

Review

# Role of alcohols in the formation of inverse microemulsions and back extraction of proteins/enzymes in a reverse micellar system

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## Abstract

In this review, we attempt to describe the structure and formation of inverse microemulsion with special emphasis on the role of alcohols in its formation. The review then focuses on the role of alcohols in the back extraction of various proteins/enzymes from the reverse micellar phase. It gives a deep insight into the most commonly used alcohols in back extraction and the factors governing its selection and the effect it has on the protein–micelle and the micelle–micelle interactions.

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*Keywords:* Reverse micelles; Proteins/enzymes; Alcohols; Back extraction; Microemulsions

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## 1. Introduction

Protein bioseparation, which refers to the recovery and purification of protein products from various biological feed streams, is considered to be one of the most important unit operations in the bioprocess industry. This is mainly because of the phenomenal development in the field of modern biotechnology and the increasing demand for more and more protein products to be purified in larger quantities. A further boost to protein bioseparation is likely to come from the developing science of proteomics.

The purification of proteins from the product streams of bioreactors and other biological feed streams is widely recognized to be technically and economically challenging. Protein bioseparation quite often becomes the limiting factor in the successful developments of protein-based products. The isolation and purification cost can be a substantial fraction of the total cost of production for most products of biological origin. Since the bioseparation cost is the major one there is thus an incentive for developing cost-effective isolation and purification processes. A myriad of protein bioseparation techniques is available. It can be classified into three broad categories:

1. high-productivity, low-resolution (cell disruption, liquid–liquid extraction, ultrafiltration, etc.);
2. high-resolution, low-productivity (ultracentrifugation, affinity separation, electrophoresis, etc.);
3. high-resolution, high-productivity (fluidized bed chromatography, membrane chromatography, monolith column chromatography).

Most conventional protein separation processes rely on a scheme, which is described as Removal, Isolation, Purification, Polishing (RIPP) [1]. Biological feed streams are generally dilute with respect to the target proteins, which need to be separated from a large number of impurities. Such a feed stream would easily overwhelm a high-resolution separation device. Hence, low-resolution high-productivity techniques are first used to reduce the volume and the overall level of process stream. This is followed by high-resolution low-productivity techniques to obtain pure target proteins. However, with the advent of high-resolution high-productivity techniques it is often possible to shorten, if not totally replace the RIPP scheme.

During the production of recombinant protein, the protein may be accumulated either in the cytoplasm, the periplasm, or the extracellular medium. The intracellular protein can also form aggregates with highly dense structures called inclusion bodies. The choice of the purification scheme will depend on these factors and on the degree of purity of the product, which depends on the further utilization of the protein. As a first step, after

cell lysis, if the protein is not secreted, the cellular material is separated from the liquid medium, using a centrifugation or a filtration step, followed by chromatographic steps. It has been demonstrated that it is possible to use liquid–liquid extraction technology to integrate the first two steps into one to obtain separation of the product from the cellular material with simultaneous concentration of the protein.

Reverse micellar extraction (RME) is an attractive liquid–liquid extraction (LLE) method for downstream processing of biological products, as many biochemicals including amino acids, proteins, enzymes and nucleic acids can be solubilized within and recovered from such solutions without loss of native function or activity. These systems also offer low interfacial tension, ease of scale-up and continuous operation. RME offers a number of unique, desirable features in contrast to aqueous two-phase extraction (ATPE) [2], which has been widely studied:

1. Partitioning behavior of biomolecules in RME can be regulated by varying the size and shape of reverse micelles. This can be easily accomplished by the self-assembling and labile nature of reverse micelles as opposed to the unchanging identity of the polymers (which is fixed upon synthesis) used in ATPE.
2. Partitioning selectivity of proteins can be achieved in RME based on the hydrophobic nature of proteins due to the fact that reverse micelles provide the hydrophobic and hydrophilic environments to solutes simultaneously.
3. Recovery of biomolecules from the reverse micellar phase can be easily facilitated by exploiting the de-assembling nature of reverse micelles in aqueous media. Moreover, the surfactants can be separated from the biomolecules by filtration [3] and can be recycled.

In contrast, efficient methods for the recovery and recycling of polymers in ATPE have not yet been developed. This problem has been solved to some extent using thermo-separating polymers; a suitable way for the recovery of polyethylene glycol (an extensively used polymer in ATPE) is not yet developed [4].

A successful RME process should include both forward and back extraction steps in their optimized conditions. In contrast to the extensive studies examining forward extraction process, back extraction has been addressed to a much lesser extent. Most of the previous studies tacitly assume that conditions, which normally prevent protein uptake in the forward transfer, would promote their release in the back transfer. That is to select a pH and salt condition that has minimal forward transfer efficiency. This assumption however, is not true [5] and results in only a low protein recovery [6]. As reviewed by Kelley et al. [7],

the overall recovery in RME is generally below 80%. Rahaman et al. [8] reported only 10–20% of alkaline protease recovery from AOT/isooctane reverse micelles, due to kinetic limitations. The enzymes are in very different environments during the LLE extraction process and thus, their activities and conformation may be affected by various factors such as ionic strength and pH of the aqueous phase and the type of solvent and co-solvent of the reverse micellar solutions [9]. In this review, we first look at the basics of microemulsions and the formation of reverse micelles followed by the extraction of proteins using reverse micelles. We then concentrate on the role of alcohols as co-solvent/co-surfactant in the formation of reverse micelles and the role of alcohols in the back extraction of protein and its effect on enzyme activity and conformation.

## 2. Extraction and back extraction processes

A RME cycle is basically composed of two processes: forward and backward extraction. In the forward extraction step, biomolecules are transferred from the initial aqueous phase to the reverse micelles. In the back extraction step, biomolecules are transferred from the reverse micelles back to a fresh aqueous strip solution. A schematic representation of protein solubilization in the reverse micelles from aqueous phase is shown in Fig. 1. In a number of recent publications, extraction of proteins (both forward and backward) has been demonstrated using various reverse micellar systems [10–18]. These studies revealed that the extraction process is often controlled by various factors such as concentration and type of the surfactant, pH and ionic strength of the aqueous phase, concentration and type of the co-surfactants, salts, charge of the protein, temperature, water content, size and shape of reverse micelle, etc. By manipulating these parameters selective separation of the desired protein from mixtures can be achieved. During the back extraction, the enzyme-loaded reverse micellar solution from the forward extraction is mixed with a fresh strip aqueous phase. The mixture is then centrifuged, and the two phases separated. Back extraction of proteins is affected by pH and salt concentration in the feed solution and in the aqueous strip phase. High ionic strength is desirable in the new aqueous phase where back extraction is performed.

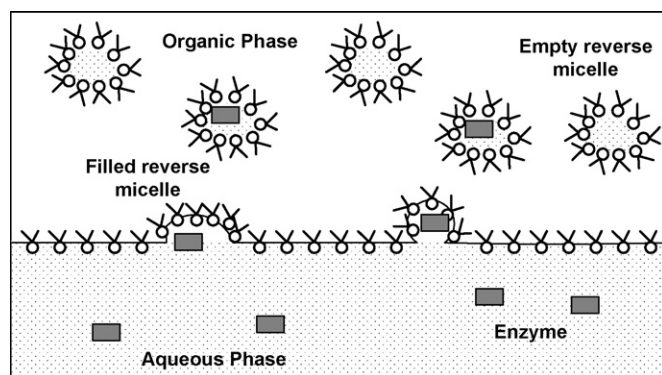


Fig. 1. Schematic representation of mechanism of protein solubilization into reverse micellar phase from aqueous phase [2].

### 2.1. Factors affecting back extraction

The distribution of protein/enzyme between an organic micellar phase and an aqueous phase is largely determined by the conditions of aqueous phase, namely, pH, ionic strength and type of salt. The parameters related to the organic phase affect the partition of proteins such as the concentration and type of surfactant, type of solvent, etc. Changes in temperature can also affect the solubilization of biomolecules. Phase transfer depends on the characteristics of proteins, namely, the isoelectric point ( $pI$ ), size and shape, hydrophobicity and charge distribution. All these factors have been explained in detail in literature review [2,19,20].

An important factor relevant to this review is the co-surfactant (co-solvent). It is a type of solvent that helps surfactants to dissolve in the organic solvent and form reverse micelles thereafter [21]. Cationic surfactants form very small micelles ( $W_0 < 3$ ); thus, a co-surfactant is added to the system to make them grow [22]. Anionic surfactants form large micelles ( $W_0 = 20–115$ ), so the addition of co-surfactant is not necessary [23,24]. Although the exact mechanism of the co-solvent remains unclear, it was proposed that co-solvent molecules might be inserted between the molecules of the surfactant [6], thereby resulting in two important effects. Firstly, the interaction between hydrophiles of the surfactant is changed. Secondly, the arrangement of surfactant molecules in the solvent is loosened; it will then be possible to overcome the steric difficulty and arrange the big surfactant molecules in a loose manner. The result of these two effects may lead to the collapse of the cohesive force of surfactant, followed by dissolution of surfactant in solvent [11]. According to Krei et al. [21] co-solvent acts by increasing the solubility of the surfactant in the organic phase and, in the case of *n*-hexanol, by stabilizing the microemulsions.

Reversed micelles formed by cationic surfactants are smaller than those formed by anionic surfactants. To enlarge the micelle size when a cationic surfactant is used, a co-surfactant, usually an alcohol, is added to the organic phase [11,22,25]. On the contrary, Krei et al. [21] have found that, in the extraction of  $\alpha$ -amylase, there is an accentuated decrease in micelle size with increasing *n*-hexanol content, especially those in the range of 5–10%. Hexanol probably reduces electrostatic repulsion between the charged surfactant head groups and gives rise to weak hydrophobic interactions between the hydrophobic tails of surfactant molecules; both effects lead to a denser packing of surfactant molecules in the reverse micelle and consequently to a reduction in  $W_0$ . Generally, not-so-short alcohols such as *n*-butanol, benzyl alcohol, *n*-pentanol, *n*-hexanol, *n*-heptanol, *n*-octanol and *n*-decanol are used as co-surfactants [10,11,19,25]. Different co-surfactants have different properties that affect the microstructures of reverse micelles. Chang and Chen [10] and Chang et al. [11] used several alcohols (*n*-butanol, *n*-pentanol, *n*-hexanol, *n*-heptanol, *n*-octanol and *n*-decanol) as co-surfactants in Aliquat 336 reverse micelles to extract  $\alpha$ -amylase and obtained the highest recovery of enzymatic activity with *n*-butanol. In their study, only low-solubility alcohols were utilized.

## 2.2. Back extraction process

Transfer of solubilized proteins from the reverse micellar phase back to an aqueous phase constitutes back extraction. The next step is the recovery and concentration of protein in a new aqueous phase. In most cases the proteins can be recovered either as a result of an electrostatic repulsion of the surfactant, choosing the convenient pH, or through a size exclusion effect by increasing salt concentration (1–2 M) in the fresh aqueous phase [26]. However, this is far from being a clear-cut situation. In several cases, the yield of back extraction decreases with factors that decrease the droplet radius of the reverse micelles, such as an increase in salt concentration or a decrease of temperature. This behavior is rationalized as the result of an increase in the protein–surfactant interaction as the distance to interfacial layer decreases, as does the water content  $W_0$  [27]. The transfer of proteins into the organic phase seems also to be in some cases an irreversible process, in the sense that the protein cannot be recovered under the conditions that are unfavorable to extraction, i.e., using a pH such that the protein bears the same charge as the surfactants and high salt concentrations. This is probably the result of a strong hydrophobic interaction of the protein with the surfactant. In these situations, the use of a polar alcohol to disrupt this interaction has been proposed [28].

Back extraction of proteins out of AOT reverse micelles is a slow, interfacial process and is governed by the coalescence of reversed micelles with the macroscopic interface [29,30]. It has been established that this mechanism can be extended to hydrophilic substances such as amino acids and salts as well [31]. Back extraction is supposed to contain several steps. The first step called the “attachment step” comprehends the successful approaching of the reversed micelle to the phase boundary. Minimum requirements of this step are: (i) free space at the phase boundary and (ii) rupture of the organic solvent (isooctane) film between the reverse micelle and interface. Also, attachment step is considered to be fast and hence not rate determining. After the attachment step, there are two ways for the reversed micelle to lose its content to the aqueous bulk phase, permeation or coalescence. The fast bilayer permeation mechanism is exclusively suitable for the exchange of water. Its driving force is the difference in osmotic pressures of different aqueous environments (micelle and bulk phase). The assumption of the permeation mechanism, i.e., diffusion of water through monolayers as well as across lipid bilayers [32], is generally regarded to be reasonable in a variety of closely related water transfer processes.

The second mechanistic way for back extraction process to occur, and the only one possible for solutes is the coalescence. The reverse micellar AOT shell will have to merge with the AOT monolayer of the macroscopic interface. This surfactant layer fusion would therefore be the first step of the coalescence process. Properties of the reverse micelles (size, internal interactions, etc.) and the macroscopic interface should influence this step. The last step of coalescence process must meet one requirement: there has to be a minimum exchange time between the bulk aqueous phase and the content of opened micelle at the phase boundary. This can be realized by either a local deformation of the interface, leading to the complete loss of the identity

of reverse micelles, or simply a sufficiently slow reformation of the micelle merged before. Then the micelle would more or less keep its old surfactant layer and change only the content. This third step is assumed to be dependent on the solute. Thus, the merging of the two surfactant layers is rate determining for back extraction of hydrophilic solutes. The properties of macroscopic interface are expected to have an important impact on the back extraction rate. The effect of varying bulk aqueous ionic strength is also seen in this light. On the other hand, properties of the reverse micelles, which affect their kinetic stability (mainly the ionic strength), should then play a role for the coalescence kinetics too. The nature of the solute, however, would be of minor importance for the merging process.

Some alternative approaches for enhanced recovery of proteins from the reverse micellar phase have been recently presented. They include: (a) use of silica particles for the sorption of the proteins as well as surfactants and water directly from protein-filled reverse micelles [33]; (b) addition of dewatering agents such as isopropyl alcohol [5] and dehydration of reverse micelles with molecular sieves to recover protein [34]; (c) formation of clathrate hydrates via pressurization [35]; (d) use of NaDEHP/isooctane/brine reverse micelles which can easily be destabilized by adding divalent cations (such as  $\text{Ca}^{2+}$ ) and subsequent release of protein into the aqueous media [36]; (e) addition of sucrose to enhance protein recovery by reducing the protein–surfactant interactions [37]; (f) back extraction with the aid of a counter-ionic surfactant [38–40] and through gas hydrates formation [41].

## 3. Role of alcohols in microemulsions

In general, alcohols play an important role in both the formation of reverse micelles and the back extraction of proteins.

### 3.1. Alcohols in the formation of inverse microemulsions

Perez-Casas et al. [42] envisaged a reasonable molecular picture of alcohol–AOT interaction in the presence and absence of reverse micelles from dynamic light scattering (DLS) and heat capacity studies. In the absence of reverse micelles, all alcohols form complexes with the free AOT molecules in solution, a process that competes with the alcohol self-association. The alcohol–AOT complex is most probably formed via an interaction between the hydroxyl group of the alcohol and the ionic head of AOT. When reverse micelles are present, two behaviors are found: (i) methanol and ethanol are located in the micellar water pools; at low AOT concentration these alcohols only interact with water, but at high AOT concentration they also form a complex with AOT molecules at the micellar interface, and (ii) for *n*-butanol, longer normal alcohols and branched chain alcohols, two different processes occur. In the first case, AOT molecules are withdrawn from the micelles to be complexed with alcohol and secondly the molecules in the bulk of the solution and alcohol molecules penetrate the micelle shell, where they also form a complex with AOT.

Microemulsions are mixtures of water, oil and surfactant, and their phase behavior has been under study for several decades.

From Shinoda's work [43] it is evident that a microemulsion is made without alcohols using only three components: water, a hydrocarbon and a single non-ionic surfactant. This proves that alcohols are not a prerequisite for the formation of microemulsions. Another view is that the reverse micelles formed with anionic surfactants, such as AOT, generally solubilize large quantities of water in the organic phase without the addition of other organic compounds [44–46]. On the other hand, cationic surfactants usually require a co-surfactant, such as an alcohol, in order to form reverse micelles [6,47–54]. Cationic reverse micelles solubilize less water than most anionic reverse micelles.

During the past years various researchers have tried to find the exact role of alcohols in microemulsions. Kahlweit et al. [55] described alcohols to act as a weak amphiphile when added to a binary water–oil mixture. Thus, the alcohol was treated as “co-solvents” that partition between the aqueous domain and the amphiphilic film. Strey and Jonstromer [56] have studied the role of medium-chain alcohols on the phase behavior of microemulsions in ternary systems; they concluded that the longer the alcohol chain length, the larger the bending elastic module of the mixed film. From spin label experiments on flexible films, Dimeglio et al. [57] concluded that short-chain alcohols reduce the rigidity of the interfacial film. Penders and Strey [58] studied the influence of *n*-octanol (denoted as  $C_8E_0$ ) on the phase behavior of ternary microemulsion system  $H_2O$ –*n*-octane– $C_8E_5$ . They expected alcohols to bring about two predominant effects: one is to change the effective hydrophilicity of the amphiphilic mixture  $C_8E_5 + C_8E_0$  and the other is the increase in efficiency (or solubilization capacity) of the amphiphilic mixture, while at the same time distorting the three-phase region of the microemulsion system. An increased efficiency can quite generally be related to a decrease in interfacial tension between the bulk water and oil-rich phases [59]. Evidently,  $C_8E_0$  plays a double role in the quaternary system  $H_2O$ –*n*-octane– $C_8E_5$ – $C_8E_0$  as “co-surfactant” and as “co-solvent”. In the role of co-surfactant,  $C_8E_0$  preferentially dissolves in the amphiphilic film, making the co-surfactant mixture ( $C_8E_5 + C_8E_0$ ) more hydrophobic. In the role of the alcohol as “co-solvent”, it exhibits itself in a change of the relative proportions of  $C_8E_5$  and  $C_8E_0$  in the amphiphilic film due to the higher monomeric solubility of *n*-octanol in *n*-octane. Thus, they concluded that the addition of  $C_8E_0$  to the ternary microemulsion  $H_2O$ –*n*-octane– $C_8E_5$  causes the three-phase region to be shifted to lower temperatures and to become distorted. The distortion of the three-phase region is explained by the fact that the solubility of  $C_8E_0$  in *n*-octane is larger than that of the  $C_8E_5$  monomers. Also on increasing the  $C_8E_0$  content the efficiency of the amphiphile mixture ( $C_8E_5 + C_8E_0$ ) is increased causing an enhancement of solubilization capacity, which is seen from a decrease in the weight fraction of amphiphile and the minimum interfacial tension. The solubilization of water and *n*-octane is larger for the surfactant mixture  $C_8E_5/C_8E_0$ , meaning that there is a strong synergistic effect of the  $C_8E_5/C_8E_0$  couple. Taking the monomeric solubilities of the amphiphiles into account, the surfactant mixture behaves like a single surfactant of longer chain length.

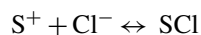
Wang et al. [60] have studied the formation of reverse micelles in isooctane with a two-tailed cationic surfactant, dioctyldime-

thylammonium chloride (DODMAC), which can be dissolved in isooctane only in the presence of an alcohol (*n*-decanol). At an alcohol concentration below 100 mM, they found that a Winsor I type system is formed where water uptake in the organic phase is near zero. With an increase in *n*-decanol concentration to 150 mM a Winsor III type system with three equilibrium phases exist. Here the water uptake in the organic phase is too low because most of the surfactant is in the middle phase. Between 150 and 175 mM of *n*-decanol, the intermediate phase disappears and most of the surfactant goes to the organic phase and the water uptake increases dramatically, an indication that reverse micelles form and the system is of Winsor II type. A further increase in *n*-decanol concentrations above 175 mM decreases the water uptake in the organic phase, while the DODMAC concentration in the aqueous phase remains constant.

To explain the decrease of water uptake with an increase in alcohol concentration above 175 mM, three phenomena are considered: (i) alcohol participates in the interfacial region of reverse micelles to screen the repulsive forces between the positively charged head groups of DODMAC. With increasing alcohol concentration, surfactant molecules pack more closely, thus reducing the water uptake and the size of reverse micelles. This result is consistent with a geometric model of a reverse micelle in which a surfactant molecule in the interfacial region is divided into a head group having an area  $a_0$  in the interface and a hydrocarbon tail having a length  $l_c$  and a volume  $v$  [53,61]. A necessary geometric condition for the formation of reverse micelles is

$$\frac{v}{a_0 l_c} > 1$$

Most cationic surfactants cannot satisfy this condition when they are alone in the organic phase. The addition of an alcohol which precipitates in the interfacial region of reverse micelles has an effect of increasing the volume of surfactant tail  $v$  and reducing the effective surface area  $a_0$ , thus facilitating the formation of reverse micelles; (ii) because DODMAC is a quaternary ammonium salt, there is an equilibrium between the ionic ( $S^+$ ) and non-ionic ( $SCI$ ) forms of the surfactant.



The ionic form of DODMAC resides in the interfacial region of reverse micelles, while the undissociated form is present in the bulk organic phase as well as in the interfacial region. The polarity of the organic phase increases with alcohol concentration, thus the organic phase becomes a better solvent for the undissociated form of the surfactant, and less surfactant is available to form reverse micelle; (iii) there is also a synergistic effect, which tends to further reduce the water uptake. The decrease of water uptake in the organic phase, which occurs due to the phenomenon (i) above, results in a decrease of dielectric constant inside the water pools [62,63]. This decrease of dielectric constant shifts the equilibrium of the surfactant from the ionic form to the undissociated form [64]. As in (ii) above, the undissociated form of the surfactant will migrate to the bulk organic phase, thus further reducing the water uptake. In this case the

importance of ionization equilibrium is stressed with emphasis given on the role of alcohols as a co-solvent.

### 3.2. Alcohols as “co-solvents”

Kahlweit et al. [55] have deduced that there is a characteristic difference between the adsorption of alcohols at the water/air and water/oil interfaces. It was shown that in aqueous solutions, the interfacial tension of water/air interface drops almost as steeply upon addition of an alcohol ( $C_iE_0$ ) as upon addition of a non-ionic amphiphile ( $C_iE_j$ ) with the same carbon number  $i$ . This implies that at water/air interfaces, alcohols adsorb almost as strongly as non-ionic amphiphiles, the driving force being essentially determined by the number  $i$ . In water/oil mixtures, however, the interfacial tension of water/oil interface decreases much slower upon addition of an alcohol than upon addition of an amphiphile, which implies that at water/oil interfaces, alcohols adsorb much more weakly than amphiphiles. This difference in adsorption can be qualitatively explained as being a result of the inverse role of the interactions between hydrophobic tails and hydrophilic heads, respectively, and the two solvents. In water-rich phase, the driving force for adsorption (as well as micellization) is basically determined by the repulsive hydrophobic interaction between the hydrocarbon tails and water [65], whereas the attractive hydrophilic interaction between the heads and water determines the solubility of the micelles.

In oil-rich phase, on the other hand, the interactions change their roles. Repulsive interactions between the head groups and non-polar solvent determine the driving force for adsorption. Although one expects the OH groups of the alcohols to be weakly hydrated, the attractive interaction between their tails and oil appears to suffice for keeping (medium chain) alcohols in the solution. With amphiphiles, on the other hand, the strong hydration of their head groups leads to a stronger repulsive interaction with oil, which reflects itself in a strong adsorption at water/oil interfaces, and the formation of (inverse) micelles. From these considerations it follows that at water/oil interfaces in the absence of amphiphiles, alcohols can only be considered as rather weakly surface-active substances. In the presence of an amphiphilic monolayer, however, one expects alcohols to dissolve in that layer due to their tendency to seek an environment with an intermediate polarity between that of oil and water which, in turn, will also affect the interaction energies between the amphiphile molecules in the monolayer and the adjacent bulk phases, and thereby the interfacial tension  $\sigma_{ab}$  between the two. This again suggests medium-chain alcohols be considered as co-solvents that distribute between the two bulk phases and interfacial layer, the distribution depending, evidently, on the carbon number of the alcohol, that of the oil, and the properties of the amphiphile.

### 3.3. Effect of alcohol chain length

It was shown that alcohols with longer hydrocarbon chains permit the formation of reverse micelles at lower concentrations than alcohols with shorter chains. This result can be explained

by the ratio of cohesive energies [66]. The quantity  $R$  is the ratio of the net interaction energy of surfactant with oil to the net interaction energy of surfactant with water, both in the presence of alcohol [67].

$$R = \frac{a_o^s - (a_o^s - a_o^a)X_a}{a_w^s - (a_w^s - a_w^a)X_a}$$

where  $X_a$  is the mole fraction of alcohol in the interfacial region of reverse micelles. The interaction energies of alcohol and surfactant with oil are  $a_o^a$  and  $a_o^s$ , respectively. Similarly,  $a_w^a$  and  $a_w^s$  are the interaction energies of alcohol and surfactant with water, respectively. The  $R$  ratio measures the tendency for the surfactant to disperse in oil with respect to its tendency to disperse in water. For  $R < 1$ , the surfactant disperses in aqueous phase and micelles are formed; for  $R > 1$ , the surfactant disperses in oil phase and reverse micelles are formed [67]. With a given surfactant,  $a_o^s$  and  $a_w^s$  are fixed; for example, for DODMAC,  $a_o^s > a_w^s$  because the number of carbon atoms of surfactant is larger than those of the  $C_5$ – $C_{10}$  alcohols and,  $a_w^s > a_w^a$  because the electrostatic interaction is much stronger than molecular interaction. To compare the effect of various alcohols on  $R$  used,  $a_w^a$  can be taken as a constant because all alcohols used have a single OH group in contact with water and their tails are in the organic phase. On the other hand,  $a_o^a$  will increase with the number of carbon atoms of the alcohol. Thus, the smaller amount of the alcohol is necessary to satisfy the  $R > 1$  condition if the hydrocarbon chain of an  $n$ -alcohol were longer.

As discussed by Bourrel and Schechter [67], the numerator of the right-hand side of above equation increases with increasing the length of alcohol chain, passes through a maximum and then decreases. For alcohols with hydrocarbon chains longer than  $C_{10}$ , the concentration of alcohol required to form reverse micelles eventually start increasing with an increase in the chain length. It is also shown that for a fixed alcohol concentration, the longer the hydrocarbon chain of alcohol, the smaller the water uptake of reverse micelles. This result is similar to the findings of Lang et al. [47]. The effect of increasing alcohol chain length on the water uptake for cationic reverse micelles is similar to that of increasing surfactant chain length [47,48]. It is seen that just above the critical alcohol concentration for reverse micelle formation the water uptake is higher for longer hydrocarbon-chain alcohols. This can be explained considering that for longer chain alcohols the critical alcohol concentration is lower, thus the screening effect between the head-groups of surfactant molecules is less and reverse micelles tend to be larger. Because the organic phase is less polar, most surfactants in the organic phase will participate in the formation of reverse micelles. In case of branched alcohols the minimum concentration of alcohol needed for the formation of reverse micelles increases in the following order: 1-heptanol < 4-heptanol < 2,4,3-pentanol. Higher concentrations of the more branched alcohols are required to shift the surfactant to the organic phase. Since branching decreases the  $a_o^a$  value, a larger amount of a branched alcohol is needed to satisfy the condition for reverse micelle formation  $R > 1$ .

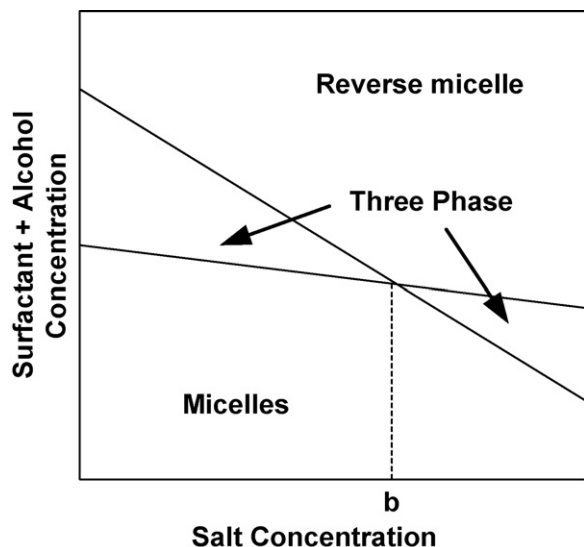


Fig. 2. Phase diagram for surfactant/alcohol/organic solvent/salt/water system [60].

### 3.4. Effect of salt concentration

Another important observation is that higher salt concentrations shift the maximum water uptake to lower concentrations of alcohol. This behavior, which is common in surfactant, alcohol, salt and solvent systems [67], is shown in the phase diagram (Fig. 2). It is seen that, at a fixed surfactant concentration, a higher salt concentration and a lower alcohol concentration are needed to begin reverse micelle formation. At a salt concentration below a certain level  $b$ , the alcohol concentration necessary for the formation of reverse micelles decreases significantly with an increase in salt concentration.

### 3.5. Alcohols as lipophilic linkers

When alcohols affect the physicochemical formulations at the interface, it may be seen as a co-surfactant. Pentanol and hexanol appear to combine with the surfactant to produce additional lipophilicity at interface. In some way a  $C_5$  or  $C_6$  alcohol is necessary to equilibrate the interfacial interaction balance of a very hydrophilic surfactant such as sodium dodecyl sulfate [68]. The second effect of the alcohol is to compete with surfactant for interfacial adsorption. Alcohol molecules enter the interfacial area and pull apart the surfactant molecules. As a consequence the interfacial surfactant concentration per unit area decreases. If surfactant adsorption is the dominant feature, then this “dilution” results in a reduction of the intensity of the properties such as interfacial order, tension lowering and solubilization [67,69]. This property of alcohols inhibits the formation of liquid crystals; for this reason alcohols are often added in ionic surfactant systems when a microemulsion is sought. In this case it may be said that alcohol behaves as a very poor surfactant; for instance,  $C_3$  and secondary  $C_4$  alcohol behave as such: they pull apart and replace the surfactant molecules but do not exhibit interactions with oil and water which are able to match the surfactant. When the chain length of alcohol increases, its interaction on the water-side of the interface remains constant (through the OH group), whereas its interaction on the oil side increases. At some length (say linear  $C_4$ ) the interaction of alcohol tail starts participating to the oil side balance, thus affecting the formulation. Beyond some length, the alcohol is no longer active in the interfacial formulation balance and essentially behaves as oil; however, it probably concentrates in the oil layer in the neighborhood of the interface and can play the role of a lipophilic linker [70].

Fig. 3 illustrates the lipophilic linker role. The lipophilic linker molecules exhibit a definite orientation because of their

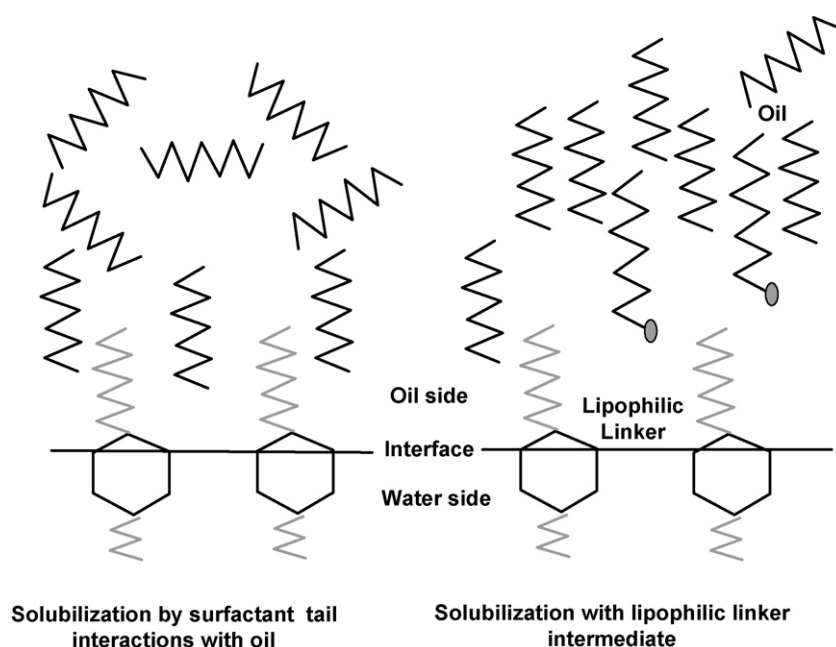


Fig. 3. Illustration of the lipophilic linker role in improving solubilization [71].

polar groups. Since the lipophilic linker molecules are located in the oil phase, they force the oil molecules of the layers next to the interface to be more ordered. This order results in enhanced interactions between the oil molecules and between the surfactant and the oil molecules. It may be said that the lipophilic linker extends the reach of surfactant tail deeper into the oil phase, thus providing extra interaction. Nevertheless, the surfactant–linker and linker–oil interactions are still loose enough to avoid the formation of liquid crystals. The lipophilic linker may be defined as an amphiphilic substance with a small hydrophilic group and a large tail, such that its overall HLB is very low. Long-chain alcohols obey this definition, and indeed they have been found to shift optimal formulation and to improve solubilization under appropriate conditions.

The chain length-dependent effect of alcohols showed the following interesting facts [70]:

1. C<sub>2</sub>–C<sub>6</sub> alcohols are adsorbed at the interface in amounts that are not negligible with respect to the surfactant. Therefore, they pull apart the surfactant molecules, without providing equivalent interaction because of their very short chain. As a result, the solubilization is low. The apparent solubilization (which considers the surfactant only) is the same as in the absence of alcohols, while the real value is lower. This indicates that the alcohol does not provide any improvement as far as the interactions with the oil phase are concerned.
2. C<sub>6</sub>–C<sub>10</sub> alcohols are in lesser amount at interface, but they provide some additional interaction on the oil side (i.e., lipophilic), thus requiring an increase of surfactant average ethylene oxide number (EON). The increased interactions on both sides results in an increase of solubilization, according to the conventional Winsor *R* reasoning.
3. The interfacial amount of long-chain alcohols (C<sub>10</sub> and above) is negligible, as may be deduced from their very small proportion at interface  $X_a$ . Their contribution to interfacial formulation was nil, since they do not require any compensation on the waterside of the interface. Thus, the increased solubilization is due to another mechanism, that is, the lipophilic linkage provided by the alcohol at oil phase boundary [71].

Dynamic light scattering measures the intensity fluctuation that occurs over short time intervals due to the Brownian motion of the micelles in solution. A pronounced effect on the diffusivity of the micelles is found with the addition of different alcohols. The diffusivity decreases for the shortest chain alcohols, methanol and ethanol, remains practically unchanged for *n*-propanol, and increases for butanol, the longer alcohols and the branched alcohols. It is also shown that the addition of methanol or ethanol does not change the kinematic viscosity ( $\eta$ ), whereas there is a sharp decrease of  $\eta$  with alcohol concentration for the other linear and branched alcohols. With increasing alcohol concentration the hydrodynamic radius ( $r_h$ ) estimated from diffusivity increases for methanol and ethanol, remains practically constant for *n*-propanol, and decreases for *n*-butanol, *n*-hexanol and the branched alcohols. For a given alcohol concentration, an

interesting behavior of  $r_h$  with alcohol carbon number and with  $W_0$  is seen. As a function of alcohol carbon number, for each *R* ratio,  $r_h$  values are close for the shorter alcohols (methanol to *n*-propanol) and then decrease markedly on going to the longer ones, where  $r_h$  is again practically constant or increases slightly. On the other hand, for any given alcohol at the same (or very close) concentration,  $r_h$  increases with  $W_0$ . It appears then that, on going from a micellar domain ( $W_0 = 10$ ) to a microemulsion region ( $W_0 = 30$ ), the size of reverse micelle increases, but their behavior with alcohol concentration or carbon number is qualitatively the same. The swelling and shrinkage of reverse micelles in the presence of short and long alcohols, together with the behavior of  $\eta$ , strongly suggest that methanol and ethanol are trapped in the water pool of reverse micelles, while the other linear and branched alcohols are dispersed in the surrounding solvent and probably also incorporated into the micellar shell [42].

Small angle X-ray scattering (SAXS) studies have been conducted to explore the role of *n*-octanol in the water/dimethyldioctylhexylethoxymalonamide (DMDOHEMA)/dodecane system. A small increase in the intensity scattered by X-ray is found in the presence of alcohol in solution. As the X-ray scattered intensity is proportional to the number of scattering objects, it is deduced that the presence of *n*-octanol in solution increases the number of aggregates. In small quantities, *n*-octanol has a co-surfactant effect [72]. Octanol molecules adsorb at the surface of small micelles and consequently, increase the surface per polar head. The specific surface area of the dodecane/water interface as well as the number of micelles is increased by this phenomenon, and as a result, the intensity on the SAXS pattern increases.

#### 4. Percolation phenomena

##### 4.1. Effect of alcohols on percolation process

Reverse micelles are highly dynamic structures whose components rearrange themselves over time and space through interactions or collisions, coalescing and re-dispersing. Factors governing the structure and stability of reverse micelles are continuing to be a crucial issue of reverse micellar systems [73]. However, the present knowledge on reverse micellar structure is almost entirely based on the bulk phase behavior. Recently, it has been shown that the percolation phenomenon in reverse micellar systems can be utilized to study the micellar stability as well as the membrane properties [74–77]. The percolation process reflects micelle–micelle interactions and can be quantified easily via the measurements of electrical conductivity of reverse micellar systems [48,74,78–84]. A microemulsion has a very low conductivity,  $10^{-9}$  to  $10^{-7} \Omega^{-1} \text{cm}^{-1}$ , which is already a significant increase compared to the conductivity of alkanes ( $\approx 10^{-14} \Omega^{-1}$ ) and is due to the fact that micelles carry charges. A known behavior occurs when water is added to the system: at a certain volume fraction the conductivity rises sharply over a narrow range and then remains practically unchanged at a considerably higher value than before the transition. A similar phenomenon is observed if temperature is increased



keeping the composition constant. This phenomenon is called percolation.

Conductivity measurements have been used to access reverse micellar formation and to probe the structural changes occurring in the systems [48,80,83]. A sharp increase in electrical conductivity caused by the percolation phenomenon well demonstrates the interaction between the micelles. The conductivity of reverse micellar systems has been measured as a function of water content ( $W_0$ ) or temperature. The cluster formation of micelles increases the conductivity over the percolation threshold, which indicates the starting point of reverse micellar droplet clustering. The percolation threshold can be varied in the presence of some additives. Some authors do not consider it a distant phenomenon but only a transition from a discrete droplet phase to a bicontinuous one [85]. It is usually considered that during percolation the droplets come in contact and ions are transferred by some kind of “hopping” mechanism and/or channels are formed through which micellar contents can be exchanged.

For percolation to occur micelles must cluster, and any alteration in the process caused by the addition of co-surfactants is reflected in the percolating temperature. It is indicated that the solubilization of proteins clearly affects the percolation process with a rapid increase in the conductivity at lower or higher water contents or temperatures, suggesting stronger or weaker attractive interactions between micelles in the presence of proteins [74–77,86].

A temperature-induced percolation has been used to study the effect of alcohols, cholesterol, and crown ethers on the percolating temperature [83]. They concluded that alcohols and cholesterol increase the percolating temperature and crown ethers decrease it. Dynamic light scattering was used to study the droplet size and interaction between AOT and co-surfactants. It was found that the radius increases linearly with water content, and that the co-surfactants increase or decrease interactions according to their structure. The short-chain alcohols increase interactions, while the long-chain ones decrease them [87]. Considering that the co-surfactants are solubilized in the micellar interface (in the case of alcohols there will be partitioning between the oil continuum and the interface), their effect on clustering and aggregation is due to the changes they cause to that interface. In view of the percolating temperatures, the conclusion is that alcohols make the interface more rigid and hence make clustering, aggregation and consequently, percolation more difficult [88]. The solubilization site of the co-surfactant is connected with the resulting fluidity of micellar interface. The long-chain alcohols increase the rigidity of the interface, which can be achieved if alcohol solubilizes in the surfactant tail region and so pushes the surfactant head groups together (Fig. 4). This solubilization site also increases the micellar curvature. Alcohol molecules have also been used to control the formation and destruction of the reverse micelles and to improve the back extraction of proteins [5,10]. The alcohol molecule is expected to be a good modifying agent for micellar membrane, but its effect has not yet been clearly understood. It remains unclear whether the alcohol molecule added into reverse micellar system is used as co-solvent or as co-surfactant in relation to their amphiphilic property. Researchers have been trying to under-

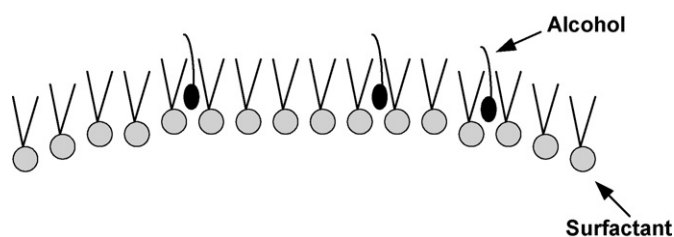


Fig. 4. Schematic representation of the solubilization site for alcohol [88].

stand the alcohol effect on the properties of micellar membrane with respect to the percolation phenomenon and also the contribution of each group (hydrocarbon, hydroxyl, halogen substituents, etc.) on alcohol molecule to the percolation process [73].

#### 4.2. Alcohol effect on micellar–micellar interactions of reverse micellar systems

Electrical conductivity ( $k$ ) varies as a function of the volume fraction of aqueous phase ( $\phi_{aq}$ ) solubilized into AOT reverse micelles in the presence of alcohols. For a reverse micellar system, the volume fraction, which corresponds to the threshold of electrical percolation, varies with species and concentration of added  $n$ -alcohol. In the case of  $n$ -butanol added reverse micellar system, the electrical percolation threshold ( $\phi_t$ ) decreases by adding  $n$ -butanol into the reverse micellar system. On the other hand, the addition of  $n$ -pentanol shows a decreasing effect on the attractive interaction between micelles [73]. This suggests that the micelle–micelle interactions are notably affected by the alcohols added into an organic solution. The percolation thresholds are also affected by the added alcohol concentration. Lang et al. [47] found that the addition of  $n$ -alcohol molecules to W/O microemulsion considerably enlarges the microemulsion range (monophasic).

The effectiveness of  $n$ -alcohols to the percolation phenomenon is explained by the variation of micellar size caused by the added alcohols and determined in low water content ( $W_0 = 20$ –30). Although the micellar size is an important factor to characterize the alcohol effect, it is not enough to explain the concentration effect and the percolation phenomena occurred at high  $W_0$  range. It is considered that the mobility of micellar membrane is changed by adding alcohols, affecting the micellar structure. The role and effect of alcohols in reverse micellar system, however, has not been well understood yet. The difference  $\Delta\phi_t (= \phi_t - \phi_p)$  indicates the effect of alcohol concentration on the percolation process, where  $\phi_t$  and  $\phi_p$  are the percolation thresholds with and without alcohol, respectively. A linear correlation is observed between  $\Delta\phi_t$  and the concentration of each alcohol. The slope,  $\beta_t$ , is a measure of the effect of alcohol addition on the attractive interaction between micelles. A positive  $\beta_t$  value means the stabilization of reverse micellar system or the decrease of micelle–micelle interactions by the addition of alcohols. On the contrary, a negative  $\beta_t$  value means the destabilization of reverse micellar system or the increase of micellar–micellar interactions. It can be said

that the  $\beta_t$  value markedly changes with the alcohol species [73].

The  $\beta_t$  values of various alcohols such as alkanols, diols, triol and halogenols are used to understand the contribution of each group of the alcohols, e.g., the CH, OH and halogen groups, to the properties of micellar membrane. Among *n*-alkanols, there seems to be a linear correlation between the  $\beta_t$  value and the number of carbon atoms, suggesting that the difference of the percolation phenomena between added *n*-butanol and *n*-pentanol is simply a part of the related behavior. Hence, it is considered that the hydrophobic sites of alcohols (hydrocarbon groups) are responsible for the decreasing micellar–micellar interaction. This result means that the addition of alcohol molecules of appropriate chain length to AOT reverse micellar system may easily control the micellar–micellar interactions, and the  $\beta_t$  value is rather convenient to evaluate and thus design the alcohol effect on micellar membrane. The  $\beta_t$  values for diols are smaller and do not seem to have a linear correlation with the number of carbon atoms, suggesting that the additional OH group decreases the  $\beta_t$  value. As for halogenols, substitution of halogen atoms increases the  $\beta_t$  value regardless of their species. Thus, whereas the hydrophobic hydrocarbon and halogen atom group decrease the intermicellar interactions proportionally to their number, the hydroxyl group increases the intermicellar interactions. A similar result in the effect of alcohols inducing  $\alpha$ -helix formation of protein or peptide was also reported [89,90].

## 5. Alcohol effect on the back extraction of proteins

The design of an effective protein separation process using reverse micellar phase requires that we know the extent to which enzymatic activity of the recovered proteins is retained following completion of the extraction/stripping cycle. Alcohol is often used, as a promoter of back extraction and has to be carefully chosen because a small structural change causes a decrease in the protein activity. The extent of back extraction of proteins with alcohol addition not always depends on the pH in the recovery phase and thus aqueous solution with a mild pH is used to recover the protein, to optimize final enzyme activity. A strong interaction between the solubilized proteins and micelles induces the micelle–micelle interaction or micellar cluster formation, resulting in a decrease of back extraction fractions. Hence, the control of micelle–micelle interaction may become a very important factor for the success of back extraction of the proteins. Alcohol is considered to be a good modifying agent for reverse micelles because alcohol molecules have amphiphilic property as a co-surfactant [73]. The influence of various alcohols on the reverse micellar system has been reported using the percolation phenomenon [73]. A number of proteins have been back extracted with the help of alcohols, each having their unique mechanism. Therefore, it is very important to study the effect of alcohols on the change in enzyme activity and conformation during the RME processes. A list of proteins and the alcohols used in their back extraction is given in Table 1.

### 5.1. Extraction of $\alpha$ -chymotrypsin from AOT–DOPA mixed reverse micellar systems

Back transfer of extracted chymotrypsin by the mixed reverse micelles of AOT–DOPA is significantly enhanced by the addition of isobutanol to the reverse micellar phase [18]. Although the back transfer of proteins in alcohol-free system is very slow, the rate of back transfer is markedly improved by adding 10 vol% alcohol to the reverse micellar phase, back transfer being accomplished in less than 2 h. This back extraction behavior is related to the frequency of fusion among reverse micelles and the rigidity of surfactant layer. Electrical conductivity measurements also support the above idea, because in a mixed surfactant ratio of 4 (AOT/DOPA), a percolation behavior is observed at the lowest temperature. The effect of added alcohol content is studied on the degree of back extraction of chymotrypsin. A small addition of alcohol improves the back transfer of chymotrypsin from the reverse micellar phase. Although the effects of pH and salt concentration on the back extraction are studied, it is surprising that both key parameters in the forward extraction are not sensitive to the back extraction. These results suggest that the main driving force of the back transfer of chymotrypsin is not the electrostatic repulsion.

To improve the degree of back extraction, it might be necessary to promote the frequency of fusion among reverse micelles and the interexchange between surfactant molecules forming reverse micelles and oriented at the bulk aqueous/organic interface [91]. The kind of alcohol significantly affects the degree of back transfer. This result also suggests that merging of reverse micelles is important to release a protein entrapped because alcohol is known to promote the exchange rate of reverse micelles. Isobutanol is the best promoter to improve the efficiency of the back extraction and the influence is gradually reduced with a longer alcohol.

### 5.2. Extraction of $\alpha$ -amylase from Aliquat 336/isooctane reverse micelles

The extraction and back extraction of  $\alpha$ -amylase was carried out in Aliquat 336/isooctane reverse micellar system in the presence of different co-solvents (*n*-alcohols) [11]. Most sections of the CD spectra of  $\alpha$ -amylase in the strip solution using *n*-butanol (as co-solvent), coincided with those of the native state spectrum, which means that the confirmation of  $\alpha$ -amylase might also be mostly recovered to its native state. For the other five alcohols (*n*-pentanol, *n*-hexanol, *n*-heptanol, *n*-octanol, *n*-decanol), however, it was shown that  $\alpha$ -amylase molecule in the strip solution will deviate from the native state; thereby implying that possibly only small parts of the  $\alpha$ -amylase activity would be able to be recovered at the end of an extraction cycle. Although it is still somewhat difficult to determine the effect of co-solvent on the change in  $\alpha$ -amylase structure, some conclusions can be drawn from the experimental results. From small to big, this effect seems to have a sequence: *n*-butanol > *n*-pentanol > *n*-hexanol  $\approx$  *n*-heptanol  $\approx$  *n*-octanol  $\approx$  *n*-decanol. The reason for this co-solvent effect is due to the intrinsic physical properties of *n*-alcohols. Due to the difference in polarity and

Table 1  
Effect of added alcohols on the percentage of back extraction of proteins/enzymes and their enzymatic activity

Protein/system	Alcohol	Back extraction (%)	Activity (%) / purification factor	Reference
$\alpha$ -Chymotrypsin (AOT–DOPA/isooctane)	None	62.8	0.034	[18]
	Isopropyl alcohol	93.8	0.031	
	Isobutanol (10%)	97.1	1.0	
	Isoamyl alcohol	90.7	0.74	
	<i>n</i> -Hexanol	84.5	0.68	
	<i>n</i> -Octanol	67.4	0.24	
	<i>n</i> -Decanol	59.9	0.10	
$\alpha$ -Amylase (Aliquat 336/isooctane)	Oleyl alcohol	37.1	0.55	[11]
	<i>n</i> -Butanol	82.7	0.91	
	<i>n</i> -Pentanol	50.0	0.64	
	<i>n</i> -Hexanol	29.7	0.25	
	<i>n</i> -Heptanol	30.4	0.27	
	<i>n</i> -Octanol	30.8	0.32	
Porcine pepsin (AOT/isooctane)	<i>n</i> -Decanol	32.4	0.29	[5]
	Isopropyl alcohol (10–15%)	100	No loss in activity	
Bovine chymosin (AOT/isooctane)	Isopropyl alcohol (10–15%)	70	No loss in activity	[5]
Hemoglobin (DOPA/isooctane)	Methanol	75	–	[91]
	Ethanol	78	–	
	Isopropyl alcohol	58	–	
	<i>n</i> -Propanol	59	–	
Bovine serum albumin (AOT/isooctane)	None	100	–	[94]
	<i>n</i> -Propanol	100	–	
	<i>n</i> -Hexanol	100	–	
	<i>n</i> -Octanol	100	–	
$\beta$ -Lactoglobulin (AOT/isooctane)	None	62	–	[94]
	<i>n</i> -Pentanol	70	–	
	<i>n</i> -Hexanol	97	–	
	<i>n</i> -Octanol	99	–	
Carbon anhydrase (AOT/isooctane)	<i>n</i> -Propanol	20	18 (activity yield)	[94]
	<i>n</i> -Butanol	25	23	
	<i>n</i> -Hexanol	57	58	
	<i>n</i> -Octanol	72	75	
Cytochrome <i>b</i> <sub>5</sub> (AOT/cyclohexane/ <i>n</i> -decanol)	<i>n</i> -Decanol	0	–	[96]
	<i>n</i> -Octanol	30	–	
	<i>n</i> -Hexanol	78	–	
	<i>n</i> -Butanol	82	–	

water solubilization of *n*-alcohols, the properties of Aliquat 336 reverse micelles with these *n*-alcohols may also have some differences. The reverse micelles with *n*-alcohols of relative higher polarity and water solubilization such as *n*-butanol may be easily formed and destroyed. Probably the interaction between enzyme and reverse micelles may be weaker [92], thus, the activity and structure of  $\alpha$ -amylase may be less affected during the RME cycle. In the case of co-solvent with lower polarity and water solubilization such as *n*-decanol, however, this interaction may become stronger and the enzyme may be easier to denature during the RME processes.

The recovery of enzyme activity also depends strongly on the co-solvent type. In the case of *n*-butanol as co-solvent, more than 80% of the total activity of  $\alpha$ -amylase in the initial aqueous phase can be recovered at the end of an extraction cycle; for *n*-pentanol, about 50% of the total activity can be maintained in the strip solution. For the other four alcohols (*n*-hexanol, *n*-heptanol, *n*-octanol, *n*-decanol), only about 30% of the total activity can

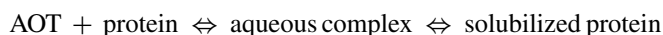
be recovered after an extraction cycle. These findings can offer a practical co-solvent selection criterion for Aliquat 336 reverse micelles which suggest that, among the six *n*-alcohols, only *n*-butanol is the best and suitable co-solvent for Aliquat 336 reverse micelles to separate and purify  $\alpha$ -amylase by RME. In conclusion though this study is limited to only one enzyme with one type of reverse micelle, results obtained probably have a more general validity.

### 5.3. Release and recovery of porcine pepsin and bovine chymosin from AOT/isooctane system

Porcine pepsin and bovine chymosin, once solubilized could not be released from AOT/isooctane systems even under conditions that ordinarily prevent uptake [5]. Their release is promoted by adding 10–15% (v/v) isopropyl alcohol (IPA) to the aqueous phase. Addition of IPA at this level allows for full release of pepsin and 70% recovery of chymosin. Also, in the presence

of IPA at this concentration the reverse micelles retain their functional integrity and forward transfer remains possible. Both uptake and release of proteins by reverse micelles are found to occur rapidly in the presence of IPA.

No study in the current literature offers a satisfactory explanation for why pepsin is retained in the reverse micelle phase when the conditions for its residence are so unfavorable. The findings of Carlson and Nagarajan [5] have implications for a new model of the mechanism of interfacial transfer of proteins between an organic reverse micelle phase and an aqueous phase. The forward and backward transfer results show that pepsin/AOT/isooctane system does not attain equilibrium; thus, a kinetic explanation appears to be required. A minimal model requires there to be two kinetic steps in series, one involving a fast equilibrium and one involving a slow equilibrium:



The second equilibrium involving the transfer of aqueous complex to the organic phase appears to be a slow process preceded by a rather rapid equilibrium to establish a “transferable” complex. Another way of looking at this is that there appears to be some mechanism involving AOT leaving the organic phase and going out to interact with protein molecules to form a complex in the aqueous phase (cloudiness in the lower phase). Once the complex is formed, it can mediate the transfer of protein into the organic phase in a second step. In the second step, the equilibrium lies far to the right so that the forward transfer process is fast but the reverse of this step is slow. The experimental fact suggests that without IPA the reverse of the second step is very slow and virtually disallows transfer of protein back to the aqueous phase (at least when salt is present). IPA addition appears to increase the rate of the reverse in this second step allowing equilibrium to be established. One may further speculate that the first step in the process involves the charge of the protein and the salt concentration. High salt or high pH values prevent complex formation and thus disallow forward transfer. The back transfer process is rate-controlled, so it is little affected by salt or pH. Thus, addition of IPA can be considered as a practical method for back extraction from the AOT reverse micelles.

#### 5.4. Extraction of BSA and cytochrome *c* using AOT reverse micellar system

The back extraction behavior of BSA and cytochrome *c* and their percolation phenomenon has been studied focusing on the formation of micellar cluster via protein–micelle interactions [93]. A relationship between protein back extraction and their percolation phenomenon by adding small amount of alcohol has also been established. The percolation process is effective for the evaluation of micelle–micelle interactions [76,77,83]. In the case of BSA, the electrical percolation threshold increases with solubilizing BSA into the reverse micelles. This indicates an increase in attractive interaction between micelles as BSA is solubilized into the reverse micelles. In contrast, for the solubilized cytochrome *c*, the percolation threshold decreases in the lower value than that of the protein-free system. These results suggest that the micelle–micelle interactions are notably affected

by protein species and concentration solubilized into the reverse micelles. Also it is believed that cytochrome *c* interacts with the AOT surfactant layer. Hence, the protein–micelle (electrostatic attractive) interactions seem to reduce the stability of reverse micellar system by decreasing electrostatic repulsive interactions between the micelles. The formation of micellar clusters shows a larger hydrophobic attraction than an electrostatic repulsive force between the micelles. It is found that BSA is easily back extracted into the new aqueous phase at the pH range above the *pI*, but the back extraction fraction of BSA decreases at the pH range below the *pI*. On the other hand, cytochrome *c* is comparatively difficult to be back extracted.

The effect of added alcohol on the time course of back extraction of BSA and cytochrome *c* shows that the addition of *n*-propanol reduces the back extraction rate than that of alcohol-free system. However, the back extraction is accelerated when *n*-hexanol or *n*-octanol are added. Therefore, a clear difference is found depending on the species of alcohols used in reverse micellar system. The back extraction rates increase with increasing number of alkyl chain per alcohol molecule added to reverse micellar system at the same alcohol concentration. This is an interesting result indicating the possibility that protein back extraction process can be controlled by alcohol addition to the reverse micellar system. These studies show two types of alcohols. The first type includes *n*-hexanol and *n*-octanol, which promotes the back extraction rate with increasing alcohol concentration. This type of alcohol has an effect of reducing interaction between the micelles. The second type includes *n*-propanol and *n*-butanol, which can slightly reduce the back extraction rate with increasing alcohol concentration. They have the tendency of increasing the interaction between the micelles. In general, back extraction of proteins is governed more by the resistance at the interface than the diffusional resistance in the reverse micellar phase and aqueous phase. The decreasing of micelle–micelle interaction accelerates the back extraction, explaining the role of alcohol on the back extraction of proteins in reverse micellar systems. It is considered that alcohol molecules added to surfactant organic solvent may act as a co-surfactant when reverse micelles are formed, because the micellar properties change in the presence of alcohols affecting the micelle–micelle and protein–micelle interactions.

#### 5.5. Extraction of BSA, carbon anhydrase and $\beta$ -lactoglobulin from AOT/isooctane system

The effect of various alcohols on the structure of proteins (BSA, CAB and  $\beta$ -lactoglobulin) and their back extraction behavior has been studied [94]. The native structure of  $\beta$ -LG, a predominantly  $\beta$ -sheet protein, is denatured to a  $\alpha$ -helical state by the addition of alcohols. The effectiveness of alcohols on protein denaturation varies markedly depending on their species. Thus, the use of an alcohol directly into the strip solution is not recommended in the back extraction of proteins and it needs an appropriate choice of alcohol molecules. The alcohol-induced protein denaturation is considered to be negligible in the concentration range 0.01–0.1% (v/v). The aggregation of alcohol

molecules is also found to be a critical factor enhancing protein denaturation [95].

A clear difference in the back extraction depending on alcohol species is observed in the case of BSA. The addition of *n*-propanol results in a slight decrease in the back extraction rates, whereas the addition of *n*-octanol accelerates the back extraction rates with the alcohol concentration. This indicates that the protein back extraction process can be regulated by a small amount of alcohol added to the reverse micellar system. In case of CAB, the addition of *n*-hexanol and *n*-octanol increases the back extraction yield (75%), whereas *n*-butanol and *n*-propanol do not show a very significant effect (20%). Alcohol additions have good effects on the activity yield as well as the back extraction fraction, indicating a decrease in the resistance of micellar or interface membrane by the alcohol molecules. High concentration of alcohols like *n*-hexanol, or *n*-octanol usually results in the destruction of reverse micelles when they are added into the surfactant organic solution. At low concentrations of alcohols, however, the reverse micelles are formed safely.

### 5.6. Extraction of hemoglobin using reverse micelles of diolelyphosphoric acid

New reverse micelles formed by diolelyphosphoric acid (DOPA) can easily extract hemoglobin, which cannot be extracted by conventional surfactant AOT. The DOPA reverse micelles are the first to show high extraction ability without a co-surfactant [91]. The difference between DOPA and AOT reverse micelles in the extraction of hemoglobin is considered to be due to the hydrophobicity of surfactant in the hydrophobic moiety. The hydrophobicity of interfacial complex formed between surfactants and hemoglobin is a key factor in extracting hemoglobin efficiently into the reverse micellar phase. DOPA can form very stable reverse micelles containing hemoglobin in isooctane because of its favorable long alkyl chains to solubilized them in such non-polar solvents; however, this point gives rise to a serious problem for the back extraction of hemoglobin. The hemoglobin extracted by the forward transfer is not released from the reverse micellar phase, even when it is contacted with a fresh aqueous solution in which the conditions would not allow hemoglobin uptake. Hence, the extraction system of hemoglobin is not considered to be an equilibrium operation. Alcohols like methanol, ethanol, isopropyl alcohol and *n*-propanol are added to the aqueous strip phase to study its effect on the back extraction. The results show that the addition of alcohol causes hemoglobin to release from the DOPA reverse micelles, but each alcohol has a distinct critical alcohol concentration (CAC) for the release of hemoglobin, and the CAC decreases gradually with increasing carbon number in the alcohol. Although a moderate addition of alcohol promotes the hemoglobin release, the reverse micelles might be destroyed by a large amount of alcohol. IPA and ethanol are found to be the most effective promoter available for the back extraction of hemoglobin.

Three dominant factors are found to facilitate the back transfer of hemoglobin from DOPA reverse micelles (Fig. 5): (i) an alcohol addition will reduce interfacial resistance for the reverse micelles containing hemoglobin, because the alcohol is known

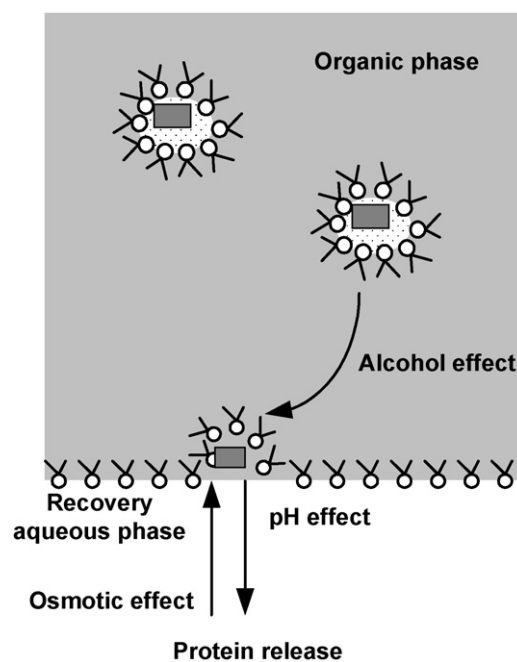


Fig. 5. Schematic diagram of the back transfer of hemoglobin from a DOPA reverse micellar phase by the addition of alcohol [91].

to promote the fusion/fission of reverse micelles [82]. However, excess alcohol induces the destabilization of reverse micelles and often causes the denaturation of proteins; (ii) another important factor is the pH of the recovery solution. An electrostatic repulsion between protein surface and surfactant head groups is required to remove hemoglobin from the reverse micellar phase; and finally (iii) the direction of water transfer, which means “from the bulk phase to water pool” or “from water pool to the bulk phase”, is a key factor in ensuring back transfer of hemoglobin and it is determined by the osmotic pressure between the bulk phase and water pool. When the ionic strength of water pool in reverse micelles is higher than that of the recovery aqueous phase, water can transport from the bulk phase to water pool. This behavior results in the swelling of water pool, and the microenvironment in water pool would be changed to be a favorable condition. Even under the favorable conditions of two of the three factors, protein release would not occur. Accomplishment of the back extraction of hemoglobin from the DOPA reverse micelles requires all three factors.

### 5.7. Extraction of recombinant cytochrome *b*<sub>5</sub> from CTAB reverse micelles using various alcohols

Proteins are usually back extracted by contacting a loaded organic phase with a new aqueous phase at high ionic strengths (up to 2 M salt). However, under these conditions no transference of cytochrome to the fresh aqueous phase is observed. In the back extraction process, most of CTAB (70%) accumulates at the interface. The protein stays in the bulk organic phase with the remaining surfactant. When alcohol is added to the biphasic system before the onset of back extraction, part of the cytochrome is back extracted to the aqueous phase [31]. This effect is dependent on the type and concentration of alcohol,

increasing with alcohols of shorter chain length. The added alcohol also causes the solubilization of surfactant in the aqueous phase, along with back extraction of cytochrome. As more CTAB is solubilized in the aqueous phase, more cytochrome is transferred. This suggests that the back-extracted cytochrome is still associated to the surfactant. Back extraction of cytochrome  $b_5$  is likely due to a decrease in the dielectric constant of aqueous medium caused by the residual presence of alcohol in the aqueous phase. It is assumed that the polar alcohols distribute into the aqueous phase more than hydrophobic alcohols. Thus,  $n$ -butanol has a more pronounced effect on the dielectric constant of water than, for instance,  $n$ -hexanol or  $n$ -decanol. As alcohol concentration increases, its effect on the dielectric constant of aqueous phase also increases. As a result of the partition of an alcohol between the bulk aqueous phase, the aqueous pool of reverse micelles and the organic phase, the incompatibility of hydrophobic/hydrophilic components of the biphasic system decreases, i.e., the organic solvent becomes less hydrophobic and the water more lipophilic [47]. This causes a weakening effect on the hydrophobic protein–surfactant interaction. It also causes an increased partition of surfactant to the aqueous phase.

Back extraction of cytochrome  $b_5$  in the presence of 20% (v/v)  $n$ -hexanol is possible, provided that the ionic strength is kept high [96]. It is known that by adding an alcohol to a water-in-oil microemulsion the size of the droplets decreases with increasing alcohol concentrations [47]. Thus, a size exclusion effect may also have occurred in this situation, since the protein is transferred to the aqueous phase only in the presence of an alcohol at moderately high ionic strength and at a pH where protein and surfactant have opposite charges. At lower ionic strengths, this electrostatic interaction is not damped, and thus the protein is not back extracted to the aqueous phase. Apparently the back extraction process is not controlled by a single factor. It is feasible that part of cytochrome is back extracted to a fresh aqueous phase, due to a size exclusion effect at high ionic strength and due to the disruption of hydrophobic interaction with the surfactant by the alcohol. This duplicity of behavior could be a consequence of the ambivalent character of cytochrome, since it may interact with surfactant through its apolar side chains in the hydrophobic surface region or with its ionic side chains in the remaining surface region. More polar alcohols like  $n$ -butanol and  $n$ -hexanol better succeed in the back extraction because they better penetrate the interfacial layer of reverse micelles and disrupt the hydrophobic surfactant–protein interaction. This interaction is very strong since surfactant is co-extracted with protein to the aqueous phase. It is likely that this behavior is a consequence of the particular structure of cytochrome  $b_5$  since, at its surface, both polar and apolar regions are present.

#### 5.8. Effect of $n$ -hexanol on cutinase–AOT interaction in reverse micelle

Cutinase encapsulated in dioctyl sulfosuccinate reverse micelle displays very low stability, undergoing fast denaturation due to an anchoring at the micellar interface [97]. Denaturation in reverse micelles is mainly the unfolding of the three-dimensional

structure since the decrease in the circular dichroism ellipticity in the far-UV range is very small. Although a co-surfactant is not needed in reverse micelles of AOT, the effect of  $n$ -hexanol in the structure of the reverse micelle and in the cutinase conformation was studied, because the stability of cutinase is highly improved in the presence of  $n$ -hexanol. In the presence of hexanol, cutinase is encapsulated in a large reverse micelle which was proved by dynamic light scattering. This large reverse micelle filled with cutinase was built from the fusion of reverse micelles according to a pseudo-unimolecular process ranging in time from a few minutes to 2 h, depending on the reverse micellar concentration.

The encapsulation of cutinase in AOT reverse micelles in the absence and presence of  $n$ -hexanol is clearly distinct. The size of reverse micelle filled with cutinase is around 10-fold larger in the presence of  $n$ -hexanol due to the slow fusion of empty reverse micelles. In addition,  $n$ -hexanol as co-surfactant changes the interfacial properties and the new interfacial characteristics do not cause denaturation. The absence of rotational mobility for cutinase in AOT/ $n$ -hexanol reverse micelles points to a cutinase location at the interface. Higher stabilities in reverse micelles are often associated to a location within the water pool. This study thus shows that proteins with low stabilities in reverse micelles can display high stabilities if the interfacial properties are changed. A location within the water pool does not seem to be a prerequisite to prevent cutinase denaturation.

#### 5.9. Analysis of protein back extraction processes in alcohol mediated AOT reverse micelles

The effects of the addition of alcohol on the back extraction of several small globular proteins such as  $\beta$ -lactoglobulin, bovine carbonic anhydrase, and lipase were studied using the reverse micellar systems [98]. The protein back extraction induced by the addition of alcohol was markedly influenced by the structural changes of reverse micelles ( $\beta_t$ ) and protein ( $m$ ). The alcohols such as  $n$ -hexanol,  $n$ -hexanol and  $n$ -octanol suppressed the formation of reverse micelle clusters (positive  $\beta_t$ ) and remarkably enhanced the back extraction of proteins. On the other hand, the alcohols such as  $n$ -propanol and  $n$ -butanol were responsible for enhancing the formation of reverse micelle clusters (negative  $\beta_t$ ) thus suppressing the back extraction of proteins. The markedly effective alcohol molecules on the denaturation of proteins (high  $m$ ) suppressed the back extraction of proteins. The  $m$  values were estimated by measuring the dependence of the free energy change of denaturation on the concentration of alcohol and were correlated well with the solvent-accessible surface area of alcohol molecules. Simple equations on the basis of these factors were constructed which easily explained the various back extractions affected by alcohol molecules, suggesting an important guide to select appropriate alcohol for bioseparation or other bioprocesses.

## 6. Conclusions

This review has pointed out several features of microemulsions, their formation and some important aspects for the implementation of liquid–liquid extraction of proteins by reverse

micelles, with emphasis on back extraction using alcohols and also the role of alcohols in the formation of inverse microemulsions. The following conclusions could be made: (i) alcohols act as a weak amphiphile when added into a binary water-in-oil mixture. They act as “co-solvents” in some cases where they partition between the aqueous domain and the amphiphilic film and as “co-surfactant” it dissolves in the amphiphilic film, making co-surfactant mixture more hydrophobic. Medium-chain alcohols are usually considered as co-solvents; (ii) the amount of alcohol added to the microemulsion system is also considered to be critical. At lower concentration of alcohol the water uptake in the organic phase maybe almost zero giving rise to Winsor I type system and at very high concentration of alcohols also the water up take decreases; (iii) for alcohols with hydrocarbon chain longer than C<sub>10</sub>, the concentration of alcohol required to form reverse micelles eventually start increasing with an increase in the chain length. For a fixed alcohol concentration, the longer the hydrocarbon chains of alcohol, the smaller the water uptake of reverse micelles.

Different kinds of alcohols with various contributing factors have been employed for successful back extraction of proteins. Although a specific quantitative or semi-quantitative index cannot be determined with respect to HLB value of surfactant or *pI* of protein, etc., for protein back extraction using alcohols, various researchers have explained their unique mechanism: (i) one of the most important role of alcohol in protein back extraction is the control of micelle–micelle interaction. Alcohols like *n*-hexanol and *n*-octanol is considered to reduce micelle–micelle interaction, whereas *n*-propanol and *n*-butanol may slightly increase the interaction between the micelles leading to reduced back extraction rates; (ii) the percolation process, a sensitive and convenient measure of micro-interface of reverse micellar systems, clearly shows the effect of a small amount of various alcohols on micelle–micelle interactions. The percolation of reverse micellar system is highly dependent on the species and concentration of the added alcohols. Alcohols suppress the formation of micellar cluster therefore improving the back extraction; (iii) alcohols with high polarity and water solubilization like *n*-butanol and *n*-hexanol better succeed in back extraction because they better penetrate the interfacial layer of reverse micelles and disrupt the hydrophobic surfactant–protein interaction, whereas use of alcohols with low polarity and water solubilization like *n*-heptanol and *n*-decanol leads to stronger enzyme–reverse micellar interaction leading to enzyme denaturation; (iv) each alcohol may have a critical alcohol concentration for a particular protein/enzyme and the CAC gradually decreases with increasing carbon number of alcohols; (v) addition of alcohols directly into the strip solution is not always recommended for certain proteins because of its denaturing effect, but it also depends on the choice of alcohol used; (vi) the best promoters improving the efficiency of back extraction may be isobutanol and isopropyl alcohol but this is not valid for all types of proteins.

In order to devise a generalized guideline for the selection of alcohols for protein back extraction, extensive study needs to be done with respect to the types of proteins and alcohol species. The generality of using certain alcohols for certain type

of proteins or surfactants in back extraction would be of considerable advantage in the reverse micellar extraction processes.

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