Supplementary Information

Creation of Linear Carbon Dot Array with Improved Optical Properties through Controlled Covalent Conjugation with DNA

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Experimental Section

Materials and methods

Citric acid (CA), branched polyethylenimine (BPEI) and HPLC purified single strand 24mer 5'-phosphorylated DNA were purchased from Sigma Aldrich. Methyl imidazole and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl) were obtained from Alfa Aesar. All chemicals required for buffer preparation and gel electrophoresis were obtained from either Sigma or Alfa Aesar and used without further purification. All chemicals were used as received. Nanopure water from Millipore was used in all experiments, including spectroscopic studies.

Synthesis of Carbon Dot (CD)

A low temperature pyrolysis approach was used to prepare amine functionalised carbon dots with precursor citric acid as carbon source and BPEI for amine functionalisation in one simple step. In a typical synthesis procedure, 0.5 g BPEI and 1.0 g CA were dissolved uniformly with 10 mL hot water in a 25 mL beaker, and then heated moderately (<200 °C) using a heating mantle. After heating a yellow color solution appeared and was allowed to cool at room temperature. The resulting solution was diluted to 25 mL and centrifuged at 8000 rpm for 20min to remove the large agglomerated particles and the unreacted organic moieties and then loaded into dialysis bags for against ultra-pure water for 48 h. The solution exhibited strong blue emission under the UV light; it indicated the formation of CDs. The purified CDs solution was stored at 4 °C for further use.

Conjugation of CD with DNA (S1 and S2)

5' phosphorylated ssDNA (S1) and (S2) (6 nmol), was activated by addition of EDC (10 µl, 0.1M) and aqueous solution of 1-methyl imidazole (10 µl, 0.2 M) at pH 6 and thoroughly mixed for 90 min at 37 °C with constant stirring. The intermediate product was purified by dialysis (MWCO-10 KDa) to remove the excess EDC, 1-methyl imidazole and unreacted DNA and CDs. To this activated 5'- phosphorimidazolide ssDNA, 3µl of amine functionalized CDs (1mg/ml) was added in the presence of (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES

buffer (10 µl, 0.2M, pH8) and kept at 55 ºC for 18 h. The intermediates and the crude reaction mixture was dialyzed to obtain the conjugates of CD-DNA [CD-$(S1)_1$ and CD-$(S2)_2$].The CD-ssDNA conjugates were annealed to get CD arrays.

Annealing of $(S1)_2$-CD and - $(S2)_2$ -CD

For hybridization of CD-$(S1)_2$ and CD-$(S2)_2$ both products were taken in equal molar ratio and annealing was performed in presence of 10 mM TE, 10mM magnesium chloride and 100 mM NaCl. The samples were first heated to 90 ºC and then slowly cooled to 20 ºC with a ramp of 0.1ºC/s and then stored at 4 ºC for further downstream characterization.

Agarose Gel Electrophoresis

CD,CD-$(S1)_2$ conjugate and [$(S1)_2$-CD-$(S2)_2$-CD]n assembly was visualized by 2% agarose gel electrophoresis in 1X tris acetate EDTA (TAE) chilled buffer pH 8 running at 90V. Gel mobility of CD-$(S1)_2$ and CD-$(S2)_2$ were found to be exactly similar. Images were acquired by a Canon d-60 digital camera. S1 and S1-S2 used as controls were premixed with Sybr Gold nucleic acid stain® whereas CD samples was visualized as such without any staining.

Characterizations

Optical Absorption and Emission Spectroscopy

UV-visible spectra were recorded on an UV-2550 spectrophotometer (Shimadzu) by scanning the sample in the wavelength range from 200 to 600 nm. Steady-state fluorescence spectra were collected on a Fluoromax-4 spectrofluorometer 45 (Horiba, Japan) at excitation wavelength of 350 nm. The fluorescence time resolved decays were measured by using time-correlated single photon counting (TCSPC) technique by a time-resolved fluorescence spectrophotometer as of Edinburgh Instruments (LifeSpec-II, UK). The fluorescence transients were detected in magic angle (54.7^o) polarization using Hamamatsu MCP PMT (3809U) as a detector. The time resolved fluorescence decays were recorded on a 100 ns window with 4096 channels, laser 375 nm, frequency 5 MHz, 5000 counts. All measurements were performed in aqueous medium.

Circular Dichroism (CD) spectra were obtained on a Jasco J-1500 spectropolarimeter in sodium phosphate buffer (200µl, 1 mM, pH 7.2). Data were expressed as degrees of ellipticity (è), in units of millidegrees (mdeg). Each sample was scanned thrice with a scan rate of 50 nm/min using micro cuvette having 1 mm path length.

Determination of Quantum Yield of Carbon Dots

For the determination of quantum yield, a solution of quinine sulphate (QY= 0.55) in 0.5 M H_2SO_4 was used as standard. The solutions of CDs in water and quinine sulphate in H_2SO_4 were prepared to have similar absorbance at the excitation wavelength. Quantum yield was calculated using eqn (1).

$$QY_x = QY_s \times (A_s/A_x) \times (I_x/I_s) \times (n_x^2/n_s^2) \quad \ldots (1)$$

Here, subscripts x and s refer to the sample and the reference material, respectively, QY to the quantum yield, A to the absorbance at the excitation wavelength, I to the integrated fluorescence intensity and n to the refractive index of the solvent.

Ninhydrin assay

The presence of primary amine group on the BPEI capped carbon dots was determined by Ninhydrin colorimetric assay. For this reaction, different volumes (0.1ml-1ml) of standard amino acid solution and CD sample were taken in labeled test tubes with 4ml with nanopure water. 1ml of 8% w/v of Ninhydrin reagent was added. The tubes were vortexed and incubated in water bath for 15 minutes. 1ml of ethanol was added to stop the reaction and absorbance was read at 570 nm. Concentrations of amino groups on CDs were determined from standard curve.

EtBr intercalation assay

For confirmation of CD-$(S1)_2$ and CD-$(S2)_2$ hybridization in array, 0.1µM EtBr was incubated with the 10ul of the array and CD-$(S1)_2$ sample and incubated for 15 minutes. Only EtBr solution was taken as control. EtBr was excited at 510nm and emission was obtained at 610nm.[1]

Fluorescence lifetime decay analysis

The fluorescence time resolved decays were measured by using time-correlated single photon counting (TCSPC) technique by a time-resolved fluorescence spectrophotometer from Edinburgh Instruments (LifeSpec-II, UK). The fluorescence transients were detected in magic angle (54.7^o) polarization using Hamamatsu MCP PMT (3809U) as a detector. The time resolved fluorescence decays were recorded on a 200 ns window with 4096 channels, laser 375 nm, frequency 5 MHz5000 counts. The longest component of all the three samples, i.e. CD, CD-$(S_2)_2$and CD arrays, τ_3 is approximately 10-12 ns with amplitude of 68-76% of total fluorescence intensity. The decay time τ_2 contribute 20-27 % and the shortest lifetime τ_1 contribute 2-4 % of total amplitude. The average fluorescence intensity decay time for CDs, CD-$(S1)_2$and CD arrays were found to be 8.54 ns, 8.66 ns and 10.34 ns respectively.

Sample	τ_1(ns)	Relative%	τ_2 (ns)	Relative %	τ_3 (ns)	Relative %	χ^2	Average Lifetime (ns)
CD	0.15	4.39	2.45	27.28	11.53	68.33	1.390	8.54
CD-$(S1)_2$	0.55	2.65	4.31	20.49	10.11	76.87	1.056	8.66
CD array	0.18	1.49	4.29	22.76	12.35	75.75	1.232	10.34

Table S1. Fluorescence lifetime of CDs, CD-$(S1)_2$and CD arrays

DNA melting studies

The control dsDNA (S1 and S2 hybrid), dsDNA and loosely added CD and self-assembled CD-DNA [CD-$(S1)_2$ and CD-$(S2)_2$] hybrid structures was studied by optical melting experiments using a Peltier controlled UV–vis spectrophotometer (Bioquest, Cecil, UK). The samples were denatured by heating from 20 to 90 °C while monitoring UV absorbance at 260 nm to observe the melting point. The absorbance data were analyzed to obtain the melting temperature (Tm) of the samples.

XRD and FTIR

Powder XRD was performed in a Rigaku TTRAX III X-ray diffractometer. FTIR spectra were recorded on a Perkin Elmer spectrometer in the range 500–4000 cm^{-1}.

HPLC

CD-DNA conjugates were analyzed by a reverse phase high performance chromatography (RP-HPLC) with controller CBM-20Alite, pump LC-20AD and PDA detector SPD-M20A (Shimadzu, Kyoto, Japan) and using a C18G column (250 × 4.6 mm). The mobile phase contained 10mM Tris.HCl buffer (pH 7.4) as eluent A and 85% acetonitrile in water as eluent B. The flow rate was 0.2 ml/min with 25±1 ºC column temperature and 10 µl injection volumes. UV detection was done at 260 nm. The elution program was as follows: eluent A: 98% to 80% from start to 30 min and 80% to 90% of A from 30 to 50 min; eluent B: 2% to 20% from start to 30 min and 20% to 10% from 30 to 50 min.

AFM

AFM image was obtained by drop casting dilute solution of CD and CD array samples on freshly prepared APS mica. The images were captured in intermittent contact mode atomic force microscopy (ACAFM) using Agilent 5500 Scanning Probe Microscope. Commercial silicon nitride cantilevers having force constant 20–80 N/m were used for the measurements (Micromesh, Bulgaria). The cantilever was oscillating at its resonance frequency ranging from 265–410 kHz.

HRTEM

The morphological characteristics of the CD and $[(S1)_2\text{-CD-}(S2)_2\text{-CD}]_n$ array samples were studied under HRTEM (Tecnai G2, F30 Transmission electron microscope, 300 kV). Samples were prepared by applying diluted solutions onto carbon-coated copper TEM grids. The grids were allowed to dry prior to measurements. The prepared grids were observed at an accelerated voltage of 200 kV.

Cell Culture

HEK293 (Human Embryonic Kidney293) cells were cultured in DMEM supplemented with 10% FBS, L-glutamine, Pen-Strep and incubated in CO_2 incubator at 37 °C in 5% CO_2. Cells were passaged by trypsinization every 3-4 days.

Cell Imaging

HEK293 cells were cultured on coverslips in 24-well plate at the density of 40,000cells/well. After 24hrs, the medium was removed, and the cells were washed using PBS. The CD and CD-array (1μg/mL) was then added to the respective well. The negative control was also prepared with cells without carbon nanoparticles. After 2hrs of incubation cells were washed three times with PBS and then fixed with 4%PFA for 20 min, followed by two washes with PBS, and mounted on slides. Imaging was done using Nikon-TiE confocal microscope. As a negative control to the experiment HEK293 cells were incubated without any carbon dot and imaged under the same setting as CD and CD-Array. Cells without any dot did not show any fluorescence in any of the channels.

MTT assay

HEK293 cells were seeded in 96-well plates at the density of 5000cells/well. After 24hrs, the medium was replaced with medium containing CD and CD-array at concentrations of 0, 1, 3, and 5μg/mL and further incubated for 24 h and 48 h. After the designated time intervals, the wells were washed with PBS, and 100μL of freshly prepared MTT (1 mg/mL) solution in culture medium was added to each well. The MTT medium solution was carefully removed after 4 h of incubation. Dimethyl sulfoxide (100μL/well) was then added into each well, and the plate was gently shaken for 5-10 min. The absorbance of MTT at 570 nm was recorded by the microplate reader. Cell viability was expressed by the ratio of absorbance of the cells incubated with CDs to that of the cells incubated with culture medium only. HEK293 cells were treated with varying concentrations of CD and CD-Array. No significant impact on cell viability was observed which confirms that these nanoparticles do not cause cytotoxicity.

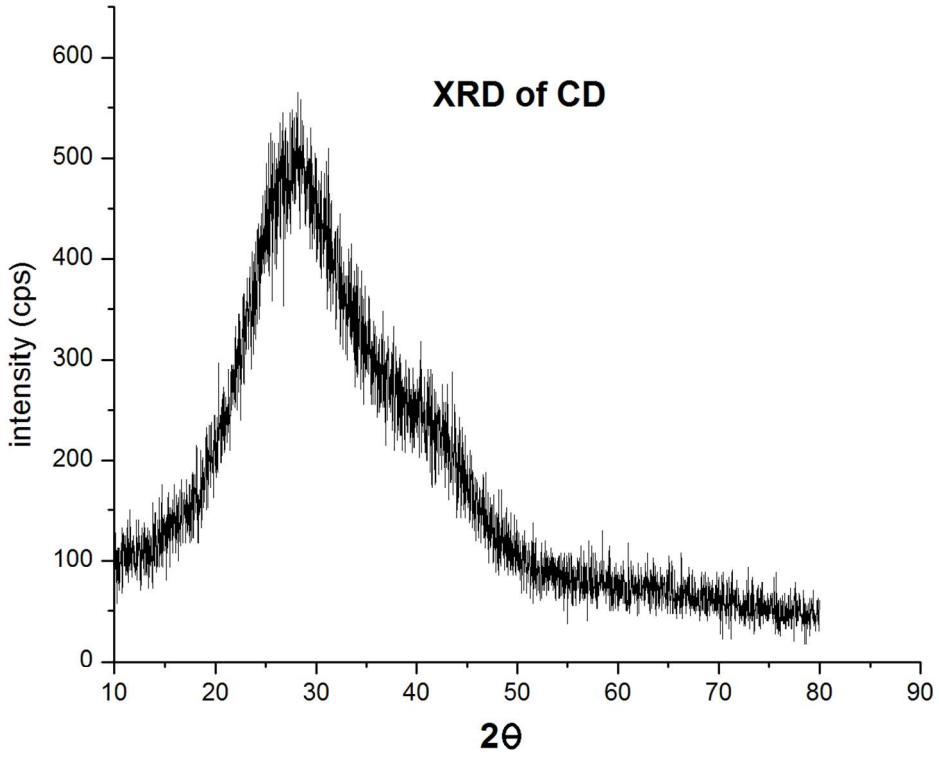

Fig. S1: Powder XRD of CD

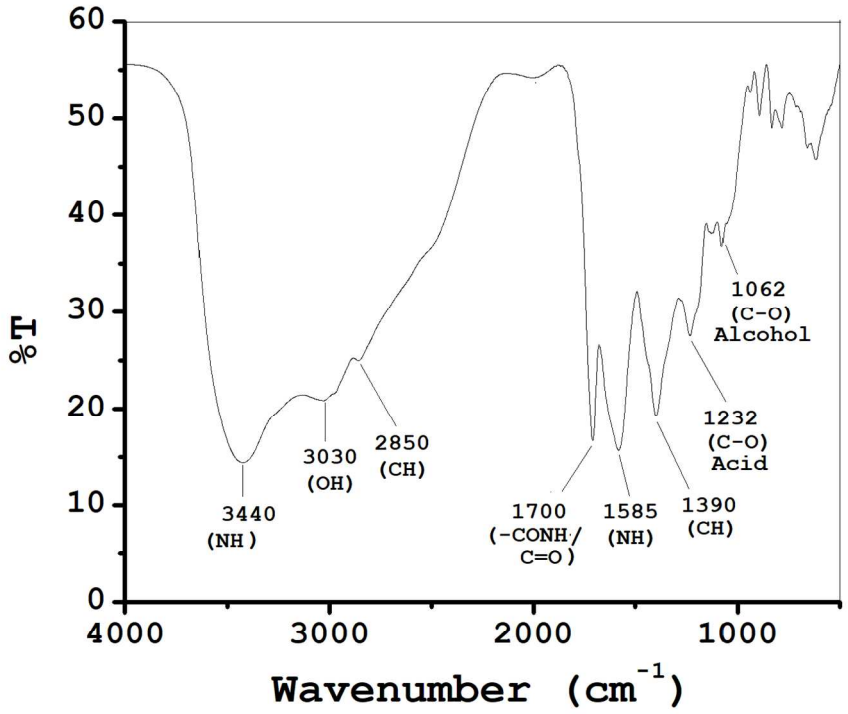

Fig. S2: FTIR Spectrum of CD

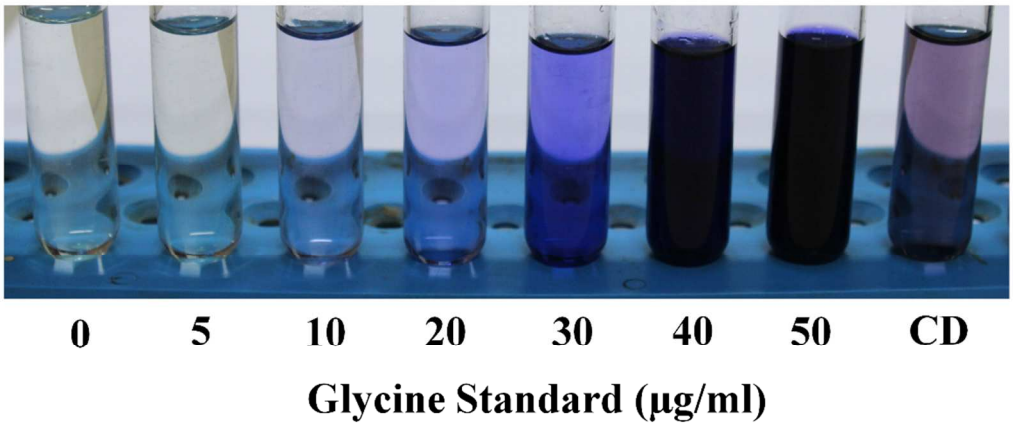

Fig S3: Ninhydrin Assay to determine concentration of amine groups on CD

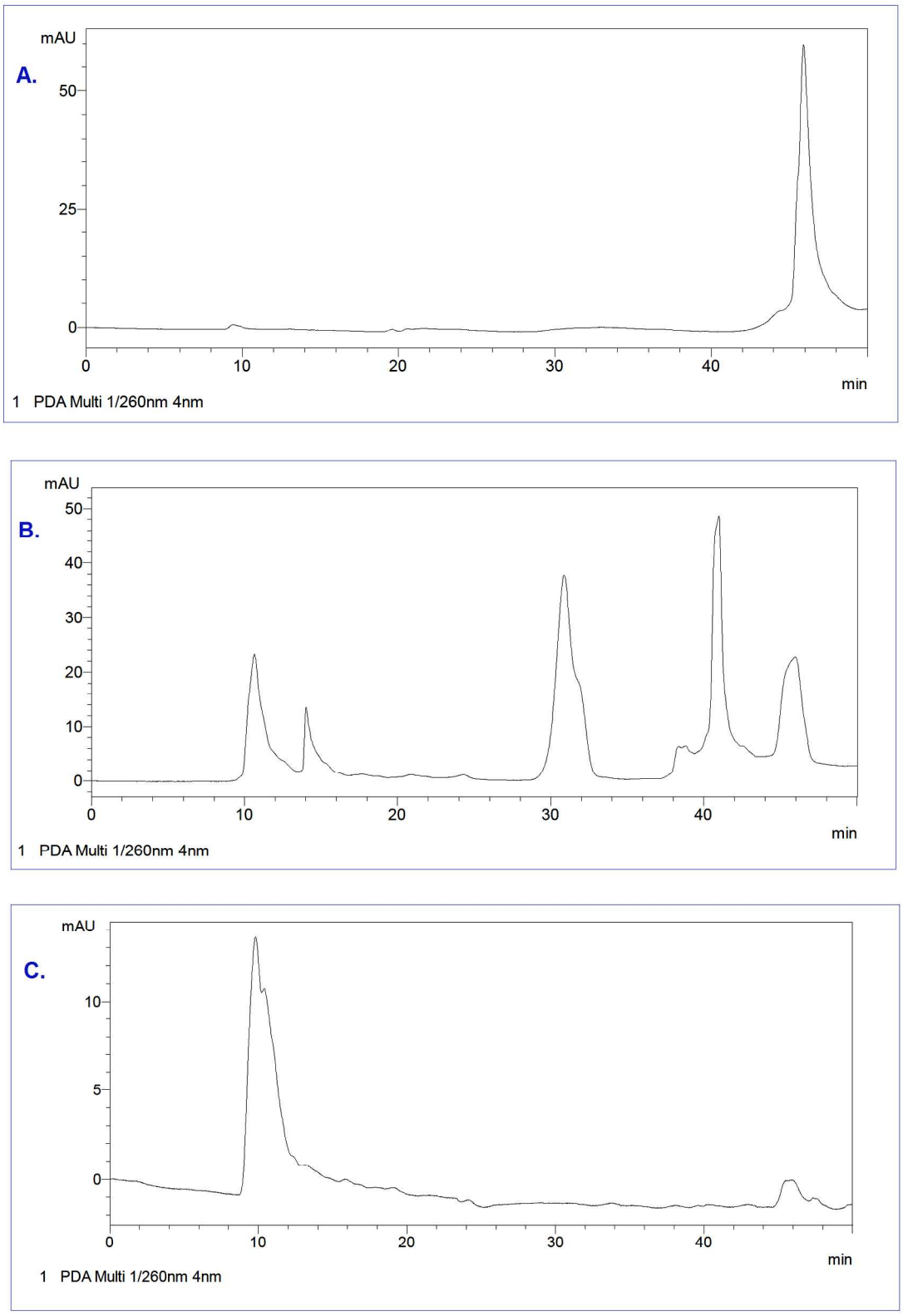

Fig. S4: HPLC profile A. Only S1 (ssDNA) B. Reaction mixtures of CD and S1: 1:2 (~10 min), unreacted CD (~14 min), 1:5 (~31 min) & 1:10 (~41 min) and S1 (~46 min). C. Purified $(S1)_2$-CD (~10 min).

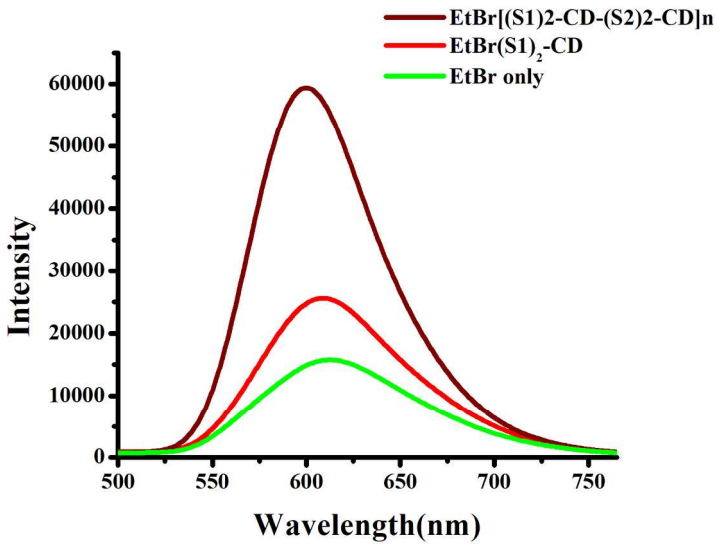

Fig. S5: Ethidium Bromide assay to show formation of double stranded DNA upon array formation following hybridization.

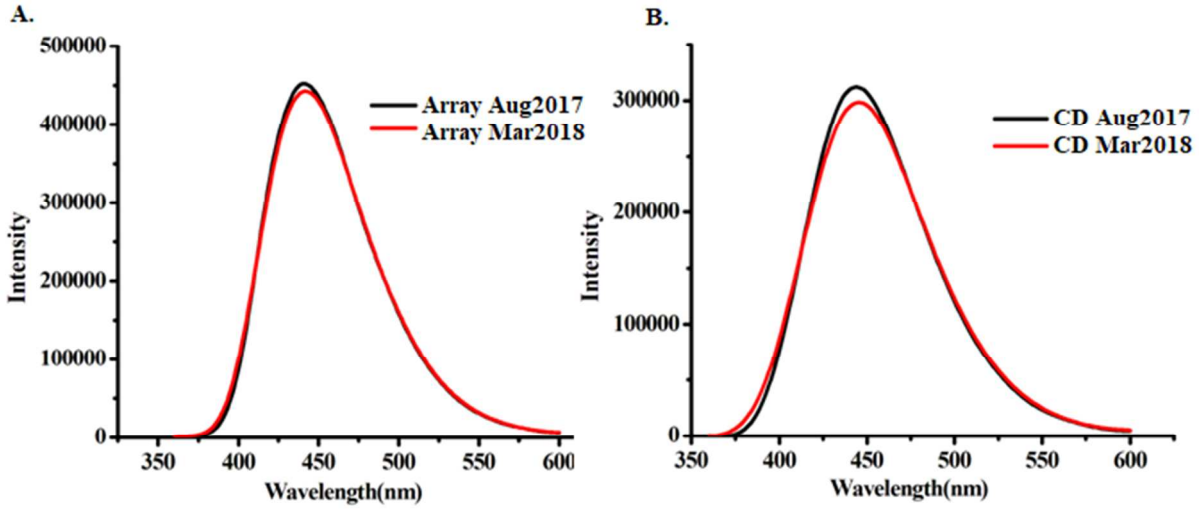

Fig. S6: Fluorescence spectra of CD and CD-DNA Array taken on August 2017 and March 2018 for comparison to check loss of fluorescence and hence stability of CD and Array.

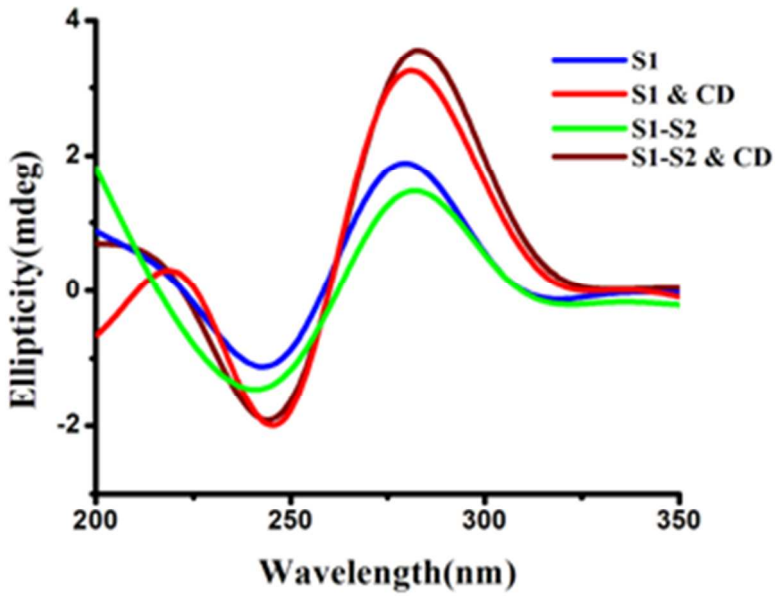

Fig. S7: Circular Dichroism Spectra of S1 and S1-S2 in presence of loosely added CD

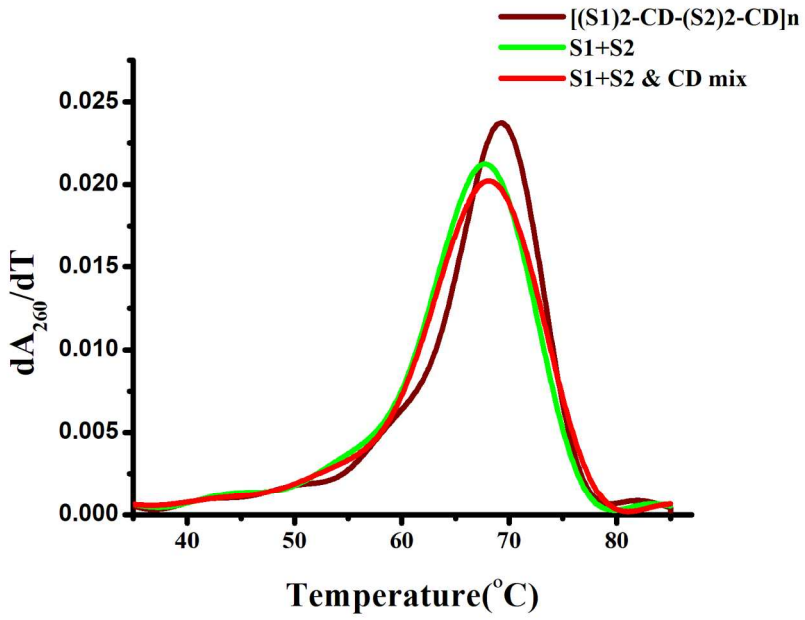

Figure S8: Thermal Melting curves of S1-S2 and $[(S1)_2$-CD-$(S2)_2$-CD]n array

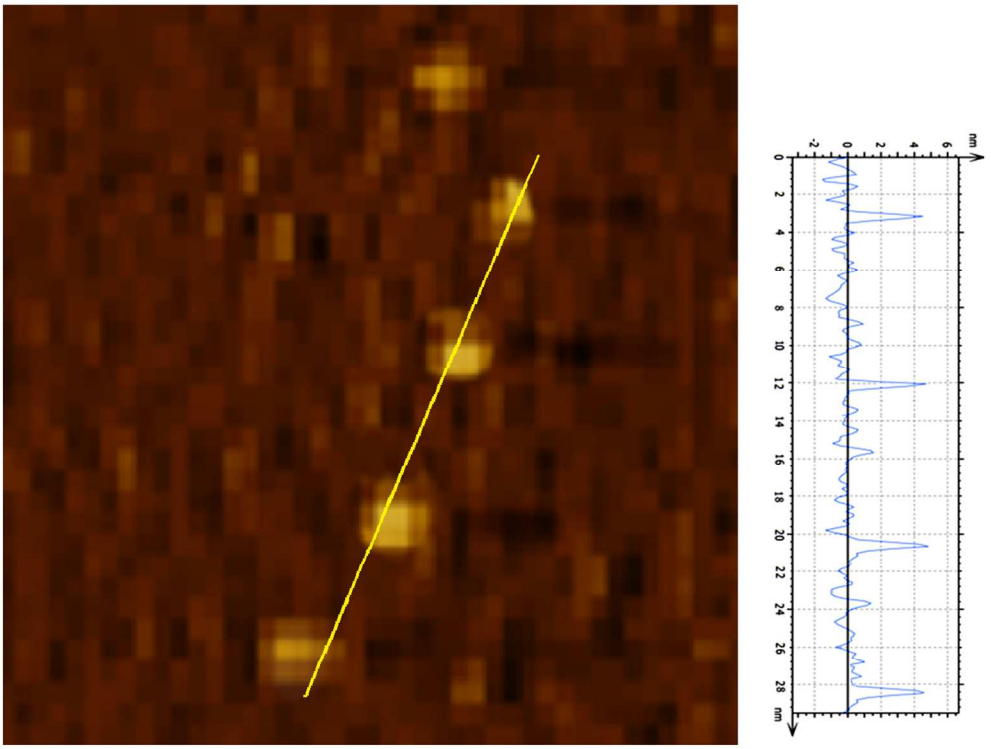

Fig. S9: AFM image of $[(S1)_2\text{-CD-}(S2)_2\text{-CD}]n$ array showing height profile and inter CD distance in array

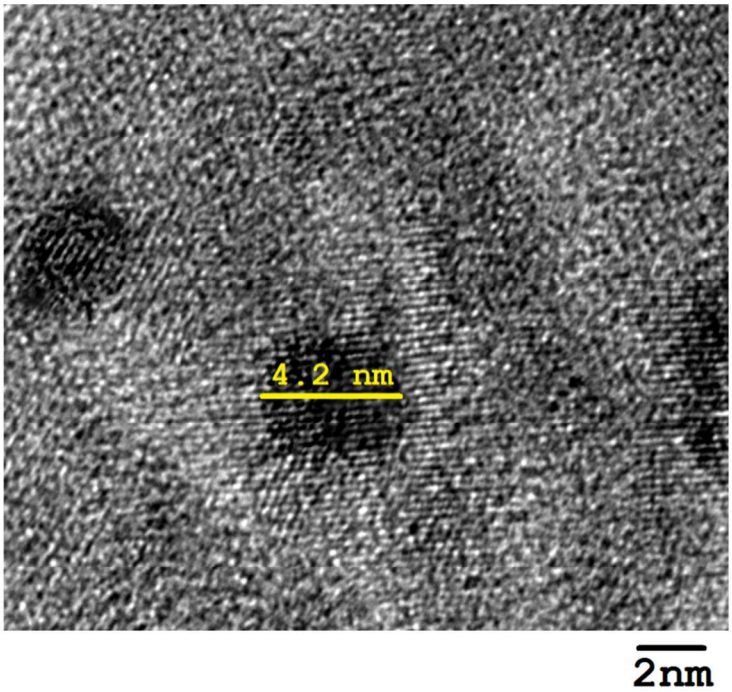

Fig. S10: HRTEM image of CD showing lattice fringes used for size calculation

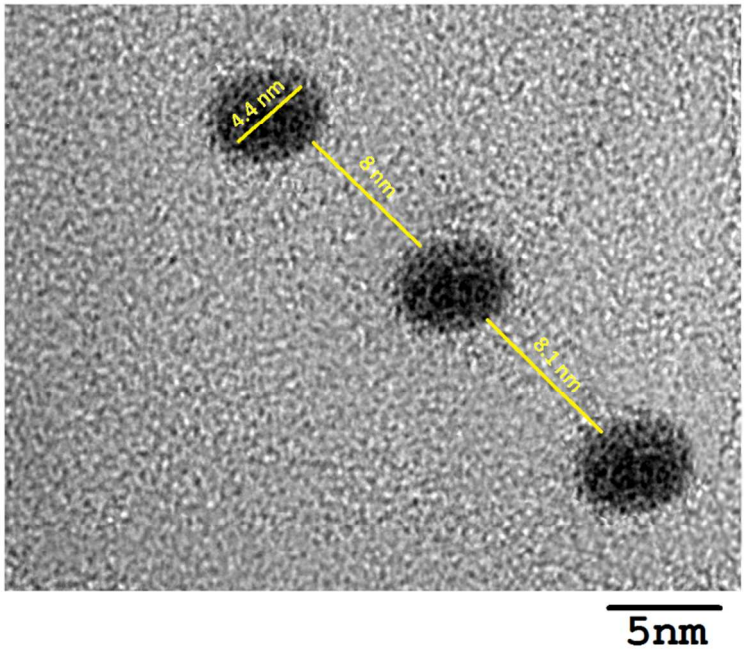

Fig. S11: TEM image of $[(S1)_2\text{-CD-}(S2)_2\text{-CD}]n$ array showing inter CD distance

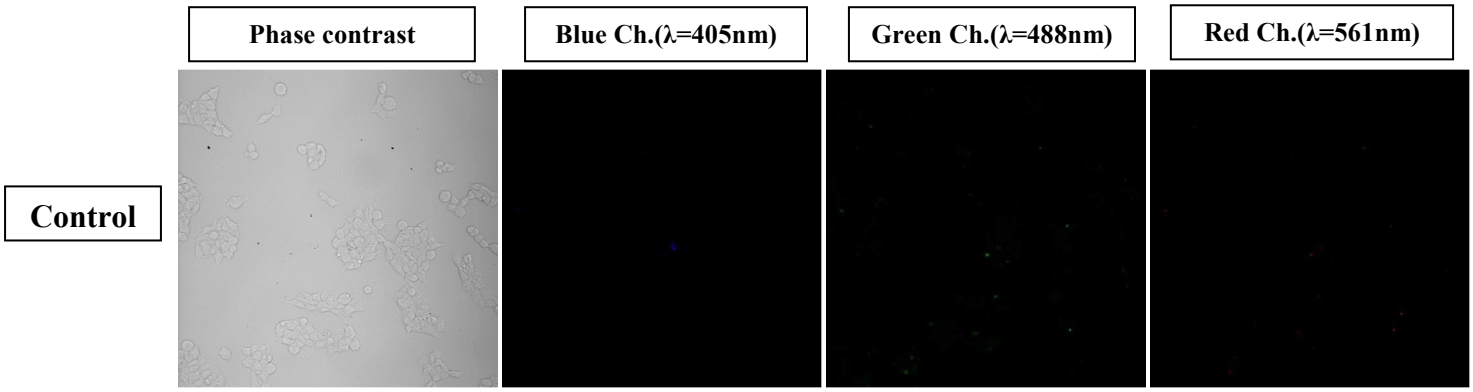

Fig.S12: Confocal images of HEK293 cells incubated without CD.

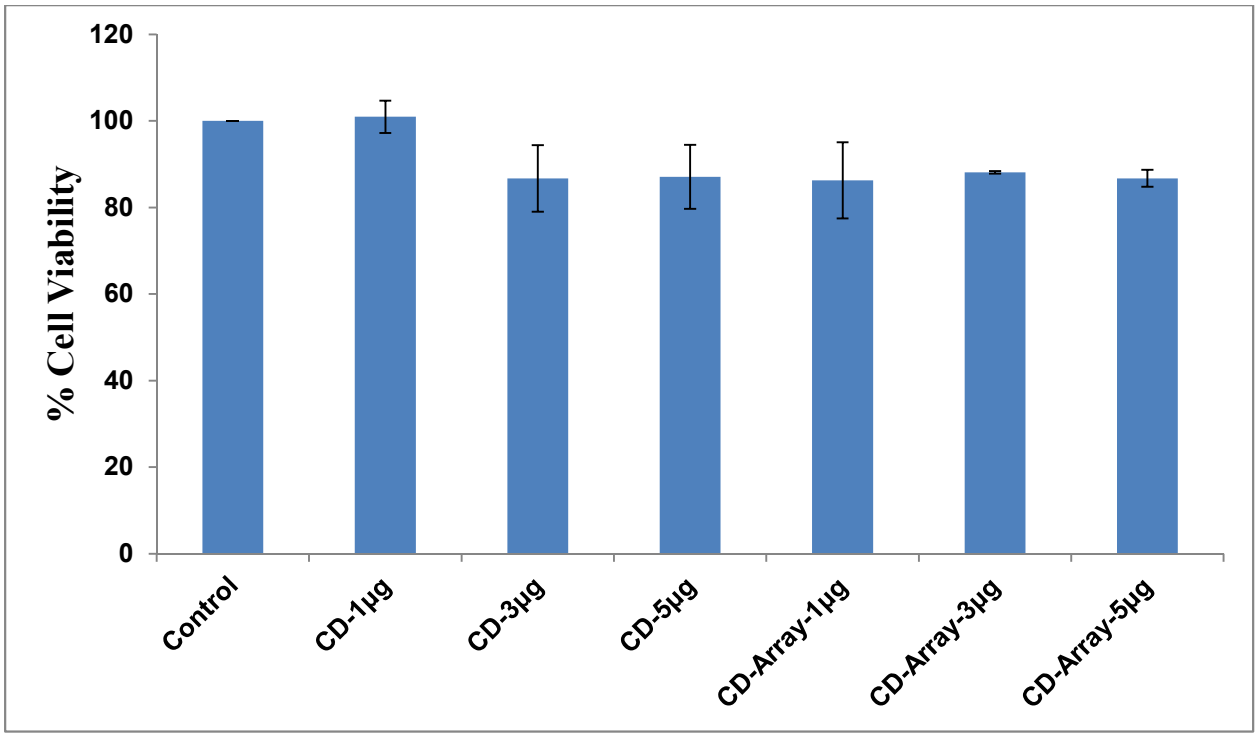

Fig.S13: Cell viability of HEK293 cells after incubation with varying concentrations of CD and CD-Array.

References

1. A.I.Dragon, E.S. Bishop, R.J.Strouse,J.R.Casas-Finet, M.A. Schenerman, C. D. Geddes,*Chem. Phys. Lett.*, 2009**, 480** , 296.