Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Implications of surface passivation on physicochemical and bioimaging properties of carbon dots

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Experimental Section

Materials

Chitosan and polyethylene glycol (PEG-4000) were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd., India. Polyethyleneimine (PEI, Mw = 25 kDa) was purchased from Sigma Aldrich, USA. Glacial Acetic acid was purchased from SD-fine chem. limited (SDFCL), India. All the solutions were prepared in ultra pure water (18 MΩ cm).

Synthesis of PEI passivated C-dots (CD-PEI) and PEG passivated C-dots (CD-PEG)

0.4 g of PEI was first dissolved in 60 mL of distilled water in a 250 mL flask. This was followed by the addition of 300 µL of acetic acid and 0.4 g of chitosan powder, the mixture was stirred vigorously to form a homogenous solution. Then the solution was transferred to hydrothermal reactor and sealed. The mixture was heated at a constant temperature of 200°C for 8 hours in a nitrifying atmosphere at 15 kg/cm^2g. When cooled to room temperature, the solution was collected and centrifuged at 9000 rpm for 20 minutes to disentangle the large, insoluble black precipitates. Finally, a dark brown colored solution was obtained.

Similarly, PEG passivated C-dots (CD-PEG) were synthesized by using 0.4 g of PEG-4000 instead of PEI and finally, a yellow colored solution was obtained.

Characterization of CDs

Absorption and fluorescence measurements were performed using a UV-vis double beam spectrophotometer (Lasany, LI-2800) and a Fluorescence spectrophotometer (Hitachi F-4600, Japan). The shape and size of the nanoparticles was visualized by Transmission electron microscope (TEM) (Model: FEI TECHNAI G2) by drop casting the dilutions onto non-shining side of carbon-coated copper TEM grids. The particle diameter was estimated through Image J software. Fourier Transform Infrared spectra (FTIR) of the samples were studied using a FTIR spectrometer (Thermo Nicolet) in the range 4000–400 cm^{-1} using KBr pellets. Elemental analysis was performed using digital field emission scanning electron microscope (FE-SEM) (Model: QUANTA 200-FEG) coupled with an energy dispersive X-ray analysis (EDAX) facility.

Fluorescence lifetime measurements were recorded by a ‘‘Fluoro Cube Fluorescence Lifetime System’’ (Horiba Jobin Yvon) equipped with Nano LED (635 nm) source and decay curves were analyzed by IBH decay analysis v 6.1 software. X-ray diffraction (XRD) patterns were obtained by advance powder X-ray diffractometer (Bruker AXS D8) using Cu-Kα radiation, λ= 1.5406A˚, range of 0°–90° at a scan rate of 0.05°/min for 40 minutes. Zeta potentials and DLS measurements were done using a Zetasizer (Malvern, Nano ZS 90). The fluorescence microscopic images were acquired through fluorescent inverted microscope (EVOS® FL Color, AMEFC 4300) under bright field, DAPI (excitation 360 nm, emission 447 nm), GFP (excitation 470 nm, emission 525 nm), RFP (530 nm excitation, 593 nm emission) light cubes, respectively.

Quantum yield measurements

Quinine sulphate was chosen as a reference having a quantum yield of 0.54 at 360 nm. Fluorescence quantum yield of CDs in water was calculated according to the following equation:

$$Q = Q_R \times \frac{I}{I_R} \times \frac{A_R}{A} \times \frac{\eta^2}{\eta^2_R}$$

where Q is the quantum yield of desired sample, I is the measured integrated emission intensity (area under the curve), refractive index being η and A means the optical density. Subscript R denotes the reference fluorophore of known quantum yield. To eliminate the chances of re-absorption effects, the absorption in the 10 mm cuvette was always kept under 0.1 at the excitation wavelength. The emission range of the samples was kept between 375-700 nm.

Photostability test

The stability in emission was evaluated by continuous excitation of the samples kept in a 10 mm cuvette inside a fluorescence spectrophotometer (Hitachi F- 4600, Japan) equipped with Xenon arc lamp. The emission of the samples was monitored within a time scan of 7200 s (2 hours).

Agarose gel electrophoresis

1.2 % agarose gel loaded with the samples was run in Tris Acetate-EDTA buffer at 85V for 40 min. The gel was visualized using a UV trans-illuminator.

SDS- PAGE (Sodium dodecyl sulphate- polyacrylamide gel electrophoresis)

Samples were loaded to a 12% denaturing gel and electrophoresis was performed at 120V for 1h using Biorad Mini-Protean electrophoresis unit. After electrophoresis, the fluorescent bands were excised and visualized under different excitation filters using inverted microscope (EVOS FL® Color, AMEFC 4300).

pK_a value estimation

The excited state pK_a values of CD-PEI were calculated according to previously reported method using the following equation:

$$pK_a^* = pH_x + \lg [(I_B - I_x)/ (I_x - I_{HB+})]$$

where I_B represents the fluorescence intensity at absolute base form, I_x is the fluorescence intensity at the pH chosen and I_{HB+} is the fluorescence intensity of absolute acid form, respectively [31].

Cell culture

A549 (human lung adenocarcinoma) cells and BHK-21(Baby hamster kidney fibroblast) cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. The cells were incubated at 37°C in 5% CO_2 under humidified conditions.

Cytotoxicity

In vitro cytotoxicity of CD-PEI and CD-PEG was evaluated using MTT cell assay kit (Himedia, CCK003). A549 and BHK-21 cells were seeded on 96-well culture plate at a density of 1 x 10^4 cells/well and incubated for 24 h at 37°C in 5% CO_2. After adequate growth of the cells, the growth medium was replaced by medium containing different concentrations of CD-PEI and CD-PEG and incubated for 24 hours. After incubation, CD-PEI and CD-PEG containing medium was removed, and replaced with 100 µL of fresh medium containing 15 µL MTT (5 mg/ml) in each well and incubated for another 4 h. The medium was then removed without disturbing the monolayer and 100 µL of solubilization solution was added to all the wells to dissolve formazan

crystals, followed by shaking for 15 min. The absorption of each well was measured at 580 nm using a microplate reader. Non-treated cells (in DMEM) were used as control and the relative cell viability (mean% ± SD, n = 3) was expressed as: $A_{test}/A_{control}$ X 100%.

Fluorescence based bioimaging

In order to explore the bioimaging efficiencies of CD-PEI and CD-PEG, A549 and BHK-21 cells were seeded in 3 cm culture dishes containing 2 mL of DMEM medium. After appropriate growth of the cells, equal concentration of 0.22 μm filtered sterilized aqueous suspension of CD-PEI and CD-PEG (5 mg/mL) were added to respective dishes under similar conditions. After an incubation of 1h, the medium containing CD-PEI and CD-PEG was removed. The cells were then washed twice with phosphate buffered saline (PBS) to remove any media traces left over. The cells were retained in PBS during optical imaging. Bioimaging efficiencies of CD-PEI and CD-PEG were assessed by examining the labeled A549 and BHK-21 cells under inverted fluorescent microscope (EVOS® FL Color, AMEFC 4300).

Quantitative analysis

For quantitative analysis $1x10^5$ A549 cells were seeded in each well. After 12 h, equal concentration of CD-PEI and CD-PEG (5 mg/mL) were added to appropriate wells and incubated for 1 h. Cells were then trypsinized and collected, followed by washing at 10000 rpm for 2 minutes. To remove any traces of unbound CDs, cells were washed twice with PBS. Fluorescence spectroscopy and quantum yield measurements were performed by preparing similar dilutions of all the samples for comparative purposes.

Sample	**Integrated emission intensity (*I*)**	**Absorbance at 360 nm (*A*)**	**Refractive index of solvent (η)**	**Quantum yield at 360 nm (*Q*)**
Quinine sulphate	796366	0.0860	1.33	0.54 (known)
CD-PEG	127103	0.1057	1.33	0.0701
CD-PEI	241753	0.1072	1.33	0.1315

Table S1. Quantum yield calculation of CDs.

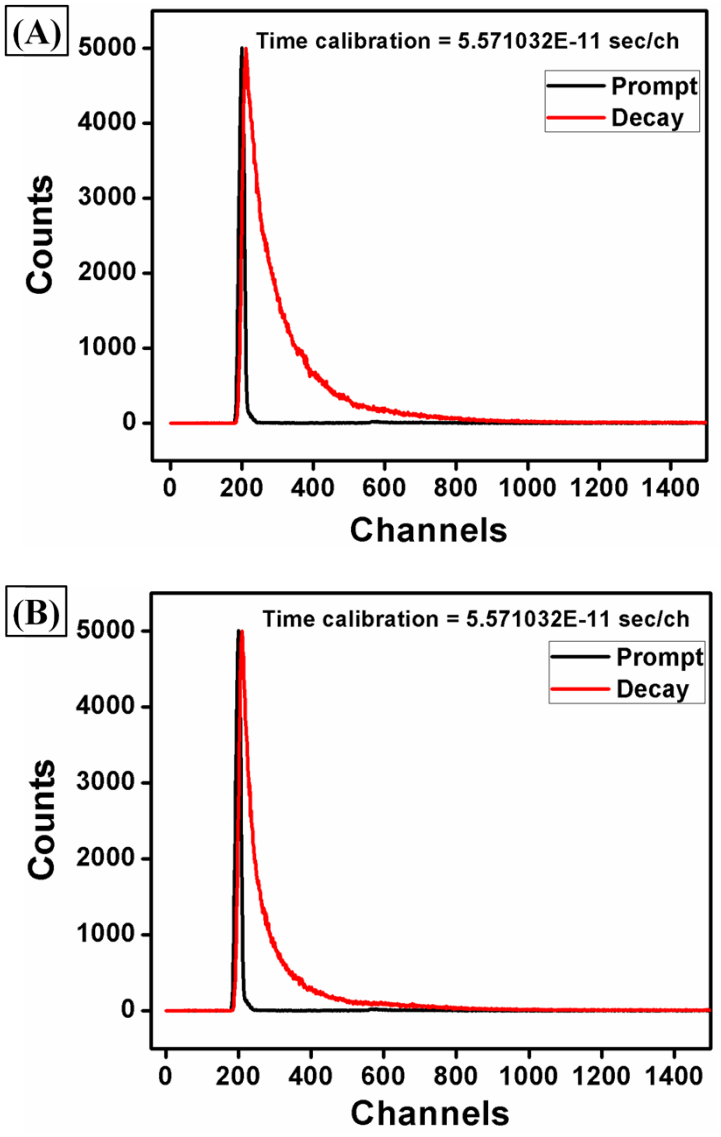

Fig. S1. Fluorescence decay curve of (A) CD-PEI (λ_{ex} = 360 nm; λ_{em} = 460 nm) and (B) CD-PEG (λ_{ex} = 320 nm; λ_{em} = 400 nm).

Sample	a_1	τ_1(ns)	a_2	τ_1(ns)	a_3	τ_1(ns)	τ_{av}(ns)	χ^2
CD-PEI	0.0912	1.221	0.6174	4.979	0.2913	10.326	6.193	1.173
CD-PEG	0.5002	3.316	0.2125	0.746	0.2873	10.470	4.825	1.219

Table S2. Tabular representation of fluorescence lifetime calculation of CDs.

Average lifetime (τ_{av}) was calculated by using the following equation:

$$\tau_{av} = a_1\tau_1 + a_2\tau_2 + a_3\tau_3$$

where τ_1, τ_2 ,τ_3 were the first, second and third component of the decay time of CDs and a_1, a_2 ,a_3 were the corresponding relative weightings(emission %) of these components, respectively.

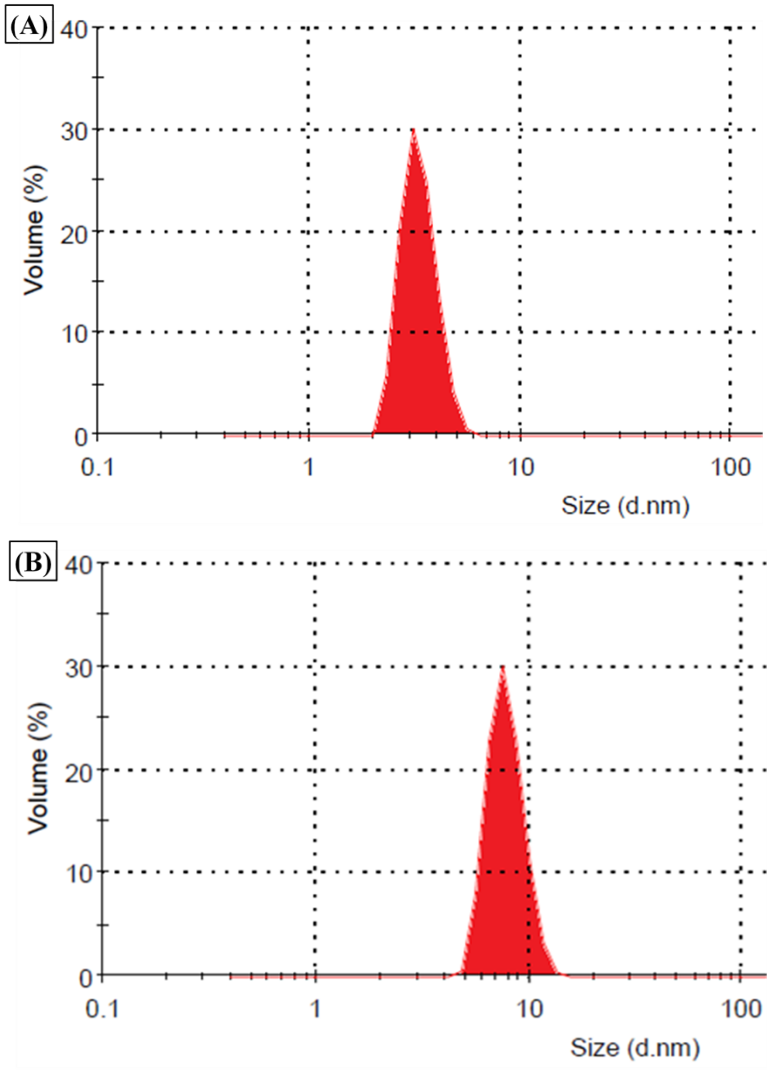

Fig. S2. DLS spectrum of (A) CD-PEI and (B) CD-PEG representing size distribution by volume.

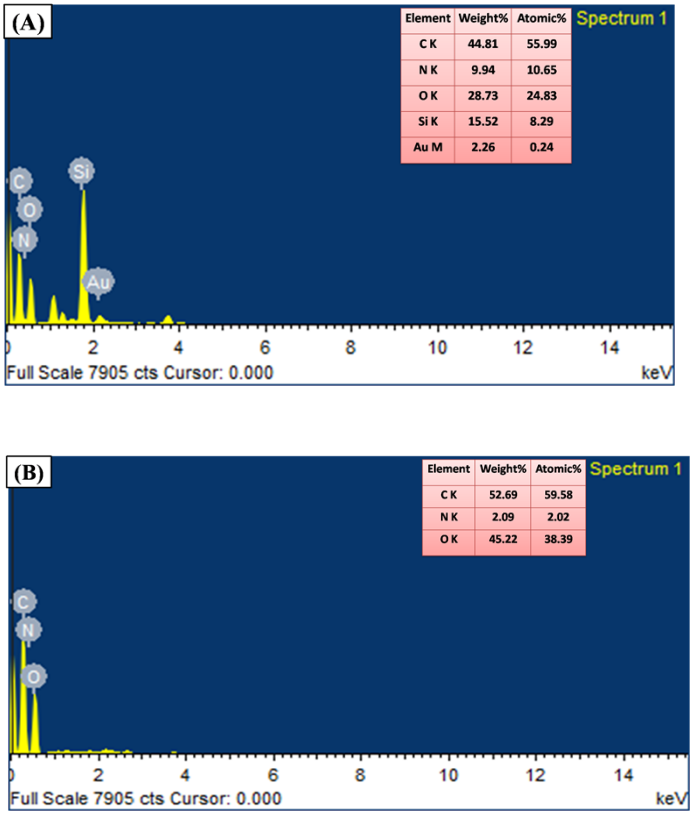

Fig. S3. EDAX spectrum and elemental composition of (A) CD-PEI and (B) CD-PEG

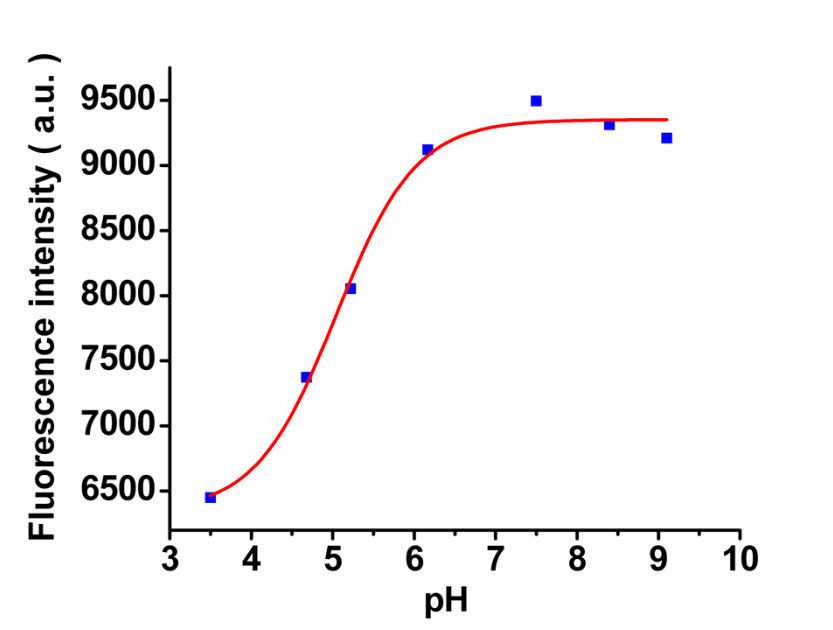

Fig. S4. Change in fluorescence intensity of CD-PEI at 460 nm (λ_{ex} = 360 nm) as a function of pH. The solid line is fit to emission intensity from pH 3.5-9.0.

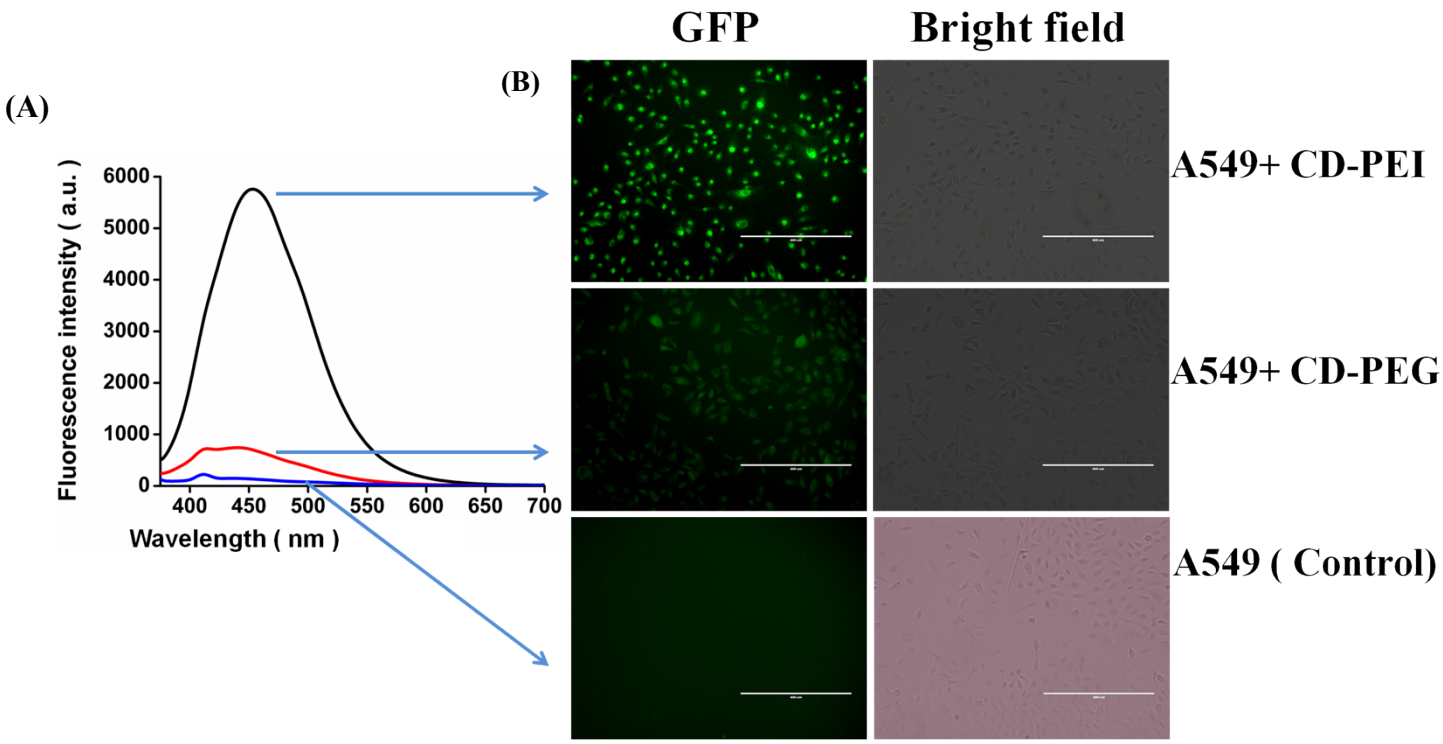

Fig. S5. (A) Fluorescence spectra of A549 cells (blue), A549 + CD-PEG (red) and A549 + CD-PEI (black).(B) Fluorescence microscopic images of A549 cells labeled with CDs.

Sample	Integrated emission intensity *(I)*	Absorbance at 360 nm *(A)*	Refractive index of solvent (η)	Quantum yield at 360 nm *(Q)*
Quinine sulphate	627215	0.0831	1.33	0.54
A549 + CD-PEI	85996	0.0939	1.33	0.0655
A549 + CD-PEG	50046	0.1024	1.33	0.0349

Table S3. Quantum yield measurements of CDs labeled A549 cells.