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Critical Review

Nanoliposomes: Historical Perspectives and Major Advances in Cancer Therapy. A critical Review

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ABSTRACT

Since the discovery of liposomes by Bangham and co-workers in 1960, giant strides in the field of liposomes as drug carriers for cancer therapy have been achieved. From the very first generation of liposomes known as conventional liposomes to the stealth liposomes which are surface decorated with polyethylene glycol attachments to protect the liposomes from phagocytic attack, and finally, a more recent nano-liposome delivery innovation which involves specific antibody targeting and stimulus sensitivity. Advanced research on enhancing the stability of liposomes through various smart surface modifications has been ongoing. Some liposomal formulations have made it from benchtop research to pharmaceutical manufacturing and marketing. Commercially available are stealth liposome formulations which have been approved for use, including Doxil® (a PEGylated liposomal formulation of doxorubicin hydrochloride). These formulations have shown increased bioavailability at the target site compared to conventional drugs.

INTRODUCTION

Nano-liposomes are nano-sized phospholipid vesicles composed of two concentric spherical lipid bilayers, entrapping a distinct aqueous compartment (Worsham et al; 2019). They have a large surface area and are relatively stable compared to larger-sized liposomes. The spherical bilayer configuration is spontaneously formed when lipids are dispersed in an aqueous medium by the introduction of external kinetic energy in a bi-phasic system. This energy could be through mixing, agitation, extrusion, or sonication from ultrasound energy (Samad et al; 2007). Chemically a surface-active agent may be introduced into the system to lower surface tension and facilitate vesicle formation (Ahmed et al; 2020). Chemical and physical interactions that influence the alignment of phospho-lipid molecules in liposome formation include van der Waals molecular interaction; steric repulsion and attraction; hydrogen dipole attraction and steric stabilization. Phospholipids are generally composed of one polar hydrophilic head (usually structured as a functional group) and two hydrophobic tails composed of hydrogen and carbon (hydrocarbon chains). Depending on the structure of the functional group liposomes are either positively charged, negatively charged, or neutral (Dufont et al; 2012; Zsadzinski et al; 2011). Phospholipids assemble based on their charges and interaction with molecules of the surrounding media which is aqueous. The molecular structure of the phospholipids, the length of the hydrocarbon tail, and the size and charge of the polar heads determine the molecular alignment and spontaneous spherical lipid formation (Akbarzadeh et al; 2013). The polar head (composed of phosphate and choline functional groups) of the lipid is attracted to the aqueous molecules of water which is composed of covalently bonded positively charged hydrogen

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atoms linked together by an oxygen atom. The hydrocarbon tail of lipid molecules is sterically repelled away from the aqueous phase molecules, whilst the polar heads are attracted to molecules of the aqueous media. Molecular interaction between the hydrophilic head groups, hydrophobic tails, and the individual molecules of aqueous solvent forming the solution foster the bilipid alignment (Silvander; 2002). The phospholipids, therefore, naturally align into a spherical arrangement with their polar ends oriented toward the aqueous compartments whilst their lipophilic hydrocarbon tails are oriented away from the aqueous compartment (Samad et al; 2007). Liposomes possess both aqueous inner and non-aqueous bilayer compartments that can act as carriers for both water-soluble and non-water-soluble drugs (Akbarzadeh et al; 2013). Liposomes which are soft drug carriers owe their versatility to their ability to house a drug molecule to its site of action for release regardless of its chemical or physical properties. Hydrophilic drugs can be conveniently dissolved in the core while lipophilic drugs with higher log P values can be entrapped in the lipid bilayer region of the liposome (Vijay et al; 2010; Moles et al; 2019). Liposomes act as carriers for a wide variety of drugs, water-soluble drugs such as anticancer agents e.g. Doxorubicin and Daunorubicin can be entrapped in the aqueous core. Non -water-soluble drugs, can be encapsulated in its bilipid layer this includes peptides, RNA, DNA, diagnostic or imaging agents, Gadolinium, and Iron. (Vemuri and Rhodes, 1995; Thakur et al; 2019).

Liposomes as nano-carriers for drug delivery can modify the pharmacokinetics and pharmacodynamics of the drug. As nano-carriers, they can successfully alter the biodistribution of the drug through the phenomenon called the "enhanced permeability effect" (Gaglardi et al; 2021). This phenomenon causes the drug to accumulate selectively at the target site alone (Gabrielle-Madelmont et al; 2003). This development greatly improves treatment outcome as a higher percentage of the drug reaches the site of action and less of the drug is lost during circulation, leading to a reduction in the overall side effects experienced in cancer therapy. It has however been realized that to have 100% drug accumulation at the target site, more strategic means must be adopted through research (Senapati et al; 2018).

Optimal drug delivery may be achieved through surface modification of the liposomes, using target ligands such as proteins, antibodies, folates, and peptides. This phenomenon is known as active targeting (Worsham et al; 2019; Khan et al; 2020). The release of the drug at the target site is the conclusive step and is, therefore, an especially important step in drug delivery; it may be assisted using stimulus-responsive liposomes called temperature-sensitive liposomes or pH-sensitive liposomes, which allow for controlled and timely release of the drug encapsulated in the liposome (Attia et al; 2019). The temperature-sensitive liposomes operate using heat in the form of high-intensity focused ultrasound which is impacted on the cancer cells once the liposomes have accumulated at the tumor site (Tharkar et al; 2019). The drug is then released from the liposomes, it is either absorbed into the cancer cells by the chemical process of intracellular uptake or into the tumor vasculature. In the case of pH-sensitive liposomes, the liposomes release the loaded drug in an environment of low pH such as the tumor target site. This review aims to discuss the historical perspective of the advancement of liposome research and the various strategies; active targeting and controlled release based on stimulus-response, with the hope of the achievement of an ideal drug delivery system, providing minimal side effects during chemotherapy and complete elimination of cancer cells (Gyamera and Kim, 2019).

Historical Perspective (Discovery of Liposomes)

The first occurrence of liposomes was in December 1932, J.Y Johnson applied for a British patent of pharmaceutical preparation called “depot” the formulation was capable of slow release of the medicament without any detriment to the patient. Depot which was intended as an intramuscular or subcutaneous injection could be prepared by combining active pharmaceutical medicament with an oily or lipid phase sourced from fat this could be in combination with aqueous fluids and emulsifiers like beeswax, vegetable wax, and petroleum wax. The slow-release property observed in the formulation, was likely due to the formation of liposomes by the suspended lipids, allowing a slow release of drug and prolonged duration of action (Bulbake et al; 2017). The formulation contained lecithin, cholesterol, and water, in a ratio that can be referred to as a baseline formula for preparing liposomes.

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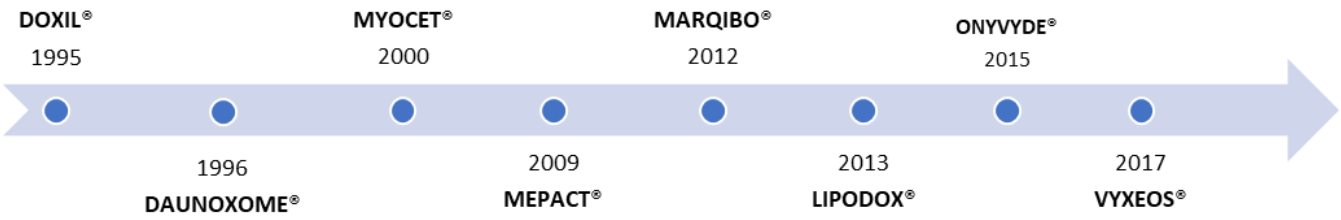

Figure 1.: Timeline showing liposomal formulations for cancer therapy and their year of FDA (Food and drug administration) approval.

Mid-19th Century

The actual discovery of liposomes was by Alec. D Bangham and co-workers in Cambridge in the 1960s. They observed that phospholipids self-assemble into bilayers when dispersed an aqueous phase and curl to form spherical shells known as vesicles. The primary interest in liposomes spurred from the fact that they resembled biological cells and can be used to study many cellular processes, also their physiological biocompatibility has made them suitable vehicles for drug delivery. Since then, liposomes have received tremendous attention as potential carriers for drug delivery during chemotherapy. Liposomes are made up of lipids that are non-toxic and bio-compatible hence very suitable as nano-carriers for cancer drugs. The continuous research on liposome technology has advanced from conventional liposomes to sterically stabilized liposomes and finally ligand-targeted liposomes (Worsham et al; 2019)

Millennial Decades

This is the decade of liposome-based chemotherapeutics which is a pharmaceutically established technology to improve the safety profile of cancer chemotherapeutics such as doxorubicin, paclitaxel, topotecan, vincristine. The liposome encapsulates these cytotoxic drugs in its internal aqueous compartment and releases them at the tumor site (Ledezma-Gallegos et al; 2020). Liposomes take advantage of poor vascularization of cancer tissues and accumulate at the target site through the phenomenon known as enhanced permeability and retention effect. (Hadjidemetriou et al; 2019). The approval of the first liposomal drug, Doxil ® (stealth liposome) in 1995 marked a major milestone in liposomal delivery.

Unfortunately based on clinical performance the high level of clinical efficacy expected from the drug was not achieved at the initial stages (Barenholzk, 2002). Since then, many more liposomal formulations have been approved and reached the market (Kamel et al 2019). Examples of such liposomes include Daunoxome® which was approved by FDA 1996 for the treatment of tumors, lymphomas, and sarcomas. Daunoxome® consist of small unilamellar vesicles made up of distearoylphosphatidylcholine and cholesterol. An increase by ten times was observed in the level of daunorubicin delivery to the cancer cells in comparison to the conventional daunorubicin. Another liposomal formulation of doxorubicin called Myocet® was approved in the year 2000. It showed high stability during systemic circulation and effective encapsulation efficiency of the drug doxorubicin. There was also increased targeting of tumor tissues and minimal toxicity. These factors observed led to a more effective drug delivery of doxorubicin by Myocet® (He et al; 2019). A more recent FDA approved liposomal formulation is Vyxeos®. Vyxeos® is a liposomal formulation loaded with cytarabine and daunorubicin used in the treatment of acute myeloid leukemia. Vyxeos® was shown to improve survival rates of older patients with secondary acute myeloid leukemia (Lancet et al; 2018; Deutsch et al; 2018).

Chemical modifications such as attachment of poly-ethylene-glycol (PEG), creation of stimuli-responsive liposomes, and antibody or peptide targeting have been adapted over years of research to improve the stability, and clinical efficacy of liposomes (Cavadas and Gonzalez-Fernandez, 2011). Mid millennial decades featured; peptide targeted-temperature sensitive liposomes being investigated in combination with focused ultrasound in the treatment of cancer under the guidance of fluorescent imaging. Peptides were covalently coupled to temperature-sensitive stealth liposomes using a method of preparation adaptable for large-scale preparation of liposomes for

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clinical trials. The active peptide targeting also ensured the accumulation of liposomes at the target site while the stimulus, thermo-sensitivity enhanced release of the drug, at the target site. The combination of active targeting and stimuli response aimed to ensure delivery of the cancer drug specifically, to the cancer tissues, thereby eliminating common side-effects and improving the clinical efficacy of the administered drug (Al-Ahmady et al; 2015). Liposome-loaded siRNA for codelivery of SN38 prodrug was investigated in a study by Bi et al, the liposomal drug delivery system was shown to be more effective in inducing tumor inhibition in xenograft tumor-bearing nude mice (Bi et al; 2018). Even more recent, is the use of enzyme, redox, light, or magnetic sensitivity to improve liposome release and cellular uptake of loaded cancer drug molecules. In a study by Enzian et al in 2020, light-sensitive liposomes were formulated and incorporated with photoactive molecules. The light which served as the remote release trigger was carefully controlled temporally and spatially. Liposomes were loaded with the fluorescence molecule calcein. A high concentration of calcein inside the liposomes encouraged quenching while the release of calcein from liposomes stopped the quenching effect hence an increase in fluorescence intensity. Effective release of calcein was observed upon irradiation at 420nm, an increase in permeability of the lipid membrane when liposomes were exposed to light (Enzian et al, 2020). In another study by Wei et al, redox-sensitive liposomes were formulated and loaded with camptothecin. An enhanced release of camptothecin was observed in reductive conditions with enhanced cellular uptake. Invitro and in vivo anticancer assays showed improved pharmacological efficacy of camptothecin (Wei et al, 2019). A combination of light, magnetic, and heat sensitivity is seen in the scientific investigation by Dorjsuren et al, where temperature-sensitive liposomes were formulated and loaded with magnetic nanoparticles for targeted delivery of anticancer drug through near infra-red triggered release and combined photothermal-chemotherapy (Dorjsuren et al; 2020).

Table 1.0 Shows liposomal formulations that have passed the clinical stage, have been approved and made it to the pharmaceutical market. (Chang and Yeh, 2012, Ferreira et al; 2019; Fukuda et al; 2017; Beltrán-Gracia et al; 2019; Bulbake et al; 2017).

MAIN METHODS OF PREPARATION OF LIPOSOMES

Mechanical Dispersion Method

The process of lipid film hydration and agitation produces multi-lamellar vesicles, which are then subjected to membrane extrusion or micro fluidization to produce unilamellar vesicles. Attempts have been made to address the problem of drug leakage from liposomes. Applications such as freeze-drying or air-drying, have been considered however during these processes care must be taken to ensure that the physical structure and the physicochemical properties of the formulated liposomes remain intact. To avoid damage to the liposomal membranes during the process of freeze-drying, cryoprotectants may be introduced. Cryoprotectants are usually used to protect tissues from damage by freeze-drying, they act by penetrating and increasing the solute content of cells, their application in the protection of liposome membranes has been considered useful (Zsadzinski et al; 2011). The introduction of gels to the interior aqueous layer of the liposome's membranes has also been considered. Liposomes may then be reconstituted after freeze-drying when needed. Most times a mixture of more than one mechanical dispersion method is engaged to achieve stable effective liposomes of uniformed sizes.

Solvent dispersion method

The two main methods are the ether injection method and the ethanol injection method. In the first method, the lipids are dissolved in an organic solvent (ether) and injected into a solution containing the anticancer drug at low pressure and a temperature range between 55-65^{o}C (Cavadas et al; 2011) The ether is then removed by creating a vacuum resulting in the formation of liposomes. The latter method involves dissolving lipids in ethanol before injecting the mixture into a buffer solution, the ethanol is then removed. This results in well-formed liposomes, however, the liposomes formed may vary in size (Immordino et al; 2006)

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Table 1. List of liposomal formulations in the market.

DRUG	LIPOSOME TYPE	LIPID COMPOSITION	INDICATION	STAGE (FDA APPROVAL DATE)	PRODUCT NAME	REFERENCES
Vincristine	PEGylated liposome	CHOLESTEROL AND EGG SPHINGOMYELIN (45; 55) Molar ratio	Metastatic malignant melanoma	Market (2012)	Marqibo®	(Silverman and Deitcher, 2013)
Doxorubicin	Liposome	DPPC, MSPC and PEG 2000-DSPE (90;10;4) Molar ratio	Non-resistible hepatocellular carcinoma	Not Approved	Thermodox®	(Lyon et al; 2017)
Doxorubicin	Liposome	HSPC, cholesterol and PEG 2000-DSPE (56;39.5)	Kaposi Sarcoma and breast cancer	Market (1995)	Doxil®	(Barenholz; 2012)
Doxorubicin	PEGylated liposome	DSPC, cholesterol and PEG 2000-DSPE (56;39.5) (molar ratio)	Kaposi Sarcoma and breast cancer	Market (2013)	Lipo-Dox®	(Chou et al; 2015)
Doxorubicin Hydrochloride	Non-PEGylated Liposome	Cholesterol and Phosphatidyl choline (45:55)	In combination with cyclophosphamide for the treatment of metastatic cancer	Market (2000)	Myocet®	(Batist et al; 2002)
Paclitaxel	Liposome	72g PC, 10.8 Cholesterol in ethanol	Gastric, ovarian, and lung cancer	Market (2015)	Lipusu®	(Wang et al; 2018)
Mifamurtide	Non-PEGylated Liposome	DOPS: POPC (3: 7 Molar ratio)	Osteosarcoma	Market (Europe) (2009)	Mepact®	(Liu et al; 2012)
Irinotecan + fluorouracil + folinic acid	PEGylated Liposome	DSPC, cholesterol, and methoxy-terminated polyethylene glycol-distearoylphosphatidyl ethanolamine (3:2:0.015)	Metastatic adenocarcinoma	Market (2015)	Onivyde®	(Ur Rehman et al; 2016)
Daunorubicin citrate	PEGylated Liposome	Distearoylphosphatidylcholine: cholesterol: daunorubicin (10:5:1)	Metastatic Breast cancer	Market (1996)	DaunoXome®	(Pillai; 2019)
Daunorubicin + Cytarabine	Liposome	Distearoylphosphatidyl choline, distearoyl phosphatidylglycerol and cholesterol (7:2:1)	Secondary acute myeloid leukaemia	Market (2017)	Vyxeos®	(Krauss et al; 2019)

Detergent Removal Method

In this method, liposomes are derived from already formed mixed detergent micelles with solubilized, phospholipids in an aqueous phase. The detergent is then removed by dialysis and the micelles become rich in phospholipids. This then leads to the formation of liposomes of similar size; however complete removal of the detergent during the process may pose a major challenge (Rajan et al; 2010).

Electro-formation Method

Electro-formation is the most used method for the formation of unilamellar liposomes. It involves the

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use of metal electrodes (Stainless steel, Copper, or Platinum) (Bellon et al; 2018). An electrical field is established by the electrode (cathode and anode). It works based on the principle of electrostatic and osmotic forces facilitating lipid swelling leading to liposome formation. Lipids such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine may be used (Behuria et al; 2020).

Table 2. Methods of preparation of liposomes for passive loading of anticancer drugs (Akbarzadeh et al 2013).

Mechanical dispersion method	Sonication French pressure cell extrusion Freeze-thawed liposomes Lipid film hydration Reconstitution of dried vesicles Micro-emulsion Membrane extrusion
Solvent dispersion method	Ether Injection Ethanol Injection Reverse phase evaporation
Detergent removal method	Dialysis Detergent (alkyl glycoside, Triton X-100 Gel-permeation chromatography Dilution
Electro-formation method	Electric field Swelling Anode Cathode
Microfluidic Method	Fluid movement Spontaneous-liposome formation
Heating Method	Phase transition temperature Heating
Supercritical fluidic Method	Supercritical fluids Cosolvent Atomization High-Pressure Vessels

Microfluidic Method

Microfluidics can be classified into two groups. Continuous-flow microfluidic and digital droplet-based microfluidics, while nanoliposomes are formed from continuous-flow microfluidics. Microfluidics enables precision in the size and size distribution of liposomes (Yu and Lee, 2009). It is particularly applied in the production of nanoliposomes. Microfluidics harnesses the principle of manipulation of liquid flow through microchannels. Incredible control of fluidic movement and mixing is relevant to achieving the required nanoparticulate size (Koloucek et al; 2020). Liposomes are formed based on a diffusion-controlled process whereby dissolved lipid spontaneously assemble into liposomes and isopropyl alcohol rapidly diffuses and forms a bi-aqueous stream at the interfacial region. Liposomes formed are within the range of 100nm-300nm (Carugo et al; 2016).

Heating Method

This is a favorable method, for liposome preparation as it does not involve the use of organic solvents. In this method, phospholipids are dispersed in a hydrating medium for about 60 minutes then heated beyond the phase transition temperature of the mixture of phospholipid for another 60 minutes. Due to the high-temperature conditions in which liposomes are formed further purification of liposomes is usually unnecessary (Nkanga et al; 2019).

Supercritical Fluidic Method

In supercritical fluid-assisted liposome formation, the supercritical fluid acts as a solvent or co-solvent for the lipid and phospholipid (Santo et al; 2014). Aqueous droplets are formed by atomization in a high-pressure vessel containing phospholipids, ethanol, and carbon-dioxide (Maja et al; 2020). The droplets are surrounded by lipid layers thereby creating liposomes (William et al 2020).

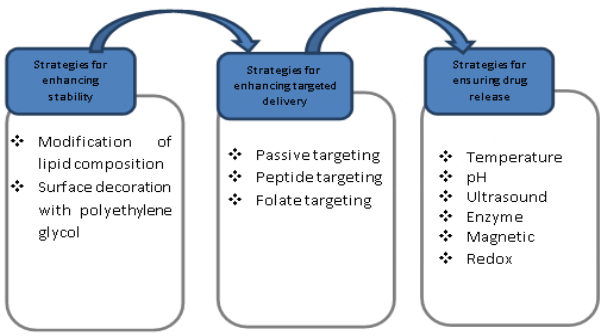

Figure 2.: Showing the transition of liposomes through various strategies to improve drug delivery efficiency.

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STRATEGIES FOR ENHANCING THE STABILITY OF LIPOSOMES

Modification of lipid composition

The complete chemical composition of liposomes has a direct effect on their pharmacokinetic properties and stability during circulation in the body. Liposomes are generally composed of lipids, phospholipids, and fatty acids (Barenholzk, 2002). The phospholipids constitute the largest percentage of the three components. Phospholipids consist of a polar head and non-polar hydrocarbon tail which makes them amphipathic a property responsible for their ability to spontaneously form stable bilayers. The inclusion of phospholipids with longer chain lengths results in higher van der Waals attraction between the lipids that make up the membrane thus promoting stronger cohesion and resulting in more stable liposomes (Chang and Yeh, 2012). The charge of the polar head is especially important in terms of stability of the liposome as this determines the level of electrostatic interaction that will exist between liposomes and other charged molecules such as encapsulated drugs. Liposomes made up of phospholipids with negative or positively charged polar heads tend to be less stable as they can interact with oppositely charged molecules and cause membrane instability, but liposomes composed of neutral phospholipids do not have this problem (Chang and Yeh 2012).

In a situation whereby a mixture of phospholipids is used as is the case most of the time a balance in net charge occurs thereby maintaining stability. The addition of fatty acids and cholesterol leads to an increase in the rigidity of the liposome bi-lipid membrane, cholesterol reduces the rotational freedom of the phospholipid hydrocarbon chains, which then leads to an overall increase in the stability of the bilayer (Varga et al; 2012).

Stealth Liposomes (Polyethylene Glycol)

One major problem with conventional liposomes is the short circulation time. Once administered intravenously into the body liposomes are instantaneously recognized by serum proteins called opsonin and the mononuclear phagocytic system. This renders them susceptible to attack (Salmaso et al; 2013). The covalent attachment of polyethylene glycol to the liposome surface creates a steric barrier that prevents the liposomes from being recognized by opsonin and the mononuclear phagocyte system (Gyamera et al; 2019). The coating of liposome surface with a hydrophilic polymer PEG- polyethylene glycol renders it almost invisible to the mononuclear phagocyte system (Chang and Yeh, 2012). The polymer increases the hydrophilicity of the liposome surface. Hydration of the surface by the polymer provides a steric barrier that prevents liposomes from interacting with opsonin and other blood serum proteins (Maurer et al; 2001). The hydrophilic polymer may be covalently attached to a component of the phospholipid membrane (usually phosphor–ethanolamine) or maybe physically adsorbed onto the surface (Immordino et al; 2006; Patel et al; 2017). The thickness of the steric barrier depends on the grafting density (the amount of PEG attached to the surface), optimal stability occurs at higher percentages of PEG coating however above 15mol % PEG the stability reduces, and an increase in PEG will lead to disruption of the bilayer (Harris and Chess, 2003; Suk et al; 2016). The drawback in this situation is that although PEG enables escape from the opsonin and mononuclear phagocytic system it may reduce the chances of the liposome binding effectively or transferring its content intracellularly at the target site. This emphasizes the need for an effective means of ensuring target specificity and release of encapsulated drugs at the target site (Chaubey et al; 2020).

STRATEGIES FOR ENHANCING LIPOSOME TARGETED DELIVERY.

Passive Targeting

The enhanced permeability effect (EPR) is the basic governing principle in the formulation of nano-lipids as drug delivery carriers; it is also referred to as passive targeting. The relationship between the accumulation of liposomes at the target site and the EPR effect is unanimous, as both contribute constructively towards achieving the accumulation of cancer agents at tumor target sites. The mechanism of the EPR effect is largely based on the anatomical and physiological defectiveness of the tumor tissues (Sharma et al; 2018). The rapid growth of tumor tissues results in the clustering of tumor cells in multiples, for the cells to thrive and further expand the neo-vasculature of the cells are altered to enable

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the supply of a large quantity of oxygen and nutrients (Shashi et al; 2012). Alterations observed in the tumor vasculature and tumor environment include,

- Irregularities in vascular shape
- The excessive leakiness of vasculature and dysfunctional lymphatic drainage
- Extensive angiogenesis
- Copious production of permeability mediators such as bradykinin

The "EPR effect" capitalizes on these abnormalities at the target site this allows accumulation of liposomes at the target site. Unfortunately, research works by Maeda Hiroshi, 2015 on the EPR effect have argued that the efficacy of the EPR effect varies widely depending on the structure, vascular density, and etymology of the tumor sites. The EPR effect does not guarantee specific and selective targeting as it has been observed that normal cells are still affected by the loaded drug when released. (Maeda, 2015).

Active Targeting

"Active targeting" as the name implies devices a means by which a nano-drug delivery system delivers a drug actively to the target site. The main aim of active targeting is to increase the amount of drug that gets to the target site and reduce drug action on non-target sites. Active targeting can be achieved through modification of the liposome surface by efficiently linking a target ligand to the liposome. Ligands such as peptides, folates, antibodies, proteins, and glycoproteins act as target ligands. These ligands act by binding to receptors largely express on cancer cells alone, the molecular weight of these ligands range from 500Da to 150KDa (Pu et al; 2019). The first reference to active targeting was by Paul Erlich in 1906, he referred to actively targeted liposomes as magic bullets, and went on to describe them as particles that selectively target diseased cells without harming normal cells. The earliest attempt at delivery of a ligand-targeted drug to cancer cells was by Matte et al in 1958. Renewed interest in the field was spurred by the lack of specificity of passively targeted nanoparticles (Al Ahmady et al; 2015; Ren et al; 2021). The first approach to active targeting was by coupling nanoparticles with antibodies. Some level of success was recorded in terms of target specificity and many antibody-targeted liposomes were successfully developed between 1995 to 2005 (Allen et al; 2002; Biri Kovacs et al; 2020)

Folate Ligands

Receptors which are surface receptors for folic acid cannot be accessed in healthy cells during systemic circulation however they are widely expressed on the cell surface of tumors (Goli Samimi and C Annunziata, 2021). This makes it possible for various types of tumors to be targeted through folate-derived peptides or antibodies. These peptides or antibodies can bind to folate receptors at tumor cells. Coupling of folate-derived peptides with nanoparticles; hence attachment to the liposome surface will ensure specific targeting of tumors during drug delivery of the respective nanoparticle (Shashi et al 2012; Singh et al 2019). In a recent study by Park et al, Folate receptor beta targeted pH sensitive liposomes were formulated and loaded with a combination of doxycycline and docetaxel. Liposomes were acid responsive and released the synergistic combination of doxycycline and docetaxel at the tumor micro-environment. The anticancer drug combination showed a repressive effect on the tumor growth via inhibition of CAPN2 expression (Park et al; 2021).

Peptide Ligands

On-going research in this field has led to the identification of many peptides with different amino acid sequences and specific tumor receptors that may be attached to liposomes for drug delivery. Drug delivery by liposomes can be observed using imaging technologies such as high intensity focus ultrasound. Peptide targeted liposomes to deliver the drug successfully to the target site however leakage of the drug is observed during blood circulation. (Kono et al; 2001). Accumulation of the drug at tumor tissue may also be observed as well as an increase in tumor tissue elimination; however complete tumor elimination was yet to be achieved through the aid of peptide ligands (Varga et al; 2012). Challenges such as poor cellular penetration of cell-targeting peptides, inefficient mechanisms of overcoming cell defense mechanisms against cytotoxic drugs after cellular internalization of the liposome, cell detoxification by metabolizing enzymes, and cell deoxyribonucleic acid repair in response to chemotherapy-induced damage alongside other more intricate challenges hamper the success of peptide targeted liposomes (Gyanani et al; 2021). More technical challenges include difficulties in achieving effective coupling of peptides to stealth

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liposomes such that the ligand function of the peptides is not affected by the hydrophilic barrier (polyethylene glycol) (Sercombe et al; 2015).

Peptides may be used to target the ligands in association with the tumor cells, they may also be used to target tumor neo-vasculature or stroma or even specific subcellular compartments of cancer cells. Peptides can either attach themselves to the surface of the cancer cell or penetrate the cancer cells to function (Maryama et al 2002). Peptides are attached covalently to liposomes by covalent coupling techniques; a peptide can either be bonded to the lipid compounds on the surface of the liposomes or attached indirectly to the polyethylene compound decorated on the surface of the liposome (Argenziano et al; 2021). The first step in constructing a peptide targeted nanoparticle is to select or create the peptide-specific for the target tumor to achieve this a peptide library is used (Mitchell et al 2020). In a study by RGD-LP targeted liposomes were prepared and loaded with doxorubicin hydrochloride. *In-vivo* imaging reflected that RGD-LP caused a clear increase in aggregation of liposomes at target site. Release of doxorubicin showed high tumor growth inhibition (Ren et al; 2021).

Identification and selection of peptide ligands

A variety of peptides are screened against the cancer cell lines to pick the target peptide (Patra et al; 2010). Peptides that show an affinity for the cancer receptor cell lines or peptides that penetrate the cancer cell lines are usually identified as homing peptides or target peptides**.** To recognize ligand peptides which, target cancer cell lines, a combinatorial peptide library screening is used (Maruyama, 2002). For example, in breast cancer a human breast cancer xenograft this tissue will be used through phage display technique (Ferreira et al; 2019) The combinatorial library consists of varying peptides in free solution linked to a solid substrate (bead) or peptides arrayed on the surface of a micro-organism. Peptides are then inserted into a bacteriophage which then displays interactions between peptides and cancer cells, hence the name phage display (Ferreira et al; 2019).

STRATEGIES FOR ENSURING DRUG RELEASE

Temperature Sensitive Liposomes

Temperature-sensitive liposomes were discovered by Yatvin et al in the mid-'70s. Since then, the concept of heat-triggered nano-drug delivery has been explored. Temperature-sensitive liposomes are made up of several specifically selected phospholipids which have a small polar group and long hydrophilic chains (Al Ahmady et al; 2015). Temperature-sensitive liposomes become porous or permeable when heated to certain temperatures this ranges between (41- 43^{o}C, Tm) and is also known as the transition temperature (Motamarry et al; 2017).

Table 3. Various peptides and their target cell receptors and cancer cell lines, each peptide has a receptor specific to it (Ferreira et al; 2019).

PEPTIDE	PROTEIN SEQUENCE	RECEPTOR CELL	CANCER CELL TYPE	REFERENCES
GE11	HAIYPRH	EGFR	Lung cancer, cervical cancer, and liver cancer	(Huang et al; 2021)
HER-2 peptide	YCDGFYACYMDV	HER-2	Pancreatic cancer	(Biri Kovacs et al; 2020)
Tripeptide RGD	αvβ3	Neo-vasculature	Head and neck cancer	(Ren et al; 2021)
Anti-tumour anti-body derived peptides	EPPT	Cancer cells	Breast cancer Ovarian cancer or Colon cancer	(Aronson et al; 2020)
Pep-1	CGEMGWVRC	IL-13Rα2	Brain cancer	(Jiao et al; 2017)

At this point the liposome membrane changes from a gel to sol state, heating causes a change in the chemical structure which increases membrane permeability (Al Jamal and Zahraq, 2012). This phenomenon can be

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applied in drug delivery by allowing liposomes to accumulate at target site then introducing hyperthermia. One reliable method of introducing hyperthermia is through "High-intensity focus ultrasound" (Wang et al; 2018). High intensity focused ultrasound engages a combination of ultrasound energy, acoustic streaming, and mild hyperthermia; the ultrasound energy causes cavitation at the lipid membrane which leads to a collapse of a vapor cavity at the lipid bilayer membrane (Kono, 2001). The concept of temperature-sensitive liposome was first proposed by Yatvin and Weisten in the late seventies, but more research was carried in the area over the last decade. The change in their supramolecular structure (transformation from gel to liquid crystalline phase) when heated above its phase transition temperature leads to its permeability (Tagami et al; 2011). The T_m of each phospholipid depends on the length and saturation of the hydrocarbon chain. The melting phase transition temperature of the liposome formed is because of the individual transition temperatures of the phospholipids (Vemuri and Rhodes; 1995). Careful selection and the proper combination of phospholipids help to achieve the right level of temperature sensitivity of liposomes (Al Jamal et al; 2012). Inclusion of a lysolipid to the bilayer enhances the complete release of liposomal content through the formation of transient pores (Langereis and Grull, 2012; ten Hagen et al; 2010; Gabrielle-Madelmont et al; 2003). Prior to the application of high intensity focus ultrasound nanoparticles must accumulate at the target tissue. Migration of liposomes towards target site is monitored by magnetic resonance imaging (MRI). This gives thermal and spatial feedback on the location of liposomes, temperature change during heating, and release kinetics of liposome content (Needham and Kim, 2000)

pH-Sensitive Liposomes

pH triggered release takes advantage of the low pH at the target site (pH at tumor tissues is usually lower than the normal physiological pH) while the pH at the tumor site ranges from between 4-6 the pH of the blood is 7.4. Liposomes are stable at the pH of the blood during circulation but unstable at a lower pH in the tumor tissue environment this phenomenon is used to promote release at the tumor tissue target sites (Betrand et al; 2009). pH-sensitive liposomes are composed of specific phospholipids that alter or change their aggregate structure when the pH is changed. One major example is the use of phospho-ethanolamine, the lipid has a small polar head group that occupies less volume and large hydrocarbon chains (giving it a cone-shaped structure), thereby preventing the formation of bi-lipid layers (lamellar phase) instead a hexagonal phase is formed on aggregation (Murthy and Karanth, 2007). The phospho-ethanolamine molecules tend to promote interaction between the amine and phosphate groups of the polar head, this leads to the formation of a reverse hexagonal structure. To prevent the interaction and allow the formation of bi-lipid layers negatively charged amphiphilic phospholipids are introduced between the phospho-ethanolamine molecules this confers a degree of repulsion between the molecules reducing the interaction and enhancing stability and formation of a spherical bi-lipid layer (Casares et al; 2019). At lower pH protonation of the negatively charged amphiphilic phospholipid reduces the stabilizing effect allowing the formation of the hexagonal phase. Other options are the use of novel photosensitive lipids and synthetic fusogenic peptides or proteins which could be included in the bilayer (Fattal et al; 2004). Release of the drug into the cytosol is in three steps, first, the liposome binds to the cell at the target site, which is promoted by the presence of receptor-specific ligands that bind to receptors on target cells. The internalization of the cell by endocytosis, once internalized the liposome faces the risk of being degraded by the lysosome, however, due to low pH in the cell destabilization of liposomes prevents this degradation form from occurring. This results in the release of more drugs at the target site for pharmacological action (Simoes et al 2004).

In a study by Kanamala et al, dual pH sensitive liposomes with low pH triggered shedable PEG was developed. PEG_{2000} was chemically coupled with phosphatidylethanolamine. Cleavage of the PEG is triggered by at the tumor's microenvironment. The reason for designing a cleavable PEG is that the polymer presents an advantage and setback. PEG prevents liposomes from recognition by the bodies reticulo endothelial system, however it also hampers effective release of liposome content at the target site. The development of cleavable PEG at the target site solves the dilemma. The developed liposomes showed effective evasion of the endo reticular system

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during blood circulation and increased liposome accumulation at target site (Kanamala et al; 2019)

Light-sensitive Liposomes

The concept of light-triggered liposome delivery relies on the supply of an ideal light source at the right frequency and wavelength to enable penetration of tissues and photosensitization of nanoparticles and therapeutic compounds loaded in liposomes (Enzian et al; 2020). Photocleavable liposomes can be used to achieve drug release in response to light at a particular wavelength usually (700nm to 1100nm). The principle of drug release is based on the disruption of the liposome membrane by decomposition through irradiation (Heidarli et al; 2017; Lee and Thompson, 2018).

In a study by Enzian et al, liposomes were formulated with photoactive molecules. Al (III) phthalocyanine chloride disulfonic acid, benzoporphyrine derivative monoacid chlorine e6 and 5,10- di-(4-hydroxyphenyl)-15, 20-diphenyl-21, 23*H*-porphyrin. Liposomes encapsulated the molecule calcein and release of calcein was observed on irradiation of the loaded liposomes (Enzian et al; 2020).

Magnetic-sensitive Liposomes

Magnetic field sensitive liposome exhibits great potential in biomedical applications as they are biocompatible and have unique characteristics. Magnetic field liposomes can be prepared in three different ways.

- The addition of hydrophobic magnetic nanoparticles within the lipid bilayer membrane of the liposome.
- It can also be prepared by the addition of hydrophilic magnetic nanoparticles within the core of the liposome.
- Coupling of magnetic nanoparticles to the surface of nanoliposomes.

Magnetic nanoparticles are drawn towards magnetic force. A unique principle is the application of magnetic field liposomes along with heat triggering. Heat is developed by a remotely applied magnetic field focused on a particular region of the body (site of action). Heat-triggered drug release depends on the magnetic characteristics of the loaded nanoparticles, the frequency, and amplitude of the magnetic field, as well as the local environment (Heidarli et al 2017).

In an investigation by Shen et al, temperature sensitive liposomes were formulated and loaded with magnetic nanoparticles. Liposomes were further loaded with doxorubicin. Liposomes were designed for near infrared laser triggered release. Magnetic resonance imaging showed an outstanding accumulation of liposomes at target site. A combination of chemo-photothermal treatment showed that the formulation remarkably inhibited tumor growth with limited bystander effect (Shen et al; 2019).

Ultrasound Sensitive Liposomes

Ultrasound-sensitive liposomes work through the disruption of the liposome membrane thereby triggering the release of its content. Acoustic parameters and factors can be intelligently manipulated to energetic enough to facilitate drug release (Harris and Chess, 2003). Ultrasound frequency may be high or low. However low ultrasound is more effective as it can penetrate deeper into the tissues. Acoustic waves with frequencies above 20kHz can rupture the structure of the liposome bilipid membrane. Membrane disruption is caused by cavitation hyperthermia and acoustic streaming (Heidarli et al 2017; Liu et al; 2020).

In a study by de Matos et al, Mistletoe lectin-1 was loaded in ultrasound sensitive liposomes. Ultrasound liposomes were formulated by encapsulating perfluorocarbon nanodroplets in stealth liposomes. Release was facilitated by cavitation through high intensity focus ultrasound. An 80% release of Mistletoe lectin-1 was achieved on application of high intensity focus ultrasound highlighting its efficacy (de Matos; 2019).

Enzyme-Sensitive Liposomes

Enzyme-sensitive liposomes are fabricated to undergo structural transformation and release the encapsulated drug when in contact with enzymes secreted rapidly by the body in a pathological state. Drug release is therefore facilitated by enzyme presence at the target site without any more triggering. The quantity of drug released is proportional to the amount of active enzyme at the site of action. The mechanism of drug release is by enzyme-mediated hydrolysis of phospholipids which disrupts the integrity of the liposome bilayer. Drug

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release is affected by several factors like enzyme isoforms, lipid assembly, lipid physical properties, liposome composition, and the presence of a lipopolymer (Heidarli et al 2017; Almeida et al; 2020).

In a study by Mock et al, secreted phospholipase A2 responsive liposomes were formulated. Phospholipase A2 functions by splitting phospholipids to give lysolipids as well as fatty acids. Phospholipase A2 is over expressed in breast and prostate cancers. Formulation of phospholipase A2 responsive liposomes will facilitate the release of its cargo at the target site due to lipid bilayer cleavage. The phospholipase A2 sensitive liposomes were loaded with doxorubicin and applied in the treatment of prostate cancer in rats. The developed formulation was found to be almost three times more effective in shrinking tumor growth compared to pegylated drug loaded liposomes (Fouladi et al; 2017).

Redox sensitive liposomes

Redox-sensitive liposomes have breakable or destructible disulfide bonds that can be cleaved if the hydrophilicity or charge of the amphiphile is altered. This can be achieved using reducing agents or removal of a crosslinker bringing about liposome lipid phase transitions. Redox-sensitive liposomes maybe are prepared by a surface coating of liposomes with chitooligosaccharides through a disulfide linker. These surface coated liposomes are stable in the body system but are automatically disrupted when in contact with cytosolic level reducing agents in its surrounding environment (Heidarli et al 2017: Wang et al; 2021).

An example of redox sensitive liposomes was reported in a study by Wang et al, redox sensitive liposomes were designed and loaded with irinotecan. The redox sensitivity was investigated by studying the release of irinotecan from liposomes in the presence of glutathione (cancer cells produce glutathione) Based on the results it was observed that irinotecan loaded redox sensitive liposomes had better antitumor activity compared with free irinotecan and conventional liposomes loaded with irinotecan (Ortega et al; 2011; Wang et al; 2021).

CONCLUSIONS

Various modifications have been achieved with the intention of improving the activity of liposomes as a biological cargo in the field of drug delivery. From minor modifications in lipid composition to more obvious adjustments which include the incorporation of smart technologies such as stimulus sensitivity and tumour targeting. Through these adjustments liposomes have been able to overcome the problem of identification during circulation by the body's macrophages through the surface attachment of poly-ethylene-glycol (Cheng et al; 2021). Currently, strategies are being applied towards tackling the problem of inefficient accumulation and release of drugs at the target site. Such strategies include active targeting (cancer cell recognition) i.e., the use of antibodies, folates, and peptides which recognize and attach directly to cancer cells in a lock and key fashion. Some strategies in liposomal delivery are still in the research while others have been approved for clinical studies. Examples of such strategies in research include trigger mechanisms for drug release; pH and temperature-sensitive liposomes, enzyme sensitive liposomes, also the use of gold nanoparticles that can absorb infrared radiation in coating liposomes to enhance stability, reduce toxicity and improve release. Thermodox, a doxorubicin loaded temperature sensitive liposomal formulation is currently approved for phase III clinical trials and on its path to final FDA approval (An et al; 2021). Intriguing observations in the drug delivery abilities of liposomes indicate promising results. Expansion in the clinical use of liposomes is expected in the future, as more sophisticated liposomal formulations are being developed (Worsham et al; 2019; An et al; 2021).

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