Polymer–metal complex micelles for the combination of sustained drug releasing and photodynamic therapy†

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Polymer–metal complex micelles (PMCMs) containing cisplatin (*cis*-dichlorodiammineplatinum(II), CDDP), an anticancer drug, were prepared by a coordination bond of CDDP to dendrimer phthalocyanine (DPc) and poly(ethylene glycol)-block-poly(L-aspartic acid) (PEG-PLAn; molecular weight of PEG segment = 12 000 g mol^{-1}; polymerization degrees of aspartic acid segment n = 68, 96). Transmission electron microscopy (TEM) and laser light scattering (LLS) exhibited formation of unimodal $PMCM_{68}$ and $PMCM_{96}$ with average sizes of 108 and 135 nm, respectively. PMCMs were very stable in 10 mM phosphate buffer solution (PBS) without NaCl to maintain their shape and size over a month. However, PMCMs slowly released CDDP when they were incubated in physiological saline PBS solution at 37 °C. Upon laser light irradiation, generation of singlet oxygen was detected by photo-luminescence observation. The PMCMs would be effective nano-devices for anticancer drug carriers with sustained drug release and photodynamic therapy (PDT).

Introduction

Polymeric micelles have received great attention as targeted drug delivery systems.[1–4] Many types of polymeric micelles are currently undergoing clinical investigation for malignant tumor treatment.[5–8] A polymeric micelle offers effective steric protection from external environments and has numerous potential advantages, such as improved bioavailability, reduced drug toxicity and side-effects, and substantial changes in drug biodistribution, including tumor targeting *via* the enhanced permeability and retention (EPR) effect.[9–11] Moreover, target-specific moieties can be incorporated into the polymeric shell to achieve active drug targeting to specific organs. In general, amphiphilic block copolymers were utilized for the formation of polymeric micelle, where the hydrophobic segments associate with each other to avoid contact with aqueous solvent. In addition to the hydrophobic interactions, recently various alternative interactions, such as metal coordination and electrostatic interactions, have been utilized to build up polymeric micelles. Representatively, block copolymer having hydrophilic and anionic segments has been utilized to prepare a polymeric micelle encapsulating cisplatin (*cis*-dichlorodiammineplatinum(II), CDDP; Fig. 1), where the driving force for the micelle formation is metal coordination interaction between Pt ions and anionic segments.[12,13] CDDP is one of the most widely used anticancer drugs. It is particularly effective in treating testicular and ovarian cancer and has increasingly been used against cervical and small-cell lung cancer.[14–16] Despite its great efficiency, the clinical application of CDDP is limited because of its severely toxic side effects, low solubility and an extremely short circulation period in blood due to glomerular excretion.[17–19] The cisplatin-loaded polymeric

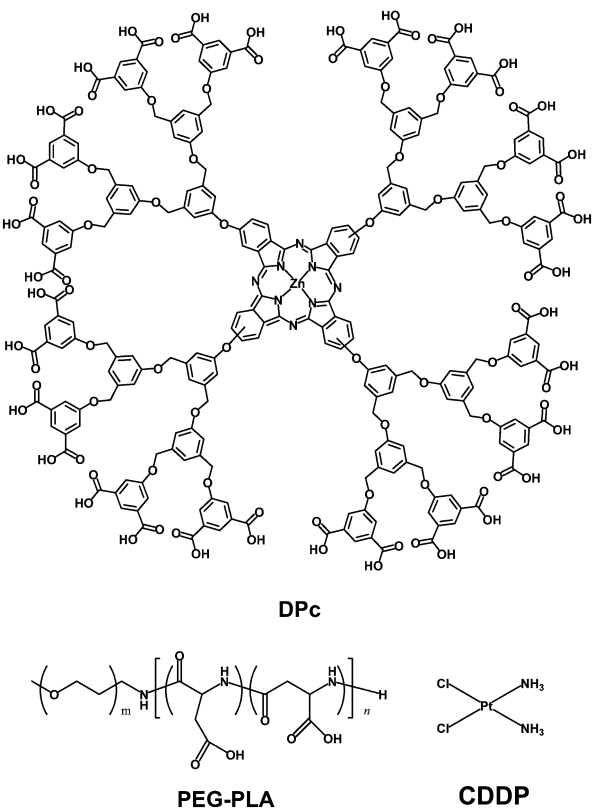

Fig. 1 Structures of DPc, PEG-PLA, and CDDP.

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micelles reported are expected to improve these problems by reduced side effects and increased tumor localization.

Photodynamic therapy (PDT) has also attracted interest as a less invasive but effective treatment for solid tumors. PDT is based on the administration of photosensitizers. Upon appropriate light irradiation, photosensitizers can be converted to excited triplet state and transfer their excitation energy or electron to intracellular oxygen. Through the above processes, reactive oxygen species (ROS) can be generated, and the oxidative destruction of malignant tissue occurs due to the high reactivity of ROS. Recently, we have reported ionic dendrimer phthalocyanine (DPc; Fig. 1) as an efficient photosensitizer for PDT.[20–23] The charged ionic surface can form polyion complex micelles by means of electrostatic interaction with oppositely charged block copolymer. The substitution of large dendritic wedges can effectively prevent the aggregate formation of the core photosensitizing unit even in the highly concentrated micellar core. The micellar formulation of DPc exhibited remarkably high photodynamic efficacy.

Recently, the combination of cisplatin with radiotherapy or PDT has been proposed as a very effective cancer treatment. For example, a combination of ionizing X-ray radiation with CDDP has been shown to lead to the enhancement of DNA damage.[24,25] Moreover, the combination of CDDP with photosensitizers, such as indocyanine green, and photofrin led to significant increments in the cytotoxic and apoptotic deaths of cancer cells.[26–30]

In this paper, we report a new type of polymeric micelle system for the combination of CDDP with PDT. Polymer–metal complex micelles (PMCMs; Fig. 2) were formed by coordination interaction of CDDP with DPc and poly(ethylene glycol)-block-poly(L-aspartic acid) (PEG-PLA*n*; molecular weight of PEG segment = 12 000 g mol^{-1}; polymerization degrees of aspartic acid segment n = 68, 96; Fig. 1), where the DPc and PEG-PLA were synthesized by a previously reported procedure.[31,32]

Experimental

Instruments

The UV–Vis spectrum was measured using a V-660 spectrometer (JASCO, Japan). ICP-MS was performed on a PQ Exell (Thermo Elemental, USA). Transmission electron microscope (TEM) images were obtained using a JEM-2100 (JEOL, Japan) operated at 200 kV. The specimens for TEM were prepared by dropping the solution onto Formvar/Carbon grid (300 mesh, Cu, 50/pKg) and then dried at room temperature for 24 h. The size of PMCMs was measured by laser light scattering (LLS) system using BI-9000AT/200SM. (Brookhaven Instruments Corporation, Center for Advanced Colloidal Materials, Dongguk University).

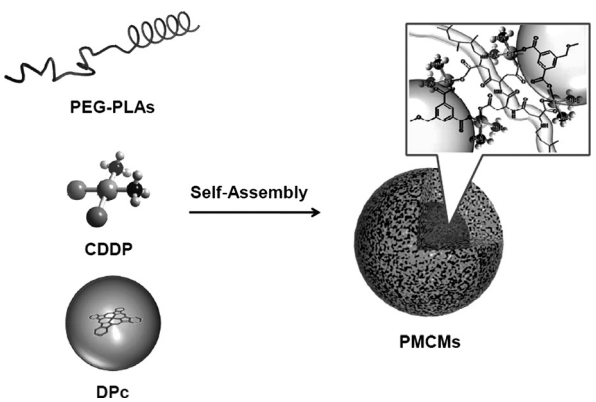

Fig. 2 Formation of PMCMs.

Materials

All chemicals were purchased from commercial sources and used as received. PEG-PLAs were synthesized by a previously reported procedure.[31,32] Briefly, the *N*-carboxyl anhydride of benzyl-L-aspartate was polymerized by initiation with CH_3O-PEG-NH_2 (12 000 g mol^{-1}) in DMF under Ar, followed by deprotection of the benzyl groups using aqueous 1 M NaOH solution. GPC measurement of PEG-PLAs exhibited single sharp peaks. From the ^{1}H NMR measurements in D_2O, the polymerization degrees of the PLA segment were determined to be 68 and 96, respectively. DPc was prepared from dimethyl-5-hydroxyisophthalate and 4-nitrophthalonitrile according to previously reported procedure. CDDP was purchased from Sigma-Aldrich.

Formation of PMCMs

To a 10 mM NaCl-free PBS solution (5 mL) containing DPc (3.89 mg, 1 eq.) and PEG-PLA_{68} (14.8 mg, 1 eq.), CDDP (1.00 mg, 48 eq.) solution in 10 mM NaCl-free PBS (5 mL) was added at once, and then stirred for 24 h at 25 °C under dark conditions to obtain a solution containing $PMCM_{68}$. For the preparation of $PMCM_{96}$, DPc (2.89 mg, 1 eq.), PEG-PLA_{96} (11.7 mg, 1 eq.) and CDDP (1.00 mg, 72 eq.) were treated in the same manner as the $PMCM_{68}$ formation.

Determination of drug loading capacity and measurement of CDDP release from PMCMs

The loading capacity of CDDP in PMCMs was confirmed by the stannous chloride method.[33] PMCM solutions were ultrafiltered for 15 min at 15 000 rpm using Amicon® centricon centrifugal devices (MW: 3000, Millipore Co., USA). The filtrate was mixed with 4 M HCl–0.4 M $SnCl_2$ solution in a ratio of 1 : 1. The absorbance at 407 nm of Pt–$SnCl_2$ complex was measured by spectrophotometer to determine the amount of Pt ions in the filtrate.

To measure the release profile of CDDP from PMCMs, the solutions of PMCMs in 2 mL of NaCl-free and saline (150 mM NaCl) 10 mM PBS were put into the dialysis bags (Spectra/Por® dialysis membrane, MWCO: 3500, Spectrum Laboratories Inc., USA) and dialyzed with 200 mL of NaCl-free and saline 10 mM PBS (150 mM NaCl), respectively, at 37 °C for 2 days. At a definite time interval, 100 μL of solution outside of the dialysis bag was sampled and treated with 9.9 mL of 2% HNO_3. Then, the concentration of Pt ions was measured by ICP-MS analysis.

Time-resolved photo-luminescence of the singlet oxygen

Time-resolved photo-luminescence from singlet oxygen relaxation was measured using a Nd-YAG pumped optical parametric oscillator (OPO) laser (B. M. Industries, OP901-355, 5 ns

FWHM pulse) as an excitation laser beam. Photo-luminescence from the sample was detected at an angle perpendicular to the excitation beam. The signals were collected through the interference filter (1270 nm, spectrogon), the cut-off filter (<1000 nm, CVI), and the germanium photodiode (EG & G, spectrogon). The signal was acquired by a 500 MHz digital oscilloscope and transferred to a computer for data analysis.

Because singlet oxygen has a very short lifetime (1–3 μs) in an aqueous medium and the solution has strong light scattering due to the large hydrodynamic volume of PMCMs, the direct observation of photo-luminescence from singlet oxygen was extremely difficult in the PBS solution. Therefore, the solvent of PMCM was diluted with ethanol to elongate the lifetime of the singlet oxygen.

Results and discussion

PMCMs were prepared by simple mixing of solutions containing PEG-PLAs, DPc, and CDDP in 10 mM NaCl-free PBS, where the formation of PMCMs was confirmed by TEM and LLS measurements. Because the driving force of PMCM formation is ligand exchange reaction of Pt atoms from chloride to carboxylates, the reaction takes place in a relatively slow manner. Therefore, the reaction mixtures were stirred for 24 h at 25 °C. When the same amounts of DPc and CDDP were mixed in 10 mM NaCl-free PBS, insoluble precipitation was occurred, indicating that the PEG-PLAs play critical roles for the formation of PMCMs. On the other hand, the mixture solution of PEG-PLAs and DPc did not afford micellar formulation, because both negatively-charged PLA segments and DPc cannot associate with each other. DPc has strong Q band absorption around 685 nm in aqueous solution. By the formation of PMCMs, Q band absorption of DPc was slightly changed to 630 nm, indicating that the microenvironment of DPc was changed (Fig. 3). This spectral change coincides with the incorporation of DPc into the micellar core when DPc formed polyion complex micelles, which has been reported in our previous paper.[34]

For the preparation of the ternary complex system, various combinations of mixing ratio should be considered. To find the optimum condition for PMCM formation, firstly the mixing ratio of DPc and PEG-PLAs was fixed to 1 : 1. Then the amounts of CDDP addition were varied from 12 to 96 equivalent amounts

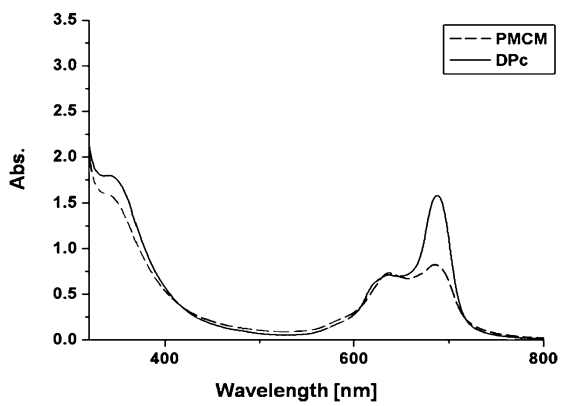

Fig. 3 Absorption spectra of DPc (5.95×10^{-5} M) and $PMCM_{96}$ (5.95×10^{-5} M) in 10 mM PBS at 298 K.

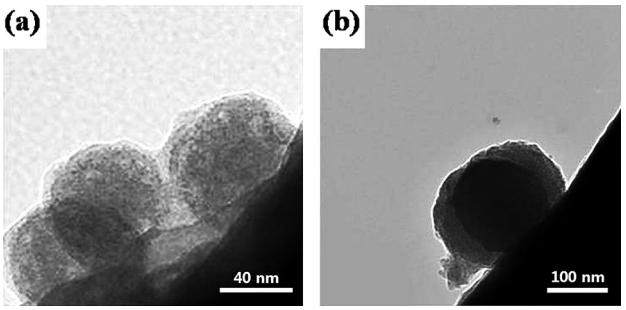

Fig. 4 TEM images of $PMCM_{68}$ (a) and $PMCM_{96}$ (b).

to those of DPc and PEG-PLAs. A unimodal distribution of $PMCM_{68}$ formation was confirmed by LLS measurement when the addition amount of CDDP was reached to 48 equivalents. For the $PEG\text{-}PLA_{96}$, 72 equivalents of CDDP were needed to form a unimodal distribution of $PMCM_{96}$ formation. Because the sizes of $PMCM_{68}$ and $PMCM_{96}$ were not increased though the amounts of CDDP were increased over 48 and 72 equivalents, respectively, those ratios of CDDP would be optimum amounts for the formation of PMCMs.

As shown in Fig. 4, globular shape of PMCMs has been confirmed by TEM measurement, where the average sizes were 97 and 140 nm for $PMCM_{68}$ and $PMCM_{96}$, respectively. LLS measurement exhibited that the average diameters of $PMCM_{68}$ and $PMCM_{96}$ were 108 nm and 135 nm, respectively, with remarkably narrow distributions, where the polydispersity indices (μ_2/Γ^2) were 0.011 and 0.028, respectively, by the cumulant method (Fig. 5).

To determine the loading amount of CDDP in PMCMs, the amount of Pt ions in the ultrafiltrate of the PMCM solutions was

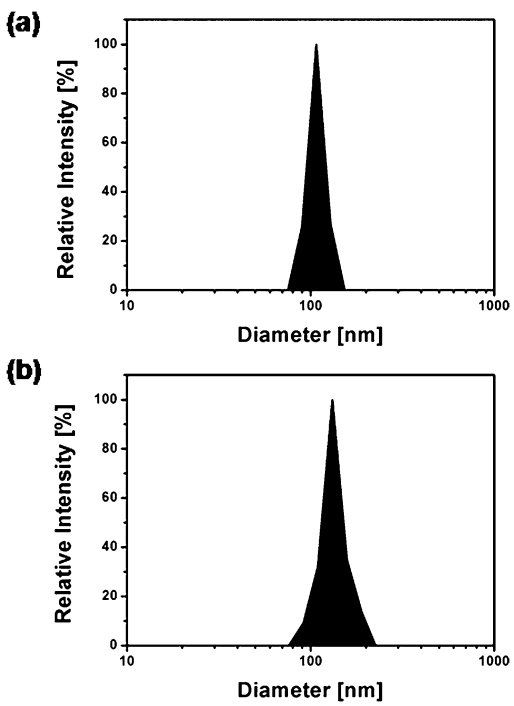

Fig. 5 Size distributions of $PMCM_{68}$ (a) and $PMCM_{96}$ (b).

measured by the stannous chloride method.[33] Because the cut-off MW of the ultrafiltration device is 3000, only free CDDP can pass through the membrane. Consequently, the weight proportions of CDDP in the residue of ultrafiltration were determined to be 24.1 ± 0.3 and 27.0 ± 1.4 wt% for $PMCM_{68}$ and $PMCM_{96}$, respectively. The residues were redispersed in a fixed amount of 10 mM NaCl-free PBS, and then again ultrafiltered to determine more accurate values of loading capacity. As a result, the loading capacities of CDDP in $PMCM_{68}$ and $PMCM_{96}$ were determined to be about 23 and 25 wt%, where the loading efficacy was 59 and 53%, respectively. This high loading capacity has large pharmaceutical merits as total dose of a formulation can be reduced.

The driving force of PMCM formation is ligand exchange reaction between the chlorides of CDDP to the carboxylates of DPc and PEG-PLAs. It is well known that the two chloride ligands of CDDP can be substituted to various reactive groups depending on the concentration of chloride ions in the surroundings.[35] When the chloride ligands of CDDP are substituted by carboxylates, the newly formed carboxylic ligands are reversibly cleavable due to their fairly low nucleophilicity. As the coordination bond forms between Pt atoms and carboxylate groups of DPcs and PLA segments, an insoluble crosslinked core can be formed and thereby hydrophilic PEG segments work as a hydrophilic shell.

The PMCMs are very stable in 10 mM PBS without NaCl and maintain their shape and size for over a month. However, they slowly released CDDP when they were incubated in physiological saline PBS at 37 °C. To measure the release profile of CDDP from PMCMs, the two types of solution, PMCMs in NaCl-free PBS and saline PBS, were dialyzed. The concentration of Pt ions in dialysate was measured by ICP-MS analysis. In the case of PMCMs in NaCl-free PBS, the amount of CDDP coming out of the dialysis bag was saturated to about 40% within 3 h. The amount of CDDP remaining in the dialysis bag coincides well with the weight proportion of CDDP in PMCMs which has been obtained by ultrafiltration, 41 and 47% for $PMCM_{68}$ and $PMCM_{96}$, respectively, indicating that non-associated CDDPs only diffused from the dialysis bag. In the case of the physiological saline condition, the CDDP concentration of dialysate continuously increased until 45 h. To remove the effect of non-associated CDDP diffusion, the difference between CDDP concentration in diaylsate under NaCl-free and physiological saline conditions were collected. As a result, the CDDP release from PMCMs continuously increased to reach 35% at 45 h driven by the ligand exchange reaction from carboxylate to chloride (Fig. 6a). Interestingly, $PMCM_{96}$ exhibits a slightly retarded release of CDDP compared to $PMCM_{68}$. The long PLA segments which combine with CDDP would result in high crosslinking density; thereby $PMCM_{96}$ might have the induction period for the release of CDDP. From the above results, drug release characteristics as well as the size of PMCMs is possibly controlled by the change of anionic chain length.

In our previous paper, we reported that DPc was an effective photosensitizer for PDT.[36–38] Photodynamic therapy is based on the accumulation of a photosensitizer in malignant tissue. The large hydrodynamic volume of PMCMs is potentially very useful for effective delivery of photosensitizers through the EPR effect.[9–11] To test the oxygen sensitizability of PMCM, the photo-luminescence of singlet oxygen was observed under the pulsed laser light irradiation with a wavelength of 615 nm To effectively detect photo-luminescence from singlet oxygen, the solution of $PMCM_{68}$ was diluted with ethanol (PBS/ethanol: 5 : 95). Upon light irradiation, $PMCM_{68}$ successfully generated singlet oxygen, which was observed by means of photo-luminescence from singlet oxygen at a wavelength of 1270 nm (Fig. 6b). The lifetime of photo-luminescence was 13 μs, which is consistent with the typical lifetime of singlet oxygen in ethanol. As regards, PMCMs have great possibility for PDT as DPc generates the singlet oxygen after forming PMCM.

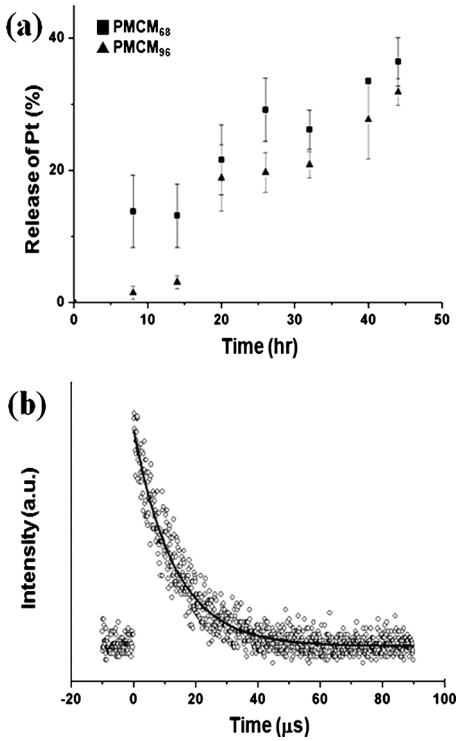

Fig. 6 (a) Release of CDDP from $PMCM_{68}$ and $PMCM_{96}$, and (b) time-resolved photo-luminescence of singlet oxygen.

Conclusions

We have prepared a new type of multi-functional nano-device to combine photodynamic therapy and anticancer drug delivery. By the simple mixing of CDDP with PEG-PLA and DPc in NaCl-free PBS solution, PMCMs were obtained *via* polymer–metal coordination interactions. Moreover, the size of PMCM could be controlled by changing the anionic chain length of PEG-PLA. PMCMs have great potential as a biomedical nano-device for combination therapy as evidenced by the sustained release of CDDP from PMCMs under physiological saline conditions and the generation of singlet oxygen under light irradiation. Further biological investigation will be accomplished in the near future.

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