Supporting Information for:

Coassembled Peptide Hydrogel Boosts Radiosensitization of Cisplatin

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Supplemental Methods

Chemicals and Materials

CDDP and Naproxen (Npx) were supplied by Dalian Meilun Biotechnology Co., LTD, and Energy Chemical respectively. Fmoc-amino acids, O-Benzotriazole-N, N, N', N'-tetramethyl-uronium-hexafluorophosphate (HBTU), and 2-Cl-trityl chloride resin were purchased from GL Biochem. Trifluoroacetic acid (TFA), diisopropylethylamine (DIEA), and triisopropylsilane (TIS) were purchased from J&K Chemical Technology. All cell lines were maintained in our lab. Cell culture mediums, penicillin, and streptomycin, fetal bovine serum, and trypsin-EDTA (0.25%) were obtained from Gibco. LysoTracker Red DND-99 and FITC Annexin V apoptosis detection kits were from Yesen Biotech and BD Bioscience Pharmingen respectively. P53 and γ-H2AX antibodies used for western blotting as well as γ-H2AX immunofluorescence analysis were obtained from Abcam or Cell Signalling Technology. Ultrapurified water was obtained from the Milli Q Plus system.

Synthesis of Npx-ffey

Npx capped short peptide derivatives Npx-ffey was prepared by the standard solid-phase peptide synthesis (SPPS) method just as we previous report. In brief, 2-chlorotrityl chloride resin was used as the phase support and different N-Fmoc protected amino acids with side chains properly protected were used as the reactants. Every amino acid would experience deprotection (20% piperidine) and acrylation (DIEA: HBTU: amino acid=2:1:1) in anhydrous DMF. After deprotecting the Fmoc of the last amino acid, Npx was dissolved in anhydrous DMF and further reacted with former peptides. Finally, the Npx-ffey was cleaved with a mixed solution (19/0.5/0.5 of TFA/TIS/H_2O), and the final crude products were purified by reverse phase HPLC (yield: 78%). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.17-8.05 (m, 3H), 7.99 (d, J = 8.2 Hz, 1H), 7.70 (s, 1H), 7.63 (d, J = 8.7 Hz, 1H), 7.57 (s, 1H), 7.28-7.10 (m, 8H), 7.02 (d, J = 8.4 Hz, 2H), 6.99- 6.83 (m, 5H), 6.65 (d, J = 8.2 Hz, 2H), 4.60 -4.53 (m, 1H), 4.48-4.41 (m, 1H), 4.40-4.30 (m, 2H), 3.86 (s, 3H), 3.76 (q, J = 7.0 Hz, 1H), 3.06-2.61 (m, 6H), 2.25 (t, J = 8.2 Hz, 2H), 1.90 (s, 1H), 1.82-1.70 (m, 1H), 1.32 (d, J = 7.0 Hz, 3H). LC-MS: M_{cal} =816.34, M_{exa} =817. NBD (4-nitro-2,1,3-benzoxadiazole)-terminated peptide sequence NBD-ffey are synthesized under the same process as mentioned above. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.49 (d, J = 9.1 Hz, 1H), 8.19 (t, J = 7.9 Hz, 2H), 8.11 (d, J = 7.7 Hz, 1H), 8.03 (d, J = 8.1 Hz, 1H), 7.21 (d, J = 6.4 Hz, 5H), 7.18-7.07 (m, 6H), 7.01 (d, J = 8.4 Hz, 3H), 6.64 (d, J = 8.5 Hz, 2H), 6.33 (d, J = 8.6 Hz, 1H), 4.52 (s, 2H), 4.32 (s, 2H), 2.90 (s, 4H), 2.77 (s, 2H), 2.63 (s, 2H), 2.27-2.21 (m, 2H), 1.88 (s, 2H), 1.72 (s, 2H). LC-MS: M_{cal} =838.29, M_{exa} =839.00

Preparation of hydrogel

Peptides Npx-ffey and CDDP were dissolved in phosphate buffer (PBS) under ultrasound and Na_2CO_3 to adjust the pH to 7.4. After heating the solution on an alcohol lamp and storing the hydrogel at 4 °C for an hour, the hydrogel N-P would be formed. The hydrogel of N was prepared following the same procedures, but Npx-ffey alone would spend at least 2 h in forming gelatinous solid. The formation of hydrogel consisted of NBD-ffey and CDDP (NBD-ffey/Pt) or NBD-ffey alone was prepared by the same method as Npx-ffey.

Rheology

A rheometer (AR 1500ex) with a 25 mm-parallel plate at the gap of 500 μm was used to perform the rheology test. About 400 μL hydrogel was needed to be characterized by the mode of frequency sweep (0.1-100 rad/s).

Preparation of TEM samples

The morphologies of N-P and N were acquired by Transmission Electron Microscopy and the observations were conducted on Philips T20ST electron microscope. 15 μL N-P or N were dropped onto a carbon-coated copper grid for 5 min, the redundant liquid was removed. Then the rest was negatively stained with 2% uranyl acetate solution for another 5 min. After removing the unnecessary dye solution, the carbon-coated copper grids were dried at room temperature for observation. To observe the distribution of cisplatin on the skeleton of gels, the sample preparation was the same as the above-mentioned steps except that the negative dye solution was not used.

FTIR spectroscopy analysis

The powder of freeze-dried hydrogels of N and N-P were pressed into KBr pellets and measured by using a Nicolet iS10 FTIR spectrometer (Thermo Scientific) with scanning wavenumber between 4000 and 400 cm^{-1}.

CD Spectroscopy

The secondary structures of N or N-P were monitored by a BioLogic (MOS-450) system. Hydrogel N or N-P was added into a 0.1 cm path-length quartz cell, the CD signals were recorded in the 190-260 nm scanning wavelength range. The raw data would be smoothed and subtracted from the blank according to the manufacturer's agreements.

Zeta potential

The zeta potential of hydrogel N or N-P was measured by dynamic light scattering (BI-200SM). 200 μM N or N-P was diluted with 10 mM NaCl solution to 1.5 mL before measurement. Each sample was detected in triplicate.

UV/Vis spectroscopy analysis

UV/Vis spectra were recorded on a TU-1810 UV spectrometer (PERSEE) by loading the samples in a 1 mm quartz cuvette. The information on different concentrations of samples was obtained from 250 nm to 450 nm.

Cellular Uptake Experiments

For confocal imaging, A549 cells were seeded on confocal microscopic dishes at a density of 2×10^5 per well. After 80% confluence, the original culture medium was removed, followed by adding the mediums containing NBD-ffey as well as NBD-ffey/Pt at an equivalent dose of 50 μM respectively. After incubation for 3 h, the lysosome staining was performed by Lyso-Tracker Red. Then the cells were rinsed with cold PBS and fixed in 4% paraformaldehyde (PFA) solution for subsequent nuclear staining with DAPI. Finally, the cells were imaged by CLSM with an oil immersion 63× objective lens (Nikon eclipse Ti2).

ICP-MS (Agilent 7800) was adapted to measure the total amount of platinum and platinated DNA in the cells. A549 cells were seeded in 100 mm^2 dishes and cultured under an atmosphere of humidified 5% CO_2 at 37 °C. After 90% confluence, the culture mediums were replaced by free CDDP (20 μM) and N-P (20 μM) containing fresh mediums. After 24 h, the cells were harvested, centrifuged, and suspended in PBS. For analyzing the total CDDP concentration, the cells would be directly digested by aqua regia. For the amount of platinated DNA, a DNA extraction kit (GenElute Mammalian Genomic DNA Miniprep Kit, Sigma Aldrich) was applied to extract the total DNA in cells. The total DNA was then digested by aqua regia. All samples were measured by ICP-MS and each experiment was repeated at least three times.

Colony formation assay

A549 cells were seeded in 6-well plates and cultured under standard conditions for 24 h. Then the cells were incubated with N-P, N, free CDDP, took PBS as the control group. After another 24 h, different formulations were replaced by fresh culture medium again and the cells were irradiated at 0, 2, 4, 6 Gy by gamma radiation of ^{137}Cs (photon energy 662 keV). The cells continued to be cultured under standard conditions until most cell colonies had more than 50 cells. Finally, the cells would be stained by 0.25 % crystal violet. The colonies with more than 50 cells were counted and taking the non-irradiated group as the 100% to calculate the surviving fraction. The software of Origin 2018 was applied to plot the survival curve. The sensitizer enhancement ratio (SER) was calculated by GraphPad Prism 5 according to the previous report[1].

γ-H2AX immunofluorescence analysis

A549 cells were seeded in confocal microscopic dishes at a density of 2×10^5 per well. After 24 h, the cell culture medium was replaced by N-P, N, and free CDDP containing culture medium and continued to culture for 24 h. After removing the excessive drugs, the cells were irradiated with gamma rays under 6 Gy. Then the cells were cultured for another hour following fixed by 4% paraformaldehyde for 30 min. Finally, the cells experience classical immunofluorescence processing methods and are observed by a fluorescence microscope (Leica DMI6000).

Comet assay

Just as we mentioned before, A549 cells were seeded in 6-well plates at a density of 3×10^5 cells per well and cultured under standard conditions. After 24 h, the cell culture medium was replaced by N-P, N, and free CDDP containing culture medium and continued to culture for another 24 h, then the cells were irradiated with gamma rays under 6 Gy. After irradiation, the cells were immediately harvested, centrifuged, and suspended in PBS. The protocol of comet assay was performed according to the published reports[2]. The DNA damage was calculated by a special comet assay software project (CASP).

Evaluation of cytotoxicity

Cytotoxicity of different formulations was evaluated by cell counting kit-8 (CCK8) in Hela, MCF-7, and A549 cancer cells. In brief, cancer cells were seeded in a 96-well plate with a density of 4,000 cells per well and allowed to adhere for 24 h. Then the cancer cells were treated with different formulations at an equivalent dose based on

CDDP. After another 24 h, different formulations were replaced by fresh culture medium again and the cells were irradiated at 6 Gy by gamma radiation of ^{137}Cs. After irradiation, cells would continue to be cultured for 24 h. At last, 10 μL of CCK8 solution was added into each well. The 96-well plate was analyzed by a microplate reader (Thermo Varioskan Flash) at a wavelength of 450 nm. Data are presented as mean ± standard deviation (n = 3).

Cell Apoptosis Analysis

The apoptosis-inducing capabilities of different formulations after 6 Gy irradiation were evaluated by a FITC Annexin V apoptosis detection kit. After incubating different formulations with A549 cells (3×10^5/well) in 6-well plates for 24 h, the cells were irradiated with gamma rays under 6 Gy. Different from comet assay, the cells would be cultured under standard conditions for 24 h after irradiation. Next, the manufacturer's instructions were used to deal with the cell samples. The samples were analyzed by flow cytometry (Invitrogen Attune NxT).

Cell cycle experiment

The experimental procedures before irradiation were the same as that of cell apoptosis analysis. 24 h after irradiation, the cells were harvested, centrifuged, and fixed with pre-cooled 70% ethanol solution to store at 4 ℃ overnight. The ethanol was removed by centrifugation and the cells were thoroughly washed with cold PBS. 500 μL pre-made propidium iodide (PI) dye (The prescription of 5 mL PI dye is as follows: Sodium citrate, 5.882 mg; PI, 250 μL; RNase, 10 μL) was added to the cells and the mixture was incubated at 37 ℃ for 10 min. The cell cycle was tested by flow cytometry and the results were analyzed by FlowJo 7.6.

***In Vitro* COX-2 Inhibition Assay**

COX fluorescent inhibitor screening assay kit (700100, Cayman Chemical) was applied to compare the *in vitro* COX-2 inhibition ability for a hydrogel of N and N-P. Following the instruction of manufactures, the fluorescence produced by the reaction between prostaglandin G2 (PGG2) and ADHP (10-acetyl-3, 7-dihydroxyphenoxazine) was measured by a microplate reader (Thermo Varioskan Flash).

Western Blot analysis

Western blot analysis was performed to evaluate the expression difference of P53 and COX-2 in A549 cells. After culturing, incubating, or irradiating cells, the cells were collected and resuspended with lysis buffer. The total protein concentrations of different formulations were estimated by the BCA kit and detailed experimental procedures referenced the previous study[3].

Statistical Analysis

We employed SPSS 17.0 software to conduct statistical comparisons by Student's t-test. Quantitative data were expressed as mean ± standard deviation (SD). P-value < 0.05 meant statistically significant.

Supplemental figures

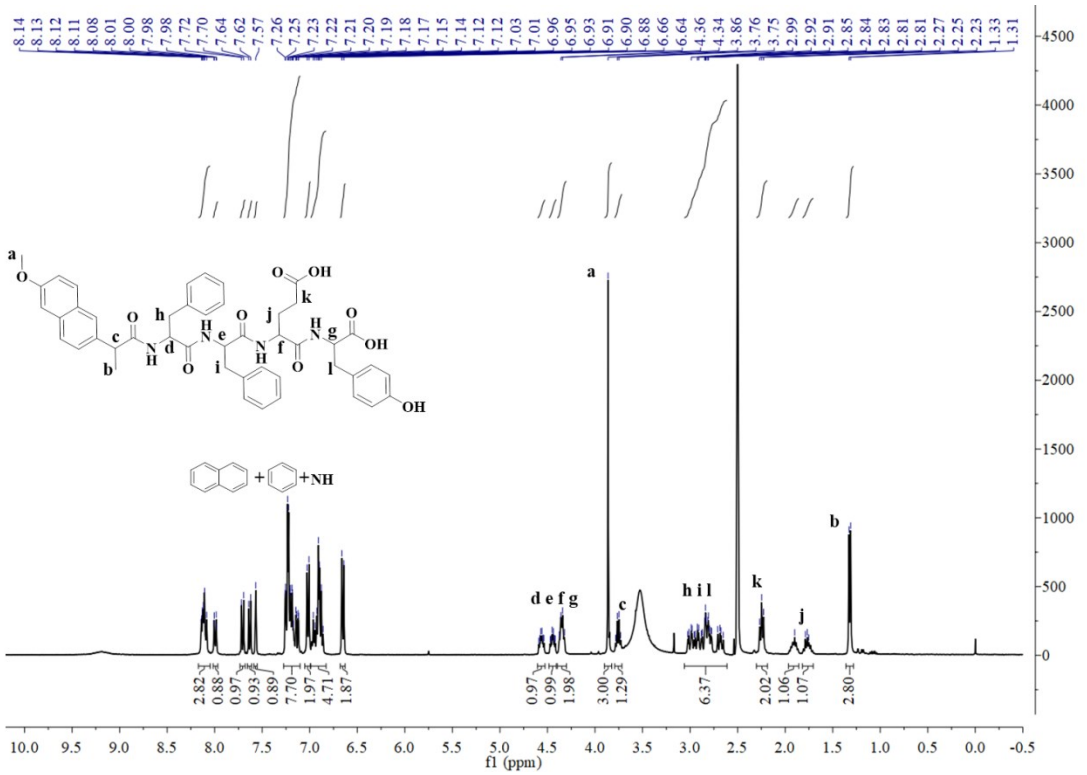


Figure S1. ^{1}H NMR spectrum of Npx-ffey in DMSO-d6

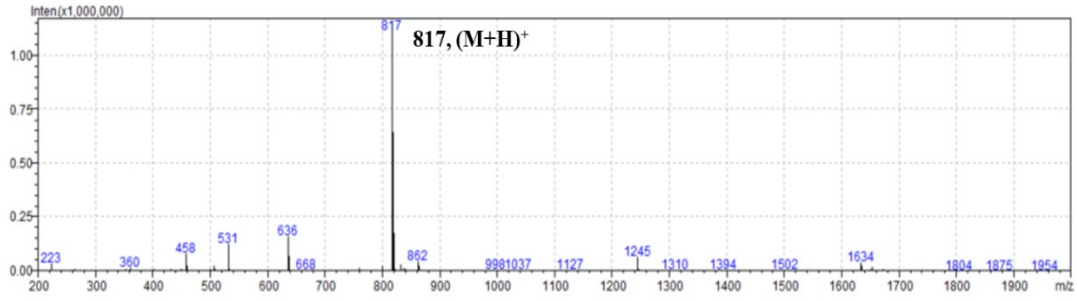


Figure S2. MS of Npx-ffey

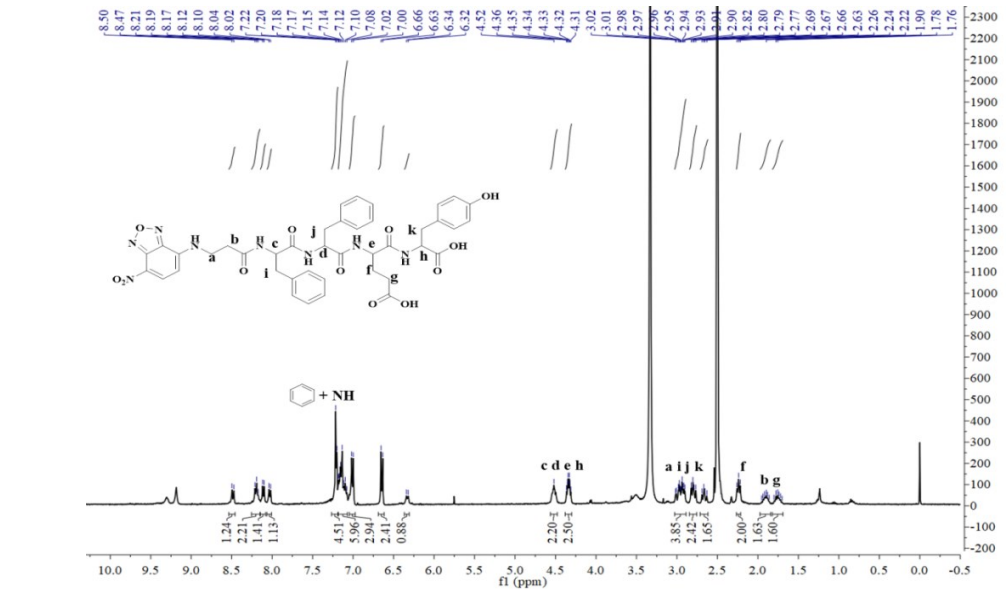


Figure S3. ^{1}H NMR spectrum of NBD-ffey in DMSO-d6

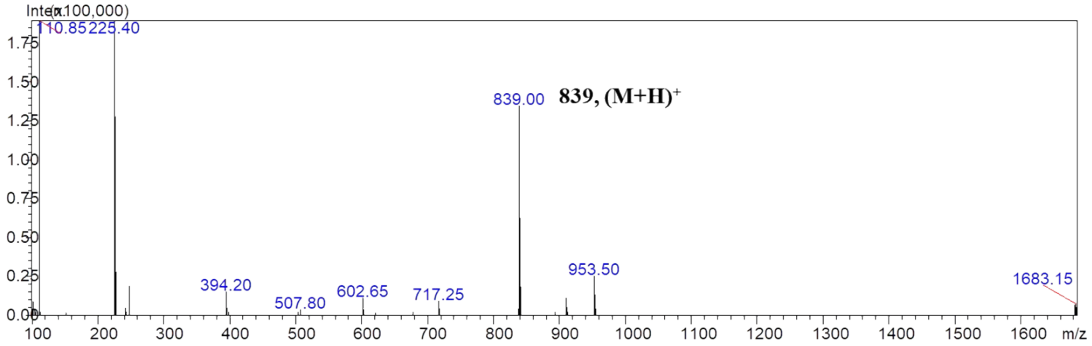


Figure S4. MS of NBD-ffey

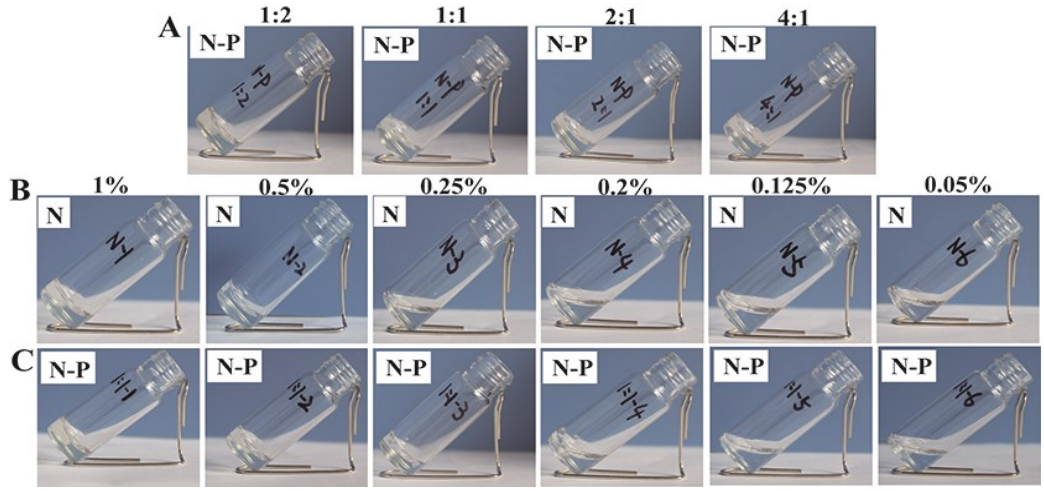


Figure S5. Hydrogels formation of (A) N-P with different mole ratios between Npx-ffey and CDDP; (B) N with different concentrations; (C) N-P with different concentrations of Npx-ffey.

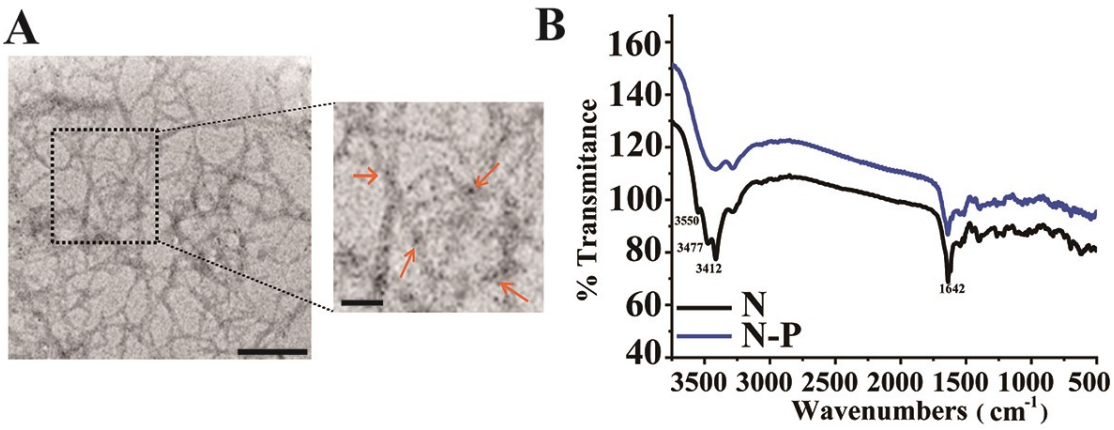


Figure S6. (A) TEM images of hydrogel of N-P (yellow arrows represent CDDP, Scale bar=100 nm); (B) FTIR spectra of N-P and N.

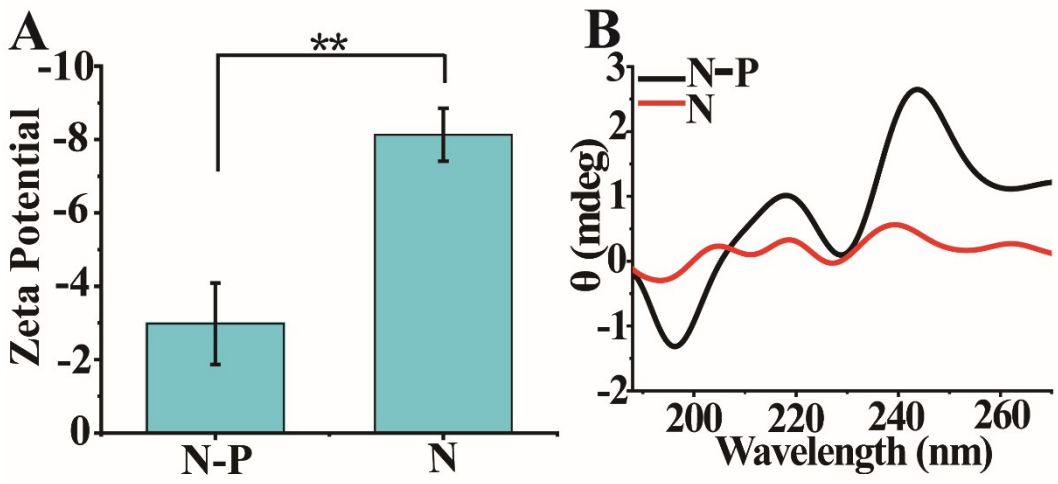
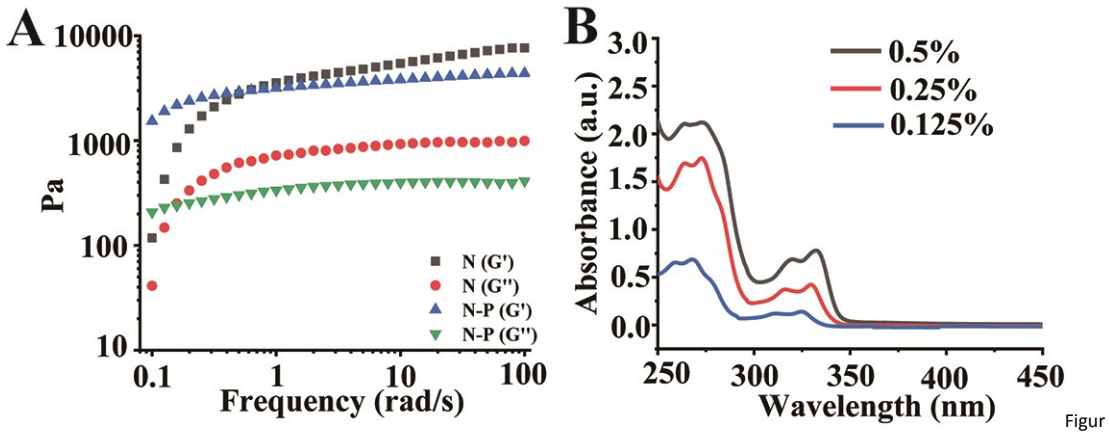


Figure S7. (A) The zeta potentials of the hydrogels of N-P and N. (B) CD spectra of the hydrogels of N-P and N. ** represents P <0.01 in (A).



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e S8. (A) Dynamic frequency sweep of the hydrogels of N-P and N; (B) UV/Vis spectrum of N-P with different concentrations.

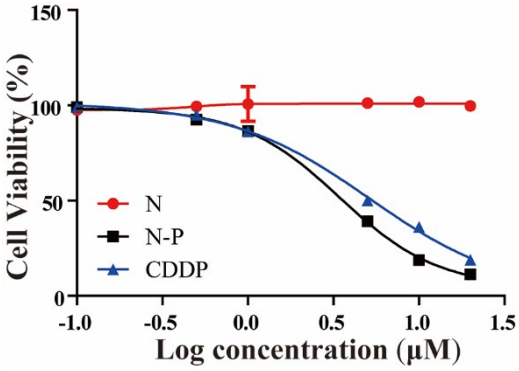

Figure S9. Cytotoxicities of different formulations against A549 cancer cells in the absence of IR.

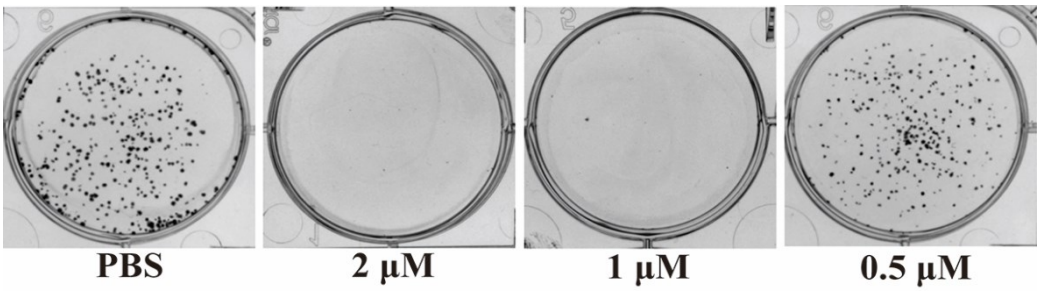

Figure S10. The comparison of colony formation between different concentrations of N-P with the PBS-treated groups.

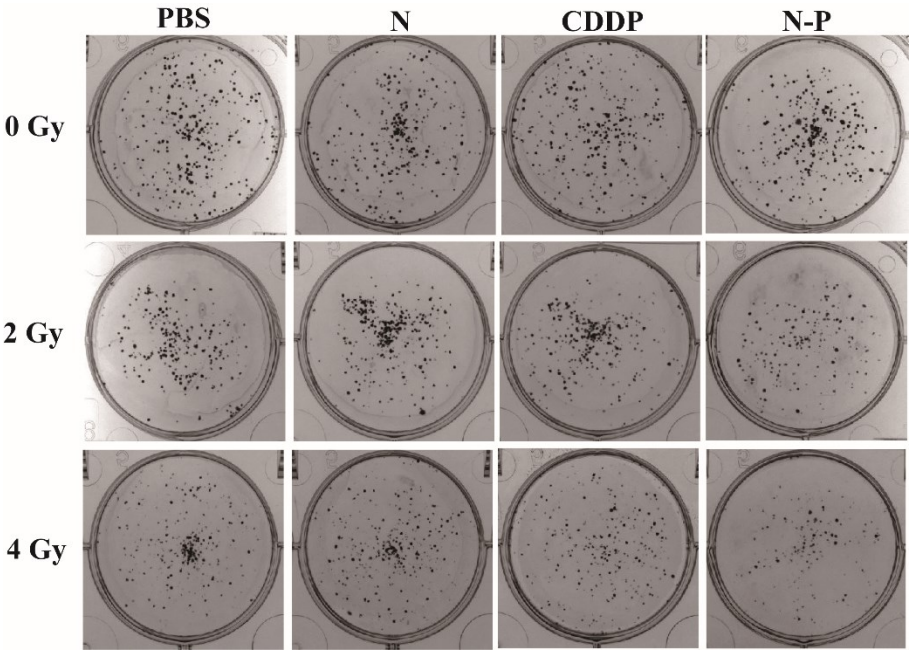

Figure S11. The degree of colony formation against A549 cells treated by PBS, free CDDP, or N-P at an equivalent dose of 0.5 μM under different radiation doses.

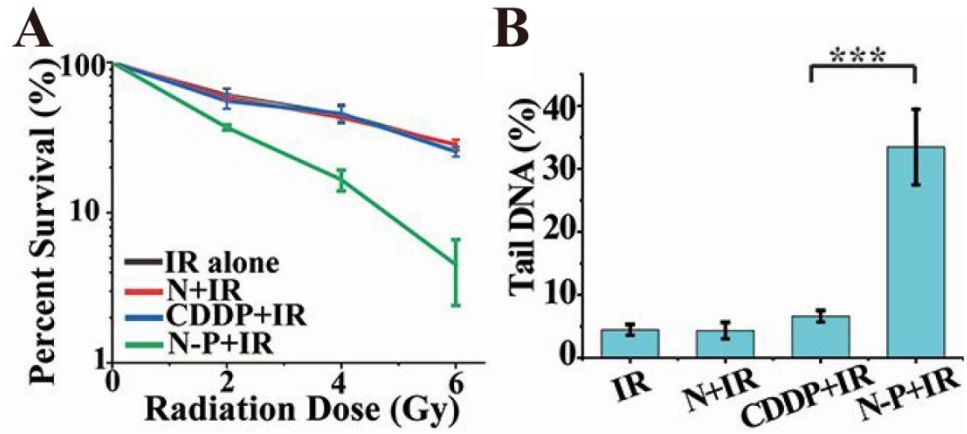

Figure S12. (A) Clonogenic assay survival curves of A549 cell samples treated with different formulations. (B) Comet assay tail DNA contents analyzed using CASP software. *** represents P <0.001 in (B).

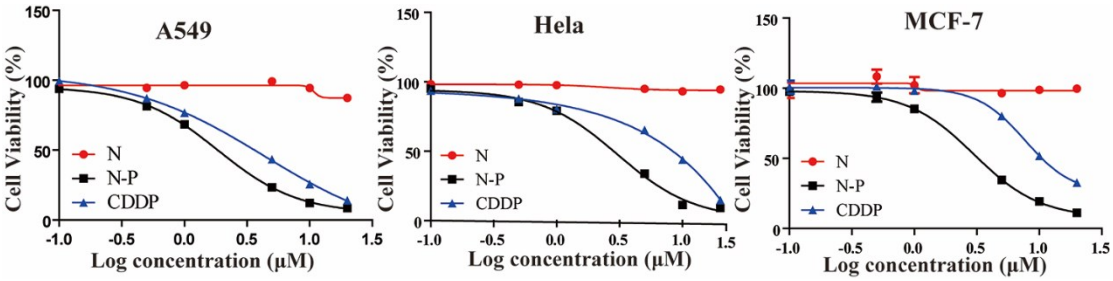

Figure S13. *In vitro* cytotoxicities of different formulations against A549, Hela, and MCF-7 cells, respectively under 6 Gy radiation.

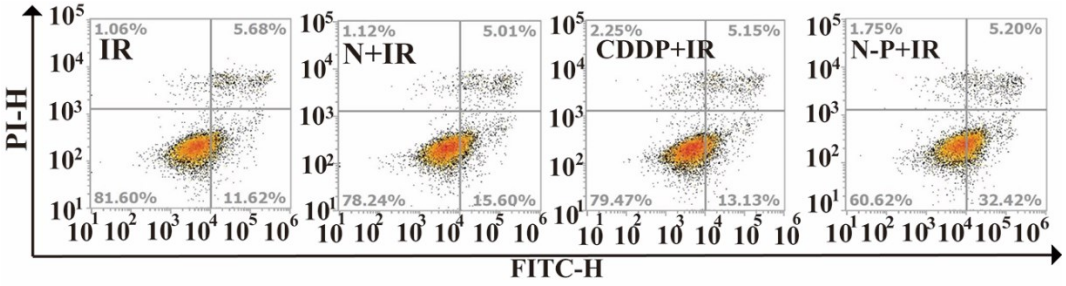

Figure S14. Flow cytometry analysis of cell apoptosis induced by various formulations under 6 Gy irradiation.

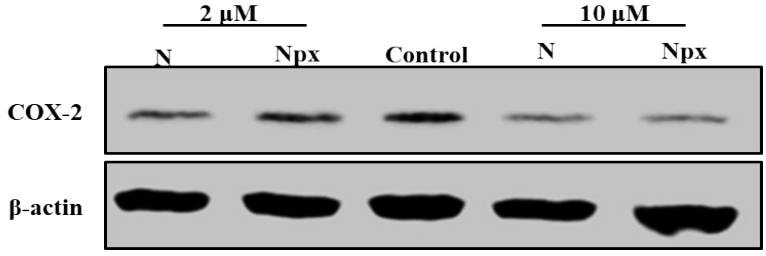

Figure S15. Effects of N and Npx on the expression of COX-2

References

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3. D. B. Cheng, X. H. Zhang, Y. J. Gao, L. Ji, D. Hou, Z. Wang, W. Xu, Z. Y. Qiao and H. Wang, *J. Am. Chem. Soc.*, 2019, **141**, 7235-7239.