Electronic Supplementary Information

Carbon quantum dots-NO photoreleaser nanohybrids for two-photon phototherapy of hypoxic tumors

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S1. Chemicals.
All chemicals were from commercial sources at the highest possible purity and used as received. All solvents used were spectrophotometric grade.

S2. Instrumentation.
UV-Vis spectra were recorded with a Varian Cary spectrometer, using quartz cells with a path length of 1 cm. Fluorescence emission spectra were recorded with a Varian Cary Eclipse spectrometer and a Spex Fluorolog-2 (mod. F-111) in air-equilibrated solutions. Hydrodynamic diameters of the nanoparticles were determined on a Malvern Nano-ZS zetasizer and a Horiba LS 550 apparatus equipped with a diode laser with a wavelength of 650 nm.

Amperometric NO detection.
NO release was measured with a World Precision Instrument, ISO-NO meter, equipped with a data acquisition system, and based on direct amperometric detection of NO with short response time (< 5 s) and sensitivity range 1 nM – 20 μM. The analog signal was digitalized with a four-channel recording system and transferred to a PC. The sensor was accurately calibrated by mixing standard solutions of $NaNO_2$ with 0.1 M H_2SO_4 and 0.1 M KI according to the reaction:

$$4H^+ + 2I^- + 2NO_2^- \rightarrow 2H_2O + 2NO + I_2$$

Irradiation was performed in a thermostated quartz cell (1 cm pathlength, 3 mL capacity) under gentle stirring. Irradiation was performed by using the monochromatic radiation of 350 nm of the Spex Fluorolog-2 (mod. F-111) or by using a 200 mW continuum laser with λ_{exc} = 405 nm. NO measurements were carried out under stirring with the electrode positioned outside the light path in order to avoid NO signal artefacts due to photoelectric interference on the ISO-NO electrode.

Chemical NO detection.
NO release was also measured by means of the well-known, highly sensitive (detection limit on the order of the picomoles) fluorimetric bioassay of Misko *et al.*,[1s] based on the ring closure of the nonfluorescent 2,3-diaminonaphthalene (DAN) with nitrite to form the highly fluorescent product 2,3-diaminonaphthotriazole (DANT). Briefly, once photogenerated in aerobic conditions NO, in the absence of other scavengers, is rapidly converted into nitrite and nitrate, its stable metabolities. Nitrite react quantitatively with DAN to give rise DANT. Aliquots of 1 mL of aqueous solutions of either compound **2** or **3b** were irradiated or kept in the

dark. Afterwards 3.5 mL of 0.31 mM DAN in 0.62 M HCl were added and solutions were stirred for 20 min at room temperature. 100 μL of NaOH 2.8 M was put into the fluorescent cuvette and the fluorescence was recorded at λ_{exc} = 360 nm.

S3. Synthetic procedures.

*Synthesis of **1**.* Following a modification of the procedure developed by Yang *et al*, carbon nano powder (< 50 nm, 1g) was refluxed in 2.6 M nitric acid (100 mL) for 12 hours and subsequently dialyzed against distilled water.[2S] The resulting solution was centrifuged and the supernatant removed. The black pellet was dried in a vacuum oven for 12 hours and refluxed in neat $SOCl_2$ for 6 hours. Excess $SOCl_2$ was removed by evaporation and the sample (100 mg) was mixed with poly (ethylene glycol)-bis-3-aminopropyl terminated MW 1500 (1.5g) in a flask heated to 110°C, stirred vigorously under argon for 72 hours. The mixture was allowed to cool to room temperature and dispersed in water. The resulting solution was centrifuged at 25000g and the supernatant was retained. The product was purified using a WAP-10 Sephadex column and the coloured fraction was retained. A solution of succinic anhydride in CH_2Cl_2 (3 mL) was combined with a solution of the amine terminated CQDs (1 mL, 45 mg/mL) and Et_3N in CH_2Cl_2 and the solution maintained at ambient temperature and stirred under Ar for 15 hours. The mixture was diluted with aqueous HCl (1 M, 30 mL) and extracted with $CHCl_3$ (3 x 30 mL). The organic phase was dried over Na_2SO_4, and the solvent removed under reduced pressure to afford a dark yellow residue. The residue was resuspended in PBS to afford a solution of carboxylic acid terminated C Dots (20 mg/mL).

*Synthesis of **2**.* The NO photodonor **2** was synthesized according to our already reported procedure.[3S]

*Synthesis of **3a-d**.* Carboxylic acid terminated CQDs **1** (0.5 mL, 20 mg/mL) in PBS were added to 1-(3-dimethylaminopropyl)-3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (5.0 mg, 25 μM) and N-Hydroxysuccinimide (2.0 mg, 10 μM). The solution was stirred at room temperature for 20 min. This stock (1 mL) was split evenly into four vials and varying amounts of **2** (2 mg 7.6 μM) in 1mL DMSO were added to each vial, the corresponding solution were stirred for 4 hours at room temperature and subsequently purified using a WAP-10 Sephadex column.

S4. Cell viability studies.

HeLa cells were cultured in Hams-F12 essential medium, supplemented with foetal bovine serum (10 % v/v) penicillin (200 μg mL^{-1}) and non-essential amino acids (0.1 mM). The cells were seeded in 384 well glass bottom plates at a density of 5 x 10^4 cells mL^{-1}, incubated overnight at 37°C in a humidified CO_2 (5%) atmosphere and spiked with concentrations of **3d** prepared in a PBS solution to allow for final concentrations of 1, 10, 25, 50, and 100 μM (with respect to the concentration of **2** on the CQD surface). Wells were also spiked with identical concentrations of **2** for comparative purposes. The cells were incubated in the dark for a further 24 h, the medium was removed and each well was washed twice with 50 μL PBS. 50 μL fresh media was added to each well and selected wells subjected to 30 seconds irradiation at 800 nm using a Lecia confocal microscope equipped with a mode locked Ti:sapphire laser generating 100fs wide pulses at a rate of 80 MHz with an average power of 80 mW. After irradiation the cells were allowed to incubate in the dark for a further 24 hrs at 37°C in a humidified CO_2 (5%) atmosphere. Cell viability was then determined by MTT assay.[4S]

S5. *In vivo* experiments

All animals employed in this study were treated humanely and in accordance with the licensed procedures under the UK Animals (Scientific Procedures) Act 1986. BxPC-3 cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum. Cells were cultured at 37°C under 5% CO_2 in air. BxPc-3 cells(1 x10^7) were re-suspended in 100 μl of matrigel and implanted into the rear dorsal of male Balb/c SCID (C.B-17/IcrHan®Hsd-Prkdcscid) mice. Tumour formation occurred approximately 2 weeks

after implantation and tumour measurements were taken every other day using calipers. Once the tumours had reached an average volume of 100 mm^3 calculated from the geometric mean diameter using the equation tumour volume = $4\pi r^3/3$, animals were randomly distributed into 2 groups (n=2). Following induction of anaesthesia (intraperitoneal injection of Hypnorm/Hypnovel), a 50 µL aliquot of **3d** (5.312 mg/Kg) in PBS was injected directly into each tumour. Where relevant animals were treated by exposure to the Ti:Sapphire source two-photon irradiation at 800 nm for three 3 min periods with a 1 min lag time between each treatment. Post treatment animals were allowed to recover from anaesthesia and tumour volume was monitored at the indicated times. The % increase in tumour volume was calculated employing the pre-treatment measurements for each group.

Supplementary bibliography

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2SP. S, T. Yang, X. Wang, H. Wang, F. Lu, P. G. Lu, L. Cao, M. J. Meziani, J. H. Liu, Y. Liu, M. Chen, Y. Huang and Y. P. Sun, *J. Phys. Chem. C. Nanomater Interfaces,* 2009, **113**, 18110
3S. F. L. Callari and S. Sortino, *Chem. Commun.*, 2008, 1971.
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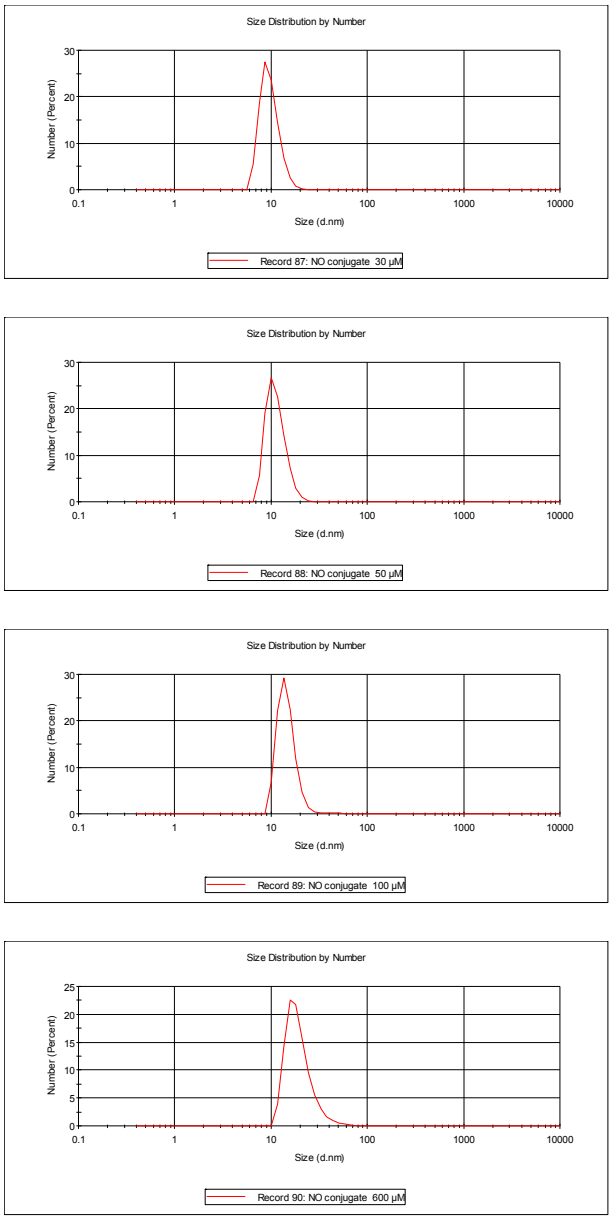


Figure S1 Dynamic light scattering for **3a-d** (from top to bottom) recorded in PBS buffer pH 7.4.

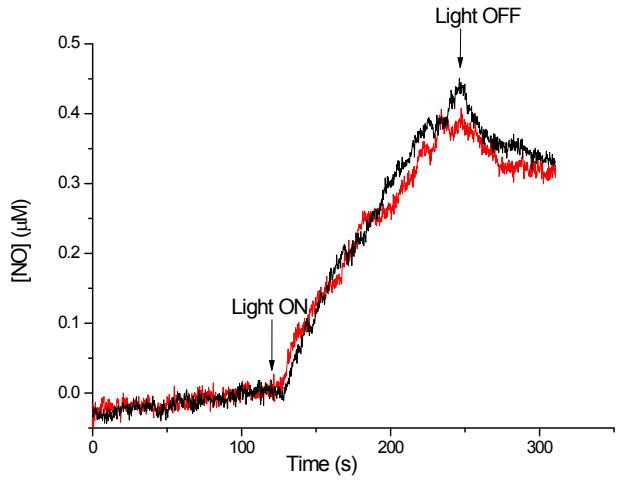
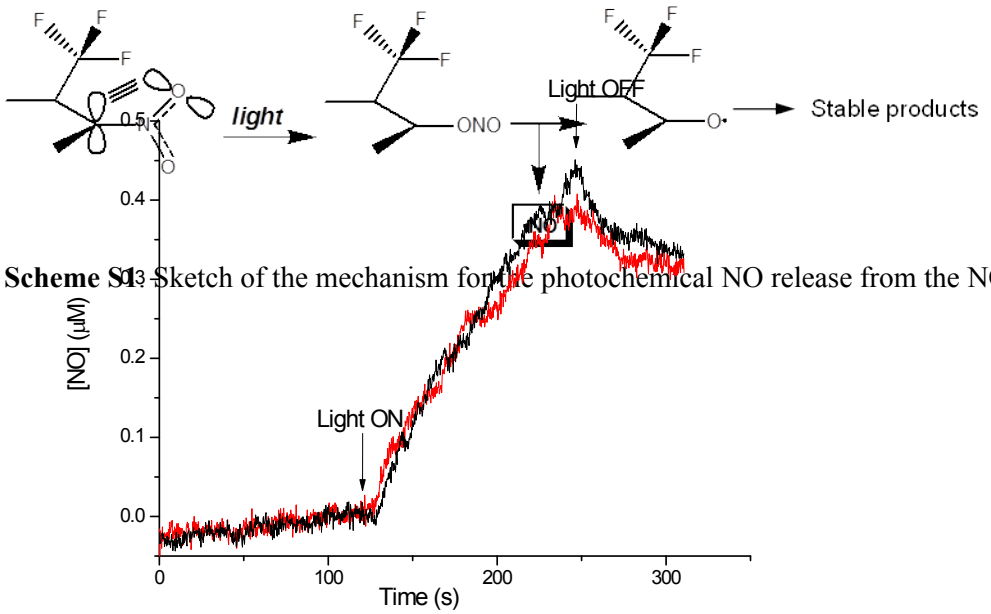


Figure S2 NO release profile and observed upon 405 nm light irradiation of aqueous solutions of **2** (black) and the CQDs **3b** (red) in PBS at pH 7.4. The two samples were optically matched at the irradiation wavelength.



Scheme S1 Sketch of the mechanism for the photochemical NO release from the NO photodonor