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Fluorescent carbon dots with tunable negative charges for bio-imaging in bacterial viability assessment

Fang Lu [a, 1], Yuxiang Song [b, 1], Hui Huang [b], Yang Liu [b], Yijun Fu [b], Jian Huang [c], Hao Li [b, **], Huihua Qu [a, ***], Zhenhui Kang [b, *]

[a] School of Basic Medical Sciences, Beijing University of Chinese Medicine, Beijing 100029, China
[b] Jiangsu Key Laboratory for Carbon-Based Functional Materials & Devices, Institute of Functional Nano & Soft Materials (FUNSOM), Soochow University, 199 Ren'ai Road, Suzhou, 215123, Jiangsu, PR China
[c] School of Biology & Basic Medical Sciences, Soochow University, 199 Ren'ai Road, Suzhou, 215123, Jiangsu, PR China

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ABSTRACT

Applying bio-imaging in bacterial viability assessment is vital to human health. Nitrogen, phosphorus co-doped carbon dots (NPCDs) with high visible fluorescence and tunable negative charges were synthesized by a feasible hydrothermal reaction. The as-prepared NPCDs possess abundant surface functional groups, excellent photostability and bio-compatibility. Especially, the surface negative charges of NPCDs can be adjusted by changing the kind of the precursors. The obtained NPCDs as a fluorescent dye could selectively stain dead bacteria instead of live ones due to electrostatic repulsion since the cell walls of bacteria possess negative charges. The NPCDs with less negative charges provides a low accurate mortality rate due to a weaker repulsion, while NPCDs with the most negative charges give a comparable mortality rate with traditional plate counting method but consumes shorter time. Our study suggests that NPCDs with highly negative charges could serve as a precise and efficient fluorescent probe to determine the living state of bacteria, widening the path for bio-imaging application in bacterial viability assessment.

1. Introduction

The assessment of bacterial viability is vital to human health. Hence, it is of high privilege to develop a fast and simple route to assess bacterial viability [1–3]. Generally, an established plate counting method is the most widely applied way to distinguish live or dead bacteria, which can also give an accurate mortality rate [4]. However, several hours or days of bacteria incubation are required using this method. Considering the cell walls of both Gram-positive and Gram-negative bacteria possess negative charges, it is possible to apply bio-imaging in bacterial viability assessment using a fluorescent dye with negative charges [5].

Nanomaterials are being widely studied in recent years [6–9]. Carbon dots (CDs), which are emerging as a novel fluorescent nanomaterial for their widespread applications in substantial areas, have aroused considerable attention owing to their dramatic properties including favorable photoluminescence (PL), excellent chemical stability and catalytic activity, water solubility and biocompatibility [10–14]. One of the most notable feature of CDs is that they can be easily surface functionalized, so that new dopant elements can be employed for the purpose of enhanced properties. The doped elements in CDs that can provide additional lone pairs of electrons or unoccupied orbital, such as nitrogen (N) and phosphorus (P), are ideal dopants to improve electronic and optical properties of CDs. Moreover, N could introduce defect sites by replacing carbon atoms, providing potential active sites to enrich chemical activities of CDs [15–18]. A series of papers have reported versatile applications of doped CDs in photocatalysis, electrocatalysis, sensors, cell imaging *etc.*, as well as the methods to synthesize CDs with N and/or P as dopants such as heating urea-derived C_3N_4 and ethylenediamine, pyrolysis of the mixture of urea and phosphoric acid, heating ammonium citrate *etc.* [19–23] However, to the best of our knowledge, studies of the relationship between the ratio of dopants and the surface charges of doped CDs have not been reported so far. Moreover, the doped CDs with

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: lihao000000@163.com (H. Li), quhuihua@gmail.com (H. Qu), zhkang@suda.edu.cn (Z. Kang).
[1] Authors contributed equally to this work.

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negative charges may be an ideal dye *via* bio-imaging in bacterial viability assessment.

Herein, we introduced that the obtained NPCDs as a fluorescent dye could selectively stain dead bacteria instead of live ones due to electrostatic repulsion, while NPCDs with more negative charges give a comparable mortality rate with traditional plate counting method but consumes shorter time. The desired NPCDs were synthesized by a one-step hydrothermal reaction of ethylenediamine and yeast extract. Compared with existing methods, the yeast extract we used is directly derived from yeast fungus, leading to an excellent bio-compatibility (over 80% alive with 500 μg/mL). The as-prepared NPCDs showed strong fluorescence and negative charges, which can be tuned by changing the involved precursor. More importantly, after a short time of incubation with *E. coli* (2 h), NPCDs can selectively stain dead bacteria rather than live ones, while the more negative surface charges gave the more accurate mortality rate (36.1%), which could compete with the traditional time-consuming plate counting method (37.6%). It is suggested that NPCDs with highly negative surface charges could serve as an efficient and accurate fluorescent probe to determine the exact mortality rate of bacteria in a short time, and widen the path to apply bio-imaging in bacterial viability assessment.

2. Experimental

2.1. Materials and characterization methods

All the chemical reagents were purchased from Sigma-Aldrich, and they were used as received. The HeLa cell line was purchased from the Cell Bank of Chinese Academy of Science.

Transmission electron microscopy images were obtained from a FEI/Philips Tecnai G^2 F20 TWIN TEM. Fourier transform infrared (FT-IR) spectra were characterized by a Bruker Fourier Transform Infrared Spectrometer (Hyperion), while the ultraviolet–visible (UV–vis) measurement used a Perkin Elmer UV–vis spectrophotometer (Lambda 750). The photoluminescence (PL) spectra were acquired using a Horiba Jobin Yvon (Fluoro Max-4) luminescence spectrometer. X-ray photoelectron spectroscopy (XPS) analysis was conducted on a KRATOS Axis ultra-DLD X-ray photoelectron spectrometer. Dynamic light scattering and zeta potential of NPCDs was measured using a ZEN3690 zetasizer (Malvern, U.K.). Laser scanning confocal microscopy (LSCM) images were collected by a Laser-scanning confocal fluorescence microscope (Leica, TCS-SP5).

2.2. Synthesis of NPCDs

Using four brand-new Teflon-lined autoclaves with the same volume (10 mL), 0.1 g yeast extract was dissolved into 6 mL deionized water each. Then four different substances were added into the solution following the sequence below:

Solution-1: 0,1 g ethylenediamine into the solution of yeast extract;
Solution-2: 0.1 g citric acid into the solution of yeast extract;
Solution-3: 0.1 g melamine into the solution of yeast extract;
Solution-4: 0.4 g ammonia water (25%–28%) into the solution of yeast extract.

All NPCDs were synthesized by one-step hydrothermal reaction of the precursors at 120 °C for 1 day to form original NPCDs solutions directly, and labeled as NPCDs-1, NPCDs-2, NPCDs-3 and NPCDs-4 corresponding to solution-1, solution-2, solution-3 and solution-4, respectively. After that, the original NPCDs solutions were dialyzed using a semipermeable membrane (MWCO 500) to remove the impurities and ions (Na^+, K^+ and Cl^-). The obtained NPCDs solutions were stored at 4 °C for further experiments.

To further study the relation between nitrogen atomic ratio and surface charge of NPCDs, CDs without doped nitrogen (CDs-0) were synthesized *via* a reported method by directly heating citric acid [24]. 2 g citric acid was added into a beaker and heated at 200 °C for 30 min. The liquated citric acid was neutralized to pH = 7 using 10 mg/mL NaOH solution to obtain an orange solution. The as-prepared CDs-0 solution was stored at 4 °C and the zeta potential of it was measured along with NPCDs-1, NPCDs-2, NPCDs-3 and NPCDs-4.

2.3. Cytotoxicity analysis

The HeLa cell line was cultured in the standard medium at 37 °C in 5% CO_2. Cells were incubated in a 96-well plate for 2 days. NPCDs were diluted to a series of known concentrations and then added to the cells. After incubation for 2 days, the relative viabilities of the cell were measured using colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays to assess the metabolic activity of cells treated as described above. The cells were lysed with acidulated sodium dodecyl sulfate (SDS). Absorbance was determined at 570 nm by a microplate reader (Bio-Rad 680, USA). At least three independent experiments were carried out to obtain the accurate data.

2.4. Bacteria culture

E. coli was shaking cultured at 180 rpm in lysogeny broth (LB) medium at 37 °C and obtained in exponential phase of cell cycle with the value of optical density at 600 nm (OD600) reaching 0.6–0.8. Then *E. coli* was washed with sterile water and centrifuged at 5000 rpm for 3 min for 3 times. Alive *E. coli* was boiled at 60 °C for 1 h to obtain dead ones, whereas 10 min for partially dead ones. *E. coli* was dispersed in the solutions of NPCDs at 37 °C after boiling treatment, and shaking cultured for another 2 h. Before LSCM analysis, all the samples of *E. coli* were washed with sterile water and centrifuged at 3000 rpm for 6 times in order to remove NPCDs that did not enter the bacteria.

3. Results and discussion

3.1. Characterization of NPCDs

Fig. 1a shows the TEM image and related size distribution of NPCDs-1, demonstrating that NPCDs-1 are uniformly dispersed with their diameters in the range of 1–6 nm. The FT-IR spectrum of NPCDs-1 is exhibited in Fig. 1b. The typical absorption peaks at 3435 cm^{-1} and 3268 cm^{-1} can be assigned to stretching vibrations of O–H and N–H of amino groups, respectively. The peak at 2927 cm^{-1} represents the stretching vibrations of methylene, while a characteristic peak of C=O stretching vibrations can be observed at 1632 cm^{-1}. The peak located at 1393 cm^{-1} can be attributed to stretching vibrations of C–N, and a peak centered at 1086 cm^{-1} corresponds to the asymmetric and symmetric stretching vibrations of C–O–C [25–28]. Fig. 1c shows the UV–Vis absorption spectrum of NPCDs-1. The absorption peak located at ~280 nm corresponds to the π-π* transition of C=C, while the peak at ~340 nm can be attributed to the n-π* transition of carbonyl/amine functional groups [19]. The FT-IR and UV–Vis spectra of NPCDs-1 demonstrate that plenty of hydrophilic functional groups exist on the surface of NPCDs-1, leading to an excellent aqueous dispersibility. As shown in Fig. 1d, NPCDs-1 can emit a strong fluorescence under the excitation from 300 nm to 460 nm, with the maximum fluorescent intensity obtained at 360 nm excitation. As the excitation wavelength elevates, the