Erythrocyte Membrane-Enveloped Salvianolic Acid B Nanoparticles Attenuate Cerebral Ischemia-Reperfusion Injury

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Purpose: Ischemic stroke is the second leading cause of death and the third leading cause of disability worldwide. Salvianolic acid B (SAB), a water-soluble phenolic acid derived from the traditional Chinese medicine Salvia miltiorrhiza, exerted protective effects on cerebral ischemia-reperfusion injury. However, the efficacy of SAB is seriously hindered by poor blood brain barrier permeability and short biological half-life in plasma. Brain targeted biomimetic nanoparticle delivery systems offer much promise in overcoming these limitations.

Methods: A brain targeted biomimetic nanomedicine (RR@SABNPs) was developed, which comprised of SAB loaded bovine serum albumin nanoparticles and functionalized red blood cell membrane (RBCM) with Arg-Gly-Asp (RGD). The characterization parameters, including particle size, zeta potential, morphology, Encapsulation Efficiency (EE), Drug Loading (DL), release behavior, stability, and biocompatibility, were investigated. Moreover, the middle cerebral artery occlusion/reperfusion (MCAO/R) mouse model was used to assess the therapeutic efficacy of RR@SABNPs on ischemic stroke. Finally, the reactive oxygen species (ROS) levels and mitochondrial membrane potential (MMP) were detected by DHE and JC-1 staining in oxygen-glucose deprivation/reperfusion (OGD/R) and H₂O₂ injured PC12 cells.

Results: RR@SABNPs exhibited spheric morphology with core-shell structures and good stability and biocompatibility. Meanwhile, RR@SABNPs can significantly prolong SAB circulation time by overcoming the reticuloendothelial system (RES) and actively targeting ischemic BBB. Moreover, RR@SABNPs had comprehensive protective effects on MCAO/R model mice, manifested as a reduced infarct volume and improved neurological and sensorimotor functions, and significantly scavenged excess ROS and maintained MMP.

Conclusion: The designed brain targeted biomimetic nanomedicine RR@SABNPs can significantly prolong the half-time of SAB, deliver SAB into the ischemic brain and exhibit good therapeutic effects on MCAO/R model mice.

Keywords: red blood cell membrane, blood brain barrier, long circulation, reactive oxygen species, mitochondrial membrane potential

Introduction

Stroke is the second leading cause of death and the third leading cause of disability worldwide, ischemic stroke contributes over 87% of stroke. The deficiency of oxygen, and glucose resulting from the occlusion of the cerebral artery causes ischemic stroke. Currently, recombinant tissue plasminogen activator is the only drug approved by the US Food and Drug Administration (FDA) for the treatment of ischemic stroke, which can quickly dissolve thrombosis and restore blood flow. However, reperfusion injury is the main obstacle to the recovery of neurological function after thrombolytic therapy, which results in nearly 40% of survivors with disability. It is no doubt that neuroprotection is very significant for the treatment of ischemic stroke. However, no neuroprotective agent has been approved by the FDA for the treatment of ischemic stroke due to the complexity of the pathological mechanisms, the limitation of blood brain barrier (BBB), the lack of specific targeting, and so on.

Salvianolic acid B (SAB), a water-soluble phenolic acid derived from the traditional Chinese medicine Salvia miltiorrhiza, has been reported to exert protective effects on cerebral ischemia-reperfusion injury via scavenging free radicals, improving energy metabolism, and reducing apoptosis and inflammation by the activation of SIRT1 signaling.
Meanwhile, SAB can improve cognitive impairment in rat transient global cerebral ischemia model by promoting the proliferation of neural stem/progenitor cells via the PI3K/Akt signal pathway. However, the poor stability of SAB in plasma and the existence of BBB hindered the application of SAB in the clinic. Thus, a transferrin receptor monoclonal antibody OX26 modified lipid carrier (OX26-BA/Sal B-NLC) was constructed to deliver baicalin and SAB, which could promote the brain delivery of SAB.

Bovine serum albumin (BSA) is widely applied in nano delivery systems of hydrophobic and hydrophilic drugs such as itraconazole and gemcitabine due to its biocompatible and biodegradability. Meanwhile, it has been reported that SAB has strong interaction with BSA, which provides a potential for the construction of SAB loaded BSA nanoparticles. However, the circulation time of nanoparticles in vivo is significantly limited by the reticuloendothelial system (RES), which resulted in a major fraction of nanoparticles distributed in the liver and spleen instead of the disease site. The shortened circulation time of nanoparticles is not conducive to the accumulation at the target site. PEGylation is a common strategy to prolong the circulation time of nanoparticles in vivo via the formulation of hydrophilic surface, and it has been reported that the presence of PEG chains on the surface of BSA nanoparticles can decrease the uptake of macrophages and extend the circulation time in vivo. However, the coating of hydrophilic material also reduces the interaction with targeted cells. Recently, a “don’t-eat-us” strategy was developed to block the RES. Red blood cell membrane (RBCM) has been widely used in nano delivery systems due to its immune escape ability. CD47, an integral membrane protein embedded in RBCM, could interact with phagocytic cells and release “do not eat me” signal. Additionally, other membrane proteins help drug nanocarriers present as “self” to the immune system. Therefore, RBCM coating can prolong the circulation time and reduce the elimination of nanoparticles by RES, thereby promoting the brain delivery of nanoparticles.

Herein, we designed a biomimetic drug delivery system (RR@SABNPs), which consists of two components: 1) SAB loaded BSA nanoparticles (SABNPs) as the inner core. 2) Arg-Gly-Asp (RGD) functioned RBCM as the outer shell. RGD-RBCM acts as a bionic camouflage with a targeted ligand to prolong nanoparticle circulation time and target ischemic BBB actively. The latter advantage is attributed to the high affinity of RGD to integrin αvβ3, which is upregulated in endothelial cells during ischemia. RR@SABNPs was successfully constructed and characterized in the current study, including particle size, zeta potential, morphology, and drug release behavior. Then, the ischemic BBB targeting of RR@SABNPs was confirmed by cellular uptake and living image analysis. Meanwhile, the pharmacokinetics of SAB in rats after intravenous administration of RR@SABNPs and anti-phagocytosis in vitro were performed to verify the capability and mechanism of RR@SABNPs to prolong drug circulation time. Moreover, the therapeutic efficacy of RR@SABNPs on ischemic stroke was assessed in the middle cerebral artery occlusion/reperfusion (MCAO/R) mouse model. Scavenging intracellular ROS and maintaining
mitochondrial membrane potential (MMP) of RR@SABNPs were confirmed in oxygen-glucose deprivation/reperfusion (OGD/R) and H$_2$O$_2$ injured PC12 cells. Additionally, the safety of the RR@SABNPs was also preliminarily examined in vivo.

**Materials and Methods**

**Materials**
Salvianolic acid B was purchased from Chengdu Desite Biotechnology Co., LTD (Chengdu, China). Bovine Serum Albumin (MW 66 kDa, purity>98%, Roche™ reagent grade) was purchased from Yu-ming Biological technology Co., LTD (Qingdao, China). RGD-mPEG2000-DSPE and FITC-mPEG2000-DSPE were purchased from Xi’an Ruixi Biological Technology Co., LTD (Xian, China). DiR was purchased from Shanghai J&K Scientific Co., LTD (Shanghai, China). TTC was purchased from Sigma-Aldrich (St Louis, United States), DAPI, JC-1, and DHE were purchased from Beyotime Biotechnology Co., LTD (Shanghai, China). All solvents used were of analytical grade.

RAW264.7, bEnd.3 and PC12 cells were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).
Healthy adult male SD rats and C57BL/6J mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All the animals were acclimated under standard laboratory conditions and had free access to standard water and food. All the procedures were conducted in accordance with the “Guiding Principles in the Care and Use of Animals (China)” and were approved by the Laboratory Animal Ethics Committee of Zhejiang Chinese Medicine University.

**Preparation of RR@SABNPs**

**Preparation of SABNPs**
The BSA nanoparticles were constructed by the desolvation method. Briefly, BSA (20 mg) and SAB (2.0 mg) were dissolved in deionized water (1 mL), and the solution was then adjusted to 8.0 with 0.1 M NaOH. Subsequently, ethanol (6 mL) was added by constant agitation at the rate of 1 mL /min with a constant flow pump. After the mixture was stirred for 30 min at room temperature, 21 μL 2% glutaraldehyde solution was added and stirred continuously at room temperature for 12 h. Finally, the mixture was rotary evaporated at 40 °C to remove the ethanol and the stabilized particles (SABNPs) were collected after being filtered through a 0.8 μm membrane syringe filter and centrifuged. Various fluorescent nanoparticles used in this study were prepared by the same method, only fluorescence probe in ethanol.

**Extraction of RBCM**
The RBCM were extracted from RBCs as previously described. Briefly, fresh heparinized whole blood was collected from male rats and then centrifuged at 3000 rpm for 5 min at 4 °C to remove the plasma, and the resulting RBCs were washed twice with cold PBS (1×). The isolated RBCs were incubated for 30 min in cold 0.25× PBS (V:V, 1:10) at 4 °C. The resulting RBCs lysate was centrifuged (12,000 rpm, 20 min) at 4 °C to remove hemoglobin, and the collected precipitate was washed until the supernatant was colorless after centrifugation. The resulting RBCM was suspended and stored in distilled water.

**Preparation of R@SABNPs and RR@SABNPs**
The collected RBCM were sonicated (100 W) for 5 min, and then extruded through 800 nm and 400-nm polycarbonate membranes at least 20 times respectively using a mini extruder (Avanti Polar Lipids Inc.). Subsequently, 0.5 mL of SABNPs (10 mg/mL) was mixed with RBCM from 0.25 mL RBCs and 0.25 mL PBS (1×), and the mixture was extruded through 800 nm polycarbonate membranes at least ten times to obtain the R@SABNPs.

In order to prepare the brain-targeted RBCM, RGD-mPEG2000-DSPE was dissolved in RBCM and incubated for 30 min at 37 °C. The mixture was centrifuged (12,000 rpm, 20 min) to remove the unreacted RGD-mPEG2000-DSPE. The collected brain-targeted RBCM was treated as described above, and RR@SABNPs were obtained by the same method. The amount of RGD-mPEG2000-DSPE on RR@SABNPs was determined indirectly using FITC-mPEG2000-DSPE.

**Characterization of the RR@SABNPs**

**Particle Size and Zeta-Potential**
Particle size, polydispersity index (PDI), and zeta-potential of different nanoparticles were measured by dynamic light scattering.
Encapsulation Efficiency (EE) and Drug Loading (DL)
The EE and DL of nanoparticles were determined according to a previous method with slight modifications. In brief, 100 μL of nanoparticles diluted with acetonitrile was ultrasonicated (10 min) and centrifuged (12,000 rpm, 10 min). The SAB amount in supernate was analyzed by high-performance liquid chromatography (HPLC). The mobile phase consists of the mixture solution of methanol and acetonitrile (v/v, 1:1), and 0.2% phosphoric acid solution at a ratio of 60:40 (v/v). A C18 column (250×4.6 mm, 5 μm) was used as the stationary phase. The injection volume was 30 μL, and the effluent was monitored at 280 nm. The values of DL and EE of nanoparticles were obtained according to the following equations:

\[
\text{EE} \, (\%) = \frac{\text{Weight of SAB in SABNPs}}{\text{Weight of the initial SAB}} \times 100
\]

\[
\text{DL} \, (\%) = \frac{\text{Weight of SAB in SABNPs}}{\text{Weight of nanoparticles}} \times 100
\]

SDS-PAGE Analysis
The protein profiles of RBCs, RBCM, SABNPs, and RR@SABNPs were determined by SDS-PAGE electrophoresis. Briefly, RBCs, RBCM, SABNPs, and RR@SABNPs were lysed with the RIPA lysis buffer containing 1 mM PMSF. The concentration of protein in the collected lysates was determined by a BCA assay, and the lysates were mixed with SDS sample buffer and heated at 100 °C for 10 min. The samples were separated by SDS-PAGE electrophoresis and stained with Coomassie Brilliant Blue.

Transmission Electron Microscope (TEM) Analysis
The morphologies of SABNPs and RR@SABNPs were observed by transmission electron microscope (TEM; H-7000; Hitachi, Tokyo, Japan). SABNPs or RR@SABNPs solution was dropped onto carbon film-coated copper grids, then the obtained sample was stained with phosphotungstic acid solution.

Laser Scanning Confocal Microscope Analysis
In order to ensure the successful modification of RGD-mPEG2000-DSPE in the RR@SABNPs, laser scanning confocal microscope was used. In this assay, FITC-mPEG2000-DSPE was mixed with RGD-mPEG2000-DSPE.

In vitro Drug Release
To evaluate the in vitro release behavior of SAB from RR@SABNPs or SAB solution, the dynamic dialysis technique was used according to the previous method. Briefly, 5% (w/v) vitamin C was added into normal saline as the release medium to prevent SAB degradation. The dialysis bags (3.5 KDa MWCO) contained 1 mL of RR@SABNPs or SAB solution (equivalent to 1 mg of SAB) were placed in 50 mL release medium with constant stirring (400 rpm) at 37 °C. 0.5 mL samples were collected at certain intervals and the same amount of fresh media pre-heated to 37 °C was supplemented. The supernatants of the collected samples were analyzed by HPLC. The experiment was repeated three times.

Stability Study of Nanoparticles
The Stability of SAB in Plasma
0.2 mL rat plasma mixed with 1.8 mL of RR@SABNPs or SAB solution were incubated at 37 °C. 0.1 mL samples were collected at certain intervals and mixed with acetonitrile. The supernatants of the mixture were analyzed by HPLC after centrifugation (12,000 rpm, 10 min). The experiment was repeated three times.

The Stability of Nanoparticles
SABNPs and RR@SABNPs were placed at room temperatures, and the particles size and PDI were measured in pre-determined time intervals.

Hemolytic Test
RBCs were collected by centrifugation (3000 rpm, 5 min) and suspended in saline solution to obtain 2% RBCs suspension. SABNPs, RR@SABNPs or RR@SABNPs mixed with 2% RBCs suspension and incubated for 4 h at 37 °C. Saline solution and trizol were used as the negative and positive control, respectively. The mixture was centrifuged (3000 rpm, 5 min), and the UV absorbance intensity of the supernatants was measured at 570 nm using a microplate reader.
Cytotoxicity Assays
bEnd.3 cells, PC12 cells, or RAW 264.7 cells were seeded into the 96-well plates at the density of 1×10^4 cells per well and cultured to 80% fusion. Subsequently, the medium was replaced with 100 μL DMEM medium containing SABNPs, R@SABNPs, or RR@SABNPs (without SAB). After 24 h, the cell viability was measured using CCK8 assay kit.

Pharmacokinetics Study
Healthy male SD rats were randomly divided into the following three groups (n=6/group): 1) SABNPs group; 2) R@SABNPs group; 3) SAB solution group. SD rats were intravenously injected with SABNPs, R@SABNPs, or SAB solution (SAB 40 mg/kg), respectively. Subsequently, the blood samples were collected at certain intervals and centrifuged (4000 rpm, 10 min). The collected plasma in the supernatant mixed with acetonitrile (HCl 1%) in equal volume. Subsequently, the mixture was vortexed and centrifuged (12,000 rpm, 10 min). The supernatant was measured by HPLC. The pharmacokinetic parameters were analyzed using PK solver 2.0.

Anti-Phagocytosis in vitro
RAW 264.3 cells were seeded into the 24-well plates (5×10^4 cells/well). Subsequently, the medium was replaced with the DMEM medium containing FITC loaded SABNPs or RR@SABNPs after incubating to 80% fusion. Finally, the medium was removed and the cells were washed twice with PBS after incubating for 1 h. The anti-phagocytosis of different nanoparticles in RAW 264.3 cells was determined and quantified by inverted fluorescence microscopy and flow cytometry.

Cellular Uptake Study
bEnd.3 cells were used to evaluate the cellular uptake of nanoparticles due to its highly expressed αvβ3. In detail, bEnd.3 cells were seeded into the 24-well plates (5×10^4 cells/well). Subsequently, the medium was replaced the DMEM medium containing FITC loaded SABNPs or R@SABNPs after incubating to 80% fusion. Finally, the medium was removed and the cells were washed twice with PBS after incubating for 4 h. The cellular uptake of different nanoparticles in bEnd.3 cells was determined and quantified by inverted fluorescence microscopy and flow cytometry.

In order to evaluate the mechanism of cellular uptake of R@SABNPs in bEnd.3 cells, RGD was pre-treated in bEnd.3 cells for 2 h. Subsequently, the medium was replaced with the DMEM medium containing FITC-loaded R@SABNPs and incubated for 4 h. The cellular uptake of R@SABNPs in bEnd.3 cells with or without RGD pre-treated were determined and quantified by inverted fluorescence microscopy and flow cytometry.

The Establishment of MCAO/R Mouse Model
Adult male C57BL/6J mice (body weight 23–25g) were anesthetized with 3% isoflurane for induction, and the anesthetization was maintained with 1.0–1.5% isoflurane in 30% oxygen and 70% nitrous oxide. During the surgery, the rectal temperature was maintained at 36.5±0.5°C using a heating pad. Then the left common carotid artery (CCA), the left external carotid artery (ECA), and the internal carotid artery (ICA) were exposed via the midline neck incision under a surgical microscope. Next, the CCA and ECA were ligated, and the ICA was temporarily suspended using microvascular aneurysm clips. After a small puncture was made in the ECA, a silicone-coated 6–0 monofilament (0.20–0.25 mm in diameter) was gently introduced into the ICA through the ECA to occlude the middle cerebral artery. Cerebral blood flow and temperature were monitored with a Laser Doppler Perfusion and Temperature Monitor (MoorVMS-LDF2, Moor Instruments, UK). A reduction in blood flow of over 70% was considered the successful occlusion of the MCA. After 1.5 h of ischemia, the monofilament was slowly withdrawn to allow reperfusion. Sham operation underwent all the surgical procedures except that the arteries were not ligated. All mice that survived until the endpoint were included for data collection.

Living Imaging Analysis
The MCAO/R model mice were administrated intravenously with PBS, DiR loaded SABNPs, and R@SABNPs after reperfusion for 16 h, and imaged at 2, 4, and 6 h via an IVIS Spectrum in vivo imaging system (PerkinElmer, USA). After the last point in time, the mice were perfused with normal saline and sacrificed. The major organs, including the
heart, liver, spleen, lung, kidney and brain, were collected and imaged. Additionally, the brains were sectioned and detected by an IVIS Spectrum in vivo imaging system.

Neuroprotective Effect of RR@SABNPs on MCAO/R Mice

Adult male C57BL/6J mice (body weight 23–25g) were randomly divided into four groups: 1) the sham-operated group; 2) the model group; 3) the SAB solution group; 4) the RR@SABNPs group. Except for the sham-operated group, all groups were subjected to MCAO/R surgery and administered saline, SAB solution (10 mg/kg), and RR@SABNPs (10 mg/kg) immediately after reperfusion and repeated once per day for the following 2 days. Neurological functions and sensorimotor functions of pre-operative and post-operative mice were monitored using the modified neurological severity score (mNSS) and the foot-fault test, respectively, as previous literature described. In detail, the mNSS test included motor (muscle status and abnormal movement), sensory systems (visual, tactile, and proprioceptive) and reflexes. The foot-fault test was used to assess the accuracy of forepaw placement on the grid as a percentage of foot faults of the stroke-impaired forepaw to total steps. Mice placed their paws on an elevated grid surface (30 × 35×31 cm) with grid openings of 2.5 cm². A foot fault was recorded when a paw slipped while moving on an elevated grid surface.

After the last neurological and sensorimotor assessment, the mice were sacrificed, and the brain was collected and cut into 7 coronal slices of 1 mm thickness. Then the brain slices were stained with 1.5% TTC. After TTC staining, brain slices were photographed, and the infarct volume was analyzed by Image J software (NIH). The infarct volume was calculated using the following formula: infarct volume (%) = [(contralateral hemisphere volume) - (non-ischemic ipsilateral hemisphere volume)] × 100/contralateral hemisphere volume.

After the mice were sacrificed, the brains were collected, fixed (4% paraformaldehyde for 24 h) and dehydrated. The obtained brains were sliced into 5 µm conventional sections using a microtome. Subsequently, the brain sections were stained with Hematoxylin and eosin (H&E) or Nissl staining solution. Finally, the sections were observed and imaged by a light microscope or physicochemical scanner after being covered with neutral resin.

Neuroprotective Effect of RR@SABNPs

Effect of RR@SABNPs on PC12 Cells Injured by OGD/R

To investigate the effect of RR@SABNPs on PC12 cells in vitro, the OGD/R model was established. Briefly, PC12 cells were seeded in 96-well plate at a density of 1×10⁴ cells/well and incubated until 80% confluence was achieved. The cells were randomly divided into five groups: control group, model group, SAB solution group, R@SABNPs group and RR@SABNPs group. Except the control group, the cells were incubated in Earle’s balanced salt solution and incubated in a specialized hypoxia incubator chamber (Stem Cell, Canada) containing an anaerobic gas mixture (95% N₂ and 5% CO₂) kept at 37°C for 6 h. Then, followed by the SAB solution, R@SABNPs and RR@SABNPs administration and reoxygenation for 24 h (SAB was normalized to 20 μg/mL). Subsequently, the culture medium was collected and centrifuged at 2000 rpm for 5 min, and 50 μL of supernatant was mixed with an equal amount of LDH-detection assay mixture according to the manufacturer’s instructions. The absorbance was determined at 560 nm using a microplate reader.

To investigate the intracellular ROS levels and mitochondrial membrane potential (MMP), OGD/R injured PC12 cells model were established as mentioned above. PC12 cells were stained with DHE and JC-1 to detect ROS and MMP, respectively. The fluorescence images were captured using an inverted fluorescence microscope. The MMP was indicated by the ratio of red/green fluorescence intensity.

Effect of RR@SABNPs on PC12 Cells Injured by H₂O₂

To further investigate the ability of RR@SABNPs to decrease the intracellular ROS levels and maintain MMP, H₂O₂-induced oxidative damage model was established. Briefly, PC12 cells were seeded in 96-well plate (1×10⁴ cells/well) and incubated until 80% confluence was achieved. The cells were randomly divided into five groups: control group, model group, SAB solution group, R@SABNPs group and RR@SABNPs group. The SAB solution, R@SABNPs, and RR@SABNPs were added to each well (SAB was normalized to 20 μg/mL). After incubation for 20 h, all cells, except within the control group, were incubated with 800 μM H₂O₂ for 4 h. LDH release in culture medium and intracellular ROS level and MMP were detected by the methods described above.
Statistical Analysis
All data were expressed as the mean ± standard deviation (SD). The data were subjected to the analysis of variance (One Way ANOVA) with the SPSS 17.0 software.

Results and Discussion
Characterization of RR@SABNPs
The ischemic brain targeted RBCM coated nanoparticles, RR@SABNPs, was prepared by following steps (Figure 1): 1) the fabrication of SAB loaded nanoparticle (SABNPs) composed of BSA that is biocompatible and biodegradable; 2) the synthesis of RGD modified RBCM by incubation of RGD-PEG2000-DSPE with extracted RBCM; 3) the fusion of SABNPs with RGD modified RBCM through mechanical extrusion. The long-term circulatory effect of RBCs is mediated by a series of membrane proteins. The difference in membrane proteins between RBCs and RBCM was detected by using SDS-PAGE electrophoresis analysis. The similar protein band of RBCs and RBCM demonstrated that the extraction of RBCM does not destroy the integrity of membrane proteins of RBCs (Figure 2A), which was helpful for membrane coated nanoparticles to obtain the inherent functions of RBCs. As shown in Figure 2B, compared with R@SABNPs, the particle size and zeta potential of RR@SABNPs presented a slight increase, respectively. The specific parameters, including particle size, polydispersity index, zeta potential, EE, and DL, were summarized in Table 1. SABNPs, R@SABNPs, and RR@SABNPs were spherical in shape, and the obvious core-shell structure of R@SABNPs and RR@SABNPs were observed in TEM images (Figures S1 and 2C) indicating the existence of RBCM coating on the surface of SABNPs. The SDS-PAGE analysis indicated that RR@SABNPs preserved the protein of RBCM (Figure 2A). To further characterize the complete coating of RBCM and the successful modification of RGD, RGD-PEG2000-DSPE was mixed with FITC-PEG2000-DSPE in the preparation of RR@SABNPs. The amount of RGD-mPEG-DSPE in

Figure 1 Schematic design of RR@SABNPs.
RR@SABNPs was 2.44±0.31 μg/mg RR@SABNPs. The occurrence of the green fluorescence surrounding SABNPs indicated the complete coating of SABNPs by RBCM and the successful insertion of FITC-PEG2000-DSPE (Figure 2D).

RR@SABNPs has good stability over time manifesting only a negligible change in particle size of RR@SABNPs over 72 h at 4 °C (Figure S2). The stability of SAB loaded in RR@SABNPs was investigated in plasma. Compared with the SAB solution, RR@SABNPs exhibited significantly protective effects on SAB from degradation (Figure 2E). The SAB release profile showed that SAB exhibited better sustained release characteristics than SAB solution (Figure 2F), which contributed to protect SAB from degradation in the circulation (Figure 2E). The blood biocompatibility of SABNPs, R@SABNPs, and RR@SABNPs was investigated by hemolytic test. As shown in Figure S3, there was no obvious hemolysis in these formulation groups. Meanwhile, the cytotoxicity of these different formulations was also evaluated in bEnd.3, RAW 264.3, and PC12 cells. Compared with the control group, no obvious cytotoxicity was observed in SABNPs, R@SABNPs, and RR@SABNPs groups (Figure S4A-C). These results indicated that SABNPs, R@SABNPs, and RR@SABNPs had good biocompatibility.

**Long Cycling Performance of RR@SABNPs**

The circulation behavior of nanoparticles in vivo is strongly associated with the RES. To confirm the ability of RR@SABNPs to evade the immune system, RAW 264.7 cells were used to evaluate the anti-phagocytosis of RR@SABNPs. As shown in Figure 3A, the fluorescence intensity of RR@SABNPs group was significantly decreased.
than that of SABNPs group, indicating that RBCM coating can reduce the clearance of nanoparticles by the RES system. The flow cytometry showed that the phagotrophy of RR@SABNPs by macrophages was reduced by about 50% compared with that of SABNPs group (Figure 3B and C). Decreased macrophage uptake signified the potential of longer circulation time in vivo, which contributed to reaching the targeted region.

To determine the ability of RR@SABNPs to prolong the half-time of SAB, the pharmacokinetic behavior of SAB in SAB solution, SABNPs and RR@SABNPs were evaluated in vivo. The concentration-time profiles of SAB in plasma were shown in Figure 3D, and the parameters were presented in Table 2. After the intravenous injection of 40 mg/kg SAB, the concentration of SAB in plasma sharply decreased from 33.51 μg/mL to 3.8 μg/mL in 10 min, and gradually decreased to 0.25 μg/mL in 6 h in the SAB solution group, which is similar with the previous literature. Compared with the SAB solution, SAB plasma concentrations in nanoparticles were significantly increased, which was attributed to the

![Figure 3](https://example.com/image3.png) The long circulation effect of RR@SABNPs. (A) The fluorescence images of FITC loaded SABNPs and RR@SABNPs uptake by RAW 264.7 cells. Scale bar, 50 μm; (B) Flow cytometry histograms of cellular uptake in RAW 264.7 cells; (C) Quantification of the mean fluorescence intensity of the flow cytometry histograms (n=3); (D) Pharmacokinetics of SAB in SAB solution, SABNPs, and RR@SABNPs in rats after intravenous administration (n=6). Data are expressed as mean ± SD; ***p < 0.001.

Table 2 The Pharmacokinetic Parameters of SAB Solution, SABNPs, and RR@SABNPs

| Pharmacokinetic Parameters | SAB Solution | SABNPs | RR@SABNPs |
|----------------------------|--------------|--------|-----------|
| C<sub>max</sub> (μg/mL)     | 33.51±10.72  | 59.16±4.61* | 80.27±18.08** |
| t<sub>1/2α</sub> (h)        | 0.014±0.007  | 0.080±0.052* | 0.098±0.171** |
| t<sub>1/2β</sub> (h)        | 0.558±0.236  | 1.828±1.178* | 30.53±15.64** |
| AUCl<sub>inf</sub> (μg/mL*h) | 10.92±5.77   | 38.94±15.48* | 1442.46±629.76** |
| MRT (mg/kg/(μg/mL))        | 0.47±0.32    | 2.46±1.94*  | 43.63±22.45** |

Notes: *p<0.05 compared with SAB solution; **p<0.05 compared with SABNPs. Data presented as mean ±SD, n=6.
Cellular Uptake of RR@SABNPs

To investigate the tropism of RR@SABNPs, the cellular uptake of RR@SABNPs was evaluated on bEnd.3 cells, which highly expressed integrin αvβ3.23 As shown in Figure 4A, a higher fluorescence in RR@SABNPs group was observed compared with SABNPs and R@SABNPs groups, indicating that RR@SABNPs can enter into bEnd.3 cells more efficiently. Flow cytometry analysis also showed the similar uptake efficiency, presenting the mean fluorescence intensity in bEnd.3 cells treated with RR@SABNPs was nearly 1.4-fold higher than that in bEnd.3 cells treated with SABNPs (Figure 4B and C). It has been reported that RGD could target integrin αvβ3 and enter the cytoplasm via receptor-mediated endocytosis.27 In order to illustrate the mechanism of cellular uptake of RR@SABNPs in bEnd.3 cells, the cellular uptake of RR@SABNPs in bEnd.3 cells with or without RGD pre-treated were evaluated. As shown in Figure 4D and E, the uptake of RR@SABNPs in RGD pre-treated bEnd.3 cells were significantly lower than that in untreated bEnd.3 cells. These results indicated that the RR@SABNPs exhibited a high affinity to integrin αvβ3 expressed cells, which contributed to RR@SABNPs precisely delivering SAB to the ischemic brain region that highly expressed integrin αvβ3.

The Ability of RR@SABNPs to Target the Ischemic Brain in vivo

To evaluate the ability of RR@SABNPs to target the ischemic brain in vivo, C57/BL6 mice were subjected to MCAO/R in the right hemisphere. Briefly, Dir loaded RR@SABNPs and SABNPs were intravenously administrated in C57/BL6 mice which received 1.5 h of MCAO and 16 h of reperfusion. Subsequently, these mice were imaged at the preset time to evaluate the distribution of nanoparticles in the brain. As shown in Figure 5A, compared with the control group, obvious fluorescence was observed in RR@SABNPs and SABNPs group, indicating the accumulation of nanoparticles in the brain. The increase of intracerebral fluorescence intensity over time indicated that the number of nanoparticles entering the brain increased, and the fluorescence was strongest at 6 h after drug administration (22 h after reperfusion). Meanwhile, enhanced intracerebral fluorescence was also observed in the mice treated with RR@SABNPs compared to the SABNPs group, suggesting that a higher amount of RR@SABNPs was accumulated in the brain via long circulation of RBCs camouflage and RGD-mediated. After 6 h, the mice were dissected, and the main organs were collected and imaged by an ex vivo image system (Figure 5B). The strongest fluorescence was observed in the liver of the mice in the SABNPs group, which was consistent with our previous study.28 However, contrary to literature reports,29 the modification of RGD in RR@SABNPs did not increase the distribution in the liver, which expressed high levels of integrin αvβ3.30 The decreased fluorescence intensity of the liver and spleen in the mice of RR@SABNPs group was attributed to the immune escape ability of RR@SABNPs endowed by RBCM decoration.31

In order to more intuitively evaluate whether RR@SABNPs can target ischemic brain tissue, the collected brain was dissected and analyzed. Figure 5D presented the representative coronal sections of the ischemic region and non-ischemic region. As shown in Figure 5D, there was fluorescence distribution in the ischemic and non-ischemic areas. The fluorescent intensity observed in the RR@SABNPs group was much higher (nearly 4-fold) than that of the SABNPs group (Figure 5D and E). Furtherly, the fluorescence intensity of the ischemic region was significantly higher in the RR@SABNPs group when compared to the SABNPs group, suggesting that a higher amount of SAB accumulated in the ischemic area (Figure 5D). In detail, the fluorescence ratio of the ipsilateral to the contralateral region increased to as high as 1.5 following RR@SABNPs administration (Figure 5F). These results indicated that the modification of RGD significantly enhanced the ischemic brain target of RR@SABNPs, which was attributable to integrin αvβ3. It has been reported that the RGD peptide sequence has a high affinity to integrin αvβ3,29,32 which is involved in angiogenesis after ischemia and expressed in the vessels of both the ischemic core and penumbra but not in the non-ischemic regions.33 Additionally, a higher fluorescence distribution in the non-ischemic area of RR@SABNPs
Figure 4 The cellular uptake of RR@SABNPs in Bend.3 cells. (A) Representative fluorescence images of different formulations uptake by Bend.3 cells. Scale bar, 50 μm; (B) Flow cytometry histograms of cellular uptake in Bend.3 cells; (C) Quantification of the mean fluorescence intensity of the flow cytometry histograms; (D) Representative fluorescence images of RR@SABNPs uptake by Bend.3 cells with or without RGD pre-treated. Scale bar, 50 μm; (E) Quantification of the mean fluorescence intensity of RR@SABNPs uptake by Bend.3 cells with or without RGD pre-treated by flow cytometry. Data are expressed as mean ± SD; n= 3; ***P < 0.001.
group which had a slight expression of integrin αvβ3, further demonstrated the advantage of longer circulation time endowed by RBCM coating.

Protective Effect of RR@SABNPs on MCAO/R Mice

To evaluate the therapeutic potential of RR@SABNPs to ischemic stroke, the mouse model of MCAO/R model was selected. As shown in Figure 6A, after reperfusion, the model mice were intravenously injected with the SAB solution, RR@SABNPs, or saline once a day for 2 consecutive days, and survival mice were executed at 24 h after the last administration. Samples were harvested and analyzed. Additionally, the neurological and the sensorimotor functions were measured by mNSS and foot fault test, respectively. The cerebral infarct volume was significantly increased after MCAO/R compared with the sham-operated group, indicating the successful establishment of MCAO/R model. With the treatment of the SAB solution, the cerebral infarct volume of MCAO/R model mice decreased slightly (from 48% to 43%), and there was no significant difference. Compared to the MCAO/R model group, the cerebral infarct volume of the RR@SABNPs group was significantly decreased (from 48% to 38%) (Figure 6B and C). Meanwhile, the neurological functions and the sensorimotor functions of the RR@SABNPs group were significantly improved, which was manifested by a decrease in the mNSS score (Figure 6D) and the number of fault steps in the horizontal grid floor (Figure 6E).
Figure 6F was a representative image of brain section of MCAO/R model mice, and peri-infarct areas (Cortex and Dentate Gyrus (DG)) were selected to evaluate pathological characteristics after treatment. As shown in Figure 6G, H&E staining revealed that the number of cells was severely decreased in the ischemic Cortex and DG regions of MCAO/R mice, while the density of cells remarkably increased in the RR@SABNPs group. To further evaluate the effect of RR@SABNPs in inhibition of neuronal loss in the brains of MCAO/R mice, a Nissl staining analysis was performed.
Compared with the sham-operated group, obvious cavities and fewer Nissen-positive cells were observed in the ischemic region of the MCAO/R group (Figure 6H). The condition had significantly improved after the RR@SABNPs administration, presenting a shrinking empty space and an increase in the number of Nisseni bodies in the penumbra area (Figure 6H). The results revealed that RR@SABNPs could efficiently inhibit neuronal loss of MCAO/R mice.

The Neuroprotective Mechanism of RR@SABNPs
To further investigate the neuroprotective mechanism of RR@SABNPs in ischemic stroke, the OGD/R injured PC12 cells model was established. The LDH release assay was used to assess the level of plasma membrane damage in a cell population. As shown in Figure 7A, OGD/R significantly increased the LDH release of PC12 cells than that of the control group. Similar to the previous reports, SAB treatment can significantly reverse the increased release of LDH.\textsuperscript{5} Compared with the SAB solution group, RR@SABNPs treatment further decreased the release of LDH, which may be contributed to the enhanced cellular uptake of soluble salvianolic acid B by nanoparticles. Excessive ROS production has been associated with impaired mitochondrial function and cell injury. Therefore, the intracellular ROS levels were evaluated.

*Figure 7* Neuroprotective mechanism of RR@SABNPs on OGD/R injured PC12 cells. (A) The LDH release of OGD/R injured PC12 cells; (B) Representative fluorescence images of intracellular ROS in OGD/R injured PC12 cells. Scale bar, 40 μm; (C) Representative fluorescence images of MMP in OGD/R injured PC12 cells. Scale bar, 40 μm. Data are expressed with mean ± SD; ***P < 0.001.
using DHE, an intracellular ROS indicator. As shown in Figure 7B, OGD/R significantly increased the fluorescence intensity of PC12 cells compared with the control group. The increased fluorescence intensity was conversed by the SAB solution, R@SABNPs, and especially RR@SABNPs. This result was consistent with the previous report that SAB could effectively remove intracellular ROS. Mitochondria are the primary source of ROS. Meanwhile, over-accumulated ROS plays a critical role in dysfunctional mitochondria. Post-ischemic reperfusion is usually accompanied by the decreasing of the MMP.34 We evaluated whether RR@SABNPs could inhibit mitochondrial dysfunction by increasing the mitochondrial membrane potential following OGD/R injury using a JC-1 fluorescence probe. As shown in Figure 7C, JC-1 emitted strong red fluorescence in the control group, indicating that JC-1 existed in the normal mitochondria in an aggregated state. After OGD/R injury, MMP decreased in PC12 cells, manifesting as decreased red fluorescence and increased green fluorescence. The RR@SABNPs administration significantly increased the MMP compared to the OGD/R group. To further verify whether RR@SABNPs can play a neuroprotective role by decreasing intracellular ROS level and increasing mitochondrial membrane potential, H₂O₂-induced oxidative damage model in PC12 cells was established. The results were presented in Figures S5-S7, and they were similar to those of the OGD/R model.

Safety Evaluation of RR@SABNPs in vivo
The effect of RR@SABNPs on the integrity of BBB was evaluated by Evans blue. As shown in Figure S8, there was no obvious Evans blue leakage in brain tissue administrated with RR@SABNPs compared with the control group, indicating that the BBB of RR@SABNPs treated mice was intact. To furtherly elucidate the biosafety of nanoparticles, H&E staining analysis of the main organs was performed. As shown in Figure 8, there was no obvious histological change in RR@SABNPs group compared with the control group. These results indicated that RR@SABNPs had good biocompatibility in vivo.

Conclusion
In this study, functionalized RBCM camouflaged SAB loaded BSA nanoparticles were developed to target the ischemic brain and treat ischemic stroke. The RBCM coating could reduce the cellular uptake of RR@SABNPs by macrophages, thereby prolonging its circulation time. The modification of RBCM with RGD endowed RR@SABNPs with ischemic BBB targeting ability via integrin αvβ3 mediated transcytosis and endocytosis, which was conducive to the specific accumulation in the ischemic brain of MCAO/R mice. Naturally, the accumulation of RR@SABNPs in the ischemic brain improved therapeutic effects for MCAO/R mice by scavenging intracellular ROS and maintaining MMP. However, there was no significant difference between SAB solution and RR@SABNPs in the cerebral infarct volume, neurological

Figure 8 The safety of RR@SABNPs in vivo. H&E staining of main organs in different groups. Scale bar, 50 μm.
functions and sensorimotor functions of MCAO/R model mice. It may be related to inadequate release and non-specific uptake of brain cells. Taken together, this ischemic brain targeted biomimetic strategy provides a potential platform for improving the long circulation performance and ischemic brain delivery of drugs. Meanwhile, the unfortunate pharmacodynamic results suggest that the importance of responsive drug release and cell and organelle targeting.

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Disclosure
The authors report no conflicts of interest.

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