Positively charged polypeptide nanogel enhances mucoadhesion and penetrability of 10-hydroxycamptothecin in orthotopic bladder carcinoma

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1. Introduction

Bladder cancer (BC) has become a serious public health problem due to its continuously rising incidence, high recurrence rate, and poor quality of life. Intravesical instillation of chemotherapy, one of common and important treatment strategies for BC, is restricted partially due to the short residence time and the low penetration ability of current antineoplastic agent formulations in clinic. Herein, a positively charged disulfide-core-crosslinked polypeptide nanogel of poly(l-lysine)-poly(l-phenylalanine-co-l-cystine) (PLL–P(LP-co-LC)) was synthesized. 10-Hydroxycamptothecin (HCPT) was loaded into the core via a facile diffusion to obtain loading nanogel (i.e., NG/HCPT). The reduction-responsive cationic polypeptide nanogel not only showed a high drug-loading efficiency, a prolonged residence time, and an improved tissue penetration capability, but also demonstrated an ability to accurately and rapidly release HCPT in bladder cancer cells. NG/HCPT exhibited superior cytotoxicity against human T24 bladder cancer cells compared to that of free HCPT in vitro. Moreover, the positively charged loading nanogel exhibited significantly enhanced antitumor efficacy and reduced side effects toward orthotopic bladder cancer model in vivo. Overall, the smart polypeptide nanogel with enhanced residence and permeability provides a promising drug delivery platform for local chemotherapy of BC.

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phenylalanine-co-L-cystine) (PLL-P(LP-co-LC)) was developed to deliver 10-hydroxycamptothecin (HCPT) into orthotopic BC cells. The loading nanogel was noted as NG/HCPT. As a derivative of camptothecin (CPT), HCPT is a very effective inhibitor of topoisomerase I that inhibits DNA replication and RNA transcription of a broad spectrum of tumor cells [19]. To verify our hypothesis, it was further examined whether the reduction-responsive cationic NG/HCPT would increase retention time and improve tissue penetrability to eventually facilitate cancer cell uptake and intracellular activation of HCPT. PLL offered a positive charge to bond with the negatively charged bladder mucosa, and thus was expected to endow NG/HCPT with mucoadhesivity [20]. Furthermore, the amphipathic PLL-P(LP-co-LC) with LL residues would allow HCPT to enter the cells in a similar way to amphipathic cell penetrating peptides (CPPs) [21]. Given the different metabolic pathways and rates of cancer cells in comparison with normal ones, malignant cells exhibit high intracellular glutathione (GSH) concentration of about 10.0 mM [22,23]. Therefore, after cell internalization, the disulfide bond in the core of NG/HCPT was selectively degraded due to the high level of intracellular GSH, which further accelerated the release of HCPT to induce enhanced cell apoptosis [24,25].

In this work, the solution properties, HCPT release profiles, cell internalization, cytotoxicity and apoptosis in vitro, and mucoadhesiveness, tissue distribution, antitumor efficacy, and systemic toxicity in vivo of NG/HCPT were systematically investigated. Encouragingly, the high drug-loading efficiency, prolonged residence time, enhanced penetrating ability, and reduction-responsive release property give NG/HCPT extremely high antitumor activities in vitro and in vivo.

2. Materials and methods

2.1. Materials

N(ε)-Benzyloxycarbonyl-L-lysine, L-phenylalanine, and L-cystine were purchased from GL Biochem Co., Ltd. (Shanghai, P.R. China). N(ε)-Benzyloxycarbonyl-L-lysine N-carboxyanhydride (ZLL NCA), L-phenylalanine N-carboxyanhydride (LP NCA), and L-cystine N-carboxyanhydride (LC NCA) were synthesized as described in our previous work with slight modification [26–29]. N,N-Dimethylformamide (DMF) was dried over calcium hydride (CaH₂) at room temperature before vacuum distillation. HCPT was purchased from Beijing Huafeng United Technology Co., Ltd. (Beijing, P.R. China). Clear 6-well and 96-well cell culture plates were obtained from Corning Costar Co. (Cambridge, MA, USA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Methyl thiazolyl tetrazolium (MTT), 4′,5′-diamidino-2-phenylindole dihydrochloride (DAPI), and o-diphenylthiophenetol (DTT) were sourced from Sigma-Aldrich (Shanghai, P.R. China) and used as received. Acetic acid, ammonium acetate, and triethylamine were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, P.R. China). 24-Gauge closed IV catheter system was purchased from Suzhou Bidi Medical Devices Co., Ltd. (Suzhou, P.R. China). All the other solvents and reagents were purchased from Sigma-Aldrich (Shanghai, P.R. China) and used as received. The purified deionized water was prepared by the Milli-Q plus system (Millipore Co., Billerica, MA, USA).

2.2. Preparation and characterization of blank and loading nanogels

2.2.1. Synthesis of disulfide-core-crosslinked polypeptide nanogel

As depicted in Scheme 1A, the synthesis of PLL–(PLP-co-PLC) was started through the ring-opening polymerization (ROP) of ZLL NCA using n-hexylamine as the macroinitiator. In brief, n-hexylamine and ZLL NCA were dissolved in anhydrous DMF in a flame-dry flask. The reaction mixture was stirred at 25 °C for 72 h. Then, LP NCA and LC NCA were added, and the mixture was stirred for another 72 h. The solution was then precipitated into an excess amount of diethyl ether. The obtained product was further washed twice with diethyl ether and dried under vacuum at room temperature for 24 h. Subsequently, the precipitate was dissolved in trifluoroacetic acid (TFA), and then hydrobromic acid/acetic acid (HBr/HAc (33 wt.%) was added. After stirred for 1 h at 25 °C, the mixture was dialyzed and freeze-dried to give a product of PLL–(PLP-co-PLC).

2.2.2. Determination of drug loading content and drug loading efficiency

NG/HCPT was prepared through a facile diffusion and dialysis method. In short, polypeptide (500.0 mg) and HCPT (250.0 mg) were dissolved in 20.0 mL of DMF by vortex and sonication. The mixture was then stirred for 2 h at room temperature. Then, 10.0 mL of deionized water was added to the solution while stirring, followed by dialysis for 24 h. The dialysis medium was refreshed five times, and the whole procedure was performed in the dark. Then, the solution was filtered and lyophilized to obtain NG/HCPT.

To determine the drug loading content (DLC) and drug loading efficiency (DLE), the freeze-dried NG/HCPT was accurately weighed and dissolved in DMF. The HCPT concentration was then analyzed by ultraviolet-visible (UV–vis) spectrophotometry using a standard curve method \((\lambda_{\text{abs}} = 365 \text{ nm})\). The DLC and DLE of NG/HCPT were calculated according to Eqs. (1) and (2), respectively.

\[
\text{DLC (\%)} = \frac{\text{Amount of drug in nanogel}}{\text{Amount of loading nanogel}} \times 100\% \quad (1)
\]

\[
\text{DLE (\%)} = \frac{\text{Amount of drug in nanogel}}{\text{Total amount of feeding drug}} \times 100\% \quad (2)
\]

2.2.3. Characterizations

Proton nuclear magnetic resonance (1H NMR) spectra were recorded on a Bruker AV 400 NMR spectrometer (Ettlingen, Germany) with deuterated dimethyl sulfoxide (DMSO-d₆) as a solvent. Fourier-transform infrared (FT-IR) spectra were measured on a Bio-Rad Win-IR instrument (Cambridge, MA, USA) using potassium bromide (KBr) method. The morphology of NG/HCPT was revealed by transmission electron microscopy (TEM) measurement on a JEOL JEM-1011 TEM (JEOL, Tokyo, Japan) with an accelerating voltage of 100 kV. To prepare the TEM sample, a small drop of NG/HCPT aqueous solution was deposited onto a 230 mesh copper grid coated with carbon, and allowed to dry at room temperature for over 24 h. The hydrodynamic radii \((R_h)\) were determined by dynamic laser scattering (DLS) measurements on a WyattQELS instrument with a vertically polarized He–Ne laser (DAWN EOS, Wyatt Technology Co., Santa Barbara, CA, USA). The scattering angle was fixed at 90°. The samples were prepared in aqueous solution at a concentration of 100.0 μg mL⁻¹. Before measurements, the solution was filtered through a 0.45 μm Millipore filter. The zeta-potential of NG/HCPT was determined by a Zeta Potential/BI-90Plus Particle Size Analyzer (Brookhaven, USA).

2.2.4. In vitro HCPT release

The in vitro HCPT release kinetics from NG/HCPT were investigated in phosphate-buffered saline (PBS; pH 7.4, Tween-80 (0.1%, W/V) without, or with 5.0 or 10.0 mM DTT. Typically, 0.1 mg of free HCPT or 1.0 mg of freeze-dried NG/HCPT was dissolved in 10.0 mL of corresponding release medium and introduced into a dialysis bag (molecular weight cut-off \((\text{MWCO}) = 3500 \text{ Da})\). The release experiment was initiated by placing the end-sealed dialysis bag into 100.0 mL of release medium at 37 °C with a continuous shaking rate of 70 rpm. At predetermined time intervals, 2.0 mL of dialysate was withdrawn and replaced with an equivalent amount of fresh medium. Sample solution was acidified with 1.0 N HCl and 20.0 μL was measured for the accumulative amount of HCPT release by high-performance liquid chromatography (HPLC; \(\lambda_{\text{abs}} = 371 \text{ nm})\).

2.3. Intracellular drug release

The cell uptake and intracellular drug release behavior of NG/HCPT were observed by confocal laser scanning microscopy (CLSM) and microplate reader toward T24 cells, a human bladder cancer cell line. For CLSM observation, T24 cells were seeded onto glass coverslips in 6-well plate at a density of 1.5 × 10⁵ cells in 2.0 mL of complete DMEM containing 10% (V/V) FBS, supplemented with 50.0 IU mL⁻¹ penicillin and 50.0 IU mL⁻¹ streptomycin per well, and cultured at 37 °C in 5% (V/V) carbon dioxide (CO₂) atmosphere for 24 h. Then the incubation medium was removed, and free HCPT or NG/HCPT in 2.0 mL of complete DMEM was added with a final HCPT concentration of 1.25 mg L⁻¹. The cells incubated with PBS were used as a control. At predetermined time intervals, the culture media were removed, and cells were washed three times with PBS. Thereafter, the cells were fixed with 4% (W/V) PBS-buff ered formaldehyde for 20 min at room temperature. The cell microages were determined by CLSM (LSM 780, Carl Zeiss, Jena, Germany).

Microplate reader determination was performed as described by Wei et al. [30]. The cells were cultured in 96-well plate and then
incubated with different HCPT formulations (1.25 mg L\(^{-1}\)) for a consistent period of time, then washed three times with ice-cold PBS solution. Cell lysate with 1% (V/V) Triton X-100 was used to treat the samples, which was followed by the addition of sodium hydroxide (NaOH) to dissolve the internalized HCPT. After these treatments, the plate was measured at 384 nm by an Infinite M200 microplate spectrophotometer (Tecan, Durham, USA). Each experiment was performed in triplicate.

2.4. In vitro cell proliferation inhibition assays

The in vitro cell proliferation inhibition capabilities of free HCPT and NG/HCPT against T24 cells were evaluated by a standard MTT assay. The cells were planted into 96-well plate at 1.0 × 10\(^4\) cells per well in 20.0 mL of complete DMEM and incubated at 37 °C for 24 h. Then, the incubation medium was removed, and free HCPT or NG/HCPT in 200.0 μL of complete DMEM with various HCPT concentrations from 0.02 to 10.0 mg L\(^{-1}\) was added. The cells without the coincubation with HCPT formulations were used as a control. The cells were subjected to MTT assays after being incubated for another 24 h. The absorbance of the solution was measured at 490 nm on a Bio-Rad 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The cell viability (%) was calculated according to Eq. (3).

\[
\text{Cell Viability} (\%) = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100\%
\]  

In Eq. (3), \(A_{\text{sample}}\) and \(A_{\text{control}}\) represented the absorbances of sample well and control well, respectively.

2.5. Apoptosis detections

The HCPT-induced apoptosis of T24 cells was assessed by flow cytometry (FCM) analysis. T24 cells were seeded in 6-well plate at a density of 2 × 10\(^5\) cells per well and incubated for 24 h. Subsequently, the incubation medium was removed, and free HCPT or NG/HCPT in 2.0 mL of complete DMEM was added with a HCPT concentration of 0.1 mg L\(^{-1}\). The cells without pretreatment were used as a control. Cells were incubated for another 24 h at 37 °C and harvested with ethylenediaminetetraacetic acid (EDTA)-free trypsin, centrifuged at 3000 rpm for 5 min, and then washed with PBS. The cells were resuspended in 0.5 mL of binding buffer and stained with 5.0 μL Annexin V-FLITC under room temperature for 10 min, followed by the addition of 5.0 μL propidium iodide (PI) on ice. At the end of coincubation, the cells were analyzed immediately by FCM analysis. The first 10,000 events were acquired by CXP analysis software V2.1 (Applied Cytometry Systems, Dinnington, UK).

2.6. Animal procedure

The male BALB/c mice at the age of 10 weeks were purchased from the Laboratory Animal Center of Jilin University. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Animal Care and Use Committee of Jilin University.

2.7. Bladder cancer model induction and histopathology determinations

Bladder cancer was induced by adding 0.05% (W/V) BBN in drinking water for 20 weeks. It was proved that murine exposure to BBN reliably induced a carcinoma histologically indistinguishable from human urothelial carcinoma [31,32]. The mice were observed once a day, weighed weekly, and palpated for bladder lesions every other day. At the end of BBN treatment, one mouse was sacrificed randomly, and necropsy was performed. The bladder and major organs (the heart, liver, spleen, lung, and kidney) were excised, fixed in 4% (W/V) PBS-buffered paraformaldehyde, and then embedded in paraffin. The sections of these embedded tissue samples were stained with hematoxylin and eosin (H&E) to assess the incidence and progressions of bladder cancer with the use of a microscope (Nikon TE2000U, Kanagawa, Japan).

2.8. Mucoadhesiveness and penetrability studies

The mucoadhesiveness and penetrability of NG/HCPT were investigated by CLSM. First, the male BALB/c mice with bladder cancer were anesthetized by an intraperitoneal injection of 10% chloral hydrate (W/V). Then, the animals received an intravesical instillation with 0.15 mL of free HCPT or NG/HCPT solution with an equivalent HCPT concentration of 1.0 mg mL\(^{-1}\) via a lubricated 24-gauge closed IV catheter system. The mice were anesthetized for approximately 2 h and monitored carefully after catheterization. At predetermined time intervals, the bladders were excised, opened, and washed five times with PBS. For the mucoadhesiveness study, tissue sections were prepared by cutting the flattened bladder into a square and washing it thoroughly with PBS again. The urothelial surfaces of these prepared bladder sections were determined by CLSM immediately. For the penetrability study, the flattened bladders were embedded in Tissue-Tek OCT compound embedding medium (Miles Inc., Diagnostics Division, Elkhart, IN, USA). Then, cryogenic sections of 6 μm thickness were sliced from the mucous membrane to the serous membrane serially using a freezing microtome (Leica CM 1900, Wetzlar, Germany). The sections were then observed by CLSM.

2.9. In vivo biodistribution

Male BALB/c mice with bladder cancer established as described above were treated with either free HCPT or NG/HCPT at an equivalent HCPT dose of 6.0 mg per kg body weight (mg (kg BW)\(^{-1}\)) by intravesical instillation (\(n = 3\) for each group) and sacrificed 6 h later. The bladder and other major organs (the heart, liver, spleen, lung, and kidney) were excised, washed, and accurately weighted. The tissue samples were homogenized with normal saline, which was acidified to pH 3.0 with acetic acid. Afterward, the tissue homogenates were extracted with two volumes of cold cetonitride/methanol (1/1, V/V). The clear suspensions were obtained by centrifugation at 15,000 rpm at 4 °C for 10 min. 20.0 μL of the clear suspension was used to determine the concentration of HCPT by HPLC. The mobile phase was a mixture of acetonitrile/aqueous buffer (67/33, V/V), in which the aqueous buffer was made up of 75 mmol L\(^{-1}\) ammonium acetate, 5 mmol L\(^{-1}\) triethylamine, and 0.5% (V/V) acetic acid [33].

2.10. In vivo antitumor efficacies

A total of 15 male BALB/c mice with bladder cancer were randomly divided into three groups (\(n = 5\) for each group). The animals were treated with PBS, free HCPT, or NG/HCPT at an equivalent HCPT dose of 6.0 mg (kg BW)\(^{-1}\) by intravesical instillation weekly for a total of 6 weeks. Cystography was used once a week for monitoring development of the bladder cancers. The body weight was measured at the same time as an indicator of systemic toxicity.

2.11. Histological and immunohistochemical analyses

One week after the last intravesical instillation of various HCPT formulations, the mice were sacrificed, and their bladders were excised, fixed in 4% (W/V) PBS-buffered paraformaldehyde, and followed by dehydration, clearing, wax infiltration, and embedding. The sections of these embedded tissue samples were used for H&E staining. Some other sections were stained with the immunohistochemical method to assess the expressions of poly (ADP-ribose) polymerase (PARP) and proliferating cell nuclear antigen (PCNA) (Abcam, Cambridge, MA, USA). The alterations of histology were examined by microscope, and those of immunohistochemistry were evaluated by CLSM.
2.12. Statistical analyses

All experiments were performed at least three times, and the results are presented as means ± standard deviation (SD). Data were analyzed for statistical significance using Student’s t-test. \( P < 0.05 \) was considered statistically significant, and \( P < 0.01 \) and \( P < 0.001 \) were considered highly significant.

3. Results and discussion

3.1. Preparations and characterizations of unloaded and loaded PLL–P(LP-co-LC)

Many kinds of copolymers that contain polypeptide blocks can be prepared by the ROP of amino acid NCA using different macroinitiators [34,35]. In this work, the PLL–P(LP-co-LC) copolymer was synthesized by the ROP of ZLL NCA, and subsequent LP NCA and LC NCA with initiator \( n \)-hexylamine, and the deprotection of benzylxycarbonyl group in the ZLL unit (Scheme 1A). The successful preparation of PLL–P(LP-co-LC) was confirmed by \(^1\)H NMR and FT-IR spectra (Fig. 1). As shown in Fig. 1A, the resonances at 4.29 ppm (b) and 4.49 ppm (k + p) were attributed to the protons in the backbone of PLL, and PLP and PLC, respectively. The signals at 2.80 ppm (l + q) were ascribed to the methylene protons of the side groups of LP and LC units, that is, \( \text{C}_8\text{H}_4\text{CH}_2\text{C}_2\text{H}_4\text{NH}_2, 2\text{H} \) and \(-\text{SCH}_2\text{NH}_2, 2\text{H} \), respectively. The peaks at 1.37 ppm (c; \(-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2, 2\text{H} \), 1.60 and 1.70 ppm (d; \(-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2, 2\text{H} \), and 2.93 ppm (f; \(-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2, 2\text{H} \)) were assigned to the methylene protons in the side chain of LL unit. The phenyl group protons gave a characteristic peak at 7.13 ppm (i) \(-\text{CH}_2\text{Ph, 5H} \). The results of FT-IR spectra were also consistent with the chemical structure of PLL–P(LP-co-LC), as the appearance of typical absorptions at 1654 cm\(^{-1}\) (\( \nu_{\text{C=O}} \)) and 1528 cm\(^{-1}\) (\( \nu_{\text{C=O} - \text{N} = \text{N}} \)) assigned to the amide bond (Fig. 1B). The molar ratio of LL, LP, and LC in PLL–P(LP-co-LC) was calculated as 11:13:6 according to the content of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) determined by elemental analyses.

HCPT was loaded into the core of PLL–P(LP-co-LC) nanogel through diffusion in a normal physiological aqueous environment. The DLC and DLE of NG/HCPT were at high levels of 30.6 and 88.2 wt.%, respectively. The resulting NG/HCPT exhibited spherical morphology with an average diameter of around 65 nm, which was obtained from TEM measurements (Fig. 2A). Furthermore, the hydrodynamic radius \( (R_h) \) of NG/HCPT detected by DLS was 65.9 ± 0.4 nm (Fig. 2B), showing a little bigger than that obtained from TEM microimage. The minimal difference is most likely due to the dehydration and systole of nanogel in the preparation of TEM sample. However, at 0–24 h, the \( R_h \) of NG/HCPT was measured to increase from 65.9 ± 0.4 to 391 ± 60.8 nm in the presence of 10.0 mM DTT. After that, the \( R_h \)-related light scattering signal could not be detected. In addition, NG/HCPT in PBS at pH 7.4 showed negligible size alteration within 48 h (Fig. 2C). The results indicated that the NG/HCPT possessed excellent stability under physiological conditions but rapid structural swelling and HCPT release in the reductive microenvironment. In addition, NG/HCPT was positively charged in PBS at pH 7.4 with zeta potential of 16.3 ± 1.4 mV, which was due, in part, to the LC unit [20]. It should be noticed that the nanoparticles with a positive surface charge could improve the interaction with biological membranes and facilitate cell endocytosis [36,37].

The in vitro HCPT release behaviors from NG/HCPT were investigated in PBS (pH 7.4, Tween-80 (0.1%, W/V)) without or with 5.0 or 10.0 mM DTT. As shown in Fig. 2D, the rapid diffusion of free HCPT from dialysis membrane confirmed the feasibility of the drug release analysis method. As a comparison, no significant initial burst release of HCPT from NG/HCPT was observed within the same time period. In the absence of DTT, a slow drug release from NG/HCPT was demonstrated, and ≤50% of the loaded drug was released during the test duration (i.e., 72 h). However, 81.2% and 95.4% of HCPT were released from NG/HCPT when the concentration of DTT increased from 5.0 to 10.0 mM, respectively. The release was accelerated by the increase of DTT concentration to 10.0 mM, analogous to the reductive intracellular microenvironment. The fast HCPT release from NG/HCPT in reductive conditions was attributed to swelling of the nanogel induced by cleavage of the disulfide bond. Due to this characteristic, it is possible that HCPT release was low in normal tissue and significantly accelerated in tumor site. The reduction-responsive profile could enhance the overall therapeutic efficacy and reduce the side effects in vivo.

3.2. Intracellular drug release and cytotoxicity

The cell internalization and release behaviors of NG/HCPT were monitored with CLSM and microplate reader toward T24 cells. For CLSM observation, T24 cells were incubated with free HCPT or NG/HCPT with an equivalent HCPT concentration of 1.25 μg mL\(^{-1}\) for 2 or 6 h. Cells treated with PBS were used as a control. As depicted in Fig. 3A, after 2 h coincubation, the HCPT fluorescence was observed in the cells incubated with free HCPT or NG/HCPT. HCPT was observed distributed in the cytoplasm just outside the nuclei of cells. There was a little higher HCPT fluorescence intensity in the cells treated with free HCPT than that of cells treated with NG/HCPT at 2 h. It was reported that HCPT molecule was transported into tumor cells through a
diffusion mechanism, while NG/HCPT was internalized presumably via the endocytosis pathway [38]. The phenomenon indicated that free HCPT could be internalized into T24 cells slightly faster by the diffusion process at 2 h. As coincubation time prolonged to 6 h (Fig. 3A), the NG/HCPT-treated cells depicted markedly enhanced HCPT fluorescence. The fluorescence distributed throughout the entire cells and accumulated especially strongly in the nuclei, which indicated that HCPT was released and delivered into the nuclei of T24 cells. However, the fluorescence intensities of cells treated with free HCPT became extremely weak and accumulated mainly in the cytoplasm.

The results were due to the cationic, LL-rich, and disulfide crosslinked properties, which gave the polypeptide nanogel great opportunities for intracellular drug delivery efficiently. The cationic NG/HCPT was adhered to the cell membrane via electrostatic interaction and entered the cells in a way similar to CPPs. It was reported that CPPs possess the ability to enter cells carrying coupled cargoes with them [39,40]. Amphipathic CPPs were one of the classes. Amphipathicity and LL residue were considered to play a crucial role in the cell translocation of this class of CPPs [21]. Two characteristics of PLL–P(LP-co-LC) were amphipathicity and the existence of LL residues. Subsequently, the intracellular reductive conditions triggered NG/HCPT to deliver and release HCPT by cleavage of the disulfide bond. Therefore, in our work, NG/HCPT could effectively enhance endocytosis, trigger rapidly intracellular HCPT release, and improve drug accumulation in nuclei.

For further confirmation, the cell uptake and release behaviors were determined by a microplate reader as described by Wei et al. [30]. The HCPT content of the cells treated with free HCPT rapidly reached its peak and slowly decreased with the extension of time. After incubation for 6 h, the content of NG/HCPT was strikingly higher than that of free HCPT (Fig. 3B), which was in agreement with the results of CLSM analyses (Fig. 3A). The similar results were reported before [41].

However, the mechanism is still not clear. After reviewing a large number of relevant literatures, we hypothesized that free HCPT, a small molecule, could enter the cells via diffusion pathway. The intracellular HCPT concentration increased rapidly, until the intracellular and extracellular concentrations reached a dynamic equilibrium. HCPT was subsequently metabolized to its carboxylate form and into glucuronide [42]. At this time, the intracellular HCPT concentration was reduced, and the extracellular HCPT molecules continued to diffuse into the cells. As the metabolic rate was higher than the diffusion rate, the total concentration of HCPT in the cells decreased. However, NG/HCPT entered inside the cells through a process named “endocytosis”. Endocytosis involves multiple stages and is a relatively long process [43]. Thus, the time to balance the HCPT concentrations inside and outside the cells was prolonged. This was exactly why NG/HCPT reached the peak levels later than that of free HCPT. The smart drug delivery system carrying large amounts of drugs into cells compensated for the metabolism. As a result, the overall HCPT value inside the cells showed a slow decline in the cells treated with NG/HCPT.

The in vitro cytotoxicity of free HCPT and NG/HCPT against T24 bladder cancer cells was estimated by a standard MTT assay. As shown in Fig. 3C, the cells treated with free HCPT or NG/HCPT exhibited cell proliferation inhibition efficacies in a concentration-dependent manner when compared with the control. At any given equivalent concentration, NG/HCPT induced more obvious inhibition in comparison with free HCPT toward T24 cells. The profound cell killing effect of NG/HCPT was probably due to the excellent cell penetration properties, improved
endocytosis as well as rapid intracellular HCPT release triggered by the high concentration of GSH in T24 cells. It was worth noting that the half maximal inhibitory concentration (IC50) value of NG/HCPT was determined as 2.7 mg L⁻¹, which was lower than that of free HCPT (i.e., 7.9 mg L⁻¹) after 24 h coincubation. It quantitatively confirmed a better cytotoxic effect of NG/HCPT than that of free HCPT, and revealed the reduction sensitive ability of polypeptides in bladder cancer cells. The reduction-responsive drug delivery behavior endows NG/HCPT with excellent potential for chemotherapy, and made it possible for NG/HCPT to have a better antitumor capability in vivo. It should be noted that the HCPT content of NG/HCPT group remained at a high level throughout the experiment process, which confirmed the findings of cytotoxicity.

The apoptotic activities of T24 cells induced by free HCPT and NG/HCPT were assessed by FCM analyses. After incubating for 24 h with different HCPT formulations at a consistent HCPT concentration of 0.1 mg L⁻¹, the cells were double stained for viability and apoptosis. As depicted in Fig. 3D, free HCPT and NG/HCPT resulted in 3.5% and 8.9% early apoptotic cells in the fourth quadrant (Q4), 12.0% and 13.6% late apoptotic cells in Q2, and 82.6% and 76.5% normal cells in Q3, respectively, which were consistent with the MTT results. The results were possibly due to the effective cell internalization through endocytosis, fast intracellular HCPT release, and a high level of drug accumulation in the nuclei. The increased apoptotic activity proved PLL–P(LP-co-LC) to be an excellent antitumor drug delivery vector.
3.3. Mucoadhesiveness and penetrability, and biodistribution

The mucoadhesiveness and penetrability of NG/HCPT were investigated by CLSM. The tumor-bearing mice were anesthetized and received an intravesical instillation of free HCPT or NG/HCPT solution through the urethra. At predetermined time intervals, the bladders were excised and prepared as bladder sections. For the mucoadhesiveness study, the urothelial surface was observed, and for the penetrability study, the full-thickness bladder wall was observed by CLSM. As shown in Fig. 4A, there was a markedly higher HCPT fluorescence intensity in the bladder wall treated with NG/HCPT than that of free HCPT at any time intervals. The strongest fluorescence intensity occurred in the NG/HCPT group at 0.5 h. The fluorescence of free HCPT group decreased rapidly, while the NG/HCPT group maintained relatively high fluorescence intensity. All these results indicated a super mucoadhesiveness of NG/HCPT. To further quantify its mucoadhesiveness, the relative optical density of HCPT was evaluated with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The optical density of free HCPT at 0.5 h was defined as “1”. The relative optical density of NG/HCPT was calculated as the ratio to free HCPT. Unexpectedly, the optical density of NG/HCPT was 1.7 times higher than that of free HCPT at 0.5 h, 2.5 times higher at 2 h, and 5.3 times higher at 6 h, which is shown in Fig. 4B.

As shown in Fig. 5A, due to the rapid simple diffusion, the HCPT fluorescence was detected in the whole layer of the bladder wall treated with free HCPT at 0.5 h. Furthermore, the fluorescence near the mucous membrane was obviously stronger. The fluorescence was limited to the vicinity of mucous membrane in the NG/HCPT group. As the time prolonged, HCPT gradually penetrated into the whole bladder wall of the group treated with NG/HCPT, and the fluorescence intensity was

![Fig. 4. Mucoadhesiveness. (A) Mucoadhesiveness of free HCPT and NG/HCPT investigated by CLSM, and (B) statistical analysis of optical density of HCPT fluorescence intensity. The scale bar in (A) represents 40 μm. Data are presented as mean ± SD (n = 3; ***P < 0.001).](image-url)
Fig. 5. Penetrability. (A) Penetrability of free HCPT and NG/HCPT investigated by CLSM, and (B) statistical analysis of optical density of HCPT fluorescence intensity. The arrow represents the penetrating direction of HCPT. The scale bar in (A) represents 40 μm. M, mucous membrane; S, serous membrane.
maintained at a high level. This phenomenon suggested that NG/HCPT could not only penetrate into the full-thickness bladder wall, but also indicated a high HCPT concentration. To further quantify the penetrability of NG/HCPT, the relative optical density of HCPT was evaluated with ImageJ software. The optical density of maximum value was defined as "1". The relative optical density of free HCPT and NG/HCPT was calculated as the ratio to maximum value, which was shown in Fig. 5B.

The in vivo biodistribution study was conducted by HPLC. Male BALB/c mice with bladder cancer were treated with free HCPT or NG/HCPT by intravesical instillation. 6 h later, the bladder and other major organs (the heart, liver, spleen, lung, and kidney) were excised, homogenized, and acidified. The tissue homogenates were extracted with two volumes of a cold mixture of acetonitrile/methanol (1/1, V/V). Then, 20.0 μL of clear suspension obtained by centrifugation was used to determine the HCPT content. As shown in Fig. S1, Supporting information, only the bladder treated with NG/HCPT provided an extremely high level of HCPT content, which was 3.2 times higher than that of the free HCPT-treated one. The phenomenon confirmed the superior mucoadhesiveness of PLL–P(LP-co-LC) nanogel again, which was also confirmed by CSLM. These results could also further explain why the in vivo antitumor effect of NG/HCPT was better than that of free HCPT. NG/HCPT was highly selectively accumulated in bladder tissues and rarely in other organs, which not only improved the efficacy but also reduced the side effects of HCPT.

3.4. In vivo antitumor efficacy

The in vivo antitumor efficiency of NG/HCPT was evaluated using male BALB/c mice with orthotopic bladder cancer. As depicted in Scheme 1B, the bladder cancer was developed by adding 0.05% (W/V) BBN into the drinking water of the mice for 20 weeks. The mice were randomly divided into three groups (n = 5 for each group), and treated with PBS, free HCPT, or NG/HCPT at an equivalent HCPT dose of 6.0 mg (kg BW)^{-1} by intravesical instillation once a week for a total of 6 weeks. The cystography and body weight were then monitored every week as indicators of tumor size and systemic toxicity, respectively, over the entire treatment period. The last cystography was given after the end of treatment as shown in Fig. 6A. All the tumors showed an irregular surface. Compared with the huge space-occupying lesion at the top surface of the bladder in the PBS group, there was a small convexity that occupied the side wall in the free HCPT group. However, no significant abnormalities were observed in the NG/HCPT group due to the smooth margin. The results indicated that the treatment with NG/HCPT induced remarkable tumor growth inhibition as compared with those with both PBS and free HCPT. The suboptimal antitumor efficacy of free HCPT was due to its quick excretion with urine. The favorable antitumor efficacy of NG/HCPT was most benefited from the significantly improved mucoadhesiveness and penetrability of PLL–P(LP-co-LC). This could eventually lead to enhanced cell uptake of HCPT, as well as improved intracellular accumulation of drug content.

![Fig. 6. Tumor inhibition in vivo. (A) Cystography, (B) intact, and (C) opened bladders after the end of treatment. (D) Body weight and (E) survival rate during treatment with PBS (control), free HCPT, or NG/HCPT. Scale bars in (A), (B), and (C) represent 1 cm. Data are presented as mean ± SD (n = 5; *P < 0.05).](image)
The intact bladders excised from the mice after the end of treatment were used to observe the outer surface. As shown in Fig. 6B, regardless of the size or general shape, the bladder treated with NG/HCPT was the most similar to that of a healthy mouse. However, the bladder treated with PBS became as hard as leather, which resulted from the tumor tissues infiltrating the muscular layer. These excised bladders were then opened to gain a better understanding of the inner surface (Fig. 6C). No appreciable neoplasms were observed in the NG/HCPT-treated bladder, which confirmed the inhibition in tumor growth of NG/HCPT. Body weight change is an important indicator of the side effects. As shown in Fig. 6D, all the tumor-bearing mice treated with NG/HCPT showed no notable body weight change during the whole therapeutic process, suggesting no obvious toxicity. The survival rate is another essential reflection of systemic toxicity. Prolonged survival time was observed both after treatment with free HCPT and NG/HCPT. The exceptionally long survival time was observed in the NG/HCPT group (Fig. 6E), which was in agreement with the tumor growth inhibition data. NG/HCPT had a sustained HCPT release and showed selective accumulation in the tumor site, which improved the tumor inhibitory effect and decreased the systemic toxicity. This indicates that NG/HCPT exhibited an advantage of biological safety.

Histopathological analysis was used to evaluate whether the bladder cancer model was successful or not. As shown in Fig. 7A, at the end of BBN treatment, the tumor cells were larger with deep color and deformity in nuclei in comparison to normal tissue cells, indicating the successful establishment of bladder cancer.

To further investigate the antitumor efficacy after the treatment with various HCPT formulations, the bladders were excised from mice and sectioned for histopathological analyses at the end of the treatment.

For H&E staining, the chromatin was dyed bluish violet with alkaline hematoxylin, and the cytoplasm and extracellular matrix were nonspecifically stained pink by acidic eosin. The nuclei of tumor cells were large and heterogeneous, and the cell morphology was clear with spherical or spindle shape. Whereas, the chromatin of necrotic cells became darker, pyknotic, even absent, and the cell morphology was not clear. As shown in Fig. 7A, large amounts of tumor cells with clear cell morphology and more chromatin invaded the muscular layer in the group of PBS, and few necrotic areas were observed, indicating active tumor growth.

**Fig. 7.** Histopathology and immunohistochemistry. (A) Histopathological (i.e., H&E) and (B) immunohistochemical (i.e., PARP and PCNA) analyses of tumor tissue sections after treatment with PBS (as a control), free HCPT, or NG/HCPT. The scale bars in (A) and (B) represent 100 and 40 μm, respectively. Relative optical densities of tumor sections from (C) PARP and (D) PCNA after treatment with PBS (as a control), free HCPT, or NG/HCPT. Data are presented as mean ± SD (n = 3; **P < 0.01, ***P < 0.001).
Fortunately, nuclei became darker and pyknotic, causing nuclear fragmentation and disappearance, and various degrees of tumor necrosis in the free HCPT and NG/HCPT groups. The areas of necrosis in the NG/HCPT group were the largest among the three groups, and extensive pyknosis and karyorrhexis were observed, indicating more significant antitumor activity in the group treated with NG/HCPT. In addition, the tumor necrosis areas were calculated with NIS-Elements imaging software (Nikon, NY, USA). It was up to 46.3% ± 2.2% in the NG/HCPT group, which is about 3.8 times larger than that of free HCPT group (P < 0.01). However, there was only 1.5% ± 1.0% in the control group treated with PBS, which was consistent with the in vivo antitumor efficiency.

PARP was one of the essential substrates cleaved by both caspase-3 and caspase-7. The presence of cleaved PARP could further detect DNA strand breaks in many cell types [44]. The expression of cleaved 25 kDa fragments of PARP was measured in bladder sections to further evaluate the tumor apoptosis by immunohistochemistry. As shown in Fig. 7B, more intensive positive signal was obtained in the bladder tissue treated with NG/HCPT compared with that of HCPT-treated one. This effect indicated more cells undergoing apoptosis in the NG/HCPT group than in the HCPT group.

PCNA was a nuclear protein that was expressed in G1-M phases of the cell cycle, but was maximally expressed in late G1-S phases [45]. G1 and G2 cell blocks, which may occur with DNA damage, might have different effects on the presence and intensity of PCNA expression because the maximal expression of the antigen occurred at different points in the cell cycle [45–47]. The expression of PCNA was also upregulated during DNA repair [45], and had been used widely to identify proliferating cells [48,49]. We performed immunohistochemical stains for PCNA in bladder sections to evaluate the cell proliferation status. As shown in Fig. 7B, the strongest and weakest fluorescence intensities occurred in the PBS and NG/HCPT groups, respectively, indicating the fastest and slowest cell growth rate of bladder cancer. All these results clearly demonstrated that NG/HCPT could efficiently deliver HCPT to bladder cancer cells, leading to inhibited tumor cells growth and increased apoptosis level in vivo, which guaranteed the high therapeutic effect in bladder cancer.

To further confirm the immunohistochemical assay, the quantification of PARP and PCNA fluorescence intensities was calculated with a similar method used to calculate mucoadhesiveness. The fluorescence intensity of control group was set as “1”. As shown in Fig. 7C, the order of PARP amounts was as follows: NG/HCPT > free HCPT > PBS (as a control). The signal of NG/HCPT group was 2.2 times higher than that of the free HCPT group. Once again, this result confirmed that more tumor cells were undergoing apoptosis in the NG/HCPT group. As shown in Fig. 7D, the order of PCNA amounts was calculated as follows: PBS (as a control) > free HCPT > NG/HCPT. The signal percentage between the NG/HCPT and free HCPT groups was 11.4%, indicating a slower cell growth in the NG/HCPT group. All of the above results confirmed the strong therapeutic effect of NG/HCPT in bladder cancer.

4. Conclusion

In summary, a reduction-responsive cationic polypeptide nanogel was successfully synthesized through the sequential ROP of ZLL NCA, and LP NCA and LC NCA, and the subsequent deprotection. Compared with free HCPT, the reduction-sensitive property made the nanogel more accurately release HCPT and promote accumulation in T24 cells in vitro. In addition, the positive surface charge, amphipathicity, and LL residues produced the mucoadhesive and permeation properties in vivo. Consequently, the intravesical instillation of NG/HCPT could significantly inhibit tumor growth in an orthotopic bladder cancer model. Certainly, the improved antitumor effect was also due to the high drug loading capability and reduction-responsive release property. This drug carrier could be extended to a variety of hydrophobic antitumor drugs. In summary, in the simple synthesis process and the promising results demonstrate that the smart polypeptide nanogel paves the way for intravesical instillation of chemotherapy for patients with NMIBC.

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Appendix A. Supplementary data

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References


