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Enhancement of cerebroprotective effects of lipid nanoparticles encapsulating FK506 on cerebral ischemia/reperfusion injury by particle size regulation

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Abstract

Delivery of cerebroprotective agents using liposomes has been demonstrated to be useful for treating cerebral ischemia/reperfusion (I/R) injury. We previously reported that intravenous administration of liposomes with diameters of 100 nm showed higher accumulation in the I/R region compared with larger liposomes (>200 nm) by passage through the disintegrated blood-brain barrier, suggesting a size-dependence for liposome-mediated drug delivery. Based on these findings, we hypothesized that regulation of liposomal particle size (<100 nm) may enhance the therapeutic efficacy of encapsulated drugs on cerebral I/R injury. Herein, we prepared lipid nanoparticles (LNP) with particle sizes <100 nm by the microfluidics method and compared their therapeutic potential with LNP exhibiting sizes >100 nm in cerebral I/R model rats. Intravenously administered smaller LNP (ca. 60 nm) exhibited wider accumulation and diffusivity in the brain parenchyma of the I/R region compared with larger LNP (>100 nm). Importantly, treatment with LNP encapsulating the cerebroprotective agent FK506 (FK-LNP) with particle sizes <100 nm showed greater cerebroprotective effects than FK-LNP with sizes >100 nm, and also significantly ameliorated brain injury. These results suggest that particle size regulation of LNP to sizes <100 nm can enhance the therapeutic effect of encapsulated drugs for treatment of cerebral I/R injury, and that FK-LNP could be a promising cerebroprotective agent.

Keywords

Cerebral ischemia/reperfusion injury; Blood-brain barrier; Lipid nanoparticles; Particle size regulation; Microfluidics; FK506

1. Introduction

Ischemic stroke is a leading cause of severe disability and mortality worldwide. Use of tissue plasminogen activator and endovascular thrombectomy with medical devices are approved to restore diminished cerebral blood flow by ischemic stroke; however, secondary cerebral ischemia/reperfusion (I/R) injury is frequently induced and results in poor outcomes for stroke patients [1]. While there is currently no globally-applicable cerebroprotective agent available to treat cerebral I/R injury, a lot of candidate drugs have been created. However, the presence of the blood-brain barrier (BBB) often results in insufficient drug distribution into the injured sites, which leads to translational failure of drug candidates [2]. Hence, development of efficient drug delivery technologies into the lesioned area is needed to improve the therapeutic efficacy of those candidate drugs.

In our previous studies, we have been demonstrated that delivery of cerebroprotective agents using nano-sized lipid nanoparticles (LNP), namely liposomes, into the lesioned area is effective for treating cerebral I/R injury [3]. Although the BBB limits liposomal entry into the brain tissue under healthy conditions, it has been reported that BBB disruption occurs around the region of I/R and vascular permeability is increased by certain mediators [4]. Based on these phenomena, intravenously administered nano-sized liposomes containing therapeutic agents were found to pass through the disintegrated BBB and be retained in the brain parenchyma, resulting in amelioration of brain injury in transient middle cerebral artery occlusion (t-MCAO) rats, a cerebral I/R model [5].

The therapeutic effects of liposomal drugs are known to be influenced by various factors, such as the release properties of encapsulated drugs, the physicochemical properties of the particle surface, as well as particle size [6]. In fact, results of our previous study showed that the therapeutic effects of liposomal neuroprotectants on cerebral I/R injury are altered by changing the release rate of encapsulated drugs, thus demonstrating the importance of optimizing the release properties for the development of liposomal neuroprotectants [7]. With regard to particle size, we also reported that liposomes with diameters of 100 nm exhibit broader accumulation in the lesioned area compared with those >200 nm in size, and that liposomes >800 nm in size cannot accumulate [8]. These findings suggest that the spaces between the disintegrated BBB are large enough to enable entry of liposomes with sizes of approximately 100 nm. Moreover, it was also reported that intracranial injection of poly(lactic-co-glycolic acid) (PLGA) nanoparticles with diameters <100 nm (i.e., 70 nm) widely diffuse in the brain parenchyma, whereas those with sizes >100 nm lack diffusivity [9]. Based on these findings, we hypothesized that intravenously administered LNP with diameters <100 nm should demonstrate superior diffusion into the I/R region after passing through the disintegrated BBB, resulting in higher cerebroprotective effects of the encapsulated drugs compared with conventional 100-nm liposomes. However, a detailed understanding of the relationship between particle size and therapeutic efficacy against cerebral I/R injury remains to be elucidated.

To prepare particle size-regulated LNP with diameters <100 nm, we employed a microfluidics method. Previously, preparation of liposomes with sizes <100 nm with high stability

and reproducibility by conventional methods, such as extrusion and sonication in the thin-film method, has been difficult. The microfluidics method, on the other hand, was demonstrated to allow for highly precise preparation of monodisperse LNP with sizes <100 nm by appropriately setting the mixing condition of an organic phase (lipids dissolved in alcohol) and an aqueous phase in a micromixer [10]. By incorporating hydrophobic drugs into the organic phase and/or hydrophilic drugs in the aqueous phase, encapsulation of drugs into the resultant LNP can simultaneously be achieved [10]. Indeed, encapsulation of small molecular drugs [11], proteins [12], and nucleic acids [13] into LNP with sizes <100 nm (ranging from 30 to 70 nm) were reported. In these systems, particle size is tunable depending on the lipid composition and production parameters (e.g., mixing speed and ratio of organic to aqueous phase). Based on these findings, the microfluidics system is also expected to be applicable for preparation of cerebroprotective agent-encapsulated LNP with diameters <100 nm.

In the present study, we investigated various conditions to prepare LNP with sizes <100 nm using the microfluidics method. Thereafter, cerebral distribution of intravenously injected LNP with varying diameters was evaluated in t-MCAO rats. In addition, we developed particle size-regulated LNP encapsulating the cerebroprotective agent FK506 (tacrolimus), which is an approved immunosuppressant and has also been reported to exhibit cerebroprotective effects via inhibition of calcineurin activation following cerebral I/R [14]. By using the LNP encapsulating FK506 (FK-LNP) with sizes <100 nm, we investigated whether particle size regulation enhances the cerebroprotective

effects of FK506 on cerebral I/R injury by comparison to FK-LNP with sizes >100 nm in t-MCAO rats.

2. Materials and methods

2.1. Preparation of lipid nanoparticles (LNP)

Distearoylphosphatidylcholine (DSPC) and distearoylphosphatidylethanolamine (DSPE)-polyethylene glycol $(PEG)_{2000}$ were purchased from NOF corporation (Tokyo, Japan) and cholesterol was purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). LNPs consisting of DSPC/cholesterol/DSPE-PEG2000 (1/1/0.1 molar ratio) were prepared by the microfluidics method using the NanoAssemblr™ Benchtop (Precision Nanosystems, Vancuver, BC, Canada). A microfluidic cartridge (NanoAssemblr™ Benchtop Cartridges) was employed, and the above lipid mixture dissolved in ethanol and phosphate-buffered saline (PBS) was injected into the cartridge as organic and aqueous phases, respectively. LNP production parameters, namely flow rate ratio (the ratio of the lipid mixture/PBS injected into the cartridge) and total flow rate (the injection speed of both organic and aqueous phases into the cartridge), were set by the Nanoassemblr® software. For fluorescence labeling of LNP, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen, CA, USA) was mixed to the initial lipid mixture to become 2.5 mol% concentration of total lipids. Thereafter, the resultant LNP suspension was purified by a PD-10 desalting column (GE Healthcare, Tokyo, Japan) equilibrated using PBS for removing residual ethanol in the final formulation (final lipid concentration: 5 mM as total lipids). The particle size and the ζ-potential of LNP were determined using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

2.2. Animals

Wistar male rats (Eight-week-old, 180-220 g) were bought from Japan SLC, Inc. (Shizuoka, Japan). All experiments with animals were assessed and approved by the Animal and Ethics Review Committee of Tokushima University.

2.3. Transient middle cerebral artery occlusion rats

t-MCAO rats were prepared as previously reported [8]. Briefly, anesthesia using isoflurane (Fujifilm Wako Pure Chemical) was induced with 3% and maintained with 1.5% during surgical operation in rats using an anesthesia machine for small animals (Model TK-4, Bio Machinery, Chiba, Japan). Body temperature was maintained at 37ºC using a temperature controller for small animals (Unique Medical, Tokyo, Japan). After incision of the cervical skin, the right internal carotid artery (ICA) was exposed. Subsequently, a silicon-coated 4.0 nylon filament 18-mm in length was gently inserted to the origin of the MCA from the ICA for induction of occlusion. The rat was recovered from isoflurane anesthesia following suture of the neck skin incision. At 1 h after the start of occlusion, the filament was withdrawn from the origin of the MCA to allow for reperfusion of the blood.

2.4. Cerebral distribution of LNP

DiI-labeled LNP (DiI-LNP) were administered intravenously into t-MCAO rats (2.5 μmol lipid/rat) immediately after reperfusion. At 3 h after injection, the brains of the rats were dissected after transcardial perfusion and fixation, and 2-mm coronal brain sections were prepared. Fluorescence in the sections derived from DiI-LNP was analyzed using an *in vivo* imaging system (IVIS; Xenogen, CA, USA).

2.5. Immunohistological analysis of intracerebral distribution of LNP

t-MCAO rats were treated according to the protocol mentioned in section 2.4., except that transcardial perfusion and fixation were performed prior to dissection. The 2-mm brain sections were embedded into optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan), followed by freezing in dry ice/ethanol bath. The 10-μm slices were then prepared with a cryostat (CM3050S; Leica Biosystems, Tokyo, Japan), and immunostaining for CD31 was performed. Briefly, the sections were performed blocking with 3% bovine serum albumin in PBS for 15 min at room temperature, followed by reaction with rabbit anti-CD31 antibody (NB100-2284; Novusbio, CO, USA) for 18 h at 4ºC. Thereafter, the sections were reacted with goat anti-rabbit IgG conjugated with Alexa fluor 488 (ab150077; Abcam, Cambridge, UK) for 30 min at room temperature. After mounting using aqueous mounting medium (PermaFluor™; Lab Vision Corporation, Fremont, CA, USA), fluorescence in the sections was analyzed using a confocal laser scanning microscope (LSM700; Carl Zeiss, Jena, Germany). Diffusion distances of DiI-LNP from the blood vessels into

the parenchyma were analyzed using the image-analysis software Image J (National Institutes of Health, Bethesda, MD, USA), for which three independent experiments were performed.

2.6. Preparation of LNP encapsulating FK506

To prepare LNP encapsulating FK506 (FK-LNP), FK506 solution in ethanol was added to the initial lipid mixture (final concentration: 3 mol% of DSPC), and the same procedure used for preparation of empty LNP was performed as described in Section 2.1. After removal of unencapsulated FK506 using a PD-10 column, the FK506 concentration in the LNP suspension was measured by HPLC (Shimadzu, Kyoto, Japan). The HPLC analyses were performed according to the conditions as previously reported [15]. The encapsulation ratio of FK506 was calculated as the percentage of the amount of encapsulated FK506 in LNP to the amount of FK506 added to the initial lipid mixture.

FK506-encapsulated PEGylated liposomes (FK-Lipo) consisting of DSPC/cholesterol/DSPE-PEG$_{2000}$=1/1/0.1 or DSPC/DSPE-PEG$_{2000}$=1/0.1 were prepared by the thin-film method as previously reported [15]. The sizes of FK-Lipo were adjusted by extrusion with a 100-nm polycarbonate membrane filter (Avanti Polar Lipids, Birmingham, AL, USA), and the FK506 encapsulation ratio was determined by HPLC.

2.7. Therapeutic experiment

FK-LNP (100 μg/kg FK506 dose) or PBS was administered intravenously into t-MCAO rats just after reperfusion. After 24 h of reperfusion, motor functional outcome was evaluated with a 21-point neurological test as previously described [16], in which healthy rats are given 21 points. Brains were subsequently dissected and sliced into 2-mm thick coronal sections, followed by staining in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Fujifilm Wako Pure Chemical) solution dissolved in PBS for 30 min at 37ºC to visualize living brain cells. The damaged brain volume was assessed using the image-analysis software Image J.

2.8. Statistical analysis

Statistical analysis was conducted by one-way analysis of variance followed by the Tukey *post-hoc* test. Data were presented as mean ± standard deviation (S.D.).

3. Results

3.1. Preparation of particle-size regulated LNP

We first prepared LNP by adjusting the production parameters, namely flow rate ratio of lipid mixture to PBS (lipid/PBS), total flow rate (TFR), and lipid composition, to prepare LNP with sizes <100 nm by the microfluidics method. Through a number of preliminary experiments, lipid/PBS flow rate ratio=1/4 and the lipid composition DSPC/cholesterol/DSPE-PEG$_{2000}$=1/1/0.1 (molar ratio) were found to be appropriate to allow for reproducible preparation of LNP with sizes <100 nm (data not shown). This lipid composition has also previously been used to prepare liposomal drugs for treating cerebral I/R injury [5, 7]. By adjusting the TFR from 2 to 21 mL/min, LNP particle sizes could be successfully regulated to have sizes ranging from 92.6 ± 11.2 nm (2 mL/min) to 56.9 ± 2.8 nm (18 mL/min) with monodispersity (Fig. 1A). The ζ-potential of each formulation was similarly neutral regardless of the TFR condition (Fig. 1B). These microfluidic conditions were employed to prepare LNP of varying sizes for the following experiments.

3.2. Influence of particle size on LNP accumulation in the cerebral I/R region

DiI-labeled LNP (DiI-LNP) were prepared to investigate cerebral distribution of intravenously-administered LNP into t-MCAO rats. By adjusting the TFR to 2 and 18 mL/min, DiI-LNP with diameters of 124.3 ± 8.8 nm (PDI: 0.24 ± 0.01; ζ-potential: -1.1 ± 0.21 mV) and 55.5

± 1.9 nm (PDI: 0.10 ± 0.05; ζ-potential: -1.2 ± 0.09 mV) were prepared, and are referred to as DiI-L-LNP and DiI-S-LNP, respectively. IVIS images of brain sections of t-MCAO rats were obtained at 3 h after each LNP injection, with the right brain hemisphere indicating the ischemic side. Results showed that accumulation of both DiI-LNP was observed in the ischemic hemisphere of t-MCAO rats (Fig. 2A). Importantly, the fluorescence was more widely observed in the DiI-S-LNP-injected group compared with DiI-L-LNP group. To further examine the influence of particle size, the intracerebral distribution of each DiI-LNP was histologically analyzed. Confocal images showed that DiI fluorescence derived from each LNP was hardly detected in the non-ischemic hemisphere (Figs. 2B and C). In the ischemic side, extravasation of each LNP from cerebral vessels was observed, although the fluorescence of DiI-L-LNP was mainly present in the vicinity of the vessels (Fig. 2B). On the other hand, significantly higher leakage of DiI-S-LNP was observed, not only around the vessels, but also in the broader area of the brain parenchyma, compared with DiI-L-LNP (Figs. 2C and D).

3.3. Encapsulation of FK506 into LNP

The chemical structure of FK506 is shown in Fig. 3A. FK-LNP with diameters of 116.4 ± 7.7 nm (PDI: 0.24 ± 0.01; ζ-potential: -1.0 ± 0.04 mV) and 60.7 ± 5.6 nm (PDI: 0.07 ± 0.02; ζ-potential: -1.1 ± 0.2 mV) were prepared by setting TFR at 2 and 18 mL/min, and are referred to as FK-L-LNP and FK-S-LNP, respectively (Fig. 3B). HPLC analyses were performed to determine the

FK506 encapsulation ratios into FK-L-LNP and FK-S-LNP to be 66.4 ± 10.9% and 74.7 ± 2.9%, respectively (Fig. 3C). For FK-Lipo prepared by the thin-film method, the FK506 encapsulation ratio into liposomes without cholesterol was 33.4 ± 2.7%, while no FK506 encapsulation was found for liposomes with cholesterol, which used the same composition as that of FK-LNP (Fig. 3D).

3.4. Therapeutic effects of different sizes of FK-LNP on cerebral I/R injury

Cerebroprotective effects of FK-LNP were investigated in t-MCAO rats. Images of TTC staining showed that treatment with FK-L-LNP suppressed brain damage (although not significantly), whereas broad brain damage was observed in the PBS-treated group (Figs. 4A and B). Importantly, intravenous administration of FK-S-LNP significantly reduced damaged brain volume compared with the PBS-group, and exhibited higher cerebroprotective effects compared with FK-L-LNP. Also, although both sizes of FK-LNP significantly ameliorated motor functional deficits induced by cerebral I/R, treatment with FK-S-LNP showed higher effects than treatment with FK-L-LNP (Fig. 4C).

4. Discussion

Our previous study demonstrated that liposomal accumulation into the cerebral I/R region by passage through the disrupted BBB occurs in a particle size-dependent manner for liposomes with diameters >100 nm [8]. Based on these findings, we hypothesized that regulation of particle size to <100 nm should increase liposomal accumulation into the cerebral I/R area, and allow for enhancement of the therapeutic effects of encapsulated drugs. We prepared LNP exhibiting diameters <100 nm by the microfluidics method (Fig. 1), and investigated their cerebral distributions in t-MCAO rats. The findings from this study demonstrate that DiI-S-LNP (ca. 60 nm) exhibited broader accumulation and diffusivity in the region of cerebral I/R following intravenous administration compared with DiI-L-LNP (Fig. 2). The present results are consistent with a previous report that found that 70-nm PLGA nanoparticles diffuse widely in the brain parenchyma after intracranial injection, while 147-nm nanoparticles cannot efficiently diffuse owing to dense brain tissue [9]. On the other hand, surface modification of synthetic nanoparticles densely with PEG was previously reported to allow for a significant increase of their diffusion through the brain parenchyma in comparison with non-PEG-modified nanoparticles, following intracranial administration into mice [17]. Based on these findings, reduction in particle size was suggested to effectively allow for accumulation and diffusivity of intravenously injected LNP into the cerebral I/R region. Moreover, PEG-coating onto the LNP may also contribute to diffusivity through the brain parenchyma.

In this study, we employed the microfluidics method to prepare FK-LNP. We previously reported that encapsulation of FK506 into liposomes brings about a significant increase in its cerebroprotective effect using 100-nm-sized PEGylated liposomes consisting of dipalmitoylphosphatydilcholine/DSPE-PEG_{2000}=20/1 (molar ratio) prepared by the thin-lipid film method [18]. In that study, the FK506 encapsulation efficiency into the liposomes was 30-40%, which dramatically decreased upon incorporation of cholesterol, consistent with the results of the present study (Fig. 3). These findings may be due to the fact that FK506 competes with cholesterol within the hydrophobic spaces in the lipid bilayer membranes, a phenomenon similar to that previously noted for liposomes encapsulating hydrophobic paclitaxel [19]. On the other hand, the microfluidics method resulted in a superior encapsulation ratio into LNP, namely >70% of the initially added FK506, with good reproducibility while reducing particle size (Fig. 3). The previous study on preparation of LNP encapsulating small interfering RNA (siRNA) by microfluidics reported that formation of an interior hydrophobic core composed of siRNA, cationic ionizable lipids, and other lipids, and subsequent homogenous coating of the core by PEG-lipids during rapid mixing allows for high siRNA encapsulation efficiency into LNP [20]. Similar to these findings for LNP encapsulating siRNA, we can speculate that FK506 molecules form a hydrophobic core with DSPC and cholesterol during microfluidic mixing, which leads to higher drug encapsulation in LNP compared with liposomes prepared by the thin-film method, although a detailed mechanism is needed to be elucidated.

Intravenous administration of FK-S-LNP significantly ameliorated cerebral I/R injury in t-MCAO rats compared with FK-L-LNP (Fig. 4). These results suggest that FK-S-LNP could widely diffuse through the brain parenchyma after passing through the disintegrated BBB and then release entrapped FK506, resulting in efficient exertion of its cerebroprotective effects against brain cells. Collectively, the present findings demonstrate the importance of regulation of LNP particle size for efficient delivery of cerebroprotective agents to treat cerebral I/R injury. To date, a number of liposomal cerebroprotective agents and their effectiveness against cerebral I/R injury have been reported [3]. Application of the microfluidics method to such cerebroprotective agents, like FK506, is expected to facilitate preparation of size-regulated nanoparticles with better drug encapsulation efficiency and higher therapeutic efficacy against cerebral I/R injury, compared with conventional liposomal agents.

In conclusion, we demonstrated that intravenous administration of LNP with diameters <100 nm prepared by the microfluidics method exhibited broader accumulation and diffusivity in the brain parenchyma around the region of cerebral I/R compared with LNP exhibiting sizes >100 nm. Also, treatment with FK-S-LNP significantly ameliorated cerebral I/R injury and showed greater therapeutic effects compared with FK-L-LNP in t-MCAO rats. These results demonstrate that particle size regulation to <100 nm should be an important factor to augment the therapeutic effects of entrapped drugs for the treatment of cerebral I/R injury. This is the first report on the enhancement of cerebroprotective effects of LNP encapsulating FK506 by particle size regulation using the

microfluidics method, and subsequent demonstration of their utility for treating cerebral I/R injury. The present results offer promise for further development of LNP encapsulating cerebroprotective agents.

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Figure legends

Fig. 1. Physicochemical properties of lipid nanoparticles (LNP) prepared by the microfluidics method.

Particle size, polydispersity index (PDI) (A) and ζ-potential (B) of LNP composed of DSPC/cholesterol/DSPE-PEG$_{2000}$=1/1/0.1 (molar ratio) were analyzed. Each LNP was prepared using a microfluidics device by changing the total flow ratio (TFR; the injection speed of both lipid mixture and PBS into the cartridge). Data are mean ± S.D. (n=3).

Fig. 2. Accumulation of different sizes of LNP into the cerebral I/R region of t-MCAO rats.

DiI-L-LNP or DiI-S-LNP were intravenously injected (2.5 μmol lipid/rat) into t-MCAO rats immediately after reperfusion following 1-h occlusion. After 3 h of injection, the brains of the rats were dissected, and coronal 2-mm brain sections were prepared. (A) DiI fluorescence in the sections were determined by IVIS (blue and red colors indicate areas of low and high fluorescence, respectively). (B, C) The frozen 10-μm brain sections were immunostained for CD31 to visualize cerebral vessels, and intracerebral distribution of both DiI-LNP in the non-ischemic and ischemic hemispheres was analyzed using a confocal laser scanning microscope. Scale bars=20 μm. Green and red colors represent cerebral vessels (Alexa488), and LNP (DiI), respectively. (D) Quantitative data on diffusion distances of each DiI-LNP from blood vessels into the brain parenchyma. Data are mean ± S.D. calculated from independent three experiments. * $P<0.05$.

Fig. 3. Preparation of particle-size regulated LNP encapsulating FK506 (FK-LNP) by the microfluidics method.

(A) Chemical structure of FK506. By setting TFR as 2 or 18 mL/min, FK-LNP exhibiting different diameters were prepared by the microfluidics method. (B) Particle size and PDI of each FK-LNP. (C) The encapsulation ratios of FK506 into FK-LNP of different sizes were calculated as the percentage of the amount of encapsulated FK506 in LNP to the amount of initially added FK506. (D) The encapsulation ratios of FK506 into liposomes consisting of

$DSPC/cholesterol/DSPE-PEG_{2000}$=1/1/0.1 (molar ratio) (cholesterol (+)) or $DSPC/DSPE-PEG_{2000}$=1/0.1 (cholesterol (-)) are shown, of which the particle sizes were 130.1 ± 2.0 and 141.3 ± 6.2 nm, respectively. Data are mean ± S.D. (n=3).

Fig. 4. Therapeutic effects of varying sizes of FK-LNP on cerebral I/R injury in t-MCAO rats.

t-MCAO rats were intravenously administered FK-L-LNP, FK-S-LNP (100 μg/kg FK506 dose), or PBS immediately after reperfusion. (A) After 24 h of reperfusion, 2-mm coronal brain sections were prepared and viable brain cells were stained in TTC solution. (B) The volume of damaged brain (white area) was evaluated using Image J. (C) The motor scores of the rats were assessed by a 21-point neurological test before dissection. Data are mean ± S.D. (n=6). ** $P<0.01$ and *** $P<0.001$ vs. PBS-treated group.

Fig. 1

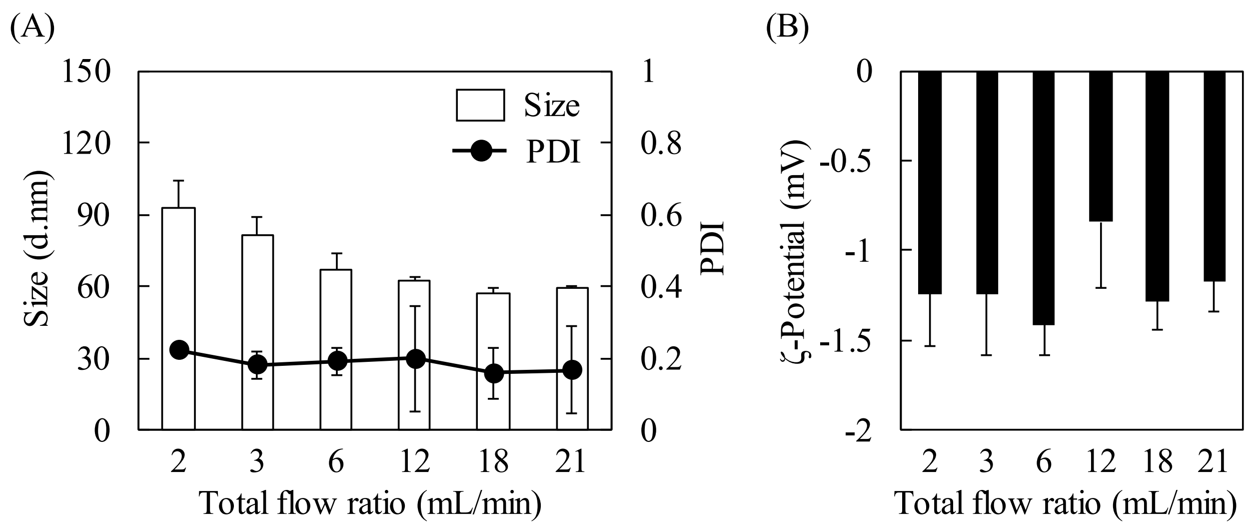


Fig. 2

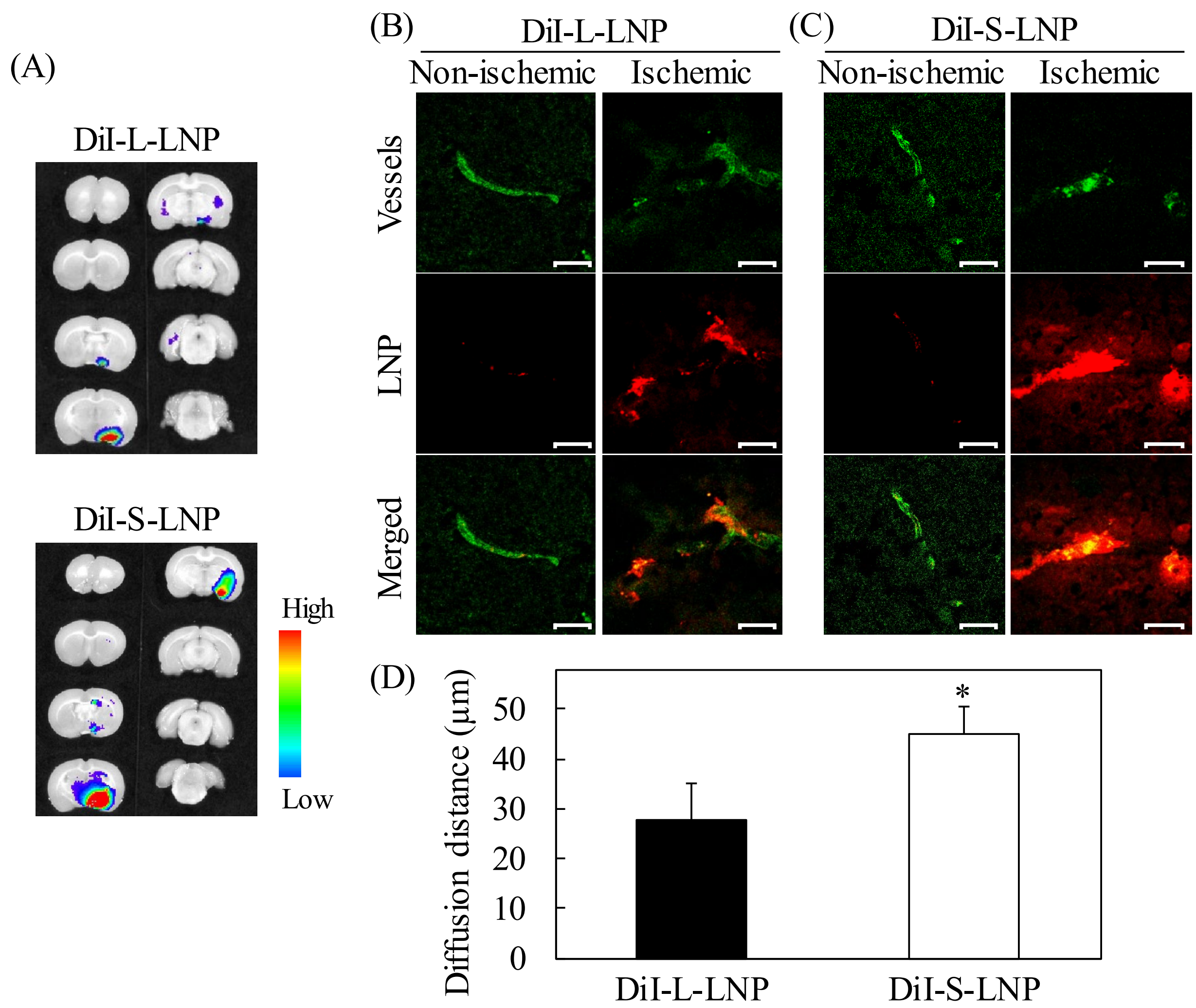


Fig. 3

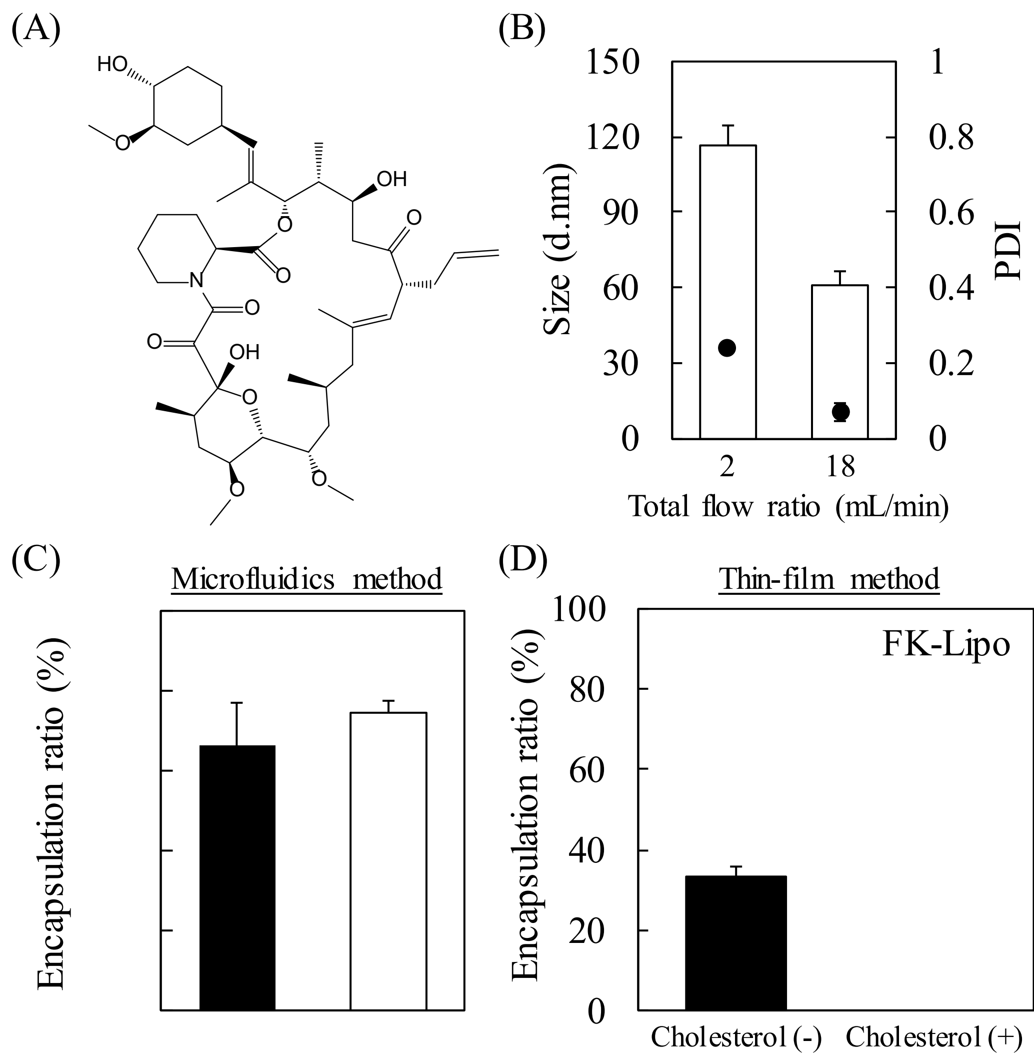
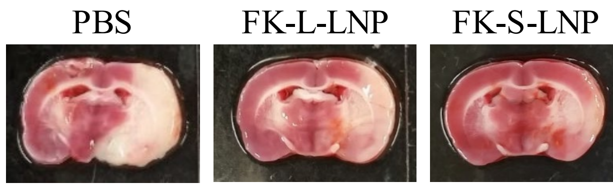
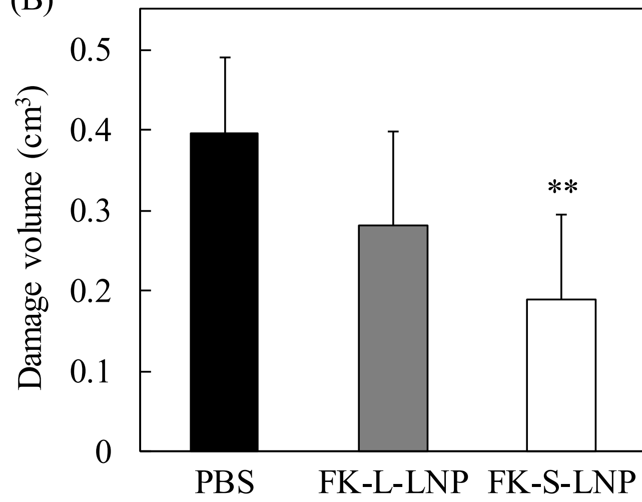


Fig. 4

(A)



(B)



(C)