Biodegradable polymer–cisplatin (IV) conjugate as a pro-drug of cisplatin (II). Biomaterials

ARTICLE *in* BIOMATERIALS · OCTOBER 2011

Impact Factor: 8.56 · DOI: 10.1016/j.biomaterials.2011.06.072 · Source: PubMed

Biomaterials 32 (2011) 7732–7739

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Biodegradable polymer – cisplatin(IV) conjugate as a pro-drug of cisplatin(II)

Haihua Xiao [a,b], Ruogu Qi [a,b], Shi Liu [a,b], Xiuli Hu [a], Taicheng Duan [a], Yonghui Zheng [a], Yubin Huang [a], Xiabin Jing [a,*]

[a] State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, People's Republic of China
[b] Graduate School of Chinese Academy of Sciences, Beijing 100049, People's Republic of China

ARTICLE INFO

Article history:
Received 16 June 2011
Accepted 28 June 2011
Available online 23 July 2011

Keywords:
Cisplatin
Platinum anti-tumor drugs
Polymer-drug conjugate
Cancer therapy
Drug delivery

ABSTRACT

A Pt(IV) complex was covalently conjugated to a new biodegradable amphiphilic tri-block copolymer, MPEG-b-PCL-b-PLL, which contains pendant amino groups, to form a polymeric pro-drug of cisplatin(II), MPEG-b-PCL-b-PLL/Pt(IV). The conjugate was assembled into nano-micelles. The Pt(IV) complex, the polymer carrier and the conjugate were characterized systematically. In vitro release experiments showed that drug release from the polymer-Pt(IV) micelles follows an acid responsive and oxidation-reduction sensitive kinetics. HPLC-ICP-MS analysis revealed that cisplatin(II) can be released from the conjugate under an acidic plus a reductive condition which is available inside a cancerous cell. In vitro MTT assay demonstrated that the polymer-Pt(IV) micelles display higher cytotoxicity against SKOV-3 tumor cells than both cisplatin(II) and Pt(IV) complex. This enhanced cytotoxicity is attributed to effective internalization of the micelles by the cells via endocytosis mechanism, which was observed by fluorescence imaging and by direct determination of the platinum uptake by the cells. This polymer-Pt(IV) conjugate is a promising polymeric pro-drug of cisplatin in micellar form. It can protect the Pt(IV) complex against blood clearance. It can enter cancerous cells via endocytosis mechanism and then cisplatin(II) can be released. Therefore, this polymeric pro-drug of cisplatin is expected to find clinical applications in the future.

1. Introduction

Among the efforts to improve the anti-cancer activity and to overcome the adverse side effects of cisplatin, carboplatin, oxaliplatin and other platinum(II)-based drugs such as nephrotoxicity, peripheral neuropathy and hearing loss in the high frequency range [1–3], much attention has been paid to platinum(IV)-based compounds. Many platinum(IV) compounds were synthesized [4–6] and their anti-cancer activities were examined. A relationship between the structure and the anti-cancer activity was proposed [7–9]. It is mostly believed that reduction of relatively inert platinum(IV) complexes to their platinum(II) counterparts is essential to exhibit anti-cancer activities [7–10]. This reduction is possible in cancerous cells because of the presence of mercaptan, glutathione and other reducing agents in cell plasma [8–10]. Therefore, they are considered as pro-drugs of platinum(II) drugs. If these platinum(IV) compounds remain their +4 valence state in the circulation system and are reduced to +2 valence state after they enter the cancerous cells, they behave as qualified anti-cancer drugs and can be administered in high dose to achieve high anti-tumor efficacy without severe side effects. For this purpose, protecting these platinum(IV) drugs from reduction before they enter the cancerous cells is essential. This is a challenge to the modern pharmaceutics.

Fortunately, polymeric drug delivery systems have several useful functions that benefit the protection and delivery of platinum(IV) drugs. Polymer conjugates can preferentially enter the cancerous tissue via EPR effect [11–13]. Polymeric micelles usually enter the cancer cells via endocytosis with considerable efficiency. The carrier polymer can be degraded and the encapsulated drugs can be released rapidly under the endosome or secondary lysosome conditions. However, few efforts have been made to develop amphiphilic block copolymer/platinum(IV) drug micelles. Zhang et al. [14] reported a polymer-platinum(IV) pro-drug for the first time. It was composed of a platinum(IV) complex with two keto-bearing ligands in the axial positions to which two terminal-amino-carrying PEG-b-PLA chains were attached via a acid responsive Schiff-base linkage. The conjugates displayed abilities to kill A2780 human ovarian carcinoma cells several times higher than free cisplatin. However, on the one hand, because the Pt(IV) atom was connected with two MPEG-PLA chains of mean molecular weight of 10 kDa, the platinum content in the conjugate is only 1 wt

* Corresponding author. Tel./fax: +86 431 85262775.
E-mail address: xbjing@ciac.jl.cn (X. Jing).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.06.072

%. On the other hand, whether the cisplatin(II) or cisplatin(IV) species were released were not mentioned at all.

More Recently, cisplatin(IV) derivative with a carboxyl group was conjugated to a polylactide derivative with pendant hydroxyl groups by Lippard and Langer [15] to form PLA-Pt(IV) pro-drugs and PLA-Pt(IV)/DTX (docetaxel) multifunctional nanoparticles. The nanoparticles with higher platinum content, typically 10% w/w, showed high enough toxicity against cancer cells compared to those containing only DTX or platinum drugs. A release mechanism of platinum species from the nanoparticles was proposed based on a faster platinum release under reductive conditions compared with that in PBS. It was thought that in PBS, platinum(IV) species were predominantly released via ester hydrolysis; upon reduction, the pendant platinum(IV) complexes on the polymer chains were reduced into platinum(II) species quickly. However, both the platinum(IV) and platinum(II) species were not identified.

Herein, we first prepared a platinum(IV) complex (complex 2 in Scheme 1(a)) as reported elsewhere [16], which consists of a COOH-functionalized axial ligand (Scheme 1(a)), and then we designed and synthesized an amphiphilic block copolymer/platinum(IV) drug conjugate (Scheme 1(b)) based on our own experience in polymer-paclitaxel [17,18] and polymer-doxorubicin [19] conjugates. The amphiphilic copolymer MPEG-b-PCL-b-PLL with L-lysine residues in the PLL block contains several free NH_2 groups to react with the carboxyl groups of complex 2. Hence, the platinum content in the conjugate can be greatly improved. Furthermore, the Pt(IV) species can be released because of the acid-responsive hydrolysis, while upon reduction, release of platinum(II) from the PEG-b-PCL-b-PLL chains was thought to be predominant. Moreover, because it is chemically attached to the polymers, initial burst release of the drug encountered by usual physical encapsulation can be avoided. Therefore, this conjugate is expected to be a potent cisplatin pro-drug.

2. Materials and methods

2.1. Materials

Monomethoxyl poly(ethylene glycol) with an average molecular weight of 5000 Da ($MPEG_{5K}$), ε-caprolactone (CL), N-hydroxysuccinimide (NHS), 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), Rhodamine B, succinic anhydride, palladium hydroxide on charcoal catalysts ($Pd(OH)_2/C$, 10% w/w) were purchased from Sigma-Aldrich. Cisplatin (purity 99%) was bought from Shandong Boyuan Chemical Company, China. The MPEG-b-PCL-b-PLL synthesis was described in the supporting information (SI). All other chemicals and solvents were used without further purification.

2.2. General measurements

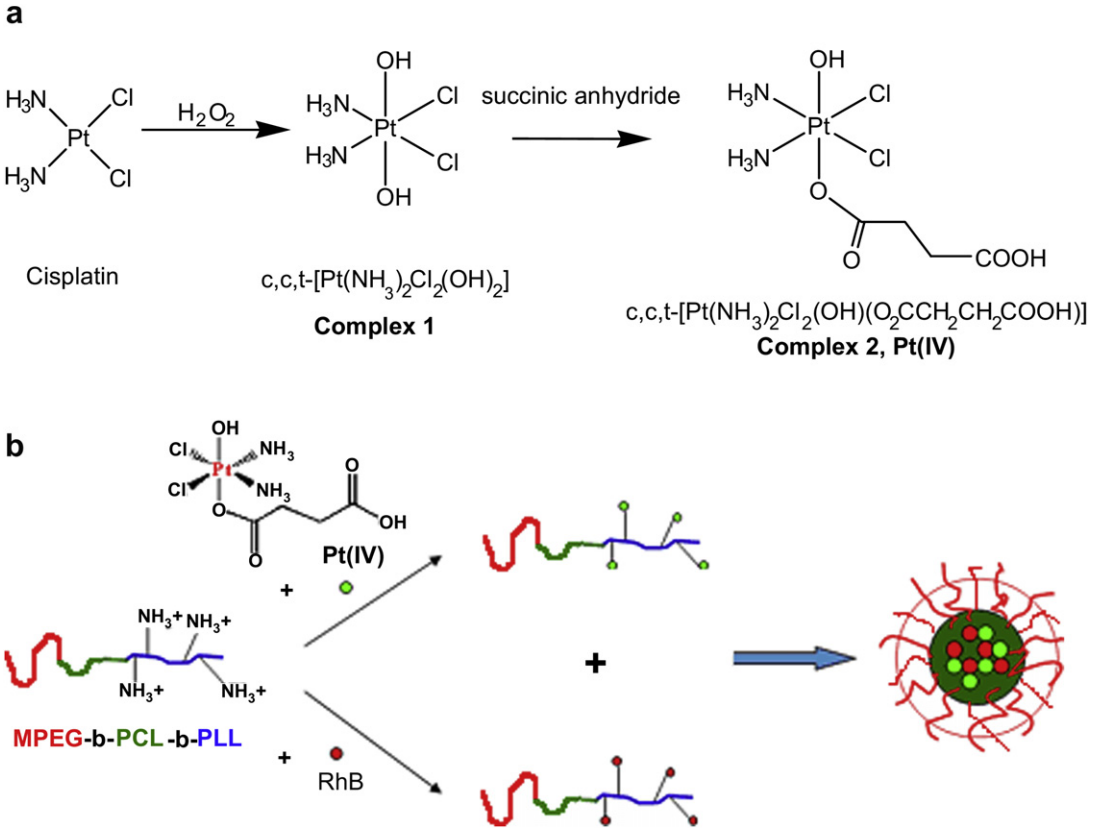
^1H NMR spectra were measured by a Unity-300 MHz NMR spectrometer (Bruker) at room temperature. Fourier Transform Infrared (FT-IR) spectra were recorded on a Bruker Vertex 70 spectrometer. Mass Spectroscopy (ESI-MS) measurements were performed on a Quattro Premier XE system (Waters) equipped with an electrospray interface (ESI). Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 6300, Thermoscientific, USA) was used to determine the total platinum contents in the polymer-Pt(IV) conjugate and samples obtained outside of the dialysis bags in drug release experiments. Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Xseries II, Thermoscientific, USA) was used for quantitative determination of trace levels of platinum. Size and size distribution of micelles were determined by DLS with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology, USA). The morphology of the polymer-Pt(IV) micelles was measured by TEM performed on a JEOL JEM-1011 electron microscope. Particle size and zeta potential measurements were conducted on a Malvern Zetasizer Nano ZS.

2.3. Synthesis of c,c,t-[$Pt(NH_3)_2Cl_2(OH)_2$] (Complex 1)

Complex 1 (Scheme 1(a)) was synthesized according to reference [16].

2.4. Synthesis of c,c,t-[$Pt(NH_3)_2Cl_2(OOCCH_2CH_2COOH)(OH)$] (complex 2)

The platinum complex 2 (Scheme 1(a)) was synthesized as previously described [16]. Briefly, to a solution of complex 1 (0.2 g, 0.6 mmol) in DMSO (16 ml) was added succinic anhydride (0.06 g, 0.6 mmol) and the reaction mixture was stirred at room



Scheme 1. Preparation of platinum complex 2 (a), MPEG-b-PCL-b-PLL/Pt(IV) and MPEG-b-PCL-b-PLL/RhB conjugates and their composite micelles (b).

temperature for 12 h. The solution was lyophilized and 10 ml acetone was added to precipitate a light yellow solid. It was washed several times with acetone and diethyl ether, and dried. The complex 2 was isolated in 50% yield. ESI-MS (negative mode): Calc. = 434.13, Found 433; ^{1}H NMR (DMSO-d_6): 6.0 (br, 6H), 2.45–2.25 (m, 4H). Anal. Calc. for $C_4H_{12}Cl_2N_2O_5Pt$ (434.13): C, 11.07; H, 2.79; N, 6.45; Found: C, 10.9; H, 2.65; N, 6.51.

2.5. Conjugate platinum complex 2 to MPEG-b-PCL-b-PLL

Platinum complex 2 was conjugated to the polymer MPEG-b-PCL-b-PLL using EDC/NHS method in aqueous solution. Briefly, EDC·HCl (0.191 g, 1 mmol) and NHS (0.115 g, 1 mmol) were dissolved in de-ionized water under stirring. Then platinum complex 2 (0.347 g, 0.8 mmol) was added into the aqueous solution. After the mixture (suspension) became clear, 0.5 g of polymer MPEG-b-PCL-b-PLL in 100 ml water was added and the reaction mixture was kept stirring at room temperature for 24 h, then it was dialyzed against water for 12 h and lyophilized to obtain MPEG-b-PCL-b-PLL/Pt(IV) conjugates.

2.6. Synthesis of Rhodamine B Labeled MPEG-b-PCL-b-PLL (MPEG-b-PCL-b-PLL/RhB)

Rhodamine B (13 mg) was dissolved in 2 ml DMSO, to which 12 mg DCC and 7 mg NHS were added. The reaction mixture was kept at room temperature for 12 h and then it was filtered. The filtrate was added into a solution of 50 mg MPEG-b-PCL-b-PLL in DMSO and the reaction was continued for 8 h. Thereafter, the mixture solution was dialyzed for 5 days to remove un-reacted Rhodamine B, and finally lyophilized.

2.7. Preparation of MPEG-b-PCL-b-PLL/Pt(IV) micelles

The pro-drug micelles were prepared by nano-precipitation method. In brief, 50 mg MPEG-b-PCL-b-PLL/Pt(IV) conjugate was dissolved in a flask with 10 ml DMF, and then 50 ml water was added dropwise into the flask under stirring to form a micellar solution. The solution was dialyzed against water to remove DMF and then freeze-dried.

The composite micelles of MPEG-b-PCL-b-PLL/Pt(IV) and MPEG-b-PCL-b-PLL/RhB were prepared in the similar method (Scheme 1(b)). 1 mg of MPEG-b-PCL-b-PLL/RhB and 9 mg of MPEG-b-PCL-b-PLL/Pt(IV) conjugates were dissolved in 3 ml DMSO in a flask under stirring, and then de-ionized water was dropped into the flask to form composite micelles. After that, the micellar solution was dialyzed against water and then freeze-dried.

2.8. Drug release from MPEG-b-PCL-b-PLL/Pt(IV) micelles

50 mg of lyophilized MPEG-b-PCL-b-PLL/Pt(IV) micelles (Pt content 5.4 wt%) was dissolved in 20 ml of phosphate buffered saline (0.1 M PBS, pH = 7.4). The solution was then placed into a pre-swelled dialysis bag with a molecular weight cutoff of 3.5 kDa and immersed into 140 ml of 0.1 mol/L PBS (pH = 7.4). The dialysis was conducted at 37 °C in a shaking culture incubator. 1.5 mL of sample solution was withdrawn from the incubation medium at specified time intervals and measured for Pt concentration by ICP-OES. After sampling, equal volume of fresh PBS was immediately added into the incubation medium. The platinum released from the micelles was expressed as the percentage of cumulative platinum outside the dialysis bag to the total platinum in the micelles.

The same drug release procedure was performed in the presence of 5 mM and 0.1 mM sodium ascorbate, respectively.

2.9. MTT (3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

SKOV-3 cells were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, and grown in RPMI 1640 (Life Technologies) supplemented with 0.03% L-glutamine and 1% penicillin/streptomycin in 5% CO_2 at 37 °C.

SKOV-3 cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 10^5 cells/well and incubated in RPMI 1640 for 24 h. The medium was then replaced by cisplatin, platinum complex 2, MPEG-b-PCL-b-PLL/Pt(IV) micelles, at a final equivalent Pt concentration from 0.1 to 125 μg/mL for the former three. The amounts of drug free polymer MPEG-b-PCL-b-PLL were equal to those in MPEG-b-PCL-b-PLL/Pt(IV) conjugates. The incubation was continued for 24 h. Then, 20 μL of MTT solution in PBS with the concentration of 5 mg/mL was added and the plates were incubated for another 4 h at 37 °C, followed by removal of the culture medium containing MTT and addition of 150 μL of DMSO to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 min, and the absorbance of formazan product was measured at 492 nm by a microplate reader.

2.10. Cellular uptake

2.10.1. Fluorescence imaging of cancer cells

SKOV-3 cells were grown in RPMI 1640 with 10% fetal bovine serum at 37 °C in 5% CO_2. The cells were seeded in 6-well plates and grown for 24 h prior to incubation with 0.2 mg/mL composite micelles (MPEG-b-PCL-b-PLL/Pt(IV)//MPEG-b-PCL-b-PLL/RhB). Live cells were imaged 6 h post-treatment using an inverted microscope (TE2000-U, Nikon). Pictures were taken with a digital camera (DXM1200F, Nikon).

2.10.2. Determination of platinum contents in the cells

SKOV-3 cells were seeded in 6-well plates. These cells were then treated with cisplatin, complex 2 and polymer-Pt(IV) conjugate with the platinum concentration in the culture medium regulated to the same value of 5 μM and subsequently incubated at 37 °C for 2 h or 6 h. After washed with PBS three times, cells were lysed by cell lysis buffer. Platinum contents in the cell lysis solution were determined by ICP-MS. The protein content in each cell sample was determined by using bicinchoninic acid (BCA) assay. The total platinum content was expressed as nano-grams of Pt per microgram of total proteins.

3. Results and discussion

3.1. Syntheses and characterization of Pt(IV) complexes 1 and complex 2

Many Pt(IV) complexes have been prepared and studied during the last decades and some of them entered clinical trials [20–22]. Studies showed that reduction potential of these Pt(IV) complexes is a key factor affecting its anti-tumor activities. Generally, too negative reduction potential means difficulty in its reduction to the active species, thus leading to low anti-tumor activity; whereas too positive reduction potential means much easy reduction to the Pt(II) counterparts, inevitably causing instability of the Pt(IV) drug under physiological conditions. Therefore, our synthesis began with cisplatin(II) [8,9]. It was oxidized with H_2O_2 into complex 1 that consisted of two hydroxyl groups at its axial positions, as shown in Scheme 1(a). One of the two hydroxyl groups was then reacted with succinic anhydride to form complex 2 with a free carboxyl group capable of reacting with NH_2 groups using EDC·HCl/NHS method. FT-IR, ^{1}H NMR and ESI-MS were used to confirm the structure of complex 2 (Figs. S1, S2 and S3 in the SI). The reason for us to choose complex 2 was that it has an ideal reduction potential (−0.49 V at pH = 7.4; −0.42 V at pH = 6.0) [16], and thus, it is stable enough to travel through the bloodstream until it reaches a tumor cell without immature decomposition. Moreover, it is more easily reduced to Pt(II) species once inside the cell because of the positive shift of reduction potential in acidic environment in the endosome compartments [16].

3.2. Synthesis of MPEG-b-PCL-b-PLL/Pt(IV) conjugate

The synthesis of tri-block copolymer MPEG-b-PCL-b-PLL was detailedly described in Scheme S1 and it was characterized by ^{1}H NMR (Fig. S4) and GPC (Fig. S5). They are used to react with the complex 2 using EDC·HCl/NHS method to form MPEG-b-PCL-b-PLL/Pt(IV) conjugate. This tri-block copolymer was chosen due to its biodegradability and amphiphilic nature. Moreover, there are several NH_2 pendant groups on its molecular chains. By increasing the degree of polymerization of the PLL and by increasing the molar ratio of Pt/NH_2, platinum content in the polymeric Pt(IV) conjugates can be controlled and enhanced. Here the platinum content is defined as the weight ratio of platinum atoms to the polymer-Pt(IV) conjugate. As is shown in Fig. 1, when the molar ratio of Pt(IV) complex 2 to the amino groups of MPEG-b-PCL-b-PLL increases from 0.5 to 4, the platinum content increases from 3.2% to 13.6%. However, when this molar ratio is beyond 4:1, drug loading does not increase any longer. This is likely due to the complete assumption of the pendant amino groups. In fact, the theoretical highest platinum content is 14.8% if all NH_2 groups are used up. The platinum content of 13.6% corresponds to the condensation rate of 92%.

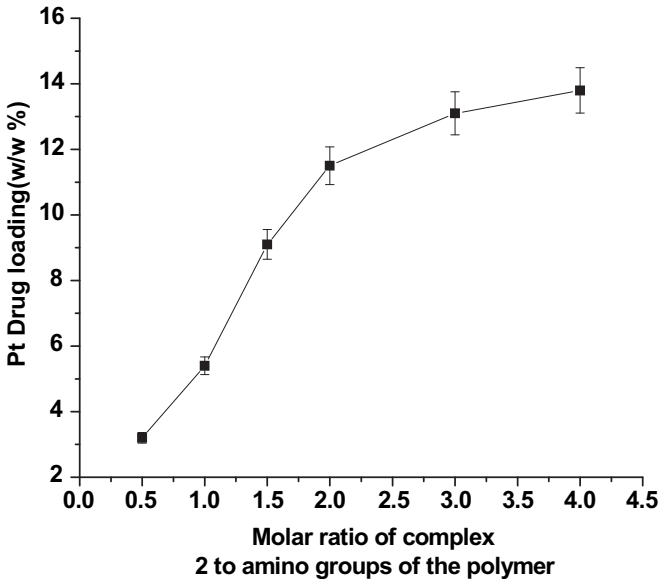


Fig. 1. Platinum drug loading as a function of the molar ratio of platinum complex 2 to the pendant amino groups of the polymer MPEG-b-PCL-b-PLL.

3.3. Characterization of MPEG-b-PCL-b-PLL/Pt(IV) conjugate micelles

The tri-block copolymer MPEG-b-PCL-b-PLL has two hydrophilic blocks (MPEG and PLL) and one hydrophobic block (PCL). The Pt(IV) complex 2 is partly dissolvable in water. When the polymer MPEG-b-PCL-b-PLL was conjugated with Pt(IV) complex 2 to form MPEG-b-PCL-b-PLL/Pt(IV), the newly formed polymeric Pt(IV) conjugate can self-assemble into micelles with the PCL-b-PLL/Pt(IV) as the inner core, and the MPEG block as the hydrophilic shell.

Critical micelle concentration (CMC) of the MPEG-b-PCL-b-PLL/Pt(IV) conjugates was determined using pyrene as a hydrophobic probe [23]. When the fluorescence emission of pyrene was fixed at 390 nm, its excitation spectra depended on concentration of MPEG-b-PCL-b-PLL/Pt(IV) conjugate. From this dependence, its CMC was calculated to be 0.01 g/L (Fig. S6). TEM and DLS were used to characterize the morphology and size of MPEG-b-PCL-b-PLL and MPEG-b-PCL-b-PLL/Pt(IV) conjugate micelles. Typical TEM pictures are shown in Fig. 2. MPEG-b-PCL-b-PLL micelles and MPEG-b-PCL-b-PLL/Pt(IV) conjugate micelles have similar spherical structure with an average diameter of about 200–220 nm and 150–160 nm, respectively. DLS measurement (Fig. 2(insets)) showed that both MPEG-b-PCL-b-PLL and MPEG-b-PCL-b-PLL/Pt(IV) conjugate micelles had a unimodal size distribution with an average diameter of 200–220 nm and 150–160 nm, in agreement with those determined by TEM images. Moreover, the size distribution of the carrier polymer micelles (Fig. 2(A, inset)) was wider than that of the drug conjugate micelles (Fig. 2(B, inset)), probably because the hydrophilic PLL block in MPEG-b-PCL-b-PLL becomes hydrophobic in MPEG-b-PCL-b-PLL/Pt(IV) after combination with Pt(IV) complex 2. Further experiment was done to study the surface zeta-potential of the polymer-Pt(IV) micelles. It was about +6.9 mV, and was greatly lower than that of the polymer micelles (+49.3 mV). This result suggests that due to the conjugation of cisplatin(IV) to the polymer, the positively charged amino groups were neutralized and the PLL/Pt(IV) segments were located in the core part of the micelles.

3.4. Drug release from MPEG-b-PCL-b-PLL/Pt(IV) micelles

Drug release experiments from the micelles were performed at different pH values and different concentrations of reducing agent sodium ascorbate. ICP-OES was used to determine the amount of platinum released. The weight ratio of accumulative released platinum to the total platinum payload in the micelles was measured as a function of release time. Fig. 3A shows the release kinetics from the MPEG-b-PCL-b-PLL/Pt(IV) conjugate at pH 5.0 and pH 7.4, respectively. The platinum release rate from the micelles at pH 5.0 was significantly faster than that at pH 7.4. When the platinum loading was 5.4 wt% (Fig. 3A), to release 50% of the total platinum payload took 20 h at pH 5.0, while it took more than 50 h at pH 7.4. This difference is attributed to the pH sensitivity of hydrolysis reaction of MPEG-b-PCL-b-PLL/Pt(IV) conjugate. It is well-known that both the polymer main chains and the linkages between the polymer backbone and the platinum(IV) complex are hydrolysable under acidic conditions.

It is believed that MPEG-b-PCL-b-PLL/Pt(IV) conjugate is actually a pro-drug. Only when the Pt(IV) species are reduced to active anti-cancer Pt(II) species like cisplatin(II), can it play a role of anti-tumor agent. This reduction is expected to take place inside the cancerous cells [8–10]. In order to mimic the reductive environment inside cells, the release experiments were carried out in the presence of two concentrations of aqueous solutions of sodium ascorbate. As shown in Fig. 3B, when the ascorbate concentration is 5 mM, which mimics the reductive intracellular environment [9]. The released platinum from the micelles is nearly 80% at 3 h. Even at 0.1 mM of ascorbate, it takes only 10 h to release 80% platinum. Moreover, compared to Fig. 3A, the platinum release is much faster

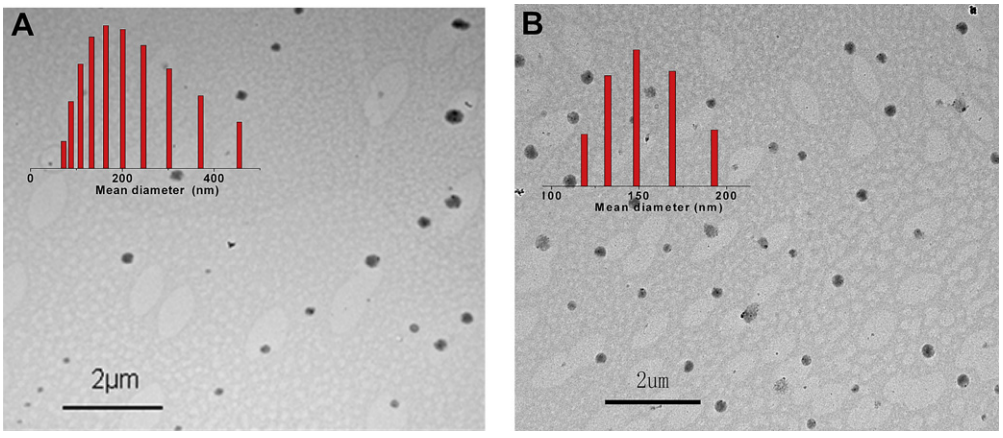


Fig. 2. TEM morphology and DLS characterization (insets) of MPEG-b-PCL-b-PLL (A) and MPEG-b-PCL-b-PLL/Pt(IV) micelles (B).

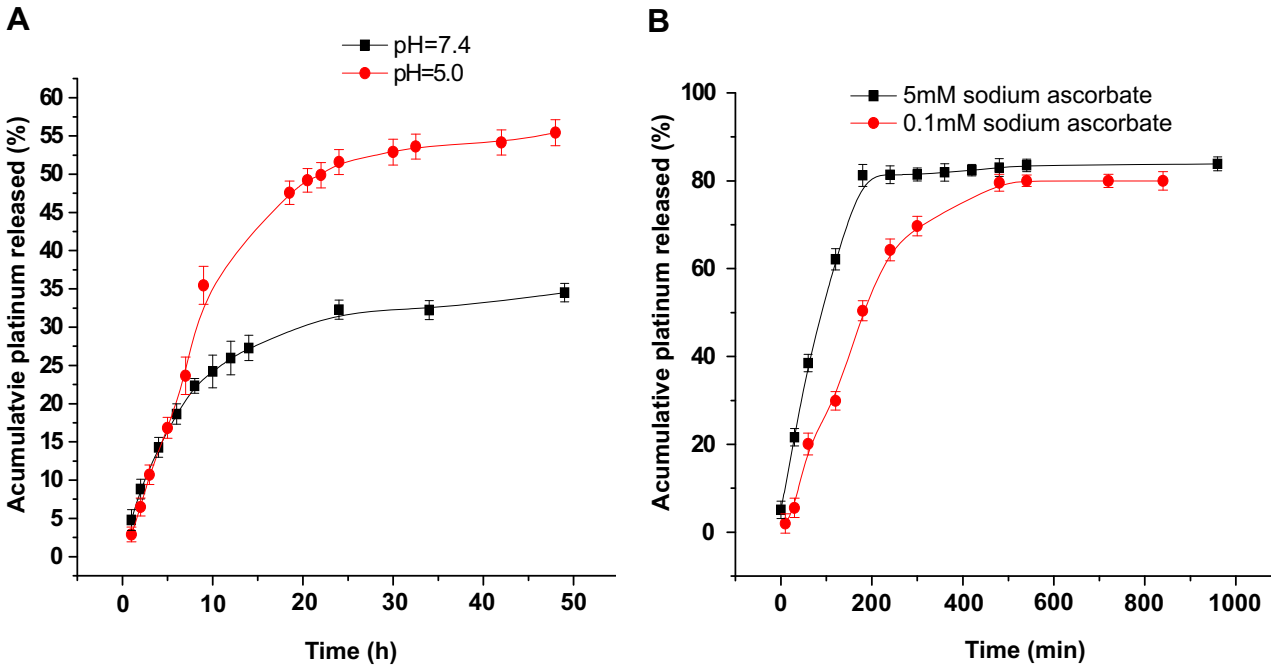


Fig. 3. Drug release profiles of MPEG-b-PCL-b-PLL/Pt(IV) micelles: (A) at pH 7.4 and 5.0; (B) at sodium ascorbate concentrations of 5 and 0.1 mM.

no matter the ascorbate concentration is 5 mM or 0.1 mM, indicating that the MPEG-b-PCL-b-PLL/Pt(IV) conjugate is more susceptible to reducing environment than pH change in the examined pH range. Because there are enough reducing agents like mercaptan, glutathione and others in the cell plasma, Pt(IV) species in MPEG-b-PCL-b-PLL/Pt(IV) conjugate can be reduced to Pt(II) species easily and can serve as an anti-cancer agent.

3.5. Released species of MPEG-b-PCL-b-PLL/Pt(IV) conjugate studied by HPLC-ICP-MS

It should be pointed out that the released platinum species above were detected by ICP-OES. This method can measure the platinum content precisely but it cannot provide any information about its ligands. To solve this problem, an HPLC-ICP-MS method [24,25] was applied to analyze the reaction products of the MPEG-b-PCL-b-PLL/Pt(IV) conjugate and its precusors. The experimental details are given in SI. Based on the analyses of cisplatin(II) (Fig. S7), Pt(IV) complex 1 (Fig. S8) and complex 2 (Fig. S9) by HPLC-ICP-MS, typical retention times (RT) of cisplatin(II), Pt(IV) complex 1 and complex 2 are deduced to be ca. 100, 85–90, and 160 s, respectively (Table S2). The HPLC-ICP-MS chromatograms of MPEG-b-PCL-b-PLL/Pt(IV) is shown in Fig. 4A. It gives a peak at ca. 102 s after reduction by 5 mM sodium ascorbate, indicating direct release of cisplatin(II) from the polymeric complex without hydrolysis (Fig. 4A(a)). Under hydrolytic condition (pH 5.0, 24 h, Fig. 4A(b)), only two peaks at 160 and 90 s are observed, indicating formation of Pt(IV) complex 1 and complex 2. Further hydrolysis (pH 5.0, 5 d (Fig. 4A(c)) leads to disappearance of the 160 s peak, implying transformation from complex 2 to complex 1. It is interesting to notice that peaks with longer retention times, i.e., those Pt complexes with longer tails were not observed. This implies that the polymeric complex is broken predominantly between the Pt atom and the succinato ligand and secondly at the amide linkage between succinic acid and lysine residue. This is just what we are expecting for a drug delivery system via endocytosis.

Based on the above results of release experiments and HPLC-ICP-MS, Fig. 4B is proposed to describe the possible pathways of the drug release from polymer-Pt(IV) conjugates, i.e., (1) hydrolysis of the Pt-carboxylato coordination bonds, the amide linkage between lysine residue and succinic acid, and the polymer main chains, especially the poly(L-lysine) blocks; (2) reduction of the central platinum atom from +4 valence to +2 valence. Among the three manners of hydrolysis, the first one is straightforward to get complex 1; the second one leads to release of complex 2; and the third one results in a longer tail on complex 2. Further hydrolysis may convert complex 2 and its derivatives into complex 1. The platinum atom in these release products is in +4 valence. Therefore, it has to be reduced to +2 valence to serve as an anti-cancer agent. In fact, only the first two were experimentally observed by HPLC-ICP-MS. It means that the polymeric amide or ester linkages are relatively more stable to hydrolysis. Reduction of Pt(IV) may take place in complex 2 and even in polymeric conjugate polymer-Pt(IV). Once this reduction occurs, the axial positions of the Pt atom will be emptied and thus cisplatin(II) will be released directly no matter its original axial ligand is succinato or a polymer chain. In fact, HPLC-ICP-MS did not find complex 1, complex 2 or complex 2 with longer tails under a reductive condition (5 mM ascorbate, Fig. 4A(a)), most probably because the MPEG-b-PCL-b-PLL/Pt(IV) conjugate is directly reduced and broken into carrier polymer and cisplatin(II). This statement is in accord with the release experiment result in Fig. 3 that the platinum species are released more rapidly under reductive conditions than under hydrolytic conditions. Therefore the following conclusion may be drawn: under intracellular conditions cisplatin(II) is the predominant product derived from the MPEG-b-PCL-b-PLL/Pt(IV) micelles.

3.6. Cytotoxicity

To examine the cytotoxicity of cisplatin, Pt(IV) complex 2, and MPEG-b-PCL-b-PLL/Pt(IV) conjugate, SKOV-3 cells were exposed to them in four doses (0.1–125 μg/ml Pt) for 24 h. MPEG-b-PCL-b-PLL polymer was similarly examined to evaluate its safety. Its amounts were equal to those in the corresponding MPEG-b-PCL-b-PLL/Pt(IV) conjugate samples (Pt content: 10% w/w). The cytotoxicity was evaluated using MTT assay and the results are presented in Fig. 5.

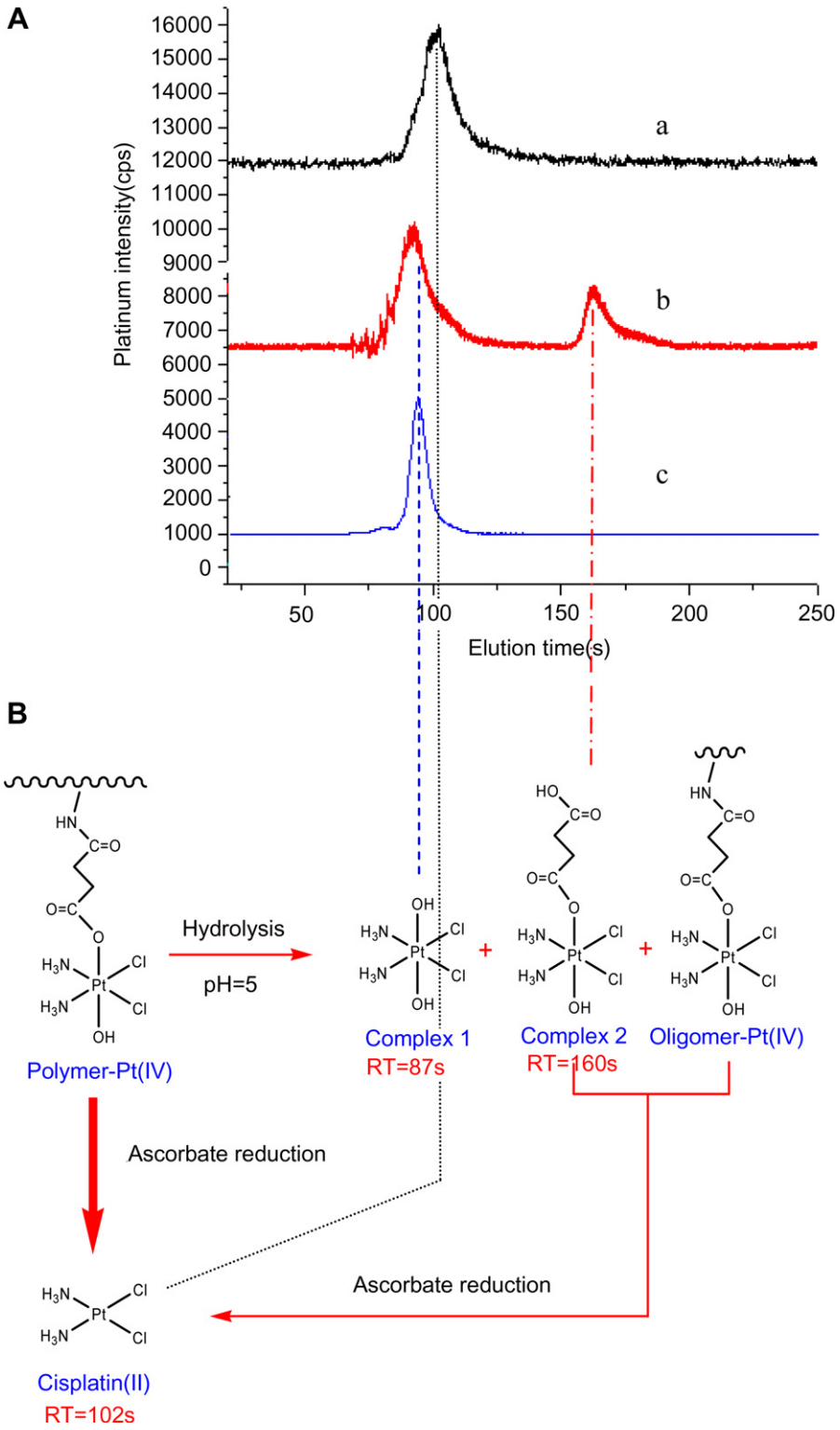


Fig. 4. (A) HPLC-ICP-MS chromatograms of MPEG-b-PCL-b-PLL/Pt(IV) micelles in 5 mM sodium ascorbate aqueous solution (a), in aqueous solutions of pH 5.0 for 24 h (b) and for 5 days (c). (B) Possible pathways of the platinum complex MPEG-b-PCL-b-PLL/Pt(IV) during ascorbate reduction and acidic hydrolysis.

First of all, very little toxicity was observed for the copolymer itself. Even at a concentration as high as 1.2 mg/mL, the cell viability is still as high as 75%. It means it is a safe drug carrier. As expected, cisplatin exhibits higher cytotoxicity (IC_{50Pt} = 36.0 μg/ml) than Pt(IV) complex 2) (IC_{50Pt} > 200 μg/ml), because of the difficulty of entering the cells and the necessity of reduction of Pt(IV) to Pt(II) of the latter. MPEG-b-PCL-b-PLL/Pt(IV) conjugate micelles (IC_{50Pt} = 5.25 μg/ml) displayed as about 7 times cytotoxicity as cisplatin. The cell viabilities at 0.1, 10, and 50 μg/ml are approximately half of those for cisplatin. This enhanced cytotoxicity of MPEG-b-PCL-b-PLL/Pt(IV) conjugate micelles can be attributed to their higher efficiency of endocytosis than small molecular cisplatin

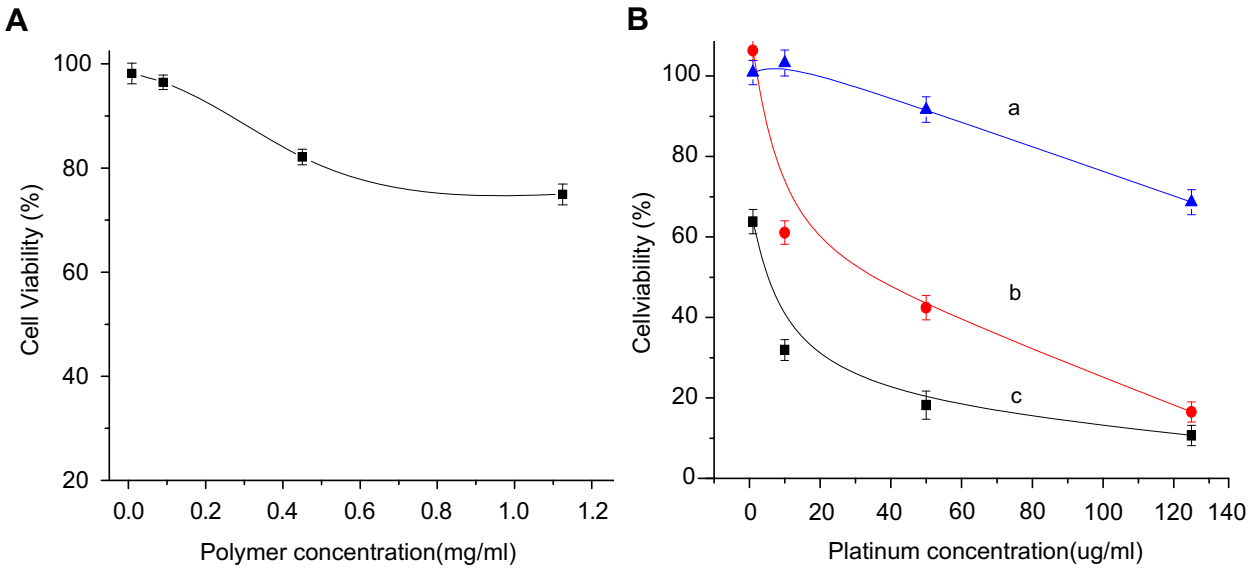


Fig. 5. Cytotoxicity of (A) Pt-free polymer MPEG-b-PCL-b-PLL, (B) Pt(IV) complex 2 (a), cisplatin (b) and MPEG-b-PCL-b-PLL/Pt(IV) micelles (c) after 24 h incubation with SKOV-3 cells. The concentration of MPEG-b-PCL-b-PLL was equal to that in MPEG-b-PCL-b-PLL/Pt(IV) micelles.

[22,26,27] and subsequent effective reduction inside the SKOV-3 cells. This conclusion can be supported by the cellular uptake experiments in the next section.

3.7. Cellular uptake

Cellular uptake by SKOV-3 human ovarian cancer cells was examined by fluorescence imaging and platinum content measurement of the cells by ICP-MS. In order to visualize the polymer-Pt(IV) conjugates taken by the cells, MPEG-b-PCL-b-PLL/Pt(IV) conjugate and a fluorescent copolymer, MPEG-b-PCL-b-PLL/RhB, were co-self-assembled into composite micelles (Scheme 1(b)). Because the two polymers have identical molecular chain structures, each of these micelles is believed to consist of the two components, not a single component, and therefore, Rhodamine B can serve as a fluorescent probe for the composite micelles. Fig. 6 shows the fluorescence micrograph of SKOV-3 cells 6 h post administration of the drugs. Because the cells were thoroughly washed, the red fluorescence (Rhodamine B) is believed to come from the micelles taken inside the cells. The intense red fluorescence in the cells indicates that the polymer-drug micelles are mainly located in cell plasma and full of cell plasma. It means that endocytosis has taken place with high efficiency.

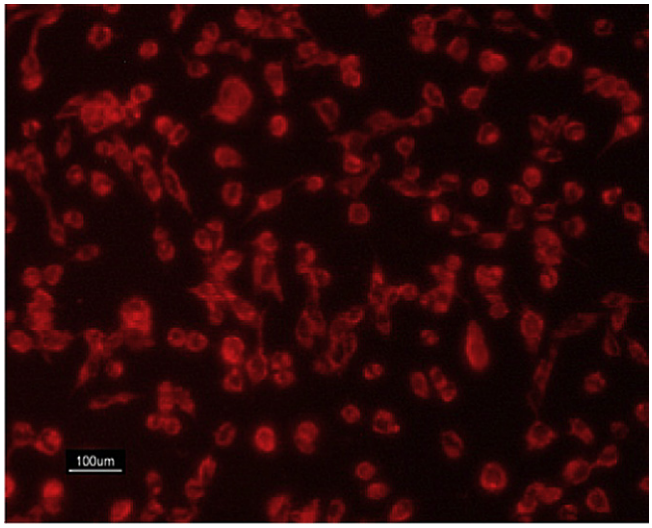


Fig. 6. Fluorescence images of SKOV-3 cells after 6 h incubation with MPEG-b-PCL-b-PLL/RhB/MPEG-b-PCL-b-PLL/Pt(IV) composite micelles.

In order to quantitatively determine the drug content taken by the cells, ICP-MS was used to determine the platinum content in the cell lysis liquid, and the platinum content is expressed as “ng of platinum per mg of total cell proteins”. The latter was determined by BCA assay. As shown in Table 1, when the cells were incubated for 2 h with cisplatin, Pt(IV) complex 2 and MPEG-b-PCL-b-PLL/Pt(IV) micelles (Pt content 10% w/w) at the equivalent initial platinum concentration of 5 μM (0.975 μg/ml), the platinum contents in the cells were 33.8, 18.6 and 141.3 ng/mg of proteins, respectively. These data implied that cisplatin enters the cells more rapidly than Pt(IV) complex 2, while the polymer-Pt(IV) micelles get internalized in the cells at a higher speed than cisplatin and Pt(IV) complex 2, in agreement with the above fluorescence observation. It is noticed that 6 h post drug treatment, the platinum contents in the cells became 112, 26.2 and 1097 ng/mg of proteins, respectively. Of the three drugs, the polymer-Pt(IV) micelles was the most effective as far as drug-internalization is concerned. This enhanced endocytosis is responsible for the highest cytotoxicity of MPEG-b-PCL-b-PLL/Pt(IV) micelles compared to the other two observed in the preceding section.

Table 1
Cellular intake of cisplatin, Pt(IV) complex 2, MPEG-b-PCL-b-PLL/Pt(IV) micelles by ICP-MS (in ng Pt/mg protein).

Sample	Cellular platinum concentration	
	2 h incubation	6 h incubation
Cisplatin	33.8	112
Pt(IV) complex 2	18.6	26.2
MPEG-b-PCL-b-PLL/Pt(IV) micelles	141.3	1097

4. Conclusion

An MPEG-b-PCL-b-PLL/Pt(IV) conjugate was prepared by covalently coupling a carboxyl-functionalized Pt(IV) complex to a biodegradable and amphiphilic tri-block copolymer, MPEG-b-PCL-b-PLL, with pendant amino groups. It could self-assemble into micelles with a mean diameter of 150–160 nm, a surface potential less than +10 mV, a CMC value of ca. 0.01 g/L, and a platinum content over 10 wt%. The micelles showed acid responsive and oxidation-reduction sensitive drug release kinetics. HPLC-ICP-MS was used to systematically analyze the platinum complexes (cisplatin, complex 1, complex 2, MPEG-b-PCL-b-PLL/Pt(IV)), under different pH values and in the presence of sodium ascorbate of typical concentrations. Based on the drug release profiles and HPLC-ICP-MS analyses, it was deduced that cisplatin is predominant in the released species of the conjugate micelles. In vitro evaluation showed that the MPEG-b-PCL-b-PLL/Pt(IV) micelles display higher cytotoxicity against SKOV-3 tumor cells than both cisplatin and Pt(IV) complex 2 because of their effective internalization by the cells. Therefore, the MPEG-b-PCL-b-PLL/Pt(IV) is a promising polymeric pro-drug of cisplatin and is expected to find clinical application in the future. The same strategy is applicable to carboplatin and oxaliplatin to develop their polymeric Pt(IV) pro-drugs.

Acknowledgements

Financial support was provided by the National Natural Science Foundation of China (Project No.21004062, 20674084, 50733003), and by the Ministry of Science and Technology of China ("973 Project", No. 2009CB930102; "863 Project", No. 2007AA03Z535).

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.06.072.

References

[1] Madias NE, Harrington JT. Platinum nephrotoxicity. Am J Med 1978;65:307–14.
[2] Thompson SW, Davis LE, Kornfeld M, Hilgers RD, Standefer JC. Cisplatin neuropathy. Clinical, electrophysiologic, morphologic, and toxicologic studies. Cancer 1984;54:1269–75.
[3] Ekborn A, Laurell G, Andersson A, Wallin I, Eksborg S, Ehrsson H. Cisplatin-induced hearing loss: influence of the mode of drug administration in the guinea pig. Hearing Res 2000;140:38–44.
[4] Giandomenico CM, Abrams MJ, Murrer BA, Vollano JF, Rheinheimer MI, Wyer SB, et al. Carboxylation of kinetically inert platinum(IV) hydroxy complexes. An entrée into orally active platinum(IV) antitumor agents. Inorg Chem 1995;34:1015–21.
[5] Reithofer M, Galanski M, Roller A, Keppler BK. An entry to novel platinum complexes: carboxylation of dihydroxoplatinum(IV) complexes with succinic anhydride and subsequent derivatization. Eur J Inorg Chem 2006;13:2612–7.
[6] Reithofer MR, Valiahdi SM, Jakupec MA, Arion VB, Egger A, Galanski M, et al. Novel di- and tetracarboxylatoplatinum(IV) complexes. Synthesis, characterization, cytotoxic activity, and DNA platination. J Med Chem 2007;50:6692–9.
[7] Hall MD, Mellor HR, Callaghan R, Hambley TW. Basis for design and development of platinum(IV) anticancer complexes. J Med Chem 2007;50:3403–11.
[8] Choi S, Filotto C, Bisanzo M, Delaney S, Lagasee D, Whitworth JL, et al. Reduction and anticancer activity of platinum(IV) complexes. Inorg Chem 1998;37:2500–4.
[9] Hall MD, Hambley TW. Platinum(IV) antitumor compounds: their bioinorganic chemistry. Coord Chem Rev 2002;232:49–67.
[10] Shi TS, Berglund J, Elding LI. Kinetics and mechanism for reduction of trans-dichlorotetracyanoplatinate(IV) by thioglycolic acid, l-cysteine, dl-penicillamine, and glutathione in aqueous solution. Inorg Chem 1996;35:3498–503.
[11] Maeda H, Sawa T, Konno T. Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. J Control Release 2001;74:47–61.
[12] Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Adv Enzym Regul 2001;41:189–207.
[13] Maeda H, Bharate GY, Daruwalla J. Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. Eur J Pharm Biopharm 2009;71:409–19.
[14] Aryal S, Hu C-M, Zhang LF. Polymer-cisplatin conjugate nanoparticles for acid-responsive drug delivery. ACS Nano 2010;4:251–8.
[15] Kolishetti N, Dhar S, Valencia PM, Lin LQ, Karnik R, Lippard SJ, et al. Engineering of self-assembled nanoparticle platform for precisely controlled combination drug therapy. Proc Natl Acad Sci USA 2010;107:17939–44.
[16] Dhar S, Daniel WL, Giljohann DA, Mirkin CA, Lippard SJ. Polyvalent oligonucleotide gold nanoparticle conjugates as delivery vehicles for platinum(IV) wareheads. J Am Chem Soc 2009;131:14652–3.
[17] Xie ZG, Guan HL, Chen XS, Lu TC, Chen L, Hu XL, et al. A novel polymer–paclitaxel conjugate based on amphiphilic triblock copolymer. J Control Release 2007;117:210–6.
[18] Xu XY, Zhang XF, Wang XH, Li YX, Jing XB. Comparative study of paclitaxel physically encapsulated in and chemically conjugated with PEG-PLA. Polym Adv Technol 2009;20:843–8.
[19] Hu XL, Liu S, Huang YB, Chen XS, Jing XB. Biodegradable block copolymer-doxorubicin conjugates via different linkages: preparation, characterization, and in vitro evaluation. Biomacromolecules 2010;11:2094–102.
[20] Ribaud P, Gouveia J, Misset JL, Mathé G. Phase I study of cis-dichloro-trans-dihydroxy-bis(isopropylamine)platinum IV (CHIP). Oncology 1986;43:78–82.
[21] Kurata T, Tamura T, Sasaki Y, Fujii H, Negoro S, Fukuoka M, et al. Pharmacokinetic and pharmacodynamic analysis of bis-acetato-ammine-dichloro-cyclohexylamine-platinum(IV) (JM216) administered once a day for five consecutive days: a phase I study. Jpn J Clin Oncol 2000;30:377–84.
[22] Latif T, Wood L, Connell C, Smith DC, Vaughn D, Lebwohl D, et al. Phase II study of oral bis (aceto) ammine dichloro (cyclohexamine) platinum (IV) (JM-216, BMS-182751) given daily x 5 in hormone refractory prostate cancer (HRPC). Invest New Drugs 2005;23:79–84.
[23] Ray GB, Chakraborty I, Moulik SP. Pyrene absorption can be a convenient method for probing critical micellar concentration (cmc) and indexing micellar polarity. J Colloid Interf Sci 2006;294:248–54.
[24] Hann S, Koellensperger G, Stefánka ZS, Stingeder G, Fürhacker M, Buchberger W, et al. Application of HPLC-ICP-MS to speciation of cisplatin and its degradation products in water containing different chloride concentrations and in human urine. J Anal Atom Spectrom 2003;18:1391–5.
[25] Galettis P, Carr JL, Paxton JW, McKeage MJ. Quantitative determination of platinum complexes in human plasma generated from the oral antitumour drug JM216 using directly coupled high-performance liquid chromatography-inductively coupled plasma mass spectrometry without desolvation. J Anal Atom Spectrom 1999;14:953–6.
[26] Duong HT, Huynh VT, de Souza P, Stenzel MH. Core-cross-linked micelles synthesized by clicking bifunctional Pt(IV) anticancer drugs to isocyanates. Biomacromolecules 2010;11:2290–9.
[27] Feazell RP, Nakayama-Ratchford N, Dai H, Lippard SJ. Soluble single-walled carbon nanotubes as longboat delivery systems for platinum(IV) anticancer drug design. J Am Chem Soc 2007;129:8438–9.