FORMULATION OF LIPOSOMES AS A DRUG CARRIER FOR BUPARVAQUONE

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FORMULATION OF LIPOSOMES AS A DRUG CARRIER FOR BUPARVAQUONE

by

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This thesis is dedicated to

My dearest husband, father, mother, family,

lecturers and friends

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LIST OF ABBREVATION

α	alpha
AUC	Area under Curve
BPQ	Bupavarquone
Da	Dalton
e.g	example given
EE	Encapsulation Efficiency
g	gram
HPLC	High Performance Liquid Chromatography
kg	Kilogram
LD	Lethal dose
LUV	Large Unilamellar Vesicles
mg	milligram
M_I	initial amount present
mins	minutes
MLV	Multi Lamellar Vesicles
mm	millimeter
mol	molarity
M_T	amount released at time,t
MWCO	Molecular Weight Cut Off
NCE	New Chemical Entity
nm	nanometer
NTDS	Neglected Tropical Diseases
PBS	Phosphate Buffer Saline
PC	Phosphotidylcholine

PDI	Polydispersity Index
RES	Reticuloendothelial System
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
rpm	rotation per minute
RT	Room Temperature
SUV	Small Unilamellar Vesicles
TEM	Transmission Electron Microscopy
WHO	World Health Organization
µm	micrometer

FORMULASI LIPOSOM SEBAGAI PEMBAWA DRUG UNTUK BUPARVAQUONE

ABSTRAK

Kajian ini merangkumi penyediaan liposom sebagai pembawa drug buparvaquone yang merupakan ubat bagi rawatan penyakit leishmaniasis. Kajian awal bertumpu untuk menggantikan penggunaan klorofom dengan etanol sebagai pelarut lipid. Keputusan yang diperoleh menunjukkan liposom boleh dilarutkan dalam etanol dan klorofom boleh dikecualikan dari formulasi. Liposom tanpa drug disediakan melalui dua kaedah berbeza iaitu kaedah tradisional Bangham dan juga kaedah suntikan etanol. Kajian awal juga dijalankan bagi mengenalpasti factor-faktor yang mempengaruhi saiz dan polidispersiti liposom yang dihasilkan. Liposom juga diuji dengan menambah kolesterol dan alpha-tocopherol sebagai penstabil partikel liposom. Kajian menunjukkan liposom kaedah suntikan etanol lebih stabil berbanding liposom kaedah tradisional Bangham. Liposom yang dihasilkan menggunakan kaedah tradisional Bangham dikecualikan bagi ujian pelepasan dan pemerangkapan BPQ disebabkan ujian fizikokimia menunjukkan ia tidak stabil dalam jangka masa panjang. Berbeza dengan liposom kaedah suntikan etanol di mana hasil kajian menunjukkan kestabil untuk 6 bulan dengan ukuran saiz dan polidispersiti masih kurang dari 0.200. Liposom tanpa drug menunjukkan saiz dan polidispersiti lebih kecil berbanding dengan liposom yang telah dimasukkan drug BPQ. Liposom bagi pemerangkapan Bupavarquone menggunakan dua formulasi. Formulasi pertama adalah lecithin 100 mg dengan kolesterol 50 mg. Formulasi kedua yang digunakan mengandungi 100mg lecithin dan 50mg alpha-tocopherol. Liposom dicirikan mengikuti beberapa ujian pencirian fizikokimia termasuk ujian

pemerangkapan dan ujian pelepasan buparvaquone (BPQ) secara *in vitro* selama 24 jam.Liposom mengandungi BPQ menunjukkan kejayaan inkorporasi ubat sebanyak 60-85%. Bagaimanapun penambahan kadar ubat yang terlalu tinggi iaitu 40 mg Buparvaquone mengakibatkan penurunan peratusan pemerangkapan BPQ dalam liposom. Bagi kajian pelepasan ubatan lebih daripada 80% ubatan telah berjaya dilepaskan dari liposom dalam masa 24 jam. Keputusan dan penemuan yang diperoleh menunjukkkan liposom mempunyai potensi yang amat sesuai untuk digunakan bagi rawatan leishmaniasis yang bertumpu di system RES. Kesimpulannya dua formulasi liposom yang mampu berfungsi sebagai pembawa drug buparvaquone telah telah berjaya dihasilkan menggunakan kaedah suntikan etanol dan boleh digunakan di masa hadapan bagi kajian kajian *in vivo* untuk mengkaji potensinya mengubati penyakit leishmaniasis.

FORMULATION OF LIPOSOMES AS A DRUG CARRIER FOR BUPARVAQUONE

ABSTRACT

This study was carried out to encapsulate the drug buparvaquone (BPQ) and to study its physiochemical properties and along with its *in vitro* release study. Initial studies focused on elimination of chloroform as lipid solvent and its substitution with ethanol. Result obtained showed that chloroform can be substituted with ethanol. Two different fabrication methods were used to produce liposomes: 1) the classic Bangham method and 2) ethanol injection method. Preliminary study was conducted to study the parameters that affect the particle size and polydispersity index (PDI). Liposomes were formulated with the addition of stabilizers, cholesterol and alpha-tocopherol. The liposomes were characterized based on physicochemical characteristics to record their effects on size and polydispersity. Two formulations obtained from the ethanol injection method were used for loading of buparvaquone whereby the first formulation with 100mg lecithin and 50mg cholesterol, the second one with 100mg lecithin and 50mg alpha-tocopherol. The study conducted for BPQ loading and BPQ encapsulation eliminated Bangham method liposomes as the stability study showed that the particle size and polydispersity increased to above 0.200 within 6 months whilst ethanol injection particles remained stable over same period of time. The BPQ loaded particles for ethanol injection method were larger in size but did not exceed 200nm. The encapsulation efficiency of liposome formulated with cholesterol and alpha tocopherol showed similar encapsulation percentage. The increase in drug: lipid ratio resulted in lower encapsulation efficiency. The drug

release study showed more than 80% of drug was released by 24 hours of study. The findings of this study suggest that production of liposome particles successfully encapsulated the drug buparvaquone. Two formulations were successfully formulated. These formulations can be used for further *in vivo* studies on Leishmania cells. It has a huge potential to be used for further development in the treatment of leishmaniasis.

CHAPTER ONE

INTRODUCTION

1.1PROBLEM STATEMENT

The high prevalence of leishmaniasis in countries like Africa, India, Bangladesh and many other countries and the emergence of resistance to conventional drugs demonstrate the need to develop less toxic and more efficient treatments. There is a limited safe therapeutic option to treat visceral leishmaniasis. Quite often, the drug treatments result in poor patience compliance and relapses occur due to the unfinished treatments and the severe adverse effects (den Boer et al., 2009 and Murray, 2001).

Currently, there are two formulations in use: sodium stibogluconate (SSG) and meglumine antimoniate. Both formulations have comparable efficacy and toxicity. They have poor oral absorption and are given via intramuscular or intravenous injections. Common side effects include nausea and vomiting, hepatitis and pancreatitis. Gastrointestinal symptoms appear a major risk factor for death during treatment with SSG.

Miltefosine is a membrane active alkyl phospholipid, originally used as an anticancer drug. It was found to have antileishmania activity in animal models in the early 1990s. It is active orally. Animal studies have shown that it has some reproductive toxicity and thus it is contraindicated in pregnancy, and needs to be used with caution in women of reproductive age. However, the long half life (seven days) makes the risk of developing parasites resistant to miltefosine a real possibility.

The use of LAB was initially limited in the early 1990s in resource rich countries to a second line drug when VL parasites persisted despite SSG treatment. The evaluation of efficacies of different Amphotericin B formulations (Fungizone, Ambisome, Abelcet, and Amphocil) in a murine model of visceral leishmaniasis showed that liposomal Amphotericin B formulation could successfully cured the infection with a 21-day course (50 mg/day) of (Khan and Owais, 2006) in the cases where leishmaniasis was unresponsive to several courses of treatment with the standard drugs (antimonials). In India some 90% of patients with visceral leishmaniasis were successfully treated with liposomal Amphotericin B in treatment regimens as brief as 5 days. The liposomal Amphotericin B thus offered a remedy for the principal drawback of all other antileishmanial agents which have a prolonged duration of treatment. Currently, AmBisome is the only liposomal product approved for the treatment of visceral leishmaniasis in adult and pediatrics and considered the first choice for patients who are unresponsive to antimonials (Croft and Coombs, 2003). Although AmBisome has been approved for the treatment of visceral leishmaniasis the drug dosage delivered is low compared to dosage required to treat visceral leishmaniasis especially in Africa.

Buparvaquone (BPQ), an approved veterinary drug for theileriosis treatment, has shown promising activities against *Leishmania* spp. (Croft et al., 1992). Althoug BPQ showed anti-leishmanial activity at nanomolar concentrations (Croft et al., 1992), it has limited *in vivo* efficacy for VL (Croft et al., 1992) and CL (Garnier et al., 2007) attributed to its poor distribution and low availability. Besides that BPQ has a low water solubility (<1 mg/L) resulting in poor solubility in biological media such as gastric fluids (Müller and Jacobs, 2002). Liposomes have been widely used

as safe and effective vehicles for improving drug delivery of drugs with poor solubility (Croft et al., 1992) and high toxicity. As highlighted above, liposomal Amphotericin B has been used to treat leishmaniasis with fewer toxic effects (Bern et al., 2006 and Sundar et al., 2011).

This study, thus aims to prepare formulations of liposome particles to encapsulate the drug buparvaquone and to study its release profile. The potential of liposome to encapsulate buparvaquone was explored to enhance encapsulation which is a common problem with buparvaquone due to its poor solubility. The liposomes produced will include two different membrane stabilizers cholesterol and alpha-tocopherol which can help prevent formation of leaky liposomes.

1.2DRUG DELIVERY

Generally, the term drug delivery refers to the various methods or systems used to ensure drugs are administered effectively into the body (Sivakumar and Rao, 2001, Karalis et al., 2010). These systems must take into account a number of needs, ranging from ease of delivery to effectiveness of the drugs. The efficacy of a drug product depends on the amount of active moiety absorbed from its formulation and how rapidly the absorption process takes place. Hence drug delivery systems also need to consider the way in which a drug is metabolized by the body. For example, some drugs are destroyed in the intestinal tract, which means that they should not be introduced to the body through conventional routes which pass through the intestine. Others may be dangerous in large amounts, which means that a time release method should be used to deliver the drug to avoid toxicity (Dass, 2008).

Nanotechnology holds a significant potential as an effective drug delivery system (Karalis et al., 2010). Nanocarriers of various geometries and composition materials such as nanocapsules, micelles, liposomes, polymeric nanoparticles, solid lipid nanoparticles have been developed for the delivery of different therapeutics (Zeng et al., 2011). Colloidal drug carriers like emulsions, liposomes and nanoparticles have generated great interest to drug delivery mainly due to their versatile nature (Date et al., 2007). The discovery of liposomes contributed to a great leap in drug delivery research (Date et al., 2007). Liposomes are most extensively investigated amongst various colloidal carriers. Knowledge of liposome behavior *in vitro* enabled more rational designs of liposomes for the specific treatment of diseases (Sabin et al., 2010). At present, a number of companies work exclusively with liposomes for the development of different therapies: antibiotics, antitumor drugs, allergic sensitization formulas, gene therapy, and so on (Huang and MacDonald, 2004).

Liposomal drug delivery systems have been extensively studied for its potential effectiveness in reducing the toxicity of drugs (Stenekes et al., 2000, Webb et al., 2007). After their discovery by Bangham, liposomes have been developed as non toxic, biodegradable and non-immunogenic drug delivery vehicles. Liposomes are versatile and unique as they have the capacity to incorporate both hydrophobic/lipophilic and hydrophilic drug (Sharma and Sharma, 1996, Huang and MacDonald, 2004).

1.3 LIPOSOMES AS A DRUG DELIVERY SYSTEM

1.3.1Liposome: Definition and Classification

Liposome was accidently found by Alec Bangham while studying blood clotting. He found that phospholipids when combined with water immediately formed a sphere. This was because phospholipids has two ends in its structure (Sabin et al., 2006). One end has the affinity towards water and the other end is water insoluble (Shailesh et al., 2009). In the early years of their applications, liposomes were prepared from various lipid classes identical to those present in most biological membranes.

Liposomes are broadly defined as lipid bilayers surrounded in an aqueous space. Classification of liposomes can be divided into three classes; multilamellar vesicles (MLV) consist of several (up to 14) lipid layers (in an onion-like arrangement) separated from one another by a layer of aqueous solution. The diameters of these vesicles are over several hundred nanometers. The second one is known as small unilamellar vesicles (SUV) which are made out of a single lipid layer of 25–50nm in diameter. Large unilamellar vesicles (LUV) are a very heterogeneous group of vesicles that are surrounded by a single lipid layer. The diameter of these liposomes ranges from 100 nm up to cell size (giant vesicles) (Riaz, 1995, Sharma and Sharma, 1996, Chatterjee and Banerjee, 2002).

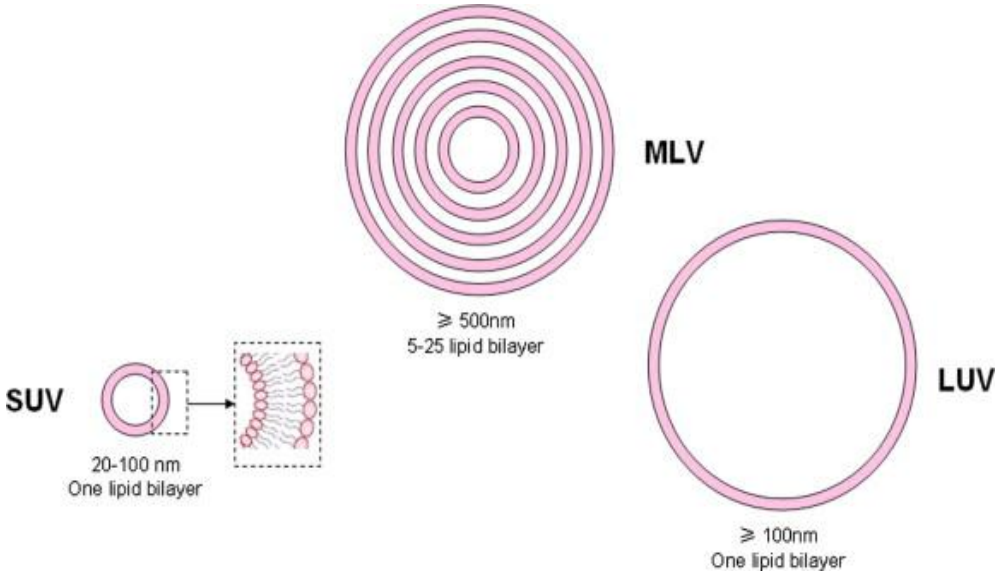


Figure1.1: Schematic representation-types of liposomes (Yang et al., 2011)

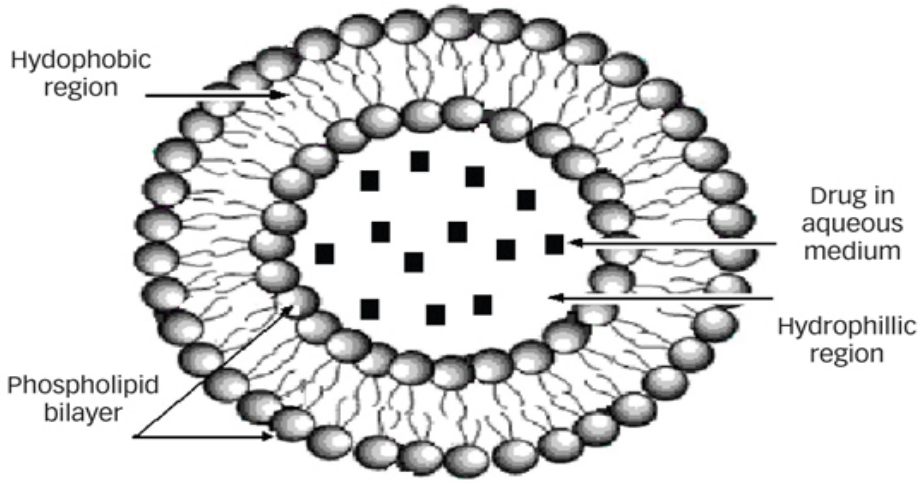


Figure 1.2: The basic structure of liposome (Pattnaik and Ray, 2009)

1.3.2 Mechanisms of Liposome Uptake and Drug Release

One common strategy for the delivery of encapsulated and/or intercalated material via liposomes exploits the pH gradients. The release of liposomes entrapped drug is also affected by various mechanisms which include passive diffusion of drug across the liposome membrane and perturbation of liposome membrane by intracellular and/or extracellular proteins in the tissue. These mechanisms operate simultaneously. Liposome uptake is by endocytosis, where liposomes are subjected to acidification along the endosomal pathway from early endosomes to lysosomes. The extent of release can be estimated and controlled by *in vitro* experiments in simulated conditions (Riaz, 1995). Phagocytosis/endocytosis occurs when endosomes take in liposomes. They fuse with lysosomes where digestion occurs, breaking open the liposomes after which it results in destabilization of the liposome bilayer releasing encapsulated material. This mechanism is necessary for mediating lipid destabilization, and therefore the release of encapsulated material in liposomes is restricted to the lumen of endosomal vesicles and the cytosol (Pollock et al., 2010).

Endocytosis of liposomes depends on their size. MLVS are endocytosed by various cells. Liposomes larger than 0.4µ cannot deliver entrapped material into cytoplasm. In general, the larger the liposomes size the faster the clearance. Large MLV are cleared mostly by liver cells. Increased clearance is desired when the RES is the site of action of the entrapped drug. Negatively charged liposomes are cleared faster than neutral or positive liposomes due to the interaction of liposomal membrane with plasma component. Intravenous administration of liposome is rapidly removed from the circulation by RES. The liver has the highest rate of

clearance followed by spleen. The discontinuous capillary walls in the liver allow the penetration of liposomes less than 0.2µ (Pattnaik and Ray, 2009).

1.3.3Phospholipids As Liposomal Matrix

Phospholipids are the basic building block and the major component of every cell membrane in the human body. How they work forming a membrane is elegant and miraculous (Memoli et al., 2000). Each phospholipid molecule has three major parts, one head and two tails. The head is made from choline, phosphate, and glycerol. The head is hydrophilic, it is attracted to water and as for the tails they are made of a long, essential fatty acid chain. These fatty acids are hydrophobic so they are repelled by water. When phospholipids are placed in an aqueous solution, the hydrophilic heads of the phospholipids form a line side by side with their tails behind. Then because the tails are hydrophobic, another phospholipid layer will line itself up tail-to-tail in response to the same aqueous environment. This natural alignment creates two-rows of tightly fitted phospholipid molecules, called a phospholipid bilayer. It is these phospholipid bilayers that form the membranes around and within every cell in our bodies (Yang et al., 2011).

One bilayer is about one 1,000th the thickness of a sheet of paper. It is these liposomal bubbles that are part of every application of liposomal drug delivery technology. Since the body is literally held together with phospholipids, these liposomal spheres are readily accepted by the body as essential building materials and their tiny size enables them to be quickly assimilated into the bloodstream for delivery throughout the body (Struppe et al., 2000, Parks et al., 2000). Liposomes can be formulated by phospholipids derived naturally or synthetically. One of the

most common natural phospholipids is phospatidylcholine (PC). It is an amphipathic molecule and is also known as lecithin (Memoli et al., 2000) which are commonly derived from egg or soybean.

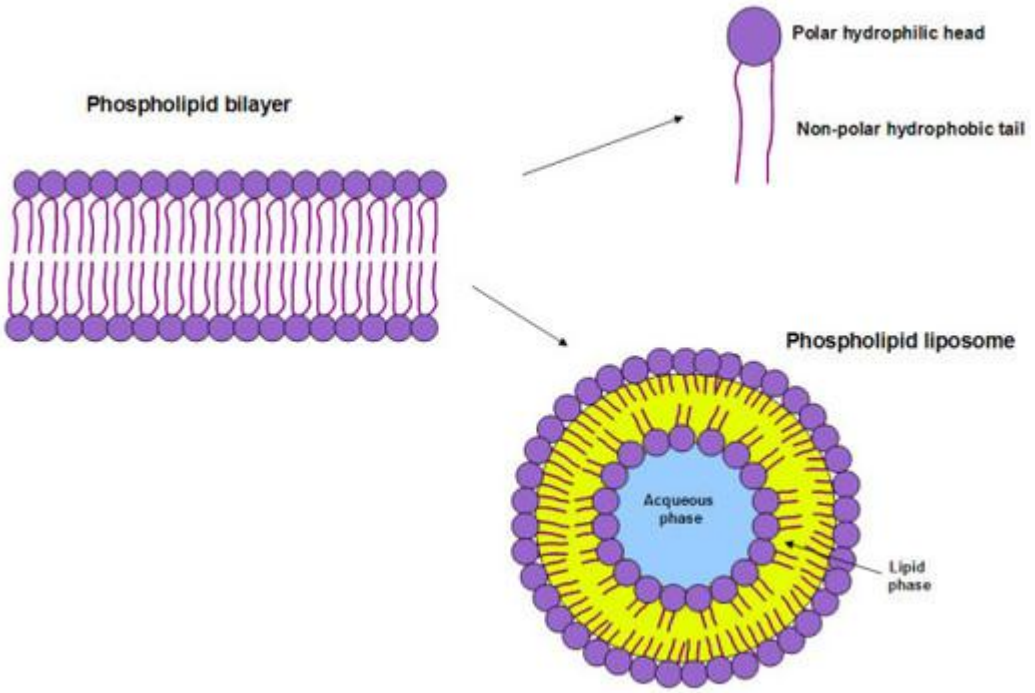


Figure 1.3: Phospholipid bilayer composed of hydrophobic non-polar tails and hydrophilic polar heads (Belhocine and Prato, 2011)

1.3.4 Phosphatidylcholine and Liposome

Phosphatidylcholines (PC) are a class of phospholipids that incorporate choline as the head group. They are a major component of biological membranes and can be easily obtained from a variety of readily available sources such as egg yolk or soy beans from which they are mechanically or chemically extracted. They are also a member of the lecithin group commonly found in animal and plant tissues.

The name "lecithin" was originally derived from the Greek term *lekithos* which means egg yolk. This name was first given by Theodore Nicolas Gobley, a French chemist and pharmacist in 1847, to the egg yolk's phosphatidylcholine he identified. Phosphatidylcholines are such a major component of lecithin that in some contexts the terms are synonymous. However, lecithin extract consists of a mixture of phosphatidylcholine and other compounds like phosphatidylethanolamine and phosphatidylisotinol (Singhvi and Singh, 2011).

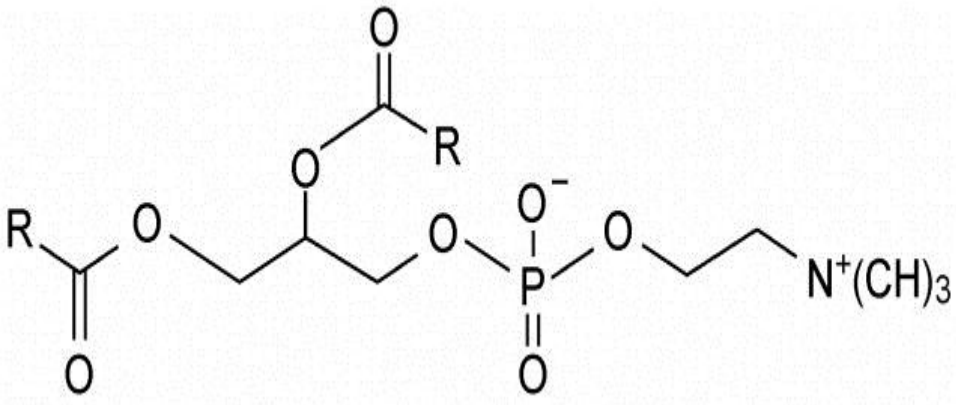


Figure 1.4: The Structure of Phosphotidylcholine(Lecithin) (Raut et al., 2012)

1.3.5 Safety and Biocompatibility of Liposomes

The use of liposomes as a drug carrier system was proposed by Gregoriadis & Ryman in the early 70's (Gregoriadis and Ryman, 1972, Kozubek et al., 2000). Since this first report, liposomes were developed as an advanced drug delivery vehicle. Liposome is regarded as a safe drug carrier as they are generally considered non-toxic, biodegradable and non-immunogenic. Incorporation of a drug with liposomes markedly changes its pharmacokinetics and lowers its systemic toxicity; furthermore,

the drug can be prevented from early degradation after introducing to the target organism (Gregoriadis and Davis, 1979). The use of liposomes for the delivery of various active compounds is recognized in relation to water solubility of the compound. When the compound is water soluble, the size and volume of the aqueous compartment of the vesicle is crucial. In contrast, hydrophobic compounds will prefer incorporation into the lipid (amphiphile) layer that constructs the vesicle.

Depending on the need, one can use SUV/LUV type or MLV type vesicles for effective entrapment and delivery of the drug to the target tissues or cells. The charge properties and interactions of the active compound with vesicle forming molecules will determine the efficacy of entrapment, i.e., the amount of the compound that can be "loaded" into a single vesicle. On the other hand, the composition of the molecules used for the formation of the vesicular structure will affect the fate of vesicles from the site of their introduction to the interaction with components of the body (e.g., surface charge, serum proteins, lipoproteins, opsonin system, and phagocytic system) and finally target cells. To be clinically successful, liposomal formulation should be able to encapsulate high amount of drug with proper loading stability. Liposomes can be made to contain an antigen, an antibiotic, an allergen, a drug substance or a gene (as in gene therapy) (Huang et al., 2006).

Depending on the requirements of each drug substance; the liposome characteristics can be modified. To reduce the rate of liposome degradation and therefore to slow down the release of the contents, the composition and size of the spheres can be modified. Liposome affinity for a given tissue can also be increased by varying vesicle composition, electrical charge or by adding receptors or adhesion factors contributing to increase the amount of drug present in the target tissues or

organ. An example of this effect is provided by doxorubicin, an expensive toxic antitumor drug that is used in human medicine. Doxil is a pegylated (polyethylene glycol coated) liposome-encapsulated form of doxorubicin. It was developed to treat Kaposi's sarcoma, an AIDS-related cancer that causes lesions to grow under the skin, in the lining of the mouth, nose, throat or in other organs. The polyethylene glycol coating results in preferential concentration of Doxil in the skin. However, this also results in a side effect called palmar plantar erythrodysesthesia (PPE), more commonly known as hand-foot syndrome. Following the administration of Doxil, small amounts of the drug can leak from capillaries in the palms of the hands and soles of the feet. The result of this leakage is redness, tenderness, and peeling of the skin that can be uncomfortable and even painful. The prevalence of this side effect limits the Doxil dose that can be given as compared with doxorubicin in the same treatment regimen, thereby limiting potential substitution. Substitution would be desirable because liposome-encapsulated doxorubicin is less cardiotoxic than unencapsulated doxorubicin. Doxil is also approved by the FDA for treatment of ovarian cancer and multiple myeloma (Basu, 2005).

1.3.6 Fabrication Techniques for the Production of Liposomes

There are various modes for liposome fabrication and a wide variety of conventional techniques that can be used to produce liposomal formulations, including Bangham, detergent depletion, ether/ethanol injection and emulsion methods (Mozafari, 2005). All methods for producing liposomes require lipids to be combined with an aqueous phase. The conventional methods generally involves a lipid solution being produced using an organic solvent prior to dispersion into the aqueous phase. However, the use of a volatile organic solvent may affect the chemical structure of an entrapped

chemical and also contribute to the toxicity and stability of the liposomes (Patel et al., 2011).

1.3.6(a) Bangham Method

Bangham method, also known as the hand shaken or thin film hydration method is one of the most traditionally used techniques for the fabrication of liposomes. The process is carried out by dissolving lipids in an organic phase, followed by the removal of the organic solvent, usually via evaporation. This results in the formation of a thin lipid film (crude liposome). The final step is the hydration of the lipid film with an aqueous media, carried by agitation to detach the swelling lamellae from the vessel surface. Then it forms a sealed spherical structure (liposomes). The solvent removal stage is time-consuming since it often requires several hours of exposure to high vacuum.

Liposomes produced via Bangham method are often several microns in size. Although Bangham method is one of the simplest methods for liposome formation, it has limited use due to low entrapment efficiency, difficulty in removing organic solvent, homogenization and is only suitable for small scale production (Mohammed et al., 2004, Mozafari, 2005). One alternative as shown in a previous study by Ran and Yalkowsky (Ran and Yalkowsky, 2001) was to produce liposomes using the thin film method by replacing the common organic solvent chloroform with halothane, a non-flammable, non-carcinogenic inhalation anesthetic (Jain et al., 2003, Santos et al., 2004).

1.3.6(b) Ethanol Injection Method

Ethanol injection method is one of the easiest and fastest method to produce liposomes. The method of preparation is carried out by injecting a small volume of ethanol containing lipid solution into a large volume of water or any other aqueous phase using a fine needle. The force by injections ensures homogeneous mixing of the lipids. The solution is then subjected to ethanol evaporation to eliminate the organic solvent to give a coarse liposomal solution. Later, liposomes are sonicated and homogenized for further downsizing (Pons et al., 1993).

1.3.6(c) Detergent Depletion Method

The detergent depletion method is a mild process for producing a wide variety of vesicle types and highly homogeneous liposomes. The method is based on the formation of detergent-lipid micelles, followed by the removal of the detergent to form liposomes. Detergent-lipid micelles can be formed by either hydrating a lipid with a detergent solution or by drying both lipid and detergent from an organic solution then adding an aqueous solution. The detergent associates with the lipid, protecting the hydrophobic sections from interacting with the aqueous phase, and thus micelles are formed rather than vesicles. Afterward, the detergent is removed from the micelle solution, using dilution by 10 to 100 fold, dialysis, column chromatography or adsorption, to form the vesicles (Betageri, 1993).

Despite dilution being the simplest method for detergent removal, the disadvantage is that the final concentration of liposomes in the solution is low and entrapment of any hydrophobic compounds is poor. The detergent might also remains in the formulation and should be removed via other methods. The size and

homogeneity of liposomes produced using detergent depletion are based on the rate at which the detergent is removed and the initial ratio of detergent to phospholipids. The method is very time consuming and equilibration of the micelles can be a slow process. Another potential disadvantage of the process is that the methods used to remove the detergent may also remove any other small hydrophilic compounds (Betageri, 1993).

1.3.6(d) Emulsion Method

The production of liposomes using emulsion method generally involves the formation of a water-in-oil emulsion through the addition of a small amount of aqueous media to a larger volume of immiscible organic solution containing the phospholipids. The mixture is agitated to disperse the aqueous media as tiny droplets throughout the organic solvent and the lipid aligns itself into a monolayer at the boundary between the organic and aqueous phases. The size of the droplets can be controlled by the agitation applied and the amount of lipid present. There must be sufficient lipid to surround the droplet or it may fuse with other droplets. The water-in-oil emulsion is transformed into a liposomal suspension through the formation of a double emulsion. The organic solution containing the water droplets is added to a large volume of aqueous media and agitated, producing a water-in-oil-in-water emulsion. A lipid monolayer also forms around the organic droplets producing aqueous cores surrounded by two lipid monolayers that are separated by an organic layer (Betageri, 1993). Unilamellar liposomes with high entrapment of the initial aqueous media can then be formed by the removal of the organic solvent, for example, by passing a stream of nitrogen through the double emulsion. The lipid, solvent composition and shaking time period were controlled so that multiple water

droplets become contained within a single organic droplet in the double emulsion. Once the organic solvent was removed, the droplets formed multiple compartments within a single lipid vesicle, usually 1–2 μm in diameter. Multivesicular liposomes provide the potential to deliver multiple compounds that are unstable when combined (Mozafari, 2005).

1.3.7 Excipients used in Formulating Liposomes

1.3.7(a) Cholesterol

Cholesterol is one of the major components found in membranes and is also an additive used widely in formulating liposomes (Sideratou et al., 2000). Their role includes membrane organization, dynamics and function (Kepczynski et al., 2008). Insufficient quantity, cholesterol aids in liposomes stability (Sivakumar and Rao, 2001). Cholesterol also increases packing density of the phospholipids molecules and reduced the bilayer permeability to ions and solutes (Semple et al., 1996). The presence of cholesterol exerts a profound influence on the properties of the lipid bilayers of the liposomes. It has been known for several decades that the addition of cholesterol to a fluid phase bilayer (mainly unsaturated lipids) decreases its permeability to water (Sivakumar and Rao, 2001). A liposome that is made from 100% unsaturated lipid in fluid phase cannot hold its encapsulated content and the encapsulated water soluble drugs will leak out over time. Therefore, the addition of cholesterol is necessary in order to prevent the leakage of the encapsulated drug from the liposomes.

Cholesterol prevents the leakage of drug as the molecules fill in the free space that was formed due to the kink in the chain of the unsaturated lipids and this

decreased the flexibility of the surrounding lipid chains. This interaction also increases the mechanical rigidity of the fluid bilayers and decreases their lateral diffusion. In contrast, the addition of cholesterol to gel phase bilayers (mainly saturated lipids) disrupts local packing orders and increases the diffusion coefficient and decreases the elasticity of the vesicle. Liposomes made from 100% saturated lipids are leak-proof in the absence of the cholesterol. One of the main reasons for addition of cholesterol to liposomes that are made from saturated lipids is to increase the phase transition temperature. The phase transition temperature (Tm) of the mixture of the lipids in the liposomes will be increased, but not eliminated, by the addition of cholesterol. Cholesterol increases the Tm indicating that the incorporation of cholesterol increases the thermal energy and changing its state from the solid-gel to the liquid crystalline phase. This enables the retention of more drugs due to its colloidal stability (Drummond et al., 2008) and increased circulatory lifetimes of liposomes (Kirby et al., 1980).

Furthermore, it has been suggested that the cholesterol increases the half-lives of circulating liposomes as a result of a dual functionality of membrane cholesterol (Patel et al., 2011). First, as mentioned previously, cholesterol decreases membrane permeability and affords the bilayer a greater resistance to destabilization by blood components. Second, it has been demonstrated that cholesterol-rich liposomes possess a lower affinity for uptake by hepatic Kupffer cells, suggesting that cholesterol inhibits the binding of serum opsonins to liposomes (Semple et al., 1996).

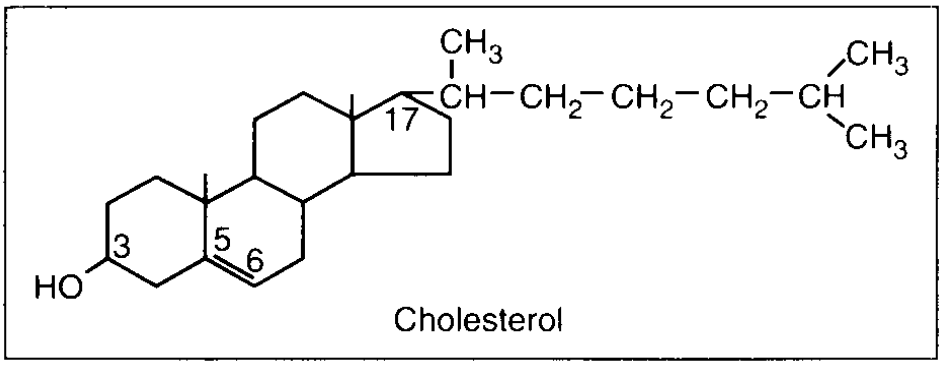


Figure 1.5: The Structure of Cholesterol (Ahmad, 1994)

1.3.7(b) α-Tocopherol

α-tocopherol, the major component of Vitamin E, is a lipid-soluble hydrocarbon compound that partitions into lipid storage organelles and cell membranes. It is an efficient scavenger of lipid peroxyl radicals and, hence, it is able to break peroxyl chain propagation reactions in cellular membranes preventing lipid peroxidation. Considerable evidence suggests that liposome-encapsulated antioxidants can be superior to the corresponding free antioxidants in this regard (Xiong et al., 2009). α-tocopherol is able to reduce the negative effects of high phospholipid concentrations and perhaps permit the use of liposome concentrations that would otherwise be toxic (CR et al., 1998). In a study with model membrane systems, α-tocopherol was shown to be intercalated into phospholipid bilayers with the long axis of the molecule oriented parallel to the lipid hydrocarbon chains, and it was able to rotate about its long axis and diffused laterally within fluid lipid bilayers. Because of its membrane stabilizing effect, α-tocopherol has been used in the preparation of liposomes for the delivery of several drugs (Suntres and Shek, 1994).

The maximum amount of α-tocopherol that can be contained in egg phosphatidylcholine or phosphatidylcholine liposomes is approximately 33%. α-tocopherol alters the membrane characteristics of liposomes by making them more stable and less permeable to aqueous solutes and highly resistant to protein-induced disruption. The suppression of protein-induced disruption is more pronounced with α-tocopherol than with cholesterol even at lower molar ratios. In addition, α-tocopherol in liposomes can undergo spontaneous intermembrane transfer to an acceptor membrane without the fusion of the protein with α-tocopherol liposomes. Thus, liposomes containing α-tocopherol (15 to 30 mol %) may be useful for delivering physiological quantities of this vitamin component or other drugs to cells in culture or to tissues *in vivo* (Sarkar et al., 2002).

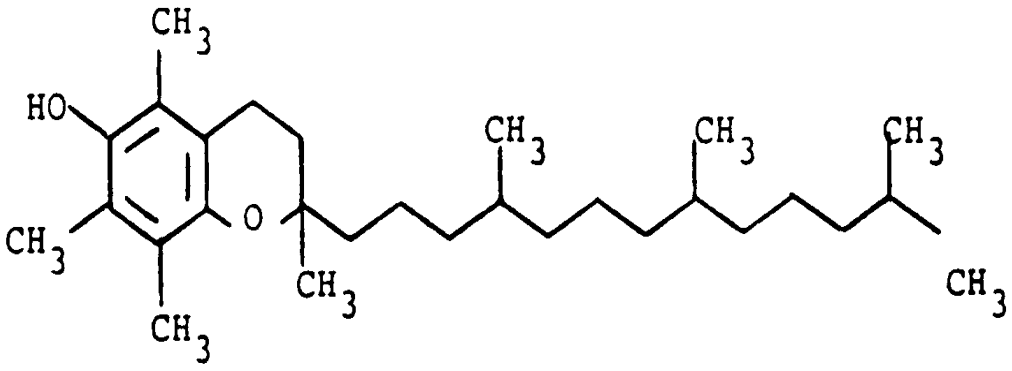


Figure 1.6: The Structure of α-tocopherol (Reto et al., 2003)

1.3.8 Advantages of Liposomes Application in Drug Delivery

One of the most important characteristics of liposomes is that they are avidly phagocytosed by macrophages and other cells of the reticuloendothelial system upon intravenous injection (Bru et al., 2002). As a result, they make excellent delivery system for many purified antigens. Some of the other properties of liposomes, notably entrapment and retention of virtually any pharmacologically active agent, structural versatility and an apparently innocuous nature, were attributes of a potentially powerful tool for the control of drug action (Huang and MacDonald, 2004).

There are many other reasons to use liposomes as drug carriers. The first is the solubilisation. Liposomes may solubilise lipophilic drugs that would otherwise be difficult to administer intravenously. Second is protection; liposome encapsulated drugs are inaccessible to metabolizing enzymes; conversely, body components (such as erythrocytes or tissues at the injection site) are not directly exposed to the full dose of the drug. Third is prolonged duration of action; liposomes can prolong drug action by slowly releasing the drug in the body. Fourth is the directing potential whereby targeting options change the distribution of the drug through the body. The fifth reason is internalization. Liposomes are endocytosed or phagocytosed by cells, where liposome dependent drugs as the case of leishmaniasis can be up taken by cells (Proulx et al., 2001) The sixth reason is amplification where liposomes can be used as adjuvant in vaccine formulation. The drawbacks of buparvaquone in treating leishmaniasis due to its solubility and hydrophobic nature make liposome a logical drug carrier of buparvaquone. Hence the formulation study of buparvaquone was carried out.

1.4 Leishmaniasis

Neglected Tropical Diseases (NTDS) caused by bacterial, protozoan and helminthes which are responsible for approximately 534,000 deaths yearly include leishmaniasis, African trypanosomiasis, dengue fever, malaria, schistosomiasis, tuberculosis, chagas disease, leprosy, lymphatic filariasis and onchocerciasis (Nwaka and Hudson, 2006). These are endemic diseases in tropical and sub tropical regions (Croft and Yardley, 2002). These tropical infections were used to be found throughout the more temperate climates of North America and Europe. These diseases were eradicated from North America and Europe as a result of good sanitation, hygiene, and vector-control methods, as well as a rise in the standards of living. Economic factor rather than climate contributes to the occurrence of these infections now considered "tropical" and is found mainly in poorer, developing countries. Countries like Iran and Afghanistan exhibit high rates of tropical disease even without tropical climate (Sharma and Singh, 2008).

Leishmaniasis is a parasitic disease caused by infection of 20 different *Leishmania* parasites. The disease is transmitted between mammalian host by the bite of *Phlebotomus* sand flies infected by the parasites (Sharma and Singh, 2008). Leishmaniasis takes several forms of occurrence and the most common forms is cutaneous leishmaniasis that holds responsible for skin sores. Whilst visceral leishmaniasis the more fatal form of the parasite infects the internal organs of the body (for example, spleen, liver, and bone marrow) (Croft et al., 2006).

1.4.1 Signs and Symptoms of Leishmaniasis

People infected by cutaneous leishmaniasis would commonly have one or more sores on their skin that over time changes in its size and appearance. The sores may end up as an ulcer with appearance similar of a volcano (a raised edge and central crater). Some people would develop sores that are covered by a scab. The degree of pain differs from case to case.

As for visceral leishmaniasis the symptoms would include fever, weight loss, and an enlarged spleen and liver. It also causes abnormal blood counts. For example, patients would usually have low blood counts for red blood cell count (condition known as anemia) or low white blood cell count, and low platelet count. Symptoms of leishmaniasis do not presume in all cases. However, after the sand fly bite, the skin sore of cutaneous leishmaniasis usually develops within a few weeks or months. For cases of visceral leishmaniasis patients usually become sick within months or even as long as years after being bitten by the leishmaniasis vector (Croft and Yardley, 2002)

1.4.2 Distribution of Disease

Each year 1.5 million cases of cutaneous leishmaniasis and 500,000 new cases for visceral leishmaniasis are recorded (Croft et al., 2006, Venkatesh et al., 2009). Leishmaniasis is found in focal areas of about 88 countries on the world map. Some of these countries have records for the world's highest occurring cases of leishmaniasis:

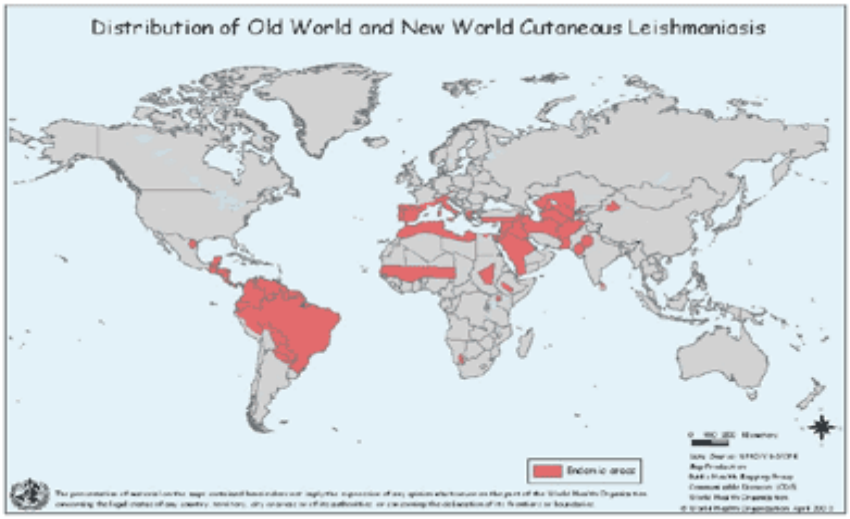
a) More than 90% of reported cases for cutaneous leishmaniasis occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia (also known as Old World) and for the New World, it includes Brazil and Peru. N.Africa, South Asia, Middle

East, Tunisia Ethiopia, India, Sudan, Bangladesh, China, Central and West Asia, Central and South America, Kenya, Mexico, Belize, Guatemala and Ecuador (Refer to Figure 1.7 a).

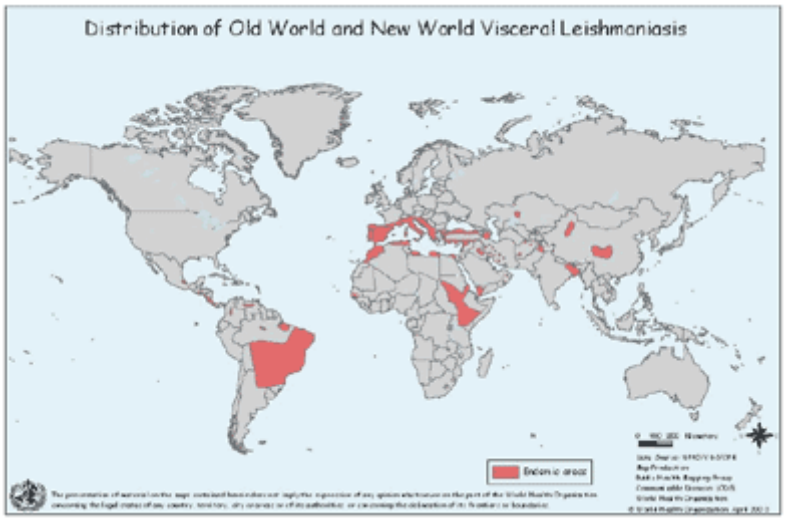
b) Over 90% of cases recorded for visceral leishmaniasis (Organization, 2014) were in countries including India, Bangladesh, Nepal, Sudan and Brazil have Venezuela, Brazil, Peru, French, Guiana, Guyana, Surinam, Panama and Costa Rica (Refer to Figure1.7 b).

1.4.3 Epidemiology

Humans are mainly infected by several *Leishmania* species that include *Leishmania donovani,* which causes visceral leishmaniasis also known as kala azar; *Leishmania tropica* and *Leishmania brasiliensis* which cause cutaneous leishmaniasis. Although it has occurrence worldwide, the predominant occurrence of visceral leishmaniasis is encountered in India, South America, Central Asia, the Middle East, and Africa. As for cutaneous leishmaniasis caused by *L tropica,* the most common encounters are along the shores of the Mediterranean, throughout the Middle East, central Africa, and parts of India (Croft et al., 2006). The form of cutaneous leishmaniasis by *L brasiliensis* is more confined to Central America and South America (Croft et al., 1991, Sharma and Singh, 2008).



(a)



(b)

Figure 1.7 Distribution of (a) cutaneous leishmaniasis and (b) visceral leishmaniasis in the world **(World Health Organization, 2014)**

Primarily a zoonotic disease, leishmaniasis can be caused by 20 species of protozoa pathogenic for humans belonging to the genus Leishmania (Quellette et al., 2004).