Antioxidant capacity of Nitrogen and Sulfur co-doped carbon nanodots

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Abstract:

Carbon nanodots (CNDs) have shown potential for antioxidative activity at the cellular level. Here we applied a facile hydrothermal method to prepare fluorescent nitrogen and sulfur (N,S-) codoped CNDs using α -lipoic acid, citric acid, and urea as precursor molecules. This work describes a comprehensive study for exploring their antioxidation activity using UV-vis absorption and electrochemistry measurements of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH $^{\bullet}$), as well as a lucigenin chemiluminescence (lucigenin-CL) assay. The lucigenin-CL assay detects superoxide anion radicals, i.e., reactive oxygen species (ROS) produced through the xanthine/xanthine oxidase (XO) reaction. The electrochemically derived relationship between the unreacted nitrogen-centered DPPH $^{\bullet}$ and CND concentrations agrees with that obtained from UV-vis measurements. A reaction pathway for the ROS antioxidative reaction of N,S-codoped CNDs is proposed. These findings should aid in the development of N,S-codoped CNDs for practical use in biomedical applications.

Keywords: carbon nanodots | nitrogen and sulfur doping | antioxidation

Article:

Introduction

Carbon nanodots (CNDs) display desirable properties for biomedical applications, including low toxicity, excellent photoluminescence, and good biocompatibility.⁽¹⁻⁵⁾ Both bottom-up and top-down approaches have been used for the synthesis of CNDs.⁽⁶⁾ Top-down approaches include chemical ablation, electrochemistry-based carbonization, and laser ablation, while bottom-up approaches include microwave irradiation and hydro- or solvothermal methods.⁽⁷⁻⁹⁾ CNDs have been used in bioimaging and biosensing because of their excellent fluorescence and low cytotoxicity.^(10,11) Photoluminescence has been attributed to the CNDs' conjugated π states, functional groups, and surface electronic states,⁽¹²⁾ and it can be tuned by changes in their compositions and structures, as well as by doping with other nonmetallic elements.⁽¹³⁾ For instance, Zhang's group has improved quantum yields by doping the CNDs with nitrogen.⁽¹⁴⁾ Prato's group has reported new procedures for the synthesis of size- and surface-controllable and structurally defined, highly fluorescent N-doped CNDs.⁽¹⁵⁾ Electron-rich N atoms can shift the CNDs' electronic states and create additional active sites, which enhances the CNDs' photoluminescence intensity.⁽¹⁶⁾ Both S-doped and N,S-codoped CNDs show enhanced fluorescence and photoluminescence quantum yields in bioimaging and sensing applications.^(13,17,18)

CNDs also show promise for protecting cells from oxidative stress. For example, superoxide or hydroxyl radicals were reported to be scavenged by CNDs that were prepared by using date molasses through microwave irradiation techniques.⁽¹⁹⁾ Recently, the antioxidation activity of the N-doped CNDs was studied by embedding the CNDs in ionic liquid solutions, and the radical scavenging activity was ascribed to their rich amino surface.⁽²⁰⁾ Chong et al. synthesized graphene quantum dots and studied the difference in their oxidation activity in cells, both with and without light.⁽²¹⁾ While some additional studies exist on the CNDs' antioxidation activity using cellular and chemical assays,⁽²²⁻²⁴⁾ a better understanding of the antioxidation properties of CNDs is needed in order to facilitate their potential applications in biomedicine. To help elucidate their mechanism of action, the antioxidation activities of N,S-codoped CNDs and their underlying antioxidation behaviors are examined herein using optical, electrochemical, and biochemical superoxide scavenging assays.

The DPPH[•]-based assay is a commonly used method to evaluate the antioxidant activity.⁽²⁵⁾ Upon reaction with antioxidants in a solution, the deep-violet color of a DPPH[•] solution changes to light yellow with its conversion to the DPPH-H complex. This reaction progress was monitored by measuring the absorbance changes at 517 nm,^(26,27) and the unreacted DPPH[•] concentration as a function of the CND concentration was evaluated by cyclic voltammetry.⁽²⁶⁾ Lucigenin-derived chemiluminescence (lucigenin-CL) measurement is another powerful tool to investigate the superoxide scavenging capability in vitro related to mitochondria-derived reactive oxygen species (ROS).⁽²⁸⁾ Xanthine can be oxidized to uric acid upon the addition of the enzyme xanthine oxidase (XO). This reaction causes the production of $O_2^{\bullet-}$.⁽²⁹⁾ Thus, XO is regarded as a biological superoxide radical source that plays a role in generating oxidative stress.⁽³⁰⁾ In the experiments presented herein, the xanthine/XO system is combined with lucigenin-CL measurements to evaluate the N,S-codoped CNDs' superoxide scavenging activity.

We used a hydrothermal method to prepare the N,S-codoped CNDs from three precursor molecules (α -lipoic acid + citric acid + urea). The purified CNDs were characterized with regard to morphology, elemental content, and optical properties. It is expected that the N and S doping

in CNDs plays a synergistic role that enhances the fluorescence intensity of CNDs and increases their antioxidative activity because of the increase in the π -system electron density, which enhances the CNDs' electron-donating ability.⁽²²⁾ Compared with the aforementioned radical scavenging studies of CNDs,^(19–26) this work reports some new findings on the antioxidation capacity of N,S-codoped CNDs for DPPH[•] free radicals and an unprecedented scavenging activity for ROS generated by the xanthine/XO system. The mechanistic reaction pathways for antioxidation are proposed based on their underlying reactivity and multiple assays using UV–vis spectroscopy and electrochemistry with DPPH[•] and lucigenin-CL. Additionally, the bioimaging of cells was performed using the CNDs' fluorescence at different excitation wavelengths, and the CNDs' biocompatibility was evaluated by a cytotoxicity study.

Experimental Section

Synthesis of N,S-Codoped CNDs. Scheme 1 shows a general route to obtaining N,S-codoped CNDs. A hydrothermal method is applied to synthesize the CNDs using α -lipoic acid + citric acid + and urea as precursors. Briefly, deionized water (20 mL) was used to dissolve sodium hydroxide (0.36 g, Aldrich) to get a basic solution. Then, citric acid (0.2 g, 99%, Acros Organics), α -lipoic acid (0.6 g, 99%, Aldrich), and urea (1.0 g, 99.5%, Aldrich) were simultaneously added to the basic solution, forming a homogeneous yellow solution. This yellow solution was transferred to a Teflon reactor (50 mL volume, PPL-lined vessel chamber kettle, 250 °C) and heated to 230 °C for 18 h. Next, the aqueous reaction solution was cooled to room temperature, and it was purified using dialysis (1000 MWCO, Fisher Scientific) against deionized water for 24 h. The water was changed three times in order to aid in the dialysis. Last, a freeze-drying method (24 h in FreeZone 6, Labconco) was used to dry the resulting product. The final N,S-codoped CNDs' yield was measured to be 41.5 mg.

Scheme 1. Illustration of the Preparation Route for Obtaining N,S-Codoped CNDs

Characterization of N,S-Codoped CNDs. We used transmission electron microscopy (TEM; Carl Zeiss Libra 120 PLUS) and atomic force microscopy (AFM; Agilent 5600LS) to quantify the size and evaluate the morphology of the N,S-codoped CNDs (on a copper grid coated with carbon for TEM and a fresh mica for AFM). Fourier transform infrared spectroscopy (FTIR; Varian 670), Raman spectroscopy (Horiba XploRA ONE), and X-ray photoelectron spectroscopy (XPS; Thermo Fisher ESCALAB 250Xi) were used to examine the chemical structure and elemental content of the N,S-codoped CNDs. UV–vis spectroscopy (Agilent Cary 6000i) and fluorescence spectroscopy (Agilent Cary Eclipse) were employed to study the optical properties of the N,S-codoped CNDs.

DPPH[•]: UV–Vis Spectroscopy-Based Assay. We used a UV–vis spectrometer to monitor the absorbance change at 517 nm and then to calculate the antioxidation activity of the N,S-codoped CNDs to DPPH[•] (Alfa Aesar).⁽³¹⁾ DPPH[•] was added into absolute methanol to obtain a solution concentration of 0.02 mg/mL for each measurement. Then, N,S-codoped CNDs with different concentrations were added into the DPPH[•] solutions and allowed to incubate in the dark for 2 h.

DPPH[•]: Electrochemistry-Based Assay. A three-electrode electrochemical cell (a gold working electrode, an Ag/AgCl reference electrode, and a platinum counter electrode; Fisher Scientific) was used to conduct the cyclic voltammetry under room temperature at different scan rates. The studied solutions (at pH = 7.4, 5 mL) contained 0.02 mg/mL DPPH[•] to which N,S-codoped CNDs were added at different concentrations, which was prepared using a 1:1 mixture solution by volume of absolute methanol (Fisher Scientific) and phosphate-buffered (PBS; pH = 7.0, Life Tech). As with the UV–vis studies, the DPPH[•] solution was incubated with N,S-codoped CNDs for 2 h in the dark prior to measurement.

Lucigenin-CL Study. Chemiluminescence (CL) was monitored with a BioTek microplate reader (Winooski, VT) at 37 °C for 30 min. For the enzyme system, the reaction mixture contained 100 μM xanthine (99%, Aldrich) and 10 mM XO (grade I, Aldrich) in 1 mL of a PBS solution (pH = 7.4) containing 0.1 mM diethylenetriaminepentaacetic acid, and the reaction was initialized by adding 5 μL of lucigenin (5 μM). The superoxide scavenging activities of the N,S-codoped CNDs were analyzed by adding the N,S-codoped CNDs at different concentrations into the xanthine/XO system to quench the Lucigenin-CL emission intensity.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT)-Based Assay. EA hy926 endothelial cells from ATCC (Manassas, VA) were cultured with Dulbecco's modified Eagle's medium (DMEM; Life Tech) containing fetal bovine serum (FBS; 10%) and penicillin–streptomycin (1%) at 37 °C in 5% CO₂ for 24 h. A 48-well culture plate was used to seed the cells, and their density was 1.5×10^5 per well. Then, Hank's balanced salt solution (HBSS media) was used to replace the culture medium containing different doses of N,S-codoped CNDs to culture cells for another 24 h. Afterward, 300 μL of a 0.2 mg/mL MTT (99%, Fisher Scientific) solution was added to each well culture plate. After incubation for 2 h at 37 °C, the cell culture plate was rinsed twice with 200 μL of PBS for each well and 300 μL of dimethyl sulfoxide was added. At room temperature, the resulting solution was shaken for 10 min. Finally, we measured the absorption at 570 nm with a BioTek microplate reader.

Multicolor Bioimaging. EA hy926 endothelial cells were seeded in an 8-chamber slide with DMEM containing 1% penicillin–streptomycin and 10% FBS at 37 °C in a 5% CO₂ incubator for 24 h. DMEM was then removed, followed by the addition of the mixture of N,S-codoped CNDs (100 μg/mL) and the DMEM medium containing 0.5% FBS into each chamber, and the solution was allowed to incubate for 24 h. After that, the slide was rinsed by PBS twice to remove an extra medium and the N,S-codoped CNDs. The CND incubated cells were fixed with 4% paraformaldehyde (Fisher Scientific). Cellular fluorescent images were obtained using an Olympus IX70 inverted fluorescence microscope.

Results and Discussion

Figure 1. Characterization of the N,S-codoped CNDs using different techniques: (A) TEM images; (B) size distribution based on the TEM image; (C) AFM topography image; (D) a representative height profile from the AFM image; (E) FTIR spectra; (F) Raman spectra; (G and H) XPS spectra (C 1s and S 2p); (I) UV-vis absorption spectra.

N,S-Codoped CND Synthesis and Characterization. TEM data (Figure 1A) and the associated size distribution (Figure 1B) indicate that the CNDs have an average size of about 3 nm. This finding is supported by AFM data (Figure 1C) and the associated height profile analyses (Figure 1D). The FTIR spectra of the CNDs (Figure 1E) display broad bands at 3100–3400 cm^{-1} , which are assigned to $\nu(\text{N-H})$ and $\nu(\text{O-H})$ stretching motions. The OH and NH functionalities add hydrophilicity to the CNDs, making them stable in an aqueous solution. The FTIR signals at 764 cm^{-1} (C–C), 1002 cm^{-1} (C–S), 1179 cm^{-1} (C–O), 1413 cm^{-1} (C=C), and 1565 cm^{-1} (C=O) were assigned.^(32,33) The Raman spectra (Figure 1F) give an I_D/I_G ratio of about 0.98, obtained from the intensities at 1347 cm^{-1} (D bands, sp^3 -hybridized) and 1562 cm^{-1} (G bands, sp^2 -hybridized), and this finding is consistent with a disordered graphite structure for the CNDs.⁽³⁴⁾ A C–S stretching transition was assigned to the 653 cm^{-1} transition.⁽³⁵⁾ XPS data (Figure 1G,H and the survey spectrum of Figure S1) corroborate the functionalities of the CNDs. On the basis of the C

1s XPS spectra, five components were detected at 289.0 eV (O=C—OH), 287.8 eV (C=N and C=O), 286.6 eV (C—N and C—O), 285.6 eV (C—S), and 284.8 eV (C=C and C—C), respectively.^(36,37) The S 2p XPS spectrum was fitted to a sum of two doublets at 165.0 eV (C—S, S 2p_{1/2}), 163.7 eV (C—S, S 2p_{3/2}), 163.0 eV (S²⁻, S 2p_{1/2}), and 161.7 eV (S²⁻, S 2p_{3/2}).^(38,39) Altogether, these data indicate that the N,S-codoped CNDs have a spherical morphology with an average size of ~3 nm, which possess a graphitic structure doped with N and S and display surface functionalities of carboxylates and amines/amides. The 270 nm band in the absorption spectrum of Figure 1I is assigned to the π – π^* transition of C=N bonds,^(40,41) and the feature between 300 and 350 nm is attributed to surface states.^(32,42) Using a quinine sulfate reference for photoluminescence quantum yield measurements,⁽¹²⁾ the relative quantum yield was measured to be 11% (Table S1) for the N,S-codoped CNDs.

DPPH•: UV–Vis-Based Assay. UV–vis spectroscopy of DPPH• was used to evaluate the antioxidation activity of the CNDs. In a methanol solution, DPPH• is a radical, and the solution presents a dense violet color.⁽²⁷⁾ Upon the addition of N,S-codoped CNDs, the solution color changes; i.e., the absorbance intensity of the DPPH• methanol solutions at 517 nm decreases (Figure 2A). The antioxidant activity can be calculated from the equation

$$\text{antioxidation activity} = \frac{A_0 - A_c}{A_0} \times 100\% \quad (1)$$

in which A_0 and A_c represent the absorbances of DPPH• at 517 nm without and with the N,S-codoped CNDs, respectively. The antioxidation activities of 16.2%, 39.0%, 70.0%, 88.4%, and 92.9% are obtained (averaged value based on three trials) at the N,S-codoped CND concentrations of 0.02, 0.03, 0.05, 0.07, and 0.10 mg/mL, respectively. The DPPH• with the N,S-codoped CND incubation reaches its steady state after 2 h, and the solution's antioxidant activity increases when the concentration of N,S-codoped CNDs increases from 0.02 to 0.07 mg/mL. A plateau in the activity is found at higher concentrations of N,S-codoped CNDs (>0.07 mg/mL; Figure 2B). The concentration dependence of N,S-codoped CNDs is consistent with that of graphene quantum dots and some other types of CNDs.^(21,24,26) Using the same hydrothermal method, CNDs from citric acid (CNDs without N,S doping) and from urea + citric acid (N-doped CNDs) were prepared and evaluated by the DPPH• test (see Figure S2). It was found that the N,S-codoped CNDs have the highest antioxidation activity. For example, at a 0.10 mg/mL incubation concentration, the antioxidation activity order is N,S-codoped CNDs (~92.9%; Figure 2B) > N-doped CNDs (~71.1%; Figure S2D) > CNDs without N,S doping (~34.6%; Figure S2B). Because the electronegativity of N (3.04 in the Pauling scale) is larger than that of C (2.55 in the Pauling scale), doping N onto a C framework creates a high positive charge density on the C atom,⁽⁴³⁾ facilitating the interaction between the N-doped CNDs and DPPH•. Furthermore, the atomic radii of N (~0.65 Å) and C (~0.70 Å) are significantly smaller than that of S (~1.10 Å); such a significant size difference may induce Stone–Wales defects and strain in the C framework.⁽⁴⁴⁾ It has been reported that more catalytic sites could be generated for the oxygen reduction reaction due to the Stone–Wales defect generation in the C framework by doping S onto the reduced graphene oxide.⁽⁴⁵⁾ The defects generated in the C crystal lattice may serve as sites for the radical scavenging reduction reactions. Moreover, considering that the addition of S increases the CNDs' polarizability (N,S-codoped CNDs' polarizability > N-doped CNDs' polarizability),⁽⁴⁶⁾ the N,S-codoped CNDs may act as a stronger electron donor, further

facilitating the radical scavenging activity. Hence, one may conclude that the high antioxidation activity of the N,S doping may result from a synergistic effect of the electronegativity difference between N and C, S-induced active defect generation, and the high polarizability of S.

Figure 2. (A) UV-vis absorption spectra after incubation of the N,S-codoped CNDs (from 0 to 0.10 mg/mL) into DPPH• solutions (0.02 mg/mL). (B) Antioxidation activity curve as a function of the N,S-codoped CND concentration. The error bars are smaller than the symbol size.

DPPH•: Electrochemistry-Based Assay. In a previous study, we used an electrochemical method to quantify how incubation with different concentrations of CNDs affects the unreacted DPPH• concentrations.⁽²⁶⁾ Without CND incubation, we found that the faradaic current for the DPPH• redox reaction is diffusion-limited in an electrolyte solution.⁽⁴⁷⁾ With CND incubation, the neutral DPPH-H is formed from DPPH• by taking up a proton from the CNDs. This process can occur through a mechanism of H-atom transfer (HAT),⁽²⁶⁾ involving surface functional groups, like -COOH, of the CNDs acting as proton donors. Higher concentrations of CNDs, but below 0.1 mg/mL, decrease the concentrations of the free radical DPPH• as the redox species in the electrolyte.⁽²⁶⁾

After incubation with N,S-codoped CNDs, the cyclic voltammograms of DPPH• present different redox peak currents in the methanolic PBS solution at different concentrations of CNDs (Figures 3 and S3). Control experiments (Figure S4), in the absence of DPPH•, do not show redox peaks in a methanolic PBS solution of 0.05 mg/mL N,S-codoped CNDs over the potential window (vs Ag/AgCl) ranging from 0.0 to 0.7 V. This result agrees with recent studies on the redox properties and electronic states of CNDs whose redox reactions usually occur at more negative (< -1.0 V) or more positive (> 0.7 V) potentials.^(48,49) Hence, the redox peaks in Figure 3 result from an electrochemical reaction with DPPH•.⁽²⁶⁾ In another control experiment that used a DPPH• solution without CNDs, the cyclic voltammograms (Figure S5) show that the redox peak currents obtained from DPPH• at a concentration of 0.01 mg/mL are much lower than that obtained from a solution with a DPPH• concentration of 0.02 mg/mL (Figure S3A). The anodic/cathodic peak current magnitudes decrease as the concentration of N,S-codoped CNDs increase in solution, further substantiating our claim that N,S-codoped CNDs react with DPPH•. Note that a one-electron-transfer process is supported by the full-width-at-half-maximum (fwhm) value of about 110 mV (at 50 mV/s in Figure S3) in the faradaic waves. A diffusion coefficient (D_0 , cm²/s) was extracted according to the relationship between the peak current, i_p (A), and the scan rate (V/s) by using the Randles-Sevcik formula, namely,^(50,51)

$$i_p = (2.69 \times 10^5) n^{3/2} A C D_0^{1/2} \nu^{1/2} \quad (2)$$

where n is the number of electrons exchanged, A is the electrode active area (0.043 cm^2), C represents the DPPH $^{\bullet}$ concentration (mol/mL), and ν is the voltage scan rate (V/s). D_0 , the diffusion coefficient of DPPH $^{\bullet}$, was found to be $2.0 \times 10^{-5} \text{ cm}^2/\text{s}$. The N,S-codoped CND concentration-dependent slopes in Figure 4A and the diffusion coefficient determined by eq 2 were used to calculate the unreacted DPPH $^{\bullet}$ concentrations in different solutions (Figure 4B). The free DPPH $^{\bullet}$ concentration was found to be 31.8, 24.8, 18.2, 12.1, and 6.6 nmol/mL at the N,S-codoped CND concentrations of 0.02, 0.03, 0.05, 0.07, and 0.10 mg/mL, respectively. In the end, we calculated the scavenging activity using the equation

$$\text{scavenging activity} = \frac{C_0 - C_c}{C_0} \times 100\% \quad (3)$$

in which C_0 and C_c are the concentration of DPPH $^{\bullet}$ without and with the incubation of N,S-codoped CNDs. The scavenging activity was calculated to be 38.9%, 52.2%, 65.1%, 76.7%, and 87.4% (Figure 4C) for 0.02, 0.03, 0.05, 0.07, and 0.10 mg/mL concentrations of CNDs, respectively. These findings are corroborated by the antioxidation activity results shown in the UV-vis spectroscopy experiments.

Lucigenin-CL Study for N,S-Codoped CNDs. Lucigenin-CL has been widely used for biological and enzyme assays, especially for in vitro and in vivo superoxide detection.^(52,53) XO can catalyze the oxidation of xanthine to uric acid and superoxide, a reaction believed to be involved in inflammatory disease.⁽⁵⁴⁾ Lucigenin is one of the CL probes used for detecting superoxide production in various cellular systems.⁽²⁸⁾ Here, we used lucigenin as a CL probe and XO as a ROS production source for measuring the superoxide scavenging activity of N,S-codoped CNDs. Figure 5A shows lucigenin-CL quenching as a function of the N,S-codoped CND concentration. The lucigenin-CL intensity decreases with increasing N,S-codoped CND concentration until it reaches a plateau at concentrations of 0.05 mg/mL and higher for N,S-codoped CNDs. The intensity of lucigenin-CL is quenched by about 59% at 0.10 mg/mL of N,S-codoped CNDs (Figure 5B). The antioxidation activity that is found for N,S-codoped CNDs in a DPPH $^{\bullet}$ solution from UV-vis and electrochemical studies is more efficient than the scavenging activity for ROS that is observed in the lucigenin-CL experiments (e.g., 90% vs ~59% for a N,S-codoped CND concentration of 0.10 mg/mL).

Figure 3. Cyclic voltammograms for the DPPH•-gold electrode system shown at different scan rates for different concentrations of N,S-codoped CNDs: 0.00, 0.02, 0.03, 0.05, 0.07, and 0.10 mg/mL.

Figure 4. (A) Linear dependence of the peak currents on the scan rates incubated with different N,S-codoped CND concentrations. (B) Reserved DPPH[•] concentrations at different N,S-codoped CND concentrations. (C) Calculated scavenging activity versus N,S-codoped CND concentration.

Figure 5. (A) Superoxide scavenging activities of different concentrations of N,S-codoped CNDs measured by xanthine/XO-system-induced lucigenin-CL. The control is without N,S-codoped CND treatment. (B) Calculated scavenging activity versus N,S-codoped CND concentration.

Figure 6 illustrates the proposed antioxidation reaction pathways for lucigenin-CL quenching by CNDs. Univalent reduction of lucigenin generates the lucigenin cation radical, and it may react with XO-derived ROS (i.e., the superoxide $O_2^{\bullet-}$) to generate lucigenin dioxetane.⁽⁵⁵⁾ Two *N*-methylacridone molecules can be formed by the decomposition of this unstable dioxetane intermediate; one of them forms as the electronically excited state and can emit a photon to relax to the ground state.⁽⁵⁶⁾ It is anticipated that the synergistic effect of N (electronegativity-difference-induced high electron density) and S (size-difference-induced defect generation and high polarization of S) doping promotes the conjugated π system to act as an electron donor (Figure 6),⁽²²⁾ allowing the N,S-codoped CNDs to work as superoxide scavengers and reduce the concentration of a XO-derived $O_2^{\bullet-}$ superoxide (Figure 6, step 1). This process will inhibit the reaction between the lucigenin cation radical and the superoxide, decreasing the formation of the dioxetane intermediate (Figure 6, step 2). Consequently, lucigenin-CL is quenched by the addition of N,S-codoped CNDs (Figure 6, step 3). Because higher concentrations of N,S-codoped CNDs result in higher superoxide scavenging activity, a lower intensity of lucigenin-CL is observed. These results are corroborated by the DPPH $^{\bullet}$ assay. To the best of our knowledge, this is the first instance where N,S-codoped CNDs have been shown to act as antioxidants in enzyme-generated ROS scavenging.

Figure 6. Schematic illustration of the reaction pathways of ROS scavenging by N,S-codoped CNDs in the xanthine/XO system and lucigenin-CL quenching process.

N,S-Codoped CND Cytotoxicity Study. The biocompatibility of N,S-codoped CNDs was evaluated by MTT-based assay. For this system, the mitochondrial activity is indicated by the MTT conversion to formazan crystals by living cells.⁽⁵⁷⁾ We performed the MTT assay in EA hy926 endothelial cells incubated with 0.02, 0.03, 0.05, 0.07, or 0.10 mg/mL of N,S-codoped CNDs. Figure 7A shows high cell viability (>90%) when incubated with 0.1 mg/mL N,S-codoped CNDs for 24 h. Figure 7B shows the formation of formazan crystals in the cells in the absence of N,S-codoped CND treatment. When the N,S-codoped CNDs were added, however, the number density of formazan crystals in the images does not change much for both low (Figure 7C) and high (Figure 7D) concentrations of N,S-codoped CNDs. These observations indicate that the N,S-codoped CNDs exhibit good biocompatibility.

N,S-Codoped CND Fluorescence and Multicolor Bioimaging. CNDs have been used as a fluorescent label in bioimaging applications because of their excitation-dependent photoluminescence.^(58,59) Figure 8A shows fluorescence emission spectra of the dissolved N,S-codoped CNDs in deionized water as a function of the excitation wavelengths. The maximum emission peak at 408 nm occurs for an excitation wavelength of 350 nm. Longer-wavelength excitation renders a red shift in the fluorescence emission spectra, which has been investigated by others.^(60,61) Microscopy is an essential tool for biological and biomedical imaging studies,⁽⁶²⁾ and in this study, an Olympus IX70 inverted fluorescence microscope was used to monitor the cellular uptake of N,S-codoped CNDs by EA hy926 endothelial cells after incubation with 0.10 mg/mL N,S-codoped CNDs for 24 h (Figure 8B–D). According to the MTT assay results, the cell viability is still over 90% after 24 h with 0.10 mg/mL N,S-codoped CNDs. The strong blue

fluorescence indicates that N,S-codoped CNDs are internalized by the EA hy926 endothelial cells. The intensity of the green and red fluorescence from cells is weaker because the intensity of emission for N,S-codoped CNDs weakens as the observation wavelength is red-shifted.

Figure 7. (A) MTT assay for cell viability values (%) measured at different concentrations of N,S-codoped CND treatment to EA hy926 endothelial cells for 24 h. EA hy926 endothelial cells under MTT assay images were taken via a microscope with different concentrations of N,S-codoped CND incubation: (B) cells without N,S-codoped CND incubation; (C) cells with 0.02 mg/mL N,S-codoped CND incubation; (D) cells with 0.10 mg/mL N,S-codoped CND incubation. All scale bars represent 200 μ m.

Figure 8. (A) Fluorescence emission spectra of the N,S-codoped CNDs in deionized water. Fluorescence images of EA. (B–D) Optical images of hy926 endothelial cells with the treatment (24 h) of 0.10 mg/mL N,S-codoped CNDs at excitation wavelengths of 360 nm (B), 470 nm (C), and 560 nm (D). All scale bars represent 50 μm .

Conclusion

This work explored the antioxidation activity of N,S-codoped CNDs by three different methods: UV–vis absorption of DPPH $^{\bullet}$, electrochemistry using DPPH $^{\bullet}$, and lucigenin-CL assay of enzyme-generated ROS. The electrochemically derived relationship between unreacted DPPH $^{\bullet}$ and the CND concentration agrees reasonably well with the UV–vis absorption dose-dependent results, and the redox reaction is explained by the HAT mechanism. We also proposed a reaction pathway for the xanthine/XO system-induced lucigenin-CL quenching by the ROS scavenging reaction of N,S-codoped CNDs. Combined with the biocompatibility and bioimaging capabilities, these results provide promise for the development of alternative treatment options not possible with traditional pharmacological and ROS scavenger approaches, suggesting a new “nanopharmacology” for more effective treatment of inflammatory disorders such as atherosclerosis.

Supporting Information

The Supporting Information is available at DOI: 10.1021/acsanm.8b00404.

Author Contributions

The manuscript was written with contributions by all authors. All authors have approved the final version of the manuscript.

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The authors declare no competing financial interest.

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