Penumbra-targeted CircOGDH siRNA-loaded nanoparticles alleviate neuronal apoptosis in focal brain ischaemia

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ABSTRACT

Background Nanoparticles (NPs) are a class of substances that can be loaded with therapeutic agents delivered to specific areas. In our earlier research, we identified a neuron-derived circular RNA (circRNA), circular oxoglutarate dehydrogenase (CircOGDH), as a promising therapeutic target for acute ischaemic stroke. This study dedicated to explore a prospective preliminary strategy of CircOGDH-based NP delivered to the ischaemic penumbra region in middle cerebral artery occlusion/reperfusion (MCAO/R) mice.

Methods Immunofluorescence in primary cortex neurons and in vivo fluorescence imaging revealed endocytosis of Poly(lactide-co-glycolide) (PLGA) poly amidoamine(PAMAM)@CircOGDH small interfering RNA (siRNA) NPs. Western blotting analysis and CCK8 assay were performed to evaluate the apoptotic level in ischaemic neurons treated with PLGA–PAMAM@CircOGDH siRNA NPs. Quantitative reverse transcription PCR experiments, mice behaviour test, T2 MRI analysis, Nissl and TdT-mediated dUTP nick end labeling (TUNEL) staining were performed to evaluate the apoptosis level of ischaemic penumbra neurons in MCAO/R mice. Biosafety evaluation of NPs in MCAO/R mice was detected by blood routine examination, liver and kidney function examination and HE staining.

Results PLGA–PAMAM@CircOGDH siRNA NPs were successfully assembled. Endocytosis of PLGA–PAMAM@CircOGDH siRNA NPs in ischaemic neurons alleviated neuronal apoptotic level in vitro and in vivo. Furthermore, mice behaviour test showed that the neurological defects of MCAO/R mice were significantly alleviated after the tail injection of PLGA–PAMAM@CircOGDH siRNA NPs, and no toxic effects were observed.

Conclusion In conclusion, our results suggest that PLGA–PAMAM@CircOGDH siRNA NPs can be delivered to the ischaemic penumbra region and alleviate neuron apoptosis in MCAO/R mice and in ischaemic neurons; therefore, our study provides a desirable approach for using circRNA-based NPs for the treatment of ischaemic stroke.

INTRODUCTION

Ischaemic stroke is a leading cause of disability and mortality worldwide and China faces a great challenge. For optimal treatment results after acute ischaemic stroke (AIS), the ischaemic penumbra tissue must be rescued in a stringent time window. Although the current guidelines for the clinical treatment of AIS recommends recombinant tissue plasminogen activator and endovascular thrombectomy (EVT) to improve functional recovery, the limited therapeutic time window and the serviceable remedy remain a question. Moreover, during the ischaemic reperfusion process in EVT, complicated pathophysiological responses can lead to neural loss and blood–brain barrier breakdown. Thus, initiatives in new neuroprotective drugs delivered to the brain still need to explore.

Data from the past decades have revealed that nanoparticles (NPs) can be effective vehicles to transport therapeutic agents, including drugs, proteins, vaccines, small
interfering RNA (siRNA) and DNA, to the brain. The rapid development of nanotechnology has yielded various types of NPs, such as polymer NPs, polymer micelles, liposomes and inorganic NPs. Poly(lactide-co-glycolide) (PLGA) is an FDA-approved polymer that has been widely used as a drug carrier because of its excellent biocompatibility, biosafety and biodegradability. PAMAM dendrimers are excellent candidate polymers because of their positive charge, which allows them to form highly stable complexes with negatively charged siRNA; thus, they are commonly used as gene delivery systems. But an emerging consensus is that beyond their gene delivery effects, PAMAM-type dendrimers (<10 nm) also exerts several toxicological actions such as haemolysis and cell death, and removes easily through the blood circulation. Thus PLGA–PAMAM NPs were assembled to minimise the usage amount of PAMAM in the core, and also decreased the strongly positive charge of PAMAM. Research has shown that PLGA biomimetic nanocarriers loaded with human fat extract can increase neurobehavioral recovery in ischaemic stroke, and PAMAM dendrimer NPs can be taken up by neurons after focal brain ischemia. Actually, there are series of NPs for RNA therapeutics delivery, NP-based targeting and delivery approaches are becoming promising strategy for effective treatments in ischaemic stroke.

Circular RNAs (circRNAs) are a type of non-coding RNAs that are found in various species and are more stable than linear RNA. They can interact with microRNAs and RNA-binding proteins that affect axon growth, neuronal apoptosis and autophagy—processes that are critical in the pathogenesis of ischaemic stroke. In our previous research, we observed that a circRNA derived from oxoglutarate dehydrogenase (OGDH), CircOGDH, was significantly upregulated in the plasma of AIS patients and penumbra neurons of middle cerebral artery occlusion (MCAO) mice. Furthermore, adenovirus-mediated CircOGDH knockdown ameliorated neuronal apoptosis in MCAO mice and ischaemic neurons. These results indicate that CircOGDH is a potential therapeutic target in patients with AIS. Moreover, studies have reported that gold NPs conjugated with CircDNMT1 siRNA are a potential treatment for breast cancer. Poly(β-amino ester) NPs loaded with CircMDK siRNA were reported as a therapeutic strategy to treat hepatocellular carcinoma. These studies indicate that NP delivery is a promising strategy for targeting circRNAs.

In this study, we assembled PLGA–PAMAM@CircOGDH siRNA NPs and demonstrated that PLGA–PAMAM-mediated in vivo delivery of CircOGDH siRNA improved penumbra neuron survival and neurological function in middle cerebral artery occlusion/reperfusion (MCAO/R) mice. Our study provides a promising nanotherapeutic strategy for circRNA-targeting agents during ischaemic reperfusion in ischaemic stroke.

**MATERIALS AND METHODS**

Detailed experimental procedures were provided in the online supplemental file.

**RESULTS**

**Design, assembly and characterisation of PLGA–PAMAM@CircOGDH siRNA NPs**

PLGA–PAMAM and PLGA–PAMAM@CircOGDH siRNA NPs were assembled as illustrated in figure 1A. The PAMAM dendrimers displayed a positive charge and formed high-stability complexes with the negatively charged siRNA via electrostatic interaction; therefore, a Zetasizer Nano ZS particle analyzer was used to examine the zeta potential of the PLGA, PAMAM, PLGA–PAMAM and PLGA–PAMAM@CircOGDH siRNA NPs. The zeta potential of the PLGA–PAMAM@CircOGDH siRNA NPs was significantly lower compared with that of PLGA–PAMAM NPs (figure 1B), indicating that PLGA–PAMAM@CircOGDH siRNA NPs were successfully assembled. The particle size distribution demonstrated a similar range of sizes for PLGA–PAMAM (165±2.8 nm) and PLGA–PAMAM@CircOGDH siRNA NPs (163±10.3 nm) (figure 1C). In addition, the morphology of PLGA–PAMAM and PLGA–PAMAM@CircOGDH siRNA NPs was characterised by transmission Electron Microscope (TEM). The TEM images (figure 1D) revealed that the two types of NPs had a different morphology, and element mapping showed that C, O, N and P are present (online supplemental figure 1E). Next, NPs were assembled using Cy3-labelled CircOGDH siRNA to form PLGA–PAMAM@Cy3-CircOGDH siRNA NPs. The extension time of PLGA–PAMAM@Cy3-CircOGDH siRNA NPs was completely inhibited, whereas that of CircOGDH siRNA alone was not. Furthermore, as NP stability is an important parameter for siRNA delivery, first, the size changes were detected in Fetal bovine serum (FBS) solution, which mimics a human physiological environment, and we found PLGA–PAMAM@CircOGDH siRNA NPs maintained at similar size (online supplemental figure 1B,C). Next, NPs were assembled using Cy3-labelled CircOGDH siRNA to form PLGA–PAMAM@Cy3-CircOGDH siRNA NPs. The extension time of PLGA–PAMAM@Cy3-CircOGDH siRNA NPs had no effect on the ultraviolet–visible (UV–Vis) (figure 1F) or fluorescence (figure 1G) spectra at different time points.

**PLGA–PAMAM@CircOGDH siRNA NPs ameliorates neuronal apoptosis in vitro**

Subsequently, the cellular uptake of PLGA–PAMAM@CircOGDH siRNA NPs was evaluated. Cy3 labelling revealed that PLGA–PAMAM@cy3-CircOGDH siRNA NPs were taken up by cortical primary neurons in vitro (figure 2A). Previous studies have reported that endocytosis is a key pathway through which NPs enter cells. Thus, the mechanism by which NPs entered neurons was investigated. Lysotracker (cherry) and cy3 (red) labelling...
Figure 1 Characterisation of PLGA–PAMAM@CircOGDH siRNA NPs. (A) Schematic illustrations of the assembly of PLGA–PAMAM@CircOGDH siRNA NPs. (B) Zeta potential of PLGA, PAMAM, PLGA–PAMAM and PLGA–PAMAM@CircOGDH siRNA NPs. Data are presented as means±SD; n=3 (Mann-Whitney U test). (C) Size distribution of PLGA–PAMAM and PLGA–PAMAM@CircOGDH siRNA NPs. Data are presented as means±SD; n=3 (Mann-Whitney U test). (D) TEM images of PLGA–PAMAM and PLGA–PAMAM@CircOGDH siRNA NPs. Scale bar=200 nm. (E) Electrophoretic mobility of siRNA and PLGA–PAMAM@CircOGDH siRNA NPs on agarose gel. Marker: 1000 bp DNA ladder. Extension time of PLGA–PAMAM@Cy3–CircOGDH siRNA NPs at 0 hour, 24 hours, 48 hours and 72 hours on the UV−Vis (F) and fluorescence spectra (G). CircOGDH, circular RNAs derived from oxoglutarate dehydrogenase; NPs, nanoparticles; PLGA, poly(lactide-co-glycolide); siRNA, small interfering RNA; UV−Vis, ultraviolet−visible; PAMAM, poly amidoamine; TEM, transmission Electron Microscope.
Figure 2  Cellular uptake of PLGA–PAMAM@CircOGDH siRNA NPs. (A) Representative fluorescent microscope images showing the transfection efficiency of PLGA–PAMAM@Cy3-CircOGDH siRNA NPs (red) in primary cortex neurons at 24 hours. Neurons were stained with Nissl (green), and nuclei were stained with DAPI (blue). Scale bar=50 µm. (B) Cell viability was determined in primary cortical neurons treated with NC, CircOGDH siRNA and PLGA–PAMAM@CircOGDH siRNA NPs. Data were presented as mean ± SD; n=3, Mann-Whitney U test. (C–E) Western blot analysis of BAX, BCL2 and cleaved caspase 3 expression levels in CON and ischaemic-reperfusion (OGD/R) neurons treated with PLGA–PAMAM and PLGA–PAMAM@CircOGDH siRNA NPs. Data were presented as mean±SD; n=3, Mann-Whitney U test. CircOGDH, circular RNAs derived from circular oxoglutarate dehydrogenase; CON, control; NC, normal control; NPs, nanoparticles; OGD/R, oxygen and glucose deprivation reperfusion; PLGA, poly(lactide-co-glycolide); siRNA, small interfering RNA; PAMAM, poly amidoamine; DAPI, 4',6-Diamidino-2'-phenylindole.
were chosen to monitor the intracellular localisation of PLGA–PAMAM@CircOGDH siRNA NPs in neurons. The results indicated that PLGA–PAMAM@cy3-CircOGDH siRNA NPs entered neurons via endocytosis beginning at 3 hours after administration and reaching a peak at 9 hours (online supplemental figure 2A). Moreover, we performed flow cytometry analysis in SH-SY5Y cells treated with PLGA–PAMAM@cy3-CircOGDH siRNA NPs and found it can be uptake by SH-SY5Y cells (online supplemental figure 3). These results demonstrate that the assembled PLGA–PAMAM@CircOGDH siRNA NPs can be taken up by cortical primary neurons and SH-SY5Y cells. To further explore the pathophysiological function of PLGA–PAMAM@CircOGDH siRNA NPs in neurons, we established oxygen and glucose deprivation reperfusion model (OGD/R) of neurons. After confirming that PLGA–PAMAM had no effect on neurons (online supplemental figure 2B), we conducted the CGK8 experiment and found that PLGA–PAMAM@CircOGDH siRNA NPs significantly ameliorated neuronal death (figure 2B). And we found that PLGA–PAMAM@CircOGDH siRNA NPs caused an increase of BCL-2/BAX ratio and a decrease of cleaved caspase-3 expression in OGD/R neurons (figure 2C–E). Our previous research indicated that knockdown of CircOGDH ameliorates neuronal apoptosis via targeting miR-5112/COL4A4 mRNA axis, next we detected COL4A4 protein expression level neurons treated with PLGA–PAMAM@CircOGDH siRNA NPs and we found that it caused a decrease of COL4A4 protein expression in OGD/R neurons (online supplemental figure 4), which was consistent with the decrease of COL4A4 mRNA expression in our previous research. Taken together, these results suggest that PLGA–PAMAM@CircOGDH siRNA NPs ameliorate neuronal apoptosis under ischaemic condition in vitro.

**PLGA–PAMAM@CircOGDH siRNA NPs downregulate CircOGDH expression levels in MCAO/R mice**

After confirming that the PLGA–PAMAM@CircOGDH siRNA NPs could be taken up by cortical primary neurons in vitro, we focused on their delivery in MCAO mice. Mice underwent MCAO for 40 min and reperfusion for 3 days; NPs were administered by tail vein injection every 24 hours during reperfusion (figure 3A). Cerebral blood flow measurement confirmed the success of the MCAO and MCAO/R models (figure 3B). We observed increased uptake of NPs in the penumbra neurons (figure 3C,D), while only a few were localised in microglia and astrocytes (online supplemental figures 5 and 6A), compared with the contralateral tissue of MCAO/R mice after PLGA–PAMAM@Cy3-CircOGDH siRNA NP injection. Furthermore, using Cy5.5-labelled PLGA–PAMAM NPs, we evaluated the biodistribution of PLGA–PAMAM NPs in vivo after tail vein injection in MCAO mice by performing fluorescence imaging. The results demonstrated that Cy5.5-labelled PLGA–PAMAM NPs mainly accumulated in the liver and kidneys, but they were also observed in the brain, within an extension time of 0.5 hour, 1.5 hours, 2.5 hours and day 3 (figure 3E, online supplemental figure 6B). Furthermore, quantitative reverse transcription PCR analysis showed that PLGA–PAMAM@CircOGDH siRNA NPs significantly downregulated the CircOGDH expression level in the penumbra tissue of MCAO/R mice (figure 3F). Taken together, these results demonstrate that PLGA–PAMAM@CircOGDH siRNA NPs can be delivered to the brain and significantly downregulate the CircOGDH expression level in the penumbra tissues of MCAO/R mice.

**PLGA–PAMAM@CircOGDH siRNA NPs improve neuron survival and neurologic function in MCAO/R mice**

Next, we evaluated the neuroprotective effect of PLGA–PAMAM@CircOGDH siRNA NPs in MCAO/R mice. Mice underwent neurological behavioural experiments on day 3 after reperfusion, and their brain tissues were harvested for analysis. After confirmed that PLGA–PAMAM exerted no effect in MCAO/R mice (online supplemental figure 7), we conducted the neurological behavioural experiments and demonstrated that MCAO/R mice injected with PLGA–PAMAM@CircOGDH siRNA NPs exhibited a decreased total time to complete the adhesive removal somatosensory test (figure 4A), a lower ratio of foot faults in the grid-walking test (figure 4B) and a lower ratio of right-biased counts in the cylinder test (figure 4C) compared with the mice in the MCAO/R+PLGA–PAMAM group. Furthermore, we showed that PLGA–PAMAM@CircOGDH siRNA NPs significantly decreased brain infarct size as revealed by T2 MRI (online supplemental figure 8A,B). Nissl staining of brain sections indicated that MCAO/R mice injected with PLGA–PAMAM@CircOGDH siRNA NPs had decreased neuron loss in the penumbra tissue compared with mice in the MCAO/R+PLGA–PAMAM group (figure 4D–H and online supplemental figure 8C). The TUNEL and Nissl co-staining assay of brain sections revealed similar results; MCAO/R mice injected with PLGA–PAMAM@CircOGDH siRNA NPs showed decreased neuron apoptosis in the penumbra tissue compared with mice in the MCAO/R+PLGA–PAMAM group (figure 4F–H). Thus, our results demonstrated that PLGA–PAMAM@CircOGDH siRNA NPs improved neuron survival and neurologic function in MCAO/R mice.

**PLGA–PAMAM@CircOGDH siRNA NPs exert no significant toxicity in MCAO/R mice**

We next performed haematological and pathological analyses to test the toxicity of these NPs in MCAO/R mice. The haematological analysis showed that the red blood cell (RBC), white blood cell (WBC) and platelet (PLT) levels fluctuated within the normal range in MCAO/R mice injected with PLGA–PAMAM@CircOGDH siRNA NPs or PLGA–PAMAM NPs (online supplemental figure 9). Biochemical analysis of the main organs was conducted by aspartate transaminase, alanine transaminase and creatinine examination, which indicated no obvious injury in MCAO/R mice injected with PLGA–PAMAM@CircOGDH.
Figure 3  PLGA–PAMAM@CircOGDH siRNA NPs downregulated CircOGDH expression level in MCAO/R mice. (A) Illustration of the in vivo experimental design. (B) Representative images showing the CBF of mice in the SHAM, MCAO and MCAO/R groups. Units of the colour scale: PUs. (C) and (D) Representative fluorescence imaging showing the cellular uptake of PLGA–PAMAM@Cy3–CircOGDH siRNA NPs (red) in the penumbra tissues of MCAO/R mice 3 days after tail injection. Nuclei were stained with DAPI (blue). Scale bar=50 µm (left), scale bar=10 µm (right). (E) In vivo fluorescence imaging of Cy5.5-labelled PLGA–PAMAM NPs in MCAO mice at 0.5 hour 1.5 hours and 2.5 hours. Units of the colour scale: PUs. (F) RT-qPCR analysis of the CircOGDH expression level in the penumbra tissues from SHAM and MCAO/R mice after tail injection of PLGA–PAMAM and PLGA–PAMAM@CircOGDH siRNA NPs on day 1. Data are presented as mean±SD; n=3, Mann–Whitney U test. CBF, Cerebral blood flow; CircOGDH, circular RNAs derived from circular oxoglutarate dehydrogenase; MCAO/R, middle cerebral artery occlusion/reperfusion; NPs, nanoparticles; PLGA, poly(lactide-co-glycolide); PUs, perfusion units; RT-qPCR, quantitative reverse transcription PCR; siRNA, small interfering RNA; PAMAM, poly amidoamine; DAPI, 4′,6-Diamidino-2′-phenylindole.
Figure 4  PLGA–PAMAM@CircOGDH siRNA NPs alleviated neuronal apoptosis in MCAO/R mice. PLGA–PAMAM@CircOGDH siRNA NPs improved behavioural recovery in MCAO/R mice 3 days after tail injection, as demonstrated by the adhesive removal test (A), grid-walking test (B) and cylinder test (C). Data are presented as means±SD adhesive removal test: n=4 in each group (Mann-Whitney U test); grid-walking test: n=8 in the SHAM group and the MCAO/R+PLGA–PAMAM@CircOGDH siRNA group, n=6 for MCAO/R+PLGA–PAMAM group (Mann-Whitney U test); cylinder test: n=7 in the SHAM group and the MCAO/R+PLGA–PAMAM@CircOGDH siRNA group, n=6 for the MCAO/R+PLGA–PAMAM group (one-way ANOVA followed by Tamhane T2 test). (D) and (E) Nissl staining showing the number of neurons in the SHAM, MCAO/R+PLGA–PAMAM and MCAO/R+PLGA–PAMAM@CircOGDH siRNA groups 3 days after tail injection. Scale bar=100 µm. For the quantification of results in (E), data were presented as mean±SD; n=3, Mann-Whitney U test. (F)–(H) TUNEL and Nissl staining of brain sections showing TUNEL-positive and intact neurons in the brains of mice in the MCAO/R+PLGA–PAMAM and MCAO/R+PLGA–PAMAM@CircOGDH siRNA groups 3 days after tail injection. White dashed lines (left) indicate potential infarct regions; yellow dashed lines (right) indicate potential penumbra regions. Scale bar=50 µm. Data were presented as mean±SD; n=3, Mann-Whitney U test. CircOGDH, circular RNAs derived from circular oxoglutarate dehydrogenase; MCAO/R, middle cerebral artery occlusion/reperfusion; NPs, nanoparticles; PLGA, poly(lactide-co-glycolide); siRNA, small interfering RNA; PAMAM, poly amidoamine; ANOVA, analysis of Variance; TUNEL, TdT-mediated dUTP nick end labeling.
siRNA NPs or PLGA–PAMAM NPs (figure 5A–C). Finally, H&E staining revealed that the main organs (the heart, liver, spleen, lungs and kidneys) displayed no observable pathological injuries on day 3 after treatment with PLGA–PAMAM@CircOGDH siRNA NPs or PLGA–PAMAM NPs in MCAO/R mice (figure 5D). Thus, our results indicated that PLGA–PAMAM@CircOGDH siRNA NPs improved penumbra neuron survival and exerted no significant toxicity in MCAO/R mice.

**DISCUSSION**

The pathological process of ischaemic stroke is complicated and involves inflammation, oxidative stress and excitotoxicity, which lead to neuronal death. The management of ischaemic stroke largely depends on intervention within a narrow time window, and rescuing the maximum number of ischaemic penumbra neurons is crucial for optimal AIS treatment. Mechanical thrombectomy is a well-established treatment used in patients with AIS for proximal large vessel occlusion within a stringent time window. Though the brain blood flow is restored, secondary damage will occur during the ischaemic reperfusion process in ischaemic region after mechanical thrombectomy. Our previous studies identified and screened a neuron-derived CircOGDH, which was identified as a potential penumbra therapeutic target in AIS. Furthermore, we found that the binding of CircOGDH to microRNA-5112 regulated the downstream COL4A4 expression level, thus to alleviate neuronal apoptotic level under ischaemic conditions in a permanent 3-hour MCAO mouse model without ischaemic reperfusion. As numerous neuroprotective agents have failed to show benefit in a Phase III clinical trial of AIS, effective and new treatment strategies that combine reperfusion raised attention. In the present study, we assembled PLGA–PAMAM NPs loaded with CircOGDH siRNA and demonstrated that they exerted a potential protective effect in ODG/R primary cortical neurons and MCAO/R mice using a series of experiments.

Neuronal apoptosis is widely recognised as a crucial factor during the pathological process of ischaemic stroke. circRNAs are a type of noncoding RNA expressed in tissue-specific, cell-specific and development-stage-specific patterns, they are abundant in the brain and are more stable than linear RNA. Increasing evidence identified that circRNAs have been implicated in the pathological process of ischaemic stroke, and circRNA-based therapeutics emerge as a potential therapy for various therapeutic areas, such as prophylactic vaccines. Our previous studies identified a neuron-derived CircOGDH, which could aggravating neuronal apoptosis and was identified as a potential penumbra biomarker in AIS. However, still many challenges remain regarding targeted delivery of circRNAs. siRNA is one of the meaningful gene silencing tool that has been widely used to control target gene expression in vivo and in vitro. Still many challenges remain regarding siRNA delivery because of the molecule’s rapid enzymatic degradation. Previous studies have reported that NPs loaded with siRNA improve therapeutic efficacy in ischaemic stroke when administered intravenously. In line with these findings, our results indicate that PLGA–PAMAM@CircOGDH siRNA NPs can protect CircOGDH siRNA from enzymatic degradation for 72 hours (according to UV–Vis and fluorescence spectra analysis). PLGA–PAMAM@CircOGDH siRNA NPs can be taken up by ischaemic neurons in vitro and penumbra neurons in MCAO/R mice. We found that PLGA–PAMAM@CircOGDH siRNA NPs preferentially accumulated in the penumbra area of MCAO/R mice, thus significantly downregulated CircOGDH expression levels in penumbra tissues of MCAO/R mice. Additionally, we demonstrated that PLGA–PAMAM@CircOGDH siRNA NPs alleviated neuronal apoptotic level in ischaemic neurons, and tail injection of PLGA–PAMAM@CircOGDH siRNA NPs significantly decreased brain infarct size and alleviated neurologic function injury and apoptotic level of ischaemic penumbra neurons in MCAO/R mice. Polymers, lipids, inorganic materials and exosomes can be used in nanostructured gene delivery systems. PLGA is an FDA-approved polymer commonly employed in gene and drug delivery systems for clinical use. Despite its excellent biocompatibility and biodegradability, PLGA is difficult to load with nucleic acids because it is negatively charged. Thus, our study assembled PLGA–PAMAM NPs. PAMAM dendrimers are widely used as a nonviral gene carrier because they exhibit positively charged amine groups on their surface and can be internalised into cells by endocytosis; however, the cytotoxic activity of PAMAM dendrimers requires careful attention, which was a limitation of our study. To address this limitation, haematological and pathological analyses were conducted. The results revealed that the biomarkers evaluated in these analyses remained within a normal range in MCAO/R mice injected with PLGA–PAMAM@CircOGDH siRNA NPs or PLGA–PAMAM NPs. Studies have reported that biomimetic NPs exert high blood compatibility and long circulation time, and many different nanoplatforms have been used as nanotherapies to provide neuroprotection in brain injury. Therefore, after confirming the protective effect of PLGA–PAMAM@CircOGDH siRNA NPs in MCAO/R mice, our future work will be dedicated to optimise our NPs to minimise the toxicity and improve the delivery efficiency targeting the neurons in the brain.

**CONCLUSION**

In summary, our data showed that we successfully assembled the PLGA–PAMAM@CircOGDH siRNA NPs and found that they could exert protective effects and preserve the integrity of CircOGDH siRNA by preventing potential degradation. We revealed that PLGA–PAMAM@CircOGDH siRNA NPs alleviated neuronal apoptotic level in ischaemic neurons, and tail injection of PLGA–PAMAM@CircOGDH siRNA NPs significantly decreased brain infarct size and alleviated neurologic function injury.
Figure 5  PLGA–PAMAM@CircOGDH siRNA NPs exert no significant toxicity in MCAO/R mice. Haematological analysis of AST (A), ALT (B) and CR (C) were evaluated 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. (D) H&E staining of the heart, liver, spleen, lungs and kidneys in SHAM, MCAO+PLGA–PAMAM and MCAO+PLGA–PAMAM@CircOGDH siRNA mice 3 days after tail injection. Scale bar=100 µm. ALT, alanine transaminase; AST, aspartate transaminase; CircOGDH, circular RNAs derived from circular oxoglutarate dehydrogenase; CR, creatinine; MCAO/R, middle cerebral artery occlusion/reperfusion; NPs, nanoparticles; PLGA, poly(lactide-co-glycolide); siRNA, small interfering RNA; PAMAM, poly amidoamine.
and apoptotic level of ischaemic penumbra neurons in MCAO/R mice. Thus, our study provides a nanotherapeutic strategy for targeting CircOGDH in ischaemic stroke, which may promote the potential clinical transformation of multifunctional nanodrugs targeting circRNA (online supplemental file 2).

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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: AACTCGTGGAGGACCAC TTG, Reverse: GAGCTTCGACTCAGGGAAAG, β-actin Forward: ACGGCCAGGTCATCACTA TTG, 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°C. 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 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 °C 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 °C, 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, 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, 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: ACCTGAGGAGGACTGCTTTTGG, Reverse: CAAGAAGGAAGGCTGGAAAAGA), CircOGDH (Forward: AACTCGTGGAGGACCATGTG, 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 2ΔΔCT method, 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, 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% CO2). (2) OGD/R: neurons were exposed to DMEM solution without glucose in an incubator containing 0% O2, 5% CO2 with balanced N2 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% CO2).

**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 $18 \times 10^4$ 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 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.
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.
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.
References


Graphical Abstract

PLGA-PAMAM
@ CircOGDH siRNA NPs

MCAO-reperfusion

through BBB

Neuron
CircOGDH
CircOGDH siRNA