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Thermosensitive liposomes modified with poly(*N*-isopropylacrylamide-co-propylacrylic acid) copolymers for triggered release of Doxorubicin

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Abstract

A novel polymer-modified thermosensitive liposome (pTSL) was developed for the delivery of Doxorubicin (DOX) for cancer therapy. Copolymers containing temperature-responsive *N*-isopropylacrylamide (NIPAAm) and pH-responsive propylacrylic acid (PAA) were synthesized via reversible addition fragmentation chain transfer (RAFT) polymerization, yielding copolymers with dual pH/temperature dependent phase transition properties. When attached to liposomes, these copolymers were membrane-disruptive in a pH/temperature dependent manner. pTSL demonstrated enhanced release profile and significantly lower thermal dose threshold when compared to traditional thermosensitive formulations, and were stable in serum with minimal drug leakage over time. These liposomes thus have the potential to dramatically reduce the risk of damage to healthy tissues that is normally associated with liposomal cancer therapy.

Keywords

Drug delivery; thermosensitive liposomes; smart polymer; poly(NIPAAm); thermal dose; hyperthermia; copolymer

INTRODUCTION

Thermosensitive liposomes (TSL) are a promising and extensively studied class of liposomes with tuneable drug release properties. TSL are composed of lipid bilayers that undergo temperature-dependent phase transitions from gel to liquid phase and are permeable at elevated temperatures, resulting in rapid release of encapsulated drug.^{1, 2} TSL systems designed for cancer therapy have gained considerable momentum, but have suffered from several deficiencies, including 1) need for heating beyond conditions that can be safely tolerated¹, and 2) unstable carriers that leak drugs prematurely at physiological conditions.^{3–6}

The standard measure of heating delivered to tissue is the thermal dose, which takes a heat treatment at one temperature and calculates the equivalent exposure time at a reference temperature of 43°C⁷:

$$t_{43^{\circ}\text{C}} = \Delta t \cdot R^{(43-T)}, \quad (1)$$

where $t_{43^{\circ}\text{C}}$ is the thermal dose of the exposure in equivalent minutes at 43°C , Δt is the duration of exposure (min), T is the average temperature of the exposure over Δt , and R is an empirical constant ($R=0.5$ at $T \geq 43^{\circ}\text{C}$, 0.25 at $T < 43^{\circ}\text{C}$). Studies have shown that the thermal dose threshold for 100% necrosis in various tissues ranges from 240 to as low as 50 equivalent min at 43°C .^{8, 9} Meshorer *et al.* (1983) found that in porcine tissue, necrosis onset occurred at $t_{43^{\circ}\text{C}} = 0.5\text{--}30$ min, with moderate damage at $t_{43^{\circ}\text{C}} = 60\text{--}240$ min, and severe damage at $t_{43^{\circ}\text{C}} > 240$ min.⁸

Traditional TSL release 50% of drug at $t_{43^{\circ}\text{C}} = 5$ min, resulting in significant risk of necrosis to healthy tissue surrounding the tumor. This drawback has led to the development of lysolipid-containing thermosensitive liposomes (LTSL).^{1, 2} Lysolipids facilitate the formation of defects and membrane openings during the phase transition, increasing bilayer permeability and causing rapid drug release.^{3, 4} However, lysolipids have been shown to rapidly desorb from the bilayer,⁴ likely resulting in loss of temperature-sensitivity *in vivo*. Indeed, premature leakage of drug *in vivo* (50% of encapsulated drug at 37°C within 5 min,⁵ ~70% within 1 hr⁶) has been observed.

To address these issues, we introduce a novel polymer-modified, non-lysolipid TSL (pTSL) decorated with a membrane-disruptive copolymer of *N*-isopropylacrylamide (NIPAAm) and propylacrylic acid (PAA) (Figure 1). The copolymer – p(NIPAAm-*co*-PAA) – is based on poly(*N*-isopropylacrylamide) (pNIPAAm), a key member of the temperature-responsive class of alkyl acrylamide polymers. pNIPAAm undergoes a sharp coil-to-globule transition and phase separation at its lower critical solution temperature (LCST) in water. This transition is spontaneous and endothermic, driven by the entropy gain from the release of H-bonded water molecules.¹⁰ The LCST can be tuned to a desired temperature range (*e.g.* $38\text{--}40^{\circ}\text{C}$) by copolymerization of NIPAAm with either a hydrophilic comonomer (raises LCST), or a hydrophobic comonomer (lowers LCST). When heated above the LCST, there is evidence that NIPAAm copolymers attached to liposomes become membrane disruptive, aiding drug release.^{11–13} Additionally, there has been evidence that the presence of long polymer chains in polymer-modified liposomes increases resistance to uptake by the reticuloendothelial system,¹⁴ and thus it can be reasonably expected that polymer-modified TSL will be long-circulating and stable *in vivo*.

pH-sensitive monomers may also be copolymerized with NIPAAm, adding a pH-sensitive dimension to the phase transition.^{10, 15–16} This pH-sensitivity is relevant in drug delivery systems targeting the endosomal compartment ($4.9 < \text{pH} < 5.5$) as well as the extracellular environment of tumors ($6.5 < \text{pH} < 7.5$).^{17–19} Carboxylic acid monomers such as acrylic acid (AA), methacrylic acid (MAA), and PAA are protonated at $\text{pH} < \text{p}K_{\text{a}}$, and the loss of net negative charge upon acidification causes an increase in hydrophobic character that aids the polymer phase transition (*e.g.* lowers LCST). AA¹³ and MAA^{17, 20–22} monomers have been copolymerized with NIPAAm using traditional free radical polymerization techniques to form random copolymers with both temperature and pH-sensitive properties. However, while these copolymers show promise, the critical transitions of AA and MAA are typically below pH 5.0, which is more acidic than the interstitial pH levels that have been measured at the tumor site.²³ Thus, NIPAAm copolymers that become membrane-disruptive at pH 6.0 – 7.0 would be better suited for triggering drug release from liposomes within tumor interstitium. Furthermore, an increase in hydrophobicity of these copolymers in mildly acidic conditions could reduce the temperature and thermal dose required for disruption of liposomes and subsequent release of encapsulated drugs.

In this study, p(NIPAAm-co-PAA) was synthesized and attached to traditional TSL. PAA contains a longer alkyl segment and higher pK_a value than AA and MAA. Polymer compositions containing PAA undergo sharp phase transitions above pH 6.0, thus making them more applicable for cancer therapy.¹⁰ Copolymers were synthesized using reversible addition fragmentation chain transfer (RAFT) polymerization, which allows for controlled and narrow molecular weight distributions and close agreement between theoretical and experimentally determined molecular weights.^{24–26} pTSL modified with these copolymers and containing Doxorubicin (DOX) - an anthracycline antibiotic used in chemotherapy - demonstrate an enhanced release profile that is both pH and temperature-sensitive, dramatically reducing thermal dose requirements for drug release when compared to traditional TSL.

EXPERIMENTAL SECTION

Materials

NIPAAm, 2,2'-azobis(2-methylpropionitrile) (AIBN), and bovine calf serum (BCS) were purchased from Aldrich. NIPAAm was recrystallized in hexane and AIBN was recrystallized in methanol prior to use. PAA and 2-dodecyl-sulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP) were synthesized according to previously published protocols.^{26, 27} 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), L- α -phosphatidyl-choline(soy-hydrogenated) (HSPC), cholesterol (CHOL) 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG-2000), and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MPPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

Polymerizations

p(NIPAAm-co-PAA) synthesis was carried out as described in previously published protocols,¹⁰ using RAFT chemistry with initiator AIBN and chain transfer agent DMP at 95:5 mol NIPAAm:PAA and [DMP]:[AIBN] at 5:1 molar (Scheme 1). The choice of NIPAAm:PAA ratio was informed by previously published light scattering experiments, which demonstrated that copolymers of NIPAAm:PAA = 95:5 undergo sharp transitions at LCST = 42°C at pH 6.5 and LCST = 28°C at pH 5,¹⁰ properties that could potentially be exploited to generate drug carriers that are pH/temperature-sensitive at physiologically relevant conditions.

In a typical synthesis, NIPAAm (2.36 g, 20.9 mmol), PAA (0.125 g, 1.10 mmol), AIBN (3 mg, 1.83×10^{-5} mol), and DMP (33.0 mg, 9.15×10^{-5} mol) were dissolved in methanol (2.50 ml, HPLC grade) in a 10-ml round bottom flask. The mixture was degassed by purging with nitrogen for 20 min, and polymerization was carried out at 60°C for 17 h. After polymerization, methanol was evaporated under a stream of air at room temperature, yielding solid polymer. The polymer was then dissolved in THF and precipitated three times into pentane. The final product was dried to constant weight under vacuum to provide 1.97 g polymer (yield, 79.3%).

Polymer characterization

Molecular weights of copolymers were determined using a gel permeation chromatograph (Viscotek) with LiBr DMF as eluent (0.01 mol/L, 1 ml/min, 60°C) and poly(methyl methacrylate) calibration standards. ¹H NMR (Bruker AC 500, methanol-*d*₄ as solvent) was used to determine copolymer composition by comparing peak areas of NIPAAm unit isopropyl C-H signal at 3.9 ppm with total peak area between 0.8 and 1.8 ppm, which includes all other C-H protons.

Liposome synthesis

Liposomes of required composition and size were prepared by the lipid film hydration and extrusion method²⁸: Lipids at the desired ratios (10 mg total lipid) were dissolved in 1 ml chloroform. Four liposomal formulations were generated: nonthermosensitive liposome (NSTL), lysolipid-containing thermosensitive liposome (LTSL), traditional thermosensitive liposome (TSL), and polymer-modified thermosensitive liposome (pTSL). Compositions were as follows: NSTL = HSPC:CHOL:DSPE-PEG-2000 at 75:50:3 molar, LTSL = DPPC:MPPC:DSPE-PEG-2000 at 90:10:4, TSL = DPPC:HSPC:CHOL:DSPE-PEG-2000 at 100:50:30:6, and pTSL with identical composition as TSL but with addition of copolymer. TSL has a melting temperature of approximately 43°C, while NSTL remains stable in mildly hyperthermic conditions.²⁹ Chloroform was removed via evaporation by applying a stream of argon, leaving a dried, thin lipid film. This film was rehydrated in 1 ml 300 mM citrate (pH 4.0), forming a suspension of multilamellar vesicles. This suspension was then extruded through two polycarbonate membranes (800 nm pore size followed by 100 nm) at 38°C using an extruder (Avanti Polar Lipids mini extruder or 10-ml LIPEX extruder, Northern Lipids, Inc.), yielding a homogeneous suspension of unilamellar vesicles (~90 nm (LTSL) or ~130 nm (NSTL, TSL, pTSL), determined by dynamic light scattering, Brookhaven Instruments Corp, model 90Plus).

Encapsulation of DOX into the liposomes was carried out using the pH gradient-driven loading protocol³⁰: Untrapped citrate was removed and pH gradient (pH 4.0 inside liposome, pH 7.5 outside) established by gel filtration through Sephadex (G-50) equilibrated with 20 mM HEPES buffer (pH 7.5). DOX was added to the liposome suspension at a DOX:lipid mass ratio of 0.2:10, and liposome-DOX was incubated at 33°C (LTSL) or 40°C (all other formulations) for 1 h. Untrapped DOX was removed via dialysis (Spectrum Labs, MWCO 1 kDa). To generate pTSL, DOX-loaded TSL were incubated with p(NIPAAm-co-PAA) at a polymer:lipid mass ratio of 1:1 at 30°C for 1hr. RAFT polymerization of NIPAAm and PAA results in copolymers with large terminal DMP groups (Scheme 1) that are expected to act as hydrophobic anchors, allowing polymer incorporation into the lipid bilayer. Unattached copolymer was removed by dialysis (50 kDa MWCO).

Liposome characterization

Encapsulation efficiency and drug release were determined by taking advantage of the self-quenching fluorescent properties of DOX³¹: DOX molecules encapsulated within the liposome are in close proximity to each other and do not produce an appreciable fluorescence signal. However when released into surrounding solution and diluted, DOX fluorescence is restored, providing a quantitative means of measuring release.

Fluorescence intensities were measured with a spectrofluorometer (Shimadzu, RF-1501, $\lambda_{\text{excitation}} = 479 \text{ nm}$, $\lambda_{\text{emission}} = 557 \text{ nm}$ for samples in HEPES). The fluorescence measured upon addition of 10% Triton-X 100, a detergent that lyses the lipid bilayer, was considered as 100% release. In a typical sample, 150 μl liposome solution was added to 20 mM HEPES buffer, with or without the addition of 150 μl T-X (final buffer volume, 3 ml). Intensities were measured prior to and after dialysis and efficiency calculated as follows:

$$\text{Encapsulation efficiency} = F_{\text{TX}} / F_{\text{TX}_0} \cdot 100 \%, \quad (2)$$

where $F_{\text{TX}} \equiv$ fluorescence after dialysis with the addition of T-X, and $F_{\text{TX}_0} \equiv$ fluorescence prior to dialysis and with the addition of T-X. To measure extent of drug release following

heating, fluorescence was measured after treatment, normalized against baseline, and compared to 100% release:

$$\% \text{ DOX released} = (F_h - F_c) / (F_{TX} - F_c) \cdot 100 \%, \quad (3)$$

where $F_h \equiv$ fluorescence after heat treatment, $F_c \equiv$ baseline fluorescence, and F_{TX} is defined as above.

Release studies in HEPES

Liposomes were incubated in a heating bath circulator (Neslab EX-7) and DOX release measured as a function of temperature and time. Typically, 150 μ l liposome samples were diluted in 20 mM HEPES (ionic strength adjusted to 154 mM with addition of NaCl, buffer adjusted to pH 7.5 or 5 by addition of 1 N HCl). Liposomes in buffer were incubated at the desired temperature and time. NTSL (pH 7.5), TSL (pH 7.5), and pTSL (pH 7.5 and pH 5) were tested, with NTSL serving as the negative control. Increases in fluorescent intensities arising from DOX release were measured in the spectrofluorometer, and release determined according to (3). The melting temperature (T_m) for each liposome formulation was defined as the temperature at which 50% DOX release was observed following 5 min incubations in HEPES. Finally, the thermal doses required for 50% release were calculated according to (1).

Stability and release studies in serum

Drug leakage from pTSL, TSL, and LTSL in serum at 37°C was monitored over time to assess the stability of each formulation. Aliquots of the liposome suspensions (volume = 100 μ l) were suspended in 20% BCS in 20 mM HEPES (3 ml total). The buffer was adjusted to pH 7.5 or 5 as needed by addition of 1 N HCl, and liposomes were incubated at 37°C for 5, 30, 60, and 90 min in a heating bath circulator (Neslab EX-7). TSL (pH 7.5), pTSL (pH 7.5 and pH 5), and LTSL (pH 7.5) were studied. Following heating, fluorescence intensities from free DOX were measured in the spectrofluorometer ($\lambda_{\text{excitation}} = 470$ nm, $\lambda_{\text{emission}} = 588$ nm), and the percentage of DOX released at each time point was determined according to (3).

Drug release in serum was also studied as a function of temperature and pH. Typically, 100 μ l liposome samples were diluted in 20% BCS in 20 mM HEPES (3 ml total). Buffer was adjusted to pH 7.5, 6.5, or 5 by addition of 1 N HCl. Liposomes in buffer were incubated at 42°C and 43°C for 3 min (45 sec and 3 min thermal doses, respectively). TSL (pH 7.5) and pTSL (pH 7.5, pH 6.5, and pH 5) were tested. Increases in fluorescent intensities arising from DOX release were measured in the spectrofluorometer, and release determined according to (3).

RESULTS AND DISCUSSION

Results of copolymer and liposome characterization are summarized in Table 1 and Table 2, respectively. Copolymers synthesized via RAFT polymerization displayed low M_w/M_n values (1.2) with $M_n = 30$ kDa. $^1\text{H-NMR}$ results indicated a final copolymer composition of 91% NIPAAm, 9% PAA. As described in previously published work,¹⁰ copolymers with identical feed NIPAAm:PAA ratio undergo sharp transitions at LCST = 42°C at pH 6.5 and LCST = 28°C at pH 5.0, demonstrating the significance of pH in determining copolymer transition behavior and potential membrane disruption capability.

Liposomes were of uniform size and low polydispersity, as determined by dynamic light scattering. All formulations showed relatively high encapsulation efficiencies, as determined

by (2). When heated in 20 mM HEPES buffer, TSL and pTSL formulations both demonstrated temperature-sensitivity, whereas DOX release from NTSL was not observed over the temperature range of interest (Figure 2, n refers to sample size).

NTSL lack DPPC – the temperature-responsive lipid component of TSL, pTSL, and LTSL – which undergoes a gel-to-liquid phase transition at 41.5°C, and thus displayed minimal release as expected. Traditional TSL demonstrated 50% release at $T_m = 43.1^\circ\text{C}$, while pTSL at pH 7.5 displayed $T_m = 39.6^\circ\text{C}$. Heating the pTSL in an acidic environment (pH 5) reduced the T_m further to 37.8°C. TSL at pH 7.5 showed minimal drug leakage at physiological temperature (4.3% at 37°C), while incorporation of copolymer resulted in an increase in leakage (13.9% at 37°C). Under acidic conditions (pH 5), drug leakage at physiological temperature was significantly increased (40.8%), demonstrating the formulation's pH sensitivity.

In order to assess the clinical potential of these liposomes, the thermal doses resulting from 5 min incubations in HEPES at the respective T_m for each liposome preparation (*i.e.* threshold temperature for 50% drug release) were calculated and compared (Figure 3). Traditional TSL exhibited a thermal dose of 5.44 min, which is expected to pose significant necrosis risks to healthy tissue *in vivo*. Incorporation of the copolymer into the lipid shell reduced the thermal dose to 0.045 min (2.73 sec), a 120-fold reduction. Under acidic conditions, thermal dose was further decreased to 0.004 min (0.23 sec), a greater than 1400 fold reduction when compared to traditional TSL. Differences across all groups were statistically significant ($p < 0.0001$ between TSL and pTSL, pH 7.5 and pTSL, pH 5; $p < 0.001$ between pTSL, pH 7.5 and pTSL, pH 5; Student's T-test).

To evaluate the timecourse of release, kinetics studies were conducted. The kinetics studies were conducted in HEPES at 40.6°C, a temperature slightly higher than the measured T_m of pTSL but less than that of TSL. As expected, TSL did not show appreciable release over 5 min of treatment (Figure 4). In contrast, pTSL at both pH 7.5 and pH 5 released > 70% of drug, with pTSL at pH 5 showing greater initial release than pTSL at pH 7.5.

To further assess the clinical potential of these formulations, DOX stability and release were evaluated in the presence of serum. While the addition of p(NIPAAm-co-PAA) in pTSL resulted in reductions in thermal dose in HEPES, it remained to be seen if the copolymer would affect the formulation's stability. To test this, pTSL were incubated at 37°C in the presence of 20% bovine serum and drug leakage monitored over time. Results were compared with drug leakage observed in traditional unmodified TSL and lysolipid-containing liposomes (LTSL). As shown in Figure 5, both TSL and pTSL at pH 7.5 were stable, releasing less than 7% of encapsulated drug following 90 minute incubations in serum. Leakage from pTSL at pH 7.5 was not statistically different from that of TSL at pH 7.5, providing evidence that the presence of the copolymer does not reduce the pTSL formulation's stability. Following 5 minute exposures in serum, leakage from LTSL at pH 7.5 was already significantly greater (18%) than leakage from TSL and pTSL at pH 7.5 ($p < 0.05$, Student's t-test). At higher incubation times, LTSL were highly unstable, with 81.4% of encapsulated DOX released following 30 min incubations in serum, a greater than 25 fold increase when compared to TSL and pTSL at pH 7.5 ($p < 0.0005$, Student's t-test). This is in agreement with other published findings which have also shown LTSL to be highly unstable under physiological conditions.⁶ Interestingly, release from pTSL at pH 5 increased with incubation time, with 17.1% of encapsulated drug being released at 5 min and 55% released at 90 min. Release from pTSL at pH 5 was significantly greater than release from pTSL at pH 7.5 for incubation times 30 min or greater ($p < 0.0005$, Student's t-test), demonstrating pH-sensitivity at 37°C and supporting release study data in HEPES.

Release properties in serum were then studied to assess whether the pH/temperature-sensitivity of pTSL observed in HEPES would be retained under physiological conditions. TSL at pH 7.5 and pTSL at pH 7.5, 6.5, and 5 were incubated in the presence of 20% bovine serum at 42°C and 43°C for 3 min, and resulting DOX release measured (Figure 5). At 42°C, 12.4% of encapsulated DOX was released from TSL following 3 min incubations. Addition of copolymer increased DOX release threefold, to 37.4%. Under increasingly acidic conditions, release was further increased to 51.4% and 63.0% at pH 6.5 and 5, respectively, a fivefold increase from unmodified TSL. At 43°C, DOX release from TSL increased to 44.9%, with increased release seen with incorporation of copolymer (59.1% and 64.1% at pH 7.5 and 6.5, respectively). Differences between all groups at 42°C were statistically significant ($p < 0.0001$, with the exception of pTSL at pH 6.5 and pTSL at pH 5, for which $p < 0.005$; Student's t-test). At 43°C, differences between pTSL groups and TSL groups were statistically significant ($p < 0.05$, Student's t-test), but not between pTSL at pH 7.5 and pTSL at 6.5.

Together, release studies in HEPES and in serum demonstrate that the addition of the pH/temperature-sensitive copolymer p(NIPAAm-co-PAA) to temperature-sensitive liposomes significantly reduces the thermal dose required to achieve sufficient release from these carriers. Results support the idea that copolymers containing NIPAAm are membrane-disruptive at elevated temperatures, and that incorporation into the TSL bilayer can lower the required threshold temperature for drug release. Furthermore, the effect of the copolymer on the threshold temperature was further enhanced at acidic conditions due to the pH-sensitivity of the monomer PAA. These findings demonstrate that copolymers with rationally designed pH and temperature-responsive properties can confer these properties to liposomes by incorporation within the lipid bilayer. The reduction in thermal dose associated with the pTSL formulation has the potential to significantly lower risk profiles *in vivo* when compared to standard TSL formulations, and tests to confirm this hypothesis are underway. Importantly, the pH/temperature-sensitivity of pTSL was retained in serum and the formulation proved stable at physiological conditions, highlighting the clinical potential of these carriers.

CONCLUSIONS

A novel thermosensitive liposome decorated with a pH/temperature-sensitive copolymer of NIPAAm and PAA was developed. Copolymers were synthesized via RAFT polymerization, yielding copolymers of narrow molecular weight distribution. When attached to liposomes, these copolymers displayed membrane-disruptive capabilities, resulting in release of encapsulated drug. Drug release in polymer-modified thermosensitive liposomes (pTSL) occurred at significantly lower temperatures when compared with traditional thermosensitive liposomes (TSL), resulting in a much lower thermal dose required for drug release. Drug release was also sensitive to pH, with pTSL at acidic pH demonstrating a lower thermal dose requirement when compared to pTSL at neutral pH. Importantly, the pH/temperature sensitivity and the ability of pTSL to reduce thermal dose requirements were retained in the presence of serum. The formulation also demonstrated stability in serum, with minimal drug leakage over time. These results suggest that: 1) pTSL can be used for temperature-triggered drug release with thermal dose requirements well under the accepted thresholds for the onset of tissue necrosis, and 2) these carriers can remain stable in circulation long enough to accumulate at the tumor, where their pH-sensitivity can then exploit the slightly acidic environment of the tumor interstitium.

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Figure 1. Illustration of DOX release from polymer-modified thermosensitive liposomes. Heating results in the collapse of polymer chains, which results in disruption of the bilayer and release of encapsulated drug.

Figure 2. DOX release in 20 mM HEPES as a function of temperature (5 min incubations), for NTSL (○, n=4), traditional TSL (●, n=4), pTSL at pH 7.5 (◆, n=4), and pTSL at pH 5 (■, n=4).

Figure 3.

Thermal doses (equivalent minutes at 43°C) required for 50% drug release for TSL (n=4), pTSL at pH 7.5 (n=4), and pTSL at pH 5 (n=4) in 20 mM HEPES. Differences across all groups were statistically significant (* $p < 0.001$, ** $p < 0.0001$, Student's T-test).

Figure 4. DOX release in 20 mM HEPES at 40.6°C as a function of time for traditional TSL ($\square$, $n=4$), pTSL at pH 7.5 ($\diamond$, $n=4$), and pTSL at pH 5 ($\blacksquare$, $n=3$).

Figure 5. Stability in the presence of 20% bovine serum at 37°C for traditional TSL at pH 7.5 (white bars, n=6), pTSL at pH 7.5 (light grey bars, n=6), pTSL at pH 5 (dark grey bars, n=7), and LTSL at pH 7.5 (etched bars, n=7). (* $p<0.05$, ** $p<0.0005$; Student's t-test).

Figure 6.

DOX release following 3 min incubations in 20% bovine serum at 42°C and 43°C (thermal dose of 45 sec and 3 min, respectively) for traditional TSL at pH 7.5 (white bars, n=7), pTSL at pH 7.5 (light grey bars, n=7), pTSL at pH 6.5 (dark grey bars, n=3), and pTSL at pH 5 (etched bar, n=3). (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$).

Scheme 1.

RAFT copolymerization of *N*-isopropylacrylamide (NIPAAm) and propylacrylic acid (PAA).

Table 1Poly(*N*-isopropylacrylamide-*co*-propylacrylic acid) characteristics

PAA amount (mol%) in feed	PAA amount (mol%) in polymer	[monomer] ₀ / [CTA] ₀ +2[AIBN] ₀	Molecular weight (<i>M_n</i>) (Da)	<i>M_w</i> / <i>M_n</i>
5.0	8.8 ^a	171.7	30,000 ^b	1.20

^a Estimated from ¹H NMR^b Determined by GPC in DMF containing 0.01 mol/L LiBr at 60°C using PMMA standards

Table 2

Liposome characteristics

Formulation	Diameter (nm)	Polydispersity	Encapsulation efficiency (%)	T_m^a (pH 7.5)	Thermal dose required (equivalent minutes at 43°C) ^b
NTSL	129.7 ± 8.7	0.005 ± 0.001	92.2 ± 8.3	N/A	N/A
pTSL	122.2 ± 1.1	0.098 ± 0.007	89.5 ± 9.0	39.8°C	0.0592
TSL	131.7 ± 13.3	0.063 ± 0.068	92.4 ± 5.2	43.1°C	4.257
LTSL	93.3 ± 9.0	0.151 ± 0.061	80.5 ± 30.6	N/A	N/A

^a T_m defined as the temperature at which 50% drug release was observed following 5 min incubations in 20 mM HEPES.

^bThermal dose in HEPES, calculated according to (1) with $T = T_m$ and $t = 5$ min. Note that DOX release from LTSL was not evaluated in HEPES in this study.