

Ultrasound-Mediated Targeted Delivery of ROS-Responsive Scavenging Nanomicelles for the Treatment of Ischemic Stroke

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Purpose: Ischemic stroke (IS) is a highly disabling and fatal cerebrovascular disease. Current vascular recanalization often triggers an explosive burst of reactive oxygen species (ROS) and a subsequent neuroinflammatory storm, leading to severe reperfusion injury. However, existing neuroprotective strategies face dual translational bottlenecks: the blood-brain barrier (BBB) restricts drug penetration, and conventional carriers cannot precisely modulate the deep ischemic microenvironment. To overcome these limitations, this study developed a dual-targeted delivery strategy combining low-intensity pulsed ultrasound with microbubbles (LIPUS-MBs) and ROS-responsive scavenging anti-inflammatory nanomicelles (TPLN).

Results: The ROS-responsive scavenging TPLN nanomicelles were successfully synthesized. In vitro experiments demonstrated that TPLN efficiently scavenged intracellular ROS, protected PC12 neurons from oxygen-glucose deprivation (OGD)-induced apoptosis, and promoted the M2 repolarization of BV2 microglia. In vivo, the acoustic cavitation of LIPUS-MBs safely and reversibly permeabilized the BBB, increasing the targeted accumulation of TPLN within the ischemic lesion of MCAO rats. TPLN effectively mitigated oxidative damage and drove microglial polarization from a pro-inflammatory M1 state to a neuroreparative M2 phenotype, interrupting the oxidative stress-neuroinflammation cycle. Consequently, this synergistic therapy reduced cerebral infarct volume, alleviated brain edema, and facilitated neurological functional recovery.

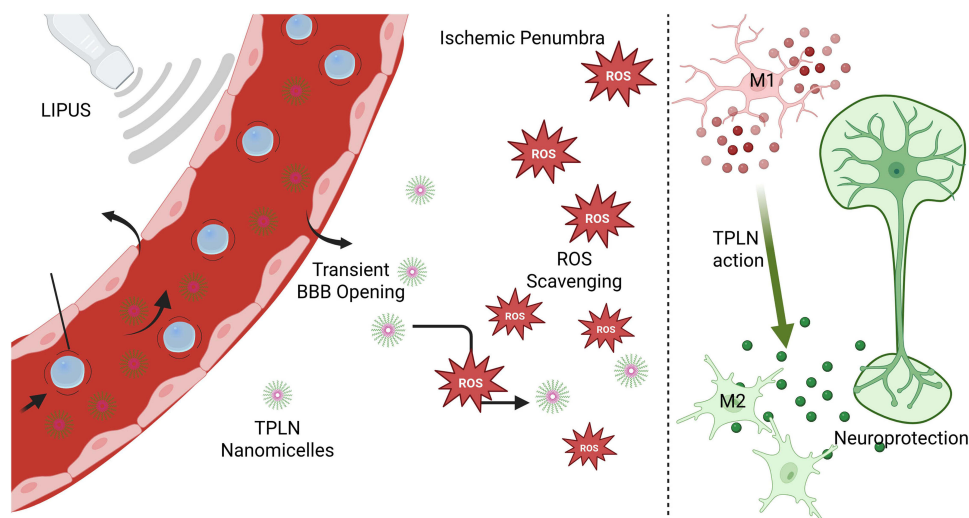
Conclusion: The dual-targeted delivery system combining LIPUS-MBs-mediated physical BBB opening and ROS-responsive scavenging TPLN chemical targeting provides a highly translatable therapeutic platform for the precision management of ischemic stroke. However, as these findings are primarily based on an acute-phase rodent model, the long-term therapeutic effects and clinical translational applicability require further investigation.

Keywords: low-intensity pulsed ultrasound, blood-brain barrier, microbubbles, oxidative stress, microglial polarization, reperfusion injury

Introduction

Ischemic stroke is the second leading cause of death worldwide, characterized by high morbidity and disability rates.¹⁻³ Current clinical management primarily relies on vascular recanalization;⁴ however, this approach frequently triggers severe secondary ischemia/reperfusion (I/R) injury.⁵ During the I/R phase, the explosive generation of ROS in the ischemic lesion not only directly damages neurons but also polarizes microglia toward a pro-inflammatory (M1) phenotype, thereby suppressing the neuroreparative (M2) phenotype.^{6,7} This vicious cycle between oxidative stress and neuroinflammation exacerbates neuronal apoptosis within the ischemic penumbra. Therefore, efficiently scavenging

Graphical Abstract



excess ROS and reversing microglial polarization have emerged as crucial strategies for remodeling the ischemic microenvironment and mitigating IS-induced damage.^{8,9}

To address these pathological mechanisms, ROS-responsive scavenging nanomedicines have demonstrated immense potential in remodeling the inflammatory microenvironment.^{9–11} However, the strict physical obstruction of BBB severely restricts drug penetration, preventing conventional neuroprotective agents and traditional nanocarriers from crossing the barrier within an effective therapeutic window. Even considering the transient, early increase in BBB permeability following I/R, the vast majority of therapeutic agents fail to achieve adequate intracranial accumulation,^{12,13} fundamentally limiting their clinical translation and therapeutic efficacy. In this study, TPLN is specifically utilized as ROS-responsive scavenging nanomicelles. The underlying mechanism relies on its unique polymeric backbone conjugated with luminol (3-aminophthalhydrazide) moieties.¹⁴ Rather than acting merely as a passive carrier, the nanomicelles structurally respond to the highly oxidative microenvironment at the ischemic site. Upon exposure to overproduced ROS, the luminol moieties undergo rapid chemical oxidation and hydrolysis. This process simultaneously consumes (scavenges) the localized excessive ROS to alleviate oxidative stress, and triggers the on-demand release of the active anti-inflammatory units to inhibit downstream inflammatory pathways.

To overcome this physical barrier, ultrasound combined with microbubbles (MBs) has been widely utilized to reversibly open the tight junctions.^{15–18} Nevertheless, the cerebral microvascular network is extremely fragile following ischemia. Traditional low-intensity focused ultrasound (LIFUS), due to its highly concentrated acoustic energy, is prone to exerting excessive mechanical stress on the lesion and inducing microhemorrhages. In contrast, low-intensity pulsed ultrasound (LIPUS) features a more uniform acoustic field and a milder energy profile, effectively preventing local energy accumulation. It can safely permeabilize the BBB in the ischemic region at a remarkably low cavitation threshold.^{19–21} While LIPUS-MBs has shown great promise in non-invasive interventions for other neurological diseases,^{22,23} its specific application for targeted drug delivery in ischemic stroke remains largely unexplored. Given its superior safety profile in fragile microenvironments, it emerges as an ideal modality for mediating the targeted brain delivery of nanomedicines.

Driven by these insights, this study developed a dual-targeted delivery strategy integrating “LIPUS-MBs-mediated physical permeabilization and nanomedicine-driven chemical targeting” (LIPUS-MBs/TPLN). LIPUS-MBs are employed to gently open the BBB at the target site, providing an efficient entry pathway for ROS-responsive scavenging anti-inflammatory nanomicelles (TPLN). Both *in vitro* and *in vivo* (MCAO rat model) evaluations demonstrated that the

specifically accumulated TPLN effectively scavenges excess ROS and robustly drives microglial polarization from the M1 to the M2 phenotype. Ultimately, immunofluorescence, TTC staining, and behavioral assessments confirmed that this system substantially reduces cerebral infarct volume and ameliorates neurological deficits, providing a highly translatable intervention paradigm to interrupt the pathological cascade of I/R injury.

Materials and Methods

Experimental Reagents and Instruments

Experimental Reagents

Chemicals and reagents were used as received unless otherwise noted. Cyanuric chloride and 3-aminophthalhydrazide (luminol) were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China), while mPEG-NH₂ was provided by Ruixi Biological Technology Co., Ltd. (Xi'an, China). Boster Biological Technology (Wuhan, China) supplied the cell culture reagents, FBS, and ELISA kits (TNF- α , IL-1 β , IL-6). Fluorescent probes (DCFH-DA, calcein-AM/PI) and assay kits for apoptosis and oxidative stress (MDA, SOD) were obtained from Beyotime Biotechnology (Shanghai, China). Primary antibodies (anti-CD86, anti-CD206, and anti-Iba-1) were purchased from Proteintech Group (Wuhan, China), and SonoVue microbubbles were acquired from Bracco Suisse SA (Switzerland). All other chemicals were of analytical grade.

Experimental Instruments

Instrumental analyses were performed using standard protocols. A Bruker AVANCE III 600 MHz spectrometer (Bruker, Germany) and a Nicolet 6700 spectrometer (Thermo Scientific, USA) were utilized to record ¹H NMR and FT-IR spectra, respectively. FEI (USA) supplied the Tecnai G2 12 transmission electron microscope (TEM) for morphological observations. Dandong Bettersize Instruments Ltd. (China) provided the Zeta potential analyzer for hydrodynamic diameter and surface charge measurements. Confocal fluorescence imaging and flow cytometry were conducted on systems from Nikon/Andor (Japan/UK) and Beckman Coulter (USA), respectively. A multimode microplate reader was obtained from Molecular Devices (USA) for absorbance and fluorescence quantification. For in vivo evaluations, RWD Life Science (Shenzhen, China) supplied the fluorescence and laser speckle blood flow imaging systems, while the UT1021 therapeutic ultrasound device essential for LIPUS intervention was manufactured by Shenzhen Dongdixin Technology Co., Ltd. (China).

Experimental Cells

Murine microglia (BV2) and rat pheochromocytoma (PC12) cell lines (Boster Biological Technology, Wuhan, China) were maintained in DMEM and RPMI-1640 media, respectively. Both media were supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere.

Experimental Animals

Male Sprague-Dawley (SD) rats (weighing 250–280 g) were purchased from the Experimental Animal Center of Chongqing Medical University (License No. SYXK (Yu) 2022–0016). All rats were housed in a temperature-controlled environment (22 ± 2 °C) under a 12-h light/dark cycle with free access to standard laboratory chow and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (Approval No. IACUC-CQMU-2024-04077) and were performed in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Experimental Methods

Synthesis of TPL Polymer

The TCT-mPEG intermediate was synthesized via nucleophilic substitution between cyanuric chloride and mPEG-NH₂ in anhydrous DCM in the presence of DIPEA, with continuous stirring at 0°C for 24 h. Following purification via silica gel column chromatography, the intermediate was reacted with luminol (molar ratio 1:5) in anhydrous DMSO containing TEA. This reaction proceeded at 65°C for 48 h under a nitrogen atmosphere. The resulting mixture was dialyzed (MWCO 2 kDa) against ultrapure water for 72 h to remove unreacted small molecules, and subsequently lyophilized to yield the final macromolecular polymer (TPL).

Preparation and Characterization of TPLN

TPLN nanomicelles were formulated via the ultrasonication-assisted self-assembly of the synthesized TPL polymer in ultrapure water. Successful chemical synthesis of the TPL backbone was confirmed by ^1H NMR and FT-IR spectroscopy. Following assembly, the hydrodynamic diameter, polydispersity index (PDI), and zeta potential of the nanomicelles were evaluated via dynamic light scattering (DLS) at room temperature, while their morphological features were visualized using TEM.

Cytotoxicity Assay

The in vitro cytotoxicity of TPLN against PC12 and BV2 cells was evaluated via a standard CCK-8 assay. Following seeding in 96-well plates, cells were exposed to gradient concentrations of TPLN (0–200 $\mu\text{g/mL}$) for 24 h. Cell viability was subsequently quantified by recording the absorbance at 450 nm post-incubation with the CCK-8 reagent.

Cellular Uptake and Targeting Behavior

Cellular internalization dynamics were profiled by incubating PC12 and BV2 cells with Cy5-labeled TPLN (Cy5-TPLN) under varying temporal (0–6 h at 50 $\mu\text{g/mL}$) and dosage (0–100 $\mu\text{g/mL}$ for 2 h) parameters. To mimic ischemic stress and evaluate active targeting capabilities, an in vitro chemical OGD microenvironment was induced via CoCl_2 (100 μM) pre-treatment. Ultimately, uptake efficiency across both normoxic and OGD conditions was visualized using CLSM and quantitatively analyzed via flow cytometry.

Endocytosis Mechanism Assays

To elucidate the specific internalization pathways of TPLN under normoxic and OGD conditions, temperature-dependent and pharmacological inhibition assays were conducted. For the temperature-dependent assay, cells were incubated with TPLN for 2 h at 4 $^\circ\text{C}$ or 37 $^\circ\text{C}$. For the endocytic inhibition assay, cells were pre-incubated with the caveolae-mediated endocytosis inhibitor genistein (200 μM) or the macropinocytosis inhibitor EIPA (10 μM) for 1 h at 37 $^\circ\text{C}$, using an equivalent volume of DMSO as the vehicle control. Subsequently, TPLN was added, and the cells were co-incubated for an additional 2 h in the presence of the respective inhibitors. Finally, all treated cells were harvested, washed thoroughly with cold PBS, and immediately analyzed via flow cytometry to determine the mean fluorescence intensity.

Apoptosis and Cell Viability

To simulate an ischemic apoptotic microenvironment, PC12 cells were challenged with CoCl_2 (100 μM). Following co-incubation with TPLN, the early and late apoptotic populations were quantitatively analyzed via flow cytometry utilizing Annexin V-FITC/PI double staining. Concurrently, overall cell viability and survival status were visualized using a calcein-AM/PI live/dead assay under fluorescence microscopy.

Intracellular ROS Scavenging

The intracellular ROS scavenging efficacy of TPLN was assessed employing a DCFH-DA fluorescent probe. An intracellular ROS overload was initially induced in PC12 cells via glutamate stimulation (20 mM, 12 h). Subsequent to TPLN treatment (50 $\mu\text{g/mL}$, 2 h) and dark incubation with DCFH-DA, the intracellular ROS levels were visually captured by CLSM and quantitatively profiled via flow cytometry.

Microglial Polarization

To evaluate microglial phenotypic transitions, BV2 cells were challenged with CoCl_2 -induced OGD and subsequently treated with TPLN. Following standard fixation and blocking procedures, the cells were immuno-stained with primary antibodies targeting CD86 (M1 marker) and CD206 (M2 marker). Post-incubation with appropriate fluorophore-conjugated secondary antibodies and DAPI nuclear counterstain, the spatial distribution of polarization markers was visually captured and qualitatively profiled via CLSM.

Anti-Inflammatory and Oxidative Stress Evaluation

To assess the immunomodulatory and antioxidant efficacy of TPLN, cell culture supernatants from OGD-injured BV2 cells were harvested post-treatment to quantify the extracellular secretion of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) via respective ELISA kits. Concurrently, intracellular protein lysates were extracted to determine

malondialdehyde (MDA) accumulation and superoxide dismutase (SOD) activity utilizing commercial biochemical assays in strict accordance with the manufacturers' instructions.

MCAO/R Model and Treatment Protocols

The middle cerebral artery occlusion/reperfusion (MCAO/R) model was established in male SD rats. Following intraperitoneal anesthesia with 2% sodium pentobarbital, a monofilament was advanced through the internal carotid artery to transiently occlude the origin of the middle cerebral artery. After 60 min of focal ischemia, the filament was carefully withdrawn to initiate reperfusion.

Successfully modeled rats were subsequently randomized into four distinct cohorts (n = 28 per group). The Sham cohort underwent identical vessel isolation without arterial occlusion. The MCAO (Model) cohort received a systemic injection of physiological saline post-reperfusion. For the therapeutic cohorts, the TPLN group was intravenously administered TPLN nanomicelles (20 mg/kg). Crucially, the combined intervention cohort (LIPUS-MBs + TPLN) received concurrent intravenous infusions of TPLN (20 mg/kg) and SonoVue microbubbles, accompanied by targeted LIPUS sonication (3.0 W/cm², 1 MHz, 40% duty cycle, 120s). Driven by our prior spatial-temporal profiling of BBB permeability, all therapeutic interventions were executed at 2 h post-reperfusion to maximize intracranial accumulation.

Evaluation of BBB Permeability

To comprehensively profile blood-brain barrier (BBB) permeability dynamics, an Evans blue (EB) extravasation assay was executed across seven distinct rat cohorts (n = 5 per group). Baseline and sonication-mediated physiological BBB integrities were established utilizing intact rats (Control, LIPUS alone, and LIPUS+MBs). Parallely, pathological BBB breakdown and USMB-augmented permeability were mapped in MCAO-challenged rats across early (2 h) and delayed (24 h) reperfusion windows, factoring in the presence or absence of targeted ultrasound interventions. Following in vivo circulation, harvested brain specimens were directly subjected to formamide extraction (37 °C, 48h). Ultimate intracranial EB accumulation was determined via spectrophotometric quantification of the resultant supernatants at 626 nm.

Biodistribution and Cellular Co-Localization

The in vivo biodistribution and lesion-targeting efficacy were mapped following the systemic administration of Cy5-labeled TPLN (20 mg/kg). At 12 h post-injection, brain specimens were excised for macroscopic ex vivo fluorescence imaging. To explicitly delineate the microscopic cellular internalization within the ischemic region, tissue sections were double-stained with DAPI and primary antibodies against NeuN (neuronal marker) or Iba-1 (microglial marker). The spatial co-localization of nanomicelles with distinct neural cell populations was visually profiled via CLSM.

Infarct Volume and Brain Water Content

At 24 h post-treatment, the brains were harvested and sectioned into 2-mm coronal slices. The slices were stained with 2% TTC solution at 37°C for 20 min in the dark. The infarct volume percentages were calculated using ImageJ software. Additionally, cerebral edema was evaluated by measuring the brain water content (BWC) using the wet-dry weight method. The ischemic hemispheres were dried at 125°C for 24 h to calculate the BWC.

Histological Evaluation and Microglial Polarization

Brain tissues from the ischemic penumbra were fixed, embedded in paraffin, and cut into 5 µm sections. Hematoxylin and Eosin (H and E) and Nissl staining were performed to assess neuronal morphology. To evaluate microglial polarization, immunofluorescence double staining was conducted. The sections were co-stained with antibodies against Iba-1 and CD86 (for the M1 phenotype) or CD206 (for the M2 phenotype). DAPI was used for nuclear counterstaining.

In vivo Anti-Inflammatory and Oxidative Stress Analysis

At 48 h post-treatment, the ischemic brain tissues were harvested and homogenized. Oxidative stress markers, including malondialdehyde (MDA) and superoxide dismutase (SOD), were measured using commercial biochemical assay kits. Additionally, the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the tissue homogenates were quantified using corresponding ELISA kits.

Neurological Behavioral Evaluation

Neurological behavioral tests were conducted to evaluate long-term functional recovery. Neurological deficits were assessed using a 5-point Longa scoring system on day 7 post-operation. Furthermore, the modified Neurological Severity Score (mNSS) was evaluated on day 14 to comprehensively assess motor, sensory, balance, and reflex functions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 10.1.2 software. Data were first tested for normality. Statistical differences among multiple groups were analyzed using one-way analysis of variance (ANOVA). All quantitative measurements are presented as the mean $\pm$ standard error of the mean (SEM). A P-value < 0.05 was considered to indicate a statistically significant difference.

Results

Synthesis and Characterization of TPLN

To overcome the physical BBB and remodel the ischemic microenvironment, a dual-targeted therapeutic strategy combining ultrasound-mediated physical BBB opening with chemical guidance was designed, as illustrated in the overall mechanism (Figure 1B). As the core of this system, an amphiphilic ROS-responsive scavenging polymer conjugate, TPL, was synthesized (Figure 1A). Luminol was selected as the functional moiety due to its specific chemical reactivity. Upon

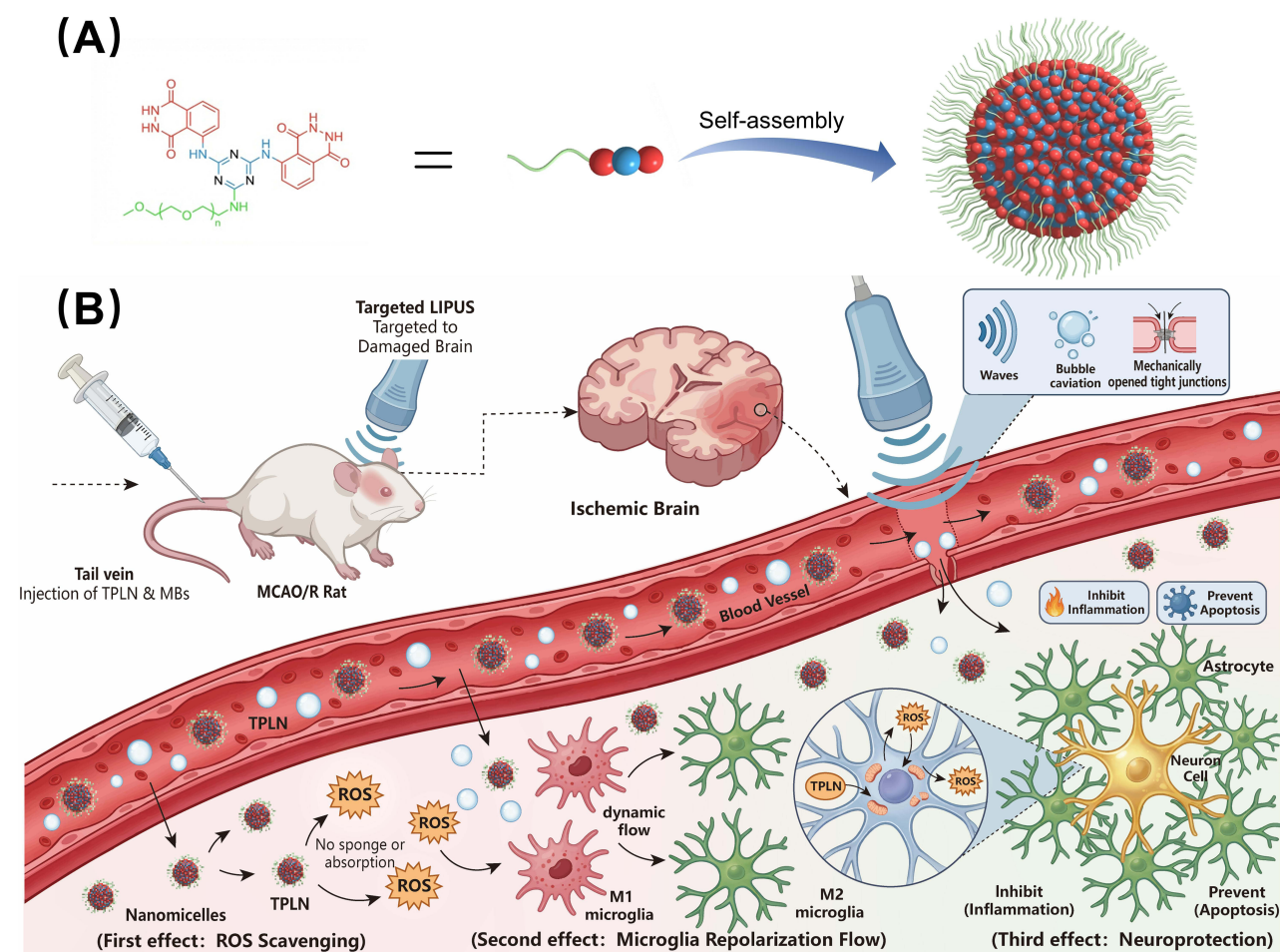


Figure 1 Schematic illustration of the dual-targeted delivery strategy for ischemic stroke treatment. **(A)** Chemical synthetic route and self-assembly process of the ROS-responsive scavenging TPLN nanomicelles. The chemical structures are color-coded: red denotes luminol, blue denotes cyanuric chloride, and green denotes mPEG-NH₂. **(B)** Schematic representation of the in vivo synergistic therapeutic mechanism: targeted LIPUS-MBs physically open the blood-brain barrier (BBB) to enhance TPLN accumulation, followed by TPLN actively scavenging ROS to modulate microglial repolarization (M1 to M2) and alleviate neuroinflammation.

encountering ROS, luminol undergoes a rapid oxidation reaction, effectively consuming these oxidative molecules.^{24,25} By covalently conjugating luminol onto the polymer backbone, this material neutralizes excess ROS in the ischemic microenvironment.

The chemical structure of the synthesized TPL polymer was confirmed by ^1H NMR and FT-IR spectroscopy. As shown in Figure 2A and B, the appearance of characteristic peaks corresponding to both the polymer backbone and the luminol moiety verified successful conjugation. In an aqueous environment, TPL polymers self-assembled into TPLN nanomicelles. TEM (Figure 2E) revealed a spherical morphology. DLS analysis (Figure 2D) indicated an average hydrodynamic diameter of 112.55 ± 9.57 nm and a PDI of 0.312 ± 0.038 . This nanoscale size facilitates systemic circulation and brain parenchyma penetration following BBB opening. Additionally, the zeta potential of TPLN was -13.03 ± 0.21 mV (Figure 2C). A slightly negative surface charge reduces non-specific protein adsorption and limits macrophage clearance, providing the necessary colloidal stability for in vivo applications.

Targeted Uptake and Neuroprotection in PC12 Cells

Before investigating the neuroprotective mechanisms, the in vitro cytotoxicity of TPLN was evaluated. After a 24 h co-incubation, both PC12 neurons and BV2 microglia maintained high viability (>80%) at TPLN concentrations up to $50 \mu\text{g/mL}$ (Figure 3A), establishing a safe dosage threshold for subsequent evaluations. The dose- and time-dependent cellular internalization of TPLN was also confirmed (Supplementary Figure S1).

Cellular uptake under ischemic conditions was assessed using an OGD model in PC12 cells. Both CLSM and flow cytometry demonstrated enhanced internalization of TPLN under OGD conditions compared to normoxic controls. CLSM images displayed higher intracellular fluorescence (Figure 3C), and flow cytometry confirmed a significant

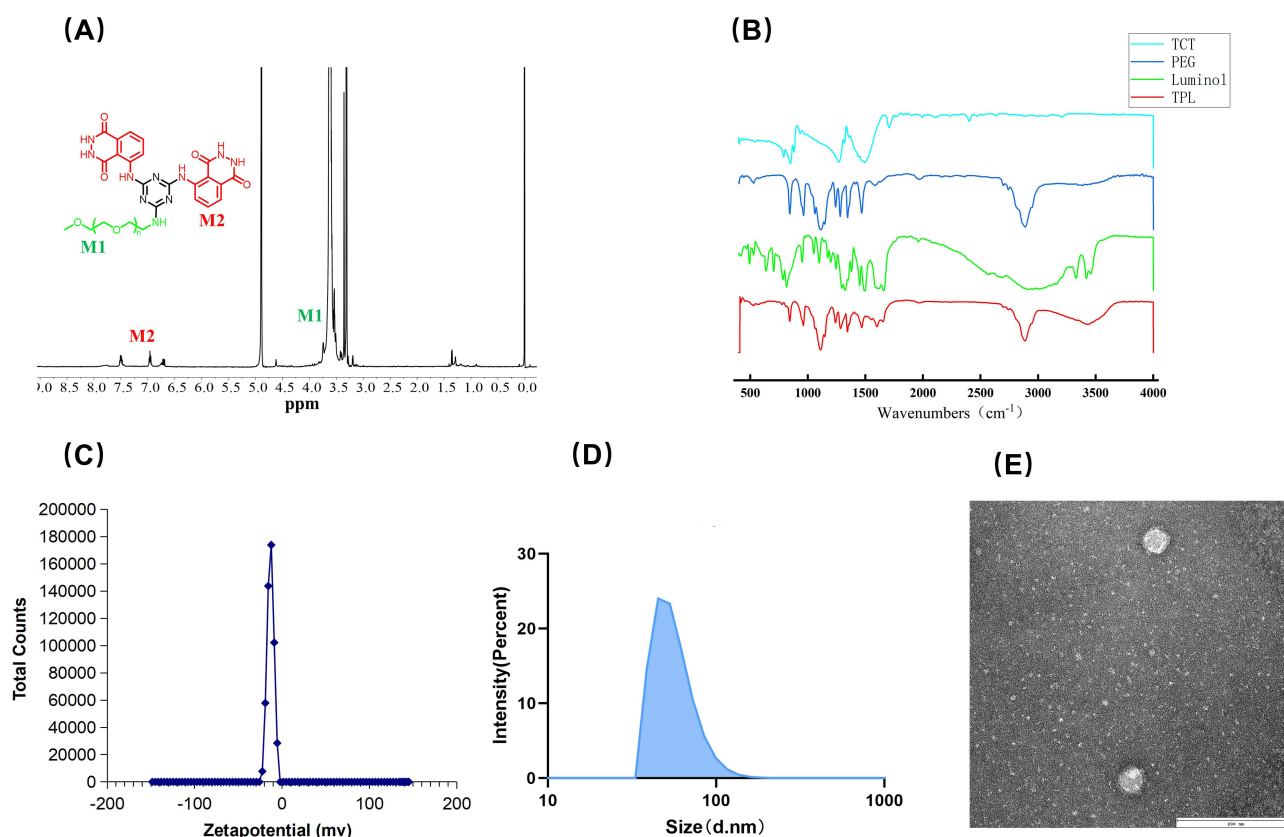


Figure 2 Synthesis and characterization of TPLN nanomicelles. (A) ^1H NMR spectrum of the synthesized TPL polymer. M1 and M2 indicate the characteristic peaks of the PEG and luminol segments, respectively. (B) FTIR spectra of TCT, PEG, luminol, and the TPL polymer. (C) Zeta potential and (D) size distribution of TPLN nanomicelles determined by dynamic light scattering (DLS). (E) Representative TEM image showing the morphology of TPLN nanomicelles. Scale bar = 200 nm. Data are presented as mean $\pm$ SD ($n=3$).

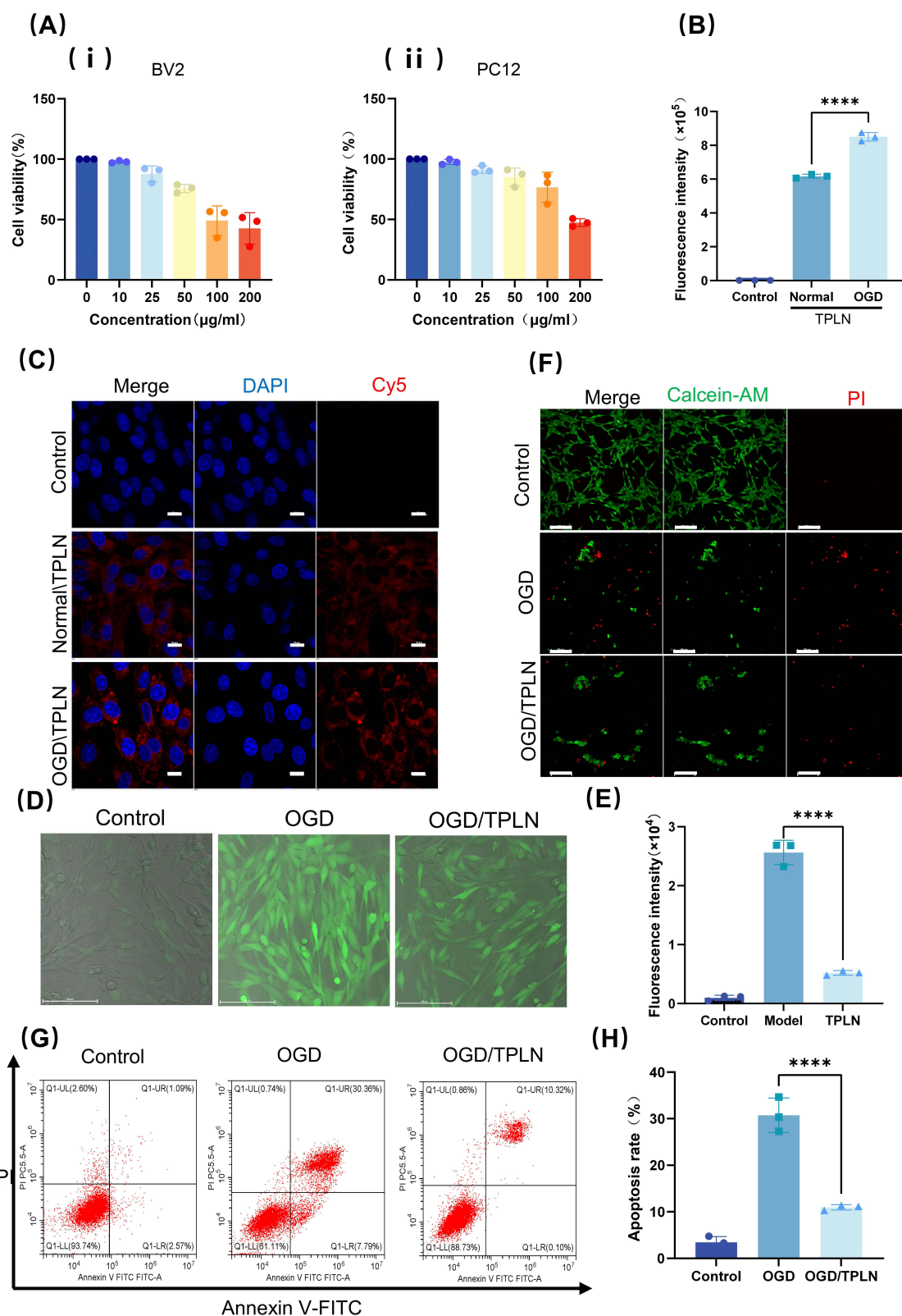


Figure 3 In vitro cellular uptake, ROS scavenging, and anti-apoptotic effects of TPLN. **(A)** Relative cell viability of (i) BV2 and (ii) PC12 cells incubated with various concentrations of TPLN. **(B)** Quantitative fluorescence intensity and **(C)** representative CLSM images showing the cellular uptake of Cy5-labeled TPLN under normal and oxygen-glucose deprivation (OGD) conditions, Scale bar = 10 μm . **(D)** Representative fluorescence images and **(E)** corresponding quantitative analysis of intracellular ROS levels. **(F)** Live/dead staining (Calcein-AM/PI) of cells following different treatments, Scale bar = 100 μm . **(G)** Flow cytometry analysis of apoptosis using Annexin V-FITC/PI double staining and **(H)** the quantified apoptosis rate. **** $P < 0.0001$.

increase in mean fluorescence intensity (MFI) (Figure 3B). To elucidate the underlying mechanisms, temperature-dependent and pharmacological inhibition assays were conducted. Incubation at 4°C significantly inhibited cellular uptake in the OGD group compared to 37°C, confirming an energy-dependent endocytosis process rather than passive membrane permeabilization (Supplementary Figure S2). Furthermore, pretreatment with the caveolae-mediated endocytosis inhibitor genistein inhibited over 85% of TPLN uptake under both normoxic and OGD conditions. The absolute contribution of this pathway increased under OGD, as evidenced by the preserved inhibition ratio despite elevated total uptake. Meanwhile, the macropinocytosis inhibitor EIPA revealed a basal contribution of ~25% that remained unchanged under OGD (Supplementary Figure S3). These data demonstrate that in damaged neurons, enhanced TPLN uptake is primarily driven by the active upregulation of caveolae-mediated endocytosis.

The intracellular reactive oxygen species (ROS) scavenging capability of TPLN was evaluated using a DCFH-DA probe (Figure 3D and E). OGD treatment induced significant ROS generation, evidenced by an intense green fluorescence signal. TPLN treatment effectively reversed this effect, visibly decreasing the fluorescence intensity. Flow cytometry quantification confirmed a reduction in MFI in the TPLN-treated group, demonstrating the efficient clearance of intracellular ROS by the nanomicelles. Because ROS accumulation during ischemia mediates cell death,²⁶ this clearance directly contributes to mitigating subsequent oxidative damage.

The protective effect of TPLN against OGD-induced neuronal injury was further evaluated.

Flow cytometry analysis (Figure 3G) showed that while OGD exposure elevated the cellular apoptosis rate, TPLN treatment significantly reduced this apoptotic population. Consistent with these findings, Live/Dead double staining (Figure 3F) revealed a decrease in PI-positive (dead) cells and an increase in Calcein-AM-positive (live) cells in the TPLN-treated group relative to the OGD group. By clearing intracellular ROS, TPLN interrupts the downstream apoptotic signaling cascades activated by oxidative stress,²⁷ thereby preserving neuronal viability.

Targeted Uptake and Microglial Repolarization in BV2 Cells

The cellular uptake of TPLN by BV2 microglia under ischemic conditions was evaluated using an OGD model. Both flow cytometry (Figure 4A) and CLSM (Figure 4B) revealed higher intracellular fluorescence intensity in OGD-treated cells compared to normoxic controls, indicating enhanced internalization. Consistent with the findings in PC12 cells, genistein inhibited over 85% of TPLN uptake, confirming caveolae-mediated endocytosis as the predominant internalization route. Interestingly, a cell-type-specific secondary mechanism was observed in microglia: while EIPA showed no effect under normoxia, it partially inhibited uptake (approximately 44%) under OGD. This suggests an additional activation of macropinocytosis in response to ischemic stress (Supplementary Figure S4). Because microglia are central mediators of neuroinflammation, this enhanced accumulation facilitates the targeted modulation of the inflammatory microenvironment.

Microglial oxidative stress was assessed by measuring intracellular MDA levels (Figure 4E) and SOD activity (Figure 4F). OGD exposure increased MDA levels and decreased SOD activity. TPLN treatment reversed these trends, effectively reducing the cellular oxidative burden. Because intracellular oxidative stress triggers pro-inflammatory microglial activation,²⁸ clearing ROS establishes a biochemical basis for subsequent phenotypic regulation.

The phenotypic polarization of BV2 cells was evaluated using immunofluorescence staining (Figure 4C and D). OGD stimulation increased the expression of the M1 marker CD86 and decreased the M2 marker CD206. Biologically, this M1-dominant state is typically associated with the release of neurotoxic cytokines and the exacerbation of oxidative stress. Conversely, TPLN treatment effectively reduced CD86 and increased CD206 expression relative to the OGD group. This shift demonstrates a robust microglial transition from the pro-inflammatory M1 phenotype toward the neuroreparative M2 phenotype. Such repolarization is crucial, as it halts the destructive inflammatory cascade and actively establishes an anti-inflammatory microenvironment necessary for resolving inflammation and promoting tissue repair after ischemic stroke.^{29,30}

To evaluate the functional consequences of this repolarization, the secretion of pro-inflammatory cytokines was quantified using ELISA. OGD stimulation elevated the levels of IL-6 (Figure 4G), IL-1 β (Figure 4H), and TNF- α (Figure 4I) in the cell culture supernatant. TPLN treatment significantly reduced the secretion of these cytokines

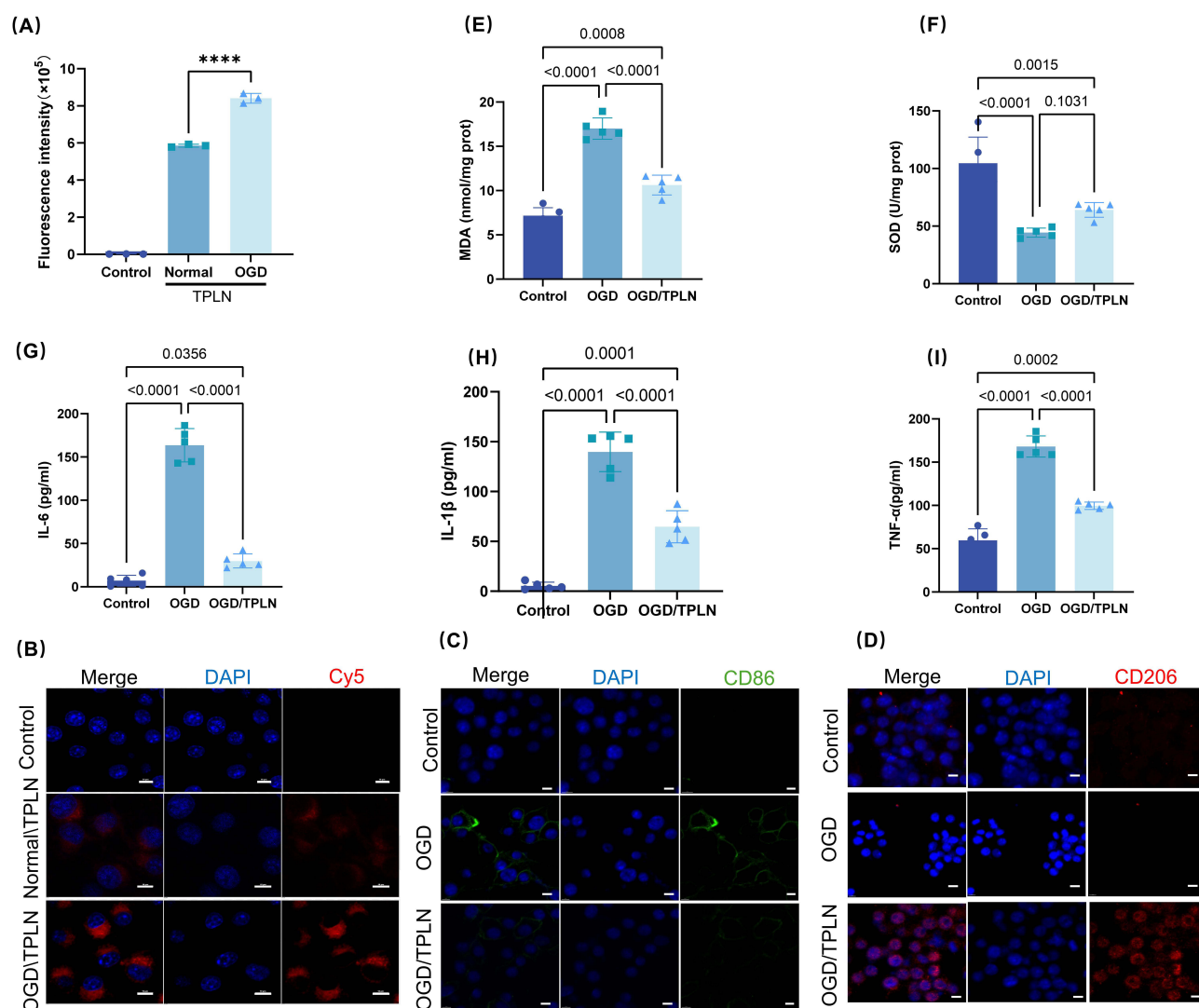


Figure 4 In vitro cellular uptake, oxidative stress regulation, and phenotypic polarization of BV2 microglia. (A) Quantitative fluorescence intensity and (B) representative CLSM images showing the cellular uptake of Cy5-labeled TPLN under normal and OGD conditions. Representative immunofluorescence images of (C) the M1 marker CD86 and (D) the M2 marker CD206. Scale bar = 10 μ m. Intracellular (E) MDA concentration and (F) SOD activity. Secretion levels of the pro-inflammatory cytokines (G) IL-6, (H) IL-1 β , and (I) TNF- α in the cell culture supernatant determined by ELISA. **** $P < 0.0001$.

compared to the OGD group, confirming its in vitro anti-inflammatory efficacy. Downregulating these mediators is essential for alleviating secondary inflammatory injury in the ischemic brain.³¹

LIPUS-MBs-Mediated BBB Opening and Targeted Delivery

The in vivo treatment protocol for the MCAO/R model is illustrated in Figure 5A. The capability of LIPUS-MBs to open BBB was evaluated via EB extravasation. Quantitative analysis (Figure 5B) revealed that while ischemia induced baseline EB leakage, LIPUS-MBs intervention significantly increased EB extravasation in the ipsilateral hemisphere compared to the untreated MCAO group. Histological evaluation (Supplementary Figure S5) confirmed that the applied acoustic parameters did not induce microhemorrhage or tissue damage. By utilizing ultrasound-induced microbubble cavitation to transiently widen endothelial tight junctions,³² this physical intervention safely augments the permeation of macromolecules into the brain parenchyma.

Brain accumulation was assessed via ex vivo fluorescence imaging following the intravenous administration of Cy5-labeled TPLN (Figure 5C). Compared to the spontaneous permeation observed in the TPLN-only group, the LIPUS-MBs

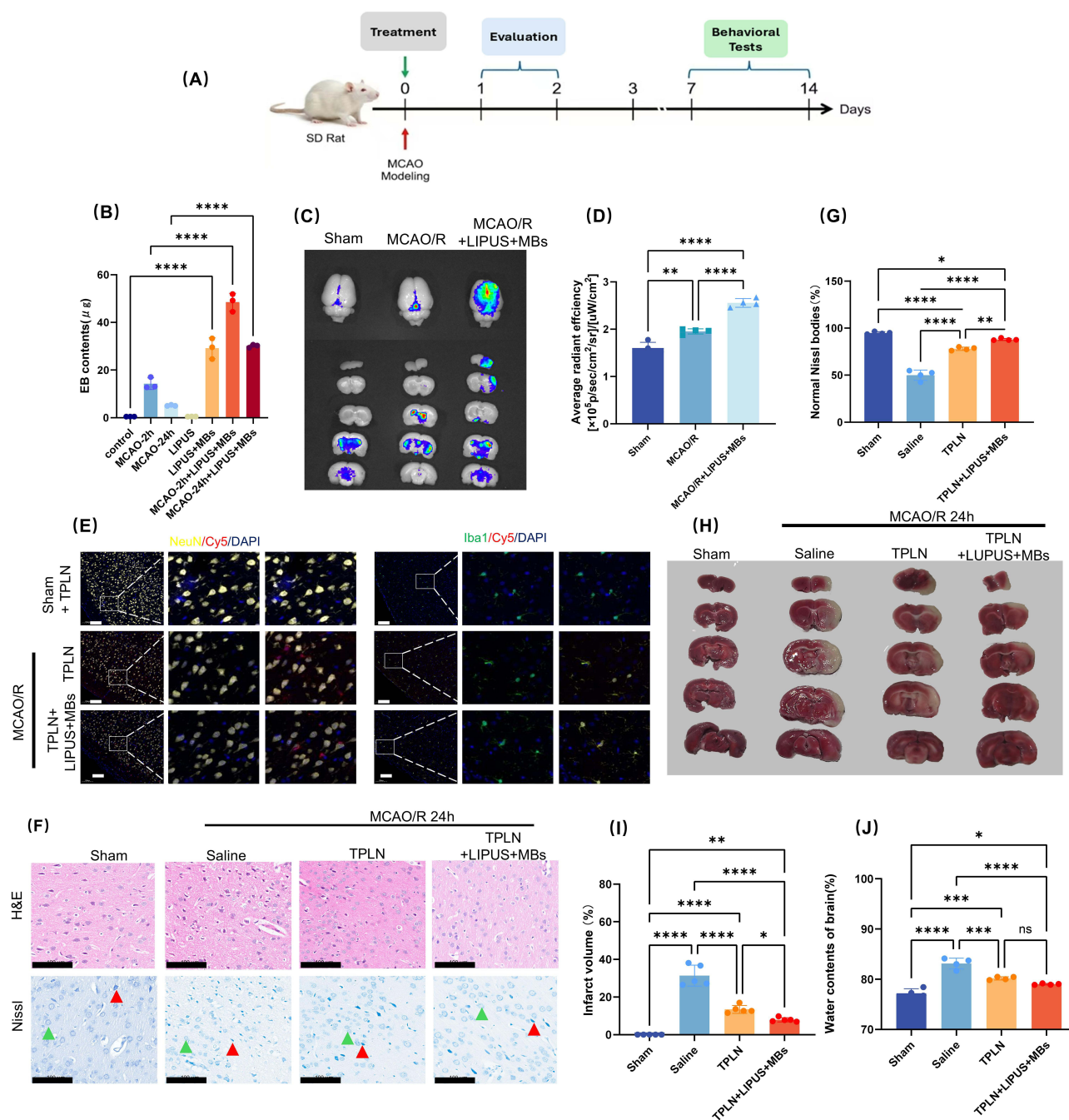


Figure 5 In vivo targeted delivery and neuroprotective efficacy of LIPUS-MBs + TPLN in MCAO/R rats. **(A)** Schematic timeline of the in vivo experimental protocol. **(B)** Quantitative analysis of Evans Blue (EB) extravasation. **(C)** Ex vivo fluorescence images and **(D)** corresponding quantitative average radiant efficiency of the harvested brains. **(E)** Representative immunofluorescence co-localization images of Cy5-labeled TPLN with neurons (NeuN) and microglia (Iba1) in the ischemic region, Scale bar = 100 μ m. **(F)** Representative H & E and Nissl staining images of the ischemic penumbra (red arrows: apoptotic bodies; green arrows: normal Nissl bodies, annotated in Nissl staining), and **(G)** the quantitative percentage of normal Nissl bodies. **(H)** Representative TTC-stained brain slices and **(I)** the quantified infarct volume. **(J)** Brain water content in different treatment groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns, not significant.

+ TPLN group exhibited a significantly higher fluorescence signal in the ischemic hemisphere. Quantitative analysis of the fluorescence intensity (Figure 5D) confirmed this enhanced intracranial accumulation.

To evaluate the cellular distribution of TPLN within the brain parenchyma, immunofluorescence co-localization staining was performed (Figure 5E). The Cy5-TPLN fluorescence visibly co-localized with NeuN (neuronal marker) and Iba-1 (microglial marker) within the ischemic lesion. This confirms that TPLN successfully crossed the ultrasound-

activated BBB and was internalized by target neurons and microglia *in vivo*. Ultimately, the combination of LIPUS-MBs and targeted nanomicelles effectively enhances specific cellular delivery to the ischemic central nervous system.^{33,34}

In vivo Neuroprotection and Functional Recovery

Histopathological alterations within the ischemic penumbra were evaluated using H and E and Nissl staining (Figure 5F). The untreated MCAO group exhibited severe tissue damage, characterized by loosely arranged cells, interstitial edema, and nuclear pyknosis, accompanied by extensive neuronal loss and the dissolution of Nissl bodies. TPLN treatment alleviated these morphological damages, with the LIPUS-MBs + TPLN group exhibiting the most preserved histological structure. Quantitative analysis of the normal neuron percentage (Figure 5G) confirmed that the combined therapy significantly increased neuronal survival. Preserving neuronal integrity is fundamental to preventing stroke-induced neurological deficits.³⁵

Cerebral infarct volume was visualized using TTC staining (Figure 5H). The untreated MCAO group displayed a distinct pale infarct area in the ipsilateral hemisphere, which was reduced following TPLN treatments. Quantitative measurement of the infarct volume (Figure 5I) confirmed that the LIPUS-MBs + TPLN group achieved the smallest infarct volume among all cohorts. Reducing the infarct size is a primary indicator of neuroprotection,³⁶ demonstrating that the enhanced accumulation of ROS-responsive scavenging nanomicelles effectively prevents ischemia-induced tissue necrosis.

Cerebral edema was evaluated by measuring the brain water content (Figure 5J). MCAO surgery significantly increased the water content in the ischemic hemisphere compared to the sham group. However, the application of LIPUS-MBs + TPLN effectively reduced this pathological swelling. Because severe oxidative stress exacerbates pathological blood-brain barrier disruption and promotes vasogenic edema,³⁷ the targeted clearance of excess ROS by TPLN mitigates this secondary process, effectively alleviating brain tissue swelling.

Functional Recovery and Molecular Mechanisms

Neurological functional recovery was evaluated using the Longa and modified Neurological Severity Score (mNSS) systems (Figure 6A and B). Following the MCAO procedure, rats exhibited severe behavioral deficits. Compared to the untreated MCAO group, the LIPUS-MBs + TPLN intervention significantly reduced both Longa and mNSS scores. Because preserving viable neurons correlates with the recovery of motor and sensory functions,³⁸ this functional restoration is consistent with the observed structural preservation in the ischemic penumbra.

To investigate the underlying antioxidant mechanism, brain tissue MDA levels (Figure 6C) and SOD activity (Figure 6D) were quantified. Ischemia significantly increased MDA accumulation and decreased SOD activity. Treatment with LIPUS-MBs + TPLN effectively reversed these trends. These results demonstrate that the accumulated nanomicelles clear excess ROS *in vivo* to mitigate oxidative damage.³⁹

The *in vivo* phenotypic polarization of microglia was evaluated via immunofluorescence staining (Figure 6E). In the pathological progression of ischemic stroke, sustained M1 activation drives secondary brain injury, whereas M2 polarization facilitates tissue remodeling and neurological recovery. Consistent with this, the untreated MCAO/R group exhibited high expression of the M1 marker and low expression of the M2 marker. Conversely, LIPUS-MBs + TPLN treatment significantly decreased the M1 signal and increased the M2 signal. This targeted shift indicates a critical microglial transition from a pro-inflammatory M1 state to a neuroreparative M2 state. Promoting this M2 polarization provides a profound mechanistic basis for regulating post-stroke neuroinflammation and mitigating long-term tissue damage.^{34,40}

The modulation of the inflammatory microenvironment was evaluated by quantifying tissue-level pro-inflammatory cytokines using ELISA. The MCAO procedure significantly increased the concentrations of IL-1 β (Figure 6F), TNF- α (Figure 6G), and IL-6 (Figure 6H). Consistent with the observed microglial repolarization, LIPUS-MBs + TPLN treatment effectively reduced the levels of these cytokines. Because downregulating these inflammatory mediators reduces secondary neuroinflammatory injury,⁴¹ these combined mechanisms confirm the robust neuroprotective effect of the dual-targeted delivery strategy.

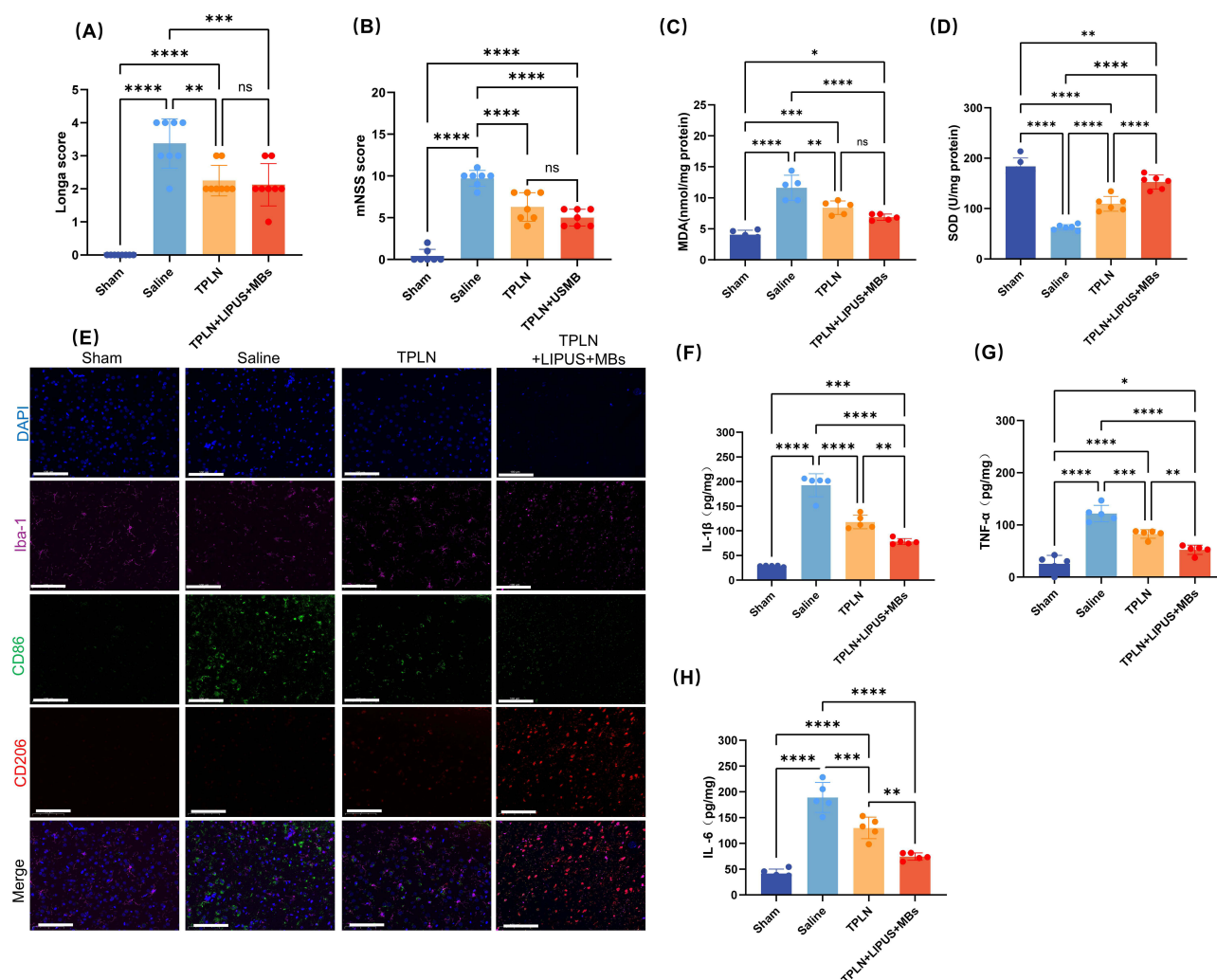


Figure 6 In vivo neurobehavioral recovery and modulation of the neuroinflammatory microenvironment. **(A)** Longa scores and **(B)** modified Neurological Severity Scores (mNSS) of the rats following different treatments. **(C)** Malondialdehyde (MDA) levels and **(D)** superoxide dismutase (SOD) activity in the brain tissue. **(E)** Representative immunofluorescence images showing the phenotypic polarization of microglia (M1 marker CD86, M2 marker CD206) in the ischemic brain tissue, Scale bar = 100 μ m. Tissue-level concentrations of the pro-inflammatory cytokines **(F)** IL-1 β , **(G)** TNF- α , and **(H)** IL-6 determined by ELISA. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant.

Discussion

Ischemic stroke reperfusion injury is driven by the overproduction of ROS and the subsequent neuroinflammatory cascade. Effective pharmacological interventions for these pathological processes are severely restricted by the physical obstruction of the BBB. To address this translational bottleneck, this study developed a dual-targeted delivery strategy combining LIPUS-MBs and ROS-responsive scavenging nanomicelles (TPLN). Our findings demonstrate that this combined system effectively permeabilizes the BBB, enhances the targeted accumulation of TPLN in the ischemic penumbra, and provides comprehensive neuroprotection through synergistic antioxidant and immunomodulatory mechanisms.

The therapeutic rationale for utilizing TPLN relies on its dual functionality as ROS-responsive scavenging nanomicelles. Unlike conventional nanocarriers that function solely as passive transport vehicles, the luminol-conjugated polymer backbone of TPLN actively neutralizes local ROS upon entry into the oxidative microenvironment. Our in vitro results confirmed that TPLN efficiently internalizes into ischemic cells and mitigates intracellular oxidative stress. By clearing these free radicals at the source, TPLN interrupts the downstream oxidative damage and apoptotic

signaling pathways. This active depletion of the oxidative burden establishes the crucial biochemical foundation for alleviating subsequent neuroinflammation.

Importantly, the application of LIPUS-MBs in this strategy extends beyond its role as a physical BBB permeabilizer. Physically, the acoustic cavitation of microbubbles driven by LIPUS provides a safe, uniform, and reversible opening of the endothelial tight junctions, circumventing the tissue damage and microhemorrhage associated with high-intensity ultrasound. Biologically, emerging evidence indicates that low-intensity ultrasound exerts intrinsic neuromodulatory and neuroprotective effects. Recent studies demonstrate that LIPUS facilitates glymphatic influx and clearance via the TRPV4-AQP4 pathway, promotes oligodendrocyte maturation and remyelination by downregulating the IL-17A/Notch1 signaling cascade, and induces neurogenesis in brain injury models.^{42–44} Furthermore, the clinical feasibility of this physical intervention is strongly supported by recent human trials, where noninvasive focused ultrasound has successfully and safely opened the BBB in eloquent structures, such as the hippocampus, in Alzheimer's disease patients.⁴⁵ As highlighted in a recent comprehensive review, transcranial focused ultrasound has emerged as a transformative, widely deployable tool for human brain disorders.⁴⁶ The convergence of these established clinical safety profiles with the intrinsic neuroprotective properties of LIPUS and the chemical targeting of TPLN underscores the high translational potential of this dual-delivery system.

The localized and transient permeabilization of the BBB by LIPUS-MBs synergizes with the active ROS-responsive scavenging capabilities of TPLN, maximizing the effective accumulation of nanomicelles within the ischemic penumbra. This enhanced penetration translates directly into robust structural tissue preservation. Our histological and macro-morphological evaluations—evidenced by H and E, Nissl, and TTC staining—demonstrate that the dual-targeted strategy significantly minimizes the infarct volume and preserves the integrity of highly vulnerable neurons. Rescuing these neurons within the critical time window prevents the irreversible expansion of the ischemic core.⁴⁷ Consistent with this structural preservation, the reduction in cerebral edema and the corresponding recovery in neurobehavioral scores confirm that maximizing drug delivery to the penumbra effectively halts the progression of stroke-induced brain injury.⁴⁸

Beyond immediate structural preservation, the accumulation of TPLN fundamentally regulates the post-ischemic microenvironment. Ischemic injury typically drives microglia toward a pro-inflammatory M1 phenotype, which secretes neurotoxic cytokines and exacerbates secondary tissue damage.⁴⁹ The efficient clearance of excess ROS by TPLN eliminates the primary biochemical trigger sustaining this pro-inflammatory state. Consequently, as evidenced by our *in vivo* immunofluorescence and ELISA data, the targeted delivery of TPLN induces a microglial phenotypic transition from the M1 state to the neuroreparative M2 state. This repolarization is accompanied by a significant downregulation of pro-inflammatory mediators, including TNF- α , IL-1 β , and IL-6. By disrupting the feed-forward cycle between oxidative stress and neuroinflammation, this immunomodulatory mechanism fosters a permissive environment for long-term tissue repair and functional recovery.³⁹

Despite the promising neuroprotective outcomes, several limitations of this study warrant further investigation. First, the therapeutic evaluations were primarily confined to the acute phase of ischemic stroke. The effects of the LIPUS-MBs and TPLN combinational therapy on long-term neuroplasticity, angiogenesis, and cognitive recovery require extended observation.⁵⁰ Second, while the immunomodulatory and anti-apoptotic effects of TPLN were confirmed, its specific intracellular pathways and downstream molecular targets remain to be fully elucidated. Future transcriptomic or proteomic analyses are required to explore these underlying mechanisms. Third, the current experiments were conducted exclusively in a rodent MCAO model; validation in large animal models is imperative for clinical translation. Finally, the acoustic parameters of LIPUS and the dosing regimen of the nanomicelles require optimization to accommodate varying stroke severities. Addressing these variables will facilitate the development of personalized intervention protocols.

Conclusion

This study presents a dual-targeted delivery platform combining LIPUS-MBs and ROS-responsive scavenging TPLN nanomicelles for the treatment of ischemic stroke. LIPUS-mediated physical permeabilization of the blood-brain barrier significantly enhances the focal accumulation of TPLN within the ischemic penumbra. By actively neutralizing intracellular ROS, the targeted nanomicelles disrupt the pathological oxidative stress-neuroinflammation cycle and drive a neuroreparative M2 microglial transition. This synergistic intervention successfully preserves structural integrity

and facilitates neurological functional recovery in MCAO models. Ultimately, this physical-chemical strategy provides a robust and translatable therapeutic framework for the precision management of ischemic stroke and related neuroinflammatory disorders.

Abbreviations

IS, ischemic stroke; ROS, reactive oxygen species; BBB, blood-brain barrier; LIPUS, low-intensity pulsed ultrasound; MBs, microbubbles; LIPUS-MBs, low-intensity pulsed ultrasound with microbubbles; MCAO, middle cerebral artery occlusion; MCAO/R, middle cerebral artery occlusion/reperfusion; OGD, oxygen-glucose deprivation; EB, Evans blue; H&E, Hematoxylin and Eosin; TTC, 2,3,5-triphenyltetrazolium chloride; MDA, malondialdehyde; SOD, superoxide dismutase; mNSS, modified Neurological Severity Score; DLS, dynamic light scattering; TEM, transmission electron microscope; CLSM, confocal laser scanning microscopy; ELISA, enzyme-linked immunosorbent assay; BWC, brain water content; I/R, ischemia/reperfusion; MFI, mean fluorescence intensity; MWCO, molecular weight cut-off; PDI, polydispersity index.

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Disclosure

The authors report no conflicts of interest in this work.

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