REVIEW ARTICLE

Carbohydrate-Functionalized Liposomes In Cancer Therapy

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Abstract: Existing cancer treatments are often accompanied by adverse side effects that can greatly reduce the quality of life of cancer patients; this sets the platform for the development and application of nanocarrier-based platforms for the delivery of anticancer drugs. Among these nanocarriers, liposomes have demonstrated excellent potential in drug delivery applications. Furthermore, the overexpression of certain receptors on cancer cells has led to the development of active targeting approaches where liposome surfaces are decorated with ligands against these receptors. Given the central role that sugars play in cancer biology, more and more researchers are integrating "glycoscience" into their anticancer therapeutic designs. Carbohydrate functionalized liposomes present an attractive drug delivery system due to their biocompatibility, biodegradability, low toxicity, and specific cell targeting ability. This review presents an overview of the preparation methods, characterization, evaluation, and applications of carbohydrate functionalized liposomes in cancer therapy.

1. INTRODUCTION

Nanomedicine is an emerging field that employs nano-sized materials to diagnose and treat diseases. A wide variety of organic, inorganic, polymeric and metallic nanostructures have been used as targeted drug delivery vehicles including, dendrimers, micelles, solid lipid nanoparticles (SLNs), carbon nanotubes, and liposomes [1]. The efficacy of these nanoparticles (NPs) as nanocarriers depends on their size, shape, hydrophobicity, surface characteristics, and other chemical and biophysical features. Ideally, carriers used for drug delivery should be non-immunogenic, non-toxic, chemically and physically stable in *in vitro* and *in vivo* settings, have restricted drug distribution to the target site, have a predictable and controllable rate of drug release, and have minimal drug leakage while circulating in the body. In addition, carriers should be easily bio-degradable and freely eliminated from the body without any side effects [1, 2]. The overall goal of targeted drug delivery is to treat the disease effectively while minimizing adverse side effects [3].

Cancer is one of the four major non-communicable diseases, *i.e.*, heart disease, cancers, lung disease, and diabetes, which are considered the leading causes of death worldwide [4]. Existing cancer therapies include surgery, chemotherapy, radiotherapy, hyperthermic tumor ablation, immunotherapy, photodynamic therapy, transplantation (applicable only for cancers of hematopoietic tissues), and gene therapy [5]. However, the existing treatment techniques present several issues, such as high-dose requirements, poor bioavailability, high toxicity, low therapeutic indices, development of multi-drug resistance (MDR), and non-specific targeting, which in turn results in adverse systemic side effects [3, 6]. Nanocarrier-based platforms enable the effective delivery of anticancer drugs into the tumor by exploiting the unique pathophysiology of the tumor and its microenvironment. Furthermore, many receptor molecules known to be overexpressed on cancer cells have been explored as docking sites for nanocarriers encapsulating anticancer-drugs and decorated with ligands against these receptors [6]. Among emerging nanocarriers, liposomes have demonstrated great potential in preclinical and clinical studies. A number of liposome-based products have been approved for the treatment of various cancers, and many others are in different phases of clinical trials. Some of the approved treatments are listed in Table **1**.

Carbohydrate based polymers have a long history in the pharmaceutical field. In traditional medicine, they have been used as immune-modulators, anti-tumor adjuvants and anti-inflammatory agents. Different carbohydrate-based polymers have been introduced into the synthesis and coating of nanostructures to impart their appealing properties such as bioavailability, biocompatibility, biodegradability, low toxicity, high chemical reactivity, and facile chemical modification to the developed nano-vehicles particularly in the delivery of anticancer therapeutic agents [12].

2. CARBOHYDRATES

Carbohydrates are organic compounds made up of carbon, hydrogen and oxygen in the proportion of 1:2:1, and the general formula used to represent many carbohydrates is $C_x(H_2O)_y$. The building blocks of carbohydrates are single sugars known as monosaccharides, such as glucose and fructose. When two monosaccharides are joined together, they

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Table 1. Commercially available liposomal treatments.

Name	Target	Drug	Nanocarrier	Ref.
Myocet™	Metastatic Breast cancer	Doxorubicin	Liposome	[7]
Abraxane	Metastatic breast cancer	Paclitaxel	Albumin bound	[8]
Doxil®	Ovarian cancer	Doxorubicin	PEGylated liposomal	[9]
Caelyx®	Kaposi's sarcoma	Doxorubicin	PEGylated liposomal	[10]
DaunoXome®	Kaposi's sarcoma	Daunorubicin	PEGylated liposomal	[11]

form disaccharides, such as sucrose (common table sugar); moreover, when chains of three or more monosaccharides are linked together, they form macromolecules called polysaccharides or glycans. Glycans are essential bio-molecules which provide structure and energy storage. In mammalian tissues, glycans are present in conjugate forms, including glycoproteins, glycolipids and proteoglycans, which are used for regulatory purposes. In terms of energy storage, cellulose found in plants and glycogen found in animals is two of the most commonly known energy-storing polysaccharides [13-16].

3. GLUCOSE UPTAKE BY CANCER CELLS

Many tumors exhibit increased glucose uptake. Several theories have been presented to explain this exacerbated glucose consumption including, the increase of hexokinase (HK) expression, the expression of modified glycan chains, the decrease of glucose-6-phosphatase (G6P) mediated glucose dephosphorylation, and the overexpression of sugar transporters [17-19]. The following sections detail each of the presented explanations.

3.1. Increased Expression of Hexokinase

It is widely accepted that cancer cells favor the anaerobic glycolytic pathway, even in the presence of sufficient oxygen, rather than oxidative phosphorylation for cellular energy generation. This results in increased glucose uptake and the fermentation of glucose to lactate, a phenomenon known as the Warburg effect [20]. This high rate of glycolysis induces the production of G6P, which through the HK reaction is a precursor of nucleotides necessary for DNA and RNA synthesis, and *via* triose phosphates derived from the glycolytic pathway (a precursor for the lipids required for membrane synthesis). The enhanced rates of energy, DNA, RNA and membrane production are all essential to match the rapid cell division that occurs during tumor growth and proliferation. In recent years, it has been established that the driving force for the increased glycolytic flux is the phosphorylation of glucose rather than sugar uptake; therefore HK, which catalyzes the first step of the glycolytic pathway (the phosphorylation of glucose) has attracted considerable attention in studies designed to clarify the molecular mechanisms underlying the high glycolytic rate associated with cancer cells [21]. Four isoforms of HK have been identified in mammalian cells, HK I, II, III, and IV. The four types of HK show high tissue specificity; with HK I being predominantly expressed in the brain and red blood cells, HK II in skeletal muscles and adipocytes, HK III has not been found to be

dominant in any tissue type and HK IV (also known as glucokinase) is specific to the liver and pancreas. According to studies conducted on the isozyme compositions of cancer cells, HK II and to a lesser extent, HK I, are highly overexpressed on malignant tumors [21, 22].

The increased activity of HKs in tumors can be attributed to two mechanisms, the first is the overproduction of this enzyme by cancer cells, and the second is the tendency of HKs to bind to the outer mitochondrial membrane. In 1981, Bustamante, Morris and Pedersen [23] inspected mitochondria from hepatoma cells and found that tumor mitochondria, unlike the mitochondria of healthy liver cells, contained bound HKs which constituted about 70% of the total cellular HK activity. Moreover, the study reported that the high glycolytic rate of the tumor cytoplasm was reduced markedly when the tumor mitochondria were removed; however, the glycolytic rate levels were restored almost entirely upon the addition of the HK-containing tumor mitochondria to the tumor cytosol. This indicated that a determining factor in this heightened glycolysis of many malignant cancers is mitochondrial bound HK [21].

3.2. Expression of Modified Glycan Chains

As mentioned earlier, glycans are carbohydrate-based polymers made by living organisms. Glycans are present in cells as free forms or conjugated to other molecules. The different classes of glycoconjugates include glycosphingolipids, proteoglycans, and glycoproteins. Some of the processes controlled by glycans in eukaryotic cells include protein folding, cell signaling, pathogen recognition, inflammation, tissue organization, and cell proliferation [16, 18]

Glycosylation is an enzymatic process that links saccharides to other saccharides, proteins or lipids [24]. Proteins can be glycosylated by the covalent attachment of a saccharide to a polypeptide backbone *via* nitrogen or oxygen linkages (N-linkage to asparagine or an O-linkage to serine or threonine). In addition, intracellular proteins can be modified with the O-linked N acetylglucosamine(O-GlcNAcylation) [24, 25]. Cellular glycosylation is a highly regulated multistep process. However, variations in glycosylation are quite common in cancer; in fact, aberrant glycosylation and the expression of modified glycan chains on tumors have been established as hallmarks of cancer progression and metastasis. The alterations of glycosylation in cancer cells include truncated O-glycans, branched N-glycans, diverse fucosylated and sialylated terminal structures, and variations in glycosphingolipid expression. Changes in glycans' expression can arise from a variety of mechanisms such as an un-

der or overexpression of glycosyltransferases and glycosidases, changes of glycosyltransferase localization within the secretory pathway (Golgi apparatus and endoplasmic reticulum), modified molecular chaperone activity, as well as metabolic alterations and donor substrate availability [24-27].

The clinical significance of analyzing altered glycans and glycosylation lies mainly in the cancer biomarker field. Several glycan antigens are currently used for detecting and monitoring the progression of tumors. CA19-9 or SLe[a] is a cancer antigen that was discovered in the serum of patients with colon or pancreatic cancers. CA125 recognizes mucin MUC16 and is used in monitoring ovarian cancer; moreover, truncated O-GalNAc glycans Tn, STn, and T attached to MUC16 from cancer cells enhance the diagnosis and monitoring of ovarian cancer. SLex-related glycans in lung and breast cancers can be used to monitor the residual cancer burden after surgery. In liver cancer, the core fucose on N-glycans can aid in early diagnosis. Other applications of glycans include glycan-targeted cancer therapies. The most successful of these trials is immunotherapy against certain tumor-associated gangliosides like GD2 in melanoma and against incompletely glycosylated mucins. The clinical trials of the N-glycan synthesis inhibitor swainsonine, unfortunately, revealed toxicity, whereas low-molecular-weight oligosaccharides of hyaluronan are proving to be useful therapeutically, particularly because they inhibit the pro-oncogenic influences of constitutive polymeric hyaluronan, namely drug resistance and signaling events induced by hyaluronan–CD44 interaction [27, 28]. Table **2** presents some of the previously mentioned carbohydrate antigens and the types of cancer they are most commonly associated with.

Table 2. Common carbohydrate antigen targets

Carbohydrate antigen	Cancer type
CA19-9 (SLe[a])	colon and pancreatic
CA125	ovarian
Truncated O-GalNAc glycans (Tn, STn, and T)	ovarian
SLex-related glycans	breast and lung
Polysialic acid (PSA)	non-small cell lung cancer
Globo-H	ovarian
SLeA	colorectal

3.3. Decrease of Glucose-6-phosphatase Mediated Glucose Dephosphorylation

The rapid proliferation of cancer cells often leads tissues to outgrow their blood supply leading to hypoxia and nutrient deficiency in the tumor microenvironment. As a result, cancer cells promote angiogenesis to increase their nutrient and oxygen supply [29]. This ability of cancer cells to continually adapt to their environment is driven by constant metabolic reprogramming. One example of this metabolic reprogramming is the Warburg effect, which was discussed earlier. However, the enhanced glucose uptake seen with the Warburg effect is not effective under hypoxic conditions because nutrient supply is insufficient. Therefore, cancer cells can activate other metabolic processes, such as glycogen mobilization, to provide intermediates for their augmented, reprogramed glycolytic pathway. A key enzyme in the regulation of the glycogenolytic pathway, preferred by cancer cells, is the glucose-6–phosphatase (G6Pase) complex, which is located at the membrane of the endoplasmic reticulum (ER) [30]. G6Pase hydrolyzes G6P into free glucose in the terminal step of gluconeogenesis and glycogenolysis. This reaction stems from the G6Pase complex, comprising the G6Pase catalytic subunit (G6PC) and the G6P translocase (G6PT), which is coupled functionally. G6PT transports G6P from the cytoplasm to the ER, where G6P is hydrolyzed by G6PC. Studies have found that G6Pase is absent in gluconeogenic tissue tumors such as hepatocellular carcinoma (HCC) and renal cell carcinoma, whereas G6Pase is overexpressed in non-gluconeogenic tissue tumors such as ovarian cancer and glioblastomas (GBMs) [31, 32]. Abbadi *et al*. [30] determined that G6Pase is overexpressed in GBMs when compared with healthy brain cells. They discovered that human-derived brain tumor-initiating cells (BTIC) use this enzyme to counteract the glycolytic inhibition induced by 2-deoxy-D-glucose (2DG) and sustain malignant progression. They proposed that the downregulation of G6Pase would make GBMs unable to survive glycolytic inhibition. Moreover, BTICs that survive G6Pase knockdown were found to be less aggressive (reduced differentiation, invasion and proliferation).

3.4. The Overexpression of Sugar Transporters

Tumor cells, like other cells, require energy for their growth and survival. In normal cells, glucose enters the cells through glucose transporter proteins [33]. There are two main families of transporters: GLUT (Na^+-independent sugar transporters or facilitative sugar transporters) and SGLT (Na^+-dependent sugar co-transporters). The main difference between the two families is that GLUTs transport sugar across the plasma membrane down the concentration gradient, conversely, SGLT proteins move sugars inside of cells against the concentration gradient. The major difference between sugar transporters in each group is the main site of expression. Each GLUT carrier shows excellent tissue specificity and displays particular features suitable for the energy requirements and proper function of the specific tissue where the GLUT is expressed. GLUT1 overexpression has been observed in many human cancers; moreover, the deregulation of GLUT1 expression was found to reflect the presence of alterations in different signaling pathways. The increased glucose uptake associated with cancer was found to be induced by activated ras or src oncogenes, which are key elements in the transduction of multiple signaling pathways. Similarly, SGLT1 induction was also found to be used by cancer cells to enhance their glucose uptake and their glycolysis so that cancer cells obtain sufficient energy for maintaining their rapid proliferation [19].

4. CARBOHYDRATE FUNCTIONALIZED NANO-CARRIERS FOR CANCER TARGETING AND THERAPY

From the previous discussion, it is evident that carbohydrates play an essential role in tumor progression and metastasis. As a result, there has been an increasing interest in functionalizing various NPs with different carbohydrate molecules and evaluating their therapeutic efficacy using various assays. The most common NPs to which carbohydrates were conjugated include, gold nanoparticles (AuNPs), quantum dots (QDs), magnetic nanoparticles (MNPs), dendrimers, various polymers, carbon nanotubes, and liposomes [34]. This review will focus on liposomes; however, some studies utilizing other nanoplatforms are presented in Table 3.

Table 3. Glyconanomaterial platforms for cancer targeting and therapy

Nanoparticle	Author (year)	Ref.
Gold nanoparticles	Brust *et al.* (1994)	[77]
	Sangabathuni *et al.* (2016)	[78]
	Zhang *et al.* (2018)	[79]
	De la Fuente (2001)	[80]
	Malek *et al.* (2014)	[81]
Magnetic nanoparticles	El-Boubbou *et al.* (2010)	[82]
	Cai *et al.* (2010)	[83]
	Lee *et al.* (2011)	[84]
	Shao *et al.* (2018)	[85]
	Conde *et al.* (2015)	[86]
Quantum dots	Ahire *et al.* (2013)	[87]
	Dalal *et al.* (2018)	[88]
	Ahire *et al.* (2015)	[89]
	Zayed *et al.* (2019)	[90]
	Santos *et al.* (2006)	[91]
Dendrimers	Sheng *et al.* (2008)	[92]
	Vannucci *et al.* (2003)	[93]
	Hulikova *et al.* (2011)	[94]
	Andreozzi *et al.* (2017)	[95]
	Studzian *et al.* (2017)	[96]
Carbon nanotubes	Hong *et al.* (2010)	[97]
	Fahrenholtz *et al.* (2015)	[98]
	Datir *et al.* (2012)	[99]
	Zheng *et al.* (2016)	[100]
	Cao *et al.* (2015)	[101]

Liposomes are artificial lipid bilayer vesicles formed of the same materials that make up cellular membranes. Liposomes are composed of different phospholipids arranged in concentric spheres surrounding a central aqueous core. The phospholipids' hydrophilic heads are directed towards the aqueous environments, whereas the hydrophobic tails are directed inwards, away from the aqueous environments. Over the past decades, liposomes have been extensively studied as drug, gene, imaging contrast agent, and antigen delivery systems. Liposomes are attractive as delivery vehicles because they are able to improve the stability, therapeutic efficiency, and pharmacokinetic properties of drugs while reducing their side effects on healthy cells. Liposomes are biocompati-

ble, biodegradable, non-toxic, and non-immunogenic. Moreover, liposomes have both lipophilic and aqueous environments making them useful for delivering hydrophobic, amphipathic, and hydrophilic drugs [18, 35, 36]

Liposomal surface functionalization unlocks the enormous potential in the application of liposomes. Various biomolecules have been conjugated to liposome surfaces for a variety of biomedical applications. One of the most significant breakthroughs in the development of functionalized liposomes was the decoration of liposome surfaces with poly(ethylene) glycol (PEG) as it imparted stealth properties onto these drug delivery systems. The presence of PEG on the surface of liposomes extends their blood circulation time while reducing their uptake by the organs of the reticuloendothelial system (RES). The presence of PEG also helps reduce vesicle aggregation, thus enhancing the stability of the liposomal formulations [37]. Carbohydrate-functionalized liposomes have been used to study carbohydrate–carbohydrate recognition events; however, the primary application of glycan-coated liposomes is to direct the targeted liposomes to cells or tissues where carbohydrate-binding proteins are upregulated [18]

Lectins are carbohydrate-binding proteins that are highly specific for sugar groups of other molecules [38]. A number of cells express the lectin receptors on their surfaces; hence when carbohydrate moieties are conjugated to liposomes, the resultant glycosylated vehicles will be recognized and endocytosed by lectin receptors [39].

Several liposome preparations have been reported that display various carbohydrates on the outer membrane surface of liposomes. The following sections present the recent advances in the preparation, characterization, and evaluation of carbohydrate-functionalized liposomes, as well as their applications as delivery systems of anticancer therapeutics.

5. CARBOHYDRATE FUNCTIONALIZED NANO-CARRIERS FOR CANCER TARGETING AND THERAPY

Several strategies have been developed and used for functionalizing the surface of liposomes with carbohydrate ligands. The two most commonly used techniques are the direct liposome formulation method and the post functionalization approach [35]. The direct liposome formulation approach involves the synthesis of the glycolipid ligand followed by the preparation of liposomes using other principal lipids. Various neoglycolipids have been synthesized by either chemical or enzymatic strategies. Espuelas *et al.* [40] synthesized a novel conjugate (Man_4K_3DOG) composed of a tetramannosyl head group connected, *via* a PEG spacer, to a lipid moiety. This amphiphilic molecule was then incorporated into the liposome bilayers. The multivalent mannose residues on the surface of the liposomes showed a much higher binding affinity for Concanavalin A than their monomannosyl analogues. Xu *et al.* [41] synthesized novel glycolipids containing 2 and 15 oligomaltose units and phosphatidylethanolamine. These novel glycolipids were characterized by Fourier-transform infrared spectroscopy (FTIR)

and proton nuclear magnetic resonance (¹H-NMR). The linear structure of the synthesized glycolipids was verified using an end-point conjugation strategy involving the selective oxidation of the reducing end groups of maltose oligosaccharides, followed by aminolysis with di-stearoyl phosphatidylethanolamine. Moreover, the glycolipids were used to form sterically stable liposomes, as determined by turbidity measurements. Zhang *et al*. [42] synthesized three types of galactosylated cholesterol glycolipid formulations (gal-PEG194-chol, gal-PEG1000-chol and gal-PEG2000-chol). The synthesized lipids were then used to prepare doxorubicin (DOX) liposomes. Flow cytometry and laser confocal scanning microscopy showed that the galactose-modified liposomes enabled the uptake of liposomes into the human liver cancer cell line HepG2 *via* the asialoglycoprotein receptor (ASGPR). Furthermore, cytotoxicity assays showed that the cell proliferation inhibition effect of galactose-modified liposomes was higher than that of the unconjugated liposomes. Garg *et al*. [43] covalently coupled β-d-1-Thiogalactopyranoside residues with dimyristoyl phosphatidylethanolamine. This glycolipid was then used to form liposomes. *In vitro* studies were conducted in order to assess ligand-specific activity. In addition, the drug release from liposomes was studied using the dialysis method and the percent cumulative drug release after 24 h was low (around 34.8%). The major limitation of the direct formulation method is that some of the carbohydrate targeting ligands may face the interior aqueous compartment of the formed liposomes, and become unavailable for interaction with their receptors [35, 39, 44]. To solve this issue, several research groups have opted for the post functionalization approach. Zhang *et al*. [45] developed a chemically selective liposome surface glycofunctionalization method based on Staudinger ligation. In the developed method, a carbohydrate derivative carrying an azide spacer was conjugated onto the surface of preformed liposomes bearing a terminal triphosphine. Similarly, Vabbilisetty and Sun [46] used chemically selective functionalization *via* Staudinger ligation to prepare two types of anchoring lipids, Chol-PEG$_{2000}$-TP and DSPE-PEG$_{2000}$-TP. The glyco-functionalization liposome surface (with latosyl azide) was evaluated, and the anchoring lipids affected the liposome size, stability, encapsulation efficiency, release capacity, and lectin binding. In another study by Garg and Jain [47], galactosylated liposomes were prepared and characterized *in vitro*. O-palmitoylgalactose (OPG) was synthesized through the esterification of galactose by the reaction of palmitoyl chloride in dimethylformamide under anhydrous catalytic conditions. The liposomes were then coated with the synthesized OPG. The maximum cellular uptake was achieved using galactosylated liposomes. Moreover, this formulation maintained significant levels of azidothymidine (AZT) in tissues that overexpressed galactose specific receptors and had an extended residence time in the body, which enhanced the half-life of AZT.

Whether liposomes are synthesized prior to the conjugation of the carbohydrate or using the generated glycolipid, all methods for liposome preparation involve the following steps: (1) drying down the lipids from an organic solvent,

(2) hydrating the formed lipid film with an aqueous medium (3) purifying the liposomes from any unencapsulated agents, and (4) characterizing the final product [48, 49]

The drugs to be encapsulated can be loaded into liposomes either during the formation of liposomes (passive loading) or after the formation of the vesicles (active loading). As a result, liposome preparation methods are classified as passive- or active-loading methods. Passive loading methods are further divided into mechanical dispersion methods (*e.g.*, lipid film hydration, micro-emulsification, sonication, French pressure cell, membrane extrusion, and freeze-thawing), solvent dispersion methods (*e.g.*, ethanol/ether injection, double emulsion, and reverse-phase evaporation) and detergent solubilization (*e.g.*, dialysis, column chromatography, and dilution) [49, 50]

The purification of liposomal products is an essential step because it enables the removal of excess components and unencapsulated drugs. This is particularly true for hydrophilic drugs, where only a small amount of the drugs is incorporated into the aqueous interior core, while the rest is left in the liposomal suspensions. Therefore, it is necessary to eliminate the free drug. Since the molecular weight of most drugs is much lower than that of liposomes, the size difference can be used as the basis for separation. Methods that rely on size exclusion include dialysis, centrifugation, and chromatographic-column separation. Although these methods are useful for liposome purification, they tend to be time-consuming and may even diminish product yield or dilute liposomes. Other methods for the elimination of unencapsulated drugs include ion-exchange chromatography, microcentrifugation, and ultrafiltration. All of these methods have their own advantages and disadvantages, and the choice of the most suitable purification method depends on the characteristics of the synthesized liposomes [51, 52].

Finally, regarding the storage of liposomes, there is still no standard method to store and guarantee the long-term stability of liposomes; however, certain guidelines have been formulated based on experimentation. For instance, the finished-formulation can be stored at physiological pH, since the rate of lipid hydrolysis is lowest at a pH of 7.4. Moreover, given that liposomes are very susceptible to temperatures that promote oxidation and leakage of the entrapped cargo, storage at 2-8 °C is ideal [53]. Liposomes, however, cannot be stored in a freezer because that will lead to the formation of ice crystals that may rupture the phospholipid-bilayers in liposomes [54]. In addition, since lipids are susceptible to photooxidation, protecting them from light during storage is highly recommended. If the nature of the drug or the requirements for the bilayer structure does not permit storage as an aqueous dispersion, then freeze-drying or concentration by evaporation might provide systems that are stable for storage. The processes governing these techniques are still not fully understood; therefore, in order to optimize storage conditions, systematic work on the fundamentals of these processes should be carried out [55].

6. CARBOHYDRATE FUNCTIONALIZED NANO-CARRIERS FOR CANCER TARGETING AND THERAPY

The properties of NPs can greatly influence their behavior *in vivo*. Morphological properties such as size and shape are determinants of the biodistribution, and the duration NPs remain in the blood circulation. Ideally, NPs should remain in circulation until they reach their target anatomical site; however, these particles will be directly eliminated from the body if recognized by the immune system [2]. Studies have shown that particles with diameters between 0.1–7 μm can be detected by the organs of the RES (liver or spleen) and are phagocytized by macrophages. However, if the particle's diameter is lower than 100 nm, it will remain within the fenestra of the endothelial lining of blood vessels, hence reducing the possibility of it being recognized and phagocytized. The smallest NPs, with diameters less than 6 nm, undergo glomerular filtration in the kidneys. With regard to shape, studies have reported that spherically shaped particles are freer to move and less likely to line up with or drop into the bifurcations of vessels or filtering organs than irregularly shaped NPs. Additionally, it was noted that cylindrically or spherically shaped NPs are internalized more promptly than their irregularly shaped counterparts. Surface properties such as hydrophobicity and surface charge, in addition to the presence/absence of surface ligands, can also influence the NPs' behavior within the biological system and potentiate significant changes in their performance [56]. For these reasons, the characterization of liposomes has focused on methods that address surface morphology. These methods include dynamic light scattering (DLS), transmission electron microscopy (TEM), high-performance liquid chromatography (HPLC), and enzymatic methods [35].

6.1. Dynamic Light Scattering (DLS)

The size distribution of liposomal drug carriers is of key interest because size affects both the liposome's *in vitro* and *in vivo* characteristics. The *in vitro* properties affected by liposomal size include encapsulation capacity, aggregation and sedimentation behavior. While the affected *in vivo* behaviors, including circulation time in the blood-stream, biodistribution, especially when targeting solid tumors, and uptake by the mononuclear phagocytic system (MPS) [57]. DLS, also known as photon correlation spectroscopy (PCS) or quasi-elastic light scattering, is a technique used to measure the size of particles in the sub-micron range [58]. This technique is based on the concept of Brownian motion or pedesis, which states that the movement of particles suspended in a fluid, *i.e.*, liquid or gas, is random because the particles are continuously colliding with the fast-moving molecules of the suspending medium. In most DLS systems, a laser of a known wavelength is focused on a dilute sample, and the intensity of scattered light is collected by a detector. An algorithm-based process is then applied to resolve the instrumental data to its constituents and simplify it to determine the particle size distribution of the sample [59].

To determine the hydrodynamic radius, D_h, of the particles, it is necessary to correlate the intensity to the diffusion coefficient of the particles. This is done using an autocorrelation function (ACF). This ACF examines the changes in scattered intensity over a period of time for a given volume of particles. In the case of a simple monodisperse particle size distribution (PSD), the ACF is a single decaying exponential function [60]. Equations 1 through 4 explain how the hydrodynamic radius of the particles is determined.

$$C(\tau) = 1 + \beta e^{-2\gamma\tau} \tag{1}$$

$$\gamma = D_t q^2 \tag{2}$$

$$q = \frac{4\pi\eta}{\lambda}\sin\left(\frac{\theta}{2}\right) \tag{3}$$

$$D_h = \frac{k_B T}{3\pi D_t \, \eta(T)} \tag{4}$$

Here, τ indicates the delay, that is, the amount of time that a duplicate intensity trace is shifted from the original intensity before the averaging is performed, and β is the correlation function amplitude at zero delay. A series of calculations yield the decay constant γ that is inversely proportional to the diffusivity of the particle. In equation (3), q, is a constant called the "scattering wave vector", this constant translates the time scale of the diffusion process into the distance scale set by the laser wavelength. Furthermore, in equation (3), θ represents the scattering angle while the index of refraction of the solvent is given by η, the thermodynamic temperature is symbolized by T, while λ represents the wavelength of the laser's light, and finally, the Boltzmann's constant is given by k_B. Once the coefficient of diffusion D_t has been determined, the hydrodynamic radius can be evaluated using the Stokes-Einstein equation (Eq. 4) [59].

Another application of DLS, one that is particularly important when dealing with carbohydrate moieties that bear charged sugars or lipid head groups, is monitoring changes in aggregation states. These charged molecules might interact with certain elements in the environment or physiologically essential ions, causing the glycoliposomes to aggregate, which in turn hinders their performance [35, 61]. As mentioned earlier, DLS determines particles' size distributions by measuring the scattering of light from particles in solution. Since larger particles move slower than smaller particles, the resulting distinctive fluctuations of scattered light could indicate the formation of carbohydrate aggregates [62].

6.2. Transmission Electron Microscopy (TEM)

TEM is a method used to resolve fine features whose dimensions are less than 100 nm in size. TEM was invented by Max Knoll and Ernst Ruska in 1931. The early transmission electron microscope consisted of a series of horizontal lenses; however, this arrangement was soon abandoned in fa-

vor of the vertically aligned lenses due to its poor resolution [63].

The first step in TEM is the preparation of the sample. This involves placing a small portion of the liposomal sample on a coated copper grid and leaving it to dry before imaging. The prepared sample is then set in a vacuum chamber in the middle of the machine. Once the sample is in position, a beam of electrons is fired through the sample from an electron gun placed at the top of the machine. The electron gun uses high voltages and electromagnetic coils to accelerate the electrons to very high speeds, which make them behave as waves (wave-particle duality). The accelerated electrons travel through the sample and out the other side where coils or photographic plates focus them to form the TEM image. The faster the electrons travel, the smaller the waves they form, and the more detailed the resulting TEM images [64].

TEM is one of the most commonly used techniques to discern the structure of NPs in general, and liposomes in particular, because it can determine the total particle size, shape, inner core, surface coating as well as the size distribution with high resolution [35, 65]. However, the sample preparation step in traditional TEM is undesirable in the analysis of liposomes, as the drying process significantly alters the liposomal structure making the TEM image unrepresentative of the sample's true structure in an aqueous environment [35]. As a result, researchers have turned to the use of cryo-TEM to accurately represent the structures of liposomes. Cryo☐TEM involves the plunge freezing of hydrated samples in liquid ethane; this causes the water in the liposomal samples to take on a frozen☐hydrated glass☐like state very close to the native state of liposomes in solution. The ability of cryo-TEM to preserve the true structure of liposomes is what made it the gold standard for liposome imaging.

6.3. High-Performance Liquid Chromatography (HPLC)

In addition to size and surface morphology, the lipid content of liposomes is an important aspect of the characterization of liposomal formulations. The phospholipid content of liposomes is usually determined using the Bartlett assay, the Stewart assay, or enzymatic reactions. A significant disadvantage of these methods is that they provide only the total lipid content rather than the specific composition of individual components [66]. In HPLC, a pump forces a solvent through a column under high pressures. The column is packed with an adsorbent or stationary phase (typically a granular material such as silica). HPLC is usually used in combination with other detectors, and the most common type of detector used with HPLC for the quantification of lipid constituents of glycoliposomes is a light scattering detector. Furthermore, enzymatic analyses of the sugar moieties functionalizing glycoliposomes are often performed in combination with phospholipid quantification *via* HPLC. Enzymatic analyses require the use of enzymes to digest the carbohydrate moieties from the surface of the liposome. The amount of sugar can then be determined by using HPLC [67, 68].

6.4. Colorimetric Methods

The amount of carbohydrate moieties on the liposome surface can be determined using colorimetric techniques such as the Seliwanoff's test, Bial's test, and the phenol-sulfuric acid method. Seliwanoff's test is used to differentiate between aldoses and ketoses. Upon treatment with concentrated acid, ketoses dehydrate rapidly to give furfural derivatives, and when condensed with resorcinol, they give a cherry red complex. This test is usually used to detect fructose, sucrose and other keto containing carbohydrates. The Bial's test is useful in distinguishing pentose sugars from hexose sugars. Pentose, such as ribose, sugars form furfural in acidic media, and condense with orcinol in the presence of ferric ions to give a blue-green complex that is soluble in butyl alcohol [69]. Finally, in the phenol-sulfuric acid test, concentrated sulfuric acid breaks down poly or disaccharides into monosaccharides that are dehydrated into furfural or 5-hydroxymethyl furfural. The furan derivative will then react with phenol forming an orange solution that has an absorbance at 490 nm [70].

7. EVALUATION OF CARBOHYDRATE FUNCTIONALIZED LIPOSOMES

Stability and binding affinity are two important aspects to consider when evaluating liposomes. Any compromises to the liposomal structure could lead to collapse or aggregation, which in turn can lead to leakage of the payload or the inability of the targeting ligand to effectively bind to its target. Therefore, evaluating the stability and binding affinity of glycoliposomes is key to their success as drug delivery vehicles.

7.1. Liposome Stability

One of the main limitations to the widespread application of liposomes is the inherent instability of phospholipids. Liposomes must be stable physically and chemically during the storage period as well as remain intact and of the appropriate size before reaching their target sites. Consequently, considerable attention has been given to the study the liposome stability, and several approaches have been developed to enhance the stability and retention of liposomes [35, 70, 71]. One of the most commonly used methods to stabilize liposomes is to modify the surface with an even more hydrophilic coating than the existing head group.

This hydrophilic coating permits the formation of clathrate hydrates (a solid network of hydrogen-bonded water molecules that form cavities that can house various molecules) on the exterior surface of the liposome, which further stabilizes the structure of the bilayer. In addition to enhancing stabilization, this water barrier also prevents liposomes from being opsonized by proteins or captured by MPS cells [72]. Therefore, using carbohydrate ligands as a hydrophilic coating for liposomes has emerged as an attractive option [73].

The main methods of evaluating liposome stability are DLS and fluorescence. DLS is used to monitor the stability of liposomes by examining the changes in the size distribu-

tion of the liposomes [74]. As mentioned earlier, the phospholipids making up the liposomal bilayers are inherently unstable; and overtime, as the bilayers degrade, they often begin to form unilamellar micelles, which aggregate with the existing liposomes to form even larger vesicles. In this case, the resulting DLS results would show an increase in the size distribution of the liposomes as well as a splitting of the distribution into two, representing lipid aggregates and large lipid vesicles. The second approach of evaluating liposome stability involves monitoring the release of an encapsulated fluorophore overtime *via* fluorescence monitoring. The process of liposome membrane disruption involves two main steps; a significant change in permeability, followed by the progress of the lysis, which eventually leads to the complete rupture of the bilayer assembly and the release of the encapsulated drug. The first step occurs due to packing distortions which lead to the formation of transient pores [75]. Domecq *et al*. [76] investigated the use of fluorescence to monitor liposome stability. The fluorescent molecular probe octadecylrhodamine B (R18) was used in this study because it exhibits self-quenched fluorescence and is able to anchor itself to the lipid bilayer of the liposome rather than being trapped in the aqueous core compartment. The addition of Triton X-100 (a surfactant) to the liposomes produces a sharp decrease in the self-quenching of R18 caused by the dilution of the probe in the bilayer. The fluorescence intensity curves showed a sudden change in slope, which corresponded to the point at which liposomes began breaking down into their component phospholipids. The interpretation of the fluorescence measurements was corroborated by DLS measurements, as the addition of Triton X-100 to the samples showed a decrease in the number of kilocounts per second (K_{cp}s) which correlated with the increase in R18 fluorescence intensity [77-101].

7.2. Binding Affinity

Another important aspect is the ability of the carbohydrates decorating the surface of the glycoliposomes to bind to their targeted receptor, particularly lectins (carbohydrate-binding proteins) effectively [35]. Various methods have been employed to assess the binding affinity of glycoliposomes to their targets, such as:

1 Agglutination assays: Agglutination is best defined as the formation of clumps of cells or particles, and in terms of glycoliposomes, agglutination is the clumping of glycoliposomes upon binding to the surface of the targeted lectin. Agglutination assays involve measuring the changes in light passing through a sample, *i.e.*, the scattering, or blockage of light. Prior to the development of DLS, agglutination assays were performed by measuring changes in turbidity. Turbidity is a measure of the cloudiness of a fluid due to the presence of suspended particles. Therefore, if carbohydrate conjugated liposomes bind to their targets, they would agglutinate, which would increase the turbidity of the sample, which, in turn, would block the passage of light. Similarly, as gly-

coliposomes bind to their targeted receptors, the way in which the light is scattered by the sample changes. This event can be visualized using DLS as a change in the size distribution compared to that of the original glycoliposome sample [102], [103].

2 Colorimetric methods: Such methods involve monitoring changes in the color, ultraviolet-visible (U-V-vis) spectrum, or fluorescence of the sample upon binding.

3 Immunoassays: Such methods rely on the use of fluorophores to assess the binding event, particularly fluorescently tagged antibodies. The tagged antibody can be specific to the carbohydrate ligand, or to the target. If the tagged antibody is specific to the ligand, it will fluoresce in the event of binding. The reverse is true if the tagged antibody is specific to the target, the bound glycoliposomes will block the antibody, meaning that there will not be a fluorescence signal, which in turn denotes a positive binding event. This type of immunoassays is referred to as inhibition assays [35, 102-104].

8. EVALUATION OF CARBOHYDRATE FUNCTIONALIZED LIPOSOMES

The literature on carbohydrate functionalized liposomes has been increasing steadily, as several research groups have investigated the use glycoliposomes in cancer therapy. Zhao *et al*. [105] investigated the targeted delivery of DOX using galactosylated liposomes. The uptake and targeting of the synthesized galactosylated liposomes were verified *in vitro* and *in vivo* using fluorescence microscopy and the Xenogen Corporation IVIS imaging system, respectively. The *in vitro* fluorescence, microscopy results showed that galactose conjugated liposomes resulted in higher specific cellular uptake by the HepG2 cells than the non-targeted liposomes. *In vivo* fluorescence imaging results yielded higher fluorescence intensities when the mice were treated with galactosylated liposomes than when they were treated with conventional liposomes. Moreover, the anti-tumor effects of DOX were more pronounced when delivered *via* galactosylated liposomes, while no significant changes were observed using the non-targeted formulations. Chen *et al*. [106] developed a novel approach for targeting B lymphoma cells using sialic acid-targeted-DOX-loaded liposomes displaying high-affinity glycan ligands of CD22. The targeted liposomes were actively bound and endocytosed by the cluster differentiation (CD22) receptors on B cells, and significantly extended the life of the mouse model xenografted with human B-cell lymphoma. Boks *et al*. [107] used glycoliposomes to target dendritic cells (DCs) with tumor antigens. The researchers synthesized liposomes containing the glycan Lewis (Le)[X,] which is highly specific for the C-type lectin receptor overexpressed by DCs. In another study, Xiong *et al*. [108] developed mannosylated paclitaxel (PTX)-encapsulating liposomes, which took advantage of the overexpression of the mannose receptor (MR) in several colon cancer cell lines such as CT26 cells. The results of the *in vitro* and *in vivo* studies showed that mannosylated liposomes had a higher uptake by CT26

Table 4. Summary of some relevant *in vitro* and *in vivo* studies.

Moiety	Payload	Tumor model, Animal strain	Main findings	Ref.
-	Cantharidin (CTD)	Human hepatocellular carcinoma (Hep-G2),	CTD encapsulation efficiency was approximately 88.9%. Liposomal CTD for liposomal CTD inhibited cancer cell growth 3-times, 6.7-times and 5.4-times better than free CTD (for 24, 48 and 72 h treatments). The *in vivo* results showed that for free CTD, the mean tumor volume of 2306.39 ± 214.28 mm^3 at day 42, while for liposomal CTD, the mean tumor size of 1807.35 ± 467.95 mm^3.	[110]
peptide D[KLAK-LAK]2 (KLA)	Paclitaxel (PTX)	Human alveolar basal epithelial adenocarcinoma (A549) and drug-resistant lung cancer A549/Taxol cells	DKD-PTX liposomes promoted cell death compared to blank DKD liposomes. Blank DKD-Lips did not affect $\Delta\psi_m$. However, for DKD/PTX-Lips, the percentage of A549/Taxol cells, with decreased $\Delta\psi_m$, was reduced to $19.29 \pm 10.81\%$. DKD-PTX liposomes had the highest efficacy in treating drug-resistant lung cancer A549/Taxol cells xenografted onto nude mice (tumor growth inhibition 86.7%).	[112]
Asp$_8$ and Folate	Doxorubicin	Breast cancer (MDA-MB-231) cells	DOX-A/F-LS treatment prolonged median survival time by 1.7, 1.4, 1.2, and 1.3-folds compared to the treatment groups of physiological saline, free DOX, DOX-A-LS, and DOX-F-LS, respectively.	[114]
ErbB2 antibody Fab	Doxorubicin	Breast cancer (HCC1954) and (MDA-MB-468) cells, Female BALB/c nu/nu mice	The cell association of Fab′-GGLG liposomes increased 10-fold in comparison to GGLG liposomes. A significantly enhanced tumor growth inhibition was observed in an ErbB2-overexpressing breast cancer-bearing mouse model.	[115]
^{111}In-EGF	Doxorubicin	Human breast Cancer (MDA-MB-468 and MCF7) cells, Female athymic nude mice	The cytotoxicity was higher for the MDA-MB-468 than the MCF7 cell lines. A 66% increase in tumor uptake in the MDA-MB-468 cell line.	[116]
iRGD	Doxorubicin	Mammary carcinoma cells (4T1), human breast adenocarcinoma (MCF-7) cell and Human Umbilical Vein Endothelial (HU-VEC) Cells	DOX from iRGD-LTSL-DOX rapidly penetrated tumor interstitial space after HIFU-triggered heat treatment.	[117]

cells, an enhanced tumor inhibition rate, and no notable *in vivo* toxicity. Minnelli *et al.* [109] compared the behavior of mannose-6-phosphate (M6P) liposomes to that of non-functionalized liposomes in breast cancer cells (MCF7) and human dermal fibroblast cells (HDF). The synthesized liposomes were loaded with the model drug calcein, and flow cytometry analyses showed increased uptake of M6P liposomes by the MCF7 cells compared to HDF cells. Moreover, the liposomes were loaded with N-hexanoyl-D-erythro-sphingosine (C6Cer), and using DLS and spectrophotometric turbidity measurements, the authors showed that ceramide loaded M6P liposomes significantly increased tumor apoptosis in MCF7 cells compared to HDF cells.

A literature survey also showed that glycoliposomes tended to give *in vitro* and *in vivo* results comparable to other actively-targeted liposomes. For example, in a study conducted by Zhang *et al.* [110], cantharidin (CTD)-encapsulated liposomes were used to treat HepG2. HepG2 cells were treated with free CTD and liposomal CTD for 24, 48, and 72 h. Liposomal CTD inhibited cancer cell growth 3-times, 6.7-times, and 5.4-times, respectively, more effectively than free CTD. The *in vivo* results showed that free CTD showed little effect on tumor growth inhibition with the mean tumor volume of 2306.39 ± 214.28 mm^3 at day 42. In contrast, the treatment with liposomal CTD was more efficacious than that of free CTD with a mean tumor size of

1807.35 ± 467.95 mm^3. Zhou *et al.* [111] investigated the use of 3-Galactosidase-30-stearyl deoxyglycyrrhetinic acid (11-DGA-3-O-Gal)-modified liposomes (11-DGA-3-O-Gal-CTD-lip) for the targeted delivery of CTD to hepatocellular carcinomas. Compared with non-targeted liposomes (CTD-lip), 11-DGA-3-O-Gal-CTD-lip showed a 64% increase in the cell proliferation inhibitory effect and increased the inhibition of HepG2 cell migration by 1.52-, 1.46-, and 2.06-fold at different concentrations. The inhibition mechanism of 11-DGA-3-O-Gal-CTD-lip on hepatocellular carcinoma was partly through cell cycle arrest at the S phase. The results of the *in vivo* tissue distribution studies showed that the liver concentration of 11-DGA-3-O-Gal-CTD-lip ($2.01 \pm 0.18\mu g/g$) was significantly higher than that of CTD-lip (0.75 ± 0.08 μg/g). This result indicated that the liposomes modified with 11-DGA-3-O-Gal could deliver the drug rapidly to the liver after intravenous administration. In both studies, the liposomal form of CTD yielded better results than free CTD. Although the cell growth inhibition in Zhang *et al.* [110] experiments was higher compared to that reported by Zhou *et al.* [111] for the 11-DGA-3-O-Gal-CTD-lip after 24 h; the *in vivo* results were just as promising, and the therapeutic effect was observed in a much shorter period.

Mitochondrion-targeting liposomes have been developed as a promising strategy to deliver anticancer drugs directly

Table 5. Summary of studies focusing on carbohydrate functionalized liposomes.

Carbohydrate Ligand	Receptor	Payload	Tumor model, animal strain	Main Findings	Ref.
Galactose	Asialoglycoprotein receptors (ASGPr)	-	Human hepatocellular carcinoma (HepG2), male ddY mice	Gal-liposomes with low Gal-C4-Chol showed a slight improvement in liver accumulation compared to conventional liposomes. The highest uptake ratio by cells was observed with 5% Gal-C4-Chol liposomes.	[118]
Galactose	Asialoglycoprotein receptors (ASGPr)	Doxorubicin	Hepatocarcinoma 22 (H22), female KM mice	PEG-GalL DOX showed a slow transfer of DOX to the liver and reduced concentrations in the liver. The inhibitory rate of PEG-GalL DOX to H22 tumors was 94% higher than free DOX other liposomal formulations. The tumor distribution of DOX revealed no difference between PEG-GalL DOX and non-targeted PEGylated DOX liposomes.	[119]
Galactose	Asialoglycoprotein receptors (ASGPr)	Doxorubicin	Human hepatocellular carcinoma (HepG2) and human colorectal adenocarcinoma (HCT-8), Balb/c-nu mice	Galactose targeted liposomes showed stronger specific cell uptake by HepG2 cells. *In vivo* results showed Gal-liposomes had higher fluorescent intensity over non-targeted liposomes in the liver tumor. Tumor progression was significantly suppressed by DOX-loaded galactosylated liposomes.	[105]
Lactose	Asialoglycoprotein receptors (ASGPr)	Doxorubicin	Human hepatocellular carcinoma (HepG2), female imprinting control region mice and athymic nude-Foxn1nu mice	*In vitro* uptake of Lac-L-calcein by HepG2 cells was four times greater than that of non-targeted liposomes. Lac-L-DOX exhibited enhanced *in vivo* cytotoxicity compared with non-targeted liposomes. The tumor inhibition of Lac-L-DOX was than L-DOX and free doxorubicin.	[120]
Chitosan	-	Paclitaxel (PTX)	Human non-small cell lung cancer (NCIH358), ovarian cancer (SK-OV-3), and breast cancer (MDA MB-231), ICR mice	The LMWC-PTX conjugate showed increased bioavailability and significant tumor growth inhibition.	[121]
Hyaluronic acid (HA)	Cluster determinant 44 (CD44)	Paclitaxel (PTX)	Human bladder carcinoma cell (RT-4 and RT-112/84), female BALB/c and SCID mice, and Fischer female rats	HYTAD1-p20 showed higher inhibitory effect against RT-4 and RT-112/84 than that of free drug, and directly interacted with CD44 expressed by bladder tumor cells.	[122]
Sucrose, maltose	-	Doxorubicin	Human hepatocellular carcinoma (HepG2), human malignant melanoma (A375P), breast cancer cells (MCF-7), bronchioalveolar carcinoma (NCI-H358) and cervical carcinoma cells (Hela)	Disaccharide-modified liposomes enhanced the intracellular uptake of liposomes into various cancer cell lines *via* lectin-mediated endocytosis. DOX-loaded disaccharide-modified liposomes showed higher cytotoxicity against various cancer cells than conventional DOX-liposomes.	[123]
Sialyl Lewis[X]	E-selectin	Cisplatin	Mouse breast carcinoma (Ehrlich-Lettre Ascites) EAT cells, adenocarcinomic human alveolar basal epithelial cells (A549) and Human umbilical vein endothelial cells (HUVEC), female Balb/c mice	Mice treated with CDDP-SLX-Lip showed an improved survival rate of 75%.	[124]
Lewis(Le)[X]	C-type lectin	Hydrophilic TLR ligand Poly I:C (Invivogen) and the antigenic peptide gp100$_{280-288}$ (YLEPGPVTA)	Human monocyte-derived dendritic cells and gp100-specific CD8^{+} T-cells	Lewis[X]- plusMPLA-modified liposomes were efficiently internalized by DC-SIGN. Targeting DCs with Lewis[X]- and MPLA modified glycoliposomes enhances antigen presentation to CD8^{+} T cells.	[107]

Carbohydrate Ligand	Receptor	Payload	Tumor model, animal strain	Main Findings	Ref.
Mannose	Mannose receptor (MR)	Plasmid DNA and RNA	Melanoma cells (B16-F10), Male C57Bl/6J mice	DCs transfected with ML/melanoma B16 RNA complexes caused five-to six-fold increased inhibition of melanoma lung metastasis. ML/melanoma B16 RNA complexes injected into mice induced the production of melanoma B16-specific T-lymphocytes, which were two-timed more efficient in B16 cell killing than control liposome.	[125]
Lactose	Asialoglycoprotein receptors (ASGPr)	-	Human hepatoma cells (HUH7 and Alexander), human colon cancer cells (FCC) and human lung cancer cells (KNS)	Cancer cells were found to have a common affinity with lactose CHP liposomes, but the mechanisms had no connection with the ASGPr of hepatocytes.	[126]
Mannose	Mannose receptor (MR)	Paclitaxel (PTX)	Colon cancer cells (CT26 and HC-T116)	CT26 cells treated with Coumarin-6-Man liposomes showed stronger fluorescence than other formulations. The cytotoxicity of PTX-Man liposomes was higher than PTX-Liposomes due to higher cell uptake by CT26 cells. The inhibition rate of PTX-Man liposomes was 69.78% compared with the control group.	[108]
Mannose	-	Calcein, C6-ceramide (C6-Cer)	Human Dermal Fibroblast cells (HDF) and breast cancer (MCF7) cells	Flow cytometry results showed increased uptake of M6P- liposomes in MCF7 cells compared to HDF. Ceramide-loaded M6P-liposomes significantly increased apoptosis in MCF7 with respect to HDF.	[109]
Chitosan	-	Docetaxel (DTX)	Colon cancer cells (HT29)	C-FL showed enhanced pharmacokinetic parameters and cytotoxic efficiency compared to uncoated liposomes.	[127]
Lactobionic acid	Asialoglycoprotein receptors (ASGPr)	Oxaliplatin	Hepatocellular carcinoma (BEL7402 HCC cell)	Fluorescence microscopy revealed higher uptake of the LA-LP by BEL7402 HCC cells. Organ distribution studies showed that modifying liposomes with lactobionic acid significantly enhanced the tumor uptake of the drug.	[128]
Chondroitin sulfate	Cluster determinant 44 (CD44) receptor	Calcein	Breast cancer cells (MDA-M-B-231)	*In vitro* drug release from uncoupled liposomes was 44.2%, while release from CS-LP was 38.3% at the end of 24 hr. In cell-uptake studies, higher fluorescence intensity was observed with the CS-LPs (5509) compared to control liposomes (3690).	[129]
Hyaluronic acid (HA)	Cluster determinant 44 (CD44) receptor	Paclitaxel (PTX)	Human lung cancer cells (A549) and Taxol-resistant lung cancer cells (A549/Taxol)	HA liposomes had a higher entrapment efficiency of 85%. The results of cellular uptake studies showed that the uptake of HA liposomes increased by 4.8-fold in A549/T cells compared with uncoated coumarin-6 liposomes.	[113]
Sialyl Lewis[X]	E-selectin	-	Chinese hamster ovary cells (CHO), colon carcinoma cells (HT29) and human umbilical vein endothelial cells (HUVEC)	Sialyl Lewis X-conjugated liposomes were the most effective tumor growth inhibitors in all three assays, inhibiting the adhesion of HT29 colon- and Lewis lung carcinoma cells by about 60–80%.	[130]
Sialic acid	Cluster differentiation 22 (CD22) receptor	Doxorubicin	Daudi Burkitt lymphoma cells and Wild-type Chinese hamster ovary cells (CHO), Nonobese diabetic-–severe combined immunodeficiency (NOD-SCID) mice and Sialoadhesin (Sn) knockout mice	Modified liposomes exhibited 33-fold higher cytotoxicity of Daudi cells than uncoupled liposomes. Liposomes with 5% ligands demonstrated an MTS greater than 100 days with 5 of 8 long-term survivors.	[106]
Galactose	Asialoglycoprotein receptors (ASGPr)	Vimentin siRNA and doxorubicin	human hepatocellular carcinoma cells (Huh7) and lung epithelial carcinoma (A549), Balb/c athymic nude mice	The biodistribution study results suggested that Gal--DOX/siRNA liposomes allowed higher DOX concentrations to reach the normal liver and hepatic tumor tissue than non-targeted liposomes and free DOX. MTT cytotoxicity assays showed that Gal-DOX/siRNA-liposomes exhibited enhanced cytotoxic effects *in vitro*.	[131]

(Table 5) contd....

Carbohydrate Ligand	Receptor	Payload	Tumor model, animal strain	Main Findings	Ref.
Galactose	Asialoglycoprotein receptors (ASGPr)	Cantharidin (CTD)	Human hepatocellular carcinoma (HepG2)	11-DGA-3-O-Gal-CTD-lip showed a 1.64-times higher cell proliferation inhibitory effect and increased the inhibition of HepG2 cell migration by 1.52-, 1.46-, and 2.06-times at different concentrations. *In vivo* tissue distribution results showed that the liver concentration of 11-DGA-3-O-Gal-CTD-lip (2.01 ± 0.18μg/g) was significantly higher than that of CTD-lip (0.75 ± 0.08 μg/g).	[111]

to tumor sites, as these have tremendous potential for killing cancer cells, especially those exhibiting anticancer MDR. Jiang *et al.* [112] developed a dual-functional DKD-PTX liposome system possessing both pH responsiveness and mitochondrial targeting properties to enhance PTX accumulation in mitochondria and trigger apoptosis in human lung cancer A549 cells, and drug-resistant lung cancer A549/Taxol cells. The cytotoxicity results showed that the DKD-PTX liposomal system promoted cell death compared to blank DKD liposomes, as the IC_{50} of DKD-PTX liposomes was 0.06 μg/mL for A549 cells and 0.66 μg/mL for A549/Taxol cells. The results of mitochondrial membrane potential measurements showed that for blank DKD liposomes, the percentage of A549/Taxol cells, with decreased $\Delta\psi_m$, was 99.28 ± 1.85%, so the blank DKD-Lips had no effect on $\Delta\psi_m$. However, after applying DKD/PTXLips, the percentage of A549/Taxol cells, with decreased $\Delta\psi_m$, was reduced to 19.29 ± 10.81%. Comparing the mitochondria-targeting efficacy of glycoliposomes to that of non-targeted liposomes, Tian *et al.* [113] designed hyaluronic acid (HA) coated liposomes encapsulating PTX and the mitochondria targeting moiety dequalinium (DQA). The *in vitro* cellular uptake of HA-PTX liposomes increased by 4.8-fold in A549/T cells compared with liposome without the HA-coating. With regard to the *in vitro* inhibitory effects, PTX liposomes were more effective than PTX solution (the IC_{50} of PTX-L was 1.30 μg/mL for A549 cells and 5.52 μg/mL for A549/Taxol cells). Moreover, HA-PTX-L was even more effective against A549 and especially A549/Taxol cells (the IC_{50} of HA-PTX-L against A549 and A549/Taxol cells was 0.1 μg/mL for both cell lines). In addition, HA-PTX-DQA liposomes induced the highest reduction of mitochondrial membrane potential, which was about 28% and 35% in treated A549 and A549/Taxol cells compared with control cells, respectively. All of these findings indicated that the developed HA-liposomes were able to interfere with mitochondrial functioning and achieve PTX accumulation in mitochondria that can initiate cell apoptosis and overcome MDR.

Table **4** presents some studies pertaining to the treatment of cancer using actively-modified liposomes. The functionalizing moieties presented in this Table do not include carbohydrates in order to provide a sense of how the performance of glycoliposomes (Table **5**) compares to other targeting moieties

CONCLUSION

Carbohydrates are one of the four major organic molecules necessary for life, they are the main source of energy for living cells, and recently carbohydrates have proven to be quite promising in drug delivery applications. Glycosylation imparts some desirable characteristics onto nanocarriers, such as bioavailability, biocompatibility, biodegradability, low toxicity, and easy chemical modification, which lead to the development of promising drug delivery systems. This review presented an overview of different glycosylated carriers used to deliver anticancer therapeutics, with a focus on glycosylated liposomes as well as techniques used to characterize them.

AUTHOR CONTRIBUTIONS

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

LIST OF ABBREVIATIONS

AuNP	=	Gold Nanoparticle
ACF	=	Autocorrelation Function
ASGP-R	=	Asialoglycoprotein Receptor
AZT	=	Azidothymidine
BTIC	=	Brain Tumor-initiating Cells
CD22	=	Cluster of Differentiation 22
CD44 CTD	=	Cluster of Differentiation 44 Cantharidin
C6Cer	=	N-hexanoyl-D-erythro-sphingosine
DNA	=	Deoxyribonucleic Acid
DOX	=	Doxorubicin
DLS	=	Dynamic Light Scattering
DC	=	Dendritic Cells
ER	=	Endoplasmic Reticulum
FTIR	=	Fourier-transform Infrared Spectroscopy
G6P	=	Glucose-6-phosphate
GD	=	ganglioside
G6Pase	=	Glucose-6-phosphatase

G6PC = Catalytic Subunit of G6P

G6PT = Translocase Subunit of G6P

GBM = glioblastomas

GLUT = Sodium Ion Independent Sugar Transporter

HK = Hexokinase

HCC = Cepatocellular Carcinoma

1H-NMR = Proton Nuclear Magnetic Resonance

HPLC = High Performance Liquid Chromatography

HepG2 = Hepatocellular Carcinoma

HDF = Human Dermal Fibroblast Cells

MUC = Mucin

MNP = Magnetic Nanoparticle

MP = Mononuclear Phagocytic System

MR = Mannose Receptor

M6P = Mannose-6-phosphate

MCF7 = Breast Cancer Cells

NP = Nanoparticle

OPG = O-palmitoyl Galactose

PCS = Photon Correlation Spectroscopy

PSD = Particle Size Distribution

PL = Phospholipid

PTX = Paclitaxel

PEG = Polyethylene Glycol

QD = Quantum Dot

RNA = Riboxynucleic Acid

RES = Reticuloendothelial System

SLN = Solid Lipid Nanoparticle

SGLT = Sodium Ion Dependent Sugar Transporter

TEM = Transmission Electron Microscopy

UV-vis spectrum = Ultraviolet Visible Spectrum

Gal = Galactose;

DOX = Doxorubicin;

CHOL = Cholesterol;

PEG = Polyethylene Glycol;

Lac = Lactose;

LMWC = Low Molecular Weight Chitosan;

CDDP = Cisplatin;

DC = Dendritic Cells;

ML = Mannosylated;

CHP = Cholesterol Pullulan;

Man = Mannose;

C-FL = Chitosan Flexible Liposomes;

LA-LP = Lactobionic Liposomes;

CS-LP = Chondroitin Sulfate Liposomes;

HA = Hyaluronic Acid;

MTS = Mean tsime of Survival.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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