Novel T7-modified pH-responsive targeted nanosystem for co-delivery of docetaxel and curcumin in the treatment of esophageal cancer

Lian Deng\textsuperscript{1,*}, Xiongjie Zhu\textsuperscript{1,*}, Zhongjian Yu\textsuperscript{1}, Ying Li\textsuperscript{1}, Lingyu Qin\textsuperscript{1}, Zhile Liu\textsuperscript{1}, Longbao Feng\textsuperscript{2}, Rui Guo\textsuperscript{2,*}, Yanfang Zheng\textsuperscript{1,*}

\textsuperscript{1}Department of Oncology, Zhujiang Hospital of Southern Medical University, Guangzhou, China
\textsuperscript{2}Key Laboratory of Biomaterials of Guangdong Higher Education Institutes, Guangdong Provincial Engineering and Technological Research Center for Drug Carrier Development, Department of Biomedical Engineering, Jinan University, Guangzhou 510632, China

* Contributed equally to this work.

*Corresponding Authors: Yanfang Zheng, E-mail: 18665000236@163.com; Rui Guo, E-mail: guorui@jnu.edu.cn.
Abstract: Although single-drug chemotherapy is still an effective treatment for esophageal cancer, its long-term application is limited by severe side effects. Nanomedicines have increasingly attracted attention because of their good biological safety, targeting and high-efficiency loading of multiple drugs. Herein, we have developed a pH-responsive nanocarrier that has high affinity for the transferrin receptor, which is overexpressed by tumor cells. The system is capable of simultaneous delivery of the chemotherapy drug, docetaxel, and the Chinese Medicine, curcumin, for treatment of esophageal cancer. This novel T7-modified targeting nanosystem releases loaded drugs when exposed to the acidic microenvironment of the tumor, and exerts a synergistic anti-tumor effect, and T7-NP-DC with docetaxel and curcumin loading of 10% and 6.1%, respectively. *In vitro* and *in vivo* studies showed that improved anti-tumor efficacy could be obtained by loading docetaxel and curcumin into the T7-modified nanocarrier without obvious toxicity or side effects, compared to drug without nanocarrier. Furthermore, the nanocarriers conjugated with T7 short peptides were more readily taken up by esophageal cancer cells compared with normal cells. Together, our findings indicate that the materials can safely exert synergistic anti-tumor effects and provide an excellent therapeutic platform for combination therapy of esophageal cancer.

**Keywords:** nanocarrier; pH-responsive; T7 peptide; docetaxel; esophageal cancer
Introduction

Esophageal cancer is the seventh most common cancer and ranks sixth in terms of cancer-related mortality worldwide. It has been estimated that around 572,000 cases led to 508,000 deaths in 2018 [1]. Although continuous scientific progress has benefited cancer patients, those with advanced lung cancer still suffer from the deficiencies of chemotherapy, which are mainly attributed to poor tumor targeting, high toxicity and side effects, short half-life, uncontrolled release, poor bioavailability and drug resistance [2, 3].

Among the commonly used chemotherapeutic agents, taxanes are a class of drugs used in advanced lung cancer and have long-lasting anti-cancer properties [4]. Of these, docetaxel (DTX) is a second generation drug in the paclitaxel family that has demonstrated promising survival benefits in esophageal cancer patients [5]. Unfortunately, single chemotherapeutic drugs have limitations and side effects that lead to lower patient compliance [6]. In order to improve the efficacy of chemotherapeutic drugs and reduce their side effects, some proprietary Chinese medicines are often added as adjuvant drugs in clinical applications. Among them, curcumin (CUR) is the most representative. As reported in the literature [7, 8], curcumin, a natural polyphenolic compound derived from the roots of turmeric, has anti-inflammatory and anti-tumor effects, and is chemosensitizing. In a phase I clinical study of curcumin plus docetaxel in patients with advanced breast cancer, better therapeutic efficacy was observed in 8 of 14 patients compared with single docetaxel therapy [9]. In addition, in other studies in ovarian, prostate and breast cancer, curcumin combined with docetaxel was found to be more effective than docetaxel alone [10-12]. Based on these previous studies, we speculate that the combination of curcumin and docetaxel has potential in the treatment of esophageal cancer.

In past decades, the inherent limitations of traditional cancer therapy have led to the development and application of various nanotechnologies to treat cancer more effectively and safely [13, 14]. The increasing interest in nanotechnology for cancer is due to its unique and attractive features, such as its utility for drug delivery, diagnosis, imaging and synthetic vaccine development, as well as the inherent therapeutic properties of some nanomaterials [15-17]. To precisely control the release of drug at the tumor site and to meet requirements for increased anti-tumor effect and reduced damage to normal tissues, research has made great contributions in
the area of stimuli-responsive release of materials (for example, pH, redox state and enzymes) [18-20]. Based on the characteristic acidity of the tumor microenvironment, Li and colleagues developed pH-responsive nanoparticles that improved the anti-tumor effect of paclitaxel on drug-resistant and metastatic breast cancer [21]. Similarly, another study on pH-responsive nanocarriers also confirmed their capability for drug-release on demand to better inhibit the activity of HeLa cells [22]. In addition, poor permeability of tumors to drugs is a major obstacle to cancer treatment. Targeted delivery of nanomedicines to tumor cells could enhance tumor detection and therapy. Very recently, Gao demonstrated that tumor penetration of nanoparticles modified with T7 peptide was 7.89-fold higher compared with unmodified nanoparticles [23]. Jiang and co-workers confirmed that T7-modified nanoparticles showed more pronounced accumulation in the tumor and curative effect compared with unmodified nanoparticles [24].

At present, most nanomedicine research is focused on single-drug treatment of cancer, which can benefit patients to a certain extent by, for example, reducing toxicity. However, administration of nanomedicines in combination with traditional clinical therapies, such as radiation, small molecule and biological drugs, will have the greatest impact. It has previously been demonstrated that co-administration of curcumin with docetaxel via nanocarrier had the potential to improve anti-tumor efficacy in breast cancer [12]. Furthermore, in a phase III trial in patients with acute myeloid leukemia, Veyons, a liposomal nanomedicine for co-delivery of cytarabine and daunorubicin, prolonged life by 6–10 months compared with standard treatment [25]. Therefore, the synergistic antitumor effect of docetaxel and curcumin in esophageal cancer may be enhanced by nanocarrier delivery.

With regard to the potential of nanomedicine for combination therapy, in the present research we report a novel T7-targeting nanosystem for co-delivery of docetaxel and curcumin with pH-responsive drug-release capability (T7-NP-DC). The stimuli-responsive release, cytotoxicity, cellular uptake, and permeability in 3D tumorspheres of T7-NP-DC were thoroughly investigated in vitro and in vivo. According to the results, the synthesized nanomedicine not only exhibits good tumor targeting, but also has good anti-tumor effect, which shows potential for accurate treatment of tumors in the future.

Materials
Fetal bovine serum (FBS), 1640 culture medium and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Life Technologies Inc. (Gibco-BRL, Gaithersburg, MD, USA). Docetaxel and curcumin were purchased from Yuanye Corporation (Shanghai, China) and Ronge Pharmaceutical Technology Development Co., Ltd. (Shanghai, China), respectively. α-Malemidyl-ω-N-hydroxy succinimidyl polyethylene glycol (NHS-PEG-MAL, MW 3500) was obtained from Jenkem Technology (Beijing, China). T7 polypeptide (SH-CHAIYPRH) was purchased from Jill Biochemical Co., Ltd. (Shanghai, China). Transferrin receptor (TfR) primary antibody was purchased from eBioscience (Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Dimethyl sulfoxide (DMSO), methanol and branched polyethyleneimine (PEI) with an average molecular weight of 1.8 kDa were purchased from Aladdin Biochemical Technology (Shanghai, China). LysoTracker was bought from Meilun Biotechnology Co., Ltd. (Dalian, China). Annexin V-FITC/PI apoptosis detection kit was purchased from Kaiji Biotechnology Co., Ltd. (Nanjing, China). Nude mice, bedding and feed were purchased from the experimental animal center of Southern Medical University (Guangzhou, China).

Methods

Preparation and characterization of nanoparticles

Synthesis of CM-β-CD

CM-β-CD was synthesized as follows: β-cyclodextrin (β-CD, 2.14 g) and NaOH (0.3 g) were dissolved in water (30 mL) and treated with 1% (w/w) aqueous monochloroacetic acid (5 mL). The mixture was stirred at 50 °C for 4 h and then the pH was adjusted to 7 with hydrochloric acid. Excess ethanol was added to the neutral solution, producing a white precipitate. The solid precipitate was filtered and dried under vacuum to give carboxymethylated β-CD (CM-β-CD).

Synthesis of CM-β-CD-PEI

CM-β-CD (1.2 g) was activated with carbonyldiimidazole (CDI, 0.36 g) in DMSO (5 mL) at room temperature for 2 h. Polyethyleneimine (100 mg) was added dropwise to the activated CM-β-CD solution and stirred at room temperature for 12 h. The mixture was purified by dialysis against water (MW 1000 Da) for 7 days followed by lyophilization to give CM-β-CD-PEI.

Synthesis of CM-β-CD-PEI-PEG
CM-β-CD-PEI (1 g) and MAL-PEG-NHS (1 g) were dissolved in water (10 mL) in an ampule, followed by addition of saturated aqueous NaHCO₃ to adjust to pH 7.5–8.5. The mixture was then stirred at room temperature. After 4 h, the mixture was purified by dialysis against water (MW 1000 Da) for 24 h followed by lyophilization to give a lavender solid.

**Synthesis of CM-β-CD-PEI-PEG-T7**

To conjugate CM-β-CD-PEI-PEG to T7, CM-β-CD-PEI-PEG (0.25 g) was dissolved in water (5 mL). T7 polypeptide solution (1 mL) was added and the pH was adjusted to 6.0–6.5. The reaction mixture was stirred at room temperature for 24 h, after which the Mal group of CM-β-CD-PEI-PEG had reacted with the thiol group of Cys-T7. Finally, the conjugate CM-β-CD-PEI-PEG-T7 was obtained by dialysis and lyophilization.

**Synthesis of CM-β-CD-PEI-PEG-T7/DTX/CUR**

CM-β-CD-PEI-PEG-T7/DTX/CUR was synthesized by the double emulsion (W/O/W) method. Briefly, CM-β-CD-PEI-PEG-T7 (40 mg) was dissolved in ethyl acetate (1 mL) followed by addition of docetaxel and curcumin solutions (1 mL). The mixture was emulsified by sonication (150 W, 60 s) to form a colloidal solution. A 4% PVA solution (2 mL) was then added to the colloidal solution and the mixture was sonicated (150 W, 90 s) to form a W/O/W double emulsion. After 4 h, CM-β-CD-PEI-PEG-T7/DTX/CUR was obtained by centrifugation at 5600 × g for 10 min at room temperature and washing twice with distilled water. CM-β-CD-PEI-PEG-T7/DTX and CM-β-CD-PEI-PEG-T7/CUR were prepared using the aforementioned method. The concentrations of DTX and CUR were measured by ultraviolet spectrophotometry. Drug-loading (DL) content and encapsulation efficiency (EE) were calculated as follows:

\[
EE (%) = \frac{A_1}{A_2} \times 100%
\]

\[
DL (%) = \frac{A_1}{B} \times 100%
\]

A₁ is the weight of drug in the carrier material, A₂ is the weight of drug added and B is the weight of carrier material.

**1H NMR**
The chemical structures of CM-β-CD-PEI-PEG-T7 and CM-β-CD-PEI-PEG were characterized by $^1$H nuclear magnetic resonance (NMR) spectroscopy (300 MHz, Varian, USA) using D$_2$O as the solvent.

**Particle size distribution and morphological characterization**

The particle sizes of CM-β-CD-PEI-PEG-T7/DTX/CUR and CM-β-CD-PEI-PEG-T7 were measured using a Zetasizer Nano ZS (Malvern) apparatus. Briefly, a 1 mg/mL sample (1 mL) was placed in a sample cell and the particle size was measured using a dynamic light scattering (DLS) laser nanoparticle analyzer at 25 °C. The morphologies of CM-β-CD-PEI-PEG-T7/DTX/CUR and CM-β-CD-PEI-PEG-T7 were characterized using transmission electron microscopy (JEOL TEM-1210).

**Drug release**

*In vitro* release of DTX and CUR from CM-β-CD-PEI-PEG-T7/DTX/CUR was studied using the dialysis membrane diffusion technique. Briefly, CM-β-CD-PEI-PEG-T7/DTX/CUR solution (1 mg/mL, 3 mL) was enclosed in a dialysis bag and placed in phosphate buffer (10 mL, pH 5.5 or 7.4) containing 10% Tween 80 at 37 °C. At a predetermined time point, a sample of the buffer (1 mL) was taken for UV-visible spectroscopy and replaced with an equal volume of fresh buffer. The cumulative release of DTX and CUR was calculated as follows:

\[
\text{Percentage release (\text{%}) = } \frac{M_1}{M_0} \times 100\%
\]

Where $M_1$ is the mass of released drug, and $M_0$ is the mass of total drug in the nanosystem.

**Cell Culture**

The non-neoplastic esophageal epithelial cell line, Het-1a, and four esophageal squamous cell carcinoma (ESCC) cell lines, KYSE150, KYSE510, Eca9706, and CaES-17, were donated by Clinical Research Central at Nanfang Hospital in Guangzhou, China. Het-1a cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin (all from Gibco, Life Technologies, CA, USA). The four ESCC cell lines were cultured in RPMI 1640 (Gibco, Life Technologies) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO2.

**RNA extraction and quantitative polymerase chain reaction (qPCR)**
Total RNA was extracted using Trizol reagent according to the manufacturer (TaKaRa Bio Inc., Japan) instructions. The RNA purity and concentration were evaluated by spectrophotometry using a nanodrop 2000c instrument (Thermo Scientific, Rockford, IL, USA) and reverse transcription was conducted using PrimeScript™ RT Master Mix (TaKaRa Bio Inc., Japan). For TfR quantification, qPCR was performed using TB Green® Premix Ex TaqTM II reagent (TaKaRa Bio Inc., Japan) on a LightCycler 480 system (Roche, Basel, Switzerland). The sequences of the primers were as follows: TfR forward, 5’-CTGCCTCTTTCTGTTGTTGT-3’ and reverse, 5’-CTTTGGCCAA AATTTGCGACG-3’.

**Examination of TfR expression by flow cytometry**

Cells (Het-1a, KYSE150, KYSE510, CaES-17 and Eca9706) in good condition were digested with trypsin, centrifuged and then washed three times with phosphate buffered saline (PBS) to remove residual medium. Subsequently, FITC-labeled primary antibody (TfR, 5 μL) was added in the dark to bind with TfR on the cell surface. After 30 min, flow cytometry was used to determine the fluorescence intensity of the cells.

**Synergistic effects of docetaxel and curcumin**

The synergistic effects of different concentrations of docetaxel and curcumin were evaluated by MTT assay. Cells were seeded in 96-well plates (KYSE150 4 × 10^3/ well, KYSE510 3.5 × 10^3/ well) and incubated overnight. The culture medium was replaced with fresh medium (100 μL) containing either single-drug (docetaxel or curcumin) or a combination of the two drugs at various ratios (DTX:CUR 2:1, 1:1, or 1:2). The DTX concentrations in KYSE150 cells were 0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 ng/mL, and in KYSE510 cells were 0, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4 and 8 ng/mL. After incubation for 48 h, cell viability was evaluated by MTT assay. The culture medium was removed and the wells treated with MTT solution (20 μL, 5 mg/mL) and 1640 medium (200 μL) without FBS for 4 h at 37°C. The MTT solution was discarded and DMSO (150 μL/well) was added to dissolve the formazan dye. The absorbance was measured by spectrophotometry at 570 nm. Cell viability and inhibitory effects were determined from the OD values. The combination index (CI) was evaluated using CompuSyn software (Version 1.0), where
CI = 1 indicated an additive effect, CI > 1 indicated an antagonistic effect and CI < 1 indicated a synergistic effect.

**Cellular uptake**

A confocal laser scanning microscope (CLSM) was used to evaluate uptake of the nanocarriers. KYSE510 cells were incubated in a confocal culture dish overnight, pre-treated with T7 or transferrin (Tf), and then treated with FITC-labeled NP and T7-NP (200 μg/mL). After incubation for 4 h, the culture medium was discarded and the cells washed three times with PBS. The cells were fixed with 4% paraformaldehyde for 30 min and then discarded and the cells washed with PBS. Finally, the cells were stained with Hoechst solution for 15 min, washed three times, and then the fluorescence intensity was observed by CLSM.

**In vitro cytotoxicity of nanomedicines**

The experimental procedures were the same as those just described, except that the added drug was replaced by the nanomedicines.

**Cell apoptosis**

To evaluate the effect of different formulations on apoptosis, cells (KYSE150 and KYSE510) were seeded in 6-well plates at a density of 3 × 10^5 cells/well for 24 h and then treated with mixtures containing equivalent DTX concentrations (2 ng/mL) for 48 h. The cells were washed twice with PBS, subjected to trypsin digestion and then stained with an Annexin V-FITC/PI apoptosis detection kit (Dojindo, Japan) according to the manufacturer’s instructions. Analysis was conducted by flow cytometry.

**3-Dimensional tumorsphere**

KYSE510 cells in good condition were digested, resuspended, and then added at a concentration of 1 × 10^4/mL to a 96-well plate containing 2% agarose. A tumorsphere was formed on the surface after 3 days culture. Following treatment with different formulations, the diameters of the tumorspheres were measured by optical microscopy at days 1, 3, 5 and 7. The following formulas were used for analysis:

\[
V = \pi d_{\text{max}} d_{\text{min}} / 6
\]

Tumorsphere rate of change (R) = Vi/V0 × 100%

Vi: represents the volume on day i.
V0: represents the volume on day 0.

**Biodistribution of nanoparticles in vivo**

To investigate the biodistribution of nanoparticles in vivo, xenograft-bearing mice were divided into 4 groups (n = 3, NP, NP-DC, T7-NP-D and T7-NP-DC). The Cy5.5-labeled nanoparticles were injected intravenously and then the fluorescence distribution in mice was observed at 2 and 8 h using an in vivo live imaging instrument.

**In vivo anti-tumor efficacy**

Male Balb/c mice (4–5 weeks, 18–20 g) were purchased from the Animal Center of Nanfang Medical University and reared in the SPF region. Cells (1 × 10⁶) in PBS (0.1 mL) were injected subcutaneously into the right flank of the mice. The xenograft-bearing mice were then divided into six groups (PBS, DTX, NP-D, NP-DC, T7-NP-D and T7-NP-DC), and the drugs were injected intravenously every other day for a total of 12 d. Tumor volumes were monitored by vernier caliper every 3 d and calculated as follows: length × width²/2.

**Biosafety in vivo**

Mice were euthanized at the end of the treatment and blood was collected for routine testing, and liver and kidney function tests. The major organs (heart, liver, spleen, lung and kidney) and tumor were harvested and fixed in 4% paraformaldehyde. Hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) were performed to evaluate the biosafety of the materials and tumor inhibitory effect (Ki-67).

**Statistical analysis**

Statistical analysis was conducted using SPSS 20 software. A one-way ANOVA test was used to compare multiple groups. P values < 0.05 were considered statistically significant.

**Results and discussion**

**TfR expression in ESCC and Het-1a cell lines**

As reported in the literature [26], TfR is overexpressed in malignant tumors. Herein, the qPCR analysis was performed to measure expression of TfR mRNA in ESCC cell lines and the non-neoplastic esophagus cell line, Het-1a. An increase of TfR mRNA expression was found in ESCC cell lines (KYSE150, KYSE510, Eca9706 and CaES-17) compared to Het-1a (Figure S1A,
*P < 0.05, ***P < 0.005). Investigation of TfR expression by flow cytometry, as shown in Figures 1B and 1C, revealed that CaES-17 cells had the highest TfR expression, while expression by KYSE150 and KYSE510 cells was also higher than Het-1a cells. In view of the high expression of TfR in malignant tumors, Zhao developed a Tf-decorated nanocarrier that exhibited impressive anti-tumor activity and more efficient cellular uptake in TfR-overexpressing lung cancer cells [27]. Other studies also demonstrated the impressive uptake efficiency and anti-tumor effect on lung cancer cells [28, 29]. We therefore speculated that we could exploit this phenomenon by designing a nanocarrier to specifically bind surface TfR and target tumors.

**Inhibitory effect of different formulations in vitro**

Adjuvants are often used in the clinic to ameliorate the side effects of single-drug treatment. Moreover, it has been reported that chemotherapy-sensitizing drugs can be used in combination with a single-drug to enhance anti-tumor activity, suggesting that patients could benefit from combination therapy. Therefore, in this study, we attempted to optimize the formulation of docetaxel and curcumin, using CompuSyn software to calculate the combination index. Firstly, the cells (KYSE150 and KYSE510) in good condition were cultured in 96-well plates and divided into three groups (DTX:CUR 2:1, 1:1 and 1:2). Then, the different formulations of docetaxel and curcumin were added on schedule. The CCK-8 assay was performed 48 h post-treatment to evaluate inhibition and synergistic anti-tumor efficacy. As shown in Figure 2, in the two cancer cell lines (KYSE150 and KYSE 510), the CI was less than 1 when the DTX:CUR ratio was 1:2. When the DTX:CUR ratio was 2:1, 1:1 or 1:2, the higher proportion of docetaxel led to weaker synergy, possibly even an antagonistic effect (Tables 1 and S1). Compared with previous studies, it was found that the combined application of curcumin and docetaxel could indeed synergistically enhance the antitumor effect [11, 12]. Hence, we finally determined that DTX:CUR(1:2) was the optimal formulation for synergistic anti-tumor activity against esophageal cancer.

**Synthesis of CM-β-CD-PEI and CM-β-CD-PEI-PEG-T7**

The synthesis of CM-β-CD-PEI-PEG-T7 is shown in Scheme 1. First, β-cyclodextrin was reacted with chloroacetic acid under basic conditions to form carboxymethyl-β-cyclodextrin (CM-β-CD). CM-β-CD-PEI was synthesized by amidation reaction of CM-β-CD and
polyethyleneimine. Reaction of the product with MAL-PEG-NHS, a coupling agent, gave CM-β-CD-PEI-PEG. The chemical structure of CM-β-CD-PEI-PEG was characterized by nuclear magnetic resonance spectroscopy (1H NMR), as shown in Figure S2. As planned, T7 was combined with CM-β-CD-PEI via NHS-PEG-MAL to construct the drug nanocarrier (CM-β-CD-PEI-PEG-T7). The NMR spectrum contained a D2O solvent peak at 4.7 ppm; proton absorption peaks at chemical shift values of 3.724 and 3.822 ppm were attributed to characteristic peaks of the T7 peptide in CM-β-CD-PEI-PEG-T7; the proton absorption peaks at chemical shifts of 5.0 and 3.2–3.6 ppm were attributed to β-CD. Together, the successful synthesis of CM-β-CD-PEI-PEG-T7 was demonstrated by 1H NMR.

**Characterization of drug-loaded nanoparticles**

According to previous studies [30], nanoparticle size can affect their excretion pathway in vivo and their ability to enter cells. It is therefore necessary to characterize the nanomedicines. The regular spherical shape of CM-β-CD-PEI-PEG-T7 before and after drug loading was scanned by transmission electron microscopy (TEM) (Figure 2A). Many small particles appeared in the nanospheres after drug loading, which was attributed to the loading of docetaxel and curcumin (Figure 2A). Subsequently, dynamic light scattering (DLS) was performed to characterize the obtained nanoparticles (Figure S2B). The particle size of CM-β-CD-PEI-PEG-T7 was approximately 100 nm. The diameter of the nanocarrier increased to 200 nm after loading of docetaxel and curcumin, which was consistent with the results from TEM.

**Stimuli-responsive (pH) drug-release in vitro**

Stimuli-responsive release of nanomedicines is based on the specific microenvironment of tumor tissue, which not only enables maximum release of drug into tumor tissue, but also reduces damage to normal tissue [31]. Herein, we tested the drug-release efficiency of the nanomedicines in a simulated tumor microenvironment. The DTX and CUR content were measured at 10% and 6.1%, respectively, using HPLC and UV spectroscopy. Normal physiological and tumor microenvironment states were simulated using PBS and a pH 5.5 solution, respectively. As shown in Figure S2, the drug-release rates were significantly enhanced in the slightly acidic environment, and the cumulative release rates of DTX and CUR reached 86.8% and 60.2%, respectively. In
contrast, in PBS alone, the cumulative release rate of DTX was decreased to 26.5%, and that of CUR was 12.3%. In previous research, the DTX-loading content of PCL-PEG reached 10.4% and almost 90% DTX was released at pH 5.5 after 24 h, which is similar to our results [32]. It is apparent that our materials also have a good drug loading rate.

**Cytotoxicity in vitro**

To further explore the anti-tumor efficiency of the nanomedicines *in vitro*, we treated esophageal cancer cells (KYSE150 and KYSE510) with different formulations and determined cell viability using the CCK-8 assay (Figures 3C and S6C), apoptosis rate by flow cytometry (Figures 3A, 3B, S6A and S6B), and necrotic area by 3D tumorsphere experiment (Figure S5). At first, there was no damage observed after treatment with different concentrations of nanoparticles, indicating good biocompatibility (Figure S4). The IC$_{50}$ values of the various nanomedicines, presented in Table S2, indicated that the best anti-tumor effect was obtained by treatment with T7-NP-DC. Moreover, the anti-tumor efficacy of free docetaxel was enhanced by loading into the T7-NP. Similar results were obtained by flow cytometry and in the 3D tumorsphere experiment. Taking the results together, the nanocarrier has good biocompatibility and has confirmed drug-delivery capability. Co-delivery of curcumin could enhance the anti-tumor effect of docetaxel on esophageal cancer.

**Cellular uptake**

The random distribution of drugs is a major obstacle to their anti-tumor effect [33]. When the therapeutic drug is loaded into a nanocarrier with a targeting effect, it will be equivalent to a smart bomb that can exert its killing-effect in specific parts of the body. Previously, numerous studies have confirmed that when the drug is loaded into a nanocarrier, significantly enhanced distribution in the tumor can be achieved, which not only increases the efficacy of free drugs, but also protects normal tissues from damage [34]. For this study, we developed a nanocarrier binding the TfR of the tumor surface.

The FITC-labeled nanoparticles were used to examine the targeting ability of T7-decorated nanoparticles to cells *in vitro*. Compared with the NP group, cellular uptake of T7-NP, with T7-modification, was greater and could be suppressed or promoted by the addition of free-T7 peptide and transferrin, respectively (Figures 4 and S7). Prior research has demonstrated that
T7-conjugated lipid nanoparticles could enhance cellular uptake compared to non-targeted nanoparticles [28]. Recently, there has been another report demonstrating the better cellular uptake of T7-decorated exosomes compared with unmodified-exosomes in the treatment of glioblastoma [35]. These findings indicate that T7-modified nanoparticles could enhance the uptake efficiency of esophageal cancer cells.

**Biodistribution of nanoparticles in vivo**

Tumor-bearing mice were divided to 5 groups (PBS, NP, NP-DC, T7-NP-D and T7-NP-DC). Cy5.5-labeled nanoparticles were injected intravenously prior to in vivo imaging. After 2 h there was no significant difference among all groups. The Cy5.5-labeled nanoparticles were distributed throughout the body, especially in the liver and kidney (Figure 5A). Examination 8 h post injection, as shown in Figure 5A, showed greatly decreased fluorescence. Nanoparticles without T7-modification had been eliminated from the tumor, while those with the modification remained in the tumor site. Our results confirm previous studies showing that the EPR effect of the nanomaterial can be improved by coupling short peptides for surface targeting [36]. The major organs (kidney, liver, lung, spleen and heart) and tumors were harvested after the mice had been euthanized, and then imaged as described earlier. The T7-NP-D and T7-NP-DC groups showed enrichment of Cy5.5-labeled nanoparticles in tumors. In the other two groups, no fluorescence was observed in the tumor tissue and only residual nanoparticles were seen in the liver and kidney (Figure 5B). A previous study confirmed that T7-conjugated nanoparticles could be co-internalized with receptor-bound transferrin [37]. In essence, nanoparticles with T7-modification have a good tumor targeting effect, indicating that the system is a good platform for delivery of anti-tumor drugs to tumor tissue.

**Anti-tumor efficacy and biosafety in vivo**

As planned, tumor-bearing mice were divided into 6 groups (PBS, NP-D, NP-DC, T7-NP-D, T7-NP-DC) and tumor volumes were measured every other day until the end of treatment. At the end of the experiment, blood was collected and major organs (lung, heart, liver, kidney and spleen) and tumors were harvested for HE staining and IHC analysis. As shown in Figure 6A, inhibitory activity was observed in all groups except the PBS group, indicating the efficacy of docetaxel toward the xenografts. Moreover, a better anti-tumor effect was seen in the T7-NP-D and
T7-NP-DC groups than in the NP-D and NP-DC groups that lacked the T7-modification, suggesting that the enhancement of anti-tumor effect was induced by decoration with the T7 targeting-peptide. Furthermore, the best inhibitory effect was obtained by the administration of T7-NP-DC according to the immunohistochemistry results (Figures 6D and 6E). In a study on melanoma, nanoparticles formed from PEG-b-PPS-b-PEI enhanced the anti-tumor effect without deleterious effects [38]. A previous study reported that nanoparticle co-delivery of docetaxel and curcumin could synergistically enhance activity against breast cancer cells [12]. Therefore, these in vivo results suggest that the combination of docetaxel and curcumin has synergistic anti-tumor efficacy.

The toxicity of the nanomedicines to blood and major organs was also investigated. After administration of the nanomedicines for 12 d, there was no weight loss and no deleterious effect on hematopoietic function among the treatment groups (Figures 6B and S8). There was also no obvious damage observed in the major organs using HE staining (Figure 7).

**Conclusion**

In summary, we have successfully built a novel T7-targeting nanocarrier with pH-responsive drug-release capability. The targeting ability, anti-tumor activity and biocompatibility of the system have been verified in vivo and in vitro. The nanomedicines not only have superior targeting ability, but also exhibit a synergistic anti-tumor effect. This study has laid a foundation for future development of combination-chemotherapy.

**Abbreviations**

CCK-8: Cell Counting Kit-8; 1H NMR: nuclear magnetic resonance spectroscopy; CLSM: confocal laser scanning microscope; TEM: transmission electron microscopy; DLS: dynamic light scattering; HE: Hematoxylin-eosin; IHC: immunohistochemistry; CI: combination index; TfR: Transferrin receptor; CDI: carbonyldiimidazole; DTX: docetaxel; CUR: curcumin; DMSO: dimethyl sulfoxide; PEI: polyethyleneimine; NHS-PEG-MAL: α-maleimidyl-ω-N-hydroxysuccinimidyld polyethylene glycol; β-CD: β-cyclodextrin; CM-β-CD: carboxymethylated β-CD; NP: CM-β-CD-PEI-PEG; T7-NP: CM-β-CD-PEI-PEG-T7; NP-D: CM-β-CD-PEI-PEG-DTX; NP-C: CM-β-CD-PEI-PEG-CUR; NP-DC:
CM-β-CD-PEI-PEG-DTX/CUR; T7-NP-D: CM-β-CD-PEI-PEG-T7/DTX; T7-NP-C: CM-β-CD-PEI-PEG-T7/CUR; T7-NP-DC: CM-β-CD-PEI-PEG-T7/DTX/CUR.

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Authors’ contributions

Lian Deng, Xiongjie Zhu performed the experiments and analyzed the data. Xiongjie Zhu wrote the manuscript. Zhongjian Yu and Longbao Feng provided some reagents for the experiments. Ying Li, Lingyu Qin and Zhile Liu helped with in vitro and in vivo assays. Rui Guo prepared the nanoparticles and make the characterization of it. Yanfang Zheng was the principle investigator and provided the expenses for the experiment.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

All animals experiments were approved by the ethical committee of Zhujiang Hospital of Southern Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interest.

Author details

1Department of Oncology, Zhujiang Hospital of Southern Medical University, Guangzhou, China. 2Key Laboratory of Biomaterials of Guangdong Higher Education Institutes, Guangdong Provincial Engineering and Technological Research Center for Drug Carrier Development,
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Figure Captions:

Table 1: Combination index (CI) of different drug formulations in KYSE150.

Figure 1: Demonstration of optimal formulations in ESCC. Following treatment with different formulations (DTX:CUR = 2:1, 1:1 and 1:2), inhibition efficacy was measured by CCK-8 assay (A, C), and then the synergistic effect was further evaluated by the combination index (CI) (B, D).

Figure 2: Characterization of nanoparticles. The size of nanoparticles (CM-β-CD-PEI-PEG-T7 and CM-β-CD-PEI-PEG-T7/DTX/CUR) was measured by TEM (A) and DLS (B).

Figure 3: Synergistic anti-tumor efficacy of nanomedicines in ESCC. Cells (KYSE150) were incubated with different treatments (DTX, CUR, NP, T7-NP, NP-D, NP-C, NP-DC, T7-NP-D, T7-NP-C and T7-NP-DC) for 48 h. Cell viability was then determined by flow cytometry (A, B) and CCK-8 assay (C).

Figure 4: Preferential cellular uptake of T7-modified nanoparticles. Cells (KYSE150) in good condition were pretreated with FITC-labeled nanoparticles composed of different treatments. The fluorescence intensity in cells was then observed using a confocal microscope. Cellular uptake of T7-decorated nanoparticles was superior to that of nanoparticles without T7-decoration.

Figure 5: Biodistribution of nanocarriers in vivo. Tumor-bearing mice were divided into 5 groups (PBS, NP, NP-DC, NPT-D and NPT-DC). After intravenous injection of Cy5.5-labeled nanoparticles, their enrichment in the body was explored using an IVIS Spectrum System (A), and the major organs were harvested and imaged (B).

Figure 6: Anti-tumor efficacy in vivo. Tumor-bearing mice were divided into 6 groups (PBS, DTX, NP-DTX, NP-DTX/CUR, T7-NP-DTX and T7-NP-DTX/CUR), treated by intravenous injection every other day for a total of 12 days. The mice were weighed (B) and tumors were harvested (A) and measured (C). Then, immunohistochemistry was conducted to investigate the expression of Ki-67 in response to various drug formulations (D, E).

Figure 7: Biocompatibility of nanomedicines in vivo. There was no significant damage observed by HE staining of heart, liver, spleen, lung and kidney after 12 days treatment with different nanomedicines.
Table 1: Combination index (CI) of different drug formulations in KYSE150

| DTX (ng/ml) | DTX:CUR 2:1 CI | DTX:CUR 1:1 CIC | DTX:CUR 1:2 CI |
|------------|----------------|------------------|----------------|
| 0.25       | 5.17357        | 0.48132          | 0.43564        |
| 0.5        | 0.73178        | 0.31493          | 0.47814        |
| 1          | 0.20394        | 0.31295          | 0.15368        |
| 2          | 0.20896        | 0.12393          | 0.1687         |
| 4          | 0.22412        | 0.18779          | 0.15239        |
| 8          | 0.31678        | 0.27078          | 0.18287        |
| 16         | 0.50756        | 0.37689          | 0.27741        |
| 32         | 0.73219        | 0.66478          | 0.35483        |
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7