Self-Assembled Folic Acid-Targeted Pectin-Multi-Arm Polyethylene Glycol Nanoparticles for Tumor Intracellular Chemotherapy

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ABSTRACT: Ursolic acid is widely used as an effective anticancer drug for the treatment of various cancers. However, its poor water solubility, short circulation time in vivo, and lack of targeting have made it a burden for clinical applications. We report a self-assembled folate-modified pectin nanoparticle for loading ursolic acid (HCPT@F-Pt-PU NPs) and embed the anticancer drug hydroxycamptothecin to achieve synergistic treatment with ursolic acid. In addition, the galactose residue of the pectin molecule can be recognized by the asialoglycoprotein receptor on the surface of the liver cancer cell, promoting the rapid penetration and release of HCPT@F-Pt-PU NPs intracellularly. In particular, the introduction of multiharm polyethylene glycol can improve the uniformity (106 nm) and concealment of the nanoparticles and avoid the early release of the drug or the toxicity to normal cells. HCPT@F-Pt-PU NPs have a high drug loading (7.27 wt %) and embedding efficiency (19.84 wt %) and continuous circulation up to 80 h, leading to more apoptosis (91.61%). HCPT@F-Pt-PU NP intracellular drug delivery will be a promising strategy.

1. INTRODUCTION

Drug resistance of tumor cells leads to complications for treatment, and radiation therapy causes unpredictable side effects. Compared to chemotherapeutic drugs, because pectin can inhibit Gal-3, the key target of cancer metastasis, pH-modified pectin may play an important role in antitumor applications. Pectin is an acidic heteropolysaccharide with a lot of biologically active functions, such as regulation of immune response, inhibition of tumor growth, and metastasis. With the development of pectin as a safe drug carrier, new pectin drug delivery systems will gradually be established. Neufeld and Bianco-Peled established the drug delivery system of pectin−chitosan loaded for loading mesalazine, curcumin, and progesterone and found that some interactions between chitosan and pectin promoted the slow release of drugs. Jung, Arnold, and Wicker took advantage of CaCl2 charge to modify pectin-coated indomethacin hydrogels; the drug release efficiency was reduced after immersion in the simulated gastric fluid for 2 h and the intestinal fluid for 3 h. In addition, the enzyme-dependent pectin−ketoprofen (PT−KP) complex could target the colon through breaking the ester bond between PT and KP, achieving drug delivery and enrichment of colon cancer. Similarly, the pectin−cysteine complex, pectin−PVP−curcumin, pectin−zein−curcumin nanoparticles, and pectin−methotrexate nanoparticles were reported.

However, a low drug loading efficiency, a short half-time, and the lack of targeting limited the application of pectin-based drug delivery systems. Commonly, the reduction of blood component interactions will induce the activation of the complement system, resulting in a decrease in the blood clearance rate of the drug carrier, namely, the stealth effect. Polyethylene glycol (PEG) is a nonionic hydrophilic polymer with stealth behavior, which reduces the aggregation tendency by spatial stability and affects the pharmacokinetic performance of drugs or carriers. PEG shielding or PEG administration could prolong the blood circulation time and increase the possibility of the drug reaching the site of action.

In this study, multiharm PEG was used to enhance the water solubility and stability of drugs, increase the enhanced permeability and retention effect, and prolong the circulation time in vivo. In addition, in order to ameliorate the enrichment density of drugs in tumors, folic acid was applied to target folic acid-receptor proteins. Simply, ursolic acid (UA) was used as a

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model drug, eight-arm PEG (8armPEG-COOH) was chemically linked to UA, and the drug was cocoupled with pectin by inducing targeted folic acid to form the folic acid-pectin-eight-arm PEG-UA conjugate (F-Pt-PU). Afterward, the hydrophobic drug hydroxycamptothecin (HCPT) was embedded in the F-Pt-PU conjugate to prepare folate-modified pectin-eight-arm PEG-based nanoparticles (HCPT@F-Pt-PU NPs). A combination of two anticancer drugs was used to improve the effect of synergistic inhibition with different mechanisms of eliminating tumors, and the active capture effect of folic acid could enhance the enrichment ability of tumor sites to achieve rapid elimination of tumor cells. The design of the F-Pt-PU conjugates and the self-assembly scheme of the HCPT@F-Pt-PU NPs are displayed in Figure 1.

2. RESULTS AND DISCUSSION

2.1. Preparation of HCPT@F-Pt-PU NPs. FTIR spectra of FA-NH2, UA, 8armPEG-COOH, pectin, and F-Pt-PU are shown in Figure 2. Pectin and F-Pt-PU displayed a strong band for the C=O group in the range of 1700–1600 cm⁻¹, and FA-NH2 and F-Pt-PU showed a wake N–H stretch in the range of 3500–3100 cm⁻¹. The one band of secondary amides (R–CO–NH–R) and unusually low-value C=O would indicate the presence of an amide functional group. 8armPEG-COOH, pectin, and F-Pt-PU characteristic absorption peaks at 1610 cm⁻¹ belong to the carboxylate (−COO⁻) asymmetric stretching vibration. The bands of pectin and F-Pt-PU at 1105 and 1000 cm⁻¹ are assigned to the ether bond stretching vibration. However, because the pectin itself contains ester bonds, which brings difficulties to the analysis, we will continue to explain this part in conjunction with the nuclear magnetic resonance test.

1H NMR spectra were recorded for free UA, 8armPEG-UA, pectin, FA-NH2, pectin-FA, the F-Pt-PU conjugate, and HCPT@F-Pt-PU NPs (Figure 3). The signals at δ 3.2–4.0 and δ 2.2–3.2 are attributed to most of the proton characteristic peaks of pectin (D2O) and FA-NH2 (D2O),
respectively. The signals of pectin-FA (D₂O) at δ 6.8, δ 7.7, and δ 8.5 are the proton characteristic peaks of folic acid, indicating the successful synthesis of pectin and folic acid. The signal at δ 6.6–2.3 is attributed to most of the proton characteristic peaks of UA (CDCl₃), δ 2.5–3.9 (4nH, −(CH₂CH₂O)ₙ−) and δ 4.2 (2H, −CH₂OC(O)O) are the methylene proton peak of 8armPEG-COOH (CDCl₃). The multiple peak of UA around δ 4.25 (1H, CH), indicating the formation of the ester bond between UA and 8armPEG-COOH. The terminal methylene proton peak of PEG moves from δ 4.13 (1H, CH) to the lower field of δ 4.17 (1H, CH), and the small peak remaining at δ 4.13 (1H, CH) indicates the presence of unreacted PEG functional groups. Most of the characteristic peaks of UA, 8armPEG-COOH, and pectin can be seen in F-Pt-PU NPs. However, proton characteristic peaks of folic acid in F-Pt-PU conjugates are very weak in ¹H NMR because of addition of folic acid. The weak peak of folic acid can be seen in F-Pt-PU NPs by ¹H NMR because hydrophilic folic acid was exposed on the surface of nanoparticles.

2.2. Physicochemical Characterization of Nanoparticles. The UA-loading rates of F-Pt-PU NPs and HCPT@F-Pt-PU NPs were 8.12 and 7.27%, respectively, and the HCPT-embedding rate was as high as 19.84% (HCPT) (Table 1). The particle size of HCPT@F-Pt-PU NPs was significantly increased (105 nm) because of HCPT. TEM images of HCPT@F-Pt-PU NPs showed the successful embedment by self-assembly (Figure 4A). The hydrodynamic diameter of HCPT@F-Pt-PU NPs was relatively concentrated (Figure 4B), and good dispersibility of HCPT@F-Pt-PU NPs was obtained because of the effect of HCPT embedding as an increased hydrophobic core. Pt-PU NPs, F-Pt-PU NPs, and HCPT@F-Pt-PU NPs were dispersed in PBS for 35 days to observe size changes. The results showed that the particle size changed little, indicating that the nanoparticles had a good stability (Figure 4C). In addition, zeta potential was measured for further evaluating the stability of nanoparticles. Functional groups which are electrically charged can affect drug carriers within living cells, and positively charged particles have influence in cell membrane infiltration and cell internalization. Obviously, as shown in Table 1, F-Pt-PU NPs and HCPT@F-Pt-PU NPs have positive surface charges (ζ = 11.87 and 13.64 mV) because of folic acid and unreacted galacturonic acid of pectin in the process of preparing nanoparticles by self-assembly. The rich galacturonic acid and folic acid carboxyl were exposed on the surface of nanoparticles to form a lot of positive charges that improved the stability of the nanoparticles.

2.3. In Vitro Drug Release. The release of HCPT@F-Pt-PU NPs at different pH values (pH 5.8, 7.4, and 8.1) was simulated in the biological fluid, and the release kinetics of UA and HCPT in HCPT@F-Pt-PU NPs was studied through HPLC analysis. As can be seen from Figure 5A, HCPT@F-Pt-PU NPs of pH 5.8 were released less than pH 7.4 or 8.0 and without burst release. Obviously, the release of UA in HCPT@F-Pt-PU NPs was pH-dependent. The addition of esterase promoted the hydrolysis of HCPT@F-Pt-PU NPs and further promoted the release of HCPT (Figure 5B). HCPT@F-Pt-PU NPs could be used as the carrier of UA and HCPT to realize relieved and controlled release and also lead to the pH-dependent passive targeting in cells.

2.4. In Vitro Cytotoxicity Evaluation of HCPT@F-Pt-PU NPs. In vitro cytotoxicity evaluation of HCPT@F-Pt-PU NPs in H22 and HepG2 cells was performed by the CCK-8 method, and free UA, HCPT, F-Pt-PU NPs, and HCPT@F-Pt-PU NPs of different concentration gradients were added into H22 and HepG2 cells to incubate for 24, 48, and 72 h, respectively. As shown in Figure 6A,C, when the drug concentration is 10 µg mL⁻¹, free UA, HCPT, F-Pt-PU NPs, and HCPT@F-Pt-PU NPs (equivalent UA) could significantly inhibit cell growth and kill cells with the extension of

Table 1. Particle Size, Zeta Potential, and Drug Loading Efficiency of Nanoparticles

| compound       | DLE-UA (wt %) ± SEM | DLE-HCPT (wt %) ± SEM | size (nm) ± SEM | zeta potential (mV) ± SEM |
|----------------|---------------------|-----------------------|----------------|---------------------------|
| 8armPEG-UA     | 18.71 ± 0.33        | 87.91 ± 5.13          | 87.91 ± 5.13   | 11.87 ± 0.22              |
| F-Pt-PU NPs    | 8.12 ± 0.58         | 105.72 ± 6.94         | 105.72 ± 6.94  | 13.64 ± 0.46              |
| HCPT@F-Pt-PU NPs | 7.27 ± 0.29        | 19.84 ± 0.60          |                |                           |

Figure 4. (A) TEM images of HCPT@F-Pt-PU NPs. (B) Hydrodynamic diameter of HCPT@F-Pt-PU NPs. (C) Size stability of Pt-PU NPs, F-Pt-PU NPs, and HCPT@F-Pt-PU NPs in PBS for 35 days at room temperature (*p < 0.05, n = 3).

Figure 5. (A) UA release kinetics with different pH in HCPT@F-Pt-PU NPs and (B) HCPT release kinetics with esterase and without esterase in PBS at pH 7.4 and 37 °C from the HCPT@F-Pt-PU NPs

Figure 6. (A) In vitro cytotoxicity of HCPT@F-Pt-PU NPs in H22 and HepG2 cells incubated with different concentration of UA for 24, 48, and 72 h. (C) In vitro cytotoxicity of HCPT@F-Pt-PU NPs in H22 and HepG2 cells incubated with different concentration of HCPT for 24, 48, and 72 h.

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incubation time. After treating for 24, 48, and 72 h, 53, 36, and 23% H22 cells survived with administration of free UA, and 38, 24, and 18% H22 cells survived after HCPT treatment; however, only 29, 8, and 1% H22 cells survived after HCPT@F–Pt–PNP treatment. Obviously, the time-dependent cytotoxicity of nanoparticles in H22 cells was better than that of free UA and HCPT. Similar results were also prominent on HepG2 cells; after treating for 24, 48, and 72 h, 17, 6, and 2% HepG2 cells survived after HCPT@F–Pt–PNP treatment.

The release of drug carriers is consistent with the release mechanism of polymer nanoparticles. The cytotoxicity of free UA, HCPT, F-Pt-PU NPs, and HCPT@F-Pt-PU NPs in H22 and HepG2 cells was different because of the chemical sensitivity of different cell lines. IC_{50} was calculated for estimating the potential drug efficacy of drug carriers (Figure 6B,D). The results indicated that IC_{50} followed the order: UA > HCPT > F-Pt-PU NPs > HCPT@F-Pt-PU NPs (Table 2).

Table 2. In Vitro Cytotoxicity Analysis (IC_{50} μg mL^{-1})

| sample             | H22       | HepG2     |
|--------------------|-----------|-----------|
| UA                 | 10.03 (1.1577) | 9.07 (1.2095) |
| HCPT               | 0.44 (0.08091) | 0.37 (0.06879) |
| F-Pt-PU NPs        | 0.10 (0.02317) | 0.09 (0.02017) |
| HCPT@F-Pt-PU NPs   | 0.04 (0.00805) | 0.04 (0.00811) |

In H22 and HepG2 cells, the cytotoxicities of HCPT@F-Pt-PU NPs were 250.8- and 226.8-fold of free UA, respectively, and were 11.0- and 9.2-fold of free HCPT. It is clarified that HCPT@F-Pt-PU NPs are more effective than free drugs. In addition, the internalization of the polymer and the strong release in lysosomes further enhanced the efficacy of the drug. IC_{50} of HCPT@F-Pt-PU NPs in H22 and HepG2 was 0.04, and the combined index (CI) of UA and HCPT was 0.04, indicating that HCPT@F-Pt-PU NPs can significantly achieve the synergistic effect of UA and HCPT.

2.5. Folate Competition. To further evaluate the role of folic acid from HCPT@F-Pt-PU NPs in cellular uptake, H22 cells were used as folic acid receptor overexpression and the tumor that lack of folic acid receptor. 0.1 μg mL^{-1} HCPT@F-Pt-PU NPs were used for detection, as shown in Figure 7; the efficacy of HCPT@F-Pt-PU NPs was inhibited by adding extra folic acid, and the cell viability changed little with the increase of folic acid concentration. It suggests that supplementation of additional folic acid has a competitive effect on folic acid-targeted nanoparticles in cells and hinders the uptake of HCPT@F-Pt-PU NPs by cell membrane overexpressing folic acid receptor cells. It is also indicated that HCPT@F-Pt-PU NPs can actively target the cells with folic acid receptor overexpression.

2.6. Cellular Uptake. To assess the ability of cellular internalization of the folic acid-targeted pectin delivery system, H22 cells were incubated with free HCPT (IC_{50}), HCPT@Pt-PU NPs (IC_{50}), and HCPT@F-Pt-PU NPs (IC_{50}) for 4 h (Figure 8). Fluorescence of HCPT (green) and DAPI (blue) was observed by confocal microscopy after administration. The
enrichment of HCPT@F-Pt-PU NPs (green fluorescence) on the cell surface is more effective than HCPT@Pt-PU NPs and free HCPT, indicating an enhanced folic acid-targeting receptor. Encapsulated HCPT maintained a higher concentration in the cytoplasm region than free HCPT. The embedded drug could be ingested through the endocytosis pathway, thus avoiding the effect of p-glycoprotein. In addition, the increased density of green fluorescence in HCPT@F-Pt-PU NPs was concentrated in the cytoplasm region near the cell membrane, which was stronger than that of HCPT@Pt-PU NPs and free HCPT. It is possibly the targeted effect of folic acid and pectin which can actively capture tumors fast and be visualized on the surface.

In addition, apoptosis was evaluated by the Annexin V-FITC/PI assay with flow cytometry for further verification of the apoptosis of cells after laser irradiation (Figure 9). Free UA could only induce partial apoptosis (11.94%), while the Pt-PU conjugate and F-Pt-PU conjugate could induce 21.99 and 44.53% after UA was connected with 8armPEG and pectin, respectively. Compared with Pt-PU, apoptosis of HCPT@Pt-PU NPs (69.29%) was significantly improved because of effective dual targeting, implying that the synergy of drugs under high loading is greater than that of drugs alone. After combining with folic acid, the target capacity of HCPT@F-Pt-PU NPs to folate receptors on the surface of cell membranes is significantly enhanced and rapidly enriched in tumor sites. Next, pectin combines with the intracellular asialoglycoprotein receptor (ASGPR) to rapidly release drugs, leading to more apoptosis (91.61%) through synergy with HCPT.

2.7. Pharmacokinetics in Mice. Intravenous injection of HCPT@F-Pt-PU NPs was performed to study the pharmacokinetics of mice carrying H22. It can be seen from Figure 10...

Figure 8. Confocal microscopy pictures of H22 cells incubated with (A) free HCPT, (B) HCPT@Pt-PU NPs, and (C) HCPT@F-Pt-PU NPs at an equivalent concentration of HCPT (IC50) for 4 h at 37 °C.

Figure 9. Annexin V-FITC/PI apoptosis detection analysis of H22 cells after 24 h of incubation by flow cytometry under the treatment of PBS, UA, the Pt-PU conjugate, the F-Pt-PU conjugate, HCPT@Pt-PU NPs, and HCPT@F-Pt-PU NPs.
that after the administration of HCPT@F-Pt-PU NPs, the concentration of UA and HCPT in plasma decreased slowly, and the existence time was longer than that of free UA, which may be due to the break of the ester bond between 8arm-PEG and UA the release caused. The presence of HCPT@F-Pt-PU NPs (80 h) in the blood was higher than free UA (7 h) and HCPT (8 h). The concentration of HCPT@F-Pt-PU NPs in plasma was higher than that of F-Pt-PU NPs, possibly because embedded HCPT enhanced the strength of the hydrophobic core, thereby reducing the hydrolysis rate of nanoparticles.

After 24 h of intravenous administration, F-Pt-PU NPs and HCPT@F-Pt-PU NPs showed prolonged clearance rates with UA levels of 22.5 and 29.7 % ID/g, respectively, and an HCPT level of 14.9 % ID/g. The blood circulation half-life of UA in F-Pt-PU NPs and HCPT@F-Pt-PU NPs was extended from 1.3 to 8.3 and 10 h, respectively, and that of HCPT in HCPT@F-Pt-PU NPs was extended from 0.7 to 5.9 h.

2.8. In Vivo Antitumor Activity of Nanoparticles. The drug delivery efficacy was evaluated by administration of free UA (10 mg kg\(^{-1}\)/mouse), HCPT (10 mg kg\(^{-1}\)/mouse), and
nanoparticles (equivalent UA) with H22 tumor-bearing mice. The tumor volume of HCPT@F-Pt-PU NPs was much smaller than that treated with free UA and HCPT injection (Figures 11 and 12A). As can be seen from Figure 12B and Table 3, the antitumor capacity was in the order HCPT@F-Pt-PU NPs > F-Pt-PU NPs > UA and HCPT. The tumor growth inhibition (TGI) value of HCPT@F-Pt-PU NPs on day 24 is up to 88.43%, and the survival rate on day 30 is 76.11%. Obviously, the TGI value and survival rate of nanoparticles are higher than those of free UA (33.82%/24 days, 0%/30 days) and HCPT (25.37%/24 days, 0%/30 days). These findings were also consistent with the above evaluation results in vitro. These results are consistent with the in vitro evaluation results.

Throughout the experiment, the average weight of the mice did not change significantly of all treated mice (Figure 12C), suggesting that nanoparticles are safe for drug delivery at such doses. In addition, tumor volume had little effect on mouse body weight.

2.9. Evaluation of the Side Effects. The parameter IgE level was used to quickly assess the type I hypersensitivity of the HCPT@F-Pt-PU NP in H22 tumor-bearing mice. In Figure 12D, free UA and HCPT showed a higher IgE level than the control group because of the poor water solubility of UA and HCPT. Drug carriers showed a similar IgE level to the control group, suggesting that nanoparticles could greatly reduce the harm of allergic reactions. The white blood cell (WBC) count was also evaluated after administration with different samples, which was usually considered as an indicator of hematological toxicity. From Figure 12E, the WBC counts of mice in F-Pt-PU NPs and HCPT@F-Pt-PU NPs were increased faster than that in free drugs, which indicated that the folic acid-targeted pectin delivery system could avoid serious hematological toxicity.

3. CONCLUSIONS

In summary, we proposed the targeted HCPT@F-Pt-PU NPs for evaluation of pharmaceutical properties on liver cancer. A satisfactory drug delivery efficiency was achieved because of the effect of targeted folic acid. The UA- and HCPT-loaded nanoparticles could be rapidly accumulated on the surface of the tumor and simultaneously inhibit tumor growth and metastasis. First, HCPT@F-Pt-PU NPs displayed exceptionally high drug loading capacities (LCs) (UA: 7.27 wt %, HCPT: 19.84 wt %) and stability owing to the unique physicochemical characteristics of pectin and 8armPEG. Second, the continuous circulation time of HCPT@F-Pt-PU NPs in the blood was up to 80 h, and the survival rate was as high as 76.11% after 30 days' treatment. Third, the enhanced cytotoxicity of HCPT@F-Pt-PU NPs was 250.8-fold (H22) and 226.8-fold (HepG2) to the free UA, respectively, and was 11.0-fold and 9.2-fold to the free HCPT, respectively. Active tumor-targeted HCPT@F-Pt-PU NPs displayed immense potential for synergistic inhibition of liver cancers.

4. EXPERIMENTAL SECTION

4.1. Materials. Folic acid (99%), 8armPEG-COOH (Mw = 10 kDa), and pectin (Mw = 100 kDa, DE = 36%) were obtained from Sigma-Aldrich. Both 8armPEG-COOH and pectin are FDA and EU food-grade products. 10-Hydroxy camptothecin (HCPT) and UA were purchased from Chengdu Preferred Biotechnology Co., Ltd. (Sichuan, China). Other reagents were purchased from Aitemon Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) was purchased from Hyclone Co., Ltd. DMEM, RPMI-1640, and...
penicillin, and streptomycin were purchased from Gibico Co., Ltd.

H22 and HepG2 cells were supplied by the Institute of Process Engineering of Chinese Academy of Sciences (CAS). 5-week-old female mice (BALB/c, 25−30 g) were provided by Beijing Hfk Bioscience Co., Ltd. The mice had free access to food and water with constantly maintaining 12 h dark and 12 h light cycles at 25 ± 2 °C. All the animal experiments were consistent with the guidelines set by the National Institutes of Health (NIH Publication no. 85−23, revised 1985) and were approved by the Experimental Animal Ethics Committee, Beijing.

4.2. Characterization. 1H NMR of the sample was performed using a Bruker 500 instrument and processed by TOPSpin software for spectral analysis. UA, 8armPEG-UA, and HCPT@F-Pt-PU NPs were dissolved in deuterated chloroform (CDCl3). FA-NH2, pectin, pectin-FA, and F-Pt-PU were dissolved in deuterium water (D2O). The transmission electron microscopy (TEM) images were obtained on a JEM-100CXa (JEOL, Japan). The HCPT@F-Pt-PU NP sample was dispersed in deionized water by ultrasonication, dropped onto a copper net using a 20 μL pipette, and air-dried. The HCPT@F-Pt-PU NP diameters and zeta potential were measured on a Zetasizer Nano ZS90 (Malvern, UK). F-Pt-PU NPs and the HCPT@F-Pt-PU NP powder were dissolved in deionized water and ultrasonically dispersed.

4.3. Synthesis of 8armPEG-UA Conjugates. The 8armPEG-UA conjugate was synthesized following our published work, and the preparation scheme of the 8armPEG-UA conjugates is shown in Figure 13. Briefly, 8armPEG-COOH (1.0 g, 0.1 mmol) was added to a round-bottom flask containing dimethyl sulfoxide (DMSO, 25 mL) and stirred to dissolve. Then, 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide HCl (EDC, 0.24 g, 1.2 mmol) was added to activate the carboxyl group of 8armPEG-COOH for 30 min. Afterward, UA (0.27 g, 0.6 mmol) and 4-dimethylaminopyridine (DMAP, 0.12 g, 1.0 mmol) were added and heated to 35 °C for 48 h under stirring. The mixture was transferred to a
dialysis membrane (MWCO 1 kDa) in a PBS solution (pH 7.4) and dialyzed for 24 h. The dialyzed external solution was replaced every 6 h four times. After dialysis, the mixture was collected and freeze-dried to obtain the 8armPEG-UA powder.

4.4. Synthesis of Folic Acid-NH$_2$. There are two carboxyl groups ($\alpha$ and $\gamma$) in folic acid. Folic acid has a good affinity with the $\alpha$-carboxyl group that leads to poor reactivity, and the reactivity of the $\gamma$-carboxyl group is relatively high.$^{36-38}$ In this study, the amino group was introduced to the $\gamma$-position to prepare folic acid-NH$_2$. As shown in Figure 14, folic acid (88.28 mg, 0.2 mmol) was added to a round-bottom flask containing DMSO (15 mL) in a 50 °C water bath. Then, EDC (76.9 mg, 0.4 mmol) and NHS (92 mg, 0.8 mmol) were added to activate the carboxyl group for 6 h. The mixture was mixed with ethylenediamine (156.26 mg, 2.6 mmol) to stir at room temperature overnight. The mixture was precipitated by the addition of excess diethyl ether, separated by centrifugation, washed several times with acetonitrile, and dried under vacuum to obtain the folic acid-NH$_2$ powder.

4.5. Synthesis of F-Pt-PU Conjugates. The preparation of F-Pt-PU conjugates is shown in Figure 15. 8armPEG-UA (1.0 g, 0.1 mmol) was added to a round-bottom flask containing pyridine (40 mL) and stirred to dissolve. Afterward, EDC (0.1 g, 0.5 mmol) was added to activate the carboxyl group for 30 min. Then, pectin (0.18 g, 1.2 mmol), folic acid-NH$_2$ (0.5 g), and DMAP (0.24 g, 2.0 mmol) were added and heated to 35 °C for 48 h under stirring. The mixture was precipitated by the addition of excess diethyl ether ($V_{\text{diethyl ether}}/V_{\text{mixture}} = 3:1$, v/v), separated by centrifugation, and washed several times with diethyl ether. The obtained precipitate was transferred to a dialysis membrane (MWCO 8 kDa) and dialyzed in a PBS solution with a pH of 7.4 for 24 h. The dialyzed external solution was replaced every 6 h four times. The mixture in the dialysis membrane was collected and freeze-dried to obtain the folic acid-NH$_2$ powder.

4.6. Preparation of Targeted HCPT@F-Pt-PU NPs. Targeted HCPT@F-Pt-PU NPs were prepared by self-assembly.$^{39-41}$ Briefly, 5 mg of F-Pt-PU conjugates and 1 mg of HCPT were dissolved in 2 mL of DMSO and added dropwise slowly to deionized water with rapid stirring for 10 min to obtain the HCPT@F-Pt-PU NP solution. The nanoparticle solution was transferred to a dialysis membrane (MWCO 3 kDa) and dialyzed against the PBS solution (pH 7.4) for 12 h. Then, the nanoparticle solution was collected and freeze-dried to obtain the HCPT@F-Pt-PU NP powder. The preparation of F-Pt-PU NPs was the same as that of HCPT@F-Pt-PU NPs without HCPT.

4.7. Delivery Measurements of HCPT@F-Pt-PU NPs. Drug delivery measurements were carried out at room temperature with a UV-2000 spectrophotometer. The measurements of UA and HCPT were taken at 210 and 254 nm, respectively. Briefly, suspensions of 8armPEG-UA (1 mg) conjugates in 10 mL of methanol–water solution (88%, v/v) were prepared, and the concentration of UA was determined using a calibration curve. The delivery efficiency of UA and HCPT in HCPT@F-Pt-PU NPs was also determined using a UV–Vis spectrophotometer. Suspensions of HCPT@F-Pt-PU NPs (5 mg) in 25 mL of dilute hydrochloric acid (5%, v/v) were hydrolyzed and centrifuged to obtain free UA and HCPT. The precipitate was dissolved in an 88% methanol solution (v/v) and a 60% ethanol solution (v/v) to determine the concentration using a calibration curve, respectively. Several UA–methanol solutions in the 20–200 μg mL$^{-1}$ concentrations were configured to paint the calibration curve of UA. The HCPT–ethanol solution in the 4–64 μg mL$^{-1}$ concentrations was configured to draw the calibration curve of HCPT. The UA LC and HCPT encapsulation capacity (EC) were estimated according to the following formulae

$$\text{LC} (%) = \frac{(\text{total UA in nanoparticles/total particle weight}) \times 100\%}{(1)}$$

$$\text{EC} (%) = \frac{(\text{total HCPT in nanoparticles/total particle weight}) \times 100\%}{(2)}$$

4.8. UA and HCPT Release from HCPT@F-Pt-PU NPs. 20 mg of HCPT@F-Pt-PU NPs was dissolved in 15 mL of the PBS medium (pH 5.0, 7.4, and 8.0) at 37 °C under gentle shaking. At determined time intervals, 2 mL of the dialysate solution at different pH was taken out, and the buffer was refreshed with 2 mL of the release medium. UA and HCPT were measured by HPLC. Measurement conditions of HCPT are the following: a C18 reverse-phase column, acetonitrile–water solution (30/70, v/v), a flow rate of 0.8 mL/min, a column temperature of 25 °C, a detection wavelength of 254 nm, and an injection volume of 10 μL. Measurement conditions of UA are the following: a C18 reverse-phase column, methanol–water (88/12, v/v), a flow rate of 0.8 mL/min, a column temperature of 25 °C, a detection wavelength of 210 nm, and an injection volume of 10 μL. In addition, esterase (30 units) was added to a dialysis bag (MWCO 3.5 kDa) as a control.

4.9. In Vitro Cellular Uptake. H22 cells were incubated in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin and incubated in a humidified incubator at 37 °C. Cellular uptake of free HCPT, HCPT@Pt-PU NPs, and HCPT@F-Pt-PU NPs was observed by confocal laser scanning microscopy (CLSM, TCS SPS, Leica). H22 cells were seeded on a confocal dish containing 4 cm$^2$ slides at a density of 1.0 × 10$^5$ cells/mL and incubated at 37 °C overnight. Afterward, the H22 cells were incubated with free HCPT (IC$_{50}$), HCPT@Pt-PU NPs (IC$_{50}$), and HCPT@F-Pt-PU NPs (IC$_{50}$) at 37 °C for 4 h. Then, the cells were rinsed with DPBS softly, resuspended, and fixed in 4% paraformaldehyde for 15 min. Then, DAPI solution (1 mL, 0.5 μg mL$^{-1}$) was added to the cells for 5 min. The DAPI solution was removed, washed with DPBS three times, and saved at 4 °C. The fluorescence distribution around the H22 cells at 488 nm was observed by confocal laser scanning microscopy to analyze the ability of the nanoparticles to capture cells.

4.10. In Vitro Cytotoxicity. In order to compare the cytotoxicity of the free drug with nanoparticles on both H22 and HepG2 cells, the CCK-8 assay was employed. H22 and HepG2 cells were seeded in 96-well plates at the same density of 5 × 10$^3$ cells/well and incubated at 37 °C, respectively. Drugs were dissolved in a small amount of DMSO and diluted with the medium. The medium was replaced with serial dilutions of free UA, free HCPT, F-Pt-PU NPs, and HCPT@F-Pt-PU NPs (equal to UA, 0.01–100 μg mL$^{-1}$) and cultured for 24, 48, and 72 h. Then, 20 μL of the CCK-8 solution was added to 96-well plates and incubated at 37 °C, which protected from light for 1 h. Samples were measured using an infinite M200 microplate spectrophotometer at 450 nm. Cell viability was normalized in the absence of the samples. The IC$_{50}$ method was used to calculate the concentration of the...
drug that inhibited 50% of cell growth. The combination index (CI) of UA and HCPT in HCPT@F-Pt-PU NPs was assessed according to the following formula

\[ CI = \frac{UA_C/UA_A + HCPT_C/HCPT_A}{C} \]

where \( UA_A \) and HCPT_A represent the IC_{50} of UA and HCPT in nanoparticles, respectively, while \( UA_C \) and HCPT_C represent the IC_{50} of free UA and free HCPT. CI < 1 indicates drug synergism, while CI > 1 indicates the antagonistic effect.

4.11. Pharmacokinetic Study. A total of 30 healthy tumor-free BALB/c female mice (5 weeks) were randomly divided into five groups, and PBS (control), free UA, free HCPT, F-Pt-PU NPs, and HCPT@F-Pt-PU NPs were injected. Blood samples were collected from the eyelids of the mice at 0, 0.1, 0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 30, 36, 48, and 72 h, and plasma was obtained by centrifugation at 4 °C for 10 min. 50 μL of 0.1 N NaOH was added to 100 μL of plasma and dissolved in the water bath at 37 °C for 15 min. Afterward, 50 μL of 0.1 N HCl was added to neutralize the mixture, and 100 μL of methanol was added to mix for 5 min. The mixture was centrifuged at 4000 rpm for 5 min, and the supernatant was dried in nitrogen. The dried supernatant containing total UA or HCPT was dissolved repeatedly with 100 μL of methanol and measured by HPLC (a C18 reverse-phase column, 60% acetonitrile–0.05% trifluoroacetic acid, 0.8 mL/min, 25 °C, 254 nm, an injection volume of 10 μL). Blood levels of UA and HCPT are plotted by percentage unit (% ID/g) after injection.

4.12. In Vivo Antitumor Efficacy. To establish a H22 subcutaneous tumor xenograft model, 30 BALB/c female mice (5 weeks) were randomly divided into five groups (n = 6) and 200 μL, \( 1 \times 10^6 \) cells/mouse H22 cells were injected in the right auxiliary flanking region. When the tumor volume reached 50–100 mm³, mice were administered with PBS (control group), UA (10 mg kg⁻¹), HCPT (10 mg kg⁻¹), and nanoparticles (equivalent UA) on days 0, 2, 4, 6, and 8 by intravenous injection. The size of tumor and the body weight of the mice were monitored at an interval of 2 days. The tumor volume of mice was calculated according to the following formula

\[ \text{Tumor volume (TV)} = \frac{(L \times W^2)}{2} \]

where L and W represent the long and wide tumor diameters (mm), respectively. The relative tumor volume (RTV) was calculated at different time points before administration, and the TGI (% TGI) of samples was calculated using the following formula

\[ \% \text{TGI} = \left[ \frac{(C - T)}{C} \right] \times 100\% \]

where C and T represent the mean tumor volume of the control group and treatment group, respectively.

4.13. Detection of Allergic Reaction. Allergic reaction testing is essential to protect current chemotherapeutic drugs from toxic side effects. A total of 30 tumor-bearing mice were randomly divided into five groups and administered every 2 days with UA of 7 mg kg⁻¹, HCPT of 6 mg kg⁻¹, and nanoparticles (equal to free UA). The blood of five groups of mice was collected, and the serum of samples was analyzed by IgE ELISA.

4.14. Statistical Analysis. Data were reported as mean values ± SD by variance analysis. The statistical significance was considered as *p < 0.05, **p < 0.01.

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Notes

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