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Reverse micelle extraction-an alternative for recovering antibiotics

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ABSTRACT

Downstream processing of antibiotics is usually conducted through conventional solvent extraction. Several limitations of the conventional solvent extraction are formation of stable emulsion, solvent loss using volatile solvents, low extraction yield, and unable to separate amphoteric antibiotics which are barely soluble in organic solvents. Reverse micelle extraction is an attractive alternative for replacing conventional solvent extraction of antibiotics. It has several advantages: high selectivity, high mass and activity recovery, simple operation, cost effective, easy to scale up, using non-toxic substances, and can design to be as environmental friendly as possible by using biosurfactants. This paper briefly introduces reverse micelle extraction and reviews several crucial factors that should be manipulated during operation to achieve optimum recovery. The process can be easily optimized by adjusting those factors.

Keywords: reverse micelle extraction; review; downstream processing

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INTRODUCTION

Antibiotics are a class of drug that are capable to destroy bacteria (bactericidal) or slow down the growth ofbacteria and organisms (bacteriostatic) [1]. Sometimes they are described as good bacteria which have a role to fight unpleasant bacteria [2]. Antibiotics are often prescribed in modern medicine as antibacterial agents to effectively cure diseases caused by bacterial infection. Antibiotic can be of naturally derived antibiotic, semi-synthetic antibiotic or synthetic antibiotic. They are usually produced through fermentation process. Their high volume but low product concentration streams make downstream processing very cost intensive. Conventionally, antibiotics are separated from broth through solvent extraction. When brought into contact with another immiscible liquid phase, antibiotics in aqueous phase tend to distribute themselves between the two phases and allow separation process to occur with ease [3]. This method is widely applied for recovering lipophilic antibiotics from broth. However, the main problem of using conventional liquid-liquid extraction is the formation of stable emulsion which makes further processing difficult [4]. Some other problems that had been identified are solvent loss using volatile solvents, low extraction yield, and unable to separate amphoteric antibiotics which are barely soluble in organic solvents [3, 5].

Other methods for separating and purifying antibiotics include liquid membrane extraction, aqueous two-phase system, chromatography, and membrane separation [6]. Liquid membrane requires stable membrane formation which hinders its commercial operation [7]. Aqueous phase two-phase system has higher cost due to the polymer/polymer system and high ionic strength needed [8, 9]. Chromatography is hard to scale up due to the equipment used, while membrane separation suffers from fouling and clogging problems. Downstream processing of

antibiotics requiresa method which is efficient, simple, cost effective, easy to scale up, using non-toxic substances, and preferably environmental friendly. An alternative that possesses these characteristic is reverse micelle extraction [10].

Introduction to reverse micelle extraction

Isolated surfactant molecules tend to arrange themselves spontaneously into ordered structures through self-assembly process. Weak noncovalent bonds such as Coulomb interaction, hydrophobic interaction, and hydrogen bonds, as well as weak covalent bonds such as coordination bonds are found to be the interactions involved during micelles formation [11]. The types of surfactant aggregates can be micelles, vesicles or reverse micelles. Their size and shape depends mainly on several factors, which can be described by packing factor, *g*:

$$g = \frac{V}{a_0 l_c}$$

V is the volume of hydrophobic chain, a_0 is the mean cross-sectional area of head group in the aggregate, and l_c is the length of the fully extended chain. The condition for reverse micelles to form is when $g > 1$. A minimum surfactant concentration should also be achieved to form functional reversed micelles. This minimum surfactant concentration is known as the critical micelle concentration (CMC).

Reverse micelles are aggregates of surfactant molecules in apolar (organic) solvents with aqueous cores trapped by their polar heads. This unique feature allows reverse micelles to solubilize water and hydrophilic molecules such as proteins and dyes. The schematic of surfactant molecule and structure of a single reverse micelle is shown in Figure 1 and Figure 2 respectively [12]. Reverse micellar system is particularly effective on extracting charged molecules due to strong electrostatic interaction between surfactant molecules and target molecules. The most common surfactants used to form reverse micelles include AOT (Aerosol OT (bis 2-ethylhexyl) sodium sulfosuccinate), SDS (sodium dodecyl sulfate), and CTAB (cetylmethylammonium bromide). These are ionic surfactants which can provide strong electrostatic interactions between surfactant head groups and target molecules. Sometimes non-ionic surfactants such as Tween 80, Tween 85, and Triton X-100 are added to the ionic surfactants to reduce the electrostatic interactions and improve the reverse micelle extraction process.

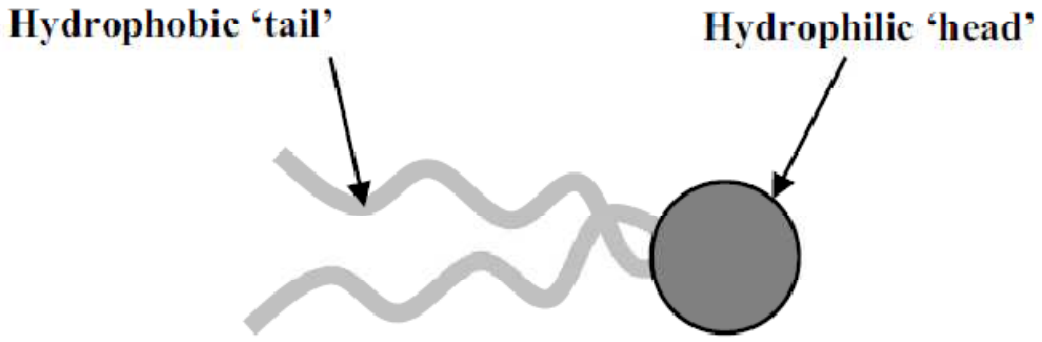

Figure 1: Schematic of a surfactant molecule [Adapted From: 12]

Reverse micelle extraction is widely studied for downstream processing of biotechnology products [13-16]. Various bio-molecules such as penicillin G [17],nattokinase[18], chitosanases[19], polyphenol oxidase [20], β-glucosidase[21], lipase [22], and laccase[23] were effectively recovered through reverse micelle extraction. The bio-molecules recovered often retain high activities because they are shielded by surfactant molecules from direct contact with organic solvents during the extraction process. This prevents the bio-molecules from denaturation. It can dissolve molecules which are normally insoluble in bulk organic solvents and it has potential for large scale continuous operation [12]. With these characteristics, reverse micelle extraction is a suitable alternative for recovering antibiotics from broth.

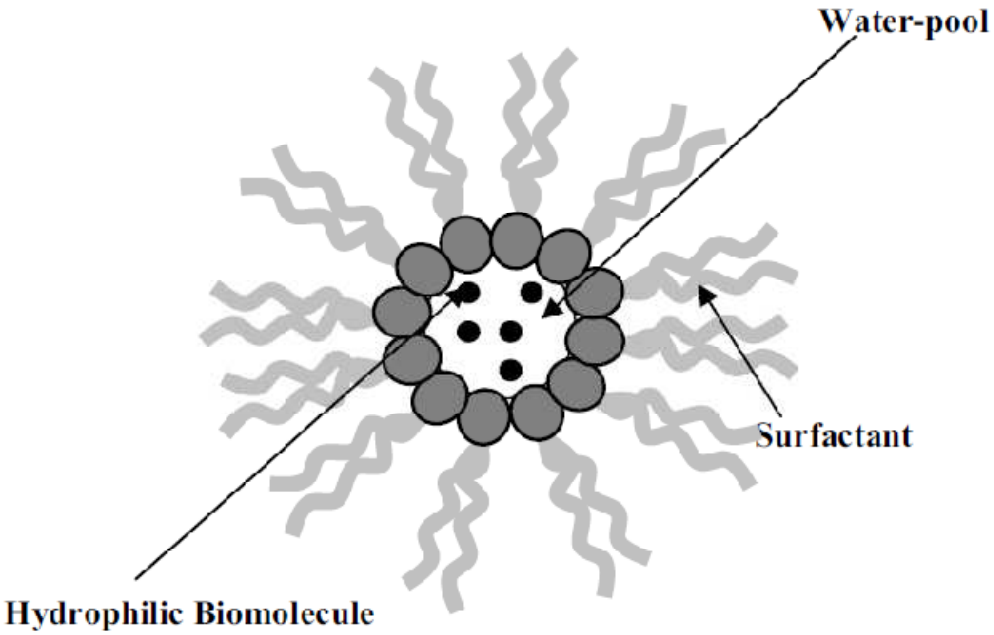

Figure 2: Structure of a reverse micelle [Adapted From: 12]

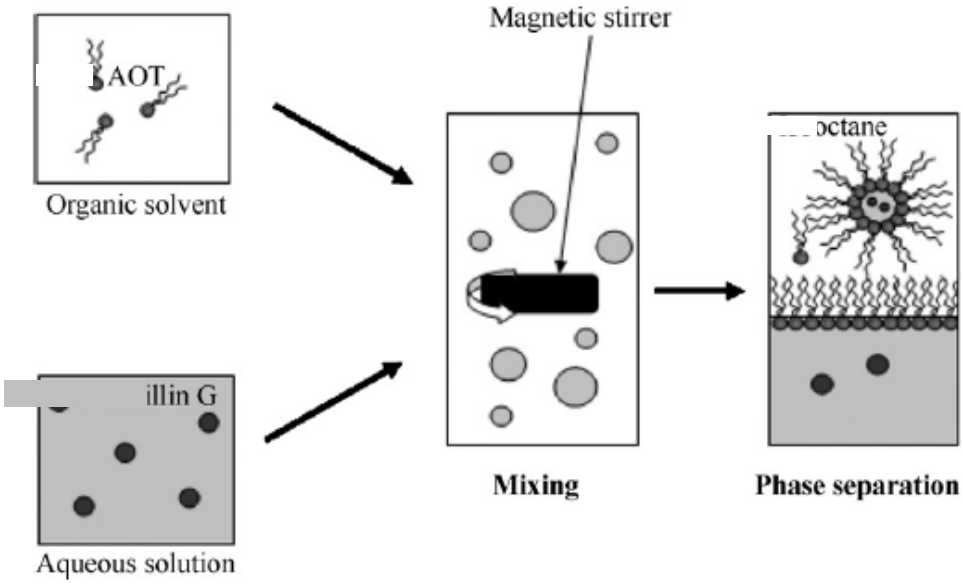

Figure 3: Forward extraction procedure [Adapted From: 27]

Forward extraction

Generally reverse micelle extraction is conducted in two steps which are forward extraction and backward extraction. During forward extraction, the aqueous phase containing target molecules are brought into contact with organic phase containing reverse micelles [24-26]. Phase mixing through external agitation for short period is required. During the mixing process, target molecules from aqueous phase are solubilized into the water cores of reverse micelles in organic phase. When extracting bio-molecules, the agitation rate must be controlled carefully because emulsion will form if the agitation rate is too high. The emulsion will hinder the extraction process and causes low

extraction yield. The schematic of forward extraction is given in Figure 3 [27]. Forward extraction efficiency is governed by several important factors which will be discussed in following sub-sections.

Effects of aqueous phase pH

Since electrostatic interaction is one of the major interactions in reverse micelle extraction, changing the solution pH will directly affect extraction performance because pH change influences the surface change of molecules in solution. The effect is particularly significant on antibiotics which are zwitterions. They are sensitive to pH and limited range of pH is applicable before they loss their activities. At pH above pI, the antibiotics become negatively charged while at pH below pI, they become positively charged [28]. Generally, solution pH is adjusted so that the molecules have opposite charge as the surfactant used to ensure extraction occurs. There often exists an optimum pH for reverse micelle extraction of protein.

A report on reverse micelle extraction of bovine serum albumin (BSA) and lysozyme using anionic AOT shows that maximum forward extraction percentages are located slightly below their pI values [29]. This trend can still be observed when non-ionic surfactants are added to the reverse micelles. Extraction of amoxicillin using AOT/TWEEN 85 mixed reverse micellar system was found to achieve maximum extraction percentage at pH lower than pI of amoxicillin [30]. When pH was increased to near pI value (4.7), the electrostatic attraction between antibiotics and surfactant molecules weakened thus reducing forward extraction percentage. Besides antibiotics surface charge, pH also affects the formation of reverse micelles by some surfactants. For example, anionic dioleylphosphoric acid (DOLPA) does not form reverse micelles at low pH range due to abundance of hydrogen ions which makes DOLPA molecules exists as undissociated form [31]. DOLPA reverse micelles only start forming at medium pH range.

Effects of surfactant concentration and type

After reaching CMC, adding more surfactant will cause more reversed micelles to form rather than substantial increase in micellar size. This allows more target molecules to be solubilized into the organic phase and therefore, better extraction performance. It can be explained using following simple model:

Antibiotics + n*Reversed micelles → Antibiotics-Reversed micelles
[Reversed micelles] = [Surfactant] / Aggregation number

First line of the model shows that antibiotic molecules react with n number of reversed micelles to form antibiotics-reversed micelle complex. Second line shows that concentration of reverse micelles equals to concentration of surfactant divided by aggregation number. Assuming that aggregation number is not related to surfactant concentration, the concentration of reverse micelles will be proportional to surfactant concentration, and so with the amount of antibiotic molecules that can be solubilized into reversed micellar phase. An optimal surfactant concentration value can often be determined.

When AOT concentration is increased, amount of penicillin G solubilized into the organic phase also increased [5]. However, stable emulsion was observed at high AOT concentration (>445g/L). Increasing CTAB concentration from 25 to 75 mM also reported to increase tannase recovery from 53% to 67% and dropped at higher CTAB concentration [28]. Forward extraction efficiency usually drops at high surfactant concentration due to intermicellar collision and micellar clustering [23].

The type of surfactants used affects forward extraction efficiency significantly. Some surfactants like AOT are capable to form stable reverse micelles at wide range of operating conditions. On the other hand, some surfactants such as zwitterionic surfactants only form reverse micelle at certain pH range. Selection of surfactant is crucial to ensure stable reverse micelles are formed during the extraction process. Mixing different surfactants also improve the forward extraction. AOT/TWEEN 85 reverse micellar system has significantly lower CMC compared to that of AOT alone [32]. This allows 120 times higher amoxicillin solubilization into the organic phase compared to both single surfactant system at low surfactant concentration [33]. Water solubilization in mixed reverse micellar system is also reported [34].

Effects of salt concentration and type

A minimum amount of salt is usually needed to form stable reverse micelles. Increasing KCl concentration up to 10g/L was found to increase penicillin extraction significantly [27]. However, the forward extraction efficiency

dropped when KCl was further increased. On the other hand, forward extraction percentage of erythromycin was found dropping when KCl was added [35]. This shows that even the same salt type may have different effects on different reverse micellar systems. The decrease of forward extraction efficiency at higher salt concentration is generally due to the Debye screening. Salts can reduce the electrostatic interaction within microemulsion droplet, causing weaker protein-surfactant charge interaction and reduce the size of microemulsion droplets [36]. This is useful for separating molecules with different size since larger molecules will be excluded by adjusting the salt concentration but when the reverse micelles become too small, the desired molecules will also be excluded and causes low forward extraction percentage.

Different salt types may give different impacts on proteins. Some cations (Na, Ca, Zn) have inhibitory effects on yeast laccase while some cations (K, Mg, phosphate) have activating or stabilizing effects [36]. The effects of KCl and $CaCl_2$ were compared and the results show that $CaCl_2$ leads to smaller reverse micelles. The system with $CaCl_2$ becomes more sensitive to stirring and form emulsion easily [27]. Another report states that adding NaCl salt gives the highest extraction of penicillin G compared to KCl and $CaCl_2$[31]. The reasons to these phenomena may lie at the valent number and equilibrium constant of the salts.

Effects of initial antibiotic concentration and antibiotic type

For a fixed amount of reversed micellar phase, the extraction capacity would be constant due to the amount of reversed micelles available for extraction. Therefore, increasing initial antibiotic concentration at fixed surfactant concentration will result in lower forward extraction percentage due to limited number of reversed micelles available for solubilization. An investigation on the effect of penicillin G concentration in kinetic study reveals that transfer efficiency is highest at low penicillin G concentration [37]. Higher initial penicillin G concentration should provide greater driving force for extraction. However, the percentage of extracted penicillin G is lower.

Different antibiotics also have different extraction percentage under similar extraction conditions. Five antibiotics (penicillin G, ampicillin, streptomycin, teicoplanin, and rifampisin) were extracted using DOLPA reverse micelles [31]. Although the trend of decreasing extraction percentage at higher antibiotic concentrations was observed, they each showed very different behaviors. This can be explained by their different antibiotic groups and molecular structures. Since antibiotics are complex structures, they may have interacted with the reverse micelles at different ways.

Backward extraction

After forward extraction, the antibiotics in organic phase need to be released into a fresh aqueous phase through backward extraction. The procedure is similar to those in forward extraction: organic phase loaded with antibiotics is mixed with fresh stripping aqueous phase. Longer time may be needed because backward extraction is more difficult and a slower process compared to forward extraction. The process involves destabilization of reverse micelles or salt out of molecules loaded in reversed micelles. Additives such as salt or co-solvents may be required to achieve high extraction yield. Backward extraction efficiency is also governed by several important factors [38-41].

Effects of pH of stripping aqueous phase

If the aqueous phase pH during forward extraction is adjusted to obtain maximum electrostatic attraction between antibiotics and surfactant head groups, then the pH of stripping aqueous phase will be adjusted to obtain the least electrostatic attraction between antibiotics and surfactant. Backward extraction of BSA and lysozyme were found higher at pH higher than their respective pI[29]. Maximum extraction of amoxicillin was located at solution pH slightly above its pI for both AOT and AOT/TWEEN 85 reverse micellar systems but almost no backward extraction was observed at pH lower than its pI[30]. Similar trend was reported by other researchers [22, 23]. These results support the first statement regarding the effects of pH.

Effects of salt concentration

Second important factor to achieve high recovery of antibiotics during backward extraction is salt concentration. Usually higher salt concentration is needed to decrease electrostatic interaction, destabilize reversed micelles, decrease electrostatic repulsion between surfactant head groups, and thus allows more protein to be released. Increasing salt concentration leads to thinner double-electric layer and smaller micelles[42]. Almost all penicillin G can be transferred to aqueous phase during backward extraction at high salt concentration within wide pH range [5]. IncreasingKClconcentration up to 12g/L enhanced the release of amoxicillin to aqueous phase due to reduction of reverse micelles size [30]. However, when higher salt concentration was used, white precipitates were observed and

lower backward extraction efficiency was obtained. This is probably due to the formation of electrostatic shield surrounding reverse micelles which hindered release of antibiotics as well as denaturation of antibiotics at high salt concentration.

Effects of surfactant concentration

The concentration of surfactant used during forward extraction affects backward extraction indirectly. Optimal lysozyme backward extraction was obtained at low AOT concentration (20mM) while BSA at higher AOT concentration (100mM) [29]. This observation was explained through difference in denaturation behavior of lysozyme and BSA. For penicillin G, highest backward extraction was reported at 25mM DOLPA [31]. This is related to forward extraction where saturation of surfactant molecules leads to formation of antibiotics-surfactant complex which are difficult to separate.

Effects of initial antibiotic concentration

Backward extraction of penicillin G achieved higher mass transfer rate at higher initial penicillin G concentration [37]. This is similar to the observation during forward extraction. Although having higher mass transfer rate, increasing initial antibiotic concentration generally decrease the backward extraction percentage due to the limited capacity of stripping aqueous phase to recover the higher amount of antibiotics contained in reverse micelles [31]. Slight decrease in backward extraction efficiency was observed when higher feed protein concentration was introduced while activity recovery was found to be near constant [21]. This situation may be related to the selectivity of the reverse micellar system under the experimental conditions investigated.

Effects of co-solvents or additives

Sometimes co-solvents or addictives are needed during backward extraction to achieve reasonable recovery efficiency. The recovery of penicillin G is very low without any additives[17]. Addition of 5% v/v hexanol was found to significantly enhance backward extraction efficiency of amoxicillin even at pH lower than its pI[30]. Addition of 10% v/v ethanol during backward extraction was found to enhance the recovery of laccase[23]. However, adding isopropanol has no significant effect on backward extraction of tannase[22]. Alcohol molecules are known to be capable of penetrating the reverse micelles, which may destabilize them. Smaller alcohols should have better destabilizing ability compared to bulky alcohols due to the penetration degree of the alcohols.

Addition of dilute chaotropecan significantly increase the recovery of kallikrein[43]. Analysis showed that chaotrope influences the size of reverse micelles to make the release of protein easier. Another method to enhance backward extraction efficiency is by adding counter-ionic surfactant. The oppositely charged surfactant molecules will neutralize the charge of surfactant shell and lead to collapse of reversed micelles [20], thus promoting the release of molecules contained inside.

CONCLUSION

Over decades of investigations, reverse micelle extraction has showed its potential as an alternative for downstream processing of antibiotics. It can selectively extract desired molecules at high extraction efficiency through electrostatic interactions and hydrophobic interactions. It is capable for scale-up and continuous operation. The costs are not high because it only requires the same equipment as conventional liquid-liquid extraction. Safer solvents can be used for extracting antibiotics which are conventional extracted through toxic solvents, besides eliminating the need of expansive de-emulsifiers which are sometime used in conventional solvent extraction process. The total extraction time of reverse micelle extraction is significantly shorter than conventional solvent extraction. Recent studies also show that biosurfactants can be used instead of synthetic surfactants. This allows greenerdownstream processing of antibiotics to be established.

The efficiency of reverse micelle extraction can be adjusted easily by changing several crucial factors. These factors are aqueous phase pH, surfactant concentration, salt concentration, and concentration of antibiotics. Aqueous phase pH affects the surface charge of antibiotics, thus it should be adjusted to allow highest electrostatic interaction and lowest electrostatic interaction possible between antibiotics and surfactant molecules during forward extraction and backward extraction respectively. However, care should be taken to avoid denaturation due to extreme pH.

Generally, increasing surfactant concentration increases the amount of reversed micelles and thus increases the extraction percentage. Usually an optimum surfactant concentration can be determined. Nevertheless, too high

surfactant concentration may cause adverse effects on extraction such as reduced extraction efficiency and more impurities extracted.

Salts are usually added during reverse micelle extraction to reduce the size of micelles. This may lead to reduced forward extraction efficiency but minimum amount of salt is sometimes needed to stabilize the reverse micelles. During backward extraction, addition of salt helps to squeeze out the molecules loaded in reversed micelles. In case of extracting antibiotics, salt concentration must be controlled carefully to avoid denaturation.

Initial antibiotic concentration also affects the final recovery of antibiotics. However, it depends on the amount of reverse micelles available for solubilization. If the amount of reverse micelles available is limited, the extraction percentage of feed with higher antibiotic concentration will be lower. Thus initial antibiotic concentration should be adjusted to achieve optimal recovery whenever possible.

Although many studies had been reported regarding reverse micelle extraction, most of them were conducted in lab scale. Further studies should be conducted to investigate the large scale operation, continuous operation, and solvent recovery. Besides that, development of green reverse micelle extraction is also in progress. With all the advantages, reverse micelle extraction should be an attractive alternative for industrial recovery of antibiotics.

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