Effective accumulation of polyion complex micelle to experimental choroidal neovascularization in rats

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Abstract **Exudative age-related macular degeneration, characterized by choroidal neovascularization (CNV), is a major cause of visual loss. In this study, we examined the distribution of the polyion complex (PIC) micelle encapsulating FITC-P(Lys) in blood and in experimental CNV in rats to investigate whether PIC micelle can be used for treatment of CNV. We demonstrate that PIC micelle has long-circulating characteristics, accumulating to the CNV lesions and is retained in the lesion for as long as 168 h after intravenous administration. These results raise the possibility that PIC micelles can be used for achieving effective drug targeting to CNV.**

Key words: Age-related macular degeneration; Choroidal neovascularization; Drug delivery system; Nanotechnology; Polyion complex micelle

1. Introduction

Exudative age-related macular degeneration (AMD), characterized by choroidal neovascularization (CNV), is a major cause of visual loss in developed countries [1,2]. Photocoagulation of the entire CNV is an effective treatment option for exudative AMD proved in large randomized control studies performed by the Macular Photocoagulation Study Group [3]. However, since most CNV extends to the subfovea, permanent central visual loss is inevitable immediately after photocoagulation. Thus, alternative treatments for CNV with minimal damage to the healthy retina, such as photodynamic therapy, are being developed [4]. In addition, compounds with anti-angiogenic properties are under intensive study for possible clinical applications [5,6]. Although studies in vivo using animal CNV models have demonstrated the favorable results of several anti-angiogenic drugs such as interferon-β and thalidomide, these drugs were not effective in inhibiting the development of CNV in humans. To develop a pharmacological therapy for CNV with minimal systemic adverse effects, it is necessary to achieve a high local concentration of the drug [7].

These results have prompted the search for an alternative drug delivery system. Macromolecules can accumulate and prolong their retention in perivascular regions of solid tumors to a greater extent than in normal tissues because newly formed vessels in solid tumors exhibit high substance permeability compared with those in normal tissues, and the lymph systems in tumor tissue are incomplete [8,9]. This effect is known as the enhanced permeability retention (EPR) effect [10]. CNV membranes have high permeability and several studies have demonstrated that macromolecules accumulate in experimental CNV presumably through the EPR effect [7].

The size of the molecules is an important factor in exerting the EPR effect. Polymeric micelles have a size range of several tens of nanometers with a very narrow distribution, similar to that of viruses and lipoproteins [8]. Thus, they accumulate in solid tumors through the EPR effect [11,12]. In addition, compared with the drug delivery system based on macromolecule conjugates, polymeric micelle can stably encapsulate chemical compounds with high efficiency [8]. On the basis on this together with their high drug-loading capacity, polymeric micelles are expected to become a novel drug delivery system. In fact, we have developed a drug delivery system with polymeric micelles encapsulating doxorubicin [13] and it is now in phase II clinical trial for the treatment of solid tumors [14].

We have recently developed a novel type of polymeric micelle formed through electrostatic interaction (polyion complex (PIC) micelle) [8,15]. Unlike polyion complexes formed from an oppositely charged pair of simple homopolymers or statistical copolymers, PIC micelles from charged block copolymers are totally water-soluble and are narrowly distributed. In this study, to investigate whether PIC micelle can be used for treatment of CNV, we examined the distribution of the PIC micelle in blood and in experimental CNV in rats.

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Abbreviations: AMD, age-related macular degeneration; CNV, choroidal neovascularization; DLS, dynamic light scattering; EPR, enhanced permeability retention; PIC, polyion complex

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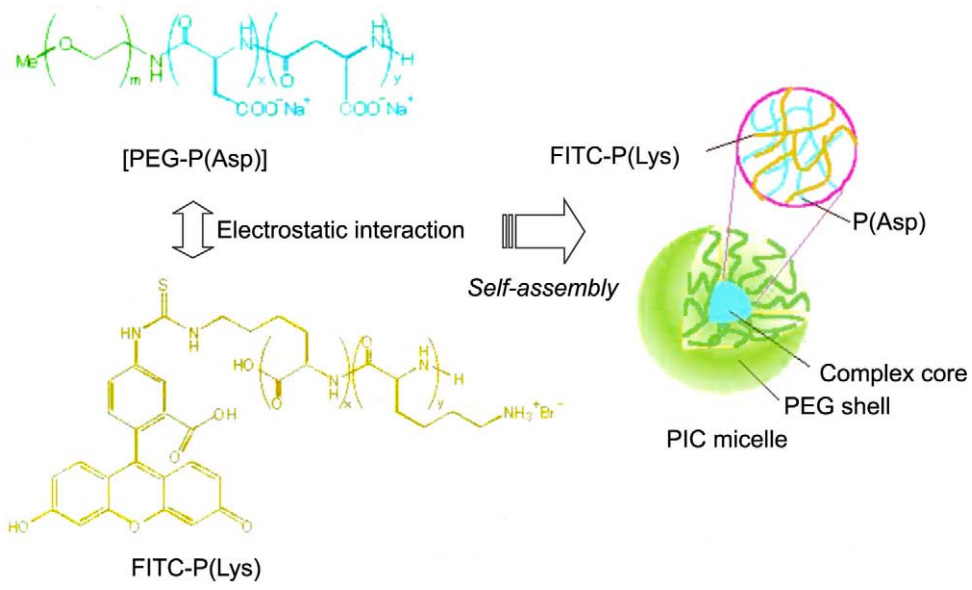

Fig. 1. Preparation and schematic structure of PIC micelle encapsulating FITC-P(Lys). PIC micelle consists of an inner complex core formed by the P(Asp) segment and FITC-P(Lys), and an outer PEG shell.

2. Materials and methods

2.1. Animals

Male Brown Norway rats weighing 100–120 g were obtained from Saitama Animal Lab (Saitama, Japan). All experiments were conducted in accordance with the Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Experimental CNV

A general anesthesia was induced with an intraperitoneal injection (1000 μl/kg) of a mixture (7:1) of ketamine hydrochloride (Ketalar®, Sankyo, Tokyo, Japan) and xylazine hydrochloride (Celactal®, Bayer, Tokyo, Japan) or by inhalation of diethyl ether. The pupil was dilated with one drop of 0.5% tropicamide (Mydrin® M, Santen Pharmaceutical, Osaka, Japan) for photocoagulation. Experimental CNV was created as previously described [16,17]. Fifty laser photocoagulations were applied to each eye between the major retinal vessels around the optic disc with a diode laser photocoagulator (DC-3000®, Nidek, Osaka, Japan) and a slit lamp delivery system (SL150, Topcon, Tokyo, Japan) at a spot size of 75 μm, duration of 0.05 s, and intensity of 200 mW.

2.3. Preparation of PIC micelle encapsulating fluorescein isothiocyanate-labeled poly-L-lysine (Fig. 1)

Fluorescein isothiocyanate-labeled poly-L-lysine [FITC-P(Lys), 100.0 mg; polymerization degree = 105, FITC = 0.004 mol/mol of Lys] and 48.6 mg of polyethylene glycol-*block*-poly-α,β-aspartic acid) [PEG-P(Asp); PEG MW = 5000 g/mol, polymerization degree of P(Asp) segment = 78] were dissolved in 10.0 and 5.0 ml of phosphate-buffered saline (PBS), respectively. PIC micelle solution was prepared by mixing the same volume (5.0 ml) of FITC-P(Lys) and PEG-P(Asp) solutions, in which the molar ratio of Lys and Asp residues was adjusted to unity. As a control, 5.0 ml of FITC-P(Lys) solution was diluted in 5.0 ml of PBS. Both PIC micelle and control solutions included the same concentration (5.0 mg/ml) of FITC-P(Lys). The average diameter and polydispersity index of PIC micelles was evaluated by dynamic light scattering (DLS) measured at 25°C, using a light scattering spectrophotometer (DLS-7000, Otsuka Electronics, Osaka, Japan) with a vertically polarized incident beam at 632.8 nm supplied by a He/Ne laser. A scattering angle of 90° was used in this study.

2.4. Accumulation of PIC micelle to CNV lesions: histological analysis

To investigate the accumulation of PIC micelle to the CNV lesions, 50 photocoagulations were applied to the right eye of a total of 23 rats. The left eyes served as non-photocoagulation controls. By tail injection, 400 μl of PIC micelle encapsulating 5.0 mg/ml FITC-P(Lys) or 400 μl of free FITC-P(Lys) at a concentration of 5.0 mg/ml ($n = 3$) was administered to rats 7 days after photocoagulation. There was high mortality in rats receiving FITC-P(Lys) after 1 h, suggesting that P(Lys) has toxicity.

After the rats were killed with an overdose of sodium pentobarbital, the eyes were immediately enucleated, snap-frozen in OCT compound 1, 4, 8, 24, and 168 h later for the PIC micelle group ($n = 4$ at each time point) and 1 h later for the FITC P(Lys) group ($n = 3$). Then frozen sections were mounted with the ProLong Antifade Kit (Molecular Probes, Eugene, OR, USA) and observed under a fluorescent microscope (model IX, Olympus, Tokyo, Japan).

2.5. Measurement of the concentration of PIC micelle in laser-treated eyes and blood

To investigate the concentration of FITC-P(Lys) in laser-treated eyes and blood, rats received PIC micelle encapsulating FITC-

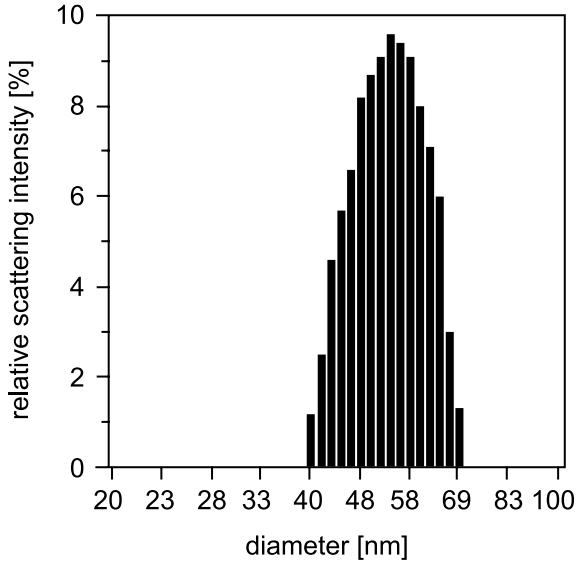

Fig. 2. DLS histogram of PIC micelle encapsulating FITC-P(Lys). The average diameter and polydispersity index of PIC micelles were evaluated by DLS measurement at 25°C, using a light scattering spectrophotometer. PIC micelle was narrowly dispersed with a size around 50.7 nm.

P(Lys) ($n = 20$) or free FITC-P(Lys) ($n = 3$) intravenously 7 days after photocoagulation. They were killed with an overdose injection of sodium pentobarbital 1, 4, 8, 24 and 168 h after receiving the PIC micelles encapsulating FITC-P(Lys) ($n = 4$ at each time point) and 1 h after receiving free FITC-P(Lys) ($n = 3$). Immediately after death, blood samples were collected and the eyes were enucleated. The blood samples were centrifuged at 12 000 rpm for 5 min and the supernatant was collected and subjected to spectrophotometric analysis. The retina/choroid was collected after the anterior segment and vitreous were removed. Then, the retina/choroid samples were homogenized in 0.1 ml of PBS and suspended in a total of 0.3 ml of PBS. The homogenates were then centrifuged at 12 000 rpm for 5 min and the supernatant was collected and subjected to spectrophotometric analysis. The fluorescence intensity was measured in a fluorescence spectrophotometer (FP-6500, Jasco, Tokyo, Japan) with an excitation wavelength of 495 nm and an emission wavelength of 520 nm. The actual concentration of FITC-P(Lys) was calculated by means of a calibration curve.

2.6. Statistical analysis

Mann–Whitney's *U*-test was used. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Preparation of PIC micelle encapsulating FITC-P(Lys)

Precipitation after mixing FITC-P(Lys) and PEG-P(Asp) solutions at 25°C was not observed, even after a period of over 2 months, suggesting the high storing stability of PIC micelle. Fig. 2 shows the size distribution of PIC micelle obtained from histogram analysis of DLS measurements. It was clear that the prepared PIC micelle had a unimodal size distribution. Also the average diameter and polydispersity index of PIC micelle were determined to be 50.7 nm and 0.046 by using the cumulant approach of DLS measurement, indicating the formation of PIC micelle with an extremely narrow size distribution.

3.2. Accumulation of PIC micelle to CNV lesions

3.2.1. PIC micelle group. Fluorescent staining was observed in the CNV lesions and also in the choriocapillaris

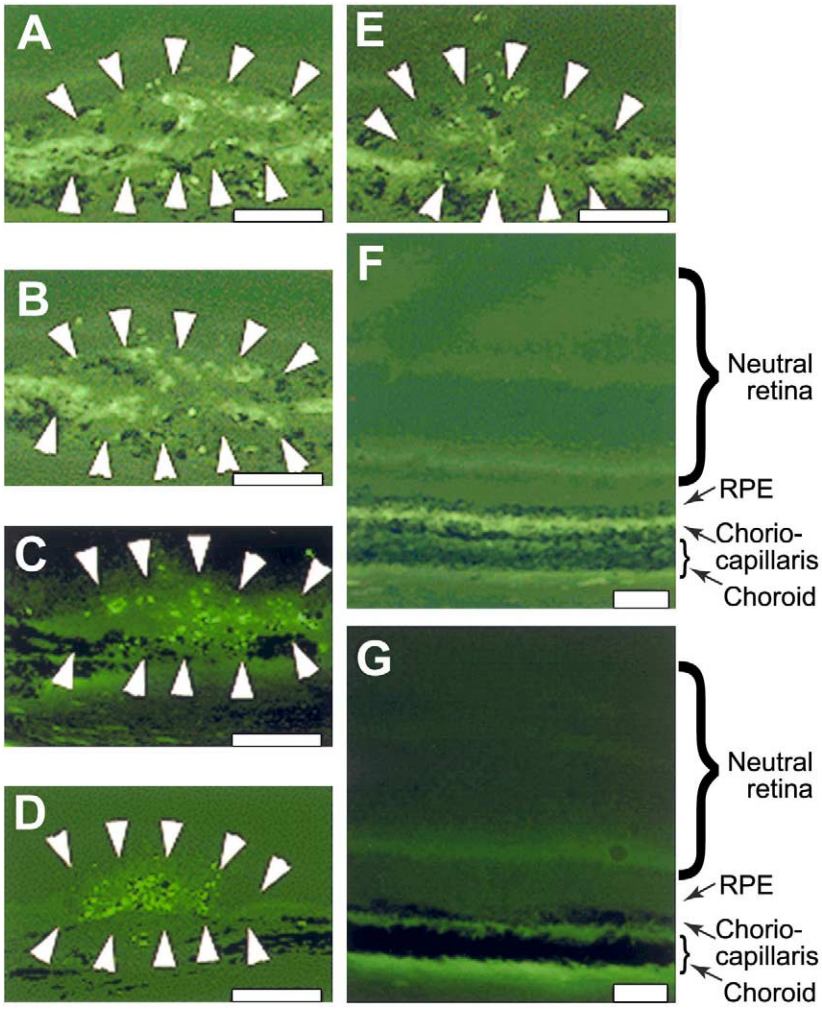

Fig. 3. Accumulation of PIC micelle to CNV lesion. The frozen sections of the CNV lesions were observed under a fluorescent microscopic 1 (A), 4 (B), 24 (C) and 168 (D) h after rats received PIC micelle-incorporated FITC-P(Lys). Note that FITC-P(Lys) initially diffuses to the CNV lesion and choriocapillaris (A,B), and becomes confined to the CNV lesion thereafter (C,D). Bright fluorescence was observed in the CNV lesions up to 168 h (D). One hour after rats received an intravenous injection of free FITC-P(Lys), FITC-P(Lys) distributes to the CNV lesion and choriocapillaris (E). Arrowheads in A–E indicate CNV lesions. Most rats died 1 h after free FITC-P(Lys) administration. In the laser-non-treated eyes, fluorescence was observed in the choriocapillaris 1 h after rats received PIC micelle (F) and the fluorescence became invisible 4 h after the injection of PIC micelle (G). Note that PIC micelle effectively accumulated to the CNV lesion throughout the studied period. Scale bar, 25 μm.

for up to 4 h (Fig. 3A,B). Twenty-four hours after the administration, accumulation of FITC-P(Lys) to the CNV lesion was more evident and fluorescence became invisible outside the photocoagulated lesion including the choroidal and retinal vasculature (Fig. 3C). The fluorescence was observed for up to 168 h (Fig. 3D). In the non-laser-treated eyes, the fluorescence was visible in the choroidal vessels for up to 4 h (Fig. 3F), and the fluorescence became invisible at 24 h and thereafter (Fig. 3G and data not shown). Light microscopic analysis revealed no abnormalities in other retinal structures.

3.2.2. Free FITC-P(Lys) group. Most rats died 1 h after the FITC-P(Lys) administration. When evaluated 1 h after intravenous administration, fluorescence was observed in the CNV lesion and choriocapillaris (Fig. 3E). In order to reduce the toxic effect of free FITC-P(Lys), rats received a lower dose (10 mg per injection) of free FITC-P(Lys). However, all rats died before 2 h after the administration ($n > 10$).

3.3. Concentration of FITC-P(Lys) in the laser-treated eyes

3.3.1. PIC micelle group. FITC-P(Lys) was detected in the retina/choroid from the laser-treated eyes as early as 1 h and the concentration peaked at 4 h and the residual FITC-P(Lys) was still evident 168 h after intravenous administration (Fig. 4). The concentration of FITC-P(Lys) was below the detectable level in the non-laser-treated eyes.

3.3.2. Free FITC-P(Lys) group. When evaluated 1 h after intravenous administration, the concentration of FITC-P(Lys) in the free FITC-P(lys) group was significantly lower compared to that in the PIC micelle group (Fig. 4).

3.4. Concentration of PIC micelle in blood

Fig. 5 shows the concentration of FITC-P(Lys) in blood after a single injection of either free FITC-P(Lys) or PIC micelle encapsulating FITC-P(Lys). The residual amount of FITC-P(Lys) in blood was 5.0, 7.8, 3.8 and 0.5% of the injected dose at 1, 4, 8, and 24 h, respectively, after intravenous injection of PIC micelles encapsulating FITC-P(Lys). After 168 h, it was below the detectable level. The concentration of FITC-P(Lys) in blood was 15% of the injected dose 1 h after free FITC-P(Lys) injection.

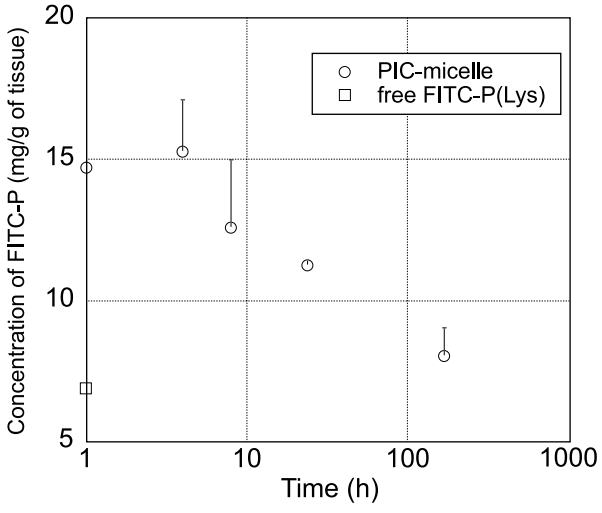

Fig. 4. Concentration of FITC-P(Lys) in retina/choroid. Concentrations of FITC-P(Lys) in retina/choroid after rats received equivalent doses of FITC-P(Lys) of PIC micelle-incorporated FITC-P(Lys). Note that FITC-P(Lys) was detected in retina/choroid from the laser-treated eyes of the PIC micelle group as early as 1 h and the concentration peaked at 4 h and was still evident 18 h after intravenous administration, whereas the concentration of FITC-P(Lys) in the free FITC-P(Lys) group was significantly lower compared to that in the PIC micelle group 1 h after injection. Most rats died 1 h after free FITC-P(Lys) administration. Error bars indicate S.D.

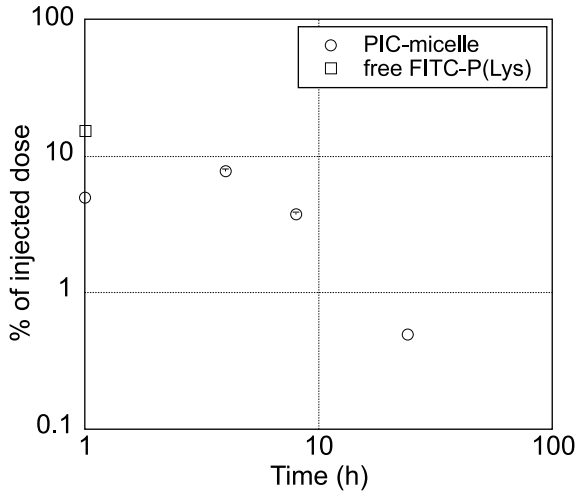

Fig. 5. Concentration of PIC micelle in blood. Concentration of FITC-P(Lys) in blood after rats received 400 μl of free FITC-P(Lys) at a concentration of 5.0 mg/ml or 400 μl of PIC micelles encapsulating 5.0 mg/ml FITC-P(Lys). The residual amount of FITC-P(Lys) in blood was 5.0, 7.8, 3.8 and 0.5% of the injected dose at 1, 4, 8 and 24 h, respectively, after intravenous injection of PIC micelles encapsulating FITC-P(Lys). Most rats died 1 h after free FITC-P(Lys) administration, whereas no rats died after intravenous injection of PIC micelles encapsulating FITC-P(Lys). Error bars indicate S.D.

4. Discussion

In this study, FITC-P(Lys) was retained in the CNV lesion for as long as 168 h after intravenous injection of PIC micelle encapsulating FITC-P(Lys) as demonstrated by histological analysis and confirmed by the measurement of the concentration in the retina/choroid of the laser-treated eyes. Because it was impossible to distinguish free FITC-P(Lys) and FITC-P(Lys) encapsulated into PIC micelle, the data presented here do not address whether FITC-P(Lys) was present in a free form or encapsulated in the PIC micelle. Together with the high mortality rate associated with free FITC-P(Lys) administration, it was not directly proven whether encapsulation of FITC-P(Lys) into PIC micelle enhanced the accumulation of FITC-P(Lys) to the CNV lesions throughout the studied period. However, it was demonstrated that a significantly higher amount of FITC-P(Lys) accumulated to the CNV lesions 1 h after the injection in the PIC micelle group compared to the free FITC-P(Lys) group. It is of note that the amount of FITC-P(Lys) in the non-laser-treated eyes was below the detectable level. In addition, because CNVs are highly permeable similar to the newly formed vessels in solid tumors, it is plausible to speculate that the PIC micelle is likely to accumulate to CNV lesions presumably through the EPR effect. This idea is consistent with recent studies that have demonstrated that macromolecules accumulate in the CNV lesion in rabbits [18].

We have reported that polymeric micelles have long-circulating characteristics [8] and that the stability of PIC micelles

in the blood stream can be controlled by the charge ratio and the length of the poly-amino acid, one of the block copolymers that constitute PIC micelles [19]. In this study, 0.5% of the injected dose was retained in the blood even 24 h after injection of PIC micelle encapsulating FITC-P(Lys). Generally, the concentration of compounds in the blood decreases rapidly to less than 0.1% of the injected dose in a few hours after intravenous injection of low molecular weight compounds such as fluorescein sodium. In fact, the concentration of fluorescein sodium 1 h after intravenous injection was at a non-detectable level (data not shown). Taken together, PIC micelle encapsulating FITC-P(Lys) also have long-circulating characteristics, which is a great advantage to exert the EPR effect.

The concentration of FITC-P(Lys) delivered by PIC micelle to the retina/choroid peaked 4 h after injection, and remained as long as 7 days at a concentration higher than 15 μg/g protein after only one intravenous injection. Because a total of 2 mg FITC-P(Lys) was administered, the total amount of FITC-P(Lys) was approximately 300 ng in the retina/choroid when it was delivered by PIC micelles. As deduced from these results, 0.02% of the injected dose accumulated to and was retained within the retina/choroid for as long as 7 days. In the blood, 5% and 0.5% of the injected dose were retained 1 and 24 h after the injection of PIC micelle, respectively. Thus, the FITC-P(Lys) in blood vs. that in the retina/choroid at 1 and 24 h after the injection was 340:1 and 45:1, respectively, suggesting that PIC micelle could be effectively targeted to the retina/choroid through the EPR effect, in spite of the low concentrations in blood. Moreover, we found that the administration of free FITC-P(Lys) was associated with high mortality, whereas administration of PIC micelles encapsulating FITC-P(Lys) was not. This is in line with previous studies which demonstrated that modification of compounds with a polymeric carrier results in a decreased adverse effect [20]. Our observation supports that the PIC micelle attenuated the adverse effect, although the underlying cause of this lethal toxicity remains to be unraveled. Such characteristics of the PIC micelle are a great advantage to achieve effective targeting of drug while reducing systemic adverse effects.

The concentration of PIC micelles increased in the liver and spleen but gradually decreased in the kidney and lung within 24 h after intravenous injection (data not shown), suggesting that the PIC micelle is mainly eliminated through the spleen.

In summary, it has been demonstrated that the PIC micelle effectively accumulates to the CNV lesion. The distribution of drug-loaded polymeric micelles in the body may be determined mainly by their size and surface properties and is less affected by the properties of loaded drugs if they are embedded in the inner core of the micelles. Since PIC micelles are demonstrated to be able to reserve a variety of drugs, enzymes [21] and DNA in the core and can serve as non-viral gene delivery vectors [9,19,22], we believe that PIC micelles have great potential for achieving effective drug targeting to CNV.

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