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Treatment of Liver Cancer Using Specifically Targeted PLGA-based Paclitaxel Nanoparticle

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Abstract

We have designed a nanoparticle drug delivery system. The system included PLGA, paclitaxel (PTX) and transferrin (TF). This system can specifically target to liver cancer cells through intravenously administration. The PLGA-PTX-TF nanoparticles show excellent liver cancer treatment effect (IC50 4.79 g/mL) and are dose dependent *in vitro*. The PLGA-PTX-TF nanoparticle also show noticeable anti-tumor efficacy *in vivo* compared to PLGA-PTX (anti-cancer rate: 79.09% *vs.* 35.47%). Therefore, PLGA-PTX-TF nanoparticles can be considered as an effective anticancer drug delivery system for liver cancer therapy for the clinical use.

Keywords: Paclitaxel • PLGA • Nanoparticles • Tumor specificity • Delivery system

Introduction

Paclitaxel (PTX) is a major anticancer drug in breast cancer [1], prostate cancer [2], ovarian cancer [3], head neck cancer [4], non-small cell lung cancer [5] and the treatment of rheumatoid arthritis [6] because of the unique microtubule stability properties [7]. PTX has poor solubility in water [8]. In order to increase PTX solubility, polyoxyethylene castor oil [9] is used to mix with PTX before intravenous administration. However, the cosolvent will cause the release of histamine in the body [10] and leach the PVC [11] to cause toxic reaction, which limited the clinical application of paclitaxel.

Poly (lactic-co-glycolic acid) (PLGA) is a kind of synthetic polymer materials, which has good biocompatibility [12], slow degradation in body [13] and the end products are non-toxicity [14]. It has been approved by FDA for the biodegradable materials used in human. Compared with polyoxyethylene castor oil, PLGA coated paclitaxel nanoparticles have the following advantages:

1) PLGA can prolong the circulation time and be more comprehensive as well as long-lasting effect on cancer cells when they enter G2/M phase.

2) PLGA has low side effects and the PLGA microspheres can greatly improve the medication safety. Therefore, in this study we choose PLGA as paclitaxel coating materials.

Drug delivery *via* nanoparticle-based carriers has been linked with biological recognition molecules to endow them with the expected function of bio-recognition and specific targeting for biomedical applications. Successful targeting with antibody will result in a specific concentration of the anti-cancer drugs at the intended tumor sites of delivery in a much more effective manner than the passive accumulation of drugs by the enhanced permeability and retention effect [15]. In view of this problem, transferrin (TF) is selected as an excellent choice.

In this article, we develop PLGA-based paclitaxel nanoparticle drug delivery system with specific targeting to liver cancer cells *in vitro* and *in vivo*. The PLGA-PTX-TF nanoparticles have excellent liver cancer treatment effect and are dose dependent *in vitro*. The PLGA-PTX-TF nanoparticle show noticeable anti-tumor efficacy *in vivo* compared to PLGA-PTX. Finally, we determine that PLGA-PTX-TF nanoparticles are effective anticancer drug delivery system for liver cancer therapy [16].

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Materials and Methods

Chemicals

Paclitaxel (J&K Scientific),1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC,Sigma-Aldrich), N-hydroxysulfosuccinimide sodium salt (Sulfo- NHS, Sigma- Aldrich),PLGA (Sigma- Aldrich), transferrin (≥ 98%, Sigma- Aldrich), Taxol (Hospira Australia Pty Ltd), MTT (Sigma- Aldrich).

Preparation of PLGA-PTX-TF nanoparticles

Hydrophobic PTX (1 mg) was dissolved into acetone solution (15 mL) and PLGA (10 mg) dissolved into 30 mL deionized water. The two components were mixed into the bottle under a reduced pressure condition. After rotary evaporation at 35°C for 1 h, the acetone in the bottle was removed and the product PLGA-PTX nanoparticles were stored at 4°C refrigerator for further use.

0.167 mm PLGA-PTX nanoparticles were mixed with 26 mm EDC and 52 mM NHS and reacted for 2 hours at room temperature with continuous shaking. The mixture was purified by Millipore filter for several times. The resulting PLGA-PTX-TF were dispersed in PBS (5 mL) and stored at 4°C for further use.

Cell imaging

HepG-2 cells were propagated in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin (1%). Then the cultured cells were trypsinized and resuspended in this DMEM at a concentration of about 1 × 105/mL. The cell suspension (100 L) was transferred to a confocal dish (35 mm). After incubation for 24 h at 37°C in 5% CO_2, the cells were carefully rinsed with PBS solution (pH 7.4). Then a colloidal solution of PLGA-PTX nanoparticles (100 L, 0.2 g/L) and the same concentration of PLGA-PTX-TF nanoparticles were added, separately. After incubation for 3 h at 37°C in 5% CO_2, the dish was rinsed three times with PBS solution (pH 7.4) and then fresh serum-free medium (1 mL) was added. The plates were incubated for another 10 min at 37°C. Differential interference contrast (DIC) was performed on an IX 71 inverted microscope with a 60 × oil immersion objective (NA 1.45; Olympus), equipped with a digital camera (DVC 1412-B/W, DVC Co., USA). The digital images were collected and analyzed using IP Lab software (BD Biosciences Bioimaging).

In vitro PLGA empty nanoparticles cytotoxicity measurement

HepG-2 cells were trypsinized and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS, 10%) and penicillin/streptomycin (1%). The cells were seeded at a density of 0.2-1.0 million cells per well in a 96-well plate. After 24 h of incubation at 37°C with 5% CO2 (the following steps were carried out under the same conditions), the cells were washed with phosphate buffered saline (PBS, 0.01 mol/L, pH 7.4). The PLGA empty nanoparticles solutions with different concentrations (10 L, PLGA concentration of 10.4 g/mL, 20.8 g/mL, 41.7 g/mL, 83.3 g/mL and 166.7 g/mL) and DMEM (90L) were added to the wells. After 24 h of incubation, the supernatant was removed, and the cells were washed with PBS for three times. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (10 L, 0.5 mg/mL) and DMEM (90 L) were then added to each well. After 4 h of incubation, the medium was discarded, and the intracellular water-insoluble formazan blue was collected by DMSO (100 L). The optical absorbance was measured at 490 nm on a Synergy H4 Hybrid Multi-Mode Microplate Reader. Each data point was collected by averaging that of ten wells, and the untreated cells were used as controls. The data analyses were conducted by Student's t-test. Differences with P values <0.05 were considered to be statistically significant.

In vitro anti-tumoral activity

HepG-2 cells were trypsinized and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS, 10%) and penicillin/streptomycin (1%). The cells were seeded at a density of 0.2-1.0 million cells per well in a 96-well plate. After 24 h of incubation at 37°C with 5% CO_2 (the following steps were carried out under the same conditions), the cells were washed with phosphate buffered saline (PBS, 0.01 mol/L, pH7.4). The PLGA-PTX nanoparticles and PLGA-PTX-TF solutions with different concentrations (PTX concentration of 0.26 g/mL, 0.32 g/mL, 1.04 g/mL, 2.1 g/mL, 4.17 g/mL, 8.34 g/mL, 16.68 g/mL, 33.35 g/mL and 66.7 g/mL) and DMEM (90 L) were added to the wells. After 1 day, 2 days and 3 days of incubation, the supernatant was removed, and the cells were washed with PBS for three times. At determined time, MTT (3-(4,5- dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) solution (10 L, 0.5 mg/mL) and DMEM (90 L) were then added to each well. After 4 h of incubation, the medium was discarded, and the intracellular water-insoluble formazan blue was collected by DMSO (100 L). The optical absorbance was measured at 490 nm on a Synergy H4 Hybrid Multi-Mode Microplate Reader. Each data point was collected by averaging that of ten wells, and the untreated cells were used as controls. The data analyses were conducted by Student's t-test. Differences with P values <0.05 were considered to be statistically significant.

In vivo tumor therapeutic effect study

Athymic nude mice (8 weeks, 20 ± 2 g) obtained from Department of Laboratory Animal Science (Peking University Health Science Center) and injected with HepG-2 liver tumor on shoulders to establish the tumor-bearing mice. The therapeutic effect of PLGA-PTX-TF nanoparticles was assessed by measuring the diameter of the tumors with a caliper. When tumors volume reached nearly 50 mm^3, the mice were randomly divided into three groups: 0.9% saline solution group, PLGA-PTX group and PLGA-PTX-TF group. After administration of different samples *via* tail vein, the tumors were measured every day until 11 days when the mice were sacrificed. The mice body weight was also measured every day with mice electronic body weighing scales.

In vivo imaging with spectra unmixing technique

Athymic nude mice (8 weeks, 20 ± 2 g) obtained from Department of Laboratory Animal Science (Peking University Health Science Center) were injected with HepG-2 liver tumor on shoulders to establish the tumor-bearing mice and then administrated through the tail vein with the PLGA-fluorescent nanoparticles (1 g/mL, 0.2 mL) and PLGA-fluorescent-TF nanoparticles (1 g/mL, 0.2 mL), respectively. *In vivo* fluorescence images of the mice were obtained with Maestro 2 *in vivo* with spectra unmixing system (Cambridge Research & Instrumentation, Woburn, MA). The excitation filter was set as 435-480 nm; the emission filter was 620 nm long-pass filter. The liquid crystal tunable emission filter (LCTF, with a bandwidth of 20 nm and a scanning wavelength range of 500-950 nm) was automatically stepped in 10 nm increments from 500-720 nm, while the CCD captured images at each wavelength with constant exposure. All animal experiments were approved by the Animal Ethics Committee of the Medical School, Peking University.

Results and Discussion

Preparation of PLGA-PTX-TF nanoparticles

In this work, we use PLGA-PTX-TF nanoparticles as the therapeutic nanoparticles, which particle sizes are almost 70 nm. The PLGA-PTX nanoparticles are synthesis by a reduced pressure condition as reported [16] and modified with transferrin by a standard procedure that had been widely used in functionalization of nanoparticles. The excessive EDC and sulfo-NHS to the carboxylic groups on PLGA-PTX nanoparticles form active ester leaving groups, which can react subsequently with free amino groups present in transferrin to form the resulting PLGA-PTX-TF nanoparticles.

Specificity targeted treatment of PLGA-PTX-TF nanoparticles to tumor cells

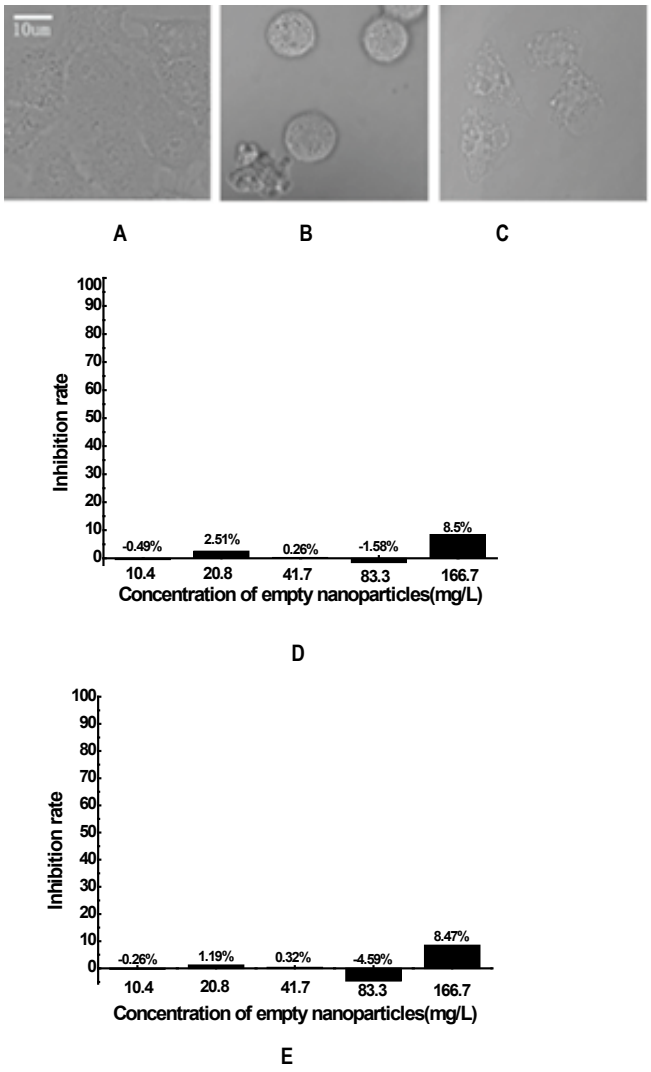
To demonstrate the binding specificity of PLGA-PTX-TF nanoparticles to tumor cells, PLGA-PTX nanoparticles and PLGA-PTX-TF nanoparticles are incubated with HepG-2 cells. After 3 h of incubation at 37°C with PLGA-PTX-TF nanoparticles and PLGA-PTX nanoparticles, it can be clearly seen that PLGA-PTX nanoparticles exhibit weak effect to the HepG-2 cells (Figure 1A and 1B), whereas PLGA-PTX-TF nanoparticles show strong excellent effect to HepG-2 cells (Figure 1C).

In vitro cytotoxicity test of PLGA empty nanoparticles

In order to test the cytotoxicity of empty nanoparticles in the living system, HepG-2 cells are grown for 24 h and 48 h in the presence of PLGA empty nanoparticles. The HepG-2 cells are exposed to DMEM with different concentrations of nanoparticles (PLGA concentration of 10.4 g/mL, 20.8 g/mL, 41.7 g/mL, 83.3 g/mL and 166.7 g/mL) and the percentages of the cells inhibition rate are quantified. Results show that at the largest concentration of PLGA at 24 h, the inhibition rate is 8.5%; and the largest concentration of PLGA at 48 h, the inhibition rate is 8.47%. Therefore, there nearly no inhibition is found based on cell proliferation count both 24 h and 48 h in empty nanoparticles group (Figure 1D and 1E), which means that the empty nanoparticles has no cytoxicity to HepG-2 cells. Meanwhile, it will not affect the evaluation of the inhibition rate of PLGA-PTX and PLGA-PTX-TF nanoparticles in the subsequent experiments.

Comparing the therapeutic effect of Taxol, PLGA-PTX nanoparticles and PLGA-PTX-TF nanoparticles

In this experiment, the *in vitro* therapeutic effects of Taxol, PLGA-PTX nanoparticles and PLGA-PTX-TF nanoparticles are evaluated by MTT method. After 3 days' administration of PLGA-PTX-TF, there is continuous inhibit effect on the proliferation of HepG-2 cells because of the biodegradability of PLGA. The maximal therapeutic effect of PLGA-PTX-TF appears at 66.7 g/mL, on which concentration cell viability is only 10.7%. However, there's no obvious therapeutic effect on HepG-2 cells in the Taxol group especially when the treatment time prolonged (Figure 2A). Comparing to the Taxol group, the anti-tumor activity of PLGA-PTX and PLGA-PTX-TF is obvious, including IC50 of PLGA-PTX-TF is 4.79 g/mL and IC50 of PLGA-PTX is 12.59 g/mL, respectively. In the PLGA-PTX group, there is weaker treatment comparing to PLGA-PTX-TF group after 3 days' administration and cell viability is 43% at 66.7 g/mL(Figure 2B). In addition, with the increase of PLGA-PTX-TF nanoparticles from 0.26 g/mL to 66.7 g/mL, the cell viability of HepG-2 cells decrease significantly, showing that it is a dose-dependent therapeutic manner (Figure 2C). That because the high concentration of paclitaxel can induce cell microtubule to form stable bundles, generating a large number of microtubule complexes. Therefore, cell division arrest in G2/M phase. In summary, PLGA-

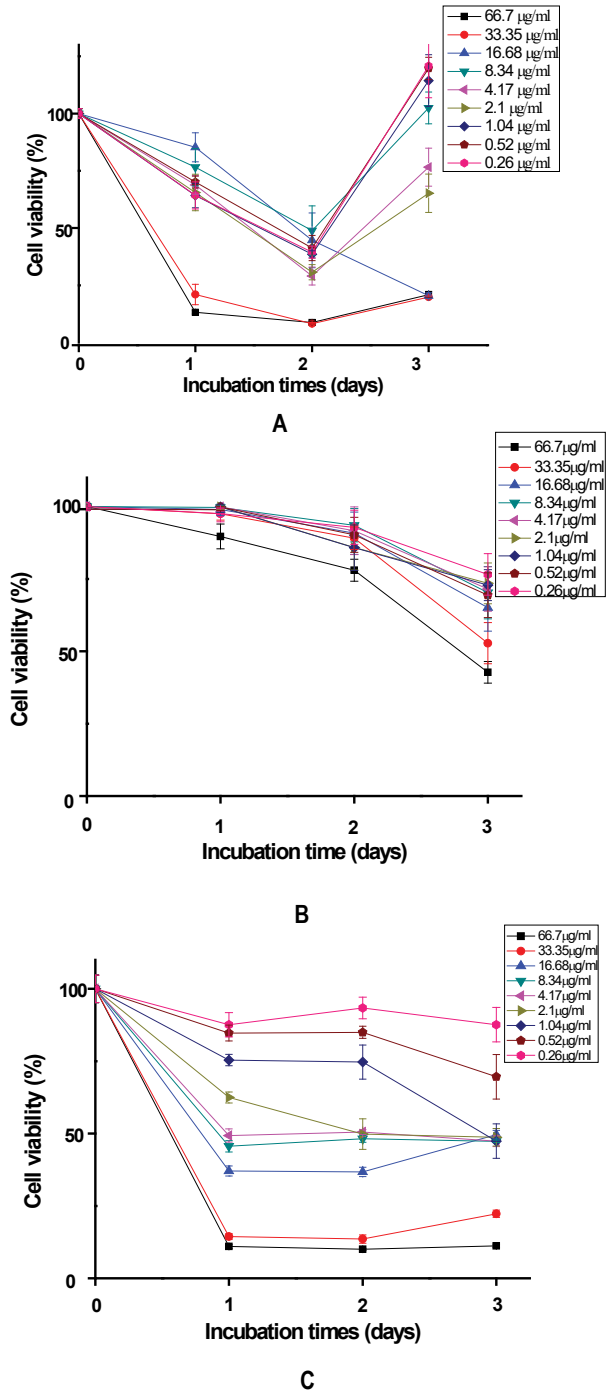


Figures 1. (**A**) *In vitro* cells therapeutic experiments of normal HepG-2 cells; (**B**) PLGA-PTX nanoparticles administrated into HepG-2 cells; **C**) PLGA-PTX-TF nanoparticles administrated into HepG-2 cells; (**D**) *In vitro* cytotoxicity test of empty nanoparticles at 24 h and (**E**) *In vitro* cytotoxicity test of empty nanoparticles at 48 h.

PTX-TF nanoparticles can effectively inhibit the growth of liver cancer cells, and can maintain the drug effect for a long period of time. Meanwhile, with the increase of the PLGA-PTX-TF nanoparticles concentration, antitumor activity is also increased.

In vivo therapeutic effect of nanoparticles in HepG-2 xenograft tumor animal models

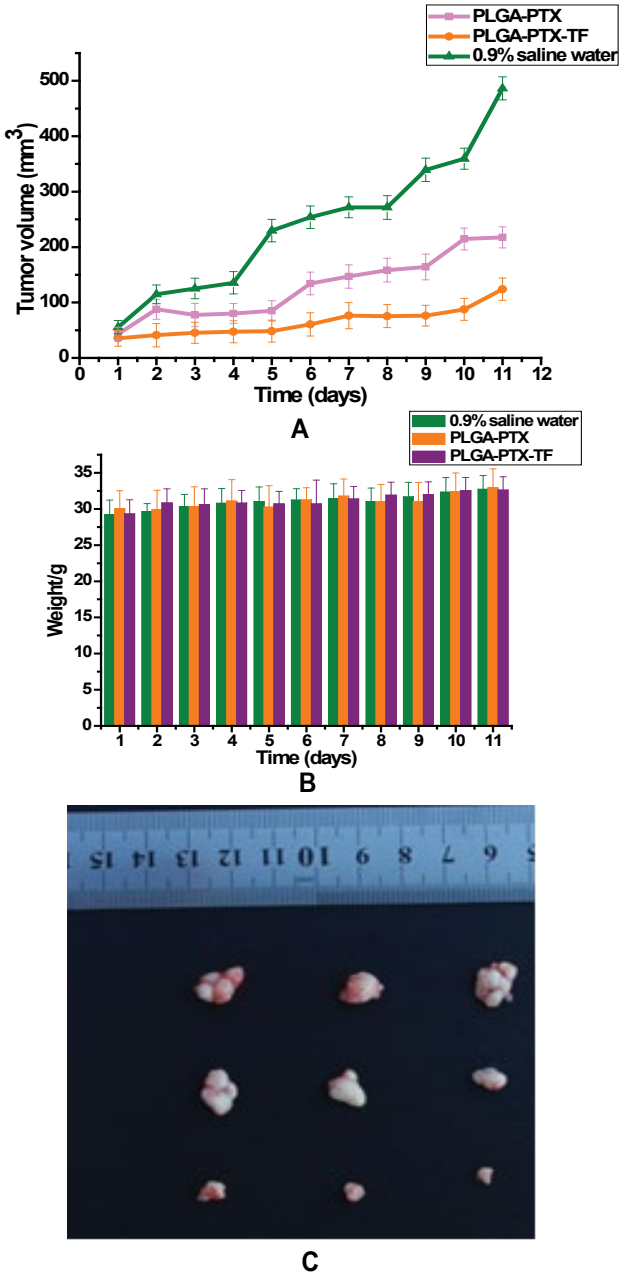
Liver cancer mice models are randomly divided into three groups and each group has 5 mice. The average weight difference between each group is less than 1 g. The same dose of 0.9% saline solution, PLGA-PTX nanoparticles and PLGA-PTX-TF nanoparticles are injected into mice *via* tail vein. The volumes of tumors are measured each day till 11 days (Figure 3A). The results show that the best treatment group is PLGA-PTX-TF nanoparticles group during the three groups with the slowest increase of tumor volume from day one to day eleven. PLGA-PTX also has the smaller tumor growth volume comparing to 0.9 % saline water group. The anti-cancer rate is 35.47% in PLGA-PTX nanoparticles group and 79.09% in PLGA-PTX-TF nanoparticles group. Therefore, the results show that PLGA-PTX-TF nanoparticles group has the obvious anti-cancer effect, mainly because of the tumor targeting property. Mice body weights in the three groups show no significant differences throughout the 11 days' study. There are slightly increase of the body weight because of the natural growth of the mice (Figure 3B). Mice are sacrificed after 11 days' study, and the tumor are completely stripped from subcutaneous tissue as shown in Figure 3C.



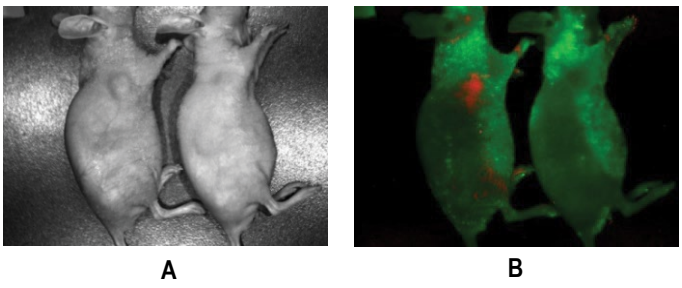
Figures 2. **(A)** *In vivo* therapeutic effect of 0.9% saline water group, PLGA-PTX nanoparticles group and PLGA-PTX-TF nanoparticles group; (**B**) Mice body weights in 0.9% saline water group, PLGA-PTX nanoparticles group and PLGA-PTX-TF nanoparticles group during the 11 days and **(C)** Subcutaneous tumors of 0.9% saline water group, PLGA-PTX nanoparticles group and PLGA-PTX-TF nanoparticles group.

In vivo imaging of PLGA-PTX-TF nanoparticles specifically targeted to tumor site.

We use fluorescent nanoparticles as we report before [16] to replace the paclitaxel under the same package process to simulate the PLGA-PTX nanoparticles and PLGA-PTX-TF nanoparticles *in vivo* targeting tumor process. In our experiments, athymic nude mice bearing subcutaneous HepG-2 tumor (Figure 4A) are administered with PLGA-fluorescent nanoparticles and PLGA-fluorescent-TF nanoparticles through intravenous injection, respectively. Images are taken at 3 h by the Maestro 2 *in vivo* imaging system. Using spectral unmixing technique, we separate the auto fluorescence background signals from the fluorescent nanoparticles signals (Figure 4B). The composite image



Figures 3. (A) *In vivo* therapeutic effect of 0.9% saline water group, PLGA-PTX nanoparticles group and PLGA-PTX-TF nanoparticles group; **(B)** Mice body weights in 0.9% saline water group, PLGA-PTX nanoparticles group and PLGA-PTX-TF nanoparticles group during the 11 days and **(C)** Subcutaneous tumors of 0.9% saline water group, PLGA-PTX nanoparticles group and PLGA-PTX-TF nanoparticles group.



A B

Figures 4. *In vivo* imaging of HepG-2 tumor-bearing mice (tumor: right shoulder inside violet circles) injected with PLGA-fluorescent-TF nanoparticles (left mouse) and PLGA-fluorescent nanoparticles (right mouse) via the tail vein. **(A)** White image of HepG-2 tumor-bearing mice and **(B)** Unmixed fluorescent images of HepG-2 tumor-bearing mice.

clearly shows the whole animal and the tumor sites which indicate that the PLGA-fluorescent-TF nanoparticles can specifically targeted to the tumor site.

On the other hand, control group injected with PLGA-fluorescent nanoparticles have relatively low accumulation in tumor sites, and the luminescent signals are too weak to be detected. From the tumor targeting experiment, it can be concluded that PLGA-PTX-TF exhibit efficiently affinity and specificity to tumor cells through transferrin receptor *in vivo*.

Conclusion

In conclusion, we have designed a nanoparticle drug delivery system with specific targeting to liver cancer cells *in vitro* and *in vivo*. The PLGA-PTX-TF nanoparticles show excellent liver cancer treatment effect. The PLGA empty nanoparticles have nearly no cytotoxicity. The PLGA-PTX-TF nanoparticle show noticeable anti-tumor efficacy *in vivo*. From the results above, we can safely draw the conclusion that PLGA-PTX-TF nanoparticles are effective anticancer drug delivery system for liver cancer therapy.

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