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Dextran–platinum(IV) conjugate as reduction-responsive carrier for triggered drug release

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Reduction-responsive nano-carriers have been confirmed to be promising for intracellular drug delivery. To develop multifunctional polymer-based drug delivery system, a novel Dextran–Pt(IV) conjugate was synthesized by conjugating Pt(IV) to the side chains of the hydrophilic dextran and used for doxorubicin (DOX) delivery. Pt(IV) conjugation could change the hydrophilicity of dextran, leading to the self-assembly of Dextran–Pt(IV) conjugates with different morphologies. Pt(IV) segments served as the key components in assembly formation and the antitumor prodrug. Under reductive environment, Pt(IV) was found to be reduced to its active Pt(II) form and cleaved from dextran, shifting the hydrophilic-hydrophobic balance of Dextran–Pt(IV) conjugate. The collapse of the assembly structure due to the partially or completely recovered hydrophilicity of dextran led to triggered release of DOX. By the combination of the released hydrophobic DOX and recovered hydrophilic Pt(II), DOX-loaded Dextran–Pt(IV) conjugate was found to be very effective for antitumor aim as demonstrated in *in vitro* cytotoxicity evaluation. This DOX-loaded Dextran–Pt(IV) conjugate system provided a new strategy to trigger release of hydrophobic and hydrophilic drugs at the same time via the single reduction-responsive control to gain the enhanced anti-tumor effect.

Introduction

In recent years, polymer-based drug delivery systems (PDDSs) have been expected to possess enhanced clinical applications due to their fine features including tunable sizes, prolonged drug circulation time as well as performable drug loading efficiency for hydrophobic drugs and so on.[1] Considerable efforts have been made to explore desirable PDDSs which can be stable in blood circulation and perfectly control drug release at tumor sites and in cancerous cells.[2] Many stimuli including enzymes, pH, redox and light have been employed to realize programmable drug release for PDDSs with stimuli-responsive linkage in polymer backbone or on side chains.[3] Typically, upon exposure to stimuli, PDDSs with responsive linkage in the backbone could undergo fast disintegration, and PDDSs with responsive linkage on the side chains could shift hydrophilic-hydrophobic balance (HHB).[4] For instance, Wu et al. reported micelles containing adenine and tertiary amine units with pH-responsive merits. In cancerous cells, acidic pH could change the HHB of micelles by protonation of tertiary amines, causing quick release of anticancer drug to obtain an enhanced *in vitro*

cytotoxicity.[5] Zhao et al. developed many photo-responsive block co-polymers containing nitrobenzyl on the side chains, and the dissociation of hydrophobic nitrobenzyl could shift the HHB of these polymers for desirable application.[6]

Among the above stimuli, reduction-responsiveness is particularly promising, because the level of reductants involving glutathione (GSH) and ascorbate in cancerous cells are largely higher than that in blood circulation.[7,8] Cleavage of reduction-responsive groups due to the high reductant concentration in cancerous cells could be used to trigger the disintegration or disassembly of PDDSs for rapid drug release. Several strategies have been reported to fabricate intelligent reduction-responsive PDDSs by using disulfide or diselenide linkage.[7,9] Oh et al. reported a copolymer consisting of pendant disulfide-labeled methacrylate polymer block.[10] In response to intracellular reductants, the pendant units could be cleaved from the polymer to change the HHB of the aggregates, resulting in burst release of the encapsulated drug. Zhang et al. reported a dual-redox responsive polyurethane system containing diselenide linkage in the backbone.[11] Compared with disulfide linkage, diselenide was proven to be more sensitive to reductive environment for triggered dye or drug release. However, several limitations of the above mentioned reduction-responsive PDDSs are still remained, such as lacking of multifunction and inability to carry more than one drug, especially for the transportation of a hydrophobic and a hydrophilic drug together for more effective combination therapy.

Platinum(II) based drugs have been widely used in clinical trials for cancer therapy, accompanying with severe side effects including nephrotoxicity, hearing loss and drug resistance.[12] As prodrug of

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hydrophilic Pt(II), the hydrophobic Pt(IV) has higher stability and lower cytotoxicity, which could be reduced to its active Pt(II) form in response to intracellular reductants at tumor site.[13] In our previous works, Pt(IV) complexes were conjugated to amphiphilic copolymer as reduction-responsive prodrugs to minimize their defects such as short blood circulation time and less uptake efficacy into aimed cells.[14, 15] In Xiao's report, Pt(IV) was bound to a tri-block copolymer through condensation between carboxyl group of Pt(IV) and pendant amine groups on the polymer, and the obtained conjugate exhibited higher cytotoxicity than cisplatin(II).[15] A polymer–(tandem drug) conjugate system was developed by Zhou et al. through conjugation a tandem-drug (Pt and demethylcantharidin) with biodegradable block polymer. The polymer conjugate also displayed enhanced cytotoxicity *in vitro* and *in vivo*.[16] From the above systems, two important characteristics brought inspiration for our new design of a unique reduction-responsive PDDS. At first, Pt(IV) itself is hydrophobic which can be used as the hydrophobic segment to construct the total structure of an amphiphilic copolymer. Meanwhile, like disulfide linkage, Pt(IV) is also reduction-responsive and could be reduced to hydrophilic Pt(II) and cleaved from the copolymer structure to recover the hydrophilic character of polymer, and the released Pt(II) further works as the active anticancer drug inside cancer cells.
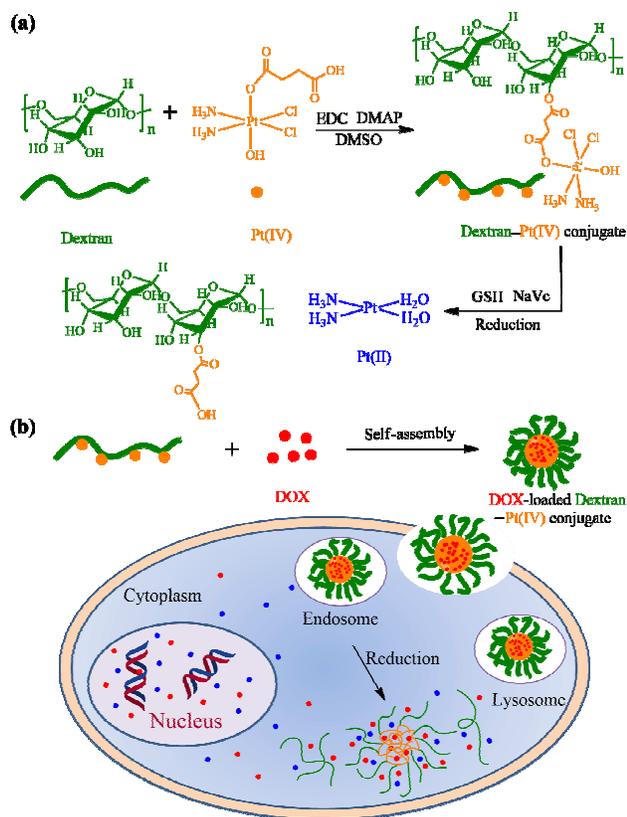
Scheme 1 (a) Synthesis and reduction reaction of Dextran–Pt(IV) conjugate, (b) intracellular reduction-responsiveness of DOX-loaded Dextran–Pt(IV) conjugate for triggered drug release in cancer cell.

Hence, in this report, we constructed an amphiphilic Dextran–Pt(IV) conjugate by grafting Pt(IV) complex to the side chains of

dextran (Scheme 1a). The polymer-drug conjugate was further used as a reduction-responsive PDDS for triggered doxorubicin (DOX) release. By changing Pt(IV) grafting ratios, the Pt(IV) conjugated to hydrophilic dextran played the role of a hydrophobic unit to enable the macromolecule conjugate to self-assemble into different morphologies. In cancer cell, Dextran–Pt(IV) conjugate could shift its HHB responsively under the condition of intracellular reductants by partial or complete cleavage of the pendant Pt(IV) to Pt(II), leading to the disintegration of the assembly and the triggered release of the encapsulated DOX. Meanwhile, the released active Pt(II) would express additional antitumor efficiency. This newly developed PDDS could make full use of the desirable multi-roles of Pt(IV) complex (hydrophobic unit, reduction-responsive group and prodrug). It should be a simple strategy to co-deliver hydrophobic and hydrophilic drugs for potential combination therapy (Scheme 1b).

Experimental

Materials

Dextran with an average molecular weight of 10000 Da (Dextran$_{10k}$), succinic anhydride, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl), 4-Dimethylaminopyridine (DMAP) and sodium ascorbate (NaVc) were purchased from Sigma-Aldrich. Cisplatin (purity 99%) was bought from Shandong Boyuan Chemical Company, China. Doxorubicin (DOX) (purity 99%) was bought from Hisun Pharmaceutical Limited Company, Zhejiang, China. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Dimethyl sulphoxide (DMSO) was distilled before use after dried with calcium hydride for 72 h.

General measurements

^{1}H-NMR spectra were recorded by a Unity-300 MHz NMR spectrameter (Bruker) (DMSO-d$_6$ as the solvent) at room temperature. Mass Spectroscopy (ESI-MS) measurements were conducted by a Quattro Premier XE system (Waters) equipped with an electrospray interface (ESI). The platinum content was determined by Inductively Coupled Plasma Optical Emission Spectrameter (ICP-OES, iCAP 6300, Thermoscientific, USA) and Inductively Coupled Plasma Mass Spectrameter (ICP-MS, X series II, Thermoscientific, USA). Fourier Transform Infrared (FT-IR) spectra were recorded using a Bruker Vertex 70 spectrameter. UV-visible electronic absorption spectra were measured on a Varian Cary 300 UV-visible spectrophotometer in 1 cm path-length cuvettes. Diameters were performed by dynamic light scattering (DLS) with a Brookhaven 90Plus size analyzer. Transmission electron microscopy (TEM) images were recorded on a JEOL JEM-1011 electron microscope.

Preparation and characterization of Dextran–Pt(IV) conjugate

Synthesis of Pt(IV) complex. *c,c,t*-[Pt(NH$_3$)$_2$Cl$_2$(OH)$_2$] was synthesized and the data was presented in the supporting

information.[15,17,18] Pt(IV) was synthesized according to reference.[15,17,18] To a suspension of *c,c,t*-[Pt(NH₃)₂Cl₂(OH)₂] (0.334 g, 1 mmol) in DMSO (5 mL) was added an equivalent succinic anhydride (0.1 g, 1 mmol). The solution was stirred at 30 °C for 12 h. Then the solution was precipitated in ethyl ether, and the light yellow precipitate was dried (Scheme S1). ^{1}H NMR: (300 MHz, DMSO-d₆, 25 °C): δH 2.33-2.39 (4H, m, -OC*H₂CH₂*C-), 5.8-5.95 (6H, m, NH₃); IR: cm^{-1} 3643 (br, OH), 1718 (sh, *CO*-OH), 1642 (sh, -PtO*OC*-C-), 538 (sh, Pt-OH); ESI-MS: (negative mode) m/z 433 [M-H]$^-$ (Fig. 1, Fig. 2, Fig. S2).

Synthesis of Dextran–Pt(IV) conjugate. A solution of dextran (100 mg, 0.01 mmol) and Pt(IV) (217 mg, 0.5 mmol) in anhydrous DMSO (10 mL) was added with DMAP (61 mg, 0.5 mmol) and EDC·HCl (958.5 mg, 5 mmol). Then, the reaction mixture was stirred at room temperature for 24 h.[19,20] After that, the solution was dialyzed (MWCO = 3500) against distilled water for 72 h to remove DMSO and un-reacted Pt(IV) and then freeze-dried for storage. The Pt content of Dextran–Pt(IV) conjugate was measured by ICP-OES.

Determination of the critical micellar concentration (CMC) of Dextran–Pt(IV) conjugate

The CMC of Dextran–Pt(IV) conjugate was determined using pyrene as a hydrophobic probe by fluorescence measurement. Every prepared glass vial was added the predetermined amount of pyrene (3.6×10^{-6}, 0.5 mL) in acetone. After the acetone was evaporated, Dextran–Pt(IV) conjugate in water (3 mL) with various concentrations was added. The mixtures were shaken with a homothermal incubator at 37 °C at a shaking speed of 100 rpm for 3 h. The fluorescence emission of pyrene was fixed at 391 nm, and the excitation wavelength was fixed at a wavelength range of 300-360 nm and recorded with a scan speed of 500 nm/min.[21,22]

Encapsulation of DOX into Dextran–Pt(IV) conjugate

DOX·HCl (20 mg) was first treated with triethylamine in DMSO (1 mL) for 0.5 h. The obtained DOX solution was mixed with Dextran–Pt(IV) conjugate (100 mg) in DMSO (2 mL) and stirred for 3 h. The mixture solution was added into 50 mL of deionized water dropwisely within 5 min and stirred continuously for 3 h to form stable DOX-loaded Dextran–Pt(IV) conjugate micelles. Then the resultant solution was dialyzed (MWCO = 3500) against distilled water for 36 h to remove DMSO and free DOX. After that, the dialysis solution was lyophilized for further analysis and usage.[4,16]

For DOX content evaluation, DOX-loaded Dextran–Pt(IV) conjugate (1 mg) was dissolved in DMSO (1 mL) for UV measurement. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated using equations below.

DLC (%) = [DOX weight in nanoparticles/total weight of nanoparticles] × 100

DLE (%) = [DOX weight in nanoparticles/initial feeding amount of DOX] × 100

In vitro drug release studies

Briefly, 4 mg of DOX-loaded Dextran–Pt(IV) conjugate was dissolved in 2 mL of the corresponding release solution and sealed into a dialysis bag (MWCO = 3500). The dialysis bag was immersed into 20 mL of the release solution including 5 mM of sodium ascorbate solution, 0.1 mM of sodium ascorbate solution, PBS (pH 7.4) and acetate buffer solution (pH 5.0). The experiment was conducted in a shaking incubator maintaining temperature at 37 °C with a shaking speed of 100 rpm. At predefined time intervals, 2 mL of the outside solution was withdrawn and replaced with the same volume of fresh solution. DOX content in the releasing medium was determined by UV-vis spectra, and Pt content was determined by ICP-MS.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Human hepatoma carcinoma HepG2 cells and human breast carcinoma MCF-7 cells were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, and maintained in DMEM (10% fetal bovine serum, 5% CO₂ at 37 °C). Cells were seeded in 96-well plates (5×10^3 per well in density) with 100 μL of DMEM medium for 24 h. Then 100 μL of the fresh media containing Dextran, Cisplatin, Pt(IV), DOX, Dextran–Pt(IV) conjugate, DOX-loaded Dextran–Pt(IV) conjugate, DOX + Pt(IV) and DOX + cisplatin were added with the same Pt content and the same DOX content. After incubation for 48 h and 72 h, 20 μL of MTT solution in PBS (5 mg/mL) was added and continuously incubated for 4 h. Then the medium containing MTT was removed, and DMSO (150 μL) was added to every well to dissolve the product crystals. After that, the absorbance was determined at 490 nm with microplate reader followed by shaking for 10 min.

Cellular uptake by confocal microscopy and flow cytometry analysis

HepG2 cells were grown in a 6-well plate at a density of 5×10^4 cells with DMEM medium containing 10% fetal bovine serum and incubated for 24 h. For the DOX-loaded Dextran–Pt(IV) conjugate group, two wells were pretreated with 10 mM glutathion (GSH) for two hours. The medium was removed and washed three times with PBS, and the other four wells were left untreated as control. Free DOX and DOX-loaded Dextran–Pt(IV) conjugate in DMEM were added to make the final DOX content at 3 μg/mL. After 0.5 and 4 h incubation, cells was fixed with 4% formaldehyde for 25 min followed by washed with PBS for three times and stained the nuclei with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI). The final observation was conducted using a laser scanning confocal microscopy (Zeiss, LSM 710). And the cellular uptake of free DOX and DOX-loaded Dextran–Pt(IV) conjugate was quantitatively determined by flow cytometry analysis.

Results and discussion

Synthesis and characterization of Dextran–Pt(IV) conjugate

For chemical grafting of Pt(IV) to polymer chain, Pt(IV) was first obtained according the references.[18] The structure of Pt(IV) was confirmed by ^{1}H NMR, FT-IR and ESI-MS (Fig. 1a, Fig. 2b and Fig. S2). Chemical shift at 5.95 ppm (Fig. 1a) was assigned to NH₃ ligand of Pt(IV), and chemical shift at 2.4 ppm belonged to the

methylene protons of succinate ligand, demonstrating the successful synthesis of Pt(IV).[18,23] Dextran–Pt(IV) conjugate was prepared using the condensation reaction between the carboxyl group of Pt(IV) and the pendant hydroxyl groups on dextran (Scheme 1a). As shown in ^{1}H NMR spectra, chemical shifts of dextran were found at 3.0-3.8 ppm and 4.3-5.0 ppm, and the intrinsic peaks of Pt(IV) were appeared at 2.4 ppm and 6.4 ppm (Fig. 1c).[24,25] The total synthesis was further confirmed by FT-IR analysis. As shown in Fig. 2, the weakened absorption band at 538 cm^{-1} and the newly appeared peaks at 1642 and 1718 cm^{-1} clearly suggested the successful synthesis of Pt(IV). After Pt(IV) conjugation, the characteristic peak of dextran at 1655 cm^{-1} still remained, and the peaks at 1732 cm^{-1} derived from Pt(IV) appeared.[26,27]
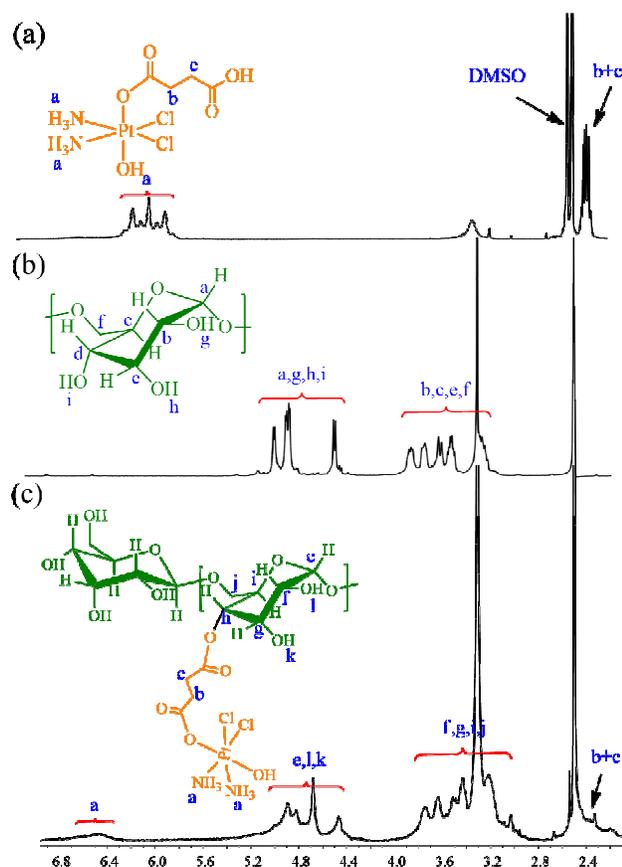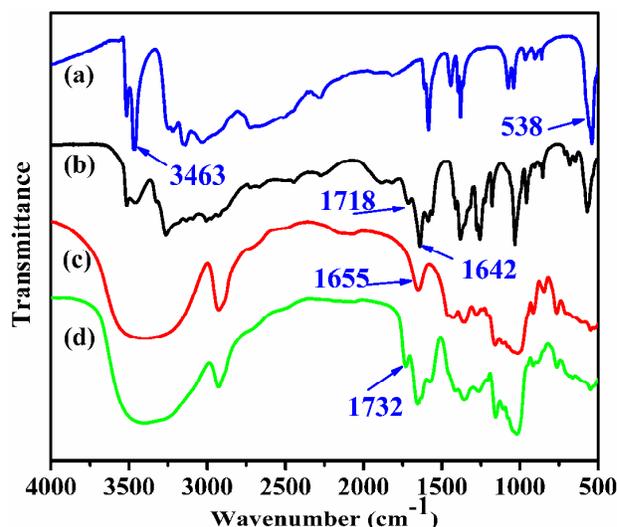

Fig. 2 FT-IR spectra of (a) c,c,t-[Pt(NH$_3$)$_2$Cl$_2$(OH)$_2$], (b) Pt(IV) complex, (c) Dextran and (d) Dextran–Pt(IV) conjugate.

The grafting ratio of Pt(IV) or the Pt content in Dextran–Pt(IV) conjugate could be regulated by varying the feeding amount of Pt(IV), which was further detected by ICP-OES.[28-30] As shown in Table 1, Pt content in Dextran conjugate increased nearly linearly with the increase of the feeding amount of Pt(IV) (Fig. S3). The average number of Pt(IV) on each dextran chain was named as n and dextran with different Pt number was defined as Dextran–Pt(IV)$_n$. Due to the introduction of the hydrophobic Pt(IV) structure, Dextran–Pt(IV) conjugate became amphiphilic which could self-assemble into nanoparticle in aqueous solution. It was found that Dextran–Pt(IV) conjugates with different Pt contents formed nanoparticles with different morphologies (Fig. 3). When the Pt content increased from 2.5 wt% to 7.8 wt% in the conjugate, micelles were found with different particle sizes (Fig. 3a-3c). Interestingly, when the Pt content reached 10.1 wt% (Dextran–Pt(IV)$_{5.76}$), clear hollow vesicle structure was confirmed (Fig. 3d). Interestingly, micellar structure was re-observed when the Pt content was further increased to 11.8 wt% (Dextran–Pt(IV)$_{6.86}$) (Fig. 3e). It is reasonable that variation of the hydrophilic-hydrophobic ratios contributed to the complicated self-assembly morphologies of dextran conjugates.[19,31]

Fig. 1 ^{1}H NMR spectra of (a) Pt(IV) complex, (b) Dextran and (c) Dextran–Pt(IV) conjugate in DMSO-d$_6$.

Table 1 Synthesized Dextran–Pt(IV) conjugate with different grafting ratios.

n^a(Dextran)/n^a[Pt(IV)]	Pt content in Dextran–Pt(IV) conjugate (wt%)^b	Diameter (nm)^c	PDI^d	n^e	EE(%)^f
1/10	2.5	120	0.284	1.31	9
1/20	4.9	150	0.221	2.60	22
1/30	7.8	146	0.271	4.34	34
1/40	10.1	157	0.208	5.76	48
1/50	11.8	186	0.235	6.86	70

^{a} Molar ratio of dextran and Pt(IV) in feed; ^{b} Pt content in Dextran–Pt(IV) conjugate determined by ICP-OES; ^{c} Size of Dextran–Pt(IV) conjugate in water measured by DLS; ^{d} Size distribution parameter. ^{e} Average number of Pt(IV) on each Dextran–Pt(IV) conjugate; ^{f} Encapsulation efficacy of DOX by Dextran–Pt(IV) conjugate.

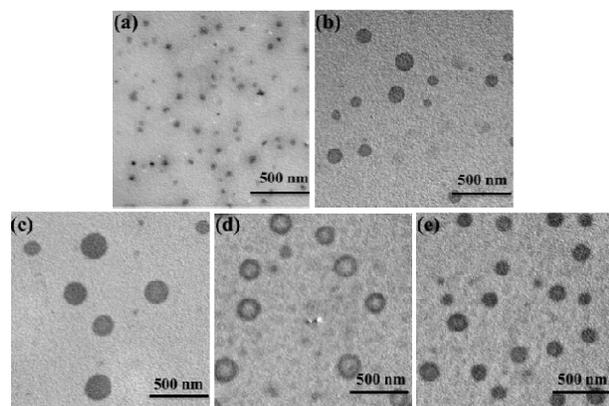# Journal of Materials Chemistry B

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Fig. 3 TEM images of Dextran–Pt(IV) conjugate nanoparticles with different Pt content: (a) 2.5 wt%, (b) 4.9 wt%, (c) 7.8 wt%, (d) 10.1 wt% and (e) 11.8 wt%.

It has to be mentioned that the pendant Pt(IV) could be reduced to Pt (II) form and cleaved from dextran backbone under reductive condition. Dramatic morphology changes of nanoparticles were assumed, since the dissociation of the pendant Pt(IV) would help the modified dextran to recover its hydrophilicity, leading to great shift of hydrophilic-hydrophobic balance (HHB) of the total polymer.[32] In order to confirm this, Dextran–Pt(IV)$_{6.86}$ conjugate was treated with 5 mM of sodium ascorbate solution (NaVc) to mimic the intracellular reductive environment.[33] As shown in the TEM images (Fig. 4b), 1 h reductive treatment had obvious effect on the morphology of nanoparticles. Compared with the starting image (Fig. 4a), most of the nanoparticles were smaller with micellar structures, while small vesicles also began to emerge. After 2 h treatment, nearly all the micelles changed to vesicle structure (Fig. 4c). Interestingly, micellar structure appeared again after 4 h reduction (Fig. 4d). Further increasing the reduction time only led to small amount of large aggregates, and most of the nanoparticles disappeared (Fig. 4e). The corresponding size distribution and change of Dextran–Pt(IV) conjugate under NaVc treatment were presented in Fig. 5. From these results, we could deduced that the reduction-responsive cleavage of Pt(IV) from polymer side chains was a time-dependent procedure. The gradual removal of Pt(IV) slowly changed the HHB of the remained Dextran–Pt(IV) conjugate, leading to the disassembly and reassembly of the nanoparticles. It is rational that after 2 h reduction, the slow dissociation of Pt(IV) decreased the Pt content in polymer from 11.8 wt% to around 10.1 wt%, and the reassembly of the reduced Dextran–Pt(IV) conjugate resulted in vesicles, in agreement with the situation in Fig. 3d. Further dissociation of Pt(IV) would continuously decrease the Pt content in polymer but increase the hydrophilicity of the conjugate. If the Pt content fell into the range of 2.5 to 7.8 wt%, reassembly of Dextran–Pt(IV)

conjugate into micelles would occur again as Fig. 3a to Fig. 3c demonstrated. It had to be mentioned that at that time point, most of the polymer–drug conjugates became highly hydrophilic and dispersed into water solution, thus the remained amount of the reassembled micelles was very few. All these results well demonstrated the HHB-type reduction-responsiveness of Dextran–Pt(IV) conjugate, which would be beneficial as a smart PDDS for triggered release of encapsulated drugs.
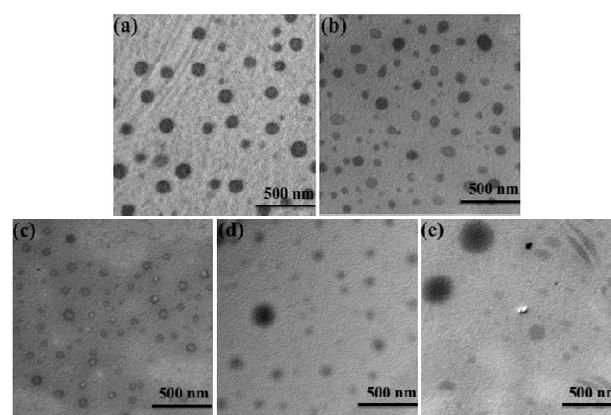

Fig. 4 TEM images of Dextran–Pt(IV) conjugate in response to 5 mM of sodium ascorbate for different times: (a) 0 h, (b) 1 h, (c) 2 h, (d) 4 h and (e) 9 h.
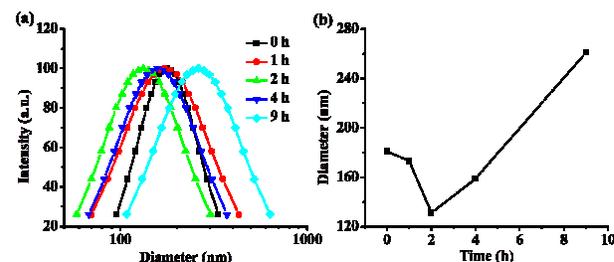

Fig. 5 (a) Size distribution of Dextran–Pt(IV) conjugate in response to 5 mM of sodium ascorbate for different times; (b) Average hydrodynamic diameter change of Dextran–Pt(IV) conjugate in response to 5 mM of sodium ascorbate with time increased.

Drug loading and release of Dextran–Pt(IV) conjugate

The critical micellar concentration (CMC) of Dextran–Pt(IV)$_{6.86}$ is about 0.038 mg/mL (Fig. S4), suggesting that Dextran–Pt(IV) conjugate should be a stable, water-soluble and reduction-responsive drug carrier. Moreover, with the conjugation of hydrophobic Pt(IV) segments, Dextran–Pt(IV) conjugate itself became the unique macromolecular prodrug. Based on these characteristics, DOX was further encapsulated into the Dextran–Pt(IV) conjugate to realize the potential combination therapy. With

Pt(IV) grafting ratio increased, the DOX-loading efficacy of Dextran–Pt(IV) conjugate increased consequently (Table 1). This might due to the gradual increase of hydrophobic interaction between Pt(IV) and DOX with the increasing grafting ratio. Dextran–Pt(IV)$_{6.86}$ conjugate was used as the reference polymer in the following experiments and DOX loading content was controlled to be 3.1 wt% with a loading efficiency of 70 wt%. Dynamic light scattering (DLS) analysis displayed that both Dextran–Pt(IV) conjugate and DOX-loaded Dextran–Pt(IV) conjugate nanoparticles had narrowly distributed sizes with 180-200 nm and 220-240 nm, respectively (Fig. 6a). Similar results were also obtained from TEM observations (Fig. 6b, Fig. 6c).[34]

Because of the reduction-responsiveness of Dextran–Pt(IV) conjugate carrier, *in vitro* drug release behaviors of DOX-loaded Dextran–Pt(IV) conjugate were investigated under reductive conditions with different concentrations of sodium ascorbate (NaVc). In these experiments, PBS (pH 7.4) and 0.1 mM of reductant were set to mimic the basic circumstance in blood, while acetate buffer solution (pH 5.0) and 5 mM of reductant were set to simulate the condition in cancer cells.[15,18] The amount of the released DOX and Pt was determined by UV-vis and ICP-MS, respectively.
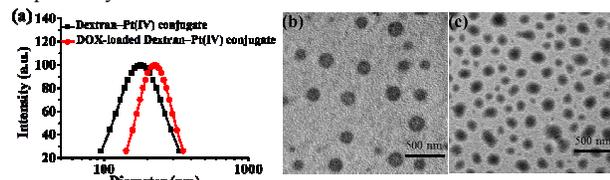

Fig. 6 (a) Hydrodynamic diameters and TEM images of (b) Dextran–Pt(IV) conjugate and (c) DOX-loaded Dextran–Pt(IV) conjugate.

In the case of DOX release, slow release kinetics was observed no matter at pH 7.4 or 5.0 without reductant involved. The released DOX from nanoparticles at pH 7.4 and 5.0 was only 10 wt% and 18 wt% after 90 h, respectively (Fig. S5a). Addition of 0.1 mM of reductant had a little effect on elevating the DOX release to a total 20 wt% after 90 h, implying the potential stability of the conjugate carrier system during blood circulation (Fig. 7a). However, DOX release was significantly accelerated when 5 mM of reductant was introduced. DOX release exceeded 20 wt% in 20 h, and the accumulative amount was more than 45 wt% after 90 h (Fig. 7a). These results further verified the reduction-responsiveness of Dextran–Pt(IV) conjugate as a carrier for triggered drug release. Under reductive condition inside tumor cells, reduction and cleavage of the pendant Pt(IV) units greatly changed the HHB of the Dextran–Pt(IV) conjugate and induced dissociation and reassembly of Dextran–Pt(IV) conjugate, resulting in rapid release of the encapsulated DOX.

Since Dextran–Pt(IV) conjugate also acted as the prodrug of active hydrophilic Pt(II) form, Pt release from the Dextran–Pt(IV) conjugate nanoparticles became an important and necessary evaluation. In fact, Pt release would not only confirm the reduction-responsiveness, but also reflect the potential anti-tumor activity of the drug carrier system. Without reductant, nanoparticles exhibited a moderate Pt release of about 23 wt% in 70 h at pH 7.4 (Fig. S5b). This was mainly owe to the hydrolysis of the ester bond between Pt(IV) and dextran, which was further proved by the increased Pt

release at pH 5.0.[35] The easier and faster hydrolysis of the ester bonds at pH 5.0 resulted in a total 37 wt% of Pt release in 70 h. Addition of 0.1 mM of reductant did not make notable increase of Pt release than pH 7.4. However, 5 mM of NaVc significantly accelerated the release (Fig. 7b). The cleaved Pt content was rapidly accumulated to 35 wt% in the first 20 h, and further increased to 57 wt% after 70 h.
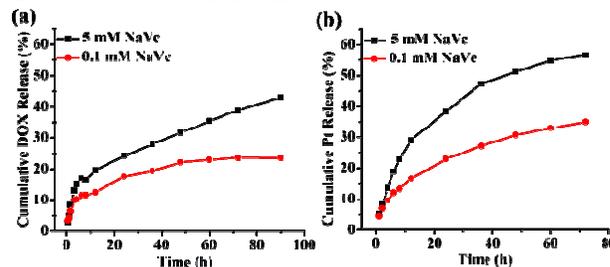

Fig. 7 (a) DOX release profiles, and (b) Pt release profiles of DOX-loaded Dextran–Pt(IV) conjugate in 5 mM and 0.1 mM of sodium ascorbate (NaVc) solution.

From Fig. 7 we also found that the speed of Pt release was much faster than that of DOX release when 5 mM of reductant was applied. It is reasonable that the reduction-responsiveness of Dextran–Pt(IV) conjugate nanoparticles realized the reduction and release of Pt segments at first. And the recovery of hydrophilicity of dextran would then induce more and more disruption of the assemblies, allowing for more amount and fast release of the encapsulated DOX. As a conclusion, the triggered release of Pt and DOX at the same time could be easily realized via a simple and reduction-responsive regulation, and the co-delivery of these two different drugs should have the potential superiority as a new combination therapy strategy to obtain a better anti-cancer effect.

In vitro cytotoxicity

The anticancer efficacy of Cisplatin, Pt(IV), DOX, DOX + Pt(IV), DOX + cisplatin, Dextran–Pt(IV) conjugate, DOX-loaded Dextran–Pt(IV) conjugate and dextran was evaluated against HepG2 cells, and the results were shown in Fig. 8 and Table 2.[36] First of all, dextran showed no cytotoxicity to cells. Compared with cisplatin, the toxicity of Pt(IV) complex was significantly alleviated. Combination of DOX and cisplatin, DOX and Pt(IV) showed higher cytotoxicity than the single drugs, demonstrating the synergistic effect of the two drugs. Dextran–Pt(IV) conjugate nanoparticles showed a slightly higher IC$_{50}$ values (50% inhibiting concentration) than that of cisplatin (Table 2), indicating that the uptake of the nanoparticles and reduction of Pt(IV) to its active Pt(II) form were time-consuming procedures.[37] In the case of DOX-loaded Dextran–Pt(IV) conjugate, the cytotoxicity was clearly higher than that of free DOX under the same DOX concentration. The further improved cytotoxicity of DOX-loaded Dextran–Pt(IV) conjugate should be mainly contributed by the reduced Pt(II) active form from conjugate and the released DOX. These results indicated that the reduction-responsive release of Pt and DOX at the same time could be successfully realized by using DOX-loaded Dextran–Pt(IV) conjugate for combination therapy and enhanced cancer treatment.

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Table 2 IC$_{50}$ values of cisplatin (μM), Pt(IV) (μM), DOX (μg), Dextran–Pt(IV) conjugate (Pt: μM), DOX-loaded Dextran–Pt(IV) conjugate (Pt/DOX: μM/μg), DOX + Pt(IV) (Pt/DOX: μM/μg) and DOX + cisplatin (Pt/DOX: μM/μg) against HepG2 and MCF-7 cells after incubation of 48 h and 72 h.

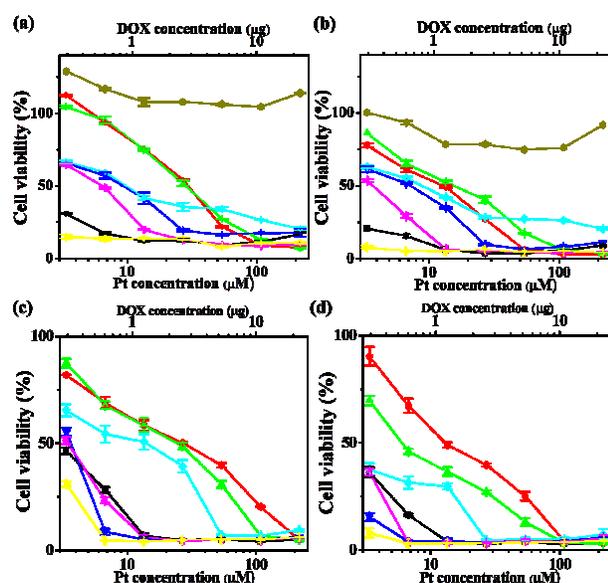|		Cisplatin	Pt(IV)	DOX	DOX + cisplatin	Pt(IV) + DOX	Dextran–Pt(IV) conjugate	DOX-loaded Dextran–Pt(IV) conjugate
HepG2	48 h	<3.37	29.91	0.82	<3.37/<0.26	6.19/0.53	29.91	9.76/0.82
	72 h	<3.37	12.82	0.73	<3.37/<0.26	3.65/0.33	16.02	7.01/0.62
MCF-7	48 h	<3.37	28.28	1.17	<3.37/<0.26	3.59/0.31	24.31	3.69/0.32
	72 h	<3.37	13.67	<0.26	<3.37/<0.26	<3.37/<0.26	6.02	<3.37/<0.26

Fig. 8 *In vitro* cytotoxicity of cisplatin (—■—), Pt(IV) (—●—), Dextran–Pt(IV) conjugate (—▲—), DOX-loaded Dextran–Pt(IV) conjugate (—▼—), DOX (—●—), DOX + Pt(IV) (—◄—), DOX + Cisplatin (—▶—) and Dextran (—●—) against HepG2 cells for (a) 48 h and (b) 72 h incubation; against MCF-7 cells for (c) 48 h and (d) 72 h.

According to the references,[38,40] MCF-7 cancer cells are slightly insensitive to free DOX. Therefore, MCF-7 cells were used to investigate whether DOX-loaded Dextran–Pt(IV) conjugate could well enhance the cancer cell sensitivity to DOX or not. As shown in Fig.8 and Table 2, DOX-loaded Dextran–Pt(IV) conjugate indeed had better cytotoxicity than free DOX. The IC$_{50}$ value for DOX-loaded Dextran–Pt(IV) conjugate was three times lower than that of free DOX after 48 h of incubation. These results indicated that DOX-loaded Dextran–Pt(IV) conjugate could make full use of the efficacy of Pt and DOX at the same time, and enhance the cells sensitivity to DOX.

Cellular uptake and internalization

Confocal laser scanning microscopy (CLSM) was exploited to further demonstrate the time-dependent uptake and triggered drug release ability of the DOX-loaded Dextran–Pt(IV) conjugate. HepG2 cells were incubated with free DOX and DOX-loaded Dextran–Pt(IV) conjugate for 0.5 h and 4 h with a DOX concentration fixed at 3 μg/mL, respectively, and the nuclei were stained with DAPI. As shown in Fig. 9, free DOX was mostly located in the nuclei after 0.5 h, while DOX fluorescence of DOX-loaded Dextran–Pt(IV) conjugate was mainly found in the cytoplasm and almost no DOX fluorescence from nuclei was observed. However, when HepG2 cells were pretreated with GSH and then incubated with DOX-loaded Dextran–Pt(IV) conjugate for 0.5 h, most fluorescence of DOX from nuclei was observed.[41] It is reasonable that the responsive reduction of Pt(IV) from the conjugate under GSH treatment triggered the corresponding quick release of DOX and subsequent diffusion into the nuclei. After 4 h incubation, most fluorescence of the DOX from DOX-loaded Dextran–Pt(IV) conjugate was confirmed in the nuclei for both the GSH un-pretreated and pretreated cells, showing the time-dependent and triggered release pattern of DOX intracellularly.[21,22] The CLSM results well demonstrated that DOX-loaded Dextran–Pt(IV) conjugate could be efficiently taken into cells. Under intracellular reduction condition, Pt(IV) segments were reduced to the active Pt(II) form and cleaved from dextran side chains to induce the disruption of Dextran–Pt(IV) conjugate structure. Thus, DOX was quickly released and accumulated into nuclei to display its activity corresponding to above cytotoxicity.

Furthermore, the ability of free DOX and DOX-loaded Dextran–Pt(IV) conjugate to enter HepG2 cells was quantitatively examined by flow cytometry measurement. Again, HepG2 cells were incubated with free DOX and DOX-loaded Dextran–Pt(IV) conjugate with the same DOX concentration for 0.5 h and 4 h, respectively.[38] After 0.5 h incubation, cells treated with DOX-loaded Dextran–Pt(IV) conjugate showed a distinct right fluorescence shift than that with free DOX (Fig. 10a), indicating an enhanced cell uptake of DOX-loaded Dextran–Pt(IV) conjugate. When the incubation time increased to 4 h, the counting level of DOX-loaded Dextran–Pt(IV) conjugate in cells was further increased, which was twice times higher than that of free DOX in cells (Fig. 10b). It has been reported that free DOX entered cells through the diffusion pathway commonly, while nanoparticles were

uptaken by cells mainly through the endocytosis pathway.[39-41] The greatly enhanced cellular uptake makes DOX-loaded Dextran–Pt(IV) conjugate to be a very desirable drug carrier to accumulate more drugs in cancer cells to gain the better therapeutic effect.
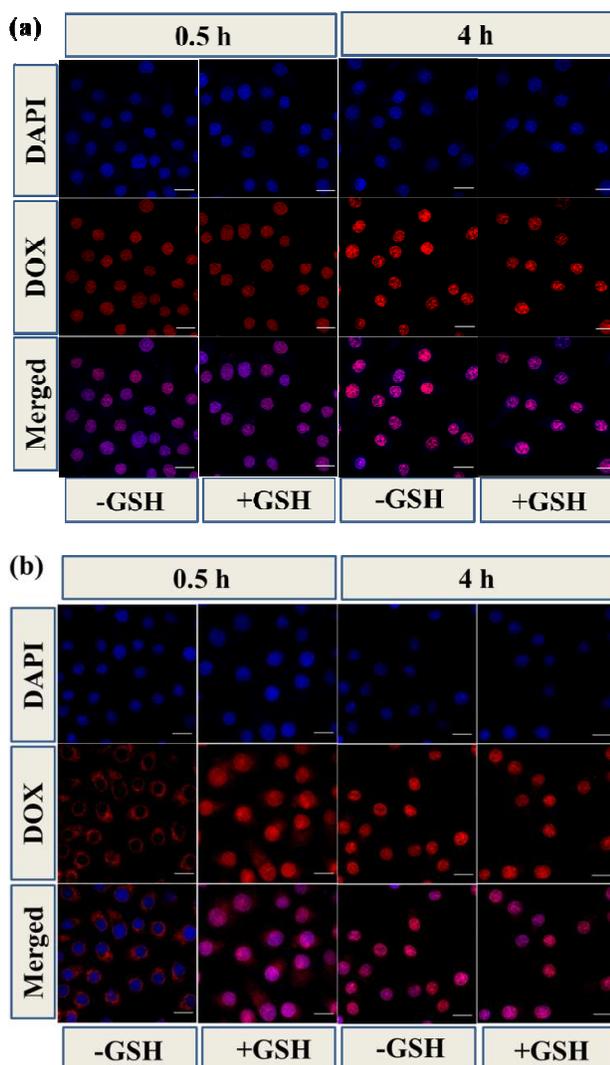

Fig. 9 CLSM images of HepG2 cells incubated with (a) free DOX and (b) DOX-loaded Dextran–Pt(IV) conjugate for 0.5 h and 4 h with and without GSH pre-treatment.
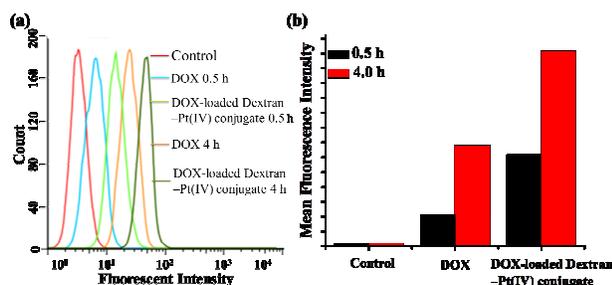

Fig. 10 (a) Quantitative analysis of free DOX and DOX-loaded Dextran–Pt(IV) conjugate uptake by flow cytometry for 0.5 h and 4 h and (b) mean fluorescence intensity of cells at 0.5 h and 4 h.

Conclusions

In summary, dextran conjugates based on Pt(IV) prodrug were successfully synthesized with desirable CMC value. The hydrophobic Pt(IV) segments changed the hydrophilicity of dextran and the obtained amphiphilic Dextran–Pt(IV) conjugate could self-assembly into stable nanoparticles as reduction-responsive drug carrier for DOX encapsulation. DOX-loaded Dextran–Pt(IV) conjugate showed triggered release kinetics for dual drugs. In the presence of reductants, the structure-stable Dextran–Pt(IV) conjugate shifted its hydrophilic-hydrophobic balance due to the time-dependent reduction and cleavage of Pt(IV) from dextran side chains, leading to the quick release of encapsulated DOX. DOX-loaded Dextran–Pt(IV) conjugate exhibited preferable cytotoxicity towards HepG2 cells due to the combined antitumor effect of DOX and Pt(II). This DOX-loaded Dextran–Pt(IV) conjugate system provided a new strategy for triggered release of the hydrophobic and hydrophilic drugs at the same time via a single reduction-responsive control to gain enhanced anti-cancer effect.

Acknowledgements

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Graphical abstract
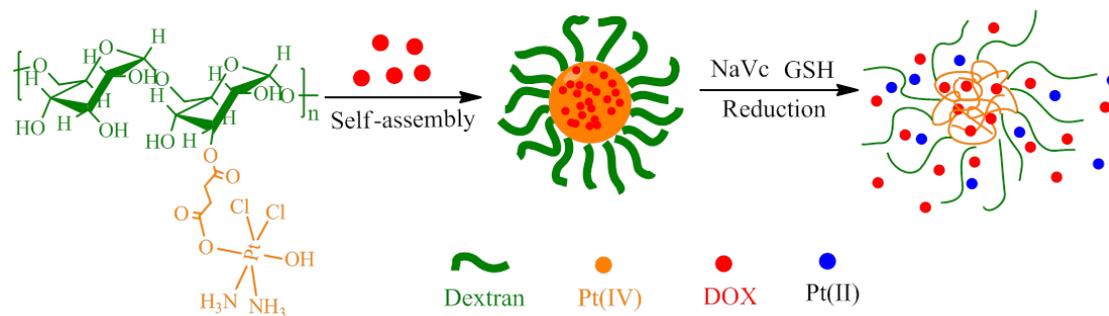
An amphiphilic Dextran–Pt(IV) conjugate was constructed by conjugation of a hydrophobic Pt(IV) prodrug to the side chains of hydrophilic dextran. The conjugate could self-assemble into stable nanoparticle as reduction-responsive drug carrier for DOX encapsulation. Under intracellular reductive condition, Pt(IV) was reduced to active Pt(II) and cleaved from dextran, which shifted hydrophilic-hydrophobic balance of the conjugate and triggered DOX release for effective combination therapy.