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Functional Surface Engineering of C-Dots for Fluorescent Biosensing and in Vivo Bioimaging

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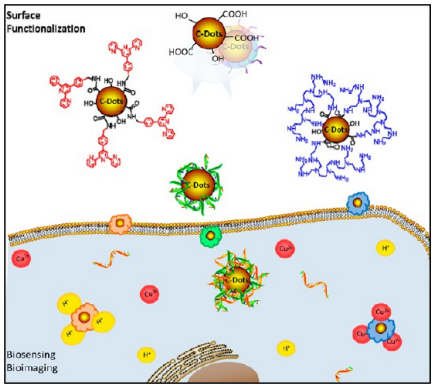
CONSPECTUS

Nanoparticles are promising scaffolds for applications such as imaging, chemical sensors and biosensors, diagnostics, drug delivery, catalysis, energy, photonics, medicine, and more. Surface functionalization of nanoparticles introduces an additional dimension in controlling nanoparticle interfacial properties and provides an effective bridge to connect nanoparticles to biological systems. With fascinating photoluminescence properties, carbon dots (C-dots), carbon-containing nanoparticles that are attracting considerable attention as a new type of quantum dot, are becoming both an important class of imaging probes and a versatile platform for engineering multifunctional nanosensors. In order to transfer C-dots from proof-of-concept studies toward real world applications such as in vivo bioimaging and biosensing, careful design and engineering of C-dot probes is becoming increasingly important.



A comprehensive knowledge of how C-dot surfaces with various properties behave is essential for engineering C-dots with useful imaging properties such as high quantum yield, stability, and low toxicity, and with desirable biosensing properties such as high selectivity, sensitivity, and accuracy. Several reviews in recent years have reported preparation methods and properties of C-dots and described their application in biosensors, catalysis, photovoltaic cells, and more. However, no one has yet systematically summarized the surface engineering of C-dots, nor the use of C-dots as fluorescent nanosensors or probes for in vivo imaging in cells, tissues, and living organisms.

In this Account, we discuss the major design principles and criteria for engineering the surface functionality of C-dots for biological applications. These criteria include brightness, long-term stability, and good biocompatibility. We review recent developments in designing C-dot surfaces with various functionalities for use as nanosensors or as fluorescent probes with fascinating analytical performance, and we emphasize applications in bioimaging and biosensing in live cells, tissues, and animals. In addition, we highlight our work on the design and synthesis of a C-dot ratiometric biosensor for intracellular Cu^{2+} detection, and a twophoton fluorescent probe for pH measurement in live cells and tissues.

We conclude this Account by outlining future directions in engineering the functional surface of C-dots for a variety of in vivo imaging applications, including dots with combined targeting, imaging and therapeutic-delivery capabilities, or high-resolution multiplexed vascular imaging. With each application C-dots should open new horizons of multiplexed quantitative detection, high-resolution fluorescence imaging, and long-term, real-time monitoring of their target.

1. Introduction

Nanomaterial has a tremendous impact on the advancement of a wide range of fields including electronics, photonics, energy, catalysis, and medicine. In the development of fluorescent nanomaterials, the discovery of semiconductor quantum dots (QDs) is considered as a major milestone. Semiconductor QDs usually have tunable and narrow emission spectral features, bright fluorescence, high photostability, and resistance to metabolic degradation in bioapplications. However, a major disadvantage limits the use of QDs because most of the high-performance QDs are composed of toxic heavy metal elements such as cadmium. Thus, extensive efforts have been paid on the development of less toxic fluorescent nanomaterials as alternatives to the semiconductor QDs.

In this regard, carbon nanomaterials have fascinating optical properties and already show encouraging performance in bioimaging and biosensing. Fluorescent carbon

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nanomaterials appear in different forms such as fullerene, carbon nanotubes, nanodiamonds, graphene, and a rising star: carbon dots (C-Dots). C-Dots were accidently discovered as a byproduct during the electrophoretic purification of single-wall carbon nanotubes (SWCNTs) fabricated by arc-discharge methods.[1] Since then, more synthesis methods were developed for lowering the cost, simplifying operation, improving quantum yield (QY), or realizing larger-scale production. The key issue for designing C-Dots lies in controlling the number of atoms within one C-Dot core. For this purpose, raw C-Dot cores can be obtained by two main approaches "top-down" and "bottom-up", which have been well summarized in the previous reviews.[2−5]

Unlike nanodiamonds, C-Dots have greater sp^2 character, which is symbolic of nanocrystalline graphite. These materials have also been referred to as carbonic nanodots because of their high oxygen content. To distinguish them from the graphene quantum dots (GQDs) which have the similar electrons confinement in π-domains with graphene[6] and can be synthesized in well-defined structures through organic processes,[7] the C-Dots discussed in this paper refer to three-dimensional carbon materials of nanometer scale in general (whether there are lattice structures or not). The as-prepared C-Dots readily exhibit quantum effects with diameters below 10 nm. Compared with bulk materials, one of the most characteristic properties of C-dots is the size dependence of the photophysical properties, which allows property control simply by nanoparticle growth.

In addition, the C-Dots show fascinating properties such as resistance to photobleaching, low-toxicity, and good stability. Unique photophysical and chemical properties rendered by C-Dots promoted development of novel imaging probes, high performance-nanosensors, and multifunctional nanocomposites for addressing challenging issues raised by biological research. Biological applications based on C-Dots have resulted in more than 220% increase in related peer-reviewed publications since 2006 (based on PubMed and SciFinder searches). Further modification of the C-Dots with tailored organic coating materials and molecules generates biologically compatible probes with QY, good stability, and low-toxicity against live species. Therefore, engineering the functional surface of C-Dots is a key bridge to connect the C-Dots with fascinating biological applications. This Account focuses on the recent achievements in functional surface engineering of C-Dots for enhancing the quantum yield, improving the stability, and lowering the cytotoxicity. We summarize developments in employing the functional C-Dots for fluorescent nanosensors and bioimaging in live cells, tissues, and animals. The last section outlines potential directions in using engineered C-Dots for multiple targeting, imaging, and therapeutic modules, and C-Dot-based nanodevices that incorporate multiple functions of imaging, drug loading, and sensing capacities within a single nanoparticle.

2. Engineering the Functional Surface of C-Dots for Biologically Available Fluorescent Probes

Carbon dot provides a solid foundation for the development sensors. However, bare C-Dots are usually not fluorescent or weakly fluorescent, and do not show biological functionality due to poor interaction with biological systems. Manipulation of the C-Dot core size, chemical composition, and structure controls the photophysical properties of the probes. Modification with coating materials may produce strong fluorescent C-Dot probes with controllable biocompatibility and stability. Further functionalization of the C-Dots with recognition biomolecules generates specific biofunctionalities on the surface. Thus, design of C-Dot-based probes usually requires several steps. Each step follows distinctive design principles aimed at controlling optical, physical, chemical, and physiological properties of the probes for biological applications.

2.1. Brightness. Although C-Dots have fascinating photoluminescence properties with λ_{ex} dependent emission intensity that is similar to semiconductor QDs, the origin is still a matter of debate. It is speculated that quantum effect, emissive traps, and mostly radiative recombination of excitons contribute to the fluorescence property of C-Dots.[4] Considering that almost all C-Dots show a high concentration of C-sp^2 hybridization, the band gap transitions corresponding to conjugated π-domains, some complex origins associated with defects in the graphene structures as well as their interconnected may also be taken into account.[6] Now, it is increasingly adopted in the relevant research community that radiative recombination of the C-Dot surface-confined electrons and holes are responsible for the observed bright photoluminescence. Sun's group has provided the experimental evidence in support of the mechanistic framework. They found that the photoluminescence quenching results with both electron donors and acceptors, which could apparently scavenge the surface-confined holes and electrons in carbon dots, respectively, to result in efficient and effective quenching of the emissions.[8] Recently, they also demonstrated that the photogenerated electrons in C-Dots could be used for reduction purposes.[9,10]

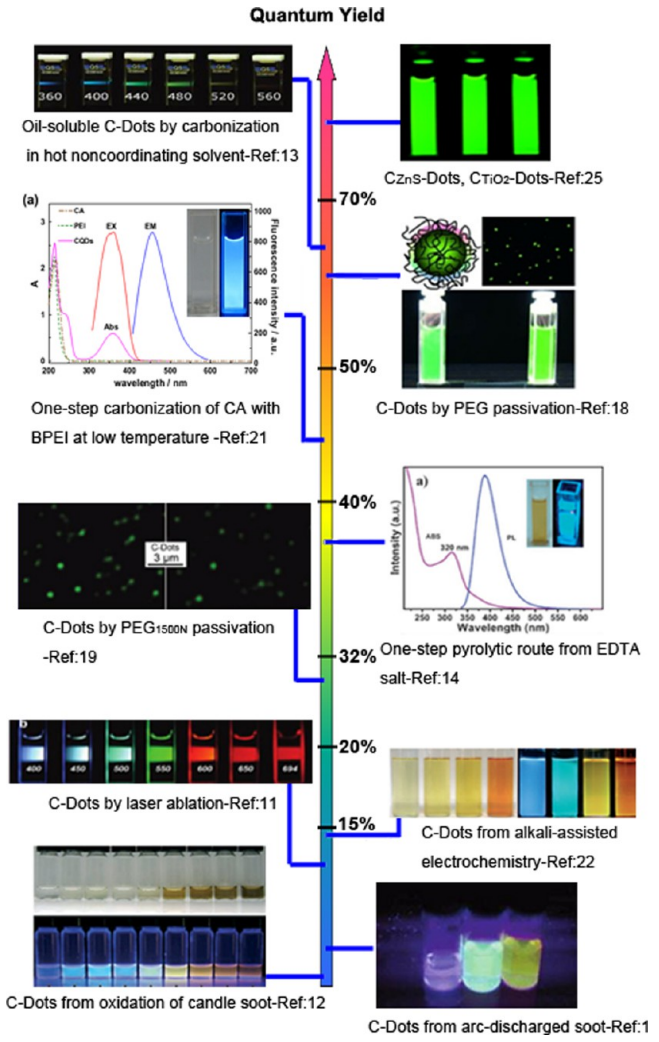

FIGURE 1. Illustration of representative efforts for improving the fluorescence intensity of C-Dots by engineering the surface since 2004. Adapted from refs 1, 11−14, 18, 19, 21, 22, and 25. Images from refs 1, 11, 13, and 19 were reproduced with permission from the American Chemical Society, refs 14 and 25 were transferred the permission from The Royal Society of Chemistry, ref 12 was obtained the Copyright 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, refs 18 and 22 were obtained the Copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, and ref 21 was transferred the Copyright (2012), with permission from Elsevier.

Since QY is relatively important for fluorescence bioimaging and biosensing, great efforts have been paid on engineering the surface functionality of C-Dots for improving the fluorescence intensity, as summarized in Figure 1.The photoluminesce QY of C-Dots from cheap carbon sources such as candle combustion soot, natural gas burners, and carbohydrates, are usually below 15%.[1,11,12] On the other hand, oil-soluble C-Dots fabricated by carbonizing carbon precursors, e.g., citric acid, in hot noncoordinating solvent possess a maximum QY up to 53% at room temperature, which is one of the highest results reported so far.[13] Similar method can also be used for the preparation of water-soluble C-Dots via changing the reaction solvent and capping agent with maximum QY of 17%.[13] Besides, water-soluble C-Dots with blue luminescence QY of 31.6−40.6% can also be synthesized by a one-step pyrolytic route from ethylenediamine-tetraacetic acid salts.[14]

The influence of ions on QY of C-Dots has also been investigated. Enhanced photoluminescence of the C-Dots has been observed due to the effect of cations (Na^+, Ca^{2+}, Al^{3+}) and anions (Cl^-, SO_4^{2-}, PO_4^{3-}).[15] Recently, Pramanik's group[16] reported a simple route to achieve C-Dots from individual biocompatible and biodegradable polysaccharides, and investigated the effect of various inorganic metal ions on the photoluminescence properties of C-Dots. The quenching (Cu^{2+}-combined C-Dots) and enhancement (Sn^{2+}-, Cd^{2+}-, and Zn^{2+}-combined C-Dots) of photoluminescence intensity is thought to be related with photoinduced electron transfer (PET) mechanism. In this model, ions with different d-electronic configuration show protoinduced-metal energy transfer (Cu^{2+}) or internal charge transfer (Sn^{2+}, Cd^{2+}, and Zn^{2+}) between the combined ions and C-Dots.

It should be mentioned that the C-Dots prepared by laser ablating method were usually not fluorescent, but the surface passivation could dramatically improve the QY of C-Dots. Yang et al. fabricated C-Dots functionalized with amino groups by hydrothermal carbonization of chitosan at a mild temperature. The QY of these water-soluble fluorescent C-Dots is about 7.8%.[17] Sun's group passivated the surface of C-Dots by oligomeric poly(ethylene glycol) diamine (PEG_{1500N}) molecules, and observed strong green fluorescence emissions with QY close to 20%. Further processing of the samples to achieve better surface passivation resulted in PEGy-lated C-Dots of fluorescence QY higher than 50%.[18,19] Recently, the use of an organosilane as a coordinating solvent to synthesize highly luminescent C-Dots with QY of 47% was reported.[20] The C-Dots, which benefit from surface methoxysilyl groups, can be transformed into water-soluble C-Dots/silica particles for bioimaging purpose. Chi and his co-workers[21] also synthesized polyamine-functionalized C-Dots with branched polyethylenimine (BPEI) in one simple step. The high fluorescence QY of 42.5% and the abundant BPEI at the surface of the spherical graphite nanocrystals suggest their promising applications in chemical sensing.

More significantly, the photoluminescent C-Dots with high QY were fabricated through doping the C-Dot core with ZnO (C_{ZnO}-Dots) or ZnS (C_{ZnS}-Dots) in aqueous solutions,

followed by the functionalized with PEG_{1500N} molecules.[23] The QYs of the obtained C_{ZnO}-Dots and C_{ZnS}-Dots were 45% and 50%, respectively, which are comparable to the commercially available CdSe/ZnS QDs in luminescence brightness. The bright C-Dots doped with ZnS (C_{ZnS}-Dots) were successfully used in live mice for cytotoxicity assays and further bioimaging.[24] More recently, the same group functionalized small C-Dots by a combination of doping with nanoscale semiconductors and organic functionalization, followed by gel column fractionation to harvest the most fluorescent C-Dots. The observed emission QYs for C_{ZnS}-Dots and C_{TiO2}-Dots were up to 78% and 70%, respectively.[25]

2.2. Stability. Compared with QDs, the C-Dots obtained by various methods are usually water-soluble with good fluorescence stability. Generally, the fluorescence stability can be divided into photostability and store-stability. Photostability means the fluorescence emission intensity keeps stable during a long-time continuous excitation, while store-stability means the fluorescence intensity of C-Dots stays constant after a long store period. For example, the fluorescence intensity of C-Dots prepared by electro-oxidation of graphite stays the same after a continuous excitation of 6 h with a xenon lamp (8.3 W).[26] The fluorescence intensity of C-Dots from the dehydration of carbohydrates by acid decreases by only 17% after 19 h of continuous excitation at 360 nm.[27] Besides, C-Dots from laser rapid passivation and from microwave assisted one-step synthesis both have good photostability during continuous excitation.[28] Other methods could also generate fluorescent C-Dots with good store-stability with a long store period of 2−6 months. Oil-soluble C-Dots from carbonizing carbon precursors in hot noncoordination solvent can be stored for 2 months with no change in its fluorescent intensity.[13] Meanwhile, water-soluble C-Dots from one-step alkali/acid assisted ultrasonic treatment or from passivation of cheap commercial lampblack can also be stored for up to 6 months.[29,30]

2.3. Biocompatibility. Although impressive progress has been made in engineering bright C-Dots probes with good stability, biocompatibility of the functionalized C-Dots is still a critical issue for further applications in live cells, tissues, and animals. Systematic cytotoxicity evaluations were carried out on both raw C-Dots and passivated C-Dots in the past few years.[31,32] Sun's group employed two kinds of raw C-Dots for cytotoxicity assay: one was commercially supplied carbon nanopowder; another was produced by the arc-discharge of graphite rods and then refluxed in HNO_3 for 12 h. The bare C-Dots were apparently nontoxic to cells up to relatively high concentration of 0.4 mg mL^{-1}. Luminescent C-Dots synthesized by the electrochemical treatment of graphite were also evaluated in terms of established cytotoxicity assay with a human kidney cell line, in which the cell viability was not affected by the particles.[26] Furthermore, Ray et al.[33] improved a soot-based approach for small-size C-Dots synthesis with diameters of 2−6 nm. The experimental results of cell viability confirmed that the C-Dots showed negligible cytotoxicity at concentrations required for fluorescence bioimaging.

The cytotoxicity of the C-Dots passivated with functional groups, such as PEG (polyethylene glycol),[24] PPEI-EI (poly(propionylethylenimine-*co*-ethylenimine)),[34] PEI (polyethylenimine),[32] BPEI (branched poly(ethylenimine)),[35] and PAA (poly(acrylic acid)),[36] was also evaluated. The PEGylated C-Dots in all available configurations[18,19] were obviously noncytotoxic up to concentrations much higher than what is necessary for cell imaging and related applications. In addition, C-Dots functionalized with PEG_{1500N} were injected into mice for toxicity evaluation up to 28 days, and the results suggested no significant toxic effects in vivo.[19] Meanwhile, experimental results indicated that the PPEI-EI-passivated C-Dots were mostly nontoxic to the cells below a relatively high threshold of carbon core-equivalent PPEI-EI concentration.[34] According to the MTT assay, free PEI sample was apparently nontoxic to HT-29 cells even at relatively high concentrations. The PEI-functionalized C-Dots were also evaluated in terms of MTT assay, and the results suggested their being more cytotoxic than PPEI-EI-functionalized C-Dots. The more ethylenimine (EI) units within PEI may be associated with lower concentration thresholds for the C-Dots to become cytotoxic, as PEI is the homopolymer corresponding to PPEI-EI with an extreme EI fraction of 100%. Free PAA nonaqueous solution was found to be harmful to cells even at relatively low concentrations (50 μg mL^{-1}). The PAA-functionalized C-Dots were generally comparable to free PAA at the same C-Dot core-equivalent concentrations, both obviously more toxic to the cells with an exposure time of 24 h, but less so when the exposure time was shortened to 4 h. Overall, surface passivation molecules of no or low cytotoxicity even at high concentrations such as PEG and PPEI-EI are suitable for C-Dots functionalization for in vivo imaging and biosensing. For molecules that are more cytotoxic including BPEI and PAA, their corresponding C-Dots may be still used in vivo if their concentrations are kept low enough and the incubation time short enough.[32]

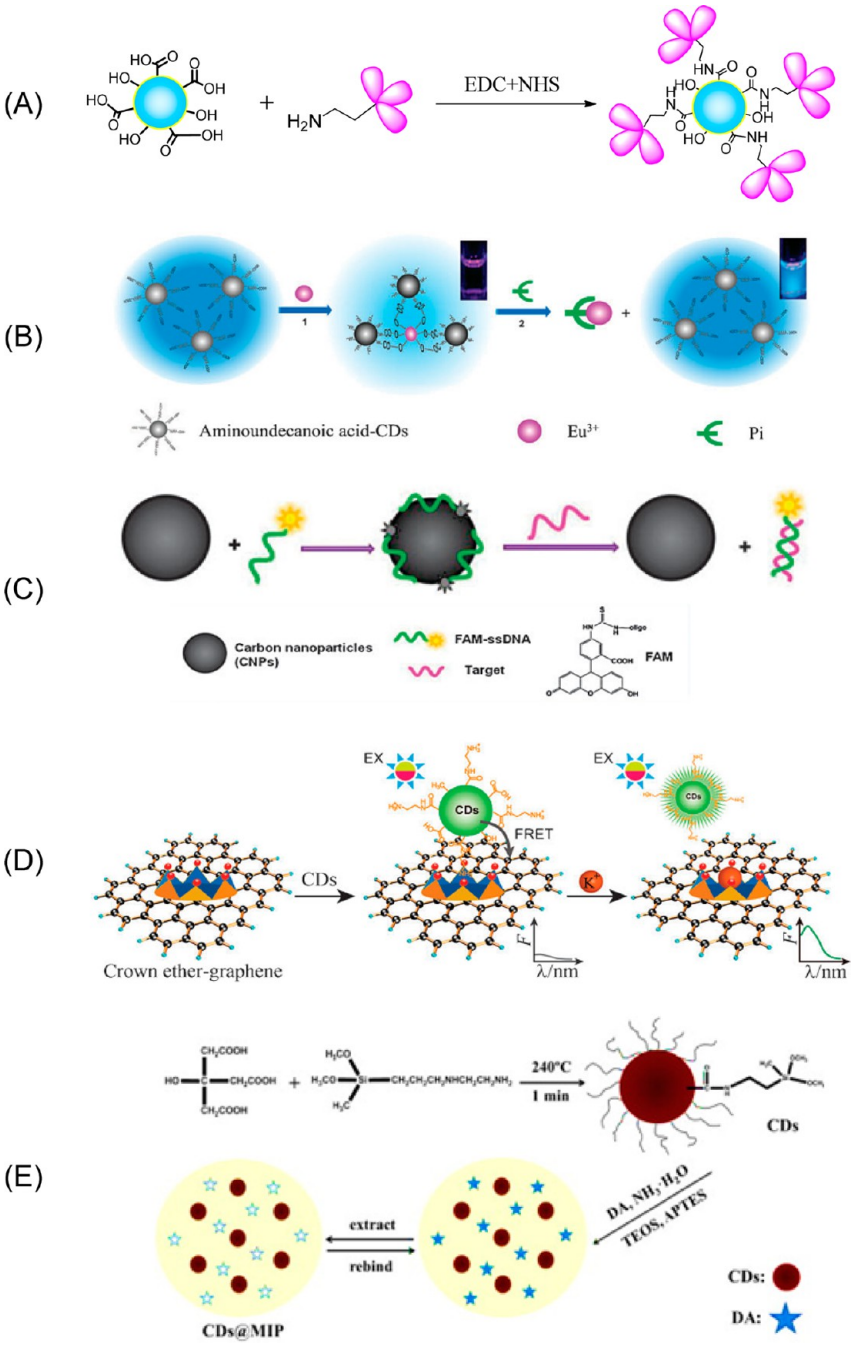

FIGURE 2. (A) Link functional groups via primary amine groups to carboxylic acid-containing C-Dots by using NHS and EDC. (B) Metal coordination between Eu^{3+} and the surface of C-Dots functionalized with carboxylate groups. Adapted from ref 43 with permission from The Royal Society of Chemistry. (C) $\pi-\pi$ stacking interaction between dye-labeled ss-DNA probe and the surface of the C-Dots. Adapted from ref 44 with permission from The Royal Society of Chemistry. (D) Primary alkyl-ammonium binding between 18C6E and the surface of aminated C-Dots. Adapted from ref 48 with permission from The Royal Society of Chemistry. (E) Sol−gel polymerization of MIP on the surface of C-Dots. Adapted from ref 49, Copyright 2012, with permission from Elsevier.

3. Designing the Functional Surface of C-Dots for Fluorescent Sensing

Advances in synthesis and surface modification technologies made C-Dots appealing platforms for engineering of biological probes with improved brightness, tunable fluorescence, enhanced water-solubility and biocompatibility. Onging work on controlling the surface properties and functionalities of C-Dots aims at transforming these materials into biologically compatible nanoprobes and biofunctional nanosensors. Some C-Dots treated by acid reflux can

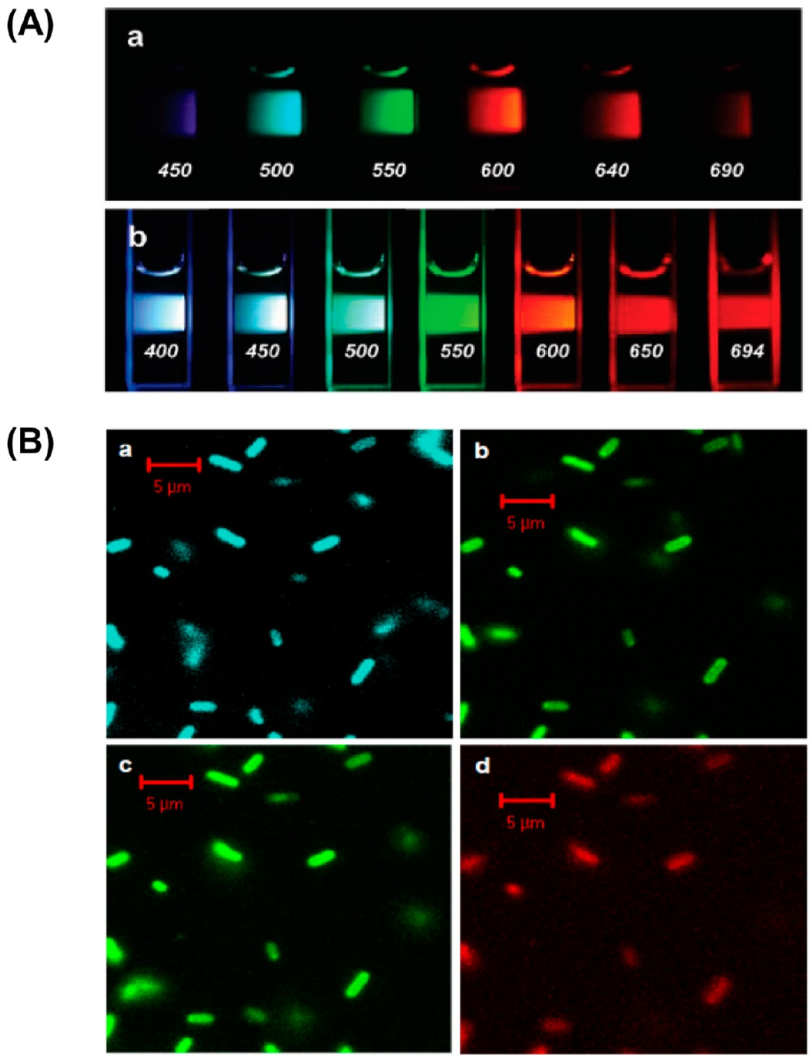

FIGURE 3. (A) Aqueous solution of the PEG_{1500N}-attached carbon dots (a) excited at 400 nm and photographed through band-pass filters of different wavelengths as indicated, and (b) excited at the indicated wavelengths and photographed directly. (B) Confocal microscopy images of *E. coli* ATCC 25922 cells labeled with the carbon dots: (a) $\lambda_{ex} = 458$ nm, detected with 475 nm long pass filter; (b) $\lambda_{ex} = 477$ nm, detected with 505 nm long pass filter; (c) $\lambda_{ex} = 488$ nm, detected with 530 nm long pass filter; and (d) $\lambda_{ex} = 514$ nm, detected with 560 nm long pass filter. Adapted with permission from ref 11. Copyright 2006 American Chemical Society.

be used directly as pH sensors due to the hydroxyl and carboxyl groups on the surface. In addition, pH-sensitive C-Dots can also be obtained after passivation by PEG,[30] *N*-acetyl-L-cysteine, or mercaptosuccinic acid,[37] and some other polymers.[32] Specific receptors are needed to conjugate with C-Dots for constructing fluorescent nanosensors with high specificity. Several available C-Dots surface functionalities are summarized in Figure 2.

One of the simplest and most popular bioconjugation methods is covalent bond formation through reactive functional groups such as primary amines,[38,39] carboxylic acids,[40] and hydroxyls.[41] For example, water-soluble C-Dots surrounded with −COOH groups can readily combine with *N*-(2-aminoethyl)-*N*,*N*,*N*′-tris(pyridin-2ylmethyl)ethane-1,2-diamine (AE-TPEA)[38] and 4′-(aminomethylphenyl)-2,2′,6′,2″-terpyridine (AE-TPY),[39] for the detection of Cu^{2+} and pH in biological systems, respectively (Figure 2A). Bovine serum albumin and lysine can be conjugated onto the surface of C-Dots through the same method and provide a "turn-off" fluorescence sensor for Cu^{2+}.[40]

The sensor has been successfully applied in human hair and tap water samples with a linear range from 2.0 nM to 1.5 μM and a detection limit of 0.58 nM. Through the same method, branched BPEI-functionalized C-Dots were designed for the detection of Cu^{2+}[35] with a dynamic range from 10 to 1100 nM and a detection limit of 6 nM. Ratiometric C-Dots pH sensors can be synthesized by heating citric acid in glycerol in the presence of

4,7,10-trioxa-1,13-tridecanediamine (TTDDA) and pH-insensitive rhodamine B isothiocyanate.[42] Recently, C-Dots covalently coupled with naphthalimide-azide[41] was reported as a highly selective and sensitive H_2S detector base on the FRET between C-Dots and the product from the reduction of naphthalimide-azide by H_2S.

Besides covalent bonding to C-Dots, coordination is another strategy. Metal ion coordination was employed to bind europium (Eu^{3+}) onto the surface of C-Dots[43] (Figure 2B) due to the affinity between Eu^{3+} and oxygen-donor atoms, which subsequently quenches the fluorescence of C-Dots. This sensor is highly selective and sensitive for phosphate detection based on restoration of the fluorescence, because Eu^{3+} has higher affinity to the oxygen-donor atoms originated from free phosphates than those from carboxyl groups on C-Dots surface. The detection range of phosphate was determined to be 0.4 to 15 μM.

The $\pi-\pi$ stacking interactions between C-Dots and DNA or aptamer (Figure 2C) can also be used as binding strategy for detection of nucleic acid.[44] The fluorescence from dye-labeled single-stranded DNA probe is quenched after adsorption onto the C-Dots surface via $\pi-\pi$ interaction. When the target DNA matches the dye-labeled DNA to form double-stranded DNA, the fluorescence is recovered. This strategy is also employed for determination of metal ions such as Hg^{2+} and Ag^{+} through T-Hg^{2+}-T and C-Ag^{+}-C base pairs, respectively.[45,46] Meanwhile, aptamer biosensors for thrombin detection were successfully constructed based on $\pi-\pi$ interactions between C-Dots and FAM (fluorescein)-[47] or up-converting phosphor-labeled aptamer.[36] This approach allowed the detection of thrombin in the range of 0–120 nM. More recently, another aptamer biosensor is constructed for thrombin based on FRET from up-converting phosphors to C-Dots.[36] The sensor showed a linear range from 0.5 to 20 nM either in a buffer solution or in spiked human serum samples, demonstrating the high robustness of the sensor in a complex biological environment.

Furthermore, primary alkyl-ammonium binding (Figure 2D) is used to modify 18-crown-6-ether (18C6E) on the surface of aminated C-Dots, which conjugated on reduced graphene oxide (rGO) at the same time.[48] The FRET efficiency between C-Dots and rGO as a function of the specific cation-ligand complexation was employed for K^{+} detection, because the FRET process was inhibited by the competition between K^{+} and ammonium for 18C6E.

Sol–gel technique is also a promising approach for engineering the surface of C-Dots with functional molecules. For example, C-Dots@MIP (molecularly imprinted polymer)

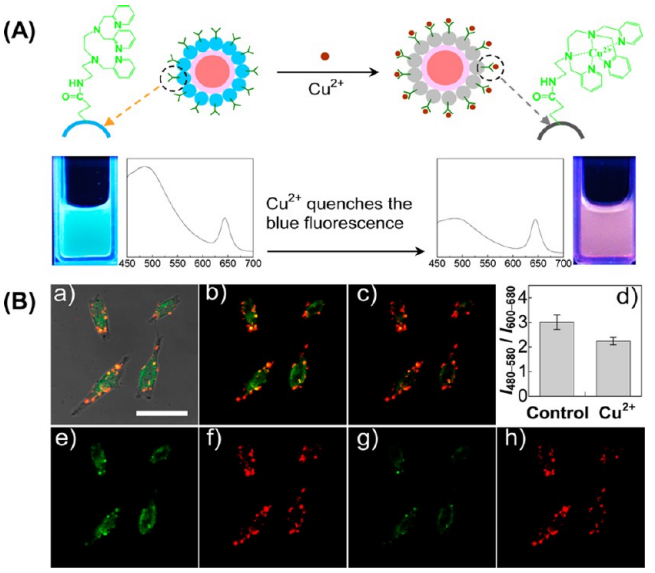

FIGURE 4. (A) Schematic illustration of the dual-emission fluorescent sensing of Cu^{2+} based on CdSe@C-TPEA nanohybrid. (B) (a) The overlay of bright-field and fluorescence images of HeLa cells incubated with CdSe@C-TPEA. (b, c) Confocal fluorescence images of HeLa cells (b) before and (c) after the exogenous Cu^{2+} source treatment. (d) Bar graph representing the integrated intensity from 480 to 580 nm over the integrated fluorescence intensity from 600 to 680 nm; values are the mean ratio generated from the intensity from three randomly selected fields in both channel. (e, g) Confocal fluorescence images obtained from the 480–580 nm channel before and after the exogenous Cu^{2+} source treatment, while (f, h) are from the 600–680 channel. Scale Bar: 25 μm. Adapted from ref 52, Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

was synthesized by one-pot room-temperature sol–gel polymerization and applied as a fluorescence sensor for the detection of dopamine in aqueous solution (Figure 2E).[49] Esteves da Silva[37] and co-workers used sol–gel method to functionalize the surface of C-Dots with PEG_{200N} and *N*-acetyl-L-cysteine and constructed a fluorescent nanosensor sensitive to micromolar level of Hg^{2+} and Cu^{2+} as well as the solution pH. Furthermore, they immobilized the functionalized C-Dots in a sol–gel matrix at the tip of an optical fiber to stabilize the photophysical and chemical properties of fluorescent C-Dots.[50] The fiber optic system allowed reversible sensing of Hg^{2+} in the submicromolar concentration range in aqueous solution and remained stable after 6 months of use.

4. Tailoring the Functional Surface of C-Dots for in Vivo Bioimaging and Biosensing

The absence of adverse effects on cell physiology and small cytotoxicity encourage further exploration of suitable C-Dot probes for in vivo applications. The pioneering work on C-Dots for bioimaging in vitro and in vivo was reported by

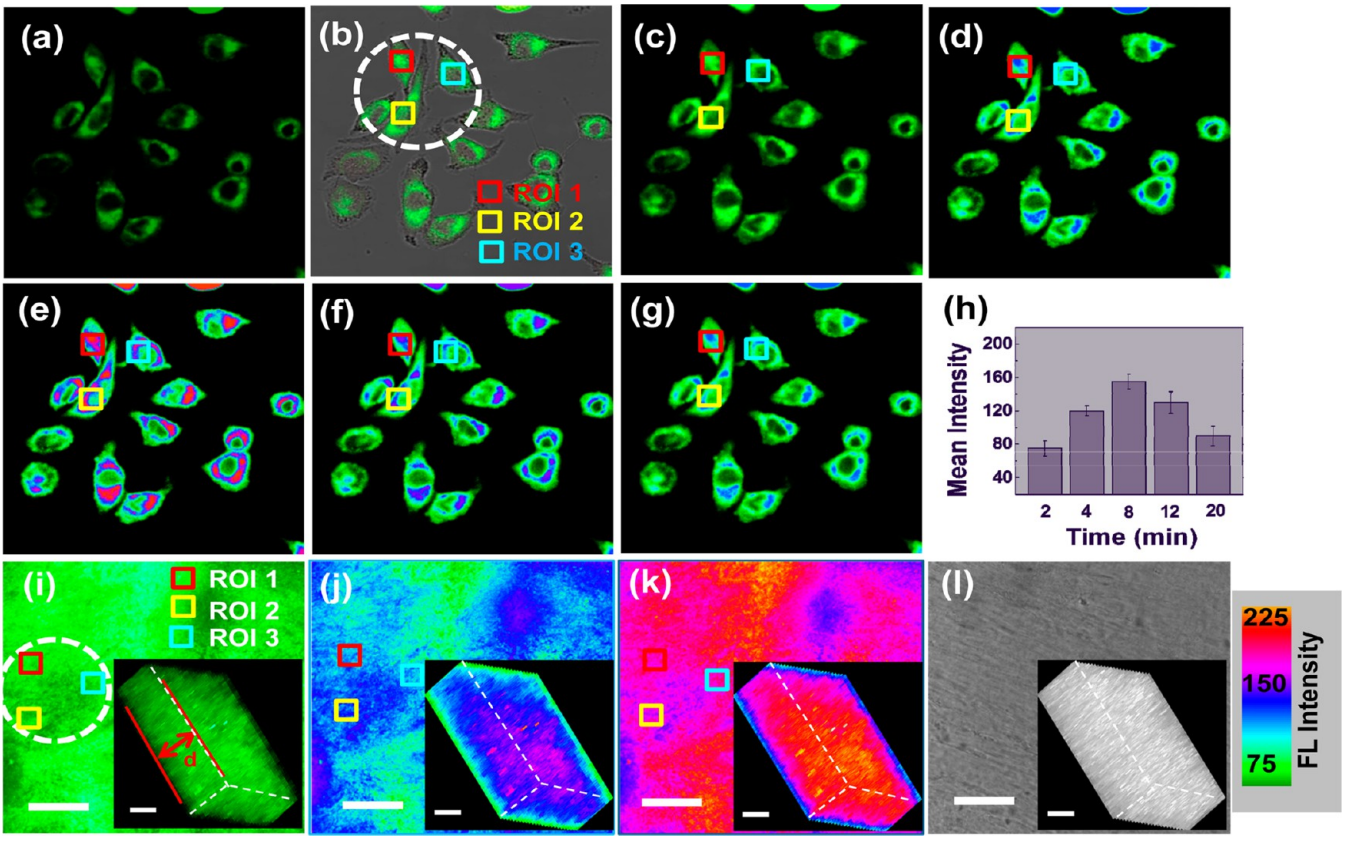

FIGURE 5. (a–g) Na^+-H^+ exchange dependent two-photon confocal fluorescence images of A549 cells. (a) Two-photon confocal fluorescence. (b) Overlapped images of A549 cells incubated with C-Dots-TPY probe. (c–e) Whole visual field of ouabain-treated A549 cells for suspended in choline CH_3SO_3-Ringer's solution for (c) 2, (d) 4, and (e) 8 min. (f, g) Na^+-dependent real-kalinization of ouabain-treated A549 cells by adding $NaCH_3SO_3$ on top of the choline CH_3SO_3-Ringer's solution for another (f) 4 and (g) 8 min. (h) Mean fluorescence intensity induced by Na^+–H^+ exchange of A549 cells. Data represent the mean fluorescence intensity of distinct fields (inset ROI 1–3). (i–l) Two-photon confocal fluorescence and bright-field images of C-Dots-TPY probe in A549 lung cancer cells tissue slice upon pH changes from pH 7.8 (i, inset), to pH 7.1 (j, inset), and then to pH 6.4 (k, inset). (i–k) Two-photon confocal fluorescence and (l) bright-field images at a depth of 150 μm. (i–k, inset) 3D two-photon confocal fluorescence and (l, inset) bright-field images accumulated along the *z* direction at depth of 65–185 μm. Scale bars: 60 μm. Reproduced from ref 39. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Sun's group.[11] In the paper, C-Dots were synthesized via laser ablation of carbon targets followed by HNO_3 reflux, and then passivation with PEG_{1500N}. No detectable fluorescence was obtained for raw C-Dots or after refluxing by HNO_3. However, bright luminescence (Figure 3A) was clearly observed after surface passivation of the C-Dots by PEG_{1500N}. Confocal microscopy images of *E. coli* ATCC 25922 labeled with the functionalized C-Dots were obtained at different excitation wavelengths (Figure 3B).

Soon afterward, C-Dots for multiphoton bioimaging in live cells were also explored.[34] The C-Dots passivated by PPEI-EI with EI fraction of ~20% were readily soluble in water, and were found to be strongly emissive in the visible with two-photon excitation in the near-infrared (800 nm). 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, and can give two-photon luminescence under excitation of 800 nm. Fluorescent images of the C-Dots synthesized in small size (2–6 nm) through a soot-based approach[33] in live cells can be obtained by incubating with EAC cells only for 30 min. The imaging results show that cells turn bright blue-green under UV excitation and yellow under blue excitation, but colorless without uptake of C-Dots. This suggests that C-Dots enter into the live cells, and are available for fluorescent bioimaging of live cells.

In order to explore C-Dots as biocompatible nanoprobes for targeting cancer cells, C-Dots were passivated by three kinds of polymers, including PEG_{1500N}, triblock polymer PEI-PEG-PEI, and 4-armed amine-terminated PEG (4-arm PEG). Then, the passivated C-Dots were further functionalized by protein transferrin through carbodiimide chemistry for targeting cancer cells. Photoluminescence images showed that functionalized C-Dots were internalized more efficiently in HeLa cells than the nonfunctionalized, and those C-Dots

passivated with PEG_{1500N} and 4-arm PEG more than those passivated with PEI-PEG-PEI. The cellular uptake experiments indicate that the design of surface functional groups permit efficient conjugation ability with biomolecules through carbodiimide chemistry.[51]

More interestingly, a tunable ratiometric pH sensor was developed for the quantitative measurement of the intracellular pH of cells based on C-Dots.[42] The amino-coated C-Dots were functionalized with pH-sensitive fluorescein isothiocyanate and pH-insensitive rhodamine B isothiocyanate, resulting in the dual-emission probe upon a single excitation. The pH sensor exhibited low cytotoxicity, good cell-permeability, and an excellent reversibility between pH 5 and 9. Thus, real-time quantitative determination of intracellular pH of intact HeLa cells and the pH fluctuations associated with oxidative stress were successfully performed.[42]

Our group first integrated C-Dots coated with a specific organic molecule AE-TPEA and CdSe/ZnS QDs and developed a ratiometric strategy for intracellular sensing of Cu^{2+} (Figure 4A).[52] The fluorescent probe can monitor Cu^{2+} in a concentration range from 5 - 200 μM at the physiological pH environment. Following uptake, the particles are found to reside in various intracellular compartments (Figure 4B). After exogenous Cu^{2+} source treatment, the fluorescence emission color of the probe turned from green-yellow to red (Figure 4B). These initial experiments on live cells demonstrate the great potential for C-Dot-based dual-emission hybrid sensors in the investigation of fundamental biological processes.

As demonstrated above, C-Dots have been widely employed for bioimaging in various live cells, because they show bright and stable fluorescence, as well as water solubility and good biocompatibility. However, it is still at an early stage to use C-Dots as imaging agents for tissues and animals. Recently, we reported the design and synthesis of a C-Dots-based probe for two-photon bioimaging of physiological pH in live cells as well as tissues (Figure 5).[39] The fluorescent emission intensity showed good linearity with pH variation in the range of 6.0−8.5, which meets the requirement for in vivo sensing of pH variation. As a result, the real-time cytosolic pH changes images were obtained in A549 cells and LCC tumor cells upon Na^+−K^+ exchanges. More interestingly, the 3D two-photon confocal fluorescence imaging along the *z* direction demonstrated that the C-Dot-TPY probe was capable of monitoring pH gradients at a 65−185 μm depth in living tissues using two-photon microscopy.

In order to increase the brightness of C-Dots and to achieve similar performance with the well-known

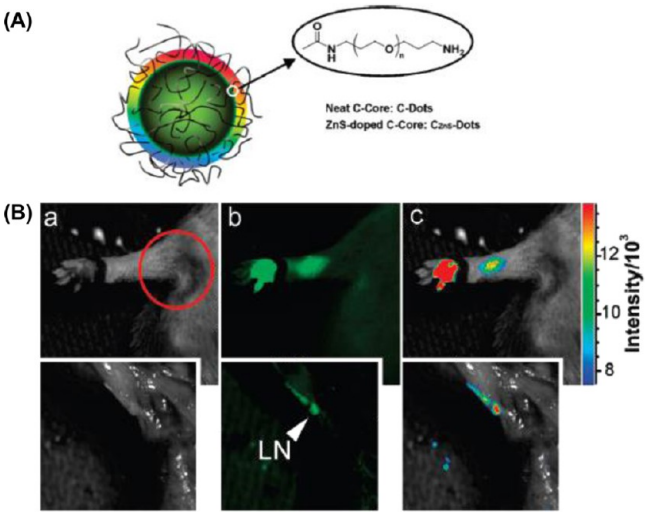

FIGURE 6. (A) Scheme for ZnS-doped C-Dots. (B) Intradermal injection of C_{ZnS}-Dots: (a) bright field, (b) as-detected fluorescence, and (c) color-coded images. Insets: dissected (in the circled area) axillary lymph node. Adapted with permission from ref 24. Copyright 2009 American Chemical Society.

CdSe/ZnS QDs in tissues and animals, the C-Dots and C_{ZnS}-Dots passivated by PEG_{1500N} were used for the first time for in vivo imaging in mice.[24] The fluorescence images of the subcutaneously injected mice exhibited bright emissions upon excitation of 470 and 545 nm from C-Dots and C_{ZnS}-Dots. The injected C-Dots in mice diffused relatively slowly, and the fluorescence faded at 24 h postinjection. The brighter green fluorescence of C_{ZnS}-Dots was further employed to track the migration through lymph vessels (Figure 6). The axillary lymph nodes were harvested and dissected and exhibited fluorescence spectral features from the C-Dots. Finally, whole-body circulation of C-Dots was studied by intravenous injection into mice. At 3 h postinjection, bright fluorescence in the urine became visible, suggesting that the intravenously injected C-Dots were primarily excreted via urine.

In another study, Sun's group[19] synthesizes C-Dots by laser ablation and passivation with PEG_{1500N} in slightly modified procedures for mice imaging (Figure 7). The green luminescence of C-Dots was generally bright and comparable to those of the commercial QDs under the same experimental conditions. The two-photon fluorescence images (800 nm excitation) of C-Dots were clearly observed in sliced liver and spleen harvested from mice 6 h after intravenous exposure to C-Dots. The amounts of C-Dots were found to be much higher in liver and spleen than those in other organs, but still relatively low in absolute concentration.

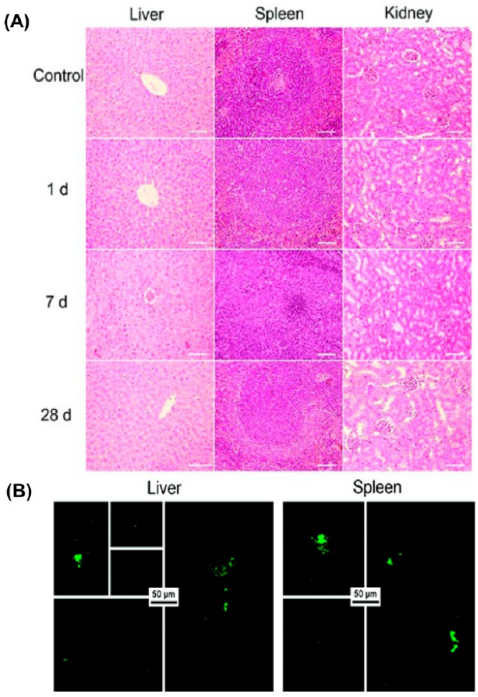

FIGURE 7. (A) Results from histopathological analyses of liver, spleen, and kidneys. (B) Fluorescence images (two-photon excitation at 800 nm) of sliced liver and spleen harvested from mice 6 h after intravenous exposure to C-Dots. Adapted with permission from ref 19. Copyright 2009 American Chemical Society.

5. Conclusions and Perspectives

As a nanomaterial for bioimaging and biosensing without a long history, C-Dot has caught a lot of attention for their easy preparation, fascinating photophysical properties, excellent stability, low-toxicity, and versatile surface chemistry. Engineering the surface functionality of C-Dots according to the multistep design and modification can generate C-Dots with high QY, good biocompatibility, and long-term stability, available for a wide variety of applications ranging from cell and target-bioimaging to biodetection for pH, metal ions, DNA, protein, and so on in live cells, tissues, and animals. With each application, C-Dots have opened new ways to quantitative detection, high-resolution fluorescent imaging, and real-time tracking of molecules.

Actually, the versatility of C-Dots provides vast flexibility in engineering the surface of probes for a variety of in vivo imaging applications such as high-resolution multiplexed vascular imaging, intraoperative image guidance, real-time cell tracking, specific interaction with biomolecules and cells, and so on. Meanwhile, recent advancements in the engineering of C-Dots probes and the promising benefits of this technology can bring a shift of focus from the synthesis of single-component probes toward the design of hybrid nanostructures composed of multiple targeting, imaging, and therapeutic modules. For example, C-Dots integrated with MRI contrast agents or radionuclides can be used for dual-mode imaging, whereas when combined with drugs or nucleic acid therapeutics C-Dots can serve as traceable delivery vehicles. In general, C-Dots can be used as universal scaffolds for the attachment of extra components and targeting ligands due to their large surface area and modular surface chemistry. Aiming at expanding C-Dots functionality even further, it is not a dream to engineer the C-Dot-based multifunctional nanodevices that promise to combine the benefits of multiple imaging modalities and incorporate the imaging, drug loading, and sensing capacities within a single nanoparticle. Yet, currently available C-Dot probes are far from being ideal, leaving plenty of room for the development of novel designs for the functional surface of C-Dots.

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BIOGRAPHICAL INFORMATION

Yang Tian received her Ph.D. degree in electronic chemistry from Tokyo Institute of Technology. After a postdoctoral training at University of Tokyo, she was appointed as a Professor in the Department of Chemistry at Tongji University, China in 2005. Her research expertise is molecular imaging, biosensor, and bionanotechnology. She has coauthored over 50 papers and book chapters.

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FOOTNOTES

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The authors declare no competing financial interest.

REFERENCES

1 Xu, X.; Ray, R.; Gu, Y.; Ploehn, H. J.; Gearheart, L.; Raker, K.; Scrivens, W. A. Electrophoretic Analysis and Purification of Fluorescent Single-Walled Carbon Nanotube Fragments. *J. Am. Chem. Soc.* **2004**, *126*, 12736–12737.

2 Esteves da Silva, G, J. C.; Gonçalves, H. M. R. Analytical and bioanalytical applications of carbon dots. *TrAC, Trends Anal. Chem.* **2011**, *30*, 1327–1336 and references cited therein.

3 Baker, S. N.; Baker, G. A. Luminescent carbon nanodots: emergent nanolights. *Angew. Chem., Int. Ed.* **2010**, *49*, 6726–6744 and references cited therein.

4 Li, H.; Kang, Z.; Liu, Y.; Lee, S. Carbon nanodots: synthesis, properties and applications. *J. Mater. Chem.* **2012**, *22*, 24230–24253 and references cited therein.

5 Shen, J.; Zhu, Y.; Yang, X.; Li, C. Graphene quantum dots: emergent nanolights for bioimaging, sensors,catalysis and photovoltaic devices. *Chem. Commun* **2012**, *48*, 3686–3699.

6 Cao, Li; M, M. J.; Sahu, S.; Sun, Y. Photoluminescence Properties of Graphene versus Other Carbon Nanomaterials. *Acc. Chem. Res.* **2013**, *46*, 171–180 and references cited therein.

7 Yan, X.; L, B.; Li, L. Colloidal Graphene Quantum Dots with Well-Defined Structures. *Acc. Chem. Res.* **2012**, DOI: 10.1021/ar300137p.

8 Wang, X.; Cao, L.; Lu, F. S.; Meziani, M. J.; Li, H.; Qi, G.; Zhou, B.; Harruff, B. A.; Kermarrec, F.; Sun, Y. P. Photoinduced Electron Transfers with Carbon Dots. *Chem. Commun.* **2009**, *25*, 3774–3776.

9 Xu, J.; Sahu, S.; Cao, L.; Anilkumar, P.; Tackett, K. N., II; Qian, H. J.; Bunker, C. E.; Guliants, E. A.; Parenzan, A.; Sun, Y.-P. Carbon Nanoparticles as Chromophores for Photon Harvesting and Photoconversion. *Chem. Phys. Chem* **2011**, *12*, 3604–3608.

10 Cao, L.; Sahu, S.; Anilkumar, P.; Bunker, C. E.; Xu, J. A.; Fernando, K. A. S.; Wang, P.; Guliants, E. A.; Tackett, K. N., II; Sun, Y. P. Carbon Nanoparticles as Visible-Light Photocatalysts for Efficient CO2 Conversion and Beyond. *J. Am. Chem. Soc.* **2011**, *133*, 4754–7457.

11 Sun, Y.; Zhou, B.; Lin, Y.; Wang, W.; Fernando, K. A. S.; Pathak, P.; Meziani, M. J.; Harruff, B. A.; Wang, X.; Wang, H.; Luo, P.; Yang, H.; Kose, M. E.; Chen, B.; Veca, L. M.; Xie, S. Quantum-Sized Carbon Dots for Bright and Colorful Photoluminescence. *J. Am. Chem. Soc.* **2006**, *128*, 7756–7757.

12 Liu, H.; Ye, T.; Mao, C. Fluorescent carbon nanoparticles derived from candle soot. *Angew. Chem., Int. Ed.* **2007**, *46*, 6473–6475.

13 Wang, F.; Pang, S.; Wang, L.; Li, Q.; Kreiter, M.; Liu, C. One-Step Synthesis of Highly Luminescent Carbon Dots in Noncoordinating Solvents. *Chem. Mater.* **2010**, *22*, 4528–4530.

14 Pan, D.; Zhang, J.; Li, Z.; Wu, C.; Yan, X.; Wu, M. Observation of pH-, solvent-, spin-, and excitation-dependent blue photoluminescence from carbon nanoparticles. *Chem. Commun.* **2010**, *46*, 3681–3683.

15 Wang, X.; Qu, K.; Xu, B.; Ren, J.; Qu, X. Microwave assisted one-step green synthesis of cell-permeable multicolor photoluminescent carbon dots without surface passivation reagents. *J. Mater. Chem.* **2011**, *21*, 2445–2450.

16 Chandra, S.; Pathan, S. H.; Mitra, S.; Modha, B. H.; Goswami, A.; Pramanik, P. Tuning of photoluminescence on different surface functionalized carbon quantum dots. *RSC Adv.* **2012**, *2*, 3602–3606.

17 Yang, Y.; Cui, J.; Zheng, M.; Hu, C.; Tan, S.; Xiao, Y.; Yang, Q.; Liu, Y. One-step synthesis of amino-functionalized fluorescent carbon nanoparticles by hydrothermal carbonization of chitosan. *Chem. Commun.* **2012**, *48*, 380–382.

18 Wang, X.; Cao, L.; Yang, S.; Lu, F.; Meziani, M.; Tian, L.; Sun, K.; Bloodgood, A. M.; Sun, Y. Bandgap-like strong fluorescence in functionalized carbon nanoparticles. *Angew. Chem., Int. Ed.* **2010**, *49*, 5310–5314.

19 Yang, S. T.; Wang, X.; Wang, H.; Lu, F.; Luo, P.; Cao, L.; Meziani, M. J.; Liu, J.; Liu, Y.; Chen, M.; Huang, Y.; Sun, Y. Carbon Dots as Nontoxic and High-Performance Fluorescence Imaging Agents. *J. Phys. Chem. C* **2009**, *113*, 18110–18114.

20 Wang, F.; Xie, Z.; Zhang, H.; Liu, C.; Zhang, Y. Highly Luminescent Organosilane-Functionalized Carbon Dots. *Adv. Funct. Mater.* **2011**, *21*, 1027–1031.

21 Dong, Y.; Wang, R.; Li, H.; Shao, J.; Chi, Y.; Lin, X.; Chen, G. Polyamine-functionalized carbon quantum dots for chemical sensing. *Carbon.* **2012**, *50*, 2810–2815.

22 Li, H.; He, X.; Kang, Z.; Huang, H.; Liu, Y.; Liu, J.; Lian, S.; Tsang, C. H. A.; Yang, X.; Lee, S. Water-soluble fluorescent carbon quantum dots and photocatalyst design. *Angew. Chem., Int. Ed.* **2010**, *49*, 4430–4434.

23 Sun, Y. P.; Wang, X.; Lu, F. S.; Cao, L.; Meziani, M. J.; Luo, P. J. G.; Gu, L. R.; Veca, L. M. Doped Carbon Nanopaticles as a new Platform for Highly Photoluminescent Dots. *J. Phys. Chem. C* **2008**, *112*, 18295–18298.

24 Yang, S.; Cao, L.; Luo, P.; Lu, F.; Wang, X.; Wang, H.; Meziani, M.; Liu, Y.; Qi, G.; Sun, Y. Carbon Dots for Optical Imaging in Vivo. *J. Am. Chem. Soc.* **2009**, *131*, 11308–11309.

25 Anilkumar, P.; Wang, X.; Cao, L.; Sahu, S.; Liu, J.; Wang, P.; Korch, K.; Tackett, K. N., II; Parenzan, A.; Sun, Y. Toward quantitatively fluorescent carbon-based "quantum" dots. *Nanoscale* **2011**, *3*, 2023–2027.

26 Zhao, Q.; Zhang, Z.; Huang, B.; Peng, J.; Zhang, M.; Pang, D. Facile preparation of low cytotoxicity fluorescent carbon nanocrystals by electrooxidation of graphite. *Chem. Commun.* **2008**, *41*, 5116–5118.

27 Peng, H.; Sejdic, J. T. Simple Aqueous Solution Route to Luminescent Carbogenic Dots from Carbohydrates. *Chem. Mater.* **2009**, *21*, 5563–5565.

28 Li, X.; Wang, H.; Shimizu, Y.; Pyatenko, A.; Kawaguchi, K.; Koshizaki, N. Preparation of carbon quantum dots with tunable photoluminescence by rapid laser passivation in ordinary organic solvents. *Chem. Commun.* **2011**, *47*, 932–934.

29 Li, H.; He, X.; Liu, Y.; Huang, H.; Lian, S.; Lee, S. T.; Kang, Z. One-step ultrasonic synthesis of water-soluble carbon nanoparticles with excellent photoluminescent properties. *Carbon* **2010**, *49*, 605–609.

30 Mao, X.; Zheng, H.; Long, Y.; Du, J.; Hao, J.; Wang, L.; Zhou, D. Study on the fluorescence characteristics of carbon dots. *Spectrochim. Acta, Part A* **2010**, *75*, 553–557.

31 Liu, J.; Anilkumar, P.; Cao, L.; Wang, X.; Yang, S.; Luo, P.; Wang, H.; Lu, F.; Meziani, M. J.; Liu, Y.; Korch, K.; Sun, Y. Cytotoxicity Evaluations of Fluorescent Carbon Nanoparticles. *Nano LIFE* **2010**, *1*, 153–160.

32 Wang, Y.; Anilkumar, P.; Cao, L.; Liu, J.; Luo, P.; Tackett, K. N., II; Sahu, S.; Wang, P.; Wang, X.; Sun, Y. Carbon dots of different composition and surface functionalization: cytotoxicity issues relevant to fluorescence cell imaging. *Exp. Biol. Med.* **2011**, *236*, 1231–1238.

33 Ray, S. C.; Saha, A.; Jana, N. R.; Sarkar, R. Fluorescent Carbon Nanoparticles: Synthesis, Characterization, and Bioimaging Application. *J. Phys. Chem. C* **2009**, *113*, 18546–18551.

34 Cao, L.; Wang, X.; Meziani, M.; Lu, F.; Wang, H.; Luo, P.; Lin, Y.; Harruff, B. A.; Veca, L. M.; Murray, D.; Xie, S.; Sun, Y. Carbon Dots for Multiphoton Bioimaging. *J. Am. Chem. Soc.* **2007**, *129*, 11318–11319.

35 Dong, Y.; Wang, R.; Li, G.; Chen, C.; Chi, Y.; Chen, G. Polyamine-functionalized carbon quantum dots as fluorescent probes for selective and sensitive detection of copper ions. *Anal. Chem.* **2012**, *84*, 6220–6224.

36 Wang, Y.; Bao, L.; Liu, Z.; Pang, D. Aptamer biosensor based on fluorescence resonance energy transfer from upconverting phosphors to carbon nanoparticles for thrombin detection in human plasma. *Anal. Chem.* **2011**, *83*, 8130–8137.

37 Gonçalves, H.; Jorge, P. A. S.; Fernandes, J. R. A.; Esteves da Silva, J. C.G. Hg(II) sensing based on functionalized carbon dots obtained by direct laser ablation. *Sens. Actuators, B* **2010**, *145*, 702–707.

38 Qu, Q.; Zhu, A.; Shao, X.; Shi, G.; Tian, Y. Development of a carbon quantum dots-based fluorescent Cu^{2+} probe suitable for living cell imaging. *Chem. Commun.* **2012**, *48*, 5473–5475.

39 Kong, B.; Zhu, A.; Ding, C.; Zhao, X.; Li, B.; Tian, Y. Carbon dot-based inorganic-organic nanosystem for two-photon imaging and biosensing of pH variation in living cells and tissues. *Adv. Mater.* **2012**, *24*, 5844–5848.

40 Liu, J.; Lin, L.; Wang, X.; Lin, S.; Cai, W.; Zhang, L.; Zheng, Z. Highly selective and sensitive detection of Cu^{2+} with lysine enhancing bovine serum albumin modified-carbon dots fluorescent probe. *Analyst* **2012**, *137*, 2637–2642.

41 Yu, C.; Li, X.; Zeng, F.; Zheng, F.; Wu, S. Carbon-dot-based ratiometric fluorescent sensor for detecting hydrogen sulfide in aqueous media and inside live cells. *Chem. Commun.* **2013**, *49*, 403–405.

42 Shi, W.; Li, X.; Ma, H. A tunable ratiometric pH sensor Based on Carbon Nanodots for the Quantitative Measurement of the Intracellular pH of Whole Cells. *Angew. Chem., Int. Ed.* **2012**, *51*, 6432–6435.

43 Zhao, H.; Liu, L.; Liu, Z.; Wang, Y.; Zhao, X.; Huang, C. Highly selective detection of phosphate in very complicated matrixes with an off-on fluorescent probe of europium-adjusted carbon dots. *Chem. Commun.* **2011**, *47*, 2604–2606.

44 Li, H.; Zhang, Y.; Wang, L.; Tian, J.; Sun, X. Nucleic acid detection using carbon nanoparticles as a fluorescent sensing platform. *Chem. Commun.* **2011**, *47*, 961–963.

45 Li, H.; Zhai, J.; Tian, J.; Luo, Y.; Sun, X. Carbon nanoparticle for highly sensitive and selective fluorescent detection of mercury(II) ion in aqueous solution. *Biosens. Bioelectron.* **2011**, *26*, 4656–4660.

46 Li, H.; Zhai, J.; Sun, X. Sensitive and selective detection of silver(I) ion in aqueous solution using carbon nanoparticles as a cheap, effective fluorescent sensing platform. *Langmuir* **2011**, *27*, 4305–4308.

47 Liu, J.; Li, J.; Jiang, Y.; Yang, S.; Tan, W.; Yang, R. Combination of π-π stacking and electrostatic repulsion between carboxylic carbon nanoparticles and fluorescent oligonucleotides for rapid and sensitive detection of thrombin. *Chem. Commun.* **2011**, *47*, 11321–11323.

48 Wei, W.; Xu, C.; Ren, J.; Xu, B.; Qu, X. Sensing metal ions with ion selectivity of a crown ether and fluorescence resonance energy transfer between carbon dots and graphene. *Chem. Commun.* **2012**, *48*, 1284–1286.

49 Mao, Y.; Bao, Y.; Han, D.; Li, F.; Niu, L. Efficient one-pot synthesis of molecularly imprinted silica nanospheres embedded carbon dots for fluorescent dopamine optosensing. *Biosen. Bioelectron.* **2012**, *38*, 55–60.

50 Gonçalves, H.; Duarte, A. J.; Esteves da Silva, J. C. G. Optical fiber sensor for Hg (II) based on carbon dots. *Biosen. Bioelectron.* **2010**, *26*, 1302–1306.

51 Li, Q.; Ohulchanskyy, T. Y.; Liu, R.; Koynov, K.; Wu, D.; Best, A.; Kumar, R.; Bonoiu, A.; Prasad, P. N. Photoluminescent Carbon Dots as Biocompatible Nanoprobes for Targeting Cancer Cells in Vitro. *J. Phys. Chem. C* **2010**, *114*, 12062–12068.

52 Zhu, A.; Qu, Q.; Shao, X.; Kong, B.; Tian, Y. Carbon-dot-based dual-emission nanohybrid produces a ratiometric fluorescent sensor for in vivo imaging of cellular copper ions. *Angew. Chem., Int. Ed.* **2012**, *51*, 7185–7189.