The Permeability of Puerarin Loaded Poly(butylcyanoacrylate) Nanoparticles Coated with Polysorbate 80 on the Blood–Brain Barrier and ItsProtective Effect against Cerebral Ischemia/Reperfusion Injury

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Puerarin (PUE) is a good candidate for treating stroke, but its low concentration in brain after administration limits its curative efficacy. The aim of the present work was to design and characterize PUE loaded poly(butylcyanoacrylate) nanoparticles (PBCN) coated with polysorbate 80 (Ps 80), and to evaluate the effect of PBCN on the permeability of PUE across the blood–brain barrier (BBB) and the effect of PUE loaded PBCN on the cerebral ischemia/reperfusion injury. PUE loaded PBCN were successfully prepared by anionic polymerization method with the mean particle size of 201.2 nm and the zeta potential of −7.72 mV. The in vitro release behavior of PUE from the nanoparticles showed a biphasic profile manner with an initial burst release followed by a sustained release. The results of pharmacokinetic and biodistribution to brain performed in mice after intravenous administration showed that the drug concentrations in blood and brain for PUE loaded PBCN were both greater than these for the free drug. Moreover, compared with free drug, the vein injection of PUE loaded PBCN exerted the better neuroprotective effect in rats with focal cerebral ischemic injury via significantly decreasing neurological deficit scores, increasing body weight, lowering brain water content, and reducing the infarct volume. The results indicated that this preparation may reduce the total dose required for the stroke therapy with concurrent reduction in dose related toxicity. All these findings suggest that PBCN could enhance the transport of PUE to brain and have a potential as a neuroprotective agent in the focal cerebral ischemic injury.

Key words puerarin; poly(butylcyanoacrylate); nanoparticle; brain biodistribution; neuroprotective effect

The management of brain related diseases is often complicated by the inability of drugs to pass the blood–brain barrier (BBB) even if some pathologies alter the permeability of this barrier. BBB is dynamic barrier impeding the passage of drugs from the blood compartment to the brain. Enhancing drug level in the brain is helpful for the treatment of brain diseases (with or without disruption of BBB), because the higher concentration could contribute to decrease drug dose, reduce drug side effects, increased drug viability, and improved quality of patient’s life.1 Stroke is the third leading cause of death worldwide, and it results in the permanently disabled of 15 to 30% of patients and accounts for socioeconomic burden due to a rise in the aging population.2–4 A thrombus occluding a brain artery is the leading mechanism underlying ischemic stroke,5 which accounts for approximately 70–85% of all stroke6 and no effective therapies are available.7 The safe and effective drugs have been the research focus of ischemic stroke therapy.

Puerarin (PUE) (Fig. 1) is a isoflavone C-glycoside found in Traditional Chinese Medicinal herbs such as Pueraria lobata (Willd), Ohwi, and P. thomsonii Benth, which is known due to its potential effects on health benefits in oriental, and has been proved to be helpful in the treatment of cardiovascular, neurological and hyperglycemic diseases.8–11 PUE have been shown to have the pharmacologic action of protecting against cerebral ischemia mediated by the inhibition of inflammatory responses, apoptosis, and neutrophil activation in vitro and in vivo.12–14 The low drug concentration of PUE in brain after intravenous (i.v.) administration results from its low lipid solubility and be a substrate of P-glycoprotein.16 Which limited the clinical advantages of puerarin, thus highlights the need for development of a suitable preparation to help it cross the BBB.

In recent years, nanoparticles (NP) as carrier have been used to deliver drugs to the brain, which provides a significant advantage to current strategies. Currently polymeric nanoparticles is the focus of attention as potential brain-targeting drug systems which could enhance the concentration and slow release of drug in the brain as well as decreasing peripheral toxicity.19 It was proven that poly(butylcyanoacrylate) nanoparticles (PBCN) coated with polysorbate 80 (Ps 80) could improve the delivery of the therapeutic agents to brain after i.v. administration.17,19,20 In this work, the PUE-loaded PBCN (PUE-PBCN) was prepared, and characterized with size, zeta

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Fig. 1. Chemical Structure of PUE
potential, and in vitro release study. The pharmacokinetics and biodistribution to brain in mice as well as the pharmacological effect on the ischemia/reperfusion injury model in rats of PUE-PBCN were evaluated.

MATERIALS AND METHODS

Materials  PUE was obtained from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). Dextran 70 (α-+Glucose) and poloxamer 188 (F68) were purchased from the Shanghai Treechem Biotech Co., Ltd. (Shanghai, China). Ps 80 was supplied by Shanghai Chemical Reagent Co., Ltd. (Shanghai, China). N-Butylcyclooctylacrylate (BCA) was provided by Beijing Suncon Medical Adhesive Co., Ltd. (Beijing, China). PUE injection was produced by Ruiyang Pharmaceutical Co., Ltd. (Zibo, China). 2,3,5-Triphenyltetrazolium chloride (TTC) was purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents and solvents used in the study were of best grade available commercially.

Animals  Kunming mice (18–22 g) and Wistar rats (220–250 g) were supplied by the Medical Animal Test Center of Shandong University. The animals were acclimatized at 25±2°C, a relative humidity of 60±5% and a 12 h dark/light cycle for 7 d before the study and fasted overnight before experiment with free access to water. All animal experiments complied with the requirements of National Institutes of Health Guide for the Care and Use of Laboratory Animals (People’s Republic of China).

Preparation and Characteristics of PBCN  The PBCN loading PUE was prepared using open anionic polymerization method according to previous reports.21) The polymerization reaction was carried out in acidic medium (0.01m HCl) containing 0.5% (w/v) of dextran 70 as stabilizer and 0.5% (w/v) of Poloxamer 188 as emulsifier. Crude PUE was first dissolved in 0.01m HCl and added to the above system. The monomer BCA was added drop by drop into the stirred medium (the weight ratio of drug to polymer was 1:2), and conducted for 4 h under magnetic agitation at 600 rpm and room temperature. Subsequently, the pH of resulting suspension was adjusted to about 6.8 with sodium hydroxide solution and kept at 1 mL/min and 25°C. The supernatant was withdrawn and evaporated to dryness under dry nitrogen atmosphere in water bath at 37°C. The dry samples were redissolved in 100 µL methanol. A 20 µL of this sample solution was injected into HPLC system for determining PUE in plasma and brain samples. The Agilent 1100 series HPLC system (Agilent Co., U.S.A.) with column C18 (4.6×150mm, i.d. 5µm, Agilent, U.S.A.) protected by RP18 (3 mm) guard column (Phenomenex, U.S.A.) was used for the analysis of PUE in plasma or brain tissue. The mobile phase consisted of acetonitrile and water at the volume ratio of 11:89 with the flow rate and the column temperature maintained at 1 mL/min and 25°C, respectively. The low limit of this method for PUE was 14.7 ng·mL⁻¹ or ng·g⁻¹.

Effect of PUE-PBCN on the Model Rats with Middle Cerebral Artery Occlusion (MCAO) and Reperfusion  The protection against cerebral ischemia of PUE-PBCN was studied by a MCAO and reperfusion model in Wistar rats. All animals were weighted and divided into 4 groups (n=12): (1) sham-operation group, (2) control group, intravenous administrated with saline solution, (3) PUE group, intravenous administrated with PUE injection (20 mg/kg), (4) PUE-PBCN group, intravenous administrated with PUE-PBCN (20 mg/kg). Saline solution, PUE or PUE-PBCN were given approximately 10 min before occlusion and immediately after reperfusion. Animals were anesthetized with 1.5% pentobarbital sodium (0.2 mL/kg) by intraperitoneal injection. The right middle cerebral artery (MCA) was occluded according to Longa’s method26) with modification. The right common carotid artery (CCA) and internal carotid artery (ICA) were exposed and dissected away from adjacent nerves. The external carotid artery (ECA) was isolated and ligated transiently, and a 4-0 monofilament nylon thread then gently inserted from the ECA lumen into the ICA until the tip occluded the origin of the MCA.
After the incision was closed, the animals were allowed to awake from the anesthesia and the filament was removed from the ICA after 1.5 h to allow MCA reperfusion. The animals were returned to their cage with free access to food and water. The rats of sham-operation group received the same protocol without the nylon monofilament inserted.

Twenty-four hours after the surgery, the neurological symptom score was determined using a five-points scale described by Longa et al. The score regulation consists of 0 grade = no neurological deficit, 1 grade = failure to extend the right forepaw fully, 2 grade = circling to the right, 3 grade = circling to the reverse of the right—the left, 4 grade = no spontaneous walking with a depressed level of consciousness. After the evaluation of neurological symptoms, the rats were weighed and the percentage of body weight decreasing was calculated from Eq. 1.

\[
\text{body weight decreasing (\%) } = \frac{\text{body weight before surgery} - \text{body weight after surgery}}{\text{body weight before surgery}} \times 100\% \tag{1}
\]

Following the neurological symptom score and body weight test, rats were sacrificed and their brain tissues were quickly removed. One half of the obtained brain tissues \((n=6)\) were used to detect the brain water content, one half \((n=6)\) for the infarct volume.

The water content was measured by comparing the wet weight with the dry weight. Brain tissues were weighted immediately after removed and after drying off in oven at 105°C for 24 h, respectively. The water content was calculated according to Eq. 2:

\[
\text{water content (\%)} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100\% \tag{2}
\]

The infarct volume was assessed by cutting each brain into six coronal sections (3-mm thick), and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 min with shaking. Unstained areas were defined as ischemia lesions. The slices were photographed with a digital camera (Canon, Tokyo, Japan), and the infarct area was measured using an image analysis system (ImageJ 1.46m). The infarct ratio (percentage of infarction) was calculated from the data of infarct volume and total coronal section according to Eq. 3:

\[
\text{ratio (\%)} = \frac{\text{infract volume (mm}^3\text{)} }{\text{total coronal section (mm}^3\text{)} } \times 100\% \tag{3}
\]

**Statistical Analysis**

Statistical analysis of the data from the physicochemical, pharmacokinetics and biodistribution studies were carried out using Student’s \(t\)-test. One-way analysis of variance (ANOVA) followed by a Dunnett’s test was used for studies of PUE-PBCN on rats with MCAO and reperfusion. A \(p<0.05\) was considered statistically significant.

**RESULTS**

**Physicochemical Characteristics of PUE-PBCN**

In order to improve the pharmacological activity of PUE on cerebrovascular diseases, PUE-PBCN coated with Ps 80 was prepared by anionic polymerization. The mean particle size of nanoparticles was 201.2 nm (Fig. 2) with polydispersity index (PDI) of 0.387, and the mean zeta potential was \(-7.72\) mV. The release experiment of PUE from PBCN was performed in phosphate buffer at pH 7.4 with dynamic dialysis method. The drug release profiles from PUE solution or PBCN suspension were shown in Fig. 3. The PUE release from PBCN showed a burst drug release (65%) within the initial 2 h, and then followed by a sustained release with the cumulative release of drug was about 80% within 24 h. As control, the release of PUE from solution was found to be much faster without obvious sustained release, approximately 92% of PUE released in 4 h.

**Studies on Pharmacokinetics and Biodistribution to Brain in Mice**

The data for pharmacokinetics and biodistribution in mice were analyzed from averaged concentrations in plasma and brain with DAS (version 2.0, supplied by the Chinese Pharmacological Society), and the pharmacokinetic behaviors of PUE and PUE-PBCN in mice by intravenous administration followed non-compartment model. Figure 4 showed the profiles of mean drug concentration in plasma versus time after i.v. administration of PUE and PUE-PBCN at dose of 10 mg/kg. As shown in Table 1, the area under the concentration–time curve \((AUC_{(0-\infty)})\) of PUE-PBCN \((9.35\pm 2.49\ \mu g/mL\cdot h)\) was 2.27-fold that of PUE injection administra-
tration (4.10±0.94 µg/mL·h, p<0.01). Moreover, the maximal concentrations (C₀) of PUE-PBCN was 1.53 fold that of PUE (31.28±6.245 vs. 20.41±4.314, p<0.01). The mean residence time (MRT₀–∞) of PUE-PBCN was 4.611±2.571 h, which was 19 times longer than that of PUE (0.244±0.056 h, p<0.01). Besides, the plasma clearance (CL) of nanoparticles was much lower compared with that of the free drug.

As represented in Fig. 5 and Table 2, the measured AUC value in brain tissue for PUE-PBCN was much higher than that for PUE. Compared with PUE injection, C₀ of PUE in brain from nanoparticles was about 1.7 fold that for PUE injection. After a single dose administration, PUE from injection was quickly removed, while PUE-PBCN showed a prolonged time of PUE in brain tissue, which could still be measured in brain at 8h.

**Effect of PUE-PBCN on Model Rats with MCAO and Reperfusion. Neurological Evaluation** Twenty-four hours after the onset of MCAO, the neurological abnormality of rats was observed and scored. The results were shown in Fig. 6A. The neurological deficit scores in MCAO rats were significantly higher than those of sham-operation group which showed no neurological abnormalities with the neurological score 0. The results demonstrated that the MCAO model with the cerebral ischemia was successfully established. The control group treated with saline solution showed severely neurological deficiencies with the mean neurological score 2.5±0.84. Treatment with PUE and PUE-PBCN at dose of 20mg/kg could significantly decrease the neurological deficiencies scores compared with the model control group (p<0.01). While rats treated with PUE-PBCN displayed lower scores (1.00±0.63) in the neurological deficit tests compared with that treated with PUE (1.83±0.41) (p<0.05).

**Body Weight** The body weight of control group showed a significant decrease compared with the sham-operation group (p<0.01). As shown in Fig. 6B, treatment with PUE or PUE-PBCN significantly inhibited the decrease of body weight. At the same dose, the body weight decreasing percentage of rats treated with PUE-PBCN (9.26±4.41) was less than that treated with PUE (11.69±2.24%) (p<0.05).

**Brain Water Content** As shown in Fig. 6C, in sham-operation group, the water content of the right hemisphere (damaged side) was similar to the left hemisphere (normal). However, it was much higher in the right hemisphere than that in the left hemisphere in control group (p<0.01). In addition, treatment with PUE and PUE-PBCN could attenuate the brain edema compared with control group (p<0.05) at 24h after reperfusion, and PUE-PBCN group showed better efficacy at the same dose compared with free drug (p<0.05).

**Infarction Volume** The infarct volume was measured using 3-mm-thick slices of the brain at 24h after MCAO reperfusion in rats by the TTC-staining in which the infarct tissue became pale and the normal tissue was red. Figure 7A showed typical photographs of coronal sections of the sham-operation group, the control group and the PUE or nanopar-

![Fig. 4. Concentration–Time Curves of PUE and PUE-PBCN after i.v. Administration in Mice (Mean±S.D., n=6)](image)

![Fig. 5. PUE Concentration in Brain at Different Times Following i.v. PUE Injection and PUE-PBCN (Mean±S.D., n=6)](image)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>PUE</th>
<th>PUE-PBCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₀</td>
<td>µg/mL</td>
<td>20.41±4.314</td>
<td>31.28±6.245*</td>
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<tr>
<td>AUC₀–∞</td>
<td>µg/mL·h</td>
<td>4.102±0.943</td>
<td>9.347±2.486**</td>
</tr>
<tr>
<td>MRT₀–∞</td>
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<td>0.244±0.056</td>
<td>4.611±2.571**</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>h</td>
<td>0.417±0.205</td>
<td>8.663±3.533*</td>
</tr>
<tr>
<td>CL</td>
<td>L/h·kg</td>
<td>2.538±0.53</td>
<td>1.131±0.279**</td>
</tr>
</tbody>
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C₀: The initial time concentration; AUC₀–∞: the area under the plasma concentration–time curve from zero to infinity; MRT₀–∞: mean residence time from zero to infinity; t₁/₂: elimination half-life; CL: clearance.
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Particles groups, respectively. In the control group, a well-defined region of ischemia was successfully induced. Administration of PUE and PUE-PBCN at the dose of 10 mg/kg significantly reduced the infarct volume compared with that of the control group ($p<0.05$). Moreover, as shown in Fig. 7B, PUE-PBCN showed a much better potency compared with PUE injection ($p<0.05$).

**DISCUSSION**

In the present study, the PUE-loaded PBCN was successfully prepared by anionic polymerization method using F68 as surfactant and Dextran 70 as stabilizer. Generally, the physicochemical properties such as particle size, zeta potential dominate the *in vitro* and *in vivo* behavior of nanoparticles. It can be seen from Fig. 2 that the mean particle diameter of PUE-PBCN was found almost in the range of 100–500 nm, indicating that the size range of nanoparticles was narrow. Particles in nanometer range can be easily transported through the circulation to different tissue of the body. $^{22}$ The smaller size of particles may be result in more nanoparticles entering the vascular endothelial cells of BBB by endocytosis. $^{27}$ The mean zeta potential of PUE-PBCN was $-7.72$ mV, which was due to the hydroxyl ions and carbonyl group of polymer present on surface of nanoparticles, $^{28}$ and the result was close to the study on curcumin loaded PBCN. $^{29}$ The zeta potential which represents the charge of the particles plays an important role in the stability of nanoparticles. $^{30}$ The electrostatic repulsion
between particles with the same electrical charge will prevent the aggregation of nanoparticles.\textsuperscript{31} The same electrical charges of PUE-PBCN could stabilize the nanoparticle system.

The biphasic profile of PUE release from nanoparticles exhibited an initial rapid release during the first 2 h, followed by a slow release. This could be attributed to the fact that some of the drug on or near the surface of nanoparticles moved out \textit{via} short diffusion path which brought about a burst drug release, and then the release of PUE from the inner core of nanoparticles might result in the second slow release phase. The results was in accordance with previous reports that drug loaded PBCN provided a controlled release pattern.\textsuperscript{32,33} The slow release of PBCN make it possible for drug to stay in blood for a long time, which can prolong the retention of PUE in the blood vessel endothelial cells of the brain, and enhance PUE to pass BBB into brain.

PUE-loaded PBCN showed significant differences in terms of the pharmacokinetic behavior from PUE injection solution (Fig. 4). The area under the plasma concentration–time curve ($AUC$) is an important parameter for pharmacokinetic analyses of a drug, which represents the total drug exposure integrated over time, and is the relationship between time and plasma concentration.\textsuperscript{29} The significantly higher $AUC$ of PUE-PBCN compared with free PUE demonstrated a long blood circulation property of nanoparticles. Additionally, for PUE-PBCN, the $t_{1/2}$ and $MRT$ in blood was significantly longer, whereas the $CL$ was smaller relative to that for the free PUE after i.v. injection. It was mainly because, Ps 80 and poloxamer 188 could form the hydrophilic protective layer at the surface of nanoparticles to repel the absorption of opsonin proteins \textit{via} steric repulsion effect, which could block the adhesion of NP to the surface of macrophages, furthermore decreased phagocytic uptake and levels in RES organs,\textsuperscript{17,35} thereby enhanced the half-life of PUE and increase the blood circulation time of nanoparticles.\textsuperscript{36}

Previous researches reported that Ps 80-coated PBCN could successfully transport many drugs into the brain.\textsuperscript{20,35,37} In our experiment, $AUC$ and $C_{p}$ of PUE in brain tissue PUE-PBCN was 4.36 and 1.72-fold that of PUE injection, respectively, demonstrating that PUE in nanoparticle system was apt to brain tissue, which could result from the brain targeting of PBCN coated with Ps 80.

Many studies indicated the protective effects of PUE on the cerebral ischemia/reperfusion injury. The protective mechanisms might be related to its ability of inhibiting hypoxia inducible factor-$\alpha$ and tumor necrosis factor-$\alpha$, activating caspase-3, improving expression of 70-kDa heat-shock protein and decreasing expression of Fas.$^{12,14,38}$ In the present study, we compared the effect of PUE-PBCN and free PUE on an animal model by MCAO and reperfusion in rats, which was frequently used to evaluate drug efficacy on cerebral ischemia and infarction.\textsuperscript{39} Many of stroke patients suffer from focal cerebral ischemia caused by middle cerebral arterial thrombosis, so this model is closed to the pathophysiology of human.\textsuperscript{5} Neurological test was used to assess the changed functions following focal cerebral ischemia, indicating that PUE-PBCN significantly decreased neurological deficit scores compared with PUE after i.v. administration (Fig. 6A). Changes in body weight, another index of ischemic brain damage, correlates with infarct size 24 h after MCAO.\textsuperscript{40} We found PUE-PBCN showed more stronger protection against body weight losing in rats than free PUE (Fig. 6B). The cerebral water content can reflect the alleviation effect on the cerebral edema of drug. Observed in our study, treatment with PUE-PBCN showed better efficacy on attenuating the brain edema caused by focal cerebral ischemia in contrast to free PUE (Fig. 6C). Above changes were also reflected in the infarct volume (Fig. 7) which is the most vigorous index of ischemic brain damage.\textsuperscript{5} After being intravenously administered, PUE-PBCN significantly reduced the infarct volume compared with PUE injection solution. In a word, PUE-PBCN showed a better effect on cerebral ischemia and reperfusion injury in rats than the free drug, which indicated possibility for higher drug concentration in brain of PUE when it was bound to PBCN.

Our research demonstrated that Ps 80 coated PBCA as a vector could enhance the delivery of PUE into the brain pass through the BBB. How PBCN bring drug into brain so far has not been fully known, the possible mechanisms are as follows: (1) Receptor-mediated endocytosis. Researches\textsuperscript{41} have proved that low density lipoprotein receptor (LDLR) is involved in the mediation of the transport of PBCN across the BBB. LDLR-related protein (LRP) such as LRP 1 and LRP 2 are expressed on the BBB, which serve as a receptor for low density lipoprotein (LDL). Ps 80 is described as a potential “lead substance” for brain targeting\textsuperscript{35} which enables particles to adsorb apolipoprotein E or B from the blood after injection. Nanoparticles combined with apolipoproteins mimic LDL to be endocytosed by the brain blood capillary endothelial cells without immediate efflux by LRP-mediated endocytosis, and then transported through the BBB into brain.\textsuperscript{42–45} (2) Passive diffusion.\textsuperscript{35} Nanoparticles bound to the inner endothelial lining of the brain capillaries results in a concentration gradient, thus the drug can be transported to brain tissue by passive diffusion following release within the endothelial cells. (3) Inhibition of the P-glycoprotein (P-gp) efflux pump.\textsuperscript{46} P-gp is an ATP-dependent efflux pump and a member of a family of intrinsic membrane proteins which express on the BBB interface and prevent the intracellular accumulation of chemotherapeutic agents.\textsuperscript{47} The overexpression of P-gp was described after focal cerebral ischemia,\textsuperscript{48} and upgrading of P-gp lead to the active efflux transport which can impede drug delivering into brain. As a inhibitor of P-gp, Ps 80 suppress the efflux transport to enhance puerarin, which is the substrate of P-gp,\textsuperscript{46} penetration into the brain and lead to higher brain levels of drugs after intra vascular injection.\textsuperscript{49}  

**CONCLUSION**

In the present study, we successfully fabricated and characterized Ps 80-coated PUE-PBCN as drug delivery system to enhance the concentration of PUE in brain. Compared to free drug, PUE-PBCN showed higher concentrations in brain of mice and better effect on MCAO and reperfusion in model rats. It can be concluded that PUE-PBCN can enhance the transport of PUE to brain and may act as a neuroprotective agent useful in the treatment of stroke.

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