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View Article Online
DOI: 10.1039/C6RA04309G

Journal Name

COMMUNICATION

Pseudo-Multicolor Carbon Dots Emission and the Dilution-Induced Reversible Fluorescence Shift

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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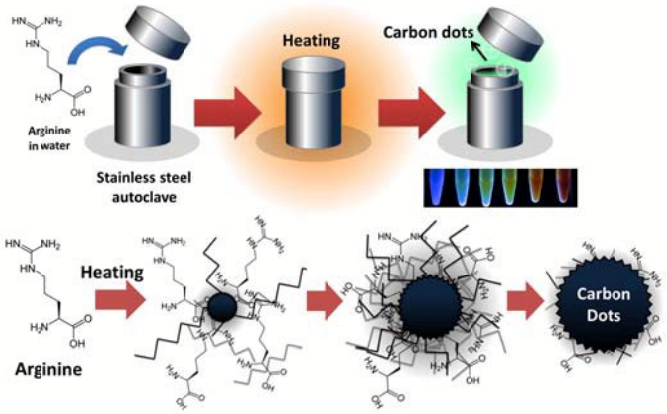
The size- and color-tunable carbon dots (CDs) that emit blue to red using a single precursor in pure water was achieved by simple concentration and reaction time control. Distinct from conventional fluorophores, a reversible spectra shift of CD was observed upon dilution which could be a result of altered CD-CD inter-particle interaction, leading to minimized self-absorption.

Carbon dots (CDs) have been proposed to be the next-generation nanomaterial and may serve as a promising alternative for both *in vitro* and *in vivo* clinical applications including drug delivery, biosensing, and bioimaging due to their environmental friendliness, excellent biocompatibility, ease of preparation, and cost-effectiveness.[1] They are luminescent and consist of an amorphous carbon or graphite structure with highly oxygenated groups at their surface. CDs have been synthesized via several approaches, such as laser ablation,[2] electrooxidation of graphite,[3] acid treatment of carbon precursors,[4] hydrothermal synthesis,[5] microwave-based pyrolysis,[6] and room-temperature catalytic dehydration of small molecules.[7] Although significant progress has been made, high-quality fluorescent CDs remain scarce. Their availability is limited by the following bottlenecks. First, most CDs possess weak fluorescence with low quantum yield (QY%) and are excited by UV light.[8] Second, few carbon nanoparticles emit longer wavelengths (red to near-IR) [9] and multiple precursors and additional extraction steps are necessary for preparation. Third, although some methods[10] report milligram-scale quantities with 5–60% QY%, these methods require high-energy radiation-based synthesis followed by surface functionalization and purification, and simple, large-scale synthesis is usually difficult. Finally, although CDs that show excitation-dependent emission have been reported,[9, 11] such behavior was distinct from that of conventional fluorophores and the detailed mechanism by which CDs generate photoluminescence is not yet fully understood.[12] Some studies have proposed that excitation-dependent emission bands might originate from the red-edge effect of inherited functionalities at the surface.[13] The effect of band-to-band (π*-π) and interstate-to-band (π*-n) induced transitions leads to multicolor emissions. Amino functional groups might act as radiative recombination centers for tunable photoluminescent emission.[14] Nevertheless, it remains difficult to determine general criteria for modulation of the fluorescent properties of CDs, as CDs synthesized from various precursors have usually been employed.



Scheme 1. Single precursor-based, one-step CD synthesis. Using a Teflon-lined autoclave reactor, we tuned the synthetic parameters (arginine concentration and reaction time) to enable multicolor CD synthesis.

Compounds possessing amino or/and carboxyl groups have been proposed to be suitable candidates, as a result of their better inter-molecular interactions to initiate nuclei formation[5, 15] during CD synthesis. Amino acids possessing both amino and carboxyl groups are compatible with biological systems and several previous studies[6, 15-16] have reported the use of amino acid as ideal initial reactants. Herein, we demonstrated that multiple fluorescent CDs with significantly improved quantum yield could be synthesized using a single precursor in pure water, simply by tuning the concentration and the reaction time without necessity to employ additional catalysts or acid

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Electronic Supplementary Information (ESI) available. See DOI: 10.1039/x0xx00000x

treatment. The high QY% (41.8 %) of CD was synthesized which enabled in vivo bioimaging excited at 633 nm. In addition, our study revealed that the CD-CD inter-particle interaction could play a substantial role in the observed fluorescence, resulting in red-shifted "pseudo-multicolor" fluorescence. This strategy also enabled systematic investigation of the reaction parameters of CD synthesis, facilitating a better understanding of the observed photoluminescence of such materials.

Table 1. Summary of the relative content, diameter, and quantum yield of synthesized CDs. Carbon dots synthesized at specified concentrations (Molarity, M) and times (hours, h) are abbreviated as $CD_{concentration,time}$. (All synthesized CD suspensions were diluted to normalize the absorbance consistent with 0.01 mg mL^{-1} quinine sulfate before QY% calculation)

	XPS					
	C [%]	N [%]	O [%]	C/(N+O)	Size (nm)	Quantum yield (%)
$CD_{1,3}$	67.39	9.23	23.38	2.07	2.1±0.8	14.6
$CD_{1,6}$	58.34	11.35	30.3	1.93	3.2±1.3	27.9
$CD_{1,9}$	51.85	11.8	36.35	1.08	4.7±1.2	32.6
$CD_{1,12}$	50.27	10.91	38.82	1.01	5.6±1.3	34.5
$CD_{2,9}$	47.48	11.48	41.04	0.90	8.7±3.7	38.4
$CD_{3,9}$	42.54	13.16	44.3	0.74	10.9±3.4	41.8

The hydrothermal approach using a Teflon-lined autoclave reactor was employed due to its flexibility for easy scale-up and simple one-step CD synthesis (Scheme 1). Besides, under high temperature and pressure, the solubility of amino acid could be further improved. After an initial screen of 10 representative amino acids with characteristic side chains (Table S1), the arginine (1M) was selected as excellent CD precursor in this study according to the achieved highest QY% (compared with the other amino acids under identical hydrothermal conditions of 180°C for 9 h). In the subsequent experiment to optimize the hydrothermal conditions, we tuned the concentration of the arginine precursor (1 M, 2 M, and 3 M (higher precursor concentrations were not examined owing to the arginine solubility)) and the reaction time (3 h, 6 h, 9 h, and 12 h). Carbon dots synthesized at specified precursor concentrations and times are abbreviated as $CD_{concentration,\ time}$. Six conditions for CD synthesis ($CD_{1,3}$, $CD_{1,6}$, $CD_{1,9}$, $CD_{1,12}$, $CD_{2,9}$, and $CD_{3,9}$) were compared and characterized by atomic force microscopy (AFM), Raman, Fourier-transform infrared (FT-IR), X-ray photoluminescence spectroscopy (XPS), and X-Ray Diffraction (XRD) (Table 1; Fig. S1-S4). The spherical CDs were well dispersed as observed in AFM images. Two characteristic bands, D and G, in Raman spectra of CDs were found in all samples and appeared featureless with increasing reaction time and concentration; this is probably due to the enhanced intrinsic photoluminescence background of CDs during synthesis. The FT-IR and deconvoluted high-resolution C1s and N1s XPS analysis confirmed the functional groups (enhanced amide I stretching and aldehyde C-H IR peaks, Fig. S2) and chemical composition of the CDs (Fig. S3). The obtained CDs presented a broad diffraction peak at 2θ= 20-22° in its XRD pattern (Fig. S4), indicating that the interlayer spacing of the (002) diffraction peak is 0.392 nm, which was larger than that of graphite (0.34 nm). The increase in d value potentially revealed an increase in heterogeneous nature attributed to the introduction of more nitrogen-, and oxygen-containing groups.[8,12] Improved crystallinity was observed in $CD_{3,9}$ and this result was consistent with the found crystalline structures using high resolution TEM (Fig. S5). The QY% was improved by the increased precursor content and the prolonged reaction time and it was inversely correlated with the chemical content (carbon(C)/(nitrogen (N) + oxygen (O)) of the CDs (Table 1).

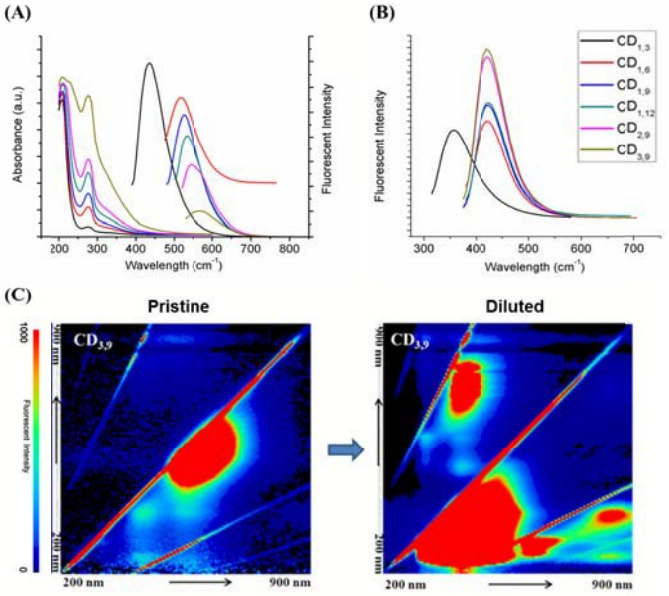

Fig. 1. (A) The absorbance spectra and the fluorescent λ_{em} maxima of six synthesized CDs (excite at λ_{ex} maxima, respectively); (B) normalized, maximal fluorescent emission peaks (CD samples diluted to the absorbance consistent with 0.01 mg mL^{-1} quinine sulfate). (C) The 3D fluorescence spectra of pristine $CD_{3,9}$ (measured right after synthesis) and that of diluted CDs. Recovered intrinsic fluorescence of $CD_{3,9}$ with enhanced fluorescence intensity was observed upon dilution. (x-axis: 200 nm-900 nm of emission wavelengths; y-axis: 200 nm-900 nm of excitation wavelengths; rainbow color gradient: florescence intensity; the HRTEM image of the $CD_{3,9}$ is shown in Fig. S5).

The CD absorption spectra showed a broad shoulder with a maximum at 280 nm, which could be ascribed to the n-π* transition of the C=O band and the π-π* transition of the conjugated C=C band (Fig. 1A). All synthesized CDs showed excitation-dependent fluorescence emission (Fig. S8), and their λ_{ex} and λ_{em} maxima were both red-shifted (from $\lambda_{ex}/\lambda_{em}$ = 380/440 nm for $CD_{1,3}$, to $\lambda_{ex}/\lambda_{em}$ =520/570 nm for $CD_{3,9}$, Fig. 1A). We also observed red and near-IR emission of CDs, using lasers excited at 488 nm (Fig. S6). Color changes from blue to red were observed under UV (365 nm) illumination (Fig. 2, pristine CDs). However, as shown in Fig. 1A, broadening of spectra and decreasing photoluminescence intensity at long wavelengths were observed. This decrease in intensity was inconsistent with the calculated QY% in Table 1. On the other hand, diluted CD suspensions (to make the absorbance consistent with 0.01 mg mL^{-1} quinine sulfate) revealed the increased fluorescence intensity exactly corresponding to QY% (Fig. 1B; Fig. 2, diluted CDs). The fluorescent emission of the CD samples was recovered after dilution. A full scan of both the excitation and the emission wavelengths (200–900 nm) of

the other synthesized CDs photoluminescence confirmed this observation (Fig. S7-S8). Interestingly, we also found suspected upconversion photoluminescence after the dilution step (Fig. 1C), but nevertheless, additional investigation is undergoing to clarify such phenomenon as it could be resulted from the excitation of the short-wavelength second-order diffraction light coexisting with long-wavelength light in the spectrometer [17]. We employed $CD_{3,9}$ suspension to further investigate the dilution-induced fluorescence spectra changes. Reversible peak shifts were observed upon dilution and re-concentration (Fig. 3). Diluted $CD_{3,9}$ revealed recovered blue emission whereas concentrated, pristine $CD_{3,9}$ has shown quenched and red-shifted fluorescence. Such phenomena are distinct from those observed in conventional fluorescent molecules (e.g. a high quinine sulfate concentration led to diminished fluorescence emission with only limited λ_{em} shifts (Fig. S9). The synthesized CDs were additionally investigated in various solvents to examine their solubility, fluorescence spectra and the effects on QY% (Fig. S10; Table S2). All synthesized CDs were soluble in pure water and MeOH; with minute solubility in DMF and DMSO for CDs synthesized under high arginine concentration and longer reaction time, but were insoluble in THF and toluene (Fig. S10). Superior QY% was found in pure water when comparing with that of the other tested solvents (Table S2). The fluorescence spectra of all soluble CDs behaved similar excitation-dependent emission patterns (e.g. $CD_{3,9}$ shown in Fig. S11). However, due to the poor solubility in most organic solvents, the potential inner filter effect of CDs in high concentration was unavailable.

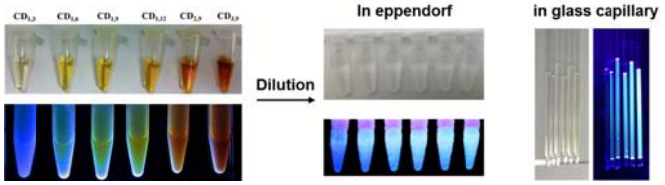

Fig. 2. Photograph of synthesized CDs (pristine) and CDs after dilution (pristine CD suspensions were diluted to normalize the absorbance consistent with 0.01 mg mL^{-1} quinine sulfate) under white light and under a UV lamp (365 nm). (diluted samples were placed both in the plastic eppendorf tube and glass capillary to verify their intrinsic fluorescence)

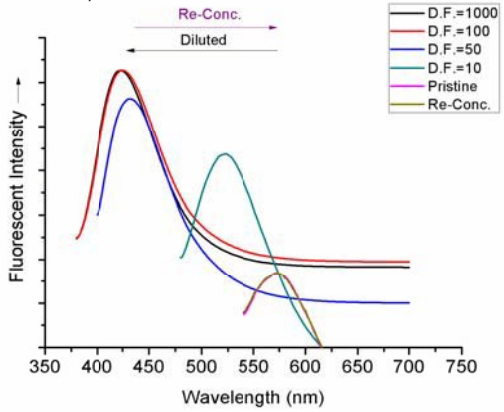

Fig. 3. Reversible fluorescence shift upon dilution and re-concentration. The red-blue shifts of maximal fluorescence emission peaks in pristine $CD_{3,9}$ with dilution factor (D.F.) of 10X, 50X, 100X, and 1000X, respectively. (The black line arrow indicates serial dilution of the $CD_{3,9}$ solution and the purple line arrow represents the 50X re-concentration of $CD_{3,9}$ sample from 50X-diluted solution).

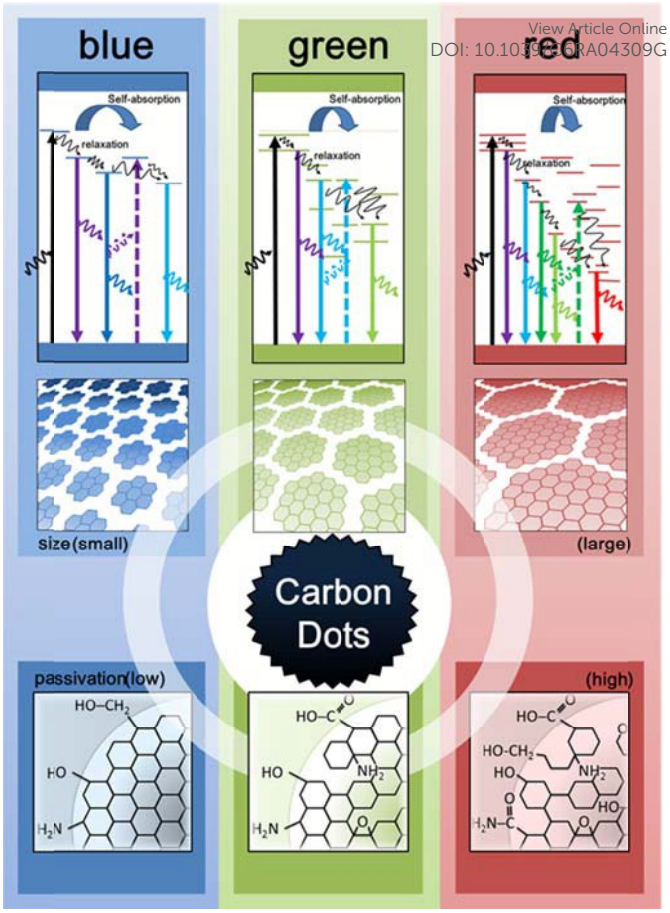

Fig. 4. Proposed hypothetical mechanism of red-shifted fluorescent CDs. The photoluminescent properties of CDs can result from quantum confinement, the sp^2 nano-domain, and surface defects created during passivation. In addition, red-shifted spectra were observed in concentrated CDs. This could be due to the heterogeneity (including size and passivation level) created during the hydrothermal process. The presence of multiple emission centers on CDs surfaces could undergo self-absorption or energy exchange with adjacent CDs (dash line), leading to the shifts of fluorescence emission to longer wavelengths.noting that the fluorescence emissions of certain pristine CDs were often compared right after synthesis without considering the inconsistency of the absorbance among CDs samples. In these cases, the "multicolor" CDs were likely inappropriately characterized.

Developing high quality multicolor CDs for bioimaging is current research hotspot. As shown in our study, CDs with different particle sizes, chemical content/functional groups and QY% could be tuned by varying the precursor concentration and reaction time. An apparent red-shift of the fluorescence maximum was observed as reaction time increased from 3 h to 6 h. The high precursor concentration and a prolonged reaction time may facilitate carbonization to enable large particle growth, increasing the graphitic domains and the status of surface passivation. We also found although there were limited fluorescent λ_{em} maxima shifts after 6 h, significant improvement in hydrophilicity (due to the surface passivation) and QY% was achieved with prolonged reaction time ($CD_{1,9}$ and $CD_{1,12}$) and increasing arginine concentration ($CD_{2,9}$ and $CD_{3,9}$). We provide here a potential model (Fig. 4) to demonstrate the possibly involved mechanisms[18]. High degree of surface passivation observed at a high arginine concentration using a long reaction time might result in the

presence of surface energy traps that become emissive upon stabilization.[19] Emission at longer wavelengths may have contributed to quantum-confinement[20] and electronic conjugate structures, the larger sp^2 nano-domain with appropriate size of band gaps.[21] Additionally, the involvement of oxygen and nitrogen may change the sp^2 domain by modulating the extent of the sp^3 carbon matrix, creating defects that alter the distribution of emissive sites at the CD surface [22] and energy levels corresponding to the maximum transition probability.[23] As a result, different surface defects may generate different emissive sites, leading to excitation-dependent and tunable long-wavelength emission behaviors. Not only size but also surface status may have contributed to such alteration in the CD photoluminescent properties. Finally, the multiple emission centers may be present at CD surfaces, due to the heterogeneity (including size and passivation level) created during the hydrothermal process. Spectral distribution and increased non-radiative trapping[24] may also occur. With concentrated CD suspensions (e.g. in the case of $CD_{3,9}$), although only red-shifted spectra could be observed at longer wavelengths, blue-region emission was actually present but quenched (potentially caused by the inner filter effect[25]). When the energy gap is matched, the two individual emitters could undergo self-absorption or energy exchange with adjacent CDs, altering the fluorescence emission spectra.

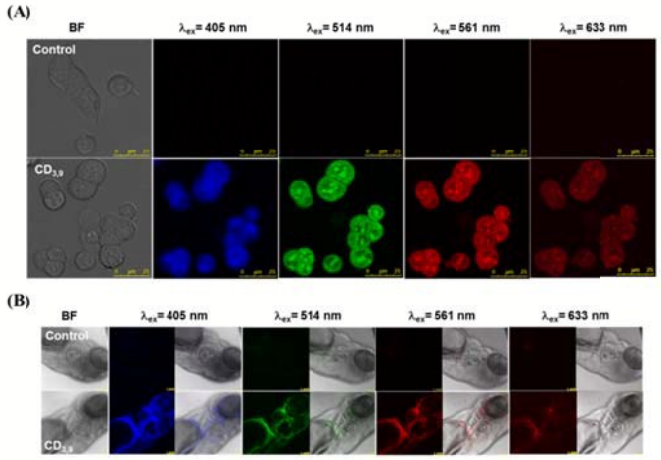

Fig. 5. (A) Confocal images of HCT116 cells incubated with $CD_{3,9}$ and (B) zebrafish treated with $CD_{3,9}$ by microinjection. The images were obtained at excitation wavelengths of 405 nm, 514 nm, 561 nm, and 633 nm, respectively (pseudo-colors were employed to represent the emission at various excitation wavelengths).

In summary, we developed a single source, one-step method for size- and color-tunable CDs. This simple strategy improves and simplifies conventional processes for synthesis of CDs, which often involve strong acids or bases as catalysts and require multiple precursors for nucleation and passivation. By tuning the hydrothermal reaction time and the precursor concentration, the QY% of CDs could be significantly improved. Most importantly, we observed a dramatic spectral shift in diluted CDs, which is distinct from typical fluorophore–fluorophore interactions. We have shown that the CD-CD inter-particle interaction could play a substantial role in the observed fluorescence and the so-called "multicolor" fluorescence might actually originate from the inner filter effect, resulting in red-shifted fluorescence (But the inherent fluorescent is in fact located in blue region). As a result, the "pseudo-multicolor" CD emission was observed. Nonetheless, detailed investigations still will be required to clarify whether the source of such heterogeneity is the distribution of optical properties between CDs, demonstrating a collective response, or between individual emitters within CDs.

Additionally, our synthesized CDs showed low toxicity, wide-range pH stability (pH 3–11) and were resistant to photobleaching for several months under ambient conditions or continuous laser irradiation (18 W, 380–760 nm) (Fig. S12-S13). The excellent QY% (41.8 %) of the $CD_{3,9}$ in water solution has facilitated the development of new biocompatible optical tracers for real-time in vivo bioimaging (Fig. 5; Fig. S14-S15), enabling the excitation at longer wavelengths (633nm) to minimize tissue damages. Intense excitation-dependent fluorescence signals could be observed throughout the zebrafish body, indicating that CDs had effectively entered the blood circulation. Such optical probe shows potential for further bio-distribution studies and drug delivery application. Finally, by taking advantage of dilution-induced fluorescence alteration resulted from CD-CD inter-particle interaction, the optical characteristics may be applied to swelling sensing of some hydrogel-based materials in the future.

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DOI: 10.1039/C6RA04309G

Table of contents entry

Reversible spectra shift of carbon dots upon dilution which could be a result of altered CD-CD inter-particle interaction.