Supporting Information

Extending the Tissue Penetration Capability of Conventional Photosensitisers: A Carbon Quantum Dot – Protoporphyrin IX Conjugate For Use in Two-Photon Excited Photodynamic Therapy

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Materials and Methods

Equipment and Reagents: Chemicals were purchased from commercial sources at the highest possible purity and used as received. UV-Vis spectra were recorded with a Varian Cary spectrometer, using quartz cells with a path a length of 1 cm. Emission spectra were recorded with a Varian Cary Eclipse spectrometer in aerated solutions, slits = 5 nm. Hydrodynamic diameters of the nanoparticles were determined on a Malvern Nano-ZS zetasizer. Quantum yields were calculated with reference to Rhodamine 6G.

Synthesis of Carbon Quantum Dots: 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 (40 mL) for 12 hours and subsequently dialyzed against distilled water. The resulting solution was centrifuged and the supernatant removed. The black pellet was dried in a vacuum oven for 12 hours and then refluxed in neat $SOCl_2$ for 6 hours. Excess $SOCl_2$ was removed by evaporation and the sample (100mg) was mixed with poly (ethylene glycol)-bis-3-aminopropyl terminated MW1500 (1.5g) in a flask heated to 110°C, and stirred vigorously under argon for three days. The mixture was allowed to cool down 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.

Conjugation of protoporphyrin IX to CQDs to form CQD-P: Synthesis of CQD-1 conjugates: Following a procedure developed by Yildiz et al, protoporphyrin IX (10.0 mg, 17.0 μM) dissolved in DMF (2.0 mL) was added to 1-(3-Dimethylaminopropyl)-3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (5.7mg, 30μM) and N-Hydroxysuccinimide (2.0 mg, 10 μM). The solution was stirred at 25^{o}C for 20 min. CQDs (5mg / mL in PBS) were added and the solution was stirred for 4 hours. The **CQD-P** conjugate was purified using a WAP-10 Sephadex column.

In vitro singlet oxygen determination: A solution was prepared containing **CQD-P** (5 μM) and DPBF (10 μM) in EtOH:H_2O (50:50). The solution was aerated for 10 mins before being irradiated with light at 365 nm delivered from a fibre optic probe (20 J/cm^2), for 60 mins. Aliquots were removed and their

absorbance recorded every 5 minutes at 410 nm using a Carey Absorbance spectrometer. The absorbance readings were corrected for residual protoporphyrin IX absorbance.

Cell viability studies: HeLa cells were cultured in Hams-F12 essential media, supplemented with foetal bovine serum (10 % v/v) penicillin (200 μg mL^{-1}), streptomycin (200 μg mL^{-1}) and non-essential amino acids (0.1 mM). The cells were seeded in 384 well plates at a density of 5×10^4 cells mL^{-1}, incubated overnight at 37 °C in a humidified CO_2 (5%) atmosphere and spiked with concentrations of **CQD-P** prepared in a PBS solution to allow for final concentrations of 0.1, 0.5, 1.0, 1.5 and 2 μM. Wells were also spiked with similar concentration of protoporphyrin IX as controls. 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. 50μL fresh media was added to each well and the selected wells were subjected to 30 seconds irradiation at 800 nm using a Leica confocal microscope equipped with a mode locked Ti:sapphire laser generating 100 fs 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 in a humidified CO_2 (5%) atmosphere. Cell viability was then determined by counting the number of viable cells per well using a microscope fitted with a counting grid. Results were reported relative to those from wells that were not exposed to drug or two-photon irradiation.

In vivo studies: All animals used in this study were treated as humanely and in accordance with licensed procedures under the UK Animals (Scientific Procedures) Act, 1986. For treatment of tumours the RIF-1 (radiation-induced fibrosarcoma) syngenic tumour model in C3H/HeN mice was employed as described previously.[18] Essentially RIF-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. Cells grown to 85% confluence were harvested by centrifugation following trypsinisation, washed in phosphate buffered saline (PBS) and finally suspended at a concentration of 1 x 10^7 cells/mL in PBS. 0.1 mL aliquots were injected intradermally into the flank of recipient 8 week old C3H/HeN mice and tumours appeared in animals within 3 weeks. Prior to treatment, animals were anaesthetised using Hypnorm/Hypnoval (1:1) administered via intraperitoneal injection. 50μL aliquots of the CQD-P preparation (30μM) were

administered by intratumoral injection. Where relevant animals were treated by exposure to the Ti:Sapphire source described above and animals were allowed to recover from anaesthesia following treatments. The effect of treatment was determined by measuring tumour volume and this was calculated using volume = $4/3(\pi R^3)$ where R was the average radius derived from the geometric mean diameter. Tumour volumes were employed to calculate the % tumour growth and this was calculated from the starting, pre-treatment volumes.

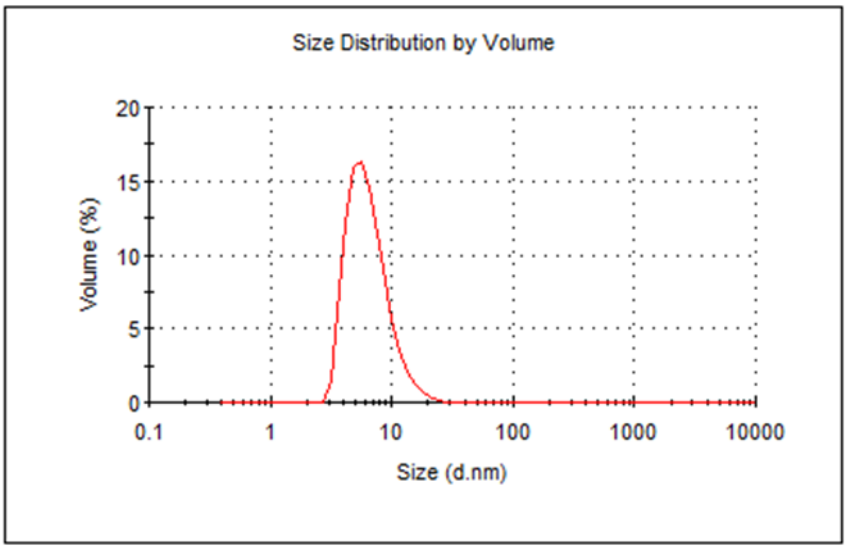

Figure S1 Dynamic Light Scattering plot for Carbon Quantum Dots recorded in water.

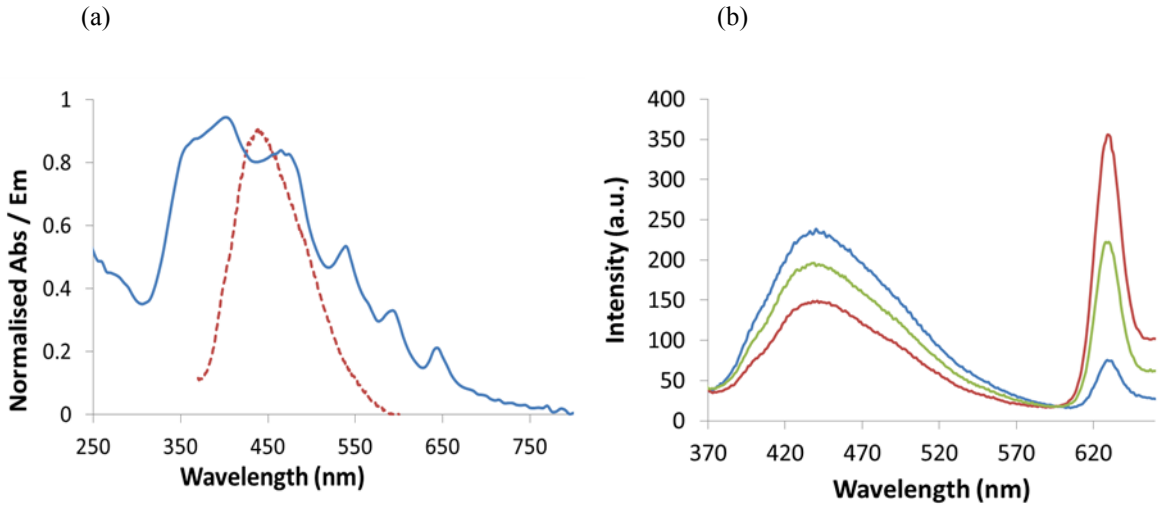

Figure S2. (a) Overlay of emission spectrum from CQDs (red line) and absorbance spectrum from Protoporphyrin IX (blue line) and (b) Emission spectra for **CQD-P** conjugate with increasing amounts of protoporphyrin IX attached: red line 30µM, green line 18µM and blue line 10µM protoporphyrin IX. λ_{exc} = 365 nm.

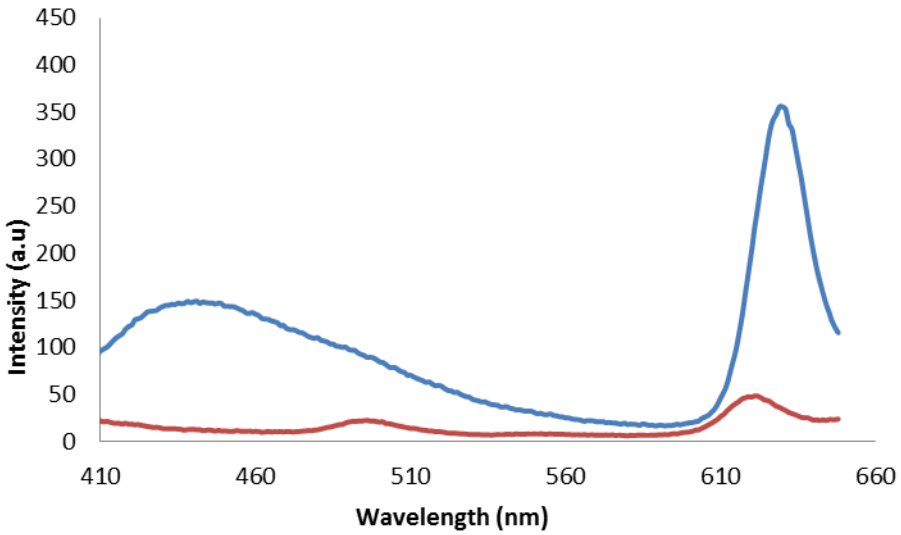

Figure S3 Emission spectra for Protoporphyrin IX alone and **CQD-P** when excited at 365 nm. Both samples contain the same concentration of Protoporphyrin IX = 18 μM

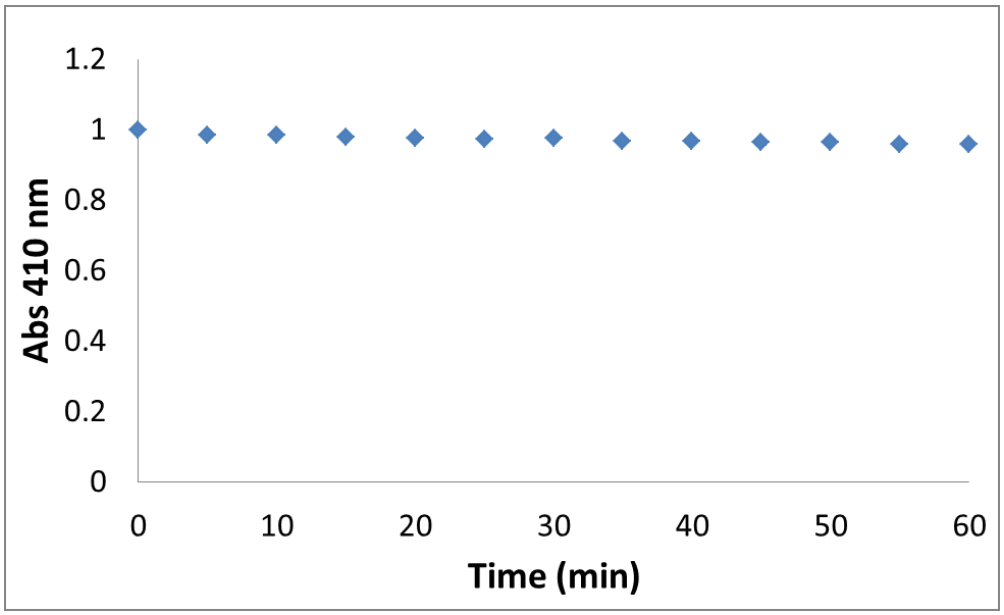

Figure S4 Plot of relative intensity of DPBF at 410 nm against time for CQDs alone.

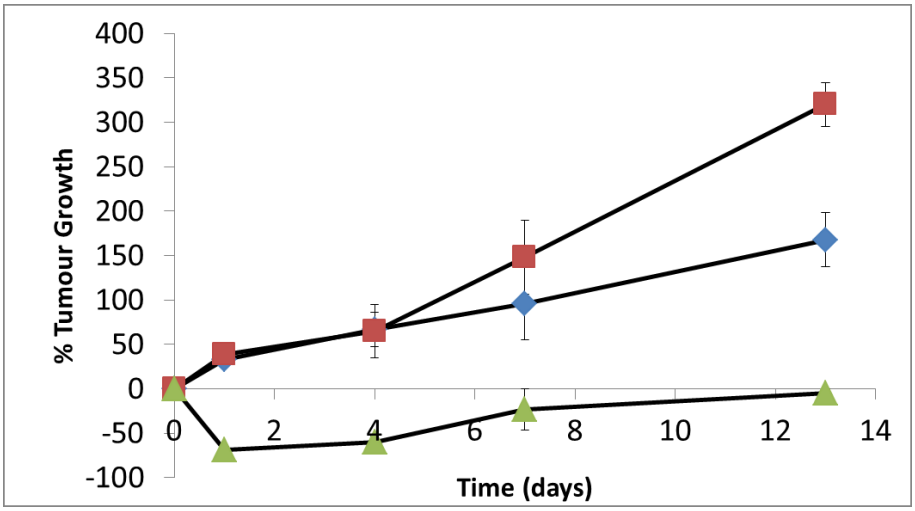

Figure S5 Plot of % tumour growth against time for mice treated with (i) two-photon light only (red squares) (ii) **CQD-P** only (blue triangles) and (iii) **CQD-P** + two-photon light (green triangles).