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# Nanoparticles accumulate in ischemic core and penumbra region even when cerebral perfusion is reduced

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ABSTRACT

The use of a liposomal drug delivery system is a promising strategy for avoiding side effects and enhancing drug efficiency by changing the distribution of the intact drug. We have previously shown that liposomal agents quickly accumulated in an ischemia–reperfusion region and ameliorated cerebral ischemia–reperfusion injury when they were injected after reperfusion in transient middle cerebral artery occlusion (t-MCAO) rats. In the present study, we hypothesized that liposomes also act effectively as a drug carrier in the ischemic state, since the integrity of the blood brain barrier is disrupted at an early stage after an ischemic event. To test this hypothesis, the cerebral distribution of fluorescence-labeled liposomes was observed in permanent MCAO (p-MCAO) rats. The liposomes accumulated in the ischemic core and the penumbra region when injected at 1 or 2 h after occlusion. The accumulation in the ischemic core region was clearly greater than that in the penumbra region, despite the cerebral blood perfusion of the core region being substantially reduced. This result suggests that drug delivery to an ischemic region using liposomes is possible even when cerebral blood circulation has not recovered. Because liposomal drug delivery systems have the potential to effectively employ a number of agents that have failed in clinical trials, they may offer an effective strategy for achieving neuroprotection in stroke patients.


## 1. Introduction

Ischemic stroke is the second most common cause of death and the leading cause of adult disability worldwide [1]. The reduction in cerebral blood flow that occurs with an ischemic event results in cerebral cell death unless rapidly reversed. Thrombolytic therapy by the injection of tissue plasminogen activator (t-PA) has been approved for the neuroprotection of the cerebral ischemic region, but the therapeutic time window is narrow, and the number of patients that this therapy is appropriate for is very small [2,3]. Hence, the development of a new and more widely applicable therapeutic strategy is required for the improvement of ischemic stroke outcome. One strategy with considerable potential is the use of a drug delivery system (DDS) to reduce ischemic damage by site-selected delivery of the drug.

Several reports have shown that the blood–brain barrier (BBB) is disrupted in the acute phase after an ischemic event [4–6]. Because this disruption causes an increase in vascular permeability, substances with higher molecular weight might be able to enter the brain parenchyma during this phase. In a previous report, we showed that FITC-dextran (150 kDa) quickly accumulated in the ischemic hemisphere of transient middle cerebral artery occlusion (t-MCAO) rats when FITC-dextran was intravenously injected immediately after reperfusion [7]. These findings suggest that BBB disruption occurs at an early stage after cerebral occlusion, and that macromolecules pass through the spaces between endothelial cells in the acute phase of cerebral ischemia. Correspondingly, nanoparticles might also be able to leak into brain parenchyma during the acute phase of cerebral ischemia. If this hypothesis is correct, nanoparticles such as liposomes are promising drug delivery carriers for neuroprotection against and diagnosis of cerebral ischemic disease.

In this study, we observed the cerebral distribution of nanoparticles in permanent MCAO (p-MCAO) rats to determine whether nanoparticles are applicable to the treatment and diagnosis of ischemic stroke.

*Abbreviations:* I/R, ischemia/reperfusion; p-MCAO, permanent middle cerebral artery occlusion; t-MCAO, transient middle cerebral artery occlusion.

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## 2. Methods

### 2.1. Animals

Male Wistar rats (170–210 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were cared for according to the Animal Facility Guidelines of the University of Shizuoka. All animal procedures were approved by the Animal and Ethics Review Committee of the University of Shizuoka.

### 2.2. MCAO rats

MCAO was induced experimentally in rats as described previously [8]. In brief, anesthesia was induced with 3% isoflurane and maintained with 1.5% isoflurane during cerebral stroke surgery by small animal anesthetizer (Model TK-4, Bio Machinery, Chiba, Japan). Rectal temperature was maintained at 37 °C with a heating pad. After a median incision of the neck skin, the right carotid artery, external carotid artery, and ICA were isolated with careful conservation of the vagal nerve. A 4–0 monofilament nylon filament coated with silicon was introduced into the right ICA and advanced to the origin of the MCA to occlude it. Silk thread was used for ligation to keep the filament at the site of insertion into the MCA. After the operation, the neck skin was closed and anesthesia was discontinued. p-MCAO rats were examined without withdrawing of the filament, while, in the case of t-MCAO, cerebral blood flow was restored by withdrawing the filament about 10 mm under isoflurane anesthesia after 2 h of ischemia. Success of the surgery was judged by the appearance of hemiparesis and an increase in body temperature at 1 h after occlusion. Sham-operated rats received the same experimental surgery without suture insertion. This surgery never induced the cerebral cell death and neurological deficit [9]. Non-operated rat did not receive any surgery.

### 2.3. Preparation of liposomes

Liposomes composed of distearoylphosphatidylcholine (DSPC) and cholesterol (2/1 M ratio) were prepared as follows. Lipids dissolved in chloroform were rotary-evaporated to form a thin lipid film. The film was then dried for over 1 h under reduced pressure. Next, the dried film was hydrated with 0.3 M sucrose buffer (pH 7.4). The liposome solution was freeze–thawed for 3 cycles with liquid nitrogen and then sonicated for 15 min at 65 °C. Finally, the particle size of the liposomes was adjusted by extrusion through 100 nm-pore size polycarbonate filters (Nuclepore, Cambridge, MA, USA). Liposome size was measured with ZETASIZER (Malvern Instruments, Worcs, UK). To monitor the cerebral distribution of liposomes, DiI-$C_{18}$ (Molecular Probes Inc., Eugene, OR, USA) was mixed with the initial lipid solution for fluorescence labeling of the liposomes.

### 2.4. Laser Doppler perfusion imaging of cerebral blood flow

MCAO rats were anesthetized as described above. A skin incision was made on the head to expose the whole skull. A whole brain scan was performed using PeriScan PIM-III (Perimed AB, Stockholm, Sweden). The laser beam was set to lambda, and the scan range was set as 2.5 × 2.5. Laser Doppler perfusion imaging was performed at 30 min after occlusion. The ratio of the total blood perfusion in the ipsilateral hemisphere to that in the contralateral hemisphere was calculated.

### 2.5. Cerebral distribution of liposomes

DiI-labeled liposomes (10 mM as DSPC, 0.5 mL) were intravenously injected into MCAO rats at 1 or 2 h after the start of ischemia. The rats were sacrificed at 1 h after the injection, and the brain was sliced into 2-mm thick coronal sections with a rat brain slicer (Muromachi Kikai, Tokyo, Japan). All sections were put on glass slides, and DiI fluorescence was measured with an *in vivo* imaging system (IVIS, Xenogen Corp., Alameda, CA).

### 2.6. Histological analysis

DiI-labeled liposomes were intravenously injected into MCAO rats at 1 h after the induction of ischemia. At 1 h after injection, brain slices were placed on an optical cutting temperature compound (Sakura, Finetech., Co. Ltd., Tokyo, Japan), and then frozen in a dry ice-ethanol bath. These frozen sections were cut into 10 μm sections with a cryostat (HM505E, Microm, Walldorf, Germany). The sections were incubated in 1% bovine serum albumin-containing PBS for 10 min at room temperature for protein blocking, in biotinylated anti-mouse CD31 rat monoclonal antibody (BD Pharmingen, Franklin Lakes, NJ, USA) for 18 h at 4 °C, and then in streptavidin-Alexa fluor 488 conjugates (Molecular Probes Inc.) for 30 min at room temperature. Finally, the sections were mounted with Perma Fluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA, USA), and their fluorescence was observed with a microscopic LSM system (Carl Zeiss, Co., Ltd., Germany).

### 2.7. Brain damage assessment in MCAO rats

The ischemic or ischemia/reperfusion damage in p-MCAO or t-MCAO rats, respectively, was assessed by morphometric analysis of the brain sections stained with TTC (Wako Pure Chemical Ind. Ltd., Tokyo, Japan). To assess cerebral cell death, p-MCAO rats were sacrificed 2 or 3 h after ischemia, and t-MCAO rats were sacrificed after 1.5 or 3 h reperfusion pre-treated with 2 h ischemia. The brains were sliced into 2 mm thick coronal sections using a rat brain slicer. The brain sections of p-MCAO and t-MCAO rats were stained with 2% TTC in PBS for 30 min at 37 °C, then fixed in 10% formalin neutral buffer solution. All sections were put on glass slides and photographed with a digital camera (OLYMPUS E-300). Damaged regions were defined as areas that were completely white.

### 2.8. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Data are presented as mean ± SD.

## 3. Results

### 3.1. Imaging of cerebral blood flow in MCAO rats

Cerebral blood flow was observed in non-operated, sham-operated, and p-MCAO rats. There were no significant differences in blood perfusion between the ipsilateral hemisphere and contralateral hemisphere in non-operated rats (Fig. 1A and D). Compared with the contralateral hemisphere, sham surgery induced approximately 20% loss of ipsilateral cerebral perfusion due to internal carotid artery (ICA) ligation (Fig. 1B and D). In contrast, MCAO reduced ipsilateral cerebral perfusion by 60% at 30 min after the onset of ischemia (Fig 1C and D). Additionally, hemiparesis and increased body temperature were observed in the MCAO-operated rats. Based on these findings, it appears that the MCAO operation induced acute ischemia in the ipsilateral hemisphere.

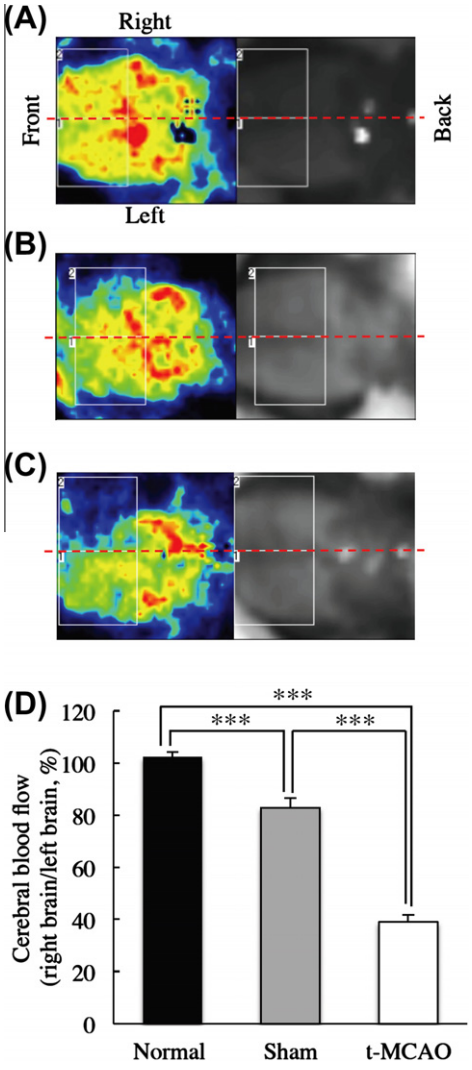


**Fig. 1.** Imaging of cerebral perfusion in MCAO rats by PeriScan PIM-III. (A) The imaging shows cerebral perfusion from the head with the whole skull exposed in non-operated rats. Laser Doppler perfusion imaging was performed at 30 min after occlusion to verify surgical success after induction of cerebral ischemia in sham-operated rats (B) and MCAO rats (C). Reduction of cerebral perfusion was observed at an early stage after surgery. Bar shows the relative levels of fluorescence intensity, ranging from low (blue), to medium (green), to high (yellow, red). The imaging data represent of four independent animal experiments, all of which demonstrated a similar profile of responses. (D) The graph shows data calculated from the photon counts of the cerebral perfusion image ($n = 4$). The vertical axis indicates the ratio of the total photon count in the ischemic hemisphere to that in non-ischemic hemisphere. MCAO surgery induced significant reduction in cerebral perfusion in the ischemic hemisphere. Significant differences are indicated as follows: $^{***}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.2. Cerebral distribution of liposomes in MCAO rats

The particle size and ζ-potential of the 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI-$C_{18}$)-labeled liposomes were 114.5 ± 9.3 nm and −0.7 ± 0.7 mV, respectively. Liposomes of this size have been shown to leak into brain parenchyma in the ischemic region of t-MCAO rats when the liposomes were injected after reperfusion [9]. In the present study, p-MCAO rats were injected with DiI-labeled liposomes at 1 or 2 h after occlusion, and fluorescence was imaged in the brain sections at 1 h post-injection of the liposomes. Higher DiI fluorescence intensity was observed in the ipsilateral hemisphere compared with contralateral hemisphere at both injection time points (Fig. 2A and B). In particular, the liposomes administered after 2 h of ischemia markedly accumulated

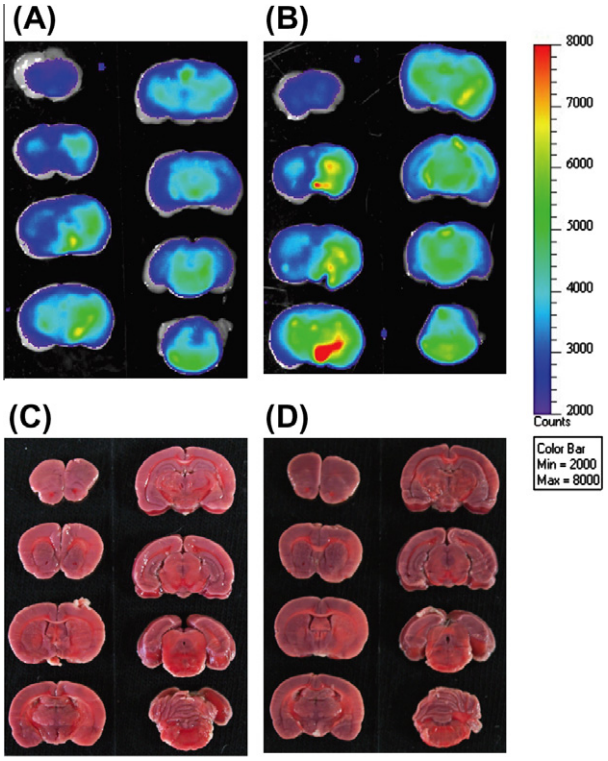


**Fig. 2.** Accumulation of liposomes in the ischemic hemisphere and brain damage under ischemic conditions. Fluorescence imaging of brain slices was performed after injection of fluorescence-labeled liposomes, followed by TTC staining. In this figure, A and B show the results of the intravenous injection of DiI-labeled liposomes in p-MCAO rats at 1 and 2 h after the onset of ischemia, respectively. The left hemispheres of the brain slices show the non-ischemic side; and the right hemispheres show the ischemic side. Bar shows the relative levels of fluorescence intensity, ranging from low (blue), to medium (green), to high (yellow, red). The brain damage following 2 (C) and 3 h (D) ischemia was evaluated by TTC staining. These data indicate the typical images out of five independent animal experiments, although all of other experiments demonstrated a similar profile of responses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the ipsilateral hemisphere (Fig. 2B). Furthermore, the fluorescence of the DiI-labeled liposomes was observed in brain parenchyma only in the ischemic hemisphere (Fig. 3), indicating that the injected liposomes had reached the ischemic region and leaked into brain parenchyma through the spaces caused by the disruption of BBB.

### 3.3. Brain damage assessment

After imaging, the brain slices were incubated with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) solution, which stains viable cells red. Prominent TTC-determined cerebral cell death was not detected at 2 or 3 h after MCAO (Fig. 2C and D). In contrast, 1.5 or 3 h reperfusion after 2 h ischemia caused drastic brain damage (Fig. 4A and B). The TTC-determined cerebral lesions gradually spread out from the ischemic core as time passed. Notably, TTC-determined cerebral cell death appeared in the striatum at an early stage after reperfusion. Taken together, before the obvious cerebral cell death appeared, nanoparticles accumulated in the ischemic core region in p-MCAO rats.

## 4. Discussion

In this study, a 60% reduction in ipsilateral cerebral perfusion was observed in the MCAO rats (Fig. 2). Because the depth limit

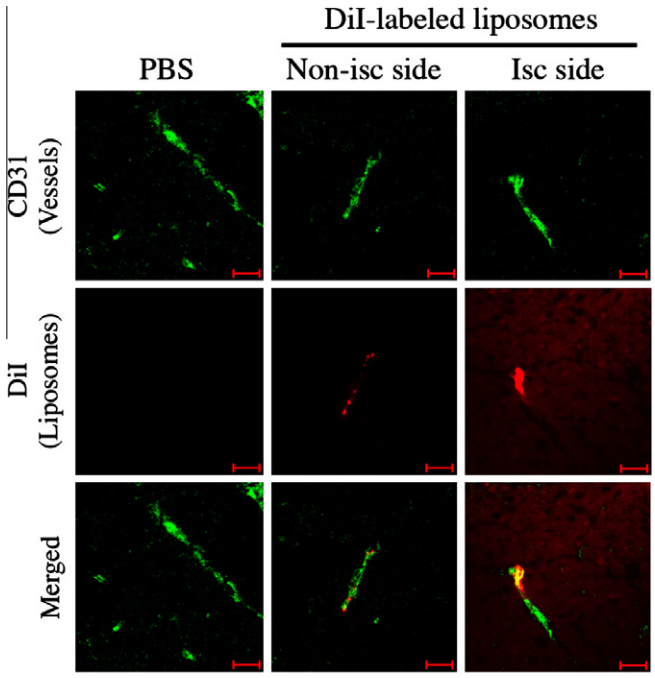


**Fig. 3.** Intracerebral distribution of liposomes in p-MCAO rats. Brain sections (10 μm) were prepared from p-MCAO rats injected with DiI-labeled liposomes. Immunostaining for CD31 was performed on the sections to visualize cerebral vessels. The fluorescence images of the sections were observed by confocal laser scan microscopy. Green shows cerebral vessels and red shows the fluorescence of DiI. Isc side indicates ischemic hemisphere; non-isc side indicates non-ischemic hemisphere. Scale bar, 20 μm.

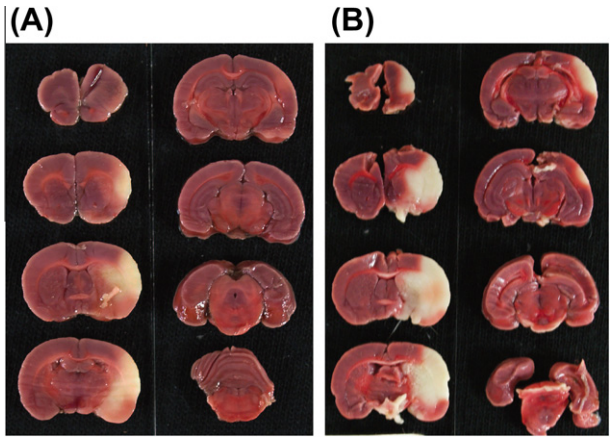


**Fig. 4.** Brain damage induced by ischemia/reperfusion in t-MCAO rats. After 2 h of ischemia followed by 1.5 (A) or 3 h (B) of reperfusion, the damaged regions were visualized by TTC staining. The left hemispheres of the brain slices are the non-ischemic side; the right hemispheres are the ischemic side. White areas show damaged regions; red areas show surviving regions. This is the representative from four independent animal experiments, all of which demonstrated similar profile of responses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the laser Doppler perfusion imaging was almost 1 mm, the detectable area was far from the ischemic core region. The penumbra region, which is salvaged if reperfusion is started before necrosis has occurred, is defined as a region of 20–40% intermediate cerebral blood flow depression [10,11]. Therefore, the surface of the ipsilateral hemisphere can be considered the penumbra region in this model.

The amount of cerebral blood flow in ischemic core regions has been found to be more than 80% lower than in the normal condition [12,13]. This reduction causes a disruption in cerebral vascular integrity, resulting in increased vascular permeability [14]. In this study, despite cerebral perfusion being greatly reduced, liposomes were able to flow into ischemic regions and accumulated strikingly in the striatum, the region that was assumed to be the ischemic core. We previously observed that liposome encapsulating hemoglobin (LEH) with 200 nm size flew to penumbra regions of ischemia model rats [15] and protect ischemic damage of the region [16]. The present results that liposomes reached to the ischemic region under reduced perfusion by ischemia are consistent with these observations.

The present results also indicate that the BBB in the ischemic region is severely disrupted in the early stage of ischemia and that nanoparticles accumulate and are retained in that region in the ischemic state. Additionally, accumulation of liposomes in the penumbra region was observed when they were injected after 1 or 2 h of MCAO. This indicates that drug delivery to an ischemic region for neuroprotection using nanocarriers is possible even when cerebral perfusion has not recovered. This finding suggests some possible new approaches to ischemic stroke therapy and diagnosis. t-PA is the only approved therapy for stroke treatment; however, the therapeutic time window is very short and the number of potential patients accepted for t-PA treatment is very small because the drug increases the risk of intracerebral hemorrhage. Thus, expansion of the therapeutic time window and protection of the neurovascular unit are desirable for improving stroke outcome [17,18]. A number of drugs injected with or before t-PA treatment have succeeded in prolonging the therapeutic time window though neuroprotection in animal studies [19–21]. However, many neuroprotectants have not been adopted for clinical use, despite clear preclinical evidence of their neuroprotective efficacy [22,23]. Major factors behind this lack of adoption are adverse side effects and insufficiency of medicinal efficacy therefore attempting to decrease these side effects and selective delivery of them are a promising strategy for designing neuroprotectants.

DDS using nanoparticles can improve drug efficiency and reduce side effects through more specific delivery and altered biodistribution; hence, DDS technology has the potential to overcome the above problems with neuroprotectants, and may lead to the reuse of compounds that have failed in clinical trials. In addition, our results indicate that a nano DDS can also be applied to the diagnosis of stroke patients. Many nanoparticle radiolabeling methods have been developed with the aim of observing nanoparticle distribution [24–26]. These techniques enable the diagnosis of a variety of diseases using a real-time *in vivo* imaging system such as positron emission tomography. Moreover, some nanoparticles, including liposomes, can be modified with functional molecules, and these carriers possess specific localized activity. In short, the intravenous injection of neuroprotectant-encapsulated and targeting molecule-modified nanoparticles may simultaneously enable both early diagnosis of and neuroprotection from cerebral stroke.

In conclusion, we have revealed that 100-nm nanoparticles accumulated in the ischemic core region when they were intravenously injected at 1 or 2 h after the onset of cerebral ischemia, despite blood perfusion in this region being dramatically reduced. This indicates that drug delivery to the ischemic region based on nanotechnology may be possible even when cerebral perfusion has not been restored by a thrombolytic agent. This finding suggests that a nano drug delivery system is a potential strategy for neuroprotection during the ischemic state in cerebral stroke.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.080.

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