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Inhibition of *MDR1* gene expression and enhancing cellular uptake for effective colon cancer treatment using dual-surface-functionalized nanoparticles

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Abstract

Nanomedicine options for colon cancer therapy have been limited by the lack of suitable carriers capable of delivering sufficient drug into tumors to cause lethal toxicity. To circumvent this limitation, we fabricated a camptothecin (CPT)-loaded poly(lactic-co-glycolic acid) nanoparticle (NP) with dual-surface functionalization—Pluronic F127 and chitosan—for inhibiting multi-drug resistant gene 1 (*MDR1*) expression and enhancing tumor uptake. The resultant spherical NPs-P/C had a desirable particle size (~268 nm), slightly positive zeta-potential, and the ability to efficiently down-regulate the expression of *MDR1*. *In vitro* cytotoxicity tests revealed that the 24 and 48 h IC₅₀ values of NPs-P/C1 were 2.03 and 0.67 μM, respectively, which were much lower than those for free CPT and other NPs. Interestingly, NPs-P/C1 showed the highest cellular uptake efficiency (approximately 85.5%) among the different drug formulations. Most importantly, treatment of colon tumor-bearing mice with various drug formulations confirmed that the introduction of Pluronic F127 and chitosan to the NP surface significantly enhanced the therapeutic efficacy of CPT, induced tumor cell apoptosis, and reduced systemic toxicity. Collectively, these findings suggest that our one-step-fabricated, dual-surface-functionalized NPs may hold promise as a readily scalable and effective drug carrier with clinical potential in colon cancer therapy.

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Keywords

Multidrug resistant gene 1; Enhanced cellular uptake; Nanoparticles; Surface functionalization; Chemotherapy; Colon cancer

1. Introduction

Colon cancer has emerged as a major public health problem, accounting for more than 1.4 million new cases and over half a million deaths worldwide each year [1]. It is the third-most common malignancy and the second-most common cause of cancer-related death [2]. Predominant among the therapeutic strategies currently available for colon cancer treatment are surgery, chemotherapy, and radiotherapy [3]. Of these modalities, chemotherapy is the most common. Recently, the combination of chemotherapy and nanotechnology, especially polymeric nanoparticle (NP)-based drug formulations, has come to be widely applied in cancer treatment owing to the controlled drug release properties, facilitated extravasation into the tumor, and reduced adverse systemic effects associated with these formulations [4-6]. However, broader application of these NP-based approaches is still seriously stymied by their low delivery efficiency, which prevents accumulation of sufficient therapeutic drug in the tumor over a sufficiently long period.

P-glycoprotein (P-gp), encoded by the multidrug resistance gene 1 (*MDR1*) gene, is over-expressed in many cancers. It is one of the main obstacles to achieve sufficient accumulation of intracellular drugs and is associated with clinical therapeutic failure in over 90% of patients with metastatic cancer [7, 8]. To suppress the drug efflux function of P-gp, researchers have developed numerous chemosensitizers, including cyclosporine A [9], verapamil [10], and P-gp siRNA [11]. Unfortunately, most have shown limited success in clinical trials, largely owing to their serious adverse effects (*e.g.*, hypotension, immunosuppression, and systemic toxicities) and lack of specificity and efficacy [12, 13]. Pluronic copolymer, a class of Food and Drug Administration (FDA)-approved biocompatible polymer, has been proposed as a promising chemosensitizer. It has a number of unique beneficial features that increase the efficacy of anticancer drug delivery. First, it can inhibit the drug efflux function of P-gp. The reason might be that the amphiphilic property created by the triblock structure of hydrophilic poly(ethylene oxide) (PEO) blocks and the hydrophobic poly(propylene oxide) (PPO) (PEO-PPO-PEO) enables it to easily insert into the plasma membrane, decreasing membrane microviscosity, and thus inhibiting P-gp activity [14,15]. This has been confirmed experimentally by Hong *et al.*, who showed that introduction of the pluronic copolymer to NPs aided in reversing MDR [16], and by Alakhov *et al.*, who further reported that pluronic copolymers induce a significant increase in drug activity in both non-MDR cells and MDR cells [17]. Second, its low cytotoxicity and weak immunogenicity make it suitable for both topical and systemic administration. Zhao *et al.* reported that systemic administration of a pluronic copolymer with Doxil enhanced drug release from liposomes and increased its therapeutic efficacy [18]. Third, the hydrophilic PEO segments tend to prevent aggregation, protein adsorption, and recognition by the reticuloendothelial system [19]. Jackson *et al.* demonstrated that Pluronic F127 remarkably

inhibited plasma protein adsorption on microparticle surfaces and the opsonization effect on neutrophil activation [20].

High intracellular drug concentrations can also be achieved through enhanced cellular uptake of NPs. It is well known that the physicochemical characteristics of NPs, such as particle size, surface charge, composition and surface hydrophobicity, affect their cellular uptake and *in vivo* distribution [21, 22]. Of these characteristics, surface charge is the factor that exerts the greatest influence on drug delivery function [23]. Generally, negatively charged and neutral NPs have a low level of cell interaction and internalization, whereas cationic NPs bind to negatively charged groups on the cell surface, facilitating cellular uptake and intracellular drug release [22]. Chitosan, a natural, biodegradable polymer, has been widely used in drug or gene delivery [24-26]. Notably, chitosan is unique among materials used in the formulation of NPs because its pKa (~6.5) closely matches that of extracellular pH values in tumors (~6.5) [27]. Thus, chitosan-functionalized NPs are more positively charged under the acidic conditions in tumors, presumably creating stronger electrostatic interactions with negatively charged tumor cells that facilitates their tumor cell adsorption and tissue retention [28]. Numerous previous reports have confirmed that chitosan functionalization significantly increase the cellular uptake and tumor accumulation of NPs [29-32].

Camptothecin (CPT), a natural plant alkaloid extracted from *Camptotheca acuminata*, is a promising antitumor agent by inhibiting the nuclear enzyme topoisomerase I. Despite of its outstanding preclinical activity against colon cancer, its hydrophobic property and lactone instability present serious obstacles in the clinic application [33]. To address these problems, many attempts have been made to improve drug delivery efficiency. CPT-polymer conjugates [34], micelle [35], microsphere [36] and liposome [37] have been explored with some success for colon cancer therapy. Previous studies have found that NPs could stabilize CPT and increase its circulatory retention in blood stream [33, 38]. Poly(lactic acid/glycolic acid) (PLGA), an FDA-approved biodegradable copolymer, can encapsulate hydrophobic drugs to form NPs with high efficiency, and has been widely used in drug delivery applications [39]. Here, we hypothesized that surface functionalization of camptothecin (CPT)-loaded PLGA NPs with Pluronic F127 and chitosan would inhibit the function of over-expressed P-gp in cancer cells and enhance CPT tumor uptake. Further, by increasing and maintaining the intratumoral drug concentration, these dual-functionalized NPs are predicted to ultimately enhance the therapeutic efficacy of CPT, as depicted in Fig. 1a. We tested these predictions by characterizing the physiochemical and *MDR1*-inhibitory properties of NPs, and investigating *in vitro* cell viability and cellular uptake, and *in vivo* anti-tumor activity and bio-distribution.

2. Materials and Methods

2.1. Materials

Poly(D,L-lactide-co-glycolide) (PLGA, Mw = 38–54 kg/mol), camptothecin, poly(vinyl alcohol) (PVA, 86-89% hydrolyzed, low molecular weight), Pluronic F127, chitosan, trehalose, sodium nitrite, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Paraformaldehyde stock solution (16%) was from Electron Microscopy Science

(Hatfield, PA, USA). Sterile defibrinated sheep blood was supplied by VWR Scientific (Philadelphia, PA, USA). Vybrant[®] MTT cell proliferation assay kit and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Vybrant Apoptosis assay Kit were obtained from Molecular Probes (Eugene, OR, USA). Matrigel was purchased from Corning Inc. (New York, NY, USA). Buffered formalin (10%) was supplied by EMD Millipore (Billerica, MA, USA). Hematoxylin and eosin were from Richard-Allan Scientific (Kalamazoo, MI, USA).

2.2. Depolymerization of chitosan and intrinsic viscosity measurement

Molecular weight of chitosan was tailored by depolymerization using sodium nitrite following a reported method [40]. Viscosity-average molecular weight of the resulting chitosan was determined as 1.8×10^4 using a 0.5 M CH₃COOH/0.2 M CH₃COONa by viscometric method [41]. The depolymerized chitosan was used in the NPs fabrication process.

2.3. Fabrication of NPs

Blank and CPT-loaded NPs were prepared by a modified oil-in-water (O/W) emulsion-solvent evaporation technique. Briefly, 50 mg of PLGA and, optionally, various amounts of Pluronic F127 and CPT were co-dissolved in 1 mL of dichloromethane (DCM)-methanol co-solvent (8:2). The resulting organic solution was added drop-wise to 4 mL PVA solutions (5%) with or without depolymerized chitosan (0.5%). The mixture was subsequently sonicated six times (10 s each time) at 70% amplitude in an ice bath using a Sonifier 450 (Branson Sonic Power, Danbury, CT, USA). This emulsion was immediately poured into 100 mL of aqueous solution containing 0.3% PVA with or without 0.03% depolymerized chitosan. The organic solvent was evaporated under low vacuum conditions (Rotary evaporator, Yamato RE200, Santa Clara, CA, USA). The NPs formed by this method were collected by centrifugation at 12,000 g for 20 min, washed three times with deionized water, re-suspended in 1 mL of aqueous solution containing trehalose (5%), and dried in a lyophilizer. The resultant NPs were stored at -20 °C in airtight container.

2.4. Characterization of NPs

Particle sizes (nm) and zeta potential (mV) of NPs were measured by dynamic light scattering (DLS) using 90 Plus/BI-MAS (Multi angle particle sizing) or DLS after applying an electric field using a ZetaPlus (Zeta potential analyzer, Brookhaven Instruments Corporation, Holtsville, NY, USA), respectively. The average and standard deviations of the diameters (nm) or zeta potential (mV) were calculated using 3 runs. Each run is an average of 10 measurements.

The morphology of NPs was observed with a scanning electron microscopy (SEM, LEO 1450VP, Zeiss, Germany). A drop of dilute NPs suspension was mounted on a freshly cleaved glass slide using carbon adhesive tape and sputter-coated with a mixture of gold and palladium (60:40) in an argon atmosphere under low pressure. The morphology of NPs was also observed with a transmission electron microscope (TEM, LEO 906E, Zeiss, Germany). A drop of NPs suspension was placed onto 400-mesh carbon-coated copper grids and dried before analysis.

The amount of CPT encapsulated in NPs was determined by measuring the intrinsic fluorescence of CPT on a PerkinElmer EnSpire multimode plate reader (Perkin Elmer, Boston, MA, USA). In a typical example, CPT-loaded NPs (3 mg) were dissolved in acidic DMSO (0.1% HCl). Then 100 μ L of the solution was transferred to a black 96-well plate. The fluorescence intensity of the CPT was measured at 430 nm emission wavelength (360 nm excitation wavelength).

2.5. Release profiles of CPT from NPs

The release behavior of CPT from NPs was conducted by the dialysis method. Briefly, NPs were dispersed in PBS to form a suspension (equal to 250 μ g of CPT). The suspension was transferred into a regenerated Cellulose Dialysis tube (molecular weight cut-off = 10,000 Da) and the sample-filled tube was closed tightly at both ends to keep each tube surface area equivalent. Then, the closed bag was put into a centrifuge tube and immersed in 15 mL PBS release medium. The tube was put in a water bath shaking at 100 rpm at 37 °C. At appropriate time points, outer solution was taken for measurement and fresh release medium was added. The amount of CPT in the outer solution was measured according to the method described in Section 2.4. All of the operations were carried out in triplicate.

2.6. Hemolysis assay

The hemolytic potential of free CPT and various NPs was tested according to the previous reports [42, 43]. Briefly, sterile defibrinated sheep blood was centrifuged at 1200 rpm for 15 min to harvest erythrocytes. The cell pellet was washed three times with PBS, and resuspended in PBS to achieve a concentration of 2% (v/v). Various NPs were suspended in PBS and diluted to different drug concentrations from 0.1 μ M to 100 μ M. For each concentration, 150 μ L suspensions were mixed with 150 μ L 2% erythrocytes. The resulting suspensions were incubated for 1 h at 37 °C in a water bath, and then centrifuged at 3,000 g for 15 min. The release of hemoglobin was determined by photometric analysis of the supernatant at 576 nm on a PerkinElmer EnSpire multimode plate reader (Perkin Elmer, Boston, MA, USA). Two control group were provided for this assay: untreated erythrocyte suspension (as negative control), and erythrocyte suspension treated with 1% Triton X-100 (as positive control).

2.7. Cytotoxicity assay

For 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide assay (MTT) test, Colon-26 cells were seeded at a density of 2×10^4 cells/well in 96-well plates and incubated overnight. The cells were incubated in the RPMI 1640 medium containing various amounts of CPT (0.1 – 100 μ M) for 4, 24, and 48 h, respectively. Free CPT was dissolved in 9:1 (v/v) medium/DMSO, and CPT-loaded NPs were suspended in medium. At the end of the incubation period, NPs-containing medium was removed and the cells were thoroughly rinsed three times with cold PBS. Cells were then incubated with MTT (0.5 mg/mL) at 37 °C for 4 h. Thereafter, the media were discarded and 50 μ L dimethyl sulfoxide (DMSO) was added to each well prior to spectrophotometric measurements at 570 nm. Untreated cells were used as negative references, whereas cells were treated with 0.1% Triton X-100 as positive controls.

Cell-attachment assays were performed to investigate the real-time cytotoxicity of NPs using electrical impedance sensing (ECIS) technology (Applied BioPhysics, Troy, NY, USA). The ECIS model 1600R was used for these experiments. The measurement system consists of an 8-well culture dish (ECIS 8W1E plate), the surface of which is seeded with Caco2-BBE cells at a density of 1×10^6 /well. Once cells reached confluence, free CPT or NPs-P/C1 were added to the wells and the CPT concentration in the medium is set as 10 μ M. Untreated cells were used as negative references, whereas cells were treated with 0.1% Triton X-100 as positive controls. Basal resistance measurements were performed using the ideal frequency for Caco2-BBE cells, 500 Hz, and a voltage of 1 V.

2.8. Cell apoptosis studies

The induction of apoptosis was studied by flow cytometry (FCM). Colon-26 cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated overnight. The cells were exposed to the culture medium (as control) and free CPT or NPs-P/C1-containing medium at equivalent CPT concentrations of 10 μ M for different periods (6 h or 18 h). After incubation, cells were thoroughly rinsed with cold PBS to eliminate excess of NPs, which were not taken up by cells. Subsequently, the treated cells were harvested using accutase and the apoptosis profiles were detected using Annexin V-FITC/PI Vybrant Apoptosis assay Kit. 5 μ L of Annexin V-FITC solution and 1 μ L propidium iodide (PI, 100 μ g/mL) were added to 100 μ L of the cell suspensions. The samples were mixed gently and incubated at room temperature for 15 min in the dark. Healthy cells were double negative, early apoptotic cells were positive for annexin V staining but negative for PI staining, and necrotic cells were only stained with PI, while late apoptotic cells were double positive. Excitation wave was set at 488 nm and the emitted green fluorescence of Annexin V and red fluorescence of PI were collected using 525 and 575 nm band pass filters, respectively. For each sample, a minimum of 5,000 events were scored by a FCM Canto™ (BD Biosciences, San Jose, CA, USA). Triplicate samples were analyzed for each experiment.

2.9. Quantitative reverse-transcription PCR (qRT-PCR)

Colon-26 cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated overnight. Blank NPs-C, blank NPs-P/C1, Free CPT (CPT, 10 μ M) or NPs-P/C1 (CPT, 10 μ M) were added to the medium. After 8 h or 16 h co-incubation, total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). The complementary DNA (cDNA) was generated from the total RNAs isolated above using the Maxima First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. Levels of MDR1 RNA expression were quantified by reverse-transcription polymerase chain reaction using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Hanover, MD, USA). The data were normalized to the internal control: 36B4. Relative gene expression levels were calculated using the 2^{-C_t} method. Sequences of all the primers used for reverse-transcription polymerase chain reaction are given in Table 3.

2.10. Intracellular NPs uptake visualization

Colon-26 cells were seeded in eight-chamber tissue culture glass slide (BD Falcon, Bedford, MA, USA) at a density of 5.0×10^4 cells/well and incubated overnight. The culture medium

was exchanged to serum-free medium containing free CPT or NPs-P/C1 with different CPT concentration (0, 50, and 100 μ M). After co-culture for 3 h, the cells were thoroughly rinsed with cold PBS for 3 times to eliminate excess of NPs and then fixed in 4% paraformaldehyde for 15 min. Images were acquired using an Olympus equipped with a Hamamatsu Digital Camera ORCA-03G.

2.11. Quantification of cellular uptake using FCM

Colon-26 cells were seeded in 12-well plates at a density of 3×10^5 cells/well and incubated overnight. The medium was exchanged to serum-free medium containing free CPT or various NPs (equal to 25 μ M CPT). Cells without treatment were used as negative controls. After incubation for different time periods (1, 3, and 5 h), the cells were thoroughly rinsed with cold PBS to eliminate excess of NPs, which were not taken up by cells. Subsequently, the treated cells were harvested using accutase, transferred to centrifuge tubes, and centrifuged at 1,500 rpm for 5 min. Upon removal of the supernatant, the cells were re-suspended in 0.5 mL of FCM buffer, transferred to round-bottom polystyrene test tubes (BD Falcon, 12 $\times$ 75 mm), and kept at 4 $^{\circ}$ C until analysis. Analytical FCM was performed using the DAPI channel on the FCM CantoTM (BD Biosciences, San Jose, CA, USA). A total of 5,000 ungated cells were analyzed.

2.12. In vivo antitumor activity evaluation

All animal care and studies were approved by Georgia State University Institutional Animal Care and Use Committee. The anti-cancer activities of CPT-loaded NPs were evaluated in mice bearing subcutaneous colon tumors. Colon-26 cells (1×10^6 cells, 0.1 mL) were injected with matrigel into C57BL/6 mice (4 weeks old, The Jackson Laboratory) subcutaneously, and drug treatments were started 8 days after inoculation. Different formulations were injected into the tail vein every 4 days. Free CPT was formulated in PBS solution containing DMSO (10%, v/v) and Tween-80 (5%, v/v), and CPT-loaded NPs were suspended in PBS. Mice were divided into five groups with each group receiving formulations as follows: (i) PBS; (ii) free CPT (CPT, 10 mg/kg); (iii) NPs-PVA (CPT, 10 mg/kg); (iv) NPs-P (CPT, 10 mg/kg), and (v) NPs-P/C1 (CPT, 10 mg/kg). The tumor volumes and body weights of the mice were measured and recorded. Tumor volume was calculated as follows: volume = $1/2LW^2$, where L is the long diameter and W is the short diameter of a tumor. Animals were euthanized when the signals of sickness, such as breathing problems, failure to eat and drink, lethargy or abnormal posture, were observed. To conduct histological analysis, tumors and the major organs (heart, liver, spleen, lung and kidney) were fixed for 2 days in 10% buffered formalin solution and embedded in paraffin. After deparaffinization, the tissue sections (5 μ m) were stained with hematoxylin/eosin (H&E). Images were acquired using an Olympus equipped with a Hamamatsu Digital Camera DP-26. The apoptosis of tumor cells was determined using 4,6-diamidino-2-2-phenylindole (DAPI) and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assays, with a commercial apoptosis detection kit according to the manufacturer's standard protocols (Roche Diagnostics, Indianapolis, IN, USA). Images were acquired using an Olympus equipped with a Hamamatsu Digital Camera ORCA-03G. Three fields of each section after TUNEL staining were chosen randomly for counting the apoptosis cells and proliferation cells. The percentage of apoptotic cells in the samples was obtained by dividing the number

of apoptotic cells (TUNEL positive cells shown as green dots) from the number of total cells in each microscopic field.

2.13. Studies on CPT distribution in tumor-bearing mice

C57BL/6 mice (4 weeks old) were used to measure CPT biodistribution. After 8 days of inoculation, tumor-bearing mice were injected via a lateral tail vein with free CPT solution or NPs-P/C1 suspension at a dose of 10 mg/kg of CPT. At 4, 24, and 48 h after injection, mice were sacrificed via carbon dioxide inhalation and 0.5–1 mL whole blood was collected via cardiac puncture. Plasma was obtained by centrifugation of the blood at 3,000 rpm for 5 min. The major organs and tumor were excised and homogenized in saline. For the determination of CPT, an aliquot of plasma or the tissue homogenate was acidified with the aqueous phosphoric acid (0.15 M), and then CPT was extracted with chloroform:methanol (4:1 volume ratio). After centrifugation of the mixture, 10 μ L of the chloroform:methanol layer was directly injected into a Shimadzu HPLC system equipped with a fluorescence detector (λ_{ex} : 375 nm and λ_{em} : 435 nm) and a reverse-phase C-18 column (5 μ m, 250 mm $\times$ 4.6 mm, Agilent). The mobile phase was composed of 35% acetonitrile and 65% acetic acid (0.1%).

2.14. Statistical analysis

Statistical analysis was performed using Student's *t*-test for *in vitro* and non-parametric Mann-Whitney test for *in vivo* data processing. Data were expressed as mean $\pm$ standard error of mean (S.E.M.). Statistical significance was represented by **P*<0.05 and ***P*<0.01.

3. Results and discussion

3.1. Fabrication of CPT-loaded NPs

We prepared CPT-loaded NPs using the emulsion-solvent evaporation technique, a common and well-established method for preparing active-substance-loaded NPs [44]. Theoretically, rapid addition of the organic phase (PLGA; optionally CPT/Pluronic F127) to the aqueous phase together with emulsifiers (PVA; optionally chitosan), with sonication, leads to the immediate formation of an oil/water emulsion based on the Gibbs-Marangoni effect (mechanical mechanism) and a capillary break-up mechanism [45]. Upon evaporation of DCM/methanol cosolvents under reduced pressure, CPT molecules are transferred to the PLGA hydrophobic core through hydrophobic interactions (the “like dissolves like” principle) and further solidified to form compacted NPs [46].

Emulsifiers presented at the interface serve to separate the oil and water phases, and they are necessary to prevent NPs aggregation [47]. PVA and Pluronic F127 are extensively utilized as emulsifiers for the fabrication of polyester NPs. PVA is a copolymer of hydrophobic poly(vinyl acetate) and hydrophilic poly(vinyl alcohol) [48]. Similarly, Pluronic F127 consists of hydrophobic PPO blocks and hydrophilic PEO blocks [14]. During the NPs formation process, the hydrophobic segments of PVA and Pluronic F127 penetrate into the organic phase and remain entrapped in the polymeric matrix of the NPs, and their hydrophilic segments surround NPs and stabilize them through steric hindrance. Chitosan is a natural, linear, cationic polymer that has long been used for surface modification of

polyester NPs to prolong pharmacological effects [49, 50]. This action of chitosan coating could be attributable to the effects of chain entanglement with PVA and pluronic copolymer. Alternatively, this effect may reflect adsorption of positively charged chitosan to the negative-charged NPs surface.

3.2. Physicochemical characterization of NPs

Particle size and zeta-potential are critical parameters because they directly impact the stability, cellular uptake, and biodistribution of NPs [45]. As summarized in Table 1, DLS measurements showed that the average hydrodynamic diameter of NPs was in the range of 258 nm to 286 nm. NPs-PVA had a diameter of 258 nm. With the introduction of Pluronic F127 (NPs-P), particle size increased to 278 nm. Interestingly, the size of NPs-P/C1, produced by further introduction of chitosan while maintaining the same PLGA/Pluronic F127 weight ratio, was much smaller than that of NPs-P. Additionally, we found that the particle size distribution of NPs-P/C1 was much narrower than that of NPs-P (Fig. 1b and Fig. 1c). For NPs-P/C generally, the particle size clearly depended on the amount of Pluronic F127 for a given PVA/chitosan condition. Table 1 also shows that NPs produced with PVA/Pluronic F127 as an emulsifier were electronegative, whereas all the chitosan-coated NPs had a positive zeta-potential (approximately +20 mV), reflecting the presence of chitosan on the surface. It has been proposed that most cells preferentially internalize slightly positively charged NPs with a size less than 400 nm [51-53]. Therefore, the properties of the NPs-P/C described above would be considered favorable for internalization into cells. For all the fabricated NPs, drug loading ranged from 5.7% to 6.6%, and the corresponding encapsulation efficiency was in the range of 86.2% to 99.3%. The introduction of chitosan onto the NPs resulted in high drug loading and encapsulation efficiency. Fig. 1b and Fig. 1c shows representative SEM and TEM images of NPs-P and NPs-P/C1, respectively, revealing that both types of NPs were nearly spherical in shape with a smooth surface morphology; a majority of unhydrated NPs possessed diameters ranging from 121 nm to 194 nm. There was no significant difference in morphology and surface roughness between these two types of NPs.

Since CPT was encapsulated in the NPs, it was expected that the optical properties of CPT would be retained in NPs. Supplementary Fig. 1a shows representative fluorescence microscopic images of NPs-P/C1. The NPs appeared as bright blue spheres, and showed good monodispersity and high photoluminescence. The fluorescence of dyes has the general effect of enlarging the size of individual NPs owing to the point spread function; accordingly, the diameters of the observed fluorescence dots were much larger than the hydrodynamic sizes indicated above. The emission properties of CPT were also well preserved following their encapsulation in NPs (Supplementary Fig. 1b). These results suggest that NPs-P/C1 successfully encapsulate CPT and exhibit great potential for use in tracking and quantifying cellular uptake using fluorescence microscopy and flow cytometry, respectively.

3.3. In vitro drug-release profile

Although CPT is an important anticancer drug for colon cancer therapy, intravenous administration of CPT causes severe side effects, such as leukopenia, thrombocytopenia,

anemia and gastrointestinal toxicities, owing to distribution of the drug throughout the body via the bloodstream [54]. Therefore, sustained release and passive tumor-targeting profiles have been proposed as approaches for minimizing the adverse effects of NPs on off-target to normal tissues [55]. In addition, the release of drugs from NPs usually depends on numerous factors related to the structure, morphology and physicochemical properties of the NPs, as well as loaded drugs [56, 57]. Since Pluronic F127 and chitosan were found to be key factors in modulating the properties of NPs, we investigated the *in vitro* release behavior of all four types of NPs. NPs loaded initially with 250 μ g CPT were suspended in PBS, and their release profiles as function of time were monitored. As shown in Fig. 2, all NPs exhibited a very similar pattern, with a slight initial burst release, followed by release of ~40% of trapped CPT within the first 30 h. Over the course of 8 days, the cumulative amount of CPT released was greater than 80% for all types of NPs. As previously reported, drug release from PLGA NPs reflects the combined effects of swelling, pore diffusion, erosion, and degradation processes [56]. Accordingly, after the initial burst release, CPT migrated from the core to the surface of the polymeric matrix and showed moderate and sustained release. NPs-P displayed a pattern of CPT release generally similar to that of NPs-PVA, but exhibited a clearly diminished initial burst and delayed subsequent release, as previously reported [58]. In contrast, the release rates of NPs-P/C1 and NPs-P/C2 were higher than that of NPs-PVA, and this difference is attributable to surface functionalization with chitosan. Chitosan is much more hydrophilic than PLGA, thus enabling the PBS solution to more easily penetrate into the NP matrix and allowing more drug release during the same period of time. This enhancing effect of chitosan on drug release by PLGA NPs has also been observed by others [50].

On the basis of the above observations, we conclude that NPs-P/C is capable of well-controlled, timed release of entrapped CPT for more than 8 days. When intravenously injected, NPs loaded with CPT would be expected to exhibit a further slowing in the rate of CPT release at early and later stages owing to the limited blood volume. Therefore, these NPs should meet the main requirements for delivering CPT over a sufficiently long period.

3.4. In vitro cytotoxicity

To determine whether PLGA-encapsulated CPT maintains its anti-cancer activity, we investigated its effects on Colon-26 cells at different time points (4, 24, and 48 h). Blank NPs-P/C1 had no effect on cell viability, even at concentration up to 500 μ g/mL (Supplementary Fig. 2). Treatment of Colon-26 cells with increasing concentrations of free CPT or CPT-loaded NPs, resulting in a significant decrease in cell viability. As seen in Fig. 3a, CPT-loaded NPs exerted a stronger anti-cancer effect at CPT concentrations above 5 μ M after 24 h of co-incubation. Moreover, CPT-loaded NPs yielded much higher anti-cancer activities compared with free CPT after 24 h and 48 h of treatment (Fig. 3a and Fig. 3b). As shown in Table 2, the IC₅₀ of CPT-loaded NPs-P/C1 toward Colon-26 cells was 2.03 nM at 24 h and 0.67 nM at 48 h. These values are 4.4- and 2.7-times lower than that obtained for free CPT at the corresponding times, and are also much lower than those for other NPs. Three factors appear to account for the enhanced cytotoxicity of NPs-P/C1: (1) they increase the aqueous solubility of CPT and maintain CPT in an active form; (2) they inhibit MDR by virtue of the introduction of Pluronic F127; and (3) they enhance endocytosis-mediated drug

uptake of drug-loaded NPs in the presence of chitosan (as described below). Accordingly, of the two types of NPs-P/C, NPs-P/C1 was selected for further experiments.

As previously reported, MTT assays are not suitable for real-time analysis of cellular transformation [59]. As an alternative, we employed ECIS, an automated, real-time analytical tool for measuring cellular proliferation, cytotoxicity, apoptosis, and attachment [60]. As shown in Fig. 4a, Caco2-BBE cells attached to the electrode surface to form a confluent monolayer with a resistance of approximately 16,000 Ohms. These cells were then incubated with free CPT (10 μ M) or NPs-P/C1 containing the same drug concentration. Untreated Caco2-BBE monolayers exhibited a continuous increase in resistance, whereas those treated with Triton X-100, used as a positive control (100% cell death), exhibited a sharp decrease in resistance. Consistent with its cytotoxic effect noted above, free CPT induced a significant reduction in the resistance of Caco2-BBE cell monolayers. Interestingly, NPs-P/C1 slightly increased resistance initially, followed by a subsequent decrease. This bimodal effect might indicate that the efficient controlled drug release behavior of NPs-P/C1 restricted the initial burst effect; in contrast, free CPT, facilitated by DMSO in the medium, could penetrate cells immediately and induce cell death. During the above-described experiments, changes in cell morphology were also investigated by direct light microscopy 38 h after treatment. As shown in Fig. 4b, untreated control Caco2-BBE cells maintained contact with each other and exhibited a flattened morphology with clear outlines. In contrast, treated groups exhibited distinct morphological features in common, including the presence of apoptotic bodies. Cells exposed to Triton X-100 or free CPT showed an irregular cell surface and lacked junctions with surrounding cells. Notably, after treatment with NPs-P/C1, vast stretches of cells were dead and detached from the wells, leaving only aggregated cells with irregular cell surfaces. These different morphologies among treatment groups might indicate different anti-cancer profiles of free CPT and NPs-P/C1. The instability of NPs in blood is one of the serious limitations for *in vivo* application. As showed in Supplementary Fig. 3, no evidence of hemolysis was seen with all the NPs below the drug concentration of 100 μ M (the highest concentrations tested in *in vitro* study), with Triton X-100 being a positive control which achieves 100% hemolysis. The results suggested that all the NPs would be non-toxic towards erythrocytes after intravenous administration.

3.5. In vitro apoptosis assay

An early event in apoptosis is flipping of phosphatidylserine (PS) from the inner to the outer layer of the plasma membrane, exposing it on the cell surface and making it accessible to annexin V, which binds PS with high affinity. Using FITC-conjugated annexin in conjunction with PI, a fluorescent nuclear stain that detects necrotic cells, it is possible to detect necrosis and/or apoptosis by FCM. Four distinct phenotypes are distinguishable using this approach: (1) viable cells (lower left quadrant), (2) early apoptotic cells (lower right quadrant), (3) necrotic cells (upper left quadrant), and (4) late apoptotic cells (upper right quadrant).

To quantitatively compare the apoptotic effect of free CPT and NPs-P/C1, we treated Colon-26 cells with free CPT (10 μ M) and NPs-P/C1 containing the same concentration of

CPT, and then stained treated cells using an Annexin V-FITC/PI Apoptosis Detection Kit. The percentage of cells corresponding to each of the four phenotypes was determined using FCM analysis software. As shown in Fig. 5, very few apoptotic cells were detected in the untreated control group. However, the percentage of viable cells decreased with increasing duration of treatment with free CPT or NPs-P/C1. The percentage of viable cells after a 4-h treatment with free CPT was lower than that observed after treatment with NPs-P/C1, suggesting that free CPT dissolved in DMSO (10%)-containing medium rapidly penetrated cells through passive diffusion across the plasma membrane, producing a high intracellular concentration and directly exerting its anti-tumor effects in the nucleus. Interestingly, treatment with NPs-P/C1 significantly decreased the percentage of viable Colon-26 cells, reducing it to 81.2% after 8 h and further reducing it to 36.2% after 16 h. These results indicate that NPs-P/C1 were efficiently internalized by cells and delayed the release of CPT to produce a sustained cytotoxic action. Importantly, the population of necrotic cells decreased sharply after treating with NPs-P/C1 for 16 h, possibly indicating that a rapid loss of cells due to NPs-P/C1-induced necrosis, with subsequent decomposition of necrotic cells into fragments. This would explain the much lower number of cells observed in wells after a 16-h NPs-P/C1 treatment compared with other treatments (data not shown).

These results reconfirm the IC₅₀ results shown in Table 2, demonstrating that NPs-P/C1 is a more effective anti-cancer agent than free CPT, inducing more necrosis and apoptosis. After penetration into cells, free CPT might be pumped out of cells by efflux pumps such as P-gp, leading to a subtherapeutic intracellular drug concentration. However, NPs-P/C1 efficiently avoids this phenomenon by virtue of their potential ability to inhibit P-gp activity, resulting in enhanced cellular uptake.

3.6. Inhibition of MDR1 gene expression

In order to determine the mechanism responsible for the efficacy of NPs-P/C1 in Colon-26 cells, we investigated expression of the *MDR1* gene using qRT-PCR. As can be seen in Fig. 6a and Fig. 6b, empty NPs-C surface-functionalized with chitosan did not inhibit *MDR1* mRNA expression. However, empty NPs-P/C1 suppressed *MDR1* mRNA expression in Colon-26 cells, an effect was apparent at 8 h and was significant after 16 h, indicating that Pluronic F127 retained its ability to inhibit P-gp function after introduction into NPs-P/C1. Interestingly, treatment with free CPT also slightly reduced *MDR1* gene expression. CPT and Pluronic F127 acted synergistically to inhibit P-gp function, with *MDR1* gene expression decreasing by approximately 42.6% and 53.6% after treatment with NPs-P/C1 for 8 h and 16 h, respectively, compared with controls.

3.7. Cellular uptake of NPs-P/C1 by Colon-26 cells

Efficient cellular uptake is a major requirement for the therapeutic efficacy of NPs. Here, taking advantage of the intrinsic fluorescence of CPT, we investigated the intracellular delivery of CPT into cancer cells. Colon-26 cells were treated with different concentrations (0, 50 and 100 μ M) of NPs-P/C1 for 3 h. As shown in Fig. 7, NP treatment induced significantly greater drug accumulation in Colon-26 cells, as evidenced by the increase in CPT fluorescence intensity. A weak signal of CPT-loaded NPs-P/C1 was detected at a CPT concentration of 50 μ M, but a much stronger signal was observed at a CPT concentration of

100 μ M. Notably, the fluorescence intensity of NPs-P/C1-treated cells was much stronger than that of the same concentration (100 μ M) of free CPT. Taken together, these results demonstrate that NPs-P/C1 are efficiently internalized by Colon-26 cells.

To further quantitatively compare the cellular uptake of free CPT and various NPs, we treated Colon-26 cells with free CPT (25 μ M) or NPs loaded with the same concentration of CPT, and investigated their cellular uptake profiles at different time points (1, 3, and 5 h). The fluorescence emission intensities of Colon-26 cells treated with free CPT or CPT-loaded NPs for 5 h are presented in Fig. 8a. Notably, the fluorescence intensity of Colon-26 cells incubated with NPs-P/C1 was 3.4-, 2.1-, and 1.4-fold higher than that in cells incubated with free CPT, NPs-PVA, or NPs-P, respectively. This indicates that surface modification with Pluronic F127 and chitosan endowed NPs with a greater ability to inhibit P-gp and enhanced cellular uptake efficiency. As shown in Fig. 8b, cellular uptake of free CPT and NPs-PVA was low. However, the introduction of an appropriate amount of Pluronic F127 and/or chitosan to the surface of NPs (NPs-P and NPs-P/C1) sharply increased the cellular uptake of NPs. Moreover, in contrast to free CPT and NPs-PVA, which showed time-dependent intracellular accumulation, there was no difference in the accumulation of NPs-P/C1 at 1, 3 and 5 h. These results indicate that cells internalized NPs-P/C1 more rapidly than free CPT and NPs-PVA. A conjoint analysis of the results shown in Fig. 8b and Fig. 8c shows that the geometric mean of the fluorescence intensity of free CPT-treated cells remained stable with increasing percentage of cellular uptake, most likely reflecting simultaneous internalization and pumping out of free CPT. In contrast, the geometric mean of the fluorescence intensity of NPs-P/C1-treated cells increased even without an increase in the percentage of cellular uptake, indicating that the cells continued to take up NPs-P/C1 and did not pump out the released CPT.

3.8. In vivo antitumor efficacy

To compare relative *in vivo* antitumor efficacy, we intravenously injected NPs-P/C1 and control formulations (free CPT, NPs-PVA, and NPs-P) into mice bearing a subcutaneous colon tumor. As to the dose of CPT, previous reports suggested that 30 mg/kg is the maximum tolerated dose for murine cancer treatment, and 10 mg/kg has often been utilized in mice experiment [33, 35]. Body weight loss is a commonly used indicator for evaluating formulation toxicity. As shown in Fig. 9a, mice treated with free CPT were in poor physical condition and exhibited a 15.1% decrease in body weight compared with mice in the PBS-treated control group, indicating the severe systemic toxicity of free CPT. By comparison, none of the NP treatments caused marked weight loss in mice, indicating the comparable safety of the various NP formulations. The tumor growth profiles for the various treatment groups are shown in Fig. 9b. Mice in the group injected with free CPT showed slightly smaller tumor volumes than those in the PBS-treated control group. Notably, tumor volumes in mice treated with NP formulations were much smaller than those in mice treated with either PBS or free CPT. For example, injection of free CPT at 10 mg/kg suppressed tumor size by 7.9% at day 12, compared with the PBS control group, whereas injection of NPs-PVA, NPs-P, or NPs-P/C1 loaded with the same CPT dose decreased tumor volumes by 24.1%, 43.2% and 50.5%, respectively. The significant suppression of tumor growth in the

NPs-P/C1-treated group could reflect the combined effect of enhanced cellular uptake and suppressed MDR.

Table 4 compares antitumor efficiency of different CPT formulations against colon cancer in previous reports. Based on the fact that there are important differences in the mice strains, cell strains and endpoints, it is difficult to make a definitive statement. Collectively considering the side effect and therapeutic efficacy, our NPs-P/C1 appear to be comparable to the best previously reported systems (*e.g.*, PEG-P(Asp(Bz-70))) that have been developed for colon cancer therapy. Tumors weights correlated well with tumor sizes (Fig. 9c). A histological analysis of tumor tissues (Fig. 9d) showed that treatment with NPs-P/C1 resulted in a remarkable decrease in the number of cancerous cells compared with the control group and other drug formulations. The antitumor efficacy of NPs-P/C1 against implanted Colon-26 cells was also evaluated using TUNEL assays, which detect apoptosis in tumor tissue. As showed in Fig. 10a, TUNEL positivity (green fluorescence) was barely detectable in the tumors of mice treated with PBS, but was clearly observed in tumors of mice treated with free CPT or various NPs. However, only a small portion of tumor tissue from mice treated with free CPT exhibited apoptotic signals. In contrast, ~68.3% of cells in tumor tissues from mice treated with NPs-P/C1 were TUNEL positive, a value significantly higher than those observed in tumor tissues from mice treated with free CPT or other types of NPs (Fig. 10b). These results indicate that NPs-P/C1 is the most efficient of the four types of formulation.

Toxicity of drug formulations is always a significant concern. To further evaluate the safety of free CPT and various NPs, we performed histochemical analyses by staining with hematoxylin and eosin. As shown in Fig. 11, no noticeable evidence of organ damage was detected in H&E-stained organ slices from PBS- or NP-treated mice. However, serious organ damage was detected in mice treated with free CPT. These pathologies included myocardial fibrillar loss and vacuolation in heart tissues, significant edema and ballooning degeneration of hepatocytes, increased number of granulocytes in spleen, tubular vacuolization and tubular dilation with hemorrhagic areas in kidney, and increased alveolar wall thickness and cellular infiltration in lung tissue. Collectively, these findings indicate that encapsulation of CPT in NPs-P/C1 greatly decreases the toxicity and adverse side effects of CPT while effectively enhancing its antitumor efficacy.

3.9. Biodistribution of CPT in tumor-bearing mice

The biodistribution of CPT in major tissues and tumors was monitored at different time points (4, 24 and 48 h) after intravenous injection of either free CPT or CPT-loaded NPs-P/C1 into mice bearing subcutaneous colon tumors. As shown in Fig. 12, NPs-P/C1 were initially localized in the lungs; at 4 h, NPs-P/C1 levels in this tissue were 14.8 $\mu\text{g/g}$ tissue, a value that dropped to 6.7 $\mu\text{g/g}$ tissue after 24 h. 48 h after injection, CPT delivered via NPs-P/C1 showed a 1.23-fold higher accumulation in tumors compared to that of free CPT. NPs also showed much lower accumulation in organs. This preferential accumulation of NPs in tumor tissue is likely attributable to the well-known enhanced permeability and retention (EPR) effect. CPT-loaded NPs-P/C1 exhibited good retention in the body, excellent tumor

localization, and prolonged release of CPT—factors that we believe contribute to its superior antitumor activity.

4. Conclusions

In the present study, we designed and constructed a dual-surface-functionalized (Pluronic F127 and chitosan) CPT-loaded PLGA nanoparticle (NP-P/C) for colon cancer therapy. NPs-P/C1 with desirable diameter and zeta-potential effectively inhibited expression of the *MDR1* gene and enhanced cellular uptake. Consequently, NPs-P/C1 exerted the strongest anti-cancer activity *in vitro* compared with other drug formulations. *In vivo* experiments demonstrated that NPs-P/C1 exhibited the highest efficacy against subcutaneous colon tumors in mice compared with free CPT, NPs-PVA and NPs-P. Collectively, our findings indicate that this dual-surface-functionalized NPs-P/C1—which is fabricated using a convenient one-step process—inhibition of P-gp function, enhanced tumor uptake and improved therapeutic efficacy, and thus could represent an effective strategy for colon cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Preparation of dual-surface-functionalized NPs using a one-step fabrication process. (a) Schematic illustration of the preparation of NPs-P/C, and cellular uptake and release of CPT into the nucleus. (b) Representative SEM, TEM, size distribution, and zeta-potential profiles of NPs-P and NPs-P/C1.

Figure 2.

In vitro cumulative release of CPT from different NPs in PBS (pH 7.4) at 37 °C. Data are presented as means $\pm$ S.E.M. (n = 3).

Figure 3.

In vitro cytotoxicity of free CPT and various NPs against Colon-26 cells after incubating for 24 h (a), and 48 h (b) based on MTT assays. Triton X-100 (0.1%) was used as a positive control to produce a maximum cell death rate (100%), whereas cell culture medium was used as a negative control (death rate defined as 0%). Cytotoxicity is given as the percentage of viable cells remaining after treatment. Each point represents the mean $\pm$ S.E.M. (n = 5).

Figure 4.

In vitro cytotoxicity of free CPT and NPs-P/C1 against Caco2-BBE cells based on ECIS technology. (a) ECIS was used to determine cell viability in real time during an extended exposure to an NP suspension (CPT, 10 μ M). As controls, ECIS was also performed on untreated cells or cells treated with Triton X-100 (0.1%) in RPMI-1640 medium. (b) Morphology of Caco2-BBE cells grown for 38 h in 8-well ECIS dishes.

Figure 5.

The apoptosis profiles of Colon-26 cells following treatment with free CPT or NPs-P/C1 (CPT, 10 μ M). The percentages of cell states are indicated in each quadrant. Lower left, viable cells; lower right, early apoptotic cells; upper left, necrotic cells; upper right, late apoptotic cells. Data are presented as means $\pm$ S.E.M. (n = 3).

Figure 6.

In vitro inhibition of the expression of the *MDR1* gene by NPs-P/C1 in Colon-26 cells at 8 h (a) and 16 h (b). Each point represents the mean $\pm$ S.E.M. (n = 3). Statistical significance was assessed using Student's *t*-test (**P* < 0.05 and ***P* < 0.01).

Figure 7.

Fluorescence images showing cellular uptake of free CPT and NPs-P/C1 in Colon-26 cells after treatment with different CPT concentrations (0, 50 and 100 μM) for 3 h. Scale bar = 10 μm .

Figure 8.

Quantification of cellular uptake of free CPT and various NPs by Colon-26 cells. (a) Flow cytometry histogram profiles of fluorescence intensity for cells treated with free CPT or NPs (CPT, 25 μ M) for 5 h. Percentage of CPT-containing Colon-26 cells (b) and quantification of CPT fluorescent intensity in Colon-26 cells (c) after treatment with free CPT or NPs (CPT, 25 μ M) at different time points (1, 3, and 5 h). Each point represents the mean $\pm$ S.E.M. (n = 3). Statistical significance was assessed using Student's *t*-test (**P* < 0.05 and ***P* < 0.01).

Figure 9.

Anti-cancer effects of free CPT, NPs-PVA, NPs-P, and NPs-P/C1 *in vivo*. (a) Changes in body weight in different treatment groups. Mouse body weight was normalized to day 0 body weight (expressed as a percentage) and is depicted as the mean of each treatment group $\pm$ S.E.M. ($n = 5$). (b) Tumor growth profiles in different groups during treatment with free CPT or various NPs. Arrows indicate the days of drug injections. Each point represents the mean $\pm$ S.E.M. ($n = 5$). (c) Tumor weights in each treatment group at the study end point. Each point represents the mean $\pm$ S.E.M. ($n = 5$). Statistical significance was assessed using non-parametric Mann-Whitney test ($*P < 0.05$ and $**P < 0.01$). (d) H&E staining of tumor tissues from mice treated with free CPT or various NPs for 12 days. Scale bar = 20 μ m.

Figure 10.

Measurement of apoptosis. (a) Representative images of double-fluorescence labeling of DAPI nuclear staining (blue) and TUNEL staining (green) in tumors after a 12-d treatment. Scale bar = 20 μ m. (b) Percentages of TUNEL-positive cells in tumor sections of PBS-, free CPT-, and NP-treated groups. Each point represents the mean $\pm$ S.E.M. ($n = 3$). Statistical significance was assessed using non-parametric Mann-Whitney test ($*P < 0.05$ and $**P < 0.01$).

Figure 11.

H&E staining of sections of major organ tissues obtained from tumor-bearing mice after a 12-d treatment with PBS, free CPT, or different NPs. Heart, liver, spleen, lung, and kidney were harvested on day 20 after tumor transplantation. Scale bar = 20 μ m.

Figure 12.

Biodistribution of CPT at 4, 24, and 48 h after intravenous injection of free CPT or NPs-P/C1 into colon tumor-bearing mice. Each point represents the mean $\pm$ S.E.M. ($n = 3$). Statistical significance was assessed using non-parametric Mann-Whitney test.

Table 1Characteristics of the CPT-loaded nanoparticles (mean $\pm$ S.E.M.; n=3).

Nanoparticles	Nanoparticle Surface	P/P Ratio ^a	Particle Size (nm)	Zeta-potential (mV)	Drug Loading (%)	Encapsulation Efficiency (%)
Blank NPs-P/C1	PVA/Pluronic/chitosan	1:0.5	274.9 $\pm$ 12.5	18.9 $\pm$ 2.0	–	–
NPs-PVA	PVA	1:0	258.5 $\pm$ 10.2	–17.1 $\pm$ 2.8	5.69 $\pm$ 0.02	86.19 $\pm$ 0.30
NPs-P	PVA/Pluronic	1:0.5	278.2 $\pm$ 4.2	–12.8 $\pm$ 1.1	5.82 $\pm$ 0.07	87.95 $\pm$ 1.06
NPs-P/C1	PVA/ Pluronic/chitosan	1:0.5	268.6 $\pm$ 14.3	21.1 $\pm$ 0.9	6.41 $\pm$ 0.04	97.05 $\pm$ 0.66
NPs-P/C2	PVA/ Pluronic/chitosan	1:1	286.9 $\pm$ 15.7	19.0 $\pm$ 4.3	6.56 $\pm$ 0.02	99.34 $\pm$ 0.31

^aP/P ratio = weight of PLGA/weight of Pluronic F127.

Table 2

IC₅₀ (μM) of nanoparticles compared to free CPT against Colon-26 cells line.

Incubation time	Free CPT	NPs-PVA	NPs-P	NPs-P/C1	NPs-P/C2
24 h	8.84	3.51	2.67	2.03	2.57
48 h	1.79	1.05	0.79	0.67	0.83

Table 3

Primers used in this study.

Primer name	Sequence	Description
MDR1-F	5'- AAAGCTGGAGCAGTTGCTGA-3'	MDR1 gene RT-PCR forward primer
MDR1-R	5'- TGCCTATCGAAATGCTGGCT-3'	MDR1 gene RT-PCR reverse primer
36B4-F	5'-TCCAGGCTTTGGGCATCA-3'	36B4 gene RT-PCR forward primer
36B4-R	5'-CTTATCAGCTGCACATCACTCAGA-3'	36B4 gene RT-PCR reverse primer

Table 4

Comparison of tumor growth inhibition after injection of different CPT formulations.

Carrier ^a	Cell strain (Source)	Days after first injection	Total dose (mg/kg)	Routes ^b	Tumor growth inhibition ^c	Ref.
PEG-P(Asp(Bz-70))	Colon 26	8	30	Single	18.5%	[35]
	(Cell Resource Center, Tohoku University)	8	15	Single	27.5%	
		8	30	10 × 3	42.1%	
HAS-DB-L	Colon 26	8	10	Single	34.6%	[37]
	(Cell Resource Center, Tohoku University)	8	20	10 × 2	Mice dead	
NPs-P/CI	Colon-26	8	20	10 × 2	66.3%	[this work]
	(CLS Cell Lines Service GmbH)	12	30	10 × 3	49.5%	

^aPEG-P(Asp(Bz-70)): poly (ethylene glycol)-poly (benzyl aspartate-70); HAS-DB-L: PEG and human serum albumin coated 3,5-bis (dodecyloxy) benzoic acid; NPs-P/CI : Pluronic F127/chitosan-functionalized camptothecin-loaded nanoparticles (weight of PLGA/weight of Pluronic F127 = 1:0.5).

^bAll animals received intravenously injection via a lateral tail vein.

^cTumor growth inhibition (T/C %) was calculated according to the following equation: T/C % = (mean tumor volume of treated group)/(mean tumor volume of control group).