Korean J. of Chem. Eng., **14**(5), 334-340 (1997)

EXTRACTION OF PROTEINS AND POLYMERS USING REVERSE MICELLES AND PERCOLATION PROCESS

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(Received 19 May 1997 • accepted 4 July 1997)

Abstract – Specific interfacial properties, which affect protein extraction in AOT and AOT-lecithin reverse micellar systems (RVMS), have been studied by using their percolation processes. Solubilization of proteins or synthetic polymers into RVMS by the injection method and its effect on the percolation processes could be easily evaluated by the measurement of electrical conductivity. The percolation process is found to be a sensitive and convenient measure of micro-interface of RVMS solubilizing various polymers or proteins, which clearly reflects the polymer(protein)-micellar and micellar-micellar interactions. The stability of RVMS or micellar-micellar interaction was dependent on the kinds, concentration and molecular weight of solubilized polymers. The value of β, defined as the variation of percolation threshold with the concentration of solubilized polymers, can be utilized to evaluate the stability of RVMS solubilizing polymers or micellar-micellar interactions. The values of β are affected by the hydrophobicity, molecular weight and absolute value of the net charge of the polymers solubilized into the AOT reverse micelles, which were evaluated by using Aqueous Two-Phase Systems (ATPS).

Key words: *Reverse Micelle, Protein-micellar Interaction, Percolation, Aqueous Two-Phase System*

INTRODUCTION

Self-assembling functional materials such as reverse micelles and liposomes have been used for separation or reaction of various biomolecules. Among the self-assembling functional materials, the reverse micelles are attractive simple model systems for actual complex biomembranes, since it has been suggested that reverse micellar structures may exist *in vivo* within bilayer membrane structure [Luisi et al., 1988; Oberholzer et al., 1995]. However, there are many problems to be solved in relation to properties of their surface or interactions between the interfaces and solubilized molecules.

A reverse micellar solution is a thermodynamically stable mixture of water, oil and surfactant where the water regions are separated from oil by a monolayer of surfactants. The reverse micelles have a capability to solubilize a variety of biomolecules into the nanometer-size water pools. Reverse micellar systems (RVMS) have various potentials such as extraction systems of proteins from fermentation broth or culture solution, hydrophobic reaction media for enzymes and preparation media for functional materials.

Using RVMS for extraction systems, however, proteins are known to suffer from denaturation and the back-extraction fraction decreases significantly when the protein-micellar interaction is too strong. An effective extraction is, therefore, achieved by controlling the above interaction in particular electrostatic, hydrophobic, steric and affinity interactions [Kuboi et al., 1991; Kelley et al., 1993].

A percolation process reflects these interactions and it can be quantified easily by the measurement of the electrical conductivity of the RVMS. Electrical conductivity measurements have been used to assess RVMS formation and to probe the structural changes occurring in such systems [Jada et al., 1989, 1990; Alexandridis et al., 1995]. A sharp increase in electrical conductivity caused by the percolation phenomenon well demonstrates the interaction between the micelles. It is generally accepted that conductivity percolation in RVMS with a spherical droplet structure is a result of reverse micellar droplet clustering [Alexandridis et al., 1995]. The conductivity of RVMS has been measured as a function of water content or temperature. The cluster formation of micelles increases the electrical conductivity after the percolation threshold which indicates the starting point of the reverse micellar droplet clustering. The percolation threshold can be varied by different additives. It has been also demonstrated that the solubilization of proteins favors the percolation process with an increase in the conductivity at lower or higher water content and temperature, suggesting stronger or weaker attractive interactions between micelles in the presence of proteins [Huruguen et al., 1991; Holovko et al., 1993].

In the previous work, we proposed the determination of the interaction bertween proteins and micelles by measuring the electrical conductivity of RVMS in their percolation processes [Kuboi et al., 1996]. The percolation phenomenon was also sensitively affected by the concentration and the kinds of proteins solubilized in the micelles. The specific interfacial parameter β, defined as the variation of percolation threshold with the concentration of solubilized protein, was considered to reflect the protein-micellar interactions.

The relation between β value and the back-extraction behav-

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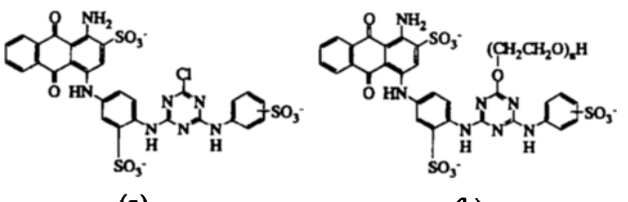
ior was also suggested. There were two types of proteins such as type A and B. Type A proteins such as BSA, have positive β values at the pH both above and below pI and for which back-extracted fractions (E_b) are comparatively higher. Type B proteins such as cytochrome c, however, in which the back-extraction process is relatively difficult, have low and negative values of β.

In this paper, to control the protein-micellar interaction, the β values of mixed RVMS, which contains lecithin as a co-surfactant, have been compared with those of AOT RVMS. In addition, to study the percolation process in detail, the solubilization of the various synthetic polymers into AOT/isooctane reverse micelles was carried out by the stepwise injection method and their percolation processes were studied by the measurements of the electrical conductivity focusing on the attractive interactions between micelles mediated by the solubilized polymers. The surface properties of polymers have been evaluated by Aqueous Two-Phase Systems (ATPS) and compared with their percolation results.

EXPERIMENTAL

1. Materials

AOT (sodium di[2-ethylhexyl] sulfosuccinate) of purity 95 % was purchased from Tokyo Kasei Co. and was used without further purification. Bovine serum albumin (BSA, pI=4.9) and cytochrome c (pI=10.1) from horse heart were purchased from Sigma Chemical Co.. Lecithin was prepared from egg phosphatidylcholine (PC; 93 %, HPLC), extracted from egg yolk and purified by Singleton's method [Singleton et al., 1965]. A buffer solution, acetic acid/acetate (pH 4-6), tris/HCl (pH 7-8) or glycine/NaOH (pH 10-11.7), was used and the concentration of each buffer salt was 10 mM. Polyethylene glycol (PEG) 1540, 4 K, 6 K, 20 K and 50 K (average molecular weight (MW): 1.5 k, 3 k, 7.5 k, 20 k and 50 kDa, respectively) were supplied by Wako Pure Chemical Industries, Ltd. (Osaka Japan) and PEG 8 K (MW : 8 kDa) was supplied by Union Carbide (New York, USA). Dextran (Dex) 60-90 K and 100-200 K (MW : 60-90 k and 100-200 kDa, respectively) were also obtained from Wako and Dex-T500 (MW : 500 kDa) was obtained from Pharmacia LKB (Uppsala, Sweden). Polypropylene glycol (PPG) 1000, 3000 (MW : 1 k and 3 kDa), polyvinyl alcohol (PVA) 500 and 1500 (MW : 22 k and 66 kDa) were purchased from Wako. Cibacron Blue F 3G-A (Cb), a kind of triazine dyes, was purchased from Fluka (Tokyo, Japan). Cibacron Blue F3G-A-PEG derivatives (Cb-PEG) were synthesized as described previously [Johansson et al., 1985]. The structural formulas of free Cb and Cb-PEG were shown in Fig. 1. The synthesized Cb-PEG derivatives were analyzed by FT/IR-230 (Jasco Co., Japan).



(a) (b)

Fig. 1. The structural formulas of used polymers (a) Cibacron Blue F3G-A (Cb) and (b) Cb-PEG.

The analytical methods employed in ATPS in respect of surface properties of polymers (surface net hydrophobicity (HFS) and net charge of Cb-PEG) were described elsewhere [Kuboi et al., 1990a; Johansson et al., 1994]. The determination method of molecular net charge is as follows.

PEG4 K (8 %)/Dex 100-200 K (8 %) was prepared to determine the charge of Cb-PEG by mixing 2 g of 40 % PEG4 K and 4 g of 20 % Dex 100-200 K with 2.5 ml of either 0.25 M Na_2SO_4 or 0.5 M $NaClO_4$. Water and sample were added to give total weight of 10 g. Two different salts were used so that ΔlogK can be determined. The systems were equilibrated by mixing and then centrifuged at 5,000 rpm for 10 min at 298 K. The free Cb and Cb-PEG were measured by the absorbance at 615 nm using a Hewlett Packard 8452A Diode Array Spectrophotometer. Amino acid (Glycine) was determined by using the reaction with fluorescamine. Fluorescence measurements were carried out with the excitation wavelength (Ex) at 390 nm and emission (Em) at 480 nm.

2. Methods

2-1. Back-extraction

The proteins were solubilized into 200 mM AOT/isooctane solution by the injection method [Shiomori et al., 1994]. The buffer solution of dissolved protein was injected into AOT/isooctane solution, and shaken vigorously until a clear solution was obtained. The value of W_o (=[H_2O]/[AOT]) of the reverse micellar solution was kept at 20 in all the back-extraction experiments. Back-extraction of the protein from the reverse micelles was carried out by contacting the reverse micellar solution containing proteins with new buffer solution containing 0.1 M KCl. Similar experiments were also carried out for AOT-lecithin mixed RVMS. The pH value in the feed solution injected into reverse micelles, pH_{inj}, was varied. The protein concentrations were determined by using a Hewlett Packard 8452A Diode Array Spectrophotometer.

2-2. Percolation Processes

The conductivity of RVMS was measured as a function of water content with a TOA Electronics Ltd. conductivity meter CM-40V and a platinum electrode. The electrode was inserted into the test tube containing the reverse micellar solution and the tube was placed in a thermostated water bath. Electrical conductivity measurements were performed with dropwise addition of an aqueous phase containing proteins to 200 mM AOT/isooctane or AOT-lecithin/isooctane solution, which was the same as in the back-extraction experiment, until the percolation phenomenon was reached. Similar experiments were also carried out for the synthetic polymers. The percolation threshold with and without proteins and polymers defined as the starting point of the sharp increase in conductivity is abbreviated as ϕ_p or $\phi_{p,p}$ according to the previous paper [Kuboi et al., 1996].

RESULTS AND DISCUSSION

1. Back-extraction of Proteins Solubilized into Reverse Micelles

After the protein was solubilized at a particular pH_{inj}, back-extraction was carried out with an aqueous phase of a given

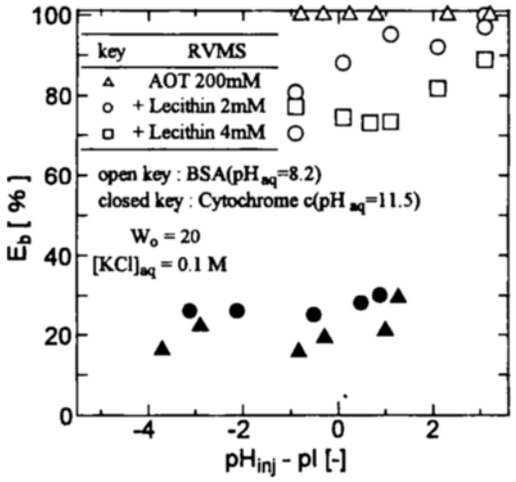


Fig. 2. Effects of pH_{inj} and RVMS on the back-extraction of proteins.

pH_{aq}. The results for BSA and cytochrome c with various pH_{inj} values are shown in Fig. 2. The fraction of the proteins back-extracted to the aqueous phase, E_b, is plotted against the pH deviation from pI (isoelectric point) of each protein. It shows that BSA is easy to be back-extracted in the AOT RVMS. In the mixed RVMS, however, E_b is comparatively lower than that of AOT RVMS. BSA is known to have locally hydrophobic binding sites. Hence, co-surfactants binding to BSA modify the surface properties of BSA and thus affect the back-extraction processes. On the other hand, cytochrome c shows a small increasing of E_b due to the addition of co-surfactant lecithin. Cytochrome c is known to have a localized plus charged site as a membrane protein. This site of cytochrome c might strongly interact to the anionic surfactant interface. Therefore, the electrostatic interaction between cytochrome c and AOT micellar interface is likely to decrease by the addition of co-surfactant.

2. Effect of Proteins Solubilized on the Percolation Processes

The conductivity of a micellar solution strongly depends on its aqueous volume fraction. The variation of the electrical conductivity of the AOT reverse micellar solution is plotted in Fig. 3 against the volume fraction of water in the organic phase, ϕ_{aq}. With increasing water content, the conductivities are at first insensitive to the water content. Then, the conductivities are seen to increase very sharply, about three orders of magnitude, when the water contents exceed respective threshold values indicating percolation phenomena. At this condition, though micellar size and protein concentration are not exactly the same as those in back-extraction, the percolation process is clearly reflecting the protein-micellar interactions. For example, an increase in the concentration of BSA shifted the percolation threshod (ϕ_p) to a higher value of ϕ_{aq}. In contrast, for the RVMS with cytochrome c, the percolation threshold has been shifted to a lower value of ϕ_{aq} than for the protein-free system. This behavior can be explained either by the presence of the strong dipole moment of cytochrome c inducing the interactions between droplets or by the hydrophobic character of the protein favoring the inter-connection between the microphases or by the charge head-group area of

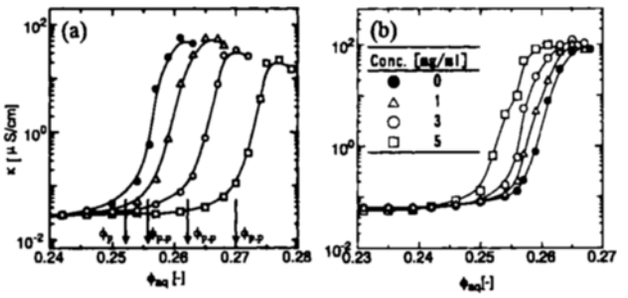


Fig. 3. Variation of the conductivity as a function of the water content in RVMS.
(a) BSA [pH 8.0], (b) Cytochrome c [pH 11.0].

the surfactant. Cytochrome c in reverse micelles has been studied by several authors [Huruguen et al., 1991; Larsson et al., 1993; Cassin et al., 1994]. There are also indications that cytochrome c interacts with the AOT surfactant layer. Therefore, the protein-micellar (electrostatic attractive) interactions seem to decrease the stability of RVMS by decreasing electrostatic repulsive interaction between micelles. The formation of micellar clusters shows a larger hydrophobic attraction than an electrostatic repulsive force between the micelles.

The percolation threshold depends on the concentration of the proteins solubilized in the micelles. The difference, $\Delta\phi_p$ ($=\phi_{p,p} - \phi_p$), shows the effect of the protein concentration on the percolation process. Here, $\phi_{p,p}$ and ϕ_p are the values of the percolation threshold with and without protein, respectively. $\Delta\phi_p$ is plotted against the concentration of BSA solubilized in the micelles under different RVMSs in Fig. 4. There is a linear correlation between $\Delta\phi_p$ and the concentration of protein (C_{pr}). The slope, β, is a measure of the effect of solubilized protein on the attractive interaction between micelles. A positive value of β means the stabilization of RVMS or the decreasing of inter-micellar interactions with the solubilization of protein. However, Fig. 4 shows that the value of the slope of the mixed RVMS are lower than that of AOT RVMS. It is shown that the stabilization effect of the RVMS decreases with solubilization of BSA in the presence of the co-sur-

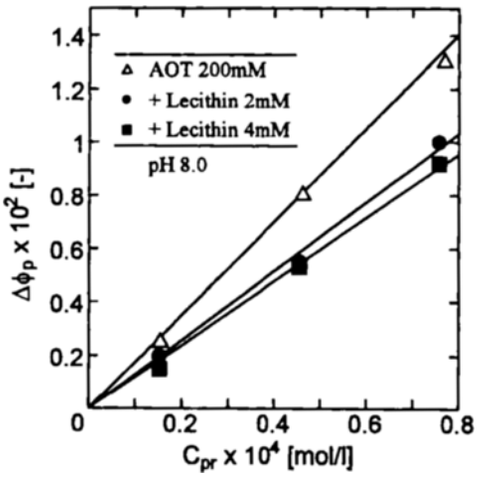


Fig. 4. Effect of BSA concentration on the percolation threshold in AOT and AOT-lecithin mixed RVMS.

factant lecithin. Hence, it can be found that the protein-micellar interactions affect either the stability of RVMS or micellar-micellar interactions and thus affect the back-extraction processes. Therefore, the back-extraction behaviors can be reasonably explained by the percolation phenomena reflecting the protein-micellar interactions.

3. Effect of Synthetic Polymers Solubilized on the Percolation Processes

Figures 5-7 show the variation of the electrical conductivity κ as a function of the volume fraction ϕ_{aq} of the aqueous phase for AOT/isooctane/water microemulsion containing synthetic polymers in the dispersed phase. These figures present several remarkable features: (I) All κ versus ϕ_{aq} variations present an electrical percolation phenomena. (II) For the RVMS without PEG, the volume fraction, ϕ_p, which corresponds to the threshold of electrical percolation, increases as the PEG molecular weight increases. This result indicates a decrease of the attractive interaction between micelles as the polymer molecular weight increases. Suarez et al. [1993] have found the same results and they explained it by the variation of the micellar size caused by solubilizing polymers. (III) But, the electrical percolation threshold is seem to increase with increasing PEG concentration as found for protein BSA. This phenomenon could not be explained only by the variation of micellar size determined in low W_o (W_o=20-30). It also means that the increasing of PEG concentration decreases the attractive interaction between micelles. (IV) The water solubility (corresponding to the percolation threshold ϕ value in the variation of κ versus ϕ_{aq} in Fig. 5-7, called $\phi_{p,p}$) increases as the PEG concentration or molecular weight increases, suggesting that the stability of RVMS increases with the solubilizing of PEG. On the other hand, PPG decreases the water solubility in the RVMS as the PPG concentration increases as seem for cytochrome c. PPG also decreased the water solubility as its molecular weight increased.

The increases of $\phi_{p,p}$, caused by the increases of polymer concentration may be concerned with a surface property of polymer solubilized in the RVMS. In case of the biopolymers, it is well known that the proteins solubilized in RVMS interact with the micellar interface through electrostatic, hydrophobic, steric and affinity interactions [Larsson et al., 1993; Kuboi et al., 1996]. The relationship between the relative percolation thresholds and solubilized polymers concentrations is also examined. $\Delta\phi_p$ is plotted against the concentration of PEG solubilized in the micelles in Fig. 8. There is a linear correlation between $\Delta\phi_p$ and the concentration of PEG and PPG. The value of β means the stabilization of RVMS with the solubilization of the polymer. A positive value of β means the stabilization of RVMS or the decrease of micellar-micellar interactions with the solubilization of polymer. We define these polymers as type A polymers with stabilization effect. On the other hand, a negative β means the destabilization of RVMS or the increase of inter-micellar interactions with polymer solubiliza-

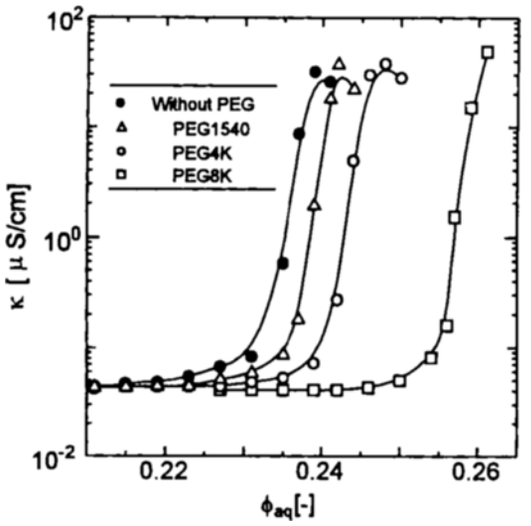


Fig. 6. Effects of the molecular weight of various polyethylene glycol (PEG) on the percolation processes of reverse micellar systems.

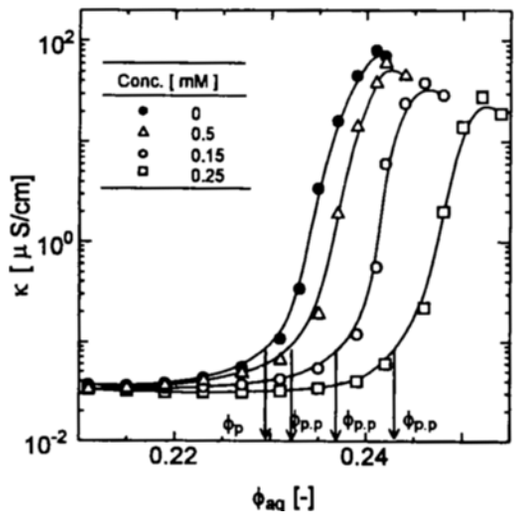


Fig. 5. Effects of polyethylene glycol 20 K (MW : 20 kDa) concentration on the percolation processes of reverse micellar systems.

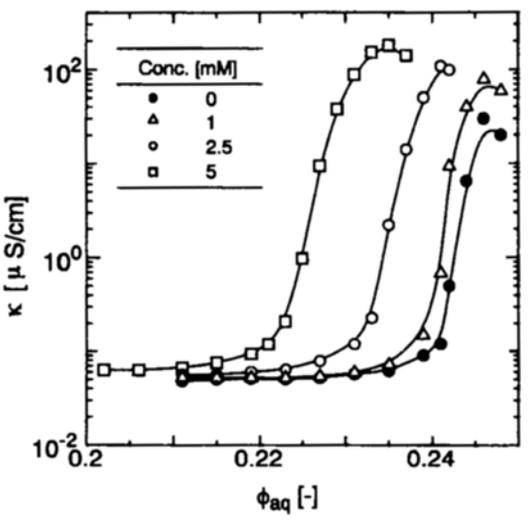


Fig. 7. Effects of polypropylene glycol (PPG 1000, MW : 1 kDa) concentration on the percolation processes of reverse micellar systems.

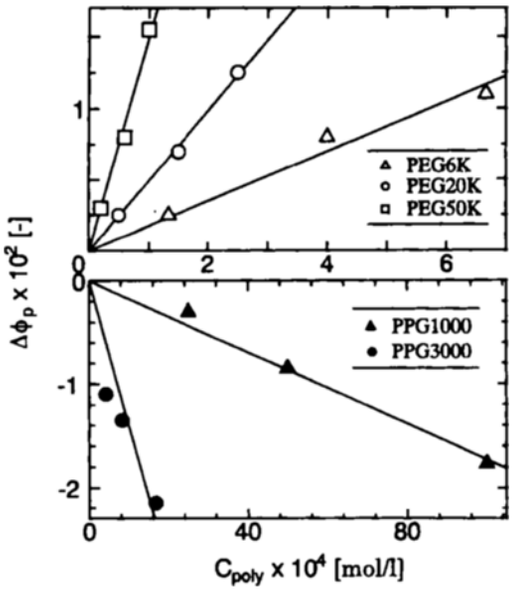


Fig. 8. Effect of polymer concentration on the percolation threshold.
(a) Polyethylene glycol (PEG), (b) Polypropylene glycol (PPG).

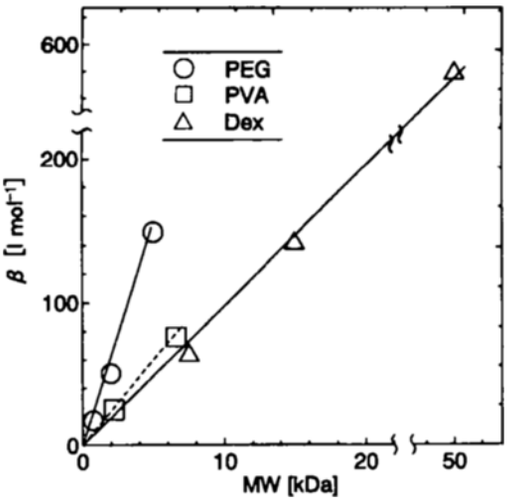


Fig. 9. Effect of molecular weight of stabilization polymers on the RVMS' stability, β.

tion. These polymers are defined as type B polymers with destabilization effect. In the case of proteins, there were also two type proteins (Type A and B) [Kuboi et al., 1996].

4. The Stability (β) of RVMS Solubilizing Various Non-Ionic Polymers

To compare with other polymers with stabilization effect, Dex and PVA were examined. As concentrations of Dex and PVA were increased, the percolation thresholds were shifted to higher values of ϕ_{aq}, and there was a linear correlation between $\Delta\phi_p$ and polymers concentration just as for PEG. The values of β are plotted against the molecular weights in Fig. 9. It shows that the values of β are increased linearly with increasing polymers molecular weight, but with differing slopes. All PEG, PVA and Dex can be used to form aqueous two-phase systems, i.e. aqueous-aqueous phase separation of two mutually incompatible components [Kuboi et al., 1990a, b]. Although each polymer has a different surface hydrophobicity, PEG is most hydrophobic and PVA is more hydrophobic than Dex. It is thought that the water structure mediated by hydration in the micellar water pools may account for the hydrophobic effect of polymer on the percolation processes. The structure and role of the water, however, in the micellar water pools have not been well understood yet, and thus should be studied further.

In the case of proteins, it has been shown that the values of β (at the isoelectric point of each protein) are proportional to the surface hydrophobicity (HFS) and molecular weight (MW) of the proteins [Kuboi et al., 1996]. Therefore, the result of Fig. 9 means that the stability of RVMS is affected by the hydrophobicity and molecular weight of the polymers solubilized into the reverse micelles.

5. Evaluation of Surface Properties of Polymers Using ATPS and Their Effect on Percolation Process

In contrast to non-ionic polymers, the values of β for proteins were affected by the pH of the protein solution injected into the reverse micelles, that was, by the electrostaic interactions between micelles and proteins [Kuboi et al., 1996]. To study the electrostatic effect on the percolation processes, the effect of the addition of Cibacron Blue F3G-A-PEG derivatives (Cb-PEG) was examined. The surface properties of Cb-PEG derivatives could be evaluated by using ATPS. The surface net hydrohobicity of polymer (HFS) was measured from partitioning studies of the polymer in ATPS composed of various molecular weights and concentrations of PEG and Dex [Tanaka et al., 1991; Kuboi et al., 1990(a)]. The net charge determination of polymer could be also made by using ATPS [Johansson et al., 1994; Albertsson et al., 1986]. The partitioning of a charged molecule or particle between the phases of ATPS depends on the salt included in the system. Partitioning such charged species in the presence of different salts permits the charge of small and large ions, including proteins, to be estimated.

The relation between the partition coefficient, K, of a molecule with a net charge Z and the specific salt used is given by Eq. (1):

$$\log K = \log K_o + (FZ/RT)\,\psi \quad (1)$$

where ψ is the electrochemical potential, Z is the net charge of the molecules, F is the Faraday constant, R is the gas constant and T is the temperature. The K_o value is essentially independent of salt i.e. the contribution to the molecule partitioning depending on the several effects except the electrostatic effect. If $(F\psi/RT)$ is substituted with γ, Eq. (1) can be expressed simply by

$$\log K = \log K_o + \gamma Z \quad (2)$$

where the constant γ depends on the salt included in the system. If the sample ion has the partition coefficient K_1 or K_2 for the salt 1 or salt 2, respectively, the difference $\Delta \log K = \log K_1 - \log K_2$ will be proportional to the net charge, Z, of the ion. This dependence can be expressed as follows:

Table 1. The surface properties of Cb-PEG derivatives.

	MW [kDa]	Δ logK [-]	Z [-]	HFS [kJmol^{-1}]	β [*l*mol^{-1}]
Cibacron blue	0.8	−0.435	−16.2	71.0	14.7
Cb-PEG 1540	2.8	−0.577	−21.5	106.1	67.8
Cb-PEG 4 K	3.8	−0.343	−12.8	122.3	60.7
Cb-PEG 8 K	8.8	−0.146	−5.4	105.7	21.9
Cb-PEG 20 K	20.8	−0.256	−9.5	64.7	226.0

$$\Delta \log K=(\gamma_1-\gamma_2)Z=\Delta\gamma Z \quad (3)$$

The $\Delta\gamma$ value can be obtained by the partitioning of an ion with the known charge. In this study, amino acid (Glycine) was used.

Z, β, HFS and MW of Cb-PEG derivatives are shown in Table 1. Cb-PEG derivatives have various surface charges (Z) depending on their average molecular weights. The β values of Cb-PEG derivatives were compared with their net surface charges. The effects of molecular weight and net surface charge on β values are shown in Fig. 10. The values of β are proportional to the molecular weights (MW) and net surface charges (Z) of the polymers, and expressed as;

$$\beta \propto (MW)f(-Z)$$

Therefore, the result of Fig. 10 means that the stability of RVMS is affected by the molecular weight and the negative value of net surface charge of the type A polymers solubilized into the AOT reverse micelles.

For the type B polymers, further study on the effects of the other surface properties of polymers such as local hydrophobicity and structural effect should be made in future.

CONCLUSION

Specific interfacial properties, which affect protein extraction in the AOT and AOT-lecithin RVMS, have been studied by using their percolation processes. The percolation process is a sensitive and convenient measure of micro-interface of RVMS solubilizing various proteins or sythetic polymers, which clearly reflects the polymer-micellar and micellar-micellar interactions. The percolation of RVMS is highly dependent on the kinds (surface properties), their molecular weights and concentrations of solubilized polymers. The values of β, defined as the variation of percolation threshold with the concentration of polymers, reflect the stability of RVMS or micellar-micellar interaction and depend on the surface properties of polymers such as surface net hydrophobicity and charge which can be evaluated by using ATPS.

The percolation processes with polymers and proteins can be utilized to evaluate and understand the protein-micellar interactions and to explain the back-extraction results for proteins. The relation between β and E_b is also applicable to the mixed RVMS containing lecithin as a co-surfactant.

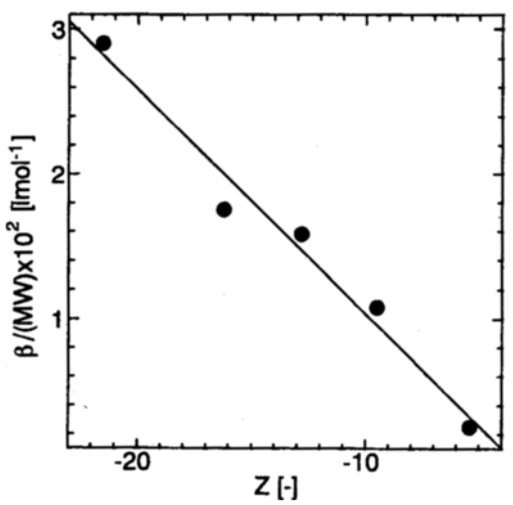


Fig. 10. Effects of net charge (Z) and molecular weight (MW) of anionic-polymers solubilized reverse micelles on the RVMS' stability, β.

NOMENCLATURE

[AOT] : concentration of AOT [M]
C_{pr} : concentration of protein [M]
C_{poly} : concentration of synthetic polymer [M]
E_b : fraction of back-extraction
100 $[Protein]_{aq}/[Protein]_{org}$ [%]
F : Faraday constant [C/mol]
HFS : hydrophobic factor of solutes [kJ/mol]
K : partition coefficient in aqueous two phase system [-]
MW : average molecular weight [-]
pH_{inj} : pH of the protein solution injected into reverse micelles [-]
pH_{aq} : pH or aqueous phase used for back-extraction [-]
pI : isoelectric point of protein [-]
[Protein] : concentration of protein [M]
R : gas constant [Jmol^{-1} K^{-1}]
T : temperature [K]
W_o : water content, molar ratio of H_2O to AOT
$[H_2O]/[AOT]$ [-]
Z : net charge [-]
β : stability parameter of reverse micellar systems [*l*mol^{-1}]
κ : electrical conductivity [μS/cm]
ϕ_p : percolation threshold without polymer [-]
$\phi_{p,p}$: percolation threshold with polymer [-]
$\Delta\phi_p$: $\phi_{p,p}-\phi_p$ [-]
ψ : electrochemical potential [mV]

Subscripts
inj : injected solution
aq : aqueous solution used for back-extraction
org : orgainc phase
pr : protein
poly : synthetic polymer

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