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Electronic supplementary information

Co-assembled hybrids of proteins and carbon dots for intracellular protein delivery

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1 Materials and Methods

Materials. Chemicals and reagents were obtained from commercial sources without further purification. CDsG and CDsA were prepared following the protocol has been reported,[S1] and then dissolved in Milli-Q water. The EGFP was acquired following the protocol has been reported,[S2] and then dissolved in Milli-Q water. Milli-Q water was collected from a Milli-Q system (Millipore, USA).

Methods. The size distribution was performed using a Zeta-sizer Nano-ZS (Malvern Instruments Ltd.). Transmission electron microscopy (TEM) images were taken by a JEOL JEM-1011 (Japan) at the accelerating voltage of 100 kV. UV−vis absorption spectra were recorded via a Shimadzu UV-2450 UV−vis scanning spectrophotometer. Fluorescence emission spectra were conducted on a LS-55 fluorophotometer. The cell confocal images were obtained using Zeiss confocal laser microscope (ZEISS LSM 700). Flow cytometry analysis was performed by a flow cytometer (Beckman, USA).

2 Characterization

2.1 Preparation of CDs and CDs-protein nanoparticles.

CDsG and CDsA were prepared following the protocol has been reported,[S1] briefly, D-Glucose and L-glutamic acid (L-Aspartic acid) were loaded into a beaker at equivalent stoichiometric ratio and then 1 M sodium hydroxide (NaOH) was added. The transparent solution was heated to 125 ^{o}C and kept for 30 min, then heated to 200 ^{o}C and maintained for 20 min. The final reaction products were completely solubilized, and then subjected to dialysis against water to remove small molecules for 1 d. The resulting product was freeze-dried to obtain brown solid.

CDs-protein nanoparticles was achieved by simple mixing of CDs with proteins solution in water and shaking overnight at room temperature. After that, the free EGFP in solution was removed by filtration.

2.2 Protein stability against enzymatic digestion.

The role of CDs in protecting proteins from enzymatic cleavage was probed by trypsin digestion analysis. Briefly, trypsin aqueous solution (0.125%) was introduced into each free EGFP or CDsG-EGFP samples with the same EGFP concentration, and the enzyme reaction was performed at 37^{o}C for 6 h. All samples were mixed with loading buffer and heated to 100^{o}C for 10 min, and then all samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), to detect the amount of remainder proteins.

2.3 Cell culture.

HeLa cells were propagated to confluence in DMEM medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS, and maintained at 37^{o}C in a humidified atmosphere of 5% CO_2 for further cell experiments.

2.4 Biocompatibility of CDsG-EGFP in vitro by MTT Assay.

Cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 8×10^3 cells per well and incubated in DMEM for 24 h. The medium was then replaced by 200 μL of DMEM containing predetermined concentrations of CDs and EGFP and CDsG-EGFP, respectively, and then incubated for 24 h, followed by MTT assays to measure the live cells. Cell viabilities were determined by reading the absorbance of the plates at 490 nm with a microplate reader. The cells incubated with DMEM were used as the control. The cell viability (%) =A sample /A control ×100 %.

2.5 In vitro protein release behaviors of CDsG-EGFP.

To measure the release properties of CDsG-EGFP, CDsG-EGFP were dispersed in different solutions at 37°C and kept under oscillation at 100 r/min. Fluorescence of EGFP will recover upon release of EGFP from CDsG-EGFP. Therefore, we detected the fluorescence of solutions at selected time intervals to quantify the protein release ratio.

2.6 Cellular uptake in vitro.

The cellular uptake measurement was investigated by CLSM and flow cytometry. Cells harvested in a logarithmic growth phase were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated in DMEM for 24 h. The medium was then replaced by 2 mL of DMEM containing free EGFP or CDsG-EGFP with 10 μg/mL of EGFP and incubated for different hours at 37°C, and further washed using PBS for 3 times.

For the CLSM, the cells were fixed with 4% of paraformaldehyde solution for 10 min. After that, DAPI was added for another 5 min incubation to locate the nucleus. Later, the cells were washed with PBS and observed using confocal laser scanning microscopy (CLSM, Zeiss LSM 700).

For the flow cytometry, the cells were collected and re-suspended in 0.5 mL PBS 7.4. Flow cytometry analysis was performed by a flow cytometer (Beckman, USA) which collected 1×10^4 gated events for each sample.

2.7 Endocytosis pathway detection of CDsG-EGFP.

Cells harvested in a logarithmic growth phase were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated in DMEM for 24 h. Then, serum-free DMEM as the control and various inhibitors including sucrose (clathrin-mediated endocytosis, 450 mM), genistein (caveolin-dependent endocytosis, 100 μM) were used in serum-free DMEM for 1 h and 4°C culturing was used to inhibit energy-dependent mechanisms. Then, CDsG-EGFP were further added for 4 h incubation. Subsequently, the cells were collected and re-suspended in 0.5 mL PBS 7.4. Flow cytometry analysis was performed by a flow cytometer (Beckman, USA) which collected 1×10^4 gated events for each sample.

2.8 Statistical analysis.

Quantitative data were expressed as mean ± standard deviation. ANOVA analysis and

Student's t test were utilized for statistical contrast. P < 0.05 was figured statistically significant and used asterisks (***,** and *) to indicate $p < 0.001$, 0.01 and 0.05, respectively.

3 Results

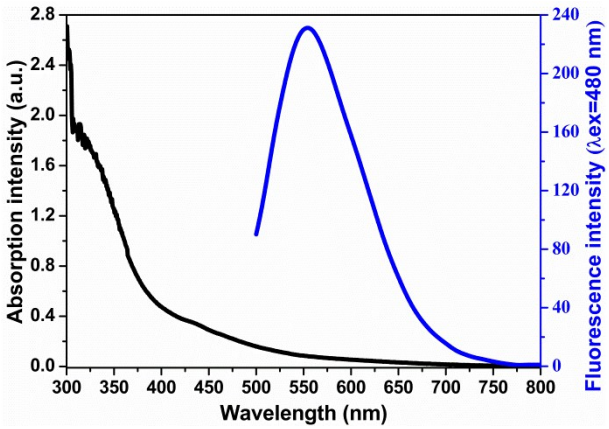

Fig. S1 Absorption and PL (excited at 480 nm) spectra of CDsG.

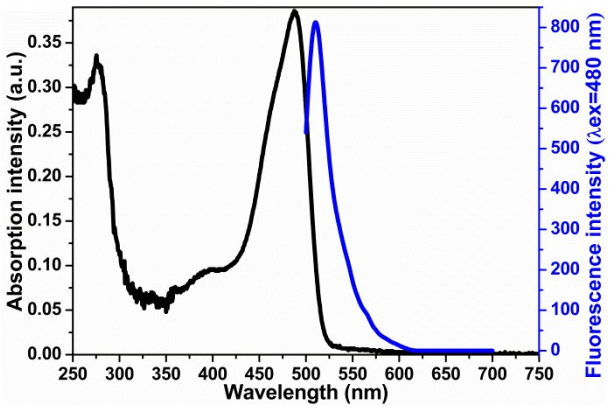

Fig. S2 Absorption and PL (excited at 480 nm) spectra of EGFP.

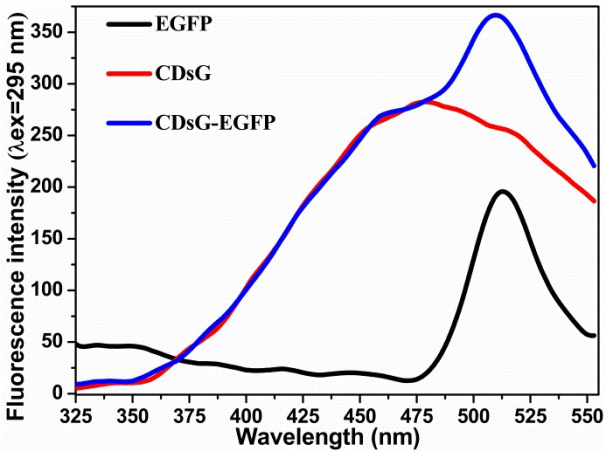

Fig. S3 PL (excited at 295 nm) spectra of EGFP, CDsG and CDsG-EGFP.

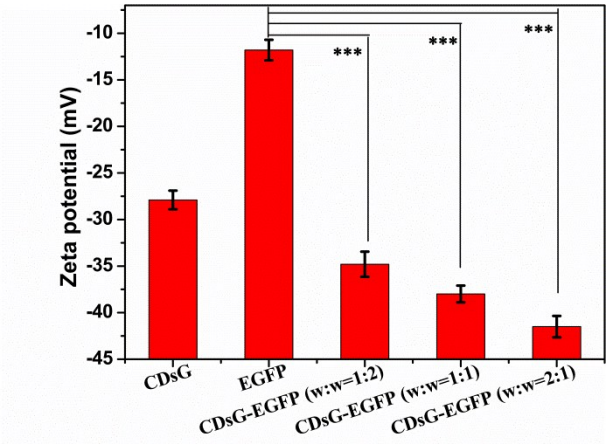

Fig. S4 Zeta potential of CDsG, EGFP and CDsG-EGFP with different weight ratio between CDsG and EGFP. Data represent mean values ± standard deviation, n=3. The analysis of variance is completed using a one-way ANOVA. Asterisks (***, ** and *) indicate $p <$ 0.001, 0.01 and 0.05, respectively.

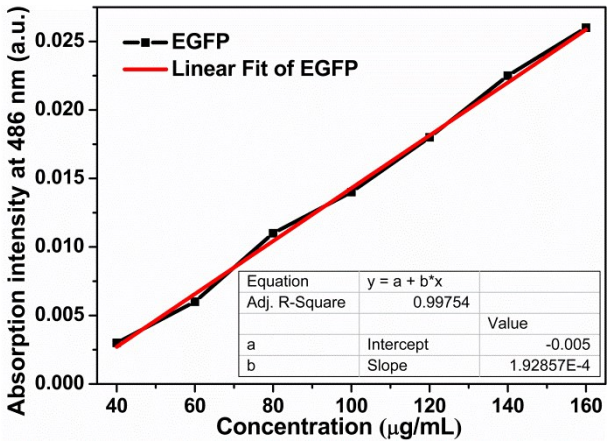

Fig. S5 Standard absorbance curve of EGFP. (The absorbance of EGFP at 486 nm in water as a function of EGFP concentration.)

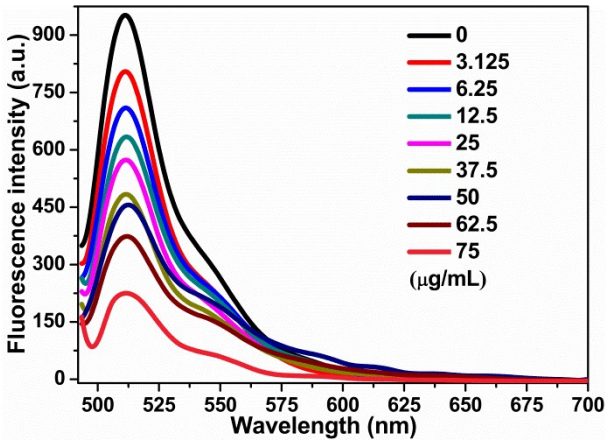

Fig. S6 PL spectra of EGFP (25 μg/mL) excited at 480 nm during the course of a titration with CDsG (0-75 μg/mL) in deionized water at room temperature**.**

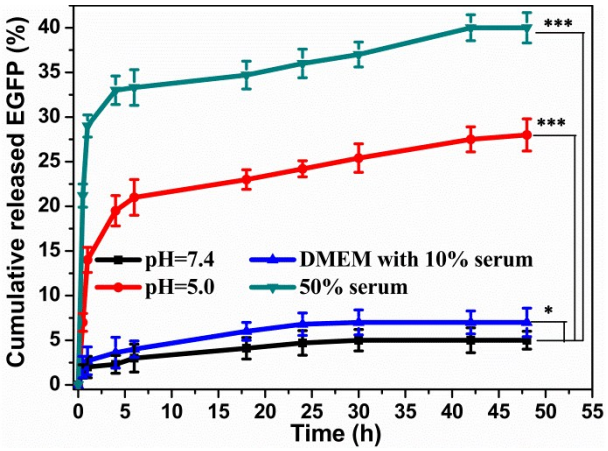

Fig. S7 EGFP release profile of CDsG-EGFP in different environments. Black line: water (pH=7.4); red line: water (pH=5.0); blue line: DMEM with 10% serum; green line: water containing 50% serum. Data represent mean values ± standard deviation, n=3. The analysis of variance is completed using a one-way ANOVA. Asterisks (***, ** and *) indicate $p < 0.001$, 0.01 and 0.05, respectively.

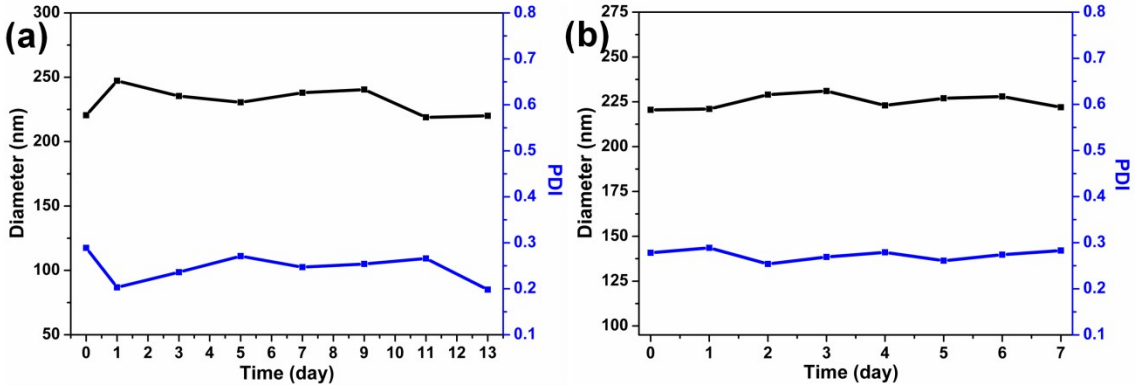

Fig. S8 Size-stability of CDsG-EGFP dispersed in water (a) and PBS (b) over different days.

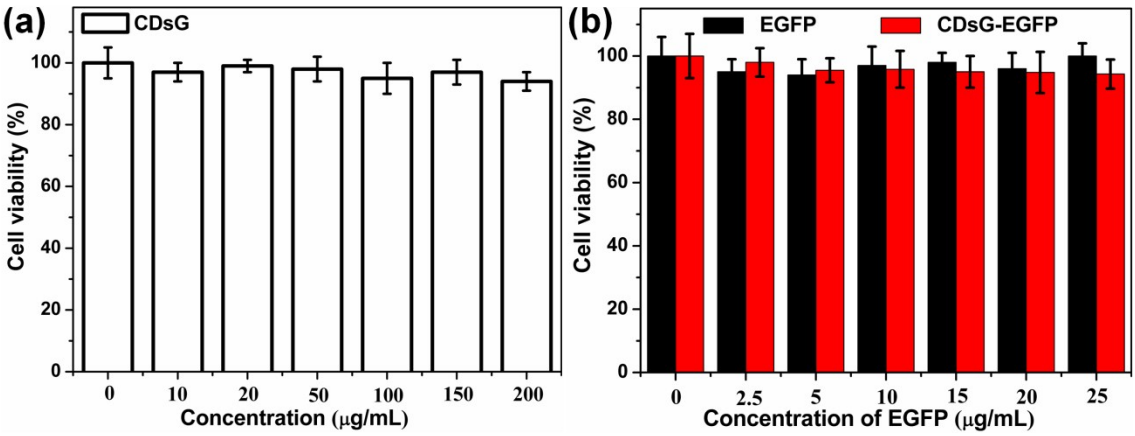

Fig. S9 In vitro cytotoxicity of CDsG, EGFP and CDsG-EGFP incubated in HeLa cells with different concentration for 24 h. Data represent mean values ± standard deviation, n=3. The analysis of variance is completed using a one-way ANOVA. Asterisks (***, ** and *) indicate $p < 0.001$, 0.01 and 0.05, respectively.

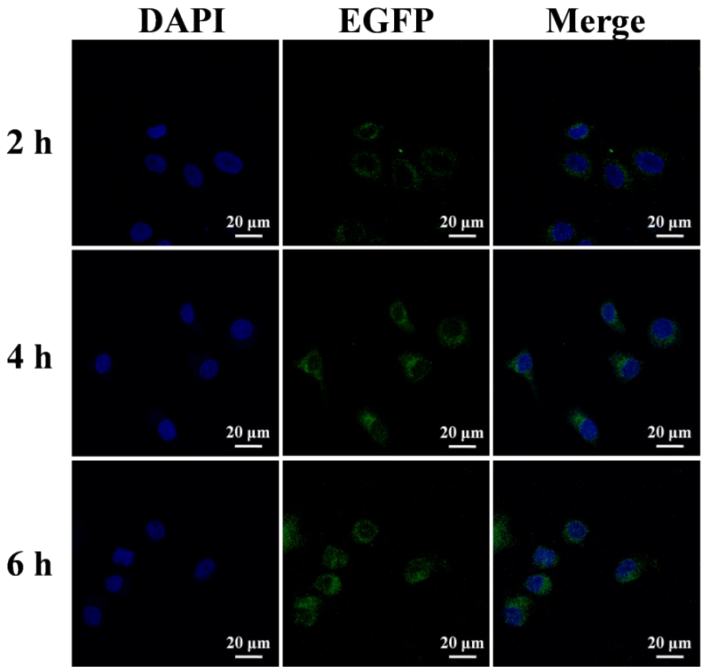

Fig. S10 Confocal microscopy images showing changes in the signal of EGFP in HeLa cells treated with CDsG-EGFP at 2, 4, and 6 h. Different imaging channels are displayed horizontally for each sample (from left to right): DAPI (405 nm excitation), EGFP channel (488 nm excitation), and merged images.

Fig. S11 Relative intensities of EGFP from CDsG-EGFP internalized by HeLa cells treated with serum-free DMEM (control), sucrose (450 mM), genistein (100 μM) at 37°C and serum-free DMEM at 4°C using flow cytometry. Data represent mean values ± standard deviation, n=3. The analysis of variance is completed using a one-way ANOVA. Asterisks (***, ** and *) indicate $p < 0.001$, 0.01 and 0.05, respectively.

Reference

S1. M. Zheng, S. Ruan, S. Liu, T. Sun, D. Qu, H. Zhao, Z. Xie, H. Gao, X. Jing and Z. Sun, *ACS nano*, 2015, **9**, 11455-11461.

S2. X. Guan, C. Li, D. Wang, W. Sun and X. Gai, *RSC Adv.*, 2016, **6**, 9461-9464.