Supporting Information for

NIR Emissive Carbon Nanodots as a Tool to Mark Ribosomal RNA and Components using Super-Resolution Microscopy

Rohan Lamba,[1,§] Abdul Salam,[1,§] Farhan Anjum,[2] Aditya Yadav,[1] Richa Garg,[1] Kush Kaushik,[1] Shagun Sharma,[1] and Chayan Kanti Nandi[1,]*

[1]School of Chemical Sciences, Indian Institute of Technology Mandi, H.P.-175075, India

[2]School of Biological Sciences and Bioengineering, Indian Institute of Technology Mandi, H.P.-175075, India

[§]Rohan Lamba and Abdul Salam equally contributed to the work

Experimental Section

Materials

All glasswares were washed with aqua regia, followed by rinsing several times with double-distilled water. 1,8-Diaminonapthalene (Merck), Acetone HPLC (SD Fine) were used for synthesis. All chemicals were used without further purification. Double-distilled (18.2 MΩ) deionized water (ELGA PURELAB Ultra) was used in the entire process.

Characterization

UV-Visible and Steady-State Fluorescence

Shimadzu UV-Vis 2450 spectrophotometer was used to analyze the spectra of synthetic carbon dots in the UV-Vis using a quartz cuvette with a 10 mm path length to obtain the spectra. The Horiba Fluorolog-3 spectrofluorometer was used to detect the steady-state fluorescence.

Fourier Transform Infrared Spectroscopy (FTIR)

The popular technique for identifying the different molecular functional groups and surface-passivated nanoparticles is infrared spectroscopy. Using Resolutions Pro FTIR Software, we employed an Agilent Technology Cary 660 FTIR spectrophotometer with a 4 cm^{-1} resolution

(Model No. K8002AA Cary 660, USA). It makes use of Diamond ATR, which increases energy throughput by around 30% in contrast to other typical FTIR systems, enabling faster data collection with greater quality and less noise. The diamond ATR plate was covered with the sample chamber's lid after a little part of the dried CNDs sample had been spread on it. Using Resolutions Pro FTIR Software, the spectrum was obtained by eliminating the background spectra from the sample spectra.

Transmission Electron Microscope (TEM)

The transmission electron microscopy (TEM) technique involves guiding an electron beam through a very thin specimen as it interacts with it. Electrons passing through the material interact, creating a picture, which is then amplified and focused onto an imaging device. The most potent magnification at the nanoscale may be used by TEM to gather information on surface characteristics, shape, size, and structure. The particle size and dispersity of the synthesized nanoparticles were measured using a TECNAI G^2 200 kV TEM (FEI, Electron Optics) electron microscope with a 200 kV input voltage. A gold grid that had been coated with carbon to generate TEM grids was cast with 50 mL of the diluted solution. The nature of the substance is examined using the SAED Pattern.

Fluorescence lifetime

The fluorescence lifetime and time-resolved anisotropy decays were assessed using the Horiba scientific Delta Flex TCSPC system with Pulsed LED Sources. For spectral value deconvolution, Ludox has been employed as an IRF. By tri-exponentially fitting the photon decays in different channels with a chi-squared value < 1.2, the fluorescence lifetime was ascertained.

Fluorescence anisotropy measurements

Using a Horiba scientific Delta Flex TCSPC (Time-Correlated Single Photon Counting) system (Serial No. 11385, Scotland) and 24 pulsed 280, 390, 455, 560, and 647 nm LED sources, the fluorescence anisotropy was determined. The deconvolution of the spectral value's instrument response function (IRF) has been measured using Ludox. As the polarizer and analyzer, two polarisers were utilized. Using DAS6's fluorescence decay analysis software, measurement decay curves were fitted using the fitting program to determine fluorescence anisotropy. Impulse Fit and

Reconvulation Fit are the fitting models utilized for Fluorescence Anisotropy. Unless otherwise noted, all measurements in this thesis were taken using the Reconvulation Fit method. With the aid of Perrin's equation and the Reorientation or Rotational Correlation Time provided by the Fitting curve, the Hydrodynamic Radius of the Rotor may be computed.

$$\theta = \frac{\eta V}{k_b T}$$

Where θ = Rotational or Reorientation Time Constant/Rotational Correlation Time, η = Viscosity of solvent or dispersion medium, V= Volume of Rotor/Molecule (Hydrodynamic), Boltzmann Constant (k_b), and T is Absolute Temperature of the NP-Medium Temperature. Reconvulation Fitting was used in fitting up all the experimental data obtained from Fluorescence Anisotropy.

Raman Spectroscopy

Raman spectroscopy is a spectroscopic technique that is used to measure the vibrational, rotational, and low-frequency modes of a substance. The energy of the laser photons is changed higher or lower as a result of interactions between the laser light and the phonons, which are molecular vibrations, in the system. The energy shift reveals details about the system's vibrational modes. It was measured on a Confocal Raman microscope (Horiba Scientific). It operates with a 532 nm laser and has a minimum spectral range of 100 cm^{-1} to 3000 cm^{-1}. Scientific CCD with a 24-by-256-pixel sensor and air cooling was employed for the measurement. The sample was prepared for analysis by applying a drop of CNDs to the glass/quartz slide, drying the sample at 30 degrees, and then completing the analysis using LabSpec 6 software.

X-Ray Diffraction (XRD)

A powder X-ray diffractometer outfitted with a 9-kW rotating anode X-ray generator and a NaI Scintillation counter-detection device was used to conduct a powder X-ray experiment. The target substance was a copper anode, and the cathode was a precise focus filament. Before the XRD experiment, the substance had already dried and solidified.

High-Resolution Mass Spectrometry (HRMS)

By injecting 1 mL of diluted materials in a capillary at 3500 V, high-resolution mass spectrum data were obtained using a Bruker Daltonik GmbH (Model - Impact HD, USA) instrument in positive mode. At 2000 V charging voltage, spectra from 100 m/z to 1250 m/z were seen in active mode. The pressure for dry nebulization is 0.3 bars.

Zeta potential

A Malvern Zetasizer Nano system with a 633 nm He-Ne laser was used to assess zeta potential. A cuvette (Malvern-DTS1061, United Kingdom) was used to measure the sample over the course of ten runs, each lasting 10 seconds. The data consisted of the average of at least five measurements.

X-ray photoelectron spectroscopy (XPS)

The C60 sputter gun and Auger Electron Spectroscopy (AES) module PHI 5000 Versa Probe II, both manufactured by FEI Inc. USA, were used in this investigation to characterize and scan the spectra for the C1s, N1s, and O1s regions. CND samples have been made ready for XPS examination by either dusting the powder over the sticky carbon conductive tape's surface or crushing the powder into a tablet. Typical XPS samples can range in size from 0.5 to 1 cm^2 and thickness up to 4 mm.

Atomic force microscopy (AFM)

CNDs sample aqueous solutions were generally dried overnight on a silicon substrate. Samples were dried before being scanned and scrutinized on the AFM stage using ScanAsyst® image optimization software. The following relevant scanning options were available: Tip velocity is (4 m/s for 2 m, 15 m/s for 5 m, and 30 m/s for 10 m) for all experiments; resonance frequency (probe) is 60-80 kHz resolution: 1:1, 512 samples each line, 256 lines. The cantilever beam of the AFM probe has a diameter of less than 100 and a very sharp tip. The probe is equipped with a piezoelectric scanner tube that moves the probe across a selected area of the CND's surface. Interatomic forces operating between the probe tip and the CND's surface cause the cantilever to flex when the surface topology (or other properties) of the CND change. A laser light reflected from the rear of the cantilever is used to gauge its deflection. The computer receives this data and generates a topographical map or other fascinating features. To examine the created CNDs sample, the dialyzed sample was first mounted on the silicon wafer using a room-temperature drying

procedure. It was then placed on the specimen stage using a specialized holder. With a pointed tip, samples were placed in the object chamber's focus, observed, and then the surfaces were examined using the NanoScope Analysis v1.40r1 application.

TGA Analysis

Thermogravimetric analysis (TGA), a technique for thermal analysis, calculates a sample's mass as a function of time and temperature. For a material whose initial weight is known, the temperature is steadily increased, and at various time intervals, weight fluctuations as a function of temperature are recorded. A Perkin Elmer Pyris 1 instrument was used to perform the thermogravimetric analysis in a nitrogen environment. Each experiment involved heating the samples to 800 °C at a rate of 5 °C per minute while allowing them to flow at a rate of 20 mL per minute.

Cell culture and cell maintenance:

Hela and HEK 293t cells were cultured in DMEM media (media components are 10% FBS, 1% HEPES buffer, 1% anti-anti, 1% Penstrep, 1% Non-essential amino acids (NEA) in T 25-flask. Cells were allowed to grow at 37 °C, with 95% humidity in 5% CO_2 incubator. Before staining (with DAN CND, and Trackers) cells were seeded on coverslip with a seeding density of 5×10^4 and allowed to grow overnight for proper adherence and morphology acquirement.

Staining, slide preparation and colocalization:

Along with DAN CND cells were stained with different Cell organelle trackers, MitoTracker Green DND was used to visualize the mitochondria and to check the colocalization with DAN CND-stained organelles, MitoTracker Green was allowed to incubate for 30 minutes with concentration of 20 nM. Further ER-Tracker green was used to visualizes the endoplasmic reticulum and to check the colocalization. The concentration of ER was 100 nM and allowed to stain the cells for 30 minutes in fix and live cells both. The colocalization was also performed with Syto RNA Select^{TM} Green Fluorescent cell stain , for this 500 nM of concentration was given and incubated for 30 minutes in both fix and live cells condition. Live cell staining was performed in 35 mm confocal live cells plates, and to fix the cells, cells were washed with 1X PBS twice and

fixed in 4% paraformaldehyde for 10 minutes. Further cells were washed with 1X PBS twice and mounted on glass slides by placing mounting media (9:1 Glycerol: PBS).

RNA digestion experiment:

HeLa cells were first treated with the medium containing 30 μg/mL RNase solution (GE) at 37 °C for 2 h, respectively, then cells were rinsed with PBS three times before CND staining for 30 min. Afterward, the confocal images were acquired for untreated and RNase-treated cells (Ex: 640 nm).

Cell viability assay

Cell viability of HeLa cells against DAN CND was performed by XTT assay, first cells were maintained as per the National Centre for Cell Science (NCCS) Pune, Maharashtra India guidelines and seeded in 96 well plates with seeding density 7 x 10^3. After proper adherence of cells and confluency cells were exposed to DAN CND for 6 hrs. After DAN CND incubation XTT reagent along with an electron coupler was added to each well and incubated for 8 hours. The optical density of each well containing cellular media along with XTT reagent and electron coupler was taken using Tecan Infinite M200 PRO multi-plate reader. Optical density was collected at 570 nm with a reference read at 650 nm. The final absorbance values were analysed concerning control and blank samples to obtain the cell viability measures.

Confocal microscopy

Prior to confocal microscopy the cells were stained with CNDs for 30 minutes after achieving the appropriate adhesion and proliferation stages to get sufficient labelling density for confocal imaging. During the RNase and DNase digestion experiment, RNase and DNase enzymes were administered for 6 hours. The cells were ultimately fixed after 10 minutes by incubation with a 4% paraformaldehyde (solution in a 1X PBS buffer). The fixed cells were then washed in 1X PBS buffer twice to remove extra agents. The coverslips were mounted on a glass slide and edges were sealed before imaging. A Nikon Eclipse Ti inverted microscope was used for the confocal microscopy, and Nikon NIS-Element software was used to take photographs. The cell samples were stimulated by the four lasers, which had wavelengths of 401, 488, 561, and 639 nm. The photons might eventually be collected by selecting the appropriate filter set.

Single-particle time trace and photon counts

To gather single-molecule time traces, CNDs were spin-coated over a pre-cleaned glass cover slip (Piranha solution followed by multi step sonication and cleaning with DI water). A 50-mW maximum power 532 nm DPSS laser was employed. On a specially made inverted optical microscope, an oil immersion Nikon TIRF objective (100x magnification and 1.49 NA) was mounted. Utilizing a 560 nm long pass dichroic from AHF Analysentechnik, the excitation and emission light were separated. The emitted light was further filtered with 680/70 band pass filter before the detector. Single-particle photon events were recorded using an Andor EMCCD iXon Ultra 897U with a frame rate of 20 Hz (50 ms exposure time) with EM Gain 300 in kinetic photon counting mode. Pixel Read out rate of 17 MHz with frame transfer enabled was used for acquisition. 5000 frames (~250 s) of 128 x 128 pixels (20.48 µm x 20.48 µm) area was recorded and saved in '.fits' format using Andor Solis software. Custom-made ImageJ macro language scripts were used to identify the region of interests (ROI) containing bright spots. Intensity fluctuation in these ROIs were then measured for the duration of the whole acquisition. Thresholding was done manually over the background signal. Signal below threshold value is considered background and above it was considered as the fluorescence signal. These time traces were then analysed further for calculating total photon counts, number of cycles, and photon/cycles. Molecules whose ROIs were overlapping were discarded from the analysis.

Super resolution radial fluctuations (SRRF) microscopy

Same microscopy setup used for the single particle data acquisition (mentioned above) was used for the SRRF data acquisition also. We acquired a time series of 2000 images with 50 ms of exposure time for each picture to conduct SRRF analysis. Using an open-source NanoJ-SRRF ImageJ plugin and a NVIDIA GeForce 1050 Ti GPU. The best pictures were produced with the default configuration of NanoJ-SRRF plugin of ImageJ with a ring radius of 0.5 for the SRRF analysis was selected. The selected radial magnification was five times the original pixel size. Before doing the SRRF analysis, drift correction was performed using the NanoJ-Core ImageJ plugin. The obtained SRRF image is then background corrected. The area of the picture where there were no fluorescence structure throughout the measurement time served as the basis for calculating the background. Cellular RNA is super-resolved in the obtained SRRF pictures, and the line profiles are shown with Gaussian fitting. The FWHM is then determined and recorded for additional study. The Andor iXon Ultra 897 has pixels that are 16 µm x 16 µm, which, when

projected at a 100x magnification, may be read as 160 nm x 160 nm for image analysis. After five times magnification using SRRF, the effective pixel size decrease to 32 nm x 32 nm.

Figures

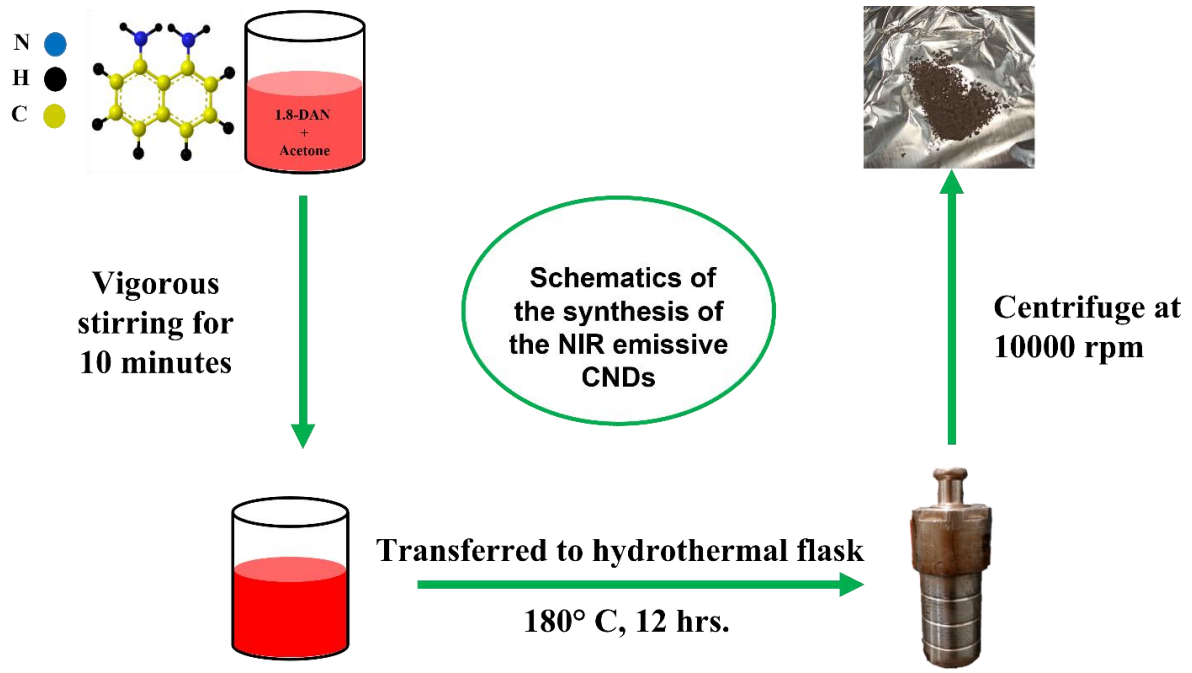

Figure S1: Synthesis steps of DAN-CNDs

Step 1: 180 mg DAN (1,8-Diaminonapthalene) was weighed in the dark and dissolved in 35 ml acetone. Then, add a small amount of acid (mixture of HCl and HNO_3) to maintain the pH at 2. Further, the mixture was shaken on a vibrator vortex for 5 minutes.

Step 2: Hydrothermal treatment to the above reaction mixture at 180 ºC for 12 hours, then after 12 hours, cool down the hydrothermal at room temperature.

Step 3: Centrifuge the crude sample for 20 minutes at 10000 rpm to collect a solid black sample that settles down during centrifugation.

Step 4: Wash the black solid several times with acetone to remove unreacted precursor, if any and dry it entirely at room temperature for further characterization in DMSO (Dimethyl sulfoxide).

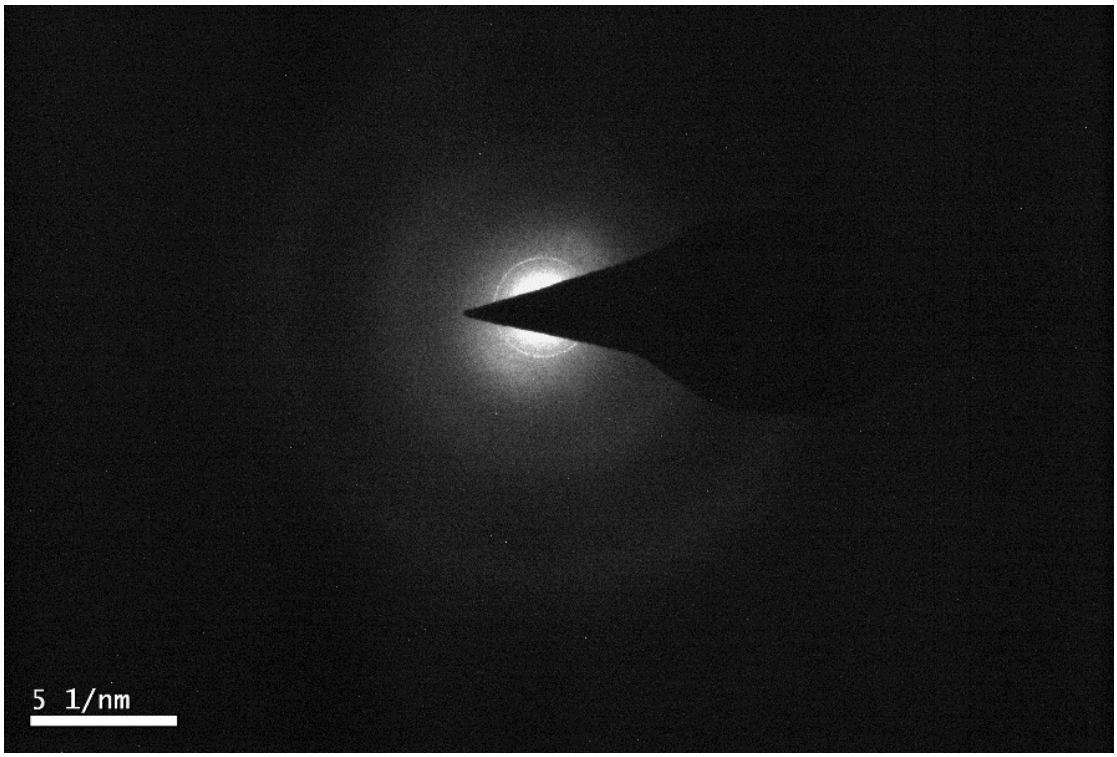

Figure S2: The selected area electron diffraction (SAED) pattern.

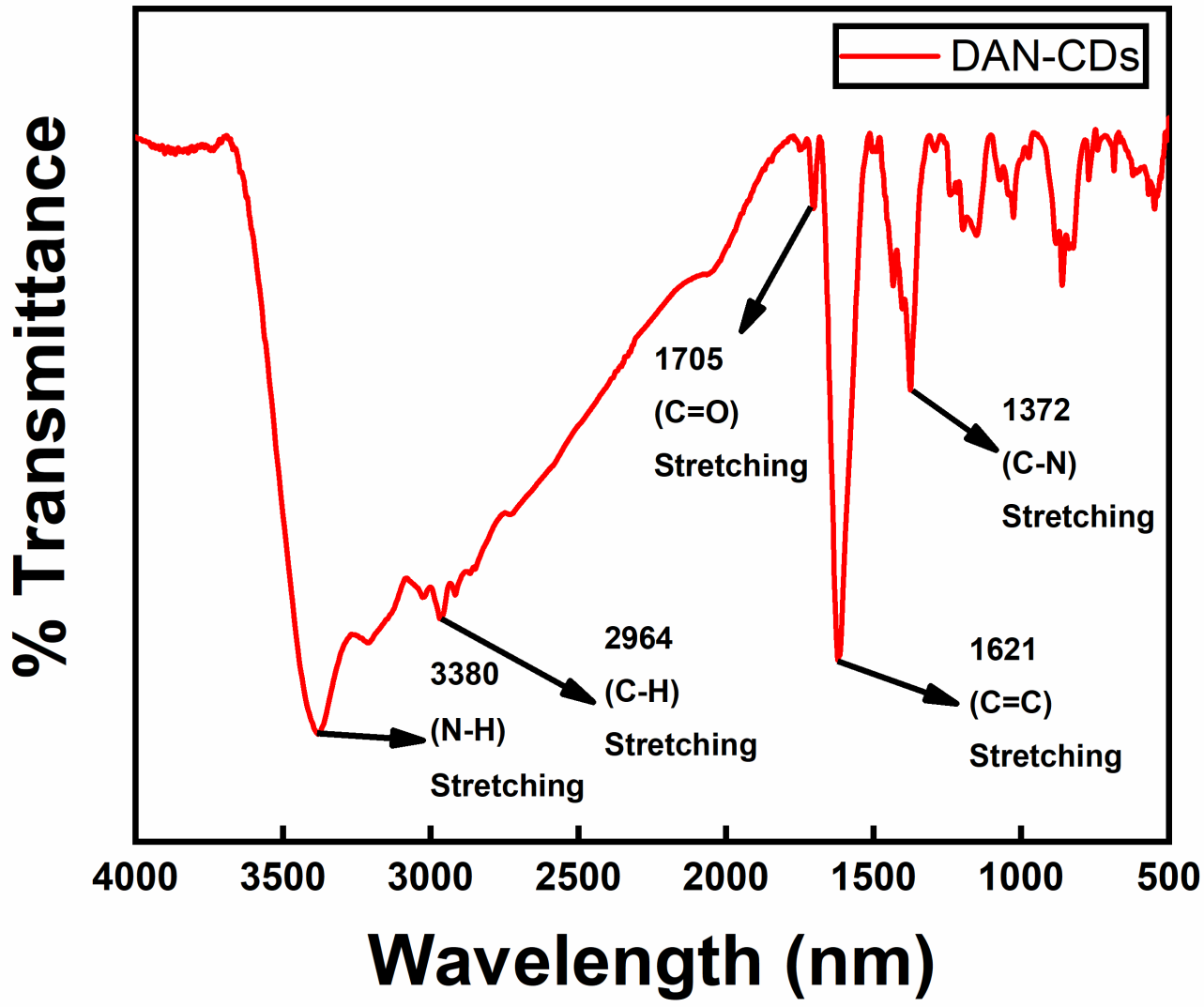

Figure S3: FT-IR spectra of DAN CNDs confirm the presence of C-H (2967 cm^{-1}), C=O (1705 cm^{-1}), C=C (1621 cm^{-1}), and N-H (3380 cm^{-1}) functional groups.

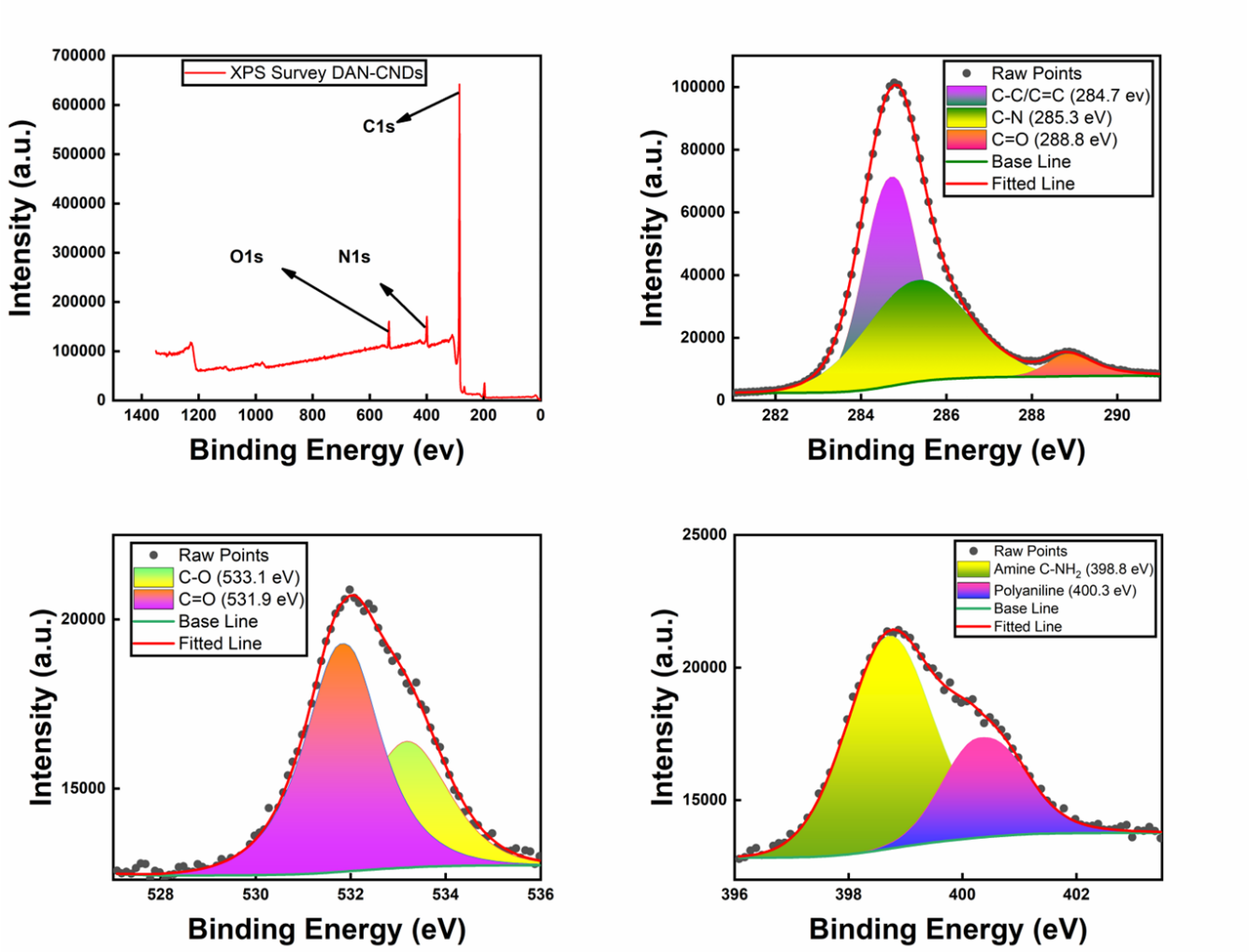

Figure S4: (a) The XPS data suggested the presence of C, N, and O in 88.95%, 7.01%, and 4.05, respectively. (b) Deconvolution of the C1s spectrum indicates three peaks for C-C/C=C, C-N, and C=O at binding energy values of 284.7, 285.3, and 288.8 eV. (c) Deconvoluted oxygen O1s spectrum with peaks at 533.1 and 531.9 eV corresponds to C-O and C=O, respectively. (d) Deconvolution of the N1s spectrum exhibited two peaks for amine ($C-NH_2$) and Polyaniline at binding energy values of 398.8, and 400.3 eV.

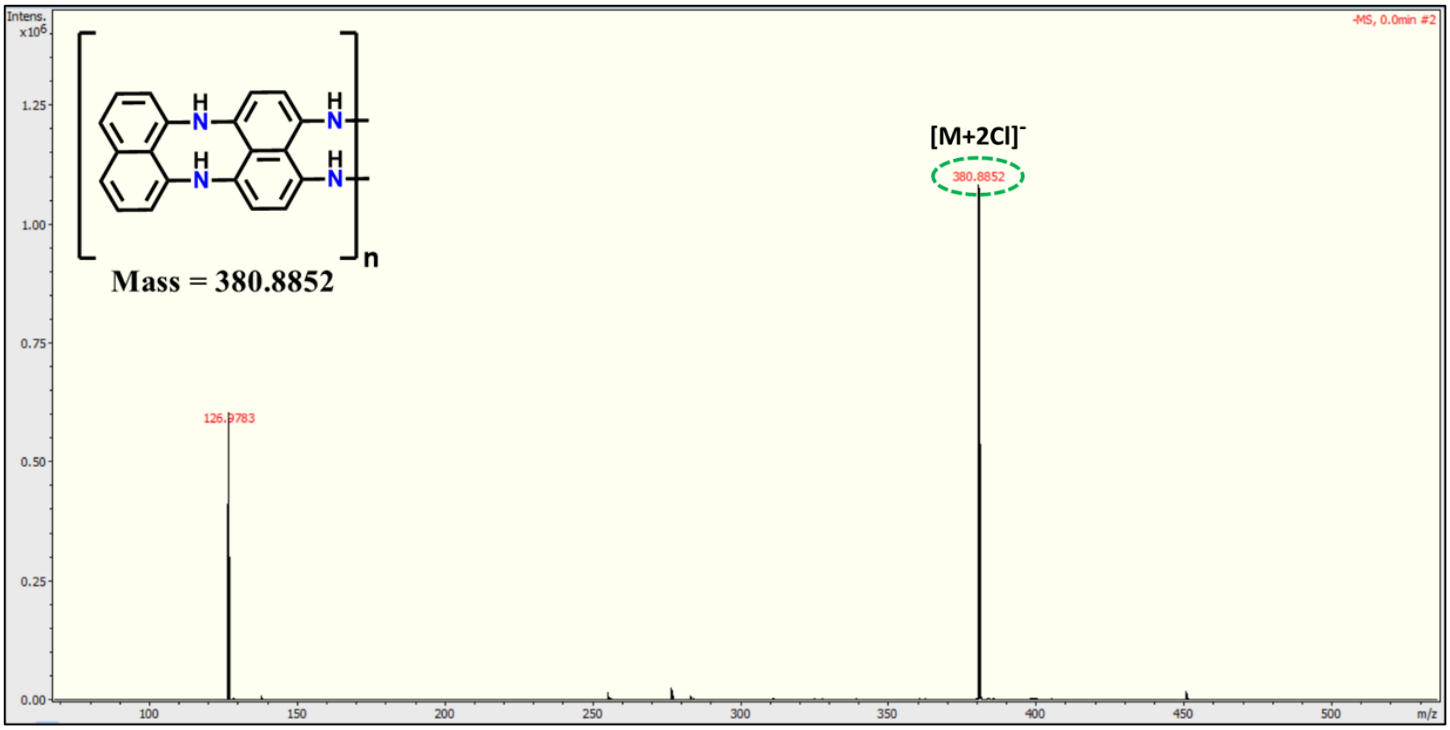

Figure S5: High resolution mass spectrometry (HRMS) confirms the presence of polyaniline unit at the surface with m/z value at 380.8852.

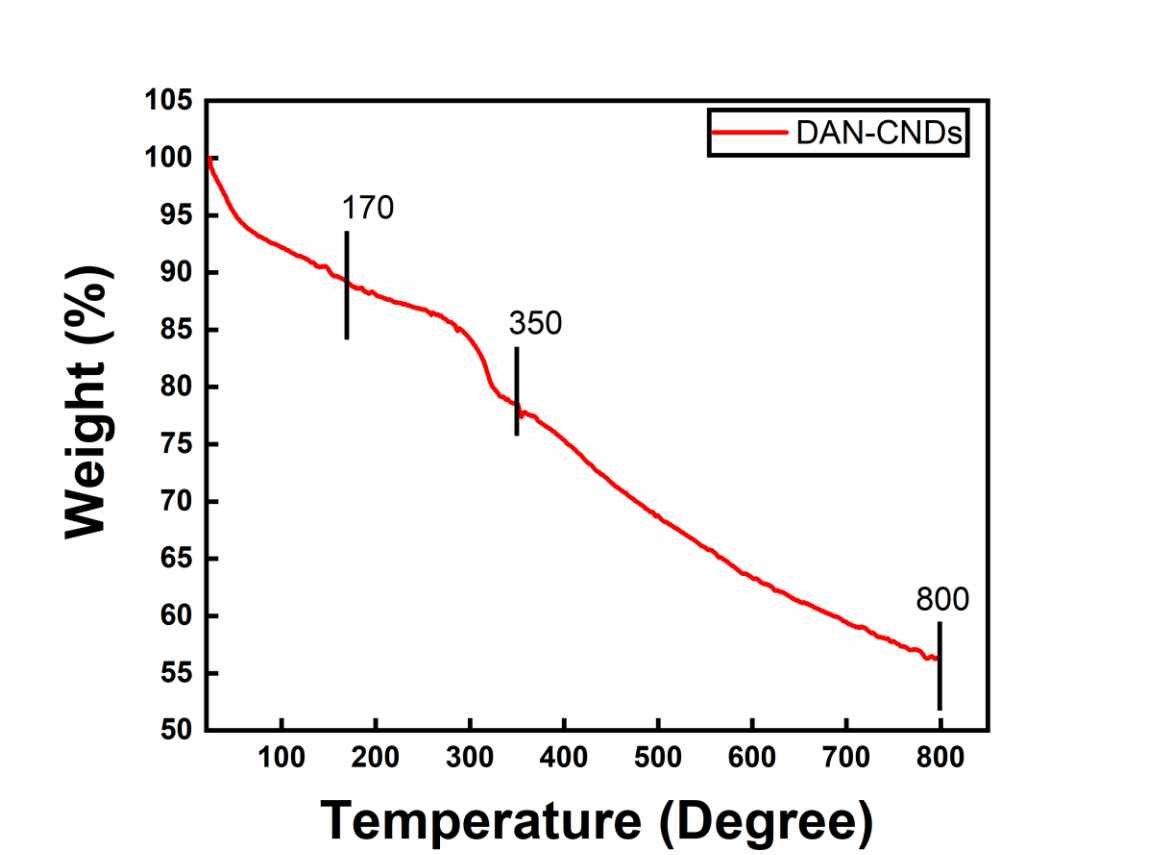

Figure S6: The TGA data shows a significant mass loss (25%) within 350 °C, indicating the presence of less thermally stable volatile organic compounds as surface functional groups. The thermally more stable CNDs like structure core structure could be interpreted with only 20% mass loss from 350-800 °C.

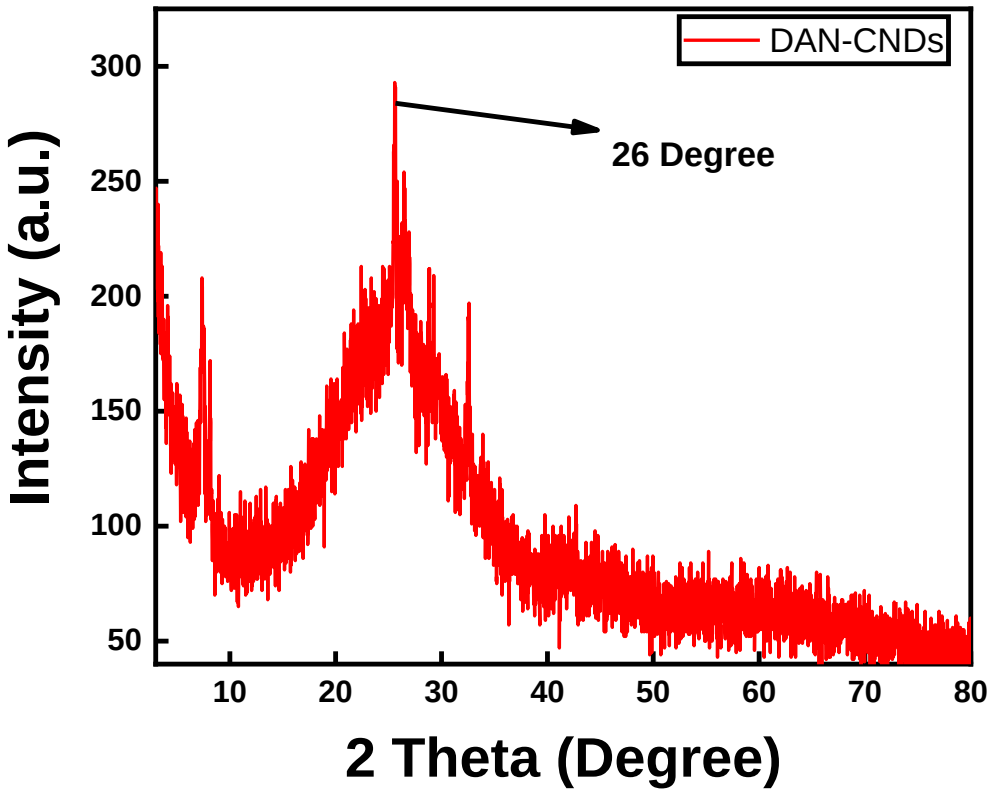

Figure S7: Powder X-ray diffraction (PXRD) of DAN CNDs shows a broad peak at ~26 degrees, suggesting the representation of 002 planes of graphitic carbon.

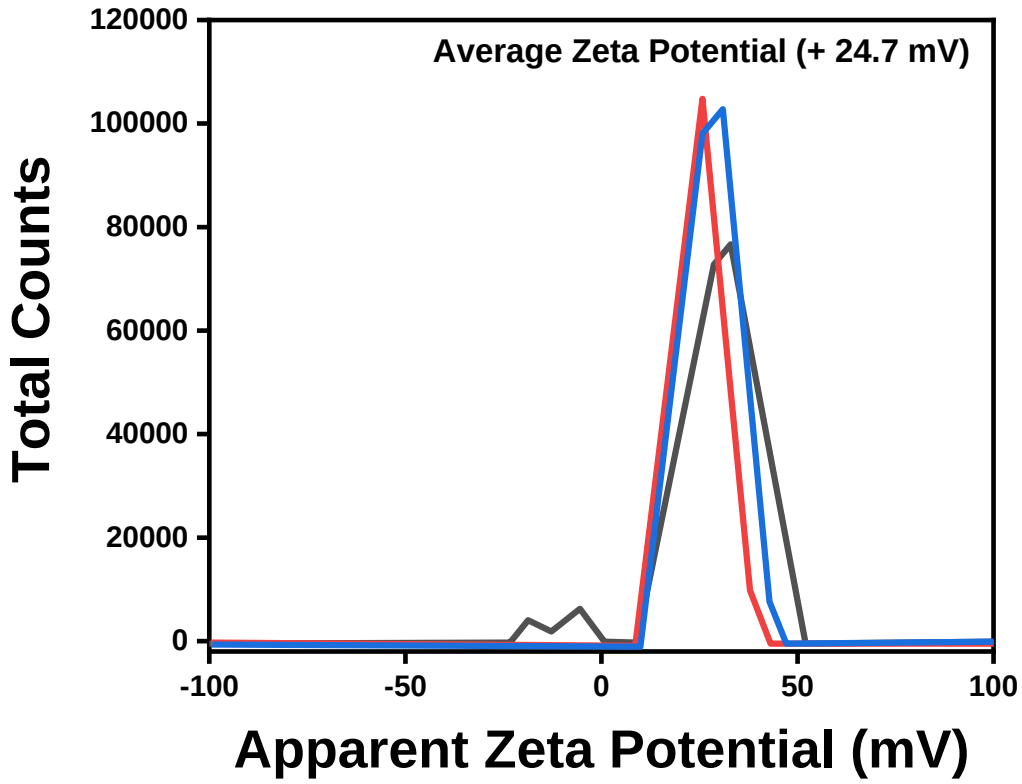

Figure S8: The average Zeta Potential of DAN CNDs was +24.7 mV, supporting the large number of -NH_2 as surface functional groups.

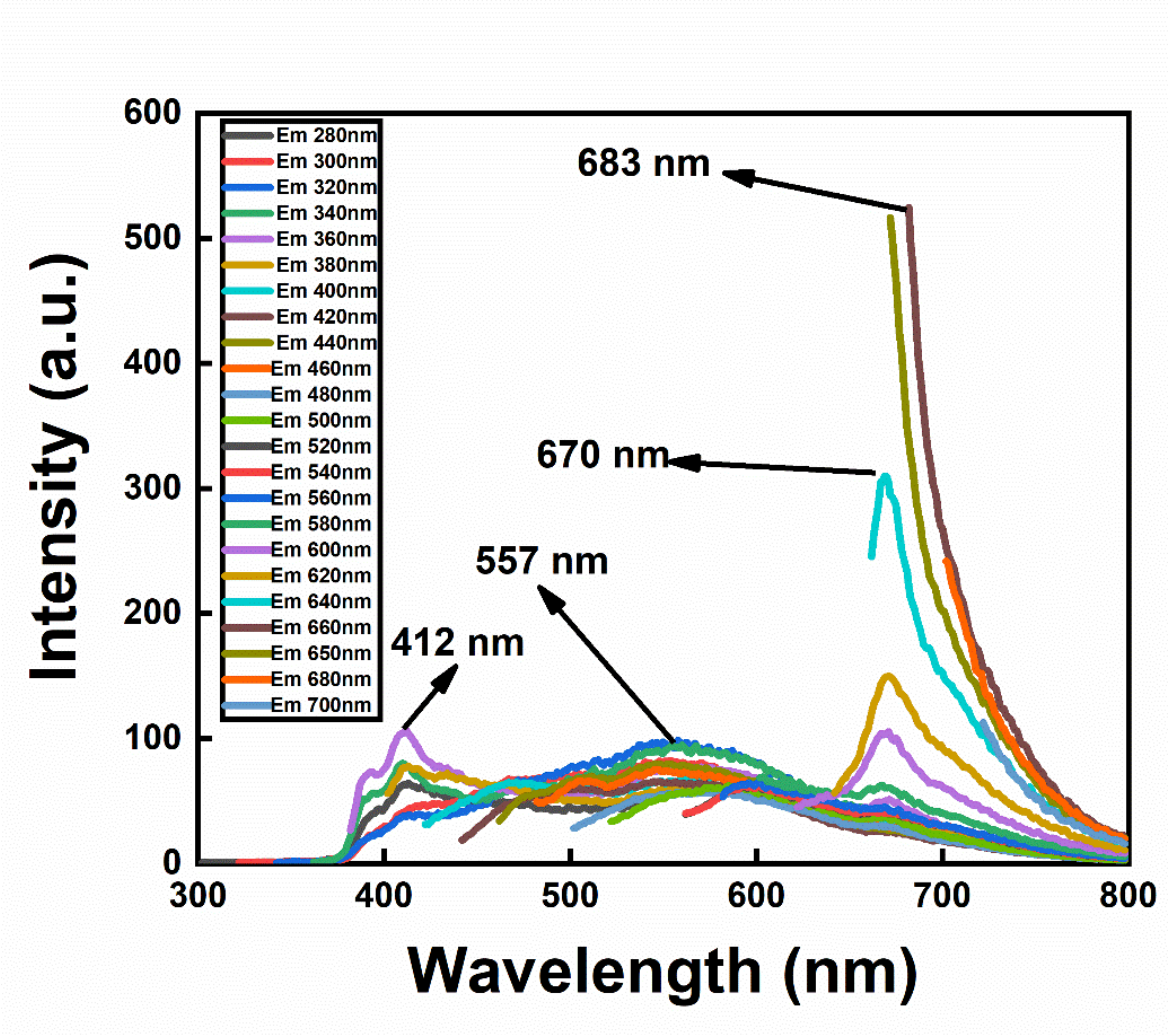

Figure S9: Excitation wavelength independent emission spectra of DAN CNDs.

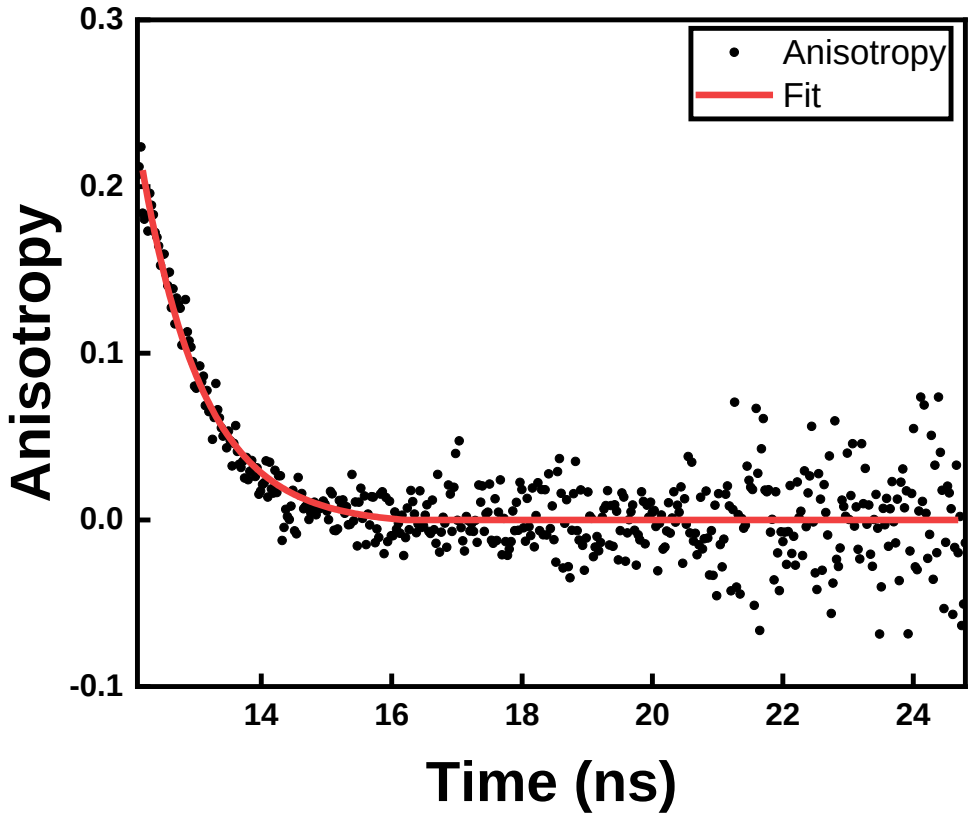

Figure S10: Fluorescence anisotropy decay for 670 nm emission yielded τ_{rot} = 0.9 ns. Such a low value of τ_{rot} indicates that the fluorescence anisotropy loss is not due to the rotation of the CNDs particles as a whole but rather due to the local reorientation motion of the fluorophore units at the DAN-CNDs surface.

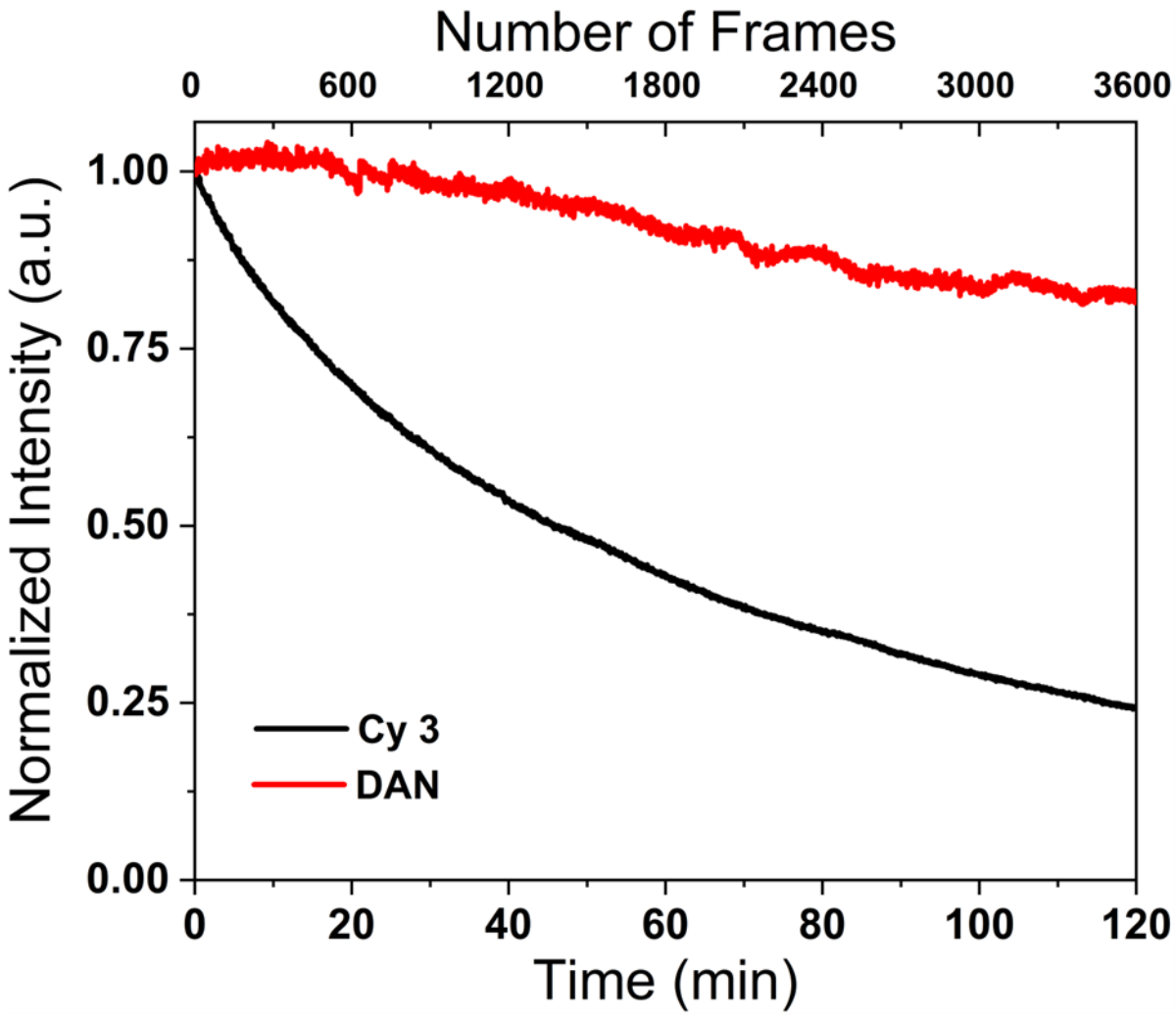

Figure S11: Time-dependent photostability experiment of DAN CND (Red) compared to commercial dye Cy3 (Black) with a 532 nm laser of 12.54 W cm^{-2} power density.

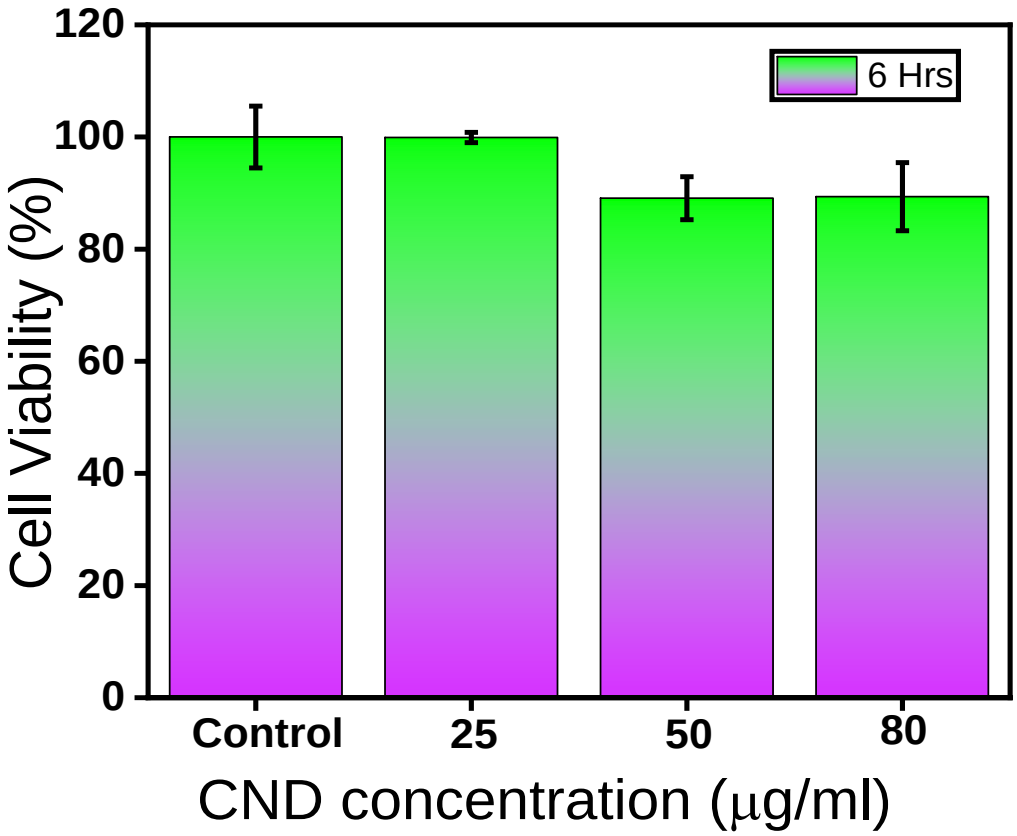

Figure S12: The cell viability of DAN CNDs calculated for 6 hours at various concentrations.

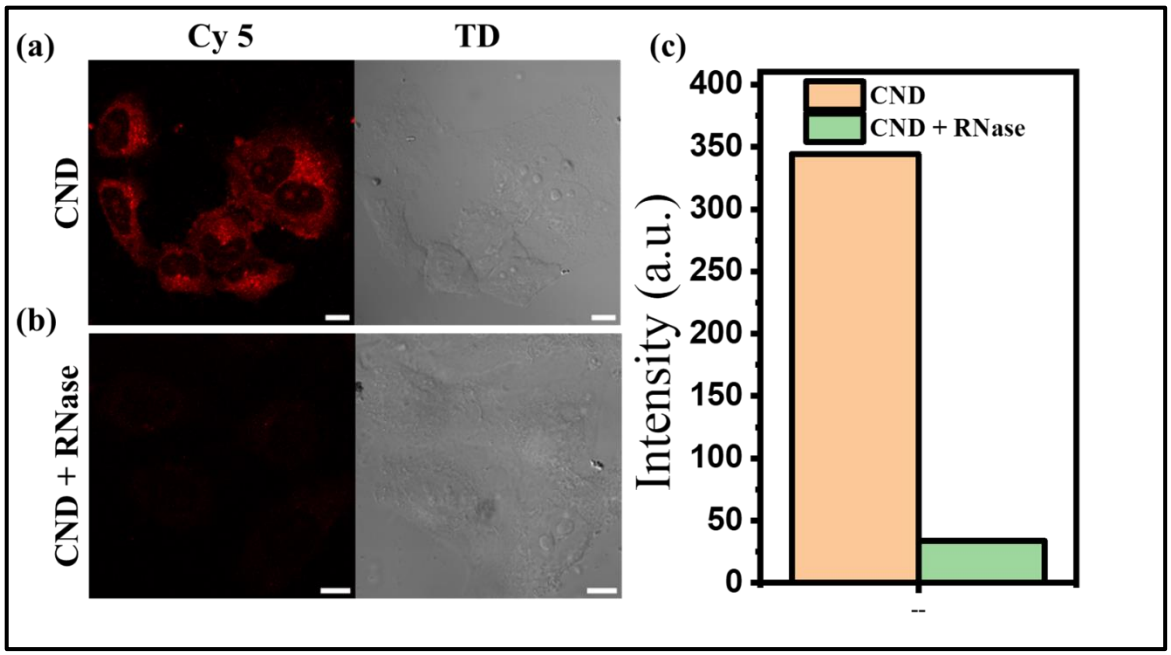

Figure S13: Confocal images of HeLa cells (a) stained by CNDs before RNase treatment and (b) after 30 μg/ml RNase treatment at 37 °C for 2 h. (c) The corresponding fluorescence intensities before and after RNase treatments showing a substantial decrease (90%) of the emission intensity. Scale bar: 10 μm

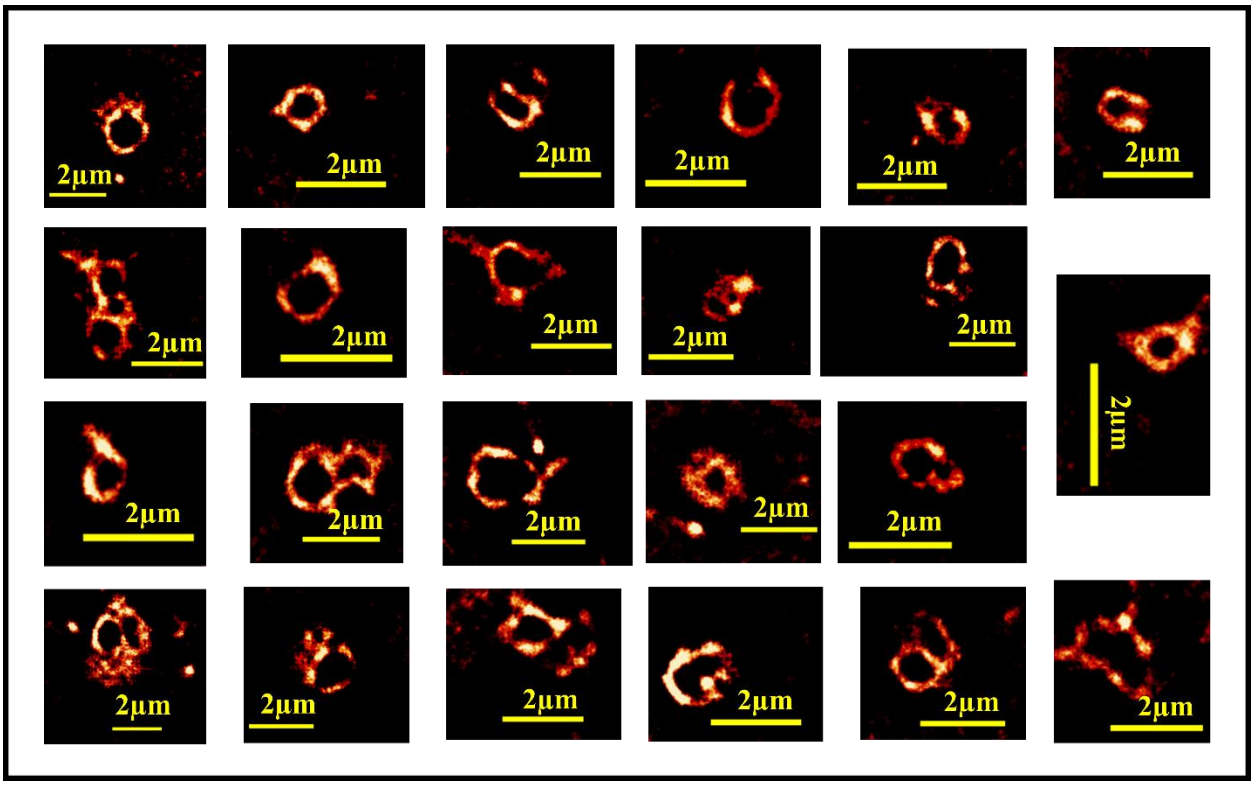

Figure S14: SRRF Images show ring-like morphology of dense fibrillar component (DFC) stained with DAN CNDs in HeLa cells.