Stepwise pH-responsive nanoparticles containing charge-reversible pullulan-based shells and poly(β-amino ester)/poly(lactic-co-glycolic acid) cores as carriers of anticancer drugs for combination therapy on hepatocellular carcinoma

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A B S T R A C T

Stepwise pH-responsive nanoparticle system containing charge reversible pullulan-based (CAPL) shell and poly(β-amino ester) (PBAE)/poly(lactic-co-glycolic acid) (PLAG) core is designed to be used as carriers of paclitaxel (PTX) and combretastatin A4 (CA4) for combining antiangiogenesis and chemotherapy to treat hepatocellular carcinoma (HCC). CAPL-coated PBAE/PLGA (CAPL/PBAE/PLGA) nanoparticles displayed step-by-step responses to weakly acidic tumor microenvironment (pH ≈ 6.5) and endo/lysosome (pH ≈ 5.5) respectively through the cleavage of β-carboxylic amide bond in CAPL and the “proton-sponge” effect of PBAE, thus realized the efficient and orderly releases of CA4 and PTX. In human HCC HepG2 cells and human umbilical vein endothelial cells, CAPL/PBAE/PLGA nanoparticles significantly enhanced synergistic effects of PTX and CA4 on cell proliferation and cell migration. In HepG2 tumor-bearing mice, CAPL/PBAE/PLGA nanoparticles showed excellent tumor-targeting capability and remarkably increased inhibitory effects of PTX and CA4 on tumor growth and angiogenesis. In conclusion, this novel nanoparticle system is a promising candidate as carrier for drugs against HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers and the third leading cause of cancer death worldwide, particularly in Southeast Asia and Africa [1–3]. Despite some recent advances that have been achieved in diagnosis and treatment of HCC, it remains a highly lethal disease due to recurrence of metastasis [4,5]. Traditional chemotherapy has not been found to be effective in prolonging overall survival of patients with HCC, meanwhile it is often associated with significant toxicities and acquired drug resistance [5,6]. Angiogenesis plays an important role in the progression of HCC, thus antiangiogenesis in its fledgling stage is believed as a promising strategy for HCC therapy [7,8]. Sorafenib, an antiangiogenic agent, has been widely used in advanced HCC patients, but shows little statistically significant improvement in overall survival [9]. A recent viewpoint is towards combination therapy, using antiangiogenesis as well as chemotherapy for synergistic effects on HCC [10,11].

Several investigations have reported that the combination treatment of paclitaxel (PTX) and combretastatin A4 (CA4), two microtubule-associated inhibitors, displayed synergistic effects on tumor cells and tumor vasculature [12,13]. In our preliminary experiments, we also observed their synergistic effects on HCC mouse model. PTX exhibited more potent activity on tumor cells than on tumor vasculature, whereas CA4 was the opposite. However, the wide-spread clinical applications of PTX and CA4, especially their combination use, would be tremendously limited due to the following problems. First, PTX and CA4 both display low bioavailability resulting from their poor solubility. Clinically, PTX is dissolved in the mixture of polyethoxylated castor oil (Cremophor® EL)/dehydrated ethanol (50/50, v/v) to increase its aqueous solubility, but Cremophor® EL has some side effects including systemic and hemological toxicity [14]. Second, systematic administrations of PTX and CA4 will bring about inevitable and serious toxicities, e.g., hepatotoxicity, nephrotoxicity and neurotoxicity due to lacking of tumor-targeted delivery. Therefore, it is very important to develop novel carriers for PTX and CA4 to improve their solubility and deliver them targeting tumors.

In this study, we designed a nanoparticle system as carriers of PTX and CA4 for their combination therapy targeting HCC. This nanoparticle system contains charge reversible pullulan-based (CAPL) shells and...
poly[β-amino ester] (PBAE)/poly[lactic-co-glycolic acid] (PLAG) cores. CAPL, synthesized from pullulan via two reactions, has many β-carboxylic amide groups in its molecular structure (Scheme 1a). According to the previous reports [15–17], β-carboxylic amide bond will break spontaneously under weakly acidic conditions such as extracellular acidity in tumor microenvironment [18], thus realize the charge reverse from negative to positive (Scheme 1b). PBAEs, as a novel kind of cationic polymers firstly developed by Langer’s group [19], show strong “proton-sponge” effect, and therefore are particularly suitable for intracellular delivery of genes, proteins, peptides, and low-molecular-weight drugs [20–22]. PBAE, containing piperidine rings in structural unit (Scheme 1c), was chosen as a material to prepare nanoparticles for carrying PTX and CA4 to improve their bioavailability and promote their intracellular delivery. However, the in vivo applications of PBAEs are limited by their poor stability in blood, which is mainly resulted from their hydrolysis under physiological conditions and possession of abundant positive charges [22]. Here, we used the following two strategies to resolve these problems. First, PLGA was added into PBAE nanoparticles to enhance their stability and adjust the release rates of encapsulated drugs. Second, CAPL was coated on the surfaces of PBAE/PLGA nanoparticles to shield their positive charges. So far, many investigations have confirmed that surface modification is perhaps an optimal strategy to improve the in vivo stability of PBAE-based drug carriers [23,24]. In addition, pullulan-based shells are likely to conduce to HCC-targeted drug delivery because polysaccharide backbone of pullulan is a natural ligand for asialoglycoprotein receptor (ASGPR) [25], which is often over expressed by HCC cells [26].

The hepatoma-targeting and stepwise pH-responsive mechanisms of CAPL-coated PBAE/PLGA (CAPL/PBAE/PLGA) nanoparticles are illustrated in Scheme 1d. First, they are preferentially accumulated in

![Scheme 1](image-url). Schematic illustrations of composition and structure of CAPL/PBAE/PLGA nanoparticles, and their mechanisms for in vivo delivery of antitumor drugs. (a) The synthesis route of CAPL. (b) The pH-responsive charge-reversal mechanism of β-carboxyl amide bond in CAPL. (c) The chemical structure of PBAE. (d) The illustration for in vivo hepatoma-targeting and stepwise pH-responsive mechanisms of CAPL/PBAE/PLGA nanoparticles.
hepatoma through the enhanced permeability and retention (EPR) effect and specific affinity for ASGPR on HCC cells. Second, \(\beta\)-carboxyl amide bonds in CAPL are cleaved in weakly acidic tumor microenvironment (pH 6.2–6.9 [27]), and subsequently detached from the nanoparticle surfaces due to the electrical repulsion between charge reversed CAPL and positively charged PBAE/PLGA nanocores. After that, PBAE/PLGA nanocores are internalized by tumor cells or vascular endothelial cells (VECs) via endocytosis and then release the encapsulated drugs via the “proton-sponge” effect, which is induced by the low pH of endo/lysosomes (pH 4.0–6.0 [28]). Moreover, CAPL/PBAE/PLGA nanoparticles can be also efficiently uptaken by hepatoma cells via ASGPR-mediated endocytosis, and followed by the intracellular release of antitumor drugs via the “proton-sponge” effect of PBAE. In this study, we also systematically evaluated the in vitro and in vivo potentials of CAPL/PBAE/PLGA nanoparticles as carriers of PTX and CA4 for combination therapy on HCC.

2. Materials and methods

2.1. Materials

Pullulan (average molecular weight 200 kDa) was obtained from Hayashibara Biochemical Laboratory, Inc. (Okayama, Japan). PLGA (average molecular weight 20 kDa, lactide/glycolide ratio 50/50) was purchased from Daigang Biomaterial Co., Ltd. (Jinan, China). PBAE (average molecular weight 28,000 relative to polystyrene standards) was synthesized by Michael-addition reaction of 4,4’-trimethyleneediaperidine and 1,4-butanediol diacrylate according to the previous report [19]. PTX and CA4 were obtained from MeiGen Biotechnology Co., Ltd. (Dalian, China). Dimethyldisulfide (DMSO, dry grade), diethylentriamine and N,N’-carbonyldimidazole were purchased from J&K Scientific Ltd. (Beijing, China). Cis-4-cyclohexene-1,2-dicarboxylic anhydride was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 1,1-dioctadecyl-3,3,3’-tetracyanomethyloxycarbonylperchlorate (Dil) and cyanine 5.5 (Cy5.5) were purchased from Fanbo Biochemicals (Beijing, China). PTX, when used, was dissolved in Cremophor® EL (BASF Corporation, Germany)/dehydrated ethanol mixture (50/50, v/v).

HCC HepG2 cells and human umbilical vein endothelial cells (HUVECs), obtained from American Type Culture Collection, were cultured respectively in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies) and RPMI 1640 medium (Life Technologies), which were supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin. BALB/c nude mice were bought from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in a specific pathogen-free environment. HCC xenograft mouse model was constructed by transplanting HepG2 cells subcutaneously to the nude mice. All animal experiments were carried out according to the protocols approved by the Tianjin Medical University Animal Care and Use Committee.

2.2. Synthesis and characterization of CAPL

AMPL was firstly synthesized according to the method previously reported [29,30]. Briefly, 500 mg of pullulan was reacted with 500 mg of N,N’-carbonyldimidazole in 40 mL of DMSO under stirring at room temperature. After 5 min, the mixture was dropwise added to 1.5 g of diethylentriamine under stirring in 10 mL DMSO and further stirred at 35 °C for 20 h. After that, the reaction solution was dialyzed against deionized water for 2 days with 6 exchanges and the dialyzed was then freeze-dried to obtain AMPL. The degree of substitution (DS) of diethylentriamine moiety in AMPL was determined by the pH-metric titration method that was described in the Supplementary Material.

Next, AMPL was carboxylated by reacting with cis-4-cyclohexene-1,2-dicarboxylic anhydride to synthesize CAPL. Typically, 200 mg of AMPL and 100 mg of cis-4-cyclohexene-1,2-dicarboxylic anhydride were dissolved in 10 mL DMSO and then stirred at room temperature for 24 h. After cooling, the reaction solution was poured into excess ethanol, thus obtained a large amount of white precipitate. This precipitate was collected and washed with ethanol for several times, and followed by drying to obtain CAPL. The Fourier transform infrared (FT-IR) spectra of pullulan, AMPL and CAPL were measured on a NEXUS 470 spectrometer (Nicolet, USA), and their proton nuclear magnetic resonance (1H NMR) spectra were recorded by a 400 MHz spectrometer (Avance III, Bruker, Germany) in D2O.

2.3. Preparation and characterization of CAPL/PBAE/PLGA nanoparticles

PLGA/PBAE nanoparticles were firstly prepared by the emulsion evaporation method. Briefly, 60 mg of PBAE and PLGA with different weight ratios were dissolved in 4 mL CH2Cl2 and then added into 20 mL deionized water, and followed by sonication for 3 min using an ultrasonic processor (UH-500A, Autoscope Instrument, Tianjin, China) to form an O/W emulsion. This emulsion was then poured into 50 mL of deionized water and continuously stirred at 500 rpm for 4 h. The obtained colloidal nanoparticle suspension was centrifuged and washed with deionized water. The product was then resuspended in deionized water and finally freeze-dried to obtain the powder of PBAE/PLGA nanoparticles. CAPL was coated on the surfaces of PBAE/PLGA nanoparticles using incubation method. Briefly, different amounts of CAPL were dissolved in deionized water which pH was adjusted to about 9.0 using triethylamine. After that, the above O/W emulsion of PBAE/PLGA nanoparticles was added into CAPL solution and incubated for 4 h under stirring at 500 rpm. The nanoparticle dispersion was then collected by centrifugation, washed with deionized water, and finally freeze-dried to obtain CAPL/PBAE/PLGA nanoparticle powder.

PBAE/PLGA and CAPL/PBAE/PLGA nanoparticles were dispersed in deionized water at concentration of 0.5 mg/mL, and their sizes, size distributions and zeta potentials were measured using an automatic particle analyzer (Zetasizer Nano ZS, Malvern, UK). The morphologies of these nanoparticles were characterized by transmission electron microscopy (TEM, Hitachi HT7700 Tokyo, Japan).

2.4. Evaluation of pH-responsive capability of CAPL/PBAE/PLGA nanoparticle

CAPL/PBAE/PLGA nanoparticles were dispersed in phosphate buffer saline (PBS) solutions with pHs of 7.4, 7.0 and 6.5 at concentration of 1 mg/mL under stirring at 100 rpm. After designated time intervals, CAPL/PBAE/PLGA nanoparticles were collected by centrifugation and washed with deionized water for at least three times. Next, zeta potentials of nanoparticles were measured in deionized water and their morphologies were observed by TEM.

2.5. Drug loading and release studies

Different amounts of PTX and CA4 were dissolved in CH2Cl2 containing PBAE and PLGA at weight ratio of 5:1 and then processed according to the emulsion solvent evaporation method as above described to prepare PTX- and CA4-loaded PBAE/PLGA (PBAE/PLGA/PTX and PBAE/PLGA/CA4) nanoparticles. CAPL was coated on the surfaces of these nanoparticles by incubation method to prepare CAPL/PBAE/PLGA/PTX and CAPL/PBAE/PLGA/CA4 nanoparticles. The loading contents and encapsulation efficiencies of PTX and CA4 in these nanoparticles were measured using ultraviolet spectrophotometry (Beckman DU-640, USA) at wave lengths of 300 nm and 240 nm, respectively. Meanwhile, Dil-loaded PBAE/PLGA (PBAE/PLGA/Dil) and CAPL/PBAE/PLGA (CAPL/PBAE/PLGA/Dil) nanoparticles, Cy5.5-loaded PBAE/PLGA (PBAE/PLGA/Cy5.5) and CAPL/PBAE/PLGA (CAPL/PBAE/PLGA/Cy5.5) nanoparticles were also prepared by the same method.

The in vitro releases of PTX and CA4 from PBAE/PLGA and CAPL/PBAE/PLGA nanoparticles were detected using PBS solutions with pHs of 7.4, 7.0, 6.5, and 5.5 as the release media. Typically, 5 mg of drug-loaded nanoparticle was dispersed in 50 mL release media and then placed in the air bath at 37 ± 0.2 °C under shaking at 100 rpm. To ensure the efficient release of PTX, 0.05% (w/v) tween-80 was added to the release media.
media. At designated time intervals, the release media were collected through centrifugation and the fresh release media were then added. Next, the amounts of released PTX and CA4 were determined by the ultra performance liquid chromatography method as follows. ACQUITY UPLC system (ACQ-BSM, Waters) and C18 analytical column (50 mm × 2.1 mm, 1.7 μm, Waters) were used with column temperature of 30 °C. The mobile phase consisted of acetonitrile and deionized water (45:55 v/v, pH 6.5) and the flow rate was 0.2 mL/min. The injection volume was 2 μL. The detection wavelengths of PTX and CA4 were 240 and 300 nm, respectively.

2.6. Cellular uptakes and intracellular locations of CAPL/PBAE/PLGA/Dil nanoparticles

The uptakes of free Dil, PBAE/PLGA/Dil and CAPL/PBAE/PLGA/Dil nanoparticles in HCC HepG2 cells were quantitatively assessed in different pH culture media by the confocal laser microscopy. Briefly, HepG2 cells were seeded onto 12-well glass slides at density of 5 × 10^4 cells per well and incubated with 0.25% trypsin and resuspended in PBS containing 1% bovine serum albumin (BSA), and then analyzed by a FACS calibur flow cytometer (Beckman Coulter, USA).

The intracellular locations of free Dil, PBAE/PLGA/Dil and CAPL/PBAE/PLGA/Dil nanoparticles in HepG2 cells in different pH culture media were observed by the confocal laser microscopy. Briefly, HepG2 cells were seeded onto 12-well glass slides at density of 5 × 10^4 cells per well and incubated in culture media (pH 7.4, 7.0 and 6.5) for 24 h. Next, the cells were fixed with 0.25% Trypsin and resuspended in PBS containing 0.1% bovine serum albumin (BSA), and then analyzed by a FACS calibur flow cytometer. (Beckman Coulter, USA).

2.7. Cytotoxicity assay

The cytotoxicities of free PTX, free CA4, PTX/CA4 mixture, and PTX/CA4 nanoparticles in HepG2 cells and HUVECs were measured in different pH culture media by MTT assay. Briefly, HepG2 cells were seeded in 6-well culture plates and incubated in culture media (pH 7.4, 7.0 and 6.5) for 24 h. Free Dil, PBAE/PLGA/Dil and CAPL/PBAE/PLGA/Dil nanoparticles were then added at the same Dil concentrations. After incubation for 12 h, the cells were washed with PBS, detached with 0.25% Trypsin and resuspended in PBS containing 0.1% bovine serum albumin (BSA), and finally analyzed by a FACs calibur flow cytometer (Beckman Coulter, USA).

2.8. Wound healing assay

HUVECs were seeded in 6-well plates in pH 6.5 culture media for 48 h to form confluent monolayers, which were then wounded by scratching with a pipette tip. After washing with PBS, HUVECs were further incubated in serum-free RPMI 1640 media containing free PTX, free CA4, PTX/CA4 mixture, and PTX/CA4 nanoparticles at PTX and CA4 concentration of 10 and 5 ng/mL, respectively. At 0, 6, 12, and 24 h, the widths of wound were recorded and representative photomicrographs were taken at 0 and 24 h.

2.9. Distribution of CAPL/PBAE/PLGA nanoparticles in HepG2 tumor-bearing mice

The hepatoma-targeting capability of CAPL/PBAE/PLGA nanoparticles was analyzed by in vivo bioluminescence imaging. Typically, HepG2 tumor-bearing nude mice were injected with normal saline (the control), free Cy5.5, PBAE/PLGA/Cy5.5 and CAPL/PBAE/PLGA/Cy5.5 nanoparticles via tail vein, and then imaged using IVIS in vivo imaging system (PerkinElmer, USA) at 6 and 24 h post administrations. Next, the mice were sacrificed by cervical dislocation, and then the tumors and major organs (heart, liver, spleen, lung, kidney) were collected for further observation.

Moreover, Dil was used as a model drug to evaluate drug accumulation characteristics in the liver, spleen and tumor tissues through delivery of CAPL/PBAE/PLGA nanoparticles. Briefly, HepG2 tumor-bearing mice were intravenously injected with free Dil, PBAE/PLGA/Dil and CAPL/PBAE/PLGA/Dil nanoparticles at the same Dil doses. At 24 h post administrations, the mice were sacrificed and the tissues were harvested for cryosectioning. The obtained 5 μm thick sections were then stained with DAPI and visualized using the confocal laser microscopy.

2.10. Evaluation of in vivo antitumor activity

HepG2 tumor-bearing nude mice were randomly divided into 5 groups (n = 6 per group) and respectively treated with normal saline (the control), free PTX, free CA4, PTX/CA4 mixture, and PTX/CA4 nanoparticles at PTX and CA4 doses of 8 and 10 mg/kg, respectively. All administrations were carried out via intravenous injection every other day for consecutive 4 times. During the treatment process, the tumor sizes and body weights were measured every 2 days. The tumor volumes were then calculated by the ellipsoidal formula according to our previous report [11]. After that, all mice were sacrificed, and the main organs and tumors were then harvested for further histological analysis.

The above tissue samples were fixed in 10% formalin, dehydrated with a graded ethanol series, embedded in paraffin, and cut to 5 μm sections. After that, sections were stained with hematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis., MO, USA) and then imaged with a fluorescence microscope (IX71, Olympus, Japan). For immunohistochemical evaluation of tumor vessels, sections of tumors were further immersed in 5% BSA for 20 min, and then followed by incubating with rabbit polyclonal antibody against CD31 (Abcam, Cambridge, MA) at 1:100 dilution at 4 °C overnight. After washing with Dako wash buffer (Dako K1492), the sections were processed with goat anti-rabbit IgG Alexa fluor 594 red (Invitrogen A11037) at 1:500 dilution for 30 min, dyed by diamino-benzidine and hematoxylin, and finally observed under a fluorescence microscope.

2.11. Statistical analysis

All values in this study are presented as means ± SD of at least three independent experiments. Statistical analysis was performed using the Student’s t-test, and P < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of CAPL

CAPL was synthesized via two-step reactions shown in Scheme 1a. Pullulan was reacted with diethylthretamine/N,N-carbonyl diimidazole to form amine-modified pullulan (AMPL), and then followed by the conjugation with cis-4-cyclohexene-1,2-dicarboxylic anhydride to synthesize CAPL. The obtained AMPL and CAPL were chemically characterized.
by FT-IR, $^1$H NMR and elemental analysis. Compared to pullulan, the signals at 1701 cm$^{-1}$ and 1573 cm$^{-1}$ in IR spectrum of AMPL (Fig. 1a) were attributed to the CO stretching vibration (amide-I) and NH deformation vibration (amide-II) of amide bond coupling diethylenetriamine to pullulan. In $^1$H NMR spectrum of AMPL (Fig. 1b), the characteristic proton shifts of $-\text{CH}_2$-$\text{CH}_2$ in diethylenetriamine moiety appeared respectively at 3.23, 2.86, 2.75, and 2.68 ppm. The DS of diethylenetriamine moiety was determined by the pH-metric titration method (Fig. 15), and was about 12.3%.

During the synthesis of CAPL, a large excess of cis-4-cyclohexene-1,2-dicarboxylic anhydride was added to confirm that the amino end groups of diethylenetriamine moiety were completely substituted. In the IR spectrum of CAPL (Fig. 1a), the signals at 1520–1780 cm$^{-1}$, which were mainly attributed to the CO stretching vibrations of carboxyl and amide groups (amide-I), and the NH deformation vibrations (amide-II), were significantly enhanced compared to AMPL. In the $^1$H NMR spectrum of CAPL (Fig. 1b), the shifts at 2.25 and 5.78 ppm were assigned respectively to the protons of $-\text{CH}_2$-$\text{CH}_2$ and $-\text{CHICH}_2$ in cyclohexene moiety. Besides that, compared to AMPL, the proton shift of $-\text{CH}_2$-$\text{CH}_2$, which was linked to the end amino group in diethylenetriamine moiety, obviously changed from 2.68 to 3.09 ppm due to the acylation of the end amino group. All above results suggested that AMPL and CAPL were successfully synthesized in this study.

### 3.2. Preparation and characterization of CAPL/PBAE/PLGA nanoparticles

We firstly prepared PBAE/PLGA nanoparticles with different PBAE/PLGA weight ratios using the emulsion solvent evaporation method, in which no emulsifier needed to be used because PBAE itself had strong surfactant activity. With PBAE/PLGA nanoparticles prepared in this study, the DS of diethylenetriamine nano- particles exhibited gradually decreased size, narrowed size distribution and increased zeta potential (Supplement Table S1), meanwhile their spherical morphology changed to be more regular (Supplement Fig. S2). Moreover, we also prepared PBAE/PLGA/PTX and PBAE/PLGA/CA4 nanoparticles with different PBAE/PLGA weight ratios, and assessed their releases in physiological saline at pH 7.4 and pH 5.5 release media. The results are shown in Supplement Fig. S3. PTX and CA4 displayed more obvious pH-responsive and orderly release properties from PBAE/PLGA nanoparticles at PBAE/PLGA weight ratio of 5/1. According to our previous report [11], the pH-responsive and orderly releases of CA4 and PTX would be beneficial to combining antiangiogenesis and chemotherapy to treat cancers. Thus, 5/1 was believed as an optimal PBAE/PLGA weight ratio to prepare PBAE/PLGA nanoparticles. The TEM image showed that PBAE/PLGA nanoparticles had a regular spherical shape with compact structure (Fig. 2a). The size of PBAE/PLGA nanoparticles, determined by dynamic light scattering method, was only 120 nm with a very narrow distribution (Fig. 2b).

CAPL was incubated with the above prepared PBAE/PLGA nanoparticles in aqueous medium at pH 9.0 to form CAPL/PBAE/PLGA nanoparticles. With CAPL/PBAE/PLGA weight ratio increasing from 1/5 to 30/5/1, the size of these nanoparticles gradually decreased and their zeta potential changed from positive to negative (Supplement Table S2), indicating that CAPL was successfully coated on the surfaces of PBAE/PLGA nanoparticles. By contrast, CAPL/PBAE/PLGA nanoparticles with CAPL/PBAE/PLGA weight ratio of 15/5/1 had a relatively small size (178.1 nm) and a high negative zeta potential ($-17.8$ mV), meantime also exhibited excellent storage stability. Their size maintained constant after storage at 4 °C for at least one week. Therefore, CAPL/PBAE/PLGA nanoparticles with weight ratio of 15/5/1 were used for the following experiments. CAPL/PBAE/PLGA nanoparticles had classic “core–shell” structure with CAPL shells from about 20 to 50 nm in thickness (Fig. 2c) and showed narrow size distribution (Fig. 2d). In addition, we further evaluated the pH-responsive capability of PBAE/PLGA nanoparticles (weight ratio 15/5/1) in both physiological saline with and without 10% FBS. The results showed that PBAE/PLGA and CAPL/PBAE/PLGA nanoparticles both exhibited excellent physical stability in physiological saline. However, in physical saline containing 10% FBS, CAPL/PBAE/PLGA nanoparticles were obviously more stable than PBAE/PLGA nanoparticles (Fig. S4).

### 3.3. Evaluation of pH-responsive capability of CAPL/PBAE/PLGA nanoparticles induced by the charge reversal of CAPL

$\beta$-carboxyl amide bond has pH-sensitive cleavage property. It is generally stable and negatively charged at the physiological pH, but can be hydrolyzed and free the amino group with positive charge in weakly acidic conditions [15–18]. This pH-sensitive cleavage is closely related to the chemical environment surrounding $\beta$-carboxyl amide bond [17]. In our preliminary experiment, we synthesized different charge reversal pullulan derivatives and found that CAPL had more obvious charge reversal capability at pH 6.5–7.0, which was basically consistent with the pH range (6.2–6.9) of tumor microenvironment [27]. After cleavage of $\beta$-carboxyl amide bond, charge reversed CAPL could spontaneously detach from the nanoparticle surfaces due to the electrical repulsion, thus

![Fig. 1. IR (a) and $^1$H NMR spectra (b) of pullulan, AMPL and CAPL.](image-url)
freed PBAE/PLGA nanocores carrying positive charges (Scheme 1d). To evaluate this pH-responsive capability of CAPL/PBAE/PLGA nanoparticles induced by the charge reversal of CAPL, we measured zeta potentials of CAPL/PBAE/PLGA nanoparticles in deionized water after storage in aqueous media respectively at pH 7.4, 7.0 and 6.5 for different times. The results are shown in Fig. 3a. Very evidently, the changes of zeta potentials exhibited a high selectivity for the medium pH. After storage in pH 6.5 aqueous medium for 2 h, the nanoparticle zeta potential varied from $-17.3$ to $9.8 \text{ mV}$, which suggested that CAPL successfully detached from the nanoparticle surfaces. Furthermore, TEM images (Fig. 3b) showed that CAPL shells almost completely disappeared after 6-hour storage at pH 6.5, further confirming that the charge reversal of CAPL induced the exposure of PBAE/PLGA nanocores.

To further evaluate this pH-responsive capability, we prepared PBAE/PLGA/Dil and CAPL/PBAE/PLGA/Dil nanoparticles, and assessed their cellular uptakes in HepG2 cells using the confocal microscopy and flow cytometry after 12-hour incubations in different pH culture media. Dil, as a fluorescence dye for lipophilic membrane, was mainly located in the cell membrane and nearly no red fluorescence was observed in the cytoplasm (Fig. 4a). However, Dil loaded by PBAE/PLGA and CAPL/PBAE/PLGA nanoparticles rapidly entered into HepG2 cells and was mainly located around cell nucleus (Fig. 4b and d). By comparison, PBAE/PLGA/Dil nanoparticles were easier to be uptaken by HepG2 cells (Fig. 4c) than CAPL/PBAE/PLGA/Dil nanoparticles (Fig. 4e) especially at pH 7.4. We believed this was due to their different cell entry mechanisms. Like other positively charged polymer nanoparticles [31,32], PBAE/PLGA/Dil nanoparticles could be very efficiently internalized by HepG2 cells through positive charge-mediated endocytosis. However, CAPL/PBAE/PLGA/Dil nanoparticles were mainly uptaken by HepG2 cells through receptor-mediated endocytosis because polysaccharide backbone of pullulan was a natural ligand for ASGPR that was often over-expressed by HCC cells [24–26]. In addition, the flow cytometry data (Fig. 4e) show that the accumulation of CAPL/PBAE/PLGA/Dil nanoparticles in HepG2 cells had significant pH-selectivity, e.g., their cellular uptake was much higher at pH 6.5 than at pH 7.4. It implied that PBAE/PLGA nanocores were freed from CAPL shells under weakly acidic condition, and then entered into HepG2 cells via positive charge-mediated endocytosis.
endocytosis. All above results further confirmed pH-responsive capability of CAPL/PBAE/PLGA nanoparticles induced by the charge reversal of CAPL.

3.4. Drug loading capability and pH-responsive drug release behavior of CAPL/PBAE/PLGA nanoparticles

The above results suggested that CAPL/PBAE/PLGA nanoparticles could be used as a novel carrier for delivering antitumor drugs targeting tumor microenvironment. Tumor vasculature is a vital component of tumor microenvironment and supports tumor growth by delivery of nutrients, oxygen and immune cells. Therefore, antiangiogenesis treatment has attracted more and more attentions recently and is believed as a promising strategy for cancer therapy [33–35]. The combination treatment of antiangiogenesis and chemotherapy has obvious synergistic inhibitory effects on the tumor growth both in vitro and in vivo, for example, PTX and CA4, two microtubule-associated inhibitors, displayed significant synergistic effects on tumor cells and tumor vasculature [12, 13]. Here, CAPL/PBAE/PLGA nanoparticles were used as carriers for PTX and CA4 to enhance their synergistic effects on HCC, and decrease their toxic and side effects on normal tissues by tumor microenvironment-responsive delivery.

Hence, we assessed the loading capabilities of CAPL/PBAE/PLGA nanoparticles for PTX and CA4. First, PBAE/PLGA/PTX and PBAE/PLGA/CA4 nanoparticles were prepared at different drug feed weight ratios and their characterizations are shown in Table 1. Compared to blank nanoparticles, PBAE/PLGA/PTX and PBAE/PLGA/CA4 nanoparticles had obviously larger sizes, but their zeta potentials maintained relatively high values. As drug feed weight ratio increased, PBAE/PLGA/PTX and PBAE/PLGA/CA4 nanoparticles both exhibited gradually increased sizes and drug loading contents, but drug encapsulation efficiencies decreased meanwhile. Based on a balanced consideration of application essentials, e.g., small size, high drug loading content and encapsulation efficiency, 9/25/5 was believed as an optimal feed weight ratio for preparations of

![Fig. 4. Cellular uptakes of CAPL/PBAE/PLGA nanoparticles in HepG2 cells after 12-hour incubations respectively at pH 7.4, 7.0 and 6.5. Confocal images of HepG2 cells incubated respectively with free Dil (a), PBAE/PLGA/Dil (b) and CAPL/PBAE/PLGA/Dil nanoparticles (d). Dil emitted red fluorescence. 4′,6-diamidino-2-phenylindole (DAPI) was used to stain cell nuclei with blue fluorescence. Flow cytometry analyses of HepG2 cells incubated respectively with PBAE/PLGA/Dil (c) and CAPL/PBAE/PLGA nanoparticles (e).](image)

### Table 1

<table>
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<th>Drug</th>
<th>Drug/PBAE/PLGA (w/w/w)</th>
<th>Sizea (d/nm)</th>
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<th>Zeta potential (mV)</th>
<th>DCb (%)</th>
<th>EEc (%)</th>
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* Size (mean diameter) and polydispersity index (PDI) were determined by dynamic laser light scattering method for at least three times.

* Drug loading content (DC) was defined as weight percentage ratio between the loaded drug and nanoparticles.

* Drug encapsulation efficiency (EE) was defined as weight percentage ratio between the loaded drug and the added drug during preparation.
both PBAE/PLGA/PTX and PBAE/PLGA/CA4 nanoparticles. After that, CAPL was coated on the surfaces of PBAE/PLGA/PTX and PBAE/PLGA/CA4 nanoparticles using above incubation method at CAPL/PBAE/PLGA weight ratio of 15/5/1 to obtain CAPL/PBAE/PLGA/PTX and CAPL/PBAE/PLGA/CA4 nanoparticles, which exhibited spherical shapes with obvious shell–core structures (Supplement Fig. S5a and S5c). The sizes of CAPL/PBAE/PLGA/PTX and CAPL/PBAE/PLGA/CA4 nanoparticles were respectively 209.4 and 218.2 nm with narrow distributions (Supplement Fig. S5b and S5d), and their zeta potentials were —16.7 and —18.2 mV. PTX and CA4 loading contents, determined by ultraviolet (UV) spectrophotometer, were 8.2% and 6.9%, respectively.

Next, we evaluated in vitro releases of PTX and CA4 from CAPL/PBAE/PLGA nanoparticles using dynamic dialysis method at different pHs. As shown in Fig. 5a and b, PTX and CA4 both displayed significant pH-responsive in vitro releases. When the medium pH decreased from 7.4 to 6.5, PTX and CA4 both exhibited gradually increased in vitro releases. It was perhaps because the drugs loaded on the nanoparticle surface layers released after CAPL shells detached from PBAE/PLGA nanocores under weakly acidic conditions. As the medium pH further decreased to 5.5, PTX and CA4 releases were dramatically accelerated, e.g., about 21.6% of PTX and 70.0% of CA4 were released at 24 h, which was due to the protonation and degradation of PBAE according to the previous reports [19–22]. It is indicated that drugs loaded by CAPL/PBAE/PLGA nanoparticles could escape from the endo/lysosomal trapping through the “proton-sponge” effect and then be released into the cytoplasm. Subsequently, we observed the intracellular locations of Dil loaded by CAPL/PBAE/PLGA nanoparticles by the confocal microscopy to monitor its endo/lysosomal escape. As shown in Fig. 5c, a large amount of Dil was successfully escaped from the endo/lysosomes in HepG2 cells after incubation with CAPL/PBAE/PLGA/Dil nanoparticles at pH 6.5 for only 2 h. From all above results, we deduced that CAPL/PBAE/PLGA nanoparticles could produce step-by-step responses respectively to weakly acidic tumor microenvironment and cell endo/lysosome, thus would be conducive to cell entry and intracellular release of the loaded drugs. In addition, CA4 exhibited more rapid release from CAPL/PBAE/PLGA nanoparticles (Fig. 5b) than PTX (Fig. 5a) perhaps due to its relatively stronger hydrophilicity. According to our previous report [11], the orderly releases of CA4 and PTX would benefit to exerting their synergistic effects of antiangiogenesis and antitumor.

3.5. In vitro synergistic effects of combination treatment of CAPL/PBAE/PLGA/PTX and CAPL/PBAE/PLGA/CA4 (PTX/CA4) nanoparticles

The cytotoxicities of PTX/CA4 nanoparticles in HepG2 cells and HUVECs were assessed compared to other treatments, including free PTX, free CA4, and PTX/CA4 mixture, using MTT assay. The growth curves of HepG2 cells and HUVECs treated with free PTX and free CA4 at different concentrations at 48 h are shown in Supplement Fig. S6a and S6b. IC_{50} values of free PTX and free CA4 were respectively 7.6 and 3.1 μg mL^{-1} in HepG2 cells, and 39.6 and 11.8 ng mL^{-1} in HUVECs. Next, we chose PTX and CA4 concentrations that were far below their IC_{50} values in HepG2 cells and HUVECs to evaluate the cytotoxicities of various treatments after 48 h. At PTX and CA4 concentrations of 2.5 and 1.0 μg/mL, PTX/CA4 nanoparticles exhibited much higher cytotoxicity than other treatments in HepG2 cells. Furthermore, there was no significant difference in the cytotoxicities of each treatment at different pH values (Fig. 6a). At PTX and CA4 concentrations of 10 and 5 ng/mL, PTX/CA4 mixture and PTX/CA4 nanoparticles both displayed evidently enhanced cytotoxicities in HUVECs compared to free PTX and free CA4, whereas only PTX/CA4 nanoparticles exhibited pH-dependent inhibitory effect on the growth of HUVECs (Fig. 6b). Compared to PTX/CA4 mixture, the cytotoxicity of PTX/CA4 nanoparticles significantly decreased at pH 7.4 and pH 7.0, but increased at pH 6.5 (Fig. 6b). We believed it was because PTX/CA4 nanoparticles were much easier to be uptaken by HUVECs at pH 6.5 owing to the exposure of PBAE/PLGA nanocores from CAPL shells. However, this difference was not obvious in HepG2 cells because CAPL/PBAE/PLGA nanoparticles could be sufficiently uptaken by HCC cells via ASGPR-mediated endocytosis even at pH 7.4 and pH 7.0. In addition, we measured the cytotoxicities of blank PBAE/PLGA and CAPL/PBAE/PLGA nanoparticles in HepG2 cells and HUVECs. As shown in Supplement Fig. S6c and S6d, nearly no evident cytotoxicities of these blank nanoparticles were observed in their used concentrations. From the above results, we deduced

Fig. 5. pH-responsive drug releases from CAPL/PBAE/PLGA nanoparticles. The in vitro release profiles of PTX (a) and CA4 (b) from CAPL/PBAE/PLGA nanoparticles in PBS with pH 7.4, 7.0, 6.5, and 5.5. (c) The confocal images of HepG2 cells after 2-hour incubation with CAPL/PBAE/PLGA/Dil nanoparticles at pH 6.5. DAPI and Lyso tracker were used to stain the nucleus and the endo/lysosomes respectively with blue and green fluorescence.
that PTX/CA4 nanoparticles had significant synergistic inhibitory effects on the growths of HepG2 cells and HUVECs.

In view that the migration of VECs plays an important role in the process of tumor angiogenesis [36], we further evaluated the inhibitory activity of PTX/CA4 nanoparticles on the migration of HUVECs at PTX and CA4 concentrations of 10 and 5 ng/mL using wound healing test. The results are shown in Fig. 6c and d. PTX/CA4 mixture and PTX/CA4 nanoparticles both exhibited significantly higher inhibitory activities on the migration of HUVECs than free PTX and free CA4, indicating that the combination of these two drugs had significant synergistic effects on tumor angiogenesis. Besides, PTX/CA4 nanoparticles still displayed slightly increased inhibition effect on the migration of HUVECs in comparison to PTX/CA4 mixture after 12 h. Thus, we deduced that the combination treatment strategy using PTX/CA4 nanoparticles might be likely to efficiently suppress tumor angiogenesis.

3.6. Distribution and accumulation of CAPL/PBAE/PLGA nanoparticles in HepG2 tumor-bearing mice

The in vivo distribution and accumulation of CAPL/PBAE/PLGA nanoparticles were detected in HCC mouse model to evaluate their hepatoma-targeting capability and drug delivery efficiency. HepG2 cells were injected subcutaneously into nude mice to construct HCC mouse model. Cy5.5, a near-infrared fluorescence dye, was loaded into PBAE/PLGA and CAPL/PBAE/PLGA nanoparticles to label them. After that, HepG2 tumor-bearing mice were injected respectively with PBAE/PLGA and CAPL/PBAE/PLGA nanoparticles via tail vein, and then imaged using in vivo imaging system. As shown in Fig. 7a, free Cy5.5 was rapidly eliminated from the body of HepG2 tumor-bearing mouse and its red fluorescence was nearly invisible at 6 h after administration. PBAE/PLGA/Cy5.5 nanoparticles obviously delayed the elimination of Cy5.5 in HepG2 tumor-bearing mouse and were mostly distributed in the liver and spleen at 24 h after administration. Meanwhile there were also small amounts of distributions of PBAE/PLGA/Cy5.5 nanoparticles in the tumor and kidney (Fig. 7b). However, CAPL/PBAE/PLGA/Cy5.5 nanoparticles began to be accumulated in the tumor at 6 h after administration, and were mostly distributed in the tumor and liver at 24 h (Fig. 7b). In addition, CAPL/PBAE/PLGA/ Cy5.5 nanoparticles exhibited significantly increased tumor accumulation and decreased liver distribution in comparison to PBAE/PLGA/ Cy5.5 nanoparticles, indicating that surface modification of PBAE/PLGA nanoparticles with CAPL could realize the efficient hepatoma-targeting delivery for antitumor drugs. In addition, we also evaluated the biodistributions CAPL/PBAE/PLGA/Cy5.5 nanoparticles in the mice bearing human breast cancer MCF-7 cells with no ASGPR expression. As shown in Fig. S7, CAPL/PBAE/PLGA/Cy5.5 nanoparticles also showed excellent tumor-targeting capability for this non-ASGPR-expressed tumor. Thus, we speculated that the main reason for the tumor accumulation of CAPL/PBAE/PLGA nanoparticles was caused by the enhancement of carrier stability in the bloodstream and subsequent effective EPR effect.

Next, we also detected tissue distributions of Dil delivered by CAPL/ PBAE/PLGA nanoparticles in HepG2 tumor-bearing mice. After intravenous injections of free Dil, PBAE/PLGA/Dil and CAPL/PBAE/PLGA nanoparticles at the same Dil doses, the livers, spleens and tumors were removed from HepG2 tumor-bearing mice at 24 h and processed by the frozen section technique. After that, the distributions of Dil fluorescence were observed under confocal microscopy. The results are shown in Fig. 7c and d. Compared to free Dil and PBAE/PLGA/Dil nanoparticles, the distribution of Dil delivered by CAPL/PBAE/PLGA/Dil nanoparticles was dramatically reduced in the liver and spleen, but its tumor accumulation was very significantly enhanced, which coincided exactly with above results. More interesting, it seemed that CAPL/PBAE/PLGA/Dil nanoparticles were much easier to reach tumor deep sites by contrast with PBAE/PLGA/Dil nanoparticles (Fig. 7c), which was very favorable for exerting synergistic effects of PTX and CA4.

3.7. In vivo synergistic effects of PTX/CA4 nanoparticles

We further evaluated synergistic effects of PTX/CA4 nanoparticles in HepG2 tumor-bearing mice. All treatments, including free PTX, free CA4, PTX/CA4 mixture and PTX/CA4 nanoparticles, were given through...
intravenous injections every other day for consecutive 4 times at PTX and CA4 doses of 8 and 10 mg/kg, respectively. After that, tumor volume changes and body weights were continuously detected for at least 18 days, and then main tissues were removed for the further histological analysis.

The tumor growth curves after various treatments are shown in Fig. 8a. Compared to the control group with physiological saline treatment, free CA4 only slightly inhibited the growth of HepG2 tumors in mice, indicating that single treatment of antiangiogenesis could not be used as an effective anticancer strategy. PTX/CA4 mixture significantly delayed the tumor growth in comparison to both free PTX and free CA4, demonstrating that the combination treatment of antiangiogenesis and chemotherapy could produce synergistic antitumor effect. Moreover, PTX/CA4 nanoparticles exhibited much stronger tumor-inhibitory activity than PTX/CA4 mixture, whereas blank CAPL/PBAE/PLGA nanoparticles did not bring about any influence on the tumor growth (data not shown). We believed this was because CAPL/PBAE/PLGA nanoparticles could efficiently deliver anticancer drugs targeting hepatoma and selectively release drugs in weakly acidic tumor microenvironment. Fig. 8b shows the changes of mice body weights after various treatments. In the control and free CA4-treated groups, the body weights of mice more or less increased with time over. However, the treatments of free PTX and PTX/CA4 mixture both caused significant decreases in the body weight, indicating that PTX, as a commonly used chemotherapeutic agent in clinic, had great in vivo toxicity. By contrast, PTX/CA4 nanoparticles obviously overcame the declining trend of the body weight, implying that CAPL/PBAE/PLGA nanoparticles effectively alleviated the in vivo toxicity of PTX through hepatoma-targeted delivery.

The micrographs of H&E stained tissue sections are displayed in Fig. 8c. Histopathological changes in the liver, e.g., vascular congestion, tissue swelling and inflammatory cell infiltration, were clearly observed in mice treated with free PTX and PTX/CA4 mixture, indicating that these treatments induced the hepatic injury. However, no pathological changes and injuries were detectable in main tissues in mice treated with PTX/CA4 nanoparticles. In addition, the phenomena of tumor

![In vivo evaluations of hepatoma-targeting capability and drug delivery efficiency of CAPL/PBAE/PLGA nanoparticles.](image-url)
necrosis and suppressed invasive tumor cells were relatively visible in PTX/CA4 nanoparticle treatment group in comparison to the control and other treatment groups. It suggested that hepatoma-targeted delivery via CAPL/PBAE/PLGA nanoparticles reduced in vivo toxicity of PTX and enhanced synergistic effects of PTX and CA4. Next, we assessed the intratumoral vascularization using immunohistochemical CD31 staining to evaluate antiangiogenesis activity of PTX/CA4 nanoparticles. As shown in Fig. 8d and e, compared to the control, all treatments significantly decreased tumor microvessel densities in HepG2 tumor-bearing mice, but PTX/CA4 nanoparticles displayed higher antiangiogenesis activity than other treatments, further confirming their enhanced synergistic effect on tumor angiogenesis.

Fig. 8. In vivo synergistic effects of PTX/CA4 nanoparticles against hepatoma. (a) The tumor growth curves of HepG2 tumor-bearing mice after tail vein injections of free PTX, free CA4, PTX/CA4 mixture, and PTX/CA4 nanoparticles (NPs). (b) The body weight changes of HepG2 tumor-bearing mice after various treatments. (c) H&E stained images of tumor paraffin sections from the heart, lung, liver, spleen, kidney, and tumor tissues in various treatment groups. (d) The immunohistochemistry images of tumor sections stained with anti-CD31 antibody. Brown color represents positive staining of VECs. (e) The comparison of microvessel densities (MVDs) in the tumors of HepG2 tumor-bearing mice with various treatments. Data are represented as mean ± SD, n = 6. *P < 0.05 and **P < 0.01 compared to the control.
4. Conclusion

CAPL/PAE/PLGA nanoparticles, which could produce step-by-step responses specifically to weakly acidic tumor microenvironment and endo/lysosome, were prepared and used as carriers of PTX and CA4 for combining antiangiogenesis and chemotherapy to treat HCC. CAPL/PAE/PLGA nanoparticles had relatively high loading capability for PTX and CA4, and efficiently realized their orderly in vitro releases. At cellular levels, CAPL/PAE/PLGA nanoparticles significantly enhanced synergistic effects of PTX and CA4 on the proliferations of HepG2 cells and HUVECs, and the migration of HUVECs. In HepG2 tumor-bearing mice, CAPL/PAE/PLGA nanoparticles exhibited an excellent hepatoma-targeting capability and remarkably increased synergistic effects of PTX and CA4 on tumor growth and tumor angiogenesis. Taken together, CAPL/PAE/PLGA nanoparticles displayed great potential as a novel delivery carrier for antitumor drugs targeting HCC.

Acknowledgments

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Appendix A. Supplementary data

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References