

Codelivery of thioridazine and doxorubicin using nanoparticles for effective breast cancer therapy

Xun Jin^{1,*}
 Bingwen Zou^{2,*}
 Li Luo^{1,*}
 Chuanhong Zhong³
 Peilan Zhang¹
 Hao Cheng¹
 Yanfang Guo¹
 Maling Gou¹

¹State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, and Collaborative Innovation Center for Biotherapy,

²Department of Thoracic Oncology, Cancer Center, West China Hospital, Medical School, Sichuan University, Chengdu, ³Department of Neurosurgery, The Affiliated Hospital of Luzhou Medical College, Luzhou, Sichuan Province, People's Republic of China

*These authors contributed equally to this work

Abstract: Cancer chemotherapy can benefit from the combination of different anticancer drugs. Here, we prepared doxorubicin (Dox)- and thioridazine (Thio)-coloaded methoxy poly(ethylene glycol)-poly(L-lactic acid) (MPEG-PLA) nanoparticles (NPs) for breast cancer therapy. These NPs have an average particle size of 27 nm. The drug loading efficiencies of Thio and Dox are 4.71% and 1.98%, respectively. Compared to the treatment of Thio or Dox alone, the combination of Thio and Dox exhibited a synergistic effect in inhibiting the growth of 4T1 breast cancer cells in vitro. In addition, the Thio- and Dox-coloaded MPEG-PLA NPs could efficiently suppress the growth of breast cancer cells in vivo. This study suggests that Thio- and Dox-coloaded MPEG-PLA NPs might have potential applications in breast cancer treatment.

Keywords: thioridazine, doxorubicin, codelivery, nanoparticles, breast cancer

Introduction

Breast cancer is one of the most common tumors in women worldwide and remains the leading cause of cancer-related death among females in less developed countries.^{1,2} Although much progress has been made in breast cancer therapeutics, ~30% of breast cancer patients treated in early stage suffer from recurrence due to therapeutic resistance, such as chemoresistance.³ Hence, it is critical to develop effective strategies to overcome drug resistance for breast cancer therapy.

Instead of single drug treatment, simultaneous administration of multiple drugs is an attractive way to maximize the therapeutic response of chemotherapy and minimize the occurrence of multidrug resistance.^{4,5} Thioridazine (Thio), a phenothiazine derivative, is a piperidine antipsychotic drug that was recently found to inhibit cancer cell growth.⁶⁻⁸ Doxorubicin (Dox), an anthracycline antibiotic, is one of the most widely applied anticancer drugs for various malignancies, including breast, prostate, and colon cancers.⁹⁻¹² However, serious side effects, such as the dose-dependent cardiotoxicity, myelosuppression, and nephrotoxicity, markedly limit its clinical use.^{13,14} The strategy of combining two chemotherapeutic agents has been proved to be able to achieve the synergetic anticancer effects. Lv et al¹⁵ used the combination of Dox and paclitaxel to treat lung cancer. Zhang et al¹⁶ combined Dox and dasatinib for breast cancer. In this study, the combination of Thio and Dox was designed to maximize the anticancer activity and minimize drug resistance.

Over the past decades, nanotechnology has made great contribution to the development of drug delivery systems.¹⁷⁻¹⁹ Encapsulation of anticancer drugs by polymeric nanoparticles (NPs) facilitates drug distribution in tumor tissues through an enhanced permeability and retention (EPR) effect that shows better pharmacokinetics profiles in vivo and reduces multidrug resistance of malignancies, leading to enhanced anticancer effects.²⁰⁻²³ Various micelles, such as MPEG poly(ϵ -caprolactone and methoxy

Correspondence: Maling Gou
 State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Collaborative Innovation Center for Biotherapy, Sichuan University, Chengdu 610041, People's Republic of China
 Tel +86 28 8516 4061
 Fax +86 28 8516 4060
 Email goumaling@scu.edu.cn

poly(ethylene glycol)-poly(L-lactic acid) (MPEG-PLA), have been extensively used as effective drug delivery systems.²⁴ MPEG-PLA, an amphiphilic polymer, is appropriate for drug delivery due to its biodegradation ability and low clearance rate. Here, we coencapsulated Dox and Thio with MPEG-PLA polymer in two steps, forming Thio-Dox-coencapsulated NPs. The prepared NPs were characterized in size distribution, morphology, and in vitro drug release behavior. The anticancer effect against breast cancer 4T1 cells was also evaluated both in vitro and in vivo. Our results suggest that the codelivery of Dox and Thio using an MPEG-PLA nanocarrier has a potential application in breast cancer chemotherapy.

Materials and methods

Materials

MPEG-PLA (MPEG:PLA molar ratio =50:50, molecular weight =4,000 g/mol) was purchased from DaiGang (Jinan, Shandong, People's Republic of China). Dox hydrochloride was provided by Melonepharma Co Ltd (Dalian, Liaoning, People's Republic of China). Thio was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, People's Republic of China). Methanol and acetonitrile were purchased from Kermel (Tianjin, People's Republic of China). All other chemicals and solvents were obtained from Kelong Chemical Reagent Factory (Chengdu, Sichuan, People's Republic of China), and they were at least of analytical grade.

Mice breast cancer cell line 4T1, mice colon cancer cell line C26, and human embryo kidney cell line HEK293 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The Ethical Committee of the West China Hospital, Sichuan University, approved this study. RPMI1640 complete medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from HyClone (Logan, UT, USA). All cells were maintained in RPMI1640 complete medium supplemented with 10% (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂ atmosphere. All mice were obtained from the Beijing HFK Bioscience (Beijing, People's Republic of China). Mice were humanely treated according to the guidelines of the Institutional Animal Care and Treatment Committee of Sichuan University, and all animal procedures were approved and controlled by the Institutional Animal Care and Treatment Committee of Sichuan University.

Preparation of Dox, Thio, and Thio-Dox-loaded NPs

Preparation of Thio NPs

To obtain the Thio/MPEG-PLA NPs, 95 mg MPEG-PLA and 5 mg Thio were codissolved in 2 mL dichloromethane,

followed by evaporation under reduced pressure in a rotary evaporator at 60°C. Subsequently, the film was rehydrated in 500 µL phosphate-buffered saline (PBS; pH =7.4), allowing the self-assembly of Thio/MPEG-PLA NPs.

Preparation of codelivery Thio-Dox NPs

To prepare the Thio-Dox NPs, 2.5 mg Dox aqueous solution was added into the Thio/MPEG-PLA solution dropwise under moderate stirring. After 20 minutes, the Thio-Dox-loaded NPs were obtained and stored at 4°C for future use.

Drug loading (DL) and encapsulation efficiency (EE) of codelivery Thio-Dox NPs were calculated from the following formulas:

$$\begin{aligned} \text{Drug loading (DL, \% w/w)} \\ = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of nanoparticles}} \times 100\% \end{aligned} \quad (1)$$

$$\begin{aligned} \text{Encapsulation efficiency (EE, \% w/w)} \\ = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of total drug used}} \times 100\% \end{aligned} \quad (2)$$

Characterization of codelivery Thio-Dox NPs

Particle size analysis

The particle size of prepared NPs was determined by Malvern Nano ZS90 (Malvern Instruments, Malvern, UK). The temperature was kept at 25°C during the measuring process. All results were the mean of three test runs.

Morphology study

The morphology of the prepared NPs was observed under a transmission electron microscope (H-6009IV; Hitachi Ltd., Tokyo, Japan). NPs were diluted with distilled water and placed on a copper grid covered with nitrocellulose. Samples were negatively stained with phosphotungstic acid and dried at room temperature.

In vitro drug release study

In vitro drug release behaviors of free Thio, free Dox, and Thio-Dox-loaded NPs were determined by dialysis method.²⁴ Briefly, 2 mL of free Thio, or free Dox solution, or Thio-Dox-loaded NPs were placed in dialysis bags (molecular weight cutoff, 3.5 kDa). The dialysis bags were incubated in 20 mL of PBS (prewarmed to 37°C and pH =7.4 and pH =5.5) or 20 mL of PBS (prewarmed to 37°C and pH 7.4) containing 10% FBS at 37°C with shaking at 100 rpm in a 50 mL tube. At predetermined time points, 2 mL of release media was collected, and the incubation mediums were replaced with fresh

incubation mediums. The amount of released drugs was quantified by high-performance liquid chromatography (HPLC, Waters 2695; Waters Corporation, Milford, MA, USA). For Dox, chromatography was performed on a Kromasil C18 column (4.6×250 mm², 5 μm for Dox). The effluents were monitored at excitation wavelength of 233 nm at 40°C. The mobile phase consisted of a gradient of methanol, dipotassium phosphate (0.01 mol/L), and acetonitrile (8:9:3, pH =3.52) with a flow rate of 1 mL/min. For Thio, chromatography was performed on a Kromasil C18 column (4.6×150 mm², 5 μm). The effluents were monitored at excitation wavelength of 263 nm. The mobile phase consisted of methanol and water (70:30) with 0.3% tetramethylethylenediamine (pH =6.0) with a flow rate of 1 mL/min. This experiment was repeated three times, and the results were shown as mean value ± standard deviation (SD).

In vitro anticancer activity

Colony formation analysis

The growth inhibition effect of Thio in breast cancer cells was examined by the colony formation analysis. Briefly, 4T1 cells harvested at ~80% confluence were suspended in RPMI1640 with 10% FBS (100 cells/well). Then, normal saline (NS) and different concentrations of Thio (1 μg/mL, 2 μg/mL, and 4 μg/mL) were added into the culture medium. After incubation for 3 weeks, cell colonies were stained with gimesa. The results were shown as mean value ± SD.

MTT assay

The cytotoxicity of Thio and Dox was determined against C26, 4T1, and HEK293 cell lines using MTT assay. Dox and Thio used in the cytotoxicity assays were prepared in normal saline. C26, 4T1, and HEK293 cells were resuspended in complete medium and seeded in 96-well tissue culture plates at a concentration of 3,000 cells/well. The cells were allowed to attach to the surface for 12 hours and then exposed to 100 μL of diluted formulations ranging from 0 μg/mL to 16 μg/mL Thio or from 0 μg/mL to 8 μg/mL Dox, compared with Thio- or Dox-loaded MPEG-PLA. After 3 days of incubation, 20 μL of MTT solution (5 mg/mL) was added to each well, and plates were continuously incubated for an additional 4 hours. The formazan crystals produced were solubilized by adding 100 μL dimethyl sulfoxide. Optical densities were read at 570 nm using a microplate reader. The cells incubated in culture medium alone served as a control for cell viability. Mean optical density values were used to estimate the cell viability.

Synergistic effect of Thio and Dox

A MTT method was used to evaluate the synergistic effects of Thio and Dox. Briefly, the half maximal inhibitory

concentration (IC₅₀) of Thio and Dox to breast cancer cell line 4T1 was tested with MTT. Then, a theoretical IC₅₀ line combined with Thio and Dox was drawn up with the IC₅₀ values of Thio (3.5, 0) and Dox (0, 0.9). To verify the synergistic effects of Thio and Dox, Thio (0.5 μg/mL, 1 μg/mL, 1.5 μg/mL, 2 μg/mL, and 2.5 μg/mL) was added into the cultured 4T1 cells in 96 well plates. The IC₅₀ of Dox to 4T1 was evaluated with MTT. The IC₅₀ of Dox to 4T1 in Thio medium was compared with the corresponding value in the straight line, and we found that a lower IC₅₀ value of Dox, combined with Thio, to 4T1 cells compared with the theoretical value calculated via the IC₅₀ line. Consistent results were observed in the Thio IC₅₀ test.

In vivo anticancer activity

To evaluate the anticancer effects of Thio-Dox NPs, the mice subcutaneously transplanted breast cancer model was established. 4T1 breast cancer cells (5×10⁵ cells/mouse) were suspended in 100 μL of RPMI 1640 basic medium and subcutaneously injected into the right flank of female BALB/c mice. On post-injective day 5, mice were randomly divided into five groups (six mice per group). At post-injective times (days 9, 12, 15, and 18), NS, Dox (5 mg/kg), Thio (10 mg/kg), Thio-Dox (5/10 mg/kg), and Thio-Dox NPs (5/10 mg/kg) formulations were given by intravenous injection. At the predetermined time points, mice were weighed, and tumor size was measured. The tumor volume was calculated with the following formula: tumor volume = length × width²/2. The mice were sacrificed at day 23. Tumors and vital organs were obtained. Tumor weight was recorded, and vital organs were stored for further pathological section analysis.

Statistical analysis

All experimental data were presented as the mean value ± SD. One-way analysis of variance was applied using SPSS software. *P*-values <0.05 were considered to be statistically significant.

Results and discussion

Antitumor effect of Thio in breast cancer

Recently, it was reported that Thio could selectively target leukemic cancer stem cells, but it had no effect on normal blood stem cells.⁶ In this study, we examined the effect of Thio on the growth inhibition of breast cancer 4T1 cells by colony formation. As expected, Thio could suppress colony formation in a dose-dependent manner as shown in Figure 1A and B. Using the concentration of 4 μg/mL of Thio, the colony formation inhibiting rate was ~100%, indicating that Thio was capable of inhibiting 4T1 cell

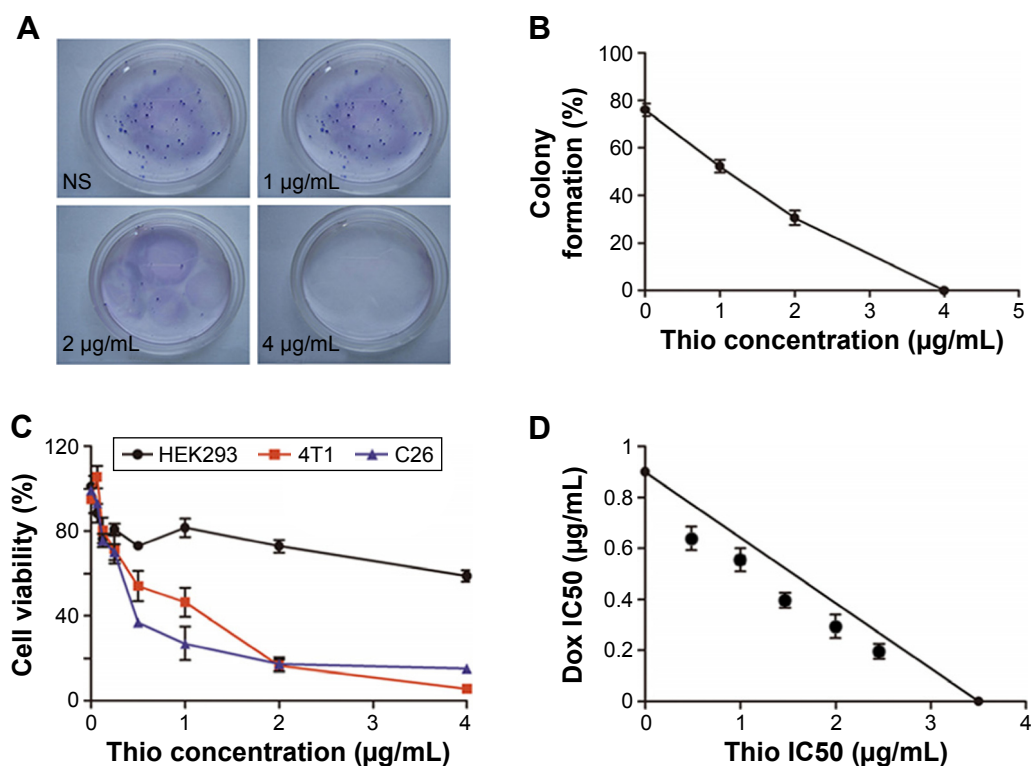


Figure 1 Anticancer effects of Thio in breast cancer.

Notes: (A) Colony formation of 4T1 cells with Thio treatment. (B) Colony formation rate of 4T1 cells using different concentrations of Thio. (C) Cytotoxicity of Thio on 4T1, C26, and HEK293 cell lines. (D) Cytotoxicity of combination of Thio and Dox on 4T1 cells.

Abbreviations: Thio, thioridazine; Dox, doxorubicin; IC50, half maximal inhibitory concentration; NS, normal saline.

growth. The cytotoxicity of Thio in several other cell lines is also shown in Figure 1C. Both 4T1 breast cancer cells and C26 colon cancer cells, but not human embryo kidney cells HEK293, were sensitive to Thio, indicating that Thio selectively killed tumor cells while had no obvious cytotoxicity in normal cells. The IC₅₀ value of Thio in 4T1 and C26 cells was 0.35 µg/mL and 0.65 µg/mL, respectively. We further investigated the synergistic effect of Thio and Dox by MTT analysis. As shown in Figure 1D, the IC₅₀ value of Thio using the IC₅₀ of Dox concentration was below the theoretical value, indicating that Thio and Dox synergistically inhibited breast cancer cell growth.

Encapsulation of Thio to MPEG-PLA NPs

NP carriers enable a better accumulation of drugs in tumor tissues through an EPR effect ability to overcome multidrug resistance with better pharmacokinetics profiles in vivo. MPEG-PLA NPs have been used to deliver several anticancer drugs due to their stability and high EE. The biodegradability of MPEG-PLA ensures the safety, and its low clearance rate would contribute to the long circulation of drugs in vivo. Therefore, we designed the Thio MPEG-PLA NPs for breast cancer treatment. The feasibility of Thio loaded to

MPEG-PLA NPs was determined by size and transmission electron microscopy analysis. As revealed in Figure 2A and B, the average size of Thio/MPEG-PLA NPs was 25.0 nm. To evaluate the anticancer effects of Thio-loaded MPEG-PLA NPs compared with free Thio, MTT analysis of free Thio and Thio-loaded MPEG-PLA NPs to 4T1 cells (1 day) was performed. As shown in Figure 2C, the IC₅₀ of free Thio and Thio/MPEG-PLA NPs was 2.9 µg/mL and 1.8 µg/mL, respectively. Therefore, the cytotoxicity of Thio was enhanced after encapsulation in vitro.

Characterization of Thio-Dox-loaded MPEG-PLA NPs

We further investigated whether Thio and Dox can be loaded on MPEG-PLA NPs. Successful combination of different chemotherapy drugs is usually based on specific properties of the prepared drug delivery system, such as successful incorporation, and sustained release. In recent years, attempts have been made to deliver chemotherapy drugs simultaneously through various delivery systems, such as micelles, liposomes, microcapsule, and inorganic NPs.²⁵ For example, codelivery of Dox and curcumin in poly(D,L-lactide-co-glycolide) NPs has been demonstrated to be an

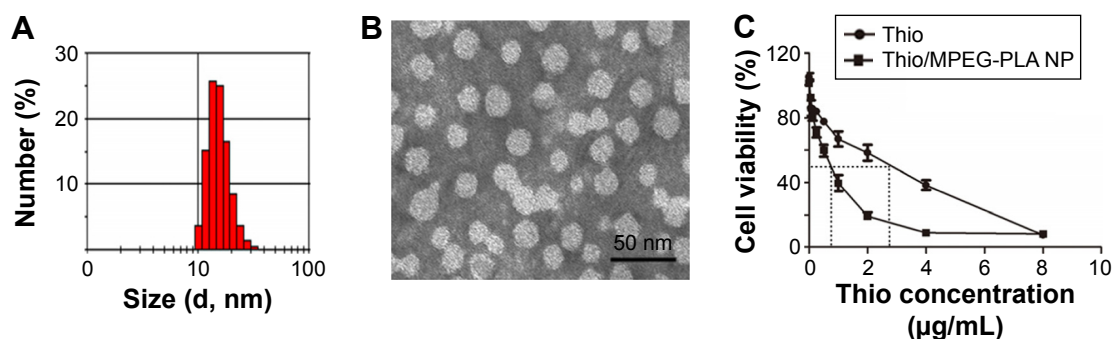


Figure 2 Characterization of Thio/MPEG-PLA NPs.

Notes: (A) Size distribution spectrum of Thio/MPEG-PLA NPs. (B) TEM image of Thio/MPEG-PLA NPs. (C) Cytotoxicity of Thio and Thio/MPEG-PLA NPs.

Abbreviations: Thio, thioridazine; MPEG-PLA, methoxy poly(ethylene glycol)-poly(L-lactic acid); NP, nanoparticle; TEM, transmission electron microscopic.

accessible method to improving anticancer effects.²⁶ Besides this, MPEG-PLA copolymer-based codelivery of Dox and paclitaxel also showed enhanced anticancer effects with an improved drug delivery system.¹⁵

In our study, we loaded Thio-Dox into MPEG-PLA NPs by a two-step self-assembly method (Figure 3A). MPEG-PLA could self-assemble into micelles and form a core-shell structure in the rehydration process in water due to its amphiphilic property. Evaporation of the Thio and MPEG-PLA codissolved solution allowed the two components to be adequately mixed. In this structure, both Thio and Dox were loaded on the NPs, forming a structure. When the in-feed mass ratio of Thio/Dox/MPEG-PLA was 1/2/40, the NPs had an EE of 96.30% (Thio) and 79.16% (Dox), and DL of 4.71% (Thio) and 1.98% (Dox).

The size of Thio-Dox/MPEG-PLA NPs was 27 nm (Figure 3B), which was much smaller than the dimensional requirement for *in vivo* application. The transmission electron microscopic image of Thio-Dox/MPEG-PLA NPs is shown in Figure 3C. NP-mediated cellular response was size dependent, and mammalian cells could efficiently uptake NPs with a size <100 nm.^{27,28} The Thio-Dox-loaded NPs with a particle size of ~20 nm were suitable for the drug delivery system. The release profiles of Thio and Dox from the MPEG-PLA NPs were studied using a dialysis method. As shown in Figure 3D and E, both Thio and Dox released slower from the NPs in pH 7.4 than in pH 5.5. The pH-dependent releasing behavior might be due to the deprotonation of the amino group of Dox and faster degradation of MPEG-PLA NPs at lower pH.²⁹ The drug release of free Thio, free Dox, and Thio-Dox/MPEG-PLA NPs in PBS containing 10% FBS is shown in Figure 3F and G. The results suggested that free Thio and free Dox released faster than the drug encapsulation by MPEG-PLA NPs. Thus Thio and Dox might be very slowly released in the plasma under normal

physiological conditions (pH =7.4) but quickly released at the solid tumor site (pH =5.5), which was expected to achieve selective release of Thio and Dox in tumor and reduce the systemic toxicity.

Anticancer effects of Thio-Dox-loaded MPEG-PLA NPs in breast cancer *in vivo*

The therapeutic effect following the treatment of Dox and Thio formulations in mice-bearing 4T1 xenografts is described in Figure 4. Compared with the tumor weight 0.832 g in NS group, the average weight of tumors in Thio-treated group and Dox-treated group was 0.538 g and 0.342 g, respectively. The formulation of Dox and Thio showed more significant tumor inhibition effect than Dox or Thio alone, as evidenced by smaller tumor volume and tumor weight (Figure 4A–C).

The anticancer activity of Dox and Thio in breast cancer was further enhanced through the NP-based drug codelivery system. The average weight of Thio-Dox group and Thio-Dox/MPEG-PLA NPs group was 0.176 g and 0.122 g, respectively. The tumor inhibitory rates were 35.4% (free Thio), 58.9% (free Dox), 78.8% (free Thio and Dox), and 85.6% (Thio-Dox/MPEG-PLA NPs) (Figure 4A–C). The increased anticancer effects of Thio-Dox/MPEG-PLA NPs is due to the better drug accumulation in tumor tissues resulting from an EPR effect, ability to overcome multidrug resistance, and better pharmacokinetics profiles *in vivo*. We further evaluated the effect of Thio and Dox on metastasis of tumor cells. As shown in Figure 4D, free Thio or Dox may inhibit the metastasis of tumor cells, while the combination of Thio and Dox revealed no lung nodules in mice. In addition, we could not observe metastatic lung nodules in mouse treated with Thio and Dox loaded to MPEG-PLA NPs, implying that the drug encapsulation by MPEG-PLA NPs may not have significant effect on breast cancer metastasis or the nonsignificant

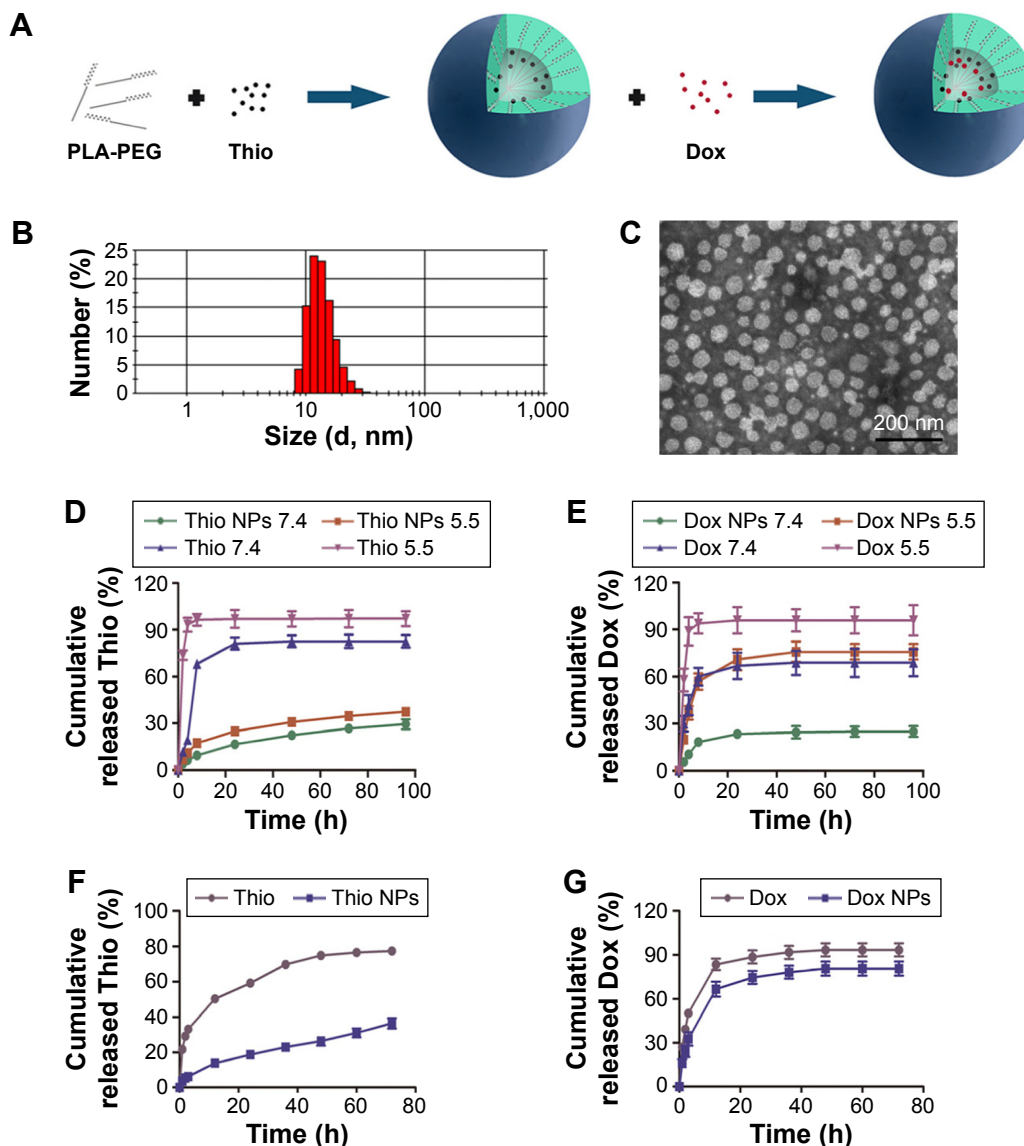


Figure 3 Preparation and characterization of Thio-Dox/MPEG-PLA NPs. **Notes:** (A) Synthesis of Thio-Dox/MPEG-PLA NPs. (B) Size distribution spectrum of Thio-Dox/MPEG-PLA NPs. (C) TEM image of Thio-Dox/MPEG-PLA NPs. (D) Drug release of free Thio and Thio/MPEG-PLA NPs in PBS (pH 7.4 and 5.5). (E) Drug release of free Dox and Dox/MPEG-PLA NPs in PBS (pH 7.4 and 5.5). (F) Drug release of free Thio and Thio/MPEG-PLA NPs in PBS containing 10% FBS. (G) Drug release of free Dox and Dox/MPEG-PLA NPs in PBS containing 10% FBS. **Abbreviations:** Thio, thionidazine; Dox, doxorubicin; MPEG-PLA, methoxy poly(ethylene glycol)-poly(L-lactic acid); NP, nanoparticle; TEM, transmission electron microscopy; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PLA-PEG, poly(L-lactic acid)-poly(ethylene glycol).

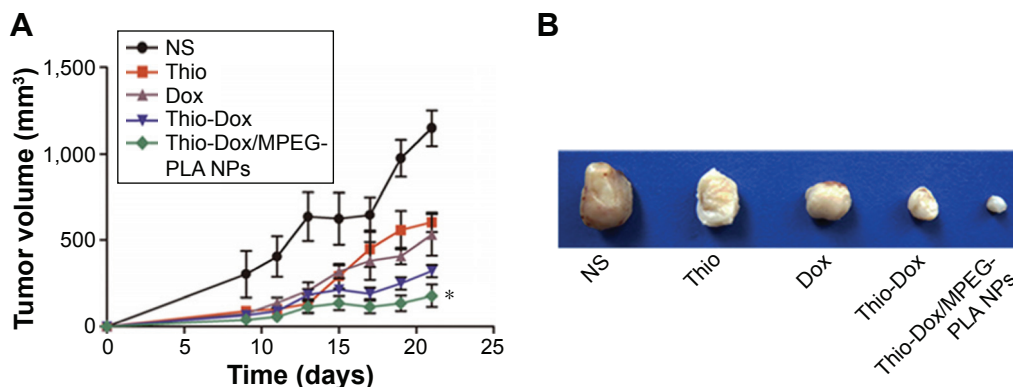


Figure 4 (Continued)

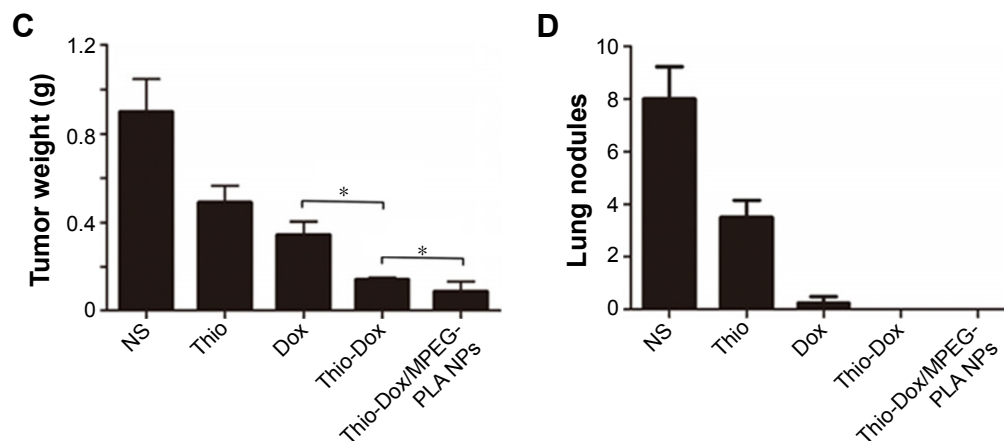


Figure 4 Intravenous injection of Thio-Dox/MPEG-PLA NPs inhibited the growth of breast cancer cells in vivo.

Notes: (A) Tumor development curve. Female mice were subcutaneously transplanted 4T1 cells on day 0. On day 5, the mice were randomized into five groups and intravenously injected with saline (control), free Thio, free Dox, free Thio and Dox, and Thio-Dox/MPEG-PLA NPs twice a week. (B) Representative photographs of tumors in each treated group on day 22. (C) Tumor weight in different groups on day 22. (D) Numbers of lung nodules in different groups. For all graphs, error bars indicate mean \pm SD, * $P < 0.05$.

Abbreviations: Thio, thioridazine; Dox, doxorubicin; MPEG-PLA, methoxy poly(ethylene glycol)-poly(L-lactic acid); NP, nanoparticle; PEG-PLA, poly(L-lactic acid)-poly(ethylene glycol).

effect on metastasis may be due to the marked inhibition of primary tumor growth.

Conclusion

In this study, Thio and Dox were coencapsulated into biodegradable MPEG-PLA NPs. Compared to free Dox and Thio, the prepared NPs were more efficient in inhibiting the growth of breast cancer cells in vitro and in vivo. Taken together, our study suggests that Thio- and Dox-co-loaded MPEG-PLA NPs might have potential application in breast cancer treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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