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Facile preparation of bright orange fluorescent carbon dots and the constructed biosensing platform for the detection of pH in living cells

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Abstract

Long wavelength (i.e., orange- to red-light) fluorescence emission and functionality are critically longing for the development and applications of carbon dots (CDs) toward biosensor and bioimaging analysis. Herein, the N-doped carbon dots (N-CDs) with bright orange fluorescence emission at 592 nm were facilely synthesized via p-phenylenediamine as a carbon precursor by microwave method. The as-prepared N-CDs exhibit favorable biocompatibility, excellent water solubility, highly optical stabilities and display sensitive pH response behavior. When the pH is decreased from 9.45-2.45, its emission fluorescence intensity will be significantly

enhanced. The pKa value of N-CDs is 5.80 and it shows linear response to the physiological pH range of 4.45-7.00. Moreover, the N-CDs also possess high selectivity of hydrogen ions over common metal ions and some bioactive molecules, excellent photostability and reversibility. Based on this, a fluorescence sensing platform is established for the detection of pH in the environment. The N-CDs were further used as the fluorescent probe for cellular imaging based on its biocompatibility and cell membrane permeability and further successfully applied to monitor pH fluctuations in live cells. It is also anticipated that the N-CDs are potential bio-nano-materials for real-time tracking of the intracellular pH especially under physiological conditions in the disease diagnosis, biosensing and biomedical fields.

Keywords: N-doped carbon dots; orange fluorescence emission; biosensor; pH detection; cell imaging

1 Introduction

Following the graphene, carbon nanotubes, Carbon dots (CDs) as an emerging luminescent carbon nanomaterial have drawn much attention on account of their

excellent optical and chemical properties [1-5]. Carbon dots constitute a fascinating class that comprises discrete, quasis pherical nanoparticles with sizes below 10 nm [6-8]. Compared to organic fluorescent dyes and the conventional semiconductor quantum dots, CDs have a unique combination of these distinct performances such as easy preparation and functionalization, highly photo stabilities, superior biocompatibility and biolabeling potentials [9-17]. Over the past decade, a number of significant breakthroughs of CDs have taken place in preparation methods, optical properties and applications. First of all, CDs are more diverse in synthesis. It evolved from the initial electrochemical method to laser ablation, hydrothermal, microwave, strong acid oxidation, surface functionalization, heteroatom doping and other synthetic methods. And the preparation is more convenient and efficient. In optical properties, the CDs displayed very weak fluorescence when they were first discovered during the separation and purification of single-walled carbon nanotubes by Xu et al.[1] And currently the quantum yield of CDs can reach up to 88% higher than the initial 1% by various chemical methods such as heteroatom doping and surface modification[18, 19]. Besides, CDs have opened a promising prospect of carbon luminescence in a wide range of technologies, such as bioimaging, optical sensing, biomedicine etc [20-25]. And CDs have already demonstrated their viability in a variety of applications. However, most of CDs showed strong blue and green-light emitting, which greatly restricted their applications in biology on account of their weak penetration capability and severe photodamage to biological tissues [20, 26, 27]. Therefore, the development of CDs with long fluorescence (i.e., orange- to red-light)

emission are critically desired toward better penetration capability, minimal damage to the biological samples and low background interference.

However, the preparation of efficient long-wavelength emission CDs is a challenging issue due to the lack of effective synthesis strategy and the uncertainty of luminescence mechanism [8, 16, 24, 27, 28]. Some attempts showed that doping was an effective method to regulate the properties of CDs.[29, 30] By the introduction of heteroatoms (e.g., nitrogen, phosphorus, boron, etc.), the electronic structure would be changed and produced n-type or p-type electron carriers. Therefore, the optical properties of CDs are closely related to the energy gap of HOMO and LUMO, which can be modulated by the amount and type of doping[29]. Nitrogen is a typical dopant because its outer layer contains five electrons, and the atomic size is similar to that of carbon atoms. The nitrogen atoms on the surface of the CDs will provide a lone-pair electrons, leading to the upward shift of the Fermi level and the change of optical properties of CDs[10, 29]. At present, various nitrogenous compounds as precursors have been used to fabricate the nitrogen doped CDs. In addition, recent studies have shown that N-doping was found to be very effective method for preparing the long emissive CDs [31, 32]. Lin et al. first prepared red fluorescence CDs with 603nm and pointed that high nitrogen content and large quantum-size are responsible for the efficient red emission [20]. Jiang et al. reported the bright-yellow-emissive N‑Doped CDs expanding to cellular imaging and bifunctional sensing [33]. Ding et al. fabricated the N‑Doped red CDs for bioimaging both in vitro and in vivo [26]. However, many currently obtained CDs require complicated separation process from

the original complex system by column chromatography or HPLC [20, 26, 33, 34]. The pretreatment procedures were to some extent troublesome and time-consuming, which present the limitation for their further application. Thus, it is of urgent need to develop an effective method to synthesized orange or red emissive CDs by simple and convenient method.

Herein, we report the effective orange-emissive N-doped CDs (i, e., N-CDs) with high quantum yield prepared from p-phenlenediamine and ethanediamine via microwave method. The synthetic method is facile and easy operation without any other separation process. The as-prepared N-CDs display excitation wavelength independence and sensitive pH response. The fluorescence intensity of the N-CDs present a remarkable enhancement when the pH is decreased from 9.45 to 2.45. The pKa value of N-CDs was found to be 5.80 and it shows linear response to the physiological range of pH 4.45-7.00. Therefore, a fluorescence analysis spectrum method was established for the detection of pH in the environment. In addition, the as-prepared N-CDs also possess excellent photo- and chemical stability, favorable biocompatibility, low toxicity, superior aqueous dispersibility and cellular labeling capability. With these superior properties, the N-CDs indicated the potential applications as a biosensing platform to monitor the physiological pH value of living cells. Scheme 1 illustrated the synthetic procedure of the N-CDs and the N-CDs-based fluorescence biosensing platform for the detection of pH in living cells.

2 Experimental Sections

2.1 Materials

Reagent-grade of p-phenylenediamine, leucine (Leu), serine (Ser), aspartic acid (Asp), histidine (His), tryptophan (Trp), glutamic (Glu), lysine (Lys), alanine (Ala), cysteine (Cys), and glutathione (GSH) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Ethanediamine and sodium chloride (NaCl) was purchased from Tianjin chemical reagent co. LTD (Tianjin, China). All reagents were of analytical grade and were used as received without further purification. Deionized (DI) water was used throughout this study. All aqueous solutions were prepared with ultrapure water (≥18.25 MΩ cm) from a Milli-Q Plus system (Millipore, Bedford, MA, USA).

2.2 Preparation of CDs

First, 0.2 g of p-phenylenediamine were accurately quantified in the beaker and 5ml ethylenediamine were added, keep stirring until the p-phenylenediamine completely dissolved. Put the solution into a microwave oven and heated for 20 min under the power of 500 W, and then the beaker was cooled to room temperature. Afterwards, the hydrochloric acid (5 M) was added into the solution, there are some air bubbles generated, then constantly to join the hydrochloric acid until bubbles disappear. The obtained solution was further purified by dialysis to remove the remaining unreacted small molecules by dialysis tubing (cutoff molecular weight ～ 1000) for roughly 24 h. Finally, the dialysis solution was further freeze-dried and obtained a fluffy sponge like lavender solid.

2.3 Characterization of CDs

The transmission electron microscopic (TEM) images were carried out on a JEOL JEM-2100 transmission electron microscope (Tokyo, Japan) at 300 kv. The elemental analysis was obtained by an Elementar Vario Micro Cube (Hanau, Germany). The X-ray photoelectron spectra were measured by an AXIS ULTRA DLD X-ray photoelectron spectrometer (Kratos, Tokyo, Japan). The Fourier transform infrared (FT-IR) spectra were recorded on a Bruker Tensor II FTIR spectrometer (Bremen, Germany) with the KBr pellet technique. The UV−Vis absorption spectra were measured on a PerkinElmer UV/VIS Lambda 365. The fluorescence spectrum and fluorescence lifetime was measured with an Edinburgh FLS 920 spectrofluorometer (Livingston, UK). The cellular imaging was carried out using confocal laser fluorescence microscopy (LSM-880, Zeiss, Germany). AFE20 pH meter (Mettler Toledo, Switzerland) was used to adjust the pH value.

2.4 Determination of quantum yield

Quantum yield (QY) of the N-CDs was determined by a relative measuring method [35]. Rhodamine B (QY = 89 % in ethanol, λ_{ex} = 495 nm) was selected as the reference. Bringing obtained parameters into the following equation:

$$\emptyset = \emptyset' \times \frac{A'}{I'} \times \frac{I}{A} \times \frac{n^2}{n'^2}$$

Where $\emptyset$ is the QY of the testing sample, I is the testing sample's integrated emission intensity, n is the refractive index (1.33 for water and 1.36 for ethanol), and A is the optical density. The prime symbol (′) refers to the referenced dye of known QY. To receive more accurate results, a series of solutions of CDs and reference were

prepared with concentrations adjusted such that the optical absorbance values were between 0 – 0.1 at 495 nm. The PL spectra were measured and the PL intensity was integrated. QYs were determined by comparison of the integrated PL intensity vs absorbance curves (refractive index, n, had also to be considered).

2.5 Cellular Toxicity Test

In vitro cytotoxicity of the N-CDs against A549 cells was assessed by the standard MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. First, A549 cells were seeded in a 96-well plate with a density of 1×10^4 cells per mL and incubated for 24 h at 37 ℃. Then removed the culture medium and treated with Dulbecco's Modified Eagle's Medium (DMEM) with different concentrations of CDs (10-50 μg mL^{-1}) for 24 h. Then, the culture medium was dislodged and 10 μL of MTT (5.0 mg mL^{-1} in PBS) was added into each well with fresh culture medium. After 4 hours incubation, the culture medium was removed again and 50 μL of DMSO was added into each well to dissolve MTT. Finally, the microplate reader (Tecan Austria GmbH, GrÖdig, Austria) was used to record the optical density of each sample at a wavelength of 490 nm.

2.6 Cellular Imaging

The potential of N-CDs in biomarkers can be displayed by the A549 cellular imaging studies. A549 cells were seeded in a 6-well plate with the density of 2×10^4 cells per well and incubated for 24 h in a humidified incubator containing 5 % CO_2 at 37 ℃. Then the cells were incubated in fresh culture medium with 50 μL ml^{-1} of

N-CDs for 4 h. Finally, the cells were washed by phosphate-buffered saline (PBS) three times. Cells imaging was performed on a confocal laser fluorescence microscopy.

2.7 Fluorescence assay of pH

The detection of pH was performed in 10 mM phosphate buffer saline (PBS containing 150 mM NaCl) at various pH. In a typical run, 100 μL of N-CDs dispersion (2.0 mg mL^{-1}) was added into 1.90 mL of PBS at various pHs. The fluorescence emission spectra were recorded after 3 min at room temperature. The measurements were conducted in triplicate.

3 Results and discussion

The bright orange-emissive N-doped CDs (N-CDs) were facilely prepared from p-phenylenediamine and ethylenediamine by microwave method. And then through dialysis, freeze-drying, and finally get fluffy pale violet powder (Fig. S1). First, the transmission electron microscope (TEM) image presented in Fig. 1a shows that the zero dimensional carbon nanoparticles was synthesized with the form of uniform size and good dispersion which reveals our success to obtain CDs. The N-CDs displays uniform nanoparticles with a relatively narrow size distribution between 3 and 6 nm and the average size is about 4.8 nm (Fig. 1b). In addition, these nanoparticles are homogeneously dispersed without apparent aggregation.

Subsequently, the optical properties containing UV-Vis absorption, fluorescence excitation and emission of the N-CDs are fully examined. As shown in Fig. 1c (blue

line), the N-CDs exhibit a very broad UV absorption in the range of 200-600 nm and with a strong characteristic absorption peaks at 245 nm, which could be ascribed to the presence of π-π* (aromatic C=C) and n-π (C-N) transitions.[7, 30, 33] The optimal excitation (black line) and emission (red line) were at 535 nm and 592 nm, respectively. Under the irradiation of 365 nm UV light, the N-CDs aqueous solution can immediately presents bright-orange photoluminescence (PL) (Fig. 1c inset). Moreover, the PL excitation curve corresponding to the emission was observed have only one peak at 535 nm (Fig. 1c), with a position quite close to that of the absorption band in the lower energy region. This result confirms the consistency of our N-CDs in the field of optical properties, and also reflects the uniformity of the sample particles in size from the side.[33] Unlike many other previously reported CDs, the N-CDs show an excitation-independent feature with a single fixed emission at 592 nm when excited it with different wavelengths (Fig. 1d). This observation implies that only one type of dominating emission state exists in the N-CDs and confirms the homogeneity of the carbon particles once again [20, 36-38].

Then, the elemental analysis, Fourier transform infrared (FT-IR) and X-ray photoelectron spectroscopy (XPS) spectra were carried out to characterize functionalization and chemical composition of the N-CDs. The elemental analysis reveals that the N-CDs mainly comprise carbon, oxygen and nitrogen and elements, and the C, O, N and H atom ratio was 45.00%, 21.56%, 26.19%, 7.25%, respectively. The XPS survey spectrum (Fig. 2b) shows three dominant peaks at 285.6, 398.0 and 532.1 eV corresponding to C 1s, N 1s, and O 1s, respectively [36]. And the results of

XPS are accordance with the experimental results of elemental analysis. The FT-IR spectrum indicates that the N-CDs mainly contain amine (3442 and 3192 cm^{-1}), methylene (2977, 2914 and 2805 cm^{-1}), aromatic C=C (1601 and 1507 cm^{-1}), and C-N (1085 cm^{-1}) and C-O (1035 cm^{-1}) functional groups or chemical bonds (Fig. 2a) [20, 31, 33]. The XPS surveys spectrum of the N-CDs further confirms the FT-IR assignments. The high-resolution XPS spectrum of C 1s (Fig. 2c) exhibits four characteristic peaks of C=C/C-C, C-N, C-O, and -CONH-with binding energies at about 284.70, 285.00, 286.30, and 288.15 eV, respectively [26, 33]. This proves that the N-CDs possess rich hydrophilic groups on the surfaces and is in accord with the FT-IR spectrum. The high-resolution XPS spectrum of N 1s could be resolved into three distinctive peaks of 398.60, 399.32 and 400.40 eV, corresponding to pyridine-like N, amino N and pyrrole-like N, respectively (Fig. 2d) [26]. This shows that nitrogen have been successfully doped into the carbon dots. The O 1s peak gives two components at 531.26 and 532.51 eV corresponding to C-OH and C=O (Fig. S2). The above analysis showed that the prepared N-CDs might have functional groups like -OH, and -NH, and nitrogen atom was also doped into the CDs.

As pH plays a crucial adjective role in many chemical and biological systems, such as the homeostasis, cellular proliferation and apoptosis, enzymatic activity, tumor growth and other physiological and pathological process, its quantitative detection is of critical importance and indispensable. The N-CDs was found to be sensitive to pH at the beginning of the experiment. The fluorescence emission spectra were performed in order to explore the optical responses of N-CDs to pH. As shown

in Fig. S3, the fluorescence emission wavelength is stable under a broad range of pH (1-13), but the emission intensity is very sensitive to the pH value. From the Fig. 3a, the fluorescence intensity increases gradually with the decrease of the pH from 9.45 to 2.45 under the excitation of 535 nm. And a well fitted sigmoidal curve is obtained from Fig. 3b by using the Boltzmann equation： F=11498.54 + 360842.79/(1+exp((pH-5.80)/0.41)) (R^2 = 0.9983, pKa = 5.80 ± 0.06). Fig. 3b (inset) shows effect linear response to the physiological range of pH 4.45-7.00. Which is based on the following equation : F=1.03-146031.71pH, R^2=0.9931. Therefore, a fluorescence analysis spectrum method was established for the detection of pH in the environment. The N-CDs is worthwhile for near-neutral cytosolic pH research. It is absolutely essential to investigated the near-neutral pH range since the normal physiological hydrogen ion concentration varies within narrow limits, which is beneficial for their further applications especially in bioimaging and biosensor.[21] Fig. 3c shows the photographs of N-CDs in the different pH PBS solutions under daylight and 365 nm UV lamp irradiation. Interestingly, the color of the N-CDs aqueous solution under daylight is from pale pink to deep pink as the pH dropped from 7.5 to 3.0. And the gradual enhancement of fluorescence can also be observed with naked eye with the decreases of the pH from 7.5 to 3.0. These results demonstrate that the N-CDs can be used to establish a biological sensing platform applying to supervise the intracellular pH quantitatively within the near neutrality range. Compared with other fluorescent pH sensor based on carbon dots，the N-CDs possess longer fluorescence emission and excitation-independent feature，which make

it more valuable for quantitative pH measurements in biological systems in the future. (Table S1).

The recognition mechanism of the N-CDs to the hydrogen ion can be attributed to the elementary speciation of nitrogen atoms (pyridine-like N, amino N and pyrrole-like N) on the surface of N-CDs. Because hydrogen ion was highly appreciated by the pyridine-like N, amino N or pyrrole-like N under acidic conditions. The range that has a sensitive response to the pH for the N-CDs is also under acidic conditions. The lone pair electrons on the nitrogen atoms could form coordination bond with the hydrogen ions in acid solution. Therefore, the pH sensitivity is introduced via the nitrogen (pyridine-like N, amino N and pyrrole-like N) moiety upon either protonation or deprotonation, especially the amino N, the majority nitrogen element forms of the N-CDs [21, 39]. So we speculated this conclusion that hydrogen ions binding with nitrogen atoms (pyridine-like N, amino N and pyrrole-like N) in N-CDs cause the significant fluorescent spectral response to pH. Through this mechanism the biological sensing platform that based on the N-CDs was established for the detection of hydrogen ion.

The fluorescence decay of N-CDs in different pH (3.0, 5.8, 7.4) measured by the time-correlated single photon counting method at 592 nm represents a bi-exponential fitting curve, and the average lifetime is calculated to be 3.16 ns, 2.94 ns, 2.65 ns, respectively (Fig. S4). The fluorescent quantum yield (QY) was determined using Rhodamine B as a reference by a widely accepted relative method [35]. As shown in Fig. S5, the relative quantum yield (QY) of the N-CDs was calculated in DI water

under excitation of 495 nm and took the Rhodamine B (QY = 89% in ethanol, λ_{ex}= 495 nm) as the reference. Bring the measured data into the above mentioned equation and get the result of 14%. Furthermore, the stability of the N-CDs is tested by measuring the changes of fluorescence intensity under continuous irradiation excitation (535 nm) with a Xe lamp for 100 min (Fig. S6). The fluorescence intensity is continuously monitored and recorded. Fig. S6 shows the time course of fluorescence intensity of the N-CDs at pH 7.4, 5.8 and 3.0 at room temperature. The experimental results reveal that the aqueous solution of the N-CDs possesses excellent photostability. And this characteristic provides a guarantee for the future application of the N-CDs to monitor pH variation in real time. In addition, the N-CDs were verified have outstanding stability under high ionic strength environments. As demonstrated in Fig. S7, the fluorescence intensity of N-CDs are observed to have no significant changes even for a high NaCl concentration of 1 M. This performance is significant because it is indispensable for N-CDs to be used under physical salt conditions in practical applications [21]. It was also found to be possible to repeatedly redisperse the dry sample (powder) in water without any aggregation, which is important for preservation and transportation [36].

In addition, we have also accomplished the selectivity and reversibility research of the N-CDs. First, it is worth noting that the N-CDs exhibits high selectivity toward hydrogen ions over competitive species such as some bioactive molecules and metal ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, Ba^{2+},Fe^{3+}, Cu^{2+}, Fe^{2+}, Al^{3+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, Ag^+, Sn^{2+}, Cr^{3+}, l-alanine, glutathione, serine, leucine, histidine,

tryptophan, aspartic acid, glutamic acid, lysine, cysteine) at pH 7.4, 5.8 and 3.0, respectively. As shown in Fig. 4, high concentrations (over their physiological concentrations) of some bioactive molecules (Fig. 4a) and metal ions (Fig. 4b) make no visible impact on the fluorescence intensity of the N-CDs at pH 7.4, 5.8 and 3.0. These results indicate that the N-CDs present a distinguished selectivity response to hydrogen ions in the presence of some bioactive molecules and metal ions with high concentrations. In addition, the influence of various metal ions and some biomolecules in the signals in the lineal region was also studied. As shown in Fig. S8 (a)-(f), a various of metal ions and biomolecules have no significant effect on the fluorescence intensity of N-CDs in the linear range of pH, which indicates that N-CDs has excellent selectivity for hydrogen ions in the linear response range. Secondly, the reversibility of fluorescent probe is very important to monitor the reversible change of the pH value of the organism. Changing the pH value of the aqueous solution of the N-CDs quickly by using concentrated aqueous sodium hydroxide and hydrochloric acid, then observing the fluorescence intensity values in acidic (pH=3.0) and neutral (pH=7.4) conditions. As shown in Fig. 5, the pH value is switched back and forth between 3.0 and 7.4, as well as the fluorescence intensity values. After several cycles, there is still 99.0% of the fluorescence intensity successfully recovered in acidic conditions. And the response and recovery times are within seconds. It is quite clear that the N-CDs exhibit a highly reversible response to pH.

Bestowed with superior optical properties, excellent selectivity to hydrogen ions and high photostability, the as-prepared N-CDs are proposed to serve as a potential

biolabeling reagent. So it is crucial to evaluate the cytotoxicity of the N-CDs to living cells by MTT assay before the cell fluorescence imaging experiments. Fig. 6 shows the viability of A-549 cells under various concentrations of N-CDs from 10 $\mu g \cdot mL^{-1}$ to 50 $\mu g \cdot mL^{-1}$. The results demonstrate that over 91.5% of cells are viable after 24 h incubation with the N-CDs (10−50 $\mu g\ mL^{-1}$). This observation confirms the very low cytotoxicity of the N-CDs and provides a guarantee for their potential use in vivo and in vitro imaging.

The cellular imaging capability of the N-CDs was preliminarily investigated for exploring the potential applications for biolabeling. After being incubated A-549 cells with the N-CDs aqueous solutions (50 $\mu g\ mL^{-1}$) at pH 7.4, 5.8 and 3.0 for 4 h, the living cells were imaged with a confocal microscope under bright-field illumination and under 514 nm laser excitation. The cells stained with N-CDs display tiny soft orange fluorescence at pH 7.4(Fig. 7c). When the pH decreases to 5.8, the orange fluorescence enhanced and N-CDs were well-dispersed in the cytoplasm and nucleus region. (Fig. 7b). When the pH drops to 3.0, the orange fluorescence is the brightest (Fig. 7a). When the pH switches from 5.8 to 7.4, the orange emission is re-quenched, demonstrating the reversibility of emission of N-CDs in the cells. When the pH switches from 3.0 to 5.8, the orange fluorescence changes are not obvious. The Figure 7 indicates that the as-prepared N-CDs can easily penetrate the cell membrane into the cell at low toxicity conditions. The brightness of the cells varies significantly at different pH, which can be the basis for determining the intracellular pH. Meanwhile, the orange wavelength emission of the N-CDs can also avoid the interference of the

cellular matrix and reduce the photo damage to the cell. These results further testify that N-CDs are competent fluorescent pH probes for reversibly monitoring the pH in live cells. And the N-CDs can be used as the potential bio-nano-materials for real-time tracking of the intracellular pH.

4 Conclusions

In summary, we report the effective orange-emissive CDs (i, e., N-CDs) with high quantum yield prepared from p-phenlenediamine and ethanediamine via microwave method. The synthetic method is facile and convenient operation without column chromatography and any other separation process. The as-prepared N-CDs display excitation wavelength independence and sensitive pH response. Additionally, the N-CDs also possess excellent photo- and chemical stability, favorable biocompatibility, low toxicity, superior aqueous dispersibility and cellular labeling capability. With these outstanding properties, the N-CDs can be used as a fluorescent pH probe to noninvasively monitor physiological pH in living cells, and because of its bright orange fluorescence which can effectively avoid the interference by autofluorescence of biological matrix and reduce the photodamage of the biological tissues by ultraviolet excitation light. It is also anticipated that the N-CDs are potential bio-nano-materials for real-time tracking of the intracellular pH especially under physiological conditions in the biosensing, disease diagnosis and biomedical fields.

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References

[1] X. Xu, R. Ray, Y. Gu, H.J. Ploehn, L. Gearheart, K. Raker, W.A. Scrivens, Electrophoretic Analysis and Purification of Fluorescent Single-Walled Carbon Nanotube Fragments, Journal of the American Chemical Society 126(40) (2004) 12736-12737.

[2] Y.-P. Sun, B. Zhou, Y. Lin, W. Wang, K.A.S. Fernando, P. Pathak, M.J. Meziani, B.A. Harruff, X. Wang, H. Wang, P.G. Luo, H. Yang, M.E. Kose, B. Chen, L.M. Veca, S.-Y. Xie, Quantum-Sized Carbon Dots for Bright and Colorful Photoluminescence, Journal of the American Chemical Society 128(24) (2006) 7756-7757.

[3] X. Gong, W. Lu, M.C. Paau, Q. Hu, X. Wu, S. Shuang, C. Dong, M.M. Choi, Facile synthesis of nitrogen-doped carbon dots for Fe(3+) sensing and cellular imaging, Analytica chimica acta 861 (2015) 74-84.

[4] W. Lu, X. Gong, M. Nan, Y. Liu, S. Shuang, C. Dong, Comparative study for N and S doped carbon dots: Synthesis, characterization and applications for Fe(3+) probe and cellular imaging, Analytica chimica acta 898 (2015) 116-27.

[5] X. Gong, Y. Liu, Z. Yang, S. Shuang, Z. Zhang, C. Dong, An "on-off-on" fluorescent nanoprobe for recognition of chromium(VI) and ascorbic acid based on phosphorus/nitrogen dual-doped carbon quantum dot, Analytica chimica acta 968 (2017) 85-96.

[6] X. Wang, L. Cao, F. Lu, M.J. Meziani, H. Li, G. Qi, B. Zhou, B.A. Harruff, F. Kermarrec, Y.-P. Sun, Photoinduced electron transfers with carbon dots, Chemical communications (25) (2009) 3774-3776.

[7] L. Wang, H.S. Zhou, Green synthesis of luminescent nitrogen-doped carbon dots from milk and its imaging application, Analytical chemistry 86(18) (2014) 8902-5.

[8] C. Yang, S. Zhu, Z. Li, Z. Li, C. Chen, L. Sun, W. Tang, R. Liu, Y. Sun, M. Yu, Nitrogen-doped carbon dots with excitation-independent long-wavelength emission produced by a room-temperature reaction, Chemical communications 52(80) (2016) 11912-11914.

[9] H. Li, Z. Kang, Y. Liu, S.-T. Lee, Carbon nanodots: synthesis, properties and applications, Journal of Materials Chemistry 22(46) (2012) 24230.

[10] Y. Wang, A. Hu, Carbon quantum dots: synthesis, properties and applications, Journal of Materials Chemistry C 2(34) (2014) 6921-6939.

[11] C. Ding, A. Zhu, Y. Tian, Functional Surface Engineering of C-Dots for Fluorescent Biosensing and in Vivo Bioimaging, Accounts of Chemical Research 47(1) (2014) 20-30.

[12] P. Roy, P.-C. Chen, A.P. Periasamy, Y.-N. Chen, H.-T. Chang, Photoluminescent carbon nanodots: synthesis, physicochemical properties and analytical applications, Materials Today 18(8) (2015) 447-458.

[13] X.T. Zheng, A. Ananthanarayanan, K.Q. Luo, P. Chen, Glowing graphene quantum dots and carbon dots: properties, syntheses, and biological applications, Small 11(14) (2015) 1620-36.

[14] J.H. Liu, L. Cao, G.E. LeCroy, P. Wang, M.J. Meziani, Y. Dong, Y. Liu, P.G. Luo, Y.P. Sun, Carbon "Quantum" Dots for Fluorescence Labeling of Cells, ACS applied materials & interfaces 7(34) (2015) 19439-45.

[15] L. Zhang, H. Wei, F. Gao, Y. Su, Y. Su, J. Zhang, Y. Ma, M. Xu, M. Xu, Z. Li, Z. Yang, Controllable Synthesis of Fluorescent Carbon Dots and Their Detection Application as Nanoprobes, Nano-Micro Letters 5(4) (2013).

[16] L. Bao, C. Liu, Z.L. Zhang, D.W. Pang, Photoluminescence-tunable carbon nanodots: surface-state energy-gap tuning, Advanced materials 27(10) (2015) 1663-7.

[17] A. Zhao, Z. Chen, C. Zhao, N. Gao, J. Ren, X. Qu, Recent advances in bioapplications of C-dots, Carbon 85 (2015) 309-327.

[18] Z. Xie, F. Wang, C.Y. Liu, Organic-inorganic hybrid functional carbon dot gel glasses, Advanced materials 24(13) (2012) 1716-21.

[19] X. Wang, L. Cao, S.-T. Yang, F. Lu, M.J. Meziani, L. Tian, K.W. Sun, M.A. Bloodgood, Y.-P. Sun, Bandgap-Like Strong Fluorescence in Functionalized Carbon Nanoparticles, Angewandte Chemie 122(31) (2010) 5438-5442.

[20] K. Jiang, S. Sun, L. Zhang, Y. Lu, A. Wu, C. Cai, H. Lin, Red, green, and blue luminescence by carbon dots: full-color emission tuning and multicolor cellular imaging, Angewandte Chemie 54(18) (2015) 5360-3.

[21] X. Gong, W. Lu, Y. Liu, Z. Li, S. Shuang, C. Dong, M.M.F. Choi, Low temperature synthesis of phosphorous and nitrogen co-doped yellow fluorescent carbon dots for sensing and bioimaging, Journal of Materials Chemistry B 3(33) (2015) 6813-6819.

[22] A.R. Chowdhuri, T. Singh, S.K. Ghosh, S.K. Sahu, Carbon Dots Embedded Magnetic Nanoparticles @Chitosan @Metal Organic Framework as a Nanoprobe for pH Sensitive Targeted Anticancer Drug Delivery, ACS applied materials & interfaces

8(26) (2016) 16573-83.

[23] X. Miao, X. Yan, D. Qu, D. Li, F.F. Tao, Z. Sun, Red Emissive Sulfur, Nitrogen Codoped Carbon Dots and Their Application in Ion Detection and Theraonostics, ACS applied materials & interfaces 9(22) (2017) 18549-18556.

[24] L. Pan, S. Sun, A. Zhang, K. Jiang, L. Zhang, C. Dong, Q. Huang, A. Wu, H. Lin, Truly Fluorescent Excitation-Dependent Carbon Dots and Their Applications in Multicolor Cellular Imaging and Multidimensional Sensing, Advanced materials 27(47) (2015) 7782-7.

[25] K. Hola, Y. Zhang, Y. Wang, E.P. Giannelis, R. Zboril, A.L. Rogach, Carbon dots—Emerging light emitters for bioimaging, cancer therapy and optoelectronics, Nano Today 9(5) (2014) 590-603.

[26] H. Ding, S.B. Yu, J.S. Wei, H.M. Xiong, Full-Color Light-Emitting Carbon Dots with a Surface-State-Controlled Luminescence Mechanism, ACS nano 10(1) (2016) 484-91.

[27] S. Qu, D. Zhou, D. Li, W. Ji, P. Jing, D. Han, L. Liu, H. Zeng, D. Shen, Toward Efficient Orange Emissive Carbon Nanodots through Conjugated sp(2) -Domain Controlling and Surface Charges Engineering, Advanced materials 28(18) (2016) 3516-21.

[28] S. Zhu, Y. Song, X. Zhao, J. Shao, J. Zhang, B. Yang, The photoluminescence mechanism in carbon dots (graphene quantum dots, carbon nanodots, and polymer dots): current state and future perspective, Nano Research 8(2) (2015) 355-381.

[29] Y. Park, J. Yoo, B. Lim, W. Kwon, S.W. Rhee, Improving the functionality of

carbon nanodots: doping and surface functionalization, Journal of Materials Chemistry A 4(30) (2016) 11582-11603.

[30] M. Xu, G. He, Z. Li, F. He, F. Gao, Y. Su, L. Zhang, Z. Yang, Y. Zhang, A green heterogeneous synthesis of N-doped carbon dots and their photoluminescence applications in solid and aqueous states, Nanoscale 6(17) (2014) 10307-15.

[31] W. Li, Z. Zhang, B. Kong, S. Feng, J. Wang, L. Wang, J. Yang, F. Zhang, P. Wu, D. Zhao, Simple and green synthesis of nitrogen-doped photoluminescent carbonaceous nanospheres for bioimaging, Angewandte Chemie 52(31) (2013) 8151-5.

[32] Z. Qian, J. Ma, X. Shan, H. Feng, L. Shao, J. Chen, Highly luminescent N-doped carbon quantum dots as an effective multifunctional fluorescence sensing platform, Chemistry 20(8) (2014) 2254-63.

[33] K. Jiang, S. Sun, L. Zhang, Y. Wang, C. Cai, H. Lin, Bright-Yellow-Emissive N-Doped Carbon Dots: Preparation, Cellular Imaging, and Bifunctional Sensing, ACS applied materials & interfaces 7(41) (2015) 23231-8.

[34] X. Gong, Q. Hu, M.C. Paau, Y. Zhang, S. Shuang, C. Dong, M.M. Choi, Red-green-blue fluorescent hollow carbon nanoparticles isolated from chromatographic fractions for cellular imaging, Nanoscale 6(14) (2014) 8162-70.

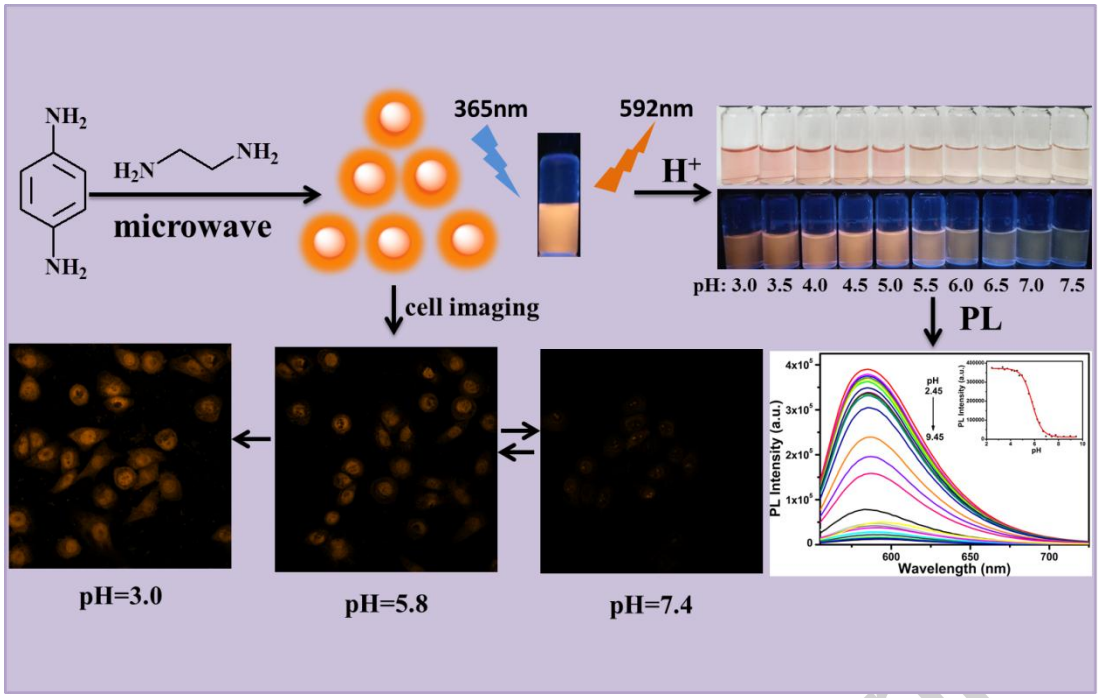
[35] M. Grabolle, M. Spieles, V. Lesnyak, N. Gaponik, A. Eychmüller, U. Resch-Genger, Determination of the Fluorescence Quantum Yield of Quantum Dots: Suitable Procedures and Achievable Uncertainties, Analytical chemistry 81(15) (2009) 6285-6294.

[36] S. Zhu, Q. Meng, L. Wang, J. Zhang, Y. Song, H. Jin, K. Zhang, H. Sun, H. Wang, B. Yang, Highly photoluminescent carbon dots for multicolor patterning, sensors, and bioimaging, Angewandte Chemie 52(14) (2013) 3953-7.

[37] S.N. Baker, G.A. Baker, Luminescent carbon nanodots: emergent nanolights, Angewandte Chemie 49(38) (2010) 6726-44.

[38] S.Y. Lim, W. Shen, Z. Gao, Carbon quantum dots and their applications, Chemical Society reviews 44(1) (2015) 362-81.

[39] W. Niu, L. Fan, M. Nan, Z. Li, D. Lu, M.S. Wong, S. Shuang, C. Dong, Ratiometric emission fluorescent pH probe for imaging of living cells in extreme acidity, Analytical chemistry 87(5) (2015) 2788-93.



Scheme 1 Illustration of the synthetic procedure of the N-CDs and the N-CDs-based fluorescence biosensing platform for the detection of pH in living cells.

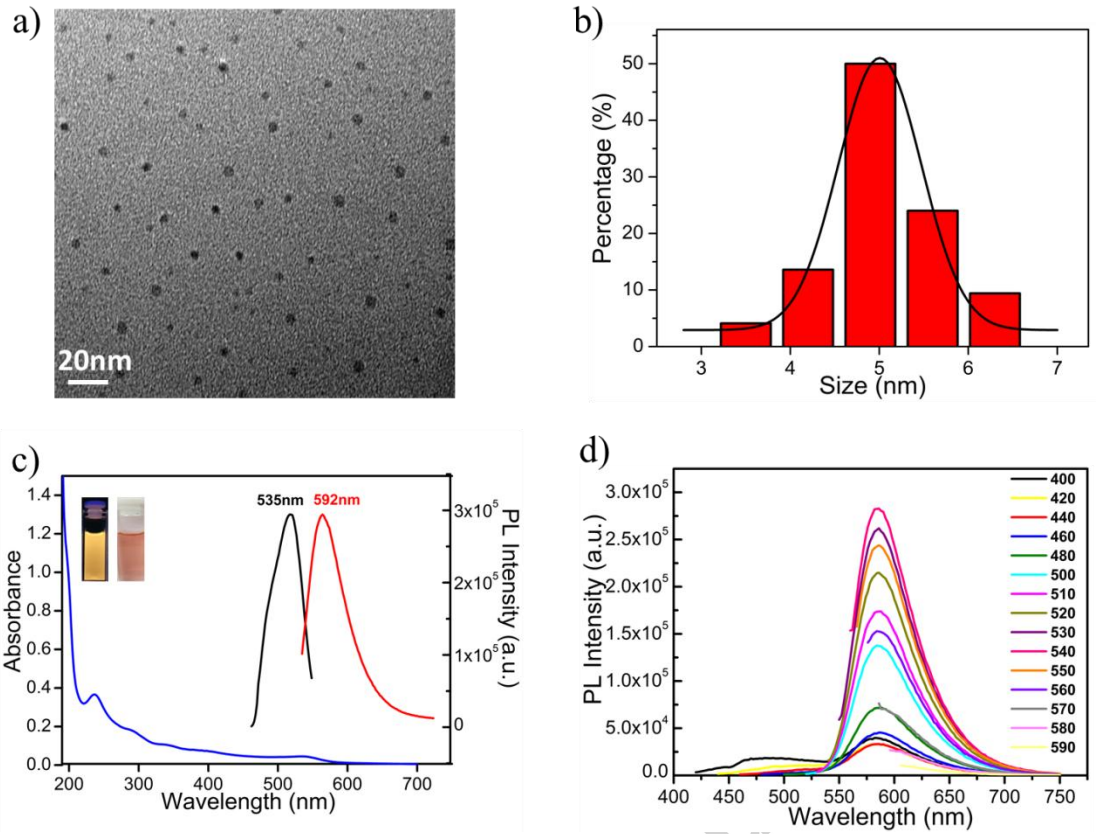


Fig. 1 (a) TEM image of the N-CDs, (b) size distribution of the N-CDs, (c) UV-Vis absorption (blue line), PL excitation (black line) and emission spectra (red line)of N-CDs. The inset displays photographs of the N-CD under daylight (right) and UV irradiation (left) in aqueous solution. (d) PL spectra of N-CDs (0.5 mg mL-1) at different excitation 400-590 nm.

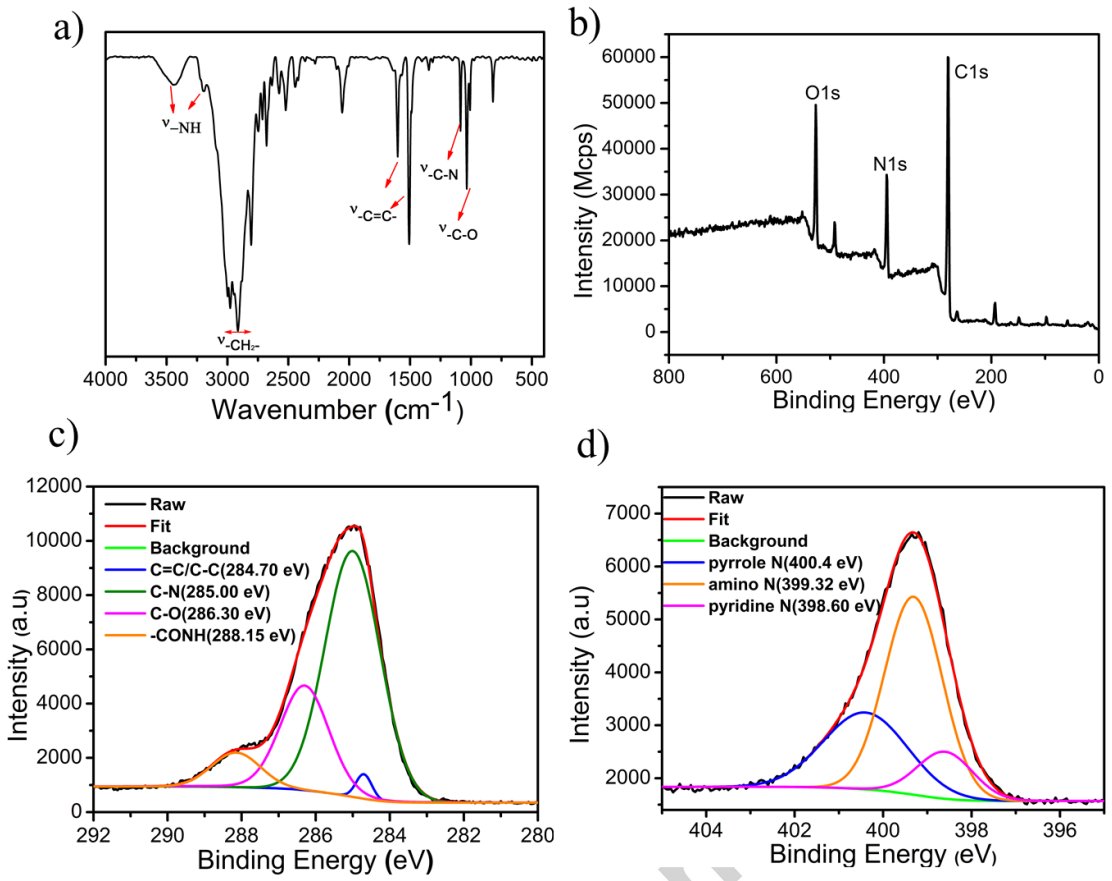


Fig. 2 (a) FTIR spectrum of N-CDs, (b) XPS survey scan of N-CDs, (c) C1s XPS and (d) N1s XPS.

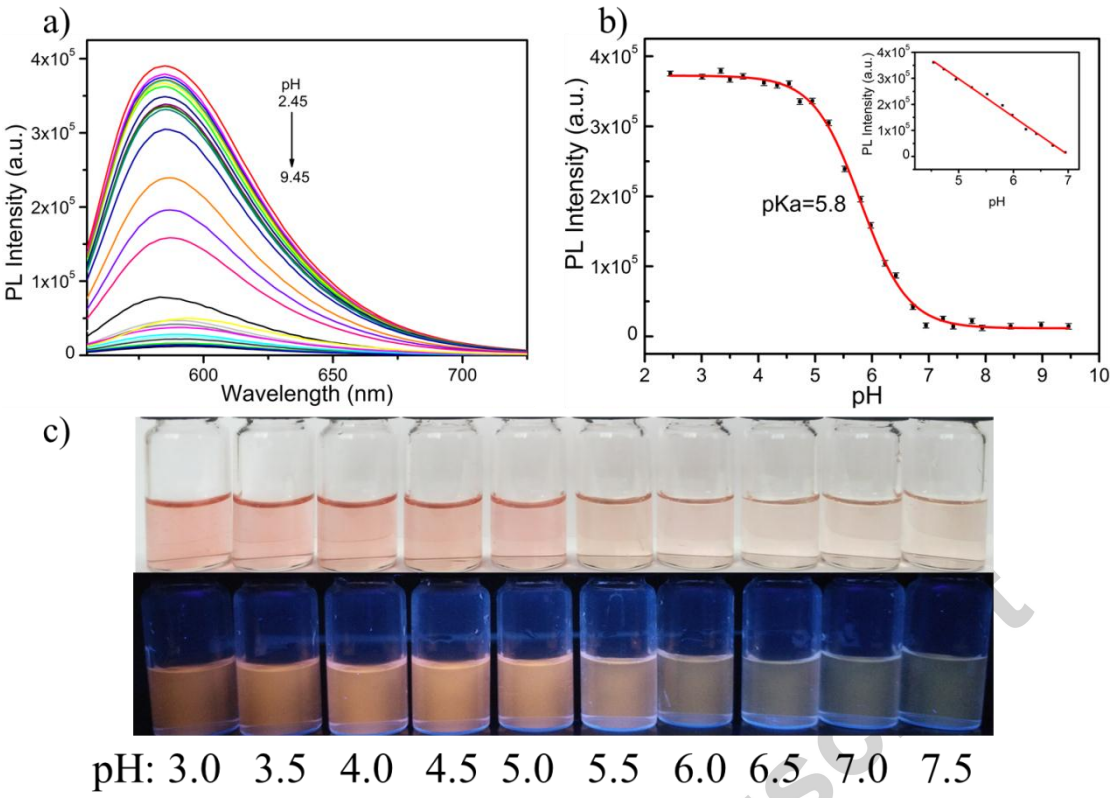


Fig. 3 (a) The PL spectrum of 0.30 mg mL-1 N-CD as pH increases from 2.45 to 9.45 at λex of 535 nm. (b) Sigmoidal fitting of pH-dependent fluorescence intensity at 592 nm. The inset displays the linear plot of N-CD against pH (4.5-7.0). (c) Photographs of N-CDs in the different pH PBS under daylight and 365 nm UV lamp irradiation.

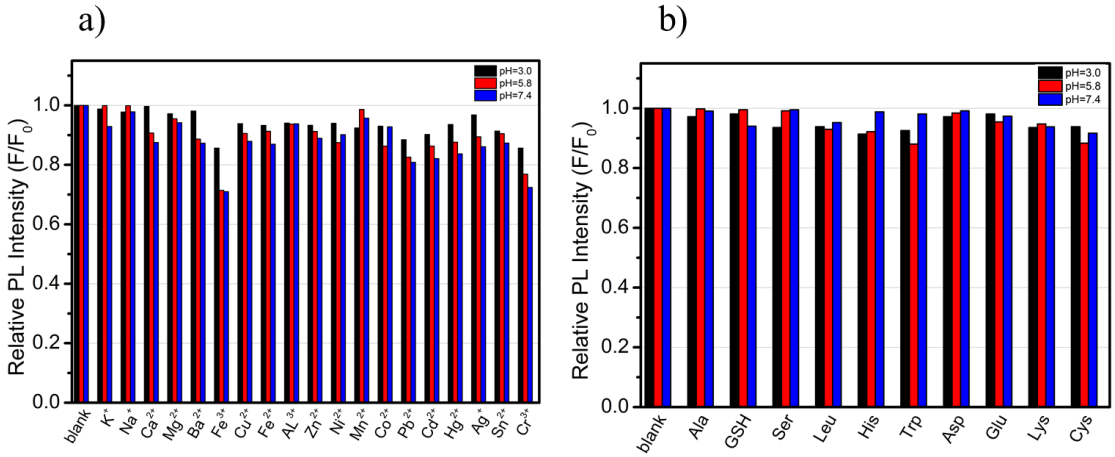


Fig. 4 Fluorescence intensity of 0.5 mg ml^{-1} N-CDs at different pH in the presence of a) metal ions and b) biological molecules.

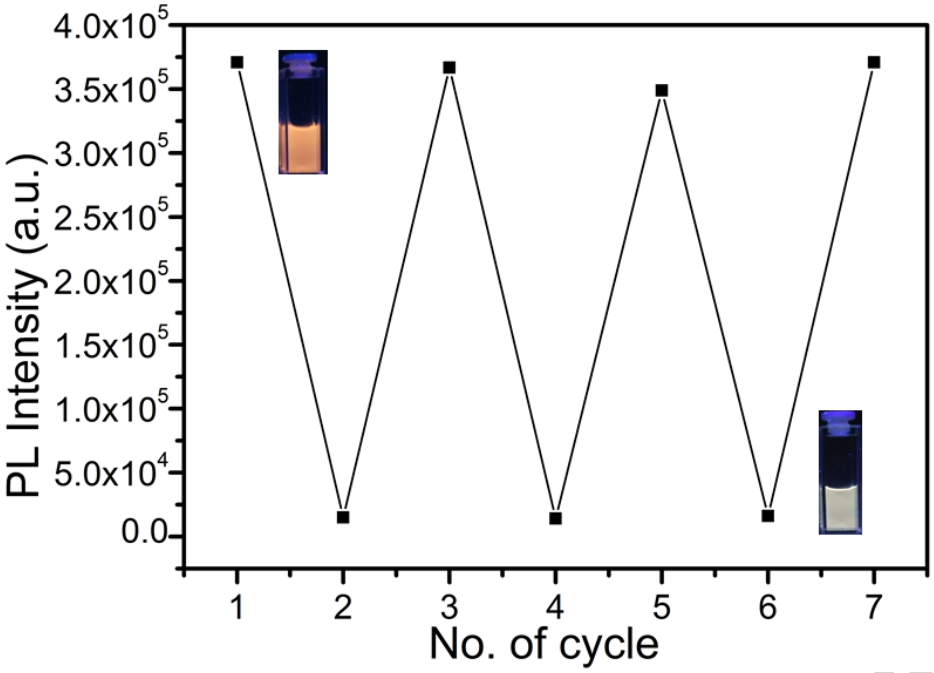


Fig. 5 Changes in fluorescence intensity of N-CDs between pH 7.4 and 3.0 at λ_{ex} =535 nm.

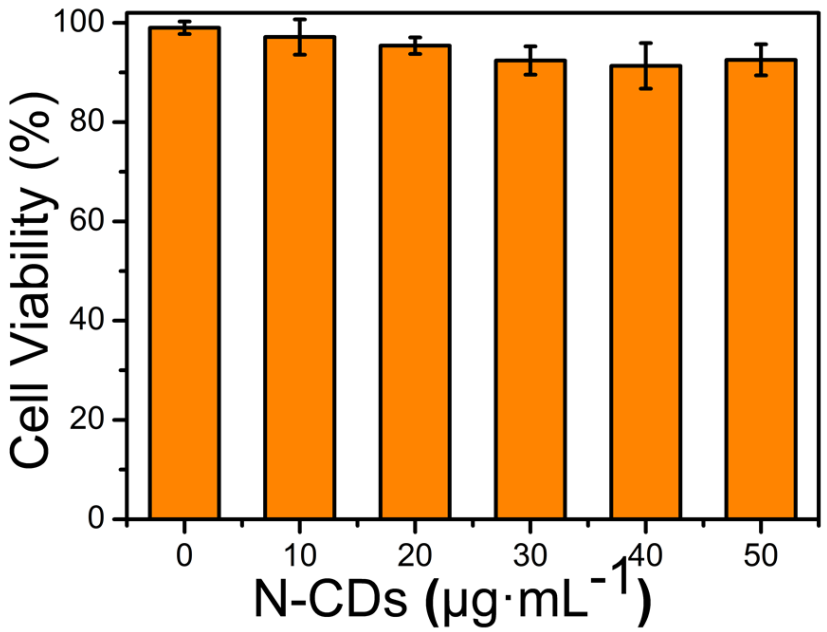


Fig. 6 Cellular cytotoxicity assessment of the N-CDs using the standard MTT assay toward A-549 cells.

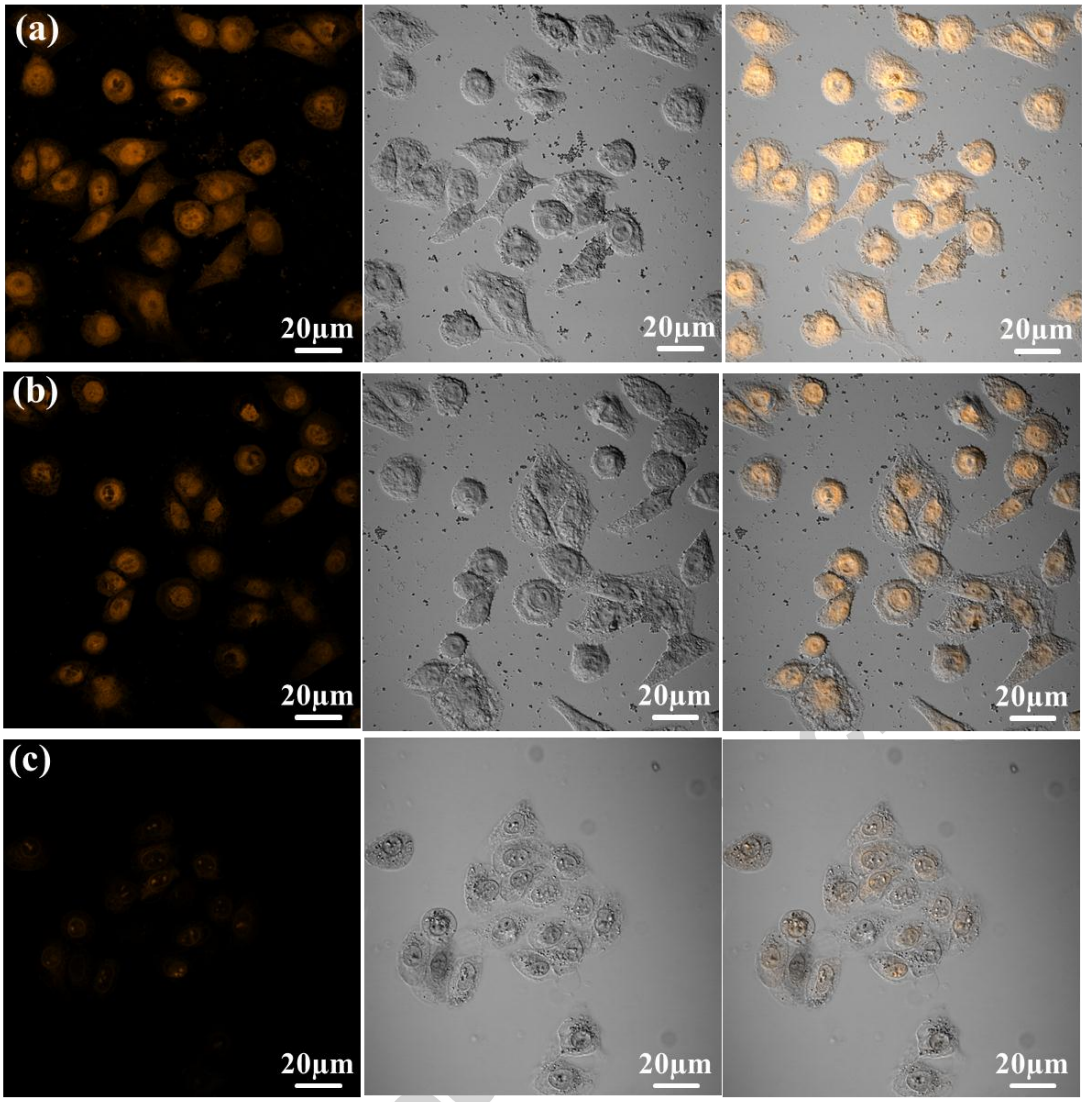


Fig. 7 Confocal fluorescence images of 40 μg mL^{-1} N-CDs in A-549 cells at different pH with the $\lambda_{ex}/\lambda_{em}$ are 514/592 nm. (a) Dark field image, bright field image, merged image of Bright and dark field image of the N-CDs at pH 3.0. (b) Dark field image, bright field image, merged image of Bright and dark field image of the N-CDs at pH 5.8. (c) Dark field image, bright field image, merged image of Bright and dark field image of the N-CDs at pH 7.4.

Highlights

- The as-prepared N-doping carbon dots (N-CDs) with a long wavelength emission at 592nm which can better applied in bioimaging and biosensor.
- The synthetic of the N-CDs were facilely prepared by microwave treatment and without column chromatography and any other separation process.
- The N-CDs with favorable biocompatibility, low toxicity, high photostability, superior aqueous dispersibility and cellular labeling capability.
- The development of a fluorescence biosensing platform for the detection of pH in living cells.