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A liposomal formulation study of 2,7-dichlorodihydrofluorescein for detection of reactive oxygen species

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Abstract

It is known that reactive oxygen species (ROS) are connected with various diseases, and many studies have examined redox conditions in the body. However, there is a problem with stability of ROS and selectivity of detection probe. In this study, we aimed to stabilize the detection probe, and have tried to encapsulate the probe in polyethyleneglycol (PEG)-modified liposomes. Dichlorodihydrofluorescein (DCDHF) is oxidized to dichlorofluorescein (DCF), a highly fluorescent product, by ROS. We tried liposomalization of DCDHF probes by the Bangham method. However, it was found that DCDHF was oxidized during preparation and converted to DCF. In contrast, when we performed to encapsulate DCDHF in the liposome after the preparation of empty liposome, the stabilized DCDHF encapsulated liposome was successfully proposed by the addition of DCDHF solution by freeze-drying and re-hydration. Furthermore, the encapsulated efficacy of PEG-modified liposomes was higher than unmodified liposomes. This DCDHF liposome was examined for reactivity with hydroxyl radical and peroxynitrite as highly ROS. It was confirmed that DCDHF liposome had a protective effect on the hydroxyl radical, though an effect of the liposomalization of DCDHF was not shown on reactivity of the peroxynitrite. Therefore, it is likely that the liposomalization of DCDHF has selectivity for certain radical species. It is hoped that these results can be applied to novel and simple diagnostics for redox detection of conditions in the body.

Keywords: Liposome; Reactive oxygen species (ROS); Peroxynitrite ($ONOO^-$); Hydroxyl radical ($^{\bullet}OH$); Dichlorodihydrofluorescein (DCDHF)

1. Introduction

It is known that lifestyle-related diseases such as cancer, brain disease, cardiac disease and diabetes, are serious problems and it related to reactive oxygen species (ROS), free radicals and peroxides, etc. (Hibbs et al., 1987; Corbett et al., 1991; Groves et al., 1995; Gadelha et al., 1997; Tagami et al., 1998). However, these ROS are essential components that play antiseptic property, physiological substance, and intracellular messenger roles at the same time (Yamamoto et al., 2003). Many studies have examined fluorescence probes that react with ROS, and spin probes that are measured by electron spin resonance (ESR) (Takeshita et al., 2002). However, there are many problems regarding stability and similar potentials of ROS in redox reactions that cannot be easily separated. Therefore, certain diseases cannot be evaluated for ROS generation. Hydroxyl radical and peroxynitrite as ROS have strong oxidative effect, and are produced from superoxide anion radical and NO in endothelial cell, respectively. Furthermore, it was known that hydroxyl radical and peroxide are excessively generated in ischemia–reperfusion and inflammation. However, hydroxyl radical and peroxynitrite as high ROS cannot be detected separately by fluorescence probes. Thus, if each ROS can be selectively detected and the relation between ROS generation and lifestyle-related diseases can be clarified, it may lead to the establishment of novel diagnostics.

Liposomes, microspheres and emulsions are typical drug carriers, and it has been suggested that liposome-encapsulated drugs stabilize in the body (Jones and Nicholas, 1991; Allen and Gregoriadis, 1988; Allen et al., 1991; Zhang et al., 2004).

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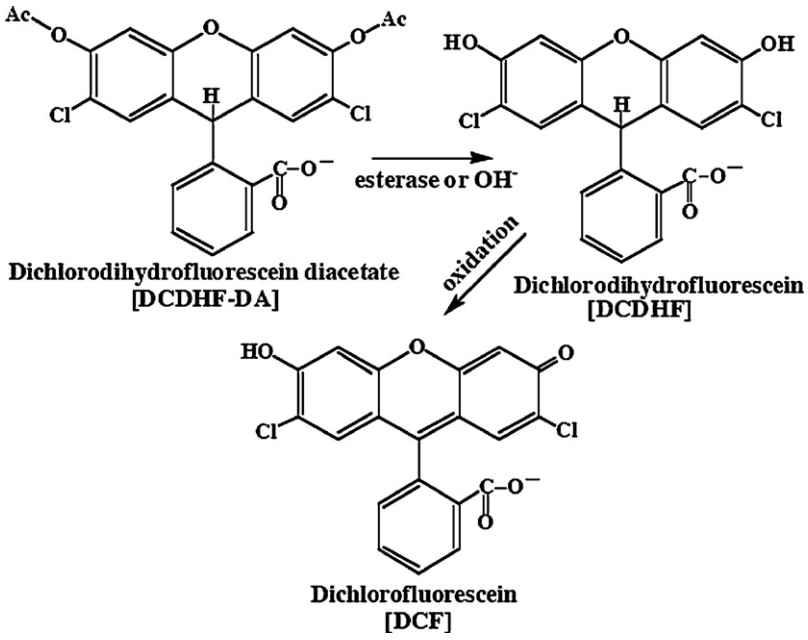

Fig. 1. Reaction of DCDHF. Following enzymatic or base-catalyzed cleavage of the diacetate groups, DCDHF is readily oxidized to the highly fluorescent product DCF.

It is therefore possible to speculate that ROS probes could be stabilized by liposomalization.

2,7-Dichlorodihydrofluorescein diacetate (DCDHF–DA) is used as an indicator of ROS. It is known that DCDHF–DA is cleaved at its diacetate groups (DCDHF) under enzymatic or basic conditions, and that DCDHF is oxidized by ROS to the highly fluorescent product dichlorofluorescein (DCF) (Fig. 1) (Kooy et al., 1997; Crow, 1997; O'Malley et al., 2004). However, DCDHF solution has same reactivity with hydroxyl radical and peroxynitrite as high ROS. Namely, DCDHF solution cannot detect selectively hydroxyl radical or peroxynitrite, and the each role of hydroxyl radical or peroxynitrite in the body is unclear. In this study, we examine the liposomalization of DCDHF and its selective reactivity with ROS.

2. Materials and methods

2.1. Chemicals

Hydrogenated soybean phosphatidylcholine (HSPC: PC purity >99%) and L-*a*-distearoylphosphatidyl DL-glycerol (DSPG:Na base, purity >99%) were purchased from NOF Co. Ltd. (Tokyo, Japan). 1-Monomethoxypolyethyleneglycol-2,3-distearoylglycerol (PEG–DSG), with PEGs of average molecular weight of 2000 (PEG-2000), 900 (PEG-900) were a gift from NOF Co. Ltd. (Tokyo, Japan). 2,7-Dichlorodihydrofluorescein diacetate (DCDHF–DA) and peroxynitrite (ONOO–) were purchased from Cayman Chemical Co. Ltd. (MI, USA). All other reagents were of the highest purity commercially available.

2.2. Preparation of liposomes

Liposomes were either prepared according to Bangham method (Bangham et al., 1965), or a freeze-drying method (Komatsu et al., 2001) and was used to encapsulate DCDHF in liposomes with DCDHF solution.

2.2.1. Preparation of DCDHF liposomes by Bangham method

Liposomes were prepared according to a modified of Bangham method. HSPC/Cholesterol/DSPG/PEG-2000/PEG-900/DCDHF–DA (100:100:60:5:10:1 μmol) were dissolved in a chloroform/methanol mixture (4:1, v/v) and the mixture was perfectly dispersed by sonication. Chloroform and methanol were evaporated to dryness under a stream of nitrogen gas. Chloroform and methanol were completely removed from the lipid film under reduced pressure and the film was hydrated with 10 ml of 9.0% sucrose in 10 mM phosphate buffer (pH 7.0) in a water bath at 75 °C for 20 min. The liposome suspension was sized by extruder method using the extruder and polycarbonate membrane filters.

Prepared liposomes were put in dialysis tubes (Sanko-junyaku, Co. Ltd. Tokyo, Japan) that were preliminarily washed with 9.0% sucrose/10 mM phosphate buffer (pH 7.0) and isotonized, and then dialyzed against this buffer for 16 h. The particle sizes and ζ potential of the liposomes were measured in samples diluted with this buffer using an electrophoretic light scattering apparatus (ELS-8000; Otsuka Electronics, Co. Ltd. Osaka, Japan).

2.2.2. Preparation of DCDHF liposomes by freeze-drying method

HSPC/Cholesterol/DSPG/PEG-2000/PEG-900 (100:100:60:5:10 μmol) were dissolved in a chloroform/methanol mixture (4:1, v/v) and the mixture was perfectly dispersed by sonication. Chloroform and methanol were evaporated to dryness under a stream of nitrogen gas. Chloroform and methanol were completely removed from the

lipid film under reduced pressure and the film was hydrated with 10 ml of 9.0% sucrose in 10 mM phosphate buffer (pH 7.0) in a water bath at 75 °C for 20 min. The liposome suspension was sized by extrusion and dialyzed against 10 mM phosphate buffer (pH 7.0) for 16 h. After dialysis, sucrose (0, 5, 9, 15, 20%, w/v) was added to the liposome suspension, and each liposome suspension (3.0 ml) was added into a vial prior to freezing at −30 °C for 12 h followed by freeze-drying for 12 h. Each liposome powder was re-hydrated with distilled water or DCDHF solution, and liposome suspensions were obtained by freeze-drying method.

Prepared liposomes were put in dialysis tubes (Sanko-junyaku, Co. Ltd. Tokyo, Japan) preliminarily washed with 9.0% sucrose/10 mM phosphate buffer (pH 7.0) and made isotonization, and dialyzed against this buffer for 16 h. The particle sizes and ζ potential of the liposomes were measured in samples diluted with this buffer using an electrophoretic light scattering apparatus (ELS-8000; Otsuka Electronics, Co. Ltd. Osaka, Japan).

2.3. Measurement of DCF concentration

Each sample was dissolved in a chloroform/methanol mixture (4:1, v/v) and made homogeneous, and then the DCF concentration calculated with a spectrophotometer at an absorbance wavelength of 500 nm.

2.4. Effect of DCDHF liposomalizaton on photostability

The photostabilities of DCDHF solution and DCDHF liposome suspension were evaluated. Each sample was added to a test tube 40 cm from a fluorescent light, then the apparatus was sealed by the cardboard covered with aluminum foil. The DCF concentration of each sample was measured at 0, 20, 40, 60 and 120 min.

DCF concentration was determined according to Section 2.3.

2.5. Reactivity of DCDHF liposome with hydroxyl radicals or peroxynitrite

Hydroxyl radicals were generated by the Fenton reaction. DCDHF solution or DCDHF liposomes (DCDHF: 100 μM) and 1 mM $FeCl_2$ (0.16 ml) were mixed and added to H_2O_2 (0–0.8 μM), and the reaction mixture was adjusted to 1.0 ml by 9.0% sucrose in 10 mM phosphate buffer. Then, the mixture solution was sampled at definite times and the DCF concentration was measured. DCDHF solution or DCDHF liposomes (0.2 ml) were added to peroxynitrite (4, 20, 40 μM), and the reaction mixture was adjusted to 1.0 ml by 9.0% sucrose in 10 mM phosphate buffer. The DCF concentration was determined according to Section 2.3.

2.6. Observation of DCDHF liposomes by confocal laser scanning microscopy

Images of DCDHF liposomes after reaction with hydroxyl radicals or peroxynitrite were obtained by confocal laser scanning microscopy (LSM-510, Carl Zeiss Co. Ltd.). DCDHF liposomes were observed after reaction with hydroxyl radicals at definite times, whereas they were observed immediately after the reaction with peroxynitrite.

2.7. Statistical analysis

Statistical analysis was carried out using Student's *t*-test and ANOVA.

3. Results

3.1. Preparation of liposomes

The particle size and ζ potentials of DCDHF liposomes prepared according to Bangham method or freeze-drying method are shown Table 1. The particle size when using Bangham method was indicated to be 138.2 ± 3.1 nm whereas it was increased by freeze-drying method (about 400 nm). The minimum particle size was showed to be 411.7 ± 86.6 nm after the addition of 9% sucrose. The ζ potential when using freeze-drying method was indicated to be half of that when using Bangham method. The ζ potential when using freeze-drying method was not related to the sucrose concentration. Freeze-drying method showed a high-encapsulation efficiency (73.3 ± 8.3%). It was shown that DCDHF liposome prepared by freeze-drying method could not detect DCF production as an oxide of DCDHF. On

Table 1
Particle size and ζ potential of DCDHF liposomes

Method	Particle sizes	ζ potential	Encapsulation efficiency
Bangham			
Empty liposome	135.7 ± 1.5	−22.4 ± 2.4	
DCDHF liposome	138.2 ± 3.1	−20.9 ± 1.8	
Freeze-drying			
Sucrose			
0	1249.1 ± 229.4	−11.0 ± 2.6	73.29 ± 8.30
5	464.5 ± 42.8	−10.2 ± 1.9	
9	411.7 ± 86.6	−12.3 ± 0.9	
15	708.6 ± 97.5	−9.6 ± 1.5	
20	620.4 ± 78.7	−10.0 ± 2.0	

DCDHF concentration: 100 μM. Particle size and ζ potential are expressed as the mean ± S.D. ($n \geqq 3$).

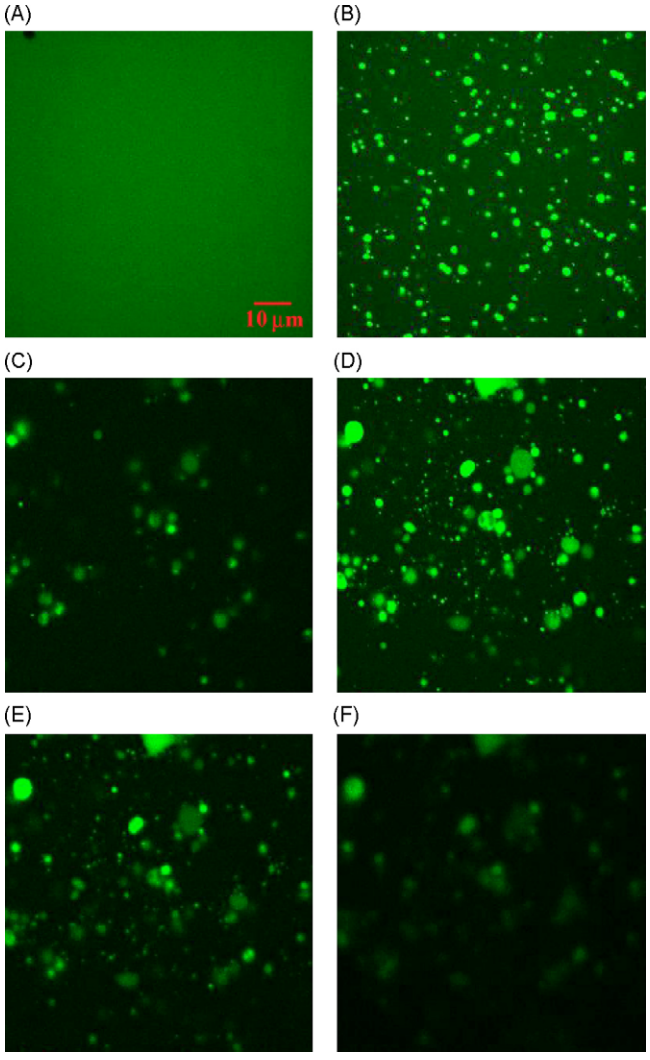

Fig. 2. Confocal laser scanning micrographs of DCDHF liposomes. The images were taken using a confocal laser scanning microscope with an argon ion laser (488 nm). (A) Image of DCDHF solution. (B) Image of DCDHF liposomes. (C–F) A series of representative vertical images of DCDHF liposomes; (C) top of liposomes; (D) the upper-side of liposomes; (E) the under-side of liposomes; (F) bottom of liposomes.

the other hand, the concentration DCF in DCDHF liposome prepared by Bangham method was shown to be 1.13 ± 0.30 μM.

3.2. Observation of DCDHF liposomes by confocal laser scanning microscopy

DCDHF solutions and DCDHF liposomes were obtained by confocal laser scanning microscopy (Fig. 2). DCDHF solutions showed widespread green fluorescence (Fig. 2A). On the other hand, DCDHF liposomes were confirmed to show strong fluorescence in DCDHF liposomes, when compared to that outside the liposomes (Fig. 2B). Furthermore, images of DCDHF liposomes cut into *z*-axis slices were obtained (Fig. 2 (C–F)). It was confirmed that the fluorescent liposomes were spheres (Fig. 2C and F).

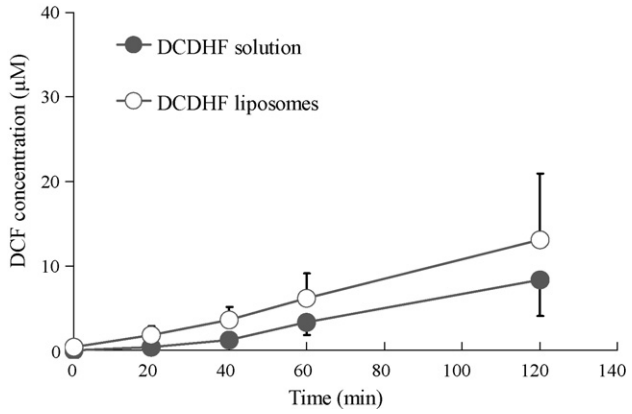

Fig. 3. Stability of DCDHF solution (closed circles) and DCDHF liposome (open circles) against light irradiation. Each point represents the mean ± S.D. of three samples.

3.3. Photostability of DCDHF

After the light irradiation of DCDHF solutions and DCDHF liposomes, the concentrations of produced DCF were measured as shown in Fig. 3. In group, it was shown that the DCF concentration rose after light irradiation. However, DCDHF liposomes showed 1.6 times the indicated value of DCDHF solution after irradiation for 120 min (no significance).

3.4. Reactivity of DCDHF liposomes with hydroxyl radicals or peroxynitrite

DCF concentrations after reaction of hydroxyl radicals with DCDHF liposomes are shown in Fig. 4. The DCF was confirmed to be produced in both DCDHF solutions and DCDHF liposomes in the presence of hydrogen peroxide, whereas DCF was not generated without hydrogen peroxide. It was shown that the production of DCF was inhibited by about 60% by liposomalization at any concentration of hydrogen peroxide.

DCF concentrations after reaction of peroxynitrite with DCDHF liposomes are shown in Fig. 5. It was shown that DCF concentrations in DCDHF liposomes were similar to those

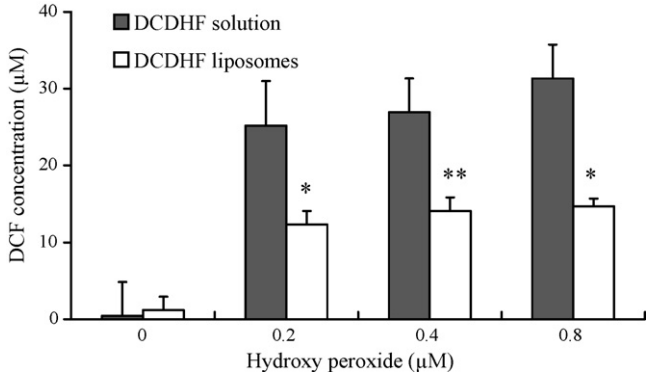

Fig. 4. Reactivity with hydroxyl radicals. Hydroxyl radicals were produced by Fenton reaction for 60 min, and hydroxyl radical production was changed by adding hydroxyl peroxide to DCDHF solution (closed columns) and DCDHF liposomes (open columns). Each column represents the mean ± S.D. ($n > 3$). Significant different from DCDHF solution: $^{*}p < 0.05$ and $^{**}p < 0.01$.

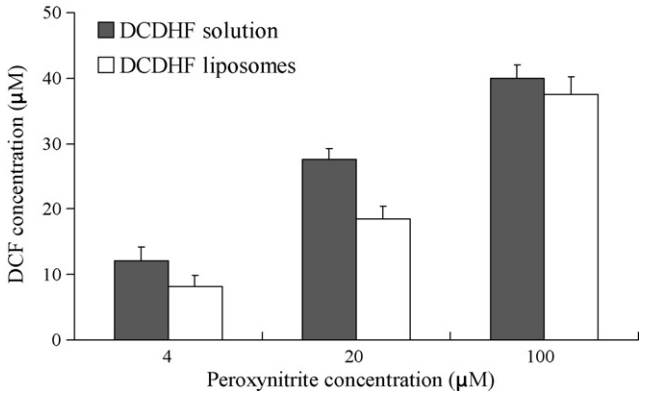

Fig. 5. Reactivity of peroxynitrite with DCDHF solution or DCDHF liposomes. Each column represents the mean ± S.D. of three samples.

of DCDHF solutions after reaction with 100 μM peroxynitrite.

Confocal laser scanning microscopic images of DCDHF liposomes after reaction with hydroxyl radicals or peroxynitrite are shown in Fig. 6. DCDHF liposomes were confirmed to encapsulate DCDHF within liposome (Fig. 6(B)). DCDHF liposomes after with hydroxyl radicals appeared the same after 10, 20, 30 min (Fig. 6 (C1)–(C3)) when compared to those shown in Fig. 6 (B). In addition, DCDHF liposomes reacted with peroxynitrite also showed the same appearance (Fig. 6(D)). On the other hand, fluorescence within DCDHF liposomes decreased after destruction of liposomal membranes by the addition of Triton-X 100 (Fig. 6(C4) and (D2)). These images show that DCDHF liposomes kept their structure intact reacting with radical species.

4. Discussion

It is known that ROS are connected with various diseases. If it is possible to detect ROS and the relation between ROS generation and lifestyle-related diseases can be clarified, it may lead to the establishment of novel diagnostics. In this study, we examine the liposomalization of DCDHF as a florescent probe to detect ROS, selectively.

The encapsulated efficacy was low in unmodified liposomes. On the other hand, PEG-modified liposomes was high (data is not shown). DCDHF liposomes prepared by Bangham method showed identical particle sizes and ζ potentials as empty liposomes. However, it was found that DCDHF was oxidized during preparation, and DCF was produced. On the other hand, DCDHF liposomes prepared by freeze-drying method suppressed DCDHF oxidation during preparation and DCF production did not occur. It is likely that this preparation method was hardly affected by temperature and did not easily oxidize as compared to Bangham method. DCDHF liposomes prepared by freeze-drying method were observed by confocal laser scanning microscopy. DCDHF solutions showed widespread green fluorescence. On the other hand, DCDHF liposomes were confirmed to have strong fluorescence only within the liposome. Furthermore, it was confirmed that the structure of fluorescent liposomes was spherical and that DCDHF existed within the

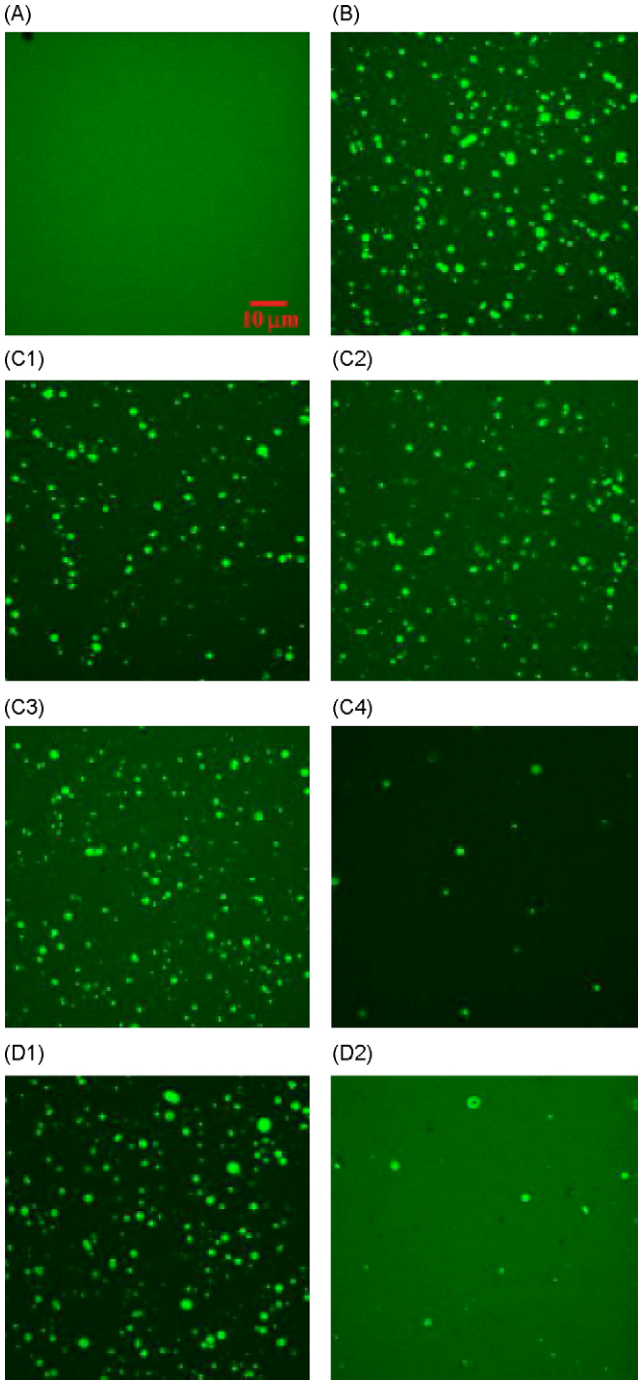

Fig. 6. Images of confocal laser scanning microscope. (A) DCDHF solution, (B) DCDHF liposome, reactivity of DCDHF liposome with hydroxyl radical for (C1) 10 min, (C2) 20 min, and (C3) 30 min after the reaction, (C4) and (D2) with addition of Triton X-100, (D1) reactivity of DCDHF liposome with peroxynitrite, added Triton X-100.

intraliposomal area and liposomal membrane. It was also shown that DCDHF liposomes were unstable against light irradiation when compared to DCDHF solution. It was speculated that the instability of DCDHF in liposomal preparations depended on the turbidity of the liposomal suspensions. In other words, it was considered that the light was scattered by liposome suspension, energy absorption occurred and the photostability of

DCDHF within the intraliposomal area and liposomal membrane was reduced. It is expected that this instability of DCDHF in liposome by light irradiation was avoided by down sizing of liposomes (no turbidity).

Next, we examined the reaction of DCDHF liposomes with hydroxyl radicals. In the reaction with hydroxyl radicals, the liposomalization of DCDHF inhibited DCF production by 60% when compared to DCDHF solution at 60 min. In contrast, DCF production after reaction of DCDHF liposomes with peroxynitrite (100 μM) was shown to be similar to that of DCDHF solution. Namely, DCDHF solution has same reactivity with hydroxyl radical and peroxynitrite, whereas it is likely that liposomal DCDHF has selectivity for certain ROS. Reactions of DCDHF liposomes with hydroxyl radicals or peroxynitrite were obtained by confocal laser scanning microscopy. The destruction of liposomal membrane was not observed during reaction of DCDHF liposomes with hydroxyl radical or peroxynitrite. It was thereby confirmed that the reaction of DCDHF with ROS occurred in intact liposomes, and it is likely that these liposomes have selectivity for peroxynitrite. We consider this difference of reactivity may be related to a difference in permeability or the half-life of the radical species. There is no fluorescence probe in the reaction of peroxynitrite only. Namely, it is not clear on the role of peroxynitrite in some disease. The determination by the combined use of DCDHF solution and its liposomal suspension was expected to detect selectively for certain high ROS and to clarify the connection of certain high ROS with some diseases.

In conclusion, we have easily and efficiently encapsulated DCDHF in liposomes by freeze-drying method. The difference in reactions of DCDHF liposomes with hydroxyl radicals and proxynitrite suggest that liposomalization of DCDHF could protect this probe in the body and have selectivity of radical species. As for application of this liposome formulation, we hope that DCDHF liposomes could be used to measure ROS products in the blood. It could then lead to the establishment of novel diagnostics.

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