

# Carbon Dot-Silica Nanoparticle Composites for Ultralong Lifetime Phosphorescence Imaging in Tissue and Cells at Room Temperature

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## Supporting Information



ABSTRACT: Compared to fluorescence imaging with short-lived emissive probes, the use of phosphorescent probes conveys the advantage of long signal persistance in time and this permits one to discriminate against interference from autofluorescence. However, the realization of room temperature phosphorescence (RTP) probes that feature ultralong emission lifetimes in aqueous solution is still a challenge. Here, we present a rational strategy for realizing ultralong RTP in air-saturated aqueous media from carbon dot-based silica composites ( $CDs@SiO_2$ ) which feature emission lifetimes as long as 1.64 s. The excellent phosphorescence properties, their small size, and their water solubility make  $CDs@SiO_2$  a promising material for biological imaging application. We demonstrate their use as efficient reporters both in plant tissue and in animal cells where strong autofluorescence poses a severe challenge for conventional, short-lifetime probes.

## 1. INTRODUCTION

Imaging and sensing with optical probes is in widespread use for the monitoring of complex biological processes, with great progress made over the past decades to improve their sensitivity, specificity, and achievable spatial and temporal resolution.<sup>1</sup> Fluorescence probes are most commonly used in these applications, and signals usually characterized according to wavelength and intensity, providing information on the identity, spatial location, and concentration of the probe.<sup>2</sup> A problem with fluorescence imaging stems from interference by endogenous background fluorescence (autofluorescence) of biological samples which spans the entire visible spectrum and thus overlaps with emission wavelengths of the reporter fluorophores. Since the emission lifetimes for the reporter signals and the autofluorescence are similar (of the order of nanoseconds), there is no way to discriminate between reporters and autofluorescence,<sup>3</sup> resulting in poor performance at low fluorophore concentrations. Devising long-lifetime luminescence probes, which can be temporally distinguished from the short-lived autofluorescence, is thus a highly desirable goal in biological imaging applications. The signal-to-noise ratio can then be increased by using short pulses of light for

probe excitation and setting appropriate time delays before the signals are collected.

Exploiting the phenomenon of room temperature phosphorescence (RTP) is a promising strategy in this respect. To obtain high-quality phosphorescence images of biological tissue, these materials need to exhibit sufficiently long phosphorescence lifetimes in their aqueous environment for the effective elimination of autofluorescence. In recent years, RTP materials have been extensively studied and broadly classified into organometallic complexes<sup>4,5</sup> and crystalline organic compounds.<sup>6,7</sup> Organometallic complexes commonly contain noble metals, such as iridium and platinum, thereby suffering from high cost and cytotoxicity. Crystalline organic compounds, on the other hand, are formed by special organic moieties with limited chemical diversity, and they require complex synthetic procedures for manufacture. In addition, successful application for phosphorescence imaging has been limited by their poor biocompatibility and dispersion proper-

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Figure 1. (a) Schematic illustration of the possible structural formation of  $CDs@SiO_2$ . (b) Snapshots of  $CDs@SiO_2$  dialyzed against deionized water (upper row), aqueous  $CDs@SiO_2$  after dialysis (middle row), solid-state  $CDs@SiO_2$  (bottom row) at different delay times from 0 to 10 s after 365 nm UV irradiation under ambient conditions, respectively.

ties in aqueous environments. The development of metal-free RTP materials featuring long lifetimes, low toxicity, good water dispersion properties, and cost-effective and facile routes for manufacture is thus timely and highly desirable.

Carbon dots (CDs) have received widespread attention as metal-free alternatives for RTP material.<sup>8-16</sup> They have numerous merits, including excellent water solubility, good biocompatibility, and facile preparation, which make them promising candidates for time-resolved fluorescence imaging. However, most reported CD-based RTP materials are confined to their solid states. For example, polymer CDs benefit from the so called crosslink-enhanced emission (CEE) effect which leads to a suppression of nonradiative transitions, leading to enhanced RTP properties.<sup>8,9</sup> Similarly, incorporating CDs into various matrices, such as poly(vinyl alcohol),<sup>10</sup> polyurethane,<sup>11</sup> KAl(SO<sub>4</sub>)<sub>2</sub>: $x(H_2O)$ ,<sup>12</sup> silica gel,<sup>13</sup> urea/biuret,<sup>14</sup> zeolites,<sup>15</sup> and boric acid,<sup>16</sup> can also protect the triplet states of CDs from which phosphorescence is emitted by restriction of intermolecular motions. Past strategies have therefore focused mostly on CDs in their solid states for RTP generation because phosphorescence can be easily quenched by water and dissolved oxygen.<sup>17</sup> Nevertheless, some noteworthy attempts have been reported to develop aqueous CD-based RTP materials. For example, Lin et al. have successfully prepared long afterglow CD-based composites dispersed in water where they exhibited a lifetime of 0.703 s, mostly consisting of delayed fluorescence.<sup>18</sup> Recently, the incorporation of CDs and cyanuric acid was shown to enhance RTP in the presence of bound water where the suspension exhibits a lifetime of 687 ms.<sup>17</sup> All these aqueous CD-based RTP materials exhibit relatively short phosphorescence lifetimes or strong signal quenching in bulk water, which impedes their potential for practical application.

Inspired by these studies, we hypothesized that a successful strategy for the production of efficient RTP from aqueous CD-based materials involves the confinement of CDs within a hydrophilic matrix, which is able to protect CDs from environmental quenchers and also provides for excellent dispersion properties in aqueous environments—key to their use in biological research. In this work, we encapsulated CDs in colloidal silica, verified that the composite dispersed readily in water, and that RTP from the dispersion was successfully achieved and stable. Figure 1a shows a schematic of the CDs encapsulated within silica networks through the conventional Stöber synthesis route,<sup>19</sup> which involves the hydrolysis and condensation of tetraethyl orthosilicate (TEOS) in the presence of CDs, water, and ammonia. During the TEOS hydrolysis step, Si–O–C bonds are formed between CDs and

silica that act as scaffolds for the nucleation and growth of the SiO<sub>2</sub> framework. The CDs are thus covalently linked into the silica network, with the silica acting as a matrix that helps the dispersion of the CDs, offering protection from environmental quenchers, such as water and oxygen. More importantly, abundant silanol groups on the composite surface endow the entire hybrid system with good hydrophilicity. Figure 1b shows RTP from both aqueous and solid-state CDs@SiO<sub>2</sub> that is seen to last for nearly 10 s and is visible to the unaided eye. The measured 1/e decay time was 1.64 s and is the longest so far reported for any aqueous CD-based RTP material. In this article, we report the first application of CD-based RTP materials for practical imaging in biological samples. We successfully resolve the phosphorescence decay from CDbased RTP materials taken up by plant samples (mung bean sprouts and onion bulb epidermal tissue) and animal cells (mouse breast carcinoma EM-6 cells), demonstrating the advantage of phosphorescent CDs@SiO<sub>2</sub> in bioimaging by eliminating the interference of autofluorescence.

#### 2. EXPERIMENTAL SECTION

**2.1. Synthesis of CDs.** Polyacrylic acid (PAA) (0.45 g) and ethylenediamine (EDA) (0.84 mL) were mixed in deionized water (60 mL) under stirring, and the solution was then transferred into a 100 mL Teflon-lined stainless-steel autoclave and subsequently heated at 200  $^{\circ}$ C for 8 h, using a modified approach of that reported in ref 8. After cooling down to room temperature, the resultant solution was dialyzed in dialysis bags (3000 Da) against deionized water for 2 days. Finally, CDs were obtained by freeze drying and then redispersed in deionized water.

**2.2.** Synthesis of CDs@SiO<sub>2</sub>. Briefly, CDs (20 mg), deionized water (50 mL), tetraethyl orthosilicate (TEOS) (8 mL), and ammonia aqueous solution (2 mL) were added to a 100 mL flask. After stirring at 100 °C for 5 h, the resulting product was dialyzed in a dialysis bag (3000 Da) against deionized water for 3 days. After filtering through a 0.22  $\mu$ m pore-sized membrane, the aqueous CDs@SiO<sub>2</sub> was obtained. The solid-state CDs@SiO<sub>2</sub> material was produced by drying the aqueous CDs@SiO<sub>2</sub> for 12 h in a vacuum drying oven heated to 80 °C.

**2.3.** Synthesis of CDs-2@SiO<sub>2</sub> to CDs-5@SiO<sub>2</sub>. For preparation of CDs-2 to CDs-5, the mole number of starting material-I was the same as the amount of PAA used in the synthesis of CDs (starting material for CDs-2: polyethylene; CDs-3: acrylic acid; CDs-4: PAA, CDs-5: none). Likewise, the mole number of starting material-II was the same as the amount of EDA used in the synthesis of CDs (CDs-2: EDA; CDs-3: EDA; CDs-4: EDA; CDs-5: none). The subsequent steps were the same as for the synthesis of CDs mentioned above. Identical synthesis conditions of preparing CDs-2@SiO<sub>2</sub> to CDs-5@SiO<sub>2</sub> were employed to ensure valid comparison with CDs@SiO<sub>2</sub>.

2.4. Synthesis of CDs@SiO<sub>2</sub>-2. Briefly, to a 100 mL flask, deionized water (50 mL), TEOS (8 mL), and ammonia aqueous



**Figure 2.** (a) FT-IR spectra of CDs,  $CDs@SiO_2$ , and  $SiO_2$ , respectively. (b) HRTEM image of  $CDs@SiO_2$ . (c) XPS patterns of CDs and  $CDs@SiO_2$ . (d–f) High-resolution XPS spectra of C 1s, Si 2p, and O 1s of  $CDs@SiO_2$ , respectively. (g) Photoluminescence (PL) emission spectra of aqueous  $CDs@SiO_2$ , excited at different excitation wavelengths. (h) Afterglow emission spectra of aqueous  $CDs@SiO_2$ , excited at different excitation spectra of solid-state  $CDs@SiO_2$  at different temperatures under excitation of 356 nm.

solution (2 mL) were added. After stirring under 100 °C for 5 h, CDs (20 mg) were added into the flask and continuously stirred under the same conditions for another 5 h. The resulting product was dialyzed in a dialysis bag (3000 Da) against deionized water for 3 days. After filtering through a 0.22  $\mu$ m membrane, the aqueous CDs@SiO<sub>2</sub>-2 was obtained.

## 3. RESULTS AND DISCUSSION

In general, effective RTP from CDs is enabled if the following conditions are met: (i) the  $n \rightarrow \pi^*$  transition from carboxylic groups in CDs enables efficient spin-orbit coupling and generates excited triplet states;<sup>20,21</sup> (ii) a high degree of crosslinking and entanglement of polymers confine the motion of CDs and thereby excited triplet states;<sup>8,22</sup> (iii) the presence of heteroatoms (N, P, and F) via doping in CDs facilitates intersystem crossing (ISC) for the introduction of RTP.<sup>9,14,23</sup> Given the above considerations, we chose PAA and EDA as starting materials for the preparation of CDs. As shown in Figure S1a,b, the produced CDs are narrowly distributed around an average diameter of 3.1 nm. The inset of Figure S1b reveals a high crystallinity of CDs with an interplanar spacing of 0.21 nm, which could be attributed to the (001) facet of graphite,<sup>24</sup> indicating that CDs consist of graphitized cores and amorphous surfaces. Fourier transform infrared (FT-IR)

spectra (Figure 2a) confirm the existence of abundant -COOH groups in the CDs.

While no phosphorescence was observed in CDs solution under room temperature, in dried samples, void of  $H_2O$ , a weak afterglow was observed, which is similar in character to what was reported.<sup>8</sup> These findings indicate that the system has excellent potential to realize RTP if one manages to protect the system from environmental quenchers such as  $H_2O$  and  $O_2$ .<sup>9</sup>

Remarkably, through the hydrolysis and condensation of TEOS in the presence of CDs, water, and ammonia, colloidal  $CDs@SiO_2$  composites could be successfully prepared by the conventional Stöber method,<sup>19</sup> and materials thus manufactured featured both strong fluorescence and phosphorescence emission. As shown in the transmission electron microscopy (TEM) image (Figure 2b), the structure of  $CDs@SiO_2$  is clearly resolved and the CDs are seen to be well dispersed in the amorphous silica framework. Moreover, scanning electron microscopy (SEM) (Figure S2a) revealed a smooth surface of  $CDs@SiO_2$ , indicating that CDs were anchored within the matrix rather than adsorbed onto the surface. This conclusion was supported by an experiment, in which we synthesized  $CDs@SiO_2-2$  by adding CDs only after the formation of SiO<sub>2</sub>. As displayed in Figure S2b, the resulting material features a



**Figure 3.** (a) Afterglow decay curve and lifetimes of solid-state  $CDs@SiO_2$  at different temperatures, monitored at 520 nm with 356 nm excitation. (b) Energy level diagram of  $CDs@SiO_2$  (Abs.: absorption; Fluo.: fluorescence; Vib.: vibration; Phos.: phosphoresence). (c) Afterglow decay curve and lifetimes of aqueous  $CDs@SiO_2$  monitored at 520 nm with 356 nm excitation. (d) Normalized UV-vis absorbance spectrum and afterglow excitation spectrum of aqueous  $CDs@SiO_2$  monitored at 520 nm. (e) Normalized fluorescence intensities (excited at 347 nm) and phosphorescence (excited at 356 nm) of aqueous  $CDs@SiO_2$  under different pH values. (f) Normalized fluorescence intensities (excited at 347 nm) and phosphorescence (excited at 356 nm) of solid-state  $CDs@SiO_2$  under different oxygen contents.

rough surface as the CDs were externally adsorbed onto the silica scaffold, and were thus less protected from the environment, resulting in poorer RTP performance, which will be discussed later.

To characterize the nature of the chemical bonding between CDs and silica, Fourier transform infrared (FT-IR) spectroscopy and X-ray photoelectron spectroscopy (XPS) were performed on the material. FT-IR spectra (Figure 2a) revealed an absorption band at 3450 cm<sup>-1</sup> assignable to the O-H stretching vibration from -COOH groups in the CDs and Si-OH of silica. A further band at 1668 cm<sup>-1</sup> is attributed to the vibration of C=O bonds in CDs.<sup>25</sup> Upon incorporation of CDs into the silica matrix, further characteristic vibrations appear for Si–O–Si (1080–1357  $\text{cm}^{-1}$ ) and Si–O–C (871  $cm^{-1}$ ).<sup>26</sup> Although the Si–O–C vibration at 871  $cm^{-1}$  is partially masked from the strong absorption band of Si-O-Si vibration present in silica, it can nevertheless be clearly distinguished in CDs@SiO<sub>2</sub>.<sup>27</sup> In addition, the existence of Si-OH, as evident from the 800 cm<sup>-1</sup> peak, confers good hydrophobicity to the material. Correspondingly, XPS spectra (Figure 2c) reveal the existence of C, N, and O in CDs and a newly emerging peak, indicating the presence of Si in CDs@ SiO<sub>2</sub>. The C 1s spectrum of CDs@SiO<sub>2</sub> (Figure 2d) can be deconvoluted into four peaks at 284.0, 284.9, 286.1, and 287.6 eV corresponding to C=C/C-C, C-O/C-N, Si-O-C, and C=O bonds, respectively.<sup>28</sup> The O 1s analysis (Figure 2e) indicates the existence of Si-O-C (532.8 eV) and SiO<sub>2</sub> (533.5 eV).<sup>29</sup> The Si 2p spectrum (Figure 2f) involves two bands that can be assigned to Si-O-C (103.0 eV) and Si-Ox (104.2 eV), respectively.<sup>26</sup> Comparing this with the highresolution XPS spectra of CDs (Figure S3a-c) proves the formation of Si-O-C and SiO<sub>2</sub> bonds in CDs@SiO<sub>2</sub>. Altogether, then, these data provide clear evidence that CDs are embedded within colloidal silica through Si-O-C bonds.

Subsequently, the optical properties of CDs@SiO<sub>2</sub> were systematically investigated. As shown in Figure 2g, aqueous CDs@SiO2 exhibits a blue luminescence with excitationwavelength-dependent PL behavior, which is similar to that of CDs solution (Figure S4a-c). Fluorescence lifetime measurements of CDs and the composite were also carried out, as displayed in Figure S5a. The PL decay curves for CDs in solution and aqueous CDs@SiO<sub>2</sub> could be fitted by a doubleexponential function, and average decay times were calculated to be 5.9 and 5.5 ns, respectively. The similar PL and decay features of CDs and CDs@SiO2 indicate that it is the CDs themselves which constitute the main luminescent species. The fluorescence quantum yield (QY) for aqueous CDs and aqueous CDs@SiO<sub>2</sub> was measured to be 7.5 and 9.8%, respectively, consistent with a previous report that the formation of C-O-Si bonds in a silica matrix enhances the PL emission.<sup>26,30</sup> More importantly, although the CDs in solution on their own do not exhibit any phosphorescence, the composite material becomes highly phosphorescent after incorporation into the silica matrix. As shown in the afterglow emission spectra of aqueous CDs@SiO<sub>2</sub> (Figure 2h), the afterglow emission maximum is centered at a wavelength of 520 nm with an obvious shoulder near 440 nm when excited at shorter wavelengths. These photoluminescence characteristics are tentatively ascribed predominantly to phosphorescence and a portion of delayed fluorescence (DF). To further confirm this deduction, the effects of temperature on the afterglow properties of CDs@SiO2 were investigated. Figures 2i and 3a demonstrate that both intensity and lifetime increased significantly as the temperature was decreased to 77 K, resulting from an effective restriction of vibrational motion and suppression of nonradiative transitions at low temperature.<sup>31</sup> In addition, a blue shift of the emission peak was observed at 77 K, indicating changes in the configuration of transitions.<sup>3</sup> Furthermore, as the temperature was increased gradually from

77 K, a small part of DF showed up, confirming the existence of a thermally assisted process;<sup>18</sup> thus, we can conclude that the observed afterglow of CDs@SiO<sub>2</sub> mainly consists of RTP and a small part of thermally activated delayed florescence (TADF) emission. Note again that pure CDs in solution do not exhibit any afterglow emission at room temperature; however, a weak emission became apparent at 77 K. From PL and afterglow emission spectra of pure CDs, and aqueous CDs@SiO<sub>2</sub> at 77 K (Figure S5b,c), the energy gap between the lowest  $T_1$  and  $S_1$  ( $\Delta E_{ST}$ ) decreased from 0.54 to 0.46 eV, after the silica encapsulation. This implies that the silica matrix effectively stabilizes the excited triplet species in CDs, facilitating both intersystem crossing (ISC) and reverse intersystem crossing (RISC) (Figure 3b).<sup>33</sup>

To gain insights into the RTP properties of aqueous CDs@ $SiO_2$ , their afterglow decay was measured. As shown in Figure 3c, the decay curve can be fitted well by a triexponential function, suggesting the presence of three decay channels responsible for the observed PL pattern. From the decay components, an average lifetime was calculated according to<sup>9,18</sup>

$$\tau_{\rm avg} = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i$$

yielding  $\tau_{\rm avg}$  = 1.64 s, the longest RTP lifetime for an aqueous CD-based material reported so far. Subsequently, we investigated the origin of RTP from aqueous CDs@SiO2. Figure 3d shows two absorption bands peaked at 270 and 325 nm, which belong to the  $\pi \to \pi^*$  transition of C=C and  $n \to$  $\pi^*$  transitions of C=O, respectively.<sup>34,35</sup> It can be seen that the afterglow excitation band overlaps partially with the absorption peak in the 300-400 nm wavelength region, indicating that the phosphorescence originates from the C=Obond in the CDs. This had previously been shown to promote the generation of intrinsic triplet excitons through intersystem crossing (ISC).<sup>8,14</sup> To confirm the validity of this assumption, a negative control experiment was performed. As presented in Table S1, a material we refer to as CDs-2 is structurally similar to CDs, but without carboxyl groups present in the raw materials. From the UV-vis absorption spectra in Figure S6, it can be seen that CDs@SiO<sub>2</sub> and CDs-2@SiO<sub>2</sub> differ in that the  $n \to \pi^*$  transition of C=O is not present in the latter, which therefore hardly emits RTP. Altogether, it appears that RTP mainly stems from the C=O bond of CDs, promoting ISC to the triplet states.

The distinctive advantage of CDs@SiO<sub>2</sub> is the outstanding RTP characteristics when the material is in solution. In previous reports, TEM, SEM, FT-IR, and XPS have been used to study the activation of RTP in CDs through embedding or immobilizing them in certain matrix materials. These point to dispersion of CDs within the silica framework and the formation of covalent bonds between CDs and silica as vital in suppressing nonradiative transitions by rigidifying the excited triplet species. This in turn leads to the promotion of  $T_1$  electrons to undergo radiative recombination, thus giving rise to RTP emission.<sup>10,16</sup> To experimentally verify the critical role of dispersion CDs within the silica framework, we made a comparison between CDs@SiO2 and a material referred to as CDs@SiO<sub>2</sub>-2. The latter was prepared by adding CDs after formation of the silica network. As revealed in Figure S7a,b, CDs@SiO<sub>2</sub>-2 shows much poorer phosphorescence performance than CDs@SiO2. To elucidate mechanistic details behind this observation, we analyzed CDs@SiO<sub>2</sub>-2 using TEM and

XPS. Figure S8a shows clearly that a few CDs were attached to the silica (the area within the white dashed line). The highresolution XPS data presented in Figure S8b-d and Table S2 show a significant decrease in the contents of Si-O-C bonds compared to CDs@SiO<sub>2</sub>. Again, the data point to a reduction in the quenching of triplet excitons in CDs through covalently crosslinked bonds. Moreover, the effects of pH on the fluorescence and phosphorescence of aqueous CDs@SiO<sub>2</sub> were investigated, as shown in Figure 3e. Both the fluorescence and phosphorescence intensities are seen to remain to within about 80% of their peak values when the pH is below 11, but there is a sudden and dramatic drop as the pH increases beyond 11. This could be due to etching of the silica in a strongly alkaline condition,<sup>36</sup> resulting in an exposure of CDs to the surrounding solution. It can be concluded that silica matrix leads to very strong enhancement and protection of RTP from CDs. In addition, the optical properties of aqueous CDs@SiO<sub>2</sub> in the presence of various metal ions were studied, respectively, in Figure S9; both fluorescence and phosphorescence intensities of CDs@SiO2 remained in an acceptable level (>80%) when confronted with metal ions, except the major quenching caused by  $Ce^{3+}$ ,  $Co^{2+}$ , and  $Fe^{3+}$  ions, but it should be noted that CDs@SiO2 in this experiment was exposed to very high concentrations (80 mM) of metal ions. So, it is fair to say that the luminescence of CDs@SiO<sub>2</sub> is relatively stable in the environment with the interference of potentially existing metal ions.

As expected, the particles were found to remain well dispersed in solution for months without any noticeable deterioration of RTP properties. We heated and thus dried samples of aqueous CDs@SiO2 to result in xerogels or powders and, as presented in Figure S10a-e, their fluorescence and phosphorescence properties were found to remain similar to those of aqueous CDs@SiO2. However, it was not possible to redisperse the material in water to form a colloidal solution because the dehydration reaction occurs between silanol groups, and the latter are critical to confer the hydrophilic behavior on the composite material. Still, the RTP of CDs@ SiO<sub>2</sub> powder was not quenched by water, as shown in Figure S11. The silica network furthermore protects the triplet excitons in CDs@SiO<sub>2</sub> from being quenched by oxygen. As shown in Figure 3f, only a small quenching effect was found for a range of O<sub>2</sub> concentrations in CDs@SiO<sub>2</sub> powder. These results suggest that the key role of the silica network lies in the protection of the excited triplet species from quenchers. On the other hand, efficient crosslinking and entanglement of CDs have previously been reported as beneficial for the formation of intraparticle hydrogen bonds, which can decrease the distance between chemical groups and confine the motion of CDs, leading to the stabilization of emissive species contributing to RTP emission.<sup>8</sup> The crosslinking of CDs via the silica matrix might thus be considered as another reason for the observed favorable RTP properties. To justify this hypothesis, we conducted a set of contrasting experiments using preparations of three further kinds of CDs in which the degree of crosslinking was varied. We denote these materials as CDs-3 to CDs-5 (see Section 2 for details). For all, identical synthesis conditions were used to those for CDs@SiO<sub>2</sub> to ensure valid comparisons. As shown in Figure S12a,b and Table S1, CDs-3based silica composites exhibit weak RTP compared to CDs@ SiO<sub>2</sub>, since the former feature significantly reduced polymer crosslinking. Both CDs-4- and CDs-5-based silica composites showed no RTP emission at all, on account of the fact that



**Figure 4.** (a) Digital photos of mung bean sprouts during and after irradiation with UV light at 365 nm, including an untreated control and CDs@ SiO<sub>2</sub>-treated sample. (b) Fluorescence and phosphorescence (time-resolved fluorescence) images of a cross section of the stem of CDs@SiO<sub>2</sub>-treated mung bean sprouts. Scale bar: 200  $\mu$ m. (c) Fluorescence and phosphorescence (time-resolved fluorescence) images of CDs@SiO<sub>2</sub>-treated onion bulb epidermal tissue. Scale bar: 200  $\mu$ m. (d) Bright-field, fluorescence, and phosphorescence (time-resolved fluorescence) images of CDs@ SiO<sub>2</sub>-treated signature bars beast carcinoma EM-6 cells. Scale bar: 50  $\mu$ m.

these structures do not feature any internally crosslinked structure. These results demonstrate that polymerization and crosslinking are essential for practical RTP properties in the developed RTP reporter systems.

In summary, the formation of CDs through the crosslinking of polymer structure and subsequent carbonization can preliminarily stabilize the intraparticle excited triplet species (C=O bonds). Then, during the process of TEOS hydrolysis, covalent reactions take place between TEOS and protonic functional groups (e.g., -COOH) in the CDs through nucleophilic attack on  $(RO)_{4-x}Si(OH)_X$  units by carboxylic acids present in the CDs.<sup>37–39</sup> The condensation is likely to be initiated on the surface of CDs, thereby generating Si-O-C bonds that act as scaffolds for the nucleation and growth of the  $SiO_2$  framework. This then permits the covalent integration of CDs into the silica network. The silica matrix permits effective dispersion of CDs in aqueous solvents and acts as a shield to prevent quenching by species such as water and oxygen. Furthermore, the formation of Si-O-C bonds decreases the  $\Delta E_{\rm ST}$  from 0.54 eV for pure CDs to 0.46 eV for aqueous CDs@SiO<sub>2</sub>, leading to both an enhancement and stabilization of RTP. Notably, and critical for applications as a reporter

system for biological systems, the existence of Si–OH confers excellent hydrophilicity on the hybrid system. In addition,  $CDs@SiO_2$  exhibits efficient and long-lifetime RTP even in airsaturated aqueous media, offering excellent potential for diagnostic applications in a wide range of environments.<sup>40,41</sup>

To demonstrate this potential, and test the biocompatibility of CDs@SiO<sub>2</sub>, we performed an MTT assay on mouse breast carcinoma EM-6 cell line (Figure S13), which verified a very low cytotoxicity of aqueous CDs@SiO<sub>2</sub> with up to 150  $\mu$ g/mL. Tests were also performed in plant samples, for which mung bean sprouts were chosen as a model system. The seeds were grown in aqueous CDs@SiO2 for 3 days, and the resulting mung bean sprouts were washed thoroughly with deionized water. No noticeable difference was seen in the growth and development of the treated samples compared to an untreated control, as evident from Figure 4a. Under UV irradiation at 365 nm, it is difficult to distinguish the untreated control group from the CDs@SiO2 treated group because of the strong endogenous autofluorescence emitted by plant tissue at the same excitation wavelength. However, when the irradiation is turned off, a strong, green, phosphorescence signal is emitted from the CDs@SiO<sub>2</sub>-treated group, indicating the successful

uptake of CDs@SiO<sub>2</sub> in the plant. The distribution of CDs@ SiO<sub>2</sub> in mung bean sprouts was studied in more detail using an inverted fluorescence microscope. Again, the tissue autofluorescence strongly interferes with the CDs@SiO<sub>2</sub> fluorescence, as shown in the cross-sectional images of the mung bean sprout stems shown in Figure 4b. The time-resolved phosphorescence images were obtained by turning off the excitation source and collecting sequential RTP images at different time intervals (e.g., 500 ms, 1 s). The images show that the CDs@SiO<sub>2</sub> localize mainly to the external cell walls and in vascular bundles. Next, we incubated onion bulb epidermal tissue as another model tissue and again exposed it to CDs@SiO<sub>2</sub> solution, followed by washing and subsequent phosphorescence imaging. As shown in Figure 4c, the phosphorescence signal again provides a clear outline of the cell walls. Finally, we tested the RTP reporter in animal cells. Figure 4d displays the intracellular fluorescence and phosphorescence signals from EM-6 mouse breast carcinoma cells after incubation with CDs@SiO2. Clearly, the reporter system is taken up by the cell line via endocytosis, suggesting a potential for live cell imaging applications. These results suggest the successful phosphorescence imaging of CDs@SiO<sub>2</sub>, both in vivo and in vitro, highlighting the advantage of longlived phosphorescence in bioimaging by eliminating the autofluorescence interference, especially under short-wavelength excitation.

## 4. CONCLUSIONS

In summary, we have successfully produced ultralong RTP from a CD-based material (CDs@SiO<sub>2</sub>) by incorporating CDs into a three-dimensional silica network through the hydrolysis and condensation of TEOS. The system benefits from the existence of surface Si-OH groups, resulting in a composite with superior water dispersion ability and which features exceptionally long RTP signal decays (1.64 s), to our knowledge longer than any reported in the current literature. Further studies revealed that the RTP originates mainly from C=O bonds present in CDs, whose major function is a facilitation of ISC to the triplet state. Moreover, the interparticle amorphous structure of CDs and the formation of covalent bonds between CDs and silica were also shown to play critical roles in the observed RTP emission signatures and act by rigidifying the excited triplet species, promoting the  $T_1$ electrons to undergo radiative recombination and suppressing nonradiative transitions. Significantly, in light of the superior ultralong-lived RTP property and water dispersibility of the CDs@SiO<sub>2</sub>, we were able for the first time to utilize CD-based RTP materials for phosphorescence imaging both in plant tissues and in animal cells, opening up a new avenue to manufacture sensor materials in biotechnological applications. The system is nontoxic, readily traverses cell walls both in plant and animal tissue, and provides for an efficient means to discriminate against tissue autofluorescence. Importantly, since SiO<sub>2</sub> is commonly used in the construction of functional biosystems, including biosensors, biomarkers, and modern therapeutics, there is widespread potential for future exploration of aqueous CDs@SiO2 as ultralong-lived RTP reporters, opening up a new avenue of research in novel probe designs.

## ASSOCIATED CONTENT

#### **S** Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemmater.9b04120.

Experimental section of imaging; characterizations; HRTEM images of CDs and SEM images of CDs(@SiO<sub>2</sub>; XPS and PL spectra of CDs; PL and RTP properties of different CD-based silica composites; MTT assay; XPS analysis of different CD-based silica composites (PDF)

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#### Notes

The authors declare no competing financial interest.

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