GRAPHICAL ABSTRACT

Core Cross-linked Poly(ethylene glycol)-*graft*-Dextran Nanoparticles for Reduction and pH Dual Responsive Intracellular Drug Delivery

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ABSTRACT A kind of core cross-linked poly(ethylene glycol)-*graft*-Dextran nanoparticles (CPD NPs) was prepared by a simple chemical cross-linking method for reduction and pH dual response drug delivery. The resultant CPD NPs are of homogeneous spherical structure with sizes from 69 ± 11 to 107 ± 18 nm. Doxorubicin (DOX) was then loaded into the CPD NPs in high efficiency, and showing typical reduction and pH dual responsive release profiles. The flow cytometric analysis and confocal laser scanning microscopy (CLSM) confirmed that the DOX-loaded CPD NPs could be internalized into cancer cell efficiently and release DOX in intracellular environment. Furthermore, cell cytotoxicity assays indicated that the CPD NPs had good biocompatibility toward both cancerous and normal cells, while the Dox-loaded CPD NPs exhibited significant inhibition of cell proliferation in various cancer cells. Therefore, this biocompatible CPD NP may have great potential for intracellular drug delivery in clinical cancer therapy.

KEYWORDS

Drug delivery

Nanoparticles

Reduction-responsive

pH-responsive

Nanomedicine

INTRODUCTION

In the past decades, many kinds of environment-sensitive polymeric nanoparticles (NPs) have been developed as smart nanocarriers for tumor-targeted delivery of anticancer drugs.¹⁻⁴ The preclinical and clinical studies have demonstrated that the polymeric nanocarriers are able to prolong circulation time *in vivo*, target specific tumor tissues via the enhanced permeability and retention (EPR) effect, and reduce side effects of drug, leading to improved therapeutic efficiency.⁵⁻⁷ Due to the distinct tumor microenvironment as compared with normal tissues, the nanocarriers are usually designed to be stable in the blood circulation while releasing the cargos in response to biological stimuli at tumor sites or intracellular space. In these cases, various stimuli-responsiveness, such as pH,⁸⁻¹¹ glutathione (GSH),¹²⁻¹⁶ temperature,^{17, 18} light¹⁹⁻²¹ and enzyme,²²⁻²⁴ have been extensively investigated.²⁵

pH and reduction dual-sensitive nanoparticles (NPs) are the most widely used stimuli-responsive drug carriers for improving intracellular anticancer drug delivery.²⁶⁻³³ On the one hand, the anticancer drugs could be rapidly released from the pH-sensitive NPs in acidic tumor tissues (pH 6.5–6.8), endosomes (pH 5.5–6.5), and lysosomes (4.5–5.5), while keeping constant in bloodstream and normal tissues (pH 7.4).^{31, 34, 35} On the other hand, the concentration of GSH (the most abundant thiol-containing peptide in animal cells) in the cytoplasm (0.5–10 mM) is 100–1000 times higher than that in the cellular exterior (about 2–20 μ M).^{6, 28} Furthermore, there is about 4-fold higher concentration of GSH in tumor tissues than in normal tissues.²⁹ Thus, redox-sensitive nanoparticles for intracellular release of various drugs can be

designed and prepared based on these GSH variations.³⁶⁻³⁸ Up to now, several kinds of pH and redox dual-response nanocarriers had been prepared for intracellular anticancer drug delivery, such as PMAA-based nanogels,³⁹ PDPA/PMAA-based capsules,²⁰ PEG-SS-PTMBPEC-based micelles⁴⁰, and so on. However, there are still many dilemmas in the development of anti-tumor nanocarrier, such as pursuing high drug loading efficiency with low toxic side effect, realizing minimal drug release in normal physiological conditions while quick release under intracellular conditions. For example, the strong shear stress and dilution action in blood circulation usually lead to premature drug release from the polymeric micelles, which thereafter induce severe side effects.⁴¹ To solve this problem, stabilization of nanoparticles by cross-linking has become an effective approach.⁴²⁻⁴⁴ It is vital to choose an adaptive cross-linker because it may directly affect the stability and properties of the obtained core-cross-linked nanoparticles. Recently, cross-linkers bearing disulfide bonds are receiving considerable attentions since that they are stable in the circulation and extracellular tissues owing to the relatively low concentration of GSH, but can immediately degrade in the more reductive intracellular environment.³⁰

Dextran is a family of hydrophilic polysaccharides that has been widely investigated for constructure of nanoparticulate drug delivery systems. Anticancer drug can either be conjugated directly onto the backbone of dextran or be encapsulated in the hydrophobic core of dextran-built micelles for systemic drug delivery.^{45, 46, 47} However, a few examples reported the preparation of core-crosslinked dextran nanoparticles for anticancer drug delivery.^{13, 48, 49} In this

study, a kind of core cross-linked PEG-*graft*-Dex nanoparticles (CPD NPs) was prepared and used for reduction and pH dual responsive intracellular drug delivery (Scheme 1). The obtained CPD NPs were of spherical morphology with stable disulfide-based core cross-linked structure. Doxorubicin (DOX) was then encapsulated into the CPD NPs in high efficiency. And the DOX-loaded CPD NPs exhibited reduction and pH dual responsive drug release profiles *in vitro*. Furthermore, the DOX-loaded CPD NPs were investigated to be efficiently internalized into cancer cell and release DOX payload upon intracellular stimuli, which thus led to high anticancer efficiencies in a number of cancer cells.

MATERIALS AND METHODS

Materials. Poly(ethylene glycol) methyl ether (mPEG2K, $M_n=2000$ g/mol), Succinic anhydride (ScA) and glutathione (GSH) were obtained from Sigma-Aldrich and used as received. Dextran (Dex, $M_n=70$ kDa, Sigma-Aldrich), 3,3'-dithiodipropionic acid (DTPA, Sigma-Aldrich), 4-(dimethylamino)pyridine (DMAP, Sigma-Aldrich), and *N*-(3-(dimethylamino)propyl)-*N*-ethylcarbodiimide hydrochloride (EDC·HCl, GL Biochem Ltd.) were used directly. Doxorubicin hydrochloride (DOX·HCl) was bought from Zhejiang Hisun Pharmaceutical Co., Ltd. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-disphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich. All the other chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and used as obtained.

Instruments and methods. ^1H NMR spectra were collected on a Bruker AV 300 NMR spectrometer in deuteration dimethyl sulfoxide ($\text{DMSO}-d_6$). Fourier transform infrared (FTIR) measurements were performed on a Bio-Rad Win-IR Spectrometer. Transmission electron microscopy (TEM) images were obtained by a JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 KV. The sizes of particles were measured by a WyattQELS dynamic laser scattering (DLS) instrument with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology).

Synthesis of carboxyl-mPEG (PEG-COOH). First, mPEG2K (20 g, 10 mmol) and ScA (5.0 g, 50 mmol) were dissolved in dichloromethane (120 mL). Then, 0.24 g of DMAP (2.0 mmol) was added into the flask. The reaction proceeded at room temperature for 48 h under stirring. After that, the solution was precipitated in 8-fold ethyl ether to yield the crude product. The crude sample was re-precipitated in excess amount of ethyl ether for another two times. The final product was obtained by drying in vacuum for 24 h (yield 78%).

Synthesis of core cross-linked PEG-graft-Dex nanoparticles (CPD NPs). 0.81 g of dextran was firstly dissolved in 20 mL of DMSO with mild ultrasonication for 4h. And then, 2.08 g of PEG-COOH was added. Meanwhile, 0.06 g of DMAP (0.50 mmol) and 1.92 g of EDC·HCl (1.00 mmol) were added into the flask. After stirring at room temperature for 24 h, DTPA was added along with the addition of 0.58 g of

EDC·HCl. After stirring for another 24 h, the above reaction mixture was dialyzed (MWCO: 3500 Da) against deionized water for 3 days. Finally, the solution was lyophilized to obtain the CPD NPs. The feeding molar ratios of dextran to DTPA are listed in Table 1.

***In vitro* drug loading and release.** Doxorubicin (DOX) was used as a model drug for *in vitro* drug loading and release. DOX-loaded CPD nanoparticles were obtained by a simple dialysis method. Briefly, DOX·HCl (5.0 mg or 10.0 mg) and CPD NPs (50.0 mg) were dissolved in 3.0 mL of DMSO. The mixture was stirred overnight and then 6.0 mL of PBS (pH 7.4) was added dropwisely into the solution. Then, DMSO was removed by dialysis against deionized water for 12 h at room temperature (MWCO: 3500 Da). The dialysis was performed in dark and the dialysis medium was renewed five times. The DOX-loaded CPD NPs were obtained by filtering the solution through a 0.45µm filter, followed by lyophilization of the filtered solution. The amount of DOX was quantified via fluorescence measurement by a PTI fluorescence spectrometer (Photon Technology International, U.S.A.) using a standard calibration curve method ($\lambda_{\text{ex}} = 480 \text{ nm}$).⁵⁰ The drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following equations:

$$\text{DLC (\%)} = \frac{\text{weight of drug in nanoparticle}}{\text{total weight of drug-loaded nanoparticle}} \times 100$$

$$\text{DLC (\%)} = \frac{\text{weight of drug in nanoparticle}}{\text{total weight of drug in feed}} \times 100$$

In vitro DOX release profiles were investigated in PBS at pH 5.5, 6.8, and 7.4 with or without the presence of GSH, respectively. The freeze-dried DOX-loaded NPs (2.0 mg) were dissolved in 3.0 mL of PBS with 0 or 10 mM GSH, and then the solutions were immediately transferred into dialysis bags (MWCO: 3500 Da). The end-sealed dialysis bag was placed in 100 mL of PBS containing 0 or 10 mM GSH at 37 °C under a constant shaking at 75 rpm. At chosen time intervals, 2.0 mL of external release medium was collected, and an equivalent volume of fresh medium was added. The amount of released DOX was analyzed by fluorescence measurements ($\lambda_{\text{ex}} = 480 \text{ nm}$) using a standard curve method. The measurements were carried out in triplicate.

Confocal laser scanning microscopic observation. The intracellular release behaviors of DOX-loaded nanoparticles were monitored by confocal laser scanning microscopy (CLSM) using a MCF-7 cell line. MCF-7 cells were seeded in a 6-well plates (1×10^5 cells per well) in 2.0 mL of complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, supplemented with 50 U mL⁻¹ penicillin and 50 U mL⁻¹ streptomycin, and cultured at 37 °C in 5% CO₂ atmosphere. After incubation for 24 h, the culture medium was removed. And then, the cells for the GSH pretreated group were treated with 10 mM GSH for additional 2 h. Cells were washed twice by PBS and incubated at 37 °C for another 2 h or 6 h with culture media containing DOX-loaded nanoparticles at a final DOX concentration of 10 mg L⁻¹ in complete DMEM. Cells without GSH treatment were used as control. Then, the culture medium was withdrawn and cells were washed three times with PBS. After that, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with PBS thrice. For staining the cell nuclei, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI, blue) for 5 min. CLSM images of cells were observed through a confocal microscope (Zeiss LSM 780).

Flow cytometric analysis. A549 and MCF-7 cells were used for analyzing the cellular uptake of DOX-loaded nanoparticles by flow cytometric analysis. Firstly, the cells were seeded in a 6-well plates at a final density of 3×10^5 cells per well in 2 mL of complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, supplemented with 50 U mL⁻¹ penicillin and 50 U mL⁻¹ streptomycin, and cultured at 37 °C in 5% CO₂ atmosphere for 24 h. And then the culture medium was removed, and the cells for the GSH pretreated group were treated with 10 mM GSH for 2h. Cells were washed by PBS twice and supplied with new culture media and DOX-loaded nanoparticles at 37 °C for additional 3 h or 6 h. The final concentration of DOX was 10 mg L⁻¹. The cells without pretreatment were used for comparison. Thereafter, the culture medium was removed and the cells were washed with PBS three times and treated with trypsin. Then, 1.0 mL of PBS was added to each well, and the solutions were centrifuged at 1000 rpm for 5 min. Finally, after removal of the supernatants, the cells were resuspended in 0.3 mL of PBS. The

analysis was performed by using a flow cytometer (Beckman, California, U.S.A.) for 1×10^4 cells.

Cell viability assays. The relative cytotoxicities of the DOX-free core cross-linked PEG-*graft*-Dex nanoparticles (CPD NPs) against L929, HepG2, A549, MCF-7, C26 and B16F10 cells were evaluated *in vitro* by a standard MTT assay. Generally, the cells were seeded into 96-well plates (7000 cells/well) in 180 μ L of DMEM with 10% fetal bovine serum, supplemented with 50 U mL^{-1} penicillin and 50 U mL^{-1} streptomycin, and then incubated at 37 °C under 5% CO_2 atmosphere for 24 h. 20 μ L of DOX-free nanoparticles solutions in complete DMEM at different concentrations (0–10 g L^{-1}) were added. The MTT assay was in progress at 37 °C for another 48 h. Then, MTT solution (5.0 mg/mL, 20 μ L) was added into each well and incubated continually for another 4 h. The culture medium was withdrawn, and 150 μ L of DMSO was added into each well to dissolve the formazan. The absorbency of the solution was measured on a Bio-Rad 680 microplate reader at 490 nm.⁵¹ Cell viability (%) was calculated based on following equation:

$$\text{Cell viability (\%)} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Where A_{sample} represents the absorbency of the sample wells, and A_{control} represents the absorbency of control wells.⁵²

The cytotoxicities of DOX-loaded nanoparticles were also tested by using a MTT assay against HepG2, A549, MCF-7, C26 and B16F10 cells. Similarly, the cells were seeded in 96-well plates at a density of 7000 cells per well in 180 μ L of DMEM with 10% fetal bovine serum, supplemented with 50 U mL^{-1} penicillin and 50 U mL^{-1} streptomycin, and further incubated at 37 °C for 24 h under 5% CO_2 atmosphere. 20 μ L of DOX-loaded nanoparticles with different DOX concentrations were then added, and the cells were incubated for another 48 h. The MTT assay was conducted similarly to the method described above for MTT assay of the DOX-free CPD NPs.

RESULTS AND DISCUSSION

Synthesis and characterization of CPD nanoparticles. Poly(ethylene glycol) methyl ether and dextran have been widely used in drug delivery system owing to their excellent biocompatibility.^{22, 31, 36, 51} Herein, the PEG grafted dextran (PD) was firstly synthesized by chemical reaction of PEG-COOH with dextran using EDC·HCl and DMAP (Scheme 1) as the coupling reagents. Then, the CPD nanoparticles with different degrees of core-crosslinking densities were prepared by cross-linking with various amounts of 3,3'-dithiodipropionic acid (DTPA) (Table 1).

The chemical structures of CPD NPs were firstly characterized by ¹H NMR (Fig 1) and FTIR (Fig S2). In ¹H NMR spectra, the resonance peaks at 3.51 and 3.23 ppm were ascribed to the methylene protons and terminal methyl protons of mPEG, respectively. The multiple peaks from 4.5 to 5.0 ppm corresponded to the protons of glucose unit in dextran. The peaks at 2.62 and 2.88 ppm were ascribed to protons from DTPA. As shown in Fig 1 and Fig S1, the spectra of CPD NPs show all essential peaks of mPEG, dextran and DTPA, which confirmed the successful preparation of CPD NPs. Additionally, the FT IR spectra (Fig S2) revealed that the PEG and DTPA have been successfully conjugated with dextran. The formation of CPD NPs was further analyzed by DLS (Table 1 and Fig 2A). It is observed that the size of nanoparticle would increase slightly with the increased amount of added cross-linker.

To further demonstrate the core cross-linked structure of CPD NPs, the size changes of nanoparticles after dilution with 10-fold *N,N*-Dimethylformamide (DMF) were measured.⁵³⁻⁵⁵ As shown in Fig 2A, the CPD NPs dissolved in DMF could

maintain their intact structures, however, their sizes increased significantly as compared to those dissolved in deionized water. This data confirm the core cross-linked structure of the resultant CPD NPs, which underwent swelling of core upon addition of DMF. Additionally, the size of CPD **1** NPs rose from 69 ± 11 to 304 ± 24 nm, while the sizes of the other three CPD NPs increased from around 100 to 200 nm.⁵⁵ This obvious difference between CPD **1** and the others demonstrated that the structure of CPD NPs would become sufficiently stable once the feed molar ratio of DTPA to repeating units of dextran reached 20%. Furthermore, the TEM image also indicated that the obtained CPD NPs were of spherical structure in aqueous media (Fig 2C).

Doxorubicin (DOX), a commonly used anti-cancer drug, was then loaded into nanoparticles. In Fig 2B, the size of drug-loaded nanoparticles became bigger than DOX-free nanoparticles, probably owing to the loading of DOX (Table 1).³⁹ The similar results can also be seen from TEM measurements (Fig 2C–E). The sizes of blank CPD **1**, CPD **1** with 3.61 wt% DOX, and CPD **1** with 6.89 wt% DOX were around 65, 120 and 150 nm, respectively, while their corresponding diameters were 69 ± 11 , 138 ± 21 and 177 ± 18 nm by DLS measurements. The sizes detected by DLS were larger than TEM results, which is possibly due to the dehydration of the nanoparticles in the TEM sample preparation process.

***In vitro* stimuli-responsive drug release.** The DOX was loaded into CPD NPs by using a typical dialysis method.^{8, 12} With addition of 10 wt% DOX, the drug loading contents (DLC) and drug loading efficiencies (DLE) of CPD NPs were detected to be

3.61–4.00% and 39.65–43.95% respectively (Table 1). By contrast, the DLC and DLE of CPD NPs with 20 wt% feeding DOX increased to 6.89–8.65% and 41.32–51.85%, respectively.^{41, 56} These results suggested that the DLC and DLE had a slight increase with the increasing amount of DTPA. That is to say, adding more cross-linker can raise DLC and DLE, which is consistent with the previous report.⁵⁰

The drug release profiles of DOX-loaded CPD NPs were then performed at different pHs with or without the presence of GSH, and the typical results are showed in Fig 3. As shown in Fig 3A, the release of DOX was suppressed with a released amount of 19.8% at pH 7.4 without GSH. Upon addition of 10 mM GSH, rapid drug release was observed with 47.5% of DOX released from the CPD NPs, which is due to the cleavage of disulfide bonds and subsequent disintegration of CPD NPs (Fig S3). In the absence of GSH, the release of DOX was also influenced by the pH value. Obviously, lower pH can promote the release of DOX from CPD NPs, which is probably due to the protonation of the amino groups of DOX and the disassociation of the electronic interaction between DOX and residue carboxyl group in DTA cross-linker at pH 5.5.⁵⁷ Similar drug release profiles were observed in DOX-loaded CPD 4 (containing 8.65 wt% DOX, Fig 3B) and DOX-loaded CPD 2 (containing 3.69 wt% DOX, Fig S4). Therefore, these results indicated that the synthesized CPD NPs can serve as pH and reduction dual response carriers for anti-cancer drug delivery.

Intracellular drug release. The cellular internalization and intracellular drug release behaviors of DOX-loaded nanoparticles were then investigated in A549 and MCF-7 cells by using flow cytometric analysis (FCA), and the results are showed in Fig 4.

After incubation for 3 h, the group incubated with DOX-loaded CPD NPs showed the lowest fluorescence intensity while significantly enhanced fluorescence was observed in the group pretreated with 10 mM GSH, indicating that the reducing conditions could enhance the release of DOX in intracellular compartment. Moreover, the group of free DOX showed even stronger fluorescence intensity either in the presence or absence of 10 mM GSH. This is probably ascribed to the intensive stability of cross-linked structure and the controlled release pattern of DOX-loaded CPD NPs.^{12, 36} To further confirm the controlled release behaviors of DOX from DOX-loaded CPD NPs, cells were incubated for a longer time (6 h). Compared to cells incubated for 3 h, obvious improvement of fluorescence intensity was noticed after incubation for 6 h, which revealed the gradual and sustained release of DOX.^{4, 6}

Similar to the FCA results, confocal laser scanning microscopy (CLSM) images show that cells with GSH pretreatment exhibit stronger DOX fluorescence intensity than that of cells without GSH pretreatment (Fig 5). The quantitative mean fluorescence intensities of DAPI and DOX in MCF-7 cells at 2 h and 6 h were measured and listed in Table 2. The mean fluorescence intensities of DAPI in all the tested groups were between 253.8 ± 4.6 and 255.0 ± 0.2 , which was almost the same. Similarly, the mean fluorescence intensities of DOX in the free DOX treated groups were almost constant in the absence or presence of GSH and no marked changes were observed when extending the incubation time to 6 h. By contrast, the fluorescence intensities of DOX in the DOX-loaded CPD 4 treated groups were 181.3 ± 44.4 and 226.8 ± 27.7 at 2 h for MCF-7 cells with and without GSH pretreatment, respectively,

and these fluorescence intensities would further increase to 240.9 ± 21.9 and 252.6 ± 7.7 for MCF-7 cells with and without GSH pretreatment, respectively, when incubating for 6 h. In general, all the above results proved that the DOX was gradually released from CPD NPs triggered by the reductive GSH.

***In vitro* cytotoxicities of blank and DOX-loaded CPD NPs.** The *in vitro* cytotoxicity of CPD NPs was evaluated by MTT assay in L929 (murine fibroblasts), HepG2 (human hepatoma cells), A549 (human lung cancer cells), MCF-7 (human breast cancer cells), C26 (murine colon cancer cells), and B16F10 (murine melanoma cells) cells. As shown in Fig S5, the viabilities of all tested cells kept over 85% after incubation with CPD NPs at different concentrations up to 1000 mg L^{-1} for 48 h. It demonstrated that CPD NPs did harmless to human or murine cell lines. The low cytotoxicity and good biocompatibility of CPD NPs are essential for anti-cancer drug delivery *in vivo*.

The *in vitro* cytotoxicities of DOX-loaded NPs were then evaluated by MTT assay against HepG2, A549, MCF-7, C26, and B16F10 cells. As typically shown in Fig 6, the cell cytotoxicities of DOX-loaded CPD NPs were almost consistent with that of free DOX. The IC_{50} of MCF-7 cells treated with DOX-loaded CPD 1–4 NPs (with 10 wt% feeding DOX) were 0.47, 0.46, 0.52 and 0.47 mg L^{-1} , respectively, which were close to that of free DOX group (0.55 mg L^{-1}). Furthermore, the viabilities of MCF-7 cells treated with DOX-loaded CPD NPs at 48 h were significantly decreased as compared to those at 24 h, indicating that the DOX could be gradually released in intracellular environment. The similar results were observed in A549, HepG2, C26

and B16F10 cells (Fig S6, S7 and Table 3). In sum, these results indicated that the CPD NPs possessed good biocompatibility and could be used for intracellular delivery of anticancer drug in various cancer cells.

CONCLUSIONS

In this work, disulfide core-linked PEG-*graft*-Dextran nanoparticles (CPD NPs) were firstly prepared by a simple chemical cross-linking reaction. The synthesized nanoparticles are spherical and monodispersed with high stable core cross-linked structure. Drug-loaded CPD NPs can release DOX in response to acidic pH or reduction environment.^{55, 58} Meanwhile, the DOX-loaded NPs exhibited high cytotoxicities in various types of cancer cells. In general, this work provides a facile method for preparation of stable core-crosslinked nanoparticles. The dense PEG surface and stable core-crosslinked structure would ensure the prolonged circulation time of as-prepared nanoparticles *in vivo*.^{59, 60, 61} Furthermore, the disulfide-based cross-linker also endows the as-prepared nanoparticles with triggered release property in reductive intracellular compartment. Nevertheless, a slight burst release of DOX was observed in the initial of drug release *in vitro*. Thus, future development will focus on enhancing the stability of DOX in nanoparticles by either direct conjugation of DOX or addition of hydrophobic moieties onto the dextran backbone.

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ALL FIGURE CAPTIONS

Scheme 1. Preparation of core cross-linked nanoparticles with pH and redox dual sensitivity.

Fig 1. ^1H NMR spectra of PEG-COOH, dextran, DTPA, and CPD **1** in $\text{DMSO}-d_6$.

Fig 2. Size and morphologies of CPD NPs with or without loading of DOX. (A) Diameters of CPD NPs dissolved in deionized water and DMF, respectively. (B) Diameters of CPD NPs (a), CPD NPs with 10 wt% feeding DOX (b), and CPD NPs with 20 wt% feeding DOX (c). TEM micrograph of CPD 1 (C), CPD 1 with 10 wt% feeding DOX (D), and CPD 1 with 20 wt% feeding DOX (E).

Fig 3. The drug release behavior of CPD 4 with 4.00 wt% DOX (A) and 8.65 wt% DOX (B) in the absence or presence of 10 mM GSH at pH 5.5 and 7.4.

Fig 4. Flow cytometric profiles for A549 and MCF-7 cells incubated with CPD **4** (containing 4.00 wt% DOX) (a), CPD **4** (containing 4.00 wt% DOX) plus pretreatment with 10mM GSH (b), free DOX (c), free DOX plus pretreatment with 10 mM GSH (d).

Fig 5. Representative CLSM images of MCF-7 cells incubated with CPD 4 (containing 4.00 wt% DOX), CPD 4 (containing 4.00 wt% DOX) plus pretreatment with 10mM GSH, free DOX, and free DOX plus pretreatment with 10 mM GSH for 2 h or 6 h, respectively. For each panel, the images from left to right show cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), differential interference

contract (DIC) images, overlays of the three images, and the 2.5 D images. The scale bars are 50 μm . DOX dosage was set at 10 mg L^{-1} .

Fig 6. *In vitro* cytotoxicity of CPD 1 (containing 6.89 wt% DOX) (a), CPD 2 (containing 7.52 wt% DOX) (b), CPD 3 (containing 8.64 wt% DOX) (c), CPD 4 (containing 8.65 wt% DOX) (d), and free DOX (e). MCF-7 cells were incubated for 24 h (A) and 48 h (B), respectively.

ALL TABLES

nanoparticles	Feeding molar ratio ^a	Size ^b (nm)	PDI	Feeding mass ratio ^c =10 : 1				Feeding mass ratio ^c =5 : 1			
				Size ^b (nm)	PDI	DLC(%)	DLE(%)	Size ^b (nm)	PDI	DLC(%)	DLE(%)
CPD 1	10 : 1	69±11	0.025	138±21	0.023	3.61	39.65	177±18	0.018	6.89	41.32
CPD 2	10 : 2	70±17	0.036	142±18	0.016	3.69	40.58	176±13	0.016	7.52	45.09
CPD 3	10 : 3	93±13	0.019	151±13	0.016	3.73	41.08	191±13	0.034	8.64	51.84
CPD 4	10 : 5	107±18	0.028	148±12	0.047	4.00	43.95	164±16	0.031	8.65	51.85

^aFeeding molar ratio of the repeating units of dextran and DTPA. ^bDiameters of CPD NPs determined by DLS at pH = 7.4. ^cFeeding mass ratios of CPD NPs and drug (DOX).

Table 1. Characterizations of CPD and DOX-loaded CPD nanoparticles.

	2 h		6 h	
	DAPI ^b	DOX ^c	DAPI ^b	DOX ^c
CPD 4 ^a	254.8±1.7	181.3±44.4	255.0±0.0	240.9±21.8
CPD 4 ^a + 10 mM GSH	255.0±0.0	226.8±27.7	255.0±0.2	252.6±7.7
DOX	254.8±1.9	252.3±8.5	255.0±0.1	249.7±12.2
DOX + 10 mM GSH	253.8±4.6	250.3±11.1	255.0±0.0	253.3±6.5

^aCPD **4** nanoparticles with 10 wt% feeding DOX. ^bMean fluorescence intensity of DAPI. ^cMean fluorescence intensity of DOX.

Table 2. Quantitative mean fluorescence intensity of DAPI and DOX in CLSM

images of MCF-7 cells at 2 h or 6 h.

entry	IC ₅₀ (mg/L)						
	HepG2 ^c	HepG2 ^d	MCF-7 ^c	MCF-7 ^d	A549 ^d	C26 ^d	B16F10 ^d
CPD 1 ^a	5.93	0.97	2.96	0.47	1.18	0.64	0.13
CPD 2 ^a	7.13	1.13	2.44	0.46	0.79	0.43	0.14
CPD 3 ^a	8.69	0.89	2.33	0.52	1.01	0.53	0.11
CPD 4 ^a	8.72	1.33	2.28	0.47	1.12	0.67	0.10
CPD 1 ^b	—	—	—	0.65	1.23	0.63	0.08
CPD 2 ^b	—	—	—	0.60	1.28	0.66	0.08
CPD 3 ^b	—	—	—	0.64	1.66	0.69	0.08
CPD 4 ^b	—	—	—	0.57	1.77	0.67	0.08
free DOX	14.11	1.49	2.27	0.55	1.28	0.86	0.09

^aNanoparticles loaded 10% DOX. ^bNanoparticles loaded 20% DOX. ^cCells treated by free DOX and DOX-loaded nanoparticles at 24 h. ^dCells treated by free DOX and DOX-loaded nanoparticles at 48 h.

Table 3. IC₅₀ Values for free DOX and DOX-loaded CPD NPs in different cancer cells measured by MTT assays.