


Folic Acid-Conjugated CuFeSe₂ Nanoparticles for Targeted T2-Weighted Magnetic Resonance Imaging and Computed Tomography of Tumors In Vivo

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Background: Development of new long-circulating contrast agents for computed tomography (CT) and magnetic resonance imaging (MRI) of different biological systems still remains a great challenge. Here, we report the synthesis of folic acid (FA)-targeted CuFeSe₂ nano-contrast agent for CT and MRI imaging in vitro and in vivo.

Methods and Results: In our study, CuFeSe₂ was fabricated through a facile and green aqueous reaction and then further aminated through silanization. The amine-functionalized CuFeSe₂-NH₂ nanoparticles enable the covalent conjugation of folate-conjugated polyethylene glycol (FA-PEG-COOH) as a targeting ligand onto their surface, which could improve the dispersion and endue the targetability of nanoparticles, respectively. The formed multi-functional CuFeSe₂-PEG-FA nanoparticles were characterized via different techniques, which exhibited outstanding dispersion, good biocompatibility and excellent FA-targeted capability. Meanwhile, the nanoparticles were quite safe in the given concentration range as confirmed by in vitro and in vivo toxicity assay. Importantly, CuFeSe₂-PEG-FA nanoparticles were successfully applied in CT/MRI dual-modality imaging in vitro and in vivo, which showed a better imaging performance and targeted capability.

Conclusion: Therefore, the constructed CuFeSe₂-PEG-FA nanoparticles have a great potential as an efficient contrast agent for dual-modality imaging of different biological systems.

Keywords: CuFeSe₂ nanoparticles, magnetic resonance imaging, computed tomography imaging, dual-modality imaging, folic acid

Introduction

Computed tomography (CT) is one of the most widely used imaging techniques in diagnostic medicine due to its high resolution, unlimited tissue penetration and cost effectiveness.^{1,2} The follow-up emerging magnetic resonance imaging (MRI) has also been identified as a powerful and noninvasive imaging technique because of its no ionizing radiation and high-quality soft tissue contrast.^{3,4} However, use of a large amount of contrast agents is often required for accurate diagnosis of various diseases because non-contrast techniques have lower sensitivity and the use of the contrast agents can help to clarify images and allow better interpretation.^{5,6} It is well-known that multimodal imaging can integrate the advantages of different imaging modalities by overcoming their individual limitations.^{7,8} CT imaging is very suitable for bone and calcification imaging and MRI can offset the poor soft-

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tissue contrast of CT, so that this CT/MRI dual-modality imaging is of significance to the clinical complementation and beneficial for improving the diagnostic efficiency of diseases.⁹ More importantly, the future of CT/MRI will greatly benefit from the development of CT/MRI dual-modality contrast agents.

Currently, clinically used control agents, eg small molecular iodinated contrast agents and gadolinium compounds, are some single-modality control agents for CT or MRI imaging, which suffer from lower sensitivity, potential allergy and renal toxicity.^{10,11} Compared with the above conventional contrast agents, nanoparticles refer to a class of materials with a one-dimensional space size <100 nm, which have special volume and structure. In addition, nanomaterials are not easily degraded by various enzymes and have advantages of low toxicity, high signal intensity, excellent targeting capability, long half-life, good surface activity and strong surface modification.¹² This indicates that nano-contrast agents have a great potential for molecular imaging. To date, some contrast agents have been fabricated for CT/MRI dual-modality imaging, including FeBi NPs, Gd chelated Au NPs, FePt NPs, Au or TaOx decorated Fe₃O₄ NPs, and NaHoF₄ NPs.^{9,13–19} These nanoparticles have displayed inspiring performance and may be used as some promising contrast agents for CT/MRI dual-modality imaging of different biological systems.

CuFeSe₂, a ternary sulfide nanomaterial, has high photothermal conversion efficiency (82%), superparamagnetic properties and effective X-ray attenuation, which makes for ideal nanoprobe for multimodal imaging and tumor photothermal therapy.³ It's reported that some nanoparticles based on CuFeSe₂ have been developed for modality imaging and photothermal therapy, eg CuFeSe₂@PTMP-PMAA nanocrystals, CFS@DOX, MPEG-PCL@CuFeSe₂ and bioactive glass scaffolds functionalized by the CuFeSe₂ nanocrystals.^{3,20–22} Furthermore, we have been engaged in the development of multimodal contrast agents based on CuFeSe₂ in recent years and successfully fabricated the nanoparticles based on CuFeSe₂ for modality imaging.^{21,23} However, the attention about molecular research based on CuFeSe₂ was not as much as we thought in the field of multimodal imaging due to complex synthetic processes, unstable performance and non-definite targetability.^{3,22} Therefore, there is currently still a need for developing CuFeSe₂-based modality contrast agents with great clinical translation potential.

Of course, an outstanding contrast agent should not only have excellent imaging performance but also own good targeting capability. For the targeted tumor imaging, it is necessary to modify nanoparticles with specific ligands that can recognize molecular signatures on the surface of cancer cells. As one of the most studied cancer-targeting ligands, folic acid (FA) has been known to target FA receptors (FAR) that are overexpressed in some malignant tumors including brain, head and neck, lung, breast, ovary, endometrium, kidney, and myeloid cancers.^{24–26} In contrast, the expression of FAR on normal tissue cell is more conservative. Therefore, the overexpression of FAR provides a theoretical basis for targeted therapy and imaging of some specific tumors. Up to now, some nanomaterials based on FA have been fabricated as potential control agents for targeted imaging and therapy of some tumors.^{27–29}

Inspired by the property of CuFeSe₂ and the targetability of FA, in our study, novel types of CuFeSe₂-PEG-FA nanoparticles were synthesized and explored as dual-modality contrast agents for CT and MRI. A series of evaluations were carried out to characterize their morphology, size, biocompatibility, toxicity and targetability. Furthermore, the developed CuFeSe₂-PEG-FA nanoparticles were used as a nanoprobe for CT/MRI dual-modality imaging in vitro (4T1 and HepG2 cells) and in vivo (4T1 tumor model).

Materials and Methods

Chemicals and Materials

Selenium powder (Se, ≥99.5%, WM = 78.96), sodium borohydride (NaBH₄, ≥99.0%, WM = 37.83), CuCl₂·2H₂O (≥99.0%, WM = 170.48), FeSO₄·7H₂O (≥99.0%, WM = 278.01), 3-aminopropyltriethoxysilan (APTES, ≥98%, WM = 221.37), dimethylformamide (DMF), folic acid (FA, ≥98%, WM = 441.4), NH₂-PEG-COOH (MW = 2000), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, ≥98%, MW = 191.7), N-hydroxysuccinimide (NHS, ≥98%, WM = 115.09) and chloral hydrate were purchased from Shanghai Macklin Biochemical Co., Ltd. Roswell Park Memorial Institute-1640 (RPMI-1640) medium, Dulbecco's minimum essential medium (DMEM) high sugar medium and fetal bovine serum (FBS) were purchased from Sigma-Aldrich. Penicillin-streptomycin solution, trypsin digestion fluid and PBS buffer (pH 7.2–7.4) were purchased from Beyotime.

Synthesis of CuFeSe₂-NH₂, CuFeSe₂-PEG-FA

The magnetic CuFeSe₂ nanoparticles were prepared according to a previously reported method.³ 39.48 mg of Se powder and 50 mg of NaBH₄ were dispersed in 100 mL of ultrapure water and stirred at room temperature at ambient conditions with the protection of argon flow. After the Se powder was completely dissolved, a 5 mL mixture of CuCl₂·2H₂O (42.62 mg) and FeSO₄·7H₂O (69.75 mg) was added. The mixture was stirred at room temperature for 2 h. The nanocrystals were separated by centrifugation, dried under reduced pressure and stored at 0–4 °C. CuFeSe₂-NH₂ nanoparticles were obtained by adding 40 µL of APTES to a suspension of 10 mg CuFeSe₂ in 2 mL DMF. The mixture was sonicated for the first 2 minutes and kept under energetic stirring for 6 h at room temperature. The nanoparticles were washed from the unreacted APTES by three centrifugation and redispersion steps in DMF.

Next, folate-conjugated polyethylene glycol (FA-PEG-COOH) was fabricated on the basis of the method in a previous study.³⁰ To modify the CuFeSe₂-NH₂ nanoparticles with FA-PEG-COOH, 5.4 µmol FA-PEG-COOH (12.39 mg), 27 µmol EDC (5.18 mg) and 27 µmol NHS (3.11 mg) were dissolved in 2 mL DMSO respectively. The three solutions were mixed and stirred for 2 h to activate the carboxyl groups of FA-PEG-COOH. Then 20 mg CuFeSe₂-NH₂ nanoparticles dissolved in DMF (10 mL) were added to the activated FA-PEG-COOH solution, and the mixture was stirred mildly for 3 h at room temperature in the dark. To ensure a maximum PEG density, the last functionalization step was achieved overnight, under vigorous stirring at 60 °C. The CuFeSe₂-PEG-FA nanoparticles were precipitated by centrifugation (10,000 rpm/min, 10 min), and purified with ethanol through several cycles of redispersion and centrifugation. The synthesis process is shown in Figure 1.

Characterization

JEM 1200EX transmission electron microscope (TEM, FEI, USA) was used to detect the morphology and energy spectrum of three nanoparticles, including CuFeSe₂, CuFeSe₂-NH₂ and CuFeSe₂-PEG-FA. The elemental composition of CuFeSe₂ and CuFeSe₂-PEG-FA was analyzed respectively by X-ray powder diffractometer (XRD, Bruker, Germany). The scanning conditions were as follows: 2θ range 10–90°, scanning rate 6°/min, and scanning step length 0.02. Various elemental contents of CuFeSe₂-PEG-FA were detected using inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent, USA). Test conditions were as follows: emission power 1.0 kW, plasma gas flow 15 L/min, auxiliary gas flow 1.5 L/min, argon carrier, detection mode for axial observation, linear calibration. Different chemical bonds in CuFeSe₂-NH₂, and CuFeSe₂-PEG-FA nanoparticles were explored by Fourier transform infrared spectrometer (FTIR, Shimadzu, Japan). The absorbance of CuFeSe₂-PEG-FA nanoparticles solution was measured with a SHIMADZU UV-2600 spectrophotometer.

In vitro CT and MRI of CuFeSe₂-PEG-FA samples were performed on IQon CT (Philips, Holland) and 3.0 T MRI (Siemens, German) respectively. As a control, in vitro CT imaging of iohexol (Yangzijiang Pharmaceutical Group Limited) at the corresponding concentration was also carried out. The scanning parameters of CT were as follows: tube voltage 120 kV, tube current 100 mAs, matrix 512×512, slice thickness 0.8 mm, and field of view (FOV) 150 mm. The parameters of MRI were as follows: repetition time (TR) 5290 ms, echo time (TE) 68 ms, matrix 256×256, slice thickness 1 mm, field of view 180 mm, number of excitations 2.

Cell Culture and Cytotoxicity Assessment

4T1 (murine breast cancer cells) and HepG2 cells were obtained from ATCC. Two cells were cultured in RPMI-

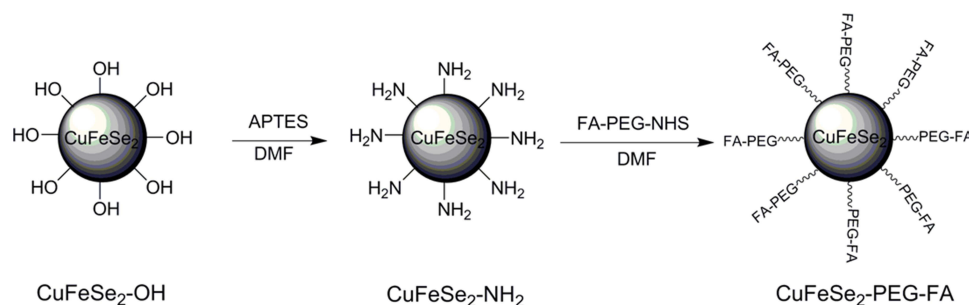


Figure 1 Schematic representation of the synthesis of multifunctional CuFeSe₂-PEG-FA nanoparticles.

1640 and DMEM respectively supplemented with 10% FBS in a cell incubator at 37 °C and 5% CO₂ conditions. The cell cytotoxicity in vitro was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 4T1 and HepG2 cells were seeded into a 96-well cell culture plate at 5000/well and then incubated for 24 h at 37 °C and 5% CO₂. RPMI-1640 solutions of CuFeSe₂-PEG-FA nanoparticles with different sizes (concentrations: 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL) were added to the wells. 4T1 and HepG2 cells were then incubated for 24 h at 37 °C and 5% CO₂, and the cell viability was calculated using a typical MTT assay.

In Vitro Targeted Assessment

4T1 and HepG2 cells (1×10^4 /mL) were seeded into a 6-well cell culture plate at 2 mL/well and then incubated overnight at 37 °C and 5% CO₂. And then, DMEM solution of CuFeSe₂-PEG-FA and CuFeSe₂ nanoparticles was added to the wells (2 mL/well, 100 µg/mL) respectively. As a control, the DMEM solution was added to the corresponding wells and incubated for 6 hours. Next, the cells were washed 5 times with PBS. Finally, the binding condition of the cells and CuFeSe₂-PEG-FA/CuFeSe₂ nanoparticles was evaluated by the inverted fiber mirror (the magnification was $\times 400$).

Furthermore, the related tests were further conducted to verify the successful modification of FA. Briefly, 4T1 cells were seeded in a 6-well plate with a density of 2×10^4 cells per well, and a blocking assay was carried out using two groups of cells. One group (A group) was treated with different concentrations of CuFeSe₂-PEG-FA (0, 50, 100, and 200 µg/mL). The other group (B group) was first treated with FA (300 µg/mL, 1.5 mL, DMEM) for 4 h, and then, different concentrations of CuFeSe₂-PEG-FA (0, 50, 100, and 200 µg/mL) dispersion were added. After incubation for 4 h, each group of cells was washed with PBS five times. Finally, the in vitro CT and MR imaging were performed.

Toxicity Assessment In Vivo

To investigate the in vivo toxicity, CuFeSe₂-PEG-FA nanoparticles (0.58 mmol Fe/kg) were intravenously injected through the tail vein of BABL/C female mice. Then, the mice (n = 15) were sacrificed at different time points post-injection (1, 3, 5, 10, and 15 d, respectively). For comparison, mice (n = 3) injected with glucose solution were used as controls and sacrificed at 15 d. The

major organs (heart, liver, spleen, lung, kidney, and muscle of left thigh) were carefully harvested, weighed, and fixed with 4% formalin solution. Finally, pathological section and hematoxylin and eosin (H&E) staining analysis were carried out to assess the in vivo toxicity of the CuFeSe₂-PEG-FA nanoparticles.

CT/MRI Dual-Modality Imaging of Cancer Cells In Vitro

The suspension of 4T1 and HepG2 cells (1×10^4 /mL, 3 mL) were seeded in T25 culture bottles respectively and incubated at 37 °C and 5% CO₂. After overnight culture to bring the cells to adhesion, the cells were washed 2 times with PBS. Subsequently, 4 mL of CuFeSe₂-PEG-FA solution (DMEM medium preparation, 200 µg/mL) was added to T25 the culture bottle and incubated for 6 hours. As a control, 4 mL of CuFeSe₂ (200 µg/mL) and DMEM solution was added to the corresponding T25 culture bottles respectively. After that, the cells were washed 5 times with PBS and digested for 2 minutes using trypsin digestion fluid. Finally, centrifugal separation was carried out (1000 rpm/min, 2 min) and heavy suspension was performed with 1 mL PBS.

CT imaging of these cell suspensions was performed using IQon CT (Philips, Holland, tube voltage = 120 kV, tube current = 100 mAs, matrix = 512×512 , slice thickness = 0.8 mm, field of view = 150 mm). CT values and effective atomic numbers were measured by the corresponding reprocessing workstation. For MRI, a Siemens Prisma 3.0 T Signa HDxt superconductor clinical MR system (Siemens, German) was used. T2 weighted imaging (T2WI) was obtained with 1 mm slice thickness, 5290/68 ms TR/TE, 180 mm FOV, 256×256 matrix and 2 NEXs.

CT/MRI Dual-Modality Imaging In Vivo

All procedures and animal experiments were approved by the Animal Ethical Committee of Southwest Medical University (2020423) and conducted in accordance with state regulations. Animal care and handling procedures were in agreement with the guidelines of the Regional Ethics Committee for Animal Experiments. The 6- to 8-week-old BABL/C female mice (average body weight: 20 g, Dashuo Animal Technology Limited, Jianyang) were subcutaneously injected with 1×10^6 4T1 cells in 0.2 mL PBS solution on the right leg root. While the tumor nodules reached a volume of 100 mm³, the mice were anesthetized by intraperitoneal injection of 5% chloral

hydrate and 0.1 mL glucose solution of CuFeSe₂-PEG-FA nanoparticles (0.58 mmol Fe/kg) was delivered into the mice via the tail vein. As a control, 0.1 mL CuFeSe₂ solution and 0.1 mL glucose solution (5%) were intravenously injected via the tail vein.

For MRI, 3.0T Prisma MR (Siemens, German) was used to scan the mice with a wrist coil. For each animal, T2WI and T2*map were obtained both before and after administration of the CuFeSe₂-PEG-FA, CuFeSe₂ nanoparticles and 5% glucose solution respectively at the time points of 2 min, 30 min, 1, 1.5, 2, 3, 4, 5, 6, 8, and 12 h post-injection (T2WI: TR = 5290 ms, TE = 70 ms, slice thickness = 2 mm, matrix = 256 × 256, FOV = 120 mm; T2*map: TR = 293.0 ms, TE = 2.98 ms, slice thickness = 2 mm, matrix = 256 × 256, FOV = 120 mm, flip angle = 60°). After the scanning, the section showing the maximum size of the tumor was selected in T2WI and T2*map for further image fusion and regions of interest (ROIs) were delineated to measure T2* values of the tumor using the MRI postprocessing workstation. The above tumor-bearing BALB/C mice were sacrificed after MR scanning at the 12 h time point. And then, the tumor tissues were carefully harvested and fixed with 4% formalin solution. Next, ICP was used to detect Fe element content in tumor tissues.

Further in vivo tumor CT imaging was conducted before and after the intravenous injection of 0.1 mL glucose solution of CuFeSe₂-PEG-FA nanoparticles (dose = 100 mg/kg). The section showing the maximum size of the tumor was selected and the ROI of the tumor was delineated to measure average CT value and effective atomic number. The scanning parameters of CT were as follows: tube voltage 120 kV, tube current 60 mAs, matrix 512 × 512, slice thickness 0.8 mm.

Data Analysis

Continuous variables (T2* values, CT values, and Fe element content in tumor tissues) were expressed as mean ± standard deviation (SD) because the distribution of data was normal. T2* values and CT values before and after injection of the CuFeSe₂-PEG-FA nanoparticles were compared using paired sample *t*-test. Group design *t*-test was used to compare the Fe contents of tumor tissue in the experimental and control group. All statistical analyses were conducted using SPSS 25. A two-sided *p* value < 0.05 was considered significant.

Results and Discussion

Characterization of CuFeSe₂-PEG-FA

The resultant nanoparticles were characterized with TEM to determine their morphology and size. Small spherical particles of CuFeSe₂ are clearly observed (Figure 2A). The crystal structure of the nanoparticles was revealed by their high-resolution TEM (HRTEM) image (Figure 2B) and the continuous selected area electron diffraction (SAED) rings in the inset (Figure 2C). TEM was also used to characterize the morphology and size distribution of the formed CuFeSe₂-NH₂ and CuFeSe₂-PEG-FA nanoparticles (Figure 2D and E). It's clear that both nanoparticles possessed a spherical shape and a uniform size distribution with a mean diameter of 9.80 nm for CuFeSe₂-NH₂ nanoparticles (Figure 2F) and 10.89 nm for CuFeSe₂-PEG-FA nanoparticles (Figure 2G), respectively. By comparison with the pristine CuFeSe₂ nanoparticles that have a size of 5.10 nm (Figure S1), the increased diameter of the nanoparticles should be associated with the APTES modification onto the particle surfaces.

The ratio of Cu/Fe/Se in CuFeSe₂-PEG-FA nanoparticles was 1:1:2, measured by ICP-OES. Meanwhile, energy dispersive X-ray spectroscopy (EDX) was performed to identify the composition and verify the presence of the three elements (Cu, Fe, and Se) in each individual nanocrystal. In supporting information (Figure S2) the EDX spectrum showed that the three elements of Cu, Fe, and Se were coexistent in the synthesized nanoparticles.

The crystal structures of CuFeSe₂ and CuFeSe₂-PEG-FA nanocrystals were further characterized by XRD. The XRD pattern confirmed the existence of CuFeSe₂, and a broad peak from 22° to 28° should be attributed to the amorphous silica. This indicated that silica was successfully coated onto the CuFeSe₂ nanoparticles surfaces (Figure 3A). Further investigation with the FTIR spectra confirmed the presence of the silica shell (Figure 3B). The sample exhibited a strong broad band at around $\nu = 1101 \text{ cm}^{-1}$, corresponding to the Si-O-Si asymmetric (ν_{as}) stretching vibration. TEM images of the core-shell structured CuFeSe₂-NH₂ nanocomposites showed that the mean diameter of the particles was 9.80 nm and the thickness of the silica shell was 4.70 nm. Stability test showed that CuFeSe₂-PEG-FA nanoparticles possessed excellent solubility and stability in different media, such as H₂O, phosphate-buffered saline (PBS), 0.9% saline (NaCl), and 5% glucose solution, as demonstrated by their similar absorbance (Figure S3A). Their excellent colloidal stability was further demonstrated by the absence of precipitates or

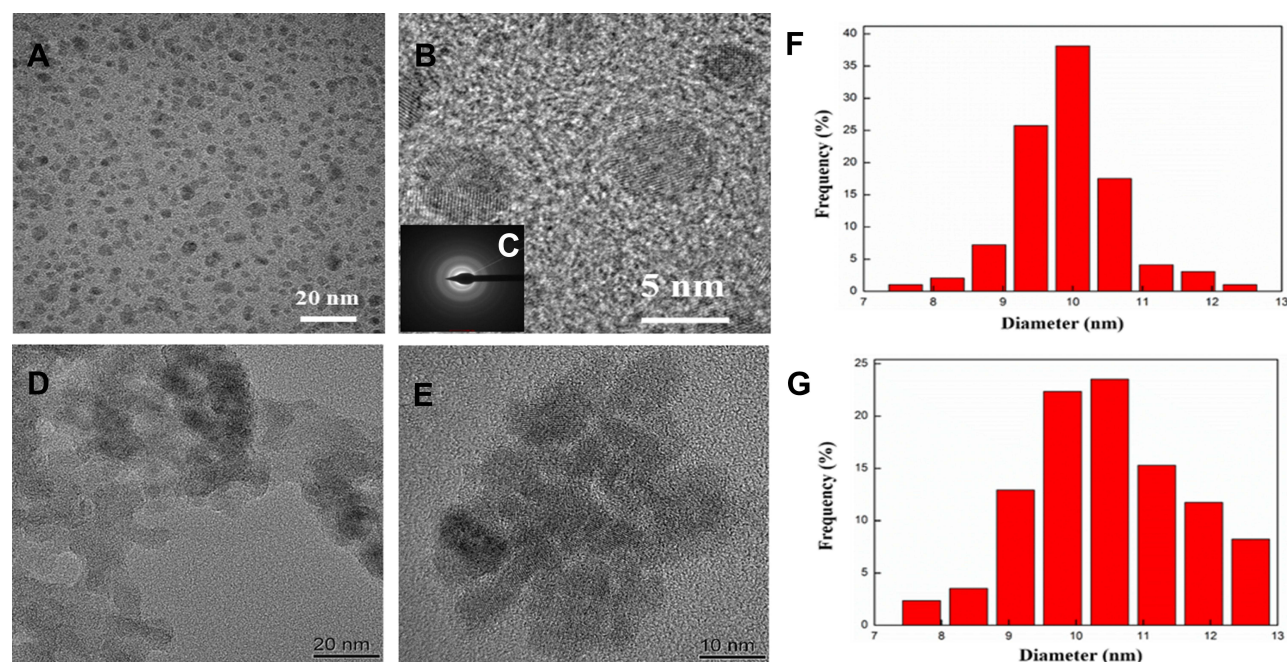


Figure 2 (A) TEM image of CuFeSe₂ nanoparticles. (B) High-resolution TEM image and (C) SAED pattern of CuFeSe₂ nanoparticles. (D) TEM image of CuFeSe₂-NH₂ nanocomposite. (E) TEM image of CuFeSe₂-PEG-FA nanocomposites. (F) The size distribution histograms of CuFeSe₂-NH₂. (G) The size distribution histograms of CuFeSe₂-PEG-FA.

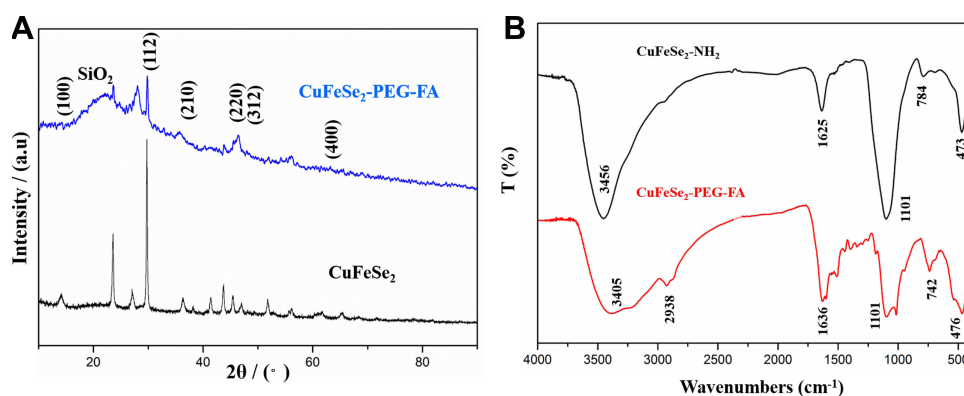


Figure 3 (A) X-ray diffraction pattern of CuFeSe₂ and CuFeSe₂-PEG-FA. (B) FTIR spectra of CuFeSe₂-NH₂ and CuFeSe₂-PEG-FA.

aggregates in their aqueous solutions during 15 days of storage (Figure S3B).

It's important to characterize the X-ray attenuation and magnetic properties of the CuFeSe₂-PEG-FA nanoparticles before their use as a dual-modality contrast agent for CT and MRI. Figure 4A showed that the effective atomic number of the targeted CuFeSe₂-PEG-FA nanocrystals and iohexol increased with the rise of the concentrations. Although the increase of the CuFeSe₂-PEG-FA nanocrystals was inferior to that of iohexol, the effective atomic number of these nanocrystals still displayed a linear upward trend (Figure 4B), which indicated that the

CuFeSe₂-PEG-FA nanoparticles had a great potential for enhanced CT imaging. Meanwhile, we also measured CT values of the targeted CuFeSe₂-PEG-FA nanoparticles, discovering that there still existed a linear upward trend between CT value and the CuFeSe₂-PEG-FA nanoparticles concentration (Figure S5). Furthermore, for the in vitro MRI imaging, it could be seen that the signal intensity of T2WI decreased with the increase of the concentration of the CuFeSe₂-PEG-FA nanoparticles (Figure 4C). Its transverse relaxation rate (r_2) was calculated to be 4.28 mM⁻¹S⁻¹ (Figure 4D). Therefore, these in vitro imaging results demonstrated the robustness of CuFeSe₂-PEG-FA

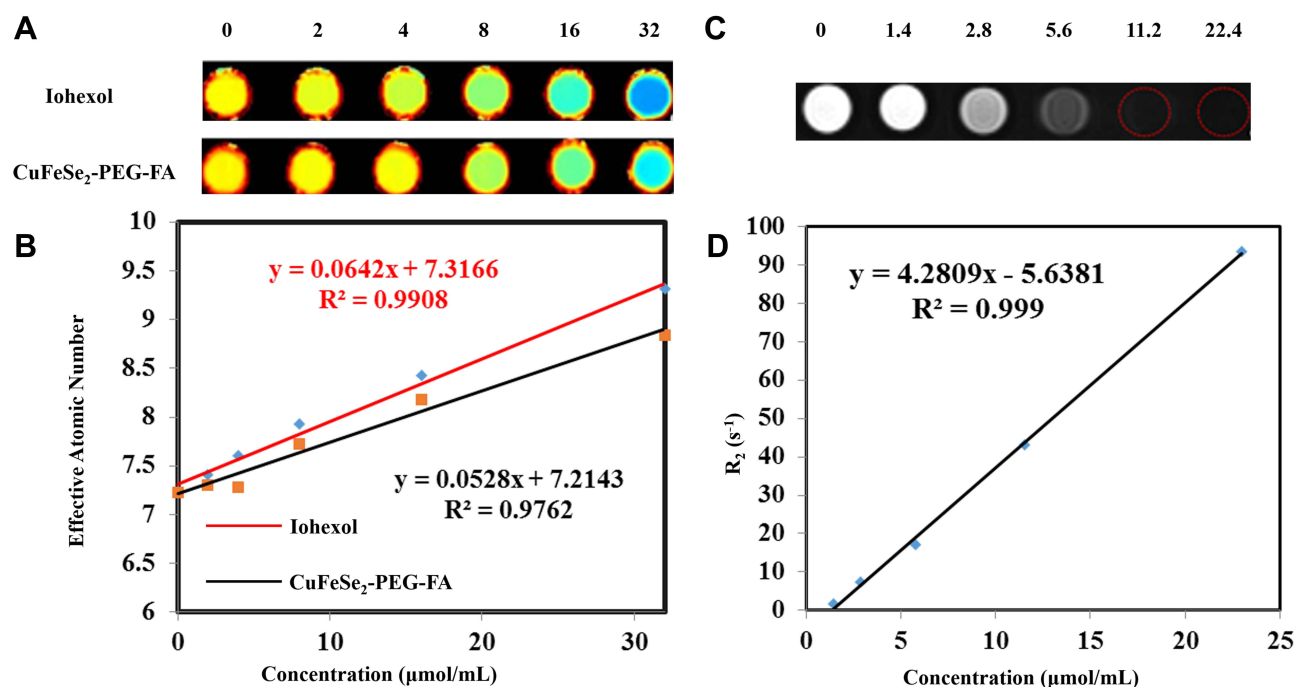


Figure 4 (A) In vitro CT imaging of iohexol and the CuFeSe₂-PEG-FA nanocrystals with the different concentration. (B) The linear correlation between the effective atomic number of iohexol and CuFeSe₂-PEG-FA nanocrystals with their concentrations respectively. (C) In vitro MRI imaging of CuFeSe₂-PEG-FA nanoparticles using different concentrations. (D) The transverse relaxation rate (r_2) was determined to be $4.28 \text{ mM}^{-1}\text{s}^{-1}$.

nanoparticles as a contrast agent for in vivo dual-modality imaging if they were safe and had good biocompatibility.

Cell Culture and Cytotoxicity Assessment

After incubation of 4T1 and HepG2 cells with CuFeSe₂-PEG-FA at the particle concentration of 200, 100, 50, 25, 12.5 and 6.25 μg/mL for 24 h, MTT assay was used to evaluate the cell viability. The results in [Figure S5](#) demonstrated that CuFeSe₂-PEG-FA had good biocompatibility, and no serious cytotoxicity toward 4T1 and HepG2 cells was observed in the range of 0–200 μg/mL, in which the cell viability was higher than 80%.

In Vitro Targeted Assessment

After 4T1 and HepG2 cells were incubated for 6 hours with the DMEM solution of CuFeSe₂-PEG-FA and CuFeSe₂ nanoparticles, we found that more targeted nanoparticles could bind to FA receptor on the surfaces of these cells compared with the control and untargeted groups ([Figure S6A–E](#)). Besides, 4T1 cells bound to more CuFeSe₂-PEG-FA nanoparticles due to FA receptor overexpression compared with HepG2 cells ([Figure S6C and D](#)). However, in the untargeted group, both

cells only bound to a few CuFeSe₂ nanoparticles and no significant difference was found between two cells ([Figure S6E and F](#)). These results confirmed that CuFeSe₂-PEG-FA nanoparticles had excellent targeted capability.

Furthermore, another related test also verified the successful modification of FA further. As is shown in [Figure S7](#), the MR signal intensity of two groups decreased with the increase of the concentration of the CuFeSe₂-PEG-FA nanoparticles due to the dose-dependence of cellular uptake ([Figure S7A and C](#)). Besides, the signal intensity of A group was lower than that of B group and MR signal intensity was significantly different between two groups at the concentrations of 100 μg/mL ($p = 0.047$) and 200 μg/mL ($p = 0.049$). Moreover, CT showed us that CT values of two groups increased with the increase of the concentration of the CuFeSe₂-PEG-FA nanoparticles and CT values of A group were higher than that of B group ([Figure S6B and D](#)). Importantly, CT values had also a significant difference between two groups at the concentrations of 100 μg/mL ($p = 0.035$) and 200 μg/mL ($p = 0.009$). What this suggests is that 4T1 cells without experiencing a blocking assay can bind to more CuFeSe₂-PEG-FA nanoparticles compared with the control group, which verifies the successful modification of FA.

Toxicity Assessment In Vivo

No death occurred and no abnormal activity was found in the mice within 15 d after they were injected with the CuFeSe₂-PEG-FA nanoparticles via the tail vein. For these mice, H&E staining indicated the microstructures of various organs including myocardial striation and muscle fibers, hepatic lobules and the hepatocytes, lymphoid follicles and germinal center, alveoli, collecting ducts and thigh muscle fibers, all exhibited normal morphology and were arranged regularly at different time points. No tissue necrosis and inflammatory response were observed compared with the control group (Figure 5).

CT/MRI Dual-Modality Imaging of Cancer Cells In Vitro

To confirm the cancer cell targeting ability of the CuFeSe₂-PEG-FA nanoparticles and validate their CT and MRI performance, 4T1 (FAR overexpression)³¹ and HepG2 cells (Less FAR expression)³² were treated with the CuFeSe₂-

PEG-FA nanoparticles at 37 °C and 5% CO₂ for 6 h. Then, CT imaging and T2WI of the cells were performed. From the T2WI and CT images of 4T1 cells treated with the CuFeSe₂-PEG-FA nanoparticles (Figure S8), MR signal intensity decreased (Figure S8A and C), and CT value (Figure S8b, 8d) increased compared with that of 4T1 cells treated with the CuFeSe₂ nanoparticles and glucose solution. What this suggests was that CuFeSe₂-PEG-FA nanoparticles may be an appropriate positive control agent for enhanced CT imaging and a negative agent for T2WI.

In contrast, from the T2WI and CT images of HepG2 cells, there was no obvious change for both MR signal intensity, and CT values between the targeted and untargeted nanoparticles due to the less expression of FAR on the surface of HepG2 cells (Figure S8A and B). For 4T1 and HepG2 cells treated with the CuFeSe₂-PEG-FA nanoparticles, the former had lower MR signal intensity ($p = 0.008$), higher CT value ($p = 0.035$) and their differences were statistically significant between two cells. This further

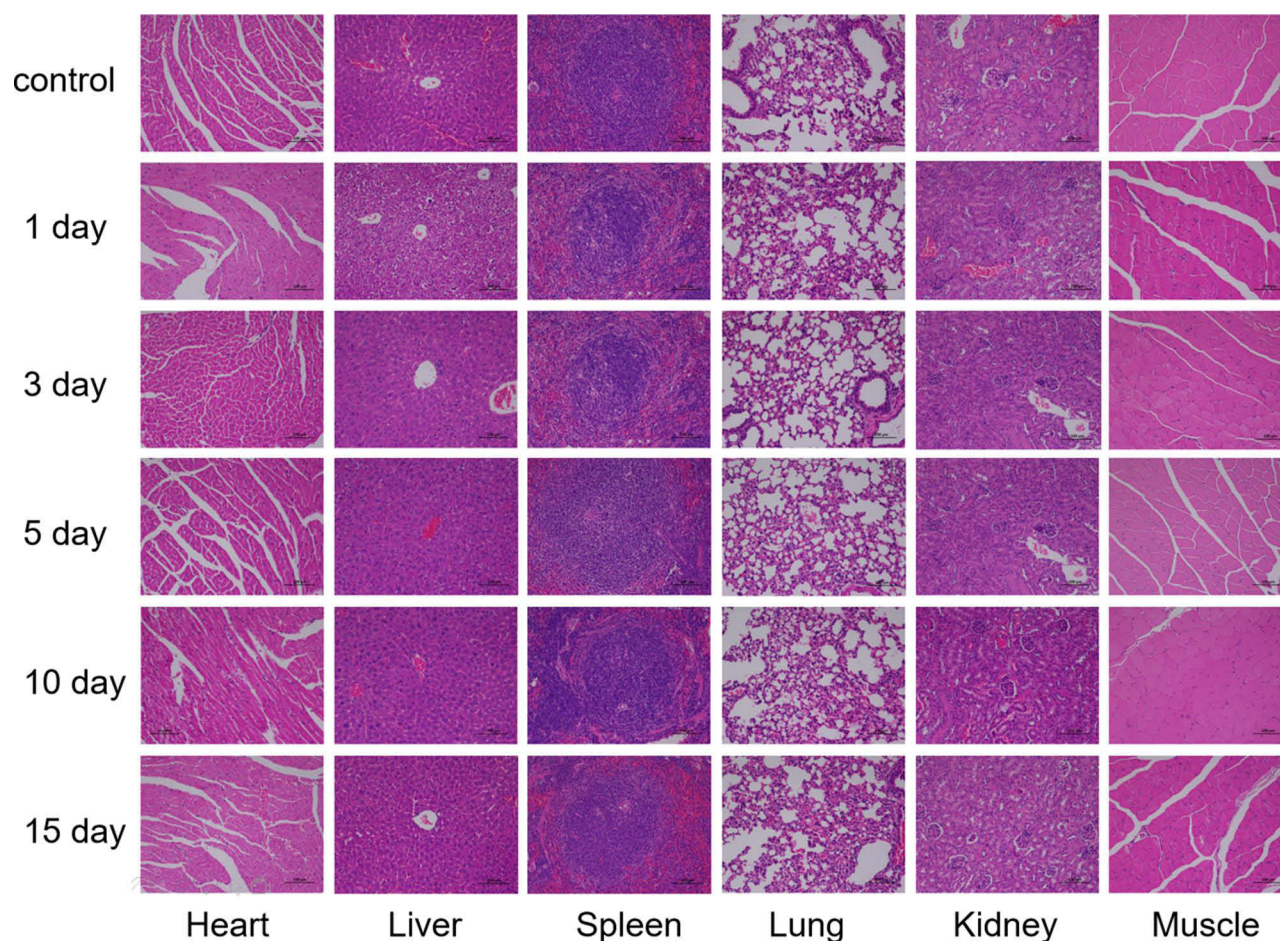


Figure 5 Hematoxylin and eosin (H&E) staining of important organs for normal mice at different time points after the injection of CuFeSe₂-PEG-FA contrast agent and 5% glucose solution via the tail vein.

illustrated the role played by the attached FA ligands that were able to direct an active targeting to 4T1 cells over-expressing FAR via a receptor-mediated pathway.

CT/MRI Dual-Modality Imaging In Vivo

First, we explored the feasibility of using CuFeSe₂-PEG-FA nanoparticles for specific MRI of tumors in vivo (Figure 6) and the same dosage of CuFeSe₂ and 5% glucose solution were used as a control. Figure 6A showed the fusion images of T2WI and T2* map for 4T1 tumor model obtained before injection, and 2 min, 30 min, 1 h, 5 h and 12 h post-injection, respectively. It could be seen that the tumor MR signal of mice injected with CuFeSe₂-PEG-FA nanoparticles gradually decreased as a function of time (the range from 0–5 h) and subsequently gradually increased (Figure 6A). Besides, a significant difference was found between T2* values of tumor tissue before injection and that at the time points of 5 h and 8 h post-injection ($p < 0.05$) (Table 1). However, no

significant difference was found between T2* values of tumor tissue before and after injection in liver and muscle tissue ($p > 0.05$) (Tables S1 and S2). The above results demonstrated that the targeted CuFeSe₂-PEG-FA nanoparticles had a great potential to be a negative control agent for T2WI.

For the untargeted group, it could be observed that the tumor MR signal had a similar trend with the targeted group (Figure 6B). However, there was no significant difference between T2* values of tumor tissue before and after injection. In sharp contrast, the tumor MR signal of mice treated with 5% glucose solution increased first and then decreased gradually (Figure 6C). No significant difference was found between T2* value of tumor tissue before and after injection, which indicated that 5% glucose solution could not reduce T2* value of tumor tissue. In addition, T2* value was significantly different for the experimental (21.42 ± 2.79) and control group (32.35 ± 4.21) ($p < 0.01$), which further confirmed that the CuFeSe₂-PEG-FA nanoparticles could

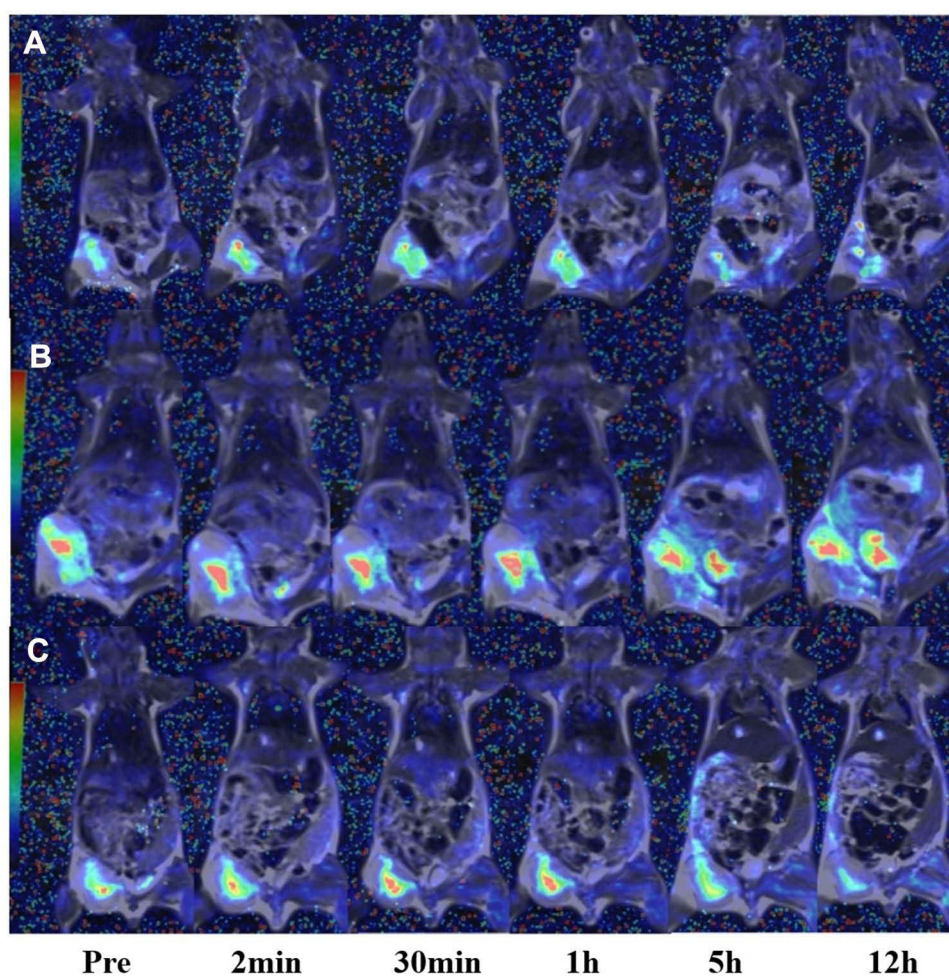


Figure 6 The fusion images of T2WI and T2* map for 4T1 tumor model, acquired at 0, 2 min, 30 min, 1 h, 5 h and 12 h after the mice were intravenously injected with the CuFeSe₂-PEG-FA nanoparticles (A), CuFeSe₂ nanoparticles (B) and 5% glucose solution (C) respectively.

Table 1 The T2* Value of Tumor at Different Time Points Before and After the Injection

Scanning Time	The T2* Value of Tumor in Experiment Group (n = 3)	p	The T2* Value of Tumor in Control Group (n = 2)	p	The T2* Value of Tumor in Untargeted Group (n = 2)	p
Pre	28.20 ± 4.27		30.88 ± 12.18		28.38 ± 6.12	
2 min	22.30 ± 4.19	0.138	32.66 ± 6.50	0.734	27.04 ± 4.37	0.729
30 min	22.16 ± 3.56	0.302	31.12 ± 10.20	0.892	28.28 ± 3.93	0.979
1 h	22.31 ± 0.27	0.126	37.61 ± 1.30	0.542	27.85 ± 4.70	0.853
1.5 h	22.19 ± 1.32	0.155	37.51 ± 2.60	0.507	24.61 ± 2.67	0.271
2 h	16.99 ± 5.18	0.052	31.53 ± 4.95	0.919	24.21 ± 3.89	0.329
3 h	21.58 ± 3.19	0.190	36.04 ± 6.96	0.395	24.53 ± 3.52	0.182
4 h	21.49 ± 4.26	0.223	34.70 ± 8.58	0.375	24.11 ± 3.21	0.159
5 h	18.14 ± 1.00	0.040	35.07 ± 4.36	0.587	22.12 ± 3.08	0.072
6 h	21.41 ± 4.79	0.261	31.03 ± 4.29	0.983	22.67 ± 1.39	0.186
8 h	21.48 ± 2.08	0.045	24.48 ± 1.40	0.555	23.15 ± 2.98	0.263
12 h	18.82 ± 3.87	0.099	25.59 ± 0.22	0.644	23.77 ± 3.82	0.291

Note: The T2* values of tumor before and after the injection were expressed as mean ± SD.

reduce the T2* value of tumor tissue after excluding the interference of 5% glucose solution.

ICP results displayed that Fe element content of tumor tissue in the experimental group (33.40 ± 1.78 mg/kg) was higher than that in the untargeted (28.53 ± 1.21 mg/kg) and control group (26.30 ± 1.70 mg/kg) and there existed a significant difference between the pairwise ($p < 0.05$) (Figure S9). What this suggested was that CuFeSe₂-PEG-FA nanoparticles could reduce the T2* value by increasing Fe content of tumor tissue.

For the CT imaging of intravenous injection, we could see that the average CT values of tumor tissue after administration of the CuFeSe₂-PEG-FA nanoparticles were higher than those before injection (Figure 7). Table S3 showed that there was a significant difference between CT values before and after injection (at the time points of 8 h and 12 h post-injection, $p < 0.05$). Hence, the CuFeSe₂-PEG-FA

nanoparticles had the power to raise CT values of tumor tissue at larger concentrations and may serve as an excellent CT contrast agent for various biomedical applications.

Conclusion

In summary, a facile approach has been developed to generate multifunctional CuFeSe₂-PEG-FA nanoparticles for CT/MRI dual-modality targeted imaging. The formed multifunctional CuFeSe₂-PEG-FA nanoparticles exhibited outstanding water solubility, cytocompatibility, hemocompatibility, and biosafety in the given concentration range. With the targeting specificity to cancer cells overexpressing FAR being confirmed, the developed CuFeSe₂-PEG-FA nanoparticles were able to be used as an efficient nanoprobe for CT/MRI dual-modality imaging of cancer cells in vitro and a xenografted tumor model in vivo via a FA-mediated

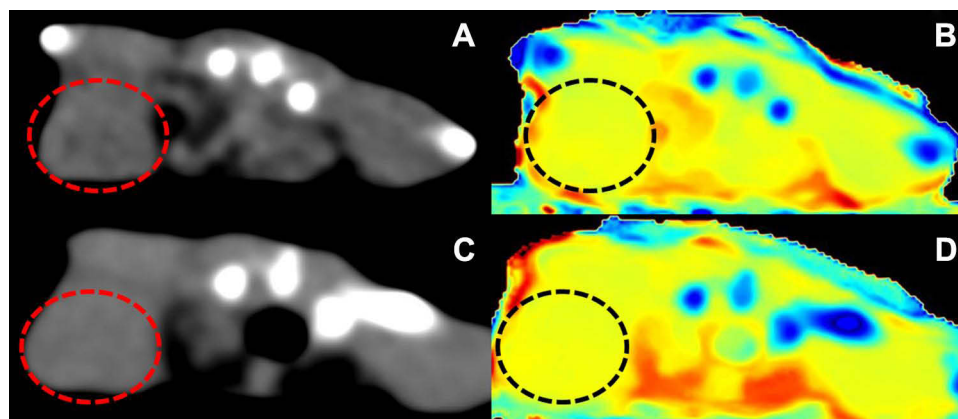


Figure 7 (A) and (C) are conventional CT images before and after intravenous injection respectively. (B) and (D) are effective atomic number images before and after intravenous injection respectively. The red and black circles represent the tumor tissues.

active targeting pathway. Our results suggested that this nanoparticle may appear as an attractive candidate for a future theranostic platform.

Abbreviations

CT, computed tomography; MRI, magnetic resonance imaging; FA-PEG-COOH, folate-conjugated polyethylene glycol; FA, folic acid; FAR, FA receptors; NaBH₄, sodium borohydride; APTES, 3-aminopropyltriethoxysilan; DMF, dimethylformamide; EDCI, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; NHS, N-hydroxysuccinimide; RPMI-1640, Roswell Park Memorial Institute-1640; DMEM, dulbecco's minimum essential medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; TEM, transmission Electron microscope; XRD, X-ray powder diffractometer; ICP-OES, inductively coupled plasma optical emission spectrometry; FTIR, Fourier transform infrared spectrometer; FOV, field of view; T2WI, T2 weighted imaging; TR, repetition time; TE, echo time; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HRTEM, high resolution transmission electron microscopy; SAED, selected area electron diffraction; EDX, energy dispersive X-ray spectroscopy.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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