

## Supplementary Information for

### Rapid chelator-free radiolabeling of quantum dots for *in vivo* imaging

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## Materials and Methods

**Materials.** Zinc acetate dehydrate, manganese sulfate monohydrate, sodium sulfide, and copper chloride dihydrate were purchased from Sigma Aldrich. 3-mercaptopropionic acid (3-MPA) was obtained from ACROS Organics. HS-PEG2000-NH<sub>2</sub>, HS-PEG5000-OCH<sub>3</sub>, and NHS-PEG5000-Mal were received from JenKem Technology USA. <sup>68</sup>Ga is eluted from a <sup>68</sup>Ga generator (IGG100, Eckert & Ziegler Isotope Products, Berlin, Germany). <sup>64</sup>Cu is received from University of Washington. Carboxyfluorescein-conjugated peptides (FAM-cys-CRV, FAM-CGKRK, FAM-cys) were purchased from LifeTein, LLC (Somerset, NJ). FAM or FAM-cys was attached through an aminohexanoic acid linker to the N-terminal amino group. The C-terminus of peptide was not blocked.

**Synthesis of ZnS QDs.** The synthesis of ZnS QDs was adapted from a previous QD protocol.<sup>1</sup> 110 mg zinc acetate was dissolved in 5 mL deionized (DI) H<sub>2</sub>O for a final concentration of 0.1 M. 298 mg 3-MPA was diluted in 20 mL DI H<sub>2</sub>O. The two solutions were mixed together and brought up to a total volume of 35 mL using DI H<sub>2</sub>O. The pH was adjusted to 10.3 ± 0.2 with 2 M NaOH aqueous solution. The transparent colorless solution was then degassed with N<sub>2</sub> for 30 min. 108 mg Na<sub>2</sub>S was dissolved in 4.5 mL DI H<sub>2</sub>O (0.1 M), added to the degassed solution, and quickly and thoroughly mixed. The final solution was heated to 50 °C for 2.5 h and then cooled to room temperature. 80 mL of ethanol was added to the solution to precipitate the QDs. The suspension was

centrifuged at 1000 g for 3 min and the supernatant was removed. The precipitate was washed two more times with ethanol and centrifuged to remove residual MPA and metal ions. The white precipitate was dissolved in DI H<sub>2</sub>O to be used directly for characterization or lyophilized for storage. The yield was typically ~ 40 mg.

The manganese-doped zinc sulfide QD was synthesized similarly by mixing manganese sulfate monohydrate (0.01 M, 1.5 mL) with zinc acetate before adding 3-MPA.

**PEGylation and peptide conjugation.** 4 mg ZnS QD powder was dissolved in 0.5 mL PBS and mixed with HS-PEG2000-NH<sub>2</sub> (40 mg, in 1 mL PBS). The solution was incubated at 50 °C overnight and purified using 10 kDa ultrafiltration filter (3000 g, 10 min) to remove the free PEG molecules. It was further washed with PBS three times. The purified QD-NH<sub>2</sub> (about 1 mL) was mixed with NHS-PEG5000-Mal (10 mg, in 400 μL PBS) and incubated at room temperature overnight. The mixture was purified using 30 kDa filter (3000 g, 10 min) and washed with PBS three times. The final volume of the QD-Mal solution was brought up to 0.5 mL. Carboxyfluorescein-conjugated peptide (FAM-cys-CRV, or FAM-CGKRK) or the control FAM-cys was then conjugated onto the surface through the reaction of thiol group from the cysteine residue to maleimide functionalized PEG. (In FAM-cys-CRV, thiol group for conjugation was from the additional cysteine between the FAM label and the CRV sequence.) 25 μL of FAM-cys-CRV, FAM-cys, or FAM-CGKRK (1 mg/mL) was added to 200 μL QD-Mal solution and incubated at 4 °C overnight. The mixture was then purified using 30 kDa filter (3000g, 10 min) and washed with PBS three times. The final volume of the QD-peptide solution was brought up to 200 μL and stored at 4 °C for *in vitro* studies.

To synthesize non-targeting QD-OCH<sub>3</sub>, 4 mg QD powder was dissolved in 0.5 mL PBS and mixed with HS-PEG5000-OCH<sub>3</sub> (40 mg, in 1 mL PBS). The solution was incubated at 50 °C overnight, purified using 30 kDa filter (3000 g, 10 min), and washed with PBS three times. The final volume after washing was brought up to 0.5 mL and stored as a stock solution at 4 °C for *in vitro* or *in vivo* studies.

**Characterization.** The hydrodynamic size distribution and zeta potential of QDs was measured using a Malvern Instruments Zetasizer Nano. The core size was visualized by transmission electron microscopy with a JEOL 1200 EX II microscope, operating at 80 kV. The elemental analysis of zinc and copper was performed using inductively coupled plasma-mass spectroscopy by Weck Laboratory, Inc. Fourier-transform infrared spectra (FTIR) were recorded using a Thermo Scientific Nicolet 6700 instrument. Thermogravimetric analyses (TGA) were obtained using a Perkin Elmer Simultaneous Thermal Analyzer (STA 6000). About 10 mg of each QD sample was heated from 30 °C to 700 °C at a heating rate of 10°C/min under a 20 mL/min oxygen flow. Absorbance and fluorescence spectra of QDs were obtained from a Beckman Coulter DU800 spectrophotometer (absorbance), a HORIBA FluoroMax-4 spectrofluorometer, or a FlexStation 3 multimode microplate reader (Molecular Devices).

**Stability test of peptide conjugation.** The stability of QD-peptide in aqueous solution (room temperature) was assessed over time (2 weeks). At each time point, detached peptide was removed by ultrafiltration (10 kDa cutoff membrane) at 14000 g for 10 min. The absorbance of purified QD-peptide was then measured by absorbance at 500 nm.

**Cation exchange.** 1  $\mu\text{L}$  of  $\text{CuCl}_2$  (1 mM) was first mixed with 49  $\mu\text{L}$  sodium acetate buffer (0.1 M, pH 5.5) and then added to 50  $\mu\text{L}$  QD-OCH<sub>3</sub> stock solution. The reaction mixture was incubated at 37 °C for 10 min and then purified by centrifugation using a 10 kDa filter (14000 g, 10 min) to remove free copper or zinc ions. The solution was washed two more times with PBS and used for measurement of size distribution or elemental analysis.

**Radiolabeling. <sup>68</sup>Ga labeling.** About 1 mL of <sup>68</sup>GaCl<sub>3</sub> (about 74 MBq) was eluted with 0.05 M HCl in a 60 mL syringe from the <sup>68</sup>Ga-generator (IGG100, Eckert & Ziegler Isotope Products, Berlin, Germany). HCl was prepared with high purity hydrochloric acid (Sigma-Aldrich, St. Louis, MO) and OmniTrace Ultra high purity water (EMD Millipore, Billerica, MA). 200  $\mu\text{L}$  of <sup>68</sup>GaCl<sub>3</sub>/HCl was first mixed with 200  $\mu\text{L}$  1 M sodium acetate pH 5.5 buffer. This solution was then added to 25  $\mu\text{L}$  QD-OCH<sub>3</sub> stock solution in the labeling vial and incubated at 37 °C for 15 min. The mixture was centrifuged using a 10 kDa filter (14000 g, 10 min) to remove free <sup>68</sup>GaCl<sub>3</sub> and washed two more times with PBS. The final solution was diluted to 100  $\mu\text{L}$  by PBS for *in vitro* experiments.

**<sup>64</sup>Cu labeling.** <sup>64</sup>CuCl<sub>2</sub> solution received from University of Washington was diluted with sodium acetate buffer (0.1 M, pH 5) to 370 MBq/mL. 18.5 MBq was added to 25  $\mu\text{L}$  QD-OCH<sub>3</sub> stock solution and incubated at 37 °C for 15 min. The mixture was centrifuged using a 10 kDa filter (14000 g, 10 min) to remove free <sup>64</sup>CuCl<sub>2</sub> and washed two more times with PBS. The final solution was diluted to 100  $\mu\text{L}$  by PBS for IV injection.

**Radio-thin layer chromatography.** Radiochemical purity was measured by Radio-thin layer chromatography (Radio-TLC) using a Whatman-31ET chromatography paper strip (GE Healthcare, Bio-Sciences, Pittsburgh PA) as the stationary phase and sodium acetate buffer (0.1 M, pH 5.5) as the mobile phase. The strip was air-dried and scanned with a radio-TLC imaging scanner (Bioscan AR 2000, Eckert & Ziegler Radiopharma Inc., Hopkinton MA).

**Stability test of Radiolabeling.** QD-OCH<sub>3</sub> samples were radiolabeled with <sup>64</sup>Cu as described above. After purification by centrifugal ultrafiltration and wash with PBS, samples were incubated with freshly obtained mouse blood at 37 °C until the indicated time points (3 h or 24 h). The samples in blood were then centrifuged again with 10 kDa filter to remove any free isotopes released from the nanoparticles. An extra wash was performed to eliminate any free <sup>64</sup>Cu adsorbed on the filter membrane or particle surface. The filtrates and samples were then measured for their radioactivity to determine the stability of the <sup>64</sup>Cu-QD-OCH<sub>3</sub> in mouse blood.

**Cellular binding/uptake study.** MCF10CA1a cells ( $1 \times 10^6$ ) were incubated with QD-cys or QD-CGKRK (normalized to same fluorescence intensity as 10  $\mu\text{M}$  FAM-CGKRK

peptide) in 300  $\mu$ L of complete culture medium. After incubation at 4 °C for 1 h, the sample-containing medium was removed by centrifugation and the cells were washed with PBS once. Flow cytometry data were then acquired on BD LSRFortessa (BD Biosciences, San Jose). Experiments were repeated three times on different days.

J774A.1 or RAW264.7 cells ( $0.3 \times 10^6$ ) were seeded in 12-well plates and cultured overnight. QD-CRV, QD-cys were radiolabeled with  $^{68}\text{Ga}$  as described above. After removal of the culture medium, cells in each well were incubated with  $^{68}\text{Ga}$ -QD-CRV or  $^{68}\text{Ga}$ -QD-cys (630 kBq) in 1 mL of complete culture medium. After incubation at 37 °C for 1 h, the QD-containing medium was removed and the cells were washed twice with PBS. Cell lysis buffer (0.3 mL, Qiagen Buffer RLT Plus) was added to each well and the cell lysates were then transferred to gamma counting tubes to measure the radioactivity.

**Blood circulation half-life.** All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Sanford Burnham Prebys Medical Discovery Institute (SBP). To measure the blood circulation half-life, manganese doped zinc sulfide ZnMnS QD or ZnMnS-OCH<sub>3</sub> (50  $\mu$ L of stock solution for each mouse, further diluted to 100  $\mu$ L by adding 50  $\mu$ L PBS) was injected intravenously via the tail vein into multiple Balb/c mice and the blood was collected into plasma separator tubes with lithium heparin (BD Microtainer) at different time points (5 min, 30 min, 1 h, 2 h, 4 h, 7 h, 10 h, 24 h) post injection. Plasma was isolated by centrifugation (15 minutes at 1500x g). The fluorescent intensities of plasma at 600 nm (Ex = 300 nm) were then measured with FlexStation 3 multimode microplate reader (Molecular Devices) and plotted against circulation time. The data were then fitted to one phase exponential decay using OriginLab 2015.

***In vivo* PET.** 4T1 breast cancer cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Orthotopic breast tumors were generated by injecting one million 4T1 cells into the mammary fat pad. Animals with a tumor size of about 8 mm in diameter were used for *in vivo* study. All animal work was approved by the Institutional Animal Care Committee of the Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA, USA).

PET/CT imaging was performed using an Inveon small animal PET/CT imaging system (Siemens, Knoxville, TN). Each mouse was injected intravenously via the tail vein with 12.1~15.3 MBq of activity under awake conditions. For imaging, mice were anesthetized using 1-2% isoflurane in medical grade oxygen 15 min prior to the scheduled PET scan. A static 15-min emission scan was acquired followed by a whole-body micro-CT scan at 3 h and 24 h post-injection. CT acquisitions were acquired using tube voltage = 80 kV, current = 500  $\mu$ A, and 275 ms exposure time with a half gantry rotation with 220 projections. After the 24 h post IV PET/CT scan, various tissues were harvest from the animals and the radioactivity was measured by a gamma counter to evaluate the biodistribution. PET images were reconstructed using an OSEM/3D-SPMAP protocol with a matrix size of 256 x 256, 18 OSEM iterations, and 2 MAP iterations. Attenuation correction was applied using the CT data which was reconstructed using a Feldkamp

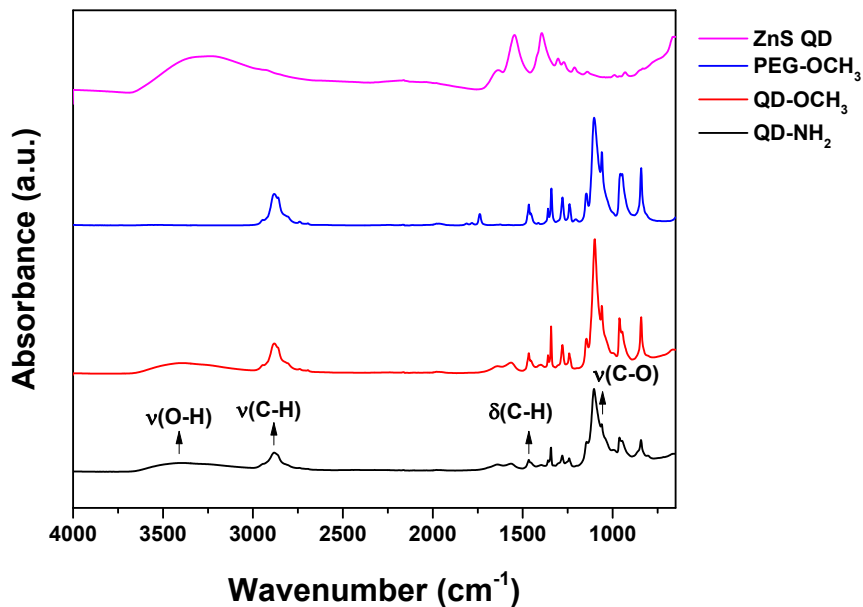
cone beam method with no down sampling, slight reduction and mouse beam hardening. PET and CT images were co-registered using Amira 6.1.

**Biodistribution.** After indicated time points post intravenous injection of radiolabeled tracers ( $^{68}\text{Ga}$ -QD was injected at 2.4~4.8 MBq per mouse for 1 h circulation), tissues were harvested from the animals and the radioactivity was measured by a gamma counter (Gamma 9000 Beckman Instruments, Fullerton, CA) to evaluate the biodistribution. The percentage injected dose per gram of tissue (%ID/g) was calculated by comparing counts to tissue weight.

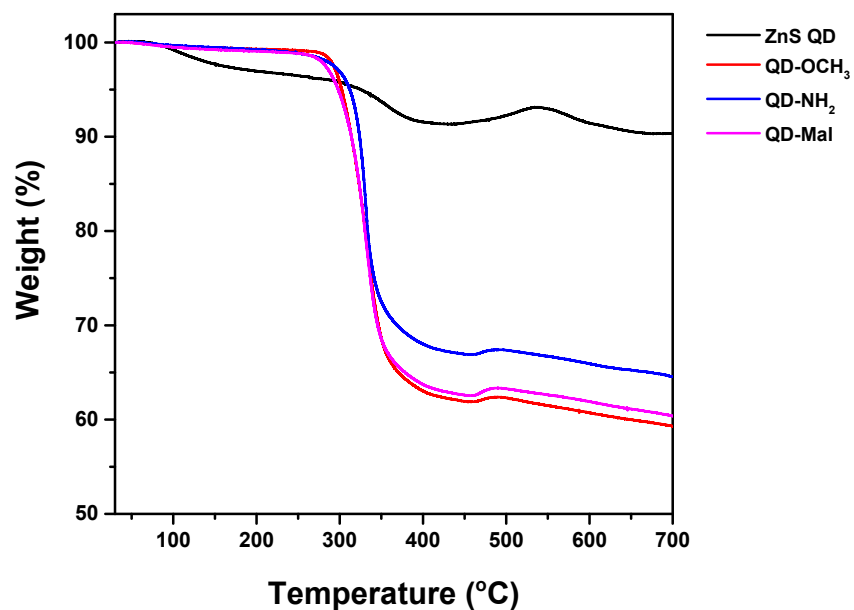
**Immunofluorescence staining.** Frozen tissue sections were first treated with PBS containing 1% BSA and 0.1% Triton X100 (blocking buffer) at room temperature (RT) for 1 h. The sections were washed three times with PBS and then incubated with primary antibodies (goat anti-FITC, rat anti-mouse CD31, and rat anti-mouse CD11b) diluted (1:200) in blocking buffer at 4 °C overnight, followed by the appropriate secondary antibodies diluted (1:200) in blocking buffer at RT for 1 h. After washing with PBS, sections were mounted in DAPI-containing mounting medium (Vector Laboratories, Burlingame, CA) with a coverslip and examined under a Zeiss LSM 710 NLO confocal microscope.

**Statistical analysis.** Experiments were performed at least three times. All data represent mean value  $\pm$  SEM, if not mentioned specifically. The significance analyses were performed using two-tailed Student's t-test as detailed in the figure legends.

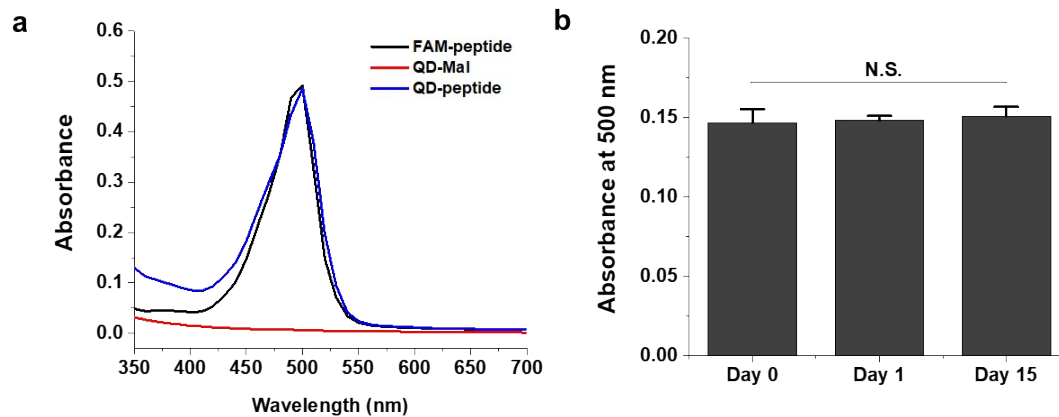
Supplementary figures:



Supplementary Fig. S1. Representative Fourier-transform infrared (FTIR) spectra of QDs and PEG, confirming surface PEGylation. The absorbance spectra of PEG and PEGylated QDs showed a broad peak at around 3400 cm<sup>-1</sup> (O-H stretching), a strong peak at around 2950 cm<sup>-1</sup> (C-H asymmetric stretching) and 2880 cm<sup>-1</sup> (C-H symmetric stretching), a peak at 1460 cm<sup>-1</sup> (CH<sub>2</sub> bending), and a peak at 1057 cm<sup>-1</sup> (C-O stretching).

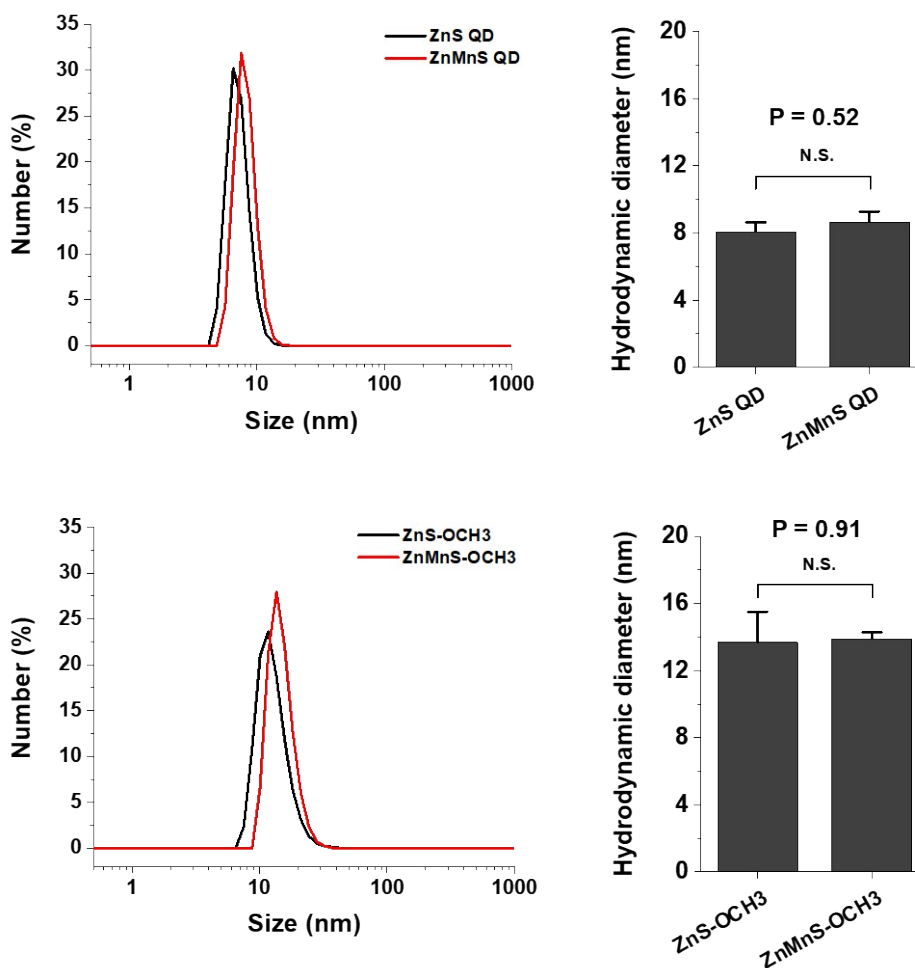


Supplementary Fig. S2. Thermogravimetric analyses (TGA) of various QDs. QD samples were lyophilized overnight prior to analyses. The weight change (%) is plotted from 30 to 700 °C. Surface PEGylation of QD-OCH<sub>3</sub>, QD-NH<sub>2</sub>, and QD-Mal was quantified as the difference in percentage weight at 700 °C compared to the non-PEGylated ZnS QD, and determined to be 31%, 26%, and 30%, respectively.

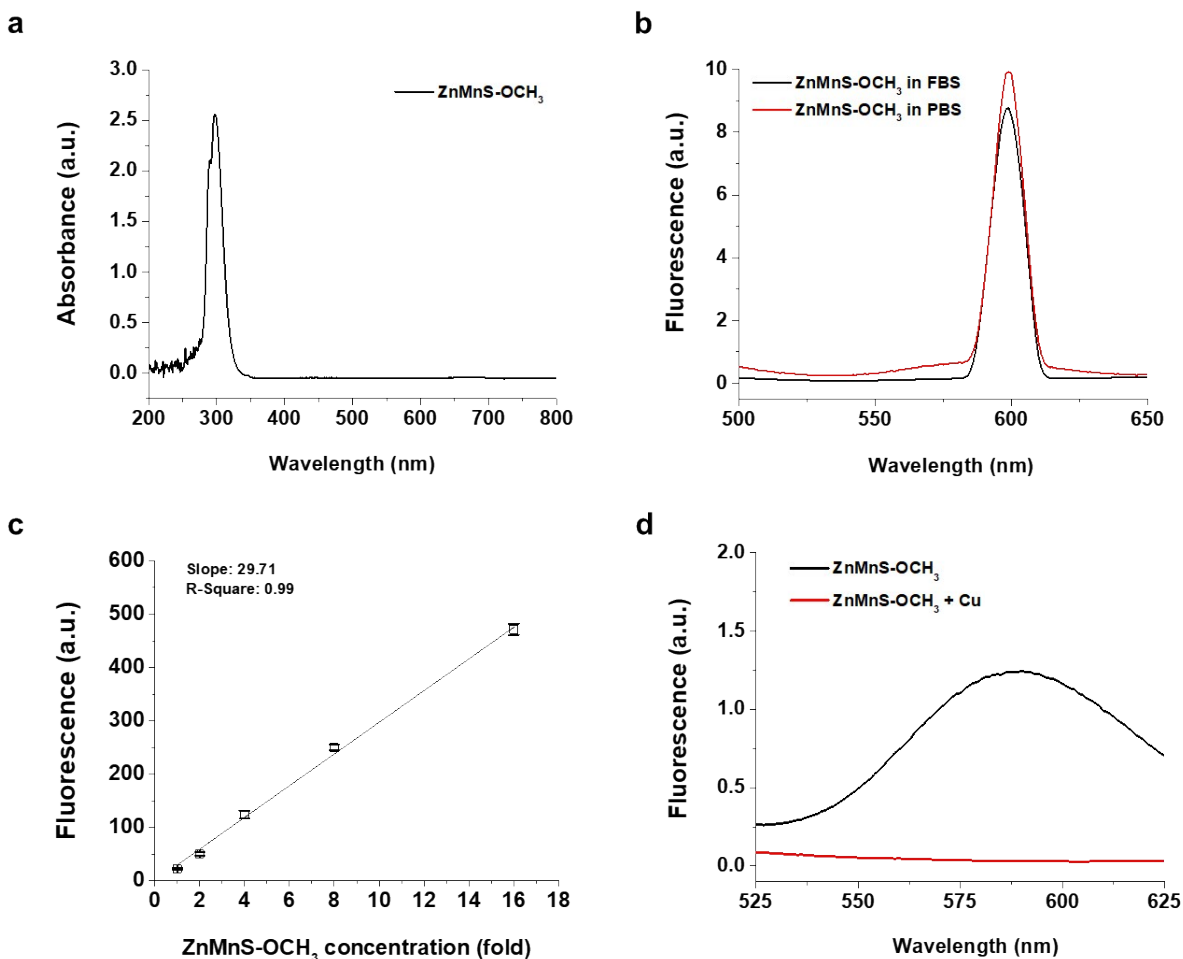


Supplementary Fig. S3. Validation of peptide conjugation on QD surface. a) UV-Vis absorbance spectra of FAM-peptide and QD-peptide showed absorption at 500 nm, compared to the non-conjugated QD-Mal. b) Stability of peptide conjugation on QD-peptide in aqueous media (room temperature) over time, measured by absorbance at 500 nm. At each time point, detached peptide was removed by ultrafiltration (10 kDa cutoff membrane) at 14000 g for 10 min. The absorbance of purified QD-peptide was then measured. Data represent mean value  $\pm$  SEM. N = 3. Two-tailed Student's t-test were performed. N.S.: not significant difference.

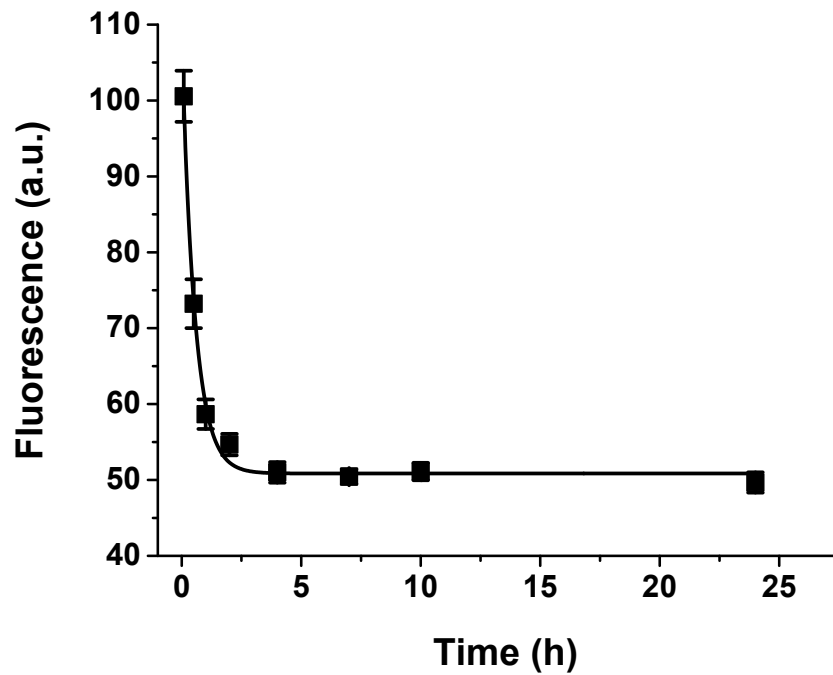




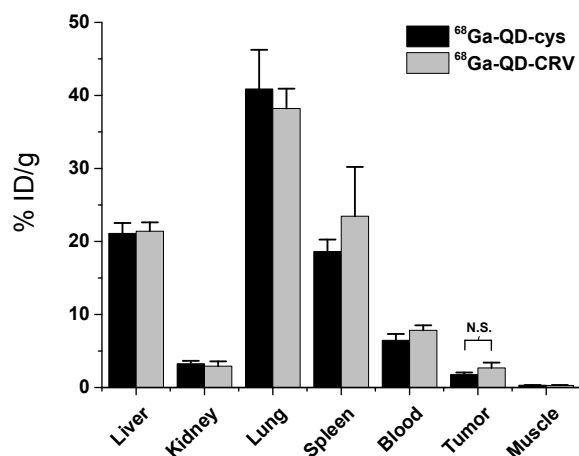
Supplementary Fig. S4. Hydrodynamic diameter of Mn-doped ZnS QD (ZnMnS QD) and PEGylated ZnMnS QD (ZnMnS-OCH3) compared to non-doped ZnS analogues measured by DLS. Data represent mean value  $\pm$  SEM. N = 3. Two-tailed Student's t-test were performed. N.S.: not significant difference.



Supplementary Fig. S5. Absorbance and Fluorescence spectra of ZnMnS-OCH<sub>3</sub>. a) Maximum absorbance of ZnMnS-OCH<sub>3</sub> at 300 nm. b) Fluorescence of ZnMnS-OCH<sub>3</sub> at ~ 600 nm with an excitation at 300 nm. FBS: fetal bovine serum. PBS: phosphate buffered saline. c) Fluorescence intensity of ZnMnS-OCH<sub>3</sub> at 600 nm at different concentrations in the range relevant to the blood circulation study. Data represent mean value  $\pm$  SEM. N = 3. Linear fitting was performed by OriginLab 2015. d) Fluorescence of ZnMnS-OCH<sub>3</sub> before and after cation exchange with copper solution.



Supplementary Fig S6. Blood half-life of non-PEGylated ZnMnS QDs. Plasma concentration of QDs was measured by fluorescence at different time points after intravenous injection into Balb/c mice. The data represent mean value  $\pm$  SEM. N = 9.



Supplementary Fig. S7. Biodistribution of  $^{68}\text{Ga-QD-CRV}$  and  $^{68}\text{Ga-QD-cys}$  in mice bearing 4T1 breast tumors. QDs were intravenously injected into animals and tissues were harvested after 1 h circulation. Radioactivity in each tissue was measured by a gamma counter. The percentage injected dose per gram of tissue (%ID/g) was calculated by comparing counts to tissue weight. All data represent mean value  $\pm$  SEM. N = 3. N.S.: not significant different. P = 0.30, Student's t test.

## References

1. Y. Nai-Qiang, L. Ling, L. Jie-Mei, L. Yan-Song, G. Mao-Gang, W. Yi-Zhi, Z. Li-Xin and X. Xiao-Liang, *Chinese Physics B*, 2012, **21**, 116101.