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Liposomal Nanoformulations as Current Tumor-Targeting Approach to Cancer Therapy

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Abstract

The liposomes present great potential for applications in targeted delivery of chemotherapeutics in the treatment of cancer. The use of liposomal drug carriers as vehicles for targeting of chemotherapeutic agents to tumor tissues is based on their advantages over other dosage forms, represented by their low systemic toxicity, their bioavailability, and their possibility to enhance the solubility of different chemotherapeutic agents, due to the ability to encapsulate both hydrophilic and lipophilic drugs. They enhance the therapeutic index of anticancer drugs by increasing the drug concentration in tumor cells through tumor targeting. The available approaches used for tumor targeting using liposomes are passive targeting, active targeting, and triggered drug release. The most advanced targeting strategies proposed for cancer treatment are the development of multifunctional liposomes, having combined targeting mechanism. In this chapter, the tumor-targeting mechanisms are described in detail as well as the possibilities to design the targeted liposomal nanocarrier in order to reach the desired target in the body and minimizing the off-target effects. Moreover, the current status of preclinical and clinical evaluation is highlighted.

Keywords: liposomes, cancer, tumor-targeting, passive targeting, active targeting

1. Introduction

The main characteristic of cancer is the existence of abnormal rapidly proliferating cells. Conventional chemotherapy is based on using chemotherapeutic agents that eliminate these uncontrollably dividing cells [1]. Most currently used anticancer agents are not able to differentiate between cancerous cells and normal ones, resulting in high systemic toxicity and side

effects [2]. Because of the severity of the side effects, often dose reduction or cessation of the treatment is necessary, rendering chemotherapy inefficient [3]. By limiting the administered dose to reduce excessive toxicity, only a small fraction of the drug will reach the target tumor site, whereas the remaining portion of the drug will be distributed to other tissues in the body. This lack of specificity toward cancerous cells translates into an insufficient amount of chemotherapeutic drug reaching the site of action [1]. Liposomal nanosystems can overcome the drawbacks of conventional chemotherapy, by increased drug delivery in the tumor tissue and lower drug concentration in normal tissues. This way, the therapeutic efficiency of chemotherapy is increased, while the toxicity and side effects are reduced [4]. Also, due to their small size, the circulation time of standard chemotherapeutic agents is often short as they are rapidly eliminated from the bloodstream by macrophages, thus reducing the effective drug concentration at the tumor site [3]. Moreover, the majority of current chemotherapeutic agents have poor water solubility and absorption, which result in low bioavailability [2]. The incorporation of the chemotherapeutic drugs in liposomal drug delivery systems offers advantages by protecting the drug from degradation, increasing its circulation time in the bloodstream and overall improving its pharmacokinetic profile [1, 2].

2. Liposomal nanoformulations for tumor targeting

2.1. Liposomes for passive tumor accumulation

Passive targeting consists in the transport of nanocarriers through leaky tumor capillary endothelium into the tumor interstitial space [5]. The underlying mechanism, which makes passive targeting possible, is the enhanced permeability and retention (EPR) effect.

It was observed that certain circumstances, such as inflammation/hypoxia, tumors, or infarcts, can determine an enhanced permeability of the endothelial lining of the blood vessel wall compared with the normal state of the tissue [6]. When reaching a given size, a tumor can no longer rely on the normal vasculature present in its vicinity to provide all the oxygen supply needed for its further proliferation. Therefore, as oxygen-deprived cells start to die, they secrete growth factors that promote the formation of new blood vessels from the surrounding capillaries, process known as angiogenesis [7]. These newly formed irregular blood vessels lack the basal membrane of normal vascular structures, thus presenting a discontinuous epithelium, which allows particles, such as nanocarriers (in the size range of 20–200 nm), to extravasate and accumulate inside the interstitial space [8]. Following permeation into the tumor, the enhanced accumulation of nanocarriers in the tumor microenvironment is favored by the poor lymphatic drainage in the tissue. In tumors, the lymphatic function is defective, resulting in minimal uptake of the interstitial fluid. Therefore, nanocarriers that have reached the perivascular space are not cleared efficiently and accumulate in the tumor interstitium [7]. This spontaneous accumulation or “passive” targeting is currently known as the EPR effect [9]. Utilization of the EPR effect is therefore an effective strategy for targeting nanopreparations, such as liposomes, to the site of a tumor and has been extensively documented using various tumor types and animal models, since its early discovery in 1980s by Matsumura and Maeda [10, 11].

Several factors have been shown to influence and favor the EPR, for example, prolonged systemic circulation that allows longer interaction of liposomes with the target, size of the liposomes, composition, and charge on the surface of liposomes [12]. Longevity in blood is achieved by coating the liposomes with polymers, such as polyethylene glycol (PEG). PEG has been shown to protect liposomes from recognition and rapid removal from the circulation by the mononuclear phagocyte system (MPS), enabling the liposomes to stay in the circulation for a prolonged period of time and allowing them to substantially extravasate and accumulate in tumors, hence giving the liposomes long-circulating properties [13]. PEG prevents opsonization by shielding of the surface charge, enhancing the repulsive interaction between polymer-coated liposomes and blood components, increasing surface hydrophilicity, and forming a polymeric layer over the liposome surface which renders them impermeable to opsonins [14, 15]. Additionally, their accumulation in the tumor is strongly linked on the size of the endothelial gaps in the capillary vasculature, which varies between 200 and 2000 nm, depending on the tumor type, its environment, and its localization [7]. An effective extravasation has been shown to occur for particles averaging from 30 to 100 nm in the case of hyperpermeable tumors such as murine colon adenocarcinoma, whereas for poorly permeable tumors (human pancreatic adenocarcinoma), only particles smaller than 70 nm proved to be effective [16, 17]. Last, the composition and charge on the surface of liposomes have impact on passive targeting. The presence of surface-charged lipids can alter the opsonization profile of the liposomes, their recognition by cells of the MPS, and hence their overall plasma circulation profile [18, 19]. While anionic or neutral liposomes escape from renal clearance, the positive surface charge of cationic liposomes leads to nonspecific interactions with the anionic species in the blood, resulting in rapid clearance from circulation by the reticuloendothelial system (RES), which reduces the EPR effect [11, 12]. Moreover, it has been reported that the aggregation of liposomes occurs with greater amounts of cationic lipids in the liposomal membrane; therefore, an optimization of the composition of the liposomal membrane is crucial for enhancing tumor penetration [20].

Conventional liposome formulation is based on the use of phospholipids and cholesterol, the last playing an essential role in the regulation of liposomal membrane fluidity, affecting vesicles permeability and stability [21]. Unmodified liposomes are rapidly eliminated from the circulation by the macrophages of RES, their main clearance sites being liver and spleen [22]. Grafting of PEG on the surface results in the formation of “stealth” or stabilized liposomes, which have improved *in vivo* stability and increased circulation time, up to 24–48 h (the long-circulating liposomes). PEG performance as stabilizer depends on chain length, optimal surface density, and optimal chain configuration. The percentage of PEGylated phospholipids necessary for stealth behavior is about 5–7% mol. with PEG 2 kDa and 15–25% with smaller PEG 350 Da to 1 kDa. Depending on the PEG density and configuration on the liposome structure, three models are possible: “mushroom” (low-polymer density), “pancake” (high-graft density), or “brush” (ideal model, ensuring efficient coverage of the surface) [11, 23].

It was reported that PEGylated egg phosphatidylcholine-cholesterol liposomes loaded with doxorubicin (DOX), having ~100 nm, passively accumulated in the tumor vessels of a multi-drug-resistant breast cancer xenograft model, exhibiting a remarkable antitumor effect, where the free DOX failed to provide any detectable therapeutic effect [24].

Mitoxantrone (MTO), an anthracenedione closely related to anthracyclines, was encapsulated in PEGylated liposomes, and efficacy studies in breast cancer model using liposomal-based MTO chemotherapeutic treatment in comparison with free MTO were realized. MTO encapsulation in liposomes limited the toxicity, which allowed the administration of higher MTO doses in the treatment of breast carcinoma on mice [25].

Recently, scientists reached the conclusion that the EPR effect is much more complex than initially defined, as it encompasses complex biological processes such as angiogenesis, vascular permeability, hemodynamic regulation as well as heterogeneities in tumor genetic profile and in the tumor microenvironment and lymphangiogenesis. As these factors differ from patient to patient and from one tumor type to another, they represent an important source of variability when considering the distribution and accumulation of liposomes in tumors. For these reasons, the sole use of the EPR effect as targeting mechanism may now be considered outdated, leaving the focus on designing actively targeted liposomes and liposomes, which combine the passive tumor accumulation with active targeting and/or stimuli sensitivity [2].

2.2. Actively targeted liposomal systems

The limitations of passive tumor targeting have been addressed by developing another kind of targeted drug delivery named active targeting. Various receptors are known to be involved in the development and progression of cancer, so they can be regarded as potential targets for the development of drug delivery systems. Liposomal drug delivery systems for active targeting are designed to have targeting moieties attached on their surface. The targeting ligands bind to the corresponding receptors or surface molecules which are overexpressed on the surface of the tumor cells or tumor vasculature [2, 26]. As a result, liposomes are internalized in the tumor cells by endocytosis and drug concentration in tumor cells is increased [12]. The targeting moieties can be monoclonal antibodies, fragments of antibodies, peptides, proteins, nucleic acids, carbohydrates, or small molecules [2, 26].

The design of liposomes for active targeting is a complex task in which various factors must be taken into account. For instance, the manufacturing material and the size of the liposomes, the type of ligand, the ligand conjugation method, and the ligand density determine the efficacy of the liposomal system both *in vitro* and *in vivo*. The affinity of a ligand for its target is greatly affected by the density of the ligand on the surface of the liposomes [26]. Generally, an increased ligand density favors the uptake of the delivery system as there is a higher probability of interaction with the target (multivalency) [27]. However, a supplementary increase in ligand density can negatively impact on ligand-substrate interactions due to improper orientation of the ligand, steric hindrance of vicinal molecules, and so on. To bind to its specific substrate, a ligand has to be in the proximity of its target, to be able to recognize and interact with it, so the design of liposomal systems with increased circulation time will favor the interaction [26]. As shown above, modifying the surface of the liposomes with PEG can prolong the blood circulation time by avoiding opsonization, but PEG with long chains can hinder the binding of the ligand to its target and PEGylation can increase the size of the liposomes. Besides PEGylation, the size of the liposomes and the surface and ligand charge have important contributions in the ligand-substrate interactions. The size of the liposomes can influence

cellular uptake and intracellular deposition. The charge of the liposomes and the ligand can determine attractive or repulsive forces, which in turn will affect the degree of conjugation. This problem can be solved by adding a spacer, like PEG. It has been shown that cationic liposomes bind to their targets and are consequently internalized to a greater extent than negatively charged particles [26].

Generally, an active targeting liposomal drug delivery system consists of the following components: (1) the liposomal carrier, (2) a hydrophilic polymer forming a protective layer around the liposome, (3) a ligand specifically targeting a certain substrate, (4) a linker molecule or a functional group that couples the ligand to the liposome, and (5) a drug encapsulated in or bound to the liposomal system. Ligands can be covalently or non-covalently bound to the surface of the liposome. The most extensively used approach is the one based on covalent binding of the ligand to the liposomes, usually done with the aid of a linker through a series of chemical reactions [28].

The ligand can be conjugated to the liposomes' components (e.g., a lipid) either prior to liposome preparation or afterwards. Usually, a pre-liposome preparation conjugation has the advantage of allowing better control of the liposomes' physicochemical properties. On the other hand, the post-liposome assembly strategy is based on coupling the ligand to the already-prepared liposomes, and is applied if the ligand changes the properties of the liposomes' components, the ligand is too large to participate in self-assembly, or has a poor stability in organic solvents [26].

Active targeting can be addressed either to tumor cells or to the tumor endothelium.

2.2.1. Active targeting of tumor cells

In targeting tumor cells, the ligand should have a high affinity for a specific receptor overexpressed by tumor cells in order to bind to the receptor and subsequently be endocytosed into the cells. The receptors most exploited in active targeting include the following discussed below [2].

The folate receptor is overexpressed in various types of cancer such as breast, ovarian, lung, colon, kidney, and brain cancers [29]. It has two isoforms: the alpha isoform, which is overexpressed in most cancers, and the beta isoform, which is expressed on the surface of activated macrophages [2]. Active drug delivery targeting the folate receptor involves conjugating folic acid to the surface of liposomes, usually through a PEG spacer between the lipids and the folate. Several liposomal systems conjugated with folic acid have been developed for the delivery of different anticancer agents, including imatinib [30], docetaxel [31], DOX [32], and daunorubicin [33]:

- a. The transferrin receptor (TfR) is a transmembrane glycoprotein involved in cellular iron uptake from transferrin (Tf), a plasma protein, by receptor-mediated endocytosis [34]. The TfR has been explored as a target for cancer treatment due to its accessibility, its pivotal role in cell growth, and proliferation and also its overexpression by various types of malignant cells [35]. Recently, nanoparticulate systems modified with Tf were proposed to deliver the

chemotherapeutic agents across the blood-brain barrier (BBB), for the treatment of brain tumors such as glioma. For instance, Tf was attached to the surface of vincristine and tetrandrine-loaded liposomes [36] and for modifying liposomes loaded with cisplatin [37]. Both liposomal formulations showed a more potent cytotoxic effect on tumor cells than the free drugs and the non-modified liposomal drugs, on C6 glioma cells *in vitro* [36, 37].

- b. The epidermal growth factor receptor (EGFR) is a 170-kD glycoprotein which belongs to the ErbB family of tyrosine kinase receptors. The EGFR plays a crucial role in cancer progression and metastasis since it activates signaling pathways responsible for promoting cell proliferation, angiogenesis, and inhibiting apoptosis [38]. Overexpression of EGFR has been observed in various types of cancer, including breast, lung, colon, ovarian, pancreatic, and kidney cancers [2]. EGFR-mediated delivery via liposomes is based on using antibodies or antibody fragments embedded in the lipidic membrane. Several anti-EGFR-liposomal systems have been reported for the delivery of DOX [39, 40]. A work describes the development of a large-scale, Good Manufacturing Practice (GMP) compliant process for manufacturing EGFR-targeted immunoliposomes loaded with DOX, using the already approved Cetuximab (C225) and PEGylated-liposomal DOX (Caelyx®). The liposomal formulation was safe, according to the results of a clinical trial [41]. Cetuximab or Cetuximab fragments (Fab') were also coupled to oxaliplatin-loaded liposomes for increased selectivity for tumor cells. Both liposomal formulations showed greater cellular uptake than untargeted liposomes in EGFR-positive cell cultures *in vitro*, and *in vivo* experiments on colon cancer-bearing mice indicated improved efficacy over untargeted liposomal oxaliplatin. Liposomes equipped with Fab' fragments bound to a higher extent to EGFR and had better uptake than liposomes coupled to Cetuximab [42].
- c. Glycoproteins expressed on the surface of cancer cells can be bound by lectins which can be used as targeting moieties on liposomes, since the bond between the two is very specific. A PEGylated-liposomal system functionalized with recombinant human E-selectin for the selective delivery of DOX to tumor cells was designed by attaching E-selectin to the PEG chains of PEG2000-DSPE through a maleimide group. When tested on two circulating malignant cell lines expressing sialylated carbohydrate groups, a significant reduction in cell viability was obtained compared to the control and empty E-selectin-coupled liposomes, which shows that the developed liposomal system could be useful in capturing and eliminating circulating tumor cells under flow conditions [43].
- d. CD44 (cluster of differentiation 44) is a transmembrane glycoprotein which contains a specific binding domain for hyaluronic acid. CD44 is involved in a series of biological processes, including proliferation, migration, growth, differentiation, and angiogenesis [44]. Various cancers, such as leukemia, ovarian, colon, gastric, pancreatic, and epithelial cancers, have been documented to overexpress CD44.

Several liposomal systems decorated with hyaluronic acid have been described in literature for the delivery of gemcitabine [45] and DOX [46] to tumor cells. Other reported methods of targeting the CD44 receptor involve using anti-CD44 monoclonal antibodies [44] or RNA aptamers (e.g., Apt1) [47].

2.2.2. Active targeting of the tumor endothelium

This type of nanosystem is capable of binding and destroying tumor vasculature and indirectly limiting the growth of the tumor cells that are supplied with nutrients and oxygen by these blood vessels. Targeting the tumor endothelium is advantageous because the nanosystems do not have to extravasate in order to reach their site of action, and can directly bind to the corresponding receptors which are easily accessible [2]; the risk of developing resistance to chemotherapy is reduced because endothelial cells have less genetic variations than tumor cells, and markers expressed by endothelial cells are not specific for any type of tumor [12]. The main targets of the neovascular endothelial cancer cells are described below:

- a. The vascular endothelial growth factor (VEGF) is produced by tumor cells in hypoxic conditions [2]. VEGF and its receptor (VEGFR) play an important role in angiogenesis, inducing the proliferation, migration, and survival of epithelial cells. Also, VEGF increases the permeability of blood vessels [48]. There are two main strategies of targeting VEGF-mediated angiogenesis, namely targeting VEGFR to reduce VEGF binding or targeting VEGF to decrease its binding to VEGFR [2]. A novel PEGylated-liposomal system functionalized with a fully human anti-VEGF 165 monoclonal antibody was proposed for paclitaxel. The PEGylated immunoliposomes showed superior antitumor activity compared to unmodified liposomes and the commercially available paclitaxel (Taxol®) in SGC-7901 human gastric cancer-bearing nude mice [48].
- b. The integrins are a family of heterodimeric transmembrane glycoproteins participating in interactions between cells or between cells and extracellular matrix. They are composed of non-covalently bound polypeptide α - and β -subunits [12]. Among these integrins, $\alpha_v\beta_3$ -integrin seems to be the most important integrin in angiogenesis. It is an endothelial cell receptor for extracellular matrix proteins, including fibrinogen/fibrin, fibronectin, vitronectin, thrombospondin, and osteopontin. Higher expression of $\alpha_v\beta_3$ -integrin has been observed in melanoma, lung, and brain cancers [2, 49]. Research has revealed that the arginine-glycine-aspartic acid (RGD) amino acid sequence is the binding site contained in all ligands bound by $\alpha_v\beta_3$ -integrin, and new RGD-containing peptides or derivatives with high affinity and selectivity for $\alpha_v\beta_3$ -integrin have recently been proposed. Moreover, the incorporation of cytotoxic drugs in nanosystems decorated with RGD-containing ligands could promote antitumor effect by offering a dual-targeting strategy against tumors [50].

A liposomal system containing DOX was engrafted with three different cyclo-RGD-based peptides: cRGDyC (Arg-Gly-Asp-D-Tyr-Cys), cRGDfK (Arg-Gly-Asp-D-Phe-Lys), and cRGDf[N-Met]K (Arg-Gly-Asp-D-Phe-[N-Methyl]Lys). The latter peptide was synthesized based on Cilengitide, the most selective inhibitor of $\alpha_v\beta_3$ -integrin currently evaluated in a phase III clinical trial for glioblastoma therapy. *In vitro* experiments regarding liposome-cell association and cytotoxicity were conducted in human umbilical vein endothelial cells (HUVEC) and emphasized the ability of RGD-targeted liposomes to associate to HUVEC through integrin-mediated endocytosis. The therapeutic efficacy of RGD-targeted liposomes was assessed in C-26 colon carcinoma tumor xenograft model in mice. Among the investigated peptides, RGDf[N-Met]K had the most potent cytotoxic effect and increased the survival of mice [50].

In another study, other three RGD-based peptides were evaluated as potential ligands, coupled to liposomes: a monomeric c(RGDfK) (moRGD), a dimeric c(RGDfK) (diRGD), and a special dimeric c(RGDfK) (P-diRGD) containing a PEG spacer between two cyclic RGD motifs. P-diRGD-modified liposomes exhibited the strongest interaction with and internalization in B16 murine melanoma cells. The targetability of P-diRGD-modified liposomes in B16-bearing mice was approximately 2.4-fold and 2.8-fold more increased than that of moRGD- and diRGD-modified liposomes [49].

2.3. Stimuli-sensitive liposomes

Stimuli-responsive liposomes have been developed with the purpose of overcoming problems associated with conventional and long-circulating liposomes, such as a slow release of the loaded drug or the incapacity to fuse with the endosome after internalization. The concept of increasing drug targeting through triggered release is based on utilizing subtle pathological changes in the tumor microenvironment and has been extensively studied in the past years for improved efficiency of liposomal drug release [12, 51]. The stimuli-sensitive nanocarriers maintain their stealth function throughout circulation, and upon arrival at the specific tumor site, undergo rapid changes, such as aggregation, disruption, and permeability changes, which trigger drug release when exposed to a particular tumor microenvironment [2, 52, 53]. In order to achieve site-specific triggered drug release, several strategies have been investigated, for example, internal stimuli that are characteristic for a tumor microenvironment (low pH, redox potential, high temperature, and enzymes) and external stimuli, such as magnetic fields, ultrasound, or light [54–56]. Both internal and external stimuli-sensitive liposomes will be addressed further, classified according to the mechanism exploited.

2.3.1. Internal stimuli

- a. The pH-sensitive triggered release is based on the degradation of the liposomal carriers followed by the release of the entrapped drug in tissues with a low pH, such as tumors, the cell cytoplasm, or the endosome [2, 12]. Although PEGylation increases the longevity of the liposomes in the circulation, in some cases it does not guarantee the escape of liposomes from endosomes, allowing the degradation of their contents prior to achieving their target. With the purpose of overcoming this problem, pH-labile linkers have been introduced between the hydrophilic PEG and the hydrophobic moiety, linkers that are cleaved upon exposure to the relatively low-endosomal pH or the acidotic tumor mass [57].

pH-sensitive dextran liposomes having 3-methylglutaryl residues (MGlu-Dex) were described. Surface modification of phosphatidylcholine liposomes with MGlu-Dex enabled obtaining highly pH-sensitive liposomes that were stable at neutral pH but were strongly destabilized in the weakly acidic pH region (pH ~5.5). *In vivo* data suggested that compared to unmodified liposomes, MGlu-Dex-ovalbumin liposomes efficiently increased the uptake of ovalbumin by dendritic cells and significantly suppressed tumor growth [58].

- b. Temperature-triggered drug delivery represents an attractive strategy in cancer therapy, because compared to normal tissues, pathological areas, such as tumors, show a distinctive hyperthermia [2]. Temperature-sensitive liposomes release the encapsulated drugs at the

melting-phase transition temperature (T_m) of the lipid bilayer/the lower critical solution temperature (polymers), temperature at which the membrane changes its permeability, disrupting to release the drug [59]. Temperature-sensitive liposomes have been widely investigated in the last decades and successfully applied in both preclinical and clinical studies in combination with heat-based therapies, such as radiofrequency ablation, ultrasound hyperthermia, and microwave hyperthermia [60]. A temperature-triggered liposomal system, ThermoDox[®] developed by Celsion Corporation (NJ, USA), has successfully demonstrated its improved efficacy during phase III clinical trials for the treatment of hepatocellular carcinoma and phase II trials for breast cancer and colorectal liver metastases [60].

- c. Enzymes, such as matrix metalloproteinases—MMPs (e.g. MMP2), phospholipase A2, alkaline phosphatase, transglutaminase, or phosphatidylinositol-specific phospholipase C, are overexpressed in tumor tissues and have been suggested as potential candidates for enzymatically triggered drug release from liposomes [61]. A hybrid liposome composed of phospholipid (DPPC) and PEGylated block-copolymer (Poloxamer 188) was described for the rapid release of encapsulated DOX in the presence of phospholipase A2 (PLA2). Drug release from liposomes was facilitated by higher PLA2 concentrations and was found to be dependent on the temperature and the presence of calcium ion, partially explaining PLA2-responsive drug release. DOX release from liposomes triggered by PLA2 exhibited enhanced cytotoxic effects on the A549 lung cancer cell line, suggesting that DPPC/P188 liposomes are a promising drug carrier for PLA2-expressing sites such as inflammatory lung cancer [62].

To overcome the fact that conventional liposomes have no mechanism for specifically releasing the encapsulated cargos inside the cancer cells, calcein-loaded liposomes containing a novel destabilization peptide (LMDP) were proposed. This peptide can destabilize liposomal membranes upon cleavage by the intramembranous proteases in cancer cells. *In vitro* tests showed that encapsulated calcein was successfully released in the presence of a membrane fraction containing an LMDP-cleavable protease, proving the responsiveness of the system to the cancer-specific protease [63].

2.3.2. External stimuli

- a. The use of activated light, made by the adjustment of parameters such as wavelength, intensity, pulse duration, and cycle, has been recognized as a promising tool for several biomedical applications, including light-triggered drug delivery [12]. Visible light, UV, and near-infrared (NIR) light have been investigated so far as triggers for the drug delivery; however, near-infrared is the most desirable for tumor targeting, since it penetrates deeper into the tissue. Thus, the preparation of porphyrin-phospholipid (PoP)-doped liposomes that are permeabilized by directly near-infrared light was described. Upon systemic administration, laser irradiation-enhanced deposition of actively loaded DOX in mouse xenografts, enabling an effective single-treatment antitumor therapy [64]. Another study reported the incorporation of an unsaturated phospholipid, such as dioleoylphosphatidylcholine (DOPC), in order to accelerate the near-infrared light-triggered DOX release in porphyrin-phospholipid

liposomes. The formulation inhibited human pancreatic xenograft growth in mice following a single intravenous administration of 6 mg kg^{-1} DOX, loaded in liposomes [65].

- b. Ultrasound-mediated drug delivery represents an attractive way to achieve noninvasive penetration into deep tissues and produce focused, controlled drug delivery [66]. High-intensity focused ultrasound (HIFU) produces local heating, which can promote phase transition of the lipids, facilitating drug release from liposomes. While HIFU is considered ideal for deeper tumors, low-frequency ultrasound (LFUS) is only appropriate for superficial tumors and has been used to trigger drug release from stealth liposomes without affecting the physicochemical properties of the drug [67]. Moreover, it was demonstrated that tumor vascular endothelium becomes more permeable after ultrasound.

A novel nanocarrier of emulsion liposomes (eLiposomes) composed of a perfluoropentane nanodroplet within the aqueous interior of a DPPC liposome, along with the anticancer drug DOX, was described. *In vitro* studies showed that the liposomes displayed good release of DOX upon the application of low-intensity ultrasound at 20 kHz, 1.0 MHz, and 3.0 MHz. This novel drug delivery system promises to provide enhanced drug delivery of DOX compared to traditional stealth liposomes and has the potential to reduce the side effects of cardiotoxicity caused by DOX [68].

- c. Magnetic-triggered drug release has received great attention in the past years, as magnetized liposomes have significant biomedical applications such as magnetic hyperthermia, magnetic transfection, and manipulation of cells and proteins [12]. Liposomes are usually magnetized by the incorporation of Fe_3O_4 or $\gamma\text{-Fe}_2\text{O}_3$, and once exposed to a magnetic field, the chemotherapeutic agent incorporated is completely released. Due to their magnetic properties, nanoscale size (approximately 10 nm), and biocompatible nature, these magnetized liposomes are also referred to as SPIONs [2]. For example, DOX-loaded magnetic liposomes were proposed as strategy for anti-colorectal cancer treatment, using a combination of chemotherapy and thermotherapy. *In vitro* cytotoxicity and hyperthermia studies were evaluated against colorectal cancer (CT-26 cells) with high-frequency magnetic field (HFMF) exposure and was found that the combination between DOX-loaded liposomes and HFMF was more effective than either hyperthermia or chemotherapy treatment individually [69].

2.4. Multifunctional liposomes

The current trend reflected by the scientific publications in the field is to develop liposomal nanoformulations that simultaneously demonstrate more than one useful function, that is, multifunctional liposomes, by combining two (longevity and targetability; targetability and stimuli sensitivity) or even all three functionalities mentioned above (longevity, targetability, and stimuli sensitivity). Thus, an ideal nanoformulation used for tumor-targeting purposes should possess the following properties: long circulation in the body, specificity for the site of the disease, sensitivity to local/external stimuli found in/applied to the tumor tissue, enhanced intracellular delivery of the drug, contrast properties to allow *in vivo* visualization, and others [70].

2.4.1. Liposomes combining *in vivo* longevity and specific target recognition

This type of liposomal formulations combine the drug delivery advantages of PEGylation, such as longevity in blood and passive tumor accumulation, with tumor cell-specific or tumor endothelium-specific delivery by ligand association at their surface. In spite of the advantages, the specific ligands attached to the surface of liposomes may increase the rate of uptake by the RES, could facilitate the development of unwanted immune response, and their amount must be optimized to ensure successful binding to the target [70].

The majority of research in this field utilizes monoclonal antibodies for the design of PEGylated immunoliposomes. Several PEGylated immunoliposomes designed for specific target of EGFR are described in Section 2.2.1. Others are designed to target the human epidermal growth factor receptor 2 (HER2), a growth hormone receptor overexpressed on the surface of certain types of breast cancer cells. HER2 antibody was used in a recent study as a targeting ligand in PEGylated immunoliposomes loaded with DOX. The formulation was tested for combination therapy in association with liposomal bevacizumab, and animal studies revealed increased accumulation of DOX at the tumor site and a significant delay of tumor growth in the combinational liposomal drug delivery group compared to free DOX, liposomal DOX, immunoliposomal DOX, and liposomal bevacizumab [71].

Several research groups developed long-circulating targeted liposomes as a strategy to transport drugs across the BBB for treating brain glioma. Thus, polyethyleneimine (PEI), a positively charged polymer, and vapreotide (VAP), a synthetic somatostatin analog, were used as targeting molecules for vinorelbine and tetrandrine. The multifunctional drug-loaded system demonstrated enhanced antitumor efficacy on glioma-bearing mice, explained by a combination of long circulation time in the blood (PEGylated lipids), enhanced transport of drugs across BBB (absorptive-mediated endocytosis by PEI, blocking the expression of P-gp protein by tetrandrine), and increased intracellular uptake by glioma cells and glioma stem cells (receptor-mediated endocytosis by VAP) [72].

Another group reported the use of stabilized peptide ligands, that is, cA7R (cyclic A7R) and ^DA7R, for multifunctional glioma-targeted drug delivery. The mentioned peptides were developed to enhance the proteolytic stability of the linear L-peptide A7R (^LA7R), which binds with high affinity and specificity to vascular endothelial growth factor receptor 2 (VEGFR2) and neuropilin-1 (NRP-1), which are overexpressed in glioma. In one study, ^DA7R, the retro-inverso derivative of ^LA7R, was associated to PEGylated liposomes to achieve multifunctional targeting of DOX to glioma. ^DA7R had similar binding affinity to its receptors *in vitro*, but ^DA7R-conjugated liposomes were superior to ^LA7R-modified liposomes in terms of antitumor efficiency *in vivo*, due to their better serum stability and higher tumor accumulation [73]. The same authors conjugated the cyclic derivative, cA7R, on the surface of DOX-loaded PEGylated liposomes, and the resulted system exhibited excellent antitumor, anti-angiogenesis, and anti-vasculogenic mimicry effects, resulting in improved therapeutic efficacy in U87 xenograft nude mice as compared to other DOX formulations (solution, non-functionalized liposomes, or liposomes functionalized with ^LA7R) [74].

2.4.2. Active-targeted, stimuli-sensitive long-circulating liposomes

Many liposomal systems described combine long circulation properties, with active targeting and stimuli-responsive drug release functions. The release of drugs from such carriers is triggered specifically at target sites either by local characteristics specific for the tumor tissue or by the application of stimuli at target tissue from outside of the body [75]. Such multifunctional approach was exploited in EGFR-targeting-thermosensitive liposomes. The liposomes were functionalized with GE11, an EGFR-specific peptide or Cetuximab antibody fragments (Fab') for comparison, and dipalmitoylphosphatidylcholine (DPPC):DSPC:DSPE-PEG:DSPE-PEG-GE11 were used to achieve thermosensitivity. The proposed liposomal formulation released DOX at temperatures above 40°C. Of the two investigated anti-EGFR ligands, Fab' was more potent in terms of cellular uptake. On breast cancer cell lines, targeted liposomes encapsulating DOX proved to be more cytotoxic than the plain liposomal DOX [76]. In another study, multifunctional liposomes with target specificity, temperature-triggered drug release, and near-infrared fluorescence imaging were designed. DOX-loaded stealth liposomes were modified with thermosensitive poly[2-(2-ethoxy)ethoxyethyl vinyl ether] chains, conjugated with the antibody trastuzumab (Herceptin, HER), and furthermore indocyanine green was incorporated for near-infrared fluorescence imaging. The group reported the excellent ability of these liposomes for association and internalization to target cells overexpressing Her-2, when heated at 45°C for 5 min [77].

2.4.3. Multifunctional liposomes for enhanced intracellular delivery

In order to improve the cytotoxicity of the chemotherapeutics loaded in liposomes, the use of cell-penetrating peptides (CPPs), which enhance the transport through the plasma membrane into cells, has been proposed [78]. Among these, the use of transactivator of transcription peptide (TATp) in the design of multifunctional liposomes has been shown to enhance cell uptake and cytotoxicity of the loaded drug, or even to increase the therapeutic efficacy against multidrug-resistant cancer cells [79, 80]. To prevent the proteolytic degradation of TAT, which might alter its targeting properties, it is necessary to shield it, usually through the use of PEG chains.

In a recent study, the advantages of formulating paclitaxel (PTX)-loaded liposomes functionalized with TAT and cleavable PEG via a redox-responsive disulfide linker (PTX-C-TAT-LP) were investigated. At tumor site, in the presence of exogenous reducing agent glutathione (GSH), PEG was detached and TAT was exposed to facilitate cell internalization. Compared to conventional stealth PTX-TAT liposomes, PTX-C-TAT-LP achieved enhanced tumor distribution and demonstrated superior delivery efficiency both *in vitro* and *in vivo* [81]. Another study reports a novel dual-functional liposome system possessing mitochondrial targeting properties and extracellular pH response which has been proved to enhance paclitaxel accumulation into the mitochondria. Peptide D[KLAKLAK]2 (KLA) was modified with 2, 3-dimethylmaleic anhydride (DMA) and combined with 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) to yield a DSPE-KLA-DMA (DKD) lipid, which, at tumor extracellular pH (~6.8), reversed the surface charge of liposomes (negative to positive), facilitating

their internalization. *In vitro* studies proved that pH-sensitive-modified liposomes exhibited improved efficacy in treating drug-resistant lung cancer A549/Taxol cells compared to conventional therapy [82].

3. Clinical experience with liposomes for cancer chemotherapy

Research on chemotherapy via liposomal drug delivery has known significant progress in the last decades, evolving from *in vitro* and *in vivo* preclinical studies on animals to numerous clinical trials. There are over 1000 clinical trials containing the terms “liposome” and “cancer,” either completed or active, according to The National Institutes of Health’s (NIH) web-based database, ClinicalTrials.gov and the EU Clinical Trial Register. There are several ongoing clinical trials investigating the efficiency of liposomal cisplatin, NDDP (cisplatin analog), paclitaxel, mitoxantrone, irinotecan, SN38 (the active metabolite of irinotecan), topotecan, lurtotecan, a camptothecin analog, vinorelbine, annamycin, docetaxel, DOX, and vincristine [83–87]. The association of chemotherapeutic drugs is a frequently used strategy in chemotherapy. In this sense, some clinical trials evaluate the synergistic cytotoxicity of a combination of two agents, such as irinotecan hydrochloride-floxuridine and cytarabine-daunorubicin in liposomal forms [86, 88].

Moreover, liposomes are the first nanoscale systems to be approved in 1995. The first liposomal system approved by the regulatory authorities for the treatment of cancer was liposomal DOX (in 1995), marketed as Doxil[®] in the USA and Caelyx[®] in Europe [89, 90]. Other liposomal DOX formulations, such as Myocet[®] and Lipo-Dox[®], have also been introduced into the market [91]. Lipo-Dox[®], Doxil[®], and Caelyx[®] are sterically stabilized liposomal DOX formulation having the same clinical indications. In contrast to these products, Myocet[®] is a non-PEGylated liposome encapsulating DOX, used to treat metastatic breast cancer in association with cyclophosphamide [90].

Other cytotoxic drugs incorporated in approved liposomal products are daunorubicin and vincristine in DaunoXome[®] and Marqibo[®], respectively [91]. DaunoXome[®] is a conventional liposomal formulation containing daunorubicin as a citrate salt, used in clinical practice in the treatment of Kaposi’s sarcoma [91]. Marqibo[®] is a sphingomyelin and cholesterol-based liposomal formulation of vincristine [90], indicated in acute lymphoblastic leukemia [91].

All aforementioned products are administered intravenously, but other routes of administration are also exploited in liposomal drug delivery. For instance, DepoCyt[®], a liposomal system containing cytosine arabinoside (a nucleoside analog of deoxycytidine), is administered spinally/intrathecally in neoplastic meningitis and lymphomatous meningitis [92, 93].

Currently, there are several liposomal systems for active targeting that are being investigated in different stages of clinical trials, but no formulation is commercially available. Most of them refer to liposomal systems modified with a transferrin receptor-targeted ligand.

For instance, MBP-426 is a liposome system conjugated with human transferrin for the delivery of oxaliplatin in patients with advanced or metastatic solid tumors that has completed a phase I clinical trial. It was also investigated in a phase Ib/II clinical trial in combination with leucovorin and 5-fluorouracil in second-line patients with metastatic gastric, gastroesophageal junction, or esophageal adenocarcinoma [94, 95].

Even though liposomes and targeting antibodies are both approved for clinical use, there are few studies on nanosystems which combine these two strategies. For example, anti-EGFR immunoliposomes encapsulating DOX have been shown to target the epidermal growth factor receptor by coupling Fab' fragments of the Cetuximab monoclonal antibody on the surface of the liposomes [96, 97]. MCC-465 is a PEGylated immunoliposome containing DOX, modified with the F(ab')₂ fragment of GAH human monoclonal antibody, for the treatment of gastric cancer [97, 98]. The delivery of DOX to the brain via liposomes has been enhanced by conjugation with glutathione. 2B3-101 is a glutathione-PEGylated-liposomal system capable of transporting DOX across the BBB by using the glutathione transporters [97, 99].

4. Conclusions

The liposomes present great potential for applications in targeted delivery of chemotherapeutics in the treatment of cancer. Based on their potential, several formulations are already approved and are clinically used in cancer treatment. However, many more have failed during the preclinical evaluation or early stages of clinical development. Therefore, future development of liposomal-based-targeted chemotherapy should comprise strategies based on deep understanding of the pathophysiological mechanism of the disease, on the preparation process and stability issues, and on the correlation between the physicochemical characteristics of the nanocarrier and its targeting ability.

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