In vivo biodistribution and toxicity assessment of triplet-triplet annihilation-based upconversion nanocapsules

Bo Tian, Qiuhong Wang, Qianqian Su, Wei Feng, Fuyou Li*

Department of Chemistry, State Key Laboratory of Molecular Engineering of Polymers & Institutes of Biomedical Sciences, Fudan University, Shanghai, PR China

ARTICLE INFO

Article history:
Received 29 June 2016
Received in revised form 6 October 2016
Accepted 7 October 2016
Available online 8 October 2016

Keywords:
Triplet-triplet annihilation (TTA)
Upconversion nanocapsules (UCNCs)
Biodistribution
Toxicity assessment

ABSTRACT

Triplet-triplet annihilation (TTA)-based upconversion nanocapsules (UCNCs) have great potential in biological and medical applications. However, there are numerous unresolved issues with respect to the safety of these novel nanomaterials. In this work, for the first time, we studied the in vivo biodistribution of UCNCs which were synthesized by co-loading platinum (II)-tetraphenyl-tetrabenzoporphyrin (PtTPBP) and boron dipyrromethene derivative (BDP) into bovine serum albumin (BSA)-stabilized soybean oil droplets, and systematically assessed the potential toxicity of UCNCs both in vitro and in vivo. The results showed that UCNCs had no significant influence on the proliferation or the migration of HeLa cells even when the dosage was increased to 12 mg/mL. The biodistribution results demonstrated that UCNCs mainly accumulated in the mononuclear phagocyte system (MPS) including the liver and spleen after intravenous injection of the nanocapsules. When mice were intravenously injected with 1200 mg/kg of the UCNCs over a period of 60 days, no noticeable toxicity was observed under these treatment conditions as shown by body weight results, histological analyses, hematological analyses and blood biochemical examinations. This research inspires further studies on UCNCs for biomedical applications.

1. Introduction

Upconversion luminescence is an anti-Stokes process in which the absorption of two or lower energy photons leads to the emission of higher energy photons [1–7]. Upconversion luminescence emission can be achieved in two main processes: lanthanide based upconversion and triplet-triplet annihilation (TTA) based upconversion. Lanthanide upconversion nanoparticles (UCNPs) show excellent upconversion luminescence under continuous wave (CW) excitation of 980 nm, and have been widely reported for various bioapplications [2–5,8,9]. In the triplet-triplet annihilation (TTA) upconversion luminescence (UCL) process, the lower energy photon excited sensitizer transfers its energy to the emitter (acceptor/annihilator), and then two of the emitters generate a higher energy photon via triplet-triplet annihilation [2,6,10]. Lanthanide based upconversion and TTA based upconversion exhibit unique luminescence properties such as large anti-Stokes shift, long luminescence lifetimes, high photostability, and absence of autofluorescence interference from biological samples for imaging of small animals [7,10,11]. Furthermore, compared to lanthanide based upconversion, TTA based upconversion shows advantages including the requirement for low excitation power density, tunable excitation wavelength and emission wavelength by independent selection of the sensitizer and emitter, intense absorption coefficient of the sensitizer and high upconversion quantum yield [2,6,10,12–19]. Therefore, TTA-UCL has attracted more and more attention as an efficient process in bioapplications [2,6,12,20–23].

Despite the encouraging results when TTA-UCL nanomaterials are used in biology and medicine, there are many unresolved issues with respect to understanding how these TTA-UCL nanomaterials interact with biological systems. In particularly, the development of these novel materials has raised concerns about their potential bio-toxicity in humans. To date, only a few studies have assessed the in vitro toxicity of TTA-UCL nanomaterials. Our group demonstrated that after incubation with 320 μg/mL of TTA upconversion nanoparticles (sensitizer/emitter: PdOEP/DPA) for 24 h, more than 90% of living cells were detected, thus indicating the low cytotoxicity of these nanocapsules [7]. Turshatov et al. studied the cell toxicity of TTA-UCL-based upconversion nanocapsules and obtained similar...
results [15]. However, these results were from in vitro studies, and the in vivo toxicity of TTA-based upconversion nanomaterials has not yet been reported [2,24]. In vivo systems are extremely complicated and the interactions between the nanomaterials and biological components can cause unique absorption, distribution, metabolism and excretion. Therefore, the data from in vitro studies may not reflect the results in vivo [25]. Hence, further animal experiments are urgently needed to assess the toxicity of TTA-UCL nanomaterials.

In the present study, in vivo biodistribution and toxicity assessment of TTA-based upconversion nanocapsules (UCNCs) was performed for the first time. The nanocapsules (hydrodynamic diameter: ~235 nm) were fabricated by co-loading platinum (II)-tetraphenyl-tetrabenzo porphyrin (PtTPBP) and boron dipyrromethene derivative (BDP) into bovine serum albumin (BSA)-stabilized soybean oil droplets. UCNCs at a dose of ~1200 mg/kg were used for the in vivo studies. Biodistribution studies based on whole body UCL imaging showed that UCNCs mainly accumulated in liver and spleen, and quickly excreted. The body weight of female Balb/c mice at 60 days post-injection of ~1200 mg/kg UCNCs was not significantly different to that in the control group. Furthermore, the data from histological assessments, hematological analyses and blood biochemical tests in female mice after injection of UCNCs indicated no evident toxic effects at the dose of ~1200 mg/kg over a period of 60 days. Our results demonstrated that UCNCs had no obvious toxicity in vitro and in vivo.

2. Materials and methods

2.1. Materials

All of the starting materials were obtained from commercial suppliers and used without further purification. Platinum (II)-tetr phenyl-tetrabenzo porphyrin (PtTPBP) was purchased from Aldrich. Boron dipyrromethene derivative (BDP) was synthesized according to published procedures [26]. Soybean oil of pharmaceutical grade was obtained from Aladdin Industrial Corporation. Bovine serum albumin (BSA) was purchased from Sigma. Fetal bovine serum (FBS) was purchased from Gibco. Cell culture reagents were purchased from Invitrogen. Methyl thiazolyl tetrazolium (MTT) was purchased from Sigma. Crystal violet was purchased from Shanghai Bogoo Biotechnology Co., Ltd., China. Phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO) were obtained from Acros. Ethanol and dichloromethane were supplied from Sinopharm Chemical Reagent Co., Ltd. (Shanghai). Deionized water was used in the experiments throughout.

2.2. Synthesis of upconversion nanocapsules (UCNCs)

Encapsulation of sensitizer (PtTPBP) and emitter (BDP) in BSA was prepared as reported previously with modifications [27]. Briefly, 0.12 mg sensitizer and 4.94 mg emitter were dissolved together in a mixed oil phase composed of 1 mL soybean oil and 2 mL dichloromethane. The dichloromethane was removed by rotary evaporation, and then, the final concentrations of sensitizer and emitter in soybean oil was 0.12 mmol/L and 7.5 mmol/L, respectively. After dissolving 250 mg BSA in 19 mL water, the oil solution was added into the aqueous solution with 5% oil volume fraction. The mixture was emulsified at room temperature using an ultrasonic processor (VC 75, Sonics & Materials, Inc.) for 30 min. The emulsion was heated at 90 °C for 1 h. Then, the droplets were dispersed in water and the resultant emulsion was filtrated with 0.8 μm membrane to obtain aseptic emulsion.

2.3. Characterization

The UV–vis absorption spectra of UCNCs, PtTPBP and BDP were measured using a Shimadzu 3000 spectrophotometer. The upconversion luminescence (UCL) emission spectra were recorded using Edinburgh FLS-920 instrument with a 633 nm short pass filter, and the excitation source using an external 0–400 mW adjustable continuous-wave (CW) laser at 635 nm (Changchun fs-optics Co., China), instead of the Xeon source in the spectrophotometer. The particle morphology of UCNCs was obtained by atomic force microscopy (AFM, Multimode 8, Bruker) and analyzed by Nano Scope Analysis software provided by Bruker. Particle sizes of UCNCs were determined by dynamic laser scattering (DLS) at 25 °C using a commercial laser light scattering instrument (Zetasizer Nano ZS90, Malvern Instruments Ltd.) at a fixed angle of 90°. DLS samples were prepared freshly before the measurement by diluting the emulsion 100 times with water. The zeta-potential was measured on a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd.) and calculated using the Dispersion Technology Software provided by Malvern. Matrix assisted laser desorption ionization-time of flight mass spectra (MALDI-TOF-MS) were measured on an AB SCIEX 5800 system.

2.4. Stability assay

To determine the stability of UCNCs in physiological solution, the sizes of UCNCs were monitored in serum and whether the dye leaked from these nanocapsules. 1 mL of UCNCs at concentration of ~60 mg/mL was mixed with 1 mL of fetal bovine serum (FBS) in a dialysis bag of the molecular-weight cutoff at 14000 Da. The dialysis was carried out in dialysate of water (1 L). The dialysate was magnetic stirred. For each time point (0 h, 2 h, 4 h, 7 h, 3 d and 8 d), 50 μL of UCNCs solution was taken out and diluted 40 times with water, which was then measured by DLS to determine the size of nanocapsules. Moreover, the emission spectra of the two samples were dialyzed before and after 8 days were recorded on Edinburgh FLS-920 instrument (λex = 635 nm).

5 mL of UCNCs at concentration of ~60 mg/mL was mixed with 5 mL of FBS in a dialysis bag of the molecular-weight cutoff at 14000 Da. The dialysis was carried out in dialysate of water (500 mL) and the dialysate was magnetic stirred for 8 days. The dialysate was extracted by ethanol and determined by matrix assisted laser desorption ionization-time of flight/time of flight mass spectrometer.

2.5. Cell uptake

The human epithelial adenocarcinoma HeLa cell line was purchased from Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS, China) and cultured in RPMI-1640 (Roswell Park Memorial Institute’s medium) culture medium containing 10% FBS and 1% penicillin/streptomycin at 37 °C under 5% CO2. Cell uptake of UCNCs was determined by UCL and fluorescence luminescence imaging. For confocal imaging, HeLa cells (1 × 104 cells) were incubated with UCNCs at the concentration of 6 mg/mL in a culture dish for 8 h. After the cells had been washed five times with PBS to remove excess UCNCs, confocal images of cells were recorded under our modified confocal laser scanning microscope (CLSM) [27]. The instrument was built on an inverted microscope (Olympus IX81, Japan) and a confocal scanning unit (PV1000, Olympus, Japan). For the confocal upconversion luminescence (UCL) imaging, the continuous wave (CW) laser at 633 nm provided the excitation light. UCL emission at 500–600 nm was collected. For the confocal fluorescence luminescence (FL) imaging, under 488 nm CW laser excitation, FL emission at
500–600 nm was collected.

2.6. Cytotoxicity assay

The cell toxicity was measured using a standard MTT assay in HeLa cells. HeLa cells were precultured in 96-well cell culture plates at $1 \times 10^4$/well for 24 h. UCNCs at a series of concentrations (0.5, 0.75, 1, 1.5, 2, 3, 4, 6 and 12 mg/mL) were added into the wells as the treatment groups. PBS was added into the other wells as the control groups. The cells were incubated for 24 h and 48 h at 37 °C under 5% CO₂. Subsequently, the original medium was removed and 100 μL fresh medium mixture with 10 μL MTT (5 mg/mL) was added to each well. After another 4 h incubation, the medium was removed and 150 μL DMSO was added to each well to dissolve the MTT crystals completely. An enzyme-linked immunosorbent assay reader (infinite M200, Tecan, Austria) was used to measure the OD570 (absorbance value at 570 nm) of each well with background subtraction at 690 nm. The following formula was used to calculate the cell viability: cell viability (%) = (mean of absorbance value of treatment group/mean of absorbance value of control group) × 100.

2.7. Cell migration assay

The cell migration assay was carried out following a reported procedure with slight modifications [28]. HeLa cells were precultured in two cell culture flasks at 1 × 10⁵/mL (4 mL/flask) for 24 h. 1 mL of UCNCs (~60 mg/mL) were added into a culture flask at final concentration of 12 mg/mL as the treatment group; 1 mL of PBS was added into another flask as the control group. After incubation for 24 h, the cells were washed with FBS-free medium for three times and moved to the top chambers of a Transwell system (Corning Inc.) at $2 \times 10^4$/well. The bottom chambers contained 600 μL/well medium supplemented with 10% FBS. After another 24 h incubation, the cells were fixed by replacing the medium in the top and bottom chambers with 4% paraformaldehyde for 30 min. The chambers were washed with PBS for three times and the cells remained on top surface of membranes were removed using cotton swab. The cells that had penetrated to the bottom surfaces of the membranes were stained with 0.1% crystal violet for 10 min. After washing the chambers with PBS for three times, the stained cells were counted using a digital microscope.

2.8. In vivo and ex vivo UCL imaging

Female Balb/c mice were purchased from the Second Military Medical University (Shanghai, China). Animal procedures were in agreement with the guidelines of the Institutional Animal Care and Use Committee. In vivo UCL imaging was performed using a modified upconversion luminescence in vivo imaging system designed by our group [29]. In this system, one external 0–400 mW adjustable 635 nm CW laser was used as the external source and a cooled electron-multiplying charge-coupled device (EMCCD, Andor DU897) as the signal collector. A 633 nm short pass filter was placed before the charge-coupled device (CCD) camera to cut the excitation light. Spectral images were analyzed by Bruker MI Software. A laser power density of 62 mW/cm² was used during imaging. For in vivo imaging, Balb/c mice were intravenously injected with UCNCs and imaged at different time points post-injection after sacrificing. For ex vivo imaging, the major organs of injected mice were collected for UCL imaging.

---

**Fig. 1.** (a) Schematic illustration of UCNC. (b) The chemical structures of PtTPBP and BDP. (c) UV–vis absorption spectra of PtTPBP (red line) and BDP (blue line) in soybean oil and UCNCs (black line) in water, respectively. (d) UCL emission spectrum of UCNCs in water excited by 635 nm laser using 633 nm short pass filter. Insert: bright-field photo (left) and UCL emission photo (right) of UCNCs in aqueous solution, and the UCL emission photo was achieved by camera using 633 nm short pass filter. (e) Hydrodynamic diameters of UCNCs dialyzed with FBS for different times (2 h, 4 h, 7 h, 3 d and 8 d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.9. Body weight measurement

Four weeks old female Balb/c mice (n = 4) were intravenously injected with UCNCs at dose of ~1200 mg/kg and marked as the treatment group. Another 4 mice with no injection were marked as the control group. The body weight of the mice in 2 groups was recorded every two days and lasted for 60 days.

2.10. Histological, hematological and blood biochemical analyses

Four weeks old female Balb/c mice were intravenously injected with UCNCs at dose of ~1200 mg/kg and scarified at various time points after injection (8, 30 and 60 days, 4 mice per time point). 4 other Balb/c mice with no injection were used as the untreated control group. The mice were fasted for 16 h before bleeding. Blood was collected from the orbital sinus of mouse by quickly removing the eyeball using an ophthalmic tweezers. Approximately 1 mL blood from each mouse was collected before it was euthanatized for hematological analysis and blood biochemical analysis. The blood samples were measured in the Shanghai Research Center for Biomodel Organisms (Shanghai, China). After blood collection, major organs (liver, spleen, kidney, heart and lung) from those mice were obtained, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. These histological sections were examined using a digital microscope.

3. Results and discussion

3.1. Synthesis and characterization of UCNCs

UCNCs were synthesized following a previously reported procedure with some modifications [27]. As shown in Fig. 1a and Fig. 1b, the hydrophobic sensitizer (PtTPBP) and emitter (BDP) were dissolved in soybean oil and the oil solution was then encapsulated by BSA. Subsequently, BSA was denatured by heating to form a stable oil-water interfacial film [30]. Finally, the concentration of UCNCs in aqueous solution was ~60 mg/mL. The concentration of PtTPBP and BDP in aqueous solution was 6.05 × 10⁻¹² mg/mL (6 × 10⁻¹⁶ mol/L) and 2.47 × 10⁻¹ⁱ mg/mL (3.75 × 10⁻¹⁴ mol/L), respectively. The main components of soybean oil, linoleic acid and oleic acid, showed reduced ability to consume the generated singlet oxygen in the upconversion system [10]. In addition, BSA contains tryptophan residues which can react with singlet oxygen [10,31]. Therefore, these components help to protect the UCL process from being quenched by singlet oxygen [10].

Successful loading of the sensitizer (PtTPBP) and emitter (BDP) into the BSA nanocapsule was confirmed by UV–vis absorption spectroscopy and the UCL emission spectroscopy (Fig. 1c and d). In the absorption spectra, the main absorption peaks of PtTPBP in the soybean oil were located at 429 and 612 nm (Fig. 1c, red line) and the characteristic peak of BDP in the soybean oil was at 518 nm (Fig. 1c, blue line). The appearance of these three peaks in the absorption spectrum of UCNCs dispersed in aqueous solution confirmed the coexistence of PtTPBP and BDP in the nanocapsules (Fig. 1c, black line). Moreover, compared to the respective spectra, the absorption peaks of both the sensitizer and emitter encapsulated in the nanocapsules did not shift significantly, indicating that neither sensitizer nor emitter aggregation occurred. Under 635 nm...
Fig. 4. In vivo UCL images of mice intravenously injected with ~1200 mg/kg UCNCs at various time points post-injection.
CW laser excitation, the UCL spectrum of UCNCs in aqueous solution was recorded by a fluorescence spectrometer with a 633 nm short pass filter. As shown in Fig. 1d, the significant UCL emission with a center band at 558 nm was observed, which further confirmed that the sensitizer and emitter coexisted in the nanocapsule. The hydrodynamic diameter of UCNCs in aqueous solution measured by dynamic laser scattering (DLS) was 235 nm (Fig. S1 in the SI). Atomic force microscope (AFM) imaging confirmed the DLS result and clearly showed core-shell structure of UCNCs (Fig. S2 in the SI). Furthermore, a representative single nanocapsule was chosen to calculate the amount of PtTPBP and BDP incubated with UCNCs at a concentration of 12 mg/mL for up to 48 h, the relative cell viability was still greater than 85% (Fig. S5 in the SI). It is worth noting that we used more than a two-fold concentration in our experiments compared to previous studies [7,15]. These results suggested no obvious cytotoxicity of UCNCs.

However, although the results of the cytotoxicity assay did not show significant cell death, this does not mean that cell function was not affected. Cell migration is a highly integrated multistep process and plays a key role in many stages from embryo to adult [32]. Therefore, it is important to determine the migratory capacity of cells following incubation with UCNCs. In brief, HeLa cells were incubated with UCNCs for 24 h and washed with FBS-free medium. The cells were collected and moved to the top chambers of a Transwell system. The medium in the top chambers contained no FBS, while the medium in the bottom chambers contained 10% FBS. If cell function was unaffected, the cells would migrate from the top chambers to the bottom chambers through the micro-porous membrane due to the differences in serum concentration. As shown in Fig. 3b and c and Fig. S6 in the SI, the cells treated with UCNCs at concentrations up to 12 mg/mL for 24 h, the number of cells which passed through the micro-porous membrane was not significantly different to that in the control group. Furthermore, cell morphology was not obviously changed after incubation with UCNCs (Fig. 3b). These results demonstrated that UCNCs did not affect cell migration capability, providing evidence for potential in vivo applications.

3.2. Stability assay of UCNCs in physiological solution

In order to study the stability of UCNCs under physiological conditions, a solution of UCNCs in serum was monitored by DLS and matrix assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF/TOF MS). As shown in Fig. 1e, the variation in size of UCNCs was less than 3% during 8 days of dialysis in PBS. Less change in DLS data of UCNCs in serum was observed before and after dialysis for 8 days. In addition, no information from BDP (Fig. S4a in the SI) was detected by mass spectroscopy in the dialysate of UCNCs (Fig. S4b in the SI), indicating no significant leakage of BDP from the nanocapsules. These results indicated excellent stability of UCNCs in physiological solution over a long period of time.

3.3. Cell uptake of UCNCs

Efficient UCL and fluorescence imaging in living cells was performed using a modified confocal laser scanning microscope (CLSM) [7] to study the cell uptake of UCNCs. HeLa cells were incubated with UCNCs at the concentration of 6 mg/mL for 8 h. After the cells had been washed five times with PBS to remove excess UCNCs, the UCL signal at 500–600 nm was collected under 633 nm CW laser irradiation (Fig. 2a). When excited with the 488 nm CW laser, a conventional fluorescence signal at 500–600 nm from the emitter BDP was observed (Fig. 2b). As seen in Fig. 2c and d, merged UCL, fluorescence and bright-field images indicated that luminescence signal distribution was correlated with successful HeLa cell uptake of UCNCs. Moreover, the UCL signal and the fluorescence signal overlapped perfectly (Fig. 2d), suggesting that the intracellular UCL emission at 500–600 nm was originated from BDP in the UCNCs.

3.4. Cytotoxicity assay and cell migration assay of UCNCs

The cellular toxicity of UCNCs was assessed using human epithelial adenocarcinoma HeLa cells. HeLa cells were incubated in a series of concentrations of UCNCs for 24 h and 48 h, respectively. The relative viabilities of the cells were determined with a standard MTT assay. After 24 h of incubation with UCNCs, the relative cell viabilities were all greater than 91% (Fig. 3a), indicating no obvious cytotoxicity on HeLa cell proliferation was observed in the presence of 0.5–12 mg/mL UCNCs (Fig. 3a and Fig. S5 in the SI). Even when incubated with UCNCs at a concentration of 12 mg/mL for up to 48 h, the relative cell viability was still greater than 85% (Fig. S5 in the SI). It is worth noting that we used more than a two-fold concentration in our experiments compared to previous studies [7,15]. These results suggested no obvious cytotoxicity of UCNCs.

3.5. In vivo and ex vivo UCL imaging of mice

In vivo and ex vivo UCL imaging of mice injected with UCNCs was performed using a modified in vivo imaging system by introducing a 635 nm CW laser as the excitation source [29]. For the imaging studies, the excitation light was cut by placing a 633 nm short pass filter in front of the CCD. At a low laser power density of 62 mW/cm², we first studied the minimum dose of TTA-based upconversion nanocapsules for in vivo UCL imaging. Female Balb/c mice were intravenously injected with UCNCs at a series of doses and imaged 5 min post-injection after sacrifice. As shown in Fig. 5 in the SI, after injection with ~60 mg/kg UCNCs, no obvious UCL signal was detected by the imaging system. When the dose was increased to ~90 mg/kg, a significant UCL signal from the liver was detected.

Fig. 5. Body weight of the mice injected with ~1200 mg/kg UCNCs (black) and without injection (red). Error bars were based on the standard deviations of four samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Therefore, the UCNCs dose of ~90 mg/kg was the minimum imaging dose under the condition of 62 mW/cm² laser power density. In particular, region of interest (ROI) analysis of the UCL signal revealed a high signal-to-noise (SNR) of 36. For the in vivo biodistribution imaging studies, female Balb/c mice were intravenously injected with UCNCs at a dose of ~1200 mg/kg, sacrificed at different time points post-injection and then imaged. Strong UCL emission was observed from the mouse liver after intravenous injection of UCNCs 5 min post-injection, demonstrating that the UCNCs were quickly absorbed by the liver (Fig. 4). Within 1 h, the signal demonstrated that the UCNCs were mainly in the liver and spleen. From 1 h to 12 h, UCL emission gradually reduced, indicating that the UCNCs were metabolized by the liver. At 24 h post-injection, no UCL emission was observed, suggesting that most of the UCNCs may have been eliminated from the mouse.

To further understand the biodistribution of UCNCs, ex vivo imaging of organs was carried out (Fig. S8 in the SI). The liver, spleen, kidney and lung from each mouse were collected and imaged by the modified imaging system. Ex vivo images of these organs showed high accumulation of UCNCs in the liver and spleen, while no appreciable UCL signal from the kidney or lung was detected. The accumulation of UCNCs in the liver and spleen was
expected, as these organs contain many macrophages, part of the mononuclear phagocyte system (MPS), and are involved in the uptake and metabolism of foreign particles [25]. At 24 h post-injection, no UCL signal was detected in these organs which was consistent with in vivo imaging results.

3.6. Body weight monitoring

A useful indicator for studying the toxicity effects of UCNCs is the fluctuation in body weight. In our study, mice were injected with and without ~1200 mg/kg UCNCs in the treatment and control group, respectively. The body weight of mice was recorded once every two days for 60 days. As shown in Fig. 5, death, obvious body weight decrease, and other signs of significant weakness were not observed in UCNCs injected mice over the 60-days period. During the period from day 2 to day 16, there were small weight differences between the mice in the two groups. However, the results of the Student t-test indicated that there was no significant difference between the treatment group and the control group (p > 0.05). After 16 days, the body weight of the mice injected with UCNCs increased in a pattern similar to that of the mice without treatment with UCNCs. These results demonstrated that the body weight of mice was unaffected by UCNCs.

3.7. Histological analysis

Histological analysis is important to evaluate whether the UCNCs caused tissue damage, inflammation or lesions. The analysis was performed using tissues obtained from the major organs including the liver, spleen, kidney, heart and lung to assess signs of the potential toxicity of UCNCs at a dose of ~1200 mg/kg over 60 days. As shown in Fig. 6, the micro-structures of these organs in UCNCs-treated mice were normal, and there were no obvious differences between the treated group and the control group. We did not observe inflammatory infiltrates of hepatocytes in the liver samples. Numerous hepatocytes had two or more nuclei, indicating active function. The red pulp contains a large number of blood cells, which was identified in the spleen samples. The nephron is the basic unit of structure and function of the kidney, and consists of the renal corpuscle and renal tubule. The renal corpuscle composes of the glomerulus and Bowman's capsule. The renal corpuseles and renal tubules were easily distinguished in the kidney samples. Cardiac muscle tissue in heart samples was normal and showed no hydropic degeneration. Moreover, no pulmonary fibrosis or other abnormal phenomena were observed in the lung samples. No necrosis was observed in the groups.

3.8. Hematological and blood biochemical analyses

To confirm the results of the histological analysis and quantitatively evaluate the influence of UCNCs in exposed mice, hematological and blood biochemical analyses were carried out. Following the injection of UCNCs, the blood is the first physiological system they interact with. The UCNCs and other nanomaterials are a similar size to viruses and large proteins, and consequently they may stimulate or suppress the activity of the immune system and affect related hematology and/or blood biochemistry factors such as white blood cells or albumin [33,34].

For the hematological analysis, the following important hematology markers were selected: red blood cells (RBC), white blood cells (WBC), platelets (PLT), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red blood cell volume distribution width-standard deviation (RDW-SD), red blood cell volume distribution width-coefficient of variation (RDW-CV) and neutrophils (NEUT). The hematological results are shown in Fig. 7 and the p values which were calculated using the Student’s t-test by comparing the treatment groups with the corresponding control groups are shown in Table S1. According to the p values, no significant differences between the treatment groups and the corresponding control groups
were observed, indicating that all hematology parameters in the UCNCs-treated groups at 8 days, 30 days and 60 days post-injection were in the normal range.

Blood biochemical analysis is used to determine whether the function of vital organs such as the liver and kidney is damaged. Liver function markers including albumin (ALB), globulin (GLB), ratio of albumin and globulin (A/G), total protein (TP), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) were assessed. All albumin and most of the globulin in serum are synthesized by the liver. ALP is used as a marker for the detection of bile duct obstruction [35]. ALT is specific for cellular injury in the liver and AST is also useful, but less specific [36]. TBIL composed of unconjugated bilirubin and conjugated bilirubin is the product of hemoglobin catabolism [37]. Therefore, many diseases can alter the concentration of these markers in serum [35,38]. As shown in Fig. 8 and Table S2, no obvious liver injury was induced by UCNCs. Indicators of kidney function including blood urea nitrogen (BUN) and uric acid (UA) were examined. These indicators in the serum of UCNCs-treated mice were also normal (Fig. 8, Table S2). Taken together, no appreciable toxicity of UCNCs was observed from the hematology and blood biochemistry results, suggesting that these UCNCs are safe under our experimental conditions for in vivo application.

### 4. Conclusions

In summary, we assessed the in vivo biodistribution and toxicity of UCNCs. These UCNCs showed excellent stability in physiological solution and no leaking of the dye was observed. The in vitro toxicity results showed that the UCNCs had no obvious effects on the proliferation of HeLa cells, and the related cell viability was greater than 85% after incubation with UCNCs at the concentration of 12 mg/mL for 48 h. Furthermore, at this high concentration, HeLa cell migration was not affected by UCNCs after 24 h incubation. The biodistribution results from in vivo UCL imaging showed that UCNCs mainly accumulated in the liver and spleen and were eliminated from the mouse within 24 h. When intravenously injected with UCNCs at a dose as high as 1200 mg/kg, the body weights of mice were not significantly affected over a period of 60 days. Histology, hematology and blood biochemistry results showed no apparent toxicity of UCNCs in treated mice. Our results provide an important reference for the future clinical application of UCNCs.

### Conflict of interest

No financial conflict of interest was reported by the authors of this paper.

### Acknowledgment

The authors acknowledge the financial support from the State Key Basic Research Program of China (2015CB931800), the National Natural Science Foundation of China (21375024, 21231004) and the Research Fund for the Doctoral Program of Higher Education (20130071110036).

### Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2016.10.008.

### References


