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Carbon dots as artificial peroxidases for analytical applications

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Abstract

Nanozymes have become attractive in analytical and biomedical fields, mainly because of their low cost, long shelf life, and less environmental sensitivity. Particularly, nanozymes formed from nanomaterials having high surface area and rich active sites are interesting since their activities can be tuned through carefully controlling their size, morphology, and surface properties. This review article focuses on preparation of carbon dots (C dots) possessing peroxidase-like activity and their analytical applications. We highlight the important roles of the oxidation states and surface residues of C dots and their nanocomposites with metal, metal oxides, or metal sulfides playing on determining their specificity and sensitivity toward H2O2. Examples of C dot nanozymes (CDzymes) for developing sensitive and selective absorption, fluorescence, and electrochemical sensing systems in the presence of substrates are presented to show their potential in analytical applications. For example, CDzymes couple with glucose oxidase and cholesterol oxidase are specific and sensitive for quantitation of glucose and cholesterol, separately, when using 3,3′,5,5′-tetramethylbenzidine as the signal probe. This review article concludes with possible strategies for enhancing and tuning the catalytic activity of CDzymes.

Keywords: Carbon dots, CDzymes, Nanozymes, Peroxidase, Sensing

1. Introduction

Many natural enzymes with high specificities and catalytic activities are popular for analytical and biomedical applications [1, 2]. For example, horseradish peroxidase (HRP) is the most widely used enzyme for sensitive and selective detection of H2O2. The HRP general mechanism is initiated from the pentacoordinated ferric heme, binding H2O2. One of the H2O2 oxygen atoms then leaves as water, while the other is retained as a ferryl group to generate compound I, featuring an Fe(IV) center coupled to a porphyrin cation radical. Compound I then accepts one electron from a second substrate molecule, yielding the enzyme native state (ferric). As to the fate of the substrate, loss of one electron, usually accompanied by loss of a proton, leads to the formation of products with different absorbance, fluorescence and electrical properties from that of the substrates. More importantly, enzyme cascades of HRP combined with various enzymes are used in many sensitive and selective assays for many important analytes. For instance, HRP-glucose oxidase (GOx) and HRP-uricase are commonly employed to develop sensing systems for detection of glucose and uric acid, respectively [3].
Nowadays, immobilized oxidative enzymes are broadly accepted as a green way to face the challenge of high amounts of micropollutants in nature. Immobilized HRP are showed better stability, and reusability as well as easy separation from reaction mixture that make them more favorable and economic in compared to free enzymes [4]. Furthermore, the combination of enzyme immobilization with prodrugs was also considered as a promising approach for biomedical application of enzyme in cancer therapy [5]. However, use of natural enzymes for developing sensing systems is sometimes limited by their high cost and short shelf lifetime. In addition, their catalytic activities are usually very sensitive to environmental conditions [6]. For example, most enzymes reach maximal catalytic activities at temperature around 37°C and pH value at around 7.0. Therefore, inexpensive artificial enzymes with high catalytic activities and excellent stabilities for analytical and biomedical applications are highly demanded.

A number of organic materials and biomaterials like DNAzymes have been recognized for quantitation of various analytes with advantages of low cost, stability, and a wider working range (pH, ionic strength, and temperature) [7]. However, the specificity and turnover number of the artificial enzymes are usually not great as that of the natural ones. As an alternative to natural and artificial enzymes, nanozymes (nanomaterial-based artificial enzymes) with high activity have been prepared and applied for various analytical and biomedical applications [8–17]. In addition, nanozymes, when compared to DNAzymes, are usually cheaper and less sensitive to changes in pH, ionic strength, and temperature. Their activity is usually size dependent; small nanoparticles with greater surface area and higher density of defects (active sites) are more active than larger ones [18]. Many metal-based nanoparticles, including Pt, Pd, Au, and Ag exhibiting peroxidase-, oxidase-, and catalase-like activities have been used to develop sensitive and selective sensing assays for detection of various analytes, such as protein, heavy metal ions, and glucose [19–27]. Some relatively cheaper nanozymes, including metal oxide (Fe3O4, CuO, CeO2, MnO2, and V2O5) nanoparticles and metal sulfide (FeS, CuS, and MoS2) nanoparticles, have been employed to develop sensitive and selective sensing systems and to fabricate various logic gates [28–36]. With the advantages of high activity, low cost, and stability, nanozymes have become very popular materials in analytical chemistry and biomedical applications [37].

Having excellent biocompatible, catalytic, mechanical, electrical, optical, and thermal properties, many carbon nanomaterials such as carbon nanotubes, carbon dots (C dots), activated carbon, and graphite have become popular as energy materials, drug delivery, sensors, field emission devices, and water splitting [38–42]. For example, C dots with CuS, CoS, and NiS nanomaterials have been shown improved light conversion efficiency by taking advantages of the conductivity of C dots [43, 44]. Core–shell carbon nanomaterials prepared from red onion skins and boron have shown efficient for water splitting, with high oxygen reduction reaction efficiency and greater stability [45].

Owing to having high surface area, great number of surface defects, stability and biocompatibility, C dots are applied for various analytical and biomedical applications [46]. In addition, they can be prepared through green and environment friendly approaches, with large-scale production [47]. Thus, we focus our discussion on C dot nanozymes (CDzymes) with peroxidase mimic catalytic activity for analytical and biomedical applications in this review article, mainly because of our own interest and their importance for detection of important analytes such as H2O2, glucose, uric acid, glutathione and cholesterol. Further information regarding nanozymes and their applications are available from several excellent review papers published in the last three years [8, 9, 48]. We briefly discuss the preparation of CDzymes and their characteristics. Examples of their analytical applications are provided to highlight their advantages and drawbacks as artificial enzymes to replace natural peroxidases. This review article concludes with the discussion about the challenges and strategies for developing ideal CDzymes.

2. Preparation of C dots

C dots refer to carbon nanomaterials with photoluminescence properties and they have received extensive attention as sensitive materials in sensing and imaging applications [49–52]. Photoluminescent carbon nanomaterials are also called in different names, including carbon quantum dots (carbon nanoparticles with sizes below 10 nm and some form of surface passivation) [53], carbon nanodots (carbon nanomaterials with sizes below 10 nm) [49], graphene quantum dots (graphene sheets with lateral size less than 100 nm) [54], carbonogenic dots (discrete carbon nanoparticles of near spherical geometry with sizes below 10 nm) [55],
and carbon nanocrystals (smaller crystals of around 2 nm in size) [56]. To make it easier for readers to follow, we use “C dots” to represent photoluminescent carbon nanomaterials in this article.

Each C dot consists of a carbonaceous core and a surface passivation layer. The carbon core can be either sp² hybridized graphene fragments or carbon composed of sp² and sp³ hybridized carbon [57]. The size range of C dots is typically from 2 nm to 100 nm. Features of C dots include large surface area to volume ratios, insufficiently coordinated surface atoms, and many unsaturated bonds. In general, each C dot has a surface passivation layer on its core to reduce the system’s Gibbs free energy. When hydrophilic carbon precursors are used, hydrophilic C dots are usually obtained, mainly because of the existence of rich hydroxyl, amino, and carboxyl groups on their surfaces. On the other hand, C dots are generally hydrophobic when using hydrophobic carbon precursors. To modulate the polarity, optical properties, chemical reactivity, and selectivity of C dots, modifiers such as oligomers, polymers, and biomolecules are used to passivate their surfaces [58, 59]. Having the advantages of biocompatibility, brightness, negligible photoquenching and photobleaching, stability against salt, and ease in preparation, low-cost C dots have become interesting materials for sensing, in vitro and in vivo imaging applications [60–67].

C dots can be prepared from various sources through top-down and bottom-up approaches [68, 69]. When a large size of solid or powder (graphite, carbon fiber, and carbon black) is available as a carbon source, a top-down approach through etching/oxidation is usually applied to obtain C dots. The structure of carbon source generally contains graphite crystallites or a large number of sp² conjugated microdomains. Because of consumption of large amount of energy, need of an expensive synthetic system, and difficulty for a large-scale preparation of C dots, the top-down approach is less popular than the bottom-up approaches like hydrothermal route and electrochemical approach. Ever since the C dots preparation from amino acids through hydrothermal approaches were demonstrated [70], this approach has become most popular for the preparation of C dots from various carbon precursors such as carbohydrates, organic acids, organic amines, and polymers. The pioneering works for preparation of C dots with different biological activities from tea and used coffee powders have led to preparation of C dots from natural sources such as fruits, grass, and trees [71–75]. It has been suggested that C dots are formed from their precursors through four steps of dehydration, condensation, carbonization, and passivation [76]. When the preparation conditions are mild, C dots usually have no obvious crystal structures, with cores consisting of either amorphous carbon or non-conjugated polymers [77, 78]. The surface groups of C dots can be introduced either directly during the preparation process or through subsequent passivation treatment with functional organic ligands after the synthesis.

3. Artificial peroxidases of C dots

Owing to having high surface area and great number of surface defects, stable and biocompatible C dots are efficient for generation of reactive oxygen species inside cells [63, 71, 72]. C dots and their hybrids with nonmetal, metal, and metal oxide possess some apparent benefits such as resistance toward inhibition or digestion by proteases. When compared to natural enzymes, CDzymes have longer shelf lifetime, less working restriction, and lower cost, however, usually have lower catalytic activity [9, 79–81]. CDzymes possess peroxidase-like catalytic activities, which have been used to develop sensitive sensing systems for detection of various analytes and for environmental monitoring. Most of the sensing systems are based on the catalytic activities of the CDzymes to oxidize H₂O₂ to form hydroxyl radicals that convert substrates to form products with different absorbance, fluorescence and electrical properties from that of the substrates [81, 82].

3.1. C dots

Most sensing systems are based on the fact that C dots catalyzed H₂O₂-mediated oxidation of a peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB), leading to a change in color (colorless to blue) and an absorbance increase at 652 nm [83]. Generally, the catalytic pathway of C dots follows a ping-pong mechanism. According to the ping-pong mechanism, C dots and its intermediate C dots* are existent in the reaction system. Once an electron-donor substrate binds to C dots, C dots* are formed. For example, H₂O₂ molecules inside cells react with C dots to form C dots*, which then return to C dots and ROS is formed. When compared to C dots without containing heteroatoms, C dots synthesized from certain precursors containing heteroatoms usually possess higher catalytic activity, mainly because of their strong affinity toward substrates and high electron transfer rate.

Similar to HRP, the activities of the CDzymes are highly dependent on their concentration, substrate
concentration, pH, and temperature. C dots prepared from candle soot by oxidative treatment with 5 M HNO₃ show peroxidase-like activity in sodium acetate buffer to catalyze the typical color reaction of H₂O₂ and TMB (Fig. 1). Electron transfer from the amino groups of TMB to the C dots has been suggested for the increase in the electron density and mobility in the C dots, which then accelerate electron transfer from the C dots to H₂O₂. As a result, the oxidation rate of TMB by H₂O₂ increases. Under the optimal conditions, the assay provides a linear concentration range of 0.010–0.4 mM, with LOD of 2.0 μM for glucose, and a linear concentration range of 0.010–0.20 mM, with LOD of 3.0 μM for uric acid. When compared to C dots (without NaBH₄ treatment), the reduced C dots provides lower catalytic activity toward H₂O₂, mainly because many ketonic carbonyl groups (–C=O) are converted to hydroxyl groups (–OH) in the reduced C dots. Relative to hydroxyl groups, ketonic carbonyl groups have higher catalytic activity for H₂O₂. C dots treated separately with phenylhydrazine, benzoic anhydride, and 2-bromo-1-phenylalanine were used to investigate the role of the functional groups in the catalytic activity of C dots [85]. It is noted that the three reagents act as selective deactivation agents to react with the –C=O, –OH and carboxyl groups (O=C–O) on the C dots, respectively. Based on the Km and the maximum initial velocity (Vₘₐₓ) values for 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), the –C=O groups are suggested to act as the catalytically active sites, meanwhile the O=C–O and the –OH groups serve separately as the substrate binding sites and catalytic-activity inhibiting residues.

When combined with gold nanorods (GNRs), C dots enable colorimetric detection of glucose [86]. The C dots prepared from litchi rind through a carbonized treatment, followed by refluxing with 5 M HNO₃ at 140 °C for 12 h exhibit peroxidase-like catalytic activity in the H₂O₂-mediated oxidation of iodide to form iodine that etches the GNRs along the longitudinal direction due to higher reaction activities at the tips of GNRs (Fig. 2). The etching of GNRs results in blue shifts in the maximum absorption wavelength from 953 to 645 nm. The shift in the maximum absorption wavelength decreases linearly upon increasing the glucose concentration in the range of 0.01–2.0 mM, with LOD of 3.0 μM. C dots prepared from wood charcoal through an electrochemical synthesis using (NH₄)₂S₂O₈ as an electrolyte possess peroxidase-like activity [87]. During electro-oxidation of wood charcoal, S₄O₆²⁻ ions produce SO₄•⁻ radicals that function as sharp
electrochemical scissors to cut down graphene sheets into very small intact sp² structures through oxidation of the C–C bonds. The as-prepared C dots provide a V_max of $7.2 \times 10^{-7}$ M s⁻¹ and K_m of 12 μM for TMB oxidation reaction, showing their high peroxidase-like activity. When combined with GOx, the C dots in the presence of TMB allow a rapid and sensitive detection of glucose, with LOD of 6.0 μM and a linear range of 10–60 μM. Oxidative C dots prepared from multiwalled carbon nanotubes through a facile oxidation reflux approach show high peroxidase-like activity in a wide range of pH values, mainly because of abundant –C=O and O=C=O– groups, and negligible –OH groups found on their surfaces [88]. The oxidative C dots provide a K_m value that is five times lower than those of C dots and even an order of magnitude lower than that of HRP. In the presence of TMB, C dots allow detection of H₂O₂ over a linear concentration range of 20–600 μM. Using GOx to oxidize glucose to form H₂O₂, the system can determine glucose concentration in diluted blood samples from Balb/c mice, with high accuracy and precision. C dots prepared from graphite using a simple wet chemical method also show peroxidase-like activity [89]. When combined with cholesterol oxidase (ChOx), and TMB as substrate, C dots has been reported to detect cholesterol over a linear concentration range of 20–600 μM, with LOD of 6.0 μM (Fig. 3).

Photosensitization is a promising avenue of oxygen activation, which can overcome the spin selection rule to transform the ground state oxygen (^3O₂) into a highly reactive singlet oxygen (^1O₂). C dots are a promising type of carbon-based photosensitizer, and nitrogen doping can further improve the oxygen photosensitization performance. Wu et al. proposed a well-developed synthetic protocol of hydrothermal treatment of citric acid and ethylenediamine for the preparation of nitrogen-doped C dots (N-doped C dots) [90]. The oxygen photosensitization performances of the N-doped C dots were first confirmed by ROS investigation with TMB oxidation as the ROS probe and EPR. After XPS analysis of the surface nitrogen doping speciation, it was found that the changes of graphitic N and pyrrolic N correlated well with the oxygen photosensitization performances of N-doped C dots. The excellent photosensitized oxygen activation makes these N-doped C dots a promising oxidase-mimicking nanozyme for photodynamic antimicrobial chemotherapy and other applications [91].
addition, N-doped C dots through strong acid oxidation of three dimensional N-doped graphene aerogel possess peroxidase-like activity for the oxidation of TMB [92]. The introduction of N atoms into benzene ring atoms can efficiently influence the spin density and the charge distribution of the carbon atoms, enhancing the density of the catalytic active sites on the graphene surface with low steric hindrance for binding TMB [93]. Meanwhile, the lone-pair of electrons in the amino groups of TMB are transferred to the surfaces of the N-doped C dots, which are also responsible for increasing their electron density and mobility. As a result, the electron transfer from the N-doped C dots to H2O2 is efficient, leading to high peroxidase-like activity of the CDzyme. The CDzyme/TMB sensing system with GOx is selective and sensitive for quantitation of glucose over a linear concentration range of 0.25–375 μM, in diluted serum and fruit juice samples. N-doped C dots prepared from organic amines, such as dimethylamine, ethylamine, and tripropylamine in the presence of H2SO4 through a microwave-assisted heating process also show peroxidase-like activity [94]. The N-doped C dots in the presence of TMB allows for the detection of H2O2 over a linear concentration range of 1–100 μM, with LOD of 0.4 μM. When combined with GOx, the colorimetric assay detects glucose with a linear concentration range of 1–5 μM and LOD of 0.5 μM. In addition, nitrogen-rich nucleobases are reported as good precursors for the large-scale and cost-effective synthesis of N-doped C dots through direct pyrolysis [95]. The dominant graphitic N species greatly boost the peroxidase-like activities of nucleobase-derived C dots. In the presence of TMB, the N-doped C dots allow for the H2O2 detection over a linear concentration range of 0.25–20 μM, with LOD of 115.5 nM. When combined with GOx, the colorimetric assay allows quantitation of glucose with a linear concentration range of 2–50 μM and LOD of 1.14 μM.

Assays based on analyte induced reduction of TMB products through H2O2 mediated oxidation have been developed for the quantitation of various analytes, such as glutathione (GSH) and ascorbic acid (AA), which possess hydrogen donating abilities [96–99]. In this case, the analyte induces a color change from blue to colorless and decreases in the absorbance at 653 nm. C dots prepared from disodium salt of ethylenediaminetetraacetic acid (Na2EDTA·2H2O) through pyrolysis were used for the quantitation of GSH [96]. The TMB oxidation products (cationic radicals) generated by H2O2 in the presence of C dots possessing peroxidase-like activity are reduced by GSH to form TMB. Upon increasing the GSH concentration, the absorbance at 653 nm decreases linearly over the concentration range of 0–7 μM. The assay exhibits an LOD of 0.3 μM for GSH and allows for the quantitation of GSH in human blood samples. C dots prepared from the latex of E. milii medicinal plant through a hydrothermal treatment at 180 °C for 3 h were used for the quantitation of GSH [97]. The prepared C dots with a quantum yield of 39.2% are resistant to high salt (ionic strength) and possess intrinsic peroxidase-like activity for TMB oxidation in the presence of H2O2. The C dots provide a small K_m value of 0.427 mM toward TMB, with a higher V_max (2.2 × 10^{-3} M s^{-1}) when compared to HRP. Having LOD of 5.3 nM and a linear concentration range 0.02–0.1 μM for GSH, the assay is capable of quantitation of GSH in human blood serum samples. C dots prepared from carbon black were applied to detect GSH down to 0.5 μM in the presence of TMB [98]. Having high selectivity and sensitivity, the CDzyme/TMB sensing system allows quantitation of GSH in complicated biological samples like cell lysates. C dots prepared from leaf extracts of neem (Azadirachta indica) through a one-pot hydrothermal method were employed for quantitation of AA [90]. When compared to HRP for H2O2, the prepared C dots exhibit a smaller K_m value (0.49 vs. 3.7 mM), revealing that the artificial nanozyme has greater affinity toward H2O2. The CDzyme allows for the detection of H2O2 down to 35.0 μM, with a linear concentration range of 0.1–0.5 mM. When TMB is the substrate, HRP and the CDzyme show similar K_m values (0.43 vs. 0.51 mM). The result reveals that the CDzyme have less affinity toward TMB than H2O2. The results from the steady-state kinetic analysis suggest a ping-pong mechanism for the oxidation of TMB by the CDzyme. The CDzyme with TMB in the presence of H2O2 allow detection of AA in the concentration range of 5–40 μM, with LOD of 1.8 μM. The sensing system can be applied for the determination of AA in real samples such as common fruits, with good accuracy and precision (Fig. 4).

CDzyme/TMB sensing systems for the quantitation of some oxidative ions such as Fe^{3+} and Ag^{+} have been realized [93]. C dots prepared by refluxing a membranous carbonized β-cyclodextrin in HNO_3 at 300 °C were used for quantitation of Ag^{+} and Fe^{3+} ions [100]. Because of the unique spatial structure of β-cyclodextrin, the carbonized blocks tend to form membranous structures, leading to the formation of high-quality C dots with a fluorescence quantum yield of 6.4%. The C dots possess peroxidase-like activity and allow colorimetric quantification of H2O2 in a linear concentration range of 2–
500 μM and LOD of 1.0 μM. By taking the advantage of strong reduction strengths of Fe$^{3+}$ and Ag$^{+}$ to reduce the TMB oxidative products, the CDzyme/TMB system is sensitive for the colorimetric detection of the two analytes, with LODs of 0.8 and 0.5 μM for Fe$^{3+}$ and Ag$^{+}$ ions, respectively. The assay is also selective towards the two analytes over the potential interfering ionic species (1 mM K$^+$, Na$^+$, Zn$^{2+}$, Fe$^{2+}$, Cu$^{2+}$, Pb$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Al$^{3+}$, and Cr$^{3+}$). A CDzyme/TMB system was developed for highly selective and sensitive detection of Hg$^{2+}$ ions in the presence of cysteine (Cys) [101]. C dots prepared from Na$_2$EDTA·2H$_2$O exhibit peroxidase-like activity to catalyze TMB to form oxidative TMB products. Similar to GSH, Cys is a powerful anti-radical biomolecule, which can reduce the as-formed cationic free radicals. Because Hg$^{2+}$ ions have strong affinity toward thiol compounds, the strength of Cys for reduction of the as-formed cationic free radicals is suppressed in the presence of Hg$^{2+}$ (Fig. 5). The CDzyme/TMB system in the presence of Cys (5 μM) is sensitive for the quantitation of Hg$^{2+}$ ions, with a linear concentration range of 0–0.31 μM and LOD of 23.0 nM. Other metal ions, such as Ag$^+$, Cd$^{2+}$, Cu$^{2+}$, Co$^{2+}$, Ni$^{2+}$, and Pb$^{2+}$ show negligible interference, revealing high selectivity of this assay.

### 3.2. C dot nanocomposites

C dot nanocomposites have been reported to possess enhanced peroxidase-like activity than that of C dots, due to the synergistic effects of different elements (N, Fe, Pt, Cu, Mo, S) [102–107]. Nitrogen- and iron-containing C dots (N,Fe–C dots) synthesized from a branched polyethylenimine (as a nitrogen source) and hemin (as an iron source) through a hydrothermal route at 180 °C show peroxidase-like activity [102]. The Fe species in N,Fe–C dots act like a Fenton’s reagent [103, 104], enhancing their catalytic activity. The N,Fe–C dots were used to develop colorimetric and fluorometric assays for the quantitation of dopamine (DA), based on DA-induced inhibition of the oxidation reaction of TMB. The colorimetric assay for DA has a LOD of 0.03 μM and a visual LOD of 0.05 μM. The analyte dependent signal response of the fluorometric assay is based on an inner filter effect from the oxidized TMB that absorbs the fluorescence (excitation/emission wavelengths 360/452 nm) of N,Fe–C dots. The fluorescent assay exhibits LOD of 20.0 nM for the detection of DA. Having high sensitivity and selectivity, the assay allows for the quantitation of DA in human serum samples. For the preparation of Pt-modified C dots (Pt–C nanocomposites), C dots were firstly synthesized from L-AA by a hydrothermal process at 180 °C, which were then added...
into H₂PtCl₆ solution in the presence of NaBH₄ under magnetic stirring for 48 h [105]. NaBH₄ acted as a reducing agent to reduce H₂PtCl₆ to form Pt on the surface of C dots, leading to the formation of Pt–C nanocomposites. The reduction current intensity of H₂O₂ generated in the Pt–C nanocomposites modified electrode in the absence of TMB is the highest, which is 4-, 3-, and 2.5-fold larger than those of blank, C dots, and Pt-modified electrode, respectively, indicating the highest catalytic activity of the Pt–C nanocomposites for the reduction of H₂O₂. When TMB was added, the current signal generated from the reduction of H₂O₂ by the Pt–C nanocomposites modified electrode decreased sharply to just about 10% of its original value. At the same time, only slight decreases were observed when separately using blank, C dots, and Pt-modified electrode. This is because electrons transfer quickly from TMB to H₂O₂ on the Pt–C nanocomposites surface, leading to decreased electron transfer from the electrode to the solution. As a result, a sharp decrease in current was observed in the presence of TMB. Due to the synergistic effects between C dots and Pt, the peroxidase-like activity of the Pt–C nanocomposite is nine and five times higher than those of C dots and Pt nanoparticles, respectively. The Pt–C nanocomposites in the presence of TMB enable visual and colorimetric detection of H₂O₂ with LOD of 0.8 μM. In the presence of GOx, the sensing system allows detection of glucose down to 1.7 μM. A facile solid-phase synthesis strategy was developed to synthesize Cu-doped CDs (Cu-CDs) using citric acid as the carbon source and Cu(NO₃)₂ as the dopant, respectively [106]. The as-prepared Cu-CDs exhibited superior peroxidase-like activity and were stable under a wide range of pH and temperatures. Consequently, the Cu-CD-based chemiluminescence sensing was applied to sensitively detect glucose with a low detection limit of 0.32 μM, and the recoveries and the relative standard deviation of the serum sample are 87.2-112.2% and 8.16% (n = 6), respectively. Notably, the proposed chemiluminescence sensing was also successfully applied for label-free detection of glucose in complex biological samples. Mo, S co-doped CQDs (Mo-CQDs) as a peroxidase mimic were used to fabricate a cascade colorimetric biosensor to detect cholesterol [107]. The Mo-CQDs possess a robust peroxidase-like activity. The Mo, S doping in the CQDs notably boosts the yield of CQDs and may facilitate the electron transfer between TMB and H₂O₂, which further enhances the catalytic activity of CQDs. The colorimetric biosensor based on Mo-CQDs and cholesterol oxidase exhibited excellent selectivity and high sensitivity for cholesterol in the range of 0.01-1.0 mM along with a detection limit as low as 7 μM. The total cholesterol concentration in the serum sample was measured with satisfactory results and read out by the naked eye, indicating the potential application in clinical diagnosis and portable test kits. Core-shell type Au nanoparticles@C nanocomposites prepared from chloroauric acid/sodium polyacrylate (precursor/soft template) and C dots (citric acid/urea as precursors under 900 W microwave irradiation) through a chemical reduction route show high peroxidase-like activity for H₂O₂ [108]. In the preparation process, Au nanoparticles are in situ reduced by C dots and assemble on the surface of sodium polyacrylate-C dots soft template. In addition, C dots act as both stabilizer and reducing agent. The Au nanoparticles@C nanocomposites have core-shell nanostructure, each with an ultrathin carbon layer of 1–2 nm. When compared to gold nanoparticles alone, the nanocomposite possesses higher peroxidase-like activity, mainly because of more efficient electron transfer between TMB and H₂O₂. K_m value of the nanocomposite for TMB (0.059 mM) is much smaller than those provided by HRP (0.43 mM) and AuNPs (0.74 mM), revealing its higher affinity towards TMB.

By taking the advantages of large surface area, good adsorption ability, and anion exchange property of layered double hydroxides (LDH), C-dot/NiAl–LDH nanocomposites exhibit high peroxidase-like activity for TMB oxidation [109]. The nanocomposite is formed through electrostatic self-assembly of negatively charged C dots and positively charged NiAl–LDH nanoplates. The peroxidase-like activity of the nanocomposite is higher than C dots and NiAl–LDH alone, showing the existence of a synergistic effect between C dots and NiAl–LDH. Compared to HRP, the nanocomposite exhibits a lower apparent K_m value for TMB, revealing its higher affinity for TMB than that of HRP, mainly because of its larger surface area, stronger adsorption ability to TMB, and higher conductivity attributed from the C dots. The CDzyme has been used to develop a colorimetric method for the detection of H₂O₂, providing a linear concentration range of 0.2–20 μM and LOD of 0.1 μM. Also, the CDzyme/TMB sensing system can quantitate H₂O₂ in milk samples, with good accuracy and precision.

Nanocomposites formed from MoS₂ quantum dots and C dots show a synergetic peroxidase-like activity for H₂O₂ [110]. MoS₂ quantum dots with an average size of 1.3 nm were obtained by thermal treatment of MoS₂ nanosheets that had been...
prepared from MoS$_2$ powder through a simple exfoliation method. C dots with an average diameter of 14.5 ± 4.6 nm were prepared by thermal pyrolysis of glucose powder. The catalytic activity of the nanocomposite for H$_2$O$_2$ was determined by monitoring the chemiluminescence (CL) generated from the rhodamine B (RB)-H$_2$O$_2$ reaction. The nanocomposite has a catalytic activity that is 7.2- and 14.3-fold higher than that of the MoS$_2$ and C dots alone, respectively. The assay allows quantitation of H$_2$O$_2$, with a linear concentration range of 1.5–460 nM and LOD of 0.4 nM. Because MoS$_2$ nanosheets enhanced the activity of ChOx for the oxidation of cholesterol, the proposed CL system allows detection of cholesterol, with a linear concentration range of 0.08–300 μM and LOD of 35.0 nM (Fig. 6). Having high sensitivity and selectivity, the assay allows quantitation of cholesterol in human serum samples.

C dot/Fe$_3$O$_4$ nanocomposites also show peroxidase-like activity for sensitive detection of H$_2$O$_2$ and AA [111]. α-Fe$_2$O$_3$ nanofibers synthesized via electrospinning and C dots prepared from citric acid/urea under microwave irradiation at a power of 750 W were used to prepare C dot/α-Fe$_2$O$_3$ hybrid nanofibers through a one-step hydrothermal reaction. The obtained C dot/α-Fe$_2$O$_3$ hybrid nanofibers were then subjected to calcination to form C dot/Fe$_3$O$_4$ nanocomposites. When compared to individual C dots, α-Fe$_2$O$_3$ nanofibers, C dots/α-Fe$_2$O$_3$ hybrid nanofibers, and commercial Fe$_3$O$_4$ nanoparticles, the nanocomposite provides the highest peroxidase-like activity for TMB. The nanocomposite has a low K$_m$ value (0.06 mM) for TMB, showing its strong affinity toward TMB. Colorimetric assays using the nanocomposite and TMB allow for the detection of H$_2$O$_2$ and AA, with LODs of 0.9 and 0.3 μM, respectively. A simpler approach was developed for the preparation of C-dot/Fe$_3$O$_4$ nanocomposite from mixing solutions of C dots and Fe$_3$O$_4$ nanoparticles [112]. The nanocomposite is formed through the interaction of the OH groups on the Fe$_3$O$_4$ nanoparticles with the OH and CO$_2$H groups on the surfaces of C dots. The nanocomposite in the presence of TMB exhibits V$_{\text{max}}$ and K$_m$ of 1.4 × 10$^{-7}$ M s$^{-1}$ and 3.5 mM for H$_2$O$_2$, respectively. The K$_m$ value is close to that of HRP. Relative to C dots and Fe$_3$O$_4$ nanoparticles, the nanocomposite provides 44.0- and 7.6-fold lower K$_m$ value, showing its stronger affinity toward H$_2$O$_2$. The CDzyme/TMB system is sensitive for the detection of H$_2$O$_2$, with a linear concentration range of 10.0 nM–1.0 mM and LOD of 1.0 nM.

Nanocomposites of C dots with CuO nanostructures also show high peroxidase-like activity for H$_2$O$_2$ [113]. Graphene oxide (GO) was firstly
prepared from graphite powder according to the Hummers and Offeman method [114]. Then, C dots were prepared from GO through a microwave-hydrothermal approach, which were then used as supports for the growth of CuO nanoneedles from copper acetate to produce C dot/CuO nanocomposites. When compared to the individual C dots and CuO nanoneedles, the nanocomposite provides a lower $K_m$ value (0.098 mM) and thus a stronger affinity toward $H_2O_2$, showing a synergistic effect from the two nanomaterials. $H_2O_2$ is adsorbed on the nanocomposite surface and then activated by the $Cu^{2+}$ to generate hydroxyl species ($\bullet$OH) that oxidizes TMB into its blue colored form (Fig. 7). The CDzyme/TMB sensing system shows a linear range of $0.5–10 \, \mu M$ and LOD of 0.2 $\mu M$ for $H_2O_2$. In the presence of GOx, the sensing system allows detection of glucose in serum samples, with a linear concentration range of $2–100 \, \mu M$ and LOD of 0.6 $\mu M$.

By taking advantage of electrical conductivity of C dots, nanocomposites were prepared from ZnFe$_2$O$_4$ and C dots to achieve high sensitivity for the electrochemical determination of DNA [115]. The ZnFe$_2$O$_4$ nanoparticles were prepared from ZnCl$_2$ and FeCl$_3$·2H$_2$O through a hydrothermal route. Then C dots prepared from GO aqueous suspension through the Hummers and Offeman approach were assembled on the surface of ZnFe$_2$O$_4$ nanoparticles through a photo-Fenton reaction. During the photo-Fenton reaction, radicals ($\bullet$OH and $\bullet$O$_2$H) were generated from Fe$^{3+}$ and $H_2O_2$. Small graphene sheets were formed through a simple exfoliation of GO in the presence of radicals and then adsorbed on the ZnFe$_2$O$_4$ surface as in situ nucleation of C dots. The resulting composites are spherical particles with diameter of about 100–120 nm, each with a thin layer (20 nm) on the particle surface. The ZnFe$_2$O$_4$/C dot nanocomposite labeled with complementary ssDNA (S3; 5’-$NH_2$-$(CH_2)_6$-ATG TCC CTC AGA CCC TTT-3’) was then used as an enzyme mimic. In the presence of target DNA (S2; 5’-ACT GCT AGA GAT TTT CCA CAC TGA CTA AAA GGG TCT GAG GGA-3’), the nanocomposite is deposited on a Pd nanowire/graphene sheet-ssNDA (S1; 5’-TGG AAA ATC TCT AGC AGT CGT-$(CH_2)_6$-SH-3’) modified glassy carbon electrode through DNA hybridization. The ZnFe$_2$O$_4$/C dot nanocomposite-ssNDA (S3) interacts strongly with the functional electrode surface, providing stability and durability to the electrode. The functional electrode shows high peroxidase-like catalytic activity for detection of $H_2O_2$ as shown in Fig. 8. Using thionine as an electron mediator, the assay allows the detection of the target DNA (S2) with a linear concentration range of 0.1 fM–5 nM and LOD of $6.2 \times 10^{-17}$ M. The reason for such a high sensitivity for the target DNA sensor is mainly due to a good pathway for electron transfer provided by the Pd nanowire/graphene sheet and high surface density of the capture probe (S1) on the electrode surface. Having such a high sensitivity and selectivity, the assay is able to quantify target DNA in human serum samples, showing their great potential for gene diagnostics.

### 4. Conclusion and outlook

CDzymes possessing peroxidase-like activity have several attractive features, including catalytic activity, ease in preparation, (photo)chemical stability, cost-effectiveness, and good biocompatibility. They...
alone or in conjunction with various enzymes have been used to develop sensing systems (Table 1) for sensitive and selective quantitation of many analytes. When compared to HRP, the CDzymes are advantageous of low cost, long shelf life, and good thermal/pH stability. Compared to colorimetric methods, fluorescence and electrochemical methods using CDzymes are more sensitive. Nevertheless, colorimetric assays can be performed free of any instrumentation (i.e., by the naked eye). Like most nanozymes, most reported CDzymes are limited to samples without containing complicated matrices, mainly because of loss in their activity once interfering species are adsorbed on their surfaces. In this case, surface modification of C dots with polymers are preferred to minimize nonspecific adsorption. When conducting surface modification, great attention must be paid to minimize the loss of the catalytic activity of C dots.

Although most of reported CDzymes do not exhibit catalytic activity as high as that of HRP, their catalytic activity can be further enhanced by careful selection of carbon precursors and passivation of their surface with active ligands. Carbon precursors having high affinity towards electron-rich substrates shall be useful for preparation of CDzymes with high catalytic activity. Since N-doping C dots have shown higher activity than C dots, C dots containing heteroatoms such as S, P, and B, with different structures shall be tested. To further enhance their activity, C dots with high surface area and great amounts of surface defects are required. Treating C dots with strong inorganic acids/bases, photolysis, and/or oxygen plasma can be efficient for varying their size, morphology, and structure.

Although CDzymes have become more popular in recent years, only ones with peroxidase-like activity have been used for developing sensitive and selective sensing systems. To expand their applications, nanocomposites formed from C dots and metal (oxide) nanoparticles, including Pt, Pd, Au, Ag, and BiOx, shall be good candidates. Based on the fact that the activity of metal nanoparticle can be tuned/changed by deposition of different metal ions, nanocomposites of C dots with metal nanoparticles containing different metal ions such as Au–Ag and BiOx–Pt nanoparticles are also good to be tested. Once CDzymes with various enzymes-like activities are available, it will be interesting to develop C dots based enzyme cascades.
| Nanocarbon | Abbreviation in Ref. | Carbon source | Synthesis method | Detection method | Target | LOD | Real sample | Ref. |
|------------|----------------------|---------------|------------------|------------------|--------|-----|-------------|------|
| C dots | C-Dots | Candle soot | Reflux with HNO₃ at 140 °C for 12 h | Colorimetric | H₂O₂ | 0.2 µM | human serum | [83] |
| t-CDs | Lampblack | | Reflux with HNO₃ at 140 °C for 12 h, and then reduction with NaBH₄ | Colorimetric | glucose | 0.4 µM | human serum | [84] |
| CQDs | Litchi rind | | Reflux with HNO₃ at 140 °C for 12 h | Colorimetric | glucose | 2.0 µM | human serum | [86] |
| E-GQDs | Wood charcoal | | Electrochemical oxidation at 5 V in the presence of 0.01 M (NH₄)₂S₂O₈ | Colorimetric | H₂O₂ | 0.9 µM | - | [87] |
| o-GQDs | Multiwalled carbon nanotubes | | Reflux with HNO₃ at 140 °C for 48 h | Colorimetric | glucose | 0.2 µM | blood from Balb/c mice | [88] |
| GQDs | Graphite powder | | Wet chemical oxidation method (sonicated for 2 h and 30 min at room temperature followed by stirring for 45 min at 90 °C.) | Colorimetric | H₂O₂ | 20 nM | - | [89] |
| N-GQDs | Graphite powder, dopamine | | Hydrothermal treatment at 75 °C for 6 h | Colorimetric | H₂O₂ | 5.3 µM | human serum, commercial fruit juices | [92] |
| CNDs | Dimethylamine | | Microwave heat-treatment for 60 s | Colorimetric | H₂O₂ | 16.0 µM | - | [93] |
| CDs | Na₂EDTA | | Pyrolysis at 400 °C for 2 h | Colorimetric | GSH | 0.3 µM | human whole blood | [96] |
| CQDs | Latexes of E. milii plant | | Hydrothermal treatment at 180 °C for 3 h | Colorimetric | GSH | 5.3 nM | human serum | [97] |
| GDs | Carbon black | | Reflux with HNO₃ at 130 °C for 24 h | Colorimetric | H₂O₂ | 10 nM | cell lysate | [98] |
| N-CQDs | Leaf extracts of neem (Azadirachta indica) | | Hydrothermal treatment at 150 °C for 4 h | Colorimetric | H₂O₂ | 35.0 µM | fresh fruit juice | [99] |
| CDs | β-Cyclodextrin | | Reflux with HNO₃ for 12 h | Colorimetric | H₂O₂ | 1.8 µM | - | [100] |
| CDs | Na₂EDTA | | Pyrolysis at 400 °C for 2 h | Colorimetric | H₂O₂ | 1.0 µM | - | [101] |

(continued on next page)
| Nanocarbon            | Abbreviation in Ref. | Carbon source                          | Synthesis method                                           | Detection method      | Target | LOD     | Real sample      | Ref. |
|----------------------|----------------------|----------------------------------------|------------------------------------------------------------|-----------------------|--------|---------|-----------------|------|
| C dot nanocomposites | N,Fe-CDs             | BPEI, hemin                            | Hydrothermal treatment at 180 °C for 10 h                  | Colorimetric          | DA     | 0.03 μM | human serum     | [102]|
| Pt-CDs               | L-ascorbic acid,     | H$_2$PtCl$_6$                          | Hydrothermal treatment at 180 °C for 4 h                   | Fluorescence Colorimetric | H$_2$O$_2$ | 0.8 μM | -               | [105]|
| AuNPs@CDs            | Citric acid, chloroa |                                      | Microwave heat-treatment for 300 s and chemical reduction route | -                     | glucose | 1.7 μM | -               | [108]|
| C-dot/NiAl--LDH      | Citric acid, Ni(NO$_3$)$_2$, Al(NO$_3$)$_3$ |                                      | Hydrothermal treatment at 200 °C for 3 h and simple mixing at room temperature | Colorimetric          | H$_2$O$_2$ | 0.1 μM | milk            | [109]|
| MoS$_2$ QDs, GQDs    | Glucose, MoS$_2$     |                                       | Pyrolysis at 180 °C for GQDs and heated at 120 °C for MoS$_2$ QDs | Chemiluminometric     | H$_2$O$_2$ | 0.4 nM | human serum     | [110]|
| CDs/Fe$_3$O$_4$      | Citric acid, Fe(NO$_3$)$_3$ |                                      | Microwave heat-treatment for 300 s and hydrothermal treatment at 140 °C for 4 h, following calcined at 500 °C for 4 h | Colorimetric          | H$_2$O$_2$ | 0.9 μM | -               | [111]|
| C-dots/Fe$_3$O$_4$   | Carbon soot, FeCl$_3$|                                       | Reflux with HNO$_3$ and mixed together in acidic media for 30 min | Colorimetric          | H$_2$O$_2$ | 1.0 nM | -               | [112]|
| GQDs/CuO             | GO, copper acetate   |                                       | Microwave heat-treatment at 200 °C for 8 min and simple mixing at room temperature | Colorimetric          | H$_2$O$_2$ | 0.2 μM | -               | [113]|
| ZnFe$_2$O$_4$/GQDs   | GO, ZnCl$_2$, FeCl$_3$ |                                       | Photo-Fenton reaction (365 nm, 1000 W)                     | Differential pulse voltammetry | DNA     | $6.2 \times 10^{-17}$ M | human serum | [115]|

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Recently, our research group has found that C dots with microstructures show light-induced peroxidase-like activity (unpublished results). Their activity is higher than individual C dots and depends on the wavelength of irradiation light. Their activity is also dependent on oxygen content, and thus it will be interesting to develop sensing systems for monitoring the oxygen content inside cells using C dot microstructures. When C dots with microstructures are used to entrap various chemicals such as metal, metal oxide, or oxidizing/reducing agents, CDzymes with different enzyme-like activities shall be available. In addition, their photo-induced catalytic activity shall be enhanced, enabling more sensitive sensing systems.

Conflicts of interest
The authors declare no conflicts of interest.

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