**RESEARCH ARTICLE**

**Furin-responsive triterpenine-based liposomal complex enhances anticervical cancer therapy through size modulation**

Yunyan Chen\(^{a,b,c}\), Mengfei Guo\(^{a,b}\), Ding Qu\(^{a,b}\), Yuping Liu\(^{a,b}\), Jian Guo\(^{a,b}\) and Yan Chen\(^{a,b}\)

\(^{a}\)Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, China; \(^{b}\)Jiangsu Provincial Academy of Traditional Chinese Medicine, Nanjing, China; \(^{c}\)School of Pharmacy, Wannan Medical College, Wuhu, China

**ABSTRACT**

The accumulation and penetration of antitumor drugs in tumor tissues are directly related to their antitumor effects. The particle size of the nanodrug delivery system is one of the most important factors for the accumulation and penetration of antitumor drugs within tumor tissues. Generally, nanodelivery systems of intermediate size (100–120 nm) are capable of efficient accumulation owing to prolonged circulation and enhanced permeability and retention (EPR) effect; however, smaller ones (20–40 nm) are effective for deep penetration within tumor tissue. Currently a conventional drug delivery system cannot possess two types of optimal sizes, simultaneously. To solve this and to enhance cervical cancer treatment, a furin-responsive triterpenine-based liposomal complex (PEGcleavable Tf-CTM/L), with Tf-CTM (transferrin-modified tripterine-loaded coix seed oil microemulsion) in core, coated with a thermo-sensitive lipid and a kind of PEG shell modified with a furin-cleavable peptide was developed to improve tumor-specific accumulation and penetration. Herein, PEGcleavable Tf-CTM/L was capable of efficient accumulation because of EPR effect. The PEG shells could timely detach under stimulation of overexpressed furin protein to solve the problem of the steric hindrance dilemma. The small-sized Tf-CTM released under stimulation of tumor microthermal environment in cervical cancer, which was efficient with regards to deep penetration at tumor sites. Notably, compared to the use of triterpine alone, PEGcleavable Tf-CTM/L promoted anticervical efficacy and displayed diminished systemic toxicity by efficient accumulation and deep penetration of antitumor drugs within tumor tissues. Our study provides a new strategy, and holds promising potential for anticervical cancer treatment.

**1. Introduction**

Cervical cancer, one of the most common malignancies, threatens women’s lives and health worldwide (Banerjee & Kamrava, 2014; Vaisy et al., 2014; Tsikouras et al., 2016; Gaffney et al., 2018). In recent years, although the human papillomavirus (HPV) prophylaxis vaccine has been developed to prevent cervical cancer, it has not been widely used in developing countries. This is mainly due to limitations in regional economic development (Shi et al., 2008; Colombara and Wang, 2013; Zeng et al., 2016; Yin, 2017). In the clinic, a combination of carboplatin and paclitaxel has been widely used, for decades, as the gold standard for cervical cancer treatment to improve the quality of life and prolong patient survival (Mabuchi et al., 2009; Angioli et al., 2014; Symonds et al., 2015; Yang et al., 2015). However, the rapid clearance of antitumor drugs in the physiological circulation, and common side effects leads to the reduced efficacy of cervical cancer treatment. The clinical treatment of cervical cancer still faces great challenges.

Fortunately, nanodrug delivery systems have significantly improved the efficacy of anti-tumor drugs, while reducing the side effects of nontargeted drug delivery systems (Akhter et al., 2013; Biswas et al., 2016; Tran et al., 2017; Zhen et al., 2017; Reinišová et al., 2019; Sun et al., 2019). However, the efficiency of nanodrug delivery systems still significantly affects the efficacy of anti-tumor treatment. Whether the delivery system could efficiently accumulate at tumor sites and deeply penetrate within tumor tissues is an obstacle that needs to be overcome. The particle size of a nano-sized drug delivery system is the most crucial factor for accumulation and penetration in tumor tissues (Ding et al., 2012; Wang et al., 2015; Scenario, 2016; Islam et al., 2017; Zhang et al., 2017; Ruan et al., 2019; Yu et al., 2019; Tang et al., 2013).

To overcome this obstacle, we developed a furin-responsive triterpenine-based liposomal complex (PEGcleavable Tf-CTM/L) capable of cleavage by using a programmed assembly strategy that triggered by the microenvironment of cervical cancer models such as the...
overexpression of furin protein (Scheme 1). Tf-CTM was coated with thermo-sensitive lipids and PEG shell modified with a furin-cleavable peptide (S1), and PEGcleavable Tf-CTM/L with intermediate size (~110 nm) was capable of efficient accumulation. Owing to enhanced permeability and retention (EPR) effect, PEG shells can predominantly improve drug accumulation at tumor sites (Zhao et al., 2016; Hardiansyah et al., 2017; Ibrahim et al., 2017; Mcmasters et al., 2017; Kenechukwu et al., 2018; Nam et al., 2018; Rahmatolahzadeh et al., 2018). However, PEG shells can reduce cellular uptake due to steric hindrance (Grosse et al., 2010; Zhu et al., 2014; Jiang et al., 2017). To overcome this steric hindrance dilemma, the PEG shell was modified with a furin-cleavable peptide and its detachment was characterized using quartz crystal microbalance (QCM) technology (Wen et al., 2012; Wang et al., 2013; Yu et al., 2013; Jin et al., 2015). After efficient accumulation at tumor sites, the PEG shells could automatically detach from the liposomal complex under the stimulation of overexpressed furin protein. Thereafter, the lipid shells could become unstable in the tumoral microthermal environment, to perform deep tumor penetration by small-sized Tf-CTM (~40 nm) (Kumar et al., 2010; Jaaks & Bernasconi, 2017). For small-sized Tf-CTM, we developed a dual-component microemulsion delivery system (Tf-CTM) comprising tripterine (anticancer drug 1) and coix seed oil (anticancer drug 2, oil phase). As previously reported, it has been verified that after transferrin modification, Tf-CTM exhibited enhanced anti-cervical cancer treatment (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018).

Thus, PEGcleavable Tf-CTM/L possessed two types of optimal sizes, simultaneously; offering a new insight into effectively improving the antitumor efficacy of the nanodrug delivery system by resolving the contradiction of optimal size of accumulation and penetration at the tumor sites.

2. Materials and methods

2.1. Chemicals and reagents

GRVRRSC (G: glycine; R: arginine; V: valine; S: serine; C: cysteine) was provided by GL Biochem Co., Ltd. (Shanghai, China). MonomethoxyPEG (mPEG) was provided by Sigma-Aldrich (St. Louis, MO). 4-Dimethylaminopyridine (DMAP), 1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine iodide (DiD) and Triethylamine (TEA) were all provided by Aladdin Co., Ltd. (Shanghai, China). Octadecanol, succinic anhydride, maleic anhydride, and tripterine (purity >98.0%) were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Coix seed oil (extracted by carbon
dioxide supercritical technology, purity >85%). RH 40 and PEG 400 were purchased from BASF Co., Ltd. (Ludwigshafen, Germany). Dipalmitylophosphatidylcholine (DPPC), stearyllysophosphocholine (S-lysoPC), and Distearoylphosphoethanolamine-PEG2000 (DSPE-MPEG2k) were provided by A.V.T Co., Ltd. (purity > 98.0%, China). Double-distilled water was purified via Milli Q purification system (Merck Millipore). All other reagents used were of analytical grade.

2.2. Animals

Nude mice (BALB/c, 22 ± 2 g) were provided by the Model Animal Research Center of Yangzhou University (Jiangsu, China). All the animals were acclimatized for at least 7 days, and raised under 12 h light/dark cycles with food/water. The protocols were approved by the Animal Experimentation Ethics Committee of Nanjing University of Traditional Chinese Medicine (Nanjing, China).

2.3. Preparation and characterization of PEGcleavable Tf-CTM/L

The preparation of PEGcleavable Tf-CTM/L was carried out in two steps as follows. Firstly, the main anti-tumor microemulsion, Tf-CTM comprising tripterine (10 mg), coix seed oil (400 mg), RH 40 (450 mg), PEG 400 (150 mg), and Tf was prepared according to our previous report (Chen et al., 2018). Secondly, by the thin-film hydration method, Tf-CTM was then encapsulated into the hydrophilic core of the liposome. 45 18.00 mg of DPPC, 4.00 mg of DSPE-mPEG2k, 1.60 mg of S-lysoPC, and 2.36 mg of furin-cleavable peptide were dissolved in chloroform. The chloroform was removed to acquire the lipid film, at 37 °C under reduced pressure condition. And then the lipid film was hydrated with 10 mL of Tf-CTM using a rotary evaporator at 50 °C (Qu et al., 2018). The obtained PEGcleavable Tf-CTM/L was successively ultrasonicated (250 W, ultrasound 3 s interval 2 s for 10 min), extruded through a membrane filter (0.45 μm), and finally purified using G-50 Sephadex column. 45 mg of PEGcleavable Tf-CTM/L was prepared without furin-cleavable peptide. DSPE-mPEG5k was used, instead of the furin-cleavable peptide, to prepare PEGuncleavable Tf-CTM/L as a control.

The surface potential and average particle size of PEGcleavable Tf-CTM/L were measured by DLS (Nano ZS, Malvern Instruments Ltd, Malvern, UK). The morphology of PEGcleavable Tf-CTM/L was characterized by TEM (JEM-200CXJEOL, Tokyo, Japan) (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). 1 mL of PEGcleavable Tf-CTM/L was firstly transferred to a dialysis bag (10 kDa MWCO), and then followed by incubation within 120 mL of PBS (pH 7.4) with 0.5% (wt%) Tween 80, under pH 7.4 at 37 °C and 42 °C. During the 0–24 h period, 50 μL of PEGcleavable Tf-CTM/L solution was taken at the predetermined time intervals with rotary shaking at 60 rpm. The concentration and the accumulative release of tripterine from PEGcleavable Tf-CTM/L was determined via HPLC.

2.4. Drug encapsulation efficiency (EE) of PEGcleavable Tf-CTM/L

The EE was calculated as follows:

\[ \text{EE}_{\text{tripterine}}(\%) = \frac{W_{\text{encapsulated drug}}}{W_{\text{feeding drug}}} \times 100\% \]

where \( W \) represents the tripterine amount of various formulations.

The content of tripterine in PEGcleavable Tf-CTM/L was determined by HPLC (1260 Infinity, Agilent Technologies) at 426 nm. The chromatographic conditions were as follows: C18 column (4.6 × 150 mm × 5 μm, Diamond); mobile phase (methanol: water = 90:10); column temperature (30 °C); flow rate (1.0 mL/min) (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018).

2.5. Drug release in vitro

As previously reported, tripterine released from PEGcleavable Tf-CTM/L was determined via dialysis method (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). HeLa 3D tumor spheroids with an appropriate size (~300 nm) were seeded in the 96-well plates with the surface of an agarose-based culture medium, under an atmosphere of 5% CO\(_2\) at 37 °C. HeLa 3D tumor spheroids with an appropriate size (~300 nm) were selected and then transferred to confocal dishes, for study after 10 days of incubation (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). HeLa 3D tumor spheroids were treated with CTM, Tf-CTM with small size and Tf-CTM/L, PEGcleavable Tf-CTM/L with large size at fluorescein isothiocyanate (FITC, 10 μM) for 8 h and then fixed with 4% (v/v) paraformaldehyde and then observed via CLSM (Z-stack tool, 5 μm interval/scan).

2.6. Mechanism of PEGdetachment studied by quartz crystal microbalance (QCM)

The characterization of the furin-cleavable PEG shell of PEGcleavable Tf-CTM/L was studied by QCM (Pang et al., 2010; Kang et al., 2014; Qian et al., 2016; Marsh et al., 2018). In this part of the study, the QCM experiment was conducted at 25 °C. The frequency change (ΔF) was recorded using a third overtone. PEGcleavable Tf-CTM/L and PEGuncleavable Tf-CTM/L were employed to investigate the furin-cleavable PEG shell. First, PEGcleavable Tf-CTM/L and PEGuncleavable Tf-CTM/L were dissolved within PBS (pH 7.4) and injected into QCM-D cells until the frequency was constant and dissipation for adsorption to the films. PBS (pH 7.4) and furin protein solution were injected successively for 1 h, and the change in frequency was observed.

2.7. Cell culture and preparation of 3D tumor spheroids

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, 10% FBS, 100 μg/mL streptomycin/penicillin) under 5% CO\(_2\). HeLa cells (1 × 10\(^5\)) were seeded in the 96-well plates with the surface of an agarose-based culture medium, under an atmosphere of 5% CO\(_2\) at 37 °C. HeLa 3D tumor spheroids with an appropriate size (~300 nm) were selected and then transferred to confocal dishes, for study after 10 days of incubation (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). HeLa 3D tumor spheroids were treated with CTM, Tf-CTM with small size and Tf-CTM/L, PEGcleavable Tf-CTM/L with large size at fluorescein isothiocyanate (FITC, 10 μM) for 8 h and then fixed with 4% (v/v) paraformaldehyde and then observed via CLSM (Z-stack tool, 5 μm interval/scan).
2.8. Intracellular delivery and cellular uptake

HeLa cells \( (1 \times 10^5) \) were seeded in six-well plates and treated with FITC-labeled PEG-cleavable TF-CTM/L for 2 h after reaching 80% of the overspread. The concentration of various formulations was calculated as FITC (10 \( \mu \)M). After incubation, HeLa cells were rinsed with PBS three times, and then collected by trypsin without EDTA to harvest in 0.2 mL of PBS via flow cytometry (Guava 6HT, Merck Millipore).

According to previous studies, the intracellular delivery of various tripterine treatments was studied (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). HeLa cells \( (1 \times 10^5) \) were seeded in 12-well plates and treated with FITC-labeled PEG-cleavable TF-CTM/L at a FITC concentration of 10 \( \mu \)M. After 2 h of incubation, HeLa cells were washed by PBS (thrice, ice cold), followed by staining with MitoTracker Red (100 nM, Yeasen, China) and LysoTracker Red (50 nM, Abcam, UK). Finally, HeLa cells were fixed via 4% (v%) paraformaldehyde (25 °C) and observed by CLSM.

2.9. Cytotoxicity

Five thousand HeLa cells were seeded in 96-well plates and cultured for 24 h. HeLa cells underwent various treatments for 24 h at predetermined concentrations (0.625 – 20 \( \mu \)g/mL) after removal of DMEM. After treatment, 50 mL containing 5 \( \mu \)L of MTT solution was then added and HeLa cells were stained for further 4 h. The formazan crystals obtained were dissolved in DMSO (100 \( \mu \)L). The absorbance (A) was measured using a microplate reader at 570 nm (Qu et al., 2015; 2017; Chen et al., 2018; Qu et al., 2018).

2.10. Cell apoptosis induction

According to a previous report, HeLa cell suspension (50 \( \mu \)L, containing 5 \( \times \)10^3 cells) was collected and then incubated with Annexin V-PE staining kit (50 \( \mu \)L, Guava, Merck Millipore) for 15 min (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). The tripterine concentrations of various formulations were 1 \( \mu \)g/mL, the incubation time was 2–6 h, and the analysis was performed, immediately, by flow cytometry.

2.11. Xenograft tumor models

HeLa cell suspension (200 \( \mu \)L, containing 2 \( \times \)10^7 cells) was injected to the right hind leg of the nude mice subcutaneously, to establish the HeLa xenograft tumor model. The tumor size was measured via vernier calipers and calculated using the following formula: \( V = \frac{L \times W^2}{2} \), where \( W \) represents a smaller vertical width and \( L \) represents a larger vertical length (Qu et al., 2018).

2.12. In vivo imaging

When the tumor volume increased to 120 mm^3, HeLa xenograft nude mice were randomly divided into four groups and designed as follows: DiD, TF-DiD-C-MEs, TF-DiD-C-MEs/L, and PEGylated TF-DiD-CMIES/L. 0.2 mL of various formulations was administered intraperitoneally to nude mice at a DiD dose of 30 \( \mu \)g/mL (Qu et al., 2018).

After isoflurane-anesthesia was performed, the near-infrared images of administered nude mice were acquired using an in vivo imaging system (PerkinElmer, USA) at predetermined time points, post-administration (Qu et al., 2018). The fluorescence measurement was performed by the region-of-interest (ROI) function, adopting the live image software. Afterward, at 12 h post-treatment, the mice were euthanized. Using an in vivo imaging system, fluorescence images of major normal organs, such as the heart, liver, spleen, lung, kidney, and tumor tissues were collected.

2.13. Antitumor efficacy and systemic safety

With an average tumor size (−120 mm^3), HeLa xenograft tumor-bearing nude mice were intraperitoneally injected with saline (negative control), tripterine, TF-CTM, TF-CTM/L-37 °C, TF-CTM/L-42 °C, PEG-cleavable TF-CTM/L-37 °C, PEG-cleavable TF-CTM/L-42 °C, and PEG uncleavable TF-CTM/L-42 °C at a tripterine dose of 1.5 mg/kg once every 2 days. The efficacy of TF-CTM/L-42 °C, PEG-cleavable TF-CTM/L-42 °C and PEG uncleavable TF-CTM/L-42 °C were also assessed 6 h after administration by immersing the tumor-bearing leg in a 42 °C water bath for 1 h (Al-Jamal et al., 2012). The body temperature of all the other animals was maintained about 37 °C throughout our studies. The body weight and tumor size were daily recorded. Blood samples were assembled from the eyeballs of mice, followed by harvesting major organs, tumor, heart, liver, spleen, lung, and kidney. All blood samples were used for blood route analysis, liver/kidney function, and cytokines/chemokines. The cytokines/chemokines, including interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12A (IL-12A), chemokine 2 (CCL-2), tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)), transforming growth factor-\( \beta 1 \) (TGF-\( \beta 1 \)), and interferon-\( \gamma \) (IFN-\( \gamma \)). The tumor index (TI) was calculated using the following formula: weight tumor/weight body and the inhibition of tumor growth was calculated using the following formula: weight tumor/weight body and the inhibition of tumor growth was calculated using the following formula: weight tumor/weight body. Hematoxylin and eosin (HE) staining of harvested organs was performed using standard protocols. Conventional immunohistochemistry was used to observe the cell proliferation of the tumors using the Ki-67 antibody staining method. Furthermore, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, KeyGen Biotech, China) was used to evaluate tumor apoptosis.

2.14. CD-31 and \( \alpha \)-SMA assay

The tumor microenvironment was characterized by \( \alpha \)-SMA and anti-CD-31 antibody. Prior to \( \alpha \)-SMA staining, tumor sections were firstly deparaffinized via dimethylbenzene three times, and then rinsed with water-alcohol solutions, followed by incubation with 0.1% Triton X-100 within PBS (15 min) and finally blocking with 1% BSA (30 min). Thereafter, the tumor sections were incubated with primary polyclonal human \( \alpha \)-SMA antibody (Abcam, UK) and then further incubated with Alexa Fluor 555-conjugated secondary antibody...
both for 1 h. Followed by washing samples with PBS thrice, staining with DAPI for 30 min, and then fixing with 4% paraformaldehyde for 15 min. All of the above operations were carried out at room temperature (25°C) and observed by CLSM. Likewise, CD-31 staining was similar to that of α-SMA. The primary antibody was changed to CD-31 antibody (Qu et al., 2017; Qu et al., 2018).

2.15. Statistical analysis

The results are described as the mean ± standard deviation (SD). Statistical significance was determined using a two-tailed Student’s t-test. The differences were considered statistically significant at *p < .05, **p < .01.

3. Results and discussion

3.1. Characterization of PEGcleavable Tf-CTM/L

PEGcleavable Tf-CTM/L was mainly comprised of active targeting Tf-CTM, lipid bilayer membrane, and PEG shell modified with furin-cleavable peptide. The particle sizes of Tf-CTM, Tf-CTM/L, and PEGcleavable Tf-CTM/L were 40.33 ± 0.26, 111.3 ± 1.07, and 115.3 ± 1.37 nm, respectively, (Table 1). As shown in Figure 1(A), Tf-CTM displayed a spherical morphology with a small particle size of approximately 40 nm. Blank Lips (without Tf-CTM as the core) with a clear lipid bilayer were 110 nm around. In particular, PEGcleavable Tf-CTM/L exhibited the structure of Tf-CTM with small particle size located inside the large-sized liposome, illustrating that Tf-CTM was capable of being encapsulated into liposome, using the thin film dispersion method, which results in the liposomal complex simultaneously possessing two optimal sizes (Qu et al., 2018). The zeta potentials of Tf-CTM, Tf-CTM/L, and PEGcleavable Tf-CTM/L were −13.6 ± 1.13, −22.5 ± 0.361, and −47.8 ± 0.591 mV, respectively, (Table 1). In comparison with TF-CTM, the zeta potential of Tf-CTM/L was significantly increased, demonstrating that Tf-CTM was mainly distributed in the liposome. In comparison with TF-CTM/L at the corresponding mass ratio, the zeta potential of the PEGcleavable Tf-CTM/L was remarkably higher, which indicated that Tf-CTM/L was mainly successfully modified by the furin-cleavable peptide with negative charge (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). The encapsulation efficiency of tripterine in CTM, Tf-CTM, Tf-CTM/L, and PEGcleavable Tf-CTM/L were 98.45, 94.50, 70.81, and 73.40%, respectively. The EE of Tf-CTM/L and PEGcleavable Tf-CTM/L were 98.45, 94.50, 70.81, and 73.40%, respectively, (Table 1). As shown in Figure 1(A), Tf-CTM displayed a spherical morphology with a small particle size of approximately 40 nm. Blank Lips (without Tf-CTM as the core) with a clear lipid bilayer were 110 nm around. In particular, PEGcleavable Tf-CTM/L exhibited the structure of Tf-CTM with small particle size located inside the large-sized liposome, illustrating that Tf-CTM was capable of being encapsulated into liposome, using the thin film dispersion method, which results in the liposomal complex simultaneously possessing two optimal sizes (Qu et al., 2018). The zeta potentials of Tf-CTM, Tf-CTM/L, and PEGcleavable Tf-CTM/L were −13.6 ± 1.13, −22.5 ± 0.361, and −47.8 ± 0.591 mV, respectively, (Table 1). In comparison with TF-CTM, the zeta potential of Tf-CTM/L was significantly increased, demonstrating that Tf-CTM was mainly distributed in the liposome. In comparison with TF-CTM/L at the corresponding mass ratio, the zeta potential of the PEGcleavable Tf-CTM/L was remarkably higher, which indicated that Tf-CTM/L was mainly successfully modified by the furin-cleavable peptide with negative charge (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). The encapsulation efficiency of tripterine in CTM, Tf-CTM, Tf-CTM/L, and PEGcleavable Tf-CTM/L were 98.45, 94.50, 70.81, and 73.40%, respectively. The EE of Tf-CTM/L and PEGcleavable Tf-CTM/L were 98.45, 94.50, 70.81, and 73.40%, respectively, (Table 1). As shown in Figure 1(A), Tf-CTM displayed a spherical morphology with a small particle size of approximately 40 nm. Blank Lips (without Tf-CTM as the core) with a clear lipid bilayer were 110 nm around. In particular, PEGcleavable Tf-CTM/L exhibited the structure of Tf-CTM with small particle size located inside the large-sized liposome, illustrating that Tf-CTM was capable of being encapsulated into liposome, using the thin film dispersion method, which results in the liposomal complex simultaneously possessing two optimal sizes (Qu et al., 2018). The zeta potentials of Tf-CTM, Tf-CTM/L, and PEGcleavable Tf-CTM/L were −13.6 ± 1.13, −22.5 ± 0.361, and −47.8 ± 0.591 mV, respectively, (Table 1). In comparison with TF-CTM, the zeta potential of Tf-CTM/L was significantly increased, demonstrating that Tf-CTM was mainly distributed in the liposome. In comparison with TF-CTM/L at the corresponding mass ratio, the zeta potential of the PEGcleavable Tf-CTM/L was remarkably higher, which indicated that Tf-CTM/L was mainly successfully modified by the furin-cleavable peptide with negative charge (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). The encapsulation efficiency of tripterine in CTM, Tf-CTM, Tf-CTM/L, and PEGcleavable Tf-CTM/L were 98.45, 94.50, 70.81, and 73.40%, respectively. The EE of Tf-CTM/L and PEGcleavable Tf-CTM/L were 98.45, 94.50, 70.81, and 73.40%, respectively, (Table 1). In comparison with TF-CTM, the zeta potential of Tf-CTM/L was significantly increased, demonstrating that Tf-CTM was mainly distributed in the liposome. In comparison with TF-CTM/L at the corresponding mass ratio, the zeta potential of the PEGcleavable Tf-CTM/L was remarkably higher, which indicated that Tf-CTM/L was mainly successfully modified by the furin-cleavable peptide with negative charge (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). The encapsulation efficiency of tripterine in CTM, Tf-CTM, Tf-CTM/L, and PEGcleavable Tf-CTM/L were 98.45, 94.50, 70.81, and 73.40%, respectively. The EE of Tf-CTM/L and PEGcleavable Tf-CTM/L were 98.45, 94.50, 70.81, and 73.40%, respectively, (Table 1).

### Table 1. Particle size and zeta potential of various tripterine treatments (n = 3).

| Formulation                | Size (nm)  | PDI       | Zeta potential (mV) |
|---------------------------|------------|-----------|---------------------|
| TF-CTM                    | 40.33 ± 0.26 | 0.146 ± 0.01 | −13.63 ± 1.13       |
| TF-CTM/L                  | 111.3 ± 1.07 | 0.311 ± 0.05 | −22.5 ± 0.361       |
| PEGcleavable Tf-CTM/L     | 115.3 ± 1.37 | 0.261 ± 0.15 | −47.8 ± 0.591       |
| PEGcleavable TF-CTM/L     | 100.6 ± 2.01 | 0.491 ± 0.26 | −30.2 ± 0.328       |

(incubated with furin protein)

3.2. Cellular uptake assay

We evaluated the uptake of various formulations in cellular uptake study. The intracellular fluorescence of the FITC/Tf-C-MEs was more intense than that of various treatments group (Figure 2(A,B)), suggesting the enhancement of endocytosis after transferrin modification (**p < .01) (Chen et al., 2018). The intracellular fluorescence of the 42°C group was more intensive compared with that of the 37°C group (*p < .05), suggesting that enhancement of endocytosis was triggered by temperature (Chen et al., 2018). In particular, the uptake fluorescence intensity of HeLa cells by the FITC/Tf-C-MEs was 568.97 ± 11.42, and that of FITC/PEGcleavable Tf-C-MEs/L under the stimulation of overexpressed furin protein (Figure 1(D), phase 4), while there was no detachment observed from PEGuncleavable Tf-CTM/L (Figure 1(E)). In addition, the detachment of the PEG shell could further solve the PEG-steric hindrance dilemma to promote cellular uptake of tripterine and enhance the efficacy of cervical cancer treatment.

3.2. Cellular uptake assay

We evaluated the uptake of various formulations in cellular uptake study. The intracellular fluorescence of the FITC/Tf-C-MEs was more intense than that of various treatments group (Figure 2(A,B)), suggesting the enhancement of endocytosis after transferrin modification (**p < .01) (Chen et al., 2018). The intracellular fluorescence of the 42°C group was more intensive compared with that of the 37°C group (*p < .05), suggesting that enhancement of endocytosis was triggered by temperature (Chen et al., 2018). In particular, the uptake fluorescence intensity of HeLa cells by the FITC/Tf-C-MEs was 568.97 ± 11.42, and that of FITC/PEGcleavable Tf-C-MEs/L under the stimulation of overexpressed furin protein (Figure 1(D), phase 4), while there was no detachment observed from PEGuncleavable Tf-CTM/L (Figure 1(E)). In addition, the detachment of the PEG shell could further solve the PEG-steric hindrance dilemma to promote cellular uptake of tripterine and enhance the efficacy of cervical cancer treatment.
(yellow fluorescence), which may be due to the release of FITC/Tf-C-MEs.

Meanwhile, tripterine can inhibit the proliferation of tumor cells through the mitochondrial targeting pathway (Yoon et al., 2014; Shweta et al., 2015; Yu et al., 2015). Mitochondria were stained with MitoTracker Red (red fluorescence) and formulations were labeled with FITC (green fluorescence) (Chen et al., 2018). Interestingly, we found

Figure 1. Characterization of PEGcleavable Tf-CTM/L. (A) TEM images of Tf-CTM, blank Lip and PEGcleavable Tf-CTM/L. The red arrows represent the liposome-internal Tf-CTM. (B) Drug encapsulation efficiency of various treatments. (C) In vitro accumulative drug release of PEGcleavable Tf-CTM/L under pH 7.4 within 24 h. Data are represented as mean ± SD; n = 3. *p < .05. (D) The dynamic process of PEGcleavable Tf-CTM/L adsorption and PEGcleavable Tf-CTM/L detachment monitored by QCM under pH 7.4. (E) The dynamic process of PEGuncleavable Tf-CTM/L adsorption and PEGuncleavable Tf-CTM/L detachment monitored by QCM under pH 7.4.
colocalization in the mitochondria of FITC/Tf-C-MEs, FITC/Tf-C-MEs/L, and FITC/PEGcleavable Tf-C-MEs/L, suggesting that FITC/Tf-C-MEs were probably retained by mitochondria as well (Figure 2(D)).

3.3. Cell apoptosis induction

For the HeLa cell apoptosis study, the concentration of tripterine was 1 μg/mL and the incubation time was 2, 4, and 6 h, respectively.

After treatment for 2 h, tripterine exhibited the strongest ability to induce apoptosis, and the apoptosis rate was 34.92% (Figure 3(A)). The apoptosis rate of various treatments was lower than that of tripterine, mainly due to the dual encapsulation of liposomes and microemulsions. The dual-encapsulation structure can delay tripterine release from PEGcleavable Tf-CTM/L and thus reduce the toxicity against nontargeted sites.

After treatment for 4–6 h, there was a significant difference between PEGcleavable Tf-CTM/L-42 °C (36.30%, 48.07%)
and PEGcleavable Tf-CTM/L-37°C (32.72%, 37.94%) in the total apoptosis rate, which suggests that enhancement of cellular uptake was triggered by temperature. Compared to PEGcleavable Tf-CTM/L-42°C (36.30%), Tf-CTM (50.35%) exhibited 1.38-fold higher apoptosis rates at 4 h, and 1.14-fold higher apoptosis rates at 6 h, respectively, (Figure 3(B,C)), which suggested that small size and modification of transferrin could enhance apoptosis of tumor cells (Chen et al., 2018).

Interestingly, we found that Tf-CTM significantly induced cell apoptosis. Meanwhile, the apoptosis rate of PEGcleavable Tf-CTM/L-42°C showed a gradually increasing trend as the delivery time increased, suggesting that PEGcleavable Tf-CTM/L-42°C had a strong potential to induce HeLa cell apoptosis. After treatment with various treatments (1.0 μg/mL of tripterine) for 2, 4, and 6 h, PEGcleavable Tf-CTM/L-42°C displayed an advantage in apoptosis induction, as shown in Supplementary Figure S2A, B, and C.

### 3.4. Antiproliferative effects in vitro

After 24 h of treatment with various tripterine formulations, as the concentration of tripterine was higher over than 2.50 μg/mL, the proliferation of HeLa cells was significantly inhibited (Figure 4). The IC\textsubscript{50} values of various tripterine formulations against HeLa cells were 1.3710 ± 0.02, 0.9524 ± 0.01, 0.8119 ± 0.02, 1.0440 ± 0.01, 0.9192 ± 0.01, 1.0520 ± 0.04, 0.9601 ± 0.02, and 1.378 ± 0.12 μg/mL, respectively. Different tripterine microemulsions such as Tf-CTM can increase the solubility of tripterine and thus improve the uptake of tripterine by HeLa cells. Compared with PEGcleavable Tf-CTM/L-37°C, PEGcleavable Tf-CTM/L-42°C exhibited stronger cytotoxicity against HeLa cells. Tf-CTM released by liposome after incubation (42°C) also presented a more potent cytotoxicity against HeLa cells.

### 3.5. Tumor penetration and treatment

As previously reported, we successfully cultivated HeLa 3D tumor spheroids (Chen et al., 2018). In this study, HeLa three-dimensional (3D) tumor spheroids were also adopted to investigate the intratumor penetration of PEGcleavable Tf-CTM/L.

HeLa 3D tumor spheroids were all compact and spherical after culture for 10 days, with a diameter of approximately 300 μm. The penetration of CTM, Tf-CTM, Tf-CTM/L, and...
PEGcleavable Tf-CTM/L in HeLa 3D tumor spheroids was investigated after treatment for 8 h (Figure 5(A)). Drug penetration was marked with blue fluorescence. The penetration of various treatments in HeLa 3D tumor spheres was obviously related to the particle size. Small-sized formulations such as CTM (32.47 ± 0.26 nm) and Tf-CTM (40.33 ± 0.26 nm) could penetrate into the interior of HeLa 3D tumor spheres, however, Tf-CTM/L (111.30 ± 1.07 nm) and PEGcleavable Tf-CTM/L (115.30 ± 1.37 nm) with large size just reached the surface around the HeLa 3D tumor spheres. The permeability of CTM and Tf-CTM with small size was significantly deeper than that of Tf-CTM/L and PEGcleavable Tf-CTM/L with large size (Wilhelm et al., 2016). We further investigated the cytotoxicity of various treatments against HeLa 3D tumor spheroids.

Figure 5. (A) In vitro penetration of FITC into the HeLa 3D tumor spheroids after incubation with CTM, Tf-CTM, Tf-CTM/L, PEGcleavable Tf-CTM/L for 8 h. Z-stack images were obtained by CLSM. Scale bar is 100 μm. (1) CTM, (2) Tf-CTM, (3) Tf-CTM/L, (4) PEGcleavable Tf-CTM/L (B) Viability of HeLa 3D tumor spheres treated with T, CTM, Tf-CTM after 24 h using CCK8 (n = 6). **p < .01. (C) Viability of HeLa 3D tumor spheres treated with Tf-CTM/L and PEGcleavable Tf-CTM/L after 24 h using CCK8 (n = 6).

Figure 6. Investigation of biodistribution (A) Distribution of NIR signal on mice treated with different DiD-labeled formulations at the predetermined intervals. (B) Fluorescence images of normal organs at 12 h post-injection. (C) Fluorescence images of tumor tissues at 12 h post-injection. (D) Quantitative analysis of fluorescence in the tumor tissues after 12 h of the administration. Data are represented as mean ± SD, n = 3. **p < .01. (i), (ii), (iii), (iv) represent DiD, Tf-DiD-CMEs, Tf-DiD-CMEs/L, and PEGylated Tf-DiD-CMEs/L, respectively.
roids (Figure 5B,C) (Chen et al., 2018). The IC$_{50}$ values of T, CTM, Tf-CTM, Tf-CTM/L-37 °C, Tf-CTM/L-42 °C, PEGcleavable TF-CTM/L-37 °C, PEGcleavable TF-CTM/L-42 °C, and PEGuncleavable Tf-CTM/L-42 °C were 260.2 ± 1.3, 100.7 ± 1.1, 79.60 ± 1.1, 327.6 ± 1.5, 236.9 ± 1.5, 150.6 ± 1.1, and 165.6 ± 2.8 µg/mL, respectively, against HeLa 3D tumor spheroids. We observed dramatically lower cytotoxicity among CTM, Tf-CTM, and PEGcleavable TF-CTM/L-42 °C, respectively, against HeLa 3D tumor spheroids. The advantage of microemulsion was more obvious than that of the composite, which is related to the deep penetration advantage of nanoparticles with small particle size and the higher uptake of tripterine by HeLa 3D tumor spheroids.

### 3.6. Biodistribution

To elucidate the biodistribution under NIR in vivo imaging system, PEGcleavable TF-CTM/L was labeled with DiD (PEGylated Tf-DiD-CMEs/L), and DiD, Tf-DiD-CMEs, and Tf-DiD-CMEs/L acted as controls (Qu et al., 2018). DiD group showed no obvious aggregation at the tumor site during the...
entire post-injection period. After treatment with Tf-DiD-CMEs, it accumulated at tumor sites. Additionally, the extension of administration time during 1–12 h resulted in the accumulation of Tf-DiD-CMEs at tumor sites becoming more obvious. After treatment with Tf-DiD-CMEs/L around 2–12 h post-treatment, strong fluorescence signals were detected around the tumor site. Notably, after treatment with PEGlyated Tf-DiD-CMEs/L, there was a significant NIR signal at the tumor site during the entire observation period (Figure 6(A)). After 12-h post-treatment, PEGlyated Tf-DiD-CMEs/L, and Tf-DiD-CMEs/L mainly accumulated in the liver and detected in the kidney, suggesting the capture of nanoparticles, mainly by the reticuloendothelial system and kidney-mediated elimination (Figure 6(B)). The tumor tissues
were harvested to analyze the fluorescence intensity qualita-
tively and quantitatively to evaluate the potential tumor-tar-
getting ability (Figure 6(C,D)). There was a significant
difference between PEGlyated Tf-DiD-CMEs/L and Tf-DiD-
CMEs in biodistribution \((/\text{C}_3/) < \cdot 01\). Notably, tumors from
mice treated with PEGlyated Tf-DiD-CMEs/L displayed the
highest intensity of fluorescence compared to the others,
suggesting that PEGlyated Tf-DiD-CMEs/L was capable of effi-
cient accumulation at tumor sites by the advantage of the
EPR effect.

3.7. Evaluation of antitumor efficacy

The antitumor efficacy in vivo of PEGcleavable Tf-CTM/L-42 °C
was investigated, with saline, tripterine, Tf-CTM, Tf-CTM/L-
37 °C, Tf-CTM/L-42 °C, PEGcleavable Tf-CTM/L-37 °C, and
PEGuncleavable Tf-CTM/L-42 °C as controls. The mice were
intraperitoneally administered with various treatments at
doses of 1.5 mg tripterine/kg once every 2 days. Compared
to the saline group, the tumor growth of mice treated with
various tripterine treatments were largely inhibited (Figure
7(A)). Due to the long circulation of PEG and temperature
triggers release of tripterine, PEGcleavable Tf-CTM/L with
water bath of 42 °C presented the strongest antitumor activ-
ity than any other formulations. Notably, the tumor inhibition
ratio of PEGcleavable Tf-CTM/L-42 °C was 80.33%, which was
1.25- and 1.29-fold higher than Tf-CTM/L-42 °C and
PEGcleavable Tf-CTM/L-37 °C, respectively (Figure 7(B)). After
treatment with PEGcleavable Tf-CTM/L-42 °C using HeLa
xenograft tumor-bearing mice, the tumor weight of
PEGcleavable Tf-CTM/L-42 °C was the lowest among the vari-
ous treatment groups (Figure 7(C)). As previously reported,
tripterine can lead to several severe side effects, including a
short survival period and a sharp reduction in bodyweight.
However, the bodyweight of tripterine and Tf-CTM-treated
mice was lower than that of the PEGcleavable Tf-CTM/L
group, and only minor fluctuations in body weight of
PEGcleavable Tf-CTM/L-42 °C were observed; mainly due to
dual-encapsulation by liposomes and microemulsions of trip-
terine (Figure 7(D)). In HE-stained tumor section among vari-
ous tripterine treatments, PEGcleavable Tf-CTM/L-42 °C
induced the largest necrosis area with tumor cell nucleus dis-
appeared, illustrating an overwhelming antitumor capacity
(Figure 7(E)). Compared with the saline group, the Ki-67-posi-
tive cells in the Tf-CTM, PEGcleavable Tf-CTM/L-37 °C, and
Tf-CTM/L-42 °C treatments were sharply reduced. Notably, only
a small amount of brown cells were found in the tumor sec-
tion of PEGcleavable Tf-CTM/L-42 °C (Figure 7(F)). In various
tripterine treatments treated tumor sections, a large number
of TUNEL-positive cells were detected. Notably, the green
fluorescence in the treatment of PEGcleavable Tf-CTM/L-42 °C
was stronger than that in the other treatments (Figure 7(G)).
Validated by HeLa xenograft tumor-bearing mice models, all the results above verified that PEG-cleavable TF-CTM/L-42°C had good antitumor efficacy by resolving a contradiction of conformity in optimal size of accumulation and penetration at the tumor sites.

3.8. Evaluation of systemic safety

Due to the toxicity, many of the antitumor ingredients were limited to clinical use. The application of tripterine also faces the following obstacles: highly toxic, narrow treatment window, and poor solubility; which severely limits the tripterine to clinical use. PEG-cleavable TF-CTM/L significantly improved the accumulation at tumor sites, while reducing the side effects of non-targeted sites. In this part of the study, we detected the samples of organs and blood to evaluate the potential toxicity of various tripterine treatments. Compared to the normal mice, PEG-cleavable TF-CTM/L-42°C had a negligible effect on white blood cell (WBC) and platelet (PLT), but slightly reduced the levels of hemoglobin (HGB) and red
blood cell (RBC) after 14 days of treatment (Figure 8(A–D)). The liver and spleen index of mice did not change significantly after treatment with PEGcleavable Tf-CTM/L-42°C (Figure 8(E,F)). In terms of HE staining of main normal organs, PEGcleavable Tf-CTM/L-42°C hardly caused pathological changes of heart, liver, spleen, lung, and kidney (Figure 8(G)). We also investigated the changes of serum aminopherase including alanineaminotransferase (ALT) and aspartate aminotransferase (AST). We also detected the changes of classic indices, including uric acid (UA), blood urine nitrogen (BUN), and creatinine (CREA) of kidney function. As previously reported, tripterine may induce acute damage to the liver and kidney injury.42-45 Compared with the normal groups, there was no significant change in the concentrations of ALT, UA, BUN, AST, and CREA among various tripterine treatments, suggesting low toxicity against the function of liver and kidney after treatment with PEGcleavable Tf-CTM/L-42°C (Supplementary Figure S3).

3.9. Cytokine determination

The potential mechanism of the anti-tumor effect of PEGcleavable Tf-CTM/L-42°C was associated with the normalization of the tumor microenvironment by tripterine (Qu et al., 2015, 2017; Chen et al., 2018; Qu et al., 2018). In this part of study, after treated with various tripterine formulations, we detected the alterations of fibroblasts, tumor vessels, and cytokines as well. Cytokines are closely associated with the occurrence and development of cancer. The

Figure 9. CD 31 and α-SMA Assay (A) Fluorescence images of tumor section stained with anti-α-SMA primary antibody after 24 h of the last administration. The red represents the TAFs and the blue represents nucleus. The bar is 50 μm. (B) Fluorescence images of tumor section stained with anti-CD 31 primary antibody after 24 h of the last administration. The red represents the tumor vessel and the blue represents nucleus. The bar is 50 μm.
determination of cytokines in the serum of tumor-bearing nude mice can reflect the antitumor mechanism of various tripterine treatments. It is well known that IFN-γ, IL-2, and IL-12A are capable of inhibiting tumor cell proliferation, blocking the angiogenesis pathway, regulating immunity, and decreasing the formation of tumor-associated fibroblasts (TAFs) (Mumm et al., 2011; Bunimovich-Mendrazitsky et al., 2016; Komohara et al., 2016; Yue et al., 2016; Jiang et al., 2017; Vourch et al., 2017; Wu et al., 2017; Ayuthaya et al., 2018; Burkart et al., 2018; Hydes et al., 2018; Kamensek et al., 2018; Lampreht Tratar et al., 2018; Posadasánchez & Vargasalarcón, 2018). In contrast, IL-10, IL-6, CCl2, and TGF-β are considered to be tumor-promoting cytokines, which could promote the proliferation of tumor cells, rebuilding of vascular networks, and so on (Alhamarneh et al., 2015; Song et al., 2015; Lau et al., 2016; Suchal et al., 2016; Kim et al., 2017; Kong et al., 2017). Compared to the saline group, the concentrations of IL-10, IL-6, CCl2, and TGF-β in serum were significantly reduced after treatment with PEGcleavable Tf-CTM/L-42 °C (Supplementary Figure S4A,C,F,H). Interestingly, PEGcleavable Tf-CTM/L-42 °C significantly improved the concentration of IFN-γ compared with the saline group (Supplementary Figure S4B,D,E). There was no noticeable change in the serum level of TNF-α after various tripterine formulations (Supplementary Figure S4G). These results further explain the antitumor mechanism of PEGcleavable Tf-CTM/L-42 °C.

3.10. CD 31 assay and α-SMA

TAFs, the major obstacles of deep tumor drug delivery, and fibroblasts were stained using α-SMA. Notably, compared to the saline group, and other tripterine treatments, the level of α-SMA was significantly reduced after treatment with PEGcleavable Tf-CTM/L-42 °C (Figure 9(A)). PEGcleavable Tf-CTM/L-42 °C played a role in suppressing tumor angiogenesis by the efficient accumulation and deep penetration within tumor tissue.

The vascular endothelial cells were conjugated with anti-CD 31 antibody, and then labeled with a secondary antibody (red fluorescence). After treatment with PEGcleavable Tf-CTM/L-42 °C, the red fluorescence sharply decreased, indicating a significant decrease in vessel density, in addition to indicating the high efficiency of anti-tumor drug accumulation and deep penetration at the tumor sites (Figure 9(B)).

4. Conclusions

In summary, we have developed a dual-encapsulation liposomal nanodrug delivery system with two optimal sizes to enhance the efficacy of cervical cancer treatment by enhancing accumulation and penetration at tumor sites. PEGcleavable Tf-CTM/L-42 °C showed stronger inhibition of xenograft tumor growth in comparison with other tripterine treatments. Meanwhile, the near-infrared images of PEGcleavable Tf-CTM/L exhibited a prominent tumor-targeting ability due to prolonged circulation and the EPR effect. Notably, Tf-CTM released from PEGcleavable Tf-CTM/L with small particle size enhanced tumor penetration in HeLa 3D tumor spheroids. More effective accumulation and deep penetration in tumor tissues enhanced the anticervical cancer effects of tripterine. This study offers a new sight and technology to effectively improve the antitumor efficacy of nano-sized anticancer Traditional Chinese Medicine by resolving a contradiction in the optimal size of accumulation and penetration in the tumor sites.

Disclosure statement

The authors report no conflicts of interest in this work.

Funding

This work was supported financially by the National Natural Science Foundation of China [81673606, 81873016] and the Science and Key Medical Talent Project of Jiangsu Province [ZDRCA 2016036].

References

Akhter S, Ahmad I, Ahmad MZ, et al. (2013). Nanomedicines as cancer therapeutics: current status. Curr Cancer Drug Targets 13:362–78. Alhamamneh O, Agada F, Madden L. (2015). Serum IL10 and circulating CD4(+/-) CD25(high) regulatory T cell numbers as predictors of clinical outcome and survival in patients with head and neck squamous cell carcinoma. Head Neck 33:415–23. Al-Jamal WT, Al-Ahmady ZS, Kostarelos K. (2012). Pharmacokinetics & tissue distribution of temperature-sensitive liposomal doxorubicin in tumor-bearing mice triggered with mild hyperthermia. Biomaterials 33:4608–17.

Angioli R, Plotti F, Luvero D, et al. (2014). Feasibility and safety of carboplatin plus paclitaxel as neo-adjuvant chemotherapy for locally advanced cervical cancer: a pilot t study. Tumour Biol 35:2741–6. Ayuthaya B, Everts V, Pavasant P. (2018). The immunopathogenic and immunomodulatory effects of interleukin-12 in periodontal disease. Eur J Oral Sci 126:75–83.

Banerjee R, Kamrava M. (2014). Brachytherapy in the treatment of cervical cancer: a review. Int J Womens Health 6:555–64. Biswas S, Kumar P, Lakhaní PM, Ghosh B. (2016). Recent advances in polymeric micelles for anti-cancer drug delivery. Eur J Pharm Sci 83:184–202.

Bunimovich-Mendrazitsky S, Halachmi S, Kronik N. (2016). Improving Bacillus Calmette-Guérin (BCG) immunotherapy for bladder cancer by adding Interleukin 2 (IL-2): a mathematical model. Math Med Biol 33:159–88.

Burkart C, Mukhopadhyay A, Shirley SA, et al. (2018). Improving therapeutical efficacy of IL-12 intratumoral gene electrotransfer through novel plasmid design and modified parameters. Gene Ther 25:93–103. Chen Y, Qu D, Fu RP, et al. (2018). A Tf-modified tripterine-loaded coix seed oil microemulsion enhances anti-cervical cancer treatment. Int J Nanomed 13:7275–87.

Colobara DV, Wang SM. (2013). The impact of HPV vaccination delays in China: Lessons from HBV control programs. Vaccine 31:4057–9.

Ding D, Wang J, Zhu Z, et al. (2012). Tumor accumulation, penetration, and antitumor response of cisplatin-loaded gelatin/poly(acrylic acid) nanoparticles. ACS Appl Mater Interfaces 4:1838–46.

Gaffney DK, Hashibe M, Kepka D, et al. (2018). Too many women are dying from cervix cancer: Problems and solutions. Gynecol Oncol 151:547–54.
reccurrent cervical cancer (CIRCCa): a randomised, double-blind, placebo-controlled phase 2 trial. Lancet Oncol 16:1515–24.
Tang L, Gabrielson NP, Uckun FM, et al. (2013). Size-dependent tumor penetration and in vivo efficacy of monodisperse drug-silica nanoconjugates. Mol Pharm 10:883–92.
Tran S, DeGiovanni PJ, Piel B, Rai P. (2017). Cancer nanomedicine: a review of recent success in drug delivery. Clin Transl Med 6:44.
Tsikouras P, Zervoudis S, Manav B, et al. (2016). Cervical cancer: screening, diagnosis and staging. J BUON 21:320–5.
Vaisy A, Lotfinejad S, Zhian F. (2014). Risk of cancer with combined oral contraceptive use among Iranian women. Asian Pac J Cancer Prev 15:5517–22.
Vourch M, Roquilly A, Broquet A, et al. (2017). Exoenzyme T plays a pivotal role in the IFN-γ production after Pseudomonas challenge in IL-12 primed natural killer cells. Front Immunol 8:1283.
Wang K, Liu Y, Yi W-J, et al. (2013). Novel shell-cross-linked micelles with detachable PEG corona for glutathione-mediated intracellular drug delivery. Soft Matter 9:692–9.
Wang J, Mao W, Lock LL, et al. (2015). The role of micelle size in tumor accumulation, penetration, and treatment. Acs Nano 9:7195–206.
Wen H, Dong C, Dong H, et al. (2012). Engineered redox-responsive PEG detachment mechanism in PEGylated nano-graphene oxide for intracellular drug delivery. Small 8:760–9.
Wilhelm S, Tavares AJ, Dai Q, et al. (2016). Analysis of nanoparticle delivery to tumors. Nat Rev Mater 1:16014.
Wu J, Cui T, Yin C. (2017). Co-delivery of doxorubicin and interleukin-2 via chitosan based nanoparticles for enhanced antitumor efficacy. Acta Biomater 47:81–90.
Yang L, Guo J, Shen Y, et al. (2015). Clinical efficacy and safety of paclitaxel plus carboplatin as neoadjuvant chemotherapy prior to radical hysterectomy and pelvic lymphadenectomy for stage IB2-IIIB cervical cancer. Int J Clin Exp Med 8:13690–8.
Yin Y. (2017). HPV vaccination in China needs to be more cost-effective. Lancet 390:1735–6.
Yoon MJ, Lee AR, Jeong SA, et al. (2014). Release of Ca²⁺ from the endoplasmic reticulum and its subsequent influx into mitochondria trigger celastrol-induced paraptosis in cancer cells. Oncotarget 5:6816–31.
Yu W, He X, Yang Z, et al. (2019). Sequentially responsive biomimetic nanoparticles with optimal size in combination with checkpoint blockade for cascade synergetic treatment of breast cancer and lung metastasis. Biomaterials 217:119309.
Yue T, Zheng X, Dou Y, et al. (2016). Interleukin 12 shows a better curative effect on lung cancer than paclitaxel and cisplatin doublet chemotherapy. BMC Cancer 16:665.
Yu J, Li X, Luo Y, et al. (2013). Poly(ethylene glycol) shell-sheddable magnetic nanomicelle as the carrier of doxorubicin with enhanced cellular uptake. Colloids Surf B Biointerf 107:213–9.
Yu X, Zhou X, Fu C, et al. (2015). Celastrol induces apoptosis of human osteosarcoma cells via the mitochondrial apoptotic pathway. Oncol Rep 34:1129–36.
Zeng Z, Yang H, Li Z, et al. (2016). Prevalence and genotype distribution of HPV infection in China: analysis of 51,345 HPV genotyping results from China’s largest CAP certified laboratory. J Cancer 7:1037–43.
Zhang F, Zhu G, Jacobson O, et al. (2017). Transformative nanomedicine of an amphiphilic camptothecin prodrug for long circulation and high tumor uptake in cancer therapy. Acs Nano 11:8838–48.
Zhao H, Li Q, Hong Z. (2016). Paclitaxel-loaded mixed micelles enhance ovarian cancer therapy through extracellular pH-triggered PEG detachment and endosomal escape. Mol Pharm 13:2411–22.
Zhen L, Shirui T, Shuan L, et al. (2017). Cancer drug delivery in the nano era: an overview and perspectives. Oncol Rep 38:611–24.
Zhu H, Dong C, Dong H, et al. (2014). Cleavable PEGylation and hydrophobic histidylolation of polylysine for siRNA delivery and tumor Gene Therapy. ACS Appl Mater Interf 6:10393–407.