Expert Opinion

Antibody-modified liposomes for cancer chemotherapy

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Background: Liposomes, phospholipids, nanosized bubbles with a bilayered membrane structure, have drawn a lot of interest as pharmaceutical carriers for drugs and genes. In particular, liposomes are widely used for drug delivery into tumors. *Objective*: In many cases, to enhance the efficacy of the liposomal drugs, drug-loaded liposomes are targeted to the tumors by means of different specific ligands, such as monoclonal antibodies. Thus, this review analyzes the application of antibody-targeted liposomes loaded with various chemotherapeutic agents and various liposomal products under development at experimental and preclinical level. *Methods*: The papers published on the subject of cancer-targeted liposomes mainly over the last 10 – 15 years are discussed. *Conclusion*: Antibody-targeted liposomes loaded with anticancer drugs demonstrate high potential for clinical applications.

Keywords: antibodies, cancer, drug delivery, drug targeting, liposomes

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1. Introduction

Fast-developing nanotechnology, among other areas, is expected to have a dramatic impact on medicine. The application of nanotechnology for treatment, diagnosis, monitoring and control of biological systems, is now often referred to as nanomedicine. Among many possible applications of nanotechnology in medicine, the use of various nanomaterials as pharmaceutical delivery systems for drugs, DNA and imaging agents is getting increased attention. Many varieties of nanoparticles are available [1], such as different polymeric and metal nanoparticles, liposomes, niosomes, solid lipid particles, micelles, quantum dots, dendrimers, microcapsules, cells, cell ghosts, lipoproteins and many different nanoassemblies.

The paradigm of using nanoparticulate pharmaceutical carriers to enhance the *in vivo* efficiency of many drugs, beginning with anticancer drugs, well established itself both in pharmaceutical research and clinical setting. The first publications on the clinical potential of pharmaceutical nanocarriers appeared long before the field of 'nanomedicine' became clearly defined; see for example papers by Gregoriadis on the carrier potential of liposomes in biology and medicine [2,3]. Numerous nanoparticle-based drug delivery and drug targeting systems have been under development for quite a long time, and several important monographs on this subject were published 15 – 20 years ago [4,5]. A number of important review articles and monographs have now been published on this subject. Recent publications summarize the most important developments in this area and specifically address the issues of nanocarriers designed to deliver drugs into certain individual sites of disease (cancer first of all) and to perform in the body various functions simultaneously [6-17]. The aim of using various nanoparticulate drug carriers is, first of all, to minimize drug degradation and inactivation following administration, prevent undesirable side effects, and increase drug bioavailability and the fraction of the drug delivered in the pathological area. In general, pharmaceutical drug carriers, especially the ones for parenteral administration, are

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expected to be biodegradable, easy and reasonably cheap to prepare, have small particle size, possess high loading capacity, demonstrate prolonged circulation, and, ideally, specifically or non-specifically accumulate in required sites in the body [18].

Some time ago, it was found that high-molecular-weight (≥ 40 kDa), long-circulating macromolecules as well as various long-circulating nanoparticulate pharmaceutical carriers are capable of spontaneous accumulations in various pathological sites, such as solid tumors and infracted areas, via the so-called enhanced permeability and retention (EPR) effect [19,20]. This effect is based on the fact that the pathological vasculature, unlike vasculature of normal healthy tissues, is 'leaky', that is, penetrable for large molecules and even for small particles, which allows for their extravasation and accumulation in an interstitial tumor space. Such accumulation is additionally facilitated by the virtual lack of the lymphatic system, responsible for the drainage of macromolecules from normal tissues, in many tumors [20]. It has been found that the effective pore size in the endothelial lining of the blood vessels in most peripheral human tumors is in the range of 200 – 600 nm in diameter, and the EPR effect allows for passive targeting to tumors based on the cutoff size of the leaky vasculature [21].

Pharmaceutical nanocarriers can be surface-modified by a variety of different moieties to impart them with certain properties and functionalities. These functionalities are expected to provide nanocarriers the following properties. First, prolonged circulation in the blood [22,23] and the ability to accumulate in various pathological areas (such as solid tumors) via the EPR effect (protective polymeric coating with polyethylene glycol [PEG] is frequently used for this purpose) [24,25]. The important property of PEG to impart the longevity in the blood to different drug carriers (first of all to liposomes) was described almost 20 years ago [26-28], although the mechanism of this phenomenon was elucidated few years later [29,30] and some alternative polymeric coatings have been suggested [31].

Second, the ability to specifically recognize and bind target tissues or cells via the surface-attached specific ligand (monoclonal antibodies as well as their Fab fragments and some other moieties, such as folate or transferrin, are used for this purpose) [32]. Again, the possibility of specific targeting of various pharmaceutical nanocarriers – liposomes, in the first turn – was considered some time ago [33,34]. Eventually, this was applied also to long-circulating PEGylated liposomes [35,36], and is now a well-established approach [37].

Third, the ability to respond to local stimuli characteristic of the pathological site by, for example, releasing an entrapped drug or specifically acting on cellular membranes under the abnormal pH or temperature in disease sites. This property could be provided by surface-attached pH- or temperature-sensitive components [38,39].

Finally, the ability to penetrate inside cells bypassing the lysosomal degradation for efficient targeting of intracellular drug targets (for this purpose, the surface of nanocarriers is additionally modified by cell-penetrating peptides) [40,41].

These are just the most evident examples. Some other specific properties can also be listed, such as an attachment of diagnostic moieties.

By virtue of their small size and by functionalizing their surface with synthetic polymers and appropriate ligands, nanoparticulate carriers can be targeted to specific cells and locations within the body after intravenous and subcutaneous routes of injection. Such approaches may enhance detection sensitivity in medical imaging, improve therapeutic effectiveness, and decrease side effects. Some of the carriers can be engineered in such a way that they can be activated by changes in the environmental pH, chemical stimuli, by the application of a rapidly oscillating magnetic field, or by application of an external heat source [42-45]. Such modifications offer control over particle integrity, drug delivery rates, and the location of drug release, for example within specific organelles. Some include the incorporation of one or more nanosystems within other carriers, as in micellar encapsulation of quantum dots [46].

2. Liposomes: properties and applications

Since the discovery that phospholipids in aqueous systems can form closed bilayered structures, liposomes have moved a long way to becoming a pharmaceutical carrier of choice for numerous practical applications. Liposomes, artificial phospholipid vesicles, can be obtained by various methods from lipid dispersions in water. Liposome preparation, their physicochemical properties and possible biomedical applications have already been extensively discussed in several monographs [22,47-50], and many different methods have been suggested to prepare liposomes of different sizes, structure and size distribution. To increase liposome stability towards the action of the physiological environment, cholesterol is incorporated into the liposomal membrane (sometimes up to 50% mol). The size of liposomes depends on their composition and preparation method and can vary from around 50 nm to > 1 μm in diameter. Multilamellar vesicles range from 500 to 5000 nm and consist of several concentric bilayers. Large unilamellar vesicles range from 200 to 800 nm, and small unilamellar vesicles are around 100 nm (or even smaller) in size and formed by a single bilayer (**Figure 1**). The encapsulation efficacy for different substances is also variable depending on the liposome composition, size, charge and preparation method. The use of the reverse phase evaporation method [51] permits inclusion of ≥ 50% of the substance to be encapsulated from the water phase into the liposomes. Furthermore, a variety of methods have been developed to obtain lyophilized liposomal preparations possessing good storage stability [52]. The *in vitro* release rate of different compounds from liposomes, including proteins of moderate molecular weight, such as lysozyme or insulin, is usually < 1% per hour

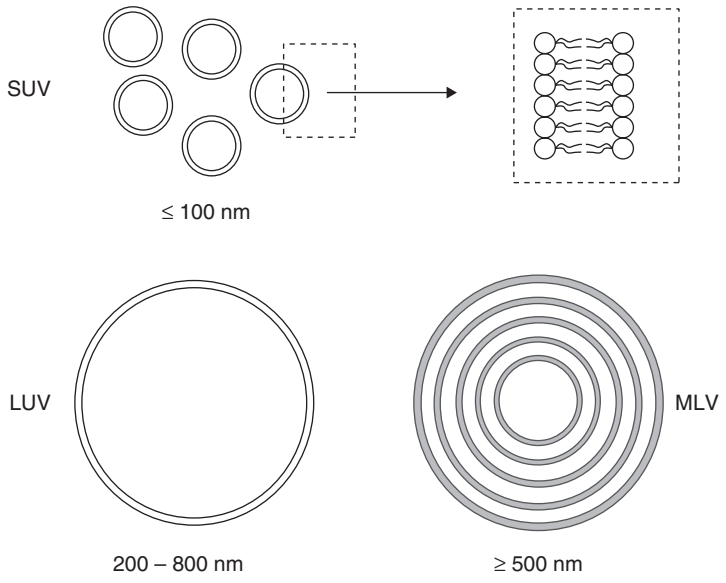

Figure 1. Main types of liposomes (in terms of their size and structure). See text for descriptions.
LUV: Large unilamellar vesicles; MLV: Multilamellar vesicles; SUV: Small unilamellar vesicles.

(assuming that the incubation temperature sufficiently differs from the phase transition temperature of a given phospholipid). *In vivo*, this parameter can vary within wide limits (minutes to hours) and depends on the liposome membrane composition, cholesterol content, and liposome location in the body.

Liposomes are biocompatible, cause no or very little antigenic, pyrogenic, allergic and toxic reactions; they easily undergo biodegradation; they protect the host from any undesirable effects of the encapsulated drug, at the same time protecting an entrapped drugs from the inactivating action of the physiological medium; and are capable to deliver their content inside many cells (the principal mechanisms of liposome–cell interaction are presented in **Figure 2**). Different methods of liposomal content delivery into the cytoplasm have been described [53]. According to one of these methods, the liposome is made of pH-sensitive components and, after being endocytosed in the intact form, it fuses with the endovacuolar membrane under the action of lowered pH inside the endosome, releasing its content into the cytoplasm. In addition, liposomes have been shown to fuse with the microscopic pores on the cell surface (which appear, for example, as a result of ischemia) [54,55] and deliver their contents including DNA into the cell cytoplasm. Liposomes modified on the surface with TAT-peptide [56], or other cell-penetrating peptides, such as Antp, penetratin or synthetic polyarginines [41] are also capable of delivering their cargo inside cells [57].

Water-soluble drugs are entrapped into the liposomal inner aqueous space (and, in case of multilammellar liposomes, into the aqueous space between bilayers), while less soluble drugs, such as paclitaxel, may be incorporated into the phospholipid membrane.

Biodistribution of liposomes is a very important parameter from the clinical point of view. As with other microparticulate delivery systems, conventional liposomes suffer from rapid elimination from the systemic circulation by the cells of the reticulo-endothelial system (RES) [58] (now also termed monocytic phagocyte system). Many studies have shown that within the first 15 – 30 min after intravenous administration of liposomes, 50 – 80% of the dose is adsorbed by the cells of the RES, primarily by the Kupffer cells of the liver.

Clinical applications of liposomes are multiple and well known (see some examples in **Table 1**). Doxorubicin in polyethylene glycol(PEG)-coated liposomes is successfully used for the treatment of solid tumors in patients with breast carcinoma metastases with subsequent survival improvement [59-61]. The same set of indications was targeted by the combination therapy involving liposomal doxorubicin and paclitaxel [62] or Doxil/Caelyx (doxorubicin in PEG-liposomes) and carboplatin [63]. Caelyx is also in Phase II studies for patients with squamous cell cancer of the head and neck [64] and ovarian cancer [65]. Clinical data showed the impressive effect of doxorubicin in PEG-liposomes against unresectable hepatocellular carcinoma [66], cutaneous T-cell lymphoma [67] and sarcoma [68]. A recent review on the successful use of Caelyx in the treatment of ovarian cancer can be found in [69]. Liposomal lurtotecan was found to be effective in patients with topotecan-resistant ovarian cancer [70]. Among other indications, one may notice the use of the liposomal amphotericin B for the treatment of visceral leishmaniasis [71] and long-acting analgesia with liposomal bupivacaine in healthy volunteers [72].

However, the main use of liposomes as drug carriers is still in cancer chemotherapy.

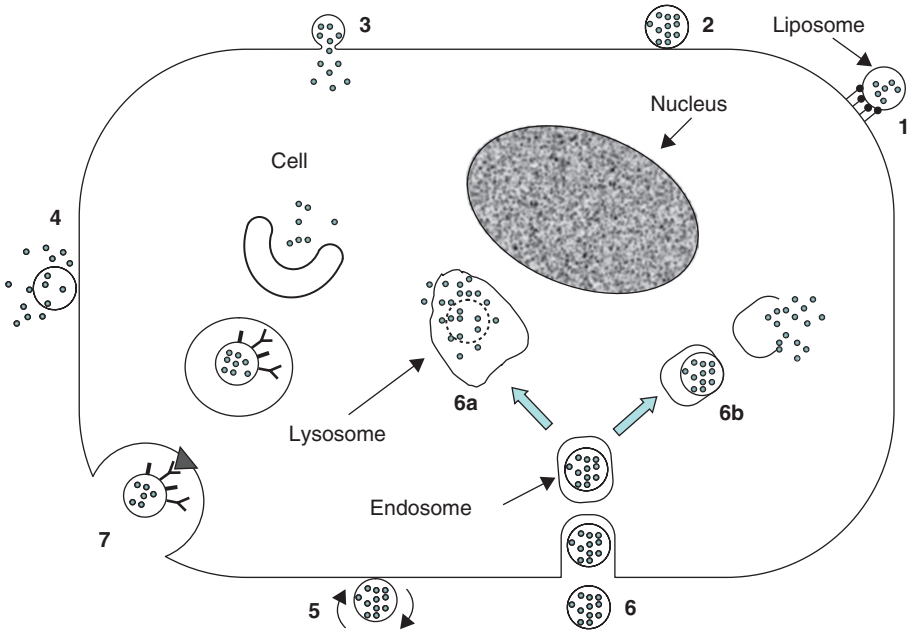

Figure 2. Liposome–cell interaction. Drug-loaded liposomes can adsorb on the cell surface specifically (**1**) or non-specifically (**2**). Liposomes can also fuse with the cell membrane releasing their contents inside the cytoplasm (**3**). Liposomes can also be destabilized by cell membrane components when adsorbed on the surface so that the released drug can enter cell via micropinocytosis or passive diffusion (**4**). Liposomes can undergo the direct or transfer protein-mediated exchange of lipid components with the cell membrane (**5**). They can also be subjected to a specific or non-specific endocytosis (**6**). In this case, liposomes can be delivered by the endosome into the lysosome (**6a**) or, they can provoke endosome destabilization, which results in drug liberation into the cytoplasm (**6b**). Drug-loaded liposomes modified with certain viral components can specifically interact with cells, provoke endocytosis, and via the interaction of viral components with the inner membrane of the endosome, release the drug into the cytoplasm (**7**). Notice, that only type (**1**) liposomes are specific immunoliposomes; most other uptake mechanisms are occurring with plain liposomes.

Table 1. Some anticancer liposomal drugs approved for clinical application or under clinical evaluation (in different countries, same drug could be approved for different indications).

Active drug (and product name for liposomal preparation where available)	Indications
All-trans retinoic acid (Altragen)	Acute promyelocytic leukemia; non-Hodgkin's lymphoma; renal cell carcinoma; Kaposi's sarcoma [268,269]
Annamycin	Doxorubicin-resistant tumors [270]
BLP 25 vaccine Stimuvax®	Non small cell lung cancer vaccine [271]
DNA plasmid encoding HLA-B7 and β2 microglobulin (Allovectin-7)	Metastatic melanoma [272]
Daunorubicin (DaunoXome)	Kaposi's sarcoma [273]
Doxorubicin (Mycet)	Combinational therapy of recurrent breast cancer [274,275]
Doxorubicin in PEG-liposomes (Doxil, Caelyx)	Refractory Kaposi's sarcoma; ovarian cancer; recurrent breast cancer, prostate cancer [273,276]
E1A gene	Various tumors [277]
Liposomes for various drugs and diagnostic agents (lipoMASC)	Broad applications [278,279]
Lurtotecan (NX211)	Ovarian cancer [280]
Platinum compounds (Cisplatin, Platar)	Germ cell cancers, small-cell lung carcinoma, head and neck cancer [281-283]
Vincristine (Onco TCS)	Non-Hodgkin's lymphoma [284]

3. Long-circulating liposomes

A serious limitation with all pharmaceutical nanocarriers, including liposomes, is that the body normally treats them as foreign particles, and thus, they become easily opsonized and removed from the circulation long prior to completion of their function. One of the drawbacks of the use of liposomes was the fast elimination from the blood and capture of the liposomal preparations by the cells of the RES, primarily, in the liver. To increase liposomal drug accumulation in the desired areas, the use of targeted liposomes with surface-attached ligands capable of recognition and binding to cells of interest has been suggested. Immunoglobulins of the IgG class and their fragments are the most widely used targeting moieties for liposomes (termed 'immunoliposomes' after the modification), which could be attached to liposomes without affecting their integrity and antibody properties by covalent binding to the liposome surface or by hydrophobic insertion into the liposomal membrane after modification with hydrophobic residues [73]. Still, despite improvements of the targeting efficacy, the majority of immunoliposomes end up in the liver, as a consequence of insufficient time for the interaction between the target and targeted liposome. Better target accumulation can be expected if liposomes can stay in the circulation long enough, thus providing more time for targeted liposomes to interact with the target. Prolonged circulation allows also for liposomes to deliver pharmaceutical agents to targets other than the RES.

Thus, one of the most important properties of any pharmaceutical nanocarrier loaded with any anticancer drug is its blood circulation longevity, and long-circulating pharmaceuticals and pharmaceutical carriers represent an important and still growing area of biomedical research [5,22,32,74,75]. There are several important reasons for producing long-circulating drugs and drug carriers. One is to maintain a required level of a pharmaceutical agent in the blood for extended time periods. Then, long-circulating drug-containing microparticulates or large macromolecular aggregates can slowly accumulate (EPR effect, also termed as 'passive' targeting or accumulation via an impaired filtration mechanism) in pathological sites with affected and leaky vasculature (such as tumors, inflammations, and infarcted areas), and facilitate drug delivery in those areas [25,76,77]. In addition, the prolonged circulation can help to achieve a better targeting effect for targeted (specific ligand-modified) drugs and drug carriers allowing for more time for their interaction with the target [75] due to higher number of passages of targeted pharmaceuticals through the target.

Chemical modification of pharmaceutical nanocarriers with certain synthetic polymers, such as PEG is the most frequent way to impart the *in vivo* longevity to drug carriers, as was first suggested for liposomes in [26,28,78-80]. Hydrophilic polymers have been shown to protect individual molecules and solid particulates from interaction with different solutes. The term 'steric stabilization' has been introduced to describe the phenomenon of polymer-mediated protection [81]. On the biological level, coating nanoparticles with PEG sterically hinders interactions of blood components with their surface and reduces the binding of plasma proteins with PEGylated nanoparticles, as was demonstrated for liposomes in [79,82-86]. This prevents drug carrier interaction with opsonins and slows down their capture by the RES [58]. Mechanisms of preventing opsonization by PEG include shielding the surface charge, increased surface hydrophilicity [87], enhanced repulsive interaction between polymer-coated nanocarriers and blood components [88], and formation of the polymeric layer over the particle surface, which is impermeable for large molecules of opsonins even at relatively low polymer concentrations [30,87]. As a protecting polymer, PEG provides a very attractive combination of properties: excellent solubility in aqueous solutions, high flexibility of its polymer chain, very low toxicity, absent immunogenicity and antigenicity, lack of accumulation in the RES cells, and minimum influence on specific biological properties of modified pharmaceuticals [89-92]. It is also important that PEG is not biodegradable and subsequently does not form any toxic metabolites. PEG molecules with a molecular weight < 40 kDa are readily excretable via the kidneys. PEG is also easily commercially available in a variety of molecular weights. PEGs, which are normally used for the modification of drug carriers, have a molecular weight of 1 – 20 kDa. There presently exist many chemical approaches to synthesize activated derivatives of PEG and to couple these derivatives with a variety of drugs and drug carriers [89,93,94].

The most significant biological consequence of nanocarrier modification with protecting polymers is a sharp increase in the carrier circulation time and decrease in its RES (liver) accumulation [26,30,32]. This fact is very important clinically, as various long-circulating nanocarriers have been shown to effectively accumulate in many tumors via the EPR effect [25,76-77,95]. From a pharmacokinetic point of view, the association of drugs with any nanocarrier has pronounced effects: delayed drug absorption, restricted drug biodistribution, decreased volume of drug biodistribution, delayed drug clearance, and retarded drug metabolism [96,97]. The presence of protective polymer on the carrier surface changes all these parameters still further [32,79].

As for other pharmaceutical carriers, different methods have been suggested to achieve long circulation of liposomes *in vivo*, including coating of the liposome surface with PEG (**Figure 3**) [26,29].

It was repeatedly shown that, similar to macromolecules, liposomes are capable of accumulating in various pathological areas with affected vasculature (such as tumor, infarcts, and inflammations) via the EPR effect [20,98]. Prolonged circulation naturally enhances this way of target accumulation. Doxorubicin, incorporated into long-circulating PEGylated liposomes (Doxil) demonstrates good activity in EPR-based tumor therapy and strongly diminishes the toxic side effects

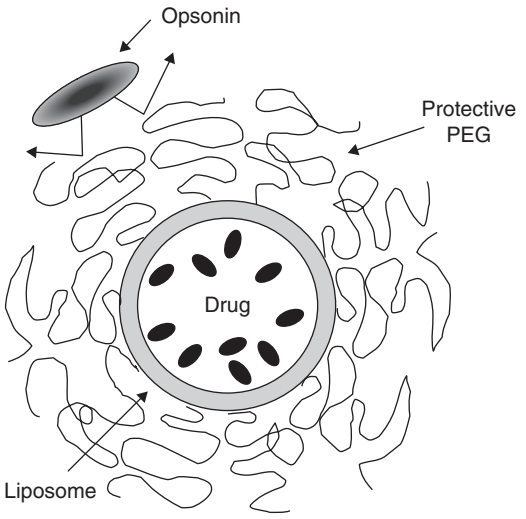

Figure 3. Long-circulating stericaly protected liposome grafted with a protective polymer such as PEG, which shields the liposome surface from the interaction with opsonizing proteins.
PEG: Polyethylene glycol.

(cardiotoxicity) of the original drug [99]. Evidently, long-circulating liposomes can be easily adapted for the delivery of various pharmaceuticals to tumor and other 'leaky' areas. It should be, however, noted here that recent evidence showed that PEG-liposomes, previously considered as biologically inert, could still induce certain side reactions via activation of the complement system [100,101].

Long-circulating liposomes are now investigated in details and widely used in biomedical *in vitro* and *in vivo* studies and have also found their way into clinical practice [22,99]. An important feature of protective polymers is their flexibility, which allows a relatively small number of surface-grafted polymer molecules to create impermeable layer over the liposome surface [23,30]. Long-circulating liposomes demonstrate dose-independent, non-saturable, log-linear kinetics, and increased bioavailability [102]. The relative role of the liposome charge and protective polymer molecular size was investigated, showing that opsonins with different molecular sizes may be involved in the clearance of liposomes containing different charged lipids [103]. PEG was also attached to the liposome surface in a removable fashion to facilitate the liposome capture by the cell after PEG-liposomes accumulate in target site via the EPR effect [20] and PEG coating is detached under the action of local pathological conditions (decreased pH in tumors). New detachable PEG conjugates are described in [104], where the detachment process is based on the mild thiolysis of the dithiobenzylurethane linkage between PEG and amino-containing substrate (such as PE). Low pH-degradable PEG-lipid conjugates based on the hydrazone linkage between PEG and lipid have also been described [39,105].

4. Administration of liposomal drugs

Liposomes as a dosage form allow for a broad variety of administration routes. In addition to the most traditional and frequent parenteral (intravenous) way of administration, some alternative approaches have also been developed or are under development, although each of these approaches has its own problems and limitations. Thus, oral administration requires high liposome stability and resistance towards acidic conditions in the stomach, and drug delivery from the gut to the blood with subsequent drug release [106]. Chitosan-coated insulin liposomes were shown to cause hypoglycemic effect in mice following oral administration [107]. Liposomes made with addition of gangliosides GM1 and GM type III are stable in different biological media and can survive the gastrointestinal tract [108]. PEG-coated liposomes were used for oral delivery of recombinant human epidermal growth factor for gastric ulcer healing [109]. The hypocalcemic effect of liposomal salmon calcitonin following oral administration was shown in [110]. PEG-liposomes are also considered for oral vaccines – ovalbumin in PEG-coated liposomes induces the best mucosal immune response of all carriers tested [111]. To improve protein and peptide bioavailability via the oral route, an oral colon-specific drug delivery system for bee venom peptide was developed based on coated alginate gel bead-entrapped liposomes [112].

After liposome freeze-drying was developed [113], aerosolized liposomal preparations become possible for lung delivery. Combined aerosol of liposomal paclitaxel and ciclosporin A gives better results in the treatment of pulmonary metastases of renal cell carcinoma in mice than each drug given alone [114]. Improved delivery of rifampicin by aerosolized liposomes to alveolar macrophages might become significant in the treatment of tuberculosis [115]. Aerosolized liposomal budesonide was effective against experimental asthma in mice [116]. Aerosoles of liposomal 9-nitrocamptothecin were non-toxic and efficiently treated melanoma and osteosarcoma lung metastases in mice [117]. Aerosolized liposomal paclitaxel effectively treated pulmonary metastases in murine renal carcinoma model [118]. Liposomes for pulmonary delivery of a potent vasodilator, vasoactive intestinal peptide, were engineered recently [119]. Nebulization was suggested recently to deliver liposomal aerosols [120]. In this particular case, a dispersion of the physical mixture of drugs and phospholipid in saline was used that spontaneously formed liposomes with the encapsulated drug. Liposomes for drug delivery to the lungs by nebulization have also been described in [121].

Because subcutaneous administration of liposomes results in their uptake by draining lymphatic capillaries at the injection site and active capture of liposomes by macrophages in regional lymph nodes, plain and ligand-targeted liposomes were suggested as good means to target lymphatics for therapeutic and diagnostic applications after subcutaneous administration [122]. Liposome uptake by lymph nodes might be increased by using biotin-bearing liposomes for preliminary

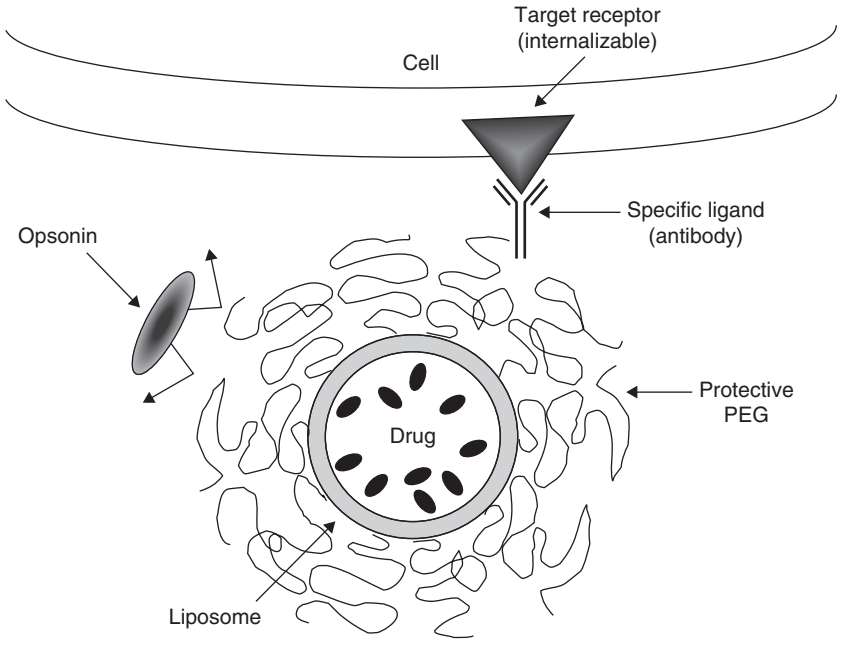

Figure 4. Long-circulating immunoliposome, modified with specific ligand (antibody) at distal tips of some grafted PEG chains. Such a liposome is protected from opsonins by PEG coating, and also specifically interacts (via the attached antibody) with target receptors on the surface of target cells (ideally, providing an increased internalization).
PEG: Polyethylene glycol.

injection and avidin for subsequent administration that aggregates biotin-liposomes and increases their uptake by lymph node macrophages [123]. Liposomes have been used for lymphatic delivery of methotrexate [124] and for magnetic resonance imaging with gadolinium-loaded liposomes [125].

An interesting example of a new approach is a combination of radio-frequency tumor ablation (tumor cell killing by applying high frequency irradiation resulting in local increase in the temperature) with intravenous liposomal doxorubicin, which resulted in better tumor accumulation of liposomes and increased necrosis in tumors [126,127].

Whatever application route is envisioned for the liposomal drugs, according to [128] the following quality control assays should be applied to pharmaceutical liposomal formulations for use in humans: i) basic characterization assays: pH, osmolarity, trapped volume, phospholipid concentration, phospholipid composition, phospholipid acyl chain composition, cholesterol concentration, active compound concentration, residual organic solvents and heavy metals, active compound/phospholipid ratio, proton or ion gradient before and after remote loading; ii) chemical stability assays: phospholipid hydrolysis, non-esterified fatty acid concentration, phospholipid acyl chain autoxidation, cholesterol autoxidation, active compound degradation; iii) physical characterization assays: appearance, vesicle size distribution, submicron range, micron range, electrical surface potential and surface pH, zeta potential, thermotropic behavior, phase transition, and phase separation, percentage of free drug; and iv) microbiological assays: sterility, pyrogenicity (endotoxin level).

5. Targeted liposomes in cancer chemotherapy

Current development of liposomal carriers often involves the attempt to combine the properties of long-circulating liposomes and targeted liposomes in one preparation [35,129,130]. To achieve better selectivity of PEG-coated liposomes, it is advantageous to attach the targeting ligand via a PEG spacer arm, so that the ligand is extended outside of the dense PEG brush excluding steric hindrances for the ligand binding to the target. Various advanced technologies are used for this purpose, and the targeting moiety is usually attached above the protecting polymer layer, by coupling it with the distal water-exposed terminus of activated liposome-grafted polymer molecule (**Figure 4**) [129,131].

As PEG-lipid conjugates used for the steric protection of liposomes and other pharmaceutical nanocarriers, and for the preparation of polymeric micelles, are derived from methoxy-PEG and carry non-reactive methoxy terminal groups, several attempts have been made to functionalize PEG tips in PEG-lipid conjugates. For this purpose several types of endgroup functionalized lipopolymers of general formula X-PEG-PE [89,132], where X represents a reactive functional group-containing moiety and PEG-PE represents the conjugate of PE and PEG, were introduced. Most of the endgroup functionalized PEG-lipids were synthesized from

heterobifunctional PEG derivatives containing hydroxyl and carboxyl or amino groups. Typically, the hydroxyl endgroup of PEG was derivatized to form a urethane attachment with the hydrophobic lipid anchor, PE, while the amino or carboxyl groups were used for the conjugation reaction or further functionalization. To further simplify the coupling procedure and to make it applicable for single-step binding of a large variety of amino group-containing ligands (including antibodies, proteins and small molecules) to the distal end of nanocarrier-attached polymeric chains, amphiphilic PEG derivative, p-nitrophenylcarbonyl-PEG-PE (pNP-PEG-PE), was introduced [131,133,134]. pNP-PEG-PE readily adsorbs on hydrophobic nanoparticles or incorporates into liposomes and micelles via its phospholipid residue, and easily binds any amino group-containing compound via its water-exposed pNP group forming stable and non-toxic urethane (carbamate) bonds. The reaction between the pNP group and the ligand amino group proceeds easily and quantitatively at pH 8.0, while excessive free pNP groups are easily eliminated by spontaneous hydrolysis. Other methods that could be used for the coupling of ligands to the distal tips of PEG chains include PEG activation with hydrazine group (in case of antibody attachment, hydrazine reacts with the oxidized carbohydrate groups in the oligosaccharide moiety of the antibody); pyridyldithiopropionate (PDP) group (after conversion of the PDP into the thiol, it reacts with maleimide groups of the premodified ligand); or maleimide group (reacts with thiol groups in prethiolated ligand) [29,135-139]. The ligand (antibody) binding to PEGylated liposomes was also performed via the PEG terminus modified with cyanuric chloride [140]. See review on various coupling techniques in [141,142].

An interesting approach to couple various ligands, such as antibodies, to liposomes including PEGylated liposomes involves a so-called 'postinsertion' technique [143]. This technique is based on the preliminary activation of ligands with any reactive PEG-PE derivative and subsequent co-incubation of unstable micelles formed by the modified ligand-PEG-PE conjugates with preformed drug-loaded plain or PEGylated liposomes. Eventually, modified ligands spontaneously incorporate from their micelles into the more thermodynamically favorable surrounding of the liposome membrane. This method was used, in particular, to prepare immuno-Doxil by modifying it with pNP-PEG-PE-modified anticancer 2C5 monoclonal antibodies [144,145].

5.1 Antibody-targeted liposomes

Antibodies are the most diverse and broadly used specific ligands for experimental targeted chemotherapy of various tumors with drug-loaded liposomes. There are multiple original papers and reviews on antibody-targeted drug-loaded liposomes in cancer [37,146-151].

Early attempts to attach antibodies to liposomes for their targeting to certain cells and tissues in the body go back to late 1970s/early 1980s, when antibody molecules and some model proteins were coupled to the surface of plain, non-PEGylated liposomes. The binding chemistry involved the use of bifunctional reagents to couple liposome-incorporated reactive groups with a protein [152,153]; protein modification with hydrophobic residues providing and efficient incorporation of the modified proteins into the liposomal membrane [154], with *N*-glutarylphosphatidyl ethanolamine becoming the most frequent modifier [155-157]; protein attachment to liposome via activated liposome-incorporated sugar moieties or via activated sugar moieties in the antibody molecule [158], and few other approaches [159,160]. Comparative studies of the preparation of immunoliposomes with the use of two bifunctional coupling agents and investigation of *in vitro* immunoliposome-target cell binding was studied in [161]. One of the first reviews on antibody-modified liposomes can be found in [73].

Antibody-modified liposomes of the 'first generation' have been used to estimate certain parameters of their interaction with target cells *in vitro* [162] and perform liposome targeting to certain model and real targets both *in vitro* and *in vivo*, such as extracellular matrix antigens or infracted areas in the myocardium [163,164]. Importantly, it was noted that the modification of antibody-bearing liposomes with PEG (to make them long-circulating) usually results in decreased binding efficacy because of steric shielding of surface-attached antibodies by the liposome-grafted PEG [35,165]. This eventually led to the development of multiple methods to attach antibodies onto the surface of PEG layer in PEGylated liposomes.

In general, antibody attachment can decrease the circulating time of liposomes because of increased uptake of the modified liposomes via Fc receptors of circulating or liver macrophages or opsonization of the liposome-tagged antibody molecules [166,167]. Whole antibodies can also trigger complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity [37]. These effects could be minimized by using antibody Fab fragments instead of whole antibodies [168]. Although Fab fragments can also accelerate liposome clearance [169], in general, Fab-liposomes circulate significantly longer than full antibody-modified liposomes [169]. In the case of antibody-modified PEGylated liposomes, even a certain decrease in the circulation time for antibody-modified PEG-liposomes still allows for their sufficiently long circulation, permitting good target accumulation. Clearly, attention should be paid not to over modify PEG-liposomes with the antibody to the level when their longevity is seriously compromised.

Interestingly, in some cases tumor accumulation of antibody-modified long-circulating liposomes is comparable with the accumulation of long-circulating liposomes without antibody attached [170-173]. However, therapeutic activity is higher for antibody-targeted liposomes. As explained in [174] using PEGylated liposomes modified or non-modified with anti-HER2 antibody, although intratumoral accumulation is similar for both preparations, antibody-modified preparations are much better internalized by tumor cells, which allows

for higher drug doses to be delivered inside cancer cells, that is, for more efficient cancer cell killing.

In some other cases, however, the liposome internalization seems unimportant. Thus, it was shown in [175] that PEGylated liposomes loaded with vincristine or doxorubicin and modified (or non-modified) with antibodies against internalizing CD19 antigen or non-internalizing CD20 antigen demonstrate therapeutic effects, which depended more on the type of the drug used than on the ability to be internalized. As expected, the cytotoxicity of targeted liposomes depended also on the rate of drug release from the liposomes [176].

An interesting phenomenon was described in [177], the authors of which have demonstrated that while non-targeted doxorubicin-containing liposomes were toxic to various cancer cells to the extent reflecting cell sensitivity to the drug, the cytotoxicity of antibody-targeted liposomes was proportional to the surface density of the surface antigen against which liposomes were targeted. The critical antigen surface concentration was ~ $4 \times 10 \times 4$ sites per single cell; beyond this value, any further increase in the antigen density was no longer important. Similar observations have also been made in [171,178]. As cancer cells are often rather heterogenous in respect to antigens they express, it was suggested in [37] to use a combination of antibodies against different antigens on a single liposome to provide better and more uniform targeting of all cells within the tumor. Alternatively, the 'bystander' effect can also be relied on [37], that is, the action of the drug released from the liposomes attached to a certain cancer cell on the neighboring cancer cells devoid of a similar receptor.

The first systematic studies with antibody-modified liposomes have been performed using both non-PEGylated and PEGylated liposomes modified with the 34A monoclonal antibody, which is highly specific towards murine pulmonary endothelial cells [136,179-183]. This approach was shown to be effective in targeting lung metastases in a murine model with the liposomal 3′,5′-*O*-dipalmitoyl-5-fluoro-2′-deoxyuridine [182].

Another antibody, which has gained popularity in cancer targeting, is the monoclonal antibody against HER2, the antigen frequently overexpressed on various cancer cells. Monoclonal anti-HER2 antibodies, including the humanized ones, as well as clinically used trastuzumab antibodies, have been used to render drug-loaded liposomes (long-circulating liposomes) specific for HER2-positive cancer cells [138,170,173,174,184,185]. This antibody was successfully used to deliver doxorubicin, both in plain and long-circulating liposomes, to breast tumor xenografts in mice, which resulted in significantly enhanced therapeutic activity of the drug. PEGylated liposomes decorated with anti-HER2 antibody were shown to undergo effective endocytosis by HER2-positive cancer cells allowing for better drug (doxorubicin) accumulation inside tumor cells with better therapeutic outcome. Compared with doxorubicin in plain PEGylated liposomes (Doxil), which normally accumulates in the tumor interstitial space, in case of antibody-targeted Doxil, more drug molecules were discovered inside cancer cells, that is, targeting with the antibody increases drug internalization by target cells.

Another promising antibody to target tumors with drug-loaded liposomes is the monoclonal antibody against CD19 antigen, which is also frequently overexpressed on various cancer cells. Anti-CD19 antibody-modified liposomes loaded with doxorubicin demonstrated clearly enhanced targeting and therapeutic efficacy, both *in vitro* and *in vivo* in mice with human $CD19^+$ B lymphoma cells [178]. Similar results have also been obtained with doxorubicin-loaded liposomes modified with antibodies against internalizable C19 antigen and against non-internalizable CD20 antigen [175]. Anti-CD19 antibodies have also been used to target doxorubicin-loaded liposomes with variable drug release rates to experimental tumors [176]. Recently, a successful attempt was made to target doxorubicin-loaded long-circulating liposomes to CD19-expressing cancer cells with single-chain Fv fragments of CD19 antibodies [186,187].

Because neuroblastoma cells usually overexpress disialoganglioside GD2, antibodies against GD2 and their Fab′ fragments have been suggested to target drug-loaded liposomes to corresponding tumors [188-190]. Fab′ fragments of anti-GD2 antibodies covalently coupled to long-circulating liposomes loaded with doxorubicin, allowed for increased binding and higher cytotoxicity against target cells both *in vitro* and *in vivo*, including in models of human tumors in nude mice and in metastatic models. GD2-targeted immunoliposomes with the novel antitumoral drug, fenretinide, inducing apoptosis in neuroblastoma and melanoma cell lines, have also demonstrated strong antineuroblastoma activity both *in vitro* and *in vivo* in mice [191]. The combination of doxorubicin-loaded PEGylated liposomes targeted with anti-GD2, and with NGR-peptides specifically binding with the tumor vasculature, produced an improved therapeutic effect by acting on both tumor cells and tumor blood vessels [190].

An interesting novel target for antitumor drug delivery by means of targeted liposomes is the membrane type-1 matrix metalloprotease (MT1-MMP), playing an important role in tumor neoangiogenesis and overexpressed both on tumor cells and on neoangiogenic endothelium. The modification of doxorubicin-loaded long-circulating liposomes with anti-MT1-MMP antibody resulted in an increased uptake of the targeted liposomes by MT1-MMP-overexpressing HT1080 fibrosarcoma cells *in vitro* and in more effective inhibition of tumor growth *in vivo* compared with antibody-free doxorubicin-loaded PEGylated liposomes [192]. It was demonstrated that anti-MM1-MMP antibody enhances the endocytic internalization of drug-loaded liposomes, thus increasing their cytotoxicity [193]. Strong action of such preparation on tumor endothelial cells was noted.

Epidermal growth factor receptor (EGFR) and its variant EGFRvIII can serve as valuable targets for intracellular drug

delivery into tumor cells overexpressing these receptors. Fab′ fragments of the monoclonal antibody C225, which binds both EGFR and EGFRvIII, and scFv fragment of the monoclonal antibody, which binds only to EGFR, were coupled to drug-loaded liposomes and allowed for substantially enhanced binding of such targeted liposomes with cancer cells overexpressing corresponding receptors, such as glioma cells U87 and carcinoma cells A0431 and MDA-MB-468. The better binding resulted in enhanced internalization and increased cytotoxicity [194]. *In vivo* therapy with such targeted drug-loaded liposomes (doxorubicin, epirubicin and vinorelbine were used as drugs) always resulted in better tumor growth inhibition than therapy with non-targeted liposomal drugs [195]. Fab′ fragment derived from the humanized anti-EGFR monoclonal antibody EMD72000 was shown to provide efficient intracellular delivery of the liposomal drugs into colorectal tumor cells [196]. The authors of this study have also shown that the attachment of the targeting moiety to PEGylated liposomes requires the length of the spacer arm to be sufficient to overcome possible steric shielding of antibody fragments by sterically protecting PEG chains. An interesting method to construct anti-EGFR-targeted liposomes was suggested in [197], where the anti-EFGR antibody (cetuximab or C225) was covalently linked to the folate-binding protein via a thioester bond and then coupled to the preformed folate-containing liposomes. Cetuximab-liposomes loaded with boron derivatives for boron neutron capture therapy were also prepared using the cholesterol-based anchor and micelle-transfer technology [198].

Various proteins of the extracellular matrix expressed on the surface of cancer cells have also been used as targets for the antibody-mediated delivery of the liposomal drugs. Thus, β_1-integrins expressed on the surface of human non-small-cell lung carcinomas were targeted by doxorubicin-loaded liposomes modified with Fab′ fragments of anti-β_1-integrin monoclonal antibodies [199]. Treatment of SCID mice with lung tumor xenografts with such liposomes resulted in significant suppression of tumor growth compared with all controls and also inhibited metastases. The idea of targeting various antigens (preferably, the internalizable ones) on the endothelial cells by antibody-liposome conjugates was tested long ago [200]. However, the approach has attracted real attention only in the last few years. Thus, liposomes modified with anti-E-selectin antibodies were successfully internalized by activated endothelial cells *in vitro* through E-selectin-mediated endocytosis [201]. Another possible target for antibody-mediated cancer therapy with drug-loaded liposomes is the epithelial cell adhesion molecule (EpCAM), which is expressed in many tumors, but not in normal cells [202]. EpCAM-targeted immunoliposomes were generated by covalent attachment of the humanized scFv fragment of the 4D5MOCB monoclonal antibody to the surface of PEGylated doxorubicin-loaded liposomes and demonstrated significantly improved binding, internalization and cytotoxicity with EpCAM-positive cancer cells. Similarly, liposomes coupled with antibodies against vascular cell adhesion molecule-1 (VCAM-1) can be effectively targeted to activated endothelial cells overexpressing VCAM-1 [203]. Liposomes loaded with cytotoxic drugs were also targeted to ED-B fibronectin using the scFv fragment of the corresponding antibody [204]. Proliferating endothelial cells have been targeted with doxorubicin-loaded liposomes modified with scFv fragments of the antibody against endoglin overexpressed on such cells [205].

Lipid-based drug carriers have also been conjugated with antibodies (or their fragments) against transferrin receptor (TfR), which is frequently overexpressed on the surface of various cancer cells. For example, such carriers were modified with the OX26 monoclonal antibody against TfR via liposome-incorporated maleimide-modified PEG(2000)-PE molecules and demonstrated strong binding with cells over-expressing TfR [206]. The same antibody was attached to daunomicin-loaded liposomes non-covalently via the avidin–biotin couple, and the modified liposomes demonstrated good accumulation in multi-drug-resistant RBE4 brain capillary endothelial cells both *in vitro* and *in vivo* [207].

Liposomes loaded with a lipophilic prodrug, 5-fluorodeoxyuridine, and modified with the monoclonal antibody, CC531, against rat colon carcinoma demonstrated good binding with target cells [208] and effective intracellular drug delivery compared with all controls [209]. Antibody CC52 against rat colon adenocarcinoma CC531 attached to PEGylated liposomes provided specific accumulation of liposomes in a rat model of metastatic CC531 tumors [210].

Non-pathogenic antinuclear autoantibodies (ANAs), which are frequently detected in cancer patients and in healthy elderly individuals, represent a subclass of natural anticancer antibodies. Previously, it was shown that certain monoclonal ANAs (such as mAbs 2C5 and 1G3) recognize the surface of numerous tumors, but not normal cells [211-213]. Nucleosome-restricted specificity was shown for some of these monoclonal ANAs, and tumor cell surface-bound nucleosomes have been shown to be their universal molecular target on the surface of a variety of tumor cells [213,214]. Because these antibodies can effectively recognize a broad variety of tumors, they may serve as specific ligands to deliver other drugs and drug carriers into tumors. These antibodies were used to prepare drug-loaded, tumor-targeted, long-circulating immunoliposomes (with doxorubicin), which demonstrated highly specific binding with various cancer cells (murine Lewis lung carcinoma, 4T1, C26, and human BT-20, MCF-7, PC3 cells) *in vitro* [144,215], significantly increased tumor accumulation in model tumors in mice including intracranial human brain U-87 MG tumor xenografts in nude mice, decreased side effects, and superior antitumor activity *in vivo* [216-218].

Doxorubicin-loaded PEGylated liposomes were also modified with Fab′ fragments of an anti-CD74 antibody via a PEG-based heterobifunctional coupling reagent and demonstrated a significantly accelerated and enhanced

accumulation in Raji human B lymphoma cells *in vitro* [219]. Anti-CD166 scFv attached to drug-loaded liposomes facilitated doxorubicin internalization by several prostate cancer cell lines (Du-145, PC3, LNCaP) [220]. ScFv fragments of antibodies against leukemia stem cells and oncogenic molecules participating in acute myeloid leukemia pathogenesis were used to target acute leukemia stem cells [221]. Doxorubicin-loaded liposomes were successfully targeted to the kidney by using Fab′ fragments of the monoclonal OX7 antibody directed against Thy1.1 antigen in rats [222]. Because fibroblast activation protein (FAP) represents a cell surface antigen expressed by the tumor stromal fibroblasts in different cancers, scFv from the antibody cross-reacting with human and mouse FAP was used to target PEGylated liposomes to tumor stromal cells [223]. Tumor necrotic zones were effectively targeted by doxorubicin-loaded liposomes modified with chimeric TNT-3 monoclonal antibody specific towards degenerating cells located in necrotic regions of tumors and demonstrated enhanced therapeutic efficacy in nude mice bearing H460 tumors [224]. Combination of immunoliposome and endosome-disruptive peptide improves cytosolic delivery of liposomal drug, increases cytotoxicity, and opens new approaches to constructing targeted liposomal systems, as shown with diphtheria toxin A chain incorporated together with pH-dependent fusogenic peptide diINF-7 into liposomes specific towards ovarian carcinoma [225].

Early clinical trials of antibody-targeted drug-loaded liposomes have already demonstrated some promising results. Thus, doxorubicin-loaded PEGylated liposomes (with a size of ~ 140 nm) modified with F(ab′)2 fragments of the GAH monoclonal antibody specific for stomach cancer were tested in a Phase I clinical studies and demonstrated the pharmacokinetics similar to that of Doxil [226].

Thus, there exists a whole set of antibodies or their fragments used for targeting liposomal anticancer drugs to tumors (**Table 2**).

5.2 Miscellaneous ligands

Because transferrin (Tf) receptors (TfR) are overexpressed on the surface of certain tumor cells, antibodies against TfR as well as Tf itself are among popular ligands for liposome targeting to tumors and inside tumor cells [227] (although TfR expression in normal cells, particularly in the liver, can compete with tumor targeting of Tf-liposomes). Recent studies involve the coupling of Tf to PEG on PEGylated liposomes in order to combine longevity and targetability for drug delivery into solid tumors [228]. A similar approach was applied to deliver into tumors agents for photodynamic therapy including hypericin [229,230] and for intracellular delivery of cisplatin into gastric cancer [231]. Tf-coupled doxorubicin-loaded liposomes demonstrate increased binding and toxicity against C6 glioma [232]. Interestingly, the increase in the expression of the TfR was also discovered in postischemic cerebral endothelium, which was used to deliver Tf-modified PEG-liposomes to postischemic brain in rats [233]. Tf [234] as well as anti-TfR antibodies [235,236] were also used to facilitate gene delivery into cells by cationic liposomes. Tf-mediated liposome delivery was also successfully used for brain targeting. Immunoliposomes with OX26 monoclonal antibody to the rat TfR were found to concentrate on brain microvascular endothelium [237].

Targeting tumors with folate-modified liposomes represents a very popular approach, as folate receptor (FR) expression is frequently overexpressed in many tumor cells. After early studies demonstrated the possibility of delivery of macromolecules [238] and liposomes [239] into living cells using FR endocytosis, which could bypass multi-drug resistance, the interest to folate-targeted drug delivery by liposomes grew fast (see important reviews in [240,241]). Liposomal daunorubicin [242] as well as doxorubicin [243] and 5-fluorouracyl [244] were delivered into various tumor cells both *in vitro* and *in vivo* via FR and demonstrated increased cytotoxicity. Recently, the application of folate-modified doxorubicin-loaded liposomes for the treatment of acute myelogenous leukemia was combined with the induction of FR using all-trans retinoic acid [245]. Folate-targeted liposomes have been suggested as delivery vehicles for boron neutron capture therapy [246] and used also for targeting tumors with haptens for tumor immunotherapy [247]. Within the frame of gene therapy, folate-targeted liposomes were used for both gene targeting to tumor cells [248] as well as for targeting tumors with antisense oligonucleotides [249].

The search for new ligands for liposome targeting concentrates on specific receptors overexpressed on target cells (particularly cancer cells) and certain specific components of pathologic cells. Thus, liposome targeting to tumors has been achieved by using vitamin and growth factor receptors [250]. Vasoactive intestinal peptide (VIP) was used to target PEG-liposomes with radionuclides to VIP-receptors of the tumor, which resulted in an enhanced breast cancer inhibition in rats [251]. PEG-liposomes were also targeted by RGD peptides to integrins of the tumor vasculature and, being loaded with doxorubicin, demonstrated increased efficiency against C26 colon carcinoma in a murine model [252]. RGD peptide was also used for targeting liposomes to integrins on activated platelets and, thus, could be used for specific cardiovascular targeting [253] as well as for selective drug delivery to monocytes/neutrophils in the brain [254]. Similar angiogenic homing peptide was used for targeted delivery to vascular endothelium of drug-loaded liposomes in experimental treatment of tumors in mice [255]. EGFR-targeted immunoliposomes were specifically delivered to variety of tumor cells overexpressing EGFR [194]. Mitomycin C in long-circulating hyaluronan-targeted liposomes increases its activity against tumors overexpressing hyaluronan receptors [256]. The ability of galactosilated liposomes to concentrate in parenchymal cells was applied for gene delivery in these cells [257]. Cisplatin-loaded liposomes, specifically binding chondroitin sulphate, overexpressed in

Table 2. Examples of antibodies (or their fragments) used to target liposomal anticancer drugs to tumors.

Targeting agent	Cell surface antigen	Drug	Model	Ref.
Anti-CD19	CD19	Doxorubicin	Namala hu-B-cell lymphoma	[175,176,178]
Anti-CD19	CD19	Doxorubicin	Human multiple myeloma, ARH and cell line	[186,187,285]
Anti-CD19, scFv	CD19	Doxorubicin	Raji human B lymphoma	[186]
Recombinant human, anti-HER2-Fab′ or scFv C6.5	HER2	Doxorubicin	HER2-overexpressing human breast cancer	[171]
Anti-HER2	HER2	Paclitaxel	HER2-overexpressing human breast cancer	[185]
Anti-HER2 Fab′ or scFv	HER2	–	Human breast BT-474 adenocarcinoma	[138,174]
Anti-hu CEA 21B2 and anti-hu CEA 21B2 Fab′	Human carcinoembrionic antigen, CEA	Vincristine	CEA-positive human gastric antigen, MKN45	[286]
MRK16	P-glycoprotein	–	Human myelogenous leukemia K562	[287]
Anti-β_1-integrin Fab′	Human β_1-integrins	Doxorubicin	Human non-small-cell lung carcinoma	[199]
CC52	CC531	Floxuridine (analog)	Rat colon carcinoma	[288]
Anti-GD_2 and anti-GD_2-Fab′	GD_2	Doxorubicin	Human neuroblastoma	[189]
Anti-GD_2	Disialoganglioside, GD_2	Fenretinide	Human melanoma	[289]
Anti-idiotype mAb, S5A8	38C13	Doxorubicin	Murine D-cell lymphoma	[290]
Anti-human E-selectin	E-selectins	–	Activated human endothelial cells	[291]
Anti-ganglioside G_{M3} (DH2) or anti-L^{Ex} (SH1)	Carbohydrate, ganglioside (G_{M3}); Lewis X (Le^x)	Doxorubicin	B16BL6 mouse melanoma and HRT-18 human colorectal adenocarcinoma	[292]
Anti-ED-B scFv (ED-B domain)	B-fibronectin (ED-B domain)	Fluorodeoxyuridylate analog	*In vitro* Caco-2 cells and *in vivo* murine F9 teratocarcinoma	[293]
chTNT	TNT	Doxorubicin	Human non-small-lung carcinoma H460	[224]
Anti-MT1-MMP-Fab′	Metalloproteinase MT1-MMP	Doxorubicin	Human HT1080 fibrosarcoma	[192,193]
Anti-CD74 LL1	CD74	Doxorubicin	Raji human B lymphoma	[219]
Anti-nucleosome 2C5 mAb	Nucleosome	–	Murine LLC, 4T1, C26	[216]
Anti-nucleosome 2C5 mAb	Nucleosome	Doxorubicin	Human BT-20, MCF-7, PC3	[144,145]
Anti-nucleosome 2C5 mAb	Nucleosome	Doxorubicin	Murine LLC, 4T1, C26; human BT-20, MCF-7, PC3	[218]
C225 mAb or Fab′	EGF receptor	Doxorubicin	Human MDA-MB-468	[195]
OX26 mAb	Rat transferin receptor	Daunomycin	RBE4 brain capillary cells, rat biodistribution	[207]
Anti-Thy-1.1 OX7 mAb	Thy-1.1	Doxorubicin	Rat mesamgial cells, biodistribution	[222]
Anti-VCAM-1	Vascular cell adhesion molecule-1	–	Human endothelial cells	[203]
scFv A5	Endothelin	Doxorubicin	Endothelial cells HUVEC, HDMEC	[205]
Anti-FAP scFv	Fibroblast activation protein	–	Tumor stromal cells	[223]
Anti-51-kDa Fab′	Parasite-specific 51-kDa protein	Doxorubicin	Mouse model of visceral leishmaniasis	[294]

-: No drug.

many tumor cells were used for successful suppression of tumor growth and metastases *in vivo* [258]. Tumor-selective targeting of PEGylated liposomes was also achieved by grafting these liposomes with basic fibroblast growth factor-binding peptide [259]. Intraperitoneal cancer can be successfully targeted by oligomannose-coated liposomes, as discussed in a recent review [260].

An interesting example of intracellular targeting of liposomes was described recently, where liposomes containing a mitochonriotropic amphiphilic cation with delocalized positive charge were shown to specifically target mitochondria in intact cells [261].

5.3 Some general considerations for engineering tumor-targeted liposomes

There are several clear aims one wants to achieve when using antibody-mediated tumor targeting of drug-loaded liposomes compared with more traditional liposome-based dosage forms: i) such liposomes should accumulate in target tumors fast and effectively; ii) the quantity of the drug delivered into the tumor by such liposomes should be higher than in the case of other delivery systems; iii) ideally, liposomal drugs should not only accumulate in the interstitial space inside tumors, but also be internalized by the target cells creating high intracellular drug concentration and allowing for bypassing multi-drug resistance.

To achieve these goals certain considerations should be taken into account when developing antibody-targeted liposomes for chemotherapy. First, the target should be identified, which is present (overexpessed) on the surface of tumor cells to be targeted in sufficient quantity providing good opportunity for the targeted liposomes to firmly bind with cancer cells [177]. Second, the specific ligand (antibody or its fragment) should be attached to the surface of the drug-loaded liposome in a way that does not affect its specific binding properties (the optimal choice should be made among the variety of coupling methods available, while keeping in mind that a method suitable for one antibody, will not necessarily be suitable for another), and in sufficient quantity to provide the multipoint binding with the target (50 – 100 antibody molecules should be coupled to the surface of a 100-nm liposome); in the case of PEGylated long-circulating liposomes the quantity of the attached antibodies should not be excessive in order not to overcompromise the liposome longevity [144,262]. Third, it is highly desirable that the targeting antibody is internalizable and facilitates the internalization of the liposome and liposome-incorporated anticancer drug [174,195]. Fourth, drug release from the liposome inside the tumor or inside the tumor cell should provide the therapeutic concentration of the drug in the target and maintain it within a reasonable period of time (few hours) [175,176]. All these properties are easy to follow and optimize in a set of standard and easy to perform *in vitro* experiments.

With all the promising data on antibody-targeted drug-loaded liposomes for cancer therapy, one needs to mention several problems associated with these systems, both from the biological and technological points of view. Biologically, one can expect certain changes in normal pharmacokinetics and biodistribution of plain and long-circulating liposomes after their modification with antibodies. These changes could result in an increased uptake of antibody-bearing liposomes by the RES. Although some early studies did not reveal big differences in biodistribution of antibody-free and antibody-modified liposomes [181,263], later it was found that antibody attachment still can accelerate the liposomes clearance and RES accumulation, especially following repeated administration [264]. It looks, however, that the use of smaller antibody fragments (such as Fab) instead of whole antibodies can minimize protein-mediated liposome clearance and uptake by the RES [187].

The presence of proteins (antibodies) on liposomes can also result in increased immunogencitty of such preparation. Thus, it was shown long ago that the administration in mice of liposomes (including PEG-liposomes) modified with IgG2a resulted in an increased production of anti-IgG2a antibodies in experimental animals [265]. This observation was later confirmed by the authors of [266].

From the technological point of view, the addition of the surface-attached antibody to the liposomal preparation will certainly result in the cost increase of the final product because of the high cost of antibodies and additional preparation step. At this moment, it is difficult to say how serious this problem could become; however, it may be taken care of by minimizing the quantity of the attached antibody (optimization issues) and by using technologies (such as postinsertion technique [198,262,267]) allowing for the minimal lost of the antibody during the attachment procedure.

6. Expert opinion

Summing up, there exist multiple important achievements in the development of liposomal preparations of anticancer drugs targeted to tumors by different tumor-specific antibodies. First, a significant number of monoclonal antibodies is now identified and engineered as chimeric or humanized antibodies, or as Fab′ or scFv fragments. Second, several reasonably simple, highly effective and reproducible methods to couple these antibodies or their fragments to the surface of plain or PEGylated drug-loaded liposomes are developed yielding antibody-modified liposomes with preservation of specific antibody affinity and capable of effective recognition of the target cells both *in vitro* and *in vivo*. Third, in many cases, antibody-targeted liposomes demonstrate better internalization by cancer cells and more effective intracellular drug delivery than other preparations, which could allow to overcome multi-drug resistance Fourth, extensive data are already accumulated, clearly demonstrating significant benefits of antibody-targeted liposomal drugs in numerous animal models, such as accelerated target accumulation, increased quantity of the drug delivered to the target, decreased side

effects associated with the administration of non-targeted liposomal drugs, and significantly enhanced therapeutic outcome. Fifth, some early clinical trials with antibody-targeted liposomal drugs have already been initiated, yielding promising results.

Adding to the list the fact that liposomes are reasonably easy to make and liposomal preparations demonstrate sufficient stability at storage and in the body, antibody-targeted drug-loaded liposomes represent very promising candidates for cancer chemotherapy, and we can expect their extensive clinical evaluation in the near future. Breast cancer and lung cancer, which have been used as targets in the vast majority of investigations, can be named as primary candidates for therapy with antibody-targeted liposomes.

Possible hurdles on the way to the actual antibody-targeted liposomal drugs for cancer could be divided into two groups: biological and technological. From the biological point of view, modification of drug-loaded long-circulating liposomes with proteins (antibodies) could change their biodistribution compared with non-modified liposomes and increase their uptake by the RES. Liposome-attached proteins can also elicit undesirable immune response. These problems could be addressed by minimizing the quantity of the liposome-attached target protein via preliminary optimization and by using less immunogenic antibody fragments rather than whole antibodies. From the technological point of view, the modification of liposomal drugs with antibodies involves an additional preparation step and increases the final cost of the preparation because of both this additional step as well as the high cost of antibodies themselves. These problems cannot be eliminated; however, they could be addressed to a certain extent by using simple techniques of quantitative incorporation of antibodies into liposomes, such as postinsertion technique.

In general, advantages seem to outweigh disadvantages, and practical applications of antibody-targeted liposomal drugs for tumor therapy should become a reality within the next few years.

Declaration of interest

The authors declare no conflict of interest and have received no payment in preparation of this manuscript.

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