Desalination and Water Treatment
www.deswater.com

17 (2010) 31–36
May

1944-3994/1944-3986
doi: 10.5004/dwt.2010.1695

Design of lipoprotein-adsorbed liposomes retaining Mn-porphyrins for SOD mimic delivery to brains

Mayuko Kanehisa, Shoichiro Asayama, Hiroyoshi Kawakami*

Department of Applied Chemistry, Tokyo Metropolitan University, 1-1 Minami-Osawa, Hachioji, Tokyo 192-0397, Japan
Tel. +81426771111(ext.)4972; Fax +81426772821; email: kawakami-hiroyoshi@c.metro-u.ac.jp

Received 23 July 2009; accepted 2 December 2009

ABSTRACT

Drug delivery to brains is becoming more and more important but is severely restricted by the blood–brain barrier (BBB). In this study, we have prepared an antioxidant for brain targeting by apolipoprotein E (ApoE), which was suggested to mediate this drug transport across the BBB. Superoxide dismutase (SOD) catalyzing the reduction from superoxide radical anion ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) is the key enzyme for the protection from oxidative stress. The oxidative stress is considered to be implicated in the pathogenesis of a number of diseases, such as Alzheimer's disease. The SOD mimic manganese porphyrins (Mn-porphyrins) have been retained by a liposome composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine, sodium oleate, and Tween-80. The Tween-80 is reported to lead to the adsorption of ApoE. The resulting liposomes were incubated with serum proteins containing ApoE. After the incubation, the protein determination proved that the amount of adsorbed proteins on the liposome surface increased almost in proportion to the amount of the Tween-80 of the liposome. Subsequently, sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) revealed that the adsorbed proteins contained ApoE. Furthermore, the cellular uptake of the liposome was examined using cells expressing low-density lipoprotein receptors which recognize ApoE. The intracellular Mn-porphyrin retained by the liposome increased in proportion to the amount of the Tween-80 of the liposome. Moreover, the brain uptake of the Mn-porphyrin was observed in our preliminary in vivo experiment, where i.v. injection of the resulting liposome was carried out. These results suggest that the Mn-porphyrin SOD mimic can be delivered across the BBB, probably after interaction with lipoprotein receptors on the brain capillary endothelial cell membranes. Our system is considered to be a promising approach for protection of the brain under oxidative stress.

Keywords: Mn-porphyrin; Liposome; SOD mimic; Brain targeting; Drug delivery system

1. Introduction

Reactive oxygen species (ROS) are considered to be implicated in the pathogenesis of a number of diseases, such as atherosclerosis, cancer, and Alzheimer's disease [1–3]. Their toxic effects are amplified by pathological events including neutrophil activation, hyperoxia, metabolism of redox-active drugs, radiation exposure, and ischemia/reperfusion. Subsequently, the overproduced ROS, such as superoxide radical anion ($O_2^{\cdot -}$), cause the membrane damage resulting from lipid peroxidation, as well as the attack on proteins and nucleic acids. Therefore, antioxidant enzymes are useful for the therapy of ROS-mediated injuries and diseases.

*Corresponding author

Presented at the Fifth Conference of the Aseanian Membrane Society Aseania 2009 "Recent Progress in Membrane Science and Technology", 12–14 July 2009, Kobe, Japan.

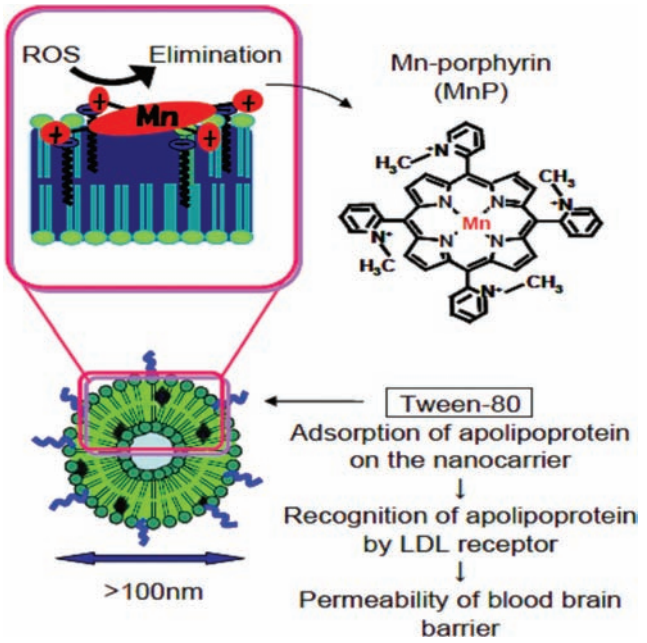

Fig.1. Design concept of the liposomes retaining Mn-porphyrin.

Superoxide dismutase (SOD) catalyzing the reduction from $O_2^{\cdot -}$ to H_2O_2 is the key enzyme for the protection of oxidative stress. As a defense mechanism against microorganisms and various exogenous compounds [4], on the other hand, phagocytic cells, i.e., neutrophils, monocytes, and macrophages, generate ROS during the respiratory burst by membrane-bound NADPH oxidase [5]. These merits and demerits of ROS have led us to create the drug delivery system using SOD to reduce oxidative stress.

Fridovich and coworkers have reported that manganese porphyrins (Mn-porphyrins) are promising compounds as SOD mimics with chemical versatility [6]. Subsequently, we have synthesized a macromolecular Mn-porphyrin for enhancing half-life in the blood circulation [7]. However, these Mn-porphyrins lack the ability to target the cells overproducing $O_2^{\cdot -}$, although the selective delivery of SOD mimics into every particular cell is promising for a radical approach [8] to various therapy.

The neural cells in the brain are weak against oxidative stress caused by ROS. In the brain, a lot of oxygen is consumed, resulting in the generation of ROS. The elimination of ROS is important for nerve diseases under oxidative conditions. Accordingly, Mn-porphyrin SOD mimic delivery to the brain is becoming more and more important but is severely restricted by the blood–brain barrier (BBB).

In this study, we have prepared an antioxidant for brain targeting by apolipoprotein E (ApoE), which was suggested to mediate this drug transport across BBB. Our attempts to develop delivery systems to improve drug (SOD mimic) transport to and across the tight endothelial cell layer of brain capillaries have been focused on the low-density lipoprotein receptor and one of its ligands, ApoE. Both are involved in the cellular uptake of cholesterol rich lipoproteins from the circulation [9], and the receptor is expressed on brain capillary endothelial cells at levels higher than those of other vessels [10].

The SOD mimic Mn-porphyrins have been retained by a liposome composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), sodium oleate (OA_{Na}), and Tween-80 [11,12]. Liposomes are phospholipid bilayer vesicles that have been studied extensively as potential drug and gene carriers. The Tween-80 is reported to lead to the adsorption of ApoE [13]. The Mn-porphyrin SOD mimic is expected to be delivered across the BBB, by the interaction with lipoprotein receptors on the brain capillary endothelial cell membranes (Fig. 1).

2. Materials and methods

2.1. Materials

DMPC was kindly furnished by the Nippon Fine Chemical Co., Osaka, Japan. OA_{Na} was purchased from the Tokyo Kasei Kogyo Co., Tokyo, Japan, and Tween-80 was purchased from the Nacalai Tesque, Inc., Tokyo, Japan. EDTA, Na_2EDTA, and HNO_3 were purchased from the Kanto Chemical Co., Tokyo, Japan. Quality KO_2 was purchased from Aldrich, Milwaukee, WI, USA. DMSO was obtained from Nacalai Tesque, Tokyo, Japan, and was dried over 3-Å molecular sieves. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co., Ltd.

2.2. Liposome preparation

Mn-porphyrins in their chloride form, 5,10,15,20-tetrakis-(*N*-methyl-2-pyridinio)porphinatomanganese was synthesized as reported in a previous paper [6].

The liposome contained cationic/anionic lipid combinations and were composed of Mn-porphyrin, DMPC, OA_{Na}, and Tween-80, as shown in Table 1.

The liposome retaining Mn-porphyrin was prepared by an ultrasonic irradiation method as reported in a previous paper [14]. At first, to form the complex of Mn-porphyrin (1 μmol) and OA_{Na} (4 μmol), they were dissolved in methanol. In addition, the methanol solution of a mixture with the Mn-porphyrin and OA_{Na} and various amount of Tween-80 dissolved in methanol were added to a chloroform solution of a lipid

mixture with DMPC (200 μmol). The chloroform solution was dried into a thin film in a round-bottom flask on a rotary evaporator. The lipids were then sonicated at 4°C for 30 min, flowed by incubation at room temperature for 1 h and filtration with 0.22-μm pore sterilized filter. The size of liposome was determined by a dynamic light scattering (NICOMP 370, Particle Sizing System, Santa Barbara, USA).

2.3. Stopped-flow kinetic analysis

The rate constant (k_{cat}) of the $O_2^{\cdot -}$ dismutation was measured with an SX.18MV stopped-flow spectrometer purchased from Applied Photophysics, Ltd., UK, according to the literature [15]. A DMSO solution of KO_2 was rapidly mixed with a HEPES buffer solution at pH 8.1 and 36°C to give $O_2^{\cdot -}$. The decay of $O_2^{\cdot -}$ was spectrophotometrically monitored at 245 nm. The rate constants were determined from the mean of at least 30 experiments with a deviation of no more than 10% from the mean.

2.4. Determination of adsorbed protein on liposome surface

The liposome (100 μM; 0.5 mL) was incubated with 10% fetal bovine serum (FBS) for 1 h at room temperature. After the incubation, the liposome was collected by centrifugation (202 G, 30 min), followed by washing with phosphate-buffered saline (PBS). The adsorbed protein was detached with 0.001% SDS in 50 mM phosphate buffer. The concentration of the detached protein in the resulting buffer was determined with Coomassie brilliant blue (CBB) G-250 using calibration curve at the absorbance at 590 nm.

2.5. SDS–PAGE

The adsorbed proteins on the liposome were separated by 12% SDS–PAGE. After the PAGE, the resulting gel was stained with CBB. The stained bands were determined with Bio-Rad Quantity One Software.

2.6. In vitro experiment

HepG2 cells were incubated in 75-cm^2 tissue culture flasks containing DMEM supplemented with 10% heat-inactivated FBS. After confluent growth, 100 μM Mn-porphyrin alone or the Mn-porphyrin retained by the liposome (2.4 mL), which was incubated with 10% FBS in the medium (15 mL), was added to the adherent cells, followed by the incubation for 1 h at 37°C. The resulting cells were detached, counted, and washed with PBS (–) to remove free Mn-porphyrins. Then, the cell lysis was obtained by adding 0.5 M HNO_3. The manganese in the lysis was determined by atomic absorption spectrometry at 403 nm using a Varian SpectrAA-640 (Varian Australis Pty., Ltd., Australia).

Table 1
Preparation of the liposomes retaining Mn-porphyrin

Mn-porphyrin loaded nanocarrier	DMPC (C14)	Tween-80	MnM2Py4P: OA_{Na}
Tween 0 mol%	200	0	1:4
Tween 0.1 mol%	200	0.2	1:4
Tween 1.0 mol%	200	2.0	1:4
Tween 1.5 mol%	200	3.0	1:4
Tween 4.0 mol%	200	8.0	1:4

2.7. In vivo experiment

Brain delivery studies of the liposome retaining Mn-porphyrin were carried out in rats (200 g, $n = 3$). The rats were injected (intravenous) with a dose of 2.2 mL kg^{-1} of 100 μM Mn-porphyrin alone or the Mn-porphyrin retained by the liposome in saline. At 2 h post-injection, the rats were sacrificed. The Mn determination of the homogenate of the brain was carried out by atomic absorption spectrometry as Section 2.6.

3. Results and discussion

3.1. Size and stability of the liposomes retaining Mn-porphyrin

Fig. 2 shows the effect of the incubation time on the particle size of the liposomes retaining Mn-porphyrin in the presence of 10% FBS. The liposome alone and the liposome containing 0–0.1mol% Tween-80 exhibited large particle size after incubation of FBS. On the other hand, the liposome containing 1.0–4.0 mol% Tween-80 exhibited stable colloidal stability after 4 h incubation. The particle size of the liposome containing 1.0–1.5 mol% Tween-80 maintained below 100 nm, and that containing 4.0 mol% Tween-80 did below 200 nm. These results suggest that the existence of Tween-80 increased colloidal stability in the presence of 10% FBS.

To examine further the colloidal stability in the presence of FBS, we incubated the liposome retaining Mn-porphyrin in the presence of 30–50% FBS. It should be noted that the liposome containing 1.0–1.5 mol% Tween-80 kept the particle diameter approximately 100 nm in spite of the higher concentration of FBS (Table 2). Therefore, the resulting liposomes are

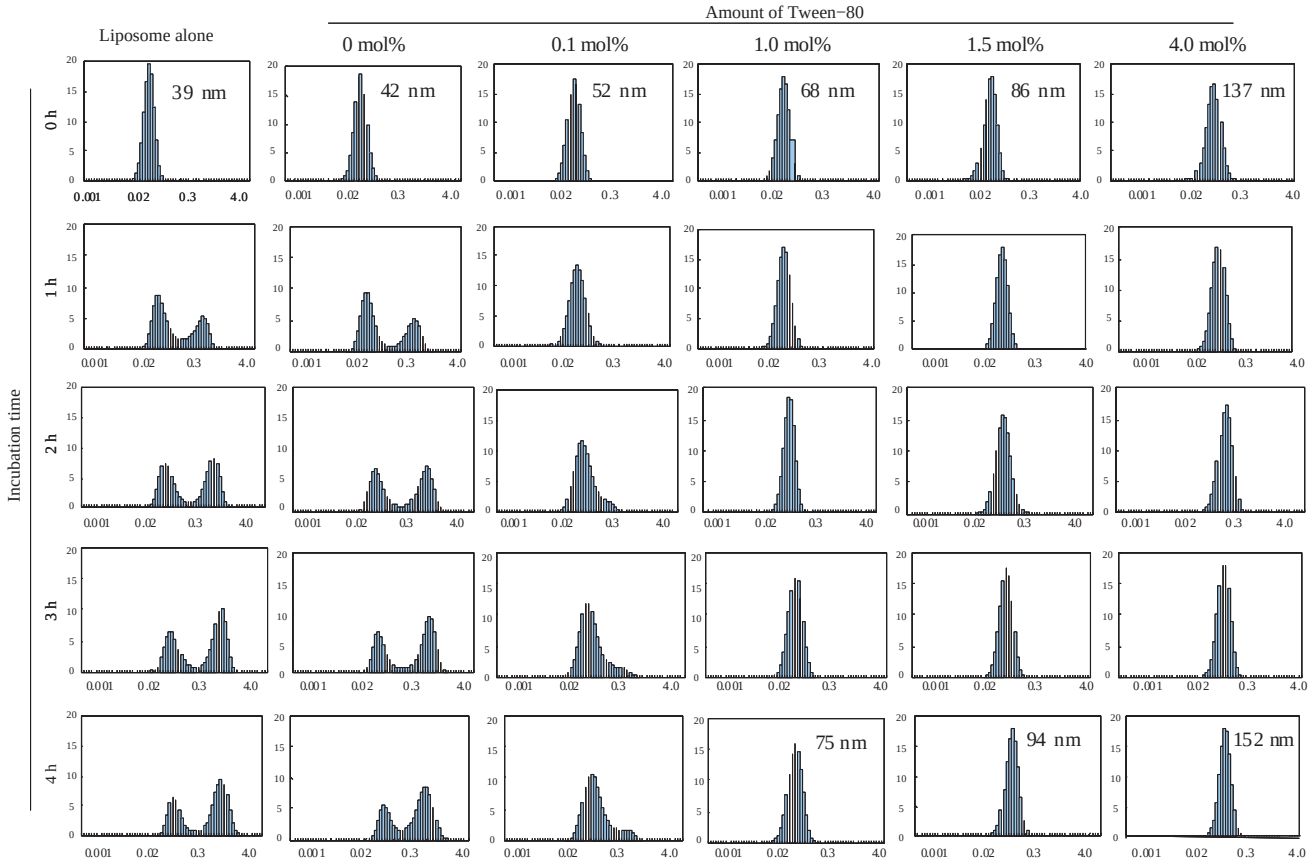

Fig. 2. Effect of the incubation time on the particle size of the liposome retaining Mn-porphyrins with various amounts of Tween-80 in the presence of 10% FBS.

expected to be stable carrier of Mn-porphyrin in blood stream. The slight increase of the particle size in proportion to the concentration of FBS suggests the protein adsorption on the surface of the liposome.

3.2. SOD activity of the liposomes retaining Mn-porphyrin

Table 3 shows the results of the SOD activities of the liposomes retaining Mn-porphyrin. The catalytic rate constants of them were measured using a stopped-flow kinetic analysis. The $O_2^{\cdot -}$ permeability through lipid bilayers as a liposome is known to be significant low [16]. Therefore, if the Mn-porphyrin had been entrapped inside the liposome or deployed into the hydrophobic bilayer, the SOD activity of the liposomes retaining Mn-porphyrin would significantly have decreased as compared with that of Mn-porphyrin. However, the SOD activities of the liposomes were significantly lower than that of Mn-porphyrin, indicating that the Mn-porphyrin may be loaded on the outer-surface of the liposome in spite of the presence of Tween-80.

3.3. Protein adsorption on the surface of the liposomes retaining Mn-porphyrin

As shown in Fig. 3, the protein adsorption on the surface of the liposome was increased in proportion to the amount of Tween-80. The amount of the adsorbed protein on the surface of the liposome containing 1.0–4.0 mol% Tween-80 was estimated to be 1.2 μg/lipid(μmol), which was consistent with approximately 0.3 ng cm^{-2} (the lipid area was estimated to be 0.7 nm^2.). From the estimated amount of the adsorbed protein as well as the increase of the

Table 2
Diameter (nm) of the liposome retaining Mn-porphyrin

	Amount of Tween-80 (mol%)				
Amount of FBS	0	0.1	1.0	1.5	4.0
0%	42	52	68	86	137
10%	231	68	74	94	152
30%	291	90	95	120	206
50%	327	80	105	132	227

Table 3
SOD activity of the liposome retaining Mn-porphyrin

Liposome retaining Mn-porphyrin	SOD activity ($\times 10^6 M^{-1}s^{-1}$)
MnP	28
Tween 0 mol%	12
Tween 0.1 mol%	8.9
Tween 1.0 mol%	12
Tween 1.5 mol%	12
Tween 4.0 mol%	8.4

diameter (Fig. 2), the monolayer adsorption of the serum proteins is considered to occur.

3.4. Characterization of the adsorbed proteins

To examine the characterization of the adsorbed proteins on the liposome containing 1.5 mol% Tween-80, which had stable size of the particle, we carried out SDS–PAGE, as shown in Fig. 4. The band of the adsorbed proteins seem to include ApoE as well as albumin, apolipoprotein A-I (ApoA-I) and globulin. The determination of the band suggests that 10–30 mol% apolipoproteins were adsorbed on the liposome.

3.5. Cellular recognition of the liposomes retaining Mn-porphyrins

We examined whether the liposomes retaining Mn-porphyrins were recognized by the cells expressing LDL receptors. Fig. 5 shows the recognition of the liposomes containing 0.1–4.0 mol% Tween-80 by the cells. The amount of the liposomes retaining

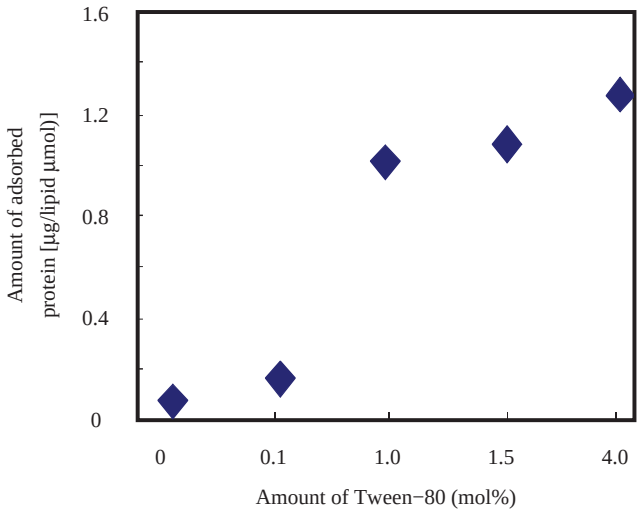

Fig. 3. Adsorption of FBS on the liposomes retaining Mn-porphyrin.

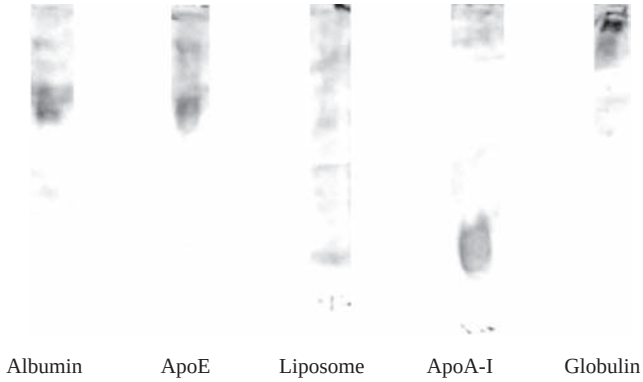

Fig. 4. SDS–PAGE of the adsorbed proteins on the liposomes retaining Mn-porphyrin.

Mn-porphyrins recognized by the cell was increased as the amount of Tween-80 increased. These results suggest that the cellular recognition of the liposome mediated by the interaction between LDL receptors on the cell and ApoE adsorbed through Tween-80.

3.6. Brain uptake of the liposomes retaining Mn-porphyrins

Finally, we examined the brain uptake of the liposomes retaining Mn-porphyrin in vivo. As shown in Fig. 6, the brain uptake of the liposomes containing 1.5% Tween-80 was significantly higher than that of the liposome with no Tween-80. These results suggest that the Mn-porphyrin on the liposome containing 1.5 mol% Tween-80 can be delivered across the BBB, probably after interaction with lipoprotein receptors on the brain capillary endothelial cell membranes.

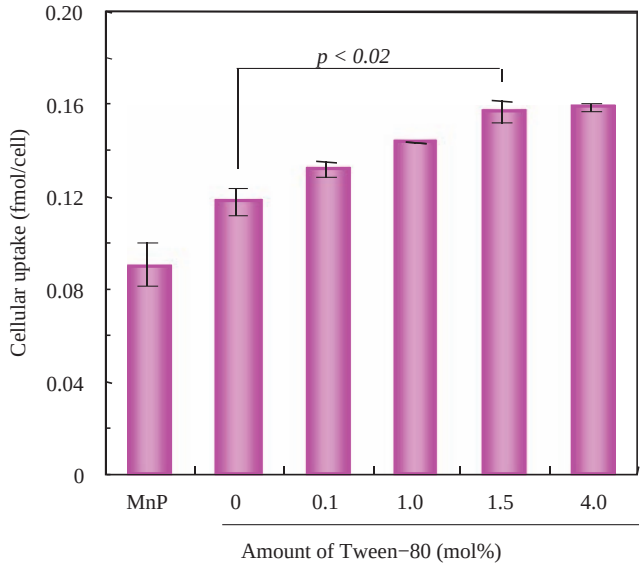

Fig. 5. Cellular recognition (HepG2) of the liposomes retaining Mn-porphyrin.

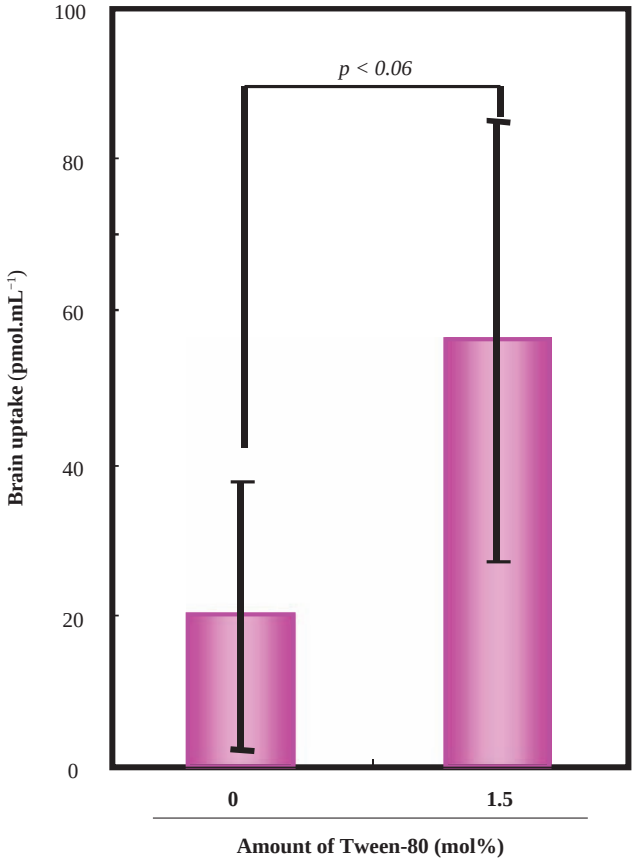

Fig. 6. Rat brain uptake of the liposomes retaining Mn-porphyrin ($n = 3$, $P < 0.06$).

4. Conclusions

In this study, we have prepared an antioxidant for brain targeting by ApoE, which was suggested to mediate this drug transport across the BBB. The resulting lipoprotein-adsorbed liposomes retaining Mn-porphyrins is considered to be delivered to brains through lipoprotein receptors on the brain capillary endothelial cell membranes. Our system is expected to be a promising approach for protection for the brain under oxidative stress.

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