Supramolecular Nanosystem Based on Pillararene-Capped CuS Nanoparticles for Targeted Chemo-Photothermal Therapy

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ABSTRACT: A smart supramolecular nanosystem integrating targeting, chemotherapy, and photothermal therapy was constructed based on carboxylatopillar[5]arene (CP[5]A)-functionalized CuS nanoparticles (CuS@CP NPs). CuS@CP NPs with good monodispersity and strong near-infrared absorption were synthesized in aqueous solution through a facile one-pot supramolecular capping method, followed by surface installation of a liver cancer-targeted galactose derivative through host–guest binding interaction. The resulting smart supramolecular nanosystem, namely, CuS@CPG, exhibited excellent photothermal ablation capability to HepG2 cells upon irradiation with laser at 808 nm. Chemotherapeutic drug, doxorubicin hydrochloride (DOX), was further loaded on CuS@CPG via electrostatic interactions between positively charged DOX and negatively charged CP[5]A to give CuS@CPG–DOX with a high drug-loading capacity up to 48.4%. The weakening of DOX–CP[5]A interactions in an acidic environment promoted the pH-responsive drug release from CuS@CPG–DOX. Significantly, this multifunctional supramolecular nanosystem showed a remarkably enhanced therapeutic effect through the combination of targeted chemotherapy and photothermal therapy upon in vitro cell study. Moreover, preliminary in vivo study demonstrated that CuS@CPG and CuS@CPG–DOX had good biocompatibility and excellent tumor inhibition effects upon near-infrared laser irradiation.

KEYWORDS: CuS nanoparticles, carboxylatopillar[5]arene, supramolecule, chemotherapy, photothermal therapy

INTRODUCTION

Photothermal therapy (PTT) based on optical absorbing agents that can convert near-infrared (NIR) light energy into hyperthermia to kill cancer cells has attracted tremendous attention in recent years.1 A number of nanomaterials have been developed as PTT agents, such as carbon nanomaterials,2 metal nanomaterials (e.g., Au nanorod, Pd nanosheet, Bi nanoparticles (NPs), etc.),3–6 metal-based chalcogenides (e.g., CuS, WS2, Bi2Se3, etc.),7–10 and heterojunction nanocomposites.11,12 Owing to the low cytotoxicity, high photothermal conversion efficiency, facile synthesis, and low cost, CuS NPs have been widely studied and used as PTT agents and contrast agents.13,14 So far, several kinds of CuS NPs were prepared using various stabilizers, such as citrate, bovine serum albumin, and melanin.15–18 We note that the synergistic combination therapy of PTT and chemotherapy has proven to be an efficient approach to enhance the therapeutic effect of cancer.19,20 To construct multifunctional nanosystems for synergistic therapy, CuS NPs should be ideally integrated with other functional components (e.g., folic acid, arginine–glycine–aspartic acid peptide, poly(ethylene glycol), etc.) as well as anticancer drugs.21–25 However, construction of these multifunctional nanosystems involves complex and tedious synthetic procedures. Therefore, developing a facile strategy to fabricate smart CuS nanosystems with multifunctionality is highly desirable.

As is well known, supramolecular macrocycles, such as cyclodextrins, calix[n]arenes, cucurbit[n]urils, and pillar[n]arenes, could form stable inclusion complexes with certain guest molecules via specific noncovalent interactions.8,6–30 The introduction of supramolecular macrocycles is an effective method to generate multifunctional inorganic NPs, expanding their applications in biomedicine.31–36 For instance, cucurbit[7]uril molecules were assembled on diaminohexane-terminated gold NPs to construct intracellular 1-adamantyl-
amine-triggered therapeutic systems; cyclodextrin-capped Au NPs were applied in the fabrication of targeted drug-delivery system. As a relatively new class of synthetic macrocycles in supramolecular chemistry, pillar-shaped pillar[n]arenes with hydrophobic cavity and two functional rims have received considerable attention in materials science and nanomedicine. They can not only serve as effective carriers of drugs and genes, but also be modified on the surfaces of gold NPs, silver NPs, CdTe quantum dots, and Fe3O4 NPs to realize multifunctionality. Among them, water-soluble carboxylatopillar[5]arene sodium salts (CP[5]A) possessing five carboxylate groups on each rim and good binding ability toward guest molecules show great potentials in the fabrication of water-soluble multifunctional nanosystems for biomedicine. Notably, CP[5]A-modified upconversion NPs (UCNPs) were prepared via the assembly of CP[5]A with ammonium bromides functionalized on UCNPs, forming a pH-responsive drug-delivery system.

Herein, we develop a facile strategy utilizing a supramolecular approach to construct multifunctional nanosystem based on CP[5]A-capped CuS NPs (Scheme 1). Hydrophilic CP[5]A-capped CuS NPs (CuS@CP) were for the first time prepared by employing CP[5]A rings as stabilizing ligands. The liver cancer-targeted galactose derivative was then assembled on CuS@CP via the host−guest interaction between pyridinium salt moiety of galactose derivative and CP[5]A cavity. Finally, anticancer drug doxorubicin hydrochloride (DOX) was loaded on the surface of CuS@CPG mainly through electrostatic interactions, resulting in a versatile nanosystem for targeted cancer chemotherapy and photothermal therapy. This study opens a new perspective on using facile supramolecular strategy to construct multifunctional nanosystem for cancer therapeutic applications.

Scheme 1. Schematic Diagram of the Preparation of CuS@CPG—DOX and Its Cellular Uptake and Drug-Release Process

EXPERIMENTAL SECTION

Materials. Na2S·9H2O and CuCl2·2H2O were purchased from Tianjin Fuchen Chemical Reagents Factory. 2,3,4,6-Tetra-O-acetyl-β-glucopyranosyl bromide, tetraethylene glycol, propargyl bromide, and NaN3 were obtained from Aladdin Company (Shanghai, China). Ion-exchange resin (Amberlite IR 120 H+) was purchased from Beijing InnoChem Science & Technology Co., Ltd. DOX was obtained from Adamas-beta. Calcein-AM and propidium iodide (PI) were purchased from BestBio, Shanghai. Tetrahydrofuran (THF), pyridine, and dimethylsulfoxide (DMSO) were purchased from Beijing Chemical Reagent Co., Ltd. Phosphate-buffered saline (PBS) was prepared according to the Chinese Pharmacopoeia (2010 Edition). CP[5]A was synthesized according to our previously reported procedure.

Characterization. Powder X-ray diffraction (PXRD) pattern was recorded on a Rigaku D/MAX2550 X-ray diffractometer. X-ray photoelectron spectra were measured on an ESCALAB 250 photoelectron spectrometer. The morphology of NPs was observed by transmission electron microscopy (TEM, Tecnai G2 S-Twin F20). The hydrodynamic diameter of nanomaterials was measured on a Zetasizer Nano ZS. NMR spectra were obtained using a Varian 300 MHz NMR spectrometer. Fourier transform infrared (FT-IR) spectra and UV−vis−NIR absorbance spectra were measured on IFS-66V/S and Shimadzu UV-1800 spectrophotometers. Infrared thermal images of dispersions were recorded by an infrared thermal imager (E40, FLIR). The fluorescence images were recorded on an inverted fluorescence microscope (Olympus IX51), and confocal laser scanning microscopy (CLSM) images were obtained on a confocal fluorescence microscope (LSM 710, Zeiss). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were measured using a microplate reader (Infiniti F200 Pro, TECAN).

Synthesis of CuS@CP NPs. Na2S·9H2O (24.0 mg, 0.1 mmol) was added into deionized water (100 mL) containing CuCl2·2H2O (16.9 mg, 0.1 mmol) and CP[5]A (28.0 mg, 0.02 mmol). After being stirred for 10 min at room temperature, the reaction mixture was heated at 90 °C for 15 min until a dark green solution was observed. Then, the CuS@CP NPs were purified by dialyzing with a cellulose ester membrane bag (molecular weight cutoff = 3500) for 3 days to remove the unreacted ions and CP[5]A. The obtained CuS@CP NPs were stored at 4 °C.
Photothermal Evaluation of CuS@CP NPs. An aqueous suspension of CuS@CP (1 mL) NPs at different concentrations was added into a quartz cuvette, which was illuminated with an 808 nm laser at an output power density of 2.0 W/cm² for 7 min. The increase in temperature was recorded every 10 s by a digital thermometer. The photothermal conversion efficiency (η) of CuS@CP aqueous solution was calculated by a method reported by Roper and co-workers. In the following equations, τi is the time constant of the sample system; T_max and T_surf are the equilibrium temperature and ambient temperature, respectively; h represents the heat transfer coefficient; S represents the surface area of the container; Q_in represents the heat dissipation from the light, which is absorbed by the solvent and quartz sample cell; I is the laser power density; and A_max is the absorbance of CuS@CP at 808 nm.

\[
t = -\tau_i \ln \theta = -\tau_i \ln \frac{T_{\text{surf}} - T}{T_{\text{max}} - T_{\text{surf}}}
\]

\[
\tau_i = \frac{m_C P_C}{h S}
\]

\[
\eta = \frac{h S (T_{\text{max}} - T_{\text{surf}}) - Q_{\text{in}}}{I (1 - 10^{-3\tau_i})}
\]

Synthesis of CuS@CPG–DOX and Drug Release. Galactose derivative (G) solution (28 μL, 25 mg/mL) was mixed with CuS@CP solution (20 mL, 174 μg/mL), and the mixed solution was stirred at room temperature for 12 h. CuS@CPG was then obtained via centrifugation. The as-prepared CuS@CPG was added into the aqueous solution of DOX (10 mL, 400 μg/mL). After being stirred for 24 h, CuS@CPG–DOX was centrifuged, washed five times with deionized water, and finally dried in a freeze dryer. The equation of drug-loading capacity is as follows:

\[
\text{loading capacity (\%) = } \frac{\text{mass of loaded drug}}{\text{mass of loaded nanoparticles}} \times 100\%
\]

UV–vis spectrophotometer was used to monitor the controlled release process of DOX. First, CuS@CPG–DOX (1.0 mg) was dispersed in PBS (1.5 mL, pH = 7.4, 5.0, and 3.5) at room temperature. The supernatant containing DOX released from the nanosystem was collected via centrifugation at predetermined time intervals. The precipitated CuS@CPG–DOX was then dispersed in fresh PBS (1.5 mL) again. The amount of DOX released from the nanosystem can be calculated by measuring the absorbance of the collected supernatant at a wavelength of 480 nm.

MTT Assay. In vitro cytotoxicity of CuS@CPG was evaluated by the standard MTT assay for both human embryonic kidney cell line (HEK293 cells) and hepatocellular carcinoma cells (HepG2 cells). The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing CuS@CPG–DOX (99 μg/mL) at 37 °C and 5% CO₂. After different time intervals (10 min, 30 min, 1 h, and 4 h), the cells were treated with 4,6-diamidino-2-phenylindole (DAPI) for 10 min to stain the nuclei, washed with PBS several times to remove excess materials and dyes, and fixed on a glass microscope slide with 4% paraformaldehyde. Finally, the blue fluorescence of DAPI and the red fluorescence of DOX were recorded on CLSM under excitation at 358 and 530 nm light, respectively.

Cellular Uptake of CuS@CPG–DOX. The effect of targeting molecule-galactose derivative (G) on cellular uptake of CuS@CPG–DOX was studied. HepG2 cells were seeded and incubated in culture dishes at a density of 1 × 10⁴ for 24 h at 37 °C and 5% CO₂. Then, the complete medium was replaced by a fresh medium containing DOX, CuS@CP–DOX, and CuS@CPG–DOX at the same DOX concentration (1.5 μg/mL), respectively. After incubation for 1 h, the cells were collected, washed three times with PBS, and stained with FITC for 30 min. Finally, the internalized fluorescence of DOX was detected by flow cytometry analysis.

In Vivo Chemo-Photothermal Therapy toward HepG2 Cells. HepG2 cells were seeded and incubated in 96-well plates at a density of 5 × 10⁵/well for 24 h. When the cells are attached to the plates completely, they were incubated in a fresh medium with different concentrations of CuS@CPG, CuS@CP–DOX, and DOX, respectively. After being incubated for 4 h, some cells treated with CuS@CPG and CuS@CPG–DOX were irradiated by an 808 nm laser (2 W/cm²) for 3 min. For comparison, others treated with DOX and CuS@CPG–DOX were without irradiation. And all of the cells were further incubated at 37 °C and 5% CO₂ conditions for another 20 h. Finally, the relative cell viabilities were evaluated by the standard MTT assay.

In Vivo Antitumor Study. In vivo antitumor efficacies of CuS@ CP and CuS@CPG–DOX were studied by HepG2 tumor-bearing nude mice. Each 6 week old male nude mouse was subcutaneously injected with 1.5 × 10⁶ HepG2 cells. When the average volume of the tumor reached about 163 mm³, the mice were divided into eight groups randomly according to the disposal condition (six mice per group): naive, saline + NIR, CuS@CP, CuS@CP + NIR, CuS@CPG–DOX, CuS@CPG–DOX + NIR, and DOX. Except naive group, saline, CuS@CP (1.07 mg/kg), CuS@CPG + NIR (1.07 mg/kg), CuS@CPG–DOX (2.07 mg/kg), CuS@CPG–DOX + NIR (2.07 mg/kg), and free DOX (1.00 mg/kg) were injected intravenously into the tumor-bearing mice on days of 0, 3, 6, and 12. After 4 h of injection, the tumor regions of saline + NIR, CuS@CPG + NIR, and CuS@CPG–DOX + NIR groups were irradiated for 5 min with an 808 nm laser at an output power density of 2.0 W/cm². Tumor size and body weight of mice were measured and recorded every 2 days. On the 22nd day after the first administration, the mice were sacrificed and their major tissues, including heart, liver, spleen, lung, and kidney, were taken for histological assay. The tissue section (5 μm thick) stained with hematoxylin–eosin (H&E) was observed under an ECLIPSE Ti-S microscope (Nikon, Tokyo, Japan). All animals used in the procedures were handled in strict accordance with the Guide for the Care and Use of Laboratory Animals, and the procedures were approved by the University Committee in the Use of Animals of Jilin University of China.

## RESULTS AND DISCUSSION

Preparation and Characterization of CuS@CP NPs. CuS@CP NPs were synthesized in water by reacting CuCl₂ with Na₂S in the presence of CP [5]. It is worth noting that water-soluble CP [5] A macrocycle possesses five carboxyl groups on each rim, so it can serve as an excellent stabilizer in the preparation of CuS NPs via the formation of carboxyl–Cu coordination bonds. Moreover, as a functional supramolecular macrocycle, CP [5] A on CuS NPs were integrated with galactose derivative (G) through the host–guest interaction to realize liver cancer targeting. The CuS@CPG was further loaded with anticancer drug DOX via electrostatic interactions, generating a
CuS@CPG−DOX nanosystem combining targeting, chemotherapy, and photothermal therapy multifunctionalities.

The characteristic peaks in the PXRD pattern suggest the hexagonal structure of the as-prepared CuS@CP NPs, as referenced by standard Cu$_9$S$_8$ phase (JCPDS Card No. 36-0379) (Figure S1). The X-ray photoelectron spectrum shows two typical peaks at 932.9 and 952.6 eV with regard to Cu 2p$_{3/2}$ and Cu 2p$_{1/2}$, respectively, which are assigned to Cu(I), and the peaks at 935.0 and 955.2 eV to Cu(II) (Figure S2). TEM image indicates that CuS@CP displays good monodispersibility in aqueous solution and has a regular morphology with an average diameter of ca. 6 nm, as calculated by statistics analysis of particle size distribution (Figure 1a,b). The hydrodynamic diameter of CuS@CP determined by dynamic light scattering is 10 nm (Figure S3), which is slightly higher than its average diameter, due to the existence of hydration of CP[S]A on CuS NPs. The presence of CP[S]A on the CuS NPs is verified by the characteristic absorption peaks at 1198, 1496, and 1587 cm$^{-1}$ in the FT-IR spectrum of CuS@CP (Figure S4), which can be assigned to the vibrations of C−O−C, Ar−H of benzene, and C=O from CP[S]A, respectively. The UV−vis−NIR absorption spectra of CuS@CP NPs solutions (Figure 1c) show a strong absorbance in the NIR region (λ = 700−1100 nm), which is attributed to the localized surface plasmon resonance of CuS@CP with vacancy. And the intensity of the absorption peak at 1000 nm is enhanced with increase in the concentration of CuS@CP NPs. Importantly, the strong absorption of CuS@CP NPs in the NIR region promises the potential application in NIR laser-induced PTT. To further investigate the photothermal properties, CuS@CP NPs solutions of different concentrations were exposed to an 808 nm laser (2 W/cm$^2$) for 7 min and pure water was used as the control. As shown in Figure 1d, the temperature of CuS@CP solutions increases markedly with increase in the concentration and irradiation time. After irradiation for 7 min, the temperature elevation of CuS@CP solution in the concentration range of...
14.5–174 μg/mL is about 10–44 °C. It is significant for photothermal ablation of cancer cells that hyperthermia (>45 °C) can be easily reached toward body tissues via irradiating CuS@CP NPs even at low concentration (14.5 μg/mL). In sharp contrast, the temperature elevation of water is only 3 °C. The photothermal conversion efficiency (η) of CuS@CP aqueous solution was calculated by a method reported by Roper and co-workers,\textsuperscript{51} where \( \tau_p \) is the time constant for heat transfer that can be determined by the plot of cooling time versus negative natural logarithm of driving force temperature (313 s) (Figure 1e,f). According to the obtained data and equations, the photothermal conversion efficiency of CuS@CP is calculated to be 34%. We further studied the photothermal stability of CuS@CP via monitoring the temperature change of the CuS@CP solution upon on/off NIR laser irradiation for five repeated cycles. As shown in Figure S5, no notable difference for temperature elevation upon NIR laser irradiation is observed after five repeated cycles, demonstrating the high photothermal stability of CuS@CP NPs.

**Biocompatibility and Photothermal Ablation Capability of CuS@CPG.** Liver cancer-targeted galactose derivative (G) with pyridine moiety was prepared according to the synthetic route shown in Scheme S1, where intermediate products and final product G were determined by NMR spectra (Figures S6–S11).\textsuperscript{55–58} There is strong host–guest complexation between the pyridine moiety of G molecule and CP[S]A ring, and their association constant (\( K_a \)) was determined to be \( (1.07 \pm 0.24) \times 10^4 \text{ M}^{-1} \) through NMR titration experiments and the nonlinear curve-fitting method (Figures S12 and S13). The G molecules were successfully assembled on CuS@CP NPs through forming a stable inclusion complex with CP[S]A capped on Cu NPs,\textsuperscript{59} resulting in a liver cancer-targeted nanosystem, that is, CuS@CPG. The biocompatibility of the CuS@CPG nanosystem was evaluated by the standard MTT assay for both HEK293 cells and HepG2 cells (Figure S14). After incubation with CuS@CPG for 24 h at concentrations from 8 to 68 μg/mL, the HEK293 cells showed a relatively higher cell viability (above 86%). And the viability of the HepG2 cells incubated with CuS@CPG at a concentration of 68 μg/mL was up to 91%. The results demonstrate that CuS@CPG has negligible cytotoxicity toward normal cells and cancer cells at low concentrations.

Upon irradiation with an NIR laser at 808 nm (2 W/cm²), the temperature elevation of cell culture containing CuS@CP and CuS@CPG at the same concentration (51 μg/mL) was recorded by real-time thermal images (Figure 2a). After irradiation for only 7 min, the temperature of both systems increased rapidly and approached 60 °C, affording their great potential for photothermal ablation. To further investigate photothermal ablation capabilities of CuS@CP and CuS@CPG nanosystems to HepG2 cells, calcein-AM (mark living cells with green emission) and PI (mark died cells with red emission) were used to differentiate live and dead cells at different conditions. As shown in Figure 2b, almost all of the HepG2 cells after 7 min irradiation with an 808 nm laser are alive, indicating that 808 nm light would not be absorbed by the pure culture and is noninvasive to cells. The activities of HepG2 cells are also not affected in the presence of CuS@CP and CuS@CPG. Under the 808 laser irradiation for 7 min, the majority of HepG2 cells treated with CuS@CP NPs and CuS@CPG NPs suffered from the ablation and died. Notably, the photothermal ablation capability of CuS@CPG is stronger than that of CuS@CP, which is attributed to the targeting effect of G molecules.

**DOX Loading and Release Behaviors of CuS@CPG–DOX.** Except for binding targeted guest molecule G, CP[S]A macrocycles on the surface of CuS NPs can further integrate anticancer drug, DOX, via intermolecular electrostatic interactions, generating a drug-loaded multifunctional nanosystem, that is, CuS@CPG–DOX. The dispersibility of CuS@CPG–DOX NPs in water was characterized by TEM, which showed certain dispersibility in water without serious aggregation (Figure S15). As shown in Figure 3a, CuS@CPG–DOX still exhibits an obvious absorption in the NIR region derived from CuS NPs. In addition, a characteristic absorption peak of DOX at about 506 nm appears in the UV–vis–NIR spectrum of CuS@CPG–DOX, demonstrating that DOX is efficiently loaded in the CuS@CPG nanosystem. The stability of the CuS@CPG–DOX system was studied by dispersing CuS@CPG–DOX NPs in PBS (pH 7.4) and simulated body fluids (Hank’s balanced salt solution) for 24 h, respectively. After centrifugation, the supernatant was measured by a UV–vis spectrophotometer. As seen in Figure S16, no DOX was detected in the supernatant; this implied that DOX molecules were firmly integrated on the surface of the nanoparticles. The loading capacity of DOX in the CuS@CPG–DOX nanosystem was about 48.4%, as calculated by the drug loading experiment (Figure S17).

To further study the pH-responsive drug-release performance, CuS@CPG–DOX was dispersed in PBS at different pHs (7.4, 5.0, and 3.5). Notably, as shown in Figure 3b, drug release from CuS@CPG–DOX is severely limited in physiological environment (pH 7.4). Upon lowering the pH of PBS from 7.4 to 3.5, the amount of released DOX significantly increases. And the total released DOX from CuS@CPG–DOX reaches 38.2 and 44.8% at pHs 5.0 and 3.5, respectively. The acid-responsive drug release of CuS@CPG–DOX is mainly caused by the weakening of host–guest interactions due to the conversion of...

Intracellular Drug-Delivery Performance of CuS@CPG−DOX. As is known, DOX can block the replication and transcription of DNA to induce cell apoptosis, so only those DOX located in the cell nucleus could effectively play the therapeutic effect. We further investigated the intracellular drug-delivery process of CuS@CPG−DOX and the distribution of DOX by CLSM. Blue fluorescent DAPI was used to mark the cell nucleus. After incubation for only 10 min, the red fluorescence of DOX began to appear in the cytoplasm of HepG2 cells (Figure 4). Upon incubation for 30 min, DOX signals overlapped with the blue signals of DAPI were observed, indicating that DOX released from CuS@CP−DOX can quickly accumulate in cell nucleus. And the intensity of red fluorescence in cell nucleus increased with increase in incubation time.

In Vitro Cellular Uptake of CuS@CPG−DOX. The targeting effect of CuS@CPG−DOX was further evaluated by flow cytometry experiments. As shown in Figure 5a,b, the mean fluorescence intensity (MFI) of DOX in HepG2 cells incubated with CuS@CP−DOX for 1 h is lower than that incubated with free DOX, indicating that less amount of CuS@CP−DOX was ingested by cells. Notably, CuS@CPG−DOX showed the largest cellular uptake, which is attributed to the existence of galactose-mediated endocytosis between CuS@CPG−DOX and asialoglycoprotein receptor overexpressed on HepG2 cells. We have further verified the target ability of the system by preincubating the HepG2 cells with lactobionic acid as a control group along with CuS@CP−DOX in CLSM analysis. As shown in Figure 6, stronger red fluorescence of DOX is observed in HepG2 cells incubated with CuS@CP−DOX than that with CuS@CP−DOX, which confirmed that the G molecules could effectively promote the endocytosis of cells toward CuS@CP−DOX NPs. After the lactobionic acid preincubation, the red fluorescence signals of CuS@CPG−DOX NPs in cells became weaker because lactobionic acid could block the asialoglycoprotein receptors and further inhibit the galactose-mediated endocytosis. Moreover, we compared the viabilities of HEK293 cells and HepG2 cells, which were incubated with CuS@CPG−DOX. As seen in Figure S18, after incubation with CuS@CPG−DOX in the concentration range of 2−32 μg/mL, the HepG2 cells show lower viability than the HEK293 cells. The results confirm that the CuS@CPG−DOX system can selectively kill HepG2 cells due to the target ability of G molecules integrated on the nanoparticles.

Synergistic Therapeutic Effects of CuS@CPG−DOX. The combined chemo- and photothermal effect in vitro from the CuS@CPG−DOX nanosystem was further investigated by the standard MTT assay. Compared to the high viability of HepG2 cells incubated with CuS@CP NPs without NIR laser irradiation, the cell viability gradually decreased with increasing concentrations of CuS@CP NPs upon NIR laser irradiation, due to the generated effective photothermal ablation (Figure 7). Furthermore, CuS@CPG−DOX nanosystem showed excellent chemotherapy effect, owing to the DOX released in the acidic conditions of HepG2 cells. Because of the limited drug release,
the cell-killing capacity of CuS@CPG−DOX was weaker than that of free DOX at high concentrations (above 32 μg/mL). However, the synergy effect of CuS@CPG−DOX + NIR combining photothermal therapy and chemotherapy affords the nanosystem comparable therapeutic effect with free DOX.

In Vivo Antitumor Assessment. The positive in vitro results inspired us to further study the antitumor efficacy of CuS@CPG and CuS@CPG−DOX NPs in the HepG2 tumorbearing nude mice. As shown in Figure 8a, the tumor size increased rapidly in the saline, saline + NIR, and CuS@CPG groups. For both groups injected with CuS@CPG−DOX and DOX, the growth of tumor had slight inhibition, indicating that the chemotherapy effects of CuS@CPG−DOX and DOX in vivo were not obvious at the usage dose. Notably, upon treatment with CuS@CPG under irradiation with an 808 nm laser, the tumor size was effectively suppressed; this demonstrated a good in vivo photothermal therapy effect of CuS@CPG. The CuS@CPG−DOX + NIR group also showed an excellent inhibition effect of tumor growth as with CuS@CPG + NIR groups after 22 day treatment. Significantly, the therapeutic efficacy of CuS@CPG−DOX + NIR was long-lasting, attributed to its combined therapy effect.

To evaluate the potential long-term toxicity of injection materials, body weight of nude mice was measured during the treatment period (Figure 8b). No notable body weight change was observed for different treatment groups with DOX, CuS@CPG, and CuS@CPG−DOX, this indicated that there was negligible toxicity of these materials in vivo. Moreover, histology analysis of the heart, liver, spleen, lung, and kidney stained with H&E were used to assess the in vivo toxicity of these materials on the major organs. No evident inflammatory lesion or tissue damage is observed in Figure 9, confirming the good biocompatibility of CuS@CPG and CuS@CPG−DOX in vivo with or without 808 nm laser irradiation.
CONCLUSIONS

In summary, a facile supramolecular assembly strategy was developed to construct multifunctional CuS@CPG−DOX nanosystem for targeted chemo-photothermal therapy. Versatile CuS NPs were successfully synthesized by employing supramolecular macrocycle, CP[5]A, as stabilizer and exhibited good water dispersibility and high photothermal conversion efficiency. Specific targeting to liver cancer cells was realized by surface functionalization of CuS@CP with galactose derivative (G) via host–guest interaction, and the as-prepared CuS@CPG nanosystem showed better photothermal ablation capability toward HepG2 cells compared to CuS@CP. Moreover, anticancer drug DOX was further loaded onto CuS@CPG via the intermolecular electrostatic interaction between CP[5]A and DOX, which exhibited excellent drug release in response to pH changes. Significantly, under irradiation with laser at 808 nm, CuS@CPG−DOX displayed a good synergistic in vitro therapeutic effect with combined targeted chemotherapy and photothermal therapy. Moreover, preliminary in vivo study showed efficient tumor inhibition capability of CuS@CPG and

Figure 8. (a) Tumor growth curves of tumor-bearing mice after treatment with saline, saline + NIR, CuS@CPG, CuS@CPG + NIR, DOX, CuS@CPG−DOX, and CuS@CPG−DOX + NIR. (b) Weight change curves of mice during the treatment period.

Figure 9. H&E images of the major organs of tumor-bearing mice after treatment with saline, saline + NIR, CuS@CPG, CuS@CPG + NIR, CuS@CPG−DOX, CuS@CPG−DOX + NIR, and DOX. The scale bar is 0.4 mm.
CuS@CPG–DOX under NIR irradiation as well as their good biocompatibility. This work clearly reveals that the marriage of supramolecular macrocycles with CuS nanoparticles is a simple yet powerful strategy to construct smart multifunctional system for biomedical applications.

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