the similarity in the molecular weight of CytA (27 kDa) and CAB (28.8 kDa). It is therefore suggested that this disagreement arises because CytA exhibits strong cytolytic activity like a detergent[19] although CAB exhibits no such activity and acts via another mode.

3.2 *Variation of CAB-lipid interactions at various pHs*

The variation in calcein release at different pHs (Fig. 1(a)) may be attributed to the conformational changes of proteins. Immobilized liposome chromatography (ILC) is an effective tool for evaluating the extent of the interaction between proteins and lipid membranes by retarding the elution behavior of the target protein.[7–9,13] ILC was used to analyze the interaction between proteins and lipid membranes. Figure 2(a) shows the elution profile of CAB on the ILC column at various pHs. At pH4, CAB was markedly retarded compared with the results obtained at other pHs. To normalize the retardation of CAB due to the interaction between proteins and lipid membranes, the specific capacity factor k_s was determined using eq. (3). Figure 2(b) shows the plots of k_s value as a function of pH. The k_s attained its maximum value at pH 4. It has been reported that the k_s values correspond well with the extent of the hydrophobic interaction between proteins and lipid membranes in the case of electrostatically neutral liposomes.[7–9] The partly-denatured proteins strongly interact with liposome membranes because of the increase in hydrophobic interaction.[7,9,13,20] The conformation of CAB around pH4 is, therefore, considered to be an intermediate state.

The local hydrophobicity of CAB (*LH*) was also evaluated at various pHs using the aqueous two-phase partitioning method. Similar to the case of k_s, *LH* showed a maximum value at around pH4, as shown in Fig. 2(b). The *LH* was evaluated on the basis of the binding of the hydrophobic probe Triton X-405 with the hydrophobic binding sites of

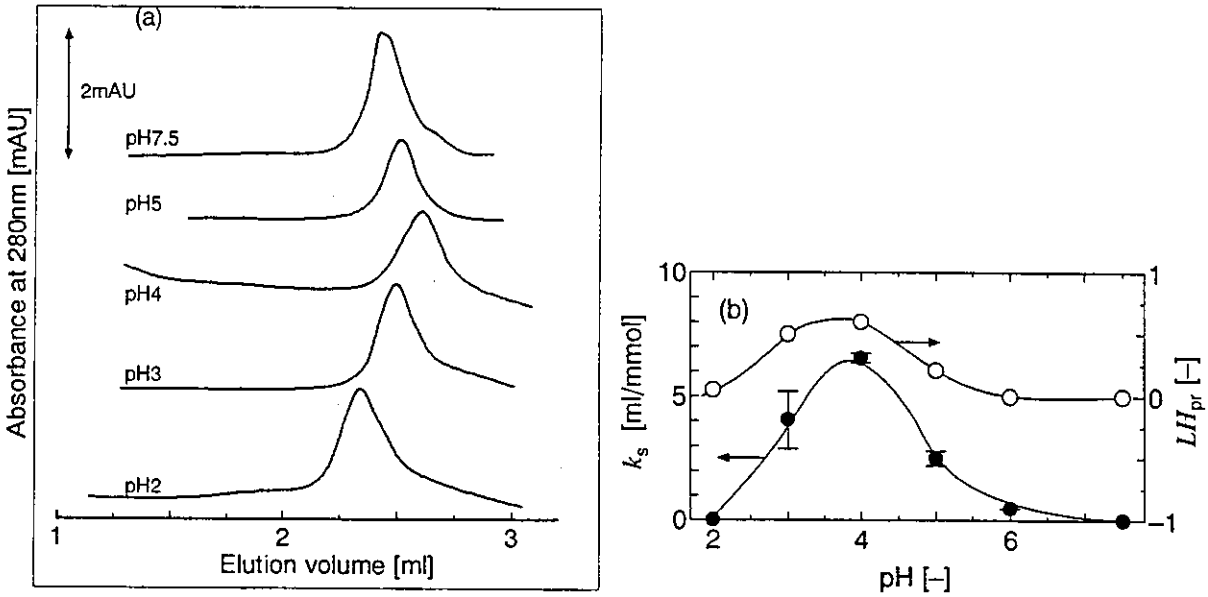

Fig. 2. (a) Elution profile of CAB in the ILC column at various pHs. (b) The pH-dependency of specific capacity factor (k_s) of CAB and its local hydrophobicity (LH_{pr}). k_s and LH_{pr} are defined in the experimental section. All experiments were performed at 25°C.

proteins. A protein in a partly denatured state has a large *LH*.[7–9,16] This is supported by the results of a binding experiment performed with a hydrophobic fluorescent probe, 1-anilinonaphthalene-8-sulfonic acid (ANS), which is an effective probe for proteins in partly denatured states such as the molten-globule (MG) state,[21–23] and the results of circular dichrorism experiments.[22] It is also known that a protein in an MG-like conformation exhibits large fluctuations.[24] As described in our series of reports, the protein conformation with high *LH* may correspond to the partly denatured state exhibiting large fluctuations.

It is concluded that the large membrane perturbation effect of CAB significantly induced the enhancement of calcein release from liposomes through the hydrophobic interaction between CAB and lipid membranes.

3.3 *Effect of lipid composition on calcein release with CAB of intermediate state*

Because calcein release is known to be affected by membrane permeability, the kinetics in our experimental systems may depend on lipid composition. To study the influence of lipid composition on calcein release, the effect of cholesterol on calcein release from liposomes was investigated in the presence of CAB at pH4 (Fig. 3). As the molar ratio of cholesterol was increased, both k_s and RF_{max} decreased. It has been reported that the addition of cholesterol leads to the stabilization of lipid membrane structures. The interaction between CAB and liposomes was considered to decrease due to stabilization of the liposomes by cholesterol.

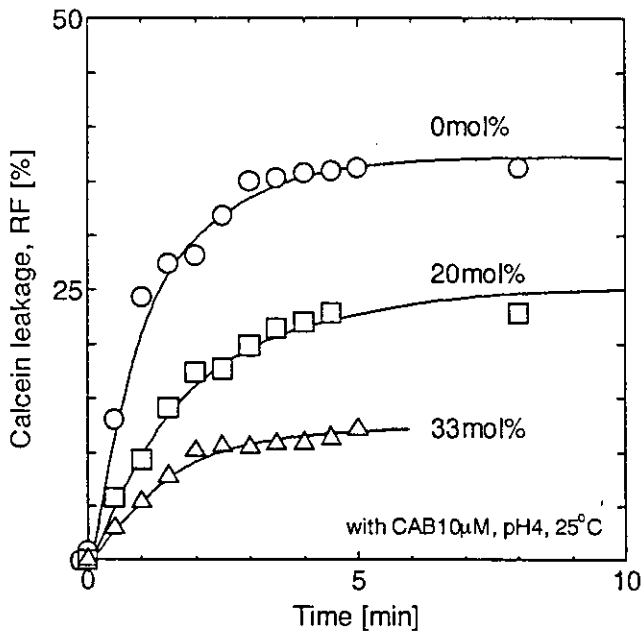

Fig. 3. Time-dependence of calcein leakage (*RF*) from cholesterol-containing POPC liposomes in the presence of CAB at pH4 at 25°C.

The effect of the phospholipid species on kinetic parameters was also studied (Fig. 4). The order of calcein release (its saturation level) was as follows: DOPC > POPC > DMPC > DPPC. POPC and DOPC have one and two unsaturated acyl-chains, respectively, whereas DMPC and DPPC have no such chains. The order of the packing density in lipid membranes is, then, DOPC < POPC < DMPC < DPPC, which is opposite to the order of calcein release. It is known that a low packing density of phospholipid makes lipid membranes unstable.[25] The changes in properties due to the addition of cholesterol and the variation in lipid composition are closely related to the membrane fluidity, which is a characteristic of membrane permeability. The influence of lipid composition on membrane fluidity was then investigated. Figure 5 shows the membrane fluidity of various liposomes evaluated using 1,6-diphenyl-1,3,5-hexatriene (DPH). The addition of cholesterol decreased the membrane fluidity, whereas a liposome composed of phospholipid with a low packing density has a large membrane fluidity. These results are consistent with previous reports.[26] It was thus shown that calcein release may be modulated by controlling membrane fluidity.

3.4 Dependence of calcein release on characteristics of proteins and membranes

On the basis of these results, the kinetics of the CAB-induced release of calcein were shown to depend on (i) the conformational state of CAB and (ii) the dynamic properties of liposomes. It is expected that such phenomena are applicable to cases with other proteins. To quantify the protein-induced release of calcein, a common parameter that can characterize both proteins and liposomes is needed. Judging from Fig. 2(a), proteins at a low pH,

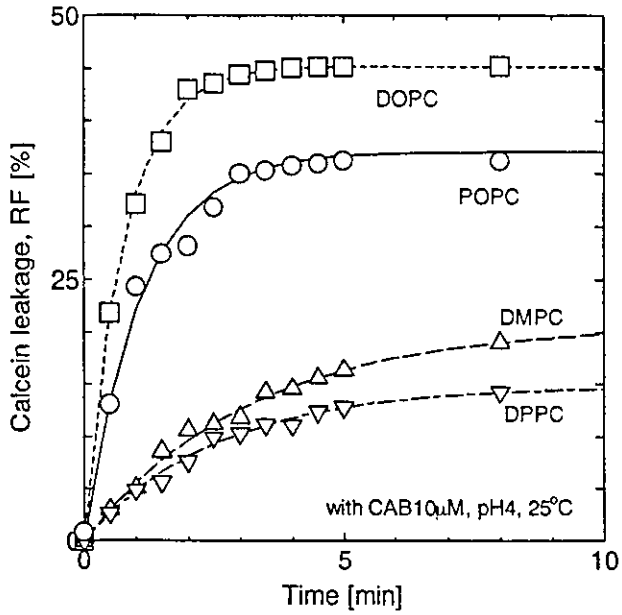

Fig. 4. Time-dependence of calcein leakage (*RF*) from various liposomes in the presence of CAB under pH4 at 25°C.

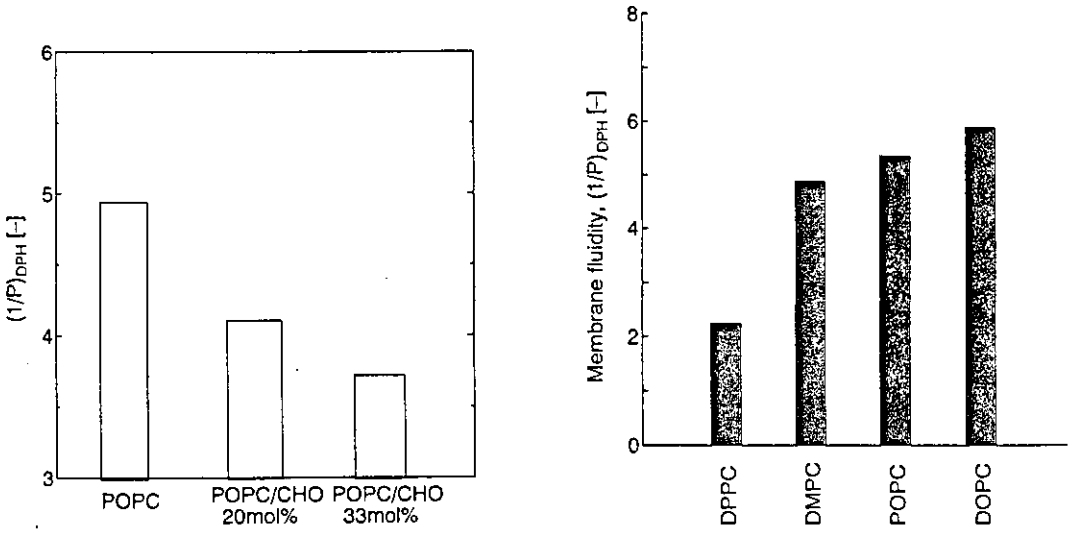

Fig. 5. Membrane fluidity of various of liposomes. Membrane fluidity was calculated from the reciprocal value of the polarization of fluorescent probe DPH. Details are in the experimental section.

where their entire surface is hydrophobic, did not interact significantly with liposomes, indicating that the conventional parameters such as the fluorescent intensity of tryptophan residues cannot be used. The *LH* of proteins is known to reflect the features of the partly denatured proteins that strongly interact with the liposomes. The *LH* was therefore selected as the indicator for the interaction between proteins and liposomes, and the extent of the interaction between proteins and liposomes was then defined as the product of the *LH* of proteins and the *LH* of liposomes, $LH_{lip} \times LH_{pr}$, from the viewpoint of the reaction rate theorem.

Figure 6 shows plots of $RF_{max} / \Phi_c (\Phi_c < 0.3)$ versus $LH_{lip} \times LH_{pr}$. There may be two categories (type A and B) of protein-induced calcein release. In both cases, a linear relationship between the values is observed. These results may reflect the identical features of each protein. Information on the secondary structure of various proteins is summarized in Table 1. The values of RF_{max} / Φ_c of proteins classified as type A, having a structure with relatively low β-sheet content or without disulfide bonds, were larger than those of proteins of type B. On the contrary, the proteins of type B tended to possess a β-sheet-rich structure or disulfide bonds. As for amyloid β protein (1–40), its RF_{max} / Φ_c was small, although neither β-sheet nor disulfide bonds were included in its structure, as shown in Table 1. The orientation of amyloid β protein (1–40) is considered to be different from that of other proteins; the detailed causes of this are still under investigation. These findings indicate that the extent of the protein-liposome interaction depends on the conformation of the proteins, which determines the identical features of the proteins.

3.5 *Model for disturbances due to proteins*

On the basisi of these results, the mechanism of calcein release from liposomes may be discussed. The single-exponential kinetics of calcein release (Figs. 1, 3, 4) indicate that the

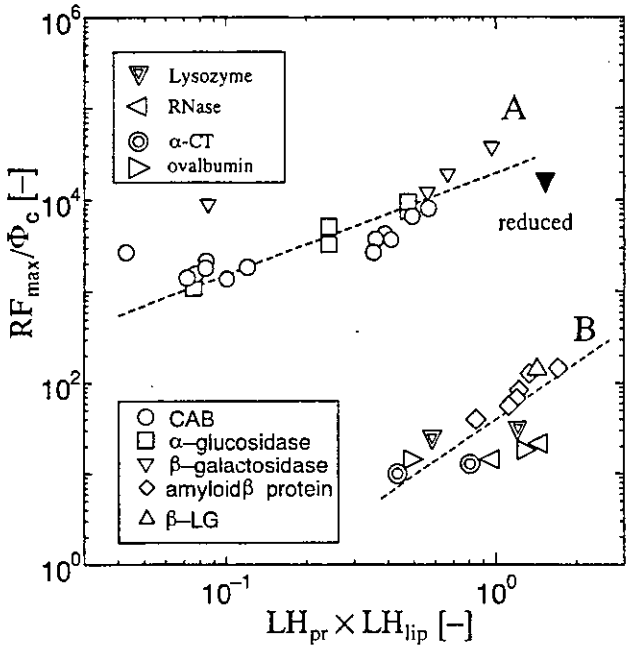

Fig. 6. Relationship between the protein-induced calcein leakage and the hydrophobic protein-liposome interaction. The solid triangle represents the reduced lysozyme.

Table 1
Molecular parameters of the proteins used.

Sample no.	Protein	MW	Fraction(%) of α-helix	β-sheet	S-S
1	carbonic anhydrae	30000	20	35	0
2	α-glucosidase	65000	—	—	0
3	α-chmotrypsin	25700	8	10	5
4	lysozyme	14300	29	16	4
5	ribonuclease A	13700	18	44	4
6	β-lactogliblin	13400	10	43	2
7	β-galactosidase	540000(4)	11.3	35.4	0
8	ovalbumin	46000	33	30	1
9	amyloid β protein (1–40)	4312	37.5	0	0

membrane association of proteins is a simple one-step process as previously reported.[18] The amount of released calcein approached a constant value within a short time (Figs. 1, 3, 4), resulting in two kinds of dependence of kinetic parameters on *LH* being observed. It has been reported that the capacity factor of proteins and lipid membranes determined by ILC is correlated with *LH* in a single relationship.[7] The difference between the calcein release

and ILC parameters arises because calcein release depends not only on the intensity of protein-liposome interactions but also on the effect of perturbations on the liposome membranes. Many researchers interpreted calcein release as the generation of either transient pores or defects.[27,28] The formation of pores depends on the molecular characteristics of the inducer. According to previous reports, the following two cases can be considered: (i) inducers with membrane-lytic activity;[18,27,28] (ii) inducers without this activity.[15]

Triton X-100 is a good example of the former case. Such a molecule (i.e., surfactant), in general, inserts into the interior of phospholipid membranes because of its hydrophobicity. Above the critical micellar concentration (cmc) in the lipid membranes, the formation of mixed micelles between phospholipids and surfactants occurs in the lipid membranes, and subsequent smaller micelles are formed from the liposomes, leading to the destruction of liposomes.[28] This process depends on the critical micelle concentration of surfactant in the lipid membranes, not on the dynamic properties of the surfactant.

In the latter case, the molecules exhibiting large fluctuations, including those shown in our results, are good examples. Although proteins in native or unfolded states cannot interact with lipid membranes (Figs. 2(a), 2(b)), ordered structures of lipid membranes were destroyed because of their large fluctuations, followed by the breakdown of the permeable barrier of lipid membranes. It is suggested that the fluctuation of proteins, therefore, plays an important role in this process.

The intramolecular force between phospholipid molecules, the so-called *microviscosity*, maintains the ordered structure of lipid membranes and is not markedly influenced by the insertion of surfactants perpendicular to the surface of the lipid membrane. The rapid saturation of calcein release induced by Triton X-100 indicates that the decrease in microviscosity occurs in the extremely limited region around the Triton X-100 molecule. It is just lateral diffusion that, in the broad region of the lipid membrane, provides a force which can overcome the microviscosity. According to reports on membrane-disturbing proteins such as the F-protein of the Sendai virus,[29] proteins that cannot intrude into the lipid membrane may be extended on the liposome membrane, so that the domain of the F-protein inserted into the membrane can move without changing its secondary structure by lateral diffusion on the membrane surface. It is suggested that the α-helix structure is suitable for lateral diffusion rather than the β-sheet structure, judging from Fig. 6. These concepts are supported by the result that the α-helix structure acts as a dynamic domain in the protein molecule.[30] In addition, for the secondary structure of proteins accessing a lipid surface with a hydrophilic surface, proteins may have a hydrophilic surface to some extent. Furthermore, the reduction in the number of disulfide bonds in the lysozyme increased RF_{max} / Φ_c (Fig. 6). Cleavage of disulfide bonds may lead to incremental changes in LH_{pr} accompanying the large fluctuations of protein molecule as a whole. It is assumed that the liposome-protein interaction is dominated by LH.

Specific secondary structures working as dynamic domains may reflect the stability of protein molecules. Their stability is, in general, controlled by (i) hydrophobic interactions, (ii) electrostatic interactions, (iii) hydrogen bonding, and (iv) other interactions including van der Waals interactions. Recently, the findings that suggest hydrogen bonding substantially determines the stability of the protein interior, have accumulated, suggesting that the

strength of hydrogen bonding may affect protein-lipid membrane interactions.[31] Therefore, hydrogen bonding would also be a key factor as well as *LH*. This problem is now under investigation.

On the basis of this discussion, it is suggested that large fluctuations of proteins enable the intense lateral diffusion of their secondary structures, such as the α-helix structure on the liposomal surface, which overcomes the microviscosity of the lipid membranes, which is then followed by the breakdown of the permeable barrier of the liposome membrane against the solutes. The permeability of calcein from the liposomes is controlled by the fluctuation of proteins, characterized by the *LH*. It has thus been shown that liposomes may be used as sensor elements for the detection of conformational changes of proteins under stress. These findings are expected to be applied to the design of liposome-based bioreactors or drug delivery systems.

Acknowledgements

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Monitoring of Protein Dynamics on Membrane Under Stress Condition Using Dielectric Dispersion Analysis

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ABSTRACT

Dielectric dispersion analysis (DDA) method was selected to the evaluation for variation of membrane properties of liposome and proteoliposomes (liposomes in the bilayer of which membrane protein is incorporated) under stress condition. For DDA, we measured dielectric spectra and performed fitting analysis with two-type function of Debye's equation against the spectra, and we obtained a characteristic frequency observed around 50 MHz (f_{c2}), which is one of the fitting parameters and corresponds to an extent of the rotational motions of lipid molecules. The change of membrane properties of the liposome was first investigated in the liposome suspension with hydroperoxide in order to investigate the effect of oxidative stress. 1-Stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine (SAPC) was herewith used as an easily-oxidizing phospholipid. In the case of SAPC liposome, the decrease of the f_{c2} value was observed, suggesting the restriction of molecular motion of the surface of SAPC liposome by the oxidation of the SAPC in the presence of hydroperoxide. The stress response of proteoliposome incorporated (Na^+, K^+)-ATPase as a membrane protein was secondly analyzed in the SAPC liposome solution. Compared with the f_{c2} value of liposome without the membrane protein, the f_{c2} value of proteoliposome was found to be small, probably, due to the restriction of lipid molecules by the incorporation of and interaction with the membrane protein. A significant decrease of the f_{c2} value was also observed under the heat and oxidative stress, together with the hydrolysis activity of ATPase. Based on the above results, the molecular motion of lipid on the liposome surface was found to be analyzed under stress conditions, even in the presence of membrane protein.

KEY WORDS

oxidative stress, proteoliposome, membrane protein, dielectric dispersion

INTRODUCTION

Recently, (membrane) proteins have been studied in the biological field for the purposes of the application to biomaterials because (membrane) proteins are nano materials well established to nano-size level. In the field of protein engineering, the investigation on the control of catalytic activity and the binding of specific target biomolecules has been developed with a point mutation of amino acid by the

gene mutation technique and a modification of polymer such as polysaccharides, dextran, and poly(ethylene glycol). These methods provide many valuable informations of the enzymatic function of cytoplasmic proteins enough to apply for the industrial usage. On the other hand, the study on the membrane protein, for the purpose of the application to the industrial usage, has not been developed since there is many difficulties in the preparation/reconstitution of membrane protein from cell membrane and evaluation of enzymatic or signal transductional function.

Liposome, which is a closed phospholipid bilayer membrane, is a useful tool for the reconstitution of membrane proteins better than another reconstitution bases (eg. Langmuir-Blodgett membrane, silicon base, and so on). So far, some reconstitution methods have been reported: (i) gel permeation chromatography method (Yoda et al., 1984), (ii) dialysis method (Urbatsch et al., 1994), and (iii) cell-to-liposome transport method (Sunamoto et al., 1990). Especially, method (iii) is found to be dependant on not only the heating temperature but also the lipid composition of liposome. This strongly suggests that the reconstitution of the membrane protein, in other words, its activity may be predominated by the ordered structure of lipid membrane. However, there is a lack of findings on this problem. The conformation of the membrane protein in the lipid bilayer seems to depend on the small number of the phospholipid molecules surrounding the membrane protein (*boundary lipid*). The conventional method that probe molecules are used does not always produce the valuable information on the above problem not only because the evaluation by the probe may include the secondary information but also because the incorporation of the probe may alter the ordered structure of the membrane. Therefore, we considered that the rapid and non-invade dielectric dispersion analysis method is a useful method for the purpose.

In this study, we prepared the proteoliposomes, mainly by the conventional method (ii), incorporated (Na^+, K^+)-adenosine 5'-triphosphatase (ATPase) as a model membrane protein. We added the heat stress and hydroperoxide to the proteoliposome suspension to alter the ordered structure of lipid bilayer. Then, we studied the relationship between the surface properties of the proteoliposomes and the hydrolysis activity of ATPase. Finally, we discuss the oxidation process of proteoliposomes with the dielectric dispersion analysis (DDA).

MATERIALS AND METHODS

For liposome preparation, 1,2-dimylistoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1-stearoyl-2-arachidoyl-*sn*-glycero-3-phosphocholine (SAPC) were used. We used 3-((3-cholamidepropyl)dimethyammonio)-1-propane sulfonate (CHAPS) as the solubilizing reagent for membrane protein. As the membrane protein for proteoliposome preparation, (Na^+,K^+)-adenosine

DMPC

SAPC

CHAPS

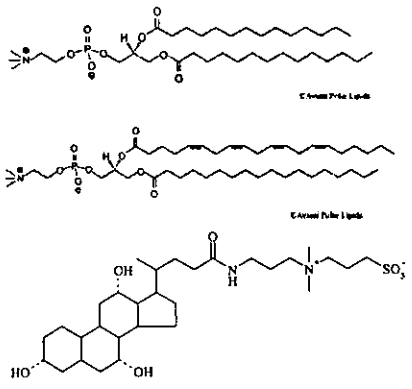

Figure 1. Molecular structure of phospholipids and detergent used in this study.

5'-triphosphatase ((Na^+, K^+)-ATPase) was purchased from Sigma (USA). Figure 1 shows the molecular structure of phospholipids and detergent used in this study.

Liposome was prepared according to the method described elsewhere (Morita et al., 2003; Kuboi et al., 1997). Briefly, dried phospholipid film was prepared on inertial surface of a flask by evaporating chloroformic phospholipid solution at 37 ℃. The lipid film was hydrolyzed with the buffer for an hour, subsequently the hydrolyzed suspension was frozen for 15 min at the condition of −80 ℃ and thawed for 15 min at the condition of 37 ℃. Repeating these operations for five times, multilamellar liposome suspension was prepared. Passing the suspension through polycarbonate membrane, suspension containing unilamellar liposomes with their diameters ca. 100 nm was prepared.

The preparation of proteoliposome was performed by dialysis of the mixture of the phospholipids, detergents, and membrane protein with cellulose tube and corresponding solvent as a dialyzing buffer. The oxidation of the samples was started by the addition of the hydroperoxide with the final concentration of 13mM. ATPase activity was assayed by evaluating the time course of the hydrolyzed phosphate from ATP induced by enzymatic activity of ATPase, according to the colorimetric method (Chifflet et al., 1988). Progress of phospholipid oxidation was evaluated by the determination of thiobarbituric acid reactive substances (TBARS) produced by the oxidation process (Quinlan et al., 1988).

The dielectric dispersion analysis was performed as follows: The sample solution was injected to the measuring cell, and capacitance (C_p) and conductance (G) were measured to determine relative permittivity (ε') and dielectric loss (ε'') according to the following formulae (1) and (2). To measure the frequency dependency of capacitance and conductance, Impedance Analyzer (4291B, Agilent Technologies) was used; the measuring frequency was ranged from 1 MHz to 1 GHz.

$$\varepsilon' = \frac{C_p}{C_0} \quad (1) \quad , \qquad \varepsilon'' = \frac{G}{2\pi f C_0} \quad (2)$$

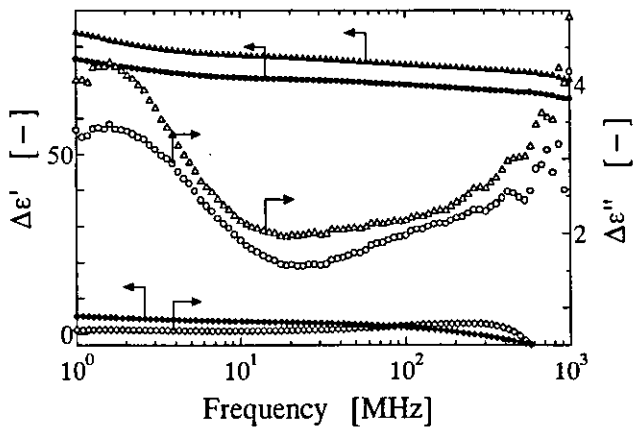

Figure 2. Relative permittivity (Δε', closed keys) and dielectric loss (Δε'', open keys) spectra of DMPC liposome (circles), proteoliposome (triangles) and CHAPS (diamonds).

Where, f and C_0 represent measuring frequency and cell constant, respectively.

Frequency dependencies of relative permittivity and dielectric loss were obtained as shown in Figure 2. Fitting analysis was performed according to two-type functions of Debye's equation (3) and (4) to obtain the dielectric parameters (Shikata and Imai, 1998).

$$\varepsilon' = \frac{\Delta\varepsilon_1}{1+(f/f_{c1})^2} + \frac{\Delta\varepsilon_2}{1+(f/f_{c2})^2} \quad (3)$$

$$\varepsilon'' = \frac{\Delta\varepsilon_1 (f/f_{c1})}{1+(f/f_{c1})^2} + \frac{\Delta\varepsilon_2 (f/f_{c2})}{1+(f/f_{c2})^2} \qquad (4)$$

RESULTS AND DISCUSSION

Dielectric property of proteoliposomes.

Conventionally, membrane properties such as membrane fluidity and hydrophobicity are measured by hydrophobic fluorescence probe including 1-anilinonaphthalene 8-sulfonate (ANS) and (trimethylammonium-)diphenylhexatriene ((TMA-)DPH). For the proteoliposomes, the above conventional method does not always give the appropriate information on membrane property because there is the possibility that hydrophobic probes may bind to the hydrophobic region in the membrane protein. Then, we consider the dielectric dispersion measurement proper because this method is non-probe and non-destructive method.

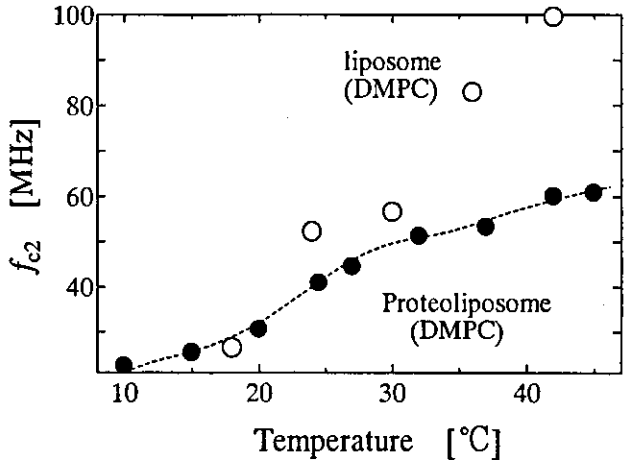

Figure 3. Temperature dependency of proteoliposome incorporating (Na^+, K^+)-ATPase with DMPC as a phospholipid (closed keys).

Figure 2 shows the dielectric spectrum of the liposome, proteoliposomes and ATPase-solubilized micells. Firstly, to check the effect of membrane protein against the dielectric dispersion phenomena of proteoliposomes, we investigated the spectrum of protein and ATPase-solubilized micelles (MPMs). In the case of cytoplasmic protein, α-chymotrypsin, the high concentration condition at around 5 mg/ml gave the significant dielectric dispersion was observed in our focused frequency range. Our results agree with the previous results in the case of lysozyme (Amo et al., 1997; Bonincontro et al., 1999). This is because the number density of dipole moment in the protein molecule is quietly small to detect the dielectric dispersion. Therefore, the concentration of membrane protein in our systems (ca. 0.025 mg/ml) should not give the significant dielectric relaxation, meaning that the aggregate of membrane protein not incorporated into lipid membranes does not contribute to the dielectric spectrum. Secondly, we investigated the effect of membrane protein solubilized in the micelles since such micelles may remain in the proteoliposome solution without aggregation. The relaxation strength ($\Delta\varepsilon'$) and characteristic frequency of the micelles and MPMs were obtained from the fitting analysis with equations (3) and (4): $\Delta\varepsilon_1$=0.352, f_{c1}=24.9 MHz, $\Delta\varepsilon_2$=2.48, f_{c2}=242 MHz at 40 mM of CHAPS. If we assume that the detergent remains as MPMs after a dialysis, the dielectric parameters of the sample should be same as the micelles. Our sample shows the similar tendency to that of the pure DMPC liposome, especially, the peculiar dispersion at around 2 MHz similar to the pure DMPC liposome. Therefore, we concluded that the dielectric dispersion analysis of the poteoliposome is possible with the same index with the conventional liposome.

The characteristic frequency exhibiting at around 50 MHz (f_{c2}) corresponds to the axial rotational

motion of phospholipid molecules in the case of the conventional liposome. We obtained the f_{c2} value of liposome and proteolipsome from Figure 2: f_{c2} = 55 MHz (DMPC liposome), f_{c2} = 50 MHz (proteoliposome). In the proteoliposome, the decrease in the f_{c2} value was observed. This is attributed to by the incorporation of the membrane protein into the lipid bilayer, which agrees with the previous findings that the denatured proteins interact with liposomal membrane induced the decrease in the f_{c2} value (Morita et al., 2003).

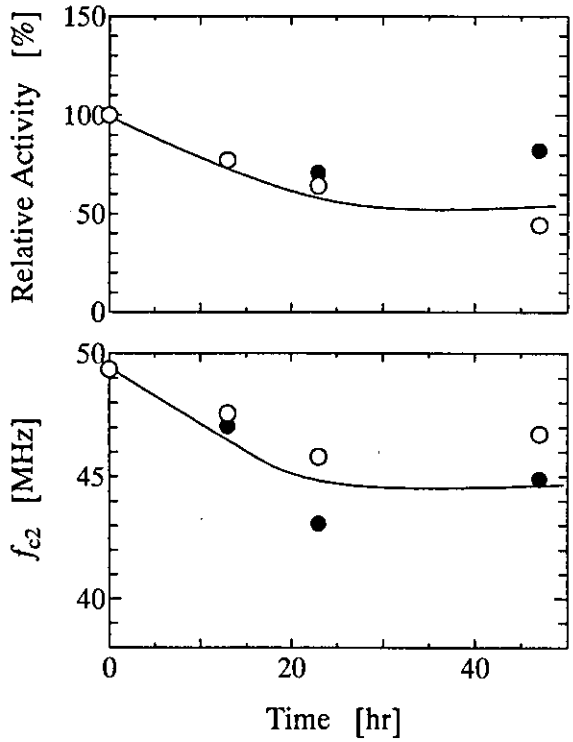

Figure 4. Time-course of (a) the ATPase activity and (b) characteristic frequency f_{c2} of proteoliposome with incubation time at 37℃ (black) and 45℃ (white).

Temperature effect against the function of the proteoliposome

The temperature effect against the f_{c2} value of (proteo)liposome was next studied in Figure 3. In the case of pure DMPC liposome, the f_{c2} value, which is an index for the membrane fluidity, shows the temperature dependency similar to the membrane fluidity. For the proteoliposome, the f_{c2} value is significantly decreased compared with those of pure DMPC liposome above the gel-to-liquid phase transition temperature (i.e. T_m=23.9°C). Above T_m, the relaxation behavior was investigated according to Arrhenius's equation (5),

$$k = 2\pi f_{c2} = A\exp(-\Delta E/RT) \qquad (5)$$

where R is the gas constant (8.314 $JK^{-1}mol^{-1}$), the macroscopic relaxation time, and therefore exponential rate constant ($k = 1/\tau = 2\pi f_{c2}$), is dependent on the activation energy. The estimated activation energy of pure DMPC and proteoliposome are 42.2 and 19.9 kJ/mol, respectively. Our estimates are comparable to the value calculated from the data for pure DMPC liposome reported by Schrader and Kaatze (47.7 kJ/mol). The activation energy is equal to the energy required to break the intermolecular bonds that are restricting the reorientation of the dipole, and thus indicates the strength of the intermolecular $PO_4^-...CH_3^+N$ bond (Smith et al., 1998). Then, the decremental change in the f_{c2} value by the incorporation of membrane protein is considered to indicate the unstability of the intermolecular bonds, in other words, the variance of the ordered structure of lipid bilayer.

To study the relationship between membrane property and the activity of membrane protein, we evaluated the hydrolysis activity of ATP. The hydrolysis amount of ATP was measured with time and the initial rate per the weight of ATPase was defined as the ATPase activity. Our result at 25°C was 1.5 U/mg and was quite low value as compared with the conventional reports (Yoda et al., 1986, Cornelius

and Skou, 1984). The unstability of intermolecular bonds between phospholipids may be one of factors for the low activity in our experimental system. The activity at 25°C was set at control to asses the temperature effect against the ATPase activity. The relative activity of ATPase was monitored at 37 and 45°C (Figure 4(b)). At 45°C, the activity was decreased to 50 %. The f_{c2} value was also monitored and the similar tendency with the case of the activity was observed as shown in Figure 4(b). We could not here conclude which is the driving force of the denaturation of ATPase, the effect of temperature itself or conformational change of ATPase induced by mobility change of phospholipids. Now we are investigating about that problem in details.

Effect of oxidative stress against the proteoliposome

For the evaluation of membrane fluidity in oxidative stress, it is not available to use (TMA-)DPH, since the probe has three double bonds in the hexatriene group and is easily affected by reactive oxygen species (ROS). We applied, therefore, DDA for the study of oxidative stress against molecular mobility of the phospholipid molecules. Figure 5(a) shows the time-course of the f_{c2} value of liposomes with each component of the phospholipids in the presence of 13mM of hydroperoxide in the suspension. Progress of oxidative reaction was confirmed by the determination of TBARS production, as shown in Figure 5(b). As the oxidation of SAPC was progressing, the decrease of f_{c2} was observed, whereas no remarkable change in the f_{c2} value was observed in pure DMPC liposomes. Generally, the oxdative reaction is progressed in the double bond of phospholipid. Since SAPC has four double bonds in its chemical structutre, SAPC is easily influenced by ROS. In the time-course of TBARS quantity, the subtle lag-time was observed, indicating the existence of the intermediate of oxidative reaction. Correspondingly, the drastic decrease in the f_{c2} value in the beginning also means the production of the oxidative intermediate. Furthermore, since the above tendency is dependent on the SAPC concentration, we concluded that the f_{c2} value is an index for the oxidation of phospholipid in this experimental condition. Simultaneously, we performed ATPase assay of proteoliposome in the same condition. The ATPase activity was immediately decreased and no activity was observed within 30 min. From the comparison of the time-course of ATPase activity and f_{c2} value in the oxidative stress, we considered that the ROS directly attack the ATPase together with phospholipid membrane although the production of the oxidative intermediate of SAPC may have something to do with the conformational change/denature of ATPase.

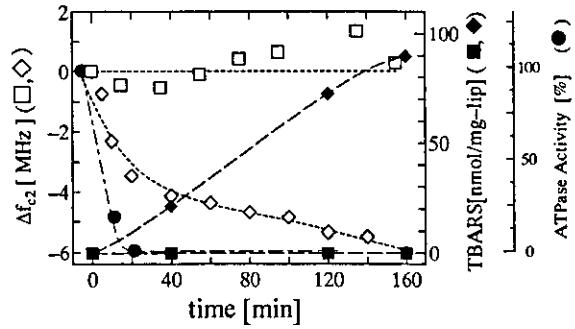

Figure 5. Time-course of the change of characteristic frequency f_{c2} of DMPC (open squares), DMPC/SAPC (3/1) (open diamonds) and produced TBARS of DMPC (closed squares), DMPC/SAPC (3/1) (closed diamonds) liposomes, and relative ATPase activity (closed circles) in the oxidation process.

CONCLUSION

In this study, DDA method was selected to the evaluation for variation of membrane properties of liposome and proteoliposome under stress condition. The change of membrane properties of the liposome was first investigated in the liposome suspension with hydroperoxide in order to investigate the effect of oxidative stress. SAPC was herewith used as an easily-oxidizing phospholipid. In the case of SAPC liposome, the decrease of the f_{c2} value was observed, suggesting the restriction of molecular motion of the surface of SAPC liposome by the oxidation of the SAPC in the presence of hydroperoxide. The stress response of proteoliposome incorporated (Na^+, K^+)-ATPase as a membrane protein was secondly analyzed in the SAPC liposome solution. Compared with the f_{c2} value of liposome without the membrane protein, the f_{c2} value of proteoliposome was found to be small, probably, due to the restriction of lipid molecules by the incorporation of and interaction with the membrane protein. A significant decrease of the f_{c2} value was also observed under the heat and oxidative stress. Based on the above results, the molecular motion of lipid on the liposome surface was found to be analyzed under stress conditions, even in the presence of membrane protein.

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An attempt at decision making in tissue engineering: reactor evaluation using the analytic hierarchy process (AHP)

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Abstract

To develop a method of human-oriented evaluation in tissue engineering and regenerative medicine, we applied the analytic hierarchy process (AHP) in the evaluation of tissue engineering reactors. For evaluating a reactor for a bioartificial liver (BAL) support system, we identified five criteria: safety, scalability, cell growth environment, mimicking native liver functions and handling. Based on these criteria, we evaluated six different types of BAL bioreactors by three panelists. Using the AHP method, we successfully ranked BAL systems for bridge use in liver transplantation in the attempt at decision making based on human-oriented evaluation.

Keywords: Analytic hierarchy process; Bioartificial liver; Decision-making; Reactor evaluation

1. Introduction

In the 21st century, tissue engineering and regenerative medicine are expected to be powerful tools for repairing damaged and/or diseased tissues and organs. Many researchers are developing new reactors, materials, methods of cultivation, cell lines, media, and so on using various chemical, biological, and engineering techniques. In this field, it is very difficult to accurately consider the evaluation of tissue engineering tools or methods because of the diversification of tissue engineering and/or regenerative processes.

The analytic hierarchy process (AHP) was developed by Saaty in the field of operation research for the analysis of decision-making analysis in a number of different areas [1]. The process involves structuring a problem from a primary objective to secondary levels of objectives. Once these hierarchies have been established, a "pairwise comparison matrix" of each element within each level is constructed. This method is suitable for multi-objective decision-making processes, when the trade-off relationship between decision criteria and alternatives exists in the process. The ranking result is the relative one. In the field of chemical engineering, the AHP method was used for the engineering problem of selecting a chemical laboratory reactor [2,3]. In the biomedical field, Cook et al. developed a rating system of allocating cadaver livers for orthotropic transplantation [4]. Hummel et al. also applied the AHP method in the multidisciplinary evaluation of medical devices, such as a heart-assist blood pump [5]. Before tissue engineering and/or regenerative processes can be applied to clinical treatment, these processes must be validated and standardized. Many studies regarding these processes have been made, and various conditions have been employed. An objective method is required for the validation and standardization of these processes.

In this article, we applied the AHP method in the evaluation of reactors to determine the efficacy of AHP methods for the validation and standardization of processes used in tissue engineering and regenerative medicine.

As sample problems for the process of multi-objective decision making, we selected the evaluation of bioartificial liver support bioreactors. A hybrid bioartificial liver (BAL) support system, composed of artificial materials and living cells, was developed for use as a bridge in patients with hepatic failure [6–10]. The liver has a number of hepatic functions such as detoxification, drug metabolism, ammonia removal, urea synthesis, and protein synthesis, which are supported by viable hepatocytes. The bioartificial liver support system is used to support the patient with hepatic failure by using the functions of living hepatocytes. In order to construct an effective BAL system, many well-designed bioreactor devices have been invented. However, it is impossible to directly compare and evaluate these reactors because

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Nomenclature

a_{ij}	pairwise comparison of element i to element j
A	Matrix of pairwise comparison
B_i	matrix of estimated weight vectors
CI	consistency index
CR	consistency ratio
D	decision vector
n	number of decision elements (natural number)
RI	average random consistency index
w_i	importance (weight) of element i
W	weight vector

Greek letter

λ_{max}	principal eigenvalue of pairwise comparison matrix

of the differences in their construction, operation, and utility. In some cases, it is possible to quantitatively evaluate the characteristics of BAL system (for example, clearance of drug, urea production rate, ammonia production rate, etc.). However, in some cases, it is very difficult to compare the different function quantitatively (i.e. it is very difficult to compare maximum cell concentration and asepsis quantitatively). In such cases, the human-oriented evaluation is useful. The fuzzy control can easily utilize the empirical knowledge gained from skilled operators. The AHP methodology is similar to fuzzy control. Using the AHP methodology, the human-oriented ranking is able to be converted into "relative number". In other words, it is possible to rank the BAL bioreactor "relatively" based on human-oriented evaluation. We chose the evaluation of BAL bioreactors as an example in a process of multi-objective decision making.

2. Methods

2.1. AHP methodology

The analytic hierarchy process (AHP) was developed by Saaty and coworkers [1–3]. It has been used in many different fields as a multi-attribute decision analysis tool with multiple alternatives and criteria. AHP uses "pairwise comparisons" and matrix algebra to weigh criteria; the decision is made by using the derived weights of the evaluative criteria.

In this study, a three-level model was employed, as shown in Fig. 1. The flow chart of AHP analysis was shown in Fig. 2. In AHP analysis, the objective is defined in level 1. In level 2, general required functions are weighed by the following process. Based on pairwise comparisons, the relative importance of a criterion to other criteria is determined

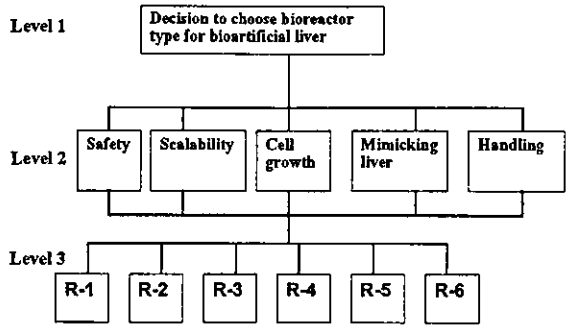

Fig. 1. Decision hierarchy employed for AHP in this study. Level 1 shows the objective of the AHP analysis. Level 2 shows the criteria of functions required for the bioreactor. Level 3 shows the criteria for the bioreactor types. R-1: collagen entrapped hollow fiber. R-2: capillary network hollow fiber. R-3: radial flow bioreactor. R-4: rigid-type multicapillary polyether polyurethane foam for/hepatocyte spheroid packed-bed module. R-5: packed-bed type reactor using reticulated polyvinyl formal (PVF) resin as a supporting material. R-6: circulatory flow bioreactor with glass fiber cloth (Cygnus).

(Table 1). Matrix A is established using this relative importance (Eq. (1)):

$$A = [a_{ij}], \quad a_{ij} = \frac{w_i}{w_j}, \; a_{ji} = \frac{1}{a_{ij}}, \; a_{ii} = 1 \tag{1}$$

Here w_i is the weight of criterion i. From the above matrix, the weights can easily be determined by solving the

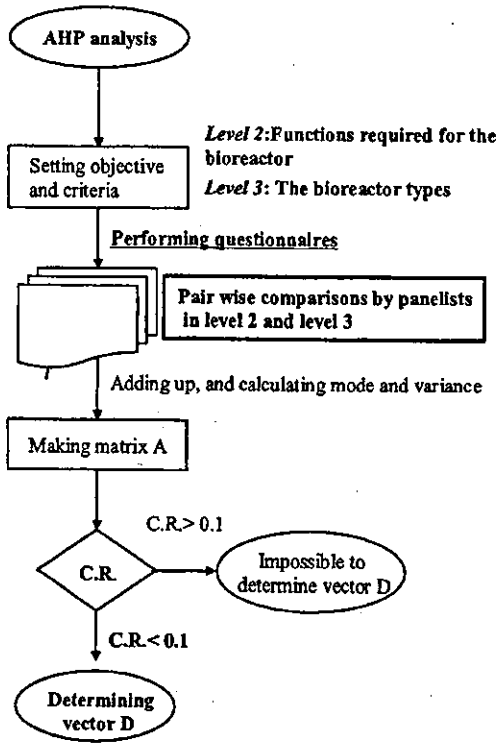

Fig. 2. Flow chart of AHP analysis in bioreactor selection for bioartificial liver support system.

Table 1
Scale of relative importance used in AHP process

Relative importance	Definition
1	Equal importance
3	Moderate importance
5	Strong importance
7	Very strong importance
9	Extreme importance
2, 4, 6, 8	Intermediate values between two adjacent judgments

following equation (Eq. (2)):

$$AW = nW, \quad W = (w_1, w_2, \ldots, w_n)^{\mathrm{T}} \tag{2}$$

where n is the natural number.

From the equation, n is determined as eigenvalue (λ_{max}) of the matrix, that is,

$$A'W' = \lambda_{max} W' \tag{3}$$

and w_i satisfies the following equation (Eq. (4)):

$$\sum_i w_i = 1 \tag{4}$$

The judgment for the satisfaction of λ_{max} is made by determining the consistency index (CI) and consistency ratio (CR) from Eqs. (5) and (6):

$$\mathrm{CI} = \frac{\lambda_{max} - n}{n - 1} \tag{5}$$

$$\mathrm{CR} = \frac{\mathrm{CI}}{\mathrm{RI}} \tag{6}$$

RI is the average random consistency index and calculated to be as follows [1,3]:

$$\mathrm{RI} = 0.58\ (n = 3),\ 0.90\ (n = 4),\ 1.12\ (n = 5),\ 1.25\ (n = 6)$$

If CR is greater than 0.1, λ_{max} is not satisfied.

The same processes are performed in level 3. The weight set for the reactors under a criterion in level 2 is determined. For example, weight set 1 (B_1) for the reactors is determined from the standpoint of safety, B_2 from scalability, and so on. Finally, the decision of reactor type is evaluated by Eq. (6):

$$D = [B_1, B_1, \ldots, B_n]W' \tag{6}$$

where D is the decision vector.

2.2. Pairwise comparisons

All criteria of pairwise comparison were based on the referenced papers. The AHP analysis in BAL evaluation, we used the various types of related reactors' Refs. [12–28].

3. Results and discussion

3.1. Case study... bioartificial liver (BAL) support system—typical and characteristic BAL reactor candidate

Many researchers have developed hybrid bioartificial liver (BAL) support systems consisting of bioreactors and living cells. In this case study, we selected six typical BAL bioreactors for AHP evaluation. Details of the selected bioreactor systems are explained below.

(1) *Collagen entrapping hollow fiber* (collagen entrapment hepatocyte spheroid bioreactor) [11,12]
Hu et al. invented the collagen-entrapped hollow fiber bioreactor. In this reactor, hepatocytes were entrapped in the luminal side of a hollow fiber bioreactor by collagen loading. The hepatocytes formed into self-assembled spheroids before collagen entrapment in stirred tanks. Toxic compounds in a patient's blood diffused through the hollow fiber ultrafiltration membrane and were metabolized.

(2) *Capillary network hollow fiber (modular extracorporeal liver support (MELS))* [13–15]
The concept of MELS is the combination of different units for extracorporeal therapy. The cell module consisted of a capillary network of hollow fibers which contain three different, but related, capillary systems, which provided three functions: medium inflow, cell oxygenation/CO_2 removal, and medium outflow.

(3) *Radial flow bioreactor* [16–19]
Application of a radial flow bioreactor in cell culture was reported by Tharakan and Chau and developed to large scale cultivation system by Yoshida et al. [16–18]. They proposed that the nutrients flow radially across an annular bed of fiber from the central feed distribution tube and the nutrients were evenly delivered in the reactor. Matsuura et al. applied the radial flow bioreactor to an artificial liver support system [19].

(4) *Rigid-type multi-capillary polyether polyurethane foam (MC-PUF)/hepatocyte spheroid packed-bed module* [20,21]
Funatsu and coworkers developed a rigid MC-PUF as a packed-bed module for an artificial liver support system [20,21]. The MC-PUF had a macroporous structure and many capillaries and could achieve a sufficient transfer volume. Primary hepatocytes quickly formed spheroids in the PUF pores and maintained the high density cultivation and high level of liver functions for more than a few weeks.

(5) *Packed-bed type reactor using reticulated polyvinyl formal (PVF) resin as a supporting material* [22–24]
The typical packed-bed BAL bioreactor is a packed-bed reactor using reticulated PVF resin as a supporting material [22–24]. Within its matrix, this supporting material had continuous interconnected pores of about 2 mm × 2 mm × 2 mm. The use of PVF resin in

a packed-bed type reactor resulted in a maximum cell density on the order of 10^7 cells/cm^3 PVF and exhibited a high level of hepatic functions for up to 1 week.

(6) *Circulatory flow bioreactor with glass fiber cloth, "Cygnus"* [25–27]

The Cygnus was originally invented for high-density recombinant cell culture (US patent 5270207). The principal characteristic of the reactor was the uniform delivery of nutrient and oxygen by vertical circulatory flow from the center of the rolled-up cloth to the peripheral area of the reactor. Enosawa and coworkers applied this reactor to a BAL system and was able to successfully prolong the survival time of a pig with hepatic failure [27].

3.2. Evaluation criteria for BAL systems

The most important problem facing those desiring to construct a BAL system is that the detailed mechanisms and required functions related to hepatic failure are still unclear [6–8]. Consequently, functions of the whole liver are seen as requisites for BAL construction. In order to evaluate BAL systems, a multi-objective evaluation is necessary; that is, the BAL system should be evaluated from several perspectives. We selected five evaluation criteria for relative and temporary BAL assessment.

(1) *Safety* (asepsis, sterilization, immunoisolation, blood cell separation, prevention of contamination)

The BAL system is a medical device. One of the most important, if not the most important, criteria for a medical device is safety. This criterion contains all safety-related items, e.g., maintaining aseptic conditions, the method of sterilization, ease of sterilization, immunoisolation for the prevention of immunoreaction, blood cell separation, and prevention of outside contamination.

(2) *Scalability* (ease of scale up, reactor size, high blood flow rate, high medium circulation rate)

The BAL system consists of living cells and mechanical materials. The cultivation vessel in the BAL system is a kind of bioreactor. In the design of the reactor, scalability is an important factor. "Scalability" includes the ease with which the scale can be increased, variable and compact reactor size, achievement of high blood flow and medium circulation rates, and other related factors affecting reactor scalability.

(3) *Cell growth environment* (high cell density, high viability, oxygen transport, nutrient transport, mass transfer)

To attain a high level of liver function, it is important to support a suitable environment for sustainable cell growth and maintenance for ensuring high density and viability. Good mass transfers of oxygen and nutrients are especially important because the rate of oxygen consumption of hepatocytes is greater than that of other cells.

(4) *Mimicking native liver functions* (three-dimensional structure, suitable scaffold, spheroid formation and maintenance, co-culture of hepatocytes with non-parenchymal liver cells)

Native liver has a variety of functions. It is difficult to determine the most important liver function in therapeutic use. Therefore, mimicking native liver functions is necessary for an effective BAL system. For mimicking, a three-dimensional structure, a suitable scaffold, and a suitable environment for spheroid formation or other mimicking factors are essential.

(5) *Handling* (user-friendly, short preparation time, ease of setup, ease of control and operation, maintenance of reactor with cells, cost, availability)

Finally, "handling" is an important factor for practical use. A complicated BAL system is not practicable even if it has a superior reactor system. The concept of "handling" includes the ease of reactor operation, short preparation time, ease of operation and setup, control, maintenance of reactor with cells, and so on. Of course, the cost and availability of the reactor are also fundamental considerations for handling.

3.3. AHP analysis for evaluation of BAL systems

To compare the selected reactors under the same conditions, we assumed xenogenic bioartificial liver support systems using primary porcine hepatocytes for bridge-use of liver transplantation for AHP evaluation. We also assumed that the same number and same quality of hepatocytes would be used in each BAL system. The pairwise comparisons were conducted for the general required functions in level 2. Table 1 lists a set of nine suggested definitions for entering a judgment value for the pairwise comparison. Using this scale, we performed the pairwise comparison between evaluation criteria. In this evaluation, we selected three experts for evaluation panelists who had different backgrounds (panelist 1, biochemical engineer; panelist 2, material scientist and panelist 3, medical researcher). The pairwise comparisons of panelists in level 2 were shown in Fig. 3. The overall tendency of evaluation ranking was similar between panelists. Medical researchers tended to think that the safety criteria was significantly important. Moreover, medical researchers seemed to suppose that the scalability was also an important factor for BAL function. These ranking results (weight vector) in level 2 are shown in Table 2. The CR value in level 2 was less than 0.1 and satisfied the consistency for panelists 1 and 2. However, the CR value in panelist 3 was higher than 0.1. Therefore, the ranking result (weight) for panelist 3 was not a reliable one. The relative importance of "safety" was highest among the criteria. "Safety" is thus the most important factor for BAL selection. In contrast, "scalability" is not so important in panelists 1 and 2.

Based on the level 2 analysis, 75 third level pairwise comparisons among the six selected BAL reactor systems were conducted for panelists 1–3. In these comparisons, all