Smart Self-assembled Nanosystem Based on Water-soluble Pillararene and Rare Earth Doped-Upconversion Nanoparticles for pH-Responsive Drug Delivery

Haihong Li, Ruoyan Wei, Guihua Yan, Ji Sun, Chunju Li, Haifang Wang, Liyi Shi, John A. Capobianco, and Lining Sun

ACS Appl. Mater. Interfaces, Just Accepted Manuscript • DOI: 10.1021/acsami.7b14193 • Publication Date (Web): 16 Jan 2018

Downloaded from http://pubs.acs.org on January 16, 2018

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.
Smart Self-assembled Nanosystem Based on Water-soluble Pillararene and Rare Earth Doped-Upconversion Nanoparticles for pH-Responsive Drug Delivery

Haihong Li,† Ruoyan Wei,‡ Gui-Hua Yan,§ Ji Sun,† Chunju Li,† Haifang Wang,*§ Liyi Shi,†‡ John A. Capobianco,⊥ and Lining Sun*,†‡

† Department of Chemistry, Shanghai University, Shanghai 200444, P. R. China.
‡ Research Center of Nano Science and Technology, and School of Material Science and Engineering, Shanghai University, Shanghai 200444, P. R. China.
§ Institute of Nanochemistry and Nanobiology, Shanghai University, Shanghai 200444, P. R. China.
⊥ Department of Chemistry and Biochemistry and Center for Nanoscience Research, Concordia University, 7141 Sherbrooke St. West, Montreal, QC H4B 1R6, Canada.
*E-mail: lnsun@shu.edu.cn (L. Sun); hwang@shu.edu.cn (H. Wang)

ABSTRACT

Exploring novel drug delivery systems (DDSs) with good stability and new structure to integrate pillararene and upconversion nanoparticles (UCNPs) into one system continues to be an important challenge. Herein we report the novel preparation of a supramolecular upconversion nanosystem via the host-guest complexation based on carboxylate-based pillar[5]arene (WP5) and 15-carboxy-\(N,N,N\)-trialkylpentadecan-1-ammonium bromides (1) functionalized UCNPs to produce WP5\(\supseteq\)1-UCNPs that can be loaded with the chemotherapeutic drug doxorubicin (DOX). Importantly, the WP5 on the surface of the drug loaded nanosystem can be efficiently protonated under acidic conditions, resulting in the collapse of the nanosystem and the drug release. Moreover, cellular uptake confirms that the nanosystem can enter human cervical cancer (HeLa) cells, resulting in drug
accumulation in the cells. More importantly, cytotoxicity experiments demonstrated the excellent biocompatibility of WP5⊃1-UCNPs without loading DOX and that the nanosystem DOX-WP5⊃1-UCNPs exhibited an ability of killing HeLa cells effectively. We also investigated magnetic resonance imaging and upconversion luminescence imaging, which may be employed as visual imaging agents in cancer diagnosis and treatment. Thus, in the present work we show a simple yet powerful strategy to combine UCNPs and pillar[5]arene to produce a unified nanosystem for dual-mode bioimaging guided therapeutic applications.

KEYWORDS: rare earth doped upconversion nanoparticles, pillararene, host-guest complexation, upconversion luminescence imaging/magnetic resonance imaging, drug release

1. INTRODUCTION

Over the past few years, a myriad of nanomaterials based on bioimaging and therapeutic applications have been constructed for the diagnosis and treatment of various kinds of cancer.\textsuperscript{1-5} Among these therapies, chemotherapy is one of the most common treatments for many cancers.\textsuperscript{6-9} Among many activating signals, pH triggers belong to the most extensively investigated stimuli based on different pH values. Moreover, intracellular pH distribution is heterogeneous in different subcellular compartments. For instance, lysosomes and endosomes are acidic, ranging from pH of 4.5 to 6.6, and the cytoplasm have a neutral pH of 7.2-7.4.\textsuperscript{10,11} Currently, advanced supramolecular self-assembled nanosystems with well-designed structures for chemotherapy have attracted considerable attention and are in demand due to the potential applications in drug delivery systems (DDSs).\textsuperscript{12,13} Various of DDSs based on supramolecular self-assembled nanosystems including micelles,\textsuperscript{14} vesicles,\textsuperscript{15} and nanocapsules,\textsuperscript{16} have been explicitly designed to have high drug loading capacity. Pillar[n]arenes,\textsuperscript{17-19} are macrocyclic host compounds synthesized from the phenolic monomer, 1,4-dialkoxybenzene. The 1,4-dialkoxybenzene units that are linked by methylene bridges at the 2,5 position (para-positions) contribute to the highly...
symmetrical pillar shaped architecture. Pillar[n]arene are macrocyclic hosts with interesting host-guest properties not only to supramolecular chemists but also to others working in a variety of fields in chemistry and materials science.\textsuperscript{20,21} Recently, research on pillar[n]arene-based supramolecular has attracted huge attention in the fabrication of nanosystems for DDSs,\textsuperscript{22-24} due to the unique rigid pillar architecture, facile chemical modification, and high drug loading capacity. It also reduces the side effects of drugs to normal tissue, since it preferentially delivers anti-cancer drugs to tumor tissues \textit{via} the enhanced permeability and retention (EPR) effects.\textsuperscript{25} Among them, carboxylate-based pillar[5]arene (WP5) has been frequently used as host molecules in host-guest chemistry studies due to their sensitive pH-responsive properties and good water solubility.\textsuperscript{24} Especially, WP5 can be protonated to the corresponding carboxylic acid derivative WP5H in acidic conditions.\textsuperscript{12} Based on this feature, WP5-based nanosystems have been extensively applied in the fabrication of acid-responsive DDSs.

Recently, upconversion nanoparticles (UCNPs) have been explored and applied in the field of biology and medicine.\textsuperscript{27-31} UCNPs are able to emit UV, visible, and near-infrared (NIR) luminescence upon excitation using NIR irradiation, which is located in the first biological window. UCNPs show a number of advantages such as narrow emission bands, low cytotoxicity, low autofluorescence, absence of photodamage, and high penetration depth of excitation light at the relatively low power density.\textsuperscript{31} Furthermore, Gd-doped UCNPs can serve as effective magnetic resonance imaging (MRI) contrast agent since the Gd element has seven unpaired 4f electrons.\textsuperscript{32-34} Thus, the integration of multimodal UCNPs and water-soluble WP5 based on host-guest chemistry provides a supramolecular upconversion nanosystem with outstanding drug delivery property and excellent imaging capability. Therefore, it is highly desirable to construct a novel supramolecular upconversion nanosystem utilizing smart host-guest chemistry.

In this study, we develop the unprecedented synthesis of pillar[5]arene-based acid-responsive supramolecular upconversion nanosystems (WP5⊃1-UCNPs) for anticancer drug delivery, upconversion luminescence (UCL) imaging and MRI. The
nansystem was developed using pillar[5]arene (water-soluble and carboxylate-based WP5) and 15-carboxy-N,N,N-trialkylpentadecan-1-ammonium bromides (1) modified UCNPs (1-UCNPs), as schematically depicted in Scheme 1. Specifically, the NaYF₄:Yb/Er was synthesized through the modified solvothermal method. To enhance the intensity of upconversion luminescence and provide T₁-magnetic resonance (MR) imaging ability, a very thin NaGdF₄ shell was coated on the surface of nanoparticles NaYF₄:Yb/Er to obtain the core–shell NaYF₄:Yb/Er@NaGdF₄ (OA-UCNPs) nanoparticles. Subsequently, the hydrophilic WP5 was assembled with 15-carboxy-N,N,N-trialkylpentadecan-1-ammonium Bromide (compound 1) modified UCNPs based on host-guest complexation between the ammonium bromide moiety of 1-UCNPs with the hydrophobic cavity of WP5, resulting in the WP5⊃1-UCNPs nanosystem. The obtained WP5⊃1-UCNPs supramolecular nanosystem show good water-stability and biocompatibility. After loading with anticancer drugs doxorubicin (DOX-WP5⊃1-UCNPs), DOX is efficiently released in an acidic environment, due to the acid-sensitive property of WP5. Cytotoxicity experiments demonstrate that the DOX-WP5⊃1-UCNPs nanosystem exhibit toxicity for HeLa cells. In addition, celluar uptake experiments suggest that the DOX-WP5⊃1-UCNPs nanosystem can enter HeLa cells with significant drug accumulation in HeLa cells. Furthermore, the supramolecular upconversion nanosystem was successfully applied for *in vitro* UCL and MR imaging.

2. EXPERIMENTAL SECTION

2.1. Materials. Rare-earth chlorides RECl₃·6H₂O (99.99%) (RE = Y, Yb, Er, Gd), 1-octadecane (ODE, 90%) and oleic acid (OA, 90%) were bought from Sigma Aldrich Co., Ltd. Methanol (CH₃OH, 99.5%), ammonium fluoride (NH₄F, 98%), sodium hydroxide (NaOH, 96%), trimethylamine (TMA, 33 wt% in ethanol), and cyclohexane were obtained from Aladdin Company. Acetonitrile (99.9%) and hydrobromic acid (47%-49%) were obtained from Alfa Aesar Ltd. Hexadecan-16-olide (97.0%) was obtained from TCI Co., Ltd. Doxorubicin hydrochloride (DOX·HCl) was obtained from Sangon Biotech.
dichloromethane ($\text{CH}_2\text{Cl}_2$) and $\text{H}_2\text{SO}_4$ were from Sinopharm Co. Ltd. Ultrapure deionized water (Millipore system) was used for all the experiments.

2.2. Preparation of 16-bromohexadecanoic Acid (1*). The synthesis of 16-bromohexadecanoic acid is shown in Supporting Information (see Scheme S1). Hexadecan-16-olide (2.5 g, 9.83 mmol) was introduced to concentrated sulphuric acid (1.50 mL) and HBr (7.50 mL) in a 25 mL flask, and heated under reflux for 72 h. The final reaction residue was added to a flask containing 50 mL of hot CH$_2$Cl$_2$ and then filtered while washing with 25 mL of saturated NaCl solution and 25 mL of deionized water and finally dried over MgSO$_4$. The white solid was obtained as a crude product and dried under vacuum. The crude product was then recrystallized from cold acetonitrile and filtered. Compound 1* was obtained (1*, 3.15 g, 95%). $^1$H NMR (DMSO-d$_6$, 500 MHz, 293 K) $\delta$ (ppm): 11.92 (s, 1H), 3.52 (t, $J$ = 6.7 Hz, 2H), 2.18 (t, $J$ = 7.4 Hz, 2H), 1.80 (m, 2H), 1.49 (m, 2H), 1.24–1.37 (m + s at 1.24 ppm, 22 H) (Figure S1).

2.3. Synthesis of 15-carboxy-N,N,N-trialkylpentadecan-1-ammonium Bromide (1). The compound 1 was synthesized using the mixed reagents of 16-bromohexadecanoic acid (3.0 g, 0.15 mmol) and 10 M equivalents of trimethylamine (Scheme S1), which were heated at 80 °C in a 100 mL teflon-lined stainless-steel autoclave for 72 h. The reaction residue was filtered to give a white solid, which was recrystallized from cold ethanol to obtain the product. (1, 1.25 g, yield 46%). $^1$H NMR (DMSO-d$_6$, 500 MHz, 293 K) $\delta$ (ppm): 11.97 (s, 1H), 3.28 (t, $J$ = 7.1 Hz, 2H), 3.09 (d, $J$ = 35.3 Hz, 9H), 2.21 (t, $J$ = 6.9 Hz), 1.66 (m, 2H), 1.48 (m, 2H), 1.06–1.24 (m+s at 1.24 ppm, 22 H) (Figure S2).

2.4. Synthesis of Ammonium Salt-based Pillar[5]arene (WP5). WP5 was prepared based on the reported synthesis,$^{35,36}$ shown in Scheme S2. The crude product was recrystallized from cold ethanol and deionized water and then dried in vacuum to obtain the product (WP5, 202 mg, yield 88%). $^1$H NMR (500 MHz, D$_2$O, 293 K) $\delta$ (ppm): 6.70 (s, 10H), 4.22 (s, 20H), 3.82 (s, 10H) (Figure S3).

2.5. Synthesis of Oleate-capped NaYF$_4$:Yb/Er@NaGdF$_4$ Core-shell Nanoparticles (OA-UCNPs). NaYF$_4$:Yb/Er@NaGdF$_4$ core-shell nanoparticles were
obtained according to our previously reported method.\textsuperscript{37} The NaYF\textsubscript{4}:Yb/Er core nanoparticles were dispersed in cyclohexane (20 mL, NaYF\textsubscript{4}:Yb/Er, 20 mg·mL\textsuperscript{-1}). The resulting product NaYF\textsubscript{4}:Yb/Er@NaGdF\textsubscript{4} (OA-UCNPs) was finally dispersed in cyclohexane (10 mL, NaYF\textsubscript{4}:Yb/Er@NaGdF\textsubscript{4}, 18 mg·mL\textsuperscript{-1}).

2.6. Functionalization of Oleate-capped-UCNPs with compound 1 (1-UCNPs). The equal volume of water was injected to the cyclohexane dispersion of OA-UCNPs (5 mL, 15 mg·mL\textsuperscript{-1}) in a 50 ml flask and stirred vigorously for 6 hours. The pH range of 2-4 was maintained in the reaction mixture by the addition of a solution of HCl (0.1 M). The solution was extracted with water three times to remove the OA. The OA free UCNPs were centrifuged in the aqueous phase and recovered as a solid pellet. Finally, 2 mL of a solution of compound 1 (20 wt%) was dispersed in water (10 mL), and the ligand free UCNPs were injected into a flask containing compound 1 and then stirred for 24 h at 25 °C. After centrifugation, the product was obtained and dispersed in deionized water (5 mL, 1-UCNPs, 10 mg·mL\textsuperscript{-1}).

2.7. Self-assembly of Supramolecular Upconversion Nanosystem (WP5⊃1-UCNPs). Specifically, 1-UCNPs (10 mg·mL\textsuperscript{-1}, 5 mL) dispersed in deionized water was injected into a 100 mL flask at room temperature, followed by the injection of a solution of WP5 (5.0 mL, 2.50 mM). The product was centrifuged and washed several times with water and finally dispersed in deionized water (WP5⊃1-UCNPs, 5 mL, 8 mg·mL\textsuperscript{-1}).

2.8. Loading of Doxorubicin (DOX) into WP5⊃1-UCNPs (DOX-WP5⊃1-UCNPs). DOX·HCl was desalted with excess trimethylamine (TMA) to generate DOX. Specifically, DOX·HCl (5 mg, 0.0086 mM) and trimethylamine (TMA, 10 µL, 33 wt% in ethanol) were injected into a flask containing 3 mL of chloroform and stirred under dark at 25 °C for 12 hours. Subsequently, the mixture was evaporated to dry the solvent on a rotary evaporator to obtain the desalted DOX (95% yield, TMA·HCl as a by-product can be removed by dialysis in the subsequent drug release). Loading of WP5⊃1-UCNPs with DOX was carried out by mixing 2 mL of WP5⊃1-UCNPs (2 mg·mL\textsuperscript{-1}) with 2 mL of DOX (1 mg·mL\textsuperscript{-1}) in 6 mL deionized water and stirring for 24 h in the dark at 25 °C. The concentrations of DOX-loaded...
WP5⊃1-UCNPs and unloaded DOX in the final solution were calculated to be 0.41 mg·mL⁻¹ and 0.19 mg·mL⁻¹, respectively. DOX-loaded WP5⊃1-UCNPs were purified in deionized water using a dialysis tube with a molecular cutoff of 10 000 Da until negligible fluorescent of DOX was detected in the dialysate. The precipitate was washed three times with water. The as-obtained precipitate was redispersed in deionized water to form a transparent solution (DOX-WP5⊃1-UCNPs, 2 mL, 2 mg·mL⁻¹) through sonication. The amount of DOX that was not loaded and found in the dialysate was quantitatively measured by monitoring the absorbance at 480 nm.

The loading efficiency of DOX was calculated using the following formula:

\[
\text{loading efficiency (\%) = } (\frac{m_{\text{DOX-loaded}}}{m_{\text{NPs}}}) \times 100
\]  

(1)

where; \(m_{\text{DOX-loaded}}\) is the mass of DOX loaded in WP5⊃1-UCNPs, \(m_{\text{NPs}}\) is mass of DOX- WP5⊃1-UCNPs.

2.9. Release of DOX from WP5⊃1-UCNPs. The release of DOX was carried out using the following procedure. DOX-WP5⊃1-UCNPs were added to a dialysis tube with a molecular cutoff of 8 000 Da and then dipped into 5 mL of phosphate buffer solution (0.1 M, pH = 4.0, 5.0, 6.0, 6.5 and 7.4, respectively) with shaking at 30 °C. The dialysate containing the released DOX was withdrawn at a preset time interval and collected, followed by the addition of an equal amount of fresh phosphate buffer solution. Additionally, the amount of DOX released from the nanosystem in the dialysate was measured by monitoring the absorbance of DOX at 480 nm.

2.10. Characterization. X-ray diffraction (XRD) patterns were carried out by using a 3 kW D/MAX2200 PC diffractometer equipped with a CuKα source at a generator power of 60 kV and 80 mA. The scan range was set from 10° to 90° 2θ at a scanning rate of 8° min⁻¹. The transmission electron microscope (TEM) test samples were made by dropping a dilute solution of nanoparticles onto a copper grid, and the TEM images were measured \textit{via} a JEM-2010F low-to-high resolution TEM operated at 120 kV. The Fourier transform infrared (FTIR) spectra were measured using an Avatar 370 spectrometer. The spectra were recorded in transmission mode using a wavenumber range of 4000 to 400 cm⁻¹. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was employed to monitor the Gd³⁺ concentration in
DOX-WP5⊃1-UCNPs. Upconversion luminescence (UCL) spectra were obtained using an Edinburgh FLS-920 fluorescence spectrometer with 980 nm laser (Connect Fiber Optics, China) excitation. UV-vis spectra were collected on a Shimadzu UV-2501PC spectrophotometer. ^1^H NMR spectra were obtained with a Bruker AVANCE/AV 500 spectrometer using the deuterated solvent as the lock and the residual solvent (D_2O, δ = 4.79) or TMS (DMSO-d6 ((D, 99.9%)+0.03% V/V TMS), δ =0) as the internal reference. Particle size distribution and Zeta potential were measured using dynamic light scattering (DLS) using a Nano-ZS (Malvern Instruments).

2.11. Relaxivity Measurement. The relaxivity of WP5⊃1-UCNPs was determined in a MRI instrument with 3.0 T magnetic field at 25 °C. WP5⊃1-UCNPs were dispersed in deionized water. The samples contained Gd^{3+} ion concentrations in the range 0-1.57 mM Gd^{3+}. The inverse longitudinal times (1/T_1) were plotted against Gd^{3+} concentration values. The relaxivity r_1 of the WP5⊃1-UCNPs was calculated from the slope of the fitted regression line. All measurements were performed in triplicate.

2.12. Cytotoxicity Assays. The human cervical cancer line (HeLa cells) were got from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and maintained in accordance to the supplier's protocols. HeLa cells were cultured in a DMEM medium (Evergreen Bio-Engineering Materials Co. Ltd., China) supplemented with 10% (v/v) fetal bovine serum (FBS, Zhejiang Tianhang Bio-Engineering Co. Ltd., China). HeLa cells were planted in 96-well plate (5000 well⁻¹) and grown in a humid atmosphere with 95% air and 5% CO_2 at 37 °C overnight to allow the cultured cells to grow against the walls of the flask.

The cell viability was investigated in vitro by a WST-8 cell counting kit (CCK-8, Dojindo Molecular Technologies Inc., Japan). The fresh culture medium (200 µL) containing 1-UCNPs, WP5⊃1-UCNPs, or DOX-WP5⊃1-UCNPs at same concentration gradient (0, 25, 50, 100, and 200 µg mL⁻¹), or DOX with corresponding concentration gradient (0, 1.5, 3, 6, and 12 µg mL⁻¹) was added to the cells at 37 °C under 5% CO_2/95% air for 24 h. Afterward, the tetrazolium salt, WST-8, was added to each well and then incubated at 37 °C under 5% CO_2 for 2 h. The optical density
(OD) at 450 nm of each well at was recorded using a microplate reader (Thermo, Varioskan Flash, Boston, MA USA). The cell viability is expressed as the percentage of \((\text{OD}_{\text{test}} - \text{OD}_{\text{blank}})/(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})\), where \(\text{OD}_{\text{test}}\) is the OD of the cells exposed to \(\text{WP5}\uparrow\text{I-UCNPs}, \text{ 1-UCNPs}, \text{DOX-WP5}\uparrow\text{1-UCNPs}, \text{or free DOX, \text{OD}_{\text{blank}} represents the OD of the well without cells, and \text{OD}_{\text{control}} represents the OD of the control, and. Sextuplets results were obtained for all experiments.

The live/dead staining kit (L-3224, Invitrogen, USA) was used to image for the live/dead status of cells. A dye mixture was used to differentiate live cells (green, excitation/emission: 495 nm/515 nm) by calcein AM from dead cells (red, excitation/emission: 528 nm/617 nm) by ethidium homodimer-1. After cells were incubated for 24 h in 96-well plates, the culture medium was replaced with the culture medium containing \(\text{DOX-WP5}\uparrow\text{1-UCNPs}, \text{DOX, WP5}\uparrow\text{1-UCNPs and 1-UCNPs. After 24 h incubation, the dyes dissolved in D-Hank’s were added to the cells and incubated at room temperature for 30 min. Finally, cells were studied under a fluorescence microscope (DMI3000, Leica, Wetzlar, Germany). The cells treated with \(\text{H}_2\text{O}_2\) (200 mM) for 15 min were used as the positive control group.

2.13. Cellular Uptake. Confocal imaging was performed with an Olympus FV1000 laser scanning confocal microscope at 980 nm NIR laser (~500 mW, Connect Fiber Optics, China) excitation. \(\text{WP5}\uparrow\text{1-UCNPs and DOX-WP5}\uparrow\text{1-UCNPs were added to the cells and incubated for 0.5, 1, 2, and 4 hours. The cells were rinsed several times with PBS (0.01 M) to remove any excess nanoparticles and kept in PBS at room temperature. UCL emission in green and red region were collected at 520-560 and 630-680 nm, respectively. Meanwhile, HeLa cells incubated with culture medium acted as the control group. All images were obtained under the same instrumental conditions.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of the Overall Nanosystem. A new theranostic nanosystem was rationally designed and synthesized by assembling the water soluble host-guest macrocycle pillar[5]arene, WP5, on the surface of lanthanide doped 1-UCNPs, shown in Scheme 1. Typically, the \(\text{NaYF}_4\cdot\text{Yb}/\text{Er}\) was synthesized through
the modified solvothermal method.\textsuperscript{35} To enhance the intensity of upconversion luminescence and provide $T_1$-MR imaging ability, a thin NaGdF$_4$ shell was coated on the surface of NaYF$_4$:Yb/Er core nanoparticles to obtain NaYF$_4$:Yb/Er@NaGdF$_4$ (OA-UCNPs) core-shell nanoparticles by epitaxial growth method.\textsuperscript{37,38} Oleate ligands were removed from the surface of OA-UCNPs and the subsequent capping of the oleate free UCNPs with compound 1 produced the hydrophilic nanoparticles 1-UCNPs by coordination of the carboxyl group of compound 1 and Ln$^{3+}$ ions of UCNPs. Subsequently, the hydrophilic WP5 was assembled with 1-UCNPs based on host-guest complexation between the ammonium bromide moiety of 1-UCNPs with the hydrophobic cavity of WP5, resulting in the WP5$\supset$1-UCNPs nanosystems.

The morphologies and structures of NaYF$_4$:Yb/Er, OA-UCNPs, 1-UCNPs, and WP5$\supset$1-UCNPs were characterized by TEM (see Figure 1). Figure 1a and 1b show the TEM data for the NaYF$_4$:Yb/Er and OA-UCNPs. From the particle size analysis of the low resolution TEM micrographs the average particle size was found to be 28 and 35 nm for the core and core-shell nanoparticles, respectively. The increase of particle size of core/shell nanoparticles is due to the growth of the NaGdF$_4$ shell around the core. Based on the differences in the sizes of the core and core-shell nanoparticles, the shell thickness was noted to be highly uniform and estimated to be 7 nm. Elemental mapping of WP5$\supset$1-UCNPs (Figure 1f) provides further evidence that the shell, NaGdF$_4$, is present. The surface of the as prepared OA-UCNPs was covered with hydrophobic oleic acid ligands so that there was no inherent aqueous dispersibility for subsequent functionalization. Thus, the oleate ligand was removed from the surface of oleate-capped core/shell UCNPs using an acid treatment process developed by Capobianco’s group.\textsuperscript{39} These oleate free UCNPs facilitate the conjugation of HOOC(CH$_2$)$_{15}$NMe$_3$Br, producing the hydrophilic 1-UCNPs. The TEM image of the 1-UCNPs show excellent monodispersibility in aqueous solution (see Figure 1c). The resulting WP5$\supset$1-UCNPs have a spherical morphology and show a mean particle size of $\sim$ 45 nm (see Figure 1d). The lattice fringes on the individual particle surfaces can be clearly identified from the HR-TEM image of WP5$\supset$1-UCNPs in Figure 1e. The distance between the lattice fringes was
determined to be 0.52 nm that corresponds to the \( d \)-spacing of the (100) lattice plane in the hexagonal NaYF\(_4\) structure. The dynamic light scattering (DLS) assay clearly display the hydrous particle diameter of 1-UCNPs and WP5\(\supset\)1-UCNPs nanosystem are 65 nm and 137 nm in water, respectively (Figure S4a and 4b). In addition, colloidal stability of the WP5\(\supset\)1-UCNPs in water was investigated by DLS analysis (Figure S4c), which suggests that the supramolecular upconversion nanosystem can be stably dispersed in water for one week. The X-ray diffraction patterns for all samples (Figure S4d) were consistent with the reference pattern of the standard hexagonal phase of bulk NaYF\(_4\) (JCPDS: 16–0334). As well the particles are highly crystalline showing that the acid treatment, the conjugation of HOOC(CH\(_2\))\(_{15}\)NMe\(_3\)Br and WP5 and the loading with DOX has not affected their crystallinity.

Figure S5 shows the FT-IR spectra of OA-UCNPs, compound 1, and 1-UCNPs. The FT-IR peaks in Figure S5a corresponded to the various stretching and bending vibrations due to the oleate molecules capping the nanoparticle surface. Specifically, the peaks at 2856 and 2926 cm\(^{-1}\) corresponded to the asymmetric and symmetric -CH\(_2\) stretches of the \( sp^3\) hybridized carbons of the oleate molecule. The peaks observed at 1467 and 1564 cm\(^{-1}\) were attributed to the symmetric and asymmetric COO\(^-\) stretches, respectively.\(^{40,41}\) Upon addition of compound 1 (HOOC(CH\(_2\))\(_{15}\)NMe\(_3\)Br) to the oleate free UCNPs to produce 1-UCNPs a change in the FTIR spectrum was observed (Figure S5c). For example, a peak at 1737 cm\(^{-1}\) was attributed to the –C=O stretch and a broad stretch was noted at approximately 3500 cm\(^{-1}\) assigned to the O-H stretching which is characteristic of compound 1 (Figure S5b). The two bands at 2855 and 2923 cm\(^{-1}\) were ascribed to the symmetric and asymmetric -CH\(_2\) stretch respectively of the long alkyl chain found in compound 1. These assignments are in excellent agreement with the FTIR spectrum of HOOC(CH\(_2\))\(_{15}\)NMe\(_3\)Br (Figure S5b).

The addition of WP5 to 1-UCNPs was revealed by the appearance of a peak at 1263 cm\(^{-1}\) that is characteristic for the WP5 (Figure S6a and 6b). This suggests that WP5 was assembled on the surface of 1-UCNPs successfully, producing WP5\(\supset\)1-UCNPs. The FT-IR spectra of DOX and DOX-WP5\(\supset\)1-UCNPs were shown in Figures S6c and S6d, respectively. The DOX displayed the characteristic band at 11
1728 cm\(^{-1}\) due to the stretching vibration of carbonyl group at the 13-keto position, and the bands at 1614 and 1585 cm\(^{-1}\) can be attributed to the two carbonyl groups of the anthracene ring. The broad band at 3327 cm\(^{-1}\) is attributed to the vibration of –OH groups which includes a contribution of the –NH\(_2\). The FT-IR spectrum of DOX-WP5⊃1-UCNPs shows the characteristic peaks of DOX providing evidence of the association of the DOX with WP5⊃1-UCNPs.

Zeta potential measurements showed that the addition of WP5 to the 1-UCNPs decreased the value from +51.0 mV to -18.5 mV demonstrating that WP5 was successfully added to the 1-UCNPs forming WP5⊃1-UCNPs (Figure S7). The decrease in the zeta potential is due to the interaction of compound 1, which is positively charged (trimethyl ammonium bromide), with the carboxylates found on the pillar[5]arene. Upon loading the DOX to form the nanosystem, DOX-WP5⊃1-UCNPs, the zeta potential increased to -3.7 mV (Figure S7), due to the amino groups of the DOX.

Thermogravimetric analysis of the 1-UCNPs, WP5⊃1-UCNPs and DOX-WP5⊃1-UCNPs showed a mass loss of 2.6, 7.7 and 11.4%, respectively (Figure S8). Specifically, the loss of 2.6% in the 1-UCNPs nanosystem is attributed to the mass of compound 1 on the UCNPs. The loss of 5.1% in the WP5⊃1-UCNPs represents the mass of the WP5 assembled on the 1-UCNPs. The loss of 3.7% in the DOX-WP5⊃1-UCNPs nanosystem is attributed to the mass of DOX loaded in the WP5⊃1-UCNPs.

3.2. Host-Guest Complexation Studies in Water. It has been well-established that negatively charged pillar[5]arene WP5 can complex with positively charged guests with suitable size.\(^{42,43}\) As expected, compound 1 containing cationic trimethylammonium group can be bound inside the cavity of WP5 to form an inclusion complex. To confirm this smart behavior, the host-guest complexation was investigated between equimolar (1.00 mM) D\(_2\)O solution of compound 1 and WP5 by \(^1\)H NMR spectrum, as shown in Figure 2. Compared with free compound 1, the resonance peaks corresponding to the protons of methylene H\(_{b-g}\) showed pronounced upfield shift and broadening upon addition of WP5. This is due to the
inclusion-induced shielding effects. Meanwhile, WP5 is deshielded by the presence of the guest compound 1, since the signals of H\textsubscript{1,3} shifted downfield. The above results reveal that the positively charged trimethylammonium moiety of compound 1 was threaded into the negatively charged cavity of WP5 to form an supramolecular inclusion complex WP5⊃1 (Figure S9), a smart behavior. As discussed previously the results obtained from the FT-IR measurements and zeta potential indicate the formation of WP5⊃1-UCNPs (Figure S5- Figure S7).

3.3. Drug Loading and Release Properties. To study the drug release behavior and evaluate the loading efficiency of the pH-responsive WP5⊃1-UCNPs nanosystem, HCl·DOX was chosen as a fluorescent anticancer model drug. After being desalted by trimethylamine (TMA), the neutral DOX was successfully loaded in the hydrophobic gap between WP5s possibly due to intermolecular electrostatic interaction and π-π stacking interaction. The DOX that did not enter between the WP5 gaps was removed by dialysis. To determine the concentration of DOX found in the DOX-WP5⊃1-UCNPs nanosystem we measured the absorbance of DOX at 480 nm (Figure S10) and established a calibration curve (Figure 3a). Using the curve and the absorbance value obtained for the nanosystem DOX-WP5⊃1-UCNPs (Figure S11) we calculated the concentration of DOX, which allowed us to calculate the loading efficiency to be 3.67 wt%. This value is in excellent agreement with the data obtained from the TGA. More importantly, although the loading efficiency of the nanosystem is low compared with other DOX delivery systems,\textsuperscript{44,45} it is still an effective delivery system based on the following cytotoxicity experiment.

To explore the pH-responsive behavior of the nanosystem, we simulated the biosystem in the buffer solution. The pH values of cytoplasm, lysosome and endosome are around 7.4-7.2, 6.5-4.5, and 6.6-5.5, respectively.\textsuperscript{46,47} So the buffer solution with certain pH value that is close to the pH in each organelle was selected for the study of release behavior. In order to investigate the release behavior of DOX from the DOX-WP5⊃1-UCNPs nanosystem, we measured the absorbance of peak at 480 nm as a function of time at different pH (phosphate buffer solution, 0.1 M, pH = 7.4, 6.5, 6.0, 5.0, and 4.0) at 37 °C. As can be observed from Figure 3b, 69% of DOX
was released from the nanosystem at pH 4.0 over a period of 24 h. At pH 5.0 and 6.0, around 45 % and 34 % of DOX was released respectively over the same period of time. However, the DOX release is much lower when pH is close to neutral conditions (such as pH 6.5 and 7.4). Therefore, the results demonstrate that DOX can be released from the nanosystem at low pH but in a relatively stable state under neutral conditions.

From the data obtained for the release of DOX and results on the investigation of the assembly of WP5⊃1-UCNPs reported in the previous sections we are now in a position to provide an explanation for the encapsulation and release of DOX. From the NMR results we have shown that the protons, H_{b-g}, of the alkyl chain of compound 1 reside in the hydrophobic cavity of the WP5. Whereas, proton, H_{h-o} and the trimethylammonium moiety protruded from the cavity. The binding affinity for such guest-host inclusion is mainly driven by the hydrophobic interactions. The WP5s found on the assembly, WP5⊃1-UCNPs, are separated by a hydrophobic gap, thus the DOX may fill these gaps via intermolecular electrostatic interaction and π-π stacking. The electrostatic interaction between the carboxyl groups in WP5 and amino group in DOX also contributed to the high drug loading content. Also, the protonation of DOX and the weakening of the interaction of WP5 and DOX at low pH would result in the accelerated drug release in acidic environment.48,49

According to the references, the association constant (Ka) of the complexation between compound 1 and WP5 was assumed to be around (1.75 ± 0.21) × 10^6 M^{-1}.35,43 It has been well established that the complex WP5 can be transformed into WP5H in the acidic condition via the protonation of carboxylate group. This results in weakening the interaction between the WP5s on the nanoparticles resulting in the drug release. In previous work we displayed the oleic acid are adsorbed on the Ln-UCNPs chemically through the coordination of COO^- group and Ln^{3+} ions on the surface of the nanoparticles. In the acidic conditions the oleate of the surface is protonated and oleic acid detaches leaving a positively charged surface. The moiety WP5⊃1 is coordinated on the Ln-UCNPs via the carboxylate group on compound 1 analogous to the oleate. Thus, it is reasonable to assume that at acidic condition both
the carboxylate group of WP5 and compound 1 were protonated (positive zeta potential.), which led to the dissociation of the electrostatic repulsion between WP5 and compound 1, subsequently leading to the release of DOX from the WP5⊃1-UCNPs nanosystem.

3.4. Upconversion Luminescence Properties. The upconversion luminescence spectra of WP5⊃1-UCNPs and DOX-WP5⊃1-UCNPs upon 980 nm excitation are displayed in Figure 4. Comparing the two spectra, it can be noted that the peak positions of two spectra remain the same; however, there seems to be a decrease for the intensity of the peaks associated with the green emission at 521 nm (⁵H₁₁/₂⁻⁴I₁₅/₂), and 540 nm (⁴S₃/₂⁻⁴I₁₅/₂) and no change in the intensity of the red emission at 654 nm (⁴F₉/₂⁻⁴I₁₅/₂). The assemblies WP5⊃1-UCNPs and DOX-WP5⊃1-UCNPs disperse readily in water (Figure 4 panels 1 and 3) forming clear (WP5⊃1-UCNPs) and light pink (DOX-WP5⊃1-UCNPs) colloidal solutions. Figure 4 (panels 2 and 4) shows the emission from the same solutions upon 980 nm excitation where it can be observed visually that the DOX-WP5⊃1-UCNPs assembly demonstrates a weaker green emission. Figure S12 shows the overlap of the absorbance of DOX with the emission of the WP5⊃1-UCNPs centered at 521 nm and 540 nm, due to the transitions of (⁵H₁₁/₂⁻⁴I₁₅/₂) and (⁴S₃/₂⁻⁴I₁₅/₂) of Er⁢³⁺ ions, respectively. The green emission intensity decreased by approximately 43%, which provides the evidence of a possible non-radiative energy transfer from the UCNPs to the DOX.⁵¹,⁵²

3.5. Magnetic Resonance Imaging (MRI) of WP5⊃1-UCNPs Nanosystem. MRI, a medical technique, that is used to image bodily organs and tissue by measuring the hydrogen proton spin relaxation time following an external radiofrequency stimulus. The basic concept of MRI involves the interaction of the hydrogen proton spins with an applied magnetic field. Gadolinium (Gd) base chelated complexes have been predominantly used for contrast enhancement especially for T₁ imaging.⁵³,⁵⁴ As displayed in Figure 5a, a positive enhancement of the magnetic resonance signal was observed in T₁-weighted and color-mapped MR images with the concentration of Gd³⁺ increasing. The brightest spot is observed for the sample with a Gd³⁺ concentration of 1.57 mM and is an indication that the assembly WP5⊃1-UCNPs may be acted as
positive $T_1$ MRI contrast agent. The efficiency of the nanosystem as $T_1$ contrast agent was investigated by measuring the proton longitudinal ($T_1$) relaxation rate and obtaining the $r_1$ value (relaxivity rate) from the slope of the plot of $1/T_1$ as a function of Gd$^{3+}$ concentration (see Figure 5b). The longitudinal relaxivity ($r_1$) was calculated to be 2.83 mM$^{-1}$·S$^{-1}$. This value is higher compared to values reported for NaGdF$_4$ nanomaterials coated with polymers or shells,$^5$ indicating that WP5$\supset$1-UCNPs can act as a potential $T_1$ MRI contrast agents.

3.6. *In Vitro* Cytotoxic Effect Against HeLa Cells. Since the biocompatibility of nanomaterials is crucial for their potential biomedical applications, the CCK-8 assay was employed to investigate the *in vitro* cytotoxicity of the nanosystem synthesized. As shown in Figure 6, after incubation with 1-UCNPs and WP5$\supset$1-UCNPs at different concentrations ranging from 0 to 200 µg·mL$^{-1}$ for 24 h, the relative viability of HeLa cells were over 96%, which demonstrates no intrinsic toxicity towards the cells since the cellular survival rate was similar to the control. The relative viabilities of HeLa cells incubated with free DOX and DOX-WP5$\supset$1-UCNPs were also investigated. When HeLa cells were incubated with DOX-WP5$\supset$1-UCNPs and DOX, respectively, the cell viability decreased gradually with samples concentrations increasing, and there was no significant difference in cell viability between the two groups of cells incubation with DOX-WP5$\supset$1-UCNPs and DOX. That is because in the acidic conditions of HeLa cells, the structure of DOX-WP5$\supset$1-UCNPs would be disassembled resulting in the release of DOX, thus leading to cell death. Such a significant cell death (72%) caused by the release of DOX from the nanosystem demonstrates the possible applicability of the WP5$\supset$1-UCNPs as a pH-responsive DDSs.

To further demonstrate the efficacy of the WP5$\supset$1-UCNPs nanosystem as a potential pH-responsive DDSs, calcein AM (mark living cells with green emission) and ethidium homodimer-1 (mark died cells with red emission) were used as dye for the HeLa cells under different conditions to investigate the effect of chemotherapy of the nanosystem. It is evident from the results in Figure 7 that WP5$\supset$1-UCNPs does not affect the viability of the cells in the concentration range 12.5-200 µg·mL$^{-1}$, since
of the HeLa cells show bright green fluorescence throughout the domain. However, after incubation with low concentration of DOX-WP5⊃1-UCNPs and free DOX (12.5 µg·mL⁻¹), a small proportion of HeLa cells were dead, and the number of HeLa cells was significantly reduced, attributed to the pH-responsive release of DOX from the DOX-WP5⊃1-UCNPs nanosystem in acid environment of the lysosome. When HeLa cells were incubated with high concentrations of DOX-WP5⊃1-UCNPs or DOX (200 µg·mL⁻¹), nearly all cells were dead and showed red fluorescence in the entire region, which effectively illustrates that the acidic conditions found in the lysosome promote the disruption of the nanosystem thus the release of the DOX.

3.7. Cellular Uptake. Cell uptake of multifunctional nanosystems is required to achieve cell imaging, enhanced delivery efficiency, and desired therapeutic effects. It has been reported in a large number of studies that such UCNPs-based DDSs are usually caught by cells via endocytosis,⁶⁶⁻⁶⁹ followed by the drug release due to the acidic conditions within lysosomes or endosomes in cancer cells.⁷⁰⁻⁷² Therefore, we speculate that the drug release of the nanosystem happens in the lysosomes or endosomes of HeLa cells.

We evaluated the cell uptake performance and cell imaging capability of WP5⊃1-UCNPs and DOX-WP5⊃1-UCNPs, utilizing laser scanning upconversion luminescence microscopy (LSUCLM). The samples were excited using a 980 nm NIR laser. Initially, HeLa cells were incubated at 37 °C with WP5⊃1-UCNPs for 0.5, 1, 2, and 4 hours, respectively, and bright UCL signals both in the green (520-560 nm) and in the red (630-680 nm) areas (Figure S13) were observed, suggesting that the WP5⊃1-UCNPs were internalized by the HeLa cells. More importantly, the merged confocal luminescence images of the green and red channel collected along the Z optical axis (Z-scan) confirm that the upconversion luminescence signals emanating from the WP5⊃1-UCNPs were localized in the cytosol region and not on the membrane surface (Figure S14).

Figure 8 shows the upconversion luminescence (excited with 980 nm) in the green (520-560 nm) and red (630-680 nm) areas of the HeLa cells incubated with the nanosystem (200 µg·mL⁻¹) at 37 °C for 0.5, 1, 2, and 4 hours, respectively. We also
observed a rose red fluorescence (550-700 nm) upon 488 nm excitation, which is characteristic of DOX. The overlay between rose red and green channels images furtherly demonstrates that both the upconversion luminescence and the rose red fluorescence were localized in the intracellular region. It is important to observe that the intensity of the emission signal attributed to upconversion luminescence from the DOX-WP5⊃UCNPs nanosystem and the fluorescence of DOX increased as a function of time. However, some of the DOX signal (rose red) did not overlap with the UCL signal completely. The phenomenon can be assigned to the fact that the release of DOX from DOX-WP5⊃UCNPs was achieved after entering the HeLa cells, which was triggered by the weakly acidic environment. Furthermore, the different permeability of DOX and UCNPs to nuclear membrane plays a significant role in the cell uptake. With increased incubation time, the DOX molecules tended to enter the cell nucleus via the nuclear pore on nuclear membrane, however, the UCNPs were unable to enter the cell nucleus.\textsuperscript{63} Based on the above results, the DOX-WP5⊃UCNPs nanosystem can be a desirable candidate for intracellular fluorescence imaging with high-contrast, and beneficial for intracellular localization of anti-cancer drug.

4. CONCLUSIONS

In summary, we have shown for the first time the pH-responsive supramolecular upconversion nanosystem WP5⊃UCNPs that was developed through the host-guest complexation based on carboxylate-based pillar[5]arene (WP5) with UCNPs. Compound 1 was conjugated rendering the UCNPs hydrophilic. We found that the DOX was loaded in the hydrophobic gaps existing between the WP5s of the assembly, WP5⊃UCNPs. In vitro cell viability assays showed that the WP5⊃UCNPs assembly did not show toxicity towards the cells, while the viability of the cells incubated with DOX-WP5⊃UCNPs is comparable to that of incubation with free DOX in equal concentration. Such a significant cell death shows the possible applicability of the nanosystem as a pH-responsive drug delivery system. Cellular uptake experiments suggest that the supramolecular upconversion nanosystems developed can enter HeLa cells, which could lead to significant drug accumulation in.
cancer cells. In addition, the nanosystem can be used as a $T_1$ MRI contrast agent as well for bioimaging making use of its upconversion properties. Therefore, the nanosystem is a promising candidate of bio-imaging-guided cancer therapy.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI:*.

Synthesis of compound 1 and WP5. $^1$H NMR spectrum of compound 1*, 1, and WP5. DLS, XRD patterns, FT-IR spectra, zeta potential, thermogravimetric analysis of UCNPs, 1-UCNPs, WP5$\supset$1-UCNPs, and DOX-WP5$\supset$1-UCNPs. The structures of 1, WP5, WP5$\supset$1 and WP5H, cartoon representation of 1, WP5, WP5$\supset$1 and WP5H. UV-vis absorption spectra of DOX, DOX-WP5$\supset$1-UCNPs, and WP5$\supset$1-UCNPs. Overlap of the luminescence emission spectrum of WP5$\supset$1-UCNPs and the absorption spectrum of DOX. CLSM images of HeLa cells after incubated with WP5$\supset$1-UCNPs. The three-dimensional images of HeLa cells after incubation with WP5P$\supset$1-UCNPs. (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

*E-mail: lnsun@shu.edu.cn (L. Sun).

*E-mail: hwang@shu.edu.cn (H. Wang).

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGEMENTS**

This work was supported by National Key R&D Program of China (No. 2016YFE0114800), the National Natural Science Foundation of China (Grant No. 21571125), and the National Basic Research Program of China (No. 19
2016YFA0201600). Prof. John A. Capobianco is a Concordia University Research Chair in Nanoscience and many thanks to Concordia University for financial support of his research. Prof. John A. Capobianco thanks the Natural Science and Engineering Research Council of Canada. Many thanks to the Instrumental Analysis & Research Center of Shanghai University.

REFERENCES


(30) Li, Y.; Gu, Y. Y.; Yuan, W.; Cao, T. Y.; Li, K.; Yang, S. P.; Zhou, Z. G.; Li, F. Y. Core-Shell-Shell NaYbF$_4$:Tm@CaF$_2$@NaDyF$_4$ Nanocomposites for Upconversion/T$_2$-Weighted MRI/Computed Tomography Lymphatic Imaging. *ACS Appl. Mater. Interfaces* 2016, 8, 19208-19216.


Scheme 1. Schematic illustration of the Synthesis of DOX-WP5→1-UCNPs nanosystem and its application for intracellular drug release, upconversion luminescence (UCL) imaging, and magnetic resonance imaging (MRI).
Figure 1. TEM images of (a) NaYF$_4$:Yb/Er, (b) NaYF$_4$:Yb/Er@NaGdF$_4$, (c) 1-UCNPs, (d) WP5⊃1-UCNPs, (e) HR-TEM image and (f) elemental mapping images of WP5⊃1-UCNPs.
Figure 2. Partial $^1$H NMR (500 MHz, D$_2$O, 293 K) spectra: (a) WP5 (10.0 mM); (b) WP5 (10.0 mM) and compound 1 (10.0 mM); (c) compound 1 (10.0 mM). The deuterated water has a chemical shift of 4.79 ppm.
Figure 3. (a) The absorbance intensity (at 480 nm) as a function of DOX concentration (0-25 µg·mL⁻¹). (b) Release of DOX from DOX-WP5⊃₁-UCNPs in phosphate buffer solution (0.1 M, pH = 4.0, 5.0, and 7.4).
Figure 4. Upconversion luminescence spectra of WP5⊃1-UCNPs and DOX-WP5⊃1-UCNPs nanosystem in water excited using 980 nm light (500 mW·cm⁻²). (Inset: 1 and 3 are the digital photo image of WP5⊃1-UCNPs and DOX-WP5⊃1-UCNPs in water, respectively. Digital photo images 2 and 4 are their corresponding upconversion emission under 980 nm NIR laser irradiation.)
Figure 5. (a) $T_1$-Weighted and color-map of magnetic resonance images of WP5$\supset$1-UCNPs nanosystem with various Gd$^{3+}$ concentrations, (b) Relaxation rate $r_1$ ($1/T_1$) as a function Gd$^{3+}$ concentrations of the WP5$\supset$1-UCNPs nanosystem.
Figure 6. Cytotoxicity assays of HeLa cells following incubation with WP5⊃1-UCNPs, 1-UCNPs, or DOX-WP5⊃1-UCNPs at different concentrations (0, 25, 50, 100, and 200 µg mL\(^{-1}\)), or free DOX at the corresponding concentrations (0, 1.5, 3, 6, and 12 µg mL\(^{-1}\)) for 24 h. HeLa cells in normal culture medium serve as the control group. Data are mean cell viability % (n = 6) ± SD. * Represents significance difference (p<0.05) in comparison with the control.
Figure 7. Fluorescence microscopy images of calcein AM/ethidium homodimer-1-stained HeLa cells (green represents living cells, red represents dead cells) incubated with the concentration range of 12.5-200 µg mL\(^{-1}\) of WP5⊃1-UCNPs or DOX-WP5⊃1-UCNPs, or DOX with the corresponding concentrations being 0.75, 1.5, 3, 6, and 12 µg mL\(^{-1}\). Scale bar: 100 µm. (Control: HeLa cells incubation with DMEM cell culture medium).
**Figure 8.** Confocal laser scanning microscopy (CLSM) images of HeLa cells following incubation with DOX-WP5\(\supset\)UCNPs 200 µg·mL\(^{-1}\) for 0.5 h, 1 h, 2 h, and 4 h at 37 °C; the rose red emission from DOX was collected at 550–700 nm using 488 nm excitation; upconversion luminescence (UCL) emission was collected by a green channel at 500–560 nm and a red channel at 600–700 nm, \(\lambda_{\text{ex}} = 980\) nm, 500 mW.