Supplementary file

Penumbra-Targeted CircOGDH siRNA-Loaded Nanoparticles Alleviate Neuronal Apoptosis in Focal Brain Ischemia

Materials and methods

Materials

PLGA-COOH (LA: GA = 50: 50, Mn =13000) was assembled, Polyamide amine (PAMAM) generation five and Tween 80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). CY5.5-NHS was purchased from RuixiBio (Xi-an, China, catalog #: R-FR-005). The water used in all experiments was distilled water (DW). The primer sequences of CircOGDH and β-actin were as followed: CircOGDH Forward: AACTCGTGGAGGACCACTTG, Reverse: GAGCTTCGACTCAGGGAAAG, β-actin Forward: ACGGCCAGGTCATCACTATTG, Reverse: CAAGAAGGAAGGCTGGAAAAGA.

Assembly of PLGA–PAMAM@ CircOGDH siRNA nanoparticles

PLGA–PAMAM was assembled as following: 21mg of PLGA-COOH were dissolved in 3 mL of acetone, the mixed solution then was stirred at room temperature until PLGA-COOH completely dissolved. 45mg of Tween 80 were dissolved in 5 mL of DW, then the dissolved PLGA-COOH mixed solution was added dropwise under stirring overnight at room temperature. PAMAM solution (generation 5, Sigma-Aldrich, St. Louis, USA, catalog #:163442-68-0) was added to the PLGA-COOH reaction mixed solution under stirring for 12 hours at room temperature. The final product PLGA–PAMAM nanoparticles were isolated by ultrafiltration using 100k MWCO centrifugal filter (Millipore, USA, catalog #: UFC901096).

CircOGDH siRNA or CY3 labeled CircOGDH siRNA (Gene Pharma, Shanghai, China) was dissolved in 500 μL of DW, then CircOGDH siRNA solution was added dropwise into PLGA–PAMAM mixed solution under stirring overnight at 4℃. The sequences of CircOGDH siRNA were as followed: 5' to 3' CACAGACAAACUUGUCAUGTT.

Characterization of PLGA–PAMAM@ CircOGDH siRNA nanoparticles

The zeta potential, size distribution and morphology of PLGA–PAMAM, PLGA–PAMAM@ CircOGDH siRNA were characterized by Zetasizer Nano ZS particle analyzer (Malvern, England) and transmission electron microscope (TEM, Hitachi H-7650, 100 kV). TEM samples were prepared by dispersing nanoparticles on copper grids. Elemental mapping of PLGA–PAMAM@ CircOGDH siRNA nanoparticles were obtained using high-resolution transmission electron microscopy (HR-TEM, JEM 2100F). The spectroscopic analysis of PLGA–PAMAM@ cy3-CircOGDH siRNA nanoparticles used UV spectrophotometry (UH 4150 Spectrophotometer, Hitachi).

Determination of siRNA complexation by gel electrophoresis assay

The encapsulation degree between CircOGDH siRNA and PLGA–PAMAM nanoparticles was evaluated by agarose gel electrophoresis assays using 1.5% agarose gel electrophoresis at 120 V for 20 min. Images was obtained using an ImageQuant LAS 500 Gel analyzer (USA).

Quick-Load® 1000 bp DNA Ladder (New England BioLabs, USA, catalog #: NO467S) was used as a DNA marker.

Animals

A total of 176 adult male BALB/c mice (22.0-25.5 g, 5 to 7 weeks) were purchased from the Institute of Laboratory Animal Science of the Chinese Academy of Medical Sciences (Guangzhou, China). Mice were housed in a strict constant temperature and humidity. Food and water were available all day and night. The study was carried out in accordance with the recommendations of the NIH Guide (NIH Publications No. 8023, revised 1978) for the Care and Use of Laboratory Animals. All experiments were carefully conducted in accordance with the guidelines for Animal Experimentation of Jinan University. The protocol was approved by the Ethics Committee of the Institute of Laboratory Animal Science of Jinan University.

Middle cerebral artery occlusion reperfusion (MCAO/R) and cerebral blood flow (CBF) measurement

Adult BALB/c mice were anesthetized with isoflurane (4% for initiating anesthesia in a chamber and 1.5% for maintaining anesthesia afterward; RWD Life Science, Shenzhen, China, catalog #: R510-22-16). A midline incision was made at the neck region and the left carotid artery, external carotid artery and internal carotid artery were isolated. The focal ischemia was induced as our described previously[1] using a filament made of nylon string coated with silicon (MSMC23B104PK100, RWD Life Science, Shenzhen, China) which was inserted into the middle cerebral artery (MCA) for 40 minutes, then the silicone tip was removed for reperfusion. Cerebral blood flow was monitored using a Laser Speckle Contrast Imaging (PeriCam PSI System, Perimed AB, Stockholm, Sweden) according to the manufacturer's instructions to confirm successful MCAO and MCAO/R. Mice were immediately put into a 37 ℃ chamber for 15 minutes and then back to a normal cages.

***In vivo* cellular uptake of PLGA–PAMAM@ CircOGDH siRNA nanoparticles**

Three days after tail intravenously injection of CY3 labeled PLGA–PAMAM@ CircOGDH siRNA nanoparticles in MCAO-reperfusion mice, mice were fixed by heart perfusion with cool physiological saline solution, followed by 30 ml of 4% paraformaldehyde (Biosharp, Hefei, China, catalog #BL539A). Then brains were collected for preparing tissue sections. Before being embedded with in optimal cutting temperature (OCT) compound, brain tissues were orderly immersed in 20%, 30% sucrose-distilled water overnight at 4 °C. Finally, brain sections were cut into 10 μm slices using a cryostat (Thermo Fisher Scientific, Waltham, MA, USA). The brain sections were incubated with the following primary antibody: Fluorescent Nissl staining (1:200; Thermo Fisher Scientific, Waltham, MA, USA, catalog #: N21483), anti-glial fibrillary acidic protein antibody (anti-GFAP; 1:100; Cell Signaling Technology, Danvers, MA, USA, catalog #: 3670S), anti-ionized calcium-binding adaptor molecule 1 antibody (Iba-1; 1:100; Abcam, catalog #: ab5076) overnight at 4 ℃, followed by incubation with a mixture of fluorescent secondary antibodies for 1 h at room temperature. Then brain sections were stained with 3,3-diaminobenzidin for 5 minutes at room temperature. Images were captured using a confocal microscope (Carl Zeiss LSM700, Vizna, Germany).

***In Vivo* fluorescence imaging of PLGA–PAMAM in MCAO/R Mice**

CY5.5-NHS (Ruixi, Xian, China, catalog #R-FR-005) labeled PLGA–PAMAM nanoparticles were assembled as following: CY5.5-NHS was dissolved in DMSO, 8μL of 10 mg/mL CY5.5-NHS solution was added to a total of 1mL PLGA–PAMAM solution. Finally, it was stirred for 12h at room temperature. The CY5.5-labeled PLGA–PAMAM nanoparticles were collected by centrifugation. 100 μL CY5.5-labeled PLGA–PAMAM nanoparticles were tail intravenously injected into MCAO/R mice. Fluorescence was monitored using the In Vivo animal imaging system (NightOWL II LB 983) at 0.5, 1.5, 2.5 h and day 3.

Mice behavioral tests

Mice were coded and were randomly divided into three groups: SHAM, MCAO/R + PLGA–PAMAM, MCAO/R + PLGA–PAMAM@ CircOGDH siRNA. Mice behavioral tests were performed by an independent investigator who was blind to the experimental groups and the data was analyzed by separate investigator.

For the grid-walking task, an elevated grid area of 32 cm × 20 cm × 50 cm (length × width × height) made of 12 mm square wire mesh was used. Mice were placed individually on the wire grid and allowed to freely move for 3 minutes. A camera was positioned beneath the grid to record stepping errors (foot faults). The numbers of foot faults and non-faults for each limb were counted. A ratio was calculated as follows: number of foot faults / (number of foot faults + number of non-faults) × 100%.

For the cylinder test, mice were placed inside a plastic cylinder (15 cm tall with a diameter of 10 cm) and videotaped for 5 minutes. The score was calculated as the ratio: (number of left hand − number of right hand) / (number of right hand + number of left hand + number of both hands).

For the adhesive removal somatosensory test, 2 small pieces of adhesive-backed paper dots of equal size (25 mm^2) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. The time for mice to remove each stimulus from the forelimb was recorded and the time exceeded 120 s were recorded as 120 s. Before surgery, animals were trained for 3 days. Once mice were able to remove the dots within 10 s, they were subjected to ischemic stroke.

MRI for mice

MRI for mice was conducted using a 9.4 tesla small animal MRI scanner (Bruker PharmaScan). Mice were anesthetized using 2% isoflurane through a nose cone, and the body temperature and respiratory rate were monitored. T2 MRI imaging was conducted at day3 after MCAO/R using a 2D fast-spin echo sequence. (T2 MRI: 2D fast-spin echo sequence (3500/33 ms of repetition time/echo time, 2 average). 17 axial slices with a slice thickness of 0.7 mm, a matrix of 256 ×256, and an FOV of 20 × 20 mm). It was positioned over the brain, excluding the olfactory bulb. Under the same scale and brain slices of MCAO mouse images, T2 MRI imaging was scanned and quantified using RadiAnt DICOM Viewer software (https://radiantviewer.com/trial).

Nissl staining

As described above[1], three days after tail intravenously injection of nanoparticles in

MCAO-reperfusion mice, mice were fixed by heart perfusion and finally, brain sections were cut into 10 μm slices using a cryostat (Thermo Fisher Scientific, Waltham, MA, USA). Nissl staining experiment was performed using the Nissl staining assay kit (Beyotime Biotechnology, Shanghai, China, catalog # C0117) following the manufacturer's instructions37 or Fluorescent Nissl dye (1:200; Thermo Fisher Scientific, Waltham, MA, USA, catalog # N21483). Brain slices were sealed with neutral gum and images were captured using light microscope (Leica DMILLED/ICC50HD, Solms, Germany). Quantification was performed using image J software (Bethesda, MD, USA). Researchers were blinded to the experimental conditions for data analysis.

Tunel staining

As described above[1], three days after tail intravenously injection of nanoparticles in MCAO-reperfusion mice, mice were fixed by heart perfusion and brain sections were cut into 10 μm slices using a cryostat (Thermo Fisher Scientific, Waltham, MA, USA). A one-step TUNEL apoptosis assay kit (Beyotime, Beijing, China, CATALOG#: C1089) was used to detect apoptosis according to the manufacturer's instructions. Brain slices were washed in PBS and subsequently incubated with 0.1% Triton X-100 in PBS for 2 min at room temperature. After washed in PBS for three times, brain slices were then incubated in TUNEL solution in the dark for 1 h at room temperature. Finally, sections were stained with Fluorescent Nissl dye (1:200; Thermo Fisher Scientific, Waltham, MA, USA, catalog # N21483) and DAPI (Beyotime, Beijing, China, CATALOG#: C1005). Images were captured using light microscope (Leica DMILLED/ICC50HD, Solms, Germany). Quantification was performed using image J software (Bethesda, MD, USA).

RNA extraction and RT-qPCR

Brain tissue was collected into a tube on the ice for RNA extraction using Trizol Reagent according to the manufacturer's instructions. For RT-qPCR, RNA was performed reverse-transcription with corresponding primers for β-actin (Forward: ACGGCCAGGTCATCACTATTG, Reverse: CAAGAAGGAAGGCTGGAAAAGA), CircOGDH (Forward: AACTCGTGGAGGACCACTTG, Reverse: GAGCTTCGACTCAGGGAAAG) (Gene Pharma, Shanghai, China) using the Prime Script RT Master Mix (Takara, Japan, catalog #:RR047A) following the manufacturer's protocol. Real-time PCR was conducted using LightCycler® 480 SYBR Green I Master (Roche, United States, catalog #:04887352001) following the manufacturer's instructions. Thermocycle conditions used in amplification: Pre incubation at 95 ℃ for 10 min, amplification using 40 cycles of 95 ℃ for 10 sec, 55-60℃ for 20sec, and 72 ℃ for 30 sec, followed by 75 ℃ to 94 ℃ with increment of 0.5 for 5 sec, finally at 40℃ for 10 sec. The comparative CT method referred to as the $2^{-\Delta\Delta CT}$ method[2], a widely used method. Relative gene expression in each group were normalized by internal control and then compared with that in corresponding control.

Primary neuron culture

Primary cortical neurons were obtained from the cerebral cortex of BALB/c mouse embryos (E18-E19) purchased from the Institute of Laboratory Animal Science of the Chinese Academy of Medical Sciences (Guangzhou, China). As described above[1], the cerebral cortex was isolated and gently pipetted and then the cell suspension was collected in a new centrifuge tube. Cells were digested in 0.125% trypsin for 15 minutes at 37 ℃ and after filtered with a 70 μm cell strainers

(Corning, New York, USA, catalog #:352350). Filtrates were collected and then centrifuged at 1000 rpm for 5 minutes. Cells were resuspended in DMEM/F-12 containing 10% FBS (Gibco, United States, catalog #: C11330500) and 1% penicillin-streptomycin (Biological Industries, Kibbutz Beit-Haemek, Israel, catalog #: 03-031-1B), and then seeded on 6-well plates pre-coated with poly-L-lysine (Sigma-Aldrich, St. Louis, USA, catalog #: P1274). Cells were cultured for 4 hours in a humidified incubator (37 °C, 5% CO2), and then medium was changed with complete medium which contained neurobasal medium (Gibco, Waltham, MA, USA) supplemented with B-27™ Supplement (Gibco, Waltham, MA, USA, catalog #: 17504-044) and 1% penicillin-streptomycin liquid. Medium was changed by half every three days. Neurons cultured after day 5 were used for experiments.

For ischemic treatments, neurons were mainly divided into two groups: (1) control (CON)：neurons were incubated in neuronal complete medium in a regular humidified incubator (37°C, 5% CO_2). (2) OGD/R：neurons were exposed to DMEM solution without glucose in an incubator containing 0 % O_2, 5 % CO_2 with balanced N_2 for 3 hours, followed by reperfusion for 24 hours. SH-SY5Y cells were obtained from American Type Culture Collection and cultured in DMEM containing 10% FBS in a humidified incubator (37 °C, 5% CO_2).

***In vitro* neuron uptake of PLGA–PAMAM@ CircOGDH siRNA nanoparticles**

Cy3 labeled PLGA–PAMAM@ CircOGDH siRNA nanoparticles were added into primary cortical neurons at day 5 for 24h, primary cortical neurons were cultured in confocal petri dishes, rinsed with PBS and fixed with 4% PFA for 15 min at room temperature. Cells were then washed in PBS twice and permeabilized with PBS containing 0.3% Triton X-100 for 20 min and blocked with 5% bovine serum albumin (BSA) for 60 min at room temperature. Cells were stained with fluorescent Nissl dye (1:200; Thermo Fisher Scientific, Waltham, MA, USA, catalog #: N21480) for 20 minutes at room temperature, followed by 3,3-diaminobenzidin for 5 minutes. After PBS washing for three times, cells were mounted and images were captured using a confocal microscope (Carl Zeiss LSM700, Vizna, Germany).

CCK8 assay

Cell viability was assessed by the Cell Counting Kit 8 (CCK8, Beyotime Biotechnology, Shanghai, China, catalog #: C0039) according to manufacturer's instruction. Neurons were seeded at 2×10^4 cells per well on 96-well plates and the OD450 was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Data were normalized and calculated by the corresponding control group.

Cellular fluorescence localization of PLGA–PAMAM@ CircOGDH siRNA nanoparticles

Intracellular localization of cy3 labeled PLGA–PAMAM@ CircOGDH siRNA was detected by fluorescence microscopy. Primary cortical neurons were cultured in confocal petri dishes for 5-6 days, and then incubated with 2.5μL 100 μg/mL of cy3 labeled PLGA–PAMAM@ CircOGDH siRNA for various times including 3h, 6h, 9h, 12h and 24h. Cells were rinsed with PBS twice and incubated with lysotracker (Life technologies, Waltham, MA, USA, catalog #L12492) for 1h in a humidified incubator (37 °C, 5% CO2), then fixed with 4% PFA for 15 min at room temperature. Cells were stained with fluorescent Nissl dye (1:200; Thermo Fisher Scientific, Waltham, MA, USA, catalog #: N21480) for 20 minutes at room temperature, followed by 3,3-diaminobenzidin

for 5 minutes. After PBS washing for three times, cells were mounted and images were captured using a confocal microscope (Carl Zeiss LSM700, Vizna, Germany).

Flow cytometry in SH-SY5Y cells

SH-SY5Y cells were seeded at 18×10^4 cells per well on 6-well plate and PLGA–PAMAM@cy3-CircOGDH siRNA, cy3-CircOGDH siRNA were added for 48 hours. After washed with cold PBS three times, SH-SY5Y cells were collected in 300 ul of PBS and used for flow cytometry (Canto, BD, USA).

Western blot analysis

Protein extracts obtained from neurons were subjected to SDS polyacrylamide gels (12%) electrophoresis and electrically transferred to a polyvinylidene difluoride membrane before incubated with specific antibodies. Primary antibodies against the following proteins were used: COL4A4 (1:1000; Proteintech), BCL-2 (catalog #: 15071S), β-actin (catalog #: 4970S), BAX (catalog #: 5023S), cleaved caspase 3 (catalog #: 9664S) (1:1000; Cell Signaling Technology). After incubation with primary antibodies overnight at 4 ℃, membranes were incubated for 1 hour with the appropriate secondary antibody (anti- Rabbit IgG H&L (HRP), catalog #: ab97051). The antibodies were visualized by enhanced chemiluminescence (ECL Plus; Wanleibio, Shenyang, China, catalog #: WLA006c). Image J software was used to quantify the band intensity, each value was normalized by β-actin. Then the ratio of COL4A4, BCL2/BAX and cleaved caspase3 in each group were compared with that in control group.

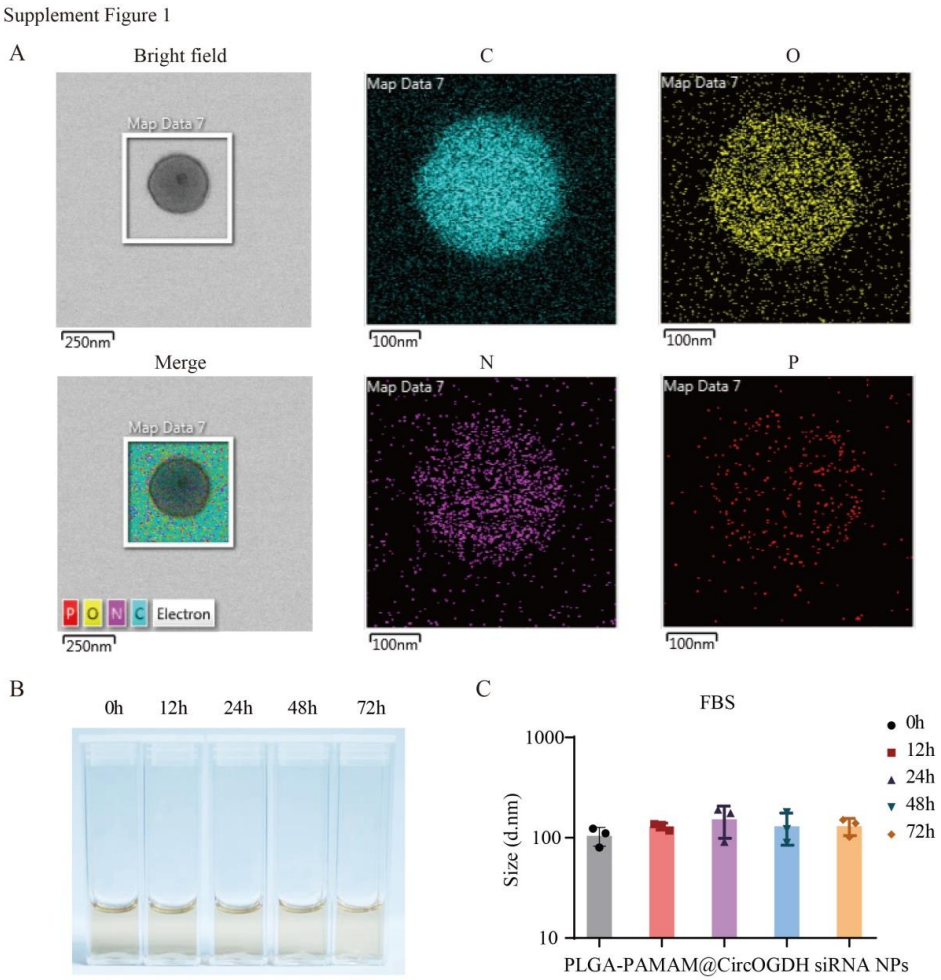
Toxicity tests of PLGA–PAMAM@ CircOGDH siRNA nanoparticles *in Vivo*

The MCAO-reperfusion mice were treated with 100μL 100 μg/mL PLGA–PAMAM@ CircOGDH siRNA nanoparticles. After treatment for 3 days, whole blood was collected and centrifuged to obtain serum for detection of RBC, WBC, PLT numbers. Hematology analysis was used to evaluate the toxicity of PLGA–PAMAM and PLGA–PAMAM@ CircOGDH siRNA nanoparticles in vivo, including effects on alanine aminotransferase (ALT), aspartate transaminase (AST) and creatinine (CREA), correspondingly, liver and kidney tissues were collected for H&E staining and pathological analysis.

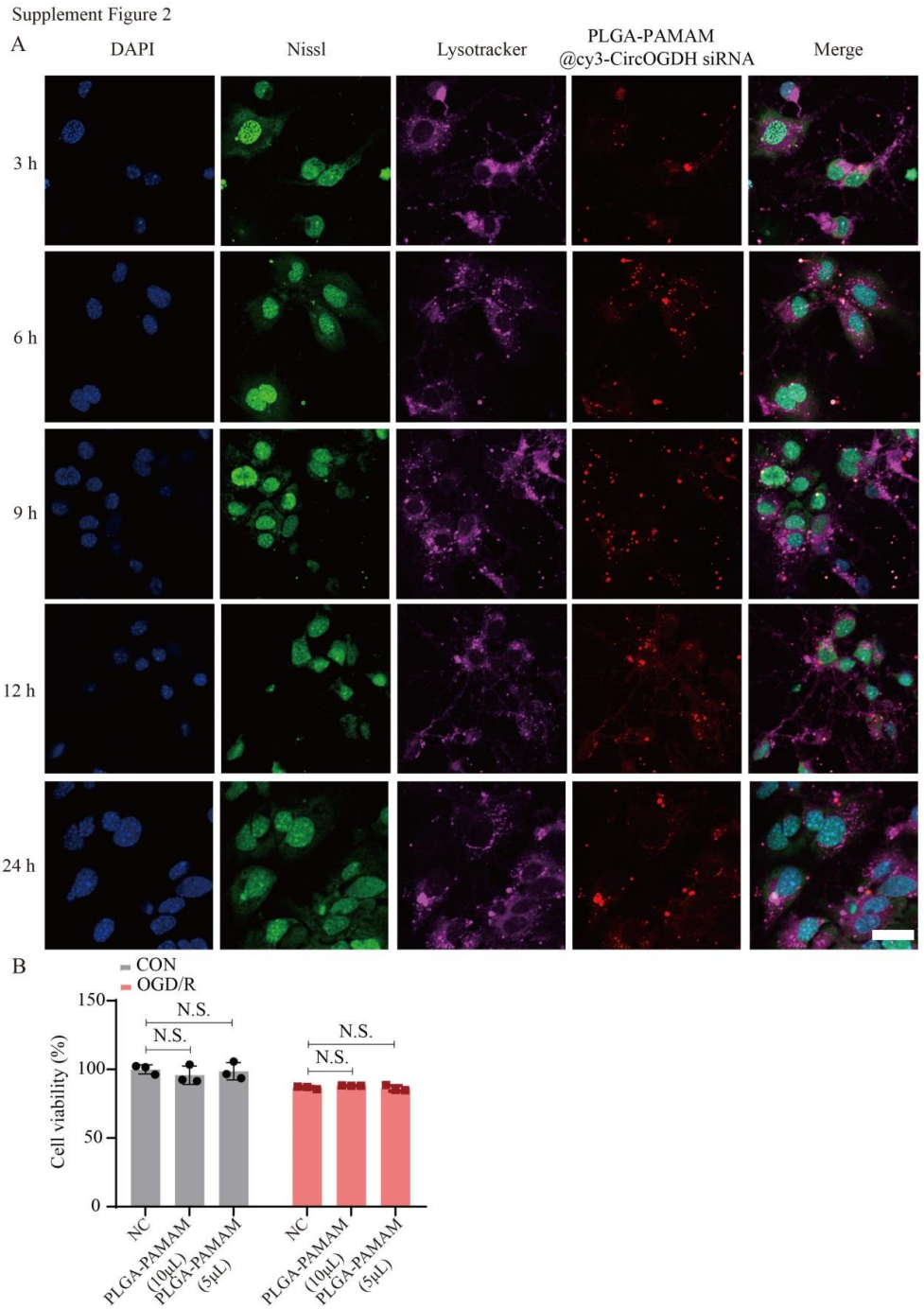
Statistics

All statistical analyses were performed using SPSS (Windows version 27.0; SPSS Inc., Chicago, IL, USA) or GraphPad Prism 8.01 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as mean ±SD. For experiments with small sample size ($n<6$), power calculations were not performed and p-values were determined by non-parametric analysis (Mann-Whitney test). Otherwise, Shapiro-Wilk test was used for normality test with a threshold of 0.05, for data with normal distribution, Student's t-tests (two-tailed) or one-way ANOVA were determined, and for data without normal distribution, Mann-Whitney test was used. P-values of 0.05 or less were considered statistically significant. All representative images were selected without bias, and had characteristics typical of the data or overall trend.

Supplementary Figure



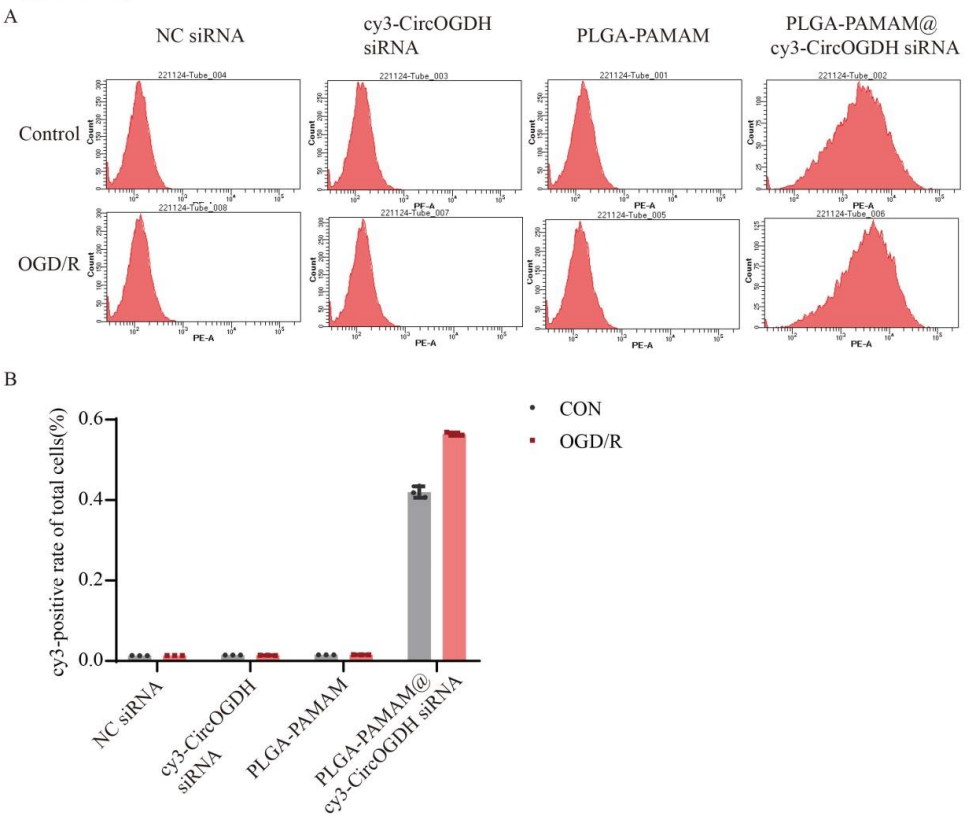
Supplementary Figure 1. Characterization of PLGA–PAMAM@CircOGDH siRNA nanoparticles. (A). Elemental mapping of PLGA–PAMAM@ CircOGDH siRNA nanoparticles. Scale bar = 100 nm. **(B-C).** Stability of PLGA–PAMAM@ CircOGDH siRNA nanoparticles in FBS solution at different time points. Data are presented as means ± SD; n = 3.



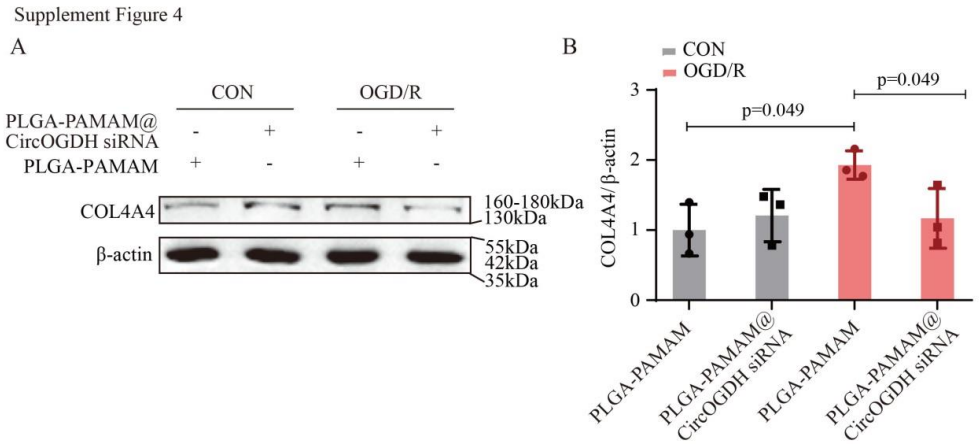
Supplementary Figure 2. Cellular uptake of PLGA–PAMAM@CircOGDH siRNA nanoparticles in primary cortical neurons. **(A)** Intracellular trafficking of PLGA–PAMAM@Cy3-CircOGDH siRNA nanoparticles (red) in primary neurons at 3 h, 6 h, 9 h, 12 h, and 24 h. Neurons were stained with Nissl (green). Lysotracker staining is shown in cherry. Nuclei were stained with DAPI (blue). Scale bar = 50 μm. **(B)** Cell viability was determined in primary cortical neurons treated with normal control (NC) and PLGA–PAMAM nanoparticles. Data were presented as mean± SD;

n=3, Mann–Whitney U test.

Supplement Figure 3

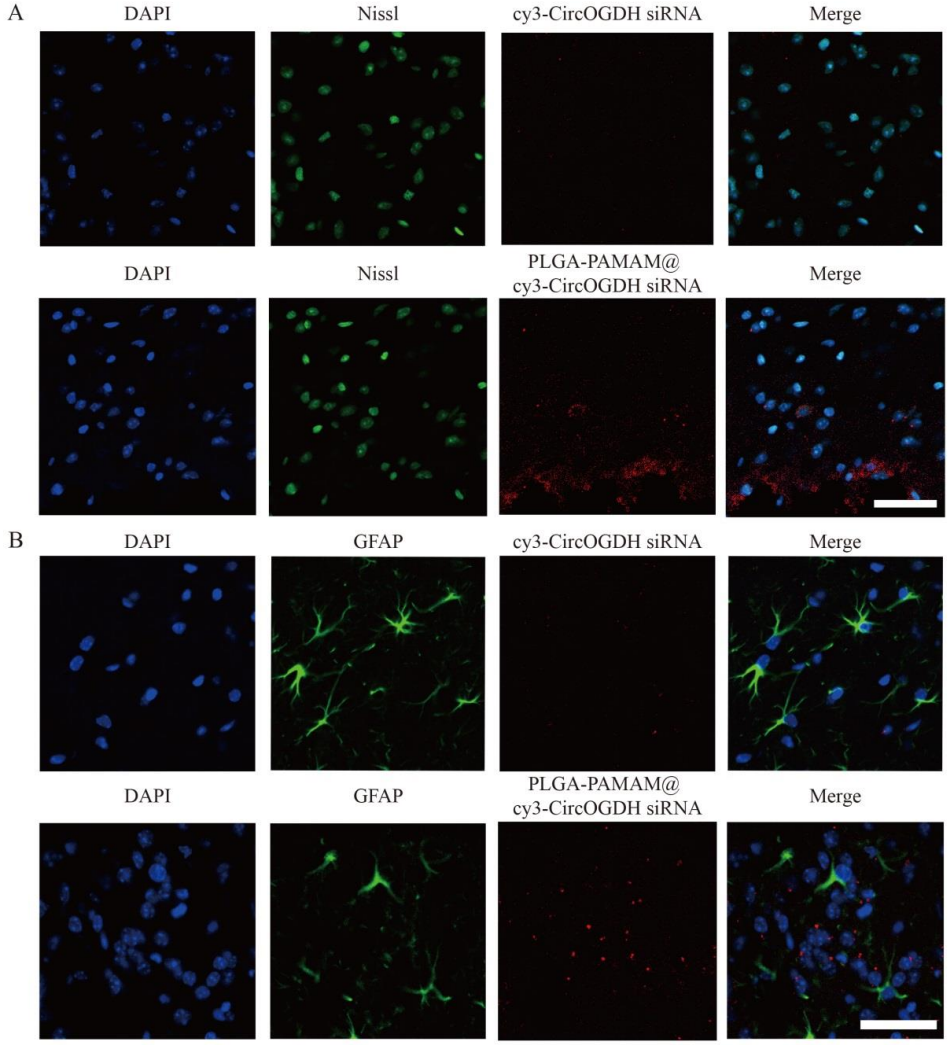


Supplementary Figure 3. Cellular uptake of PLGA–PAMAM@CircOGDH siRNA nanoparticles in SH-SY5Y cells. (A-B). Detection of the cy3-positive cells percentage in four groups by flow cytometry analysis. Data were presented as mean± SD; n=3.

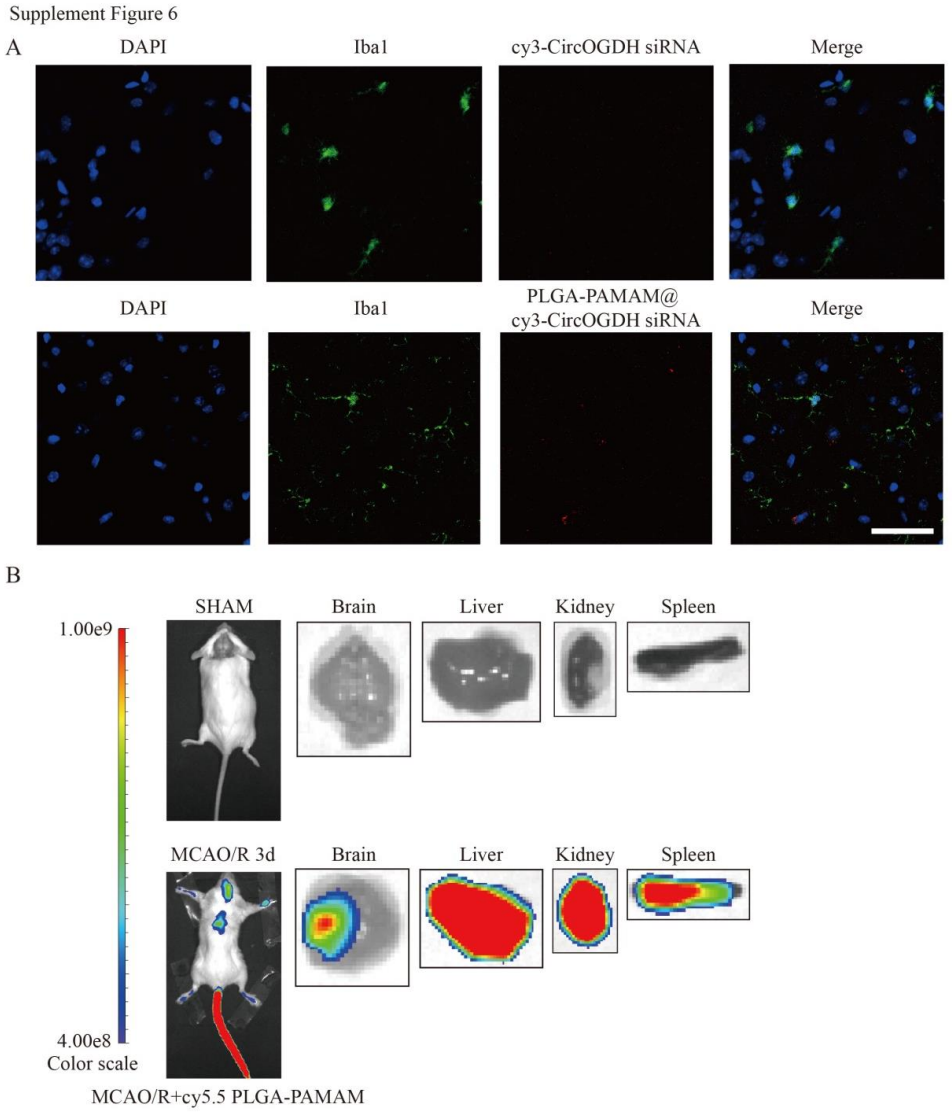


Supplementary Figure 4. PLGA–PAMAM@CircOGDH siRNA nanoparticles downregulated COL4A4 protein expression level. (A-B). Western blot analysis of COL4A4 expression level in control (CON) and ischemic-reperfusion (OGD/R) neurons treated with PLGA–PAMAM and PLGA–PAMAM@CircOGDH siRNA nanoparticles. Data were presented as mean± SD; n=3, Mann–Whitney U test.

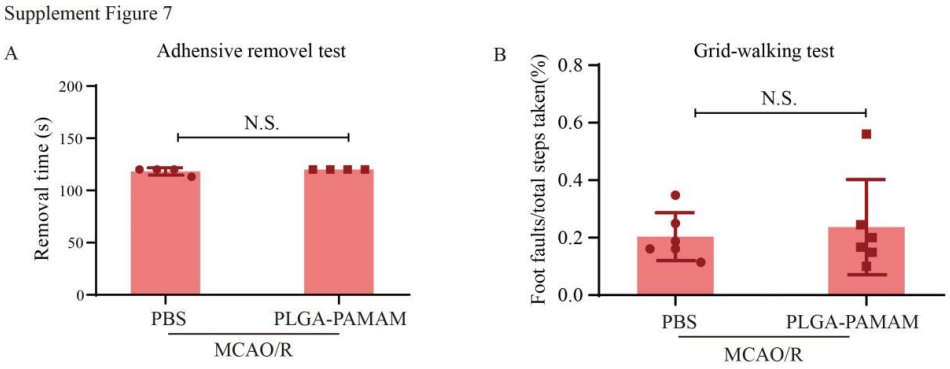
Supplement Figure 5



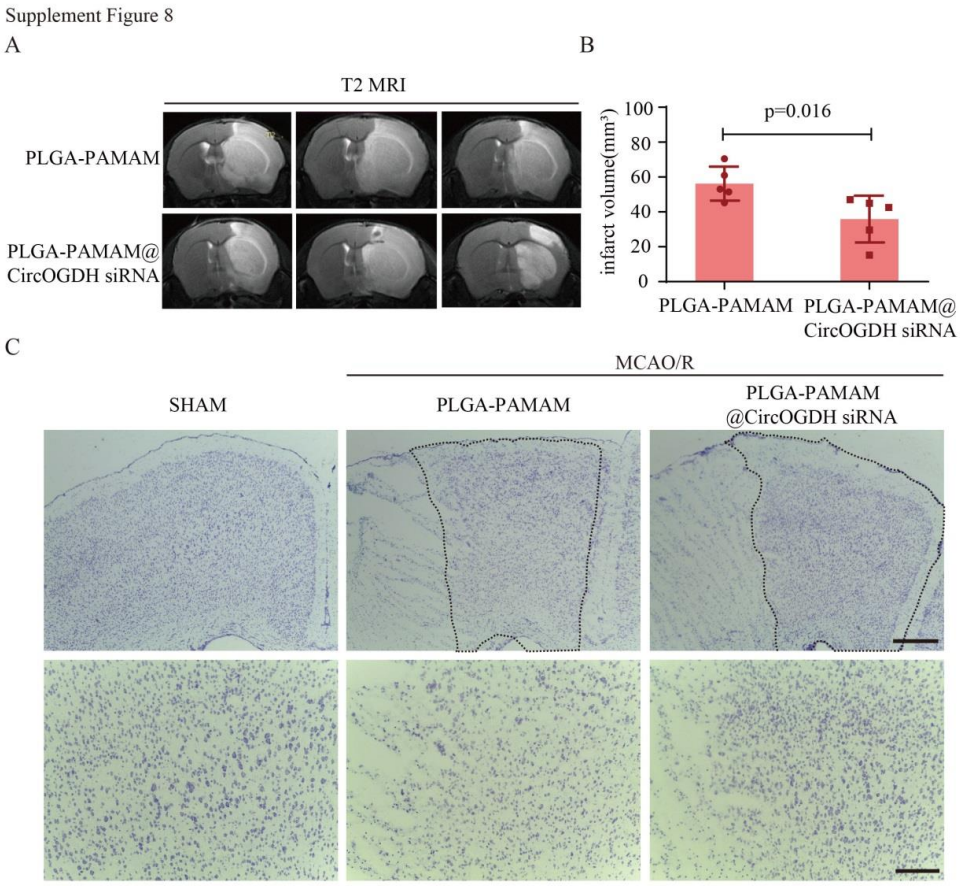
Supplementary Figure 5. Uptake of PLGA–PAMAM@CircOGDH siRNA nanoparticles in penumbra tissue cells of mice brain. (A). Immunofluorescence experiments showed the localization of PLGA–PAMAM@cy3-CircOGDH siRNA nanoparticles (red) with Nissl staining (green) in the penumbra tissue of mice brain. Nuclei were stained with DAPI. Scale bar, 50 μm. **(B).** Immunofluorescence experiments showed the localization of PLGA–PAMAM@cy3-CircOGDH siRNA nanoparticles (red) with GFAP staining (green) in the penumbra tissue of mice brain. Nuclei were stained with DAPI. Scale bar, 50 μm.



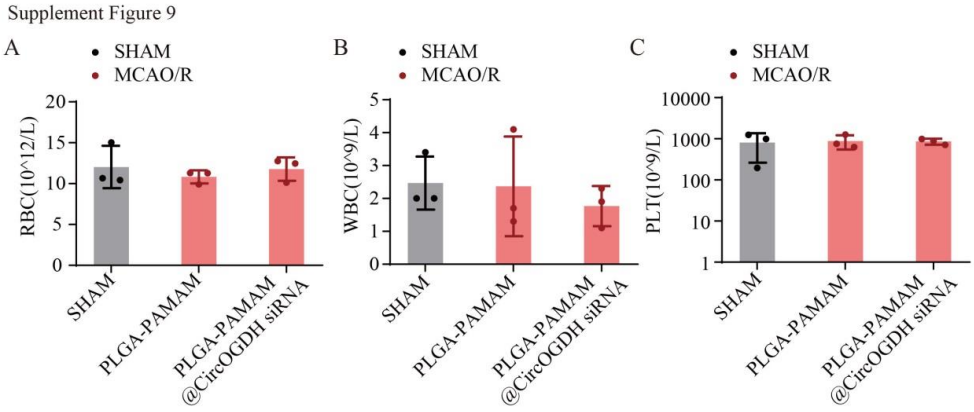
Supplementary Figure 6. Uptake of PLGA–PAMAM@CircOGDH siRNA nanoparticles in mice. (A). Immunofluorescence experiments showed the localization of PLGA–PAMAM@cy3-CircOGDH siRNA nanoparticles (red) with Iba1 staining (green) in the penumbra tissue of mice brain. Nuclei were stained with DAPI. Scale bar, 50 μm. **(B).** *In vivo* fluorescence Imaging of Cy5.5-labelled PLGA–PAMAM nanoparticles in MCAO/R mice at day 3. Units of the color scale: perfusion units (PUs).



Supplementary Figure 7. PLGA–PAMAM nanoparticles showed no effect in MCAO/R mice. (A–B). PLGA–PAMAM nanoparticles showed no effect in MCAO/R mice 3 days after tail injection, as demonstrated by the adhesive removal test (A), grid-walking test (B). Data are presented as means ± SD. Adhesive removal test: n = 3-4 in each group; grid-walking test: n = 4 in the SHAM group, n = 6 in the MCAO/R+PLGA–PAMAM group, Mann–Whitney U test.



Supplementary Figure 8. (**A–B)** Representative images showing T2 MRI in MCAO/R mice after microinjected with PLGA–PAMAM and PLGA–PAMAM@ CircOGDH siRNA nanoparticles for three days. Data were presented as mean±SD; n=5 for each group, Mann–Whitney U test. (**C)** PLGA–PAMAM@CircOGDH siRNA increased intact neuron number in MCAO/R mice. Nissl staining showing the number of neurons in the SHAM, MCAO+PLGA–PAMAM, and MCAO+PLGA–PAMAM@CircOGDH siRNA groups 3 days after tail injection. Scale bar = 500 μm (upper); scale bar = 200 μm (lower).



Supplementary Figure 9. PLGA–PAMAM@CircOGDH siRNA increased intact neuron number in MCAO/R mice. **(A–C).** Hematological analyses of RBC numbers **(A)**, WBC numbers **(B)**, and PLT numbers **(C)** were performed in the SHAM, MCAO+PLGA–PAMAM, and MCAO+PLGA–PAMAM@CircOGDH siRNA groups 3 days after tail injection. Data were presented as mean± SD; n=3, Mann–Whitney U test.

330 **References**

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