Supporting Information

Highly Fluorescent Ribonuclease-A-Encapsulated Lead Sulfide Quantum Dots for Ultrasensitive Fluorescence *in Vivo* Imaging in the Second Near-Infrared Window

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1. Cell Culture

All different cell lines (*i.e*. human embryonic kidney 293T, gastric epithelial GES-1, A375 human malignant melanoma cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific) at 37°C under 5% CO_2. The cells were collected by trypsinization and placed on a vented cap cell culture flask for culture and passage.

2. MTT Assay

MTT (3 (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide) assay was used to examine the toxic effect of the PbS Qdots on cell growth following our established procedures.[1] Cells were seeded in a 96-well plate at a density of 1×10^5 cells per well and cultured for 24 h with complete medium or Qdot-preconditioned medium containing 1.3, 2.6, 13 and 26 nM of the Qdots, respectively. The MTT stock solution (5 mg/mL) was added to each well and incubated for another 4 h. After removal of the media, 200 μL of DMSO was added to dissolve the formed formazan crystals and the absorbances at 570 nm were measured with a background correction at 630 nm using a microtiter plate reader (BioTek). Cell viability (CV) of the healthy cell as the control was defined as 100%, and CVs of the treated groups were expressed as a percentage (%) of the control value.

3. Measuring ROI intensity using *Image J* 1.48.[2-4]

A procedure for measuring the fluorescence intensity of a region of interest (ROI intensity) in **Figure S4**:

(a) Use a selection tool (*i.e*. circular area with diameter d = 44 pixels) to completely cover a sample well;

(b) Click *Set Measurements* and tick three options of *Area*, *Integrated Density* and *Mean Gray Value*;

(c) Click *Measure* to show relevant values;

(d) Select 6 dark regions around a glowing well as the background;

(e) Transfer the data from *Results* to an *Excel* worksheet;

(f) Use the following equation to determine the ROI intensity:

ROI Intensity = **Integrated Density - Area of Selected Region** × **Mean Fluorescence of Background**

Note that ROI intensity of **Figure 6** and average fluorescence of **Figure 7h** are calculated using this protocol.

4. Estimation of the number of PbS formula units and equivalent molecular weight of the PbS Qdot.

(a) Volume of the PbS Qdot core based on an average diameter of 4.35 nm obtained from TEM image:

$$V_{Qdot} = (4/3)\ \pi\ r^3 = (4/3) \times 3.14 \times (4.35\ nm/2)^3 = 43.08\ nm^3$$

(b) Volume of the face centred cubic (fcc) PbS unit cell (a_0 = 0.5934 nm).

$$V_{uc} = {a_0}^3 = (0.5934\ nm)^3 = 0.2090\ nm^3$$

(c) As each fcc unit cell contains four PbS formula units, hence the number of PbS formular units (N) contained by the Qdot core can be calculated as follows:

$$N = 4 \times (43.08/0.2090) = 824$$

The equivalent molecular weight of the Qdot core can thus be calculated as:

$$MW = N \times \text{PbS formular weight} = 824 \times (32.06 + 207.2) = 197{,}150\ g/mole.$$

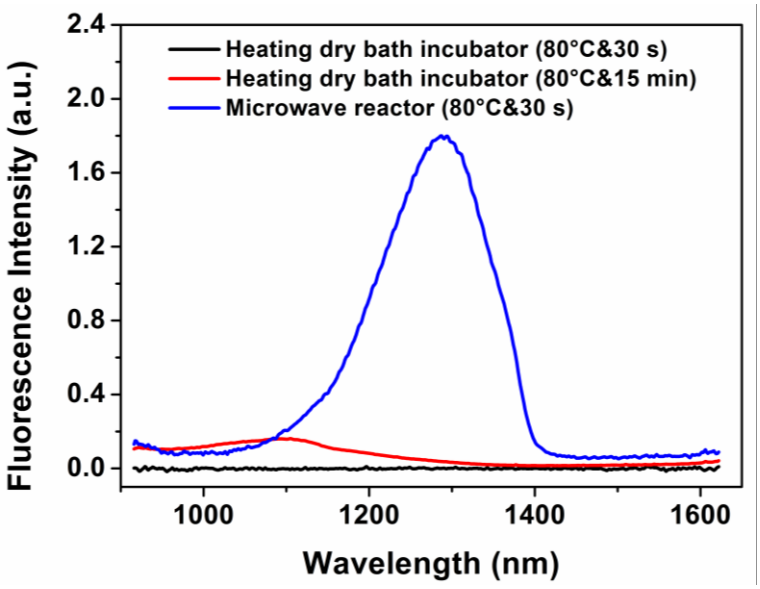

Figure S1. Comparison of fluorescence spectra of RNase-A@PbS Qdots prepared at 80^{o}C using different heating methods: a dry metal bath incubator for 30 s (black line) and 15 min (red line) and by a microwave reactor for 30 s (blue).

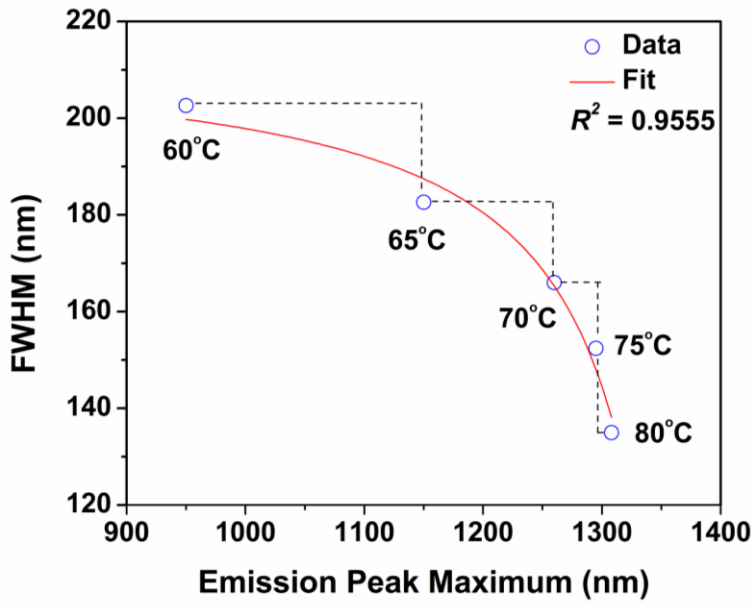

Figure S2. FWHM of emission spectra *versus* peak maximum of the PbS Qdots prepared at different temperatures. Data points are fitted with a rational function (R^2 = 0.9555) *via* the equation (Δ denotes the difference of FWHMs between two neighbouring data points):

$$\Delta = \frac{210.036 - 0.154\lambda}{1 - 0.00072\lambda} \qquad \text{(S-1)}$$

The FWHM and growth temperature is linearly but negatively correlated: it decreases by ~17 nm per 5°C of increasing temperature.

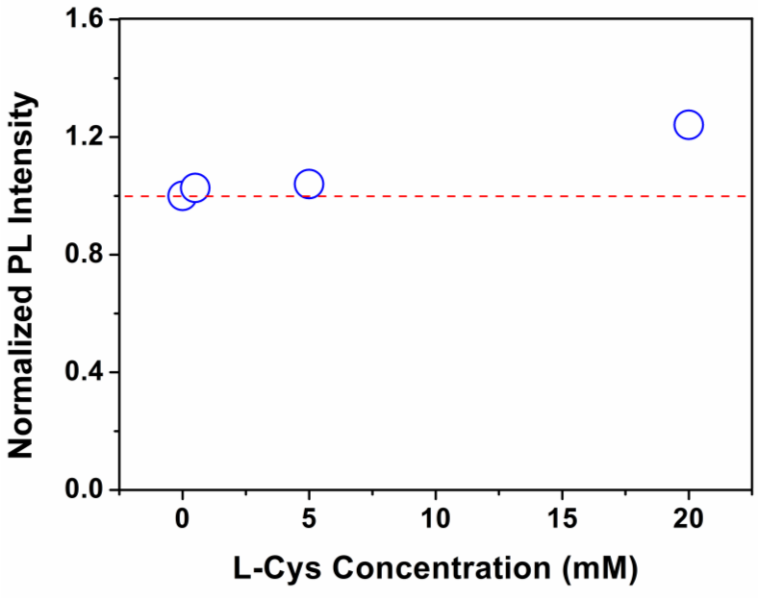

Figure S3. Effect of *L*-cysteine (*L*-Cys) concentration on the normalized fluorescence intensity of the RNase-A@PbS Qdots.

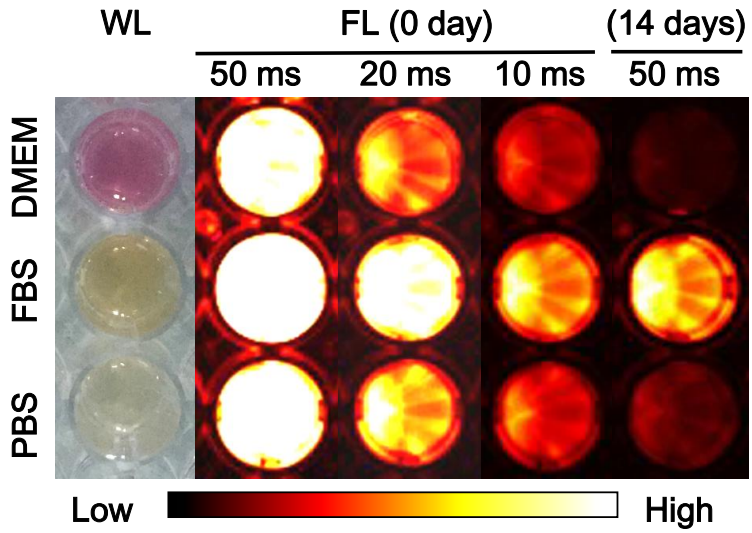

Figure S4. Stability of the RNase-A@PbS Qdots in different biologcial media over a course of 14 day storage. White-light (WL) and false-color fluorescence (FL) images of the RNase-A@PbS Qdots (~26 nM) in Dulbecco's PBS (pH 7.2), fetal bovine serum (FBS, pH 7.1) and Dulbecoo's modified eagle media (DMEM, pH 7.6). A 24-well plate loaded with the specimens is placed in a home-built NIR-II imaging prototype equipped with an 1100 nm long-pass filter and an InGaAs CCD camera, excited at 808 nm with an exposure time of 10, 20 and 50 ms over a two-week period.

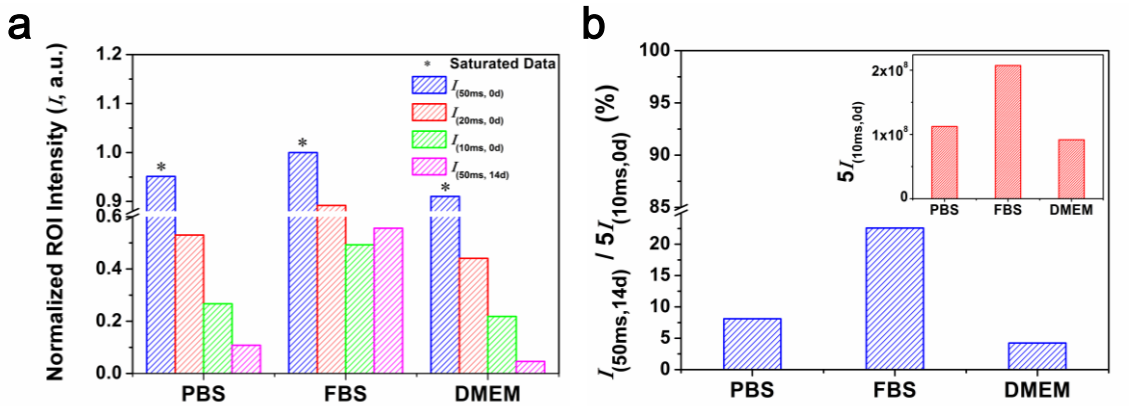

Figure S5. Qualitative analysis of fluorescence stability of RNase-A@PbS Qdots. (**a**) Normalized 'region-of-interest (ROI)' intensity (*I*) extracted from the fluorescence images of the RNase-A@PbS Qdots in three common media (PBS, FBS and DMEM) taken at the beginning (day-0, with 10, 20 and 50 ms exposure times) and the end (day-14, with 50 ms exposure) shown in **Figure S4**. The ROI itensity is extrated using the method given in the above section. (**b**) Bar chart representations of the retained Qdot intensity over a 14-day storage in the media relative to their respective day-0 values. Note that five-fold of the day-0 intensities (with 10 ms exposure) are used as the equivalent day-0 intensities with 50 ms exposure to avoid the image saturation problem (the absolute values are given in the inset). The initial ROI intensities with 50 ms exposure time appear to be saturated. Hence the real value is approximated as five-fold that of the corresponding image taken at an exposure time of 10 ms. Whilst all Qdots gradually lose their fluorescence over time, the one in FBS fades more slowly and still retains ~23% of its original fluorescence after storage of 14 days (*versus* ~7.5% and ~4.5% retained by those in PBS and DMEM).

5. The mechanism behind high colloidal stability of RNase-A@PbS Qdots in FBS.

The freshly prepared RNase-A@PbS Qdot is negatively charged with zeta potential (ZP) of -22.2 mV in the reaction solution (pH ~11), but lose its charge when being transferred into different media wherein ZPs increase to -2.5, -7.0 and -0.24 mV for PBS, FBS and DMEM, respectively (**Table S1**). The charge loss leads to colloidal instability and aggregation caused by the invalidation of electrostatic stabilization, confirmed by dynamic light scattering (DLS) measurements.[1] The Qdots show a predominant species with a hydrodynamic diameter (D_h) of ~98 nm after dialysis in pure water (pH ~7), indicating cluster formation (**Figure S6**). In FBS, two species with D_hs of 8.2 nm (~23%) and 91 nm (~74%) are observed, which can be assigned to isolated individual serum proteins and clustered/lightly-aggregated Qdots, respectively. The relatively larger D_h of the Qdots in FBS may be also due to serum proteins adsorbed on the QD surface (*aka* protein corona) by Langmuir adsorption mechanism.[5, 6] In contrast, the Qdots in PBS and DMEM exhibit even larger species with D_hs of 1011 and 305 nm, respectively. The extra-large size cumulants of >4.2 μm indicates large-scale agglomeration/aggregation (**Figure S7**), a well-known cause of fluorescence quenching. Accordingly, the Qdots fade more rapidly in PBS and DMEM than in FBS.

References

(1) Song, L.; Ho, V. H. B.; Chen, C.; Yang, Z. Q.; Liu, D. S.; Chen, R. J.; Zhou, D. J. *Adv. Healthcare Mater*. **2013**, *2*, 275.

(2) Burgess, A.; Vigneron, S.; Brioudes, E.; Labbé J-C; Lorca, T.; Castro, A. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 12564.

(3) McCloy, R. A.; Rogers, S.; Caldon, C. E.; Lorca, T.; Castro, A.; Burgess, A. *Cell Cycle* **2014**, *13*, 1400.

(4) Potapova, T. A.; Sivakumar, S; Flynn, J. N.; Li, R; Gorbsky, G. J. Mitotic. *Mol. Biol. Cell* **2011**, *22*, 1191.

(5) Cedervall, T.; Lynch, I.; Lindman, S.; Berggard, T.; Thulin, E.; Nilsson, H.; Dawson, K. A.; Linse, S. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 2050.

(6) Shang, W.; Nuffer, J. H.; Dordick, J. S.; Siegel, R. W. *Nano Lett*. **2007**, *7*, 1991.

(7) Hong, G.; Robinson, J. T.; Zhang, Y.; Diao, S.; Antaris, A. L.; Wang, Q.; Dai, H. *Angew. Chem., Int. Ed*. **2012**, *51*, 9818.

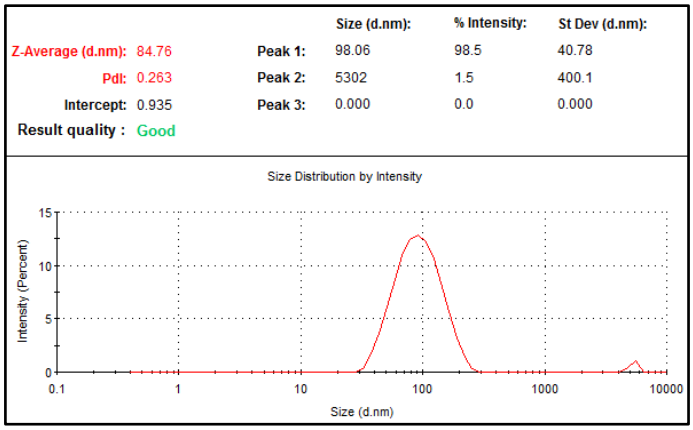

Figure S6. DLS graph of the as-prepared RNase-A@PbS Qdots under dialysis against pure water.

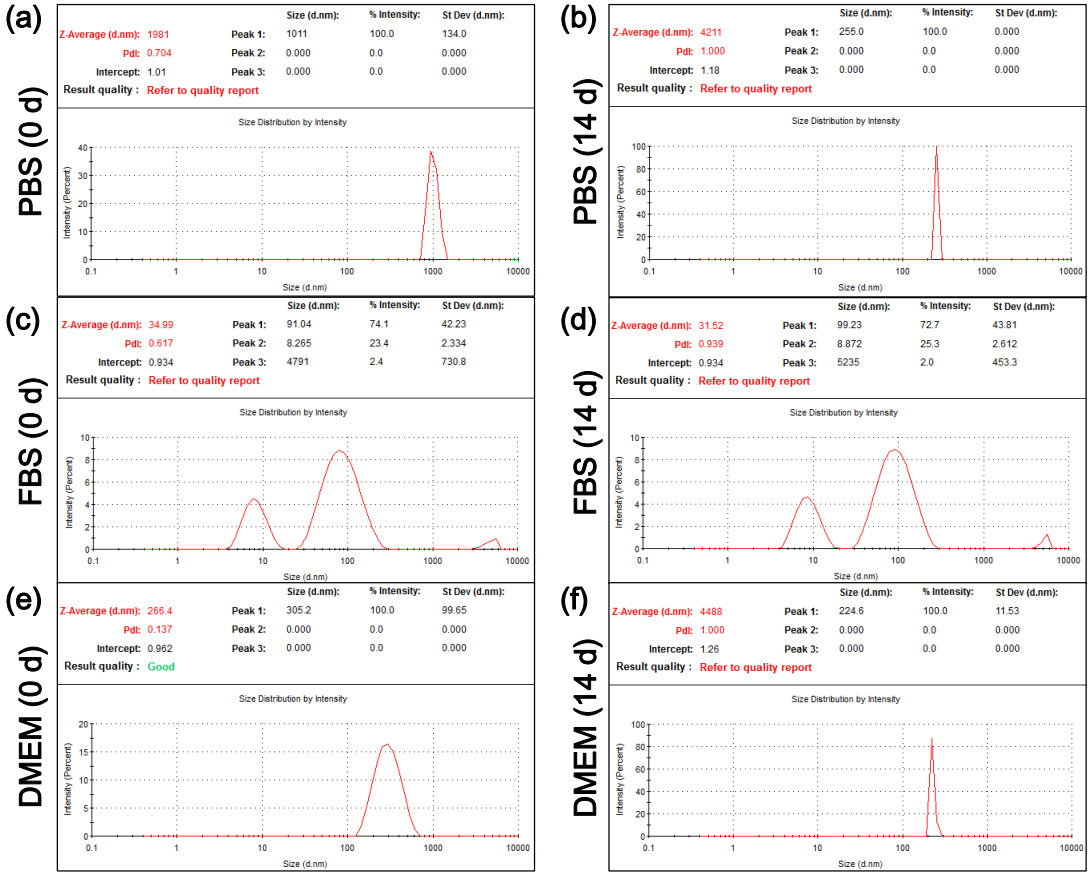

Figure S7. DLS graphs of RNase-A@PbS Qdots at day-0 (a, c, e) and day-14 (b, d, f) in different media: Dulbecco's phosphate buffered saline (PBS, a and b), FBS (c, d) and DMEM (e, f).

Table S1. Zeta potentials (ZPs) of RNase-A@PbS Qdot in three different media over a two-week storage.

Medium / Storage	PBS (pH 7.2)	FBS (pH 7.1)	DMEM (pH 7.6)
0 day	-2.5 ± 27	-7.0 ± 13.5	-0.24 ± 11
14 days	-5.4 ± 10.4	-2.4 ± 14.1	-5.0 ± 9.4

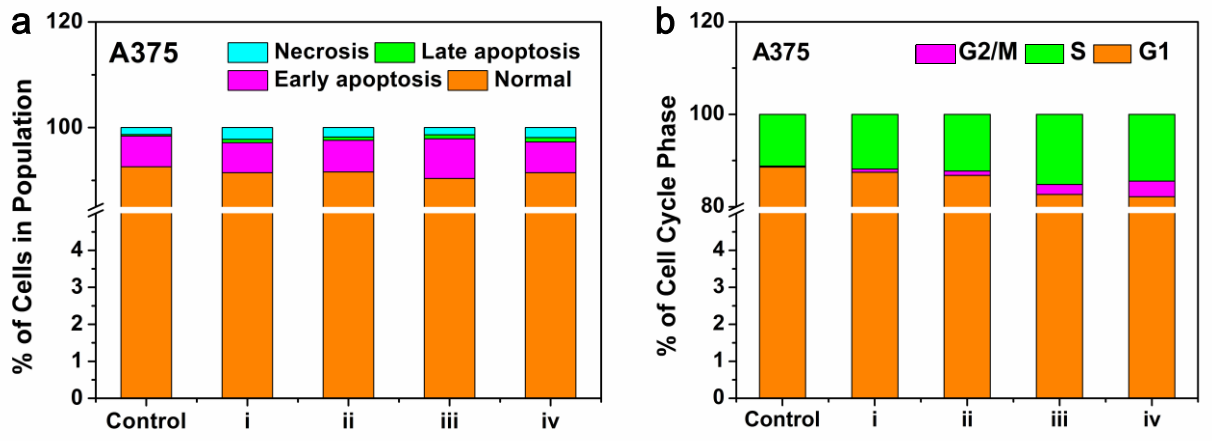

Figure S8. Apoptosis/necrosis and cell cycle distribution of A375 cells after 24 h incubation with various concentrations of the RNase-A@PbS Qdots (control = 0, i = 1.3, ii = 2.6, iii = 13 and iv = 26 nM). (**a**) Percentage of normal, early and late apoptotic, and necrotic cells. (**b**) Percentage of cells in each phase of cell cycle.

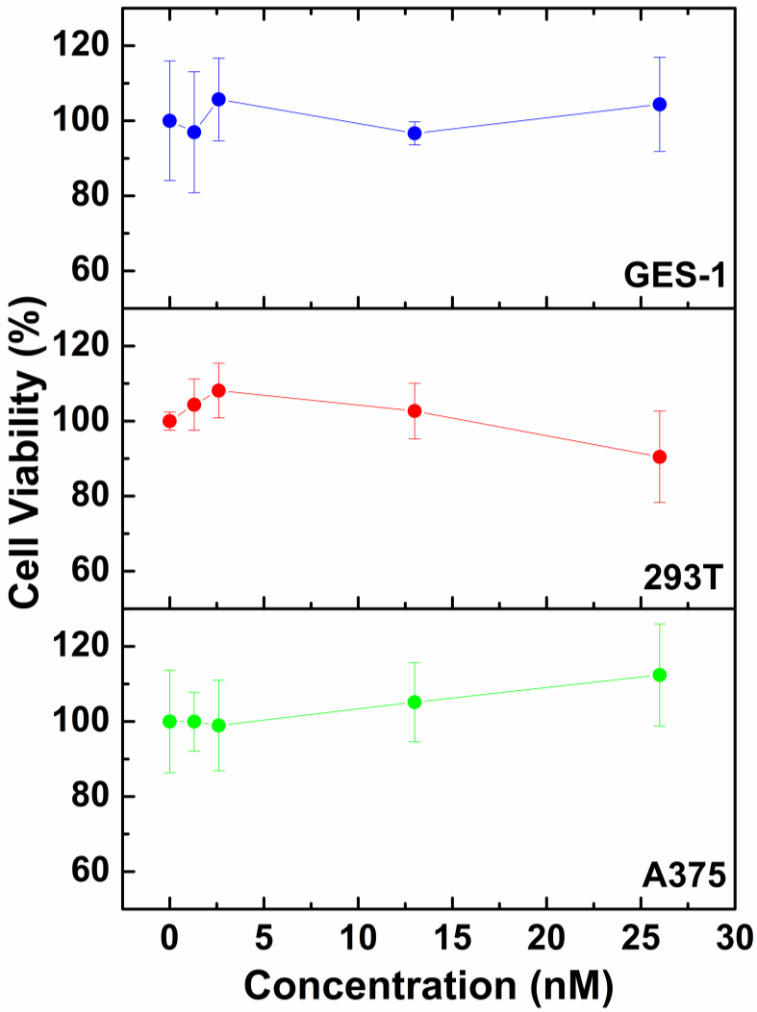

Figure S9. Percentage cell viabilites of three different cell lines (each of the healthy controls is normalized to 100) *i.e.* GES-1 (top), 293T (middle) and A375 (bottom) cells after 24 h incubation with varying concentrations of RNase-A@PbS Qdots ranging from 0 to 26 nM.

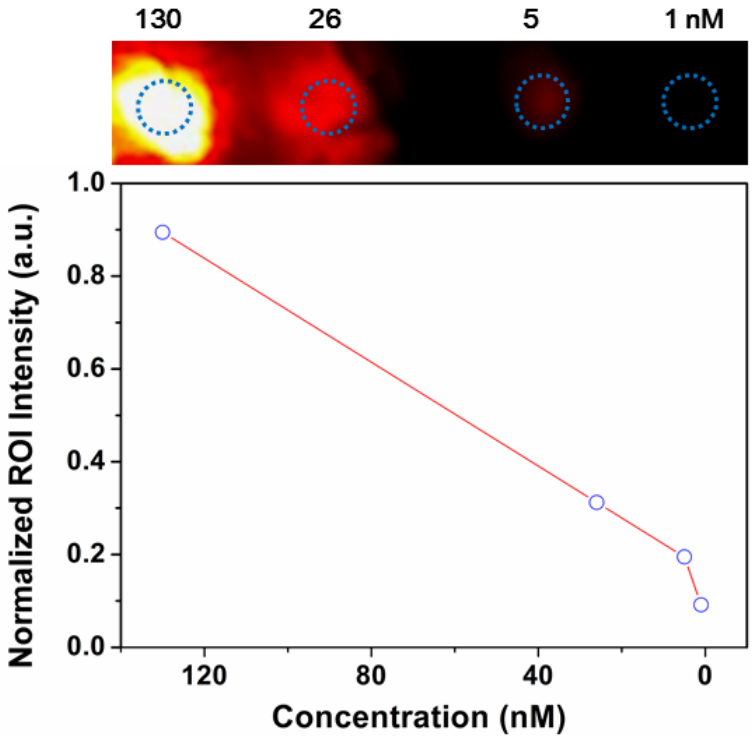

Figure S10. *Ex vivo* fluorescence imaging measurement of the Qdot threshold concentration. Upper panel: NIR-II fluorescence image of a pork muscle after injecting 50 μL of RNase-A@PbS Qdot with different concentrations (1, 5, 26 and 130 nM) at the depth of 0.5 cm (excitation: 808 nm; power density: 15 mW/cm^2; exposure time: 100 ms). Lower panel: A plot of the ROI intensity (indicated in the circle) as a function of the Qdot concentration.

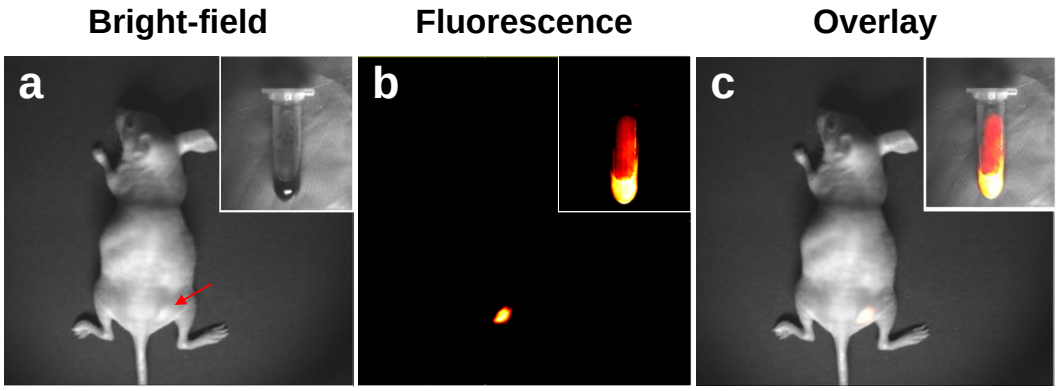

Figure S11. (a) Dorsal bright-field (BF) image of a nude mouse submuscularly injected with 20 μL of RNase-A@PbS Qdot (~26 nM) into its leg muscle. The red arrow indicates the injection site. (**b**) NIR-II fluorescence image of the mouse under NIR illumination (808 nm diode laser). (**c**) Overlay of optical and fluorescence images. The insets of Figure S11a to S11c display BF, fluorescence and overlay image of the Qdots in a sample vial, respectively.

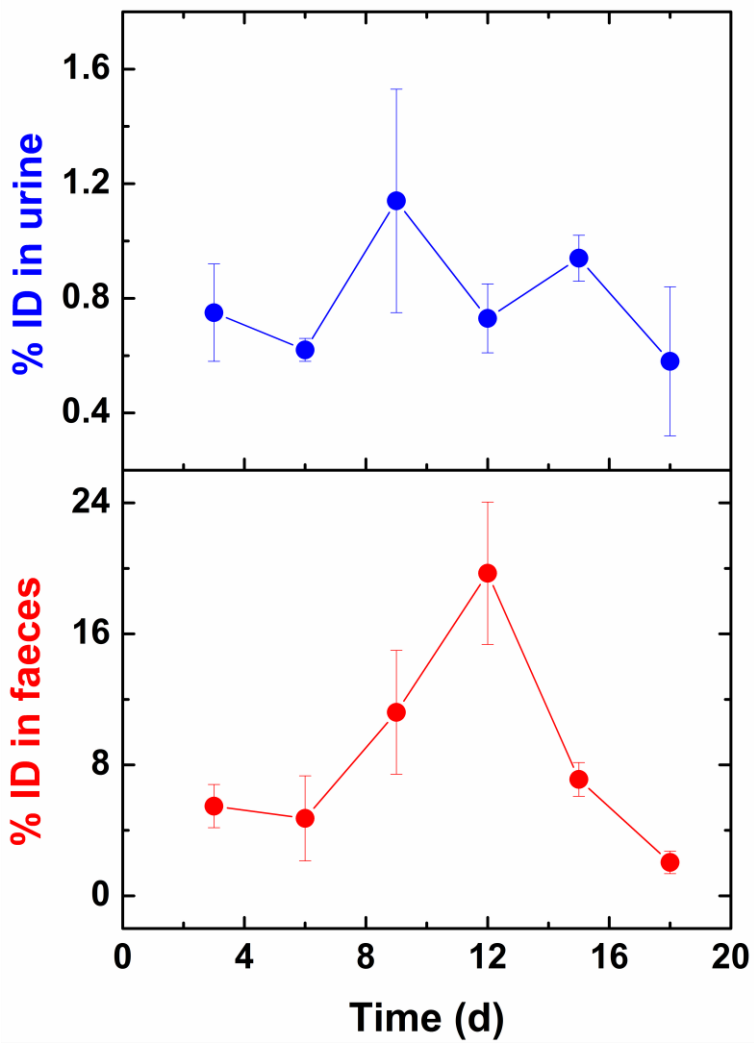

Figure S12. Urinary (top panel) and fecal (bottom panel) excretion of RNase-A@PbS Qdots over an 18-day period, as determined by ICP-MS. The mouse's feces and urine have been collected every 3 days during the experiment period (n = 2 mice), and dissolved in hot HNO_3 (~70°C) with stirring overnight. The %IDs of excreted PbS Qdots are given as the percentage ratios of Pb concentration to the Qdots initially injected.[7]