One step synthesis of efficient red emissive carbon dots and their bovine serum albumin composites with enhanced multi-photon fluorescence for in vivo bioimaging

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Abstract
Efficient red emissive carbon dots (CDs) in aqueous solutions are very scarce for high performance bioimaging applications. In this work, we report a one-step solvothermal treatment to synthesize pure red emissive CDs (FA-CDs) from citric acid and urea in formic acid without complicated purification procedures. Photoluminescence quantum yield (PLQY) of 43.4% was observed in their dimethyl sulfoxide solutions. High PLQY up to 21.9% in aqueous solutions was achieved in their bovine serum albumin (BSA) composites (FA-CDs@BSA) with significantly enhanced multi-photon fluorescence. The strong surface electron-withdrawing structure of FA-CDs caused by the high content of C=O groups contributes for their pure red emission. Owing to the significantly enhanced single and multi-photon red fluorescence and enlarged particle sizes after composing with BSA, in vivo tumor imaging and two-photon fluorescence imaging of blood vessels in mouse ear have been realized via intravenous injection of FA-CDs@BSA aqueous solutions.

Introduction
Carbon dots (CDs) are a kind of zero-dimensional carbonaceous nanomaterial, which are composed of sp²- and/or sp³-hybridized carbon domains, and decorated by surface functionalities. Due to their ultra-small size (usually less than 10 nm), simple synthesis, low toxicity, well-documented biocompatibility, and outstanding luminescent properties, CDs have attracted wild applications, particularly in bioimaging and biomedical fields.¹,² Noninvasive in vivo fluorescence (FL) imaging calls for an ideal working spectral window in red to NIR range of 650–1450 nm³. Therefore, CDs with strong emissions in red to NIR region in aqueous solutions are highly desired. Until now, efficient blue and green emissions with photoluminescence quantum yield (PLQY) higher than 60% have been reported in several CDs systems, while efficient pure red emissive CDs are still scarce. For example, Jiang and coworkers reported red emissive nitrogen- and fluorine-doped CDs from citric acid, urea, and ammonium fluoride by solvothermal treatment in dimethylformamide (DMF), followed by post alkali treatment.⁶ These CDs exhibited red emission with PLQY of 9.8% in aqueous solutions. Zhang and coworkers prepared p-phenylenediamine based CDs by solvothermal method.⁷ The red emissive CDs can be separated through silica gel column chromatography with PLQY of 31.4% in ethanol. Fan and coworkers reported red emissive CDs with large amino acid from 1,4,5,8-tetraminoanthraquinone and citric acid through solvothermal method followed by purification.
through silica gel column chromatography\(^8\), PLQY of 10.1% was measured in their DMF solutions. It can be seen that the reported efficient red emissive CDs were fabricated in multiple steps including complicated purification procedures, such as silica-gel column chromatography. More seriously, high PLQYs of the red emissions from these CDs were always obtained in their organic solutions\(^9,10\), but be significantly decreased in water\(^11–13\), which greatly hinder their application for in vivo bioimaging in aqueous system.

It has been demonstrated that surface chemical structures play an important role in the luminescence properties of CDs\(^14,15\). In our previous work, we reported orange emissive CDs with large-sized conjugated sp\(^2\)-domain through increasing the carbonization processes between citric acid and urea under solvothermal treatment in DMF\(^16\). Enhanced PLQY of 46% in ethanol was realized through surface metal cation-functionalizing of these CDs, but they exhibited a low PLQY of 6% in water. We further demonstrated solvents with electron-rich (S = O/C = O) groups, such as dimethyl sulfoxide (DMSO), DMF, N-methyl-2-pyrrolidone (NMP), can facilitate the enhancement of the red emissions from CDs\(^17\). It can be inferred that increasing the surface electron-rich groups is beneficial for pure red emissive CDs with high PLQY.

In this work, we developed a one-step solvothermal treatment to synthesize pure red emissive CDs from citric acid and urea in formic acid (FA) without complicated purification procedures. PLQY of 43.4% was observed in their DMSO solutions. Comparing the CDs synthesized from citric acid and urea in different organic solvents under the same solvothermal treatments, the FA solvothermal prepared CDs (FA-CDs) contained the highest content of C = O groups on their surface, resulting in uniform strong electron-withdrawing surface structure, which account for their pure red emission. The red emission from FA-CDs was relatively weak in water but can be effectively enhanced after composing with bovine serum albumin (BSA) with PLQY up to 21.9% in their composite (FA-CDs@BSA) aqueous solutions. The prepared FA-CDs and FA-CDs@BSA exhibit low cytotoxicity and renal excretion. Moreover, the FA-CDs@BSA can accumulate in the tumor tissue through the blood circulation with a clearly red FL tumor imaging in a long observation time window (>6 h). Furthermore, significantly enhanced two- and three-photon red emissions were observed in FA-CDs@BSA aqueous solutions. Two-photon FL imaging of mouse ear vessels has also been achieved via intravenous injection of FA-CDs@BSA aqueous solutions. To our knowledge, this is the first time of realizing in vivo two-photon FL imaging in mammals based on CDs\(^13,18–21\).

**Results**

FA-CDs were synthesized from citric acid and urea by a one-step solvothermal treatment in FA. Specifically, citric acid (2 g), urea (4 g), and 20 mL FA were reacted at 160 °C for 4 h under solvothermal condition. The reacted dark red-brown solution was added to 40 mL ethanol, shaken well, and then centrifuged at 10,000 r min\(^{-1}\) for 5 min and repeated 2–3 times. The precipitate was freeze-dried to obtain the black product of FA-CDs.

To investigate the effect of solvents in the solvothermal treatments on the morphologies, chemical structures and optical properties of CDs, acetic acid (AcOH), acetone (DMK), and DMF were chosen as solvents to prepare CDs under the same solvothermal treatments, which were AcOH-CDs, DMK-CDs, and DMF-CDs, respectively, as shown in Fig. 1.

The morphologies of the four samples were characterized using transmission electron microscopy (TEM) and atomic force microscopy (AFM). As seen in the TEM images (Fig. 2a–d), these samples show similar average particle diameters of about 5 nm with uniform dispersion. Similar well-resolved lattice fringes with an interlayer spacing of 0.26, 0.24, 0.25, and 0.21 nm can be observed in their high-resolution TEM (HRTEM) images, indicating all these samples exhibit similar graphite-like core structures\(^21–25\). The particle sizes of AcOH-CDs, DMK-CDs, DMF-CDs, FA-CDs from TEM and AFM images (Fig. 2e–h) are similar in the range of 1–7 nm.

X-ray photoelectron spectroscopy (XPS) was carried out to investigate the chemical structures of the four samples. The full survey of the XPS spectra of the four samples
reveal the presence of C, N, O, as shown in Fig. 2i. The atomic content in the four samples is shown in Table 1. It can be seen that DMK-CDs, AcOH-CDs and DMF-CDs contain the highest carbon, oxygen and nitrogen content, respectively. The high resolution C 1 s, N 1 s, and O 1 s XPS spectra of the four samples reveal the presence of C = C/C (284.8 eV), C − N/C − O (286.8 eV), and C = O/COO (288.4 eV) bonds (from C 1 s XPS spectra); C − N = C (399.5 eV), N − H (400.7 eV) bonds (from N 1 s XPS spectra); and C = O (531.2 eV), C − O/O − H (532.6 eV) bonds (from O 1 s XPS spectra). By comparison, the C = O content in FA-CDs (17.43%) and DMF-CDs (16.26%) are...
much higher than that in AcOH-CDs (9.44%) and DMK-CDs (7.17%), while the N−H and C−O/O−H content in DMF-CDs (10.48% and 7.70%, respectively) are higher than that in FA-CDs (5.30% and 7.21%, respectively). Given the electron-withdrawing character of C=O groups and proton-donating character of −NH and −OH groups, the surface chemical structure of FA-CDs is more electron-withdrawing than that of DMF-CDs. According to morphology and XPS analysis, the FA-CDs exhibit the strongest electron-withdrawing surface structure among the four samples.

The UV-visible absorption spectra of the four samples in aqueous solutions were measured (Fig. 3a). AcOH-CDs and DMK-CDs display absorption bands in UV region peaked at 337 nm and 361 nm, respectively. DMF-CDs exhibit a much broader absorption bands, with peaks at 340, 420, and 554 nm, while FA-CDs have a main absorption band at 556 nm. As shown in Fig. 3c, d, AcOH-CDs and DMK-CDs in aqueous solutions display similar single cyan luminescence centers in excitation-emission maps. DMF-CDs exhibit multiple luminescence centers covering from blue to red regions, with the main emission center in green region at 550 nm (Fig. 3e). In contrast, FA-CDs exhibit a main red emission center at 640 nm (Fig. 3f). Figure 3b shows the corresponding normalized maximum emission spectra of the four samples in aqueous solutions at 368, 375, 432, and 556 nm excitation, respectively. Considering the strongest electron-withdrawing surface structures of FA-CDs, it can be inferred the high content of C=O groups and low content of proton-donating −NH and −OH groups account for their pure red bandgap emission.

To further investigate the influence of C=O, −OH and −NH groups on the surface environments, absorption and emission spectra of FA-CDs were measured in water and DMSO solutions, as shown in Fig. 4a. From protic water solvent to aprotic DMSO solvent, the main absorption band of FA-CDs was red-shifted from 556 nm to 576 nm with a shoulder band centered at 600 nm and an emerging small absorption band at 700 nm. The red emission from FA-CDs in water was relatively weak with PLQY of 6.0%, while its intensity can be 15-fold enhancement in DMSO with PLQY up to 43.4% (Table 2). DMSO is aprotic solvent with good deprotonation ability due to the S=O groups with strong electron-withdrawing ability. It had been demonstrated that deprotonation of the surface can enhance the surface electron-withdrawing environment of CDs, leading to redshifted absorption band, and enhanced emission intensity by inhibiting the energy dissipation of hydroxyl groups. It can be inferred that the weak red emission from FA-CDs in water is due to the surface protonation caused by water molecules, while the redshifted absorption bands with enhanced red emission are due to the surface deprotonation caused by DMSO molecules. Figure 4d shows the FL decay curves of FA-CDs in water and DMSO solutions at 640 nm measured under excitation at 510 nm. FA-CDs exhibit a considerably longer average FL lifetime in DMSO than that in water, indicating much lower energy dissipation, which agrees well with their FL properties.

Temperature-dependent absorption and emission spectra of FA-CDs in water and DMSO were further measured to investigate the affection of surface protonation and deprotonation processes on their optical properties (Fig. 4b, c, e, f). For FA-CDs in water (Fig. 4b, e), the absorption band gradually increased and red-shifted from 529 nm to 561 nm from 0 to 90 °C (Fig. 4b), while their red FL intensity first gradually increased until 60 °C and then slightly decreased upon further heating to 90 °C (Fig. 4e). In contrast, the absorption bands and red emission of FA-CDs in DMSO were gradually decreased upon heating to 90 °C (Fig. 4c, f). The ratios of the maximum intensities of absorption (Iabs) and emission (Iem) bands at different temperatures to the maximum intensities of that at 20 °C were given in Fig. 4g. The opposite temperature dependent optical behaviors of FA-CDs in water and DMSO can be explained by the protonation and deprotonation processes caused by water and DMSO molecules, respectively, through intermolecular hydrogen bonds between solvent molecules and surface groups on FA-CDs. In water, the hydrogen bonds between H2O molecules and the surface carbonyl groups on FA-CDs lead to surface protonation, which account for the quenched red FL. Upon increasing temperature, the hydrogen bonds were weakened, leading to the enhanced red-shifted absorption band and enhanced red emission at temperature below 60 °C. At the temperature higher than 60 °C, the gradually decreased FL intensity can be due to thermal vibration induced energy losses. In DMSO, the deprotonation process caused by hydrogen bonds between DMSO molecules and the surface proton-donating (−NH, −OH, −COOH) on FA-CDs can be weakened upon increasing temperature, leading to decreased absorption bands and red emissions. The hydrogen bonds between DMSO molecules and the surface proton-rich groups (−NH, −OH, −COOH) on FA-CDs can be further demonstrated by their temperature dependent 1H NMR spectra (Fig. 4h). The proton signals of Ar−H (7.8 ppm), amide (8.12 ppm), −CHO (8.5–10 ppm) and −COOH (10.36, 10.49, 11.87, 12.05, 13.53 and 14 ppm) were detected in the 1H NMR spectrum of FA-CDs in DMSO-d6 at 25 °C. Upon heating to 60 °C, the proton signals of the hydrogen bonds donor groups of amide and −COOH moved to 8.06 ppm and 9.97, 10.13, 11.78, 11.96, 13.45, 13.94 ppm, respectively, while the proton signals of Ar−H, −CHO were almost unchanged, indicating temperature dependent hydrogen
Fig. 3 Absorption and emission spectra of AcOH-CDs, DMK-CDs, DMF-CDs and FA-CDs

a Normalized UV–vis absorption spectra of AcOH-CDs, DMK-CDs, DMF-CDs and FA-CDs in dilute aqueous solutions.

b The corresponding normalized maximum emission spectra of AcOH-CDs, DMK-CDs, DMF-CDs and FA-CDs in dilute aqueous solutions at 368, 375, 432 and 556 nm excitation, respectively. Excitation-emission maps of

c AcOH-CDs,

d DMK-CDs,

e DMF-CDs and

f FA-CDs in dilute aqueous solutions.
bonds between DMSO molecules and the surface $\text{−NH}$ and $\text{−COOH}$ groups on FA-CDs.

Based on the results above, it can be concluded that the red bandgap emissions from CDs are not only determined by the particle sizes or the carbonization content of their cores, but also the surface electron-withdrawing environment. High content of surface electron withdrawing groups ($\text{C=O}$) on FA-CDs and DMF-CDs cause narrowed bandgaps, leading to redshifted absorption and emission bands. DMF-CDs contain more proton-donating $\text{−NH}$ groups than FA-CDs, which weaken their surface electron-withdrawing environment, leading to insufficient redshifted emission centers with maximum emission in green light region. Although AcOH-CDs contain the highest oxygen content, while most of oxygen atoms are in the form of hydroxyl or ether groups and the content of $\text{C=O}$ groups is much smaller, leading to much more weakened surface electron-withdrawing environment.
structure. By comparing the differences of the surface chemical structures of the four samples and the four solvent molecules in the solvothermal treatments, it can be inferred that the aldehyde group in FA and their acid nature play a significant role in the formation of high content of C=O groups and low content of −NH and −OH groups on the surface of FA-CDs. Although DMF molecules also contain aldehyde group, the weak alkaline nature of DMF solvent result in the prepared DMF-CDs containing much higher −NH groups than FA-CDs, which might be the reason for their different optical properties.

To achieve enhanced red emission in aqueous solutions, BSA, a wildly used biocompatible protein, was selected to modify FA-CDs. 3 mL FA-CDs aqueous solutions (0.02 mg mL\(^{-1}\)) was added to 3 mL BSA aqueous solutions with BSA concentrations of 0.02–6 mg mL\(^{-1}\). The mixed solutions were heated at 50 °C for 10 min to give rise FA-CDs and BSA composites (FA-CDs@BSA) with different composing ratios. Figure 5a, b shows the absorption and emission spectra of FA-CDs@BSA aqueous solutions at different composing ratios (FA-CDs: 0.01 mg mL\(^{-1}\)). As increasing the BSA concentrations from 0 to 3 mg mL\(^{-1}\), the absorption bands of FA-CDs
gradually increased and red-shifted from 556 nm to 571 nm, while their red emission exhibited 8-fold enhancement with PLQY up to 21.9% in aqueous solutions, as shown in Fig. 5c and Table 2. In contrast, BSA modified DMF-CDs also exhibited enhanced emission, while their highest PLQY is just 7.3% in aqueous solutions (Table 2).

It is interesting to find that the multiphoton induced red FL from FA-CDs can be obviously enhanced after combing with BAS in aqueous solutions. As shown in Fig. 5d, e, g, h, under 1150 nm and 1550 nm fs pulse laser excitation, the red emission intensities of FA-CDs and FA-CDs@BSA aqueous solutions were found to increase linearly with the square and cube of laser power, respectively (Fig. S1), indicating the two- and three-photon FL behaviors. The two- and three-photon FL of FA-CDs@BSA aqueous solutions was 15-fold and 12-fold enhancement than that of pure FA-CDs aqueous solutions under 1150 nm fs pulse laser excitation at 3.5 mW and 1550 nm fs pulse laser excitation at 5 mW, respectively. These results indicated that combination with BSA played an important role in protecting FL quenching by water molecules to realize both enhanced single and multi-photon FL from FA-CDs in aqueous solutions.

The combination structure of FA-CDs@BSA were further analyzed. The zeta potential measurements showed that both FA-CDs and BSA were negatively charged with zeta potentials of −31.12 mV and −17 mV, respectively, while the zeta potential value became −21.55 mV of the combination of FA-CDs and BSA (FA-CDs:0.01 mg mL⁻¹, BSA:1 mg mL⁻¹), indicating that FA-CDs and BSA formed complexes (FA-CDs@BSA) through electrostatic interactions.

The conformational changes of BAS proteins before and after combining with FA-CDs were investigated by circular dichroism spectroscopy. As shown in Fig. S2, the circular dichroism signals of BAS were changed slightly after combining with FA-CDs, indicating no significant conformational changes of BAS in FA-CDs@BSA. Considering that wrapping on FA-CDs could cause significant conformational changes of BAS, it can be inferred that BAS molecules were absorbed on the surface of FA-CDs through electrostatic interactions. The morphology of FA-CDs@BSA was investigated by TEM, as shown in Fig. S3. BAS is a globulin with a Stoke radius of approximately 3.5 nm. The high contrast dots with sizes of about 4.2 nm correspond to FA-CDs, while the low contrast particles with sizes from 20 to 100 nm can be assigned to BAS clusters. It can be seen that all BAS clusters contain several FA-CDs, which are the FA-CDs@BSA. It can be inferred that FA-CDs@BSA are the combination of multiple FA-CDs and BAS molecules with much larger particle sizes, in which BAS molecules are adsorbed on the surface of FA-CDs by electrostatic interactions.

Femtosecond transient absorption (TA) spectroscopy were carried out to explore the excited state dynamics of FA-CDs in water and DMSO, and FA-CDs@BSA in water (Fig. 6). The ground state bleaching (GSB) signals of FA-CDs in water, FA-CDs in DMSO and FA-CDs@BSA in water were observed at 520 nm, 600 nm and 580 nm, respectively, which agree with their steady-state absorption spectra in general. Their positive excited state absorption (ESA) signals were observed at 700 nm, 730 nm and 750 nm, respectively, which exhibited the similar decay lifetimes with their corresponding GSB signals. Comparing the TA kinetic traces of their GSB signals, it is clear that FA-CDs in water show an extremely fast decay within 4.21 ps, while FA-CDs in DMSO show a much a longer lifetime to thousands of ps. The ultra-fast decay of FA-CDs in water can be attributed to the rapid electrons transfer quenching of the excited state by water molecules. After combining with BAS, FA-CDs@BSA in water exhibited a much longer lifetime than that of FA-CDs in water, further demonstrating the combination of BAS can effectively avoid the energy losses in aqueous solutions by preventing the interactions between the C=O groups on FA-CDs and water molecules.

The cytotoxicity of FA-CDs and FA-CDs@BSA (mass ratio of FA-CDs and BSA: 1:100) was assessed in SMMC-7721, Huh-7 and MDA-MB-231 cells. As shown in Fig. 7a, b and Fig. S4, FA-CDs and FA-CDs@BSA barely affected cell viability at concentrations containing FA-CDs up to 250 μg mL⁻¹; thus, they are of low cytotoxicity.

Due to the red fluorescent properties and low cytotoxicity, we further examined the biocompatibility and biodistributions of FA-CDs and FA-CDs@BSA in mice. Red FL imaging of main organs (heart, liver, spleen, lungs and kidneys) before and after (0.5, 3, 6, 12, 24 and 48 h) intravenous injections of FA-CDs and FA-CDs@BSA aqueous solutions were performed, as shown in Fig. 7c. Based on changes of the red FL signals in the main organs, it can be seen that both FA-CDs and FA-CDs@BSA could be excreted from the mice via liver and kidney in 48 h after intravenous injections. In contrast, FA-CDs@BSA exhibited a longer circulation time than pure FA-CDs, which can be due to their increased particle sizes after combining with BAS.

The feasibility of in vivo FL tumor imaging of mice using FA-CDs and FA-CDs@BSA were also tested. After intravenous injection of 0.2 mL FA-CDs@BSA aqueous solutions (FA-CDs: 0.2 mg mL⁻¹, BSA: 20 mg mL⁻¹) or FA-CDs aqueous solutions (0.2 mg mL⁻¹) into nude mice with 4T1 tumor, each mouse’s entire body developed a bright red FL over time, as shown in Fig. 8a. At 4 h post injection, red FL intensity was significantly reduced throughout the body, whereas the red FL signal in the tumor region of the FA-CDs@BSA treated mouse could last in much higher intensity for much longer time than...
that of the FA-CDs treated mouse. As seen in Fig. 8b, after 12 h of injection, FL signal contrast (I_T/I_N) of the red FL intensities at the tumor site (I_T) and the near tissue (I_N) in the FA-CDs@BSA treated mouse can reached a value higher than 2, which can last for a long observation time window in more than 6 h. In comparison, the FL signal ratio in tumor site of the FA-CDs treated mouse is much smaller in the whole observation period. It can be inferred that combination with BSA can increase the particle sizes of FA-CDs@BSA, which promote longer blood circulation and higher accumulation of FA-CDs@BSA in the tumor site through enhanced permeability and retention (EPR) effect, leading clearly red FL tumor imaging performance.

Considering the significant two-photon red FL of FA-CDs@BSA in aqueous solutions, in vivo two-photon red FL imaging of mouse ear vessels was carried out based on FA-CDs@BSA. Mouse was given intravenous injection of 0.2 mL FA-CDs@BSA aqueous solutions (FA-CDs: 0.2 mg mL$^{-1}$, BSA: 20 mg mL$^{-1}$). Green (506–593 nm) and red (604–678 nm) channel emissions from the mouse ear were collected under 1150 nm fs laser excitation at 4.5 mW. The green signal was the second harmonic signal from 1150 nm fs laser of collagen fibers in the mouse ear. A one-minute video of two-photon red FL imaging for the mouse ear under 1150 nm fs laser excitation was recorded before and after intravenous injection of FA-CDs@BSA aqueous solutions (Video S1). As shown in Fig. 8c, significant two-photon red FL signal from FA-CDs@BSA can be clearly detected in the mouse ear vessels at 15 s post injection. Moreover, after 40 mins of injection, bright