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Antitumor Activity of a Unique Polymer That Incorporates a Fluorescent Self-Assembled Metallacycle

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Abstract

Despite the well-known anticancer activity of mono- and multi-nuclear platinum complexes, studies of the antitumor performances of platinum-based supramolecular coordination complexes are rare. Herein, we report on the synthesis of a four-armed amphiphilic copolymer **Pt-PAZMB-*b*-POEGMA** containing a theranostic metallacycle **M**, in which the tetraphenylethene derivative acts as an aggregation-induced emissive fluorescent probe for live cell imaging and the 3,6-bis[trans-Pt(PEt₃)₂]phenanthrene (**PhenPt**) is an anticancer drug. This copolymer was further self-assembled into nanoparticles of different sizes and vesicles depending upon the experimental conditions. The impact of the morphology and size of the assemblies on their endocytic pathways, uptake rates, internalization amounts and cytotoxicities were fully investigated. The self-assemblies were further employed to encapsulate doxorubicin (DOX) to achieve a synergistic anticancer effect. Controlled drug release was also realized via amphiphilicity changes and was driven by a glutathione-induced cascade elimination reaction. The DOX-loaded nanoparticles of around 50 nm in size exhibited an excellent antitumor performance as well as a low systemic toxicity, due to an enhanced permeability and retention effect.

TOC Graphic

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ASSOCIATED CONTENT

Supporting Information

Experimental details, NMR spectra, and other materials. These materials are available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

INTRODUCTION

Over the past few decades, discrete supramolecular coordination complexes (SCCs) with well-defined size, shape and geometry have received increasing attention not only for their aesthetic attributes but also because of their wide applications in sensors, catalysis, amphiphilic self-assembly, host–guest chemistry, supramolecular polymers, and biomedicines.¹ Many sophisticated 2D metallacycles and 3D metallacages have been developed through the judicious choice of metals and ligands.² Due to the anticancer activity of organometallic ruthenium and platinum complexes, therapeutic SCCs, including these metals as the acceptors, have been prepared in recent years.³ Different from the clinical drugs, such as cisplatin, imaging capability can also be integrated into these SCC platforms by simply choosing fluorescent ligands as the coordination donors. This subsequently helps to trace the delivery and release of the organometallic drug *in vitro* and *in vivo*.⁴ Nevertheless, for biomedical applications the stability of the SCCs in a physiological environment needs to be improved, because the metals (*e.g.* Fe, Cu and Zn) and amino acids present in blood can potentially dissociate the SCCs and, thereby, causing side effects as well as erroneous imaging results. The limited solubility and often poor tumor accumulation of the SCCs are the other major obstacles. Physical encapsulation by copolymers and chemical modification of SCCs have emerged as the two effective ways to solve these problems. In particular, the post-modification of SCCs with amphiphilic copolymers is a good method to fabricate drug delivery systems (DDSs), because this can enhance the solubility and the stability of a given SCC in aqueous medium as well as allows to load other anticancer drug(s) into the polymeric scaffold, with a possibility of having a synergistic anticancer effect. Moreover, stimuli-responsiveness can be incorporated into these systems to amplify the therapeutic efficiency and to reduce the possible side effects.⁵

Herein, we develop a theranostic amphiphilic polymer **Pt-PAZMB-*b*-POEGMA**, containing glutathione (GSH)-responsive diblock copolymers as the arms and an aggregation-induced emissive metallacycle **M** as the core (Figure 1). This polymer further self-assembled into nanoparticles **NPs** of different sizes (**NP1** and **NP2**) and vesicles **VC** *via* a re-precipitation, dialysis and a double-emulsion technique, respectively. Due to their different morphology and size, these assemblies follow distinct endocytic pathways and have different internalization rates and cytotoxicities towards HeLa cells. Notably, the **NPs** and **VC** are capable of encapsulating neutral doxorubicin (DOX) and doxorubicin hydrochloride (DOX-HCl), respectively. The amphiphilicity of the polymer **Pt-PAZMB-*b*-POEGMA** was altered via a glutathione (GSH)-triggered cascade elimination, resulting in the disassembly of the nanostructures and the subsequent release of the loaded chemotherapeutic agent. The *in vitro* and *in vivo* studies demonstrated that the **NPs** with a diameter of 50 nm are the best candidates to co-deliver **PhenPt** and DOX in a synergistic manner that can effectively suppress tumor growth with a negligible systemic toxicity.

RESULTS AND DISCUSSION

Synthesis and Self-Assembly of **Pt-PAZMB-*b*-POEGMA**

As shown in Figure 1, **Pt-PAZMB-*b*-POEGMA** was synthesized via an amidation reaction of **NHS-PAZMB-*b*-POEGMA** with a metallacycle **M**, where the precursors were obtained via a reversible addition-fragmentation chain-transfer polymerization reaction (Scheme S1 and Figure S1–S4) and via a literature adopted procedure,^{6c} respectively. The material was fully characterized by multinuclear NMR (¹H and ³¹P) and gel permeation chromatography (GPC) analyses. The sharp singlet at 9.07 ppm with concomitant ¹⁹⁵Pt satellites in the ³¹P{¹H}NMR spectrum of **Pt-PAZMB-*b*-POEGMA** support the presence of **M** in this material. Likewise, the ¹H NMR resonances in the range of 6.50–9.00 ppm are diagnostic for the metallacyclic core of the polymer. Moreover, in the GPC curves the number-averaged molecular weight was increased to 89.7 kDa upon the formation of **Pt-PAZMB-*b*-POEGMA** (Figure 2a), which is about four fold higher than that of the precursor **NHS-PAZMB-*b*-POEGMA** (20.1 kDa) and thereby supporting the formation of a four-armed copolymer.

For both **M** and **Pt-PAZMB-*b*-POEGMA**, the emissions of the TPE-based dipyriddy donors are significantly enhanced (Figures 2b and S10) relative to that of the free ligand. This is because in these materials the Pt←Pyridyl coordination interactions somewhat restricted the intramolecular rotations of the TPE units and impart a better emission.⁶ The polymer also exhibits an aggregation induced emission property. As shown in Figure 2b, the emission intensity of an acetone solution of the polymer gradually increased upon the incremental addition of water. In contrast, the emission of an acetone solution containing only **M** decreased when the water fraction (f_w) increased from 0 to 40%, then increased with a further increase of f_w (Figures 2b and S9). The reason is that the solubility of **M** is poor in both pure acetone and water, while it can be better dissolved in their mixture. It should also be noted that **Pt-PAZMB-*b*-POEGMA** exhibited a much stronger AIE effect (Figure 2b) than free **M**, which is likely due to its polymeric structure.

By using fluorescence as a probe, the critical aggregation concentration (CAC) of the amphiphilic polymer **Pt-PAZMB-*b*-POEGMA** was determined to be 9.47 $\mu\text{g/mL}$ (Figure 2c), which is much lower than that of the precursor **NHS-PAZMB-*b*-POEGMA** (CAC = 33.6 $\mu\text{g/mL}$) (Figure S11). Nanoparticles (NPs) with diameters of around 50 (for **NP1**) and 500 nm (for **NP2**), were obtained from **Pt-PAZMB-*b*-POEGMA** by employing a reprecipitation and a dialysis method, respectively, and were characterized by transmission electron (TEM) (Figures 2d and 2e) and atomic force (AFM) microscopies (Figures 2j and 2k). Dynamic light scattering (DLS) studies revealed that the average diameter of **NP1** and **NP2** are 49.8 ± 3.2 and 429 ± 51.8 nm, respectively (Figures S14 and S15). In contrast, vesicles (**VC**) of 0.8–3.0 μm in diameter were obtained when **Pt-PAZMB-*b*-POEGMA** was subjected to a double emulsion technique. This material was also characterized by TEM (Figure 2f), AFM (Figure 2l) and DLS (Figure S16). The membrane thickness and height of **VC** are 7.2 ± 0.8 nm and 13 nm, respectively, as obtained from the TEM and AFM measurements. In the vesicles, the **POEGMA** segments construct the hydrophilic outer and inner layers, while the **PAZMB** portions forms the hydrophobic core layer. Due to the AIE effect of the TPE units, the solution of **NP1**, **NP2**, or **VC** are highly emissive (Figure S13) and were further characterized by confocal laser scanning microscopy (CLSM) (Figures 2g–h). The colloidal stability of these nanostructures was evidenced by the fact that neither morphology nor size change was observed over a period of two months.

Size-Dependent Endocytosis and Anticancer Effect of the Nanostructures

Nanostructures depending upon their morphology and size can enter into cells via several different endocytic pathways. This not only affects the uptake efficiency, but also influences their intracellular fate and subsequently results in different pharmacological activities of the loaded cargos (such as dyes, drugs, or genes).⁷ The internalization pathways of **NP1**, **NP2**, and **VC** into HeLa cells were investigated using a flow cytometry (FCM) technique, in the presence of various endocytosis inhibitors. The results suggest that the cellular uptake for all assemblies is greatly inhibited at 4°C (Figure 3a), indicating that these pathways are energy-dependent.^{7a} The uptakes of **NP1** and **NP2** into HeLa cells were also effectively suppressed in the presence of sucrose (Suc), while negligible effects were observed for genistein (Gen) and amiloride-HCl (Ami). These data collectively indicate that the **NPs** are mainly internalized *via* a clathrin-mediated endocytic pathway,^{7b} allowing the **NPs** to undergo an intracellular endo/lysosomal transportation. This was further supported by the CLSM images that exhibit a good overlap between the green fluorescence of the TPE units and the red fluorescence of the Lyso tracker Red dye (Figures 3c and d). In contrast, a 41.3% decrease in the cellular uptake was observed for **VC** in the presence of Ami, suggesting that a macropinocytosis mediated pathway drives the internalization of **VC**.

The time-dependent internalization of **NP1**, **NP2**, and **VC** was further studied by FCM, CLSM and inductively coupled plasma mass spectrometry (ICP-MS). Compared to the large-sized **VC**, a faster uptake rate and a higher intracellular accumulation were observed for both **NPs**, as evidenced from the FCM (Figure 3b) and ICP-MS data. Likewise, the amount of platinum in the cells treated with **NP1**, **NP2**, and **VC** for 4 h was 79.3 ± 8.2 , 70.7 ± 6.7 and 6.4 ± 0.8 ng/ 10^6 cell (Figure S17), respectively, revealing that the uptakes of **NP1** and **NP2** by HeLa cells are more efficient than that of **VC**. In the CLSM images, we also

observed that the green fluorescence intensity of the cells treated with either **NP1** or **NP2** was higher compared to those examined in the presence of **VC** (Figures 3c–e). This also supports the above results.

The nano-assemblies displayed a better photo stability compared to the conventional fluorophores. As shown in Figure 3f, a 11.4% loss of fluorescence signal was observed for **NP1** after 40 scans, while the red fluorescence of Lyso tracker Red completely disappeared after only 15 scans. This suggests that the latter fluorescence was photo bleached by the excitation light at this low working concentration; while for **NP1**, the AIE active metallacycles were not photo bleached or photo-oxidized under a similar condition, thus maintaining its emission.

The anticancer mechanism of platinum-based drugs relies on their ability to coordinate with the purine bases of DNA, interfering with the DNA repair mechanisms, causing DNA damage, and subsequently inducing cell apoptosis.⁸ To mimic the coordination interactions between **PhenPt** and DNA, several control experiments were performed. Guanosine triphosphate (GTP) was utilized as a competitive ligand to study the disassembly of the metallacycle **M** via fluorescence spectroscopy.^{4a} A significant fluorescence quenching was observed after incubating an aqueous solution of **M** with GTP (Figure S18), suggesting that GTP is a suitable donor for **PhenPt** and can disassemble the metallacycle. Next, the metallacycle **M** was gradually added to a solution containing calf-thymus DNA (ctDNA) and the relative viscosity (η/η_0) of the resulting solution was increased, indicating the formation of ctDNA/Pt coordination complex (Figure S19). The interactions between **M** and DNA were also examined by a fluorescence displacement experiment including ethidium bromide (EB) as a control. The fluorescence intensity of the ctDNA/EB complex was decreased upon a gradual addition of **M**, pointing towards the competitive binding of **M** to ctDNA (Figure S20). These studies demonstrate that the metallacycles in the nanostructures are able to crosslink with DNA.

The anticancer efficacies of **NP1**, **NP2**, and **VC** were evaluated using a 3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. For the precursor **NHS-PAZMB-b-POEGMA**, negligible changes in the relative cell viability were observed even when its concentration reached 1.00 mg/mL (Figure S21). The half maximal inhibitory concentrations (IC_{50}) are 2.89 ± 0.31 and 5.84 ± 0.63 μ M for **NP1** and **NP2** (Figure 3g), respectively. These values are much lower than that of **VC** ($IC_{50} > 20$ μ M), but are comparable with that of **PhenPt** ($IC_{50} = 1.06 \pm 0.15$ μ M), demonstrating that the anticancer efficacy of the organometallic drug was maintained in the nanoparticles, due to their rapid and efficient internalizations in the cells.

GSH-Responsive Disassembly and Controlled Drug Release

Notably, the 2-azido-methylbenzoate (**AZMB**) units of the polymer **Pt-PAZMB-b-POEGMA** are self-cleavable groups and can be easily removed via a cascade elimination reaction triggered by the intracellular GSH (Figure 1).⁹ As a consequence, the amphiphilicity of the polymer backbone was altered, resulting in a disassembly of the nanostructures. The GSH-responsive detachment of the **AZMB** units was first studied in a

dialysis medium by monitoring an absorption band centered at *ca.* 250nm that corresponds to the absorption of the released byproduct, 1-isoinidolinone. In the absence of stimulus, the absorption of the dialysis solution remained constant (Figure 2s), while the elimination percentage of **AZMB** gradually increased in the presence of 10.0 mM GSH over a period of 48 hours. Likewise, in the ^1H NMR spectrum of **NHS-PAZMB-*b*-POEGMA**, the peaks for the **AZMB** units, ranging from 7.25 to 8.10 ppm, entirely disappeared after 48 hours (Figure S22), suggesting that a complete elimination was accomplished. Further characterization of this process was performed via GPC studies, where a decrease in molecular weight from 89.7 to 58.3 kDa was observed after the copolymer solution was exposed to GSH for 24 h (Figure 2a). TEM images indicated that **NP1**, **NP2**, and **VC** split into irregular aggregates after being exposed to GSH for 4 h, likely indicating a partial cleavage of the **AZMB** units (Figures 2m–o). However, a prolonged incubation to 24 h resulted in a complete disassembly of the nanostructures (Figure 2p–r).

These GSH-responsive nanostructures were further employed as DDSs to encapsulate antitumor drugs and to achieve a synergistic anticancer effect. The hydrophobic cores of **NP1** and **NP2** are suitable platforms to load neutral DOX, whereas the hollow cavity of **VC** can encapsulate DOX·HCl. Only slight changes were observed in their sizes after loading the drugs (Table S1), while no obvious size variation was detected in a PBS containing 10% fetal bovine serum over 48 h at 37 °C. This indicates that these drug-loaded nanostructures are stable in this medium (Figure S26–28). GSH-responsive drug release was observed due to the disassembly of the nanocarriers (Figures S29–31). The pH-responsive drug release was also realized for DOX-loaded **NP1** and DOX-loaded **NP2** due to the protonation of the amine group of DOX.

By taking advantage of the intrinsic fluorescence emission of DOX, CLSM was employed to confirm the accumulation of DOX-loaded nanostructures. After being internalized by endocytosis and transported into endo/lysosomes (Figure S32), the green fluorescence of **NP1** and the red fluorescence of DOX were observed in the cytoplasm after a 4 h treatment. By extending the culture time to 24 h, the loaded DOX was successfully released due to the disassembly of the nanostructures. The released DOX accumulated into the nuclei, where the anticancer activity took place (Figure S33). Both **NHS-PAZMB-*b*-POEGMA** and the cleaved product indolin-1-one are nontoxic at all concentrations that were used in this study (Figure S21). Thus, any influence on cell viability from the DOX-loaded nanostructures is due to the toxicity of DOX and **PhenPt**. The IC_{50} values of free DOX, DOX-loaded **NP1**, DOX-loaded **NP2**, and DOX-loaded **VC** were determined to be 0.91 ± 0.10 , 4.75 ± 0.52 , 9.08 ± 0.89 and $>25 \mu\text{M}$, respectively (Figure S34). In order to calculate the combination index (CI) to confirm the presence of any synergistic effect, **NP3** of about 50 nm in diameter was prepared from the precursor **NHS-PAZMB-*b*-POEGMA**. The size and drug loading content of DOX-loaded **NP3** are comparable to those of DOX-loaded **NP1**; although the IC_{50} value of the former was increased to $6.61 \pm 0.74 \mu\text{M}$, likely due to the absence of **PhenPt**. The CI value was calculated to be 0.79 for DOX-loaded **NP1**, demonstrating a good synergistic anticancer effect present in the nanomedicine.

In Vivo Drug Delivery and Antitumor Treatment

Nanomedicines with diameters ranging from 20 to 200 nm preferentially leak into tumor tissues via a permeable tumor vasculature and are then retained in the tumor due to the reduced lymphatic drainage.¹⁰ Among these nanostructures, **NP1** appears to be the best candidate to deliver DOX *in vivo*, because its size falls within the optimal range. Moreover, the “brush like” PEG chains on the surface of **NP1** can prevent them from being adsorbed by proteins and being cleared from the body by the reticuloendothelial system (RES). These give the **NPs** a better opportunity to extravasate from the tumor vessels.¹¹ The pharmacokinetics of cisplatin, DOX·HCl, **NP1**, and **NP2** were examined in mice by intravenous (*i.v.*) injection (Figure 4a). The circulation half-life of **NP1** was 3.16 ± 0.28 h, which is much higher than those of cisplatin, DOX·HCl and **NP2** (0.27 ± 0.04 , 0.34 ± 0.05 and 0.53 ± 0.47 h, respectively), thereby confirming that the blood circulation time of **NP1** is relatively long. Biodistribution studies also showed that only a small amount of cisplatin, DOX·HCl and **NP2** appeared in the tumor region after 24 h post injection. In contrast, **NP1** resulted in remarkably higher tumor accumulation (Figure 4b). CLSM images (Figure 4c), *ex vivo* fluorescence images (Figure 4d) and multicellular spheroids staining (Figure 4e) also demonstrated that **NP1** exhibited a high tumor accumulation and an excellent tumor penetration capability due to its suitable size.

To evaluate the *in vivo* antitumor efficacy, HeLa tumor-bearing mice were intravenously injected with different formulations, including PBS, cisplatin, **NP1**, DOX·HCl, DOX-loaded **NP3**, and DOX-loaded **NP1**. The tumors treated with PBS grew exponentially over time and exhibited an average tumor volume of 1566 mm³ after 21 days (Figure 4f). For the mice administered with cisplatin or blank **NP1** (0.150 mg Pt/kg), a very limited delay of the tumor growth was observed, due to the low drug dosage. In the DOX·HCl-treatment group, a moderate tumor inhibition was achieved, with the mean tumor volume being 752 mm³ after 21 days. Due to the EPR effect and GSH-triggered drug release, DOX-loaded **NP3** exhibited a higher antitumor efficiency than free DOX·HCl. Notably, the DOX-loaded **NP1** formulation vastly outperforms all the other groups in reducing tumor volume. This is likely due to the aforesaid synergistic anticancer effect. After treatment, the tumors were also excised and weighed. Accordingly, the tumor weight and inhibition rates (%) were 5.74, 19.5, 45.9, 62.3 and 81.6% for cisplatin, **NP1**, DOX·HCl, DOX-loaded **NP3**, and DOX-loaded **NP1**, respectively (Figure S35), further suggesting a better antitumor activity of DOX-loaded **NP1**. The therapeutic efficacy of DOX-loaded **NP1** was also examined by a hematoxylin/eosin (H&E) staining and a nuclear non-histone protein (Ki67) staining. Among these therapeutic groups, the tumor tissues from the mice treated with DOX-loaded **NP1** exhibited the highest level of necrosis and the fewest proliferating cells, indicating a massive remission of proliferative activity (Figure 4h).

To assess the systemic toxicity of DOX-loaded **NP1**, we also compared the impact of different formulations on body weight, survival rate, organ histology and blood chemistry. Serious body weight fluctuations were observed for mice treated with free DOX·HCl, reflecting the huge side effects of DOX. In contrast, the group treated with DOX-loaded **NP1** did not show a significant body weight loss over the experimental period, indicating that a much reduced systemic toxicity can be achieved via the nano-formulation (Figure

S36). In comparison with other groups (24, 27, 31, 28 and 38 days for PBS, cisplatin, **NP1**, DOX·HCl, and DOX-loaded **NP3**, respectively), the median survival of the mice treated with DOX-loaded **NP1** was significantly extended (Figure 4g), due to its excellent antitumor efficacy and low systemic toxicity. Histological analysis of major organ slices of the mice treated with free DOX showed severe cardiotoxicity, which was characterized by the notable vacuolization in the cardiomyocytes (Figure S37). For DOX-loaded **NP1**, the H&E staining images of the heart, liver, spleen, lung and kidney revealed that all of the organs were normal. Moreover, blood biochemistry and hematology surveys were conducted after the administration of DOX-loaded **NP1** (Figure S38) on the 15th day with alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, red blood cells, white blood cells, creatinine and blood urea nitrogen. Compared with the normal mice, all parameters were within normal range, further confirming that no liver or renal dysfunction were induced by the DOX-loaded **NP1**.

Conclusions

In summary, we developed a theranostic platform consisting of a highly fluorescent metallacycle **M** and four GSH-responsive copolymeric arms. In the metallacycle, the emissive TPE-based bispyridyl ligands are the donors and act as a spectroscopic handle for live cell imaging, while the acceptor **PhenPt** units are employed as an anticancer drug. By using either a reprecipitation, dialysis or a double emulsion method, further self-assemblies of **NP1**, **NP2**, and **VC** were successfully prepared from this amphiphilic polymer. Due to differences in the morphology and size, these nanostructures exhibited different cytotoxicities towards HeLa cells. Furthermore, **NP1**, **NP2**, and **VC** can encapsulate DOX or DOX·HCl, while the release of the encapsulated drug was realized via a GSH-responsive elimination reaction. *In vitro* and *in vivo* investigations demonstrated that a codelivery of DOX and **PhenPt** is possible with a synergistic anticancer effect. Given these results, this work provides valuable information and methodology for the rational design of the next generation DDSs based upon supramolecular structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Synthesis of **Pt-PAZMB-*b*-POEGMA** and its GSH-triggered amphiphilicity reversion mechanism. (b) Cartoon illustration of the cellular uptake of DOX-loaded nanostructures self-assembled from **Pt-PAZMB-*b*-POEGMA**.

Figure 2.

(a) GPC curves of (I) **Pt-PAZMB-*b*-POEGMA**, (II) **Pt-PAZMB-*b*-POEGMA** before purification, (III) **Pt-PAZMB-*b*-POEGMA** after GSH treatment, and (IV) **NHS-PAZMB-*b*-POEGMA**. (b) Fluorescence intensity of **Pt-PAZMB-*b*-POEGMA** (•) and **M** (•) at 525 nm vs. f_w of the aqueous mixtures. Inset: a fluorescent photo of **Pt-PAZMB-*b*-POEGMA** in mixtures of THF and water with different f_w values. (c) CAC value determination of **Pt-PAZMB-*b*-POEGMA** by using pyrene as a probe. Inset: Tyndall effect of the aqueous solution when the concentration of **Pt-PAZMB-*b*-POEGMA** was higher than its CAC value. TEM (d, e, f), CLSM (g, h, i), and AFM (j, k, l) images of **NP1** (d, g, j), **NP2** (e, h, k), and **VC** (f, i, l). TEM images of **NP1** (m, p), **NP2** (n, q), and **VC** (o, r) after GSH treatment for 4 h (m, n, o) and 24 h (p, q, r). (s) Elimination of **AZMB** in the absence and presence of GSH (10.0 mM).

Figure 3.

(a) Cellular internalization of **NP1**, **NP2**, and **VC** by HeLa cells in the absence and presence of various inhibitors (* $p < 0.05$). (b) Mean fluorescent intensity of the HeLa cells after treatment with **NP1**, **NP2**, and **VC** for different time periods (** $p < 0.01$). CLSM images of the HeLa cells incubated with (c) **NP1**, (d) **NP2**, and (e) **VC** for 8 h. (f) Signal loss of fluorescent emission of **NP1** and Lyso tracker Red with increasing number of scans. Inset: CLSM images of living HeLa cells after different number of scans. (g) Cytotoxicity of **NP1**, **NP2**, **VC**, and **PhenPt** towards HeLa cells after 24 h incubation.

Figure 4.

(a) Blood circulation time of **NP1**, **NP2**, DOX-HCl, and cisplatin in normal mice after *i.v.* injection. (b) Tissue distributions of **NP1**, **NP2**, DOX-HCl, and cisplatin in the main organs at 24 h post-injection. (c) CLSM images of the intratumor distribution of **NP1**. (d) Fluorescence images of isolated organs separated from HeLa tumor-bearing mice at 24 h post injection of **NP1**. (e) CLSM images showing *in vitro* penetration of **NP1** and **NP1** in HeLa multicellular spheroids. (f) Tumor growth inhibition curves and (g) survival rate of the mice bearing HeLa tumors after different treatments. (h) H&E and Ki67 analyses of tumor tissues after various formulations: (I) PBS, (II) **NP1**, (III) cisplatin (IV) DOX-HCl, (V)

DOX-loaded **NP3**, (VI) DOX-loaded **NP1**. The dosage of DOX and Pt in these administrations kept 5.00 mg/kg and 0.150 mg/kg, respectively.

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