



A high stable pH-temperature dual-sensitive liposome for tuning anticancer drug release

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ABSTRACT

In order to improve the targeting and availability of liposomes to cancer cells, the temperature sensitivity of 1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and the pH sensitivity of PASP in PASP-g-C8 are incorporated in a drug delivery system. A composite pH-temperature dual-sensitive liposomes (CPTLPs) was obtained as an efficient drug delivery system. The bionic bilayer is self-assembled by cholesterol/cationic temperature-sensitive lipids as base layer and pH-sensitive octylamine grafted poly aspartic acid (PASP-g-C8) as anchors coated outside. Cytarabine (CYT) was chosen as a model drug. SEM and DLS were used to observe the morphology characteristics of CPTLPs in different micro environment. The results demonstrated that the CPTLPs remained active in both normal (pH7.4 and 37 °C) and tumor tissues (pH 5.0 and 42 °C). As a stable colloidal system, the zeta potential of CPTSLs was -41.6 mV. In vitro drug-release experiments, the CTY encapsulated dual-sensitive liposomes, CPTSLs(+), not only have significant pH-temperature sensitivity but have more prolonged release in vitro than control groups. MTT tests results indicated that the cell apoptotic effects induced by CPTSLs(+) were nearly 30% higher than the naked drug CTY in HepG2 cells, and 20% lower apoptotic in vero cells. The CPTSLs(+) sustained a stable emulsion form, less toxic effects on normal cells, and exhibited a good pH-temperature sensitivity, thus expected to be a promising tumor targeting drug delivery.

1. Introduction

Cancer has posed a grave threat to human health in the past 20 years [1]. As the mainstay in the treatment of various cancers, chemotherapy plays a vital role, but still faces many challenges, such as side effect, high elimination rate in the blood circulation and the requirement for frequent administration to maintain effectiveness [2]. In order to solve these problems, a myriad of nano-drug delivery systems including controlled release polymers [3], polymeric micelles [4–6] and dendrimers [7–9] have been developed [10]. These systems can enhance the targeting efficiency toward tumor tissues, reduces the undesired toxicity to healthy tissues, elongates the drug circulation lifetime in the bloodstream [11], and achieve drug positioning, timing, and targeted release [12].

Despite the aforementioned have significant advances in cancer treatment, one drawback that still exist in these nano-drug delivery systems is their relatively low stability. A low stability may cause the dissociation of the self-assembling drug carriers as well as undesired drug leaching and toxicity. Liposomes are hypotoxicity colloidal systems consisting of a cell membrane-like phospholipid bilayer shell with

an aqueous internal core [13,14]. These systems are widely explored in pharmaceutical research to reduce the toxicity of the drug candidates [15] and enable effective drug delivery of lipophilic, hydrophilic and amphiphilic drugs [16]. It is found that liposomes can target the reticulo-endothelial system (RES) and enhance the in vivo stability of drugs [17]. Conventional liposomes are not popular in drug carriers as a result of their poor efficiency of targeting tumor cells, relatively low stability. In order to solve these shortcomings, most research into liposomes focuses on the modification of the composition of the liposome bilayer and surface chemistry [18]. These strategies include passive, long-circulating PEGylated liposomes [19–21] ligand targeted liposomes [22–24] and stimuli-responsive liposomes [25,26].

Introducing some pH-sensitive polymer into the liposome to form pH-sensitive liposome is promising to obtain a high-target drug carrier [27]. Compound thermo-sensitive liposomes [28] were prepared by some thermal lipid material such as DMPC, DPPC and DSPC, their phase transition temperature is 24 °C, 42 °C and 55 °C respectively. They release the anticancer drug by changing the liposome membrane permeability when the lesion was local heated. Generally speaking, the above single-sensitive liposomes have both great targeting and

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availability on cancer cells. However, in comparison with single-sensitive liposomes, there are some obvious advantages of multiple corresponding including the simple and feasible preparation process, the effective and versatile properties [29], and sensitivities respond on cancer cells. As a drug carrier system, it would be preferable if liposomes are both responsive to different factors such as pH, and temperature. It has been reported that a series of dual-sensitive liposomes were synthesized. Ta et al. [30] synthesized copolymer poly (N-NI-PAAm-PAA) with temperature-sensitive N-isopropyl acrylamide (NI-PAAm) and pH-sensitive polyacrylic acid (PAA), then modified it to the liposome surface for the liposome with pH/temperature sensitivity. Guo and Kim [31] designed a liposome suspended in gold nanoparticles and temperature-sensitive polymer mixture solution. These dual-sensitive liposomes exhibited sensitivity to temperature and pH variations, but the main shortcomings of these dual-sensitive liposomes as tumor targeting drug carriers are their complex and tedious preparation process while still lack desired biological stability.

In a previous article, pH sensitive liposomes (OPLPs) with octylamine-graft-poly aspartic acid (PASP-g-C₈) coated outside were prepared [32]. The OPLPs not only enhanced the stability of the drug carrier, but also had a great response to acidic pH. Based on the OPLPs, a desired less toxic composite temperature and pH dual-sensitive liposomes has been further investigated in this work. The aim of this study was to enhance the drug carrier targeting by adding the temperature sensitivity, and reduce toxicity as well as cost of the OPLPs concurrently on the basis of keeping both its pH sensitivity and biological stability. To realize the goals, cholesterol and cationic temperature-sensitive lipids (DPPC, DC-Chol) were chosen to prepare the bilayer, and PASP-g-C₈ was coated outside to prepare a composite pH-temperature dual-sensitive liposomes (CPTSLs) (Scheme 1). The molecular structure of DPPC, DC-Chol and cholesterol are shown in Scheme S1. CPTSLs were then tested for stability, pH-temperature sensitivity, toxicity to normal cells and targeting of cancer cells. Meanwhile, pH-temperature dual-sensitive liposomes (PTSLs) were also obtained with cationic temperature-sensitive lipids (DPPC, DC-Chol) as controls. Transmission electron microscopy (TEM), dynamic light scattering (DLS) were applied to observe the change of the morphology and particle size of CPTSLs. Moreover, the anti-tumor drug CYT was chosen as the model drug. The pH and temperature sensitivity and in vitro cytotoxicity of CPLPs were assessed.

2. Experiment

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Cordent Pharma Switzerland LLC. (Switzerland). 3β [N- (N',

N'-Dimethyl aminoethane)-carbamoyl] cholesterol (DC-Chol) was purchased from Absin Bioscience Inc. (China). Cholesterol was purchased from Beijing Aoboxing Biotechnology Co. Ltd. (China). Polysuccinimide (PSI) was purchased from Luoyang Mining Run Environmental Protection Material Co Ltd. (China). Octylamine was purchased from Beijing Kehua Jingwei Tech. Co Ltd. (China). Cytarabine (CYT) was purchased from Shanghai TCI Chemical Industrial Development Co Ltd. (China). All other chemicals were of analytical grade and were commercially obtained.

2.2. Preparation of CPTSLs and PTSLs

30 mg of DPPC, 6 mg of DC-Chol and 1.5 mg of cholesterol were dissolved in 3.5 mL CH₃Cl, and then 1 mL of 12 mg/mL model drug CYT was added. The mixture was ultrasound for 2 min to form W/O emulsion. Afterwards, 12 mL PASP-g-C₈ aqueous solution in the required concentration was added to form a W/O/W emulsion. The optimization for the preparation process of pH-temperature sensitive liposomes please see the supplementary information. Finally, CH₃Cl was removed by vacuum rotary evaporation at 30 °C for 1 h and W/O/W emulsion was gained. The preparation of the PTSL was the same as described above CPTSL only with 30 mg DPPC, 7.5 mg DC-Chol without cholesterol.

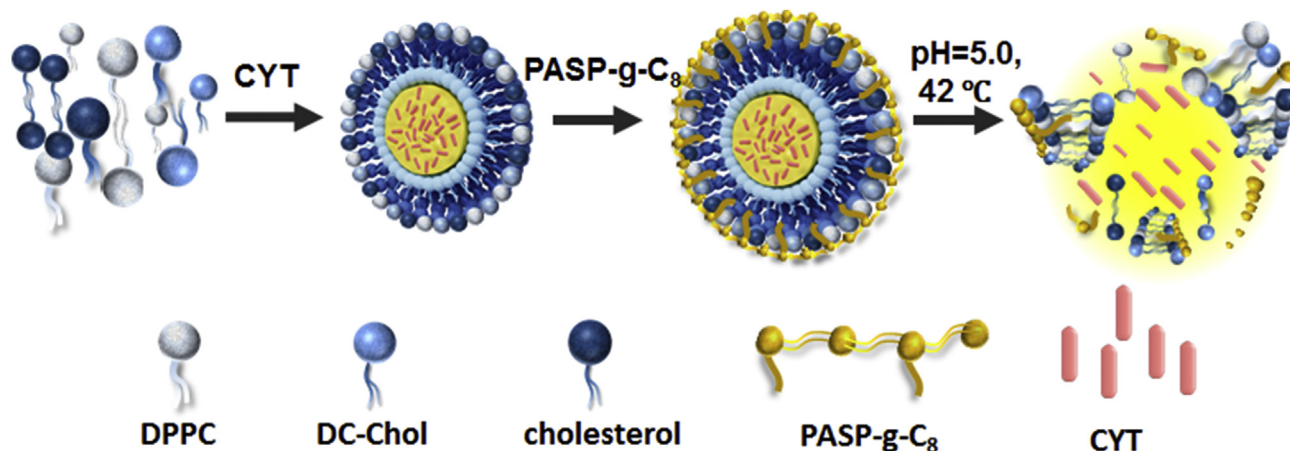
2.3. Characterization of CPTSLs and PTSLs

Particle size, polydispersity index and zeta potential of synthetic materials were determined with dynamic light scattering (DLS) (Mastersizer 2000, UK) and the intensity of the He-Ne laser light (658 nm) scattered by the samples was detected at an angle of 90°. The mean diameter of the particles was calculated using the Stokes-Einstein equation. The morphology of PTSLs and CPTSLs was observed by transmission electron microscopy (TEM) (Tecnai G2 FEI, Netherlands). The samples were dripped onto copper grids with films, and observed by TEM after drying.

The liposomes encapsulated CYT were diluted 25x, and their absorbance values of were measured at 280 nm by using microplate reader (Thermo absystems). The encapsulation efficiency (EE) was obtained from the absorbance values at 280 nm according to Eq. (1).

$$EE \% = \frac{C_e}{C_e + C_f} * 100\% \quad \text{Eq. (1)}$$

where C_e and C_f are the concentration of CYT kept inside or outside of liposomes, respectively.



Scheme 1. Schematic of PASP-g-C₈ modified composite pH- temperature sensitive liposomes.

2.4. The pH - temperature sensitivity test of the CPTSLs and PTSLs

A reverse dialysis method was used to detect the *in vitro* release of PTSLs and CPTSLs. The release medium with 200 mL PBS at pH 5.0 and pH 7.4 was added to two beakers separately. The corresponding PBS (10 mL) was injected into a dialysis bag (MWCO 3.5 kDa). Then prepared PTSLs or CPTSLs was added into beakers outside the dialysis bag. These beakers were placed into water at a temperature of 37 °C and 42 °C respectively. Two mL of the inside fluid was removed from dialysis bags every 1 h. The absorbance was measured by microplate spectrophotometer at 280 nm and the cumulative release rates at every predetermined time interval were calculated. The material characteristics were monitored after incubation for 15 min to observe the changes of PTSLs and CPTSLs after exposing to different pH solution and temperature. The changes of the particle size and the morphology of PTSLs and CPTSLs were correspondingly investigated with a light-scattering spectrophotometer (Mastersizer 2000, UK) and transmission electron microscopy (TEM) (Tecnai Spirit Bio Twin 120 kV, FEI, USA).

2.5. *In vitro* cell culture and drug treatments

HepG2 and L02 cells were cultured with Dulbecco's modified Eagle's medium (DMEM) and 1640 medium supplemented 10% FBS, respectively. Cultures were maintained in 96-well plates at a density of 1×10^6 cells/well at 37 °C in 7% CO₂ atmosphere. After 12 h, the medium was removed and replaced with fresh medium mixed with prepared PTSLs(+) and CPTSLs(+). The Concentration (20, 10, 5, 2.5 μM), incubation time (24 h, 48 h) and pretreatment temperature (42 °C, 37 °C) of PTSLs(+) and CPTSLs(+) were explored to investigate the CYT to HepG2 and L02, and the cell activity was detected by MTT method.

2.6. Liposome-cell interaction

HepG2 and L02 cells were cultured in an 8-well slide at a density of 2.5×10^4 cells per well and incubated at 37 °C in 7% CO₂ atmosphere. After 12 h, the medium was removed and the cells were treated with fresh medium mixed with prepared 20 μM rhodamine B isothiocyanate modified CPTSLs(+). After 4 h, medium mixed rhodamine B modified-CPTSLs(+) were removed and washing steps were performed. The slide was transferred to the by the qualitative confocal laser scanning microscopy (Carl Zeiss, Germany) immediately and images were recorded at 42 °C.

3. Results and discussion

3.1. Characterization of the PTSLs and CPTSLs

Pathological tissues, such as inflammatory or solid tumor tissues, are characterized by increased vascular permeability [33,34]. Therefore, the size and surface charge of liposomes are important aspects to ensure the adequacy of liposomes as a carrier system for drugs [35]. The morphology of PTSLs and CPTSLs were shown in Fig. 1. The samples were spherical, well distributed, without fragmentation or aggregation indicating that both PTSLs and CPTSLs were stable as a complete sphere *in vitro*. The zeta potential not only reflects the charged condition of the colloidal dispersion, but also represents the particle dispersion degree. It is generally acknowledged that when the absolute value of zeta potential exceeds 30 mV, repulsion will be the main acting force between particles, and thus they will not easily coagulate [36]. The average zeta potentials of PTSLs and CPTSLs were -41.6 mV and -89.0 mV, respectively. It shows that the obtained nanomaterials were both a stable colloidal system during blood circulation. and the CPTSLs shows more outstanding stability than the PTSLs. As shown in Table 1, the average individual particle sizes of the PTSLs were 124.1 nm (PDI = 0.210), while the CPTSLs were 90.0 nm

(PDI = 0.210), which demonstrated that the PTSLs and CPTSLs are so small for the particles to accumulate in tumor tissue selectively. The drug encapsulation efficiency of the liposomes prepared under neutral conditions was $71.77 \pm 3.1\%$, $70.91 \pm 1.5\%$ and drug loading was $7.1 \pm 0.2\%$, $5.8 \pm 0.1\%$ for PTSLs and CPTSLs respectively. The results showed that liposomes prepared under neutral conditions exhibited high drug encapsulation efficiency (> 70%) and satisfied drug loading, which indicated that the addition of lower concentration of DC-Chol than that of PTSLs did not affect the properties of CPTSLs.

We restricted the size of liposomes to about 100 nm enhances extravasation from tumor blood microvessels as well as interstitial transport, leading to the accumulation and retention of liposomes in solid tumors [37,38]. The size of the prepared nanoparticles may importantly influence their fates after administration [39]. DLS measurements further demonstrated that the two prepared nanoparticles had a narrow-ranged size distribution (Fig. 1a, b, while the size of CPTSLs were smaller (Table 1) than that of PTSLs, indicating that CPTSLs were more stable and much more targetable.

3.2. The pH-temperature sensitivity contrast of the PTSLs and CPTSLs

The release curve of CYT from PTSLs and CPTSLs in (Fig. 2a and b) showed that both the PTSLs and CPTSLs had significant pH-temperature sensitivity. In the whole process of drug release, the percentage of total release rate always There is a marked change under certain environment. The CYT release rate increased to 100% in 42 °C, pH 5.0 microenvironment. However, the CYT release rate was only 60% in the environment of 37 °C, pH 7.4, both PTSLs and CPTSLs have great pH - temperature sensitivity. What's more, comparing the release rate of CYT from PTSLs and CPTSLs (Fig. 2c,d), it is necessary for 6 h of CPTSLs to achieve the 100% CYT release rate. The PTSLs only need 3 h to achieve 92% release rate in 42 °C, pH 5.0 environment. When drug was loaded in PTSLs, it could be quickly released in this environment. The result demonstrated that CPTSLs were more stable and more conducive to drug release for a long time in the blood circulation.

Furthermore, the changes of morphology and structure of PTSLs and CPTSLs with pH and temperature were studied, which was further proofed the above results. The particle size and its distribution of the PTSLs and CPTSLs tended to increase with rising temperature and lowering pH (Fig. 3). This suggested that PTSLs and CPTSLs became more unstable under 42 °C and pH 5.0. The results accorded with the *in vitro* drug-release curve (Fig. 2). When comparing the particle size change of PTSLs and CPTSLs under different pH and temperature, we found that the particle size changes of PTSLs were more obviously compared to CPTSLs, which showed that CPTSLs were relatively stable and more favorable for long time *in vitro* release.

The morphological shape in different pH values and temperature for 15min was observed by TEM (Fig. 4). At pH 7.4, 37 °C, the PTSLs and CPTSLs both exhibited complete vesicle forms, maintain good round shape, and the modifier of PASP-g-C8 was coated on the surface of liposome outer layer (Fig. 4a1, b1), while the liposomes had a tendency to melt clearly, they though still maintain nearly round shape (Fig. 4a2, b2), when the pH was reduced to 5.0 and the temperature was maintained at 37 °C; PTSLs and CPTSLs can maintain the spherical conformation when the temperature was increased to 42 °C and pH was maintained at 7.4, their volume increased (Fig. 4a3, b3). At 42 °C and pH 5.0, the sizes of PTSLs and CPTSLs increased even more, and liposome started cracking and fragments of liposomes can be observed from (Fig. 4a4). By contrast, when temperature and pH both changed, the CPTSLs were able to maintain a better shape (Fig. 4b4) than PTSLs. The results indicated that CPTSLs are more stable compared to PTSLs.

We considered that there are four mechanisms could explain this phenomenon; (1) The protonated PASP-g-C₈ exhibited a relative low hydration capability at neutral pH, whereas the protonated amino group of DPPC and DC-Chol showed a higher hydration capability, thus increasing the immiscibility between DPPC, DC-Chol and PASP-g-C₈

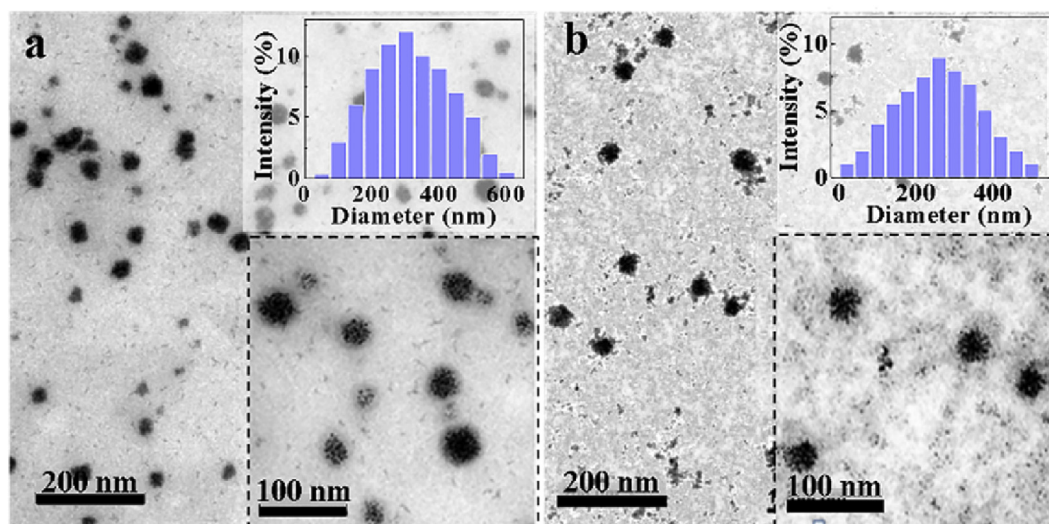


Fig. 1. Characterization of CYT-loaded formulations. The TEM images and size distributions (inside) of PTSL (a) and CPTSL (b) the two liposomes are presented. The scale bar in TEM images represents 500 nm.

Table 1

Mean size, zeta potential and EE of each kind of modified liposome.

Sample	Size (nm)	PDI	zeta potential (mV)	EE (%)
PTSL	124.1 ± 12.6	0.210	-41.6 ± 4.1	71.77 ± 3.1
CPTSL	90.9 ± 8.1	0.247	-89.0 ± 7.6	70.91 ± 1.5

Data are presented as mean ± SD (n = 3). PDI: Polydispersity index.

and resulting in the formation of a heterogeneous system. (2) When the pH decreased, the complementary lipid (DC-chol for example) and the amino and phosphate groups of DPPC could be protonated, thus weakening the interaction between DC-chol and DPPC and eliminating

the stabilizing effect of the complementary lipid. (3) When the temperature increased, the dehydration of the head group of DPPC will change its geometric shape; (4) When the pH and temperature changed together, a cooperative effect of protonation of carboxyl groups and dehydration of diethylene glycol chains on the copolymer chains can generate the pH and temperature-dependent content release from the liposomes., the When compared with the component of CPTSLs, PTSLs carried more positively charged DC-chol as well as more negatively charged PASP-g-C₈. Therefore, PTSLs were more likely to collapse when the conformation of PASP-g-C₈ changed.

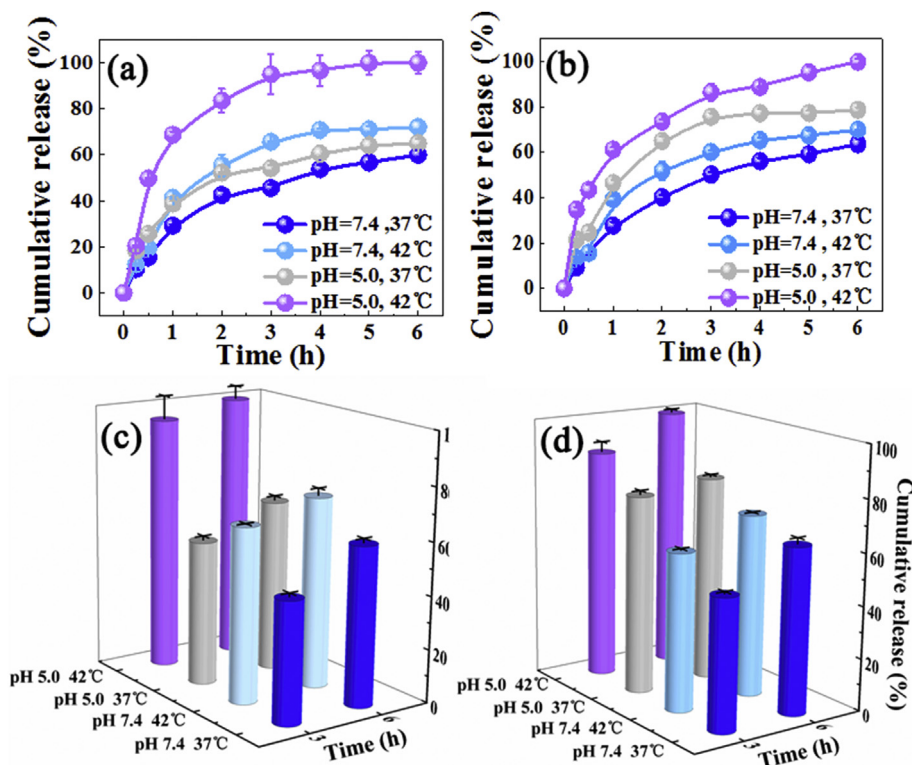


Fig. 2. Double-controllable release curve of CYT from the prepared PTSLs (a) and CPTSLs (b), the release column chart in 3 h and 6 h from PTSLs(c) and CPTSLs(d).

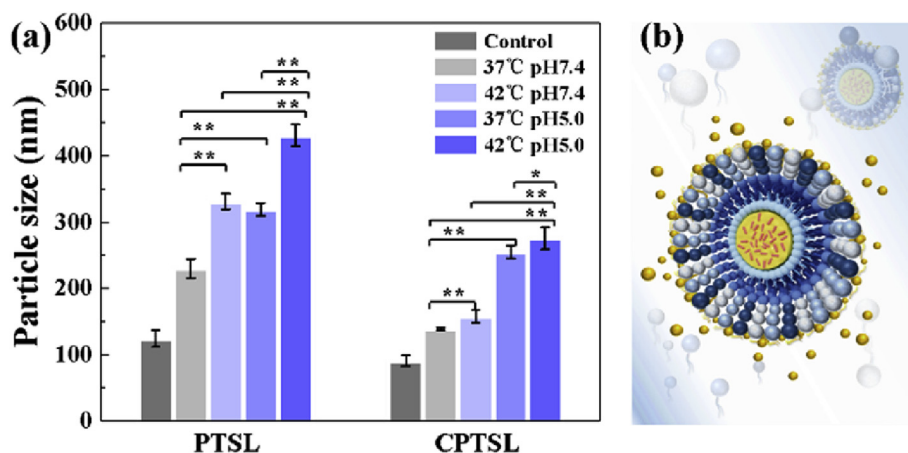


Fig. 3. Average particle size of two different modified liposomes after different pH treatments. Mean values \pm SD followed by same letter are not significantly different (* $p < 0.05$, ** $p < 0.01$).

3.3. In vitro cytotoxicity studies

3.3.1. PTSLs and CPTSLs inhibitory effect on tumor cells

The half maximal inhibitory concentration value (IC_{50}) for CYT varied from 37 ± 6.1 nM in HepG2 to 72 ± 9.1 nM in L02 [40,41]. Based on the date of IC_{50} and drug loading rate of these two liposomes (about 7%), the naked CYT group was prepared by mixed of DPPC, DC-Chol and CYT, and the weight percentage of CYT is 7%. Inhibitory effects of PTSLs and CPTSLs at different drug concentrations on human hepatoma cell in vitro were shown in Fig. 5a. PTSLs and CPTSLs loaded CYT (PTSLs(+)) and CPTSLs(+) shown stronger inhibition to tumor cells than the naked CYT, which may benefit from the changes in the cell membrane permeability of tumor cells. The tumor-suppressing effect of PTSLs(+) and CPTSLs(+) increased may because both PTSLs(+) and CPTSLs(+) have a size distribution of 100 nm, and these liposomes particles were easily penetrate in tumor cells. Tumor cell viability of CPTSLs(+) decreased from 60% to 30% with the increase of drug concentration, indicating that the increase in drug concentration is conducive to tumor inhibition. The survival ratio of CPTSLs(+) at a concentration of $10 \mu\text{M}$ was almost twice than that of the naked CYT. Furthermore, the survival rate of tumor cells after administrating CPTSLs(+) was lower than PTSLs(+) showing that the inhibitory effect of CPTSLs(+) on tumor cells was better than PTSLs(+).

Fig. 5b shows the inhibitory effect of PTSLs and CPTSLs on HepG2 at different culture time in vitro. With the extension of incubation time from 24 h to 48 h, PTSLs(+) and CPTSLs(+) inhibitory effect on the

HepG2 increased, while the inhibitory effect of CYT was still around 80%. PTSLs(+) and CPTSLs(+) not only inhibit on tumor cells but have desirable sustained releasing effect, and thus help constant drug release in tumor cells. The survival rate of tumor cells after administrated CPTSLs(+) was lower than PTSLs(+), and the inhibitory effect of CPTSLs(+) on tumor cell was more significant when culture time increased twofold. At the same time, CPTSLs without CYT (CPTSLs(-)) have no inhibitory effect on tumor cells, no matter how long incubation time, and tumor cell survival rates are close to 100% indicating CPTSLs(-) have no effect on tumor cells, while, tumor cell survival rates of PTSLs(-) decreased with the culture time. The results above confirmed that the inhibitory effect of CPTSLs(+) on tumor cells was better than PTSLs(+).

The effect of different temperature pretreatment on tumor cells was shown in Fig. 5c. As shown in Fig. S1, the temperature-tumor survival rate was 94% and 92% after 37 °C and 42 °C pre-treatment, and these date for L02 cell was 87% and 85%, respectively. The results shown that the inhibition of tumor cells by the temperature itself was very low. After PTSLs(+) dosing, the tumor cell survival rate after 42 °C pre-treatment was 55% lower than 37 °C (the 37 °C pre-treatment was 70%) and the tumor cells survival rate after administrating CPTSLs(+) was 50% and 70% after 42 °C and 37 °C pre-treatment, respectively. In addition, the tumor cells survival rate after naked CYT dosing was 80%, no matter of the 42 °C or 37 °C pre-treatment. The 42 °C pre-treatment did not harm the cell itself, but promoted PTSLs(+) and CPTSLs(+) drug release, enhanced the anti-tumor effects. Comparing the tumor

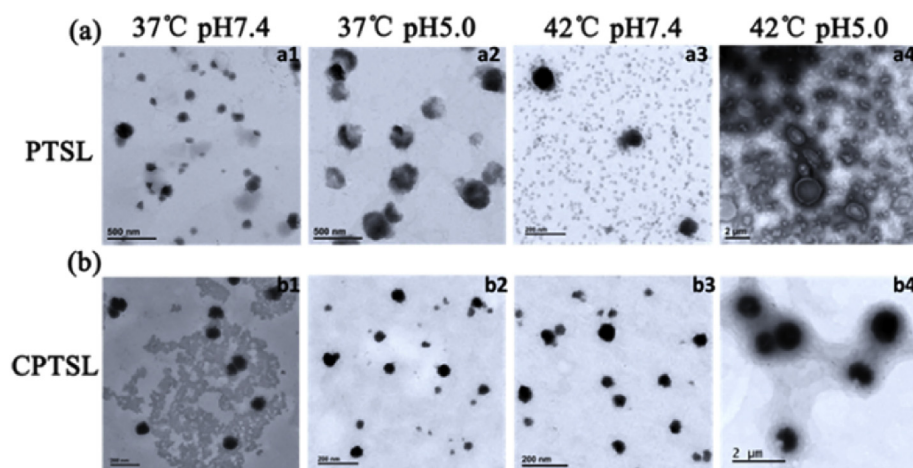


Fig. 4. TEM images of PTSL (a) and CPTSL (b) in the presence of pH 7.4, pH 5.0 and 37 °C, 42 °C. Scale bars are marked on each picture.

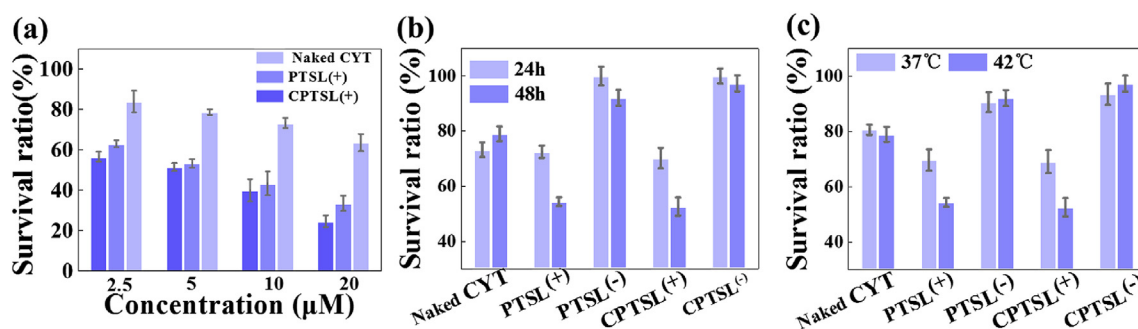


Fig. 5. In vitro tumor inhibition studies for cell viability of HepG2 treated with two different pH-temperature sensitive liposomes: (a) concentration-tumor inhibition (All the groups were treated at 42 °C for 24 h, the added concentration of liposomes is 2.5, 5, 10, 20 μM, respectively); (b) time-tumor inhibition (All the groups were treated with 10 μM liposomes at 42 °C for 24 h or 48 h); (c) pre-treatment of temperature-tumor inhibition for 48 h (All the groups were treated with 10 μM liposomes for 48 h). the naked CYT group was prepared by mixed of DPPC, DC-Chol and CYT, and the weight percentage of CYT is 7%.

cells survival rate after blank PTSLs(–) and CPTSLs(–) on tumor cells, the inhibition of tumor cells by the carrier itself was very low, especially the tumor cell viability after administrating CPTSLs(–) is close to 100%, proving once again that both drug carriers had almost no effect on tumor cells.

3.3.2. PTSLs and CPTSLs toxic effects on normal cells

In order to prove the safety of targeted formulations, separate complete toxicity studies to normal cells were displayed in Fig. 6. As expected, the result (Fig. 6a) shown with the decrease of CYT concentration, the L02 cells toxicity after naked CYT, PTSLs(+) and CPTSLs(+) separately treated L02 cell were also dropped and at the same time, causing by PTSLs(+) and CPTSLs(+) were lower than naked CYT. When the CYT concentration was 5 μM, normal L02 cell survival rate of PTSLs(+) and CPTSLs(+) was higher 10% than naked CYT. Apparently, the normal cells survival rate of PTSLs(+) and CPTSLs(+) was higher than naked CYT which illustrated that the targeted formulations reduced the toxicity to normal cells and the trend is more obvious at a lower CYT concentration. What's more, the normal cells toxicity of CPTSLs(+) were lower than PTSLs(+) the reason is that the addition of cholesterol reduced the content of DC-Chol in liposomes, and thus reduced the toxicity of cationic liposome.

Fig. 6b shows that with the extension of incubation time from 24 h to 48 h, the L02 cell toxicity of naked CYT increased by nearly 5% and there were no apparent increases for PTSLs(+) and CPTSLs(+). Comparing PTSLs(–), L02 cell survival rates administrated CPTSLs(–) are close to 100% and there was no change with the extension of time. The results proved that PTSLs(+) and CPTSLs(+) possess relatively low cytotoxicity, which can be ignored.

Toxic effects by pre-treatment temperature study was shown in Fig. 6 c, as the mortality of L02 cell being induced by naked CYT maintained at 80% without any obvious change after 1 h pretreatment

in 37 °C and 42 °C respectively. The pretreatment have no any side effect on L02 cell. When comparing the pretreatment temperature, it was found the L02 cell survival rate of pre-treatment in 42 °C slightly less than 37 °C, but this slight fluctuation did not cause significant toxicity on L02 and it can be ignored. In 42 °C, the cell survival rates of CPTSLs(+) was higher than that of PTSLs(+) indicated that CPTSLs(+) were more stable than PTSLs(+).

3.3.3. Liposome-cell interaction

As described above, MD-MAA50-LT-modified liposomes exhibited dual-signal-responsive properties in which the liposomes enhanced pyranine release under mildly acidic pH and elevated temperature To further illustrate the mechanism of normal and tumor cells circumvented by CPTSLs(+), the distribution of CYT after treatment with Lipo-s and Lipo-c were determined, respectively. The uptake of rhodamine B isothiocyanate embedded CPTSLs(+) in normal somatic cells L02 (Fig. 7 a) and tumor cells HepG2 (Fig. 7 b) were investigated by the qualitative confocal laser scanning microscopy (Carl Zeiss, Germany). The images bright field images were shown in Fig. S2. The results indicated that the release amount of rhodamine B isothiocyanate in HepG2 was much higher than that in L02 after treatment cells for 4 h. Hence, as shows in Fig. 7 c, the more acidic and higher temperature conditions in the cancer cells were beneficial for the release of CYT, thereby effectively circumvent the normal cells.

4. Conclusions

A novel pH-temperature sensitive drug carrier was prepared by combined cholesterol, cationic thermo-sensitive lipids in the bilayer with pH-sensitive PASP-g-C₈ coated outside so that the cost and cytotoxicity is low than that of PTSLs. The morphologies of PTSLs (+) and CPTSLs (+) were spherical, well distributed, without phospholipid

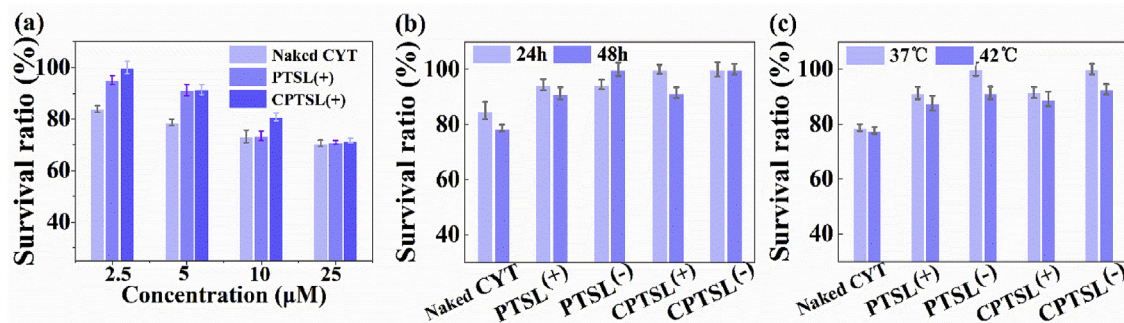


Fig. 6. In vitro cytotoxicity studies for cell viability of L02 treated with two different pH- temperature sensitive liposomes: (a) concentration-cytotoxicity (All the groups were treated at 42 °C for 24 h, the added concentration of liposomes is 2.5, 5, 10, 20 μM, respectively); (b) time-cytotoxicity (All the groups were treated with 10 μM liposomes at 42 °C for 24 h or 48 h); (c) pre-treatment of temperature-cytotoxicity. (All the groups were treated with 10 μM liposomes for 48 h).

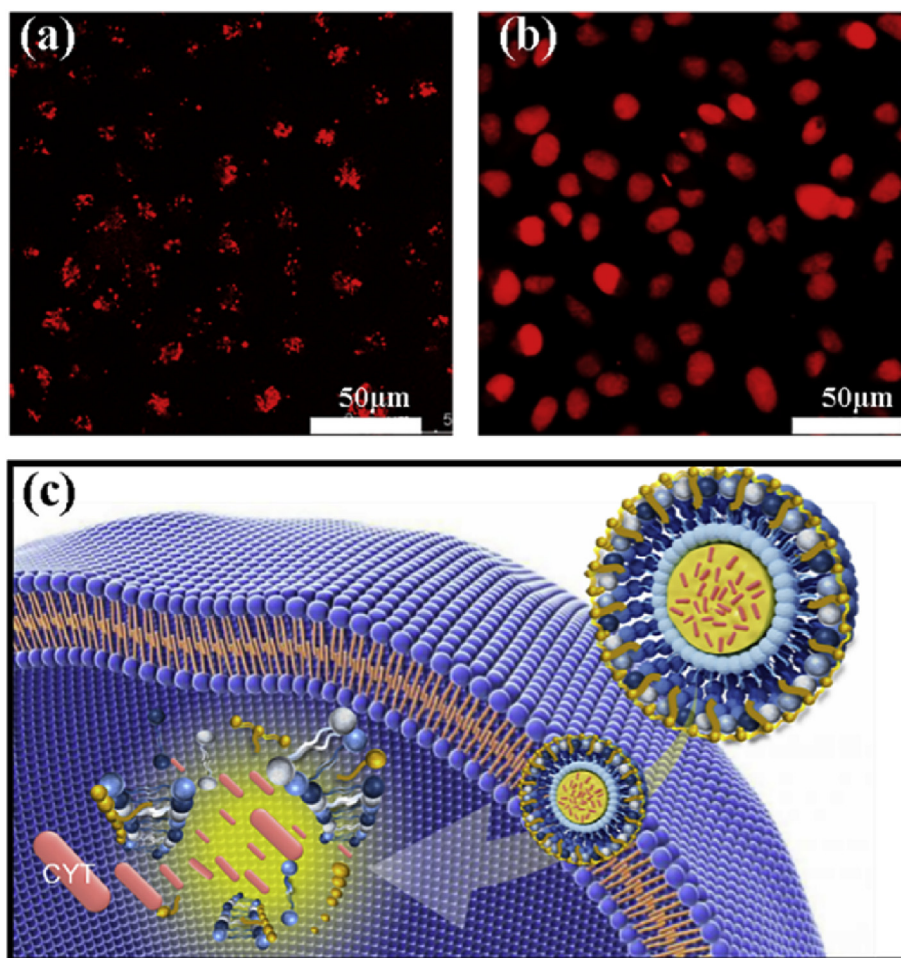


Fig. 7. The related mechanism in L02 and HepG2 cells by CPTSLs (+) in 42 °C pretreatment. (a) Live-cell confocal images obtained after treating L02 cells for 4 h. The L02 cells showed a higher pH than HepG2 cells. (b) Confocal images obtained after treating HepG2 cells for 4 h. (c) Schematic illustration of intracellular release of the novel dual-sensitive liposomes due to the pH/temperature-responsive way.

debris. The average particle size of the CPTSLs (+) and PTSLs (+) were 90.9 ± 8.1 nm and 124.1 ± 12.6 nm, and the drug encapsulation efficiency under neutral conditions was $71.77 \pm 3.1\%$, $70.91 \pm 1.5\%$, separately. The results demonstrated that the CPTSLs (+) was a more stable colloidal system than that of PTSLs (+), enhanced the enrichment of drug to the tumor area and was longer preserved in vitro. Compared with PTSLs, bio-stability tests show that, the particle sizes of CPTSL had smaller changes after 15 min of water bath at pH 7.4 and pH 5.0 at the temperature 37 °C and 42 °C respectively. CPTSLs (+) were more stable and more effective to drug release for a long time in vitro than PTSLs (+). MTT test results demonstrated that the mortality rate induced by CPTSLs (+) of tumor cell was 30% higher than naked CYT. The survival rate on normal cell L02 was almost to 100% also showing that CPTSLs (+) were safe and without toxicity to normal cells. The preparation of functional liposomes appears to be the promising way to obtain multi-sensitive drug delivery system. Further research is required to improve targeting and anti-cancer effects of the drug targeting carrier.

Declaration of competing interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.synbio.2020.05.004>.

Abbreviations

DMPC	1, 2-dimyristoyl-sn-glycero-3-phosphocholine
DSPC	1, 2-distearoyl-sn-glycero-3-phosphocholine
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DC-Chol	3β [N-(N', N'-Dimethyl aminoethane)-carbamoyl]cholesterol
PASP-g-C ₈	octylamine-graft-poly aspartic acid
CYT	Cytarabine
CPTSLs	composite pH-temperature dual-sensitive liposomes
PTSLs	pH-temperature dual-sensitive liposomes
PTSLs (+)	PTSLs loaded CYT; CPTSLs (+), CPTSLs loaded CYT

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