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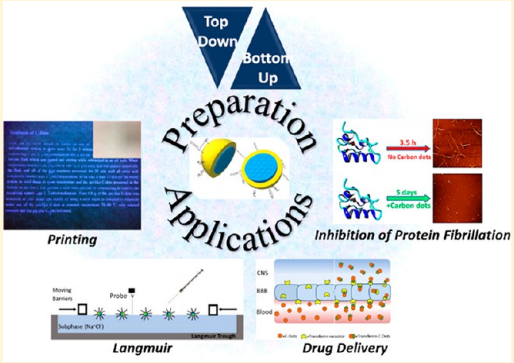
Carbon Dots: Diverse Preparation, Application, and Perspective in Surface Chemistry

Yiqun Zhou, Keenan J. Mintz, Shiv K. Sharma, and Roger M. Leblanc*

Department of Chemistry, University of Miami, Coral Gables, Florida 33146, United States

ABSTRACT: Carbon dots (CDs) are a novel class of nanoparticles with excellent properties. The development of CDs involves versatile synthesis, characterization, and various applications. However, the importance of surface chemistry of CDs, especially in applications, is often underestimated. In fact, the study of the surface chemistry of CDs is of great significance in the explanation of the unique properties of CDs as well as the pursuit of potential applications. In this feature article, we do not only introduce the development of CDs in our group but also highlight their applications where surface chemistry plays a critical role.



INTRODUCTION

The significance of surface chemistry has been increasing tremendously. Understanding the surface properties of nanoparticles (NPs) helps to learn the interactions between NPs and biological systems[1] because the surface chemistry studies are aimed at correlating the properties of nanomaterials such as size, chemical functionality, surface charge, and composition with biomolecular signaling, biological kinetics, transportation, and toxicity in both cell culture and animal experiments. In addition, surface chemistry study of NPs is eminently required to create a range of molecular architectures and functionalities at the surface. At the same time, tools developed for investigating surfaces and interfaces can be employed to grasp the complexities of molecular behavior at interfaces from a different range scale of NPs.[2] The type of NPs we obtain is determined by its source whereas surface chemistry controls its surface properties and enables the required functionalization for vast applications.[3,4]

Among all types of NPs, investigation of carbon dots (CDs) are a promising area which has gained much interest over the last 10 years or so. The discovery of CDs could be traced back to a report by a Clemson research group in 2000 on the observation of green photoluminescent emissions from functionalized carbon nanotubes.[5] There were many suspicions about the origin of the observed emissions, with a primary suspect being "carbonaceous impurities" including carbon nanoparticles, for which Haddon's research group did some nice studies. Consequently, carbonaceous nanoparticles in carbon nanotube samples for functionalization became a major target in the Clemson research group, primarily for the "battle" with Haddon's research group. And it was not until 2006 that the name "carbon dots" first showed up in a paper published by Sun et al.[6] Since then, CDs have received a great deal of research attention because of their favorable properties that include low toxicity, excellent optical properties, and easily functionalized surfaces.[7,8] The optical properties are of particular interest because of their tendency to exhibit an excitation-dependent emission of photoluminescence (PL) and relatively high fluorescence quantum yields (QY).[9,10] The excitation-dependent emission provides a versatile probe and the ability to use CDs in diverse applications. These properties, particularly the excitation-dependent emission, are frequently studied in order to improve the understanding of CDs.[11]

CDs are prepared through two main pathways: top-down and bottom-up.[12] The top-down approach involves bulk carbon precursors such as ash, soot, and graphene. These bulk materials are cut down to NPs through a variety of methods (e.g., electrochemistry and chemical oxidation). Bottom-up approaches involve small organic molecules as precursors. The commonly accepted mechanism involves the ionization and polymerization of the precursors, which is followed by carbonization. Varieties of methods used for the bottom-up approach include hydrothermal/solvothermal, microwave, and ultrasonication.[13] CDs prepared by either approach are characterized in the same pattern. The main areas of characterization for CDs are based on their optical properties, surface functionality, and morphology. These characterizations are accomplished through common techniques such as UV/vis, fluorescence, Fourier transform infrared (FTIR), and X-ray photoelectron spectroscopies (XPS), atomic force (AFM) and transmission electron microscopies (TEM), and zeta potential measurements.[14] Thorough characterization is important for assessing the capabilities of CDs in their potential applications.

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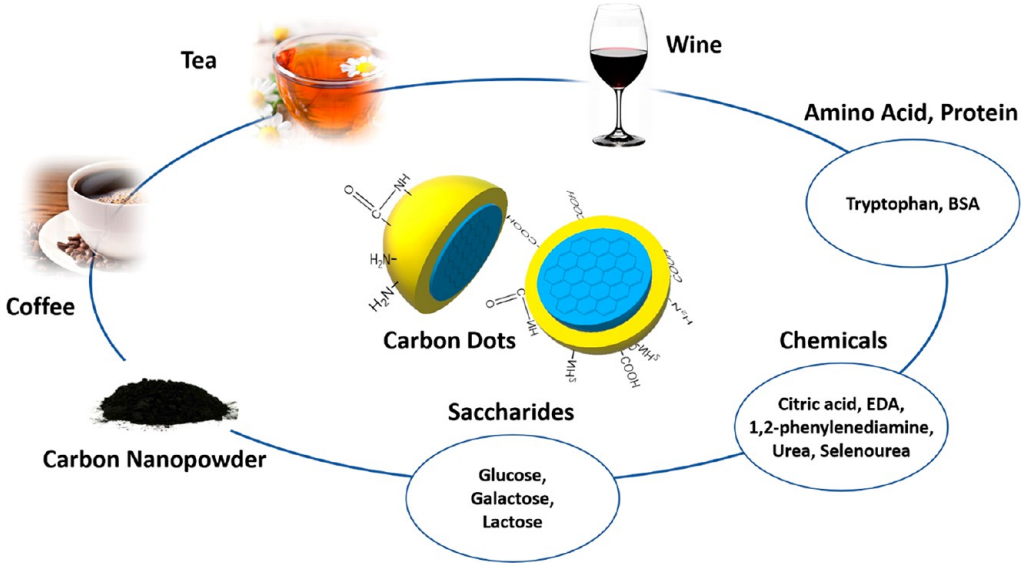
Scheme 1. Variety of Precursors of CDs Synthesis in Our Group



The application of CDs stretches across a variety of fields. They have been commonly used in bioimaging, sensing, and light-emitting diodes (LEDs).[15−17] They have also been used in energy and catalysis applications.[18,19] Additionally, because of their easily functionalized surface, they have shown capability in drug delivery.[20,21] However, despite numerous potential applications, surface chemistry is a key factor that determines the applicability of various experimental assumptions and promotes the development of CDs. In our study, the surface chemistry indicates not only the interfaces between two different phases but also the biological membrane or the surface of CDs. For example, the blood−brain barrier (BBB) is an important membrane barrier. Molecules are well selected to cross the BBB. The investigation of such molecules, including whether they can cross the BBB and its mechanism, can also be categorized into surface chemistry, and also it is meaningful to apply the mechanism to design the optimal CD-based drug delivery system (DDS) to overcome the BBB. In addition, the study of hair colorant using CDs will be another good example to show the interaction between CDs and hair proteins because not all CDs showed the potential as permanent hair colorant. Furthermore, the conjugation of drugs with CDs by forming a covalent bond or a simple electrostatic interaction can also be categorized into surface chemistry.

Herein, in this feature article, we summarize the study of the Langmuir surface technique. Also, we used CDs as a model to shed light on the importance of surface chemistry in the applications of CDs including printing, cosmetics, drug conjugation, and drug delivery across the BBB. Moreover, an interesting application is that CDs can specifically target the bones of zebrafish. Even though the mechanism might be related to the bone surface or microenvironment, it remains to be elucidated. Furthermore, as an important parameter of CDs, PL is favorably explained from surface state theory. However, the mechanism of PL is still under debate among surface state, core state, molecular state, and quantum confinement theories. Therefore, there are many studies related to the surface chemistry of CDs. Clearly, the surface chemistry of CDs is of great significance, which has never been systematically studied before. A deeper understanding of the surface chemistry can help not only in studying the molecules' behaviors at the water−air interfaces but also in designing the appropriate CDs according to the specific requirement for any application.

CARBON DOTS

Synthesis of Carbon Dots. *Synthesis Approaches.* There are many methods used to prepare CDs, making it an important consideration along with the choice of precursor. Hydrothermal methods are popular because of high availability and "green" nature of water. These methods will commonly heat up to between 160 and 200 °C in a sealed autoclave or with a reflux condenser.[14] The resulting CDs are attractive because the method ensures that they will have high water dispersibility and oxygen-containing functionalities. Generally, the mechanism that is proposed for this process is the ionization of the precursor, followed by polymerization and then carbonization into CDs.[22,23] Hydrothermal methods are almost exclusively used in bottom-up approaches with small molecules as precursors. An important exception to this is the synthesis of black CDs, which uses carbon nanopowder and is refluxed in concentrated sulfuric and nitric acids.[24]

Microwave preparations are popular because of their rapid reaction. Most CDs formed from a microwave-mediated method require less than 10 min.[25,26] This enables researchers to try more reactions as well as optimize existing preparations with ease. There is not a notable difference between the properties of CDs obtained from hydrothermal and microwave methods because both typically produce CDs with blue emission and high QY. This indicates that precursors are a more important factor in the preparation of CDs. In our laboratory, a microwave-mediated preparation was used to prepare carbon nitride quantum dots (CNQDs) and CDs from citric acid and *p*-phenylenediamine. Both of them displayed high QY (ca. 50%), but this is believed to be due to the nitrogen doping from the precursors as opposed to the microwave method.[27]

Ultrasonication is also one technique reportedly used for the synthesis of CDs aided by ultrasonic energy with an inexpensive apparatus. Kang and co-workers reported a N-doped CD synthesis via ultrasonication with glucose and aqueous ammonia as precursors.[28] They speculated that the synthesis driven by ultrasonic energy is due to the generation

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Table 1. Summary of CDs Synthesized in Our Group Together with Their Synthesis Approaches, Starting Materials, and Reaction Conditions

CD species	synthesis approaches	starting materials	reaction conditions
black CDs	top-down hydrothermal	raw carbon nanopowder	H_2SO_4/HNO_3, 110 °C for 15 h
gel-like CDs	bottom-up solvothermal	1:14 citric acid/EDA molar ratio	temperature, 160 °C; time, 50 min; protective gas, argon
orange CDs	bottom-up ultrasonication	1:25 citric acid/OPD molar ratio	solvent, water; sonication frequency, 42 kHz; time, 1 h; energy input, 252 kJ
saccharide-based CDs	bottom-up hydrothermal	saccharides (glucose, galactose, and lactose)	temperature, 200 °C; time, 5 h
tryptophan CDs	bottom-up hydrothermal	tryptophan and EDA or urea	temperature, 180 °C; time, 4 h; protective gas, argon
BSA-based CDs	bottom-up hydrothermal	bovine serum albumin, Evans blue	temperature, 160 °C; time, 4 h
tea, coffee, and wine CDs	bottom-up microwave	tea, coffee, wine	power, 700 W; time, 7 min
carbon nitride dots	bottom-up microwave or hydrothermal	citric acid and urea, thiourea, selenourea, or formamide	power, 700 W; time, 7 min; or temperature, 180 °C; time, 4.5 h; protective gas, argon

and violent collapse of small vacuum bubbles, which is caused by alternating high and low pressures in the reacting solutions resulting from ultrasonication. The cavitation led to high-speed impinging liquid jets, deagglomeration, and strong hydrodynamic shear forces, which could further carbonize the intermediate initially generated by the dehydration of glucose and ammonia mediated by ultrasonication. In addition, with the help of an ultrasonication bath, CDs have been successfully synthesized with various starting materials including a polyamide resin,[29] sucrose,[30] and citric acid.[31] Also, it was applied to the fabrication of a CDs/BiOBr composite to enhance the visible-light photocatalysis,[32] which exhibits a broader application of the method.

There are several other methods used to form CDs, especially for top-down approaches. These include arc discharge, electrochemical oxidation/deposition, and laser ablation. These methods almost exclusively utilize top-down approaches because they can apply large and intense energies that are needed to break down larger carbon structures into CDs.[33] The QY of these CDs is often not competitive with bottom-up approaches; however, the range of emission wavelengths is usually wider.[6,34]

Carbon Dots Synthesized in Our Group. In our group, on the basis of the synthesis techniques mentioned above, around 12 types of CDs have been synthesized using either top-down and bottom-up approaches. The starting materials varied from daily diet such as ham, cheese, tea, coffee, and wine to laboratory synthetic chemicals including citric acid, 1,2-ethylenediamine (EDA), *o*-phenylenediamine (OPD), urea, and saccharides (Scheme 1). For example, black CDs were prepared from raw carbon nanopowder via a hydrothermal route being oxidized by sulfuric and nitric acids overnight. It was a typical top-down method used to modify the surface of raw carbon nanopowder by converting C═C to carboxylic groups. As a result, by quantitative titration with a NaOH aqueous solution, we calculated the content of carboxylic groups on the surface of CDs (5.8 mmol/g).[35] In contrast, gel-like CDs were prepared from citric acid and EDA with a solvothermal method. However, because of the presence of EDA, gel-like CDs contain $-NH_2$, which made the quantitation of −COOH harder. However, a fluorescence study using fluorescamine to determine the $-NH_2$ could accurately analyze the content of $-NH_2$ on the surface of gel-like CDs (0.105 mmol/mg) without any interference of −COOH.[36] Most obtained CDs exhibit short-wavelength emission, but the desire to broaden the application and extend the emission wavelength to red, which will benefit bioimaging in vivo, stimulated the development of various CDs in our group. Table 1, describing the syntheses of all types of CDs, is illustrated below.

Purification of Carbon Dots. *Dialysis.* Dialysis is a common and effective purification technique for CDs according to their size or molecular weight. However, in many studies related to CD synthesis, the purification procedure was either ignored or performed without considering the particle size.[37] Therefore, the obtained CDs may still be mixed with starting materials and other reagents if the purification process was not complete. In addition, if the particle size or the molecular weight is rather small, then most CDs can pass the dialysis membrane and disperse into water. In our group, before dialysis, black CDs were previously measured with mass spectroscopy using the ESI technique, and the molecular weight of black CDs was observed to be higher than 860 Da. Thereafter, black CDs were purified with dialysis tubing with a high molecular weight cut off (MWCO).

Size-Exclusion Chromatography. Size-exclusion chromatography (SEC) is another useful purification and separation method based on the particle size and in some cases the molecular weight. In general, smaller molecules are likely to be trapped in the pores of stationary phase while larger molecules cannot enter those pores. Instead, larger molecules will bypass the pores to flow through the SEC column faster than do the smaller molecules. Because of the formation mechanism of CDs using the bottom-up approach, the particle size will increase from the starting materials to the resulting CDs. Therefore, after synthesis and rough purification to remove the large clusters, CDs will be the first separate eluent from the column. In our group, many CDs have been purified through the column such as the gel-like CDs and orange CDs that originated from citric acid and OPD via ultrasonication. Another important function of SEC is the separation of the mixture of compounds based on different sizes because we know CDs are not homogeneous. The SEC technique has been applied by Messina and co-workers to separate CDs prepared using citric acid monohydrate and urea as the precursor exposed to microwave irradiation.[38] From the collection of each eluent, four fractions with different morphological and optical properties were observed. With the help of SEC, we obtained only one separate fraction from the orange CDs prepared with the ultrasonication method,[39] which demonstrated the relatively uniform size of such CDs. However, when we applied SEC to separate the CDs from the same starting materials via microwave, there were three separate fractions acquired with different PL colors shown in Figure 1.[40] As

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confirmed by AFM and TEM, we found that the size of three fractions matches the eluent order from the SEC column (5, 3, and 2 nm for fractions 1, 2, and 3, respectively).

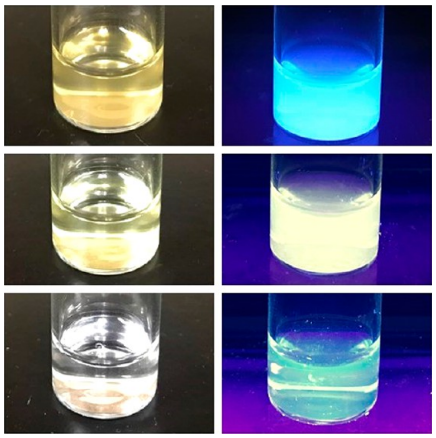

Figure 1. Aqueous dispersions of three CDs fractions with a concentration of 0.1 mg/mL (top-down: fractions 1, 2, and 3) after purification by SEC. (The left column is under regular light; the right column is under UV light at 365 nm.) (Reproduced with permission from ref 40. Copyright 2019 Elsevier.)

Thin Layer Chromatography. Thin layer chromatography (TLC) is a chromatography technique used to purify and separate a nonvolatile mixture. According to the polarity of mixture and developing solvents, TLC can be categorized as normal-phase and reversed-phase TLC. For normal-phase TLC, the stationary phase is usually a thin layer of absorbent material such as silica gel and alumina,, and the mobile phase often indicates various organic solvents. However, for reversed-phase TLC, the stationary phase involves C_8 or C_{18} silica gel and the selection of mobile phase can be expanded to polar solvents even including water and electrolytes. Therefore, TLC can be used to separate compounds with versatile polarities, and the separation with TLC is convenient to operate and efficient in obtaining individual fractions. Nonetheless, the importance of TLC in the separation is gradually overlooked with the development of new chromatography techniques such as high-performance liquid chromatography (HPLC) and solid-phase extraction. And even though the new separation techniques bring advantages such as high speed, high loading amounts, and less manual operation, they are also not easy to access.

Therefore, TLC was employed in our research to separate gel-like CDs to study the uniformity of composition. A reverse-phase TLC was used with water/acetonitrile (v_w/v_a 3/7) as the developing solvent. Thereafter, four separate fractions were obtained with different PL colors. Characterized by various techniques, we observed that the PL behaviors of two fractions were different from that of the original gel-like CDs. To be specific, the fluorescence spectrum of gel-like CDs revealed an excitation-dependent PL while there were two separate fractions showing excitation-independent PL. Their comparison is shown in Figure 2. The PL mechanism is usually a hot topic of debate between surface states and the quantum size effect. However, from another point of view, whether CDs are uniform NPs with various surface states or contain heterogeneous fractions with each individual PL behavior might yield another answer to the typical excitation-dependent PL.[36] Therefore, the greatest significance of this study via the separation of gel-like CDs is the straightforward illustration of various inner compositions, which provides another explanation of the PL mechanism. In addition, it is the first and so far the only report of the separation of CDs using TLC.

Electrophoresis. Electrophoresis can provide separation based on size and charge, and CDs usually possess some distribution for both of these properties. Electrophoresis could be used to separate different CDs fractions, as TLC has been used in our laboratory, or it could be used to separate CDs from their precursors, as has been done for tryptophan CDs (Trp-CDs) in our group and for other CDs elsewhere.[41,42] Because it is more tedious than dialysis or SEC, it is commonly used as a last resort after other purification methods have proven ineffective. Electrophoresis does provide the unique advantage of potentially being able to modify the properties of precursor/surface of CDs through changing the pH of buffer. This can allow wide versatility in the separation efficiency of CDs through electrophoresis.

Characterization of Carbon Dots. *UV/Vis.* CDs are frequently characterized by their optical properties. These include UV/vis absorption and fluorescence spectroscopies.

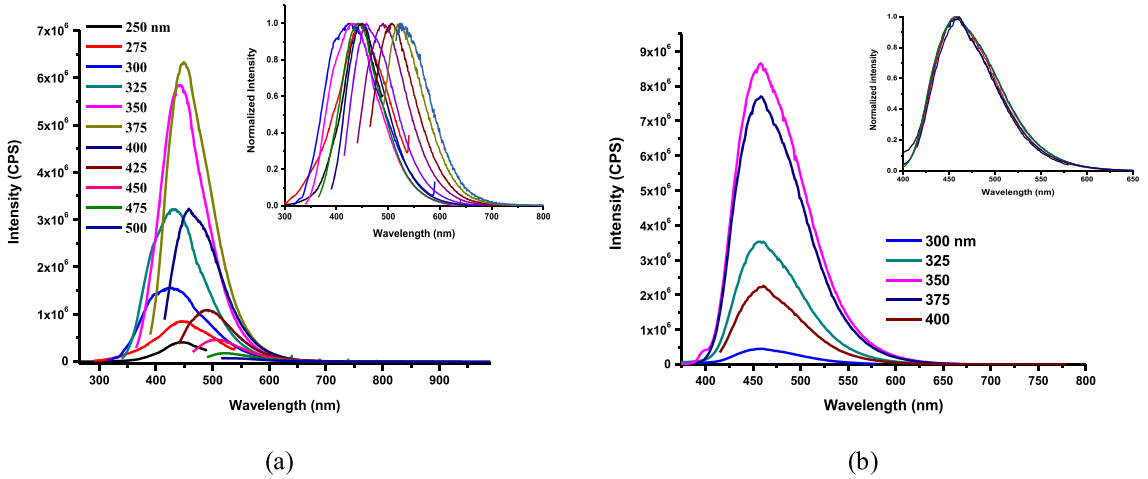

Figure 2. Fluorescence spectra of (a) gel-like CDs and (b) their separate fraction (fraction 2). (Reproduced with permission from ref 36. Copyright 2018 John Wiley and Sons.)

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The absorption spectra of CDs usually have two common features; these are the $\pi-\pi^*$ transitions of the C═C bond (ca. 250 nm) and the $n-\pi^*$ transition of the C═O bond (ca. 350 nm).[43] These peaks are common for a large majority of CDs. There are some other peaks of interest from the UV/vis absorption for CDs. Orange CDs formed from sonication display a peak in the "low-energy" region at around 400 nm which can be attributed to the NO_2 absorption cross section.[39] From UV/vis, we can also see the evidence of precursor or precursor fragment on the surface of CDs. This is the case for Trp-CDs, where the absorption band of tryptophan (280 nm) can be clearly seen in the Trp-CDs after purification through electrophoresis. A similar observation was made for BSA-CDs because the product possesses long-wavelength absorption (550 nm) which is unusual for CDs and can be attributed to a fragment of the precursor dye, Evans blue, on the surface of BSA-CDs.[44]

The absorption properties of CDs are unique and the two bands at around 250 and 350 nm are characteristic, making this technique a fast indication of the presence of CDs. As an example, the UV/vis absorption spectrum of gel-like CDs is shown in Figure 3. The data obtained from UV/vis is analyzed in conjunction with the data from fluorescence spectroscopy, which we will discuss next.

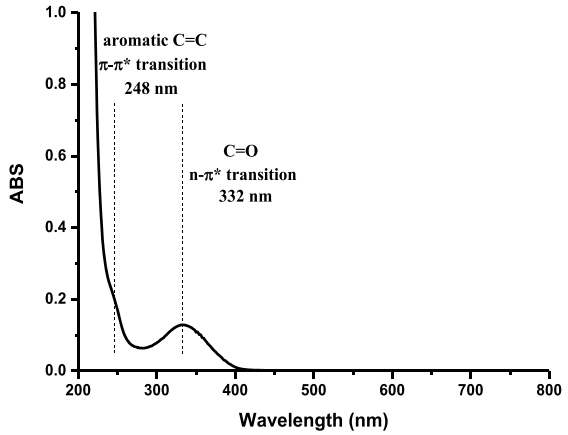

Figure 3. UV/vis absorption spectrum of a gel-like CD aqueous dispersion. (Reproduced with permission from ref 36. Copyright 2018 John Wiley and Sons.)

Fluorescence. The PL properties of CDs are perhaps their most characteristic property. The mechanism by which CDs emit light is unclear (current theories will be discussed later), and because this is the case, the emission of light from CDs is referred to by the generic term, PL. CDs' PL commonly possesses the unique trait of excitation-dependent emission.[6] This is different from other luminescent species, so it has attracted a great deal of interest. In recent years, excitation-independent CDs have also been formed, such as orange CDs.[39] The possible causes of these intriguing properties will be discussed in detail later. The color of PL emission of CDs is also of interest. CDs most frequently possess blue or green emission (400−500 nm).[14,45] The QY for CDs of this color have become quite high in recent years (above 50%).[11] As the color of CD emission has increased to yellow and orange (up to 600 nm), the QY has generally decreased. A need remains to increase QY in this range for applications such as bioimaging. Furthermore, the longest wavelengths of emission commonly obtained for CDs are around 625 nm.[46,47] This is at the beginning of the red color in the spectrum of light but can be improved by shifting to or past 700 nm.

FTIR. The surface of CDs is commonly characterized using FTIR and XPS. The surface functionalization of CDs usually involves oxygen- and nitrogen-containing functional groups such as carboxylic acids, alcohols, and amines.[48] The surface characterization of CDs is important in determining their potential applications, particularly for drug delivery. However, it appears that FTIR probes mostly the surface of CDs, and it is hard to discriminate the origin of the peaks. FTIR provides a qualitative picture of the surface of CDs, but for a more quantitative description of the surface of CDs, XPS is used.

XPS. XPS is a valuable tool for the investigation of CDs. XPS data should always be correlated with FTIR to validate both methods. It can also provide an atomic ratio of the surface of CDs. In addition, it can show the particular functional groups present for an atom based on the characteristic binding energies for the different functional groups. XPS has been used for our CDs systems to examine the surface functionalities of the particles. For black CDs, XPS data showed the surface consist of 54.6% carbon and 43.8% oxygen. This showed that nitric acid does not contribute N to the surface of CDs through nitrogen doping. Furthermore, it was shown that 23% of the O 1s signal originated from −COOH groups.[49] For gel-like CDs, XPS was used to examine the effect of reaction temperature on the preparation of CDs.[43] This allowed the atomic content to be compared with other characteristics such as the optical properties. The elemental information XPS provides makes it a valuable tool, but it is also limited by its analysis depth. XPS has been shown to provide data to within 100 Å of a material's surface,[50] whereas FTIR can reach a depth of 2 μm.[51] Thus, XPS is not able to provide core structure information for CDs. However, XPS is a powerful and important tool for the characterization of CDs, especially to determine the application potential of CDs and to be able to modify their surface.

AFM/TEM. AFM and TEM are two common techniques used to characterize the size of CDs. It is necessary to use them in conjunction with each other because TEM supplies excellent *XY*-plane information, and because CDs are so small, AFM is able to provide only the height of CDs along the *z* axis. The combination of both techniques can provide 3D information and enable us to learn that CDs are spherical. CDs' topographical features have minor variations based on the preparation method. We have found that our CDs vary in size from 1 to 8 nm with some size distribution for each system.[24,44] Smaller CDs are of interest for their increased likelihood to cross barriers such as cell membranes and the BBB. AFM and TEM can also be used to study interesting features such as the aggregation of the particles and the formation of monolayers as we have observed for CDs made from lactose.

Zeta Potential. Another characterization technique for CDs is the zeta potential. This measurement is important to know the colloidal stability of the particles in solution. A large absolute value (>20 mV) indicates that there will be interparticle repulsion leading to well-dispersed colloidal solutions. When the zeta potential is closer to zero, the particles are much more likely to experience agglomeration in solution. The black CDs have exhibited the largest zeta potential value (−38 mV) as a result of the abundance of carboxylic functionality.[49] Other CDs such as gel-like CDs and

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BSA-CDs are much closer to zero (−13 and −8 mV, respectively) because of the presence of both amine and carboxylic groups.[43,44] This technique provides important information of the surface electronic environment of CDs.

Photoluminescence. *Quantum Yield.* CDs' optical properties are important for many applications, and they have been extensively studied in order to understand and improve. We have previously discussed the importance of the emission wavelength for applications such as bioimaging. QY is also an important parameter in optimizing CDs. It has been frequently reported that heteroatom doping (e.g., N, B, or P) is a useful method to increase the CDs' QY.[3] CDs doped with EDA (gel-like CDs: 33%) and their separate fraction (55%) have achieved the highest QY in our group.[36,43] The QY for CDs with blue emission has been reported to be up to 90%.[52] Although there may be some warranted skepticism regarding these values, there is little left to improve for the QY of blue- or green-emitting CDs. On the basis of this, we have turned our attention to increasing the QY of CDs for the long-wavelength emission of light.

Photoluminescence Mechanism. The PL of CDs remains one of the least understood aspects of CDs. CDs commonly display excitation-dependent emission, which is a novel characteristic. However, in recent years, excitation-independent emission has been reported for CDs. In our laboratory there are examples such as orange CDs and the fractions of TLC-separated gel-like CDs.[36,39] There is some debate and disagreement as to what leads to the excitation dependence. The three main ideas currently proposed include quantum confinement effect, molecular states, and surface states. The theory of quantum confinement effect in CDs was investigated because metal-based quantum dots (QDs) are well known to possess PL based on this mechanism.[53] There are multiple papers published using this explanation for the PL mechanism of CDs, but it is not very common because many CD systems simply do not possess the data to support this theory.[11] Another common theory proposes the synthesis of different molecular fragments which are attached to the surface of CDs in the synthesis process.[54] This explanation is called the molecular state. There are many papers with evidence to support this theory, but the scope of this explanation is limited to only some preparations. Several CD preparations using citric acid have used this explanation for the PL mechanism.[55,56] Additionally, there have been some studies which used a fluorescent precursor in the synthesis method to explain their CDs' PL using the molecular state.[57] These claimed that the precursor/precursor fragment is attached to the surface of CDs. Although there is strong evidence for this explanation in some CDs systems, it is certainly limited to the precursor and does not completely explain the excitation-dependent emission that CDs usually possess. The third and most common theory used is surface-state-controlled PL.[7] The frequent use of this explanation has caused the general acceptance of this theory for the PL mechanism of CDs.[3] However, when surface-state-controlled PL is offered as an explanation for the PL of CDs, it is not stated if the concept can be directly applied to CDs (which commonly do possess band gaps) or if adjustments are necessary. Recently in our group we found that our gel-like CDs can be separated into four different photoluminescent fractions, each possessing unique PL properties. Two possess an excitation dependence, and two do not. On the basis of XPS and polarity information from TLC, it was concluded that the surface functionalization played an important role in controlling the PL behavior.[36] More work is needed in this area to completely elucidate the mechanism by which CDs emit light.

Unique Properties of Carbon Dots. *Temperature Effect.* Many research groups have found that the properties of CDs, including the fluorescence QY and particle size, can be tuned by different reaction temperatures considering the carbonization of CDs.[58] In our group, four different temperatures (120, 140, 160, and 180 °C) were applied to the same starting materials (citric acid/EDA 1:14 molar ratio) to synthesize gel-like CDs.[43] Various characterization methods revealed gradual increasing or decreasing patterns of different properties of these CDs. For example, with the temperature increasing from 120 to 160 °C, the QY increased by up to 33%. However, because of the increase in the degree of carbonization, the QY decreased when temperature continued to increase from 160 to 180 °C. In addition, the particle size decreased upon the increase in temperature from 120 to 180 °C, which was confirmed by both AFM and TEM measurements, and the reason was simply stated as being the longer carbonization of CDs synthesized at higher temperature. From XPS, we observed a decrease in the content of oxygen with the reaction temperature increasing, which revealed that increased temperature facilitated the desorption of oxygen-containing carbon moieties (i.e., O−H and O−C═O groups). Furthermore, temperature also has a certain effect on the synthesized CDs. For example, because of the presence of a gel network, gel-like CDs shrunk at cold temperature but expanded at higher temperature.

Solvent Effect. Another interesting effect found in some CDs is a solvent effect, which means that CDs in different solvents will exhibit different PL behaviors, including the shift of PL peaks or excitation-dependent or excitation-independent PL. One typical example is orange CDs synthesized by our group.[39] The as-obtained orange CDs were then dispersed in different common solvents including water, methanol, acetone, and tetrahydrofuran (THF) in decreasing order of the polarity index, and we found that there was a blue shift in the PL color (Figure 4). Additionally, we observed that the PL intensity and QY increased with the polarity index decreasing, which was hypothesized by the higher dispersity of orange CDs in organic solvents due to the hydrophobic functional groups on the surface such as C═C and C═N. Therefore, orange CDs can

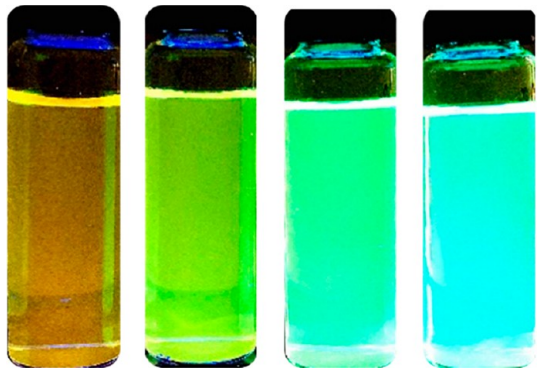

Figure 4. Solvent effect of orange CDs under the irradiation of UV light (365 nm). From left to right, CDs were dispersed in water, methanol, acetone, and THF. (Reproduced with permission from ref 39. Copyright 2018 MDPI.)

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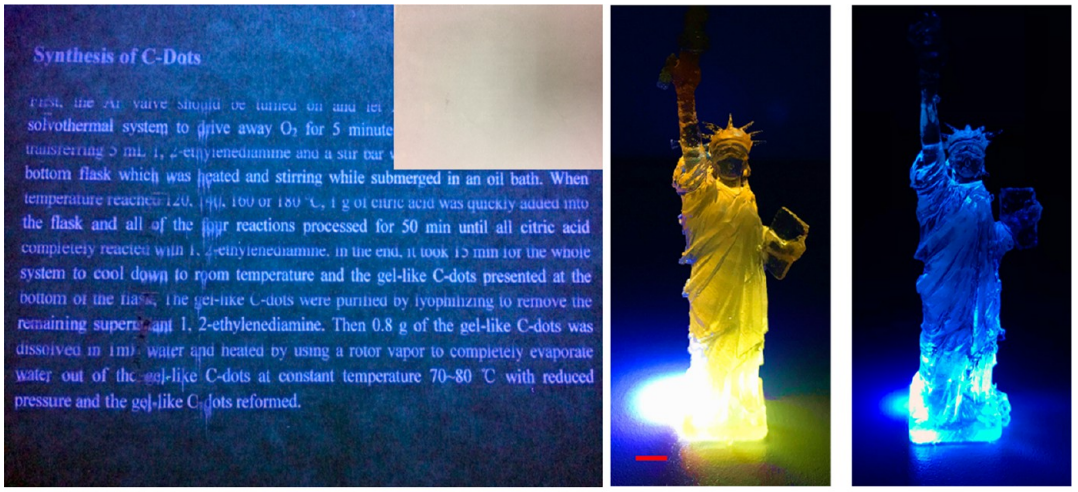

(a) (b)

Figure 5. (a) Text printing irradiated under UV light (365 nm), with irradiation under white light shown in the inset in the upper right corner. (Reproduced with permission from ref 43. Copyright 2017 John Wiley and Sons.) (b) Three-dimensional printing of the Statue of Liberty with the CD-SAP conjugate (left) and the control without orange CDs (right). (Reproduced with permission from ref 39. Copyright 2018 MDPI.)

be considered to be one of the few reported hydrophobic CDs species.

WIDE APPLICATIONS OF CARBON DOTS

Inhibition of Protein or Peptide Fibrillation. The study of age-related neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease has highlighted amyloid deposits caused by ordered peptide or protein fibrillation as the novel target for therapeutic treatment. Therefore, the most effective preventative and therapeutic strategy for these diseases is to inhibit or delay the fibrillation process. Among various current treatments, increasing attention has been paid to the effect of NPs on protein fibrillation as a result of the tunable physical/chemical properties of NPs such as the size, components, and surface charge. A few examples of capped quantum dots (QDs) have shown an inhibition effect upon protein fibrillation, but the conjugation with ligands changes the size and surface properties of the QDs. Another major issue for QDs comes from their toxicity due to their heavy metal composition. As a green alternative of QDs, CDs have many characteristic advantages over traditional QDs including small size, high PL and water dispersity, ease of preparation and functionality, low toxicity, and good biocompatibility. For any practical application in biological systems, CDs will inevitably contact the peptides and proteins and may change their conformations. Therefore, the effect of CDs on the peptide or protein conformation and fibrillation is worthy of exploration.

In our group, both human insulin and amyloid-β peptide 42 (Aβ 42) were used as models for analyzing the inhibition effect of black CDs on the protein and peptide fibrillation. Fluorescence and circular dichroism spectroscopies and atomic force microscopy (AFM) were applied to monitor the kinetics of human insulin and Aβ 42 fibrillation. Fluorescence spectroscopy, using thioflavin T (THT) as a probe, indicated that CDs added could inhibit human insulin fibrillation. The greater the concentration of CDs, the longer the lag phase prior to the formation of insulin fibrils. However, it was speculated from the fluorescence spectra that CDs could interact with the Aβ monomer at a very early stage before the critical nucleation concentration was reached. Additionally, these circular dichroism spectra demonstrated that CDs had a significant inhibiting effect on human insulin fibrillation by prolonging the lag phase time. Also, it was seen that CDs could partially suppress the fibrillation of Aβ 40 and 42. Molecular dynamics simulations showed that the hydrophilic surface of CDs can help with Aβ fibrillation inhibition. AFM images of samples withdrawn at different incubation times revealed an inhibiting effect of CDs on fibrillation followed by a concentration-dependent pattern, which was also consistent with the results obtained from the kinetics of ThT fluorescence and circular dichroism spectra. Thus, the CDs presented in this work display excellent potential for the treatment of Alzheimer's disease.

Printing. *Text Printing.* Since the discovery of CDs, their high PL has attracted much attention. The application of high PL can be of great help in daily life. For example, CDs have been applied in text printing via plenty of techniques including handwriting, inkjet printing,[59] relief printing,[60] screen printing,[61] intaglio printing,[60] and microtrace transferring.[60] Also, because most CDs are excitation-wavelength-dependent and highly photoluminescent, CDs were usually used for multicolor printing.[62] Besides being used as ink, CDs could also be applied in making a thin film to present the effect of printing. For example, CDs could be mixed with polymers such as poly(vinyl alcohol) (PVA) to form a transparent composite film as ink-free substrates for patterns, as reported by Chen and co-workers.[60] However, there are also many research groups that merely mentioned the potential use of the ink,[63] and in most research groups involved in the design of text printing using CDs, some details in printing or modifying the printer are either hidden or complicated.[64] Nonetheless, our group reported how we used the gel-like CDs to prepare the ink for text printing, and the printing was conducted in a commercial inkjet printer on nonfluorescent paper. After printing, the text can be seen only through UV light with a narrow wavelength of 365 nm (Figure 5a). On the contrary, in ambient light, the words did not show until the ink dried and oxidized for 3 years. Also, different from most CDs,[65] the gel-like CDs can be erased by water easily without leaving any trace, and the paper

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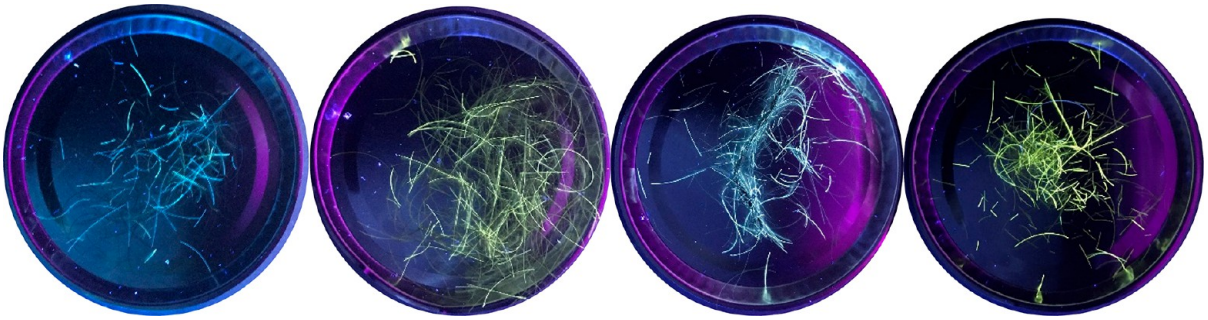

Figure 6. Various carbon dots as hair colorants on hair excited under 365 nm light. (Left to right) Hair alone, O-CD-doped hair, fraction 1 doped hair, and fraction 2 doped hair.

can be reused, which will greatly reduce the cost of text printing.

Three-Dimensional Printing. In addition to 2D text printing, 3D printing is a hot topic in the contemporary world. However, because of the limitations in 3D printing, ink materials are restricted. In our group, we discovered that CDs in aqueous solution could be absorbed into a superabsorbent polymer (SAP).[39] Then the SAP naturally lost water and could be ground into powder to be the feedstock for 3D printing. With the embedding of CDs into an SAP, CDs and the SAP have been used for the first time as the ink materials for 3D printing. Figure 5b shows the Statue of Liberty printed by the CDs-SAP conjugate (left) and the control without CDs (right).

Cosmetics. *Hair Colorant.* Hair is a protein filament that grows from follicles found in the dermis. On the surface of hair, the interaction between CDs and protein can contribute to the discovery of a permanent hair colorant made of CDs. The work was inspired by a study reported in 2018 when Huang and co-workers prepared graphene oxide (GO) and reduced graphene oxide (r-GO) mixed with chitosan at various loading levels as hair dyes.[66] In the study, they sprayed as-prepared dye solutions on hair and observed that the coating of both GO and r-GO could render new properties to the hair such as antistatic performance and heat dissipation. On the basis of known properties of GO and r-GO, these coatings may have even more functionalities including UV protection, antibacterial qualities, and odor absorption. After hair was dried, the coating of both compounds remained adhered to the hair and resistant to washing with shampoo because of the contribution of chitosan, which met the requirement for being a permanent hair colorant. In addition, the change in hair color in terms of shades and gradients was achieved by tuning the loading level of dopants. Without any doubt, the use of GO or r-GO as an alternative to traditional hair dyes reduces the organic solvents or toxic molecules in the ingredients, which protects hair from damage caused by undesired chemicals. However, the selection of hair color can be tuned only in a narrow range from dark yellow to black, and the hair-coloring process relied on chitosan, which is widely used to form hydrogels and might be able to inhibit the exhibition of many properties of GO and r-GO.

Therefore, considering many excellent properties including high water dispersity, tunable PL, abundant surface functional groups, and nontoxicity, we believed that CDs could be an even better alternative to conventional hair dyes. In our group, 12 CD species were synthesized using different approaches including hydrothermal, solvothermal, microwave mediation, and ultrasonication. The purification methods are composed of dialysis, a solvent wash, and SEC, and the starting materials covered laboratory-fabricated compounds to daily consumed products including wine, tea, coffee, and cheese. When the CDs were applied to human hair, after rinsing we found that only three types of CDs could make the hair "glow" under UV light (365 nm). The three types of CDs were orange CDs and two separate fractions of the CDs prepared with citric acid and OPD using a microwave method. In comparison, these three CDs species have higher hydrophobicity, which was confirmed by their low absolute zeta potential value. After drying hairs doped with the three CDs species with a commercial hair dryer, we still observed the fluorescence of hair of the same intensity by the naked eye as shown in Figure 6. When dry hair was washed with water or methanol, we did not see the elimination of fluorescence, which confirms the stability of CDs on hair and demonstrates that CDs can be used to make permanent photoluminescent hair colorants.

■ SURFACE CHEMISTRY OF CARBON DOTS

CDs are well known for their core−shell structure.[67] The surface of CDs is important to the explanation of the PL mechanism as well as to understanding many activities related to PL such as sensing and imaging.[67] Also, because there exist abundant hydrophilic moieties on the surface of most reported CDs, they typically have good water dispersion capability. However, to meet the requirement of applications such as hair coloring, the surface of CDs has to be hydrophobic. Thus, the surface of CDs needs to be engineered to meet different applications. In addition, CDs are promising drug nanocarriers because of their nontoxicity, good biocompatibility, and excellent PL. Drug delivery is usually realized through the conjugation between the functional groups on the surface of CDs and drug molecules via forming covalent bonds or via the electrostatic force, which usually depends on the moiety species on the surface of CDs. Furthermore, because of the presence of a specific drug or molecule, the application of CDs can be expanded to a certain extent to include drug delivery across the BBB.[21] Therefore, the study of the surface chemistry of CDs is fundamental and significant for further expanding the investigation of the characterization and application of CDs.

Langmuir Monolayer Technique for the Surface Study of Carbon Dots. When Irving Langmuir introduced the Langmuir technique, it soon gathered attention among many researchers. The Langmuir monolayer technique is essentially a molecular film at the air−water interface.[68] This technique is popular because it presents a unique methodology that helps to determine the chemical and physical behavior of a

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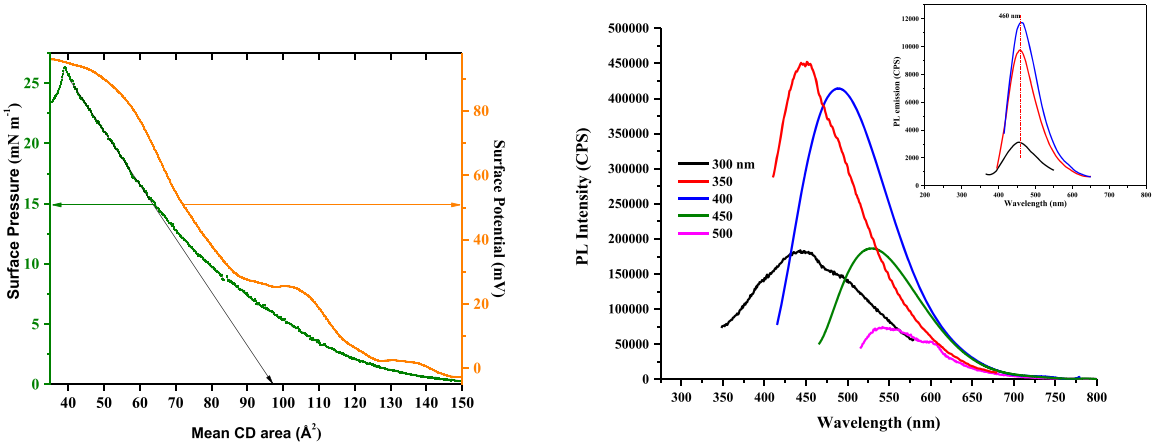

Figure 7. (a) Surface pressure−area (green) and surface potential−area (orange) isotherms of saccharide-based CDs (concentration 0.2 mg·mL^{-1}). (b) PL emission spectra of a CDs aqueous dispersion and Langmuir monolayer (inset) excited at different wavelengths. (Reproduced with permission from ref 78. Copyright 2019 ACS.)

monolayer of amphiphilic molecules at the phase boundary.[69] In recent years, the application of surface chemistry and spectroscopy of Langmuir monolayer and Langmuir−Blodgett (LB) film techniques has grown in diversity.[70,71] An LB film is a monolayer or multilayer transferred from the air−water interface to a solid substrate by vertical dipping.[70,72] It is also a useful tool in verifying the orientation of the molecules at the air−water interface. In addition, the Langmuir technique is a simple model which can help to establish a quantitative relationship between the amount of adsorbate on the surface and the pressure in the gas phase above it. In principle, the Langmuir isotherm contains all the parameters needed to do this and provides a good first approximation.[73]

The Langmuir film technique has been extensively studied as a model system for two-dimensional matter,[74] the cell membrane,[75] and more recently for use in nanoscience and nanotechnology. The use of the Langmuir isotherm technique for studying the surface behavior of CDs is comparatively new. This technique is membrane mimetic chemistry. It continues to be widely used because it is a simple model which can help to deduce quantitative relationships between the amount of adsorbate on the surface and the surface pressure in the gas phase above it. The air−water interface provides a unique methodology for studying matter in two dimensions. By taking advantage of this unique technique, not only molecular arrangement but also molecular orientation and aggregation can be modulated in a two-dimensional manner.[76] Such modulations are dependent on the properties of the analyte on the surface and/or functional materials themselves.[77]

There are many other reasons to employ the Langmuir monolayer film technique in nanochemistry. One of the features is related to the elaboration of nanotechnology growth-controlled nanoscale systems. In a broad sense, nanobiotechnology requires the organization of atoms and molecules into a two- or three-dimensional space. The competency of nanofabrication strategies and contemporary developments of methods permitting direct characterization on the molecular scale open up advanced routes in the development of self-organized nanostructures. Distinctive geometry of the air−water interface has enabled the measurement of certain macroscopic properties of these systems (such as surface pressure−area (π−A) isotherms), and the experimental difficulties ingrained in gathering the data interface have restricted spectroscopic studies. An analysis of the surface structures and features of the interface is very important to collating and understanding the properties of nanosystems. An analysis of the molecular and supramolecular biological systems, such as cell membranes, can be an outstanding model for developing "smart nanostructures" based on the molecular self-assembly of biological macromolecules. The potential of two-dimensional molecular self-assemblies is clearly explicated by Langmuir monolayer films of lipid molecules.

Saccharide-Based Carbon Dots. Since CDs were discovered, none of the CDs synthesized until now, to our knowledge, have been reported to form Langmuir monolayers. Recently, we found that CDs can form a stable Langmuir monolayer at the air−subphase interface,[78] and we measured the surface pressure−area and surface potential−area isotherms, which are shown in Figure 7a. In this work, the hydrothermal carbonization of saccharides such as lactose (LacCDs), glucose (GluCDs), and galactose (GalCDs) was performed using a bottom-up preparation method. Traditionally, CDs have excitation-dependent PL emission.[79] This is mainly because of multiple emissive centers in the CDs. Some argue that the excitation dependence results from separate particles which emit differently in the CD population. Our work showed that the PL behavior of the CDs at air−water interface has different properties than in the bulk phase (Figure 7b). At the interface, the PL of CDs was excitation-independent. The excitation-independent emission of CDs at the monolayer may have been originated from the complex surface states. This is because at the monolayer the CDs are oriented uniformly as compared to in solution. Moreover, in a 2D monolayer, the surface states with similar properties align in a specific direction, giving the monolayer a more uniform emission.

β-Galactosidase-CDs Conjugate Air−X-gal Interfaces. The conjugation of NPs with protein/enzyme molecules, in recent years, to achieve unique properties has attracted many researchers, but the conjugation of CDs with enzymes has not been performed so far. Previously, we studied the surface

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properties of an enzyme, β-galactosidase, at the air−water interface.[80] We were interested in the surface properties of the conjugate of the β-galactosidase and CDs. We used the Langmuir monolayer technique to comprehend the surface properties of the conjugated enzyme. Figure 8 shows the

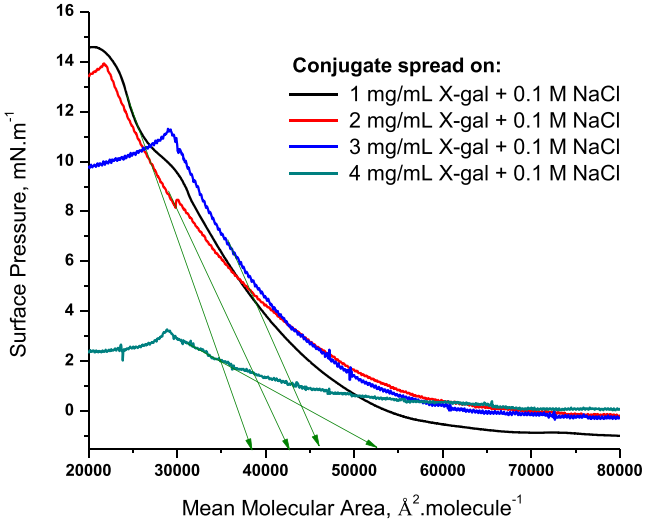

Figure 8. Langmuir isotherms of the β-galactosidase-CD conjugate (0.06 mg/mL) using 5 mL of X-gal at different concentrations (1, 2, 3, and 4 mg/mL) mixed with 25 mL of 0.1 M NaCl solution as the subphase. The conjugate spread volume was 45 μL at room temperature.

Langmuir isotherms of the β-galactosidase-CD conjugate with different concentrations of X-gal. We found that the limiting molecular area keeps on increasing with the increase in X-gal concentrations on the subphase. This observation can be explained by the interaction of the enzyme-CDs conjugate and X-gal. We also found that the conjugate was highly stable at the air−subphase interface, which reinforces the suitability of the use of the conjugated enzyme for biosensing applications. We conjugated the gel-like CDs with the enzyme, β-galactosidase, by using a mediator, EDC. Surprisingly, in the bulk phase, we found that the stability of the enzyme was increased appreciably. The enzyme activity did not diminish with relocation of the enzyme to room temperature for more than 2 months.

Membrane Barrier. There exist many types of membrane barriers in organisms. Among them, the most widely investigated are cell membranes and the BBB. They protect cells, tissues, and organs from being damaged by unwanted ions, molecules, and pathogens. However, because of the presence of these barriers, targeted drug delivery is hard to achieve, especially in the treatment of cancer and neurodegenerative disorders.

Oncology. In general, the cell uptake of NPs usually depends on the size, surface charge, and surface chemistry of NPs. All of these factors also play crucial roles in biosensing, cell labeling, intracellular delivery, and in vivo imaging. In addition, recent studies report that CDs could be internalized in the cytoplasm, especially in endosomes/lysosomes, but also in the mitochondria or endoplasmic reticulum. The authors hypothesized that the mechanism of CDs entering cells within less than 2 h is a nonendocytic pathway that is possibly due to their small size and hydrophilicity. There are also few reports on the CDs present on the cell membrane or strongly positively charged CDs reaching the cell nucleus,[81] which benefited from the strong, stable PL of CDs. Also, because of biocompatible, nontoxic, tunable surface functional groups, CDs have been used as a promising carrier in conjugation with drugs through electrostatic interaction or a typical EDC/NHS amidation reaction to realize targeted drug delivery in vivo and in vitro, which was not achieved by the traditional QDs as a result of the toxic composition. The drug that has been loaded onto CDs most commonly is doxorubicin (Dox). Tang et al. reported that a CD-Dox conjugate showed time-dependent drug release at an acidic pH level, which stimulates the lysosomal uptake in the cells of cancerous tissue.[82] Li and co-workers loaded Dox onto CDs, which were previously prepared with citric acid and urea, and the CD-Dox conjugate did not show an appreciable negative effect on the normal cells.[83] On the contrary, it revealed a strong killing effect on cancer cells, which probably was due to the higher drug release amount caused by the pH difference in normal and cancerous cells. Then an in vivo study using a liver cancer mouse model demonstrated the suitability of CDs as a bioimaging probe, the stability of the CD-Dox conjugate in an in vivo environment, and enhanced drug efficacy toward cancer cells. In addition to Dox, other drugs have also been used such as cisplatin (iv). Zhao and co-workers reported the fabrication of a pH/redox dual-responsive CD conjugate (CDs-RGD-Pt(IV)-PEG) with cisplatin(IV) (Pt(IV)) as a prodrug, RGD peptide as an active targeting ligand, and monomethoxypolyethylene glycol (mPEG) as a coating via a tumor extracellular pH (6.5−6.8) responsive benzoic−imine bond.[84] The conjugate could be constantly tracked by multiple PL of CDs, and the benzoic−imine bond and RGD peptide responded to the tumor extracellular pH and integrin $\alpha_v\beta_3$ on cancer cells, respectively, which eventually contributed to the enhanced uptake by cancer cells. After the internalization of the conjugate, prodrug cisplatin(IV) was reduced to cytotoxic cisplatin in the reductive cytosol of cancer cells to show therapeutic effects. In our group, two type of carbon nitride dots have been synthesized for bioimaging applications.[27] The dots could selectively enter the cytoplasm of SJGBM2 tumor cells and emit bright PL in wavelengths of the red region, which is beneficial to bioimaging considering the short-emissive autofluorescence of many organs. Also, it is beyond the reach of most CDs.

Furthermore, we have prepared a conjugate of CDs with a targeting ligand, transferrin, and two anticancer drugs, epirubicin and temozolomide, through a triple conjugate reaction.[85] In vitro studies were performed with glioblastoma brain tumor cell lines SJGBM2, CHLA266, CHLA200 (pediatric), and U87 (adult). The efficacy of the triple conjugated system (dual drug conjugation along with transferrin) was compared to those of dual conjugated systems (single drug conjugation along with transferrin), nontransferrin CDs−drugs, and free drug combinations. Because of the overexpression of transferrin receptors at the surfaces of many types of cancer cells,[86] the presence of transferrin on the surface of CDs serving as a targeting ligand can increase the cell penetration. As a result, transferrin-conjugated samples displayed the lowest cell viability even at a lower concentration. Among the transferrin conjugated samples, the triple conjugated system (CDs-trans-temo-epi (C-DT)) was more strongly cytotoxic to brain tumor cell lines than dual conjugated systems (C-dots-trans-temo (C-TT) and C-dots-trans-epi (C-ET)). C-DT increased the cytotoxicity to 86% in

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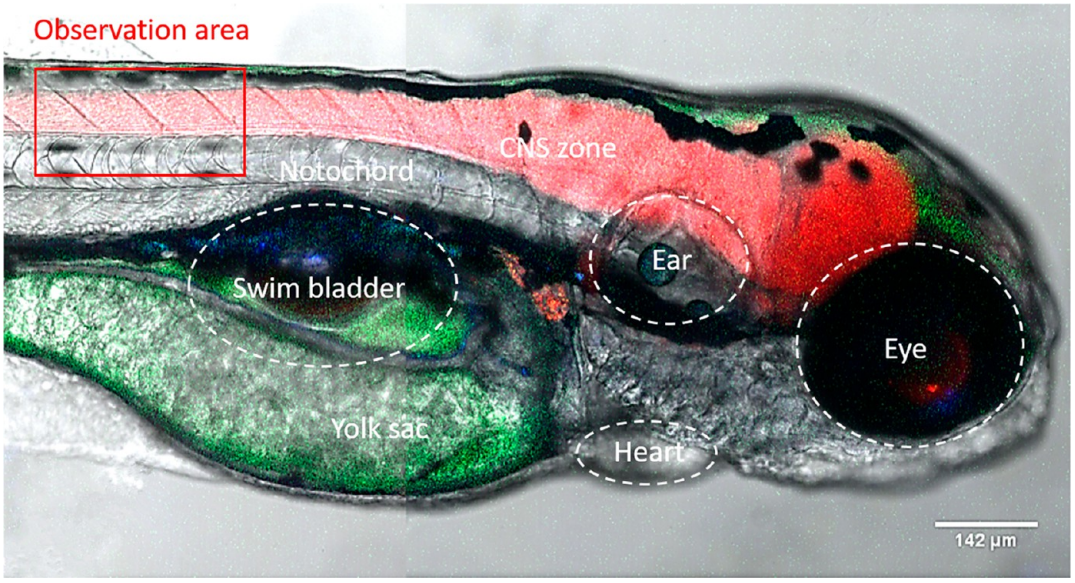

Figure 9. Light micrograph of the zebrafish body 6 days after fertilization. The red box is the major observation area for studying the CDs across the BBB. (Reproduced with permission from ref 43. Copyright 2019 Elsevier.)

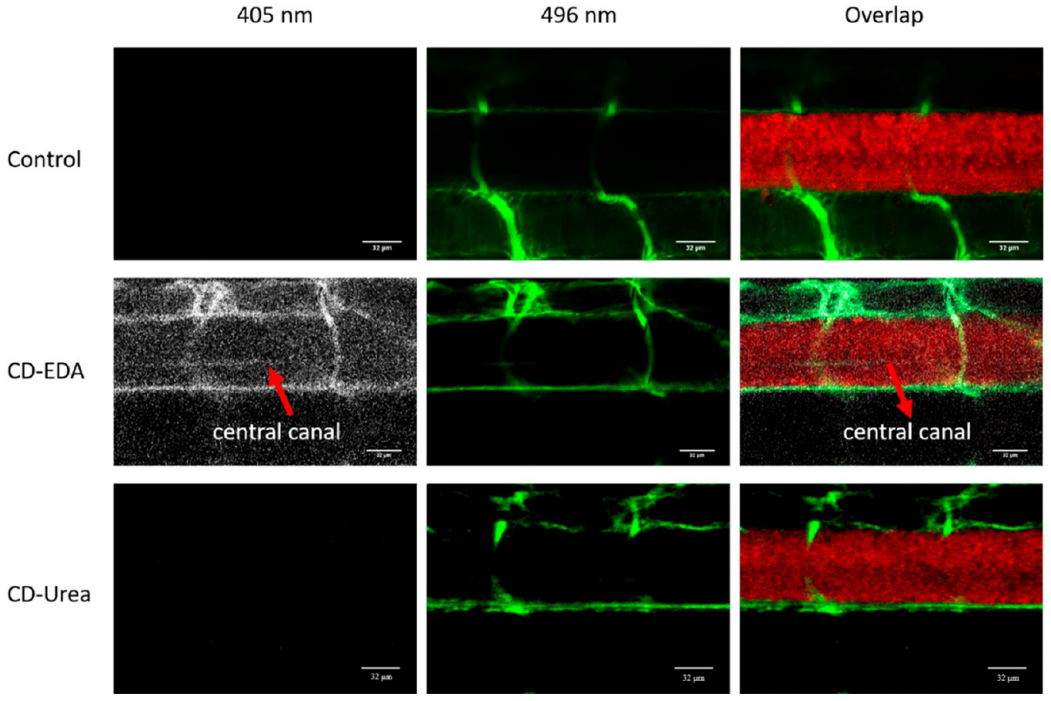

Figure 10. Confocal microscopy images of a 6-day-old transgenic zebrafish larva expressing mcherry (585 nm) in the central nervous system. The larvae were injected with either 10 000 MW fluorescein dextran dye (496 nm) alone (control, top row), a combination of dye and CD-EDA (second row), or a combination of dye and CD-urea (third row). Fluorescence from trp-CDs (405 nm) that crosses the blood−brain barrier can be seen in the central canal that is highlighted with the red arrows. (Reproduced with permission from ref 42. Copyright 2019 Elsevier.)

SJGBM2 at 0.01 μM while C-ET and C-TT reduced it only to 33 and 8%, respectively. Triple conjugated C-DT increased the cytotoxicity, and the two-drug combination in C-DT displayed a synergistic effect.

BBB. Being one of the most important components in the central nervous system (CNS), the BBB prevents most unwanted pathogens, toxins, and large, hydrophilic, highly charged molecules from entering the brain. However, it also becomes an obstacle for the drug designed for the treatment of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. Considering the side effect of traditional therapeutic approaches, NP-mediated treatment seems promising and significant.[87] Because of the small size (1−10 nm) and abundant surface functional groups, CDs have an incomparable advantage over other NPs in serving as the drug delivery carrier either via passive diffusion or active transport. In our group, we have explored the potential of CDs crossing the BBB by itself using zebrafish as the biological model. The anatomy of mcherry-expressed transgenic zebrafish is shown in Figure 9. CDs were injected into the heart of

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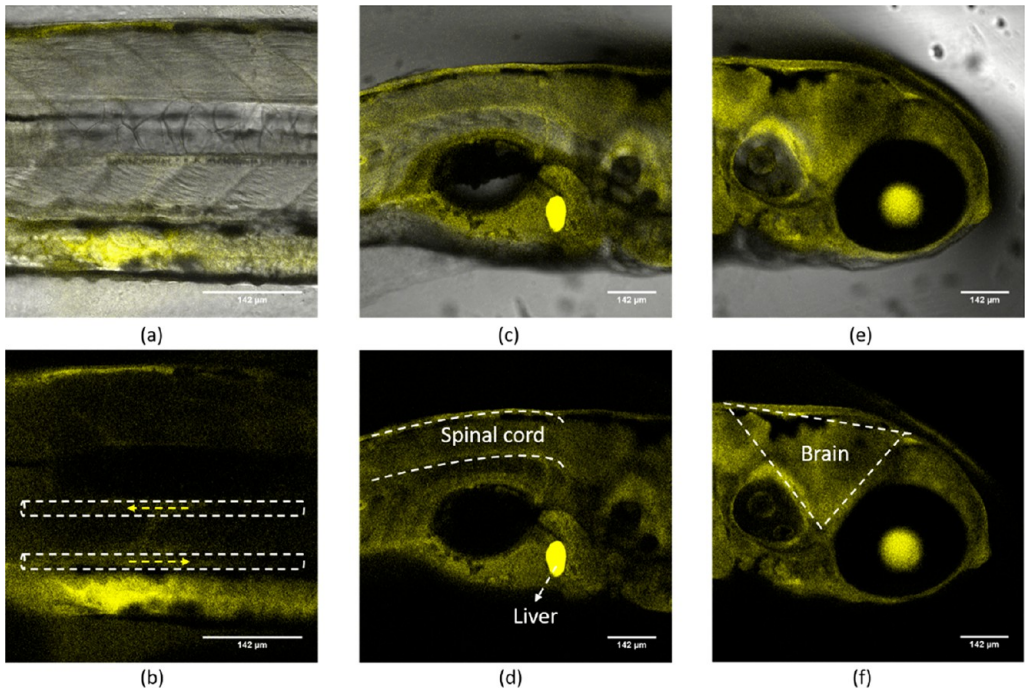

Figure 11. Confocal images of a CD aqueous dispersion (0.1 mg/mL) into zebrafish blood (a, b) and CNS including the spinal cord (c, d) and brain (e, f). (a, c, e) Overlapped images under both white light and excitation at 405 nm. (b, d, f) Images under excitation at 405 nm.

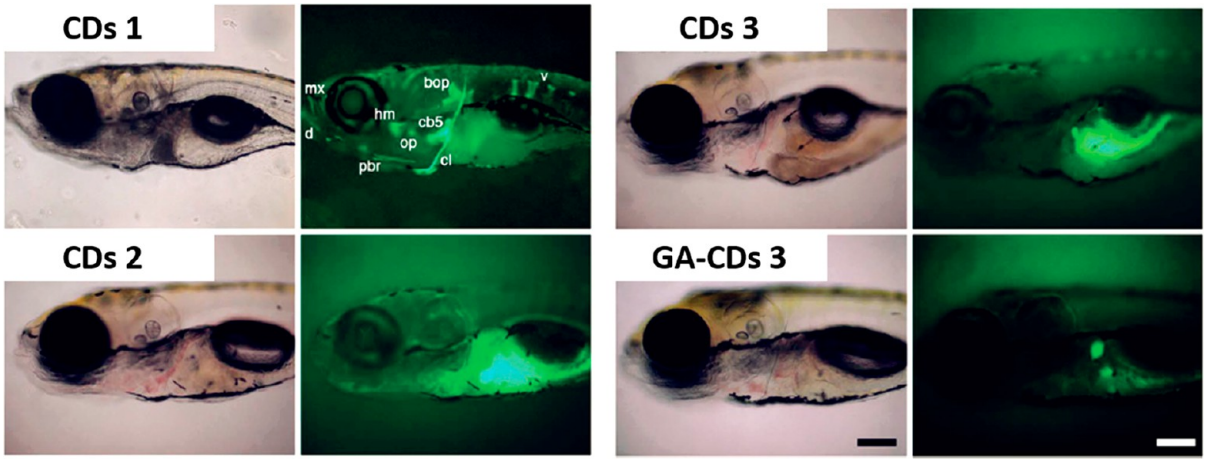

Figure 12. Transmitted and fluorescent images of 8-day-old larvae injected 6 days after fertilization with black CDs (CDs 1), citric acid (CDs 2), glycerin (CDs 3), and glutamic acid-conjugated glycerin-derived CDs (GA-CDs 3). In the image of CDs 1, bones are dentary (d), maxilla (mx), posterior branchiostegal ray (pbr), hyomandibula (hm), opercle (op), ceratobranchial 5 (cb5), clei-thrum (cl), basioccipital articulatory process (bop), and vertebrae (v). Images of CDs 2 and 3 were overexposed to demonstrate that they do not bind to bones. The autofluorescence seen in the gut tissues is a result of image overexposure. In the image of GA-CDs 3, the two stained structures correspond to primitive kidneys (pronephros). The scale bar is 100 μm. (Reproduced with permission from ref 49. Copyright 2017 The Royal Society of Chemistry.)

zebrafish, and the heart will pump out CDs along with the blood. We observed the central canal of the spinal cord (observation area in Figure 9) to examine the presence of CDs in the CNS from blood. As the first trial, the black CDs alone did not cross the BBB.[21] However, because of the presence of transferrin receptors on the BBB,[88] the black CDs were conjugated with transferrin and the conjugate eventually successfully overcame the BBB via receptor-mediated endocytosis. However, because of the abundance of tryptophan receptors on the BBB, we employed tryptophan to fabricate CDs, and the CDs made from tryptophan and EDA surprisingly crossed the BBB, which was confirmed by observation of the central canal of the spinal cord (Figure 10).[42] This result was explained by the residue of tryptophan on the surface of CDs, and the mechanism could be transporter-mediated transport. Therefore, we have proven that bare CDs could cross the BBB. Then different CDs were also administered. Among them, orange CDs exhibited the best effect. In this case, instead of injection, a 5-day-old zebrafish was soaked in the CD aqueous dispersion. After one night, the zebrafish was observed under a confocal microscope and was found to be stained with CDs all over its body (Figure 11). Most importantly, we observed the CDs in the CNS, and the explanation is hypothesized to be due to the small size (2 nm), low zeta potential, and amphiphilicity. Therefore, even though CDs did not localize in one area of the body, the observation

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that CDs crossed the BBB though the passive diffusion mechanism was significant because it may eliminate the need for injection, which would reduce the pain and death of zebrafish caused by injection.

Bone Targeting. Traditional methods to deliver drugs to bones for the treatment of mineralization disease such as osteoporosis can also disrupt homeostasis in other tissues. To overcome this problem, it is critical to develop a novel DDS and precisely deliver drugs exclusively to the bones. As we introduced previously, CDs have abundant surface functional groups on the surface, a small size, and a high PL, so they are suitable biomarkers for the targeted study. In our group, the black CDs do not have high QY. However, when the black CDs were injected into the yolk sac of zebrafish, we found that the bones of zebrafish larvae were specifically lighted up.[48] The use of zebrafish as the model has two major advantages. First, zebrafish larvae have developing bones, which are easy to target. Second, zebrafish larvae are transparent, which will be beneficial for observation. To examine the specificity of such CDs in targeting the bones, CDs prepared from three other methods were used as the control.[49] The precursors of CDs 2 and 3 were citric acid and glycerin, respectively, and CD 3 was conjugated with glutamic acid (GA) to increase the bone-binding affinity. As a result, in Figure 12, we observed that only the black CDs (CD 1) can target the bones of zebrafish, which revealed the specificity of black CDs.

The mechanism of the increasing PL in the bones might be related to the interactions of black CDs with the bones. There were two hypotheses put forward. First, the PL of black CDs could be enhanced by the bone microenvironment due to the abundance of calcium in the form of hydroxyapatite (HT). HT could have interacted with the black CDs by the electrostatic force between Ca^{2+} and the negative charge of black CDs. However, there was no evidence to prove that the increase in the PL of the CD-HT mixture was due to their interaction. HT itself has fluorescence, and the fluorescence spectrum of the mixture was possibly the simple overlap of each individual spectrum. Furthermore, the addition of Ca^{2+} to the CD solution did not show the enhancement of PL for black CDs, which confirmed that the interaction with HT was not the reason for the increasing PL of bones. Another mechanism is relevant to cartilage. During embryo development, cartilage mineralization plays a key role in the formation of vertebrate bones, and cartilage contains a large amount of chondroitin sulfate, which may trap circulating black CDs as the tissue mineralizes. Therefore, the interaction between black CDs and chondroitin sulfate sodium salt (CSSS) was analyzed with fluorescence spectroscopy. However, similarly, CSSS has fluorescence with the maximum emission peak at 493 nm. It is not convincing to account for the increasing PL intensity and maximum peak shift of the CD-CSSS mixture by the interaction between black CDs and CSSS, and the mechanism remains to be understood.

In addition, black CDs have extensive carboxyl groups but no amine moieties on their surface, so the choice of chemicals that could be delivered by black CDs is limited. To increase the cargo carrier capacity and enhance the bone-binding affinity, the surfaces of black CDs have been modified by conjugating with EDA and GA, respectively. Before conjugation, the content of carboxyl moieties had been measured by a classic acid−base titration.[35] To start with, 6.0 mg of black CDs was dissolved in 60.0 mL of water and titrated with a 5.5×10^{-4} M NaOH solution (the NaOH solution used in titration was freshly prepared and standardized by KHP). Upon the arrival of the end point, 63.42 mL of NaOH solution was used, which is around 0.03488 mmol of NaOH. Assuming only the carboxylic group on black CDs reacted with NaOH, it is estimated that 5.8 mmol of carboxyl groups is available for each gram of black CDs. On the basis of this quantification, the conjugation between black CDs with EDA and GA was followed.[49]

To conjugate black CDs with EDA, 8.34 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added to 2.0 mL of a 2.5 mg mL^{-1} black CD aqueous dispersion in PBS buffer at pH 7.4. After 20 min, 2.6 mg of EDA was added to the mixture, which was stirred at room temperature for 3 h. The product of the reaction was subjected to an SEC column (Sephacryl S-300) for purification. The sample was lyophilized to obtain solid EDA-modified CDs and then characterized by standard methods. To conjugate black CDs with GA, similarly, 8.34 mg of EDC was added to 2.0 mL of a 2.5 mg mL^{-1} black aqueous dispersion in PBS buffer at pH 7.4. After 20 min, 10.66 mg of GA was added, and the mixture was stirred at room temperature overnight. The resulting dispersion was then dialyzed with a semipermeable membrane dialysis bag (MWCO 3500) against pure water for 2 days to remove the unreacted GA. The conjugates were obtained after the removal of water by lyophilization and then characterized by standard methods.

Finally, compared to the control, neither modification significantly changed the black CDs deposition in calcified bones (Figure 13): the abdominal injection of 20 ng of EDA-

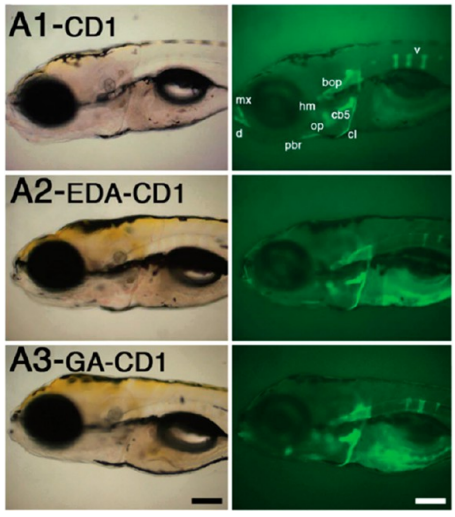

Figure 13. Chemical modification of black CDs does not significantly affect their bone-binding properties. Transmitted and fluorescent images of 8-day-old larvae injected 6 days after fertilization with black CDs (CD1), EDA- (EDA-CD1), or GA-modified black CDs (GA-CD1). The names of bones are indicated only in the CD1 image, as described in Figure 8, but are present under all conditions. The scale bar is 100 μm. (Reproduced with permission from ref 49. Copyright 2017 The Royal Society of Chemistry.)

or 25 ng of GA-modified CDs into the 6-day-old larvae resulted in strong and highly specific bone fluorescence 2 days after injection. Under all conditions, the number of vertebrae labeled by the black CD preparation was the same, although in some cases the vertebrae appeared to be thinner. We attributed these differences to the natural variation between individual

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larvae in our population,[89] as all types of individuals were observed under all conditions tested. Together, these results suggest that carboxyl or amine group surface modifications do not affect the black CD binding affinity and specificity for bones.

CONCLUSION AND FUTURE PERSPECTIVE

Conclusions. CDs can be prepared from a wide variety of precursors through many methods. Because of the abundant diversity of CDs in our group and around the world, the importance of proper purification and characterization cannot be understated. The characterization of CDs reveals unique and not well understood PL properties as well as surface functionalization which allows unique applications. CDs have shown themselves to be a useful material in many fields. Their stable PL properties provide for unique applications in 2D and 3D printing. Because of their surface groups and properties, they are also a promising tool in drug delivery for the treatment of cancer and other maladies, specifically for targeting the bones and crossing the BBB. CDs have further applications in nanomedicine because they have shown to inhibit the formation of Aβ plaques, which are directly associated with Alzheimer's disease. The use of CDs in cosmetics as a hair colorant or a nail polish additive illustrates the diversity of applications in which CDs can be applied. Because of this wide application, it is important to know more about the fundamental properties of CDs including their PL mechanism and, specifically for black CDs, their mechanism of bone targeting. The exciting development of CDs forming Langmuir monolayers could provide a unique way to study CD properties. New characterization methods will also help to provide insight into CDs and develop their potential for the future.

Future Perspective. Inspired by unique properties of each type of CDs, we will study the synergistic effect caused by conjugating two or more types of CDs. Also, to achieve better bioimaging while avoid the autofluorescence of tissues, the development of long-emissive, especially red-emissive CDs will be focused on. In the end, because of the small size and large surface area to volume ratio, CDs will be tried as an initiator for the polymerization process of some known polymers. Therefore, the future perspective of the development of CDs in our group can be summarized into three categories: (1) construction of multifunctional drug delivery system by conjugating two types of carbon dots; (2) the development of red-emissive CDs; and (3) the use of CDs as an initiator to trigger polymerization. The detailed plan of each project is demonstrated as below.

Conjugation between Carbon Dots. On the surface of CDs there exist either or both amine and carboxylic groups, so besides conjugation with drugs, they can also conjugate with each other. The significance of such conjugation results in a new type of CDs possessing many properties of different individual CDs. For example, black CDs can specifically target the bones of zebrafish, but they do not have high QY. The gel-like CDs have high QY, but they cannot be delivered to the bones. Then the conjugation between these two types of CDs is hypothesized to enhance the PL and drug loading. In addition, because of the wide distribution of orange CDs all over the zebrafish, the conjugation of orange CDs and black CDs is predicted to be located in bones by soaking up zebrafish instead of injection.

Moving forward, focusing on the conjugation of CDs with other CDs or organic molecules, it is important to increase the possibilities for conjugation. This can be done by the use of bifunctional linkers. For instance, EDA can be easily conjugated to black CDs to create amino functionalization. This would allow greater possibilities of conjugation for black CDs by providing amino groups in addition to the already present carboxylic groups. Amino acids such as cysteine, lysine, and aspartic acid could be used to add a variety of functionality using the same method. The potential of CDs in drug delivery would greatly increase when there are fewer limitations on the organic molecules which can be conjugated to CDs.

Red Photoluminescence Carbon Dot Preparation. CDs' red emission is an area which has received much attention recently. While improvements have been made in this area, there is still a need to increase the wavelength of CD emission as well as the QY of the long-wavelength emission. Lanthanides are a class of metals which are known to possess long-wavelength emission (up to 1500 nm for some lanthanides). This ability to emit light usually comes from the electron transitions between the D and F orbitals.[90] Because lanthanides are poor absorbers of light, their emission is usually observed only when there is a ligand coordinated to the metal which can absorb light and transfer energy to the metal. This energy transfer provides the excitation of the metal's electrons, enabling the emission of light.[91] To obtain the red emission of light, our goal is either to incorporate a lanthanide metal into CDs or to coordinate preprepared CDs with a lanthanide so that CDs can act as the "antenna" and absorb energy which can be transferred to the metal, enabling red emission. For this purpose, we will focus on thulium and samarium, which possess emission peaks at 800 and 650 nm, respectively. Success in this area would open many new applications for CDs, with emission in the entire visible spectrum of light able to be achieved.

Polymerization Using Carbon Dots. We predict that CDs can also be used as initiators in the polymerization process. Specifically, polyaniline, polypyrrole, and the copolymerization of polyaniline and polypyrrole from monomers can be obtained using CDs and UV light. This is because CDs have many unshared or unpaired electrons and the positively charged aniline is attracted to the CDs. This strategy will be helpful in designing the synthesis route for the polymerization of monomer through colloidal synthesis by an economic, efficient and green method. Moreover, this will shorten the preparation steps of ready-to-use material and will open a new route for polymerizing monomers with new properties and lead to economically efficient process applications. The newly synthesized polymers can be used as supercapacitors in bioanalytical applications, in sensors, and as chemiresistors. Furthermore, the mechanism of the catalytic activity of CDs can be investigated by masking the −COOH, −OH, or C═O groups on the surface of CDs.

AUTHOR INFORMATION

Corresponding Author

*E-mail: rml@miami.edu.

ORCID

Yiqun Zhou: 0000-0002-6594-9925
Keenan J. Mintz: 0000-0001-7193-6947
Shiv K. Sharma: 0000-0001-8223-7789
Roger M. Leblanc: 0000-0001-8836-8042

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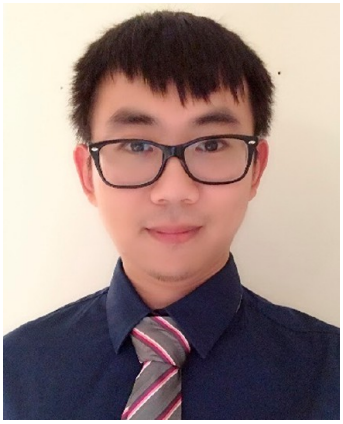
Notes

The authors declare no competing financial interest.

Biographies



Yiqun Zhou is a postdoctoral fellow in Prof. Leblanc's group at the University of Miami. He received his B.S. (2014) in chemistry from Shaanxi University of Science and Technology, and he completed his Ph.D. in chemistry in Prof. Leblanc's group at the University of Miami in the spring semester of 2019. His current research interests include the development of red-emissive carbon dots, printing (text printing and 3D printing), drug delivery using zebrafish as a model (transport across the blood–brain barrier, specific bone targeting), thermoelectricity, and photocatalysis.

Keenan J. Mintz is a Ph.D. candidate in the group of Prof. Roger M. Leblanc in the Department of Chemistry at the University of Miami. He received his B.S. in chemistry (2015) from Florida International University. His current research interests include the role of surface states in the photoluminescence of carbon dots as well as developing red emissive carbon dots for bioimaging.

Shiv K. Sharma is a lecturer in the Department of Chemistry at the University of Miami. He received his B.Sc. (2008) and M.Sc. (2012) in chemistry from Tribhuvan University, Kathmandu. He completed his Ph.D. from the University of Miami in 2018. He works in the surface chemistry of enzymes, incorporating enzymes with nanoparticles with applications in biosensing.

Roger M. Leblanc received his B.S. in chemistry in 1964 from Universite Laval, Canada, and his Ph.D. in physical chemistry in 1968 from the same university. From 1968 to 1970, he was a postdoctoral fellow in the laboratory of Prof. George Porter, FRS, in the Davy Faraday Research Laboratory at the Royal Institute of Great Britain. He was a professor from 1970 to 1993 in the Department of Chemistry and Biology at Universite du Quebec a Trois Rivieres, Canada. During this period, he was chair from 1971 to 1975 in the same department and director from 1981 to 1991 at the Photobiophysics Research Centre. In 1994, he moved to the University of Miami, where he has been a professor in the Department of Chemistry since then to the present. At the University of Miami, he was chair of the Department of Chemistry from 1994 to 2002 and he was appointed as chair from 2013 to the present. He was also one of the three editors of *Colloids and Surfaces B: Biointerfaces* from 1998 to 2013. During his early career as a scientist, his research interest was photosynthesis and photoconductivity using surface chemistry and spectroscopy. His current research interest is to apply 2-dimentional (2D) surface chemistry combined with spectroscopy and microscopy to investigate the properties of nanomaterials (graphene oxide, carbon dots, and quantum dots) and the fibrillation process of amyloidogenic protein (insulin, amyloid-β peptide, and islet amyloid polypeptide). He is also interested in designing and developing biosensors with high sensitivity and selectivity for diseases diagnose. He has published more than 520 scientific articles in peer-review journals. As a professor, he has supervised more than 100 M.S. and Ph.D. students.

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