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Monitoring of membrane damages by dialysis treatment: Study with membrane chip analysis

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ABSTRACT

The liposome immobilized on indium tin-oxide electrode was prepared as a ''Membrane Chip'' to evaluate the liposome–liposome interaction. Three kinds of neutral liposomes entrapping the fluorescence probe, calcein, were utilized as a sensory liposome. Twenty four kinds of liposomes were used to construct a membrane library based on the calcein release behavior. The loading of liposome including the unsaturated phospholipid into the dialyzer induced the variation of surface state of liposome membrane. We analyzed the above liposome by a comparison of it with the membrane library constructed by Membrane Chip. The liposome after its dialysis treatment was found to show the membrane property of the liposome with domain-like structure prior to the interaction with amyloid β protein. Also the above liposome showed the oxidized liposome-like membrane property. In conclusion, we demonstrated a membrane library-based method to evaluate the surface state of the model biomembranes with unclear surface property.

Keywords: Membrane Chip; Liposome; Oxidation; Amyloid β protein

1. Introduction

The hemodialyzer has been developed for the biomedical device to remove the insufficient components from the blood stream. There are various types of membranous lipid vesicles in the blood stream. The loading of blood stream is recognized as the stimuli to generate the reactive oxygen species (ROS), leading to the damage of biomaterials present at blood stream [1]. Proteins and biomembrane are subjective to ROS. The damaged proteins could interact with the lipid membrane, dependent of the membrane property such as membrane fluidity or hydrophobicity [2–4]. In the blood stream, these interactions can result in the aggregation or disfunction of biomaterials. The method to evaluate the surface state of the cell membrane was, then, demanded.

Recently, an intact immobilization method of liposome has been investigated [3–5]. Furthermore, the immobilization technique of liposome has been extended to the sensor system using the liposome such as the immobilized liposome chromatography (ILC) [2,3,6], and the immobilized liposome sensor [7–9], and the direct observation system using giant vesicles immobilized on electrodes [10–13]. These analytical methods have been indicated to be the promising method to study the peptide–lipid membrane interactions [12,13] or membrane–membrane interactions [7].

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If the membrane–membrane interaction can give the information on the surface state of the lipid membrane, these information might be useful to study the membrane-related disease such as Alzheimer's disease. There is, however, little report to evaluate the surface state of lipid membrane by the previous method including the Membrane Chip [14].

The major objective in this study is to show the strategy with Membrane Chip for an evaluation of the surface state of model biomembranes treated by the dialyzer. The library with 24 kinds of liposomes was prepared to compare the membrane property. The liposome composed of the unsaturated phospholipid was used as a model biomembrane. We investigated the membrane damage of liposome by its dialysis treatment and furthermore its relationship with the interaction between the damaged liposome and a model protein, amyloid β protein as a causative protein in Alzheimer's disease.

2. Materials and methods

2.1. Materials

The phospholipids used were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC (saturated)), 1,2-dilinoreoyl-*sn*-glycero-3-phospho-choline (DLPC (unsaturated)), 1-stearoyl-2-arachidonyil-*sn*-glycero-3-phosphocholine (SAPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE), Egg yolk phosphatidyl-ethanolamine (EPE), which were purchased from Avanti Polar Lipids (Birmingham, Wales, UK). Indium tin-oxide (ITO) electrodes for the immobilization of the liposomes were purchased from BAS Co. Ltd. (Tokyo, Japan). sphingomyelin (SM), diacyl glycerol (DAG), cholesterol (Ch), stearic acid (SA), linoreic acid (LA) and 16-mercaptohexadecanoic acid was obtained from Sigma Aldrich (St. Louis, MO, USA). Amyloid β protein with 40 amino acid residues (Aβ(1-40)) was purchased from the Peptide Institute (Osaka, Japan). *N*-Hydroxysuccinimide (NHS) was purchased from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). The fluorescence probe, calcein, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimid hydrochloride (WSC), 1,6-diphenyl-1,3,5-hexatriene (DPH) and pyrene were purchased from Dojindo Laboratories (Kumamoto, Japan). The nonionic detergents Triton X-100 and cholesterol were purchased from Sigma (New York, NY, USA). All other chemicals of analytical grade were purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Liposome preparation

As for the liposomes to be immobilized on the ITO electrode, three kinds of liposomes were prepared from POPC/EPE, DOPC/EPE, or DPPC/EPE, as previously described [4–8]. In brief, three kinds of lipid solutions, DPPC/EPE (99/1 mol%), DOPC/EPE (99/1 mol%) and POPC/EPE (99/1 mol%) were prepared in chloroform. They were dried in a 100 ml round-bottom flask by rotary evaporation under reduced pressure. The lipid film was dissolved in chloroform and the solvent was evaporated again to obtain a homogeneous lipid thin film. The obtained lipid film was kept under high vacuum for at least 3 h and then hydrated with 100 μM calcein solution (pH 7.5) at room temperature to form multilamellar vesicles. After five time freezing–thawing treatment, the liposome size was adjusted by the extrusion of the suspension through polycarbonate membrane (pore size: 100 nm). Finally, the free calcein was removed by a gel permeation chromatography (Sepharose 4B, 11 cm × 1 cm). As for the sample liposomes to be added on the liposome-immobilized ITO electrodes, liposomes listed in Table 1 (no. 1–24), were prepared in PBS buffer (Na_2HPO_4 21 mM, KH_2PO_4 1.5 mM, NaCl 140 mM, KCl 2.7 mM, pH 7.4) in the similar preparation manner to the above, except for the POPC/oxidized Ch (POPC/oxCh, no. 5) and DMPC/oxidized SAPC (DMPC/oxSAPC, no. 24).

For the preparation of POPC/oxCh and DMPC/oxSAPC, POPC/Ch liposomes (66/33 mol%) and DMPC/SAPC (80/20 mol%) were oxidized by mixing with $CuSO_4/H_2O_2$ (1 mM) solution overnight. This liposome suspension was mixed with chloroform to extract both phospholipid and oxidized cholesterol by Folch's method [15]. A lipid thin film of POPC/oxidized Ch was again formed and hydrated with the Tris-HCl buffer. The size of the liposomes was adjusted to 100 nm in diameter with the extrusion method after the above-mentioned freezing–thawing treatment.

2.3. Preparation of Membrane Chip

The liposome was immobilized on the solid surface according to the previous reports [4–8]. In short, a self-assembled monolayer (SAM) using 16-mercaptohexadecanoic acid was formed on ITO (1 cm × 1 cm). To activate the SAM membrane, the above electrode was immersed for 3–4 h in a solution consisting

Table 1
Summary of lipid composition and the phase state of liposome

No.	Liposome	Mol%	Phase
1	DPPC	100	s_o
2	DMPC	100	l_d+l_o
3	POPC	100	l_d
4	POPC/SM	50/50	l_d+s_o
5	POPC/oxCh[a]	66/33	n.d.[c]
6	POPC/SM/Ch	33/33/34	l_d+l_o
7	POPC/Ch	66/33	l_d+l_o
8	DMPC/SA	60/40	l_d+l_o [d]
9	DMPC/SA	80/20	l_d+l_o [d]
10	SM	100	s_o
11	SM/Ch	50/50	l_o
12	DOPC	100	l_d
13	DOPC/DPPC	50/50	l_d+s_o
14	DOPC/DPPC/Ch	33/33/33	l_d+l_o
15	DOPC/DAG	90/10	n.d.[e]
16	DMPC/LA	70/30	l_d [d]
17	DLPC (saturated)	100	l_d
18	DLPC (unsaturated)	100	l_d
19	DLPC/Ch	66/33	l_d+l_o
20	DLPC/Ch	50/50	l_d+l_o
21	POPC/SM/Ch	41.8/24.2/33	l_d+l_o
22	POPC/SM/Ch/other[b]	23.3/13.5/33.4/29.8	n.d.[c]
23	DMPC/SAPC	80/20	n.d.[c]
24	DMPC/oxSAPC	80/20	n.d.[c]
25	Dialized DMPC/SAPC	80/20	n.d.[c]

[a] Oxidized cholesterol (oxCh) was prepared by Floch's method (see Materials and Methods).
[b] ''others'' mean POPA (1.5 mol%), POPG (9.5 mol%), and DPPE (18.8 mol%).
[c] n.d.: not determined,
[d] literature [17].
[e] Soybean PC/DAG showed 2 or 3 phases including l_d (L_α) phase. l_d: liquid-disordered phase, l_o: liquid-ordered phase, s_o: solid-ordered phase.

of dioxane/distilled water (90/10, vol%) solution containing 17 mM NHS, 17 mM WSC. The liposome entrapping calcein then bound to the SAM layer by the amino conjugate method. After 1 h, the ITO electrodes immobilizing three kinds of liposomes were rinsed with the PBS buffer.

2.4. Membrane Chip analysis

Twenty-five kinds of liposome samples of 4 ml (10 μM lipid) were applied to the surface of the ITO electrode containing the above three kinds of immobilized DOPC-, DPPC-, and POPC-liposomes (see abbreviations). As an index of membrane–membrane interaction, the fluorescence intensity of calcein on the electrode surface (I) was evaluated by using an Olympus IX-51-11 FL/PH-S fluorescence microscope and an ORCA-ER C4742-95 CCD camera (Hamamatsu Photonics, Japan) connected to an Image analyzer. The observed change of calcein fluorescence ($\triangle I = I - I_0$) after 15 min later was used as a measure for the extent of liposome–liposome interaction. Since the initial fluorescence (I_0) depends on the number density of the immobilized liposome on the electrode surface [7], the signal (calcein relase, RF) was defined as below:

$$\mathrm{RF} = \Delta \mathrm{I}/I_0 = (I - I_0)/I_0. \quad (1)$$

2.5. Dialysis treatment of liposome solution

Dialyzer with hollow fibers was made by Toray Industries, Inc [16]. The total volume of 10 ml of liposome (10 μM-lipid) was loaded through the dialyzer by 1 ml/min of flow rate at 25°C. After 3 h later, 4 ml of liposome solution was applied to the Membrane Chip.

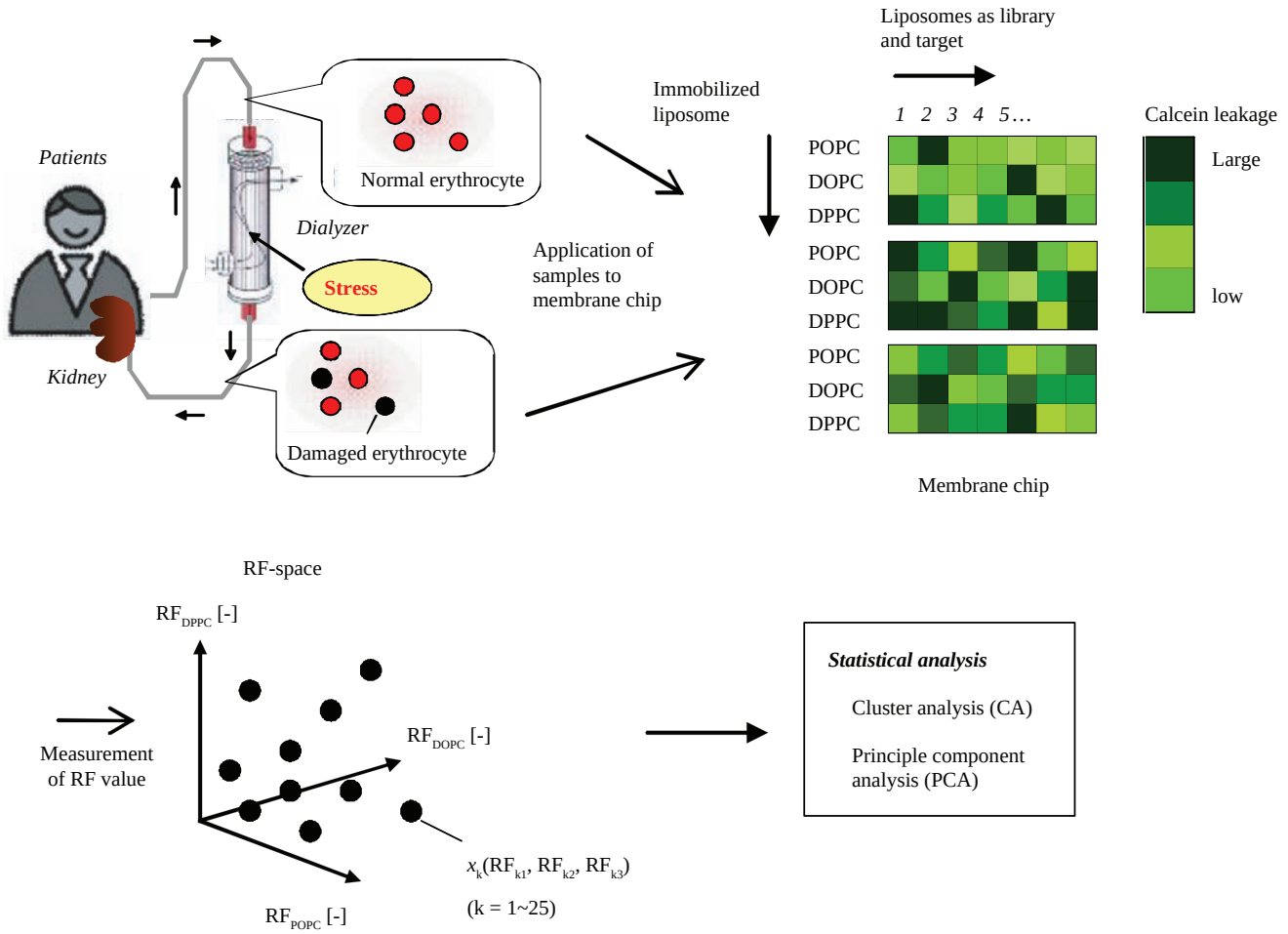

Fig. 1. Overview of monitoring for model biomembrane with membrane chip analysis. The total volume of liposome (10 μM-lipid) was 10 ml. The liposome solution was circulated in the dialyzer by 1 ml/min of flow rate. The number of liposome at the step of Membrane Chip is listed in Table 1. The RF_{POPC} means the extent of interaction induced by the addition of liposome to the immobilized-POPC liposome. The statistical analysis of the data in RF-space gives Fig. 2.

3. Results and discussion

3.1. Membrane chip analysis of the dialyzed liposome

Firstly, the membrane library was constructed with a Membrane Chip for the evaluation of the surface state of the target liposome. The addition of liposome to the immobilized-liposome electrode resulted in the calcein release, indicating the liposome–liposome interaction. The RF value for 24 kinds of liposomes listed in Table 1 were measured and displayed in the 3-dimensional space (Fig. 1). The scattering of data in the RF-space did not give the direct interpretation of interaction model even with a multiple regression analysis. The data obtained here was analyzed with a principle component analysis (PCA). We classified the liposomes in terms of the calcein release induced by the interaction between liposomes. The principle component scores, PC1 and PC2, are the unified variables synthesized from the RF values for immobilized-POPC, DOPC, and DPPC liposomes. Therefore, both principle component scores included the meaning of calcein release.

In the theory of PCA, the principle component score could be described as $PCi = \omega_{ij}RF_j$ (i = 1: POPC; 2: DOPC; 3: DPPC), i.e.,

$$PC1 = \omega_{11}RF_{POPC} + \omega_{12}RF_{DOPC} + \omega_{13}RF_{DPPC} \quad (2)$$

$$PC2 = \omega_{21}RF_{POPC} + \omega_{22}RF_{DOPC} + \omega_{23}RF_{DPPC} \quad (3)$$

where ω_{ij} is the weighted proportion with a condition of $\omega^2_{i1} + \omega^2_{i2} + \omega^2_{i3} = 1$.

The proportion ω_{ij} can be determined by the eigen value problem of the dispersion matrix or the correlation matrix of the data set $\{x_k \mid x_k = (RF_{k1}, RF_{k2}, RF_{k3})$,

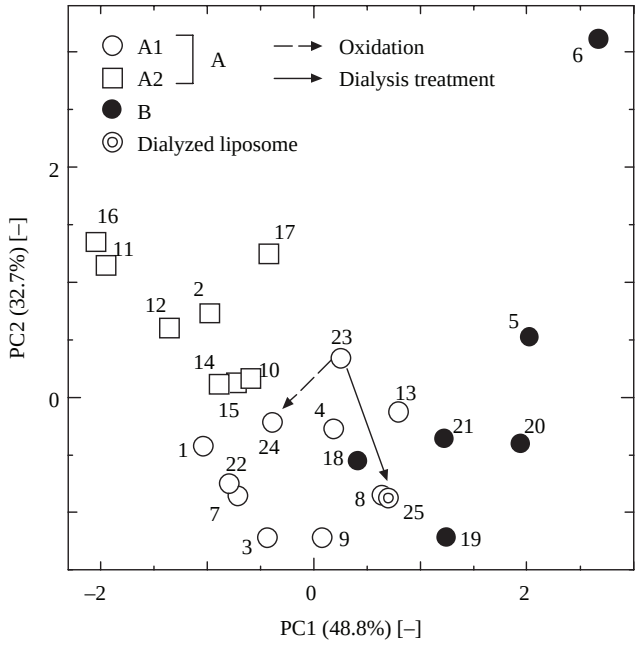

Fig. 2. Monitoring of membrane treated by a dialyzer with a Membrane Chip System. The data shown in Fig. 1 was analyzed with a CA based on the Ward's method. According to the Milligan's test with a Monte Carlo simulation [18], the classification of a data set with 25 samples into two or three groups is reasonable. The data obtained here was classified into two main groups A and B, followed by the subgroups A_1 and A_2 in A. The arrow from no. 23 to 24 means the oxidation of DMPC/SAPC liposome by $CuCl_2/H_2O_2$ (1 mM). The arrow from no. 23 to 25 means the dialysis treatment of DMPC/SAPC liposome for 3 h.

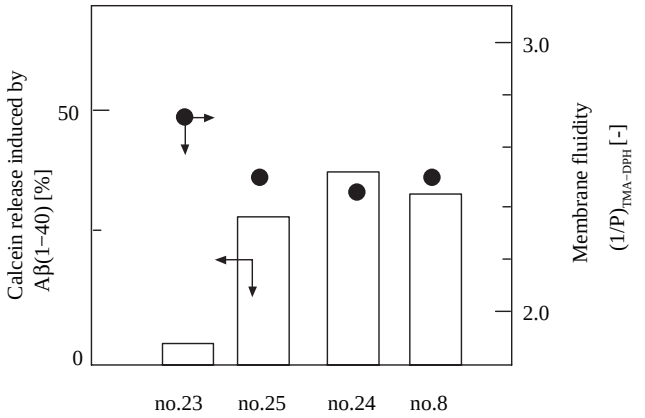

Fig. 3. Comparison of membrane properties of target model biomembrane with those of liposomes in membrane library. Calcein release experiment was performed by mixing the liposome entrapping calcein (100 mM) and Aβ(1-40) (10 μM). After 20 min later, the fluorescence intensity of calcein was measured (excitation: 490 nm; emission: 520 nm). The intensity measured after the disruption of liposome by detergent (Triton X-100) was set as 100% of calcein release. The membrane fluidity was measured with a hydrophobic fluorescence probe, DPH. The mixture of DPH (1 μM) with the liposome (250 μM) was prepared to measure the depolarization of DPH fluorescence (excitation: 360 nm; emission: 430 nm). The reciprocal value of polarization P of DPH was defined as the membrane fluidity.

$k = 1$–24}. The PC1 and PC2 values are thought to be the generalized parameters for RF values used here because PCi ($i = 1,2$) are connected with the RF value in Eqs.(2) and (3). From the PCA, $\omega_{11} > 0$, $\omega_{12} > 0$, and $\omega_{13} < 0$ were obtained, indicating that PC1 might imply the calcein release dependant of the phase state of membrane surface. POPC and DOPC showed l_d phase and DPPC showed s_o phase (Table 1). The permeation behavior under l_d or l_o phase condition depended on the membrane fluidity. The anomaly strong permeation was, however, observed in the liposome with the mixture l_d+l_o phase. Thus, it is considered that PC1 is the index for the surface state of liposome membrane enough to induce the characteristic interaction with the immobilized liposomes [14].

The accumulated proportion of PC1 and PC2 was 81.5%, implying both parameters being enough to explain the trend of the interaction between liposomes. Additionally, a cluster analysis (CA) based on Ward's method was performed to classify the interaction between liposomes. According to the Milligan's test with a Monte Carlo simulation [18], the classification of a data set $\{x_k\}$ with 25 samples into two or three groups is reasonable. The data obtained here was classified into two main groups A and B, followed by the subgroups A_1 and A_2 in A. Using both PCA and CA, we constructed the membrane library to evaluate the surface state of the target liposome (Fig. 2).

The ratio of unsaturated phospholipids in erythrocyte reaches up to 53% [19]. A binary DMPC/SAPC system (no. 23) was selected as a target liposome since a saturated DMPC and unsaturated phospholipid SAPC were included. The plot of liposome (no. 23) changed to no. 25 by the dialysis treatment for 3 h. The data (no. 25) was found to be close to the plot of the DMPC liposome incorporating stearic acid (SA, 40 mol%) (DMPC/SA, no. 8). According to the previous report [19], DMPC/SA liposomes prefers to interact with amyloid fibrils of Aβ(1-40) as a causative protein in Alzheimer's disease. It is, then, expected that liposome (no. 25) can interact with the amyloidgenic protein such as Aβ(1-40). In other words, the dialysis treatment is considered to lead to the change in membrane property of liposome in terms of the interaction with Aβ(1-40).

On the other hand, the elution of erythrocyte through the dialysis could result in the oxidative

damage because of the generation of ROS in dialyzer [1]. DMPC/SAPC liposome (no. 23) might be oxidized by the treatment with dialyzer. DMPC/oxSAPC liposome as a control was also applied to the Membrane Chip for its assignment in PC plane, indicating that DMPC/oxSAPC liposome was assigned as no. 24 in Fig. 2. Oxidation of SAPC in DMPC/SAPC liposome resulted in the movement of plot in PC-plane (from no. 23 to 24). Both the liposome of no. 24 and no. 25 were assigned into group A_1, it is considered that both liposome might show the similar surface state although both were separate in PC-plane.

In the following, a comparison of the propensity of both liposomes was performed to clarify the membrane property of the liposome (no. 25).

3.2. Evaluation of membrane property of dialyzed liposome

In order to confirm the interaction between the liposome after a dialysis treatment and amyloidgenic protein, we measured the calcein release from the liposome in the presence of Aβ(1-40). The calcein release induced by the protein is an index for the interaction between the liposome and protein [6]. Fig. 3 shows the calcein release from various liposomes induced by Aβ(1-40). Calcein release from liposome (no. 25) was clearly observed whereas no significant release was observed in no. 23. DMPC/SA liposome (no. 8) also showed the significant calcein release (~30%). Fatty acids such as SA can interact with Aβ(1-40) via the electrostatic interaction between the positively charged lysine at 16th or 28th position in peptide backbone of Aβ(1-40) [20]. The SA-lysine interaction might result in the membrane perturbation. On the other hand, in the case of the DMPC/oxSAPC liposome (no. 24) as a control of oxidatively damaged liposome, a significant interaction between the liposome and Aβ(1-40) was observed, which agrees with the report by Koppaka et al. [21].

To investigate the influence of the dialysis treatment against the membrane property of liposome, we evaluated the membrane fluidity of liposomes (Fig. 3). The decrease in membrane fluidity was observed by the dialyzer treatment (no. 23 to 25). Also, the oxidation of liposome membrane resulted in the decrease in membrane fluidity (no. 23 to 24). This decrease in membrane fluidity might result from the generation of 4-hydroxy-2-nonenal (HNE) with single fatty acid, as an oxidative metabolite of SAPC, within a liposome membrane. The incorporation of a single fatty acid (SA) into DMPC liposome membrane resulted in a decrease in membrane fluidity (no. 8), which is consistent with the above finding.

The heterogeneous lipid composition sometimes forms a unique membrane structure called as ''microdomain'' which is one of index to characterize the liposome membrane [22]. It is likely that the liposome with microdomains favors to interact with Aβ(1-40) [23]. We measured the index for microdomain formation within a liposome membrane using the fluorescence probe, pyren. The eximer/monomer ratio (E/M) of pyren embedded in liposome membrane is corresponding with the domain formation. DMPC/SA(40 mol%) liposome (no. 8) showed the high E/M ratio, indicating the presence of microdomain on its membrane (data not shown), consistent with previous report [17]. It is considered that the liposome (no. 25) could show the domain structure on its membrane. Previously, it is likely that the liposome with domain structure could strongly interact with amyloidgenic proteins such as Aβ(1-40) [23]. This prediction is consistent with the result obtained from the calcein release experiment.

From the results, we concluded that the dialysis treatment of DMPC/SAPC liposome (no. 23) could induce the domain-like structure to favor the interaction with Aβ(1-40) and induced the similar characteristic to an oxidatively damaged membrane. We successfully demonstrated the efficacy of a membrane library for the assignment of the liposome with unclear property. It is expected that the assignment of the target membrane into the specific liposome within a membrane library could give the valuable information on the membrane surface of target. The interpretation of the membrane property for the target liposome based on a membrane library can be called as ''*annotation analysis*'' according to the conventional genome analysis. Since the erythrocyte includes the unsaturated phospholipids up to 53% [19], its oxidation via the elution in a dialysis column might affect its stability in blood stream and the erythrocyte–protein interaction. Therefore, it is important to monitor the surface state of membrane with a Membrane Chip from the viewpoints of the medical application. The strategy presented in Fig. 1 would be a promising method to monitor the damage of the membrane component such as the erythrocyte in blood stream and their interaction with the other amyloidgenic proteins such as β_2-microglobulin which is a causative protein of dialysis amyloidosis.

4. Conclusions

The analysis of membrane–membrane interaction with a Membrane Chip could give the membrane library enough to interpret the membrane property of target liposome with unclear property. The efficacy of the annotation analysis for a clarification of membrane

property would depend on the quality of membrane library. The model erythrocyte liposome was treated by a dialyzer. The annotation analysis with a Membrane Chip could give the insight that the liposome dialyzed showed the similar property to those of the oxidized liposome or liposome with microdomain enough to interact with amyloidgenic proteins.

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