ABSTRACT: Nanozymes are nanomaterials with enzyme-like activities, which have been developed for inflammatory disease therapy by reactive oxygen species (ROS) scavenging. The application of nanozymes in ulcerative colitis (UC) treatment not only inherits the merits of small molecular antioxidants (e.g., 5-aminosalicylic acid) to scavenge ROS but also achieves catalytic recycle instead of stoichiometric consumption. However, current therapies usually ignore the repair of mucosa, the first line of defense, whose damage increases the risk of infections. Herein, a multifunctional nanozyme hydrogel is designed and verified both as an ROS scavenger and a mucosal healing enhancer for UC therapy. The chitosan-coated CeO2 nanozyme (CCNZ) not only possesses excellent ROS-scavenging ability but also exhibits satisfactory antibacterial capacity. After gelation with alginate, the optimized CCNZ1:Alg1.5 nanozyme hydrogel exhibits multiple functions, including inflamed site targeting, supporting cell growth, ROS scavenging, and antibacterial activity, which alleviates UC better than a clinical medication 5-aminosalicylic acid by even a single-dose treatment. This study reveals that a nanozyme providing mucosal healing is promising for UC therapy with excellent potential for clinical application and enriches the nanozyme research of treatment for diseases.

INTRODUCTION

Ulcerative colitis (UC), one of the typical inflammatory bowel diseases, is a chronic inflammatory disease accompanied by weight loss, rectal bleeding, diarrhea, and abdominal pain. After the mucosal barrier is disrupted in the gut, the innate immune cells, such as polymorphonuclear leukocytes, macrophage, and lymphocyte, migrate to inflamed sites, which engulf invading pathogens and release reactive oxygen species (ROS). However, persistent oxidative stress with uncontrolled overproduction of ROS will result in tissue injury, leading to the initiation and progression of UC. Until now, almost 60% of UC patients have received oral treatment with 5-aminosalicylic acid (5-ASA), which has shown antioxidation activities to various degrees. Although it is a conventional drug for UC therapy, the side effects from 5-ASA generally hinder the sufficient doses to ensure the efficacy. Moreover, the lack of inflamed site targeting results in the further decrease of therapeutic concentration in the gut. Therefore, new therapeutics are urgently needed for UC therapy.

Recently, nanozymes have attracted much attention in various disease therapies including UC therapy, which can catalytically scavenge ROS with their intrinsic enzyme-like activities. For example, Prussian blue and MnO2 nanozymes with multiple enzyme-like activities have been fabricated to effectively relieve colitis. The Pt@PCN222-Mn nanozyme was designed to mimic the superoxide dismutase (SOD)/catalase (CAT) cascade reactions for better ROS elimination and achieved effective colitis therapy. Meanwhile, to improve gut specificity, negatively charged nanozymes were designed to target the inflamed colonic epithelium which is characterized with the accumulation of positively charged proteins. For instance, the negatively charged ceria nanozyme grown on sheets of montmorillonite was able to target the inflamed colonic sites for UC therapy. While these studies have shown the promising therapeutic efficacy of nanozymes because of their advantages over natural enzymes or antioxidant drugs, the mucosal healing has not been achieved with nanozymes yet in UC therapy. Mucosal inflammation is observed in about 45% of patients with UC in clinical remission, which could be attributed to the ROS-induced inflammation and the activity loss of...
epithelial antimicrobial peptides. Mucosal healing is considered as a crucial endpoint in the treatment of UC patients. Therefore, it is necessary to design nanoenzymes with mucosal healing activity for better UC therapy. To fulfill this need, herein we reported the development of a CeO2-encapsulated chitosan (Cs) and alginate (Alg) hydrogel (CCNZ&Alg). To distribute CeO2 uniformly, we designed a Cs-coated CeO2 nanozyme (CCNZ), which maintained the SOD- and CAT-like activities of the pristine nanozyme and would eliminate excessive ROS and exert antibacterial activity. After gelation with Alg, the dual-crosslinked hydrogel CCNZ&Alg would not only provide an extracellular matrix-like microenvironment to favorably support cell growth for mucosal healing but also enable the inflamed site targeting because of its overall negative charges (Scheme 1). Additionally, the nanozyme hydrogel was stable in stomach and would gradually degrade in colon after oral administration. Encouragingly, a single-dose treatment with the nanozyme hydrogel achieved better therapeutic efficacy than 5-ASA under the same dosage, demonstrating the promise of combining anti-inflammation with promoted mucosal healing in UC therapy.

RESULTS AND DISCUSSION

Synthesis and Characterizations of the Chitosan-Coated CeO2 Nanozyme. The CeO2 nanozyme was chosen in this work because of its several advantages compared with other nanozymes, such as multienzyme-mimicking activities; good stability in stomach after oral administration; and satisfactory biocompatibility. The CeO2 nanozyme was prepared using the NH4OH precipitation method and further coated with Cs (Figure 1a). The chitosan-coated CeO2 nanozyme was designated as CCNZ. The formation of CCNZ was confirmed by transmission electron microscopy (TEM) imaging, showing uniform and discrete CCNZ with a particle size of about 5 nm (Figure 1b). A hydrodynamic diameter of about 10 nm in aqueous solution was observed (Figure S1). The crystalline features of CCNZ were characterized by using X-ray diffraction (XRD). As shown in Figure 1c, the characteristic peaks of CeO2 (2θ = 28.5, 32.9, 47.4, and 56.8°) were observed, further confirming the successful synthesis. Figure 1d shows the thermogravimetric analysis (TGA) curve of CCNZ in the range of 100–850 °C under a nitrogen atmosphere. The result was consistent with the inductively coupled plasma (ICP) characterization that the content of CeO2 in CCNZ was 60 wt % (Table S1). In addition, the Fourier-transform infrared (FT-IR) spectra (Figure 1e) showed characteristic peaks of Cs at 3445 (νOH and νNH absorptions), 1076 (C−N stretching vibration), and 1029 cm⁻¹ (C−O−C stretching vibration) from CCNZ, demonstrating the successful coating of Cs and the intact...
structure of Cs after coating. The ratio of Ce$^{3+}$ and Ce$^{4+}$ on the surface of CCNZ was calculated according to X-ray photoelectron spectra because the enzyme-like activities are dependent on it. As shown in Figure 1f,g, CCNZ showed a high ratio of Ce$^{3+}$ on the surface. It confirms the presence of both valences on CCNZ, which was not obviously influenced by the coating with Cs. In addition, the elements on the surface of CCNZ contain C, N, O, and Ce.

**ROS-Scavenging Activities of CCNZ.** CeO$_2$ nanoparticles can be coated by polymers with different terminal groups, which influence the functions of nanoparticles and tune the enzyme-like activities for biomedical applications. Therefore, the ROS-scavenging activity of CCNZ was further investigated. First, the SOD-like activity was correlated with the elimination of $\cdot$O$_2^-$, which was generated from riboflavin with light irradiation. As shown in Figure 1h, the $\cdot$O$_2^-$-scavenging activity of CCNZ exhibited a dose-dependent manner, and the $\cdot$O$_2^-$ elimination of 80 μg/mL CCNZ was close to 80% compared with that of the control group. In addition, Figure S2a shows that the $\cdot$O$_2^-$ elimination capacity of CCNZ was close to citric acid-coated CeO$_2$ which has been reported. The CAT-like activity of CCNZ was investigated by monitoring the dissolved O$_2$ from H$_2$O$_2$ decomposition, which exhibited time–concentration dependency relation (Figure 1i), increased by the reaction with H$_2$O$_2$. The H$_2$O$_2$ elimination of 80 μg/mL CCNZ was close to 60% compared with that of the control group (Figure S2b,c). These results showed that CCNZ retained the excellent SOD- and CAT-like activities of pristine CeO$_2$.

**Negative Surface Charge and Antibacterial Ability of the Nanozyme Hydrogel.** To increase the efficiency of inflammation targeting, the surface charge of the nanozyme hydrogel should be regulated to the negative charges, which facilitated adhesion to the inflamed colonic sites with positive charges. The positive charges can be attributed by the cationic proteins attached on the inflamed colonic sites after the damage of the mucosal barrier. Meanwhile, the infectious risks should also be inhibited during UC therapy. We hypothesized that the negatively charged surface and antibacterial effects of the nanozyme hydrogel not only exhibit...
mucosal targeting capacity for increasing intestinal adsorption but also decrease the infectious risks (Figure 2a). After regulating the ratio of CCNZ and alginate, the nanozyme hydrogel with different formulations (CCNZ:Alg, CCNZ:Alg1.5, CCNZ:Alg2, and CCNZ:Alg3) were formed (Figure 2b). We measured the zeta potential and antibacterial ability of these hydrogels. As shown in Figure 2c, the zeta potentials of CCNZ and CCNZ1:Alg1 were positive (+56.23 ± 2.06 and +21.2 ± 0.1 mV, respectively), while the zeta potentials of CCNZ1:Alg1.5, CCNZ1:Alg2, and CCNZ1:Alg3 were all negative charges (−28.1 ± 1.01, −41.9 ± 1.57, and −57.3 ± 1.19 mV, respectively). Overall, with decreasing the ratio of CCNZ, the negative charge increased. It was observed that CCNZ1:Alg1 and CCNZ1:Alg1.5 nanozyme hydrogels have superior antibacterial effects for *Staphylococcus aureus* and slightly antibacterial effects for *Escherichia coli* compared with...
the control group (Figure 2d,e). Note that the antibacterial effect of CCNZ was concentration-dependent (Figure S3), demonstrating that chitosan still exhibits an excellent antibacterial effect after coating. Therefore, combining the results of zeta potential and antibacterial properties, CCNZ:Alg1.5 was chosen as the optimal nanozyme hydrogel for further investigation.

The detailed structure of CCNZ:Alg1.5 was then studied. The Cs&Alg hydrogel was also studied as a comparison. The scanning electron microscopy (SEM) images of the cross sections of the Cs&Alg hydrogel and CCNZ:Alg1.5 nanozyme hydrogel are shown in Figure 2f–i. The internal morphologies of the hydrogels were observed. Interestingly, the CCNZ:Alg1.5 nanozyme hydrogel was more compact than Cs&Alg, which was due to the multivalent chelation between metal nanoparticle chelate alginate.22 The nanozyme hydrogel formed a tight arrangement by double crosslinking, resulting in smaller voids compared with the Cs&Alg hydrogel. In addition, the corresponding energy-dispersive spectroscopy (EDS) elemental mapping confirmed that the elements of O, C, Na,
and Ce are dispersed uniformly in the nanozyme hydrogel. Figure S4 shows that the weight percentage ratio of Ce is 14.3 wt % in the CCNZ1:Alg1.5 nanozyme hydrogel, which is close to the theoretical value (19.4%, Table S1). Meanwhile, the storage modulus ($G'$) and loss modulus ($G''$) were monitored over the frequency sweep (Figure 2j). It suggests that the $G'$ values are larger than $G''$ values for both Cs&Alg and CCNZ&Alg, confirming the formation of elastic hydrogels. Also, the $G'/G''$ values of the CCNZ1:Alg1.5 nanozyme hydrogel are larger than those of the Cs&Alg hydrogel, indicating that CCNZ1:Alg1.5 has better mechanical properties. The result was consistent with SEM imaging, which showed that the double network is formed in the nanozyme hydrogel.

To investigate whether the formation of the hydrogel affects the catalytic activity of CCNZ, the H$_2$O$_2$ elimination of CCNZ1:Alg1.5 was examined. As shown in Figure 2k, identical efficiency of CCNZ1:Alg$_{1.5}$ compared with that of CCNZ was observed at 19−24 h, and a slightly lower efficiency of the

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**Figure 4.** UC therapy with the nanozyme hydrogel compared with 5-ASA. (a) Illustration of DSS-induced UC mouse model and administration. (b) Daily body weight recorded over 11 days under different treatments. (c) Colon length of indicated treatments. (d) Representative images of colons of all treatment groups. (e) Fluorescence intensity of colon 0.5 h after injecting 0.7 mg of DCFH-DA per mouse. (f) Ce element contents from the colon of indicated treatments. (g) Histological examination of indicated treatments, H&E-, Masson’s trichrome-, and AB-PAS-stained pathological colonic sections of mice. Scale bar: 1 mm; and 100 μm. Data are presented as mean ± standard error of the mean (n = 6). *$P < 0.05$ vs DSS group, **$P < 0.01$ vs DSS group. Ns means no significance.
former was observed at 1–6 h (Figure S5), which demonstrated the retained ROS-scavenging activities. In practice, the nanozyme hydrogel has to stay in stomach for several hours. Therefore, the nanozyme hydrogel was kept in 0.9% NaCl (pH 1.2) for 4 h, and then, the H₂O₂ elimination efficiency was measured under neutral conditions (Figure S6). The result shows that the enzyme-like activity would not be influenced after passing through stomach, showing the possibility for oral administration.

**Biocompatible and Multifunctional CCNZ:Alg1.5 Nanozyme Hydrogel.** The cell viability of CCNZ and nanozyme hydrogels toward RAW 264.7 was determined to investigate their biocompatibility by using CCK8 (Cell Counting Kit-8) assays. Figure 3a shows that CCNZ did not inhibit the growth of RAW 264.7 cells within 100 μg/mL after coculture for 1 day. In addition, the transwell cell culture system was used to evaluate the potential cytotoxicity of the nanozyme hydrogel, as shown in Figure S7a. The result of Figure S7b indicates that no cytotoxicity was observed for nanozyme hydrogels of 1 or 10 mg/mL. Based on the excellent biocompatibility, we further examined the ROS-scavenging ability in cultured RAW 264.7 cells. Cells were incubated with CCNZ for 2 h, followed by 30 min of incubation with H₂O₂. The intracellular ROS level was then monitored by using a ROS-sensitive probe [2′,7′-dichlorofluorescin diacetate (DCFH-DA)] and evaluated by using laser scanning confocal microscopy (Figure S8) and flow cytometry (Figure 3b), respectively. In the presence of ROS, the nonscavenging probe (DCFH) was oxidized into its fluorescent form [2′,7′-dichlorofluorescin (DCF)]. After being treated with H₂O₂, the green DCF fluorescence represents the content of ROS in cells. The ROS level after CCNZ treatment was less than that of the H₂O₂ group, indicating that CCNZ can reduce the cellular ROS level and protect them from ROS injury, which benefits cell proliferation and growth in UC therapy. Generally, the sustained release of CCNZ from the nanozyme hydrogel in a physiological environment is shown in Figure S9. After being covalently grafted with the fluorescein isothiocyanate (FITC) fluorescence probe, the released CCNZ was monitored by using laser scanning confocal microscopy and flow cytometry, respectively. The fluorescence intensity of CCNZ in cells increased over time after being treated for 0–100 min. In addition, based on fluorescence image analysis, the released CCNZ was uptaken by RAW 264.7 cells.

To further investigate the growth of cells in the nanozyme hydrogel, RAW 264.7 cells were directly seeded on the CCNZ:Alg1.5 nanozyme hydrogel. Calcein-AM/PI staining (Figure 3c) illustrated the live/dead cells after culturing on the nanozyme hydrogel for 1 day. The results show that the majority of cells were stained in green (live cells), and few cells were stained in red (dead cells), indicating that cells were growing well on the CCNZ:Alg1.5 nanozyme hydrogel. Meanwhile, RAW 264.7 cells cocultured with the CCNZ:Alg1.5 nanozyme hydrogel in the transwell cell culture system, and the results of Calcein-AM/PI staining show excellent cytocompatibility (Figure S7c). Furthermore, as shown in Figure 3d, subcutaneously implanted CCNZ:Alg1.5 nanozyme hydrogel was used to demonstrate the host inflammatory response and the ability of cell growth in vivo. The samples were extracted after 7-day postimplantation and examined histologically. Hematoxylin and eosin (H&E) staining (Figure 3e) showed mild inflammatory responses that occurred in two groups (pointed with black arrows), indicating the typical foreign body response without excessive inflammation. Moreover, the CCNZ:Alg1.5 nanozyme hydrogel (black dashed box) maintained structural integrity and filled with cells including immune cells (pointed with blue square) and fibrocyte cells (pointed with green triangle), revealing that the robust mechanical structure provided a suitable microenvironment to the cells. In general, the CCNZ:Alg1.5 nanozyme hydrogel not only exhibited excellent cytocompatibility but also provided a robust mechanical microenvironment to support cell growth, compared with the Cs&Alg hydrogel. In addition, the negative surface charge of the CCNZ:Alg1.5 nanozyme hydrogel is able to target and adhere to the positively charged surface of inflamed colon epithelium. Fluorescent CCNZ was then obtained by covalently conjugating FITC to the NH₂ groups of the CCNZ (Figure 3f). Preferentially targeted and adhered to inflamed mucosa was further validated in vivo. Dextran sulfate sodium (DSS)-induced mice and untreated healthy control mice were orally administered with the FITC-conjugated CCNZ:Alg1.5 nanozyme hydrogel, sacrificed 12 (Figure 3g) and 24 h (Figure S10) later, respectively, and the fluorescence intensity was quantified (Figure 3h). The DSS group showed a higher fluorescence intensity than the control group, demonstrating more nanozyme hydrogel adhesion. It is obvious that the nanozyme hydrogel enhances the residence time in UC therapy. In short, the UC-targeting nanozyme hydrogel with satisfactory biosafety has been obtained and verified.

**In Vivo UC Therapy.** Based on the multifunctional nanozyme hydrogel, including ROS scavenging, antibacterial activity, excellent biocompatibility, excellent site targeting, and cell growth supporting, the acute DSS-induced UC model was utilized to investigate the therapeutic efficacy. As shown in Figure 4a, mice were subjected 8 days to drinking water containing 2% (w/v) DSS, which directly causes toxicity to colonic epithelial cells and compromises mucosal barrier, followed by the oral administration of CeO₂, Cs&Alg hydrogel, 5-ASA, CCNZ1:Alg1.5, and CCNZ1:Alg2 nanozyme hydrogel, respectively, once a day for 3 consecutive days (days 8, 9, and 10). 5-ASA is a first-line medication for UC therapy, which can remarkably suppress ROS. Meanwhile, the CCNZ1:Alg2 nanozyme hydrogel has a lower ratio of CCNZ than the CCNZ1:Alg1.5 nanozyme hydrogel. After that, the colon was collected, the length of which and pathological studies were analyzed to study the therapeutic efficacy. The body weight of all groups was recorded during the whole experiment, and the result shows that the weight of healthy mice increased, while that of all DSS-induced mice decreased, as shown in Figure 4b, demonstrating the successful onset of UC. After treatment for 3 days, the body weight of mice treated with the CCNZ1:Alg1.5 nanozyme hydrogel gradually recovered. Besides, all DSS-induced mice showed shortened colon length, but the CCNZ1:Alg1.5 nanozyme hydrogel exhibited significant protection against the shortening of colon length, and the CCNZ1:Alg2 nanozyme hydrogel also slightly protected from shortening. The colon lengths of other groups (i.e., 5-ASA, CeO₂, and Cs&Alg hydrogel) were similar to those of the DSS group (Figure 4c), and the representative images and all images of colon are shown in Figures 4d and S11. In addition, consistent with the ex vivo fluorescence intensity of colon after intraperitoneal injection of 0.7 mg DCFH-DA per mouse (Figures 4e and S12), the DSS-induced groups showed high intensity, while the CCNZ1:Alg1.5 nanozyme hydrogel
significantly decreased average radiant efficiency. This result
demonstrated that the inflammatory response was effectively
alleviated after ROS scavenging by the nanozyme. Moreover,
as shown in Figure 4f, more abundant Ce was examined in the
colon after being treated with the CCNZ1:Alg1.5 nanozyme
hydrogel compared to the CeO2 group, indicating that the
nanozyme hydrogel efficiently adhered to inflamed mucosa and
exerted healing efficacy. The result is in accordance with the
fluorescence images of colon after treatment with the FITC-
conjugated CCNZ1:Alg1.5 nanozyme hydrogel as mentioned
above (Figure 3f–h).

Besides, to further evaluate the therapeutic efficacy, the
histological examination was performed and the results are
shown in Figure 4g. H&E staining indicated the presence of
severe glandular defects, inflammatory cell infiltration, and
mucosal ulceration in the inflamed colon sections after DSS
treatment. In contrast, lots of intestinal crypts present a single
straight tubular gland with intact structures, which are open to
the mucosal surface in the control group. Compared with the
DSS group as well as the other treated groups, the
CCNZ1:Alg1.5 nanozyme hydrogel group exhibited much
lower inflammatory cell infiltration and had a sound structure.
Besides, fibrosis response in the DSS-induced UC model was
shown by Masson’s trichrome staining.25 In the DSS group,
much collagen deposition (blue) was observed. However, the
areas of collagen deposition were measurably decreased after
nanozyme hydrogel treatment. Importantly, the goblet cells and mucus were stained by Alcian blue-periodic acid-Schiff
(AB-PAS) staining to investigate their integrity. Goblet cells
secrete mucus that have a well-appreciated role in barrier
maintenance, preventing pathogens from invading the mucosa
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induced colitis. Among all the treatment groups, the numbers

Besides, to further evaluate the therapeutic efficacy, the histological examination was performed and the results are shown in Figure 4g. H&E staining indicated the presence of severe glandular defects, inflammatory cell infiltration, and mucosal ulceration in the inflamed colon sections after DSS treatment. In contrast, lots of intestinal crypts present a single straight tubular gland with intact structures, which are open to the mucosal surface in the control group. Compared with the DSS group as well as the other treated groups, the CCNZ1:Alg1.5 nanozyme hydrogel group exhibited much lower inflammatory cell infiltration and had a sound structure. Besides, fibrosis response in the DSS-induced UC model was shown by Masson’s trichrome staining.25 In the DSS group, much collagen deposition (blue) was observed. However, the areas of collagen deposition were measurably decreased after nanozyme hydrogel treatment. Importantly, the goblet cells and mucus were stained by Alcian blue-periodic acid-Schiff (AB-PAS) staining to investigate their integrity. Goblet cells secrete mucus that have a well-appreciated role in barrier maintenance, preventing pathogens from invading the mucosa to cause intestinal inflammation. The numbers of goblet cells and mucus thickness were significantly decreased in DSS-induced colitis. Among all the treatment groups, the numbers

of goblet cells and mucous thickness were recovered after CCNZ1:Alg1.5 nanozyme hydrogel treatment. As previously
mentioned, it is important to consider mucosal healing in UC,
and the CCNZ1:Alg1.5 nanozyme hydrogel shows an excellent ability to promote mucosal healing. In short, the CCNZ1:Alg1.5 nanozyme hydrogel not only targeted to inflamed colon and decreased inflammatory response but also promoted mucosal healing. Notably, there was no obvious histological abnormality of viscera (heart, liver, spleen, lung, and kidney) after therapy, suggesting that the nanozyme hydrogel possessed good histocompatibility (Figure S13).

CCNZ1:Alg1.5 Nanozyme Hydrogel for Single-Dose UC Therapy. Clinically, reduction of treatment frequency and dose is an approaching way to lower the incidence of side
effects. Based on the excellent therapeutic efficacy of the
CCNZ1:Alg1.5 nanozyme hydrogel, we further studied whether a single-dose treatment could also relieve the UC. Mice were subjected 8 days to drinking water containing 2% (w/v) DSS as the same method as mentioned above (Figure 5a). After single-dose administration, the body weight of all groups was recorded (Figure 5b). The result showed that treatment with the CCNZ1:Alg1.5 nanozyme hydrogel gradually relieved the UC. Excitingly, the CCNZ1:Alg1.5 nanozyme hydrogel still protects against shortening of the colon length (Figure 5c), as demonstrated by the corresponding images of colon in Figure S14. H&E-stained colonic pathological sections of mice are shown in Figure 5d, which indicate that the CCNZ1:Alg1.5 nanozyme hydrogel group with single dose showed less glandular defects, less inflammatory cell infiltration, and promoted mucosal healing. Collectively, the in vivo data showed that the CCNZ1:Alg1.5 nanozyme hydrogel exhibited highly therapeutic efficacy even for single dose.
In summary, we developed a nanozyme hydrogel with ROS-scavenging activities and mucosal healing capacity for UC therapy, which exhibits higher therapeutic efficacy than 5-ASA and the state-of-the-art CeO\(_2\) nanozyme. Even though nanozymes have been widely studied, few studies focus on promoting mucosal healing, which plays an important role in UC therapy. By using the dual-cross-linking hydrogel, the CCNZ&Alg nanozyme hydrogel was successfully fabricated. The antibacterial activity and surface charge were rationally regulated by different ratios of CCNZ and Alg. By taking advantage of excellent inflamed site-targeting ability and antibacterial capacity, the nanozyme hydrogel could enhance the residence time and eliminate interference from foreign bacteria in the inflamed sites. With the optimal formulation of the nanozyme hydrogel, the obtained CCNZ\(_{2/3}\)Alg\(_{1/3}\) nanozyme hydrogel was capable to scavenge ROS, exert antibacterial activity, target to the inflamed sites, and support cell growth. For UC therapy, the nanozyme hydrogel not only decreased inflammatory response but also promoted mucosal healing. In addition, the reduction of treatment frequency and dose of the nanozyme hydrogel can also reserve the therapeutic effects and decrease the side effects. Therefore, we propose that the CCNZ\(_{2/3}\)Alg\(_{1/3}\) nanozyme hydrogel could provide an innovative treatment strategy for UC therapy with excellent potential for clinical application. Furthermore, the design-on-demand strategy could be encouraged and achieved with the rational design and synthesis in this work for a broader spectrum of diseases.

## METHODS

### Materials

Cerium nitrate hexahydrate (99.95%), ethylene glycol (AR, 98%), ammonium solution (AR, 25–28%), 2,7’-dichlorofluorescein diacetate (DCFH-DA), DSS, and 5-ASA were obtained from Aladdin (Shanghai, China). Chitosan and sodium alginate were obtained from Macklin (Shanghai, China). Deionized (DI) water (18.2 MΩ-cm, Millipore) was used to prepare aqueous solution. Cell Counting Kit (CCK)-8 assay (Dojindo Laboratories, Japan) was utilized to determine cell viability.

### Characterization

TEM imaging was carried out using a Tecnai F20 microscope (FEI, Field Electron and Ion Company) at an acceleration voltage of 200 kV, and SEM imaging was carried out using a Quanta 200 scanning electron microscope (Quanta 200) at an acceleration voltage of 10 kV. Zeta potential and dynamic light scattering (DLS) were measured on a Nanosizer ZS90 (Malvern). XRD spectra were measured using a Rigaku Ultima diffractometer with 2° min\(^{-1}\) using Cu Kα radiation. FTIR spectra were recorded using a Thermo Nicolet FTIR NEXUS 870 spectrometer. The dissolved oxygen was monitored using a dissolved oxygen meter (SevenExcellence Multiparameter, METTLER TOLEDO Co. Ltd.). The amounts of Ce in CCNZ and colon tissues were analyzed by ICP atomic emission spectroscopy (ICP-AES) (Thermo Scientific). Stained cells were analyzed by laser scanning confocal microscopy (OLYMPUS) and flow cytometry (BD Biosciences).

### Preparation of CCNZ and CCNZ&Alg Nanozyme Hydrogels

The CCNZ was prepared by the NH\(_4\)OH precipitation method. In brief, 10 mL of glycol solution of 12.6 mg/mL Ce(NO\(_3\))\(_2\)·6H\(_2\)O was added into 10 mL of 0.1% acetic acid solution of Cs under vigorous stirring over 5 min at room temperature. Then, 1.6 mL of ammonium hydroxide was quickly injected into the mixture solution under vigorous stirring conditions. After 12 h, the formed CCNZ was collected by centrifugation and washed by DI water, until neutralization of the filtrate solution to physiological pH 7.4. Finally, the products were dispersed in 0.1% acetic acid solution. The nanozyme hydrogels with different compositions (CCNZ\(_{2/3}\)Alg\(_{1/3}\), CCNZ\(_{2/3}\)Alg\(_{1/0}\), CCNZ\(_{2/3}\)Alg\(_{0}\), and CCNZ\(_{2/3}\)Alg\(_{1}\)) were formed with different ratios of CCNZ and alginate (1:1, 1:1.5, 1:2, and 1:3 w/w %), respectively. The mixture was shaken for 10 min.

### SOD- and CAT-Mimicking Activity of CCNZ

The SOD-mimicking catalytic activity of CCNZ was investigated with the nitro blue tetrazolium (NBT) probe, and \(^{15}O_{2}\) was produced by irradiating riboflavin. Typically, NBT (0.1 mg mL\(^{-1}\)), ethylenediaminetetraacetic acid (0.1 M), riboflavin (1.2 mM), and different concentrations of CCNZ or citric acid-coated CeO\(_2\) were mixed in phosphate-buffered saline (PBS) (0.1 M, pH 7.2) at 37 °C for 5 min. Then, the mixtures were irradiated with a light-emitting diode lamp for 2 min. The absorption at the wavelength of 590 nm of the mixed solution was obtained using a microplate reader.

### ROS-Scavenging Ability of RAW 264.7 Cells

After seeding RAW 264.7 cells at a density of 7 × 10\(^4\) cells in 96-well plates for 24 h, CCNZ with different concentrations (0–100 μg mL\(^{-1}\)) was added and incubated for 24 h, followed by washing cells with PBS. The CCK-8 assay was used to evaluate cell viability. For the nanozyme hydrogel, different concentrations of the nanozyme hydrogel were seeded in the transwell cell culture system, the cell viability was evaluated by CCK-8. Besides, after staining with Calcein-AM/PI, the images were obtained with laser scanning confocal microscopy.

### Cell Culture

The RAW 264.7 cells were cultured in the high-glucose Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and 1% penicillin–streptomycin under a humidified atmosphere of 5% CO\(_2\) at 37 °C.

### Cell Viability

After seeding RAW 264.7 cells at a density of 2 × 10\(^5\) cells in six-well plates for 24 h, CCNZ was added and further incubated for 2 h. Then, 200 μM H\(_2\)O\(_2\) in a fresh medium was added and cultured for 30 min except for the control group. After being incubated with 10 μM DCFH-DA for 20 min and washed with PBS to remove excessive probes, the cells were observed with laser scanning confocal microscopy. Besides, after being stimulated with H\(_2\)O\(_2\) and incubated with DCFH-DA, the cells were collected by centrifugation and analyzed by flow cytometry.

### Cell Growth on the Nanozyme Hydrogel

In order to investigate the ability of the nanozyme hydrogel to support cell growth, RAW 264.7 cells with a density of 2 × 10\(^5\) cell mL\(^{-1}\) were cultured on the nanozyme hydrogel in 6-well plates for...
24 h. Then, after being stained with Calcein-AM/PI, the images were obtained with laser scanning confocal microscopy.

**Antibacterial Activity of the Nanzyme Hydrogel.** The samples were challenged with *E. coli*/*S. aureus* bacteria at a final concentration of $1 \times 10^5$ CFU mL$^{-1}$. In a 15 mL centrifuge tube, bacteria and the nanzyme hydrogel (100 μL) were cocultured for 4 h at 37 °C. The antibacterial activity was studied using a spread plate, which seeded 50 μL of bacteria and the nanzyme hydrogel stock suspension onto a spread plate. The plate was incubated at 37 °C for 24 h to form viable colony units. Photographs were taken to analyze the antibacterial activity.

**Animal Studies.** The anesthetic, surgical, and post-operative care protocols were examined and approved by the Animal Ethics Committee of Nanjing Drum Tower Hospital and conducted under the Institutional Committee of Care and Use of Laboratory Animals. Male C57BL/6 mice, with an average bodyweight of 22 g, from the Experimental Animal Center of Nanjing Medical University, were used in this study.

**Biocompatibility and Cell Growth In Vivo.** The in vivo host response and cell growth ability were evaluated through subcutaneously embedding the CCNZ$_2$:Al$_{1.5}$ nanzyme hydrogel or Cs&Alg hydrogel in mice. After 7 days, all mice were sacrificed and the skins where samples were implanted were excised. Then, the inflammatory response and cell growth ability were measured by H&E staining of the skin tissues.

**DSS-Induced Model of UC.** 22 g male C57BL/6 mice were acclimatized for 1 week before inclusion in this work, and each group had six mice. Mice were fed with sterile water containing 2% (w/v) DSS for consecutive 7 or 8 days. On the 8th day, all groups were replaced with sterile water without DSS, and the mice were orally administered with S-ASA, CeO$_2$, Cs&Alg, CCNZ$_2$:Al$_{1.5}$, and CCNZ$_2$:Al$_5$, nanzyme hydrogel, respectively, for treatment. After 3 days’ administration or 1 days’ administration with 2 days’ blank to medicine, all mice were sacrificed. Meanwhile, their colon and internal organs (heart, liver, spleen, lung, and kidney) were collected, sectioned, and stained with H&E, Masson, and AB-PAS. The colon length, in vivo anti-inflammation efficiency, fibrosis responses, mucosal healing efficiency, and biocompatibility were recorded.

**Ex Vivo ROS Evaluation of the Nanzyme Hydrogel.** For ex vivo ROS evaluation, the UC model of mice followed the same protocol as mentioned above. After the intraperitoneal injection of DCFH-DA (0.7 mg per mouse) for 0.5 h on day 11, the ROS-scavenging efficiency of different indicated groups was evaluated by collecting the colons. The fluorescence images of the whole colons were recorded using a PerkinElmer in vivo imaging system with an excitation wavelength of 465 nm and an emission wavelength of 520 nm.

Inflammation targeting of the nanzyme hydrogel: first, the CCNZ (20 mg mL$^{-1}$) was labeled with the FITC fluorescence probe (2 mg mL$^{-1}$) under magnetic stirring for 12 h at 4 °C in darkness. Then, the CCNZ was dialyzed against DI water for 24 h in a dialysis bag with a molecular weight cutoff of 1000 Da at 4 °C in darkness and collected by centrifugation (12,000 rpm and 10 min) to obtain the FITC-labeled nanzyme hydrogel. Finally, the UC model of mice was established following the same protocol as mentioned above, and the FITC-labeled nanzyme hydrogel was orally administrated. The fluorescence images of the whole colons were recorded using a PerkinElmer in vivo imaging system with an excitation wavelength of 465 nm and an emission wavelength of 520 nm. Besides, the Ce element in the colon tissue was investigated by ICP-AES. First, the parts of colons of all groups were processed by freeze-drying. Then, the products were immersed in concentrated nitric acid for 12 h at 60 °C. The solution was diluted 10 times before being examined by ICP-AES.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.1c00583.

DLS profile of CCNZ, elimination efficiency of $O_2^-$, digital photographs of the remaining bacteria-inoculated agar plates, EDS, H$_2$O$_2$ elimination of CCNZ$_2$:Al$_{1.5}$ nanzyme hydrogel treated in pH 6.8 and 1.2 for 4 h, cytotoxicity of the nanzyme hydrogel evaluated by using a transwell cell culture system, confocal images of RAW 264.7 cells, release of CCNZ from the nanzyme hydrogel, fluorescence images of colon after treatment with the FITC-conjugated CCNZ$_2$:Al$_{1.5}$ nanzyme hydrogel, images of the colons with indicated treatments and schematic of the method for the measurement of colon length, fluorescence images of colon 0.5 h after injecting 0.7 mg of DCFH-DA per mouse, H&E-stained histology sections, images of the colons with single-dose treatment, and ICP characterization of CCNZ (PDF).

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Author Contributions
C.C. and Y.C. contributed equally to this work. C.C. and H.W. designed the experiments. C.C. synthesized the materials, performed the cellular experiment, and performed the in vitro and in vivo study and analyzed the data. Y.C. designed the in vivo animal model and performed the in vivo study. S.Z. assisted in synthesizing the materials. Q.W. contributed to statistics (nanozymes): next-generation artificial enzymes. S.L. contributed to animal model and performed the cellular experiment, and performed the statistics (nanozymes): next-generation artificial enzymes.

Notes
The authors declare no competing financial interest.

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