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Review article

Recent advances on thermosensitive and pH-sensitive liposomes employed in controlled release

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ABSTRACT

Nanotechnology has recently gained lots of interest in drug delivery due to its potential to improve the therapeutic outcomes of various diseases. Particularly, a wide range of different nano-sized vesicles has been investigated for drug delivery. Among them, one of the most attractive and well-investigated nanocarriers are liposomes. Although liposomes have several advantages such as low toxicity, biodegradability and biocompatibility as well as accumulate in tumor site *via* enhanced permeability and retention (EPR) effect, inefficient drug delivery to the target cells could affect the therapeutic purpose of most of conventional liposomal formulations. Therefore, new systems of drug release including stimuli-responsive liposomal have been introduced for the improvement of the efficacy and release payloads in a site-specific manner. Stimuli-responsive liposomes stay stable in blood stream circulation but are activated in response to internal or external stimuli. This review highlights the development of thermosensitive and pH-sensitive liposomes, focusing on liposomal compositions and the effects of the synthetic polymers on their drug release behavior. Furthermore, *in vitro* and *in vivo* applications of these formulations will be discussed.

1. Introduction

Nanotechnology can be defined as a technology that focuses mainly on the synthesis, manipulation and the study of structures and devices in a nanometer size range. There are optimistic stances that the application of nanotechnology in medicine will bring remarkable improvements in the diagnosis and treatment of different kinds of diseases [1,2]. One of the most important application-oriented fields in nanotechnology is nanomedicine, which is a promising aspect of the application of nanotechnology from the diagnosis to treatment of many kinds of diseases in medicine. Nanomedicine has incredible potentials to improve the conventional therapies by developing ingenious nanodevices for drug delivery purposes, which represents the most relevant application of nanoparticles [3]. Drug delivery systems based on nanoparticles have the capability for encapsulation a wide range of therapeutic moieties such as hydrophilic and hydrophobic drugs, protein-based drugs, peptides and nucleic acids [4,5]. The entrapment of these molecules inside the nanocarriers improves their solubility and stability in the biological environment [6,7]. The release of drug molecules could be in a controlled manner which has benefits like maintenance of drug concentration in systemic circulation or some stimulus at the target site could trigger the drug release [8,9]. The nanocarrier surface is usually modified to enhance the circulation time in blood and influence the biodistribution [10,11]. Nanoparticles, because of their sub-cellular and sub-micron size, can extravagate through the endothelium in inflammatory sites, such as tumors, or penetrate microcapillaries. This phenomenon allows therapeutic agents efficient delivery to target sites and toxicity reduction of free drug to non-target organs [11,12]. Also, when the nanocarriers are functionalized with some of the contrast agents such as superparamagnetic iron oxides, they have shown significant benefits in diagnostic applications [13]. Currently, clinically approved nanoparticles have consistently proved to

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decrease the toxic side effects of their cargoes, associated with traditional chemotherapy regimens [14,15]. These nanoparticles loaded with therapeutic agents have exhibited limited long-term success clinically, emphasizing the need to develop new and innovative strategies to improve their treatment efficacy by means of adding functional elements [16]. Over the last several decades, many investigations have been conducted on various nanoparticle platforms, including polymeric micelles, polymer-therapeutic conjugates, dendrimers, liposomes and nucleic acid-based nanoparticles for their applications in therapeutic purposes. Liposomes and polymer-drug conjugates are two dominant classes of nanoparticles accounting for the majority of available therapeutic nanoparticles in clinical use [17,18]. Lipid vesicles were the first nanotechnology-based drug delivery systems, which were first reported by Bangam et al. in the 1960s and then known as liposomes [19]. Liposomes are spherical vesicles that have an internal aqueous core surrounded with a single or multiple concentric lipid bilayers [20,21]. The first formulations were made of naturally occurring lipids, while currently they may also contain synthetic lipids and surfactants not existing in biological systems [22,23]. The ability of the encapsulation a diverse range of both lipophilic and hydrophilic agents is a unique feature of these liposomal systems. Increasing of the cellular penetration of hydrophilic molecules could be achieved through the more entrapment efficiency of them within an inner aqueous phase, whereas hydrophobic molecules are contained in the lipid bilayer membrane to be loaded the therapeutic moieties [24]. These sphere-shaped vesicles vary in size from a several micrometers to a few nanometers. But, liposomes with the size of between 50 and 450 nm are usually being used in medicine [22].The size of vesicle is a crucial parameter which determines the nanocarriers clinical success. Findings showed that liposomes with larger sizes were cleared from the blood circulation more quickly and did not escape from the reticuloendothelial system (RES) [25]. Liposomes resemble cell membrane in terms of structure and composition and consider as a significant candidate for the improvement of drug delivery systems [26]. Furthermore, liposomes as drug-delivery systems suggest various advantages such as biocompatibility, high loading capacity, increased half-life, low toxicity, good solubilization and stability of incorporating drugs and preventing the degradation of the medicine in the physiological environment [27,28]. Despite these advantages, they have demonstrated several pharmacological implications and problems over the years. A major disadvantage of the conventional liposomes is that the mononuclear phagocyte system (MPS) in the host immune system quickly recognized them that subsequently rapidly cleared by RES system in the liver and spleen [29]. To overcome the aforementioned limitations, several strategies have been taken. The development of stealth or long-circulating liposomes functionalized with polyethylene glycol (PEG), as an example of the hydrophilic polymers, was a major progress took place in the 1990s. Coating of liposomal surface with PEG has shown a significant prolong circulation time and a toxicity reduction of encapsulated drugs [24]. Doxil® in the USA and Caelyx in Europe were the first FDA-approved stealth or long-circulating PEG-coated liposomal doxorubicin (DOX) nano-drug used most often to treat the patients who had advanced AIDS-related Kaposi's sarcoma in order to tackle the failure of previous chemotherapy or intolerance therapies [30]. Table 1 shows that a good number of liposomal delivered drugs are available for clinical applications and additional ones are being used in differentclinical trial stages. Despite these promising aspects, the efficacy of these systems did not improve as expected due to passive and slow release of drug from these liposomes [31]. Furthermore, tumor vascular permeability is also significantly variable across various tumor types that leads to unpredictable liposomal extravasation into the tumoral tissue [32]. Thus, in order to improve these shortcomings, new strategies have been employed to design liposomal systems that respond to a specific stimulus and then release their payload in the target site [33]. Below we will discuss on the most representative thermosensitive and pH-sensitive liposomes and their design, release behavior of drug, thermally and pH triggered mechanisms.

1.1. Stimuli-responsive liposomes

Poor availability or uncontrolled release of the encapsulated drug and low stability in humans often limits the clinical applications of liposomes as drug carriers. Thanks to the development of various stimuli-responsive delivery platforms, precise spatial and temporal control over therapeutic payload release have been made possible [69,70]. Stimuli-responsive liposomes release entrapped cargo when being exposed to an exogenous or endogenous stimulant present at the target site. Some of the pathological changes such as low pH value within the tumoral tissues, higher concentration of glutathione or various levels of specific enzymes in different tissues are considered as endogenous stimuli that minimize the exposure of surrounding normal tissues to the drug because these triggers are absent in normal human tissues [71,72]. The pH-sensitivity properties at the cellular level could be used to release the encapsulated drug from the carriers into late endosomes or lysosomes or to promote the escape of nanoparticles from the lysosomes to the cell cytoplasm. At the tissue level, one could potentially use the particular microenvironmental changes related to pathological conditions such as inflammatory or infectious diseases or ischemia [73]. Extracorporeal physical stimuli have been employed for controlled drug delivery. For example, the targeted delivery of therapeutic molecules can be magnetically guided to a diseased cells by using supermagnetic iron oxide nanoparticles [74]. Sustained release of drug can also be conveniently obtained by light, ultrasound and temperature sensitive liposomes. Release of drug from stimuli responsive liposomes in response to particular endogenous or exogenous stimuli, provides more accurate control over the delivery and doses of drug in the target site [75].

2. Thermosensitive liposomes

In order to enhance the efficacy of cancer treatment, mild hyperthermia (HT), refers to increasing of tissue temperature up to 43 °C, has long been used together with chemotherapy and radiation therapy [76]. The application of hyperthermia (HT) in combination with thermosensitive liposomes (TSL) could improve the therapeutic efficiency of drugs by different mechanisms: (i) controlling drug release from TSL into the tumoral vascular and interstitial space, (ii) enhancing the accumulation of liposomes in the tumoral tissue by increasing blood follow and tumor vasculature permeability, (iii) increasing permeation of the cell membrane and susceptibility to released drug; and (iv) producing a direct cytotoxic effect on tumoral cells [77]. Although the tumor cells are not intrinsically more sensitive than normal cells to the heat injury, they are stressed inside the tumoral microenvironment by limited supplies of nutrients, low oxygen tension or hypoxia and acidic conditions, therefore less able to tolerate high temperature compared to normal cells. Tumoral cells with disorganized vascular network have difficulty dissipating heat, which can provide new avenues for selective targeting of cancer cells with heat. Therefore, the combination of mild HT with TSL has a great potential [77].

2.1. Current developments in thermosensitive liposomes for controlled drug delivery

2.1.1. Traditional thermosensitive liposomes (TTSL)

Yatvin et al. in 1978 suggested the first TSL formulation which is known as traditional thermosensitive liposomes (TTSL) with the aim of treating bacterial infections [78]. TTSL have been developed over the next few decades and their component phospholipids can undergo phase transitions as a response to heat. TSLs presenting phase transition temperature (Tm) at which the lipid physical state changes from a solid gel ordered phase to a highly permeable liquid crystalline phase, creating boundaries that resulted in drug release *via* the membrane

Table 1
Clinically approved liposome-based products. In addition, a selection of products present in clinical trials.

	Product——————————Approved products	Released drug	Indications	Administration	Year approval	Lipid composition	Company	Ref.
1	Doxil	Doxorubicin	Ovarian cancer, kaposi' s sarcoma breast cancer	i.v.	1995	HSPC: cholesterol: PEG_{2000}-DSPE	Sequus pharmaceuticals	[34,35,36,37]
2	Abelcet	Amphotericine B	Aspergillosis	i. v.	1995	DMPC: DMPG	Sigma-Tau pharmaceuticals	[38]
3	DaunoXome	Daunorubicin	HIV-related kaposi's Sarcoma	i. v.	1996	DSPC and cholesterol	NeXstar pharmaceuticals	[39]
4	Depocyt	Cytarabine	Neoplastic and lymphomatous meningitis	i. t.	1999	DOPC, DPPG, cholesterol and Triolein	Pacira Pharma	[40,41]
5	Myocet	Doxorubicin	Metastatic breast Cancer	i. v.	2000	EPC: cholesterol	Enzon pharma	[42]
6	Mepact	Mifamurtide	Osteosarcoma	i. v.	2004	DOPS: POPC	Takeda pharmaceuticals	[43,44]
7	Exparel	Bupivacaine	Pain management	i. v.	2011	DEPC, DPPG, cholesterol and Tricaprylin	Pacira pharmaceuticals	[45,46]
8	Marqibo	Vincristine	Acute lymphoblastic leukemia	i. v.	2012	SM: cholesterol	Talon Therapeutics	[47,48]
9	Onivyde	Irinotecan	Pancreat cancer	i. v.	2015	DSPC: $mPEG_{2000}$:DSPE	Merrimack pharmaceuls	[49,50]
	Products in clinical trials							
10	INX-0076	Topotecan	Relapsed solid tumors	i. v.	Phase I	Cholesterol and sphingomyelin	Inex pharmaceuticals	[5,51]
11	OSI-211	Lurtotecan	Ovarian cancer Squamous cell cancer of head and neck	i. v.	phaseII	HSPC and cholesterol	OSI pharma	[52]
12	Endotag-I	Paclitaxel	Pancreatic cancer Triple negative breast cancer	i. v.	Phase II	DOTAP: DOPC	Medigene AG	[53]
13	Alocrest	Vinorelbine	Non-small cell lung cancer and breast cancer	i. v.	Phase I	Sphingomyelin cholesterol	Spectrum pharmaceuticals	[54]
14	Thermedox	Doxorubicin	Primary/metastatic liver cancer Recurrent chest wall breast cancer	i. v.	phaseIII	DPPC, Myristoyl stearyl phosphatidylcholine and DSPE-*N*-[amino (polyethylene glycol) -2000]	Celsion	[55,56,57]
15	Arikace	Amikacin	Lung infections	Aerosol	phaseIII	DPPC and cholesterol	Transave Inc.	[58,59,60]
16	annamycin	L-annamycin	Acute lymphocytic leukemia Acute myeloid leukemia	i. v.	Phase II	DMPC and DMPG	Aronex pharmaceuticals	[61,62]
17	SPI-077	Cisplatin	Solid tumors	i. v.	Phase II	Soybean phosphatidycholin cholesterol	Alza	[63,64]
18	LEM-ETU	Mitoxantrone	Different cancers	i. v.	Phase I	DOPC, cholesterol and cardiolipin	Neopharmlabs	[5,51]
19	T4N5 liposlotin	T4 endonucle V	Xeroderma pigmentosum	Topical	Phase III	Egg lecithin	AGI DermaticInc.	[65,66]
20	LEP-ETU	Paclitaxel	Different cancers	i. v.	Phase II	DOPC, Cardiolipin cholesterol	Neopharmlas	[67,68]

permeability. Lipids in the gel phase are ordered and condensed. The hydrocarbon tails become fully extended, and the lipid head groups are highly immobile at the water interface. Upon raising the temperature, the lipid head group mobility increases, with further increases in temperature and close to Tm changes in the orientation of C−C single bond in hydrophobic chains take place and switch from a trans to gauche state. Leaky interface regions formation at boundaries started between solid and liquid lipid domains [79]. Thus, permeability of lipid bilayer increases at the interfaces, which has been signified as an abnormal peak in the ion permeability through the lipid membrane at the Tm. At temperatures above Tm, the bilayer is present in the liquid phase. Each lipid molecules of the bilayer are confined to a two-dimensional plane, but they can freely move within the plane. Thus, the lipid membrane becomes completely fluidized and permeable. The loaded drugs are able to leak out of the TTSL during the phase transition [80]

The initial versions of TTSL were comprised of dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC) with a transition temperature higher than normal body temperature (42–44 °C) which is ideal for HT applications. This formulation increased drug release, but the rate and amount of drug released from liposome was small [80]. Addition of lipid components such as hydrogenated soy phosphocholine (HSPC), had a positive influence on the amount and drug release rate [81].

TTSL have been further developed by incorporation of cholesterol to the lipid bilayer which enhanced the stability of liposome and reduced the leakage of the entrapped drug from the liposome when exposed to serum [82]. However, the addition of a cholesterol molecule could increase the Tm that resulted in a negative effect on both of encapsulation efficiency and release rate of drug in a certain temperature [81]. Following the development of stealth PEGylated liposomes, many studies have focused on how to increase the permeability of the liposomal membrane and extend the circulation time in blood by preparation of stealth TTSLs. The presence of GM1or DSPE-PEG2000 in TSL formulation leading to increased MPS evasion and effective delay in tumor growth [83]. Li et al. showed that the appropriate PEG concentration required for maintaining TSL stability and temperature sensitivity at mild HT was 5 mol% DSPE-PEG 2000. Destabilization of lipid membrane is caused by the heterogeneous structure of DSPE-PEG2000 and enhanced the release of the encapsulated content without significantly affecting the Tm [84]. Improved sensitivity was obtained from DPPC/HSPC liposomes containing both cholesterol and PEG, but the release of drug from these liposomes was relatively low [81].Alternatively, Hossann et al. proposed a new formulation for TSL with a prolonged blood circulation time without the use of PEGylated lipids, based on inclusion of DPPGOG lipid into DPPC: DSPC liposomes. The DPPGOG based formulation showed significant increase in content release upon heating [85,86]. Promising results have been obtained by the introduction of cationic lipids in the liposome. TTSL were actively targeted to tumor tissue by adding 7.5% or 10 mol % of DPTAP into their formulation. As a result, a medium positive surface charge was observed compared to the small negative charge of most other thermosensitive liposomes. This strategy provides better targeting ability towards angiogenic endothelial and tumor cells [87].

Li et al. constructed VD-TSL (vincristine and doxorubicin loaded thermosensitive liposomes) delivery system. Co-encapsulation of drug in liposomal carrier is shown in Fig. 2 A. The biodistribution and pharmacokinetic profile of TSL were determined by the administration of Cy5-loaded liposomes to MCF-7 tumor-bearing mice. The intensity of fluorescence in mice reduced rapidly post injection. However, fluorescence signal could be detected at 12 h subsequent to administration of Cy5-TSL/HT and cy5-TSL. Cy5-TSL/HT showed strong tumoral inhibitory activities among other formulations (Fig. 1B1).HT increased TSL accumulation within the tumors, and the accumulation was doubled with any increase in temperature from 39 to 42 °C. 12 h post injection, organs were removed and analyzed on the fluorescent imager. The tumors receiving Cy5-TSL/HT showed a higher fluorescence intensity than those of other groups owing to the synergistic reaction of the EPR effect of tumors and the HT effect (Fig. 1B2) [88].

In another study, a novel formulation of idarubicin temperature-sensitive liposome (IDA-TSL) was synthesized to improve IDA retention at physiological temperature and fast triggered release at 42 °C. An intravital fluorescence microscopy imaging showed an effective *in vivo* triggered IDA release by applying mild HT (42 °C) and higher IDA uptake by cancer cells. The mean difference between IDA-TSL with or without mild HT in *in vitro* studies and favorable *in vivo* triggered release by applying mild HT indicates rapid and profound tumor response to IDA-TSL plus HT compared to free drug [89]. DSPC-DPPC lipids by different ratio of DPPC (50, 60, 70 or 80%) used to prepare four liposomal formulations containing a constant amount of DSPE-PEG2000 (5 mol %) for all TSLs. Except for TSL 50, within a seconds a complete and total DOX release occurred at 42 °C, which showed the slow drug release (66% after 1 h of HT). As opposed to *in vitro* studies, a rapid burst release and leakage of DOX was observed upon injection for all TSLs. Therefore, prediction of an optimized DOX-TSL formulation merely based on *in vitro* test is challenging. Hence, a wide range of *in vivo* experiments in combination with blood kinetics modelling is necessary to be taken into account [90].

2.1.2. Lysolipids containing thermosensitive liposomes (LTSL)

Anyarambhatla and Needham (1999) introduced lysolipid-containing thermosensitive liposomes (LTSL) to encourage the rapid drug release subsequent to 10 s by decrease the phase transition temperature. They noted that amalgamating 10 mol% of MPPC lysolipids in the liposomal lipid complex (DPPC:DSPE-PEG2000 at a molar ratio of 90:4) was able to reduce the Tm ratio from 41.90 to 41 °C [91]. Lowering the thermal dose thresholds play an important role in temperature-triggered drug release in comparison to the traditional thermosensitive liposomes. For clinical use, mild hyperthermia < 43 °C is suggested since a higher temperature than normal body temperature could cause a thermal damage to healthy tissues [92,93]. Lysolipids contain a larger head group than their single hydrocarbon tail, which giving them a positive spontaneous curvature and a tendency to form micelle structures. When the temperature approaches Tm, lateral movement of lipids increases that leads to lysolipid accumulation at the grain boundaries and facilitate stabilized defects (nanopores) formation in the bilayer. It has also been noted that DSPE-PEG2000 has an intrinsic negative curvature because of the small PE head group. Thus, it has an ability to form micelles. In the current case, the structures organized into hexagonal II phase. Therefore, DSPE-PEG and lysolipids can help to form nanopore structures in the lipid bilayer, causing rapid release of encapsulated drugs [93]. The lysolipid-based TSL formulation established by Needham and Dewhirst (trade name: ThermoDox) is the only TSL to be evaluated in late-stage clinical trials and was created by Celsion. Recently, ThermoDox is under trial apace to treat primary and secondary liver tumors with additional clinically available heating methods like High Intensity Focused Ultrasound (HIFU). Biological components such as serum proteins might have an impact on lysolipid desorption from the liposome bilayer. Banno et al. showed that the dissociation of approximately 70% of lysolipid within 1 h post injection of LTSL, could be mediated by the presence of cellular membrane pools and plasma protein. LTSL mediated delivery released approximately 20% less drug after 1 h following incubations at temperatures above Tm, showing the negative effect of lysolipid loss from LTSL on the thermal-sensitivity of the liposomes [94]. Al-Ahmady et al. investigated the created effects of protein corona on the temperature-triggered drug release properties of the TTSL and LTSL after their *in vivo* recovery from the blood circulation of CD-1 mice. In biological media, nanoparticles interact with plasma proteins and are covered by protein corona. *Ex vivo* DOX release in the presence or absence of unbound plasma proteins was evaluated at 42 °C. (i.e TSL coated with protein corona). LTSL showed an ultrafast and complete DOX release under the different conditions tested. On the contrary, after incubation with plasma

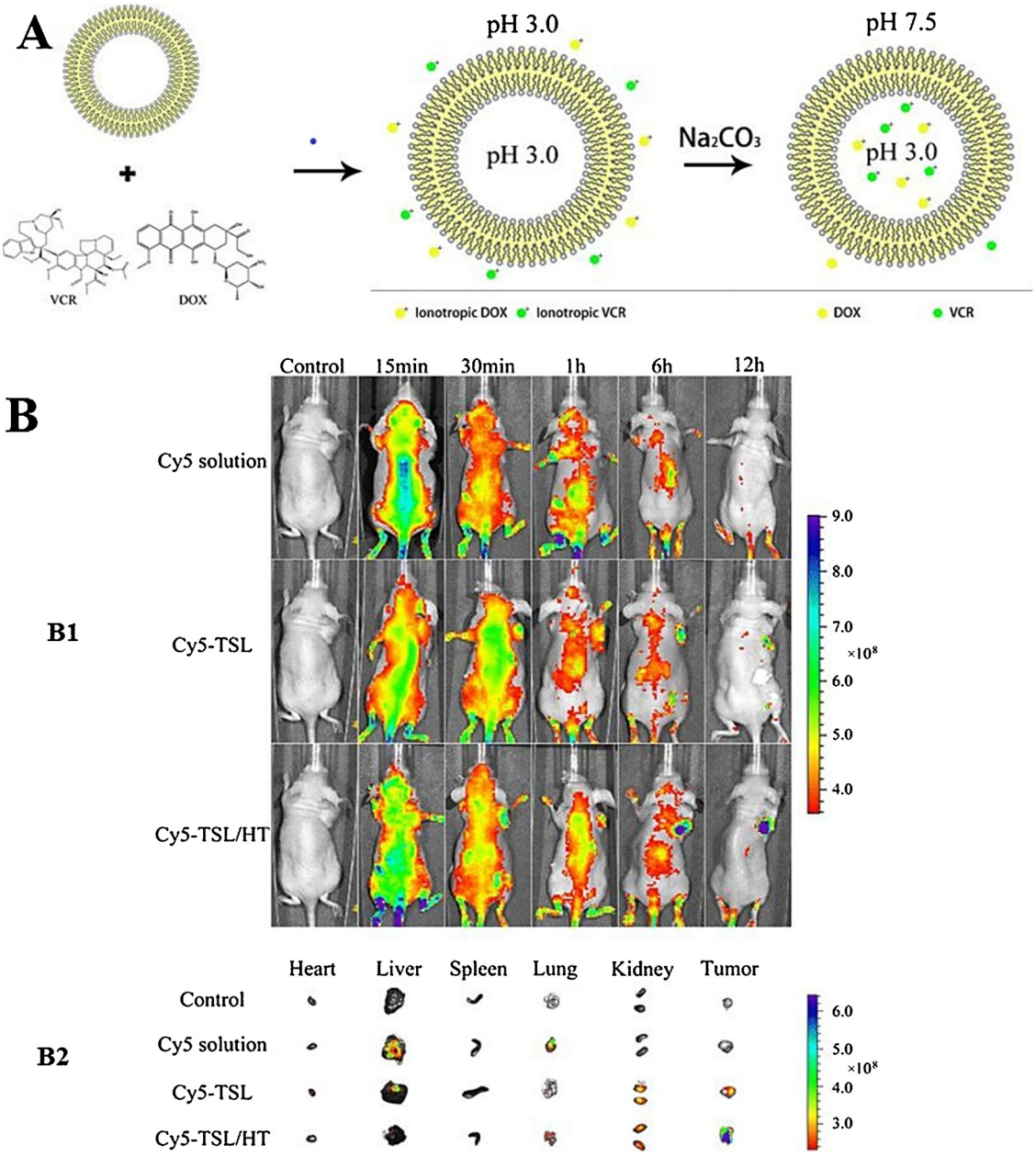

Fig. 1. Drug loading process (A). Biodistribution of Cy5 in MCF-7 tumor-bearing nude mice with different times and various formulations (2B1). Fluorescence detection of excised mice organs at the endpoint of observation (2B2).
This figure was obtained with permission from Ref. [88].

proteins DOX release from TTSL was very slow and incomplete. These observations illustrated that corona formation had a considerable effect on the release profile of TSL and is very much dependent on the protein content of drug-releasing environment and TSL structural composition [95]. The incorporation of DSPE-PEG lipids in LTSL membrane was shown to prevent the interaction of serum proteins with lipid membrane. Despite that, ~20–30% leakage of DOX occurred after 30 min incubation of LTSL with serum at 37 °C, indicating higher serum stability as compared to other TSL formulation [96,97]. HT with LTSL delivery system led to tumoral growth retardation in five tumoral types (colon HCT116, ovarian SKOV-3, squamous cell FaDu, mammary 4T07 and prostate PC-3) as compared to traditional TSL and non-thermosensitive liposomes (NTSL) [98]. In a preclinical research by Manzoor et al., intravital fluorescence imaging *via* the FaDu tumor model showed an enhanced Dox buildup and extended tumor penetration that was a result of LTSL injection into the preheated tumors. This injection resulted in a 3.5-fold greater Dox level compared to free drug and about a 78 μm drug penetration at two sides of the blood vessels, which was twice the penetration depth of typical Doxil liposomes [99]. In another study, Deng et al. designed peptide iRGD-modified LTSL-DOX to investigate the anti-tumoral effects using HIFU. *In vivo* studies indicated that after HIFU-triggered heat treatment, DOX was rapidly released from these liposomes and promote cellular apoptosis. The HIFU exposure time was 10 min and lag time between LTSL injection and HIFU exposure was short, suggesting the potential usefulness of this strategy for clinical applications. Following the evolution of LTSL, other types of TSLs with similar design principles have also been reported in the literature.

Tagami et al. created a hyperthermia-activated cytotoxic (HaT) liposomal formulation containing DOX, made of Brij 78 (molar ratio: 96:4) and DPPC. This non-ionic surfactant is made of a single acyl chain conjugated to a PEG moiety, which can substitute for lysolipid and

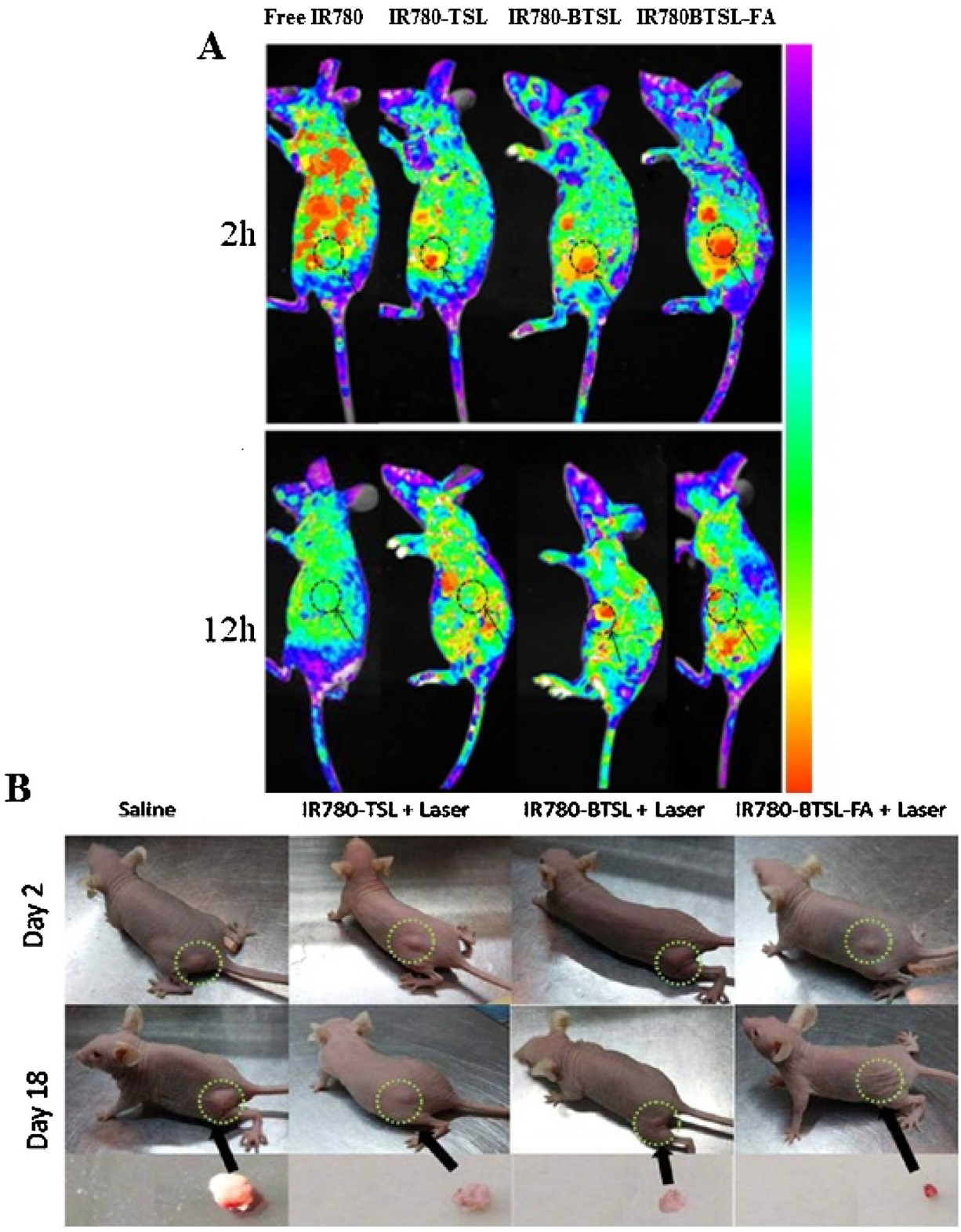

Fig. 2. (A). *In vivo* images taken at 2 and 12 h after injection of various formulations into tumor-bearing mice *via* the tail vein. The nude mice treated with IR780-BTSL-FA, IR780-BTSL and IR780-TSL were subjected to near-IR laser irradiation for 15 min. (B). Representative photographs of KB tumor bearing mice models and tumor excision on 18 days after treatments.
This figure was obtained with permission from Ref. [154].

DSPE-PEG2000. Thus, it can provide both the pore-formation and the steric stabilization to LTSL. In comparison to the LTSL, the HaT formulation showed an increase in DOX delivery to EMT-6 heated tumors at 43 °C. One treatment with HaT (3 mg DOX/kg) with hyperthermia showed intensified regression of tumors when compared to the LTSL [100]. For DOX loading in the HaT-II formulation, a Cu^{2+} gradient was used in place of the pH gradient method. In comparison to the LTSL and HaT, the clearance of DOX was reduced by a 2.5-fold. An enhancement

in drug delivery was also observed 2-fold and 1.4-fold for LTSL and HaT, respectively [101]. PA imaging was performed to study the endogenous O2 values between 30 min to 5 h post-treatment in the heated tumor before and after intravenous injection of mice either saline or HaT-DOX. They found a correlation between the changes in tumor size and the changes in SO2 of the tumor. HT-HaT-DOX formulation showed a significant drop in oxygen saturation in SO2 (10%) compared to the HT-saline control with 90% of treatment demonstrating significant tumor regression [102]. DPPG2–based formulation is another example for this class of TSL, which could increase circulation time. In comparison to PEG liposomes, in which 10 mol% of PEG lipids can be incorporated to liposome membrane, DPPG2 with the desired Tm of 42 °C can amount to 70 mol%, therefore this lipid was used to replace the function of DSPE-PEG2000 to increase release properties of TSL. Hexadecylphosphocholine (HePc) was added to DPPC:DSPC:DPPG2 liposome which function as an antitumor drug and structurally relate to MPPC lysolipids with better chemical and metabolic stability. This liposome system behave in a similar way to LTSL, resulting in 90% CF release after incubation for 5 min in fetal calf serum at 42 °C [103]. In recent studies, a doxorubicin-filled DPPG2-TSL combined with HT was evaluated in cats with feline soft tissue sarcoma. Strong therapeutic effects with tumor stabilization for doses of 0.2 mg/kg and objective tumoral responses without systemic side effects for doses of 0.6 mg/kg was observed [104] (Table 2 shows some examples of thermosensitive liposomes used in both *in vivo* and *in vitro* drug delivery).

2.1.3. Polymer-modified thermosensitive liposomes

One of the most effective methods for heat sensitizing of the liposome is to incorporate synthetic and naturally occurring polymers in lipid composition, in which the liposome membrane undergo a temperature-disruptive effect because of conformational changes in response to temperature change. Thermosensitive polymers can obtain a temperature-responsive functionality to nonthermosensitive liposomes or increase thermal sensitivity of TSLs [116]. Temperature-sensitive polymers used to modify liposomes prominently exhibit a lower critical solution temperature (LCST) which corresponds to their coil-globule transition and phase separation [117] Under the polymers' LCST, hydrogen bonding forces between water molecules and polymer chains are sufficient to solubilize the polymer. Above the LCST, the efficiency of hydrogen bonding is reduced, leading to less hydrated polymer chains and phase separation [118]. Thus, the LCST behavior mainly relying on the hydrogen binding abilities of the constituent monomer units, and can be adjusted within a desired range by tailoring hydrophobic and hydrophilic monomer content. When temperature-sensitive polymers incorporated into liposomes, the phase transition resulted in membrane disruption and promote drug release near the LCST [119]. Table 3summarizes different examples of polymer-modified thermosensitive liposomes.

Poly (*N*-isopropylacrylamide) (NIPAM) that derived from a family of poly (*N*-substituted- acrylamide) has emerged as one of the most extensively investigated temperature sensitive polymers. The NIPAM exhibits an LCST at around 32 °C which is a highly desirable temperature in biomedical fields since it is near to the body temperature of 37 °C [120]. An increase of hydrophobic monomers such as ODA and NDDAM, results in LCST decrease [121,122]. Additionally, copolymerization with a hydrophilic polymer (*e.g.* AA or AAm) increases LCST [123,124]. The first attempts focused on the modification of NIPAM by attaching p-(NIPAM-ODA) polymer into liposome bilayer *via* the hydrophobic group of ODA. The long alkyl chains of ODA used for fixation of polymer to the liposome surface. As, solely NIPAM has an LCST of 32 °C, for p-(NIPAM-ODA) an LCST occurs at 27 °C due to the hydrophobic nature of ODA. The surface modification of thermosensitive DPPC liposomes and nonthermosensitive egg phosphocholine (EPC) liposomes with an ODA and a copolymer of NIPAM results in enhanced release of fluorescent dye encapsulated within liposomes at temperatures above the polymer LCST, but the minimum release was observed below the LCST in both formulations. A synergy between the destabilization of the lipid membrane induced by the polymer and the inherent thermosensitivity of the DPPC liposomes has been suggested to be one of the main reasons of more extensive release occur from the DPPC liposomes in comparison to EPC liposomes [122]. Poly (NIPAM-ODA)-coated DOPE liposomes, showed an improved release of calcein at 40 °C. DOPE lipid has a tendency to form a hexagonal phase with a destabilizing effect on lipid membrane, which could be neutralized by using hydrated NIPAM chains. The stability effects of polymer decreases by dehydration of the polymer chains at temperatures above the LCST and thereby the liposome started to be unstable and then the drug is released [125]. The early studies of polymer-modified TSLs employed polymers, which have LCST below physiological temperature. Thus, not clinically feasible. Later studies have introduced new copolymers of NIPAM with LCST around body temperature. Hayashi et al. showed that by free radical polymerization with AAM monomers, the LCST of NIPAM could be tuned [126]. The LCST of p(NIPAM) raised from 32 °C to 39 °C, 47.2 °C and 53.2 °C when copolymerized with 10% AAM, 20% AAM and 30% AAM, respectively. The effect of comonomer type on the efficiency of release was studied by synthesizing three copolymers of

Table 2
An overview of thermosensitive liposomes employed both, *in vitro* and *in vivo*.

	Liposomal composition	Encapsulated agent	Bioassay	*In vivo* tumoral model	Ref
1	DPPC: DSPC	Bleomycin	*In vitro*	–	[105]
2	DPPC: MPPC	Arsenic Trioxide	*In vitro*	–	[106]
3	DPPC: DSPC : DSPE-PEG	Idarubicin	*In vitro*	Melanoma	[89]
			In vivo	(BLM cells)	
4	DPPC: DSPC: DSPE-PEG	Doxorubicin	*In vitro*	Murine sarcoma	[90]
			In vivo	(BFS-1 Cells)	
5	DPPC: MPPC: DSPE-PEG	Vinorelbine	*In vitro*	–	[107]
6	DPPC: DSPE-PEG_{2000}: MSPC	Doxorubicin Vincristine	*In vitro*	Breast cancer	[88]
			In vivo	(MCF-7 cells)	
7	DPPC: chol: DSPE-PEG	5-Fluorouracil	*In vitro*	Colorectal adenocarcinoma	[108]
			In vivo	(HT-29 cells)	
8	DPPC: DPPG: MSPC; DSPE-PEG	Cisplatin	*In vitro*	Cervical carcinoma	[109]
			In vivo	(ME-180 cells)	
9	DPPC: DSPC:$DPPG_2$	Gadolinium(Gd) based contrast agents	*In vitro*	–	[110]
10	DPPC: MSPC: DSPE-PEG_{2000}: DSPG	Paclitaxel	*In vitro*	Lewis lung carcinoma (LLC)	[111]
			In vivo		
11	DPPC: DSPE-PEG_{2000}: EPC : MSPC	Docetaxel	*In vitro*	–	[112]
12	DPPC: chol: Brij 78	Doxorubicin	*In vitro*		[113]
13	DPPC: DSPC: DSPE-PEG_{2000}: DPTAP	Carboxyfluorescein	*In vitro*	Murine melanoma	[114]
			In vivo	(B16BL6 cells)	
14	DPPC: DSPC: DSPE-PEG: short chain-glucosylceramide	Doxorubicin	*In vitro*	–	[115]

Table 3
Examples of polymer-modified temperature-sensitive liposomes.

	Components	Temperature-sensitive polymer	Active material	Stage of study	Ref
1	DOPE	P (NIPAM-ODA)	Calcein	*In vitro*	[138]
2	EPC	P (Apr-NIPAM) -$2C_{12}$	MTX	*In vitro*	[139]
3	EPC: DOPE	P (NIPAM-NDDAM)	Calcein	*In vitro*	[121]
4	DPPC, HSPC, chol, DSPE-PEG_{2000}	P (NIPAM-PAA)	Doxorubicin	*In vitro*	[140]
5	DPPC	$C_{12}H_{25}$-PNIPAH-CooH PnBA-PNIPAM	–	*In vitro*	[141]
6	DPPC, Chol, dimyristoylphosphatidic acid	$2C_{12}$-P (NIPMAM-NIPAM)	Doxorubicin Calcein	*In vitro*	[142]
7	DPPC, HSPC, chol, DSPE-PEG_{2000}	P (NIPAM-AAM)	Doxorubicin	*In vitro/In vivo*	[143]
8	DPPC, HSPC, chol, DSPE-PEG_{2000}	P (NIPAM-AAM)	Doxorubicin	*In vitro*	[123]
9	EPC; chol-DSPE-PEG_{2000}-Gd	P (EOEOVE-ODVE)	Doxorubicin	*In vitro/ In vivo*	[144]
10	EPC, DOPE, chol, DSPE: PEG_{2000}	P (EOEOVE-ODVE)	Doxorubicin Rhodamine	*In vitro/ In vivo*	[145]
11	DOPE, EPC	P (EOEOVE-ODVE)	Calcein	*In vitro*	[146]
12	EPC	$2C_{12}$-P (Apr-NIPAM) $2C_{12}$-P (DMAM-NIPAM) $2C_{12}$-P (NIPMAM- NIPAM)	Calcein	*In vitro*	[127]
13	DPPC, DSPE-PEG_{2000}, chol	Elastin-like polypeptide	Doxorubicin Calcein	*In vitro/ In vivo*	[136]
14	DPPC, DSPC, chol, DSPE-PEG_{2000}	Elastin-like polypeptide	Ciprofloxacin	*In vitro*	[137]
15	DPPC	Poloxamer 188	Doxorubicin Calcein	*In vitro/ In vivo*	[147]
16	DPPC, MSPC, DSPE-PEG_{2000}	Poloxamer 188	Oxaliplatin	*In vitro/ In vivo*	[132]

NIPAM with an LCST close to 40 °C, but with various enthalpy of transition (ΔH): p-(NIPAMAM-NIPAM)-2C12, p-(APr-NIPAM)-2C12, p-(DMAM-NIPAM)-2C12. To understand the effect of structural differences on the interaction of polymer with the liposome membrane and amount of drug released from liposome, all three polymers were attached to EPC liposomes. Although LCSTs of three polymers determined by differential scanning calorimetry (DSC) and cloud point were almost identical, they showed different enthalpy of transition (ΔH) which is related to the destruction of water molecules surrounding the hydrophobic group. The results indicate that calcein release from polymer-modified TSL increased with higher polymer ΔH in the following manner: Apr < DMAM < NIPMAM. Liposomes having p-(NIPAMA-MNIPAM)-

2C12 shoed rapid release at 42 °C, but also a certain amount of content release at 37 °C (40% within 15 min) [127]. Pippa et al. used p (NIPAM) in the form of an end-functionalized C12H25-PNIPAM-COOH polymer for modifying the conventional DPPC liposomes structure and their drug release properties. The structural and physicochemical behavior of these polymer-modified TSLs was dependent on the composition and the molar ratio of p(NIPAM). DSC experiments revealed the effect of p(NIPAM) incorporation on the thermotropic behavior of DPPC liposomes. The drug encapsulation and the release were found to depend mostly on the thermotropic properties of p(NIPAM). Thus, by changing the ratio of DPPC/NIPAM components, as well as molecular weight of the p(NIPAM) chains the thermosensitivity and pharmacokinetics of the incorporated drug could be modulated [128]. The addition of p(EOEOVE-ODVE) polymer with a LCST near 40 °C into the EPC:CHOL:DSPE-PEG2000 results in less than 10% leakage of DOX at 37 °C and about 90% of DOX release after incubation at 45 °C for 1 min.

Below the LCST (40 °C), the interaction between PEG groups and partially dehydrated poly(EOEOVE) chains at the liposome surface reduced their interaction with the lipid membrane. Above the LCST, the interaction of fully dehydrated polymer and PEG chains results in dehydration of PEG chains *via* H-bond formation, which enhances their interaction with lipid membrane and induce vesicle destabilization. High stability at body temperature and high temperature-sensitive properties enable use of this liposomal formulation for *in vivo* applications [129]. Poloxamers are another type of thermosensitive polymers widely known by the trade name of pluronics, a class of nonionic triblock copolymers containing main hydrophobic block of poly (propylene oxide, PPO) between two hydrophilic poly (ethylene oxide, PEO) chains. In an aqueous media, poloxamer molecules stay as individual copolymers at temperatures below their critical micelle temperature (CMT). At the higher temperatures above the CMT, the block copolymer molecules become more hydrophobic and form micelle structures with the hydro- phobic block PPO forming the core of micelle. This behavior was successfully employed to impart temperature sensitivity to liposome. Below the CMT, poloxamer-containing liposome do not associate with the lipid bilayer. At temperatures above the CMT, poloxamer molecules can partition into the lipid bilayer, causing membrane disruption and release of drug from liposome [130]. The ability of pluronic F-127-modified DOPC:cholesterol liposomes for delivery of fluorescent marker to CT-26 tumor cells was investigated. Cell-bound markers elevated when tumors were exposed to heating (30–42 °C), with tumors received temperature-sensitive liposomes and mild hyperthermia showed 2.5-fold greater dye delivery compared to controls [131]. Oxaliplatin-loaded TSL with an encapsulation efficiency of more than 90% and complete drug release within 10 min at 42 °C were obtained by adding poloxamer 188 to the conventional TSL formulation. The distinguished antitumoral activity of TSL with poloxamer was also shown in nude mice [132].

In addition to synthetic polymers, thermally responsive biopolymers have received considerable attention in the design of TSL. Elastin-like polypeptides (ELPs) are an example of thermally responsive biopolymers composed of repeated pentapeptide that have shown promising potential in cancer therapy, because of their capability to deposit and switch conformation in heated tumor tissues. ELPs are present in soluble form below their transition temperature, which are stabilized because of H-bonds formation with water molecules. As the temperature approaches above the transition temperature, a conformational change from a random coil to a β-turn occurred due to intramolecular hydrophobic interactions of ELPs [133,134]. ELP-modified thermosensitive liposomes (ELP-TSL) have been synthesized by covalent conjugation of peptide to DOX-loaded liposomes. Higher cellular uptake of these liposomes was observed after heating of tumor cells at the transition temperature of peptide due to ELP molecule dehydration on the surface of liposome. ELP concentration on the surface of liposome was not sufficient to induce drug release which might be related to the rigidity of liposomes used and ELP length [135]. To overcome this drawback, ELP was incorporated to the thermosensitive liposome consisted of DPPC:CHOL:DSPE-PEG2000 by covalently linkage of a monostearyl hydrocarbon tail to ELP. The results showed that ELP-TSL

Table 4
Targeted thermosensitive liposomes.

	Targeting ligand	Target	Bioassay	Encapsulated cargo	Ref.
1	Trastuzumab (antibody)	HER-2	*In vitro*	Calcein Rhodamine	[155]
2	Trastuzumab (antibody)	EGFR-2	*In vitro/In vivo*	Doxorubicin Indocyanine green (ICG)	[152]
3	GE11 peptide and Fab fragments of cetuximab	EGFR	*In vitro*	Doxorubicin Calcein	[151]
4	cNGR-peptide	CD13 positive cancer cells	*In vitro*	Doxorubicin	[156]
5	CRGD-peptide	Tumors and angiogenic endothelial cells	*In vitro/in vivo*	Doxorubicin	[157]
6	CREKA-peptide	Clotted plasma proteins in the tumoral vessels	*In vitro/In vivo*	Doxorubicin Carbocyanine dye Cy7	[158]
7	iRGD-peptide	$Dv\beta_3$-positive cells	*In vitro/ in vivo*	Doxorubicin	[159]
8	peptide	Bone regeneration	*In vitro*	Penta peptide of the parathyroid hormone- related protein (PTHrP 107-111)	[160]
9	Folate	Epidermoid carcinoma	*In vitro/ in vivo*	Doxorubicin	[154]
10	AS 1411 aptamer	Nucleolin reseptors	*In vitro*	Gd-DTPA (contrast agent)	[161]

released more than 95% DOX in less than 10 s at 42 °C, while maintained DOX retention at 37 °C, which might be explained by less rigid lipid bilayer and shorter ELP chain length. A significant tumor growth regression was observed after intravenous administration of these liposomes in mice bearing SCC-7 tumor in combination with HIFU in the first 2 days after the treatment due to the intravascular drug release [133,136].

In another study, ELP-TSL has been used for the delivery of Ciprofloxacin. Incorporation of ELP into liposome bilayer enables thermally-controlled drug release and caused significant bacterial killing in the mild hyperthermia ranges, suggesting that ELP-TSL may have clinical utility against musculoskeletal infections [137].

3. Targeted thermosensitive liposomes

Another approach to enhance efficiency and bioavailability of TSLs is the conjugation of specific targeting ligands such as folate, antibodies and peptides to the surface of TSLs [148–150]. Targeting molecules that have been successfully used are summarized in Table 4. A novel multifunctional temperature-sensitive liposome have been designed and functionalized with Fab fragments and GE11 peptide as anti EGFR ligands for targeted DOX delivery. EGFR is overexpressed on the surface of cancer cells and results showed that Fab conjugated TSL can effectively bind to them compared to the GE11 conjugated TSL. Calcein-loaded fab-TSL proved to be physiologically stable with a heat-triggered release > 40 °C. The combination of Fab modification and hyperthermia significantly increased tumoral cell cytotoxicity results of DOX-loaded TSL [151]. Kono et al. investigated the influence of antibody trastuzumab (Herceptin) conjugation indocyanine green (ICG)-incorporation on the PEGylated liposomes modified with EOEOVE copolymer. Strong fluorescence intensity was achieved for the tumor treated with these liposomes after heat treatment at 44 °C for 10 min, demonstrating an effective accumulation at tumoral tissues *via* the specific interaction between the antibody and its target cells [152]. In another study, paclitaxel-loaded temperature-sensitive liposomes (PTX-TSL) were generated by adding K237 peptide to the surface of liposomes. K237-modified PTX-TSL in combination with HT showed higher toxicity against SKOV-3 cells and HUVECs compared with free PTX and PTX-TSL which was mainly dependent on cellular uptake increasing through binding of K237 peptide to the receptors on the surface of these two cells [153]. NH4HCO3-containing TSL which is defined as thermosensitive bubble-generating liposome (BTSL) was developed and combined with conjugated FA ligand and photothermal agent (IR780) to improve diagnostic and therapeutic functions. *In vivo* biodistribution analysis revealed significant potential for BTSL and BTSL-FA to improve delivery of drug to the tumor site compared to TSL system, both at 2 and 12 h owing to the hyperthermia-triggered release of BTSL system. Furthermore, enhanced drug accumulation in the tumor tissue occurred for IR780-BTSL-FA compared to IR780-BTSL at 2 h. Even at 12 h, high fluorescence intensity around the tumor site was observed due to high tumor targeting efficiency of BTSL-FA. An antitumor efficacy studies in KB tumor-bearing mice models showed that the IR780-BTSL-FA in combination with near-IR laser irradiation strongly suppressed tumor growth. Thus, the effect of laser photothermal heating was significant [154]

4. pH-sensitive liposomes

Of late, pH-sensitive liposomes are preferred to conventional liposomes because they can successfully deliver gene fragments and drugs to the cytoplasm *via* the endocytic pathway [162]. For the first time, Yatvin et al. in 1980 used these types of liposomes for drug delivery [163,164]. It was later discovered that elusive pH variations in dissimilar locations like the tumor extracellular environment are useful when designing pH-sensitive liposomes for specific cancer cell targeting. These two factors are also advantageous in improving cellular internalization and controlling drug delivery in various cancers [165]. Furthermore, by adding fusogenic-like compounds the pH-sensitive liposomes could be formulated. These liposomes permit the drug to be released into the cytoplasm by interacting and assisting in fusion or destabilization of target membranes under the acidic conditions of the target tissue [166,167].

The purpose of pH-sensitive liposomes is to release drug loads at acidic pH, while in blood and normal tissues the extracellular pH is about 7.4. Nevertheless, different pathological sites (*e.g.*, primary tumors, local ischemia, inflammation and infection) possess different pH profiles that can lower pH levels to about 6.5–7.2. Tumor interstitial has a pH lower than 6.5 and this situation makes it problematic to make engineered liposomes [168,169]. Under other conditions, upon reaching the tumor sites, the pH-sensitive liposomes internalize *via* the endocytic pathway and are trapped inside the endosomal and lysosomal compartments. These liposomes, with their mild pH level, can destabilize at the endosomal stage due to their fusogenic potential, and this averts drug degradation and sequestration which encourages drug release into the cell cytoplasm [170]. The pH-sensitive liposomes are able to deliver proteins and peptides, oligonucleotides, antisense, plasmids, antibodies and drugs [171]. According to the obtained results by Fattal et al., encapsulation of antisense oligonucleotides, as a kind of disrupting gene expression molecule, into the pH-sensitive liposomes makes them possible to be used for treatment of genetic disorders or infections. Nonetheless, antisense oligonucleotides possess a weak cytoplasmic delivery and intracellular penetration; and consequently for example anionic pH-sensitive liposomes were designed as smart delivery systems. The encapsulated contents of the liposomes produced

Table 5
Recent studies on pH-sensitive liposomes.

	Liposomal formulation	Encapsulated Cargo	Bioassay	*In vivo* tumoral model	Targeting ligand	Ref
1	DOPE, HSPC, CHEMS, chol, $mPEG_{2000}$-DSPE	Dox	*In vitro*	–	–	[173]
2	PEG-Hz-PE	TAT peptides	*In vitro* *In vivo*	Lewis lung carcinoma (LLC)	TAT peptide	[174]
3	HSPC, CHEMS, PEG, di ethylenetriamine Pentaacetic acid-modified phosphatidylethanolamine	Calcein	*In vitro*	–	–	[175]
4	PEt O2-CHEMS, SPc, chol, DSPE-PEG	Dox	*In vitro*	–	–	[176]
5	SPc, chol, synthetic smart lipids (HHG2C18-L and PEGHG2C18-L)	Temsirolimus	*In vitro* *In vivo*	Murine renal carcinoma (A498 cells)	–	[177]
6	PE, chol, CHEMS oleic acid, Linoleic acid	Docetaxel	*In vitro*	–		[178]
7	HSPC, DOPC, chol, PEG_m-$PDPA_n$-PEG_m	Dox	*In vitro*	–	–	[179]
8	SPc, chol, DSPE-PEG, mPEG-CHEMS, mPEG-HZ-CHEMS	Paclitaxel	*In vitro* *In vivo*	–	–	[180]
9	DPPC, mPEG-P (HPMA-g-His) -chol	Dox	*In vitro* *In vivo*	Human colorectal	–	[181]
10	DOPE, CHEMS, DSPE-PEG_{2000}	Paclitaxel	*In vitro*	–		[182]
11	PC, Chol, DOTAP, DSPE-PEG, malachite green Carbinol base (MG)	Dox	*In vitro* *In vivo*	Epidermoid carcinoma (KB cells)	Folate	[183]
12	DOPE, HSPC, CHEMS, chol, DSPE	Dox	*In vitro* *In vivo*	Breast carcinoma (MCF7-cells)	Estrone	[184]
13	SPC, Chol, DSPE-PEG_{2000} PEG_{5000}-HZ-PE	Paclitaxel	*In vitro* *In vivo*	Murine mammary carcinoma (4T1 cells)	R8 peptide	[185]
14	SPc, chol, DSPE-PEG, [D]-H_6L_9 Peptide	Paclitaxel	*In vitro* *In vivo*	Colon adenocarcinoma (C26 cells)	CRGD peptide	[186]
15	SPC, Chol	Paclitaxel	*In vitro* *In vivo*	Murine hepatocellular carcinoma (HepG2cells)	CPP, hyaluronic acid	[187]
16	DOPE, CHEMS, DSPE-PEG_{3400}	Dox	*In vitro* *In vivo*	Breast adenocarcinoma (MDA-MB-231) cells	alendronate	[188]
17	SPC, Chol, DSPE-PEG	Dox	*In vitro* *In vivo*	Colon adenocarcinoma (HT 29 cells)	STP peptide	[189]
18	Chol, SPC, DSPE-PEG_{2000}, DSPE-SS-PEG_{5000}	Paclitaxel	*In vitro* *In vivo*	Murine melanoma tumor (B16F1)	TAT peptide	[190]
19	SPC, Chol, DSPE-PEG_{2000}	Paclitaxel	*In vitro* *In vivo*	Murine melanoma (B16F10)	CPP and cRGD peptide	[191]

from phosphatidylethanolamines (PE) are freed into the endosomal system in acidic conditions. These types of liposomes enhance the cytoplasmic delivery of oligonucleotides after endocytosis, while are capable of remaining stable in plasma [172] (Table 5 shows examples of recent studies on pH-sensitive liposomes).

5. The pH-sensitive components

According to the triggering mechanism of pH-sensitivity, a number of pH-sensitive liposomes have been designed. In general, the structure of these liposomes consist of phospholipids such as phosphatidylethanolamine (PE) or its compounds, including *e.g.* carboxylic group that are used to stabilize liposomes at neutral pH [192]. PE is commonly utilized in liposomes with a slightly hydrated and small head group, which is different from the respective acyl chains that possess a cone shape. This cone shape encourages inverted hexagonal phase (HII) formation because of its robust intermolecular interactions [193,194]. A stable bilayer structure at the physiological pH is formed because of the interaction of PE with amphiphilic molecules with a protonable acidic group [195]. Moreover, a commonly used method for liposome destabilization is the inclusion of phosphatidylserine and phosphatidylglycerol which have a negative charge into the bilayer of pH-sensitive liposomes [193,196]. One of the most common components of pH-sensitive liposomes is DOPE. When the liposomes containing DOPE with a weakly acidic amphiphilic such as phosphatidylserine (Ps), cholesteryl hemisuccinate (CHEMS) and phosphatidylglycerol (PG) are located in an acidic environment, they are destabilized and release their cargo *via* the enhanced liposomal fusion with the endosomal membrane [197].

6. pH-sensitive polymers

Another strategy to prepare the sensitive liposomes is to anchor polymers to liposomal bilayer membrane [198]. In comparison to the PE-based formulations, synthetic polymers present various desirable characteristics such as simplicity of preparation, low immunogenicity and structure versatility [199,200]. These polymers usually contain long hydrophobic chains which enables attachment to the lipid bilayer and carboxylic acid groups to obtain sensitivity [201,202]. Depends on the polymer used in pH-sensitive liposome, some polymers could destabilize the phospholipid bilayer, while others cause the fusion of the liposome with endosome/lysosome membranes [203,204]. Some examples of pH-sensitive polymers are summarized in Table 6. NIPAM-based co-polymers are largely used pH-sensitive polymers. These polymers are able to destabilize membrane at acidic pH since they accept protons and their backbones become relatively hydrophobic, while in high pH values or neutral pH they are deprotonated and become hydrophilic [205,206]. The EPC:Chol liposomes containing co-polymers of NIPAM showed an increased *in vitro* release of entrapped fluorescent markers and amphipathic drugs upon acidification. In comparison to the non-pH-sensitive liposomes, a higher cytotoxicity in j774 cells was achieved by araC-containing randomly alkylated NIPAM-anchored liposomes [207]. It has been previously demonstrated that liposomes coated with terminally alkylated NIPAM co-polymer can to some extent a steric stabilization of liposomes, thus prolonging the blood circulation time. However, due to limited *in vivo* performance of these liposomes, PEG–lipid co-incorporation into liposome was evaluated. As anticipated, prolonged circulation time was achieved, blood clearance profiles were similar to those of stealth liposomes without NIPAM [208] Many studies have focused on the interaction between polymers such as polymers poly(ethyl acrylic acid)s with liposomes. Lu et al. prepared poly (ethylacrylic acid) (PEAA) liposomes containing

Table 6
Some examples of pH-sensitive polymers.

Polymer	Lipids	Marker/ encapsulated drug	Ref.
PEAA	PC/chol	Calcein	[209]
PEAA	EPC/DMPE	Calcein	[216]
sucPG	EPC/PG	Calcein	[213]
sucPG	DPPC,DOPE,MPLA	Ovalbumin	[217]
DODA-P(NIPAM-Co-MAA)	EPC/chol/DSPE-PEG2000/DSPE-PEG-maleimide	HPTS/DPX	[218]
P(NIPAM-Co-MAA-Co-oDA)	EPC/chol	HPTS/DPX	[202]
$^{14}_{C}$-DODA-P(NIPAM-Co-MAA)^{3}-H-CHE	EPC/chol/DSPE-PEG	Radio-lableled polymer	[219]
PPZ(EEE,ABA, $C_{18}(EO)_{10}$)	EPC/chol	HPTS/DPX	[220]
Poly(styrene-co-maleic acid) (SMA)	DSPC/SMA	Calcein	[221]
MGlu-HPG	EPC/DOPE MPLA	Peptides derived from Ovalbumin (OVA-I/*II*)	[222]
CHex PG-PE	EPC, PEG/PE	Bleomycin	[214]

different phosphatidylcholines and cholesterol. The release of liposomal content was dependent on the molecular weight of PEAA. Over 80% of calcein was released when the molecular weight exceeding 8.4 kDa. Moreover, the PEAA liposome permeability could be modified by changes in the phosphocholine and cholesterol content and by adding different surface charges to the liposome. The calcein release rate was reduced with increasing in the percentage of cholesterol and acyl chain length of phosphatidylcholine (DMPC > DPPC > DSPC) [209]. Poly phosphazenes (PPZ) as a novel class of inorganic polymers were synthesized based on a skeletal structure of alternating nitrogen and phosphorous atoms. Every phosphorous atoms bearing two side groups, which play an important role in tuning PPZ properties. While more work remains to be done with this class of polymer, the major advantage is that PPZ could be rendered biodegradable with the introduction of hydrolytically labile substituents, such as rendered amino acid esters on their backbone [210,211]. Modified poly(glycidol)s (PGs) consist of PEG-like backbone and carboxyl groups on the side chains, have been made. These derivatives have demonstrated an ability to control the interaction between polymer backbone and lipid bilayer in a pH-dependent manner [212]. Kono et al. investigated the ability of succinylated PG-modified liposomes which made up of succinylated PG: EPC (3:7 M ratio) to deliver calcein into the monkey kidney cv-1 cells. The occurrence of fusion between these liposomes and cell membranes was 2.5-fold higher than with EPC liposomes [213]. In a more recent study, bleomycin (BLM)-loaded pH-sensitive liposomes modified with PEG-PE and CHexPG-PE were prepared for intracellular drug delivery. The PEG-PE/ CHexPG-PE-introduced liposomes were taken up more effectively by tumoral cells 2.5-fold than liposomes without CHexPG-PE.After intravenous injection, these liposomes strongly suppressed tumoral growth in tumor-bearing mice. However, severe toxicity was observed in mice treated with high doses of BLM-loaded pH-sensitive liposomes, indicating the entrapment of these liposomes by MPS, due to discover and recognition of hydrated PEG chains on the surface of liposome. The toxicity to the liver and spleen was significantly reduced by increasing the PEG length on the liposome surface, although lung toxicity remained [214]. Hyaluronic acid (HA)-modified pH-sensitive liposomes that have both pH sensitivity and targeting properties for cell expressing CD44 were developed. For evaluation of pH-responsivity, 2-carboxycyclohexane-1-carboxylated (CHex) or 3-methyl glutarylated (MGlu) units were introduced to HA (Fig. 3). DOX was efficiently delivered into CD-44 expressing cells by CHex-HA-modified liposomes compared to HA-modified or MGlu-HA-modified liposomes, whereas the same liposomes were not taken up effectively by cells expressing CD44proteins less [215].

6.1. Mechanisms of intracellular delivery mediated by pH-sensitive liposomes

In comparison to the more known phospholipids, PE has a cone shape that averts the forming of the lamellar phase and takes up a lower volume [223]. A robust intermolecular interaction between the polar head groups and the amino and phosphate groups of the cone shape of PE create an inverted hexagonal phase higher than the phase transition temperature. At physiological pH, the stable liposomes are produced; however, the protonation of the carboxylic groups of the amphiphilics at low acidic pH reducing their stabilizing effect on PE bilayers. This leads to a lapse in PE molecules, wherein they return to the hexagonal phase, thus causing liposomal destabilization [224]. In comparison to the non-pH-sensitive liposomes, pH-sensitive liposomes are internalized, which could be because of the PE-containing liposomes' strong ability to adhere to cell membranes. This adherence is a result of these liposomes with weak head group hydration that leads to aggregation [225,226]. Intracellular and internalization delivery *via* the pH-sensitive liposomes involve many steps. Firstly, the endocytic pathway internalizes the liposomes after cell binding. Before to mature into late endosomes, they are kept in early endosomes. However, the trapped particles within the endosome in due course find their way to the lysosome. Here, active enzymatic degradation occurs and inadequate drug delivery to the intracellular targets occurs. The drug degradation on the lysosomal level could be avoided if the pH-sensitive liposomes would be able to destabilize at this stage that finally increased the chance of drug entry into the cytosolic or nuclear targets [227]. Lower acidic pH during endosome maturation in pH-sensitive liposomes enables intracellular drug delivery probably by the lysosomotropic agents *via* averting endosome acidification. Various molecular mechanisms have been hypostasized to bring about intracellular drug release from pH-sensitive liposome. The first hypothesis suggests that pH-sensitive liposomes could destabilize the endosomal membrane by pore production, causing cytoplasmic delivery. Another hypothesis suggests that when the liposome has become destabilized, the encapsulated molecules probably disperse into the cytoplasm *via* the endosomal membrane. The third hypothesis suggests that liposome fusion with the endosomal membranes could initiate cytoplasmic delivery [228]. However, the first and latter hypothesis are most probably based on the fusogenic properties of PE.

6.2. Application of pH-sensitive liposomes

6.2.1. Chemotherapy

There are many different treatment applications for the pH-sensitive liposomes including chemotherapy *etc.* In a study, TATp-modified liposomes have been reported for their ability to accumulate in cancer tissues and ischemic areas through the enhanced EPR effect. These liposomes carrying TAT peptide moieties, which sterically shielded with pH-cleavable hydrazone bond between PEG chains and PE. TATp-modified pH-sensitive liposomes were accumulated in targeted site through the EPR effect, but the lowered pH inside the tumor or ischemic cells remove their PEG coating and allow for the enhanced cell penetration *via* the now-exposed TATp moieties [174]. Although the EPR effect is an effective strategy for accumulation of nanoparticles in tumor cells, the intrinsic barriers, including high density of collagen impaired penetration of therapeutic agents into tumors, thus limiting the

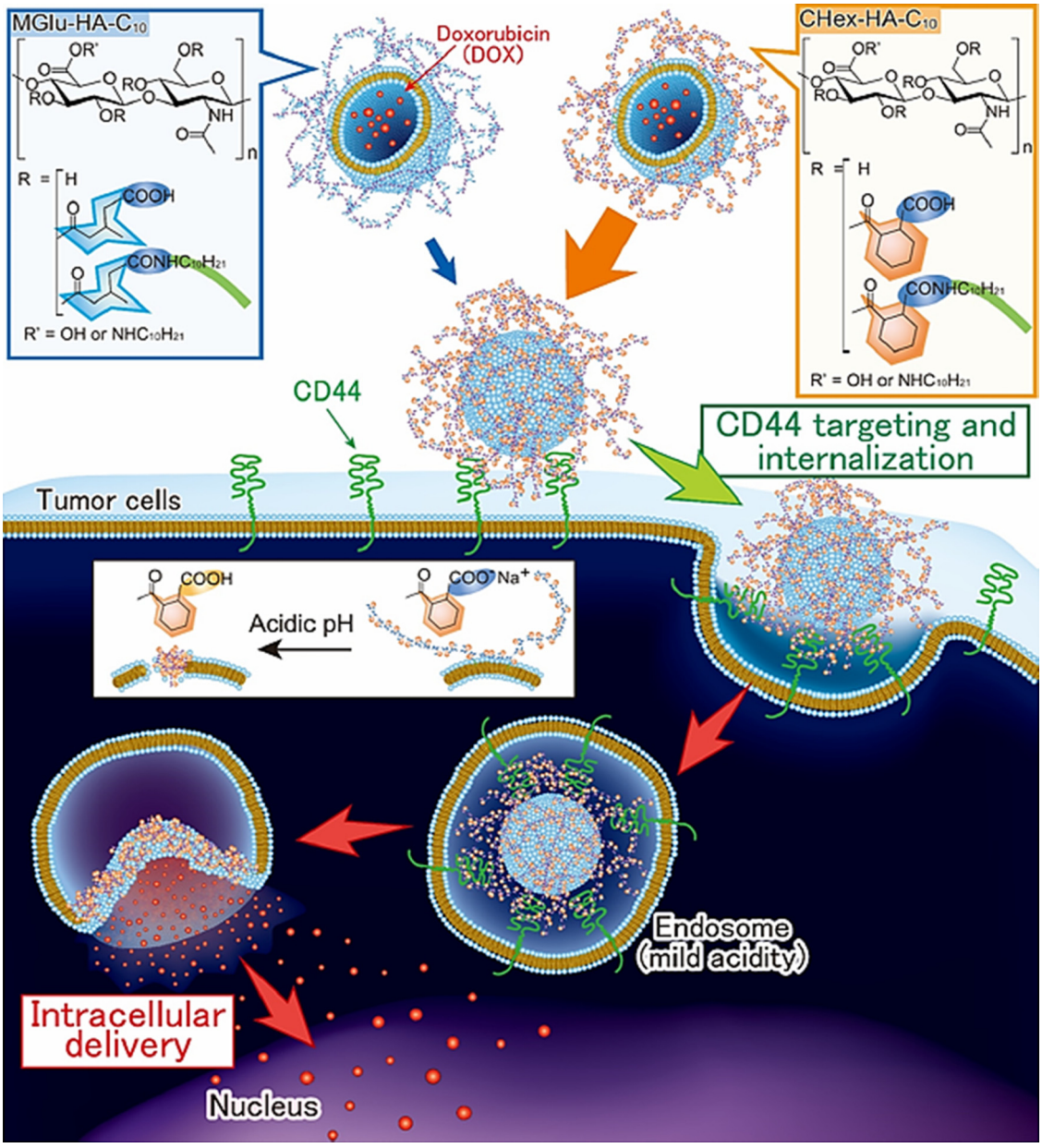

Fig. 3. For CD44 expressing cell-specific intracellular drug delivery, the liposomes were modified by hyaluronic acid (HA)-based pH-sensitive polymer. Liposome internalization occurred by the endocytosis and encapsulated in the endosome. The low acidic environment caused the destabilization of the liposome membrane. Fusion or destabilization of the endosome lead to the release of drug into the endosome and movement to the cytosol.
This figure was obtained with permission from Ref. [215].

anticancer efficacy of nanoparticles. Zhang et al. prepared the pH-sensitive cleavable liposomes (CL-Lip) composed of long pH-sensitive PEG derivative, R8 peptide and PTX. Losartan was administered in order to inhibit type I collagen, which could improve penetration of liposomes and oxygen distribution intensity in tumors. When the nanocarriers accumulated in the tumor environment through the EPR effect, the acidic pH of tumoral cells cause hydrazone bond to be hydrolyzed and then the R8 peptide could become exposed and internalization of the liposomes occurs *via* the mediation of R8 peptide. The results showed the combination therapy composed of PTX-CL-Lip and losartan displayed higher antitumor efficacy compared to PTX-CL-Lip alone [185]. A DOX-encapsulated pH-sensitive liposomes comprised of DOPE:HSPC: CHEMS: CHOL: ES-PEG-DSPE and targeted with estrone (ES-pH-sensitive liposome) were developed [184]. *In vivo* observations in tumor bearing female Balb/c mice showed that there was a strongly suppressed growth of tumor in mice administered with estrone-targeted pH-sensitive liposomes compare to the non-pH-sensitive targeted liposomes (ES-SL) and free DOX owing to accelerated DOX release from endosomes through receptor-mediated endocytosis and finally increasing the therapeutic efficacy. The major complication of doxorubicin therapy is cardiotoxicity, which hampers its extensive applicability. In the present study, Dox concentrations in the heart were significantly lower with DOX-loaded ES-pH-sensitive liposomes than ES-SL and free DOX after 3h [184]. PEG-coated pH-sensitive and PEG-folate-coated pH-sensitive liposomes encapsulating the complex radioactive 159 Gd-DTPA-BMA were synthesized in order to study the *in vivo* cytotoxicity and anticancer activity in Ehrlich tumor-bearing mice. The obtained results showed that the tumor volume remained less in Ehrlich tumor-bearing mice under treatment with radioactive formulations, indicating that ionizing radiation has played a significant role in cytotoxic activity against solid tumor cells. Moreover, biochemical or hematological changes were not significant in ten mice treated with these

formulations except a higher cytotoxicity on hepatocytes which needs further studies [229]. In order to integrate the competence of PEGylated liposomes for prolonged circulation time, anionic liposome for lower hematotoxicity and cationic liposomes for enhanced intracellular delivery in the tumor tissues, Mo et al. developed zwitterionic oligopeptide-based pH-sensitive liposomes composed of $HHG2C_{18}$-L and $PEGHG2C_{18}$-L encapsulated with temsirolimus (CCI-779). Both of formulations displayed the ability of charge conversion to the surrounding pH for enhanced cellular uptake at the tumor milieu and the positive effect on endo/lysosome-escape and mitochondrial targeting, therefore an increased antiproliferation and apoptosis. CCI-779-loaded HHG2C18-L and CCI-779-loaded PEGHG2C18-Lshowed a half maximal inhibitory concentration (IC_{50}) of approximately 3 μg/ml and 5 μg/ml at pH 6.5, indicating that 1.67-fold and 1.60-fold improved, respectively as compared with that of pH 7.4. *In vivo*, PEGHG2C18-L and HHG2C18-L had significantly higher antitumor efficacy and blood persistence in comparison with pH-sensitive liposomes [177]. RGD-coated pH-sensitive liposomes made up of PE:chol:CHEMS:RGD-CHE (6:35:15:15 M ratio) were prepared to increase the efficiency of docetaxel treatment. *In vitro* cellular uptake and cytotoxicity of RGD-modified pH-sensitive liposome containing docetaxel/coumarin-6 were evaluated using A549, HepG2 and MCF-7 cells. RGD-coated pH-sensitive liposomes displayed greater cellular uptake and cytotoxicity compared to non-coated ones. The obtained results demonstrated that RGD-coated pH-sensitive liposomes had significant fluorescence intensities, that indicated effective *in vivo* tumor targeting [230]. The switchable nano delivery system, which functionalized by peptide STP and targeting capability of VEGFR2, was constructed. *In vivo* imaging assays were carried out to evaluate the delivery efficiency in HT-29 tumor bearing mice. As shown in Fig. 4(a–d) pH-sensitive liposome functionalized by peptide STP containing DiR (STP-LS-DiR) began to accumulate in tumor tissues after 4 h while non-targeting liposome with DiR encapsulated (LS-DiR) had not been achieved. Along with time, an increased accumulation of STP-LS-DiR at tumor site was observed compared to LS-DiR owing to dualfunctional acceleration. Almost none fluorescence signal monitored in PBS injection. The *ex vivo* fluorescent imaging demonstrated an increased drug delivery efficiency of STP-LS towards VEGFR2 at acidic tumor microenvironment (Fig. 4e–f). The histological analysis about paraffin section of HT-29 tumor cells were performed to determine the vasculature-targeting efficacy of DOX-loaded STP-LS at mild acidic environment. Tumors treated with DOX-loaded STP-LS lead to higher fluorescence signal as compared to LS-DOX and PBS treated ones, which expressed high vasculature-targeting efficacy of switchable liposomes at tumor site (Fig. 4g–i). [189].

In another study, Zhao et al. designed DOX-loaded tumor-specific pH-responsive peptide H7K(R2)2-modified liposomes (DOX-PSL-H7K (R2)2) and their antiglioma activity was evaluated in mice. The anticancer activity of this formulation is associated with the tumor-specific pH-responsive peptide H7K(R2)2 which could respond at an acidic tumor microenvironment, with cell penetrating peptide (CPP) characteristics. DOX-PSL-H7K(R2)2 significantly inhibited tumor growth at pH 6.8 compared with that in DOX-loaded pH-responsive liposomes (DOX-PSL) which could be a promising strategy for enhanced cancer chemotherapy *via* the response stimuli at the mildly acidic pH in the tumor microenvironment [231].

6.2.2. Gene delivery

The key for successful gene therapy is to design a multifunctional carrier that could overcome different obstacles including endosomal membrane, cell membrane and nuclear membrane [232,233]. In another report, pH-sensitive liposomes containing DOPE and dioleyl succinyl glycerol (DOSG) (1:1 M ratio) encapsulated with pUCSV2CAT DNA have developed. The pH-sensitive liposomes undergo destabilization and become a fusion competent upon encountering the low pH environment within the endosomes, thus resulting in the release of encapsulated DNA inside the cytoplasm of the cells. In comparison, destabilization of pH-insensitive liposome does not take place in the endosome and would be a less effective system for cytoplasmic delivery of the encapsulated DNA. The obtained results showed the importance of acid sensitivity of the liposomes for an effective gene delivery to target cells. DOPE:DOSG liposome has the most sensitivity to pH and displayed a highest transfection efficiency. DOPC:DOSG liposome release 50% of its contents at pH 5.0 with less activity compared to DOPE:DOSG liposome [234]. In another study, both *in vitro* and *in vivo* transfection of a pH-sensitive mannosylated cholesterol derivative containing lipoplexes (Man-His lipoplexes) was investigated. Enhanced transfection activity and cellular uptake were achieved by Man-His-lipoplexes compared to Man-lipoplexes and bare-lipoplexes, which indicate that macrophages take them up through mannose receptor-mediated endocytosis [235]. Khalil et al. proposed a multifunctional envelope-type nano device consisting pDNA (MEND) have been modified by adding glutamic acid-alanine-leucine-alanine (GALA) and octaarginine peptide (R8) for delivering gene to the liver. Modification with GALA considerably increase gene expression levels in the liver regarding to the negative core R8-MEND. Quantification of the amount of DNA delivered to nucleus and liver cells showed that the positive core R8-MENDs significantly increased the amount of pDNA regardless of the presence or absence of GALA, which could be attributed to a difference between methods used to modify Stearylated-octaarginine (STRR8). Higher level of gene expression was achieved regarding to the negative core R8-MEND, especially the R8-GALA-MEND that could be explained through an increased gene expression rate per pDNA in the presence of GALA. As compared to similar system, including DOTAP, R8-GALA-MEND lead to an increased gene expression which suggested that the developed system is more useful for gene delivery to liver [236]. A new approach used for preparing multifunctional gene carriers is layer-by-layer technique. Li et al. described a multifunctional pH-sensitive gene delivery system, which promoted long circulation time, but prevented from uptake of gene carriers by tumor cells due to using of polyethylene glycol. In order to increase the condensation of DNA into a cationic core, protamine was used. In another study, layer-by-layer technique was applied to prepare the CMCS-cationic liposome-coated DNA/protamine/DNA complexes for having a long circulation time. The cytotoxicity and *in vitro* transfection as well as *in vivo* evaluation studies was performed on HepG2 cells and in tumor-bearing mice, respectively. This carrier showed a high serum stability while loaded DNA remains protected from nuclease digestion [237]. Guo et al. developed a cationic lipid containing an acid labile ortho ester linker for condensation of plasmid DNA into cationic lipoplexes in collaboration with a cone-shaped helper lipid DOPE. The gene delivery efficiency of DOC/ DOPE/DNA lipoplexes was evaluated in the CV-1 cells and in intratracheally administered CD-1 mice. After incubation at acidic pH, the acid-stable DC-chol/DOPE/DNA lipoplexes showed a decreased gene delivery by 5- to 10-fold compared to the DOC/ DOPE/ DNA lipoplexes [238]. In another study, two optimized complexes of pH-sensitive PEGylated liposomes and DC-chol/DOPE liposomes were prepared to evaluate the factors affecting pDNA transfection efficiency of these liposomes. DC-Chol to DOPE molar ratio is the key factor influencing the transfection efficiency of DC-Chol/DOPE liposomes. (DC-Chol/DOPE 2:3, and 1:2 M ratio) showed higher transfection efficiency compared to DC-Chol/DOPE (1:1 M ratio). In addition, DOPE is another important factor in pDNA transfection because it may induce lamellar to a hexagonal phase transition, which could facilitate escape from endosome and increase transfection efficiency. PEGylation can be used to extend the lifetime of liposomes in blood, but it had a negative influence on pH-sensitivity of complexes of DC-Chol/DOPE liposomes and pH-sensitive PEGylated liposomes. Although pH-sensitive PEGylated liposomes exhibits undesirable transfection efficiency compared to DC-Chol/DOPE liposomes, an increased accumulation in tumor tissues was observed in the administered complexes of DC-chol/ DOPE (2:3) liposomes/ pH-sensitive 1% PEGylated liposomes compared with DC-Chol/ DOPE liposomes [239].

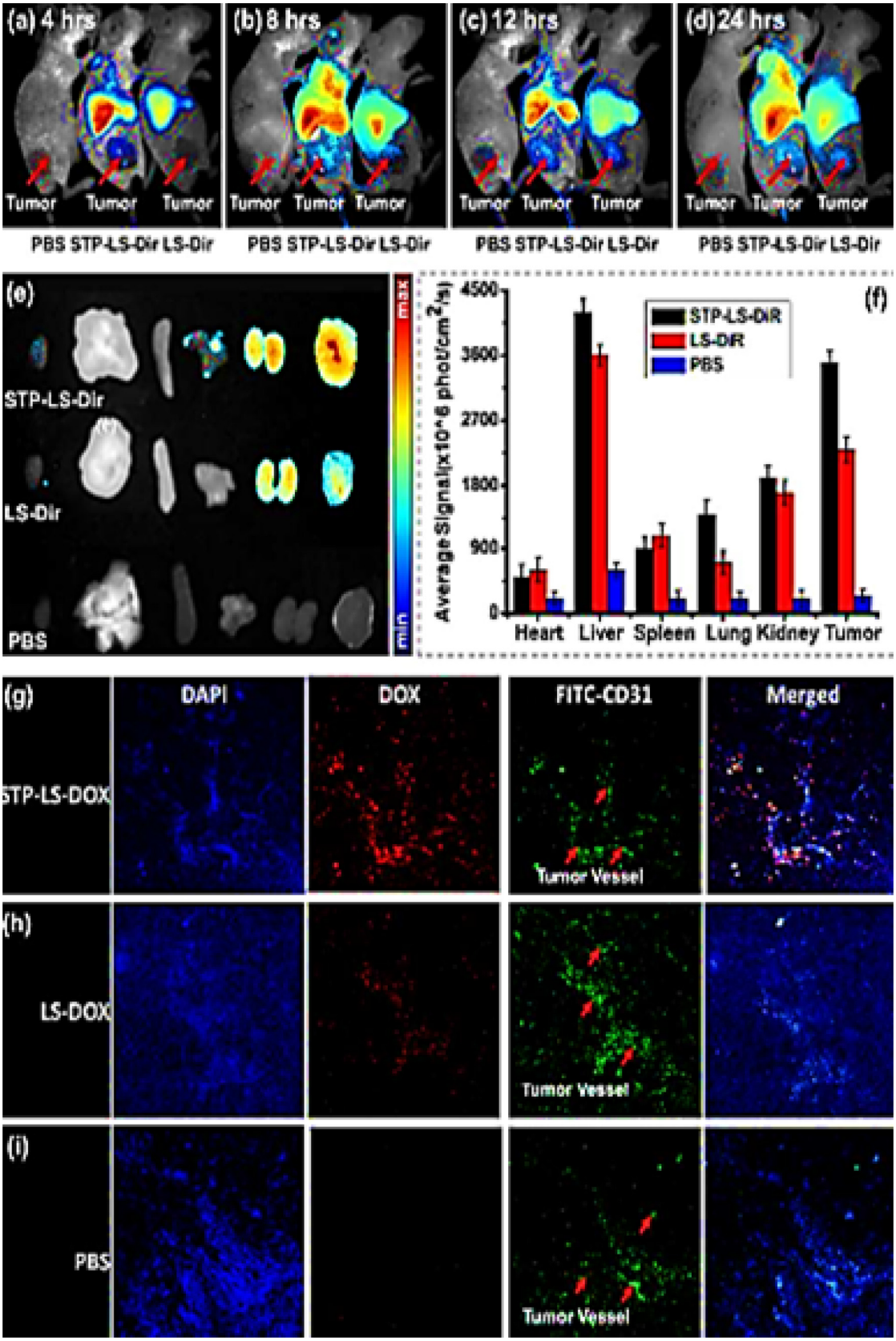

Fig. 4. *In vivo* and *ex vivo* imaging of targeted delivery to HT-29 tumor cells by STP-LS and LS. (a–d) Biodistribution and tumor accumulation of PBS, LS-DiR and STP-LS-DiR with different times. (e) *Ex vivo* fluorescence tumor imaging and organ accumulation. (f) Quantitative fluorescence intensity *ex vivo*. High fluorescence signal was observed for tumortreated with STP-LS-DiR. (g–i) paraffin section of HT-29 tumor cells treated in various conditions.
This figure was obtained with permission from Ref. [189].

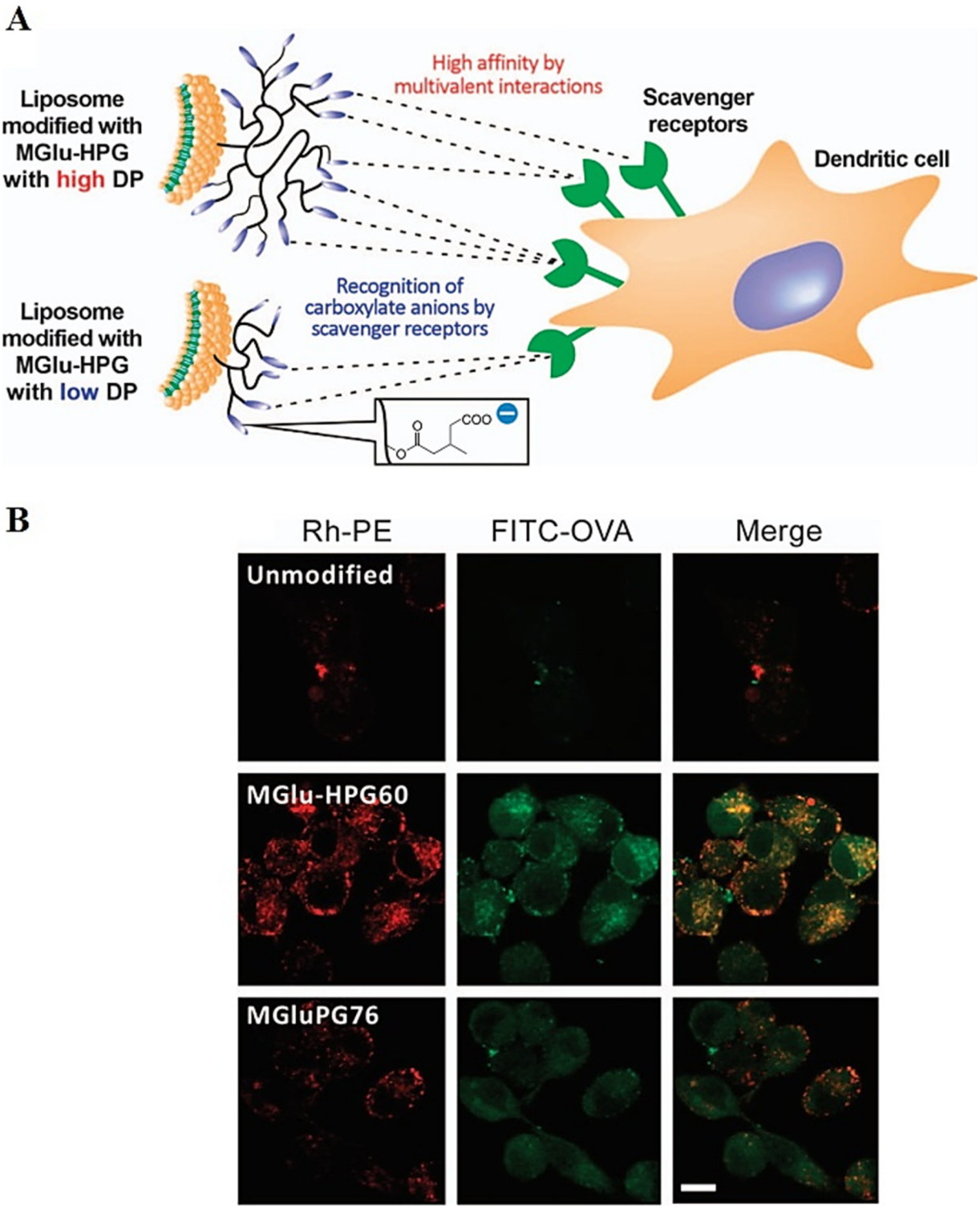

Fig. 5. (A) Schematic illustration for the interaction of MGlu-HPG-modified liposomes with dendritic cells. (B) Confocal laser scanning microscopy (CLSM) images of DC2.4 cells treated with Rh-PE-labeled and (FITC)–OVA-loaded liposomes modified with MGlu HPG60, MGluPG76 or plain liposomes.

6.2.3. Vaccine delivery

The pH-sensitive liposomes have become important tools for therapeutic and prophylactic vaccine delivery. They induce the immune response, reduce side effects such as toxicity and act as vehicles for small sized peptides [240]. In order to defend against new upcoming pathogens, novel vaccination strategies are needed [241]. Antigen-specific immune responses can be activated by Dendritic cells (DCs) to activate the DC cells and present the antigen in two main pathways. In the first pathway for induction of the humoral immunity, the lysosomal exogenous antigen is degraded and access to the major histocompatibility complex (MHC) class II molecules. In the second pathway, the endogenous antigen is exported into the cytosol of DCs. Therefore, anigen-specific cytotoxic T leukocytes (CTLs) is inducted by the loading of antigen onto MHC class I molecules that facilitated with proteasome degradation (Fig. 6) [242]. The role of pH-sensitive liposomes containing epitope peptide is to induce effective cytotoxic T lymphocyte

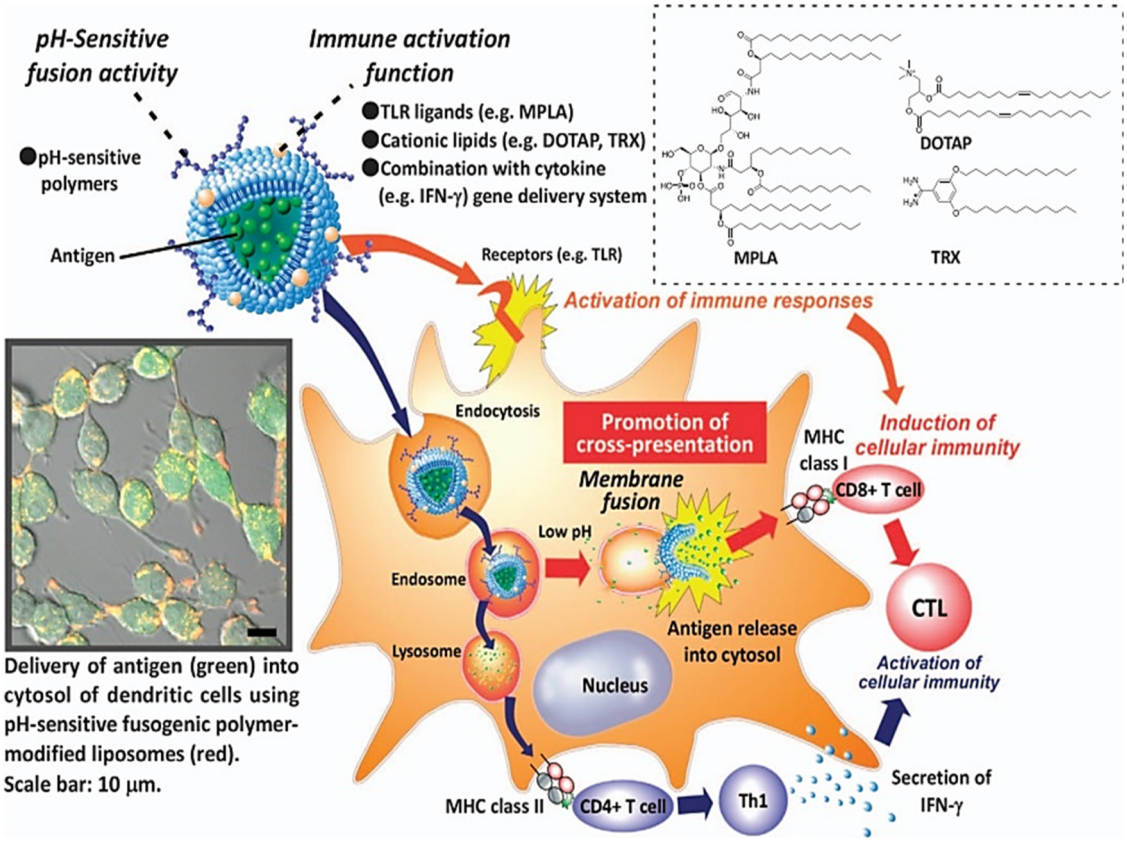

Fig. 6. Fluorescence microscopic images of the liposomal vaccine with immune activation function and pH-sensitive activity. Imagery shows the antigen-loaded pH-sensitive liposomes were functionalized with polymers and adjuvant molecules or systems. Through the endocytic pathway, the cells were internalized. Imagery shows that the liposomes are responsible to transfer the majority of the antigen into the cytosol *via* fusion with the endosomal membrane because of the acidic pH inside the endosomes. Hence, cross-presentation is stimulated, which resulted in cellular immunity induction. Microscopy imagery illustrates DC2.4 cells treated with Rhodamine-PE/fluorescein isothiocyanate (FITC)-OVA co-loaded MGlu-HPG liposomes. The interaction between the adjuvant molecules and receptors activate the dendritic cells that initiate the immune response. Additionally, the cellular immunity is also activated by IFN-γ gene-transfected dendritic cells or IFN-γ produced by Th1 cells. FITC-OVA location is indicated by green fluorescence and the liposomes location is indicated by red fluorescence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The figure was obtained with permission from Ref. [242].

(CTL) responses were examined by Chang et al. The indication of CTL responses by these liposomes blocked the tumor formation from Hantaan nucleocapsid protein transfected B16 melanoma cells in C57BL/6 mice and retarded the growth of pre-inoculated tumors [243]. Lee and coworkers studied peptide antigen delivery route mediated by fluorescein isothiocyanate (FITC)-conjugated H-2Kb CTL epitope-encapsulated pH-sensitive liposomes. By the immunization with these liposomes, a significant effect on the activation of CTL responses against delivered antigen was observed. Thus, for development of prophylactic or therapeutic vaccines, as an effective peptide adjuvant, pH-sensitive liposomes might be considered as a good candidate [244]. Vyas et al. developed the carboxyl- pH-sensitive liposomes encapsulated with terminal 19 kDa fragment of merozoite surface protein-1 of Plasmodium falciparum (PFMSP-119) using EPC and oleyl alcohol (OALc) for destabilization of lipid membrane. The significant immune responses were obtained with PFMSP-119-loaded pH-sensitive liposomes as compared to conventional liposome. Moreover, after immunization of BALB/c mice with PFMSP-119-loaded pH-sensitive liposomes high levels of IgG antibodies were detected as compared to PFMSP-119-loaded conventional liposomes and alum adsorbed formulations which provided direct evidence for acceptable immune-adjuvant action of pH-sensitive liposome [245]. The pH-sensitive fusogenic liposomes encapsulated with ovalbumin (OVA) were developed and modified with SucPG and MGluPG to delivery of antigenic proteins into dendritic cells cytosol. MGluPG-modified and SucPG-modified liposomes have carboxyl groups in their polymer side chains, which provide a negative charge on the surface of these liposomes. Thus, they are preferentially taken up by dendritic cells through interaction with scavenger receptors, suggesting a higher association of these polymer-modified liposomes to antigen-presenting cells in the body.Activation of CTLs was induced more efficiently by MGluPG-modified liposomes than the SucPG-modified liposomes. Thus, MGluPG liposomes might be proved to be a valuable asset for cancer immunotherapy and mucosal vaccination [246]. The pH-sensitive SucPG-modified liposomes loaded with ovalbumin (OVA) have been prepared as a vaccine carrier. OVA-specific IgG 1, IgG 2 and IgG 3 antibodies were significantly enhanced after immunization with SucPG-modified liposomes loaded with OVA, while the immunization with SucPG-unmodified liposomes loaded with OVA induced only OVA-specific IgG 1 antibody responses. In comparison to alum, which is a weak inducer of cell-mediated immune, higher levels of both Th1-type (IFN-γ) and Th2-type (IL-4) cytokines produced by spleen cells of mice immunized with OVA-containing SucPGmodified liposomes. Thus, pH-sensitive fusogenic SucPG-modified liposomes have the potential to be used as an antigen delivery system to stimulate both humoral (Th1) and cellular (Th2) immunities [217]. In order to deliver OVA into the dendritic cells different pH-sensitive polymers such as MGlu-LPG and MGlu-HPG were used to modify DOPE/EYPC liposomes. These pH-sensitive liposomes were taken up more efficiently by DCs and induced stronger OVA-specific cellular immune responses following subcutaneous or nasal administration compared to

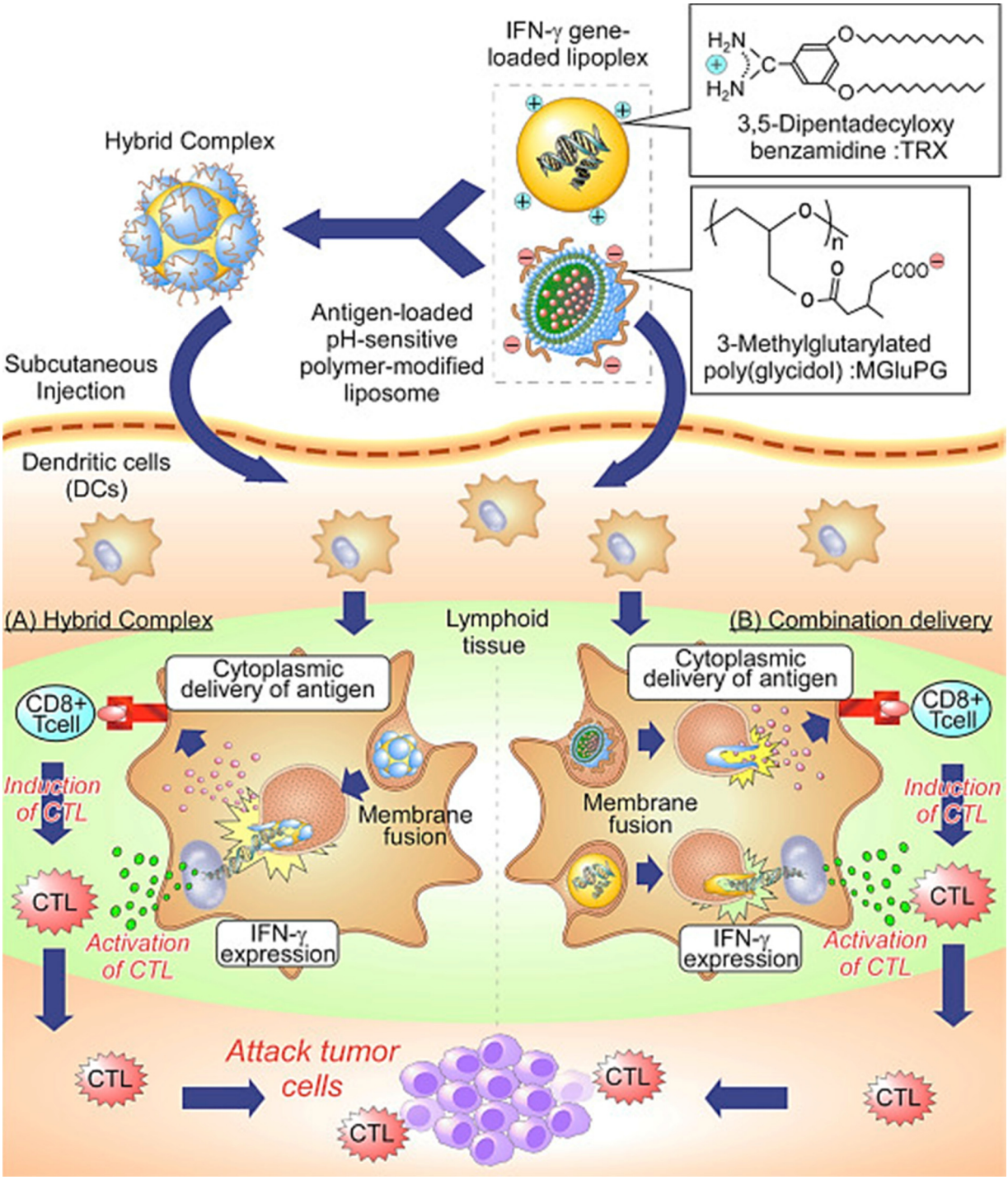

Fig. 7. Illustration of co-delivery system of IFN-$_\gamma$ gene and ovalbumin molecules (OVA) for effective induction of antitumoral immunity. (A) MGluPG-modified liposomes is used to co-delivery of lipoplexes containing OVA/IFN-$_\gamma$ gene and IFN-$_\gamma$ gene. (B) Combination delivery of lipoplexes and MGluPG-modified liposomes containing IFN-$_\gamma$ gene. Subcutaneously injected MGluPG-modified liposomes or lipoplexes transferred IFN-$_\gamma$ gene and OVA into cytosol of DCs *via* membrane fusion in response to acidic endosomal pH. DCs could induce and activate CTLs, which might be an effective approach for induction of antitumoral immunity.
This figure was obtained with permission from Ref. [253].

unmodified liposomes. Administration of the MGlu-HPG-modified or MGluPG-modified liposomes induced suppression of tumor growth and cured 50–75% of the mice. These results indicate that pH-sensitive polymer-modified liposomes can induce cellular immunity strong enough to kill the OVA-expressing tumor cells and result in tumor rejection and regression. [247]. MGlu-HPG forms more domains with hydrophobic nature at weakly acidic environment compared to MGluPG with the same degree of polymerization (DP). Thus, these liposomes exhibit an increased membrane disruption ability at acidic pH than MGluPG. MGlu-HPG showed higher fusion ability and cellular association with increasing degree of polymerization than the linear polymer backbone indicating that bulkier polymer-modified liposomes might be recognized by scavenger reseptors on the DCs efficiently owing to three-dimensional backbone structures (Fig. 5A). After the internalization into the cells, MGlu-HPG-modified liposomes encapsulating OVA exhibited higher fusion activity and deliver their

contents efficiently into the cytosol of DC2.4 cells than the MGluPG-modified liposomes (Fig. 5B) [248].For clinical applications, it is desirable to use pH-sensitive polymers with biodegradable properties for the preparation of pH-liposomes. Therefore, instead of a synthetic polymer poly(glycidol), pH-sensitive dextran derivatives having 3-methylglutarylated residues (MGlu-Dex) has been developed. OVA-entrapped MGlu-Dex modified liposomes was delivered into the cytosol of DCs efficiently. The effects of the different molecular weights of dextran and MGlu-residue content on their immune-inducement effects have been estimated. OVA-loaded MGlu56-Dex70k-C10-modified liposomes exhibited highly efficient cellular association to DCs and different molecular weights of dextran did not influence the immune stimulation effect [249]. Cationic lipids with an amidine group can act as adjuvant through interaction with not only G protein coupled receptors, but also toll-like reseptor4 (TLR4) [250,251]. Based on these studies cationic lipid 3, 5-didodecyloxybenzamidine (TRX) incorporated into (MGlu-HPG)-modified liposomes to increase antigen-specific immune responses. The cellular association of the liposomes enhanced by the addition of TRX to the membrane of liposome a high level of cytokine production (IFN-γ, IL-10, TNF-αand IL-6) from DCs was induced relied on their contents of TRX. (MGlu-HPG)-modified liposomes with higher TRX delivered entrapped OVA molecules into endosomes/lysosomes and rather than cytosol of DCs [252].

MGluPG-modified liposomes were complexed with IFN-$_{\gamma}$ gene-containing lipoplexes having OVA through electrostatic interactions. The hybrid complexes were able to co-delivered OVA and gene into DC2.4 cells through membrane fusion in response to endosomal acidic pH. This study examined the administration of hybrid complexes or OVA-loaded MGluPG-modified liposomes to mice bearing E.G7-OVA and its effects on tumoral growth was monitored. Subcutaneously injected hybrid complexes in tumor bearing mice resulted in decreasing of tumor volumes maybe due to the induction of OVA-sepecifc CTLs. However, antitumoral effects induced by hybrid complexes and MGluPG-modified liposome were same (Fig. 7A). Next, lipoplexes containing IFN-$_{\gamma}$ gene and OVA-loaded MGluPG-modified liposomes were added to DC2.4 cells simultaneously without premixing. Combination administration delivery method induced a much stronger antitumoral than to the MGluPG-modified liposomes as well as survival time of mice were increased to 60 days. (Fig. 7B). Immunization with the liposome-lipoplex combination showed more effective tumor specific immune response than the hybrid complexes delivery method [253].

Recently, adjuvant molecules (CpG-DNA and TRX) were introduced to the MGlu-HPG-modified liposomes using two complexation method Pre-mix and Post-mix). In pre-mix method, CpG-DNA was mixed with a thin membrane of liposomal lipids and in post mix method preformed liposomes was added to CpG-DNA. The post-mix method promoted higher cellular immune response when compared with the pre-mix. In comparison to the conventional pH-sensitive polymer-modified liposome-based systems, both of these methods showed a more effective antitumor effect against tumor-bearing mice [254].

7. Conclusion

Liposomes have become one of the most important carrier systems for improving the therapeutic agents delivery, mainly based on their smart behavior regarding temperature and pH conditions. To enhance the therapeutic efficacy and overcome their limitations, modified formulations have been proposed including stimuli-responsive liposomes. Recent advancements in the development of thermosensitive liposomes have been very promising and leading to further improvements. In this review, we focused on various components used in the design of TsLs. The polymers can either add to TsL to overcome problems including improvement of their temperature sensitivity or stable TSL designing which is dependent on the polymer ingredient itself. Another promising approach in the field of TsLs is the conjugation of target-specific ligands, which can increase drug retention in tumors and improve the therapeutic outcomes of liposomal chemotherapy. Additionally, cytoplasmic delivery of proteins and peptides, plasmids, ribozymes and anticancer drugs to cells can potentially increase by using pH-sensitive liposomes. However, for a formulation to be clinically viable, various substantial properties such as serum stability, prolonged circulation time *in vivo* and efficient pH-triggered release are crucial. Recent advances in liposomes modified with different pH-sensitive polymers solved problems mentioned above. All these properties make polymer-modified thermosensitive liposomes very attractive as carriers for therapeutic components with intracellular targets. In addition, development of multifunctional pharmaceutical nanovesicles that combine pH-sensitive liposomes with different release mechanisms, including temperature-sensitive, light-sensitive, ultrasound-responsive, redox-responsive and magnetic-responsive could be helpful for particular applications. The pH-sensitive liposomes might be used in various diseases and disorders, especially in clinical evaluations and assessments, thus offering exciting possibilities to move from the laboratory bench to the use in the real world.

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