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Temperature-sensitive polymers to promote heat-triggered drug release from liposomes: Towards bypassing EPR

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ABSTRACT

Heat-triggered drug release from temperature-sensitive nanocarriers upon the application of mild hyperthermia is a promising approach to achieve site-specific delivery of drugs. The combination of mild hyperthermia (41–42 °C) and temperature-sensitive liposomes (TSL) that undergo lipid phase-transition and drug release has been studied extensively and has shown promising therapeutic outcome in a variety of animal tumor models as well as initial indications of success in humans. Sensitization of liposomes to mild hyperthermia by means of exploiting the thermal behavior of temperature-sensitive polymers (TSP) provides novel opportunities. Recently, TSP-modified liposomes (TSPL) have shown potential for enhancing tumor-directed drug delivery, either by triggered drug release or by triggered cell interactions in response to heat. In this review, we describe different classes of TSPL, and analyze and discuss the mechanisms and kinetics of content release from TSPL in response to local heating. In addition, the impact of lipid composition, polymer and copolymer characteristics, serum components and PEGylation on the mechanism of content release and TSPL performance is addressed. This is done from the perspective of rationally designing TSPL, with the overall goal of conceiving efficient strategies to increase the efficacy of TSPL plus hyperthermia to improve the outcome of targeted anticancer therapy.

Contents

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1. Introduction

Site-specific and controlled drug delivery to diseased tissues in order to increase drug concentration at the target site, while reducing exposure of healthy tissues, is a holy grail in drug delivery. Especially chemotherapeutics agents that cause toxic effects on normal cells benefit from this approach. Nanocarriers are useful tools not only to formulate a variety of drugs with different physicochemical properties [1–3], but also to alter and improve pharmacokinetics of associated compounds [4–10]. The conception of the enhanced permeation and retention (EPR) effect, which proposes that nanoparticles can extravasate due to the leaky nature of a tumor vasculature, and retain there because of impaired lymphatic drainage [11,12], brought hopes that the therapeutic efficacy of chemotherapeutics can be improved via preferential accumulation of nanoparticles inside tumors. Since then, EPR-based tumor targeting has become the central dogma in development of nanoparticles to treat solid cancers and an immense number of studies on different types of nanoparticles were performed. However, only a few were eventually clinically approved [13]. Importantly, the step forward achieved with nanotechnology has not necessarily been an improved antitumor outcome, but mostly reduced side effects and less post treatment complexities [14,15], which are indeed valuable benefits for patients and should not be ignored.

1.1. Opportunities with trigger-responsive nanocarriers

Among the variety of factors that have impact on the success of nano-vehicles in clinic, tumor heterogeneity, limited penetration and poor distribution of extravasated nanoparticles into depth of tumors and poor cellular delivery of bioavailable drug are the most important hurdles [16,17]. For successful passive targeting of a tumor while relying on EPR, nanoparticles need to be stable and unrecognizable by the host immune system, and circulate relatively long (i.e. hours to days) in blood to increase the probability of extravasation during passage through a tumor. However, while these prerequisites are desired when the injected nanoparticle is in blood, a different set of characteristics are needed when arrived inside the target area, e.g. a tumor, where free, bioavailable drug molecules have to reach the target. In fact, despite ample evidence showing that compounds associated with nanoparticles accumulate to a higher degree than free drugs in a tumor, the lack of release, and therefore impaired cellular interaction, negatively impact cellular drug delivery and consequently the anti-tumor effect. This, in part, contributes to the clinical failure of many nanodrugs, or in outweighing the improved patient comfort over the therapeutic efficacy of clinically accepted nanodrugs.

The contradicting characteristics of nano-carriers brought more attention towards the so-called smart drug delivery systems, which are enabled to release or interact with target cells on demand in the target tissue. Other than ligand-modified nanoparticles that can interact with target cells, nanoparticles could be designed to be sensitive to a variety of stimuli to stimulate interacting with cells or release payload on demand. These stimulus could be endogenous such as reduced pH [18–20] or elevated enzymatic [21] or redox activity [22], or could be applied externally such as magnetic and electric fields [23,24], ultrasound [25–27], light [28,29] or heat [30–32].

Compared to endogenous stimuli in which the kinetics of compound release are mostly slow and heterogeneous, exogenous stimuli provide a high degree of spatiotemporal control and a faster drug release can be induced. Additionally, endogenous trigger-sensitive nanoparticles mainly dependent on EPR for passive accumulation inside tumors, and as stipulated above the functionality of EPR is under debate. Application of external stimuli such as heat [30–32] or sonoporation [33–35] can be applied independent of tumor pathophysiology to induce local release of content. More so, these external stimuli can also be used as vascular permeability enhancer to increase extravasation of nanoparticles [33,34].

Among different external stimuli mild hyperthermia is a convenient and advanced approach that not only could be used to induce heat-triggered responses of temperature-sensitive nanoparticles but also has therapeutic potential. Mild hyperthermia has been shown to increase sensitivity of tumor cells to chemotherapy or radiotherapy [36,37], increase tumor cell death in hypoxic regions [38–40] (regions in which radiation is known to be less effective), diminish DNA damage repair [41], and activate the immune system [42–45]. Hyperthermia is currently being used in clinical practice in combination treatment setting [46–48]. More details of therapeutic potentials of hyperthermia has been documented by Issels et al. [49].

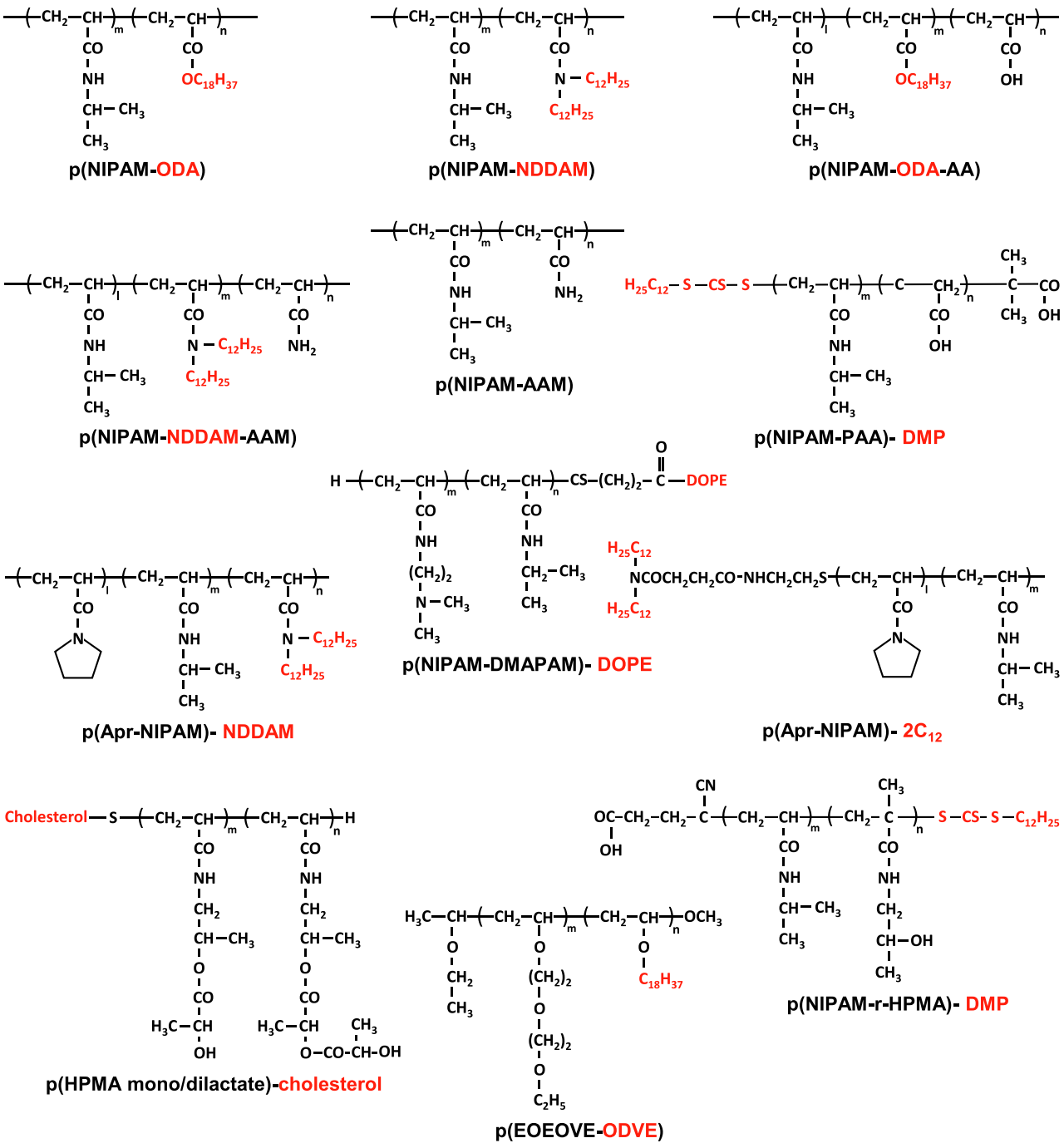

Fig. 1. Chemical structures of synthetic temperature-sensitive polymers (TSP), exhibiting LCST and have been employed for induction or enhancing thermal-triggered release from liposomes. Lipophilic groups that work as anchor units to fix copolymers on lipid membrane are represented in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Increased perfusion and enhanced vascular permeability, by increasing pore size in tumor vasculature, are two key features of applying mild hyperthermia (around 42 °C) on tumor that are in favor of delivering nanoparticles [30,50–52] or free drugs [53,54] to tumor tissue. Besides, mild hyperthermia increases cellular permeability thus facilitate cellular drug delivery [41].

Therefore, combination of mild hyperthermia with temperature sensitive nano-drugs not only provides spatiotemporal controlled targeted drug delivery but also enhances drug accumulation and antitumor response. For more details we refer readers to [55].

1.2. Lipid-polymer nanocarriers for hyperthermia-mediated triggered release

Hyperthermia (HT), as described above, could be used to trigger release from nanocarriers. An increasing number of nanocarrier formulations are studied of which lipid-based are the most advanced. Recently also polymer-based and lipid-polymer hybrid systems come in focus.

An important advantage of polymers and lipids is the possibility to select, or design, new lipids or polymers to create a surface that

has temperature dependent phase transition behavior at mild hyperthermia range (41–42 °C). Polymers and lipids have been employed in fabrication of temperature-sensitive polymeric nanoparticles or temperature-sensitive liposomes (TSL, here referred to a liposomal preparation in which temperature dependent response derives from the transition behavior of lipids). While composition of TSL centered around 1,2-dipalmitoyl-*sn-glycero*-3-phosphocholine (DPPC, transition temperature (Tc) of 41 °C) as the main lipid there are a variety of temperature-sensitive polymers (TSP) that could be custom synthesized and used for the preparation of nanoparticles. However, temperature-sensitive polymeric nanoparticles exhibit fairly slow kinetics of drug release at mild hyperthermia (e.g several hours to days for complete drug release [56]), whereas TSL exhibit a fast rate of drug release that fits with the hyperthermia setting in clinic in which duration of heating and maximum temperature reached are limited. In addition to that, liposomes, because of the aqueous interior, are efficient carriers for triggered release drug delivery. The combination of flexibility, high loading capacity and precise tuning to HT rendered liposomes successful and led TSL to reach late stages of clinical investigation [57].

On the other hand, polymers have a broad spectrum of chemistry and show expansive responses to a variety of stimuli [58,59]. Thermal response of polymers derives from changes in polymer solubility in aqueous solution at different temperature and can be classified into two kinds of polymers: (A) polymers exhibiting Lower Critical Solution Temperature (LCST), which become insoluble when ambient temperature exceeds LCST and the polymeric solution becomes turbid or cloudy (Cloud Point (CP) is alternatively being used). And, (B) polymers exhibiting Upper Critical Solution Temperature (UCST), which are insoluble at ambient temperature but become soluble when temperature exceeds UCST. When Kono [60] combined the thermal response of TSP exhibiting LCST with the carrying capability of liposomes, it was found that temperature-sensitive polymer-modified liposomes (TSPL, here referred to liposomes with a thermosensitive or a non-thermosensitive lipid membrane that are modified with temperature-sensitive polymers) can release encapsulated hydrophilic compounds at elevated temperature in comparable rates as TSL. Preparation of TSPL is relatively easy and the advanced knowledge of copolymer chemistry enables precise tuning of the transition temperature (i.e. the LCST) to trigger drug release. Fig. 1 illustrates chemical structures of different synthetic copolymers exhibiting LCST that have been used in design of TSPL. In principle, a temperature-sensitive copolymer consist of hydrophilic and hydrophobic polymer chains. By adjusting the content of each component or using a different copolymer chemistry-one can tune the LCST. For instance, by increasing the hydrophilic content or through addition of more hydrophobic moieties to a copolymer backbone the LCST will be increased or decreased, respectively.

Despite the promising potential benefits of TSPL, there are several concerning challenges that need to be addressed. The vast number in variations possible in a TSPL, such as lipid composition, different copolymers with different physical and chemical characteristics, ratios and combination of different lipids and polymers make application of TSPL complicated. Application TSPL has previously been reviewed by Kenji Kono in 2001 [61]. Here, in addition to the primary studies, recent advances and progresses made in design and application of TSPL for heat-triggered drug release have been described. The current manuscript aims at providing a comprehensive overview of different TSPL studied with in-depth analysis of different aspects related to TSPL, such as formulation, preparation and mechanisms and kinetics of release, in order to illustrate a clear picture of potentials and applicability of TSPL in heat-triggered drug delivery and to improve their application based on lessons learned from development of TSL.

2. Modification of liposomes with temperature-sensitive polymers

As mentioned above, combination of copolymers with phospholipids may provide an optimal platform for generation of nanocarriers for controlled delivery of compounds. Addition of copolymers to a liposome membrane could be done by: A) post-insertion of the copolymer into preformed liposomes or by B) addition of TSP, if soluble in organic phase, to a lipid mixture in an organic solution.

While post-insertion is convenient and does not limit remote loading of drugs into liposomes only the outer surface of liposomes will contain TSP. Remote loading of drugs is a method which makes use of a driving force, e.g. gradient or pH difference, to load nanoparticles with a compound. It has been shown that modification of both inner and outer surfaces of a liposome membrane with a copolymer results in a sharper and stronger heat-triggered release at the LCST of the copolymer, compared to modification of liposomes with the post-insertion technique [62]. However, presence of heat-sensitive polymers in the liposome bilayer imposes limitations on remote loading of a drug, which mostly requires heat.

The main approach used to decorate liposomes with TSP is through the addition of a lipophilic anchor. These anchor units are specifically added to the copolymer to promote incorporation into the liposomal membrane and to fix the copolymer on the surface of the membrane (Fig. 2). However, Han et al. found that NIPAM copolymers can be fixed on the liposome surface without addition of specific anchoring residues via the hydrophobic isopropyl groups of NIPAM [63,64]. Alternatively, electrostatic interaction between cationic charged liposomes and negatively charged NIPAM-*co*-MAA has also been employed for coating of liposomes with TSP [65] (Fig. 2).

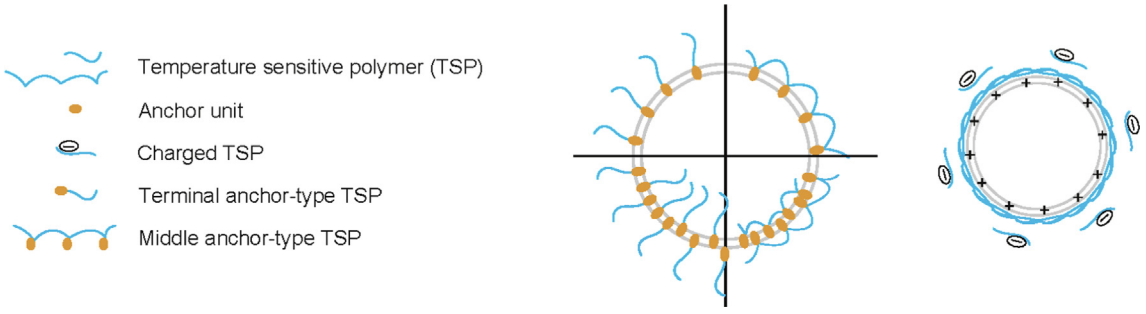

Fig. 2. Schematic illustration of different design features and decoration of liposomes with different temperature-sensitive polymers.

Addition of anchor units to copolymers could be done in two different ways (Fig. 2): I: Middle anchor-type, in which several anchor units such as octadecylacrylate (ODA) [60,66,67], or N,N-didodecylacrylamide (NDDAM) [68,69] or phospholipids [70] are added to the polymeric backbone as an additional comonomer during copolymerization. II: Terminal anchor-type, in which anchor units such as phospholipid [71], 2-dodecyl-sulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP) [72], two dodecyl groups ($2C_{12}$) [68,73,74], or cholesterol [75] are added to a synthesized copolymer via a functional group at the end of the copolymer chain.

As stated earlier increasing the hydrophobic content of a copolymer results in decreased LCST. Therefore, addition of anchor units reduces the LCST of copolymer in solution. However, important is to consider that once a copolymer is incorporated in a liposome membrane the temperature at which TSPL exhibit transition behavior will be close to the LCST of the anchor free copolymer in solution. In fact, when the lipophilic residue is lodged in and stabilized in a lipid membrane this residue is phased out and contributes to a lower degree to the thermal behavior of the attached copolymer. This makes LCST adjustment more complex and restricts extrapolating of thermal behavior of a copolymer in solution to predict thermal behavior once fixed on a liposome surface. In addition to chemistry and molecular weight of copolymers and anchor units, the type of anchoring also intervenes in this thermal behavior difference. Takei et al. [76] suggested that with the middle anchor-type, multi point grafting of NIPAM restricts conformational freedom of copolymer chains and disturbs dehydration and hydrogen bonding of the copolymer with water molecules, whereas with the terminal anchored type the copolymer chains are not subjected to additional conformational restrictions and thus demonstrates a higher extent of decrease in hydrophilic properties upon exposure to heat. For instance, APr-NIPAM (79.8:20.2 mol%, Mn 8300) represents a sharp coil to globe transition at the cloud point (CP) of 38 °C. Addition of 1.5 mol percent NDDAM as middle anchor-type resulted in significant reduction of the CP to 25.6 °C in APr-NIPAM-NDDAM (79.4:18.5:2.1 mol%, Mn 7740). But when APr-NIPAM (81.6/18.4 mol%, Mn 4100) was conjugated with 2C12 at the copolymer end the LCST was reduced from 39.6 °C to 36.4 °C. When these copolymers were decorated on the surface of DOPE (1,2-Dioleoyl-*sn-glycero*-3-phosphoethanolamine) liposomes it was expected that heat-triggered release from TSPL starts at temperatures close to the LCST of the anchor free copolymer. However, liposomes modified with APr-NIPAM-NDDAM showed 26 % release upon 15 min incubation at 25 °C, while the same content release from liposomes modified with p(APr-NIPAM)-2C12 was achieved at 38 °C. More importantly, temperature dependent release of liposomes modified with terminal anchored copolymer was significantly sharper than liposomes modified with middle anchored copolymer [68]. It seems that thermal behavior of end terminal anchored copolymer is more in favor of heat-triggered drug release.

3. Mechanism of release

To understand the mechanisms of drug release from TSPL it is important to understand the mechanic of thermal response of TSP representing LCST. A temperature-sensitive polymer is soluble in water below the LCST due to formation of hydrogen bonds between hydrophilic parts of the copolymer and water molecules and therefore represents an expanded coil. Increase of temperature weakens the hydrogen bonding between the hydrated polymer chains and water molecules and polymer chains start to lose hydrophilicity and gradually become dehydrated and start to exhibit hydrophobic behavior. Increasing the hydrophobicity of a copolymer is entropically unfavorable for water molecules. To compensate this, water decreases the surface of contact with the hydrophobized polymer chain by phasing out the polymer from the solution. During this process expanded polymeric chains (coil) shrunken into dehydrated globule [77,78].

Both increase of hydrophobicity and geometrical changes upon shrinkage play role in disturbing the integrity of the liposomal membrane decorated with TSP and affect drug release. However, the function of TSP and effect on release of content from liposomes is strongly dependent on the nature of the liposomal membrane and differs depending on liposome composition. Three kind of liposomes, including thermosensitive liposomes (TSL), non-thermosensitive liposomes (non-TSL) and DOPE-containing liposomes, could be modified with TSP but with different kinetics and mechanisms of thermal response release that will be addressed later.

3.1. Anchor type, membrane state and thermal response

Ringsdorf [79] was the first to engraft NIPAM with middle anchor octadecyl/pyrene groups on the surface of liposomes and studied the thermal behavior of polymers. Pyrene groups are spatially sensitive fluorophore probes that exhibit different emission spectrums as monomers (emission peaks at 375–405 nm) or when two or more fluorophore are spatially closed (emission peaks at 375–405 nm similar to monomer plus addition broad emission centered around 460–480 nm which is called excimer). It was shown that when modification was done on liposomes composed of DMPC (1,2-dimyristoyl-*sn-glycero*-3-phosphocholine) (Tc: 23 °C) fluorescence spectra of pyrene changed by increasing tem-

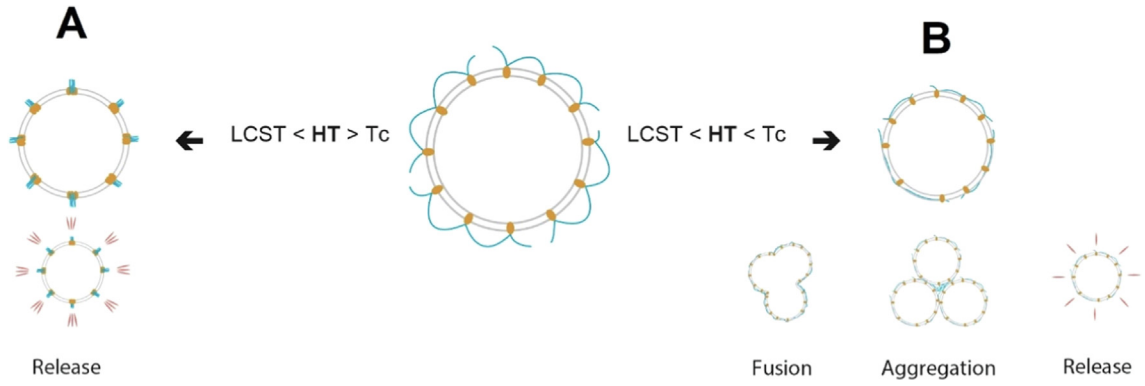

Fig. 3. Schematic illustration of the thermal behavior of middle anchor-type polymers when decorated on the surface of liposomes with different thermal states, with the lipid membranes being either in liquid-like phase (A: Tc < HT) or in solid-like phase (B: Tc < HT).

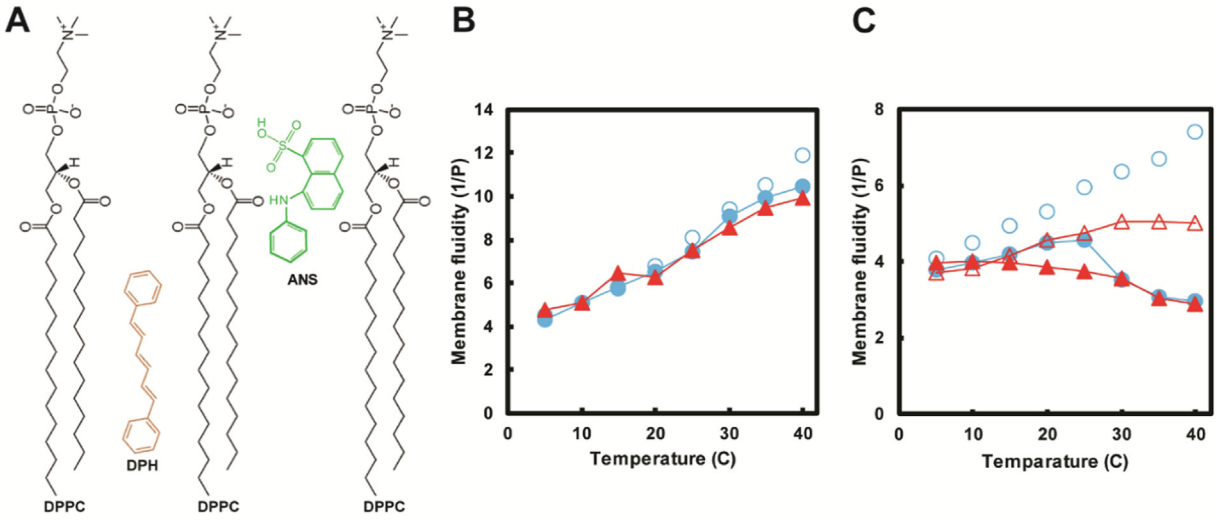

Fig. 4. Schematic illustration of DPH and ANS orientation in a phospholipid membrane (A). Membrane fluidity (1/P) of different liposomal preparations including plain EPC liposomes (○), copolymer-modified EPC liposomes (●), plain DOPE/EPC liposomes (0.64/0.36, mol/mol) (△), and copolymer-modified DOPE/EPC liposomes (0.64/0.36, mol/mol) (▲) as a function of temperature, monitored by DPH (B) or ANS (C). The concentrations of lipid and fluidity marker (DPH or ANS) were 0.4 mM and 4.4 mM, respectively. (B and C were adopted with permission from Kono et al. 1999 [69]).

perature from 26 °C to 34 °C. At the LCST of copolymer (32 °C), the expanded polymer chains shrink, polymer-loops between anchors collapse and force anchor groups containing pyrene to move towards each other. The number of pyrene pairs close enough to form excimers increase, which results in an enhanced excimer emission centered at 480 nm. Upon cooling, the polymer retrieves the hydrated expanded structure forcing anchor units and paired pyrenes apart from each other and the fluorescence spectra returns back to ground state. At this temperature range these happen due to mobility and freedom of lipids in lateral diffusion because DMPC is in liquid-like state. When the phospholipid DSPC (1,2-distearoyl-*sn-glycero*-3-phosphocholine) is used as liposomal lipid no pyrene pairing and spectral changes occur at the LCST of the polymer but pyrene approximation happens when temperature exceeds above Tc of phospholipid (i.e. at 56 °C). However, unlike DMPC liposomes, cooling down of polymer-DSPC liposomes that were exposed to temperatures above 56 °C does not result in reversible relocation of anchor units because below the Tc of the phospholipid, at which the membrane exhibits solid or gel-like phase, anchor units cannot diffuse laterally to separate paired pyrene (the concept is illustrated in Fig. 3).

Hayashi et al. [80] observed that when liposomes composed of DPPC (Tc: 41 °C) or DSPC (Tc: 55 °C) were modified with NIPAM-ODA (99:1, mol/mol, LCST: 30 °C) significant aggregation and fusion were observed in the liposomal suspension at temperatures between LCST of the co-polymer and Tc of the lipid membrane. However, at temperatures above the Tc of the phospholipids, at which transition behavior of lipids is completed, phospholipids are mostly at liquid ordered phase, and the membrane becomes more stable and less permeable compared to the state at the Tc [81]. Less significant aggregation of liposomes was seen, despite the hydrophobic nature of the surface anchored copolymer above LCST. When DLPC (1,2-dilauroyl-*sn-glycero*-3-phosphocholine, Tc = −2°C), that represents liquid-like phase under experimental condition was used as lipid, no significant association between polymer-modified liposomes took place at all experimental temperatures (Fig. 3A).

These results reveal that copolymer chains fixed on the surface of liposomes at the gel-like phase promote aggregation and fusion of the liposomes by hydrophobic interactions between copolymer chains and/or between copolymer chains and liposome membranes at temperatures above LCST (Fig. 3B). At these temperatures interaction of dehydrated polymer chains with the liposome membrane disturbs the integrity of the membrane and makes the membrane permeable for releasing encapsulated small molecules.

On the other hand, copolymers that are fixed on the surface of liposomes exhibiting liquid-like phase have more lateral mobility compared to gel-like state, therefore dehydrated copolymer chains form inter-bilayer aggregates upon shrinkage of the copolymer above the LCST. Formation of such aggregates in the bilayer reduces exposure of hydrophobic copolymer chains on the surface of liposomes, which results in less aggregation between copolymer-modified liposomes (Fig. 3A). In agreement with this is the observation that thermoresponsive polymers are more effective in temperature ranges in which the lipid membrane is in a gel like phase rather than liquid phase. For instance when DSPC is used the release of polymer-modified liposomes is much greater than polymer-modified EPC (Egg phosphatidylcholine, Tc: ~0 °C) [67].

3.2. Temperature-dependent interaction of polymers and phospholipids

Fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), and 8-anilino-1-naphthalenesulfonic acid (ANS) provide helpful information in studying the interaction of copolymers and liposome membranes. The inverse polarization of fluorescence probes is an indicator of membrane fluidity [82]. While ANS provides information on fluidity of the surface of a lipid membrane, with DPH one can track the fluidity of hydrophobic regions of a membrane (Fig. 4A). Monitoring the membrane fluidity of plain EPC, poly-EPC and poly-EPC/DOPE (36:64 mol%) by DPH revealed a monotonous increase of membrane fluidity by increasing the temperature, which occurs without significant differences in liposomes of different makeup [69] (Fig. 4B). However, when fluidity changes, as a function of temperature, were tracked by ANS it was found that for plain EPC membranes fluidity increases linearly with increasing temperature from 4 to 40 °C. For poly-EPC liposomes from 4 to 20 °C fluidity increases linearly but above 25 °C, a temperature that corresponds to the LCST of the used copolymer, fluidity reduces drastically implying adsorption of the hydrophobic copolymer chain on the surface of liposomes, which reduces the lateral mobility of membrane phospholipids (Fig. 4C). Plain EPC/

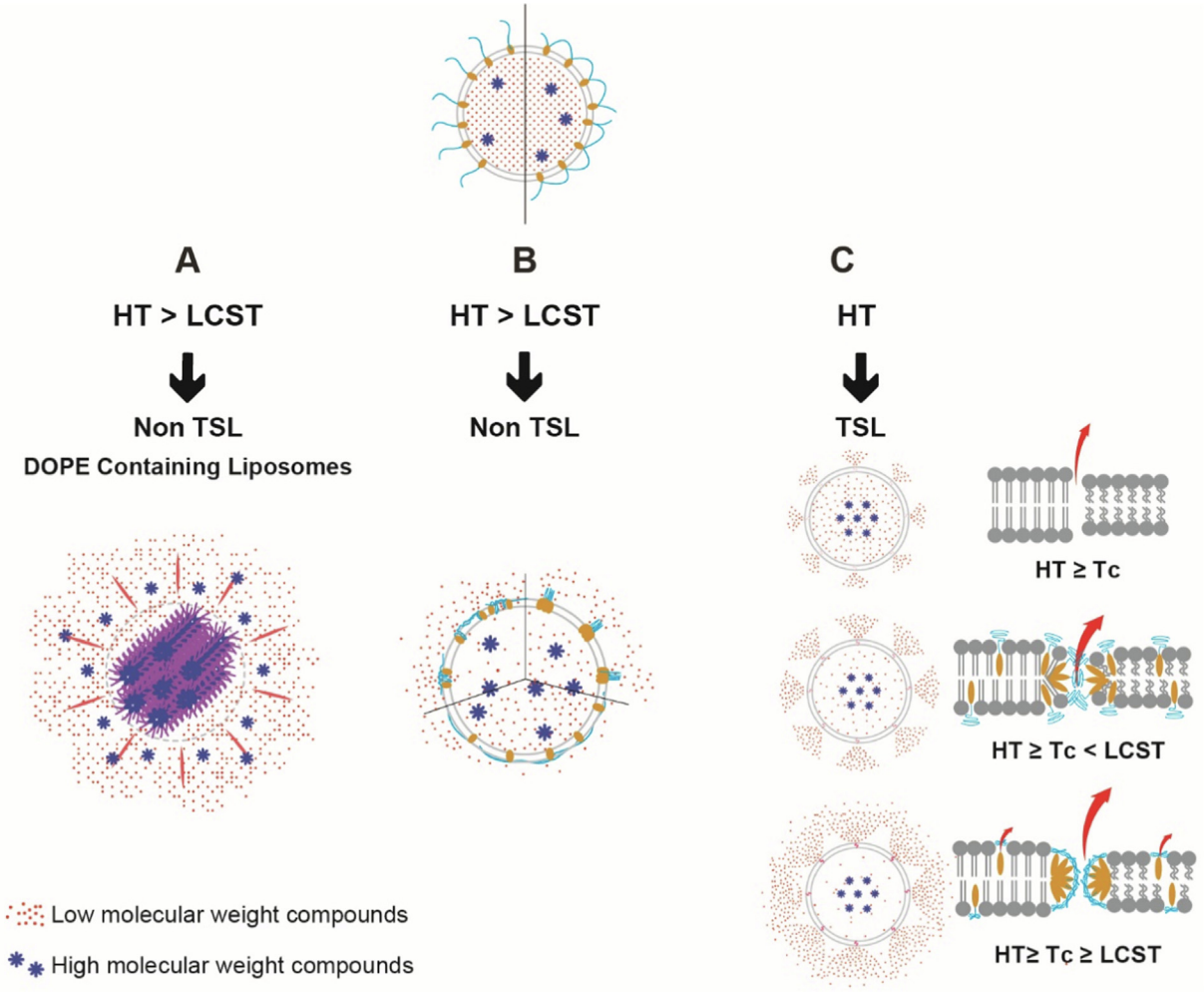

Fig. 5. Schematic illustration of mechanisms of heat-triggered content release from liposomes with different lipid compositions. In TSPL containing DOPE thermal behavior of TSPL is govern by the thermal activity of TSP where transition of TSP impose structural pressure on liposome membrane and induce transition of liposome membrane from bilayer to hexagonal phase and disintegrates liposomes (A). In TSPL composed of non-temperature sensitive lipids (non-TSL), the thermal behavior of TSPL is governed by the TSP, however, the thermal response is dependent on anchor type and the state of liposome membrane (B). When TSPL is composed of temperature sensitive liposomes (TSL) the thermal behavior of TSPL is mainly governed by the thermal activity of TSL (C): At temperatures above Tc of TSL and below LCST of TSP release occurs at Tc and TSP supports grain boundaries that facilitates drug release. When hyperthermia is above both Tc and LCST, major release takes place from grain boundaries of TSL and transition behavior of TSP facilitates and accelerates drug release from TSL.

DOPE liposomes exhibit less surface fluidity compared to plain EPC liposomes most likely due to the unfavorable geometrical inverted-cone shape structure of DOPE while in a bilayer phase a cylindrical shape is favorable. Incorporation of copolymer into this membrane increases the membrane packing and reduces the membrane fluidity even further. In contrast to poly-EPC liposomes, in poly-EPC/DOPE liposomes no increased fluidity as a function of temperature takes place. The gradual reduction of membrane fluidity starts at lower temperatures of around 15 °C indicating that a DOPE containing membrane is more sensitive even to partial conformational changes of TSP in response to heat (Fig. 4C). In addition to the packing stress imposed to membrane by the geometry of DOPE, given the fact that thermosensitive copolymer chains lose their hydrogen binding with water molecules at elevated temperatures the strong hydrogen binding site of PE is a great substitute that enhances the interaction of copolymer chains with the lipid membrane which imposes more packing stress into the membrane. This also contributes to the reduced fluidity and high sensitivity of DOPE liposomes to heat [69].

Comparing membrane fluidity measured by DPH to those obtained by ANS clearly reveals that in liposome that do not undergo phase transition, surface-anchored TSP collapse and interact with surface of liposomes in response to heat as indicated by ANS. However, the heat-hydrophobized chains of copolymer do not penetrate deep into lipophilic part of bilayer as indicated by DPH measurements.

In addition to the state of lipids at the LCST of a copolymer, the geometry of lipids also impacts on heat-triggered release from TSPL. The geometry of DOPE molecules is not in favor of a stable bilayer but tends to form a hexagonal phase. Therefore, DOPE is mainly used in combination with other lipids; DOPE may act as a lipid helper to induce fusion at low pH [83]. It was found that liposomes containing DOPE could be stabilized at physiological pH by incorporation of anchor units of TSP into the lipid bilayer. Interestingly, such liposomes exhibit burst drug release at the LCST of the used copolymer. In liposomes composed of EPC and modified with EOEOVE-*b*-ODVE, 90 % release of calcein (MW: 622.55 g/mol) could be obtained within 10 min incubation at 40 °C, whereas the same liposomes without copolymer retain 90 % of their encapsulated FITC-Dextran (average MW 4000) after 30 min incubation at 40 °C. In contrast, release from DOPE:EPC (70:30) triggered by thermosensitivity of EOEOVE-*b*-ODVE is not dependent on the molecular size of the encapsulated payload and both calcein or FITC-Dextran was released in a virtually similar manner [79]. This

implies that release from DOPE containing liposomes at the LCST of the copolymer is not because the membrane is permeabilized, but takes place because the liposome is ruptured (Fig. 5A) and enables all kind of payloads to be released. This is a unique feature of this kind of TSPL, whereas in TSPL composed of non-thermo sensitive lipids (Fig. 5B) or those composed of temperature-sensitive lipids (Fig. 5C), release rate is affected by molecular size of the encapsulated payload and compounds with large molecular weight cannot be released. Such release pattern of DOPE congaing TSPL is importantly different from a phospholipid bilayer going through solid-like to liquid-like transitions during which permeability increases but integrity is maintained (Fig. 5C). As stated earlier the geometry DOPE is not in favor of a bilayer phase. Furthermore, phosphatidylethanolamine provides ample hydrogen binding sites for interaction with dehydrated copolymer chains at elevated temperatures. Such interactions result in rigidifying of the DOPE-membrane as fluidity of the polymer-modified DOPE liposome membrane reduces when the temperature is increased. Taken together, at the LCST of the copolymer, the dehydrated chains shrink and interact with PE imposing structural pressure on the bilayer, making the bilayer unstable and finally inducing transition of this bilayer to hexagonal phase and complete destruction of the liposome occurs.

3.3. Polymer-phospholipid combination in thermosensitive liposomes

In addition to sensitizing liposomes to heat, TSP can also synergistically promote both rate and magnitude of release from temperature-sensitive liposomes. However, in such TSPL, copolymers play a dual role depending of the Tc of the used lipid. While transition behavior of polymers, and interaction with a lipid membrane, can permeabilize this membrane and induce release, their presence on the surface of TSL also facilitates release from grain boundaries formed in the lipid membrane during phase transition of the lipids. If during temperature increased transition of the used lipid occurs, close to or sooner than the LCST of the used copolymer, the Tc of the lipid determines the release temperature. In such setting TSP mainly play a supporting role in increasing the magnitude of release (Fig. 5C). Whereas, when used lipids do not undergo phase transition during working temperatures (i.e. 20–45 °C) such as EPC or DSPC the temperature at which significant release starts will be close to the LCST of the polymer [67] (Fig. 5B). It is also worth mentioning that release from DPPC-based TSPL at 41 °C is much greater than release from TSPL consisting of DSPC or EPC or polymer free DPPC-TSL at this temperature, suggesting a synergistic effect of the former combination. To understand the supporting role of TSP on release from TSL it is important to know that bare DPPC liposomes do not show significant temperature-triggered release in serum-free buffer. But when modified with non-temperature-sensitive AAM-ODA copolymer [60], or with PEG-DSPE, a significant release occurs at the Tc of DPPC in this condition, which implies the role of hydrated polymeric chains in enhancing drug release from TSL by stabilizing and supporting the grain boundaries formed in TSL during phase transition of lipid membrane.

Another difference in release behavior from TSPL composed of DPPC compared to EPC-based TSPL is the effect of polymer concentration on the profile of temperature-dependent release. While increasing the concentration of polymer in DPPC liposomes reduces premature release at temperatures below Tc and increases the magnitude of dye release from these liposomes (resulting in sharper temperature dependent release curves), in EPC liposomes increasing the polymer concentration increases the release rate but also decreases the temperature of onset of release, broadening the temperature dependent release curve [60].

The mechanism of release from TSPL varies depending on phospholipid composition, state of lipid bilayer and even type of anchor units. Understanding the mechanisms of release from a TSPL is not only crucial to predict the release behavior of TSPL, but also important in tuning and optimizing TSPL formulation. However, the complexity of TSPL response to changing temperature and the vast number of parameters influencing this behavior makes it difficult to predict outcome.

It is worth noting that the mechanisms described here are mainly related to synthetic polymers. Mechanism of temperature sensitivity of liposomes modified with biopolymers will be described later in the section on biopolymers.

4. Polymers for sensitization of liposomes to hyperthermia

Different synthetic copolymers and biopolymers have been employed for induction or enhancing heat sensitivity of liposomes (i.e. thermal content release or thermal triggered cellular association). Here, modifications and application of heat sensitive polymers that have shown successful thermal triggered release once decorated on liposomal membrane will be discussed in details.

4.1. N-isopropylacrylamide (NIPAM)-based copolymers

4.1.1. NIPAM anchored with octadecylacrylate (p(NIPAM-ODA))

The concept of temperature-triggered drug release from liposomes by means of temperature-sensitive polymer-modified lipo-

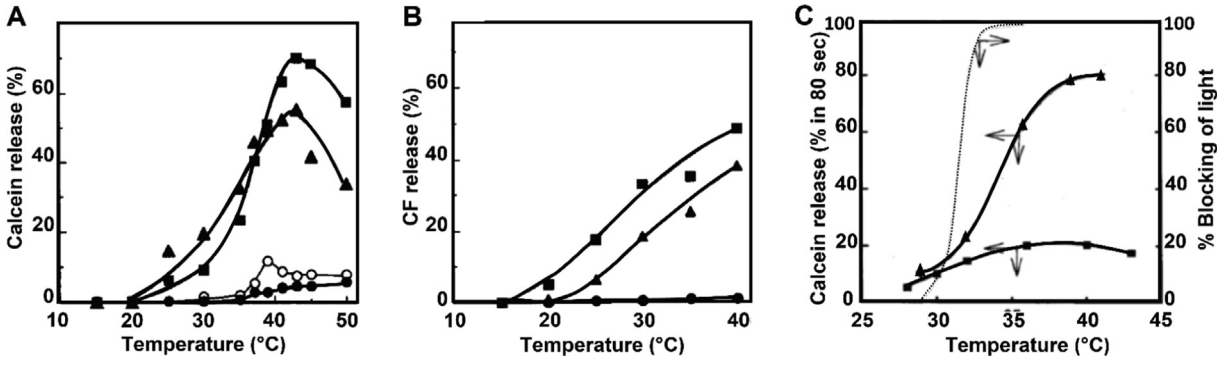

Fig. 6. Temperature-dependent release of encapsulated water-soluble dye from different TSPL. Panel A represents temperature dependence of calcein release from plain DPPC liposomes (●), DPPC liposomes modified with p(NIPAM-ODA) prepared by incubation in aqueous solution containing 1 mg (▲) or 5 mg (■) of copolymer, and DPPC liposomes modified with p(AAM-ODA) by incubation in aqueous solution containing 1 mg of the copolymer (○). Panel B represents Temperature dependence of CF release from plain EPC liposomes (●), EPC liposomes modified with p(NIPAM-ODA) prepared by incubation in aqueous solution containing 1 mg (▲) or 5 mg (■) of copolymer. Panel C represents Temperature dependent release from EPC (■) and DOPE (v) liposomes modified with p(NIPAM-ODA) in weight ratio of 0.1 polymer to lipid. Dotted line represents changes in turbidity of copolymer solution in PBS, at pH 8.0. (A and B were adopted from Kono et al. 1994 [60] and C was adopted from Kim et al. 1998 [85].

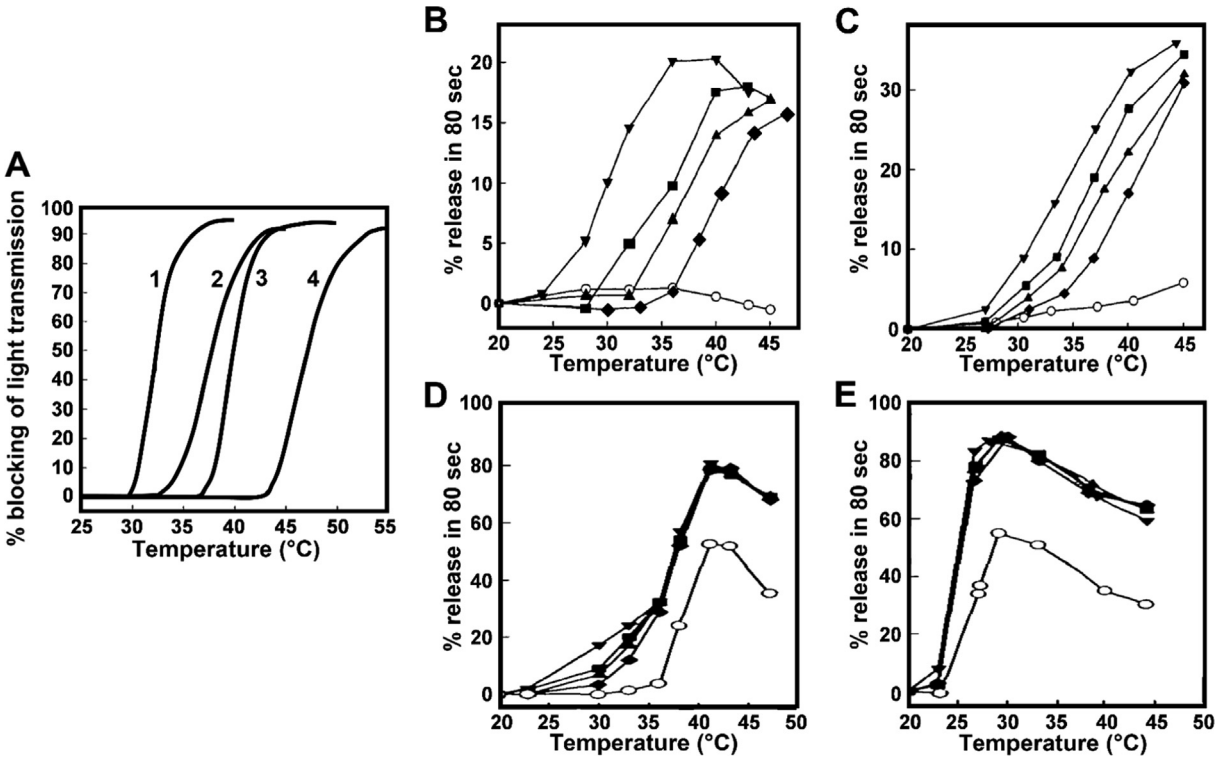

Fig. 7. Effect of AA content on thermal behavior of p(NIPAM-ODA-AA) copolymers in solution (A) or when incorporated into different liposomal membranes (B-E). Panel A represents change of turbidity of p(NIPAM-ODA-AA) in PBS (pH 7.4) with temperature. Plots 1, 2, 3, and 4 correspond to the turbidity changes of polymer solution of which the AA contents are 0, 1.09, 2.15, and 3.19 mol%, respectively. The concentration of polymers was 5 mg/ml in each, and the heating rate was 1 °C/min. Panels B-E represent temperature-dependent calcein release from EPC liposomes (B), DSPC liposomes (C), DPPC liposomes (D) and DMPC/DPPC (5: 5, w/w) liposomes (E) mixed with no copolymer (○), copolymer 1 (▼), copolymer 2 (■), copolymer 3 (▲), or copolymer 4 (◆).The ratio of the copolymer to lipid was 0.1. (reprinted with permission from Kim et al. 1997 [67]).

somes (TSPL) was first experimentally proven by Kano et al. [60] These liposomes were modified with p(NIPAM-ODA) (Fig. 1), in which NIPAM plays as thermoresponsive unit and ODA works as the anchor unit to stabilize the polymer in the outer surface of liposomes composed of either DPPC (Tc: 41.5) or EPC (Tc: bellow 0 °C) and encapsulating hydrophilic dyes (Fig. 6A and B). Modification of liposomes with p(NIPAM-ODA) (LCST: 27 °C) synergistically enhanced the thermoresponsiveness of DPPC liposomes and sensitized EPC liposomes to heat. However, these liposomes showed different profiles of dye release. In poly-DPPC liposomes significant release of encapsulated calcein or carboxyfluorescein (CF) started at around 25 °C reaching maximum release at around 41 °C, equal to the Tc of DPPC, followed by a decline in release at higher temperatures (Fig. 6A). Such pattern of content release, although more pronounced, is similar to what could be observed by heating PEGylated liposomes composed of DPPC [84] in serum free buffer.

Poly-EPC liposomes, which go through phase transition at working temperatures above 0 °C, release negligible amounts of calcein (MW: 622.5 g/mol) upon heating, while the release of a smaller molecules, i.e. CF (MW: 376.3 g/mol), started at around 25 °C, and increased by increasing the temperature in an almost linear manner (Fig. 6B). In addition to the different temperature-dependent release pattern, the magnitude of CF release from poly-EPC liposomes at 41 °C was 20 % smaller than what observed from poly-DPPC liposomes.

Later, the same group obtained more promising results by modifying liposomes composed of DOPE with p(NIPAM-ODA) (LCST: 30 °C) in which release was controlled by the LCST of the polymer through transition of the vesicle membrane from bilayer phase to H_{II} phase [66] (Fig. 5). DOPE alone cannot form stable liposomes, however inclusion of p(NIPAM-ODA) not only stabilized DOPE liposomes at room temperature (RT) but also sensitized these liposomes to heat [66,85]. p(NIPAM-ODA)-DOPE liposomes showed an onset of release at 30 °C and complete release was achieved within few seconds of incubation at 40 °C [66]. However, this TSPL shows around 90 % release during 1 min incubation at 35 °C, which halts use in the clinical setting. The same observation was also reported by Kim et al. [85] where increasing the DOPE content in EPC liposomes increases the sensitivity of liposomes to the thermal response of p(NIPAM-ODA), reaching 80 % calcein release within 25 *sec* of incubation at 40 °C. As could be seen in Fig. 6C TSPL composed of DOPE exhibit a significantly higher heat sensitivity than TSPL composed of EPC. Unlike poly-DPPC liposomes, in which release is mainly govern by the transition behavior of the lipid membrane, release from DOPE containing liposomes is triggered by the transition behavior of the co-polymer. Since the LCST of a copolymer can be controlled by adjusting the ratio of hydrophobic and hydrophilic blocks, DOPE containing vesicles are great candidates to be used for preparation of TSPL with favorable responsiveness to heat.

As stated earlier, one possible solution to increase the onset temperature of release is increasing the LCST of the copolymer by increasing the hydrophilic content. Kim et al. [67] studied copolymerized NIPAM with acrylic acid (AA) and synthesized different p(NIPAM-ODA-AA) (Fig. 1) with increasing AA contents of 0, 1.09, 2.015 and 3.19 mol% (Fig. 7A). These co-polymers exhibit a sharp coil to globe transition at elevated temperatures and the temperature at which the turbidity of co-polymer solution started to increase was 29, 33, 37 and 43 °C, respectively. When co-polymers of different AA content were fixed on the outer surface of different liposomes made of DSPC (Tc: 55 °C) (Fig. 7B), EPC (Tc: bellow 0 °C) (Fig. 7C), DPPC (Tc: 41 °C) (Fig. 7D) or DPPC:DMPC (50:50 mol, Tc around 28 °C) (Fig. 7E) different release patterns

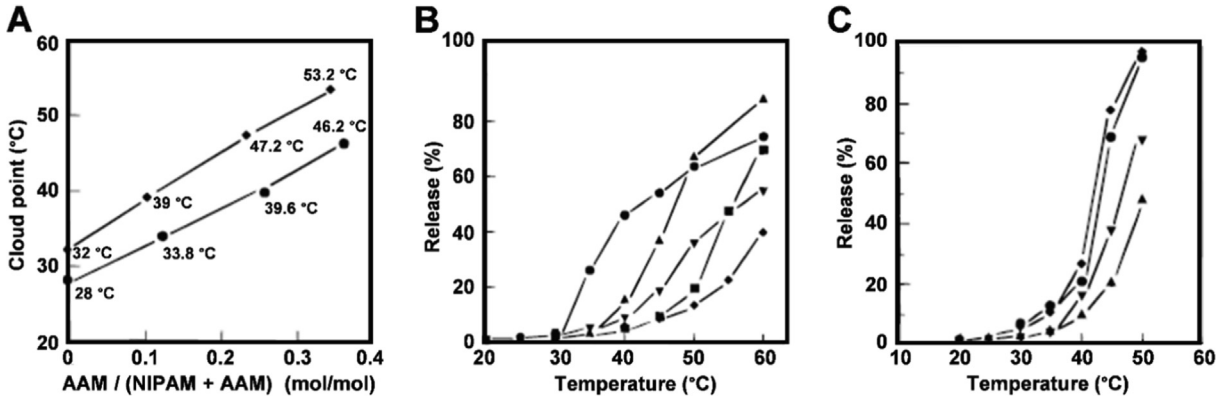

Fig. 8. Lower critical solution temperature of p(NIPAM-AAM) (◆) and p(NIPAM- NDDAM-AAM) (●) as a function of AAM content (A). Panel B represents percent release of calcein from liposomes modified with p(NIPAM-NDDAM) (98.9/1.1 mol%) (●), p(NIPAM-NDDAM-AAM) (86.7/12.3/1.0 mol%) (▲), p (NIPAM-NDDAM-AAM) (73.5/25.5/1.0 mol %) (■), and p (NIPAM-NDDAM-AAM) (63.0/35.9/1.1 mol%) (◆) and plain liposome (▼) in 10 mM Tris-HCl-buffered solution containing 140 mM NaCl and 0.1 mM EDTA at pH 7.4. TSPL was prepared by post-insertion of copolymers into liposome by incubation of preformed EPC/DOPE (4/6, w/w) liposomes with copolymer (0.5 mg/mg lipid). Panel C compares release property of liposomes modified with p(NIPAM-NDDAM-AAM) (86.7/12.3/1.0 mol%) on outer surface (▲, ▼) with post-insertion of copolymer or on both inner and outer surfaces (●, ◆) of membrane by addition of copolymer to the organic solution of lipids. EPC/DOPE liposomes (4/6, w/w) were modified with either 0.5 mg copolymer/mg lipid (▼, ◆) or 1 mg copolymer/mg lipid (▲, ●). Percent release after 1 min incubation is shown. Adopted with permission from Kono et al. 1999 [62] Copyright 1999, American Chemical Society.

were observed. The temperature of release from poly-EPC and poly-DSPC liposomes was directly correlated with the LCST of the co-polymer. In poly-DPPC liposomes it was found that below 36 °C, at which bare DPPC liposomes start to release calcein, LCST of the polymer and temperature of release from polymer-modified liposomes are directly correlated. However, above 36 °C all poly-DPPC liposomes exhibited identical temperature-release patterns and release magnitudes. In poly-DPPC/DMPC liposomes release occurred at phase transition of the lipid membrane (28 °C) and regardless of the polymer type, all liposomes exhibited identical release magnitude and pattern.

These observations imply that when during heating lipid membrane does not undergo phase transition, it is the TSP that induces triggered release, but if a lipid membrane undergoes phase transition behavior sooner than TSP, then TSP only plays a supporting role in enhancing of the release.

It was also found that at 40 °C release of calcein from the more rigid liposomes made of DSPC, while the lipid membrane is in a gel-like phase, was almost two folds higher than poly-EPC, in which the membrane is in a liquid-like phase. Worth mentioning is that at same condition poly-DPPC liposomes released almost 80 % of encapsulated calcein, which was 2-folds greater than the release from poly-DSPC liposomes. However, despite the higher magnitude of release from DPPC-based TSPL, compared to those composed of DSPC or EPC, the release behavior could not be controlled by manipulation of the LCST of the co-polymer.

4.1.2. NIPAM anchored with N,N-didodecylacrylamide (p(NIPAM-NDDAM)):

Kono et al. [69] synthesized a copolymer of NIPAM and *N,N*-didodecylacrylamide (NDDAM), in which NDDAM was randomly conjugated as the anchoring units (Fig. 1). The water insoluble copolymer exhibited a coil–globule transition around 28 °C when incorporated into EPC membranes. To investigate the impact of lipid composition on thermal behavior of TSPL, liposomes with different ratios of EPC and DOPE were modified with p(NIPAM-NDDAM). It was shown that presence of DOPE increases the affinity of the copolymer to the membrane, which is most likely due to hydrogen bond formation with the head groups of DOPE. Such increased interaction enhances the effect of the copolymer on decreasing membrane fluidity around the LCST and resulted in enhanced release from DOPE containing liposomes. TSPL composed of EPC exhibited 70 % release after 5 min incubation at 35 °C which was virtually equivalent to the observed release from TSPL composed of DOPE:EPC (64:36 mol%) after 5 min incubation at 30 °C. Clearly such release at temperatures below the physiological temperature is not suitable for drug delivery.

To overcome this limitation, increasing the LCST by increasing acrylamide content was aimed and Poly(*N*-isopropylacrylamide-*co*-N,N didodecylacrylamide-*co*-acrylamide), p(NIPAM-NDDAM-AAM) with various content of AAM were successfully synthesized by Kono et al [62] (Fig. 1). They observed that content of AAM in a copolymer and CP correlated linearly (Fig. 8A), and among the series of copolymers NIPAM-AAM with a ratio of 86.6:10.4 exhibits a CP of 39 °C, which is suitable for in vivo delivery purposes. As expected, addition of NDDAM decreases the CP of a copolymer in solution. However, it has been shown that once the lipophilic anchors incorporate into a lipid, membrane copolymers behave differently compared to what is observed in solution. In this setting coil to globe transition takes place at temperatures close to what is seen with anchor free copolymer [60,62], Therefore, calcein release from DOPE:EPC (60:40 mol%) modified with p(NIPAM- NDDAM-AAM) (86.7: 1:12.3 mol%), with a CP in solution of 33.8 °C, starts at around 40 °C close to CP in solution of p(NIPAM-AAM) (86.6:10.4 mol%, CP 39 °C) (Fig. 8B). These optimized preparations show good stability at 35 °C, while 5 min incubation at 40 or 45 °C resulted in 30 % and 80 % calcein release, respectively. In addition to this, the copolymer is soluble in organic solution and could be added to the chloroform solution of a lipid mixture at the beginning of liposome preparation. Therefore, both inner and outer surfaces of liposomes could be decorated with the copolymer. These liposomes exhibit almost two folds greater heat-triggered release at a temperature range of 40–45 °C compared to those modified by post-insertion of copolymer into preformed liposomes (Fig. 8C).

4.1.3. NIPAM co-polymerized with acrylamide (p(NIPAM-AAM)):

To enable a TSPL with useful stability at 37 °C and triggered release at a temperature slightly above that, preferably in a range of 39–42 °C, Han et al. [63] synthesized a number of p(NIPAM-AAM) with different AAM content to achieve a copolymers with an LCST slightly above physiological temperature. Although these copolymers lack specified anchoring units, the copolymers were

successfully attached to both inner and outer surface of liposomes presumably via the hydrophobic isopropyl groups. They proceeded by using p(NIPAM-AAM)-modified TSPL for controlled delivery of doxorubicin (DXR). It should be noted that due to presence of a temperature-sensitive polymer on these liposomes use of remote loading that requires heating was impossible and DXR was loaded passively by hydrating lipid film with a 1.73 M DXR solution. p (NIPAM-AAM) (83:17 mol%), which exhibits a useful LCST of around 40 °C, drastically enhanced the release of DXR from cholesterol containing TSL composed of DPPC, HSPC and Chol, (56:28:17 mol%) Fig. 9A. While 5 min incubation at 37–38 °C in presence of 50 % serum resulted in around 10 % release, DXR release of up to 65 % was achieved over 5 min of incubation at 39 °C. In addition it was found that inclusion of 3 % mPEG 2000-DSPE into the TSPL does not impact on onset and sharpness of release, while it reduces protein adsorption on the liposome surface Fig. 9B and improves the colloidal stability of the TSPL at 37 °C in the presence of 50 % serum Fig. 9C.

Addition of thermoresponsive copolymers is expected to decrease protein adsorption on TSPL and consequently increases the colloidal stability of TSPL in serum via the steric hindrance effect of fully hydrated copolymers at temperatures below the transition temperature of the copolymer [61,86]. However, Han et al. [64] show that while modification of TSPL by p(NIPAM-AAM) decreases protein adsorption compared to bare liposomes, the copolymer is less effective than PEG against protein adsorption. Nonetheless, the lowest protein association was found in TSL containing both PEG and NIPAM-AAM that makes it a great candidate for tumor targeting and the therapeutic activity was investigated further in B16F10 melanoma bearing mice [64]. PEG-TSPL showed the highest magnitude of drug release amongst the different preparations, reaching 65 % release of encapsulated DXR during 5 min incubation at 38 °C in vitro. Accordingly, higher levels of inhibition of tumor growth compared to free DXR or TSL modified with only PEG2000 or only NIPAM-AAM was observed once mild hyperthermia of 42 °C was applied for 10 min, 1 h after injection of liposomal DXR (6 mg/kg). Intravenous treatment with PEG-TSPL followed by hyperthermia was significantly more effective than PEG-TSPL without hyperthermia [64].

4.1.4. NIPAM co-polymerized with propylacrylic acid (p(NIPAM-PAA)):

Copolymers may be used to generate liposomes responsive to pH and temperature. Ta et al. [72] copolymerized NIPAM with pH responsive propylacrylic acid (PAA) via reversible addition-fragmentation chain transfer (RAFT) polymerization. Copolymers containing 91 % NIPAM and 9 % PAA showed pH dependent thermal response with LCST values of 42 °C and 28 °C at pH values of 6.5 and 5 respectively. RAFT polymerization resulted in a large terminal 2-dodecyl-sulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP) (Fig. 1) group to anchor copolymer into outer surface of preformed DXR loaded PEGylated TSL composed of DPPC, hydrogenated Soy phosphatidylcholine (HSPC), cholesterol (chol), and mPEG 2000-DSPE (54:27:16:3 mol%) via post insertion. Temperature-dependent DXR release curve reveals that in absence of serum at pH 7.5, the PEGylated PSTLs start to release DXR at 36 °C which is 5 °C lower compared to PEGylated TSL. The temperature of 50 % release during 5 min from copolymer-modified and non-modified liposomes were calculated to be 39.6 °C and 43.1 °C, respectively. The DXR leakage from PEGylated PSTLs and PEGylated TSL during 5 min incubation at 37 °C was 13.9 % and 4.3 %, respectively. Clearly, addition of copolymers improves heat responsiveness of the PEGylated TSL, and requires a 120-fold lower thermal dose (equivalent min at 43 °C) to induce a 50 % drug release in 20 mM HEPES at pH 7.5. However, incorporation of thermosensitive polymers into liposomes also results in premature release at 37 °C.

Importantly, in the presence of 20 % bovine serum PEGylated PSTL and PEGylated TSL released less than 7 % of encapsulated DXR during 90 min incubation at 37 °C, indicating stabilization of the liposomes by serum components. Lysolipid containing liposome (LTSL, DPPC/MPPC/DSPE-PEG-2000 (90:10:4 mol ratio)) on the other hand released over 80 % of the payload within 30 min 37 °C. Serum also reduced the magnitude of release at 42 °C.

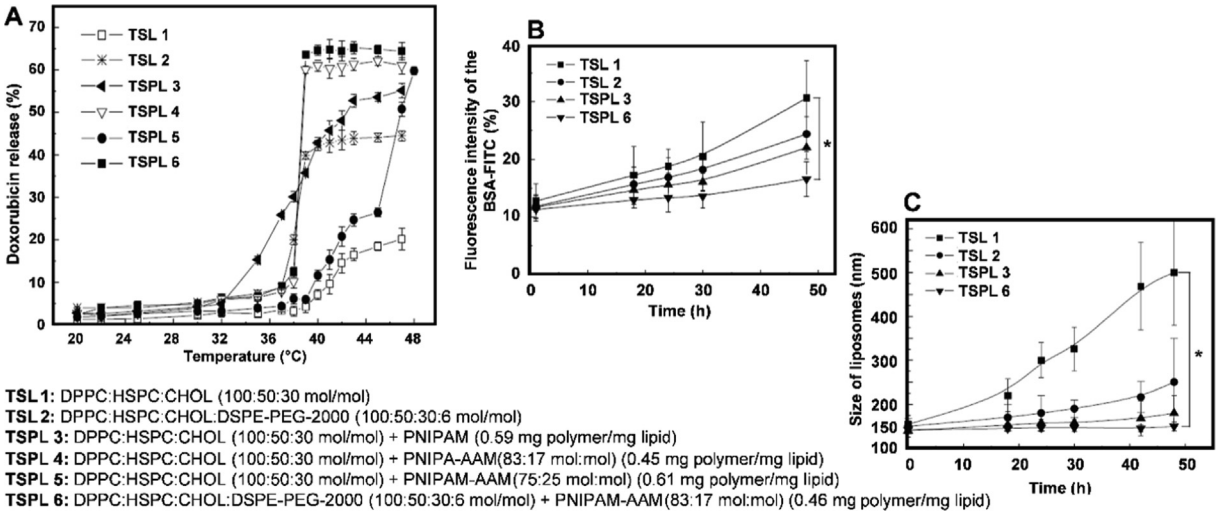

Fig. 9. Temperature-dependent drug release and effect of serum on TSPL modified with p(NIPAM-AAM). Panel A plots the temperature-dependent release of doxorubicin, during 5 min incubation at different temperatures in presence of 50 % serum, from different temperature-sensitive liposomal preparations modified with different polymers. Panel B represents fluorescence intensity of the BSA-FITC adsorbed on different liposomal preparations suspended in Tris–HCl buffered solution containing BSA-FITC after different incubation times at 37 °C. Panel C represents particle size of liposomal suspension in presence of 50 % serum after different time of incubation at 37 °C. TSL 1 and TSL 2 are liposomes without TSP, and TSPL-3, TSPL 4 and TSPL 5 were modified with p(NIPAM-AAM) with NIPAM:AAM mole ratios of 100:0, 83:17, 75:25 with LCST in solution of 33, 40, and 47 °C, respectively. TSL 2 and TSL 6 are PEGylated counterparts of TSL 1 and TSPL 4. Data is shown as mean ± SD (n = 3), *P < 0.007, Student's *t*-test). Adopted with permission from Han et al. 2006 [63].

In addition to heat, PEGylated TSL may also respond well to changes in pH. At 37 °C and in presence of 20 % serum, 5 % drug release over 1 h incubation at a pH of 7.5 improved to 40 % release at a pH 5.

Overall, addition of NIPAM-PAA to TSL reduces the thermal dose necessary to achieve an efficient drug release from this formulation of TSL, while in addition to external heat-triggered release the PEGylated PSTL is also capable of releasing the payload in a highly acidic environment (4.9 < pH < 5.5) such as the endosomal system [72] or mild acidic environment (6.5 < pH < 7.5) such as in the tumor interstitial space [87,88].

These promising results prompted Ta et al. [87] to evaluate the therapeutic efficacy of this PEGylated TSPL loaded with DXR as the therapeutic agent and MnSO4 as contrasting agent for magnetic resonance (MR) guided tumor therapy by application of focused ultrasound (FUS) mediated heating at 43 °C (MRgFUS). Although no experimental result on MR imaging after injection was reported and MR thermometry was used to monitor heating, it is known that co-encapsulation of a drug with a MR contrasting agent enables real-time monitoring of drug release through visualization of local changes in contrasting agent signal [89,90].

On the other hand, FUS provides a high degree of temporal control by creating reproducible and predictable heating via adjusting acoustic intensity and duty cycles making MRgFUS an attractive strategy for efficient drug delivery.

Ta et al. [87] observed that heating with FUS resulted in higher release rates compared to heating in a water bath, which could be a result of mechanical stress of inertial cavitation effect, in which a shock wave is created from sudden collapse of a void or bubble in liquid. However, since content release was not observed in non-thermo responsive liposomes exposed to FUS this is not a likely reason. However, it is expected that the higher release is triggered by heat, and especially from overheated "hot pockets" resulted from non-uniform heating by FUS compared to the rather uniform, but slowly decreasing, heating with a water bath.

Cells incubated with PEGylated PSTL with 5 min applied hyperthermia of 43 °C revealed comparable toxicity as what is observed with free DXR. In vivo studies revealed that application of a 5 min heat (43 °C) applied by FUS, 6 h post injection increases the antitumor effect of DXR (5 mg/kg) when encapsulated in PEGylated PSTLs rather than in PEGylated TSL, or injected as free DXR. Besides, PEGylated PSTLs plus hyperthermia showed higher antitumor activity than without hyperthermia. Worth mentioning is that accumulation of DXR was statistically identical in groups of mice that received PEGylated PSTLs or PEGylated TSL. These results imply that with this setting extravascular drug release had been accomplished.

4.1.5. NIPAM co-polymerized with N-(2-hydroxypropyl) methacrylamide and anchored with 2-dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid (p(NIPAM-r-HPMA)-DMP):

It has been shown that by copolymerization of NIPAM with hydrophilic N-(2-hydroxypropyl) methacrylamide (HPMA) via RAFT copolymerization the LCST could be increased and tuned precisely by adjusting the HPMA content [91]. Mo et al. [92] copolymerized a series of p(NIPAM-r-HPMA)-DMP (Fig. 1) among which NIPAM: HPMA (94:6 mol %), which exhibit a sharp transition at an LCST of 42 °C and employed these polymers for modification of DXR-loaded liposomes composed of DPPC, HSPC, Chol and mPEG2000 (55:25:15:3 mol) by post-insertion of the copolymer via the terminal DMP anchor during 1 h incubation with liposomes at 25 °C. Temperature dependent release curve of the optimized formulation (polymer/lipid = 1 %, wt/wt) over 2 min incubation in PBS reveals DXR release starts at temperatures around 37 °C, which reaches to 50 % release at 40 °C and the maximum release was achieved at 42 °C. Nevertheless, release over 1 h incubation at 37 °C in PBS pH 7.4 was less than 20 %, whereas 70 % release was achieved through 1 min incubation at 42 °C. By contrast, non-modified TSL showed more stability at 37 °C, and only 40 % release over 30 min incubation at 42 °C was obtained. Such burst release at 42 °C resulted in a greater cellular uptake and toxicity of PEGylated-TSPL compared to PEGylated-TSL. It was also shown that burst release of DXR results in higher and deeper penetration of DXR into both in vitro tumor spheroids and in vivo tumor model. The sharp release property of this preparation makes it a great candidate for intravascular release strategies where theoretically nanoparticles have only few seconds of exposure to HT during passage through tumor [93]. However, this has not been tested for this formulation and instead extravascular release was aimed by applying HT for 5 min 24 h post injection. For in vivo studies PEGylated-TSPL were also decorated with p(HPMA-r-APMA)-DMP conjugated with Cyanine 7.5 (Cy7.5), that can provide heat upon 808 nm laser irradiation and enables simultaneously real time infrared thermal imaging. In therapeutic efficacy studies in 4 T1-bearing mice receiving multiple doses (days 1, 3, 5, 7, and 9) of 5 mg/kg DXR, PEGylated-TSPL inflicted the highest antitumor effect.

4.1.6. NIPAM copolymerized with N,N′-dimethylaminopropylacrylamide and anchored with DOPE (p (NIPAM-DMAPAM)-DOPE)

In order to sensitize liposomes to heat, Wang et al. [71] copolymerized NIPAM with N,N′-dimethylaminopropylacrylamide (DMAPAM) and conjugated the copolymer to DOPE as the terminal anchor unit (p(NIPAM-DMAPAM)-DOPE) (Fig. 1). Copolymerization with the hydrophilic DMAPAM successfully increased LCST of NIPAM, where at molar ratio of 95 % NIPAM, and 5 % DMAPAM (MW: 5500) the copolymer of exhibits a sharp transition at LCST of 40 °C.

Unlike a mixture of DOTAP:DOPE lipid (30:70 mol%) that does not form liposomes, inclusion of copolymer conjugate helped liposome formation and DOTAP:DOPE: p(NIPAM-DMAPAM)-DOPE (30:65:5 mol%) formed stable liposomes with less than 10 % release at temperatures below 30 °C. The TSPL showed aggregation and fairly slow release at temperatures around the LCST of the copolymer. While after 60 min incubation about 20 % release was seen at 37 °C, it takes 20 min to achieve 75 % release at 42 °C. Clearly, a liposomal polymer system like this requires either long times of hyperthermia or high temperatures to induce release, which either causes serious necrosis in the affected area or is clinically unpractical. However, at elevated temperatures drastic reduction in fixed aqueous layer thickness (FALT) was observed that makes this preparation more suitable for heat-mediated liposome-cell interaction and intracellular heat-mediated release. Similar capabilities were found when liposomes composed of EPC:-cholesterol (50:50 mol%) were modified with *N*-isopropylacrylamide-*co*-N, N-dimethylacrylamide, NIPAM-DMAM (70:30 mol%, LCST 37.8) conjugated to DOPE [94].

4.2. N-acryloylpyrrolidine-based copolymers

4.2.1. Acryloylpyrrolidine copolymerized with NIPAM (p(APr-NIPAM)):

Poly(acryloylpyrrolidine) is a thermo responsive polymer with an LCST at ca. 50 °C that cannot be used for hyperthermic drug delivery. However, by copolymerization with NIPAM a proper LCST could be achieved. Kono and coworkers [68] copolymerized a series of p(APr-*co*-NIPAM) with different compositions and found a linear correlation between the LCST of the copolymer and NIPAM content (Fig. 10A), which provides a precise control in synthesis of a copolymer for a desired LCST. Copolymer were also modified in two different types regarding the anchors: a) Middle anchor-type polymer with addition of NDDAM (around 2 mol%) as an addi-

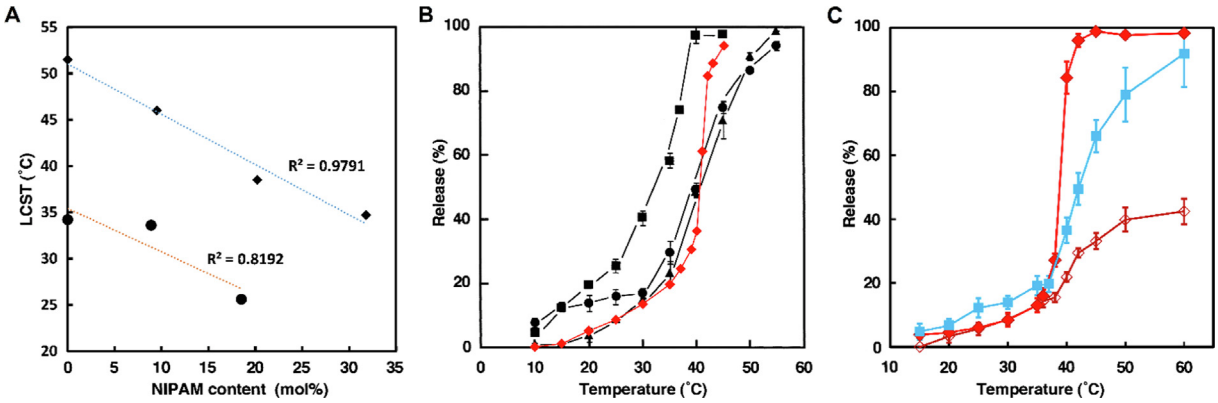

Fig. 10. Impact of copolymer composition on LCST and temperature-triggered release from liposomes. Panel A illustrates lower critical solution temperature of p(APr-NIPAM) as a function of NIPAM content in absence of NDDAM anchor units (◆) or in presence of about 2 % of NDDAM anchor units in p(APr-NIPAM-NDDAM) (●). Panel B shows percentage release of calcein from different liposomal preparations after 15 min incubation in 10 mM Tris-HCl, 140 mM NaCl and 1 mM EDTA solution (pH 7.4) at different temperatures. DOPE liposomes were modified with middle anchor type p(APr-NIPAM-NDDAM) copolymer with various APr/NIPAM/NDDAM mol ratios of 97.9/0/2.1 (▲), 88.7/8.9/2.4 (●), and 79.4/18.5/2.1 (■) or with terminal anchor type poly(APr-*co*-NIPAM)-2C12 (81.6:18.4 mol/mol) copolymer (◆). Panel C represents percentage release of calcein from DOTAP liposomes modified with poly(APr-*co*-NIPAM)-2C12 (◆, ◇) or poly(APr-*co*-NIPAM)-2C12 and 4 mol% PEG550-2C12 (■) in absence (◆, ■) or presence (◇) of 10 % v/v serum. Adopted with permission from Kono et al. 1999 [68] and Kono et al. 2002 [95].

tional comonomer p(APr-NIPAM-NDDAM) (Fig. 1), and b) Terminal anchor-type polymer by addition of didodecyl group p(APr-NIPAM)-2C_{12} (Fig. 1). As expected addition of hydrophobic NDDAM results in a significant reduction of the LCST of the copolymer in solution, but it also disturbs the linear correlation between copolymer APr/NIPAN ratio and LCST (Fig. 10A). While copolymerization of APr with 20 % NIPAM reduced the LCST by 15 °C and resulted in a copolymer with a LCST of ca. 39 °C (APr-NIPAM, 79.8:20.2 mol%) grafting copolymer with 2.1 % NDDAM dramatically reduced the LCST of APr-NIPAM-NDDAM (79.4:18.5:2.1 mol%) to 25 °C whereas introduction of terminal anchor slightly reduced the LCST of p(APr-NIPAM)-2C_{12} (APr:NIPAM 81.6:18.4 mol/mol) to 36.4 °C. This indicates that terminal anchoring provides greater control and prediction over triggering release by heat.

However, once these copolymers were installed on both inner and outer surfaces of a DOPE membrane release started at lower temperatures especially for those modified with middle anchor type copolymers. As could be seen in (Fig. 10B) modification of liposomes with copolymers of APr-NIPAM-NDDAM (88.7/8.9/2.4 mol%, LCST 33.6 °C) and APr-NIPAM-NDDAM (79.4:18.5:2.1 mol% LCST 25.6 °C) resulted in significant premature drug release even at temperatures below the copolymer LCST.

Modification of DOPE liposomes with p(APr-NIPAM)-2C_{12} resulted in a better release pattern with massive release at a narrow temperature range with good conformity with the transition behavior of the copolymer in solution. Calcein loaded DOPE liposomes modified with p(APr-NIPAM)-2C_{12} (APr:NIPAM, 81.6:18.4 mol%) exhibited a burst release of encapsulated calcein (above 70 %) within 1 min at 42 °C with a limited release of 20 % in buffer during 15 min incubation at 37 °C. Meanwhile liposomes modified with APr-NIPAM-NDDAM (79.4:18.5:2.1 mol% LCST 25.6 °C) releases over 70 % of its payload during 15 min incubation at 37 °C.

It has later been shown that methotrexate (MTX) loaded liposomes, composed of EPC and modified with this p(APr-NIPAM)-2C_{12}, exhibit higher toxicity against CV1 cells compared to unmodified liposomes upon application of 42 °C HT, while toxicity at 37 °C was even lower than unmodified liposomes [73]. It was found that at 37 °C, at which surface-anchored copolymers are mostly hydrated, polymer-modified liposomes associate less than bare liposomes with cells. At 42 °C, at which copolymers have undergone coil-globe transition, the liposome surface became hydrophobic and interacts with cells at a higher level compared to bare liposomes. However, it should be noted that association of liposomes modified with 7.2 mol% copolymer within 3 h incubation at 37 °C was approximately 70 % less than the non-modified counterpart. These results imply a minor inhibitory effect of the copolymer on cell interaction bellow the LCST where it is expected that hydrated copolymers provide a steric hindrance effect as is observed with PEGylation.

As stated earlier DOPE liposomes modified with both kind of middle and end terminal anchored copolymers showed some extent of leakage at temperatures below the LCST, which was more significant for the middle anchor type. It is likely that partial dehydration of polymer chains starts at temperatures below the LCST and such dehydrated units tend to interact with liposome membrane or adjacent cell surface, causing premature release or cellular interactions at normothermic condition.

To overcome these problems Kono and coworkers [95] investigated the effect of PEG inclusion on behavior of DOPE liposomes modified with p(APr-NIPAM)-2C_{12} (APr:NIPAM, 80:20 mol%). Addition of 10 mol% PEG550 conjugated to a didodecyl group (PEG550-2C_{12}) effectively improved the release profile (in buffer) by decreasing release at 36 °C from 25 % in non-PEGylated liposomes to less than 10 % in PEGylated counterpart during 15 min, while PEGylation hardly affected the release rate above the LCST. Temperature dependent release studies indicate that inclusion of PEG550-2C_{12} slightly increases the onset temperature of release, and sharpens the heat-triggered dye release in a more narrow temperature range compared to non-PEGylated counterpart.

It is known that the ethylene oxide units of PEG provide hydrogen bonding sites for the amide groups of NIPAN [96] that can compensate the loss of hydrogen binding in partially dehydrated segments of the copolymer and inhibit dehydrated segments to interact with the lipid membrane. However, above the LCST, when the whole copolymer becomes hydrophobic, PEG cannot inhibit copolymers to interact with the lipid membrane. Once PEG (2000)-2C12 was used calcein release was strongly reduced with a complete loss of temperature sensitivity at 5 mol% of PEG (2000)-2C12. It could be concluded that PEG plays a buffering role on hydrogen binding. At low MW the buffering capacity is only enough for compensation of hydrogen binding for partially dehydrated copolymers, but at high MW the buffering capacity is enough to completely inhibit the copolymer transition.

For the first time the effect of serum on polymer-assisted thermoresponsive liposomes was evaluated. It was found that presence of 10 % serum significantly reduced temperature-sensitivity of DOPE liposomes modified with p(APr-NIPAM)-2C_{12}. After 5 min incubation at 42 °C, copolymer-modified liposomes released 95 % or 30 % of the encapsulated calcein in absence or presence of serum, respectively. An optimum amount of 4 mol% PEG 550 was found to partially retrieve the sensitivity of the copolymer-modified liposome. However, serum widened the temperature dependent release curve of PEGylated copolymer-modified liposomes with 20 % release at 37 °C, 50 % release at 42 °C and 90 % release at 60 °C.

4.3. (2-ethoxy)ethoxyethyl vinyl ether (EOEOVE) -based copolymers

Like NIPAM-based copolymers, poly(*N*-vinylethers) exhibit thermoresponsive behavior derived from the dehydration of polymer chains at the LCST and similar to NIPAM-based copolymers the LCST could be tuned by copolymerization with hydrophilic (increase) or hydrophobic (decrease) comonomers. Kono and coworkers [79] synthesized copolymers of EOEOVE with different Mw as the temperature-sensitive moiety, and 4–5 mol% of octadecyl vinyl ether (ODVE) that acts as an anchor moiety (EOEOVE-*b*-ODVE) (Fig. 1). Living cationic polymerization enabled them to synthesize copolymers with a precise controlled MW and architecture, which is not possible by commonly used radical polymerization.

It was found that, in water, increasing the molecular weight has no impact on efficiency of polymers in disruption of water association as all tested polymers showed similar transition enthalpies of 14–15 J/g for the conformational transition. However, increasing MW results in decreased LCST and polymers with number average molecular weights (M_n) of 5300, 8300, and 15,800 exhibited endotherms centered at 45, 41, and 40 °C, respectively. In contrast, when microcalorimetric behavior of copolymers of (EOEOVE-*b*-ODVE) was tested in the presence of EPC and water, all copolymers underwent transition at similar temperatures of ca. 36–39 °C. But, the enthalpy of transition and sharpness of endotherms increased by increasing MW as copolymers with M_n of 6900, 9300, and 16,700 had transition enthalpies of 8.5, 13 and 16 J/g, respectively, indicating higher transition efficiency with longer copolymers.

When liposomes composed of DOPE:EPC (7:3) were modified by different copolymers calcein release below 30 °C or at 45 °C from all preparations were virtually identical and all exhibited a burst release at 40 °C. However, the shortest copolymer caused a slightly higher release at 35 °C, while liposomes modified with the longest copolymer exhibited a much lower release at this temperature. Most importantly, the magnitude of release at HT condition was found strongly dependent on copolymer MW, where copolymer with M_n of 16,700 D caused almost a complete release within 1–3 min of incubation at 40 °C, but liposomes modified with the two other copolymers with Mn of 6900 and 9300 revealed a slower release rate reaching a maximum of 50 % after 10 min at this temperature.

Tracking the emission of pyrenecarboxaldehyde (PyCHO) to analyze changes in hydrophobicity of polymeric domains by increasing the temperature revealed a direct correlation between polymer MW and hydrophobicity at temperatures above the LCST. In addition to that while the sharp jump of hydrophobicity observed for EOEOVE, Mn of 16100, started at 42 °C, polymers with Mn of 5500 and 9000 showed a slower hydrophobic transition at higher temperatures between 44 and 46 °C, indicating faster and stronger degree in formation of hydrophobic domains in polymers with higher MW. In addition to the more efficient dehydration, or hydrophobization, of long copolymers at LCST, it is likely that the higher degree of conformation freedom in longer copolymer chain provides a stronger interaction with the liposome membrane. Altogether, higher MW resulted in more efficient heat-triggered dye release from both EPC and DOPE:EPC liposomes compared to copolymers with lower MW.

As stated earlier NIPAM copolymers cannot trigger an efficient release of hydrophilic dyes from EPC liposomes upon application of HT [60,67,74], whereas with DPPC or DOPE liposomes a complete release could be achieved [60,66,67]. Kono et al. found that EOEOVE-*b*-ODVE copolymers are capable of triggering higher degree of calcein release from EPC liposomes compared to what was observed with NIPAM. It was found that in similar MW ranges EOEOVE polymers provide more hydrophobic domain than NIPAM polymers during coil to globe transition, which enables EOEOVE to destabilize EPC liposomes above the LCST more efficiently than NIPAM [79].

This promising results prompted Kono and colleagues [97] to investigate the potential applicability of EOEOVE-*b*-ODVE with a MW of around 18 K_D to sensitize liposomes to heat using non-thermoresponsive liposome formulations of EPC:Chol (50:45 mol/-mol) with or without 4 mol% mPEG5000-DSPE. Copolymer was added at different concentrations of 0.5–2 mol% to the lipid mixture and DXR was loaded remotely.

In non-PEGylated preparations increasing the copolymer content reduced the temperature that liposomes start to release. While in liposomes containing 0.5 mol% copolymer exponential drug release starts at ca. 40 °C, liposomes with copolymer contents of 1 and 2 mol% released started at lower temperature around 34–36 °C, and exhibited around 25 % DXR release during 10 min of incubation at 37 °C (Fig. 11A). With all copolymer concentrations almost complete DXR release was achieved within 1 min at 45 °C. Addition of mPEG5000-DSPE to liposome formulations greatly improved the temperature dependent release behavior of liposomes modified with different concentrations of copolymer, by increasing the sharpness and the magnitude of release while at the same time reduced DXR leakage at 37 °C to negligible values. To some extent PEG unifies the effect of copolymer concentration on thermal behavior of liposomes (Fig. 11B).

It worth mentioning that addition of 5 mol% mPEG2000 into TSPL decorated with p(APr-NIPAM)-2C12 resulted in complete suppression of copolymer thermal response [95] while EOEOVE-*b*-ODVE does not lose its activity in presence of PEG, indicating superior potency of EOEOVE polymer in destabilizing liposomes in response to heat.

Due to similarity of EOEOVE with PEG it is expected that EOEOVE may also improve the pharmacokinetics of copolymer-modified liposomes at temperatures below LCST. However, it was observed that while incorporation of this copolymer (2 or 4 mol %) to EPC/Chol liposomes did not make a significant improvement in pharmacokinetics, PEGylation improved the circulation life time of copolymer-modified liposomes. Interestingly, addition of 2 mol% copolymer to PEGylated liposomes resulted in an slightly faster clearance rate compared to liposomes decorated only with 4 mol % PEG polymer.

Kono and coworker also considered the proper time to apply hyperthermia to optimize treatment with thermoresponsive polymer-modified liposomes. In this regard a dose of 6 mg/kg DXR encapsulated in different liposomal formulations were injected into mice bearing C26 colon carcinoma and one course of 10 min HT of 45 °C was applied at 3, 6 or 12 h after injection. The authors reported a greater antitumor efficacy with PEGylated copolymer-modified liposomes only when HT was applied. In addition, HT at 6 or 12 h was found a bit more effective than HT at 3 h post injection.

Due to the nature of EPR effect it is clear that at later time points more liposomes have accumulated in a tumor, therefore obtaining a higher efficacy by applying HT at moments with higher intratumoral level of DXR is justified. However, the biodistribution study

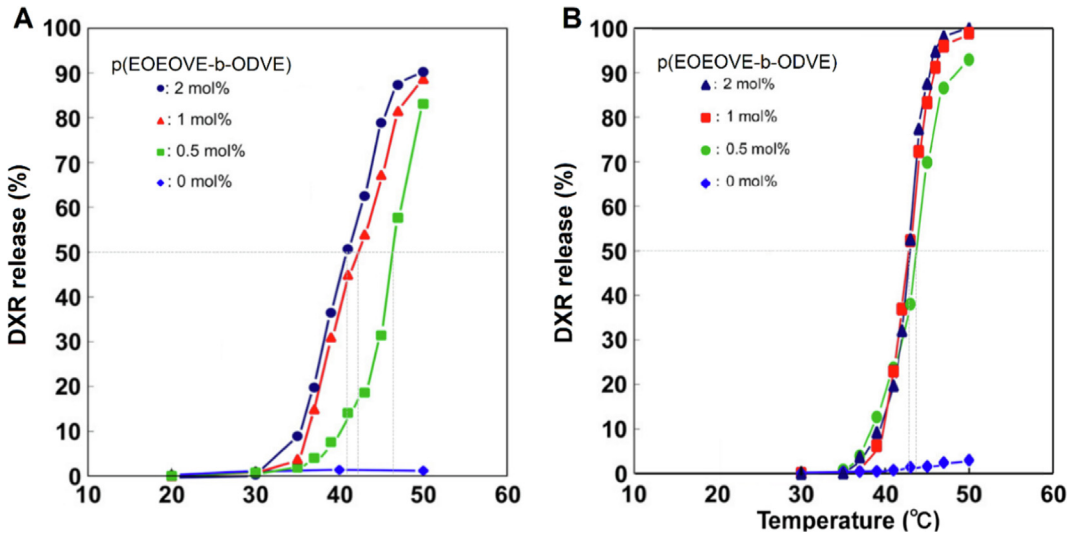

Fig. 11. Temperature-dependent release of doxorubicin (DOX) from TSPL composed of (A): EPC/Chol (50/45, mol ratio) and (B): EPC/Chol/PEG5000-DSPE (50/45/4, mol ratio) liposomes modified with different contents of p(EOEOVE-ODVE). Liposomes were diluted in PBS pH:7.4 and percentage of release over 3 min incubation at different temperatures was measured. Adopted with permission from Kono et al. 2010 [97].

shows that at 3 h after injection blood level of PEGylated copolymer-modified liposomes is at least 3 times higher than at 12 h. Therefore, it could be concluded that despite the fast release of DXR from this thermoresponsive liposome under in vitro condition, intravascular drug release, in vivo, is not beneficial with this liposome, probably because of suppression of release by blood proteins. It can be concluded that extravascular drug release is a better choice for this preparation.

In another study this TSPL (EPC:Chol: p(EOEOVE-ODVE): mPEG2000-DSPE: maleimide-PEG-PE: (50: 45:0.1:2:2 mol%)) was loaded with DXR by ammonium sulfate gradient and was decorated with trastuzumab, via the maleimide functional group at distal end of PEG-DSPE molecules, to target human epidermal growth factor, and also indocyanine green (ICG) for near-infrared fluorescence imaging [98]. Incorporation of ICG enabled monitoring of liposome accumulation in tumor by of near-infrared fluorescence imaging which displayed 2.3 times stronger fluorescence and longer retention time for targeted TSPL compared to non-targeted TSPL. In addition, it was observed that fluorescence intensity from tumor reached to constant level 7 h after liposome injection. Importantly, the impact of ICG incorporation into liposome on release behavior was also evaluated and it was found that increasing ICG content above 7.9 g ICG/mol Lipid results in greater premature release at 36–37 °C while reduces the thermal response at 43 and 45 °C. In mouse model of SK-OV3 tumor, targeted MTPL was found modestly more effective in suppression of tumor compared to non-targeted MTPL either in absence or presence of 10 min HT of 44 °C applied by radiofrequency oscillator 7 h after intravenous administration.

Following this polymer-modified liposome was equipped with an MR contrasting agent, polyamidoamine G3 dendron-based lipids having gadobenate dimeglumine (Gd^{3+}) chelate residues (G3-DL-DOTA-Gd), for real time MRI. After injection of liposomes of EPC:Chol:mPEG5000-DSPE: copoly(EOEOVE-ODVE):G3-DL-DOTA-Gd (42/42/4/2/10 mol%) into mice bearing C26 tumor, monitoring of tumor accumulation by MRI revealed that accumulation of liposomes in tumor increased over time reaching a maximum level at 8 h after injection. Therefore, when a 10 min HT of 44 °C was applied at this moment (i.e. 8 h after injection) the most effective treatment was obtained [99].

Such multi functionality of these liposomes provides an efficient patient-optimized (personalized) chemotherapeutic approach. This concept was further investigated by preparation of a multi-modal thermosensitive polymer-modified liposomes (MTPL) composed of EPC:DOPE:Chol:mPEG2000-DSPE:copoly(EO EOVE-ODVE): Rhodamine-PE (23.4: 54.6:15:4:2:1 mol%) and encapsulated with remotely loaded DXR via a MnSO4 (300 mM) gradient [100]. In vitro release characteristics of liposomes have not been reported, however, behavior of liposomes in tumors and healthy organs was traced using fluorescence and MR imaging techniques. Pharmacokinetics results revealed that maximum intratumoral concentration of liposomes was reached at 4 h after injection and remained constant up to 12 h after injection. Based on this a 15 min HT of 42.5 °C was applied on the tumor 12 h after injection by means of high-power RF pulses from the MR scanner. Injection of MTPL plus HT showed a greater trend in reducing tumor growth compared to MTPLs without HT. Later, Kokuryo et al. [101] combined chemotherapy using this MTPL with heat and high–linear energy transfer (LET) radiotherapy using carbon ion irradiation. They observed that irradiation has no impact on tumor accumulation of TSPL. However, combination of heat triggered drug release from MPTL and high-LET beam radiotherapy resulted in greater tumor suppression compared to single or combination of two treatments, when tumor growth in mice model of C26 tumor was monitored for 8 days after treatments.

Recently Yuba et al. [102] modified their targeted TSPL by replacing trastuzumab and ICG with cyclic Arg-Gly-Asp-Phe-Cys peptide (cRGDfC), and 10 mol% G3-DL-DOTA-Gd, respectively. Cyclic RGD peptides have frequently been used for targeting $\alpha_v\beta_3$ integrin receptor that is overexpressed on tumor neovasculatures as well as some tumor cells. Anti-vasculature targeting is known as an approach that can bypass EPR and reduces the dependency of tumor targeting on EPR [103–105]. In C26 tumor-bearing BALB/c nude mice RGD-TSPL exhibited superior tumor localization than non-targeted TSPL, as was shown by MRI (Fig. 12A–C) reaching plateau 3 h after the injection.

Therapeutic efficacy studies revealed that while in absence of heat both targeted and non-targeted TSPL caused limited antitumor effect, addition of HT to the treatment significantly reduced the tumor growth rate where RGD-TSPL was found more effective

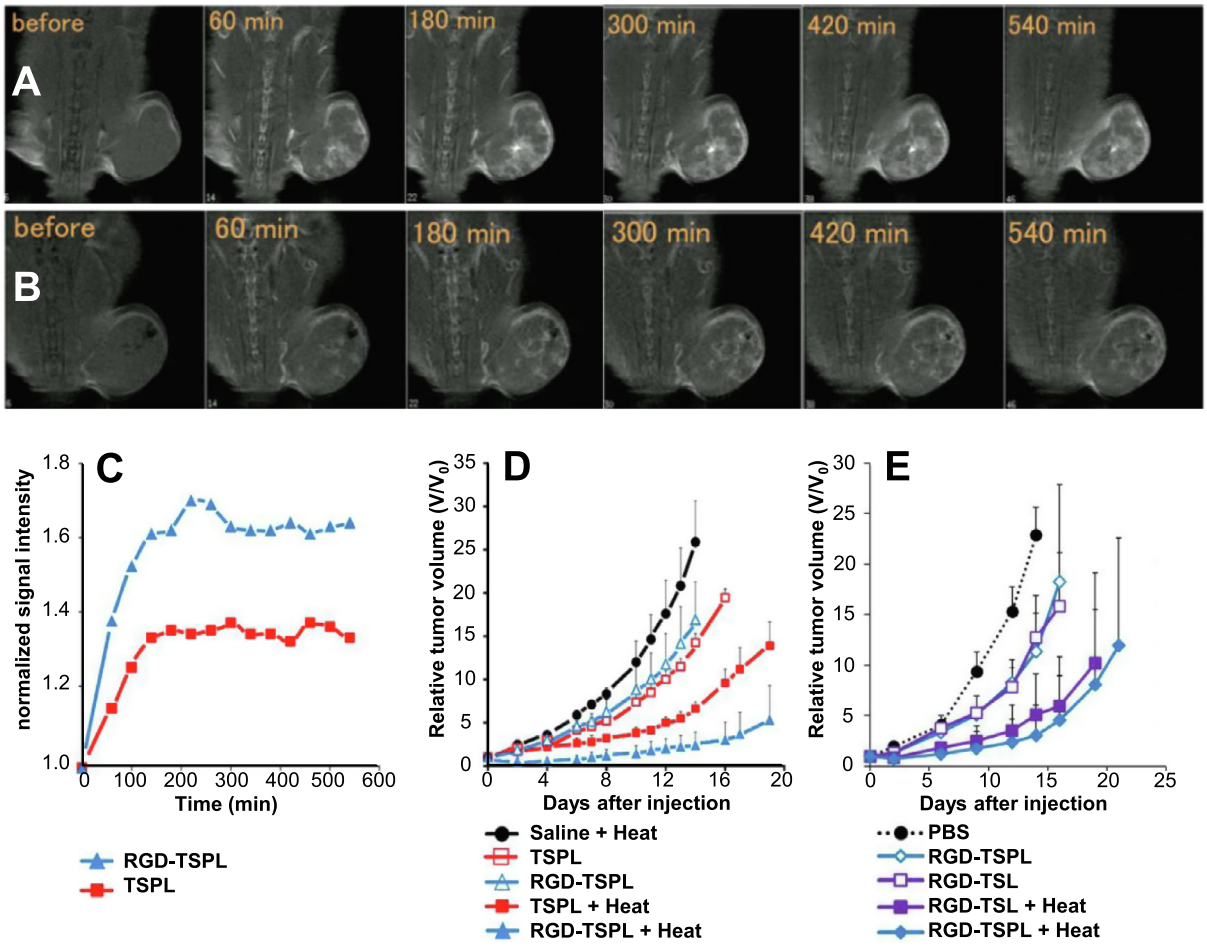

Fig. 12. Tumor accumulation and therapeutic efficacy of RGD-TSPL. Panels A and B represent T1-weighted images captured at different time points before and after injection of TSPL containing Gd-DOTA – DL, either modified with RGD peptide (RGD-TSPL) or non-modified (TSPL), respectively. Panel C illustrates normalized signal intensity as a function of time at the site of tumor site after administration of different TSPL. Panels D and E represent the impact of single iv injection of DXR (6 mg/kg) encapsulated in targeted and non-targeted TSPL or TSL on growth rate of C26 tumor model with or without application of heat (10 min HT of 43 °C) 8 h after administration. Reprinted with permission fromYuba et al. 2021 [102] Copyright 2021, American Chemical Society.

(Fig. 12D). However, when RGD-TSPL was compared against RGD-TSL (without polymer) composed of DPPC:HSPC:Chol:m PEG2000-DSPE, 54:27:15:4) similar anti-tumor effects were observed in both NT and HT conditions.

4.4. Poly(N-(2-hydroxypropyl) methacrylamide) (HPMA)

HPMA polymers are water soluble, biodegradable, and biocompatible polymers that because of the capability to circulate long in blood have been researched widely as macromolecular drug carriers especially for chemotherapeutics [106–108]. It has been shown that by copolymerization of HPMA-monolactate (CP 65 °C) and HPMA-dilactate (CP 13 °C) a thermoresponsive copolymer could be obtained in which the CP is tunable by adjusting monolactate/dilactate ratio of the copolymer [109].

Hennink and coworkers [75,110] synthesized a series of HPMA copolymers containing different ratios of HPMA-monolactate and HPMA-dilactate by radical polymerization using thiocholesterol as a chain transfer agent (CTA), which provides a cholesterol unit at the terminus of the copolymer to act as anchor unit (p(HPMA mono/dilactate)-cholesterol) (Fig. 1). The CP of the copolymer decreases by increasing HPMA-dilactate content, due to the higher hydrophobicity of the dilactate side group over the monolactate side group, with great linear correlation (r^2 = 0.99) [75].

Once copolymers with similar M_n around 11 k_D, containing different ratios of mono/dilactate, were incorporated into liposomes composed of DOPE/EPC/polymer (70:25:5 mol%) onset temperature of release was significantly higher than the CP of the copolymer. For instance, liposomes modified with a copolymer with a CP of 11.5 °C required 10 min incubation at 42 °C for complete release, while with a copolymer with a CP of 25 °C a temperature of 52 °C was needed for a complete release [110]. It is likely that hydrophobicity of the dehydrated copolymer chains at LCST is insufficient to interact and permeabilize the liposomal membrane. However, what has been neglected was the fact that the shift it thermal behavior of the copolymer, once fixed on liposomes surfaces compared to copolymer solution, is mainly attributed to incorporation of anchor units into the lipid membrane, while in a solution such hydrophobic parts play an important role in hydrophilicity loss of the copolymer by heat which was consistent with previous observations [79].

It was also found that the CP of a copolymer is not dependent on MW of copolymer. However, increasing the MW greatly reduces the onset temperature of DXR release when incorporated into liposomes. For instance, copolymers with identical mono/dilactate ratios of about (50:50 mol%) but Mn of 6.5, 10.0 and 14.5 k_D exhibit CPs of 20, 19 and 18 °C respectively, but induction of release from these liposomes starts at ca. 47, 42 and 37 °C, respectively. This indicates that longer polymer chains act more effectively in desta-

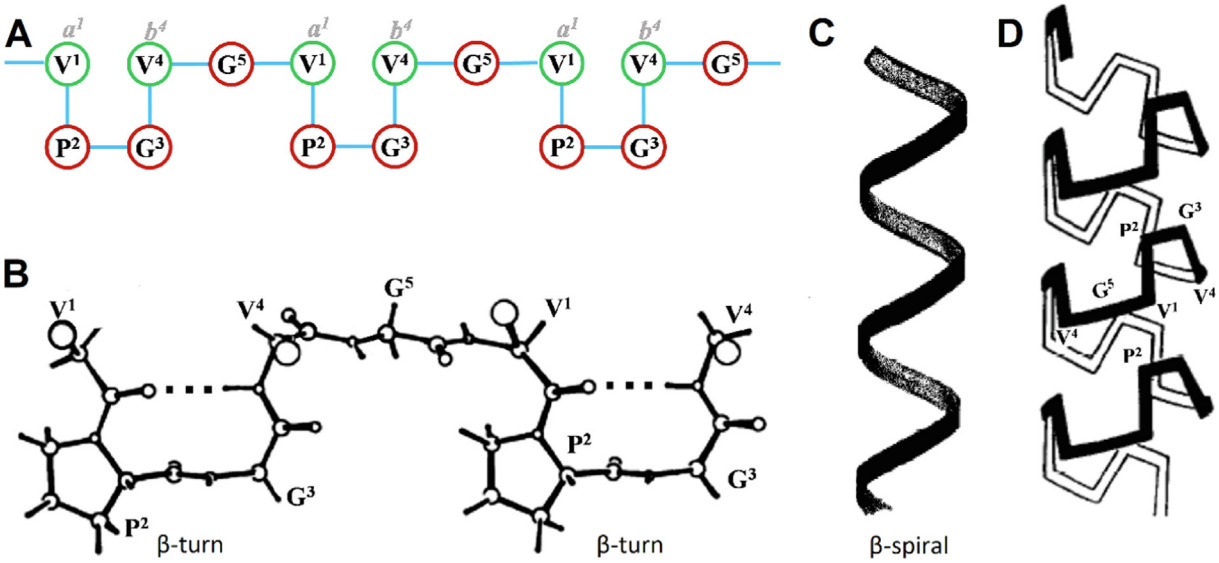

Fig. 13. Schematic representation of the molecular structure of elastin like polypeptide chain of Poly(Val[1]-Pro[2]-Gly[3]-Val[4]-Gly[5]) consists of repeats of VPGVG (A). At elevated temperatures the polypeptide forms β-turns stabilized by hydrogen bonds between Val[1] C=O (amino acid a[1]) and Val[4] NH (amino acid b[4]) in each repeat (B), and wraps-up into a β-spiral structure (C). The β-turns function as spacers between the turns of the helical structure as illustrated in (D). Reprinted with permission from Urry 1983 [115] and Urry 1992 [116].

bilizing liposome membranes. Therefore, p(HPMA mono/dilactate)-cholesterol with a CP of 19.0 °C and a Mn of 10.0 kDa was identified as the most efficient copolymer to induce release at HT of around 45 °C, while keeping the liposome stable at body temperature [75].

Later van Elka et al. [111] synthesized p(HPMA monolactate/dilactate)-cholesterol with a monolactate/dilactate ratio of 43:57 and a Mn of 85 k_D that exhibited a CP of 16 °C. liposomes composed of DOPE:EPC:copolymer (75:25:5 mol%) revealed a relatively slow doxorubicin release reaching 100 % over 10 min incubation at 47 °C, which makes application of this copolymer non-practical for HT drug delivery purposes. Although this preparation exhibited weak aggregation at 37 °C, in absence or presence of serum, in vitro platelet activation study on human blood showed no sign of liposome-induced platelet activation and suggesting that this formulation is safe for systemic administration.

4.5. Biopolymers

In addition to thermosensitive synthetic polymers, thermosensitive biopolymers have recently gained attention for hyperthermia-triggered drug release. Unlike synthetic biopolymers such as poly- and oligo-peptides can be readily synthesized with a defined chain length of defined sequence that provides a great control over tailoring transition temperature. Besides, pharmaceutical products of biopolymers face less complexity related to biodegradability and potential toxicological profile compared to synthetic polymers. Among the variety of biopolymers with sensitivity to heat, elastin-like polypeptide and leucine zipper motif have been employed for triggering drug release from liposomal carriers.

4.5.1. Elastin-like polypeptides (ELP)

ELPs, originally identified from human tropoelastin [112], are biopolymers of repeating pentapeptide of $(a$-Pro-Gly-b-Gly$)_n$ in which the *a* residue is valine or isoleucine and the *b* residue, termed the 'guest residue', can be any amino acid except proline (Pro) [113]. ELPs undergo thermal inverse phase transition exhibiting reversible self-association and phase separation resulting from formation of type II β-turns in response to heat in a narrow temperature window of less than 2 °C.

Despite some controversies on the mechanisms underlying LCST behavior of ELPs (described by Smits et al. [114]) the most compelling explanation is that by increasing the temperature the polypeptide loose water molecules while formation of hydrogen binding between the Pro and Gly residues results in formation of II b-turns in each pentapeptide repeat that consequently results in induction of conformational changes from a random coil to β-spiral structures (Fig. 13).

The transition temperature of ELPs is dependent on molecular weight, concentration, and presence of co-solutes and most importantly amino acids in *a* and most particularly *b* residues. Like other synthetic thermoresponsive polymers increasing the hydrophobicity of the guest residue results in decreasing the transition temperature of the ELP.

Shin and coworkers [117] explored the thermosensitivity of ELPs in sensitizing liposomes. They synthesized p(Val-Pro-Gly-Val-Gly)$_{20}$ equipped with a Lys residue and conjugated it to the distal end of DSPE-PEG2000-NHS. The polymer exhibited a transition temperature of 42.5 °C and 40 °C when measured in PBS or serum, respectively. However, DXR loaded liposomes composed of HSPC: Chol:DSPE-PEG2000-ELP (59:39:2) did not show any triggered DXR release upon hyperthermia, which is likely due to the distance from the lipid bilayer, but tended to aggregate at 42 °C. Such change in surface properties made these liposomes especially suitable for cell targeting in which ELP moieties act as targeting ligands that are switched on by HT. As expected, polymer-modified liposomes exhibited a heat-mediated cell interaction.

Later Kim and coworkers [118] used p(VPGVG)$_3$ (ELP3) and conjugated this directly to an stearyl group (C18) at the *N*-terminus, for anchoring in the lipid bilayer, and amidized the C-terminal (SA-ELP3-NH2). Remotely loaded DXR liposomes composed of DPPC: Chol: mPEG2000-DSPE: SA-ELP3-NH2 (76:21:3:1 mol ratio) exhibited a burst DXR release in a temperature range of 39–42 °C (≥95 % release at 42 °C within 10 s) in the presence of 20 % serum, which is comparable to the release rate from LTSL (Fig. 14A). On the other hand, while LTSL released around 60 % of the content within 30 min incubation at 37 °C, the polymer-modified TSL have

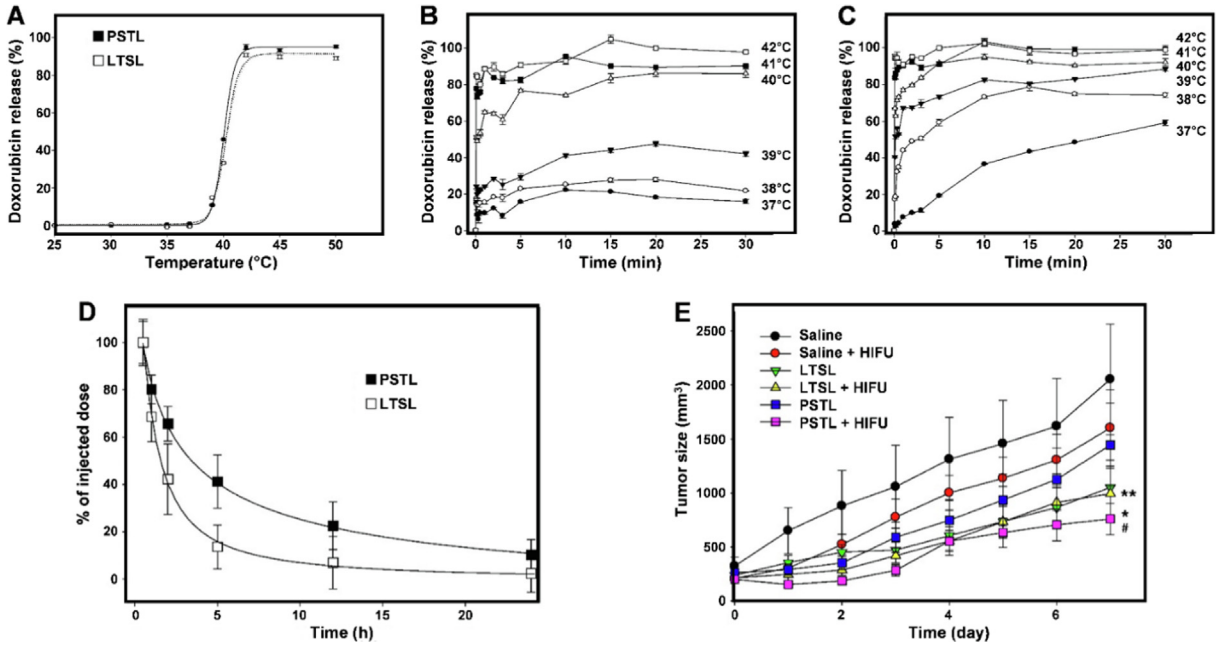

Fig. 14. Comparison of release kinetics, pharmacokinetics and therapeutic efficacy of PEGylated PSTL modified with ELP and lyso lipid containing TSL (LTSL). Panel A plots temperature dependent release of doxorubicin from PEGylated PSTL and LTSL after 5 min incubation at different temperatures. Panels B and C represent time dependent release of doxorubicin from PSTL (B) and LTSL (C) incubated at different temperatures. Release studies were performed in presence of 20 % serum. D represents the circulation life time of doxorubicin in plasma of tumor-free BALB/c mice mice received a single i.v. dose of (5 mg/kg) liposomal doxorubicin (Data are mean ± S.D. (n = 5)). Panel E represents antitumor efficacy of doxorubicin loaded PSTL and LTSL, with or without application of heat by HIFU. Liposomes were administered i.v. (5 mg /kg) into tumor-bearing BALB/c mice. Adopted with permission from Kim et al. 2013 [118].

an appropriate stability at 37 °C and the percentage of release remained around 20 % within 30 min (Fig. 14B and C).

The higher stability of this TSPL resulted in longer blood circulation ($t_{1/2}$ = 2.03 ± 0.77 h) than LTSL ($t_{1/2}$ = 0.92 ± 0.17 h) after an i.v dose of 5 mg DXR/kg into BALB/c mice (Fig. 14D).

Antitumor efficacy was evaluated after a single-dose treatment (5 mg DXR/kg) and HIFU- generated HT of 43 °C applied for 1 h right after injection. It was found that TSPL have better trend in slowing the growth rate of a subcutaneous murine squamous cell carcinoma SCC-7 tumor model compared to LTSL (Fig. 14E). However, it has to be noted that 43 °C is not an optimum temperature for maximum release from LTSL. In fact DXR release from LTSL at 43 is suppressed by 50 % compared to the release at 41–42 °C [119,120] this may partly explain why addition of HT to treatment with LTSL resulted in no advantages. Both treatments with LTSL with or without application of HIFU heat were completely identical respect to slowing tumor growth (Fig. 14E).

Further studies on optimizing the combination of ELP on TSL showed that transition behavior of ELP-lipid conjugate is inversely correlated with concentration of lipopeptide conjugate in solution, and molecular weight of the peptide (Fig. 15A) [121]. For example while SA-ELP3 (3 repeats of VPGVP) exhibited LCST values between 40 °C and 52 °C in concentration range of 1 mM to 0.01 mM the SA-ELP6 (6 repeats of VPGVP) exhibited LCST values between 27 °C and 37 °C in the same concentration range. Moreover, increasing concentration or molecular weight also sharpen the transition behavior. This also applies on ELP-conjugate when incorporated into the TSL lipid membrane. The higher the amount of ELP or the longer the ELP, the lower the temperature of the onset of release and the higher premature drug leakage.

In addition to ELP-related factors they also optimized their TSPL respect to lipid composition. It has been shown that addition of low mol% of DSPC to DPPC-based liposomes improved the thermal response of PTSL (Fig. 15B, compare lines 1 and 4) by improving sharpness and magnitude of temperature-triggered drug release. Here also was found that combination of DPPC:DSPC (75:25 mol %) is beneficial presumably by improving the formation of grain boundaries [122]. Next to addition of DSPC, it was also found that incorporation of 15 mol% cholesterol into DPPC liposomes (Fig. 15B, see lines 1–3) or 10 % into DPPC:DSPC (75:25 mol%) (Fig. 15B, see lines 4 and 5) are optimal to increase stability at 37° of ELP modified TSPL while keeping their thermal drug release sharp and complete [121].

Later a promising polymer-modified liposomes for theranostics was developed in which 2 % of a SA-ELP (the sequence of ELP has not been reported) was post inserted into preformed co-loaded DXR (loaded remotely) and Gadobenate dimeglumine (Gd-BOPTA, loaded passively) liposomes composed of DPPC:DSPC:Chol: mPG2000-DSPE (61.5:20.5:15:3 mol%), (Gd-DXR- TSPL). Encapsulation of gadolinium as a contrast agent enabled monitoring of DXR release at the tumor site by MRI when HIFU-induced mild hyperthermia was applied [123]. Gd-DXR- TSPL exhibited a better release profile compare to the lysolipid containing liposomes (Gd-DXR-LTSL). The TSPL released less than 10 % DXR during 3 h incubation at 37 °C in the presence of 10 % serum and the onset temperature of release was 2 °C higher than what was observed for LTSL. Besides, it seems that increasing the SA-ELP content and addition of DSPC improved the temperature-dependent release properties of this preparation compared to the previously reported preparation made of DPPC: Chol: mPEG2000-DSPE [118].

4.5.2. Coiled coil polymers

Coiled coils are superhelix structures formed by arrangement two or more α-helices. Coiled coils motifs are abundant in many fibrous proteins or transcription factors and are involved in assembly of higher order protein structures. Coiled coils structures offers a rich molecular tool box and have been exploited in different strategies for drug delivery purposes that has recently been well reviewed by Utterström et al. [124]. Coiled coils have been used

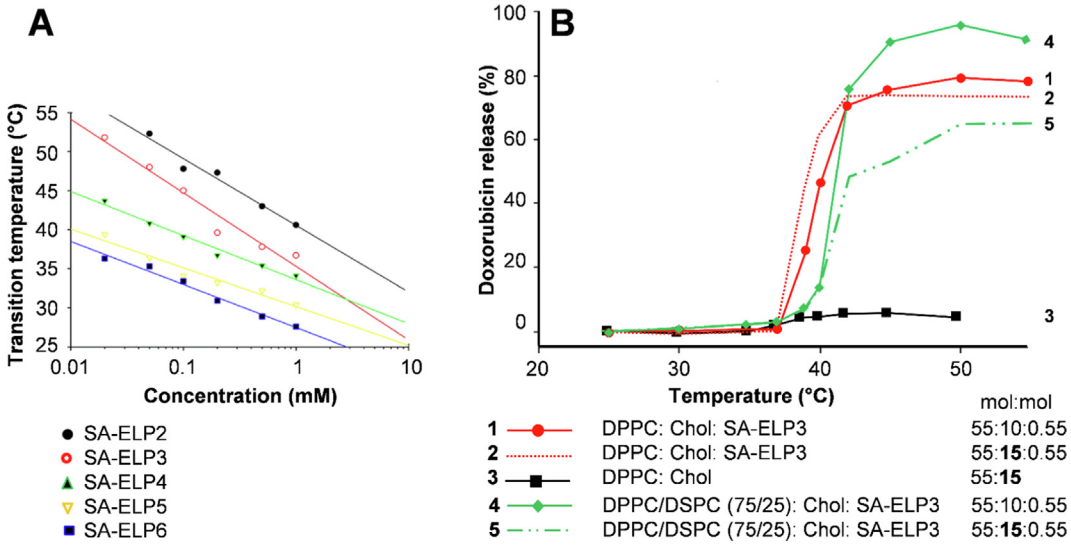

Fig. 15. Thermal behavior of ELP-lipid conjugates (SA-ELP) in solution (A) or when incorporated into different liposomal membranes (B). Panel A represents transition temperature of ELP-lipid conjugates (SA-ELP) in solution as a function of lipopeptide concentration and molecular weight of the peptide (number of repeats of ELP units). Solution of ELP-lipid conjugates were heated at a constant rate of 1 °C/min and the transition temperature was defined as the temperature at which the solution reached 50 % of transmittance. Panel B represents temperature dependent release of doxorubicin from different liposomal preparations. The amounts of doxorubicin release were measured after 5 min incubation at a desired temperature. Adopted from Park et al. 2014 [121] under the Creative Commons licenses.

for enhancing drug delivery by liposomes by facilitating cellular uptake [125,126] or pH-responsive drug release [127].

The leucine zipper motif is a common three-dimensional structural motif in proteins and originally found in DNA binding domains [128]. Leucine zipper is a subtype of coiled coils, in which two or more alpha helices wound around each other and self-assemble into a bundle, a super coil termed a coiled-coil. Leucine zippers are repeats of 7 amino acid sequence [*abcdefg*]n that form α-helical structures (Fig. 16). Where *d* (mostly leucine) and *a* residues are hydrophobic amino acids, and *e* and *g* residues are charged, and Proline is not typically observed. Under biological conditions hydrophobic interactions between *a* and *d* residues or electrostatic interactions between oppositely charged *e* and *g* residues (in heterodimeric leucine zipper) stabilize the coiled-coil, but above a certain temperature the peptide monomers adopt a reversible random coil conformation resulting in a reversible dissociation of the coiled-coil [129]. This transition temperature (melting temperature, Tm) is dependent on the peptide sequence and could be tuned by changing the amino acid sequence. For instances, a leucine zipper motif of (VSSLESK)6 exhibits a Tm of 95 °C, but by addition of lysine residues (indicated in bold) in a motif with sequence of $(VSSLESK)_2$ $(VS\mathbf{K}LESK)_1$ $(\mathbf{K}S\mathbf{K}LESK)_1$ $(VS\mathbf{K}LESK)_1$ $(VSSLESK)_1$ a Tm of 40 °C could be achieved [130]. Such reversible melting upon application of heat makes leucine zippers attractive tools in designing of delivery systems with temperature-triggered function.

Al-Ahmady et al. [131] exploited the above mentioned leucine zipper motif (T_m 40 °C) aiming at improving temperature-triggered DXR release from TSL composed of DPPC:DSPC:DSPE-PEG2000 (90:10:5 mol). Hypothetically, the stabilized coiled-coil can act as a closed pore inserted in the liposome membrane that opens and allows drug release upon dissociation by exposure to heat. UV Circular Dichroism (CD) analysis revealed that the leucine zipper peptide was successfully incorporated into lipid membrane without affecting the colloidal properties of the liposome. Both free and liposome-incorporated motifs formed a predominantly α-helix conformation at 6 °C, but by increasing the temperature underwent melting with a Tm of 46.3 ± 2.3 °C and 40.95 ± 0.1 °C, respectively. Interestingly, while free peptide unfolded irreversibly the liposome incorporated peptides refolded to the ground α-helical structure upon cooling. While DSC analysis showed that incorporation of different amounts of peptide does not impact on transition temperature of the liposomes, fluorescence anisotropy studies showed that incorporation of peptides increases the membrane fluidity at the bilayer interface independent of T_c of lipid (as observed with ANS anisotropy), but the membrane fluidity at the hydrophobic acyl chain region (as observed with DPH anisotropy) slightly deceases by peptide incorporation at temperatures below the Tc of the lipid, when a bilayer is in gel phase. Presence of the peptide in the membrane increases the DPPC-d62 acyl chain order parameters in a concentration dependent manner, as was shown by solid-state NMR studied at temperature above main transition using DPPC-d62 as the deuterated reporter lipid.

Studies of DXR release over time at 37 °C in the presence of 50 % CD-1 mouse serum, revealed no significant difference in release from peptide-modified liposomes and plain liposome within the first 1 h. However, DXR leakage during 24 h significantly reduced by increasing the peptide content of liposomes. Such stabilizing effect of peptide incorporation was also observed at 42 °C where liposomes with different peptide: lipid molar ratios of 1:100, 1:200,1:600 released about 45, 62 and 65 % of their DXR content within 5 min, whereas plain liposomes released over 80 %. Apparently incorporation of leucine zipper motif rigidifies the TSL membrane and negatively impacts on release from TSL composed of DPPC:DSPC. Such rigidification has also been observed with other peptide motifs that interact with bilayer membranes such as viscotoxin A3 [132] or a peptide fragment of the VP3 hepatitis A protein [133] and laminin [134].

Formulations of DPPC with low DSPC content are known to have a fast release property suitable for intravascular release [56]. Therefore, it could be concluded that incorporation of the leucine zipper negatively impacts on the capability of the TSL to release fast by rigidifying the membrane. Besides, it was not clear whether the dissociated coiled-coil can switch on the release. In vivo, without hyperthermia, it was also found that incorporation of peptide resulted in longer circulation time. At 1 h after injection DXR in

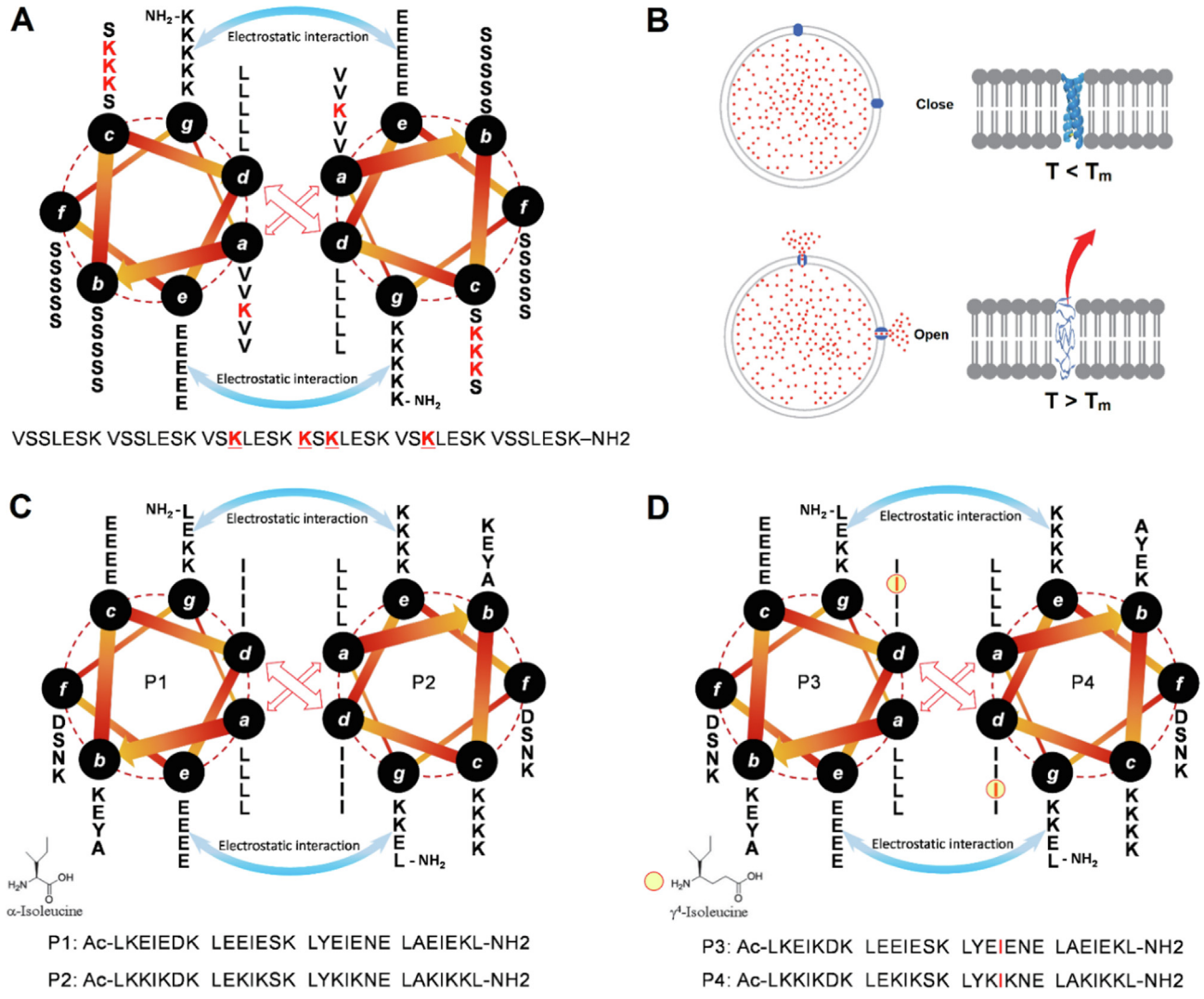

Fig. 16. Helical wheel diagrams and proposed mechanism of drug release from TSPL modified with coiled coil polymers. Panel A illustrates a wheel diagram representing molecular conformation of homodimeric coiled coils of a leucine-zipper peptide of (VSSLESK)2 (VSKLESK)1 (KSKLESK)1 (VSKLESK)1 (VSSLESK)1 at temperature below the transition temperature (40 °C) (reprinted by permission from Al-Ahmady et al. 2012 [131] Copyright 2012, American Chemical Society.). Panel B is a schematic presentation of liposome modified with leucine-zipper peptide at temperatures below and above the transition temperature of the peptide motifs. Panels C and D represent wheel diagram of heterodimeric coiled coils of native peptides (C) and the mutated peptides (D) in which α-isoleucine residues at the 'd' position of the third heptad was replaced by γ-isoleucine (indicated in red-cycled). This mutation resulted in decreasing of transition temperature from 67 °C to 40 °C in the mutated motifs reprinted by permission from Jadhav et al. 2013 [135]. In A, C and D, at temperatures below transition temperature, the self-assembled α-helices of homodimeric or heterodimeric wrap around each other supported by electrostatic interactions and form coiled coils with hydrophobic interhelical core which represents a closed pore. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

serum of mice receiving plain liposomes or the peptide-modified liposomes (1:200), was 37 % and 50 % of the injected dose, respectively.

Later Gopi and coworkers [135] designed, synthesized and utilized another heterodimeric coiled-coil composed of two mutated peptides in which α-isoleucine residues at the '*d*' position of the third heptad was replaced by γ-isoleucine [135] and found that such mutated motifs formed a heterodimeric coiled-coil with Tm of 40 °C, whereas other heterodimeric combinations containing the non-mutated motifs exhibited much higher T_m (>55 °C). The selected heterodimer with the mutated motif and the heterodimer of the parent non-mutated motifs (T_m 67 °C) were inserted in liposome membranes at the molar ratio of 10: 1 (lipid: peptide) by mixing the peptides with lipids composed of EPC: mPEG2000-DSPE (98: 2 mol%) followed by hydration in 100 μM CF in PBS buffer (10 mM phosphate, 150 mM NaCl, and pH 7.4) and sonication. The peptide-modified liposomes showed minimal CF leakage during 24 h incubation at 25 °C. However, at 42 °C only liposomes modified with the muted heterodimer show release, indicating pore opening and release at T_m of the designed coiled-coil. Nonetheless, the release rate was very slow and it took 24 h to reach a maximum release. The results indicate that release through thermally opening peptide pores cannot provide the required burst of release over a limited period of HT and such slow release does not fit in the current settings of HT treatments.

5. Discussion

Here, we comprehensively described and analyzed different liposomal systems in which thermal sensitivity is regulated or enhanced by temperature-sensitive polymers. Meaningful relations between the performed studies were made, not only to depict a better mechanistic insight with respect to thermal function of TSP on TSPL, but also to provide information required for designing predictable and applicable preparations for a precise spatiotempo-

rally controlled drug delivery to tumors. In fact, integration of a functional polymeric system into a liposomal system imposes great complexity that makes TSPL quite different from TSL. In the following, we discuss some parameters that have to be taken into account during the design of a TSPL.

5.1. Biological fluids and thermal response of TSPL

An important factor that has great impact on biological activity of any nanosystem is the interaction of components derived from the biological media with the particle surface. Other than the impact of such interactions on biodistribution behavior of injected nanoparticles, it has been shown that adsorption of serum proteins also impacts on temperature-dependent release from TSPL. Unlike TSL, in which the presence of serum proteins mainly boosts heat-triggered drug release, in TSPL, serum significantly suppresses thermal activity of TSP and reduces the magnitude of drug release, while serum can also act positively and reduce premature drug leakage from TSPL. Important is to realize that a TSPL that is designed based on behavior of copolymers in serum-free buffer, behaves different to a large extend when exposed to biological environment. More so, behavior of polymers when embedded in a lipid layer is also different from behavior in solution. Together, the unpredictability of polymer behavior and the multitude of interactions and responses complicate the design of TSPL.

5.2. Pharmacokinetics and PEGylation

One of the greatest challenges in developing TSL is premature content release from these liposomes upon injection, which greatly reduces the availability of liposomal payload at the tumor site. A good example is the lysolipid-containing TSL (LTSL) formulation, which releases a considerable portion of its content directly when exposed to serum under physiological conditions. This aspect has brought doubts about the capability of LTSL to deliver enough drug to the heated site and in fact may contribute, at least in part, to the clinical failure of TSL [55]. In addition to premature drug release, thermoresponsive polymer-modified liposomes need to be optimized to prevent recognition by the RES as well since aggregation due to changes in surface hydrophilicity occurs that may result in to a fast clearance rate and low availability at the tumor site. Like with other nanoparticles, PEGylation is a solution to reduce protein binding and increase circulation time of TSPL. Although one may argue that at temperatures below the LSCT, a coating of hydrated TSP can provide some degree of stealthiness, it was shown that TSP are not efficient enough [64]. Therefore, for a long-circulating preparation, PEG is required. Besides, proper concentration of PEG was found beneficial in improving thermal release from TSPL by sharpening the temperature dependent release curve, decreasing premature drug release at temperature below LCST, and by normalizing the effect of polymer concentration on release [72,95,97]. Kono et al. [95] observed a broad endotherm (ΔH = 43 mJ) between 20 and 30 °C in a solution of PEG(550)-$2C_{12}$ and an endotherm with ΔH of 262 mJ centered around 32 °C for a solution of Poly(NIPAM)-$2C_{12}$. When both polymers were mixed, only one endotherm centered on 32 °C with a ΔH of 315 mJ was obtained. Appearance of one endotherm in this mixture implies a mutual interaction between PEG and NIPAM polymers. The similarity of the ΔH in the mixture to the sum of both when alone in solution (43 + 262 = 305) implies that both polymers undergo transition at 32 °C. Analysis of release in serum free buffer revealed that addition of >2 mol% PEG 550 reduced heat-triggered release from DOPE containing TSPL at the LCST of the used copolymer and calcein release was massively suppressed when 1 mol% PEG2000 was added to the TSPL formulation. However, when release was studied in the presence of 10 % serum PEG exhibited concentration dependent supporting effect, where highest release rate at 40–42 °C was observed with TSPL containing 4 % PEG550. Clearly, despite the negative impact of PEG on stabilizing DOPE containing TSPL in serum free medium PEG improves thermal release from TSPL by inhibiting the suppressing effect of serum through reduction of protein binding. This positive effect has been observed in other studies with other lipid compositions as well [64].

5.3. Sharpness of thermal release vs application mode of TSPL

It needs to be taken into account that although copolymers can exhibit sharp LCST or CP as determined by optical density measurements, the thermal behavior as could be seen by calorimetric analysis starts far before the LCST. Worth mentioning is that using calorimetric thermal analysis not only provides a better insight into thermal behavior of TSP, by showing the temperature at which thermal transition starts, but also this method could be done in the presence of both copolymers and liposomal lipids and fill the gap between the LCST of copolymer and anchored–copolymer when measured via optical density measurements. Thereby, it provides a more precise estimate of copolymer behavior when incorporated into a liposomal membrane. This in one hand necessitates using thermal calorimetric analysis to determine copolymer transition behavior, but also implies that a copolymer designed to undergo transition at a desired temperature range of 41–42 °C may starts transition behavior at 37 °C. In most studied copolymers, the onset temperature of transition is below or at best very close to 37 °C. Considering the fact that during the transition hydrophobicity of copolymers increases, even the partially dehydrated copolymer chain are more prone to protein binding and opsonization and consequently RES uptake. Kim et al [118,123] developed a polymer-modified liposomal preparation with excellent stability (i.e. minimal leakage) in presence of serum at 37 °C, while LTSL released >60 % content within 30 min of incubation at this temperature. However, once this PEGylated PSTL was injected into mice only a two times longer circulation half-life compared to the leaky LTSL was observed. The biodistribution data show that polymer-modified liposomes are cleared rapidly from the blood by liver and in greater extent spleen. Therefore, it is not surprising that this TSPL could not make a significant leap in treatment of tumors compared to administration of LTSL [118]. In other words, while premature release from LTSL limits the availability of liposomal drug at the tumor site, with polymer-modified liposomes such limitation could be attributed to a fast clearance of liposomes by RES. Therefore, this needs to be taken into account in design of TSPL.

When designing TSPL it is important to consider and recognize the release approach which is aimed for. There are two main modes or approaches for heat-triggered drug release (Fig. 17) and each requires different characteristics of the nanosystem, which have also to be aligned with the applicable hyperthermia setting in the clinic.

5.3.1. Extravascular drug release:

In the extravascular drug release approach heat is applied to trigger the response of thermoresponsive nanoparticles when nanoparticles are accumulated inside tumor (Fig. 17A). Therefore, it is best to apply heat when nanoparticles reach the highest intratumoral concentration (Fig. 17a1 and a2). Since this accumulation is EPR based, important is the long circulation life time of TSPL in blood to maximize passive targeting of the tumor. Therefore, as illustrated in Fig. 17a1 and a2 drug release from both kind of slow or fast release nanosystems is efficient and all delivered drug becomes bioavailable as long as a complete release, from the all the liposomes present, could be achieved within the duration of the applied thermal dose, e.g. 1 h of 42 °C. When this approach is intended design of the formulation needs to be focused on pro-

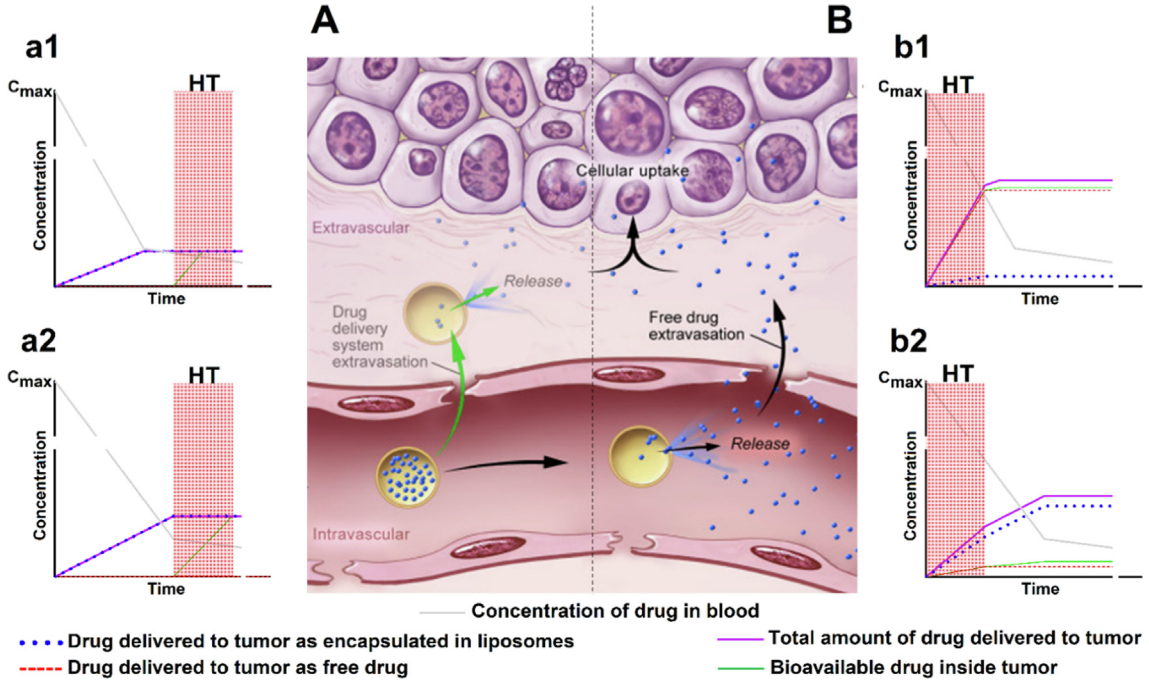

Fig. 17. Schematic representation of circulation time and drug delivery to tumor in two settings of (A) extravascular drug release or (B) intravascular drug release when either fast release (a1 and b1) or slow release (a2 and b2) nanoparticles are used. In extravascular setting (A) the majority of drug that is delivered to tumor is in liposomal form. Therefore, the fast release preparation (a1) accumulates less than the slow-release counterpart (a2) inside tumor due to its leaky nature and shorter circulation half-life. However, in both preparations during a course of hyperthermia (HT) all encapsulated drug will become bioavailable unless the slow-release preparation requires more than an applicable HT duration to release completely. With extravascular release what determines the level of drug bio-availability is the circulation life time whereas fast release is not necessarily demanded. In intravascular release (B) the majority of drug that is delivered to tumor is in the form of free drug. Therefore, despite the longer circulation time of the slow release preparation (b2) it is the fast release counterpart (b1) that deliver more to the tumor because more is released during the short transit time through tumor vasculature. A slow-release preparation can deliver more drug into tumor in an intravascular setting (b2) compared to extravascular setting (a2) because of enhanced EPR but the delivered drug is not fully bioavailable. On the other hand, a fast release preparation is more successful when is used in intravascular setting. To avoid complexity of the schematic graphs the decline phase of intratumoral concentrations was not included in the time axis, and the time axis represents the time that concentrations reach and stay at a maximum before going to the decline phase. The graphic was reprinted and modified from ten Hagen et al. 2021 [93] under Creative Commons license.

longed circulation and not per se rapid release. Sharpening the release rate is actually not advised as this is mainly accompanied by premature drug release or accelerated RES uptake.

5.3.2. Intravascular drug release

Intravascular drug release is aimed at creating a steep gradient of drug concentration between blood and tumor interstitium based on which free drug molecules can diffuse out of the circulation and reach the tumor interstitium (Fig. 17B). To maximize drug delivery, and thus concentration in the blood in the tumor, fast and significant amount of release during a short period of time is needed as passage through a tumor and exposure of nanoparticles to heat is relatively short [93]. Furthermore, hyperthermia has to be applied when intravascular concentration of injected TSPL is at C_{max}. Therefore, nanoparticles have to be able to release 100 % of content in a matter of few seconds. The payoff is premature release, though. This has been tested with LTSL, which despite a fast clearance rate, could facilitate great DXR delivery and therapeutic effect in animal models when intravascular drug release is aimed [136]. Important is to take into account that because of the intrinsic leaky nature of such ultrafast releasing liposome these liposomes are not a proper candidate to be used for extravascular release (Fig. 17a1 vs b1). There might be a limitation to intravascular drug release from TSPL that needs to be addressed. Thermal behavior of TSPL accompanied with release and aggregation due to rendering the liposomes surface hydrophobic. Therefore, it is likely that application of HT when TSPL concentration in blood is at Cmax could result in clotting of blood, occlusion or embolization and blockage of tumor vessels that is detrimental for EPR effect and consequently the drug delivery [137].

5.4. Optimizing triggered drug delivery by TSPL

The current trend in nanosystem-mediated cancer therapy is exploiting new physical or pharmacological tools that can enhance the EPR effect, or to develop new drug delivery systems that rely less on EPR-based accumulation such as vascular targeting. These have nicely and extensively been discussed previously [12,137,138]. Interestingly, mild hyperthermia has both effects; while intravascular drug release can bypass EPR accumulation of nanocarriers by delivering of free drug molecules instead, it is also a physical EPR enhancer that facilitates nanoparticle accumulation inside tumors by increasing blood flow and vasodilation (enhanced perfusion), and increases vascular permeability. Besides, next to pharmacological and immunological adjuvant effects of mild hyperthermia it greatly enhances distribution and penetration of delivered drug inside the tumor interstitium and enhances therapeutic efficacy [136]. What is important is how to gain all these benefits? As stated earlier, the timing of injection and heat application determines mode of drug release from TSPL, i.e. extravascular vs intravascular drug release. In principal when application of heat and TSPL injection take place concomitantly or close to each other (Fig. 17B) one can benefit from intravascular drug release, enhanced EPR effect and depending on kinetics of release and duration of heat extravascular drug release. Whereas application of heat when maximum passive accumulation in tumor is achieved (Fig. 17A), only triggers extravascular drug release and enhances drug distribution inside tumors. Yet such approach entirely relies on EPR to target tumors and does not benefit from EPR enhancing effect of mild hyperthermia. In addition, a recent study of Al-Jamal and Kostarelos [139] showed that heating of tumor after accumu-

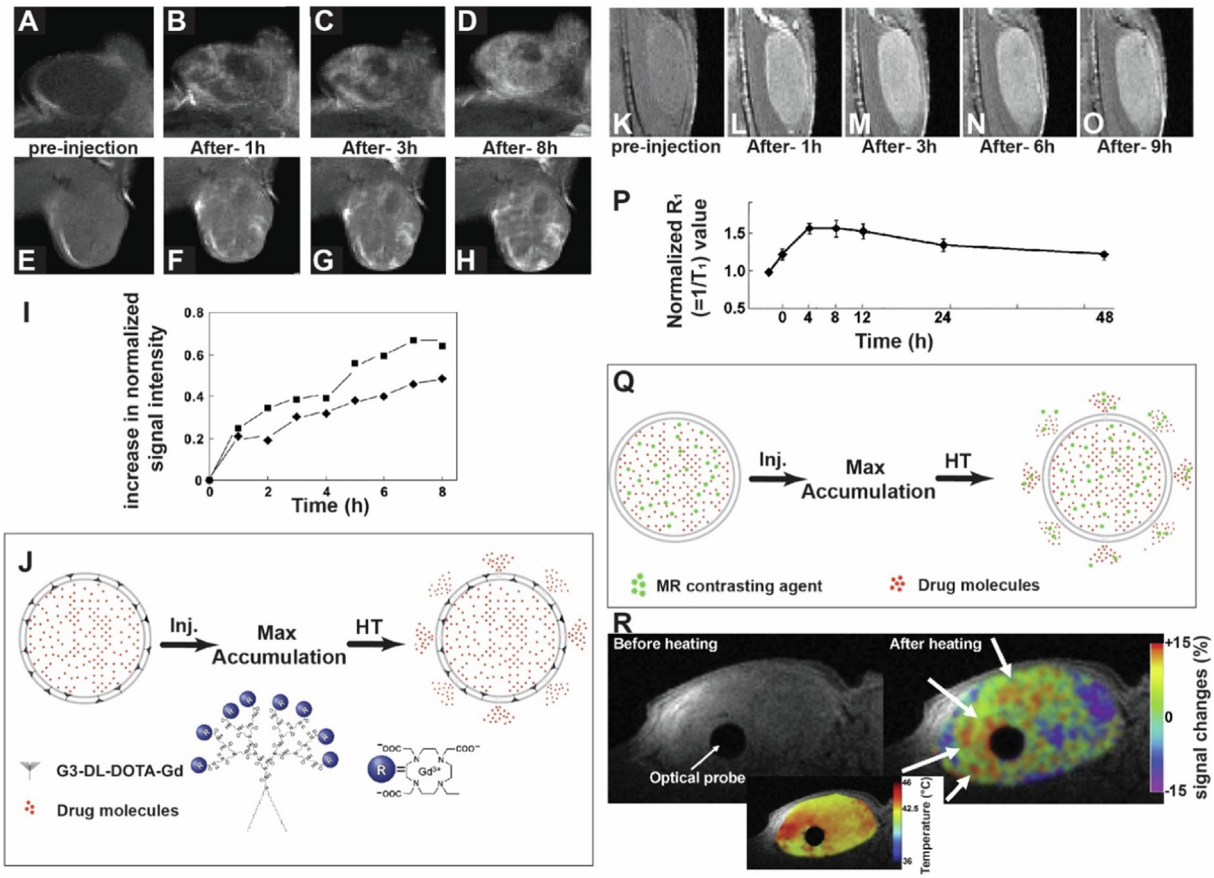

Fig. 18. Theranostic application of TSPL. Panels A-H depict tumor accumulation of liposomes with average diameters of 110 nm (A–D) or 48 nm (E–H) labeled with MR contrasting agent G3-DL-DOTA-Gd. Images of tumors were captured prior to injection of liposomal preparations and at different time points after the injection. (I) Plots the increase in the area-averaged MR signal intensity in tumor of BALB/c nude mouse injected with liposome of 110 nm (squares) or 48 nm (diamonds) as a function of time. MR signal intensities of tumors were normalized by MR signal intensities of muscle of each mouse. Panel J represents the schematic illustration of theranostic application of a temperature-sensitive liposomes in which liposomes are labeled via incorporation of contrasting agent in lipid membrane. (Adopted with permission from Kono et al. 2011 [99]). Panels K-O represent horizontal T1-weighted images acquired over a period of 9 h after liposome administration. The horizontal direction shows the elapsed time after administration. The signal intensity in the tumor increased smoothly in the first 6 h after administration. Dynamics of MTPL accumulation in tumor was evaluated by measuring R1 in the tumor for 48 h after MTPL administration (P). R1 was normalized by its value before administration. In the tumor, R1 was highest between 4 and 12 h after administration, and at 24 h remained at 86 % of the peak value. Panel Q schematically illustrates the theranostic application of a temperature-sensitive liposomes in which liposomes are labeled via encapsulation of water soluble, small molecular weight MR contrasting agent inside liposomes. Panel R represents the enhancement of MR signal after RF heating and the corresponding proton resonance frequency (PRF) temperature map acquired after the heating period. A dose of MTPLs was administered intravenously. The MR image before heating was acquired at 12 h after administration. The signal alteration map (right, shown in color scale), which is the subtraction of the before and after heating images, has been superimposed onto the after-heating T1-weighted image. The arrows point to the area where the signal intensity increased most after heating. (Adopted with permission from Kokuryo et al. 2015 [100]).

lation of TSL increases the clearance rate of drug from tumor. This concept has been illustrated in the graphical abstract of this review.

However, one may optimize the extravascular drug release by means of theranostic approaches to find the most efficient moment to apply heat. Kono and coworkers [97] labeled the membrane of their formulated TSPL (see section4.3) with G3-DL-DOTA-Gd, that is incorporated into lipid membrane, and tracked liposome accumulation in tumor. They could successfully track and visualize the accumulation of liposomes inside tumor (Fig. 18A–H) and showed that upon i.v. administration of TSPL with different sizes maximum accumulation was achieved after 8 h (Fig. 18I). This time point could be considered as an optimum time to apply heat and trigger drug release (Fig. 18J). In another study Kokuryo et al. [100] labeled their multifunctional TSPL with Mn^{2+} as an encapsulated contrasting agent. Upon injection into tumor-bearing mice, the intratumoral concentration of liposomes increases gradually (Fig. 18K–O) and reached to a maximum at 4 h post injection and remained constant up to 12 h after injection (Fig. 18P). Based on this results they chose 12 h post injection as an optimum moment for application of heat (Fig. 18P). The advantage of using encapsulated tracer, which is released similar to the encapsulated drug, over labeling liposomes with a lipid tracer is the possibility of evaluating drug release and distribution inside tumors. Fig. 18R clearly shows how heat-trigger released drug has distributed inside tumor. However, a significant degree of premature release during circulation of liposomes in blood may occur increasing the background signal and thus reducing imaging sensitivity. In the study of Kokuryo et al. [100] this has been reflected as a high signal in the kidney of animals which was about 2.5 and 1.6 folds greater that the normalized signal obtained at 4 h post injection in tumor and liver of animals, respectively.

As a matter of fact, the possibility of bypassing EPR regained more attention with the intravascular drug release approach. However, while performance of TSPL for extravascular drug release have been studied with precise and controlled manners such as MRI-guided triggered drug release, TSPL in an intravascular drug release have not yet been evaluated. Interestingly, while some fast-releasing TSPL were compared with LTSL as a reference or control with respect to release kinetics or therapeutic efficacy, in vivo antitumor activity was evaluated in extravascular release treatment models. It is important to mention that, although LTSL is a

well-known preparation this liposome is not suitable for extravascular drug release. Besides, it should be noted that the formulation of LTSL (Thermodox) that is under clinical evaluations contains monostearoylphosphatidylcholine (MSPC). Therefore, LTSL containing shorter monopalmitoylphosphatidylcholine (MPPC), which are more leaky, are not a proper representative of LTSL to be used as control. Selection of inappropriate controls or an impolitic setting may impair judgment of TSPL performance.

6. Summary of stage of temperature sensitive liposomes in clinic

As stated earlier the only heat sensitive liposome that has been utilized in human is LTSL containing DXR (ThermoDox®, Celsion corporation) that is being investigated in an intravascular thermal release setting. In the first clinical trial on 24 patients (nine with HCC and 15 with metastatic liver tumors from nine other primary sites) ThermoDox® was combined with Image-guided radiofrequency ablation (RFA) which is the first-line therapy in patients with early-stage unresectable hepatocellular carcinoma. A statistically significant dose–response effect, suggesting activity, was observed and the maximum tolerated dose was determined to be 50 mg liposomal DXR/m^2. Based on these promising result ThermoDox® proceeded directly into Phase III clinical evaluation (HEAT study, NCT00617981), which was conducted as an international, multicentered, randomized control trial at 79 global sites on 701 patients with inoperable HCC tumor sized around of 3–7 cm where combination of ThermoDox®+ RFA (a single 30-min intravenous infusion of ThermoDox®, 50 mg/m^2, starting 15 min before RFA) was compared against RFA alone. Although the HEAT study showed that combining LTLD to RFA is safe, the primary end point in improving of progression free survival (PFS) was not met. Retrospective analysis of data obtained from the HEAT trial showed a marked improvement in PFS and a statistically significant improvement in OS in patients received ThermoDox® plus RFA for ≥45 min. In addition, it was also found that combining ThermoDox® with RFA extended the treatment area, and had impact on micrometastases that are more frequently associated with these tumors [140]. These positive results encouraged the investors to launch another clinical trial (OPTIMA, NCT02112656), which was optimized based on lessons learned from the HEAT trial. In the OPTIMA study 550 patients with solitary HCC lesions ≥3 cm to ≤7 cm randomly received either ThermoDox® (50 mg/m^2 in 30 min intravenous infusion) plus standardized RFA (dwell time ≥ 45 min) or the RFA plus sham infusion. In addition OS was set as the primary endpoint while PFS was the secondary outcome measure (https://clinicaltrials.gov, NCT02112656). However, after the second interim analysis the sponsor decided to terminate the study because the futility boundary had been crossed [140]. Possible reasons for failure was comprehensively reviewed by Allen et al and us [55,141].

Importantly, these trials used thermal ablation and thermal drug release concomitantly, where thermal release will mainly happens in margins of heated area of tumor where the temperature is in mild hyperthermia range. It is known that ablation reduces blood flow through heated area by clotting and blockage of vessels. In addition, in ablative temperature (>55 °C) TSL releases less drug. Together, the condition of drug delivery to heated tumor is compromised since the ablated tumor area receives less chemotherapeutics. Therefore in other clinical trials either only the thermal release was exploited or ablation is added to treatment after the course of thermal release which fits more with release characteristics of TSL.

TARDOX is a phase I trial (NCT02181075) in which safety and feasibility of ThemoDox® was assessed for treatment of primary or secondary liver tumors but instead of RFA ablation mild hyperthermia (39–45 °C) was applied non-invasively by focused ultrasound (FUS). Combination of ThermoDox® with FUS hyperthermia was found clinically feasible, safe, and resulted in enhanced DXR delivery and response in tumors that were confirmed incurable [142–144].

In another Phase I/II clinical trial (DIGNITY, NCT00826085) infusion of ThermoDox® was combined with an approved therapeutic microwave heating of the chest wall for 60 min for treatment of recurrent regional breast cancer. The DIGNITY trials aimed at evaluating the maximum tolerated dose, bioequivalence/pharmacokinetics, and efficacy of hyperthermia and ThermoDox® in patients with local–regional recurrent breast cancer. The study is completed but the results have not been published yet.

In another ongoing clinical trial (NCT02536183) ThermoDox® is combined with MR-HIFU for treatment of children with relapsed/refractory solid tumors, which may include but are not limited to rhabdomyosarcoma and other soft tissue sarcomas, Ewing's sarcomas, osteosarcoma, neuroblastoma, Wilms' tumor, hepatic tumors, and germ cell tumors. Part one of this study was designed to determine the pediatric MTD/recommended phase II dose (RP2D) of ThermoDox® combined with MR-HIFU ablation in a traditional dose escalation study. In second part of the study instead of ablation, mild hyperthermia induced by MR-HIFU in an expanded cohort is aimed. In another pilot study of ThermoDox® combined with MR-HIFU for treatment of relapsed solid tumors (NCT04791228) the treatment was designed to heat up tumor at mild hyperthermia range by MR-HIFU following ThermoDox® infusion, and after that ablation therapy (>55 °C), where feasible and safe, will be applied. The other ongoing clinical trial (PanDox, NCT04852367) evaluates the combination of ThermoDox® and mild hyperthermia induced by HIFU against pancreatic cancer. This phase I study aims at determining whether a single dose injection of Thermodox (50 mg/m^2) administered in a 30 min infusion concurrently to FUS-induced mild hyperthermia and continuation of FUS for no longer than two hours after the infusion will increase DXR delivery to pancreatic tumors in comparison with injection of free DXR without heat.

7. Conclusion

The addition of polymeric components into a liposomal system could give TSPL unique features compared to TSL. Important however is to realize that the system gets more complex and thus harder to predict. More so, TSPL exhibit an additional thermal controlled response which is temperature-triggered cell interaction [71,97], and therefore polymers could function as a ligand that is switched on by application of external heat. In addition, temperature-sensitive polymers can sensitize non-TSL liposomal preparations to heat, which means that a broad range of lipid compositions, without restriction based on thermoresponsiveness, could be used for liposome preparations. Alongside the availability of various copolymers one can thus precisely tune thermal behavior of a large range of liposomes to release efficiently at a desired temperature. One important practical advantage of TSPL over TSL is the possibility of temperature-triggered release of high molecular weight compounds which is not possible with TSL. Therefore, we believe that TSPL are a promising drug delivery system that merits further attention.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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