Nitrogen-Doped Carbon Quantum Dot Stabilized Magnetic Iron Oxide Nanoprobe for Fluorescence, Magnetic Resonance, and Computed Tomography Triple-Modal In Vivo Bioimaging

Xiaoli Liu, Hui Jiang, Jing Ye, Chunqiu Zhao, Shengping Gao, Changyu Wu, Changhui Li, Jincheng Li, and Xuemei Wang*

1. Introduction

Medical imageology is an important part of clinical medicine since it can make accurate diagnosis for the location and nature of the lesion which is essential for the follow-up treatment. Currently, medical imaging diagnosis techniques mainly include X-ray diagnostic (such as computed radiography, digital radiography, and digital subtraction angiography), X-ray computed tomography (CT), photoacoustic and ultrasound imaging, magnetic resonance imaging (MRI), and positron emission tomography. Among them, fluorescence (FL), magnetic resonance (MR), and CT imaging are the most routinely applied imaging diagnostic techniques. However, each modality suffers from several defects and cannot provide complete information. For instance, in vivo FL imaging possesses a high sensitivity but a low spatial resolution. CT imaging, on the other hand, provides a high spatial resolution and 3D tomography information for the tissues structure of interest, while its application is usually restricted by the poor soft-tissue contrast resulting from the low sensitivity. In addition, MRI provides superior contrast resolution of soft tissue, but it is less sensitive than FL imaging. Therefore, developing the FL/MR/CT triple-modal imaging strategy which combines the merits of every imaging modality would conducive to improving the accuracy and sensitivity of disease diagnosis.

The key issue for realizing triple-modal imaging lies in rationale design of the triple-modal imaging contrast agents. Superparamagnetic iron oxide nanoparticles (such as Fe3O4 NPs) can be used as MRI contrast enhancement agents because they show good biocompatibility and superior shortening effect on transversal relaxation time (T2) of protons. Although so many approaches for producing Fe3O4 NPs have been proposed, the maintenance of colloidal stability of Fe3O4 NPs is still an essential issue for further biomedical applications. Therefore, much effort has been devoted to improve the chemical stability of Fe3O4 NPs. The common approach is to modify the surface of Fe3O4 NPs with different materials. These materials mainly include two categories: organic materials (such as polyethylene glycol, dextran, polyamine, and chitosan) and inorganic materials (such as silica, Au, Ag and carbon). Among these, carbon quantum dots (CQDs) are promising candidates since they offer the Fe3O4 NPs an excellent colloidal stability, taking advantage of the electrostatic repulsion conferred by CQDs themselves. In addition to the dispersion effect, CQDs also show other appealing advantages, namely, low cost, low toxicity, high surface area, quantum confinement effect...
excellent water solubility, easy functionalization, and superb optical properties (size- and wavelength-dependent, stable, and non-blinking FL emission).\textsuperscript{[30–33]} So the successful preparation of C-Fe$_3$O$_4$ QDs and to explore whether the synergistic effect between CQDs and Fe$_3$O$_4$ brings about the new CT imaging performance in addition to the inherent MR and FL imaging performance are matters worthy of attention.

To date, a variety of methods have been presented to synthesize fluorescent CQDs, which are usually divided into two types: top-down and bottom-up strategies.\textsuperscript{[34–39]} Generally, most of the resultant fluorescent CQDs have a relatively low quantum yield,\textsuperscript{[40,41]} which hinder their application in FL imaging. Recently, there have been several methods to improve the quantum yield of fluorescent CQDs, including doping (such as nitrogen-doping,\textsuperscript{[42,43]} phosphorus-doping,\textsuperscript{[44]} boron-doping,\textsuperscript{[45]} sulfur-doping,\textsuperscript{[46]} nitrogen/sulfur codoping,\textsuperscript{[47,48]} zinc oxide-doping, and zinc sulfide-doping\textsuperscript{[49,50]}) and surface passivation (such as passivated by 4,7,10-trioxa-1,13-tridecanediamine\textsuperscript{[51]} and oligomeric poly(ethylene glycol) diamine\textsuperscript{[52]}). Among these, nitrogen-doping (N-doping) is a common method,\textsuperscript{[53]} because N has similar size and valence electron number as carbon and N-doping can tune the electronic, physical, and chemical properties of CQDs.\textsuperscript{[54]} Despite several approaches have been reported for fabricating N-doped CQDs, most of them use the nitrogen source and carbon source separately.\textsuperscript{[55,56]} Some N-containing carbonizable species are also applied to prepare N-doped CQDs,\textsuperscript{[57]} but they require complex procedures and strong acid or base treatment. Poly-$\gamma$-glutamic acid ($\gamma$-PGA) is a natural anionic biopolymer produced by Bacillus subtilis through fermentation with good water-solubility and biocompatibility,\textsuperscript{[58]} which can be used as carbon and nitrogen precursors simultaneously. If C-Fe$_3$O$_4$ QDs can be synthesized by a simple method, a viable FL/MR/CT triple-modal imaging contrast agent could be expected.

Herein, in this study the synthesis of C-Fe$_3$O$_4$ QDs by using $\gamma$-PGA as a precursor and stabilizer has been explored via a facile one-pot hydrothermal approach (Scheme 1). The as-prepared C-Fe$_3$O$_4$ QDs yield good monodispersity, excellent

\begin{center}
\includegraphics[width=\textwidth]{Scheme_1.png}
\end{center}

\textbf{Scheme 1.} The illustration of formation process of C-Fe$_3$O$_4$ QDs and their applications for multimodal imaging in tumor-bearing nude mice.
colloidal stability, tunable FL, high quantum yield, and high $r_2$ value. Furthermore, under the combined synergistic effect of hybrid, the C-Fe$_3$O$_4$ QDs also show observable X-ray attenuation effect. All FL, MRI, and CT contrast performance of the obtained C-Fe$_3$O$_4$ QDs are further evaluated for in vivo bioimaging, which indicate that the as-prepared C-Fe$_3$O$_4$ QDs are promising candidates for tumor bioimaging/or diagnostics.

2. Results and Discussion

2.1. Synthesis and Characterization

The size and morphology of obtained products were characterized by field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM). As shown in Figure 1a, the as-prepared C-Fe$_3$O$_4$ QDs show good dispersion. Figure 1b–d shows the TEM images of C-Fe$_3$O$_4$ QDs with CQDs of about 3 ± 0.38 nm and Fe$_3$O$_4$ NPs of about 8 ± 0.62 nm, respectively. The Fe$_3$O$_4$ NPs appear darker and CQDs are slightly lighter in the color because CQDs have a lower electron density with respect to that of Fe$_3$O$_4$ NPs. Meanwhile, the image in Figure 1d shows a lattice spacing of 2.122 Å, which can be assigned to the (100) facet of graphite carbon,[59] and the inter-fringe distances of 2.560 and 4.851 Å match the (311) lattice plane and the (111) lattice plane of inverse spinel structured magnetite, respectively.[60,61] The selected-area electron diffraction (SAED) pattern in Figure 1e reveals crystalline nature of the Fe$_3$O$_4$ NPs. The lattice spacings (calculated from SAED rings) are consistent with those of bulk magnetite (Joint Committee on Powder Diffraction Standards, JCPDS: 19-0629).[62] The dynamic light scattering (DLS) results (Figure 1f) indicate that the as-prepared C-Fe$_3$O$_4$ QDs possess a hydrodynamic diameter of about 28 nm in phosphate-buffered saline (PBS) for one day with a polydispersity index of 0.149, being in agreement with the results of TEM characterization, as the hydrodynamic diameter is generally larger than geometric diameter owing to hydration effect.[63] The C-Fe$_3$O$_4$ QDs still present a narrow size distribution with average diameter of about 38 nm after 30 d. Besides, the zeta potentials ($\zeta$) of C-Fe$_3$O$_4$ QDs in neutral PBS after 1 and 30 d are −43.2 and −41.6 mV, respectively. Compared with C-Fe$_3$O$_4$ QDs, the bare Fe$_3$O$_4$ NPs exhibit large hydrodynamic diameters (>2 μm after 30 d) and obvious aggregation phenomena in PBS, which can be seen from the TEM and the DLS results of Fe$_3$O$_4$ NPs in Figure S1 (Supporting Information). It is implied that the Fe$_3$O$_4$ NPs are well stabilized by CQDs. All the results above indicate that C-Fe$_3$O$_4$ QDs are surrounded by hydrophilic negatively charged groups which improve the dispersibility and stability of C-Fe$_3$O$_4$ QDs in PBS.

The powder X-ray diffraction (XRD) pattern of C-Fe$_3$O$_4$ QDs is shown in Figure 2a. The low and broaden diffraction peak at around 25.28° can be readily indexed to graphene (002) plane.[64] The result also reveals the presence of magnetite Fe$_3$O$_4$ (JCPDS card No. 19-0629). The strongest diffraction peak at 35.73° is attributed to the (311) crystal plane, and the other representative peaks at around 30.40°, 37.23°, 43.17°, 54.56°, 57.34°, and 62.77° are assigned to the (220), (222), (400), (422), (511), and (440) crystal planes of Fe$_3$O$_4$, respectively.[65] These peaks are closely consistent with the SAED results. Additionally, the intrinsic structure of C-Fe$_3$O$_4$ QDs was investigated by Raman spectroscopy (Figure 2b). Three characteristic peaks at around 356, 501, and 674 cm$^{-1}$ are assigned to the $E_g$, $T_{2g}$, and $A_{1g}$ vibrational behaviors of magnetite, respectively,[66] further confirming that the as-prepared product contains the magnetite Fe$_3$O$_4$ phase. On the other hand, Raman spectrum also exhibits another two fundamental bands for carbon. A characteristic G band at $\approx$1568 cm$^{-1}$ corresponds to $E_{2g}$ mode of graphite and is assigned to the in-plane vibration of sp$^2$ carbon atoms,[67] while the other characteristic D band at $\approx$1387 cm$^{-1}$ is attributed to

![Figure 1.](image-url)
Figure 2. a) XRD pattern and b) Raman spectrum of C-Fe3O4 QDs.

the presence of sp³ defects. The relative intensity of the two characteristic bands (Iq/Ic) is 0.64, indicating that the carbon in the product has a similar structure as graphite. Besides, it also demonstrates that the carbon formed has some defects mainly resulted from the surface oxidation and N-doping, which will be beneficial for its application in FL imaging.

C-Fe3O4 QDs were characterized by using Fourier transform infrared (FT-IR) spectroscopy in order to identify their chemical nature. As shown in Figure 3a, the characteristic peaks of CQDs include O–H/N–H stretching at 3206 cm⁻¹, =C–C=H stretching vibrations at 3038 cm⁻¹, C–H stretching vibration at 2813 cm⁻¹, C=N stretching vibration at 1900 cm⁻¹, C=O stretching vibration of carboxylic moiety at 1663 cm⁻¹, N–H in-plane bending vibration of the polycyclic aromatic hydrocarbons at 1578 cm⁻¹, C–N in-plane stretching vibrations of aromatic amine groups at 1401 cm⁻¹, C–O stretches C-Fe3O4 QDs at 1324 cm⁻¹, asymmetric stretching vibrations of C–NH–C at 1002 cm⁻¹, and C–H out-of-plane bending vibrations of pyridine at 709 cm⁻¹. Compared with CQDs, the C-Fe3O4 QDs also include the above functional groups, but the positions of some characteristic bands are redshifted and the sharpness of these peaks is changed. Besides, the band relating to Fe–O–Fe stretching vibration of C-Fe3O4 QDs is shifted to higher wave-numbers of 618 cm⁻¹ compared to that of 570 cm⁻¹ reported for the stretching vibration of Fe–O–Fe in bulk Fe3O4. This indicates that the coordination environment of various functional groups in C-Fe3O4 QDs is changed and Fe3O4 has been successfully covalently bounded to CQDs on the surface. The above results of FT-IR spectra reveal that the C-Fe3O4 QDs mainly contain polycyclic aromatic and aromatic CN groups, which confirm the successful doping of N. Besides, the results also suggest that the product obtained is also modified with various polar function groups such as carbonyl, amino, hydroxyl, and other functional groups, which makes C-Fe3O4 QDs surface hydrophilic. X-ray photoelectron spectroscopy (XPS) measurement further confirms elemental composition. Figure 3b shows full-range XPS spectrum of the C-Fe3O4 QDs and CQDs, corresponding to the binding energies of C 1s, N 1s, O 1s, Fe 2p and C 1s, N 1s, O 1s, respectively. As shown in the high-resolution spectrum of C 1s in Figure 3c, four binding energy peaks at 284.7 eV (C=C/C=O), 285.8 eV (C–N), 287.6 eV (C=O), and 288.8 eV (O–C=O) can be found, which are agreement with the FT-IR results discussed above. Compared with CQDs, the C-Fe3O4 QDs show an additional peak at 283.3 eV assigned to C–Fe, which also indicates that C-Fe3O4 QDs has been successfully covalently bounded to CQDs on the surface. The high-resolution spectrum of Fe 2p (Figure 3d) reveals that the Fe 2p peaks at 711.0 eV (Fe 2p 1/2) and 718.8 eV (Fe 2p 3/2) assigned to the Fe3O4 NPs also shows the existence of Fe3O4 in the relevant nanocomposite. Besides, the Fe 2p XPS spectrum of Fe3O4 NPs shows the presence of Fe3O4 at 718.8 eV assigned to the +III oxidation state of Fe, which indicates that the bare Fe3O4 NPs without the synergetic protective effect of CQDs were partially oxidized. In addition to FT-IR and XPS, the solid-state cross-polarization, magic-angle spinning 13C nuclear magnetic resonance (13C CP-MAS NMR) spectrum was also used to distinguish the chemical structure of CQDs. As shown in Figure S2 (Supporting Information), the carbon atoms belonging to aliphatic chains, aromatic rings, and carbonyl groups are observed at around 45, 130, and 200 ppm, respectively. Based on the deconvolution for the region of aliphatic chains, the presence of CH3, CH2, CH, C–N, and C–O could be indicated. As shown in the high-resolution spectrum of unsaturated carbons, the signals at 135 and 150 ppm could be assigned to the C of pyridine and pyrrole. Moreover, peaks at around 170 and 200 ppm could be ascribed to the presence of carbonyl. The 13C CP-MAS NMR spectrum confirms the validity of theoretical analysis again.

The optical properties of C-Fe3O4 QDs were investigated by UV–vis absorption and FL spectra. As shown in the UV–vis absorption spectrum in Figure 4a, an obvious absorption band at 272 nm is observed which can be attributed to π–π* electron transition of the aromatic sp² bond and another absorption peak at 406 nm assigned to the π–π* electron transition of C–N or C=O bond in C-Fe3O4 QDs can also be found. Besides, we observe that the C-Fe3O4 QDs dispersion gives a strong FL
emission peak at around 464 nm when excited at 360 nm. The C-Fe$_3$O$_4$ QDs aqueous solution exhibits very slight yellow color in daylight and bright blue-green FL under the radiation of 365 nm UV lamp, respectively (inset in Figure 4a). Figure 4b,c shows the detailed FL emission spectra and the corresponding normalized FL emission spectra of C-Fe$_3$O$_4$ QDs aqueous solution at different excitation wavelengths, respectively. Unlike others, the emission wavelength of C-Fe$_3$O$_4$ QDs shows nearly no shift when the excitation wavelength is lower than 410 nm, while the emission wavelength redshifts from 471 to 544 nm as the excitation wavelength changed from 420 to 510 nm. The maximum emission wavelength of C-Fe$_3$O$_4$ QDs reaches about 410 nm as excitation wavelength is set at 467 nm. Although the exact mechanism of FL emission remains to be investigated, evidence to date suggests that FL mechanism involves two main hypotheses, i.e., emissive traps and electronic conjugate structures.$^{[82,83]}$ As shown in the results discussed above in Figure 4a, the absorption band at around 272 nm, corresponding to the $\pi-\pi^*$ electron transition of aromatic sp$^2$ bond, leads to nearly no observed FL signal. The other absorption peak is centered at around 406 nm owing to the n–$\pi^*$ transition, in other words, trapping of excited state energy by the surface states results in strong FL emission.$^{[84]}$ So it is reasonable to assume that the abundant doped N atoms as well as $\equiv$OH and $\equiv$COOH moieties on the surface of C-Fe$_3$O$_4$ QDs, which act as energy traps, endow C-Fe$_3$O$_4$ QDs with strong fluorescent characteristics. The FL lifetime of the C-Fe$_3$O$_4$ QDs was measured by time-resolved photoluminescence measurements.

---

**Figure 3.** a) FT-IR spectra of CQDs and C-Fe$_3$O$_4$ QDs; b) XPS spectra of the CQDs and C-Fe$_3$O$_4$ QD; c) high-resolution C1s XPS spectrum of the CQDs and C-Fe$_3$O$_4$ QDs; d) high-resolution O1s XPS spectrum of the C-Fe$_3$O$_4$ QD; e) high-resolution N1s XPS spectrum of the CQDs and C-Fe$_3$O$_4$ QDs and Fe2p; f) XPS spectrum of the Fe$_3$O$_4$ and C-Fe$_3$O$_4$ QDs.
The luminescence decay curve is very well fitted to a double-exponential function as shown in Figure 4d. Observed lifetimes are $\tau_1 = 1.07$ ns and $\tau_2 = 4.63$ ns. Such short FL lifetime indicates the radiative recombination nature of excitations. The average FL lifetime is calculated to be $\tau_{av} \approx 4.22$ ns. Therefore, the as-prepared C-Fe$_3$O$_4$ QDs have potential applications in bioimaging. In order to further investigate the FL properties of C-Fe$_3$O$_4$ QDs, the FL quantum yield and the effect of external factors on the FL were investigated. FL quantum yield of the C-Fe$_3$O$_4$ QDs is calculated to be about 21.6% by using quinine sulfate as a reference. Although Fe$_3$O$_4$ has no FL property, while it shows no significant effect on the FL quantum yield of the C-Fe$_3$O$_4$ QDs (the FL quantum yield of CQDs is to be about 22.2%). This quantum yield value is higher than previous reports (typically <20%). The higher quantum yield may be ascribed to the existence of specific nitrogen containing functionalities in the carbon matrix which favors the radiative relaxation pathways. Thus, the quantum yield and FL intensity of C-Fe$_3$O$_4$ QDs are greatly enhanced. The C-Fe$_3$O$_4$ QDs exhibit excellent photostability, since FL intensity of the C-Fe$_3$O$_4$ QDs remains quite stable under the irradiation with a Xe lamp (excitation wavelength: 410 nm) for 30 min (Figure 4e). Similarly, the C-Fe$_3$O$_4$ QDs aqueous solution also shows extra-high FL intensity stability after the sample has been treated with different concentrations of NaCl (Figure S3a, Supporting Information) or stored at 4 °C for 3.5 months (Figure S3b, Supporting Information). Therefore, the C-Fe$_3$O$_4$ QDs are particularly valuable for real clinic applications in bio-imaging under...
physiological conditions. Photos of C-Fe₃O₄ QDs aqueous solution in the presence and absence of an external magnet clearly demonstrate the excellent magnetic properties. As observed in Figure 4, all C-Fe₃O₄ QDs are attracted to one side of the bottle (Figure 4f, inset), illustrating the effective magnetic separation process. The magnetic nature of the as-prepared C-Fe₃O₄ QDs were further characterized by using a vibrating sample magnetometer. As shown in Figure 4f, the magnetization M (H) curve of C-Fe₃O₄ QDs shows no hysteresis and the saturation magnetization (Mₛ) is 62 emu g⁻¹ at room temperature. These results demonstrate that the as-prepared C-Fe₃O₄ QDs possess the remarkable superparamagnetic properties and high Mₛ value, which make them uniquely suitable for use as enhanced MRI contrast agents in further nano-biomedical applications.

2.2. In Vitro Cytotoxicity

On the basis of the above studies, we have further explored the in vitro toxicity of the C-Fe₃O₄ QDs before they were used for cellular and in vivo imaging applications by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Human cervical carcinoma (HeLa) cells were incubated with different concentrations of C-Fe₃O₄ QDs (0, 5, 10, 25, 50, 100, 150, 200, 300, 400, and 500 μg mL⁻¹) for 24 and 48 h, respectively (Figure 5a). It is clear from the MTT assay data that C-Fe₃O₄ QDs do not show any serious acute cytotoxicity at high concentration up to 500 μg mL⁻¹, even after 48 h of incubation, indicating the excellent biocompatibility in the given concentration range. Considering that C-Fe₃O₄ QDs will be finally injected intravenously, it is equally important to check the hemocompatibility of the material. As shown in Figure 5b,c, the C-Fe₃O₄ QDs have no or negligible hemolysis effect with the concentrations varied from 15.625 to 500 μg mL⁻¹ and all the hemolysis rates are lower than 0.5%, indicating the excellent blood compatibility of C-Fe₃O₄ QDs. It is thus inferred that the C-Fe₃O₄ QDs are cytocompatible and hemocompatible, which is critical to ensure the safety in their further in vivo bioimaging applications.

2.3. Confocal Fluorescence Imaging

To look for the possible internalization of C-Fe₃O₄ QDs, the in vitro FL imaging was performed on HeLa cells. As shown in Figure 6a, the control group treated without C-Fe₃O₄ QDs shows no FL signal. However, after incubation with 50, 100 or 150 μg mL⁻¹ C-Fe₃O₄ QDs for 24 h, green FL can be observed in cytoplasm, but not in the nucleus (Figure 6b–d). Besides, the results also indicate that the increasing C-Fe₃O₄ QDs concentrations in cell incubation media enhance the cellular uptake of C-Fe₃O₄ QDs. Furthermore, the HeLa cells treated with C-Fe₃O₄ QDs
QDs show no substantial change in morphology. All these results above demonstrate that the as-prepared C-Fe₃O₄ QDs can be promising candidates for use in FL bioimaging.

2.4. In Vivo FL Bioimaging by Using C-Fe₃O₄ QDs

Figure 7a shows the results from a FL bioimaging study performed on a CRI Maestro in vivo fluorescence imaging system. Diluted C-Fe₃O₄ QDs sample and PBS (as control) were filled in the two eppendorf (EP) tubes, respectively. Notably, due to the large number of surface energy traps, the FL signal depends on the excitation wavelength when the sample is excited at wavelengths >420 nm, which is consistent with the FL measurements (Figure 4b). In the in vivo imaging of the HeLa tumor-bearing nude mice, for comparison purpose, we applied 420 and 440 nm as excitation wavelengths, respectively. The FL images were acquired at designated time points after the PBS of C-Fe₃O₄ QDs was intravenously injected into mice. At 2 h postinjection, strong FL signals corresponding to C-Fe₃O₄ QDs in tumor can be seen under the two excitation wavelengths (Figure 7b). The strong FL signal intensity of tumor should be related to the known passive enhanced permeability and retention (EPR) effect. Compared to images acquired under excitation at 440 nm (part B), the ones taken under excitation at 420 nm (part A) exhibit better signal and background separation. In addition to the FL signal from tumor, emissions from the bladder area could also be observed, suggesting that the intravenously injected C-Fe₃O₄ QDs are mainly excreted through urine. Later, the FL intensity of tumor site decreases slowly with time, which may be due to the fact that the C-Fe₃O₄ QDs undergo a further metabolism process. All these results indicate that C-Fe₃O₄ QDs are able to be employed for in vivo FL bioimaging of tumors.

2.5. T₂-Weighted MR Imaging

The potential of applying C-Fe₃O₄ QDs as an MRI contrast agent was further tested on a 7.0 T MRI scanner. As expected, with the increase of Fe concentration, the T₂-weighted MR image displays an obvious signal intensity decrease (Figure 8a). Besides, by linear fitting of the reciprocal of the T₂ relaxation time versus the Fe concentration (Figure 8b), the r² of the C-Fe₃O₄ QDs is estimated to be 154.10 ± 4.9% after injection of C-Fe₃O₄ QDs (Figure 8d). These observations further confirm that as-prepared C-Fe₃O₄ QDs could be an effective T₂-weighted MRI contrast agent for tumors.

2.6. X-Ray CT Imaging

Considering of the above observations, we have further explored the in vitro X-ray attenuation potency of the as-prepared C-Fe₃O₄ QDs with different concentrations. As shown in Figure 9a, it is surprising to find that the C-Fe₃O₄ QDs could absorb X-ray and the brightness of CT images is enhanced with the increase of C-Fe₃O₄ QDs concentration. The line slope of Hounsfield unit (HU) values versus the C-Fe₃O₄ QDs concentration is calculated to be about 26.1 HU L g⁻¹ (Figure 9b).

As is known, materials that have high atomic weight such as gold,[91] platinum,[92] transition-metal dichalcogenides,[93] and lanthanides[94] are usually served as CT contrast agents because X-ray attenuation effect is usually proportional to the cube of the atomic number (z³),[95] and the X-ray mass attenuation coefficient is also associated with the K-edge energy value so that the electron-dense materials generally provide superior images. Since the atomic number of iron (26) is not high enough to be
used as a CT contrast agent, the CT contrast performance of C-Fe$_3$O$_4$ QDs here may be associated with change of the iron K-edge energy value. The suitable K-edge energy value may be brought by the doped nitrogen atoms in the C-Fe$_3$O$_4$ QDs.\textsuperscript{[96]}

Then, in vivo CT images of HeLa tumor-bearing nude mice were acquired before and 24 h after intravenous injection of C-Fe$_3$O$_4$ QDs solution prepared in PBS. It is evident that an obvious CT contrast enhancement in the tumor site could be observed in both transversal and sagittal CT images at 24 h postinjection (Figure 9c), which conforms to the results of aforementioned in vivo FL and MR bioimaging. By plotting the average CT signal intensity of tumor site as a function of the injection time (Figure 9d), we can see that the average CT signal intensity of tumor site is increased by 82 ± 4.1% after injection of C-Fe$_3$O$_4$ QDs solution. These results further prove that the as-prepared C-Fe$_3$O$_4$ QDs can be used as a potential contrast CT agent.

2.7. Biodistribution of C-Fe$_3$O$_4$ QDs

With the excellent contrast performance of C-Fe$_3$O$_4$ QDs, we further analyzed organ biodistribution of the C-Fe$_3$O$_4$ QDs by inductively coupled plasma mass spectrometry (ICP-MS) method. As shown in Figure 10, the C-Fe$_3$O$_4$ QDs present appreciable localization in the kidney after injection, consistent
with the urine excretion pathway (Figure 7b). Furthermore, the amount of C-Fe$_3$O$_4$ QDs in tumor is also much higher than those in other organs. The high accumulation of C-Fe$_3$O$_4$ QDs in tumor could be attributed to well-known EPR effect. All these results have demonstrated that the C-Fe$_3$O$_4$ QDs can be used as a potential contrast agent for triple-modal FL/MR/CT imaging of tumors.

3. Conclusion

In summary, in this contribution we have described the preparation of multifunctional C-Fe$_3$O$_4$ QDs nanoprobes via a new and facile one-pot hydrothermal approach for FL/CT/MRI triple-modal bioimaging applications. This synthesis process endows the as-prepared C-Fe$_3$O$_4$ QDs with excellent water dispersibility, FL property (i.e., maximum FL quantum yield about 21.6%), photostability, superparamagnetic property, and biocompatibility in the given range of concentrations. $T_2$ relaxometry measurements indicate that the as-prepared C-Fe$_3$O$_4$ QDs have a high $r_2$ value of about 154.10 mM$^{-1}$ s$^{-1}$. Most importantly, the X-ray attenuation measurements reveal that the as-prepared C-Fe$_3$O$_4$ QDs have an obvious X-ray attenuation effect. The in vivo FL, MR, and CT bioimaging studies demonstrate that the developed C-Fe$_3$O$_4$ QDs can be used as a potential contrast agent for triple-modal FL/MR/CT imaging of tumors. Based on the above features, the C-Fe$_3$O$_4$ QDs could be exploited for multimodal imaging in biomedical research.

4. Experimental Section

Materials: γ-PGA with a molecular mass of ≈130 kDa was synthesized from Bacillus subtilis NX-2 through the batch fermentation according to Liang et al.\textsuperscript{[97]} Nitrogen gas was used to create an oxygen-free atmosphere. Ammonium hydroxide solution (30%), ferrous chloride tetrahydrate (FeCl$_2$·4H$_2$O), ferric chloride hexahydrate (FeCl$_3$·6H$_2$O), nitric acid, MT, dimethyl sulfoxide (DMSO), and paraformaldehyde (PFA) were obtained from Sigma-Aldrich. Dulbecco’s modified eagle media (DMEM), penicillin-streptomycin, fetal bovine serum (FBS) were purchased from Invitrogen Co. All reagents were of analytical grade and deionized water was used throughout the experiments. HeLa cell lines were provided by Shanghai Institute of Biological Science, Chinese Academy of Science (China).

Synthesis of Multifunctional C-Fe$_3$O$_4$ QDs: The C-Fe$_3$O$_4$ QDs were synthesized by a facile one-pot hydrothermal approach. Briefly, FeCl$_3$·6H$_2$O (0.1920 g) and FeCl$_2$·4H$_2$O (0.1765 g) were completely dissolved in deionized water (20 mL) and γ-PGA solution (50 mL, 10 g L$^{-1}$) was subsequently added to the reactor. Nitrogen was bubbled into the reactor throughout the reaction. The mixed solution was vigorously stirred for 3 h by using a mechanical stirrer. Afterward, the mixture was heated to 60 °C through the water bath and titrated...
with ammonium hydroxide solution to pH 10. After this, the mixture was aged at 60 °C for another 5 h. Later, the mixture was transferred to a teflon-lined autoclave and maintained at 180 °C for 8 h. The autoclave was cooled naturally after the reaction completed and the obtained solution was dialyzed against deionized water for one day. Finally, the as-prepared C-Fe3O4 QDs were collected by freeze-drying and can be dissolved again for further applications.

Characterizations of C-Fe3O4 QDs: The FE-SEM image was obtained from a JSM-6700F scanning electron microscope (JEOL, Japan). The TEM images were obtained from a transmission electron microscope (JEOL JEM-2100, 200 kV). Size distribution and ζ potential were measured at 25 °C in PBS by DLS technique with a Zetasizer NanoZS instrument (Malvern, U.K.). The XRD patterns were obtained from an X-ray diffractometer (B8 Advance, Bruker, Germany) with a Cu Ka radiation source (λ = 1.5418 Å) operating at 30 mA and 40 kV. Raman spectra were recorded with a Renishaw inVia Reflex Raman Spectrometer (England). FT-IR spectra was studied on a Thermo Nicolet iS10 FT-IR Spectrometer (USA). The XPS was measured with a PHI Quantera II X-ray photoelectron spectrometer (ULVac-Phi, Japan) with an Mg Kα excitation source (1253.6 eV). The 13C CP-MAS NMR experiments were performed with a Bruker AVANCE III 400MHz WB Solid-state NMR spectrometer, MAS at 12 kHz in 4 mm rotors. The UV–vis absorption spectra was performed on a Biomek 35 spectrophotometer (Thermo Co. Ltd., US) and FL spectra was measured on a RF-S301PC fluorometer (Shimadzu, Tokyo, Japan), respectively. Room temperature magnetism was probed with a superconducting quantum interference device magnetometer (MPMS-XL-7). The iron content of C-Fe3O4 QDs was determined by using ICP-MS (Agilent 7500A, Palo Alto, CA). The luminescence lifetime was measured by a Fluorolog-3 fluorometer (Shimadzu, Tokyo, Japan) with a 633 nm excitation wavelength. The amplitude-weighted average FL lifetime was determined by the following equation:[98]

\[
\tau_{aw} = \frac{A_1 \tau_1^2 + A_2 \tau_2^2}{A_1 \tau_1 + A_2 \tau_2}
\]  

(1)

Where \( A_i \) denoted fractional weights of various decay time components \( \tau_i \) of the multieponential fitting. The FL quantum yield of C-Fe3O4 QDs was determined by the published procedure.[99] Briefly, quinine sulfate (with a standard quantum yield of 54% in 0.10 M H2SO4 at 360 nm) was used as a reference. The quantum yield was obtained by using the following equation:

\[
\Phi_a = \Phi_b \frac{L}{n} \times \frac{A_w}{A_a} \times \frac{n^2}{n^2} \times \frac{R}{R}
\]  

(2)

Where \( \Phi \) was the FL quantum yield, \( I \) was the integrated FL intensity, \( n \) was the refractive index of the respective solvents, and the subscript \( a \) and \( R \) stood for the sample and quinine sulfate, respectively.

Cell Culture and Cell Cytotoxicity Assessment: HeLa cells were cultured in DMEM supplemented with FBS (10%) and penicillin–streptomycin (1%) at 37 °C with 5% CO2. The cell growth inhibitory effect of C-Fe3O4 QDs was measured by MTT assay. HeLa cells (1 × 10^4) were seeded into 96-well cell culture plates. After an overnight cell adhesion, different concentrations of C-Fe3O4 QDs (800, 600, 400, 200, 100, and 50 μg mL\(^{-1}\)) were incubated with DMEM for 8 h. After that, the cells were washed with PBS twice and then incubated with MTT solution (5 mg mL\(^{-1}\), 20 μL) for 4 h. Afterward, DMSO (150 μL) was added to dissolve the resulting formazan crystals and the optical density at 490 nm was measured by a Bio-Rad Model 680 microplate reader (USA). Each sample was tested in five replicates.

Hemolysis Assay: The human whole blood stabilized by ethylenediamine tetraacetic acid was provided by the local hospital. Red blood cells (RBCs) were isolated from blood sample (2 mL) by centrifuging at 10 000 rpm for 5 min, then washed with PBS (10 mL, pH 7.4) five times until supernatant was clear and colorless. After that, the purified RBCs were resuspended in PBS (20 mL). Subsequently, the resultant RBCs suspension (0.4 mL) was incubated with PBS of C-Fe3O4 QDs (1.6 mL) with a series of concentrations, and the as-prepared RBCs suspension (0.4 mL) mixed with PBS (1.6 mL) and deionized water (1.6 mL) were used as the negative control group and the positive control group, respectively. After coincubation at 37 °C for 4 h, the mixtures were centrifuged at 10 000 rpm for 5 min and the absorbance values of supernatants at 541 nm were measured by UV–vis spectrophotometry. The hemolysis ratio was calculated by using the following equation:

\[
\text{Hemolysis(%)} = \frac{A_{\text{sample}} - A_{\text{control}(-)}}{A_{\text{control}(+)} - A_{\text{control}(-)}}
\]  

(3)

Where \( A_{\text{sample}}, A_{\text{control}(+)} \) and \( A_{\text{control}(-)} \) were the absorbance of the sample, the negative control, and the positive control, respectively.

Confocal Fluorescence Imaging: HeLa cells (1 × 10^5) were grown on the cover slips which was put in the wells of 6-well plates. After an overnight cell adhesion, the cells were washed with PBS twice and then incubated with different concentrations of C-Fe3O4 QDs for one day. After that, the cells were washed with PBS for three times and fixed with PFA solution (4%, 1 mL) for about 15 min. After washing twice with PBS again, the slides were mounted and FL images were taken under a confocal laser scanning microscope (Leica TCS SP5) with a 63×oil-immersion objective lens. The FL signal of C-Fe3O4 QDs was excited at 488 nm.

Animal Experiments: All animal experiments were conducted in accordance with regulations of southeast university and the Animal Care & Use Committee. For tumor model, HeLa cells (5 × 10^5 in 50 μL) were subcutaneously injected into the legs of each female BALB/c nude mice (four-week-old) which were obtained from Model Animal Research Center of Southeast University. The mice were used for experiments until tumor diameter reached at least 5.0 mm.

In Vivo and In Vivo FL Bioimaging by Using C-Fe3O4 QDs: To evaluate the excitation wavelength dependent imaging capability of C-Fe3O4 QDs, certain concentration of C-Fe3O4 QDs was placed in one EP tube and the other EP tube containing PBS was used as a control. Both the EP tubes were positioned on a rack and imaged with a CRI Maestro in vivo fluorescence imaging system (excitation, 420, 440 nm; emission, 520, 570 nm). For in vivo FL bioimaging by using C-Fe3O4 QDs, HeLa tumor-bearing nude mice were intravenously injected with PBS or C-Fe3O4 QDs (100 μL, 1 mg mL\(^{-1}\)) after being anesthetized with 5% isoflurane/O2 (v/v). Then, the nude mice were positioned on a CRI Maestro in vivo fluorescence imaging system (excitation, 420, 440 nm; emission, 520, 570 nm) and the FL images were captured before and at different time intervals after injection.
In Vivo and In Vivo T2-Weighted MR Imaging: In vitro T2-weighted images and T2 transverse relaxation time of C-Fe3O4 QDs were acquired by using a 7.0 T MRI scanner (Bruker Biospin, Germany). Dilutions of C-Fe3O4 QDs with different iron concentrations (by ICP-MS measurement) were filled in an array of EP tubes for T2-weighted MRI. T2-weighted images were obtained by adopting the following parameters: repetition time (TR) of 3000.0 ms, echo time (TE) of 9.0 ms, flip angle (FA) of 90°, section thickness of 1.0 mm, field of view (FOV) of 5.00 cm and matrix (MTX) of 256 × 256 pixels. The r2 was determined by the curve fitting of 1/T2 versus the iron concentration. For further in vivo MRI, anesthetized HeLa tumor-bearing nude mice were injected with PBS of C-Fe3O4 QDs (100 μL, 4 mg mL−1) via tail vein. In vivo MRI experiments were conducted on the same MRI scanner (7.0 T, Bruker Biospin, Germany), using the following sequence: TR of 1386 ms, TE of 9.0 ms, flip angle (FA) of 90°, section thickness of 1.0 mm, FOV of 2.50 cm and MTX of 256 × 256 pixels. The mice were imaged prior to and 24 h after injection.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
This work was supported by the National High Technology Research & Development Program of China (Grant No. 2015AA020502), the National Natural Science Foundation of China (Grant Nos. 81325011, 21327902, and 21175020) and the Jiangsu Natural Science Foundation (Grant No. BK20161413).

Received: June 20, 2016
Revised: July 17, 2016
Published online: August 31, 2016