Supporting Information

Towards Understanding High Quantum Yield Carbon Dots: From Molecular Fluorophores to Spherical Organic Nanocrystals

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EXPERIMENTAL SECTION

Materials: All Glass wares were washed with aqua regia (3 HCl: 1 HNO₃), followed by rinsing several times with double distilled water. Citric acid anhydrous, TRIS (tris(hydroxymethyl)aminomethane), L-cysteine, were purchased from Sigma Aldrich. Double distilled 18.3 mΩ deionized (DI) water (Elga Purelab Ultra) was used throughout for the preparation of solutions.

Synthesis of TPDCA and OPCA: For synthesis of TPDCA, 0.025 mol powders mixtures of each citric acid anhydrous and L-cysteine were mixed in a beaker and heated up to 150 °C in an oven for 2 hours. The resulting orange colored solid obtained after 2 hours was dissolved in 25ml of cold deionized water and suctioned filtered carefully, filtrate was collected. The TPDCA crystals were obtained by heating the filtrate up to 90°C and there after keeping for crystallization at room temperature for 24 hours. The obtained TPDCA crystals were washed several times with cold water to remove any impurity before further analysis. For the synthesis of OPCA, equal amount of Tris(hydroxymethyl)aminomethane (TRIS) was used instead of L-cysteine and similar procedure was followed.

UV-Vis Absorption spectroscopy: The UV-Vis absorption spectra were recorded using Shimadzu UV-Vis 2450 spectrophotometer. The spectra were collected using a quartz cuvette of 10 mm path length and volume 1 ml. All the measurements were repeated at least three times.

Transmission Electron Microscope (TEM): The particle size and dispersity of the sample were checked using a TECNAI G2 200 kV TEM (FEI, Electron Optics) electron microscope with 200 kV input voltage. TEM grids were prepared by placing 5 µL diluted and well sonicated sample solutions on a carbon coated copper grid and evaporated the solution at room temperature completely. Precautions were taken to avoid contamination from various sources like dust particles and glasswares. The lattice spacing was calculated by measuring the Fast Fourier Transform (FFT) image in the HRTEM mode and then inversing the FFT to the real space (lattice image).

Nuclear Magnetic Resonance (NMR): ¹H and ¹³C NMR spectra were recorded on Joel JNM ECX-500 FT-NMR spectrometer using D₂O as solvent and TMS as internal standard. Data are reported as follows: chemical shift in ppm (δ), multiplicity (s = singlet, d= doublet, br = broad singlet, m = multiplet), coupling constant J (Hz) integration, and interpretation.
**High Resolution Mass Spectroscopy (HRMS):** High Resolution Mass spectral data was recorded using Bruker Daltonik GmbH (Model – Impact HD, USA) instrument in positive mode by injecting 1mL of diluted sample in capillary at 3500 V. Active mode scanning of spectra has been recorded at 2000 V charging voltage from 250m/z to 1250m/z. (Nebulized pressure = 0.3Bar, dry gas = 4L/min at 200°C temperature dry heater).

**X-ray Diffraction (XRD):** Single crystal X-ray data were collected on Agilent Super Nova diffractometer, equipped with multilayer optics monochromatic dual source (Cu and Mo) and Eos CCD detector, using Mo-Kα (0.71073 Å) radiation at temperature 150 K. Data acquisition, data reduction and analytical face-index based absorption correction were performed using the program CrysAlisPRO.6 The structure was solved by Direct methods with ShelXS7 program and refined on F2 by full matrix least squares techniques with ShelXL7 program in Olex2 (v.1.2) program package.

**Fluorescence Correlation Spectroscopy:** Approx. 50µl sample solutions (~nanomolar concentrations) were drop-casted on cleaned glass coverslips and placed on an inverted confocal microscope (A1R+, Nikon, Japan) using a 1.4 NA oil-immersion 60X objective. A 488 nm gas laser was used to excite the sample at room temperature (25 °C) using appropriate dichroic and filters in the optical path. The emission beam was directed through a side-port of the microscope delivering it to a Hybrid Photomultiplier Detector Assembly single photon counting module (PicoQuant GmBh. Berlin, Germany). The fluorescence fluctuations were analyzed within a point region of interest (ROI) of the sample. The following general autocorrelation function (for n number of diffusion species) was used for fitting and calculating diffusion coefficients.

\[
G(t) = \left[ 1 + T \left( \frac{1}{\tau_{Tripl}} - 1 \right) \right] \prod_{i=1}^{n} \frac{\rho[i]}{1 + \frac{t}{\tau_{Diff}[i]}} \left( \frac{1}{1 + \frac{t}{\tau_{Diff}[i]}} \right)^{0.5}
\]  

… (1)

Where \( \tau_{Tripl} \) and \( \tau_{Diff} \) are average time of triplet blinking and diffusion through the confocal volume. \( T \) is triplet contribution and \( \rho[i] \) is the contribution of \( i^{th} \) species. Our study used the single species model with triplet contribution. The instrument parameter \( \kappa \) was calibrated with standard fluorophore (Atto488) with a known diffusion coefficient. All data were analyzed using the SymPhoTime 64 software (PicoQuant GmBh).
Steady state and Time Resolved Fluorescence Spectroscopy: Steady state fluorescence was measured using Horiba Fluorolog-3 Spectrofluorometer. All the experiments were performed at room temperature. The fluorescence was measured in 1 ml quartz cuvette. The fluorescence lifetime, time resolved emission spectra (TRES) and time resolved anisotropy decay were measured using Horiba scientific Delta Flex TCSPC system with Pulsed LED Sources. Ludox has been used as an IRF for de-convolution of the spectral value. The photon decays in different channels were fitted tri-exponentially with a chi-squared value <1.2 in order to calculate fluorescence lifetime. TRES was plotted by taking 50 slices of the 3D plot at 13 channel (~0.35ns) interval. Time resolved anisotropy decay was fitted with a single exponential function to calculate the rotational correlation time. Approximately 3 ml sample solution has been used for all measurements.

Quantum Yield Calculation: Quantum yield measurements were obtained with the help of Horiba Quanta-φ F-3029 integrating sphere mounted in the sample compartment of the Fluorolog-3 Horiba Jobin Yvon spectrofluorimeter. All the data were recorded at room temperature, from dilute solutions (~absorbance <0.1) of the C-dot sample keeping the excitation and emission slit width to be 1 nm each. The excitation wavelength was kept 340 nm. Data was processed by software supplied by Horiba-Joblin-Yvon, from which the quantum yield of the sample in solution, Φ, were calculated via the formula:

$$\Phi=100\% \times \left[ \frac{E_{\text{sample}}-E_{\text{solvent}}}{A} \right] \frac{R_{\text{solvent}}-R_{\text{sample}}}{R_{\text{solvent}}-R_{\text{sample}}}$$

Where $E_{\text{sample}}$ and $E_{\text{solvent}}$ are the areas under the emission spectra of the sample solution, and of the pure solvent, respectively. Likewise, $R_{\text{solvent}}$ and $R_{\text{sample}}$ are the areas under the reflectance spectra of the solvent, and of the sample solution, respectively. ‘A’ refers to area balance factor i.e. the ratio of the used integration time constant for emission and reflectance process. All areas were calculated by taking into account the sphere correction factors.
Figure S1: Reaction mechanism of (a) TPDCA and (b) OPCA formation.
Figure S2: Crystal structure of TPDCA as confirmed by single crystal XRD. (a) Asymmetric unit (b) unit cell structure of TPDCA.
Figure S3: (a) $^1$H NMR spectra (500 MHz, D$_2$O, $\delta$) of TPDCA [6.87-6.82 (d, 2H, CH), 5.57-5.56 (d, H, CHCOOH), 3.59-3.95 (m, 2H, CH$_2$)]. The protons of the carboxylates groups are dissociated (in D$_2$O) at neutral pH and therefore remain absent in the NMR spectra. (b) $^{13}$C NMR spectra TPDCA shows signature of nine different carbon atoms.
Figure S4: The HRMS spectra of (a) TPDCA and (b) OPCA shows their characteristic spectra both with a molecular weight of 241Da.

Figure S5: UV-Visible spectra of (a) TPDCA and (b) OPCA.
Figure S6: TRES spectra TPDCA at different pH. Spectral migration (Red shift) was observed at low pH (pH3-pH5) while the spectra remained unaltered with time at extreme pH..

Figure S7: TRES spectra OPCA at different pH. Spectral migration (Red shift) was observed at pH 2- pH 4, while no shift was observed at other extreme pH conditions.
Figure S8: FCS spectra and calculated diffusion coefficient of TPDCA at different pH.

Figure S9: FCS spectra and calculated diffusion coefficient of OPCA at different pH.
Figure S10: Time Resolved Anisotropy decay and calculated rotational correlation time of TPDCA at different pH.

Figure S11: Time Resolved Anisotropy decay and calculated rotational correlation time of OPCA at different pH.
Figure S12: Time Resolved Anisotropy decay and calculated rotational correlation time of (a) Human Serum Albumin (HSA) and (b) Tryptophan

Figure S13: Aggregation of OPCA molecule. (a) Nanocrystals formed on the TEM grid during drop drying process looks similar to carbon nanoparticles. The nanocrystals shows crystallinity in HRTEM with a lattice spacing ($d_{h,k,l}$) of 0.25nm.
Figure S14: Transmission electron microscopic image of (a) methylene succinic acid and (b) maleic acid nanocrystals. A concentration of 10mg/ml was used for drop-casting on the carbon coated copper grid. Nanocrystals with spherical morphology, narrow particle size distribution (3-6nm) and a crystal lattice with 0.21nm / 0.23nm d_{h,k,l} spacing (which is commonly attributed to graphitic carbon) were observed.
Figure S15: Excitation dependent to excitation independent emission of (a,b) TPDCA and (c,d) OPCA with pH change. Higher excitation dependence is observed at lower/acidic pH in both.