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Self-assemble pH-responsive dextran-g-Poly (lactide-co-glycolide)-g- histidine copolymer micelles for intracellular delivery of paclitaxel and its antitumor activities

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pH-sensitive copolymers have been widely used in drug delivery system to endow selectively drug release in tumor sites. Here in, dextran (DX) was conjugated with Poly (lactide-co-glycolide) (PLGA) and histidine (His) to prepare a pH-response nanocarrier, dextran-g-Poly (lactide-co -glycolide)-g-histidine (HDP) micelles, for the delivery of antitumor drugs. Different grafted ratio HDP micelles were synthesized and prepared successfully and confirmed by the critical micelle concentration (CMC) and particle size distribution (PSD). *In vitro* drug release showed that the release behaviour of paclitaxel (PTX) loaded HDP micelles was pH-dependent. All blank micelles were nontoxic *in vitro* cytotoxicity assay. In MTT assay, PTX-loaded HDP micelles with medium grafted ratio (MHDP) showed the highest cytotoxicity against MCF-7 cells. Cellular uptake experiments revealed that these pH-sensitive micelles could be taken up effectively and delivery PTX into cytoplasm to reach antitumor effect. NIR fluorescence image experiment demonstrated that HDP micelles could specifically accumulate in tumor sites via enhance permeability and retention (EPR) effect to reduce its systematically toxicity. *In vivo* antitumor activities showed that HDP micelles could effectively inhibit tumor growth and prolong survival time compared with free PTX solution. These results confirm that the biocompatible pH-response HDP micelles are a novel nanocarrier for the intracellular delivery of PTX.

1.Introduction

Over the last few decades, cancer is a great challenge to human's health[1-4]. Chemotherapy plays a significant role in the treatment of cancer[5-7]. However, serve side effects and systemic toxicity often limits its clinical application[8, 9]. Thus, reduce adverse effects and times of administration, increase therapeutic effect is becoming the main problem in drug delivery system (DDS). Polymeric micelles (PMs), which consisting of hydrophilic side chains and hydrophobic core, is playing an important part among nano-sized drug delivery system[10-12]. On the one hand, they can self-assemble in aqueous condition, on the other hand, they can increase drug encapsulation efficiency (EE %) and drug loading content (LC %) via hydrophobic interaction[13-15] to enhance its solubility. Last but not the least, because of their nano-sized, they can accumulate in tumor sites via the passive "enhanced permeability and retention (EPR) effect" and escape from the reticuloendothelial system (RES)[16-19].

PMs can self-assemble in aqueous solution over the critical micelle concentration (CMC) because of its amphiphilic. Among all the hydrophilic chains, Poly (ethylene glycol) (PEG) is the most widely used in the synthesis of PMs carrier due to it could avoid phagocytosis of RES and prevent hydrophobic compounds from the absorption of blood proteins to prolong their circulation time *in vivo*[20, 21]. However, few functional groups to modify is becoming the main obstacle of PEG. In contrast, dextran (DX), a homopolysaccharide of glucose, has many hydroxyl groups on the surface of dextran, so it is easy to modify various functional groups on the surface[22]. Besides this, due to its unique properties, such as biodegradability, wide availability, and nonfouling property[23], which properties are similar to PEG, it attracted considerable interest for highly desirable for anticancer drug delivery. As for hydrophobic

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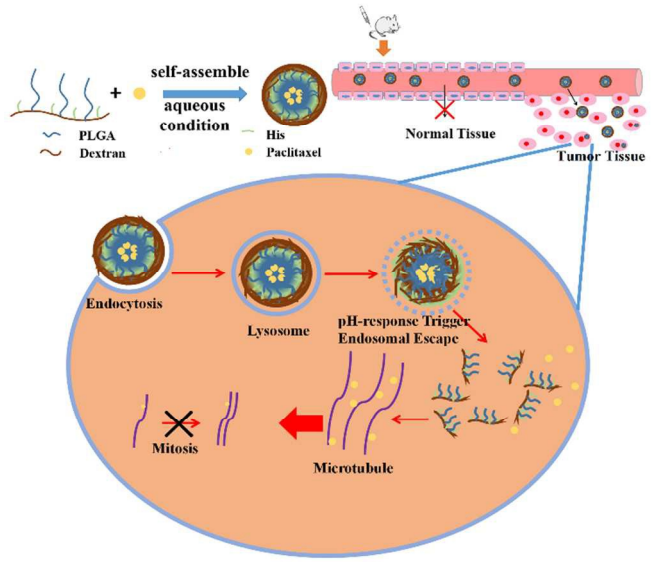
chain, Poly (lactide-co-glycolide) (PLGA) is a FDA approval biodegradable polymer which has been widely used for synthesis of nanoparticles for sustained, controlled and targeted drug delivery[24]. PTX has been widely used in clinical to treat varies of tumours including breast, ovarian, non-small cell lung, prostate, and gastric cancers[25]. The chemical conjugation of DX-PLGA has been reported to delivery PTX previously[26] However, a major barrier of this preparation is inefficient drug release, which may significantly decrease drug's therapeutical effect. The rapid drug release is indispensable for the preparation to specifically release drugs to reach therapeutical efficiency.

How to delivery therapeutical agent efficiently is a major hot plot of research. Although PMs can reach tumor sites via EPR effect, how to



Scheme 1 schematic diagram of self-assembly HDP micelles and mechanism of HDP micelles to enhance antitumor activities.

release drugs rapidly in tumor and reach therapeutic effect is another problem. As we all know, after internalization, PMs are challenging against varies of intracellular barriers[27]. The pH of tumor microenvironment is lower contrasting with normal tissues and in blood, which is about 6.0 to 5.0 in intracellular early lysosomes and late lysosomes, respectively[28]. In order to prevent degradation in lysosome and release drug in cytoplasm, the PMs should be biocompatibility and pH-sensitivity. It is necessary to find an appropriate biomaterial which have pH-sensitive. Histidine (His) has attracted extraordinary interest in pH-response groups due to its biodegradable, biocompatible and amphoteric nature[29-31]. His-based copolymer micelles can protonation in acid environment because of the imidazole ring, followed by releasing drug to cytoplasm[32, 33]. Yao and his co-workers have synthesized histidine modified dextran-g-cholesterol copolymer, which showed excellent pH-sensitive feature and better cell cytotoxicity against tumor cells[34]. In this study, histidine was used to modify Dex-PLGA (HDP) to endow pH-sensitivity. As is shown in scheme 1, HDP micelles could self-assemble in aqueous solution and reach tumor via EPR effect. Then HDP micelles would be internalized into tumor cells via endocytosis and packaged by lysosomes. After that, the micelle disassemble rapidly due to pH-response protonation of imidazole rings of His, which lead to instability of lysosome membrane and enhanced permeability of membrane. Finally PTX could release from micelles rapidly and escape from lysosome to reach cytoplasm, followed by accumulating in microtubule to exert its antitumor effects. The loading capability and acid-response release of PTX were demonstrated. The characteristics of HDP micelles such as particle size distribution, *in vitro* drug release, cell cytotoxicity and cellular uptake were tested. Furthermore, the *in vivo* antitumor activities was also investigated. This pH-sensitive micelles exhibit excellent abilities in intracellular drug delivery.

2. Materials and methods

2.1 Materials

Dextran (Dex, Mn=10kDa); N-α-T-butoxycarbonyl-L-histidine (Boc-His-OH) were purchased from Aladdin industrial corporation. Poly (lactic-co-glycolic acid) (PLGA, L/G=70/30, 1.5KDa) was purchased from Shandong daigang biotechnology CO. Ltd, China, 1-(3-Dimethylamino -propyl)-3-ethylcarbodiimide hydrochloride (EDC) and 4-dimethylamino -pyridine (DMAP) were purchased from Shanghai Medpep Co. Ltd, China. Paclitaxel (PTX) was supplied by melone biotechnology CO. Ltd (Dalian, China). 3-（4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Coumarin 6 was brought from Sigma-Aldrich (MO, USA). Dulbecco's modified Eagle medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Gibco BRL (Maryland, USA). All the other chemicals and buffer solution components were analytical grade.

2.2 Synthesis of Dex-g-PLGA (DP)

The synthesis procedure was described as previously with some changes[26]. In brief, Dex(50 mg, 0.005 mmol), PLGA(113.1 mg, 0.0754 mmol), EDC(28.8 mg, 0.15 mmol), DMAP(18.3 mg, 0.15 mmol) were dissolved in 10 ml of dry dimethyl sulfoxide (DMSO) in a flask with N_2 atmosphere protection. The mixture was stirred at room temperature for 72h. After reaction, the mixture was submitted in a dialysis membrane (MWCO: 3.0 KDa) and dialyzed against deionized water for 3 days. The final solution was lyophilized and Dex-PLGA (DP) was received.

2.3 Synthesis of Boc-His-OH modified Dex-g-PLGA(HDPB)

DP (112 mg, 0.004 mmol), Boc-His-OH (5.58mg, 0.024 mmol), 1-ethyl-3-(3-dimethyll -aminopropyl) carbodiie hydrochlide (EDC·HCl 93 mg, 0.484 mmol), 4-dimethylaminopyridine (DMAP) (5.9 mg, 0.0484 mmol) were dissolved in 10 ml of DMSO. The mixture was stirred at room temperature for 3 days. Then, the solvent was removed by dialysis against deionized water for 3 days, followed by lyophilized.

2.4 Deprotection of HDPB copolymer

HDPB (100 mg, 3.57 mmol) was dissolved in 20 ml of dichloromethane (DCM) and trifluoroacetic acid (TFA) mixed solvent (DCM: TFA 6: 4) in a flask. The mixture was reacted for 40 min at 40°C, followed by removing the organic solvent using rotatory evaporator. The obtained copolymer HDP was characterized

by ^{1}H nuclear magnetic resonance (NMR) spectrometry (400 Hz, Bruker, Germany).

2.5 Characterization of HDP micelles

The critical micelle concentration (CMC) of different copolymer was determined using a fluorescence spectrophotometer with pyrene as a hydrophobic probe[35]. Briefly, pyrene solution in acetone (6.0×10^{-4} M) was prepared, 1 ml solution was added to each brown volumetric flask and the solution was then evaporated completely under a gentle nitrogen. Copolymer solution (concentration ranging from 10^{-4} -10^{-1} mg·ml^{-1}) was added to reach a final pyrene concentration of 6.0×10^{-7} M, respectively. Then the solution was ultrasonic for 30 min and kept at room temperature overnight. The fluorescence intensity was measured using a fluorescence spectrophotometer (LS55, PerkinElmer, USA). The excitation spectrum was recorded from 305 to 350 nm with an emission wavelength of 390 nm. The intensity ratio of peak (I_1, 334 nm) and peak (I_3, 337 nm) in the emission spectrum was analysed for calculation of the CMC.

2.6 pH-responsive behaviour of HDP micelles

HDP copolymer was dispersed in PBS solution and 5 ml of the solution was taken and adjusted its pH from 7.4 to 4.0. The change of the particle size was observed by dynamic lighting scanning using a Zetasizer (Nano ZS, Malvern Co. Ltd, UK). Each sample was measured for 3 times.

2.7 Preparation and characterization of PTX-loaded HDP micelles

PTX-loaded micelle was prepared using a simple dialysis method[36]. Briefly, 10 mg of copolymer and 2 mg of PTX were dissolved in 2.0 ml DMSO. The mixture was stirred at room temperature for 24h and then added dropwise into 10 ml PBS at pH 7.4. The organic solvent was removed using dialysis method against water for 24h. Finally the solution was filtered and lyophilized. Drug loading content (LC %) and encapsulation efficiency (EE %) of PTX-micelle was measured as described previously with minor modification[37]. Briefly, 0.5 ml micelle solution was mixed with 9.5ml of DMSO and disrupted by ultrasonic treatment in an ice bath for 3 min to dissolve all PTX. After filtration through a 0.22μm filter. The solution was measured. The amount of PTX was calculated from the calibration curve, which had a linear range between 1-20 μg ml^{-1} using high performance liquid column (HPLC) method and the analytes were eluted using acetonitrile-water(50:50, v/v) at 227 nm of detection wavelength. The encapsulation efficiency (EE %) and drug loading capability (DL %) was calculated as the formula shows.

EE %= weight of the drug in micelles/weight of the feeding drug× 100

DL %= weight of the drug in micelles/ weight of the feeding copolymer and drug×100

2.8 *In vitro* release of PTX from HDP micelles

The release profiles of PTX from micelles were investigated using a dialysis bag (MWCO 3.5 KDa) at 37 °C. In brief, 2.0 ml of micelle solution was transferred to a dialysis bag and then place in conical flask with 100 ml different PBS solution (at pH 7.4 and 5.0) with 0.5% Tween 80 to meet sink condition. Both flasks were placed in shaking incubator at a stirring speeding of 100 rpm. At predetermined intervals, 1.0 ml of samples were taken and equal volume of fresh buffer were added to maintain the total volume. Concentration of PTX were measured using a HPLC method as described previously. The cumulative percentage drug release (Er) was calculated as follows:

$$E_r(\%) = \frac{V_0 C_i + V_0 C_n}{M_{PTX}} \times 100$$

In this formula, M_{PTX} represents the amount of PTX in the micelle, V_0 is the total volume of the release medium, C_i is the concentration of PTX in the *i* th sample.

2.9 *In vitro* cytotoxicity assays

The cytotoxicity of blank micelles and PTX-loaded micelles against MCF-7 cells were evaluated by the standard MTT assay[38]. In brief, the cells were seeded in 96-well plates at a destiny of 1×10^4 per well to allow cell attachment. The cells were then incubated with free PTX, blank micelles and PTX-loaded micelles in a concentration gradient at 37°C. After 24h, 48h and 72h, 10 μl MTT (5 mg ml^{-1}) was added in each well and further incubated for 4h. The medium in each well was removed and 100 μl of DMSO was added to dissolve the internalized purple formazan crystals. The absorbance was recorded at 490nm using a BioRed microplate reader (MK3, Thermo, USA). The relative cell viability (%) was calculated as follows:

$$\text{Cell viability (\%)} = \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \times 100$$

Where $A_{control}$ and A_{sample} are the absorbance in the absence and in the presence of sample treatment respectively, and A_{blank} in the absorbance of the medium.

2.10 Cell uptake of coumarin loaded HDP micelles

The cell uptake of micelles was observed by fluorescence microscopy and flow cytometry methods. Coumarin-6 was loaded into the micelles using the same method as described previously. MCF-7 cells were seeded in 6 well plates at a density of 2×10^4 per well and incubated of 48h at 37 °C. Free coumarin-6 and coumarin-loaded micelles (10.0 μg ml^{-1} of coumarin-6) in serum–free medium was added and incubated for 0.5, 2 and 4h. After incubation, the cells was washed using phosphate buffered saline (PBS) for 3 times to remove the regents. Then Hoechst 33258 (10 ug ml^{-1}, 15min) was used to visualize the nuclei. In the end, cells were viewed by fluorescence microscopy.

In order to analyse cell uptake of different regent quantitatively, flow cytometry was also used. MCF-7 cells were seeded in 6 well plates at a density of 2×10^4 and incubated of 48h at 37 °C. Free coumarin-6 and coumarin-loaded micelles(10.0 μg ml^{-1} of coumarin-6) in serum–free medium was added and incubated for 0.5, 2 and 4h, the cells were washed three times with cold PBS (pH 7.4), harvested

and resuspended in 0.5 ml PBS for the flow cytometrix analysis. All measurements were detected in triplicate.

2.11 *In vivo* antitumor activity studies

In order to evaluate *in vivo* antitumor activity of PTX-loaded micelles, nude mouse xenograft model bearing H22 cells was prepared. When the tumor volume is approximately 100 mm^3, the mice were divided randomly into three groups with different treatment by injecting 0.2 ml of formulation via the tail vein on days 0, 2, 4, 6, 8, 10, 12. 14, 16. The mice were treated with one of the following regimens (n=9): (a) 0.9% sodium chloride solution (i.v., q2d ×8) (b) paclitaxel solution (12.5 mg/kg PTX, i.v., q2d × 8), (c) HDP/PTX (10mg/kg PTX, i.v., q2d × 8) (c) HDP/PTX (20 mg/kg PTX, i.v., q2d × 8). The tumor volume and body weight in mice were measured every 2 days with Vernier calipers and tumor volume were calculated using the formula as follows:

$$V = \frac{a \times b^2}{2}$$

Where a and b are the biggest and smallest diameter, respectively. At the end of the experiment, the mice were sacrificed and the tumor were harvested and measured. The inhibition rate (IR %) of tumor growth was calculated using the equation:

$$IR\% = \frac{W_n - W_t}{W_n} \times 100$$

Where W_n and W_t mean the tumor weight of negative control group and treated group. At day 14, three rats of each group were sacrificed for measuring the tumor size, other six rat were used to measure the survival curves. The survival time of all rats were recorded each day until the day of all rats' death. All the animal experiments were performed in accordance with the Experimental Animal Administrative Committee of Shenyang Pharmaceutical University.

2.12 *In vivo* NIR fluorescence imaging of HDP micelles

In order to assess the *in vivo* biodistribution of HDP micelles in nude mice bearing H22 tumor cells. *In vivo* fluorescence imaging study was used and 1, 1'-dioctadecyl-3, 3, 3', 3'- tetramethyl indotricarbocya- nine Iodide (DiR, Invitrogen, USA) was chosen as fluorescence probe to load into the HDP micelles. The preparation method of DiR-loaded HDP micelles were in the same way as PTX-loaded micelles. The nude mice bearing H22 tumor cells were injected intravenously via tail vein at a dose of 0.2 ml DiR-loaded HDP micelles. At various time point the mice were anesthetized and imaged using an *in vivo* image system (Carestream, USA).

2.13 Statistical analysis

All of the data are presented as the mean ± standard deviation (SD) and analysed using SPSS software. Statistical analyse were examined using a One-way analysis of variance (ANOVA) and comparison among groups using independent sample *t*-test. A value of $P < 0.05$ was considered statistically significant and all the data were tested as least 3 times.

3. Result and discussion

3.1 Synthesis and characterization of HDP copolymers

The characterization of HDP was shown in Fig.1. His and PLGA were grafted on the Dex's surface via ester reaction. In this investigation, Dex could be invoked as a hydrophilic group and PLGA was used as a hydrophobic group. His was grafted on Dex to render it pH-sensitive. Peaks of 4.1 and 1.5 ppm corresponded to the protons away from glucosidic linkage in glucose unit and methyl of PLGA. The peaks at 8.2 ppm, 7.6 ppm belonged to histidine. In ^{1}H-NMR spectrum of HDP, there is no peaks in 13.0 ppm, indicating that HDP has been synthesized successfully and the unreacted PLGA and Boc-His-OH have been removed. In order to evaluate its pH-sensitive, we synthesized three different grafted ratio HDP through controlling the molar ratio of the carboxyl groups of Boc-His-OH and hydroxyl groups of dextran and used dextran-PLGA copolymer as control. The grafted ratio was determined by calculating the relative intensity ratio between the protons of hydroxyl groups of dextran appearing at 4.1 ppm, the peak of histidine appearing at 7.6 ppm and -CH_3 groups of PLGA at 1.5 ppm by ^{1}H NMR spectrum of HDP. When the molar ratio of the hydroxyl groups of dextran to the carboxyl group of Boc-His-OH was 1:1, 1:2 and 1:4, the grafted ratio was 15%, 27% and 39%, named LHDP, MHDP and HHDP, respectively. The gafted ratio of PLGA and His-OH was shown in Table 1.

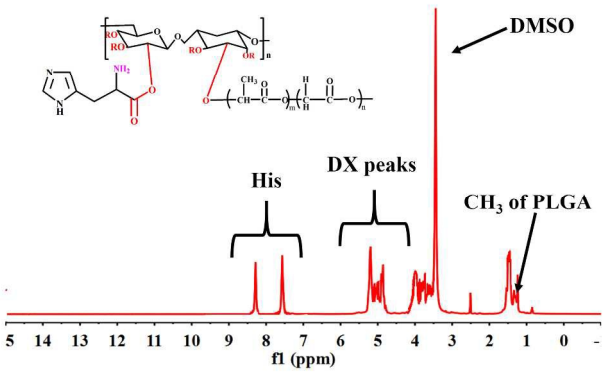


Fig.1: Typical ^{1}H-NMR spectrum of HDP copolymer in DMSO

Table 1 Grafted ratio of PLGA and His-OH in DX chain from different formulation

	GR of PLGA[a](%)	GR of His-OH[b](%)
DP	16	-
LHDP	16	15
MHDP	16	27
HHDP	16	39

a: GR of PLGA= $A_{1.3} \times 0.33/A_{4.0}$ b: GR of His-OH=$A_{7.6}/A_{4.0}$

The CMC is one of the most important parameters influencing the aggregation of copolymer micelles and diluting stability in hydrous solution. In this study, CMC value of different grafted ratio copolymer was determined by fluorescence spectroscopy using pyrene as a hydrophobic fluorescence probe. As is shown in Fig.2a, the fluorescence intensity ratio of I_{337}/I_{334} could increase dramatically when the nanoparticle forms by self-assembly. The CMC value of DP, LHDP, MHDP and HHDP were 53.13,36.39, 25.06 and 22.80 μg ml^{-1}, respectively, which is significantly lower than those of other low molecular weight surfactants[39]. The CMC value decreased as the grafted ratio increased and among all of them, HHDP showed the lowest CMC concentration because the more hydrophobic groups grafted, the more compact when the micelle formed, which result in the lower CMC. The pH-sensitive feature of HDP copolymer was investigated by the size change at different pH value. As is illustrated in Fig.2b, when the pH was over 7.4, the average particle size remained almost unchanged. However, stepwise decrease pH value of the solution resulting in an increase in average size between pH 7.4 and 4.0, and the particle size increased from 235.3 nm to 433.7 nm. This pH-response behaviour was because the protonated of imidazole rings, leading to the swollen of micelles and an increase in the average size[40].

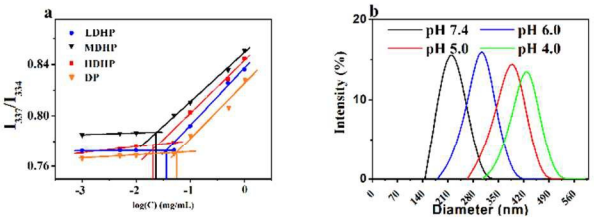


Fig.2 characterization of HDP copolymer micelles. (a): CMC determination (b): particle distribution of HDP micelles in different pH value.

3.2 Formation and characterization of PTX-loaded HDP micelles

Preparation of different micelle using a simple dialysis method without adding other agent. His-OH served as a pH-sensitive part, where it was hydrophobic in pH 7.0 and hydrophilic when the pH is below 6.0. PTX was physically incorporated into the micelle core, which named as DP/PTX, LHDP/PTX, MHDP/PTX, and HHDP/PTX. Owing to His-OH's pH-response character, PTX-loaded micelle could sustained release drug in normal condition and rapid release in acidic condition. DP micelles were prepared using the same method as before. The average particle sizes, EE (%) and DL (%) of the PTX-loaded micelles are summarized in Table 2.

Table 2 Characterization of PTX-loaded HDP micelles

	Size(nm)	EE[a] (%)	LC[b] (%)
DP	252.4±0.12	88.65±3.1	14.78±0.22
LHDP	235.3±0.03	87.05±2.9	14.50±0.21
MHDP	183.9±0.04	75.63±3.5	12.61±0.22
HHDP	152.2±0.06	70.55±4.3	11.76±0.25

[a] EE (%) = encapsulation efficiency. [b] LC (%) = drug loading content

It was found that compared with DP micelles, the average sizes of HDP micelles with different grafted ratio decreased as the grafted ratio increased. This was probably because that the hydrophobic strength of micellar core resulting in the decrease of the size. In addition, the decrease of EE% and LC% was probably because PLGA and His-OH are both in the micellar core in normal pH, strong hydrophobicity will form a weak exclusion to prevent PTX loaded into the micelle.

3.3 pH-response drug release of PTX from HDP micelles

The PTX release from DP, LHDP, MHDP and HHDP micelles were investigated in PBS(containing 0.5% w/w Tween 80) with different pH 7.4 (physiological pH) and pH 5.0 (lysosomal pH). PTX was a water-insoluble drug, Tween 80 was added to increase its solubility to achieve sink conditions. As is shown in Fig.3, the release behaviour of PTX from both DP and HDP micelles in different pH value showed a biphasic pattern characterized with a relatively rapid drug release followed by a sustained drug release. Specifically, at pH 7.4, no more than 40% of PTX released from HDP micelles after 8 h. In contrast, there was approximately 57.5% of PTX release from micelles after 8 h at pH 5.0. Release data for LHDP and MHDP showed a significant difference ($P<0.05$) at pH 5.0, which was probably due to the different grafted ratio of His-OH in the dextran chains. There were too much of histidine modified on the dextran in HHDP, which would make the hydrophobic core so compact that prevent the drug release from micelles. This phenomenon would result in the insufficient drug release from HHDP/PTX compared with MHDP/PTX. Furthermore, the rapid drug release from the MHDP micelles in pH 5.0 might also be related to the swollen hydrophobic core and made PTX close to the surface of the micelles and diffused into the medium[41]. Interestingly, there were no significant differences among the other micelles neither at pH 7.4 nor 5.0, respectively. This suggested that the HDP micelles had pH-sensitive ability and PTX-loaded micelles could release slowly in normal tissue but rapid release from micelles in acidic condition. This would make sure that HDP micelles could deliver PTX effectively intracellular and reached a better therapeutical effects. In comparison, there was no significant difference ($P<0.05$) of DP/PTX micelles in the release behaviour no matter in pH 7.4 or 5.0. This was because there was no pH-sensitive material modified on the surface of the

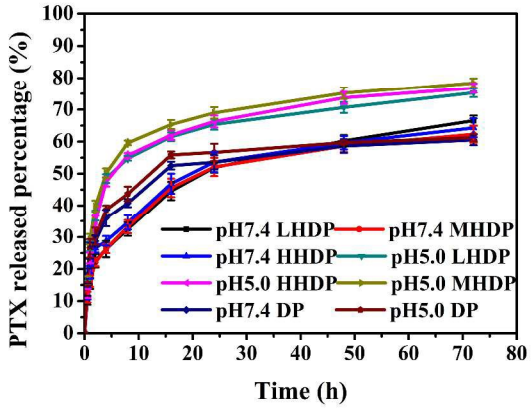


Fig.3 pH-sensitive release of PTX from HDP micelles at 37°C. (n=3)

dextran and the release environment didn't influence its release behaviour. However, there was approximately 55.0 % and 45.0% of drug released from the DP micelles and HDP micelles in normal environment (pH 7.4), respectively. This result was probably because compared with HDP, there was no histidine groups modified on the surface of dextran, which lead to the weaker hydrophobic interaction between PTX and DP, followed by rapid release compared with HDP micelles. Overall, prepared HDP micelles can distinguish lysosome pH from physiological pH by increasing drug release. In normal environment, His-OH was hydrophobic and located in the inner core of the micelles. However, when the pH was lower at about 5.0, His-OH was protonated and became hydrophilic. Finally, it located in the outer shell of the micelles. For this reason, HDP micelles' structure is not as stable as before and PTX was released from the micelles.

3.4 *In vitro* cytotoxicity assay

The cell viability of DP and HDP blank micelles and PTX-loaded micelles was evaluated using the MTT method. As for blank DP and HDP blank micelles, we used MCF-7 cell to investigate its cytotoxicity. The cell viability of blank micelles was measured after a 72h incubation. As is shown in Fig.4a, blank micelles with different grafted ratio were nontoxic and the cell viability was all over 90% at

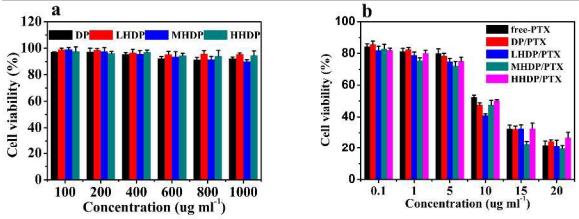


Fig. 4. a: *In vitro* cytotoxicity of blank micelles treating with MCF-7 cells b: PTX-loaded HDP micelles and free PTX in MCF-7 cells (n = 3).

different concentration (10-1000 μg ml^{-1}), which indicated that both DP and HDP copolymer were nontoxic and biocompatible. It could be used as drug delivery system for anticancer drugs.

The cytotoxicity of PTX-loaded micelles and free-PTX was also observed using the same method. As is shown in Fig.4b, cell viability was decreased as the dose increased, which showed significantly dose-dependent feature. The IC_{50} values of DP/PTX, LHDP/PTX, MHDP/PTX, HHDP/PTX and free-PTX groups were 9.58±1.45, 7.81±1.03, 7.01±0.98, 9.02±0.97 and 10.14±1.70 μg ml^{-1}, respectively. MHDP/PTX showed the lowest IC_{50} value, suggesting that the cytotoxicity of MHDP/PTX was higher than other groups. This was probably because that more His-OH on the dextran chains might make hydrophobic core so compact that prevented it contacting with the outer environment, resulting in the difficulty of protonated of imidazole rings, followed by decreasing drug release and reducing its cytotoxicity.

3.5 *In vitro* cellular uptake studies

Coumarin-6 (C6) was used as a fluorescence probe to evaluate the cellular uptake of different formulation and C6 solution was used as a negative control. As is shown in Fig.5a, it could be observe weak green fluorescence signals in MCF-7 cells treated with different formulation for 0.5 h. Then, the intracellular C6 was increased and the green signals became stronger after incubated for 2h. However, the fluorescence intensity was dramatically increased in the cytoplasm and a large amount of C6 was released from micelle and strong fluorescence signal was observed. This result indicated that the cellular uptake of different micelles had a time-dependent manner and C6 could efficiently release to the cytoplasm. However, after incubation for 4h, MHDP had the strongest fluorescence signal compared with other groups. This interesting result was in keeping with *in vitro* release assay and confirm that more His-OH would prevent drug releasing from inner core of the micelle.

In order to examine the cellular uptake quantitatively, flow cytometry was also assayed in this study. As is shown in Fig.5b and 4c, it could be found that the cellular uptake of different micelle was time-dependent and MHDP micelles had a maximum value in any time

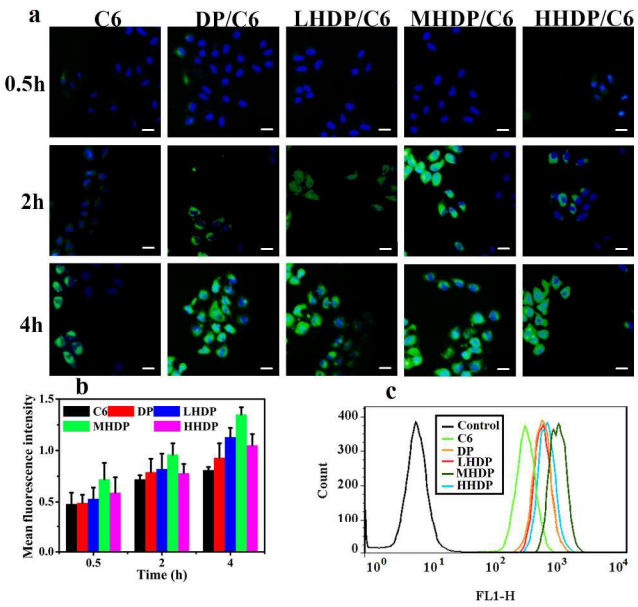


Fig.5 (a) Fluorescence microscopy images of MCF-7 cells incubated with different formulation at different time point. Green and blue colors indicate Coumarin-6 and Hoechst 33258, respectively. (b) Flow cytometry measurement of cellular uptake of different formulation at different time point. (c) Fluorescence intensity of different formulation at 4h.

point. Cellular uptake of C6 in MHDP was 1.20-, 1.17- and 1.19-fold higher than that in DP, LHDP and HHDP in MCF-7 cell at 4h, respectively. However, there was no significant difference between DP, LHDP and HHDP at any time point. This result was probably because MHDP could release C6 rapidly in tumor microenvironment (pH 5.0) due to its appropriate grafted ratio to endow pH-response comparing with DP and LHDP[34]. However, the grafted ratio of histidine in HHDP is much higher than others, which resulted the hydrophobic inner core so compact that preventing the drug release from the micelles. This result was in line with *in vitro* release and cytotoxicity assay.

In general, the above results from qualitative and quantitative analysis suggest that the uptake behaviour of DP and HDP micelle in cell was time-dependent. The accumulation of C6 in tumor sites was significantly increased due to its pH-response character. Furthermore, different grafted ratio HDP micelles has an important influence in drug release.

3.6 *In vivo* antitumor activity studies

In order to evaluate the *in vivo* antitumor activity, nude mice bearing H22 tumor cells was used in this study. When the tumor sizes were reached appropriately 100 mm^3, different drugs were given through tail vein. MHDP/PTX was used for the *in vivo* antitumor activity studies. As is shown in Fig.6a, all other groups appeared no significant difference in tumor volumes in the first 6 days except control groups, while there was different inhibition rate in tumor growth after 6 days, namely all treatment groups effectively inhibited the tumor growth in different degree. In 16 days, there was a significant different between PTX group and HDP/PTX micelles groups ($P<0.05$), but there was no significant difference between HDP/PTX micelles (10mg/kg) and HDP/PTX micelles (20mg/kg) group ($P>0.05$). These results indicated that HDP micelles could effectively inhibit tumor growth compared with PTX solution group probably due to its specifically accumulation in tumor sites and rapid release to reach a high concentration of PTX in tumor cells. Body weight changes were shown in Fig.6b, body weight of PTX solution group decreased dramatically compared with other groups, which probably because systematically toxicity of PTX. However, there were no significant weight changes in control, HDP/PTX (10mg/kg) and HDP/PTX (20mg/kg) groups, which indicated that HDP micelles could accumulate in tumor sites and reduce cytotoxicity of PTX to prolong its survival time. After 16 days, the mice were sacrificed and the tumor was harvested and weighted to calculate the tumor inhibition rate.

The Kaplan-Meier survival curve was used as a method to further verify the *in vivo* antitumor efficacy of different PTX formulation as presented in Fig.6c, HDP/PTX micelles (20mg/kg) group was 38 days,

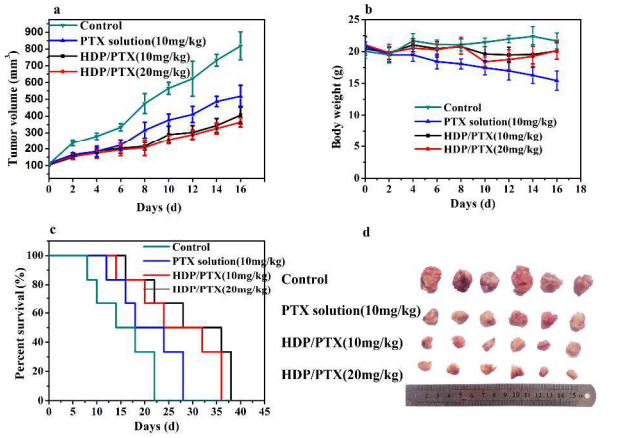


Fig.6 The mean tumor volume (a), body weight (b), Kaplan-Meier survival curve of different formulation (c) and tumor inhibition rate (d) of nude mice bearing H22 tumor cells on intravenous administration of 0.9%NaCl solution, PTX solution (10mg/kg), HDP/PTX micelles (10mg/kg) and HDP/PTX micelles (20mg/kg), respectively(n=6).

which was longer than control groups (22 days), PTX group (28 days), , HDP/PTX micelle (10mg/kg) group (36 days). As is shown in Fig.6d, tumor inhibition rate were 36.7%, 65.4% and 75.6% for PTX solution, HDP/PTX micelles (10mg/kg) and HDP/PTX micelles (20mg/kg), respectively. Tumor inhibition rate of both HDP/PTX groups were beyond 60%, which were considered to be an effective treatment.

All of these *in vivo* antitumor activities indicated that HDP/PTX micelles could effectively enhance therapeutical effect of tumor, which was in consistent with the experiment above. Those findings offered powerful evidence for HDP/PTX micelles as a novel antitumor preparation.

3.7 *In vivo* NIR fluorescence imaging of HDP micelles

DiR reagent was used as a fluorescence probe and MHDP copolymer was used to evaluate the *in vivo* biodistribution in nude mice bearing H22 tumor cells. As is shown in Fig.7, on the first 6h, the fluorescence signal of free DiR group was observed in the whole body after administration through tail vein. After that, most of the DiR was eliminated through liver and the signal became weak, only some

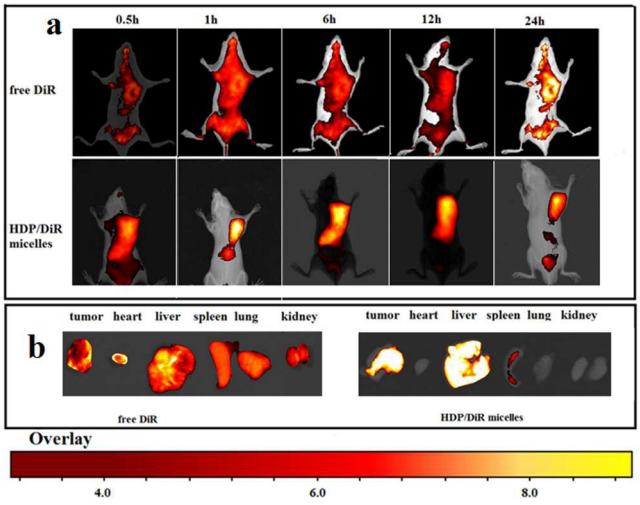


Fig.7 a:*In vivo* whole body imaging of H22 tumor-bearing nude mice after free DiR solution and DiR/HDP micelles administration at different time point with the same dose of DiR (20 μg ml^{-1}). b: The ex vivo optical images of tumors and organs of tumor-bearing mice sacrificed at 24 h

organs which have abundant blood flow remained strong fluorescence and the tumor sites only showed a slightly fluorescence intensity. In comparison, HDP/DiR micelles group showed the strong signal in tumor sites and liver. This signal became maximum in 6h in tumor. Signal in liver became weaker than before in 12h, while the fluorescence intensity in tumor decreased little. These results demonstrated that HDP micelles could specifically accumulate in tumor sites via EPR effect and rapid release in tumor acidic environment in order to reduce its systematically toxicity and reach its better therapeutical effect.

4. Conclusions

In this study, a novel pH-sensitive biodegradable HDP copolymer with different grafted ratio were synthesized successfully and PTX-loaded HDP micelles with pH-response feature was prepared to deliver PTX to tumor sites via EPR effect. *In vitro* assays, HDP micelles showed significantly pH-response release behaviour and blank micelles showed excellent biocompatible and nontoxicity. Among three different grafted ratio HDP copolymers, MHDP showed dramatically pH-triggered drug release, highest cytotoxicity, efficient internalization and highest apoptosis rate. *In vivo* studies HDP micelles showed excellent tumor inhibition rate and prolong survival time. NIR fluorescence image further demonstrated HDP micelles could specifically accumulation in tumor sites and rapid release in acidic tumor microenvironment. These results indicated that HDP copolymer is a biocompatible, tumor specifically accumulative and pH-response nanocarrier and suitable for delivering hydrophobic anticancer drugs to tumor tissues.

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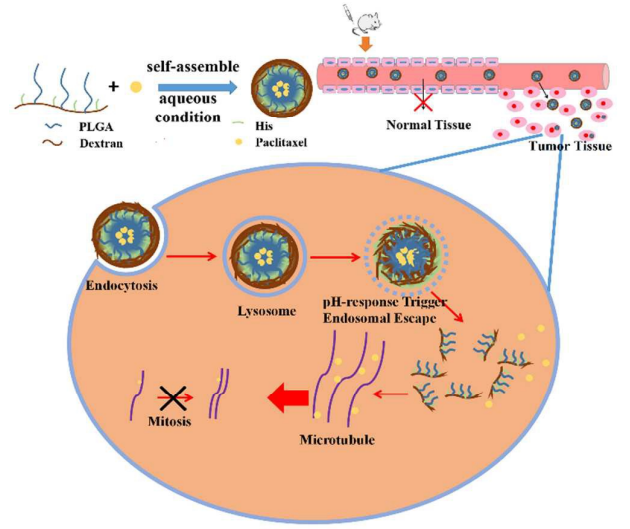
pH-sensitive copolymers have been widely used in drug delivery system to endow selectively drug release in tumor sites. Here in, dextran (DX) was conjugated with Poly (lactide-co-glycolide) (PLGA) and histidine (His) to prepare a pH-response nanocarrier, dextran-g-Poly (lactide-co -glycolide)-g-histidine (HDP) micelles, for the delivery of antitumor drugs. Different grafted ratio HDP micelles were synthesized and prepared successfully and confirmed by the critical micelle concentration (CMC) and particle size distribution (PSD). *In vitro* drug release showed that the release behaviour of paclitaxel (PTX) loaded HDP micelles was pH-dependent. All blank micelles were nontoxic *in vitro* cytotoxicity assay. In MTT assay, PTX-loaded HDP micelles with medium grafted ratio (MHDP) showed the highest cytotoxicity against MCF-7 cells. Cellular uptake experiments revealed that these pH-sensitive micelles could be taken up effectively and delivery PTX into cytoplasm to reach antitumor effect. NIR fluorescence image experiment demonstrated that HDP micelles could specifically accumulate in tumor sites via enhance permeability and retention (EPR) effect to reduce its systematically toxicity. *In vivo* antitumor activities showed that HDP micelles could effectively inhibit tumor growth and prolong survival time compared with free PTX solution. These results confirm that the biocompatible pH-response HDP micelles are a novel nanocarrier for the intracellular delivery of PTX.



Scheme 1 schematic diagram of self-assembly HDP micelles and mechanism of HDP micelles to enhance antitumor activities