Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Novel multifunctional nanospheres of $Zn_{1/3}Fe_{8/3}O_4$@Ag: synthesis, properties and application for multi-modality tumor imaging

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1. Experimental

1.1 Materials

All reagents of analytical grade purity were purchased from Shanghai Chemical Reagents Co. and used without further purification. The ethanol was redistilled with the traditional Mg-I_2 system to increase the purity above 99.95%. The phosphate buffer solution (PBS, 0.1 M, pH 7.4) was prepared by using double distilled water (Millipore Ltd. USA).

1.2 Synthesis of the multifunctional nanospheres of $Zn_{1/3}Fe_{8/3}O_4$@Ag

Step 1: A 0.7 mmol amounts of $ZnCl_2$, 1.3 mmol $FeCl_2 \cdot 4H_2O$ and 4 mmol of $FeCl_3 \cdot 6H_2O$ were dissolved in a mixture of diethylene glycol (DEG) and diethanolamine (DEA) (2:1, v/v) in a flask under protection with nitrogen gas. Separately, 16 mmol of NaOH was dissolved in a mixture of DEG and DEA (2:1, v/v). The solution of NaOH was added to a solution of metal chlorides with stirring at room temperature, causing an immediate colour change from bright yellow to deep green-

brown. After 1h, the temperature of the solution was raised during 1 h to 200°C and then kept constant for 8h in the temperature of 200°C. The solid product was isolated by cooling the reaction mixture to room temperature and centrifuging. A black solid was obtained and washed with ethanol twice and a mixture of ethanol and ethyl acetate (1:1, v/v) three times to remove the excess of DEG and DEA.

Step 2: 1 mL of $AgNO_3$ aqueous solution (34 μg/mL) was freshly prepared and used for the synthesis of Ag NCs. 1 mL of GSH aqueous solution (38 μg/mL) was added dropwise into the $AgNO_3$ solution and let to react with silver ions until it became clear. The bulky molecular structure of GSH as the ligand could play a critical role in the gelation with Ag^+ ions, leading to the formation of the relevant stable hydrogel complex[1]. Afterwards, The complex mixture was added into superparamagnetic iron oxide nanospheres ($Zn_{1/3}Fe_{8/3}O_4$) mixture and incubated at 37°C about 7 days. The product was diluted with water (15 mL) and washed three times by centrifugation (30 min, 8000 rpm) and redispersion in water (5 mL). At last, the multifunctional nanospheres of $Zn_{1/3}Fe_{8/3}O_4$@Ag could be readily obtained.

1.3 Characterization of the multifunctional nanospheres of $Zn_{1/3}Fe_{8/3}O_4$@Ag

The morphology, microstructure and magnetic property of the as-prepared $Zn_{1/3}Fe_{8/3}O_4$@Ag nanospheres were characterized by using JEM-2100 transmission electron microscope operating at 200 kV and scanning electron microscopy (SEM, JEOL JSM-5900) with EDS accessory. The PL spectra was performed on a Hitachi F-7000 fluorescence spectrophotometer. The MR imaging and relaxivity performance of $Zn_{1/3}Fe_{8/3}O_4$@Ag nanospheres were tested by using a Siemens Avanto MRI scanner

system with a magnetic field of 7.0 T and a Mico-MRI (PharmaScan, Bruker, Germany). CT images were collected using a small animal X-ray CT scanner (SOMATOM Emotion, Siemens, Germany).

1.4 Cell culture

HepG2, HeLa and K562 cancer cells (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were maintained in DMEM/High glucose medium containing 10% fetal bovine serum and antibiotics (100 mg/mL streptomycin and 100 IU/mL penicillin) at 37 °C in a CO_2 incubator.

1.5 *In vitro* cytotoxicity studies of the multifunctional nanospheres of $Zn_{1/3}Fe_{8/3}O_4$@Ag

The viability of cells was measured by the MTT method. Cancer cells (HepG2, HeLa and K562) and normal cells (L02) were seeded in 96-well plates at a concentration of 1.0×10^4 cells/well, respectively, and incubated over night at 37°C in a 5% CO_2 humidified environment. Then the cells were separately treated with different concentration （1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 and 160.0 μg/mL) of $Zn_{1/3}Fe_{8/3}O_4$@Ag. The final of $Zn_{1/3}Fe_{8/3}O_4$@Ag in each well of the cancer cells were treated with a final concentration of (dissolved with DMEM/High glucose medium), separately. The control groups were cultivated under the same condition without Nanospheres. After all samples were cultured for 48 hours, 20 mL of 5 mg/mL MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added and incubated for additional 4 h. Subsequently, the plate was centrifuged at 1000 rpm for 10 min and the supernatant was discarded, followed by addition of 150 mL of DMSO

into each well and gently shaking in the shaker incubator at 37 °C for 10 min. After this, the optical density (OD) at 492nm was recorded. The cell viability was expressed as follows:

Cell viability (%) = [A]test/[A]control×100%, where [A] represents the absorbance value at 492nm. Each experiment was repeated at least three times.

1.6 Confocal fluorescence microscopic studies in HeLa cells

Confocal fluorescence microscopy (Leica TCS SP2) measurements at an excitation wavelength of 590 nm were used to assess the method potential for bio-imaging of cancer cells treated with $Zn_{1/3}Fe_{8/3}O_4$@Ag. Thus, HeLa cell cultures were incubated with 160 μg/mL $Zn_{1/3}Fe_{8/3}O_4$@Ag following the above protocol. For control experiments, DMEM (high glucose) medium was used instead of $Zn_{1/3}Fe_{8/3}O_4$@Ag. After 24 h, both freshly prepared cell cultures were dropped on rigorously cleaned glass plates and immediately examined by confocal fluorescence microscopy.

1.7 Construction of the xenografted tumor mouse model

BALB/c female athymic nude mice, age-matched (four weeks of age) and weight-matched (18–22g), were purchased from Peking University Health Science Center. All experiments involving mice were approved by the National Institute of Biological Science and Animal Care Research Advisory Committee of Southeast University, and experiments were conducted following the guidelines of the Animal Research Ethics Board of Southeast University. The mice were randomly assigned to groups for experimental purposes. They were maintained in clean facilities with a 12-hour light/dark cycle and received water and food through a semi-barrier system.

Subcutaneous tumor models were generated by the subcutaneous inoculation (0.10 mL volume containing 5×10^7 cells/mL media) of Hela cells in the right side of their abdomen using a 1-mL syringe with a 25G needle. Tumor growth was monitored until a palpable size of about 1.0 cm was reached in all directions.

1.8 Statistics

Data were expressed as the means ± SD (standard deviation) from at least three independent experiments. One-tailed unpaired Student's t-test was used for significance testing, and $p< 0.05$ is considered significant.

References

1. Gao, S.; Chen, D.; Li, Q.; Ye, J.; Jiang, H.; Amatore, C.; Wang, X., Near-infrared fluorescence imaging of cancer cells and tumors through specific biosynthesis of silver nanoclusters. *Scientific reports* **2014,** *4*, 4384.