Supporting Information

Transformation of Viral Light Particles into Near-Infrared Fluorescence Quantum Dots-Labeled Active Tumor-Targeting Nanovectors for Drug Delivery

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Experimental Section

Materials.

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO. DiO was obtained from BeyotimeR Biotechnology, CellMask™Deep Red Plasma Membrane, SYTO 82 and LysoTracker Green were purchased from Thermo Fisher Scientific. Primary antibody against Pseudorabies virus (PrV) was purchased from Abcam. Dylight 649 conjugated secondary antibody was purchased from EarthOx. Doxorubicin hydrochloride (Dox) was obtained from Sangon Biotech.

Animals.

All animal procedures were approved by the Animal Care and Use Committee of Wuhan University. Healthy female nude mice (BALB/c) were purchased from Hunan SJA Laboratory Animal Co., Ltd.

Cell Culture and Purification of Light Particles (L-particles).

PK15 and HeLa cells were cultured in DMEM supplemented with 10% (v/v) FBS at 37 °C, 5% CO_2. PrV was propagated in PK15 cells in DMEM supplemented with 2% (v/v) FBS at 37 °C, 5% CO_2 for 48 h to replicate L-particles. After two freeze-thaw cycles, the infected PK15 cells were centrifuged at 5000 rpm for 30 min to remove

cell debris. Then, the supernatant was concentrated at 30000 rpm for 2.5 h in a Type 45 rotor (Beckman) by ultracentrifugation. The precipitation was resuspended in 1×phosphate buffer solution (1×PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and dispersed extensively by vortex for 10 min. Afterwards, virus was purified by 30%-55% sucrose density gradient centrifugation at 26000 rpm for 2.5 h in an SW41 rotor (Beckman). The upper band was collected, resuspended in 1×PBS and centrifuged at 25000 rpm for 3 h to remove sucrose. L-particles was resuspended in 1×PBS and stored at -80 °C for further use.

Characterization of L-particles.

Transmission electron microscopy (TEM). The purified L-partilces (10 μL) and PrV virions (10 μL) were dropped on carbon-coated copper grids for 10 min and subsequently drained on filter paper, respectively. The samples were negatively stained with 10 μL of 2% sodium phosphotungstate for 5 min and then washed with ultrapure water thrice. The prepared samples were examined by transmission electron microscope (H-7000 FA, Hitachi).

Protein electrophoresis. L-particles and mature virions were incubated with 5×loading buffer and heated at boiled water to decompose. Afterwards, the proteins of prepared samples were separated by SDS-PAGE gels and stained with Coomassie brilliant blue (CBB). The SDS-PAGE gels were imaged by Gel imager.

Fluorescence colocalization. Purified L-particles were dropped on glass coverslips (22 nm×22 nm) rinsed with plasma, and then heated to fix. L-particles were incubated with 100 μL of the primary antibody against PrV at 37 °C for 1.5 h after washing with 1×PBS thrice. Then, the sample was shaken thrice in 1×PBS for 5 min at room temperature to remove redundant primary antibody. The sample was incubated with Dylight 649 conjugated secondary antibody at 37 °C for 40 min and washed thrice with 1×PBS. Subsequently, viral DNA was stained with 100 μL of 5 ng/mL SYTO 82 at room temperature for 40 min. Moreover, viral envelope was labeled with 100 μL of 5 μM DiO at room temperature for 40 min and washed with 1×PBS for use. The prepared samples were observed by confocal microscope.

Characterization of QDs, QDs Labeled L-particles (QDs@L-particles) and L-particles Loaded with Dox (Dox@L-particles).

Flow cytometry. 100 μL of L-particles (1 mg/mL, protein content) and 100 μL of different concentrations of QDs (0.0375-0.45 mg/mL) were added to 0.4 cm electroporation cuvettes and electroporated using Bio-Rad Gene Pulser Xcell Electroporation System (Hercules, CA). Each sample was electroporated with 500 V/cm for a single pulse. After incubation at 37 °C for 30 min, the excess Ag_2Se QDs were removed by ultrafiltration. Finally, the labeled QDs@L-particles were resuspended in 1×PBS. Similarly, 100 μL of L-particles (1 mg/mL, protein content) and 100 μL of different concentrations of Dox (5-40 μg/mL) were added to 0.4 cm electroporation cuvettes for the same procedures. The samples were detected by flow cytometer (FACSAria III BD). The results were analyzed by Flowjo software.

High resolution transmission electron microscopy (HR-TEM). Ag_2Se QDs (5 μL) were drying on an ultrathin carbon-coated copper grid. QDs@L-particles (10 μL) were dropped on an ultrathin carbon-coated copper grid for 10 min and then negatively stained with 10 μL of 2% sodium phosphotungstate for 10 sec. The prepared samples were examined with a high resolution transmission electron microscope (JEOL JEM-2100).

Characterization of QDs. The diameter of Ag_2Se QDs was calculated by Nano Measurer 1.2 software. The UV-vis absorption spectra and fluorescence emission spectra of Ag_2Se QDs were acquired by spectrometer (Shimadzu UV-2550) and Fluorolog-3 spectrophotometer (Horiba Jobin Yvon Inc.), respectively.

Fluorescence colocalization. The envelopes of QDs@L-particles and Dox@L-particles fixed on glass coverslips were labeled with 100 μL of 5 μM DiO at room temperature for 40 min and washed with 1×PBS for use.

Preparation and Characterization of L-particles Labeled with QDs and Loaded with Dox (QDs&Dox@L-particles).

Preparation of QDs&Dox@L-particles. 0.3 mg/mL of Ag_2Se QDs and 20 μg/mL of Dox were added into 100 μL of L-particles (1 mg/mL, protein content) in 0.4 cm

electroporation cuvettes. The mixture was electroporated with a single pulse of 500 V/cm by Bio-Rad Gene Pulser Xcell Electroporation System (Hercules, CA). After incubating at 37 °C for 30 min, the excess free Ag_2Se QDs and Dox were removed by ultrafiltration to obtain QDs&Dox@L-particles.

Fluorescence colocalization. The envelopes of QDs&Dox@L-particles fixed on glass coverslips were stained with 100 μL of 1.5 mg/mL CellMask™ Deep Red plasma membrane at room temperature for 30 min and washed with 1×PBS for use.

Cell Viability and Proliferation Assays.

Cell viability. HeLa cells were incubated with different concentrations of L-particles and QDs@L-particles (0-40 μg/mL, protein content) for 48 h or with 1×PBS, Ag_2Se QDs, L-particles and QDs@L-particles (20 μg/mL) for different hours. Supernatants were replaced by 100 μL of 1×PBS and 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT). After further incubating at 37 °C, 5% CO_2 for 4 h, the liquids were removed and 200 μL of dimethyl sulfoxide (DMSO) was added. The UV absorbance was measured at 490 nm.

Proliferation assays. HeLa cells were seeded into 12-well plates and then incubated with PBS, Ag_2Se QDs, L-particles and QDs@L-particles (20 μg/mL) for 0-96 h. Subsequently, HeLa cells were digested and counted by hemocytometer.

Blood Analysis and Histological Examination.

Age- and weight-matched female BALB/c mice were intravenously injected with 1×PBS, L-particles, Ag_2Se QDs, and QDs@L-particles (100 μL, 1 mg/mL, protein content, n = 5) *via* the tail vein. The injection was repeated every 3 days for up to 5 times. The body weights of mice were measured every 3 days. After 3 weeks, the mice were euthanized. 200 μL of blood samples from each mouse were collected for the further whole blood analysis. The organs harvested from each mouse were fixed in 4% buffered paraformaldehyde for 24 h, and then embedded, sliced, stained with H&E. The samples were observed with a CCD camera (Nikon digital sight DS-U3) mounted on an inverted fluorescence microscope (Ti, Nikon).

Cellular Uptake Assays.

HeLa cells planted in a 35 mm glass-based dish in advance were incubated with microvesicles (MVs) labeled with Ag_2Se QDs and loaded with Dox (QDs&Dox@MVs) or QDs&Dox@L-particles at 4 °C for 15 min, respectively. After washing redundant QDs&Dox@MVs or QDs&Dox@L-particles, HeLa cells were incubated at 37 °C for 1 h. The prepared samples were observed and imaged by a spinning-disk confocal microscope. The statistic of the internalization of QDs&Dox@MVs and QDs&Dox@L-particles were calculated by Image Pro Plus (IPP) software.

Establishment of Xenografts in Nude Mice.

HeLa cells (5×10^7 cells in 1 mL 1×PBS) were inoculated subcutaneously into the flanks of the female BALB/c nude mice. The size of xenografts was measured every 3 days. Once the tumor volumes, which were calculated by the formula ($width^2\times length)/2$. reached approximately 150 mm^3, the mice were divided into different groups randomly.

***In Vivo* Tumor-Targeting Study.**

1×PBS, L-particles, mixture of Ag_2Se QDs and Dox, QDs&Dox@MVs and QDs&Dox@L-particles (100 μL, 1 mg/mL, protein content) were injected *via* the tail vein of the tumor-bearing mice. After 0.5, 1, 2, and 4 h, the nude mice were imaged by CRI Maestro *in vivo* fluorescence imaging system (channel DeepRed). Afterwards, the mice were euthanized and the major organs and tumors were harvested and imaged by CRI Maestro *in vivo* fluorescence imaging system. The fluorescence intensity of each tumor was calculated by Maestro 3.0 software.

Dox Intracellular Release from QDs&Dox@L-particles.

HeLa cells planted in a 35 mm glass based dish in advance were treated with 5 μg/mL LysoTracker Green at 37 °C, 5% CO_2 for 30 min and then washed with 1×PBS thrice. Then, HeLa cells incubated with QDs&Dox@MVs and QDs&Dox@L-particles at 4 °C for 15 min, respectively. After washing redundant QDs&Dox@MVs and QDs&Dox@L-particles, HeLa cells were incubated at 37 °C for 1 h. The prepared

samples were observed and imaged by a spinning-disk confocal microscope.

Antitumor Effects of QDs&Dox@L-particles *In Vitro* and *In Vivo*.

HeLa cells planted in a 96-well plate in advance were incubated with DMEM supplemented with 10% (v/v) FBS, L-particles, free Dox, QDs&Dox@MVs and QDs&Dox@L-particles (20 μg/mL) at 37 °C, 5% CO_2 for 1 h. Supernatants were replaced by 100 μL of DMEM supplemented with 10% (v/v) FBS and then incubated 37 °C, 5% CO_2 for 2-48 h. Supernatants were replaced by 100 μL of 1×PBS and 20 μL of MTT. After further incubating at 37 °C, 5% CO_2 for 4 h, the liquids were removed and 200 μL of DMSO was added. The UV absorbance was measured at 490 nm.

To evaluate the antitumor effect of QDs&Dox@L-particles *in vivo*, tumor-bearing mice were randomly separated into five groups (5 mice in each group) and intravenously injected with 1×PBS, L-particles, free Dox, QDs&Dox@MVs and QDs&Dox@L-particles, respectively. The injection was repeated every 3 days for up to 5 times. The body weights and volume of the tumors were measured every 3 days. After 30 days, the mice were euthanized. Tumors were harvested, photographed and weighed with a microbalance. Subsequently, tumors were fixed in 4% paraformaldehyde for 24 h, and then embedded, sliced, stained with 50 μL TUNEL (Roche) reaction mixture at 37 °C for 60 min. The cell nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI). The samples were observed and imaged by confocal microscope.

Fluorescence Imaging.

DiO/LysoTracker Green, Dox/SYTO 82, Dylight 649/CellMask and Ag_2Se QDs were excited by 488 nm, 561 nm, 640 nm and 488 nm laser (DPSS, USA), respectively. The emission signals were separated by 525/50 nm, 617/73 nm, 685/40 nm and 732/68 filters (Chroma) and recorded by an EMCCD (Andor iXon DU897U single photon detector).

Statistical Analyses.

Line profile, representing the intensity and distribution of every signal of each

channel on the line, was obtained by IPP software. The colocalization efficiency of fluorescent signals from two channels was quantified by intensity correlation analysis performed by Image J. The degree of colocalization was proved by Manders coefficient for red signals in thresholded image (tMr), Manders coefficient for green signals in thresholded image (tMg), and intensity correlation quotient (ICQ) values. tMg and tMr suggested the percentage of green/red signals colocalized with red/green signals in the image. ICQ value indicated the correlation intensity of two channels and ranged from -0.5 to 0.5 (+0.1 to +0.5 implies a strong covariance).

Supporting figures

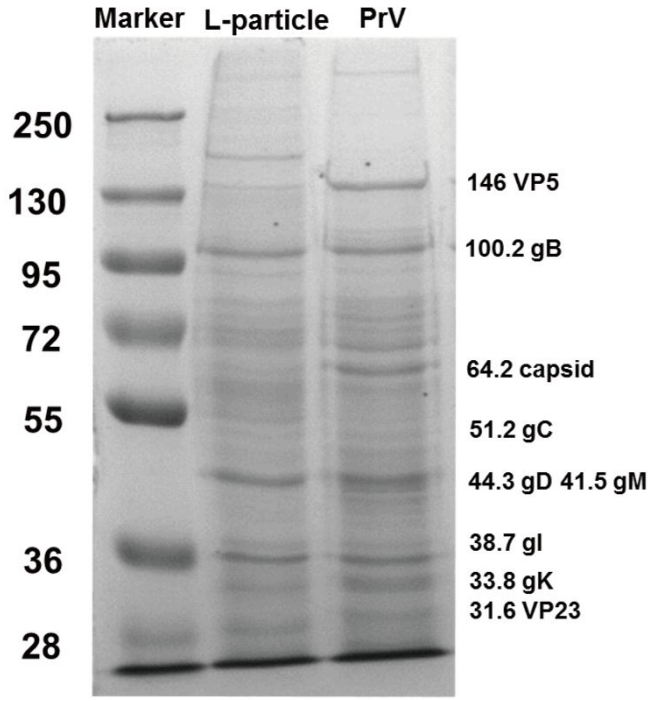

Figure S1. Presence analysis of proteins derived from L-particles of PrV and mature PrV virions.

The polypeptide profile revealed that L-particles contained several envelope proteins, such as gB, gC, gD, gM, gI and gK, as well as PrV virions. But, L-particles were lack of capsid proteins such as VP5 and VP23 which were observed in the PrV virions.[1]

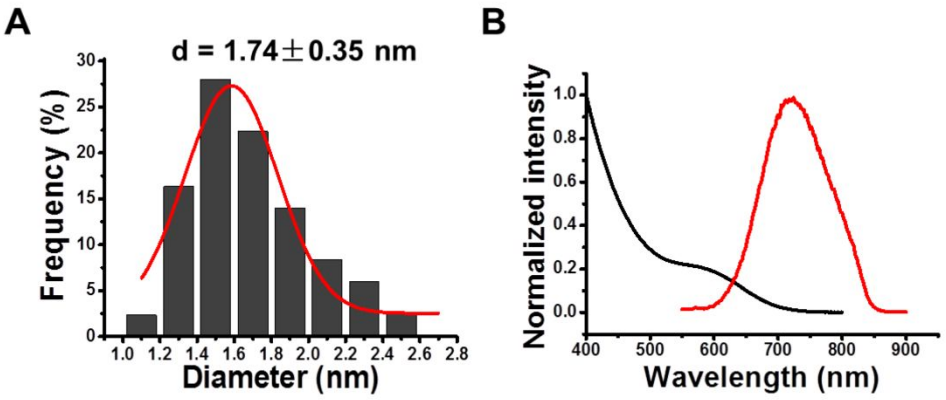

Figure S2. A) Diameter distribution of the Ag_2Se QDs according to TEM images (n = 290). B) UV-vis absorption spectra and fluorescence emission spectra of Ag_2Se QDs.

The average diameter of Ag_2Se QDs was 1.74 nm. The photoluminescence spectrum of Ag_2Se QDs showed an emission peak at 725 nm and the absorption spectrum exhibited an absorption peak at 590 nm.

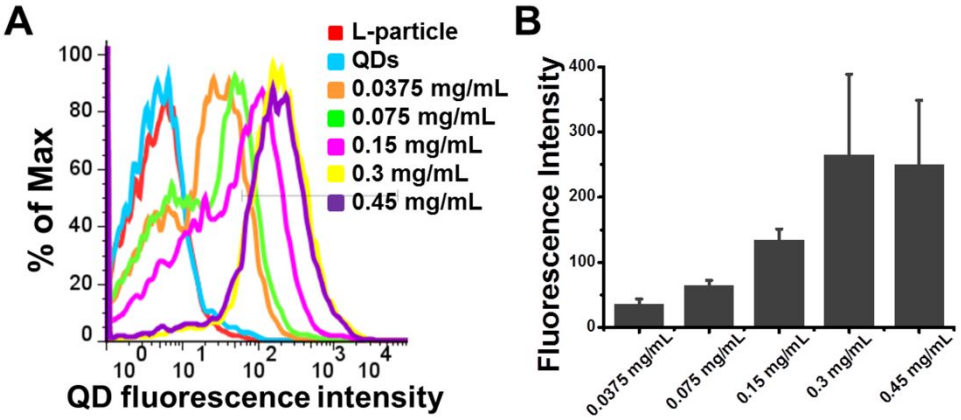

Figure S3. The working concentration of Ag_2Se QDs was optimized by flow cytometry. A) Labeling efficiency of L-particles electroporated with different concentrations of Ag_2Se QDs. B) Average fluorescence intensity of L-particles electroporated with different concentrations of Ag_2Se QDs (n = 10000).

As shown in **Figure S3**, both the labeling efficiency and average fluorescence intensity increased as the concentration of Ag_2Se QDs increased, 0.3 mg/mL was chosen as the working concentration. The results showed that *ca*. 80% of L-particles were labeled with Ag_2Se QDs, which is corresponding to the colocalization results.

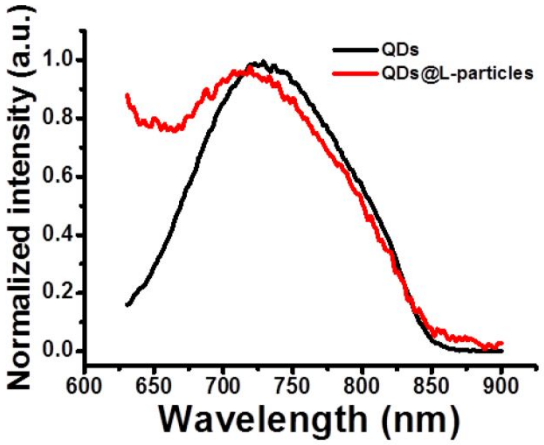

Figure S4. Fluorescence spectra of QDs (black) and QDs@L-particles (red).

Compared with Ag_2Se QDs, the peak positions of fluorescence spectra of QDs@L-particles almost did not shift, suggesting that labeling of L-particles with Ag_2Se QDs did not affect fluorescence properties of Ag_2Se QDs.

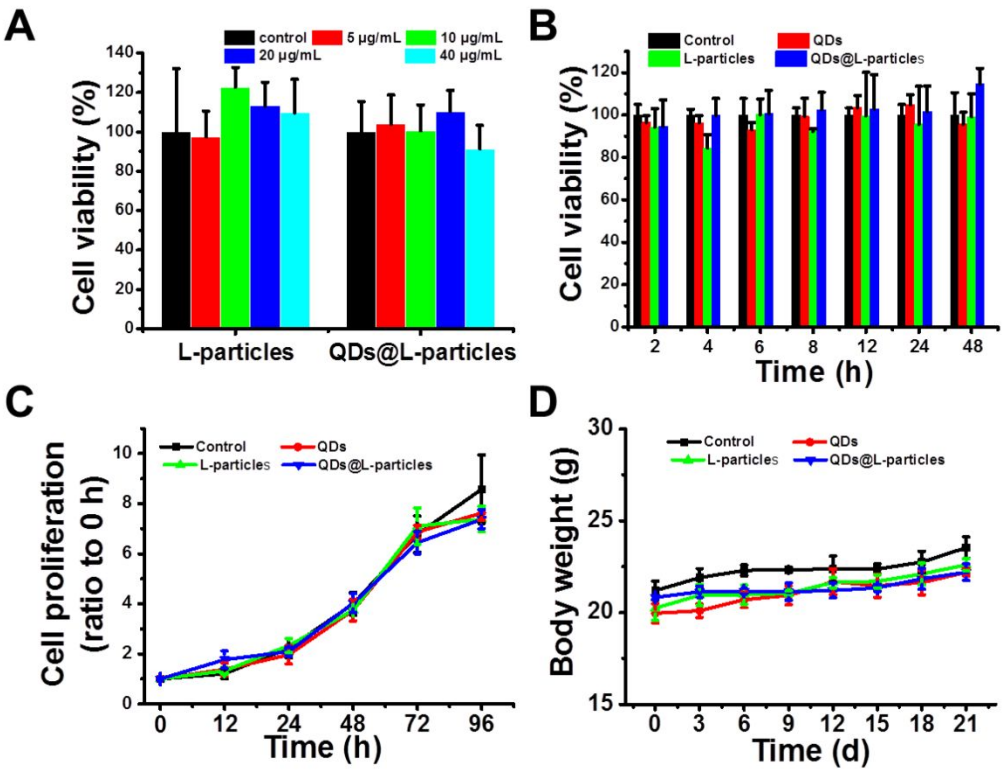

Figure S5. A) Cell viability of HeLa cells incubated with DMEM containing 10% (v/v) FBS (control), Ag_2Se QDs, L-particles and QDs@L-particles for 48 h (n = 9). B) Cell viability of HeLa cells incubated with DMEM containing 10% (v/v) FBS (control), Ag_2Se QDs, L-particles and QDs@L-particles for different hours (n = 9). C)

Proliferation of HeLa cells after incubating with DMEM containing 10% (v/v) FBS (control), Ag_2Se QDs, L-particles and QDs@L-particles for different hours (n = 3). D) Body weights of mice after intravenously injecting 1×PBS (control), Ag_2Se QDs, L-particles and QDs@L-particles (n = 5).

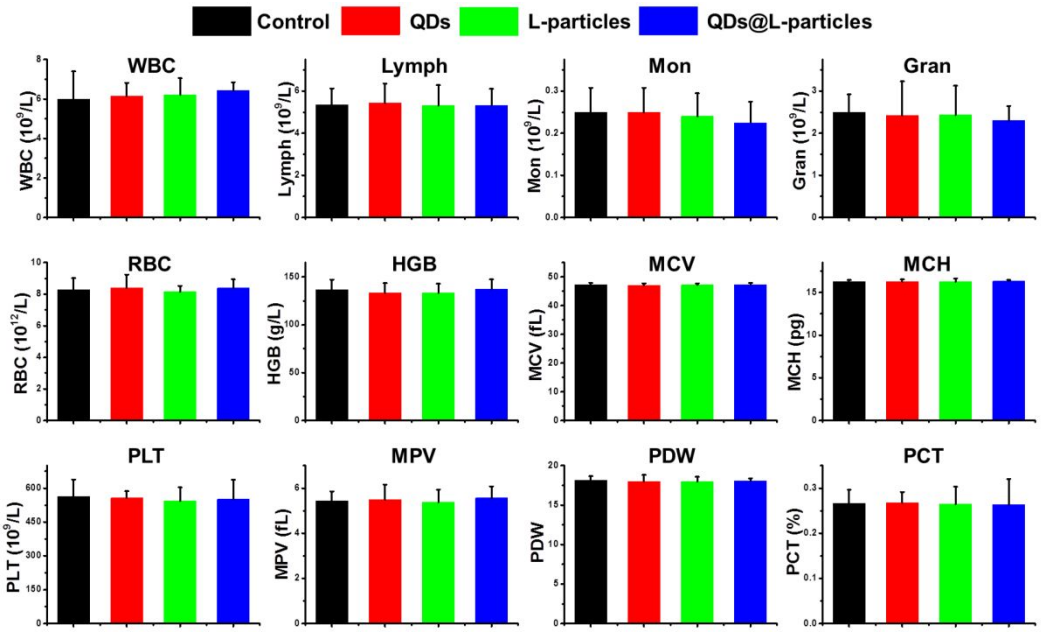

Figure S6. Whole blood cell analysis of mice after intravenously injecting 1×PBS (control), Ag_2Se QDs, L-particles and QDs@L-particles for three weeks (n = 5). WBC, white blood cell; Lymph, lymphocyte; Mon, monocyte; Gran, granulocyte; RBC, red blood cell; HGB, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; PLT, platelets; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit.

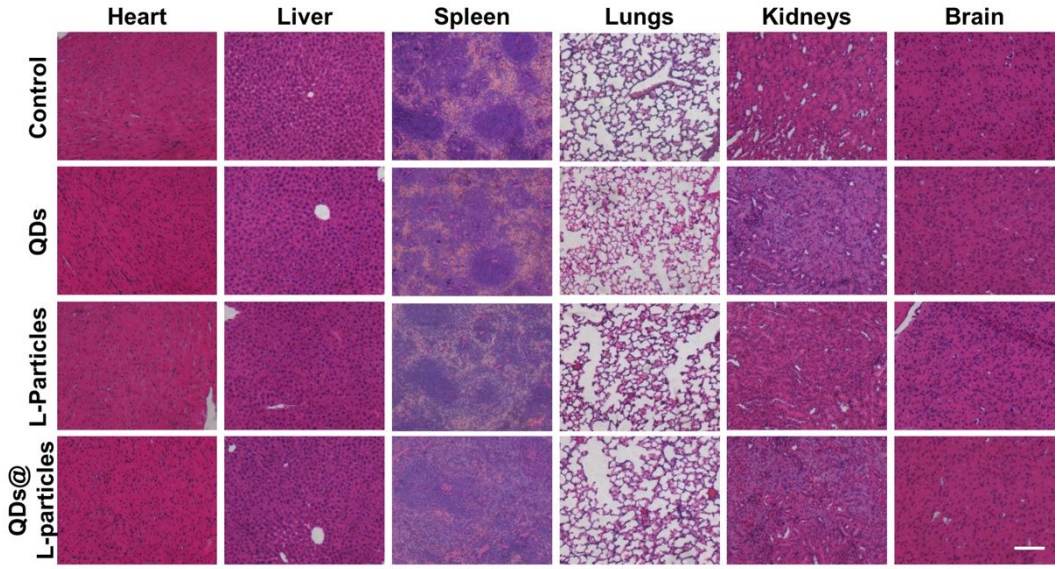

Figure S7. H&E staining of major organs, including heart, liver, spleen, lungs, kidneys and brain, dissected from mice intravenously injecting 1×PBS (control), Ag_2Se QDs, L-particles and QDs@L-particles for three weeks, respectively. Scale bar: 100 μm.

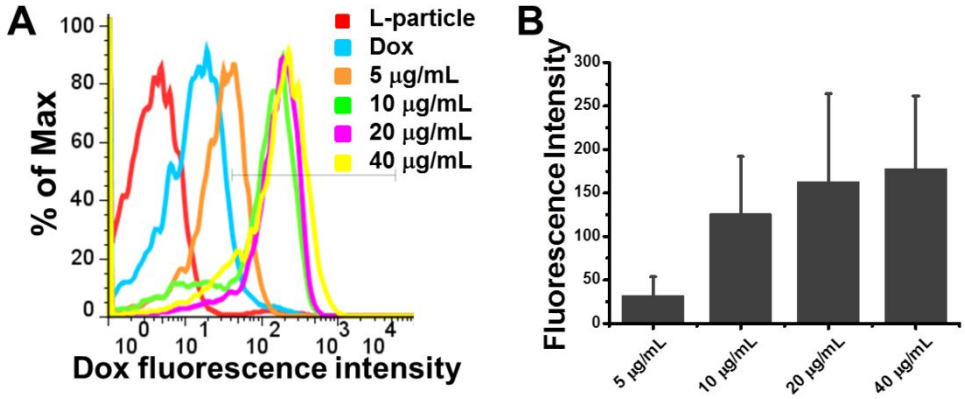

Figure S8. The working concentration of Dox was optimized by flow cytometry. A) Loading efficiency of L-particles electroporated with different concentrations of Dox. B) Average fluorescence intensity of L-particles electroporated with different concentrations of Dox (n = 10000).

As shown in **Figure S8**, both the loading efficiency and average fluorescence intensity increased as the concentration of Dox increased, 20 μg/mL was chosen as the working concentration. The results showed that *ca*. 80% of L-particles were loaded with Dox, which coincides to the colocalization results.

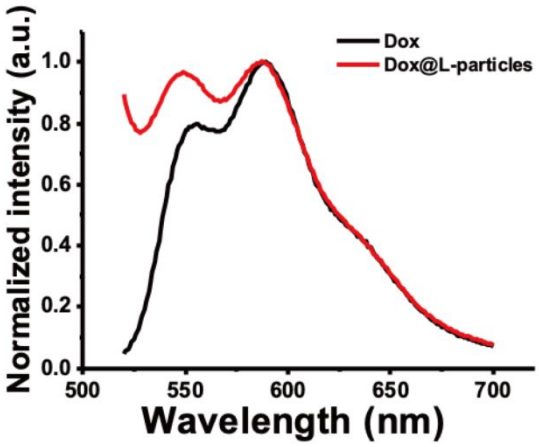

Figure S9. Fluorescence spectra of Dox (black) and Dox@L-particles (red).

The peak positions of fluorescence spectra of Dox@L-particles did not shift compared with free Dox, suggesting that loading L-particles with Dox did not affect fluorescence properties of Dox.

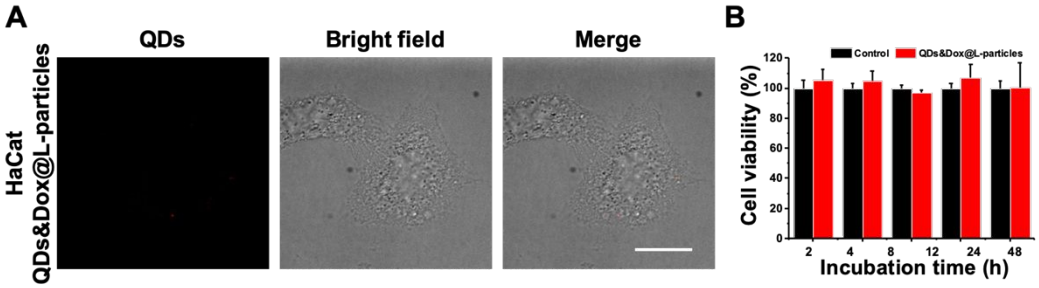

Figure S10. A) Confocal images of HaCat cells incubated with QDs&Dox@L-particles. B) Cell viability of HaCat cells treated with DEME containing 10% FBS (control) and QDs&Dox@L-particles for different hours (n = 9). The cell viability of control was set as 100%. Scale bar: 20 μm.

As shown in the **Figure S10A**, no obvious Ag_2Se QDs signals were observed, indicating that QDs&Dox@L-particles could not attach at non-tumor human epithelial cells (HaCat cells). Meanwhile, QDs&Dox@L-particles exhibited no significant cytotoxicity to HaCat cells (**Figure S10B**), suggesting that QDs&Dox@L-particles could actively target tumor cells specifically.

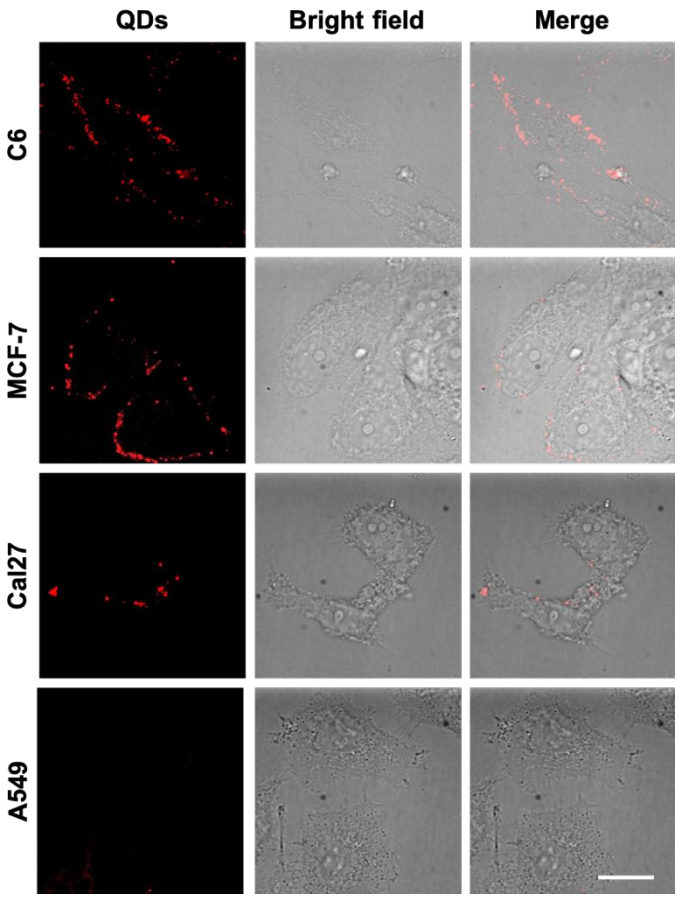

Figure S11. Confocal images of different tumor cells incubated with QDs&Dox@L-particles. Scale bar: 20 μm.

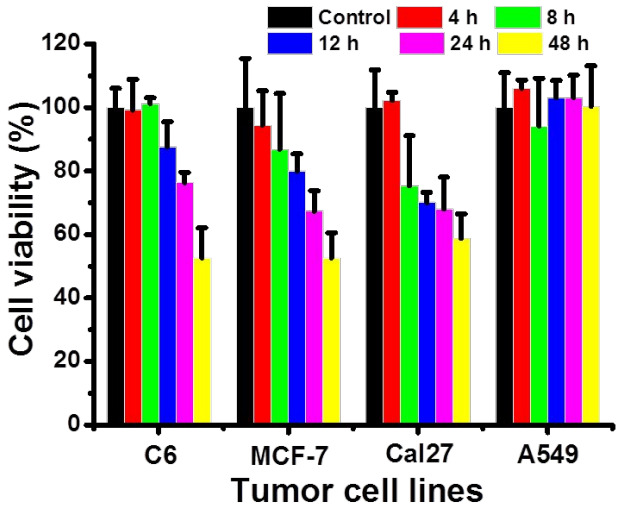

Figure S12. Cell viability of different tumor cells treated with DEME containing 10% FBS (control) and QDs&Dox@L-particles for different hours (n = 9). The cell viability of control was set as 100%.

As shown in **Figure S11**, QDs&Dox@L-particles attached at the plasma membrane of C6 (glioma cancer cells), MCF-7 (breast cancer cells) and Cal27 cells (oral cancer cells), while no obvious fluorescence signals of QDs&Dox@L-particles were observed at the surface of A549 cells (lung cancer cells). The cell viability of C6, MCF-7 and Cal27 cells incubated with QDs&Dox@L-particles decreased rapidly, demonstrating that QDs&Dox@L-particles could actively target to these cells and release antitumor drug accurately and effectively (**Figure S12**). However, the cell viability of A549 cells incubated with QDs&Dox@L-particles was similar to control group, indicating that QDs&Dox@L-particles could not attach and enter A549 cells. Thus, compared to A549 cells, C6, MCF-7 and Cal27 cells were more sensitive to QDs&Dox@L-particles, indicating that QDs&Dox@L-particles showed selectivity to different cancer cells.

According to references, the attachment and internalization process of PrV is a complex process including several viral envelope proteins (gC, gD, gB, gH and gL)[1-4] and cellular receptors (HveA, HveB, HveC, HveD and 3-O-sulfated heparan sulfate).[5-7] Thus, the origin of selectivity may be caused by the expression level of cellular receptors of PrV.

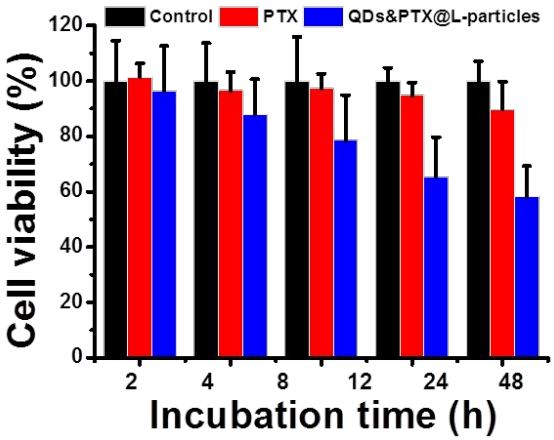

Figure S13. Cell viability of HeLa cells treated with DEME containing 10% FBS (control), paclitaxel (PTX) and QDs&PTX@L-particles for different hours (n = 9). The cell viability of control was set as 100%.

In order to further demonstrate the general applicability of L-particles, L-particles were loaded with Ag_2Se QDs and another type of cancer drug paclitaxel

(PTX) (QDs&PTX@L-particles) and then incubated with HeLa cells. The results showed that the cell viability of HeLa cells incubated with QDs&PTX@L-particles decreased to *ca.* 50%, which is similar to the results of QDs&Dox@L-particles, indicating that L-particles could actively target to HeLa cells and release antitumor drug effectively.

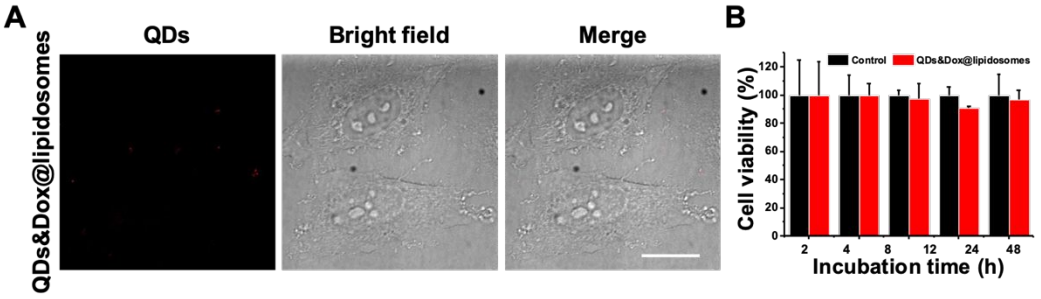

Figure S14. A) Confocal images of HeLa cells incubated with QDs&Dox@lipidosomes. B) Cell viability of HeLa cells treated with DEME containing 10% FBS (control) and QDs&Dox@lipidosomes for different hours (n = 9). The cell viability of control was set as 100%. Scale bar: 20 μm.

Lipidosomes labeled with Ag_2Se QDs and loaded with Dox (QDs&Dox@lipidosomes) were used as a negative control. As shown in **Figure S14A**, no significant fluorescence signals of Ag_2Se QDs were observed, indicating that unmodified lipidosomes could not attach and further enter HeLa cells. Furthermore, the toxicity of QDs&Dox@lipidosomes has also been measured (**Figure S14B**). The results showed that QDs&Dox@lipidosomes exhibited no significant cytotoxicity to HeLa cells, suggesting that QDs&Dox@L-particles could actively target tumor cells without any further modification.

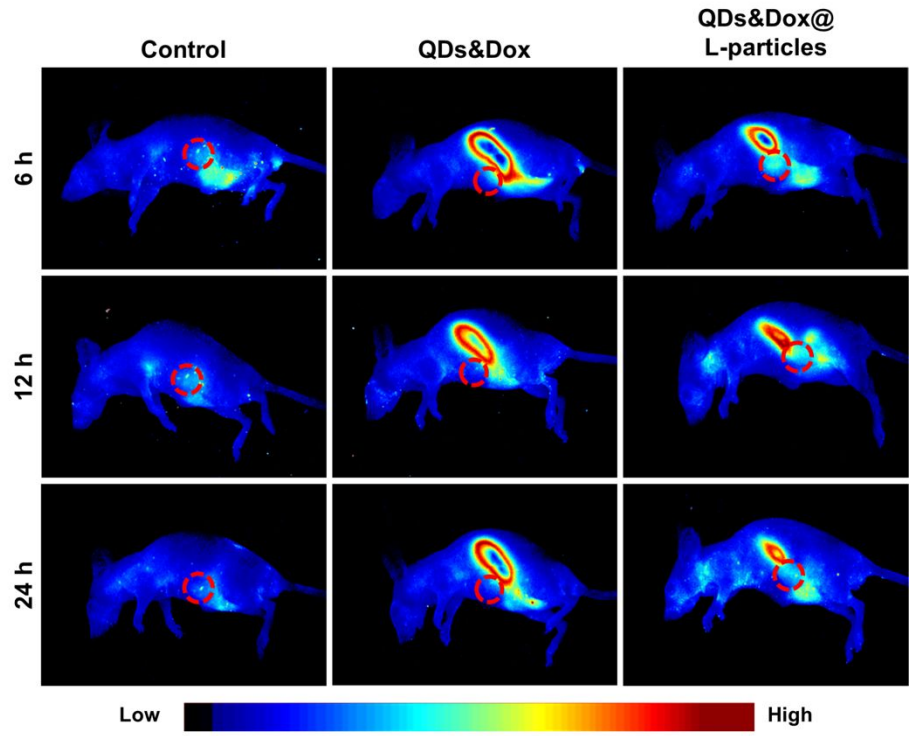

Figure S15. Photostability of multi-functional materials *in vivo* near-infrared fluorescence (NIR) fluorescence images of tumor-bearing nude mice intravenously injected with 1×PBS (control), mixture of Ag_2Se QDs and Dox, and QDs&Dox@L-particles. The red circles indicated the location of tumors.

The NIR fluorescence signals of QDs&Dox@L-particles could be detected 24 h after intravenous injection, indicating that QDs&Dox@L-particles were stable in the complicated *in vivo* environment.

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