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Acid Degradable Cross-Linked Micelles for the Delivery of Cisplatin: A Comparison with Nondegradable Cross-Linker

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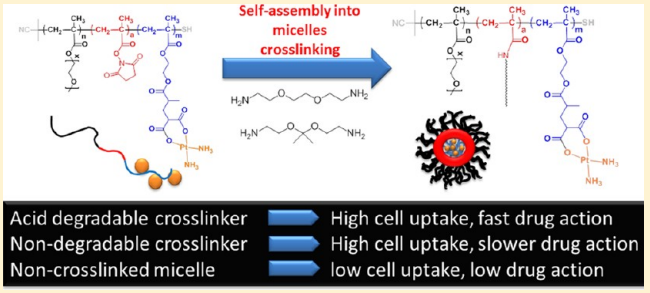
Supporting Information

ABSTRACT: Well-defined and nontoxic cross-linked polymeric micelles, containing either permanent or acid degradable cross-linkers, were employed for efficient intracellular delivery of cisplatin. The self-assembled structures were generated from triblock copolymers of poly(oligo(ethylene glycol) methyl-ether methacrylate)-*block*-poly(*N*-hydroxysuccinic methacrylate)-*block*-poly(1,1-di-*tert*-butyl 3-(2-(methacryloyloxy)ethyl) butane-1,1,3-tricarboxylate) (POEGMEMA-*b*-PNHSMA-*b*-PMAETC) loaded with cisplatinum. The polymeric micelles were subsequently cross-linked via a reaction between pendant activated esters at the nexus core of the triblock copolymer using acid degrdabale ketal diamino cross-linkers. An in vitro study confirmed that both uncross-linked and cross-linked micelles prior to the loading of the platinum drug were nontoxic against OVCAR-3 cells even at high polymer concentration (around 300 μg mL^{-1}). The drug loaded cross-linked platinum polymeric micelles were superior to the uncross-linked platinum polymeric micelles in terms of cytotoxicity against OVCAR-3, due to a higher cellular uptake. Although there was no significant difference in cytotoxicity of cross-linked platinum polymeric micelles using different cross-linkers (permanent and acid cleavable) after 72 h of exposure, the difference was noticeable after 24 h of incubation, highlighting a much higher activity for acid degradable cross-linked micelles with conjugated platinum drugs. Moreover, the clonogenic assay suggested that cross-linked micelle loaded platinum drugs, in contrast to uncross-linked micelles, can effectively inhibit the OVCAR-3 cell regrowth for an extended period of time (10 days), even at very low micellar concentrations. In summary, acid degradable linkers ensure high cellular uptake compared to uncross-linked micelles but also lead to a faster drug action in comparison to a permanently cross-linked micelle.



KEYWORDS: *platinum drugs, polymeric micelles, cross-linked micelles, acid degradable nanoparticles, cellular uptake*

INTRODUCTION

cis-Diaminedichloroplatinum(II) (cisplatin, CDDP) is one of the oldest chemotherapy drugs available and has been in widespread use to treat a wide range of solid tumors including ovarian, cervical, head and neck, and non-small-cell lung cancer.[1−8] Treatment is usually limited, however, by side-effects such as nephrotoxicity, emetogenesis, and neurotoxicity.[1,9] Inside cells, cisplatin undergoes aquation to form $[Pt(NH_3)_2Cl(OH_2)]^+$ and $[Pt(NH_3)_2(OH_2)_2]^{2+}$. Its platinum atom binds covalently to the N^7 position of purines to form 1,2- or 1,3-intrastrand cross-links and interstrand cross-links. These cisplatin−DNA adducts result in various cellular responses including replication arrest, transcription inhibition, cell-cycle arrest, DNA repair, and apoptosis.[1,10]

Recent advances in functional nanoparticles self-assembled from amphiphilic block copolymers have led to the development of smart nanocarriers that can enhance the delivery efficiency of anticancer drugs, proteins, genes, and imaging agents.[7,11−18] Polymeric micelles encapsulation of drugs, in contrast to administration of free drugs, allows an increased circulation time in the bloodstream and an effective accumulation in vascularized solid tumors due to enhanced permeability and retention (EPR) effect.[19] Furthermore, the surface of nanoparticles can be modified to prevent the recognition by the reticuloendothelial system (RES) even though the RES can detect such polymeric micelles and eliminate them from blood circulation.[19−21] An ideal drug delivery system should have advantages such as high stability in the bloodstream, effective protection of drugs from unwanted and nonspecific binding, and triggered release of loaded drugs inside targeting cells. On the other hand, polymeric micelles are based on a dynamic equilibrium, with a tendency to dissociate at low concentrations, especially upon intravenous administration.[22,23] This leads to the low delivery efficiency in targeting sites and loss of EPR effects. Therefore, stabilization of polymeric micelles becomes an important strategy that has recently attracted a vast number of studies.[18,24−29]

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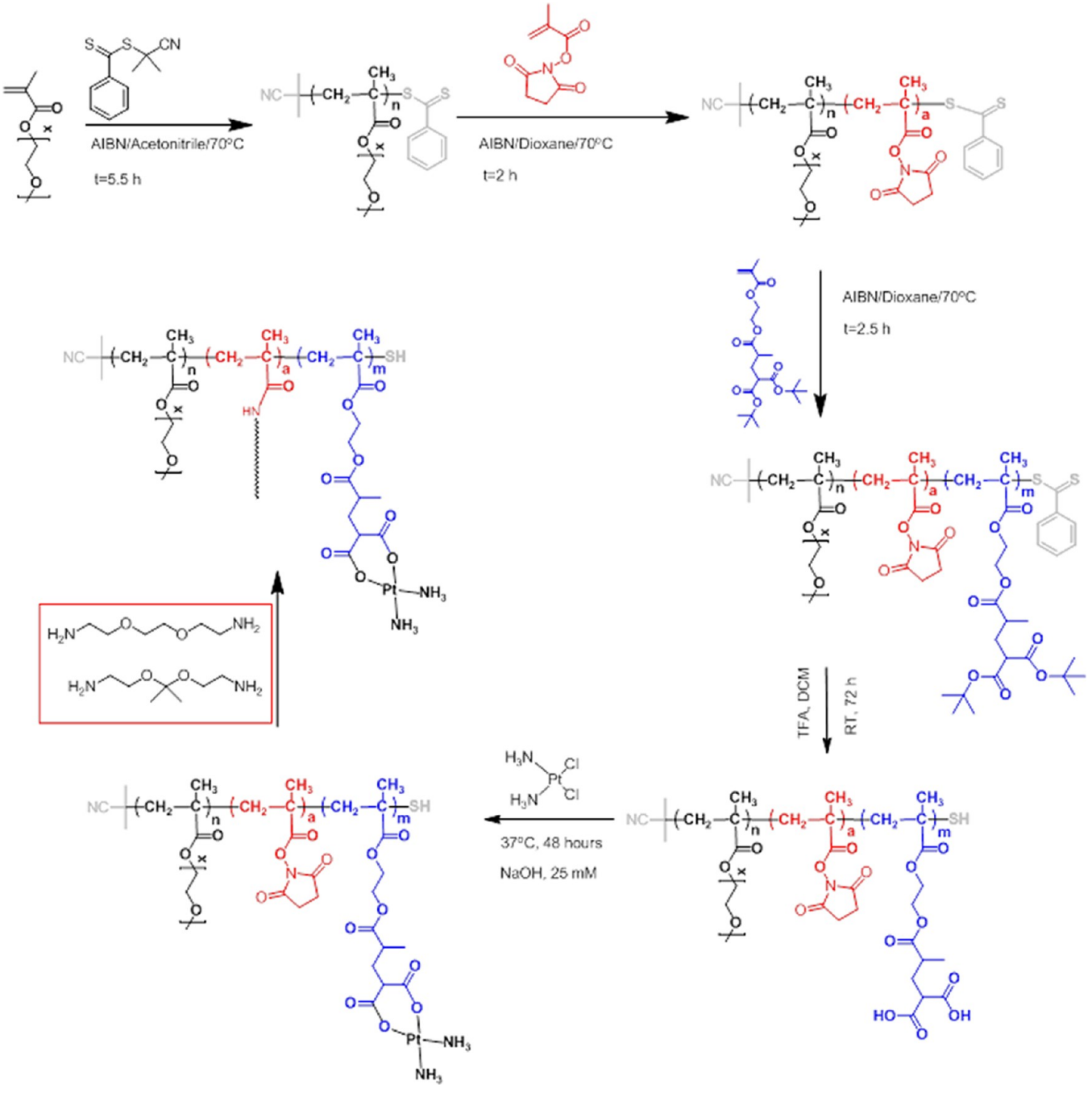
Scheme 1. Synthetic Scheme of Acid Degradable Cross-Linked Nanoparticles Containing Cisplatinum Drugs



Although it has been reported in our previous studies that more stable polymeric micelles were observed with longer hydrophobic block length[13] or with longer alkyl spacer chain (distance between polymer backbone and cisplatinum drugs),[11] they are not stable enough to tolerate the massive dilution involved by intravenous administration of drugs. In this scenario, cross-linking is a powerful tool to stabilize self-assembled structures. The high structural integrity can ensure high cellular uptake even at the lowest concentration, resulting in improved IC_{50} values of the drug.[30] However, cross-linkers should be able to degrade in the targeted sites because permanent cross-linking can limit drug release and potentially the clearance of the drug carrier.

Numerous different cross-linking techniques have been investigated using a range of different chemistries.[28,31] Although micelles are commonly cross-linked via the addition of a bifunctional reactive agent, cross-linking can in addition be achieved using a radical pathway following the addition of a divinyl cross-linker.[16,18,32,33] Cross-linking of micelles can be carried out by different ways such as core cross-linking,[26] shell cross-linking,[34,35] and nexus cross-linking.[36,37] Of these techniques, cross-linking on the nexus, that is, at the interface between core and shell, is more versatile since it allows the use of either hydrophilic or hydrophobic cross-linkers. In addition, it does not limit the loading capacity of the core while the possibility of intermicellar cross-linking is reduced.

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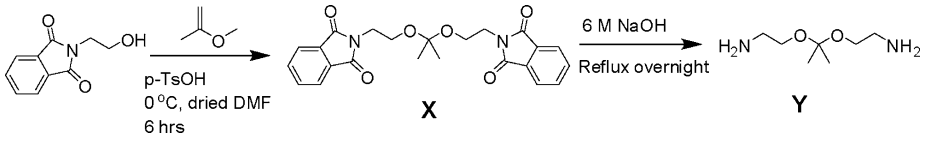
Scheme 2. Synthesis of Diamine Acid Degradable Cross-Linker



Although cross-linking of micelles has numerous benefits, the ultimate degradation of a stable nanoparticle would be desirable to enable its clearance from the body. An added benefit would be the triggered degradation inside the micelle, which simultaneously leads to a burst in drug release. The differences of pH in intracellular endosomes (pH 5.0−5.5), lysosomes (pH 4.0−4.5), and extracellular compartments (pH 7.4)[38] can act as a trigger for intracellular drug release, if an acid cleavable cross-linker is used in the system. For instance, acid degradable acetal-type cross-linkers such as 3,9-divinyl-2,4,8,10-tetraoxaspiro[5.5]undecance[39] and di(2-acryloyloxyethyoxy)-4-hydroxyphenylmethane[18] have been utilized to produce cross-linked micelles. These micelles appear to be stable at physiological pH but quickly degrade into unimers at low pH values. Another example of acid-labile linkage is the reaction of a benzaldehyde and a primary amine to generate "stealth" polycationic micelles that are stable at a high pH environment but can disassociate at endosomal pH (5.0−6.0).[40] Imine-type cross-linker can further lead to the "burst" release of doxorubicin (DOX) at endosomal pH.[41]

Herein, we describe the synthesis of cross-linked polymeric micelles carrying platinum drugs using an acid-cleavable ketal diamine cross-linker. The polymeric micelles were obtained by self-assembly of the triblock copolymer prepared via RAFT polymerization. Incorporation of a block with an activated ester as a pendant group between the shell and core-forming blocks allows cross-linking at the interface between the hydrophobic core and the hydrophilic shell (Scheme 1). In addition, a permanent cross-linker was used for comparison to evaluate the effect of the degradability on drug release and in vitro studies. ^{1}H NMR spectroscopy, dynamic light scattering (DLS), transmission electron microscopy (TEM), and size exclusion chromatography (SEC) were employed to characterize the cross-linked micelles. The differences between uncross-linked micelles and cross-linked micelles using both cross-linkers were investigated in the light of the drug release, cytotoxicity, cellular uptake, and colony formation.

EXPERIMENTAL PART

Materials. Unless otherwise specified, all chemicals were reagent grade and were used as received: Di-*tert*-butyl malonate (Aldrich, 98%), ethylene glycol dimethacrylate (EGDMA, Aldrich, 98%), potassium carbonate (Univar, anhydrous), 18-*crown*-6 (Sigma-Adrich, 99%), tetrahydrofuran (THF, anhydrous, 98%, Aldrich), diethyl ether (Et_2O anhydrous, Ajax Finechem, 99%), petroleum ether (BR 40−60 °C; Ajax Finechem, 90%), ethyl acetate (ETOAc, Ajax Finechemicals, 99.5%), *N*,*N*-dimethylacetamide (DMAc; Aldrich, HPLC grade), magnesium sulfate (Ajax Finechem, 70%), toluene (Aldrich; purum), 1,4-dioxane (Sigma-Aldrich, 99%), dichloromethane (DCM) (Ajax Finechem, 99%), trifluoroacetic acid (Sigma-Aldrich, 99%), chloroform-*d* ($CDCl_3$; Cambridge Isotape Laboratories), *cis*-dichlorodiaminoplatinum(II) (CDDP; Sigma-Aldrich; 99.9%), *N*-succinimidyl methacrylate (NHSMA, Aldrich, 99%), *N*-methyl-2-pyrrolidone (NMP, Sigma-Aldrich, 98%), 2-[2-(2-aminoethoxy)-ethoxy]ethan-1-amine (Sigma Aldrich, 97%, HPLC grade), and *p*-toluenesulfonic acid (Sigma-Aldrich, 98.5%).

2,2-Azobisisobutyronitrile (AIBN; Fluka, 98%) was purified by recrystallization from methanol. Oligo(ethylene glycol) methylether methacrylate (OEGMEMA; MW = 300 g mol^{-1}; Aldrich) was deinhibited by passing through a column of basic aluminum oxide.

The RAFT agent 2-(2-cyanopropyl)dithiobenzoate (CPDB) was synthesized according to literature[42,43] and recrystallized from toluene to yield fine pink powder. Deionized (DI) water produced by a Mili-Q water purification system has a resistivity of 17.9 mΩ/cm.

Synthesis. *Synthesis of Acid-Degradable Amine Bearing Cross-Linker.* Compound X in Scheme 2 was synthesized as described previously with some minor modifications. In a round-bottomed flask, *N*-(2-hydroxyethyl)phthalimide (3.0 g, 15.7 mmol) was dissolved in anhydrous DMF (40 mL), and *p*-toluenesulfonic acid (30 mg, 0.157 mmol) was added. The solution was cooled to 0 °C using an ice bath, and 2-methoxypropene (1.5 mL, 15.7 mmol) was carefully added to the solution. The reaction mixture was then stirred for 1 h while keeping the temperature at 0 °C to avoid loss of the highly volatile 2-methoxy propene. The flask was then connected to a trap, and the reaction mixture was heated to 45 °C under high vacuum for 6 h to remove the methanol formed during the reaction. Finally, the reaction mixture was cooled to room temperature and triethylamine (4 mL) was added to quench the reaction. To facilitate further purification, acetic anhydride (0.8 mL) was added to convert any unreacted alcohol groups into the corresponding acetate and the reaction mixture was allowed to stir overnight. DMF was then removed under reduced pressure, resulting in colorless crystals that were washed three times with ethyl acetate, yielding a thin white powder. Yield: 48%.

^{1}H NMR (300.17 MHz, $CDCl_3$, 25 °C): δ (ppm, from $CHCl_3$ signal at 7.26 ppm) 1.26 (6H, s, CH_3−C), 3.60 (4H, t, CH_2−O), 3.82 (4H, t, CH_2N), 7.4−7.8 (10H, dt, C_6H_5).

^{13}C NMR (75.48 MHz, CDCl3, 25 °C): δ (ppm from TMS) 170.1, 132.2, 123.2, 113.7, 58.0.1, 42.9, 26.5. ESI-MS. Calcd $[M + H]^+$ ($C_{23}H_{22}N_2O_6$) m/z = 422.15; found $[M + Na]^+$ = 445.3.

Compound Y (1 g, 2.4 mmol) was deprotected in 6 M NaOH (6 mL) by heating the reaction mixture at reflux overnight. The product was extracted with $CHCl_3$/iPrOH (1/1) mixture three times, and the fractions were combined and dried over anhydrous $MgSO_4$. The organic layer was filtered and evaporated in vacuo. Yield: 78%.

^{1}H NMR (300.17 MHz, $CDCl_3$, 25 °C): δ (ppm, from $CHCl_3$ signal at 7.26 ppm) 1.36 (6H, s, CH_3−C), 1.57 (bs, 4H, NH_2), 2.84 (4H, t, CH_2−NH_2), 3.46 (4H, t, CH_2−O).

^{13}C NMR (75.48 MHz, $CDCl_3$, 25 °C): δ (ppm from TMS) 113.7, 64.2, 42.9, 26.5.

Synthesis of Monomers with Pendant Carboxylic Functional Groups (1,1-Di-tert-butyl 3-(2-(methacryloyloxy)ethyl) Butane-1,1,3-tricarboxylate MAETC). 1,1-Di-*tert*-butyl 3-(2-(methacryloyloxy)ethyl) butane-1,1,3-tricarboxylate (MAETC) monomer (Scheme 3) was synthesized according to the previous publications.[11,13] The purified monomer was characterized by ^{1}H and ^{13}C NMR spectroscopy, DEPT-135, DEPT-90, and ESI-MS.

^{1}H NMR (300.17 MHz, $CDCl_3$, 25 °C): δ (ppm) = 6.12 (s, 1H,H_1), 5.58 (s, 1H, H_2); 4.34 (t, 4H, H_4 and H_5, J = 1.08 Hz); 3.2−3.25 (dd, 1H, H_9, J = 6.45 Hz, J = 8.91 Hz); 2.45−2.57 (qq, 1H, H_6, J = 6.99 Hz); 2.15 (dd, 1H, H_8, J = 6.54 Hz); 1.9 (dd, 4H, H_8 and H_3, J = 6.12 Hz); 1.43 (s, 18H, H_{10}); 1.18 (d, 3H, H_7, J = 7.02 Hz).

^{13}C NMR (75.48 MHz, CDCl3, 25 °C): δ (ppm) = 175.37 (C_g), 168.41 (C_n), 168.21 (C_p), 166.99 (C_d), 135.79 (C_c), 126.01 (C_a), 81.50 (C_o), 62.33 (C_e), 61.99 (C_f), 51.64 (C_m), 37.08 (C_h), 31.96 (C_l), 27.79 (C_q), 18.18 (C_b), 17.30 (C_k).

ESI-MS: theoretical m/z for $C_{21}H_{34}O_8$, 414.23; experimental m/z 437.1 (Na^+).

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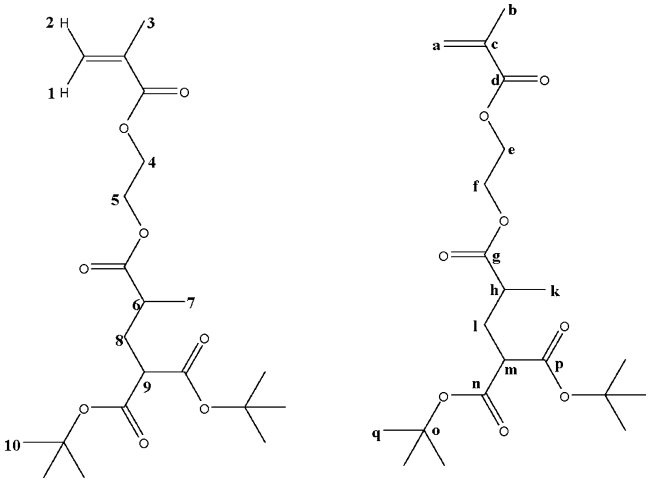
Scheme 3. MAETC Label for ^{1}H NMR (left) and ^{13}C NMR (right)



Polymer Synthesis. RAFT Polymerization of OEGMEMA Using CPADB RAFT Agent. OEGMEMA (2.5 g, 0.83 × 10^{-2} mol), CPADB (0.023 g, 0.83 × 10^{-4} mol), and AIBN (0.0027 g, 1.66 × 10^{-5} mol) were dissolved in acetonitrile (16.6 mL) in a glass bottle equipped with a magnetic stirrer bar to give [OEGMEMA]/[CPADB]/[AIBN] = 100:5:0.2 and [OEGMEMA] = 0.5 mol L^{-1}. The bottle was then sealed with a rubber septum and thoroughly deoxygenated using nitrogen purging for 30 min in an ice bath before being placed in a preheated oil bath at 70 °C. After 5.5 h, the polymerization was terminated by placing the sample in an ice bath for 5 min. Acetonitrile was removed under reduced pressure, and the concentrated reaction mixture was purified by dialysis against methanol to remove any traces of monomer and unreacted reactants using a membrane with molecular weight cutoff of 3500 Da. The solvent was evaporated, and the final polymer was characterized by ^{1}H NMR and SEC (DMAc). ^{1}H NMR (300.17 MHz, $CDCl_3$, 25 °C) δ (ppm) = 4.2−4.0 (2*n*H, $CH_3COOC\mathbf{H}_2CH_2O$), 4.0−3.8 (11*n*H, $C\mathbf{H}_2$ of OEGMEMA monomer), 3.6−3.4 (3*n*H, $C\mathbf{H}_3$ of chain end of OEGMEMA), 1.4−1.2 (3*n*H, $C\mathbf{H}_3$ of the main chain), 1.2−0.9 (2*n*H, $C\mathbf{H}_2$ of the main chain) (Figure 1). *n* is the degree of polymerization (DP_n) of POEGMEMA. The conversion of 59% was obtained after 5.5 h polymerization. The molecular weight of the POEGMEMA macroRAFT agent was measured to be 14 200 g mol^{-1} (PDI = 1.16) by SEC (DMAc), which is in good agreement with the theoretical molecular weight $M_{n(theo)}$ = 17 921 g mol^{-1}.

Synthesis of Poly(oligo(ethylene glycol) methylether methacrylate)-block-poly(N-hydroxysuccinic methacrylate) (POEGMEMA-b-PNHSMA). POEGMEMA-*b*-PNHSMA was prepared by the chain extension of POEGMEMA ($M_{n(theo)}$ = 17 921 g mol^{-1}, $M_{n(SEC)}$ = 14 200 g mol^{-1}, PDI = 1.16) using *N*-hydroxysuccinic methacrylate (NHS-MA) as a monomer. Briefly, in a glass vial, the POEGMEMA macroRAFT agent (0.5 g, 0.28 × 10^{-4} mol), NHS-MA (0.26 g, 0.14 × 10^{-2} mol), and AIBN (0.0009 g, 0.56 × 10^{-5} mol) were dissolved in 2.78 mL of *N*-methyl-2-pyrrolidone (NMP) to lead to [NHS-MA]/[MacroRAFT]/[AIBN] = 50:1:0.2 and [NHS-MA] = 0.5 mol L^{-1}. The vial was capped with a rubber septum and copper wire. The vial was then deoxygenated using nitrogen purging for 30 min and placed in a preheated oil bath at 70 °C. The polymerization was terminated by placing it in an ice bath for 5 min. The polymerization solution was precipitated twice in cold diethyl ether to yield pink brittle polymer. The final polymer was analyzed by SEC (DMAc) and ^{1}H NMR. The conversion of 22% was observed after 2 h of polymerization. The molecular weight ($M_{n(SEC)}$) of POEGMEMA-*b*-PNHS-MA was 19 200 g mol^{-1} and PDI was 1.24 measured by SEC (DMAc). This molecular weight was in good agreement with the theoretical value ($M_{n(theo)}$ = 19 934 g mol^{-1}), which was calculated using the conversions obtained by ^{1}H NMR.

^{1}H NMR (300.17 MHz, $CDCl_3$, 25 °C) δ (ppm) = 4.2−4.0 (2*n*H, $CH_3COOC\mathbf{H}_2CH_2O$), 4.0−3.8 (11*n*H, $C\mathbf{H}_2$ of OEGMEMA monomer), 3.6−3.4 (3*n*H, $C\mathbf{H}_3$ of chain end of OEGMEMA), 3.0−2.8 (4*a*H, $C\mathbf{H}_2$ of NHS), 2.4−2.2 (3*a*H, $C\mathbf{H}_3$ of NHSMA), 1.4−1.2 (3*n*H, $C\mathbf{H}_3$ of the main chain), 1.2−0.9 (2*n*H + 2*a*H, $C\mathbf{H}_2$ of the main chain) (Figure 1). *n* and *a* are the degrees of polymerization (DP_n) of POEGMEMA and PNHSMA, respectively.

Synthesis of Triblock Copolymer Poly(oligo(ethylene glycol) methylether methacrylate)-block-poly(N-hyroxysuccinic methacrylate)-block-poly(1,1-di-tert-butyl 3-(2-(methacryloyloxy)ethyl) butane-1,1,3-tricarboxylate) (POEGMEMA-b-PNHSMA-b-PMAETC). The triblock copolymer was prepared by chain extension of the diblock copolymer POEGMEMA-*b*-PNHSMA ($M_{n(theo)}$ = 19 934 g mol^{-1}, $M_{n(SEC)}$ = 19 200 g mol^{-1}, PDI = 1.24) using MAETC as a monomer. Briefly, in a glass vial, MAETC (0.06 g, 1.4 × 10^{-4} mol), POEGMEMA-*b*-PNHS-MA (0.029 g, 1.4 × 10^{-6} mol) as a macroRAFT agent, and AIBN (0.5 × 10^{-4} g, 2.89 × 10^{-7} mol) were dissolved in 0.29 mL of dioxane to result in [MAETC]/[macroRAFT]/[AIBN] = 100:1:0.2 and [MAETC] = 0.5 mol L^{-1}. The vial was capped with a rubber septum and copper wire. It was then deoxygenated using nitrogen purging for 45 min and then placed in a preheated oil bath at 70 °C. The sample was taken after 2.5 h of polymerization. In order to quench the polymerization reaction, the vial was placed in an ice bath for 5 min. The final copolymer was characterized by SEC (DMAc) and ^{1}H NMR.

^{1}H NMR (300.17 MHz, $CDCl_3$, 25 °C): δ (ppm) = 4.4−4.2 (4*m*H, $O\mathbf{CH}_2\mathbf{CH}_2O$), 4.2−4.0 (2*n*H, $CH_3COOC\mathbf{H}_2CH_2O$), 4.0−3.8 (11*n*H, $C\mathbf{H}_2$ of OEGMEMA monomer), 3.6−3.4 (3*n*H, $C\mathbf{H}_3$ of chain end of OEGMEMA), 3.3−3.1 (*m*H, *tert*-Bu-OOCC**H**CH_2COO-Bu-*tert*), 3.0−2.8 (4*a*H, $C\mathbf{H}_2$ of NHS), 2.8−2.6 (*m*H, OOCC**H**CH_3−CH2), 2.4−2.2 (3*a*H, $C\mathbf{H}_3$ of NHSMA), 2.3−2.0 (m*H*, $OOCCHCH_3C\mathbf{H}_2CH(COO\text{-Bu-}tert)_2$), 2.0−1.6 (*m*H+3*m*H, $OOCCHCH_3C\mathbf{H}_2CH(COO\text{-Bu-}tert)_2$ and $C\mathbf{H}_3$ attached to the backbone of MAETC), 1.6−1.3 (18*m*H, $OOCCHCH_3CH_2CH(COO(C\mathbf{H}_3)_3)_2$), 1.4−1.2 (3*n*H, $C\mathbf{H}_3$ of the main chain), 1.2−0.9 (2*n*H + 2*a*H + 2*m*H, $C\mathbf{H}_2$ of the main chain) (Figure 1). *n*, *a*, and *m* are the degrees of polymerization (DP_n) of POEGMEMA, PNHSMA, and PMAETC, respectively.

The conversion of 40% was observed after 2.5 h polymerization. The molecular weight ($M_{n(SEC)}$) was 37 550 g mol^{-1} and PDI was 1.29 measured by SEC (DMAc) while the theoretical molecular weight ($M_{n(theo)}$) was 28 600 g mol^{-1} using ^{1}H NMR to quantify the ratios between blocks.

Deprotection of Block Copolymers. Deprotection of the *tert*-butyl groups of POEGMEMA-*b*-PNHSMA-*b*-PMAETC was carried out similar to the procedure described in previous publications.[11,13] Briefly, the triblock copolymer (0.2 g) was fully dissolved in dichloromethane (DCM, 0.5 mL). Trifluoroacetic acid (3 equiv compared to *tert*-butyl group) was then added in the polymer mixture, followed by stirring at room temperature for 72 h. The final reaction mixture was subsequently dialyzed against acetone−water (1:1) using a tubular membrane with 3500 Da as molecular weight cutoff (MWCO). Acetone was then removed by dialysis against DI water. Subsequently, the remaining solution inside the bag was freeze-dried to yield a waxy polymer. The deprotected copolymer was characterized by ^{1}H NMR.

^{1}H NMR (300.17 MHz, $CDCl_3$, 25 °C): δ (ppm) = 4.4−4.2 (4*m*H, $O\mathbf{CH}_2\mathbf{CH}_2O$), 4.2−4.0 (2*n*H, $CH_3COOC\mathbf{H}_2CH_2O$), 4.0−3.8 (11*n*H, $C\mathbf{H}_2$ of OEGMEMA monomer), 3.6−3.4 (3*n*H + *m*H, $C\mathbf{H}_3$ of chain end of OEGMEMA and *tert*-Bu-OOCC**H**CH_2COO-Bu-*tert*), 3.0−2.8 (4*a*H, $C\mathbf{H}_2$ of NHS), 2.8−2.6 (*m*H, OOCC**H**CH_3−CH2), 2.4−2.2 (3*a*H, $C\mathbf{H}_3$ of NHSMA), 2.3−2.0 (*m*H, $OOCCHCH_3C\mathbf{H}_2CH(COO\text{-Bu-}tert)_2$), 2.0−1.6 (*m*H+3*m*H, $OOCCHCH_3C\mathbf{H}_2CH(COO\text{-Bu-}tert)_2$ and $C\mathbf{H}_3$ attached to the backbone of MAETC), 1.4−1.2 (3*n*H, $C\mathbf{H}_3$ of the main chain), 1.2−0.9 (2*n*H + 2*a*H + 2*m*H, $C\mathbf{H}_2$ of the main chain) (Figure 1). *n*, *a*, and *m* are the degrees of polymerization (DP_n) of POEGMEMA, PNHSMA, and PMAETC, respectively.

Polymer−Platinum Conjugates. Conjugation of *cis*-dichlorodiamino platinum(II) to malonic acid of the deprotected triblock copolymer POEGMEMA-*b*-PNHSMA-*b*-PMAETC was prepared following the method described in previous publications.[11,13] In a typical experi-

ment, CDDP (10 mg) was suspended in 10 mL of distilled water and mixed with silver nitrate ($[AgNO_3]/[CDDP] = 1.955$) to form the aqueous complex. The solution was stirred in the dark at room temperature for 4 h. White precipitate of silver chloride was observed indicative of the proceeding reaction. The mixture was then centrifuged at 9000 rpm for 20 min to remove the AgCl precipitates, and the supernatant was purified by passing it through a 0.22 μm filter. Polymers with carboxyl functional groups (25 mg, dissolved in 2 mL of NaOH (1 mg mL^{-1}) to be deprotonated) were added to the above prepared CDDP aqueous solution and left to react in a water bath at 37 °C for 12 h with gentle shaking The prepared conjugate was purified by ultrafiltration using Sartorius Vivaspin 6 centrifugal filter devices with a molecular weight cutoff of 3000 Da, followed by freeze-drying yielding a yellow powder.

Self-Assembly of Triblock Copolymer and Platinum Drug Conjugates into Micellular Structure. The platinum drug conjugated POEGMEMA-*b*-PNHSMA-*b*-PMAETC (60 mg) was dissolved in DMF (3 mL), which is a good solvent for the hydrophobic and the hydrophilic block. Distilled water (10 mL) was added dropwise using a syringe pump (3 mL h^{-1}) to 3 mL of conjugate in DMF (20 mg mL^{-1}) under moderate stirring at room temperature. The mixture was then dialyzed against DI water for 24 h using a dialysis membrane (MWCO 3500 Da) to remove DMF. The targeted final polymer concentration was 5 mg mL^{-1}. Care needs to be taken to avoid the hydrolysis of the NHS ester by carefully monitoring the pH value of the solution.

Synthesis of Cross-Linked Micelles. In a typical experiment, a polymer solution (5 mg mL^{-1}; 4 mL) in distilled water was employed to perform the cross-linking process. The synthesized acid degradable amine-bearing cross-linker (compound Y or 2-[2-(2-aminoethoxy)-ethoxy]ethan-1-amine, 0.5 mg, 3.3×10^{-3} mmol) was added, and the mixture was then stirred at an ambient temperature overnight. The cross-linked nanoparticles were purified using membrane dialysis (MWCO 3500 Da) for 48 h against distilled water to remove the unreacted cross-linker. A part of the solution was freeze-dried and characterized by SEC (DMAc) and DLS to confirm the cross-linking process. In this study, two types of cross-linkers including 2-[2-(2-aminoethoxy)ethoxy]ethan-1-amine and acid degradable amine-bearing cross-linker were used.

Critical Micelle Concentration. The critical micelle concentration (CMC) was measured by fluorescence spectroscopy using pyrene as a fluorescent probe following an established procedure.[44] Briefly, a stock solution of pyrene was made by dissolving pyrene (1 mg, 5 μmol) in acetone (200 mL) to form a 2.5×10^{-5} M solution. The pyrene solution (48 μL) was dropped into empty vials, and the acetone was evaporated overnight under reduced pressure. A stock solution with micelles and cross-linked micelles was serially diluted with deionized water starting with the concentration of 0 to 100 μg mL^{-1}. Each polymer solution (2 mL) was transferred to a vial containing pyrene and stirred overnight. The final concentration of pyrene in the polymer solutions was 6×10^{-7} M (which is less than the pyrene saturation concentration in water).[45] Fluorescence measurements were carried out using an excitation wavelength of λ = 237.96 nm, using a 2.5 nm slit width for excitation and a 2.5 nm slit width for emission. Emission wavelengths were scanned from 350 to 450 nm. The intensities of I_1 (372 nm) to I_3 (383 nm) vibronic bands were evaluated for each sample, and the ratio of these were plotted against the log of the concentration of each polymeric sample.[46] The critical micelle concentration (CMC) was taken as the intersection of two regression lines calculated from the linear portions of the graphs.

Release of Cisplatin from Micelles. Micelles and cross-linked micelles (2 mL) containing a platinum drug were dialyzed against pH buffer solutions (pH 7.4 and 5.5) (200 mL) at 37 °C. 0.9% of NaCl was added into these buffer solutions to trigger the platinum drug release. Dialysis membranes with molecular weight cutoff of 3500 Da were used to allow equilibrium of the free platinum drug from inside and outside the dialysis membrane. Aliquots of 1 mL were taken at different times (24 and 72 h). The amount of released Pt was determined using inductively coupled plasma mass spectroscopy (ICP-MS). To a 10 mL centrifuge tube, 1 mL of the dialysate was diluted 5 times with 2% aqua regia (HCl/HNO_3 = 3:1). The solution was then digested at 60 °C for 3 h and then cooled to room temperature. The concentration of Pt released from the conjugate was expressed as a ratio of the amount of platinum in the releasing solution (the solution outside the dialysis membrane) and that in the initial sample. The percentage of Pt released was calculated using the equation below:

$$\%\ \text{Release} = \frac{V_{\text{total}}(t) \times C + Y}{Z}$$

where $V_{\text{total}}(t)$ is the remaining volume in the releasing container at time t in mL; C is the concentration of platinum determined from ICP-MS in μg mL^{-1}; Y is the amount of platinum that has already been collected in μg; and Z is the total amount of platinum at t = 0 present in the dialysis bag in μg.

Cell Culture. The human ovarian cancer cell line OVCAR-3 was grown in RPMI-1640 [2×10^{-3} M glutamine, 1.5 g L^{-1} sodium bicarbonate, 0.010 M 2-hydroxyethylpiperazinesulfonic acid (HEPES), 4.5 g L^{-1} glucose, and 10^{-3} M sodium pyruvate] medium supplemented with 10% fetal bovine serum (FBS). The cells were grown in a 5% CO_2 atmosphere in an incubator at 37 °C.

Cytotoxicity Assay. The sulforhodamine B (SRB) assay established by the U.S. National Cancer Institute for rapid, sensitive, and inexpensive screening of antitumor drugs in microplates was employed to screen the cytotoxicity and antitumor activities of polymers and polymeric platinum drugs, respectively.[47] Human ovarian cancer cells (OVCAR-3) diluted in 100 μL of RPMI-1640 medium (2 mM L-glutamine, 1.5 g L^{-1} sodium bicarbonate, 10 mM HEPES, 4.5 g L^{-1} glucose, 1 mM sodium pyruvate) were seeded into the wells at a concentration of 3000 cells/well. The microtiter plates were incubated for 24 h at 37 °C and then exposed to various doses of polymers and micelles for 24 and 72 h before being assayed for cell growth inhibition. Cell cultures were fixed with TCA (10%, w/v) and incubated at 4 °C for 1 h. The wells were then washed five times with tap water to remove TCA, growth medium, and low molecular weight metabolites. Plates were air-dried and then stored until use. TCA-fixed cells were stained for 30 min with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed five times with 1% (v/v) acetic acid to remove unbound dye. Subsequently, the cultured plates were air-dried until no conspicuous moisture was visible before bound dye was shaken in 100 μL of 10 mM Tris base for 5 min. The absorbance at 570 nm of each well was measured using microtiter plate reader scanning spectrophotometer (BioTek's PowerWave HT Microplate Reader and KC4 Software). Each sample was replicated three times.

Cellular Uptake. Cellular uptake experiments were performed according to a previously described method with some modifications.[48] OVCAR-3 cells were seeded into a 12-well plate at 16×10^3 cells per well and incubated for 24 h. The cells were treated with polymer−Pt micelles including uncross-linked micelles and cross-linked micelles using different cross-linkers. After 24 h incubation at 37 °C, the medium was removed and rinsed with cold PBS (1 mL × 3). The cells were trypsinized and incubated with HNO_3 (68%, v/v) at 65 °C for 20 h.

Platinum content uptake was determined using inductively coupled plasma mass spectrometer (ICP-MS). A four-point standard curve was plotted between intensity versus a serial dilution of a certified reference standard ranging from 1 to 1000 ppb. The reported result of the sample is the average of three replicates.

The amount of polymer taken up by the cells was qualitatively and quantitatively determined using fluorescence microscopy and fluorescence reader, respectively. To label polymers, fluorescein-*o*-methacrylate (2% mol of POEGMEMA) was copolymerized with OEGMEMA before chain extending with NHS-MA and then with the synthesized monomers such as MAETC to yield triblock copolymer. The block copolymer was conjugated to CDDP and formed micelles following the procedure mentioned above. OVCAR3 was seeded into 12 well plates at a concentration of 16 000 cells per well and incubated for 24 h. Those cells were subsequently treated with the fluorescein-labeled micelles for 2 and 15 h at 37 °C. All cells were washed 3 times with PBS before observing under microscopy. Cell uptake pictures

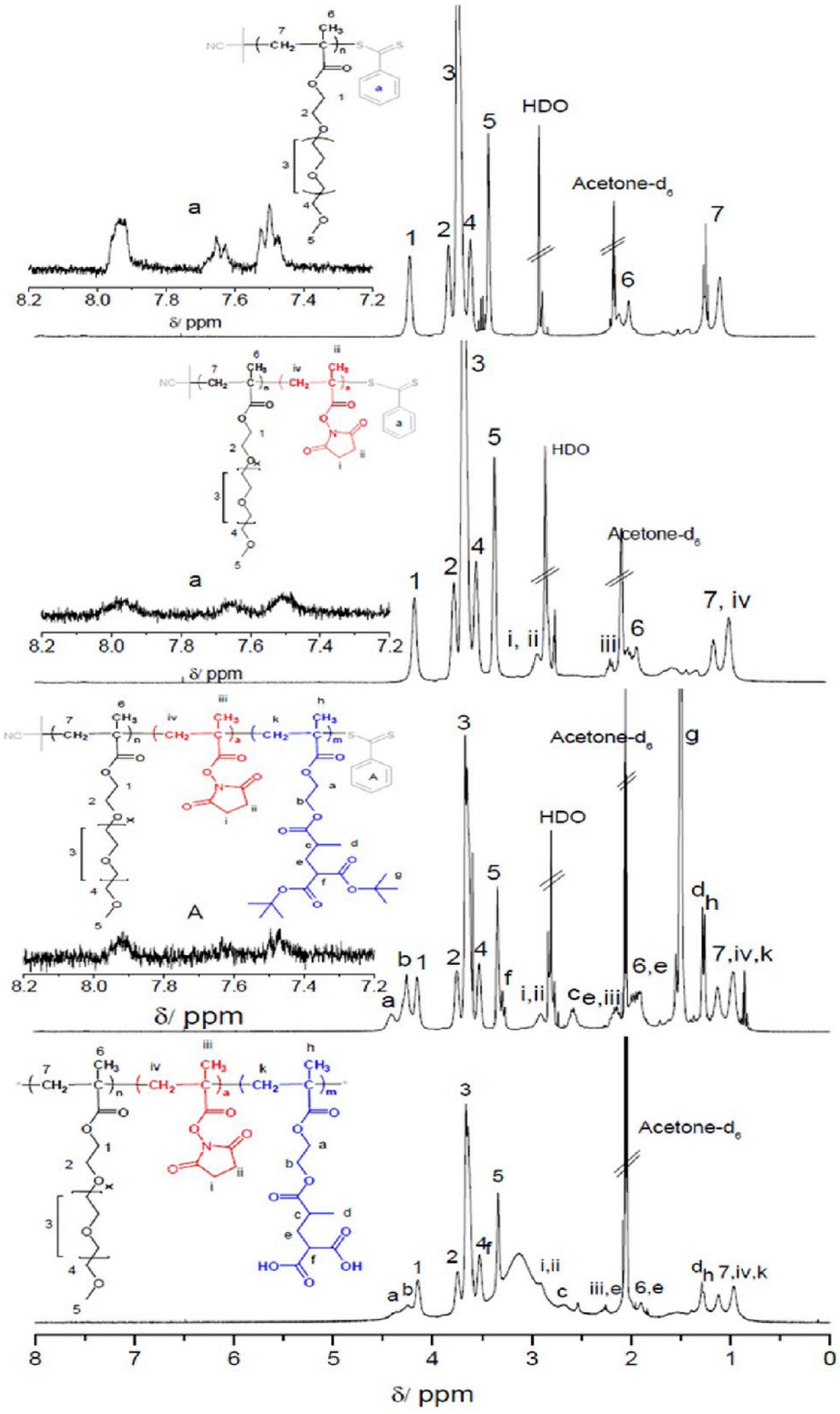


Figure 1. ^{1}H NMR (acetone-d_6) of the macroRAFT agent, the diblock copolymer, the triblock copolymer, and the deprotected triblock copolymer.

were acquired by using a fluorescence microscope with mercury lamp of λ_{ex} 535 nm and λ_{em} 590 nm to track the fluorescence micelles. Fluorescence intensity of treated cell solution was measured by fluorescence reader at λ_{ex}= 535 nm and λ_{em} = 590 nm.

Colony Formation Assay. Unlike the cell proliferation assay, colony formation assay measures the productive integrity of the cells following withdrawal of drug treatment. The assays were performed as described by Liebmann et al.[49] with some modifications. Briefly, OVCAR-3 cells were exposed to triblock copolymer (100 μg mL^{-1}), uncross-linked platinum polymeric micelles (2.1 μM Pt [correlative to 5.5 μg mL^{-1} Pt−polymer conjugates, <CMC value] and 34.8 μM Pt [correlative to 91.2 μg mL^{-1} Pt−polymer conjugates, >CMC value]), and acid-sensitive cross-linked platinum polymeric micelles (2.3 μM Pt [correlative to 6 μg mL^{-1} Pt−polymer conjugates, <CMC value] and 32.5 μM Pt [correlative to 85.2 μg mL^{-1} Pt−polymer conjugates, >CMC value]) for 72 h and then washed with phosphate buffer solution (PBS). Single survived cells were then plated in six well plates with fresh PRMI 1640 medium, and the medium was changed every 3 days. Following 10 days of incubation, the cells were washed twice with cold PBS and incubated with methanol for 30 min at room temperature to fix the cells. Methanol was evaporated, and cells were stained with 0.1% crystal violet for 3 min. The excess crystal violet was washed five times with tap water and air-dried overnight. The data were calculated based on eqs 1 and 2.

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Table 1. Triblock Copolymer Synthesis for *cis*-Platinum Conjugation

polymers	M_n^{theo} (g mol^{-1})[a]	M_n^{SEC} (g mol^{-1})[a]	PDI	no. of RU, $N_{OEGMEMA}$	no. of RU, N_{MANHS}	no. of RU, N_{MAETC}
$POEGMEMA_{50}$	17921	14200	1.16	59		
$POEGMEMA_{50}$-*b*-$PMANHS_{11}$	19934	19200	1.24	59	11	
$POEGMEMA_{50}$-*b*-$PMANHS_{11}$-*b*-$PMAETC_{40}$	37550	28600	1.29	59	11	40

[a] M_n^{SEC} determined by SEC (DMAc) using PSt calibration; M_n^{theo} calculated by ^{1}H NMR.

$$\text{Plating efficiency (PE)} = \frac{\text{no. of colonies formed}}{\text{no. of cells seeded}} \times 100\% \quad (1)$$

$$\text{Surviving fraction (SF)} = \frac{\text{no. of colonies formed after treatment}}{\text{no. of cells seeded} \times \text{PE}} \quad (2)$$

Analyses. *Size Exclusion Chromatography (SEC).* SEC was implemented using a Shimadzu modular system comprising a DGU-12A degasser, a LC-10AT pump, aSIL-10AD automatic injector, a CTO-10A column oven, a RID-10A refractive index detector, and a SPD-10A Shimadzu UV/vis detector. A 50 × 7.8 mm guard column and four 300 × 7.8 mm linear columns (500, 10^3, 10^4, and 10^5 Å pore size, 5 μm particle size) were used for the analysis. *N*,*N*′-dimethylacetamide (DMAc) (HPLC grade, 0.05% w/v of 2,6-dibutyl-4-methylphenol (BHT), 0.03% w/v of LiBr) with a flow rate of 1 mL min^{-1} and a constant temperature of 50 °C was used as the mobile phase with an injection volume of 50 μL. The samples were filtered through 0.45 μm filters. The unit was calibrated using commercially available linear polystyrene standards (0.5−1000 kDa, Polymer Laboratories). Chromatograms were processed using Cirrus 2.0 software (Polymer Laboratories).

Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker ACF300 (300 MHz) spectrometer, using $(CD_3)_2SO$, CD_3OD, or $CDCl_3$ as solvents. All chemical shifts are stated in ppm (δ) relative to tetramethylsilane (δ = 0 ppm), referenced to the chemical shifts of residual solvent resonances (^{1}H and ^{13}C). The number of scan of ^{1}H NMR was 16 as default for all polymer samples. For ^{195}Pt NMR measurement, ^{195}Pt resonances were externally referenced to Na_2PtCl_6 at 0 ppm. Spectra were obtained using a broadband observed 5 mm probe with *z*-axis gradient capability. The Bruker pulse program zgmultiscan was modified to execute a very short delay time (set at d_1 = 2 ms) followed by a hard 90° pulse. The experiment was run in increments of 20 000 scans (ns = 20 000) over a 130 kHz sweep width (9 ms acquisition time). A loop counter parameter 13 = 100 was incorporated such that the initial iteration of 20 000 scans was repeated 90 times to give an accumulated number of 2 000 000 scans (FIDs from each iteration were automatically combined and Fourier transformed to produce the frequency domain spectrum).

Dynamic Light Scattering (DLS). The average hydrodynamic diameters D_h and size distributions of the prepared micelle solution in an aqueous solution (1 mg mL^{-1}) were measured using a Malvern ZetasizerNano ZS instrument equipped with a 4 mV He−Ne laser operating at λ = 632 nm, an avalanche photodiode detector with high quantum efficiency, and an ALV/LSE-5003 multiple tau digital correlator electronics system. The samples were filtered to remove dust using a microfilter (0.45 μm) prior to measurement.

Transmission Electron Microscopy (TEM). Analyses were performed using a JEOL 1400 TEM with a beam voltage of 100 kV and a Gatan CCD for acquisition of digital images. Samples were prepared by placing a droplet of a 1 mg mL^{-1} polymer solution on a formamide and graphite coated copper grid. Excess solution was drained after 60 s using filter paper. To negatively stain the samples, a droplet of 2% (w/v) phosphotungstic acid solution was placed on the copper grid for 30 s before being drained with filter paper.

Thermogravimetric Analysis (TGA). Thermal decomposition properties of polymers were recorded using a Perkin-Elmer Thermogravimetric Analyzer (Pyris 1 TGA). Measurements were conducted over a temperature range of 30−700 °C with a programmed temperature increment of 20 K min^{-1}.

Inductively Coupled Plasma−Mass Spectrometer (ICP-MS). The Perkin-Elmer ELAN 6000 inductively coupled plasma−mass spectrometer (Perkin-Elmer, Norwalk, CT, USA) was used for quantitative determinations of platinum. All experiments were carried out at an incident ratio frequency power of 1200 W. The plasma argon gas flow of 12 L min^{-1} with an auxiliary argon flow of 0.8 L min^{-1} was used in all cases. The nebulizer gas flow was adjusted to maximize ion intensity at 0.93 L min^{-1} as indicated by the mass flow controller. The element/mass detected was ^{195}Pt, and the internal standard used was ^{193}Ir. The replicate time was set to 900 ms and the dwell time to 300 ms. Peak hopping was the scanning mode employed, and the number of sweeps/readings was set to 3. Ten replicates were measured at a normal resolution. The samples were treated with aqua regia solution at 90 °C for 2 h to digest platinum.

RESULTS AND DISCUSSION

Synthesis of POEGMEMA-*b*-PNHSMA-*b*-PMAETC Triblock Copolymer via RAFT Polymerization. The main focus of this work was to investigate any measurable benefits on cell growth inhibition when using an acid degradable cross-linker compared to a permanent cross-linker. The present work follows on from a series of previous studies, in which novel dicarboxylato ligands were synthesized for effective *cis*-platinum drug conjugation[13] showing that increased spacer length had a favorable influence on micelle stability, cellular uptake, and anticancer activities.[11] Cross-linking of the micelle was considered therefore the next logical step. In order to prepare cross-linked micelles, the triblock copolymer, POEGMEMA-*b*-PNHSMA-*b*-PMAETC, was synthesized via RAFT polymerization. Poly(oligoethylene glycol methacrylates) [POEGMEMA] homopolymer was first synthesized in acetonitrile using 2-(2-cyanopropyl) dithiobenzoate (CPDB) as the chain transfer agent and AIBN as an initiator at 70 °C to target the molecular weight of 14 kDa (Scheme 1). The polymerization was quenched at a monomer conversion of 60−70% to maintain high end group fidelity. The conversion of the monomer was calculated using ^{1}H NMR spectroscopy by comparing the intensity of vinyl proton peaks (6.1 and 5.6 ppm) to the aliphatic proton peaks (1.1−1.3 ppm).

The homopolymer POEGMEMA was then purified by dialysis against methanol and followed by SEC analysis in DMAc and ^{1}H NMR spectroscopy in acetone-d_6. ^{1}H NMR analysis shows the characteristic peaks of POEGMEMA at 4.2, 3.9−3.5, 3.3, 1.9, and 1.3−0.9 ppm attributed to CH_2O ester, CH_2O ether, CH_3O, CH_3−C, and CH_2−C backbone, respectively (Figure 1). The presence of the RAFT end group was confirmed by ^{1}H NMR analysis using the chemical shift at 7.2−8.2 ppm (benzyl group) (Figure 1). The POEGMEMA macroRAFT agent employed for the block copolymerization was obtained upon a monomer conversion of 50% ($M_{n(theo)}$ = 17 921 g mol^{-1}, $M_{n(SEC)}$ = 14 200 g mol^{-1}, PDI of 1.16).

The POEGMEMA macroRAFT was then chain extended with the activated ester monomer, *N*-succinimidyl methacrylates, at 70 °C for 2 h using *N*-methyl-2-pyrrolidone (NMP) as a solvent (Scheme 1). The resulting block copolymer,

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POEGMEMA-*b*-PMANHS, had a PDI of 1.24 and the successful chain extension was confirmed by SEC analysis showing the shift in the SEC traces from low molecular weight (14 200 g mol^{-1}) to higher molecular weight (19 200 g mol^{-1}). The monomer conversion was determined by ^{1}H NMR based on comparison of the intensities of vinyl proton peaks (6.1 and 5.6 ppm) to the characteristic peaks for methylene groups of the succinimidyl functionality at 2.9 ppm. The theoretical molecular weight (19 924 g mol^{-1}) corresponding to 11 repeating units was in good agreement with the molecular weight observed by SEC in DMAc (19200 g mol^{-1}), based on polystyrene standards (Table 1). The presence of RAFT end groups of POEGMEMA-*b*-PMANHS was confirmed by ^{1}H NMR with the benzyl peaks located at 7.2−8.2 ppm (Figure 1).

Subsequent chain extension of the POEGMEMA-*b*-PMANHS macroRAFT agent with the synthesized monomer (MAETC) was carried out in 1,4-dioxane after prior testing of the solvent for peroxides, which can potentially harm the RAFT end group (Scheme 1).[50] After 2.5 h of polymerization, the monomer conversion reached 40%, which was determined by ^{1}H NMR by comparing the vinyl proton peaks (6.1 and 5.6 ppm) to the methyl peak at 2.6 ppm (H_6 in the Synthesis of Monomers section). SEC confirmed that the chain extension process was successfully evidenced by a shift toward higher molecular weight (Figure 2). The deviation between the

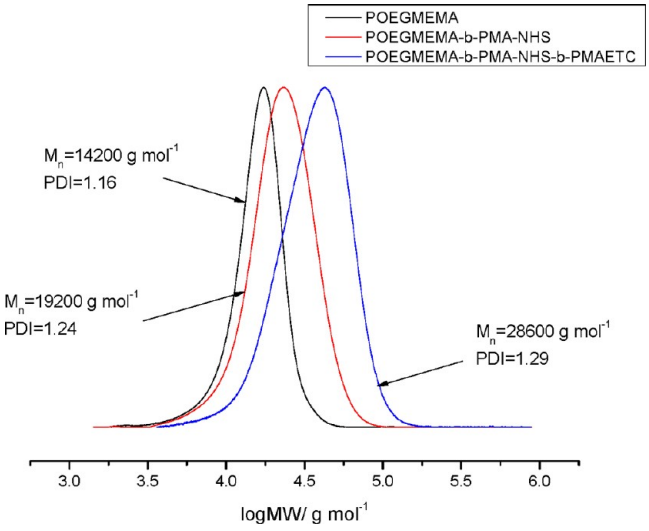

Figure 2. SEC traces of the POEGMEMA macroRAFT agent, the POEGMEMA-*b*-PMA-NHS diblock copolymer, and the triblock copolymer, POEGMEMA-*b*-PMANHS-*b*-PMAETC.

triblock copolymer molecular weight observed by SEC and the one calculated from ^{1}H NMR could be the result of the different hydrodynamic volumes for the triblock copolymer and the polystyrene used for the SEC calibration. This phenomenon has been repeatedly reported with similar polymers.[11,13] The SEC peak is not asymmetric, which is the result of an accumulation of potential side reactions that can occur during RAFT polymerization. These side reactions become more pronounced with increasing numbers of blocks and cannot be avoided. They are discussed in detail elsewhere.[19,51] Figure 1 depicts the ^{1}H NMR spectrum of the homopolymer (POEGMEMA), the diblock copolymer (POEGMEMA-*b*-PMANHS), and the triblock copolymer (POEGMEMA-*b*-PMANHS-*b*-PMAETC) in acetone-d_6.

Deprotection and Platinum Drug Conjugation. The triblock copolymer POEGMEMA-*b*-PMANHS-*b*-PMAETC was deprotected using TFA and purified via dialysis against a mixture of acetone and water (1:1) followed by pure water. ^{1}H NMR confirmed a successful deprotection as evidenced by the absence of the methyl peaks at 1.5 ppm (Figure 1). In addition, the molecular weight, measured by SEC, shifted to a higher value, in opposition to the expected molecular weight loss (Figure 3). This phenomenon has been repeatedly observed with similar polymers and can be explained by the increase in hydrodynamic volume, which was been investigated earlier.[11]

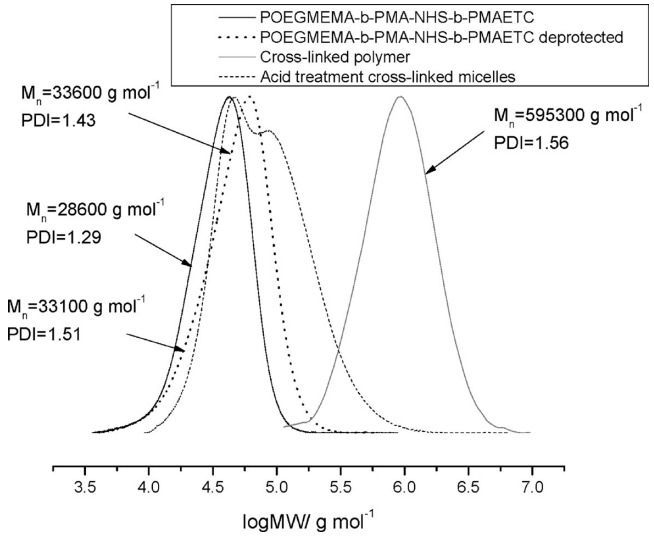

Figure 3. SEC traces of uncross-linked polymer, cross-linked polymer, and cross-linked polymer after treatment with acid.

The deprotected triblock copolymer, POEGMEMA-*b*-PMANHS-*b*-PMAETC, was then conjugated to CDDP following an established procedure.[11,13,14] The necessary deprotonation of the carboxylic acid groups using NaOH led to fully water-soluble polymers. In the next step, the polymers were incubated with the prepared *cis*-diamminediaqua platinum(II) complexes to form a macromolecular metal complex. The excess platinum drug was eliminated via dialysis against water for 24 h. The platinum loading efficiency onto polymers was measured using TGA and ICP-MS. The platinum loading was measured to be 70% based on the dicarboxylic groups, which is comparable with the results reported in our previous studies. Full platinum conjugation was restricted by steric hindrance and the entropically unfavorable stretching of the polymer chain.[11,13] The structure of the platinum complex, as displayed in Scheme 1, was confirmed using ^{195}Pt-NMR.[13] Noticeably, the originally fully water-soluble triblock copolymer has now amphiphilic character capable of self-assembling into shell−core nanostructures.

Self-Assembly in Aqueous Solution and Cross-Linking of Micelles. The platinated copolymer was freeze-dried and then redissolved in a good solvent for all three blocks, here DMF. Slow addition of DI water led to the collapse of the hydrophobic part of the block copolymer leading to aggregate formation with POEGMEMA as the water-soluble shell. The DMF was removed via dialysis against DI water. The self-assembly in water was confirmed by DLS and TEM (Figure 4). Results observed from DLS depicted an aggregate size of around 90 nm. TEM analysis confirmed the presence of

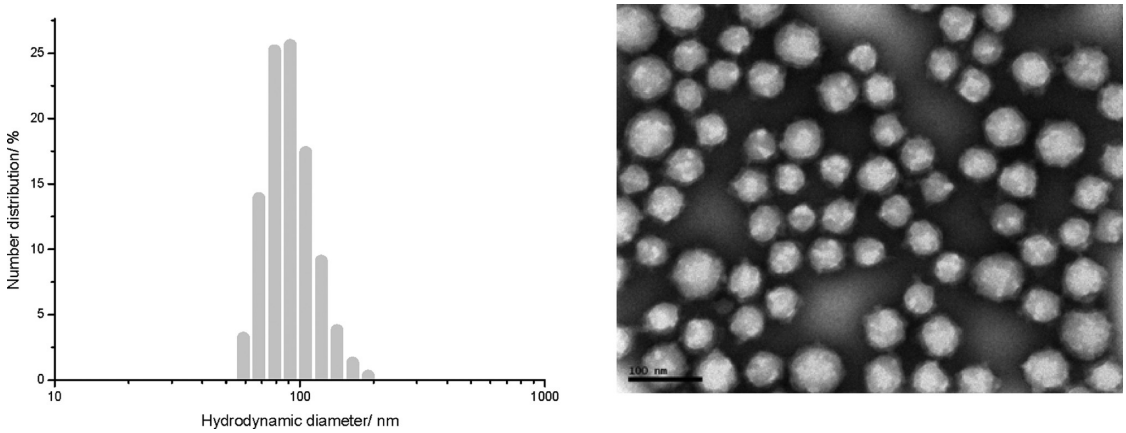

Figure 4. DLS and TEM of self-assembled micelles (polymer concentration of 1 mg mL^{-1} in water). Phosphotungstic acid was used as a negative stain, and the scale bar = 100 nm.

spherical micelles with a slightly smaller size, which is expected due to the dehydrated state (Figure 4).

The micelle stability has been identified as one potential contributing factor to allow the safe delivery of the drug loaded carrier into the tumor cell.[11,30] Lengthening of the hydrophobic block leads to micelles with a lower CMC but also led to better cellular uptake.[11] Considering the massive dilution occurring in vivo, cross-linking of the micelle might add further stabilization.[30]

In this study, the reaction between diamines and the activated esters, *N*-succinimidyl methacrylates, which are located at the nexus between the core and the shell, was utilized to cross-link the micelles. The cross-linked micelles were characterized by DLS and SEC in DMAc as DMAc is a good solvent to dissolve both blocks. Two different types of cross-linkers were employed (Scheme 1 and Table 2), which

Table 2. Summary of Platinum Polymeric Micelles Used in This Study

micelles	cross-linker	notes	CMC[a] (μg mL^{-1})
uncross-linked platinum polymeric micelles	N/A		25
cross-linked platinum polymeric micelles 1	2-[2-(2-aminoethoxy) ethoxy]ethan-1-amine	permanent cross-linker	
cross-linked platinum polymeric micelles 2	2,2′-(propane-2,2-diylbis(oxy)) diethanamine	acid degradable cross-linker	

[a]Measured using fluorescence spectroscopy with pyrene as a probe.

can either lead to permanently cross-linked micelles or result in micelles that are degradable under acidic conditions. Successful cross-linking with the acid degradable cross-linker 2,2′-(propane-2,2-diylbis(oxy))diethanamine was confirmed by a significant shift in molecular weight from the triblock copolymer (33 600 g mol^{-1}) to the cross-linked micelles (595 300 g mol^{-1}) (Figure 3). Moreover, the SEC curve in Figure 3 revealed the absence of free unimers (uncross-linker triblock copolymer) in the cross-linked sample.

To investigate their pH-sensitive characteristics, the micelles cross-linked with the acid degradable cross-linker were treated with acid (pH 5.5) for 72 h. The acid treatment of cross-linked copolymers led to the formation of free unimers in DMAc as shown in Figure 3. Although a bimodal distribution was observed for the cleaved copolymers, the SEC traces revealed a significant shift from high molecular weight (595 300 g mol^{-1}) of cross-linked polymer to low molecular weight (33 100 g mol^{-1}) polymer.

The hydrodynamic size of the micelles (both uncross-linked and cross-linked) in aqueous solution or in DMAc was determined using DLS analysis (Figure 5). In aqueous solution,

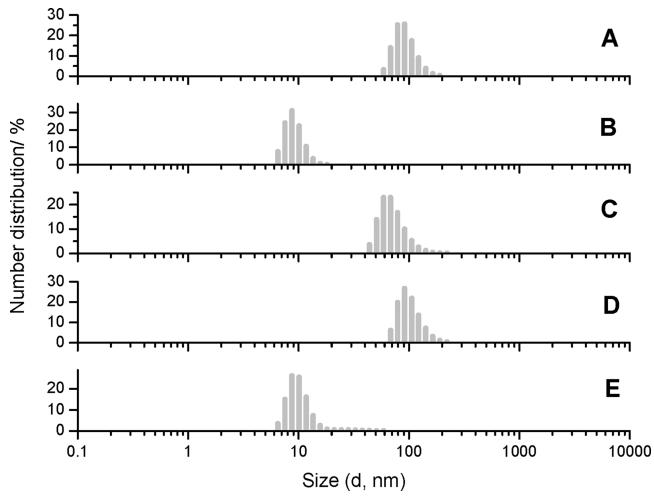

Figure 5. Hydrodynamic diameters measured by DLS of uncross-linked and cross-linked micelles before and after treatment with acid: (A) uncross-linked micelles in water; (B) uncross-linked micelles in DMAc; (C) cross-linked micelles in water; (D) cross-linked micelles in DMAc; and (E) cross-linked micelles after treatment with acid for 72 h in DMAc. All samples were measured at the concentration of 1 mg mL^{-1}.

the hydrodynamic diameter of both uncross-linked and cross-linked micelles was measured to be around 90 and 70 nm, respectively. The smaller size observed for the cross-linked micelles was attributed to the contraction of the core during the cross-linking reaction.[39] While the cross-linked micelles retained their size in DMAc, the uncross-linked micelles dissociated to the underlying triblock copolymers with a size of around 8 nm. After treatment with acid (pH 5.5) for 72 h, the sizes of cross-linked micelles in water were similar to uncross-linked micelles since the self-assembled structure was maintained because of the amphiphilic nature of the block

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copolymer. However, the formation free block copolymer unimers was clearly observed in DMAc with a hydrodynamic diameter of 9 nm indicating the destruction of the core−shell nanoparticle (Figure 5).

Cross-linking with the cross-linker 2-[2-(2-aminoethoxy)-ethoxy]ethan-1-amine led to similar results, but now the treatment with acid does not lead to disassociation in a good solvent such as DMAc; thus, a stable core−shell nanoparticle was created.

Pt Release from Platinum Polymeric Micelles. The aim of this work was to investigate the effect of the type of cross-linker on drug release and the effect on cell viability. Three types of micelles were, therefore, investigated utilizing the same triblock copolymer (Table 2). While no drug release occurs in phosphate buffer solution, the release of cisplatin from the polymer can be initiated by the addition of NaCl.[52] Around 30% and 20% of platinum drugs were released from uncross-linked micelles and cross-linked micelles after 24 h, respectively, using physiological concentrations of chloride at pH 7.4 (Figure 6). The release of platinum drugs from platinum polymer conjugates is triggered by the presence of chloride ions, which will lead to ligand exchange of the platinum complex from carboxylato ligand to chloride ligand. The drug is then cleaved from the polymer as *cis*-diaminedichloro platinum(II) (CDDP), which is the commercially available and FDA approved anticancer drug cisplatin. CDDP is then quickly converted to the active species, the *cis*-diaminediaquo platinum(II) complex.[1] The differences in drug release between both cross-linked micelles at pH = 7.4 after 24 h is negligible although there is a pronounced delay in release after 72 h (Figure 6). Reduced mobility of the polymer could potentially restrict the diffusion of the drug resulting in a slower release rate.[30]

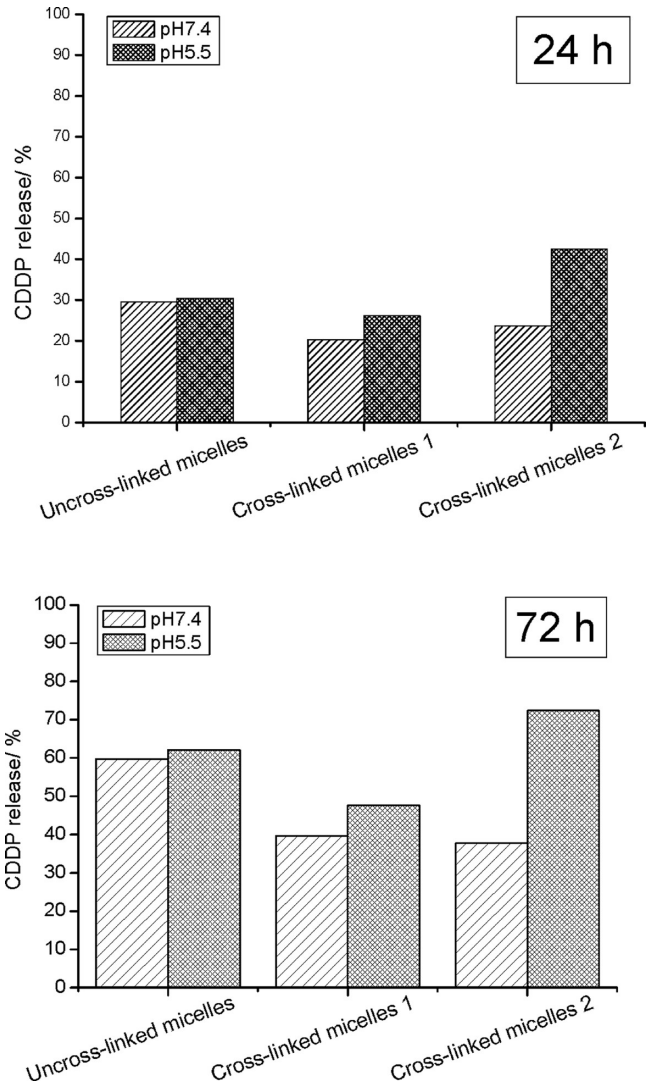

Figure 6. Release of CDDP from platinum polymeric conjugates/micelles in different pH buffer saline solutions (0.9% NaCl). The Pt amount was determined by ICP-MS.

The primary objective of this study was a triggered platinum drug release in an endosomal/lysosomal acidic environment (pH 5−5.5). Therefore, the release of the platinum drugs from the uncross-linked polymeric micelles and cross-linked polymeric micelles containing either permanent or acid degradable cross-linkers (Table 2) was investigated using equilibrium dialysis against two different pH buffer saline solutions at 37 °C. The rate of release of the Pt species at pH 5.5 for the acid-cleavable micelles was twice as much as that at pH 7.4, whereas the pH value did not have an effect when employing noncleavable micelles. It should be noted here that the release in acidic conditions is in general slightly higher since lower pH values lead to the protonation of the carboxylic acid after drug release, which lowers the density of negative charges along the polymer chain, thus promoting release. It can be concluded from these results that cross-linking using an acid degradable cross-linkers has two advantages: Cross-linking delays the release at pH 7.4, which enables better protection of the drug during systemic circulation or in the extracellular fluid of normal tissues. Furthermore, the release is accelerated once the drug carrier is in an acidic environment such as malignant tissues where fluid pH is often lower.[53] Moreover, numerous studies have shown that micelles are mainly internalized into cells via endocytosis, with the result that these micelles are located within the acidic endosomes and lysosomes before being able to enter the cytoplasm.[54]

Cytotoxicity of Polymers. The cytotoxicity of uncross-linked micelles and cross-linked micelles was examined before platinum drug conjugation against human ovarian cancer cells (OVCAR-3), which was later used to test the activity of platinum−polymer conjugates. The samples were sterilized under UV for 30 min prior to incubation with the cell lines. The sulforhodamine B (SRB) assay was employed to determine the cytotoxicity of the polymers for a period of 72 h. Different concentrations of the polymers ranging from 0 to 600 mg mL^{-1} were exposed to OVCAR-3. The uncross-linked micelles appeared to be nontoxic against OVCAR-3 (cell viability above 90%) in the concentration range of 0−600 μg mL^{-1} while the cross-linked micelles led to slightly higher toxicities, but only at high concentrations (Figure 7). The toxicity was almost negligible for either uncross-linked or cross-linked micelles as long as the polymer concentrations remained below 300 μg mL^{-1}, which is well above the concentrations typically employed in drug delivery systems. Again, it seems that the RAFT end group does not introduce any significant toxicity.[55]

In Vitro Activity of Polymeric Micelles. To evaluate the cytotoxicity of uncross-linked platinum loaded polymeric micelles and cross-linked platinum loaded polymeric micelles, human ovarian OVCAR-3 cells were exposed to various doses of the drug loaded carrier (equivalent to 0−110 μM Pt) for 24 and 72 h. The cytotoxicity was examined using the sulforhodamine B (SRB) microculture colorimetric assay, and the results

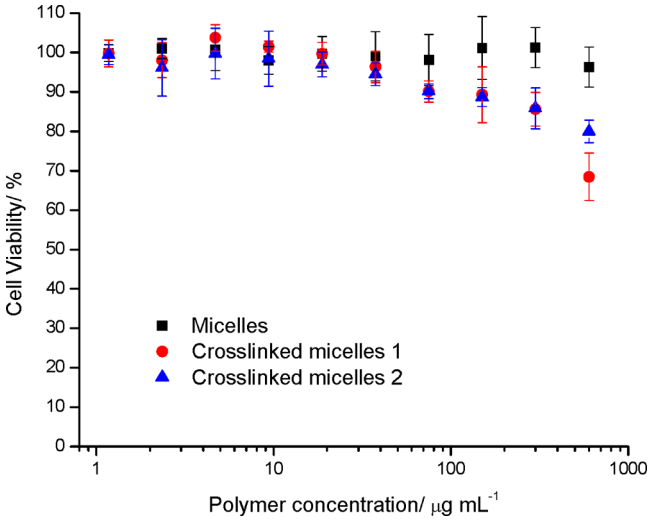

Figure 7. Percentage of living OVCAR-3 cell after being exposed to block copolymer micelle solutions. The micelles used in these experiments were self-assembled from amphiphilic protected triblock copolymers.

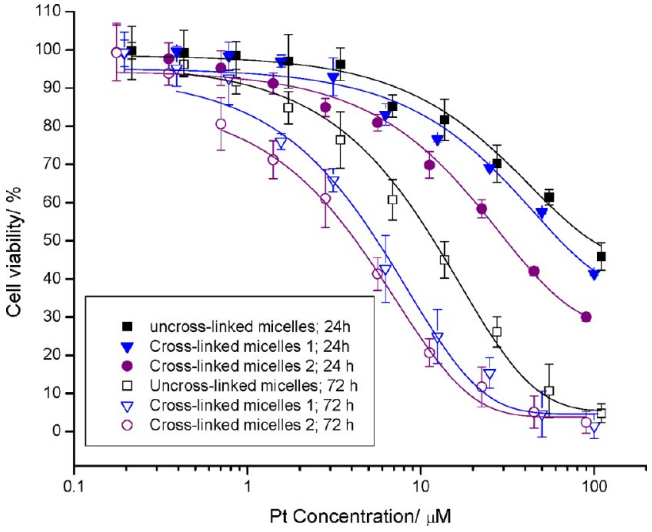

Figure 8. Cell viability (%) after exposure to uncross-linked platinum polymeric micelles and cross-linked platinum polymeric micelles against OVCAR-3 cells.

are presented in Figure 8 and Table 3. The concentration of block copolymer was equivalent to the amount of polymer present in the platinum drug loaded micelles as shown in Table S1, Supporting Information.

All three drug carriers prior to loading, uncross-linked polymeric micelles and cross-linked polymeric micelles, did not show any cytotoxicity at concentrations up to 300 μg mL^{-1} (Figure 7). The polymer concentrations used for drug loaded micelles is well below this threshold, and any cell death observed should be the result of the action of the platinum drug.

All platinum loaded polymeric micelles showed significant cytotoxicity (Figure 8). As expected, cell death increases with increasing incubation time from 24 to 72 h. For comparison, cell death of the FDA approved drug carboplatin, which

Table 3. IC_{50} Values of Uncross-Linked Platinum Polymeric Micelles and Cross-Linked Platinum Polymeric Micelles against OVCAR-3 Cells

compounds	IC_{50}, μM	
	24 h	72 h
uncross-linked platinum polymeric micelles	90	12
cross-linked platinum polymeric micelles 1	80	6.0
cross-linked platinum polymeric micelles 2	35	5.0
carboplatin		16.7

resembles the structure of the platinum complex on the polymer, has been additionally investigated. An IC_{50} of 16.7 μM for this drug was recorded (Table 3).

The cross-linked platinum loaded polymeric micelles exhibited a higher efficiency than the uncross-linked platinum loaded polymeric micelles against OVCAR-3 cells (Figure 8). The difference was even more significant after 24 h. For example, the IC_{50} value of the cross-linked platinum polymeric micelles (35 μM) was three times lower than the one of the uncross-linked platinum polymeric micelles (90 μM) after 24 h while the IC_{50} value after 72 h was still half the value (Table 3). The superior cytotoxicity of cross-linked platinum polymeric micelles may be attributed to higher cellular uptake (Table 4). An earlier study revealed that, at low micelle concentration, the micelles cannot maintain their integrity, especially when in contact with cell growth media. The cellular uptake below the CMC then drops significantly, preventing the uptake of the drug carrier.[30]

Another interesting result was the difference in cytotoxicity of the cross-linked platinum polymeric micelles using various cross-linkers (i.e., permanent vs acid cleavable). Although similar activity of both systems was observed after 72 h incubation with OVCAR-3 cells, the micelles with acid degradable cross-linker were found to be twice as active compared to the permanent cross-linked platinum polymeric micelles (Table 3). Obviously, the advantage in acid degradable micelles lies in an earlier onset of activity, which could be assigned to a faster drug release in an acidic environment.

It should be noticed that the original idea to design the platinum drug delivery carrier based on polymeric micelles is to enhance plasma retention, to prolong circulation times, and to have a better cellular uptake into solid tumors, in vivo,[7,19,21,23,56−58] compared to carboplatin or cisplatin. The enhanced in vitro cytotoxicity was, hence, unexpected and suggests that polymeric micelle delivery of platinum drugs may significantly improve their efficiency, possibly through enhanced cellular uptake. This is discussed in detail in the next section.

Cellular Uptake. The cellular uptake of uncross-linked platinum polymeric micelles and cross-linked platinum polymeric micelles was carried out using OVCAR-3 as tumor cells. To label the polymer, fluorescence-*o*-methacrylate was first introduced into the hydrophilic block of block copolymers by copolymerizing with OEGMEMA. The assumption was made that a small amount of fluorescence in the block copolymer would not change their polymer properties in terms of behavior in solution as well as loading efficiency. ICP-MS was utilized to determine the amount of platinum drugs taken up by cells while the amount of polymer was quantified by fluorescence spectroscopy.

The cellular uptake was carried out following the procedure described in our previous study.[11] Briefly, OVCAR-3 was

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Table 4. Cellular Uptake with OVCAR-3 Human Ovarian Cancer Cells for 24 h

	Pt content[a] (ng Pt/10^6 cells)		$[polymer]_{theo}$[b] (ng polymer/10^6 cells)		[polymer][c] (ng polymer/10^6 cells)	
polymer −drug conjugates	5 $\mu g\ mL^{-1}$	60 $\mu g\ mL^{-1}$	5 $\mu g\ mL^{-1}$	60 $\mu g\ mL^{-1}$	5 $\mu g\ mL^{-1}$	60 $\mu g\ mL^{-1}$
micelles	4.5 ± 2.5	60.1 ± 4.5	60.5	807.9	50.3 ± 7.1	810.7 ± 7.4
cross-linked 1	21.2 ± 1.6	121.4 ± 3.2	285.0	1632	290.3 ± 8.5	1710.5 ± 5.1
cross-linked 2	28.6 ± 4.5	145.9 ± 4.0	384.7	1961	340.2 ± 4.7	1874.8 ± 9.2

[a]Pt content determined by extracting 10^6 cells, followed by Pt content analysis via ICP-MS. [b]Theoretical polymer concentrations were calculated based on the Pt content determined by ICP-MS and the loading efficiency (70% as mentioned in the platinum loading section). [c]Actual polymer concentrations were measured by fluorescence spectrometry of the labeled polymer.

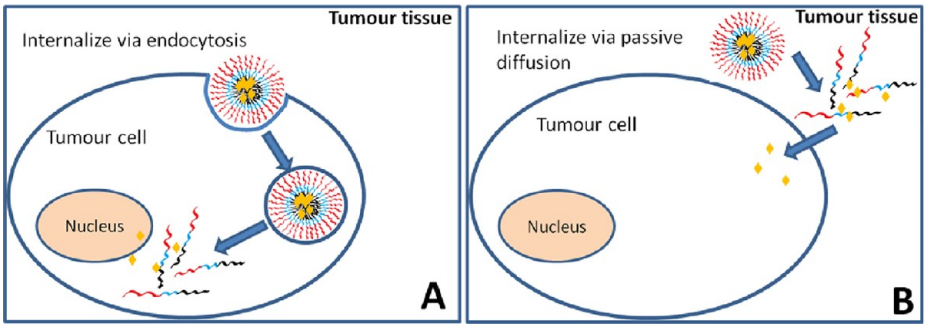

Figure 9. Scheme of the hypothesized mechanisms for platinum polymeric micellar uptake: (A) via endocytosis and (B) via passive diffusion.

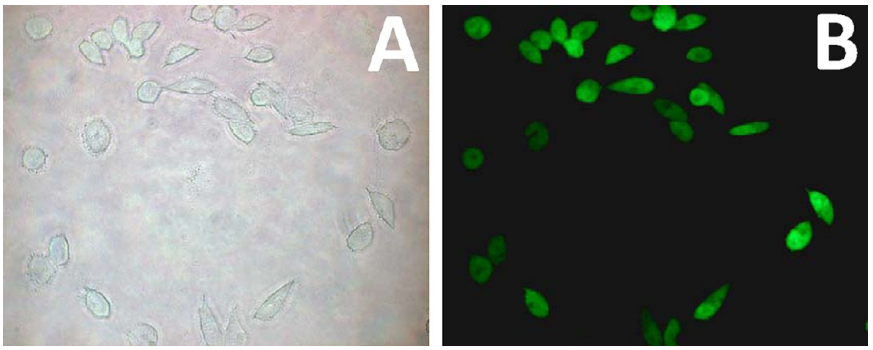

Figure 10. Fluorescence microscopy images of cross-linked platinum polymeric micelles incubated with OVCAR-3 for 2 h at 37 °C. (A) image of OVCAR-3 taken under halogen light and (B) image of OVCAR-3 taken under fluorescence light.

seeded overnight prior to incubation with the fluorescent polymers for 24 h. Subsequent intensive washing with PBS allowed the complete removal of residual micelles. Thus, the intensity of fluorescence or the amount of platinum drugs observed was equivalent to the amount of micelles or platinum drug taken up by the cells.

Two possible scenarios can occur when platinum drug loaded polymeric micelles are incubated with tumor cells (Figure 9): The drug may have been released from polymeric micelles as cisplatin prior to the uptake of the drug carrier leading to passive diffusion into the tumor cell, like any other small molecule drug (Figure 9B). Alternatively, the platinum polymeric micelles could be endocytosed before release occurs, which is the preferred pathway for the acidic endosomes and lyzosomes to take effect on the acid degradable linker (Figure 9A). In order to prove which mechanism is dominant in the current system, both platinum content and polymer concentration are determined inside the cells.

As seen by the green fluorescence of the polymer inside the cells (Figure 10), the carrier has been internalized into the OVCAR-3 cells. The cells were extracted and subject to ICP-MS analysis to determine the amount of platinum that had been taken up. Meanwhile, the fluorescence intensity in the cell growth media was monitored to quantify the amount of polymer that had been taken up by the cell. Table 4 summarizes the amount of polymer and platinum that was found inside the cell. Since the amount of platinum per amount of polymer is known, the measured platinum content could be correlated to the theoretical amount of polymer inside the tumor cell. In fact, this theoretical amount was found to be in very good agreement with the measured amount obtained from the fluorescence intensity. It is remarkable that the observation that the cross-linked platinum polymeric micelles were taken up twice as efficiently as the uncross-linked platinum polymeric micelles, and the deviation was even more significant with the micelle concentration of 5 mg mL^{-1}, which is below CMC values (Table 2). As highlighted above, the stability of the micelle was found to have a noticeable effect on cellular uptake, which furthermore led to an increased toxicity in an earlier study.[30] On the other hand, no significant difference was observed in cellular uptake of cross-linked micelles using permanent and acid degradable cross-linkers.

Colony Formation. Tumors in vivo do not grow as monolayers but as colonies.[59] It is, therefore, necessary to

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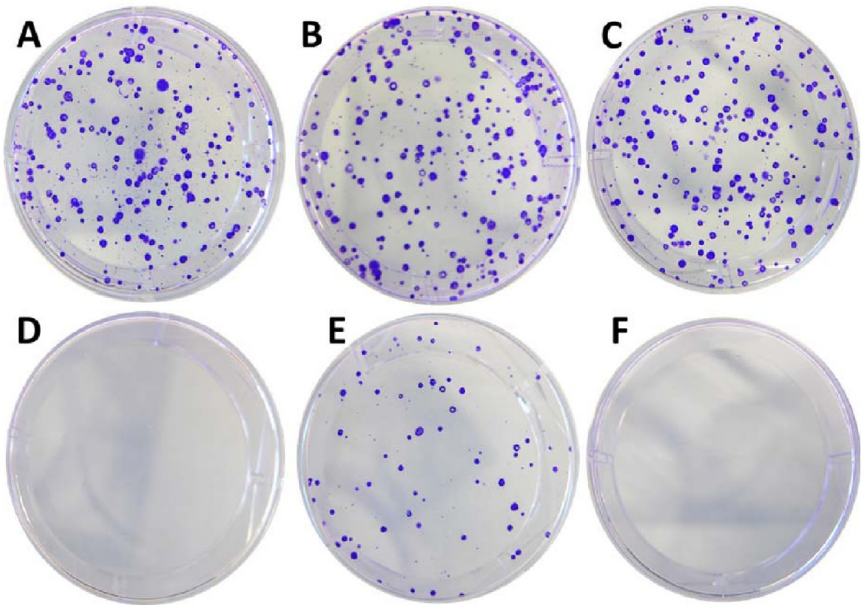

Figure 11. Colony formation of OVCAR-3 cells (A) without treatment (control), (B) empty triblock copolymers micelles at 100 μg mL^{-1}, (C) Pt-loaded uncross-linked micelles below CMC values, (D) Pt-loaded uncross-linked micelles above CMC values, (E) Pt-loaded cross-linked micelles below CMC values, and (F) Pt-loaded cross-linked micelles above CMC values.

examine whether the platinum loaded drug carriers can inhibit colony formation in human ovarian cancer OVCAR-3 cells in a long-term assay. For this purpose, a clonogenic assay was used to evaluate the effects of cell regrowth after exposing them to platinum polymer conjugates for 72 h. Contrary to the SRB assay, that only estimates cell growth inhibition relative to a control, this assay is perhaps more representative of a clinical situation, where regrowth of cancer tumors is common after treatment with chemotherapy.[11] This in vitro cell survival assay was employed following the procedure described by Franken et al.[60] Similar to the SRB assay, OVAR-3 cells were incubated for 3 days with the uncross-linked platinum polymeric micelles and the cross-linked platinum polymeric micelles with acid degradable cross-linkers at two different platinum concentrations (<CMC and >CMC values). These two concentrations of polymers were chosen because they led to significant differences in cellular uptake and cytotoxicity and hence could affect colony formation. After 72 h of drug treatment, a set of single alive cells were isolated, and they were regrown in PRMI 1640 medium containing 10% FBS for 10 days with fresh medium change every 3 days. The colonies were fixed using methanol for 30 min before being stained with crystal violet solution (0.1% in DI water) for 5 min. The colony formation assay was assessed via the surviving fraction, which is the ratio of colonies formed after treatment and the number of cells seeded that can become colonies. The polymers without platinum drugs appeared not to inhibit colony formation of OVCAR-3 cells at the concentration of 100 μg mL^{-1} while both uncross-linked micelles and cross-linked micelles showed restriction of the number of colonies formed (Figure 11). Table 5 revealed that both uncross-linked and cross-linked platinum polymeric micelles inhibit the colony formation fully at higher CMC values. The drug loaded micelles are taken up by the cells and remain lodged for an extended period of time, releasing the drug slowly over a period of several days. Interestingly, there was a significant difference in the surviving fraction of uncross-linked platinum polymeric micelles and cross-linked platinum polymeric micelles. Only less than 20% inhibition can be seen with as little as 2.1 μM of platinum concentration from uncross-linked platinum polymeric micelles compared to 70% in the case of cross-linked platinum polymeric micelles. These results, thus, suggest that cross-linked platinum polymeric micelle treatment is highly effective in suppressing the colony-forming ability of human ovarian cancer OVCAR-3 cells, probably due to a higher cellular uptake.

Table 5. Plating Efficiency and Surviving Fraction of OVCAR-3 after 3 Days of Exposure to Polymer and Polymer−Pt Conjugates Followed by 10 Days of Incubation in PRMI 1640 Medium[a]

samples	Pt concentration, μM	Pt−polymer conjugate, μg mL^{-1}	surviving fraction (SF)
uncross-linked micelles	2.1	5.5	0.82
	34.8	91.2	0
cross-linked micelles 2	2.3	6.0	0.3
	32.5	85.2	0
blank micelles	0	0	1.05
control	0	0	1
carboplatin	8.5		0.23
	241		0.11

[a]Plating efficiency (PE) was 78.4%.

CONCLUSIONS

Cross-linked micelles bearing platinum drugs were successfully synthesized via RAFT polymerization and cross-linking at the nexus between the core and the shell using permanent and acid degradable cross-linkers. This work shows that the stability of the micelles increased considerably after cross-linking, leading to an increased cellular uptake at very low concentrations. As a result, the anticancer activity of cross-linked platinum polymeric micelles is significantly enhanced compared to the uncross-linked ones. Moreover, SEC and DLS experiments proved that

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cross-linked platinum polymeric micelles were able to degrade at lysosomal pH (pH = 5.5), which triggers an accelerated drug release within the tumor cells. This effect is particularly pronounced after 24 h while the behavior of micelles cross-linked with degradable and nondegradable linkers is similar after 72 h incubation time. In vitro experiments also revealed that cross-linked platinum polymeric micelles effectively suppressed colony formation even at very low concentrations while the uncross-linked micelle below the CMC disassembles, which leads to lower uptake.

■ ASSOCIATED CONTENT

Supporting Information

Further details are given in Table S1. 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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