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Liposomes as Triggerable Carrier for Intracellular Drug Delivery

Neeraj K. Sharma and Vimal Kumar*

Department of Phytopharmaceutical & Natural Products, Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, India

Abstract: Administration of drugs is often associated with basic problems such as toxicity, instability and improper biodistribution. Encapsulation of the drug in a suitable carrier such as liposomes is one of the well recognized methods that protects the drug from the body milieu to improve stability, safety and targeting efficiency of the drug towards the target site. However, while encapsulation provides a favorable biodistribution, low toxicity and improved stability of drugs, delivery carriers should be capable of releasing encapsulated material at the appropriate site to exert their activity. Effective intracellular drug delivery is desired for many therapeutic agents those having specific molecular targets in the cytoplasm, nucleus, or other subcellular compartments of a cell such as mitochondria. Several nanocarriers that are designed to release drugs at desired sites have been developed so far. Among these, exhaustive research has established liposomes as an effective triggerable drug carrier. This mini review covers various aspects of triggering modalities examined to date such as temperature, pH, enzymes and light using liposomes.

Keywords: Drug delivery, liposomes, pH sensitive, photosensitive, thermo sensitive.

INTRODUCTION

Liposomes have been exhaustively explored as a drug delivery system for over 35 years resulting in a number of clinical applications including cancer chemotherapy, severe fungal infections, gene delivery and imaging. They are efficacious because of their ability to (i) encapsulate a therapeutically appropriate drug loading, (ii) prolong and sustain circulation time of drug (iii) accumulate drug at the target site, (iv) release their payload at levels sufficiently high enough to generate a therapeutic response and (v) improve bioavailability of the drug. These lipid based nanomedicines can be tailored for targeted delivery to various specific receptors [1, 2]. The advancement in this area during past 15 years has developed several approved therapeutic products based on liposomal technology. The clinical applications of liposomes are well known (Table **1**) [3]. Conventional therapeutic agents distribute throughout the body leading to a relatively low concentration at the target site, resulting in unwanted side effects to the healthy tissues. Now the scenario has been shifted towards site-directed delivery of drugs in pharmaceutical research [6]. Hence, a suitable drug delivery approach is desirable for targeted delivery, improved drug uptake and release in therapeutic acceptable amount. To solve these problems, wide varieties of multifunctional and stimuli-responsive carriers have been studied specially for localized drug delivery in recent years and results have been promising [7-9]. The most common fate of drug delivery systems is endocytosis that mediates through lysosomes and ends up by dumping entrapped material into the cytosol. This pathway constrains the drug release into the cytosol effectively. Therefore, the direct delivery of the drug to cytosol or bypassing this endocytic pathway would be highly recommended [10]. Effective intracellular drug delivery is also critical for drugs which have specific molecular targets inside a cell. The targets can be located in the cytoplasm (glucocorticoid receptors, therapeutic agents such as 5-Fluorouracil), nucleus (Deoxy ribo nucleic acids, antisense oligonucleotides, therapeutic agents such as doxorubicin), mitochondria (anti-oxidants) or other orgenelles of a cell. Furthermore, efflux transporters such as multidrug resistance proteins and P-glycoproteins present serious problem by extensive efflux of drug from the cell; hence cytosolic delivery is required [11]. Plasmid DNA has its site of action in nucleus. Successful gene delivery has serious limitation whether plasmid DNA is able to localize and integrate with the nuclear or mitochondrial DNA. These therapeutic entities suffer also from enzyme degradation present in cytosol and nucleus. Suitable delivery system is needed in order to protect them from the action of proteases and nucleases. Beside this the plasmid DNA has half-life of only 60 to 90 min. in cytosol, hence protection from degradation is critical for enhanced gene expression [12].

LIPOSOMES FOR CYTOSOLIC DELIVERY OF THERAPEUTICS

Liposomes have been established as a potential drug delivery carrier for cytosolic delivery because of the enormous potential of their structure and compositions [13]. In order to reach the site of action, drug has to cross a series of membrane barriers present in the cells and significant portion of the therapeutic agents is lost at each subsequent step. These obstacles are represented by the cellular association and inactivation of drug after internalization of the drug carriers by

*Address correspondence to this author at the Department of Phytopharmaceutical & Natural Products, Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, India; Tel: + 91-9824148514; Fax: 02717- 241916; E-mail: cognosy@gmail.com

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Table 1. List of commercially available liposomal products [2-5].

Type of Formulation	Active Drug	Brand Name	Manufacturer	Indication
Liposomes	Amphotericin B	Fungisome	Lifecare Innovations (India)	Fungal infections
Liposomes	Amphotericin B	Ambisome	Gilead sciences	Fungal and protozoal infections
Liposomes	Daunorubicin	DaunoXome	Gilead sciences	Kaposi's sarcoma
Pegylated liposomes	Doxurubicin	Doxil/Caelyx	Orthobiotech Schering-plough	Refractory Kaposi's sarcoma; ovarian cancer; recurrent breast cancer
Pegylated liposomes	Doxurubicin	Lipodox	Sun Pharma (India)	Ovarian cancer Multiple myeloma HIV-related Kaposi's sarcoma
Liposomes	Doxurubicin	Myocet	Zeneus pharma Sopherion therapeutics	Combinational therapy of recurrent breast cancer
Sustained release liposomes	Cytarabine	DepoCyt	Skyepharma Enzon	Lymphomatous meningitis
Depot liposomal injection	Morphine	DepoDur	Pacira Pharmaceuticals	Postsurgical analgesia
Liposomes	Vincristine	Marqibo	Talon Therapeutics	Ph- acute lymphoblastic leukemia
Sustained release liposomes	Vincristine	Onco TCS	Inex Pharmaceuticals Corporation	Non-Hodgkin's lymphoma

endocytosis followed by several processes. The commonest mechanism for liposomes to get into the cells is the association with the cell membrane and then internalization into cells by means of endocytic pathway. The efficiency of drug-carriers for cytosolic delivery of therapeutics is limited mainly by their improper interaction with cell membranes and the endocytic disruption of carriers. Early endosomes consist of the vesicles containing the therapeutic agents coming from the cell surface. Late endosomes followed by lysosomes, receive the internalized materials from early endosomes. Lysosomes as the last parts of the endocytic pathway contain the hydrolytic enzymes which digest the contents of the late endosomes. Therefore, the endosomal escape of the therapeutics is necessary before lysosome mediated digestion of the therapeutics occurs. After that cytoplasmic translocation of drug or drug-carrier to nucleus or any other cellular organelle; and the nuclear/organellar uptake is a critical step (Fig. **1**) [14]. Incorporation of steric stabilizers such as polyethylene glycol (PEG) lipids significantly improves the circulation half life and biodistribution of liposomes. Anchoring with targeting ligands which can bind to specific receptors on cell membranes is a promising approach for enhancing cell recognition and association of drug-carriers with cell membrane. This improves uptake of drug-carriers to the cell membrane by means of receptor-mediated endocytosis. Besides this triggering of delivery vehicle is essential and critical for endosomal escape. This has opened numerous avenues for research into development of strategies to enhance the endosomal escape of drug-carriers in order to improve the efficiency of cytosolic drug delivery [15]. These triggering processes should have low toxicity, reliability, low cost, and activation within the early endosome to allow efficient transfer of material to the cytoplasm of target cells [1].

DELIVERY ENHANCEMENT VIA TRIGGERED RELEASE

Photosensitive Liposomes

One of the safe and effective approaches to stimulate the release of encapsulated compounds from liposomes is light, due its control over spatial and temporal delivery of the radiation. Destabilization of the lipid membrane components by light-induced isomerization, cleavage, or polymerization is responsible for photochemical activation of liposomal content release [16]. Photoisomerizable moieties most frequently used for light-controlled release of liposomal contents are based on azobenzene in the form of 1,2-bis(4-n-butylphenylazo-4′- phenylbutyroyl)-L-α-phosphatidylcholine (Bis-azo PC) [17]. Photoisomerizable liposomes have also been prepared using retinoyl-phospholipids. Liposomal content release was also accomplished by photoisomerization of spiropyran [18, 19]. Controlled release by photocleavage typically involves photoinduced cleavage of naturally occurring lipids called plasmalogens by photodynamic sensitization [20]. The UV-induced cross-linking polymerization of 1,2-bis[10-(2',4'-hexadienoyloxy)-decanoyl]-sn-phosphatidylcholine (Bis-sorbPC) in liposomes comprising cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), and PEG2000-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), caused an over 100-fold increase in the permeability of an encapsulated fluorescent marker [21, 22]. In a very recent study, a novel photosensitive egg yolk phosphatidylcholine (PC) liposomes were formulated by modifying the liposomal surfaces with hydrophobically modified Poly(vinyl alcohol)–epoxypropoxy coumarin conjugate (HmPVA-EPC). 5(6)-carboxy fluorescein was used as a fluorescence marker and decanoyl chloride (DC) as a hydrophobic pendant for the hydrophobic modification of poly vinyl alcohol (PVA). Under UV irradiation (λ = 254 nm), fluorescence marker was readily released

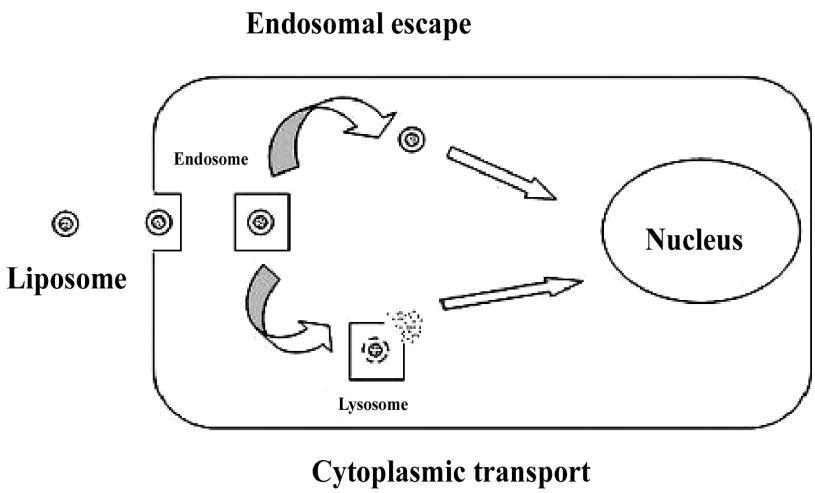

Fig. (1). A diagrammatic representation for the internalization of therapeutics encapsulated into the cell through endocytosis and subsequent endosomal escape.

for 60 min from EPC liposomes (lipid/HmPVA-EPC ratio 1:0.1) due to the photo-dimerization of EPC residues [23]. Various drug delivery systems have been exhaustively examined for targeted intracellular delivery of small-molecular chemotherapeutic drugs for the treatment of cancer. In this manner, therapeutic efficacies were enhanced as well as side effects minimized. A variety of anticancer drugs act as DNA-toxins that bind to nuclear DNA or its associated enzymes to employ their cytotoxic effect on cancer cells [24]. After entering into tumor cells, they need to be further delivered to the nucleus for therapeutic effect. For the design of vehicles capable of delivering drugs to the nucleus, particularly for *in vivo* applications, recently a novel class of phototriggerable liposomes composed of dipalmitoyl phosphatidylcholine (DPPC) and Photopolymerizable diacetylene phospholipid ($DC_{8,9}PC$) was formulated. Phototriggering of these liposomes using visible light was examined to explore these formulations for *in vivo* applications and the effect of released anticancer drugs on cellular toxicity was assessed. Very initial experiments with calcein loaded liposomes containing various ratios of DPPC:$DC_{8,9}PC$ and 4 mol% distearoyl phosphoethanolamine (DSPE)-PEG2000 showed significant release of calcein. On the basis of these results, studies with doxorubicin (DOX) loaded liposomes were performed. The effect of 514 nm laser treatment on DOX release, and cellular toxicity by released DOX were examined. Prepared liposomes were stable and after 514 nm laser treatment wavelength-specific release was observed by DOX loaded liposomes. Laser treatment of co-cultures containing DOX-loaded liposomes and cells (Raji and MCF-7) resulted in at least 2–3 fold enhanced cell killing as compared to untreated samples. To the best of our knowledge, this was the first report demonstrating improved cell killing after light-triggered release of an encapsulated anticancer agent from photosensitive liposomes [25].

Liposomes in Treatment of Cancer Using Photodynamic Therapy

An additional therapeutic tool is provided by photodynamic therapy (PDT) for the treatment of certain non-melanoma skin cancers. In this treatment photosensitive drugs are delivered to skin lesions followed by targeted light exposure. Generation of reactive oxygen species, eventually leads to cell death. Actinic keratosis and non-melanoma skin cancers are now being treated using PDT [26, 27]. The limiting factor in the success of PDT is the poor penetration of photosensitizers through biological barriers like skin and cell membranes. Long circulatory and targeted liposomes are more effective in this respect because conventional liposomes suffer from short plasma half-life that is insufficient for an adequate level of tumor uptake [28]. To solve this problem, aminolevulinic acid (ALA) and its esterified derivatives ALA-Hexyl ester (He-ALA) and ALA-Undecanoyl ester (Und-ALA) were delivered using liposomal formulations. All formulations showed good entrapment with stable vesicles upon storage for 1 week at 4^0C [29]. Beside this PDT is an innovative, evolving approach for treating neovascular diseases of the eye [30]. One recent use of liposomes in this field was carried out by formulating verteporfin encapsulated in cationic liposomes (CL-VTP). Results are comparable between CL-VTP and visudyne® in the treatment of choroidal neovascularization. PDT using CL-VTP was associated with less retinal damage compared to visudyne® [31].

Gold-Embedded Photosensitive Liposomes for Drug Delivery

The liposomes encapsulated with gold nanoparticles are easily accessible to light irradiation, provide a useful tool for controlled release of drugs in specific areas such as the eye and skin. Deeper tissues of the body could be accessed by proper adjustment of laser wavelength. In three different approaches, hydrophobic nanoparticles were embedded into the lipid bilayer, negatively charged hydrophilic nanoparticles were encapsulated in the core of liposomes, and lipid functionalized gold particles were localized on the inner and the outer surface of the liposomes [32]. Recently, mechanism of light-induced changes and functionality for liposomes embedded with gold nanoparticles was investigated. Phase transitions in distearoyl phosphatidylcholine (DSPC)/DPPC

liposomes upon heating were analyzed using real time small angle X-ray scattering. Gold nanoparticles absorb light energy and convert it to heat, cause lipid phase transition from gel to fluid phase. Internalization of gold nanoparticle-loaded liposomes into the human retinal pigment epithelial cell line ARPE-19 cells followed by light-triggered release of hydrophilic fluorescent probe (calcein) was demonstrated with fluorescence-activated cell sorting [33].

pH Sensitive Liposomes

PSLs have been developed to improve efficiency of the cytoplasmic delivery of various therapeutics. After endocytic internalization, acidic environment of the endosome destabilizes and/or fuses the liposomes with the endosomal membrane and then releases their contents into the cytoplasm escaping transfer to the lysosomes. So far, a number of pH-sensitive liposomes have been prepared using unsaturated PE and amphipathic stabilizers. They are more effective as a cytoplasmic delivery system but stability is poor compared to conventional liposomes [34]. Role of inclusion of DOPE in their composition was investigated. Results with cells pretreated with metabolic inhibitors or lysosomotropic agents clearly indicate that DOPE-containing liposomes are internalized essentially by endocytosis and that acidification of the endosomes is not the only mechanism involved in the destabilization of the liposomes inside the cell [35]. The most studied class of pH sensitive liposomal formulations consists of unsaturated PE combined with mildly acidic amphiphiles, such as oleic acid or Cholesterylhemisuccinate (CHEMS), but *in vivo* applications have been limited by their moderate stability and/or rapid removal by the mononuclear phagocyte system after intravenous administration. This study demonstrated that pH-responsiveness can be imparted to PEGylated liposomes by anchoring hydrophobically modified copolymers of N-isopropylacrylamide (NIPAM) and methacrylic acid at their surface [36-39]. Liposomal ability to promote pH-triggered cytoplasmic delivery of loaded material, establishes them as a favorable delivery systems for *in vivo* targeting of therapeutic moeites to tumors [40].

pH Sensitive Liposomes in Treatment of Cancer

PSLs composed of DOPE and CHEMS in 3:2 molar ratio were formulated and N-acetylglucosamine derivative of bovine serum albumin (N-Ac-BSA) was used as a ligand for binding to the asialoglycoprotein receptor. Fluorescence-activated cell sorting and confocal microscopy were applied to monitor the association of liposomes with chicken hepatoma. Reported delivery system showed remarkable potential in receptor-mediated targeting to chicken hepatoma cells [41]. pH-sensitive sterically stabilized liposomes to promote the release of DOX were also formulated. They were able to increase the cytotoxicity of DOX *in vitro* against B lymphoma cells. In order to develop more stable PSLs formulations, various molar ratios of the membrane rigidifying lipid, hydrogenated soy phosphatidylcholine (HSPC) and/or cholesterol (CHOL), were added to the lipid composition and mixture of DOPE/HSPC/CHEMS/CHOL/mPEG2000-DSPE at a molar ratio of 4:2:2:2:0.3 and DOPE/HSPC/CHEMS/CHOL at a molar ratio of 4:2:2:2 showed the best drug retention and pH-sensitivity [42]. In another study, stealth pH-sensitive liposomes containing cisplatin (SpHLs-CDDP) were formulated and the tissue distribution compared with free cisplatin (CDDP) was evaluated in solid Ehrlich tumor-bearing mice. Studies revealed that longer circulatory SpHLs-CDDP led to a higher accumulation of CDDP in SpHLs-CDDP administrated tumors [43]. Multidrug resistance and drug toxicity represent major obstacles to cancer chemotherapy. In order to overcome these, acute toxicity studies were performed for SpHLs-CDDP after their intraperitoneal administration in male and female mice with a single administration of free CDDP (5, 10 and 20 mg/kg) or SpHLs-CDDP (7, 12, 30, 45 and 80 mg/kg). The results revealed that SpHLs-CDDP can eliminate CDDP-induced toxicity and is thus a promising candidate for intraperitoneal chemotherapy [44]. Use of pH-responsive polymers like high-molecular weight poly(styrene-co-maleic acid) can also provide pH-sensitive property to liposomes by conformational transition from a charged extended structure to an uncharged globule below its pK1 value. Cytosolic delivery of bio-active molecules through endosome destabilization was mediated by spherocyte formation. The high-molecular weight poly(styrene-co-maleic acid)-loaded liposomes efficiently delivered 5-FU within colon cancer cells (HT-29) with improved cytocompatibility compared to conventional liposomes. This resulted in enhanced apoptosis due to increased availability of the drug at the cellular level and established the clinical potential of styrene-co-maleic acid-based vesicles [45]. Immunoliposomes (ILs) directed by monoclonal antibodies are promising vehicles for tumor targeted drug delivery. Development of a long-circulating formulation of PSLs with EGFR antibody attached was designed and tested using human non-small cell lung cancer cell line (A549) and BALB/c-nu/nu mouse tumor model. This suggests that treatment of Ab-PSLs with gemcitabine resulted in an increased apoptosis of tumor cells leading to tumor growth inhibition [46]. Another work was represented by pH-sensitive ILs formulated by including a terminally alkylated copolymer of NIPAM in the liposomal bilayer and by coupling the anti-CD33 monoclonal antibody to target leukemic cells. The content release inside the endosomes was mediated through a polymer structural change following receptor-mediated internalization. Flow cytometry and confocal microscopy analysis demonstrated that the pH-sensitive ILs were efficiently internalized by various CD33+ leukemic cell lines while limited interaction was found for liposomes anchored with an isotype-matched control antibody. Finally, the pH-sensitive ILs-CD33 formulation exhibited the highest cytotoxicity against HL60 cells, confirmed the role of the NIPAM copolymer in promoting the escape of intact arabinoside in the endosomes [47].

Gene Delivery Using pH Sensitive Liposomes

Liposomes are phospholipid bilayer vesicles with potential application in gene delivery. Novel pH-sensitive liposomes with different combinations of cationic / anionic lipids were formulated and evaluated for intracellular gene delivery. FR targeted pH-sensitive liposomes composed of dimethyldioctadecylammonium bromide/CHEMS/ folate-poly-PEG-PE), combined with polylysine-condensed plasmid DNA, were shown to mediate FR-specific delivery of a luciferase reporter gene into KB cells in the presence of 10%

serum. The findings suggested that cationic lipid-containing pH-sensitive liposomes, combined with FR targeting, can be employed as effective vehicle for intracellular gene delivery [48]. Dendritic cells (DCs) are potent antigen presenting cells, useful for cancer immunotherapy. Complexes of lipoplexes with pH-sensitive fusogenic liposomes, which comprise polymers based on poly(glycidol) with carboxyl groups were formulated and evaluated for transfection of a murine DC line DC2.4. In addition, no effects were observed on transfection or cell association after anchoring with ligands such as transferrin and mannan. It was found that endosomal escape is critical for transfection into DC2.4 cells. These complexes with pH-sensitive fusogenic polymers exhibited higher transfection activity toward DC2.4 cells compared to some commercial agents and hence may be useful as a gene vector for DCs [49]. Antisense oligonucleotides are specially effective for the treatment of viral infections, cancer and inflammatory diseases due to their ability to inhibit gene expression. However, delivery is associated with serious problems like poor stability in biological medium and weak intracellular penetration. Therefore, liposomes made of PE, able to release their contents in response to acidic pH within the endosomal system while remaining stable in plasma, can improve the cytoplasmic delivery of oligonucleotides after endocytosis [50]. In another experiment, proliferation of the friend retrovirus was specifically inhibited by the env mRNA complementary oligonucleotide encapsulated in liposomes compared with the same oligonucleotide incubated free where lack of antiviral activity was observed. Focus immunoassay or reverse transcriptase assay was employed to ascertain virus inhibition. In conclusion, protection of oligonucleotides against degradation and their more pronounced intracellular delivery was made possible with pH sensitive liposomes containing DOPE [51].

Peptide/Protein Delivery by pH Sensitive Liposomes

Long circulating classical liposomes or pH-sensitive stealth liposomes were used to increase circulation lifetime and impart intracellular delivery capacity for effective delivery of a therapeutic peptide to its nuclear site of action. Cellular uptake of peptide-loaded liposomes was also investigated in three cell lines: Hs578t human epithelial cells from breast carcinoma, MDA-MB-231 human breast carcinoma cells and WI-26 human diploid lung fibroblast cells using confocal microscopy and flow cytometry. Results revealed the important benefit of the pH-sensitive formulation over the conventional PEGylated formulation in terms of delivery of hydrophilic materials to the cytoplasm [52]. Highly pH-sensitive liposomes modified with 3-methylglutarylated poly(glycidol) of linear (MGlu-LPG) or hyperbranched structure (MGlu-HPG) were formulated. After subcutaneous or nasal administration, more effective generation of OVA-specific cytotoxic T cells were observed compared with unmodified liposomes. Furthermore, tumor burden was remarkably reduced after administration of the polymer-modified ovalbumin (OVA)-loaded liposomes to mice bearing E.G7-OVA tumor but showed slight effect on tumor growth. Results advocated them as promising vehicle of antigens for efficient cancer immunotherapy [53].

Thermosensitive Liposomes

Early liposomes have been employed for localized delivery of chemotherapy but technology has thereafter shifted towards more attractive stimuli-responsive liposomal approach. They have provided site-specific chemotherapy with triggered drug release at the target site; hence greater control over spatial and temporal therapy. Temperature - sensitive liposomes (TSLs) were first formulated by Yatvin and coworkers [54]. Mild hyperthermia (HT) can be used to improve liposomal chemotherapy by increasing vascular permeability in solid tumors and thus increase levels of liposomal accumulation, and deliver thermosensitive liposomes that release their contents by triggering by hyperthermia. By merging these two approaches, drug delivery to tumors can be remarkably enhanced [55, 56]. More recently, several attractive strategies for the development of thermo-sensitive vesicles include formulation with phospholipids having phase Tm between 41 and 42 ^{o}C or with leucine zipper sequence peptide which has dissociative, unfolding properties to optimize drug release under mild hyperthermia. Thermosensitive liposomes with long circulating properties have also been formulated using PEG or with novel lipid 1.2-dipalmitoyl-sn-glycero-3-phosphoglyceroglycerol [57-61].

Traditional Thermosensitive Liposomes for the Delivery of Chemotherapeutic Drugs

LTSLs were incubated *in vitro* over a range of temperatures and durations, and the amount of DOX released was measured as compared to NTSLs. For *in vivo* experiments, liposomes and free DOX were administered i.v. in s.c. murine adenocarcinoma tumors and mice were exposed to pulsed-high intensity focused ultrasound after 0 and 24 h. Combinations of the exposures and drug formulations were evaluated for doxorubicin concentration and growth inhibition in the tumors. Viable clinical results were produced by this strategy [62]. In another case, novel TSLs (40^{o}C) containing DOX together with local HT were formulated and evaluated for the treatment of solid growing rat rhabdomyosarcomas. Tumor inhibition and systemic toxicity were evaluated by comparing with free DOX with or without hyperthermia. Repeated treatments with DOX-liposomes + HT showed a statistically significant ($p<0.05$) tumor growth delay with minimum systemic toxicity as compared to free DOX [63]. Radiofrequency ablation was combined with lyso-thermosensitive liposomal DOX for developing a cure for medium and large tumors in hepatocellular carcinoma. Limiting factor in the curative efficacy of this therapy was unclear tumor margins [64]. Copolymers containing temperature-responsive nisopropylacrylamide (NIPAAm) and pH-responsive propylacrylic acid (PAA) were attached to formulate novel polymer-modified thermosensitive liposomes for the delivery of DOX for cancer. This formulation improved release profile and significantly ($p<0.0001$) lowered thermal dose threshold when compared to convetional thermosensitive formulations [65]. An improved thermosensitive formulation composed of DPPC and Brij78 loaded with DOX significantly altered stability in serum at 37 ^{o}C confirmed by cell based assays, and enhanced drug release rates at 41-42 ^{o}C as compared to lyso-lipid temperature sensitive liposomes. Mild hemolytic activity with comparable blood compatibility was displayed by Brij78-

liposomes and low TSLs [66, 67]. In another study, adriamycin (ADM)-encapsulated TSLs and conventional liposomes were formulated and evaluated for temperature-dependent drug release. More than 90% of loaded ADM was released from thermosensitive formulation within 30 min at 42 °C. However conventional liposomes released less than 3% in 120 min with the same condition as revealed by *in vitro* study. Body distribution and anti-tumor efficacy was evaluated in C6 glioma bearing mice model. The maximum brain concentration and survival time of mice were improved remarkably compared to ADM solution and liposomes-ADM, respectively [68]. TSLs were also developed to enhance targeting and antitumor effect of semi-synthetic therapeutic agent like vinorelbine bitartrate. A stable formulation that quickly releases drug at 42 ^{o}C was employed for *in vivo* experiments on lung tumor model. Overall inhibition of tumor was higher using temperature-sensitive liposomes group compared to the normal injection group [69]. Low TSLs and Non TSLs were developed and dual-labeled using ^{3}H-cholesteryl hexadecyl ether lipid and loaded with ^{14}C-DOX. Highest drug accumulation in tumors was observed using LTSLs due to ultrafast DOX release kinetics at 42 ˚C immediately after HT [70]. Magnetic resonance imaging controlled focused ultrasound HT was used in combination with thermosensitive liposomal DOX. DOX concentrations in heated tumors were found 26.7 times higher than in unheated tumors ($p = 0.017$) [71]. Delivery of chemotherapeutic agents after encapsulation in PEGylated liposomes provides advantages like frequent dosing, lower accumulation in healthy tissues and enhanced concentration in tumor periphery due to the EPR effect [72]. Based on this strategy combination of long circulating liposomes with thermosensitive properties was developed to deliver epirubicin hydrochloride (EPI) aiming at antitumor therapy. According to the *in vitro* results characterized by the fluorescence method, more than 90% of loaded EPI was released from EPI-low TSLs within 4 minutes at 43°C. The results of the pharmacokinetics study in rats revealed that EPI-low TSLs prolong the circulation time and enhance the *in vivo* performance compared to non thermal formulation and EPI-solution [73]. Another recent example was represented by combining stealth liposomal technology combined with hyperthermia mediated release by PEGylated cationic TSLs [74].

Gel Loaded Thermosensitive Liposomes for the Delivery of Chemotherapeutic Drugs

To reduce dosing frequency and sustain the drug action cytarabine-loaded liposomes were formulated and embedded in biodegradable and biocompatible chitosan-beta-glycerophosphate thermosensitive solution that has the property to gel at body temperature. This system sustained the release of encapsulated drug for more than 60 h as compared to drug-loaded liposomal suspension (upto 48 h). Formulation showed a higher t (1/2) (28.86 h) and AUC 2526.88 mug/mL h compared with cytarabine-loaded liposomal suspension and C-GP containing free cytarabine in rats as revealed by pharmacokinetic studies. Thus, these results showed that the formulation converted into gel form at body temperature and sustained the delivery of cytarabine [75]. Another in situ gel system was characterized by thermoreversible gel (Pluronic® F127 gel) for controlled release and improved antitumor drug efficiency of liposomes-containing paclitaxel (PTX). Result of increased viscosity of liposomal gel has the effect of creating a drug reservoir and hence longest drug-release period compared with liposomes, general gel, and commercial formulation Taxol® were observed with *in vitro* release experiment. Treatment with PTX-loaded liposomal 18% Pluronic F127 resulted into cytotoxicities, intercellular fluorescence intensity, and drug concentration in KB cells much higher compared to conventional liposomes [76].

Targeted Thermosensitive Liposomes for the Delivery of Chemotherapeutic Drugs

Magnetic hyperthermia-triggered drug release was achieved with targeted temperature sensitive magnetic liposomes recently for thermo-chemotherapy [77]. Recently folate receptor (FR) targeted thermo-sensitive magnetic liposomes were also formulated to explore the benefits of combination of drug targeting and magnetic hyperthermia-triggered drug release [78]. Formulation with human epidermal growth factor receptor 2 HER2 specific affibody (ZHER2:342-Cys) conjugated thermosensitive liposomes (HER2+affisomes) were developed. DOX-loaded HER2+ affisomes improved accumulation of DOX in human breast adenocarcinoma cells (SK-BR-3) by 2- to 3-fold compared to control liposomes. Brief exposure of liposomes–cell complexes at 45 °C prior to the onset of incubations improves cytotoxicity for affisomes and control liposomes. Under similar conditions, Doxil® however showed lower toxicity. Therefore, results revealed that HER2+affisomes demonstrate both targeting and triggering potential and may serve as effective delivery carrier for breast cancer treatment [79, 61].

Ultrasound Triggered Liposomes

Low frequency ultrasound (LFUS) to trigger the release of therapeutic agent nano sterically stabilized liposomes was evaluated for *in vivo* activity on BALB/c mice with C26 colon adenocarcinoma tumors in a footpad. The group treated by liposomal CDDP combined with LFUS had the best therapeutic efficacy compared to free cisplatin with or without LFUS, or liposomal CDDP without LFUS, or LFUS alone, or no treatment [80]. A different kind of work was reported for the preparation of a self-assembly liposomes-loaded microbubble by conjugating liposomes to microbubble surface with the help of covalent thiol-maleimide linkages [81]. These loaded complexes ensure a high drug payload and transportation along with drug release from bubble surface and/or from disrupted liposomes with the help of US [82]. Liposomes-microbubble complexes loaded with PTX were explored as possible ultrasound-triggered targeted chemotherapy against breast cancer. The amount of released payload from complex upon US exposure was found significant. It was found that complex plus US was effective for inhibition of tumor growth compared to complex without US both for *in vitro* and *in vivo* studies [83]. Combination of two consecutive steps — heating and sonication in the presence of microbubbles resulted in the release of cell-impermeable dye (TO-PRO-3) from the TSLs and was followed by evaluation of intracellular uptake. Problems associated with the cell impermeable drugs like clearance and/or degradation

in blood and no uptake by cells can be effectively solved using this formulation [10].

CONCLUSIONS

The development of delivery modalties for endosomal escape is an important area of research in therapeutic delivery of biologicals. Different strategies for endosomal escape use different distinguished approach. A safe endosomal escape agent should possess no immunogenicity and toxicity, have high efficiency, ease of application and production, and low cost large-scale manufacturing. 'Pharmaceutical' liposomes are under developed area which includes various suggested applications, and is supported by results from early clinical applications and clinical trials of different liposomal drugs. However, formulation-based research approach towards the goal of rapid, cytoplasmic release of materials from liposomal carriers has developed slowly. Recent advances with new classes of materials suggest that molecular-based approaches may offer new hope for the development of rational delivery mechanisms.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Declared none.

ABBREVIATIONS

ADM	=	Adriamycin
ALA	=	Aminolevulinic acid
He-ALA	=	ALA-Hexyl ester
Und-ALA	=	ALA-Undecanoyl ester
Bis-azo PC	=	1,2-bis4-n-butylphenylazo-4′-phenylbutyroyl)-L-α-phosphatidylcholine
Bis-sorbPC	=	1,2-bis[10-2',4'-hexadienoyloxy)-decanoyl]-sn-phosphatidylcholine
CHOL	=	Cholesterol
CHEMS	=	Cholesterylhemisuccinate
CDDP	=	Cisplatin
DOPE	=	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOPC	=	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine
DPPC	=	Dipalmitoyl phosphatidylcholine
DSPC	=	Distearoyl phosphatidylcholine
DSPE	=	Distearoyl phosphatidylethanolamine
DC	=	Decanoyl Chloride
DOX	=	Doxorubicin
EGFR	=	Epidermal Growth Factor Receptor
EPI	=	Epirubicin Hydrochloride
5-FU	=	5-Fluorouracil
FR	=	Folate Receptor
KB	=	Human Oral Carcinoma Cells
ILs	=	Immunoliposomes
LFUS	=	Low frequency ultrasound
NIPAM	=	N-isopropylacrylamide
OVA	=	Ovalbumin
PTX	=	Paclitaxel
PC	=	Phosphatidylcholine
PDT	=	Photodynamic Therapy
$DC_{8,9}PC$	=	Photopolymerizable Diacetylene Phospholipid
PSLs	=	pH-sensitive Liposomes
PEG	=	Polyethylene Glycol
HmPVA-EPC	=	Polyvinyl alcohol)–epoxypropoxy coumarin conjugate
PAA	=	Propylacrylic acid
SPC	=	Soy phosphatidylcholine
SpHL	=	Stealth pH-sensitive Liposomes
TSLs	=	Thermosensitive Liposomes
Tm	=	Transition Temperature
CL-VTP	=	Verteporfin loaded Cationic Liposomes

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