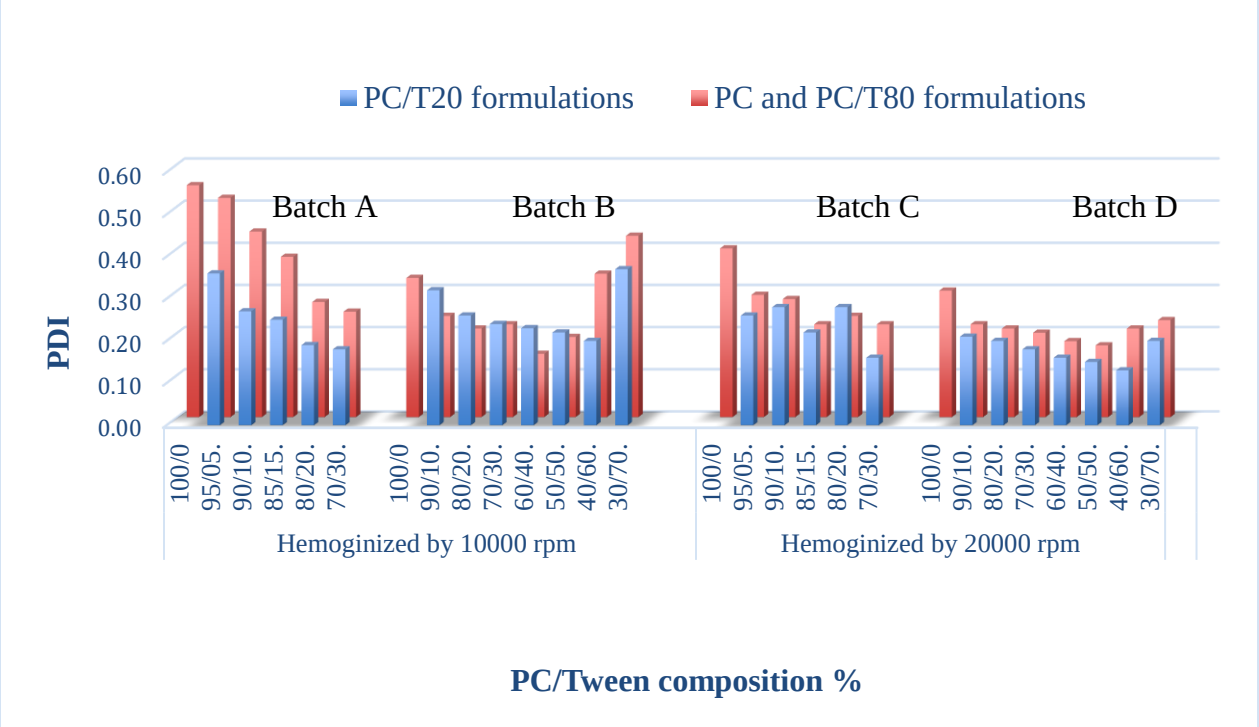


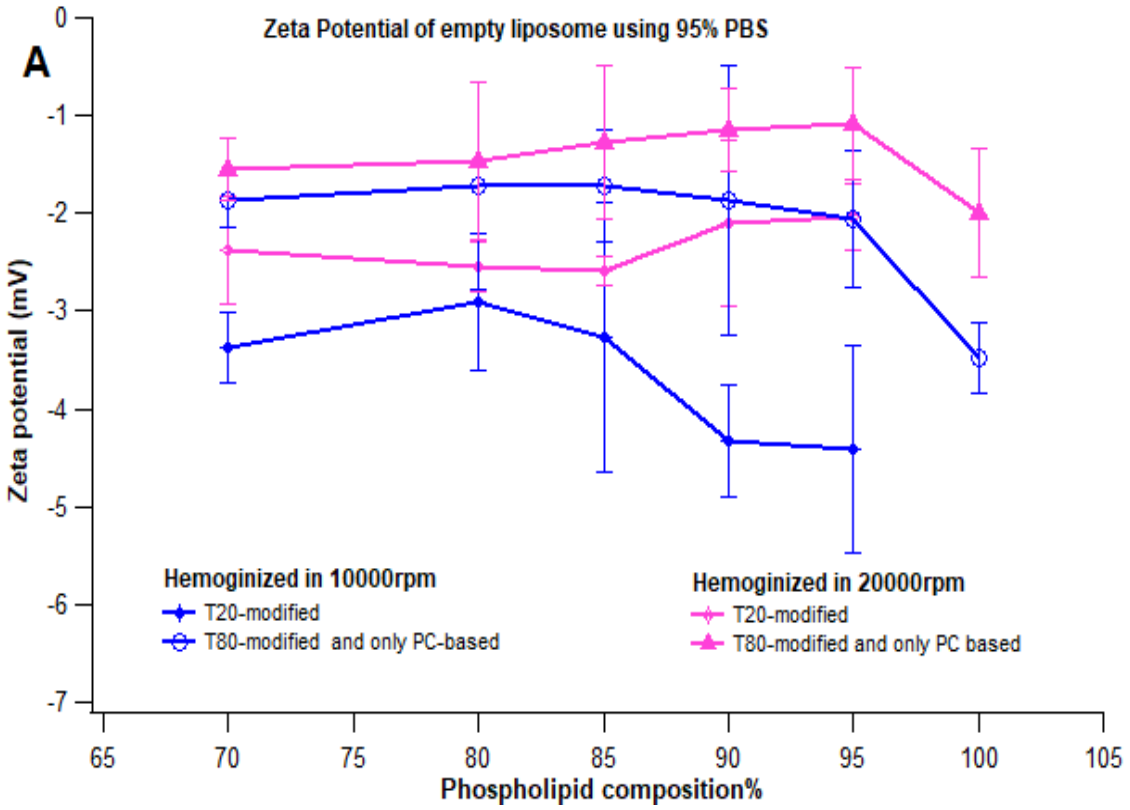
Figure 3.2: *The PDI value of all empty liposomal formulations. Formulations based on only PC and PC/T80 (red colour) and Blue coloured bars for formulations based on PC/T20. Batch A and Batch B formulations prepared in 95% PBS and homogenised using medium speed 10000rpm and 20000rpm, respectively. While batches C and D are prepared in 98%PBS and homogenised using 10000rpm and 20000rpm respectively (n=3).*

In all liposomal formulations, regardless of their phospholipid composition, the average PDI values were below 0.6 and ranged from 0.12 to 0.56, illustrated in Figure 3.2. This confirms that our liposomes were homogenous. Neither of the homogenization rates strongly influences the vesicle structure, but a slightly smaller PDI value was obtained when utilizing the highest

homogenization speed (20000rpm). Particularly in formulations in batch D, there was no significant PDI difference noticed, and the highest PDI value was 0.2 in both tween-based formulations at lipid/tween composition 30/70. In the drug delivery system, lipid-based nanocarriers, such as liposomal formulations with PDI value of 0.3 and below, are considered acceptable and indicates as a monodisperse particle[65]. Accordingly, formulations on batches D were producible for our desired PDI values and were satisfactory for further drug loadings study.

Effect on surface charge

All liposomal formulations showed a negative surface charge and zeta potential ranged from -1.15 to -5.88.The surface charge of the liposomes was also affected by the passage number and homogenization pressure[61]. This correlates with our findings where the higher homogenization rates lead to ZP increase towards zero. The plotted figure 3.3 (A and B), clearly shows that the absolute value of the surface charge in both figures decreases towards zero in response to an increase in homogenization rates. This implies that liposome size had a direct effect on ZP values of liposomes[66].



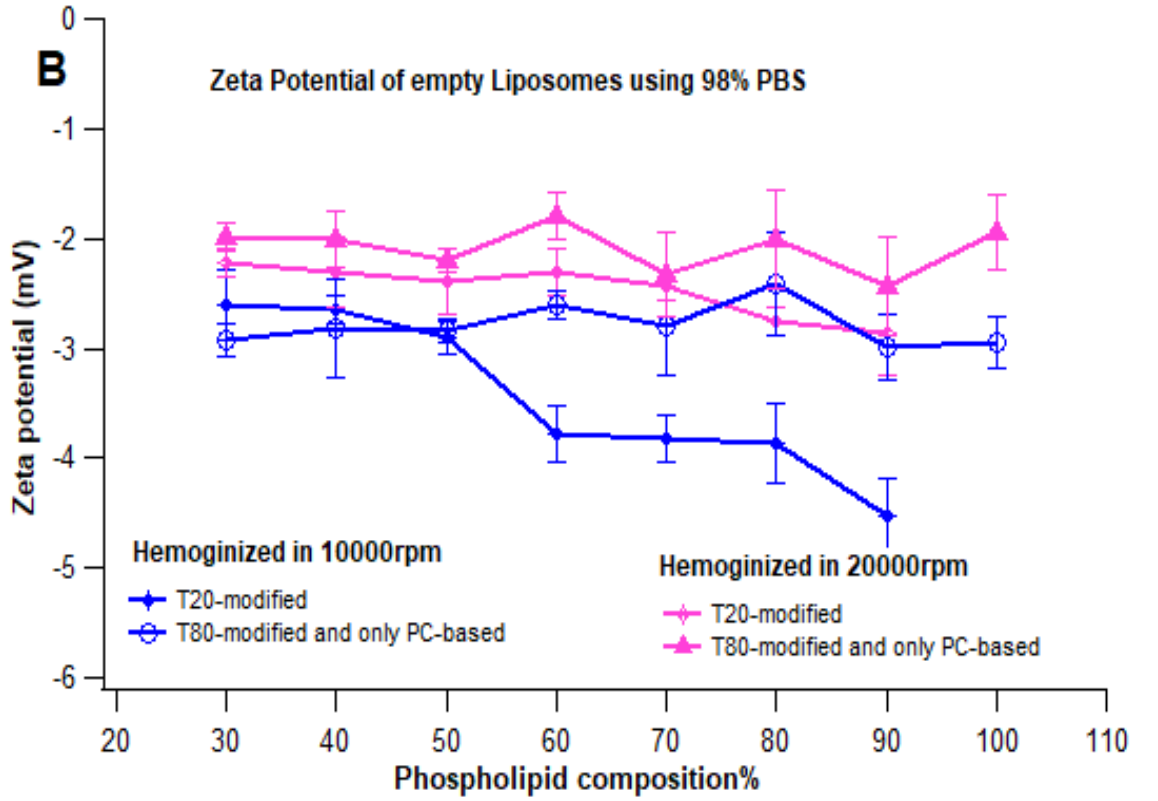


Figure 6.3: Zeta potential(mV) of empty liposomal formulations. *All liposomal formulations prepared in 95% water phase (A) and liposomal formulations prepared in 98% water phase (B) (n=3). Lilla color showed zeta potential of formulations homogenized by 20000rpm while blue color for formulations homogenized in 10000rpm.*

3.1.2 Influence of Lipid and surfactant concentration on (size, PDI, and ZP)

In addition to mechanical pressure, liposome constituents also have an impact on liposomal characteristics. PLs are the main component of the liposomal membrane. Hence, they can directly affect liposomes particle size[67]. However, additives such as surfactant also influence on vesicle size.

In our case, we had liposome formulations composed of only lipid and lipid in combination with surfactants. As shown in Figure 3.1 the mean particle size of the different formulation decreases with decreasing phosphatidylcholine concentration. For instance, regardless of their homogenization rate in all batches, formulations consisting of 100% PC had a larger vesicle size, while with a decrease in the concentration of PC, the vesicle size gradually decreases. The smallest

vesicle size was obtained on batches D at 40/60 PC/T20 and 50/50 PC/T80 composition, and both produce 37nm and 77nm, respectively. A comparable finding was reported by *Taghizadeh* and his group, where the larger and smaller liposome vesicles were obtained when using 95% and 50 % PC amount, respectively [68]. Similar findings are also reported by *Gardouh et al.[38]*, where the study revealed smaller vesicle sizes were obtained as the lipid ratio decreased.

In the present study, the presence of surfactant also had a noticeable impact on the physicochemical characteristics of the liposomal formulations. The result showed that a gradual decrease in a vesicle with surfactant concentration. We noticed that the higher the surfactant concentration, the smaller the mean vesicle size obtained. This was due to the fact that surfactant monomers were taken up into the liposome bilayer without cracking up the vesicle. And, steric repulsion, which contributed by the surfactant molecules, also inhibit or reduce the aggregation of the vesicles[69]. Experimental report from [69], revealed that liposome vesicle size reduced with increase of Tween 80 concentration from (0.2-0.8 molar ratio). This finding correlates greatly with our result where the vesicle size decreases in Tween/PC molar ratio range from 0.03 to 0.60. Similar report by *Kaur et al.* were also exhibited when liposomes were manufactured using Tween (20, 60 and 80) the particle size reduction were observed in response to surfactant concentrations[70].

However, at a certain level, with the increase of surfactant concentration, we noticed that the vesicle size and PDI increase, as demonstrated in Figures (3.1 and 3.2). This size and PDI increment were observed in both Tween-based formulations in PC/T80 (40/60) and PC/T20 (30/70) compositions, respectively. This could probably be due to the micellization process, as both surfactants are water-soluble, and with increase in concentration, the incorporation of Tween in PC bilayers becomes saturated, inducing the micellization of liposomes[20]. In this case, it is vital to take into consideration of the surfactant characteristics (carbon chain length, saturation, and HBL) as these had a significant role in vesicle size formation. The trend in increasing vesicle size could be associated with carbon chain length difference as Tween 80 has a longer fatty acid chain (C-18) than T20 (C-12 [71]. Thus, due to the longer carbon chain Tween 80 being more surface-active with lower critical micellization concentration (CMC) [22].This leads to aggregation and micelle formation. A similar observation was also noted when the molar ratio of Tween 80 increased above 0.8, where the liposomes can be dissolved entirely, results in rupture of liposomal membrane structure [69], which further leads to vesicle aggregation.

Although the same procedure was followed in manufacturing liposomal formulations based on T20 and T80, surfactant T20 produced a smaller vesicle size compared to T80 based formulations. For instance, results from batch D displayed that when using T20, the largest vesicle size 125.10 ± 0.61 nm and the smallest 36.7 ± 1.31 nm were obtained in 90/10 and 40/60 PC/T20 composition sequentially. At a similar PC/Tween composition, T80 made a vesicle size 127.70 ± 1.23 nm and 93.66 ± 1.69, respectively. This explains that surfactant difference also influences vesicle size. The smaller size vesicles had a potentially significant advantage as they can have a chance to escape the reticuloendothelial system (RES) in the liver and spleen, resulting in prolonged blood circulation and reach the target site[28].

Generally, the PDI value of surfactant-based liposomes shows that each surfactant produces a narrow range of PDI values in a range of 0.13-0.36 and 0.21-0.52 for T20 and T80 based formulations, respectively. However, in comparison to T20-based liposomes, T80 based formulations had slightly higher PDI value. Although the particle size does not correlate with PDI, the contrary was observed on formulations in 98% PBS (both batch B and D). We observed that the PDI value decreases as particle size decreases. However, these findings were also in agreement with *Garg et al.[70]*, report where PDI value was related to particle size. Garg found that the larger the vesicle size, the larger the PDI value obtained, whereas the smaller vesicle size showed smaller PDI value[70].

For further comparison of the Tween based liposomal formulations, it is important to consider the surfactant characteristic nature as these have a significant impact on physicochemical characteristic and pharmacokinetic-pharmacodynamics properties of lipid-based delivery systems[39].Although both Tweens (T20 and T80) share a common backbone, they have differences, including their carbon chain length and hydrophile-lipophile balance (HLB) values. T20 with (HLB 16.7) has a shorter carbon chain C-12, while T80 (HLB 15) has a longer carbon chain C-18 [72].Studies reported that the HLB influences vesicle reduction. Whereas the surfactant HLB value increases, the vesicle size decreases, and vice versa [39]. This thought correlates with our result well. As T20 had a larger HLB value than T80, the liposome vesicle size produced using T20 was significantly smaller than that of PC/T80 formulations. These findings are also in agreement with the finding by *Gupta, M. et al.* showed that comparison of four surfactants (Span 40 (HLB 5.7), Span 20 (HLB 8.6), Span 20 (HLB 4.3) and Span 60 (HLB 4.7)) were revealed that

the vesicle size led to increase with an increase in the HLB value(72). This could be due to the influence of surface free energy, which might reduce as the hydrophobicity rises [39].

Overall, regardless of the water phase, the achieved result showed that homogenization speed had significantly impacted our liposome's physicochemical characteristics. According to the obtained results, we noticed that four variables, particularly the phospholipid concentration, surfactant/phospholipid molar ratio, water phase, and homogenization speed had significant impact on the liposomal physiochemical parameters. For further drug encapsulation, we were taking into consideration of vesicle size, PDI, and ZP. However, as the zeta potential in all formulations was below -7, we excluded ZP from this evaluation. Based on the result, we obtained the smallest particle size and lowest PDI in formulations of batch D. Therefore, we selected batch D to encapsulate our drug for the following formulations with lipid composition (100,90,80,70 and 50) % as listed in (Table 2.5A)

3.2 Physicochemical characterization of drug loaded liposomes

The physiochemical characteristics of the various drug-loaded formulations were also determined and listed in Tables (3.1 and 3.2). Overall, after drug encapsulation, it was found that the presence of LUM had a direct effect on the increment of mean vesicle size and PDI values liposome formulation compared to the unloaded liposome. Additionally, we also noticed that the particle size increases with an increase in drug concentration. For instance, formulation without surfactants (Table 3.2) consisting of a drug concentration (0.67, 1.02, and 2.04 mg/ml) showed increased particle size ranges (127.0, 129.5, and 138.9) nm, respectively. This correlates with drug entrapment efficiency, where the entrapment efficiency increases with an increase in liposome vesicle size. However, larger liposomes have drawbacks as they have a short circulation half-life since they are rapidly excreted from the circulation by the RES[28].

Table 3.1: Effect of LUM concentration encoded in PC/T20 liposome formulations, on the entrapped drug amount (LC%), encapsulation efficiency (EE%), particle size, PDI and ZP

Batches	**PC /T20 composition** (%)	**LUM: PC Ratio**	**LUM Con. (mg/ml)**	**EE%**	**LC%**	**Particle size (d.nm+ SD)**	**PDI**	**ZP (mV)**
T20HL8		1:10	1.84	63.3	5.7	101.00 ± 0.90	0.30 ± 0.12	- 6.06 ± 0.45
T20HL8a	90/10	1:20	0.92	57.0	2.5	97.10 ± 0.23	0.12 ± 0.01	- 5.00 ± 0.78
T20HL8b		1:30	0.61	54.7	1.6	92.98 ± 0.81	0.08 ± 0.01	- 4.37 ± 0.63
T20HL9		1:10	1.63	57.6	4.9	89.00 ± 0.20	0.20 ± 0.02	- 5.18 ± 1.21
T20HL9a	80/20	1:20	0.82	51.2	2.2	95.50 ± 0.41	0.22 ± 0.01	- 4.18 ± 1.57
T20HL9b		1:30	0.54	53.8	1.5	92.50 ± 0.42	0.08 ± 0.03	- 4.18 ± 1.58
T20HL10		1:10	1.43	60.8	4.0	87.60 ± 0.49	0.13 ± 0.01	- 2.23 ± 0.71
T20HL10a	70/10	1:20	0.71	54.9	1.9	81.66 ±0.55	0.12 ± 0.01	- 2.56 ± 0.47
T20HL10b		1:30	0.47	52.0	1.2	80.00 ± 0.51	0.16 ± 0.02	- 2.58 ± 0.47
T20HL11		1:10	1.02	68.7	3.4	115.00 ± 1.65	0.36 ± 0.12	- 2.36 ± 1.25
T20HL11a	50/50	1:20	0.51	59.9	1.5	97.62 ± 1.07	0.21 ± 0.01	- 2.46 ± 1.18
T20HL11b		1:30	0.34	61.1	1.0	57.64 ± 0.31	0.29 ± 0.06	- 1.80 ± 0.91

All formulations in both Tweens had revealed a slight vesicle size increment compared to blank liposomes. The increase in mean particle size with increasing drug loading was correlated with the entrapment of drug molecules into the hydrophobic region of the vesicle, which then causes the vesicle bilayer to become apart from each other [65], leading to an increase in particle size. Similarly, in empty liposomes, vesicle size decreases with an increase in surfactant concentration. However, in contrast to empty liposomes, we obtained a larger vesicle size at a lower surfactant concentration at PC/tween (50/50) composition. This size gain could associate with an accumulation of vesicles that forms aggregates, and eventually, size increases. Our findings were also in agreement with those reported by *Bnyan et al.,* which stated that increasing surfactant concentration will increase the number of vesicle formation, leading to a larger volume of the hydrophobic bilayer [39].

Table 3.2 : Effect of LUM concentration encoded in PC/T80 and surfactant-free formulations, on the entrapped drug amount (LC%), encapsulation efficiency (EE%), particle size, PDI and ZP

Batches	**PC /T20 composition (%)**	**LUM: PC Ratio**	**LUM Con. (mg/ml)**	**EE%**	**LC%**	**Particle size (d.nm+ SD)**	**PDI**	**ZP (mV)**
PCHL7		1:10	1.84	49.6	5.0	138.90± 0.62	0.20 ± 0.02	- 7.48 ± 0.17
PCHL7a	100/0	1:20	0.92	45.0	2.3	129.50 ± 1.81	0.30± 0.02	- 6.52 ± 0.49
PCHL7b		1:30	0.61	40.2	1.3	127 ± 1.01	0.21 ± 0.02	- 6.49 ± 0.39
T80HL8		1:10	1.63	65.0	5.9	125.90 ± 0.50	0.23 ± 0.03	- 6.92 ± 1.74
T80HL8a	90/10	1:20	0.82	57.3	2.6	110.40 ± 0.89	0.21 ± 0.01	- 5.83 ± 1.03
T80HL8b		1:30	0.54	53.6	1.6	99.80 ± 0.71	0.24 ± 0.01	- 4.84 ± 1.09
T80HL9		1:10	1.43	59.9	5.0	104.00 ± 0.61	0.12 ± 0.04	- 5.61 ± 1.13
T80HL9a	80/20	1:20	0.71	52.0	2.3	88.60 ± 0.91	0.15 ± 0.01	- 5.11 ± 0.33
T80HL9b		1:30	0.47	54.7	1.5	84.61 ± 0.80	0.11 ± 0.01	- 4.36 ± 1.19
T80HL10		1:10	1.43	62.3	4.2	88.91 ± 0.78	0.26 ± 0.02	- 4.08 ± 0.21
T80HL10a	70/30	1:20	0.71	59.6	1.9	80.90 ± 0.70	0.23 ± 0.01	- 3.81 ± 0.21
T80HL10b		1:30	0.47	56.6	1.2	78.40 ± 0.40	0.20 ± 0.01	- 3.80 ± 0.53
T80HL11		1:10	1.02	71.5	3.6	132.10 ± 1.30	0.41 ± 0.19	- 3.48 ± 0.61
T80HL11a	50/50	1:20	0.51	61.2	1.5	126.40 ± 1.10	0.31 ± 0.08	- 3.17 ± 0.75
T80HL11b		1:30	0.34	63.0	1.0	98.60 ± 0.70	0.20 ± 0.08	- 3.15 ± 0.79

Another important parameter, the polydispersity index (PDI) of the drug-loaded formulation, was also determined and listed in Tables 1 and 2. The PDI value for all formulations ranges from 0.12 ± 0.01 to 0.36 ± 0.12, which indicates that our formulations were a homogenous population of liposome vesicles. As shown in Tables (3.1 and 3.2), it was difficult to correlate PDI value with Lumefantrine concentration as the obtained PDI value was similar for all formulations with a comparable PC/tween composition. In comparison to empty liposomes, no significant PDI difference was recognized. Still, a slight increment of 0.05 and 0.16 PDI value were noticed in the cases of surfactant-free and 50/50 formulations, respectively. This may correlate with a particle size as we obtain larger vesicle sizes on these formulations. The PDI value in all formulations was

reproducibly revealed from 0.08 to 0.40. The highest PDI value were obtained on batches T20HL11 (0.36) and T80H11 (0.40) were both in similar lipid/tween (50/50) composition. This finding also agrees with obtained particle size and size distribution result illustrated in Figure 3.4.

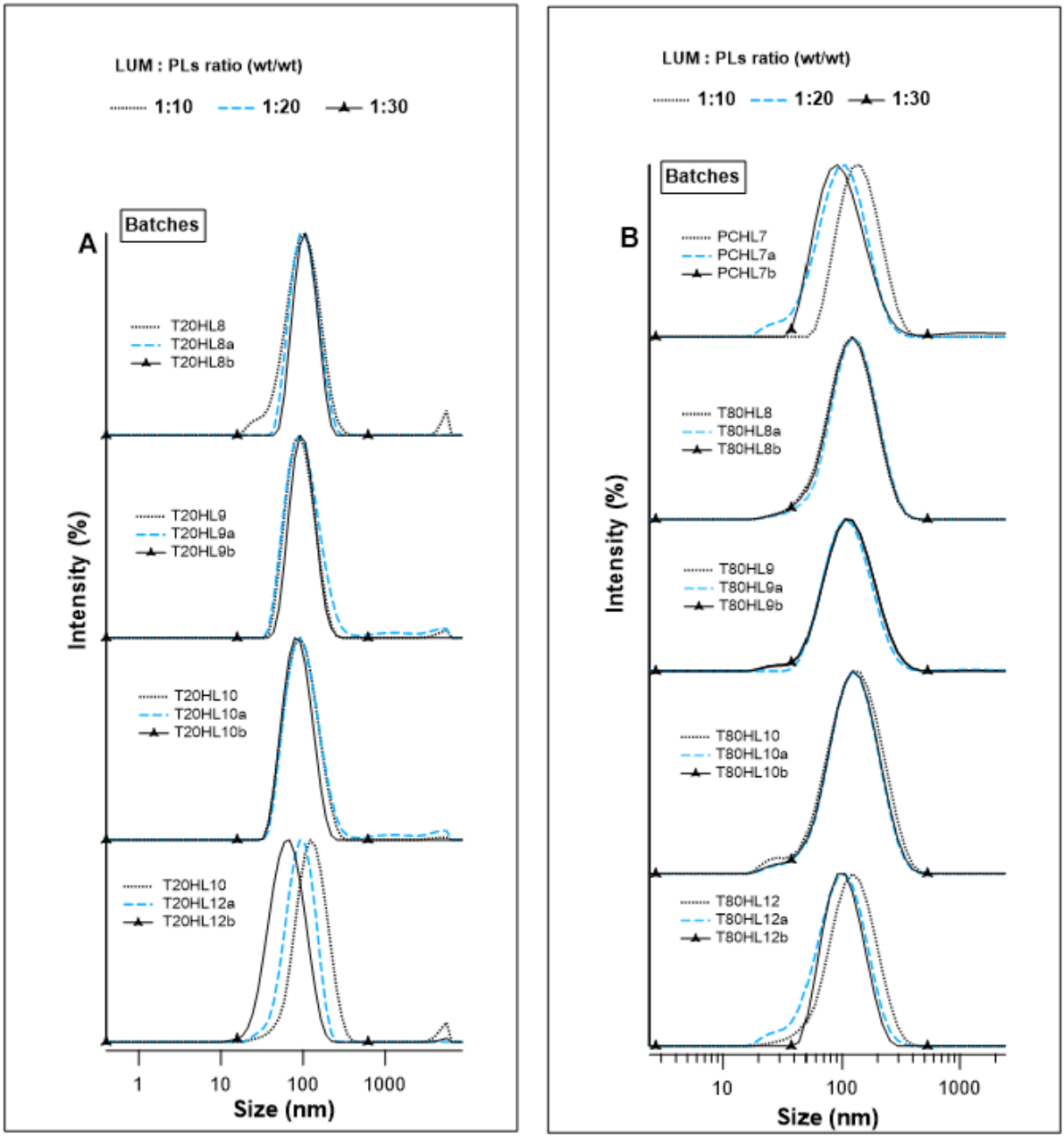


Figure 3.4: *size distribution of all LUM loaded liposome formulations . liposomes modified of T20 (A), liposomes modified of T80 and surfactant free baches(B).(n=3)*

After the encapsulation of lumefantrine, a significant shift of zeta potential was observed listed in Tables (3.1 and 3.2). ZP of the different liposome formulations was studied to determine the surface charge property of drug incorporated liposomes. For surfactant-free liposomes, the highest ZP value was around -7.48 ± 0.17 mV in a drug concentration of 2.04mg/ml, whereas the lowest ZP for both Tween-based liposome is obtained at 0.34mg/ml drug concentration were - 3.15 ± 0.79 and - 3.26 ± 0.91 mV for T20 and T80, respectively. This finding clearly expresses that higher lumefantrine concentrations had presented a more negative surface charge on the liposomes.

Compared to empty liposomes, the ZP of drug-loaded liposomes shows higher negativity. Zeta potential of empty liposome was almost neutral ranged (-2.95 ± 0.15 to -2.05 ± 0.46mV)(Fig.3.3), but with the encapsulation of lumefantrine, the ZP value was raised to a range from - 2.30 ± 0.91 to - 7.48 ± 0.17 (Table 3.1 and 3.2). We also notice that drug concentration influences the surface charge value. For instance, LUM-loaded formulations composed of only PC (at a drug concentration of 1.84mg/ml) showed an increase in negative surface charge by approximately 200% compared to empty liposomes. While at the lowest 0.34mg/ml drug concentration, the ZP showed an increase by approximately 16% and 15% in T20 and T80 modified liposome formulations, respectively. This indicates that the drug concentration impacts the surface charge of liposomes, where the surface charge of liposomes increases with increased drug concentration. Hence, the gain of a negative surface charge will improve the physical stability of the formulations by preventing their fusion and aggregation due to electrostatic repulsion.[39].

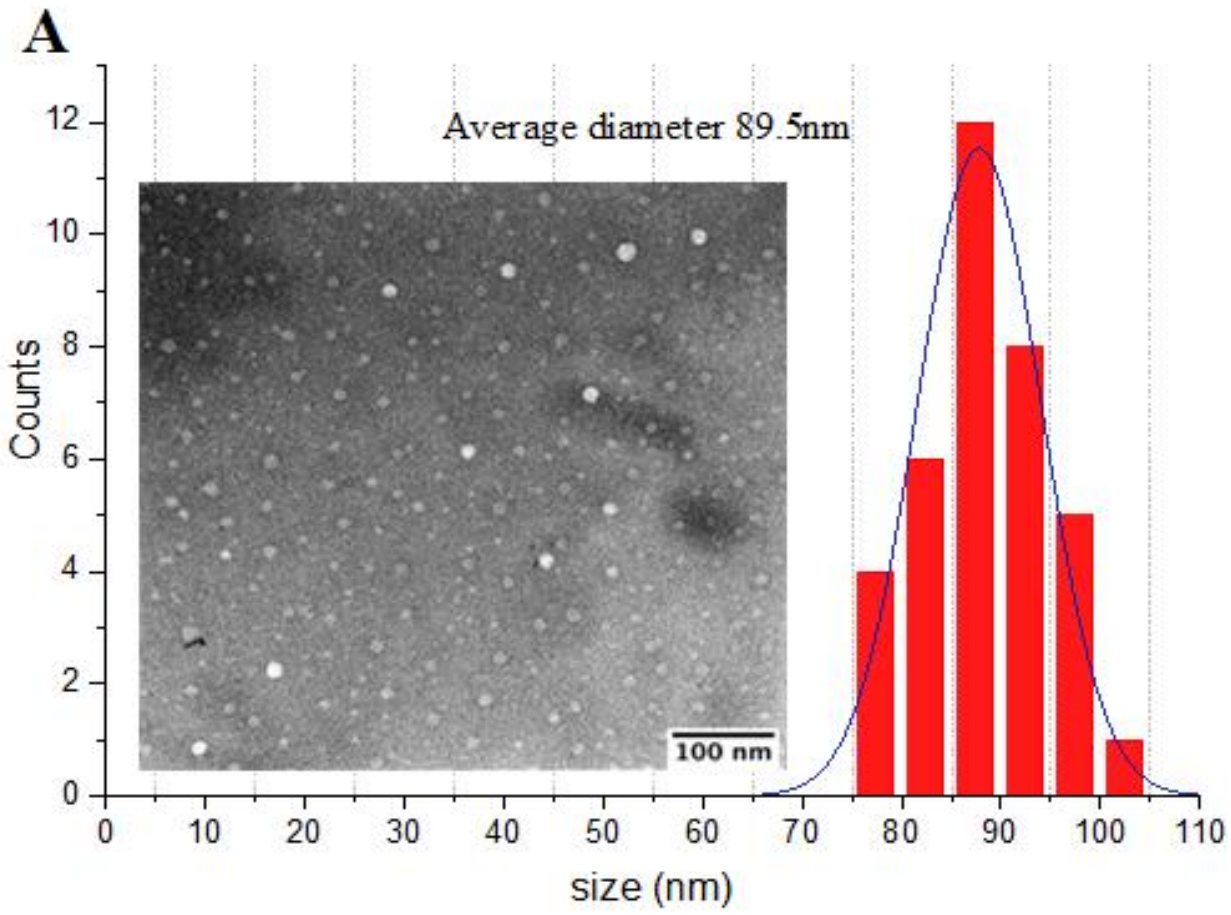
Generally, in this work, we observed that drug concentration directly affects vesicle size and surface charges of liposome formulations. Because in all formulations, we observed that an increase in drug concentration causes a corresponding increase in liposome vesicle size as well as an increase in surface charges. This finding also correlates to entrapment efficiency, where higher EE% is obtained with increasing in vesicle size.

3.3 Morphology determination

The surface morphology of the drug-loaded liposomal dispersions was examined by TEM at different magnifications (figure 3.5). In this case, the TEM image was obtained from baches T20HL8 and T80HL8 composed of 1.84mg/ml lumefantrine concentrations. The micrograph confirmed that the vesicles were spherical, mostly uni-vesicular liposomes. This indicates that bath

sonication on this method successfully converted MLVs liposomes into SUVs. According to Figures 3.5 (A and B), the mean diameter of particles perceived by TEM was approximately 89.5nm and 109nm, respectively, obtained from T20 and T80 based formulations.

However, the diameter of particles observed by TEM was relatively smaller than from particle size obtained using the DLS analyzer. Thus, vesicle size obtained from TEM analysis for batch T20HL8 and T80HL8 revealed a size decrease by 11.4% and 13.4%, compared to the DLS measurement, which was 101 and 125.9 nm, respectively. This size difference was related to the formation of the hydration of the vesicle layer throughout the liposomal dispersed in water. DLS gives the hydrodynamic size, which is the size of the nanoparticle plus the liquid layer around the particle[63], while the liposome analyzed using TEM were taken after drying on the copper grid are empty of these hydration layers.



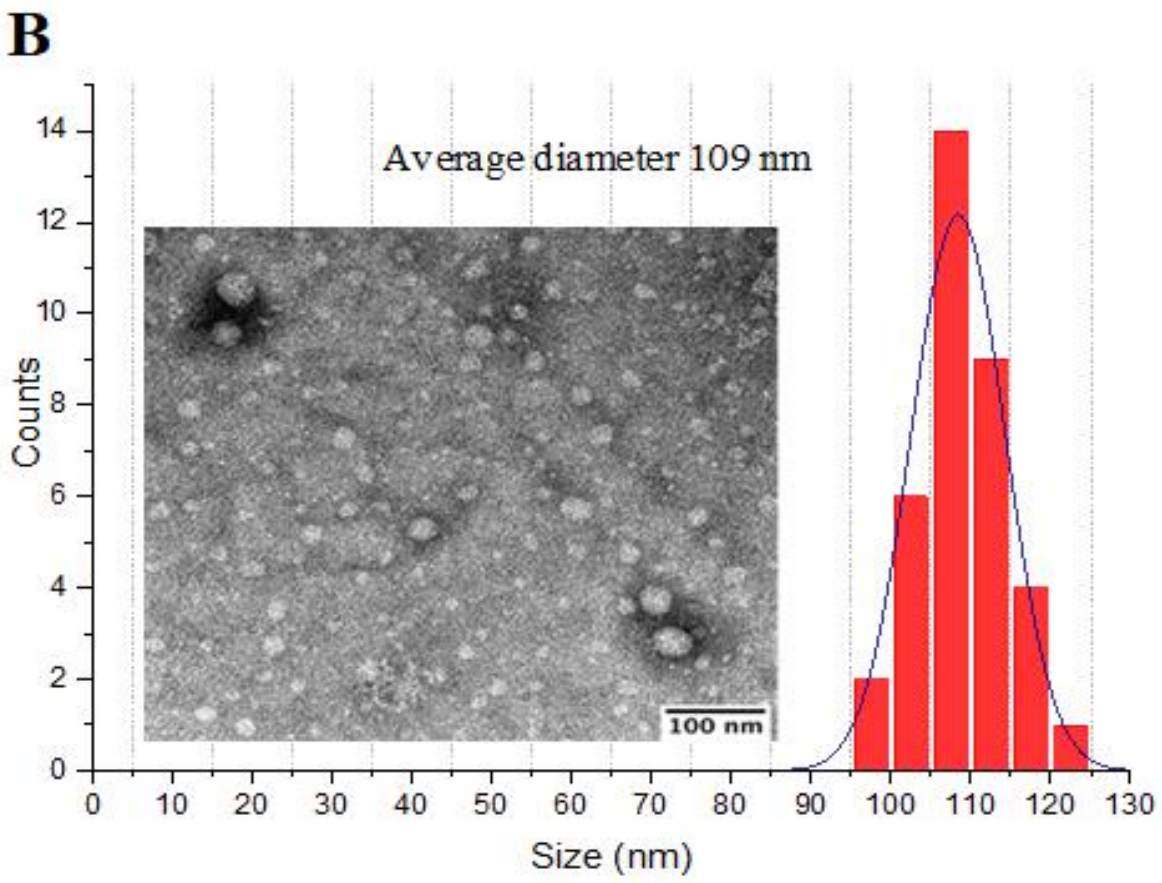


Figure 3.5. *Visualization of liposomal vesicles by transmission electron microscopy :composed of 1.84 mg/ml lumefantrine loaded liposome based on PC/T20 (A) and PC/T80(B) respectively.*

3.4 pH of liposomal formulations

The pH value of both empty and drug-loaded liposomes was determined using a pH /conductometer at 20℃. The pH of empty liposomes showed that T20 modified liposomes obtained a higher pH value compared to T80 formulations in similar PC/tween composition. This indicates pH differs among the surfactants, where Tween 80 has a lower pH value of 5-7 compared to Tween 20 with a slightly higher pH value ranged 6-8[73, 74]. The pH reading of empty T20-based liposome formulation remains slightly acidic to neutral ranging from 6.5 to 7, while T80 and surfactant-free liposomes were slightly acidic ranged from 6.80 to 7.22 and 6.20, respectively (figure 3.6). In all empty surfactant modified liposomes, pH decreases with decrease PLs concentration.

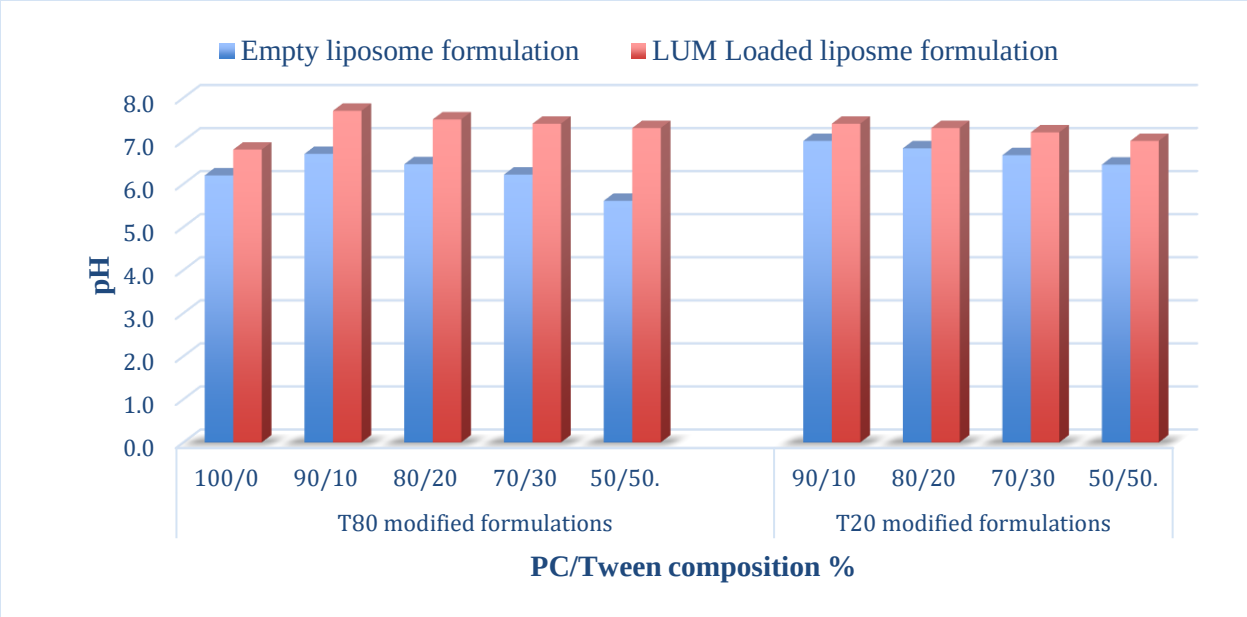


Figure 3.6: *pH value of empty and drug loaded liposome formulations. (n=3) at* 20℃

Moreover, the presence of LUM in liposome vesicles induces increases in pH of all formulation despite Lumefantrine concentrations or lipid concentrations. On the contrary to empty liposomes, the pH value of T80-modified liposomes was higher, a slightly basic with a range from 7.3 to 7.7 in a range corresponding to normal blood pH value (7.4). In contrast, the pH value of T20 modified liposomes revealed a range from (6.9-7.3) with an increase in tween concentration. This pH improvement in T80 formulations correlates to the amount of LUM as the obtained drug encapsulated efficiency percent is higher in T80 liposomes than in T20 formulations.

3.5 Standard curve preparation

A standard calibration curve for lumefantrine was prepared, and a linear relationship was plotted between peak area and concentration. Since lumefantrine is a highly lipophilic drug (logP=9.19), we dissolved it in the mobile phase acetonitrile. The regression presented a linear relationship over the concentration range of 0.0003 - 0.05 mg/mL versus the peak area. The retention time for lumefantrine obtained is around one minute, as shown in **Appendix 1**. The linear regression equation was obtained to be $Y = 10092x - 5.6979$ with a correlation coefficient R^2 of 0.9984 (Figure 3.7). The area of Lumefantrine incorporated in liposome vesicles is determined using the UHPLC technique.

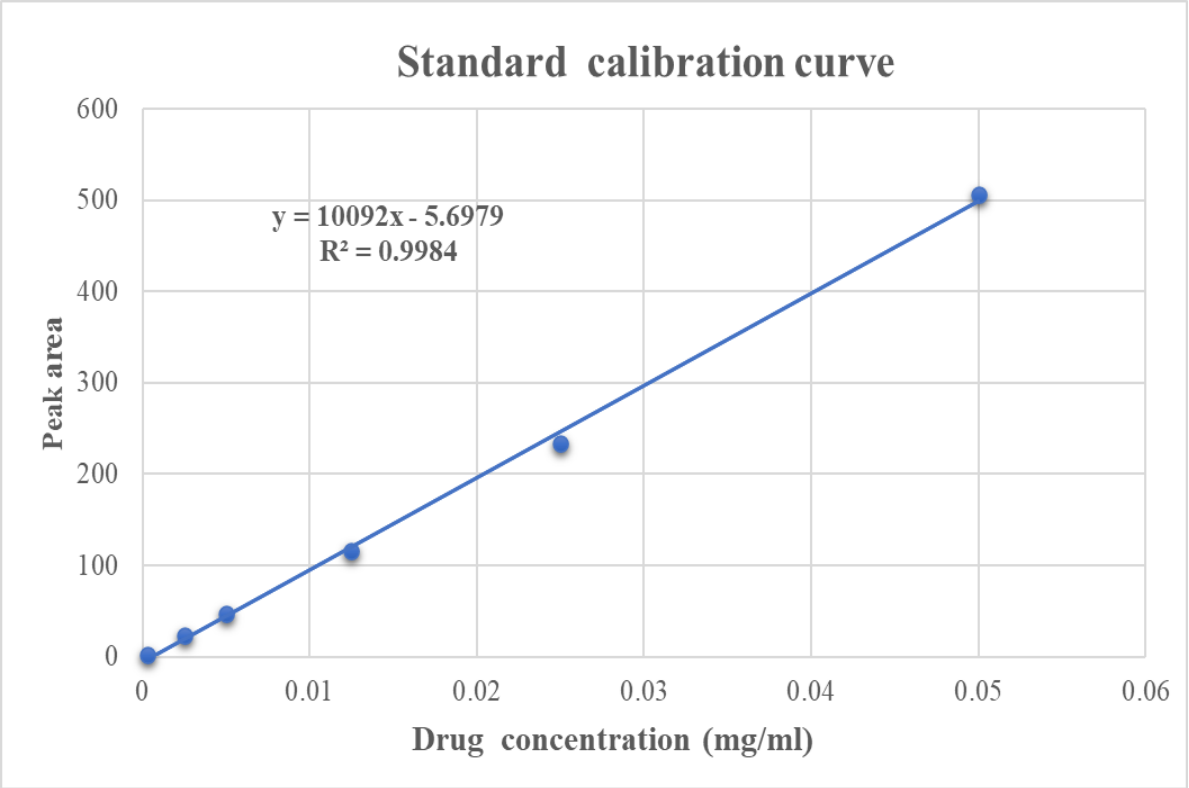


Figure 3.7*. The standard curve of Lumefantrine drug at 355 nm.*

3.6 Encapsulation and drug loading determinations

Encapsulation efficiency and drug loading capacity of Lumefantrine loaded liposomal formulation were calculated using UHPLC equipped with a UV detector at 355nm. For assuring the desired therapeutic effect, sufficient drug entrapment was required. However, the efficiency of drug entrapment in liposomes will be affected by the drug's physicochemical properties. The entrapment efficiency of liposomal formulations composed of varying concentrations of PL and surfactants were analyzed and illustrated in Figures 3.8A and 3.8B.

Figure 3.8 : *Drug entrapment efficiency of liposomal formulations.T20 modified liposome formulations(A), surfactant free and T80 modified liposome formulations (B)*

The EE% of the liposomes was significantly altered by the presence of surfactants and their drug to lipid ratio. In all formulations, regardless of their phospholipid composition, we obtain the highest entrapment efficiency at drug: lipid (1:10 ratios). According to figure 3.8B, the highest entrapment efficiency, 71.5%, was obtained from batch T80HL11 at a 1.02 mg/ml drug concentration. While, at similar drug concentration in the presence in PC/T20 samples ,68.7% of drug entrapment was obtained from batch T20HL11 (figure 3.8A). On the contrary, the liposomes in surfactant-free formulations batch PCHL7 achieved the lowest drug entrapment, 49.6%, at 2.04 mg/ml drug concentrations.

This trend could be associated with the difference in the rigidity and permeability of the formed lipid-bilayer [39]. Our phospholipid PC has a high gel to liquid-crystalline phase transition temperature at 55°C, and the liposome membrane formed from such PLs was highly rigid and less permeable causes to less entrapment of lumefantrine. In contrast, with the addition of surfactant, the liposome bilayer will get soften as the surfactant molecule inserted into the liposome phospholipid bilayer membrane leads to higher drug entrapment[75]. However, above certain concentrations, some surfactants will also increase vesicle membrane fluidity and permeability caused for easy leakage of incorporated drug molecules. A similar report by *Jain et al*. and *Chaudhary, Kohli et al.* revealed that drug entrapment efficiency decreased with an increase in surfactant concentration [76, 77].

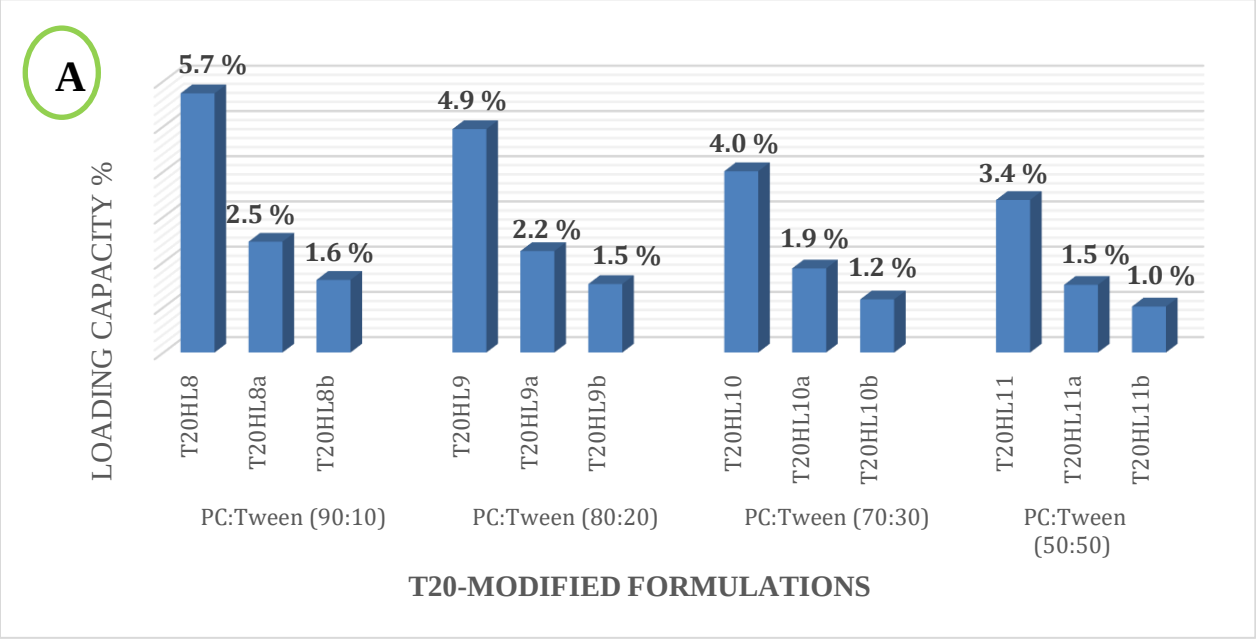
Conversely, the highest lumefantrine efficiency using high concentration surfactant in lipid/surfactant ratio (50/50) was 68.7% and 71.5 % using T20 and T80 presented in figure 3.8(A and B), respectively. This could correspond to the vesicle size increases due to an accumulation of vesicles. Studies explicated that increasing surfactant concentration will enhance the number of vesicles formed, leading to an increase in the volume of the hydrophobic bilayer region available to house a hydrophobic drug[39].

Compared to the entrapment efficiency of formulations based on tween(20 and 80), T80 liposomes were generally higher than those in T20 liposomes. For instance, in drug/lipid ratio of (1:10) at a drug concentration of (1.84, 1.63, 1.43, and 1.02) mg/ml, the entrapment efficiency of the T80 was higher by (4.3%, 3.6 %, 5.3% and 4.1%) respectively. This entrapment difference among the

two surfactants may be attributed to the earlier described differences in surfactant nature, such as carbon chain length and HLB.

Some reports revealed that surfactants with a longer carbon chain were able to enhance the solubility of a lipophilic drug in the lipid bilayer, Consequently, the drug entrapment efficiency of will increases[39]. In our case, the hydrophobic tail for T80 was almost twice longer than T20 (C18 vs. C12). Thus, at equivalent PC/tween molar ratio, drug entrapment in liposomes based on T80 revealed relatively higher than T20-modified formulations, which was associated with their carbon chain difference. Finding by *Shoukry et al*. also showed that the drug entrapment efficiency comparison using tween (20 and 80) was relatively lower EE% while using tween 20 compared to tween 80[78].

Another factor for the entrapment difference between the two surfactant-based formulations could be the difference in HBL value. However, surfactant HLB value influence on drug encapsulation still depends on the lipophilicity of the drug molecule. Several literatures reported that using surfactants with high HLB value enhanced encapsulation efficiencies of hydrophilic drug, whereas surfactants with low HBL value improves entrapment of lipophilic drug molecules[39, 78]. Thus, our result can be justified as lumefantrine was highly lipophilic (log P=9.19) and was expected to achieve higher drug entrapment using tween 80.



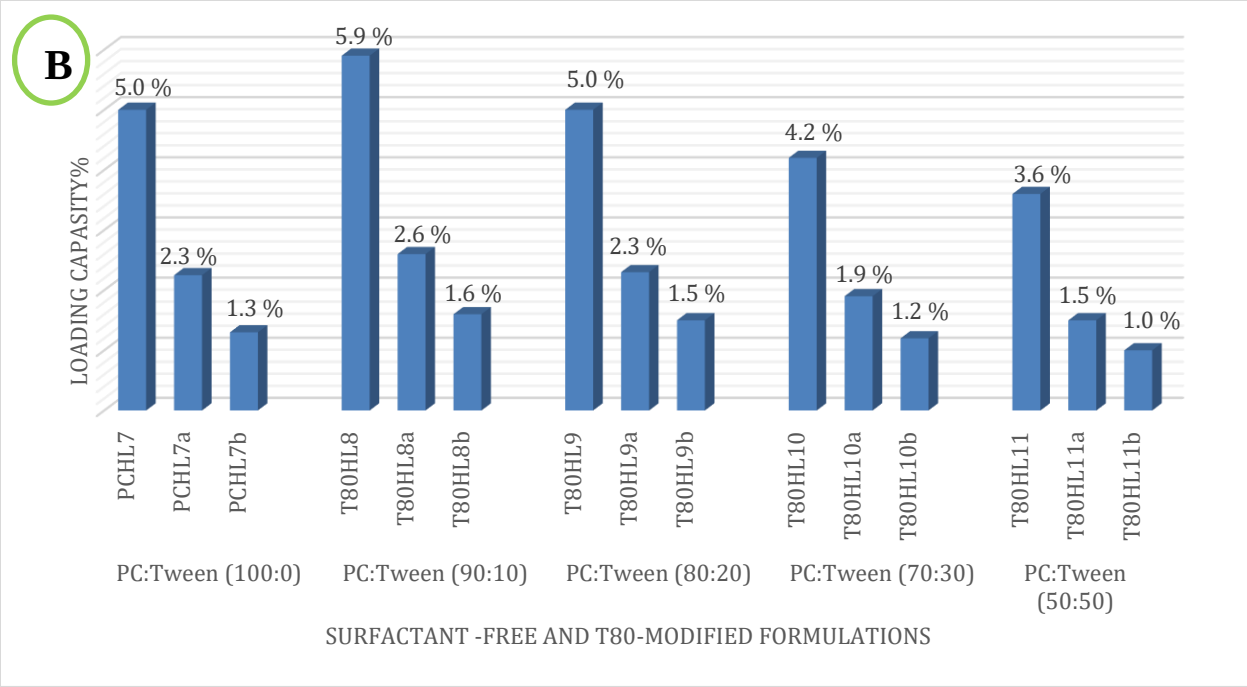


Figure 3.9*: Drug loading capacity of drug loaded formulations; T20 modified liposome formulations (A), surfactant free and T80 modified liposome formulations (B)*

Another critical parameter in the development of nanoparticle drug delivery systems is the loading capacity efficiency of the drug. The loading capacity (LC) of a drug is described as incorporating the drug molecule into the liposomes. The obtained LC% of all formulations ranges from 1 to 5.9%, illustrated in Figures 3.9 (A and B). The highest drug loading percent of lumefantrine was in formulations on drug /lipid ratio(1:10) ratio where Batch T80HL11 revealed 5.9%, followed by T20HL11 and PCHL7, with 5.7 and 5.0%, respectively. The low drug loading can be attributed to several factors, including the carrier and drug properties, size and chemical interaction between the drug and carrier[79]. In our case, it is clearly shown that the LC is dependent on liposome constituent and drug concentration.

The result showed that loading capacity decreases with a decrease in PLs and drug concentration. This could be correlated to the lipophilic drug nature as the surfactant amount increases; the vesicle membrane bilayer will be softened and high permeable, ending in significant leakage of the drug to the external aqueous phase during the production process. Another essential factor that commonly helps to explain the drug LC could be the influence of vesicle size. As the vesicle size of the nanocarrier increases, the entrapped volume increases[80]. These finding agree with our

results where formulation composed of equivalent PC/Tween composition but in different drug ratio revealed a slightly larger LC% from those larger sized formulations than the corresponding small sized vesicles.

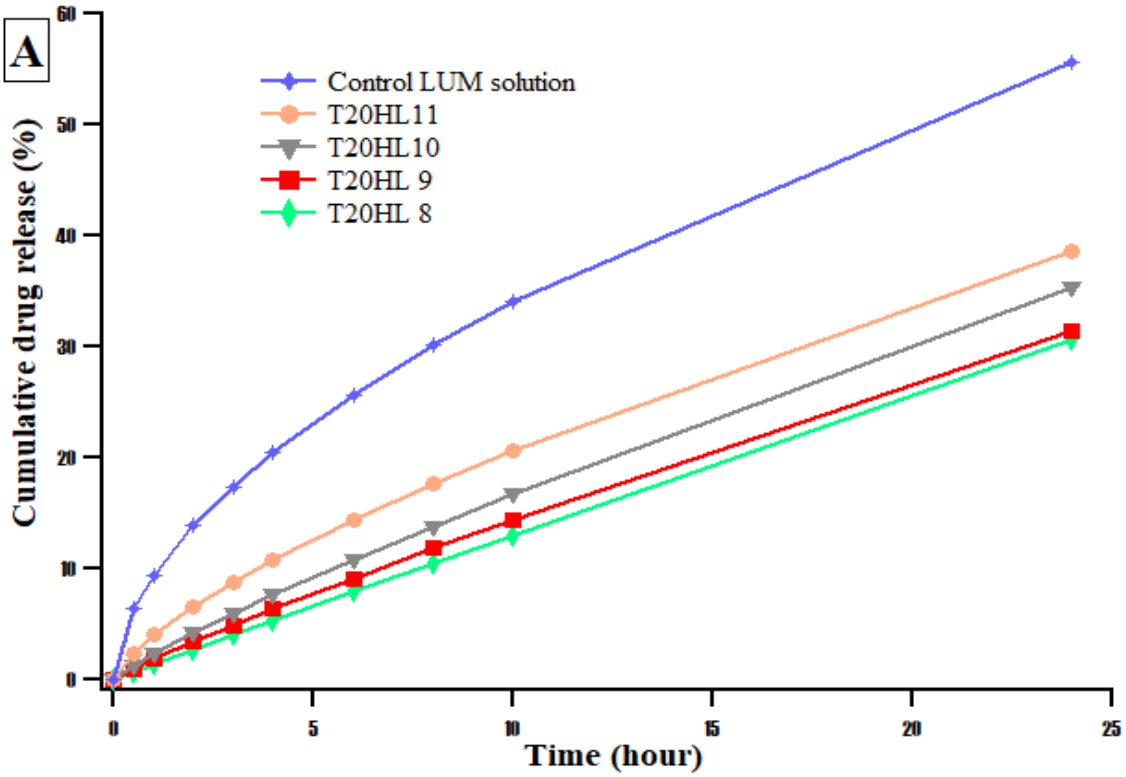
Overall, the most promising formulations regarding drug EE% and LC% were batch T80HL11 and T20HL11. Both displayed significantly higher lumefantrine encapsulation efficiency and loading capacity compared to the other liposome formulations presented in Tables (3.1 and 3.2). Also, both formulations contain 1.02 mg/ml lumefantrine encoded in (50/50)% PC/tween compositions.

After drug loading analysis, our next focus was to determine the entrapped drug release and to do stability studies. Therefore, since formulations with drug/lipid ratio (1:10) showed higher lumefantrine encapsulation efficiency than the other formulations displayed in Tables (3.1 &3.2) these formulations containing 1:20 and 1:30 drug/PC were withdrawn from further analyses. Hence formulations containing 1:10 drug /PC were chosen for further drug release, freeze-drying, and stability studies. Additionally, empty liposomes with similar PC/Tween composition as LUM loaded liposomes were studied for physical stability

3.7 In vitro release study of lumefantrine

The cumulative percentages of lumefantrine released from liposome were conducted in vitro over a period of 24 hours using the dialysis bag (molecular weight cut-off: 20 000 kD) containing the appropriate volume of lumefantrine-loaded liposome formulations. The *in vitro* drug release was carried out according to *Panwar, P., et al.*[62], method first. However, due to the high lipophilic nature of LUM, we encountered difficulties in determining the *in vitro* release of lumefantrine using this method. Therefore, the method was improved with some modifications. Dialysis bag was placed in a flask containing 25ml of release medium of glycol (7%), methanol 35%, and PBS 58% at pH 7.55. The obtained experimental data were analyzed by nonlinear regression by *Ritger-Peppas*.The cumulative DR% is illustrated in Figure 3.10 (A and B) for both T20 and T80 modified liposomes, respectively.

The release profile of LUM from the T20-liposome (Figure 3.10A) revealed that the drug molecules appeared to be released in a sustained release pattern. During the initial half hour, a very slow release of lumefantrine was observed in all Tween 20 based batches. Approximately (0.7, 1.0,1.3 and 2.4 %) of the drug is released from batches T20HL8, T20HL9, T20HL10 and T20HL11 respectively. However, with time the drug release increases gradually, and the release rate was continuously increased up to 24 hours. Our findings showed that the release rate increases with surfactant concentration. The formulations at lipid/tween composition (50:50) had the faster and highest drug release than lipid/tween composition (90:10). Of all T20-based formulation, T20HL11 has the highest drug release, 38.6%, followed by T20HL10 (35.3%), T20HL9 (31.4%), and batch T20HL8 30.7%, where these were composed of lipid/tween (50/50), (70/30), (80/20) and (90/10) respectively. This indicates that release rate is dependent on the surfactant concentration.



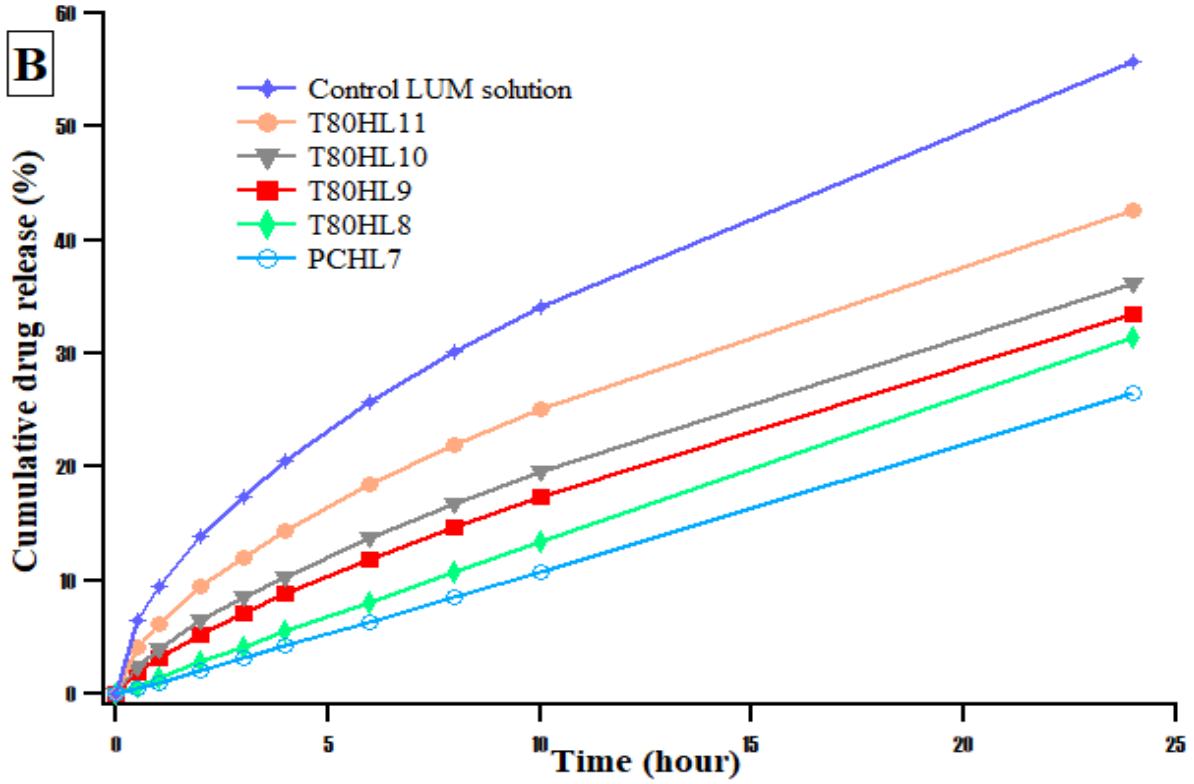


Figure 3.10: *In vitro release profiles of Lumefantrine liposomal formulations. In a release medium containing mixtures of glycol (7%), methanol 35%, and PBS 58% at 37± 0.2℃. LUM release from T20-modified liposomes (A), release from. Surfactant free T20 and T80-modified liposomal (B)*

Figure 3.10B also shows the release profile of lumefantrine from the T80-liposome and surfactant-free formulation. The highest cumulative drug release in 24 hours were obtained for T80HL11 (42.7%), followed by T80HL10 (36.1%,), T80HL9 (33.4%) and T80HL8 (31.5%). In contrast the lowest release, approximately 26.6% were obtained from surfactant free PCHL7. This slow release from only PC based formulation could correlate to phospholipids rigidity, where drug molecules present in the core of the liposomes took time to diffuse out from the internal core region to the outer releasing medium[39].Additionally, drug release is also affected by vesicle size. Thus, smaller particles can release their entrapped content fast. This is due to the fact that smaller particles have a larger surface area; consequently, most of the drug-associated particles would be near the particle surface, driving for fast drug release. In contrast, larger particles had large cores, which provide a slow diffuse of encapsulated drug [81]. This thought agrees with our result where batch PCHL7 had the biggest particle size 138.90± 0.62 (Table 3.2) and revealed the least DR%.

Studies suggest that the presence of surfactant in a formulation could affect both pharmacokinetics and pharmacodynamics properties of lipid-based delivery systems, such as enhancing drug release, circulation time, and cellular uptake [39]. Also, in this study, we observe that surfactant-based liposomes, despite their surfactant concentration, have slightly higher drug release than surfactant-free formulation. This may be attributed to the fact that surfactants are highly soluble in the aqueous medium and gives an additive solubility effect for lumefantrine.

Another important observation from the study was that the rate and extent of lumefantrine release from T80-based liposomes were higher than in both surfactant-free and T20-based formulations. The reason behind this could relate to the previously described nature and characteristic differences among the two surfactants. Previous studies suggest that parameters such as carbon chain length and transition temperature (Tc) also impact drug release and drug entrapment efficiency [39, 82]. Drug release increases with an increase in the surfactant concentration, carbon-chain length, and decreased Tc.

Although each surfactant shares a similar head group, T80 is composed of long-chain fatty acids in comparison to T20. Thus, the long C-chain of T80 will be more disruptive upon insertion into the liposome bilayer than the T20 tail [72].Consequently, a more significant release effect of encapsulated drug formed based on T80 was obtained. In contrast, T20 had a shorter carbon chain has effect on the later release of encapsulated drugs.

Moreover, the phase transition temperature (Tc) difference among the T20 and T80 could also be another reason for the release rate difference as Tc impacts the rigidity and permeability of the formed vesicle bilayer. T20 possesses a higher Tc (76℃) [74], and forms less permeable vesicle bilayer. In contrast, T80 possesses a TC of (65℃) [83] and forms less permeable vesicle bilayer. In contrast, T80 possesses a TC of (65℃) [80] and forms a more permeable bilayer than T20-based formulations. This correlates significantly with our findings, where the drug release rate of T80 formulations is higher than T20 formulations at the lipid /tween ratio. A similar result was revealed in other studies, where the release rate of Noisome formulations prepared using various surfactant Span (20, 40, 60 and 80) showed that higher drug release was obtained from Span 20 and Span 80 as they possess lower Tc and forms a slightly higher permeable vesicle compared to Span 40 and Span 60) [82].

In summary, the release rate for all liposomal formulations, regardless of their lipid or surfactant composition, is slightly low compared to Lumefantrine solution control. Compared to control, the DR% of T80 based formulation is lowered by 26%, 35%, 39%, and 43% from lower to higher surfactant concentration. Similarly, when using T20 formulation showed a decrease by 31%, 35%, 43% and 44%. The sustained effect could be due to the presence of the drug in the core of the liposomes [39], which takes time to diffuse out from the internal core region to the outer releasing medium. Also, the low release of LUM could be related to the drug solubility characteristics as LUM is a highly lipophilic drug (log P=9.19) where it will stay within the hydrophobic core of liposomes instead of releasing to the hydrophilic media [63]. This sustained drug release pattern will strongly indicate that Lumefantrine will take time to release from the nanocarrier, and therefore it will be available in the circulation system for a prolonged time. Consequently, the slow release will be advantageous in the clearance of the remaining parasite. Nevertheless, there are some drawbacks as it cannot have a rapid effect on malarial parasite eradication.

The sustained release of LUM can be associated with the trap of LUM in the liposome core. Accordingly, the obtained liposomal formulations can be considered as highly attractive nano-carriers for prolonged drug delivery for hydrophobic drugs to achieve different therapeutic objectives. For a general drug delivery, it is desirable that liposomes should stay in blood for long time [84]. Thus, sterically stabilized liposomes produced by incorporating a non-ionic surfactant have also a significant role to improve liposomal stability and enhances their circulation times in the blood. The establishment of a steric barrier improves the efficacy of encapsulated agents by reducing in vivo opsonization with serum components, and the rapid recognition and uptake by the RES [85].

Additionally, as clearance is inversely related to the particle size, SUVs liposomes are prone to enhance for the longest blood circulation [35]. In this case, all our formulations are small-sized vesicles liposome with a mean particle size range of 57-139nm (Tables 3.1 and 3.2). This indicates that these formulations were convenient for *i.v* injection as they had a chance to escape the RES resulting in prolonged blood circulation and reach the target site. Moreover, the slow or prolonged lumefantrine release also had potential benefits, including reducing the dosing frequency, improving patient compliance, and enhancing therapeutic efficiency.

3.7.1 The lumefantrine release kinetics

The release mechanism of lumefantrine were studied by *Ritger-Peppas* kinetic model, also known as power law[86]. The model parameters were calculated using the nonlinear regression method (Table 3.3), and these parameters were further used to find the fit model of the experimental data using the empirical transport equation (5).

$$\frac{M_t}{M_\infty} = kt^n \qquad \text{Equation } \mathbf{5}$$

Where M_t *and* M_∞ are the cumulative drug release at time t and infinite time,respectively. Parameter n is the diffusional exponent, which indicates the drug release mechanism, and the value of k symbolizes the drug release rate from the nanocarrier.

According *to Ritger and Peppas*, the drug release mechanism is ruled by the Fickian diffusion-controlled release when n = 0.5, and at n = 1.0, partition-controlled drug release, and when the value of n between 0.5-0.85 anomalous (non-Fickian) transport. The release constant (k) is directly proportional to the diffusion constant and hence depends on the physical and structural properties of both the drug and the carrier[87].

Table 3.3: Values of kinetic estimated parameters for the prepared lumefantrine loaded liposome formulations

	Parameters for *Ritger-Peppas* kinetic model	
Formulations	**n**	**k**
T20HL8	0.981	1.358
T20HL9	0.889	1.860
T20HL10	0.841	2.317
T20HL11	0.714	3.995
PCHL7	1.034	0.994
T80HL8	0.977	1.410
T80HL9	0.746	3.125
T80HL10	0.697	3.936
T80HL11	0.608	6.175
Control	0.538	9.442

According to Table 3.3, the highest k value was obtained from batch T20HL11 and T80HL11 which both formulations have a higher release rate, while the lowest k value was from surfactant

free formulation PCHL7. According to the table the k value increases with increase in surfactant concentration.

Overall, the lumefantrine release fitted using nonlinear regression hints a partition-controlled release for batches with a high n value (0.85-1) where the drug partitions between the lipid and release media. Then, the drug diffuses out of the dialysis bag [88]. While formulations with n value ($0.5 < n < 0.85$) hints an anomalous transport mechanism. In contrast, when the value of n is around 0.5, a diffusion-controlled drug mechanism carried by random molecular motion associated with forces such as concentration gradient.

3.8 Stability study of Drug-loaded and unloaded liposomes

The Liposomes stability is another crucial factor to the drug delivery system's efficacy as drug retention and releases determine the entrapped drug release amount and time[89]. Hence, we evaluate the stability studies for both empty and drug loaded liposomes. To determine physical stability and evaluate the shelf life, the samples were stored at dark 4℃ refrigerator for two months. The physical stability of liposomal formulations was characterized by determining vesicle Size, PDI, ZP and pH at day 1, 30 and 60. Liposomal formulations were also visually inspected after formulation to evaluate their colour and consistency difference during storage conditions. Chemical stability of LUM loaded liposome was also performed by lyophilization process.

3.8.1 Physical stability of liposomal formulations

3.8.1.1 Visual appearance stability

The visual appearance of freshly prepared unloaded liposome dispersion ranges from concentrated milky to slightly bluish shade, decreasing the PL composition and particle size. The bluish shade turbidity indicates that the liposome samples were homogenous[17]. However, after one month of storage, the turbidity appearance showed an insignificant decrease. In contrast, by the end of the second month, a very significant color change was observed. Liposome formulations without surfactant showed phase separation were a flat gray color on the top and aggregate on the bottom. In contrast, no aggregate formation was observed in Surfactants-based formulation. However, appearance changed from very light milky to an almost water-colored solution with increasing surfactant concentration were noticed.

The visual appearance of freshly optimized liposome formulations for drug loaded liposomes varied from dark yellow to orange color regarding surfactant concentration. Formulations without surfactant PCHL7 obtain a very dark yellow color. In tween-based formulations, the color varied from dark yellow to slightly yellow color with increased surfactant concentrations. However, no significant color change was noticed among T20 and T80 formulations. Appendix II displays pictures of T80 based formulations stored at 4℃ for two months.

By the end of the first month, all the liposomal formulations were relatively stable physically. There was no significant appearance change in all formulations. However, by the end of the second month, a color change had been observed. LUM loaded liposomes turned their color from dense yellow to very light-yellow color in all formulations despite their compositions. Formulations composed of the highest surfactant concentration become a lighter yellow color in comparison to other batches. Appendix II presents a picture obtained for only lipid and T80 based formulation. Furthermore, in T20 liposomal formulations, similar changes as in T80 liposomes were also observed.

Moreover, only surfactant-free formulation batch PCHL7 showed a slight separation, and a yellow-free drug was settled at the bottom of the glass. This occurrence correlates with particle size analysis which showed an increment by approximately 58.2 % from the initial measure 139.90 ± 0.50nm to 221.30 ± 5.62 nm after two months. This finding proves the role of surfactant as an excellent stabilizer against aggregations in liposomal formulation[23].

3.8.1.2 The particle size and PDI determination

The characteristics change of lumefantrine loaded liposomes in terms of mean vesicle size, PDI, and Zeta potential values for two months storage are listed in Table 3.4. The obtained result showed a slight but not notable vesicle size change in the first month. This increment in particle size was higher for surfactant-free liposomes batch PCL7 and batches T80LH12 and T20HL12, where both formulations are composed of (50/50) lipid/tween ratio. The vesicle size increase was around 4-16%. However, by the end of the second month, further size increments in all were observed. Batch T20HL11 performs extraordinary vesicle enlargement by over 400% from the initial measure, followed by batch T20HL10, T20HL9 and T20HL8, and vesicle size batch rises by approximately

(80, 64, and 35) %, respectively. This associates to the fact smaller vesicle size liposomes are prone to fusion and aggregation [39].

Table 3.4: Evaluation of physical stability of Lumefantrine loaded liposomes stored at 4°C for two months

	AFTER 24 HOURS			AFTER 1 MONTH			AFTER 2 MONTHS		
Batches	Size (nm)	PDI	ZP(mV)	Size (nm)	PDI	ZP (mV)	Size (nm)	PDI	ZP(mV)
T20HL8	92.76	0.21	-6.06	101.00	0.08	-5.10	125.54	0.16	-4.75
T20HL9	75.5	0.18	-5.18	95.49	0.08	-4.18	124.10	0.23	-3.99
T20HL10	73.25	0.13	-2.56	87.58	0.16	-3.82	132.30*	0.44	-2.23
T20HL11	71.48	0.23	-2.46	81.56	0.29	-2.22	357.57**	0.56	-1.20*
PCHL7	139.89	0.31	-7.48	157.10	0.37	-5.52	221.30*	0.42	-3.72*
T80HL8	95.65	0.23	-6.92	103.10	0.29	-5.83	116.50	0.33	-4.75
T80HL9	89.88	0.24	-5.61	101.81	0.31	-5.11	124.10	0.35	-3.99
T80HL10	84.22	0.26	-4.08	89.91	0.36	-3.80	132.30*	0.44	-2.93
T80HL11	76.02	0.28	-3.77	79.47	0.39	-3.38	455.55**	0.56	-1.02*

* Increase by over 50% ** Increase by over 100% from initial value

Thus, variation in particle size could be recognized on the plot of size particle distribution (SPD) illustrated in (Figure 3.12). From the figure , we perceive that after two-month storage all batches but particularly formulation with lipid/tween composition (50/50) and surfactant free formulation showed a broad peak which indicates a poly-dispersed particle compared to the initial SPD value. This is highly correlated with particle size increment, especially in the two last-mentioned baches, as both increase particle size by over 100% each. Thus, physical instability in liposomal vesicle size changes occurs due to aggregation/fusion and loss of entrapped drug due to leakage[89].

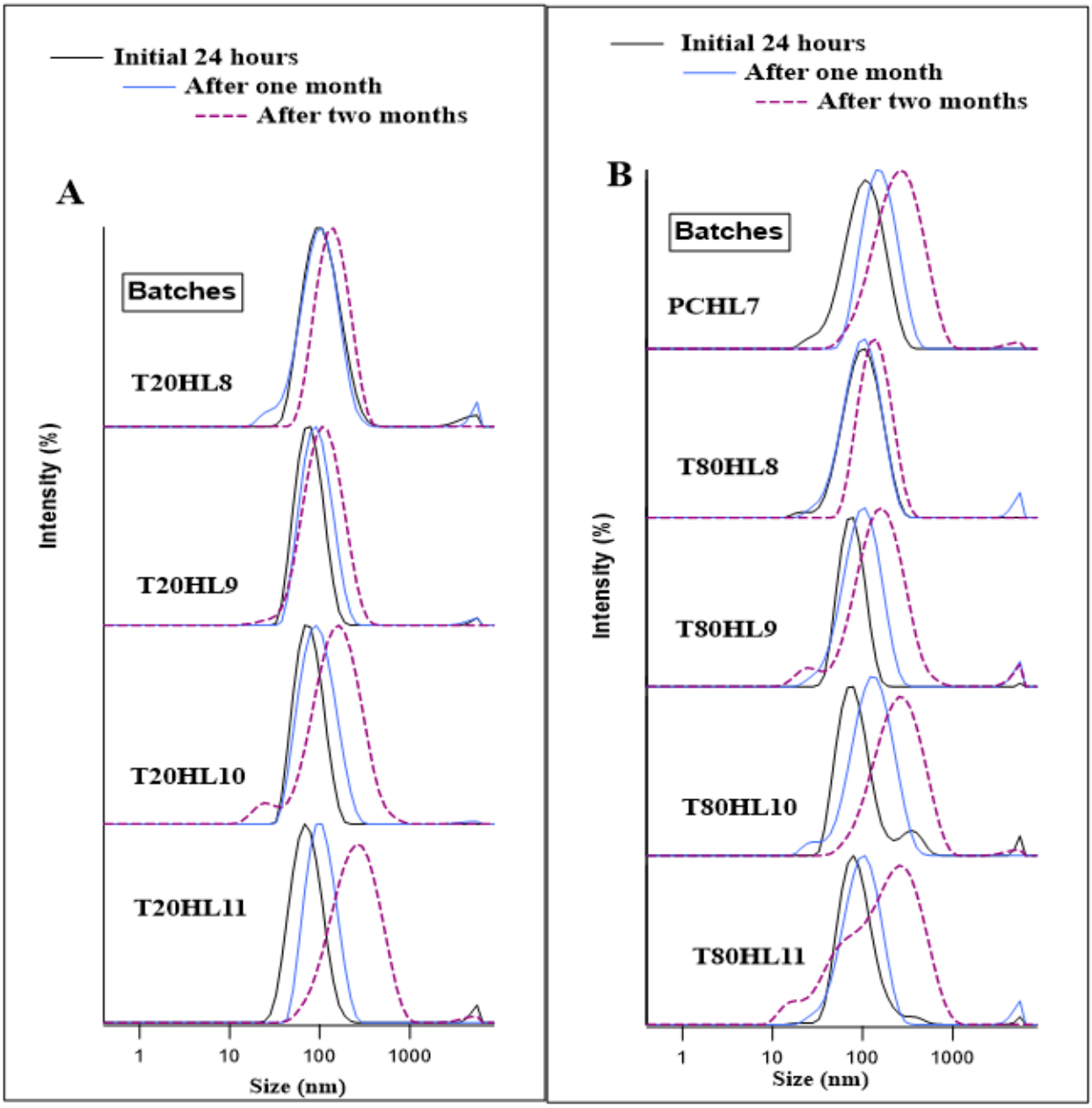


Figure 3.12*:* Stability size distribution of LUM loaded liposome formulations :*stored at* 4℃ *for two months (n=3)*

The particle size, PDI, and ZP of unloaded liposomes after two months of storage are also determined (Table 3.5). Similarly, as drug-loaded liposomes by the end of the first month, no significant change was noticed in all formulations. While the measure after two months revealed that mean vesicle size increases in all formulations, particularly in batches composed with only

PC and PC/tween 70/30 and 50/50. PDI value also shows an increase which associates with vesicle size increase.

Table 3.5*: Evaluation of Size, ZP and PDI stability of empty liposomal formulation stored at 4°C for two months*

	AFTER 24 HOURS			AFTER 1 MONTH			AFTER 2 MONTHS		
Batches	Size (nm)	PDI	ZP(mV)	Size (nm)	PDI	ZP (mV)	Size (nm)	PDI	ZP(mV)
PC98T20H8	91.1	0.3	-4.52	97.1	0.35	-3.86	106.31	0.33	-3.12
PC98T20H9	89.3	0.24	-3.86	95.3	0.3	-3.42	109.1	0.35	-3.02
PC98T20H10	79.11	0.22	-3.83	82.4	0.25	-4.24	117.90*	0.44	-1.03
PC98T20H11	48	0.19	-2.74	76.2	0.22	-3.24	348.13**	0.56	-0.14*
PC98H7	130	0.30	-5.9	116	0.28	-5.71	194.10*	0.49	-2.42*
PC98T80H8	98	0.32	-5.91	109	0.11	-5.86	116.3	0.33	-3.35
PC98T80H9	94	0.26	-4.79	108	0.16	-4.66	119.1	0.35	-3.29
PC98T80H10	81	0.24	-4.65	82.4	0.37	-4.31	127.60*	0.44	-2.03
PCT9880H11	74	0.22	-3.57	125	0.35	-3.41	315.43**	0.56	-0.52*

* Increase by over 50% ** Increase by over 100% from initial value

Zeta potential is a parameter usually used for the determination of dispersion stability[89]. ZP values of both drugs loaded, and unloaded liposomes stored for two months were also analysed listed in Tables (3.4 and 3.5). All formulations were remined in negative surface charge. But a slight increase towards zero is observed with first month. After the second month storage the ZP value of all formulations were increased toward zero but in particularly formulations composed of high surfactant concentration achieved a higher increase by approximately 50% which indicates instability of the formulations. Thus, the lower ZP value may correlates to the fact that the charge on lipids was an important parameter influencing liposomal behaviour[89]. In this way, the high surface potential might contribute to liposome physical stability by reducing the rate of aggregation and fusion of liposomes during storage. Thus, it was well in agreement with the observed size and PDI increase of formulation.

3.8.1.3 pH stability of liposomal formulations

The pH of liposomal formulations stored at 4℃ for two months was also analysed and plotted as shown in Figures (3.13 and 3.14) both for drug loaded and empty liposome, respectively. The pH measurement was performed to ascertain the pH stability of liposomal formulations with time. pH

measurement after one month shows a significant decrease to slightly acidic compare to the initial measure, which was in neutral to slightly basic condition. However, after two months of storage, the pH range reduces gradually in all formulations regardless of either drug or PLs composition. This may relate to the visual appearance of the formulations. The turbidity of liposomal formulations decreases with time, which indicates probably precipitation of drug or liposome constituents. Additionally, as liposomes are in an aqueous form, there are possible tendency related to phenomena of oxidation and hydrolysis of lipids and drug [90].

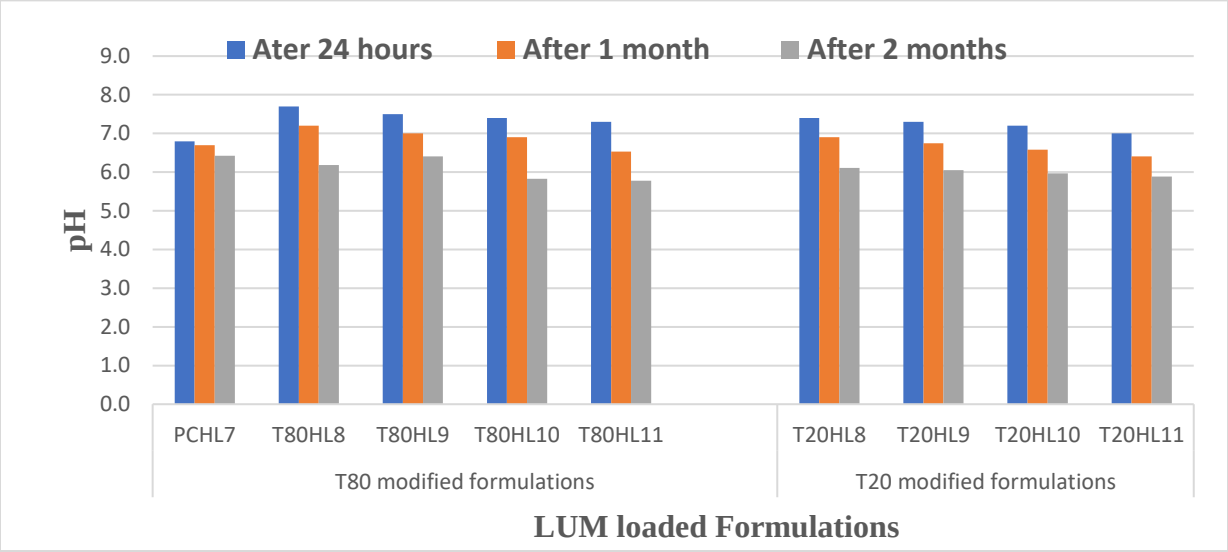


Figure 3.13 **:** *pH stability of drug loaded liposomal formulations; stored at 4℃ for 2 two months (n=3)*

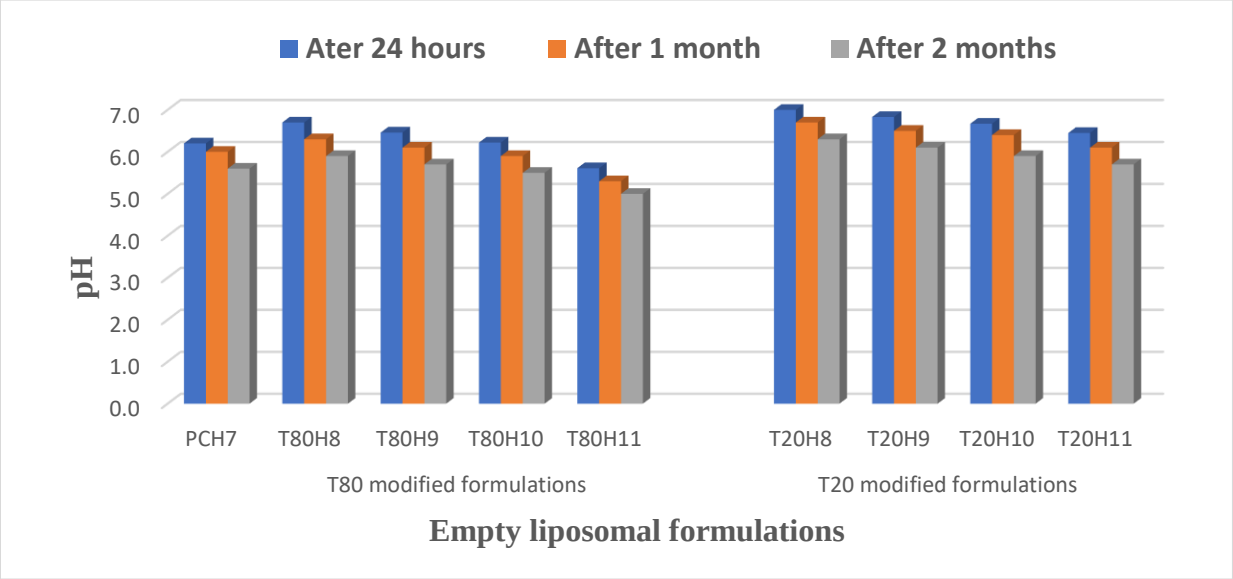


Figure 3.14: *pH stability of unloaded liposomal formulations; stored at 4℃ for 2 two months (n=3)*

3.8.2 Lyophilization process

Liposomal formulations in an aqueous form are a relatively unstable colloidal system. Consequently, there are several challenges like degradation by oxidation or hydrolysis and aggregation or fusion of liposomes during storage[10].Therefore studies suggest that such instability dilemmas can be resolved by storing the liposomal formulation in a dry state by the lyophilization process[25]. In our study, selected LUM loaded samples were freeze-dried without the addition of cryoprotectants, and the dry lyophilized cake was reconstituted with water.

The impact of lyophilization process on the stability of liposomes was determined by comparing the mean vesicle size, PDI, and encapsulation efficiency before and after freeze-drying, as shown in Tables 3.6Thus, the result revealed that the freezing-drying process has a small effect on the liposome vesicle size, leads to a slight increase in the average size. After freeze-drying, the mean vesicle size ranged from 93 to 109 nm, displaying a relative size increment compared to the mean vesicle size before freeze-drying, which was in a range from 70-92nm.Particularly formulation on batch, T20HL11 added a very size increment by 32% compared to freshly made liposomes (Table 3.6).

Table 3.6 : Effect of lyophilization on the encapsulation efficiency (EE%) , loading capacity (LC%), particle size, PDI and ZP of drug loaded liposomal formulations

Batches	EE%	DL%	Size (nm)	PDI	ZP (mV)	EE%	LC%	Size(nm)	PDI	ZP (mV)
T20HL8	62	6.2	92.76	0.2	-6.06	54.2	5.4	109.50 *	0.1	-3.11
T20HL9	61	6.1	75.5	0.2	-5.18	52	5.2	99.10 *	0.2	-2.48
T20HL10	58.1	5.8	72.25	0.1	-4.18	51.2	5.1	93.93 *	0.2	-1.02
T20HL11	66.1	6.7	70.48	0.2	-2.58	55.2*	5.6	93.15*	0.1	-0.61
PCHL7	48.4	4.8	115	0.4	-7.5	39.3*	3.9	119.5	0.3	-3.7
T80HL8	65.5	6.5	92.7	0.3	-6.9	56.4	5.6	110.5	0.2	-3.6
T80HL9	61	6.1	89.2	0.2	-5.6	53.7	5.4	102.2	0.2	-1.4
T80HL10	57.9	5.8	87.2	0.1	-4.1	51.2	5.1	99.9	0.1	0.1
T80HL11	70.3	7.1	71	0.1	-3.5	55.2*	5.6	93.8*	0.1	-0.5

* Change by over 15% from initial value

A similar size increment from liposomal formulations based on T80 was also obtained .The highest size gain is recorded on batch T80HL11 by approximately 32%. Thus, vesicle size increase after freeze-drying was correlated with decreased drug entrapment efficiency, possibly due to leakage under the lyophilization process[91].

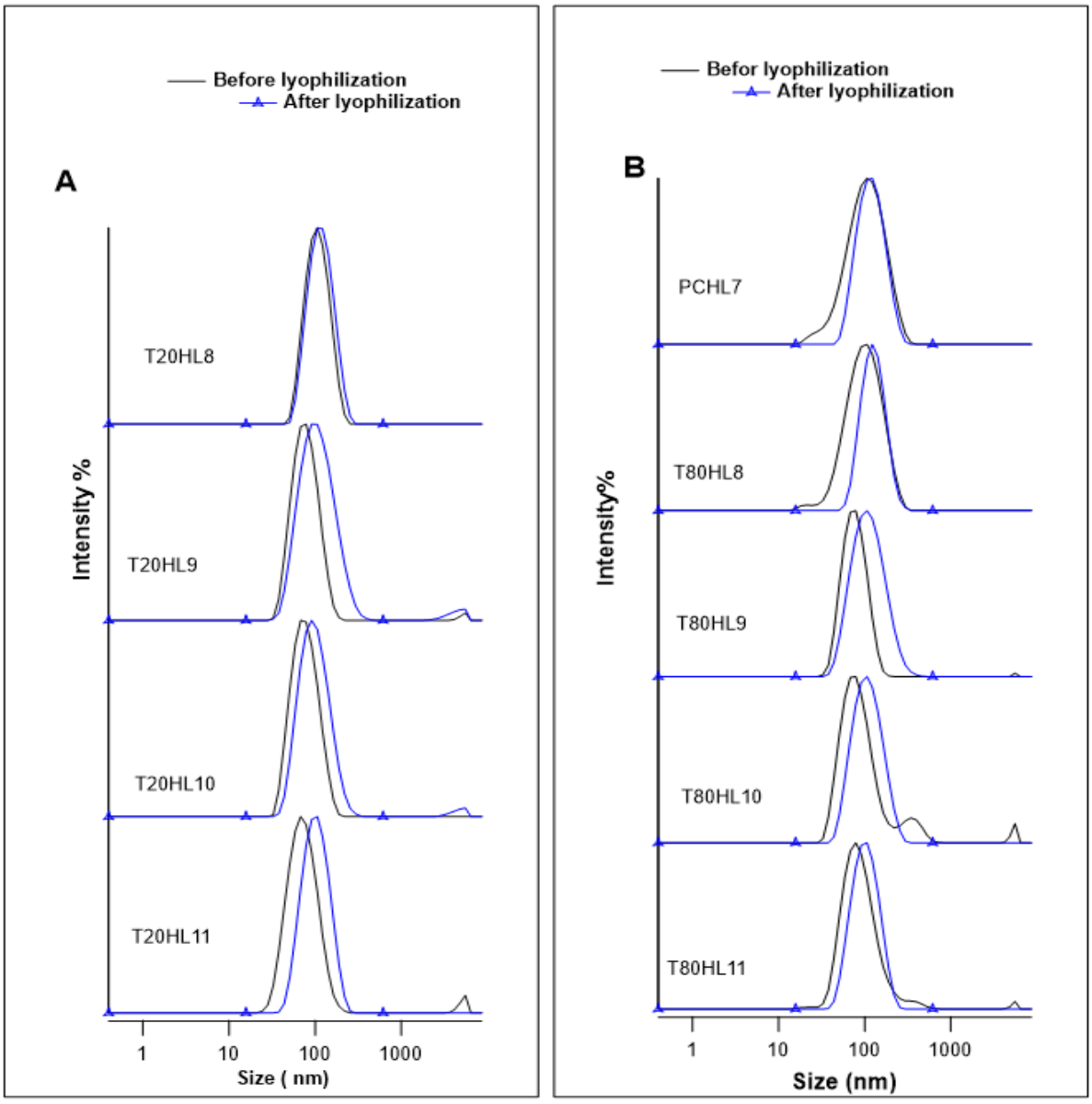
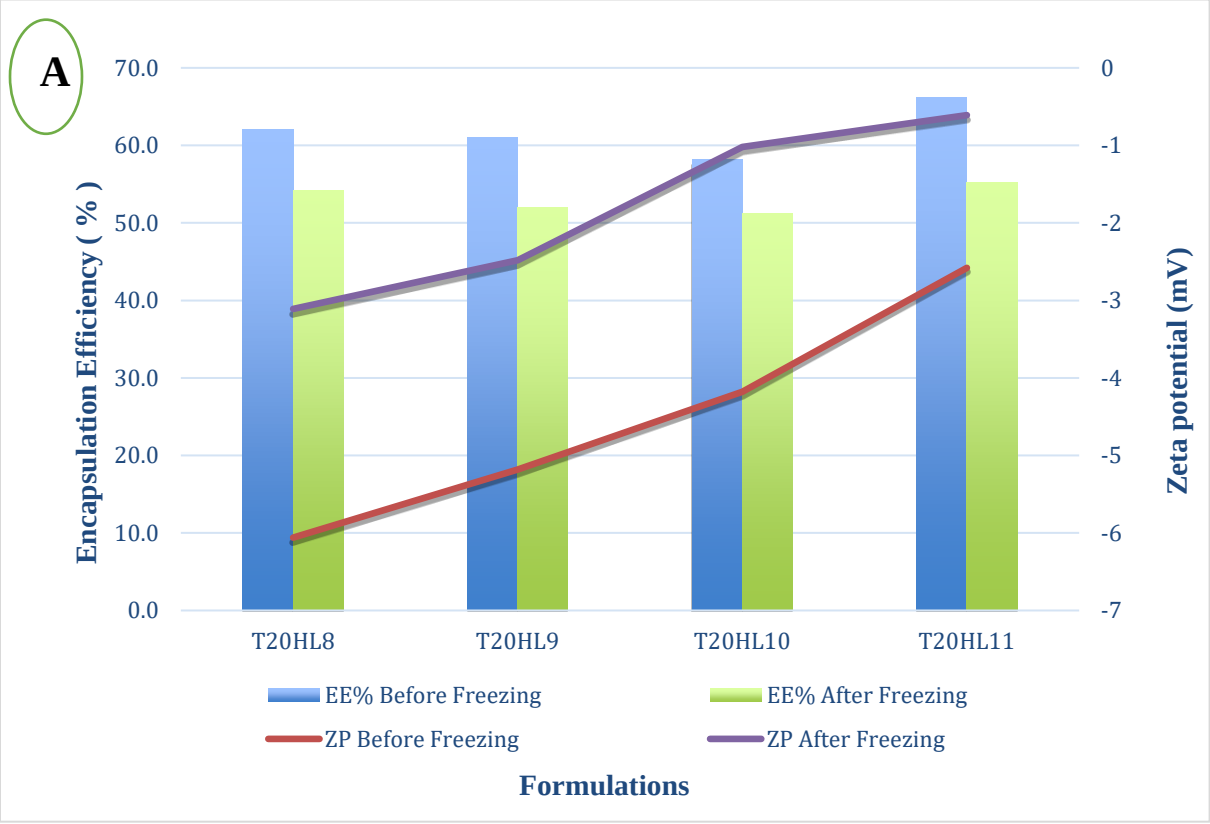


Figure 3.15: *Size distribution of LUM loaded liposome formulations for before and after freeze drying. T20-modified formulations(A), surfactant-free and T80-based formulations (B).*

The particle size distribution (PSD) of freshly prepared liposomal dispersion and the reconstituted liposomes based on Tween (20 and 80) are also plotted in Figures 3.15 (A &B), respectively. The plot showed a narrow and sharp peak, indicating that most particles were monodispersed. However, the PSD of formulations on Baches T20HL11, T80HL11, and PCHL7 are broad and flat, which is in good agreement with the obtained mean vesicle size.

Interestingly a very change in zeta potential in all formulations after freeze-drying is observed. Figures 3.16 (8A and B) clearly revealed that the ZP of liposomes after reconstitution improved towards zero in a range nearly by 49% to 100% correlated to the freshly prepared liposome values. The reason for the lower zeta potential may associates with EE% reduction probably due to leakage of drug during drying process.



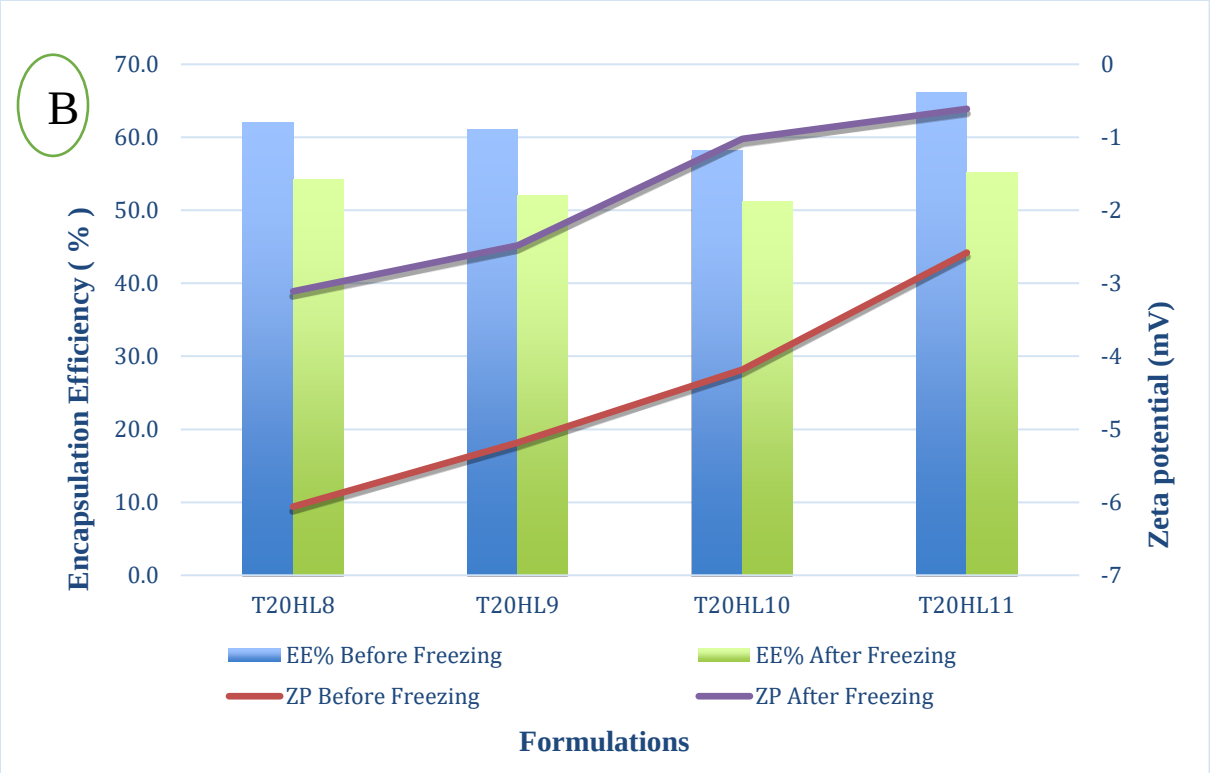


Figure 3.16: *Entrapment efficiency (EE%) and Zeta Potential (ZP) of liposomes before and after freeze drying. EE% and ZP of T20 modified liposome formulations (A) EE% and ZP of surfactant free and T80 modified liposome formulations(B).*

Figure 3.16 (A and B) also shows the entrapment percent of Lumefantrine retained in freeze-dried liposomes compared to EE% values before lyophilization. According to figures, it is clearly presented that the entrapped amount of Lumefantrine in all formulations was affected by the lyophilization process. The highest entrapment reduction by over 15% was achieved on batches PCHL7, T20HL11, and T80HL11.

All liposomal formulations, regardless of their composition, are affected by the lyophilization process. According to the obtained result, we recognize that the mean vesicle size is slightly increased, and entrapment efficiency decreased. The reasons back to the increase in vesicle size and leakage of the encapsulated material after freeze-drying could be related to the damage that happened by freezing which tended to destroy the membrane results in liposome membrane fusion [90, 91]. Hence, the probable aggregation of liposomes could prevent by adding cryoprotectants. Cryoprotectant's protective effect has been explained by their ability to interact with the

phospholipids' polar head groups and preserve the membranes while water in the membrane bilayer is removed during the sublimation process [91]. Protective agents such as saccharides can protect the liposome membranes against possible fracture and rupture [31] that might induce a change in size distribution and a loss of the encapsulated material. A similar finding by *Glavas-Dodov et al.* also reported that the process of lyophilization, without cryoprotectant, resulted in particle size enlargement and drug content leakage. However, formulations with the addition of saccharose vesicle bilayer were found to be more stable and less permeable to the encoded drug, and the particle size of liposomes was not affected during the freeze-drying process[91].

4 Conclusion

In the first part of this thesis, SUVs liposome was produced using a thin film method. Liposome formulations were prepared in a water phase (95% and 98%), and a high-pressure homogenizer in a different homogenization speeds is utilized. Liposomes prepared in 98% water phase using homogenization rate (20000rpm) provided reliable size reduction for our goal to *i.v* administration, and those were chosen for further drug loading formulations.

Secondly, LUM-loaded liposomes with different lipid and surfactant compositions were prepared and assessed for entrapment efficiency and loading capacity. The LUM entrapment efficiency within the liposome was found to be highest in liposomal formulations prepared based on T80, followed by T20 -modified liposomes, and the least entrapment efficiency in surfactant-free liposomes.

The in-vitro test of liposomal formulations was also conducted using a dialysis bag. The drug release revealed that the type and amount of surfactant influence the release effect. Formulations composed of the highest surfactant concentration showed the highest drug release while formulation without surfactant revealed least release.

A comparison among the two surfactants (T20 and T80) based formulations and surfactant-free liposomes were assessed, and no significant differences were noticed among the surfactants, but a slightly lower drug release were observed in surfactant-free formulations. In general, the highest DR% were obtained in a formulation containing T80, followed by T20 and surfactant-free formulations, respectively.

As part of stability study, lyophilization process were also done and physiochemical analysis of liposomes and entrapment efficiency of drug both before and after reconstitution were performed. A slight decrease in drug entrapment, probably due to leakage and vesicle size increment, was perceived in all formulations. While liposomes in an aqueous form stored at 4℃ showed no significant change in the first month, however after day 60 change in vesicle size, particularly in batches, composed of higher surfactant concentration and surfactant-free formulations were noticed.

In summary, we have made progress towards establishing a method for producing and testing lumefantrine liposomes and identifying a formulation that effectively can incorporate LUM. However, in-vitro release study, we have yet to succeed at the ideal formulation, and as we are still evaluating factors that influence all areas of drug encapsulation and drug release. Therefore, further studies are required to reach the intention of a formulation suitable for in vivo studies.

5 PERSPECTIVES

Liposomal formulations produced in this project will serve as a base to further work for the Lumefantrine parenteral delivery system. However, due to time restrictions, some studies have not been fulfilled. For instance, the freeze-drying analysis for chemical stability is assessed by reconstituting the dried powder after one day. However, to evaluate the long-term stability, it would be required to perform by storing the freeze-dried liposome for several months before reconstitution. Additionally, it would be essential to understand why the entrapment efficiency of liposomes after lyophilization tended to decrease, alternatively, by developing liposomal formulations composed of protectants.

Due to the highly lipophilic nature of LUM, we have encountered difficulties in determining the in-vitro release study of Lumefantrine. Therefore, the recently improved method should be evaluated. Moreover, liposomal formulations composed of different lipid/surfactant compositions and varying lumefantrine concentrations should be studied for their in vivo behaviour in terms of drug release, clearance, and biodistribution comparison with in-vitro drug release findings can be verified.

Reference list

1. Patra, J.K., et al., *Nano based drug delivery systems: recent developments and future prospects.* Journal of Nanobiotechnology, 2018. **16**(1): p. 71.
2. Shakeel, K., et al., *Development and in vitro/in vivo evaluation of artemether and lumefantrine co-loaded nanoliposomes for parenteral delivery.* Journal of Liposome Research, 2019. **29**(1): p. 35-43.
3. Patel, K., V. Sarma, and P. Vavia, *Design and evaluation of Lumefantrine - Oleic acid self nanoemulsifying ionic complex for enhanced dissolution.* Daru : journal of Faculty of Pharmacy, Tehran University of Medical Sciences, 2013. **21**(1): p. 27-27.
4. Hua, S., *Physiological and Pharmaceutical Considerations for Rectal Drug Formulations.* Frontiers in pharmacology, 2019. **10**: p. 1196-1196.
5. Homayun, B., X. Lin, and H.-J. Choi, *Challenges and Recent Progress in Oral Drug Delivery Systems for Biopharmaceuticals.* Pharmaceutics, 2019. **11**(3): p. 129.
6. Gulati, N. and H. Gupta, *Parenteral Drug Delivery: A Review.* Recent patents on drug delivery & formulation, 2011. **5**: p. 133-45.
7. Cano-Sarabia, M. and D. Maspoch, *Nanoencapsulation*, in *Encyclopedia of Nanotechnology*, B. Bhushan, Editor. 2014, Springer Netherlands: Dordrecht. p. 1-16.
8. Akbarzadeh, A., et al., *Liposome: classification, preparation, and applications.* Nanoscale research letters, 2013. **8**(1): p. 102-102.
9. Ahmad, M.U., *Lipids in Nanotechnology*. 2015: AOCS. 294: p 171-175.
10. Nkanga, C., et al., *General Perception of Liposomes: Formation, Manufacturing and Applications*. 2019.
11. Li, J., et al., *A review on phospholipids and their main applications in drug delivery systems.* Asian Journal of Pharmaceutical Sciences, 2015. **10**(2): p. 81-98.
12. van Hoogevest, P., et al., *Role of phospholipids in the oral and parenteral delivery of poorly water soluble drugs.* Journal of Drug Delivery Science and Technology, 2011. **21**(1): p. 5-16.
13. Alagumuthu, M., D. Dahiya, and P. Singh Nigam, *Phospholipid—the dynamic structure between living and non-living world; a much obligatory supramolecule for present and future.* AIMS molecular science, 2019. **6**(1): p. 1-19.
14. Alberts B, J.A., Lewis J, et al., *Molecular Biology of the Cell. 4th edition.*, in *The Lipid Bilayer*. 2002, New York: Garland Science; 2002. : https://www.ncbi.nlm.nih.gov/books/NBK26871https://www.ncbi.nlm.nih.gov/books/NBK26871.
15. Bozzuto, G. and A. Molinari, *Liposomes as nanomedical devices.* International journal of nanomedicine, 2015. **10**: p. 975-999.
16. van Hoogevest, P. and A. Wendel, *The use of natural and synthetic phospholipids as pharmaceutical excipients.* European Journal of Lipid Science and Technology, 2014. **116**(9): p. 1088-1107.

17. Editors: Basu, S.C., Basu, Manju *Liposome Methods and Protocols*. Vol. 199 of the series Methods in Molecular Biology. 2002. 249, p. 2-12.
18. Sigmaaldrich, M. *18:0-20:4 PC 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine*. 2021 [cited 2021 11.03.2021]; Available from: https://www.sigmaaldrich.com/catalog/product/avanti/850469c?lang=en&region=NO.
19. Lipids, A.P. *What Is The Transition Temperature Of The Lipid?* 2021; Available from: https://avantilipids.com/tech-support/faqs/transition-temperature.
20. Chen, W., et al., *Determination of the Main Phase Transition Temperature of Phospholipids by Nanoplasmonic Sensing.* Scientific Reports, 2018. **8**(1): p. 14815.
21. Kerwin, B.A., *Polysorbates 20 and 80 Used in the Formulation of Protein Biotherapeutics: Structure and Degradation Pathways.* Journal of Pharmaceutical Sciences, 2008. **97**(8): p. 2924-2935.
22. Ahmed, M.O., *Comparison of impact of the different hydrophilic carriers on the properties of piperazine-containing drug.* European Journal of Pharmaceutics and Biopharmaceutics, 2001. **51**(3): p. 221-225.
23. Grabarek, A.D., et al., *What Makes Polysorbate Functional? Impact of Polysorbate 80 Grade and Quality on IgG Stability During Mechanical Stress.* Journal of Pharmaceutical Sciences, 2020. **109**(1): p. 871-880.
24. Nair, L.M., et al., *Determination of polysorbate 80 in parenteral formulations by high-performance liquid chromatography and evaporative light scattering detection.* Journal of Chromatography A, 2003. **1012**(1): p. 81-86.
25. Gregory, G., *Liposome Technology, Liposome Preparation and Related Techniques*. Third ed. Vol. I. 2006: CRC Press. 324.
26. Vemuri, S. and C.T. Rhodes, *Preparation and characterization of liposomes as therapeutic delivery systems: a review.* Pharmaceutica Acta Helvetiae, 1995. **70**(2): p. 95-111.
27. Devrim, B. and A. Bozkır, *Chapter 7 - Nanocarriers and Their Potential Application as Antimicrobial Drug Delivery*, in *Nanostructures for Antimicrobial Therapy*, A. Ficai and A.M. Grumezescu, Editors. 2017, Elsevier. p. 169-202.
28. Brandl, M., *Liposomes as drug carriers: a technological approach*, in *Biotechnology Annual Review*. 2001, Elsevier. p. 59-85.
29. Fröhlich, M., V. Brecht, and R. Peschka-Süss, *Parameters influencing the determination of liposome lamellarity by 31P-NMR.* Chemistry and Physics of Lipids, 2001. **109**(1): p. 103-112.
30. Bulbake, U., et al., *Liposomal Formulations in Clinical Use: An Updated Review.* Pharmaceutics, 2017. **9**(2): p. 12.
31. Melis Çağdaş, A.D.S., Seyda Bucak, *Liposomes as Potential Drug Carrier Systems for Drug Delivery.* 2014: p. 51.
32. Kanásová, M. and K. Nesměrák, *Systematic review of liposomes' characterization methods.* Monatshefte für Chemie - Chemical Monthly, 2017. **148**(9): p. 1581-1593.

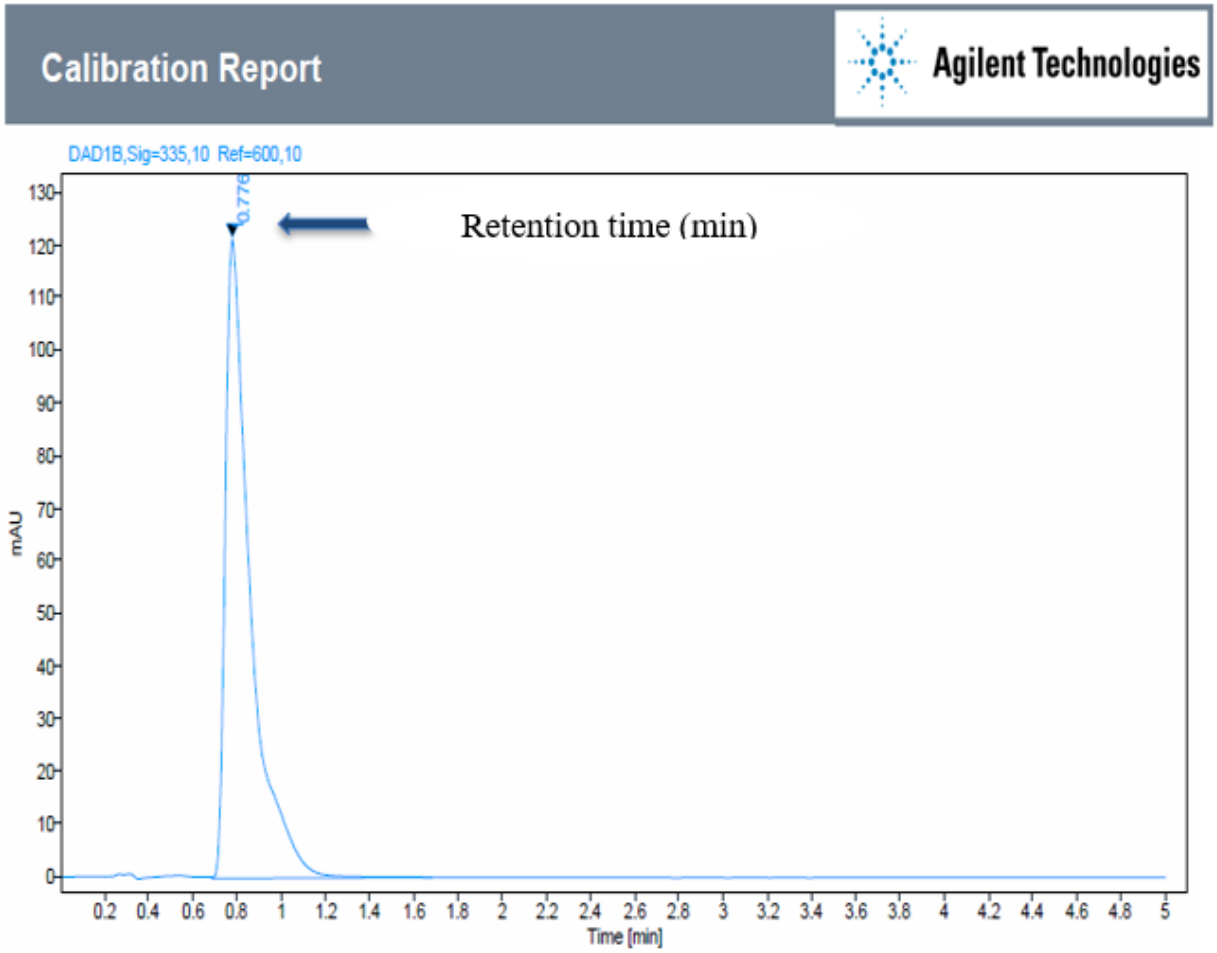
33. Date, A.A. and M.S. Nagarsenker, *Parenteral microemulsions: An overview.* International Journal of Pharmaceutics, 2008. **355**(1): p. 19-30.
34. Lin, M. and X.-R. Qi, *Purification Method of Drug-Loaded Liposome*, in *Liposome-Based Drug Delivery Systems*, W.-L. Lu and X.-R. Qi, Editors. 2018, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 1-11.
35. Nimesh, S., R. Chandra, and N. Gupta, *Advances in Nanomedicine for the Delivery of Therapeutic Nucleic Acids*. 2017, Cambridge, UNITED KINGDOM: Elsevier Science & Technology. 241; p 174-176.
36. Suk, J.S., et al., *PEGylation as a strategy for improving nanoparticle-based drug and gene delivery.* Advanced drug delivery reviews, 2016. **99**(Pt A): p. 28-51.
37. Sandeep Kalepu*, S.K.T., Sudheer Betha And Mohanvarma M, *Liposomal drug delivery system - A Comprehensive Review.* 2013.
38. Shaker, S., A.R. Gardouh, and M.M. Ghorab, *Factors affecting liposomes particle size prepared by ethanol injection method.* Research in pharmaceutical sciences, 2017. **12**(5): p. 346-352.
39. Bnyan, R., et al., *Surfactant Effects on Lipid-Based Vesicles Properties.* Journal of Pharmaceutical Sciences, 2018. **107**(5): p. 1237-1246.
40. Voigt, N., et al., *Surfactants, not size or zeta-potential influence blood–brain barrier passage of polymeric nanoparticles.* European Journal of Pharmaceutics and Biopharmaceutics, 2014. **87**(1): p. 19-29.
41. Suh, K.N., K.C. Kain, and J.S. Keystone, *Malaria.* CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne, 2004. **170**(11): p. 1693-1702.
42. WHO. *Malaria*. 2020 [cited 2021 07.03.2021]; Available from: https://www.who.int/news-room/fact-sheets/detail/malaria.
43. Britannica, T.E.o.E. *Malaria*. 2020 2020, October 2 [cited 2021 07.03.2021]; Available from: https://www.britannica.com/science/malaria.
44. Chaudhary, D.A., *Defeat Malaria*. First Edition ed. 2010: Kuldeep Jain for Health Harmony 159 : p 8-17.
45. Chiranjeev Sharma, S.K.A. *Recent Advances in Antimalarial Drug Discovery — Challenges and Opportunities*. An Overview of Tropical Diseases, Amidou Samie, IntechOpen 2015; December 2nd 2015:[Available from: https://www.intechopen.com/books/an-overview-of-tropical-diseases/recent-advances-in-antimalarial-drug-discovery-challenges-and-opportunities.
46. Gaur, D., C.E. Chitnis, and V.S. Chauhan, *Advances in Malaria Research : Recent Advances in Malaria : Recent Advances in Malaria*. 2016, Somerset, UNITED STATES: John Wiley & Sons, Incorporated. 592: p 1-5.
47. CDC, C.f.D.C.a.P. *Biology, Lifecycle* 2021 [cited 2021 07.03.2021]; Available from: https://www.cdc.gov/malaria/about/biology/index.html.
48. World Health, O., *Guidelines for the Treatment of Malaria*. 2010, Albany, SWITZERLAND: World Health Organization. 195: p 13-23.

49. Antony, H.A. and S.C. Parija, *Antimalarial drug resistance: An overview.* Tropical parasitology, 2016. **6**(1): p. 30-41.
50. Shibeshi, M.A., Z.D. Kifle, and S.A. Atnafie, *Antimalarial Drug Resistance and Novel Targets for Antimalarial Drug Discovery*. Infection and Drug Resistance, 2020. **13**: p. 4047-4060.
51. Parija, S. and I. Praharaj, *Drug resistance in malaria.* Indian Journal of Medical Microbiology, 2011. **29**(3): p. 243-8.
52. Krishna, H.M.S.l.S., *Treatment and Prevention of Malaria ,Antimalarial Drug Chemistry, Action and Use*. 2012: Springer Basel AG. 315; p104-217.
53. Verbeken, M., et al., *Stability-indicating HPLC-DAD/UV-ESI/MS impurity profiling of the anti-malarial drug lumefantrine.* Malaria journal, 2011. **10**: p. 51-51.
54. Mvango, S., et al., *Nanomedicines for Malaria Chemotherapy: Encapsulation vs. Polymer Therapeutics.* Pharmaceutical Research, 2018. **35**: p. 237.
55. Nyunt, M.M., et al., *Artemether-Lumefantrine Pharmacokinetics and Clinical Response Are Minimally Altered in Pregnant Ugandan Women Treated for Uncomplicated Falciparum Malaria.* Antimicrobial Agents and Chemotherapy, 2016. **60**(3): p. 1274-1282.
56. Kotila, O.A., et al., *Experimental determination of the physicochemical properties of lumefantrine.* Afr J Med Med Sci, 2013. **42**(3): p. 209-14.
57. PubChem. *National Center for Biotechnology Information :Lumefantrine*. 2021 [cited 2021 March 8]; Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Lumefantrine.
58. Bangham, A.D., M.M. Standish, and J.C. Watkins, *Diffusion of univalent ions across the lamellae of swollen phospholipids.* Journal of Molecular Biology, 1965. **13**(1): p. 238-IN27.
59. Müller, M., S. Mackeben, and C.C. Müller-Goymann, *Physicochemical characterisation of liposomes with encapsulated local anaesthetics.* International Journal of Pharmaceutics, 2004. **274**(1): p. 139-148.
60. Cullis, P.R., et al., *Generating and loading of liposomal systems for drug-delivery applications.* Advanced Drug Delivery Reviews, 1989. **3**(3): p. 267-282.
61. Chung, S.K., et al., *Factors influencing the physicochemical characteristics of cationic polymer-coated liposomes prepared by high-pressure homogenization.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2014. **454**: p. 8-15.
62. Panwar, P., et al., *Preparation, characterization, and in vitro release study of albendazole-encapsulated nanosize liposomes.* International Journal of Nanomedicine, 2010. **5**: p. 101-108.
63. Parashar, D., A. N. P, and M. R. S. R, *Development of artemether and lumefantrine co-loaded nanostructured lipid carriers: physicochemical characterization and in vivo antimalarial activity.* Drug Delivery, 2016. **23**(1): p. 123-129.
64. Sigmaaldrich, M. *Phosphate buffered saline*. 2021 [cited 2021 03.04.2021]; Available from:

https://www.sigmaaldrich.com/catalog/substance/phosphatebufferedsaline1234598765?lang=en&region=NO.
65. Danaei, M., et al., *Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems.* Pharmaceutics, 2018. **10**(2).
66. Najlah, M., et al., *Development of Injectable PEGylated Liposome Encapsulating Disulfiram for Colorectal Cancer Treatment.* Pharmaceutics, 2019. **11**(11): p. 610.
67. Tefas, L.R., et al., *Development of antiproliferative long-circulating liposomes co-encapsulating doxorubicin and curcumin, through the use of a quality-by-design approach.* Drug Design, Development and Therapy, 2017. **11**: p. 1605-1621.
68. Taghizadeh, S.M. and S. Bajgholi, *A new liposomal-drug-in-adhesive patch for transdermal delivery of sodium diclofenac.* Journal of Biomaterials and Nanobiotechnology, 2011. **2**: p. 576.
69. Xia, S. and S. Xu, *Ferrous sulfate liposomes: preparation, stability and application in fluid milk.* Food Research International, 2005. **38**(3): p. 289-296.
70. Kaur, P., et al., *Development, optimization and evaluation of surfactant-based pulmonary nanolipid carrier system of paclitaxel for the management of drug resistance lung cancer using Box-Behnken design.* Drug Delivery, 2016. **23**(6): p. 1912-1925.
71. Maulvi, F.A., et al., *Effect of surfactant chain length on drug release kinetics from microemulsion-laden contact lenses.* International Journal of Pharmaceutics, 2017. **524**(1): p. 193-204.
72. Cipolla, D., et al., *Modifying the Release Properties of Liposomes Toward Personalized Medicine.* Journal of Pharmaceutical Sciences, 2014. **103**(6): p. 1851-1862.
73. PubChem. *National Center for Biotechnology Information (2021). Polyoxyethylene sorbitan monooleate*. 2021 [cited 2021 May. 8]; Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Polyoxyethylene-sorbitan-monooleate.
74. Sigmaaldrich. *TWEEN® 20*. 2021 [cited 2021 May 01]; Available from: https://www.sigmaaldrich.com/catalog/product/sial/p1379?lang=en&region=NO.
75. Sarfraz, M., et al., *Development of Dual Drug Loaded Nanosized Liposomal Formulation by A Reengineered Ethanolic Injection Method and Its Pre-Clinical Pharmacokinetic Studies.* Pharmaceutics, 2018. **10**(3): p. 151.
76. Jain, S., et al., *Transfersomes—A Novel Vesicular Carrier for Enhanced Transdermal Delivery: Development, Characterization, and Performance Evaluation.* Drug Development and Industrial Pharmacy, 2003. **29**(9): p. 1013-1026.
77. Chaudhary, H., K. Kohli, and V. Kumar, *Nano-transfersomes as a novel carrier for transdermal delivery.* International Journal of Pharmaceutics, 2013. **454**(1): p. 367-380.
78. El-Laithy, H.M., O. Shoukry, and L.G. Mahran, *Novel sugar esters proniosomes for transdermal delivery of vinpocetine: Preclinical and clinical studies.* European Journal of Pharmaceutics and Biopharmaceutics, 2011. **77**(1): p. 43-55.
79. Ashok Bohara, R. and N.D. Thorat, *Hybrid Nanostructures for Cancer Theranostics*. 2018, San Diego, UNITED STATES: Elsevier. 263; P 141-142.

80. Muppidi, K., et al., *Development and Stability Studies of Novel Liposomal Vancomycin Formulations.* ISRN Pharmaceutics, 2012. **2012**: p. 636743.
81. Redhead, H.M., S.S. Davis, and L. Illum, *Drug delivery in poly(lactide-co-glycolide) nanoparticles surface modified with poloxamer 407 and poloxamine 908: in vitro characterisation and in vivo evaluation.* Journal of Controlled Release, 2001. **70**(3): p. 353-363.
82. A. Namdeo, N.K.J., *Niosomal delivery of 5-fluorouracil.* Journal of Microencapsulation, 1999. **16**(6): p. 731-740.
83. Sigmaaldrich. *TWEEN® 80*. 2021 [cited 2021 May 01]; Available from: https://www.sigmaaldrich.com/catalog/product/sigma/93781?lang=en&region=NO.
84. Blume, G. and G. Cevc, *Liposomes for the sustained drug release in vivo.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1990. **1029**(1): p. 91-97.
85. Sercombe, L., et al., *Advances and Challenges of Liposome Assisted Drug Delivery.* Frontiers in pharmacology, 2015. **6**: p. 286-286.
86. Ritger, P.L. and N.A. Peppas, *A simple equation for description of solute release I. Fickian and non-fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs.* Journal of Controlled Release, 1987. **5**(1): p. 23-36.
87. Unagolla, J.M. and A.C. Jayasuriya, *Drug transport mechanisms and in vitro release kinetics of vancomycin encapsulated chitosan-alginate polyelectrolyte microparticles as a controlled drug delivery system.* European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 2018. **114**: p. 199-209.
88. Bruschi, M.L., *Strategies to Modify the Drug Release from Pharmaceutical Systems*. 2015, Cambridge, UNITED KINGDOM: Elsevier Science & Technology. 199 :p 40-42.
89. Anderson, M. and A. Omri, *The Effect of Different Lipid Components on the In Vitro Stability and Release Kinetics of Liposome Formulations.* Drug Delivery, 2004. **11**(1): p. 33-39.
90. Franzé, S., et al., *Lyophilization of Liposomal Formulations: Still Necessary, Still Challenging.* Pharmaceutics, 2018. **10**(3): p. 139.
91. Glavas-Dodov, M., et al., *The effects of lyophilization on the stability of liposomes containing 5-FU.* International Journal of Pharmaceutics, 2005. **291**(1): p. 79-86.

Appendix I: UHPLC spectra of lumefantrine



Figure; Appendix I:*UHPLC spectra of lumefantrine for standard curve preparation. The lumefantrine dissolves in acetonitrile at a concentration of 0.005mg/ml, and the detected area was 46.401 at retention time around 0.8 minutes.*

Appendix II: Picture of Lumefantrine loaded liposome formulations

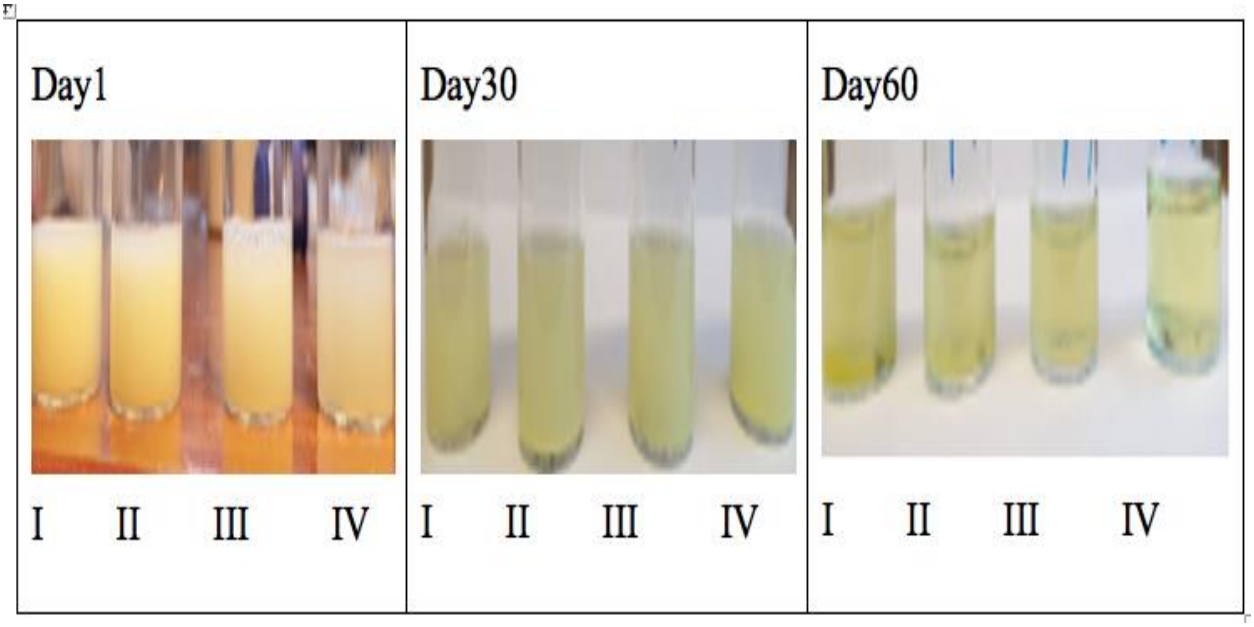


Figure Appendix II : shows *picture of surfactant free and PC/Tween 80 based lumefantrine liposomal formulations , PCHT7 (I), T80HL7 (II), T80HL9 (III) and T80HL11 (IV) stored* stored at 4℃ for 60 days.