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Enzyme-Responsive Liposomes for the Delivery of Anticancer Drugs

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Abstract

Liposomes are nanocarriers that deliver the payloads at the target site, leading to therapeutic drug concentrations at the diseased site and reduced toxic effects in healthy tissues. Several approaches have been used to enhance the ability of the nanocarrier to target the specific tissues, including ligand-targeted liposomes and stimuli-responsive liposomes. Ligand-targeted liposomes exhibit higher uptake by the target tissue due to the targeting ligand attached to the surface, while, the stimuli-responsive liposomes do not release their cargo unless they expose to an endogenous or exogenous stimulant at the target site. In this review, we mainly focus on the liposomes that are responsive to pathologically increased levels of enzymes at the target site. Enzyme-responsive liposomes release their cargo upon contact with the enzyme through several destabilization mechanisms: a) structural perturbation in the lipid bilayer, b) removal of a shielding polymer from the surface and increased cellular uptake, c) cleavage of a lipopeptide or lipopolymer incorporated in the bilayer, and d) activation of a prodrug in the liposomes.

Introduction

Among different nanoparticle-based drug delivery systems such as polymersomes, dendrimers, and magnetic nanoparticles^{1, 2}, liposomes have gained much popularity in the last few decades, particularly, in cancer therapy. Several characteristics have turned liposomes into suitable *in-vivo* nanocarriers: the natural phospholipids are biodegradable, biocompatible, nontoxic, and weakly immunogenic³. Liposomes encapsulate a broad range of drugs, and release the payloads at a target site, resulting in therapeutic drug concentration at the diseased site and reduced toxic effects in healthy tissues⁴. In addition, liposomes increase bioavailability and reduce clearance of drugs by increasing circulation and residence time in the body⁵. In this review, we discuss different types of enzyme-responsive liposomes which can enhance drug delivery in cancer treatment.

Structure of Liposomes

Liposomes are self-assembled vesicles with single or multiple phospholipid bilayers. Due to the amphiphilic nature of the constituent phospholipids, the liposomes can carry both hydrophilic and lipophilic drugs⁶. The hydrophilic drugs are encapsulated in the aqueous

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core or conjugated to the membrane surface. Hydrophobic drugs are incorporated into the lipid bilayer of the vesicles⁵. A schematic illustration of a liposome and two common phospholipids used in the formulation of liposomes are shown in Figure 1.

Liposomes are composed of natural or synthetic phospholipids. The lipid composition of liposomes has a significant impact on their stability, physicochemical, and pharmacokinetic properties. Depending on the type of phospholipids, liposomes may be cationic, anionic, or neutral at the physiological pH⁵. For example, liposomes composed of dialkyl phosphatidylcholine or phosphatidylethanolamine are neutral, while vesicles made of phosphatidylserine and phosphatidylglycerol have a net negative charge⁵ (Figure 2). The surface charge has a significant effect on the stability of liposomes both in the formulation and serum. Incorporation of other lipophilic compounds such as cholesterol into the bilayer decreases the fluidity of membrane and reduces premature drug leakage⁷.

***In-vivo* Stability of Liposomes**

Unilamellar liposomes are generally between 50–250 nm in diameter³, and able to deliver their cargo to the targeted tissues. However, the classical liposomes have a very short blood circulation time due to serum protein opsonization and uptake by the reticular endothelium system⁸. One common approach to increase the circulation half-life of liposomes is to attach polyethylene glycol (PEG) on the surface (PEGylation), introduced by Klibanov et al.,⁹ and further developed by several other researchers^{10–14}. The grafted hydrophilic polyethylene glycol on the surface of liposomes offers steric hindrance and prevents the adsorption of opsonins and subsequent endocytosis by the phagocytes. Moreover, steric repulsion induced by surface PEG layer prevents liposome aggregation and increases stability. Therefore, these sterically stabilized liposomes, termed as stealth liposomes, have a prolonged blood circulation time³. The half-life of circulation for liposomes with PEG with the molecular weight range 1900–5000 is increased to 9–16 hours¹⁵. Doxil®, introduced in 1995, is the first FDA-approved PEGylated liposome for the treatment of ovarian cancer, Kaposi's sarcoma, and breast cancer¹⁶.

Passive Targeting vs. Active Targeting

Passive targeting of liposomes

Liposomes passively accumulate in the target tissues through penetration retention effect^{17, 18}. Vasculatures in tissues affected by the tumor, infection, and inflammation have higher permeability and larger pores relative to the vasculatures in healthy tissues. Thus, drug-loaded liposomes in the range 60–150 nm extravasate from the vasculatures into the diseased tissues, and subsequently release their cargo at the desirable site of action¹⁹. In addition, the retention of liposomes is enhanced in the tumor tissues due to poor lymphatic drainage²⁰. These properties have made drug-loaded liposomes superior to free drugs which unselectively distribute into both healthy and diseased tissues. Upon arrival of liposomes at the target site, drug releases through passive diffusion across the bilayer lipid. Released drug diffuses in the interstitial fluid of the target tissue and is subsequently up-taken by the target cells¹⁹.

Ligand-targeted Liposomes

In passive targeting, the liposomes can enter normal tissues and release their drug in the vicinity of healthy cells, resulting in off-target toxic effects. This problem is alleviated by conjugating targeting ligands on the surface of liposomes. The ligand can be an antibody, peptide, glycoprotein, carbohydrate, or a ligand which recognizes and binds to a specific antigen or receptor overexpressed (or selectively expressed) on the diseased cells. If the ligand stimulates receptor-mediated internalization, target cells uptake the liposomes via endocytosis and the loaded drug is released inside the cell¹⁹. The receptor-mediated endocytosis of the ligand-targeted liposomes has important implications for the intracellular delivery of therapeutic agents such as macromolecules²¹.

The targeted liposomes are associated with better therapeutic outcomes of anti-cancer drugs in animal tumor models compared to non-targeted vesicles^{22, 23}. However, some reports also suggest that the targeted liposomes are not more effective *in vivo* compared to non-targeted counterparts²⁴. Premature leakage of the encapsulated drugs, lack of internalization, low density of antigens on the target cells, and low permeability to the target tissues may decrease the effects of targeting²¹. Due to the high cost of development, no ligand-targeted liposomes have reached the market yet. However, a few phase I and II clinical trials are in progress involving transferrin-targeted oxaliplatin liposomes (MBP-426) and glutathione-coated, doxorubicin liposomes (2B3-101)⁵.

Stimuli-responsive Liposomes

The targeted liposomes, upon reaching the intended sites, release the encapsulated drugs through passive diffusion, and this process is often slow. A strategy to enhance the release rate of the encapsulated drugs is to design environmentally-responsive liposomes. These liposomes do not release their cargo until an endogenous or exogenous stimulus induces structural changes in liposomes. A rapid release of the encapsulated drug ensures its therapeutic concentration at the target site and reduces the possibility of developing drug resistance. Endogenous stimuli are pathological changes in the target cancer tissues, such as reduced pH²⁵, overexpression of specific enzymes, and abundance of reducing agents⁴. Lack of these triggers in healthy tissues prevents the release of drugs from liposomes, and therefore, protects healthy cells from toxic effects of anti-cancer drugs. In addition, liposomes can be rendered responsive to exogenous or remote triggers such as heat²⁶, light²⁷, ultrasound²⁸, and magnetic field²⁹. The targeting ability of the stimuli-responsive liposomes can be enhanced by incorporation of suitable ligands into liposomes³⁰.

In this review, we mainly focus on enzyme-responsive liposomes and specifically their applications in drug delivery. Enzyme-responsive liposomes offer several advantages over other stimuli-responsive vesicles. The drug release is triggered by an enzyme that exists in the targeted tissue, and therefore, the liposomes do not need an external equipment to trigger the drug release. This can be beneficial in a clinical setting³¹. Localized release of the encapsulated drug reduces the side effects. In addition, enzyme-responsive liposomes can be designed to produce bioactive molecules following enzyme digestion. These molecules may exhibit synergistic therapeutic effects or facilitate the uptake of the drug at the target tissue³². Furthermore, the amount of drug release from enzyme responsive liposomes is

proportional to the concentration of the active enzyme and the severity of the pathological condition at the target site.

Enzyme-Responsive Liposomes

In several pathological conditions, such as inflammation, infection, and cancer, the concentrations of several enzymes are elevated. This biochemical abnormality can be used as the trigger to design nanocarriers which undergo structural transformation leading to the release of the encapsulated payload. This strategy has been used to develop enzyme-responsive nanoparticles from polymers, gold, quantum dots, and liposomes³². In the following sections of this review, we summarize the enzyme-responsive liposomal formulations that are triggered by different extracellular and intracellular enzymes.

Extracellular Enzymes

Secreted Phospholipase A2

Secreted phospholipase A2 (sPLA₂) increases in various inflammatory diseases, atherosclerosis, and cancers. It has also been shown that sPLA₂ is increased in the prostate, breast, and pancreatic cancers^{33–41}. Therefore, sPLA₂ responsive liposomes can be a promising carrier for the targeted delivery of anticancer agents to the tumor tissues. Particularly, sPLA₂ responsive liposomes are promising for prostate cancer since sPLA₂ IIA increases up to 22 fold in high-grade and metastatic prostate tumors^{34, 42, 43}. One example of sPLA₂ responsive liposomes for the treatment of prostate cancer was reported by Mock et al. Doxorubicin-loaded sPLA₂ responsive liposomes were prepared with 1,2-distearyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearyl-*sn*-glycero-3-phosphoethanolamine (DSPE), cholesterol, and DSPE-PEG₂₀₀₀. The liposomes were approximately 2.5 times more effective in reducing tumor growth in a mouse model of human prostate cancer compared with conventional sterically stabilized liposomes, containing DSPC, cholesterol, and DSPE-PEG₂₀₀₀⁴⁴.

The sPLA₂ is also increased in infections due to the secretion of the enzyme by several bacteria⁴⁵. Antibiotics-loaded sPLA₂-responsive liposomes offer several advantages over conventional liposomes in the treatment of infections: they release high concentrations of antibiotics at the site of infection where bacteria secrete sPLA₂, and the amount of drug release from liposomes is regulated by the concentration of the enzyme at the target site. Therefore, more amount of drug is released from liposomes in more severe infection (which is associated with higher concentrations of sPLA₂). A recent example of such liposomes has been demonstrated by Thamphiwatana et al. Doxycycline loaded liposomes, stabilized by chitosan-modified gold nanoparticles (AuChi), showed approximately six times more antibacterial effect against *Helicobacter Pylori* in vitro compared to empty AuChi liposomes. The antibacterial effect was decreased when a PLA₂ inhibitor was added to the cell culture, suggesting that the release of doxycycline was dependent on the sPLA₂ activity⁴⁶.

sPLA₂ Mechanism of Action—sPLA₂ is a Ca²⁺-dependent esterase⁴⁷. It hydrolyzes phospholipids at the *sn*2-fatty acyl ester position, producing free fatty acid and lysophospholipid^{48, 49}. sPLA₂-mediated hydrolysis of the phospholipids incorporated into

liposomes disrupts the integrity of the lipid bilayer and releases the encapsulated payloads (Figure 3). The different isoforms of sPLA₂ display altered substrate selectivity and hydrolytic activity. For example, Zhu et al. have shown that sPLA₂ group III is more potent in degrading liposomes than sPLA₂ IIa in vitro⁵⁰. Also, incorporation of cholesterol into PEG-free liposomes had a different effect on the contents release depending on the type of sPLA₂ isoform. The addition of cholesterol in the liposome bilayer had an inhibitory effect on the contents release by sPLA₂ group III compared to sPLA₂ IIa⁵⁰. The sPLA₂ IIa isoform is active specifically toward anionic lipid membranes, while other sPLA₂ isoforms (such as those derived from snake venom, mammalian sPLA₂ V and X groups) hydrolyze both anionic and zwitterionic lipid membranes^{48, 51}.

sPLA₂ has higher hydrolytic activity toward aggregated forms of lipids, such as bilayers and micelles compared to lipid monomers⁵². Physical properties and composition of the aggregated lipids have a substantial impact on the activity of sPLA₂^{53–56}. For example, the liposomes made from phospholipids with short acyl chains and anionic polar head groups are more susceptible to hydrolysis^{44, 50}. Particularly, phosphatidylethanolamine and phosphatidylglycerol are the most susceptible to hydrolysis by sPLA₂⁵⁰. Zhu et al. have also demonstrated that incorporation of DSPE or distearoylphosphatidylglycerol (DSPG) into liposomes (containing DSPC, cholesterol, and DSPE-PEG₂₀₀₀) increased the release of encapsulated content in the presence of FBS 10% (v/v) and sPLA₂ to approximately 1.4 and 1.6 times more, respectively, compared to the vesicles lacking DSPE and DSPG over 108 h⁵⁰. The addition of an sPLA₂ inhibitor to this media decreased the release from liposomes containing DSPE or DSPG to the extent similar to the control liposomes without sPLA₂. These results confirm that the release from these liposomes is mediated by sPLA₂⁴⁴. Apart from the lipid components, grafted PEG on the surface of liposomes increases the hydrolytic activity of sPLA₂^{50, 57–60}. Incorporation of DSPE-PEG increased the degradation and subsequent release of 6-carboxyfluorescein from liposomes independent of the enzyme isoform and incorporated phospholipids⁵⁰. It has been suggested that the presence of lipopolymer (lipid-PEG) changes the microscopic lateral organization of the lipid bilayer, resulting in increased enzymatic activity of sPLA₂⁵⁷.

In addition to disruption of the lipid bilayer and subsequent release of the drug, other mechanisms have been proposed to regulate drug uptake from sPLA₂ responsive liposomes. Quach et al. have suggested that phospholipase A₂ receptor (PLA2R1) might play a role in the uptake of sPLA₂ responsive liposomes⁶¹. PLA2R1 is a transmembrane glycoprotein and responsible for internalization of sPLA₂ which ultimately results in the degradation of the enzyme. PLA2R1 also regulates various biological functions, such as cell proliferation, cell migration, lipid metabolism, and cytokine production⁶². In addition, it has been recently shown that PLA2R1 might act as a tumor suppressant⁶³. Knockdown of PLA2R1 in prostate cancer cells led to increased uptake of sPLA₂ responsive liposomes compared to sterically stabilized liposomes. Although the mechanism is not yet clear, one possible explanation is that PLA2R1 has a regulatory effect on sPLA₂. This was substantiated by the observation that inhibition of PLA2R1 increased the expression of sPLA₂ group IIA which was associated with increased drug and liposome uptake⁶¹. Whether PLA2R1 has a direct effect on the uptake of sPLA₂ responsive liposomes has not yet been established.

Another factor that affects drug uptake from sPLA₂ responsive liposomes is the products of phospholipid hydrolysis, including lysophospholipids and free fatty acids. These products synergistically enhance the permeability of cell membranes^{59, 64}, leading to increased cellular uptake of the drugs. The hydrolysis products enhance permeability by integrating into lipid membranes and causing defects (by lateral phase separation) or inducing a curvature stress in the membrane⁵⁹. Interestingly, the accumulation of the hydrolysis products in the lipid bilayer and subsequent changes in the morphology of the membrane further increases sPLA₂ activity⁶⁵.

sPLA₂ Sensitive Lipoid Prodrug—The acyl chain at *sn*-2 position in a phospholipid (or ether phospholipid) can be replaced with a lipophilic drug, cleavable by sPLA₂. These lipoid prodrugs form liposomes and accumulate at the target site, where a high concentration of sPLA₂ liberates the active drug from the liposomes. These liposomes are more stable and have overcome some problems associated with conventional liposomes, including limited efficiency of lipophilic drug encapsulation and release^{66,67}. Such liposomal prodrugs have been prepared for some drugs, such as chlorambucil⁶⁸, prostaglandine⁶⁹, and retinoic acid⁷⁰ (Figure 4).

One example of liposomal prodrugs was developed by Arouri and Mourtisen⁶⁶. They prepared liposomes which contained 1-O-Stearyl-2-RAR-C6-*sn*-glycero-3-phosphoglycerol as a C6-RAR prodrug (Figure 4A) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). RAR, 4-(4-octylphenyl)-benzoic acid, is a selective antagonist for the retinoic acid receptor β ^{71, 72} and inhibits cell growth⁷⁰. In the lack of sPLA₂, C6-RAR prodrug had IC₅₀ of 110 μ M in the MT-3 breast carcinoma cell line. However, upon addition of sPLA₂, IC₅₀ decreased to 10 μ M, indicating that the prodrug was hydrolyzed to C6-RAR and Lyso-O-SPG (both are toxic with IC₅₀ of approximately 20 μ M in the cell cultures without sPLA₂)⁶⁶. In addition, premixing of prodrug and DPPC had an additive effect on the IC₅₀ of the mixture in the presence of sPLA₂. DPPC also accelerated the hydrolysis rate of the prodrug, probably due to increased heterogeneity of the membrane⁶⁶. Although the *in-vivo* study of the effectiveness of these liposomes was not demonstrated, such lipoid prodrug can be a very promising delivery model for toxic lipophilic drugs.

Interestingly, liposomes themselves can act as prodrugs if they release cytotoxic lysolipids, including antitumor ether lipids (AELs) under enzymatic digestion. In this approach, liposomes are prepared from proAELs (which are phospholipids with one non-hydrolyzable ether bond at *sn*-1 position and are hydrolyzed to AELs by sPLA₂, Figure 5). AELs exhibit cytotoxicity and inhibit cell growth^{73, 74}. However, the toxicity of AELs is limited to the target site since a high concentration of sPLA₂ (enough for degradation of liposomes) is found only in the tumor site. It has also been demonstrated that proAEL liposomes lacked hemolytic effect and systemic toxicity compared to a potent AEL (ET-18-OCH₃) *in vivo* and *vitro*^{75, 76}.

Overall, liposomes are good carriers for targeted delivery of lipid-based prodrugs to tumor sites. Prodrugs are converted into active drug at the tumor site by sPLA₂. Such delivery systems protect healthy tissues against the toxic effects of the drug.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are Zn^{2+} and Ca^{2+} dependent proteolytic enzymes responsible for the degradation of extracellular matrix⁷⁷. Beyond their physiological functions such as angiogenesis, wound healing, and tissue remodeling, these enzymes are overexpressed in several inflammatory diseases, cardiovascular diseases, and cancers⁷⁸. MMPs play important roles in growth, invasion, and metastasis of tumors^{79–81}. Particularly, MMP-2 and MMP-9 are found in breast⁸², colorectal⁸³, pancreatic⁸⁴, and lung tumors⁸⁵.

Several MMP-sensitive peptides have been designed and incorporated into liposome formulations. These peptides act as linkers between the lipids and the polymer. The polymer prevents the uptake of liposomes through a shielding effect. Upon contact with MMPs at the target site, the peptide is cleaved, leading to the detachment of the polymer and subsequent uptake of liposomes. One example of such MMP-responsive liposomes was reported by Zhu et al.⁸⁶ containing two types of lipopolymers: mAb 2C5-PEG(3400)-MMP2 cleavable peptide-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and TATp-PEG(2000)-1,2-dioctadecanoyl-*sn*-glycero-3-phosphoethanolamine (DSPE; Figure 6). The former lipopolymer, with a longer chain of PEG, functions as a protective shield over the latter lipopolymer containing the TAT peptide. TAT enhances the endocytosis-mediated uptake of the liposomes. The mAb 2C5 is a targeting moiety for cancer cells. Cleavage of the peptide (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln) at the target site liberates the long-chain PEG and removes its shielding effect. The hydrolysis leads to the exposure of TAT peptide and increased uptake of liposomes⁸⁶.

Liposomes containing a galactosylated cholesterol derivative have also been prepared for delivery of chemotherapeutics to the hepatocellular carcinoma (HCC)⁸⁷. These liposomes are taken up by the cancer cells upon binding of galactose to the asialoglycoprotein receptors. To target the liposomes to the hepatocellular tumor, PEG was attached to the phospholipid DOPE via an MMP-2 cleavable peptide linker (Figure 7). The PEG group shields the galactose ligand on the surface of liposomes and prevents their cellular uptake. However, the elevated MMP-2 concentration in HCC cleaves the linker, leading to the release of PEG. Subsequently, the exposed galactose ligand binds to the asialoglycoprotein receptor on the surface of HCC cells⁸⁷. Terada et al. have shown that the uptake of Gal-PEG-DOPE (0.5%) liposomes were significantly enhanced in the presence of more than 5 $\mu\text{g/mL}$ hMMP-2 in HepG2 cells⁸⁷. The liposomes were also stable in the serum for more than 24 hours⁸⁷.

Another strategy to prepare MMP-responsive liposomes is the incorporation of MMP-cleavable lipopeptides into the lipid bilayer. Elevated MMP concentration at the target site cleaves the lipopeptide, leading to destabilization of the membrane and leakage of the liposomal contents. It has been demonstrated that lipopeptides with the sequence Stearoyl-GPQGIAGQR(GPO)₄GG form collagen-mimetic triple helices on the surface of liposomes^{88–91}. The triple helical structure makes the peptide a good substrate for collagenase MMPs while imparting resistance to cleavage by noncollagenase MMPs (MMP-7 and MMP-10) and other proteolytic enzymes. MMP can cleave between the amino acids glycine and isoleucine⁸⁹. Liposomes containing 30% of such lipopeptide and 70% of

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine released their contents in the presence of MMP-9 in a concentration and time-dependent manner^{88, 89}. In these formulations, the type of phospholipid also affects the release of liposomal contents. Incorporation of phospholipids containing acyl chains mismatched with the acyl chain of lipopeptide results in more contents release. Lipid mismatching causes greater demixing of phospholipids and lipopeptide,^{88, 91} and reduces the ability of the membrane to recover quickly when defects are formed by the cleavage of triple helices⁸⁸.

Urokinase Plasminogen Activator

The concentration of urokinase plasminogen activator (uPA), a serine protease, is elevated in a number of human cancers, such as breast, colon, bladder, and ovarian tumors⁹². The uPA contributes to angiogenesis, tumor progression, and metastasis⁹³. The enzyme cleaves the Arg-Ser bond in peptides containing the consensus sequence Ser-Gly-Arg-Ser-Ala⁹². Therefore, liposomes containing such uPA-cleavable peptide can release their encapsulated payload upon contact with uPA at tumor sites.

Basel et al. developed uPA responsive liposomes in a hyperosmotic medium, stabilized with a copolymer cage on the surface⁹⁴. The copolymer contained the uPA cleavable peptide and poly (acrylic acid) anchored to cholesterol. After incorporation into liposomes, the copolymer was cross-linked with ethylenediamine. This crosslinked copolymer cage conferred osmotic pressure resistance to the unstable liposomes containing the hyperosmotic contents. Upon incubation with uPA in a medium of physiological osmolarity, the copolymer degraded, and the osmotic swelling of liposomes released their contents. The lipid bilayer composition and cross-linking of copolymer affected the elasticity and water permeation of the liposomes.⁹⁴.

Elastase

Elastase is a protease secreted from azurophil granules of activated neutrophils and is involved in inflammatory diseases⁹⁵ such as arthritis⁹⁶, acute lung injury⁹⁷, cystic fibrosis,⁹⁸ and tumors⁹⁹ of lungs¹⁰⁰, breast¹⁰¹, and skin¹⁰². Elastase-sensitive fusogenic liposomes were first developed by Pak et al¹⁰³ from DOPE conjugated to N-acetyl-Ala-Ala via an amide linkage (N-Ac-AA-DOPE is an elastase substrate). Conjugation of Ala-Ala with DOPE changed the shape of the phospholipid from inverted cone to cylindrical form, leading to the formation of bilayer vesicles. Cleavage of the dipeptide by elastase at the target site restored the fusogenic property of DOPE which led to the destabilization and fusion of the liposomes. The liposomes also contained 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) to confer positive charges to liposomes after the cleavage of the anionic AA-DOPE. Positively charged liposomes have the greater fusogenic capability with negatively charged cell membranes. Pak et al. have shown that pretreatment of DOTAP/N-Ac-AA-DOPE/PE liposomes with active proteinase K (which has a similar substrate specificity to elastase) simulated lipid mixing and fusion with either the target liposomes or cells (RBC ghosts) *in vitro*¹⁰³. The same research group developed a similar liposomal model from the more sensitive and selective elastase substrate N-methoxy-succinyl-Ala-Ala-Pro-Val-DOPE¹⁰⁴. Replacement of DOTAP with 1,2-dioleoyl-3-dimethylammonium-

propane (DODAP) conferred positive charges to the liposomes at lower pH following enzymatic cleavage and increased the fusogenicity. These liposomes enhanced the intracellular delivery of water soluble encapsulated contents at pH 5 when pretreated with elastase. These results suggest that elastase triggerable fusogenic liposomes can be useful for the cytoplasmic delivery of drugs¹⁰⁴.

Prostate-Specific Antigen (PSA)

Prostate-specific antigen (PSA) is a serine protease found in high concentrations in the prostate tumor tissues¹⁰⁵. PSA is also present in the serum of prostate cancer patients but is enzymatically inactivated^{105, 106}. Thus, PSA positive extracellular matrix of prostate tissue is a suitable trigger for the release of anticancer agents. A number of PSA-activated prodrugs have been developed from PSA-cleavable peptides with very selective antitumor activity *in vitro* and *in vivo*^{107–110}.

Xiang et al. prepared siRNA loaded liposomes containing a peptide (ACPP) with three domains¹¹¹: the cell penetrating polyarginine (GR₈GC)¹¹², the PSA-cleavable HSSKYQ, and the polyanionic DGGDGGDGGDGG. In this peptide, the polyanionic domain interacts with polyarginine via electrostatic interaction and shields its cell-penetrating effect. The high concentration of PSA at the target site cleaved the peptide, resulting in the detachment of polyanionic domain and subsequent activation of the cell penetrating peptide (CPP, Figure 8). The cationic polyarginine peptide also promoted endosomal escape and enhanced the cytoplasmic delivery of liposomes. The targeting of these liposomes was further enhanced by attaching the folate moiety on the surface. Folate binds to the prostate-specific membrane antigen (PSMA), a glycoprotein transporter upregulated in prostate cancer cells¹¹³. In the presence of PSA, the uptake of the liposomes containing both ACPP and folate was highest (*in vitro*) compared to the control vesicles with one or no ligand¹¹¹. Furthermore, the presence of both folate ligand and the PSA-responsive peptide significantly enhanced the RNA-mediated gene silencing and growth inhibition of the cancer cells. *In vivo* studies showed selective and prolonged accumulation of the dual-functionalized liposomes in mice prostate tumors (subcutaneously 22Rv1 xenograft). The liposomes reduced the tumor growth significantly¹¹¹. This system is very promising for highly specific delivery of therapeutic agents to prostate cancer.

Intracellular Enzymes

Cathepsin B

Cathepsin B, a lysosomal protease of the papain family, is overexpressed in several pathological conditions and malignancies, including the brain, breast, colon, prostate, and lung cancers¹¹⁴. The enzyme enhances extracellular matrix degradation through interaction with cystatins and annexin II tetramer. Cathepsin B also affects the activity of other proteases such as MMPs, uPA, and plasmin¹¹⁴.

Increased levels of cathepsin B have been exploited to activate liposomal fusogenicity at the target sites. These liposomes were composed of DOPE, a fusogenic non-bilayer forming phospholipid. At the physiologic pH, DOPE adopts an inverted hexagonal H_{II} phase¹¹⁵.

However, incorporation of a polymer-lipid conjugate stabilizes the structure of liposomes containing DOPE in a lamellar phase. In Romberg's study, the fusogenic liposomes were stabilized by poly hydroxyethyl L-glutamine (PHEG) anchored to the N-succinyl-diocetadecyl amine (DODASuc-PHEG, Figure 9)¹¹⁶. The incorporation of PHEG prolonged the blood circulation half-life of the liposomes in rats¹¹⁷. PHEG is degradable by the proteases cathepsin B, pronase, and papain. Therefore, the authors hypothesized that at the high concentrations of these enzymes at the target site would cleave the peptides, leading to structural change, destabilization, and fusion of liposomes. In the *in-vitro* study, the presence of 36 µg pronase E/µmol total lipid led to complete release of the liposomal contents following 24 hours. The release from control liposomes containing DOPE/PEG-DSPE was not different by pronase E¹¹⁶. The results indicate that shedding of the polymer from the surface is necessary for the destabilization of the liposomes and release of the contents. It would also be interesting to investigate the contents release from these liposomes in the presence of cathepsin B.

Since cathepsin B is mainly found in the acidic environment of lysosomes, liposomes responsive to both acidic pH and cathepsin B have been developed for targeted intracellular delivery of therapeutic agents. In this approach, pH-sensitive liposomes are prepared from unsaturated phosphatidylethanolamines, such as DOPE (non-bilayer forming lipid) and a stabilizer, such as oleic acid, cholesterol hemisuccinate, and palmitoyl homocysteine¹¹⁸. The stabilizers are acidic amphiphiles, negatively charged at physiological pH. Therefore, due to electrostatic repulsion, liposomes can form a stable bilayer structure at physiological pH. However, at lower pH, the carboxylate moieties of these stabilizers are protonated. The neutralization of the negative charge reverses liposomes from lamellar phase to hexagonal phase and releases the entrapped contents^{119, 120}. To increase the plasma stability of pH-sensitive liposomes containing DOPE/cholesterol hemisuccinate, Zhang et al. incorporated 15 mol% of mPEG₂₀₀₀-DSPE in the bilayer¹²¹. The mPEG-lipid conjugate further stabilized the lamellar phase and reduced the pH-sensitivity of the liposomes^{122, 123}. Therefore, Zhang et al. developed formulations in which mPEG was attached to DSPE either via a disulfide bond or cathepsin B-cleavable peptide. It was hypothesized that the cleavage of mPEG groups would restore the pH sensitivity of liposomes. However, in their study, the release rates were not significantly different between liposomes with cleavable and non-cleavable mPEG at pH 5.5 in the presence of cathepsin B and dithiothreitol. This might be due to the remaining lipid anchor (modified DSPE) which inhibited hexagonal H_{II} transition and release of entrapped doxorubicin¹²¹. Moreover, insufficient cleavage of PEG might have inhibited the release of the encapsulated drug from PEG cleavable liposomes in acidic pH.

Conclusions

Liposomes are nanocarriers with a lot of potential to deliver therapeutic agents to the diseased sites. In the last few decades, several liposomal drugs have been marketed and are commercially available⁵. Several strategies have been employed to enhance the targetability of liposomal formulations. For the environmental stimuli-responsive liposomes, the release of the payload is triggered by a stimulus at the target site. Enzyme-responsive liposomes release their cargo upon exposure to an upregulated enzyme in the targeted tissue. The most

common examples of upregulated enzymes in cancerous or inflammatory tissues are sPLA₂ and proteases, such as cathepsin B.

Different strategies have been developed for liposomal contents release in the presence of enzymes. Usually, a biocatalytic reaction at the membrane surface induces perturbation and disrupts the integrity of the lipid bilayer. The structural perturbation releases the encapsulated cargo at the diseased site. In another strategy, liposomes are stabilized by a polymer coating, degradable by a specific enzyme at the target site. Enzymatic cleavage of the polymer destabilizes liposomes and releases the encapsulated contents. Enzyme-responsive liposomes may also carry a ligand that further enhances targeted delivery. Liposomal prodrugs are also of considerable interest for enzyme responsive delivery. In this approach, liposomes contain drug molecules chemically conjugated to the lipids of the bilayer. The active drug is released from liposomes after the linker is cleaved by an enzyme in the target site. This strategy offers advantages for delivery of highly toxic lipophilic drugs that show low loading efficiency by conventional encapsulation methods.

Although enzyme responsive liposomes have been successfully developed and tested in vitro, the translation into clinical studies is lacking. The complex design, sophisticated pharmaceutical development, and difficulties in scale-up make the translation of enzyme responsive liposomes from bench to bedside challenging. In addition, the efficacy of enzyme responsive liposomes, similar to other endogenous stimuli-responsive nanocarriers, may be different from one patient to another due to different levels of the trigger at the target tissue¹²⁴. Therefore, future studies need to address the applicability and effectiveness of these novel formulations in chemotherapy.

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Abbreviations

PEG	polyethylene glycol
sPLA₂	Secreted phospholipase A ₂
DSPC	1,2-distearyl- <i>sn</i> -glycero-3-phosphocholine
DSPE	1,2-distearyl- <i>sn</i> -glycero-3-phosphoethanolamine
DSPG	distearoylphosphatidylglycerol
PLA₂R1	phospholipase A ₂ receptor
DPPC	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
AELs	ether lipids
MMPs	Matrix metalloproteinases
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine

HCC	hepatocellular carcinoma
uPA	urokinase plasminogen activator
PHEG	poly hydroxyethyl L-glutamine
DODAP	1,2-dioleoyl-3-dimethylammonium-propane
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
PSA	Prostate-specific antigen
CPP	cell penetrating peptide
PSMA	prostate specific membrane antigen

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Figure 1. Schematic representation of a drug-loaded liposome, and structure of two common phospholipids. **(A)** Hydrophilic drugs are encapsulated in the liposomal core or conjugated to the surface and the hydrophobic drugs partition into the lipid bilayer. **(B)** 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), **(C)** 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE).

A. Phosphatidylethanolamine

B. Phosphatidylcholine

C. Phosphatidylglycerol

D. Phosphatidylserine

Figure 2.

(**A**) Neutral phospholipid and (**B**) anionic phospholipids.

Figure 3.
(A) Drug release from sPLA₂ sensitive liposomes, and (B) sPLA₂ mediated hydrolysis of DSPE.

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Figure 5. Schematic structure of ProAELs which form liposomes. sPLA₂-mediated hydrolysis of ProAEL releases AEL and fatty acid in the tumor tissue.

Figure 6. MMP-responsive liposomes. The MMP-cleavable peptide is hydrolyzed between Gly and Ile. Cleavage of this peptide removes the long PEG chain from the surface of liposomes and exposes the targeting ligand.

Figure 7. MMP-responsive liposomes targeted to hepatocellular carcinoma (HCC). In the tumor tissue, a high concentration of MMP-2 cleaves the linker and removes PEG from the surface of the liposome. This results in the exposure of galactose on the surface and subsequent uptake of liposomes by HCC cells.

Figure 8. PSA responsive liposomes. The liposomes are decorated by a peptide with three domains (cell penetrating, PSA-cleavable, and polyanionic) and a targeting ligand (folate). Cleavage of the peptide by PSA liberates the polyanionic domain and activates cell penetrating peptide, leading to increased uptake of the liposomes by prostate cancer cells.

Figure 9.

Fusogenic liposomes stabilized by DODASuc-PHEG (A). In the presence of pronase E, the polyamino acid is degraded (B), leading to the destabilization and fusion of liposomes (C). Conversion of liposomes from the lipid bilayer phase to the inverted hexagonal H_{II} phase releases the encapsulated contents (adapted from Romberg B et al., 2008¹¹⁶).