

参赛学生姓名 : Ningxuan Zhu

中 学 : The Experimental High School Attached to
Beijing Normal University

省 份 : Beijing

国 家 / 地 区 : China

指导老师姓名 : Ying Yang

指导老师单位 : The Experimental High School Attached to
Beijing Normal University

论 文 题 目 : Dual-Enzyme Activity Carbon Dots Design
in Microglial Polarization for Inflammatory
Therapy After Subarachnoid Hemorrhage

Dual-Enzyme Activity Carbon Dots Design in Microglial Polarization for Inflammatory Therapy After Subarachnoid Hemorrhage

Ningxuan Zhu

Abstract

Subarachnoid hemorrhage (SAH) is a type of severe hemorrhage strokes, featuring high mortality rate, high disability rate, and difficulties in prognosis improvement clinically. Clinical studies indicates that early brain injury (EBI) is a major cause of mortality of SAH patients and delayed neurological deficit. Therefore, developing targeted therapeutic strategy EBI after SAH and promoting neurological function repair, have research value and clinical significance on optimizing SAH clinical prognosis and improving therapeutic efficacy.

In recent years, nanomaterials have become a research hotspot of the treatment of EBI after SAH. Based on the therapeutic strategy of nanomaterials, we can directly target core molecular pathological events of intervening oxidative stress and neurological inflammation in EBI, which enabling timely and effective early intervene before irreversible organic lesions developing in brain tissue. However, most current mainstream therapeutic schemes focus more on single molecular pathetic events, which limits therapeutic targets and intervention levels and often results in relatively low therapeutic efficiency, incomplete inflammatory regulation and poor long-term prognosis in patients. This makes it hard to achieve efficient treatment of EBI after SAH, urgently requiring developments in multi-targeting and synergistic enhancing therapeutic system.

Based on the information above, this project focuses on the core regulatory effects on phenotype polarization of microglia in the process of neurological inflammation, building multi-function nanomaterial therapy system that both contains SOD enzymes and CAT enzymes activity. By surface-conjugating TGN short peptide possessing high affinity for the blood-brain barrier (BBB), the materials are endowed with precise brain-targeting capability. This multi-function nanosystems can function dual therapy effects:

on one hand, it effectively mitigates neuronal injury caused by oxidative stress; on the other hand, it precisely intervenes in inflammatory signal pathway and down regulating proinflammatory factors expression, thereby remodeling a favorable microenvironment in brain tissue and finally achieving synergistic therapeutic effects on SAH early brain injury. The new nano treatment system built in this paper could provide significant theoretic backup towards SAH safe and efficient therapy and simultaneously offers a new beginning of research approach and clinical reference for target and efficient treatment in various brain injuries.

Key words: Carbon dots; Subarachnoid hemorrhage; Inflammation; Oxidative stress

Table of Contents

Abstract.....	2
Table of Contents	4
Chapter 1 Introduction.....	5
1.1 Research background and significance.....	5
1.2 Research Progress on Subarachnoid Hemorrhage.....	6
1.3 Research purpose and primary content.....	9
Chapter 2 Experimental materials and methods	12
2.1 Experimental materials and reagents.....	12
2.2 Equipment and Devices	12
2.3 Experimental methods	12
Chapter 3 Experimental results and discussion	17
3.1 The structure and morphology of CDs.....	17
3.2 Antioxidant activity of CDs.....	19
3.3 Anti-inflammatory activity of CDs.....	21
3.4 In vitro biocompatibility	22
3.5 Cross blood-brain barrier.....	23
Chapter 4 Discussion	24
4.1 Conclusion.....	24
4.2 Outlook.....	24
References	26
Acknowledgement	30

Chapter 1 Introduction

1.1 Research background and significance

Subarachnoid hemorrhage (SAH) is a severe subtype of hemorrhagic stroke. Its core pathogenesis is after the intracranial arterial rupture, vast of bloods rapidly flows into subarachnoid space, breaking down the normal homeostasis of the physiological environment, and subsequently induces a series of secondary brain injury^[1-2]. This disease is obviously characterized by abrupt onset, rapid progression, high mortality, and high disability rates. In accordance with the clinical statistics, patients of SAH suffer an acute-phase mortality rate about 30%-40%, and 50% of surviving patients will retain different extent of neurological function deficits, severely threatening human health and life security, which has become the key point and difficulty in clinical diagnosis and treatment in neurology (**Fig.1**).

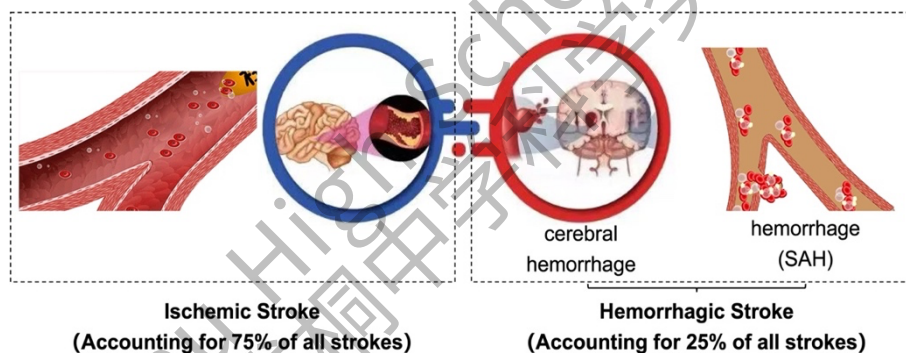


Fig.1 Classification of hemorrhagic stroke

SAH initiates not a solitary pathological event, but a cascade of intricate, interlinked multistep pathophysiological reactions that synergistically mediate the onset and progression of cerebral injury. According to the time window of occurrence, SAH can be specifically classified into early brain injury (EBI) and delayed brain injury (DBI)^[3]. In the two stages of injury mentioned above, EBI is the initial acute brain tissue injury process, and its injury levels directly determine the acute-phase prognosis of patients. More importantly, it also induces early mortality and subsequent delayed neurological deficits of clinical SAH patients core initiative factor. Indicated by clinical research, about 70% mortality of SAH patients occur in 72 hours after morbidity, and severity of neurological dysfunction of surviving patients is closely associated with the

severity of early brain injury ^[4]. Therefore, intervention of EBI has become the key entry point to improve prognosis of SAH patients.

Currently, clinical therapy of SAH still mainly bases on symptomatic and supportive treatment and prevention of complications, specifically including controlling intracranial pressure, relieving cerebral vasospasm, and preventing complications such as infection and hydrocephalus. However, this type of therapeutics mainly focus on mitigating symptom and preventing injuries from further deterioration, which is hard to reverse neuronal injury and apoptosis and has the problems of unsatisfactory neurological recovery outcomes and limited further prognosis of patients ^[5]. Based on these difficulties, deeply exploring pathogenesis of EBI after SAH, developing new therapeutic strategies of EBI, and providing effective intervention methods for neurotological recovery can not only compensate deficit in current clinical therapy, but also has a clinical value and research significance in decreasing mortality of SAH patients, improving neurotological function prognosis, and enhancing patient's survival quality.

1.2 Research Progress on Subarachnoid Hemorrhage

Abundant clinical and fundamental research has demonstrated that the occurrence and development of SAH is not a single mechanics, but a result from multiple parthenogenesis function of oxidative stress, destruction of brain blood barrier (BBB), and neurotological inflammation ^[6]. Specifically, as one of the cores initiating mechanisms, the mainly presence of SAH are components of blood stimulate brain tissues to produce vast reactive oxygen species (ROS), breaking down the balance between oxidant and antioxidant systems, and thus trigger the apoptosis of neurons ^[7].

The destroy of BBB leads to the components of plasma extravasate into the brain tissue, inducing vasogenic edema, jeopardizing injury of brain tissues. The inflammation reaction releases pro-inflammatory factors by activating microglia, forming “inflammatory cascade”, continuously amplifying the effects of brain injury. The mechanics mentioned above together form the core pathogenetic network ^[8].

In recent years, nanomaterials have gradually become the research concentration of stroke therapy areas due to their excel biocompatibility, high specific surface areas,

controllable drug loading capacity, and targeted delivery capacity^[9,10]. Based on the therapeutic strategy constructed by nanomaterials, researcher can precisely target key molecular events about brain tissue lesion, directly intervene oxidative stress, and inflammation reaction, achieving accurate and efficient intervention to SAH, breaking through the limitations of inefficiency in traditional drug delivery, difficulties in crossing BBB. In the research in this area, several teams have made breakthrough achievements. For examples, Shi et al. firstly build a nanomaterials delivery system loaded with Edaravone that achieves targeted accumulation of drugs in brain lesion tissues by optimizing particle size and surface characteristics of nanocarrier, mitigating injury caused oxidative stress, and thus significantly the neurotological function of experimental stroke models in animals, providing new approach for antioxidative treatment for SAH^[11]. Zhou et al. has developed an oligosaccharide-based nanomaterial (TPCD NPs) with BBB penetration capability and dual antioxidant and anti-inflammatory activities^[12] (**Fig.2**). This nanosystem, developed by covalently conjugating free radical-scavenging compounds with anti-inflammatory components, can actively target the inflammatory microenvironment after SAH. After penetrating BBB, it accumulates at lesion sites, effectively reduces neuronal apoptosis in the ischemic penumbra, and significantly improves motor function in mice, while also exhibiting favorable biosafety. These findings provide a novel strategy for multi-target intervention in SAH. These findings further demonstrated the therapeutic potential of nanomaterials in the treatment of SAH^[13].

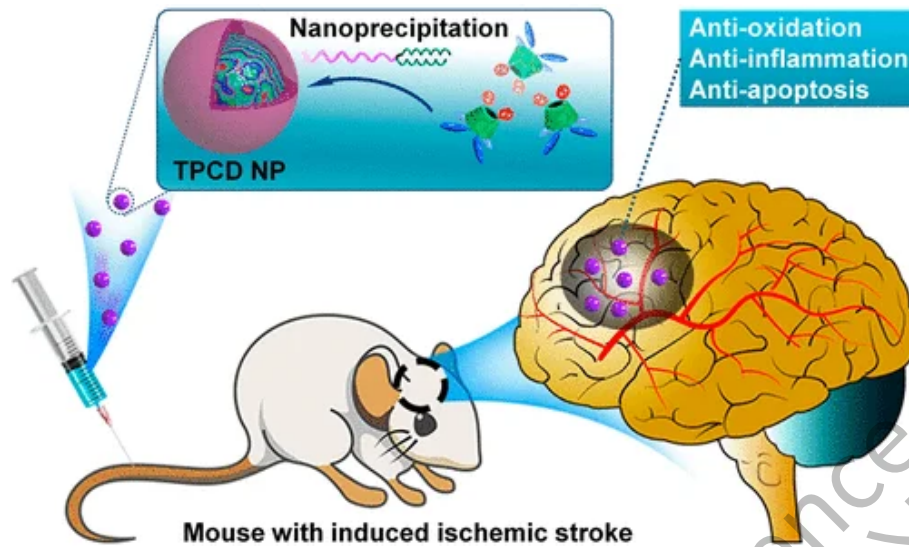


Fig.2 Research on nanomaterials used in therapy of ischemic stroke

Although treatment strategy of nanomaterials show excel advantages in the in SAH intervention, most of current research still concentrate on single events of suppressing oxidative stress or neurotological inflammation, while parthenogenesis characterizes multi-steps and various targets functioning together. Single target intervention is hard to fully inhibit the proccession of brain injury. Therefore, this type of strategy still consists of problems of limited therapeutic efficiency, unideal model animal prognosis, and difficulties in transmission of clinical application. As resident cells in the central nervous system, microglia play an important role in neuroinflammation after SAH^[15]. Due to the high plasticity of microglia, it is easy for it to differentiate into phenotypes with opposing functions when responding to the microenvironment: M1 phenotypes (promote inflammatory factor expression) and M2 phenotypes (promote anti-inflammatory factor expression). M1 phenotypes microglia is regarded as inflammatory cells, and high-level inflammatory factors and massive ROS released by it can trigger neuroinflammatory cascade, resulting in destroyed BBB and neuronal degeneration, as a crucial cause of secondary injury following intracerebral hemorrhage^[16]. Instead, M2 phenotypes microglia can promote the release of anti-inflammatory factor, becoming the key to central nervous system injury repair by mitigating partial inflammation and participating in tissue remodeling. As a result, effective regulation of phenotypes polarization of M1/M2 microglia must have an effect in reaction process of SAH

neuroinflammation. If combining strategy in suppressing oxidative stress based on this theory, it is an opportunity to further improve inflammatory microenvironment, promoting neurofunction repair after EBI following SAH.

Carbon dots (CDs) is a zero-dimensional carbon nanomaterial composed of sp^2/sp^3 carbon core and an outer shell of oxygen/nitrogen-containing functional groups, with a size of less than 10 nm. Due to its excellent optical performances, ideal biocompatibility, and ease of surface engineering, CDs have significant application prospects in biomedicine fields [17]. Along with the deeper research, applying methods of element doping, particle size regulation and surface functionalization to endow CDs with enzyme-like activity has shown wide application potential in treatment of oxidative stress-induced diseases [18]. Although there is lack of report about the work in respect to treatment using CDs with enzyme-like activity to regulate microglia's phenotype polarization, currently some research has indicated that CDs-based materials can effectively decrease the inflammatory factor level in the inflammation microenvironment [19]. Work above has demonstrated the inhibiting effect of CDs towards inflammatory factors in inflammation microenvironment, indirectly proving that CDs has influenced phenotype polarization of microglia. However, the specific functioning mechanisms remained to be explored.

1.3 Research purpose and primary content

This topic focuses on the clinical treatment deficits of EBI following SAH, combining the core function of oxidative stress and inflammatory reaction in the disease's pathenogenetic progress, aiming to design and synthesize CDs material containing activity of both SOD and CAT enzyme. By conjugating BBB-specific short peptides onto the CDs surface, enabling efficient brain-targeting functionality of CDs, to solve the problems of traditional medicine of poor BBB permeability, low targeting efficiency, and limited therapeutic efficacy. Based on the ultra-high enzyme-like activity of dual-enzyme activity CDs, combining with the small size effect and excellent electron donor/acceptor properties that the CDs contain intrinsically, and by catalyzing the interconversion of intracellular ROS valences, it can efficiently and specifically eliminate the ROS excessively accumulating in the cells and block the damage to brain

cells caused by oxidative stress. At the same time, by precisely regulating the free radical level in the cells, it can furtherly inhibit abnormal activation of inflammatory signaling pathways, downregulate the expression of pro-inflammatory factors, and correct the imbalance microglia under pathological conditions to achieve the optimal dynamic balance between the two, which finally reaches synergistic therapeutic efficacy against EBI following SAH and provides the clinical therapy of EBI following SAH with new thinking, methods, and candidate materials.

The prioritized research content basically focuses on the construction of target materials, performance validation, and treatment mechanisms, and could be specifically divided into these 3 aspects: The first is to design and synthesize the brain-targeted CDs with dual-enzyme activity, specify the synthesis parameters of CDs, identify the optimal synthesis conditions, and thus prepare CDs exhibiting dual-enzyme activity, high purity, good dispersibility, and biocompatibility; Simultaneously, to achieve efficient conjugation of the short peptide TGN to the surface of CDs, demonstrate the conjugation efficacy, characterize the physicochemical properties of CDs after conjugation, and ensure the good brain-targeting ability and dual enzyme activity of the CDs. The second aspect is the research concentrating on dual-enzyme activity that CDs scavenge ROS and protect against cellular oxidative stress injury via in vitro cellular experiments, exploring the catalysis activity of CDs at different ROS concentration and different reaction time, specify the underlying mechanisms that CDs eliminate excessive ROS in cells through valence state conversion; Also, to demonstrate protecting effects of CDs toward brain cells suffering oxidative stress injury, overseeing the change of survival rate of cells and relative parameter of oxidative stress, clarify the influences and drugs dependence of CDs antioxidation. The third is the study of dual-enzyme CDs regulating inflammatory response and microglial phenotypic balance, utilizing cells experiments and molecular biology methods, to analyze changes in the expression levels of pro-inflammatory factors and specify its molecular mechanisms inhibiting inflammatory reaction; Furthermore, we explore the regulation of microglial phenotypic conversion by CDs, detect the expression levels of two phenotypic markers, and verify whether CDs can achieve an optimal dynamic balance of microglia under

pathological conditions (Fig.3).

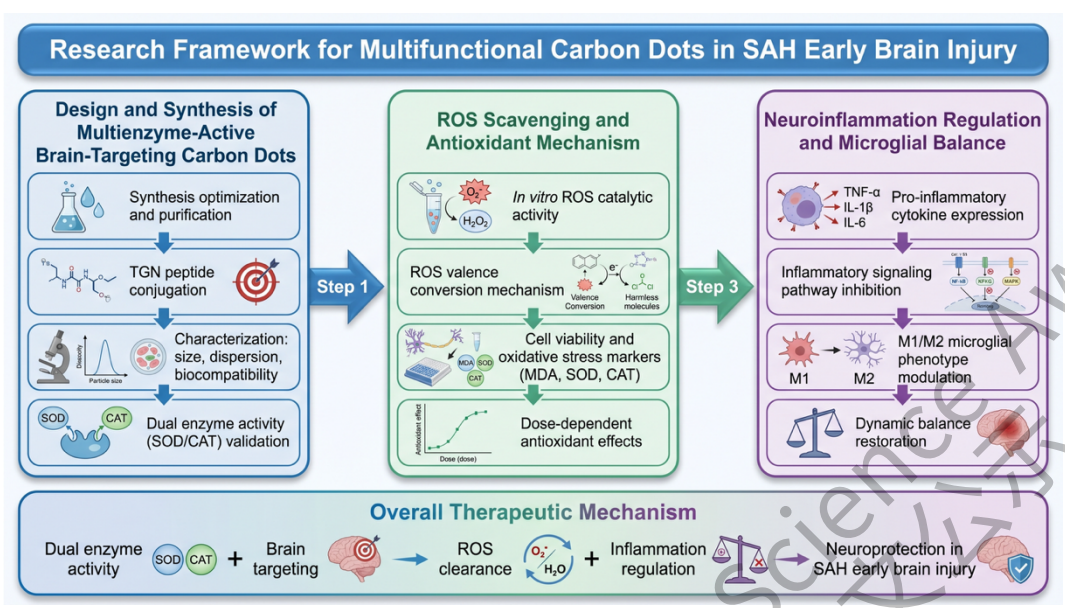


Fig.3 Research Framework

Chapter 2 Experimental materials and methods

2.1 Experimental materials and reagents

Chlorogenic Acid, N, N-Dimethylformamide, N- Hydroxysuccinimide, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), Salicylic acid, Dimethyl sulfoxide, and Lipopolysaccharide are purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Methanol, Ethanol, Petroleum ether, Dichloromethane, Ethyl acetate, Ferrous sulfate, and Hydrogen peroxide are purchase from Sinopharm Chemical Reagent Co., Ltd. Polypeptide TGN is customized by company. ELISA assay kit is purchased from Shanghai Biyuntian Biotechnology Co., Ltd. Phosphate buffer saline, Dulbecco's Modified Eagle Medium (DMEM), and Fetal bovine serum (FBS) are purchased from Hyclone (Shanghai, China). SOD assay kit and ROS assay kit (DCFH-DA) are purchase from Shanghai Beyotime Biotechnology Co., Ltd. Human Tumor Necrosis Factor- α (TNF- α), Human Interleukin-6 (IL-6), and Human Interleukin-1 β (IL-1 β) ELISA assay kit are purchased from Shanghai Biyuntian Biotechnology Co., Ltd.

2.2 Equipment and Devices

This paper uses JEM-2100 TEM to evaluate the appearance of the sample. X-ray diffraction (XRD) measurements were performed on powdered samples using an X'Pert PRO diffractometer with Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$), operating at a scanning speed of $2^\circ (2\theta)$ per minute, and NEXUS-870 spectrometer is used to collect Fourier-transform infrared (FT-IR) spectra. Electron paramagnetic resonance (EPR) spectra is obtained from Bruker EMXnano X-band benchtop CW EPR spectrometer that manipulate in 9.4 GHz X-band and measured in 25°C with magnetic field scan range of 10-600mT. Ultraviolet-visible (UV-Vis) absorption spectra were recorded by Shanghai MAPADA spectrophotometer (UV-1800PC). In the experiments, absorbance is determined by Molecular Devices microplate spectrophotometer (SpectraMax 190). Nikon inverted fluorescence microscope TS2R-FL is used to examine cell conditions.

2.3 Experimental methods

(a) Synthesis of dual-enzyme CDs: CDs were synthesized via a simple one-pot thermal

method. In brief, the process firstly dissolves chlorogenic acid (0.5g) in 25 mL N, N-Dimethylformamide, removing the solution to Teflon-lined stainless-steel autoclave, and black crude product was obtained after heating at 180 °C for 4 h and naturally cooling to room temperature. Then, using the mixture of dichloromethane and methanol as eluent, we purified crude products by silica gel column chromatography. After removing solvent and finishing further dried under vacuum, we finally acquired purified CDs. Furthermore, we used peptide TGN to chemically graft onto these CDs, to achieve brain-target capacity, and package CDs with low molecular weight PEG (average $M_n=400$), to enhance it solubility and stability. Eventually, the powder is named as Dual-enzyme CDs (**Fig.4**).

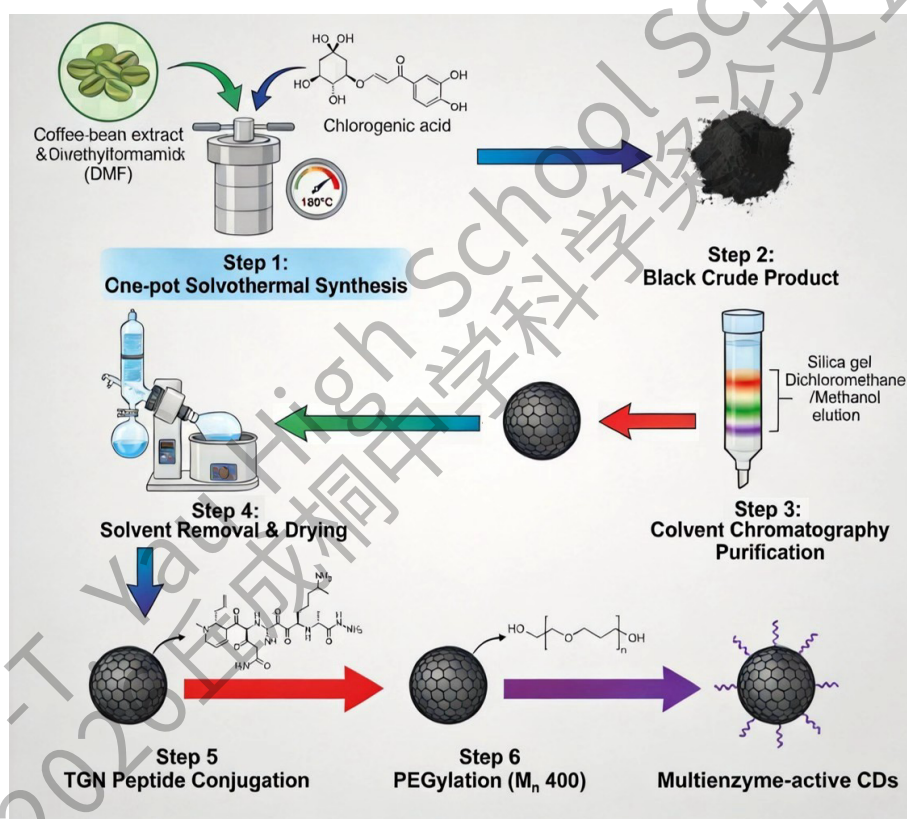


Fig.4 Synthesis pathway of Dual-enzyme activities CDs

(b) Cells incubating: This article uses the model of Human fetal liver cell line (L02), Human umbilical vein endothelial cells (HUVEC), Human hepatoblastoma cell line (HepG2), Human brain microvascular endothelial cells (HBMEC), Human neuroblastoma cell line (SH-SY5Y), BV-2 murine microglial cells and Rat pheochromocytoma cell line (PC12). Every cells are cultured in DMEM medium

(supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum) in a constant-temperature incubator at 37°C with 5% CO₂ and are passaged every 2-3 days.

(c) CDs enzyme activities assay: SOD-like enzyme activity assay (WST-8 method) adopted total SOD enzyme assay kit to conduct experiments. Starting with specimen preparation: Separately prepare 50, 100, 200, 250, and 300 µg/mL CDs-TGN, and assay kit preparation: WST-8 enzyme work solution conformation (taking 50 samples as an example): The working solution was prepared by mixing 7550 µL, WST-8 400 µL, enzyme solution 50µL in the ice to keep; Preparation of reaction initiation solution (40X): 25µL of stock solution and 975µL of deionized water were mixed. The blank groups and the sample groups were set in the 96-well plate, incubated in 37° for 30 min, and the absorbance A was read by the microplate reader. Every group of the data is present by average value ± standard error, analyzed statistically by Two-way ANOVA. The specific volume ratios of the samples in the above method were determined according to the manufacturer's instructions of the kit.

CAT-like activity assay was measured by microplate reader method. The procedure started with sample preparation: CDs-TGN were separately prepared in the concentration of 50, 100, 200, 250, and 300 µg/mL. Then, the assay kits were constitution: The chromogenic substrate and 250 mM H₂O₂ were prepared at the ratio of 1: 10000. The sample and the solution were orderly added into the 1.5 mL plastic tube, mixed thoroughly and quickly by the pipette, and the system reacted under 25° in 5 min. Catalase reaction termination solution is added with inversion mixing, and the following procedures needed to be completed in 15 min. 40 µL catalase assay buffer is added in a clear plastic centrifuge tube, added and mixed with 10 µL of the reaction system that had been terminated and mixed. 10 µL of the 50 µL system in the previous step were added in to every well of the 96-well plate, and 200µL of chromogenic working solution was added. The absorbance A was read after incubating under 25°C for 15-20 min using microplate reader at 520 nm. The specific volume ratios of the samples in the above method were determined according to the manufacturer's instructions of the kit.

(d) In vitro antioxidative activity assay: Antioxidative activity assay adopts electron

paramagnetic resonance (EPR) spectroscopy, separately measuring the elimination level.

Elimination of superoxide anion ($\bullet\text{O}_2^-$): Using PBS buffer as solution, the 10 mM xanthine solution and the 1 U/mL xanthine oxidase solution are prepared. 100 μL of xanthine solution and 100 μL of xanthine oxidase solution are taken, and 120 μL of DMPO and 880 μL PBS are added, incubated in reaction for 10 min and tested with sample.

Elimination of hydrogen peroxide (H_2O_2): The positive control group: 50 μL of 1mM CTPO, 50 μL of H_2O_2 are added with 1mL of 10 mM PBS to be incubated in 10 min, then drawn into a capillary tube, sealed with petroleum jelly for measurements. (The sample group: 1 μL of deionized water is used to replace 400 μL of the sample solution)

Elimination of cell ROS assay: Firstly, 1×10^5 HUVEC cells are cultured overnight in the glass-bottom confocal dishes. Pure DMEM is used to prepare CDs-TGN into the 300 $\mu\text{g/mL}$ disperse solution, and 1mL of the 300 $\mu\text{g/mL}$ CDs-TGN is taken for pretreatment into the confocal dishes. Then, 1 $\mu\text{g/mL}$ of LPS solution diluted by PBS is used for stimulation in 90 min, and PBS is used for repeated washing for three time.

(e) In vitro antiinflammation assay: In the experiment in vitro antiinflammation assay adopts enzyme-linked immunosorbent assay (ELISA). 4×10^4 HepG2 cells are cultured in the 24-well plate. After 24h, original culture medium are wasted, and 300 $\mu\text{g/mL}$ of CDs-TGN are used to incubate for 1 hour, washed by PBS for three times. Then, 1 $\mu\text{g/mL}$ of LPS culture medium is added to induce for 6h, and the supernatant is taken for 1000 rpm centrifuge which are used for ELISA.

TNF- α : The experiments start with setting standard well, blank well, and sample well. standard sample concentration of 0, 5, 10, 20, 40, and 80 pg/mL in each well were added with 50 μL of to create standard curve. The sample well is added with 10 μL of 300 $\mu\text{g/mL}$ CDs-TGN and 40 μL of sample dilution solution. 100 μL of detection antibody marked by horseradish peroxidase (HRP) are added into every standard well and sample well, sealing the reaction well by plate sealer, incubated in 37° incubator for 60min. 20X washing buffer is prepared by diluting the buffer into distilled water at the ratio of 1: 20. The liquid is wasted and dried by absorbent paper. Washing solution

is added into every well and allowed to stand for 1 min, discarding washing solution and drying it by absorbent paper, and the plate washing procedures are repeated for 5 times. 50 μL of substrate A and B are added into every well, incubated in the dark for 15 min. 50 μL of termination solution are added into every well, measuring OD value in every well at the wavelength of 450 nm within 15 min.

IL-1 β : All the procedures of IL-1 β follows the same of that of the TNF- α .

IL-6: All the procedures of IL-6 follows the same of that of the TNF- α and IL-1 β .

(f) Cross blood-brain barrier assay: By building blood-brain barrier model, the effect of CDs and CDs-TGN on the penetration rate of this model is tested. The specific procedures are: HBM cells (20×10^4 cells/mL, 500 μL) are placed on chamber (Corning, μm), and the lower chamber is incubated in 700 μL of culture medium, replacing the old medium every 2 days. Transepithelial electrical resistance is measured using epithelial voltammeter, which is available when the resistance is bigger than 250 $\Omega \cdot \text{cm}^2$. U87 cells are plated on the lower chamber at 8000 per well with 500 μL of medium. After 24 h, CDs and CDs-TGN at the same concentration were added to the upper chamber and incubated for 24 h. The uptake of CDs and CDs-TGN in the lower-chamber cells was observed under a fluorescence microscope, and the penetration rate was calculated.

(g) Cytotoxicity assay: 1×10^4 HepG2 cells and L02 cells are cultured in the 96-well plate. After incubated for 12h, CDs-TGN is prepared into the dispersed solution in accordance with the concentration grade of 0, 50, 100, 150, 200, 250 and 300 $\mu\text{g/mL}$ cultured in DMEM. Removing the original medium, 100 μL of DMEM dispersed solution newly containing materials are added for incubating in 24 h. At the end of incubation, 10 μL of 5 mg/mL of MTT solution are added, and then the system is incubated in the 37°C incubator for 4h. Carefully discarding the liquid in the plate, dimethyl sulfoxide (DMSO) is added, and the absorbance at 570 nm is measured after shaking on a microplate.

Chapter 3 Experimental results and discussion

3.1 The structure and morphology of CDs

To verify the successful synthesis of the dual-enzyme CDs and to clarify the structural basis for subsequent TGN conjugation and biological evaluation, the as-synthesized CDs were first characterized by TEM, HRTEM, XRD, FT-IR, XPS, and UV-Vis absorption spectroscopy. In **Fig. 5a**, results of transmission electron microscope (TEM) show that CDs are monodisperse ellipsoidal particles, with relatively narrow particle size distribution range. **Fig. 5b** is the high-resolution transmission electron microscope (HRTEM) of CDs, where we are able to observe the lattice fringes obviously inside, with corresponding lattice spacing of 0.18 nm, staying in accordance with the crystal plane of graphitic carbon [20]. Statistical data of size distribution diagram shows that the average diameter of synthesized CDs is 1.74 nm (**Fig. 5c**).

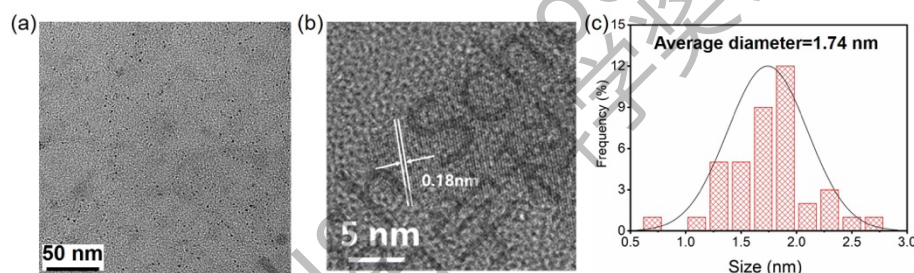


Fig. 5 TEM image (a), HRTEM image (b), and size distribution (c) of CDs

Subsequently, XRD analysis on CDs are performed as shown in **Fig. 6a**. The peak centered at $2\theta=21^\circ$ is relatively broad, matching the interlayer spacing of graphitic carbon (0.34 nm) [21]. In addition, around the position of 31° and 45° , we observed sharp impurity peaks, and we attribute this unusual phenomenon to the inorganic salt containing metallic components in the ingredients [22].

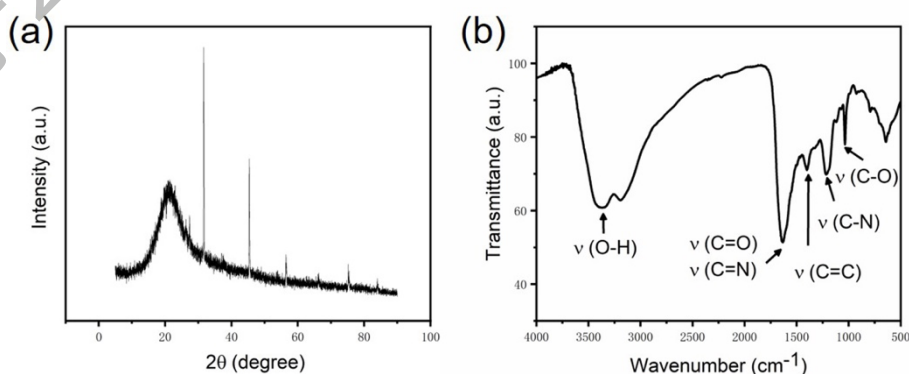


Fig. 6 XRD spectrum (a) and FT-IR spectrum (b) of CDs

In order to investigate the chemical composition of CDs, we further characterized the sample via FT-IR and XPS. From the FT-IR spectrum of CDs (**Fig. 6b**), we could see the characteristic peaks at 1025 cm^{-1} , 1213 cm^{-1} , 1400 cm^{-1} and 1636 cm^{-1} can correspond to the vibration at C-O, C-N, C=C, and C=O/C=N bond, with the according analysis results of XPS [23]. According to the **Fig. 7a**, CDs mainly consist of three elements of Carbon (285.09 eV), Oxygen (532.62 eV), and Nitrogen (400.73 eV), with molecular content of 70.47%, 28.31%, and 1.22%. At the high-resolution spectra of C 1s (**Fig. 7b**), the characteristic peaks at 284.69 eV, 286.04 eV, and 288.17 eV separately belong to C-C, C-O, and C=O bonds; The high-resolution spectra of O 1s (**Fig. 7c**) contains C=O (531.25 eV) and C-O (532.82 eV) bond; That of N 1s appears characteristic peak at 398.74 eV and 400.14 eV, which correspond to Pyridinic nitrogen and nitrate (**Fig. 7d**). The results above indicate that the surface of CDs prepared by chlorogenic acid contains abundant and diverse function groups and chemical bonds, which is critical to the conjugation with polypeptide subsequently.

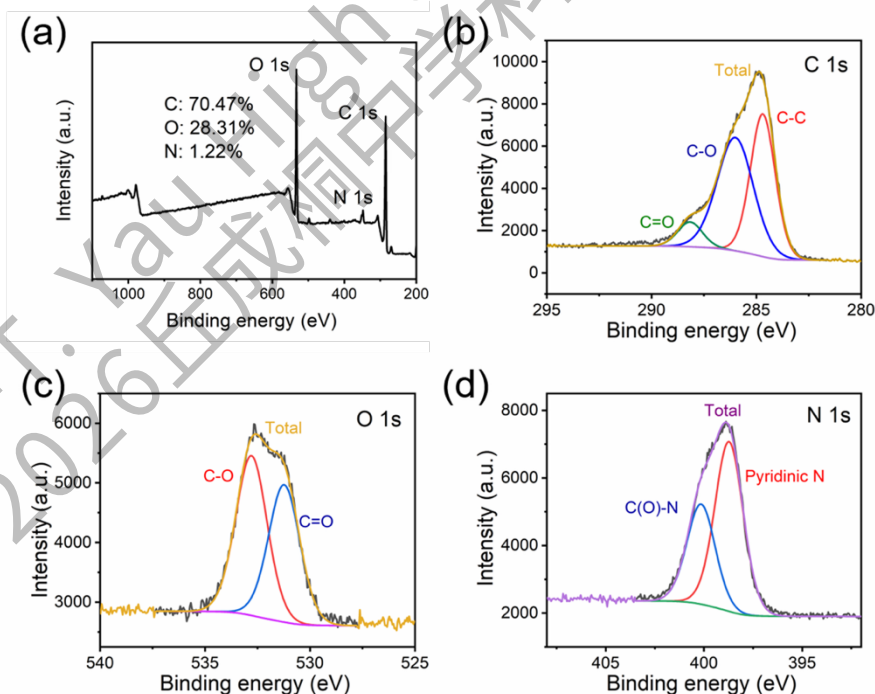


Fig. 7 (a) XPS spectrum and high-resolution (b) C 1s, (c) O 1s, and (d) O 1s spectra of CDs.

Fig. 8 is the absorption spectrum of CDs in ethanol, which, the absorption peak at

252 nm and 288 nm separately present $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transition [24]. The strong absorption at 252 nm is attributed to the $\pi\rightarrow\pi^*$ transition of the sp^2 -hybridized carbon domains (C=C), while the peak at 288 nm originates from the $n\rightarrow\pi^*$ transition of surface functional groups containing lone-pair electrons, such as C=O and C=N. These absorption features confirm the coexistence of a graphitic carbon core and abundant oxygen/nitrogen-containing surface groups, which is well consistent with the XRD, FT-IR, and XPS results. In addition, the absorption intensity gradually decreases with increasing wavelength, and no obvious absorption peak is observed in the visible region, indicating that the CDs are well dispersed in ethanol without significant aggregation. Such UV-Vis absorption behavior also suggests that the CDs possess complex surface states and graphitic domains, which may contribute to their intrinsic fluorescence and potential for bioimaging [24]. These results indicate that the as-synthesized CDs not only possess a graphitic carbon core but also are rich in surface functional groups, providing a structural basis for subsequent TGN conjugation and enzyme-like activity.

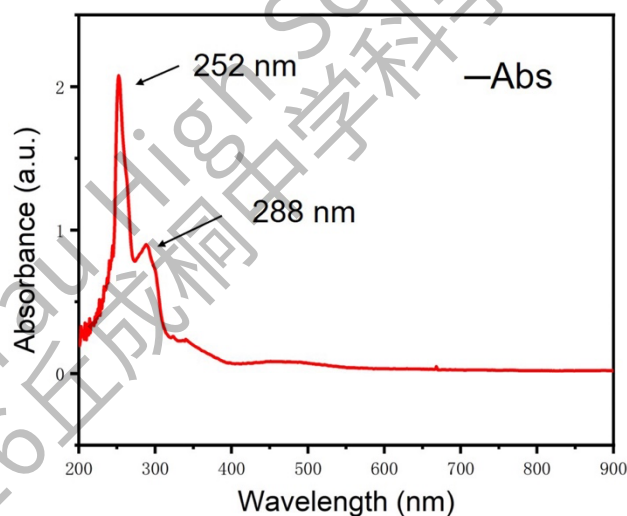


Fig. 8 UV-Vis spectrum of CDs.

3.2 Antioxidant activity of CDs

For determining the effects of dual-enzyme activities CDs system on ROS elimination function, the oxidation activity of the materials system is compared systematically [25].

$\bullet O_2^-$ is produced by xanthine/xanthine oxidase system, and the capacity to eliminate superoxide radical anion is evaluated by the characteristic EPR signals formed by DMPO trapping. Compared to the system without the materials, the DMPO- $\bullet O_2^-$

characteristic signals was mitigated in different concentration of dual-enzyme activities CDs, with the weakest signal in the high concentration of the disposed materials system, indicating its superior capacity in eliminating $\bullet\text{O}_2^-$. After adding CDs, CTPO characteristic signals decreases significantly, while the disposed materials system in high concentration shows a lower signal, meaning that the capacity of H_2O_2 decomposition and O_2 production of dual-enzyme activities CDs increases with rising concentration (**Fig. 9**). 200

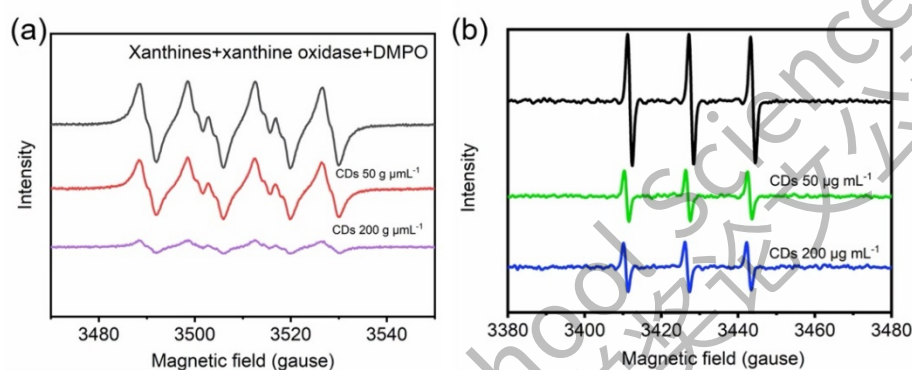


Fig. 9 EPR spectra of dual-enzyme activities CDs scavenging of superoxide anion (a) and (b) hydrogen peroxide

Subsequently, regulatory effects of dual-enzyme activities CDs on oxidative stress and inflammatory reaction are evaluated on cells level. Due to the excessive production of ROS, oxidative stress and inflammatory reactions are directly induced. DCFH-DA detector is furtherly adopted to test ROS level in the cells where green fluorescence intensity can be used to reflect ROS level [26]. After LPS stimulation, green fluorescence is observed in HUVEC cells with significant increasement, indicating a vast accumulation of ROS. HUVEC cells delt with CDs show a declination in green fluorescence, which is similar with that delt with CDs-TGN. This indicates that theses two materials can both scavenge ROS within cells, and CDs after modification are not affected in the aspect of antioxidative functions (**Fig. 10**).

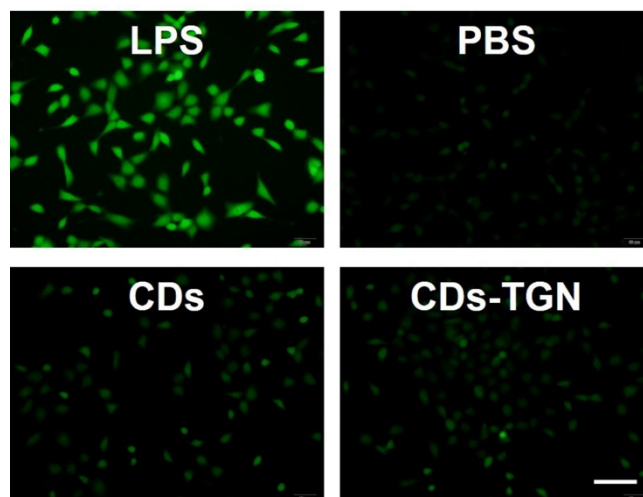


Fig. 10 Fluorescence staining images of DCFH-DA detector testing ROS in HepG2 cells

3.3 Anti-inflammatory activity of CDs

In the development of inflammation, oxidative stress could activate inflammation-related signals, stimulating the releasement of proinflammatory factors, thus amplifying inflammatory reactions [27]. Therefore, the level of TNF- α , IL-1 β , and IL-6 in the cell culture supernatant is measured. LPS stimulation significantly promotes the secretion of the three inflammatory factors. Compared to the LPS group, low concentration of CDs disposed group obviously decreases the level of TNF- α , IL-1 β , and IL-6, with further decrease under high concentration of CDs (**Fig. 11**). Notably, there is no big change in the inhibitory effect of CDs-TGN on the three inflammatory factors. The results mentioned above commonly indicates that dual-enzyme activities CDs can both eliminate excessive ROS and inhibit the spread of proinflammatory factors, and thus mitigate the inflammatory reactions related to SAH.

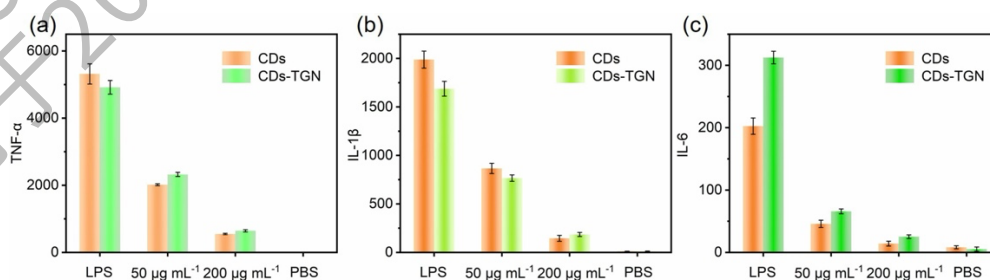


Fig. 11 ELISA analysis of different inflammatory factors level of (a) TNF- α , (b) IL-1 β , (c) IL-6 in different materials systems

3.4 In vitro biocompatibility

Before conducting cells experiments, in vitro bio capability of dual-enzyme activities CDs is evaluated. After incubating 24h the L02 and HepG2 cells separately with 0-300 $\mu\text{g mL}^{-1}$ of dual-enzyme activities CDs, cell viability is maintained above 95% (**Fig. 12**). These results indicate that dual-enzyme activities CDs feature a high biosafety.

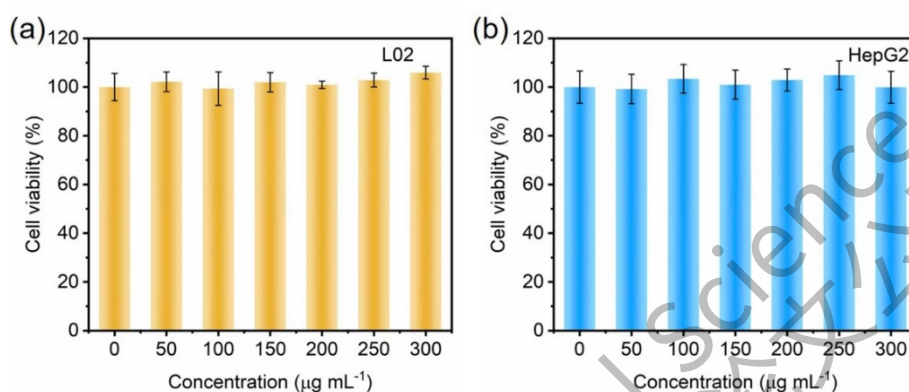


Fig. 12 MTT analysis results of L02 cells (a) and HepG2 cells (b) incubated with CDs-TGN in different concentration

In addition, to investigate the cell uptake behavior of dual-enzyme activities CDs, the analysis adopts CLSM for the cell sample. According to **Fig. 13**, the intrinsic fluorescence properties of CDs-TGN could present fluorescence at 405 nm excitation, observed fluorescence after incubated in the concentration of 100 $\mu\text{g/mL}$ with HepG2 cells together in separately 10, 20, 30 min. At 20 min, fluorescence phenomenon could be observed in the figure, indicating that CDs-TGN have already been uptake. Along with the time, the fluorescence intensity is becoming more evident, presenting the increase in phagocytosis.

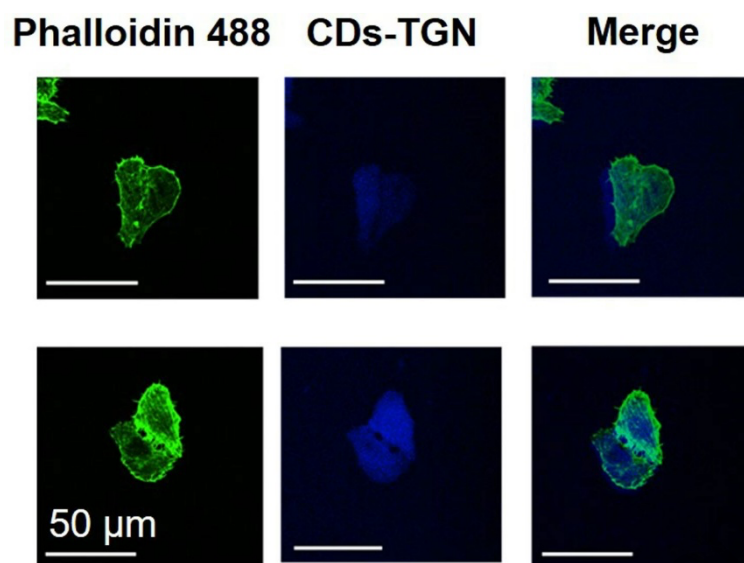


Fig. 13 Cellular phagocytosis effects of CDs-TGN under different time conditions

3.5 Cross blood-brain barrier

The principal obstacle in brain drug delivery is the BBB, which isolates the brain parenchyma from the peripheral circulatory system and makes it difficult for drugs to enter brain tissue. Therefore, the ability to cross BBB is a key point to treat cerebral hemorrhage. We thus build an in vitro BBB model (**Fig. 14a**), measuring drug permeability of different materials system to cross BBB model. Shown in **Fig. 14b**, the permeability of CDs under both different concentrations is relatively low, while the permeability of the CDs-TGN is 5 times than that of CDs, with the maximum permeability of 54.27% ($50 \mu\text{g mL}^{-1}$). This result indicates that CDs-TGN can effectively permeate BBB, thereby maximizing the dual-enzyme, anti-inflammatory, and antioxidant effects of CDs.

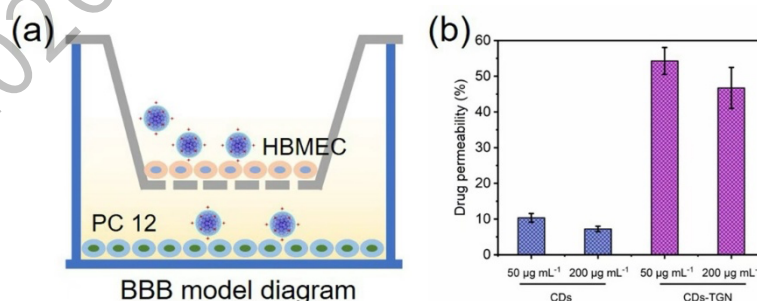


Fig. 14 Schematic diagram of BBB model (a); drug permeability of CDs and CDs-TGN under different concentrations

Chapter 4 Discussion

4.1 Conclusion

SAH is a severe subtype of hemorrhagic stroke with high mortality and disability, and early brain injury (EBI) is the core factor determining long-term prognosis. Developing a treatment vehicle to intervene EBI process and promote neurological function repairment has significant clinical and research value for improving the outcomes of SAH patients. Although present intervening strategy based on nanomaterials can pathological process of oxidative stress and neurological inflammation, exist protocols have a simple target, which is hard to achieve regulation from multi-pathway, limiting therapy results. Therefore, it is an urgent need to develop novel therapeutic system with multi-target synergy.

Based on this, this research successfully synthesized brain-targeting CDs nanomaterials modified by TGN short peptide simultaneously with double enzyme activities of SOD and CAT. This system eliminates free radicals to mitigate oxidative stress injury and synergistically treats EBI after SAH by regulating inflammatory factor levels, inhibiting excessive neuroinflammation, and remodeling the brain microenvironment. The development results can not only provide theoretical evidence for the development of therapeutic drugs targeting SAH, and also offer new design and reference of other nano-therapeutic protocol for diseases related to brain injury.

4.2 Outlook

The targeting double enzyme activities CDs constructed in this paper effectively breakthrough the limitation in single target therapy in conventional treatment for EBI in SAH, accomplishing synergistically regulation in oxidative stress and neurological inflammation, providing novel developmental direction of SAH precise and efficient therapy. Based on the existing achievement in this paper, further systematical research can be intensified, analyzing the in-depth molecular mechanisms on this multi-functional nanomaterials regulating polarization of microglia and the inhibition pathway of neurological inflammation, specifying the metabolism patterns, functioning efficiency and biosafety boundaries of the materials. Simultaneously, future research

can optimize ratio parameters and modification techniques of nanomaterials, enhancing the BBB permeability, target precision and biocapability, mitigating potential biotoxicity in the materials, allowing the adaptation of nano-therapeutic system with complex intracranial pathological microenvironment, laying experimental and theoretical foundation for subsequent in vivo animal experiments and clinical translations.

From the insight of application extension, we hope to jump out the limitation of single pathological mechanisms, to explore the intervening effects of multifunctional nano-systems on secondary injury including cerebral vasospasm, brain edema, and delayed neurological deficit following EBI in SAH, constituting multi-stages and multi-pathology therapeutic strategy covering SAH. At the same time, novel technology can be integrated into multimodal imaging and smart responsive drug release based on design of this research, developing treatment platform integrating diagnosis, monitoring, and targeting therapy, achieving precise visualized treatment of EBI in SAH. In addition, the construction of materials and therapeutic mechanisms in this paper can provide developing framework toward various nervous system injury disorders such as traumatic brain injury and ischemic brain injury, which can further conduct study on cross-disease adaptability. In this way, by enlarging the application circumstances of multifunctional nanomaterials on the field of neurological injury repayment, fostering clinical transition and industrial development of nano-targeting therapeutic technology on brain diseases.

References

- [1] Liu, J. H.; Sun, K.; Chen, Q. S.; Ding, L. S., Advances in astrocytes in aneurysmal subarachnoid haemorrhage. *Frontiers in Surgery* 2025, 12, 1511242.
- [2] Kanamaru, H.; Suzuki, H., Therapeutic potential of stem cells in subarachnoid hemorrhage. *Neural Regeneration Research* 2025, 20(4), 936-945.
- [3] Lauzier, D. C.; Athiraman, U., Role of microglia after subarachnoid hemorrhage. *Journal of Cerebral Blood Flow & Metabolism* 2024, 44(6), 841-856.
- [4] Du, W. Y.; Chen, J. H.; Cheng, G. F.; Yang, Y.; Shi, Y. Q.; Wang, Y. H., Ferroptosis mechanisms in early brain injury after subarachnoid hemorrhage. *Brain Research* 2026, 1881.
- [5] Wang, W.; Wang, Y.; Gao, L. S., Stem cells treatment for subarachnoid hemorrhage. *Neurologist* 2025, 30(2), 80-86.
- [6] Zhang, Z. Y.; Gao, W. Z.; Chen, Y. L.; Lv, T.; Zhang, F. C.; Jin, Y. C.; Li, J.; Wang, J. Q.; Jia, F.; Zhang, X. H., Alisol a mitigates neuroinflammation and oxidative stress after subarachnoid hemorrhage through SIRT3-driven eprogramming of mitochondrial metabolism. *Molecular Neurobiology* 2026, 63(1), 452.
- [7] Yang, J. S.; Wu, Q. W.; Lan, S.; Yuan, K. K.; Sun, B. W.; Meng, Y. X.; Xu, S. C.; Shi, H. Z., Peroxiredoxin-5 alleviates early brain injury after subarachnoid hemorrhage by reducing oxidative stress. *Brain Research Bulletin* 2024, 217, 111087.
- [8] Solár, P.; Brázda, V.; Bares, M.; Zamani, A.; Emamiaref, P.; Joukal, A.; Kubícková, L.; Krocka, E.; Hasanová, K.; Joukal, M., Inflammatory changes in the choroid plexus following subarachnoid hemorrhage: the role of innate immune receptors and inflammatory molecules. *Frontiers in Cellular Neuroscience* 2025, 18, 1525415.
- [9] Liu, Y.; Li, J. Y.; Guo, H. J.; Fang, C.; Yang, Q. L.; Qin, W.; Wang, H.; Xian, Y.; Yan, X. B.; Yin, B. X.; Zhang, K., Nanomaterials for stroke diagnosis and treatment. *Iscience* 2024, 27(11), 111112.
- [10] Wan, G.; Gu, L. G.; Chen, Y. Y.; Wang, Y. Q.; Sun, Y.; Li, Z. W.; Ma, W. B.; Bao, X. J.; Wang, R. Z., Nanobiotechnologies for stroke treatment. *Nanomedicine* 2025,

20(11), 1299-1319.

- [11] Bao, Q.; Hu, P.; Xu, Y.; Cheng, T.; Wei, C.; Pan, L.; Shi, J., Simultaneous blood-brain barrier crossing and protection for stroke treatment based on edaravone-loaded ceria nanoparticles. *ACS Nano* 2018, 12(7), 6794-6805.
- [12] Yuan, J.; Li, L.; Yang, Q.; Ran, H.; Wang, J.; Hu, K.; Pu, W.; Huang, J.; Wen, L.; Zhou, L.; Jiang, Y.; Xiong, X.; Zhang, J.; Zhou, Z., Targeted treatment of ischemic stroke by bioactive nanoparticle-derived reactive oxygen species responsive and inflammation-resolving nanotherapies. *ACS Nano* 2021, 15(10), 16076-16094.
- [13] Xu, Y. T.; Chen, A. Q.; Wu, J. H.; Wan, Y.; You, M. F.; Gu, X. M.; Guo, H. X.; Tan, S. W.; He, Q. W.; Hu, B., Nanomedicine: An emerging novel therapeutic strategy for hemorrhagic stroke. *International Journal of Nanomedicine* 2022, 17, 1927-1950.
- [14] Lazaridis, C.; Rusin, C. G.; Robertson, C. S., Secondary brain injury: Predicting and preventing insults. *Neuropharmacology* 2019, 145, 145-152.
- [15] Shi, L. P.; Liu, S. Y.; Chen, J. L.; Wang, H.; Wang, Z. B., Microglial polarization pathways and therapeutic drugs targeting activated microglia in traumatic brain injury. *Neural Regeneration Research* 2026, 21(1), 39-56.
- [16] Zhang, X.; Sun, N.; Zhu, M. M.; Huang, Y., Single-cell RNA sequencing reveals microglial proliferative bias and neuroinflammatory communication reprogramming following traumatic brain injury. *Frontiers in Neurology* 2026, 17, 1789863.
- [17] Yang, M.; Han, Y. Q.; Bianco, A.; Ji, D. K., Recent progress on second near-infrared emitting carbon dots in biomedicine. *ACS Nano* 2024, 18(18), 11560-11572.
- [18] Shi, J. Y.; Zhang, Y.; Fang, X. Y.; Fan, X.; Li, J.; Zhou, C. H.; Xia, Z. N.; Pang, D. W.; Liu, C., Photoswitchable antioxidant and prooxidant activities of Mg-doped carbon dot nanozymes as antibacterial and anti-inflammatory agents. *ACS Applied Materials & Interfaces* 2025, 17 (18), 26467-26479.
- [19] Shi, X. F.; Jia, X. L.; Wu, Z. F.; Zhang, Y.; Chen, X. P.; Xiong, H. M.; Mao, Y. Q., Carbon dots derived from ginger for inhibiting inflammatory osteolysis.

Nanoscale 2025, 17(47), 27269-27280.

- [20] Dong, C.; Xu, M.; Huang, J.; Li, F.; Wei, P.; Tedesco, A. C.; Bi, H., Dynamic thermosensitive solid-state photoluminescent carbonized polymer dots as temperature-responsive switches for sensor applications. ACS Applied Nano Materials 2020, 3(11), 10560-10564.
- [21] Huang, J.; Dong, C.; Xu, J.; Xuan, J.; Cheng, Q.; Bi, H., Nitrogen and chlorine co-doped carbon dots with synchronous excitation of multiple luminescence centers for blue-white emission. New Journal of Chemistry 2021, 45(16), 7056-7059.
- [22] Hu, X.; Wang, S.; Luo, Q.; Ge, B.; Cheng, Q.; Dong, C.; Xu, J.; Ding, H.; Xu, M.; Tedesco, A. C.; Huang, X.; Zhang, R.; Bi, H., Synthesis of Sn nanocluster@carbon dots for photodynamic therapy application. Chinese Chemical Letters 2021, 32(7), 2287-2291.
- [23] Xin, X.; Liu, J.; Liu, X.; Xin, Y.; Hou, Y.; Xiang, X.; Deng, Y.; Yang, B.; Yu, W., Melatonin-derived carbon dots with free radical scavenging property for effective periodontitis treatment via the Nrf2/HO-1 pathway. ACS Nano 2024, 18(11), 8307-8324.
- [24] Han, J.; Choi, S.; Hong, J.; Gang, D.; Lee, S.; Shin, K.; Ko, J.; Kim, J.-U.; Hwang, N. S.; An, Y.-H.; Gu, M.; Kim, S.-H., Superoxide dismutase-mimetic polyphenol-based carbon dots for multimodal bioimaging and treatment of atopic dermatitis. ACS Applied Materials & Interfaces 2024, 16(19), 24308-24320.
- [25] Dong, J.; Wang, Q.; Gu, T.; Liu, G.; V. Petrov, Y.; Baulin, V. E.; Tsivadze, A. Y.; Jia, D.; Zhou, Y.; Yuan, H.; Li, B., Rapamycin functionalized carbon dots: Target-oriented synthesis and suppression of vascular cell senescence. Journal of Colloid and Interface Science 2024, 660, 534-544.
- [26] Dong, C.; Wang, S.; Ma, M.; Wei, P.; Chen, Y.; Wu, A.; Zha, Z.; Bi, H., Inhibition of oxidative stress in vivo through enzyme-like activity of carbon dots. Applied Materials Today 2021, 25, 101178.
- [27] Ma, Y.; Zhao, J.; Cheng, L.; Li, C.; Yan, X.; Deng, Z.; Zhang, Y.; Liang, J.; Liu, C.; Zhang, M., Versatile carbon dots with superoxide dismutase-like nanozyme activity and red fluorescence for inflammatory bowel disease therapeutics. Carbon

2023, 204, 526-537.

2026 S.-T. Yau High School Science Award
仅用于2026丘成桐中学科学奖论文公示

Acknowledgement

In this paper, the primary acknowledgement is given to Dr. Ying Yang, who instructed me free of charge in topic researching, theoretical analysis, and thesis writings. When deciding the paper's topic, Dr. Ying Yang provided me with novel insights toward development in hemorrhagic strokes and carbon dots materials, which helps me to narrow down my research focus on subarachnoid hemorrhage and nano medicine. Dr. Ying Yang also professionally analyzed the theory of organic synthesis of reactions, blood-brain barrier targeting, and inflammation regulations in this paper. Being my instructor, Dr. Ying Yang had shown great specialty in chemistry, helping me to organize the framework of synthesis pathway. Dr. Ying Yang helped me to overcome the difficulties in learning organic chemistry, medicine chemistry, and biocompatibility, which provides strong theoretical basis for my further research. In the final paper writing process, Dr. Ying Yang assisted me with contents polishing, in which she inspected detailly with scientific validity in language usage. She suggested me with meaningful improvement and revision for my paper, from abstract to the final discussion, and she was rigorous in language precision. I would like to thank gratefully Dr. Ying Yang for her selfless supports and efforts.

I also thank the uncompensated guidance from Dr. Dong Chen, the instructor of my experimental work in Ningbo University of Finance & Economics. In this paper, Dr. Dong Chen offered me professional instruction in thesis theory establishments, with his precious experience in carbon dots materials fields, supporting me both theoretically and technically. I read a lot of Dr. Dong Chen's articles in carbon dots materials synthesis from daily ingredients, inspiring me to purify carbon dots from familiar compounds, chlorogenic acid and coffee, to efficiently synthesize the ideal antioxidative drugs for subarachnoid hemorrhage. Besides, Dr. Dong Chen helped me in preparing for experimental materials and reagents, accessing to operation of experimental devices and equipment, designing the experimental procedures, and analyzing experimental data. In the laboratory, we repeated experiments restlessly, and Dr. Dong Chen never hesitated to explain every theory and to discover the most accurate data, which I truly appreciate his attitudes of scientific rigor, metacognitively fostering me to persist in those seemingly tedious experiments. The primary obstacle in experiments is carbon dots dialysis, in which we failed to obtain the carbon dots with ideal water solubility and we had difficulty in chemical modification. To come over these, we adopted column chromatography to purify the carbon dots and fixed synthesis parameters in the modification section to reduce parallel numbers of the parameters.

Beyond the academic supports from my instructors, I am really grateful to my school that backups student's capacity in scientific research and basic knowledge obtaining, and also the atmosphere where academic discussion is strongly fostered with a cohort of scientific enthusiasts who are always passionate discuss with me. I would also thank to the supports of

my teachers and competition coaches in schools, because they really enabled me to get passionate in chemistry, biology, and humanity, letting me to consider how my research could become beneficial and significant to human well-being, with basic knowledge and profound subject contents that we never suspended to acquire. Finally, I genuinely appreciate the help from my parents and families, that, they are the persons I could trust when encountering experimental failures and frustration in my academic and daily life. Without their backups, I would never have the opportunities to achieve my dreams in science.

At last, I would acknowledge the usage of AI algorithm model when producing visible experimental procedures in 2D style, to detailly present the steps we take in real lab work. In this paper, we only used AI for creating the figure of our research framework and experimental procedures. The following are the records of conversations using Xingliu AI, which, the Fig. 3 is created in <https://www.xingliu.art/canvas?projectId=6046c7d09a5f41d7bcadd49b68da7ac7> and the Fig. 4 is created in <https://www.xingliu.art/canvas?projectId=6a584ccbc38b4f909991913c874594a3>. With the assistance of Xingliu AI, I was able to visualize the full research framework and the reaction process of carbon dots synthesis, including, for examples, how the peptide was conjugated on crude carbon dots and how the nano systems function multiply. I am really looking forward the development in visualization of AI tools, which, if used properly, would therefore benefit every researcher in staying connected with readers by conveying information visibly.