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Layer-by-Layer Assembled Polypeptide Capsules for Platinum-Based Pro-Drug Delivery

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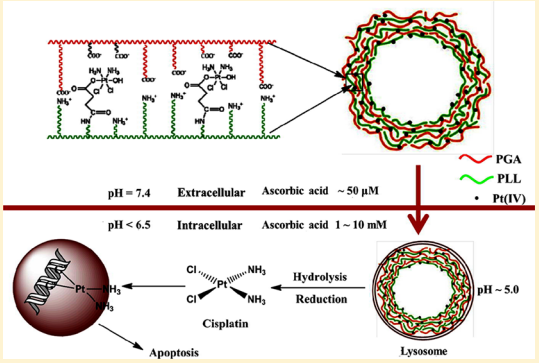
Supporting Information

ABSTRACT: Platinum(IV), a pro-drug of platinum(II), was conjugated to poly(L-lysine) (PLL), and then assembled with poly(glutamic acid) (PGA) through a layer-by-layer (LbL) approach on colloidal silica templates. After removal of the templates, biodegradable PGA/PLL-Pt(IV) multilayer capsules (diameter = 0.5 μm) with 10 μg of platinum incorporated into each bilayer were obtained. Under acidic and/or reductive conditions, the amount and rate of platinum released from the capsules were increased, which are desirable traits for platinum-based anticancer drug delivery systems. Furthermore, in vitro evaluation showed that the PGA/PLL-Pt(IV) multilayer microcapsules displayed higher cytotoxicity (IC_{50Pt} = 3.5 μg/mL) against colon cancer cells CT-26 than that of free cisplatin (IC_{50Pt} = 8.6 μg/mL). This enhanced cytotoxicity was attributed to the effective internalization of the capsules by the cancer cells, which was observed by confocal laser scanning microscopy (CLSM) imaging.



INTRODUCTION

The ability of platinum(II) to inhibit the growth of cancer cells by interfering with transcription and other DNA-mediated cellular functions has been elucidated over the past 30 years.[1] Platinum(II) is a widely used antitumor agent against a variety of solid tumors, such as testicular, breast, ovarian, bladder, lung, head, and neck cancer.[2,3] However, a number of side effects, such as nephrotoxicity, cumulative neurotoxicity, and ototoxicity, can occur which greatly limit its clinical applications.[4] In order to develop more reliable platinum anticancer agents, much attention has been paid to Pt (IV) complexes. Pt (IV) complexes with octahedral structure are known to have less toxicity compared to their Pt (II) counterparts,[5−8] but need to be reduced to the bioactive Pt (II) form by biological reductive agents, such as glutathione, ascorbic acid, and cysteine.[9,10] Therefore, Pt (IV) complexes can be considered to be pro-drugs of Pt (II) complexes. If such Pt (IV) complexes could retain their +4 valence state in the circulatory system but are then reduced to +2 valence state when they enter cancer cells, high antitumor efficacy without severe side effects could be attained. From this point of view, it is of great significance to increase cellular uptake and to protect Pt (IV) from immature reduction before they enter cancer cells.

Several types of inorganic nanoparticles have been applied to Pt (IV) pro-drug delivery.[11−14] However, detailed biocompatibility studies have shown that inorganic nanoparticles can cause severe systemic toxicity at high concentrations in vivo.[15,16] Polymeric nanocarriers, such as polymer particles, polymer micelles, polymer−drug conjugates, and polymer capsules are seen as promising alternative materials for drug delivery.[17,18] Polymeric nanocarriers usually enter the cancer cells with considerable efficiency and preferentially enter the cancerous tissue via the enhanced permeability and retention (EPR) effect.[19−21] Some efforts have been made to deliver Pt (IV) pro-drugs through polymer particles and polymer−drug conjugates.[22−25] Among the various polymeric nanocarriers, layer-by-layer (LbL) assembled polymer capsules are particularly attractive for drug delivery,[26] as the capsules can be loaded with biologically active cargos, degraded via various mechanisms, engineered with low-fouling properties, and have the potential for targeting.[27−31] However, the effective delivery and controlled release of chemotherapeutics using capsules remains a challenge, mainly due to leakage of small cytotoxic cargos from the inherently semipermeable LbL carrier systems.[32,33] Recent methods toward the controlled delivery of small cytotoxic cargos via LbL assembled polymer capsules include multiple compartments, mesoporous templates, and polymer−drug conjugates.[34−36] Due to limited solubility and

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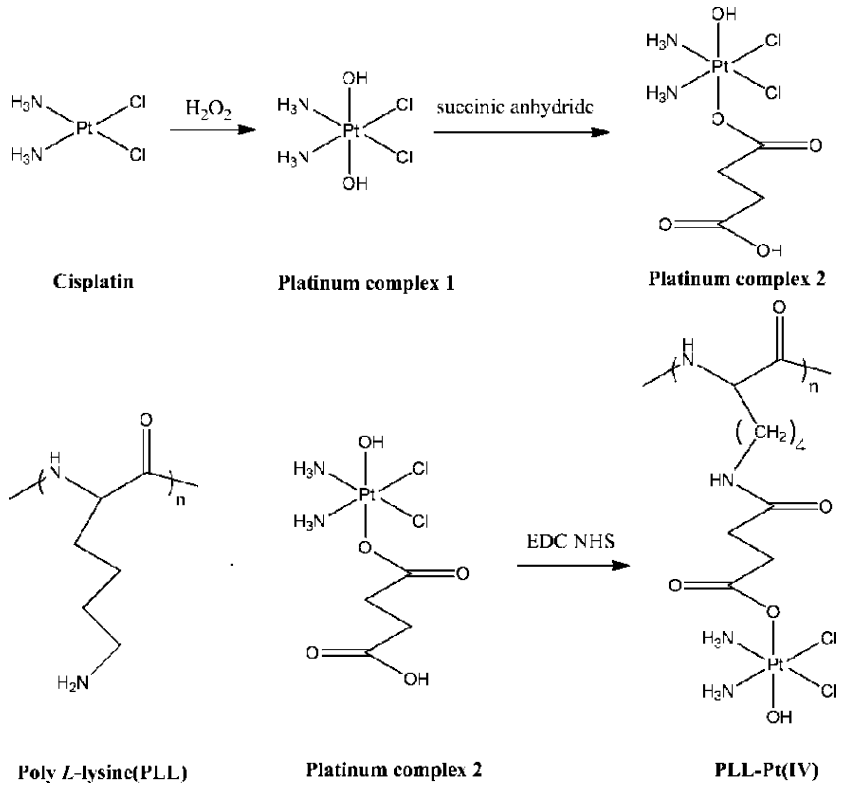
Scheme 1. Preparation of Platinum Complex 2 and PLL-Pt(IV) Conjugate



the absence of functional groups, it can be difficult to encapsulate platinum(II) into polymer capsules at high loadings and to effect controlled release.[37]

In this work, we used LbL polypeptide capsules as a carrier for platinum drug delivery. As a pro-drug of cisplatin(II), a Pt (IV) complex with a carboxyl group was synthesized and conjugated to the side chains of poly(L-lysine) (PLL), resulting in a polypeptide−drug conjugate, PLL-Pt(IV). PLL-Pt(IV) was then assembled with poly(glutamic acid) (PGA) through an LbL technique on a colloidal support into biodegradable, controllably drug-loaded, and environmentally responsive polypeptide capsules. Platinum release from the capsules was found to be greatly enhanced under low pH and reductive conditions. These platinum-loaded LbL capsules exhibited effective internalization by CT-26 colon cancer cells and showed higher cytotoxicity than that of free cisplatin, indicating that the platinum-loaded polypeptide microcapsules could be an option for platinum delivery in vivo and have potential applications in targeted intracellular cancer therapy.

EXPERIMENTAL PROCEDURES

Materials. Hydrogen peroxide (H_2O_2), hydrofluoric acid (HF), ammonium fluoride (NH_4F), *N*-hydroxysuccinimide (NHS), 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl), *N,N′*-dicyclohexyl carbodiimide (DCC), Rhodamine B, and succinic anhydride were purchased from Sigma-Aldrich and used as received. Cisplatin (purity 99%) was bought from Shandong Boyuan Chemical Company, China. Silica microspheres (0.5 and 1.5 μm) were purchased from Wuhan Sphere Scientific Corporation. Poly(L-lysine) (PLL, M_w 7000−9000 g/mol) and poly(glutamic acid) (PGA, M_w 8000−10 000 g/mol) were synthesized according to the reported method.[38] Water used in all experiments was prepared in a Millipore purification system and had a resistivity higher than 18 MΩ cm. All other chemicals were purchased from Sigma-Aldrich and used as received.

Methods. ζ-Potentials were measured on a Zetasizer 2000 (Malvern) instrument. Film thicknesses were determined using spectroscopic ellipsometer (Horiba Jobin Yvon). Typical measurements were carried out between 400 and 800 nm with a 2 nm increment. Layer thicknesses were extracted with the integrated software by fitting the experimental data with a classical wavelength dispersion model. For scanning electron microscopy (SEM) and transmission electron microscopy (TEM) measurements, 1 μL of a concentrated capsule solution was placed on a clean silicon wafer slide (or TEM grid) and allowed to dry. SEM samples were sputter-coated with gold and analyzed with a Philips XL 30 microscope at voltages of 10 kV. TEM images were obtained from a JEOL JEM-1011 electron microscope. Confocal laser scanning microscopy (CLSM) images were taken with an Olympus confocal system. Inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 6300, Thermoscientific, USA) was used to determine the platinum contents in samples. Energy-dispersive X-ray (EDX) analysis was carried out using NORAN Instruments Voyager. ^{1}H 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).

Synthesis of Pt (IV) Complex 2. Pt (IV) complex 2 (Scheme 1) was synthesized as described previously.[25] Briefly, succinic anhydride (100 mg, 1 mmol) was added to a DMSO solution (10 mL) of Pt (IV) complex 1 (c,c,t-[Pt-

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$(NH_3)_2Cl_2(OH)_2]$) (334 mg, 1 mmol), and the reaction mixture was stirred at 40 °C for 12 h. The solution was lyophilized, and acetone (10 mL) was added to precipitate a light yellow solid. The solid was washed three times with diethyl ether, and then dried to obtain platinum(IV) complex 2 with a yield of 62%. ESI-MS (negative mode): Calc. = 434, Found 433; ^{1}H NMR (DMSO-d_6): 6.00 (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, 10.95; H, 2.63; N, 6.52 (Figures S1−S3 in Supporting Information).

Preparation of PLL-Pt(IV) Conjugate (PLL-Pt(IV)). Pt (IV) complex 2 was conjugated to PLL via amidation in aqueous solution. Briefly, EDC.HCl (47 mg) and NHS (27 mg) were dissolved in deionized water with stirring. Pt (IV) complex 2 (37 mg) was then added into the above aqueous solution. After the mixture became clear, PLL (50 mg) in water (50 mL) was added, and the reaction mixture was stirred at room temperature for 24 h. Finally, the solution was dialyzed against water for another 24 h and lyophilized to obtain PLL-Pt(IV) conjugate (PLL-Pt(IV)).

Preparation of PLL-RhB and PLL-FITC Conjugates (PLL-RhB and PLL-FITC). Rhodamine B (6 mg), DCC (7 mg) and NHS (4 mg) were dissolved in 2 mL of DMSO. After 30 min, the solution was added into a DMSO solution (10 mL) of PLL (40 mg). The reaction was kept for 24 h at room temperature and the solution then dialyzed for 3 days against water to remove unreacted Rhodamine B. The RhB-labeled PLL conjugate (PLL-RhB) was finally obtained after lyophilization.

FITC (2 mg) was added to a water (50 mL) solution of PLL (50 mg) and the reaction mixture was kept stirring at room temperature for 12 h. The solution was then dialyzed against water for another 24 h and lyophilized to obtain the FITC-labeled PLL conjugate (PLL-FITC).

Multilayer Assembly on Planar Supports. Silicon wafer slides (10 × 10 mm^2) were treated with a mixture containing 2 mL of ethanol, 400 μL of 3-aminopropyltriethoxysilane (APTS), and 100 μL of 28% ammonia solution for 2 h to create positively charged surface. The slides were then rinsed thrice with ethanol, then water, and dried under a stream of nitrogen. The obtained wafer slides were sequentially immersed into PGA and PLL solutions (1 mg/mL) at pH 7.0. A period of 15 min was allowed for the deposition of each layer. Finally, the slides were rinsed with water at pH 7.0 three times and dried with nitrogen.

Multilayer Assembly on Colloidal Templates. Approximately 5 mg of silica spheres (0.5 or 1.5 μm) were treated with a mixture containing 1 mL of ethanol, 200 μL of APTS, and 60 μL of 28% ammonia solution for 3 h to create positively charged surface. The modified silica spheres were added to the PGA solution (1 mg/mL, pH was adjusted to 7.0 with 0.01 M of NaOH) with constant shaking for 15 min. After that, the particles were isolated by centrifugation (600 *g* for 3 min) and washed with 1 mL of water (pH = 7.0). The particles were redispersed, centrifuged, and washed three times. The PLL layer was then adsorbed to the particles using the same procedure above. The same cycle was repeated until three PGA/PLL bilayers were deposited (named $(PGA/PLL)_3$). For the assembly of drug-loaded or fluorescent-labeled capsules, PLL solution was substituted with PLL-Pt(IV), PLL-RhB, or PLL-FITC conjugate solution (1 mg/mL, pH = 7.0) to obtain $(PGA/PLL\text{-}Pt(IV))_3$, $(PGA/PLL\text{-}RhB)_3$, or $(PGA/PLL\text{-}FITC)_3$ microcapsules.

The hollow $(PGA/PLL)_3$, $(PGA/PLL\text{-}Pt(IV))_3$, $(PGA/PLL\text{-}RhB)_3$, and $(PGA/PLL\text{-}FITC)_3$ microcapsules were obtained by dissolving the silica sphere templates in HF/NH_4F solution (pH = 5.0) for 1 min, followed by three centrifugation (1000 *g* for 15 min)/water washing cycles.

Drug Release from $(PGA/PLL\text{-}Pt(IV))_3$ Capsules. A solution of 1.5 mL $(PGA/PLL\text{-}Pt(IV))_3$ capsules (0.5 μm) in a dialysis bag (molecular weight cutoff = 3.5 kDa) was immersed into 15 mL of PBS (0.1 mol/L, pH = 7.4 or 5.0). The dialysis was conducted at 37 °C in a shaking culture incubator. 0.5 mL of sample solution was withdrawn from the outer PBS medium at specified time intervals and measured for Pt concentration by ICP-OES. After sampling, an equal volume of fresh PBS was immediately added to keep the total volume of the outer phase constant. The platinum released from the capsules was expressed as the cumulative amount (percentage) of platinum outside the dialysis bag relative to the total amount of platinum in the capsules.

The same drug release procedure was also performed in the presence of sodium ascorbate (5.0 mM or 0.1 mM) in the dialysis bag.

MTT Assay. The cytotoxicity of cisplatin, Pt (IV) complex 2, $(PGA/PLL)_3$ capsules, and $(PGA/PLL\text{-}Pt(IV))_3$ capsules (0.5 μm) was assessed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Colon cancer cell line CT-26 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum, 0.03% L-glutamine, and 1% penicillin/streptomycin at 37 °C under 5% CO_2. The cells were split into 96-well plates (1×10^4 cells per well), and after 12 h incubation, cells were exposed to the solutions of cisplatin, Pt (IV) complex 2, $(PGA/PLL)_3$ capsules, and $(PGA/PLL\text{-}Pt(IV))_3$ capsules at six different concentrations. After further incubation for 24 h (37 °C, 5% CO_2), 20 μL of MTT in PBS (5 mg/mL) was added to each well and the plates were incubated for another 4 h at 37 °C, followed by removal of the culture medium containing MTT. 150 μL of DMSO was added to each well to dissolve the formed formazan crystals. The plates were then shaken for 10 min, and the absorbance of formazan was measured at 492 nm by a microplate reader.

Cell Internalization. Colon cancer cell line CT-26 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum, 0.03% L-glutamine, and 1% penicillin/streptomycin at 37 °C under 5% CO_2. The cells were seeded in 6-well plates (1×10^5 cells per well) and allowed to adhere overnight. Cells were then incubated with LysoTracker Red (1 μg/mL) in RPMI 1640 medium at 37 °C for 1 h, then incubated with $(PGA/PLL\text{-}FITC)_3$ capsules (0.5 μm) for 12 h. After three washes with PBS, 4% paraformaldehyde was added for another 30 min at 37 °C. Finally, nuclei were counterstained with the blue dye 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/mL) for 15 min at room temperature. Cellular uptake was observed with an Olympus FV1000 confocal laser scanning microscope (CLSM) imaging system (Japan).

RESULTS AND DISCUSSION

Synthesis of PLL-Pt(IV) Conjugate. It is difficult to encapsulate cisplatin(II) in polymer capsules or other polymeric nanocarriers due to the limited solubility and the absence of any functional groups.[37] To solve this problem, a soluble cisplatin pro-drug Pt (IV) complex 2 with a carboxyl group was synthesized. Then, the pro-drug was covalently

conjugated to a biodegradable and biocompatible polypeptide PLL to form the PLL-Pt(IV) conjugate. Controllable Pt content loading was realized by adjusting the grafting ratio of platinum complex 2 to PLL (Scheme 1). In the experiments described in this paper, the grafting ratio of Pt (IV) complex 2 to PLL was fixed at 10%, and the Pt content as measured by ICP-OES was 10.9% (w/w).

PGA/PLL-Pt(IV) Multilayer Film Assembly on Planar Supports. Direct structural analysis of the assembled multilayers on silica microspheres was hindered by a lack of appropriate analytical apparatus. As an alternative, alternating layers of PGA/PLL were sequentially deposited on silicon wafer slides, featuring a positively charged surface, using an LbL technique. In situ spectroscopic ellipsometry experiments were carried out to study the thickness variation as a function of the deposited layers of PGA/PLL multilayer films at pH 7.0 on these planar supports. As shown in Figure 1, the PLL/PGA

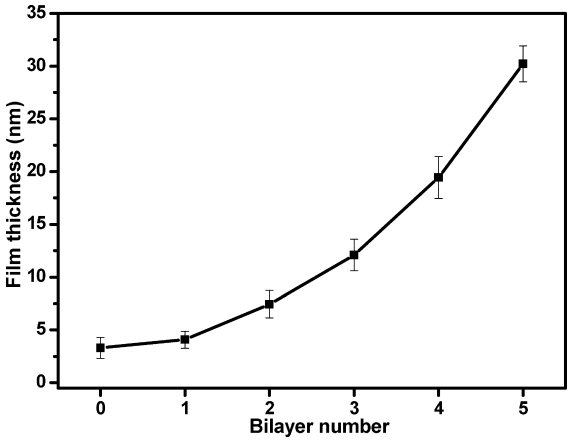

Figure 1. Film thickness of the PGA/PLL multilayer assembled on planar supports.

multilayer films exhibited a nonlinear growth tendency, which was quite different from the linear growth behavior of normal polyelectrolyte films.[39] This discrepancy could be explained by the fact that the growth of such multilayer films is entropy-driven and is an endothermic complexation process as evidenced by previous reports.[40−43]

PGA/PLL-Pt(IV) Multilayer Film Assembly on Colloidal Templates. Similar assembly behavior could be assumed with the microsphere system. Using the same conditions as those employed for planar supports, PGA/PLL bilayers were also deposited onto colloidal silica microspheres. Since PLL is positively charged (PI = 9) and PGA is negatively charged (PI = 3.5) at pH 7.0, the stepwise growth of the multilayer on the surface of silica spheres could be easily monitored by checking the ζ-potential change of the spheres after each deposition step (Figure 2). The original ζ-potential of the APTS-treated silica spheres was about +65 mV. During the polymer deposition procedure, the surface charge of the silica spheres alternated between −75 mV and +40 mV, as the PGA and PLL layers were successively deposited. This result indicated that after adsorption the charge on the surface of each layer was overcompensated, facilitating the adsorption of the next oppositely charged polypeptide, forming the multilayer film on the silica spheres.

The successful preparation and characterization of the PGA/PLL coated microspheres made a good foundation for the

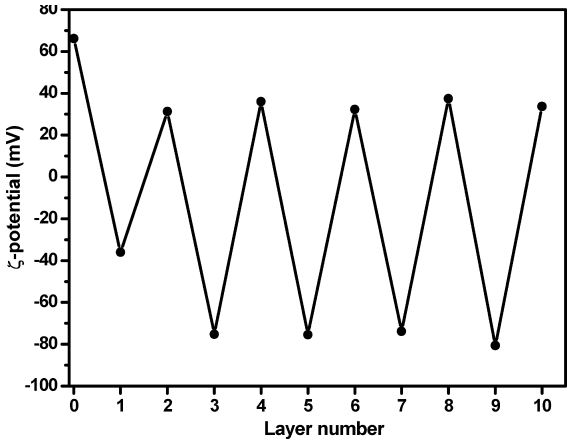

Figure 2. Variation in the ζ-potential for the deposition of PGA/PLL-Pt(IV) multilayer films on silica particles.

introduction of the PLL-Pt(IV) polymer−drug conjugate. This was achieved by simply replacing PLL with PLL-Pt(IV) using the same LbL assembly technique with PGA on silica templates. The assembly of the PGA/PLL-Pt(IV) multilayer on colloidal templates was observed by TEM and SEM (Figure 3). All the microspheres had a uniform morphology and did not show any obvious change in size after film coating. Compared with uncoated silica microspheres (Figure 3a), the polypeptide-treated microspheres (Figure 3b) showed a higher electron density on the shell, indicating successful surface coating. After removal of the silica templates, $(PGA/PLL\text{-}Pt(IV))_3$ capsules were obtained and the size of capsules could be easily adjusted by changing the size of the starting templates. In this work, $(PGA/PLL\text{-}Pt(IV))_3$ capsules with diameters of 0.5 and 1.5 μm were prepared. The larger-sized capsules were for the convenience of investigations on structure and composition. The typical collapsed structure (with folds and creases) of a range of polyelectrolyte capsules[44−46] was also examined (Figure 3c,d,f). The close contact between the capsules was due to the drying process required for TEM and SEM analyses. No shrinkage or breakage of the hollow capsules indicated that the $(PGA/PLL\text{-}Pt(IV))_3$ coating was stable under the dissolution conditions of the silica core.

In order to further confirm the structure of $(PGA/PLL\text{-}Pt(IV))_3$, RhB-labeled PLL (PLL-RhB) was used in the LbL multilayer assembly for confocal laser scanning microscopy (CLSM) imaging. The red fluorescence in the CLSM image confirmed the hollow vesicle-like structure of the $(PGA/PLL\text{-}RhB)_3$ capsules (Figure 3e). The capsule size of approximately 1.5 μm suggested that the resultant hollow capsules exhibited the same shape and size as the original silica microsphere templates.

Energy-dispersive X-ray (EDX) was used to analyze the chemical constitution of the capsules. As shown in Figure 4, the existence of a Pt peak clearly demonstrated the successful incorporation of Pt (IV) onto the silica microspheres. Si signal disappearance after template removal verified the complete dissolution of the silica cores. The Pt peak remained after dissolution of the Si core, confirming that the conjugation between Pt (IV) and PLL was stable under the silica dissolution conditions.

The amount of Pt in the LbL multilayer assembly system was determined using ICP-OES measurements (Figure 5). After deposition of a PGA/PLL-Pt(IV) bilayer, the Pt concentration

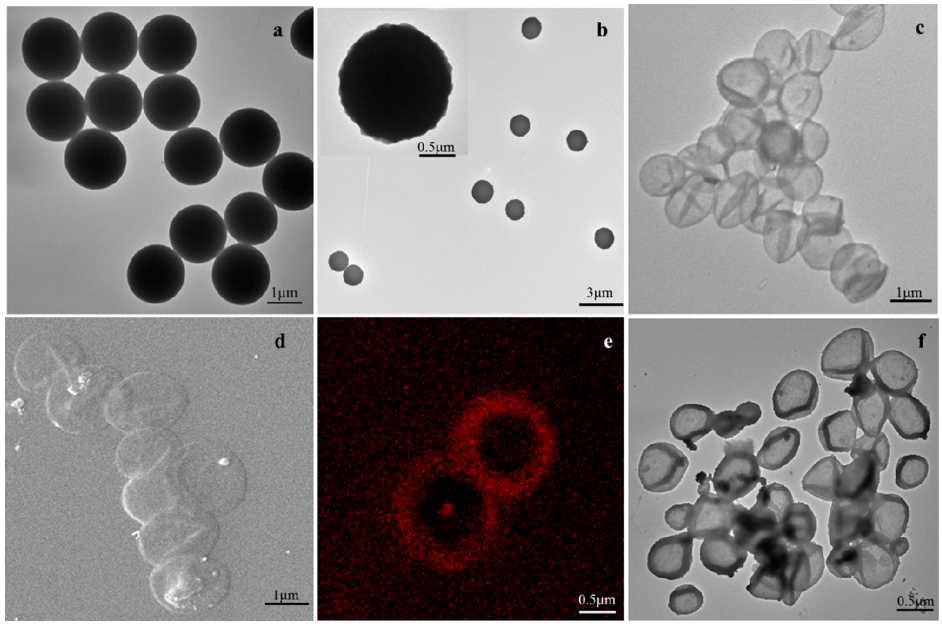

Figure 3. Images of the particles and capsules. TEM images of silica particles before (a) and after (b) coating with $(PGA/PLL\text{-}Pt(IV))_3$ films; TEM (c), SEM (d), and CLSM (e) of 1.5 μm $(PGA/PLL\text{-}Pt(IV))_3$ capsules and TEM (f) of 0.5 μm $(PGA/PLL\text{-}Pt(IV))_3$ capsules.

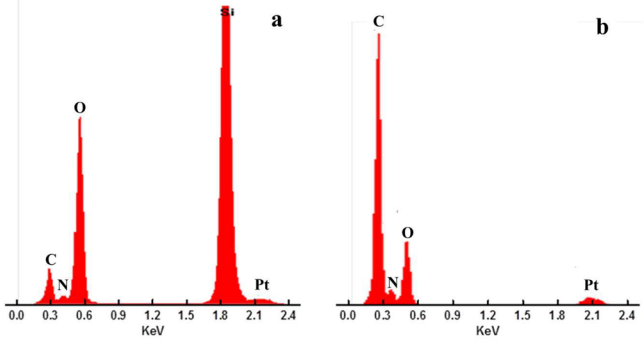

Figure 4. EDX analysis of the particles and capsules. (a) Silica particles coated with $(PGA/PLL\text{-}Pt(IV))_3$ films. (b) $(PGA/PLL\text{-}Pt(IV))_3$ capsules after silica template removal.

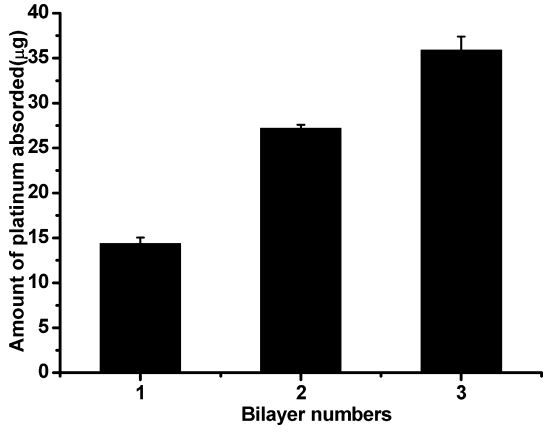

Figure 5. Amount of platinum incorporated per bilayer in the PGA/PLL-Pt(IV) multilayer capsules.

in the supernatant was measured. Compared to the total amount of Pt applied for assembly, the amount of Pt adsorbed onto the silica microspheres (0.5 μm) could be calculated. The amount of Pt incorporated in each bilayer on silica microspheres followed an approximately linear trend (~10 μg per bilayer), which implied that the amount of incorporated drug could be precisely controlled.

It was assumed that the varied size of $(PGA/PLL\text{-}Pt(IV))_3$ capsules would not lead to any property changes; but for practical applications, capsules with smaller diameter (such as 0.5 μm) would be more suitable for drug delivery via blood circulation and aid cell internalization. In these regards, only the $(PGA/PLL\text{-}Pt(IV))_3$ capsules with diameter of 0.5 μm were used in the following biorelated experiments.

Drug Release from $(PGA/PLL\text{-}Pt(IV))_3$ Capsules. Drug release experiments from the $(PGA/PLL\text{-}Pt(IV))_3$ capsules were performed with variable pH and different concentrations of reductive agent (sodium ascorbate). Again, ICP-OES was used to determine the amount of released Pt. The weight ratio of cumulative released Pt to the total Pt payload in the $(PGA/PLL\text{-}Pt(IV))_3$ capsules was calculated as a function of release time. Figure 6a shows the release kinetics for the $(PGA/PLL\text{-}Pt(IV))_3$ capsules at pH 5.0 and pH 7.4, respectively. Overall, the rate of Pt release was much greater from the capsules at pH 5.0 than at pH 7.4. Almost 40% of the Pt was rapidly released during the initial 10 h at pH 5.0 and Pt continued to be released to >60% in 120 h at pH 5.0. In contrast, less than 25% Pt release was detected after 120 h at pH 7.4. The reason for faster platinum release rate from the $(PGA/PLL\text{-}Pt(IV))_3$ capsules under acidic conditions was thought to be the increased hydrolysis of the amide or ester bonds between the Pt(IV) pro-drug and PLL. Acidic degradation of the polypeptide layers could also have contributed to some extent to this rapid release behavior. This character ensured long-term circulation of $(PGA/PLL\text{-}Pt(IV))_3$ capsules without significant Pt loss before reaching the tumor site, followed by increased Pt release in the acidic environment of the cancer cells.

As described above, only when the Pt (IV) species are reduced to Pt (II) species like cisplatin(II) can they display antitumor activity. The reduction of Pt (IV) pro-drug is more likely to take place inside cells which possess a reductive environment, such as the presence of glutathione or other

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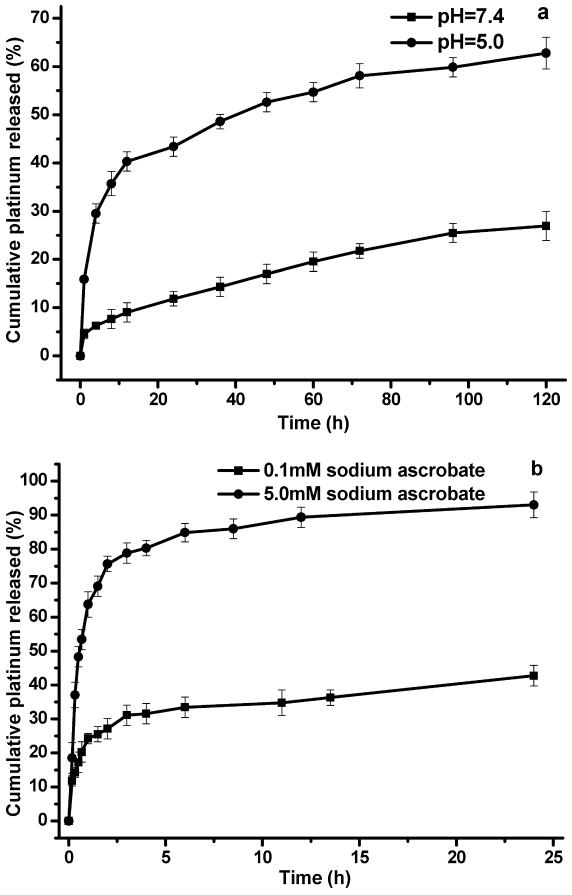


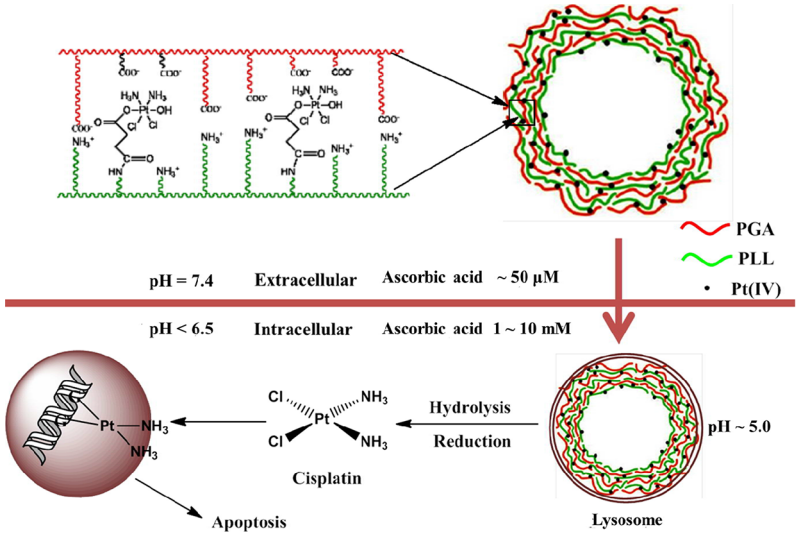
Figure 6. Platinum release profiles of $(PGA/PLL\text{-}Pt(IV))_3$ capsules. (a) In PBS at pH = 7.4 and pH = 5.0; (b) in the presence of 0.1 and 5.0 mM of sodium ascorbate.

reductive agents. Therefore, the $(PGA/PLL\text{-}Pt(IV))_3$ capsules could be considered a pro-drug of cisplatin(II). Under reductive conditions, Pt (IV) complexes in the $(PGA/PLL\text{-}Pt(IV))_3$ capsules could be reduced to the active Pt (II) species and released from the carrying capsules (Scheme 2). In order to mimic the reductive environment in blood plasma and within cells, release experiments from the $(PGA/PLL\text{-}Pt(IV))_3$ capsules were carried out in the presence of sodium ascorbate. As shown in Figure 6b, a higher concentration of reductive agent induced much faster Pt release. About 75% of the total Pt amount was released from the capsules within 2 h in the presence of 5 mM of sodium ascorbate, (corresponding to the same concentration as GSH within tumor cells[47]). This was nearly three times faster than with 0.1 mM of sodium ascorbate (the ascorbic acid concentration in blood plasma[48]). This suggested that Pt (II) could be selectively released from $(PGA/PLL\text{-}Pt(IV))_3$ capsules under reductive conditions in vivo, providing a potentially excellent intracellular targeting delivery system for platinum drugs.

When 5 mM of sodium ascorbate was applied, enhanced Pt release at pH 7 compared to that at pH 5 (in the absence of sodium ascorbate) was observed. This suggested that the $(PGA/PLL\text{-}Pt(IV))_3$ capsules were more sensitive to a reductive environment than to pH, which could be attributed to the difference in rate between reduction and hydrolysis. These results indicate that platinum drug could be well-protected by the $(PGA/PLL\text{-}Pt(IV))_3$ capsules during blood circulation, but exhibit burst release after internalization into cancer cells.

In Vitro Cytotoxicity. Next, the cytotoxicity of $(PGA/PLL\text{-}Pt(IV))_3$ capsules, together with cisplatin, Pt (IV) complex 2, and $(PGA/PLL)_3$ capsules, was examined against colon cancer cells CT-26 using the MTT assay after incubation for 24 h at 37 °C (Figure 7). Nearly 90% cell viability was observed for the $(PGA/PLL)_3$ capsules, even at high concentrations, indicating that the $(PGA/PLL)_3$ capsule was a safe drug delivery system. As expected, cisplatin exhibited a higher cytotoxicity (IC_{50Pt} = 8.6 μg/mL) than Pt (IV) complex 2 (IC_{50Pt} > 10 μg/mL), since Pt (IV) needs to be reduced to Pt (II) to become bioactive. Surprisingly, the $(PGA/PLL\text{-}Pt(IV))_3$ capsules exhibited a somewhat higher cytotoxicity (IC_{50Pt} = 3.5 μg/mL) than cisplatin. It is proposed that this enhanced cytotoxicity was due to effective higher intracellular accumulation of Pt caused by a high degree of internalization of the $(PGA/PLL\text{-}Pt(IV))_3$ capsules. In contrast to free cisplatin, which is known to

Scheme 2. Schematic Illustration of the Structure of $(PGA/PLL\text{-}Pt(IV))_3$ Microcapsules and Intracellular Release of Cisplatin(II)



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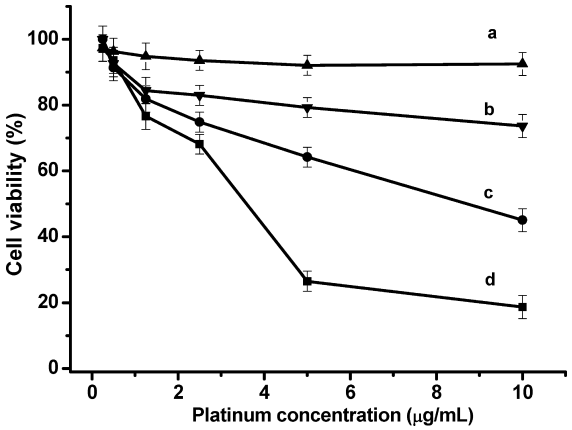

Figure 7. Cytotoxicity of different drug systems after 24 h incubation against CT-26 cells at 37 °C, 5% CO_2. (a) $(PGA/PLL)_3$ capsules, (b) Pt (IV) complex 2, (c) cisplatin, and (d) $(PGA/PLL\text{-}Pt(IV))_3$ capsules.

enter cells by passive diffusion, the $(PGA/PLL\text{-}Pt(IV))_3$ capsules were internalized with high efficacy (supported by the cellular internalization experiments in the next section). It is possible that reduction of the capsules in the cell plasma and hydrolysis in late endosomes or lysosomes could bring about a higher concentration of bioactive Pt (II) species inside the cancer cells, affording a higher cell-killing capacity than the pure drug (cisplatin).

Cell Internalization. Previous reports have demonstrated that submicrometer-sized drug-loaded LbL polymer capsules can be internalized by several different cell types (e.g., dendritic cells, monocytes, and colorectal cancer cells) via macropinocytosis or phagocytosis and display increased cytotoxicity than the corresponding low-molecular-weight drugs alone.[49−51] To correlate the observed cytotoxicity with cellular uptake and release of platinum, the internalization and intracellular distribution of the $(PGA/PLL\text{-}FITC)_3$ capsules was observed by CLSM after 12 h incubation with CT-26 cells (Figure 8). $(PGA/PLL\text{-}FITC)_3$ capsules here served as the fluorescent probe (green fluorescence) in the place of $(PGA/PLL\text{-}Pt(IV))_3$ capsules. To visualize the endocytic compartments, lysosomes were labeled with LysoTracker Red (red fluorescence). The CLSM images clearly revealed the internalization of $(PGA/PLL\text{-}FITC)_3$ capsules, with obvious green fluorescence visible in the cells after 12 h incubation. The majority of the internalized $(PGA/PLL\text{-}FITC)_3$ capsules were confirmed to be localized in the lysosomal compartments; colocalization of the red and green fluorescence was observed. These results implied that cell internalization and fusion with lysosomes were feasible with the drug-loaded $(PGA/PLL))_3$ capsules with a diameter of 0.5 μm. Compared with free cisplatin(II), a greater amount of Pt complex could be transported into the cancer cells in the form of capsules, achieving a higher intracellular concentration of Pt. The active Pt (II) could then be released under the acidic environment of the lysosomes and the reductive environment of the cytoplasm, which might be the reason for the increased efficiency of tumor cell kill of the $(PGA/PLL\text{-}Pt(IV))_3$ capsules.

CONCLUSIONS

This paper provided the first detailed investigation of layer-by-layer assembled polymer capsules for platinum-based pro-drug delivery. $(PGA/PLL\text{-}Pt(IV))_3$ microcapsules, with 10 μg of platinum incorporated into each bilayer, were obtained through

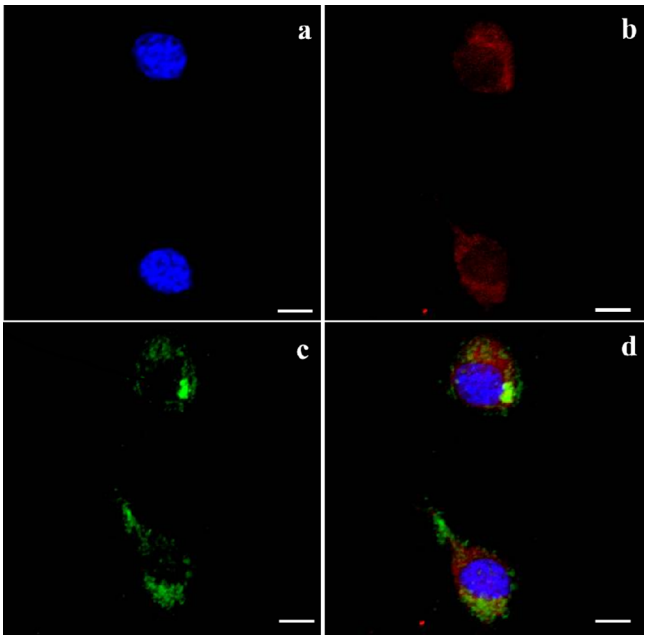

Figure 8. CLSM images of CT-26 cells with internalized $(PGA/PLL\text{-}FITC)_3$ capsules (0.5 μm) for 12 h at 37 °C, 5% CO_2. (a) Nuclear DNA was stained blue with DAPI. (b) Late endosomes and lysosomes were stained with LysoTracker Red. (c) Green fluorescence corresponds to $(PGA/PLL\text{-}FITC)_3$. (d) Overlaid image. All scale bars = 10 μm.

LbL assembly of PGA and a PLL-Pt(IV) conjugate on colloidal silica templates. The $(PGA/PLL\text{-}Pt(IV))_3$ microcapsules showed enhanced Pt release under low pH and reductive conditions. In vitro evaluation showed that these $(PGA/PLL\text{-}Pt(IV))_3$ microcapsules had higher cytotoxicity against CT-26 tumor cells than free cisplatin, likely due to the enhanced cell internalization of Pt in the form of capsules. These biodegradable polypeptide capsules could also provide an excellent vehicle for delivery of other pro-drugs or insoluble anticancer drugs. In an attempt to further improve the therapeutic efficacy, future studies will be focused toward surface functionalization of the drug-loaded LbL capsules with targeting ligands for the promotion of enhanced cell uptake and specificity. In vivo evaluation of the platinum-loaded polymer capsules described herein is also underway.

ASSOCIATED CONTENT

Supporting Information

IR, ^{1}H NMR, and ESI mass spectra of Pt (IV) complex 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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