Liposome and Their Applications in Cancer Therapy

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ABSTRACT

Liposomes, the vesicles of phospholipid bilayer, can encapsulate both hydrophilic and lipophilic drugs and protect them from degradation. Liposomes have been extensively studied and continue to create intense interest in research since their discovery in the mid-1960s. Since then, liposomes have been considered to be the most successful nanocarriers for drug deliver and have made their way to the market. Currently, a number of liposomal formulations are on the marker for cancer treatment and many more are in pipeline. This review discusses about the liposome components, methods of preparation, drug encapsulation mechanism and the potential therapeutic applications of liposomes in cancer therapy.

Keywords: Liposomes, Drug delivery, Cancer, Doxil, LipoDox, Myocet

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INTRODUCTION

Cancer is the major health concern of the century because of the leading cause of death worldwide (Fitzmaurice et al. 2015; Torre et al. 2015). It kills millions of people every year and its burden continues to rise at an alarming rate globally (Stewart and Wild 2014). Cancer is the uncontrolled growth of cells, which occurs due to the accumulation of genetic mutations and aberrant signaling of various pathways related to the growth and survival of the cells (Bhardwaj et al. 2014; Tyagi et al. 2014; Deshmukh et al. 2015; Srivastava et al. 2015a; Srivastava et al. 2015b). The complexity at genetic and phenotypic levels in cancer cells leads to the clinical diversity and therapeutic resistance in cancer cells. Chemotherapy is most commonly used treatment among a variety of approaches currently being used for the treatment of cancer, which, however, possesses several limitations and side effects (MacDonald 2009; Ramirez et al. 2009; Iwamoto 2013). According to an estimate, more than 90% cancer drugs exhibit poor bioavailability and pharmacokinetics (Iwamoto 2013). Therefore, there is a prerequisite to develop appropriate drug delivery systems, which can improve the bioavailability, pharmacokinetic properties and can deliver the active drug molecules to the site of action, without affecting the healthy cells.

To overcome the limitations of conventional chemotherapy, a number of nanocarrier delivery systems have been developed and extensively used for drug delivery to cancer cells (Tyagi et al. 2011; Tyagi et al. 2013; Arora et al. 2015). Nanocarriers have larger surface area as compared to bigger particles, which can be easily modified to encapsulate large amount of drug, to increase the blood circulation time and to enhance the accumulation of drugs in solid tumors via the enhanced permeability and retention (EPR) effect as well as selective targeting of tumor cells (Tyagi and Ghosh 2011; Allen and Cullis 2013; Bozzuto and Molinari 2015).

Nanocarriers also improve the solubility, bioavailability and pharmacokinetics properties of chemotherapeutics (Gregoriadis and Florence 1993; Bozzuto and Molinari 2015; Pattni et al. 2015). Currently, a variety of nanocarriers such as liposomes, polymeric nanoparticles, micelles, nanotubes, etc are already in the market, or under research and evaluation for cancer treatment (Sutradhar and Lutful 2014). This review summarizes the types of methods used for the preparation of liposomes, mechanism of drug loading and potential therapeutic applications in cancer therapy and provides current information on the liposomal products, which are either in clinical use, or clinical trials.

LIPOSOMES

Bangham (Bangham et al. 1965) for the first time observed that phospholipids in aqueous medium forms closed bilayer structures. Later, these closed bilayer structures were termed as liposomes by Sessa (Sessa and Weissmann 1968). The liposome comprises of an aqueous compartment surrounded by one, or more lipid bilayers (Gregoriadis and Florence 1993; Pattni et al. 2015).

Initially, liposomes were used to study the physical behavior of biological membranes like lipids orientation in bilayer, physiochemical characterization of lipids and ion transport across bio membranes (Bangham 1972; Gregoriadis and Florence 1993). However, now liposomes are extensively used for drug delivery as they meet all the requirements of a good delivery vehicle. Liposomes are biodegradable, biocompatible, and stable in colloidal solutions (Akbarzadeh et al. 2013; Allen and Cullis 2013). Liposomes protect the drug from degradation and reduce drug-related nonspecific toxicity and can be produced and formulated easily for the target specific delivery (Bitounis et al. 2012; Bozzuto and Molinari 2015).

TYPES OF LIPOSOMES

Liposomes can be classified on the basis of size and the number of phospholipid membrane layers (Akbarzadeh et al. 2013; Pattni et al. 2015) as depicted in Figure 1.

Multilamellar Vesicles (MLV): These liposomes are composed of a number of concentric phospholipid bilayer membrane separated by aqueous phase. These are big in size and may be up to 5 μm.
Small Unilamellar Vesicles (SUV): These liposomes are composed of aqueous compartment enclosed by a single lipid bilayer. The size of these liposomes may be in the range of 20-100 nm.

Large Unilamellar Vesicle (LUV): These liposomes are also composed of a single lipid bilayer surrounding aqueous compartment. The size of these liposomes is in the range of 100-250 nm.

**Figure 1- Classification of liposomes based on the lamellarity:** (A) Multilamellar Vesicles (MLV) is composed of many lipid bilayers and ranges from 1-5 µm in size. (B) Large Unilamellar Vesicle (LUV) is in the size range of 100-250 nm with single lipid bilayer. (C) Small Unilamellar Vesicles (SUV) consists of a single phospholipid bilayer surrounding the aqueous phase with size range 20-100 nm.

**COMPONENTS OF LIPOSOMES**

The major components of liposomes are phospholipids and cholesterol, major constituents of natural bio membranes. The chemical properties of these lipids control the behavior of liposomes.

**Phospholipids**

The most common phospholipids used for the preparation of liposomes are natural (egg, or soy) phosphatidylcholine, or synthetic phosphatidylcholine (PC). The natural phospholipids such as egg, or soyabean phospholipid contain substantial levels of polyunsaturated fatty acids making them less stable than the synthetic equivalents (Jing Li et al. 2015). The molar percentage of phospholipids varies from 55 to 100% of total liposomal components (Bozzuto and Molinari 2015; Jing Li et al. 2015). The most common phospholipid component of liposomes is 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) is an example of a functional phospholipid used to conjugate other polymers like polyethylene glycol (PEG) (Laouini et al. 2012; Marques-Gallego and de Kroon 2014; Jing Li et al. 2015) (Figure 2B). The type, molar percentage and packing orientation of phospholipids determine the ultimate shape and size of the liposomes (Farge and Devaux 1992; Jing Li et al. 2015).

The orientation of phospholipids in liposome bilayer depends upon the length of lipid molecules and the size of head groups (Laouini et al. 2012; Jing Li et al. 2015). The phase transition temperature (Tc) of phospholipids is also an important criterion to choose phospholipid for the preparation of liposomes (Laouini et al. 2012; Bozzuto and Molinari 2015; Jing Li et al. 2015). The phase transition temperature is defined as the temperature at which the lipid physical state converts from an ordered gel phase to a disordered liquid crystalline phase. The conversion of phases depends on hydrocarbon chain length, degree of saturation, charge, and head group species (Bitounis et al. 2012; Laouini et al. 2012; Bozzuto and Molinari 2015).
The use of phospholipids with higher phase transition temperatures generates bilayers, which are more stable (Ellens et al. 1986). This decreases the possibility for premature leakage of encapsulated components; however, considerations must be made to ensure that encapsulated drugs can still escape the liposomes once they reach the target site of action. On the other hand, if the phase transition temperature of the selected phospholipids is too high, denaturation of the encapsulated drugs may occur during the sizing, or loading processes (Bitounis et al. 2012; Laouini et al. 2012; Jing Li et al. 2015). Therefore, a good balance must be met to guarantee that the selected lipids have phase transition temperatures that prevent premature leakage of components but enable processing to occur at temperatures that are harmless to all liposomal components.

Figure 2 - Chemical structures of common liposomal components: (A) 1, 2-distearoyl-sn-glycero-phosphocholine (DSPC) (B) Cholesterol and (C) 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine polyethylene glycol (DSPE-PEG).

**Cholesterol**

The rotational freedom because of flip-flop movements in phospholipids generates liposomes of leaky properties. Cholesterol is the main component added in the liposomal formulations to stabilize the bilayer of liposomes (Laouini et al. 2012; Magarkar et al. 2014). Depending upon the rigidity and fluidity of bilayer, the molar percentage of cholesterol varies from 30-45% of total liposomes components (Kirby and Gregoriadis 1980; Laouini et al. 2012) as it provides membrane fluidity, elasticity, permeability and stability to liposomes (Kirby and Gregoriadis 1980; Magarkar et al. 2014). The chemical structure of cholesterol is presented in Figure 2C.

The polar head of cholesterol is aligned with the polar head of the phospholipids of lipid bilayer. Due to hydrophobic properties of cholesterol, it resides in the interior portion of lipid bilayers and serves to fill the gap created because of imperfect packing of phospholipid molecules. The packing of cholesterol within phospholipid bilayers prevents the flip-flop of membrane components and the movement across the membranes (Kirby and Gregoriadis 1980; Farge and Devaux 1992). Cholesterol also provides the rigidity to liposomes as it prevents the phase transition of lipid bilayers, and thus reduces the leakage of encapsulated drugs (Manes and Martinez 2004). Therefore, the percentage of cholesterol used for the preparation of liposomes also affects the ultimate phase transition temperature of the bilayer. Some studies have suggested that the cholesterol also helps in protecting the lipid bilayer from hydrolytic degradation (Simon et al. 1982). Depending on the final application of liposomes, many other components in addition to phospholipid and cholesterol have been used. Depending upon the component used, liposomes can be neutral, negative, or positively charged. The charge on the surface of liposomes plays an important role in deciding the fate and application of the liposomes (Miller et al. 1998; Tyagi et al. 2011). PEG is the other commonly used liposome component typically incorporated to increase the blood circulation times because of its stealth properties and has shown broad applications (Miller et al. 1998; Immordino et al. 2006; Tyagi and Ghosh 2011; Tyagi et al. 2013).
METHODS OF PREPARATION

There are several methods for the preparation of liposomes such as solvent removal, detergent removal, emulsion removal and ethanol injection (Laouini et al. 2012; Bozzuto and Molinari 2015). The type of preparation methods influences the properties of liposomes, including their shape, size, stability and drug loading efficiency. Thin lipid film hydration, or solvent removal method is the most common and first described method for liposome preparations (Akbarzadeh et al. 2013; Bozzuto and Molinari 2015). Briefly, the lipids are dissolved in chloroform and/or methanol mixture. The concentration of lipids is typically in the range of 10-20 mgmL$^{-1}$ depending on the solubility of lipids. The solvent is subsequently removed by a rotary evaporator under reduced pressure to produce a thin film of lipids. The thin film so formed is desiccated for required time, followed by hydration. Hydration of the dry lipid film is accomplished by adding aqueous solution, which has the osmolarity in physiological range. After completion of hydration, the liposomes of multilamellar vesicles (LMV) in the size range of 200-1000 nm are produced (Laouini et al. 2012; Akbarzadeh et al. 2013). These MLVs are broken down into smaller liposomes by sonication, or extrusion.

Sonication is generally performed in water bath type sonicators and the temperature of water is maintained above the Tc of lipids. Sonic waves disrupt the outer layers of gaint liposomes and produce small unilamellar vesicles (SUV), ranging between 20-100 nm in diameter (Laouini et al. 2012; Akbarzadeh et al. 2013). The final size of liposomes not only depends upon the sonication time and energy but also upon many factors, including lipid composition, concentration and suspension volume. Alternatively, the liposomes are passed through the extrusion assembly containing a polycarbonate membrane of definite size to reduce the size of MLVs. This process is also performed under high pressure and at a temperature above the lipid Tc. The final size of extruded liposomes tends to be close to the filter pore size. Extrusion through filters with 100 nm pores yields large unilamellar vesicles (LUV) of reproducible size.

ENCAPSULATION OF DRUGS INTO LIPOSOMES

The methods of drug encapsulation in to the liposomes can be divided into two sub groups. The passive loading in which drug encapsulation occur during the vesicle formation process and the active loading in which drug is entrapped after the formation of vesicles.

Passive loading

Passive loading is to encapsulate the drug during the formation of liposomes. The hydrophilic drugs are loaded within the internal core of the liposomes by mixing with the hydrating buffer used to hydrate the thin lipid film during the formation of liposomes. Lipophilic drugs are mixed with other liposome components during the preparation of thin dry film of lipids and ultimately loaded into lipid bilayers. The un-entrapped drug molecules are removed from liposome suspension by dialysis, or gel-filtration chromatography (Tyagi et al. 2011; Tyagi et al. 2013).

The encapsulation efficiency depends on lipid concentration, liposome size, choice of lipids, etc. The encapsulation efficiency of water-soluble compounds, which do not interact with the lipid bilayer, is relatively low if loaded by passive method and proportional to the aqueous volume enclosed in the liposomes (Tyagi et al. 2013). Large vesicles will have higher encapsulation efficiency than small vesicles (Akbarzadeh et al. 2013). While the drug that interacts with lipid bilayer, such as lipophilic compound, normally have better encapsulation rate.

Therefore, several strategies have been developed to improve the encapsulation efficiency by linking lipophilic chain to drug molecule to increase its lipophilicity and better partition into the lipid bilayer (Sutradhar and Lutfil 2014; Bozzuto and Molinari 2015). Choice of lipid composition is also critical for better loading efficiently by this method. For example, to load highly negatively charged nucleotide compounds, such as antisense or siRNA, selection of cationic lipid will greatly improve the encapsulation efficiency due to enhanced drug/lipid interaction.
Active loading

Certain weakly acidic, or alkaline drug molecules are loaded into preformed liposomes by active loading, or remote loading method. This process is driven by an electrochemical potential created by the pH, or ion gradients established across the lipid bilayer of the liposomes (Akbarzadeh et al. 2013; Bozzuto and Molinari 2015). The pH, or ion gradients are created during the liposomes preparation by using a buffer of specified pH and ion concentration. The external pH of liposomes is then exchanged with another buffer of different pH, or ion concentration through dialysis, or size exclusion chromatography. After creating the pH gradient across the liposomes membranes, drug is loaded by mixing with liposomes typically at a temperature above the phase transition temperature of the lipids to ensure the fluidity and efficient transport across the bilayer. The drug molecules interact with the ions within liposomes and get charged. The charged drug molecules are not capable to come out and remain entrapped within lipidome core.

Doxil™, liposomal doxorubicin, is the ideal example of the active loading by pH gradient method (Lasic et al. 1992; Haran et al. 1993). The active loading of doxorubicin by pH gradient method is shown in Figure 3. As shown in the figure, when the gradient of citrate buffer liposome is more than 1000 times to the citrate buffer of medium then a pH gradient is created. Doxorubicin, a weak base, is in equilibrium between an ionized state and a non-ionized state. The latter can cross the lipid bilayer, become ionized in the high proton intraliposome environment and leads to high efficient accumulation of doxorubicin inside the liposome. The another example of pH-gradient method is the loading of chloroquine diphosphate into liposomes (Qiu et al. 2008). In another study, oxymatrine- major active alkaloid constituent extracted from the traditional Chinese herb medicine *Sophora flavescens*, used for treating hepatitis B in clinical therapy in China, was also loaded into liposomes by pH gradient method (Du and Deng 2006).

**Figure 3- Active loading of drugs into liposomes:** Liposomes were prepared by hydrating in citrate buffer (●) and then external phase was exchanged with Na2CO3 (●) to create a pH gradient. (C) The neutral form of the externally added drug (●) can cross the bilayer and is protonated (■) and trapped inside the vesicles.

APPLICATIONS OF LIPOSOMES IN CANCER

Liposomes have been successfully used in cancer therapy. Although, the application of liposomes in the field of cancer therapeutics has been extensively studied and deserves a broad assessment but this is outside the scope of this review. However, the most successful applications of liposomes in cancer therapeutics are discussed here. A number of different liposomal formulations of anti-cancer agents have been shown to deliver the drug at the site of solid tumors with minimum toxicity as compared to free drug (Allen and Cullis 2013; Sutradhar and Lutfi 2014). Currently, there are a many products in the market and in clinical development for use as anti-cancer drug delivery vehicle (Allen and Cullis 2013) (Table 1). Doxil, a PEGylated liposomal formulation, is the first liposomal product that was approved by the FDA for the treatment of kaposi’s sarcoma in AIDS patients (James et al. 1994; Barenholz 2012). Doxil (US), or Caelyx (outside-US) is a PEGylated liposomal formulation encapsulating anticancer
drug doxorubicin commercialized by Johnson & Johnson. In 2011, an imbalance between the demand and supply of Doxil was observed as the manufacturing unit was shut down temporarily due to some quality control issues (Berger et al. 2014; Chou et al. 2015). To address the Doxil shortage in USA, FDA allowed temporary importation of LipoDox. LipoDox is the same liposomal formulation as Doxil in USA and made in India by Sun Pharma and in 2013, FDA approved the first generic version of Doxil, made by Sun Pharma (Berger et al. 2014; Chou et al. 2015).

In a study, it was observed that Doxil was also active against refractory ovarian cancer, and later approved by the FDA for the treatment of recurrent ovarian cancer also (Muggia 1997; Barenholz 2012). Recently, it has been approved for the treatment of breast cancer (Barenholz 2012) in USA and for the treatment of multiple myeloma in combination with velcade in Europe and Canada (Blade et al. 2011; Barenholz 2012). DaunoXome, the registered trademark of Galen, is the liposomal formulation of daunorubicin approved by the FDA for the treatment of AIDS related kaposi’s sarcoma (Cooley et al. 2007; Petre and Dittmer 2007). Myocet, the registered trade mark of Cephalon, is a non-PEGylated liposomal formulation of doxorubicin. Myocet in combination with cyclophosphamide was approved for the treatment of metastatic breast cancer in Europe but was not yet approved by the FDA for use in the United States (Batifet et al. 2001).

The Sopherion Therapeutics in the United States and Canada is conducting a pivotal phase III global registrational trial of Myocet in combination with Herceptin (trastuzumab) and Taxol (paclitaxel) for the treatment of highly aggressive HER2-positive metastatic breast cancer (Baselga et al. 2014). The liposomal formulation of vincristine made by Talon was registered under trade name of Marqibo. Marqibo was approved in 2012 by the FDA for the treatment of acute lymphoblastic leukemia (Sarris et al. 2000; Rodriguez et al. 2009).

Celator Pharmaceuticals Inc developed CPX-351, a liposomal formulation of cytarabine and daunorubicin. The CPX-351 showed promising results in phase III clinical trial on the patients with secondary acute myeloid leukemia (AML) by improving the induction response over 40% (Riviere et al. 2011; Cortes et al. 2015). Previously in phase II trial, CPX-351 had already showed a survival benefits and the data on over survival could be expected in the first quarter of 2016 (Lancet et al. 2014). Another liposomal formulation of Celator contains irinotecan Hcl and floxuridine and registered as CPX-1. The CPX-1 completed phase II clinical trial on the patients with advanced colorectal cancer (Batist et al. 2009). MM-398 is a liposomal sphere encapsulating irinotecan developed by Merrimack pharma. MM-398 is being evaluated in the clinical trials for its ability to treat various cancers, which are resistant to chemotherapy such as pancreatic, colorectal, lung and glioma (Ko et al. 2013; Roy et al. 2013; Saif 2014). Another liposomal formulation developed by Merrimack pharma is MM-302, which encapsulates doxorubicin. MM-302 is designed for selective uptake of drug into tumor cells while sparing off healthy tissues. MM-302 contains a novel antibody-drug conjugated on the surface that specifically targets cancer cells overexpressing the HER2 receptor. Currently, MM-302 is being evaluated in phase I clinical trials for its ability to treat advanced metastatic HER2-positive breast cancer (Geretti et al. 2015). MBP-426 is transferrin receptor targeted liposomal formulation of oxaliplatin designed by Mebiopharm. MBP-426 is being evaluated in phase II clinical trial for the treatment of patients with gastric cancer (Suzuki et al. 2008; Goldberg et al. 2013).

Lipoplatin is the liposomal formulation of cisplatin designed by Regulon Inc. and currently, it is being evaluated in phase III clinical trial for the patients with non-small cell lung cancer (Fantini et al. 2011). Another liposomal formulation Stimuvax is designed as anti-MUC1 cancer vaccine by Oncothyreon to treat non-small cell lung cancer and presently is in phase III clinical trial (Bradbury and Shepherd 2008; Fantini et al. 2011; Broglio et al. 2014). The thermo sensitive liposomal formulation of doxorubicin, called ThermoDox (Celsion) is under phase III clinical trial to treat the patients with primary hepatocellular carcinoma, in phase II for refractory chest wall breast cancer and colorectal liver metastasis (Poon and Borys 2011; Staruch et al. 2011).
CONCLUSION

Liposomes have revolutionized cancer therapy by their broad clinical applications. Liposomes overcome the limitations of conventional chemotherapy by improving the bioavailability and stability of the drug molecules and minimizing side effects by site-specific targeted delivery of the drugs. Liposomes were the first nanotechnology-based drug delivery systems approved for the clinical applications because of their biocompatibility and biodegradability like features. Some liposome-based drug delivery systems are already in the market and many more are undergoing research and clinical trials. So far, liposomes have established themselves in nanocarriers-based drug delivery systems as evident by the successful clinical applications of liposomal formulations in anti-cancer therapy.

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