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Rapid Microwave-Assisted Synthesis of Ultra-bright Fluorescent Carbon Dots for Live Cell Staining, Cell-Specific Targeting and in Vivo Imaging

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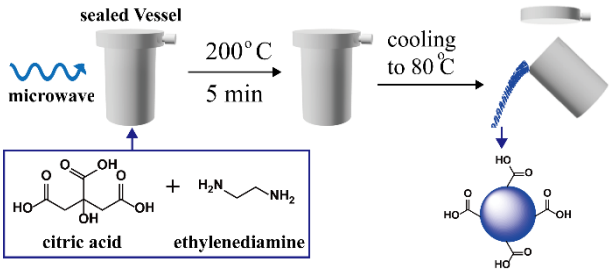
Highly fluorescent carbon dots (CDs) with quantum yields up to 96% were rapidly synthesized within 5 min via microwave irradiation with controllable temperature. Multifunctional bioimaging including live cell staining, cell-specific targeting and *in vivo* imaging were further demonstrated by using high quality and low cost CDs as contrast agents.

Fluorescent probes are particularly useful for high quality optical imaging in a variety of studies in cell biology and biomedical sciences.[1] The existing commonly used probes are organic dyes and semiconductor quantum dots (QDs).[2] However, organic dyes have poor photo-stability and most of them are expensive while QDs typically contain toxic heavy metal, which limit their *in vivo* use.[3] As an alternative, fluorescent carbon dots (CDs) have drawn intense interests due to low cost, less toxicity and adjustable emissions that make them promising candidates as optical probes in bio-imaging applications.[4] Since discovered accidentally from purification of carbon nanotubes in 2004,[5] numerous natural and synthetic substances have been actively explored to make fluorescent CDs through various methods, such as laser ablation,[4a, 6] electrochemical oxidation,[7] chemical oxidation,[8] hydrothermal decomposition[9] and microwave pyrolysis.[10] Among above-mentioned methods, microwave pyrolysis is regarded as a fast, low cost and effective method to fabricate fluorescent CDs. Benefiting from rapid and uniform heating by microwave irradiation (MI), the reaction can be accomplished within several to tens of minutes, and the reported quantum yields (QYs) could reach more than 30%.[10c] Although such QYs might afford fluorescent bio-imaging, however, CDs typically emit blue fluorescence, and in this region cell and tissues have also strong fluorescence which would affect optical imaging quality.



Scheme 1 Schematic diagram for synthesizing CDs via microwave-assisted heating with controllable temperature.

Thus, improving fluorescence brightness is still required to cover strong auto-fluorescence background of cells and tissues for obtaining high quality images. In addition, most of current reports on CDs have been focusing on synthesis methods, characterization and demonstration of cell uptake or staining,[11] but relatively few have applied CDs to cell receptor-targeting[12] and *in vivo* imaging.[13] Therefore, it is of great significance to make full use of highly fluorescent and biocompatible CDs and expand them to high quality multifunctional bio-imaging.

In this paper, we report a rapid synthesis of fluorescent CDs with QYs up to 96% via MI and then demonstrated the highly fluorescent CDs as excellent and low cost probes for live cell staining, cell-specific targeting and *in vivo* imaging. The formation of CDs was based on the condensation reaction and then carbonization of citric acid (CA) and ethylenediamine (EDA), with the detailed experimental procedures in ESI. Scheme 1 shows the synthetic diagram and reaction formula. It is noting that our synthesis methods is different with most of microwave-assisted methods published previously in the synthesis of CDs. They generally employed a domestic microwave oven and conducted the aqueous reaction in an open system with the temperature less than 100 °C.[10] By contrast, we conducted the synthetic reaction in a sealed vessel placed into the microwave digestion furnace with controllable temperature and pressure. Upon microwave irradiation, the temperature of reaction solution was rapidly elevated to 200 °C. It has been demonstrated that high temperature could favour the carbonization process and thus produced highly fluorescent CDs (QYs up to 80%), but the reaction time by the hydrothermal methods required more than 5 h.[9b]

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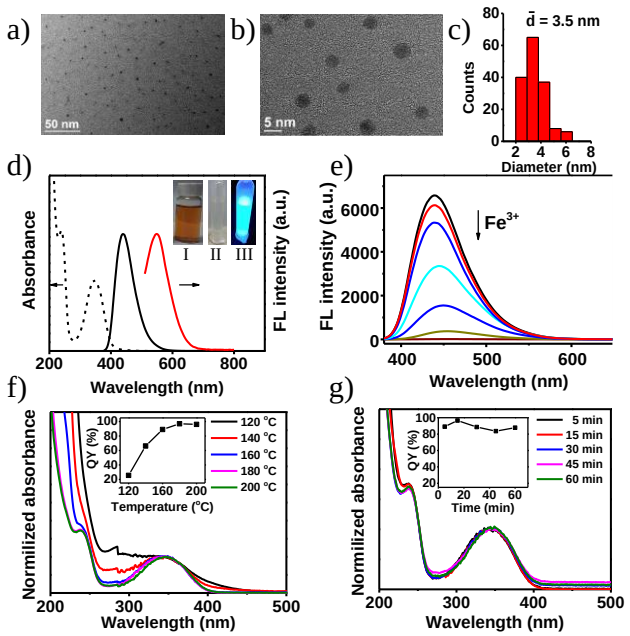

Fig. 1 (a, b) TEM images of CDs with different magnification. (c) Size distribution diagram of the CDs. (d) UV-Vis and fluorescence emission spectra of the CDs. The emission peaks are centered at 440 nm and 534 nm using 360 nm and 488 nm excitation, respectively. The insets show the photographs of crude solution under daylight (I) and the 4000-fold diluted solution under daylight (II) and 365 nm UV excitation (III). (e) Fluorescence quenching after the addition of Fe^{3+} ions with different concentration (0-3 mM). The UV-vis spectra and QYs (the insets) of CDs synthesized by MI for 15 min at different temperatures (f) and at 180 °C for different MI times (g), respectively.

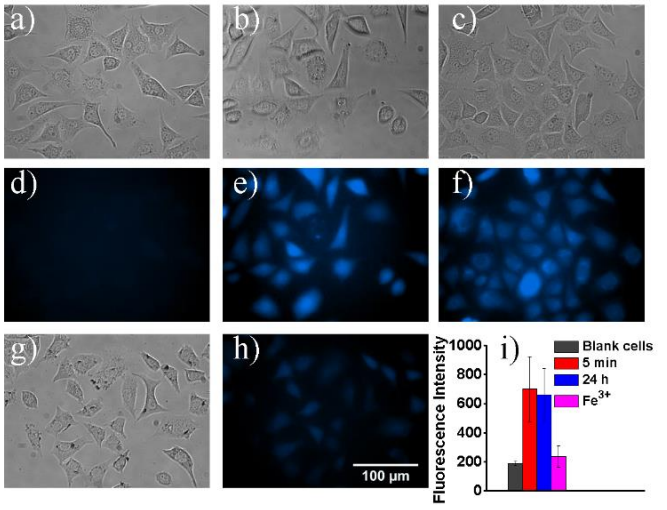

Fig. 2 Bright field (a-c, g) and fluorescence images (d-f, h) of 3T3 cells under different conditions and the intensity histogram (i) of fluorescence images. (a, d) without CD incubation as a negative control. (b, e) after 5 min incubation with CDs. (c, f) after 24 h incubation with CDs. (g, h) after 24 h incubation with Fe^{3+} (0.7 mg/mL) and then 5 min incubation with CDs.

By microwave irradiation, however, we here could accomplish the synthesis process in a sealed vessel within 5 min. Fig. 1a, b show Transmission Electron Microscopy (TEM) images of the CDs under different scale. The resulting CDs show a uniform dispersion and relatively narrow size distribution ranging from 2-6 nm with an average size of 3.5 nm (Fig. 1c). The UV-Vis spectrum of the CDs in Fig.1d shows a peak at around 345 nm and another absorption peak at 240 nm attributable to the π–π* transition of the CDs.[14] A bright fluorescence emission with a peak at around 440 nm was observed upon excitation at 360 nm (Fig. 1d). Consistent with previously reported CDs through MI approach,[10] the CDs also exhibit excitation-dependent fluorescence emission (Fig. 1d and S1), pH-dependent fluorescence intensity (Fig. S2) and fluorescence quenching effect in the presence of Fe^{3+} ions (Fig. 1e and Fig. S2). As can be seen in these figures, the fluorescence intensity decreased significantly in the solutions with too low (< 3) or high (> 9) pH values, while high selective quenching toward Fe^{3+} among meal ions tested suggested that the CDs could be developed as a promising sensor for detection of Fe^{3+}. Despite their common fluorescence feature with previously reported CDs, however, our CDs have higher fluorescence QYs up to 96%. Moreover, CDs here have high production yields (~60%) likely owing to efficient carbonization at high temperature in the sealed vessel. As shown in the insets in Fig.1d, the 4000-fold dilution of crude solution still exhibit strong fluorescence under 365 nm UV excitation. We then examined the effect of reaction temperature and time on the synthesis of CDs. With an increase of temperature, the resultant CD solution deepened gradually in colour from nearly colourless to light yellow and then dark yellow (Fig. S4), accompanied by the occurrence of the peak at around 240 nm in UV-vis spectra and an increase in QYs (Fig.1f). Upon increasing the temperature to 180 °C, as shown in the inset in Fig.1f, the QYs could reach more than 80%. At 180 °C, however, increasing reaction time from 5 min to 1 h would not significantly improve the QYs of CDs as shown in Fig. 1g, suggesting the importance of temperature for producing fluorescent CDs.

To confirm small highly fluorescent CDs for potential biomedical application, we first conducted fluorescence imaging of live cells stained with CDs. Fig. 2a-f show bright field and fluorescence images without incubation of CDs (as a control) and after incubation of 5 min and 24 h with CDs, respectively. From these images, a uniform staining and bright fluorescence for live cells could be observed. The MTT assay showed that 3T3 cells retained viability above 90% after the incubation of CDs with concentrations up to 0.8 mg/mL for 24 h, indicating their low cytotoxicity (Fig. S3). In addition, we found that the cells after 5min incubation with CDs had almost the same fluorescence intensity with those after 24 h incubation (Fig. 2i), suggesting a rapid uptake of CDs in live cells likely owing to their small size. Fig. 3g and h show the bright field and fluorescence images of live cells after 24 h incubation with Fe^{3+} and then 5 min incubation with CDs. A significant decrease in fluorescence intensity suggested a potential and promising application for detection of Fe^{3+} in live cells.

Next, we attached Arginyl-Glycyl-Aspartic acid (RGD) peptides to fluorescent CDs to target integrin receptors on live cell membrane. The used RGD peptide is a cyclic pentapeptide, cyclo(RGDfC), which can specifically recognize cell surface receptor integrin αvβ3.[15] Fig. 3a and b show 3D reconstruction of confocal fluorescence images of HeLa cells (typically with overexpressing integrins[16]) labelled with RGD modified-CDs by excitation with 405 nm and 488 nm laser. A series of optical sections along the z-axis was given in ESI (Video S1) for clear observation. Because of the excitation-dependent fluorescence emission, the CDs could be observed under the two fluorescence channels. Fig. 3c show the overlap of two colour images after projecting 3D images from z-series. Due to the composition of blue and green colour, dot-shaped

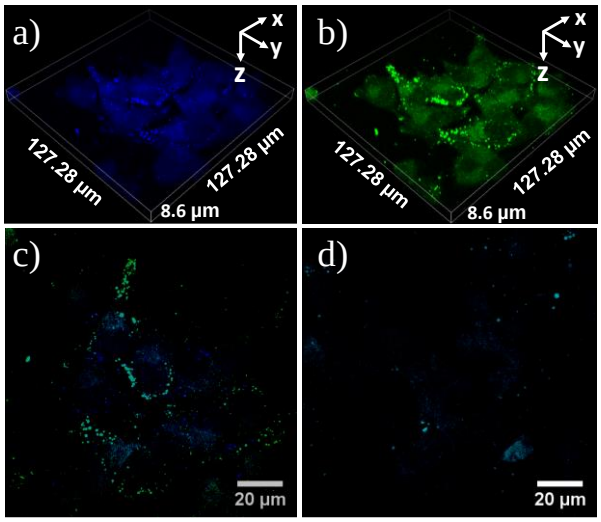

Fig. 3 3D reconstruction of confocal fluorescence images of HeLa cells labelled with RGD modified-CDs when excited with 405 nm (a) and 488 nm laser. The two-color overlap of the projected images from z-series for HeLa cells (c) as shown in a and b, and MCF-7 cells (d) incubated with the same concentration of RGD modified-CDs.

appeared cyan colour and thus confirmed the existence of CDs, Also, we found that the CDs were accumulated both on cell membrane and in the cytoplasm, which was reasonably attributed to the endocytosis of RGD-CDs mediated by αvβ3 integrin receptor.[17] In contrast, we did not observe any fluorescence signal on MCF-7 cells (integrin αvβ3 negative[18]) incubated with the same concentration of CDs (Fig. 3d), suggesting specifically targeting of RGD-CDs to live cells.

To further make use of strong fluorescence feature of CDs, we finally performed *in vivo* fluorescence imaging of HeLa tumor bearing nude mice intravenous injected with CDs using the 405 nm laser as an excitation light source, with the detailed experimental procedures in ESI. As shown in Fig.4, despite strong auto-fluorescence background in body tissue in blue region, intense fluorescence from CDs was observed in the tumor site (red circle) at 30 min after the injection, and then increase gradually up to 6 hours (Fig.4c). In addition, strong fluorescence could also be observed in the bladder (white circle), indicating high efficient renal clearance. Furthermore, the *ex vivo* imaging and biodistributions of CDs were

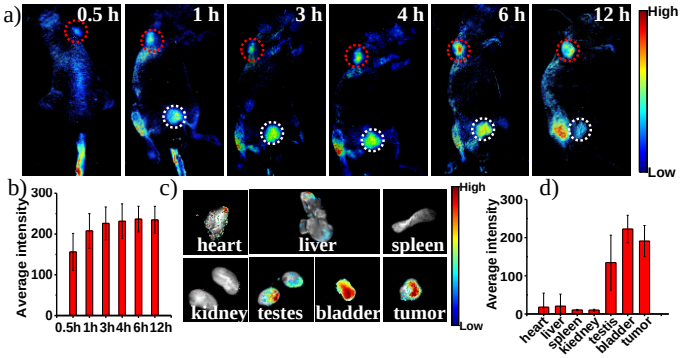

Fig. 4 (a) *In vivo* fluorescence imaging of HeLa tumor bearing mice intravenously injected with CDs at 0.5 h, 1 h, 3 h, 4 h, 6 h and 12 h after injection. Red and white circles indicate tumor and bladder sites, respectively. (b) Quantification of fluorescence intensity of tumor site. (c) *Ex vivo* fluorescence imaging of heart, liver, spleen, kidney, testes, bladder and tumor. (d) Quantification of fluorescence intensity.

performed. The mice injected with CDs were sacrificed at 24 hours post injection, and the main organ, such as heart, liver, spleen, kidney, testes, bladder and tumor, were collected. Fig. 4c shows that *ex vivo* imaging of CDs using fluorescence imaging system when excited with a 405 nm laser. The very strong fluorescence signal could be found in tumor, indicating that the CDs had significant passive targeting capability due to the Enhanced Permeability and Retention (EPR) effect at the solid tumor site in the systemic blood circulation for the high tumor uptake. The organ uptake and biodistribution were quantitatively analyzed by fluorescence intensity in Fig. 4d. It is clear that the bladder and tumor show high uptake, consistent with the imaging results. It is noting that liver show negligible signal, which show the CDs can escape the reticuloendothelial system (RES), and thus induce fast renal excretion. Taken together, the data suggested that a high tumor uptake via the passive targeting effect and renal clearance was realized for CDs.

In conclusion, we have developed a rapid synthesis of highly fluorescent CDs with QYs up to 96% within 5 min by microwave irradiation-assisted methods with controllable temperature. The resultant CDs have small size and narrow distributions. Benefiting from their high fluorescence brightness and small size, the CDs have been demonstrated as excellent contrast agents for high quality bio-imaging including live cell staining, cell-specific targeting and *in vivo* imaging with high efficient renal clearance.

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