Electronic supplementary information

Two photon excitable graphene quantum dots for structured illumination microscopy and imaging application: lysosome specificity and tissue-dependent imaging

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Materials

Neem roots were bought from the local market of Bhavnagar, India. DMEM culture media with L-glucose, Sodium bi carbonate, Phosphate Buffer Saline (PBS), Fetal Bovine Serum, Penicillin Streptomycin, 4% Paraformaldehyde (PFA), Vectashield h1000 (Mounting agent), 50 mM Ammonium chloride and Lyso Tracker Deep Red were purchased from Sigma-aldrich. 26 mm X 76 mm Microscopy glass slides and 22 mm X 22 mm (170 ± 5) µm square Cover glasses were purchased from Thor labs.

Experimental Section

Synthesis of GQDs

Neem leaf extract (1 g), obtained by grinding the Neem roots into a fine powder using a commercial mixer grinder, were boiled in Milli-Q water at 80 ^{o}C for 1 hour, and followed by centrifugation at 12000g for 20 min to remove any residual solids. The supernatant liquid was filtered through a 0.22 mm membrane for further removal of any trace solid residues present in the solution. After that this solution was used for hydrothermal reaction. The hydrothermal reaction was performed in a stainless steel autoclave at a temperature of 350 ^{o}C for 12h. The obtained GQDs were a mixture of a transparent brown suspension and black precipitates. The precipitate was removed by centrifugation at 30000g for 30 min and the supernatant was collected and washed for three times with Milli-Q water. The GQD solution was then dialysed using a filter (cutoff 4.0 kDa) for 12 h and dried in lyophilizer.

Quantum Yield calculation equation

The quantum yields were calculated using the following equation when both sample (GQDs) and reference were excited at a point where they have the same absorbance. Here Φ stands for

quantum yield, η for refractive index, I for integrated fluorescence intensity for sample and reference.

$$\Phi_s = \Phi_{ref} \times \left(\frac{\eta_s^2}{\eta_{ref}^2}\right) \times \left(\frac{I_s}{I_{ref}}\right)$$

Powder X-ray diffraction (XRD): Powder X-ray diffraction patterns were collected in the ange of 5°–50° with a Philips X'pert X-ray powder diffractometer using Cu Kα (λ = 1.54178 ´°A) radiation.

Dynamic light scattering (DLS)

The average size and size distribution of the nanocarriers were measured at 25°C by DLS using a Brookhaven instruments Zetapals.

Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectroscopic measurements were carried out using a PerkinElmer GX spectrophotometer. The spectra were recorded in the range 400–4000 cm^{-1} in KBr media.

Raman Spectroscopy

Raman spectra were recorded using a Horiba LABRAM HR excited by a 514-nm laser.

Transmission electron microscopy (TEM)

Transmission electronic microscope (TEM) images were collected using a JEOL JEM 2100 microscope operated at 200 kV. The morphology of GQDs were studied by placing a dilute sample on the TEM grids (lacey carbon formvar coated Cu grids (300 mesh)) using transmission electron microscopy. No additional staining was used.

Up conversion fluorescence measurement

UC fluorescence spectra of GQDs were measured by LS-55 fluorescence spectrophotometer (Make: PerkinElmer Co., USA) with an external 980 nm laser (as a light source) instead of the internal excitation source. The maximum power of the laser was 120 mW.

Tissue Culture Experiments

RAW cells were seeded on Cover slips (22 mm X 22 mm, 170 ± 5 μm square Cover glasses) and placed in six-well plates with DMEM culture medium containing (10% FBS and 1% Penicillin Streptomycin) for 24 hours at 37°C, 5% CO2. After 24 hours when 70% confluency was achieved the cells were washed with DMEM culture medium. After that, the cells were then washed three times with culture medium. Next the cells were washed two times with phosphate buffer saline. After carrying out the live cell uptake of the GQDs of diverse concentrations (400 nM to 1 μM) for 12 hours, the cells were washed with DMEM media, and then the cells were fixed with 4% PFA for 15 minutes and washed three times with PBS buffer and then the coverslips were mounted using mounting medium (Vectashield h-1000). The coverslips were then sealed using nail varnish before imaging.

Cytotoxicity study of GQDs by MTT assay

Cytotoxicity of GQDs on human RAW cells was determined by traditional MTT assay. The cells were seeded at a density of 10^5 /well plated in 96-well plates. Cells were typically grown to 60–70% confluence, rinsed in phosphate-buffered saline (PBS), and placed into serum-free medium overnight prior to treatments. After overnight incubation, the cells were treated with GQDs at concentration of 10, 25, 50, 75 and 100 μg/ml. After 24 h, the medium was removed, and 100 μL of fresh medium was added along with 10 μL of MTT (5 mg/mL in PBS) and incubation was continued for 4 h at 37 °C. Afterwards MTT solution was removed, and the purple crystals were solubilized in 1.0 mL of DMSO. The absorbance was recorded at

wavelength of 550 nm in Elisa Plate Reader. Cell viability was quantified by MTT assay where the viable cells were determined by the reduction of the yellow MTT into purple formazan product by mitochondrial dehydrogenase present in metabolically active cells. The experiment was done in triplicate.

Microscopy experiments

Deconvolution Wide field Microscopy: To remove the out of focus blur from stacks of acquired Z-Stack images, deconvolution of raw widefield images obtained using the OMX-SIM (Conventional Wide Field Microscopy mode) was carried out using the Soft Worx software.

Cellular uptake of GQDs (Single colour Imaging)

Single colour widefield experiments of GQDs were performed for diverse concentrations from 400ng to 1 μg. The uptake was carried out over 12 hours. They were excited at 488 nm and emission was collected at 500 to 550 nm. The widefield microscopy conditions maintained were; Z stack thickness (Sections 40 to 80), section spacing (0.250 to 0.500), and thickness of the sample (10to 12). Exposure times were between 29 to 30 and the %T was in the range of 40 to 60.

Two Photon Microscopy

Two photon microscopy experiments for GQDs were carried out using the Zeiss LSM 510Meta confocal microscope. Live cell uptake was carried out over 12 hours. The GQDs were excited at 975 nm and emission collected between 500 to 550 nm. Two photon images were acquired for GQDs (400 nM and 1 μM) cellular uptake on to RAW cells. The data processing was again carried out using FIJI and LSM software.

Colocalization Experiments (Multi colour imaging)

GQDs and Lyso and Mito Tracker Deep Red and Hoechst Colocalization experiments were performed using Lyso Tracker Deep Red or Mito Tracker Deep Red in the particular experiments. For colocalization experiments, RAW cells were incubated with GQDs (50 μg) for 12 hours and then the cells were incubated with Lyso or Mito Tracker Deep Red (750 nM) for 20 minutes. The cells were washed regularly three times with DMEM culture media and PBS (three times), fixed with 4% PFA and mounted. The GQDs were excited at 488 nm and the emission was collected in the FITC Channel (500 to 550 nm) and the Lyso or Mito Tracker Deep Red or Hoechst was excited at 644 nm and emission was collected in the Alexa Fluor 647 Channel (> 650 nm).

SIM Microscopy - Single colour, Colocalisation and Dual colour experiments

Structured illumination microscopy (SIM) carried out by using the Delta Vision OMX-SIM. The Z stacks acquired during the imaging were post-processed by using the reconstruction option of Soft Worx. For single colour experiments, the GQDs were excited at 488 nm and the emission was collected at FITC Channel (500 to 550 nm). The Structured Illumination (SI) experimental condition employed for running the SI experiment for single colour Experiments were mainly dependent on the thickness of the Z stack (Sections 85 to 100), section spacing (0.150 to 0.250), thickness of the sample (10 to 12). The dual colour experiments were performed in combination with Hoechst.

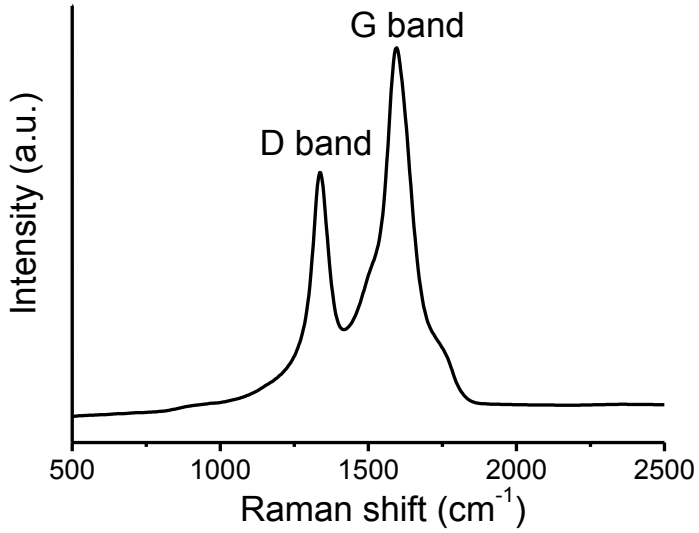

Fig. S1 Raman spectrum of GQDs. The well-known features of carbon materials in Raman spectra are the G band (~1595cm^{-1}), which is usually assigned to the E_{2g} phonon of sp^2 bonds of carbon atoms, and the D band (~1340 cm^{-1}) as a breathing mode of k-point phonons of A_{1g} symmetry, which is ascribed to local defects and disorders, particularly the defects.

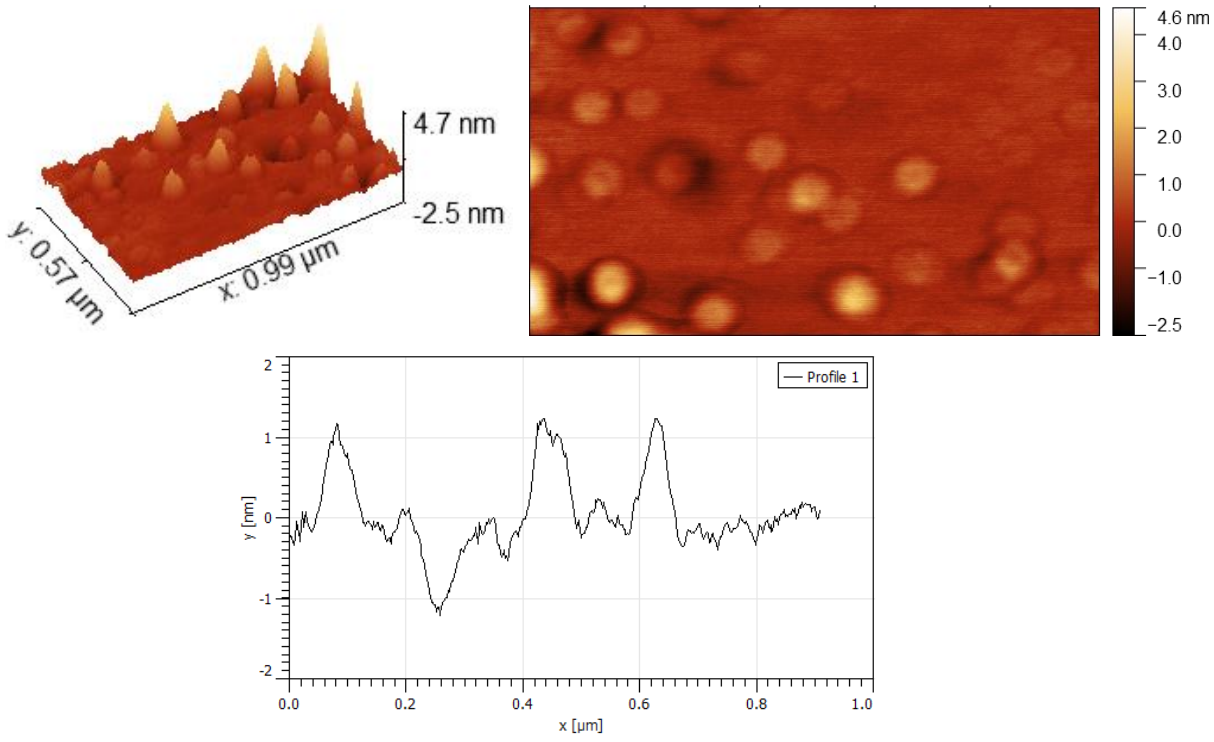

Fig. S2 AFM images of GQDs (top) and its height profile (below).

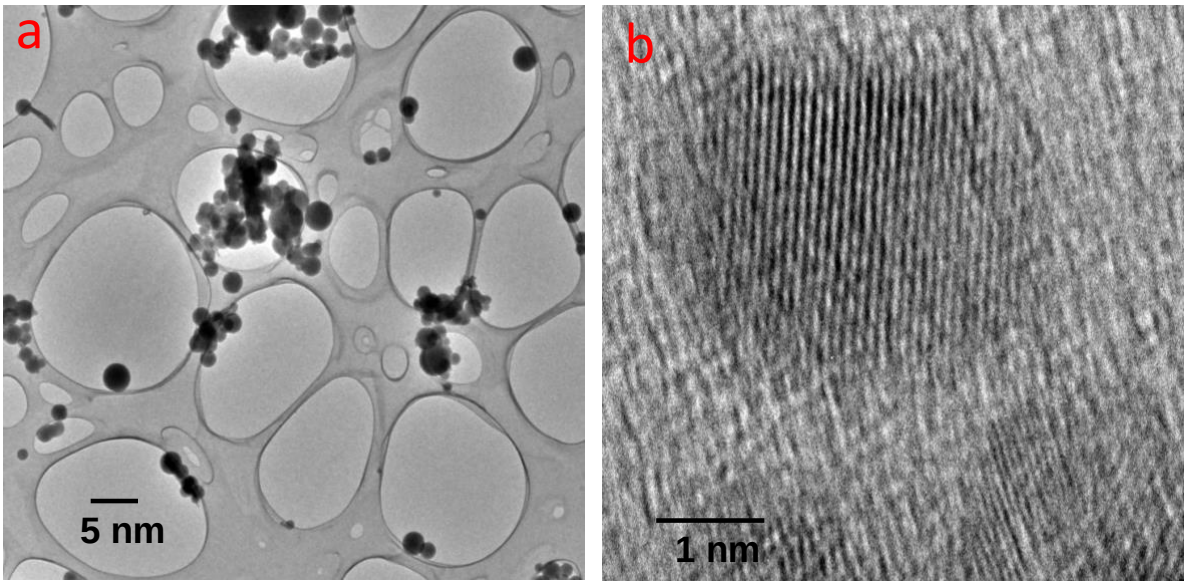

Fig. S3 (a) TEM image of GQDs and (b) HRTEM image of GQDs.

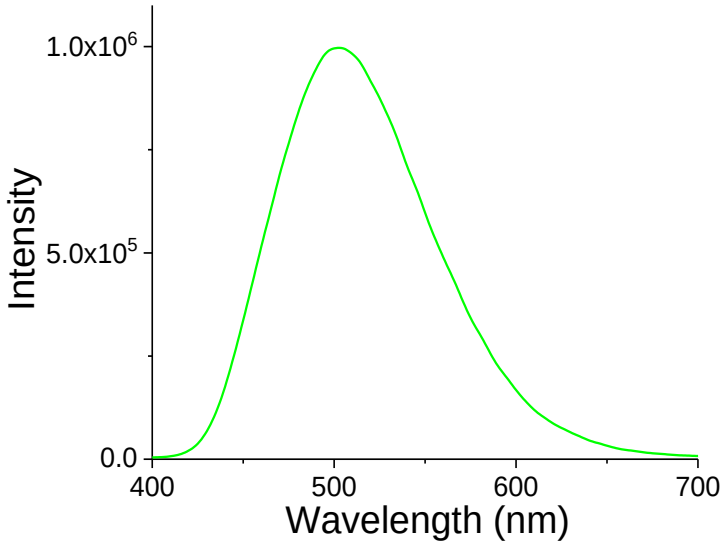

Fig. S4 Two-photon photoluminescence (excitation wavelength = 980 nm) of GQDs aqueous solution with different excitation power.

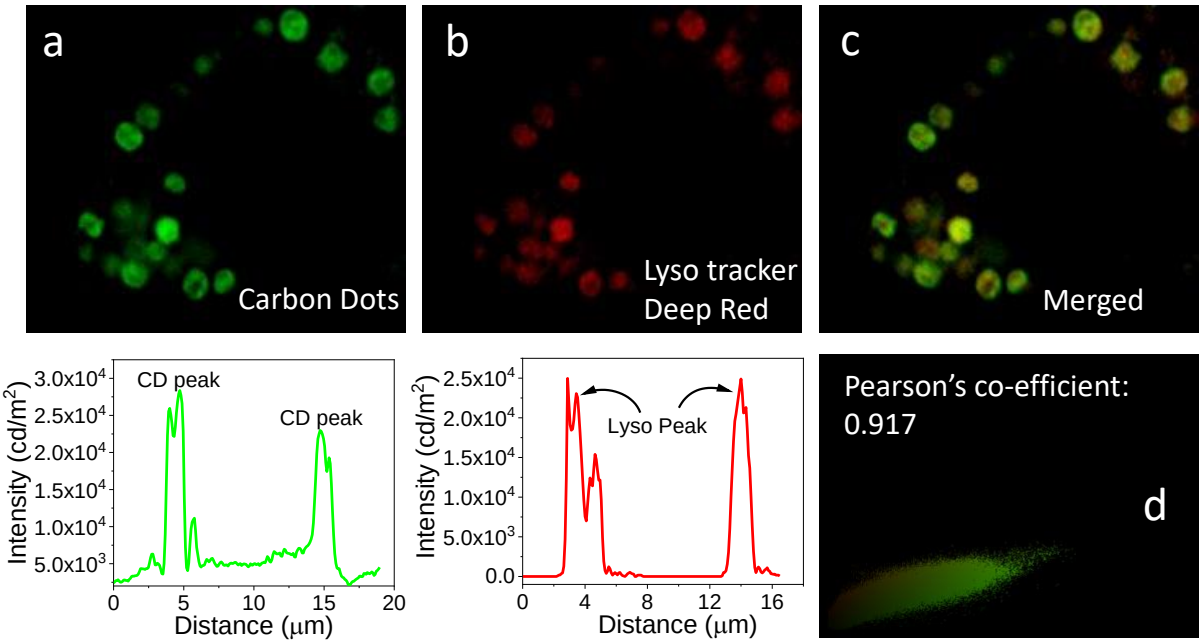

Fig. S5 Colocalization experiments of intracellular localization of GQDs using Lyso Tracker probes in MCF 7 cell line: Wide-field microscopy images of in cellular emission of GQDs (panel a) with intensity along the traced line shown underneath. Emission from Lyso Tracker Deep Red (panel b) and intensity along the same line shown below. Panel C shows the overlap of green and red fluorescence, indicating lysosomal localization of GQDs. Panel d shows the Pearson coefficient = 0.917.

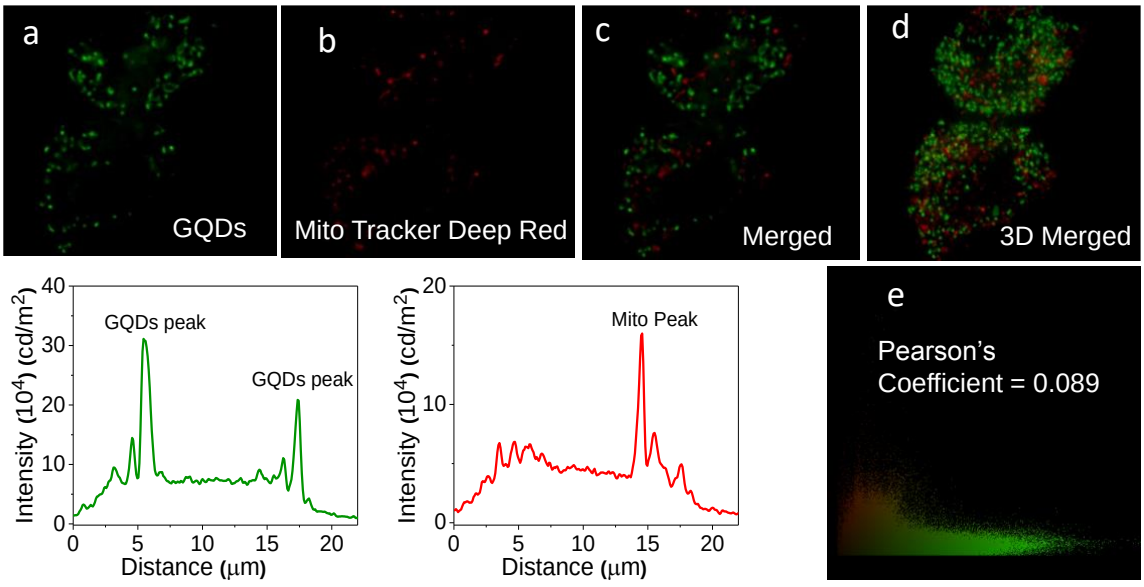

Fig. S6 Colocalization experiments of Intracellular localization of GQDs using Mito Traker Deep Red probes: Wide field microscopy images of in cellulo emission of GQDs (Panel a) with intensity along traced line shown underneath. Emission from Mito Traker Deep Red (Panel b) and intensity along the same line shown below. The overlap of the intensity is shown in Panel c. Panel c shows no overlap of the green and red fluorescence, indicates that the GQDs are not localised over mitochondria. Panel d is the 3D view. Panel e shows the Pearson co-efficient = 0.089, also supports that.

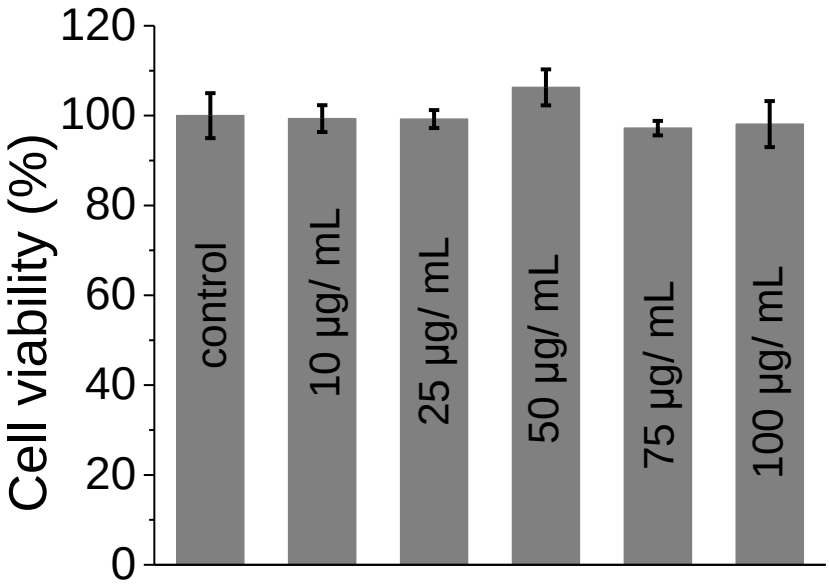

Fig. S7 Cell viability of RAW cells: Cells after 24 h exposure to a concentration range of GQDs, determined using the MTT assay. Data represent mean ± standard deviation of three replicates. The cell viability levels remained stable as compared to a control group, no decrease below 97 % was observed after exposure (24 h) to different concentrations of GQDs. The concentrations used are particularly higher than the range used in medical applications such as drug delivery or bio imaging, encouraging that these type of nano-carrier composition are usable for biomedical applications. This confirms that the synthesized GQDs are biocompatible.