Expert Opinion

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Liposomal cancer therapy: exploiting tumor characteristics

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Importance of the field: More than 10 million people worldwide are diagnosed with cancer each year, and the development of effective cancer treatments is consequently of great significance. Cancer therapy is unfortunately hampered by severe dose-limiting side effects that reduce the efficacy of cancer treatments. In the search for more effective cancer treatments, nanoparticle-based drug delivery systems, such as liposomes, that are capable of delivering their drug payload selectively to cancer cells are among the most promising approaches.

Areas covered in this review: This review provides an overview of current strategies for improving the different stages of liposomal cancer therapy, which involve transporting drug-loaded liposomes through the bloodstream, increasing tumor accumulation, and improving drug release and cancer cell uptake after accumulation at the tumor target site.

What the reader will gain: The review focuses on strategies that exploit characteristic features of solid tumors, such as abnormal vasculature, overexpression of receptors and enzymes, as well as acidic and thiolytic characteristics of the tumor microenvironment.

Take home message: It is concluded that the design of new liposomal drug delivery systems that better exploit tumor characteristic features is likely to result in more efficacious cancer treatments.

Keywords: cancer, drug delivery, drug targeting, ligand, lipid vesicles, liposomes, nanomedicine, nanoparticles, tumor

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1. Introduction

Cancer is a disease that originates from normal healthy cells of multicellular organisms. Upon accumulation of several critical mutations, normal cells may develop into malignant cancer cells characterized by rapid and uncontrolled proliferation [1]. The fact that cancer originates from an organism's own cells means that cancer cells are identical to normal cells in most respects, which makes it difficult to find drugs that are selectively toxic to cancer cells while being non-toxic to healthy cells. Most cancer drugs in current use target the high proliferation rate of cancer cells by acting on cellular targets associated with cell division. For example, action mechanisms of anticancer drugs include: disruption of DNA replication and transcription, inhibition of enzymes involved in DNA synthesis, and interference with the synthesis or breakdown of mitotic spindles involved in cell division. Naturally, these drugs also act on healthy cells and therefore cause severe dose-limiting side effects that hamper the efficacy of cancer chemotherapy. To overcome this problem, two main approaches are being attempted to improve the specificity of cancer chemotherapy. One approach involves the discovery of new anticancer drugs with improved specificity towards cancer cells. The other approach utilizes various types of drug carriers as tumor targeting vehicles that are able to transport the cancer drugs to the cancerous tissue and thereby achieve higher levels of tumor drug concentration relative to drug concentration in healthy tissue. Occasionally, these

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Article highlights.

- Liposomal cancer therapy utilizes liposomes as drug carriers for transporting anticancer drugs to tumors, and thereby increases drug accumulation.
- Polymers such as PEG are attached to the liposome surface to avoid rapid clearance from circulation and achieve long blood circulation times.
- Long circulating liposomes accumulate in tumor tissue by exploiting the leaky vasculature and poor lymphatic drainage that are characteristic of solid tumors.
- Ligands that bind to receptors or antigens that are overexpressed in tumor tissue can be attached to liposomes in an attempt to further increase tumor accumulation.
- Ligands may also increase cancer cell uptake of liposomal drugs by receptor-mediated endocytosis of intact liposomes.
- Tumor-specific drug release and prodrug activation can be achieved by exploiting the thiolytic and slightly acidic environment of solid tumors, or by enzymes that are overexpressed in tumors.
- Kinetics of tumor accumulation, cellular internalization and drug release affect the efficacy of liposomal formulations.

This box summarises key points contained in the article.

two approaches overlap, as many emerging anticancer drugs with improved specificity, such as DNA, RNA and peptide-based drugs, often require a drug delivery system to protect them from degradation in the bloodstream and to facilitate efficient cellular uptake into cancer cells [2,3]. Numerous types of drug carriers are being pursued for cancer therapy, including polymeric nanoparticles, liposomes, micelles and dendrimers [4]. The focus of this review is on liposomal drug delivery systems for cancer chemotherapy.

Liposomes were discovered by Bangham and co-workers in 1965 [5], and were soon after recognized as promising drug carriers [6,7]. Since then, substantial progress in the field of liposomal drug delivery has been achieved and first-generation liposomal formulations such as Doxil® (PEGylated liposomal doxorubicin; Centocor Ortho Biotech Inc., Horsham, PA, USA), Myocet® (non-PEGylated liposomal doxorubicin; Sopherion Therapeutics, Princeton, NJ, USA) and DaunoXome® (non-PEGylated liposomal daunorubicin; Diatos, Paris, France), have entered the clinic [8-10]. Although these formulations have improved some aspects of cancer treatment, there is still ample room for improvement of liposomal cancer therapeutics, and significant advances are continuously achieved.

At the most fundamental level, liposomal cancer therapy involves three steps, namely: encapsulation of drugs in the liposome interior or within the lipid bilayer; targeting of the liposomes to tumor tissue or circulating cancer cells; and delivery of the drugs to cancer cells. This review focuses on steps 2 and 3, concerning tumor targeting and delivery of drugs to cancer cells, and focuses on current methods for achieving these goals by exploiting histological and histochemical characteristics of cancerous tissue that distinguish tumors from healthy tissue. In other words, tumor targeting and drug release by externally applied stimuli such as magnetism and heating, for example, as well as technical aspects related to encapsulation of drugs in liposomes, fall outside the scope of this review.

2. Tumor targeting

Tumor targeting, the ability of engineered materials to accumulate predominantly in tumor tissue, can be considered the first criterion that liposomes must satisfy as drug delivery vehicles for cancer therapeutics. Liposomal drug delivery systems can be designed to accumulate in tumors by two different strategies, commonly referred to as passive targeting and active targeting.

2.1 Passive targeting by the enhanced permeability and retention effect

Passive targeting exploits the leaky vasculature and poor lymphatic drainage that characterizes many malignant tumors. Nanoparticles that are too large to penetrate the barrier constituted by endothelial cells in normal blood vessels but small enough to diffuse through the abnormal gaps that exist between endothelial cells of tumor blood vessels will extravasate into the extracellular space in tumor tissue. As a result of the diminished lymphatic drainage in tumors, the liposomes are retained after extravasation. In combination, these two characteristics make tumor vasculature function as a sieve that filters out nanoparticles and causes them to accumulate in tumor tissue. This principle of tumor targeting is known as the enhanced permeability and retention (EPR) effect, and is utilized as a tumor targeting principle for liposomal as well as other nanoparticulate and macromolecular drug delivery systems [11,12]. Passive targeting of liposomes to tumor tissue by the EPR effect is illustrated in **Figure 1A**.

The EPR effect is a progressive phenomenon that requires many passages of nanoparticles through tumor vasculature to achieve substantial tumor accumulation. For this reason, early liposomal formulations were unable to take advantage of the EPR effect because they were recognized as foreign particles by the cells of the reticuloendothelial system (RES) mainly present in the liver and spleen, and rapidly cleared from the blood circulation before getting a chance to accumulate in tumors [13]. A major breakthrough was achieved by the discovery that incorporation of first ganglioside GM1 [14], and later to an even larger extent polyethylene glycol (PEG) lipids [15,16], increased the blood circulation time of liposomes significantly. This discovery enabled tumor accumulation by the EPR effect and as a result improved antitumor efficacy of liposome-encapsulated cancer drugs [17,18]. Since the discovery that PEG-lipids increase blood circulation time of liposomes, various other polymers have been investigated for their ability to improve liposome circulation time [19-22], but PEG remains the polymer of choice thus far.

The structure of PEG-lipids and the incorporation into liposomes are shown in **Figure 2**. The most widely held belief

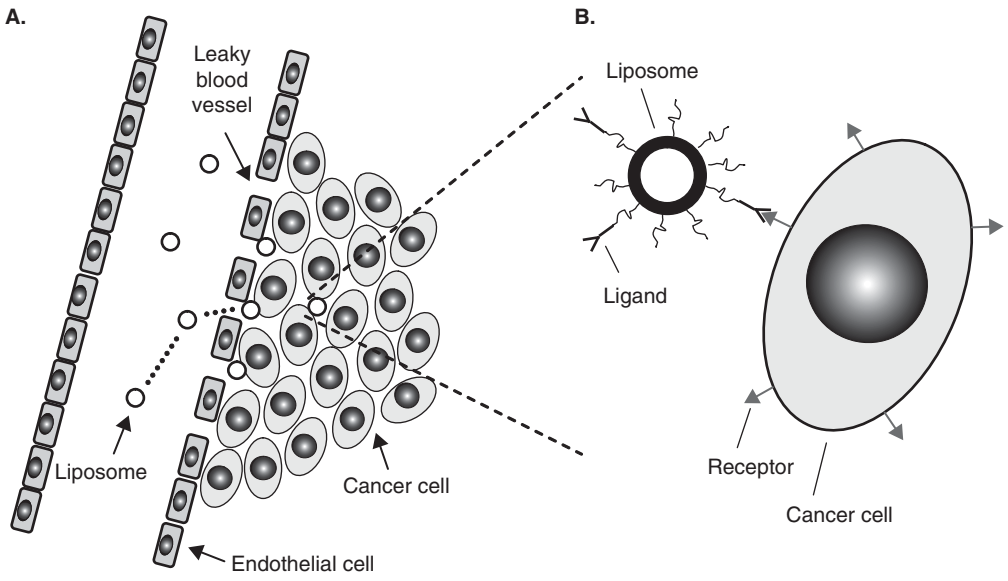

Figure 1. Targeting mechanisms in liposomal cancer therapy. A. Passive targeting by the enhanced permeability and retention effect. The gaps between endothelial cells of the tumor blood vessels lead to extravasation of liposomes into the tumor tissue, and the poor lymphatic drainage results in liposomes being retained inside the tumor. **B.** Active targeting. Ligands attached to liposomes bind to receptors or antigens that are overexpressed by cancer cells or other tumor-associated cells such as endothelial cells of the tumor vasculature.

regarding the mechanism of increased circulation time is that PEG grafted to the liposome surface creates a steric barrier that opposes the adsorption of opsonins to the liposome surface [23,24]. Opsonins are proteins of the immune system that attach to foreign particles and increase the subsequent uptake by macrophages. The notion that PEG increases circulation time by decreasing protein binding is supported by both *in vitro* studies that have demonstrated a screening effect of PEG against protein adsorption to liposome surfaces [24-26], as well as some *in vivo* studies where low protein binding in the bloodstream has correlated with longer circulation times [27]. By contrast, results from other studies have shown that the presence of bound serum proteins did not result in increased macrophage uptake, and that pre-incubating the liposomes with serum actually lowered macrophage uptake [28]. In accordance, it has also been shown that the uptake by liver cells of polystyrene microspheres coated with PEG containing block copolymers was higher before adsorption of plasma proteins than after adsorption [29]. These results have led to an alternative explanation for the ability of PEG to extend blood circulation time of liposomes. Rather than minimizing the adsorption of all serum proteins, it is speculated that certain serum proteins adsorb to the liposome surface in spite of the PEG coating, and subsequently act as nonspecific dysopsonins that, along with the PEG polymers, prevent the adsorption of opsonin proteins. However, current knowledge of protein adsorption to liposomes and other nanoparticles *in vivo* is not sufficient to elucidate the exact mechanism of the extended circulation. Regardless of the mechanism, however, it is certain that PEG significantly increases the circulation time of liposomes, to the point where substantial tumor accumulation by passive targeting occurs.

An unfortunate side effect observed with Doxil [30] and other PEGylated liposomal formulations [31] is that it causes hypersensitivity reactions in some patients, with symptoms such as shortness of breath, facial swelling, headache, chills, hypo- and hypertension, chest pain and back pain. It has been demonstrated that the negatively charged PEG-lipids activate the complement system, which may be linked to the observed hypersensitivity reactions in some patients [32]. Recently, it has also been shown that the complement activation mechanism of PEG-lipids can be abolished by methylation of the PEG-lipid phosphate group, which removes the negative charge [33].

The upper cutoff size for liposomes to be able to extravasate through the leaky vasculature and into tumor tissue has been investigated for several different tumor types. PEGylated liposomes as well as latex beads of different sizes were injected intravenously and extravasation followed by fluorescence microscopy [34,35]. The results showed that cutoff size ranged from 200 nm to 1.2 μm and varied with both tumor type and tumor location, whereas most of the tumor models had a pore cutoff size between 380 and 780 nm.

2.2 Active targeting by tumor-specific ligands

Cancer cells and tumor-associated tissue such as cells of the tumor vasculature overexpress certain cell surface receptors and antigens in comparison with healthy cells, and this difference in expression levels is being used as another tumor targeting principle in liposomal cancer therapy, known as active targeting [36]. Active targeting involves the attachment

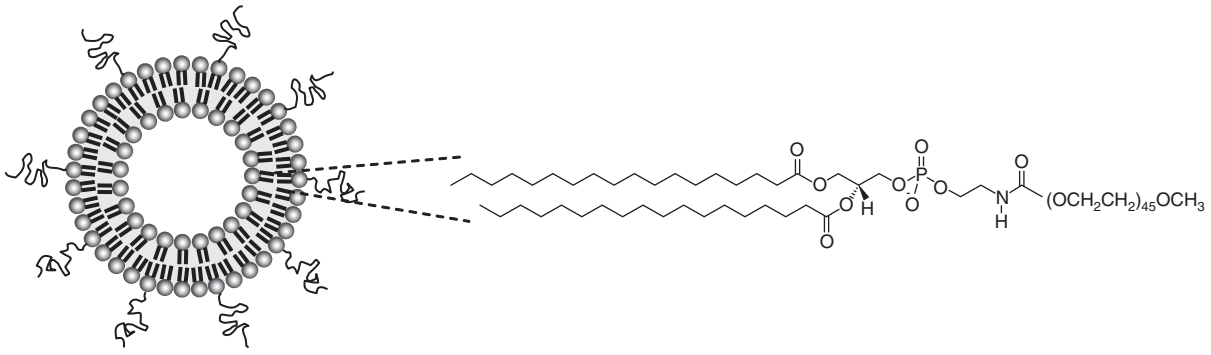

Figure 2. Schematic illustration of a liposome containing PEG-modified lipids, and the molecular structure of a PEG-lipid.

of ligands to the liposome surface that bind to cancer-specific receptors and antigens by molecular recognition. In this way, the liposomes are designed to bind in a cell-specific way rather than relying on passive targeting by the EPR effect alone. The ligand-mediated binding to tumor tissue receptors and antigens may, among other things, result in improved tumor accumulation [37,38]. The active targeting principle is shown schematically in **Figure 1B**.

As far as solid tumors are concerned, it is important to realize that ligand-targeted liposomes are transported to the tumors in the same manner as passively targeted liposomes. Only after arriving in the tumor tissue are the ligands able to bind to the cancer cells. This means that ligand-targeted liposomes must have both long blood circulation times and efficient binding to tumor targets for appreciable tumor accumulation to occur. The combination of long circulation time and active targeting with tumor-specific ligands presents a challenge. In early studies with ligand-targeted liposomes the targeting ligands were attached directly to the liposome surface along with PEG-lipids. However, using this type of construct it was found that the PEG polymers created a steric barrier that obstructed ligand–receptor binding at the cancer target site [39]. Ligands are now usually attached to the distal end of the PEG chain to enable the ligands to extend outside the PEG surface layer, and thereby expose the ligands to the receptors they target, as illustrated in **Figure 3**.

The importance of allowing the ligands to extend outside the PEG polymer coating was clearly demonstrated by Lee and Low [40], who varied the length of the PEG chain to which a folate ligand was attached. When the PEG spacer length was increased from 23 to 250 Å they observed a 37-fold increase in liposome uptake by folate receptor-expressing KB cells *in vitro*. Furthermore, *in vivo* experiments by Blume *et al.* [41] showed that it was possible to combine long circulation time in the bloodstream with efficient target binding by attaching the ligands to the distal ends of the PEG chains. Although it is possible to obtain long circulating ligand-targeted liposomes, the attachment of ligands to PEG often decreases blood circulation time [42,43]. This is to be expected in cases where normal healthy cells that the liposomes encounter also contain receptors for the ligands, if the ligands increase unspecific binding to non-target tissue, or if the presence of ligands increases opsonization and macrophage uptake. The decrease in blood circulation times sometimes observed for ligand-targeted liposomes is naturally a disadvantage with respect to tumor accumulation. Furthermore, tumor targeting with high binding affinity ligands may result in incomplete tumor penetration because of an effect known as the binding site barrier. That is, the ligand-containing liposomes bind strongly to the first cancer cells encountered after extravasation from the blood vessels, and are thereby prevented from diffusing through the tumor tissue and reach cancer cells located further away from blood vessels [44,45]. It has also been suggested that the first liposomes that bind to targets near blood vessels may block the penetration of extra liposomes to deeper tumor regions [46]. Consequently, active targeting does not necessarily result in increased tumor accumulation, and even if increased tumor accumulation does occur, this does not necessarily result in increased anticancer efficacy if the increase in drug concentration takes place only near tumor blood vessels.

2.2.1 *Antibody targeting*

Various ligand types have been identified as having tumor-specific binding properties, including: antibodies, peptides, proteins, small molecule receptor ligands and carbohydrates [4]. Among the ligands used for active targeting, antibodies have superior specificity towards tumor tissue, whereas instability during storage and higher production costs are disadvantages of antibodies.

Antibodies (also known as immunoglobulins) are proteins used by the immune system to identify foreign objects in the body. They consist of two heavy and two light polypeptide chains that are organized in a Y-shaped structure, as illustrated in **Figure 4**. The distal end of both the light and the heavy chains varies considerably among different antibodies, and these regions account for the ability of antibodies to recognize and bind to different target antigens. The binding specificity of antibodies can be utilized for active tumor targeting, either by liposome attachment of whole monoclonal antibodies

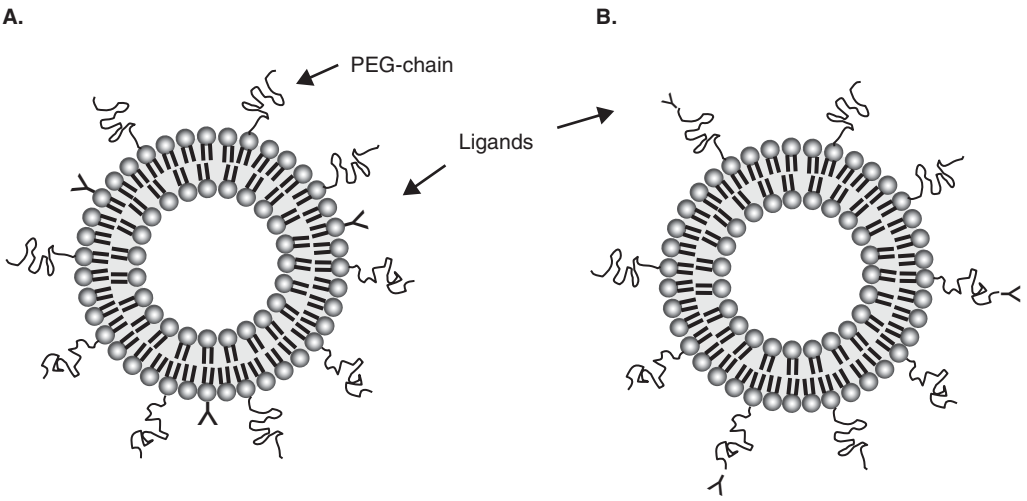

Figure 3. Two methods of attaching ligands to liposomes. A. Ligands are attached directly to the liposome surface. In this case the ligands are screened from efficient interaction with their target receptors or antigens, unless the PEG-lipids are designed to dissociate from the liposomes when reaching the tumors. **B.** Ligands are attached to the distal ends of PEG-lipids, which allows efficient interaction with target receptors and antigens. However, this way of exposing the ligands to the surroundings may decrease the blood circulation time.

(mAb), or antibody fragments such as antigen-binding fragment (Fab′) or single chain variable fragment (scFv), which still contain the antigen-binding region. An advantage of using antibody fragments is that whole antibodies result in shorter blood circulation times owing to the presence of the Fc region. Circulation times comparable to liposomes without ligands can be achieved when using scFv or Fab′ antibody fragments, resulting in improved accumulation and efficacy against both solid tumors [47,48] and B-cell lymphoma [49,50], as compared with both untargeted liposomes and liposomes targeted with whole antibodies. The shorter circulation times of whole antibody-targeted liposomes is a consequence of the Fc region, which acts as an antigen and antibody binding site, especially for macrophages in the liver and spleen [51]. The disadvantage of using Fab′ and in particular scFv fragments is that they are considerably less stable, resulting in shorter shelf-life of liposomes targeted with these proteins.

In recent years, several *in vivo* studies have demonstrated the potential of antibody-targeted liposomes to increase the anticancer efficacy, as compared with passively targeted liposomal formulations. In a study by Park *et al.* [52], doxorubicin-loaded liposomes containing Fab′ or scFv HER2 antibody fragments showed significantly increased antitumor efficacy against HER2 overexpressing breast cancer xenografts in mice as compared with non-targeted liposomes of identical composition. In a later study, the underlying mechanisms responsible for the observed improved antitumor efficacy of HER2-targeted liposomes was examined [53]. It was shown that the incorporation of the HER2 targeting moiety did not change the biodistribution, nor did it change the amount of drug accumulated in the tumor. However, the liposomal drug distribution within the tumor was different, as the antibody-targeted liposomes were located inside the tumor cells, whereas untargeted liposomes were predominantly located in the extracellular space and in macrophages. A sixfold higher intracellular concentration was measured, resulting from receptor-mediated endocytosis on binding to the HER2 receptor, and it was therefore concluded that the enhanced antitumor activity was related to increased cancer cell drug uptake, rather than increased tumor accumulation.

Similarly, Hatakeyama *et al.* [54] studied Fab′-antibody fragment-modified Doxil liposomes targeted towards membrane type 1 matrix metalloproteinases, which are involved in tumor angiogenesis and expressed on tumor cells as well as angiogenic endothelial cells. Compared with non-targeted Doxil, Fab′-containing Doxil liposomes showed increased cellular uptake *in vitro* and resulted in increased survival rates of tumor-bearing mice. However, tumor accumulation of Fab′-targeted liposomes was similar for non-targeted liposomes, indicating that the enhanced efficacy also in this case was related to the observed increase in cellular uptake resulting from the Fab′ modification, and not a result of increased tumor accumulation.

Another recent study using doxorubicin-loaded liposomes (Doxil) targeted to cancer cell surface-bound nucleosomes with 2C5 antibodies demonstrated a twofold increase in tumor accumulation in a murine breast cancer model, as compared with Doxil liposomes not containing the antibody or containing non-tumor-specific IgG proteins [55]. Moreover, the *in vivo* efficacy towards breast cancer (4T1), colon cancer (C26) and prostate cancer (PC3) models in mice was significantly improved. The same type of 2C5 antibody-targeted doxorubicin liposomes also displayed significantly improved efficacy towards Lewis lung carcinoma in another study [56]. Earlier *in vitro* experiments demonstrated that the cellular uptake of 2C5-modified and doxorubicin-loaded liposomes

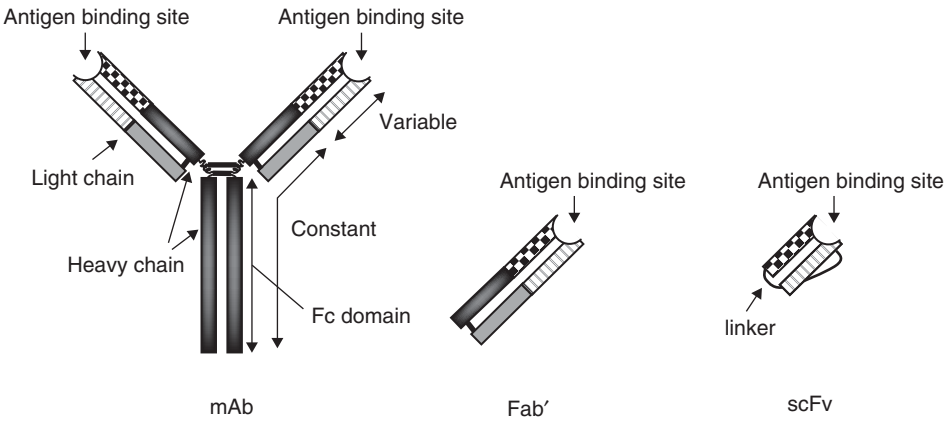

Figure 4. Structure of a monoclonal antibody (mAb) and two types of antibody fragment (Fab′ and scFv).

was increased three- to eightfold and the cytotoxicity as measured by IC_{50} values increased five- to eightfold as compared with doxorubicin liposomes without antibodies [57,58]. Thus, it seems likely that the increased cell uptake and cytotoxicity of the 2C5-targeted liposomes plays an important role in the increased *in vivo* efficacy observed for the 2C5-modified Doxil fomulations in addition to the increased tumor accumulation. Furthermore, the anticancer potential of the 2C5 antibodies themselves may be a third contributing factor for the increased efficacy [59], in addition to the increase in tumor accumulation and cellular uptake.

2.2.2 *Folate receptor targeting*

The folate receptor is an integral membrane protein involved in the cellular uptake of folic acid by receptor-mediated endocytosis. Folic acid is a vitamin necessary for the biosynthesis of DNA and the involvement in DNA synthesis is a likely explanation of why folate receptors are overexpressed in several cancer types. The overexpression by several cancer types, in combination with the fact that most normal tissues, except in the kidney, contain very low levels of folate receptor that is accessible to liposomes in the circulation, explains why folate receptors is one of the most studied receptors for ligand-based active targeting [60,61]. Moreover, folate is a small and innocuous molecule that is easily conjugated to lipids through its γ-carboxyl group without loss of binding affinity.

Gupta *et al.* [62] compared the tumor accumulation and anticancer efficacy of 5-fluorouracil (5FU) encapsulated in folate-targeted liposomes and non-targeted liposomes using a melanoma model in mice. They found an ~ 1.4 increase in tumor accumulation as well as improved anticancer efficacy of the folate-targeted liposomes. *In vitro* experiments also showed a much higher melanoma cell uptake of 5FU when loaded in folate-targeted liposomes.

By contrast, a study by Yamada *et al.* [43] showed only minor but statistically insignificant differences in antitumor efficacy between non-targeted and folate-targeted doxorubicin-loaded liposomes in a lung carcinoma model in mice. They carefully evaluated the *in vitro* and *in vivo* effects of attaching folate ligands to PEG spacers that were either shorter or longer than the unconjugated PEG chains used to achieve stealth properties. Also, the effect of folate concentration was investigated. Although increased cancer cell uptake and cytotoxicity of the folate-containing liposomes were demonstrated *in vitro*, the antitumor efficacies of folate targeted and non-targeted liposomes were similar *in vivo*. As a lower blood circulation time was observed for the folate-targeted liposomes than for non-targeted liposomes, it is possible that the increased targeting anticipated from tumor-specific binding of the folate-targeted liposomes was counteracted by an increased liposome clearance before the liposomes reached the tumor tissue.

A particularly interesting finding with folate-targeted liposomes is the ability of folate-containing liposomes to bypass the P-glycoprotein-mediated multi-drug resistance mechanism, which is one of the major obstacles in cancer treatment. This was observed in a study with doxorubicin-loaded folate-targeted liposomes, which showed increased intracellular doxorubicin concentrations and increased cytotoxicity of folate-targeted liposomes towards multi-drug resistant M109-HiFR lung cancer cells, as compared with untargeted doxorubicin-loaded liposomes [63]. It was demonstrated that the intracellular drug concentration of the folate-targeted liposomes was unaffected by addition of the P-glycoprotein inhibitor verapamil, which was in sharp contrast to administration of free doxorubicin. Hence, the results demonstrate the ability of folate-targeted liposomes to bypass the P-glycoprotein efflux pump, and it seems likely that this ability is related to the receptor-mediated endocytosis mechanism, which delivers the liposome-encapsulated drugs deeper inside the cells, away from the P-glycoprotein efflux pumps located in the plasma membrane.

2.2.3 *Transferrin receptor targeting*

Transferrin receptors are found in all nucleated cells, but are overexpressed in rapidly dividing cells such as many cancer cells. The overexpression in rapidly dividing cells is probably related

to the fact that iron is required for the functioning of ribonucleotide reductase, which is an enzyme involved in DNA synthesis [64]. The overexpression of transferrin receptor on several cancer cells enables active tumor targeting by using transferrin or transferrin receptor antibodies as liposome-targeting ligands [65]. Furthermore, transferrin receptors are located at the cell surface where they mediate uptake of iron bound to transferrin glycoproteins by means of receptor-mediated endocytosis, and targeting liposomes to transferrin receptors therefore facilitates cellular uptake of liposomes by receptor-mediated endocytosis, similarly to folate receptor targeting.

This potential has been demonstrated in studies showing increased cellular uptake of transferrin-targeted doxorubicin-loaded liposomes in C6 glioma cells *in vitro* [66], as well as increased antitumor efficacy against a human hepatoma mouse model *in vivo* [67]. Increased antitumor efficacy by transferrin targeting of oxaliplatin-containing liposomes against Colon26 mouse colon carcinoma has also been reported [68]. Interestingly, a recent study has shown that delivery of doxorubicin into multi-drug resistant human small cell lung cancer cell by transferring-mediated endocytosis to some extent was able to circumvent the P-glycoprotein efflux pump of the multi-drug resistant cells [69]. Another study by Wu *et al.* [70] used a combination of transferrin targeting and co-encapsulation of doxorubicin and an inhibitor for the P-glycoprotein efflux pump (verapamil) to overcome multi-drug resistance in leukemia cells. This formulation significantly increased doxorubicin sensitivity and it was shown that both the transferring-mediated cellular uptake and co-delivery of verapamil played important roles in overcoming multi-drug resistance. Thus, it appears that the ability of transferrin receptor targeting and folate receptor targeting to overcome P-glycoprotein efflux pump-related multi-drug resistance are similar, which supports the hypothesis that receptor-mediated endocytosis of drug-loaded liposomes is a promising avenue towards overcoming the major challenge of multi-drug resistance.

2.2.4 Targeting angiogenic blood vessels in tumors

When tumors reach a size of a few millimeters they need to establish their own blood supply in order to receive the nutrients and oxygen required for further growth. The creation of new tumor blood vessels, angiogenesis, takes place by recruiting new blood vessels from the pre-existing vasculature [71,72]. Angiogenic blood vessels in tumors express several proteins that are absent or present in much lower numbers in established blood vessels, and are consequently attractive targets for ligand-targeted liposomal cancer therapy. Compared with targets that are located on the cancer cell surface, such as folate and transferrin receptors, the targeting of tumor vasculature has certain advantages. First of all, antigens on the tumor vasculature are more accessible than antigens on the cancer cell surface, as liposomes do not have to penetrate the tumor to reach the antigens. Second, endothelial cells of the tumor vasculature are more genetically stable and do not develop drug resistance to the same extent as cancer cells. Third, killing of a relatively small number of endothelial cells in the blood vessels can lead to killing of a large number of cancer cells that these vessels support.

Ligands for targeting angiogenic blood vessels include peptides containing the RGD (Arg-Gly-Asp) or NGR (Asn-Gly-Arg) sequences. The RGD sequence has been found to bind to $\alpha_V\beta_3$ integrin and $\alpha_V\beta_5$ integrins of tumor vasculature, whereas the NGR sequence binds to an endothelium-associated form of aminopeptidase N, which is an enzyme involved in angiogenesis and tumor growth [73-75].

The potential of tumor vasculature targeting was demonstrated in a recent study by Murphy *et al.* [76], who used doxorubicin-containing liposomes targeted by a lipid-anchored cyclic RGD peptide. The antiangiogenic effect was visualized by confocal microscopy in a pancreatic tumor model, which showed a substantial disruption of the tumor vasculature. In addition, the efficacy of the RGD-targeted liposomal formulation was evaluated with respect to both primary tumor growth and metastases in both pancreatic and renal cell carcinoma models in mice. A substantial increase in efficacy towards metastasis was recorded for the RGD-targeted formulation, whereas the effects on primary tumors were more modest, although still better than untargeted liposomes and free doxorubicin. Visualization of binding sites of the RGD liposomes demonstrated that binding to primary tumors occurred only at a subset of blood vessels located near the tumor periphery. In correspondence with this observation, apoptosis was observed only at the tumor periphery, illustrating that integrin targeting to neovasculature is most effective in treating metastasis, whereas the effect on primary tumors with pre-established vasculature is more limited because integrin is not expressed in the vasculature of the tumor interior.

Another study by Hölig *et al.* [77] used phage display to select RGD peptides with high affinity for endothelial and melanoma cells. The peptide sequences with the highest binding affinities were used to prepare lipopeptides by conjugating the peptides to phospholipids, and were subsequently incorporated into doxorubicin-loaded liposomes. An unfortunate effect of peptide incorporation was that the lipopeptides significantly decreased blood circulation time at a concentration of 1 mol%. Nevertheless, a liposomal doxorubicin formulation containing only 0.1 mol% lipopeptides resulted in improved efficacy in a C26 colon carcinoma mouse model compared with untargeted liposomes and free doxorubicin.

The ability of NGR peptides to target tumor vasculature and improve therapeutic outcome has also been demonstrated, in a study by Pastorino *et al.* [78]. They used doxorubicin-loaded liposomes targeted by NGR peptides to aminopeptidase N present on angiogenic tumor vessels of neuroblastoma xenografts in mice. It was found that tumor accumulation was at least 10-fold higher for targeted liposomes compared with untargeted liposomes, and that the increased tumor accumulation was abolished when inhibiting

the NGR binding sites by co-injecting a large excess of free NGR. Notably, the increase in tumor accumulation was substantially higher than what most studies targeting cancer cell surface antigens have demonstrated, which might be a reflection of the fact that tumor vasculature is more easily accessible for binding of ligand-targeted liposomes. Furthermore, the targeted doxorubicin formulation resulted in substantial tumor regression as well as a reduction in blood vessel density. It was observed that the liposomes were able, to some extent, to penetrate into the tumor interstitium in spite of the NGR ligands. Thereby a dual targeting of both cancer cells in the tumor interior, as well as the tumor blood vessels supporting the cancer cells, was achieved, and it was speculated that this dual targeting effect contributed to the impressive antitumor efficacy.

2.2.5 Combined tumor cell and tumor vasculature targeting

Targeting towards both tumor vasculature and cancer cell surface antigens has been achieved by using a combination of two different liposomal preparations containing different targeting ligands [79]. This study examined the effect of treating neuroblastoma-bearing mice with a combination of two liposomal formulations composed of: i) doxorubicin-loaded liposomes targeted to disialoganglioside receptor GD2 (which is overexpressed on the surface of cancer cells of neuronal origin) by attachment of GD2 antibodies or their Fab′ fragments; and ii) doxorubicin-loaded liposomes targeted to endothelial cell-specific aminopeptidase A (CD13) present in angiogenic tumor blood vessels, by an NGR peptide ligand. Combined treatment where first liposomal formulation i) and then liposomal formulation ii) were administered, each at half doxorubicin doses, showed a substantial improvement in lifespan and some long-term survivors, whereas the lifespan was much shorter for mice receiving treatment with either liposome formulation on its own.

Another study used hybrid polymer/liposome particles to achieve temporal release of first an antiangiogenesis agent (combrestatin) followed by a chemotherapy agent (doxorubicin), where both were delivered in the same formulation [80]. To achieve temporal release, doxorubicin was conjugated to PLGA polymers, and this conjugate was encapsulated in liposomes containing the lipophilic combrestatin in the bilayer. After tumor accumulation by the EPR effect, the outer liposome shell first released combrestatin, leading to vascular shutdown and causing the PLGA–doxorubicin conjugate to become trapped inside the tumor. The *in vivo* antitumor efficacy was evaluated against both melanoma and lung cancer models in mice. The formulation was superior to liposome/PLGA particles containing either combrestatin or doxorubicin alone and was also superior to co-administration of particles containing each drug alone, thus demonstrating that both the temporal release and combined targeting of tumor cells and tumor vasculature were important for the improved anticancer efficacy of the formulation.

2.2.6 Active targeting with multiple ligands

The number of antigens or receptors on tumor target cells is naturally important for targeting specificity of ligand-targeted liposomes. This was demonstrated in a study using anti-HER2-modified liposomes to target HER2-expressing breast cancers, where it was found that a receptor density of 10^4 receptors/cell resulted in comparable therapeutic efficacy of targeted and non-targeted liposomal formulations, whereas the efficacy of targeted liposomes strongly exceeded the efficacy of non-targeted liposomes in models expressing higher levels of HER2 antigens [52]. Cancer cells frequently overexpress more than one type of receptor, and it may therefore be beneficial to attach two or more different ligands to liposomal formulations, as this method increases the total number of receptors available for liposome–ligand binding on the cancer cells. This strategy has been used to target liposomes conjugated with both CD19 and CD20 antibodies to B lymphoma cells expressing both CD19 and CD20 epitopes [81]. The results showed that the binding, uptake and cytotoxicity of liposomes containing both ligands were greater than liposomes targeted with either ligand individually.

The benefits of targeting with multiple ligands was also demonstrated in a study by Saul *et al.* [82], who used a dual ligand strategy to enhance the targeting selectivity of doxorubicin-loaded liposomes towards KB cells, which overexpressed both folate receptor and the epidermal growth factor receptor (EGFR). Liposomes containing both an antibody targeting the EGFR as well as ligands targeting the folate receptor were prepared, and the cytotoxicity to KB cells was compared with KB cells where one or both receptors were blocked in order to simulate normal healthy cells that express only one or no receptors. By selecting the number of ligands attached to the liposomes such that the density of each individual ligand alone was too low to induce significant toxicity to cells expressing only one of the two receptors, it was shown convincingly that the dual ligand strategy improved selectivity towards the cancer cells expressing both receptors, while sparing cells expressing a single or no receptor. However, in relation to targeting with multiple ligands, one should realize that the increased number of total ligands on the surface of liposomes may induce a greater immune response, which could lead to a reduced circulation time or adverse effects.

2.2.7 Other tumor tissue characteristics for ligand targeting

In addition to the targets discussed above, several other receptors and antigens have been used for ligand-based targeting of liposomes to tumors, including: vasoactive intestinal peptide receptors overexpressed in breast cancer [83], hyaluronan receptors overexpressed by several cancer types [84-86], sigma receptor overexpressed by melanoma, non-small-cell lung carcinoma, breast cancers of neural origin and prostate cancer [87], and urokinase plasminogen activator receptor overexpressed especially in prostate and breast cancers [88], among others.

3. Drug release and cancer cell uptake

Once the drug-loaded liposomes have arrived at the tumor site, the drug must be released from the liposomes and delivered to the cancer cells to have a therapeutic effect. As most anticancer drugs act on intracellular targets, the drugs must be taken up by the cancer cells to exert their therapeutic activity. Drug release may take place either extracellularly in the tumor interstitium, followed by cancer cell uptake of free drugs, or by uptake of intact liposomes, followed by drug release from liposomes inside the cancer cells, as illustrated in **Figure 5**.

3.1 Extracellular release in the tumor interstitium

In the following, various approaches for achieving extracellular drug release into the tumor interstitium are discussed. This release mechanism requires subsequent cancer cell uptake of free drug, and is consequently suitable only for cell membrane permeable drugs or drugs amenable to active uptake mechanisms by cancer cells.

3.1.1 Release by diffusion

The simplest form of liposome release is diffusion, which relies on the encapsulated drug being able to diffuse through the lipid bilayer wall of the liposomes and into the tumor interstitium. After being released into the tumor interstitium, the drug may be taken up by cancer cells. Simple diffusion is the most likely release mechanism of the clinically approved Doxil formulation containing the chemotherapeutic drug, doxorubicin. In the preparation of Doxil, a transmembrane gradient is used to load doxorubicin into the liposomes, and it is believed that the transmembrane proton gradient is disrupted in the tumor environment, causing doxorubicin to be released [89]. Although this form of tumor drug release works for the Doxil formulation, it has been measured that only 40 – 50% of the liposome-encapsulated doxorubicin accumulating in tumors is bioavailable during a 7-day period after injection [90]. A prerequisite for diffusion to be a feasible release mechanism is that the drug is sufficiently hydrophobic to cross the lipid bilayer wall of liposomes. Consequently, passive diffusion is not a viable release mechanism for hydrophilic lipid bilayer impermeable drugs, as has been demonstrated by *in vivo* studies using liposomes of identical lipid composition as Doxil, but loaded with the lipid bilayer impermeable drug cisplatin instead of doxorubicin [91-93]. In spite of the fact that large concentrations of liposomal cisplatin accumulated in tumor tissue, these studies failed to show a significant therapeutic effect, and it was concluded that the hydrophilic cisplatin remained inside the liposomes in the tumor interstitium [94]. In support of this conclusion, a recent study used ultrasound to trigger the release of cisplatin from liposomes, and found that < 3% was released in the absence of ultrasound treatment, whereas 70% was released when ultrasound was applied. Predictably, ultrasound-triggered release also resulted in a dramatic improvement in the antitumor efficacy [95]. These examples illustrate that drug release by diffusion is associated with the major challenge of keeping a lipid bilayer permeable drug stably encapsulated while the liposomes circulate in the bloodstream, and at the same time achieve appreciable drug release when reaching the tumor target site.

3.1.2 Release by tumor specific enzymes

As discussed in the previous section, diffusion-based release is a viable release mechanism only for a subclass of anticancer drugs that are able to diffuse through the lipid bilayer of liposomes; membrane impermeable drugs require other release mechanisms. One way to overcome the limitations of passive diffusion is to design liposomes to be degradable by tumor-specific enzymes.

Several enzymes are overexpressed in cancerous tissue, many of which are involved in tumor invasion and metastasis [96]. Enzymes of particular interest in relation to enzymatic drug release from liposomes are those that catalyze the degradation of lipids used to prepare liposomes. In addition, it is critical that the enzymes are secreted into the extracellular tumor interstitium where passively targeted liposomes accumulate.

3.1.2.1 Release by phospholipase A_2

Secretory phospholipase A_2 ($sPLA_2$) is one prominent example of a secreted enzyme that is capable of liposome degradation, and is being pursued for tumor-specific enzymatic release of liposomal anticancer drugs [97,98]. $sPLA_2$ is a family of small secretory (extracellular) ~ 14 kDa lipases that comprises several subtypes, of which $sPLA_2$ type IIA is the most intensively studied. It has been found that $sPLA_2$ type IIA is overexpressed in numerous human cancer types, including prostate [99], pancreatic [100], stomach [101], colon [102] and liver cancers [103], and it appears that overexpression of $sPLA_2$ is more pronounced in invasive cancers than in benign cancers [104].

Interestingly, the catalytic activity of $sPLA_2$ is higher towards aggregated phospholipids such as liposomes and micelles than towards lipid monomers, and the activity is highly dependent on membrane charge, lipid composition and the physical state of the lipids. As a consequence, it is possible to design liposomes to be more or less susceptible to phospholipase A_2 hydrolysis by appropriate choice of the phospholipids making up the liposomes. For example, a highly favorable property in relation to $sPLA_2$-mediated release in tumors is the fact that PEGylated liposomes are more susceptible to $sPLA_2$ hydrolysis than non-PEGylated liposomes [105]. It has also been demonstrated that $sPLA_2$ hydrolysis from liposomes similar in composition to the commercial preparation Doxil is very low owing to high cholesterol content, whereas lipid compositions optimized to be degradable by $sPLA_2$ can result in a rapid release of drugs such as doxorubicin and cisplatin [97,106]. The $sPLA_2$-degradable liposomal formulations of cisplatin also resulted in

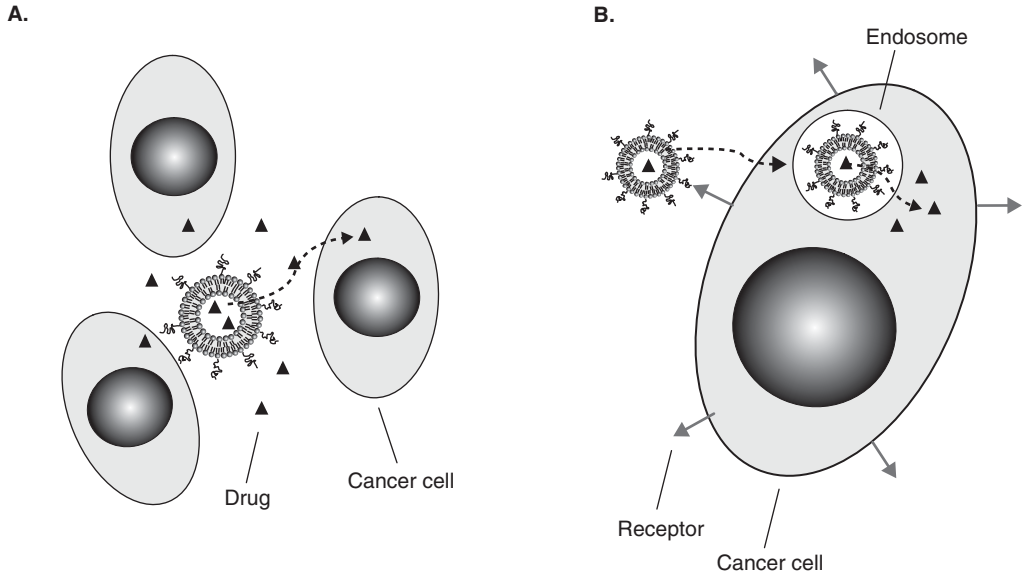

Figure 5. Schematic illustration of the two most common drug release and cancer cell uptake mechanisms. A. Liposome-encapsulated drugs may be released in the extracellular tumor interstitium followed by cancer cell uptake of the released drugs. **B.** The liposomes and encapsulated drugs are taken up by endocytosis followed by intracellular release of drugs.

significantly improved anticancer efficacy in an *in vivo* breast cancer xenograft model in mice [97,98]. As mentioned previously (see Section 3.1.1), cisplatin encapsulated in liposomes with the same lipid composition as Doxil was not released *in vivo* and showed poor efficacy, which highlights the potential of release by tumor-specific enzymes.

In addition to the release of drugs encapsulated in the aqueous liposome interior, $sPla_2$ has also been used to release and activate anticancer drugs and prodrugs incorporated into the lipid bilayer. Several anticancer lipid prodrugs have been synthesized previously [107], which were investigated as liposome formulations in Colo205 xenograft models in mice [108]; however, this first generation of prodrugs was only weakly efficacious at prolonging tumor growth. New generations of prodrugs have been synthesized with structures suitable for attaching more cytotoxic drugs [109,110], and these are now under investigation in cell and animal studies.

3.1.2.2 *Release by matrix metalloproteinases*

Matrix metalloproteinases (MMPs) are a group of secreted enzymes that serve various physiological functions related to the degradation of the extracellular matrix. More than 20 human MMP subtypes have so far been identified, and certain subtypes such as MMP-2 and MMP-9 are overexpressed by a broad range of cancers [111,112]. As the name implies, MMPs are proteinases that degrade peptides and proteins, and the use of MMPs for tumor-specific liposomal drug release is consequently not as straightforward as for phospholipase A_2 because MMPs are not active towards the lipids that are normally used to prepare liposomes. However, by incorporating lipopeptides into the liposome membrane it is possible in several ways to destabilize the liposome and obtain drug release. Srivastava and co-workers have reported the use of MMP-9-degradable lipopeptides to destabilize liposomes and has shown this to be a viable method for drug release [113]. Hashida and co-workers showed that MMP-2 can be used to unmask the PEG coating of liposomes and provide a way of targeting macrophages in the tumor interstitium by using a galactosylated liposome formulation [114]. Harashima *et al.* [115] have published articles on lipid–peptide–PEG conjugates incorporated in liposomes that on MMP activation interacted strongly with cancer cell membranes, which resulted in high transfection activity in HT1080 cancer cells and in a mouse model. A conclusion that can be drawn from the above-mentioned studies is that the MMPs are capable of penetrating the liposome PEG layer and hydrolyzing a peptide below this layer. This is not necessarily surprising, but even so notable because of the sizes of MMP-2 (72 kDa) and MMP-9 (92 kDa), which are considerably larger than $sPLA_2$ that is known to penetrate through the PEG layer [97]. This indicates that enzymes in general are able to hydrolyze substrates hidden under the liposome PEG layer, which is, to a large extent, a prerequisite for using enzymes as triggers of liposomal drug delivery systems. Other examples involving the synthesis of MMP-cleavable lipopeptides and subsequent incorporation into liposomes for *in vitro* MMP-triggered release of liposome encapsulated model drugs have been reported recently [116,117].

3.1.3 *Release by thiolytic environment of tumors*

It is well documented that tumors contain a thiolytic environment, which can be exploited to achieve tumor-specific

release of thiolytically cleavable drug conjugates. The thiolytic environment originates from both chemical components such as glutathione released into the tumor interstitium by dead cells in tumors [118], as well as overexpression of various thiolytic enzymes such as thioredoxin [119]. Recent studies have used the thiolytic tumor environment to release lipid-conjugated prodrugs of mitomycin C (MMC) [118,120]. The prodrugs were prepared by conjugating MMC to the head-group of diglycerides by means of a thiolytically cleavable dithiobenzyl linker, and the conjugates were incorporated into the lipid bilayer of PEGylated liposomes. In addition to obtaining tumor-specific release by designing a thiolytically cleavable lipidated prodrug, the incorporation into the lipid bilayer was necessitated by the fact that earlier attempts to incorporate MMC into the aqueous liposome interior were unsuccessful, probably because of the relatively high hydrophobicity of MMC. Furthermore, as MMC is a poor substrate for the P-glycoprotein that is implicated with multi-drug resistance in cancers, this formulation would be promising for treatment of tumors showing P-glycoprotein resistance. *In vitro* experiments showed that MMC was released on addition of a variety of thiolytic agents, resulting in cytotoxicity levels close to the cytotoxicity of free MMC. *In vivo* experiments compared the antitumor efficacy of the MMC liposomal formulation with the efficacy of a doxorubicin-containing formulation towards a doxorubicin-resistant as well as a less resistant lung cancer model in mice. The MMC-containing liposomes showed clear improvement of antitumor efficacy as well as a lower toxicity when compared with free MMC. Compared with doxorubicin-containing liposomes, the MMC liposomal formulation showed clearly improved efficacy towards the doxorubicin-resistant tumor model, whereas the antitumor efficacy was comparable in the less resistant tumor model [120]. Likewise, the MMC liposomal formulation also displayed improved efficacy as compared with doxorubicin liposomes in a multi-drug resistant ovarian cancer model [118].

3.2 Uptake of intact liposomes by endocytosis

Anticancer drugs displaying poor cancer cell uptake in their free form are not suitable for extracellular release. Instead, they may be taken up as intact liposomes by various endocytosis mechanisms, and subsequently released inside the cancer cells. In principle, cancer cell uptake by endocytosis is straightforward, as plain liposomes are susceptible to endocytosis [121]. However, it has been shown that liposome surface modification with PEG, necessary for prolonged blood circulation time, inhibits liposome–cell interaction and decreases endocytosis significantly [122]. As a consequence, various strategies for mediating endocytosis are being used.

3.2.1 *PEG-shedding*

One option for combining long circulation time and efficient endocytosis of cancer target cells is the use of sheddable PEG coatings. This strategy involves the attachment of PEG to the lipid anchor by means of labile bonds designed to be cleaved on exposure to tumor-specific characteristics such as low pH, reducing agents or enzymes [123]. For example, measurements have shown that the pH in the tumor interstitium is more acidic than normal tissues, with values ranging from ~ 6.6 to 7.2, compared with ~ 7.1 to 7.4 in normal tissue [124,125], and this characteristic feature of many tumors can potentially be exploited to trigger the cleavage of acid labile PEG-lipids.

Owing to the rather low pH difference between tumor tissue and healthy tissue, the design of PEG-lipids that are specifically cleaved by tumor acidification is challenging. Furthermore, acidification of tumors is linked to hypoxia, which results in elevated glycolysis activity and elevated production of lactic acid. Hypoxia, and consequently acidic pH, is therefore most pronounced at tumor areas furthest away from blood vesssels, which are also the least accessible to intravenously administered liposomes. However, with respect to the low pH difference, it has been shown that tumor pH can be lowered further by parenteral administration of glucose, which increases the production of lactic acid and decreases tumor pH [126]. In a variety of different tumors, the mean pH value was 6.97 without glucose administration, whereas it was 6.13 when glucose was administered. In comparison, mean pH values in non-cancerous tissue were 7.25 and 7.14, respectively. Although the authors are not aware of liposomal formulations taking advantage of this effect, it appears that co-administration of glucose and acid labile liposomes can potentially be exploited to achieve tumor-specific shedding of PEG or drug release from liposome formulations.

As mentioned in Section 3.1.2.2, shedding of PEG can also be achieved by tumor-specific enzymes. Hatakeyama *et al.* [115] synthesized PEG-lipids that contained a matrix metalloproteinase cleavage site inserted between the lipid acyl chains and the PEG polymer. The rationale behind this design was to achieve tumor-specific MMP-catalyzed removal of the PEG coating in order to increase cancer cell uptake of liposomes and thereby improve the transfection efficiency of encapsulated DNA. *In vivo* experiments showed that the use of MMP-cleavable PEG-lipids resulted in lower tumor accumulation than liposomes containing traditional PEG-lipids. Nevertheless, the transfection efficiency was comparable, indicating an improved specific transfection efficiency of the liposomes that did arrive at the tumor site.

A third method for cleaving off the PEG coating involves a two-stage approach, as demonstrated recently by McNeeley *et al.* [127]. In this study, a liposomal formulation containing cysteine-cleavable PEG-lipids was incorporated into a liposomal formulation, which also contained folate ligands attached to the liposome surface (i.e., shielded by the PEG polymer). On addition of cysteine as a cleaving agent, it was shown that *in vitro* cellular uptake was increased.

3.2.2 *Uptake by receptor-mediated endocytosis*

As mentioned briefly in Section 2.2, the attachment of targeting ligands to the liposome surface that bind to

internalizing receptors on cancer cells may facilitate cancer cell uptake of intact liposomes by receptor-mediated endocytosis, as long as the targeting ligands extend outside the PEG polymer cloud. Binding of liposome-conjugated ligands to cancer cell surface receptors is the first step in receptor-mediated endocytosis. Next, the plasma membrane forms an endocytotic vesicle that eventually detaches from the membrane and moves into the cell interior. The internalized vesicle, called an endosome, now goes through a series of events during which the pH continually drops, and several degradation enzymes are activated. Eventually, the endosome is delivered to lysosomes where the pH has now dropped to ~ 5.0 – 5.5 [128]. As a result of the harsh environment experienced by the liposome-encapsulated drugs when cellular uptake occurs by receptor-mediated endocytosis, this uptake mechanism is suitable only for drugs that are resistant to endosomal/lysosomal degradation enzymes and acidic pH [129]. Alternatively, liposomes may be designed to escape the endocytosis pathway at an early stage by incorporating pH-sensitive lipids or other molecules into liposomes, which are capable of provoking endosomal drug release in response to endosome acidification [130].

3.2.3 *Uptake by cell-penetrating peptides*

A relatively new approach to intracellular delivery of liposome-encapsulated drugs involves the application of cell-penetrating peptides. Cell-penetrating peptides are a class of short peptides having sequences derived from viral proteins capable of translocating macromolecules and even small particles across cell membranes [131,132]. They typically consist of < 30 amino acids and are rich in positively charged arginine and lysine residues, and some also contain hydrophobic amino acids and are amphipathic [133]. The uptake mechanism of cell-penetrating peptides is still debated, but it appears that cellular uptake may involve several different mechanisms, including clathrin-mediated endocytosis, macropinocytosis or transduction, depending on various factors such as the peptide density and the size of the molecules or particles attached to the peptides [134-136].

It has been demonstrated that the attachment of cell-penetrating peptides facilitates intracellular delivery of intact liposomes as large as 200 nm [137], and *in vitro* studies have demonstrated improved cell uptake and activity of liposome-encapsulated DNA and RNA-based therapeutics by attachment of cell-penetrating peptides [138]. Improved transfection has also been demonstrated *in vivo* by intratumoral injection of TAT peptide-modified DNA–liposome complexes [139].

An important difference between cell-penetrating peptides and ligands used for active targeting is that the interaction of cell-penetrating peptides is not cell specific, which complicates the systemic administration of cell-penetrating peptide-modified liposomes as the cell-penetrating peptides will interact strongly with healthy cells encountered in the bloodstream before accumulating in tumors. To overcome this problem, cell-penetrating peptides have been attached to the surface of liposomes sterically stabilized with acid labile PEG-lipids. In this way, the cell-penetrating peptides are shielded from interacting with cells until encountering a low pH environment, such as in tumors, where the PEG polymer is cleaved off and exposes the cell-penetrating peptides. The concept of achieving improved transfection by de-shielding of cell-penetrating peptides on exposure to low pH has proved to be viable *in vitro* on exposure to pH 5.0 [140] and *in vivo* by intratumoral injection [141].

Tseng *et al.* [142] investigated the effect of modifying doxorubicin-containing liposomes with TAT peptides on both *in vitro* cytototoxicity and *in vivo* antitumor efficacy of intravenously administered liposomes. Although a markedly improved intracellular delivery of doxorubicin was measured *in vitro*, neither the *in vitro* cytotoxicity nor the antitumor efficacy was improved as compared with plain unmodified doxorubicin-loaded liposomes. Fluorescence microscopy indicated that only a limited amount of drugs was released into the cytoplasm and nucleus, pointing to limited endosomal escape of this formulation as a possible explanation for the lack of efficacy improvement. However, a recent study has shown that the cell-penetrating peptide octaarginine is capable of mediating endosomal escape of liposomes, whereas endosomal escape of liposomes modified with octalysine is much more limited [143]. Consequently, intracellular drug release appears to depend on the type of cell-penetrating peptide, and it may be possible to formulate more efficacious formulations using different peptides. However, at this point it seems unlikely that the use of cell penetrating peptides will find use for systemic administration of liposomes unless a strategy where the peptides are masked by PEG coating that is specifically shed at the tumor target site is used.

4. Kinetic considerations of liposome accumulation, cellular internalization and drug release

Drug bioavailability in the cancerous tissue depends on the degree of liposome accumulation, intratumoral distribution, cellular internalization and drug release from the liposomes. Pharmacokinetic changes influence the toxicity and efficacy of the liposome-delivered drugs. It is therefore important to understand liposome pharmacokinetics and drug pharmacodynamics in order to develop liposomal drug delivery systems that can release drugs specifically in the tumor tissue with a release rate that matches the efficacy profile of the drug carried. This was demonstrated in a recent study, where the release rate of the common chemotherapy drug, vincristine, was controlled by varying the drug-to-lipid ratio of the formulation [144]. In these formulations, the drug released more slowly from liposomes containing higher intraliposomal vincristine concentrations, presumably owing to precipitation inside the liposomes. By using different drug-to-lipid ratios, the half-life of vincristine release from the liposomes was varied from 6 to 117 h, and the liposomal formulations

were used to examine the relationship between drug release rate and antitumor efficacy in an MX-1 human tumor xenograft model in mice. It was shown that antitumor activity was most pronounced at intermediate release rates (15.6 h in this study), whereas lower as well as higher release rates resulted in a lower anticancer activity. It is likely that the influence of release rate on anticancer efficacy is particularly pronounced for cell cycle-specific drugs such as vincristine, whereas it is expected to be less critical for drugs that are not cell cycle specific, where a burst release may sometimes be preferred.

Interestingly, active targeting does not always result in an overall higher accumulation of liposomes in tumors, but there may be important differences in the time it takes to achieve maximum accumulation. Kamaly *et al.* [145] has recently reported that folate-targeted liposomes accumulate within 2 h to the same degree as non-targeted liposomes within 24 h in a xenograft model where IGROV-1 cells were used to induce tumors in nude Balb/c mice. It seems that the targeted liposomes bind to tumor cells within the first few times of passing through the tumor vasculature, whereas non-targeted liposomes need the long circulating properties to achieve the same degree of accumulation through the EPR effect. However, this fast binding seems to be problematic as it has been shown that a strong binding to the first line of encountered cancer cells in the interstitial compartment seems to block further accumulation and tumor penetration. Furthermore, the intratumoral distribution seems to be hampered when using targeted liposomes [145-147], which may be devastating for obtaining efficient treatment of solid tumors with targeting strategies.

The kinetics of cancer cell internalization of liposomes that are bound to surface receptors may be another highly important factor. A fast internalization will provide room for the next liposomes to extravasate into the extracellular space in the tumor. The kinetics of the endocytosis is therefore highly important and will change depending on which receptors are targeted by the immunoliposomes. When combining the faster accumulation with design of a specific release profile this difference in accumulation kinetics has an impact on the maximum drug concentration that can be achieved in the tumor and therapeutic drug concentrations are more readily obtained. The choice of drug release strategy is therefore very important as some strategies have a relatively slow response to changes in the environment whereas others respond instantly. An example is the pH-sensitive systems where designs based on chemical hydrolysis of a chemical bond may have a slow response compared with liposome systems that change confirmation owing to protonation, for example, oleic acid-based liposomes or pH-sensitive lipopeptides such as liposome-anchored GALA [148]. In the latter systems conformational changes occur instantaneously as a response to a change in pH and thus have a very different response profile.

The optimal kinetic profiles for the various parameters described above are not easily obtainable, although liposomal drug delivery systems offer the necessary tools. When developing new liposome-based drug delivery systems one particular problem is that the optimization of drug release both *in vitro* and *in vivo* is a difficult task as the knowledge of drug pharmacodynamics in relation to drug delivery is often very limited. Thus, what release profile is being aimed for is not always known, which makes it difficult to design and optimize the kinetic parameters of liposomal drug delivery systems.

5. Concluding remarks

A number of significant advances have moved the field of liposomal drug delivery forward during the last four decades. The ability of PEG coating to prolong the blood circulation time of liposomes in order for significant tumor accumulation to occur was a major achievement, and played an important role in the development of the clinically approved Doxil formulation, as it accounts for the ability of Doxil to accumulate in tumor tissue by the EPR effect. With respect to drug release and cancer cell uptake once accumulation in the tumor has occurred, Doxil relies on a delicate balance of doxorubicin being fairly stably encapsulated in the bloodstream and yet able to be released by passive diffusion in the tumor interstitium. This principle is specific to doxorubicin and similar drugs, and cannot be transferred to other drug types. Also, considering the high tumor accumulation ability of Doxil, one might expect that the modest (although important) improvement in anticancer activity observed for Doxil as compared with free doxorubicin may be improved by more advanced liposomal formulations. Current approaches for reaching this goal include the use of ligand-targeted liposomes for active targeting and receptor-mediated cancer cell uptake of drug-loaded liposomes, as well as drug release by tumor enzymes or physicochemical characteristics of the tumor microenvironment. Both of these approaches appear to be promising avenues towards more efficacious liposomal formulations that may also be applicable to a broader range of anticancer drugs. However, keeping in mind that these strategies have been pursued heavily for the last decade, it may be concluded that current knowledge of the tumor environment and how the liposome formulation is altered by biomolecule adsorption during blood circulation is not sufficient to exploit the full potential of the many interesting ideas being developed in this field.

6. Expert opinion

The major developments in the liposomal cancer therapy field have, to a large extent, followed the challenges faced at the different stages of transporting liposome-encapsulated drugs first through the bloodstream, then into tumors and finally into tumor cells. PEGylation and stabilization by cholesterol prevented the rapid liposome clearance and drug release in the bloodstream, and facilitated tumor accumulation, but unfortunately the high degree of stabilization in the bloodstream hampers the drug release to cancer cells after accumulation.

The result is that highly stable formulations such as Doxil are limited to delivering doxorubicin and potentially other drugs that can be loaded with a pH gradient. At present there is intense interest in developing liposomal formulations that retain the long circulating properties of PEGylated formulations, but contain extra functionalities that improve the performance of liposomes after accumulation at the tumor site. For example, ligand targeting is a very active research area, which is being investigated for the ability to improve the targeting efficiency even further than is possible by passive targeting. Ligand targeting has shown highly increased accumulation when targeted towards receptors or antigens that are readily accessible from the bloodstream, such as circulating cancer cells or endothelial cells of blood vessels. However, apart from a few exceptions there is little indication in the literature that active targeting results in a significant increase in tumor accumulation as far as targeting towards the extracellular tumor environment is concerned. Furthermore, the tendency of ligand-targeted liposomes to accumulate predominantly near tumor blood vessels and failure to penetrate into deeper tumor regions is a disadvantage of ligand-targeted liposomes. A more promising feature of ligand targeting is the ability to improve cancer cell uptake by receptor-mediated endocytosis. This has been demonstrated even for lipid bilayer-permeable drugs such as doxorubicin, which is among the drug types expected to benefit least from active cancer cell uptake mechanisms. Moreover, the ability to bypass P-glycoprotein multi-drug resistance mechanisms is particularly interesting, as multi-drug resistance is one of the main problems in cancer chemotherapy. Another interesting feature of ligand-targeted liposomes is the ability to direct liposomes to different targets. For example, the authors find the ability to target simultaneously tumor vasculature endothelial cells and cancer cells a highly promising approach. In this way, the disruption of blood flow by the vascular-targeted liposomes may be effective towards cells that ligand-targeted liposomes are not particularly effective against, such as drug-resistant cancer cells, cancer cells not expressing receptors, and cancer cells located far away from blood vessels.

Liposomal formulations of prodrugs that are activated by tumor-specific enzymes or characteristics of the tumor microenvironment is another exciting field that the authors expect will result in more efficacious liposomal formulations. By combining the ability of a prodrug that may have specificity to rapidly proliferating cells with a tumor-specific activation mechanism, and a liposomal carrier with targeting specificity to cancerous tissue, it is reasonable to expect the total cancer specificity to be dramatically amplified.

The liposomal cancer therapy field has moved towards increasingly more complex formulations, as an increasing number of functionalities have been incorporated into the liposomes. Also, hybrid particles where liposomes are combined with other drug delivery systems such as polymeric nanoparticles are emerging as new advanced drug delivery systems, and it seems likely that this trend will continue. Perhaps the utilization of hybrid particles will go hand in hand with an increased focus on intracellular targeting to specific organelles, which is likely to gain increased focus in the near future.

It is important to realize, however, that liposomes were the first nanoparticle-based drug delivery systems to reach the market, but they are now competing with multiple other materials in the nanomedicine field, such as nanorods, dendrimers, polymersomes and hydrogels. All the different materials try to exploit the same characteristics at the tumor target site and suffer from the same difficulties. Our ability to engineer and characterize highly advanced nanoparticle systems is impressive, but our lack of knowledge on how the biological environment alters material properties is also substantial. The authors strongly believe that more research should be focused on addressing the *in vivo* behavior of the designed materials to exploit fully the engineering capabilities. This means that if a nanoparticle-based drug delivery system is designed to have a specific release mechanism such as enzymatic hydrolysis or pH triggering, one should not only show that this is possible *in vitro*, but also address whether this is actually the mechanism of drug release *in vivo*. Such studies are extremely important to develop our capabilities within this area and are not addressed sufficiently. Liposomes in general have one advantage over other types of nanomedicine materials, in that the knowledge on how to encapsulate drugs, and the stability and toxicity is substantially higher in relation to drug delivery. The authors strongly believe that investigations on new materials should go hand in hand with a focus on the *in vivo* behavior to the same level as where the liposome field is today.

Declaration of interest

T Kaasgaard and TL Andresen have previously worked for the liposomal drug delivery company LiPlasome Pharma, but have no commercial interests in the technologies described in this review.

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