Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/materialsB

Journal Name

ARTICLE

A polymer–(multifunctional single-drug) conjugate for combination therapy

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Combination therapy based on polymer–drug conjugate is one of the exciting tides in polymeric drug delivery systems. A single polymer carrier attaching two or more drugs has advantage because it provides an platform for synergistic agent action. To expand the concept of combination therapy using a single polymer–drug conjugate, we report a polymer–(multifunctional single-drug) conjugate strategy, in which three different drugs (platinum, azidyl radical and DMC) and two different type of therapies are rationally integrated and then conjugated to an amphiphilic block copolymer. When this polymer–(multifunctional single-drug) conjugate is internalized by cancer cell via endocytosis, the three integrated drugs are expected to be activated and execute therapeutic functions in a sequential fashion under extracellular (UVA irradiation) and intracellular (endosomes/lysosomes) environments to amplify the signals of cancer treatment, especially in cisplatin-resistant cancer cells.

Introduction

Polymer therapeutics have been regarded as successful anticancer nano-medicines.[1] Maeda's discovery of the enhanced permeability and retention effect and the modular model for the polymer–drug conjugate first recognized by Ringsdorf, have been the important basis needed for the early steps of designing polymer therapeutics for anticancer purpose.[2] Numerous studies are presently available on polymer–drug conjugate on carrying a single drug and, specifically a result from clinical studies.[3] However, only very recently have these applications been extended to combination therapy. Owing to the complex molecular basis of cancer, any single-drug could not have the incomparable mechanisms for an effective and sustained therapy. Combination therapy modulates different signaling pathways in diseased cells, thereby improving the therapeutic profiles and, decreasing the likelihood of cancer cells' drug resistance.[4] Combination therapy based on polymer–drug conjugate may play an important future role in polymeric drug delivery systems as cancer treatment and diagnosis.

As of now, polymer–drug conjugate for combination therapy has encompassed at least three type of systems (Fig. 1): polymer–drug conjugate A plus free drug B (Type 1),[5] polymer–drug A conjugate plus polymer–drug B conjugate (Type 2)[6] and single polymeric carrier conjugating two drugs individually (Type 3).[7] Recent studies have shown that a single polymer carrier attaching two drugs has advantage because it provides a platform for simultaneous drug delivery, allowing synergistic agent action.[8] To expand combination therapy strategy with a single polymer–drug conjugate, we have recently reported a polymer–(tandem drug) conjugate for combination chemotherapy, in which two therapeutic agents were firstly integrated into a tandem single-drug and then conjugated to a polymer carrier (Type 4).[9] However, the challenges, such as combination of different type of therapies, delivery of multiple synergistic drugs, controllable drug release kinetics, precise drugs' ratio and simple preparation procedure for batch production still exist for clinical application of combination therapy based on polymer–drug conjugate.[10]

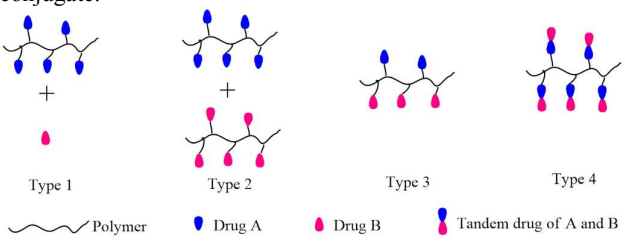

Fig. 1 Types of combination therapy based on polymer–drug conjugate.

Inspired by the above tandem drug and several other compounds such as mitaplatin, that kill cancer cell through two disparate signal pathways,[11] we hypothesized that more than two drugs or different type of therapies can be rationally integrated into a multifunctional single-drug for combination

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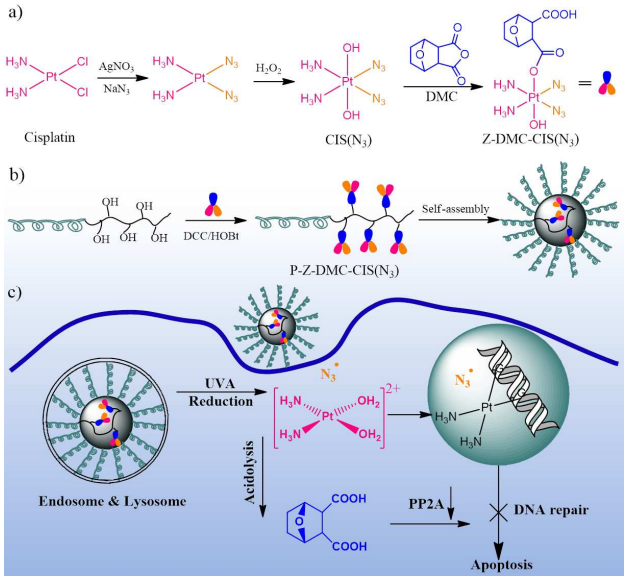
Electronic Supplementary Information (ESI) available: Additional figures as described in the text. See DOI: 10.1039/x0xx00000x

therapy. Pt(IV)-azide complexes are a class of radiation-activated pro-drugs which can kill cancer cell by dual mechanisms, biologically active Pt(II) and azidyl radicals under mild UVA irradiation.[12] DMC is an inhibitor of PP2A which is a ubiquitously expressed protein essential for nucleotide excision repair and DNA damage-induced defense mechanism.[13] Herein, we bind a Pt(IV)-azide complex ($CIS(N_3)$) and demethylcantharidin (DMC) to form a multifunctional single-drug Z-DMC-$CIS(N_3)$, in which three different drugs (platinum, azidyl radical and DMC) and two different type of therapies (chemotherapy and radiation therapy) are rationally integrated. To overcome the common drawbacks of small molecular drugs such as poor bioactivity and short blood circulation time, the multifunctional single-drug with a carboxyl group was further conjugated to a biodegradable amphiphilic block copolymer bearing pendent hydroxy groups to prepare a polymer–(multifunctional single-drug) conjugate (P-Z-DMC-$CIS(N_3)$) and micelle. It is well known that nano-micelle can be effectively internalized by cancer cell via endocytosis. When this polymer–(multifunctional single-drug) conjugate micelle is internalized by cancer cell via endocytosis, the three integrated drugs are expected to be activated and execute therapeutic functions in a sequential fashion under extracellular (UVA irradiation) and intracellular (endosomes/lysosomes) environments to amplify the signals of cancer treatment, especially in cisplatin-resistant cancer cells (Scheme 1).



Scheme 1 a) Synthesis of multifunctional single-drug Z-DMC-$CIS(N_3)$, b) conjugation of the single-drug onto biodegradable amphiphilic block copolymer mPEG-*b*-P(LA-*co*-MPD) to prepare polymer–(multifunctional single-drug) conjugate micelle, c) possible pathways after the polymer–drug conjugate micelles enter the cisplatin-resistant cancer cells.

Experimental

Materials

Sodium azide (NaN_3), Hydrogen peroxide (H_2O_2), N,N′-Dicyclohexyl carbodiimide (DCC), N-Hydroxybenzotrizole (HOBt), 5, 5-dimethylpyrroline N-oxide (DMPO), L-Tryptophan (Trp) were purchased from Sigma-Aldrich. Cisplatin (purity 99%) was bought from Shandong Boyuan Chemical Company, China and demethylcantharidin (purity 99%) was bought from Nanjing Zelang Biomedical Company, China. All other chemicals were purchased from Sigma-Aldrich and used as received.

General measurements

^{1}H NMR spectra were measured by a Unity-400 MHz NMR spectrometer (Bruker) at room temperature. Fourier Transform Infrared (FT-IR) spectra were recorded on a Bruker Vertex 70 spectrometer. Mass Spectroscopy (ESI-MS) measurements were performed on a Quattro Premier XE system (Waters) equipped with an electrospray interface (ESI). Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 6300, Thermoscientific, USA) was used to determine the total platinum contents in the polymer-drug conjugate and samples obtained outside of the dialysis bags in drug release experiments. Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Xseries II, Thermoscientific, USA) was used for quantitative determination of trace levels of platinum. Particle size and size distribution of micelles were determined by DLS with a vertically polarized HeeNe laser (DAWN EOS, Wyatt Technology, USA). The morphology of the polymer-drug conjugate micelles was measured by TEM performed on a JEOL JEM-1011 electron microscope. UV-visible electronic absorption spectra were recorded on a Varian Cary 300 UV-visible spectrophotometer in 1 cm path-length cuvettes. UVA irradiations were carried out using a xenon lamp source (CEL-S500, AuLight, China) equipped with a UVA filter (340-375 nm) for a parallel light. The power outage was measured at 10 mW cm^{-2} using a power meter (FZ-A, AuLight, China).

Preparation and characterization of P-Z-DMC-$CIS(N_3)$ micelle

Synthesis of Z-DMC-$CIS(N_3)$. c,c-$[Pt(NH_3)_2(N_3)_2]$ and $CIS(N_3)$ were prepared as previously reported.[14] Z-DMC-$CIS(N_3)$ was synthesized as following procedures. Briefly, demethylcantharidin (100 mg, 0.6 mmol) was added to a solution of $CIS(N_3)$ (208 mg, 0.6 mmol) in DMSO (10 mL), and the reaction mixture was stirred in dark at room temperature for 12 h. The solution was lyophilized and acetone (10 mL) was added to form a precipitate of a pale yellow solid. It was washed for several times with acetone and diethyl ether, and dried to afford 263 mg Z-DMC-$CIS(N_3)$, yield = 85%. Z-DMC-$CIS(N_3)$ was characterized using FTIR (Fig. S1), ^{1}H NMR (Fig. S2), ESI-MS (Fig. S3), and elemental analysis (Table S1). *Caution!* Although no problems were encountered during this work, heavy metal azides are known to be heat and shock-sensitive detonators. Therefore, it is essential that any platinum azide compounds are handled with care.

Synthesis of P-Z-DMC-$CIS(N_3)$. mPEG-*b*-P(LA-*co*-MPD) were prepared as our previously reported.[15] Z-DMC-$CIS(N_3)$ was conjugated to mPEG-*b*-P(LA-*co*-MPD) using DCC/HOBt method. Briefly, Z-DMC-$CIS(N_3)$ (50 mg), DCC (50 mg) and HOBt (20 mg) were added into the DMF (5 mL). After stirring for 30 min, mPEG-

b-P(LA-co-MPD) (100 mg) in CH_2Cl_2 (20 mL) was added and the reaction mixture was kept stirring at room temperature for 24 h. The solution was filtered and the filtrate was precipitated in ether. The solid was redissolved and reprecipitated in ether twice, giving a light yellow solid P-Z-DMC-CIS(N_3).

Preparation of P-Z-DMC-CIS(N_3) micelle. P-Z-DMC-CIS(N_3) micelle was prepared by nano-precipitation method. In brief, P-Z-DMC-CIS(N_3) (50 mg) was dissolved in a solution of DMF (5 mL), and then water (25 mL) was added dropwisely into the flask under stirring to form a micellar solution. The solution was dialyzed against water to remove DMF and then freeze-dried. The nano-micelle size was obtained from DLS. The morphology and micelle size were further characterized using TEM. Platinum content of the nano-micelle was determined by ICP-OES.

Drugs release from P-Z-DMC-CIS(N_3) micelles

Radiation responsiveness of Z-DMC-CIS(N_3) and P-Z-DMC-CIS(N_3). Aqueous solutions of Z-DMC-CIS(N_3) and P-Z-DMC-CIS(N_3) were UVA irradiated (365 nm, 10 mW/cm^2) for the indicated periods of time (0 min to 60 min), and the UV–vis spectra of the aqueous solutions were taken. For stability in the dark, aqueous solutions of Z-DMC-CIS(N_3) and P-Z-DMC-CIS(N_3) in distilled water were kept in the dark and UV–vis spectra were taken at over days.

Radiation-reduction of Z-DMC-CIS(N_3). Aqueous solutions of Z-DMC-CIS(N_3) were UVA irradiated (365 nm, 10 mW/cm^2) for 0 min, 30 min and 1 h respectively. The ESI-MS (negative mode) of the aqueous solutions were taken.

UV-vis of reactions with L-Trp. Solutions of Z-DMC-CIS(N_3) and P-Z-DMC-CIS(N_3) (20 μM Pt) with various mol equivalents of Trp were prepared. The UV-vis spectra were recorded in the presence and absence of UVA irradiation (365 nm, 18 J/cm^2). Note: all solutions of L-Trp alone were photostable under the same conditions.

^{1}H NMR of reactions with L-Trp. D_2O solutions of 4 mM Z-DMC-CIS(N_3) or P-Z-DMC-CIS(N_3) (in Pt concentration) with 8 mM DMPO were prepared in the absence and presence of 1 mM L-Trp. The ^{1}H NMR spectra were recorded in the presence and absence of UVA irradiation (365 nm, 18 J/cm^2).

Platinum release profiles. Lyophilized P-Z-DMC-CIS(N_3) micelles (5 mg) were hydrated in PBS (2 mL, 0.1 M, pH 7.4 and pH 5.0), placed into a pre-swelled dialysis bag (M_WCO = 3500) and immersed into PBS (48 mL). The dialysis was conducted at 37 °C in a shaking culture incubator. Samples were kept in the dark or monitored upon intermittent UVA irradiation (365 nm, 5 J/cm^2 each time). 1 mL was withdrawn at specified time intervals from the dialysate and measured for Pt using ICP-OES. After sampling, fresh PBS (1 mL) was added to the dialysate. The platinum released from the micelles was expressed as a percentage of cumulative platinum outside the dialysis bag to the total platinum in the micelles as a function of release time.

***In vitro* studies**

Cell culture. Cisplatin sensitive lung A549 human carcinoma cells (A549S) and cisplatin resistance A549 human carcinoma cells (A549R), and fibroblasts L929 normal cells were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, and grown in DMEM (Life Technologies) supplemented with 0.03% L-glutamine and 1% penicillin/streptomycin in 5% CO_2 at 37 °C. A549R cells were maintained with 2 μg/mL cisplatin. For MTT assay, the resistant cells were cultured in cisplatin free medium for 10 days before conducting cytotoxicity assay.

PP2A activity assay. Cultured A549S cells were plated in flasks. When the cells were 80% confluent, the media was replaced with media containing different drugs (50 μM DMC, 50 μM CIS(N_3), 50 μM Z-DMC-CIS(N_3), 50 μM P-Z-DMC-CIS(N_3)) or an equivalent volume of vehicle. For control samples, incubation was performed in the dark for 6 h (a short duration, sufficient for drug uptake, but insufficient for post exposure modification or cell death). For the UVA irradiated samples, cells were incubated for 4 h in the dark before UVA irradiation (365 nm, 5 J/cm^2), and then incubated in the dark to entire time of 6 h. Then the cells were washed 3 times in a 0.9% normal saline solution. Tissue protein extraction reagent (T-PER) (CWBiotec) solution was added to the cells, and cells were prepared for protein extraction. Lysates from each treatment group were assayed using a Malachite Green Phosphatase assay specific for serine/threonine phosphatase activity (Ser/Thr phosphatase assay kit; Invitrogen). The relative activity of PP2A was calculated according to the following equation: PP2A activity = (mean experimental phosphate amount / mean control phosphate amount) ×100 (%).

Cell viability assay (MTT). Cytotoxicity was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. L929, A549S and A549R cells were seeded in 96-well plates and incubated in DMEM overnight. The medium was then replaced by cisplatin, DMC, Z-DMC-CIS(N_3) (with or without 500 μM Trp) and P-Z-DMC-CIS(N_3) (with or without 500 μM Trp) at a final Pt concentration (or DMC concentration) from 3.375 to 216 μM. MTT assay was performed in the absence and presence of UVA irradiation (365 nm) during 24 h, 48 h and 72 h incubation time. For control samples, incubation was performed in the dark. For the samples with one UVA irradiation, cells were incubated for 4 h in the dark before the UVA irradiation (5 J/cm^2), and then incubated in the dark for the rest of time. For the samples with intermittent UVA irradiation (twice for 48 h, thrice for 72 h, 5 J/cm^2 each time), cells were incubated for 4 h in the dark before UVA irradiation was performed once a day, and then incubated in the dark for the rest of time. MTT solution (20 μL) in PBS (5 mg/mL) was added and the plates were incubated for another 4 h at 37 °C, followed by removal of the culture medium containing MTT and addition DMSO (150 μL) to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 min, and the absorbance of formazan product was measured at 490 nm by a microplate reader. Treatment groups for each cell line were replicated three times.

Cell internalization. A549S cells and A549R cells were seeded in 6-well plates (1 × 10^5 cells per well) and allowed to adhere overnight. Cells were incubated with LysoTracker Red (1 μg/mL) in RPMI 1640 medium at 37 °C for 1 h, then grown with cisplatin, Z-DMC-CIS(N_3) and P-Z-DMC-CIS(N_3) for 4 h. After three washes with PBS, 4% paraformaldehyde was added for another 30 min at 37 °C. Finally nuclei were counterstained with the Hochest (1 μg/mL) for 15 min at room temperature. Cellular uptake was

observed with an Olympus FV1000 confocal laser scanning microscope (CLSM) imaging system (Japan).

Platinum uptake. A549S cells and A549R cells were inoculated in 6-well plates. The cells were then treated with cisplatin and P-Z-DMC-CIS(N_3) with the Pt concentration in the culture medium regulated to the same value of 50 μM, 100 μM or 200 μM. They were incubated at 37 °C for 4 h. To quantitatively determine Pt intake by cells, the cell samples were washed three times with ice-cold PBS to remove surface-bound drugs first, and then incubated with 1.5 mL of 0.15 M sodium chloride (pH 3.0 was adjusted by acetic acid) for 3 min at 4 °C, then rinsed with 2 mL of cold PBS, harvested by scraping in ice-cold PBS, and finally centrifuged. Thereafter, the cell pellet were lysed by adding 200 μL cell lysis buffer and then the cell lysis solution was freezed at -20 °C for 20 min and thawed at room temperature. 100 μL of the cell lysis solution for each sample was used directly to measure the Pt content by ICP-MS. The other 100 μL of the cell lysis solution was used to determine the protein content in each cell sample by using bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The platinum content was expressed as nano-grams of Pt per milligram of total proteins.

Results and discussion

Preparation and characterization of polymer–(multifunctional single-drug) conjugate micelle

Pro-drug Z-DMC-CIS(N_3) was obtained by ring-open reaction of DMC with a Pt(IV)-azide complex (CIS(N_3)) (Scheme 1a). FTIR, ESI-MS, ^{1}H NMR and elemental analysis confirmed the structure of Z-DMC-CIS(N_3) (Fig. S1-S3, Table S1). It is hypothesized that this pro-drug converts to cisplatin(II), azidyl radicals and DMC after UVA irradiation once it enters the cancer cell, in which azidyl radicals and cisplatin(II) cause protein and DNA damage, and DMC blocks damaged DNA from repairing. In this sense, Z-DMC-CIS(N_3) is a multifunctional single-drug which integrates three different drug functions with clear ratios (cisplatin(II): azidyl radicals: DMC = 1: 2: 1) and two type of therapies (chemotherapy and radiation therapy).

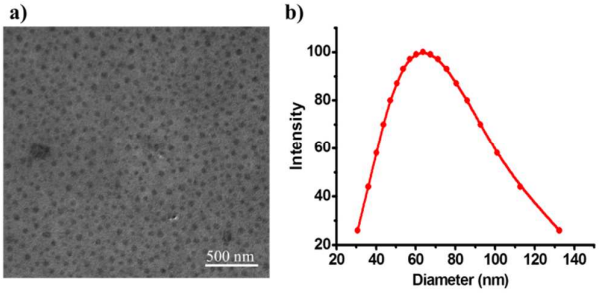


Fig. 2 a) TEM morphology and b) DLS characterization of the polymer–(multifunctional single-drug) conjugate micelle.

Biodegradable amphiphilic block copolymer mPEG-*b*-P(LA-*co*-MPD) (Fig. S4) with pendant hydroxyl groups was chosen as the carrier for the multifunctional single-drug. DCC/HOBt condensation method was used to conjugate Z-DMC-CIS(N_3) with carrier to form polymer–(multifunctional single-drug) conjugate (P-Z-DMC-CIS(N_3)) (Scheme 1b). Compared with the bare mPEG-*b*-P(LA-*co*-MPD), typical peaks at δ = 4.74, 3.30 and 1.48 ppm assigned to the protons in Z-DMC-CIS(N_3) were detected, confirming the successful synthesis of P-Z-DMC-CIS(N_3) (Fig. S5). The newly formed polymer–(multifunctional single-drug) conjugate can self-assemble into micelle because of its amphiphilic character (Fig. S6). Results showed that the micelle assumed a spherical shape, the mean diameters were about 50 nm determined by TEM or 65 nm determined by DLS, and no aggregation was observed (Fig. 2). Platinum content was determined by ICP-MS to be 12.1 wt%.

Sequential drugs release from polymer–(multifunctional single-drug) conjugate micelle

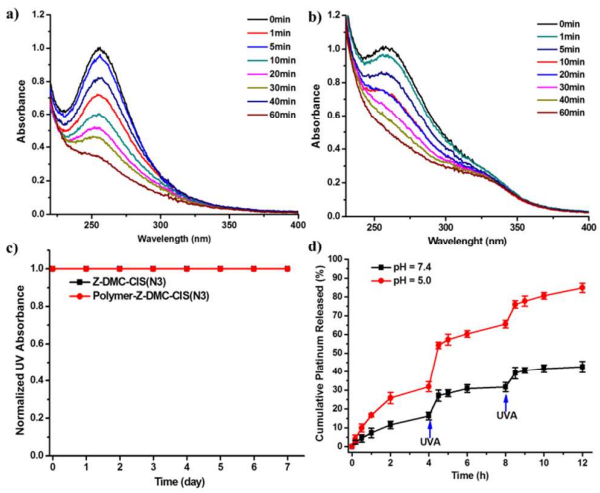


Fig. 3 UV-vis spectra of a) Z-DMC-CIS(N_3) and b) P-Z-DMC-CIS(N_3) upon UVA irradiation (365 nm, 10 mW/cm^2) for the indicated periods of time (0 min to 60 min), c) in-dark stability of Z-DMC-CIS(N_3) and P-Z-DMC-CIS(N_3) in distilled water over days, d) platinum release profiles of P-Z-DMC-CIS(N_3) in PBS (pH 7.4 and 5.0) in the presence of intermittent UVA irradiation (5 J/cm^2 each time).

Small multifunctional single-drug and polymer–(multifunctional single-drug) conjugate share the same intense N_3→Pt ligand-to-metal charge-transfer (LMCT) UV–vis absorbance centered at 258 nm (Fig. 3a,3b), with similar UV–vis spectra to the previously reported Pt(IV)–azide complexes.[16] Irradiation of P-Z-DMC-CIS(N_3) with UVA light (365 nm, 10 mW/cm^2) resulted in a rapid drop of the peak at 258 nm, indicating fast destruction of the N_3→Pt bond. In this condition, the Pt(IV) specie was reduced to the biologically active Pt(II) form and dissociated from the carrier.[17] To confirm the radiation responsiveness of the polymer–(multifunctional single-drug) conjugate, Pt release profiles were monitored upon intermittent UVA irradiation (365 nm, 5 J/cm^2 each time) at pH 7.4 and 5.0 (which mimics the pH values in endosomes and lysosomes) (Fig. 3d). As expected, Pt release was faster at pH 5.0 than at pH 7.4 from P-Z-DMC-CIS(N_3) due to the acidolysis of the Pt/DMC or DMC/polymer ester bonds. Regardless of pH, the release speed of Pt was much faster upon UVA irradiation than that in

the dark. For example, at pH 5.0, more than 25% of the total Pt could be released as swiftly as in half an hour upon each intermittent UVA irradiation. After that, less than 10% of Pt could be released in the dark during the next 4 h treatment. This phenomenon displayed an excellent "on-off" remote control over radiation responsiveness of the polymer–(multifunctional single-drug) conjugate. We found Pt release to be of our particular interest. It was much more susceptible to UVA irradiation than to pH change. The radiation-reduction to release active cisplatin(II) is supposed to be prioritized over acidolysis to release Pt(IV) from the conjugate when it was internalized within endosomes/lysosomes and exposed to UVA irradiation. It is also found that the UV–vis spectra of aqueous solution of P-Z-DMC-CIS(N_3) stored in the dark remained unchanged up to 7 days (Fig. 3c). These results proved that P-Z-DMC-CIS(N_3) was extremely stable in the dark and ideal for long-term storage while highly sensitive towards UVA irradiation for radiation therapy.

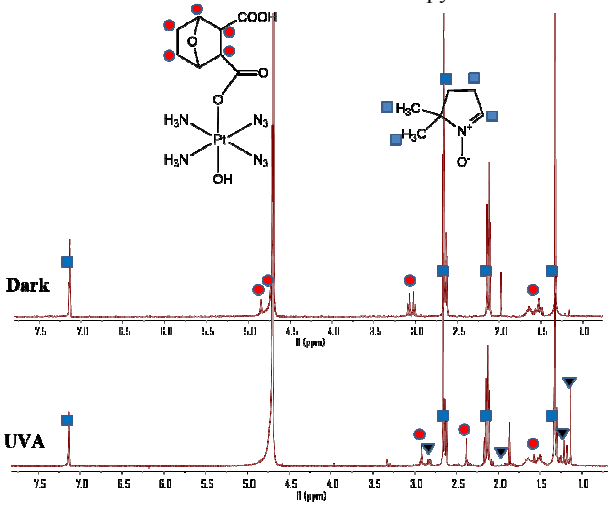


Fig. 4 ^{1}H NMR spectra of a D_2O solution of Z-DMC-CIS(N_3) (4 mM) and DMPO (8 mM) in the absence and presence of UVA irradiation (18 mJ/cm^2). Assignments: DMC peaks (●), DMPO peaks (■), DMPO photoproducts (▼).

Recent studies suggested that irradiation of Pt(IV)–azide complexes will release not only Pt(II) but also azidyl free radicals simultaneously which can cause damage to all biomolecules (protein, lipid and DNA).[18] In order to confirm the existence of azidyl radicals, we investigated the interaction of 5, 5-dimethylpyrroline N-oxide (DMPO) with Z-DMC-CIS(N_3) in the presence and absence of irradiation by NMR spectroscopy, since such radicals can be readily trapped by DMPO.[12] The intensity of ^{1}H NMR peaks for DMPO (10 mM) decreased in a D_2O solution containing Z-DMC-CIS(N_3) (5 mM) after irradiation with UVA (365 nm, 18 mJ/cm^2). Several new peaks corresponding to radiation-products from Z-DMC-CIS(N_3) and DMPO appeared, suggesting that azidyl radicals were released from Z-DMC-CIS(N_3) upon irradiation and trapped in the presence of DMPO (Fig. 4, Fig. S9). Similar DMPO radiation-products could also be found in P-Z-DMC-CIS(N_3) solution with DMPO after UVA irradiation, pointing to azidyl radicals release from the polymer–(multifunctional single-drug) conjugate (Fig. S10).

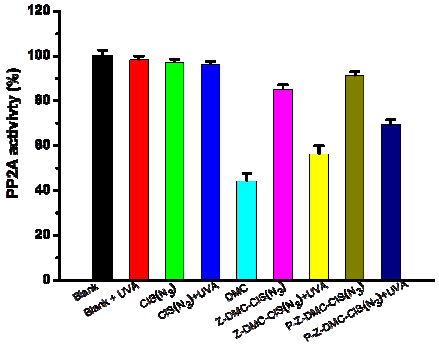


Fig. 5 PP2A activity of A549S cells exposed to Cis(N_3), DMC, Z-DMC-CIS(N_3) and P-Z-DMC-CIS(N_3) with or without UVA irradiation (5 J/cm^2).

The potential for DMC release to specifically inhibit PP2A was investigated with a PP2A activity assay.[19] As shown in Fig. 5, there were no observable effects on PP2A activity of A549S cells exposed to Cis(N_3) in the presence or absence of UVA irradiation. However, while cells were exposed to DMC, the value of PP2A activity decreased to 42% over its original level, indicating the specific PP2A inhibition of DMC. In the case of Z-DMC-CIS(N_3), the PP2A activity decreased to 83% over its original level in the dark, and further decreased to 54% upon UVA irradiation (365 nm, 5 J/cm^2). This result implied a faster DMC release from Z-DMC-CIS(N_3) upon irradiation reduction, corresponding to the ESI-MS results (Fig. S7). It is notable that A549 cells which were exposed to P-Z-DMC-CIS(N_3) decreased the PP2A activity to 92% of its original level in the dark, whereas they decreased to 68% upon UVA irradiation, illustrating that DMC was barely hydrolyzed in the dark but dissociated largely from the polymer chain in the presence of UVA irradiation. All these demonstrated that irradiation is the key stimulant towards DMC release from the polymer–(multifunctional single-drug) conjugate. The radiation reduction which preferentially occurred could subsequently improve the acidolysis of DMC. It is reasonable that after radiation-reduction release of cisplatin(II) and azidyl radicals, the DMC groups could be exposed directly to acidic environment within endosomes/lysosomes to accelerate their hydrolysis process.

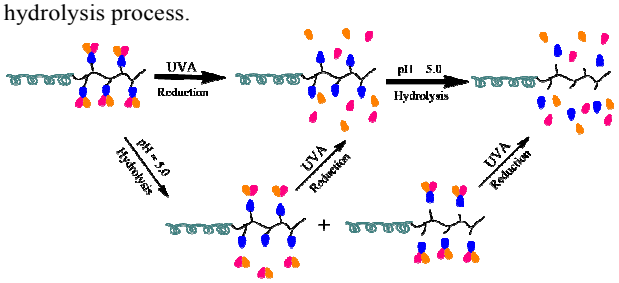


Fig. 6 Supposed drug sequence behaviors of the polymer–(multifunctional single-drug) conjugate.

Based on the above results and analyses, we recounted the drug behaviors of the polymer–(multifunctional single-drug)

conjugate in cancer cell in Fig. 6. The three active drugs could be released from the polymer–drug conjugate in a sequential fashion in the extracellular (UVA irradiation) and intracellular environments (endosomes/lysosomes). First upon UVA irradiation, released cisplatin(II) would attack nuclear DNA and lead to DNA damage by intra-strand cross-links.[20] Azidyl radicals generated simultaneously could also induce protein and DNA damage by oxidation. DMC was subsequently released by acidolysis within acidic endosomes/lysosomes to block the repair process for damaged DNA through PP2A inhibition. Therefore, we hypothesize that the polymer–(multifunctional drug) conjugate with sequential drug exposures and functions will enhance its efficacy on cancer treatment.

***In vitro* cell cytotoxicity evaluation on cisplatin resistance**

Studies have demonstrated that reduced drug uptake and increased DNA repair are two main mechanisms of cisplatin resistance.[21] The unique characteristics of the polymer–(multifunctional single-drug) conjugate provide us an excellent platform for a deeper investigation into anti-cancer efficacy especially in cisplatin-resistant cancer cells. For these reasons, the cytotoxicity of the polymer–(multifunctional single-drug) conjugate was evaluated *in vitro* using cisplatin-sensitive lung cancer cells A549S and cisplatin-resistant cells A549R.

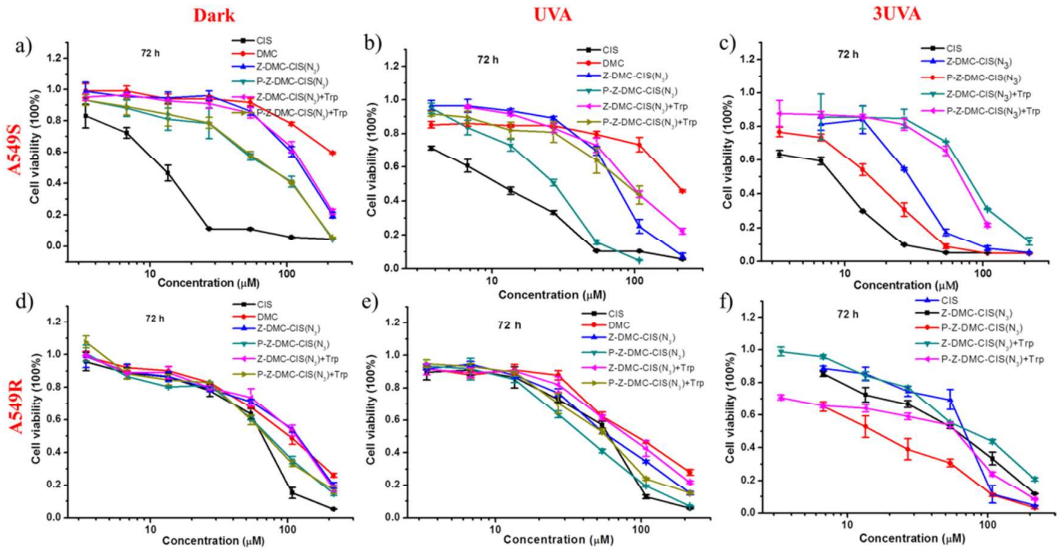


Fig. 7 *In vitro* cytotoxicity curves of cisplatin, DMC, Z-DMC-CIS(N_3) (with or without 500 μM Trp) and P-Z-DMC-CIS(N_3) (with or without 500 μM Trp) against A549S and A549R cells in the absence and presence of intermittent UVA irradiation (365 nm, once or thrice, 5 J/cm^2 each time) during 72 h incubation time.

Table 1 IC_{50} values of cisplatin, Z-DMC-CIS(N_3) (with or without 500 μM Trp) and P-Z-DMC-CIS(N_3) (with or without 500 μM Trp) against A549S and A549R cells in the absence and presence of intermittent UVA irradiation (365 nm, once or thrice, 5 J/cm^2 each time) during 72 h incubation time.

Samples	Cisplatin	Z-DMC-CIS(N_3)	P-Z-DMC-CIS(N_3)	Z-DMC-CIS(N_3) + Trp	P-Z-DMC-CIS(N_3) + Trp
Cells	A549S/A549R	A549S/A549R	A549S/A549R	A549S/A549R	A549S/A549R
IC_{50} (Dark)	12.1/65.8	128.8/118.2	70.8/73.0	135.7/134.8	74.6/74.0
IC_{50} (UVA)	11.4/61.0	73.1/60.5	27.2/40.3	93.5/81.3	86.5/57.2
IC_{50} (3UVA)	8.4/68.7	29.2/59.0	15.0/14.6	77.4/75.5	68.9/58.7

To exhibit the remote controllable radiation responsiveness of the conjugate, MTT assay was performed in the absence and presence of intermittent UVA irradiation (365 nm, once or thrice, 5 J/cm^2 each time to avoid radiation damage) during 72 h incubation time (Fig. 7, Fig.S12-14). Cell viability reached over 95% after UVA irradiation in the absence of any drug (Fig. S11). With or without UVA irradiation, A549R cells showed notably high resistance to cisplatin, and the resistant factor (RF) increased from *ca.* 5.4 in the dark to *ca.* 8.2 after irradiation for three times (Table 1). In all conditions, P-Z-DMC-CIS(N_3) was more cytotoxic than small single-drug Z-DMC-CIS(N_3). It was notable that after thrice intermittent UVA irradiation, the IC_{50} value of P-Z-DMC-CIS(N_3) in A549R cells (14.6 μM) is even comparable to cisplatin in A549S cells (8.4 μM) and the RF for the polymer–(multifunctional drug) conjugate was as low as 0.97. When the drug-treated cancer cells were co-incubated with excess tryptophan (Trp, 500 μM), an ubiquitous amino acid in many proteins,[22] the cell death in P-Z-DMC-CIS(N_3) group was dramatically reduced with significantly elevated IC_{50} values. Quenching of the azidyl radicals by Trp may greatly suppress the original activities of those free radicals on biomolecules (protein and DNA) in cancer cells (Fig. S8, S9). This observation clearly proved the generation of azidyl radicals

via UVA irradiation and also indicated the anti-cancer activity of azidyl radical on cancer cells. All these results revealed that the polymer–(multifunctional single-drug) conjugate has great potential for overcoming tumor acquired resistance to cisplatin.

Cellular uptake

Diffusion has been considered as a pathway for cisplatin to penetrate plasma membrane. Recently, studies have indicated that cisplatin entered cells by endocytosis and other mechanisms.[23] It is known that reduced uptake of cisplatin resulting from an endocytic defect is one of the main reasons for tumor cell's resistance to cisplatin.[24] To investigate how polymer–(multifunctional single-drug) conjugate increases toxicity in cisplatin-resistant cancer cells, late endosomes and lysosomes in A549R cells and A549S cells were labelled with red LysoTracker (Fig. 8, Fig. S15-16). We observed that there were less endosomes/lysosomes in A549R cells compared to A549S cells treated with cisplatin, confirming the endocytic defect of cisplatin uptake of A549R cells. Similar phenomenon was found in the single-drug Z-DMC-CIS(N_3) group. Interestingly, the red fluorescence intensity in A549R cells was comparable to A549S cells when they were treated with P-Z-DMC-CIS(N_3).

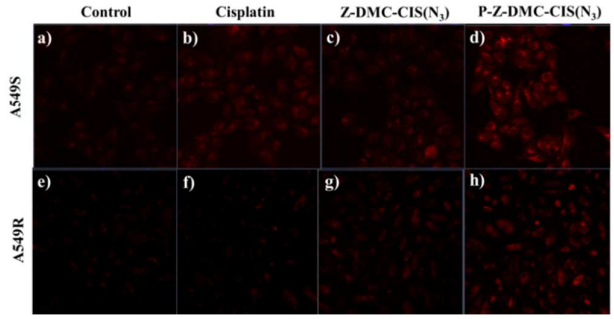


Fig. 8 CLSM images of late endosomes and lysosomes (labelled with red LysoTracker) in A549S cells (a, b, c, d) and A549R cells (e, f, g, h) treated with (a, c) control medium, (b, f) cisplatin, (c, g) Z-DMC-CIS(N_3) and (d, h) P-Z-DMC-CIS(N_3).

We believe that polymer–(multifunctional single-drug) conjugate reactivates endocytosis through its unique nanoscale properties.[25] This was also confirmed by the cellular platinum uptake assay. After 4 h cisplatin incubation, the Pt uptake by A549R cells was much less than that by A549S cells, implying the cisplatin resistant character of A549R cells. It is noteworthy that the Pt uptake in P-Z-DMC-CIS(N_3) treated A549S and A549R cells was comparable, and 10-fold compared to cisplatin in A549R cells (Fig. 9). All the results proved that polymer conjugation of small single-drug is very significant for cisplatin resistance reversal, corresponding to the above MTT results. The enhanced sensitivity of P-Z-DMC-CIS(N_3) in cancer cells after UVA irradiation could be easily understood upon the above discovery and discussion. Polymer conjugation would not only reactivate endocytosis of cisplatin-resistant cells to increase drug accumulation but also provide endosomes/lysosomes microenvironment subsequently to activate the three integrated drugs. Acidolysis of DMC from the polymer chain after radiation-reduction of cisplatin(II) and azidyl radicals within endosomes/lysosomes enhanced the inhibitory effect on repairing the damaged DNA and made cisplatin-resistant cancer cells more sensitive to the administrated drugs.

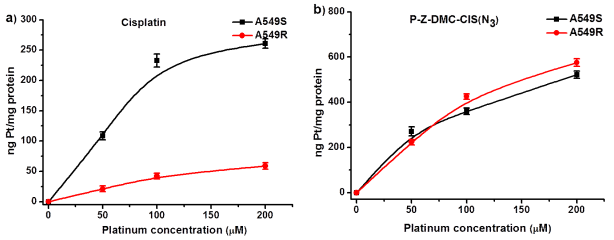


Fig. 9 Platinum uptake in A549S and A549R cells. Cells were incubated for 4 h with media containing different concentrations of a) cisplatin and b) P-Z-DMC-CIS(N_3).

Conclusions

We report a polymer–(multifunctional single-drug) conjugate strategy for combination therapy, in which three drug functions and two types of therapies were integrated into a multifunctional single-drug first and then attached to a polymeric carrier. When the polymer–(multifunctional single-drug) conjugate is internalized by cisplatin-resistant cell, all the drugs can be sequential triggered release from the polymer chain within reactivated endosomes/lysosomes under UVA irradiation to kill cancer cell and overcome cisplatin resistance. Comparing with other types of combination therapy based on polymer–drug conjugate, the polymer–(multifunctional single-drug) conjugate has several features: i) combination of types of therapies in a single polymer–drug conjugate, ii) delivery of multiple drugs to the targets of interest with definitive ratios and high drug loadings, iii) inter-synergisms of all drugs, iv) ability to control drug exposure spatially and temporally via different responsiveness, and v) simplified synthesis procedure by a single conjugation, and all these features are indispensable for batch production. This polymer–(multifunctional single-drug) conjugate approach bridges between the design of polymer therapeutics and the biomaterials, a promising strategy for clinical application in the near future.

Acknowledgements

The work is supported by the National Natural Science Foundation of China (No. 51403198, 51321062 and 21174143), Ministry of Science and Technology of China (863 Project, No. SS2012AA020603), "100 Talents Program" of Chinese Academy of Sciences (No. KGCX2-YW-802), and Jilin Provincial Science and Technology Department (No. 20150520019JH).

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