pH-sensitive nanomedicine based on PEGylated nanodiamond for enhanced tumor therapy

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Experimental Section

Preparation of ND-PEG

The synthetic route for the conjugation of ND, H_2N-PEG-COOH and DOX has been shown in scheme 1. Carboxylated ND (1mg) was weighed up, a mixture of ND and MES (1mL, 0.1M, pH 5.8) was sonicated at room temperature for 30 min. After then, EDC (0.2 mg) and NHS (0.25mg) were added in sequence and the system was stirred for 6h at room temperature. The reaction mixture was centrifuged to remove the supernatant liquid and the residue was dispersed into the BBS (1mL, 0.1M, pH 8.4) buffer solution. The suspension was socinated for 5 min with adding HN-PEG-COOH (0.3mg) and kept stirring at room temperature for 12h. The nanoparticle ND-PEG was acquired by centrifugating the reaction mixture and washing three times with deionized water. The repeatedly washed ND-PEG was dried under vacuum at room temperature.

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 (1mg/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、2、4、10、14 and 20 μmol/L respectively. Taking 2 mL H_2N-PEG-COOH BBS 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 H2N-PEG-COOH was determined by calculating the changes in H2N-PEG-COOH concentration before and after the conjugating using a fluorometer at 485 nm. The amount of H_2N-PEG-COOH grafted onto ND was nearly (150±10) μg per milligram nanodiamond.

Cell uptake of time-dependent by flow cytometer

MCF-7 cells (2×10^5) were seeded in 3.5 cm culture dishes and incubated overnight. The cells were treated with ND-PEG/DOX (5 μg/mL) for 1h、3h、5h、7h and 16h, respectively. Then the cells were harvested with trypsin and PBS. The samples were analyzed by flow cytometer (FACS Calibur, BD, USA). The fluorescence from the ND-PEG/DOX 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 (BD Biosciences).

Cell Uptake Mechanism

MCF-7 cells were seeded into 35 mm cell culture dish at a density of 2×10^5 cells/mL with complete medium and incubated for 16-20h at 37℃. After a 30 min pretreatment with the inhibitors, the cells were treated with 5μg/mL of ND-PEG/DOX at the presence of various inhibitors for 2h respectively: 450 mM sucrose, 78 mM Sodium azide, 10 mM Methyl-beta-cyclodextrin. Besides, in order to confirm whether the temperature dependency mechanism, one group of the cells were treated with 5μg/mL of ND-PEG/DOX for 2h at 4℃. After washing thrice with cold PBS (pH 7.4), the cells were digested and the mean fluorescence intensity was observed by flow cytometry.

Morphologic Observation

The cell morphology was analyzed by optical microscope. HepG2 cells, HeLa cells and MCF-7 cells were cultured into 3.5 cm cell culture dishes with complete fresh medium for 18h. After complete adhesion, cells were treated with kinds of nanoparticles and incubated for another 48h and 72h, respectively. When the treatment was done, the cells were observed with optical microscope and the pictures were captured.

Scheme and Figures:

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

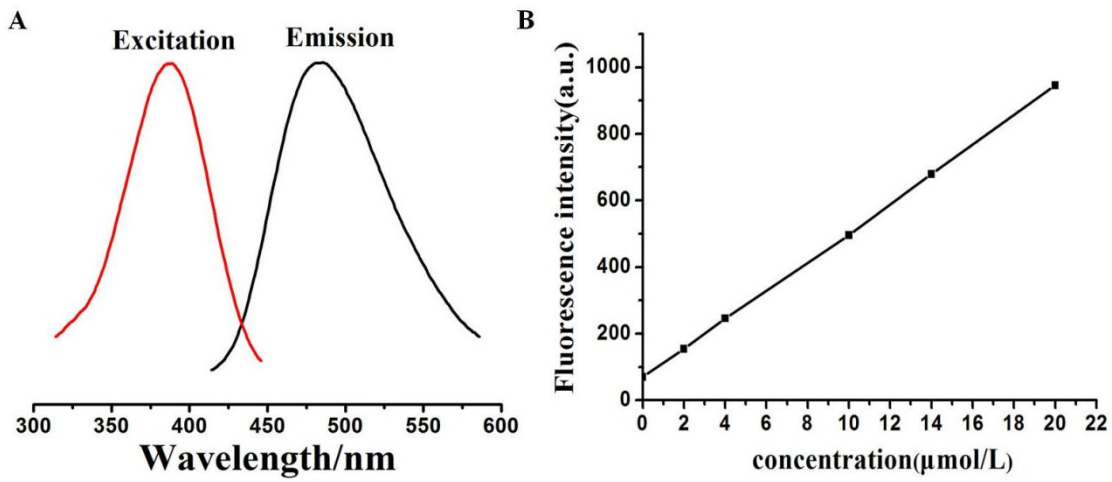


Fig.S1The interaction between H_2N-PEG-COOH and fluorescamine. (A) Fluorescence excitation and emission profiles of the fluorescent product resulting from the reaction with excitation and emission wavelengths of 388and 485 nm, respectively. (B) Standard curve of the interaction between H_2N-PEG-COOH and fluorescamine.

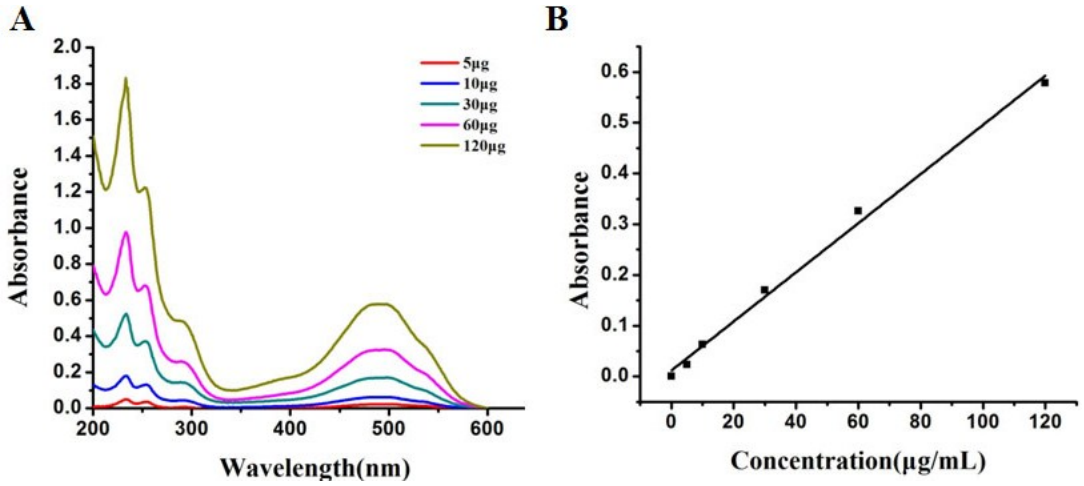


Fig. S2 Standard curve of DOX (A) UV–Vis spectra of DOX dilution curves. (B) Linear regression of absorbance versus concentration

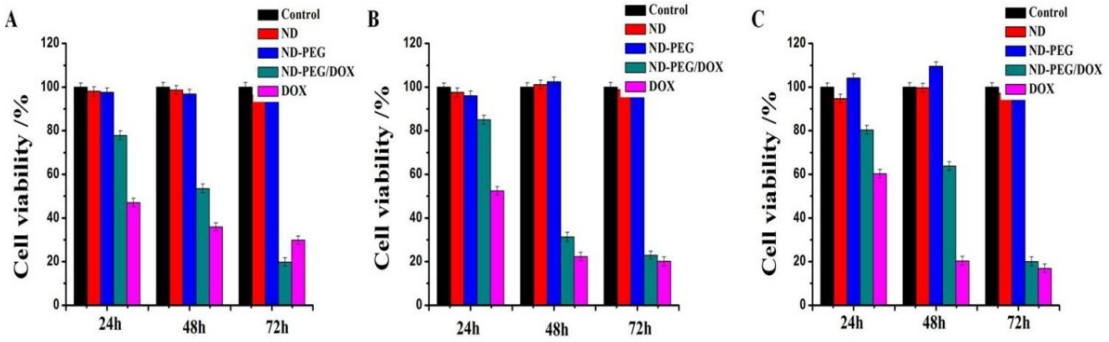


Fig. S3 Effect of free DOX and NP/D on HepG2, HeLa and MCF-7 cell viability for different time was measured by MTT assay. (A) HepG2, (B) HeLa and (C) MCF-7. Experiments were repeated three times and data are presented as the mean ± SD (for each group, n = 6).

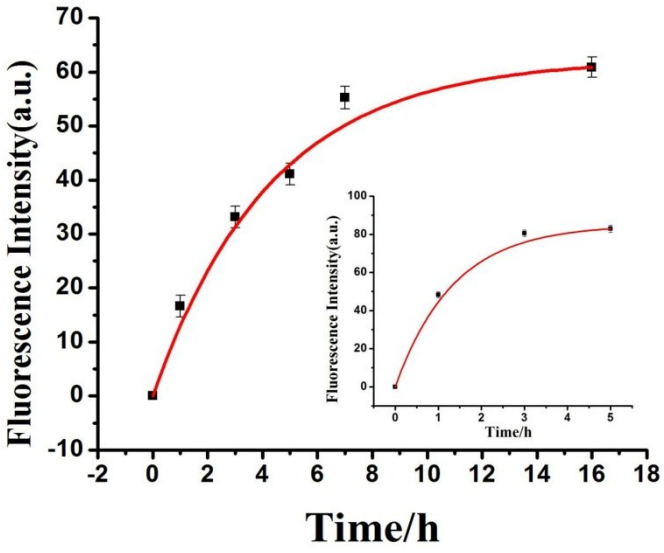


Fig. S4 The kinetics of the cellular uptake of NP/D and free DOX

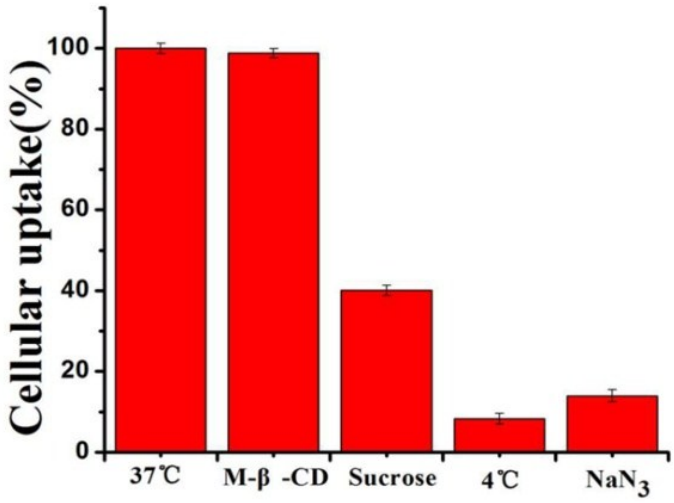
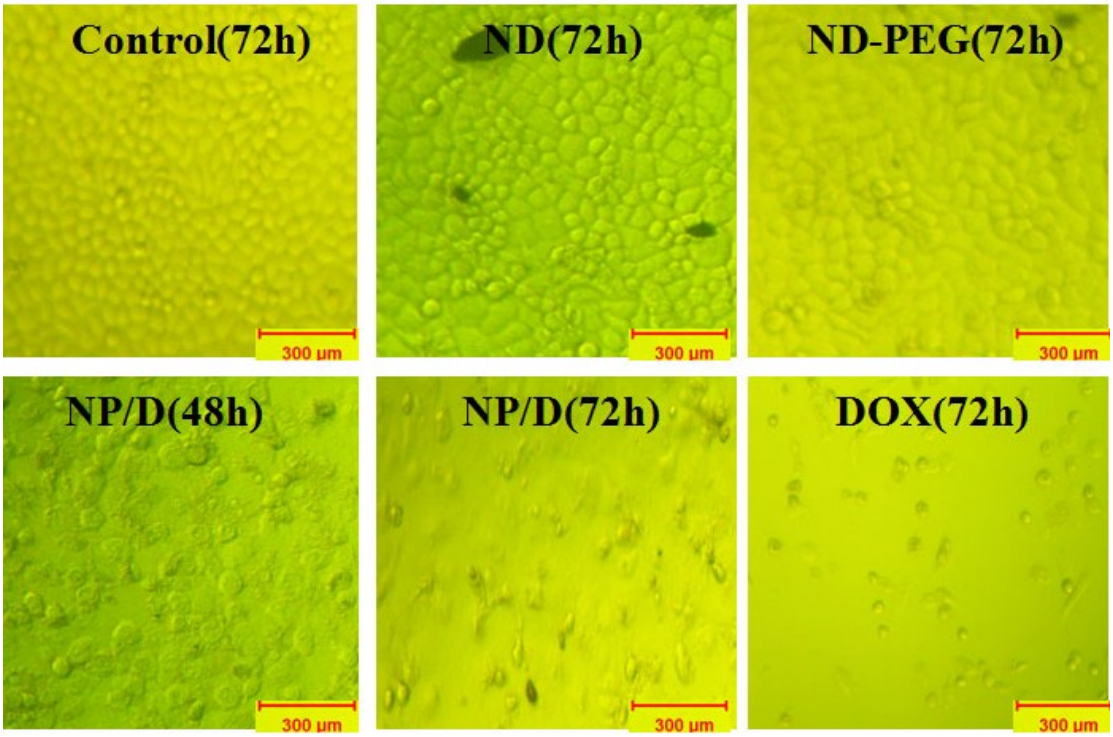
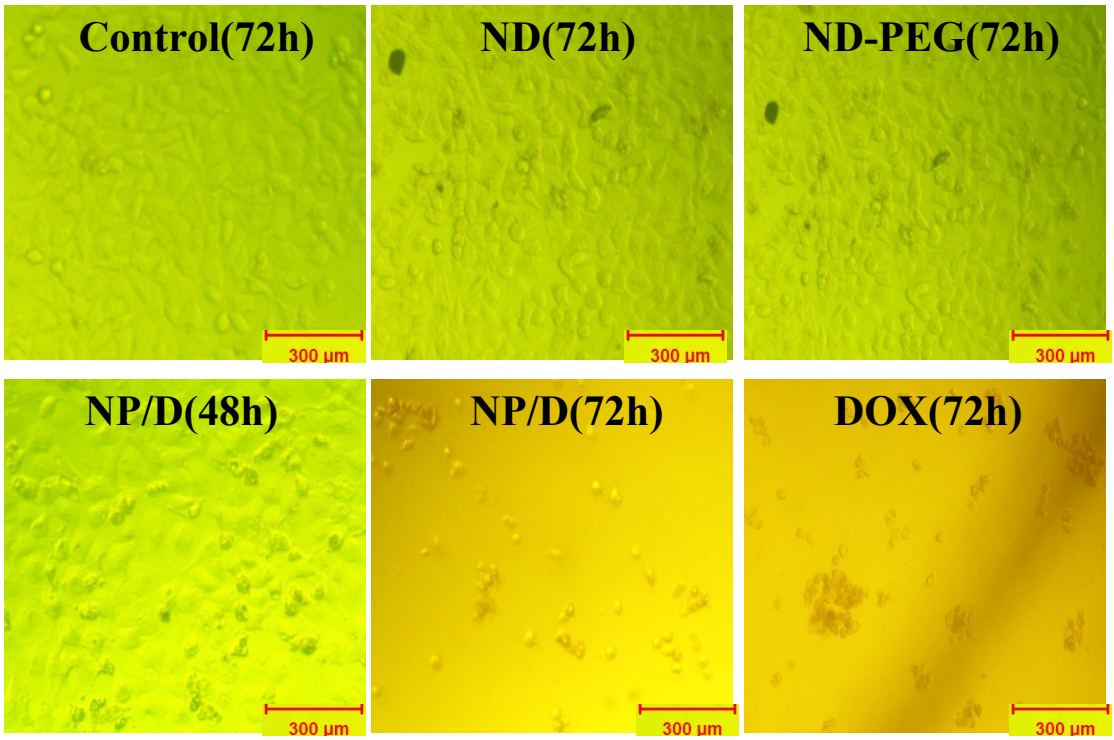


Fig. S5 Quantitative analysis on the uptake mechanism of ND-PEG/DOX nanoparticles by MCF-7 cells.

A (HepG2 cells)



B (HeLa cells)



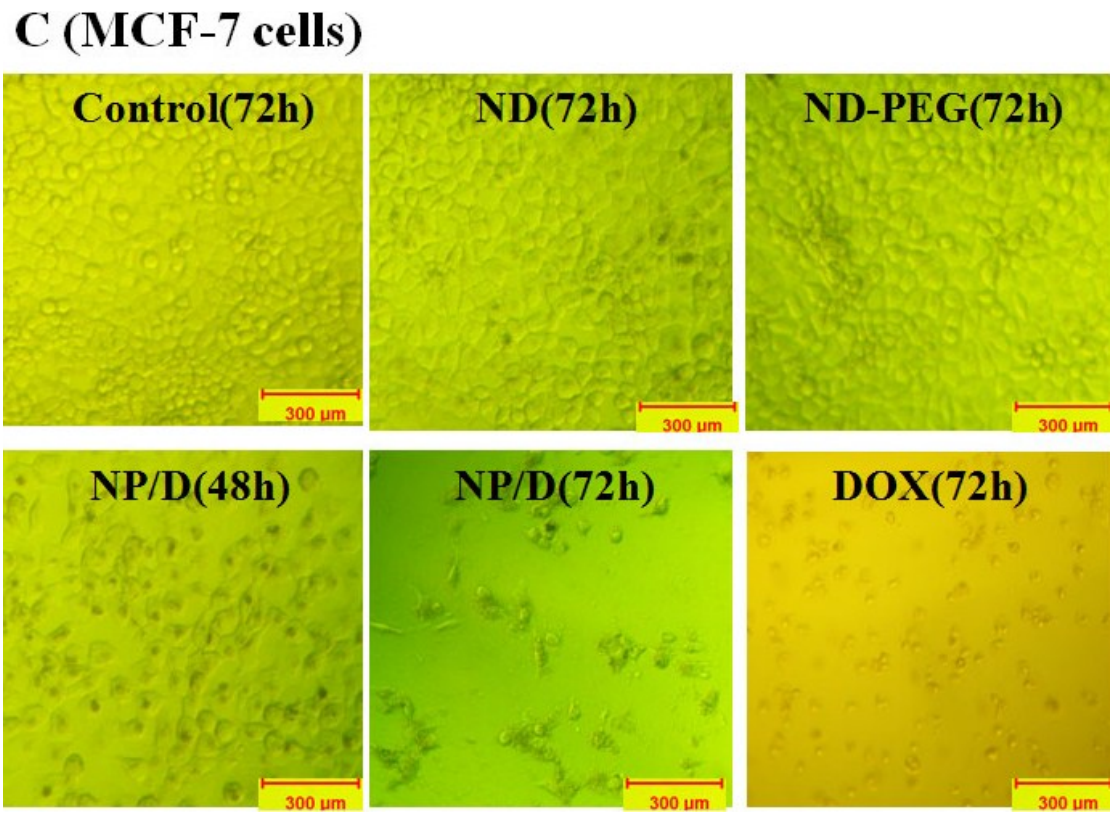


Fig. S6 Microscopy images of cells incubated with nanoparticles for 48h and 72h ((A) HepG2, (B) HeLa and (C) MCF-7). scale bar = 300 μm

References

1 Y. Dong, R. X. Cao, Y. Q. Li, Z. Q. Wang, L. Li and L. Tian, *RSC Adv.,* 2015, **5**, 82711-82716.