Synergistic Cisplatin/Doxorubicin Combination Chemotherapy for Multidrug-Resistant Cancer via Polymeric Nanogels Targeting Delivery

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Supporting Information

ABSTRACT: Combination chemotherapy has been proposed to achieve synergistic effect and minimize drug dose for cancer treatment in clinic application. In this article, the stimuli-responsive polymeric nanogels (<100 nm in size) based on poly(acrylic acid) were designed as codelivery system for doxorubicin and cisplatin to overcome drug resistance. By chelation, electrostatic interaction, and \( \pi - \pi \) stacking interactions, the nanogels could encapsulate doxorubicin and cisplatin with designed ratio and high capacity. Compared with free drugs, the nanogels could deliver more drugs into MCF-7/ADR cells. Significant accumulation in tumor tissues was observed in the biodistribution experiments. The in vitro antitumor studies demonstrated the superior cell-killing activity of the nanogel drug delivery system with a combination index of 0.84, which indicated the great synergistic effect. All the antitumor experimental data revealed that the combination therapy was effective for the multidrug-resistant MCF-7/ADR tumor with reduced side effects.

KEYWORDS: nanogel, combination chemotherapy, stimuli-responsive, multidrug-resistant, CDDP, doxorubicin

1. INTRODUCTION

Combination chemotherapy could bring many advantages for cancer therapy, such as synergistic therapeutic effect and overcoming drug resistance, and also was successfully applied in clinics. Nevertheless, differences in the pharmacokinetic profiles and biodistributions of different drugs usually made it extremely difficult for dosing and scheduling optimization. This problem was particularly acute for cancer drugs, which often had a wide spectrum of cytotoxicity. The adoption of nanodelivery vehicle could carry more multiple therapeutic agents to tumor but less accumulation in undesired sites, resulting improved therapeutic efficacy but minimized side effects.

Cisplatin (CDDP), a potent DNA chelating agent, induced tumor cells apoptosis by covalent binding with DNA purine bases to form DNA adducts that could inhibit cellular transcription and replication.\(^8,9\) Doxorubicin (DOX), a topoisomerase II (TOP\(_2\)) inhibitor, could restrain the DNA remodeling through intercalating duplex DNA and inhibiting the activity of TOP\(_2\). Inhibition of TOP\(_2\) pathway by DOX was independent of DNA damage by CDDP. In addition, DOX could further inhibit the repairing of CDDP-damaged DNA by TOP\(_2\). Therefore, the combination of DOX and CDDP could overwhelm the cellular repair mechanisms.\(^10,11\) This combination chemotherapy has shown synergistic effect in phase III clinical trials.\(^3,12-15\) However, both DOX and CDDP exhibited severe side effects and drug resistance in clinic.\(^16\)

The advantages of combination therapy and the disadvantages of free drugs have promoted the development of nanosized drug delivery systems (DDSs).\(^17-20\) Codelivery of DOX and CDDP has been achieved by many DDSs. Examples include a polymer-caged nanobin (PCN),\(^11\) nanosized brush-arm star polymers (BASPs),\(^21\) polysaccharide-based nanoparticles,\(^22\) double-helix nucleic acids,\(^23\) carbon nanotubes,\(^24\) and oligonucleotides assembled Au nanorods.\(^25\) In some cases,\(^11,21,24,25\) CDDP was in the form of derivative that requires time-consuming and laborious preparation steps; then some new drug delivery systems were demanded for easy and effective way to load the anticancer drugs. Usually, the polymeric nanogels, especially stimuli-responsive ones, were designed and prepared as one of the most promising nanoparticulate DDSs.\(^26,27\) Our group has developed some...
nanogel drug delivery systems by using methacrylic acid as monomer and N,N-bis(acryloyl)cysteamine (BAC) as cross-linker, which could undergo degradation in tumor cells and be excreted via metabolism.\textsuperscript{28,29} Herein, we developed a new type of uniform stimuli-responsive nanogels with acrylic acid (AA) as monomer, which could provide lots of free carboxylic groups and supply more sites for complexation with DOX/HCl and CDDP via strong electrostatic interaction and chelation, respectively. Because of the great affinity between them, we could easily modulate the ratio of the drugs loaded in the nanogels. This kind of nanogel was used for synergistic CDDP/doxorubicin combination chemotherapy; the in vivo antitumor experiments showed that the combination therapy was greatly effective for multidrug-resistant MCF-7/ADR tumor with reduced side effects.

2. MATERIALS AND METHODS

2.1. Materials. Acrylic acid and N,N-bis(acryloyl)cysteamine were purchased from Sigma-Aldrich. 1-Ethyl-3-[(3-dimethylaminopropyl)-propyl]-carboxydimide hydrochloride (EDC-HCl), N-hydroxysuccinimide (NHS), glutathione reduced (GSH), folic acid (FA), doxorubicin hydrochloride (DOX/HCl), and cisplatin were offered by Shanghai Aladdin Chemistry Co. Ltd. t-Boc-NHPEG-NH\textsubscript{2} was obtained from Laysan Bio, Inc. 2,2-Azobis(isobutyronitrile) (AIBN) was purchased from Sinopharm Chemical Reagent Company and used after recrystallization (ethanol). Laysan Bio, Inc. 2,2-Azobis(isobutyronitrile) (AIBN) was purchased from Sinopharm Chemical Reagent Company and used after recrystallization (ethanol). 17β-Estradiol 90-day release pellets (NE-121) were supplied by Innovative Research of America. All reagents were of analytical purity and used without further purification if not mentioned.

2.2. Preparation of PAA-Based Nanogels. Reflux-precipitation polymerization (RPP)\textsuperscript{30} was used to prepare the PAA-based nanogels. The typical synthesis procedure was as follows: AA (0.3 g, 4.16 mmol), BAC (60 mg, 0.23 mmol), and AIBN (7.2 mg, 0.044 mmol) were dissolved in acetonitrile (60 mL) with ultrasonic agitation. The reaction mixture was then quickly heated to 95 °C (the temperature of oil bath) and kept at this temperature for 1 h. After centrifugal separation (12 000 rpm, 8 min), purification (acetonitrile, 10 mL) with ultrasonic agitation. The reaction mixture was then quickly heated to 95 °C (the temperature of oil bath) and kept at this temperature for 1 h. After centrifugal separation (12 000 rpm, 8 min), purification (acetonitrile, 10 mL) was repeated, and the PAA-based nanogels were obtained as white powders.

2.3. Modification of the Nanogels. The NH\textsubscript{2}-PEG-folate was synthesized from t-Boc-NHPEG-NH\textsubscript{2} in accordance with a reported procedure.\textsuperscript{3} Then, the surface of PAA nanogels was modified with NH\textsubscript{2}-PEG (M\textsubscript{w} = 1000) and NH\textsubscript{2}-PEG-folate by carbodiimide coupling. PAA nanogels (30 mg) were dispersed in deionized water (20 mL); EDC-HCl and NHS were added to the solution (20 mL) at 4 °C. After stirring for 3 h, NH\textsubscript{2}-PEG (30 mg) and NH\textsubscript{2}-PEG-folate (15 mg) were added to the mixture, which was continued to react overnight. The product was separated by ultracentrifugation (12 000 rpm, 3 min), purified by deionized water (3 × 5 mL), and followed by lyophilization.

2.4. Redox-Triggered Degradation of PEG-PAA Nanogels. The degradation of the nanogels was studied under reducing condition. Typically, 5 mg of modified nanogel was dispersed in 10 mL of phosphate buffer solution (PBS, pH 7.4) with 10 mM GSH. The mixture was then kept in a shaking bed (37 °C, 200 rpm) for 24 h. Finally, the mixture was filtered (0.45 μm filter) and analyzed with gel permeation chromatography (GPC).

2.5. Loading of Drugs into Modified PAA-Based Nanogels. DOX/HCl, CDDP, or DOX/HCl together with CDDP were loaded into the modified PAA-based nanogels in a simple way. First, 10 mg of dry nanogels was dispersed in 20 mL of PBS (pH 7.4) with the aid of ultrasonic bathing, while DOX/HCl, CDDP, or DOX/HCl and CDDP were dissolved in water (2 mL). Subsequently, the drug solution was added into the nanogel solution, and the mixture was stirred for 24 h. Afterward, the drug-loaded nanogels were obtained by ultrafiltration (12 000 rpm) and washing with water (3 × 5 mL) to remove free drugs and the surface adsorbed drugs. All the supernatant was collected to measure the drug content. The DOX content in nanogels was calculated by subtracting the amount of DOX in supernatant (determined by a UV–vis spectrophotometer, 480 nm) from the DOX in initial solution.\textsuperscript{31} The concentrations of the CDDP were measured with ICP. The drug loading capacity (DLC) and drug loading efficiency (DLE) were calculated by the following equations:

\[
\text{DLC (\%, DOX)} = \frac{\text{initial wt of DOX} - \text{wt of DOX in supernatant}}{\text{wt of drug loaded nanogels}} \times 100\%
\]

\[
\text{DLC (\%, CDDP)} = \frac{\text{initial wt of CDDP-wt of CDDP in supernatant}}{\text{wt of drug loaded nanogel}} \times 100\%
\]

\[
\text{DLE (\%)} = \frac{\text{wt of DOX or CDDP in nanogels}}{\text{wt of DOX or CDDP in feed}} \times 100\%
\]

2.6. In Vitro Drug Release. To determine the release profiles of DOX and CDDP, the dual-drug loaded nanoparticles (about 10 mg), dried by lyophilization, were dispersed in 12 mL of phosphate buffer (pH 7.4) or acetate buffer (pH 5.0). Then the mixture was divided into six fractions (2 mL for each) and subsequently put into dialysis bags (MWCO = 14 kDa) with corresponding medium (20 mL; pH = 7.4 with GSH; pH = 7.4 without GSH; pH = 5.0 with GSH; pH = 5.0 without GSH). The release experiment was believed to start as soon as dialysis bags were placed in the release medium. At selected time intervals, 5 mL of release medium was taken out and replenished with same volume of fresh media to keep the volume of release medium unchanged. The concentrations of DOX and CDDP were measured with a UV–vis spectrophotometer and ICP, respectively. All the data represent the mean of three separate experiments.

2.7. Cell Culture and In Vitro Cell Assays. Cell Culture. Multidrug-resistant cell line of human breast adenocarcinoma, MCF-7/ADR cells (American Type Culture Collection, Manassas, VA), were cultured in DMEM with 10% fetal calf serum (FCS), 100 IU/mL penicillin G, and 100 mg/mL streptomycin sulfate at 37 °C in a humidified 5% CO\textsubscript{2} atmosphere. To maintain the drug resistance characteristics of MCF-7/ADR cells, the cells were treated with 1 μg/mL of free DOX weekly, and cultured for 1 week in DOX-free medium before in vitro cell assay. Identities of the cell line were confirmed by STR testing in 2013.\textsuperscript{32}

CCK\textsubscript{8} Assay. Cell viability was evaluated with Cell Counting Kit-8 following the manufacturer’s instructions. MCF-7/ADR cells were seeded in 96-well plates (100 μL, about 4000 per well) and cultured for 12 h. Then, the DMEM was replaced by indicated concentrations of drugs and incubated for 48 h. After discarding the drug solution and rinsing with PBS (100 μL), CCK-8 solution (10 μL) was added and incubated for 2 h. Finally, absorbance at 450 nm was measured to calculate the number of viable cells. The relative survival rate was normalized to the untreated controls after background subtraction.

Apoptosis Analysis. Apoptosis analysis was carried out with Annexin V/PI apoptosis kit (R&D Systems) according to the manufacturer’s instructions. Briefly, MCF-7/ADR cells were treated with different drugs for 48 h. Then the cells were double stained with Annexin V-FITC and PI, and cell apoptosis was measured with a flow cytometer.

2.8. Intracellular Drug Delivery. The cellular uptake of free DOX, FA-PEG-PAA-Gs-60 with DOX (DOX-NGs), CDDP/DOX dual loaded FA-PGG-PAA-Gs-60 (CDDP/DOX-NGs), and CDDP/DOX dual loaded CDDP-PAA-Gs-60 (CDDP/DOX-NFNGs) was observed on confocal laser scanning microscopy observation and flow cytometry. For CLSM, cells were seeded in 6-well plates and cultured overnight. Then the medium was replaced with free DOX, DOX-NGs, CDDP/DOX-NGs, and CDDP/DOX-NFNGs containing DMEM. After a selected period of time, the cells were rinsed with PBS (3 × 1 min). Nucleus and lysosome were labeled with 4,6-diamidino-2-phenylindole (DAPI) and Lyso-tracker Green DND-26 solution.
Scheme 1. Schematic Illustration of the Preparation, Surface Modification, Dual-Drug Loading, and Drug Releasing of the Designed PAA-Based Nanogels

Table 1. Characterization of Various PAA-Based Nanogels with Different Reaction Parameters

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<th>BAC (mg)</th>
<th>AIBN (mg)</th>
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<th>T (°C)</th>
<th>D_{292} (nm)</th>
<th>D_{DLS} (nm)</th>
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respectively, for 10 min. The samples were washed with PBS (3 × 1 min) and added 1 mL of PBS for CLSM observation. For flow cytometry, MCF-7/ADR cells were seeded in 6-well plates (about 2 × 10⁵ cells per well) and cultured overnight. The cells were treated with free DOX, DOX-NGs, CDDP/DOX-NGs, and CDDP/DOX-NFNGs for selected time intervals. After conventional treatment, the cells were resuspended in PBS for flow cytometry analysis.

2.9. Serum Stability and Hemolysis Assay. The stability of the FA-PEG-PAANGs and dual drug-loaded nanogels in serum was studied by DLS. The nanogels were first dissolved in PBS, then 10% FBS (fetal bovine serum) was added. DLS was carried out at 0.5 and 48 h. The hemolysis assay of nanogel solutions was evaluated as previously described. Fresh orbital blood from BALB/c nude mice was first removed serum, blood platelet, and white blood cell by first dissolved in PBS, then 10% serum was added. DLS was carried out at 0.5 and 48 h. The hemolysis assay of nanogel solutions was evaluated as previously described. Fresh orbital blood from BALB/c nude mice was first removed serum, blood platelet, and white blood cell by centrifugation (2000 rpm, 5 min). The red blood cells (RBCs) were washed with PBS (3 × 5 mL) and diluted with 10-fold volume of PBS. Then, 0.3 mL of RBCs suspension was mixed with (i) 4 mL of PBS as a negative control, (ii) 4 mL of deionized water as a positive control, (iii) 4 mL of FA-PEG-PAANGs solution (in PBS) at concentrations of 500, 1000, and 1500 µg/mL, and (iv) 4 mL of CDDP/DOX-NGs solution (in PBS) at concentrations of 500 µg/mL. The mixtures were kept at 37 °C with gently shaken (200 rpm) in a shaking bed. After 3 h, all samples were centrifuged (2000 rpm, 8 min), and the supernatant absorbance was measured at 540 nm by a UV−vis spectrophotometer. The hemolysis rate was calculated by following formula: hemolysis rate (%) = \frac{D_{\text{mean}} - D_{\text{nc}}}{D_{\text{pc}} - D_{\text{nc}}} × 100%. In this formula, D_{\text{mean}} means absorbance for test samples, D_{\text{pc}} means absorbance for negative control sample, and D_{\text{nc}} means absorbance for positive control sample.

2.10. Animals and in Vivo Evaluations. Female BALB/c nude mice, 20–25 g and 6–8 weeks old, were housed under standard conditions and taken care of according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. The use of all mice was approved by local Ethics Committee. 17β-Estradiol 90-day release pellets were subcutaneously implanted into the back of mice 5 days before tumor cell injection.

In Vivo Biodistribution and ex Vivo DOX Fluorescence Imaging. Female BALB/c nude mice bearing MCF-7/ADR tumor were used for in vivo real-time fluorescence imaging and ex vivo imaging. Cy7-labeled nanogels, dual-drug loaded nanogels, and free DOX were dispersed in physiological saline and then administered to mice via tail vein injection (2.5 mg/kg on DOX basis). For in vivo biodistribution of the prepared nanogels, the mice were captured at selected time points on In Vivo Xtreme (Bruker, excitation: 690 nm; emission: 720 nm). For ex vivo fluorescence imaging, the mice were killed at 2, 8, and 24 h postinjection. Major organs and tumor were dissected from mice, washed with PBS, and captured on In Vivo Xtreme (excitation: 480 nm; emission: 560 nm).

In Vivo Antitumor Activity Evaluation. BALB/c nude mice bearing MCF-7/ADR tumors were randomly divided into different groups (seven for each group) at day 8 after subcutaneously cell injection. Mice were injected intravenously with different drugs (2.5 mg/kg on DOX basis) and physiological saline. Drugs were administered once a week and lasted for 4 weeks. Every 4 days, tumor size was measured using Vernier calipers and body weight was recorded. The tumor volume (mm³) was calculated according to the following formula: \( V = a \times b^2/2 \), where \( a \) and \( b \) are the maximal and minimal diameters of tumor, respectively.

3. RESULTS AND DISCUSSION

3.1. Preparation of PAA-Based Nanogels. The PAA-based nanogels (PAANGs) were synthesized via reflux-precipitation polymerization (RPP) with acrylic acid as monomer and BAC as cross-linker. After preparation of the nanogels, their surfaces were modified with PEG and FA for long life circulation time and targeting drug delivery, respectively. The detailed procedure for the preparation, drug loading, and releasing can be seen in Scheme 1.

To obtain nanogels with a suitable morphology and size, we systematically investigated the key reaction parameters, including the amounts of BAC and AIBN, the volume of acetonitrile, and the reaction temperature. The corresponding results are listed in Table 1. As the reaction temperature increased from 75 to 95 °C, the morphology changed from...
sphere (Figure 1A,C) to raspberry-like shape (Figure 1B,E and Figure S1B). When the amount of initiator AIBN increased (compare PAANGs-40B with PAANGs-40A), a raspberry-like shape (Figure 1D) could be obtained. When the volume of acetonitrile increased (compare PAANGs-60 with PAANGs-40C), sphere shape could be formed again (Figure 1F and Figure S1C). Therefore, the raspberry-like shape was reasonably resulted from fast polymerization rate. Considering the role of size played in the biodistribution and the fenestration size for main organs, it was reported that ideal size of nanovectors for tumor should be $70^{−200}$ nm.35,36 Therefore, sample PAANGs-60, smaller than 100 nm in drying regime observed in TEM and about 180 nm in swelling state detected in DLS, was chosen as dual-drug carrier for further investigations.

**3.2. Modification of the Nanogels.** Surface PEG decoration is the most frequently used to avoid a fast recognition by the immune system and escape from reticuloendothelial system (RES) elimination by stealth effect. Thus, the nanocarrier could prolong the in vivo circulation time and enhance the accumulation by enhanced permeability and retention (EPR) effect in tumor tissues.37,38 However, the sterically hindering cloud on the surface of nanoparticles also brings out the problem of poor cellular internalization, which will severely reduce intracellular dosages of anticancer drugs and limit their efficiencies. The strategy of active targeting ligand modification for receptor-mediated endocytosis is an important solution method to this challenge.39,40 In this work, folate was used as an active targeting ligand for folate receptor (FR), which was overexpressed in many tumors including MCF-7/ADR tumor.41

PEG-NH$_2$ and FA-PEG-NH$_2$ were decorated onto the surfaces of the PAANGs-60 through carbodiimide-mediated coupling reaction. FT-IR spectroscopy (Figure S2) confirmed that PEG-NH$_2$ and FA-PEG-NH$_2$ had been successfully conjugated onto the nanogels. The strong absorbing peak at 1100 (red dashed line) of PEG could be found in the PEG modified nanogels (FA-PEG-PAANGs, blue in the FT-IR spectra) and FA-PEG-NH$_2$ (red in the FT-IR spectra), compared to PAANGs-60 (black in the FT-IR spectra). The characteristic absorption bands (black dashed lines) from benzene ring of FA could be found in FA-PEG-PAANGs.

Fluorescence spectrum was applied to further confirm the FA decoration (Figure 2A). Compared with PEG-PAA-NGs, FA-PEG-PAANGs (in PBS) showed a strong fluorescence emission peak of FA at approximately 455 nm (excited at 365 nm). The amount of folate moiety on the nanogels surface was about 6.7 wt % measured by a fluorescence spectrophotometer (ex: 365 nm) according to the folate calibration curve (Figure S3).

However, compared with the PAANGs-60 nanogels, the size and morphology of FA-PEG-PAANGs did not change much (Figure S4A,B). The hydrodynamic diameter (by DLS) rose from 191 to 255 nm, for the fact that hydrophilic PEG chains were extended in aqueous solution (Figure 2B). The surface charge of modified nanogels reduced from $−28$ to $−17$ mV (Figure S4C) due to the PEG decoration and the shielding effect from PEG corona.

**3.3. Redox-Triggered Degradation of FA-PEG-PAANGs Nanogels.** The concentration of glutathione (GSH), which could reduce disulfide bonds, was reported to be about 1000 times higher in cells ($2^{−10}$ mM) than that in extracellular milieu or blood ($2^{−20}$ μM).42−44 Moreover, some tumor tissues showed about 4-fold even higher concentrations of GSH than normal tissues.45 It was also reported that numerous γ-interferon-inducible lysosomal thiol reductases

![Figure 1. TEM images of PAA-based nanogels with different reaction parameters: (A) PAANGs-20A, scale bar is 1 μm; (B) PAANGs-20B, scale bar is 2 μm; (C) PAANGs-40A, scale bar is 1 μm; (D) PAANGs-40B, scale bar is 0.5 μm; (E) PAANGs-40C, scale bar is 1 μm; (F) PAANGs-60, scale bar is 0.5 μm.](image1)

![Figure 2. (A) Fluorescence emission spectra of PEG-PAANGs and FA-PEG-PAANGs. (B) Dynamic light scattering curves of PAANGs (PAANGs-60) and FA-PEG-PAANGs.](image2)
(GILTs), existing in endosomes and lysosomes, were capable of reducing disulfide bonds. Our prior work found that the BAC-cross-linked PMAA nanogels could degrade by protease for the amido bonds existing in the BAC units. Accordingly, the disulfide bonds contained in our designed BAC-cross-linked nanogels could be broken down by either GSH or GILT as well as the protease. In this work, degradation of FA-PEG-PAANGs was investigated in a simulated environment of tumor cells with 10 mM GSH. The molecular weight of disassembled FA-PEG-PAANGs was analyzed by GPC (Figure S5). The degraded product had a very low molecular weight ($M_n = 1150$) and narrow molecular weight distribution ($M_w/M_n = 1.10$). This result indicated that the disulfide bonds in FA-PEG-PAANGs could be efficiently broken down by 10 mM GSH and led the network of nanogel to be quickly degraded into low molecular weight fractions which would easily be discharged from the body.

3.4. Drugs Loading and in Vitro Release Study. The amino group existing in DOX structure has a $pK_a$ of 8.6, while the $pK_a$ of free carboxyl groups in PAA chains is about 4.8. Therefore, DOX could efficiently loaded into PAA via electrostatic interactions as well as $\pi-\pi$ stacking interactions. In our investigation of DOX loading ability in different drug concentration solutions, high drug loading efficiency (DLE, $\sim 96.35\%$) was found even when DOX concentration reached 0.1 mg/mL, indicating high affinity between DOX and PAA. In addition, relative high drug loading content (DLC) of about 42.13% could achieve with high DOX concentration ($\geq 0.5$ mg/mL). The Pt atom in CDDP could coordinate with the carbonyl groups in weak alkaline solution via chelation reaction. To simplify the process, CDDP and DOX were loaded into the nanogels at the same time. The experimental results (Table 2) showed that the addition of CDDP during DOX loading did not influence the DLE of DOX. With increase in concentration, the CDDP loading capacity increased to as high as 37.05%, but the drug loading efficiency reduced slightly when the concentration of CDDP reached 0.45 mg/mL (78.32% compared to 88.24% in 0.25 mg/mL with DLC of 18.1%). Above all, the FA-PEG-PAANGs-60 could efficiently encapsulate CDDP and DOX with high DLC in weak alkaline solution, also avoided organic solvents and sophisticated loading procedure. In view of the fact that certain number of free carboxyl groups were essential to keep the drug loaded nanogels hydrophilic enough to be stable in physiological condition, FA-PEG-PAANGs-60 loaded DOX and CDDP with DLC of 18.1% ($D_{\text{LE}}$ of 92.64%) and 18.1% ($D_{\text{LE}}$ of 88.24%), respectively, were chosen for further investigations.

One of the key points of DDSs for cancer therapy is controlled release of the drugs at tumor tissue. It is well-known that $pH_\text{w}$ of tumor (about 6.5) is slightly lower than normal tissue and blood. Furthermore, intracellular endolysosomes usually have strong acidity ($pH \sim 5$). The interaction intensity between DOX and PAA is much stronger in neutral or weak alkaline media than in acidic media. Thus, the DOX could maintain inside the nanogels during blood circulation but could escape from the nanogels in tumor cells. Similarly, the CDDP could be liberated from the nanogels in the form of reactive aqua species via aquation in acidic media. Further to the disassembly of nanogels with GSH or GILT/protase that had discussed above, the FA-PEG-PAANGs should have good properties for controlled intracellular drug release. The controlled GSH/pH dual stimuli-triggered drug release of CDDP/DOX-NGs was carried out in vitro. The percentages of DOX and CDDP released from the nanogels in different conditions are shown in Figure 3. At physiological pH ($\sim 7.4$), there was only about 13.11% of DOX released from the FA-PEG-PAANGs after 24 h, while in tumor intracellular environment (pH 5.0, 10 mM GSH), as much as 91.55% of

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<th>CDDP (mg/mL)</th>
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<th>DOX DLC (%)</th>
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Figure 3. (A) DOX release profiles of CDDP/DOX-NGs in different conditions. (B) CDDP release profiles of CDDP/DOX-NGs in different conditions.
DOX could escape from the carrier. When in GSH-free acid condition, only 51.25% of DOX could release, suggesting that the disassembly of nanogels could greatly help the liberation of the payload. The release behavior of CDDP was similar to that of DOX, but more slowly, this might due to that the CDDP was released from the COOH complex via aquation, while DOX was released upon protonation, which is a faster process compared to aquation.

3.5. Serum Stability and Hemolysis Assay. Another important issue for DDSs was their stability in blood. Thanks to the excellent dispersion stability of the as-prepared prepared nanogels in water, the serum stability and hemolysis assay were carried out. As illustrated in Figure 4A, either the blank carrier FA-PEG-PAANGs or the dual drug-loaded nanogels CDDP/DOX-NFs showed no aggregation in 10% FBS. After storing in 10% FBS for 48 h, the hydrodynamic diameters of the nanogels almost kept the same. The results of hemolytic activity showed that the blank carrier did not cause any hemolysis even up to 1000 μg/mL (Figure 4B and Figure S5). The drug-loaded nanogels exhibited higher hemolytic activity than that of the blank carrier, but it is also in a good level, and their hemolytic activity might be overrated due to the absorption of loaded DOX at 540 nm in calculating hemolysis.

3.6. Intracellular Drug Delivery. The cellular internalization and intracellular drug release behavior of DOX were first investigated by CLSM (Figure 5A). No signal of DOX (in red) were observed in cells with free DOX both after 1 and 3 h as a result of drug resistance. Free DOX was believed to enter MCF-7/ADR cells in a flip-flop mechanism.54 But it was actively pumped out by the overexpressing transmembrane protein P-glycoprotein (P-gp) once it entered into the cells. Therefore, intracellular DOX accumulation was rapidly decreased. Both DOX-NFs and CDDP/DOX-NFs could efficiently deliver DOX into the MCF-7/ADR cells, and they could not be pumped out by the transmembrane protein P-glycoprotein (P-gp) effectively. As shown in Figure 5A, DOX mainly located in lysosome (imaged in yellow as the result of green and red overlapping) and partly entered into nucleus (imaged in aubergine as the result of blue and red overlapping) in 3 h. Just as we known, the nanoparticles were usually internalized into the cells via endocytic pathways and then be transported to lysosome.55 Therefore, DOX encapsulated in the nanocarriers could lead to high cellular accumulation. In addition, the nucleus distribution of doxorubicin was studied by extending the incubation time to 15 h. Compared with free DOX group that showed only few DOX in cytoplasm, both CDDP/DOX-NGs and DOX-NFs groups had many DOX in nucleus (Figure S7A). Meanwhile, the cellular uptake of DOX was quantitatively measured by flow cytometry (Figure 5B). Being consistent with the results of CLSM analysis, only 1.6% of cells in free DOX group had DOX signal at 1 h, while 69.5% and 73.1% of cells were with DOX signal for DOX-NFs group and CDDP/DOX-NFs group, respectively. At 3 h, 96.2% and 98.7% of MCF-7/ADR cells had strong DOX fluorescence when treated with DOX-NFs and CDDP/DOX-NFs, respectively.

The role of FA played in intracellular delivery was also investigated. The CDDP/DOX-loaded PEG-PAANGs that without FA moiety in the surface (CDDP/DOX-NFs) was found to be less efficient. After incubation for 3 h, only 77.8% of DOX in CDDP/DOX-NFs group was internalized into the cells, comparing with 97.0% for CDDP/DOX-NFs (Figure S7B). The study with a confocal laser scanning microscopy (Figure S7A) found that the CDDP/DOX-NFs group had much less DOX entered into cell, and also most of them did not get into nucleus after 15 h incubation.

3.7. In Vitro Therapeutic Efficacy. The cytotoxicity of various drug formulations and the carrier with increasing concentrations (from 0 to 120 μg/mL) was evaluated by MCF7/ADR cells for 48 h using CCK8 assay. All drug formulations exhibited enhanced cytotoxicity to MCF7/ADR cells with dose increased (Figure 6A). The carrier, FA-PEG-PAANGs, showed nontoxicity up to a relative high dose (120 μg/mL), indicating it had a good biocompatibility. As expected, the free DOX displayed very weak killing potency to MCF7/ADR cells in low dose, as a result of drug resistance that had discussed above. Meanwhile, DOX-NFs and CDDP/DOX-NFs showed much higher efficiency compared to corresponding groups (free DOX and free CDDP/DOX combination) because the nanogels internalized by the cells through the endocytic pathway.55

The IC_{50} values (half-maximal inhibitory concentration), calculated from dose responsive curve, are summarized in Table 2. The results showed that CDDP/DOX-NGs had much lower IC_{50} values than free DOX, DOX-NFs, CDDP-NFs, and free drugs DOX-CDDP combination, showing that CDDP/DOX-NFs was more potent in killing MCF7/ADR cells. For free DOX, the IC_{50} value was 48.42 μg/mL. When DOX was loaded into the carrier, the IC_{50} values decreased greatly, about half of free DOX. To analyze the synergistic effect and antagonistic or
additive effects of two drugs, the combination index (CI) is usually introduced. The CI could be calculated according to the formula $CI = C_1/C_{x1} + C_2/C_{x2}$, where $C_1$ is the concentration of drug 1 requires to cause a certain effect in combination therapy, $C_{x1}$ is the concentration of drug 1 that generates the identical effect alone, $C_2$ is the concentration of drug 2 needed to achieve the certain effect in combination therapy, and $C_{x2}$ was the concentration of drug 2 that reaches the same effect alone. When $CI > 1$, it implies antagonistic effect; when $CI = 1$, the two drugs have an additive effect; while $CI < 1$, synergistic effects were indicated. In this work, IC_{50} values of DOX-NGs, CDDP-NGs, and CDDP/DOX-NGs were used to calculate CI. According to Table 3, IC_{50} of DOX-NGs and CDDP-NGs in MCF7/ADR cells was 17.21 and 24.30 μg/mL, respectively. In their combination therapy, when the survival rate was 50%, the concentration of DOX and CDDP was both 8.50 μg/mL. Therefore, the CI of DOX and CDDP when delivered by nanogels was $8.50/17.21 + 8.50/24.30 = 0.84$, which indicated a synergistic effect.

To further confirm the anticancer activity of our drug-loaded nanogels, cell apoptosis analysis was performed. Our results showed that DOX-NGs and CDDP/DOX-NGs treated cells

Figure 5. Cellular internalization and subcellular localization of free DOX, DOX-NGs, and CDDP/DOX-NGs after incubation with MCF-7/ADR cells for 1 and 3 h: (A) CLSM images and (B) flow cytometric analysis.
had 49.96% and 82.98% of apoptosis, respectively, whereas only 17.94% were apoptotic after treatment with equivalent concentration of free DOX (Figure 6B). However, the empty nanogels showed almost no apoptosis, indicating their biocompatibility. These results strongly indicated the higher cytotoxic efficiency of CDDP/DOX-NGs than that of free DOX against MCF7/ADR cells.

3.8. In Vivo Biodistribution. The biodistribution of FA-PEG-PAANGs in major organs as well as in tumor tissue was assessed by intravital real-time fluorescent imaging. Cyanine 7 (Cy7)-labeled FA-PEG-PAANGs and PEG-PAANGs were administered intravenously to mice bearing MCF-7/ADR tumor. As shown in Figure 7A, at 2 h postinjection, fluorescence signal of Cy7 (ex: 720 nm; em: 790 nm) was found mainly in liver and tumor, as the result of RES recognition and EPR effects, respectively. Afterward, fluorescence signal of Cy7 dramatically increased in the tumor, while rapidly receded in other organs. At 24 h postinjection, only the tumor tissue showed very strong fluorescence signal, indicating that the persistent targeting accumulation, possibly aided by the positive cell targeting of FA and the stealth effect of PEG corona. If the nanogels were not modified with FA, they also accumulated in tumor for EPR effect (Figure 7A), but their accumulation in tumor was not as efficiently as FA-PEG-PAANGs at the first 4 h.

In order to study the biodistribution of drugs, fluorescent imaging of DOX in isolated organs (including heart, liver, spleen, lung, and kidney) and tumors were obtained on In Vivo Xtreme. The mice were treated with free DOX and CDDP/DOX-NGs via tail vein intravenous injection and sacrificed to provide isolated organs and solid tumors after selected time intervals. For free DOX, fluorescence signal of DOX (ex: 535 nm; em: 580 nm) was very strong in liver and kidney at first 4 h, but as time went on, almost all the DOX signal faded off and only little could be seen in tumor and kidney (at 8 h postinjection) as shown in Figure 7B. This result suggested that the free DOX were primarily delivered to liver and kidney as foreign bodies and had a quick clearance out of body. However, compared with free DOX, liver and kidney from CDDP/DOX-NGs group had very weaker fluorescence at first 4 h. Consistent with the results of intravital real-time fluorescent imaging, more DOX accumulated in tumor but less in other organs were observed for CDDP/DOX-NGs at 8 h postinjection.

3.9. In Vivo Antitumor Activity Evaluation. From the results above, the stimuli-sensitive biodegradable CDDP/DOX-
NGs had the advantages of prolonged blood circulation and favorable tumor accumulation. So they might possess superior antitumor ability but low side effects for normal organs. Therefore, in vivo antitumor efficacy was further evaluated on a MCF-7/ADR breast tumor mouse xenograft model.

As shown in Figure 8 and Figure S8, the mice treated with free chemotherapeutic agents (DOX/HCl, CDDP or their combination) displayed obvious body weight loss, suggesting severe systemic toxicity. But when the drugs were encapsulated in FA-PEG-PAANGs, either single loaded or dual loaded, no difference in body weight compared with the control group (PBS and FA-PEG-PAANGs) were found, indicating the carrier itself had no systemic toxicity, and it also could efficiently mitigate the systemic toxicity of free drugs. The group treated with nanocarrier (FA-PEG-PAANGs) alone, as well as the PBS control group, could not inhibit tumor growth (Figure 8C,D), indicating that the therapeutic effect could be observed only in the groups with chemotherapeutic agents. When DOX was administered as free drug, no inhibitory effect on MCF-7/ADR tumor was observed as we respected because of being efficiently pumped out by overexpressing P-gp. The combination therapy of free drugs (DOX and CDDP) improved the inhibitory effect of DOX. But when DOX or CDDP was delivered by FA-PEG-PAANGs, smaller tumor sizes could be found compared with the free drug groups (Figure 8C,D and Figure S8). This stronger inhibitory effect was most possibly related to the enhanced tumor accumulation of the FA-targeting nanosized carrier. Strongest tumor-inhibitory effects could found in the group of FA-PEG-PAANGs loaded both CDDP and DOX. The antitumor efficacy in vivo was consistent with the results of in vitro experiments. No side effects, such as infection and diarrhea, were observed in the nanogel groups, indicating that the nanogel system were well tolerated. Above all, FA-PEG-PAANGs could be used as a hopeful nanovector for chemotherapeutics and overcome drug resistance.

4. CONCLUSIONS

In summary, a new type of uniform PAA-based nanogels with multiple stimuli-responsive properties was prepared in a convenient way. DOX could be loaded into the nanogels by strong electrostatic and stacking interactions with high DLE (93.0%) and DLC (48.9%), while CDDP could efficiently conjugate with COOH by chelation interactions (DLE 78.0% and DLC 35.2%). Both drugs displayed a dual stimuli-triggered release behavior with high release content (91.6% and 82.5%, respectively, in 24 h). Synergistic CDDP/doxorubicin combination chemotherapy could achieve by loading both drugs into the nanogels, and the respective content of both drugs could be regulated. DOX and CDDP were efficiently delivered into MCF-7/ADR cells and showed potent antitumor activity in vitro. Furthermore, significant inhibition of tumor growth and reduced drug-related multiorgan toxicity side effects were observed in vivo due to the advantages of prolonged blood circulation, favorable tumor accumulation, and triggered drug release. Not only hydrophilic cationic drugs but also hydrophobic drug could be efficiently loaded into this system (the
study for combination delivery of sorafenib and DOX is in progress). Therefore, this nanogel delivery system could be extended for a broad range of combination therapy and holds great promise as an effective tool to treat drug-resistant tumors.

**ASSOCIATED CONTENT**

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**REFERENCES**

Characterization method for all experiments; magnified TEM images of PAANGs-40B, PAANGs-40C, and PAANGs-60; calibration curve of folate; TEM and surface charge analysis of PAANGs-60 and FA-PEG-PAANGs; GPC results of the FA-PEG-PAANGs degradation; nuclease distribution of doxorubicin studied CLSM; study the role of FA; in vivo antitumor activity evaluation of free CDDP (PDF).

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**Notes**

The authors declare no competing financial interest.

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**RESULTS**


