**Supporting Information for**

# Low-dose X-ray Responsive Diselenide Nanocarriers for Effective Delivery of Anticancer Agents

Lianxue Zhang[1,#], Shitong Zhang[1,#], Jiaying Xu[1,#], Youyun Li[1], Jinlin He[3], Ying Yang[1], Tien Huynh[2], Peihong Ni[3], Guangxin Duan[1], Zaixing Yang[1,*], Ruhong Zhou[1,2,4,*]

[1]*State Key Laboratory of Radiation Medicine and Protection, School for Radiological and Interdisciplinary Sciences (RAD-X) and Collaborative Innovation Center of Radiation Medicine of Jiangsu Higher Education Institutions, Soochow University, Suzhou 215123, China*

[2]*Computational Biology Center, IBM Thomas J Watson Research Center, Yorktown Heights, NY 10598, USA*

[3]*College of Chemistry, Chemical Engineering and Materials Science, State and Local Joint Engineering Laboratory for Novel Functional Polymeric Materials, Soochow University, Suzhou, 215123, China*

[4]*Department of Chemistry, Columbia University, New York, NY 10027, USA*

[#] These authors contributed equally;

[*]Corresponding authors: zxyang@suda.edu.cn (Z. Yang); ruhongz@us.ibm.com (R. Zhou)

## 1. Materials.

Selenium powder, sodium borohydride, hydrogen peroxide ($H_2O_2$, 30%), isophorone diisocyanate (IPDI), dibutyltin dilaurate (DBTDL), 11-bromoundecanol were analytical grade products purchased from Aladdin Reagents. Tetrahydrofuran (THF) (A.R., Sinopharm Chemical Reagent) was dried over KOH for at least two days and then refluxed over a sodium wire with benzophenone as an indicator until the color turned purple. Poly(ethylene glycol) monomethyl ether (mPEG) ($M_w$ = 2000) was from Aladdin Reagents and dried by azeotropic distillation from toluene.

## 2. Synthesis procedures

### 2.1 Synthesis of di(1-hydroxylundecyl) diselenide.

Sodium borohydride (1.0 g, 26.4 mmol) in 10 mL water was added with magnetic stirring to selenium (1.0 g, 12.6 mmol) suspended in 15 mL water under $N_2$ flow at room temperature. After the initial vigorous reaction had subsided (10 min), additional equiv. of selenium (1.0 g, 12.6 mmol) was added. The mixture was stirred for 15 min and then warmed briefly on the steam bath to complete the dissolution of the selenium. Then a solution of 6.33 g 11-bromoethanol (25.2 mmol) in 25 mL anhydrous THF was injected into it. The reaction was performed at 50 °C for 24 h and the obtained solution was extracted three times with 20 mL of $CH_2Cl_2$ and dried with anhydrous $Na_2SO_4$. Then the product was purified by column chromatography with a 4:1 mixture of $CH_2Cl_2$ and ethyl acetate as eluent. A yellow transparent liquid was obtained with a yield of 61%. $^1$H-NMR (300 MHz, $CDCl_3$, $\delta$) (ppm): 3.63 (4H, t, HOC$\underline{H}_2$), 2.90 (4H, t, SeSeC$\underline{H}_2$) 1.72-1.28 (36H, m, HOCH$_2$ (C$\underline{H}_2$)$_9$CH$_2$SeSe); LC-MS: calculated 500.53, found 500.16.

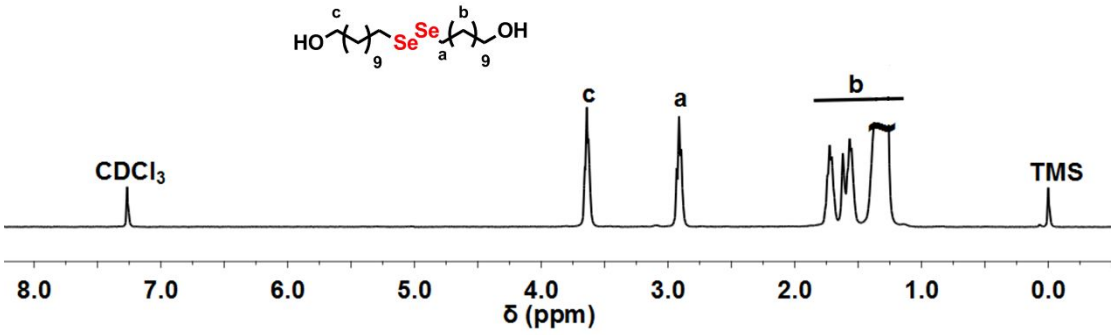


**Figure S1.** $^1$H NMR spectrum of di(1-hydroxylundecyl) diselenide in $CDCl_3$.

**2.2 Synthesis of benzyl-terminated PUSeSe.**

0.2 g $HOC_{11}SeSeC_{11}OH$ (0.40 mmol) and 10 mg of DBTDL (0.016 mmol) were dissolved in 2 mL of anhydrous THF and sealed with a rubber plug. The flask was then degassed by $N_2$ for 20 min. A solution of 0.0978 g (0.44 mmol) IPDI in 2 mL anhydrous THF was injected into the flask under $N_2$ flow. The system was transferred into an oil bath at 50 °C to react for 2 h with stirring. 0.048 g (0.44 mmol) benzyl alcohol was then dissolved in 1 mL of anhydrous THF and injected into the flask under $N_2$ flow and the reaction was carried out at 50 °C for 12 h. The solvent was then removed by rotary evaporation and the residual liquid was precipitated by cold methanol three times. A yellow powder of PUSeSe polymer was obtained after vacuum drying.

For the determination of the degree of polymerization ($n$) of hydrophobic PUSeSe block by end-group analysis, part of the reactive IPDI-terminated PUSeSe was withdrawn and reacted with benzyl alcohol to achieve the benzyl-terminated PUSeSe. According to the $^1$H NMR spectrum of PUSeSe shown in Figure S2, the degree of polymerization ($n$) is calculated to be 11 by the following eq 1, where $A_c$ and $A_h$ represent the integral values of peak c and peak h, respectively. The chemical structure, molecular weight, and molecular weight distribution ($Đ$) of amphiphilic diselenide-inserted triblock copolymer (Se-polymer) were characterized using $^1$H NMR and GPC analysis (Figure S3). From the $^1$H NMR spectrum of Se-polymer shown in Figure 1B in the maintext, the molecular weight of Se-polymer is then determined to be 11820 g mol$^{-1}$ ($\overline{M}_{n,\,NMR}$) according to eq 2, where 722.2 is the theoretic molecular weight of each repeating unit of PUSeSe. Moreover, the GPC analysis of Se-polymer in Figure S3 indicates that the molecular weight and molecular weight distribution ($Đ$) of Se-polymer are 11950 g mol$^{-1}$($\overline{M}_{n,\,GPC}$) and 1.24, respectively. All these evidences demonstrate the successful synthesis of amphiphilic Se-polymer.

$$n = A_c/A_h \tag{1}$$

$$\overline{M}_{n,\,NMR} = 722.2\times n + 4000 \tag{2}$$

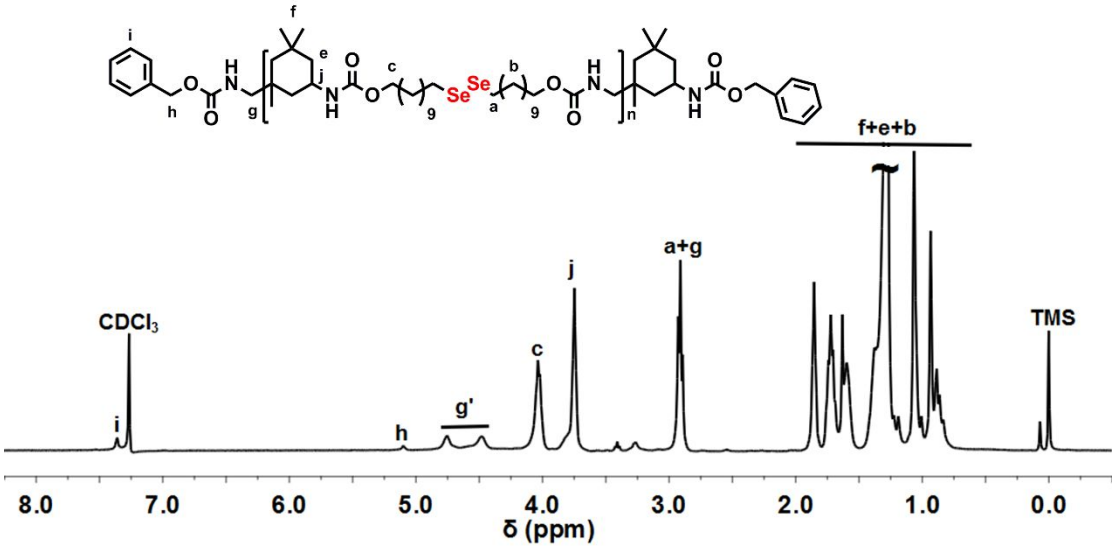


**Figure S2.** $^{1}$H NMR spectrum of PUSeSe in $CDCl_3$.

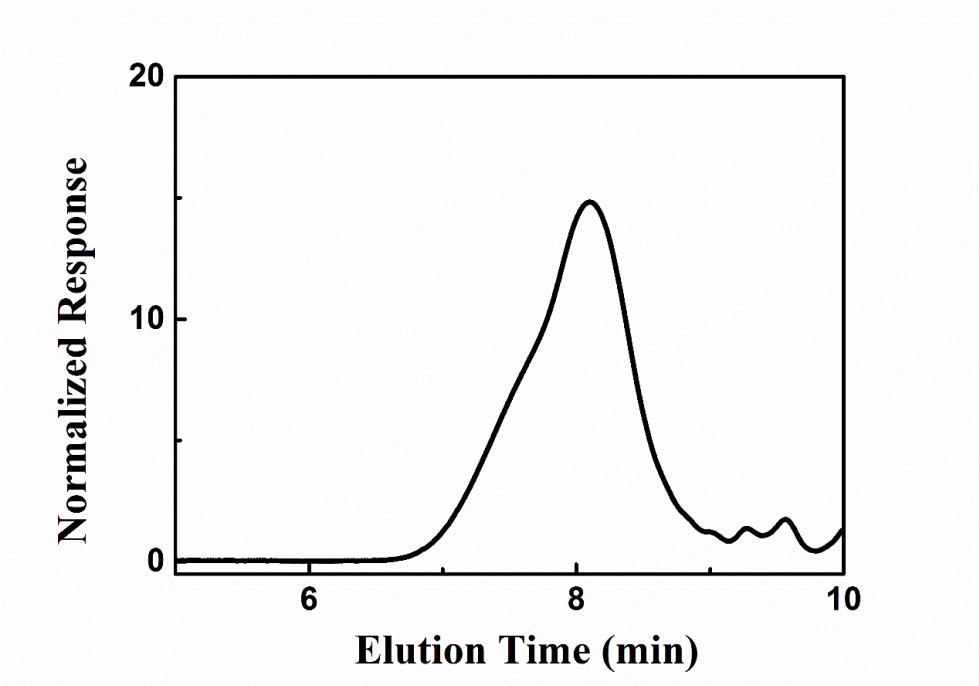


**Figure S3.** GPC plot of Se-polymer in DMF.

### 3. The critical aggregation concentration (CAC) of Se-polymer

The CAC of Se-polymer was measured by a fluorescence probe method. 50 μL of predetermined pyrene solution in acetone ($6 \times 10^{-6}$ mol $L^{-1}$) was added into a series of ampules, and then the solvent of acetone was removed via vacuum evaporation. Different concentrations of the Se-polymer aqueous solutions (5 mL) were added to each ampule and stirred for 48 h at 25 °C. After that, a fluorescence spectrophotometer

analyzed the mixture solutions to detect the variational fluorescence strength of pyrene. The CAC value was determined as the concentration of the cross-over point in the low concentration range (Y-axis: the intensity ratio of $I_{383}$-to-$I_{373}$; X-axis: the emission spectra range).

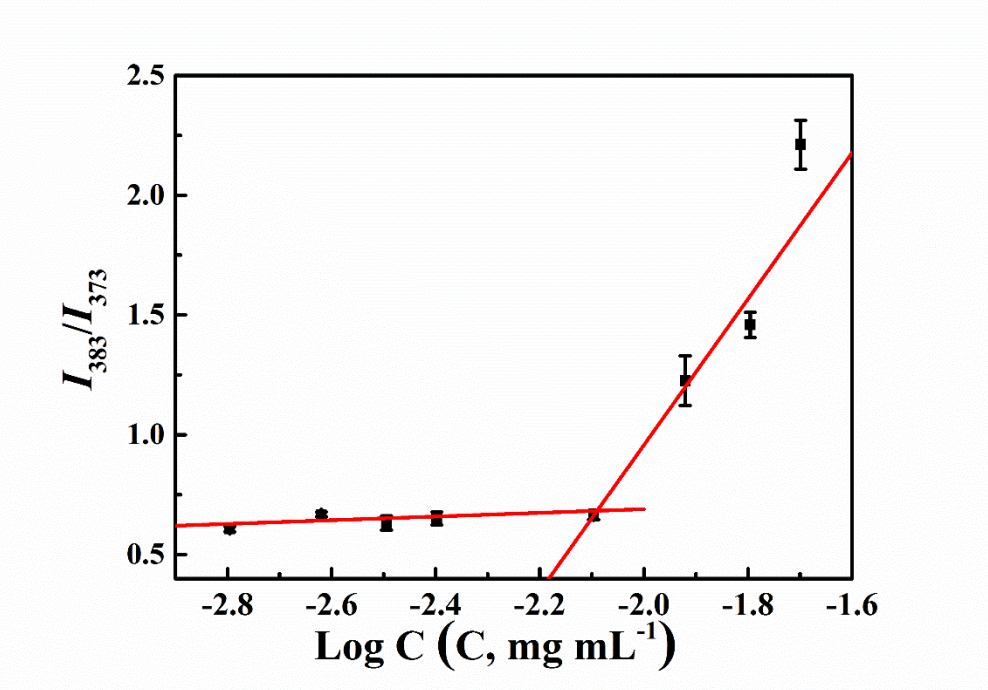


**Figure S4**. Intensity ratios ($I_{383}/I_{373}$) as a function of logarithm concentration of Se-polymer in aqueous solution.

**4. Effect of $H_2O_2$ and X-ray irradiation on the fluorescence intensity of Nile red.**

We have to first test the stability of the model NR compound under experimental conditions. Preparing 80 mL of NR solution (THF: water = 4:1, 0.1 mg mL$^{-1}$) and the solution was treated with four different ways and measured by fluorometer, such as: 10 Gy X-ray, 20 Gy X-ray, 100 μM $H_2O_2$, 10 Gy X-ray and 100 μM $H_2O_2$. The experiments were carried out in three parts.

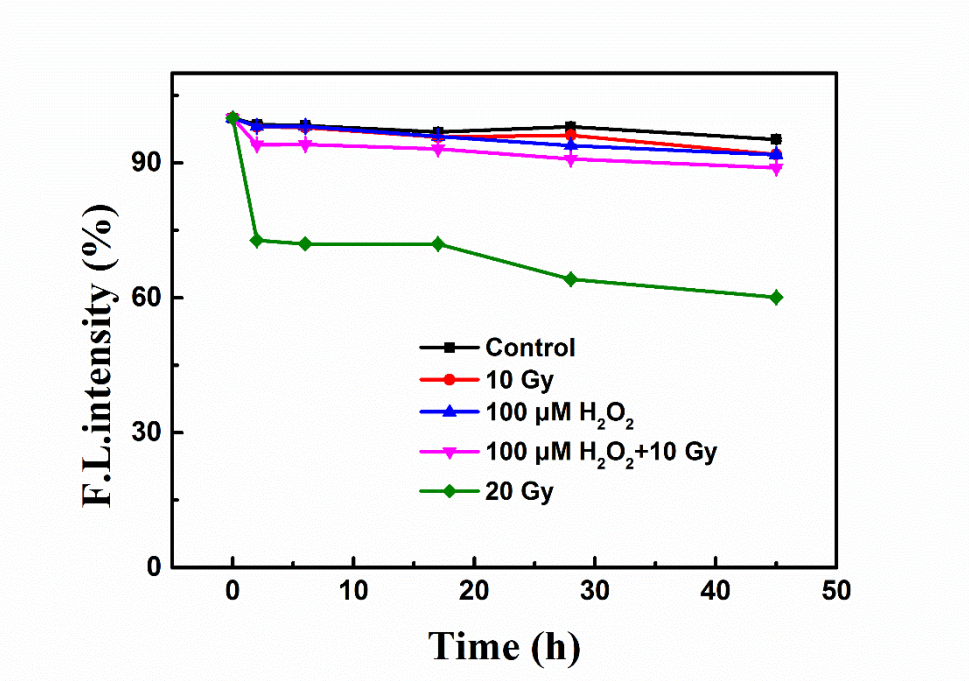


**Figure S5.** The stability of NR under $H_2O_2$ and X-ray irradiation conditions.

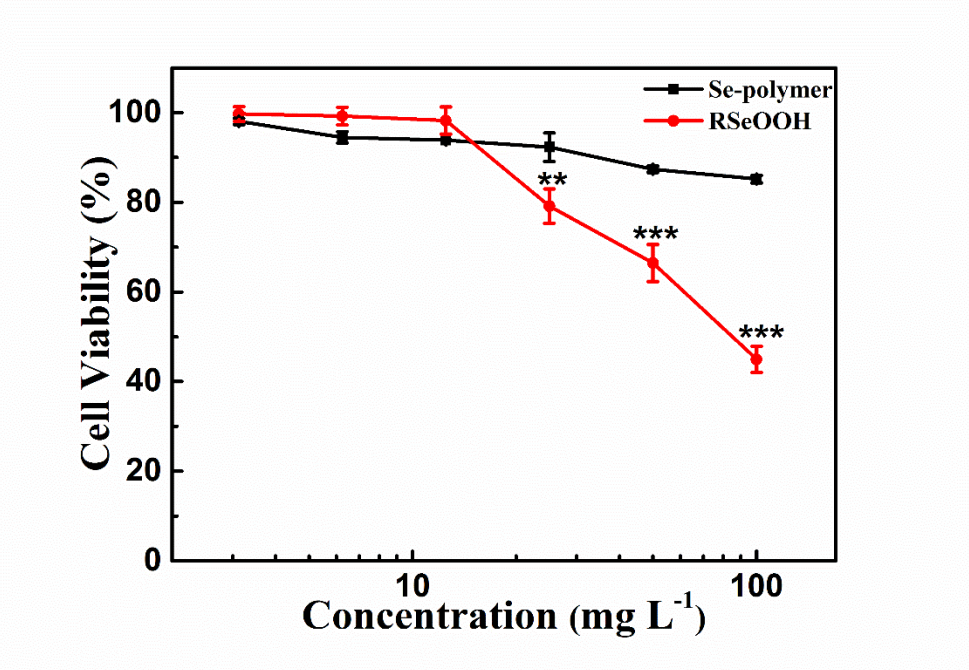


**Figure S6.** *In vitro* cytotoxicities of Se-polymer and RSeOOH at different concentrations in 4T1 cells.

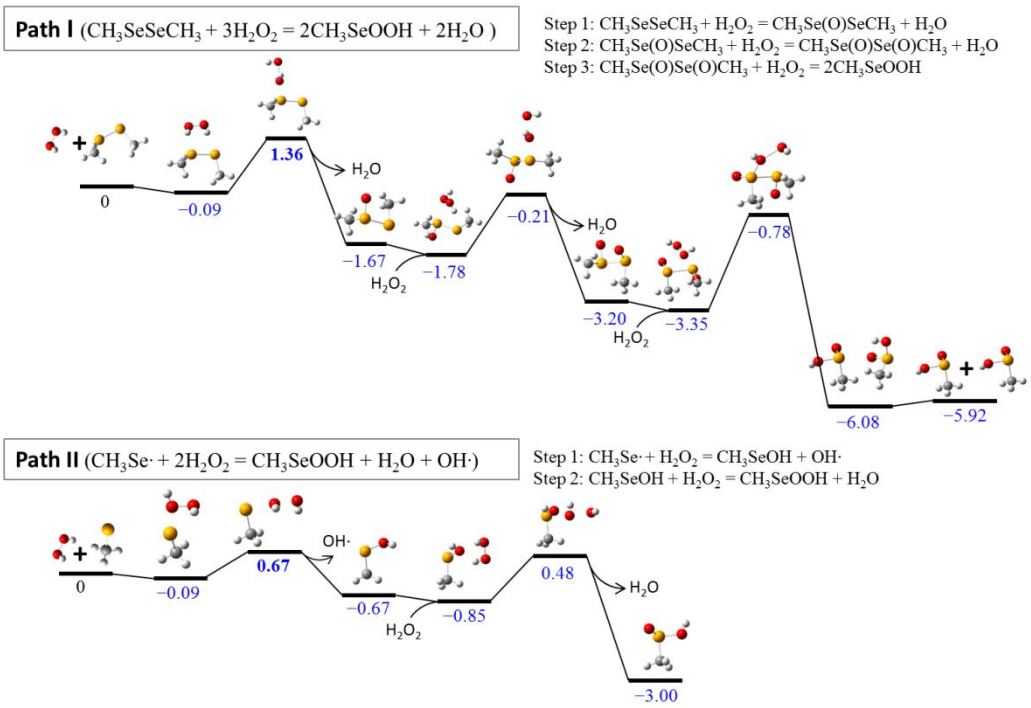


**Figure S7.** Energy profiles (relative-energy values are in eV) for the favorable reaction routes of Path I and Path II. The energy sum of the free reactants is set as zero. White, H; gray C; red, O; orange, Se.

## 5. Cellular uptake of D-NPs

The cellular uptake of D-NPs was measured by a flow cytometer. The cells were exposed to D-NPs in each treatment medium. After exposure, the cells were washed twice with PBS, and subsequently harvested using trypsin and centrifuged at 800 r/min for 5 min. Finally, the cells were resuspended in 500 μL PBS for flow cytometry.

To study the uptake pathways of D-NPs, 4T1 cells were preincubated with low temperature (4 °C) and various endocytosis inhibitors for 30 min, including chlorpromazine (CPZ, 10 μg $mL^{-1}$), cytochalasin D (Cyto D, 2 μM), Dyngo-4a (3 μM), methyl-β-cyclodextrin (β-CD, 10 mM) and genistein (40 μM). The culture medium was replaced by fresh FBS-free medium with 10 μg $mL^{-1}$ D-NPs. Samples were maintained for 2 h under a 5% $CO_2$ atmosphere at 37 °C. The effect of low temperature (4 °C) was also studied: cells were preincubated for 0.5 h and incubated with D-NPs for 2 h. Meanwhile, the cells treated with D-NPs at 37 °C were used for control studies. After

exposure, the cells were washed twice with PBS and harvested using trypsin and centrifuged at 800 r/min for 5 min. Finally, the cells were resuspended in 500 μL PBS for flow cytometry. And the results show that D-NPs was mainly internalized by clathrin-dependent endocytosis routes, since the inhibitors, β-CD, CPZ and Dingo-4a, performed the most significant inhibition effect.

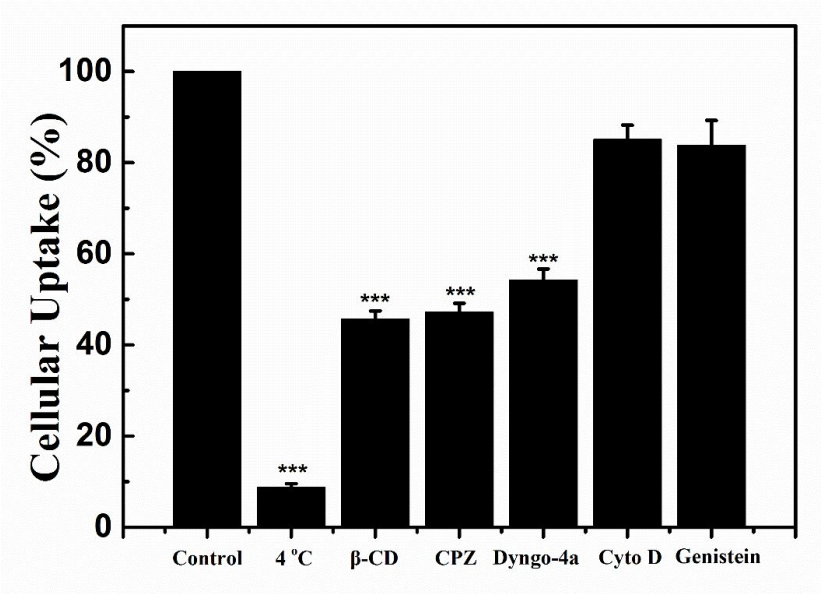


**Figure S8.** The measurements of D-NPs uptake ratio into 4T1 cells to illustrate the inhibitory effects of 4 °C, β-CD, CPZ, Dyngo-4a, Cyto D and genistein, compared with normal cellular uptake at 37 °C (control)**.**

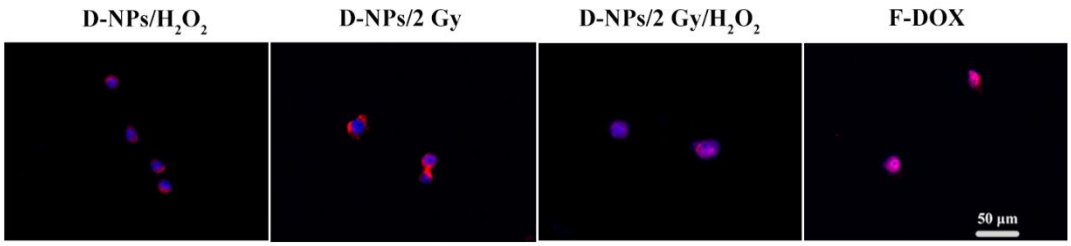


**Figure S9.** Fluorescent microscopy images of 4T1 cells treated with F-DOX (1.25 μg $mL^{-1}$), D-NPs, D-NPs combined with 2 Gy X-ray or 100 μM $H_2O_2$ or both. For each panel, the images from left to right show the cell nuclei stained by Hoechst 33342 (blue), DOX fluorescence in cells (red), and overlays of the blue and red images. Each scale

bar is 50 μm.

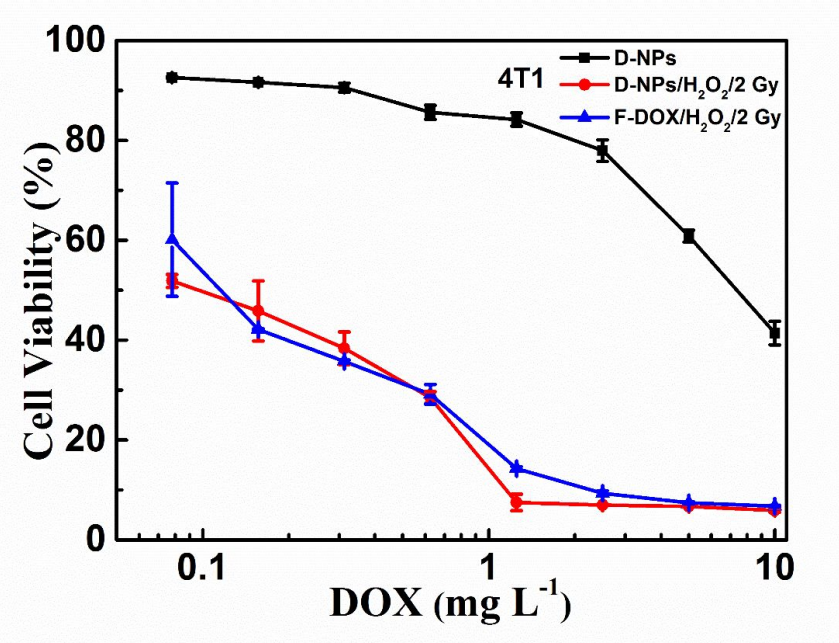


**Figure S10.** Cell viabilities of 4T1 cells treated with different methods. The half maximal inhibitory concentrations ($IC_{50}$s) of D-NPs and F-DOX against 4T1 cell under irradiation and oxidative stress are 0.13 and 0.11 mg $L^{-1}$, respectively. Data are presented as average ± standard deviation, $n$ = 3.

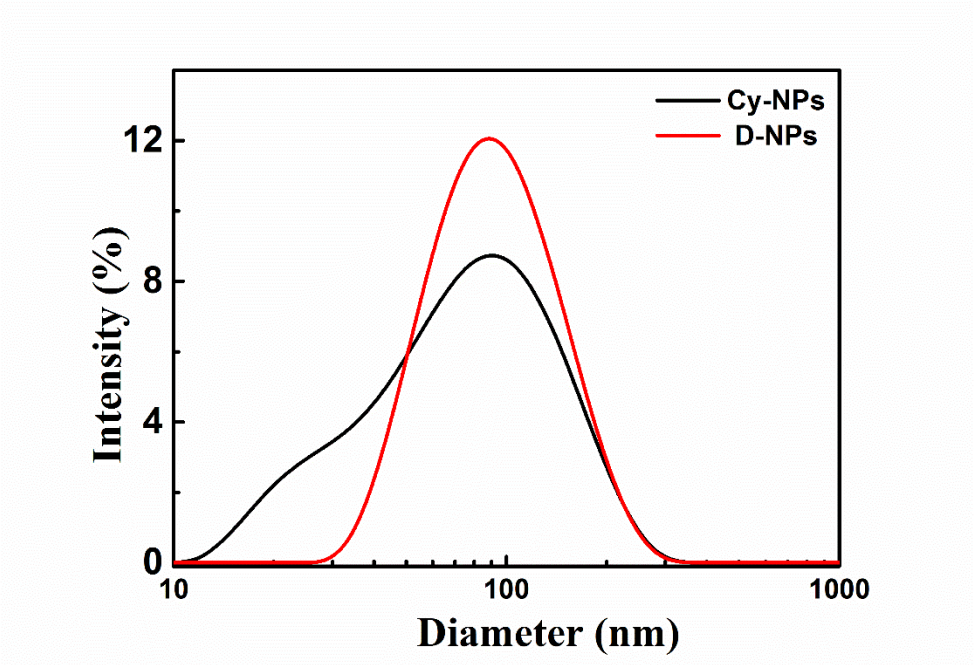


**Figure S11.** Size distribution of intact D-NPs and Cy-NPs.

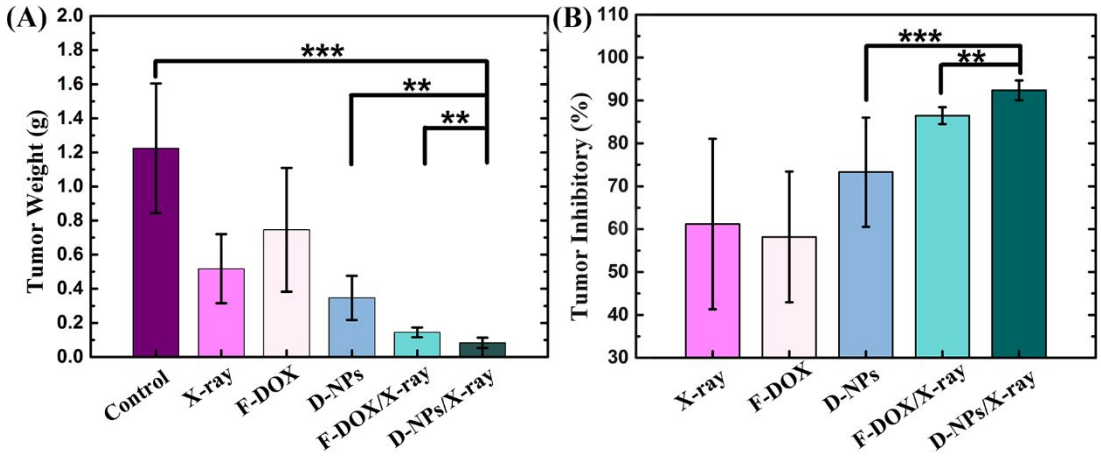


**Figure S12.** (A) Mean weight of tumors separated from mice. (B) The tumor inhibitory rate (TIR). Statistical significance: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *n* = 5.

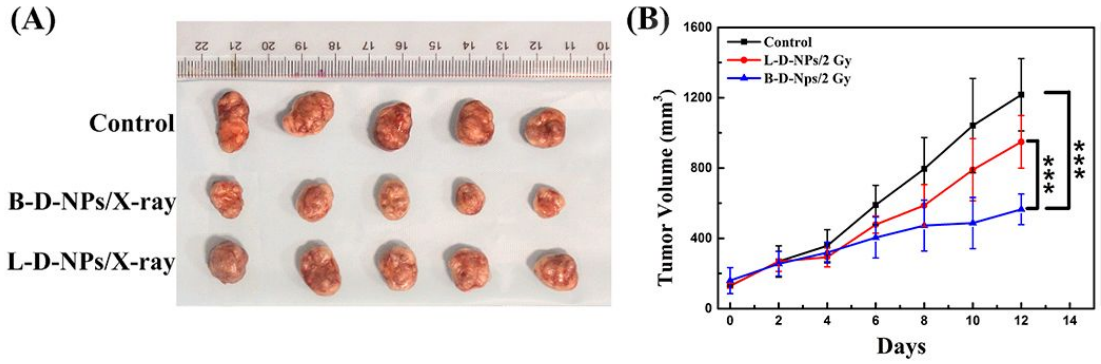


**Figure S13.** (A) *In vivo* anticancer activity; (B) Changes of tumor volume. Statistical significance: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *n* = 5. The distinct treatment efficacy illustrated the effective cargo release in "B-D-NPs/2Gy" group. Additionally, the rate of cargo release from D-NPs is quite slow in the absence of X-rays, as suggested by studies on the *in vivo* distribution of loaded cargos (Figure 5A in main text) and the reduced cytotoxicity of D-NPs (Figure 4 in main text). Thus, X-ray plays a crucial role in triggering the nanocarrier disassembly.

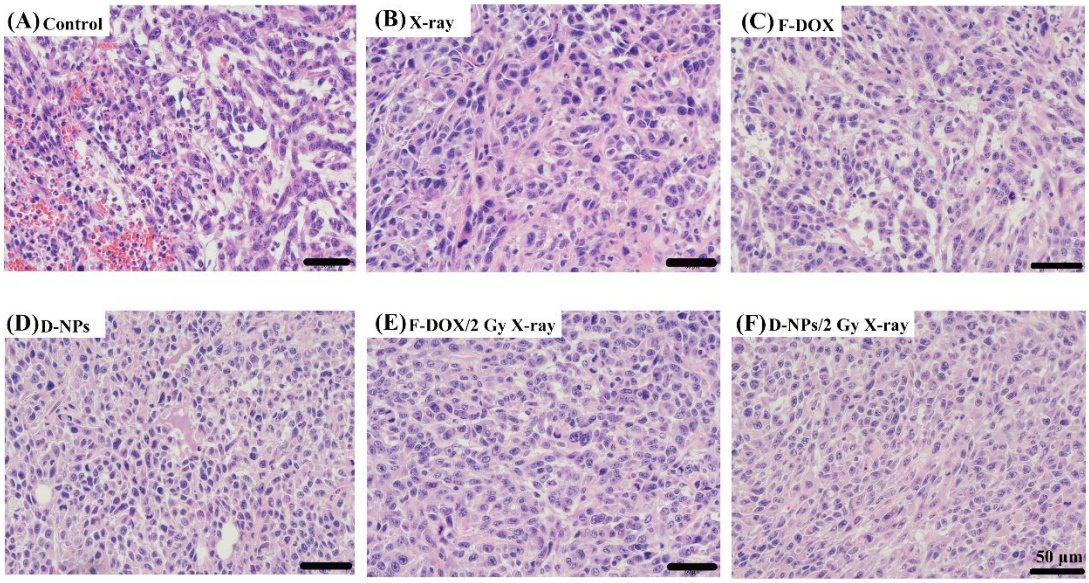


**Figure S14.** Histopathological (H&E) analysis of tumor tissue sections from 4T1-bearing mice treated with (A) control, (B) X-ray, (C) F-DOX, (D) D-NPs, (E) F-DOX/ 2 Gy X-ray, and (F) D-NPs/2 Gy X-ray. (magnification×400).

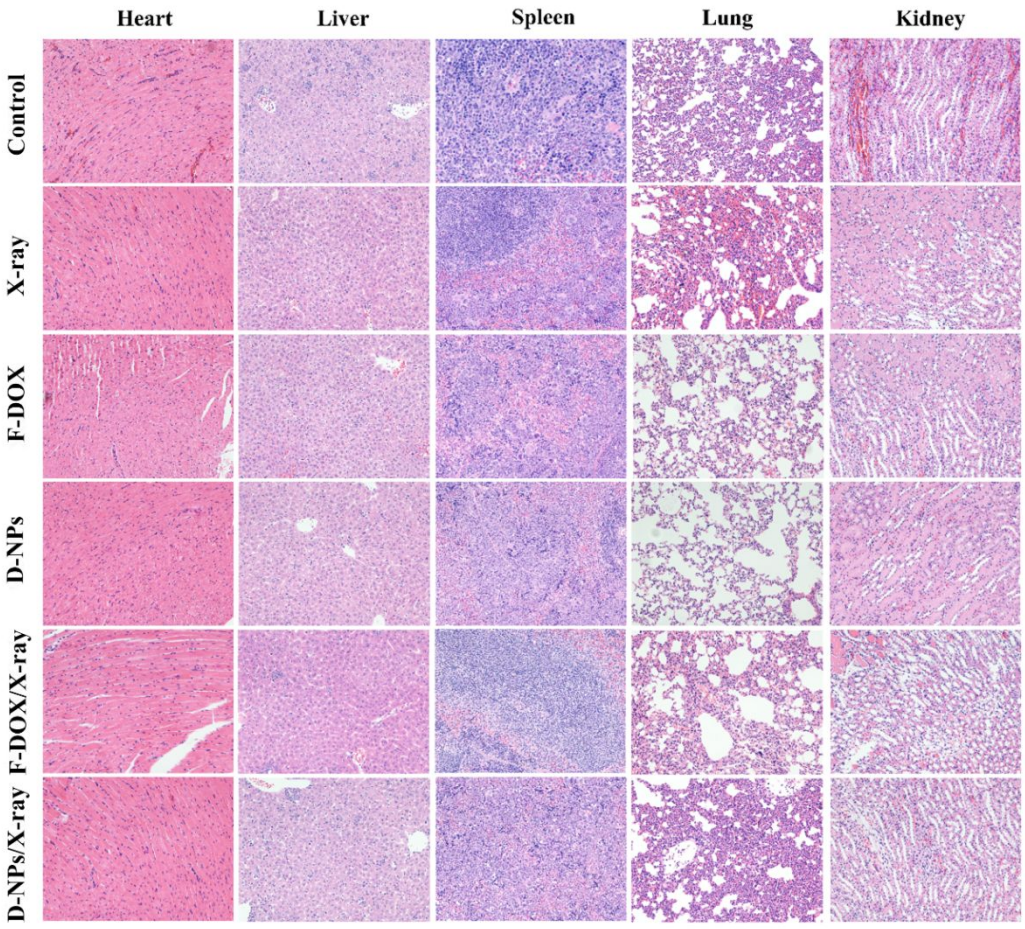


**Figure S15.** Histopathological (H&E) analyses of internal organs (i.e., the heart, liver,

spleen, lung, and kidney) sections from 4T1-bearing mice treated with various formulations (magnification×200).

**Table S1.** Characterization date of molecular weights and PDIs of Se-polymer after different treatment.

| Groups | $\bar{M}_n$ (g mol$^{-1}$) | PDI |
|---|---|---|
| Control | 12079 | 1.1 |
| 2 Gy X-ray | 12619 | 1.1 |
| 100 μM $H_2O_2$ | 9178 | 1.3 |
| 100 μM $H_2O_2$/2 Gy X-ray | 7002 | 1.2 |

$\bar{M}_n$ determined by GPC (eluent: THF).