Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information for

Ultrafast Synthesis of Nitrogen-Doped Carbon Dots via Neutralization Heat for Bioimaging and Sensing applications

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1. Materials and instrumentations

Glucose, ethylenediamine, phosphoric acid, ethanol, citric acid, sucrose and boronic acid (BA) were analytically pure and obtained from the local chemical reagent company. Phenylboronic acid (PBA) was purchased from Heowns Business License. Graphene oxide (GO) was purchased from Jicang Nano Technologies, Nanjing, China. Ovalbumin was obtained from Sigma-Aldrich, Shanghai, China. Cytochrome c, hemoglobin from bovine blood and myoglobin from equine are purchased from Solarbio Technologies. Milli-Q grade water was used throughout.

Transmission electron microscopy (TEM) images were recorded with Tecnai G2 F20, FEI Co. (America) operated at an accelerating voltage of 200 kV. The size distribution of N-Cdots was measured by calculating more than 200 particles. Infrared spectra were measured by the Bruker TENSOR 27 Fourier Transform Infrared Spectroscopy. X-ray photoelectron spectroscopy (XPS) analysis was performed by Kratos Axis Ultra DLD spectrometer fitted with a monochromatic Al K X-ray source (hv 1486.6 eV), hybrid (magnetic/electrostatic) optics, and a multi-channel plate and delay line detector. Fluorescence spectra were performed by a Hitachi FL-4500 fluorescence spectrometer. UV-Vis absorption spectra of N-Cdots were recorded by a UV-2450-visible spectrophotometer (Shimadzu, Japan) with 1×1 cm quartz cuvette along 1 cm length. The development of zebrafish embryo cultured with Cdots was observed with a fluorescence microscope (Olympus BX51, Japan) equipped with a digital camera. Confocal imaging of zebrafish was performed with a Leica Tcs SP5 confocal microscope (Germany) with the filter of excitation and emission at 488 nm and 520 nm. The temperature was measured by a mini infrared thermometer (Hongchen Electronic Technology, China).

2. Experimental section

2.1. Preparation of N-Cdots

N-Cdots were synthesized using neutralization heat, with glucose as the precursor. In brief, 200 mg of glucose was dissolved in 15 ml of ethylenediamine, and then 8 ml of phosphoric acid was added to the mixture with vigorous stirring to generate reaction heat. The mixture was stirred until it displayed a uniform color of pale yellow. 20 ml of water was added in the mixture, which was then centrifuged at 12 000 rpm for 10 min. The yellow-brown supernatant was collected. The N-Cdots was purified by adding 30 ml of ethanol to the solution, and collecting the supernatant by centrifugation at 12000 rpm for 10 min. [1] Ethanol and excess ethylenediamine were removed by rotary evaporation. The solution was then further

purified by dialyzing with a dialysis membrane (MWCO=1000) against water; the N-Cdots were obtained using vacuum lyophilization technique and could be dissolved again for biosensing and bioimaging.

2.2 Preparation of Boronic acid (BA)- and phenylboronic acid (PBA)-modified Cdots

20 mg of N-Cdots was dissolved in 10 mL of phosphate buffer solution (10 mM, pH 8.0), then 10 ml of 100 mM BA or 30 mM PBA solutions was added. The mixture was shook for 5 min to mix well and stood for 30 min. Then the solution was purified by dialyzing against water; BA-Cdots and PBA-Cdots were collected by the vacuum lyophilized technique.

2.3. Animal model

Zebrafish was used as a model in this study. All procedures that used animals were approved by the Institutional Animal Care Committee of Nankai University. Zebrafish were cultured in aquaria at 28.5°C with a 10/14-hour dark/light cycle. Embryos were collected after culturing for 14 h at 28.5°C. The embryo and larva are staged by hour post fertilization (hpf). All embryos were grown in 0.003% 1-phenyl-2-thiourea (PTU, Sigma-Aldrich, St. Louis, MO, USA) to prevent pigmentation from generating and mediate visualization until 72 hpf.

2.4. Fluorescence imaging of zebrafish embryos

AB strain fertilized embryos were cultured in regular tank water at 28.5 °C. The embryos were added in 24-well plates (15–20 embryos per well). N-Cdots, BA-Cdots and PBA-Cdots were added to each well with the concentration at 1.2 mg mL^{-1}. After cultured for 2 h, zebrafish embryos were rinsed with tank water three times for 5 min each to remove excess Cdots. The fluorescent images of the embryos were taken at 2, 9, 24, 32, 48, 72 hpf with a fluorescence microscope (Olympus BX51, Japan), of which excitation and emission are 470–490 nm and 515 nm long passes. Confocal imaging of zebrafish embryos at 2 hpf was performed with a Leica Tcs SP5 confocal microscope (Germany) with the filter of excitation and emission at 488 nm and 520–620 nm, respectively.

2.5. Sensing ovalbumin with PBA-Cdots and BA-Cdots as probes

10 ml of PBA-Cdots or BA-Cdots solution at the concentration of 0.1 mg mL^{-1} was fully mixed with 10 ml of 0.02 mg mL^{-1} graphene oxide (GO) solution. Ovalbumin was dissolved in phosphate buffer solution (10 mM, pH 7.4) with the final concentration at 0, 0.022, 0.044, 0.22, 0.88, 1.76, 2.2, 3.52, 4.4 mg mL^{-1}. Then 1 mL of Cdot-GO solution was mixed with 1 ml of ovalbumin at different concentrations. The fluorescence intensity was measured in 10 min.

3. Results and discussions

3.1. Optimization of N-Cdots synthesis

There are two key points in transforming glucose into N-Cdots with neutralization heat. One is the types of base and acid used as the heat source, and the other is the optimal ratios of glucose, base and acid. For the sake of safety and to obtain high heat generation, concentrated phosphoric acid was selected as the acid because of its appropriate acidity, and low volatility and corrosivity. Three types of bases, i.e. ethylenediamine, NaOH, and triethylamine, were used to investigate the effects of different bases on the emissions of the prepared Cdots. The fluorescence intensities of the Cdots obtained using the three bases were quite different, with maximum emission wavelengths of 498 nm, 468 nm and 454 nm, for the use of ethylenediamine, NaOH, and triethylamine, respectively (Fig. S1a). When NaOH was replaced by ethylenediamine, a 10-fold enhancement in emission was observed (Fig. S1a). The Cdots derived from triethylamine showed the lowest emission.

The neutralization heat carbonized the glucose; ethylenediamine, a primary amine containing two free amino groups, which can link to glucose, was used as a passivation reagent to enhance the fluorescence intensities of the Cdots similar to those of hydrothermal Cdots. [2-5] Triethylamine is a tertiary amine with three ethyl groups linked to the nitrogen atom and no active site that can react with glucose for the formation of N-Cdots; less heat is released from triethylamine, which could result in the incomplete formation of Cdots. NaOH has the strongest alkalinity, so it released the highest heat. However, NaOH does not contain nitrogen, so the species prepared using NaOH are “undoped” Cdots, similar to the nitrogen-free Cdots prepared via hydrothermal procedures with glucose as the precursor. [6] Such Cdots have fluorescence, but the emission is low. [6] N-Cdots are often brighter than undoped Cdots. [7-10] We therefore speculated that nitrogen atoms were successfully doped into the Cdots, enhancing their fluorescence.

Once the base and acid for the preparation of N-Cdots were selected, their ratios were optimized using a simple variable method. First, 8 ml of phosphoric acid and 15 ml of ethylenediamine were used while the amount of glucose was tested in the range of 100–800 mg. As shown in Fig. S1b, the fluorescence intensity of N-Cdots from 200 mg glucose achieved the maximum fluorescence. Therefore, the amount of glucose of 200 mg was selected for further experiments. The ratio of glucose/phosphoric acid was fixed at 200 mg/8 ml, while the amount of ethylenediamine was varied from 5~25 ml. The fluorescence intensity increased at first, but decreased gradually with the maximum emission at 15 ml (Fig. S1c). Thus, the ratio of

glucose/ethylenediamine was confirmed to be 200 mg/15 ml for further experiment. The amount of phosphoric acid was also optimized with the optimal ratio of glucose/ethylenediamine. When 8 ml phosphoric acid was used, the maximum fluorescence intensity appeared (Fig. S1d). Therefore the final ratio of glucose/ethylenediamine/phosphoric acid was confirmed to 200 mg/15ml/8 ml.

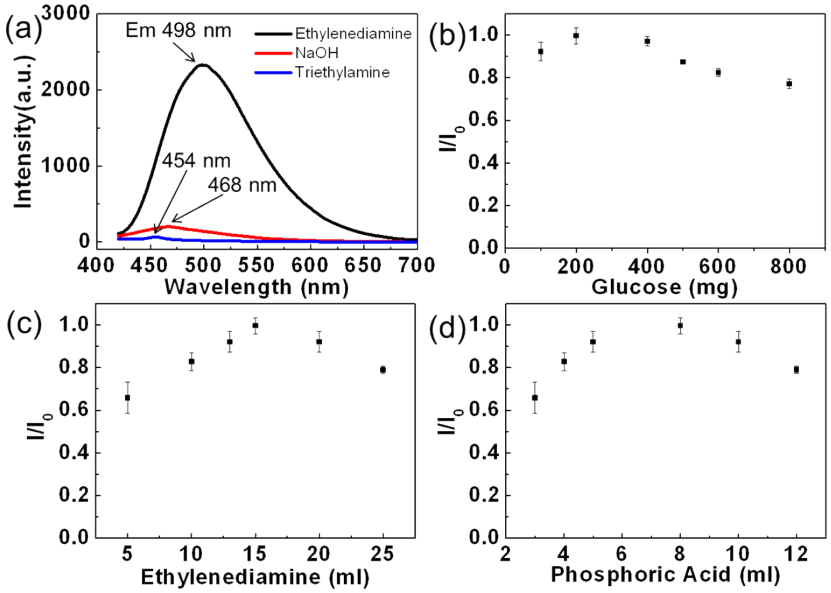

Fig. S1. (a) Fluorescence spectra of Cdots derived from ethylenediamine (15 ml), NaOH (17.92 g), and triethylamine (62 ml) with 8 mL of phosphoric acid and 200 mg of glucose as precursor. Relative fluorescence intensities of Cdots prepared with (b) different amount of glucose, 15 ml of ethylenediamine, and 8 ml of phosphoric acid, (c) different amount of ethylenediamine, 200 mg of glucose, and 8 ml of phosphoric acid, (d) different amount of phosphoric acid, 200 mg of glucose and 15ml of ethylenediamine. All the values are the strongest fluorescence intensities under excitation at 400 nm.

3.2. Characterization of N-Cdots

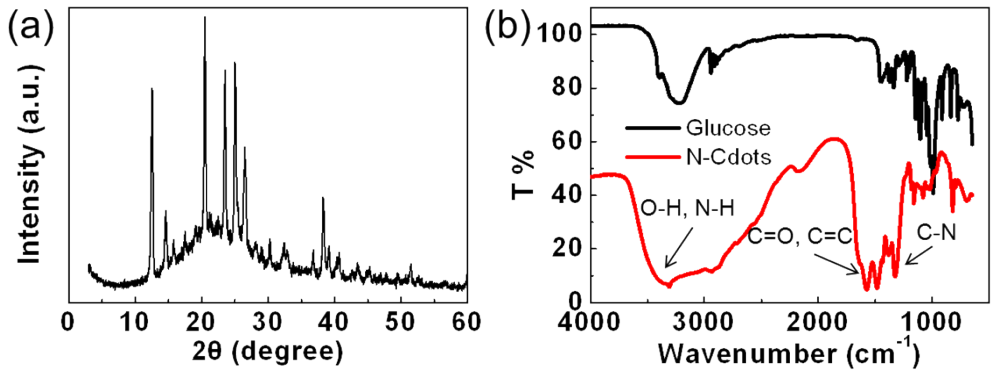

Fig. S2. (a) XRD pattern of N-Cdots; (b) FTIR spectra of N-Cdots and glucose.

3.3. Boronic acid (BA) and phenylboronic acid (PBA) mediated fluorescence enhancement.

To confirm the specificity of the interactions, the emissions from Cdots prepared via hydrothermal approach were tested with and without BA. The fluorescence of Cdots derived from citric acid by the neutralization heat and those from hydrothermal treatment of glucose are not enhanced by BA or PBA (Fig. S3), indicating that those Cdots cannot coordinate with BA or PBA. Citric acid does not have adjacent hydroxyl group; thus BA or PBA has no affinities with citric acid-derived Cdots. Similarly, the fluorescence of hydrothermal Cdots from glucose was not enhanced by BA and PBA, because the hydrothermal process lasted for 12 h; the adjacent hydroxyl group structures were destroyed.

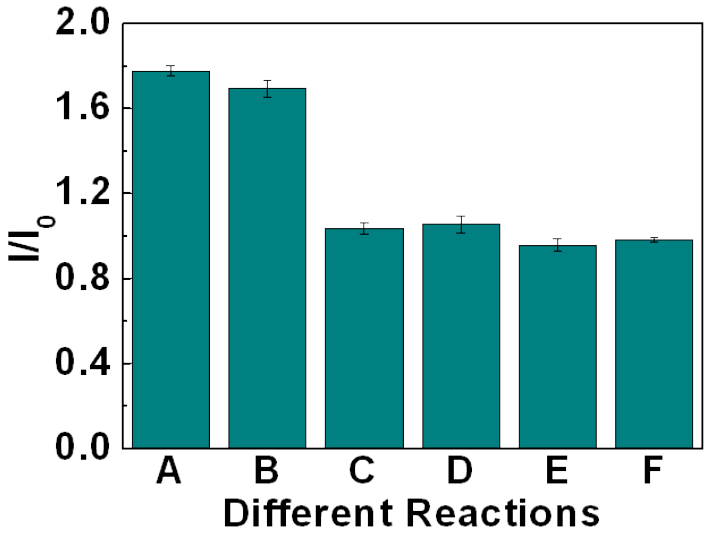

Fig. S3. Fluorescence changes of different Cdots reacted with BA or PBA. (A) N-Cdots mixed with BA; (B) N-Cdots mixed with PBA; (C) citric acid-derived Cdots mixed with BA; (D) citric acid-derived Cdots mixed with PBA; (E) glucose-derived Cdots, obtained via the hydrothermal method for 12 h, mixed with BA; and (F) glucose-derived Cdots, obtained via the hydrothermal method for 12 h, mixed with PBA.

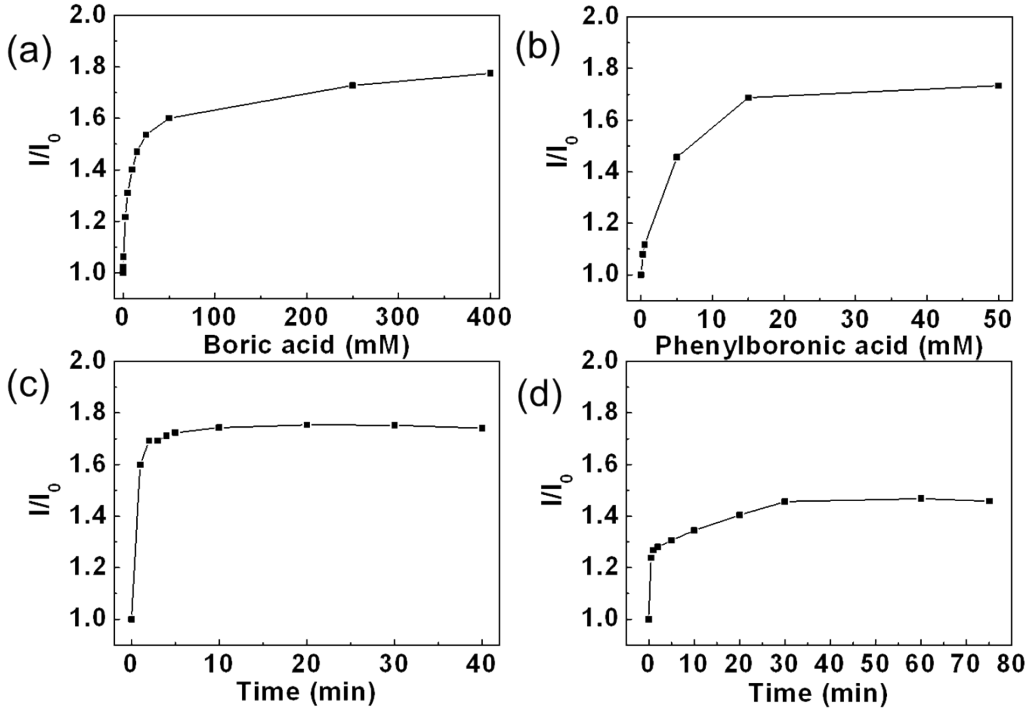

Fig. S4. Concentration-dependent fluorescence of N-Cdots with (a) BA and (b) PBA. Time-dependent fluorescence of N-Cdots with (c) BA (250 mM) and (d) PBA (15 mM) under excitation at 400 nm.

3.4. Influence of photo, salt and pH on the emission of N-Cdots

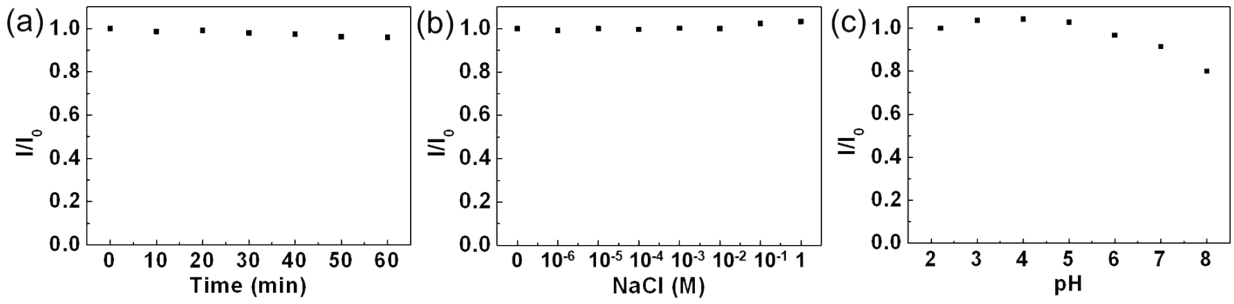

Fig. S5. (a) Time-dependent emission of N-Cdots at different illumination time; (b) Salt concentration-dependent emission of N-Cdots with different concentrations of sodium chloride; (c) pH-dependent emission of N-Cdots in disodium hydrogen phosphate-citric acid buffer at the value of 2~8. The concentration of N-Cdots was 0.05 mg mL^{-1}.

3.5. Fluorescent imaging of zebrafish embryo

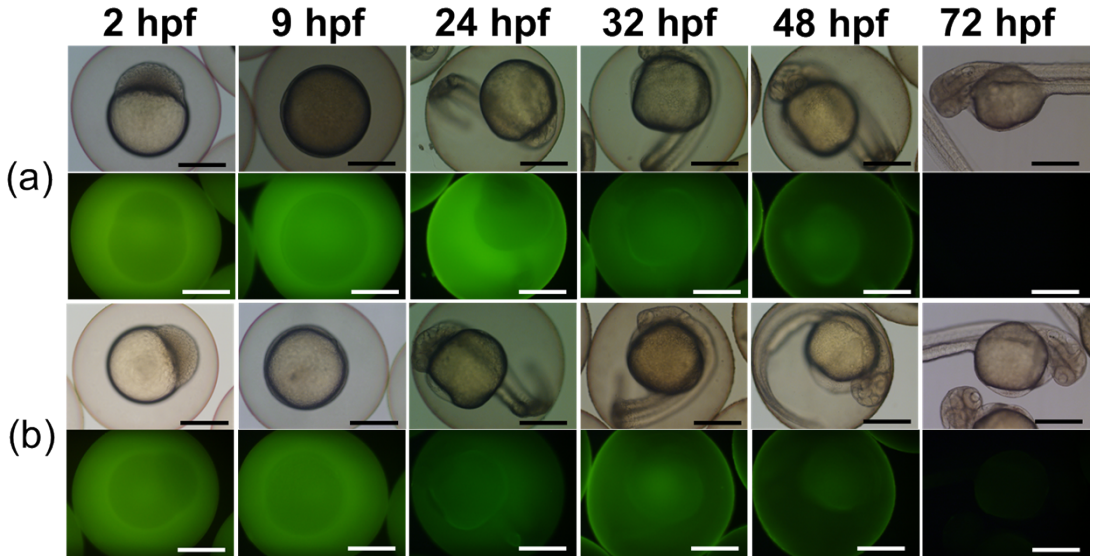

Fig. S6. Bright field (upper) and fluorescent images (lower) of zebrafish embryos at different growth stages, cultured with: (a) N-Cdots, and (b) BA-Cdots. The concentrations of N-Cdots and BA-Cdots were 1.2 mg mL^{-1}. Scale bar: 500 μm.

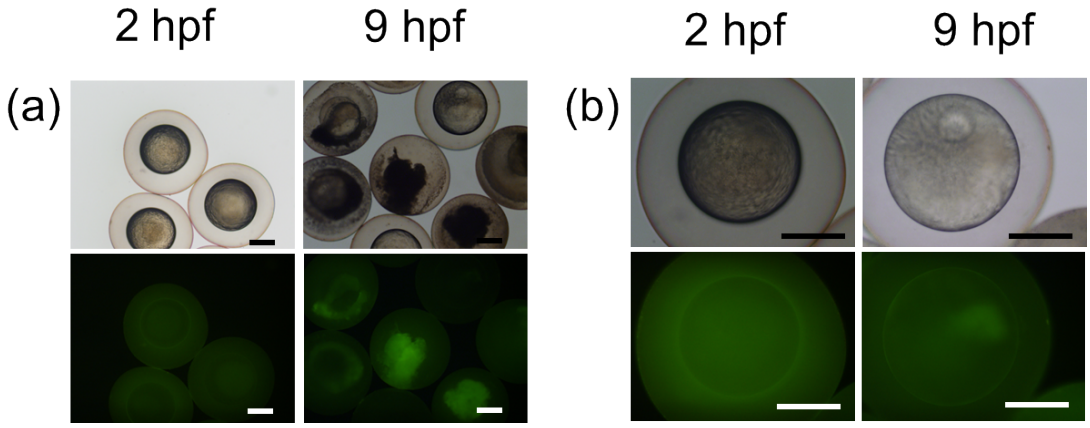

Fig. S7. Bright field and fluorescent images of zebrafish embryo at different growing stages cultured with PBA-Cdots. Scale bar: 500 μm.

3.6. Cytotoxicity study

The cell viability was evaluated on the 4T1 cells using CCK8 assay. Briefly, the cell was seeded into 96-well culture plates in culture medium at a density of 5×10^5 cells per well. Cdots were diluted at concentration of 0.01, 0.05, 0.50, 1.00 mg mL^{-1} and introduced to cells to incubate of 24 hour after 4T1 cells reached 90-95 % confluence. Cells cultured in the free medium were taken as positive control, while Triton x-100 was added to medium as negative control. 10 μL CCK-8 was added to each well and

incubated for another 1 h. The cell incubation process was carried out at 37 °C with 5 % CO_2. The absorbance at 490 nm was measured for the calculation of the cell survival rate. The experiment was performed under identical conditions three times. The cytotoxicity study showed that N-Cdots and BA-Cdots had the similar cell viabilities and were suitable for bioimaging.

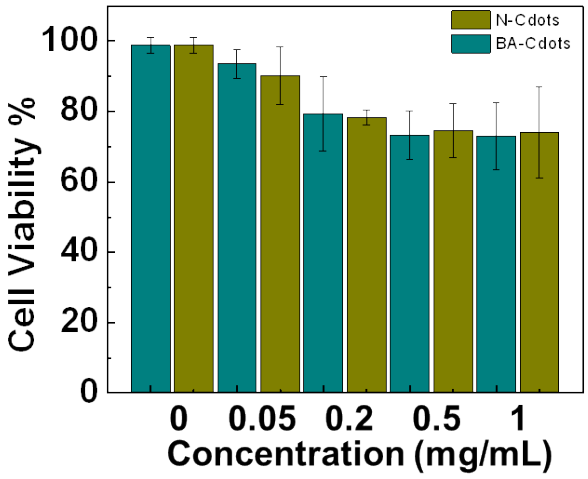

Fig. S8. Cell viability of 4T1 cells incubated with N-Cdots and BA-Cdots. Data were presented as the mean ± the standard deviation (SD).

3.7. Reactions of ovalbumin with BA-Cdots and PBA-Cdots

Ovalbumin showed weak fluorescence under the same excitation wavelength (Fig. S9a), and its background fluorescence had been deducted. In the absence of GO, the fluorescence intensity increased little when ovalbumin was added to PBA-Cdots or BA-Cdots (Fig. S9b and c). The maximum improved efficiency was found to be improved only 0.03 at the ovalbumin concentration of 2.2 mg ml^{-1} because of the surface energy transfer.

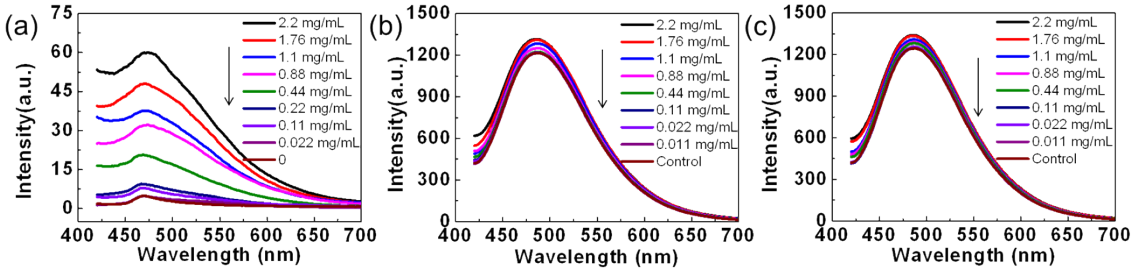

Fig. S9. Fluorescence spectra of: (a) ovalbumin, (b) the mixture of PBA-Cdots and ovalbumin; (c) the mixture of BA-Cdots and ovalbumin with the concentration of ovalbumin varied from 0 to 2.2 mg mL^{-1} under the excitation of 400 nm.

3.8. Fluorescence efficiencies of BA- and PBA-Cdots with GO

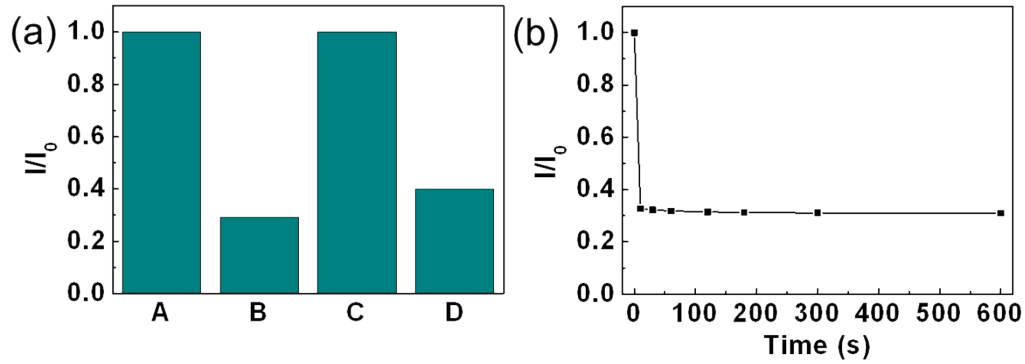

Fig. S10. (a) Fluorescence efficiencies of BA-Cdots and PBA-Cdots with and without GO; (A) BA-Cdots without GO; (B) BA-Cdots with GO; (C) PBA-Cdots without GO; (D) BA-Cdots with GO;. (b) Time-dependent fluorescence of GO/BA-Cdots. The concentrations of BA-Cdots and PBA-Cdots were 0.05 mg ml^{-1}, and that of GO was 0.01 mg ml^{-1} under excitation at 400 nm.

3.9. Selectivity of PBA-Cdots with other proteins

Four proteins were chosen to react with GO/PBA-Cdots, only ovalbumin could recover its fluorescence (Fig. S11) and could be used to build a "turn on" biosensor for ovalbumin.

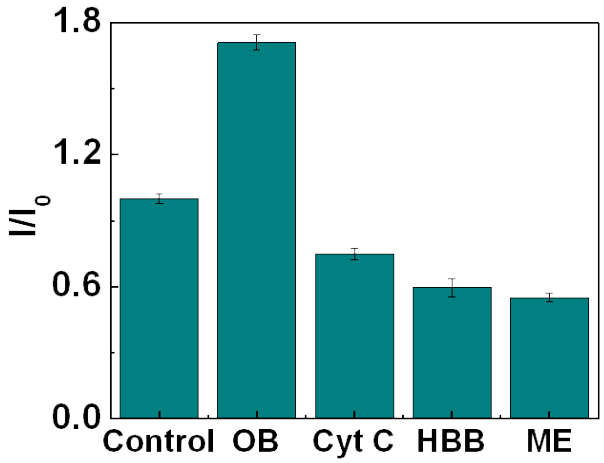

Fig. S11. PBA-Cdots/GO sensor treated with different proteins, ovalbumin (OB), cytochrome c (Cyt C), hemoglobin from bovine blood (HBB), Myoglobin from equine (ME) under the excitation of 400 nm.

3.10. Quantum yield of N-Cdots , BA-Cdots, PBA-Cdots

Quinine sulfate dissolved in 0.1 M H_2SO_4 (with the known quantum yield of 0.54 at 360 nm) was chosen as a standard to calculate the quantum yield of Cdots with the following equation:

$$\Phi_x=\Phi_{std}(I_x/I_{std})(\eta^2_x/\eta^2_{std})(A_{std}/A_x)$$

Φ stands for quantum yield, I is the measured integrated emission intensity, η is the refractive index,

and A is the optical density. The subscript std refers to the referenced fluorophore with known quantum yield. In order to minimize re-absorption effects, absorption was kept below 0.05 at the excitation wavelength of 360 nm.

Table S1. Fluorescence quantum yield of N-Cdots , BA-Cdots and PBA-Cdots.

Sample	*I*	*Abs*	η	*QY*
Quinine sulfate	736.855	0.011	1.33	0.54 (known)
N-Cdots	29.463	0.011	1.33	0.02
BA-Cdots	64.703	0.011	1.33	0.05
PBA-Cdots	60.894	0.011	1.33	0.04

3.11. Comparison with other methods

Table S2. Fluorescence quantum yield of N-Cdots , BA-Cdots and PBA-Cdots.

Method	Limit of Detection	Linear Range	Reference
Counter immune electrophoresis	0.31 mg mL^{-1}	——	[11]
Rolling circle amplification	——	10^{-12}-10^{-7} g/ml	[12]
Suspension arrays	0.25 ng/mL	2 orders of magnitude	[13]
Western blotting technique	0.5 ng ml^{-1}	0.0005-0.033 mg ml^{-1}	[13]
BA-Cdots/GO biosensor	8 μg mL^{-1}	0.022–1.76 mg mL^{-1}	This work

4. Supplementary Reference

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