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Preparation and *in vitro* evaluation of an acidic environment-responsive liposome for paclitaxel tumor targeting

Lianqin Wang *

Qilu University of Technology, No. 3501, Daxue Road, Jinan 250353, China

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ABSTRACT

Paclitaxel (PTX) is an important cancer chemotherapeutic drug. To ameliorate the disadvantages of paclitaxel, this study designed liposomes to load paclitaxel, adding the acid-sensitive material cholesteryl hemisuccinate (CHEMS) to increase the accumulation of the drug in the tumor site. To begin, we used a high-performance liquid chromatography (HPLC) method to determine the content of PTX and the encapsulation efficiency. Then, we prepared paclitaxel-loaded acid-sensitive liposomes (PTX ASLs) by a thin-film dispersion method. We investigated the physical and chemical properties of the liposomes. The particle size was 210.8 nm, the polydispersity index (PDI) was 0.182 and the ζ-potential was –31.2 mV. The liposome shape was observed by transmission electron microscopy (TEM), and the results showed that the liposomes were round with a homogenous size distribution. The release characteristics of the liposomes *in vitro* were studied via a dynamic dialysis method. The results showed that the prepared liposomes had acid sensitivity and sustained release properties. An *in vitro* cellular uptake assay of MCF-7 cells showed that the cell uptake of coumarin-6-loaded acid-sensitive liposomes was significantly higher than that of free coumarin-6. The cytotoxicity of the PTX ASLs was significantly higher than that of paclitaxel. In conclusion, these results showed that the prepared liposomes had clear acid-sensitive release characteristics and a higher cell uptake rate and cytotoxicity than free PTX. The system is very suitable for targeted cancer therapy with paclitaxel.

1. Introduction

As a serious disease that threatens human health, cancer has gained wide attention in the field of medicine. Due to increased environmental pollution, population aging and other issues, the morbidity and mortality of cancer are also increasing. According to the World Health Organization, the total number of cancer patients will reach 75 million by the 2030s [1,2]. For the treatment of cancer, chemotherapy still plays an important

* Qilu University of Technology, No. 3501, Daxue Road, Jinan 250353, China. Tel.: +86 531 89631120.
E-mail address: qluwlq@163.com.
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role. Paclitaxel is a natural product extracted from taxus, with broad-spectrum anti-tumor effects [3]. Paclitaxel causes an anti-tumor effect mainly through the promotion of cell tubulin polymerization and inhibition of its depolymerization [4]. However, the solubility of paclitaxel is poor; it can only be dissolved up to 20 mg in 1 L of water [5]. Additionally, the low cell selectivity of paclitaxel can cause toxicity in normal cells. Therefore, it is necessary to study new paclitaxel carriers to ameliorate the above shortcomings.

Liposomes are preparations composed of lipid bilayers with a liquid core that can thus encapsulate both hydrophobic and hydrophilic drugs [6]. As a drug carrier, liposomes can deliver various drugs such as anticancer drugs, antibacterial agents, and peptide hormones, among others [7]. Liposomes have the following characteristics: the composition of the material is mainly phospholipids and cholesterol, which have high affinity due to their similarity to biological membranes. When drugs are encapsulated in liposomes, the liposomes can reduce kidney excretion and extend drug action time. Studies have shown that some drug-loaded liposomes can stay in the circulation up to several days [8]. Furthermore, the entrapped drug is more stable under the protection of the lipid bilayers. Liposomes are mainly phagocytically uptaken by macrophages, but macrophages accumulate less in the heart and kidney, so this can reduce drug toxicity in the heart and kidney [9]. In addition, some studies have demonstrated that liposomes applied to traditional Chinese medicine also achieved good results [10].

In recent years, acid environment-responsive liposomes have become a research focus for medical and pharmacological scientists because of their smart release behavior and their potential clinical implications for the delivery of drugs to target sites [11]. Acid-responsive liposomes are relatively stable at physiological pH, but in acidic conditions, the liposomes lose stability, promoting the release of their contents [12]. The pH range of tumor tissue is 5.0~6.5, which is lower than that of normal tissue (pH 7.0~7.4). Therefore, the stability of the liposomes in the tumor tissue decreases, which leads to increased drug release and overall higher bioavailability. Cholesteryl hemisuccinate (CHEMS) is a good acid-environment-responsive material; it is composed of succinic acid and cholesterol β-alcohol based on esterification [13]. The many acid-sensitive properties of CHEMS arise from the protonation of carboxyl groups, changes in spatial structure, and molecular interactions between phospholipids [14]. Therefore, the addition of CHEMS in the preparation of liposomes can form acid environment-responsive liposomes to achieve increased drug release behavior at tumor sites.

2. Materials and methods

2.1. Materials

Paclitaxel (>99.5% purity) was purchased from Dalian Meilun Biological Technology Co. Ltd. (Dalian, China). Phosphatidylethanolamine and cholesterol were from Beijing Biodee Biological Technology Co. Ltd. (Beijing, China). CHEMS was from TCI (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were acquired from HyClone Laboratories (Logan, UT, USA). Dimethylsulfoxide (DMSO), penicillin–streptomycin solution and coumarin-6 were purchased from Sigma–Aldrich (St. Louis, MO, USA). All water used in experiments was Milli-Q ultrapure water (Millipore, Ireland). All other chemicals and reagents were of analytical grade.

2.2. Cell cultures

The human breast adenocarcinoma cells (MCF7) were supplied by the School of Pharmaceutical Science, Shandong University (Shandong, China), and were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) and 1% penicillin–streptomycin solution in a humidified atmosphere of 95% air and 5% CO_2 at a temperature of 37 °C.

2.3. Preparation and characterization of liposomes

The acid-sensitive liposomes were prepared by a thin-film dispersion method [15]. Briefly, 6.5 mg of phosphatidylethanolamine, 3.0 mg of cholesterol, 4.0 mg of CHEMS and 0.9 mg of paclitaxel were added into a round-bottom flask and dissolved in 3 ml of chloroform. The solvent was evaporated to dryness on a rotary evaporator (Digital Water bath SB-651, EYELA, Japan) at 40 °C to obtain a uniform and transparent film. The mixture was vacuum-dried overnight to remove residual solvent. Then, 4 ml of pH 7.4 PBS was added at a hydration temperature of 60 °C with continuous stirring for 0.5 h. The coumarin-6-loaded liposomes (C6 ASLs) were prepared using the above method and were kept away from light. The surface morphology was observed using a transmission electron microscopy (TEM) system (JEM 1200EX, JOEL, Japan). The liposomal suspension was dropped onto a copper grid and stained with 0.5% phosphotungstic acid for 10 s and dried in air before being examined. The particle sizes, polydispersity indexes (PDIs) and ζ-potentials were measured by a dynamic light scattering method using a Zetasizer Nano ZS90 instrument (Malvern, Westborough, MA). The liposomal suspension was diluted with PBS buffer (pH 7.4) to a concentration of 1 mg/ml before measurement. Each sample was determined in triplicate at 37 °C.

2.4. Encapsulation efficiency

To determine the entrapment efficiency of the ASLs, 100 μl of PTX-loaded liposomes was added to 1.9 ml of phosphate buffer solution (PBS, pH 7.4) containing 0.5% (w/v) Tween-80. The solution was vibrated by a vortex vibrator G560E Vortex-Genie (Scientific Industries Inc., USA) to let the free drug dissolve thoroughly and then centrifuged at 14,000 rpm for 10 min using a Hermle Z216MK high-speed refrigerated centrifuge. To evaluate the amount of non-encapsulated DTX, the supernatant was analyzed by HPLC with an Agilent 1200 HPLC system (Agilent, USA) at a UV absorption wavelength of 227 nm. The encapsulation efficiency (EE) was calculated according to the following equations [16]:

EE (%) = amount of PTX encapsulated in liposomes/
amount of total PTX added in the preparation × 100%.

2.5. *In vitro drug release*

The *in vitro* release study was determined by a dialysis method [17]. Precisely 1 ml of paclitaxel solution or PTX-loaded liposomes was placed in a dialysis bag (8000–14,000 Da), and the dialysis bags were placed in 40 ml of release medium (PBS containing 1 mol/L sodium salicylate [18], pH 7.4 or pH 5.0). The release studies were carried out while maintaining a constant temperature (37 °C) in a water bath with shaking (100 rpm). Five hundred microliters of the release medium was withdrawn at designed time intervals (0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h), and the same volume of medium was added. The PTX amount of each sample was analyzed by HPLC at λmax = 227 nm. The mobile phase was acetonitrile : water (V : V) (55:45).

2.6. *In vitro cellular uptake*

To analyze the drug concentration in MCF7 cells, we used coumarin-6 (C6) as the fluorescence probe, which is lipophilic and can be entrapped in liposomes. MCF7 cells were harvested with 0.25% trypsin–EDTA solution and seeded onto 6-well assay plates (Corning, USA) at 2 × 105 cells/well for 12 h. Prior to the experiment, cells were washed twice with PBS to remove the non-adherent cells. Then, the free C6 and C6 ASLs were diluted with serum-free DMEM to a concentration of 1 μg/ml C6, and the control group had the same volume of DMEM added. After incubating for 4 h at 37 °C, cells were washed twice with cold PBS and fixed with 4% paraformaldehyde. Cellular uptake images were captured with an inverted fluorescence microscope (Eclipse Ti, Nikon, Japan). The 3D fluorescence intensity graph was analyzed by ImagePro Plus software. The higher the fluorescence intensity, the higher the Z-axis height; the rainbow-colored bar means the higher the fluorescence intensity, the warmer color it will be (from blue to red). To further quantify the uptake efficiency of the cells, MCF7 cells were collected and transferred into flow tubes (Falcon, Corning, USA), suspended with PBS at 5 × 106 cells/ml and analyzed by a flow cytometer (FACS Calibur, BD Biosciences, USA) using the FITC filter. Each sample was determined in triplicate, and the data were analyzed with FlowJo 7.6 software.

2.7. *In vitro cytotoxicity*

A Cell Counting Kit 8 (CCK-8) (Bestbio, Shanghai, China) was used to test the cytotoxicity [19]. Briefly, MCF7 cells were seeded in 96-well plates (Corning, USA) at a density of 5×10^3 cells/well/100 μl at 37 °C and cultured in complete medium (DMEM supplemented with 10% FBS, and 1% penicillin–streptomycin) at 37 °C for 24 h. Then, the medium was replaced by empty ASLs, PTX solution, or PTX ASLs diluted with complete medium and incubated for 24 h or 48 h at 37 °C, after which the CCK-8 solution (10 μl/well) was added and cultured for another 1 h to let the formazan generate. The final solution absorbance was scanned by a microplate reader (Bio-Rad, USA) at 450 nm. Relative cell viability (R) was calculated as follows

R (%) = A test/A control × 100%,

where A test and A control were the absorbances of the cells treated with the test solutions and the blank complete medium as a negative control, respectively. The IC50 (half maximal inhibitory concentration) of the test groups was calculated by SPSS 10.0 software.

2.8. *Statistical analysis*

Data were statistically analyzed by SPSS 10.0 software using Student's t-test and reported as mean ± SD. $P < 0.05$ was considered as a statistically significant difference.

3. Results and discussion

3.1. *Preparation and characterization of liposomes*

PTX ASLs were prepared by a thin-film dispersion method. The particle size of the liposomes was 210.8 ± 12.1 nm, the polydispersity index (PDI) was 0.182 ± 0.015 and the zeta potential was –31.2 ± 3.5 mV. The carboxyl group of CHEMS (pKa 5.8) was deprotonated in pH 7.4 and the phosphate group of PE was also ionized in neutral environment, therefore leading to high negative charge that accumulated on the surface of liposomes and resulted in low zeta potential. The prepared liposomes had a light blue opalescent appearance (Fig. 1C); the small size and narrow size distribution mean that the liposomes were a homogenous colloidal dispersion system (shown in Fig. 1A). Taking into account the purpose of use, the preparation method is based on drug encapsulation rate and drug loading standards; although the exclusion method can narrow the diameter and distribution of liposomes, it tends to form small unilamellar vesicles (SUV) instead of large multilamellar vesicles (LUV) by stirring, which can entrap more lipophilic molecules (like paclitaxel). It will therefore enhance the drug loading percent of liposomes for pharmaceutical use. Nano-scaled size is crucial to anti-cancer nanomedicines. The PTX ASLs investigated have small particle size (~200 nm) and PDI of <0.2, which is favorable for intravenous delivery of anti-cancer drug and for the particles to accumulate in tumor tissue selectively via the EPR (enhanced permeability and retention) effect.

Nano-scale sizes are crucial to anti-cancer nanomedicines. The PTX ASLs investigated have a small particle size (~200 nm) so that the particles can accumulate in tumor tissue selectively via the EPR (enhanced permeability and retention) effect. As shown in the TEM image (Fig. 1B), the liposomes were spherical, and no aggregation was observed, which can be attributed to their low zeta potential (<–30 mV), indicating that the negatively charged liposomes can repel each other and prevent aggregation or precipitation [20]. Furthermore, the negatively charged zeta potential reduces nonspecific cellular uptake and improves the stability of the liposomes during blood circulation.

3.2. *Drug encapsulation efficiency study*

Drug encapsulation efficiency (EE %) plays an important role in clinical application, especially for anti-cancer drugs. When

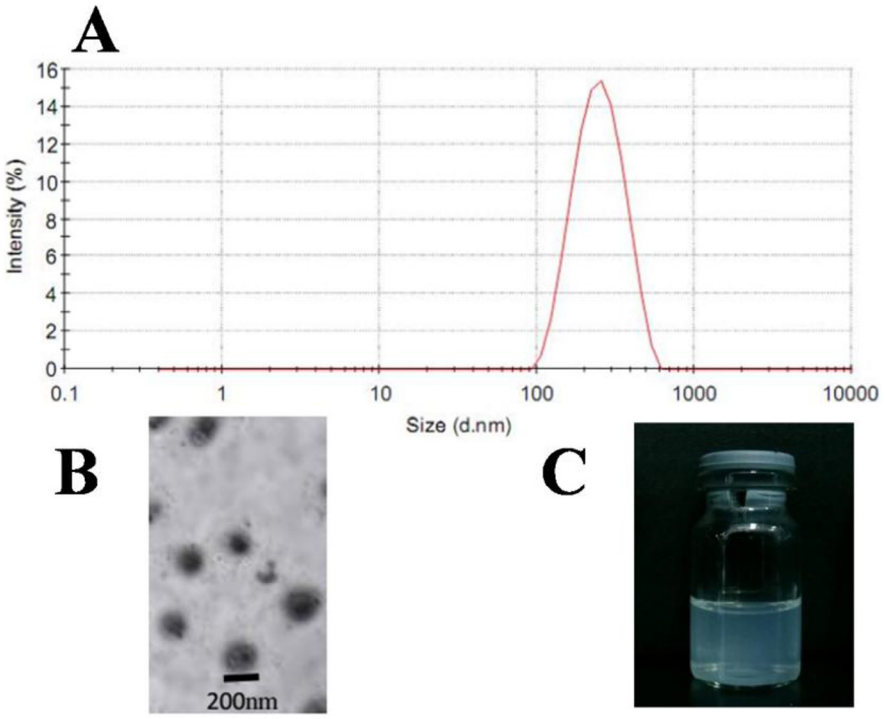

Fig. 1 – (A) The particle size distribution, (B) TEM photograph and (C) appearance of liquid suspension in front of a dark pad of PTX ASLs.

loaded with PTX, the EE% of the PTX ASLs was 82.52 ± 0.63%, whichmeans that most of the PTX added was entrapped in liposomes and at a higher level than in other reported PTX-loaded liposome formulations [21,22]. The high EE % will contribute to high therapeutic efficiency and low toxicity to normal organs and tissues.

3.3. *In vitro PTX release assessment*

The *in vitro* release profiles of PTX from the PTX ASLs at 37 °C in PBS at pH 7.4 and pH 5.0 are shown in Fig. 2 and are compared with the Taxol® group. The release profile of Taxol® showed a more obvious initial burst release than the PTX ASL

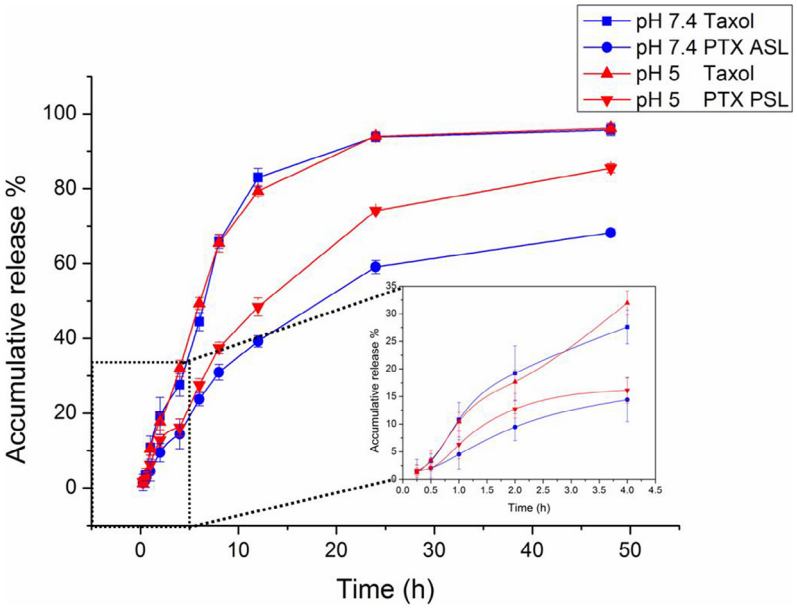

Fig. 2 – *In vitro* PTX release from Taxol® and PTX ASLs at pH 5.0 and pH 7.4.

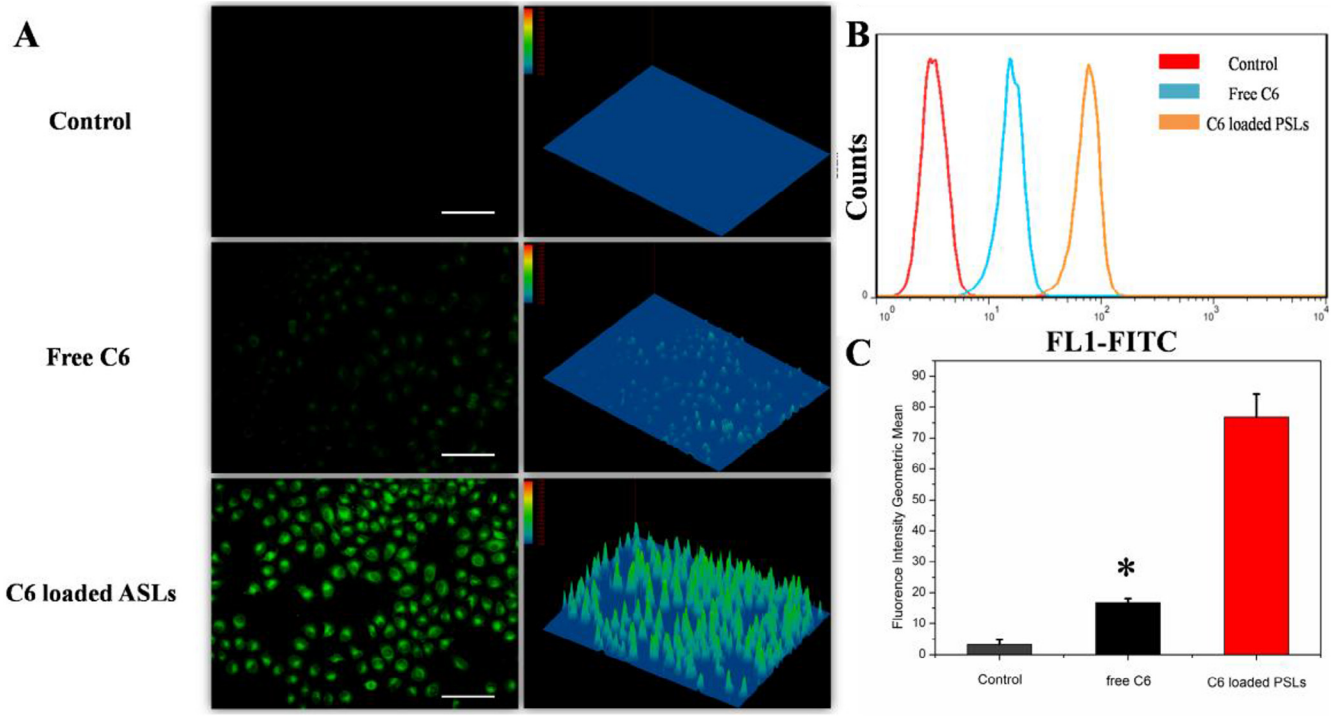

Fig. 3 – (A) Fluorescence micrographs (left) and 3D fluorescence intensity pictures (right). Scale bars represent 200 μm. (B) Flow cytometry histograms measured in FL1 channel with FITC filter. (C) Geometric mean fluorescence intensity analyzed by FlowJo software, '*' means $P < 0.05$ versus free C6. n = 3, data are shown in mean ± SD.

group, of 27.61 ± 3.04% and 31.97 ± 2.19% of its loaded cargo released within 4 h and 95.71 ± 1.47% and 96.18 ± 1.28% in 48 h at pH 7.4 and pH 5, respectively. No significant difference was found for the release of PTX from that of Taxol® at different pH values over the entire study period. Nevertheless, the *in vitro* release of PTX from the PTX ASLs was 68.2 ± 0.98% and 85.47 ± 1.21% at pH 7.4 and pH 5 in 48 h. There was a significant increment of PTX release at pH 5 compared to pH 7.4 ($P = 0.003$). The faster release of PTX at pH 5.0 showed that the acidic environment could expedite the PTX release from the PTX ASLs, demonstrating that the ASLs have acidic environment-responsive characteristics. At pH 7.4, the moderate and slower release behavior compared to Taxol® demonstrated the ASLs can be highly stable in a neutral environment, which can reduce the release of PTX from carriers in systemic circulation and contribute to more cargo accumulating at tumor sites. The smart environment-responsive capability of ASLs may be attributed to the molecular conformation change of CHEMS and its molecular interaction with PE at different pH levels.

3.4. *Cellular uptake assay*

The cellular uptake graph and flow cytometry analysis are illustrated in Fig. 3. Coumarin-6, a lipophilic green fluorescent marker with a high fluorescence intensity and a low leakage rate, can be loaded into the liposomes to evaluate the cellular uptake [23]. As observed in Fig. 3A, green fluorescence was detected both in free C6 and C6-loaded ASLs, demonstrating that the free C6 and C6 incorporated into ASLs can both be internalized into MCF7 cells. However, the green fluorescence intensity was remarkably increased following 4 h incubation with C6-loaded ASLs compared with free C6, and the 3D fluorescence intensity graph illustrated a higher Z-axis height and warmer colored peak pattern compared with free C6. Flow cytometry analysis was conducted to further confirm the cellular uptake efficiency of free C6 and C6-loaded ASLs by measuring the intensity of green fluorescence in MCF7 cells (Fig. 3B). The results were consistent with the fluorescence microscopy graphs and 3D fluorescence intensity pictures shown above, which also demonstrated a significant increase in the intensity of the green fluorescence. The statistical parameter (geometric mean of fluorescence intensity) (Fig. 3C) was calculated in the cells incubated with C6-loaded ASLs (76.83 ± 3.79) and was nearly 4.6-fold higher than in cells exposed to free coumarin-6 solutions (16.71 ± 1.42), meaning a significant difference versus free C6 ($P = 0.003 < 0.05$). All of the results above demonstrate that the C6-loaded ASLs have significantly higher cellular uptake efficiency compared with the C6 solution. As a lipophilic small molecule (MW 350.43), C6 can pass through the cell membrane through the passive diffusion pathway into the cells. However, the negatively charged ASLs can interact with cationic proteins embedded in the cell membrane to achieve positive adsorptive endocytosis, which is an energy-dependent pathway and is much more effective than free C6 [24].

3.5. *Safety of the carriers and* in vitro *therapeutic effect of PTX ASLs*

The MCF7 cell viability was determined by the CCK-8 method as described in Section 2.7. The main component of CCK-8,

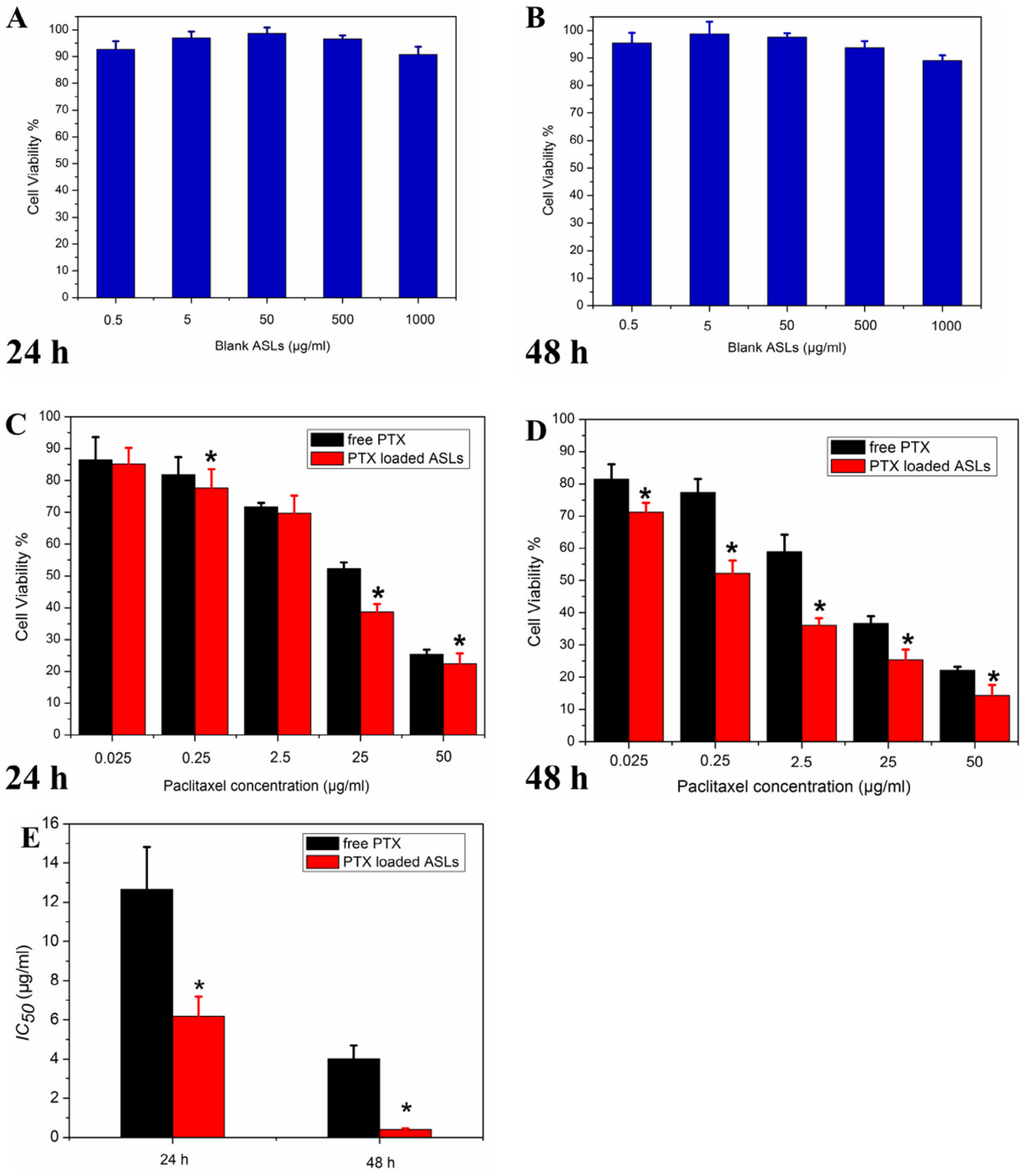

Fig. 4 – *In vitro* cell viabilities of and half maximal inhibitory concentrations (IC_{50}) under 24 h and 48 h exposure to blank and paclitaxel loaded ASLs determined by CCK-8 method. Results were shown by mean ± SD and n = 5. '*' stands for P < 0.05 versus free PTX group.

WST-8, is a water-soluble tetrazolium salt. Succinate dehydrogenase in living cells' mitochondria reduces the exogenous WST-8 to water-soluble formazan, a function dead cells do not have, and the yellow formazan has a wavelength of maximum absorption at 450 nm, which can be detected by microplate reader. The CCK-8 solution is highly stable and free of cytotoxicity and can be added directly to the sample. Since formazan is highly water soluble, no additional reagents are required. It has higher sensitivity and better reproducibility than MTT, XTT and WST-1 in detecting cytotoxicity. The cytotoxicity of the empty ASLs was examined to evaluate the safety of the carriers. As illustrated in Fig. 4A and B, the viability of MCF7 cells was no less than 85% after exposure to different concentrations of blank ASL suspensions for 24 h or 48 h. The data

suggested that the liposome carriers have negligible toxicity to cells and good biocompatibility and were safe enough for *in vitro* experiments and *in vivo* administration. The *in vitro* therapeutic effect was examined by treating MCF7 cells with ASLs loaded with various concentrations of PTX (0.025, 0.25, 2.5, 25 and 50 μg/ml) for 24 h and 48 h. Generally, an increased concentration of PTX (from 0.025 to 50 μg/ml) and a longer incubation time (from 24 h to 48 h) create a dose- and time-dependent cell-killing effect of PTX. For example, cells exposed to 0.025~50 μg/ml free PTX resulted in an 86.43 ± 7.23%~25.36 ± 1.39% survival rate (Fig. 4C); however, cell viability decreased to 85.16 ± 5.06%~22.32 ± 3.35% (Fig. 4D) when incubated for 48 h at the same PTX concentration. A significant decrease in cell viability was observed for the PTX ASLs group compared with free PTX at 0.25, 25, or 50 μg/ml for 24 h (Fig. 4C) and at different concentrations for 48 h (Fig. 4D) ($P < 0.05$). The IC50 values (Fig. 4E) for PTX ASLs (6.17 ± 1.02 μg/ml, 0.4 ± 0.05 μg/ml) were much lower than those for free PTX (12.66 ± 2.15 μg/ml, 4.02 ± 0.67 μg/ml) after incubation for 24 h and 48 h, respectively. Significant differences of the IC50 values were observed between PTX ASLs and free PTX at all of the incubation times ($P < 0.05$). The IC50 results showed that PTX ASLs have better antiproliferation efficiency than free PTX, which was consistent with the cell viability analysis above. The cytotoxicity of the PTX ASLs was mainly determined by the velocity and quantity of PTX molecules transported into the carcinoma cells. PTX, a chemotherapy drug with low molecular weight, should be transported into the cytoplasm to bind to tubulin to produce the cytotoxic effect. However, the main endocytosis pathway of PTX ASLs is based on an absorption-mediated endocytosis, which is much faster and more effective than the mechanism of PTX, which is passive diffusion. Additionally, effective cellular internalization was observed in the cell uptake assay (Section 3.4). Moreover, when endocytosed into the intracellular lysosome, the PTX ASLs can quickly unload their cargo due to the acid-responsive characteristics of PTX ASLs thereby leading to more drugs accumulated in the carcinoma cells compared with free PTX. Therefore, the results contributed to an enhanced cytotoxicity and therapeutic efficiency against carcinoma cells *in vitro*.

4. Conclusion

An acid-responsive paclitaxel-loaded liposomal drug delivery system (PTX ASLs) was successfully fabricated in this study. The small particle size (~200 nm) and narrow particle size distribution (PDI ~0.2) of the PTX ASLs contributed to an enhanced EPR effect, which increased the accumulation of the PTX ASLs in tumor sites. The low zeta potential (<–30 mV) led to high stability in blood circulation. Acid environment-triggered release profiles demonstrated that when successfully delivered to a tumor, PTX can be rapidly freed from the ASL carriers due to the phase transition behavior of lipid bilayers when anchored with CHEMS, a pH-responsive molecule. Cellular uptake results showed that when entrapped in ASLs, coumarin-6 can be delivered into tumor cells much more efficiently than free C6, which conveyed the idea that the carriers have excellent cell internalization ability. The improved *in vitro* antitumor efficacy of PTX ASLs is mainly a result of physicochemical properties of liposomes, namely, their nanoscale size and negatively charged surfaces. The clathrin-mediated endocytosis pathway greatly enhanced their cell internalization compared to free PTX. In addition, the pH-accelerated release profile of the PTX ASLs contributed to the triggered release of paclitaxel in endosomes and lysosomes with a pH of 6.5~5.0, which enhanced the bioavailability of paclitaxel delivered into tumor cells. These properties of PTX ASLs ensure a precise and safe tumor-targeting drug delivery system for breast carcinoma therapy, with largely improved therapeutic efficacy and controlled release profiles.

Conflicts of interest

The authors declare that there is no conflicts of interest.

REFERENCES

[1] Boyle P, Levin B. World cancer report 2008. IARC Press, International Agency for Research on Cancer; 2008.
[2] Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. CA Cancer J Clin 2005;55(2):74–108.
[3] Sun M, Gao Y, Zhu Z, et al. A systematic *in vitro* investigation on poly-arginine modified nanostructured lipid carrier: pharmaceutical characteristics, cellular uptake, mechanisms and cytotoxicity. Asian J Pharm Sci 2017;12(1):51–58.
[4] Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly *in vitro* by taxol. Nature 1979;277(5698):665–667.
[5] Konno T, Watanabe J, Ishihara K. Enhanced solubility of paclitaxel using water-soluble and biocompatible 2-methacryloyloxyethyl phosphorylcholine polymers. J Biomed Mater Res A 2003;65(2):209–214.
[6] Li J, Wang X, Zhang T, et al. A review on phospholipids and their main applications in drug delivery systems. Asian J Pharm Sci 2015;10(2):81–98.
[7] Chang HI, Yeh MK. Clinical development of liposome-based drugs: formulation, characterization, and therapeutic efficacy. Int J Nanomedicine 2012;7(4):49–60.
[8] Torchilin VP, Omelyanenko VG, Papisov MI, et al. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochim Biophys Acta 1994;1195(1):11–20.
[9] Chang M, Lu S, Zhang F, et al. RGD-modified pH-sensitive liposomes for docetaxel tumor targeting. Colloids Surf B Biointerfaces 2015;129:175–182.
[10] Li DC, Zhong XK, Zeng ZP, et al. Application of targeted drug delivery system in Chinese medicine. J Control Release 2009;138(2):103–112.
[11] Karanth H, Murthy RSR. pH-Sensitive liposomes-principle and application in cancer therapy. J Pharm Pharmacol 2007;59(4):469–483.
[12] Hong MS, Lim SJ, Oh YK, et al. pH-sensitive, serum-stable and long-circulating liposomes as a new drug delivery system. J Pharm Pharmacol 2002;54(1):51–58.
[13] Hafez IM, Cullis PR. Cholesteryl hemisuccinate exhibits pH sensitive polymorphic phase behavior. Biochim Biophys Acta 2000;1463(1):107–114.
[14] Fan Y, Chen C, Huang Y, et al. Study of the pH-sensitive mechanism of tumor-targeting liposomes. Colloids Surf B Biointerfaces 2017;151:19–25.

[15] Mo R, Sun Q, Li N, et al. Intracellular delivery and antitumor effects of pH-sensitive liposomes based on zwitterionic oligopeptide lipids. Biomaterials 2013;34(11):2773–2786.
[16] Gill KK, Nazzal S, Kaddoumi A. Paclitaxel loaded PEG 5000–DSPE micelles as pulmonary delivery platform: formulation characterization, tissue distribution, plasma pharmacokinetics, and toxicological evaluation. Eur J Pharm Biopharm 2011;79(2):276–284.
[17] Shahin M, Soudy R, Aliabadi HM, et al. Engineered breast tumor targeting peptide ligand modified liposomal doxorubicin and the effect of peptide density on anticancer activity. Biomaterials 2013;34(16):4089–4097.
[18] Yang X, Li Y, Li M, et al. Hyaluronic acid-coated nanostructured lipid carriers for targeting paclitaxel to cancer. Cancer Lett 2013;334(2):338–345.
[19] Chang Y, Yang ST, Liu JH, et al. *In vitro* toxicity evaluation of graphene oxide on A549 cells. Toxicol Lett 2011;200(3):201–210.
[20] Pistone S, Rykke M, Smistad G, et al. Polysaccharide-coated liposomal formulations for dental targeting. Int J Pharm 2017;516(1):106–115.
[21] Jiang L, Li L, He X, et al. Overcoming drug-resistant lung cancer by paclitaxel loaded dual-functional liposomes with mitochondria targeting and pH-response. Biomaterials 2015;52:126–139.
[22] Eloy JO, Petrilli R, Topan JF, et al. Co-loaded paclitaxel/rapamycin liposomes: development, characterization and in vitro and in vivo evaluation for breast cancer therapy. Colloids Surf B Biointerfaces 2016;141:74–82.
[23] Zuo T, Guan Y, Chang M, et al. RGD (Arg-Gly-Asp) internalized docetaxel-loaded pH sensitive liposomes: preparation, characterization and antitumor efficacy in vivo and in vitro. Colloids Surf B Biointerfaces 2016;147:90–99.
[24] Kievit FM, Zhang M. Cancer nanotheranostics: improving imaging and therapy by targeted delivery across biological barriers. Adv Mater 2011;23(36):H217–H247.