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Carbon Dots for Multiphoton Bioimaging

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Two-photon fluorescence materials have attracted much recent attention for their many promising applications, especially in the growing field of biomedical imaging.[1-5] Among the best performing two-photon fluorescence materials are semiconductor quantum dots such as CdSe and related core—shell nanoparticles.[6-8] These quantum dots have been demonstrated in various optical imaging experiments in vitro and in vivo.[6,9,10] At the same time, however, heavy metals as the essential elements in available high-performance semiconductor quantum dots have prompted serious health and environmental concerns in the community and beyond. Therefore, the search for benign alternatives has become increasingly important and urgent. Recently, we found and reported[11] that nanosized pure carbon particles may be surface-passivated to exhibit bright photoluminescence in the visible. These photoluminescent carbon dots (C-Dots, Figure 1a) are of two distinctive features: one is that the underlying carbon particles are very small (sub-10 nm); and the other is that the particle surface is passivated by organic or other molecules via either covalent linkages or chemical adsorption.[11] Mechanistically, the carbon-based photoluminescence has been attributed to passivated defects on the carbon particle surface acting as excitation energy traps.[11,12] Here we report that the C-Dots also exhibit strong luminescence with two-photon excitation in the near-infrared. The estimated two-photon absorption cross-sections of the C-Dots are comparable to those of available high-performance semiconductor quantum dots. In addition, the two-photon luminescence microscopy imaging of the C-Dots internalized in human cancer cells is demonstrated.

The C-Dots were prepared as previously reported.[11] Poly-(propionylethylenimine-*co*-ethylenimine) (PPEI-EI, with EI fraction ~20%) was used as the surface passivation agent. The C-Dots thus prepared were readily soluble in water to yield a colored aqueous solution. Shown in Figure 1b is a representative AFM image of the C-Dots on mica surface, from which feature sizes of generally less than 5 nm are identified.

The specimen for optical microscopy was prepared by first dropping a small aliquot of the aqueous solution on cover glass and then evaporating the water. A Leica confocal fluorescence microscope equipped with an argon ion laser and a femtosecond pulsed Ti:sapphire laser was used. The C-Dots were found to be strongly emissive in the visible with either the argon ion laser excitation (458 nm) or the femtosecond pulsed laser for two-photon excitation in the near-infrared (800 nm). As compared in Figure 2, the one- and two-photon luminescence images for the same scanning area match well. The C-Dots were photostable under the two-photon imaging conditions (upon repeated 800 nm excitations equivalent to generating the image in Figure 2b for at least 3000 times, no meaningful changes in emission intensities).

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Supporting Information Available: Complete ref [11] and histograms of luminescence intensities in the images. This material is available free of charge via the Internet at http://pubs.acs.org.

The same microscope setup was used to measure the two-photon spectra. For the same specimen (C-Dots deposited on cover glass), the observed spectra vary slightly from spot to spot, reflecting the inhomogeneous nature of the sample. A representative two-photon luminescence spectrum of average C-Dots is shown in Figure 3 (left). Its bandwidth is comparable with that in the one-photon spectrum of the C-Dots on surface (458 nm excitation), but significantly narrower than that of the solution-phase spectrum (400 nm excitation, Figure 3 (left)). These results again suggest inhomogeneity in the sample. The immobilization of the dots on surface might have allowed the measurement of small fractions in which the emissive species or sites are more homogeneous, with the narrower luminescence bands for both one- and two-photon excitations.

The two-photon in nature for the luminescence with the pulsed infrared laser excitation was confirmed by the dependence of observed luminescence intensities on the excitation laser power. The luminescence signals were collected with an external detector on the confocal microscope, and the laser powers for excitation were determined by using a precision power meter in the focal plane (thus free from effects of reflection and transmission losses associated with all optical components in the system). As also shown in Figure 3 (right), the quadratic relationship between the excitation laser power and the luminescence intensity is obvious, thus confirming that the excitation with two near-infrared photons was indeed responsible for the observed visible luminescence of C-Dots.

The two-photon absorption cross-section $\sigma_2(\lambda)$ of C-Dots was estimated by determining the two-photon luminescence intensities of the specimen and a reference under the same experimental conditions: $\sigma(\lambda) = \sigma_{2,\mathrm{ref}}(\lambda)\,(\langle F(t)\rangle/\langle F_{\mathrm{ref}}(t)\rangle)/(\Phi/\Phi_{\mathrm{ref}})$, where $\langle F(t)\rangle$'s represent averaged luminescence photon fluxes (or experimentally observed emission intensities), Φ's are luminescence quantum yields, and the subscript ref denotes values for the reference compound. By using rhodamine B as the reference,[13] the two-photon absorption cross-sections of C-Dots at different excitation wavelengths were calculated from the experimental results. At 800 nm, the average σ_2 value for the C-Dots was 39 000 ± 5000 GM (Goeppert-Mayer unit, with 1 GM = 10^{-50} cm^4 s/photon). It makes the C-Dots comparable in high-performance to other two-photon luminescent nanomaterials.[6,8,14,15] For example, the two-photon absorption cross-section for CdSe quantum dots at 800 nm varies in the range of 780–10 300 GM, depending on the particle sizes.[8] For CdSe/ZnS core—shell quantum dots (fluorescence at 605 nm), the two-photon absorption cross-section was estimated to be on the order of 50 000 GM.[6]

In an exploratory experiment to demonstrate the potential of C-Dots for cell imaging with two-photon luminescence microscopy, human breast cancer MCF-7 cells were cultured in terms of the established protocol.[16] Upon incubation with the C-Dots in an aqueous buffer at 37 °C, the MCF-7 cells became brightly illuminated when imaged on the fluorescence microscope with excitation by 800 nm laser pulses. As shown in Figure 4, the C-Dots were able to label both the cell membrane and the cytoplasm of MCF-7 cells without reaching the nucleus in a significant fashion. The translocation of the C-Dots from outside the cell membrane into the cytoplasm is temperature dependent, with no meaningful C-Dots internalization observed at 4 °C. While endocytosis is likely, an understanding of the internalization mechanism still requires more investigations. In addition, a better accumulation of C-Dots in the cell (even in the nucleus) may be achieved by C-Dots coupled with membrane translocation peptides such as TAT (a human immunodeficiency virus-derived protein), which facilitates the translocation of the tissue by overcoming the cellular membrane barrier and enhances the intracellular labeling efficiency.[17,18] This is being pursued along with a comparison between one- and two-photon luminescence imaging of cells labeled with C-Dots.

In summary, C-Dots are strongly two-photon active, with the pulsed laser excitation in the near-infrared to result in bright luminescence in the visible. The estimated two-photon absorption cross-sections of the C-Dots are comparable to those of the best-performing semiconductor quantum dots or core—shell nanoparticles already reported in the literature. Available results from exploratory experiments of luminescence imaging in vitro suggest that the C-Dots are internalized into the human breast cancer cells likely through endocytosis, demonstrating the potential of the C-Dots in cell imaging with two-photon luminescence microscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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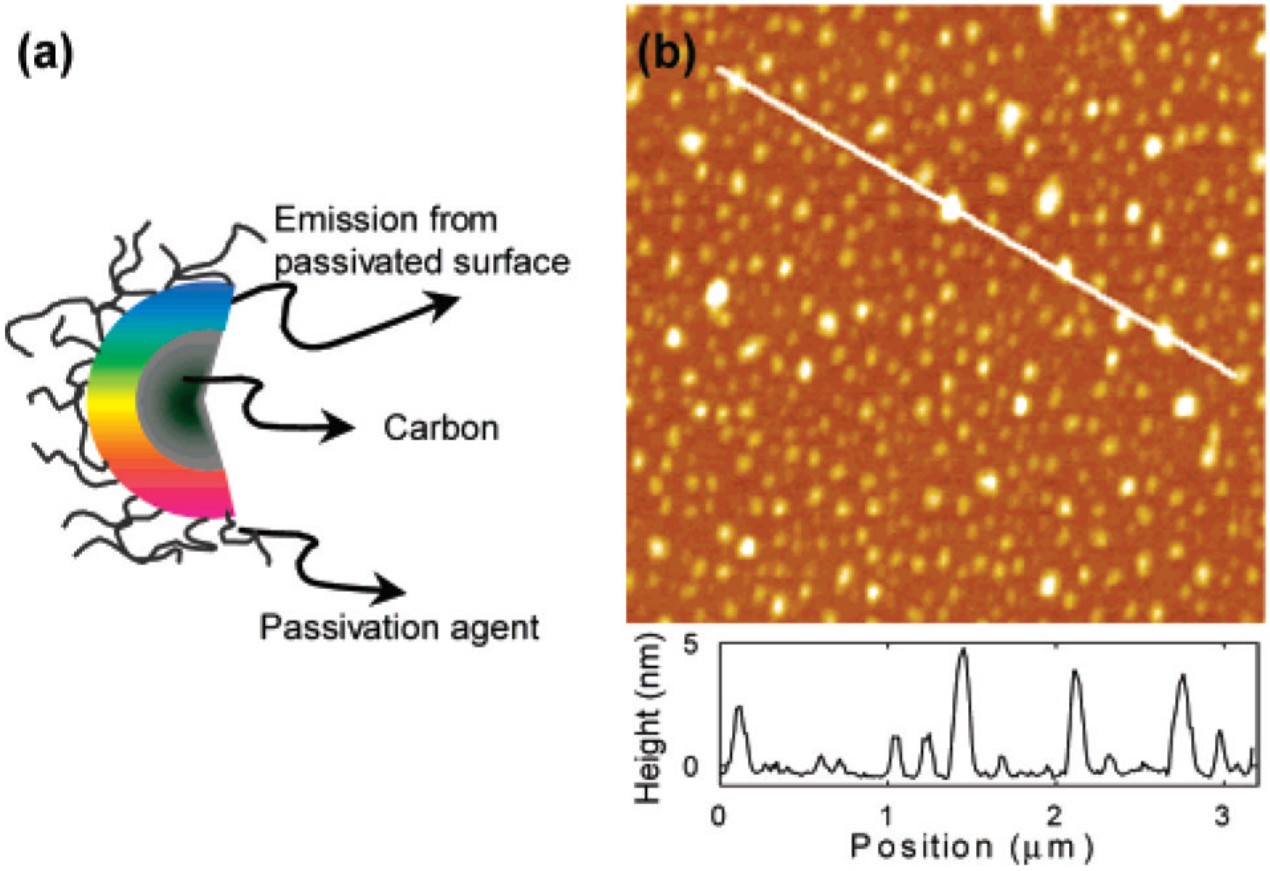

Figure 1.
(a) The C-Dot structure; (b) AFM topography image of C-Dots on mica substrate, with the height profile along the line in the image.

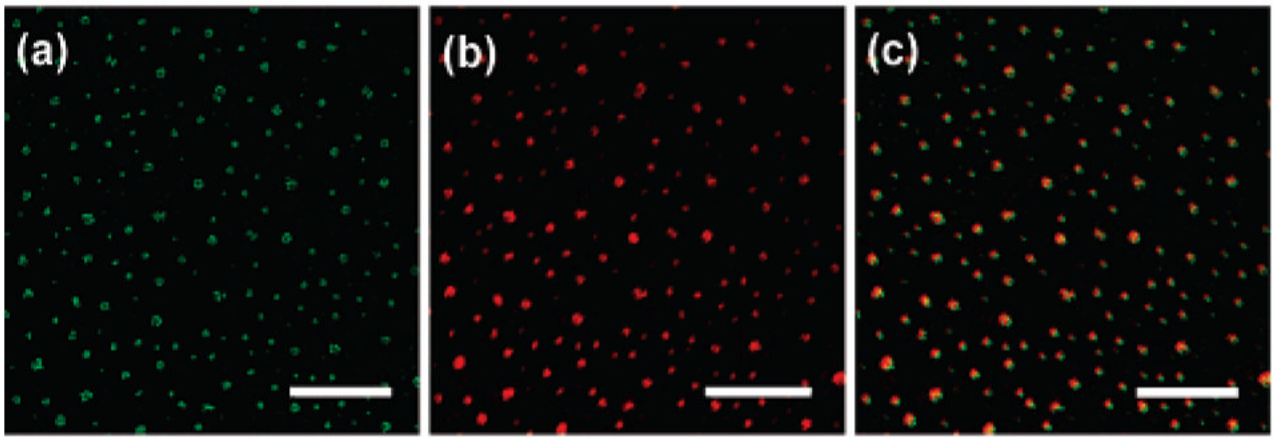

Figure 2.
Luminescence images (all scale bars 20 μm) of the C-Dots with (a) argon ion laser excitation at 458 nm and (b) femtosecond pulsed laser excitation at 800 nm; (c) is an overlay of (a) and (b).

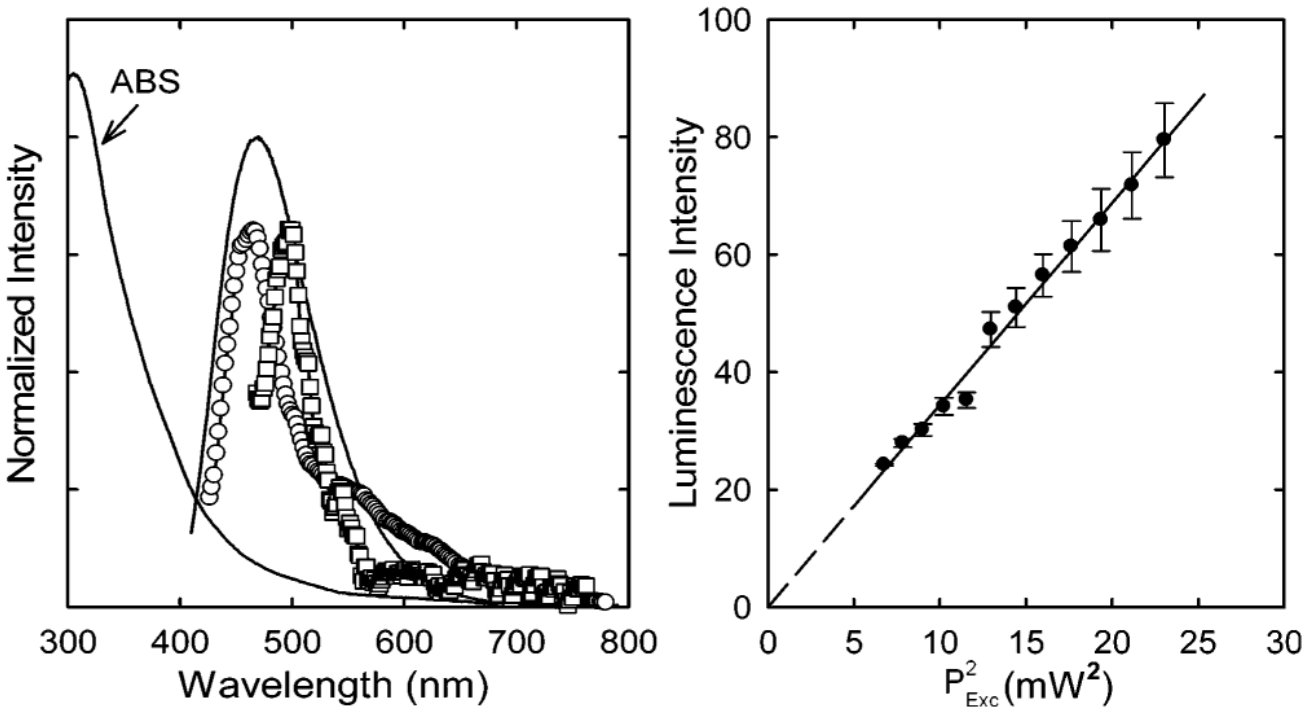

Figure 3.

(Left) The one-photon (□, 458 nm excitation) and two-photon (○, 800 nm excitation) luminescence spectra of the C-Dots on glass substrate (prepared with infinite dilution, and optical spot diameter ~500 nm, covering multiple dots immobilized on the substrate) are compared with solution-phase absorption (ABS) and luminescence (solid line, 400 nm excitation) spectra. (Right) The quadratic relationship of the observed two-photon luminescent intensity of the C-Dots on glass substrate with the excitation laser power at 800 nm (P_{Exc}, as measured at the focal plane).

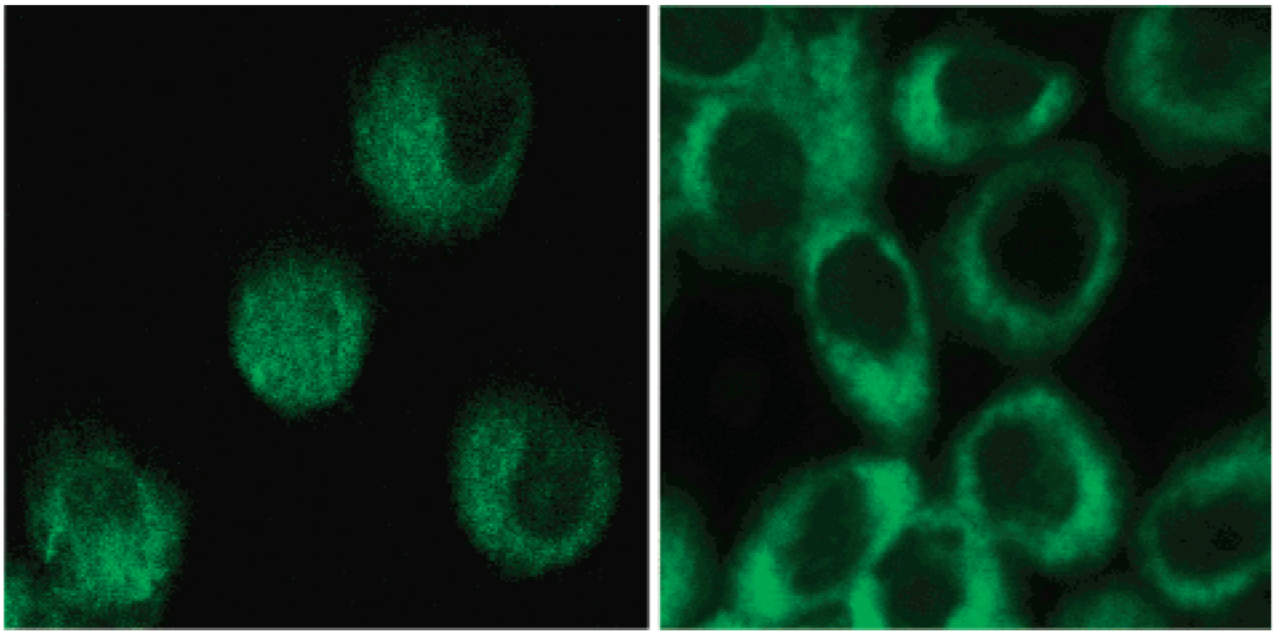

Figure 4.

Representative two-photon luminescence image (800 nm excitation) of human breast cancer MCF-7 cells with internalized C-Dots. More details on the cell experiment: MCF-7 cells (approximately 5×10^5) were seeded in each well of a four-chambered Lab-Tek coverglass system (Nalge Nunc) and cultured at 37 °C. All cells were incubated until approximately 80% confluence was reached. Separately, an aqueous solution of the C-Dots (0.9 mg/mL) was passed through a 0.2 μm sterile filter membrane (Supor Acrodisc, Gelman Science). The filtered solution (20–40 μL) was mixed with the culture medium (300 μL) and then added to three wells of the glass slide chamber (the fourth well used as a control) in which the MCF-7 cells were grown. After incubation for 2 h, the MCF-7 cells were washed three times with PBS (500 μL each time) and kept in PBS for the optical imaging.