Direct Macromolecular Drug Delivery to Cerebral Ischemia Area using Neutrophil-Mediated Nanoparticles

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Abstract

Delivery of macromolecular drugs to the brain is impeded by the blood brain barrier. The recruitment of leukocytes to lesions in the brain, a typical feature of neuroinflammation response which occurs in cerebral ischemia, offers a unique opportunity to deliver drugs to inflammation sites in the brain. In the present study, cross-linked dendrigraft poly-L-lysine (DGL) nanoparticles containing cis-aconitic anhydride-modified catalase and modified with PGP, an endogenous tripeptide that acts as a ligand with high affinity to neutrophils, were developed to form the PGP-PEG-DGL/CAT-Aco system. Significant binding efficiency to neutrophils, efficient protection of catalase enzymatic activity from degradation and effective transport to receiver cells were revealed in the delivery system. Delivery of catalase to ischemic subregions and cerebral neurocytes in MCAO mice was significantly enhanced, which obviously reducing infarct volume in MCAO mice. Thus, the therapeutic outcome of cerebral ischemia was greatly improved. The underlying mechanism was found to be related to the inhibition of ROS-mediated apoptosis. Considering that neuroinflammation occurs in many neurological disorders, the strategy developed here is not only promising for treatment of cerebral ischemia but also an effective approach for various CNS diseases related to inflammation.

Key words: brain targeting, catalase, neutrophils, PGP, ischemic stroke.

Introduction

Macromolecular drugs, including peptides and proteins, have been shown to be one of the promising treatments of neurological illness [1]. However, the inherent instability in plasma and the weak penetration through the blood brain barrier (BBB) of macromolecular drugs have hindered their further success [2]. Therefore, restricted entry of macromolecular neurotherapeutics to the brain has been a major bottleneck in the treatment of brain-related diseases, especially ischemic stroke (IS) [3]. Even though BBB integrity can be partially compromised during stroke, the reduction in blood supply to the ischemic area and subsequent “no-reflow” seriously impedes drug transport [4, 5]. Hence, an efficient macromolecular drug delivery system with brain targeting ability is highly desired to treat IS.

It has been reported that an ischemic cascade which involves overproduction of reactive oxygen species (ROS), failure of endogenous antioxidants to detoxify these entities, abnormal recruitment of inflammatory cells, and initiation of remarkable levels of cell apoptosis leads to severe brain damage and infarction [6]. Antioxidant enzymes can protect neurocytes from ROS-induced injury and have the potential to improve therapeutic outcomes for IS patients. As a promising neuroprotectant, catalase (CAT) can convert hydrogen peroxide, a representative ROS, to water and molecular oxygen with high turnover rates. It is an alternate source of
achieve brain targeting drug delivery at sequential time points following IS.

CXCR2, a classic chemoattractant receptor, was chosen as a targeting receptor in this study because it is prominently expressed on phagocytic cells, especially neutrophils [23]. As an endogenous degradation product of extracellular collagen, the tripeptide agonist N-acetyl Pro-Gly-Pro (Ac-PGP) exhibits high binding affinity and specificity to the CXCR2 receptor and a low risk of immunogenicity [24]. Ac-PGP has been recognized to be a key chemoattractant in inflammatory diseases and plays an important role in neutrophil influx in inflammatory conditions [25]. Hence, PGP can probably serve as a neutrophil anchoring peptide for brain targeting drug delivery.

Polymeric NPs are the most widely studied protein encapsulating system, which promote the stabilization and delivery efficiency of biopharmaceutical agents [26]. To improve drug loading and targeting of phagocytic cells in the circulatory system, complexation and assembly of proteins with oppositely charged polymers is very important [27]. In the present study, dendrigraft poly-L-lysine (DGL) generation 2 was used as the vector due to its excellent biodegradability and synthetic controllability as well as its excess of positive charge [28]. DGL was coupled with the targeting ligand PGP and with N-succinimidyl 3-(2-pyridyldithio)-propionate SPDP for active neutrophil targeting and improved stability [29-32], yielding a PGP-PEG-DGL-PDP polymer. CAT was modified with cis-aconitic anhydride to increase the charge density for better complexation with PGP-PEG-DGL-PDP through electronic interactions [33-35]. The system, cl PGP-PEG-DGL/CAT-Aco NPs, is illustrated in Figure. 2A.

In the system, cross-linkage of the polymer fixes the structure of the NP, suppresses dissociation and increases drug stability [29]. Modification with PGP ligands can endow the NPs with effective and selective susceptibility to phagocytosis in the circulation because of their high affinity to the receptors expressed on the surface of neutrophils. In principle, it is expected that proteins will be released from the carrier cells in the brain if they are not degraded by cellular proteases. The targeting ability of the constructed protein delivery system can be realized in three steps: first, arriving at the brain subregion by crossing the BBB; second, targeting to ischemic brain by neutrophil-mediated inflammatory migration; third, entering the cerebral neurocytes by transient intercellular connections and exosomes (Scheme 1). Thus, the targeting efficiency and the therapeutic efficacy of protein-based drugs for IS...
could be significantly improved. Considering that neuroinflammation occurs in many neurological disorders, the strategy presented here offers broad potential applications for the treatment of brain diseases.

Methods

Materials and Animals

All reagents were purchased from Sigma unless otherwise indicated. Recombinant Interleukin 8 Receptor Beta (IL8Rb) was from Cloud-Clone Corp (Product no. RPC006Hu01, Wuhan, China). DGL containing 48 primary amino groups (generation 2) was purchased from COLCOM (Montpellier Cedex, France). a-Malemidyl-u-N-hydroxysuccinimidyl polyethylene glycol (NHS-PEG-MAL, MW 3500) was obtained from JenKem Technology (Beijing, China). PGP peptide (Ac-Pro-Gly-Pro-His-His-Lys-Cys) was synthesized by China Peptides Co., Ltd. (Suzhou, Jiangsu, China). SPDP was purchased from ProteoChem, Inc. CAT from bovine liver was purchased from Calbiochem (CA, USA). BCA Protein Assay Kit was purchased from Beyotime Co. (Nantong, China). Dihydrorhodamine 123 (DHR) was purchased from AAT Bioquest Inc. (Sunnyvale, USA). TUNEL Assay Kit was obtained from Roche Diagnostics Corp.

The human promyelocytic leukemia cell line HL-60 was purchased from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). Adult male Sprague-Dawley rats (250–300 g), male C57BL/6 mice (25-30 g) and male BALB/c nude mice (25-30 g) were purchased from Sino-British SIPPR/BK Lab. Animal Co., Ltd. (Shanghai, China). The animal experiment protocol was approved by the Animal Experimentation Ethics Committee of Fudan University.

Synthesis and Characterization of PGP-PEG-DGL-PDP

The primary amino groups on the surface of DGL were specifically reacted with the NHS groups of the bifunctional PEG. NHS-PEG3.5K-MAL was dissolved in PBS 7.4 and then added drop-by-drop to stirred DGL (5 mg/ml) in PBS 7.4 solution and reacted for 1 h at a 5:1 molar ratio at room temperature. The PEG-DGL conjugate was obtained by purification using an ultrafiltration membrane (cutoff = 5 kDa). The MAL groups in PEG-DGL could specifically react with the PGP thiol groups. Thus, the PEG-DGL was mixed with PGP peptide at a 1:2 molar ratio (PEG-DGL: PGP) in PBS 7.4 for 24 h at room temperature, and the reaction product was purified with an ultrafiltration membrane (cutoff = 5 kDa) to obtain PGP-PEG-DGL. A two-fold molar excess of

![Scheme 1. In vivo processes of the circulatory neutrophil-mediated multi-brain targeting drug delivery system during brain ischemia.](http://www.thno.org)
SPDP dissolved in DMF was added to the PGP-PEG-DGL and allowed to react for an additional 1 h under gentle stirring in PBS with EDTA (100 mM Na₂PO₄, 1 mM EDTA, PH 7.4) at room temperature. A portion of the amino groups of DGL were substituted with pyridyldithiopropionyl (PDP) groups and purified with an ultrafiltration membrane (cutoff = 5 kDa) to obtain PGP-PEG-DGL-PDP polymer in a 10nM pH 7.4 Tris buffer. The above-purified polymer was freeze-dried for preparation of NPs. Then, 3 mg of polymer was redissolved in D₂O and analyzed in a 600 MHz spectrometer.

**Characterization of the PGP/CXCR2 Interaction Using SPR**

Binding was monitored with surface plasmon resonance (SPR) using a BIACORE X™ system. Anti-GST was deposited on a CM5 sensor chip (GE Healthcare) through amide-linkages. Recombinant IL8Rb/CXCR2 with two N-terminal GST tags (10 μg/ml in running buffer composed of 50 mM HEPES, pH 7.0, 0.01% CHS, 0.1% CHAPS, and 0.33 mM synthetic phospholipid blend [dioleoyl] DOPC:DOPS [7:3 wt/wt; Avanti polar lipids])[36] was then applied to the chip coated with anti-GST Ab at a flow rate of 5 L/min. Free PGP and cl PGP-modified NPs were dissolved in running buffer at a defined concentration and flowed over the CXCR2-immobilized CM5 chip to record resonance changes to assess binding affinity.

**Preparation of Dendrimer/CAT-Aco NPs**

cis-Aconitic anhydride (cis-Aco) was selected to react with free amino groups on the surface of CAT to enhance the negative charge on the protein surface. Briefly, cis-aconitic anhydride was dissolved in DMSO and added to CAT (5 mg/ml) in a pH 8.5 Na₂CO₃ solution at a 1:5 weight ratio (cis-Aco:CAT). N,N-diisopropylethylamine was added to the mixture, and the mixture was mechanically stirred at room temperature for 2 h. CAT-cis-aconitic anhydride (CAT-Aco) was purified from low molecular weight residuals using gel filtration with a Sephadex G-25 column in 10 mM Tris pH 7.4 and then lyophilized. CAT-Aco was mixed with different DGL derivatives (PEG-DGL, PGP-PEG-DGL, PEG-DGL-PDP, PGP-PEG-DGL-PDP) to electrostatically form NPs. CAT-Aco and the DGL derivative were dissolved in 10 mM pH 7.4 Tris at room temperature [37]. DTT (2 mg/ml) was added to the SPDP-modified DGL derivative (DGL 2.5 mg/ml) solution at a 1:40 volume ratio (DTT:DGL) and immediately vortexed for 15 min to reduce the disulfide bond of PDP to a thiol and then added dropwise to the stirred enzyme solution (1 mg/ml) to achieve a 2.5:1 weight ratio (DGL:CAT-Aco). The mixture was mechanically stirred at room temperature for 20 min. Then, DTT and released 2-thiopyridone were removed with dialysis against 5 L of Tris for 1 h. The SPDP-modified dendrimer/CAT-Aco NPs were cross-linked by oxidation of thiols introduced in the side chains of the lysine units of DGL to obtain cl (cross-linked) NPs during the dialysis process. Non-cl NPs were prepared using the same method described above, except the DGL derivative solution (without PDP groups) was not mixed with DTT.

**Characterization of Dendrimer/CAT-Aco NPs**

The encapsulation of CAT-Aco in DGL NPs was examined using an acrylamide gel shift assay, with naked CAT-Aco as the control. NPs composed of different weight ratios of PGP-PEG-DGL were mixed with appropriate amounts of 2% loading buffer and then electrophoresed on an 8.0% acrylamide gel containing 5 mM Tris and 50 mM glycine, at pH 8.3, under non-denaturing conditions (in the absence of SDS) to preserve the binding. The protein bands were stained with rabbit polyclonal anti-CAT antibody (Ab 6573, Abcam; 1:1000) and visualized with Immobilon Western Chemiluminescent HRP substrate (Millipore).

cl PGP-PEG-DGL/CAT-Aco NPs were freshly prepared with a specified ratio (2.5:1, DGL to CAT-Aco, w/w). The mean diameter and zeta potential of the NPs were determined with a Zeta Potential/Particle Sizer Nicomp 380 ZLS system (PPS Nicomp Particle Size System, USA). The morphology of the cl PGP-PEG-DGL/CAT-Aco NPs was examined under a high-resolution transmission electron microscope (TEM) (JEM-2010HT, Japan).

**Cell Culture**

HL-60 cells (a human promyelocytic leukemia cell line) were cultured in Iscove's modified Dulbecco’s minimum essential medium (IDMEM; Gibco Ltd, Paisley, UK) with 20% FBS and 1% penicillin-streptomycin (PS), and differentiated into PMN-like cells by adding 1.3% DMSO for 96 h as previously reported [24]. PC12 (a neuron-like rat pheochromocytoma cell line) neuronal cells were maintained in RPMI 1640 medium (Corning Cellgro, Mediatech, USA) supplemented with 10% FBS and 1% PS and 5% nonessential amino acids. Cells were maintained in an incubator in a controlled atmosphere of 37 °C, 95% relative humidity and 5% CO₂.

**Cellular Uptake and Mechanism Studies In Vitro**

HL-60 cells were added into each well of a 24-well plate (2.0×10⁶ cells/well) and then were co-incubated with FITC-labeled CAT alone or with

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different NPs at a CAT concentration of 20 μg/ml for 30 min at 37 °C in medium containing 10% FBS. After being washed with PBS twice, the cells were dispersed in DMEM and analyzed using flow cytometry (FACSCalibur, BD, USA).

The inhibition effect of different inhibitors on the cellular uptake of NPs by HL-60 cells was also measured. The cells were treated as described for the above procedures. After checking the morphology under a microscope, free PGP (0.5 mM), SB225002 (2 μg/ml), phenylarsine oxide (1 μM), filipin III (6 μg/ml) or colchicine (2 μg/ml) was added to each group of wells. HL-60 cells were incubated at 37 °C for 10 min. At the end of the incubation, the cells were rinsed with PBS twice and cultured in fresh medium. Subsequently, FITC-labeled PGP-PEG-DGL/CAT-Aco NPs with different inhibitors (the concentration of inhibitors was the same as that used for pre-incubation) was added to the corresponding group of wells. After 30 min of incubation with a DGL concentration of 100 μg/ml at 37 °C, the cell suspension was collected. The cells were washed three times with PBS and analyzed using flow cytometry. At least 10,000 events (cells) were analyzed for each sample.

**Intracellular Trafficking Studies**

To investigate the integrity of the NPs in the cells, first, DGL derivatives were labeled with BODIPY and CAT was labeled with FITC, and then, cl PGP-PEG-DGL/CAT-Aco NPs were prepared with the labeled molecules. To investigate the subcellular co-localization of NPs in the cells, BODIPY-labeled CAT was applied to complex with DGL, while the acidic endosomes were labeled with LysoTracker Green.

HL-60 cells were plated in 2-cm² dishes at a density of 2.5 × 10⁶ cells/dish and were then pretreated with LysoTracker Green (100 nM) for 60 min to label the acid compartments, followed by incubation with DAPI (0.05 mg/ml) for 5 min. After the nuclei were stained, the cells were incubated with BODIPY-labeled cl PGP-PEG-DGL/CAT-Aco NPs at a CAT concentration of 100 μg/ml for 5 min, 15 min 30 min, or 60 min. The mixture was harvested and then centrifuged at 500 g for 5 min at 4 °C. After being washed with PBS twice, cells were dispersed in PBS and fixed using Glycerol Jelly Mounting Medium. In addition, cells were treated with double dye-labeled cl PGP-PEG-DGL/CAT-Aco complexes as described previously.

**Cell-to-Cell Contact for NP Transfer**

HL-60 cells were plated in 6-well plates (3 × 10⁷ cells/well) and incubated with cl PGP-PEG-DGL/CAT-Aco NPs labeled with CAT-BODIPY (0.25 mg/ml DGL) for 1 h and were then cultured in 2 ml of DMEM supplemented with 4 μl of DiO (10 mg/ml) for 30 min at 37 °C. After being washed with HBSS twice, cells were dispersed in HBSS and added to PC12 neuronal cells. The appearance of NP-laden NPs in the cells (PC12 cells) was monitored with confocal microscopy at various time points.

**Exosome-facilitated NP Transfer**

HL-60 cells labeled with DiO were loaded with BODIPY 650/665-labeled NPs in the same manner as described above. Then, cells were supplemented with 20 μl of IFN-γ (20 ng/ml) and 1 μl of LPS (1 mg/ml) in 2 ml of HBSS solution for 2 h at 37 °C. Meanwhile, the cells were observed with confocal microscopy. Subsequently, the suspension was collected and filtered twice through low protein-binding 0.45 μm filters and then purified by gel filtration on a Sephadex G-25 column in 10 mM PH 7.4 Tris buffer to remove IFN and LPS. Isolated exosomes with encapsulated NPs were added to PC12 neuronal cells, and the dynamics of NP accumulation in receiver cells were observed with confocal microscopy.

**Model of Focal Cerebral Ischemia**

Transient MCAO mouse models were constructed according to a previously reported protocol, with minor modifications. Briefly, after 12 h of fasting, adult male BALB/c nude or C57BL/6 mice (25-30 g) were anesthetized with 10% chloral hydrate (6 ml/kg) via intraperitoneal (i.p.) injection. The left external carotid artery was incised under an operating microscope, and a monofilament nylon thread (Sunbio Biotech, China) was inserted through this incision into the internal carotid artery and further until it reached the bifurcation of the middle cerebral artery (MCA), a distance of up to 9-10 mm. A rounded tip coated with poly-L-lysine was used to occlude the entrance to the MCA and block the blood supply to the ipsilateral hemisphere of the mouse brain. After occlusion for 1.5 h, the filament was slowly withdrawn, and 1 ml of saline was administered via i.p. injection.

Adult male Sprague-Dawley rat (250-300 g) MCAO models were also constructed according to the protocol described above.

**Uptake of NPs by Neutrophils in vivo**

To quantify the uptake of NPs by granulocytes, NPs labeled with CAT-BODIPY 650/665 (7.5 mg/kg DGL) were injected into rats subjected to MCAO. Whole blood specimens were taken from rat eyes after 5 min, 15 min and 30 min. The red blood cells were lysed using RBC Lysis Buffer (eBioscience, San Diego, CA).
CA), and the cell suspension was washed with PBS. Then neutrophils were separated from total white blood cells by flow cytometry based on cellular size and granularity in the dot plot of forward scatter versus side scatter as previously described [38, 39]. The combination efficiency of the neutrophils with fluorescent NPs were measured by calculating BODIPY650/665 positive percentage of neutrophils with flow cytometry.

Qualitative assessment of NP internalization by neutrophils was carried out in vivo. Fluorescence-labeled NPs were injected into rats. At 15 min post-injection, granulocytes were isolated from abdominal aortic blood samples using OptiPrep™ Density Gradient Medium (Axis-Shield PoC AS, Oslo, Norway). Neutrophils with NPs were isolated with fluorescence activated cell sorting (FACS). The uptake of NPs was observed and recorded with confocal microscopy and TEM.

In Vivo Imaging

Male nude mice with IS were used for brain targeting evaluation. CL Dendrimer/CAT-Aco NPs were labeled with Bodipy 650/665 (Molecular Probes) and were then injected to the nude mice at a dose of 200 μg DGL/mouse 24 h post-establishment of MCAO model. At defined time points, fluorescence images of the brain were acquired using an in vivo imaging system (IVIS Spectrum, Caliper, USA). Mice were sacrificed, and the brains were dissected 3 h after injection to check the relative accumulation. In addition, the IS nude mice were administrated with Bodipy 650/665-labeled NPs as described above. After 1 h, the main organs were dissected for ex vivo fluorescence imaging.

Transport across the BBB In Vivo

To understand the targeting mechanism of CL PGP-PEG-DGL/CAT-Aco NPs, an immunofluorescence assay was performed. Bodipy 650/665-labeled NPs were intravenously administered to IS mice 24 h after reperfusion. Approximately 1 h later, mice were anesthetized, and their hearts were perfused with PBS followed by 4% formaldehyde in PBS for further study. Then, the brains were removed, fixed overnight in 4% formaldehyde, dehydrated in 15% sucrose solution until subsidence (12 h), and then in 30% sucrose until subsidence (24 h). Brains were frozen with O.C.T (Sakura, Torrance, CA, USA) at 80 °C and cut into 20-μm slices. Sections were blocked with 5% goat serum at 37 °C for 1 h. Microvessels were labeled with anti-CD31 antibody, neurons were stained with anti-MAP2 antibody, and DAPI was used for nuclear counterstaining. The distribution of fluorescence was visualized with a confocal microscope.

In Vivo MRI Studies

In vivo MRI images were acquired in C57 mice at 3 h, 24 h and 72 h post-establishment of the stroke model. Animals were anesthetized by inhalation of 2% isoflurane and then positioned in the magnet of a 7.0 T small animal MR scanner (Bruker PharmaScan, Germany). The mice were kept warm with a heating pad, and their respiration rates were monitored. Axial fast spin-echo T2-weighted magnetic resonance (T2W-MR) (repetition time ms /echo time ms: 3000/36, 1 average) images were acquired to show the ischemic territories. Mice were injected i.v. with NPs, PBS, or CAT-Aco alone (250 U/mouse) immediately following MRI, and MR examinations were repeated 48 h after treatment. The infarct volume visualized in the T2W images was quantified using the NIH Image J program and normalized to the volume of half the contralateral hemisphere.

Statistical Analysis

The data are presented as the mean ± standard deviation unless otherwise indicated. Student’s t-test and one-way analysis of variance (ANOVA) were applied to determine statistical significance between two groups and among more than two groups, respectively. A value of P < 0.05 was considered to be significant.

Results and Discussion

Preparation and Optimization of CAT-Aco NPs

Characterization of PGP-PEG-DGL-PDP

PGP-PEG-DGL-PDP was synthesized as illustrated in Fig. 1A. In 1H NMR spectra, the peaks between 1 and 2 ppm represented the protons of the three CH2 units in lysine (Figure 1B, a). Peaks near 3.6 ppm indicated repeat PEG units (Figure 1B, b). The disappearance of the characteristic peak of the MAL group at 6.7 ppm resulted from the reaction between the MAL group in PEG and the thiol group in the PGP peptide. The peak at approximately 7.6 ppm and 6.8 ppm represented the protons in the PGP histidine units, indicating the presence of PGP peptide (Figure 1B, c). The short peaks between 7.0 and 8.5 ppm in the 1H NMR spectra of PGP-PEG-DGL-PDP represented the pyridine ring in PDP (Figure 1B, d). Thus, the 1H NMR spectra confirmed the successful synthesis of PGP-PEG-DGL-PDP.

Interaction of the PGP Peptide with Receptor

The preparation process of CL PGP-PEG-DGL/CAT-Aco NPs is illustrated in Figure 2A. The binding
affinity of PGP peptides with the targeted receptors is crucial in this innovative design. SPR was conducted to assess the capacity of PGP peptide to bind CXCR2 immobilized on a BIACore sensor chip coated with a lipid bilayer. As shown in Figure 2B (insert), the interaction between PGP and CXCR2 was dose dependent, which indicated that the PGP peptide was capable of binding to CXCR2 immobilized on the sensor chip. Free PGP and cl PGP-PEG-DGL/CAT-Aco NPs displayed similar binding affinity to CXCR2, and their equilibrium dissociation constants (KD) were 2.719x10^{-7} and 1.576x10^{-7} M, respectively. The SPR results indicated that conjugation of the PGP peptide on the surface of the NP did not alter its binding affinity with CXCR2.

Characterization of Dendrimer/CAT-Aco NPs

As shown in Table S1, the size and zeta potential of the NPs were approximately 100 nm and 5.0 mV, respectively. Conjugation of the peptide ligand did not significantly affect the properties of the NPs. The particle size of the cl NPs was approximately 100 nm, with a positive charge, and favorable for adsorption to the negatively charged membrane of phagocytic cells and subsequent internalization [40]. The transmission electron micrograph (Figure 2C) showed that cl PGP-PEG-DGL/CAT-Aco NPs had a well-formed spherical shape.

The results of a gel shift assay shown in Figure 2D indicate that modified CAT could be partially encapsulated by a non-cl vector but completely encapsulated by the cl vector. Compared with naked CAT-Aco, there was no electrophoretic shift when the weight ratio of DGL to CAT-Aco was greater than 2.5, because the mobility of the CAT-Aco was significantly slowed.

Besides the qualitative assessment by acrylamide gel shift assay, we also measured the amount of protein entrapped by the dendritic polymers by CAT-Aco complexation efficiency [41]. Ultracentrifugation was used to separate CAT-loaded nanoparticles from the aqueous suspension medium. Absorbance of free CAT-Aco (at 404 nm) in the supernatant was tested and the corresponding concentration of free CAT-Aco was determined with a standard curve. The complexation efficiency of was calculated using the formula as reported previously [1]. In the present study, the complexation efficiency of CAT-Aco in cl PGP-PEG-DGL/CAT-Aco NPs was 70.69±0.46%, which was much higher than that in non-cl PGP-PEG-DGL/CAT-Aco NPs of 58.83±2.28%.

Figure 1. (A) Synthetic route for PGP-PEG-DGL-PDP. (B) The 1H-NMR spectrum of PGP-PEG-DGL-PDP in D_{2}O at 600 MHz.
Cytotoxicity of NPs in Neutrophil-like Differentiated HL-60 cells

HL-60 neutrophils were used as an in vitro model to evaluate the cytotoxicity of non-cl and cl PGP-PEG-DGL/CAT NPs (Figure S1A). The concentration of DGL ranged from 20 to 500 μg/ml. The cells treated with cl NPs showed higher viability (lower cytotoxicity) than those treated with non-cl NPs. No apparent decrease in cell viability was observed when the concentration of cl NPs was higher than 500 μg/ml. The percentage of viable cells after treatment with cl NPs remained higher than 70% at NP concentrations lower than 200 μg/ml. The cytotoxicity of non-cl NPs increased when the concentration was lower than 80 μg/ml but decreased when the concentration of DGL was higher than 80 μg/ml. This might be caused by the release of DGL, which can interact with negatively charged cellular membranes and other large-molecules through its polycation, as documented in the literature [42].

Preservation of Catalase Activity Against Degradation in Carrier Cells

The ability of different NPs to protect the enzymatic activity of CAT inside neutrophils was
assayed (Figure S1B&C). HL-60 cells were incubated with CAT alone or PGP-PEG-DGL/CAT-Aco complex for 1 h, washed with PBS, and then incubated with CAT-free media for different lengths of time. The activity of CAT released into the media and retained in the cells was determined by examining the rate of hydrogen peroxide decomposition. The results clearly indicated that cross-linked NPs showed significant protection of CAT inside neutrophils compared to free CAT and non-cl NPs. CAT loaded in non-cl NPs was degraded in cells to a lesser extent than the “naked” enzyme (Free CAT-Aco) before 4 h. In medium, the enzyme activity of both cl NPs and non-cl NPs increased with time. Preservation of CAT enzymatic activity in neutrophils by the NPs was primarily due to Protection from the acid in endocytic compartments in the cells by the “proton sponge effect” of the amino groups on the surface of the NPs [43].

**Targeting Mechanism Investigation In Vitro**

Comparison of the Uptake of Different CAT-Aco NPs by HL-60 cells

The cellular uptake of FITC-labeled “naked” CAT and various DGL vector/CAT-Aco NPs were compared in HL-60 cells using a flow cytometric analysis. From the results shown in Figure 3A & S2, we can find that the uptake of CAT was enhanced after encapsulating into NPs. The effect was further facilitated by the modification of PGP and the cross-linking of NPs. Thus, the cl NPs were selected and studied in further experiments.

**Mechanism of Cell Uptake of NPs**

HL-60 cells have been intensively applied as a reliable cell line for evaluation of the functional regulation of neutrophils [44]. To clarify the endocytic mechanism of cl PGP-PEG-DGL/CAT-Aco NP uptake, HL-60 cells were pretreated with different endocytic pathway inhibitors. As shown in Figure 3B, the results of the competition assay revealed that the addition of free PGP peptide [25] and SB225002, a CXCR2 receptor antagonist [45], significantly inhibited the cellular uptake of cl PGP-PEG-DGL/CAT-Aco NPs, which indicated that the PGP modification is crucial for effective internalization of NPs by neutrophils through specific binding to CXCR2 receptors on HL-60 cells. Other inhibitors involved in various endocytic processes were also studied. PhAsO, an inhibitor of clathrin-dependent endocytosis [46], also remarkably decreased the cellular uptake of cl PGP-PEG-DGL/CAT-Aco NPs. In contrast, filipin III [47], an inhibitor of caveolin-mediated endocytosis, which is relevant to the surface charge of the NPs, had no effect on the uptake of cl PGP-PEG-DGL/CAT-Aco NPs. Meanwhile, treatment with colchicine, which could signify particle size-related micropinocytosis, inhibited the uptake of NPs. Hence, the results show that clathrin-mediated endocytosis and micropinocytosis were primarily responsible for the internalization of cl PGP-modified NPs by neutrophils.

**Figure 3.** (A) Flow cytometric determination of fluorescence intensity within HL-60 cells treated with FITC-labeled catalase NPs with or without modification of PGP ligand. (B) Inhibition of the cellular uptake of cl PGP-PEG-DGL/CAT-Aco NPs. The data represent the mean ± SD, n=3. **P<0.01, compared with control group. (C) Confocal images of HL-60 cells incubated with cl PGP-PEG-DGL/CAT-Aco NPs for 15 min and 60 min. Green: FITC-labeled catalase. Red: BODIPY 650/665-labeled DGL. The scale bar represents 2 μm. (D) Confocal images of HL-60 cells exposed to cl PGP-PEG-DGL/CAT-Aco NPs for 5 min, 15 min, 30 min and 60 min. Green: LysoTracker Green used to label acidic endosomes. Red: BODIPY 650/665-labeled catalase. Bars represent 2 μm.
Intracellular Trafficking of NPs

The integrity of drug-loaded NPs in carrier cells is very important for delivery of the enzyme to the brain. To investigate the structure of cl PGP-PEG-DGL/CAT-Aco NPs in HL-60 cells, we labeled CAT with FITC and DGL with BODIPY 650/665 and observed their intracellular localization in HL-60 cells with confocal microscopy (Figure 3C). As expected, with the cl NPs, total co-localization of the polymer and CAT was observed inside HL-60 cells after 15 min of incubation. Interestingly, when the NPs were incubated with cells for 60 min, a portion of them were dissociated and separation of the CAT and DGL vector fluorescence could be observed, which was presumably due to cleavage of NP disulfide bonds while they were in the cells [29, 33].

Co-localization of the NPs and acidified endosomes was detected using confocal microscopy (Figure 3D). Fluorescently labeled CAT was used in cl PGP-PEG-DGL/CAT-Aco NPs and acidified endosomes were stained with LysoTracker Green. Initially, cl NPs were found to be mostly distributed in the cytoplasm (5 min), and then, they partially co-localized with acidic endosomes at 15 min and 30-60 min while mainly presented in the cytoplasm. The results indicated that cl PGP-PEG-DGL/CAT-Aco NPs might not be primarily taken up by HL-60 cells via endosome-lysosome pathways. This intracellular process might shield most proteins from degradation by cellular proteases. Endosomal escape of cl NPs was in agreement with the results reported by many other investigators concerning the “proton sponge” effect induced by amino groups on the surface of NPs [9, 48]. In addition, the acidified endosomes might to a certain degree induce charge conversion of modified CAT (CAT-Aco) to the original protein (CAT) [33].

Brain-targeting Effect In Vitro

Transwell migration assay was used to mimic the fate of neutrophils containing nanoparticles migrate across a monolayer of endothelial cells (HUVECs) and then deliver the nanoparticles to neuron cells [49, 50]. HUVECs were grown to confluence on porous membranes for 9 days followed by co-cultured with PC12 neuronal cells for 3 days. PC12 neuronal cells were labeled with cell tracer (CFSE) in the lower chamber. Meanwhile, BODIPY 650/665-labeled NPs were loaded with neutrophil-like differential HL-60s stained with HOECHST. Then the HL-60 cells were collected and added to the upper chamber above confluent HUVECs. IL-8 was added into the lower chamber to establish a chemotactic gradient for neutrophil migration across a monolayer of endothelial cells (HUVECs). Quantitative measurement of BODIPY-positive cells by flow cytometry was shown in Figure S3. It was found that the number of neutrophils and neuron cells containing NPs significantly increased in lower chamber with the stimulation of IL-8. The results demonstrated that inflammation promotes the transendothelial migration of NPs via neutrophils, which were contributable to the upregulation of nanoparticle uptake by neuron cells. Confocal microscopy images also clearly showed that NPs (red) released from neutrophils (blue) were co-localized with neuron cells (green) when IL-8 was added to the lower chamber. (Figure S4).

Cell-to-Cell Interaction for the Transfer of NPs

When neutrophils carrying NPs are recruited to the sites of inflammation in the brain, it is of interest to know how the enzyme-loaded NPs are delivered into neuronal cells. A time course of NPs labeled with CAT-BODIPY 650/665 transferred from HL-60 cells to PC12 neuronal cells was observed using confocal microscopy. HL-60 cells stained with DiO dye (green), collected and then placed on top of PC 12 neuronal cells (contact). Accumulation and co-localization of HL-60 lipids (green) with NPs (red) in non-stained PC12 neuronal cells were manifested in yellow (Figure 4A, shown with arrows). The data revealed that cell-to-cell contact facilitated the transfer of NPs along with lipid compartments from HL-60 cells to PC12 neuronal cells.

Effect of Exosomes during the Transfer of NPs

To investigate whether exosomes facilitate the transfer of NPs from neutrophils to neuronal cells, DiO (green)-labeled HL-60 cells were pre-incubated with NPs labeled with CAT-BODIPY 650/665 (red) and were visualized using confocal microscopy after two hours of incubation with or without stimulation of lipopolysaccharide (LPS) and interferon gamma (IFN-γ). Few exosomes were observed in non-activated neutrophils; meanwhile, in LPS- and IFN-γ-activated neutrophils, exosomes were clearly secreted from the cells, suggesting release of NPs from the carrier cells at the inflammation site (Figure 4B). The exosomes with encapsulated NPs released from neutrophils into the extracellular medium were harvested and purified. Then, the isolated exosomes were added to neuron-like differentiated PC12 neuronal cells. The dynamics of NP accumulation within receiver cells were visualized with confocal microscopy (Figure 4C). Images clearly showed that the exosomes could be adsorbed to the surface of the receiver cells and fuse with the cellular membrane.
Discharge of NPs into the cytoplasm of neuronal cells resulted in diffuse fluorescence. The phenomenon could be explained by NP incorporation into exosomes, which can increase binding of the drug to lipid membranes of receiver cells.

**Evaluation of the In Vivo Targeting Effect**

**Uptake of NPs by Neutrophils In Vivo**

Flow cytometry, transmission electron microscopy and confocal microscopy were utilized to elucidate the role of circulating leukocytes, mainly neutrophils, in transporting NPs to the brain. Circulating neutrophils were isolated using RBC Lysis Buffer from the blood of rats given cl PEG-DGL/CAT-Aco and cl PGP-PEG-DGL/CAT-Aco NPs. The extent of fluorescence-labeled NP endocytosis was detected with flow cytometry over time after injection of the NPs (Figure 5A & S5). Cellular uptake results reflected that cl PGP-PEG-DGL/CAT-Aco NPs were rapidly internalized by neutrophils 5 min postinjection followed by overtime elimination. It was evident that cl PGP-PEG-DGL/CAT-Aco NPs displayed significantly higher cellular uptake than unmodified cl PEG-DGL/CAT-Aco NPs, especially at shorter time points.

To obtain further evidence to confirm endocytosis of NPs by neutrophils, we isolated neutrophils from rat blood 15 min post-administration of NPs labeled with CAT-BODIPY using OptiPrep™ Density Gradient Medium. The neutrophils with NPs were isolated using FACS. The presence of NPs in the endocytic compartments of cells was identified by TEM (Figure 5B). Confocal microscopy images illustrated that the components of NPs, both DGL vector and CAT, could be simultaneously detected in neutrophils, indicating that NPs were effectively taken up by neutrophils (Figure 5C).

**Evaluation of NP Brain targeting effect**

The brain targeting efficiency of cl PGP-PEG-DGL/CAT-Aco NPs and cl PEG-DGL/CAT-Aco NPs was compared using two parallel experiments in nude mice pre-treated with transient MCAO. Extravasation of CAT in vesicular structures was observed mostly in ischemic regions 1 h after treatment with cl PGP-PEG-DGL/CAT-Aco NPs (Figure 5D). The distribution of cl PGP-PEG-DGL NPs was also found to a relatively greater extent in healthy brain capillaries in stroke mice, which could be explained by the fact that CXCR2, as a PGP-specific receptor, is also expressed on brain microvascular endothelial cells [51]. It is worth noting that a high combination of cl PGP-PEG-DGL/CAT-Aco NPs with endothelial cells is beneficial for treatment of cerebrovascular disorders, including IS, due to the protection of the BBB from oxidative damage.

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**Figure 4.** (A) Transfer of NPs from HL-60 cells to PC12 neuronal cells through intercellular fusion. Bar represents 10 μm. (B) Facilitated release of NPs from activated HL-60 cells. Bar represents 10 μm. (C) Kinetic transport of NPs via exosomes from HL-60 cells to PC12 neuronal cells. Bar represents 20 μm.
Figure 5. (A) Flow cytometry assay of NP endocytosis by neutrophils isolated from MCAO rat plasma at different times. The data shown represents the mean ± SD, n=5, P<0.05, compared with the cl PEG-DGL/CAT-Aco group. (B) TEM images of NPs inside the circulatory neutrophils of MCAO rats after injection of cl PGP-PEG-DGL/CAT-Aco. Some typical NPs are indicated by arrows. (C) Fluorescence microscopy images of NPs taken up by neutrophils in rat blood after i.v. injection of double dye-labeled cl PGP-PEG-DGL/CAT-Aco NPs. Bars represent 5 μm. (D) Distribution of BODIPY 650/665-labeled CAT in the brain subregion after administration of different cl dendrimer/CAT-Aco NPs. Blood vessels were stained with anti-CD31 (green). (E) Uptake of fluorescence-modified CAT into neurons of MCAO mice after injection of cl NPs. Neurons were labeled with MAP-2 (green). Red represents the drug. I.H., ischemic hemisphere; C.H., control hemisphere. Bar represents 10 μm.

Furthermore, the fluorescence of cl PGP-PEG-DGL/CAT-Aco NPs, which was co-localized with neutrophils, was higher than that of cl PEG-DGL/CAT-Aco NPs in brain ischemic area (shown as arrows). It depicted that PGP modified NPs, which was effectively taken up by neutrophils, showed higher drugs accumulation in ischemic subregion. (Figure S6). The phenomenon presumably attributes to the transendothelial migration of neutrophils with NPs. Co-localization of the fluorescence-labeled CAT in cl PGP-PEG-DGL/CAT-Aco group and MAP2 antibody-labeled neurons
was observed, indicating entry of translocated vesicular structures into recipient cells in MCAO mice (Figure 5E). Unsurprisingly, the amount of labeled enzyme in cl PGP-PEG-DGL/CAT-Aco group was much more than that in cl PEG-DGL/CAT-Aco group.

Images of NPs loaded with BODIPY 650/665-labeled CAT are shown in Figure 6A. The in vivo imaging results reflected that the protein drug, loaded in cl PGP-PEG-DGL/CAT-Aco NPs, accumulated in brain in 2 h followed by a slow elimination (Figure 6A). Semi-quantitative ROI analysis revealed that an obviously enhanced brain targeting efficiency of cl PGP-PEG-DGL/CAT-Aco NPs compared with NPs without PGP decoration (Figure 6B). The ex vivo images of the distribution of NPs in main organs were acquired 1 h after i.v. injection. Compared with cl PEG-DGL/CAT-Aco NPs, cl PGP-PEG-DGL/CAT-Aco NPs showed increased accumulation in the brain. There was no obvious difference in other organs between two groups (Figure S6). The result confirmed that the modification of PGP and its rapid binding with the neutrophils in the blood accounts for the targeting specificity of the NPs to the brain. In order to further elucidate the effect of neutrophils on the delivery of drug-loaded NPs into the ischemic brain, we depleted neutrophils using anti-Gr-1 antibody as reported previously [21, 52]. MCAO models were constructed two days after neutrophils depletion via i.p. injection with anti-Gr-1 antibody in nude mice. BODIPY 650/665-labeled cl PGP-PEG-DGL/CAT-Aco NPs were intravenously administrated to IS mice 24 hours after reperfusion. The in vivo brain fluorescence images (Figure 6C) demonstrated that the injection of anti-Gr-1 antibody could significantly reduce the brain distribution of NPs in MCAO mice, which confirmed the importance of neutrophils during the delivery of NPs to the ischemic brain. Based on the above results, we concluded that cl PGP-PEG-DGL/CAT-Aco NPs not only enhanced enzyme accumulation in the brain but in the ischemic subregion and cerebral neurocytes in response to stroke.

Therapeutic Efficacy of NPs in MCAO Mice with Ischemia/Reperfusion Injury

The therapeutic efficiency of the neutrophil-mediated neuroprotectant delivery system was evaluated in a mouse MCAO model of IS. To assess the efficacy of cl PGP-PEG-DGL/CAT-Aco NPs in treating brain injury, we used in vivo T2W-MRI as a non-invasive imaging technology to delineate the ischemic volume 48 h after different treatments. The images in Figure 6D depict the ischemic territory as a hyper-intensive signal corresponding to cerebral edema after transient focal cerebral ischemia. In the mice that received cl PGP-PEG-DGL/CAT-Aco NPs at 3 h, 24 h and 72 h after reperfusion, the infarct volume was significantly reduced to approximately 68.5%, 57.7% and 33.1% of the PBS group (P<0.001), respectively. The result was closely related to the general neurological score acquired before MRI, which showed significant reduction to approximately 68.1%, 55.8% and 48.3% of the PBS group (P<0.001) at the same time point after cl PGP-PEG-DGL/CAT-Aco NPs administration (Figure S8). Both evaluation experiments jointly referred to the point that the effect of cl PGP-PEG-DGL/CAT-Aco NPs was much better than that of the cl PEG-DGL/CAT-Aco NPs (P<0.05 at 3 h and P<0.01 at 24 h and 72 h). Furthermore, we presumed that the therapeutic efficiency of cl PGP-PEG-DGL/CAT-Aco NPs improved with time, primarily due to the increased amount of CAT delivered by the neutrophils recruited to the ischemic area via inflammatory response. This is of considerable importance for extension of therapeutic time window in acute cerebral ischemia.

To verify that the significant neuroprotective effect of NPs was a result of CAT-mediated ROS scavenging, the ROS levels in ischemic brains were detected by injection of the hydrogen peroxide-sensitive mitochondrial probe DHR, which can be oxidized by ROS to fluorescent rhodamine 123 (RH123). The level of ROS in ischemic brains 24 h post-treatment was evaluated by visualizing the RH123 fluorescence. The therapeutic effect was further evaluated by quantifying the apoptotic cells in the ischemic area via TUNEL staining. The RH123 and TUNEL signals were distributed widely in the ischemic area of the mice injected with PBS or CAT-Aco alone, while fewer and less intense signals were found in mice injected with NPs, especially the NPs decorated with PGP. There was a significant difference in the percentage of RH123-positive cells between the mice treated with cl PGP-PEG-DGL/Cat-Aco NPs and those treated with cl PEG-DGL/Cat-Aco NPs as well as in the percentage of TUNEL-positive cells. The reduced percentage of RH123-positive and TUNEL-positive cells was interpreted to indicate that inhibition of the ROS-dependent apoptosis pathway played an important role in the efficacy. Hematoxylin and eosin (HE) staining indicated that nearly all of the neurocyte nuclei were pyknotic in the ischemic area (I). Above all, high fluorescence intensity from RH123 and TUNEL was localized in the ischemic area (I) marked with HE staining (Figure S9).
Figure 6. (A) In vivo fluorescence images of the brains of nude mice subjected to MCAO after administration of cl dendrimer/CAT-Aco NPs labeled with CAT-BODIPY; ex vivo NIR fluorescence image of harvested brain 3 h post-treatment. (B) Semi-quantitative ROI analysis of the average fluorescence intensity from the BODIPY 650/665-labeled NPs in ischemic brain (mean ± SD, n=3). *P<0.05, **P<0.01 vs. cl PEG-DGL/CAT-Aco. (C) In vivo fluorescence images of nude mice brain after cl PGP-PEG-DGL/CAT-Aco NPs administration with or without the injection of anti-Gr-1 antibody (mean ± SD, n=3). NPs were labeled with CAT-BODIPY. ***P<0.01, ****P<0.001 vs. the healthy. (D) In vivo T2W-MRI images of ischemic mouse brain at 48 h post-injection of PBS, CAT-Aco alone (250 U/mouse), cl PEG-DGL/CAT-Aco and cl PGP-PEG-DGL/CAT-Aco (mean ± SD, n=6). *P<0.05, **P<0.01, ***P<0.001 vs. the PBS group.

Conclusion

In this study, we successfully constructed novel cl PGP-PEG-DGL/CAT-Aco NPs for delivery of macromolecular protein-based drugs, and the NPs exhibited an excellent multi-brain targeting effect. The effect was attributed to the strong binding efficiency of PGP ligands on the surface of the NPs with neutrophils in the blood, the ability of neutrophils to migrate through the BBB, and the tropism of
leucocytes to the inflammation site and the cells in the brain. Realization of the purpose of these NPs depends on high protein drug loading and encapsulation efficiency, specific and high internalization of NPs by phagocytic cells in the circulation, sufficient protection of the protein-based drug from degradation within carrier cells, targeted delivery to the diseased sites in the brain and triggered release at the site of inflammation. The process and mechanisms were comprehensively illustrated in vitro and in vivo in our study. Administration of cl PCP-PEG-DGL/CAT-Aco NPs significantly reduced the infarct volume of MCAO mice and enhanced the therapeutic outcome of cerebral ischemia. Considering that neuroinflammation occurs in many neurological disorders, the strategy reported here offers broad potential applications for treatment of brain diseases. We believe that the developed delivery system is not only promising for efficient treatment of IS but may also be an effective approach for various CNS diseases related to inflammation.

Abbreviations
cross-linked, cl; catalase, CAT; cis-aconitic anhydride, cis-Aco; CAT-cis-Aco, CAT-Aco; nanoparticle, NP.

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Supplementary Material
Supplementary figures and tables. http://www.thno.org/v07p3260s1.pdf

Competing Interests
The authors have declared that no competing interest exists.

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