One-step Synthesis of Highly Luminescent Nitrogen-doped Carbon Dots for Selective and Sensitive Detection of Mercury(II) Ions and Cellular Imaging

Ying LIU, Mei LIAO, Xueling HE, Xia LIU, Xingming KOU, and Dan XIAO

*1 College of Chemistry, Sichuan University, Chengdu 610064, P. R. China
*2 Experimental Animal Center, Sichuan University, Chengdu 610207, P. R. China
*3 College of Life Science, Sichuan University, Chengdu 610064, P. R. China
*4 College of Chemical Engineering, Sichuan University, Chengdu 610065, P. R. China

In this paper, nitrogen-doped carbon dots (N-CDs) with high quantum yield (QY) of 40.5% were prepared through a facile and straightforward hydrothermal route. The as-prepared N-CDs exhibited excellent photoluminescence properties, good water-solubility and photostability, negligible cytotoxicity and favourable biocompatibility. Such N-CDs were found to serve as an effective fluorescent sensor for selective and sensitive detection of Hg^{2+} in a wide linear response concentration range of 0 - 8 μM with a limit of detection (LOD) of 0.087 μM and could be applied to the determination of Hg^{2+} in environmental water samples. The corresponding mechanisms were discussed in detail. Moreover, another attractive finding was that the N-CDs showed satisfactory performance in bioimaging before and after the addition of Hg^{2+} in human lung cancer PC14 cells. With excellent sensitivity, selectivity and biocompatibility, such cheap carbonmaterials are potentially suitable for monitoring of Hg^{2+} in environmental applications and promising for biological applications.

Keywords Nitrogen-doped carbon dots, fluorescence, mercury ion measurement, bioimaging, intracellular sensing

(Received March 13, 2015; Accepted May 22, 2015; Published October 10, 2015)

Introduction

There is no doubt that Hg^{2+} has brought forth serious problems to environmental and human health. As a consequence, exploring cost-effective, sensitive and selective analytical methods for determining trace amounts of Hg^{2+} in environmental and biological systems is especially important. In recent years, optical sensors based on nanotechnology like functionalized nanomaterials of gold (Au), silver (Ag) and semiconductor nanomaterials have been developed and become popular, due to their accuracy, great simplicity and on-site measurement of Hg^{2+}. Unfortunately, relatively complicated synthesis routes or the involvement of expensive or even hazardous regents are needed. Therefore, it is worthwhile to develop new nanomaterials for the sensitive and selective detection of Hg^{2+} through simple, cost-effective and low-toxic synthetic routes.

Fluorescence materials, such as semiconductor quantum dots and organic fluorophores, have drawn much attention in the growing field of biomedical imaging. However, heavy metals as the essential elements in semiconductor quantum dots could pose a serious hazard to organisms and the environment, and organic fluorophores have poor photostability for imaging. Therefore, the search for biologically and environmentally benign alternatives is necessary. Due to their favorable chemical inertness, low toxicity, excellent biocompatibility, stable photoluminescence and extensive applications related to photocatalysts, medical diagnosis, photovoltaic devices, bioimaging and sensors, carbon dots (CDs) have been drawing a great deal of attention since they were first discovered by Xu and coworkers when they purified single-walled carbon nanotubes. To date, many methods have been developed to synthesize CDs. Among them, hydrothermal synthesis strategy is regarded as a simple, direct, and efficient way to obtain CDs. However, most of the prepared CDs have low photoluminescence (PL) quantum yield (QY) if no further passivation and/or doping is made, which limits their further use. As a result, improving the QY and expanding the applied range remain ongoing challenges. Doping carbon nanomaterials with heteroatoms is an effective strategy for fulfilling the above mentioned requirements. The nitrogen (N) atom, having a comparable atomic size and five valence electrons for bonding to carbon atoms, has been generally employed for chemically doping carbon nanomaterials, which is expected to improve their photoluminescence properties. Huang et al. prepared the nitrogen-contained carbon nanoparticles with strawberry juice, which can sensitively and selectively detected Hg^{2+}, but the QY was still low (QY = 6.3%).

Here, we report a straightforward and low-cost synthetic one-step route to prepare a kind of N-CDs with QY as high as 40.5% by using citric acid monohydrated as carbon resource and ammonia as nitrogen resource via hydrothermal treatment. Moreover, the fluorescence intensity of the obtained N-CDs could be effectively quenched by Hg^{2+} and not affected by other...
metal ions. Based on these experimental results, we obtained a highly sensitive and selective Hg²⁺ fluorescence probe with a detection limit of 0.087 μM, which can be applied to detect the trace amounts of Hg²⁺ in the aqueous media. Furthermore, negligible toxicity, good photostability and perfect biocompatibility of the as-prepared N-CDs mean it can be used for cellular imaging and sensing Hg²⁺ in living cells, which expands the application range of the N-CDs.

Experimental

Reagents and chemicals
Citic acid monohydrated (CA), ammonia (NH₃·H₂O, 25 wt%), sodium hydroxide (NaOH), mercuric nitrate (Hg(NO₃)₂) were supplied by Chengdu Huatao Chemical Reagent and Glass Equipment Co., Ltd. (Chengdu, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sangon (Shanghai, China). Reagents were of analytical grade and used as received without further purification. Doubly distilled water (DDW) was used throughout the experiments.

Apparatus
The size and morphologies were characterized by transmission electron microscopy (TEM) and high resolution TEM (HRTEM) on a Tecnai G2 F20 field emission HRTEM (FEI, USA) with the 200 kV accelerating voltage. X-ray diffraction (XRD) characteristic was carried out on a Tongda TD-3500 X-ray power diffractometer (Dandong, China) using Cu Kα (λ = 0.15148 nm) as radiation source. X-ray photoelectron spectroscopy (XPS) analysis was conducted on an Axis Ultra DLD spectrometer (Kratos, UK) with monochromatic Al Kα source (1486.6 eV). Fourier transform infrared (FT-IR) spectra were recorded on the basis of KBr pellets on a Thermo Nicolet 6700 FT-IR spectrophotometer (Vernon Hills, IL). Fluorescence spectra were obtained by a Hitachi F-7000 fluorescence spectrometer (Tokyo, Japan). The ultraviolet-visible (UV-vis) absorption spectra were performed with a UV1100 UV-visible absorption spectrophotometer (Techcomp, China). The zeta potential for N-CDs was measured on a Nano-ZS Zetasizer ZEN3600 (Malvern, UK). The lifetime measurements were operated on a Horiba TemPro 01 fluorescence lifetime system (Glasgow, UK) with a 370 nm NanoLED pulsed diode excitation source.

Procedures
Preparation of CDs and N-CDs. CDs were fabricated based on the previous report with a little modification (Supporting Information). The blue fluorescent N-CDs were synthesized by CA and NH₃·H₂O through a hydrothermal route. Typically, 0.5 g of CA and 4 mL of NH₃·H₂O were mixed with 5 mL of DDW in a 25 mL Teflon-lined stainless steel autoclave, then gradually heated to 200°C and kept for 5 h. When cooled down to room temperature, the obtained brown color solution was heated to 100°C for 1 h to vaporize excess ammonia and later subjected to a dialysis bag (retained molecular weight: 100 – 500 Da) for purifying. Subsequently, the N-CDs were collected by removing the large dots through centrifugation at 12000 rpm for 10 min and followed by rotary evaporation. Finally, the product was dried under vacuum before dispersing in DDW water as stock solution and then stored at room temperature.

Fluorescence detection of Hg²⁺. A certain volume of N-CDs stock solution was mixed with 1 mL of phosphate buffered solution (PBS) (50 mM, pH 7), then a calculated amount of Hg²⁺ ions was injected into the mixture. Afterwards, the mixture was diluted to the volume of 5 mL with DDW and shaken thoroughly, in which the final concentration of N-CDs was 10 μg mL⁻¹, PBS was 10 mM. After incubating for 20 min at room temperature, the fluorescence spectra were measured at 350 nm excitation wavelength (λex) and 442 nm emission wavelength (λem) with slit width of 5 and 2.5 nm for excitation and emission respectively. All of the PL intensity measurements were conducted in triplicate.

Cytotoxicity assays. The cytotoxicity assessment of N-CDs in human lung cancer PC14 cells (PC14 cells) was carried out according to MTT assays. It is a colorimetric method, which can indirectly reflect the numbers of living cells. In short, PC14 cells were first seeded at a density of 3 × 10⁴ cells per well in 96-well plates and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 24 h until the cells formed a sparsely distributed layer. The cells were then treated with a range of concentration of N-CDs for 24, 48 and 96 h in a final volume of 200 μL. Control tests were performed without N-CDs under the same conditions. Next, MTT solution (20 μL, 5 mg mL⁻¹) was added to each well and incubated for another 4 h. After carefully discarding the supernatant, the formazan crystals were fully dissolved with the addition of dimethyl sulfoxide (DMSO, 150 μL) to each well and incubation in the shaker incubator for 10 min with gentle shaking. Finally, using a Multiskan FC microplate reader (Thermo, USA), the optical density (OD) was recorded at the wavelength of 490 nm. Each assay was replicated three times.

Cellular imaging. PC14 cells were still proposed for cellular imaging. In brief, PC14 cells were seeded on a glass bottom and cultivated for 24 h at 37°C under 5% CO₂. The culture medium was then substituted with fresh culture medium containing 20 μg mL⁻¹ N-CDs and cultivated for an additional 24 h. The supernatant was discarded before imaging with BX53microscopy (Olympus, Japan). Subsequently, fresh medium containing 40 μM Hg²⁺ was introduced into the cells and incubated for 1 h, followed by imagining again.

Results and Discussion

Characterization of the as-prepared N-CDs
UV-vis absorption and fluorescence spectra of the N-CDs. As
shown in Fig. S1A-a (Supporting Information), there were two absorption bands centered at about 245 and 336 nm in the UV–vis spectra, which belonged to the $\pi-\pi^*$ transition of the C≡C bond and n–$\pi^*$ transition of the C=O bond, respectively.$^{31}$ Figure 1 displays the fluorescence emission spectra variation of the N-CDs with the change of $\lambda_{ex}$ from 300 to 390 nm. The maximum $\lambda_{em}$ remained unchanged at 442 nm and the maximum $\lambda_{ex}$ was 350 nm. The excitation-independent PL behavior may be the result of less surface defects.$^{31}$ Meanwhile, such behaviors can avoid the autofluorescence during practical application such as cell imaging.$^{31}$ As shown in Fig. S2 and Table S1 (Supporting Information) using quinine sulfate as a reference,$^{32}$ the QY of N-CDs was calculated to be 40.5% in 10 mM pH 7 PBS solution with the $\lambda_{ex}$ of 350 nm, which was higher than those reported in the literature for N-CDs$^{29,33,34}$ and the non-doped CDs (QY $\approx$ 18%) prepared under the same conditions.

**TEM and XRD.** The morphology of the synthesized N-CDs from TEM (Fig. 2) was nearly spherical and well dispersed with the average size of 3.7 nm based on statistical analysis of 350 individual particles. Furthermore, no lattice fringes were observed in the corresponding HRTEM image (inset of Fig. 2), which illustrated the N-CDs were amorphous in nature. Consistent with HRTEM resolution, XRD pattern (Fig. S3, Supporting Information) exhibited a broad diffraction peak at $2\theta = 23.7^\circ$, which was attributed to the amorphous carbon phase.$^{35}$

**XPS.** As shown in Fig. 3A, the CDs mainly contain O and C elements. In sharp contrast, the N-CDs clearly show the presence of N elements. There were three peaks at 285, 400, 532 eV in the XPS spectra of the N-CDs and they can be assigned to C 1s, N 1s, and O 1s, respectively,$^{36}$ which can indicate the successful doping of N atoms into the CDs by our one-step hydrothermal treatment procedure. Consistent with FT-IR, the high resolution scan of the C 1s spectrum for N-CDs (Fig. 3B) and CDs (the inset of Fig. 3B) also confirm the existence of corresponding groups. The three peaks at 284.6, 285.7 and 287.9 eV would respectively correspond to C–C/C≡C, C–N and C=O bonds.$^{37,38}$ Two peaks shown in the N 1s spectrum (Fig. 3C) at the binding energy of 399.5 and 401.4 eV can be respectively attributed to C-N-C and N-H.$^{39}$ Furthermore, the

---

**Fig. 2** TEM image of N-CDs. Inset panels show size (diameter) distribution (up) and the HRTEM image (down).

---

**Fig. 3** (A) XPS survey spectra of N-CDs and CDs. (B) High-resolution scan of C 1s for N-CDs and CDs (the inset). N 1s (C) and O 1s (D) spectra of the N-CDs.
potential of such N-CDs (Fig. S4B, Supporting Information) for Hg\textsuperscript{2+} was based on quenching the fluorescence of N-CDs, so the effect on the maximum emission wavelength after adding the Feasibility of N-CDs-based sensor for Hg\textsuperscript{2+} below.

Effect of the concentration of N-CDs. The sensing of N-CDs for (10\textsuperscript{μ}g mL\textsuperscript{-1}) and Hg\textsuperscript{2+} (20μg mL\textsuperscript{-1}) was complicated after 20 min and remained stable in the following 40 min (Fig. S6A, Supporting Information). So the measurements were carried out after 20 min in the following experiments.

Effect of reaction time. The reaction between N-CDs and Hg\textsuperscript{2+} was based on quenching the fluorescence of N-CDs, so the concentration of N-CDs should fall within the linear response range.\textsuperscript{32,33} As shown in Fig. S6B (Supporting Information), the maximum quenching efficiency occurred in the pH of 6 and 7. Considering the application of N-CDs in biosystems, pH = 7 was chosen for the subsequent experiments. Sensitivity of the sensing system. Under the optimal conditions as discussed above, the linear response range of N-CDs to Hg\textsuperscript{2+} was investigated at the λ\textsubscript{em} = 442 nm. From Fig. 4A, it is clearly observed that the PL intensity was highly sensitive to the concentration of Hg\textsuperscript{2+}, the higher the concentration of Hg\textsuperscript{2+}, the lower the PL intensity of N-CDs. A good linear relationship between the quenching ratio and Hg\textsuperscript{2+} concentrations in the range of 0 to 8 μM was presented (the inset of Fig. 4A) and followed the Eq. (1):

\[
\frac{(F_0 - F)}{F_0} = 9.961 \times 10^{-10} C + 0.01490 \quad (R^2 = 0.9959)
\]

where \(F_0\) and \(F\) are the PL intensity of N-CDs at 442 nm in the absence and presence of Hg\textsuperscript{2+}, and \(C\) (mol L\textsuperscript{-1}) stands for the concentration of Hg\textsuperscript{2+}. The LOD for Hg\textsuperscript{2+} ions was evaluated to be 0.087 μM using the equation DOL = 3\(S\sigma\), where \(\sigma\) is the relative standard deviation and \(S\) is the slope of the calibration curve (\(\sigma\) was 0.29% obtained from a series of 11 blank tests), which was much lower than the previous report.\textsuperscript{32,33} The mechanism of fluorescence quenching of N-CDs-Hg\textsuperscript{2+} system. Comparing Figs. S1A-a and S1A-b (Supporting Information), when Hg\textsuperscript{2+} was mixed with N-CDs, the position of the peak has not obviously changed. Therefore, the mechanism of Hg\textsuperscript{2+} quenching the PL of N-CDs can be preliminarily inferred as dynamic quenching.\textsuperscript{34} In addition, the PL properties of CDs with Hg\textsuperscript{2+} was also
investigated. As shown in Fig. 4B, the PL intensity of CDs weakened with the increase of Hg\textsuperscript{2+} concentration and the inset shows a linearity between (F\textsubscript{0} – F)/F\textsubscript{0} and Hg\textsuperscript{2+} concentration. However, the LOD was calculated to be 0.25 μM according to its calibration curve (F\textsubscript{0} – F)/F\textsubscript{0} = 4.604 × 10\textsuperscript{-3}C + 0.005390 (R\textsuperscript{2} = 0.9962) (ε was 0.38% obtained from a series of 11 blank tests), which was much higher than the LOD of N-CDs. The improved PL performance for the Hg\textsuperscript{2+} detection using N-CDs could be attributed to the N-doping-induced modulation chemical and electronic charateristics.\textsuperscript{45} Moreover, similar to the fluorescence CDs,\textsuperscript{46} it was deduced that Hg\textsuperscript{2+} can quench the fluorescence of N-CDs by reason of promoting non-radiative electron transfer process and photoinduced electron transfer properties of N-CDs. Fluorescence lifetime experiments were applied to further estimate the the eletctron transfer and exciton recombination process of the N-CDs-Hg\textsuperscript{2+} system. As shown in Fig. S7 (Supporting Information), the fluorescence lifetime of the N-CDs was 9.03 ns through fitting, indicating a fast exciton recombination process. However, when Hg\textsuperscript{2+} was added, the fluorescence lifetime of N-CDs solution obviously decreased to 4.42 ns, which confirmed the quenching type was dynamic quenching, and simulneously proved that a rapid electron transfer process in the N-CDs-Hg\textsuperscript{2+} system occurred.

Selectivity of the sensing system. The selectivity and competition experiments for N-CDs and CDs were completed in the absence and presence of various metal ions including 20 μM of Hg\textsuperscript{2+}, 40 μM of Fe\textsuperscript{3+} and 100 μM of Ba\textsuperscript{2+}, Ca\textsuperscript{2+}, Cd\textsuperscript{2+}, Cr\textsuperscript{3+}, Co\textsuperscript{2+}, K\textsuperscript{+}, Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, Ni\textsuperscript{2+}, Ag\textsuperscript{+}, Pb\textsuperscript{2+} and Fe\textsuperscript{3+}, under the same conditions. For N-CDs, it is encouraging that Hg\textsuperscript{2+}, rather than other ions, has a significant effect on the PL intensity of N-CDs even at the much higher concentration for the other metal ions (Fig. 5A). Moreover, taking into account the cross reactivity, all of the possible interference metal ions for the nanoprobe was explored in the absence and presence of Hg\textsuperscript{2+}. As indicated in Fig. 5A, apparently, the present analytical method for the detection of Hg\textsuperscript{2+} was still effective. However, Ag\textsuperscript{+}, Fe\textsuperscript{3+}, Cu\textsuperscript{2+} and Cd\textsuperscript{2+} also showed significant fluorescence decreases except Hg\textsuperscript{2+} (Fig. 5B), which indicated that CDs had poor selectivity to Hg\textsuperscript{2+}. The better selectivity of N-CDs for Hg\textsuperscript{2+} could be due to the stronger binding affinity with carboxylic, hydroxyl and amino groups on the surface of N-CDs.\textsuperscript{29} In addition, in order to further enlarge the application of N-CDs to biosystems, several representative amino acids were also investigated as potential interferences. As shown in Fig. S8 (Supporting Information), there were negligible PL quenching effects on the N-CDs. All these results clearly highlighted that the N-CDs-based PL sensor exhibited excellent selectivity for the measurement of Hg\textsuperscript{2+} over other related species and could meet the requirements for potential environmental and biological applications.

Analytical applications
Detection of Hg\textsuperscript{2+} in real water samples. The nanoprobe of Hg\textsuperscript{2+}-responsive N-CDs was firstly used to detect the real water samples including mineral water and tap water. The water samples were filtered through a 0.22 μm membrane to discard the potential interferences. It is worth mentioning that no Hg\textsuperscript{2+} was detected in the two water samples by the inductively coupled plasma atomic emission spectrometry (ICP-AES) technique. Hence, the standard addition method was employed to assess the water samples. As listed in Table 1, the recoveries for the two water samples were 96.6 – 105.5%, demonstrating to some extent that the present measuring method for Hg\textsuperscript{2+} was credible and applicable to practical applications.

Cytotoxicity tests and intracellular imaging of Hg\textsuperscript{2+}. For an outstanding fluorescence marker in biological imaging, not only good optical properties but also low toxicity is required. The penetrated cytotoxicity of the obtained N-CDs was evaluated through treating with PC14 cells by MTT assay (Fig. S9, Supporting Information). Results indicated that the cell VR was almost to be 100% even incubated with ultrahigh concentration (1200 μg mL\textsuperscript{-1}) of N-CDs for 48 h and was still more than 80% at the concentration of 400 μg mL\textsuperscript{-1} for 72 h, suggesting the N-CDs’ negligible cytotoxicity and good biocompatibility. The N-CDs were further used in cell imaging in vitro (Fig. 6). It can be seen from Fig. 6B that in the areas of membrane and cytoplasm of the PC14 cells, a strong blue fluorescence image was observed after incubation with N-CDs for 24 h. On the contrary, in the central region related to the nucleus, the fluorescence was relatively weak (the inset of Fig. 6B),
suggesting that the genetic disruption would not occur. Fresh culture medium containing exogenous $40 \mu M$ Hg$^{2+}$ was then cultivated with the cells for another 1 h. As shown in Fig. 6D, intracellular fluorescence obviously weakened. As expected, these results make the probe possible to semiquantitatively detect Hg$^{2+}$ in living cells.

Conclusions

In summary, the N-CDs with good photostability, water-solubility and quantum yield, negligible cytotoxicity and favorable biocompatibility was successfully synthesized by using CA and NH$_2$H$_2$O as precursors through a facile, straightforward hydrothermal method. Such N-CDs can be employed as an effective sensor for sensitive and selective detection of Hg$^{2+}$ in a wide linear response concentration range of $0 - 8 \mu M$ with an LOD of 0.087 $\mu M$ and its applicability was confirmed by the determination of Hg$^{2+}$ in real water samples with the recoveries of 96.6 - 105.5%. Furthermore, the N-CDs can be used as an effective fluorescence marker of living cells, which have been proved by satisfactory performances of the N-CDs in bioimaging before and after the addition of Hg$^{2+}$ in PC14 cells.

Aknowledgements

The authors would like to express their sincere thanks to the Analytical & Testing Centre of Sichuan University.

Supporting Information

The preparation of CDs, UV-vis of N-CDs (Fig. S1), the QY of N-CDs and CDs (Fig. S2 and Table S1) are given here. Some of the charaternional such as XRD, FT-IR and zeta potential (Figs. S3 - S4), and the stability (Fig. S5) of the N-CDs or CDs are displayed. In addition, factors affecting the Hg$^{2+}$ detection are shown in Fig. S6. Table S2 and Fig. S7 provide a more detailed explanation for the mechanism of fluorescence quenching of the N-CDs-Hg$^{2+}$ system. Figures S8 and S9 indicate the selectivity for amino acid and cytotoxicity of N-CDs. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References
