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Communication

Quantitative and biosafe modification of bifunctional groups onto carbon dots by click chemistry

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1. Experimental

1.1 Materials

Glucose (99%) was purchased from Innochem Technology Co., Ltd. (Beijing, China). Rhodamine B (RhB, >99.0%), N,N'-carbonyl diimidazole (CDI, 99%), N-hydroxysuccinimide (NHS, 98%), mono-propargylamine (98%), 3-azido-1-propanamine (>95.0%), dichloromethane (DCM, ≥99.9%), sodium ascorbate (99%) and tert-butanol (≥99.5%) were purchased from Aladdin Chemical Co., Ltd. (China). 1-(3-dimethylaminopropyl)-3-ethylcarbondiimide hydrochloride (EDC, 98.5%) and $CuSO_4 \cdot 5H_2O$ (99%) were purchased from Macklin Biochemistry Ltd. (Shanghai, China). Sulfo-Cy7 azide (Cy7) was purchased from Xi'an Ruixi Biological Technology Co., Ltd. (China). Deionized water was produced by ultrapure water systems (Heal Force, Shanghai, China). Dialysis bag (retained molecular weight: 3500 Da) was obtained from Beijing Solarbio Science & Technology Co., Ltd.

1.2 Synthesis of single/double fluorophore modified carbon dots

1.2.1 Preparation and alkynylation of glucose-based CDs

5 g glucose was dissolved in 45 mL deionized water, transferred into an autoclave, sealed, and kept at 180 ℃ for 12 hours. Next, the autoclave was naturally cooled down to room temperature, and the solid and liquid products were separated by vacuum filtration. The CDs suspended in the liquid portion were purified by dialysis, and then was collected after freeze-drying.

The next step is the alkynylation reaction of CDs, and the reaction degree is compared under solvent system and H_2O system, respectively.

(a) Solvent system: At the initial stage of the reaction, N_2 was pumped to remove air from the system. 100 mL anhydrous DCM, 17.5 mg glucose-based CDs and 32 mg CDI were added orderly into a three-port flask, and stirred at room temperature for 1 h to activate the carboxyl group. Then 10 μL mono-propargylamine was added and the reaction continued at room temperature for 15 h. The resulting mixture was placed in a dialysis bag (Mw cutoff: 3500 Da) to remove excess unreactants and solvents, and then freeze-dried to get the final

product, labelled as DCM-CDs-C≡C.

(b) H_2O system: 20 mg glucose-based CDs, 7 mg NHS and 17.5 mg EDC were added to 200 mL distilled water successively, and stirred at room temperature for 1 h to activate the carboxyl group. Then 4.2 μL mono-propargylamine was added and stirred at room temperature overnight. The resulting mixture was placed in a dialysis bag (Mw cutoff: 3500 Da) to remove excess unreactants and solvents, and then freeze-dried to get the final product, labelled as H_2O-CDs-C≡C.

1.2.2 Azidation of RhB

Similarly, the degree of RhB azidation is compared under solvent system and H_2O system, respectively.

(a) Solvent system: N_2 was pumped to remove air from the system at the beginning of the reaction. 50 mL anhydrous DCM, 48 mg RhB and 32 mg CDI were added in a three-port flask, and stirred at room temperature for 1 h. Then 14 μL 3-azido-1-propanamine was added and stirred at room temperature for 15 h. The product was labelled as DCM-RhB-N_3.

(b) H_2O system: 29.7 mg RhB, 7 mg NHS, and 17.5 mg EDC were added to 50 mL distilled water successively, and stirred at room temperature for 1 h. Then, 6.1 μL 3-azido-1-propanamine was added and stirred at room temperature for another 15 h. The product was labelled as H_2O-RhB-N_3.

1.2.3 Click chemistry between CDs and RhB

The alkynylated CDs (DCM-CDs-C≡C) and RhB-N_3 solution (H_2O-RhB-N_3) were mixed in proportion. Then two times the volume of tert-butanol, appropriate copper sulfate pentahydrate, and sodium ascorbate were added and stirred at room temperature for 24 h. The product is named as CDs-RhB. Five groups of samples were prepared in parallel. The ratio of RhB-N_3' mole to CDs-C≡C' mass is 0.52, 0.76, 1.54, 3.08, and 4.16, respectively ($\times 10^{-3}$ mmol/mg). According to the results of quantitative analysis, the molar ratios of alkynyl group on DCM-CDs-C≡C, azide group on H_2O-RhB-N_3, copper sulfate pentahydrate and sodium ascorbate were 3:1:0.6:1, 2:1:0.4:2, 1:1:0.2:1, 1:2:0.2:1, and 1:2.7:0.2:1, respectively. After dialysis, the resulting solution was added with appropriate amount of ultrapure water to keep CDs concentration in the five samples consistent (0.035

mg/mL). Then the fluorescence emission spectra of the five groups of samples were tested.

1.2.4 Click chemistry between CDs and RhB/Cy7

The alkynylated CDs (DMC-CDs-C≡C) was divided into five equal parts and mixed with appropriate RhB-N_3 and Cy7-N_3 respectively. Click chemical reaction was also carried out according to the above steps. The molar ratio of alkynyl group, azide group, copper sulfate pentahydrate, and sodium ascorbate was 1:1:0.2:1. The azide molar ratios of RhB and Cy7 were 5:1, 2:1, 1:1, 1:2 and 1:5, respectively. After the reaction, the five groups of samples were treated with dialysis, and then the solution with the same concentration (0.013 mg/mL) was prepared for fluorescence spectra test. The product is labeled as CDs-RhB/Cy7. See Scheme 1 for the preparation process.

Noting: the above molar ratios refer to the contents of alkyne and azide groups.

1.3 Characterizations

(High resolution) transmission electron microscope (TEM/HRTEM) images were obtained on JEM-2100F and Tecnai G2 F20 S-Twin. The Fourier-transform infrared spectroscopic (FTIR) studies were carried out using the FTIR Frontier (Bruker TENSORII, Germany) instrument. The spectra were collected by transmission method. Before testing, the samples and KBr were placed in an oven for drying treatment. The whole operation was carried out under an infrared lamp to prevent moisture absorption and fully remove the influence of moisture on test results. The scanning range is 4000-400 cm^{-1} and the resolution is 4 cm^{-1}. ^1H NMR spectra were obtained at a frequency of 600.13 MHz on Bruker Avance III (600 MHz). The chemical shifts of ^1H NMR were referenced to that of maleic acid (～6.25 for ^1H). The azide reaction degree of RhB was quantitatively analyzed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS, Ultraflex TOF/TOF, Bruker Germany). The detection range was 60-1520 g/moL. Positive ion reflection mode was applied. The fluorescence spectra (PL) were performed with a HITACHI F-7000 Spectrophotometer. The ex- and em- slit is 10 nm.

1.4 Cell culture

The human cervical carcinoma cell line Hela cells was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM-H,

with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 units/mL penicillin at 37 ◦C with 5% CO_2 and digested when reaching almost 80% confluence with 0.25% trypsin/EDTA.

1.5 In *vitro* cytotoxicity assay

To demonstrate the biosafety of CDs-RhB/Cy7, we used CCK-8 assay to detect Hela cells viability at different concentrations and at different times of intervention. Cells in the logarithmic phase (100 μL, 1×10^4 /mL) were seeded in a 96-well culture plate and cultured overnight in an incubator at 37 °C in 5% CO_2 for sufficient adherence. Various concentrations (0.70 μg/mL and 1.75 μg/mL) of CDs-RhB/Cy7 in DMEM-H solution were added to the cell plate as the treated group, and the control group was treated with an equal volume of PBS. Replace the medium with various concentrations of DMEM-H every 24 h. After 24 h、48 h、72 h and 108 h, the cell culture medium was discarded and replaced with 100 μL fresh medium without CDs-RhB/Cy7. Then, 10 μL of CCK-8 solution was added to each well of the 96-well plate and incubated at 37 °C for 2 h. The absorbance at 450 nm was then measured using a microplate reader (KHB ST-360, China). All the results were analyzed using analysis of variance. Statistical significance was considered at $p < 0.05$.

1.6 Apoptosis assay

Hela cells were seeded in a 12-well plate at a density of 1×10^5 cells/well. After treated with various concentrations (0.70 μg/mL and 1.75 μg/mL) of the CDs-RhB/Cy7 for 24h, the cells were harvested in binding buffer. Then, the cells were stained with Annexin V and Propidium Iodide at 25 °C for 20 min in the dark. The Annexin-V and Propidium Iodide-stained Hela cells were analyzed via flow cytometry (Beckman Coulter Gallios, USA).

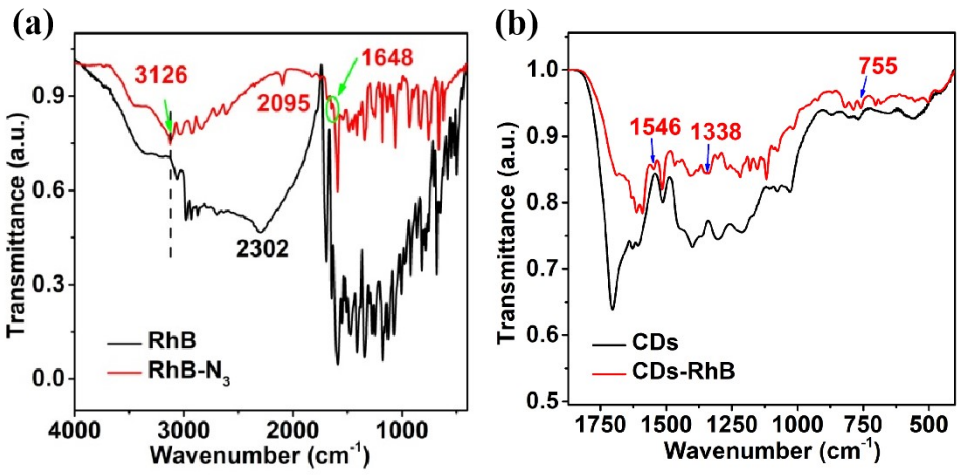

Figure S1. FTIR spectra of (a) RhB and RhB-N_3; (b) CDs and CDs-RhB.

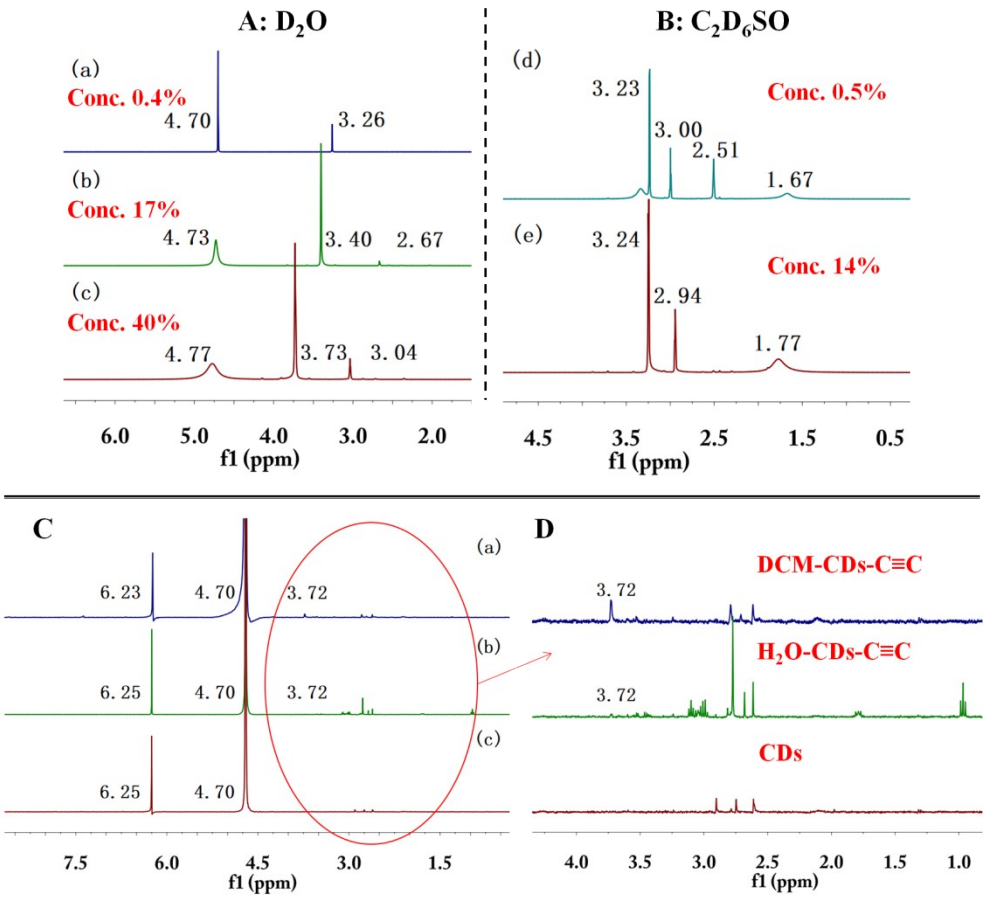

Figure S2. ^{1}H NMR spectra of mono-propargylamine in (A) D_2O and (B) C_2D_6SO with different concentrations; (C) ^{1}H NMR spectra of (a) DCM-CDs-C≡C, (b) H_2O-CDs-C≡C, and (c) CDs; (D) a partial enlargement of Figure C.

The concentrations of the samples in Figure S2C: 0.1 mL (2 mg/mL, D_2O) maleic acid, 4 mg DCM-CDs-C≡C/H_2O-CDs-C≡C/CDs, and 0.5 mL D_2O are mixed uniformly before testing.

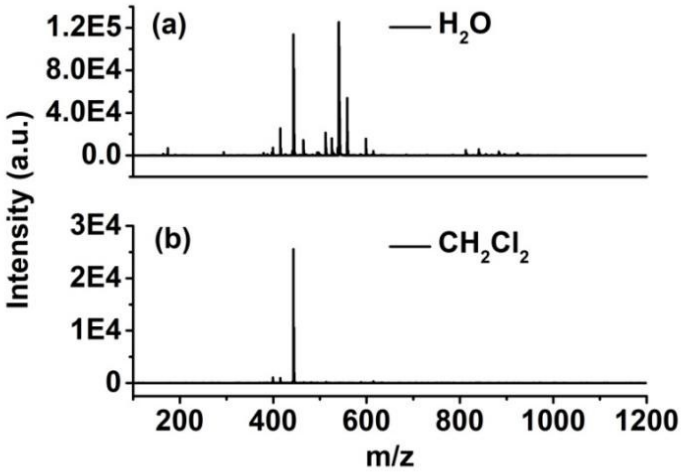

Figure S3. MALDI TOF MS spectra of (a) H_2O-RhB-N_3 and (b) DCM-RhB-N_3.

HCCA was selected as the matrix and mixed with the sample in a volume ratio of 1:1. Then the mixture dropped on the target plate.

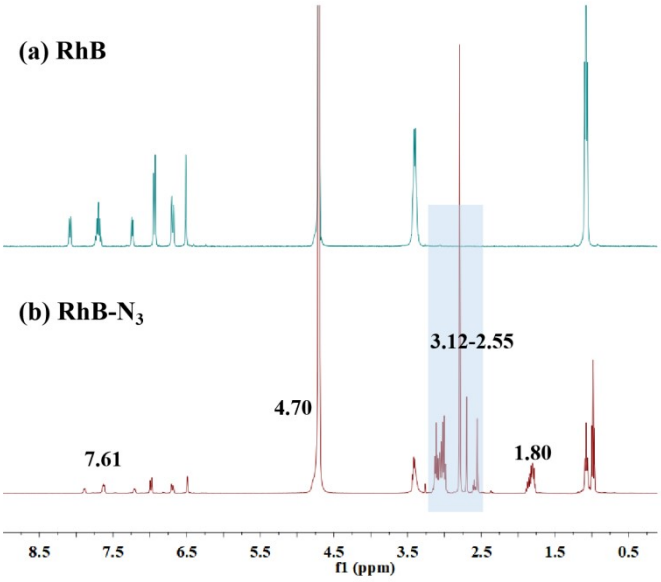

Figure S4. ^{1}H NMR spectra of (a) RhB and (b) RhB-N_3 in D_2O.

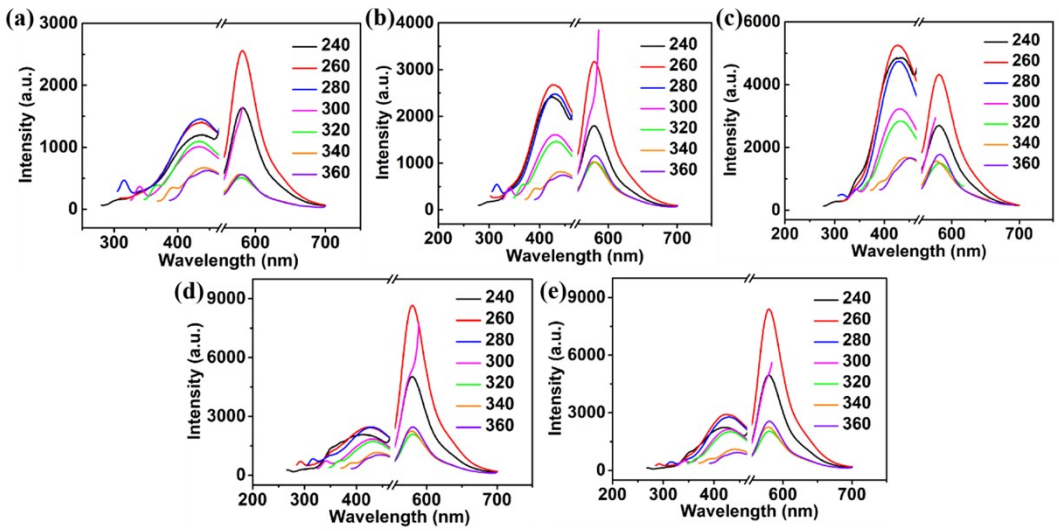

Figure S5. PL spectra of CDs-RhB with different ratios of RhB-N_3' mole to CDs-C≡C' mass: (a) 0.52, (b) 0.76, (c) 1.54, (d) 3.08, and (e) 4.16, $\times 10^{-3}$ mmol/mg, ex- and em- slit: 10 nm.

After the click chemistry reaction between CDs and RhB, the products were fully dialyzed and added moderate water to keep the solutions at the same concentration (0.035 mg/mL) for PL test.

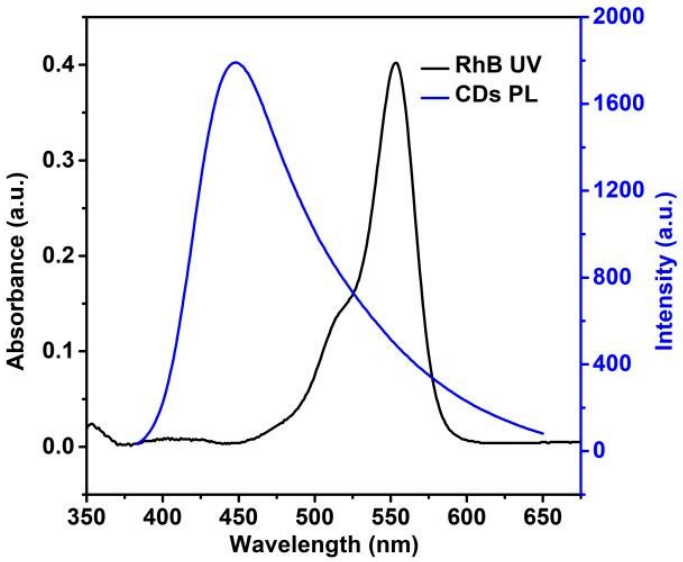

Figure S6. The UV-vis spectrum of RhB and PL curve of CDs.

RhB was dissolved in water for UV-vis testing. The excitation wavelength of CDs is 340 nm.

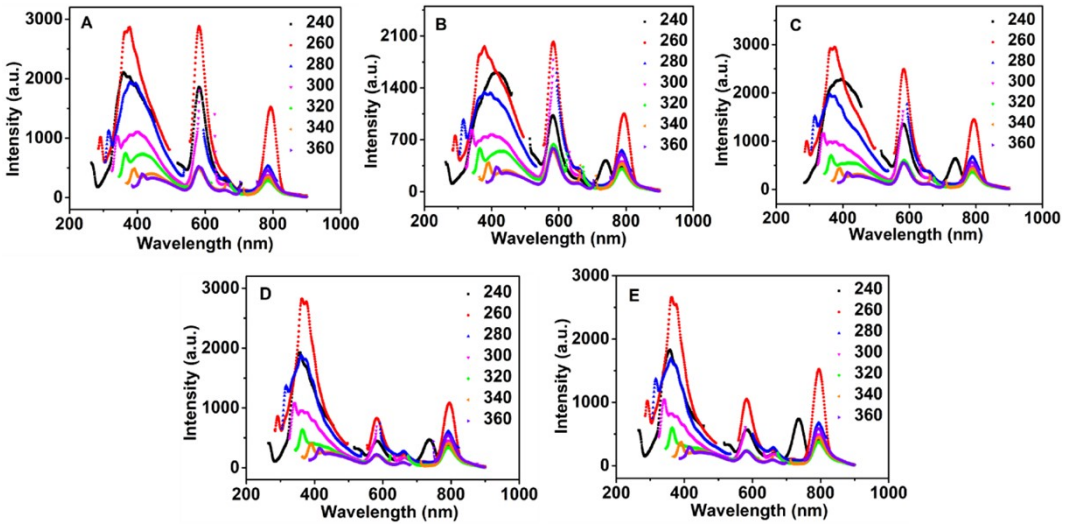

Figure S7. PL spectra of CDs-RhB/Cy7 with different molar ratios of RhB-N_3 to Cy7-N_3: (A) 5:1, (B) 2:1, (C) 1:1, (D) 1:2, and (E) 1:5, ex- and em- slit: 10 nm.

After the click chemistry reaction between CDs and RhB/Cy7, the products were fully dialyzed and added moderate water to keep the solutions at the same concentration (0.013 mg/mL) for PL test. According to experience and literatures (ACS Nano 2016: 10, 484-491; Chemistry A European Journal 2013: 19, 2276-2283; Talanta 2021: 221, 121372), the intrinsic emission peak of CDs is usually located at ca. 440 nm at the optimal excitation wavelength. While in the above fluorescence spectra, there are strong emission peaks below 400 nm, which may be due to noise or Raman scattering caused by low solution concentration.

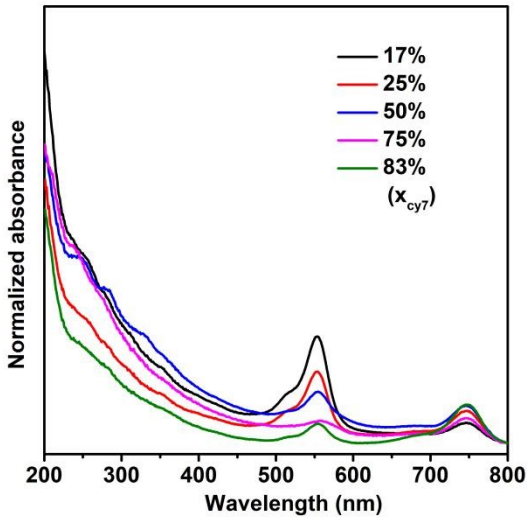

Figure S8. UV spectra of CDs-RhB/Cy7 with the increase of x_{Cy7}.

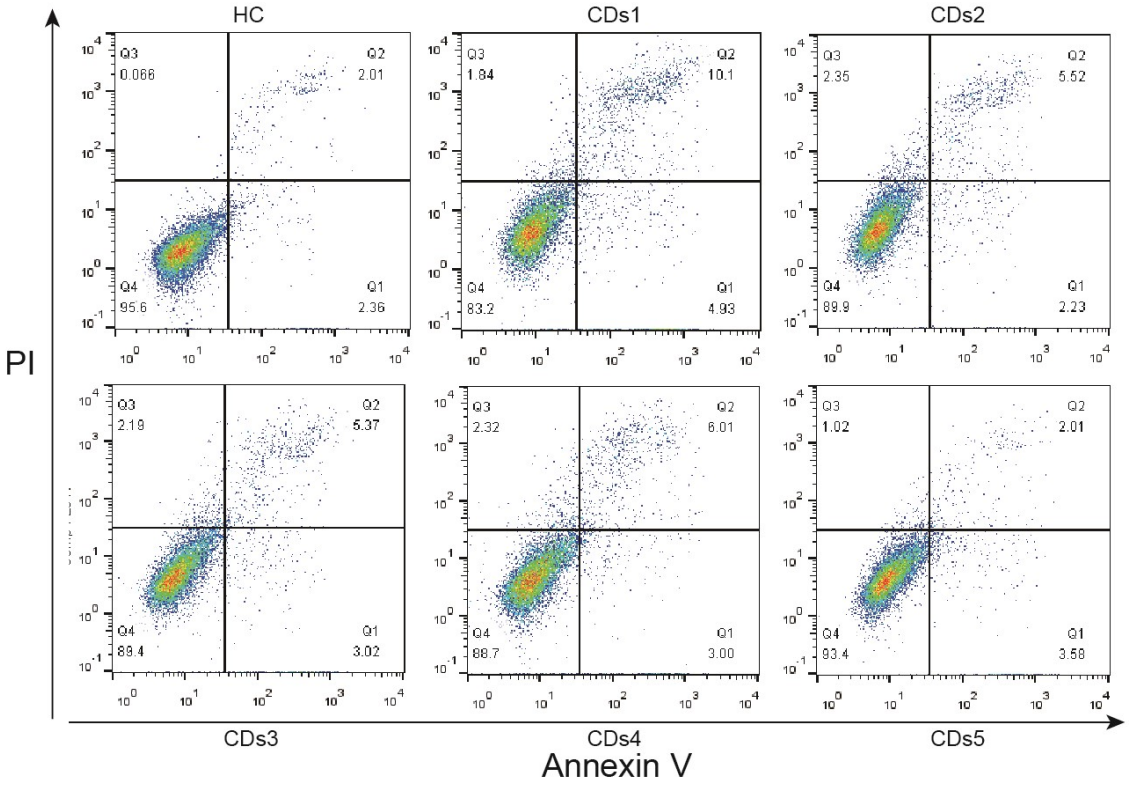

Figure S9. Effects of CDs-RhB/Cy7 (1.75 μg/mL) on Hela cell apoptosis, detected by flow cytometry. CDs1-CDs5 represent CDs-RhB/Cy7 with different molar ratios of RhB-N_3 to Cy7-N_3, 5:1, 2:1, 1:1, 1:2 and 1:5, respectively.