Expert Opinion

Antibody-targeted liposomes in cancer therapy and imaging

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Background: Targeted liposomes can be broadly defined as liposomes that are engineered to interact with a particular population of cells with the objective of delivering a payload or increasing their retention within the targeted cell population by means of a chemical interaction with cell-surface molecules or other tissue-specific ligands. *Objective*: The authors review recent advances in the field with an emphasis on pre-clinical studies and place them in the context of historical developments. *Methods*: The review focuses on immunoliposomes (antibody-mediated targeting) as these constructs are presently the most prevalent. *Conclusion*: The field has advanced in tandem with advances in liposome design and antibody and protein engineering. Targeted liposomes have been used in diagnosis to deliver magnetic resonance contrast agents and radionuclides for magnetic resonance and nuclear medicine imaging, respectively. They have been used in gene therapy to deliver a variety of gene expression modifiers, including plasmids, anti-sense oligonucleotides and short interfering RNA. Targeted liposomes provide a delivery advantage over untargeted liposomes not because of increased localization to tumor sites but because of increased interaction with the target cell population once localized to the tumor site. The increased interaction can take on the form of fusion with the cellular membrane or internalization by endocytosis. To the extent that the spatial distribution of targeted liposomes within a solid tumor may become more non-uniform than has been found for untargeted liposomes, this may be a drawback. However, systematic comparisons of the spatial distribution in tumors of targeted versus untargeted liposomes have yet to be performed. The majority of reported studies have been in the area of chemotherapy delivery. Their use in radionuclide and chemo- and radio-sensitizer delivery is just emerging. Multifunctional liposomes containing 'layered functionalities' could potentially be the future direction in targeted liposome-based therapy.

Keywords: immunoliposomes, liposomes, targeted liposomes

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1. Introduction

1.1 Distinction between targeted and untargeted liposomes

A broad definition of targeted liposomes is liposomes that are engineered to interact with a particular population of cells with the objective of delivering a payload or increasing their retention or residence time within the targeted cell population by means of a chemical interaction with the cell population or with related ligands. The most prevalent approach to accomplishing this is by using liposomes that have antibodies or antibody-based constructs (e.g., fragments or single chain variable regions) conjugated on their surface (i.e., immunoliposomes) [1-3]. This review focuses primarily upon targeting by immunoliposomes. Such liposomes demonstrate

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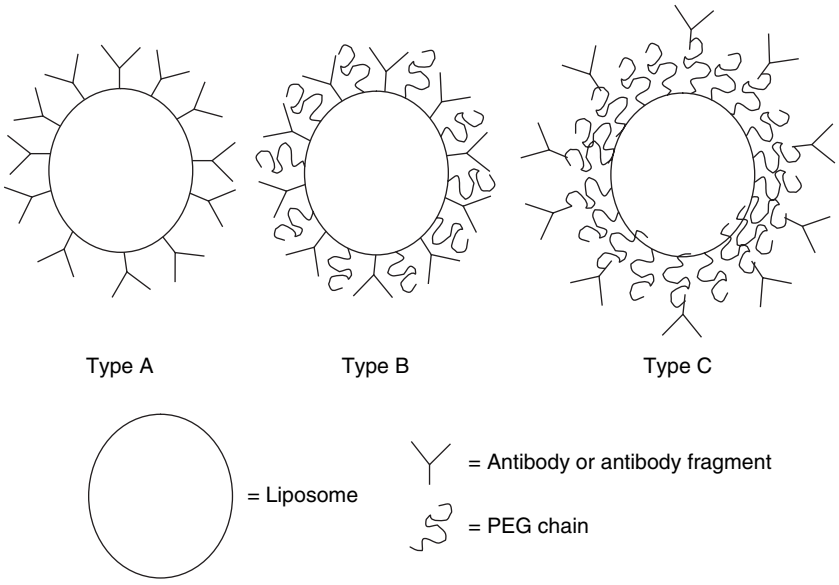

Figure 1. Illustration of different types of immunoliposomes.

specific binding to target cells, *in vitro*, followed by internalization via an endocytic pathway [4]. Untargeted liposomes do not exhibit specific binding to targeted cells, rather non-specific uptake may be observed through a variety of mechanisms that include: adsorption, endocytosis, and lipid exchange [5].

1.2 Brief history of the design/structure of targeted liposomes in drug delivery

The development of liposomes capable of homing to target cells has been an objective since the mid-70s [6,7]. Early studies on targeted liposomes resulted in three main structures (**Figure 1**) [8]: i) type A liposomes, where the antibodies are directly conjugated on the lipid headgroups resulting in immunoliposomes that exhibit specificity in binding but low blood circulation times; ii) type B liposomes that in addition to antibodies, PEGylated lipids (lipids conjugated to polyethylene glycol [PEG]) are also included on the liposome membrane, resulting in longer circulation times, although with obstruction of specific binding that depends on the extent of steric interference by the polymer chains; and iii) type C liposomes in which the cell-targeting antibodies are attached on the free termini of PEGylated lipids.

In particular, initial reports of type A antibody-conjugated liposomes demonstrated targeting, *in vitro* [2,4,9-14], but because these initial constructs were rapidly sequestered by the reticuloendothelial system (RES), attempts to demonstrate targeting *in vivo* following intravenous injection were largely unsuccessful [15-17]. Longer blood circulation times of immunoliposomes *in vivo* combined with successful targeting was demonstrated with PEG-coated (type B) liposomes. Targeting was reported against infarcted rabbit myocardium using an antimyosin antibody that targeted myosin released from necrotic cells [18]. Antibody conjugation of liposomes in this study was based on direct attachment of antibodies to the liposomal bilayer surface using antibodies that were derivatized with *N*-glutaryl phosphatidyl ethanolamine. Increasing the fraction of PEGylation resulted in lower specific binding, *in vitro*. However, this was compensated by the longer circulation times of PEGylated immunoliposomes *in vivo*. PEGylation at 4% mole PEG (5000 MW) resulted in higher accumulation at the target compared with non-PEGylated immunoliposomes. To increase specific binding while also retaining long circulation times, an alternative approach – type C liposomes – in which the antibody is attached via a thioether bond to the PEG terminus, were first developed in the mid-1990s [3,19-21]. These demonstrated improved binding to cells *in vitro*, which was achieved by binding to the terminus of the PEG chain; the binding affinity of the construct was shown to be minimally affected by the length of the polymer chain [3,20]. Immunoliposomes presently in widespread use and research investigation follow this latter design.

2. Chemical and physical aspects of targeted liposomes

Targeted liposomes are structures comprising a phospholipid membrane, an encapsulated aqueous phase (usually) containing the therapeutic agent, surface-grafted polymer chains, and surface-grafted targeting ligands. Each component of the targeted liposomes, aside from the targeting ligands, are briefly reviewed below (Section 2.3), in terms of their effect influencing the fate of liposomes *in vivo*, and, therefore, the targeting properties of liposomes.

2.1 Chemical and physical characteristics of liposome membranes relative to drug delivery

Liposomes are closed, self-assembled structures defined by one or more bilayer membranes that consist of amphiphilic molecules such as double-tailed phospholipids. The lipid membrane's physical properties, particularly permeability and fluidity, greatly influence the performance of liposomes *in vitro* and *in vivo*, and these properties can in principle be specifically tailored by choosing different combinations of lipid mixtures [22]. In general, membrane permeability is minimal to charged ions, whereas uncharged polar molecules can diffuse much faster across the bilayer. Depending on the fluid state of the liposome membrane (gel phase, liquid-crystalline phase, and several other phases of mixed lipids with intermediate fluidity), encapsulated contents may be more permeable with increasing membrane fluidity, assuming homogeneous liposome membranes. In heterogeneous liposome membranes, defined as membranes where lipid lateral phase separation occurs, or when single-component membranes are heated at the glass transition temperature (temperature above which lipids undergo a phase change from solid behavior to a more liquid-like behaviour [Tg]), permeability may show an unusual increase. This increase originates from the interface of the membrane heterogeneities. At these interfaces, gain boundaries are formed creating membrane defects due to poor lipid packing with lower effective melting temperatures [22-24].

Because of the flexibility and variety of liposome membranes, and their inherent characteristic to present an interface to the biological milieu that is similar to the immediate surface of cells, liposomes have evolved into major drug delivery carriers that combine several advantages: i) due to their relatively large aqueous core, liposomes exhibit high drug-to-carrier ratios; ii) therapeutic compounds can be encapsulated into the aqueous core or entrapped into the lipophilic membrane without any requirements for chemical modification for attachment to the carrier, that in several cases may interfere with the drug's activity; iii) lipids are not toxic themselves to the doses usually administered [25], in fact liposomal encapsulation has been used to reduce the toxicity of otherwise toxic drugs; and iv) in immuno-liposomes, conjugation of targeting ligands to the liposome surface may result in multivalency and increase in the ligand binding efficacy [20].

2.2 Liposome preparation methods

With liposomes for drug delivery applications, size and lamellarity are important parameters and require careful control. Specific and narrow size distributions are necessary, as they influence to a great extent the biodistribution of liposomes. The preparation of unilamellar liposomes is desired to maximize the aqueous encapsulated volume where the drug is entrapped, and to retain control of drug release across the single lipid bilayer membrane. Commonly, lipid suspensions are formed by addition of an aqueous phase to dry lipid layers followed by vortexing. To control size and lamellarity the suspensions are either sonicated or extruded. During sonication, cavitating high-energy ultrasound ruptures and fragments the lipid membranes, which assemble with other lipid fragments in order to minimize water exposure of their hydrophobic edge. When the size of these assembled lipid fragments is large enough to seal with itself at a curvature with less energy than the energy of the exposed hydrophobic regions, then a liposome is formed. Sonicated liposomes are mostly unillamelar [22]. Extrusion through polycarbonate membranes of well-defined pores is another method to control liposome size. Extrusion is performed at temperatures above the Tg of lipids. Unilamellarity is variable with this method and depends on the lipid composition, as well as the membrane pore size [26].

Other liposome preparation methods include reverse phase evaporation, and detergent dialysis. Reverse phase separation [27] introduces a way to develop liposome suspensions with high encapsulated aqueous volumes, and to entrap a large fraction of the aqueous material that needs to be encapsulated. The encapsulation yield with this method has been reported to be as high as 62%. In this process, inverted micelles surrounding the aqueous phase are formed in an excess of an organic solvent. The aqueous phase contains the material for encapsulation by the liposomes. After the organic solvent is slowly removed, a gel-like viscous phase is formed. Upon further removal of the organic solvent, some of the inverted micelles disintegrate, resulting in release of their aqueous phase and the presence of excess phospholipids. In this new polar environment, the excess lipids assemble around the remaining micelles forming closed bilayers (i.e., liposomes). A disadvantage of this method when the aim is to develop liposomes with encapsulated proteins is the possibility of protein denaturation due to the presence of the organic solvent.

Detergent dialysis is another method where upon formation of mixed detergent–lipid micelles, dialysis is used to remove the detergent molecules [28]. This process results in the formation of liposomes, and also in removal of non-encapsulated small molecular weight molecules from the liposome suspension. The rate of detergent removal affects the liposome size distribution. Rapid removal results in smaller liposome sizes. Lipids enriched in detergents have the tendency to fuse when in solution, so as the available time for fusion is decreased with faster detergent dialysis, less time is available for fusion and smaller liposome sizes are formed. This is a mild method that would not easily perturb the stability of even the most sensitive proteins.

Different preparation methods result in different liposome sizes. The smaller sizes of liposome suspensions are formed by sonication, larger sizes up to a few tenths of nanometers in diameter are formed by detergent dialysis, low pressure extrusion and reverse phase evaporation [29]. Other methods such as ethanol injection and high pressure homogenization result in small liposome sizes and are described in detail in

the above-cited edited books. As type C immunoliposomes are made after preparation of the intact liposomes, liposomes prepared by these different methods are equally amenable to PEGylation and antibody conjugation.

2.3 The impact of liposome physical properties in drug delivery

After the intravenous administration of liposomes, long liposome circulation times are required to increase the probability that liposomes will interact with cancer cells. This is required both for circulating micrometastatic disease or endothelial cells in the tumor vasculature, and for vascularized tumors. In the first case, although the target is readily accessible, the binding kinetics might be slow and longer presence of the targeted liposomes in the target vicinity may increase their binding efficacy. Likewise, in the second case, the rapid removal of liposomes from the circulation will reduce binding to endothelial cell targets. In the latter case, as liposome extravasation from the bloodstream through the leaky neovasculature and into the tumor interstitium is a random event, long circulation times will increase the probability that the circulating liposomes will encounter tumor vasculature, extravasate and react with components of the tumor interstitium or directly with tumor cells. During circulation in the blood, serum proteins act in a variety of ways to destabilize the circulating liposomes and orchestrate their action with circulating cells of the RES in order to remove liposomes from circulation. These mechanisms involve insertion into or adsorption of proteins on the liposome membrane, resulting in membrane destabilization and content release or presentation of liposomes, due to the adsorbed proteins, in a form identifiable by circulating cells of the RES and fast removal from circulation. In the case of immunoliposomes, additional factors such as recognition of the Fc region of tumor-targeting antibodies by circulating macrophages leads to fast liposome clearance (see Section 2.4) [30-33].

Several physical properties that are shown to be of great importance in determining the fate of liposomes *in vivo* and, consequently, in affecting their efficacy as drug delivery carriers include: liposome size, surface charge, membrane fluidity and PEGylation.

In general, particles of sizes > 250 – 300 nm in diameter do not exhibit long circulation times compared with smaller particles, and seem to accumulate to a great extent in the spleen [34-36]. Liposomes with diameters > 300 nm have been shown to rapidly accumulate in the spleen and to a great extent (exceeding 40% of the administered dose) [37,38]. The splenic arrest has been attributed to 'physical entrapment' of liposomes within the splenic sinusoids, possibly combined with potential activation of the complement system that (due to the large liposome size and greater available surface area) results in faster liposome identification by circulating macrophages [39]. For drug delivery applications, liposome sizes of ~ 100 nm in diameter are used. This is an empirically optimum value between acceptable circulation times and adequate aqueous encapsulated volume that is available for drug loading [25].

A negative charge on liposomes was shown in several studies to activate the complement system due to electrostatic adsorption of complement proteins on the liposome surface that would shorten their circulation times [32]. Although PEGylation significantly screens the charged liposome surface, even the anionic charge of the phosphate oxygen moiety of the membrane lipids right at the connection point of methoxy-PEG to the phospholipid headgroup was recently shown to play a role in complement activation [40]. Cationic liposomes also electrostatically interact with negatively charged serum proteins. In cases where liposomes are used in gene therapy for the delivery of short DNA/RNA fragments, lipoplexes may result in toxicities due to enhanced cytokine production that has been attributed to the enhanced uptake of lipoplexes by Kupffer cells localized on the hepatic endothelial walls followed by triggering of immune responses that result in the production of inflammatory cytokines [41]. A decrease in lipid membrane fluidity by incorporation of high Tg lipid components seems to also decrease the liposome clearance times, probably due to a decrease in the kinetics of adsorption of destabilizing proteins on the gel-phase lipid membranes [42].

Decoration of liposome surfaces with long chains such as PEG [43], monosialoganglioside or phosphatidylinositol [32], significantly increases the circulation times of liposomes. Presently, PEG is the most common polymer used in liposomes, mainly due to its relatively lower cost in production and its approval for clinical use [32,44]. In particular, for PEG, it is suggested that it provides a 'steric barrier' that interferes *in vivo* with the interactions of liposomes with approaching proteins, with neighboring cells and also with other liposomes. Specifically, PEGylation, depending on the extent of grafted polymers and their effective length [45], seems to delay the kinetics of protein adsorption for proteins of particular sizes and shapes [46]. However, in measurements where the total protein adsorption from plasma is monitored, the extent of PEGylation does not seem to play a significant role [47]. Secondly, the slower liposome clearance that is observed *in vivo* in PEGylated liposomes with high grafting PEG densities (leading to extended brush-like polymer chains) is suggested to occur due to the role of PEG as a steric barrier to approaching macrophages that identify adsorbed proteins [46]. Commercial formulations such as STEALTH® (ALZA) liposomes are PEGylated with relatively high surface grafting densities (5% mole for 2000 MW PEG). Thirdly, in a recent study, the role of PEGylation in preventing liposome aggregation during circulation *in vivo* and opposing the formation of larger aggregates that would be sequestered faster was clearly demonstrated [48].

However, in cases of the administration of PEGylated liposomal doxorubicin, exactly because of the long liposome

circulation times that allow for extended interactions with the complement proteins, complement activation has been connected to cases of hypersensitivity reactions (e.g., tachycardia, chest pain and dyspnia) [49,50].

Liposome interaction with the RES leads to the accumulation of carriers in normal organs such as the liver and spleen. The liver in particular is expected to significantly influence the maximum administered tolerated dose. It is expected that targeted liposomes due to their higher uptake by cancer cells will lead to lower required administered doses with improved killing efficacy and lower toxicities.

2.4 Immunolabeling of liposomes: coupling chemistries and their impact on circulation time and drug delivery

The conjugation of antibodies to the PEG chain terminus of PEGylated liposomes has been shown to influence the fate of liposomes *in vivo*. Different coupling chemistries for the conjugation of antibodies to liposomes have been extensively explored and thoroughly reviewed elsewhere [19,32]. Briefly, the major parameters that are considered in choosing an appropriate conjugation strategy are the coupling efficiency, the ability to achieve the required antibody density on the surface of liposomes, and the need to insure that the coupling methodology does not interfere with the retention of encapsulated therapeutics or other agents. The most popular chemistries for the attachment of antibodies to the free terminus of PEG chains, are: i) the method that employs a hydrazine group on the PEG chain that reacts with the oxidized carbohydrate groups of the oligosaccharide portion of the antibody; ii) the method where a maleimide group reacts with thiolated antibodies; iii) the pyridyldithioproprionate (PDP) group method which is almost the reverse version of the previous strategy. Upon activation of the PDP to produce a thiol, liposomes are coupled to antibodies that are previously labeled with maleimide moieties; and iv) the biotin-avidin method, where biotinylated PEG lipids are included into the liposome membrane and can be directly immunolabeled in a two-step approach where the addition of avidin is followed by the introduction of biotinylated antibodies. In another approach, biotinylated liposomes can be used for three-step targeting. In this approach, a bispecific (biotinylated) antibody that is slowly internalized by cells is introduced to bind to cancer cells, followed by avidin, and, then biotinylated liposomes are introduced and are expected to specifically target the prelabeled tumors [51].

The maleimide and PDP strategies are the most highly efficient coupling methods. In general, the coupling of antibodies directly on the liposome surface (on the phospholipid headgroup) is sterically obstructed if PEGylated lipids are present on the membrane. Also, a 'post-insertion' method for the formation of antibody-labeled lipids has been explored [52,53]. In this approach, antibody conjugation proceeds on PEG lipid micelles that are then introduced into the liposome suspension and are shown to become incorporated into preformed PEGylated liposomes. Is it not clear if this process is driven by preferential partition of antibody-labeled PEGylated lipids into the liposome bilayer or by an exchange between the PEGylated lipids that are initially incorporated into the bilayer membrane and the PEGylated lipids conjugated to antibodies that initially form micelles.

In vivo, complete-IgG-labeled liposomes, although exhibiting stronger binding efficacy, may result in short circulation times for liposomes, due to fast identification and uptake of the Fc fragment by circulating macrophages [33]. In particular, when complete antibodies are used, increasing densities of liposome grafted antibodies result in faster clearance of immunolabeled liposomes from circulation [21]. However, it is possible to optimize the antibody surface density so that adequately long circulation times are achieved without loss of targeting *in vivo*. In a different approach, antibody engineering has been employed for the development of antibody fragments (Fab′, scFv) that lack the Fc portion and which, when conjugated to the free termini of PEGylated lipids, seem to result in liposome circulation times that are identical to PEGylated non-immunolabeled liposomes [54]. In addition to tumor-accumulated doses, immunolabeled liposomes can improve drug bioavailability, at the cell level, as they exhibit substantially higher uptake by the targeted cancer cells compared with non-targeted liposomes *in vivo* [55]. Even non-internalized immunoliposomes that are bound to the surface of cancer cells may contribute to improved tumor retention over time. In particular, after clearance of circulating liposomes from the blood pool, the concentration gradient between the tumor interstitium and the blood pool is reversed, thus liposomes may diffuse back into the bloodstream resulting in depletion of liposomes from the tumor, unless liposomes are immunolabeled and therefore bound to cancer cells.

If the fraction of a targeting construct that is cell associated under conditions of excess target is taken as a measure of its reactivity against the target cell population, then a comparison of the reactivity of intact antibodies and immunoliposomes may be made. The reactivity of intact antibody is typically close to 100%, but that of immunoliposomes is in the range of 2 – 5%. For example, the reactivity of the anti-HER2/*neu* antibody, trastuzumab is close to 95%. However, the reactivity of trastuzumab immunoliposomes is 2.0 – 4.5% [20,56]. The drop in reactivity is probably not so much a result of steric hindrance or interference in binding due to neighboring surface PEG chains, but most likely a result of the disproportionally large volume of liposomes (100 nm in diameter compared with the characteristic length of 7 – 21 nm for antibodies), and correspondingly, the reduced diffusivity of these large constructs relative to antibodies. Of course, the advantage of liposome-mediated targeted

delivery is the very high drug-to carrier ratio that can be achieved compared with drug-labeled antibodies. This difference in 'loading' compensates for the lower binding efficacy of immunoliposomes and the advantage of immunoliposomal drug delivery becomes more apparent in cases of relatively low expression of molecular targets on the surface of cancer cells.

2.5 Possible complications and toxicity

The toxicity of immunoliposomes themselves is generally minimal. Some potential complications related to targeted immunoliposomes include – as noted above – immune responses such as the Human Antibody Mouse Antibody response [30], caused mainly due to the recognition of the murine Fc region of murine-derived antibodies. Although this does not lead to toxicity, such immune recognition leads to diversion of the chemotherapeutic agent, which could itself lead to toxicity. As noted above, immune recognition of the antibody as a foreign protein is being overcome by using lower molecular weight targeting moieties such as Fab′ and, scFv fragments that eliminate the Fc region of the targeting construct responsible for immune activation.

Crossreactivity with normal organs is another potential source of complications or toxicity. It is common for cancer cells to overexpress antigens/receptors that are relatively downregulated in healthy cells. However, although there is ongoing research to identify tumor-specific antigens for selective targeting of cancer cells, for many cases such receptors may not exist. *In vitro*, immunoliposomes exhibit high association with target cells that can be orders of magnitude greater than the association of non-targeted liposomes. It is this specificity of immunoliposomes, and their potential to result in lower toxicities that makes these structures promising for therapy. However, healthy cells that also express the targeting receptors (such as growth factor receptors that are common in targeted cancer therapy) will also associate with immunoliposomes, but to a significantly lower extent, depending on the receptor expression level. This lack of target uniqueness may contribute to toxicity *in vivo*.

Aside from accumulation due to crossreactivity, the major toxicity sites for liposomes are their common organ accumulation sites, which include the liver, spleen and bone marrow [57,58].

3. Use/findings in preclinical models

Immunoliposome studies have been performed, *in vitro*, to demonstrate cell targeting for subsequent *in vivo* applications, to evaluate different approaches to immunoliposome engineering, to assess immunoliposomal formulations of cytotoxic antitumor agents and to evaluate cell transfection strategies. The majority of work has been performed in tumor targeting.

3.1 Increased circulation time

Drugs that are rapidly cleared from the circulation require frequent administration to maintain circulating concentrations at a therapeutic level. Liposomal formulations of such rapidly clearing drugs have made it possible to reduce the frequency of administration. Immunoliposomal formulations have this benefit, as well as increased delivery to the target cells. Drummond *et al.* improved the pharmacodynamic and antitumor properties of a histone deacetylase inhibitor by encapsulating it in ErbB2 (HER2/*neu*)-targeted immunoliposomes [59]. By doing so they found that they could replace daily administration of the HDAC inhibitor LAQ824 with weekly administration of either the targeted or untargeted liposomal formulation. Studies of tumor-bearing mice 24 h after single treatment with liposomal LAQ824 (Ls-LAQ824) showed > 10% of the injected drug in the circulation, compared with undetectable blood levels 15 min after administration of LAQ824; the clearance rate *in vivo* was reduced by a factor of 164. Tumor uptake of Ls-LAQ824 and the immunoliposomal, ErbB2-targeted (using the F5 antibody) formulation (ILs-LAQ824) was > 3% of the injected drug per gram of tumor. This yielded a 5- to 10-fold increase in tumor histone acetylation over that obtained following free LAQ824 and resulting reduced levels of tumor ErbB2 mRNA. The immunoliposomes were generated using the micellar insertion (after-loading) strategy [60,61]. In these studies, the HDAC inhibitor was loaded into liposomes using an after-loading or 'remote-loading' technique [62,63]. Previous methods for liposome drug loading have been variable, with excellent results for liposomal anthracyclines [63], but more problematic efficiency and/or stability with other drugs [25]. In this technique, liposomes are formed with an entrapped solution containing poorly permeable ionic species such as substituted ammonium salts of poly(phosphate) or sucrose octasulfate and then transferred to a buffered solution containing the drug. The resulting transmembrane potential induces spontaneous accumulation of weakly basic drugs into the liposome interior, producing packing, gelation or crystallization of drug within the liposome. The technique enables highly robust encapsulation of a number of chemical classes into stabilized liposomes including epirubicin, vinca alkaloids, ellipticines and camptothecins; the resulting constructs are notable for extremely efficient loading (~ 100% of added drug is encapsulated), very high drug yields (10^4 – 10^5 drug molecules per particle) and marked stability *in vivo* [54,64,65].

3.2 Interstitial versus intracellular localization

Evidence obtained over a number of *in vivo* studies suggests that antibody conjugation on liposomes does not necessarily lead to increased tumor accumulation over antibody-free liposomes. The accumulation of immunoliposomes in tumors is postulated to be governed by the same principles that lead to liposomal tumor accumulation, extravasation

from tumor blood vessels followed by passive diffusion within the tumor interstitium. The improved antitumor activity of immunoliposomes over liposomes can be explained by the differences in intratumoral localization or internalization [66-68]. Confirmation of this is provided by the studies of Mamot *et al.* [54] and Kirpotin *et al.* [55]. In the studies by Mamot and co-workers, liposomes were loaded with the water-soluble fluorescent dye ADS645WS, with or without antibody fragments, and administered in tumor-bearing mice as a single intravenous injection. At 24 h post-injection, tumors were removed, disaggregated and washed for analysis. Confocal microscopy of the harvested tumor cells showed that anti-EGFR immunoliposomes had accumulated throughout the cytoplasm in a pattern consistent with receptor-mediated endocytosis (92% of analyzed cells). Liposomes lacking the antibody fragment showed only minimal binding or uptake, which was not significantly different from background (< 5% of analyzed cells). Quantitative analysis by flow cytometry demonstrated a sixfold greater uptake of the immunoliposomes over non-targeted immunoliposomes. In terms of overall tumor accumulation, expressed as percentage of the injected dose per gram of tumor, both liposomes and immunoliposomes showed the same high concentrations (up to 15% injected does per gram) of doxorubicin in tumors. The antitumor effects of the immunoliposomal construct was significantly ($p < 0.001 – 0.003$) superior to both the free drug and the liposomal drug [54]. Consistent with these results, the studies reported by Kirpotin *et al.*, demonstrated that anti-HER2/*neu* immunoliposomes (made using Fab′ fragments or single-chain variable region (scFv) construct on the distal end of PEG) did not alter the biodistribution relative to PEG liposomes in terms of pharmacokinetics and tumor localization in a BT-474, HER2/*neu* overexpressing xenograft mouse model. The intracellular accumulation of liposomes was shown by colloidal gold microscopy and flow cytometry to occur with immunoliposomes, but not with liposomes; flow cytometry demonstrated a sixfold greater intracellular accumulation of immunoliposomes relative to liposomes. PEG liposomes were found predominantly in the extraceulluar tumor stroma or in macrophages. A similar pattern of stromal accumulation without cancer cell internalization was observed for anti-HER2/*neu* immunoliposomes in non-HER2/*neu* overexpressing breast cancer (MCF7) xenografts. These studies are consistent with a mechanism for immunoliposomal delivery involving two phases. In the first, sterically stabilized liposomes slowly accumulate in tumor tissue, reaching high tumor levels due to the enhanced permeability and retention effect. An antibody–antigen interaction is not required for this step and does not seem to facilitate it. In the second phase, non-targeted liposomes remain in the interstitial space and are subjected to decomposition, degradation or phagocytosis, with eventual release of the drug. In contrast, immunoliposomes bind to and internalize in tumor cells via ligand–receptor interactions [54].

3.3 Internalizing versus non-internalizing immunoliposomes

Based on the discussion in Section 3.2, it could be concluded that immunoliposomes will exhibit a therapeutic advantage over untargeted liposomes only if the antibody–liposome construct is internalizing. Studies by Sapra and Allen [69] suggest that this is not necessarily the case. Using sterically stabilized (PEGylated) immunoliposomes (SILs) against two different B cell lymphoma associated antigens, CD19 (internalizing) and CD20 (non-internalizing or very slowly internalizing), they showed that therapeutic efficacy also depended on the encapsulated chemotherapeutic agent. Treatment of murine B cell lymphoma with anti-CD20-vincristine (VCR)-loaded immunoliposomes led to significantly better therapeutic outcome (49 day mean survival) compared with non-targeted liposomal VCR (32 day mean survival), free VCR (39 day mean survival), or anti-CD20-doxorubicin loaded immunoliposomes (34 day mean survival), but not better than anti-CD19-VCR loaded immunoliposomes (66 day mean survival). The anti-CD19-doxorubicin-immunoliposome yielded a 46-day mean survival time. Treatment with a mixture of anti-CD19 and anti-CD20 doxorubicin immunoliposomes yielded a mean survival time of 49 days – statistically the same as the anti-CD19-doxorubicin immunoliposome. In contrast, the combination of anti-CD19 and anti-CD20 immunoliposomes for VCR delivery yielded 70% (5/7) cure (> 150-day survival) compared with 29% (2/7) and 42% (3/7) cure for anti-CD20 and anti-CD19 immunoliposomes, respectively. Studies performed *in vitro* demonstrated that anti-CD19 immunoliposomes internalized rapidly at 37°C, but not at 4°C; no specific internalization was observed with anti-CD20 immunoliposomes.

3.4 The role of drug release rate in immunoliposome efficacy and toxicity

The observations described in the previous section can be explained, in part, by the different rates of release of VCR compared with doxorubicin. Although the difference in efficacy may also relate to expression levels of CD19 versus CD20 and their uniformity across B lymphoma cell populations, results described by Sapra *et al.* and Allen *et al.* [69,70] suggest that the difference in release rates is the dominant explanation. The study by Sapra compared the efficacy of doxorubicin and VCR loaded anti-CD19 SILs, *in vitro* and *in vivo*. Two anti-CD19 SIL constructs were examined: one using intact anti-CD19 IgG2a and one using the Fab′ fragment. The latter is monovalent and free of the immunogenic Fc portion of the intact antibody. Both constructs showed greater efficacy, *in vitro* and *in vivo* against B lymphoma than non-targeted liposomes. The intact antibody construct cleared more rapidly than the Fab′ construct, which demonstrated a pharmacokinetic profile similar to that of non-targeted (sterically stabilized) liposomes. The leakage half-times for VCR and doxorubicin

were unaffected by antibody or antibody fragment conjugation, and were 6.8 and 90 h, respectively. VCR-loaded SILs were superior to those loaded with doxorubicin. Fab′ SIL gave a better therapeutic outcome than the intact antibody-conjugated SIL for doxorubicin, but not for VCR. The longer residence time of Fab′ SIL was considered important relative to the slow release rate of doxorubicin for the results obtained with doxorubicin, and the rapid release of VCR made residence time in circulation less relevant so that both intact antibody and Fab′ SIL exhibited adequate circulation time relative to the release kinetics of VCR.

In a study designed to specifically examine the role of drug release rate on SIL efficacy and toxicity, Allen *et al.* [70], developed targeted and untargeted sterically stabilized liposome constructs exhibiting different rates of doxorubicin release to determine whether the toxicity of a doxorubicin liposome with intermediate release rate can be overcome using SIL. Therapeutic studies were performed in the human B cell lymphoma xenograft mouse model. Phosphatidylcholine/cholesterol liposomal formulations of doxorubicin having fast, intermediate and slow drug release were prepared by altering the fatty acyl chain length or degree of saturation of the phosphatidylcholine component. The clearance rate of the different liposomal constructs was confirmed to be similar by measuring liposomal lipid clearance kinetics. The release half-time of doxorubicin for the different constructs varied from 2 to 315 h. The clearance rate of doxorubicin from the circulation was found to depend upon the construct doxorubicin release rate. No toxicity was observed using liposomes with the slowest drug release rates; the therapeutic activity was superior to other constructs when targeted with anti-CD19. Liposomes with the most rapid release rate were also non-toxic, but did not exhibit therapeutic efficacy. Constructs with intermediate drug release rates had variable toxicity and efficacy. The toxicity of these constructs could be reduced or eliminated by targeting; the efficacy of targeted intermediate clearance rate constructs was intermediate between those for liposomes with the fastest and slowest drug release rates.

3.5 The impact of target cell antigen density

The relationship between the therapeutic ability of immunoliposomes and target cell antigen density was investigated by Hosokawa *et al.* [68]. Liposomes were loaded with doxorubicin using the pH gradient method [71,72], thiolated $F(ab')_2$ fragments of GAH antibody and then PEG were conjugated with the liposomes through thioether linkages [73]. The growth inhibition of 10 different cell lines with different target antigen densities (as evaluated by flow cytometry) was tested *in vitro* and *in vivo* using each cell line in a subrenal capsule nude mouse model [74]. Each cell line was also tested against an antibody-free liposomal formulation. After correcting for the different doxorubicin sensitivity of each cell line, tumor growth inhibition was minimally dependent upon cell line antigen density once the antigen density exceeded 4×10^4 sites/cell. In contrast, liposomes not conjugated to the antibody showed a tumor growth inhibition that was related to the doxorubicin sensitivity.

3.6 Post-insertion loading and layered surface functionalities

As noted earlier, antibody conjugation of immunoliposomes can be accomplished by conjugation of the antibody to the phospholipid bilayer or on the distal end of PEG molecules. Both approaches require that the antibody be incorporated into the liposome during liposome formation. Iden and Allen described a 'post-insertion' technique which enables antibody conjugation to already formed liposomes [31,52,53,60,61,75]. This approach involves the incubation of micelles that have previously had antibody conjugated onto the distal end of PEG chains. The incubation of such antibody-labeled micelles with intact liposomes for 1 h at 60°C leads to PEG exchange and transfer of antibody-conjugated PEG chains into the formed liposome, with negligible leakage of liposome contents. This approach has been used to convert commercially available liposomal doxorubicin (Doxil®; ALZA Corp.) into immunoliposomal doxorubicin. In an extensive series of binding and characterization assays, no difference between these immunoliposomes formed by conventional approaches and the post-insertion approach was observed [53]. Using the same post-insertion approach on commercially available, doxorubicin-loaded (Doxil) liposomes, Lukyanov *et al.* [76] used longer (PEG_{3400}) PEG chains to form the antibody-coated micelles so that the PEG_{2000} chains on Doxil would not interfere with the antigen binding of the antibody. In this study, the authors noted a 20% loss of doxorubicin from Doxil, but found that this loss was more than compensated for by the increased delivery of doxorubicin associated with the specific binding of immunoliposomes. The authors noted that the binding of liposomes to target cells on a per-antibody basis is reduced approximately 15-fold compared to the original non-modified antibody, most likely due to steric hindrance caused by the liposome surface. On the other hand, on a per liposome basis, the binding constant is even higher than that of an individual antibody by approximately fivefold due to a multipoint binding of the liposome to the target cell [77].

As a further step, immunoliposomes have been suggested with 'multiple layers' of surface functionalities resulting in different responses depending on the exposed surface layer [78]. A layer of long PEG chains, which is necessary for long blood circulation times, is designed to be cleaved first, and these liposomes are suggested to be combined with surface-grafted PEG chains of shorter length with targeting ligands on their free termini that will be exposed after localization close to the target cells, and that are also cleavable (in a later stage) to eventually expose a liposomal

surface coated with fusogenic peptides in order to enhance cell permeation of the delivered encapsulated contents by liposomes.

3.7 Gene delivery

Immunoliposomal formulations have also been developed to accomplish gene transfer with high selectivity for the targeted cells [79,80]. Tan *et al.* [81] have described a method of conjugating liposomes with plasmid DNA-bound antibody by incubating the pDNA, the antibody and the liposome. The resulting attachment is non-covalent and adequate for cell transfection *in vitro*. The approach has the advantage of avoiding conjugation – potential for damaging antigen-binding sites. The resulting carriers are ~ 500 nm in diameter. The relatively large size could potentially limit the blood circulation times of these carriers *in vivo*. Antibodies against the transferrin receptor and also against E-selectin, expressed on activated or inflamed endothelial cells were used to transfect endothelial cells.

scFv fragments have also been used to target liposomes for gene therapy [82-84]. The approach entailed covalently conjugating the scFv to the liposome via a cysteine at the 3′ end of the protein and a maleimide group on the PEGylated liposome. The scFv was reactive against the transferrin receptor. The transfection rate of this construct (TsPLP) *in vitro* and *in vivo* was compared with a liposome–DNA complex (lipoplex) that was unPEGylated and untargeted (LP), a PEG-coated LP (PLP), and a targeted unPEGylated LP (TsLP). The transfection efficiency of TsLP was greater than TsPLP and LP, which were similar but greater than PLP *in vitro*. However, the order of transfection efficiency *in vivo* after systemic administration via the tail vein was TsPLP > TsLP > LP or PLP, with TsPLP-mediated exogenous gene expression in tumor being twofold higher than when mediated by TsLP [83]. The TsPLP construct has also been used to modulate gene expression using antisense oligonucleotides [85,86] and short interfering RNA [87].

3.8 Prodrug and enzyme inhibitor delivery

One approach to targeted tumor cell killing – the antibody-directed enzyme prodrug therapy (ADEPT) strategy – was implemented using immunoliposomes by Koning *et al.* [88]. The ADEPT strategy involves delivery of an enzyme to targeted cells via its conjugation to antibody against the target cells (or by engineering an enzyme–antibody fusion protein [89]), followed by a prodrug that becomes toxic only after it has been acted upon by the enzyme [90]. In a variation of this strategy, Koning and co-workers demonstrated immunoliposome-mediated, targeted transfer of the lipophilic prodrug dipalmitoyl-fluoro-deoxyuridine (FUdR-dP) to the membrane of the rat colon adenocarcinoma cell line, CC531. Subsequent internalization of cell membrane-associated FUdR-dP by constitutive endocytosis or pinocytosis led to hydrolysis and intracellular delivery of the FUdR. A dipalmitoylated derivative of FUdR was synthesized and FUdR-dP was incorporated into the liposome bilayer. A murine IgG_1 antibody (CC52) against rat colon adenocarcinoma cells was coupled either directly to the liposome surface or to the distal end of the PEG chains via a hydrazide-PEG-DSPE [3,91]. Immunoliposomes delivered a 30-fold higher level of FUdR-dP (as measured by counting tritium-labeled FUdR) to CC531 cells after 3 h incubation, compared with liposomes without antibody. As demonstrated by colloidal gold microscopy, immunoliposome internalization was not necessary for the transfer of FUdR-dP from the liposome bilayer to the cell membrane. Neither the rate of transfer of FUdR-dP nor the hydrolysis of cell-associated FUdR-dP was reduced by the presence of 4 mol% PEG_{2000} on the surface of bilayer-antibody coupled immunoliposomes or by the coupling of antibody to the distal end of the PEG chains (CC52-Hz-PEG). However, the presence of PEG did reduce the total amount of FUdR delivered to cells – this was thought to be due to a lower apparent antibody density arising from PEGylation. Despite this, PEG immunoliposomes were still much more efficient at delivering FUdR than liposomes without antibody or free FUdR.

Liposomal formulations of drugs that are substrates for P-glycoprotein have demonstrated the ability to overcome the multi-drug resistance phenotype. Huwyler *et al.* [10] demonstrated that by using immunoliposomes loaded with digoxin – a substrate of P-glycoprotein – it was possible to deliver 25-times more of the drug intracellularly than was possible with free digoxin.

3.9 Contrast agent delivery for imaging

Immunoliposomes have also been used as carriers of contrast agents for MRI [92] and also to deliver radionuclides for diagnostic radionuclide imaging or targeted radionuclide therapy [93-100]. In the case of MRI, liposomal contrast agent formulations have been developed by passively encapsulating contrast metals that have been chelated into a soluble chelator [101]. Alternatively chelating moieties are modified with a hydrophobic group such as phosphatidyl ethanolamine or steraliamine and then anchored into the liposomal surface during or after liposome preparation [102]. Another approach that increases the amount of metal contrast (e.g., Gd) agent that can be loaded onto liposomes involves the use of polychelating amphiphilic polymers (PAP). Such polymers can be loaded with multiple metal atoms via the main chain-attached multiple chelating groups the metal chelate-loaded PAP can then be incorporated into the liposomal bilayer via the lipid residue on one terminus. The advantage of this approach is that a single lipid anchor can carry a polymer molecule with multiple metal-loaded chelating side groups on the liposome surface, thereby increasing the metal contrast agent delivered per carrier [103,104]. Taking advantage of this approach, Erdogan and co-workers prepared Gd-loaded PAP-containing liposomes and additionally modified them with the

2C5 antibody against a cancer-associated nucleosome antigen that is released by apoptotically dying cancer cells and subsequently expressed on the surface of neighboring cancer cells [105,106], and demonstrated the ability of such liposomes to specifically bind to tumor cells *in vitro* and bring increased quantities of Gd to tumor [103].

3.10 Radionuclide delivery for imaging and therapy

The same antinucleosome antibody and multi-DTPA liposomal construct has also been used to image liposomes loaded with the gamma-emitting radionuclide indium-111 with a gamma camera. Targeted delivery of therapeutic radionuclides using immunoliposomes is only now emerging, and has been used in cases when the conjugation chemistry of a radioactive atom to an antibody ligand does not possess adequate stability [107]. Liposomes have also been investigated in efforts to retain the alpha-emitting daughters of nanogenerator radionuclides [56,108,109].

In diagnostic radionuclide imaging, internalization of diagnostic radionuclides – in particular radiometals – into target cells will lead to prolonged retention of the radionuclide at the tumor site, and clearance from other sites will provide increased contrast over time. In MRI, 'smart' imaging agents have been produced that require intracellular or pericellular localization for signal enhancement. Both scenarios would benefit from the use of targeted liposomes.

3.11 Neovasculature and endothelial cell targeting

Antibody-free PEG liposomes that are loaded with cytotoxic drugs accumulate in tumors and exhibit antitumor effects because of their long circulation half-life and the enhanced vascular tumor permeability [110]. An alternative strategy to achieving an antitumor effect would be to target the neovasculature that supplies tumors with oxygen and nutrients rather than targeting the tumor directly [111,112]. Völkel and co-workers investigated targeting of immunoliposomes to endothelial cells using a scFv′ of an antibody against human endoglin (CD105) – an antigen that is upregulated on proliferating endothelial cells associated with tumor neovascularization. scFv immunoliposomes showed rapid and strong binding to human endoglin-expressing endothelial cells, but no binding was observed against various endoglin-negative cell lines and blood lymphocytes. The immunoliposomes showed serum and plasma stability *in vitro* at 37°C; doxorubicin-loaded scFv immunoliposomes showed differential cytotoxicity against endoglin-expressing cell lines.

Using both tumor targeting and endothelial cell-targeting doxorubicin-loaded SILs [113] were used to examine whether the combination of tumor cell and endothelial cell targeting constructs would yield better therapeutic results than each construct individually. SILs against the disialoganglioside receptor GD_2 were used to target neuroblastoma cells directly; SILs coupled to NGR peptides that target the angiogenic endothelial cell marker aminopeptidase N were used to target tumor neovasculature. Antitumor effects were significantly improved in highly aggressive neuroblastoma-bearing mouse models chosen to mimic the metastatic spread observed in patients with advanced-stage neuroblastoma. The tumor-targeting SIL inhibited tumor growth and led to a significant increase in lifespan (7/8 mice alive > 120 days) when treatment was started 24 h after intravenous tumor cell inoculation. A delay in the start of treatment substantially reduced the treatment efficacy so that 1/8 mice showed long-term survival (> 120 days) and no mice showed long-term survival when treatment was initiated 5 and 10 days post tumor inoculation, respectively. A statistically significant improvement in survival ($p < 0.006$ compared with tumor-targeting SIL, and $p = 0.0009$ compared with antineovasculature SIL) was observed when the antitumor SIL administered at 5 days post tumor cell inoculation was combined with the antineovasculature SIL administered at 19 days. The average body weight of mice receiving combined treatment was not reduced, indicating that the side effects of the combination treatment were minimal.

3.12 Intratumoral distribution

The spatial distribution of tumor-specific antibodies within solid tumors is influenced by a binding site barrier [114] and by interstitial fluid pressure within the tumor [115]. Kostarelos *et al.* examined the penetration of untargeted liposomes into prostate cancer cell spheroids and showed that penetration is highly dependent upon the surface charge, composition and size of the liposomes [116]. Surface interactions between strongly cationic liposomes and tumor cells led to an electrostatically derived barrier, which inhibited cationic liposome penetration. Electrostatically neutral liposomes with fluid bilayers or liposomes with a relatively low charge molar ratio and enhanced fusogenicity provided improved penetration and distribution within spheroids. Liposomal constructs with a high overall affinity generally yielded highly non-uniform spheroid penetration, and *vice versa*, constructs with good spheroid uniformity showed reduced spheroid accumulation.

Davies *et al.* examined the impact of external beam radiation on the distribution of liposomal doxorubicin in a murine osteosarcoma xenograft model. Radiotherapy (8 Gy, single fraction) increased the tumor uptake of doxorubicin by a factor of 2 to 4. The drug penetrated further from the tumor vessel and was found in the central portions of the xenograft [117].

Although not experimentally demonstrated, it may be anticipated that immunoliposomes will present an additional barrier to liposomal penetration associated with binding and internalization to tumor cells at the periphery of the tumor.

4. Use/findings in clinical studies

A Phase I clinical study has been reported for PEGylated (5000 MW) immunoliposomes with encapsulated doxorubicin [118]. Immunoliposomes (MCC-465, 143 nm in diameter) were conjugated to $F(ab')_2$ fragments of the human monoclonal antibody GAH that has been shown to positively react with the great majority of cancerous stomach tissues. MCC-465 were administered to patients with metastatic or recurrent stomach cancer as a 1 h infusion every 3 weeks for up to six cycles, and the dose-limiting toxicities were myelosuppression and appetite loss. The pharmacokinetics of MCC-465 were different from free doxorubicin, but comparable to Doxil. The maximum tolerated dose of MCC-465 (45.5 mg/m^2) was similar to that of Doxil (50 mg/m^2) for the study conditions.

5. Conclusions

Targeted liposomes are drug delivery constructs that result from the integration of several distinct technologies. Progress in the development of targeted liposomes for cancer therapy has, therefore, been heavily dependent upon progress in each of the related technologies. The first question in the development of such constructs: where and how to best place the targeting moiety, has been addressed. Starting with the sterically stabilized, long circulating (i.e., PEGylated) liposomes, investigators have found that the optimal configuration for targeting *in vivo* is provided by placing the antibody or related structure on the free termini of PEGylated lipids. Successful targeting *in vivo* will also depend upon the surface charge of the liposome and on the length of the PEG chain and on the PEG density in the liposomal bilayer. Cationic liposomes associate with cells following the endosomal entry that is triggered by electrostatic attraction to the locally negatively charged plasma membranes. In several cases, these liposomes are shown to directly fuse with the cell's endosomal membrane, making them ideal for the delivery of anionic macromolecules such as in gene therapy applications [119-121]. However, the overall cationic charge may trigger undesirable interactions with other cells *in vivo* (such as Kupffer cells), leading to the activation of immune responses and toxicities [41]. Anionic liposomes may cause complement activation and result in hypersensitivity reactions. Zwitterionic liposomes are preferred, as they are less likely to interact with cells in circulation and, therefore, more likely to reach their target cell population. PEGylation seems to strongly affect not only the interactions of liposomes with circulating proteins, but also with cells, and other approaching liposomes. Empirical work has shown that PEG chains with a molecular weight in the range of 2000 to 5000 are optimal when included at densities in the 2 – 5 mole% range corresponding to extended polymer 'brushes' on the liposome surface.

Studies have shown no difference between targeted and untargeted liposomes in overall tumor concentration following intravenous injection. Targeted liposomes provide a delivery advantage over untargeted liposomes, not because of increased localization to tumor sites, but because of increased interaction with the target cell population once localized to the tumor site. The increased interaction can take on the form of fusion with the cellular membrane or internalization by endocytosis. To the extent that the spatial distribution of targeted liposomes within a solid tumor may become more non-uniform than has been found for untargeted liposomes, this may be a drawback. However, systematic comparisons of the spatial distribution in tumors of targeted versus untargeted liposomes have yet to be performed.

Developments in antibody and protein engineering have also had an impact on targeting liposomal constructs. Initial constructs employed intact antibodies or fragments obtained by the enzymatic digestion of intact antibodies as the targeting moieties. The advent of genetically engineered recombinant proteins has made it possible to use engineered scFv constructs that may be produced on a large scale.

Targeted liposomes have been used in diagnostics to deliver contrast agents and radionuclides for MRI and nuclear medicine imaging, respectively. They have been used in gene therapy to deliver a variety of gene expression modifiers, including plasmids, antisense oligonucleotides and short interfering RNA. The majority of reported studies have been in the area of chemotherapy delivery. Their use in radionuclide and chemo- and radio-sensitizer delivery is just emerging. Multifunctional liposomes containing 'layered functionalities' could potentially be the future direction in targeted liposome-based therapy.

Targeted liposomes have only recently emerged as viable candidates for tumor imaging and therapy. Clinical studies using targeted liposomes are still at early stages. The diversity of preclinical studies and applications that may be found in the literature using such constructs reflects their flexibility and their potential future use in cancer diagnosis and therapy.

6. Expert opinion

6.1 Present state of targeted liposomes

The advantage of targeted liposomes in delivering therapeutic agents to cancer cells, *in vivo*, has been amply demonstrated. Surprisingly, they are still at the early stages of entering the clinic. This may reflect the emphasis in ongoing preclinical development as new targeting technologies and drug delivery strategies are devised. In addition, the emphasis on clinical investigation and regulatory approval of simpler, untargeted or passively targeted, liposomes may be drawing the resources for clinical implementation away from targeted liposomes.

Targeted liposomes also present scientific and developmental challenges that are still being addressed. The fundamental properties of unconjugated liposomes (e.g., their size, surface charge, PEGylation and membrane fluidity) that largely determine their fate *in vivo* have been identified, and their effect on liposome biodistributions and pharmacokinetics is studied and understood to a great extent. However, targeted liposomes, due to the presence of surface-conjugated ligands (including proteins or protein fragments) introduce additional complexities on their interactions with the biological milieu that require further evaluation. A greater understanding of these interactions will result in better design at the molecular and supramolecular level of targeted liposomes for maximum target specificity and reduced toxicity.

6.2 Where will targeted liposomes be most prevalent; what need will targeted liposomes satisfy?

Given the preclinical findings, it is close to inevitable that targeted liposomes will eventually be found in routine clinical use. Initial applications will likely be in the delivery of agents or agent combinations that critically depend upon efficient intracellular localization for antitumor efficacy, such as chemotherapeutic compounds and radionuclide emitters of short-range particles. As our understanding of cellular signaling and intracellular trafficking improves, one can envision targeted liposomes that, in addition to cell targeting, are engineered to concentrate in a particular subcellular compartment. Such engineering will be implemented to improve therapeutic targeting or to alter cellular function. Advances in protein engineering for custom-made, cost-affordable ligands, combined with the development of targeted liposome structures with 'layered functionalities' that become activated depending on their immediate environment, should result in targeted liposomes with improved *in vivo* performance. Targeted liposomes are particularly, if not uniquely, suited to such engineering, as they provide an extended biologically compatible platform that can incorporate the molecular machinery to achieve these objectives. The trend towards individualized patient treatment, wherein therapy is chosen based upon the expression of particular markers in a given patient, will provide a compelling rationale for the movement towards targeted liposomes. In the future, one may envision a modular drug delivery paradigm in which a liposomally encapsulated therapeutic is conjugated in real-time or within a short-time period to the appropriate targeting ligand for administration to a patient whose tumor expresses the conjugate antigens or is driven by a particular signaling pathway.

6.3 What is the major hindrance for approval of targeted liposomes, and how will it be overcome?

The potential engineering and design advantages of targeted liposomes noted above also make approval more challenging. As vehicles for drug delivery, targeted liposomes are complex constructs with multiple distinct and highly interdependent structures. A balance must be achieved between preserving a favorable circulation half-life, ensuring the reactivity of the targeting ligand and preserving retention of the payload. These functions are interdependent, and optimization of one will impact the others. Correspondingly, regulatory approval requires an understanding of the potential toxicity of each component and possibly the need to independently evaluate the toxicity of each component, in case the construct is catabolized or disassembled *in vivo*. Under such a regulatory constraint, approval would be easiest for a targeted liposomal construct employing an already approved antibody on an already approved untargeted liposome delivering an already approved agent.

The need to demonstrate large-scale good manufacturing practice (GMP) to obtain approval for commercial use is also a hindrance. The large-scale production of untargeted liposomes that are of a defined size distribution and a consistent composition has been largely solved. The large-scale production of antibodies has also been resolved. As these are biologically derived drugs, each will present with its own GMP scale-up challenge. A GMP conjugation methodology, with associated quality control that can be scaled up must be established for targeted liposomes. The complexity of the issues discussed above as well as the cost aspects will be major hurdles for clinical application of immunoliposomes.

As noted above, targeted liposomes already build upon FDA-approved structures, and despite the hurdles outlined above, this advantage, and the widespread clinical familiarity with antibodies and liposomes, will help move targeted liposomes to approval faster than other analogous artificially engineered constructs. The scale-up and GMP production challenges, although significant, are engineering challenges that will be met with further research and development. Investment in such efforts will have to come primarily from industry. In light of this, the present emphasis on, and already existing pipeline of, passively targeted liposomes may be the major hindrance to the approval of targeted liposomes. It may be necessary that these simpler but less effective constructs gain widespread acceptance and approval before the more complex, targeted liposomes become commercially available for human use.

Declaration of interest

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