Near-infrared chemiluminescent carbon nanogels for oncology imaging and therapy

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Abstract
Carbon nanogels (CNGs) with dual ability of reactive oxygen species (ROS) imaging and photodynamic therapy have been designed with self-assembled chemiluminescent carbonized polymer dots (CPDs). With efficient deep-red/near-infrared chemiluminescence (CL) emission and distinctive photodynamic capacity, the H2O2-driven chemiluminescent CNGs are further designed by assembling the polymeric conjugate and CL donors, enabling an in vitro and in vivo ROS bioimaging capability in animal inflammation models and a high-performance therapy for xenograft tumors. Mechanistically, ROS generated in inflammatory sites or tumor microenvironment can trigger the chemically initiated electron exchange luminescence in the chemical reaction of peroxalate and H2O2, enabling in vivo CL imaging. Meanwhile, part of the excited-state electrons will transfer to the ambient H2O or dissolved oxygen and in turn lead to the type I and type II photochemical ROS production of hydroxyl radicals or singlet oxygen, endowing the apoptosis of tumor cells and thus enabling cancer therapy. These results open up a new avenue for the design of multifunctional nanomaterials for bioimaging and antienoplastic agents.
1 | INTRODUCTION

Nanogels, nanosized hydrogel particles, are ideal candidates for their application in nanomedicine due to their large surface area for bioconjugation, long time of circulation in blood, and the possibility of being actively or passively targeted to the desired sites like tumor or other specific organ.1–3 On the one hand, smart nanogels with multifunction and novel properties can respond to diverse medically relevant stimuli like pH, temperature, ionic force, and redox environment, and so forth by changing their volume, refractive index, and hydrophilicity–hydrophobicity.2,3 On the other hand, combined with the comprehensive properties of hydrogels and nanomaterials, nanogels have aroused widespread concern in the nanomedical fields such as bioimaging and cancer therapy owing to their response to these physiologically related stimuli such as pH, temperature, ionic force, and redox environment.4–6 Through electrostatic interactions, reverse miniemulsion, desolvation/coacervation, hydrophobic interaction, or cross-linking of monomers, nanogels can be easily entrusted with new applicability for imaging, guided therapy, triggered drug release, or hyperthermia by incorporating the monomers with other nanomaterials.1,2,9,10 Among the spatiotemporal resolution bioimaging models, such as photoluminescence (PL), computed tomography (CT), and photoacoustic (PA) imaging, chemiluminescence (CL) imaging has been exploited as an ultrasensitive method for quantification and localization of chemical analytes in the living body due to their unnecessary autofluorescence interference.11–19 Generally, reactive oxygen species (ROS) derived from the metabolism of oxygen by living organisms have been extensively proved to be reactive and signaling molecules.20–23 To monitor the oxidation biology in organisms, CL imaging has been considered as an important biochemical analysis tool via the detection of the inflammatory response product (hydrogen peroxide, H2O2) in the physiological environment.17,24–26 In addition, combined with the photosensitizer that can produce cytotoxins, such as singlet oxygen (1O2), hydroxyl radicals (•OH), and superoxide radicals (•O2−) under optical excitation, nanogels can be used for photodynamic therapy (PDT) of cancer by effectively and specifically killing the tumor cells.1–3,27–30 Ideally, it is promising to develop long-wavelength CL nanogels as a stimulus-response system, achieving the simultaneous diagnosis and treatment in inflammatory-responsive diseases.

Carbon dots (CDs), a kind of 0D carbon-dominated nanomaterial consisting of sp2/sp3 carbon skeleton and abundant functional groups/polymer chains, have attracted extensive attention due to their novel advantages of high PL quantum yield (QY), tunable emission wavelength, high photostability, and biocompatibility.31–40 Among these different CDs, carbonized polymer dots (CPDs) made up of carbon cores and the connected polymer chains are considered with the novel polymer-like properties, enabling their promising application in various fields.31,32,41–44 In addition, bright deep-red/near-infrared (NIR) emissive CDs with narrow full width at half maximum (FWHM) has repeatedly proved that optical imaging quality is remarkably improved due to the minimal background noise, increasing high color-purity display, minimum autofluorescence, and light scattering by tissues, offering great imaging contrast and spatial resolution.42,43,45–47 In recent years, the novel property of polymer-like structure and deep-red/NIR emission for CPDs have been indicated by the opportunity to accurately identify and kill specific bacteria with the light-active cytotoxins in spatial-resolution bioimaging.45–48 Thereby, it is significant to take advantage of the novel polymer-like structure and luminescence property of CPDs to design more smart nanomaterials for various applications.

In this paper, highly biocompatible and self-illuminating carbon nanogels (CNGs) with the dual ability of ROS imaging and PDT have been designed with self-assembling chemiluminescent CPDs. For the formation of the CNGs, five kinds of monodispersed CPDs have been derived from different biomass materials (houttuynia, honeysuckle, moxa, perilla, and taxus). These CPDs exhibit polymer-like property and can self-assemble into CNGs through the hydrophilic and hydrophobic interactions. Meanwhile, these CNGs present efficient deep-red/NIR CL emission in the system of (bis [2,4,5-trichloro-6-carbopentoxyphenyl] peroxalate, CPPO) and H2O2 and distinctive PDT capacity. Thereupon, these CPDs can act as monomers of a self-illuminating platform via participating in the self-assembly of the amphiphilic polymeric conjugate (Pluronic 127, Fl27) and CL donors (CPPO), precipitating H2O2-driven water-soluble chemiluminescent carbon nanogels (CCNGs). Mechanistically, ROS generated in the inflammatory sites can trigger the chemically initiated electron exchange luminescence (CIEEL) between the CPDs and intermediate originated from the reaction of peroxalate and H2O2, enabling in vitro and in vivo CL imaging. Meanwhile, the production of 1O2, •OH, and •O2− from the CCNGs under self-illumination can efficiently induce the apoptosis of tumor cells, thus inhibiting.

KEYWORDS

cancer therapy, carbon nanogels, chemiluminescence, inflammation imaging
tumor growth, resulting in cancer therapy. The CNGs exhibit in vivo imaging quality in detecting inflammation in diverse animal models and further are proven to be an antitumor nanomedicine with selective and potent antitumor efficacy with a desirable safety profile.

2 EXPERIMENTAL SECTION

2.1 Materials

All chemicals and reagents were purchased from Sigma-Aldrich unless otherwise specified and were used without further purification. The fresh plants (houttuynia, honeysuckle, moxa, perilla, taxus) were purchased from Zhejiang Taobao Network Co., Ltd. and cultivated for 3 days.

2.2 Preparation of the CPDs

The CPDs were synthesized by the leaves of houttuynia, moxa, perilla, taxus, and the flowers of honeysuckle, respectively. Fresh plant leaves or flowers were collected and dried at 60°C to remove the water and 1.0 g dried leaves or flowers were added in 20 ml of ethanol solution (A.R.). Next, the mixture solution was transferred to a poly (tetrafluoroethylene) Teflon-lined autoclave (30 ml) and heated in an oven at 140°C for 4 h. Subsequently, the reactor was cooled to room temperature. The resulting solution was centrifuged (8000 r/min, 10 min) and the supernatant solution was collected. The final solution was obtained from the supernatant solution through 0.22 µm polyethersulfone membrane to remove large particles. Then the crude products were purified via dry silica column chromatography using a mixture of ethyl acetate and petroleum ether as the eluent. After collecting black powder by removing the solvent under reduced pressure, different dried CPDs were obtained for further characterization.

2.3 Preparation of the CNGs and chemiluminescent CNGs

To prepare the CNGs, the CPDs were homogeneously dissolved in trichloromethane for 1 mg/ml in a flask. Then another 1 ml of water was added to the CPDs solution. After the solvent was removed by a vacuum pump, the CNGs aqueous solution can be obtained. To prepare the chemiluminescent CNGs, the CPDs were dissolved in trichloromethane with a concentration of 1 mg/ml by ultrasonic, the pluronic F-127 (30 mg/ml) and bis[2,4,5-trichloro-6-carbopentyloxyphenyl] oxalate (CPPO, 3 mg/ml) were dissolved in trichloromethane by vortexing. Then, 1 mg CPDs (1 mg/ml), 30 mg F-127 (30 mg/ml), and 1 mg CPPO (1 mg/ml) were homogeneously mixed in a flask. After the solvent was removed by a vacuum pump, the dried mixture was dissolved in 3 ml Milli-Q water to prepare the CCNGs with a concentration of 333 µg/ml (based on CPDs).

2.4 In vitro CL characterization of the CCNGs

For the quantitative analysis, 50 µl of CNGs (333 µg/ml based on CPDs) were placed in a black 96-well ELISA plate, then 50 µl of H2O2 was added (Sigma-Aldrich, 323381) with different concentrations (0, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, and 50 µmol/L). After quick mixing, the plate was immediately put into the in vivo imaging system (IVIS) (Xenogen) to acquire luminescent signals with an exposure time of 30 s at every 5 min within 1 h with an open filter.

2.5 In vivo inflammatory bioimaging with the chemiluminescent CNGs

The mouse model of peritonitis was used to image endogenous H2O2. Briefly, the C57BL/6J mice were injected intraperitoneally with 200 µl Zymosan A (2 mg/ml, Z4250, Sigma-Aldrich) for 48 h. For the inhibitor study, mice were treated with 200 µl GSH (200 mg/kg) for 24 h intraperitoneally after Zymosan A treatment. The control mice were treated only using 200 µl saline. After 48 h of the Zymosan A treatment, anesthetized mice (2% isoflurane in oxygen) were treated with intraperitoneal injection of 200 µl CCNGs-1 (333 µg/ml based on CPDs). Then, the mice were immediately put into the IVIS imaging chamber (Xenogen) to acquire luminescent signals with an exposure time of 3 min at every 5 min with an open filter at 670 nm. The phosphate buffer saline (PBS) was used in the control group.

2.6 Wound-healing assay

A549 cells (1 × 10⁵/well) were seeded in 12-well plates to reach 95% confluence, and then cells were scratched with 10 µl sterile pipette tips. After washing with PBS twice, the cells were treated with CCNGs-1 at a concentration of 5, 10, and 20 µg/ml (based on CNDs-1) in RPMI 1640 medium with 2% FBS for 24 h. Wound width was observed with a microscope (Leica) after CCNGs-1 treatment for 0 and 24 h at ×200 magnification. Scratch areas were calculated by ImageJ (National Institutes of Health). Fold change of migration area was calculated as: Scratch area at 24 h/Scratch area at 0 h.
2.7 | Invasion assay

Invasion ability of A549 cells after treatment with CCNGs-1 was conducted with Transwell (3422; Costar) and Matrigel matrix (354248; BD Biosciences). Briefly, the upper chamber was precoated with 25 μl Matrigel matrix. Two hundred microliters cell suspension was added (1 × 10^5/well) in FBS free RPMI 1640 medium to the upper chamber and the lower chamber was added with 600 μl RPMI 1640 medium containing 10% FBS. After incubation with CCNGs-1 at a concentration of 5, 10, and 20 μg/ml (based on CNDs) for 24 h, the invasive cells in the lower chambers were fixed by 4% paraformaldehyde and stained by 0.5% crystal violet solution (Sigma-Aldrich). The stained cells were counted in five random fields with a microscope (Leica) at ×200 magnification.

2.8 | Xenograft tumor growth and therapy

After being acclimatized for 1 week, the A549 cells (1 × 10^7) in 200 μl FBS free RPMI 1640 medium (50% Matrigel) were injected under the right forelimb armpit to the mice. Mouse weight and tumor volume were monitored every 3 days, and the tumor volume was calculated as follows: (short diameter^2 × long diameter)/2. When the tumor grew to an average volume of 100 mm^3, the mice were randomly divided into control group and CCNGs group according to the tumor size and weight. The mice in the CCNGs-1 group were treated with 100 μl CCNGs-1 (333 μg/ml based on CPDs-1) by intertumoral injection every 3 days. The mice were euthanized until a total tumor volume of 1000 mm^3 was achieved or tumors became ulcerated.

2.9 | Ethical approval

All animal experiments were approved by The First Affiliated Hospital of Zhengzhou University under Protocol No. 2019-KY-008.

3 | RESULTS AND DISCUSSION

3.1 | Synthesis of CPDs and their assembling CNGs

In this study, five different kinds of plants commonly used as Chinese herbal medicine are selected for the preparation of CPDs. And the five biomass materials, namely houttuynia, honeysuckle, moxa, perilla, and taxus, are found to be perfect carbon sources for achieving the deep-red/NIR emissive CPDs. As depicted in Section 2, five kinds of monodispersed CPDs can be obtained through the solvothermal treatment of houttuynia, honeysuckle, moxa, perilla, and taxus (Figures 1A and S1). In addition, it is found that these CPDs can serve as monomers to be assembled into CNGs, when these CPDs are translated from ethanol to water. For convenience, these CPDs derived from houttuynia, honeysuckle, moxa, perilla, and taxus have been named as CPDs-1, CPDs-2, CPDs-3, CPDs-4, and CPDs-5, respectively. As shown in Figure 1B, the transmission electron microscopy (TEM) image of these CPDs-1 exhibits dot-shape with a monomodal size distribution ranging from 2 to 25 nm, similar to CPDs-2, CPDs-3, CPDs-4, and CPDs-5 (Figure S2), respectively, and the high-resolution TEM (HRTEM) image and the corresponding selected area electron diffraction (SAED) patterns of the CPDs-1 illustrate a well-resolved lattice spacing of 0.252 nm, corresponding to the (110) interplanar spacing of graphitic carbon structure. Atomic force microscopy (AFM) images imply that the height of these CPDs is smaller than 10 nm (Figures 1C and S3). Meanwhile, the X-ray diffraction (XRD) patterns of these CPDs show a main peak at around 23.1°, which can be attributed to the graphite (002) lattice spacing (Figure 1D). The Fourier transform infrared (FT-IR) spectra illustrate the chemical composition and functional groups of these CPDs. As shown in Figure 1E, the peaks around 3400 and 3200 cm\(^{-1}\) can be attributed to the stretching vibration of O—H and N—H. The peaks at 2900 and 1400 cm\(^{-1}\) are derived from the stretching and bending vibration of sp\(^3\) C (C—O/C—N). And the peaks at 1670 and 1600 cm\(^{-1}\) imply the existence of the stretching and skeletal vibration of sp\(^2\) C (C=C/C=N), respectively. Especially, the peaks around 2800 cm\(^{-1}\) can be ascribed to the vibration of —CH\(\_\)—, clearly indicating the existence of the alky long-chains on the surface of these CPDs. When the solvent for dispersing these nanoparticles is changed from ethanol to highly polar water, these CPDs can further self-assemble to form larger CNGs by hydrophobic interaction as illustrated in Figure 1A.

As shown in Figure 1F, the CNGs-1 formed by CPDs-1 illustrates granuliform shape with average diameter of 50 nm and similar lattice spacing of 0.252 nm, signifying that only the size changes, but the structure core does not change, similar to the other CNGs-2, CNGs-3 CNGs-4, and CNGs-5 formed by CPDs-2, CPDs-3, CPDs-4, and CPDs-5, respectively (Figure S4). And the AFM images reveal that the height of CNGs-1 also increases to around 50 nm (Figure 1G). Meanwhile, the corresponding PL intensities of the CNGs in water are much lower than those of the CPDs in ethanol, indirectly verifying that the nanoaggregate of the CPDs is formed in water and further leads to the aggregation-caused quenching (ACQ) phenomenon (Figures 1H and S5). Besides, the dynamic light scattering (DLS) images suggest that the hydrodynamic diameter of these CNGs is around 100 nm, which was larger than that
observed in TEM image owing to the polymer chains on the surface of CPDs (Figures 1I and S6).

To further investigate the mechanism of self-assembly, the chemical element and structure of these CPDs are employed to be characterized with nuclear magnetic resonance (NMR) and X-ray photoelectron spectroscopy (XPS). As shown in Figure 1J, the $^1$H NMR spectra of CPDs-1 illustrate the obvious signals of aromatic H, pyrrole H, and pyridine H, similar to CPDs-2, CPDs-3, CPDs-4, and CPDs-5 (Figure S7). And the $^{13}$C NMR spectra also confirm the obvious existence of polymer alkyl chain in these CPDs. Thus, the slight
deviations of the height and diameter between these CPDs are attributable to the various degrees of polymerization induced by the different surface groups. Due to the low contrast of surface polymer chains, only carbon cores can be observed in their HRTEM images. In addition, the full XPS spectra of these CPDs clearly illustrate three typical peaks, corresponding to the elements of C, N, and O and the high-resolution XPS C1s envelope can be deconvoluted into three Gaussian peaks corresponding to sp² C, sp³ C, and (C=O)—O (Figures 1K and S8). Furthermore, the O1s envelope can be deconvoluted into one Gaussian peak of carbonyl O, indicating hydrophilic polar chains also exist on the surface of these CPDs (Figures 1L and S9). On the basis of above characterization, it can be deduced that these CPDs consist of carbon cores and hydrophilic and hydrophobic groups on their surface (Figure 1M), which can act as conjugated blocks for further self-assembly by the hydrophobic interaction.

### 3.2 Chemiluminescent CPDs and photodynamic CNGs

Except the self-assembly property, these CPDs exhibit novel PL and CL emission, and their corresponding CNGs illustrate high photodynamic ability. As shown in Figure 2A, all these CPDs in ethanol solution exhibit very broad absorption from ultraviolet (UV) to NIR light region with deep-red/NIR PL emission under UV light excitation. Herein, these CPDs solutions show excitation-independent PL emission with peak at around 670 nm, narrow FWHM of around 20 nm, and weak shoulder emission of about 720 nm, implying localized-state-emission character because of the existence of the vibrational fine structure in PL spectra (Figure S10). In addition, the CL of these CPDs also exhibits the same feature of deep-red/NIR emission and narrow FWHM in a conventional peroxalate-H₂O₂ system. As shown in Figure 2B, when these CPDs are mixed with the CPPO and H₂O₂, bright and persistent deep-red/NIR CL can be perceived by naked eyes. Similar with their PL emission, the CL emission of these CPDs also presents deep-red/NIR emissive spectra with peak at around 670 nm and narrow FWHM of about 20 nm. The CL decay spectra of these CPDs keep consistent with their corresponding steady-state PL spectra, manifesting the emission of these CL systems is originated from the recombination of localized-state excitons under the chemical excitation (Figure S11). Moreover, the PL intensities of these CPDs in water/ethanol mixtures gradually become weakened with the increase of water content (Figures 2C and S12), and the PL lifetime of these CPDs gradually decreases with the increase of water content (Figures 2D and S13), also proving high polar water promotes the polymerization of CPDs into large aggregates of CNGs. In addition, the similar absorption of these CPDs and CNGs indicates there is no change in the optical and electronic-state property of the CPDs and their aggregated state (Figure S14). Furthermore, the absolute PL QYs of these CPDs under 410 nm excitation are determined to be 0.59%, 0.42%, 1.13%, 0.55%, and 1.01% for CPDs-1, CPDs-2, CPDs-3, CPDs-4, and CPDs-5, respectively (Figures 2E and S15). With lucigenin-H₂O₂ reaction as a reference, their corresponding CL QYs are calculated to be 3.98 × 10⁻³, 7.68 × 10⁻⁴, 6.21 × 10⁻³, 3.70 × 10⁻³, 4.62 × 10⁻³ einsteins/mol for CPDs-1, CPDs-2, CPDs-3, CPDs-4, and CPDs-5, respectively (Figures 2F and S16), which are comparable with values in ever reported NIR CL reporters.

According to the characterizations and previous report, the PL mechanism of the efficient deep-red/NIR emission for the CPDs can be deduced. For these five CPDs, similar emission peak of 670 nm and PL lifetime of around 6.0 ns well fitted by monoexponential function can be observed (Figures 2D and S13), indicating the similar PL emission center in these CPDs. The XPS N1s spectra of these CPDs show two peaks assigned to pyrrolic N and graphitic N, indicating that the deep-red/NIR emission of these CPDs may be originated from the N-related localized state from the carbon cores (Figures 2G and S17). Meanwhile, the ultra narrow FWHM of around 20 nm and extra shoulder emission from the CPDs also demonstrate the typical localized-state-emission character. In addition, when these CPDs transform into CNGs in water, these CNGs illustrate similar emission peak at 670 nm while their corresponding decay curves present a decreased lifetime owing to the elevated aggregation degree. Combined with the diversity of the CPDs and CNGs observed from TEM and AFM images, it can be concluded that the CPDs with hydrophobic groups on their surface exhibit diverse polymer-like behavior and can form nanogels with different sizes, resulting in their distinct-different photophysical properties. Moreover, these CPDs exhibit intense self-absorption with a great overlap between their absorption and emission, which is critical to the ability to produce ROS for their self-illumination (Figures 2H and S18). In this case, their ROS production ability in aqueous solution was evaluated under light irradiation. As shown in Figure 2I, the ROS such as oxygen-independent type I photochemical free radicals (∗—O₂ and •OH) and oxygen-dependent type II photochemical single oxygen (‘O₂) can be effectively produced in aqueous solution with light illumination, which all can be used as effective cytotoxins for PDT with these CNGs (Figure S19). On the basis of the above characterization, a possible transition model is proposed...
to demonstrate the ability of bioimaging and PDT of these CNGs (Figure 2J). After light or chemical excitation, the ground-state electrons of the CPDs will be excited to the nitrogen-related localized state. Part of electrons will transit to the ground state through a radiative recombination and produce deep-red/NIR light emission, contributing to the bioimaging. In aqueous solution, the monodispersed CPDs will polymerize into large CNGs and induce strong self-absorption and ACQ through energy transfer (ET) process, which is conducive...
to increase the intersystem crossing rate. Thus, part of excited-state electrons will transfer to the H₂O and ambient dissolved oxygen and further lead to the production of hydroxyl radicals and singlet oxygen, endowing CNGs with the ability of self-illumination PDT.

### 3.3 Design and synthesis of the ROS-responsive chemiluminescent CNGs

On account of the polymer-like and splendid CL property, these CPDs are attempted to nanoassemble with amphiphilic polymeric conjugate (Pluronic 127, F127) and CL donors (CPPO) as monomers, further preparing ROS-responsive chemiluminescent CNGs. As shown in Figure 3A, it is found that these CPDs can nanoprecipitate with CPPO and F127 to form hydrophilic nanogels as demonstrated in Section 2. When these chemiluminescent CNGs are exposed to H₂O₂, the reaction between CPPO and H₂O₂ yields the energetic 1,2-dioxetanedione intermediate, which can exchange electron and excite the CPDs by the process of CIEE. Then the nearby photosensitizer (PS) molecules of CPDs excited by intermediate will undergo intersystem crossing from the excited localized state to the ground state or transfer electron

![FIGURE 3](image-url) Design and synthesis of the ROS-responsive chemiluminescent CNGs. (A) Schematic illustration of the preparation of the chemiluminescent CNGs (CCNGs). (B) Illustration of the principle for CL emission and 1O₂, •OH and •−O₂ generation of these CPDs as photosensitizer (PS) in the presence of H₂O₂. (C) The TEM images of the CCNGs-1 and the HRTEM images for the nanoparticles with different sizes. (D) The photograph of these CCNGs aqueous solution under sunlight. (E) DLS distribution of these CCNGs. (F) The CL intensities of these CCNGs with and without the presence of 50 μmol/L H₂O₂ PBS solution, inset: the according photograph captured with 30 s of these CCNGs with and without the presence of H₂O₂. The CL emission spectra (G) and dynamic CL intensities (H) of these CCNGs detected under by adding 1 ml CCNGs aqueous solution (10 mg/ml) into 1 ml H₂O₂ (200 mmol/L) (EM slit = 20 nm, PMT voltage = 950 V). (I) The corresponding CL QYs of these five hinds of CCNGs. CL, chemiluminescence; CNG, carbon nanogels; DLS, dynamic light scattering; HRTEM, high-resolution transmission electron microscopy; PBS, phosphate buffer saline; ROS, reactive oxygen species; TEM, transmission electron microscopy.

- **A:** Schematic illustration of the preparation of the chemiluminescent CNGs (CCNGs).
- **B:** Illustration of the principle for CL emission and 1O₂, •OH and •−O₂ generation of these CPDs as photosensitizer (PS) in the presence of H₂O₂.
- **C:** TEM images of the CCNGs-1 and HRTEM images for the nanoparticles with different sizes.
- **D:** Photograph of these CCNGs aqueous solution under sunlight.
- **E:** DLS distribution of these CCNGs.
- **F:** CL intensities of these CCNGs with and without the presence of 50 μmol/L H₂O₂ PBS solution, inset: the according photograph captured with 30 s of these CCNGs.
- **G:** CL emission spectra and dynamic CL intensities of these CCNGs detected under by adding 1 ml CCNGs aqueous solution (10 mg/ml) into 1 ml H₂O₂ (200 mmol/L) (EM slit = 20 nm, PMT voltage = 950 V).
- **I:** Corresponding CL QYs of these five hinds of CCNGs.
to H₂O and oxygen, further generating the deep-red/NIR emission or cytotoxic ROS. With the most abundant ROS (H₂O₂) at oxidation biology in organisms, the CCNGs can evoke the deep-red/NIR self-irradiation, which can be used to overcome the limitation of penetration depth in traditional fluorescence imaging and PDT (Figure 3B). As shown in Figure 3C, the TEM and HRTEM images reveal that the CCNGs-1 have poly-dispersed morphology with a wide size ranging from 10 to 1000 nm, as similar as all the other CCNGs (Figure S20). By optimizing the different images, it can be found that these CCNGs are composed of two kinds of nanoparticles. In these CCNGs, the large particles with diameters from 100 to 1000 nm is deduced to be self-assembled by the CPDs, CPPO and F127, and the smaller particles with diameters from 10 to 100 nm are self-assembled by F127 and CPPO. These CCNGs prepared by these CPDs can be well dispersed in water and illustrate clear apparent color under sunlight (Figures 3D and S21). In addition, the DLS of CCNGs illustrate two number weighted hydrodynamic diameters ranging from 10 to 100 nm and 100 to 1000 nm (Figure 3E), which are corresponding to their TEM images.

The CL properties of these CCNGs in aqueous solution are further investigated with the IVIS. As shown in Figure 3F, the CL signals of these 50 μl CCNGs (10 mg/ml) in the presence of 1 ml H₂O₂ (50 μmol/L) are far higher than their corresponding blank, elucidating a high response sensitivity to exogenous ROS for these CCNGs. Moreover, the CL emission spectra after adding these CCNGs into H₂O₂ aqueous solution have been also measured. As illustrated in Figure 3G, these CCNGs exhibit deep-red/NIR CL emission with peak at around 680 nm with the narrow FWHM of around 20 nm, which are the same with their PL emission. Meanwhile, the CL emission of these CCNGs can persist over 40 min, which is very beneficial to long persistent glowing and deep penetration of NIR emission, these CCNGs are first employed to be used for activatable CL bioimaging of ROS in vivo and in vitro as illustrated in Figure 4A. Meanwhile, the practicability of these CCNGs for in vitro imaging of exogenous H₂O₂ is assessed by the IVIS in the cell plate (Figure S23). These CL images have been captured after adding these CCNGs into different concentrations of H₂O₂ in PBS solution. As shown in Figure 4B, these CL intensities of these CCNGs after added into H₂O₂ solution present a significant linear relationship with increasing the H₂O₂ concentration from 0 to 50 μmol/L. H₂O₂ is a major ROS generated in living organisms, overproduction of which is closely associated with the inflammation-associated diseases such as cancer, arthritis, chronic obstructive pulmonary diseases, and neurodegenerative diseases. Thus, these CCNGs can be applied for the detection and diagnosis of these diseases through monitoring the low concentration of H₂O₂ by the inflammatory imaging. Herein, the inflammatory mouse models have been established through intraperitoneal injection with Zymosan A, which can induce acute peritonitis in mouse body and further generate excessive H₂O₂. Waiting for 24 h, the deep images of mouse models have been subsequently captured for 3 min after the injection of the 300 μl CCNGs-1 (333 μg/ml based on CPDs-1). The CL diagnostic signal of Zymosan A-treated mice is almost 2.5 times higher than that for the control mice (Figures 4C, S24, and S25). After the peritonitis mice were remedied with an antioxidant glutathione (GSH), CL signal intensities of the Zymosan A/GSH-treated mice show a 40% reduction (Figure S26). As an inflamed-to-normal contrast signal, the enhanced-to-reduced CL intensity can efficiently monitor the variation of inflammatory disease in living animals. In addition, the CL emission of these CCNGs in the presence of H₂O₂ in PBS can be monitored by the IVIS for about 1 h. The CL intensities measured by the IVIS with every 5-min interval illustrate an exponential decrease and long monitoring time of more than 40 min (Figure 4D). Hence, the dynamic CL of the CCNGs has been further tested in inflammatory mouse models. After the injection of 0.5 ml CCNGs-1 (333 μg/ml based on CPDs-1), real-time imaging reveals substantially strong and sustainable luminescence in Zymosan A-treated mice. As illustrated in Figure 4E, the in vivo CL signals also last more than 40 min and the CL intensity presents an exponential decrease within the 40 min. These investigations indicate the CCNGs can be used as inflammation-responsive imaging agent in living body, which is potential for the long-term bioimaging and disease monitoring. Finally, the inflammation imaging has been also proved by the histological analyses of hematoxylin and eosin (H&E)-stained sections of peritoneal tissue. As shown in Figure 4F,G, normal microstructure can be observed at the H&E-stained sections with the straight luminal surface, tightly packed tubules closely approximating the muscularis mucosae and numerous goblet cells. It is found

3.4 In vitro and in vivo ROS-responsive CL imaging of inflammation

With the merit of long persistent glowing and deep penetration of NIR emission, these CCNGs are first employed to...
that these CL intensities consist with inflammation score under different conditions, which is a common parameter for evaluating the degree of colitis (Figure 4H). Similar positive correlations can be also observed with the H₂O₂ levels and parietal peritoneum thickness as well as the inflammation score (Figures 4I,J). The correlation analyses further affirm the increased endogenous ROS lead to the CL enhancement (Figure 4K). Overall, these above results display that these biocompatible biomass-derived CCNGs have considerable potential as effective nanoprobes for in vivo bioimaging of different inflammatory disorders, which are associated with high ROS expression.
3.5 In vitro and in vivo antitumor activity of the chemiluminescent CNGs

In the tumor microenvironment, ROS like H₂O₂ also tend to be highly expressed. Hence, in addition to as nanoparticles for in vivo bioimaging, the CCNGs have great potential to be as nanomedicine for antitumor applications. As shown in Figure 5A,B, the cytotoxicity of CPDs-1 and CCNGs-1 is firstly investigated. Herein, nearly 100% viability for A549 cells can be obtained when the concentration of the CPDs-1 is up to 40 µg/ml, indicating the biocompatibility of the CPDs. In comparison, the CCNGs-1 illustrate a higher cytotoxicity and the IC₅₀ value (inhibitory concentration of 50% cell death) is determined.

**FIGURE 5** In vitro and in vivo antitumor activity of the chemiluminescent CNGs. (A) Relative viabilities of cells incubated with various concentrations of CPDs-1. (B) Relative viabilities of cells with various concentrations of CCNGs-1. The flow cytometry effects (C) and representative quantitative data (D) of ROS concentration in A549 cells treated and untreated with CCNGs-1 incubated by DCFH-DA as probes. (E) The microscopic images (left) and representative quantitative fold change (right) of area of A549 cells after treatment with CCNGs-1 by wound-healing assay. (F) The microscopic images (left) and representative number (right) of cell migration of A549 cells after treatment with CCNGs for 24 h by transwell assay. Scale bars, 100 µm. (G) The representative digital photos of mice of the control group and the CCNGs-1-treated group. Scale bars, 2 cm. Changes of mice body weight (H) and tumor volume (I) of nude mice recorded every 3 days after different treatments. Scale bar, 1 cm. (J) Digital photos (left) and representative quantitative weight (right) of tumors removed from the killed mice. Microscopic images of H&E-stained sections of liver and kidney isolated from mice stimulated with DSS for nude mice of the control group (K) and the CCNGs-1-treated group (L). Scale bars, 100 µm. Data in (B)–(E), (G), and (J) are means ± SEM (n = 6). CCNG, chemiluminescent carbon nanogels; CNG, carbon nanogels; CPD, carbonized polymer dots; ROS, reactive oxygen species.
to be 10.452 μg/ml, indicating the potential application of cancer therapy. Thereupon, the ROS performance of A549 cells after treatment with CCNGs-1 is estimated by using commercial ROS probes (2′,7′-dichlorodihydrofluorescein diacetate, DCFH-DA). As illustrated in Figure 5C,D, the ROS profiles of CCNGs-1 in the presence of indicator DCFH-DA illustrate a continuous change, indicating the slow interaction between the CCNGs-1 and ROS in these cells. In the process, the emission of DCFH-DA is aroused and rapidly reduced with the CCNGs-1, implying the reaction between CCNGs-1 and H₂O₂. Then, the emission of DCFH-DA is slowly boosted with time, reaching equality or enhancement in 180 min, revealing the controlled-release effect and highly efficient ROS conversion of CCNGs-1.

As a proof of principle, the CCNGs-1 are further applied as nanomedicine for the self-illumination PDT of cancer on the basis of their outstanding type I and type II photochemical ROS production capacity. As shown in Figure 5E, the wound-healing assay of A549 cells after treatment with CCNGs-1 has been conducted and the corresponding fold changes of migration area treated with different concentrations of CCNGs-1 after 24 h have been calculated. In these pictures, the scratch area of cells treated with CCNGs-1 illustrates an obvious inhibition of wound healing, in which the fold change area with CCNGs-1 (20 μg/ml) is about twofold higher than that without CCNGs-1. Meanwhile, the invasion ability of A549 cells after treatment with CCNGs-1 has been conducted after fixed and stained in chamber to obtain the invasion assay. As shown in Figure 5F, the CCNGs-1 obviously cripple the invasion ability of A549 cells, in which the number of cell migration with 20 μg/ml CCNGs-1 is about half of that without CCNGs-1.

In addition to cell and tissue validation, the solid anticancer efficiency of the CCNGs-1 has been further evaluated in the tumor-bearing mice, in which the body weight, tumor long diameter, and short diameter have been recorded every 3 days during a treatment period of 33 days. The toxicological studies in vivo have been estimated with the CCNGs-1 as medicine and illustrate obvious diversity between the control group and the CCNGs-1-treated group (Figure S27). As shown in Figure 5G, all the mice are alive and the tumor volumes of CCNGs-1-treated group are smaller than those of the saline control group after intratumoral administration, indicating the potential therapeutic effect of CCNGs-1. As shown in Figure 5H, a moderate increase occurs in the body weight of both the CCNGs-1-treated group and the saline control group and their body weights increase to almost the same degree, validating that there are no noticeable side effects in all the treatments. Meanwhile, the tumor long diameter and short diameter of the CCNGs-1-treated group increase more slowly than those of the control group (Figure S28). Thereby, the lower degree of tumor volumes increase can be calculated and it is obviously indicated the antitumor efficacy of CCNGs-1 (Figure 5I). Subsequently, tumors are completely ablated at Day 33 and the tumor weight has been further investigated. As shown in Figure 5J, the CCNGs-1-treated group exhibited significantly reduced tumor weight compared with the control group. The safety profile of CCNGs-1 administered to mice is further investigated, in which the H&E-stained sections of liver and kidney have been obtained from all the mice at Day 33 after treatment. As shown in Figure 5K,L, the H&E-stained sections of the CCNGs-1 treated group show no notable injuries, as similar as those of the control group (Figure S29). Consequently, these results validate a good safety profile of CCNGs-1 for intravenous administration.

3.6 | Mechanism of the CCNGs-mediated antitumor activity

The possible mechanism of CCNGs-mediated antitumor activity has been further probed in A549 cells. As shown in Figure 6A, the CCNGs-1-induced apoptosis of A549 cells in a dose- and time-dependent manner is performed. It can be found the A549 cells exhibit higher apoptosis rate with the increase of the dose of CCNGs-1 and the treatment time, indicating that apoptosis might be the major cause of cell death (Figure 6B). Meanwhile, the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) assay and cleaved caspase-3 illustrated different cell apoptosis in the sections of tumor tissues from CCNGs-1-treated mice and control saline mice (Figure 6C). The ROS performance of tumor tissue after treatment with CCNGs-1 is evaluated by another ROS probes (dihydroethidium, DHE). As shown in Figure 6D, after incubating CCNGs-1, the tumor illustrates more ROS performance, demonstrating the ROS-induced generation of O₂ by CCNGs-1 under oxidative conditions. Furthermore, the proliferative activity of tumors has been also assessed by H&E-staining tests. As depicted in Figure 6E,F, compared with the control groups, the CCNGs-1-treated mice show more severe apoptosis or necrosis of tumor cells and the blood vessels are destroyed to barely visible in tumor tissue (Figure S30). These results strongly validate that CCNGs-induced apoptosis is mediated by the mitochondrial pathway of apoptosis.

To test this hypothesis, the Western blot analysis has been performed, revealing a significant increase in caspase-3 and bax level after treating A549 cells
FIGURE 6  Mechanism of CCNGs-mediated antitumor activity. Flow cytometric analysis (A) and representative quantitative data (B) of apoptosis rate. (C) TUNEL Cle-Cas3 and DAPI labeling assay (left) and quantitative analysis (right) of cell apoptosis in paraffin sections. (D) Confocal microscopy images (left) and quantitative analysis (right) illustrating ROS in A549 cells incubated by DHE probe. Scale bars, 100 μm. Microscopic images of H&E-stained sections of the tumor removed from the killed mice of the control group (E) and the CCNGs-1-treated group (F). Scale bars, 1 mm (left) and 100 μm (right). (G) Representative Western blot bands (left) and quantitative analysis (right) of total and cleaved caspase-3 in A549 cells after treatment with/without CCNGs-1 for 24 h. (H) Schematic illustration of the antitumor mechanisms of CCNGs-1. Data in (B)–(E), and (G) are means ± SEM (n = 6). CCNG, chemiluminescent carbon nanogels; H&E, hematoxylin and eosin; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.
with CCNGs-1 (Figures 6G and S31). In the caspase-mediated apoptosis, caspase-3 is a downstream execution molecule responsible for the initiation of apoptosis. The activation of these molecules collectively revealed that CCNGs-1 induced cell death through the apoptotic pathway. Together, these data demonstrate that CCNGs-induced cell apoptosis is largely mediated via the intrinsic mitochondrial pathway. Hence, a possible antitumor mechanism can be described as follows: When the CCNGs enter the tumor site, they can enter cells through endocytosis. Endogenous ROS of H₂O₂ in tumor cells will trigger the CIEEL to generate self-illumination. The internal radiation will in turn promote CCNGs to produce cytotoxic ROS including oxygen-independent type I photochemical free radicals (•–O₂ and •OH) and oxygen-dependent type II photochemical single oxygen (¹O₂). Furthermore, these active substances activate caspase, which can mediate apoptosis and induce cell death via the apoptotic pathway (Figure 6H).

4 | CONCLUSION

In summary, we have prepared five kinds of CPDs as luminescent agents which exhibit novel efficient deep-red/NIR PL and CL emission with narrow FWHM of 20 nm. Because of their polymer-like property, these CPDs can self-assemble into CNGs in aqueous solution and thereby ROS-responsive CCNGs are further designed and prepared by these CPDs with amphiphilic polymeric conjugate and CL donors. The luminescence of CCNGs is positively related to the levels of environmental H₂O₂ and offers high detection sensitivity and specificity, superior imaging quality, and satisfactory safety for in vitro and in vivo bioimaging applications. Moreover, ROS as active substances can effectively activate the apoptotic pathway and induce cancer cell death. And the CCNGs can be simultaneously functionalized as an effective photodynamic nanotherapy for tumors by expressing high ROS levels without the need for external excitation. Considering the multiple advantages of simultaneous diagnosis and treatment, these CNGs are promising to be developed for future clinical translation.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS STATEMENT

All animal experiments were approved by The First Affiliated Hospital of Zhengzhou University under Protocol No. 2019-KY-008.

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**SUPPORTING INFORMATION**

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