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Preparation of superoxide dismutase LIPOzyme in hollow fiber membrane module

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A B S T R A C T

Liposome-loaded membrane module (LLM) was prepared in hollow fiber module (HF-LLM), where the liposome was loaded into the hollow fiber membrane. The filtration property of the LLM was characterized. The oxidized and fragmented superoxide dismutase (SOD) was applied to the prepared LLM to separate the peptide to give a SOD-like activity on the liposome membrane, resulting in the recovery of the specific peptide. It was found that the SOD-like activity could be obtained in the SOD LIPOzyme prepared, resulting in the effective elimination of the superoxide in the HF-LLM.

Keywords: Membrane module; LIPOzyme; Membrane stress biotechnology; Antioxidative enzyme

1. Introduction

The role of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals, has become an important issue to understand the potential functions of the biological system. They may damage cellular macromolecules and may participate in the apoptosis at their higher concentration though they sometimes act as an activator of the biological activity at the lower concentration. Recently, the relation of ROS with the hemodialysis has attracted many attentions because of its serious damage to the patient. The contact of leukocytes with dialysis membranes gives a generation of the significant quantities of ROS, relating to many diseases such as arteriosclerosis [1]. The surface modification with vitamin E has recently been achieved to improve the antioxidant properties of dialysis membrane [2], resulting in the reduction of the oxidative stress in clinical use [3–6]. Hemolipodialysis using dialysis solution with liposome and vitamin C/E has also reported to related with the reduction of oxidative stress [7]. However, it seems that there could be some limitations such as non-catalytic natures and a new type of the dialyzer should be established to improve the conventional hemodialysis operation.

Liposome, a closed phospholipid bilayer membrane, has a nano-order interface (~5 nm), harboring both hydration layer and low-*k* (hydrophobic) layer on its surface. It has been reported that the liposome could recognize the (bio)molecules through the combined interactions such as electrostatic and

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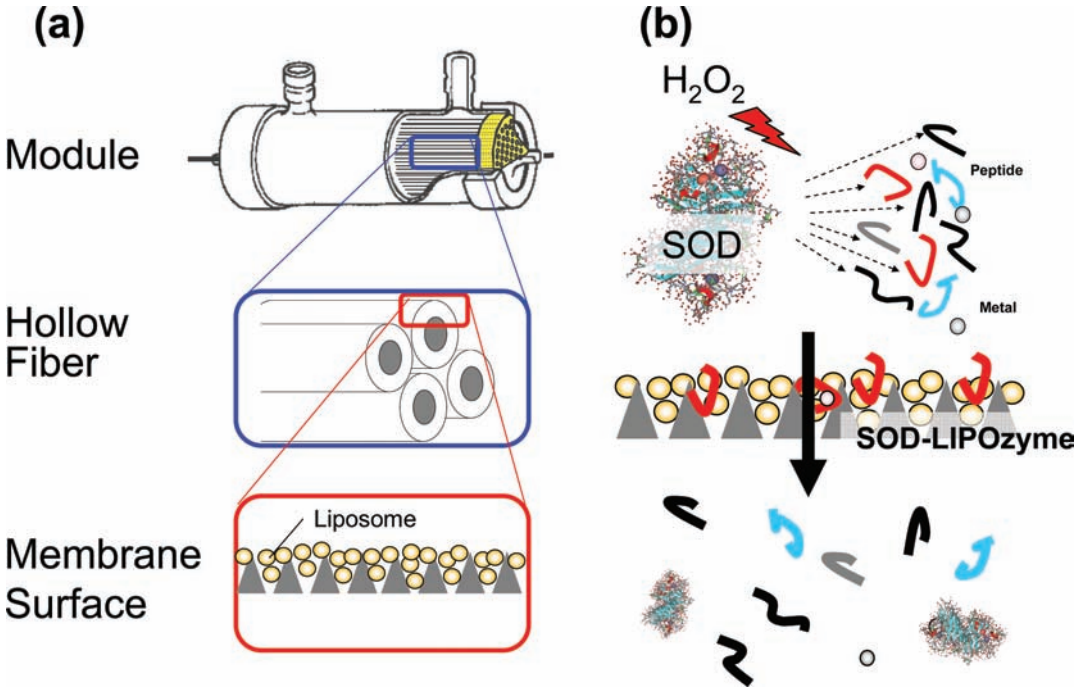

Fig. 1. Conceptual illustration of (a) hollow fiber liposome loaded membrane (HF-LLM) and (b) SOD fragment separation using HF-LLM for preparation of SOD LIPOzyme-module.

hydrophobic interaction, together with the stabilization of hydrogen bonds of molecules [8–10]. Some new aspects of the liposome membrane itself, which could be induced under stress condition, have been recently reported [11]: (a) molecular chaperone-like function to assist the protein refolding [12–14], (b) protein translocation across the membrane [15], (c) function as a mediator/initiator of membrane fusion [16–18], and (d) LIPOzyme functions (Liposome+Enzyme) [19–26]. It is expected that the use of the liposome (or LIPOzyme) would enable us to design and develop the liposome-based hemodialysis system.

The immobilization of such a liposome is an important technique for the above-mentioned purposes. It has recently been reported that the liposome can be utilized as a molecular recognition element in several analytical methods, such as (i) immobilize liposome chromatography (ILC)/immobilized liposome membrane (ILM) [8,27] and (ii) immobilized liposome sensor (ILS) [28,29]. In these immobilized liposome matrix, the liposome was immobilized via (a) physical entrapment method [30], (b) antigen–antibody method [31], (c) hydrophobic ligand method [32], (d) covalent binding method [33] and so on. It has recently been reported that the physical accumulation of the liposome in the ultrafiltration membrane for the purpose of the measurement of the partitioning of solutes such as drugs and also other small biomolecules [34]. Hollow fiber membrane module could be a powerful tool to physical immobilization of the liposome for the sophisticated hemodialysis system utilizing the LIPOzyme function. Considering the morphology of the membrane itself, polysulfone (PS) hollow fiber membrane, produced in Toray, is an ''asymmetric porous membrane'' [35]. It is thought that the liposome could be easily immobilized through the loading into such a porous space and could be utilized for the recognition of the specific peptide from the peptide mixture of oxidized and fragmented SOD solution as schematically shown in Fig. 1.

In this study, the design of the liposome loaded membrane module (LLM) equipped with hollow fibers was investigated. The liposome was first loaded into the porous space of the membrane module and the separation of the oxidized and the fragmented SOD was performed according to the previous report [22,24,25]. The SOD-like activity of the LLM loading SOD LIPOzyme was finally investigated to discuss a possible application for hemodialysis.

2. Experimental

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from NOF Co. Ltd. (Nagoya,

Japan). Other reagents of analytical grade were purchased from Wako.

2.2. Liposome preparation

The phospholipid film was dissolved in chloroform/methanol. After the solvent was evaporated, the resulting thin film was dried for at least two hours under a vacuum. The lipid film was hydrated by pure water to form the multilamellar vesicles. The solution of the multilamellar vesicle was frozen in dry ice-ethanol (−80°C) and incubated in the water bath above the phase-transition temperature. The above freezing-thawing treatment was repeated five times and the obtained liposome solution was then passed through two stacked polycarbonate filters of 50-nm pore size by using an extrusion device to adjust the liposome size. The above liposome solution was also treated under the ultrasonication in order to obtain the smaller size liposome. The size of liposomes was analyzed with a DLS-700 Ar system (Otsuka Electric Co. Ltd., Japan) equipped with an argon laser and the average size of the liposomes, prepared by polycarbonate filters with 50-nm and by ultrasonication, was 60 ± 1 and 30 ± 1 nm, respectively.

2.3. Membrane module operation

Membrane module with hollow fibers (100 fiber membranes, surface area: 62.8 cm^2), used for the housing of the liposome loading membrane, were made by Toray Industries, Inc. The membrane module was connected to the silicone tube with the inner diameter of 2 mm. The total volume of the module and the lines were determined by the preliminary experiment and was found to be 7 ml. The peristaltic pump was equipped in the flow line of silicone tube. A manometer was set at the side of the filtrate. Before the sample loading, the water solution was applied to wash the possible contamination by the impurities. The liposome suspension was applied to the membrane module system from dialysate side to blood side with a flow rate of the 0.5–2.0 ml/min. The manometer was equipped with the blood side to monitor the pressure drop across the membrane. After the liposome was loaded into the hollow fiber membrane module, the oxidized and fragmented SOD was applied together with 10 μM Cu and Zn ions. The protein concentration in the reservoir and in the outlet channel was measured as a function of the operational time. In the experiment of the SOD-like activity measurement, the xanthin and xanthin oxidase mixture together with WST1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) was applied to the liposome-loaded membrane module and the absorbance originated from the formazan converted from WST was monitored.

2.4. Analytical methods

Analysis of Cu,Zn-SOD fragmentation by H_2O_2. Cu,Zn-SOD (2 μM) was incubated with H_2O_2 (2 mM) in phosphate buffer (pH 7.4) at 37°C for 12 h. The enzymatic activity and protein concentration of fragmented SOD were determined after the incubation of SOD with H_2O_2. The SDS-–PAGE technique was used to analyze SOD fragmentation.

For the SOD activity, a highly water-soluble tetrazorium salt, WST-1, produces a water-soluble formazan dye upon reduction with a superoxide anion, where the rate of the reduction with O^{2-} is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD. The absorption spectrum of WST-1 formazan was measured at 450 nm, and the SOD activity as an inhibition activity can be quantified through the decrease in color development [36].

The BCA Protein Assay Kit was used to determine the protein concentrations. The protein was precipitated in a cold acetone solution to separate it from contaminants, so that a more accurate estimation of protein content in the sample could be obtained. The mixture was then centrifuged at 15,000 rpm for 20 min. Pellets solubilized in 50 μl H_2O were added to 1,000 μl of BCA reagent solution and incubated for 30 min at 37°C, after which the absorbance at 562 nm was measured. A standard curve was set up to analyze the protein concentrations [37].

For the reverse-phase HPLC, a Shimadzu (Kyoto, Japan) HPLC system equipped with an FCV-10AL pump, a DGU-20A3 degasser, an SPD-10A UV-VS detector, and an LC-10AD liquid chromatograph was used. Elution profiles were monitored at 220 nm on the UV detector. The mobile phase of acetonitrile/water (v/v 7/3) with a flow rate of 1 mL/min was applied at 25°C. An STR ODS-M column (0.46 cm × 15 cm), in which the particle surface was octadecylated, was used throughout this study.

3. Results and discussion

3.1. Basic characteristics of liposome loading membrane with hollow fiber module

The loading of the liposome into the hollow fiber membrane module, consisting of PS membrane, was first investigated. The experimental setup of the membrane module is shown in Fig. 2. The suspension of the POPC liposome (30 nm) in the reservoir was

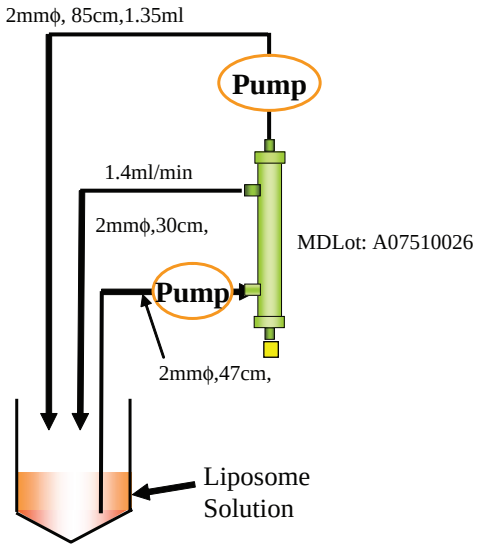

Fig. 2. Experimental setup for liposome loading into membrane module.

introduced into the membrane module with 100 hollow fibers and the filtrated solution was re-circulated into the reservoir solution. The relative flow rate of the filtered flow and by-passed flow was set at 9:1. A typical clearance of the liposome solution is shown in Fig. 3. At the flow rate of 1.0 ml/min, almost all the liposome was found to be loaded into the membrane module. It has been reported that the PS hollow fiber membrane has a gradient channel (assymetric porous membrane). Although the dialysate (outer) side of the membrane has porous structure with wide size, its inner (blood)

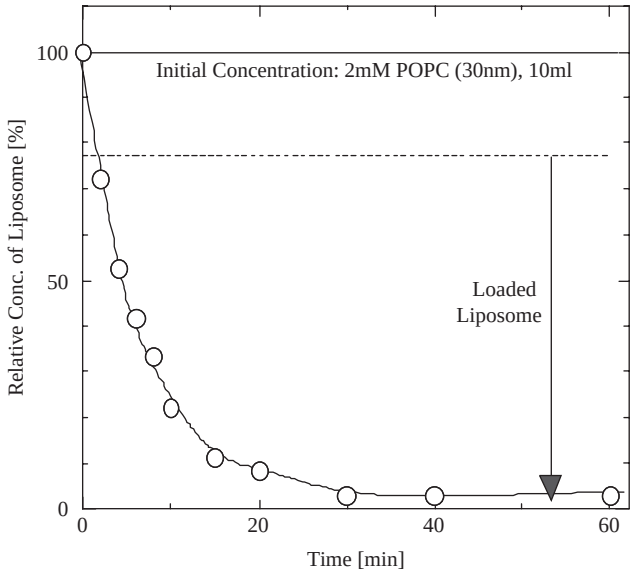

Fig. 3. Typical clearance behavior of liposome solution after treatment in membrane module.

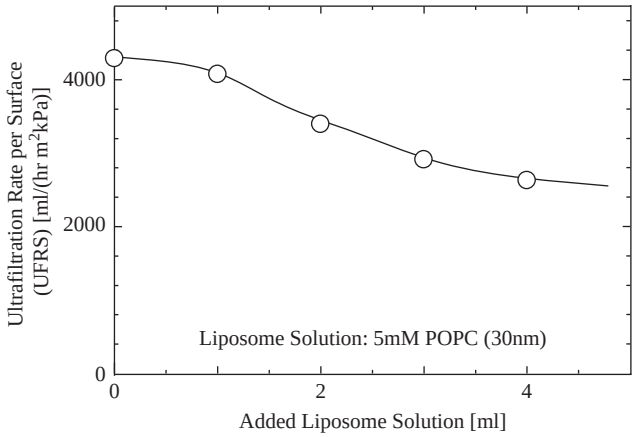

Fig. 4. Effect of loaded amounts of POPC liposome on the flow rate of water in hollow fiber membrane module.

side has nano-order size with 10–20 nm [35]. The above results show that the liposome with 30 nm could be entrapped inside a porous PS membrane.

3.1.1. Filtration property

Filtration property of the membrane module after liposome loading was also investigated by varying the flow rate of the membrane module system. Fig. 4 shows the effect of the amounts of loaded liposome on the ultrafiltration rate per surface area, UFRS. Although the increase of the loaded amounts of liposome reduced the UFRS value to 60% of the original value, a significant decrease was not observed. The UFRS value was analyzed at different flow rate as shown in Fig. 5. A significant reduction of the control module was not observed in this experimental range. On the contrary, the reduction of the UFRS values was observed when the POPC liposome was loaded into

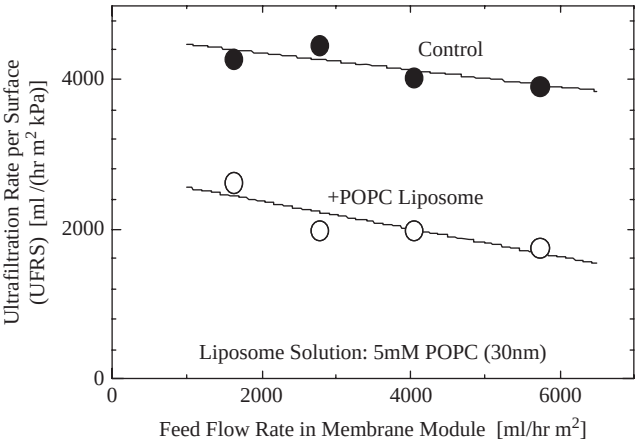

Fig. 5. Filtration property of hollow fiber membrane module and liposome loaded membrane module.

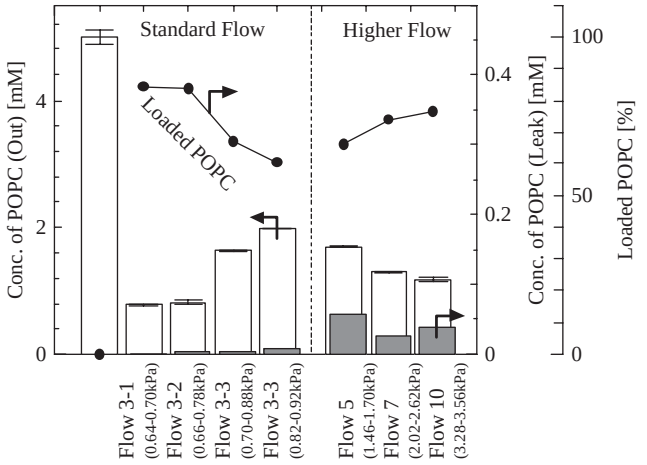

Fig. 6. Leakage of phospholipid from HF-LLM at different pressure flow rates and pressures. liposome: POPC liposome, 30 nm.

the membrane module. Although the UFRS value was reduced to approximately 50% of the control membrane module, the clogging-up of the membrane was not observed.

3.1.2. Leakage of POPC liposome

The concentration of the POPC in the reservoir and in the output of the membrane module system was studied. Fig. 6 shows the concentration level of POPC in the reservoir (open rectangle) under the lower pressure. At the lower pressure less than 0.78 kPa, more than 80% of POPC liposome was loaded into the membrane module. The loaded liposome was reduced with the increase of the flow rate or pressure, where the percentage of the POPC concentration was reduced to 60% of the originally-loaded POPC concentration. At this pressure range more than 0.8 kPa, the leakage of the POPC from the output channel of the membrane module was observed although the amounts of the leaked POPC was not so significant. A response of the filtration characteristics has also been investigated at the higher flow rate under higher pressure range (strong filtration experiment). Although the POPC concentration in the reservoir was increased with the increase of the flow rate, the leaked POPC in the filtrate side was distinctly observed, resulting in 10% leakage of the loaded POPC.

The reduced flow rate could be caused by the packing of the liposome into the small spaces of the PS membrane module under the higher pressure droplet, implying the disruption of the liposome at the above condition. However, once the pressure of the membrane module was released to standard pressure, the above filtration property was reversiblly recovered to the original state. The above rsults imply that the loaded liposome could not be disrupted under the high pressure condition but could be leaked from the narrow channel of the blood side membrane. It was thus found that the liposome with more than 30 nm could be loaded into the hollow fiber module of the PS membrane without significant variation of the filtration properties of the membrane module.

3.2. Separation of SOD fragment using liposome-loaded membrane module

It has previously been reported that the superoxide dismutase (SOD) was oxidized and fragmented in the presence of hydrogen peroxide [20,22,38]. The fragmented SOD has recently shown to be reactivated by adding the liposome together with the supplemented metal ions [22] because of the recognition function of liposome itself through the electrostatic and hydrophobic interactions and the stability of the hydrgen bonds [25]. The SOD fragment recruited on the liposome surface could induce the original SOD-like functions (SOD LIPOzyme function) [22,24,25]. The fragment of the SOD could also be recognized by the liposome loaded into the hollow fiber membrane module.

The SOD fragment solution was applied to the LLM with optimal flow rate. All filtration mode or 90% filtration (with 10% circulation path) were applied to this experimental set-up. The SOD (2 μM) was treated with 2 mM hydrogen peroxide for 12 h as schematically shown in Fig. 1b. The oxidized and fragmented SOD, together with 10 μM Cu and Zn ions, was applied to the LLM module. The concentration of the SOD fragment solutiuon in the reservoir was measured as a function of the circulation time. A typical dependence of the SOD adsorption was shown in Fig. 7. The concentration level of the SOD framgment solution was gradually reduced, resulting that the value of the concentration was approached to the saturated value. The difference between the hypothetical concentration by the sample dilution. The final concentration shows the adsorption of the SOD fragments on the liposome loaded into the hollow fiber membrane module. The liposome was found to adsorb approxymately 10% of all the SOD fragment. The characteristics of the adsorpted SOD fragments was furthermore investigated by using the RP-HPLC, where the specific fragment was suggested to be adsorbed on the liposome surface from the chromatogram (data not shown). The chromatogram of the oxidized and fragmented SOD has previously been reported, where the specific fractions at retarded fraction were found to be interacted with the liposome through the ultrafiltration and

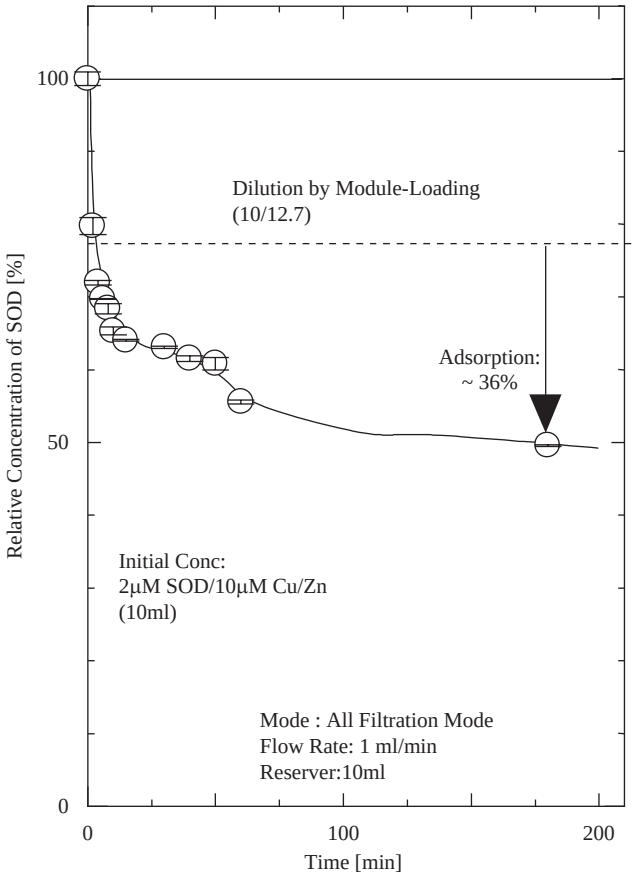

Fig. 7. Adsorption of fragmented SOD using liposome-loaded hollow fiber module.

RP-HPLC analyses [22]. The above results are well corresponding with the previous findings. The obtained results on the adsorption of the SOD fragment on the different types of the zwitterionic liposome were summarized in Fig. 8. The adsorption of the SOD fragment was not so different in the case of the POPC liposome with 30 and 50 nm. It has been reported that the adsorption of the SOD fragment tends to be correlated with the membrane fluidity (local hydrophobicity) of liposome in the batch liposome system [24]. The membrane fluidity of the POPC liposome was increased with the decrease of the liposome size [12]. The above results and previous findings imply that the adsorption of the SOD fragment could also be governed by another factor relating to the physical size of the liposome (under investigation). The highest value was obtained in the case of the POPC/Ch liposome. The effect of the type of the liposome on the adsorption of SOD fragment has previously been reported, where the POPC/Ch liposome was shown to adsorb the SOD fragment because of its characteristics to stabilize the hydrogen bond of the SOD peptide fragment. The

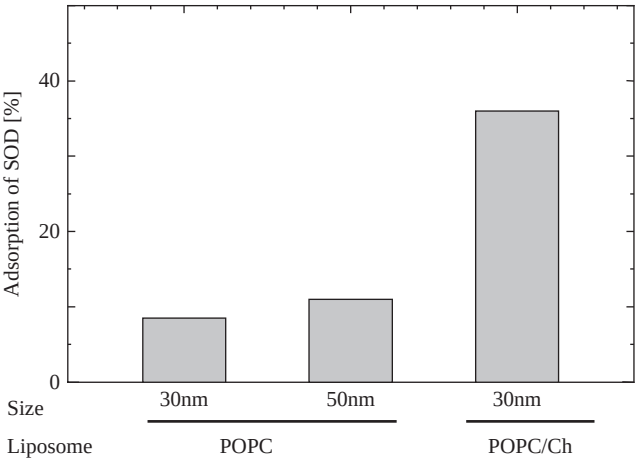

Fig. 8. Comparison of percentage of adsorption of oxidized and fragmented SOD in HF-LLM.

above findings on the adsoprtion of the SOD fragment on LLM are well corresponded with our previous findings in the batch liposome system.

It was thus found that the SOD fragment was adsorbed on the liposome loaded into the membrane module, implying that the LLM with SOD fragments could act as a SOD LIPOzyme.

3.3. Possibility of reduction of oxidative stress by using hollow fiber membrane module loading SOD LIPOzyme

It has been reported that the vitamin-E modified membrane was employed as a possible candidate in designing the hemodialysis membrane module [2,3]. Recent reports imply that the vitamin E modified membrane in clinical uses could reduce the oxidative stress [4–7]. However, there are some potential problems, such as (i) low efficiency of the inhibitory role of the vitamin E modified membrane, (ii) less sustainable use owing to non-catalytic antioxidant, and so on. In the present study, it has been shown that the SOD-LIPOzyme can be directly prepared by using the hollow fiber membrane module through the filtration of the liposome and the adsorption of the potentially-active SOD fragment on the loaded liposome surface. The preliminary experiment on the SOD-like activity in the hollow fiber membrane module loading the SOD-LIPOzyme shows the possible elimination of the oxydatie stress (such as superoxide and hydrogen peroxide) after the treatment of the oxidants-abundant water solution. The present results imply that the liposome loaded into the hollow fiber membrane module could catalytically show the SOD-like function, to convert the superoxide to hydrogen peroxide. The liposome is known as a biocompatible material, suggesting

the less toxocity of the LLM itself. The present results show that the SOD LIPOzyme could have a potential to use it for the elimination of the ROS during the hemodialysis. Various kinds of additional values could be integrated by varying the LIPOzyme functions under the control of the liposome membrane and, also, the environmental stress condition. Although further investigation is needed more in details, the possible significance of the LLM loading SOD LIPOzyme was thus shown through the experiment on the (i) liposome loading, (ii) peptide separation, and (iii) SOD-like activity measurement.

4. Conclusion

LLM was found to be directly prepared in HF-LLM, where the liposome was loaded into the hollow fiber membrane. The oxidized and fragmented SOD was applied to the prepared LLM to separate the peptide to give a SOD-like activity on the liposome membrane, resulting in the recovery of the specific peptide. It was found that the SOD-like activity could be obtained in the SOD LIPOzyme prepared, suggesting the effective elimination of the superoxide in the HF-LLM.

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