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Octaarginine-modified liposomes enhance the anti-oxidant effect of Lecithinized superoxide dismutase by increasing its cellular uptake

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Abstract The anti-oxidant enzyme superoxide dismutase (SOD) has the potential for use as a therapeutic agent in the treatment of various diseases caused by reactive oxygen species. However, achieving this would be difficult without a suitable delivery system for SOD. We previously reported that PC-SOD, in which four molecules of a phosphatidylcholine (PC) derivative were covalently bound to each dimer of recombinant human CuZnSOD, was a high affinity for the cell membrane (R. Igarashi et al, *J. Pharmacol. Exp. Ther*. 271 (1994) 1672-7). Here, we show that an octaarginine (R8) modified liposome equipped with PC-SOD (R8-LP (PC-SOD)) enhances its anti-oxidant effect. High-density R8-modified liposomes can stimulate macropinocytosis and are taken up efficiently by cells as demonstrated in a previous study (I.A. Khalil et al. *J. Biol. Chem*. 281 (2006) 3544-51). Flow cytometry analyses showed that R8-LP (PC-SOD) was taken up by cells more efficiently than PC-SOD. Moreover, R8-LP (PC-SOD) liposomes were found to scavenge superoxide anions (O_2^-) very efficiently. These results suggest that the efficient cytosolic delivery of PC-SOD by R8-modified liposomes would enhance the anti-oxidant effects of PC-SOD.

Key words: Lecithinized superoxide dismutase (PC-SOD); Octaarginine-modified liposomes; Anti-oxidant therapy; Reactive oxygen speices (ROS); Mitochondria.

Introduction

Reactive oxygen species (ROS) are mainly produced in the mitochondrial respiratory chain and are associated with a variety of diseases, including cancer, neurodegenerative diseases and aging [1,2,3,4]. Superoxide dismutase (SOD), a major anti-oxidant enzyme, is known to be involved in defending a host from ROS. Three forms of SOD have been identified in humans. Copper, Zinc SOD (CuZnSOD) is located in the cytosol, manganese SOD (MnSOD) in the mitochondria and extracellular SOD (EC-SOD) in the extracellular space [5,6,7]. SODs catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important anti-oxidant defense in nearly all cells that are exposed to oxygen. Accordingly, SOD has attracted interest as a therapeutic candidate for the treatment of various diseases that are caused by ROS.

Clinical applications of SOD have been limited because of its low cell-targeting and short half-life in the blood circulation [8]. Therefore, a drug delivery system for SOD would be highly desirable, and many researchers have attempted to achieve SOD protein therapy [9,10,11]. For example, the chemical modification of SOD with polyethylene glycol (PEG) prolongs its half-life in the blood circulation [12]. However, PEG-modified SOD has not yet been applied to treating clinical diseases

because of its low cell-targeting characteristics and poor cell-membrane affinity. To overcome these problems, we developed PC-SOD, in which four molecules of a phosphatidylcholine (PC) derivative were covalently bound to each dimer of recombinant human CuZnSOD [13] and showed that PC-SOD possessed a high affinity for the cell membrane and its blood half-life was much longer than that for unmodified SOD [14]. Moreover, PC-SOD exerted strong anti-oxidant effects in vivo [15,16,17] and is in clinical trials for treating interstitial pneumonia and colitis ulcerosa [18,19,20].

In an alternate approach, we developed octaarginine-modified liposomes (R8-LP) for efficient intracellular delivery and showed that high-density R8-LP stimulates macropinocytosis and is subsequently efficiently taken up by cells [21]. Furthermore, R8-LP was used to successfully deliver the green fluorescence protein to the cytosol, nucleus and mitochondria, when an optimal lipid composition for each organelle was used [22,23,24]. Hence, it would appear that R8-LP equipped with PC-SOD (R8-LP (PC-SOD)) would efficiently deliver SOD to the cytosol, thus enhancing its pharmacological potency.

In this study, we attempted the intracellular delivery of PC-SOD using R8-LP and scavenging ROS within living cells, as shown in Fig.1. R8-LP (PC-SOD) contains a high density of surface-modified R8, which promotes efficient internalizing by cells.

The carrier can escape from endosomes to the cytosol, where PC-SOD scavenges intracellular O_2^-. We first prepared R8-LP (PC-SOD) and optimized its characteristics. Intracellular uptake efficiency was then analyzed by flow cytometry and intracellular trafficking was observed using confocal laser scanning microscopy. Finally, the anti-oxidant effect of the preparation was evaluated to demonstrate that our strategy of integrating PC-SOD and R8-LP would be useful for anti-oxidant therapy.

Materials and methods

Materials.

1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine (DOPE) was purchased from Avanti Polar lipids (Alabaster, AL, USA). Phosphatidic acid (PA) was purchased from Sigma (St. Louis, MO, USA). Stearyl octaarginine (STR-R8) was obtained from KURABO INDUSTRIES Ltd. (Osaka, Japan). SOD and PC-SOD were prepared, as previously reported [13]. Alexa Fluor-488 labeled PC-SOD (Alexa488 PC-SOD) was prepared using an Alexa Fluor 488 Protein Labeling Kit (Invitrogen Corporation, Carlsbad, CA, USA), as described in the Supplementary material. HeLa human cervix carcinoma cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). Dulbecco's modified Eagle medium (D'MEM) and LysoTracker Red DND-99 were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Dihydroethidium was purchased from WAKO (Osaka, Japan). Hoechst33342 was purchased from Dojindo Laboratories (Kumamoto, Japan). Fetal bovine serum (FBS) was purchased from Thermo Scientific (Waltham, MA, USA). All other chemicals used were commercially available reagent-grade products.

Preparation of liposomes equipped with PC-SOD.

Liposomes equipped with PC-SOD [LP (PC-SOD)] were prepared by the lipid

film hydration method. Lipid films were formed on the bottom of a glass tube by evaporating a chloroform solution containing 550 nmol of lipids [DOPE/PA = 7 : 2 (molar ratio)] and 1.8 nmol PC-SOD. Next, 250 μL of 10 mM HEPES buffer (HB, pH 7.4) was added to the lipid film, followed by incubation at room temperature for 15 min to hydrate the lipids [total lipid concentration, 2.2 mM]. The lipid film was then sonicated for 1 min in a bath-type sonicator (85 W, Aiwa Co., Tokyo, Japan) to produce the LP (PC-SOD). To construct R8-modifeid LP (PC-SOD) [R8-LP (PC-SOD)], a solution of STR-R8 (15 mol% lipids) was added to a suspension of LP (PC-SOD).

The diameters of the particles were measured using a quasi-elastic light scattering method, and the ζ potentials were determined electrophoretically using laser doppler velocimetry (Zetasizer Nano ZS; Malvern Instruments, Herrenberg, Germany). To prepare liposomes equipped with Alexa488 PC-SOD, lipid films containing 138 nmol lipids and 112.5 pmol Alexa-488 labeled PC-SOD were prepared [total lipid concentration, 0.55 mM]. SOD activity was measured using an SOD activity Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), as described in the Supplementary material.

Cell cultures and transduction studies.

HeLa cells were maintained in D'MEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg). The cells were cultured under an atmosphere of 5% CO_2/air at 37ºC. One day before transduction, 2 x 10^5 cells were seeded on a 6-well plate (Corning, NY, USA) for flow cytometry analysis or a 35 mm glass base dish (IWAKI, Tokyo, Japan) for wide-field fluorescence microscopy and confocal laser scanning microscopy. In the case of quantifying cellular uptake and microscopy observation (Figs. 2 and 3), samples were added to the cells in serum-free medium. After a 1-hr incubation, the medium was removed. The cells were then washed once with ice-cold phosphate-buffered saline (PBS (-)) and twice with ice-cold PBS (-) containing heparin (20 U/mL). The cells were analyzed by flow cytometry, wide-field fluorescence microscopy or confocal laser scanning microscopy. For quantification of levels of intracellular O_2^- (Fig. 4), samples were added to the cells in serum-free medium. After a 1-hr incubation, 1 mL of D'MEM with serum was added to the cells, followed by a further 2-hr incubation. The O_2^- levels in cells were determined by flow cytometry.

Quantification of the cellular uptake of PC-SOD by flow cytometry.

The extent of cellular uptake for PC-SOD was determined using Alexa488

PC-SOD, by flow cytometry. PC-SOD and R8-LP (PC-SOD) (final concentration of Alexa488 PC-SOD, 72 nM) were incubated with cells, followed by washing with PBS (-)/heparin as described above.

The cells were then trypsinized, suspended in D'MEM with serum, precipitated by centrifugation (1,800*g*, 4ºC, 3 min) and washed with PBS (-)/heparin. After resuspension in 0.5 mL of PBS (-) containing 0.5% bovine serum albumin and 0.1% NaN_3, the cell suspension was filtered through nylon mesh to remove cell aggregates and dust, after which, the cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA). The cells were excited by 488 nm light from an Ar laser. The fluorescence detection channel was set to 530 nm FL1 filter. The cellular uptake of PC-SOD was expressed as mean fluorescence intensity, calculated using the CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Intracellular visualization of PC-SOD by wide-field fluorescence microscopy.

To visualize intracellular PC-SOD delivery, the cells were observed by wide-field fluorescence microscopy. Cells were incubated with PC-SOD and R8-LP (PC-SOD) (final concentration of Alexa488 PC-SOD, 72 nM), and then washed with PBS (-)/heparin as described above. The medium was then replaced with fresh medium with serum containing Hoechst33342 (final concentration, 1 μg/mL), followed by

incubation 10 min to stain the nuclei. After the incubation, the cells were washed with phenol red-free medium containing serum.

Images were acquired using a Nikon ECLIPSE TE-2000-U wide-field fluorescence microscope equipped with a Nikon Plan Apo 40x0.6 objective (Nikon Corporation, Tokyo, Japan). Control of the microscopy and the acquisition of digital images were performed with the NIS-Elements software program (Nikon). A mercury lamp was used for illumination. Blue (Hoechst33342) and green fluorophores (Alexa488 PC-SOD) were excited with light filtered through 350/50 and 492/18 excitation filters, respectively. Fluorescence was collected in the epi direction. The fluorescence was passed through a dichromatic mirror, reflecting at the exciting wavelength (82100v2bs; Chroma Technology Corp., Rockingham, VT, USA), and were further filtered from residual excitation light by bandpass filters (450/30 and 535/30, respectively). Image sequences were captured with an electron multiplier charge coupled camera (ImagEM; Hamamatsu Photonics, Hamamatsu, Japan). A Z-series of images were acquired in approximately 0.4 μm steps, and were subjected to the Richardson–Lucy algorithm-based 3D-deconvolution thereafter to minimize out-of focus blur.

Intracellular observation of PC-SOD using confocal laser scanning microscopy

We observed carries on an optical section within living cells using confocal laser scanning microscopy to obtain more advanced images of the intracellular trafficking of carries. The cells were incubated with PC-SOD and R8-LP (PC-SOD) (final concentration of Alexa488 PC-SOD, 72 nM), and then washed with PBS (-)/heparin as described above. Twenty minutes before the acquisition of fluorescence images, LysoTracker Red DND-99 was applied to the medium with serum at a final concentration of 1 μM to stain the acidic organelles (e.g., endosomes and lysosomes). After the incubation, the cells were washed with phenol red-free medium containing serum.

The cells were excited with 488 nm and 568 nm light from an Ar/Kr laser. A series of images were obtained by confocal laser scanning microscopy (LSM510 META; Carl Zeiss Co., Ltd., Jena, Germany) equipped with an Axiovert 200M inverted microscope, an oil-immersion objective lens (Plan-Apochromat 63/NA1.4) and a dichroic mirror (HFT488/568). The two fluorescence detection channels (Ch) were set to the following filters: Ch1: LP585 (red, LysoTracker Red DND-99), Ch 2: BP 505-550 (green, Alexa488 PC-SOD).

Quantification of intracellular O_2^- levels by flow cytometry.

Intracellular O_2^- was detected using dihydroethidium, a selective fluorescent probe dye for O_2^- and analyzed by flow cytometry. Cells were incubated with PC-SOD, LP (PC-SOD) and R8-LP (PC-SOD) (final concentration of PC-SOD, 100 units/mL), as described above. The cells were then washed with PBS (-) and incubated with 1.25 μM dihydroethidium for 30 min, followed by flow cytometry analysis as described above. The cells were excited with 488 nm light from an Ar laser. The fluorescence detection channel was set to 585 nm FL2 filter. O_2^- levels were expressed as the mean fluorescence intensity, which was calculated using the CellQuest software. We defined the M1 and M2 regions in the fluorescence-intensity histogram of dihydroethidium (Fig 4A) to distinguish between normal cells (M1 cells, the value for DHE-fluorescence intensity is similar in the case of non-treatment) and O_2^- level decreased cells (M2 cells, the DHE-fluorescence intensity is lower than that of non-treated cells). O_2^- level decreased cells were calculated as follows;

O_2^- level decreased cells (M2 cells) (%) = (number of M2 cells / number of total cells) × 100

Results and discussion

Preparation of R8-LP (PC-SOD)

We attempted to construct R8-LP (PC-SOD) containing an endosome-fusogenic lipid composition (DOPE/PA = 7:2 (molar ratio)) [25]. It has been reported that high density R8-LP is taken up efficiently by cells via macropinocytosis [21]. Moreover, R8-LP, composed of DOPE and PA, can achieve efficient endosomal escape, thus enhancing cytoplasmic delivery [25]. We first checked the effect of PC-SOD on the liposome preparation (Fig S1 in Supplementary material). The diameters of LP (PC-SOD) with different contents of PC-SOD were comparable (about 150 nm), suggesting that PC-SOD has no effect on the liposome preparation. We also compared the characteristics between LP (PC-SOD) and liposomes containing unmodified SOD (LP (SOD)). The findings indicate that the diameter of LP (SOD) was larger in size and more heterogeneous than that of LP (PC-SOD) (Table S1 in Supplementary material). These results indicate the importance of phosphatidyl choline in PC-SOD in a liposome preparation for SOD.

Next, the SOD activity of LP (PC-SOD) was measured (Fig S2 in Supplementary material). The results showed that the SOD activity of LP (PC-SOD) was similar to naked PC-SOD, suggesting that SOD activity was not lost during the preparation of the liposomes. Finally, we determined the optimal conditions for

R8-modification (Fig. S3 in Supplementary material). Small positively charged liposomes were formed at higher than 15 mol% of R8-modification, while aggregation occurred at a10 mol% R8-modification, probably due to electrostatic neutralization. Based on this result, we chose 15 mol% for R8-modification as the optimal condition for producing R8-LP (PC-SOD). Using these procedures we were able to successfully construct R8-LP (PC-SOD) with SOD activity. The physicochemical properties of LP (PC-SOD), R8-LP (PC-SOD) and R8-LP are summarized in Table 1.

Evaluation of the cellular uptake of PC-SOD

It is assumed that the cytoplasmic delivery of PC-SOD would contribute to its dismutase activity, because CuZnSOD is largely located in the cytosol [7]. Here, we measured the cellular uptake of PC-SOD by flow cytometry (Fig. 2A) and the intracellular trafficking of PC-SOD was observed using wide-field fluorescence microscopy (Fig. 2B). R8-LP (PC-SOD) containing Alexa488 PC-SOD was added to HeLa cells, and the cells were evaluated one hour later. Figure 2A (a) indicates the fluorescence intensities of Alexa488 PC-SOD inside the cells. The cellular uptake of PC-SOD was calculated as the mean fluorescence intensity of the flow cytometry histogram shown in Fig.2 A (a) (Fig. 2A (b)). The cellular uptake of R8-LP (PC-SOD)

was increased significantly compared with that of PC-SOD (Fig. 2A (b)). Moreover, we confirmed that R8-LP (PC-SOD) was taken up by cells, similar to R8-LP without PC-SOD (Fig. S5 in supplementary material), although the ζ potentials of R8-LP and R8-LP (PC-SOD) are different (Table 1).

Intracellular observations using wide-field fluorescence microscopy also revealed that the cellular uptake of R8-LP (PC-SOD) was higher than that of PC-SOD (Fig. 2B). In the case of R8-LP (PC-SOD), numerous green signals were detected inside cells and these signals were observed as dot forms (Fig. 2B (b)). On the other hand, signals for PC-SOD were diffuse and were not detected as dot forms (Fig. 2B (a)). Igarashi et al reported that PC-SOD accumulates in the cell membrane more effectively than unmodified SOD [13]. Taking this report into consideration, we hypothesized that most PC-SOD would diffuse to the cell membrane (Fig. 2B (a)). To validate this, we next analyzed the intracellular trafficking of PC-SOD, using optical sectioning images obtained by confocal laser scanning microscopy.

Intracellular observation of PC-SOD using confocal laser scanning microscopy

To analyze the intracellular trafficking of PC-SOD in detail, we utilized confocal laser scanning microscopy for advanced intracellular observation (Fig. 3). The

key feature of confocal laser scanning microscopy is its ability to acquire in-focus images from selected depths, a process known as optical sectioning. For interior imaging, the quality of the image is greatly enhanced over wide-field fluorescence microscopy, because image information from multiple depths in the specimen is not superimposed. In the case of R8-LP (PC-SOD), numerous green dots were observed in cells, and most of these were outside the acidic compartment (Fig. 3 (D-F)), suggesting that the carriers were delivered to the cytosol after endosomal escape. In the case of PC-SOD, the majority were observed on cell membranes, with only a few in cells (Fig. 3 (A-C)). This observation indicates that monomolecular PC-SOD has a high affinity to the cell membrane, and the few PC-SODs observed in cells would be taken up by the cell membrane by passive endocytosis. These results suggest that R8-LP has the ability to enhance the intracellular uptake efficiency of PC-SOD.

Evaluation for dismutation activity of R8-LP (PC-SOD)

Intracellular O_2^- levels were analyzed by flow cytometry using dihydroethidium (a selective O_2^- fluorescent probe). As shown in Fig. 4A, the fluorescence intensity of dihydroethidium was decreased by treatment with R8-LP (PC-SOD), whereas no changes were detected in the case of PC-SOD or R8-LP. To

evaluate the anti-oxidant effect, we estimated the percentages of O_2^- level decreased cells as described in Materials and methods (Fig. 4B). In the case of R8-LP (PC-SOD), number of O_2^- level decreased cell dramatically increased (44.6%). These results indicate that the scavenging efficiency of intracellular O_2^- in R8-LP (PC-SOD) is much superior to that in PC-SOD.

As shown in the evaluation of the cellular uptake of PC-SOD, it was found that R8-LP (PC-SOD) was taken up by almost all cells (Fig. 2A (a)). However, O_2^- level decreased cells were detected in no more than approximately half of the cells (Fig. 4). It is assumed that endosomal escape would not be sufficient for achieving effective PC-SOD delivery. We recently reported that a liposome surface modification with the pH-sensitive fusogenic peptide, GALA, enhanced endosomal escape and permitted cytosolic delivery to be efficiently achieved [25,26,27]. Accordingly, it would be expected that the modification of GALA with R8-LP (PC-SOD) would show stronger anti-oxidant effects. Studies concerning this are currently in progress.

Conclusion

We succeeded in developing an R8-LP equipped with PC-SOD. Flow cytometry analyses showed that R8-LP (PC-SOD) was taken up by cells more

efficiently than naked PC-SOD. Moreover, intracellular observations using confocal laser scanning microscopy showed that R8-LP (PC-SOD) has the ability to deliver PC-SOD to the cytosol. Finally, the findings reported herein confirm that R8-LP (PC-SOD) dramatically and effectively scavenges superoxide anions (O_2^-). These results suggest that the efficient cytosolic delivery of PC-SOD by R8-modified liposomes enhance the anti-oxidant effect of PC-SOD.

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Figure legends

Figure 1. Schematic diagram of the scavenging of O_2^- by R8-LP (PC-SOD).

R8-LP, a liposome that is surface-modified with a high density of octaarginine (R8), is efficiently internalized by cells. The carrier can escape from endosomes to the cytosol. R8-LP equipped with PC-SOD (R8-LP (SOD)) scavenges intracellular O_2^-. CuZnSOD, Copper, Zinc superoxide dismutase; CAT, catalase; PC-SOD, lecithinized superoxide dismutase.

Figure 2. Evaluation of the cellular uptake of PC-SOD.

A, The cellular uptake of PC-SOD was evaluated by flow cytometry. (a) The histogram plot shows the fluorescence intensities of Alexa488 PC-SOD within cells after the transduction of PC-SOD [blue line] and R8-LP (PC-SOD) [green line]. Black line indicates non-treated cells. (b) The cellular uptake of PC-SOD (MFI) was calculated based on the data shown in Fig.2 A (a). Data are represented as the mean ± S.D. (n = 3) *Significant difference ($p < 0.01$) was calculated by one-way ANOVA, followed by Bonferroni correction post hoc test. MFI, mean fluorescence intensity.

B, PC-SOD in cells was visualized using wide-field fluorescence microscopy: PC-SOD (a) or R8-LP (PC-SOD) (b). Nuclei were stained with Hoechst33342 prior to observation. The obtained images were processed by Richardson‐Lucy algorithm-based

3D-deconvolution. Scale bars, 20 μm.

Figure 3. Intracellular observation of PC-SOD using confocal laser scanning microscopy.

PC-SOD (A-C) and R8-LP (PC-SOD) (D-F) were incubated with HeLa cells. In this experiment, Alexa488 PC-SOD was used for the intracellular observation of PC-SOD. Acidic compartments (e.g., endosomes and lysosomes) were stained with LysoTracker Red DND-99, and then observed by confocal laser scanning microscopy: (left panels; green) Alexa488 PC-SOD, (middle panels; red) LysoTracker Red DND-99 and (right panels) merge images. Alexa488 PC-SOD appeared as yellow clusters when it was localized in acidic compartments. Scale bars, 10μm.

Figure 4. Evaluation for anti-oxidant effect of PC-SOD in living cells.

Intracellular O_2^- levels were analyzed by flow cytometry using dihydroethidium (selective O_2^- fluorescent probe) as described in Materials and methods.

A, The histogram shows the fluorescence intensities of dihydroethidium after the transduction of PC-SOD ([1] blue line), R8-LP ([2] red line) and R8-LP (PC-SOD) ([3] green line). Black line [4] indicates non-treated cells. M1, normal cells

(DHE-fluorescence intensity is similar value in the case of non-treatment); M2, O_2^- level decreased cells (DHE-fluorescence intensity is low).

B, O_2^- level decreased cells were calculated based on the data shown in Fig.4 A. Data are represented as the mean ± S.D. (n = 3). *Significant difference ($p < 0.01$) was calculated by one-way ANOVA, followed by Bonferroni correction post hoc test.

Table 1. Physicochemical characteristics of liposomes.

Data are represented as the mean ± S.D. (n = 3).

Figure 1

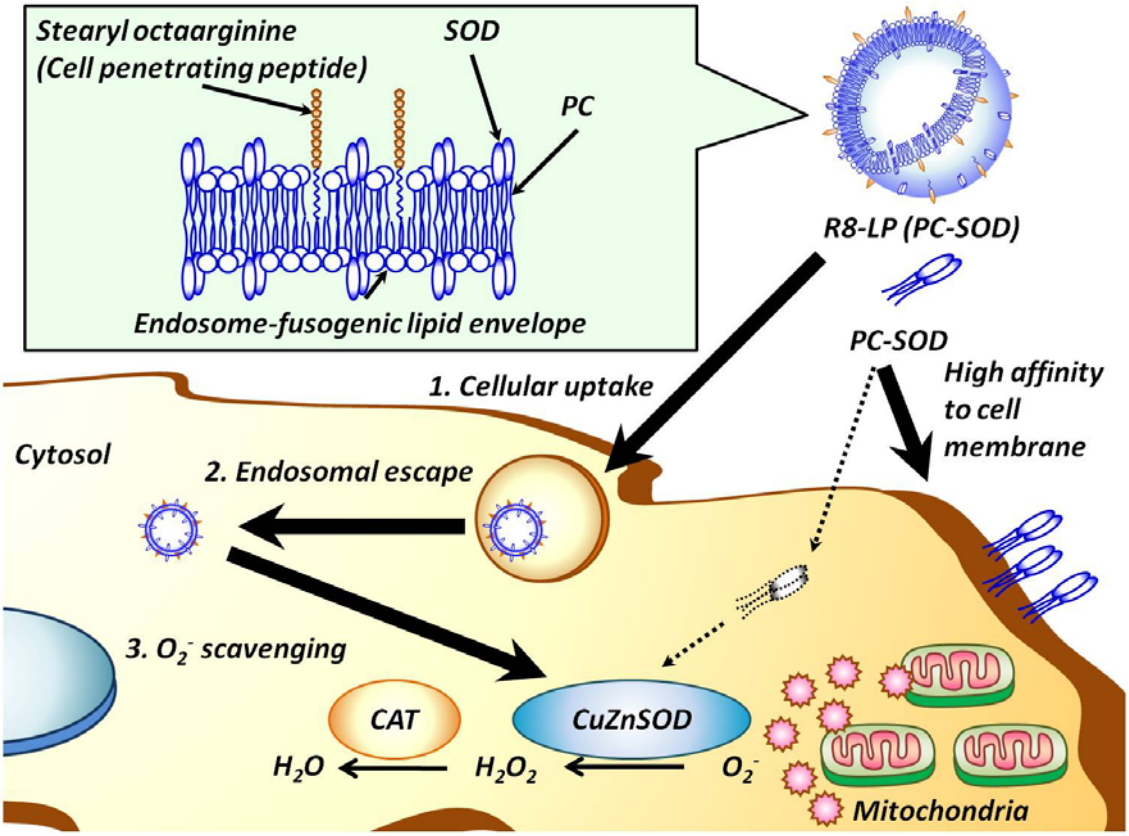

Stearyl octaarginine
(Cell penetrating peptide)
SOD
PC
Endosome-fusogenic lipid envelope
R8-LP (PC-SOD)
PC-SOD
High affinity
to cell
membrane
1. Cellular uptake
Cytosol
2. Endosomal escape
3. O_2^- scavenging
CAT
CuZnSOD
H_2O
H_2O_2
O_2^-
Mitochondria

Figure 2

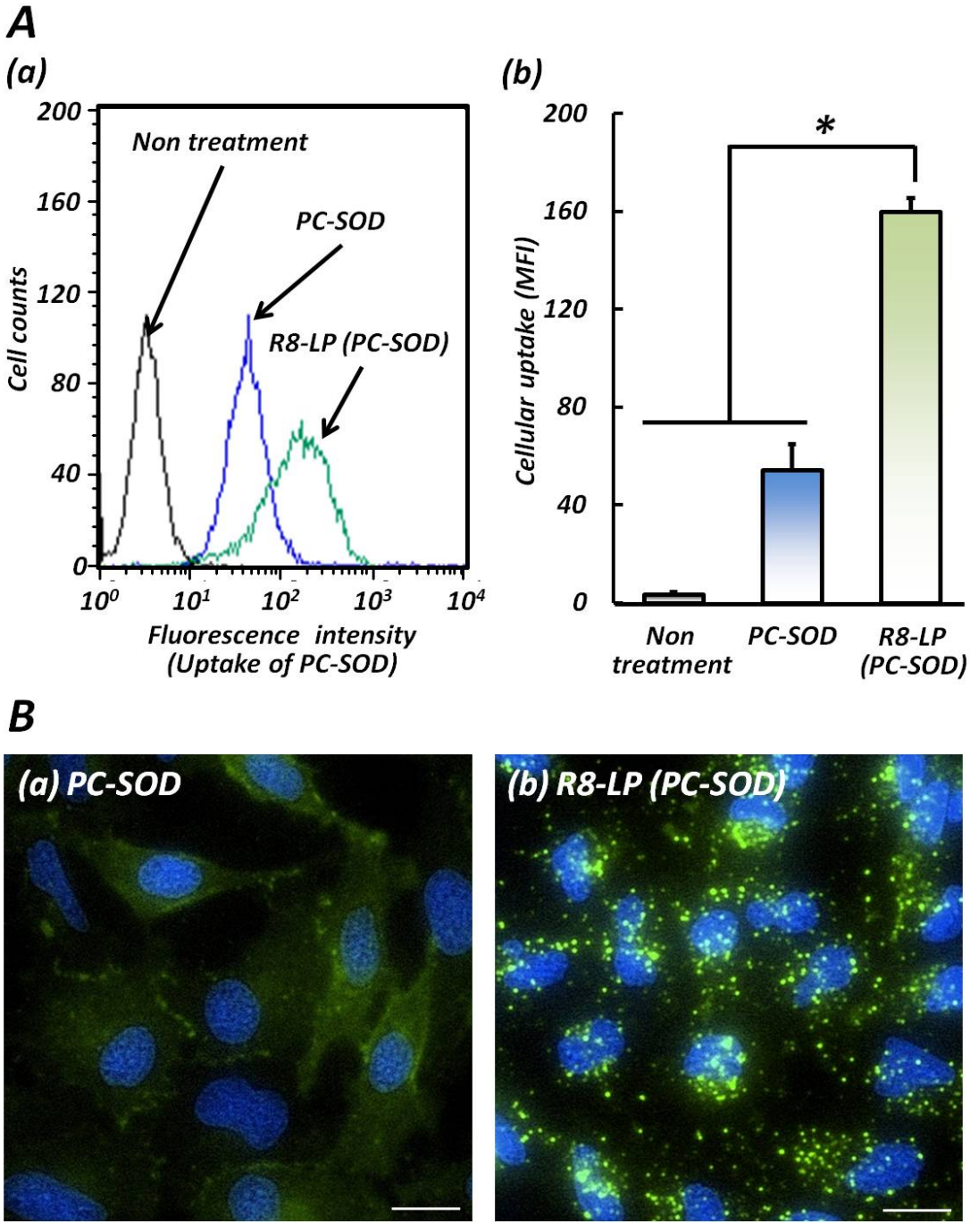

A
(a)
Non treatment
PC-SOD
R8-LP (PC-SOD)
Cell counts
200
160
120
80
40
0
10^0
10^1
10^2
10^3
10^4
Fluorescence intensity
(Uptake of PC-SOD)
(b)
*
Cellular uptake (MFI)
200
160
120
80
40
0
Non treatment
PC-SOD
R8-LP (PC-SOD)
B
(a) PC-SOD
(b) R8-LP (PC-SOD)

Figure 3

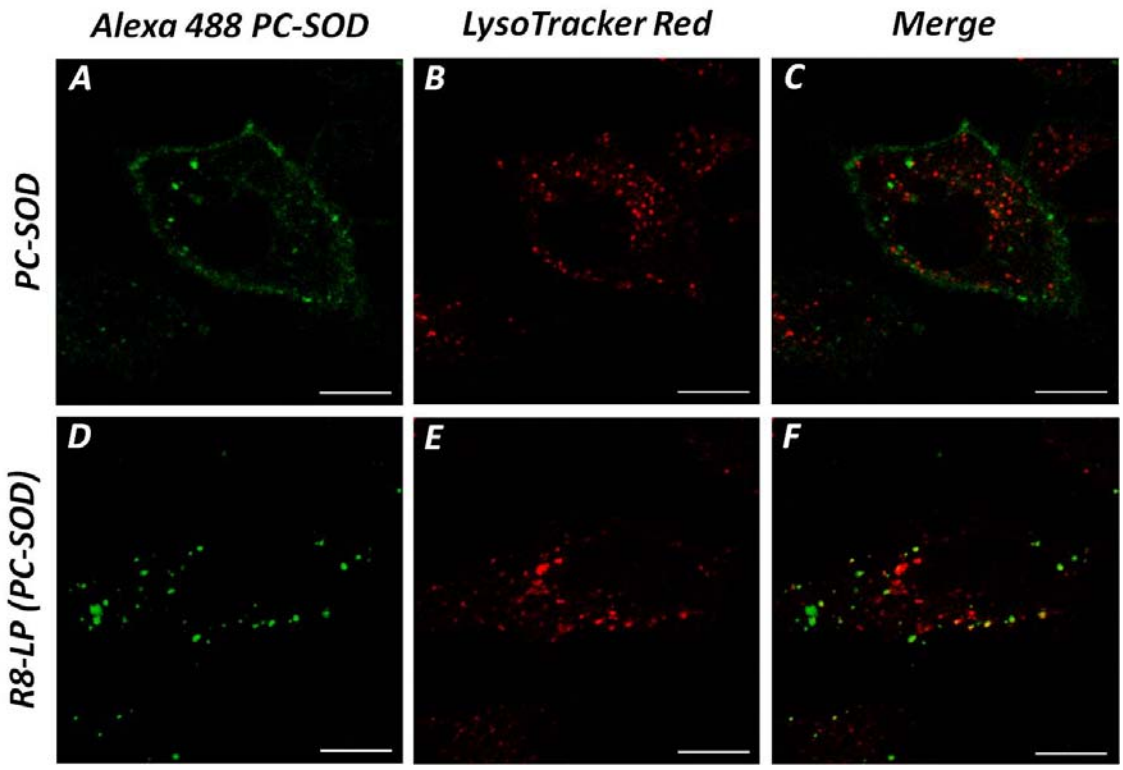

Alexa 488 PC-SOD
LysoTracker Red
Merge
A
B
C
PC-SOD
D
E
F
R8-LP (PC-SOD)

Figure 4

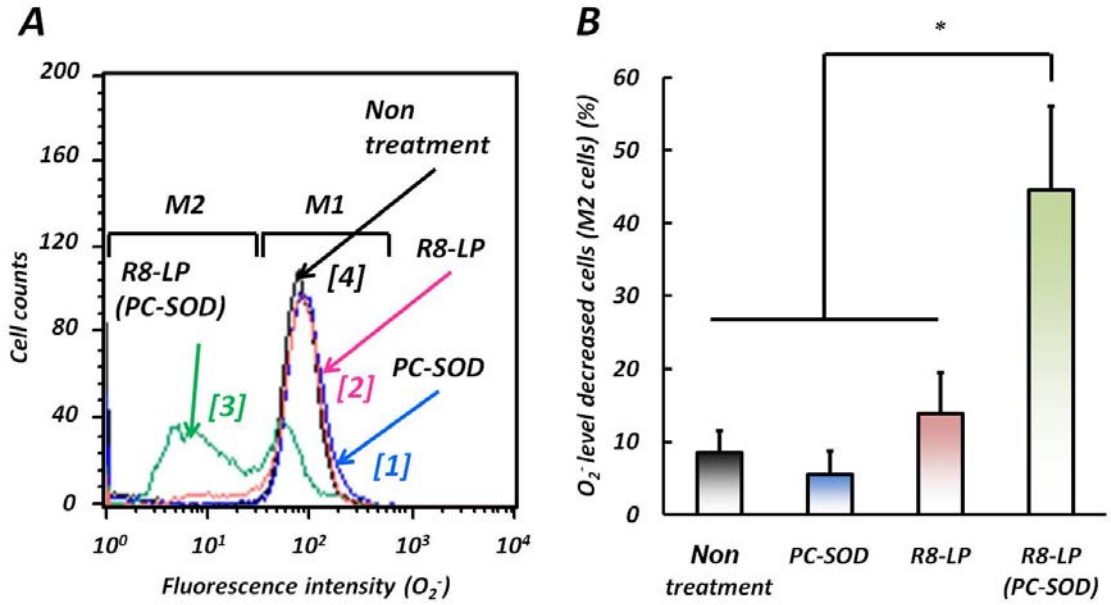

A
200
160
120
80
40
0
Cell counts
Non
treatment
M2
M1
R8-LP
(PC-SOD)
[4]
R8-LP
PC-SOD
[2]
[3]
[1]
10^0
10^1
10^2
10^3
10^4
Fluorescence intensity (O_2^-)
B
*
60
50
40
30
20
10
0
O_2^- level decreased cells (M2 cells) (%)
Non
treatment
PC-SOD
R8-LP
R8-LP
(PC-SOD)

Table 1

	Diameter (nm)	*ζ potential (mV)*
LP (PC-SOD)	*141 ± 19*	*-29 ± 13*
R8-LP(PC-SOD)	*170±7*	*20 ± 4*
R8-LP	*150 ± 39*	*45 ± 3*

Supplemental Material

1. Materials and methods

1.1. Preparation of liposomes equipped with SOD

Liposomes equipped with SOD [LP (SOD)] were prepared by the lipid film hydration method. Lipid films were formed on the bottom of a glass tube by the evaporation of a chloroform solution containing 550 nmol of lipids [DOPE/PA = 7 : 2 (molar ratio)] and 1.8 nmol SOD. Next, 250 μL of 10 mM HEPES buffer (HB, pH 7.4) was applied to the lipid film, followed by incubation at room temperature for 15 min to hydrate the lipids. The lipid film was then sonicated for approximately 1 min in a bath-type sonicator (85 W, Aiwa Co., Tokyo, Japan).

1.2. Measurement of diameter and ζ potential

Particle diameters were measured using a quasi-elastic light scattering method, and ζ potentials were determined electrophoretically using laser doppler velocimetry (Zetasizer Nano ZS; Malvern Instruments, Herrenberg, Germany).

1.3. Evaluation of SOD activity

In vitro SOD activity of PC-SOD or LP (PC-SOD) was determined by the xanthine (XA) -xanthine oxidase (XOD) method using an SOD activity Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) [Y. Oyanagui et al, *Anal. Biochem.* 142 (1984) 290-296]. PC-SOD or LP (PC-SOD) was added to XA and a nitroblue tetrazolium cocktail, and XOD was then added. In the presence of XOD, XA reacts with O_2, and generates O_2^-. SOD activity is determined from the inhibitory activity of this reaction.

1.4. Preparation of Alexa Fluor 488 labeled PC-SOD

PC-SOD was labeled with Alexa Fluor-488 using an Alexa Fluor-488 Protein Labeling Kit (Molecular Probes, Eugene, OR, USA) and was purified by gel filtration. After purification, the samples were subjected to SDS-PAGE, and the fluorescence of Alexa Fluor-488 in PC-SOD was detected using an FLA2000 fluorescent-image analyzer (Fuji Film, Tokyo, Japan). We also stained the SDS-PAGE gel with coomassie brilliant blue (CBB) to detect the presence of proteins.

1.5. Quantification for cellular uptake of R8-LP (PC-SOD) labeled by NBD-lipids.

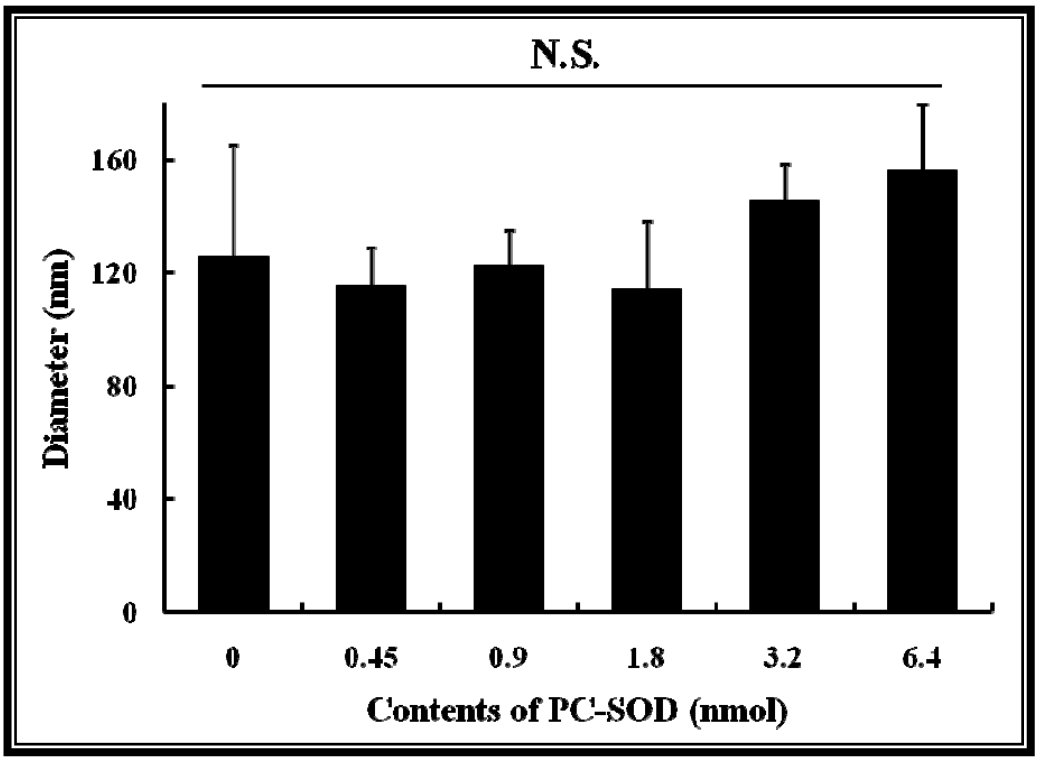
The extent of cellular uptake of liposomes was investigated using NBD-lipids, by flow cytometry. LP, R8-LP and R8-LP (PC-SOD) labeled with 1 mol% NBD-DOPE were incubated with cells (final concentration of liposome, 28 μM). The cells were then analyzed by flow cytometry as described in Materials and methods of the Main text.

2. Supplemental data

2.1. Effect of PC-SOD to liposome preparation

We examined the effect of PC-SOD on liposome preparations as described below. Lipid films containing 550 nmol of lipids [DOPE/PA = 7 : 2 (molar ratio)] and different amounts of SOD (0 - 6.4 nmol) were used in the liposome preparation. The diameters of the resulting suspension are summarized in Fig. S1. Diameters of LP (PC-SOD) with different content of PC-SOD were comparable (about 150 nm), suggesting that PC-SOD has no effect on the nature of the liposome preparation.

Fig. S1. Effect of PC-SOD on liposome preparations



The graph shows the diameters of LP (PC-SOD) with different contents of PC-SOD. Data are represented as the mean ± S.D. (n = 3). Significant differences were calculated by one-way ANOVA, followed by Student-Newman-Keul post hoc test. N.S., no significant differences.

2.2. Characteristics of liposomes equipped with PC-SOD or SOD

We prepared liposomes equipped with PC-SOD and SOD, LP (PC-SOD) and LP (SOD), respectively. The diameters and ζ potentials of these preparations are summarized in Table S1. LP (SOD) was larger in size and more heterogeneous than LP (PC-SOD). This result suggests that naked SOD disturbs the liposome preparation, while phosphatidyl choline in PC-SOD is beneficial in stabilizing the liposome preparation.

Table S1. Physicochemical characteristics of liposomes.

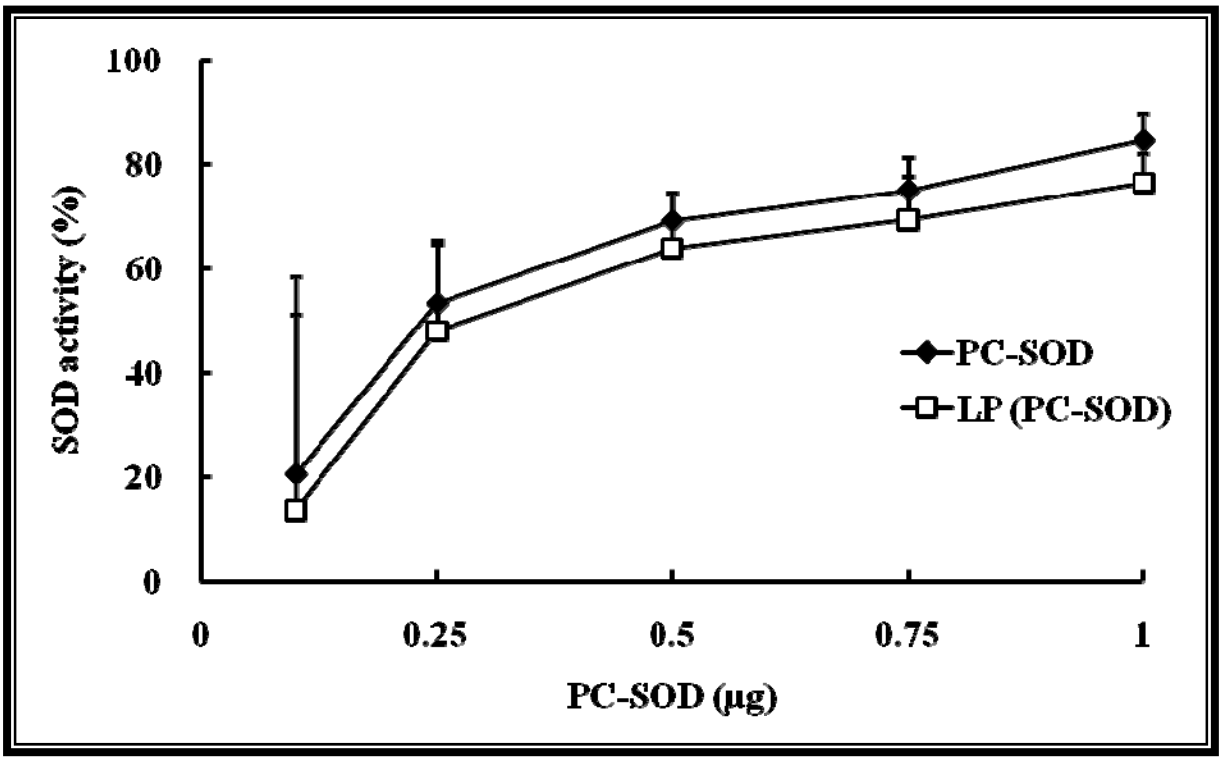
	LP (PC-SOD)	LP (SOD)
Diameter (nm)	141±19	203±134
ζ potential (mV)	-29±13	-32±21
PDI	0.27±0.06	0.41±0.04

Polydispersity index (PDI) is an indicator of the particle-size distribution. The PDI has a small value, when the particle-size distribution is homogeneity. Data are represented as the mean ± S.D. (n = 3).

2.3. Evaluation for SOD activity of LP (PC-SOD)

SOD activity of PC-SOD or LP (PC-SOD) was measured as described in Materials and methods (Fig. S2). The SOD activity of LP (PC-SOD) was similar to that of PC-SOD, suggesting that SOD activity was not lost during the preparation of the liposomes.

Fig. S2. Evaluation of SOD activity

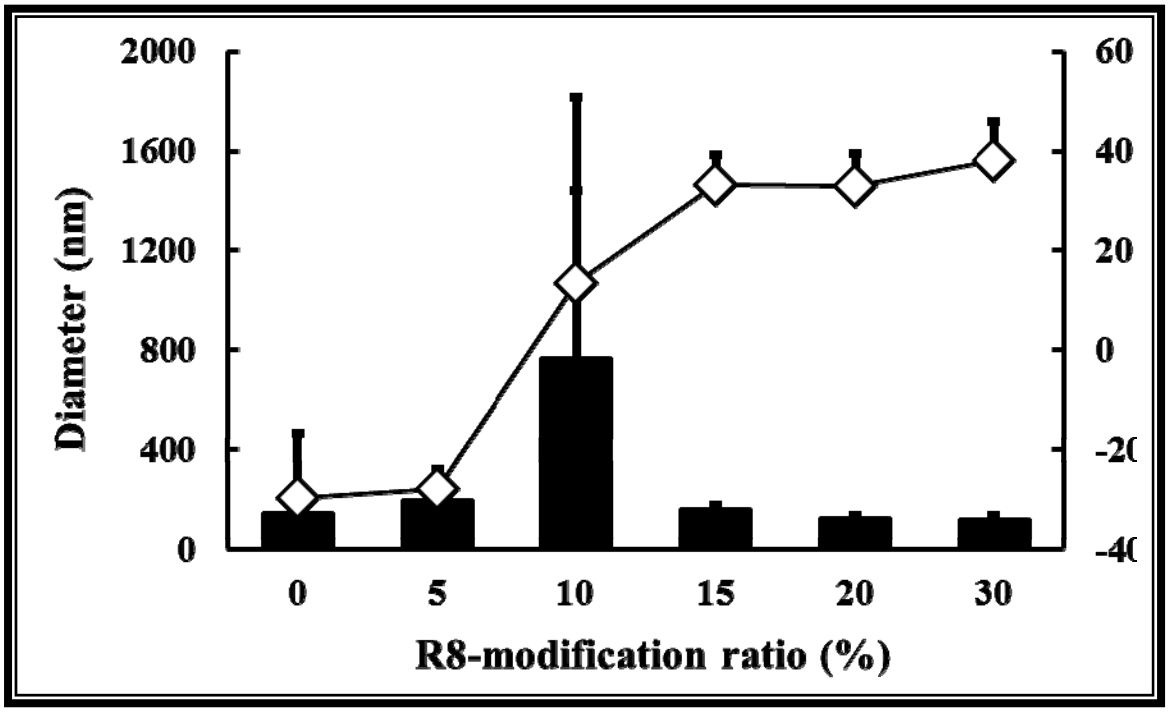


The graph shows the SOD activity of PC-SOD and LP (PC-SOD). Closed diamonds and open squares represent SOD activities of PC-SOD and LP (PC-SOD), respectively. Data are represented as the mean ± S.D. (n = 3).

2.4. Modification of R8 to liposome-surface

We determined the optimal conditions for R8-modification as described below. After the preparation of LP (PC-SOD), STR-R8 was added to the liposome suspension in a series of STR-R8 / LP (PC-SOD) ratios (0 – 30 mol% of STR-R8 per total lipids of liposomes). The diameters and the ζ potentials of the resulting suspension were then determined. Small positively charged liposomes were formed at higher than 15 mol% of R8 modification (Fig. S3). Aggregation occurred at 10 mol% of R8-modification, probably due to electrostatic neutralization.

Fig. S3. Diameters and ζ potentials of R8-modified liposomes

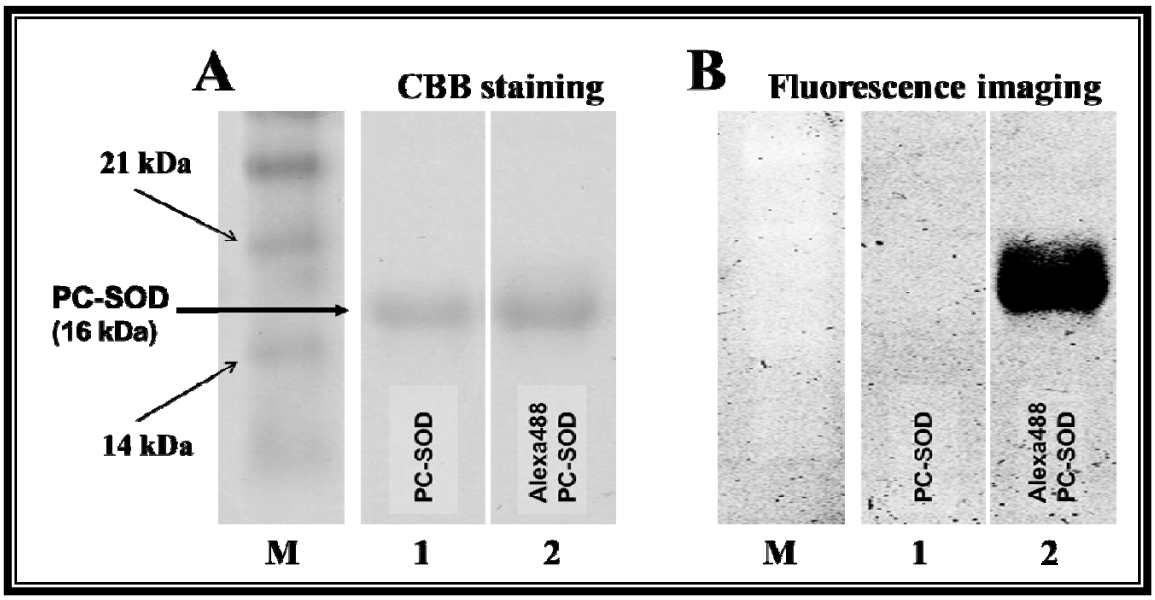


The graph shows the diameters and ζ potentials of R8-modified liposomes. Closed bars and open diamonds represent diameters and ζ potentials, respectively. Data are represented as the mean (n = 3).

2.5. Conjugation of PC-SOD with Alexa Fluor-488

The Alexa Fluor-488 reactive dye contains a tetrafluorophenyl (TFP) ester moiety that is more stable in solution than the commonly used succinimidyl ester. TFP esters react efficiently with primary amines of proteins to form stable dye-protein conjugates [S. Arttamangkul et al, Mol. Pharmacol. 58 (2000) 1570-1580]. We confirmed the conjugation of PC-SOD with Alexa Fluor-488 using SDS-PAGE (Fig. S4). Lanes 1 and 2 indicate PC-SOD and Alexa Fluor-488 labeled PC-SOD (Alexa488 PC-SOD), respectively. In CBB staining (Fig. S4 A), bands detected at about 16 kDa were considered to correspond to the PC-SOD moiety, because SOD consists of two units, each of which has a molecular weight of about 16 kDa. In the fluorescence imaging of Alexa Fluor-488 (Fig. S4 B), a band at about 16 kDa was detected in lane 2. These results show that PC-SOD was conjugated with Alexa Fluor-488.

Fig. S4. SDS-PAGE analysis for verifying the conjugation of PC-SOD with Alexa Fluor-488

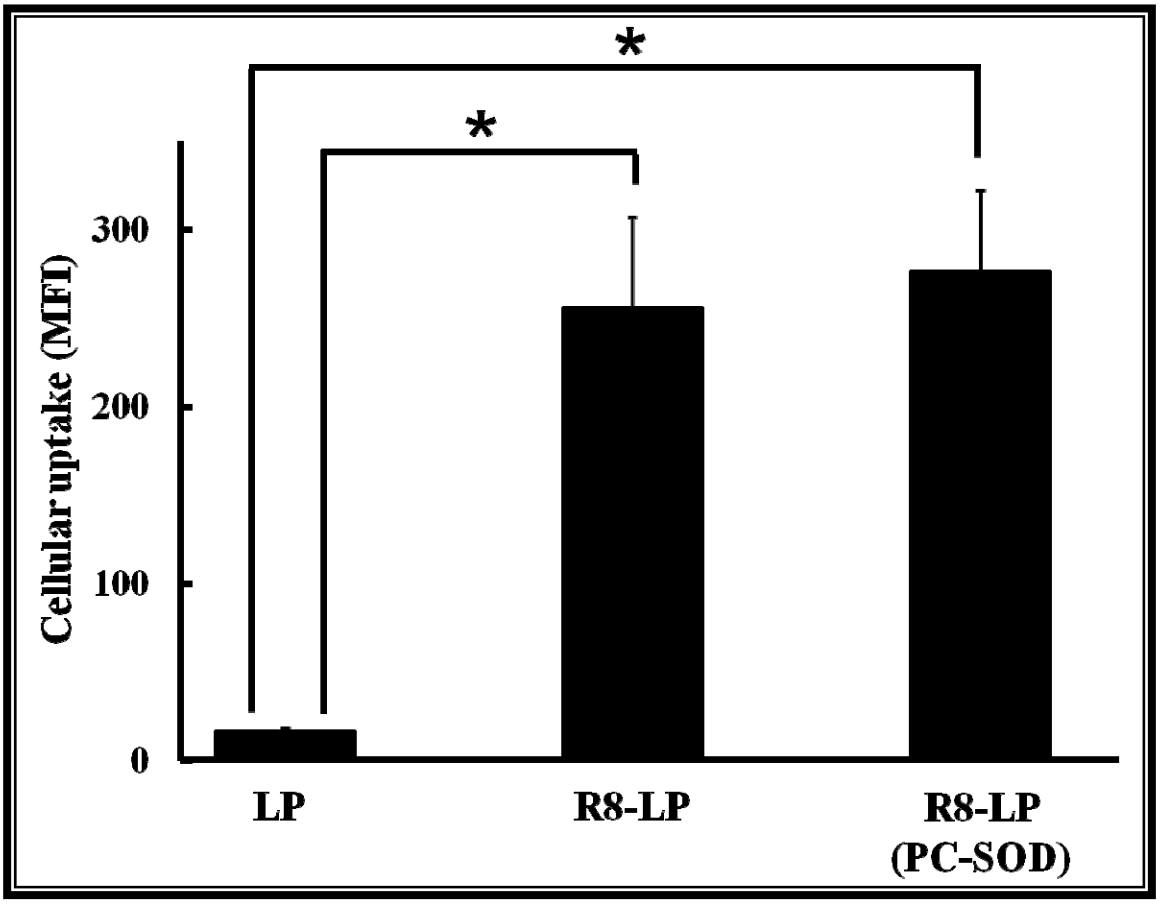


PC-SOD (lane 1) and Alexa488 PC-SOD (lane 2) were analyzed by SDS-PAGE, followed by CBB staining for protein detection (A) and fluorescence imaging for the detection of Alexa Fluor-488 (B). M indicates a protein molecular weight marker.

2.6. Evaluation of the cellular uptake of R8-LP (PC-SOD) labeled with NBD lipids

We evaluated the cellular uptake of R8-LP (PC-SOD) labeled with NBD-lipids, by flow cytometry (Fig. S5). The results showed that R8-LP (PC-SOD) was effectively taken up by cells, similar to R8-LP without PC-SOD, indicating that R8 modification greatly contributes to the higher cellular uptake of PC-SOD.

Fig. S5. Evaluation of the cellular uptake of carriers by NBD lipids



The cellular uptake of carriers (MFI) was calculated based on the fluorescence intensities of NBD-lipids in carries within cells, after the transduction of LP, R8-LP and R8-LP (PC-SOD). MFI, mean fluorescence intensity. Data are represented as the mean ± S.D. (n = 3). Asterisks indicate significant differences, as evidenced by one-way ANOVA, followed by the Student-Newman-Keul post hoc test ($p < 0.01$).