

N, S, and P-Co-doped Carbon Quantum Dots: Intrinsic Peroxidase Activity in a Wide pH Range and Its Antibacterial Applications

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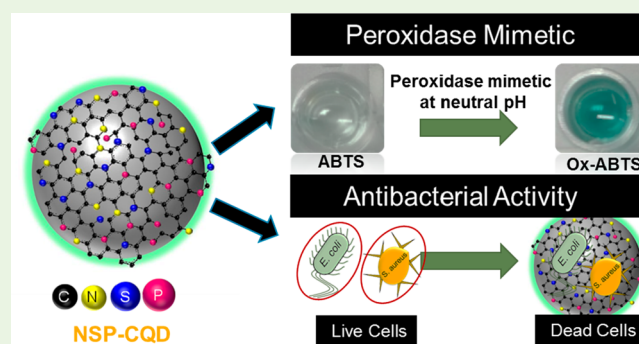
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ABSTRACT: Nanozymes have drawn significant scientific interest due to their high practical importance in terms of overcoming the instability, complicated synthesis, and high cost of protein enzymes. However, their activity is generally limited to particular pHs, especially acidic ones. Herein, we report that luminescent N, S, and P-co-doped carbon quantum dots (NSP-CQDs) act as attractive peroxidase mimetics in a wide pH range, even at neutral pH, for the peroxidase substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the presence of H₂O₂. The synergistic effects of multiple heteroatoms doping in CQDs boost the catalytic activity in a wide pH range attributed to the presence of high density of active sites for enzymatic-like catalysis and accelerated electron transfer during the peroxidase-like reactions. A possible reaction mechanism for the peroxidase-like activity of CQDs is investigated based on the radical trapping experiments. Moreover, the multifunctional activity of NSP-CQDs was further utilized for antibacterial assays for both Gram-negative and Gram-positive model species, including *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), respectively. The growths of the employed *E. coli* and *S. aureus* were found to be significantly inhibited due to the peroxidase-mediated perturbation of cell walls. The present work signifies the current advance in the rational design of N, S, and P-co-doped CQDs as highly active peroxidase mimics for novel applications in diverse fields, including catalysis, medical diagnostics, environmental chemistry, and biotechnology.

KEYWORDS: N, S, and P-co-doped carbon quantum dots, nanozymes, peroxidase mimetic, antibacterial assay, free radical



1. INTRODUCTION

Enzymes can catalyze a wide range of reactions with high catalytic activity and extreme surface specificity under physiological conditions and have been broadly used in diverse industrial fields, including chemical, agricultural, food, and pharmaceutical industries.^{1,2} However, they are susceptible to deactivation, relatively expensive to produce with high purity, and difficult to recycle, all of which limit their practical utilization.³ To resolve these limitations, enzyme-mimicking nanomaterials, so-called “nanozymes,” with remarkable performance have recently been designed and developed. Diverse metal, or carbon-based nanostructures,^{4–6} including Pt nano-clusters, Cu²⁺-functionalized carbon nitride nanoparticles,⁷ carboxyl-functionalized graphene oxide (GO–COOH),⁸ hemin-functionalized WS₂ nanosheets,⁹ silica-supported Au nanoparticles (NPs),¹⁰ Ni–Pd hollow nanoparticles,¹¹ Co₃O₄ NPs,¹² (BiO)₂CO₃ composites,¹³ and V₂O₅ nanowires,¹⁴ have been reported to mimic the complexity and functions of native enzymes.^{15,16} The unique and inherent characteristics of nanozymes, such as large surface area and high density of active sites, are considerably advantageous for achieving significant enzyme mimetic activity in practical applications.¹⁷

Unlike natural enzymes, nanozymes offer various beneficial properties, such as flexibility in composition and structural design, less consumption, have tunable catalytic activity, economic synthesis, and robustness to denaturation.⁹

Among the various catalytic activities mimicked by nanozymes, peroxidase activity has been the most widely studied owing to its diverse range of practical applications, such as biochemical analysis, wastewater treatment for reducing toxicity and biomedical diagnosis.¹⁴ However, until now, the practical utilization of peroxidase-mimicking nanozymes has been limited by the incorporation of noble metal NPs, such as Pt and Au, for achieving high catalytic activity, complicated and time-consuming synthetic protocols, aggregation of nanozymes, low water solubility, and limited activity only present in acidic pH conditions. In addition, nanozymes'

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activity is strongly dependent on the availability and reactivity of surface-active sites and electron transport properties.¹⁵ To this end, surface engineering to proliferate catalytic active sites and enhance electron transfer activity along with facile and economic synthesis is essential to boost the catalytic efficiency over a broad range of pHs, which can open this field to valuable advancements in the future.

In this context, carbon quantum dots (CQDs) with the features of a nanocrystalline core and a conjugated π -system have become ideal candidates to be employed as enzyme mimetic owing to excellent electron transfer ability,^{18,19} unique optical and physicochemical properties.²⁰ Moreover, abundant oxygenous functional groups on the CQDs surface such as C=O , O=C-O- , and -O-H can also provide many advantageous catalytic and substrate binding sites for intrinsic peroxidase activity with high efficiency.¹⁸ CQDs are much more robust and can be obtained at gram scale with ease in structural engineering and have presently been updated in their optical properties via a known method of doping. The synergistic effects of multiple heteroatom doping in the carbon lattice can instantaneously afford a high density of active sites with the generation of new properties for enhanced catalytic activity.^{2,21} More significantly, the strong interaction between the heteroatom-doped CQD components and the surficial functional groups can also effectively improve their catalytic performances due to the accelerated intermolecular electron transfer.²² N-doping can tune local features of CQDs including charge delocalization between neighboring carbon and thus provide enhanced chemical reactivity.²³ The P-doping of CQDs could increase the electron delocalization and structural defects with the generation of new charge sites due to the interruption of charge consistency of carbon atoms.²⁴ The S doping can tune the density of excited states and enhanced polarizability, structural defects, and spin density of CQDs.^{13,24} Moreover, the relatively larger atomic size of P and S with different electronegativities than carbon consequently introduce a high density of active sites as structural defects for enzymatic-like catalysis.^{13,25} N, S, and P co-doping may provide enhanced carrier separation and thus peroxidase-like activity. Although diverse techniques have been employed to synthesize heteroatom-doped CQDs, none of them are based on the fast, economic, and easy synthesis. To the best of our knowledge, facile synthesis of N, S, and P-co-doped CQDs and their application in peroxidase-mimicking antibacterial assays have not been reported. Therefore, the development of facile and low-cost heteroatom doped fluorescent CQDs is highly desirable and advantageous for enzyme mimetics and related applications.

In addition, bacterial contaminations are some of the most serious human healthcare challenges throughout the world.²⁶ Thus, a great deal of scientific efforts have been made against bacterial contaminations.²⁶ Antibacterial agents, such as antibiotics, metal NPs (especially noble metal NPs), composite NPs, quaternary ammonium compounds, and enzymes, have been extensively investigated.^{26–29} However, these materials possess risks related to environmental concerns, poor biocompatibility, high cost, and antibiotic resistance.³⁰ Therefore, low-cost, sustainable, and effective antimicrobial agents for long-term applications are in great demand. Despite tremendous biomedical applications, the development of multifunctional CQDs as antibacterial agents is still lacking, and there have been only a few reports regarding the antibacterial ability of CQDs and graphene QDs.^{26,31–33}

However, most of them exhibit their antibacterial activity at acidic pHs but have poor activity under physiological conditions around neutral pH.

Herein, we explored fluorescent N, S, and P co-doped CQDs (NSP-CQDs) as nanozymes to mimic peroxidase activity. NSP-CQDs were synthesized via a simple microwave treatment to have the photoluminescence (PL) quantum yield of $\sim 25\%$ and resistance to photobleaching. Interestingly, as-synthesized NSP-CQDs act as peroxidase mimetics to catalyze the oxidation of peroxidase substrate 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) with H_2O_2 , thereby producing a green color, as shown in Figure 1. Moreover, NSP-

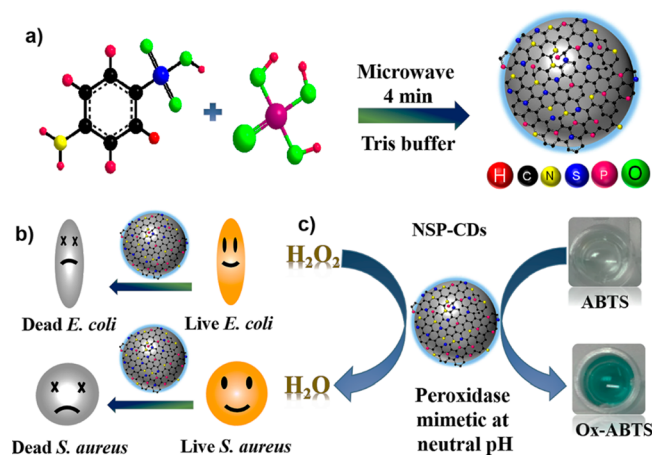


Figure 1. Synthesis and multifunctionality of NSP-CQDs. (a) Schematic illustration of N, S, and P codoped-CQDs synthesis. (b) NSP-CQDs as an antibacterial agent. (c) NSP-CQDs as a peroxidase mimetic under neutral pH conditions.

CQDs exhibited their catalytic activity in a wide pH range, including neutral pH, presumably due to the facilitated electron transfer during peroxidase-mediated reactions. In addition to their catalytic activity, NSP-CQDs exhibit considerable antibacterial activity toward both Gram-positive and Gram-negative bacteria. The fast and facile synthesis of NSP-CQDs provides a step toward biological applications and catalytic activity along with valuable strategy for the multifunctional materials.

2. EXPERIMENTAL SECTION

2.1. Materials. All reagents were of analytical grade and used as-obtained. Phosphoric acid, sulphanilic acid, and ammonia solution used for the synthesis of CQDs were purchased from Merck Chemicals, India. Tetraethylorthosilicate (TEOS), terephthalic acid (TA), ABTS, 3,3',5,5'-tetramethylbenzidine (TMB), isopropyl alcohol (IPA), or methyl alcohol (MA), benzoquinone (BQ), sodium hydroxide, and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich. All experiments were performed in deionized (DI) water. All buffer salts used were purchased from Sigma-Aldrich.

2.2. Synthesis of NSP-CQDs. The NSP-CQDs were synthesized using a facile microwave technique. At first, 0.01 mol of sulphanilic acid and 0.01 mol of tris buffer were mixed and ground in a mortar and pestle. Then, 0.1 mol phosphoric acid was added and stirred well in a 100 mL conical flask. This conical flask was irradiated for 4 min in a domestic microwave oven at 450 W power. The excess phosphoric acid was removed via neutralization with ammonia. The supernatant containing NSP-CQDs was collected via centrifugation at 6000 rpm for 20 min and used for further characterization and applications.

2.3. Material Characterizations. Scanning electron microscopy (SEM) imaging was conducted with a JEOL, JSM-7500F instrument.

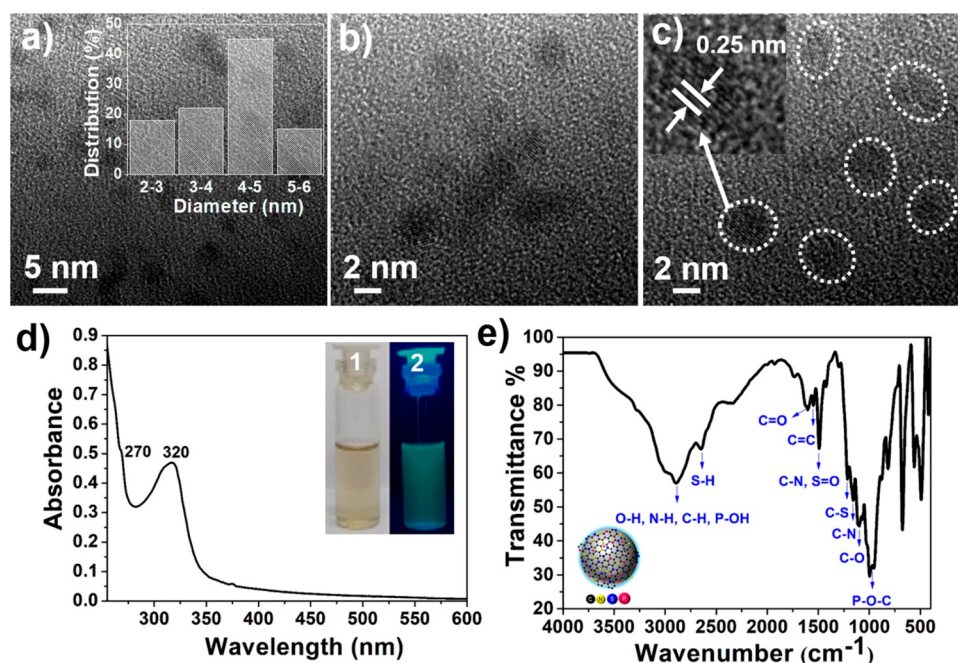


Figure 2. Morphological and physiochemical characterization of the NSP-CQDs. (a) Low-resolution TEM image of NSP-CQDs with their corresponding diameter distribution histogram in insets; (b) high resolution TEM image of NSP-CQDs, showing spherical morphology and well-dispersed nature; and (c) HRTEM image of NSP-CQDs, showing their crystalline nature. Inset: zoom image of a CQDs showing lattice fringes. (d) UV-vis adsorption spectra of NSP-CQDs. Inset shows the NSP-CQDs under the influence of visible (1) and UV (365 nm) light irradiation (2). (e) FTIR spectrum of NSP-CQDs.

The morphological and structural characterization of NSP-CQDs was performed using a Tecnai 20 G2 transmission electron microscope (TEM) and high-resolution TEM (HRTEM) at an accelerating voltage of 300 kV. Samples for TEM and HRTEM analysis were prepared by drop casting the aqueous solution of NSP-CQDs onto a carbon-coated copper grid and dried overnight in a vacuum furnace at 30 °C. One drop of NSP-CQDs was deposited onto glass slides and dried at 80 °C and examined using a Nikon optical microscope. X-ray photo electron spectroscopy (XPS) data were acquired with an ESCA⁺ omicron nanotechnology oxford instruments. The Fourier transform infrared spectra (FTIR) were recorded in solid state with a Bruker Fourier transform infrared spectrometer (Vector 22 model). Surface zeta potential as well as dynamic light scattering analyses were carried out using a Zetasizer Nano-ZS (Malvern Co.). Fluorescence spectra were recorded in the aqueous medium using a PerkinElmer LS55 fluorescence spectrometer. Fluorescence lifetime decay measurements were obtained with an EasyLife II fluorometer system. The UV-vis spectroscopy measurements of samples were recorded with a PerkinElmer Lambda 35 spectrometer at room temperature.

2.4. Peroxidase-Mimicking Activity Measurements. The peroxidase activity of the as-prepared NSP-CQDs was determined by performing representative oxidative reaction of the colorimetric substrate (ABTS) with H₂O₂. In a typical procedure, a solution containing 20 μ L ABTS (60 mM), 20 μ L NSP-CQDs (0.5 mg/mL), and 20 μ L H₂O₂ (10 mM) in 140 μ L of sodium acetate buffer (50 mM adjusted at diverse pHs) was incubated for 10 min at room temperature. Subsequently, the NSP-CQDs were separated from the reacted solution by 3 min centrifugation at 10,000 rpm, and the supernatant was subjected to analyze the progress of the reaction via representative images. The absorption intensity of the solution was evaluated in a scanning mode at 417 nm using a microplate reader (Synergy H1, BioTek, VT). Steady-state kinetic assays of NSP-CQDs were also performed with TMB and H₂O₂ as substrate. The experiment was performed by varying one substrate while keeping the other component constant. The concentration of NSP-CQDs used for kinetic assay was 50 μ g/mL.

2.5. Mechanistic Investigation of the Peroxidase-Mimicking Activity of NSP-CQDs. The formation of the reactive intermediates

during the peroxidase-like H₂O₂ decomposition with NSP-CQDs was examined through radical trapping experiments. IPA or MA (hydroxyl radical ($\bullet$ OH) scavenger) and BQ (superoxide radical ($\bullet$ O₂⁻) scavenger) were introduced in the typical ABTS oxidation reaction system.³⁴ The influences of the radical scavengers on the reaction system were evaluated by measuring the absorbance intensity change centered at 417 nm.

Second, the TA probe was used for determining hydroxyl radicals because it can form 2-hydroxyterephthalic acid showing a characteristic emission around 430 nm by reacting with $\bullet$ OH. The experiments were conducted by mixing a solution containing 100 μ L TA (10 mM), 100 μ L NSP-CQDs (diverse concentrations), and 100 μ L H₂O₂ (100 mM) in 700 μ L of sodium acetate buffer (100 mM adjusted at pH 5.5) and incubated for 12 h at room temperature without any light. Their fluorescence spectra were then recorded at 315 nm excitation using a microplate reader.

2.6. Bacterial Culture and Antibacterial Assay. *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were propagated in a Luria-Bertani (LB) medium at 37 °C under shaking conditions (200 rpm) overnight. Then, 100 μ L of the grown *E. coli* or *S. aureus* culture was inoculated with 10 mL of fresh LB with testing samples including NSP-CQDs only (100 μ g/mL), H₂O₂ only (1 mM), both NSP-CQDs and H₂O₂ (100 μ g/mL and 1 mM, respectively), or nothing (control) and subsequently incubated at 37 °C for 12 h. Afterward, the growth inhibition of bacterial cells was evaluated by measuring the change in absorbance intensity centered at 660 nm using a microplate reader. The antibacterial activities of the NSP-CQDs were also evaluated by the colony counting method.³⁵ Briefly, 50 μ L of each sample (NSP-CQDs only, H₂O₂ only, both NSP-CQDs and H₂O₂, and distilled water as a positive control) was added to 950 μ L of grown bacterial cultures and stored at 37 °C. After 1 day, 100 μ L of each sample was placed on an LB-agar plate by the spreading method and cultured for 1 day to observe the number of bacterial colonies. Dosing concentrations for NSP-CQDs and H₂O₂ were set to 100 μ g/mL and 1 mM, respectively.

Cell viability after the treatment of samples (NSP-CQDs only, both NSP-CQDs and H₂O₂, and distilled water as a positive control) was also visually assessed using the propidium iodide (PI) fluorochrome

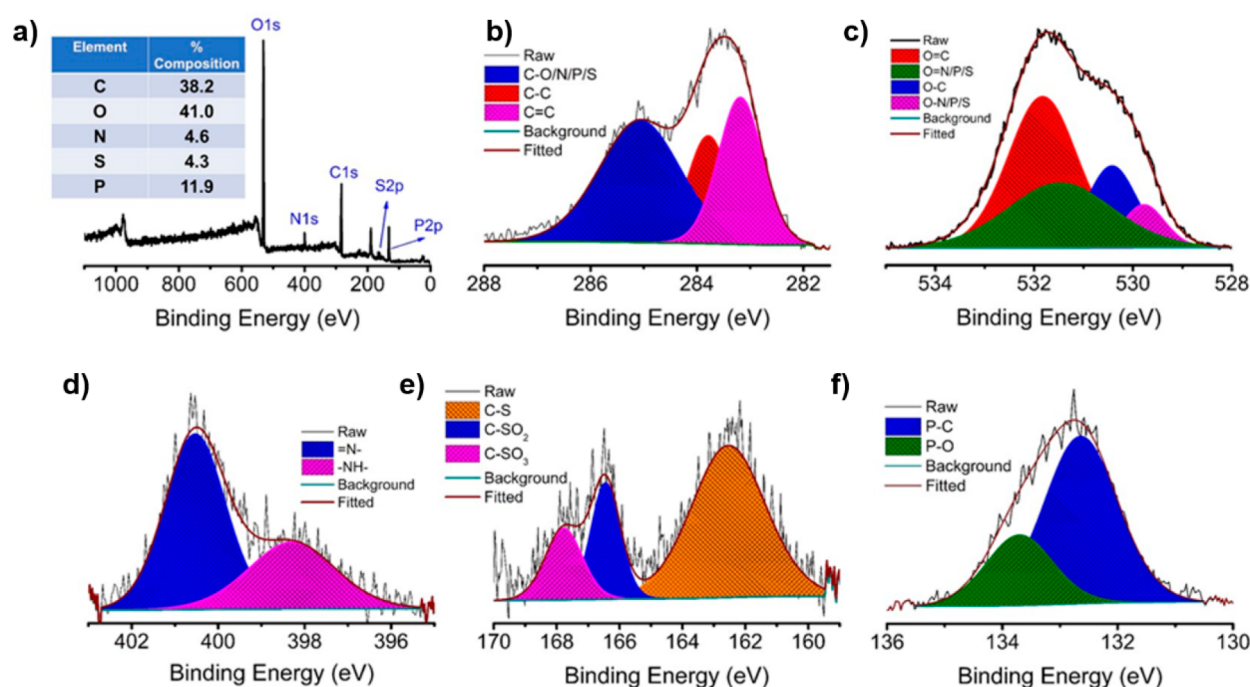


Figure 3. N, S, and P doping analysis of NSP-CQDs. (a) Wide survey XPS spectrum. High-resolution XPS spectra of (b) C 1s, (c) O 1s, (d) N 1s, (e) S 2p, and (f) P 2p in NSP-CQDs.

uptake assay. Since PI solely penetrates bacteria with altered/damaged membrane and exhibits significant fluorescence enhancement upon binding nucleic acids, bacteria with damaged membranes show red fluorescence corresponding to PI stain. The fluorescent images were recorded using a fluorescence microscope (Nikon Eclipse 80i, Japan).

3. RESULTS AND DISCUSSION

To develop a highly efficient functional material for bioanalytical applications, the choice of biologically relevant precursor and facile synthetic method is crucial. To synthesize NSP-CQDs, we used a simple microwave technique, irradiating a 1:10 mixture of sulphanilic acid and phosphoric acid for 4 min. A schematic illustration of the NSP-CQDs synthesis is shown in Figure 1a. Tris buffer and sulphanilic acid were used as sources for nitrogen, carbon, and sulfur. Phosphoric acid was used as a source of phosphorus. A series of intermolecular dehydration condensations between sulphonyl and amino groups of compounds followed by carbonization resulted in a crystalline backbone of CQDs intrinsically surface-functionalized with O-, N-, S-, and P-containing functional groups.³⁶ The resulting CQDs combine the advantage of uniform doping from the N, S, and P and surface functionalization. The as-synthesized NSP-CQDs solution was relatively homogeneous and remained clear at room temperature over one year. The NSP-CQDs were tested as nanozymes for mimicking peroxidase (Figure 1c) and as an antibacterial agent for both Gram-positive and Gram-negative bacteria (Figure 1b).

3.1. Morphological and Structural Characterizations of NSP-CQDs. The morphology, size, and surface structure of the NSP-CQDs were characterized by TEM and HRTEM. TEM images of the NSP-CQDs in Figure 2a,b show the spherical nature and excellent dispersibility of NSP-CQDs. The NSP-CQDs are 2–6 nm in diameter, as calculated statistically by counting 100 particles; a representative histogram is shown in the inset of Figure 2a. The majority of the NSP-CQDs were in

the size of 4–5 nm. The HRTEM image in Figure 2c illustrates the presence of well-resolved crystalline lattice fringes with a lattice spacing of 0.25 nm, which corresponds to the (100) facet of graphitic carbon.³⁷

UV–vis and fluorescence studies were acquired to explore the photophysical properties of NSP-CQDs. NSP-CQDs show excellent solubility in water without applying any further treatment or sonication. The aqueous solution of NSP-CQDs shows brownish yellow appearance (inset 1: Figure 2d) and changed its color to strong yellowish green under UV irradiation at 365 nm (inset 2: Figure 2d). Figure 2d shows two representative peaks at 270 and 320 nm in the UV–vis spectrum, originating from the π – π^* transition of graphitic domains and n – π^* transition of the excited defect surface states induced by the N, S, and P heteroatoms.^{38,39} The positive zeta potential of NSP-CQDs (~ 20.30 mV) was analyzed in aqueous phase at pH 7.4, which further confirms the surface functionalization on NSP-CQDs. Aggregations in NSP-CQDs solution were not observed even after 1 year of storage at ambient conditions. Excellent stability of NSP-CQDs in aqueous phase is attributed to the presence of highly positive surface functionalities, and consequently strong electrostatic repulsion between the NSP-CQDs.

FTIR spectrum in Figure 2e shows that NSP-CQDs possess different functionalities on their surface. A broad band from 3314 to 2801 cm^{-1} corresponds to the merged stretching vibrations O–H, N–H, C–H, and P–OH.^{38,40–45} The band at 2645 cm^{-1} reveals the presence of S–H stretching vibrations.^{40,41,44} The weak bands at 1600 and 1540 cm^{-1} are attributed to the C=O and C=C stretching vibrations, respectively. The medium band at 1492 cm^{-1} corresponds to the C–N and S=O stretching vibrations.^{40,41,44} The strong and merged bands at 1213, 1157, 1104, and 992 cm^{-1} correspond to the C–S, C–N, C–O, and P–O–C stretching vibrations, respectively. The bands between 600 cm^{-1} to 500 cm^{-1} correspond to the aromatic stretching and C–S/C–O

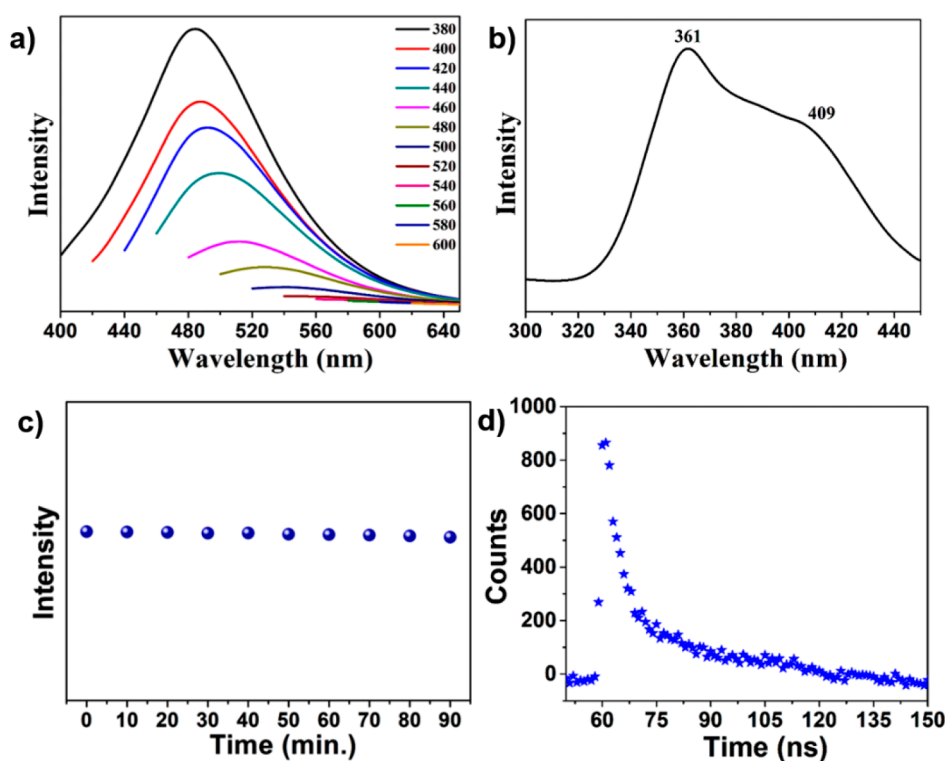


Figure 4. Optical characterization of NSP-CQDs. (a) Fluorescence emission spectra of NSP-CQDs excited at different wavelengths from 380 to 600 nm with an increment of 20 nm, (b) excitation spectrum at 485 nm emission wavelength, (c) evaluation of PL intensity of NSP-CQDs against continuous irradiation of UV light (365 nm) for different time intervals, and (d) fluorescence decay spectrum at 380 nm excitation wavelength.

bending vibrations.^{38,40–45} The FTIR analysis results imply the presence of $-\text{OH}$, COO^- , $-\text{C}-\text{SO}_x-$, $-\text{P}-\text{O}-\text{C}-$, and $=\text{N}-\text{C}-$ groups in the NSP-CQDs.

The presence of N, S, and P in NSP-CQDs and their chemical compositions were further confirmed by XPS measurements. The XPS survey scan spectrum of NSP-CQDs in Figure 3a indicates the presence of four peaks at ~ 283.45 , ~ 533.10 , ~ 401.5 , ~ 159.6 , and ~ 132.9 eV attributed to characteristic peaks of carbon (C 1s), oxygen (O 1s), nitrogen (N 1s), sulfur (S 2p), and phosphorus (P 2p), respectively. The NSP-CQDs are composed of C, O, N, S, and P elements with composition to be 38.2%, 41.0%, 4.6%, 4.3%, and 11.9%, respectively, indicating a relatively high density of N, S, and P doping. Figure 3b–f describes the high-resolution short scanned XPS spectra of C 1s, O 1s, N 1s, S 2p, and P 2p, respectively. Short scanned C 1s spectra as displayed in Figure 3b can be well deconvoluted into three components ~ 283.19 , ~ 283.79 , and ~ 285.06 eV, corresponding to $\text{C}=\text{C}$, $\text{C}-\text{C}$, and $\text{C}-\text{O}/\text{C}-\text{N}/\text{C}-\text{S}$ bonding, respectively.^{46,47} The short scan high-resolution XPS survey for O 1s region (Figure 3c) shows the presence of four major peaks at ~ 529.76 , ~ 530.40 , ~ 531.44 , and ~ 531.81 eV, corresponding to the binding energy of $\text{O}-\text{N}/\text{P}/\text{S}$, $\text{C}-\text{O}$, $\text{O}=\text{N}/\text{P}/\text{S}$, and $\text{C}=\text{O}$, respectively.⁴⁸ The short scan of N 1s (Figure 3d) shows two major peaks at ~ 400.53 and ~ 398.21 eV, which reveals the presence of pyrrolic and pyridinic nitrogen, further confirming the successful doping of nitrogen in NSP-CQDs as fused ring structures.⁴⁹ The S 2p short scan spectra (Figure 3e) at binding energy ~ 162.50 , ~ 166.44 , and 167.76 eV reveal the presence of $\text{C}-\text{S}$, $\text{C}-\text{SO}_2$, and $\text{C}-\text{SO}_3$, respectively, which further corroborate the incorporation of sulfur in NSP-CQDs.⁴⁰ The P 2p short scan can be well deconvoluted into

two major peaks (Figure 3f) at ~ 132.6 and ~ 133.7 eV, corresponding to the $\text{P}-\text{C}$ and $\text{P}-\text{O}$, respectively.⁴⁵ The comparative study of XPS and FTIR confirm the presence of N, S, and P in the CQDs.

Subsequently, the optical properties of the NSP-CQDs were investigated. NSP-CQDs show excitation-dependent PL, which is a typical feature of CQDs. The optimal emission was centered at 484 nm upon 380 nm of excitation wavelength. The spectral position of the emission peaks linearly depended on the excitation wavelength from 380 to 600 nm, as shown in Figure 4a, with a continuous increment of 20 nm attributed to the existence of energetically different emissive traps. NSP-CQDs have two PL excitation bands centered at 361 and 409 nm, as shown in Figure 4b, indicating the presence of manifold emitting centers.⁵⁰ Because fluorescence stability is critical for all practical applications, the fluorescence stability of NSP-CQDs was investigated against the continuous irradiation of UV light. NSP-CQDs emissions are relatively stable, and attenuation of the PL intensity was not observed, even after 90 min of continuous UV light irradiation (365 nm), as shown in Figure 4c. The combined effect of doping, composition, and abundant functional groups contributed significantly to protecting NSP-CQDs against photodegradation and provided excellent photostability.

The PL quantum yield at 380 nm of excitation wavelength using quinine sulfate as a reference was determined to be $\sim 25\%$, which is higher than that of many reported CQDs.^{31,51–53} The high PL quantum yield can be explained on the basis of the synergistic effects between heteroatoms (i.e., N, S, and P) and the availability of a range of functional groups, which enhanced the density of surface energy traps.³⁹ Time-resolved PL measurement of the NSP-CQDs was

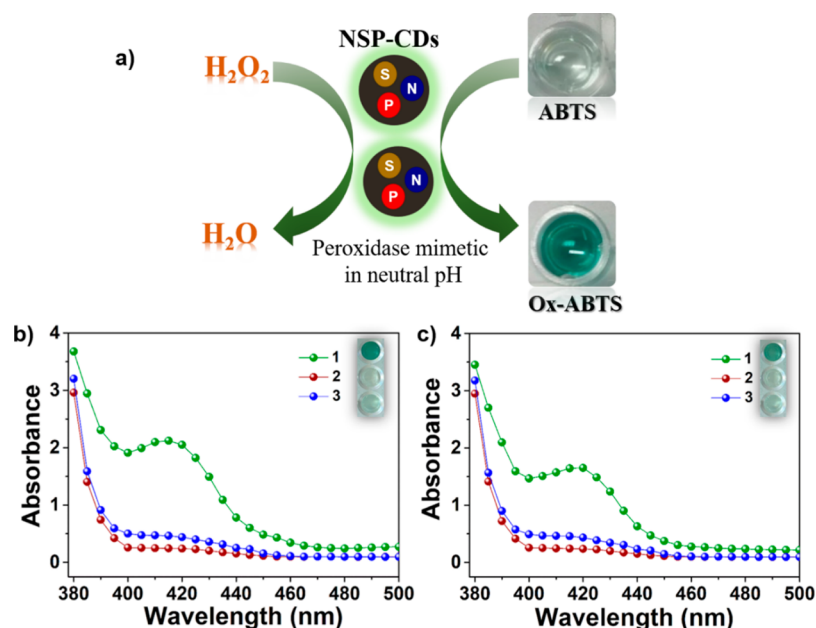


Figure 5. Catalytic activity of NSP-CQDs. (a) Schematic illustration of peroxidase-mimicking via ABTS oxidation. Peroxidase-like activity of NSP-CQDs under different parameter settings: (b) pH = 4 and (c) pH = 7. The absorbance spectra and digital images (insets) show visual color changes of ABTS in different reaction systems: (1) ABTS+H₂O₂+NSP-CQDs, (2) ABTS+H₂O+NSP-CQDs, and (3) ABTS+H₂O₂.

analyzed in triplicate at 380 nm excitation and fitted using a triexponential function with an acceptably reduced χ^2 value of 1.087, as shown in Figure 4d. The lifetime was determined to be 9.47 ns with an average of 5.5 ns.

The exact origins of the optical properties of CQDs and the mechanism behind their tunable emissions have remained a matter of debate until now because it is difficult to precisely depict and evaluate the comprehensive PL mechanism due to the limited availability of a variety of CQDs.⁵¹ Many models and hypotheses have been proposed, such as quantum size effects, radiative recombination of photogenerated electron–hole pairs, edge effect, and electronic conjugate structures.⁵¹ Several factors, including surface functionalization/passivation, multielement doping, and different surface defect states, are believed to contribute significantly to excitation-dependent emissions.⁵¹ It has been proposed that the heterogeneous distributions of π -conjugated sp^2 islands based on their size in a nonconjugated sp^3 matrix (termed as defects) create discrete energy levels within CQDs, consequently leading to their tunable PL properties.⁵⁴ Besides, binding heteroatoms, such as N, P, S, and B, and chemical composition (ratio of C:H:O) have been suggested to significantly influence the PL properties of CQDs. The introduction of heteroatoms confers additional functionalities and also tunes the radiative recombination of electron–hole pairs.⁵⁴ Shifting in emission peak positions and high quantum yield are attributed to the degree of intrinsic defect states (conjugated π - and nonconjugated- π -domains) throughout the carbogenic core.

3.2. Peroxidase-Like Activity of NSP-CQDs. Emergent CQDs are promising for various potential novel applications. We expect NSP-CQDs to exhibit a significant amount of peroxidase-like activity, possibly due to the fact that heteroatoms enhance the electronic charge transfer and surface areas to provide active sites for catalytic activity.^{55,56} To examine this, peroxidase-like activities of NSP-CQDs were determined through representative peroxidase-mediated ABTS oxidation reactions with H₂O₂ (Figure 5a), and the responses

were evaluated using absorption spectroscopy (Figure 5). The experimental results showed that the NSP-CQDs had high ABTS oxidation, as shown in Figure 5b,c, and much more intense colorimetric responses consequently took place. A characteristic broad absorption peak for oxidized ABTS was observed at 417 nm. As previously observed, a slight color change from colorless to green was observed when using only H₂O₂ without the presence of a catalyst.⁵⁷ Similar to previously reported peroxidase-like nanozymes,⁵⁸ the activity of NSP-CQDs was dependent on pH, temperature, ionic strength, and concentration of NSP-CQDs (Figure S1 of the Supporting Information, SI). Importantly, the NSP-CQDs exhibited high activity in a broad pH range; in particular, over 50% catalytic activity was maintained at pH 7 compared to the maximum at pH 4 (Figure S1a). This observation confirms that NSP-CQDs can act as peroxidase mimetics even under physiological conditions, which is highly beneficial for utilization in general biological and medical applications. The peroxidase activity of NSP-CQDs was further demonstrated by the steady-state kinetic measurements. With the appropriate concentration range of TMB and H₂O₂, the typical Michaelis–Menten curves were obtained (Figure S2). The two key parameters, Michaelis constant (K_m) and maximal reaction velocity (V_{max}), were obtained with the Lineweaver–Burk equation and listed in Table S1. The results of this investigation indicated that as-synthesized NSP-CQDs yielded a similar level of K_m toward TMB compared with that from other recent peroxidase-mimicking CQDs. The results of this investigation indicated that as-synthesized NSP-CQDs yielded one of the best K_m toward TMB compared with those from other recent peroxidase-mimicking nanozymes or even natural HRP.

The peroxidase-like activities of NSP-CQDs may be attributed to their characteristic electron transfer properties and the presence of “active sites” on their surface.⁵⁹ According to FTIR and XPS data, NSP-CQDs have abundant N-, O-, S-, and P-containing substructures, which are responsible for increasing charge carrier density and charge transfer toward the

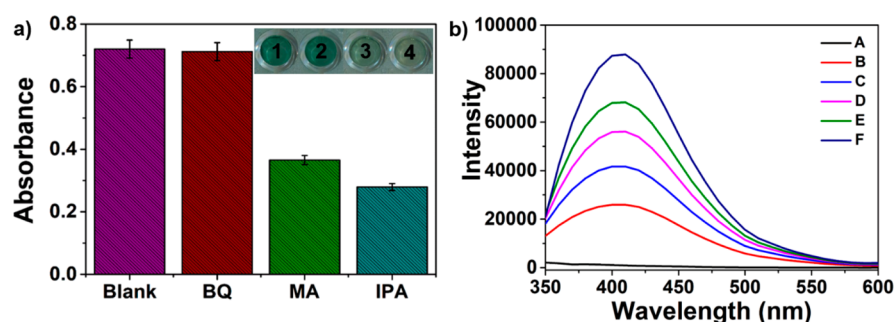


Figure 6. (a) Comparison of absorbance change centered at 417 nm with or without scavengers (IPA, MA, and BQ) in the catalytic reaction system. The inset shows the color change in the presence of scavengers: (1) control, (2) with BQ, (3) with MA, and (4) with IPA. (b) Fluorescence spectra for different concentrations of NSP-CQDs in the reaction system: A: control, B: 30 $\mu\text{g/mL}$, C: 60 $\mu\text{g/mL}$, D: 100 $\mu\text{g/mL}$, E: 150 $\mu\text{g/mL}$, and F: 300 $\mu\text{g/mL}$. These spectra are used to monitor $\cdot\text{OH}$ by determining the concentration of 2-hydroxyterephthalic acid via the oxidation of TA by $\cdot\text{OH}$ radicals.

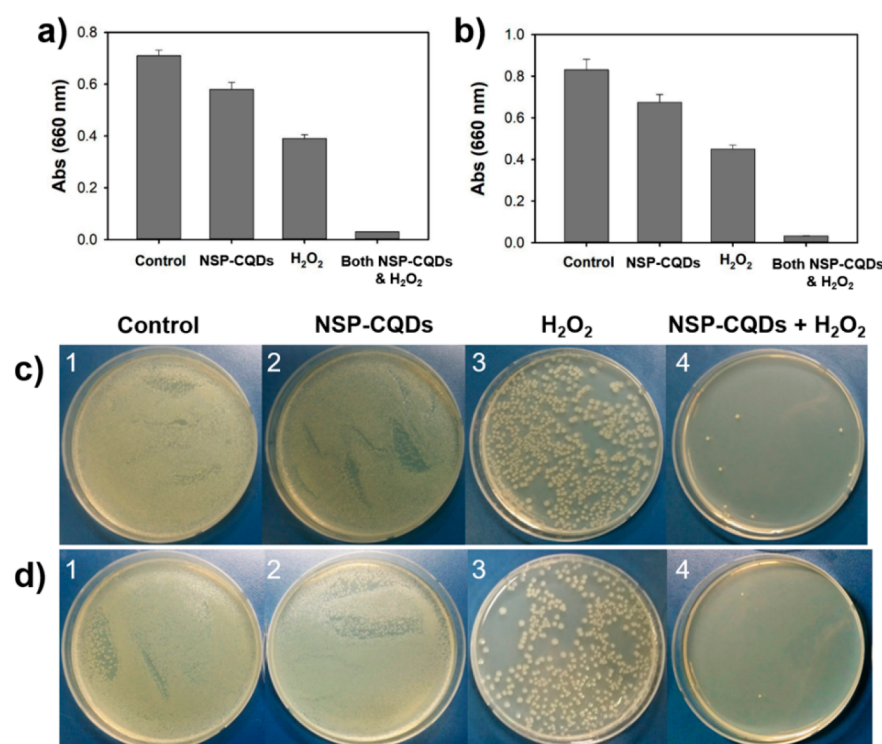


Figure 7. Antibacterial activity of NSP-CQDs. Absorbance at 660 nm of bacterial suspension of (a) *E. coli* and (b) *S. aureus* after 12 h growth with NSP-CQDs and H_2O_2 and NSP-CQDs alone. Photographic images of antibacterial activity of NSP-CQDs via colony counting method. (c) (1) Bacterial colonies of *E. coli*, (2) *E. coli* treated with NSP-CQDs, (3) *E. coli* treated with H_2O_2 , and (4) *E. coli* treated with NSP-CQDs in the presence of H_2O_2 . (d) (1) Bacterial colonies of *S. aureus*, (2) *S. aureus* treated with NSP-CQDs, (3) *S. aureus* treated with H_2O_2 , and (4) *S. aureus* treated with NSP-CQDs with H_2O_2 .

edge.⁴⁹ This led to a reduction in energy gap, and enhanced the charge transfer. This means that NSP-CQDs could facilitate the electron transfer between H_2O_2 and ABTS during the redox process.¹² ABTS was adsorbed to the surface of NSP-CQDs via surficial charge transfer and hydrophilic interactions.¹⁸ This complex formation accelerated the electron transfer between NSP-CQDs-ABTS and H_2O_2 , consequently increasing the rate of the redox reaction. As a result, NSP-CQDs exhibited a substantial amount of catalytic activity at both neutral and acidic pHs.

The oxidase-, catalase-, or superoxide dismutase (SOD)-like activity of NSP-CQDs was also investigated.⁶⁰ Oxidase activity was evaluated using ABTS substrate in the absence of H_2O_2 .

For evaluating catalase activity and identifying hydroxyl radical ($\cdot\text{OH}$) generated from the peroxidase-mediated H_2O_2 decomposition, TA was employed, which forms highly fluorescent 2-hydroxy terephthalic acid by reacting with $\cdot\text{OH}$. For evaluating SOD activity, cytochrome *c*, which exhibits a red color by reacting with superoxide radical ($\cdot\text{O}_2^-$) that is produced during the oxidation of xanthine by xanthine oxidase. The results show that the NSP-CQDs did not exhibit any meaningful oxidase-, catalase-, and SOD-like activities in our assay conditions (Figure S3), confirming that NSP-CQDs majorly serve as peroxidase mimic to mediate the decomposition of H_2O_2 .

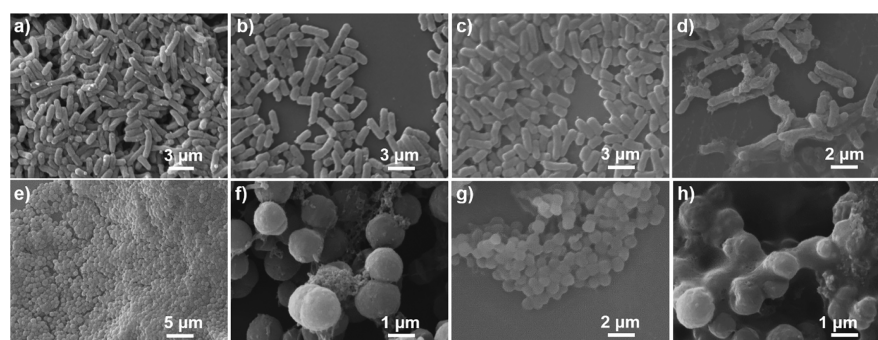


Figure 8. Morphologies of *E. coli* and *S. aureus* before and after exposure to NSP-CQDs. SEM images of (a) *E. coli* and (b) *E. coli* treated with NSP-CQDs, (c) *E. coli* treated with H₂O₂, (d) *E. coli* treated with both NSP-CQDs and H₂O₂, (e) *S. aureus*, (f) *S. aureus* treated with NSP-CQDs, (g) *S. aureus* treated with H₂O₂, and (h) *S. aureus* treated with both NSP-CQDs and H₂O₂.

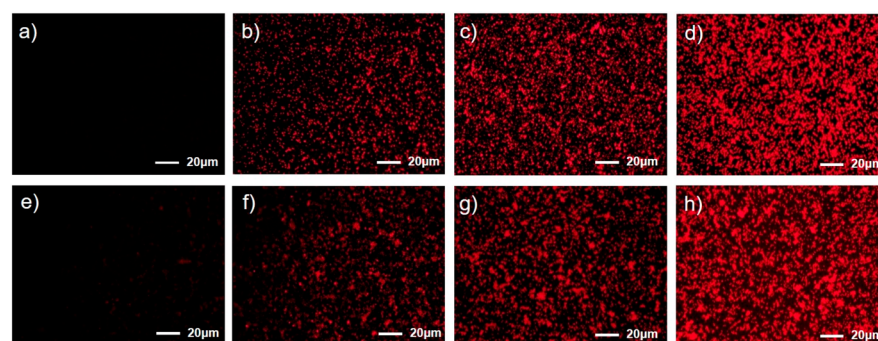


Figure 9. Staining images of membrane-disrupted dead bacteria by visualizing PI uptake. Fluorescence microscopy images of dead (a) *E. coli*, (b) *E. coli* after treatment with NSP-CQDs, (c) *E. coli* treated with H₂O₂, and (d) *E. coli* after treatment with NSP-CQDs in the presence of H₂O₂. Fluorescence microscopy images of dead (e) *S. aureus*, (f) *S. aureus* after treatment with NSP-CQDs, (g) *S. aureus* treated with H₂O₂, and (h) *S. aureus* after treatment with NSP-CQDs in the presence of H₂O₂.

To gain a deeper insight into the catalytic activity of NSP-CQDs and generation of the $\cdot\text{OH}$ through the decomposition of H₂O₂ during the peroxidase-like reactions, a radical scavenger study was performed. Three radical scavengers (IPA and MA, which both act as $\cdot\text{OH}$ scavengers, and BQ, which acts as an $\cdot\text{O}_2^-$ scavenger) were added to a reaction system containing NSP-CQDs (0.1 mg/mL), ABTS (6 mM), and H₂O₂ (1 mM) in a sodium acetate buffer (50 mM, pH 5.5) for the typical oxidation of ABTS. The results clearly show that there was a significant decrease in the absorbance at 417 nm as well as a corresponding green color intensity, both of which come from the oxidized ABTS, in the presence of IPA and MA, demonstrating the production of $\cdot\text{OH}$ during the oxidation of ABTS with NSP-CQDs (Figure 6a). However, in the presence of BQ, there was only a slight decrease in absorbance, confirming that the formation of $\cdot\text{O}_2^-$ could be ignored. $\cdot\text{OH}$ production during the NSP-CD-mediated catalysis was further confirmed by monitoring the respective fluorescence of terephthalic acid (Figure 6b). It can also be seen that the emission intensity increased as the amount of NSP-CQDs increased, showing that the formation of $\cdot\text{OH}$ was proportional to the amount of NSP-CQDs. This suggests that NSP-CQDs mediate the production of $\cdot\text{OH}$. From the above observations, we can conclude that NSP-CQDs possess the capability of producing $\cdot\text{OH}$ from H₂O₂, which can be utilized in various fields, including the antibacterial field.

3.3. Antibacterial Activity of NSP-CQDs. Peroxidase activity has been widely utilized in antibacterial applications, where H₂O₂ is converted into $\cdot\text{OH}$, attacking the bacterial cell membrane to disrupt it.¹⁰ On the basis of the strong peroxidase

activity of NSP-CQDs under physiological conditions, the antibacterial activity of NSP-CQDs was evaluated against both Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. aureus*. By measuring the absorbance at 660 nm (Abs 660) of the overnight-grown bacterial cultures prepared with NSP-CQDs with or without H₂O₂, we evaluated the level of growth inhibition and thus determined their antibacterial activities. The experiments clearly revealed that as-synthesized NSP-CQDs excellently inhibited the growth of both *E. coli* and *S. aureus* with the assistance of H₂O₂, which is likely due to the effective generation of $\cdot\text{OH}$ by the peroxidase activity of NSP-CQDs (Figure 7a,b).

In contrast, other samples (only NSP-CQDs, only H₂O₂, and control) did not yield significant antibacterial activities. The colony forming unit method on a LB-agar plate also showed that NSP-CQDs with the assistance of H₂O₂ were very efficient in suppressing the growth of both bacteria, whereas almost no reduction was observed from NSP-CQDs only or control samples, showing lawns of bacterial colonies on the plate (Figure 7c,d). With H₂O₂ only, some inhibition was observed, but hundreds of colonies were still visible on the plate. It is known that H₂O₂ itself has a marginal level of antibacterial activity for preventing bacterial contamination; however, high doses of H₂O₂ are harmful to healthy tissue and even delay wound healing.⁶¹ In this regard, peroxidase-mimicking NSP-CQDs with relatively low levels of H₂O₂ could be considered as a promising candidate for biomedical applications to prevent bacterial contaminations.

Observations of the morphological changes of bacteria before and after the treatment of NSP-CQDs-H₂O₂ are

important to elucidate the mechanism of cell death. For this purpose, SEM analyses were performed. The results of this investigation clearly show that NSP-CQDs with H_2O_2 induce changes in cell morphology, particularly in the membranes of both bacteria, which were seriously ruptured and destroyed (Figure 8a,d and e,h). This is presumably due to oxidation of the lipid membrane because $\cdot\text{OH}$ and H_2O_2 can oxidize and destroy it.¹⁰ In the presence of other samples, such as NSP-CQDs only and H_2O_2 only, no significant morphological changes were observed (Figure 8b,c and f,g). The results of this investigation are consistent with the previous peroxidase-based antibacterial assay results,¹⁰ suggesting that NSP-CQDs with H_2O_2 are highly efficient for bacterial killing.

To further examine the feasibility of NSP-CQDs- H_2O_2 for bacterial killing, we also performed a fluorescent cell viability assay to visualize membrane-disrupted dead cells. PI dye is membrane-impermeant, so it can only enter through damaged bacteria membranes, giving them a red fluorescence. As shown in Figure 9, negligible red fluorescence was seen in the positive control samples, where only *E. coli* and *S. aureus* were incubated without any additions, indicating that most bacteria were alive. After treatment with NSP-CQDs- H_2O_2 , red fluorescence became apparent, demonstrating the excellent bacteria killing capability of NSP-CQDs with the assistance of H_2O_2 through membrane disruption. These results confirm that NSP-CQDs with the assistance of H_2O_2 could efficiently kill the bacteria, possibly by the generation of $\cdot\text{OH}$ due to their peroxidase activity.

4. CONCLUSIONS

In summary, N, S, and P codoped-CQDs were synthesized via a simple domestic microwave technique to produce nanozymes. The NSP-CQDs possess intrinsic peroxidase activity, such as that of a classical peroxidase substrate, ABTS, with H_2O_2 . The intrinsic peroxidase activity is attributed to the heteroatom doping and facilitated electron transfer. Interestingly, NSP-CQDs exhibit catalytic activity over a broad pH range. NSP-CQDs as peroxidase mimetics present considerable advantages over natural enzymes, including low cost, facile synthesis, high stability, and catalytic activity over a broad pH range, making them suitable for utilization in immunoassays, biotechnology, and clinical diagnosis. The use of NSP-CQDs has been extended to antibacterial materials, and the antibacterial properties of NSP-CQDs were evaluated in both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. This work represents a significant step forward in the research on multifunctional material as an effective enzyme mimetic and antibacterial agent.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsbiomaterials.0c00831>.

Effects of parameters on the catalytic activity for ABTS oxidation by NSP-CQDs; steady-state kinetic assay of NSP-CQDs, evaluations of oxidase-, catalase-, and SOD-like activity of NSP-CQDs; table for the comparison between NSP-CQDs and other peroxidase-mimicking nanozymes and HRP; and additional references (PDF)

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Notes

The authors declare no competing financial interest.

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