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Core Cross-Linked Block Ionomer Micelles as pH-Responsive Carriers for *cis*-Diamminedichloroplatinum(II)

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Abstract

Benefits of the frequently prescribed platinum (II) chemotherapy drugs are compromised by undesirable side effects, poor pharmacokinetics and development of drug resistance. Polymer micelles derived from amphiphilic block copolymers, offer a novel macromolecular platform for carrier based delivery of such compounds. Soft polymeric nanocarriers were synthesized by template-assisted method involving condensation of the poly(ethylene oxide)-*b*-polymethacrylate anions by metal ions into core-shell block ionomer complex micelles followed by chemical cross-linking of the polyion chains in the micelle cores. The resulting micelles can efficiently incorporate cisplatin with a high loading capacity (up to 42 % w/w). Core cross-linking stabilized the micelles against structural disintegration and prevented premature drug release. The reversible cisplatin entrapment involved the carboxylate groups of the micellar core. The drug was released in a pH- responsive manner, without loss of its biological activity. The stable cross-linked polymer micelles can potentially improve platinum (II) drug disposition with improved therapeutic potential.

Keywords

cisplatin; block copolymers; cross-linking; drug delivery; micelle

INTRODUCTION

Nano-sized drug carriers like polymer conjugates, liposomes, micelles, dendrimers, inorganic or other solid particles, and others have begun to make important contributions to cancer therapy over the last decade [1]. Advantages of such nanocarriers lie in their ability to improve drug solubility, prolong systemic drug half-life, provide means for sustained and environmentally responsive drug release, enable tumor specific delivery, reduce

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immunogenicity and systemic side effects, suppress development of drug resistance and deliver simultaneously two or more drugs for combination therapy [2].

Polymer micelles of core-shell structure formed by amphiphilic block copolymers have been actively studied as carriers for delivery of low molecular mass drugs, proteins, nucleic acids, and imaging agents [3]. Their unique properties include small size (10 to 100 nm in diameter), regulated, pH- and temperature-responsive drug release, systemic stability and unique disposition characteristics in the body resulting in superior performance over other colloidal delivery systems [4]. Interestingly, such polymeric micelles have been shown to accumulate more readily in tumors than larger nanoparticulates, including liposomes [2].

Doubly hydrophilic diblock copolymers, in which one of the blocks is ionic (“*block ionomers*”), can self-assemble upon interaction with oppositely charged species resulting in the formation of block ionomer complexes (BIC) also termed “polyion core micelles” [5, 6]. Neutralization of the polyion charges leads to formation of hydrophobic domains, which segregate in aqueous media into the micelle core, while water-soluble nonionic segments, such as poly(ethylene oxide) (PEO), prevent aggregation and macroscopic phase separation, forming the micelle corona. Such nano-assemblies have allowed encapsulation of various charged therapeutic molecules, including large entities such as proteins and nucleic acids, into the micellar core. These structures are capable of a high loading capacity (up to 50%) with the ionic block providing for pH- and salt-sensitivity to control the triggered release of the therapeutic agent [7, 8].

Metal complex therapeutics can be incorporated into such BIC either by polymer-metal complexation, or through a combination of electrostatic, hydrophobic, and hydrogen bonding interactions. Drug incorporation can be achieved through direct micellization of the block ionomer with the metal-containing drug in aqueous media [9]. A covalent coordination between the metal and polyion side chain of the polymer drives the polymer-drug self-assembly into micelles. In the presence of abundant counterions, the reversible drug-polymer interaction is lost leading to disintegration of the micelle structure and release of the active drug. Indeed, PEO-poly(amino acid) micelles incorporating anticancer drug, cisplatin (*cis*-diamminedichloroplatinum(II)), were shown to have favorable pharmacokinetics, tumor-specific accumulation, improved anti-tumor activity and reduced non-specific side effects, relative to the free drug [10–13].

Premature disintegration of the micelles upon systemic administration, however, may decrease their effectiveness as drug delivery vehicles. To address this problem we and others proposed to cross-link the micelle structure to increase their stability. The core [14–16] or the shell [17–19] of the micelles were successfully cross-linked resulting in increase of micelle stability towards dilution and environmental challenges such as changes in pH, ionic strength, solvent composition, and shear forces. This strategy combines an advantage of having a reinforced micelle structure and applicability of such cross-linked micelles as a generic nanocarrier. One recent example described is polymeric micelles of PEO-*b*-poly(methacrylic acid) (PEO-*b*-PMA) copolymer with stably cross-linked ionic cores (*cl*-micelles) synthesized using template-assisted procedure [20]. Such micelles behave as nanoscale ionic gels (“nanogels”), capable of swelling in response to environmental changes (pH or ionic strength). Furthermore, due to its polyionic character the core of *cl*-micelles can encapsulate oppositely charged therapeutic molecules [21, 22].

The current study explores the potential of these *cl*-micelles as delivery carriers for cisplatin (CDDP). Towards this aim, the physicochemical properties of the micelles, their CDDP loading capacity and the release of the drug from the micelles were investigated. Altogether we demonstrate that remarkably high amount of CDDP can be incorporated into *cl*-micelles,

while allowing reversible pH-sensitive release of drug in pharmacologically active form, which can be effectively controlled by changing the composition of the micelle core.

MATERIALS AND METHODS

Materials

PEO-*b*-PMA diblock copolymer ($M_w/M_n=1.45$) was purchased from Polymer Source Inc., Canada. The block lengths were 170 and 180 repeating units for PEO and PMA respectively. The concentration of carboxylate groups in the copolymer samples was estimated by potentiometric titration. CDDP was purchased from Acros Organics. 1,2-Ethylenediamine (ED), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and other chemicals were purchased from Sigma-Aldrich (St Louis, MO) and were used without further purification.

Synthesis of the cross-linked micelles

The general procedure for the synthesis of cross-linked PEO-*b*-PMA micelles (*cl*-PEO-*b*-PMA) has been described earlier [21]. PEO-*b*-PMA/ Ca^{2+} complexes were prepared by mixing an aqueous solution of PEO-*b*-PMA with a solution of $CaCl_2$ at a molar ratio of $[Ca^{2+}] / [COO^-] = 1.3$. The EDC and ED were then added at appropriate molar ratios to the dispersion of PEO-*b*-PMA/ Ca^{2+} BIC to achieve the desired theoretical extent of cross-linking. The extent of cross-linking was controlled by the ratio of amine functional groups to carboxylic acid groups. The reaction mixture was allowed to stir overnight at room temperature. After completion of the reaction, 1.5 molar excess amount of EDTA was added followed by dialysis, first, against 0.5% aqueous ammonia, and then against distilled water to remove Ca^{2+} ions and byproducts of the cross-linking reaction.

Preparation of CDDP-loaded micelles

The aqueous dispersions of *cl*-PEO-*b*-PMA were mixed with an aqueous solution of CDDP (1 mg/ml) at pH 9.0 at a 0.5 molar ratio of CDDP to carboxylate groups of the micelle followed by incubation at 37 °C for 48 h. Unbound CDDP was removed by ultrafiltration using Centricon® Plus-20 centrifugal filter units (MWCO 30,000Da, Millipore).

Electrophoretic mobility and size measurements

Electrophoretic mobility measurements were performed using a “ZetaPlus” analyzer (Brookhaven Instrument Co.) with a 30 mW solid-state laser operating at a wavelength of 635 nm. The ζ -potential of the particles was calculated from the electrophoretic mobility values using built-in software employing the Smoluchowski ζ -potential model. Effective hydrodynamic diameters (D_{eff}) of the particles were measured by photon correlation spectroscopy in a thermostatic cell at a scattering angle of 90° using the same instrument equipped with a Multi Angle Sizing Option (BI-MAS). All measurements were performed at 25°C. Software provided by the manufacturer was used to calculate the size of the particles and polydispersity indices (PDI). The mean values of D_{eff} were calculated from the measurements performed at least in triplicate.

CDDP assay and release studies

The Pt(II) content in the micelles, was determined by an ion coupled plasma-mass spectrometer (ICP-quadrupole-MS, Varian 810-MS). For the detection of Pt(II), two isotopes Pt194 and Pt195 were chosen because of their equal abundance (33.0% and 33.8%, respectively), using Holmium as the internal standard. Standard curves in the range of Pt(II) concentrations from 2 ng/ml to 100 ng/ml were generated using platinum atomic absorption standard. Appropriate dilutions of the test samples were prepared in 0.1 N HCl. Data were

acquired and processed using the ICP-MS expert software version 2.1 b103 (Varian). The release of the Pt(II) complexes from the *cl*-PEO-*b*-PMA, in phosphate buffered saline (PBS, pH 7.4, 0.14 M NaCl), acetate buffered saline (ABS, pH 5.5, 0.14 M NaCl), phosphate buffer (pH 7.4), or acetate buffer (pH 5.5), was determined by equilibrium dialysis method using a membrane with 3,500 Da cutoff. The concentration of Pt(II) released was determined by ICP-MS, expressed as a percentage of the total Pt(II) available and plotted as a function of time.

Oligonucleotide synthesis, purification, and characterization

Oligonucleotides d(CGGTCGGCTGGC) and d(GCCAGCCGACCG) were synthesized by the Core Synthetic Facility of the Eppley Institute for Cancer Research at UNMC, purified by high-performance liquid chromatography, desalted by column chromatography on a G-10 Sephadex column, and lyophilized to dryness. The extinction coefficients of oligonucleotides were calculated in water from the tabulated values of the monomer and dimer nucleotides at 260 nm and 25°C [23]. These values were then adjusted for the random coil state at 80°C as reported earlier [24]. The following extinction coefficients at 260 nm and 25°C in $\text{mM}^{-1} \text{cm}^{-1}$ were obtained: 77.6 for d(CGGTCGGCTGGC) and 86.0 for d(GCCAGCCGACCG). The duplex formation was achieved by 1:1 mixing of the complementary strands. The duplex was lyophilized to dryness and redissolved in the assay buffer containing 20 mM NaCl sodium chloride and 10 mM sodium phosphate, pH 7.4.

Circular Dichroism (CD) Spectropolarimetry

CD measurements were done using Aviv (model 202SF) CD spectrometer (Lakewood, NJ, USA). The spectrum for the single strand d(CGGTCGGCTGGC) was recorded from 220 to 350 nm in 1 nm increments at 5 °C using a quartz cell with pathlength of 1 cm and oligonucleotide concentration of 25.6 μM .

Temperature-dependent UV spectroscopy (UV melts)

UV melting curves were recorded using a thermoelectrically controlled UV/Vis Aviv 14DS or a Lambda 40-Perkin Elmer spectrophotometer, interfaced to a PC computer for data acquisition and analysis. Absorbance at 260 nm was monitored as the temperature was scanned at a heating rate of 1 °C/min. Analysis of the shape of melting curves yields the transition temperature, T_M , which are the midpoint temperature of the order-disorder transitions, as well as van't Hoff thermodynamic parameters [25]. The oligonucleotide duplex at a concentration of 6 μM in phosphate buffer solution (10 mM, pH 7.4, 20 mM NaCl) was incubated for 24 h at 25 °C in the dark, either alone, with free *cl*-micelles, CDDP-loaded *cl*-micelles, or released Pt(II) complexes. The samples of released Pt(II) species were obtained by incubating CDDP-loaded *cl*-micelles separately for 24 h in the same buffer, followed by centrifugal filtration and removal of the macromolecular complexes.

Intracellular Pt(II) accumulation

A2780 cells were seeded in duplicate 25-cm² flasks, allowed to reach 80–90% confluence, and treated with free CDDP or CDDP-loaded micelles for 4, 8, or 24 h (37 °C) at CDDP equivalent concentrations of 50 μM . Following incubation, each flask was rinsed three times with PBS, trypsinized, aliquoted in duplicate 1.5-ml microcentrifuge tubes and pelleted by centrifugation. One of each duplicate cell pellets was wet ashed by overnight incubation in 70% nitric acid at 60 °C and analyzed for Pt(II) content by ICP-MS. The other duplicate was lysed using M-PER mammalian protein extraction reagent (Thermo Scientific, Franklin, MA) and quantified for total protein using micro BCA protein assay kit (Pierce, Rockford,

IL) as per the manufacturer's instructions. Whole cell uptake was expressed as ng Pt / mg cellular protein.

DNA platination

Duplicate 25-cm² flasks containing A2780 cells (80–90% confluence) were treated with free CDDP or CDDP-loaded micelles for 24 h (37 °C) at a CDDP equivalent concentration of 15, 30 and 60 µg/mL. Following incubation, each flask was rinsed three times with PBS, trypsinized, and aliquoted in 1.5-ml microfuge tubes and pelleted by centrifugation. Cellular DNA was isolated using Qiagen DNeasy tissue kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. Upon determination of the DNA concentration, the remaining solution was wet ashed in 70% nitric acid at 60 °C and analyzed for Pt(II) content by ICP-MS. DNA platination was expressed as ng Pt / µg DNA.

Cytotoxicity Assay

Cytotoxicity of CDDP-loaded polymer micelles was assessed against human ovarian A2780 and 2008 carcinoma cell lines and nasopharyngeal KB cancer cell line by a standard MTT assay as described previously [21]. Briefly, 5,000 cells per well were seeded in a 96-well microtiter plates and allowed to adhere for 24 h prior to the assay. Cells were then exposed to various doses (0–200 µg/ml on CDDP basis) of either CDDP alone, polymer micelles alone, or CDDP-loaded micelles for 48 h at 37°C. Following drug treatment, the cells were washed with PBS, and maintained in RPMI 1640 medium with 10% FBS for additional 24 h. To estimate cell survival, 25 µl of MTT indicator dye (5 mg/ml) was added to each well and the cells were incubated for 2 h at 37 °C in the dark. The resulting formazan crystals were solubilized in 100 µl of 50% DMF–20% SDS solution overnight at 37 °C. Absorption was then measured at 570 nm in a microplate reader (SpectraMaxM5, Molecular Devices Co., USA) and the cell viability calculated as $A_{\text{sample}}/A_{\text{control}} \times 100\%$. Based on the results of the test, the IC₅₀ values (the concentration which kills 50% of cells) were calculated using four parameter log fitting with GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). Each experiment was repeated at least three times on separate occasions and the final IC₅₀ values expressed as mean ± SD.

RESULTS AND DISCUSSION

Synthesis and characterization of core cross-linked micellar nanocarriers

The PEO-*b*-PMA micelles with cross-linked ionic cores (*cl*-micelles) were synthesized using a two-step procedure [21]. PEO-*b*-PMA block copolymers were first condensed by reacting with Ca²⁺ into the micelles with PEO shell and PMA/Ca²⁺ complex core; the cores were then cross-linked using a bifunctional cross-linking agent (1,2-ethylenediamine) in the presence of a water-soluble carbodiimide (EDC), followed by extensive dialysis to remove Ca²⁺ and cross linking byproducts. The resulting *cl*-micelles are entirely hydrophilic nanogel-like structures with hydrodynamic diameter of 100–200 nm and narrow size distribution (PDI < 0.1). They have been described as having swollen cores of a cross-linked PMA network surrounded by a PEO shell [21, 22]. Varying the molar ratio of the amine-containing cross-linker to the carboxylic groups in PEO-*b*-PMA/Ca²⁺ complexes allows for generation of *cl*-micelles with different number of cross-links in the cores. Four samples of *cl*-micelles with targeted degree of cross-linking in the cores of 10, 20, 40, and 70% were obtained from the same stock solution of PEO-*b*-PMA/Ca²⁺ template in this manner. These numbers represent the nominal extent of cross-linking that could occur; however, the actual degree of cross-linking is significantly less due to several types of side reactions [26]. Table 1 illustrates the dependence of size and ζ-potential of *cl*-micelles on the targeted degree of cross-linking. As expected, *cl*-micelles with lower degrees of core cross-linking exhibited significant swelling compared to the precursor PEO-*b*-PMA/Ca²⁺ micellar templates ($D_{\text{eff}} \approx$

90 nm). For *cl*-micelles with higher degree of cross-linking (40% and 70%) the swelling of the cores seems to be suppressed and their overall size remained nearly the same as that of the precursor. We have previously described these structures as hydrogel-like soft nanomaterials, nanogels [26]. Indeed, *cl*-micelles swelled considerably as pH increased due to increased ionization and repulsion between the PMA chains in the cross-linked cores of the micelles (Supplementary content, Figure S1). The increased number of counterions associated with the charges on the polymer network give rise to an extra osmotic pressure (Donnan effect), which also tends to expand the network. The swelling was accompanied by an increase in the net negative charge of the particles. As expected, the *cl*-micelle dimensions in response to pH strongly depended on the cross-linking density. The micelles with lower degrees of core cross-linking swelled to a much larger volume than the highly cross-linked micelles. Notably, the swelling was completely reversible: the protonation of the carboxylic groups at low pH resulted in reduction in the osmotic swelling pressure inside the cores and an overall decrease in the size of the micelle (Figure S1).

Incorporation of CDDP into *cl*-micelles

We previously demonstrated that CDDP can be efficiently encapsulated in the cores of *cl*-micelles at pH 9.0, when the PMA cores are fully swollen and carboxylic groups are completely ionized [27]. In the present study CDDP was incorporated into *cl*-micelles using a similar procedure [26]. The characteristics of CDDP-loaded *cl*-micelles with various degrees of cross-linking are presented in Table 1. As expected, drug loading was accompanied by a decrease in both the size and net negative charge (ζ -potential) of the *cl*-micelles. This is consistent with the neutralization of the PMA chains upon CDDP binding to carboxylate groups, resulting in reduction of the osmotic swelling pressure inside the cores of *cl*-micelles. Previous studies demonstrated that further increases in the molar ratio of CDDP to carboxylate groups upon loading did not lead to greater drug loading or further changes in the particle size [26]. This suggests that CDDP-loaded *cl*-micelles have reached their most condensed state. The magnitude of the micelle condensation associated with CDDP loading depended on the degree of polymer cross-linking and can be expressed in terms of the volume reduction, V_r , calculated as a ratio of the hydrodynamic volumes of *cl*-

micelle before and after CDDP loading at a given pH $\left(V_r = \left(D_{eff}^{empty} \right)^3 / \left(D_{eff}^{CDDP-loaded} \right)^3 \right)$ (this value is also discussed below as maximal swelling ratio of CDDP-loaded *cl*-micelles upon drug release). For example, at pH 7.0 the maximal volume reduction ($V_r = 1.72$) was observed for *cl*-micelles with the lowest degree of cross-linking (10%). The volume reductions induced by CDDP loading in *cl*-micelles with higher degree of cross-linking (40% and 70%) were less distinct ($V_r = 1.18$ and 1.12, respectively). This may be due to a less pronounced swelling of the original unloaded *cl*-micelles with higher degree of cross-linking. Notably, the decrease in the volume reduction of the *cl*-micelles with targeted cross-linking degrees from 10% to 70% correlated with the decrease in the CDDP loading capacity of these micelles (Table 1). This difference cannot be simply explained by the decrease in the total amount of carboxylate groups that can bind CDDP because in all cases the portion of these groups consumed to form the amide bonds appeared to be less than 10% [26]. More likely, the condensation of the *cl*-micelles with high degree of cross-linking may have limited the volume available for CDDP loading. Furthermore, the spatial distribution of the cross-links in the *cl*-micelles with high degree of cross-linking may result in lower accessibility of the bulk of the carboxylate groups to CDDP. Indeed, CDDP-loaded micelles still retained a negative ζ -potential (Table 1), suggesting that not all available carboxylate groups were consumed upon CDDP binding. It is likely that CDDP binding and subsequent collapse of the ionic cores of *cl*-micelles with different degrees of cross-linking, involve appearance of steric constraints and possible variations in the packing of drug molecules, which play important roles in determining the degree of drug loading.

CDDP-loaded *cl*-micelles maintained their dispersion stability and exhibited no aggregation or precipitation for a prolonged period of time (up to several months). It is of interest to note that binding of CDDP to the precursor PEO-*b*-PMA block copolymer, resulted in the formation of micellar aggregates with a diameter of approximately 50 nm and narrow size distribution (PDI = 0.08). However, these micelles had low dispersion stability and precipitated over 24 h, perhaps, because the resulting CDDP/PMA complex was too hydrophobic and the PEO segments in the corona of the micelles were unable to stabilize the particles in dispersion.

Figure 1 presents the dependence of the ζ -potential and D_{eff} of CDDP-loaded *cl*-micelles with 20% degree of cross-linking as a function of pH. Similar behavior was exhibited by CDDP-loaded *cl*-micelles with other degrees of cross-linking (Figure S2). It is seen that as the pH decreased from 8 to 6 the ζ -potential of CDDP-loaded micelles increased, which is consistent with gradual protonation of the carboxylate groups in *cl*-micelles. At pH 6, the CDDP-loaded micelles show a slightly negative ζ -potential (-1.99 ± 2.0 mV), suggesting that the negative charge of PMA was nearly completely neutralized by CDDP and protons. Notably, in contrast to the empty *cl*-micelles, the size of CDDP-loaded micelles did not change in this pH range (Figure 1B). This suggests, that despite changes in the osmotic swelling pressure, CDDP-loaded *cl*-micelles reached their elastic limit and did not collapse, possibly, because of the steric hindrance of Pt-PMA adducts, which occupy larger volume than the carboxylic groups of PMA chains. Furthermore, the difference in swelling behavior may originate from different extent of cross-linking in the empty and drug-loaded *cl*-micelles. It was previously reported that transition metal cations added to fully neutralized polyacrylate gel act as cross-linkers, which was manifested by an increase of the elastic modulus of the gel [28, 29]. It is likely that binding of CDDP to PMA chains in the core of *cl*-micelles also result in additional cross-linking of PMA segments through the formation of -COO-Pt-COO- bridges between network chains, which further hinders swelling/deswelling of CDDP-loaded *cl*-micelles.

Unexpectedly, further decrease of pH to 5.0 resulted in substantial change of ζ -potential of CDDP-loaded micelles, which became positive (10.33 ± 1.38 mV) (Figure 1A). This behavior can be explained by partial dissociation of Pt-PMA complexes under acidic conditions. Since the pK_a of PMA is 5.5 [27], at pH 5.0 some fraction of carboxylate ligands is protonated, which makes them better leaving groups and results in their partial displacement by water (Scheme 1). Under these conditions only 0.4% of the Pt(II) was released from the *cl*-micelles over a period of 24 h (these experiments were carried out in the absence of buffering salts to avoid coordination of other anions like phosphates or acetates to Pt [30, 31]). Therefore, nearly all Pt(II) species remained bound to the polymer chains either through the coordination with two carboxylate groups or as cationic diamine-monoaqua Pt(II)-PMA adducts. As a result CDDP-loaded *cl*-micelles acquired a net positive charge at pH 5.0. Despite an increase in charge density of the network and possible disruption of the interchain -COO-Pt-COO- bridges, there was no volume change of the CDDP-loaded micelles upon pH decrease from 6.0 to 5.0 (Figure 1B). However, it is important to note that at pH 5.0 the size of CDDP-loaded *cl*-micelles was significantly larger than that of the empty *cl*-micelles (Figure 1B and Figure 2S). This suggests that CDDP-loaded *cl*-micelles are not fully collapsed, and reinforces that CDDP binding to the ionic cores of the *cl*-micelles strongly affects their pH-responsive swelling behavior.

Release of Pt(II) complexes from *cl*-micelles

The release of the Pt(II) complexes from the *cl*-micelles was studied by equilibrium dialysis at 37°C at either pH 7.4 (PBS) or pH 5.5 (ABS), which reflect conditions encountered in plasma and in intracellular compartments (lysosomes), respectively. This release usually proceeds *via* ligand exchange reactions between the carboxylate groups of PMA and other

biologically abundant anions, such as chloride, phosphate, carbonate, and acetate [30, 31]. Figure 2 presents the kinetic profiles of Pt(II) species release from *cl*-micelles in PBS and ABS. Regardless of the degree of cross-linking, all types of *cl*-micelles displayed slow and sustained release of Pt(II) species. The lack of a burst release suggested that CDDP incorporation proceeded mainly through PMA-Pt complex formation, with minimal contribution from displaceable physically entrapped drug.

The release rates strongly depended on the cross-linking degree. Specifically, at pH 7.4 CDDP-loaded *cl*-micelles with high density of cross-links exhibited considerably faster release rates than the *cl*-micelles with lower density of cross-links. For example, *cl*-micelles with 70% degree of cross-linking released $20.9 \pm 3.1\%$ of Pt(II) species during 24 h whereas the micelles with 10% degree of cross-linking released only $9.2 \pm 0.5\%$ during the same period (Figure 2A). This observation may be ascribed to the differences in the structure of the core of CDDP-loaded *cl*-micelles. The release of Pt(II) species represents a multistep process. It includes penetration of chloride or other nucleophile ions into the micelle core, followed by their exchange with carboxylic groups in Pt(II) complexes, diffusion of Pt(II) species out of the micelles, and physical changes in polymer network (e.g., tortuosity). Since the micelles with low density of cross-linking can accommodate more CDDP compared to highly cross-linked micelles (Table 1), their core may be more dense, which may hamper the in and out diffusion of water, chloride ions and Pt(II) species.

Moreover, the drug release rates may also depend on the disentanglement/relaxation of the polymer chains within the network. In order to obtain an insight into the nature of the release, the kinetic data were analyzed in terms of the power law model that was applied by Peppas et al. [32] to describe the drug release kinetics in pH-sensitive hydrogels. According to this model, the amount of the released drug is expressed as a function of time as $M_t/M_\infty = kt^n$, where M_t is the cumulative amount of drug released at time t , M_∞ is the total amount of loaded drug, k is a kinetic constant and n is the diffusional exponent, indicative of the release mechanism. The systems with spherical geometry and purely Fickian drug diffusion have $n = 0.43$, the systems where the solute release is mainly controlled by the chain relaxation have $n = 0.85$, while the systems exhibiting “anomalous transport” involving both diffusion and relaxation have n ranging from 0.43 to 0.85. This latter appeared to be the case of all CDDP-loaded *cl*-micelles at pH 7.4 since their n values decreased from 0.77 to 0.55 as the degree of cross-linking increased (the correlation coefficients of all fits exceeded 0.99). Since at pH 7.4 the CDDP-loaded *cl*-micelles with lower degree of cross-linking are more swellable they undergo greater chain relaxations upon drug release and are expected to release the drug through the relaxation-controlled mechanism slower than the *cl*-micelles with higher degree of cross-linking, which are less swellable. (In other words there may be a greater contribution of the relaxation-controlled mechanism with the more swellable loaded micelles). This is supported by the dependence of the maximal swelling ratio V_r of the CDDP-loaded *cl*-micelles on the degree of cross-linking shown in Figure 3. Therefore the rearrangement of polymer chains of the network in more collapsed CDDP-loaded *cl*-micelles present an additional barrier for the release of the Pt(II) species.

Surprisingly, a completely different pattern of Pt(II) species release was observed under acidic conditions at pH 5.5 (Figure 2B). Specifically, *cl*-micelles with lower degrees of cross-linking displayed faster release rates compared to *cl*-micelles with higher degrees cross-linking. For example, during 24 h the *cl*-micelles with 10% and 70 % degree of cross-linking released $33.0 \pm 5.5\%$ and $12.8 \pm 1.5\%$ of loaded Pt(II), respectively. The cumulative release of Pt(II) species from *cl*-micelles with lower degree of cross-linking (10% and 20%) was faster at pH 5.5 than at pH 7.4. On the other hand, it was practically the same for *cl*-micelles with 40% degree of cross-linking, while the *cl*-micelles with 70% degree of cross-linking released the drug slower at pH 5.5 than at pH 7.4 (in the latter case only $35.0 \pm 1.2\%$

of Pt(II) species were released over the 144 h at pH 5.5 compared to $55.3 \pm 0.6\%$ at pH 7.4). Apparently, such decay in the drug release rates for the highly cross-linked micelles can be also related to their swelling behavior under the acidic conditions (Figure 3). At pH 5.5 the CDDP-loaded *cl*-micelles with 70% degree of cross-linking have higher maximal swelling ratio V_r and undergo greater chain relaxations upon drug release than the *cl*-micelles with lower degree of cross-linking. The release data at pH 5.5 were also fitted using the power law model described above. The calculated values of n increased from 0.68 to 0.77 as the degree of cross-linking increased suggesting increased involvement of the chain relaxation-controlled release mechanism.

Interestingly, the release of Pt(II) species from *cl*-micelles was also observed in chloride-free conditions in the presence of phosphate or acetate ions (10 mM) (Figure S3). In these cases the overall release rates were significantly less than in the chloride-containing medium. For instance, the CDDP-loaded *cl*-micelles with 20% degree of cross-linking released during 24 h only 3% of Pt(II) in chloride-free phosphate buffer, pH 7.4 compared to 11% in the same buffer containing 0.14 M NaCl. However, at pH 5.5 the Pt(II) release rates greatly increased even in the chloride-free conditions. These data suggest that Pt(II) species release from *cl*-micelles can occur inside the cells where the chloride concentration is much lower (4–50 mM) than in the blood and extracellular body fluids (100–110 mM) [33].

Altogether, we believe that the unusual pH-responsive drug release behavior of CDDP-loaded *cl*-micelles with various degrees of cross-linking can prove to be useful for rational design of effective anticancer nanomedicines. Such delivery systems must stably incorporate drug and minimize its release in systemic circulation but efficiently release their payload at the disease site. Compared to extracellular pH 7.4, the interstitial tumor space and inflammatory tissues (pH $\approx$ 6.4–7.2 [34]) as well as the intracellular endocytotic vesicles (pH $\approx$ 4.0 to 6.0) are known to be acidic. Hence, a dynamic delivery system with a slow drug release in extracellular milieu and enhanced release of the drug at the pathological site can be designed. The observed pH-dependent release characteristics of these *cl*-micelles with lower cross-linking degree are therefore of a real advantage. While systemic release of the Pt(II) species from these *cl*-micelles (e.g., with 20% targeted degree of cross-linking) is expected to be slow, an accelerated pH-triggered release of the drug will occur at low pH in a tumor tissue. Moreover, our previous work demonstrated that *cl*-micelles enter cancer cells *via* endocytosis and are translocated into acidic lysosomal compartments [35], where the drug release from *cl*-micelles with low degree of cross-linking in the core can be further enhanced.

Does the released drug retain its activity?

As a next step, it was essential to determine whether the CDDP-loaded *cl*-micelle can release biologically active Pt(II) species. Anticancer activity of CDDP is associated with its ability to covalently bind to cellular DNA, forming primarily intra-strand cross-links to adjacent purine residues (GG or AG) with the 1,2-intrastrand d(GpG) cross-link being the major adduct (65%) [36]. This distorts DNA structure and promotes its specific interactions with cellular HMG proteins. Upon binding with the HMG proteins the platinated GG sites become shielded from excision repair, which inhibits DNA replication and transcription and eventually leads to the cell apoptosis. Typically, the level of CDDP adducts correlates positively with the response to the treatment [37]. Incorporation of these adducts in a DNA helix causes bending, unwinding of the DNA, and partial disruption of favorable base-pair stacking interactions adjacent to the platination site. This is accompanied by a decrease in the thermal stability of the host DNA duplex exhibited by a shift of the melting temperature, T_M , of the helix-coil transition of platinated DNA towards lower temperatures compared to the unplatinated host duplex. Therefore, the activity of Pt(II) species released from CDDP-loaded *cl*-micelle can be assayed by recoding UV melting profiles of a model DNA duplex

and determining the T_M after its exposure to CDDP-loaded *cl*-micelles vs. free drug. For this purpose we used the DNA 12-mer duplex d(CGGTCGGCTGGC)/d(GCCAGCCGACCG), which contains three CDDP targeted GG sites. Guanine rich sequences are known to form inter- or intramolecular G-quadruplexes via Hoogsteen hydrogen bonding. However, the possibility of formation of such G-quadruplexes by d(CGGTCGGCTGGC) strand was excluded by CD spectroscopy (Figure S4). Specifically, there were no G-quadruplexes in the antiparallel or parallel conformations centered at 292 nm or 260 nm respectively in the CD spectrum of the duplex at 5 °C (actually, this spectrum was similar to the spectrum of the random coil state at 90 °C).

The UV melting curves were recorded in 10 mM sodium phosphate buffer supplemented with 20 mM NaCl to mimic the intracellular environment. Under these conditions, the DNA duplex unfolds in a monophasic transition with T_M of 59.1 ± 0.5 °C. After incubation of the duplex with CDDP for 24 h at a [drug]/[nucleotide] ratio of 0.2, the duplex showed lower hyperchromicity and reduced T_M (57.4 ± 0.4 °C), which was consistent with the thermal destabilization of the helical structure typical for platinated duplexes [38, 39]. Notably, exposure of the DNA duplexes to the higher concentrations of CDDP led to an even greater T_M depression (Figure S5), suggesting that a higher number of GG-sites were modified.

Furthermore, the duplexes were incubated with empty or CDDP-loaded *cl*-micelles (20% of cross-linking) as well as with Pt(II) species released and separated from *cl*-micelles over a period of 24 h. The T_M values and corresponding thermodynamic parameters are summarized in Table 2. The analysis of these data suggests that the melting behavior of the DNA duplex was not affected by the unloaded *cl*-micelles. However, a substantial decrease in T_M was observed upon incubation of the duplexes with the Pt(II) species released from CDDP-loaded *cl*-micelles. The observed decrease in thermodynamic stability of the platinated duplexes was enthalpy driven, as is evident from lower unfolding enthalpies (Table 2), and is in agreement with previous reports [38, 39]. In addition, practically no changes in the duplex T_M were detected upon the DNA exposure to CDDP-loaded *cl*-micelles at $t = 0$ h when Pt(II) species had not been yet released. These results clearly suggest that the CDDP-loaded *cl*-micelles liberate biologically active Pt species that are able to form Pt(II) DNA adducts under the conditions mimicking the intracellular environment.

Cellular uptake and *in vitro* cytotoxicity of CDDP-loaded *cl*-micelles

The anti-tumor affect of platinum drugs involves many cellular events, starting from drug accumulation in the tumor cells. The ability of *cl*-micelles to efficiently internalize in cancer cells [35] makes them an attractive vehicle for intracellular delivery. To examine accumulation of CDDP-loaded *cl*-micelles in cancer cells, the total Pt(II) content was measured in human ovarian carcinoma A2780 cells following their exposure to 50 μ M free CDDP or CDDP-loaded *cl*-micelles (with 20% degree of cross-linking) by ICP-MS. The data on time-course of Pt(II) accumulation normalized for the total cellular protein are presented in Figure 4A. The net accumulation was substantially higher in cells treated with CDDP-loaded *cl*-micelles compared to cells treated with the free drug.

Next, we examined whether increased intracellular uptake of Pt(II) with the CDDP-loaded *cl*-micelles translated into an increase in the amount of drug that reached its target. For this purpose, the extent of nucleus DNA platination was measured after 24 h exposure of A2780 cells to various concentrations of CDDP-loaded *cl*-micelles or free drug (Figure 4B). It was found that the Pt(II)-DNA adduct levels in cells treated with CDDP-loaded *cl*-micelles were decreased compared to the free drug. These data suggest that despite an increase in the total Pt(II) accumulation, the pool of bioavailable drug delivered with the *cl*-micelles was substantially reduced. Such reduction is consistent with the slow release of Pt(II) species from the *cl*-micelles. Furthermore, the drug ability to react with the DNA may be affected by

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differences in intracellular trafficking of free and micelle-associated drug. Experimental evidence suggest that cellular uptake of free CDDP can occur via passive diffusion and active uptake by a number of transport proteins [40]. The CDDP or products of its hydrolysis that evade detoxification and efflux processes in the cytosol can enter the nucleus and bind to DNA. There are two possible routes by which Pt(II) species can enter the cells exposed to CDDP-loaded *cl*-micelles. One route involves the extracellular release of Pt(II) species followed by their entry into the cells or cross-reaction with extracellular proteins such as serum albumin. Since CDDP-loaded *cl*-micelles displayed slow release of Pt(II) species in PBS containing a physiological concentration of chloride ions at pH 7.4 ($11.4 \pm 1.1\%$ after 24 h for CDDP-loaded *cl*-micelles with 20% degree of cross-linking) only a small fraction of Pt(II) species can be passively transported from extracellular media into the cytosol. Alternatively, as was previously demonstrated, *cl*-micelles can enter cancer cells via endocytosis [35] where Pt(II) species can be liberated after acidification in the endocytic vesicles. Such vesicles may serve as reservoirs of the drug available to the cell over a period of time and may also protect the drug from reaction with the cytosolic nucleophiles. Eventually, Pt(II) species can enter the cytoplasm and then nucleus. Although the mechanisms of Pt(II) endosomal export remains to be determined, it may be facilitated through the function of metal transporters on the lysosomal membrane [41]. However, the rate of release of Pt(II) species from *cl*-micelles at acidic conditions is still relatively slow ($21.8 \pm 4.4\%$ during 24 h). Thus, the cytosolic drug pool may be reduced compared to the free drug treatments due to vesicular sequestration of *cl*-micelles and slow release of Pt(II) species. This may explain a decreased DNA platination upon of treatment of cells with CDDP-loaded *cl*-micelles.

As the cytotoxicity of platinum complexes is based upon DNA platination it was not surprising that we also found reduced cytotoxicity of CDDP-loaded *cl*-micelles against panel of human cancer cell lines. MTT assay was used to assess the cytotoxicity, and calculated IC₅₀ values for each formulation are summarized in Table 3. Incorporation of CDDP into *cl*-micelles resulted in substantial increase of IC₅₀ values compared to the free drug and it was incubation time-dependent. Since unloaded *cl*-micelles did not affect the cell survival in the entire range of concentrations the cytotoxic activity of CDDP-loaded *cl*-micelles was mediated by the released Pt(II) species. It is worth mentioning that after 48 h of treatment of A2780 cells there were no significant differences in the IC₅₀ values between *cl*-micelles with different extent of cross-linking (Table S1). We interpreted these data by the differences in pH-responsive release behavior of CDDP-loaded *cl*-micelles with various extent of cross-linking. Since micelles with higher degree of core cross-linking exhibited substantially lower rates of drug release at acidic conditions (Figure 2B), it is likely that the contribution of Pt(II) species liberated from such *cl*-micelles inside the cells into overall cytotoxicity was even less than in the case of *cl*-micelles with lower density of cross-linking. This argument is also supported by our observation that uptake of *cl*-micelles with higher degree of cross-linking (70%) was suppressed compared to *cl*-micelles with lower degrees of core cross-linking (20%) (Figure S6). However, the release of CDDP into extracellular media was greatly potentiated as the cross-linking density was increased (Figure 2A), which may result in an increased uptake of the released low molecular mass Pt(II) species into the cytosol. In the static conditions used in MTT assay these two opposite release trends (intracellular and extracellular) may balance each other and, hence, mask the differences in cytotoxic activity between CDDP-loaded *cl*-micelles with different degrees of cross-linking. Taken together these data suggest that although *cl*-micelles loaded with multiple Pt(II) complexes are capable to boost Pt(II) accumulation in cancer cells, intracellular release of Pt species appears to be a critical parameter, because only free platinum complexes are therapeutically effective. Therefore, further efforts need to be focused on engineering the *cl*-micelles having additional mechanisms for triggering the intracellular drug release. One of the approaches can exploit the intracellular degradation of *cl*-micelles. This can be achieved

by the use of reversible cross-links in the ionic cores of the *cl*-micelles that are stable outside the cells but undergo cleavage in the intracellular environment. Cross-links containing reductively labile disulfide bonds or oligopeptide linkers prone to cleavage by the lysosomal cysteine proteases may be suitable in this context. We recently demonstrated that *cl*-micelles with disulfide bonds in the ionic cores (cystamine was used as a biodegradable cross-linker) were rapidly degraded in the presence of the reducing agent [26, 42], which in turn facilitated the release of the incorporated drug. Specifically, the release rate of Pt(II) species was approximately 2.7-fold higher in the presence of 10 mM of glutathione than in the non-reductive environment. This difference in the release kinetics led to considerable increase of cytotoxicity: degradable CDDP-loaded *cl*-micelles displayed nearly five-fold higher cytotoxic activity than non-degradable *cl*-micelles [42]. Despite of reduced *in vitro* cytotoxicity of CDDP-loaded *cl*-micelles, one can expect substantial benefits in using such micellar carriers *in vivo* caused by altered (relative to free drug) pharmacokinetics and an ability of *cl*-micelles to accumulate in tumor tissue due to the enhanced permeability and retention (EPR) effect. The prolonged release of Pt(II) species from CDDP-loaded *cl*-micelles sequestered in either tumor interstitium or in vesicular compartments within the tumor cells may facilitate sustaining an effective local concentration of the drug at the tumor site and, thus, increase *in vivo* effect of the drug against solid tumors. The studies in this direction are currently underway and will be reported elsewhere.

CONCLUSION

The benefits of the frequently prescribed Pt(II)-based anti-cancer drugs are compromised by their undesirable side effects, poor pharmacokinetics and development of drug resistance. Core cross-linked polymer micelles appear to be promising and versatile macromolecular platform for carrier-based delivery of such compounds. The advantages of these systems include 1) simplicity of their formulation with CDDP using ion exchange properties of the ionic cores of *cl*-micelles, 2) high level of drug incorporation (up to 42% w/w), and 3) high stability of the resulting formulations in aqueous dispersion. CDDP-loaded *cl*-micelles displayed pH-sensitive release of Pt(II) species in sustained manner that can be effectively controlled by adjusting the degree of cross-linking of the core. The released Pt(II) species retained their activity, as was manifested by their ability to reduce thermal stability of a model DNA duplex, and were able to form Pt adducts with nuclear DNA in the cancer cells. We believe that the rational design of the overall structure of the cross-linked polymer micelles and, in particular, of their ionic cores might be an effective tool to tune the drug release rates, to affect tumor/host cell interactions, and achieve desirable therapeutic effect. The strategy of the induced cleavage of the cross-links will also result in degradation of the *cl*-micelles and release the individual copolymer chains, which can eventually be removed from the body by the renal clearance once the drug is delivered. Given the similar geometry and coordination state of the central platinum, and presence of identical leaving groups in several other potent anticancer platinum analogs, *cl*-micelles could allow similar efficient and reversible drug incorporation. Such drug-carrier conjugates are likely to acquire new physicochemical, biochemical and pharmacological characteristics, thereby offering the possibility of reducing their chemotherapy associated side effects, increasing the drug bioavailability at the target sites and minimizing development of drug resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(**A**) ζ -potential and (**B**) effective diameter, D_{eff} , of CDDP-loaded *cl*-micelles (solid bars) and empty *cl*-micelles (empty bars) as function of pH of the external solution. Targeted degree of cross-linking of *cl*-micelles is 20%. Data represented as mean $\pm$ SD, * $p < 0.05$ ($n=3-5$)

Figure 2. Pt(II) release from *cl*-micelles with different degree of cross-linking of the core (■-10%, ○-20%, ●-40%, and □-70%) in (A) PBS, pH 7.4; (B) ABS, pH 5.5. Data are mean $\pm$ SEM.

Figure 3.

Plot of the maximal swelling ratios, V_r , of the CDDP-loaded *cl*-micelles at various pH as a function of targeted degree of cross-linking: (○) pH 7.0; (■) pH 5.0. Dashed line corresponds to $V_r = 1$.

Figure 4.

(A) Whole-cell Pt accumulation following incubation with 50 μ M CDDP (open bars) or CDDP-loaded *cl*-micelles (diamond bars) and (B) extent of DNA platination after 24 h exposure to various concentrations of CDDP (open bars) or CDDP-loaded *cl*-micelles (diamond bars), in A2780 cells as measured by ICP-MS. Targeted degree of cross-linking of *cl*-micelles is 20%.

Scheme 1.

(A) Conversion of CDDP into its various aqua derivatives in aqueous solution. (B) Schematic representation of the structure of the cores of CDDP-loaded *cI*-micelles at neutral and acidic pH.

Table 1
Physicochemical characteristics of *cl*-micelles with various degrees of cross-linking before and after CDDP loading at pH 7.0.^a

Targeted degree of cross-linking	<i>cl</i> -micelles			CDDP-loaded <i>cl</i> -micelles			
	<i>D_{eff}</i> (nm)	PDI	ζ-potential (mV)	<i>D_{eff}</i> (nm)	PDI	ζ-potential (mV)	Loading capacity ^b (w/w)
10%	175.0 ± 21.1	0.116 ± 0.04	-34.7 ± 3.2	102.9 ± 20.4	0.122 ± 0.06	-14.9 ± 4.3	0.42 ± 0.03
20%	124.1 ± 2.5	0.085 ± 0.03	-21.8 ± 3.2	94.7 ± 13.6	0.116 ± 0.07	-12.4 ± 4.9	0.29 ± 0.02
40%	107.0 ± 7.8	0.081 ± 0.02	-18.6 ± 0.2	91.3 ± 11.0	0.091 ± 0.03	-10.6 ± 1.9	0.21 ± 0.01
70%	100.1 ± 8.4	0.071 ± 0.03	-21.5 ± 1.6	89.2 ± 7.4	0.089 ± 0.04	-10.8 ± 1.9	0.16 ± 0.02

^a Effective diameter (*D_{eff}*), polydispersity index (PDI) and ζ-potential were determined at pH 7.0. Data presented as means (n=3) ± SD.

^b Pt content in *cl*-micelles was determined by ICP-MS. Loading capacity is expressed as mass of incorporated CDDP per mass of drug loaded *cl*-micelles (w/w). CDDP-loaded micelles were prepared at R = 0.5 and pH 9.0.

Table 2

Changes in the thermodynamic profiles of model DNA duplex as a result of interaction CDDP-loaded *cl*-micelles (20% of targeted cross-linking).^a

Sample	T _M (°C)	ΔT _M (°C)	ΔH _{vit} ^b (kcal/mol)	ΔG° (kcal/mol)	TΔS (kcal/mol)
free duplex	59.1 ± 0.1		71.1 ± 2.6	4.7 ± 0.2	66.4 ± 2.4
duplex + empty <i>cl</i> -micelles	58.8 ± 0.4	0.2 ± 0.4	73.5 ± 2.3	4.8 ± 0.2	68.7 ± 2.2
duplex + CDDP-loaded <i>cl</i> -micelles at t=0 h	58.5 ± 0.7	0.2 ± 0.7	66.8 ± 5.6	4.3 ± 0.3	62.5 ± 5.2
duplex + CDDP-loaded <i>cl</i> -micelles at t=24 h	55.5 ± 0.9	3.6 ± 1.0	56.2 ± 3.0	3.2 ± 0.3	53.0 ± 2.7
duplex + released Pt-species	56.7 ± 0.8	2.3 ± 0.7	66.3 ± 2.0	4.0 ± 0.3	62.4 ± 1.7

^a All measurements were performed in 10 mM phosphate buffer (pH 7.4), 20 mM NaCl. Duplex concentrations were 6 μM.

^b Thermodynamic parameters were derived from van't Hoff analyses of UV melting curves (n=3) [25]

Table 3

IC₅₀ values (CDDP equivalents in µg/ml) of CDDP and CDDP-loaded *cl*-micelles in different cell lines.^a

Sample	Incubation time	A2780	2008	KB
Free CDDP	48h	0.252 ± 0.05	0.22 ± 0.04	0.603 ± 0.25
	24h	1.10 ± 0.50	0.44 ± 0.05	ND
CDDP-loaded <i>cl</i> -micelles	48h	6.24 ± 0.96	4.46 ± 0.92	11.481 ± 2.50
	24h	11.63 ± 1.22	13.82 ± 3.28	ND

^aThe IC₅₀ values represent the means (n=3) ± SD. Targeted degree of cross-linking of *cl*-micelles is 20%.