**Electronic Supplementary Information**

# Nonblinking Carbon Dots for Imaging and Tracking Receptors on Live Cell Membrane

Qian Wang, [a] Zhenzhen Feng, [b] Hua He,*[a] Xiang Hu, [a] Jian Mao, [a] Xiaoliang Chen,[a] Lihua Liu, [a] Xiaoyun Wei, [a] Di Liu, [a] Simin Bi, [a] Xiaojuan Wang [a], Baosheng Ge, [a] Daoyong Yu, [a] Fang Huang *[a]

[a] State Key Laboratory of Heavy Oil Processing and Center for Bioengineering and Biotechnology, China University of Petroleum (East China), Qingdao, 266580, China

[b] Technical Center of Qingdao Customs, 99 Huanghe East Road, Huangdao District, 266500, China.

E-mail addresses: huahe@upc.edu.cn, fhuang@upc.edu.cn

## Experimental

**Chemicals materials.** All chemical agents were acquired commercially and utilized without subsequent purification processing. 2,2'-Diamino-1,1'-binaphthalene ($C_{20}H_{16}N_2$) and Citric acid ($C_6H_8O_7$) were purchased from Macklin (Shanghai, China). Anhydrous ethanol and other reagents were supplied by Aladdin Reagent Co. Ltd (Shanghai, China). Human cervical cancer Hela cells, colorectal cancer CT26 cells and human embryonic kidney 293E cells were obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China). Carboxyl-CDs (item No. 102260), Amino-CDs (item No.102305) and imidazolyl-CDs (item No.101820,) were purchased from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China).

**Synthesis of CDs.** 0.21 g citric acid (CA) and 0.092 g 2,2'-Diamino-1,1' binaphthalene were dissolved in 10 mL anhydrous ethanol and then transferred into a sealed digestion vessel of the microwave digestion furnace (CEM, America). The reaction power, temperature, time of the microwave digestion system can be programmed by the user. When set at 800 W power, the reaction temperature was rapidly elevated to 200 $^{o}$C and kept at 200 $^{o}$C for an hour. After 1 h of reaction, the solution was cooled below 80 °C and taken out from the reaction vessel. The resulting solution was firstly subjected to dialysis (500 Da cutoff) to remove unreacted reaction chemicals and concentrated with a rotary evaporator. Thereafter, the sample solution was freeze-dried to the powder. The yield of the CDs was calculated to be ~39.6% by the formula $Yield = \frac{m_{CDs}}{m_{CA} + m_{DBP}}$. The CDs was weighted and dispersed in ultrapure water for further use.

**Characterization of CDs.** Transmission electron microscopic (TEM) images were recorded on a JEM-2100 electron microscope at 200 kV. Atomic force microscopic (AFM) images were obtained using a Nanoscope IV Multimode AFM (Digital Instruments, Santa Barbara). The hydrodynamic size of CDs was measured with dynamic light scattering (DLS, ZEN3600, Malvern Instruments, UK). Ultraviolet-visible (UV-Vis) absorption spectra were acquired from 200–800 nm using a Shimadzu UV-2450 spectrophotometer. Fluorescence spectra were recorded on the Horiba FluoroMax-4 spectrometer. Fourier transform infrared (FTIR) spectra were recorded on a NICOLET 6700 IR spectrometer (Thermo Scientific). The XRD pattern was collected using a XD-

2×/M4600. X-ray photoelectron spectroscopy (XPS) measurements were performed on a VG-Scientific ESCALAB 250 spectrometer with a monochromatic Al Kα X-ray source at 1486.6 eV. Raman spectra were obtained by a Renishaw via a micro spectrometer with an excitation wavelength of 785 nm laser. Prior to fluorescence scanning, the spectrophotometer was calibrated by using a Xenon lamp scan and a water Raman scan. After calibration, the xenon lamp peak maximum was at 467 nm, while the water Raman peak maximum occurred at 397 nm for excitation. Fluorescence lifetime was acquired by using a FS5 fluorescence spectrometer (Edinburgh instruments). The emission slit width of 0.02. Data acquisition was made with the technique of time correlated single photon counting (TCSPC). Quantum yields (QYs) of CDs were measured using rhodamine 6G in ethanol solution (QY = 95%) as reference standards. All optical measurements were performed at room temperature under ambient conditions.

**Single particle imaging**. Coverslips (24 x 50 x 0.17 $mm^3$) were first cleaned by sonication in NaOH solution (0.1 M), ethanol, and ultrapure water, each for 15 min, then dried with nitrogen and stored in a clean vessel for future use. A small drop of CDs in diluted aqueous solution was carefully coated onto the coverslips. The dispersion density of CDs was controlled at ~ 0.1 particle/ $\mu m^2$. Single particle imaging was performed on a Nikon total internal reflection fluorescence microscopy (TIRFM). The CDs on a coverslip were excited by 532 nm laser in conjunction with 570 nm dichroic mirror and 590 nm long-pass filter. The excitation power was about 150 W $cm^{-2}$. The image was taken by an Andor DU897 EMCCD (16 µm/pixel) using 100 × objective combined with a 1.5 × magnification changer lens, and the resultant effective dimension for each pixel was about 106 nm. The electron-multiplying gain of EMCCD was set to 300 and the pixel readout rate was set to 1 MHz at 16 bits.

**Single particle fluorescence analysis.** We first corrected the uneven background by using the rolling ball method (10 pixels radius) in the Image J software. The average intensity was collected from 7 × 7 $pixels^2$ (742 × 742 $nm^2$) around the maximum of a single spot. To avoid the crosstalk of multiple particles into an intensity trajectory, any fluorescent spot within seven pixels of another fluorescent spot was omitted from analysis. The background traces from regions (7 × 7 $pixels^2$) of interest (ROI) with no CDs were extracted for comparison.

**Conjugation of CDs to goat anti-mouse IgG.** To conjugate CDs with goat anti-mouse IgG, we used the protocol as reported in our previous work.[1] Briefly, the CDs (50 mg/mL) were firstly activated with EDC (100 mg/mL) and NHS (100 mg/mL) in methanol (30 μL) at room temperature for 30 min. The activated CDs were added to the PBS buffer (pH 7.4) containing goat anti-mouse IgG antibody (2.5 mg/mL) for > 4 h incubation. The amount of methanol was kept below 4− 5% of total reaction volume. The solution was repeatedly ultrafiltered using a filtering membrane with a molecular weight (MW) cutoff of 100 kDa against PBS buffer. The purified CD-IgG conjugates were stored at 4 °C for use. The fluorescence correlation spectroscopy (FCS) and DLS measurement was conducted to evaluate the conjugation between CDs and IgG.

**Cell culture and receptor tracking.** All cell lines were cultured with high Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C where the $CO_2$ level was kept constant at 5%. In the experiment of endocytosis of CDs, cells are incubated with CDs for 5 h, and then the cells were imaged. For CXCR4 receptor tracking, HeLa cells expressing CXCR4 were incubated for 24 h, and washed for once before staining. 200 μL DMEM supplemented with 20% FBS was added in each well, which was performed as a blocking solution at 37°C for half an hour. Then, 120 μL (2.5 μg/mL) CXCR4 primary antibody (Biolegend, USA) was dropped and incubated 1 h at 37 °C. Thereafter, the residual primary antibody was removed. 100 μL CDs -labelled goat anti-mouse IgG secondary antibody (~ 1 μg/mL) was then added and incubated for 15 min at room temperature. Subsequently, the remnant of CDs-IgG was washed away to avoid nonspecific adsorption. For single receptor tracking, the image stack of CD-labelled CXCR4 receptors was first obtained and the diffusion coefficient and displacement were calculated with the Trackmate plugin.[2]

**MTT assays.** Cytotoxicity of CDs was assessed by MTT (3-(4, 5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide) assays against Hela cells, CT26 and 293E cells. Cells were seeded in 96-well plates with a density of $5\times10^3$ cells/well in 100 μL DMEM containing 10% FBS. The plates were then incubated at 37 °C for 24 h. The cells were washed with PBS buffer and then, 100 μL fresh medium containing of different concentrations of CDs were added. The cells were continued to incubating for 24 h. After that, 20 μL of MTT solution (5 mg/ mL) was added to each well and the plates were further incubated for 4 h at 37 °C. The precipitated formazan was

dissolved in 150 µL dimethyl sulfoxide. The absorbance at 570 nm was measured using a microplate autoreader (Molecular Devices, M2e). The percentage cell viability at different concentrations of CDs was illustrated by assigning non-treated cells to 100% viability.

**Movie S1-S6.**

**Movie S1**. Fluorescence imaging of DBP-CDs. Exposure time: 283 ms/frame. Excitation laser: 532 nm.

**Movie S2**. Fluorescence imaging of amino-CDs. Exposure time: 283 ms/frame. Excitation laser: 532 nm.

**Movie S3**. Fluorescence imaging of imidazolyl-CDs. Exposure time: 283 ms/frame. Excitation laser: 532 nm.

**Movie S4**. Fluorescence imaging of carboxyl-CDs. Exposure time: 283 ms/frame. Excitation laser: 532 nm.

**Movie S5**. Fluorescence imaging of EGFP-CXCR4 on a live cell membrane. Exposure time: 200 ms/frame. Excitation laser: 488 nm.

**Movie S6**. Fluorescence imaging of CD-labelled CXCR4 on a live cell membrane. Exposure time: 283 ms/frame. Excitation laser: 532 nm.

**Table S1 and S2.**

**Table S1** Fluorescence quantum yield of different DBP-CDs

| Samples | QY (%) |
|---|---|
| Amino-CDs | 3.30 |
| Carboxyl-CDs | 1.09 |
| Imidazolyl-CDs | 5.41 |
| DBP-CDs | 10.03 |

Note: Quantum yields (QYs) of CDs were measured using rhodamine 6G in ethanol solution as reference standards

**Table S2** Precursors for synthesis of carbon dots

| No. | Compound 1 | Compound 2 | Benzene Number | Blinking | Ref. |
|---|---|---|---|---|---|
| 1 | Citric acid | Ethylenediamine | 0 | Yes | [3] |
| 2 | Chitosan | Polyethylene glycol | 0 | yes | [4] |
| 3 | Sodium citrate | Sodium thiosulphate | 0 | yes | [5] |
| 4 | Chrysanthemum buds | ethylenediamine | 0 | yes | [6] |
| 5 | Diaminopropionic acid | Cyanoguanidine | 0 | Yes | This Work |
| 6 | Acetic acid | o-phenylenediamine | 1 | Yes | This Work |
| 7 | Phthalic acid | Diaminomaleonitrile | 1 | Yes | This Work |
| 8 | Citric acid | 1,8-Diaminonaphthalene | 2 | Yes | This Work |
| 9 | Terephthalic acid | o-phenylenediamine | 2 | Yes | This Work |
| 10 | Salicylic acid | 1,8-Diaminonaphthalene | 3 | Yes | This Work |
| **11** | **Citric acid** | **2,2'-diamino-1,1'-binaphthalene** | **4** | **No** | **This Work** |

**Figure S1-S14**

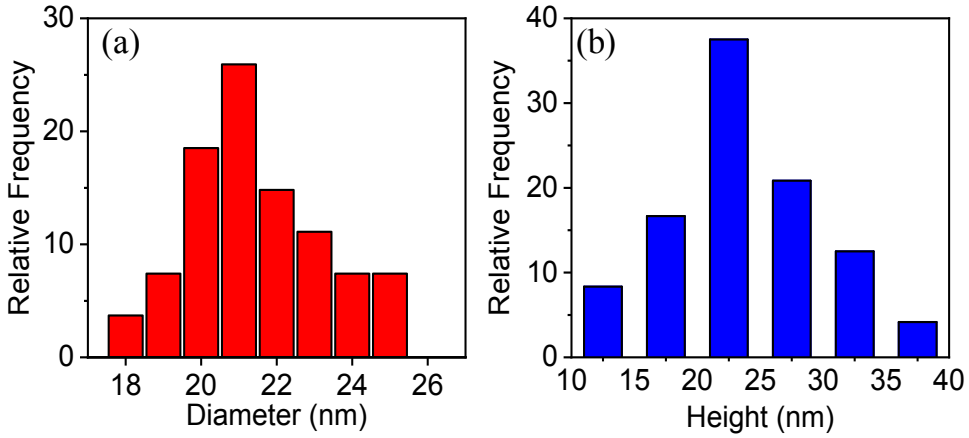


**Fig. S1.** The size or height distribution of CDs determined from (a) TEM and (b) AFM images.

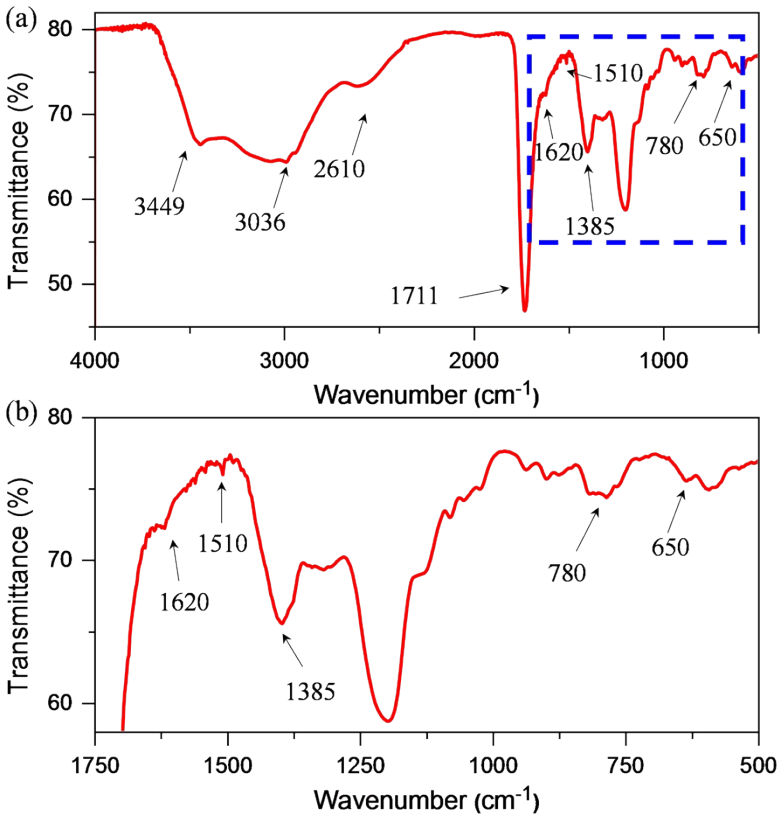


**Fig. S2** (a) FTIR spectrum of CDs and (b) the enlarged view of the region as marked in (a).

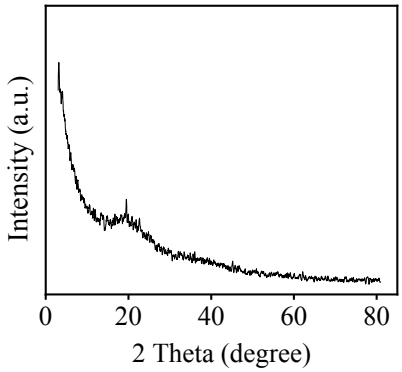


**Fig. S3** XRD profile of DBP-CDs.

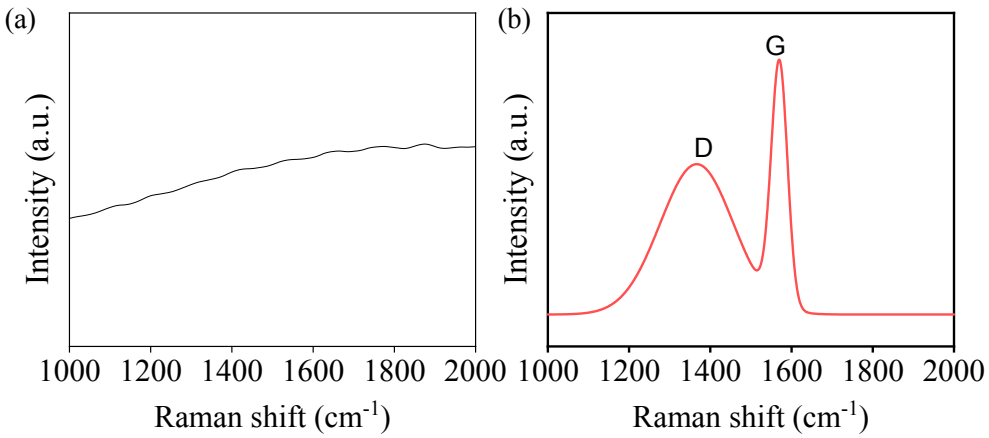


**Fig. S4** Raman spectra of (a) CA-EDA CDs and (b) our CDs.

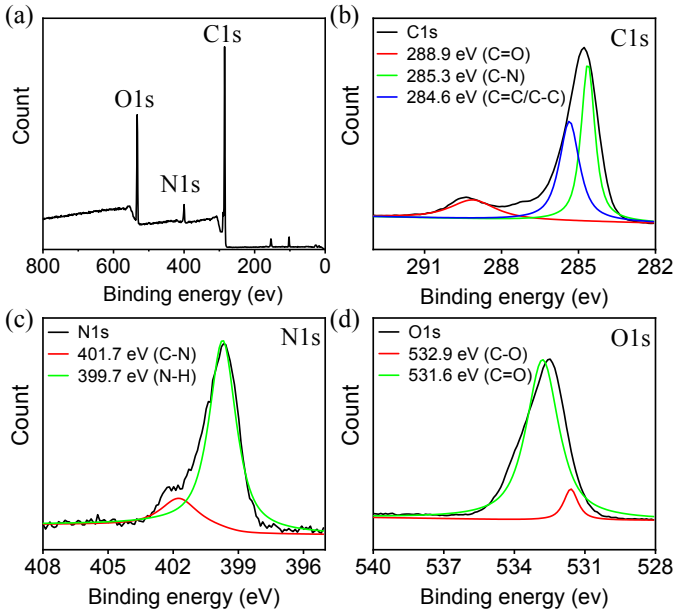


**Fig. S5** XPS spectra of DBP-CDs. (a) Survey profile, (b) C1s, (c) N1s and (d) O1s.

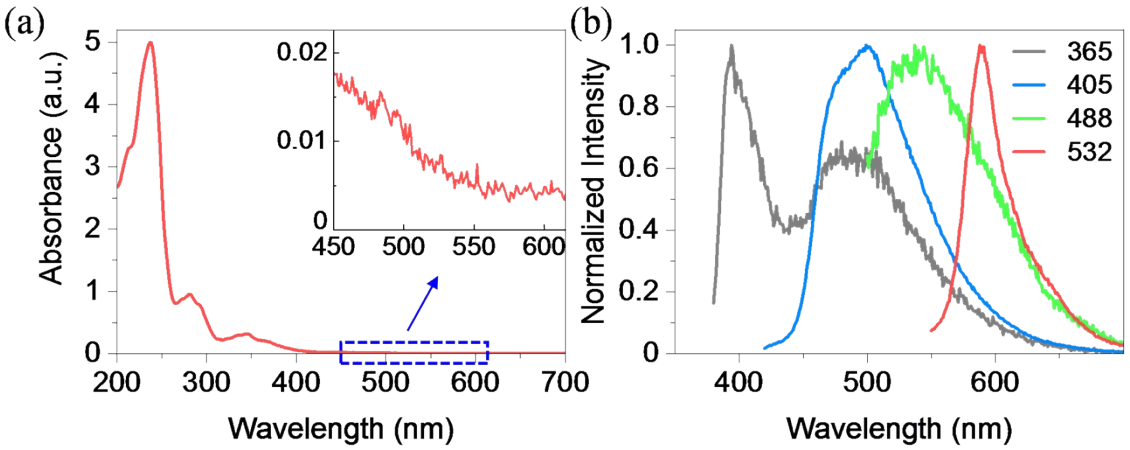


**Fig. S6** (a) UV-Vis absorption and the enlarged view of the region (inset) and (b) normalized fluorescence spectra of CDs excited at different wavelengths.

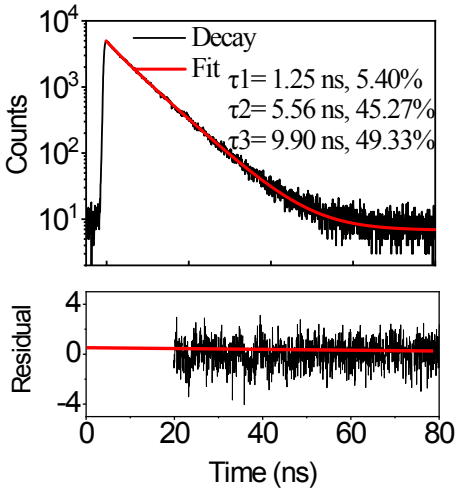


**Fig.S7** Fluorescence decay curves (black) and fitted curves (red) of DBP-CDs, excited at 532 nm. The decay curves were fitted to a triexponential decay function. The average lifetime was calculated by using the equation, $\tau = A_1\tau_1 + A_2\tau_2 + A_3\tau_3$. $A_1$, $A_2$, and $A_3$ are the fractional contributions of the lifetime components $\tau_1$, $\tau_2$, and $\tau_3$.

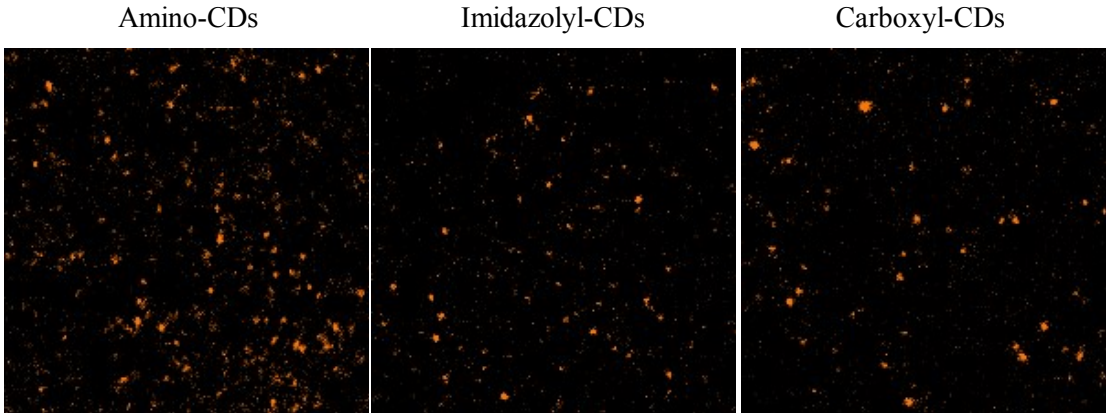


**Fig. S8** Single molecule fluorescence imaging of different commercial CDs by TIRFM, (a) Amino-CDs, (b) Carboxyl-CDs, (c) Imidazolyl-CDs. Exposure time: 287 ms/frame. Excitation laser: 532 nm.

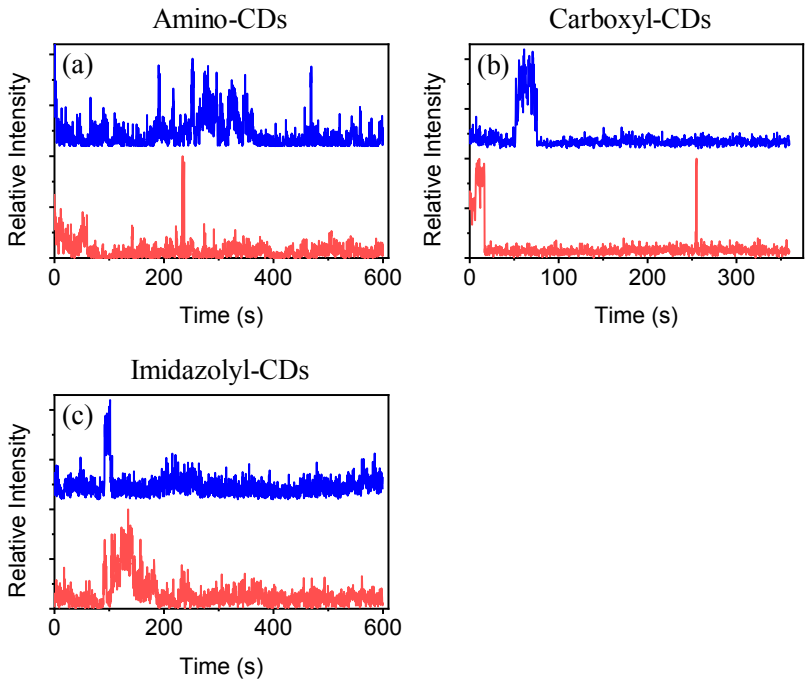


**Fig. S9** Single particle intensity trajectories for different CDs. Two random intensity trajectories change over time for single (a) Amino-CDs, (b) Carboxyl-CDs, (c) Imidazolyl-CDs. All CDs were excited with 532 nm laser. Exposure time were 287 ms.

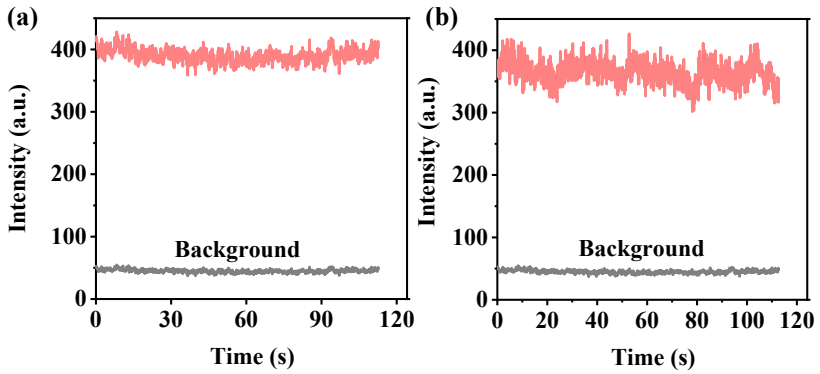


**Fig. S10** (a, b) Intensity trajectories for two random CDs (red) and background regions(grey).

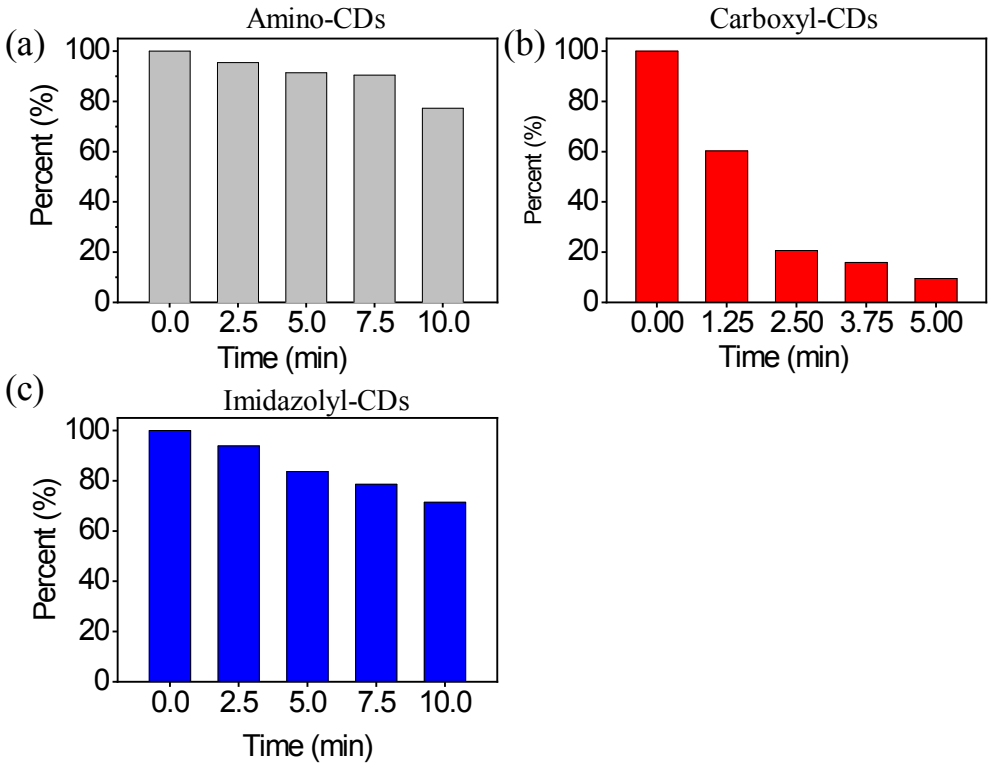


**Fig. S11** Number fraction of different CDs during continuous illumination. (a) Amino-CDs number change over time, (b) Carboxyl-CDs number change over time, (c) Imidazolyl-CDs number change over time. All CDs were excited with 532 nm laser. The number of CDs on 256 ×256 pixels$^2$ region was counted and normalized as a function of illumination time. Exposure time were 287 ms.

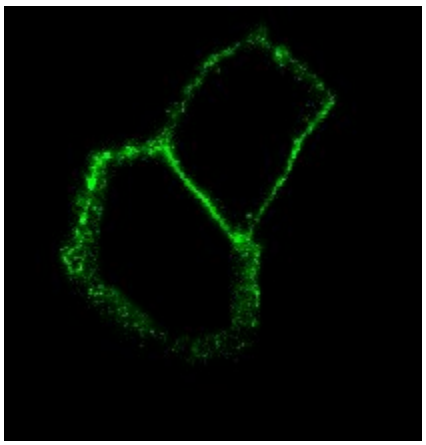

**Fig. S12** Confocal images of CXCR4-EGFP-transfected Hela cells, which indicates that the CXCR4-EGFP are mostly expressed on cell membrane. The EGFP was excited with 488 nm laser.

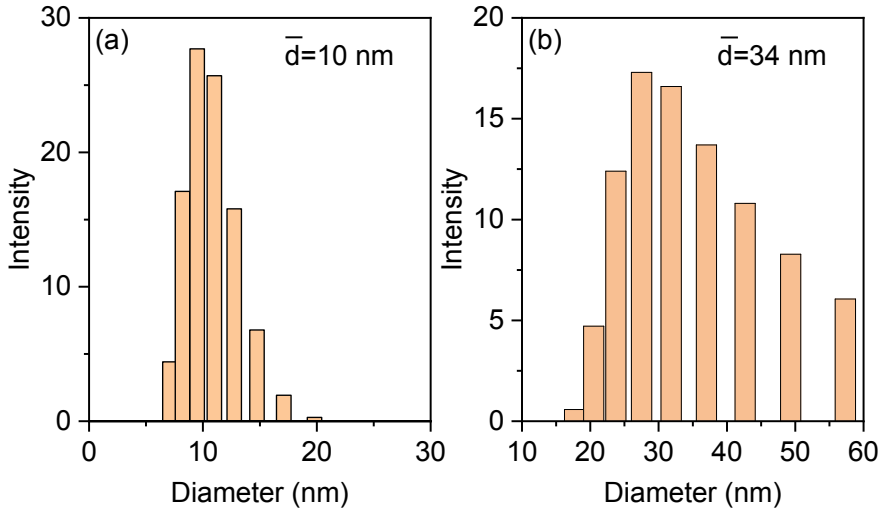


**Fig. S13** Dynamic light scattering results of (a) goat anti-mouse IgG, (b) goat anti-mouse IgG conjugates with CDs.

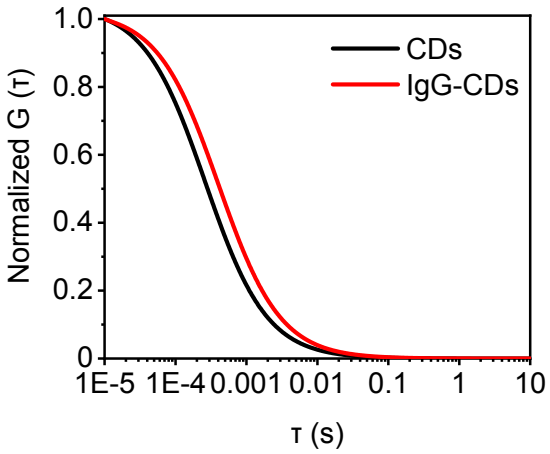


**Fig. S14** Normalized Fluorescence correlation spectra of CDs and IgG-CDs.

To confirm the binding ratio of CDs to IgG, we also performed the fluorescence correlation spectroscopy (FCS) measurement for the CDs and the CD-IgG conjugates. As discussed in our previous work,[1] FCS is an ideal tool for measuring molecular diffusion coefficients in a tiny volume.[7] The autocorrelation curve is expressed as $G(\tau) = \frac{1}{N} \cdot \frac{1}{\left(1+\frac{\tau}{\tau_d}\right)} \cdot \frac{1}{\sqrt{1+\left(\frac{\omega_0}{z_0}\right)^2 \frac{\tau}{\tau_d}}}$, where $N$ is the average number of fluorescent particles in the excitation volume, $\omega_0$ and $z_0$ are the lateral and axial radii at which the intensity of the focused beam is decreased by $e^{-2}$, respectively, $\tau_d$ is the characteristic diffusion time that relates to the diffusion coefficient $D$ by $\tau_D = {\omega_0}^2/4D$. For a spherical particle, $D$ is inversely proportional to its hydrodynamic radius R according to the Stokes-Einstein equation, $D = \frac{kT}{6\pi\eta R}$, where $k$ is the Boltzmann constant, $T$ is the temperature, and $\eta$ is the viscosity of the solution. Accordingly, $R$ is directly proportional to $\tau_d$. After conjugated to IgG, the $\tau_d$ value of the CDs increased from 519 to 716 μs, corresponding to a factor of ~1.38. Since the average size of CDs determined by TEM is ~ 21 nm, the size of IgG -CDs can be estimated as ~31 nm. The increase by ~10 nm corresponds approximately to the size of the IgG, which suggests a labeling ratio of ~1 between IgG and CDs.

## References


1. H. He, X. Liu, S. Li, X. Wang, Q. Wang, J. Li, J. Wang, H. Ren, B. Ge and S. Wang, *Analytical chemistry*, 2017, **89**, 11831-11838.
2. J.-Y. Tinevez, N. Perry, J. Schindelin, G. M. Hoopes, G. D. Reynolds, E. Laplantine, S. Y. Bednarek, S. L. Shorte and K. W. Eliceiri, *Methods*, 2017, **115**, 80-90.
3. H. He, X. Wang, Z. Feng, T. Cheng, X. Sun, Y. Sun, Y. Xia, S. Wang, J. Wang and X. Zhang, *Journal of Materials Chemistry B*, 2015, **3**, 4786-4789.
4. S. Khan, N. C. Verma, A. Gupta and C. K. Nandi, *Scientific reports*, 2015, **5**, 1-7.
5. N. C. Verma, S. Khan and C. K. Nandi, *Methods and applications in fluorescence*, 2016, **4**, 044006.
6. L. Bu, T. Luo, H. Peng, L. Li, D. Long, J. Peng and J. Huang, *Microchimica Acta*, 2019, **186**, 1-12.
7. P. Zhang, L. Li, C. Dong, H. Qian and J. Ren, *Analytica chimica acta*, 2005, **546**, 46-51.