Smart pH-responsive and high doxorubicin loading nanodiamond for in vivo selective targeting, imaging, and enhancement of anticancer therapy

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Experimental section

Preparation of ND-PEG

ND (5 mg) was first weighed exactly and dispersed in MES (5 mL, 0.1 M, pH 5.8) buffer solution for sonication at room temperature for 1 h. Then EDC (1.0 mg) and NHS (1.25 mg) were added successively and the solution was kept stirring vigorously at room temperature for 6 h. After that, the precipitate was obtained by centrifuging the solution at a speed of 15000 rpm for five minutes to remove any unreacted EDC and NHS. Then the activated ND was resuspended into the BBS (5 mL, 0.1 M, pH 8.4) buffer solution and sonicated for a moment followed by adding H_2N-PEG-

COOH (1.5 mg). The solution was kept stirring vigorously at room temperature overnight. The final product, ND-PEG, was obtained by centrifuging the solution at a speed of 15000 rpm for five minutes and purifying it with pure water for thrice to remove unbound H_2N-PEG-COOH. Finally, the ND-PEG was placed in a vacuum drying oven and protected from light.

The grafting amount of H_2N-PEG-COOH onto ND

The grafting amount of H_2N-PEG-COOH onto ND was measured as previously reported by us.[1] Fluorescamine stock solution was prepared in acetone (1 mg/mL), protected from light and stored refrigerated when not in use. The H_2N-PEG-COOH stock solution was prepared by dissolving 1 mg of H_2N-PEG-COOH in 10 mL of borate buffer solution (pH 8.4) and taken out of corresponding amount to achieve the concentrations of 0, 4, 10, 14 and 20 μmol/L, respectively. Taking 2 mL H_2N-PEG-COOH solution and adding fluorescamine in them as a 1:1 stoichiometric ratio (illustrated in Scheme. S1), the mixture solution was then eddied ten seconds immediately and stood five minutes. The absorption and fluorescence emission of the fluorescent product formed between fluorescamine and H_2N-PEG-COOH was initially explored by fluorescence spectroscopy with the excitation and emission wavelengths of 388 and 485 nm (Fig. S1A). A standard curve was plotted using H_2N-PEG-COOH concentrations (μmol/L) as the abscissa, and the fluorescence intensity as the ordinate (Fig. S1B). The linear regression equation ($F=43.716c + 67.489$, $R^2=0.9998$) was obtained. The amount of grafted H_2N-PEG-COOH was determined by the difference value of total H_2N-PEG-COOH and remanent H_2N-PEG-COOH. The amount of H_2N-PEG-COOH grafted onto ND was nearly (150±10) μg per milligram nanodiamond.

Stability

The stability of NPDC with a concentration of 0.5 mg/mL in the PBS (pH 7.4) buffer solution was determined at 4 °C for a period of up to 150 days. Samples were kept in plastic vials and

protected from light. The liquid supernatant of samples was obtained by centrifugation and measured the absorbance at 480 nm of DOX at set intervals. Drug leakage (%) was also evaluated as the percentage of free drug in the nanoparticles suspension increased.

The effect of Na_3Cit medium on DOX adsorption

To verify the important role of Na_3Cit medium on DOX adsorption, a PB medium with the pH of 8.65 was used instead of Na_3Cit medium. Specifically, duplicate of ND-PEG (1mg) were dispersed into the medium of PB (pH 8.65) and Na_3Cit (1.0M, pH 8.65) to sonicate for 1 h at room temperature followed by adding DOX (200 μg) and mixed thoroughly, respectively. After shaking for 6 h, the solution was centrifuged to remove any non-adsorbed DOX. The product was obtained by washing three times with deionized water and finally placed in a vacuum drying oven and protected from light. The amount of DOX adsorbed was determined by calculating the change in DOX concentration before and after the adsorption using a UV-Vis spectrophotometer at 480 nm. The amount of DOX loading on ND-PEG in Na_3Cit medium is 170.0 ± 1.26 μg/mg, while it is 50.4 ± 2.03 μg/mg in the PB medium (pH 8.65). So the result demonstrated that Na_3Cit medium played an important role in the adsorption of DOX onto PEGylated nanodiamond, which led to high drug loading of NPDC.

Cell morphology

MCF-7 cells were seeded in a 35 mm per dish (1.5×10^5) overnight and treated with media alone or with DOX, ND-PEG or NPDC with 5 μg/mL of DOX equivalent. The treated cells were incubated at 37 °C for 48 h and 72 h in a humidified atmosphere containing 5 % CO_2, respectively. The cellular morphology was observed using an Optical microscope.

Cell uptake

MCF-7 cells were plated at a density of 2×10^5 per 35 mm Petri dish in complete medium for 16-

20 h. Thereafter, the cells were treated with NPDC（5 μg/mL）for 1 h, 3 h, 5 h, 7 h and 16 h, respectively. After treatment with or without NPDC, the cells were collected and washed with ice-cold PBS. The samples were analyzed by flow cytometer. A minimum of 10000 cells were analyzed. The fluorescence from the NPDC was excited at a wavelength of 488 nm and the emission was collected in the red light signal range. The fluorescence intensity was quantified by Cell Quest software.

Scheme and Figures:

Scheme. S1 Reaction of fluorescamine and H_2N-PEG-COOH, yielding a highly fluorescent product.

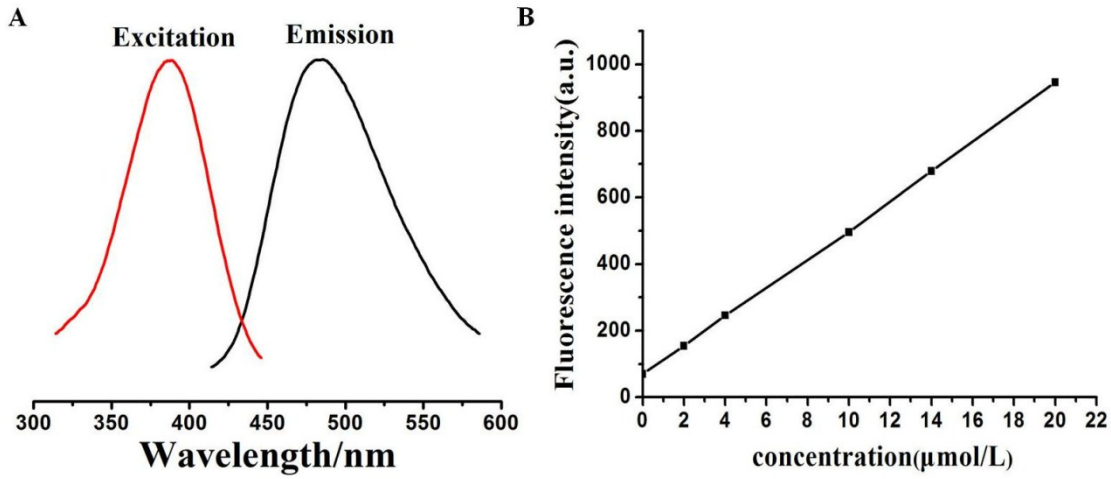


Fig. S1 The interaction between H_2N-PEG-COOH and fluorescamine. (A) Fluorescence excitation and emission profiles of the fluorescent product resulting from the reaction with excitation and emission wavelengths of 388 and 485 nm, respectively. (B) Standard curve of the interaction between H_2N-PEG-COOH and fluorescamine.

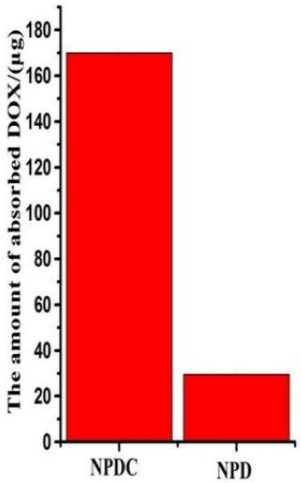


Fig. S2 The difference in loading DOX on ND-PEG carriers, where one is in the sodium citrate medium (NPDC), the other is deionized water (NPD).

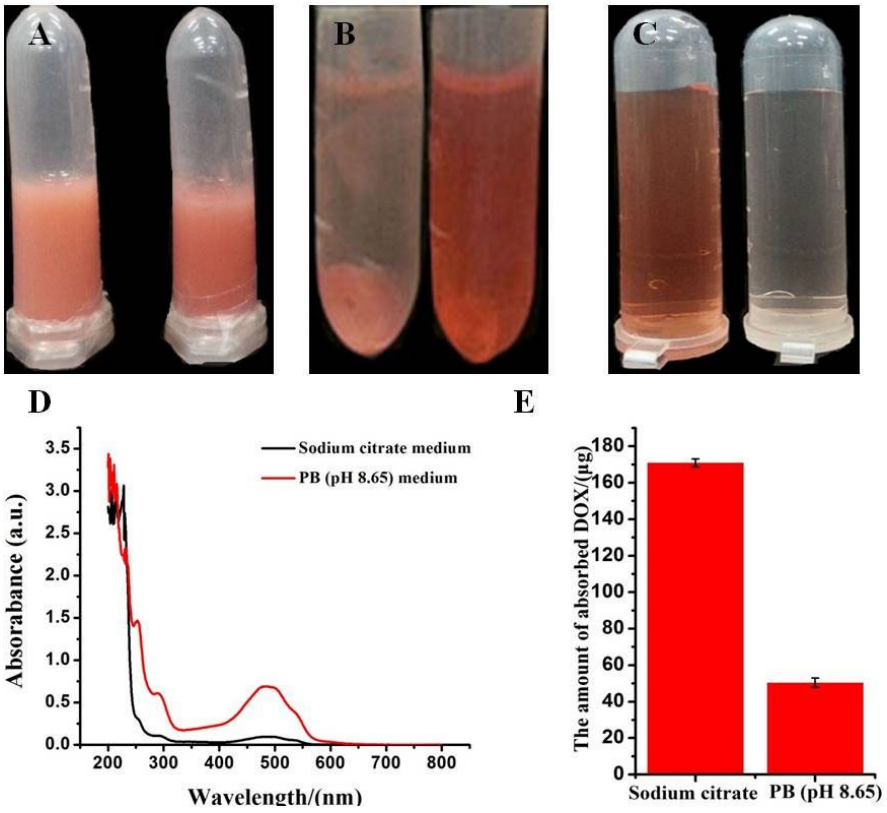


Fig. S3 The effect of Na_3Cit medium on DOX adsorption. (A) The initially mixture solution of ND-PEG and DOX in Na_3Cit medium (left) and PB medium with the pH of 8.65 (right). (B) The images of ND-PEG and DOX in Na_3Cit medium (left) and PB medium with the pH of 8.65 (right) after shaking for 6 h and centrifuging for 5 min. (C) The supernatant after centrifuging and washing for thrice with deionized water (in Na_3Cit (right) and PB medium with the pH of 8.65 (left). (D) UV–Vis spectra corresponding to (C). (E) The loading amount of DOX on ND-PEG carriers, where one is in the Na_3Cit medium, the other is in the PB medium.

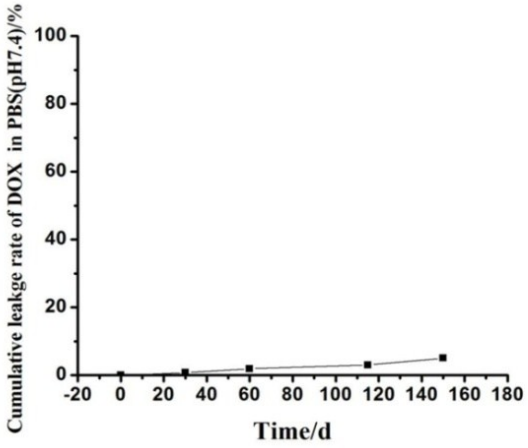


Fig. S4 The stability of ND-PEG-DOX/Na_3Cit in the PBS (pH 7.4) buffer solution

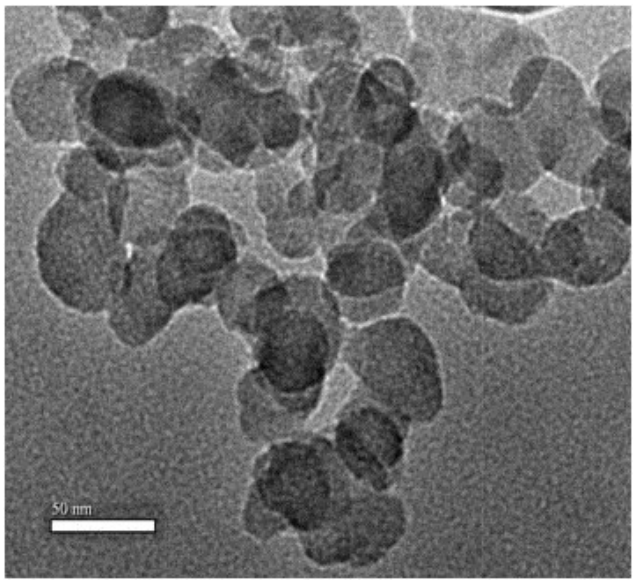


Fig. S5 Typical transmission electron microscopic images of DOX in Na_3Cit solution. The scale bars represent 50 nm.

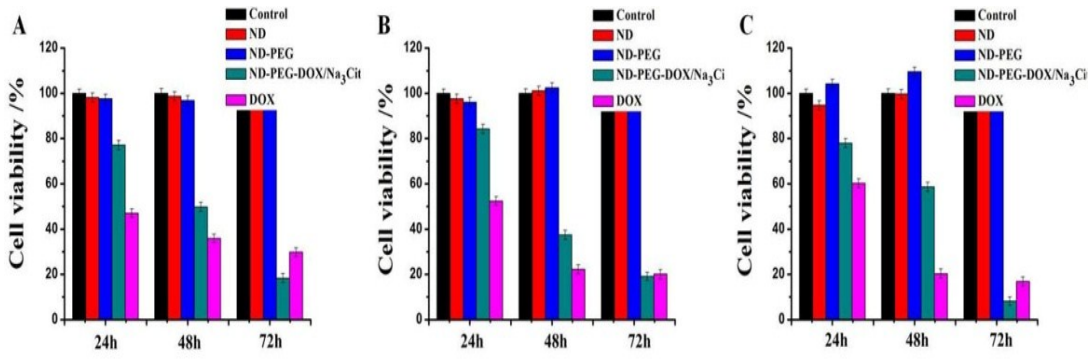
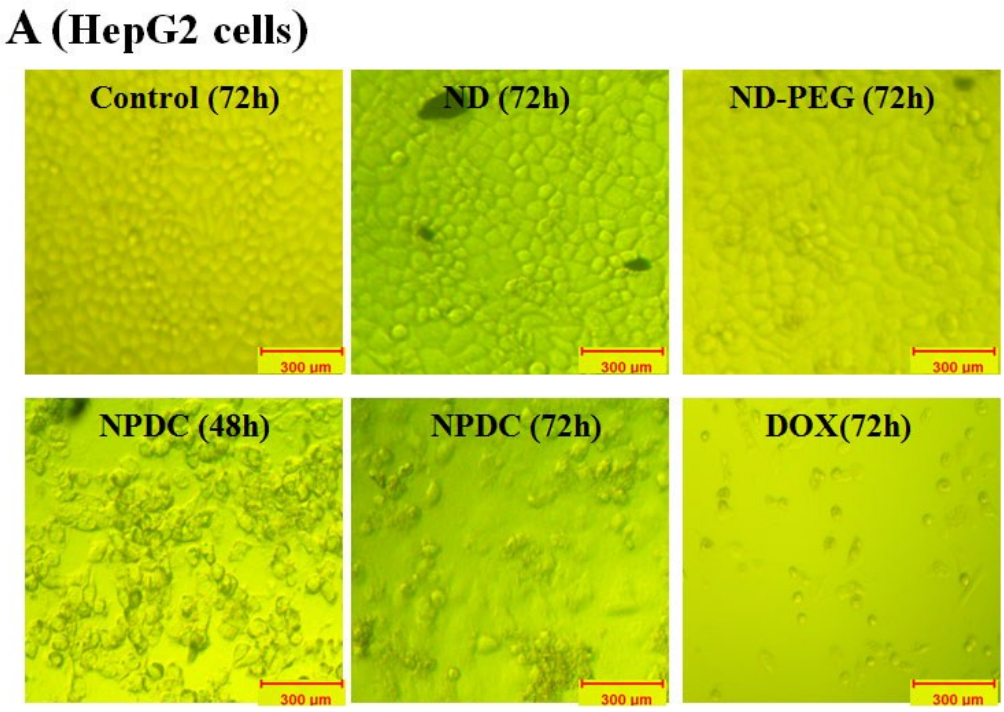


Fig. S6 Effect of free DOX and NPDC on HepG2, HeLa and MCF-7 cells viability for different time was measured by MTT assay. (A) HepG2, (B) HeLa and (C) MCF-7. Experiments were repeated three times and data are presented as the mean ± SD (for each group, n = 6).

It can be seen that HepG2, HeLa and MCF-7 cells were inhibited by both free DOX and NPDC in a time-dependent mode from Fig. S6A, Fig. S6B and Fig. S6C. Compared with the group treated with DOX, the cytotoxic effect of NPDC displayed lower on all three cells than free DOX at the incubation time of 24 h and 48 h, but obviously exhibited a little higher cytotoxic effect on HepG2 and MCF-7cells than that of free DOX and had a similar cytotoxic effect on HeLa cells to free DOX when the incubation time for 72 h.



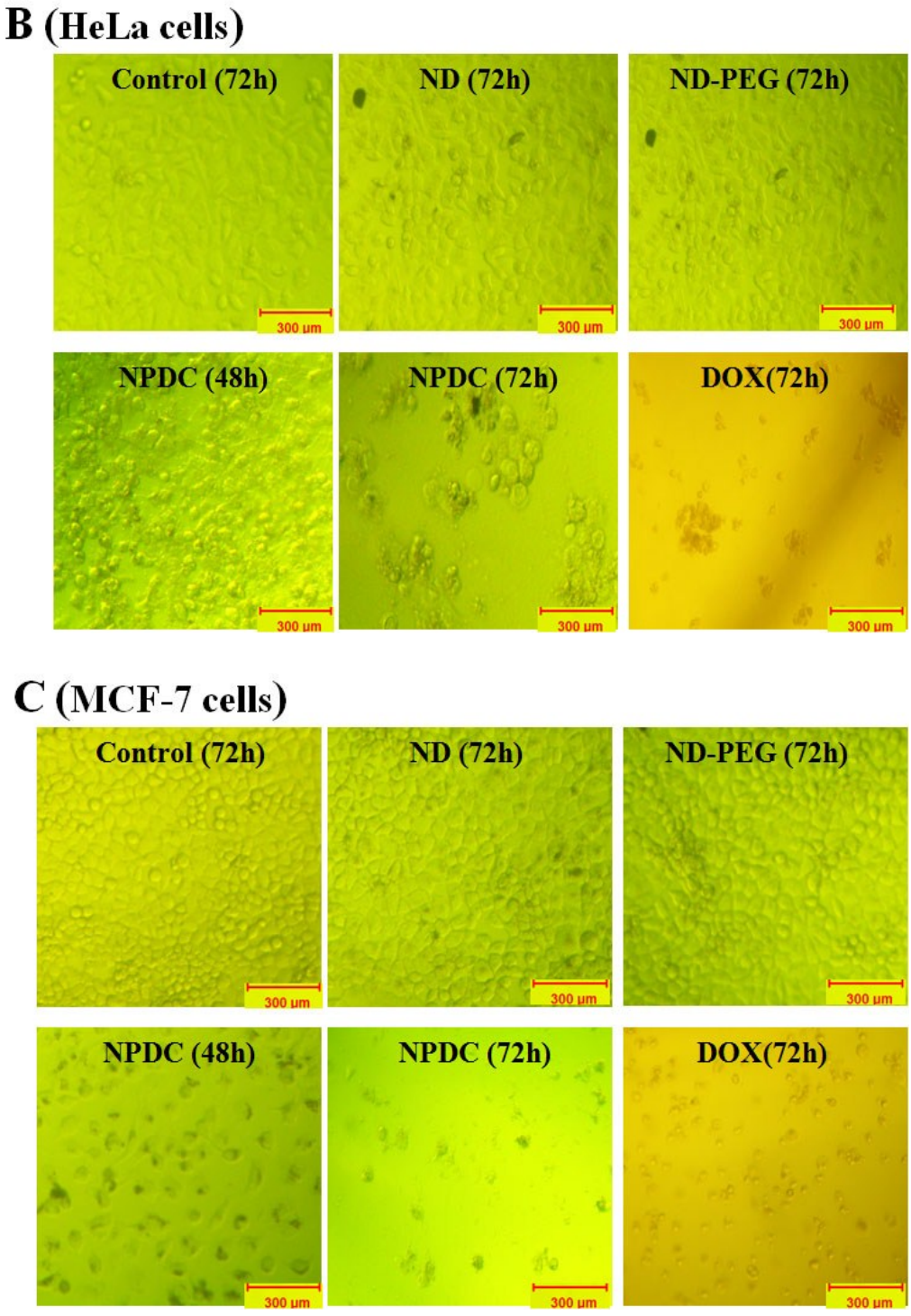


Fig. S7 Microscopy images of cells incubated with different nanoparticles, cells cultured with NDs (29.41 µg/mL), ND-PEG (29.41 µg/mL), NPDC (5 µg/mL of DOX equivalent) and DOX (5µg/mL), scale bars: 300 µm.

The interaction of ND-PEG, NPDC and free DOX with HepG2, HeLa and MCF-7cells were investigated by Optical microscopy studies. Fig. S7 shows the optical images of HepG2, HeLa and MCF-7cells after incubation with ND-PEG, NPDC and free DOX dispersed into cell culture

medium, respectively. As can be seen, HepG2, HeLa and MCF-7cells exposed to ND-PEG retained the normal morphology, showing that the ND-PEG was nontoxic. However, NPDC showed an apparently toxic effect toward the cells. After 48 h incubation with NPDC and free DOX, the optical images showed that the cell structure was severely damaged and an obvious decrease in cell number was apparent upon treatment with free or released DOX after incubating 72 h.

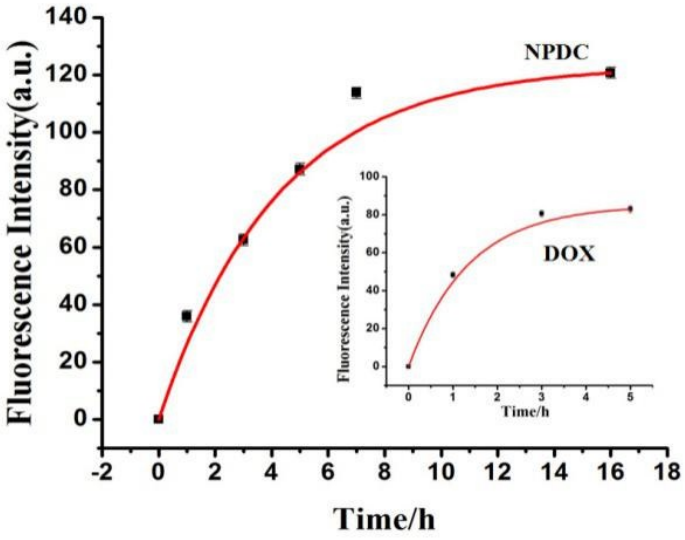


Fig. S8 The kinetics of the cellular uptake of ND-PEG-DOX/Na_3Cit and DOX

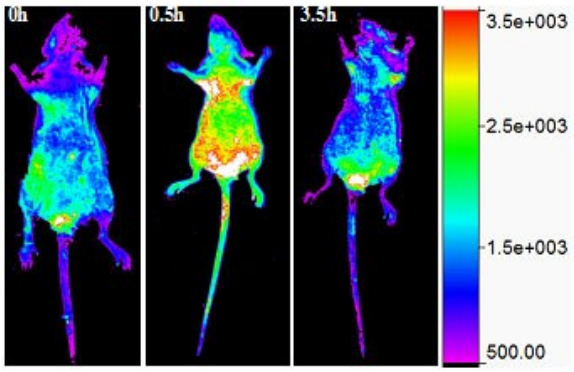


Fig. S9 In vivo images of tumor-bearing mice at various time after intraperitoneal injection of NPDC.