Liposomes for Intravenous Drug Targeting: Design and Applications

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Abstract: Drug targeting with liposomes has been studied for over 25 years and has demonstrated its value in clinical practice. This mini review offers an overview of the design and application of liposomes for i.v. drug targeting. Two approaches are outlined: passive and active targeting. The former approach is based on liposomes with prolonged circulation and selective target localization properties, while in the latter approach specific targeting ligands are coupled to the liposome surface in order to achieve enhanced interaction with target cell membranes.

I. INTRODUCTION

Successful treatment of life-threatening and chronic diseases by intravenous (i.v.) administration of therapeutic agents often involves relatively high and frequent dosing. Due to rapid elimination or a large volume of distribution, many drugs poorly accumulate at target sites while large amounts are wasted or unintendedly localize at healthy tissue sites. As a consequence, a systemic treatment approach is frequently limited by toxicity and therefore characterized by a low benefit/risk ratio. For decades research has been focusing on the possibility of encapsulating drugs in carrier vehicles that take their drug load specifically to the target sites in the body, meanwhile protecting it against rapid degradation and/or elimination and preventing undesired localization in non-diseased organs ('drug targeting').

Among a variety of drug carrier systems, liposomes (small, biocompatible lipid-bilayer vesicles, see Fig. (**1**)) have been investigated extensively and the preclinical and clinical findings have demonstrated their versatility to accommodate a large variety of drugs for a wide range of therapies [1,2]. The attraction of liposomes as drug carrier system was initially based on expectations of good biocompatibility, low toxicity and a lack of immune system activation or suppression. These assumptions were based on the fact that liposomes are typically composed of natural lipids that form bilayers with structural resemblance to cell membranes. Although reality turned out to be more complex, the approval of several liposome-based pharmaceutical products in the last decade illustrates a growing acceptance of the liposomal delivery system as an important parenteral drug formulation.

A breakthrough in the liposome research field has been the finding that i.v. injected liposomes have the ability to spontaneously localize into sites of pathology ('passive targeting') [3,4]. It was this finding that facilitated clinical development of liposomes for therapeutic purposes. In certain cases however, it remains desirable to couple a specific targeting ligand to the surface of the liposomes to achieve receptor-mediated target cell binding ('active targeting') [3]. This mini review aims at providing the reader with a condensed overview of the design and application of liposomes for i.v. drug targeting. Both the passive and active strategy will be discussed.

II. PASSIVE TARGETING

One of the important barriers limiting the application of liposomes for intravenous drug targeting has been a short blood circulation time resulting from rapid and efficient recognition and removal from blood by cells of the mononuclear phagocyte system (MPS), particularly those in the liver and spleen. This immune system's first line of defense consists of macrophages specialized in nonspecific elimination (phagocytosis) of all exogenous material in the circulation, including liposome particles. Plasma proteins like antibodies and other so-called opsonins recognize and adhere to liposomal bilayers, provoking the uptake of liposomes by MPS-macrophages [5,6]. This MPS-directed behavior of liposomes has been successfully exploited to achieve selective delivery of antimicrobials in models of intracellular infections caused by pathogens localized in MPS cells [7]. However, in the majority of diseases, the rapid sequestration by the MPS often eliminates the intended beneficial effects and moreover can pose considerable risk of toxicity to these cells [8,9].

Therefore, the introduction of liposomes exhibiting prolonged circulation by virtue of their capability to oppose rapid MPS uptake represents a milestone in liposomal drug delivery research. These newer forms of liposomes (referred to as long-circulating liposomes (LCL)) are actively being investigated worldwide and the results have substantially expanded the role of liposomes in developing new therapeutics (see Table 2). The key factor responsible for the increased interest in liposome drug delivery is the observation that LCL spontaneously and selectively accumulate at sites of enhanced vascular permeability that are

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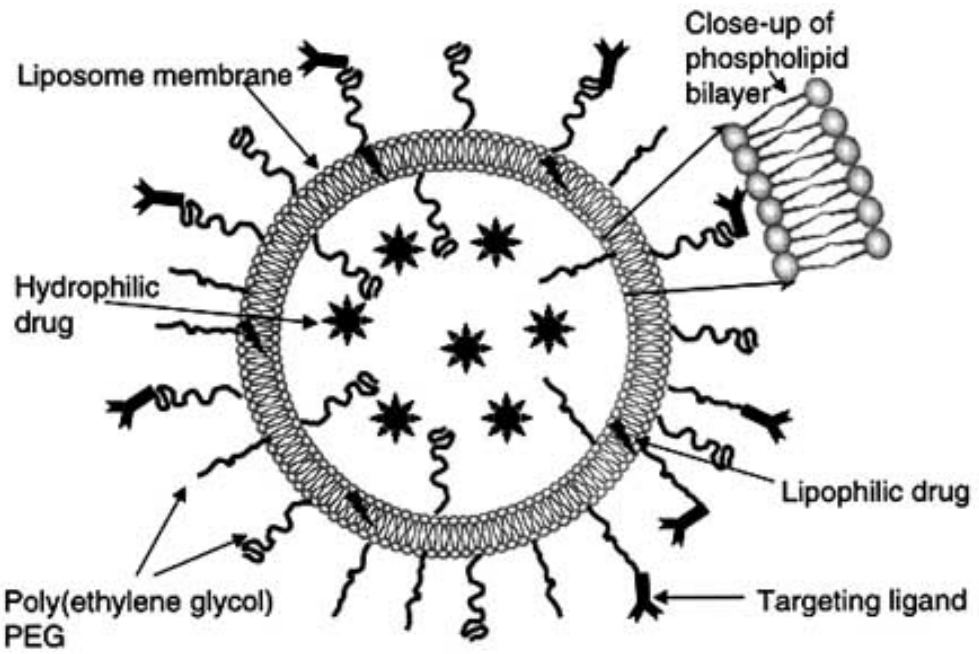

Fig. (1). Schematic drawing of a PEGylated liposome with targeting ligands and incorporated drug.

fortunately present in diseased tissues like tumors and areas of infection and inflammation [3,4]. This phenomenon is usually referred to as 'passive targeting'. The explanation for the fascinating passive targeting effect is straightforward: since LCL are generally smaller than the 'pores' that appear in the endothelial linings at pathological sites, their prolonged circulation property increases the chance that they extravasate into the extravascular space. Retention of LCL at these sites will lead to accumulation and the creation of a relatively high local drug concentration [10].

The ability to passively target drugs to extravascular sites of pathology via LCL is dependent on a combination of:

1. Prolonged blood circulation, providing ample opportunities to encounter the region of disease.

2. Adequate access to the pathological tissue and target cells therein.

3. Ability of the LCL to interact with target cells and to deliver the encapsulated drug in an active form.

Each aspect is briefly discussed below.

II.1 Prolonged Circulation

Qualitatively, the mechanism behind the approaches taken to enhance the residence time of liposomes in the blood compartment is generally explained by reduction of the adsorption of various blood components onto the liposomal surfaces (e.g., adsorption of proteins interacting with one or more receptors on the MPS-macrophage cell surface, a process termed opsonization). Thus liposome types able to resist rapid opsonization are likely to show prolonged blood circulation times [11,12].

One of the first major advances in prolonging blood residence was made possible through careful studies of the dependence of MPS-uptake on liposomal lipid composition [13]. These studies led to the findings that small (i.e., less than about 100 nm in diameter), neutral and rigid (i.e., composed of fully saturated lipids and a high cholesterol content) liposomes can exhibit prolonged circulation, but only at relatively high lipid doses [13,14]. This success in generating LCL has been exploited for development of the marketed liposomal formulations of the anticancer drug daunorubicin and the antifungal drug amphotericin B [15,16]. (Table 1). It is believed that the use of highly cohesive bilayers inhibits interaction of plasma proteins with the liposome particles. Consequently, the opsonic proteins are not able to induce the surface modifications which otherwise would 'mark' the liposomes for MPS uptake. Another approach to create LCL utilized the inclusion of specific glycolipids such as monosialoganglioside Gm1 or phosphatidylinositol (PI). It was hypothesized that these glycolipids act through creating a carbohydrate 'shield' over negatively charged groups located underneath [17]. Overall, these methods achieved some success, but are all dependent on rigid liposome bilayers, which can impose a limitation when fluid bilayers are needed to achieve appropriate drug release rate profiles *in vivo*.

A more recent development to prepare LCL with less restriction to lipid bilayer composition is based on modification of the liposome surface with hydrophilic polymers to protect the lipid surface of the liposome against

protein adsorption and consequent uptake by mononuclear phagocytes. A list of polymer coatings investigated over the years is presented in Table 1. Stable coating with hydrophilic polymers is generally achieved by coupling the polymers to lipid anchor molecules that can insert into the liposome bilayer. The hydrophilic part of the conjugate is believed to form a repulsive steric barrier that can 'hide' the liposome bilayer from plasma proteins [18,19,20].

Table 1. Polymers with Capacity to Extend the Circulation Time of Liposomes

Polymer	Ref.
Poly(ethylene glycol)	[4]
Poly(acrylamide)	[21]
Poly(vinyl pyrrolidon)	[21]
Poly(acryloyl morpholine)	[22]
Poly(2-methyl-2-oxazoline) and Poly(2-ethyl-2-oxazoline)	[23]
Poly(vinyl alcohol)	[24]
Hydroxypropylmethylcellulose	[24]

At present, by far the most extensively explored coating polymer is polyethylene glycol (PEG). The PEGylation strategy is often referred to as 'steric stabilization' or 'Stealth technology'. Typically an incorporated molar amount of 5% proves to be sufficient to achieve prolonged circulation [25]. In rats the plasma half-life of a 100 nm PEG-coated liposome is around 20 – 24 hrs while in humans a half –life of 45 hrs can be realized [26]. PEG surface modification has been shown to have important advantages over the other methods to obtain prolonged circulation behavior [27]. One important claimed advantage is that PEG-liposomes possess -within certain limits- dose-independent log-linear blood concentration-time profiles [28]. This permits dose escalation without complications arising from changes in pharmacokinetic behavior. Another advantage is the possibility of varying the lipid composition without affecting circulation time and tissue distribution, which provides an ability to optimize the liposome physicochemical properties for drug loading and release [29].

II.2 Localization at Pathological Sites

Besides rapid uptake by MPS-macrophages, another significant barrier for i.v.-injected particulate systems is the endothelial lining between the vascular space and extravascular target tissue. In most tissues the vascular system is lined with a continuous layer of endothelial cells often supported by a basement membrane. This barrier virtually excludes extrasavasation of particles such as LCL, except for a few selected sites where the endothelial lining is discontinuous. Fortunately, it has been found that regions of increased capillary permeability include pathological sites such as tumors and the sites of infection and inflammation. LCL have been shown to extravasate into these pathological areas [3,4]. The mechanism(s) of extravasation (often referred to as 'enhanced permeability and retention (EPR) effect') is not well understood but can be thought of simply as 'leakage in the plumbing' [10]. Inflammatory processes are accompanied by locally increased vascular permeability. In case of tumors, the angiogenesis process results in tumor blood vessels with increased permeability. Up to 10% of the injected LCL dose has been shown to localize in such sites of pathology, suggesting potential for substantial improvements in efficacy of encapsulated therapeutic agents that are active towards these pathologies.

II.3 Therapeutic Availability

Conceptually, many agents can benefit from enhanced delivery to the pathological target and/or reduced distribution to healthy tissues. At present abundant literature is available showing that many therapeutic agents indeed profit from encapsulation in LCL by passive targeting resulting in enhanced localization in diseased tissues (Table 1). These results suggest broad applicability of LCL for drug delivery. For most LCL-entrapped drugs improved efficacy requires a liposomal composition capable of retaining the drug in the LCL during prolonged circulation but releasing it once the LCL have accumulated in the site of pathology, the latter aspect being referred to as therapeutic availability. These requirements have apparently been met by several formulations, as they show striking therapeutic activities in animal disease models, and in some cases in humans.

As LCL are designed to be stable in the circulation, release of the active ingredients at the target site can not be taken for granted (see also Fig. (**2**), option 1). If the LCL remain intact at the target site, the release of drug will be a time-consuming process and local therapeutic levels of active drug are only slowly achieved. Fortunately, at sites of inflammation and tumors enzymes are active that can cause degradation of the liposome phospholipid bilayer [30]. Especially LCL without polymer coating are expected to be affected by enzymatic degradation. A hydrophilic polymer coating may hamper the degradation process, although recently, it has been shown that inclusion of PEG in the lipid bilayers appears to enhance enzymatic degradation [31].

When enzymatic degradation at the target site is insufficient, drug release may be achieved with triggering by external means. For over two decades researchers have been investigating the possibility of using thermosensitive liposomes. This approach is based on release of drug from the liposome as a result of enhanced fluidity of normally rigid liposome bilayers, when the target tissue is heated above the transition temperature of the lipid composition. Several studies show that this concept can indeed result in increased therapeutic efficacy of a liposomally encapsulated drug [32,33]. Another potential mechanism for drug release within the target site is uptake and intracellular processing of drug-LCL by local phagocytes. Intracellular degradation of the LCL bilayers may liberate the drug, which may subsequently diffuse out of the endosomal compartment and become active in the cytoplasm of the phagocyte. Successful targeting and modulation of macrophage populations at inflamed tissue has been achieved with long-circulating liposomes containing clodronate that can cause cell death

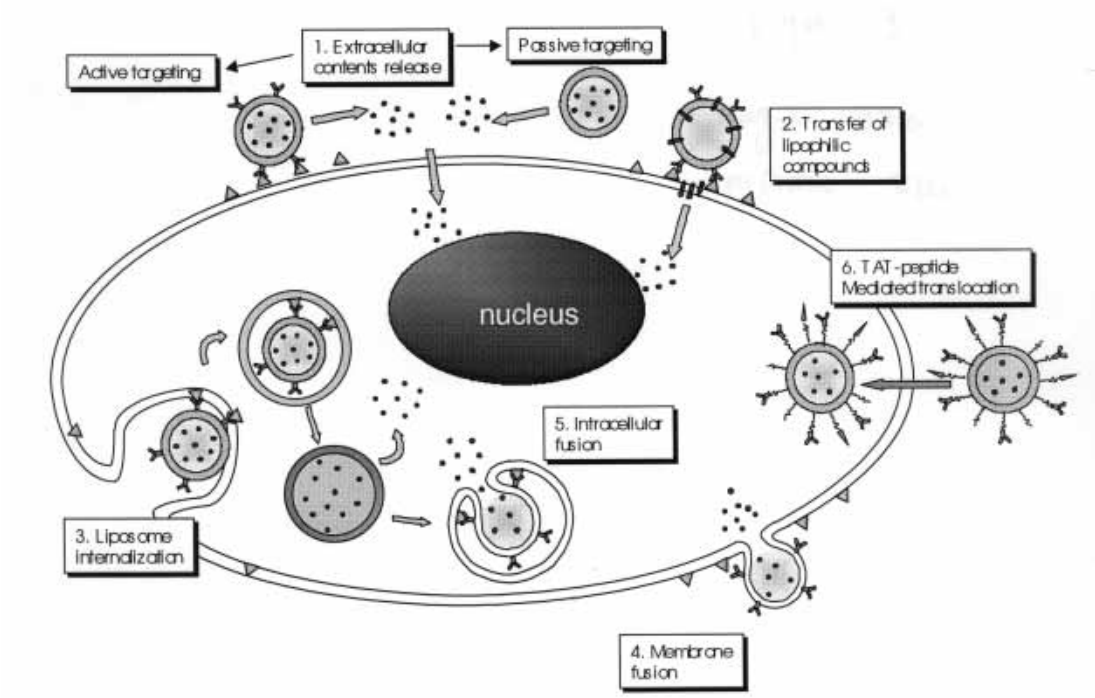

Fig. (2). Potential ways of cytosolic drug delivery with passively and actively targeted liposomes.

when it becomes intracellularly available [34,35]. Additionally, when a drug is able to pass membranes, it may eventually diffuse out of the phagocyte into the extracellular environment and thereby becomes available for interaction with the intended target cells. This concept of 'macrophage-mediated drug release' has been exemplified with the antitumor drug doxorubicin [36].

It should be mentioned that drug release within the target site is not always required. This is the case when the LCL are used for diagnostic purposes. LCL-based formulations containing isotopes for scintigraphic imaging of infection and inflammation have been shown to represent promising radiopharmaceuticals in nuclear medicine [37,38]. Table 2 shows the developmental status of the various liposomal pharmaceuticals that are currently in clinical and preclicinical studies.

Taking together, passive targeting and improved therapeutic behavior of LCL-encapsulated drugs, is based on selective but non-specific extravasation into pathological tissues accessible from the circulation due to a locally increased vascular permeability. In most cases mentioned in Table 2, drug release takes place extracellularly. Subsequently, the released drug is able by itself to reach the therapeutic intervention site (often intracellular), which often will require passive diffusion over membrane barriers. However, when target cells are not localized in the extravascular space but, for example in the blood circulation, the localization process requires more sophisticated strategies such as specific carrier-target cell recognition. In addition, an increasing number of new drug molecules, especially the new biotechnology derived agents such as proteins and nucleic acids, can not readily pass cell membranes due to their hydrophilicity and relatively high molecular weight. These molecules require liposomal carriers that are able to deliver the entrapped drug to the subcellular target compartment (often the cytoplasm or nucleus). In these situations surface-conjugated targeting ligands and/or membrane-translocating functionalities for intracellular delivery have to be included in the LCL system. Both ligand-mediated active targeting of liposomes and new approaches to obtain cytosolic drug delivery are discussed in more detail below.

III. ACTIVE TARGETING

Active targeting of liposomes refers to the conjugation of site-directing ligands to the surface of liposomes to obtain specific binding to cell receptors on the surface of the target cells. Active targeting aims at improving the therapeutic availability of liposomal drugs to target cells within the pathological site and to minimize undesired side-effects to non-target cells within the pathological tissue. Although 'active targeting' may suggest that liposomes are actively seeking their targets, resulting in increased amounts of drugs delivered at the diseased sites, this is far from reality. Ligand-mediated binding of liposomes to target cells only occurs when the intravenously administered liposomes 'passively' encounter a target cell. Target cells located in the circulation can be expected to be readily accessible. Target cells outside the vasculature are more difficult to reach and

liposomes need to extravasate before being able to bind [80-82]. As extravasation of liposome particles is the rate-limiting step in the targeting process, the presence of targeting ligands on the surface of liposomes is not likely to further enhance the amount of drug delivered at the target site. Nevertheless, the presence of targeting ligands on the surface of liposomes can be beneficial by binding to specific target cell receptors leading to improved cellular internalization of the liposomal drug contents. There are different methods available for attaching targeting ligands to PEG-LCL. The most popular methods are based on ligand attachment to the terminal end of PEG (Fig. (**1**)) [83-85]. For successful ligand-directed targeting of liposomes both the choice of the targeting ligand as well as the cell surface epitope to which the ligand will be directed are critically important.

III.1 Choice of Targeting Ligand

For active targeting of liposomes any ligand with specificity for cell-surface receptors that are selectively

Table 2. Current Developmental Status of Intravenous Liposome Products

Status	Active ingredient (product name)	Therapeutic field	Reference
Commercially available	Doxorubicin (Doxil/Caelyx™)	Oncology	[39,15]
	Daunorubicin (Daunoxome™)		[39,15]
	Amphotericin (Ambisome™)		[40,41]
Clinical study Phase III	Tretinoin (Atragen™)	Oncology	[42]
	Nystatin (Nyotran™)	Fungal infections	[43]
	Amikacin (Mikasome™)	Fungal infections	[44]
	Prostaglandin E1 (Ventus™)	Acute Respiratory Distress Syndrome	[45]
Phase II	Cisplatin (SPI-77)	Oncology	[46]
	NDDP, a cisplatin analogue		[47]
	Vincristine (ONCO-TCS)		[48]
			[49]
Phase I	Annamycin	Oncology	[50]
	Paclitaxel		
Preclinical, animal study	Gentamicin	Bacterial infections	[51]
	Ceftazidime		[52]
	Streptomycin		[53]
	Kanamycin		[54]
	Cefoxitin		[55]
	Clofazimine		[56]
	Rifampicin		[57]
	Isoniazid		[57]
	Irinotecan (CPT-11)	Oncology	[58]
	Boron derivatives for Boron Neutron Capture Therapy		[59,60]
	GL147211C, topoisomerase I – inhibiting camptothecin analogue		[61]
	TNF-alpha		[62]
	Adriamycin		[63]
	Interleukin-2		[64]
	Epirubicin		[65]
	MTP-PE, muramyl tripeptide derivative		[66]
	CNDAC, beta-D-arabino-furanosylcytosine derivative		[67]
	20(S)-Camptothecin	Leishmaniasis	[68]
	Amarogentin		[69]
	Atovaquone		[70]
	N-methylglucamine antimoniate		[71]
	Superoxide Dismutase	Arthritis	[72]
	Metotrexate		[73]
	Indomethacin		[74]
	Clodronate		[75]
	Cyclosporin	Transplantation	[76]
	Busulphan		[77]
	Hemoglobin	Red blood cell substitute	[78]
	Albuterol	Inflamed airways	[79]
	^{99}Tc radioactive tracer	Scintigraphic detection of tumors/inflamed tissue	[26]

expressed on the surface of the target cell population can be used, as long as chemical conjugation of the ligand to the liposomal surface is feasible without loss of receptor specificity and/or affinity. Frequently used ligands for this purpose are antibodies [86-88], as they can easily be raised against a variety of antigens and often show high selectivity and affinity for their antigen. Besides antibodies other ligands have also been studied, such as vitamins [89], peptides [90] and aptamers [91,92].

An important aspect to consider while choosing the appropriate targeting ligand is its immunogenicity. Some ligands, especially those produced in other species, can be recognized as 'foreign' by the immune system of the patient especially when the ligands are conjugated to the distal ends of the PEG chains of LCL (Fig. (**1**)) [93]. This ligand-mediated immune recognition may oppose the MPS-avoiding characteristics of LCL, resulting in increased clearance rates of i.v.-administered targeted liposomes. For instance, the presence of whole antibodies exposing the constant parts (Fc) of the antibody on the surface of liposomes makes these liposomes highly susceptible to Fc-receptor-mediated phagocytosis by cells of the MPS [94]. To prevent this Fc-mediated immune-recognition, antibody fragments such as Fab' and scFv molecules, lacking the constant part of antibody molecules are frequently used. Important to note is that ligands with low intrinsic immunogenicity may become strongly immunogenic when conjugated to the surface of liposomes. In most cases chemical modification of the targeting ligand is required for covalent conjugation to the liposomal surface. Such modifications may also lead to a increased immunogenicity [95].

Obviously, the affinity of the liposome-conjugated ligand for the target receptor is also an important aspect. Binding to the target receptors should be strong enough to retain the liposomal carrier to the surface of the target cells. As multiple targeting ligands are often conjugated to the surface of liposomes, affinity is in most cases not a problem. Even targeting ligands with low affinity for their receptor can be used to obtain strong binding to target cells due to the multivalent character of the targeted liposomes. However, very high affinity interactions between liposomes and target cells should be prevented as this may hamper the distribution of targeted liposomes within the pathological site. This so-called 'binding site barrier' phenomenon has been described for antibodies [96] and scFv molecules [97]. Similarly, this phenomenon was offered as an explanation for the observation that immunoliposomes targeted to solid tumors in a nude mice xenograft model were primarily located in the perivascular zones after systemic administration [98,99].

III.2 Choice of Target Receptor

In choosing the most suitable target receptor, several requirements have to be met. First, the target receptor should be expressed in sufficient amounts to allow accumulation of pharmacologically active drug levels in the pathological tissue. Second by, the target receptors should be qualitatively or at least quantitatively different from receptors found in a healthy tissue. Although cell surface receptors that are exclusively expressed under pathological conditions are scarce, disease-related overexpression of receptors is often found. For example, overexpression of adhesion molecules is common in inflamed tissue [100,101] and overexpression of growth factor receptors is often found in tumor tissue [102,103]. Third by, the target receptor should not be shed from the surface of target cells and should be readily accessible to the ligand-directed liposomes. Fourth by, if cytosolic delivery of liposomal drug is required, receptor-mediated internalization of liposomes is highly desired (see Fig. (**2**), option 3). It should be realized that targeting of liposomes to receptors with known internalizing capacities does not necessarily guarantee internalization of the liposomes. Binding of the targeting ligand may occur to specific epitopes on the internalizing receptor which do not trigger internalization [104].

III.3 Cytosolic Drug Delivery

Cytosolic access is problematic with many new biotherapeutic molecules (e.g. proteins, (poly)peptides and nucleic acids). Although ligand-mediated binding of liposomes to cell surface receptors can increase the cellular uptake of liposome-encapsulated drugs, the internalization process itself is not sufficient to yield an enhanced therapeutic effect as long as the entrapped drug is not delivered to the (sub)cellular intervention site. In most cases, the drug needs to be delivered into the cytosol in order to become effective. Some of the delivery strategies leading to cytosolic drug delivery are depicted in Fig. (**2**).

III.3.1 Cell Membrane Fusion

In case of targeting to receptors that do not internalize the liposomal drug carrier, cytosolic drug delivery can be obtained by fusion of the membranes of the cell-bound liposomes with the plasma membrane of the target cells (Fig. (**2**), option 4). Such fusogenic liposomes have been constructed simply by fusing liposomes with Sendai virus particles. The virus-liposome fusion products retain fusogenic activity and can be used for the cytosolic delivery of liposome entrapped hydrophilic compounds into cells [105,106]. Although these fusogenic virosomes have been used for many applications, among which are gene delivery and vaccination purposes as well, specific targeting of these fusogenic vesicles to predefined cell populations remains problematic as the viral receptors present on the virosomes determine which type of cells can be targeted [107,108]. Target-sensitive liposomes have been constructed whose bilayers destabilize upon target cell binding [109,110]. This binding-induced destabilization results in extracellular release of liposome-entrapped compounds. Therefore, this strategy is not suitable for the delivery of biotherapeutics that require cytosolic delivery but may be useful to improve the therapeutic availability of small, membrane-permeant drugs at target sites.

III.3.2 Endosomal Escape

When target cell binding results in internalization of the targeted liposome particles, the majority of the liposomes

will face degradation in the endocytic/lysosomal pathway. This delivery route may be useful to obtain cytosolic delivery of drug molecules that can resist lysosomal degradation and diffuse out of the endosomal and/or lysosomal compartments once the liposomes have been degraded, as has been reported for doxorubicin [36]. However, in many cases delivery into the endosomal pathway results in degradation of the liposome-entrapped drug. To prevent lysosomal degradation and to allow endosomal escape of the liposome-entrapped drug into the cytosol of target cells, endosomolytic functionalities should be incorporated (Fig. (**2**), option 5). These functionalities should induce membrane-perturbing activity preferentially in the low pH environment of endosomal compartments. Several proteins with pH-dependent membrane-perturbing activity have been identified in biological systems and some of them used to obtain enhanced cytosolic delivery are listed below.

Hemolysins

Bacterial hemolysins are proteins with membrane pore-forming capacities that are produced by a variety of bacteria [111]. In most cases, the pore-forming activity compromises the integrity of cells resulting in cell death. However, one of these hemolysins, listeriolysin O (LLO) secreted by the intracellular pathogen *Listeria monocytogenes* exclusively attacks membranes at low pH. Its function is to allow the escape of *Listeria monocytogenes* from the host's phagocytic vacuoles into the cytosol. Incorporation of LLO into liposomes has resulted in efficient cytosolic delivery of co-entrapped compounds from internalized liposomes without measurably harming the cells [112]. As the pores formed by such hemolysins are rather big, ranging in size from 15-35 nm dependent on the type of hemolysin, this approach will be essentially suitable for the cytosolic delivery of bulky macromolecules such as DNA.

Viral Fusion Proteins

Several enveloped viruses, among which is the human influenza virus A, enter cells by the process of receptor-mediated endocytosis, routing the viral particles into the endosome. The low pH within the endosomes triggers the fusion of viral envelopes with the endosomal membrane, thereby releasing the viral nucleocapsids into the cytoplasm of host cells. In case of the human influenza virus both the adhesion of virus particles to the host cell membrane, which triggers internalization, and the low pH-induced membrane fusion reaction are mediated by the viral spike glycoprotein hemagglutinin (HA) [113,114]. The envelopes of influenza viruses have been solubilized, purified and reconstituted into vesicles [115,116]. These so-called 'influenza virosomes' bearing both the HA and the neuraminidase (NA) spike proteins retain fusogenic activity exclusively at low pH and have been used as carriers for the delivery of normally membrane-impermeable substances into the cytosol of cells via the endocytic pathway [114-119]. However, as these virosomes have a tropism for sialic acid-bearing cells similar to the native virus, targeting of influenza virosomes to specific cell types is hampered. Recently, we have targeted influenza virosomes towards ovarian carcinoma cells by virtue of virosome-conjugated antibodies (Fig. (**3**), unpublished results). This was accomplished by incorporating poly(ethylene glycol) (PEG) conjugated to phospholipids into the virosome membrane. We demonstrated that this PEG-layer on the surface of influenza virosomes shields the interaction of HA with ubiquitous sialic acid residues and at the same time serves as spatial anchor for antibody attachment. In this way, virosome binding to cells was exclusively antibody mediated without

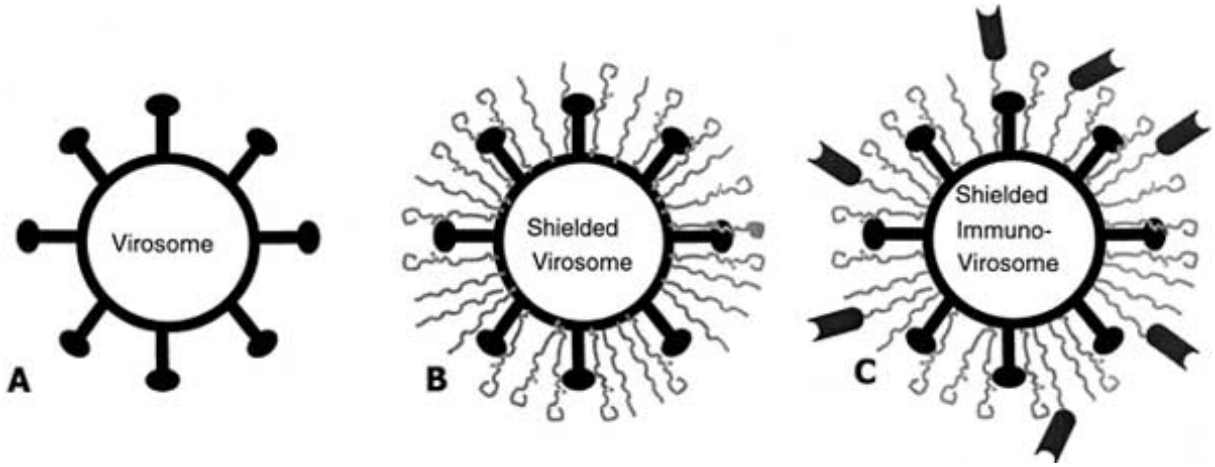

Fig. (3). Antibody-redirected targeting of influenza virosomes. Unmodified influenza virosomes expose on their surface many copies of the hemagglutinin membrane protein, which contain the binding pocket for sialic acid residues (A). Poly(ethylene glycol) grafted at high densities on the surface of influenza virosomes can effectively shield the viral spike proteins, thereby preventing HA from interacting with sialic acid residues (B). Conjugation of antibody-Fab' fragments at the distal ends of the surface-exposed PEGG chains results in specific binding of virosomes to target cell that is predominantly mediated by the exposed Fab' fragments and not by the HA proteins (C).

loss of fusogenic activity. Such antibody-redirectedinfluenza virosomes may be useful carriers for the cytosolic delivery of otherwise membrane-impermeant therapeutic compounds via the route of receptor-mediated endocytosis. Antibody-directed virosomes are expected to be immunogenic due to the presence of viral proteins bearing highly antigenic determinants. This immunogenicity can provide adjuvant-activity when these carriers are used for the delivery of antigens for the purpose of vaccination [120].

pH-Dependent Viral Fusion Peptides

The use of pH-dependent fusion peptides represents another approach to obtain cytosolic delivery of biotherapeutic molecules with targeted SSL via the route of receptor-mediated endocytosis [121]. Synthetic peptides derived from viral fusion proteins are expected to be less immunogenic than the original fusion proteins as they lack the major antigenic determinants. This is even more true when these peptides are entrapped inside liposomes [122]. In addition to reduced immunogenicity, peptides have the advantage that they can be readily synthesized at a large scale without the need for laborious purification procedures. Studies with synthetic peptides resembling the native sequence of the influenza virus N-terminal domain of the HA2 subunit have clearly demonstrated that such peptides are able to destabilize both model membranes (such as liposomes) and natural membranes in a pH-dependent manner [123-125]. Fusion peptide-induced lipid mixing between liposomes has been demonstrated, indicating that these peptides have fusogenic capacities. Influenza virus-derived fusion peptides have been successfully used to enhance the liposomal and endosomal escape of both DNA [122] and bacterial toxin fragments (personal observation) after cellular uptake of targeted liposomal formulations with co-encapsulated fusogenic peptides. This approach of cytosolic drug delivery is particularly effective as it combines targeting with efficient cytosolic delivery. The targeting step provides specificity and ensures delivery of the entire liposomal drug package into the target cells. In addition, the encapsulated fusogenic peptide triggers endosomolytic activity resulting in cytosolic drug delivery.

III.3.3 Membrane Translocating Peptides

Recently, protein transduction domains (PTD) within proteins have been identified that possess the ability to traverse biological membranes [126]. Although the exact mechanism is unknown, transduction occurs in a receptor- and transporter-independent fashion and appears to target the cell membrane directly. Several studies have demonstrated that proteins and even large colloidal particles can be shuttled into cells when conjugated to such PTDs [127]. In addition, it was recently demonstrated that conjugation of the protein transduction domain of HIV-TAT to the distal ends of PEG-chains, which were anchored into liposomal bilayers results in translocation of entire liposome particles into target cells (Fig. (**2**), option 6) [128]. This transduction process occurred even at low temperatures and in the presence of metabolic inhibitors. Although preliminary, this study shows that PTDs can be used to enhance the intracellular delivery of liposomes into cells. Unfortunately, as the transduction process is independent of specific receptors the PTD-mediated transduction is non-specific and cannot discriminate between cell types. It remains to be investigated whether PTD-mediated transduction of proteins and/or particulate carriers can be limited to specific cell types.

IV. FINAL REMARKS

So far, long circulating liposomes appear to offer a range of opportunities for i.v. targeting to pathological sites. The multifaceted capabilities of liposomal formulations seem to continuously provide researchers with new opportunities for drug targeting. The flexibility of the system allows the design and development of liposome systems for the delivery of a wide range of drug molecules; from small stable drugs to larger, fragile biotherapeutics. However, liposomal preparations that are clinically investigated or commercially available, mainly exploit the passive targeting effect for reaching tumors or sites of infection/inflammation. Obviously, the more sophisticated targeted liposome designs face a more complicated development route to reach clinical practice. Several complicating factors may play a role: for instance, any additional modification to a given drug carrier system requires thorough investigation of its influence on the safety profile of the delivery system as a whole. Another issue concerns the observation that modification of the surface of long circulating liposomes with targeting ligands or other functionalities often enhances immunogenicity and/or jeopardizes *in vivo* behavior. The latter aspect means that disappointing *in vivo* behavior may obscure each *in vitro* success yielding improved active targeting or cytosolic delivery.

We hope that this review provides the reader with some insight in the design and development of liposomal delivery systems, and envisage that the versatility of the liposomal drug carrier will continue to offer ample opportunities for the development of new and improved liposome systems, applicable in the treatment of life-threatening and chronic diseases.

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