# SUPPORTING INFORMATION

# Synergic Effect of Doxorubicin Release and Two-Photon Irradiation of $Mn^{2+}$-Doped Prussian Blue Nanoparticles on Cancer Therapy

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## Experimental part

**Materials.** All chemical reagents were purchased and used without further purification: sodium hexacyanoferrate(II) decahydrate (Alfa aesar, 99%), iron(III) chloride hexahydrate (Sigma-aldrich, 97%), manganese(II) chloride tetrahydrate (Sigma-aldrich, 99%), doxorubicin hydrochloride (Fluorochem), phosphate buffered saline (PBS) (Sigma), ultra-pure water.

**Characterization methods.** Infrared spectra were recorded on KBr pellets on a Spectrum Two DTGS spectrometer model by Perkin Elmer. UV-Vis spectra were collected on a V-650 spectrophotometer model from JASCO. Transmission Electron Microscopy (TEM) observations were carried out at 100 kV (LaB6 JEOL 1400 Plus – 120kV). Samples for TEM measurements were deposited from solutions on copper grids. Nanoparticles' size distribution histograms were determined using enlarged TEM micrographs taken at magnification from 50 K to 100 K on a statistical sample of *ca*. 300 nanoparticles. Scanning Electronic Microscopy (SEM/EDS) analyses were performed on a FEI Quanta FEG 200 instrument. The powders were deposited on an adhesive carbon film and analyzed under vacuum. The quantification of the heavy elements was carried out with the INCA software, with a dwell time of 3 µs. X-ray diffraction powder patterns were recorded using a PANalycal X'pert MDP-Pro diffractometer in Bragg Brentano geometry with Ni filtered Cu-Kα radiation (λ = 1.5418 Å). Measurements were performed at room temperature in a 2θ range of 10°-60° using a step size of

0.033° (2θ) and a counting time per step of 150 s. Zeta potential values were recorded on Malvern nanoseries, Zetasizer NanoZS (Model ZEN3600) in a DTS1060C Zetacell in water at 25°C with an equilibration time of 120 s with automatic measurement and data were treated by Zetasizer software using a Smoluchowski model.

**Synthesis of $Mn^{2+}$-doped Prussian blue nanoparticles (1):** At 25 °C, an aqueous solution of $Na_4[Fe(CN)_6]$ (10 mL, 11.25 mM) and a mixed solution of $FeCl_3 \cdot 6H_2O$ and $MnCl_2 \cdot 6H_2O$ (10 mL, 7.6 mM and 0.76 mM, respectively) were simultaneously added, at 2 mL $h^{-1}$ rate, into 100 mL of ultra-pure water, under stirring, using a syringe pump. After complete addition, the mixture was stirred one more hour before being centrifuged at 35,700 g during 15 min. The supernatant was removed and the nanoparticles were washed twice with water and ethanol before being dried under vacuum.
IR (KBr): ν(O-H) = 3630 $cm^{-1}$ (coordinated water), ν(O-H) = 3428 $cm^{-1}$ (crystallized water), ν(C≡N) = 2082 $cm^{-1}$ ($Fe^{III}$–C≡N–$Fe^{II}$), δ(O–H) = 1611 $cm^{-1}$ (crystallized water), ν($Fe^{II}$-CN) = 600 $cm^{-1}$, δ($Fe^{II}$-CN) = 505 $cm^{-1}$.
EDS: Na/Mn/Fe = 15.76/5.10/79.15
Estimated formula for **1**: $Na_{0.38}Mn_{0.12}Fe[Fe(CN)_6]_{0.91}$.
**Doxorubicin loading (1@DOX):** At room temperature, 15.1 mg of nanoparticles **1** were suspended in an aqueous doxorubicin solution (18 mL, 0.12 mg. $mL^{-1}$) for 24 hours under stirring (300 rpm) protected from light. The mixture was centrifuged at 35,700 g during 20 min. Nanoparticles were washed twice with ultra-pure water (18 mL) before being dried under vacuum. After each centrifugation, the supernatant was collected and analyzed by UV-visible spectroscopy for quantification of doxorubicin loading into the nanoparticles (Fig. S1a). A calibration curve (Fig S1b) was thus first performed with doxorubicin in water at concentrations ranging from 0.1 mg $mL^{-1}$ to 0.005 mg $mL^{-1}$ ($R^2$ = 0.9992).
IR (KBr): ν(O-H) = 3630 $cm^{-1}$ (coordinated water), ν(O-H) = 3427 $cm^{-1}$ (crystallized water), ν(C≡N) = 2080 $cm^{-1}$ ($Fe^{III}$–C≡N–$Fe^{II}$), δ(O–H) = 1612 $cm^{-1}$ (crystallized water), ν($Fe^{II}$-CN) = 600 $cm^{-1}$, δ($Fe^{II}$-CN) = 502 $cm^{-1}$.
EDS: Na/Mn/Fe = 12.39/5.24/82.37.
Estimated formula for **1**: $Na_{0.28}Mn_{0.12}Fe[Fe(CN)_6]_{0.88}@DOX_{0.04}$
**Doxorubicin release**: At room temperature, 11 mg of **1@DOX** were suspended into PBS (4 mL) and placed in a dialysis membrane (Specta/Pro® Dialysis membrane MWCO: 3.500). The membrane was immerged into 20 mL of PBS under magnetic stirring (800 rpm) and protected from light. For each time-point (1 h, 2 h, 4 h, 8 h, 24 h, 48 h), dialysate was entirely removed and replaced by 20 mL of PBS. All solutions from the dialysis were analyzed by UV-visible spectroscopy. A calibration curve was thus first performed with doxorubicin in PBS at concentrations ranging from 0.1 mg.$mL^{-1}$ to 0.001 mg.$mL^{-1}$ ($R^2$ = 0.9982) (Fig. S1b).

**Computational Part**
Monte Carlo simulations were performed at 300 K in order to evaluate the amount adsorbed at saturation for doxorubicin in the PBA structure with a chemical composition closed to the experimental one. Using a home-made code, $5\times10^6$ steps for equilibration and $5\times10^6$ steps for production were considered for calculations using Universal Force Field (UFF) combined with partial charges for both the doxorubicin molecules and the solid. Following the strategy already used in other solids,[1] ESP charges were extracted from DMol[3] calculations for doxorubicin molecules while qEq partial charges (corresponding to the partial charges obtained from the electronegativity equalization method) were considered for the solid framework and formal charges for the compensating cations. Concerning the Lennard Jones interactions, the Lorentz-Berthelot rules were applied with a cut-off

radius fixed at 12.5 Å. Ewald summation for the electrostatic part was handled to increase the convergence for the energy calculations.
The structures considered for the solids have already been presented in the literature. The resulting interactions obtained from Monte Carlo simulations are reported in Figure S7.

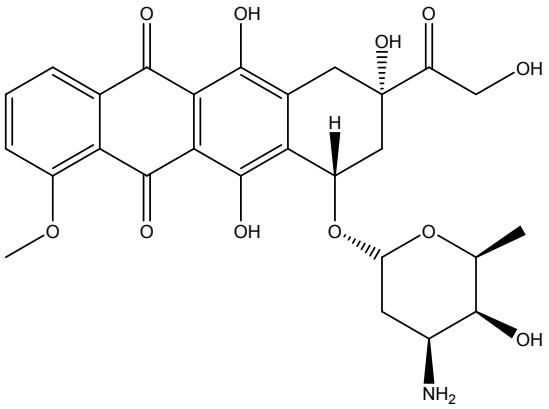
**Cell culture:**
Human breast adenocarcinoma MDA-MB-231 cell line was used (ATCC). Here this cell line that stably expresses GFP inducing the visualization of green nuclei was generously donated by Dr. Peter Coopman. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotic (0.05 mg $mL^{-1}$ gentamycin). These cells were grown in humidified atmosphere at 37°C under 5% $CO_2$.

***In vitro* cytotoxicity assay:**
MDA-MB-231 cells were seeded in 96-well plate. After 24 hours of cell growth, cells were treated with PB nanoparticles (**1**, **1@DOX**) at 50 µg.$mL^{-1}$ concentration. Toxicity was evaluated through cell counting after 1 day, 2 days, and 3 days of treatment. Cells were visualized with Leica DM.IRB microscope; green fluorescence was excited at 480 nm, cells were counted using ImageJ program.

**Evaluation of chemo-photothermal effect under TPE laser:**
MDA-MB-231 cells were seeded in 384-well plates. After 24 hours of cell growth, cells were treated with PB nanoparticles (**1**, **1@DOX**) at 50 µg $mL^{-1}$), and free doxorubicin. 24 hours after treatment, cells were visualized with LSM-confocal microscope; green fluorescence was excited at 488 nm. Then cells were irradiated with pulsed laser at 808 nm (3.7 W, 5% of total laser power) for 10 min. Cells were imaged immediately after irradiation and 24 h after irradiation. For quantification, cells were counted using ImageJ program.

**Evaluation of chemo-photothermal effect under SPE laser:**
MDA-MB-231 cells were seeded in 96-well plates. After 24 hours of cell growth, cells were treated with PB nanoparticles (**1**, **1@DOX**) at 50 µg $mL^{-1}$ concentration. Twenty four hours after treatment, cells were visualized with Leica DM.IRB microscope, green fluorescence was excited at 480 nm. Then cells were irradiated with OPE laser (Kamax innovative system) at 808 nm (2.5 W $cm^{-2}$) for 30 min. Cells were imaged 24 h , 48 h and 72 h after irradiation. For quantification, cells were counted using ImageJ program. Cell death was detected by propidium iodide (PI) and then visualized with Leica DM.IRB microscope, red fluorescence was excited at 488 nm.

# Figures




**Scheme 1.** Chemical structure for doxorubicin.

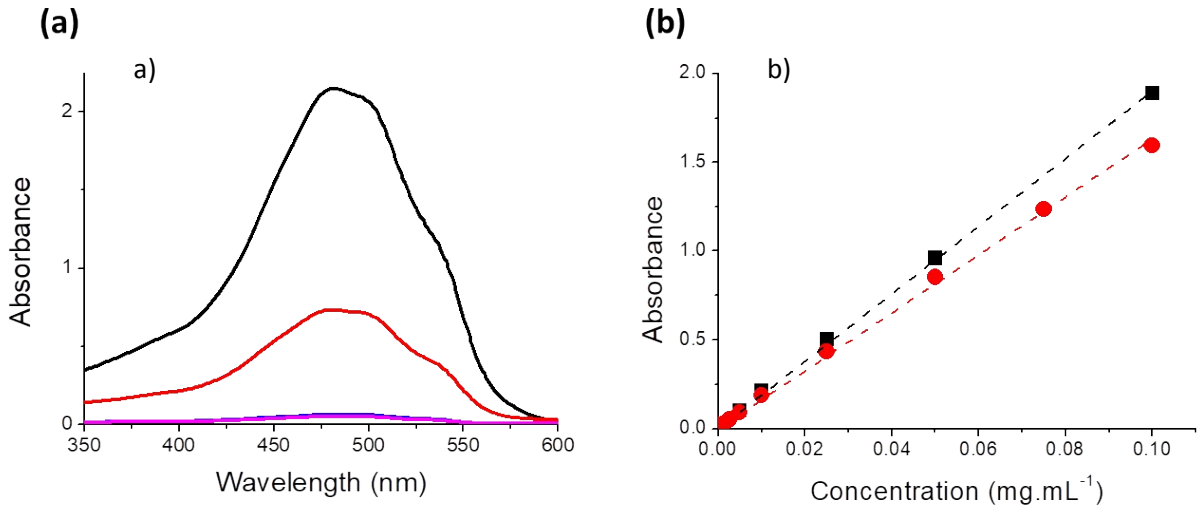



**Figure S1.** a) Absorption spectra for the initial solution (0.12 mg.mL$^{-1}$) of DOX loading (black curve) as well as the filtrate and washing solutions after the nanoparticles removing (red for the first filtrate, blue for the second and pink for the third); b) Calibration curves for the doxorubicin in water (black curve) and in PBS (red curve).

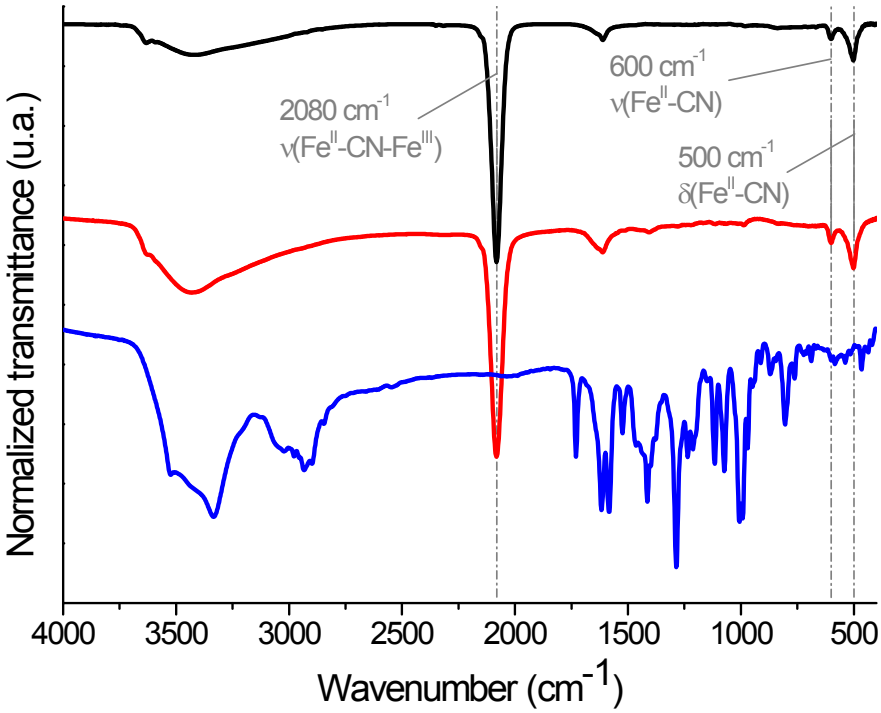



**Figure S2**. FTIR spectra for **1** (black) and **1@DOX** (red) and doxorubicin (blue).

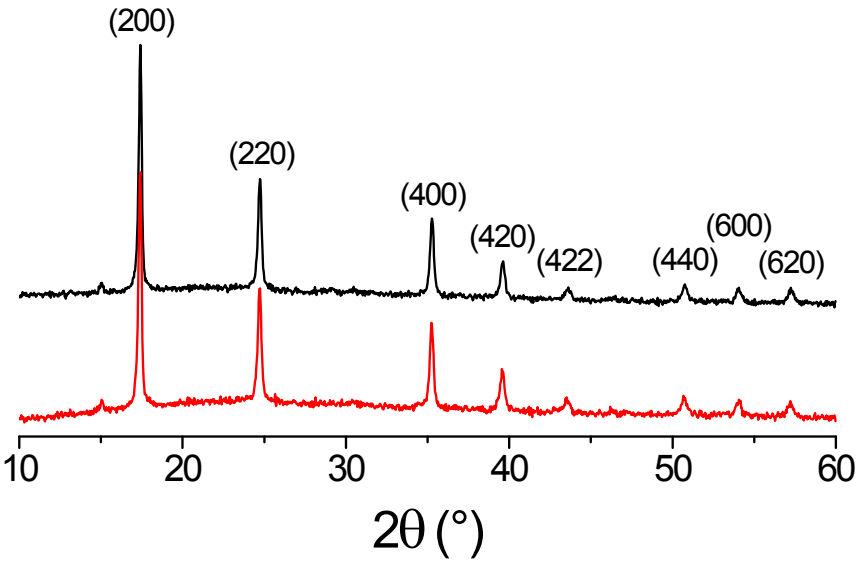



**Figure S3**. PXRD patterns of **1** (black) and **1@DOX** (red).

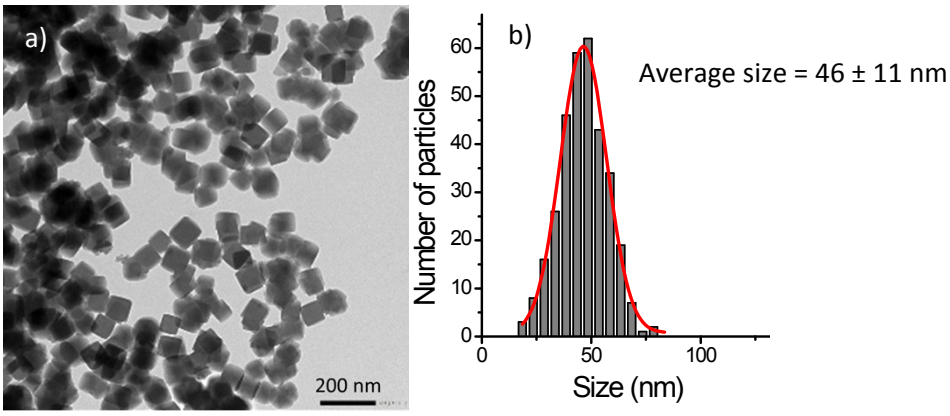



**Figure S4**. a) TEM image and b) size distribution for **1**.

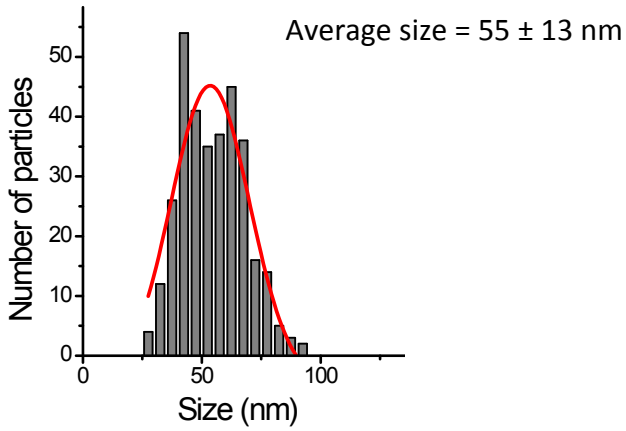



**Figure S5**. Size distribution for **1@DOX**.

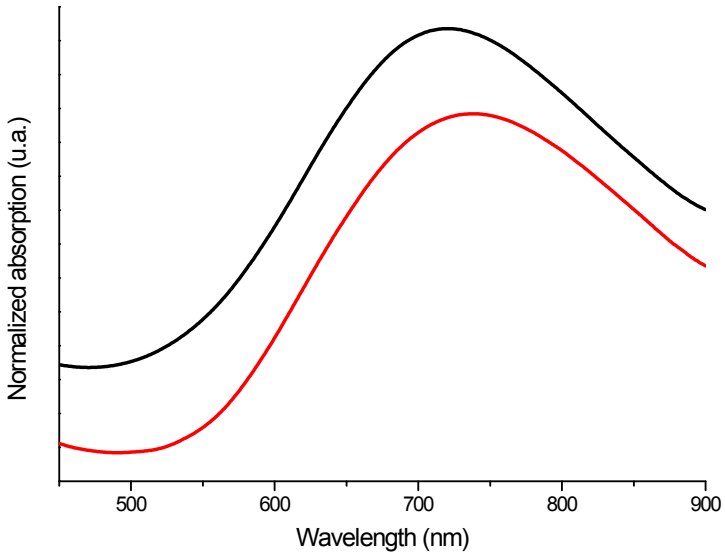



**Figure S6**. Absorption spectra in water for **1@DOX** (red curve) and **1** (black curve).

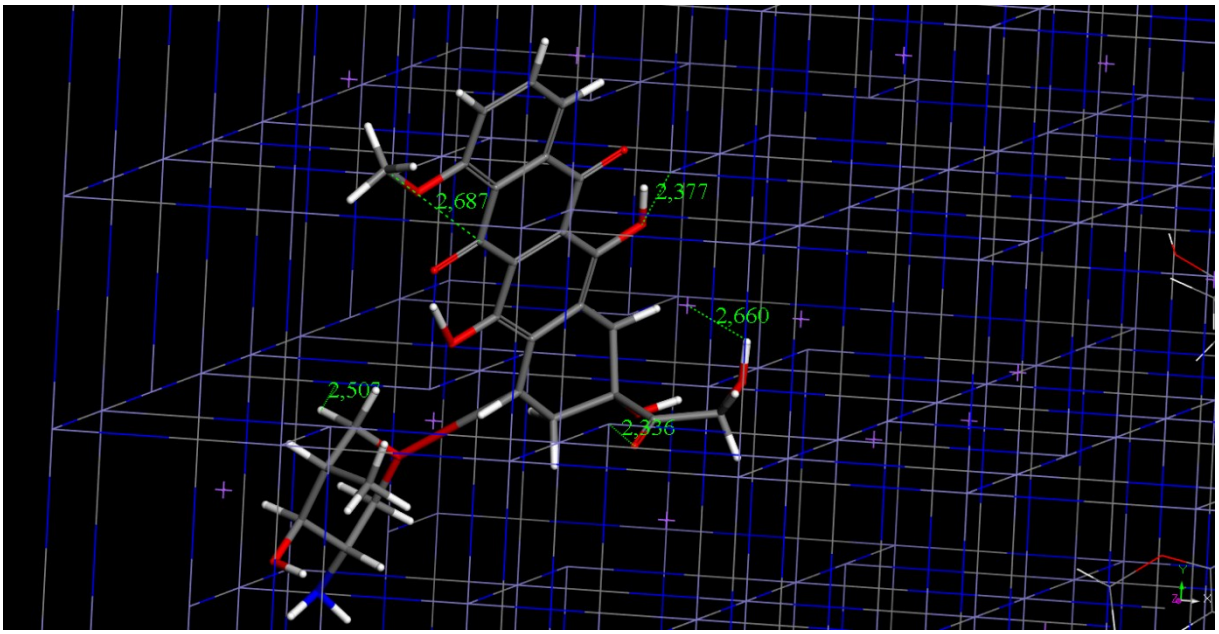



**Figure S7**. Snapshot showing main interactions between doxorubicin molecules and PBA framework demonstrating distances between doxorubicin and unsaturated metal centers and cyano groups. As observed, strong interactions can be seen between oxygen from doxorubicin and the unsaturated metal centers, as well as between methyl groups from doxorubicin and C≡N. Relatively, low interactions are observed between the cations $Na^+$ and the doxorubicin.

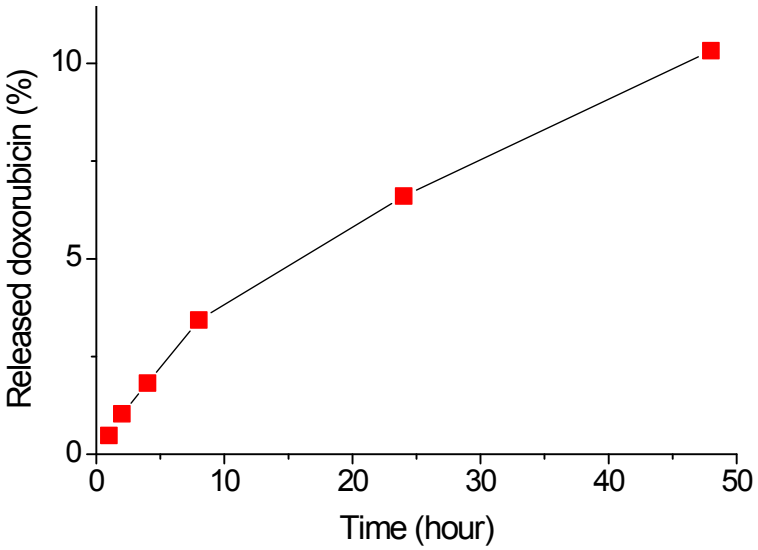



**Figure S8**. The DOX release experiments for **1@DOX**. Line is a guide for eye.

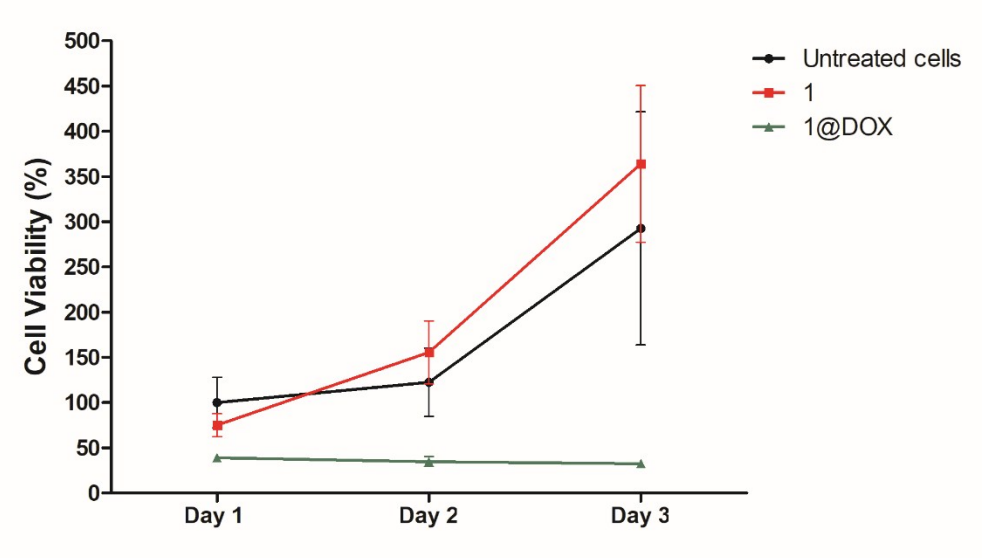



**Figure S9**. Cell viability (%) of MDA-MB-231 cells treated with 50 μg $mL^{-1}$ concentration of PB nanoparticles (**1** and **1@DOX**), toxicity was evaluated at different time periods of incubation. Data are presented as (mean ± SEM), n=3.

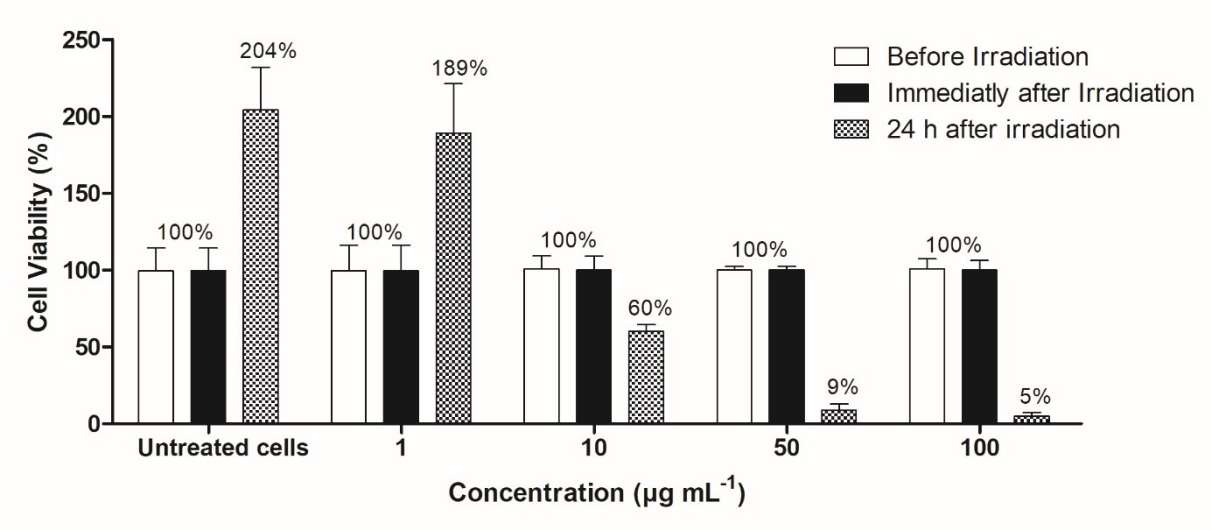



**Figure S10.** Cell counting (%) of living MDA-MB-231 cells treated with different concentration of **1@DOX** nanoparticles before irradiation, immediately after irradiation and 24 h after irradiation with a TPE laser at 808 nm (3.7 W, 5% of total laser power) for 10 min. Data are presented as (mean ± SEM), n=3.

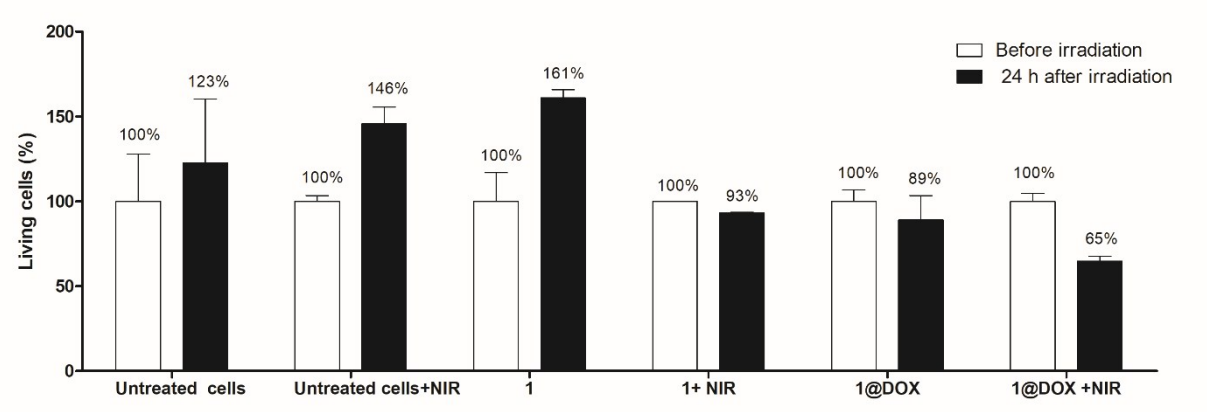



**Figure S11.** Cell counting (%) of living MDA-MB-231 cells treated with nanoparticles (**1** and **1@DOX**) at 50 µg $mL^{-1}$ concentration before irradiation and 24 h after irradiation with SPE at 808 nm (2.5 W $cm^{-2}$) for 30 min. Data are presented as (mean ± SEM), n= 3.

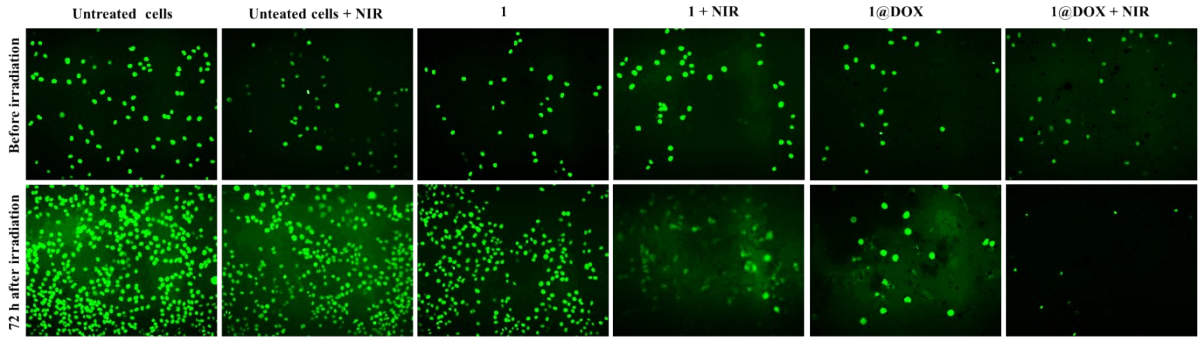



**Figure S12.** Fluorescence imaging of living MDA-MB-231 cells treated for 24 h with nanoparticles **1** and **1@DOX** at a concentration of 50 µg $mL^{-1}$ before irradiation and 72 h after irradiation with SPE laser at 808 nm (2.5 W $cm^{-2}$) for 30 min.

References

1. S. Rojas, I. Colinet, D. Cunha, T. Hidalgo, F. Salles, C. Serre, N. Guillou and P. Horcajada, *ACS Omega*, 2018, **3**, 2994.