PROTEIN EXTRACTION USING REVERSE MICELLES

By

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DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not currently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

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DEDICATION

This thesis is dedicated to my parents and my husband.

ABSTRACT

Reverse micelles are self-organized aggregates formed by a surfactant in a non-polar solvent or oil. The presence of a water pool in the polar core of reverse micelles is of considerable advantage in protein extraction. A lot of researches have been done with ionic reverse micelles applied in protein extraction. However, this ability of non-ionic reverse micelles has not been fully understood and therefore requires more research.

In this project, different surfactants (anionic AOT, cationic CTAB, non-ionic triblock L61 copolymer) were investigated for their ability to form RM and for their application in protein extraction. It was found that lysozyme could be extracted using an AOT RMS, but not with a CTAB RMS. For the first time, an aromatic solvent, *p*-xylene, was used for the extraction of lysozyme and it was found that the AOT in *p*-xylene RM system resulted in the higher lysozyme activity (73.81 %) compared to an AOT/isooctane RM system (43.2 %). The effect of different salts (KCl, KF, KBr) on the FE and BE of BSA was investigated using the CTAB in mixture of 1-bromooctane, 1-hexanol and petroleum ether. The results indicated that KCl gave the highest extraction efficiency of 64 % as compared to around 40 % with both cases of KF and KBr. The secondary structure of extracted BSA was maintained with KCl only. L61 pluronics polymers was investigated for its reverse micelles forming ability and it was established that small reverse micelles with a maximum W_0 of 4 was formed. Because of the small size of L61 reverse micelles, lysozyme could not be extracted but was precipitated out when combined with the co-surfactant AOT . The activity of the recovered lysozyme from the precipitate was maintained (66 % as compared with native lysozyme). Moreover, if L61 was used as a co-surfactant with AOT reverse micelles, extraction efficiency was improved (88 %) and the activity of the extracted lysozyme was increased (56 %) as compare to extraction with an AOT system alone (46 %). These studies thus gives useful insights in the role of individual and mixed surfactant systems in the extraction and precipitation of proteins.

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LIST OF ABBREVIATIONS

AOT	-	Docusate sodium salt
AQP	-	Aqueous phase
BE	-	Backward extraction
BSA	-	Bovine serum albumin
CD	-	Circular Dichroism
CMC	-	Critical micelle concentration
CTAB	-	Hexadecyltrimethylammonium bromide
FE	-	Forward extraction
HLB	-	Hydrophilic-lipophilic balance
IR	-	Ultrafast infrared spectroscopy
LLE	-	Liquid-liquid extraction
MW	-	Molecular weight
ORP	-	Organic phase
PPG	-	Poly (propylene glycol)
PEG	-	Poly (ethylene glycol)
RM	-	Reverse micelles
RMS	-	Reverse micellar system
RME	-	Reverse micellar extraction
UV	-	Ultraviolet
Vis	-	Visible
W_0	-	Water content
E_f	-	Forward extraction efficiency
E_b	-	Backward extraction efficiency

1.INTRODUCTION

1.1 Downstream processing

Downstream processing is the isolation and purification of biosynthetic products such as proteins, enzymes, monoclonal antibodies etc. from crude mixtures of bacterial or animal cells cultures or from the fermentation broth (Andrew, 1991). In industrial fermentation, downstream processing is the last stage before the final purified products are obtained (Fig. 1.1).

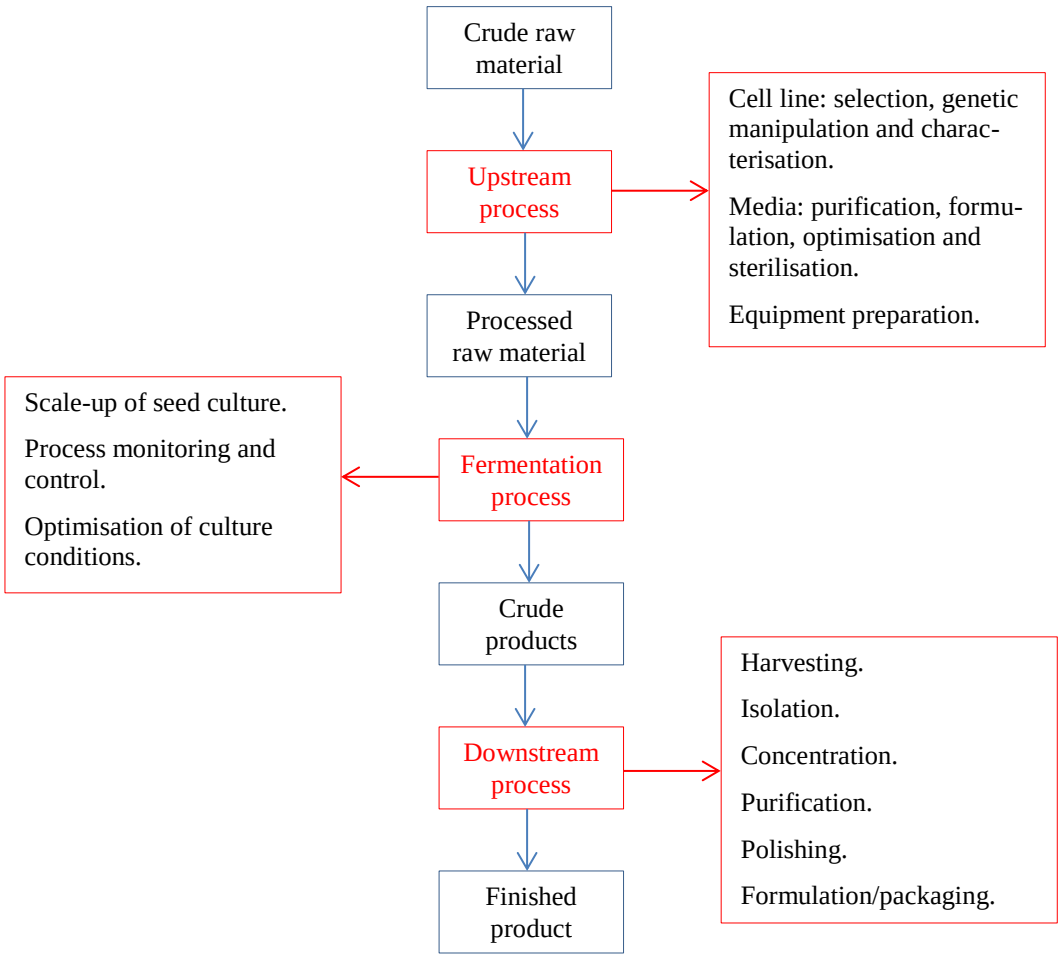

Figure 1.1: Basic steps in an industrial fermentation process (Andrew, 1991).

Biological products are important for many applications including biotransformation, diagnostics, research and development, and in the food, pharmaceutical, and cosmetics

industries. Among them, proteins are currently the most important biopharmaceuticals (Christi, 1998). Principally, the actual production of recombinant proteins typically takes place in fermentation tanks (where bacterial cells are used) or bioreactors (where mammalian cells are used), and are usually scaled up to tens of thousands of gallons. However, the purification systems (downstream processing) have not been able to keep up with this scale up as downstream processing involves a number of steps (Buchacher, 2006; Kelly, 2005). In addition, after fermentation, most of the protein products are present in very low concentration, along with large number of impurities that may only be slightly different from the product themselves (Prasad, 2010). These products are thermo-labile, and quite sensitive to chemical substances and environmental conditions such as pH and salt concentration (Sadana, 1998). As proteins are mainly targeted towards therapeutic applications, they are required at very high purity; therefore, it is crucial that more research is done in order to investigate newer methods for downstream processing (Clarke, 2013).

Downstream processing involves a number of steps such as reduction in bulk, concentration enrichment, removal of impurities (*e.g.* toxins from therapeutic products), enhancing protein stability and reducing protein degradation (Belter, 1988). Christi and colleagues (1998) published a scheme for downstream processing technique described as RIPP, which stands for Removal, Isolation, Purification and Polishing. Firstly, a large number of impurities need to be removed from biological feed streams in order to isolate the target proteins. Low-resolution, high-productivity techniques are normally included in this step (removal and isolation). Secondly, protein products are purified by using high-resolution, low-productivity techniques which falls under the purification stage. And finally, protein products are polished by using high-resolution, high-productivity techniques aimed at high product purity. In summary, these techniques must combine high productivity with high selectivity of separation, and must be feasible at mild operating conditions (Tab. 1.1).

Table 1.1: Techniques for downstream processing stage according to RIPP scheme (Christi, 1998)

High-productivity, low-resolution	**High-resolution, low-productivity**	**High-resolution, high-productivity**
Cell disruption Precipitation Centrifugation Liquid-liquid extraction Microfiltration Ultrafiltration Supercritical fluid extraction	Ultracentrifugation Packed bed chromatography Affinity separation Electrophoresis Supercritical fluid chromatography	Fluidized bed chromatography Membrane chromatography Ultrafiltration Monolith column chromatography

Companies that supply the biotechnology industry with tools for protein purification are perpetually trying to increase the speed, lower the cost, and improve productivity. However, they have many challenges to overcome. Firstly, the number of steps in the separation process varies from protein to protein along with technologies used for efficient separations. Each unit operation will bring about a physical change that will alter the product concentration and/or degree of purity. The increase in the number of steps for the separation of products results in increased time and cost of the overall process. For example, dilute speciality products typically incur high purification costs which may range from 50% to 90% of the total production costs (Clarke, 2013). Products, produced in low concentrations such as vitamins (e.g. vitamin B_{12}, a few mg/L) require processing of large volumes per mass of product, thereby necessitating large process equipment for purification, with concomitantly high capital and operating costs. The low concentrations also mean that more recovery steps are needed, with attendant cumulative losses. Secondly, processing of speciality products may require costly processing in dedicated equipment, to achieve the level of purification required, e.g. chromatography. Many of these speciality products are labile, so the range of purification operations is limited and less costly options may not be suitable. Thirdly, the finished protein products loose their activity because of temperature and pH changes during the process leading to an inactive protein (Clarke, 2013).

Recent developments in biotechnology have opened up new avenues towards the production of many biomolecules of importance in research, pharmaceutical/clinical and industrial usage. Highly selective and economical separation methods are becoming more and more important in commercial processes. Although, there has been considerable research in the area of production of biological products, the technology for separating them from the media has not kept pace with the upstream processing (production of biomolecules). The separation of many biomolecules from the product stream is still performed by batch mode, small scale processes such as column chromatography, salt and solvent precipitation and electrophoresis for which scale up poses considerable problem, making them uneconomical unless the product is of high value (Harikrishna, 2002). Although, the affinity based chromatographic separations have excellent selectivity and are being carried out on a large scale; for the most part, such systems operate discontinuously and the economy of scale has not often been realized (Ohlson, 1989). In view of the fact that product recovery costs becomes critical in the overall economics of modern biotechnological process, there has been an increased interest in the development of efficient and scalable downstream processing methods for separation, concentration and purification of biomolecules.

Developing a downstream process to recover a biological product in large quantities occurs in two stages, namely, design and scale-up. The process design is concerned with selection of steps for separation of a product from contaminants and impurities, and normally requires a series of purification steps, each removing some of the impurities and bringing the product closer to the final specification. These form the basis for scale-up and define the type of equipment, its size, and the operating parameters (Milton, 2007).

There are six groups of downstream processing operations which are, removal of insoluble, cell disruption, extraction, concentration, purification and formulation (Prasad, 2010). The aim of these operations is to obtain the product of interest in its native state. In the extraction stage,

there are several techniques that are popular in biotechnology industry, which includes LLE, supercritical fluid extraction, aqueous two-phase extraction and RME (Prasad, 2010). Among them, LLE shows distinct advantages.

1.2 Liquid-liquid extraction

1.2.1 Principle

LLE is the transfer of certain components (solute) from one phase to another when immiscible or partially soluble liquid phases are brought into contact with each other (Mazzola, 2008).

This process is described for continuously extracting substances from a first liquid with a second liquid in such a way that both are able of form two phases separated from each other (Fig. 1.2). These phases define a liquid-liquid interface. Contact of the two liquids is comprised in an apparatus in order to define a plurality of mixing chambers and one settling chamber at least. The liquids are continuously shaken in the mixing chambers to support mixing in the state of being near the liquid-liquid interface (Xu *et al*, 2001).

There are two requirements for LLE to be feasible: firstly, component(s) to be removed from the feed must preferentially distribute in the solvent; secondly, the feed and solvent phases must be substantially immiscible. Compared with other techniques, LLE belongs to a group of high-productivity, low-resolution group which is represented in Tab. 1.1.

LLE can be carried out in a number of ways such as batch extraction, stage extractions, differential extractions, and fractional extraction with a stationary phase or moving phase (Prasad, 2010).

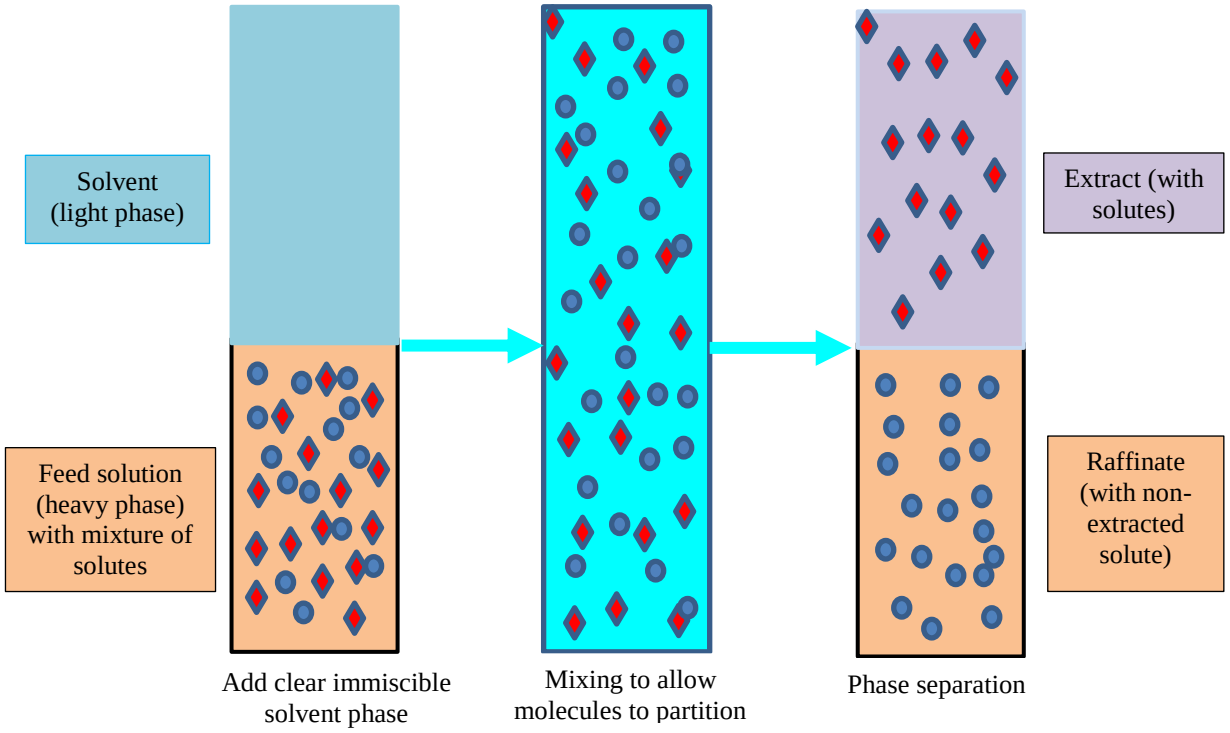

Figure 1.2: Liquid – liquid extraction principle

However, batch extraction may not be suitable for large scale extractions as it does not extract the product in pure form and to achieve a desirable yield it requires a number of steps which is not economical (Prasad, 2010). In such situations, stage extractions may be the preferred choice in the industry.

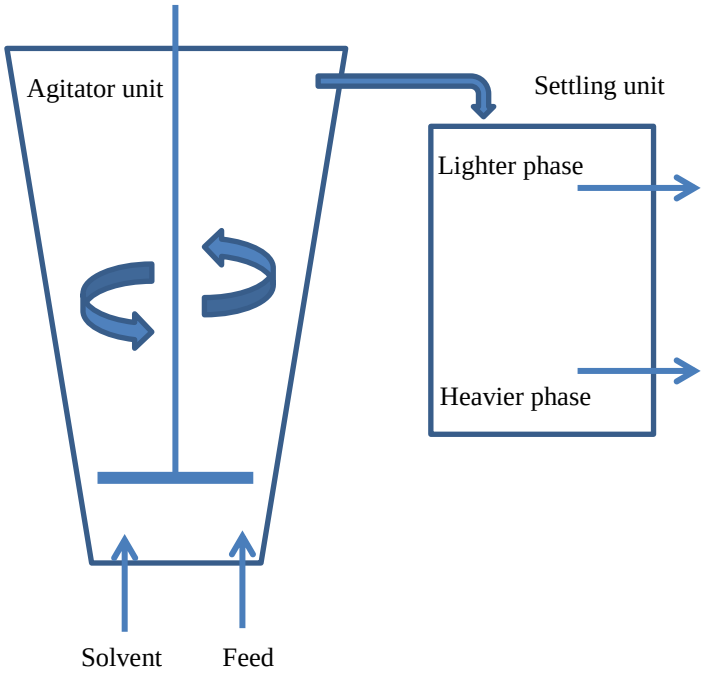

Figure 1.3: Diagrammatic representation of batch extractor

1.2.2 Stage extraction

In the stage extraction process, an agitator and a settler combine as a single unit and work continuously. Therefore, the desired product is isolated by repeated extraction in cascade of separation equipment, which is important when batch extraction is not efficient. Principally, stage extraction is similar to batch extraction as presented in Fig. 1.3.

1.2.3 Differential extraction

In differential extraction processes, the biological fluid and solvent continuously flow past one another in the equipment having a tubular geometry. Two phases usually enter in opposite direction into the chamber with an arrangement to ensure intimate mixing of the two phases. Process of flow of liquid past one another and settling into two separate phases depends on the density difference. There are different types of differential extractors, but in general, in all the extractors, biological AQP flows slowly down the tube and the solvent liquid flows past the heavy phase in opposite direction. The principle can help to increase contact-time of two phases in order to improve the extraction efficiency (Fig. 1.4) (Prasad, 2010).

1.2.4 Fractional extraction

Fractional extraction is a continuous steady state process in which the biological phase and solvent phase move counter currently separating a continuous feed with the product of interest. A fractional separator not only isolates the product of interest but also purifies it. In this type of extraction, the biological feed enters in the middle zone of the column that contains the product of interest with impurities, polar solvent enters at the top of the column and extracts the product of interest, and a straight-chain hydrocarbon enters at the bottom of the column that preferentially extracts the non-polar organics. Product stream leaves the column through the

outlet situated at the bottom and the impurities leave the column through an outlet situated at the top of the column (Fig. 1.5) (Prasad, 2010).

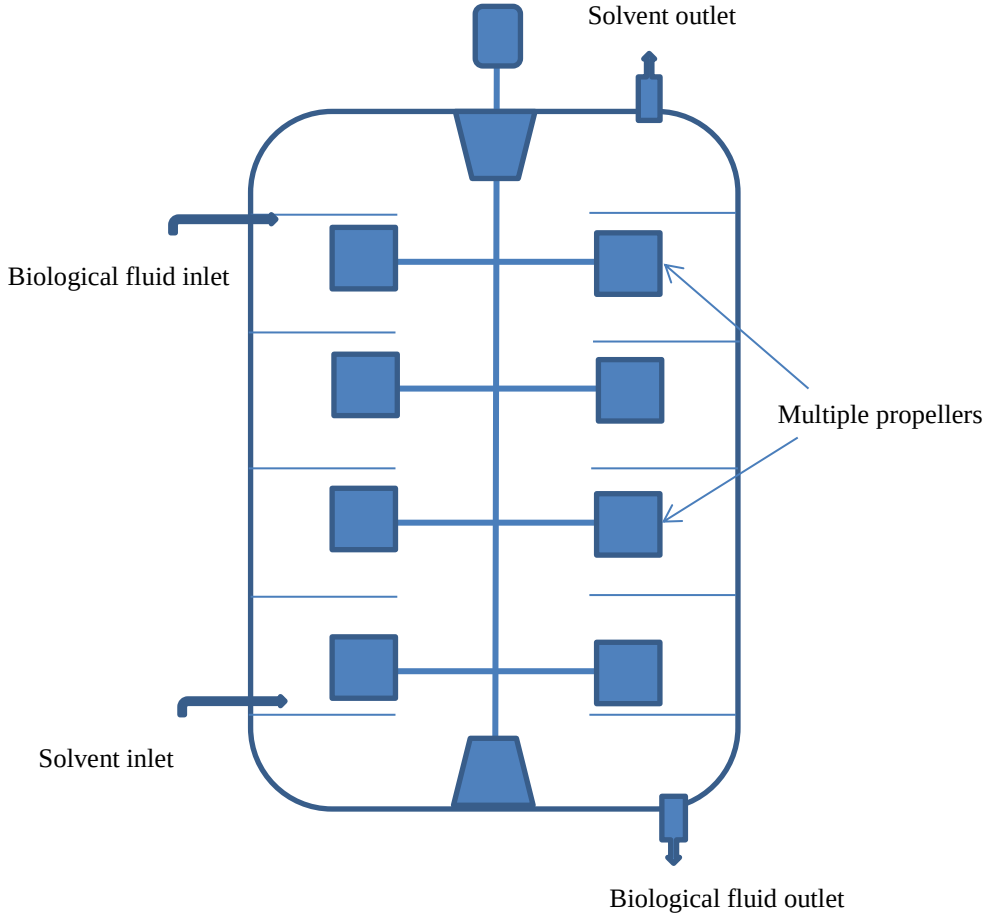

Figure 1.4: Diagrammatic representation of differential extractor.

1.2.5 Applications

The LLE technique has been used for the separation of rare metals based on the differences in their ability to form a chemical complex between the extracted subject and the particular metal species, and thus, the extractability and separation ability obtained were highly dependent on the chemical structure of the extracted subjects (Nishihama, 2001).

Biofuel and chemicals produced by biological processes such as fermentation and algae often require LLE as the first step in recovery and purification. Many of these chemicals have a higher boiling point compared to water and form azeotropes, resulting in high energy requirements for distillation. LLE often offers a process which significantly reduces energy requirement, and as

such can provide a cost effective process which has minimal energy utilization, a key factor for effective biofuel technology (Biotechnology, 2014).

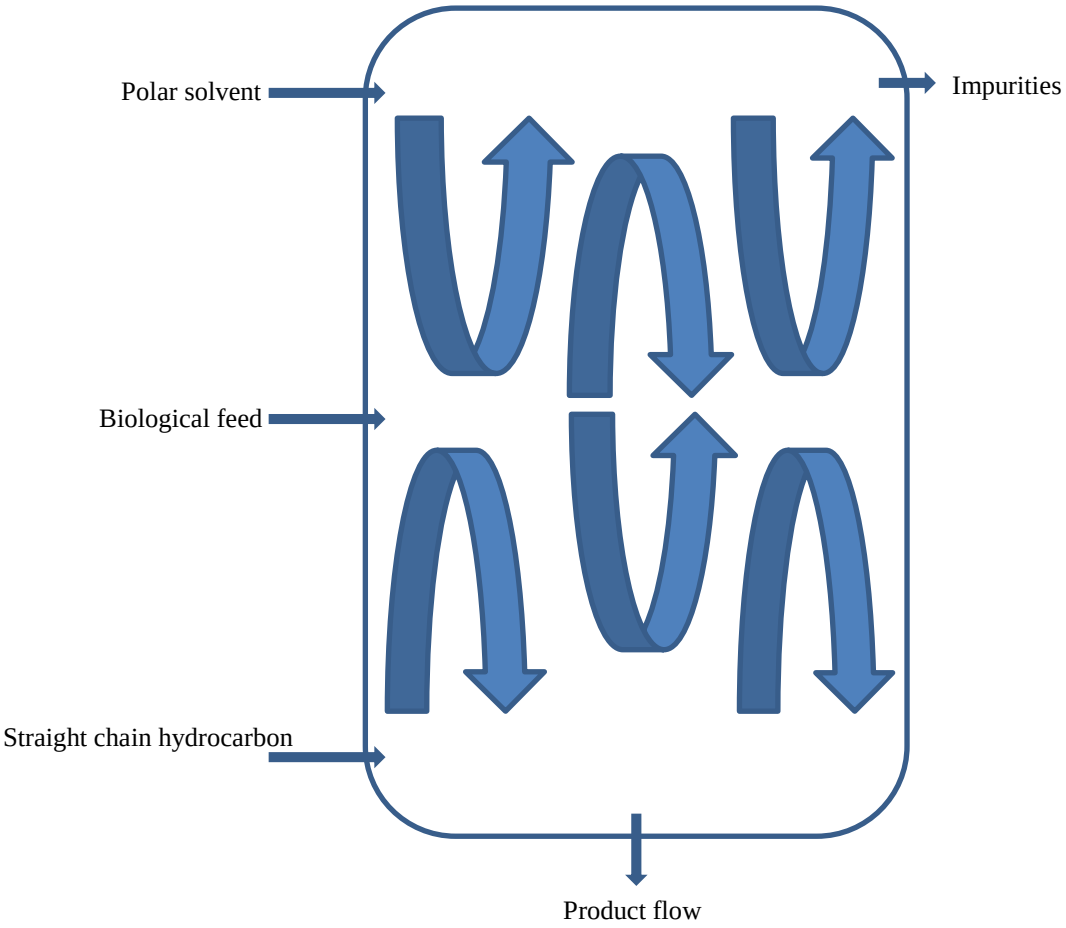

Figure 1.5: Diagrammatic representation of the fractional extractor

LLE also has many successful applications in the pharmaceutical industry because of its inherent flexibility and its suitability for processing heat-sensitive products (Crowell, 1997), examples include purification of recombinant pharmaceutical proteins such as human insulin-like growth factor 1 (IGF-1) (Hart, 1995), intracellular human recombinant interferon a1 (rhIFN-a1) (Guan, 1996), antibodies as IgG using an enteric coating polymer, Eudragit S100, as a ligand carrier (Kamihira M. K., 1992), glucose-6-phosphate dehydrogenase as the first enzyme in the pentose phosphate pathway (Hasegawa, 1992), and penicillin (Guan, 1996).

LLE is widely used as a pre-treatment technique for separation and pre-concentration of both organic and inorganic compounds from aqueous samples. However, it has a few drawbacks, such as emulsion formation and use of large volumes of solvents, which makes LLE expensive and labour intensive (Pena-Pereira, 2009). However, in comparison with other techniques, LLE is known as a suitable method for processing because of advantages such as handling large capacities with less energy consumption (for example, separation of paraffinic and aromatics in the oil industry; protection of heat sensitive products due to special environmental conditions or sensible temperatures, (as in vitamin production) and separation of small contents of high-boiling impurities, mostly in aqueous solutions. In the normal thermal separation techniques, the complete water contents have to be withdrawn by a very energy-intensive evaporation process (for example: elimination of phenol from aqueous waste). In summary, LLE is one important separation technique which has the ability to be scaled up easily, to be operated continuously, and to be highly selective (Simon, 2005). In this work, a LLE method in batch mode based on RM for the extraction of proteins is investigated as a preliminary study.

Kelly and colleagues has presented ‘tomorrows protein extraction needs’, Fig. 1.6 (Kelly, 2005). One of the methods described by them was RM method for protein extraction which is, principally, a type of LLE using surfactant in solvent as nano-particles. The next section describes the rationale behind this method for protein extraction.

1.3 Surfactant (surface active agent)

1.3.1 Characteristics

Surfactant is an abbreviation for surface active agent, which literally means active at a surface. In other words, a surfactant is characterised by its tendency to absorb at surfaces and interfaces. The term interface denotes a boundary between any two immiscible phases; the term surface indicates that one of the phases is a gas, usually air (Holmberg, 2002).

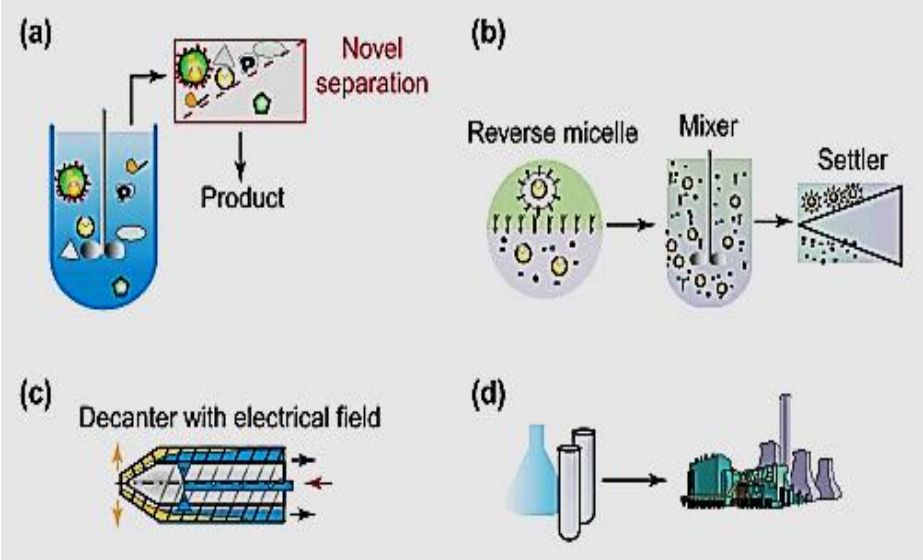

Figure 1.6*:* The protein extraction needs for tomorrow.
a) selective separation methods; b) scale-up of separation technologies to meet technical and economic requirements; c) the combination of traditional and novel methods and (d) cost-effective large-scale processes (Keller, 2001).

The driving force for a surfactant to adsorb at an interface is to lower the free energy of that phase boundary. The interface free energy per unit area represents the amount of work required to expand the interface. The term interfacial tension is often used instead of interfacial free energy per unit area. Thus, the surface tension of water (71.99×10^{-3} N/m at 25^0C) is equivalent to the interfacial free energy per unit area of the boundary between water and the air above it. When that boundary is covered by surfactant molecules, the surface tension (or the amount of work required to expand the interface) is reduced. The denser the surfactant packing at the interface, the larger the reduction in surface tension (Holmberg, 2002).

Generally, surfactants consist of a non-polar hydrocarbon tail, which is not easily dissolved in water, and a polar head group (either charged or uncharged), which is dissolved well in water. Therefore, surfactants combine hydrophilic and hydrophobic properties in one molecule, or surfactants are amphiphilic.

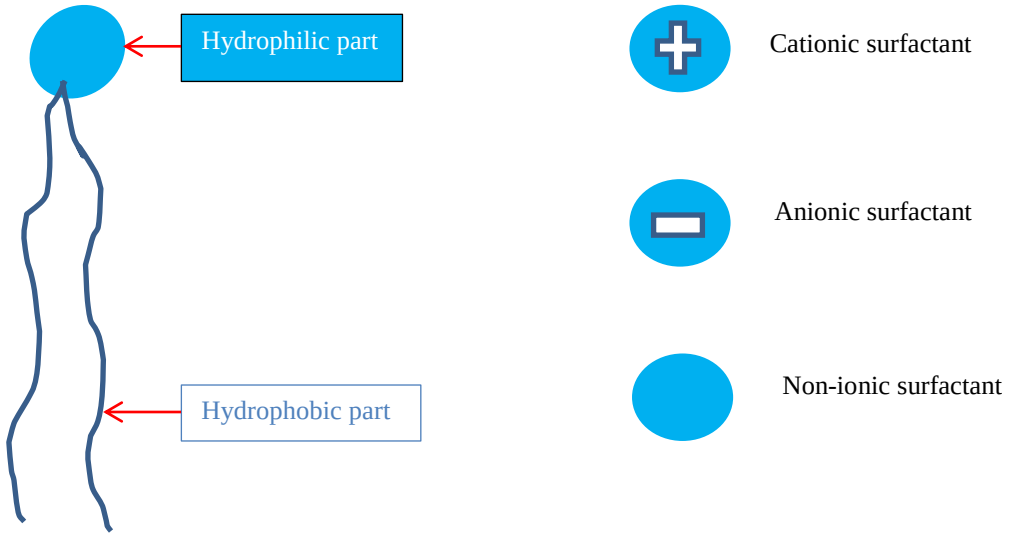

Figure 1.7: Short-chain surfactant molecular with hydrophilic head and hydrophobic tails.

One of the novel and principal reasons for the exploitation of surfactants is their significant ability to manipulate the properties of surfaces and interfaces. The applications of surfactants in industry are quite diverse, and also have a vast practical importance based on their specific characteristics (Tab. 1.2). Surfactants may have several advantages in the production and processing of foods, pharmaceuticals, agrochemicals, personal care and laundry products, mineral ores, petroleum, fuel additives and lubricants, coatings and adhesives, paints, and in photographic films (Schramm, 2003). Furthermore, they can also be found in a wide range of biological systems application, medical applications, soil remediation techniques, and other applications in environment, health, and safety (Manisha, 2009).

Table 1.2: Surfactant characteristic for various applications (Myers, 2006).

Application	Characteristics
Detergency	Low critical micelles concentration (CMC), good salt and pH stability, bio-degradability, desirable foaming properties
Emulsification	Proper HLB, application in environment and biology (safety).
Lubrication	Chemical stability, adsorption at surfaces
Mineral flotation	Proper adsorption characteristics on the ore(s) of interest, low cost.
Petroleum recovery	Proper wetting of oil-bearing formations, microemulsion formation and solubilisation properties, ease of emulsion breaking after oil recovery.
Pharmaceuticals	Biocompatibility, low toxicity, proper emulsifying properties.

1.3.2 Type of surfactant

The primary classification of surfactants is made on the basis of the charge of the polar head group. It is common practice to divide surfactants into four classes of anionic, cationic, non-ionic and zwitterion.

1.3.2.1 Anionic surfactants

They are the most commonly used surfactants which account for about 60% of the world production (13 million tons per year, worth $24.33 billion in 2008) (Liu, 2013). One main reason for their popularity is low manufacturing cost. Currently, AOT is considered as one of the most popular surfactants from this group for protein extraction (Fig. 1.8) (Myers, 2006). It is also a common ingredient in consumer products, especially laxatives of the stool softener, and as an emulsifier, dispersant, wetting agent and an adjuvant in pesticide formulation (Schor, 2010).

The cationic counter ions in the hydrophilic head most commonly used are sodium and potassium for water solubility, magnesium and calcium for oil solubility, and various amine/alkyl amines salts which makes the products both oil and water soluble. Anionic surfactant consist of alkyl-benzene sulfonates (detergents), lauryl sulphate (foaming agent), soaps (fatty acid), dialkyl sulfosuccinate (wetting agent), and lignosulfonates (dispersants).

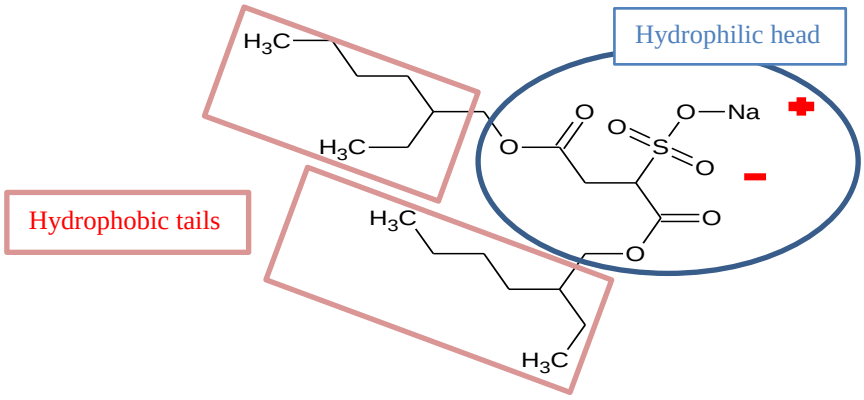

Figure 1.8: Structure of AOT molecule.

1.3.2.2 Cationic surfactants

Although cationic surfactants account for only 5-6 % of the total surfactant production, they are very useful for some specific uses based on their properties (Salager, 2002). Their positive charge allows them to adsorb onto negatively charged substrates which makes them suitable for application in fabric and hair rinsing (antistatic behaviour and softening action) and in inks and wood pulp dispersion (emulsifiers). They also act as bactericides and are therefore used to clean asepsis surgery hardware, formulate heavy duty disinfectants for domestic and hospital use, and sterilize food bottle in dairy and beverage industries (Salager, 2002). Most used cationic surfactants contain nitrogen compounds such as quaternary ammoniums and fatty amine salts, with one or several long chain of alkyl type, often coming from natural fatty acids. In general, these surfactants are more expensive than anionic ones, because of the high pressure hydrogenation reaction required to synthesis them (Salager, 2002). One of the most popular surfactant in this group is CTAB with more studies have reported (Fig. 1.9) (Bramer, 2007; Tah, 2011; Wang, 2014).

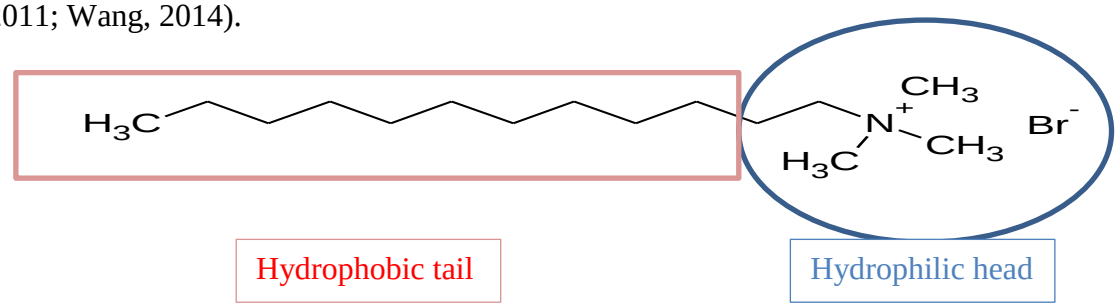

Figure 1.9: Structure of CTAB molecule

Common synthetic non-ionic surfactants are polyoxyethylene alcohol, alkylphenol ethoxylate, polysorbate 80, propylene oxide-modified polymethylsioxane, etc. (Salanger, 2002). Non-ionic surfactants are commonly used in paints, cosmetics, foodstuffs, and petroleum production additives industries because of their high biodegradability and low toxicity as compared to other surfactants.

Recently, triblock copolymers commonly called pluronics have received increasing attention from many researchers (Kabanov, 2002; Henda, 2013). Pluronics are commercially available non-ionic macromolecular surface active agents that are also known under their non-proprietary name “poloxamers” (Nace, 1996). Pluronics block copolymers are synthesized by sequential addition of propylene glycol (PG) and ethylene glycol (EG) monomers in the presence of an alkaline catalyst, such as sodium or potassium hydroxide (Alexandridis, 1995). According to their structure, pluronics are also known as poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) triblock copolymers which are denoted as PEG-PPG-PEG or $(EG)_{n1}(PG)_{m}(EG)_{n2}$.

There are different types of pluronics depending on their copolymer composition (PPG/PEG ratio) and MW (PEG and PPG block length) (Alexandridis *et al.*, 1994). PEG-PPG-PEG block copolymers find applications in industries such as detergency (Schmolka, 1977; Bahadur *et al.*, 1991), dispersion stabilization, foaming, emulsification, lubrication, formulation of cosmetics (Schmolka, 1980; Schmolka, 1984) and inks (Winnik *et al.*, 1992). They are also used in drug solubilisation and controlled release (Lin and Kawashima, 1985; Yokoyama, 1992; Guzman *et al.*, 1992; Kabanov *et al.*, 1992), and in burn wound covering (Henry and Schmolka, 1989). In addition, several researches have investigated the application of pluronics in protecting microorganisms against mechanical damage (Murhammer and Goochee, 1990; Zhang *et al.*, 1992; Orlon, 1992).

In the pluronics grid (Fig. 1.10), the copolymers along the vertical lines have the same PPG/PEG composition ratio, while the copolymers along the horizontal lines have PPG blocks of the same length (Carl, 1991).

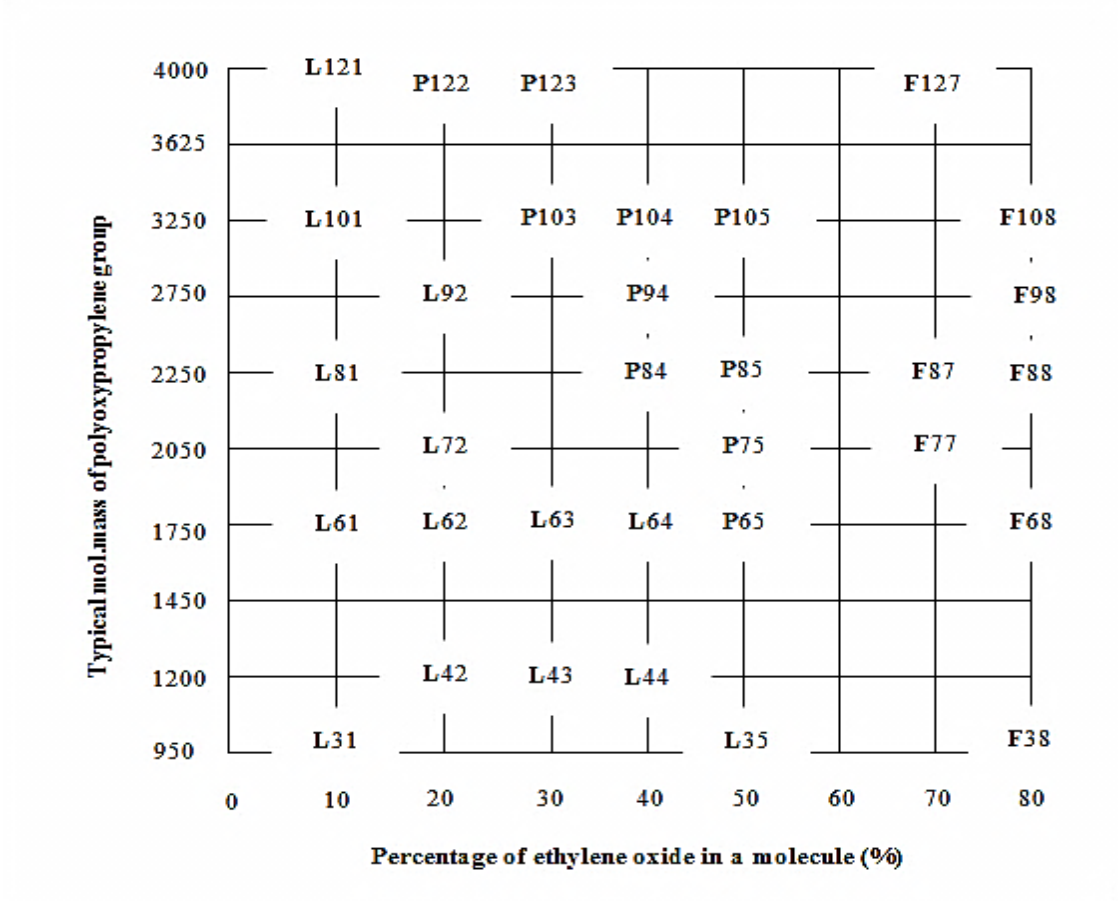

Figure 1.10: Pluronics PEG-PPG-PEG copolymers arranged in the "Pluronics grid" (Alexandridis, 1995).

Pluronics is named with a letter followed by a number. The letter denotes the physical form of pluronics (L means liquid, P means paste and F means flake). The first one or two numbers present the MW of the PPG block while the last number reveals the weight fraction of the PEG block. For example, pluronics L61, L81 and L121 are all liquid (L) and have the same percentage of PEG (10%), but different MW of PPG (in the order of 2000, 2800, and 4000) (Alexandridis, 1995).

1.3.2.3 Zwitterionic surfactant

Zwitterionic surfactant exhibits both anion and cationic characteristics in one single molecule. This is the case of synthetic products like betaines or sulfobetaines and natural substances such as amino acids and phospholipids. The positive charge is almost always carried by an ammonium group, but can also be a phosphonium ion, and the negative charge is often a carboxylate, sul-

phate, or sulfonate. The specific character of having both positive and negative charge in the head group gives zwitterionic surfactants good solubility in water with little sensitivity to salts and temperature (Bluestein, 1982).

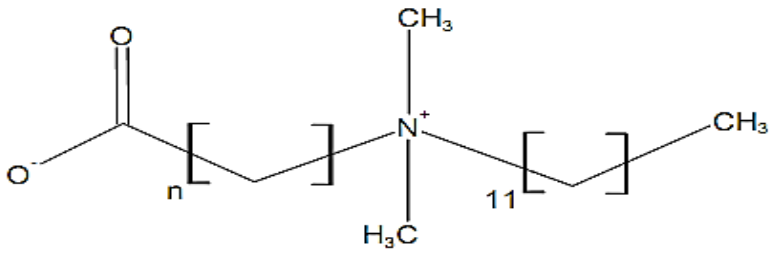

Figure 1.11: Structure of zwitterionic n-12 betaine surfactant

Zwitterionic surfactants, particularly the amino acid ones are quite biocompatible, and are used in pharmaceutical and cosmetic industries (Salager, 2002). They are also used as topical microbicides in biological testing to determine whether they will be effective against sexually transmitted diseases without irritating the mucosal tissue (Wong, 2002). Zwitterionic surfactants can also act as pathogens inactivate factor by disrupting cell walls and solubilizing membranes, or inhibiting specific enzymes and coating non-envelope viruses to prevent infection (Watanabes, 1994).

1.4 Surfactant micellization

Surfactants in solution tend to form aggregates, called micelles (Holmberg, 2002). Micelle formation, or micellization, can be viewed as an alternative mechanism to adsorption at the interface for removing hydrophobic groups from contact with water, thereby reducing the free energy of the system. It is an important phenomenon since surfactant molecules behave very differently when present in micelles than as free monomer in solution. Only surfactant

monomers contribute to surface and interfacial tension lowering their dynamic phenomena, such as wetting and foaming.

Surfactants can form different structures in solution based on the type of surfactant, and solution. A few common structures that surfactant self-assembly leads to includes spherical micelles, cylindrical micelles, surfactant bilayers, RM, bi-continuous structures and vesicles (Fig. 1.12). Micelles are formed when the surfactants are dissolved in a polar solvent such as water. They have a hydrocarbon chain interior whereas the polar head groups face the water. They can have a spherical or cylindrical form depending on the ratio between the radius of the hydrocarbon core and the length of the extended alkyl chain. Surfactant can also form bilayers or a lamellar phase whose hydrocarbon core has a thickness of 80 % of the length of the two extended alkyl chains. Reverse micelle adopts the opposite formation of micelle with the polar head groups making up the water core and the alkyl chain with non-polar solvent making up the continuous medium. In addition, when two curvature of opposite sign connect with each other, a bi-continuous structure is formed. Similarly, when two distinct water compartments connect together, one forms the core and other forms the external medium which are called vesicles.

The critical micelle concentration (CMC) is the most important characteristic of a surfactant. It is the concentration at which micelles start to form. Micelles are already generated at very low surfactant concentrations in water. A CMC of 1 mM means that the micelles will only exist beyond this concentration and only monomers will be present below 1 mM. At the CMC point, micelles have a significant change in some physical properties with increasing concentration (Fig. 1.13).

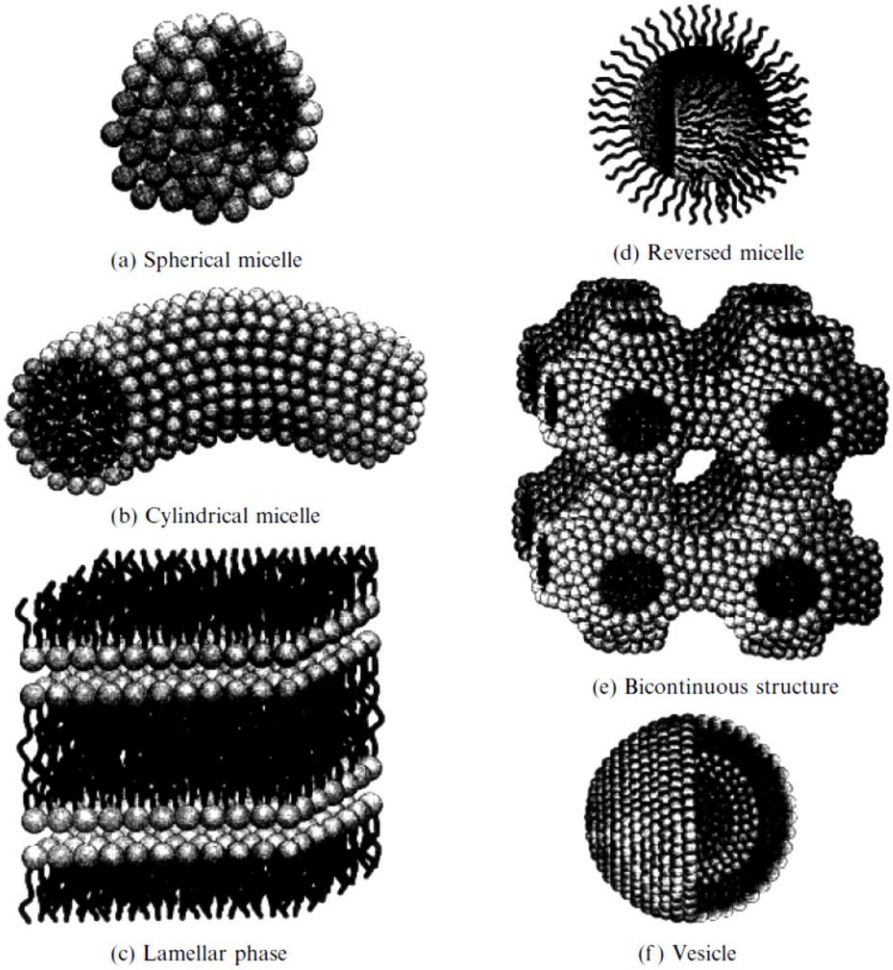

Figure 1.12*:* Different structure of surfactant in solution (Evansm, 1994).

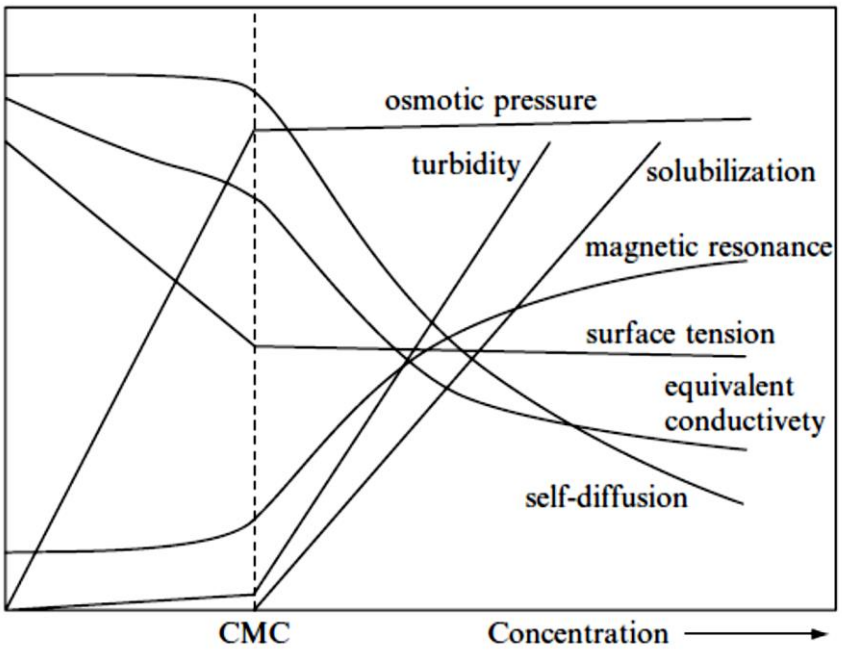

Figure 1.13: Schematic representation of the concentration dependence of some physical properties for solutions of a micelles-forming surfactant (Lindman, 1980)

The two most common and generally applicable techniques for CMC determination are surface tension and solubilisation (Holmberg, 2002). For an ionic amphiphilic, the conductivity offers a convenient approach to obtain the CMC. However, as a very large number of physicochemical properties are sensitive to surfactant micellization, there are numerous other possibilities, such as self-diffusion measurements such as NMR (nuclear magnetic resonance) and fluorescence spectroscopy (Capek, 2003; Khan, 2008; Law, 2012).

1.4.1 Micelle system

Micelles are formed at the CMC, which is detected as an inflection point when physicochemical properties such as surface tension are plotted as a function of concentration (Fig. 1.14). The main reason for micelles formation is the attainment of a minimum free energy state. The main driving force for the formation of micelles is the increase of entropy that occurs when the hydrophobic regions of the surfactant are removed from water and the ordered structure of the water molecules around this region of the molecule is lost (Langevin, 1992).

Most micelles are spherical in shape with size ranging from 3-50 nm and contain between 60 and 100 surfactant molecules (Attwood, 2008). The aggregation number, the number of molecules present in a micelle once the CMC has been reached, in case of ionic surfactants is in the range of 10-170 and 30-10,000 for non-ionic surfactants. CMC of ionic surfactants, are generally 10^{-3}-10^{-2} M higher as compared to non-ionic surfactant which usually falls in the range 10^{-4}-10^{-3} M (Attwood, 2008).

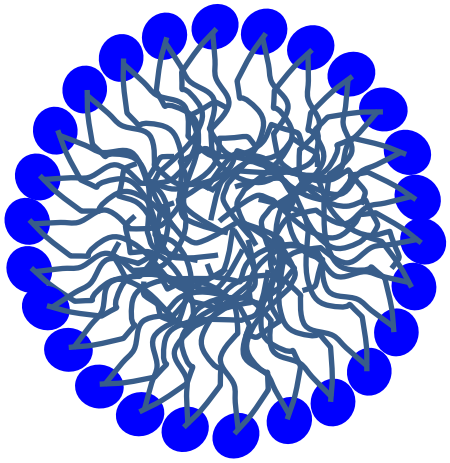

Figure 1.14: Structure of micelles of short chain surfactant with the hydrophobic core composed of the hydrocarbon chains and the hydrophilic heads in contact with aqueous solution.

1.4.2 Reverse micellar system (RMS)

RM is self-organized aggregates formed by a surfactant in non-polar solvent or oil (Fig. 1.15). They form water pools in nanometre size by the solubilisation of water in their polar cores (Liu, 2008). Most RM have a spherical shape which are usually formed by water (0-10 %), a surfactant (< 10 %), and an organic solvent (80-90 %) (Harikrishna, 2002). RM are also called water-in-oil microemulsion, namely, Winsor II emulsion (Liu, 2008). The organic solvent occupies the major part of the RMS and influences greatly the water solubilisation capacity of RM. The size of RM, which affects the extraction efficiency, also depends on the nature of the organic solvent. Co-solvents also termed as co-surfactant favour the formation of stable RM by improving the solubility of surfactants in organic solvents (Harikrishana, 2002).

The advantages of a RMS are thermodynamic stability (no phases separation with time), spontaneous formation, low interfacial tension (< 10^2 mN/m), transparent nature (nanometre size < 100 nm), large surface area (10^2-10^3 m^2/cm^3), viscosity comparable with pure organic solvent, and dynamic in nature (constant collision and fusion with each other and the contents inside RM exchange) (Sun, 2005).

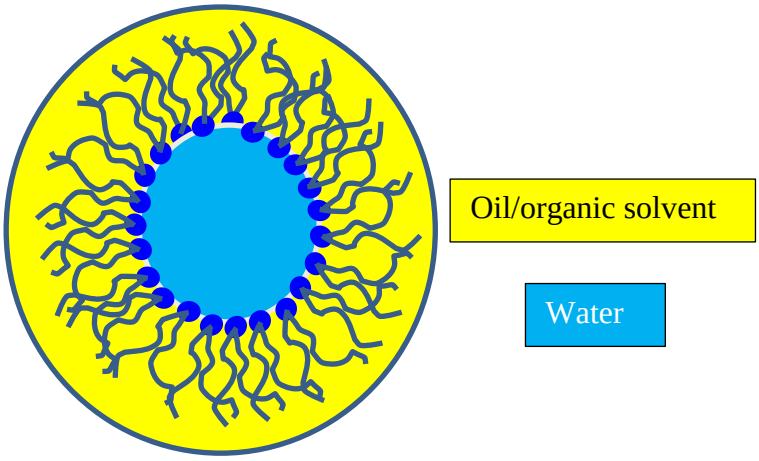

Figure 1.15: Structure of RM of short chain surfactant with their hydrophobic moieties in contact with the solvent and the hydrophilic moieties turned away from it.

Three types of RMSs were investigated in this work namely anionic RM (AOT RM), cationic RM (CTAB RM) and non-ionic RM (pluronics RM).

1.4.2.1 Anionic AOT reverse micelles

AOT is a common anionic surfactant used in the study of reverse micelle facilitated extraction of biomolecules. It is widely known to form RM in nonpolar organic media above an AOT concentration of approximately 10^{-3} M, and has the ability to entrap relatively large amounts of water (until water content W_0= [H_2O]/ [AOT] ~50 for the AOT/heptane system) in the micellar core (Abuin, 2002). The surface area of the surfactant, the RM size, and the behaviour of water in the inner core of the RM have been studied using small angle neuron scattering (SANS), small angle X-ray scattering (SAXS) and other light scattering measurements (Eicke and Rehak, 1976; Kotlarchyk *et al.*, 1985; Sheu *et al.*, 1986; Bardez *et al.*, 1995; Karukstis *et al.*, *1996;* Perez-Casas *et al.*, 1997; Erlinger *et al.*, 1999). An equation to calculate the radius of an empty reverse micelle has been derived assuming that the quantity of water is equally distributed among all RM, and that the micelles have a spherical form (Eicke and Rehak, 1976). The surface region engaged by an AOT surfactant molecule was found to be approximate1y between 0.5 and 0.6 nm^2 (Eicke and Rehak, 1976). The amount of water in a reverse micellar phase is often

expressed in terms of the water uptake number, W_0, which is the moles of water per mole of surfactant (Karpe and Ruckenstein, 1991). The RM were predicted to be spherical in shape at W_0 = 10, but an increase in the water content (W_0 > 13) leads to the formation of elongated shapes (Bardez *et al., 1995).*

Light scattering studies of AOT reverse micellar phases have found that these adopt different size and shapes in the ORP (Sheu *et al.,* 1986). It has been reported that the high interfacial shear upon mixing of a protein containing AQP – water or buffer solution, and a surfactant containing ORP – polar solvent solution causes emulsification and results in an uneven distribution of biomolecules (Paradkar and Dordick, 1993) in RM. The aggregates with large amounts of water (W_0 > 15) are known to contain both droplets and random structures (Perez-Casas *et al.,* 1997). Thus, the modelling of protein solubilisation was attempted as a function of the concentration of the protein containing RM and the free RM (Woll *et al.,* 1989; Karpe and Ruckenstein, 1991). The water-enriched layer formed around the AOT head groups reduces the water activity in such a way that it promotes binding of the peptide to the head groups (Hou, 2007). Until now, several enzymes and proteins, including lipase (Aires-Barros and Cabral, 1991), lysozyme (Chou & Chiang, 1998), and chymotrypsin (Shin *et al*., 2004) has been extracted by using AOT RMS by forward and backward extraction procedures.

The motivation to use an anionic surfactant AOT in this study has been the enormous amount of published data on the physic-chemical properties of AOT RM and the ease of RM formation (Fan *et al.*, 2008; Vinogradov *et al.*, 2003; Kinugasa *et al.*, 2002; Naoe *et al.*, 1999). However, reports have shown that in many cases AOT causes loss of enzyme activity due to strong electrostatic and hydrophobic interaction with solubilized enzymes/proteins. Therefore, non-ionic surfactants such as Brij and Triton-X were used as co-surfactant in the mixed RMS with AOT to overcome this problem (Liu, 1998; Verma, 2013). This cooperation helps to maintain the activity of the recovered enzyme or protein after extraction due to the decrease of electrostatic interaction between AOT and protein with a good extraction yield.

1.4.2.2 Cationic CTAB reverse micelles

A common surfactant of this group currently used in protein extraction is CTAB (Uskokovic *et al.*, 2005). Several different microemulsions based on CTAB such as CTAB/hexane/water (Sui *et al*., 2004), CTAB/2-octanol/water (Hua *et al*., 2003), CTAB/1-butanol/*n*-octane/water (Li *et al*., 2003; Giannakas *et al*., 2004), CTAB/*n*-pentanol/hexane/water (Chen *et al*., 2003; Palazzo *et al*., 2003) and CTAB/*n*-butanol/isooctane/water (Chen *et al*., 2001) have been investigated. It was envisaged that CTAB-based RM would require a co-surfactant and short chain alcohols in order to forma stable microemulsion (Goncalves *et al*., 2000; Xu *et al*., 2002). The reason is most cationic surfactants, including CTAB, cannot satisfy the condition of $v/(a_0 l_c) > 1$ (a_0 is the area in the interface, v and l_c is the volume and length of hydrocarbon tail of surfactant molecule), when they are alone in the ORP. The addition of an alcohol which precipitates in the interfacial region of RM has an effect of increasing the volume of surfactant tail v and reducing the effective surface area a_0, thus facilitating the formation of RM (Ninham, 1984; Mitchell, 1981). *N*-butanol or *n*-pentanol is often used as co-surfactant, increasing the polarity of the surfactant and helping to stabilize the reverse micelle solution (Carpenter *et al*., 2000). Currently, CTAB has been used in the extraction of DNA (Cota-sanchez, 2006), BSA (Zhang *et al*., 2002), xylose reductase and xylitol dehydrogenate from *Candida guilliermondii* (Cortez *et al*., 2004) and lipase (Nandini *et al*., 2009).

1.4.2.3 Non-ionic pluronics PEG-PPG-PEG reverse micelles

As a copolymer surfactant, pluronics have several fundamental advantages in protein biotechnology as compared to ordinary surfactants such as AOT, CTAB, and Tweens. Copolymers have very low toxicity (Schmolka, 1992; Chen-chow, 1981) and are large molecules that can be designed to be intrinsically multifunctional and thus combined either covalently or non-covalently with drugs to overcome multiple problems such as solubility, stability and

permeability. These polymers are also capable of interacting with and modifying the activity of various endogenous drug transport systems within the body, thus affecting drug delivery (Kabanov, 2002).

As a non-ionic surfactant, pluronics have similar advantages as other non-ionic surfactants in comparison with the ionic surfactant for protein extraction using RM. Its low electrostatic interaction with proteins leads to limited or no denaturation of extracted protein (Ayala, 1992; Goklen, 1985; Verma, 2013).

PEG-PPG-PEG block copolymers can form RMs in the organic solvent. According to Svensson and Olsson (1999) they are soluble in aromatic solvents such as xylene but without forming aggregates. If a small amount of water is added to the surfactant solution, the PEG block becomes more hydrophilic resulting in the forming of RM (Fig. 1.16). However, evidence of the reversed micelle formation using pluronics in the literature is very limited.

Christenson and co-operators (1980) studied the interaction between water and EO of poly(ethylene glycol) dodecyl ether in the hydrocarbon media by NMR and light scattering. They concluded that there was no association at low water content however, at a water/EG ratio between 0.6 and 1.4, organisational structures started to form. At higher water content, a water-rich core was formed. The chemical shift of EG increases quickly first and then tends toward a constant with a further increase in the amount of water. Therefore, it can be concluded that water plays a critical role in the reverse micelle formation of non-ionic surfactants in the organic solvent.

Su and co-workers (2003) investigated the water induced reverse micelle formation of pluronics L92 in *p*-xylene by FTIR spectroscopic technique. They reported that L92 did not form RM when Z (the molar ratio of water to EG) is less than 0.35. For $0.35<Z<1.3$, spherical micelles were formed. Moreover, for $Z>1.3$, the shape of RM could be non-spherical. The formation of

RM was explained by the strengthening of the intermolecular interactions of copolymers, which originated from the water-water interaction.

The water-induced reverse micelle formation of pluronics L64 ($EG_{13}PG_{30}EG_{13}$) in *o*-xylene was investigated by Chu and co-workers (1995). This copolymer did not form RM in *o*-xylene in the absence of water or even in the presence of small amounts of water (up to ~0.15 water molecules per EG segment). However, RM, with a hydrated PEG core and a PPG corona were shown by scattering techniques at higher water concentration (Svensson, 1999).

Alexandridis and Andersson (1998) also studied the behaviour of L64 in *p*-xylene. They concluded that L64 was miscible with *p*-xylene at 25°C in all proportion and formed an isotropic solution (L2 region) along the copolymer-oil axis of the ternary phase diagram, and up to two water molecules per EG segment could be solubilised in the L64/solution.

Recently, Henda and colleagues reported the effect of temperature and solvent mixtures on the rheological properties of pluronics F68 (Henda, 2013). They reported that the rheological properties and the conformational change of F68/*p*-xylene/water depend strongly on water content. This solution begins with a Newtonian behaviour - constant viscosity while the injection speed changes (Odell, 1988), for little amounts of water and lose this behaviour to non-Newtonian liquid plastic behaviour with increasing water concentrations, where they have assumed the non-Newtonian behaviour to the existence of the RM.

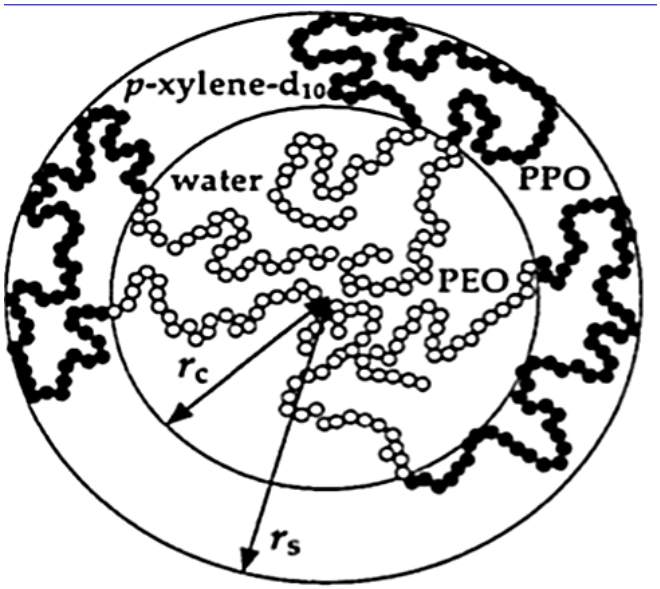

Figure 1.16: Model of the two-sphere micelle with the water and the PEG in the core and the *p*-xylene-d_{10} solvated PPG chains in the corona (Svensson and Olsson, 1999).

1.5 Reverse micelles for protein extraction

Recently, the application of the RM techniques has been receiving attractive attention from researchers since this system has many advantages and high potential compared to other separation techniques. Numerous research studies have been carried out for proteins (Gochman-Hecht and Bionco-Peled, 2006), non-protein thiol compounds (Dong and Hao, 2010), enzymes (Carneiro-da-Cunha *et al.*, 1996; Debanth *et al.*, 2007), plasmid DNS (Streiner *et al.*, 2007), organic dyes (Pandit and Basu, 2002), amino acids (Dovyap *et al.*, 2006), and amylase (Manav *et al.*, 2008) using this extraction system. It has been proved that biological compounds can be solubilized within the polar core of the RM and recovered from such solutions without the loss of native activity (Goklen, 1985; Prasad, 2010). In addition, the application of RM for protein separation can be used in downstream processing for large-scale separation of bio-molecules from fermentation mixtures (Andrew, 1991).

The ability of RMS in selecting a target protein from a mixture has not been completely understood yet, but is known to be affected by pH, type and concentration of salt, type of

solvent, temperature, type and concentration of surfactant, and the incorporation of bio-affinity ligands (Goklen *et al*., 1987; Kelly *et al*., 1993).

Luisi and colleagues first pointed out the possibility of the RM solubilisation of proteins during protein separation (Luisi *et al.*, 1979). Then, Dekker (1986, 1989) and Goklen (1985, 1987) developed this process systematically for the purpose of practical use. Later on, many researchers demonstrated factors such as water content (the molar ratio of water to surfactant, namely, W_0) and micelle size (Hai *et al*., 2008), pH of AQP and ionic strength (Rho *et al.*, 2004), type and concentration of surfactant (Shin *et al.*, 2002), and co-surfactant (Lee *et al.*, 2004) affect the protein solubilisation based on the interaction between protein and RM. Furthermore, protein-reverse micelle systems have been proposed for the study of structure and dynamics of large biomolecular systems, taking benefit of the fast tumbling time of protein-containing RM dissolved in low viscosity non-polar solvents like CO_2 and isooctane (Wand *et al*., 1998; Lefebvere *et al*., 2005; Pitre *et al*., 1993). Proof of principle studies using ubiquitin, α-chymotrypsin, and flavodoxin have shown that the protein structure does not change significantly, although there are some changes in the dynamics (Wand *et al*., 1998; Lefebvre *et al*., 2005; Pitre *et al*., 1993). Early studies of proteins in RM have also shown that the proteins are active and that the enzymatic activity is largely maintained (Creagh *et al*., 1993). The confinement of proteins in small volumes of RM also forces the folding of proteins of marginal stability (Peterson *et al*., 2004). The highly electrostatic environment in the water core of RM is known to change the thermodynamics and dynamics of water (Faeder *et al*., 2000). Nowadays, RM can be used as reaction systems for separation of proteins (Nishii, Y., *et al*, 2002), protein refolding (Ono *et al*, 2005; Wu *et al*, 2006), enzymatic catalysis (Zhang *et al*, 2007) and solvent-based extraction of proteins (Liu *et al*, 2007).

Currently, most reports prove that the protein extraction method by RM has several advantages. Protein extraction using RM offers a low-cost model based on the ability to recycle the organic solvents and surfactants (Gochman-Hecht and Bianco-Peled, 2006; Mohd-Setapar *et al.*, 2009).

Also, the capacity of soluble protein in the polar cores of RM which mimic the physiological environment of the body can preserve the native conformation of the protein (Moore and Palepu, 2007). Moreover, this technique is easy to scale up and can have a continuous operation (Hu and Gulari, 1996).

Due to these advantages, there is a growing interest in RM for future applications in biotechnology at industrial scale, such as selective extraction of proteins or enzymatic catalysis in organic media.

1.5.1 Methods of protein solubilisation using reverse micellar systems

The three common methods used to incorporate proteins into RM are injection of a concentrated aqueous protein solution, addition of dry lyophilized protein to a reverse micellar solution, and phase transfer between bulk AQP and surfactant-containing ORP (Matzke, 1992).

In the injection method, the protein which is already solubilized in a concentrated aqueous stock solution (typically, 7% by volume) is added to the surfactant containing organic solvent (dry or slightly hydrated). The resulting mixture is vigorously shaken until an optically transparent solution is obtained. This method allows controlling of the amount of water present in the system. In this method, RM is forced to form with the protein already inside. Hence, reverse micelle size do not significantly affect the protein solubilisation. For small RM sizes, the injection method solubilized more protein than the dry-addition method. This procedure, which is commonly used in bio-catalytic applications, is simple and most effective (Fig. 1.17) (Matzkw, 1992).

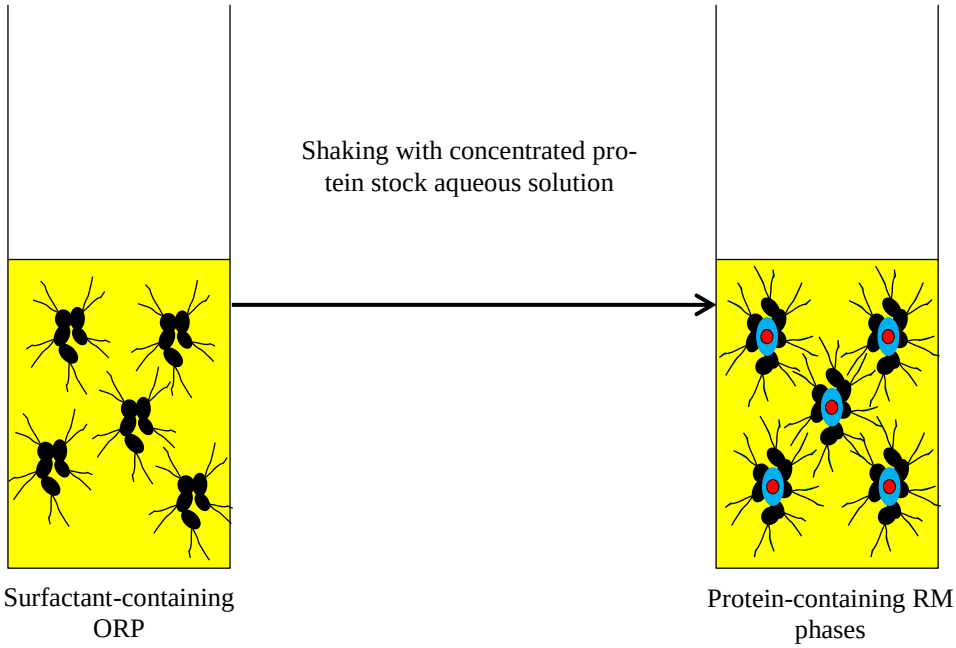

Figure 1.17: Injection method for incorporating protein inside RM

The dry addition method consists of the initial introduction of the required amount of water into the surfactant solution in an organic solvent in order to attain the required surfactant hydration degree (Fig. 1.18). A dry (lyophilized) protein can be dissolved in the resulting solution under vigorous shaking. This method is commonly used in bio-catalytic application and is well suited for hydrophobic proteins. One of the drawbacks of this method is prolonged contact between the enzyme/protein molecule and the organic solvent/surfactant that may lead to a partial denaturation of the protein/enzyme (Krishna, 2002). In this method, protein solubilisation is strongly dependent on RM size. In fact, the protein is solubilized only when the diameter of the RM is either similar to or larger than that of the protein. This is because of the large energy barrier for solubilisation of a large protein in small micelles which is difficultly overcome. But, in contrast, for a larger reverse micelle, since the RM are not required to rearrange its contents to incorporate a protein, the energy barrier is lower resulting in protein solubilisation (Krishna, 2002).

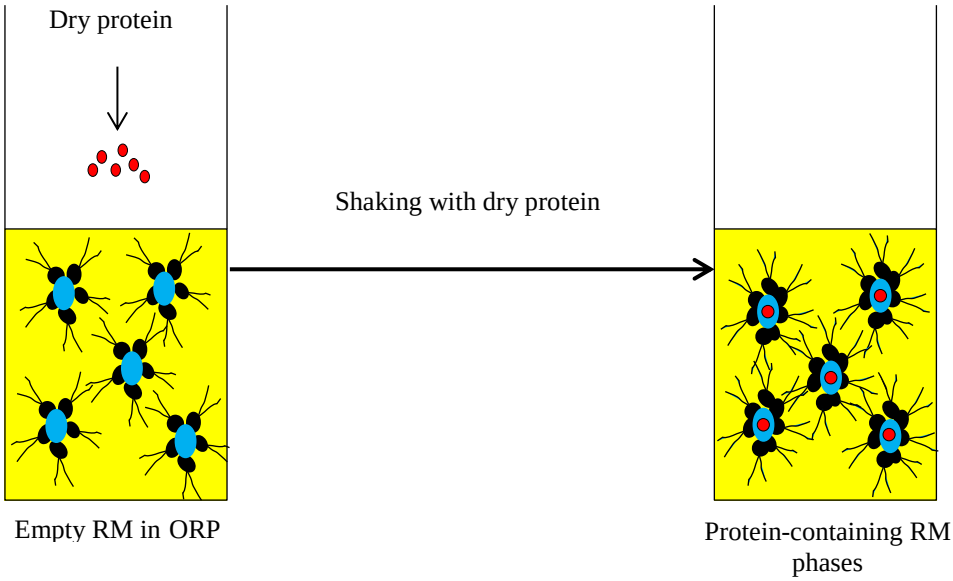

Figure 1.18: Dry addition method for protein solubility inside RM

The phase transfer method is principally similar to injection method, except the ratio between AQP and ORP is normally 1:1 (Fig. 1.19). In this method, two bulk phases (aqueous and organic) are mixed together until equilibrium is obtained by sharking or rotating. Under certain conditions, the protein molecules are transferred from the AQP to the surfactant containing ORP. Although, the enzyme solubilisation is slow, large amount of solute solubilisation is possible with minimum values of water content (Krishna, 2002). The phase transfer method is suitable for extraction of solutes from dilute aqueous solutions. The pH of the AQP, size and isoelectric point of the protein and the surfactant type were shown to have significant effect on the protein solubilisation by phase transfer method. The main driving forces for solubilisation of biomolecules in RM are the hydrophobic and electrostatic interactions. The different interactions established between the solute and the RM interface can be explored to selectively separate solute.

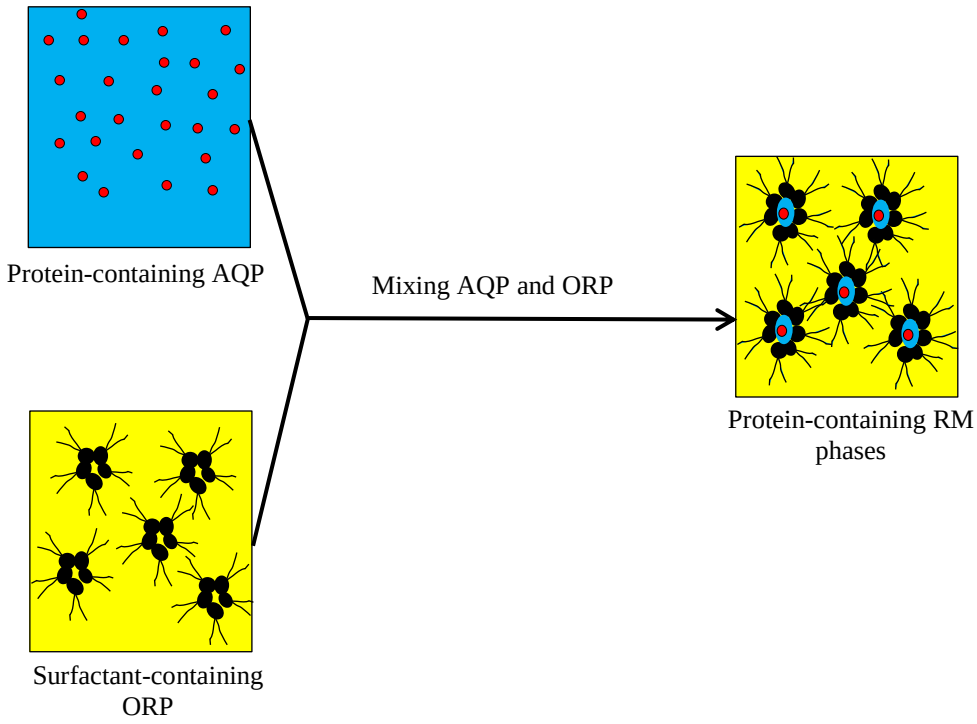

Figure 1.19: Phase transfer method for protein solubility inside RM

1.5.2 Protein extraction using reverse micellar system by the phase transfer method

The phase transfer method for protein extraction using RMS consists of two fundamental steps, namely, forward extraction and backward extraction (Fig. 1.20).

During FE, protein is transferred from an aqueous solution into a reverse micelle ORP. This process involves diffusion of proteins from aqueous solution to interface, formation of protein containing RM at the interface and transfer of protein containing RM into the ORP. Protein can be located either inside the water core or at the surface or at the corona of RMS (Fig. 1.20). Key factors that determine protein partitioning into a reverse micelle phase during FE are the electrostatic interactions between the charged protein and the polar head of the surfactant, hydrophobic interactions between the non-polar region of the proteins and the surfactant tail region, and lastly the size of the protein (Pires *et al.*, 1993) (Hong, 2000). Wolbert and colleagues reported that as protein size increases, the partition of protein into RM becomes much

more difficult (Wolbert, 1989). The reason is larger protein requires larger RM than what would be thermodynamically stable. This can be solved by the addition of co-surfactant or by manipulating the ORP by co-solvent. Also, larger proteins require a larger number of charged residues on their surface in order to be transferred into RM. This could be achieved by maintaining a higher difference between the pH of the AQP and the pI of the protein (a higher net charge on the protein). FE efficiency is controlled by various process parameters such as concentration and type of surfactants, pH and ionic strength of the AQP, concentration and type of co-surfactant, salts, charge of the protein, temperature, water content, size and shape of RM. By manipulating these parameters selective separation of the target protein can be achieved (Wolbert, 1989).

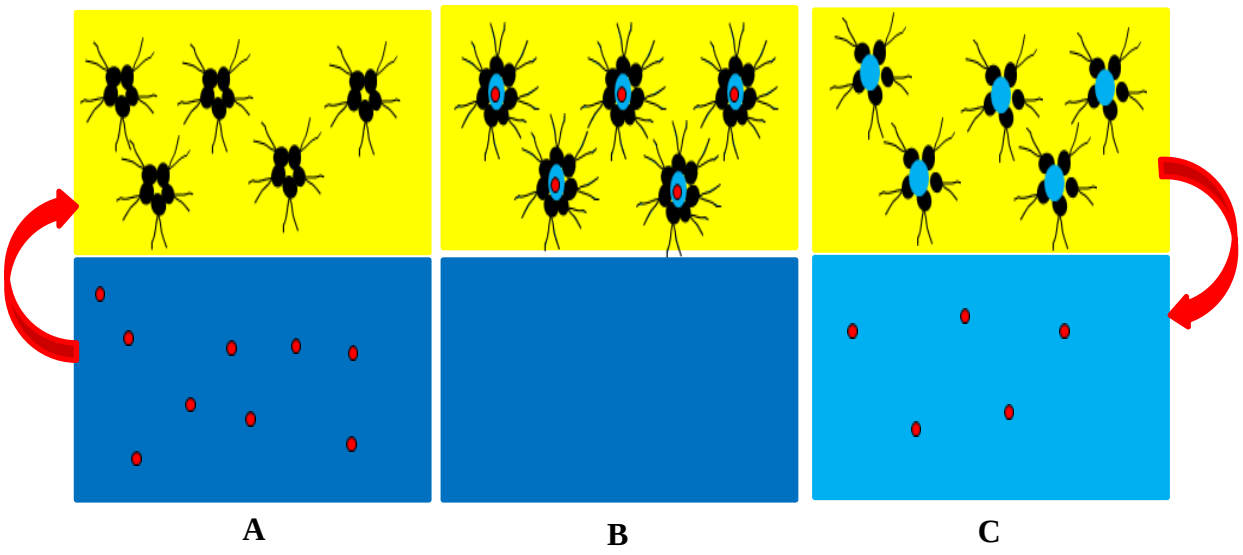

Figure 1.20: Forward extraction and backward extraction. Yellow box: organic phase contains RM. Blue (bold and light) box: aqueous phase contains proteins.
A. Organic phase and aqueous phase at the beginning.
B. Forward extraction.
C. Backward extraction.

During BE, protein is released from RM into a new AQP (stripping phase) (Liu, 2008). This process involves coalescence of protein filled RM with the interface to transfer protein to AQP. However, it is often more difficult to obtain protein from water-in-oil microemulsion solution. Consequently, this back extraction process often faces problems, such as low yield and co-extraction of surfactant. Different methods have been investigated in order to improve this

process, such as changing the ionic strength of the AQP (Andrews *et al*., 1994), decreasing concentration of surfactant (Hayes, 1997), absorbing protein using silica gel (Leser *et al*., 1993), precipitating anionic surfactant using multivalent cations (Ziyi *et al*., 1996), expulsion of protein with a counter-ionic surfactant or co-surfactant (Hayes *et al.*, 1998), and bio-affinity separation (Okazaki *et al.,* 1997; Adachi *et al.*,1997). It was reported that the BE could be enhanced (more than 10 times) with the addition of counter-ionic surfactants (Jarudilokkul, 1999). The counter-ionic surfactant was reported to interact with the oppositely charged surfactant molecules and facilitate the release of protein back into the stripping phase. The pH of the stripping solution was also adjusted to prevent the protein-surfactant interaction (similar charges on the surfactant and protein). Salt was also added at high concentration in order to reduce electrostatic interaction between the surfactant and protein (Kinugasa, 1992).

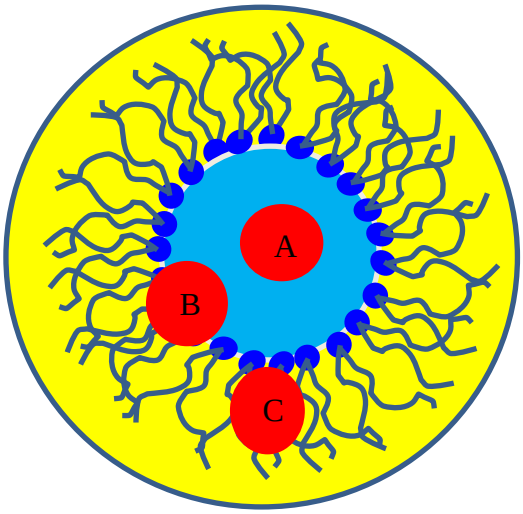

Figure 1.21: The location of solubilized protein inside RM
A) Inside water core. B) At the surface C) Inside the corona.

1.5.3 Effect of process parameters on protein extraction using reverse micellar system by phase transfer method

1.5.3.1 Aqueous phase pH

The surface charge on a protein can be determined by the pH of the AQP depending on the pI of the protein. At a pH below the pI, protein has an overall positive charge. Contrary to this the surface charge on the protein is negative when the pH is higher than the pI. As explained in section 1.4.1, solubilisation of proteins in RM is found to be dominated by electrostatic interaction between the charged protein and the inner layer of the surfactant head groups. Therefore, pH plays an important role in the extraction of protein using RM. If an anionic surfactant is used to form a RMS, then pH below pI is preferred for FE and a pH above pI is favoured during the BE. This is because at pH below pI, protein has a net positive charge which is opposite to the charge on the anionic surfactant leading to an increased electrostatic interaction between the protein and the RM, thus resulting in transfer of proteins from the AQP to the ORP. Similarly, at pH above pI, protein has a net negative charge which is similar to the charge on the anionic surfactant resulting in electrostatic repulsion which allows the protein to be expelled from the reverse micelle leading to back extraction. For large proteins such as α-amylase (MW 48,000 Da), the pH of the aqueous solution needs to be much higher than pI of protein to increase the number of charged groups on protein in order to provide higher energy for transferring protein from AQP into the RM phase (Wolbert, 1989). However, for small proteins whose size is smaller than the size of the water pool inside RM, solubilisation occurs as soon as the net charge is opposite to that of the reverse micelle interface (Gokle, 1987).

1.5.3.2 Ionic strength and type

Generally, it was observed that as the ionic strength of the aqueous solution increases, the protein intake capacity of the RM decreases (Aires-Barros, 1991). Two reasons were given to explain this phenomenon. Firstly, increasing the ionic strength decreased the Debye length that is measured as the electrostatic effect. Therefore, the electrostatic interaction between the charged protein molecules and the charged surfactant head groups of RM is reduced. Secondly, increasing the ionic strength reduces the electrostatic repulsion between the charged head groups of the surfactants in RM, thereby decreasing the size of the RM. Smaller RM will have larger curvature, which increases the density of the surfactant monolayer near the surfactant head groups, resulting in a gradual expulsion of protein molecules residing inside the RM, which is termed as a 'squeezing-out' effect. Although, lower ionic strength is preferred for the protein transfer from the AQP to the ORP, experiments cannot be performed at very low ionic strengths as the solution becomes cloudy under those conditions (Krishna, 2002). The effect of bile salts (sodium taurocholate - NaTC and 3-1-propane sulfonate - CHAPS) on percolation and size of AOT RM was studied by Yang and co-workers. They reported that the addition of bile salt help increase water uptake leading to decrease in percolation temperature and increase in the size of AOT RM. The hydrodynamic radius increased six fold in the presence of 10 mM CHAPS and doubled in the presence of 5 mM NaTC (Yang, 2003)

In addition, the type of ions also plays an important role in determining the partition behaviour of proteins in RM. Naoe and co-workers (2002) reported an increased recovery efficiency of lysozyme from RMS when guanidium salt was added to the AQP (Naoe, 2002). Kinugasa studied the effect of ion species on RMS of protein and reported the classification of ions as water structure former and water structure breaker (Kinugasa T. K., 2003). The effect of cation species (KCl, $BaCl_2$ and $CaCl_2$) on lysozyme extraction using AOT RMS was also studied (Nishii, 2004). The highest yield of lysozyme extraction was obtained at low salt concentration

of 0.3 M, for KCl, $BaCl_2$ and $CaCl_2$. Increase in salt concentration resulted in decreased extraction efficiency due to a competition between positively charged proteins and positively charge salt (Ba^{2+} or K^{+}or Ca^{2+}) for negatively charged AOT.

1.4.1.2 Surfactant type

The protein distribution is mainly dependent on the interaction between the protein and the surfactant head groups. pH of the protein solution decides the charge on the protein surface leading to the behaviour of protein with RM stabilized by charged surfactants. In addition to the charge, other surfactant-dependent parameters such as the size of the RM, the energy required for enlarging the RM, and the density of the inner surface of the RM may also influence the protein distribution. Protein denaturation and low BE yield was observed in case of RM of ionic surfactants (Battistel, 1988; Syeinmann, 1986; Harikrishana, 2002). Many studies have also been reported on the protein transfer using non-ionic surfactants such as Tween-85, Span-60, and Triton-X-100 (Naoe, 1998; Yamada, 1994; Ayala, 1992; Vasudevan, 1996). It was demonstrated that non-ionic surfactant has an apparent advantage over the ionic surfactant due to the absence of strong charges at the aqueous/organic interface, which provides a suitable environment for the protein.

1.4.1.3 Surfactant concentration

The concentration of surfactant has been shown to have little effect on the structure and size or aggregation number of the RM. However, it changes the number of RM formed, which in turn increases the protein solubilisation capacity of the RM. At surfactant concentration above a certain value, the reverse micellar interactions may occur, which leads to interfacial deformation of RM and percolation of solute (Harikrishna S. S., 2002).

1.4.1.4 Size of reverse micelles

RM size is dependent on the ratio of water to surfactant molecules. The monodispersed, small sized RM can accommodate proteins of only certain dimensions. Hence RM size may be conveniently used to include or exclude certain proteins. However, it was noted that several micelles can regroup to form larger micelles when certain operating conditions were altered (Prichanont, 2000; Gerhardt, 2002). It was also hypothesized that a protein can create around itself new larger RM of a requisite size to facilitate solubilisation (Wolf, 1979).

1.4.1.5 Co-surfactant

The use of co-surfactant, also termed as co-solvent may enhance the solubilisation kinetics, stability of a reverse micelle, and even selectivity. Reports on the addition of medium-chain length alcohols like iso-propanol and hexanol for improving the extraction efficiency has been reported (Mathew, 2007). Addition of co-surfactant is needed for non-ionic surfactants to increase the extraction efficiency (Zhang, 2002; Mathew, 2005).

1.6 Reverse micelles and protein refolding

Many recombinant proteins have been successfully produced in bacteria (Choi and Lee, 2004; Makrides, 1996; Gerigk *et al.*, 2002; Jeong and Lee, 1999; Jeong *et al.*, 2004; Mijts and Schmidt-Dannert, 2003). However, it has been reported that improper folding, protein aggregation (inclusion body formation), and lack of proper eukaryotic post-translational modifications (PTMs) of the ectopically expressed proteins could potentially affect their conformation, stability, and function (Braun, 2002; Christendat, 2000).

Eukaryotic expression systems, such as *Saccharomyces cerevisiae*, can produce proteins in a more native eukaryotic environment in comparison with prokaryotic systems. However, the common problem of this system is very low yield (Lesley, 2002; Yee, 2002).

For expressing physiological proteins, including therapeutic proteins, mammalian cell lines, such as CHO and HEK293, are widely used because they offer a cellular environment closer to the native conditions. However, the yield of this system is very low. The expression levels of purified recombinant proteins are usually in the microgram quantity (Durocher *et al.*, 2002), and expression cost of mammalian cell proteins is quite high.

All the above drawbacks in recombinant protein production require the need to develop successful commercial processes for protein refolding. One strategy for successful refolding is to prevent non-specific aggregation by using reagents that either inhibits aggregation or aid refolding. Sugar (Sundari *et al.*, 1991), proline (Kamar *et al.*, 1998), cyclodextrins (Sharma *et al.*, 2001) micelles (Tandon *et al.*, 1986), RM (Hashimoto *et al.*, 1998) and liposomes (Yoshimoto *et al.*, 2008) have been reported to prevent the irreversible aggregation of partially refolded proteins, and they are also known to facilitate protein refolding.

Based on a mild and efficient microenvironment in organic solvents that contain bio-molecules, RM has attracted attention in not only protein extraction, but also for protein refolding (Fig. 1. 22). RM is an ideal system for protein refolding due to several advantages. Firstly, reversed micelles provide nano-structured aqueous droplets constituting water pools in an organic solvent, so that proteins solubilized in the water pool can maintain their high structural conformation and activity (Peterson *et al.*, 2004). Secondly, RM isolates denatured proteins in an "individual compartment" and minimizes the side processes resulting in protein aggregation (Vinogradov *et al.*, 2003). Thirdly, RM, as geometrically restricted spaces, can subject the protein to a squeezing force and facilitate the collapse of denatured protein, which is similar to chaperone-assisted protein folding *in vivo* (Luisi, 1985).

RM can be used as a host for unfolded proteins because the size of their water core are comparable to the size of proteins and can be controlled by adjusting the W_0 (water content) (Yang, 2008; Hagen *et al.*, 1990; Hashimoto *et al.*, 1998; Gato *et al.*, 2000; Chen *et al.*, 2002).

Consequently, complete renaturation of high-concentration proteins can be obtained by RM at optimized conditions (Hashimoto *et al.*, 1998; Gato *et al.*, 2000; Ono *et al.*, 2005), and the method has been extended to the renaturation of recombinant protein expressed as inclusion bodies (Vinogradow *et al.*, 2003; Sakono *et al.*, 2004). RM are homogeneous and approximately spherical (Gochman-Hecht *et al.*, 2006) and the structure of the protein-containing RM can be described by the water-shell model, picturing protein molecules residing in the interior of the water droplet surrounded on all sides by water (Grandi *et al.*, 1981).

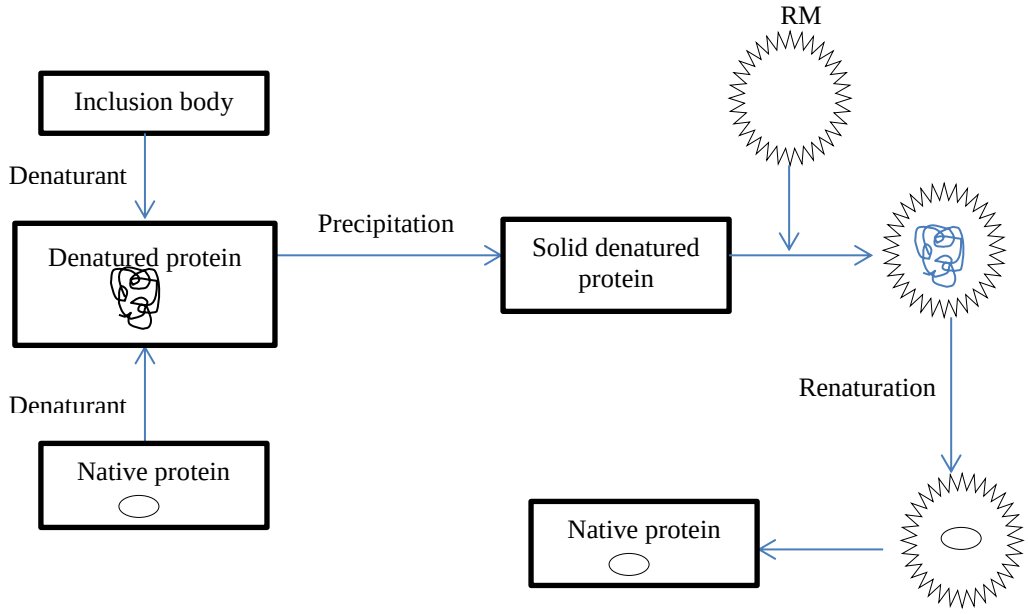

Figure 1.22: Schematic diagram of protein refolding by RM (Hashimoto *et al.*, 1997).

Goto (2000) successfully refolded 4.8 mg/ml of ribonuclease in AOT RM. Furthermore, other protein refolding methods such as dialysis (Ono *et al.*, 2005) and addition of molecular chaperone (GroEL) (Sakono *et al.*, 2004) were introduced to RM, and this was found to improve the refolding rate. However, due to the strong interactions between the surfactant and the proteins, the refolded proteins solubilized in AOT RM had to be recovered by precipitation using cold acetone (Goto *et al.*, 2000; Sakono *et al.*, 2004; Ono *et al.*, 2005). Moreover, not all proteins can be refolded in AOT RM because some proteins, such as carbonic anhydrase B, form a

precipitate which is denatured at the aqueous-oil interface by the strong interaction with AOT molecules (Sakono *et al.*, 2004).

1.7 Protein precipitation using reverse micelles

In protein extraction using RM, the formation of a water insoluble protein-surfactant precipitate at the aqueous-organic interface causes a loss of protein and is considered as a common drawback (Dekker, 1990). Precipitation of proteins using reverse micelle system was studied and developed as a new alternative separation technique. Shin and co-workers (2003) reported recovery of lysozyme from precipitate made by adding AOT directly into the aqueous solution. Later on, they were also successful in recovering cytochrome *c* and haemoglobin by the same method (Shin *et al.*, 2004). In comparison with other surfactant mediated techniques, the surfactant concentration here is considerably low and surfactant works as a precipitating ligand to form an insoluble protein-surfactant complex.

Within the surfactant-enhanced extraction systems, surfactant precipitation brings about significant advantages in comparison with reverse micelle extraction method such as simplified process, lower amount of surfactant required per mole of purified protein and short processing time (instant formation of protein-surfactant complex) (Shin *et al.*, 2003). Surfactant precipitation is a convenient alternative to conventional LLE that uses organic solvents because enzymes often show a low solubility, or lose their activity in organic media (Krei and Hystedt, 1992). Nevertheless, non-micellar surfactant-mediated separation is new and not fully understood. In view of its considerable potential, there is a definite need for more studies that would be able to shed more insight into its strengths and drawbacks, and in time lead to an improved design of such a separation system.

Due to these advantages, there is a growing interest in RM for future applications in biotechnology at industrial scales, such as selective extraction and precipitation of proteins, which has been explored in this work.

1.8 Proteins

1.4.2 Lysozyme

Lysozyme is one of the best-characterized hydrolases. Lysozyme can catalyse the hydrolysis of 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in peptidoglycan and between *N*-acetyl-D-glucosamine residues in chitodextrins (Yosimuka *et al.*, 1988; Peters *et al.*, 1989). One lysozyme molecule is made up of a single polypeptide chain consisting of 129 amino acid residues with MW of approximately 14.7 kDa (Canfiels, 1963). Lysozyme is highly stable in acidic solution and retains its activity even after heating to 100°C for 1-2 minutes due to the four-disulfide bonds in its chemical structure (Abraham, 1939; Brown, 1964).

The dimensions of crystalline lysozyme was projected to be 4.5 nm x 3.0 nm (Lye *et al.*, 1994), and its mean molecular diameter was estimated to be around 4 nm (Battistel, 1988). Lysozyme contains 18 positive and 11 negatively charged amino acids (Barlow and Thornton, 1986), and has an isoelectric point (pI) of 11.0 (Alderton *et al.*, 1946). It was the first enzyme to have its entire 3D structure determined with the aid of X-ray crystallography (Philips, 1967).

The primary structure of lysozyme consist of four pairs of cysteine that will form disulphide bridges between positions, 6 and 127, 30 and 115, 64 and 80 and 76 and 94 in its tertiary structure (Fig. 1. 23)

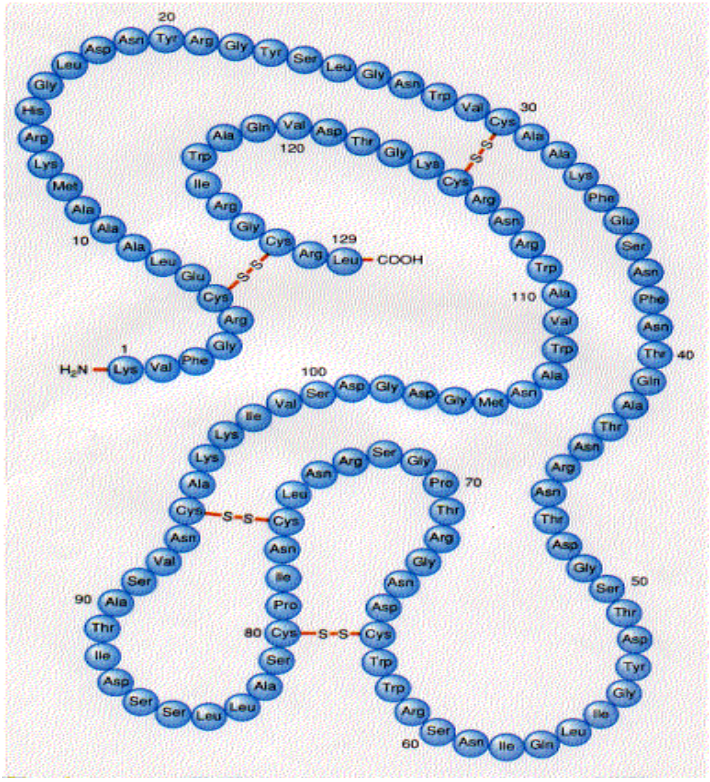

Figure 1.23: The amino acid sequence of egg white lysozyme (John Willey & Sons, 1990).

In nature, lysozyme is present in mucosal secretions such as saliva, tears and mother's milk, as well as in viruses, bacteria, phage, plants, insects, birds, and reptiles. They are also present in chicken egg-white at high concentration (about 3%) (Abeyrathne *et al.*, 2013).

Lysozyme plays a fundamental role in the immune system of an organism as it helps fight against bacterial infections. Due to its antimicrobial activity, lysozyme has been considered as a natural food preservative and in the antimicrobial treatment of fresh fruits, vegetables, seafood, and tofu. Lysozyme also finds application in wine products as an inhibitor for the development of bacteria that produces lactic acid. In pharmacy, lysozyme is used to increase the defence mechanism of the body against bacterial infections (lozenges for the treatment of sore throat and canker sores; eye drops and solutions for the decontamination of contact lenses). Furthermore, lysozyme is also known to possess beneficial effects on the skin such as facilitating wound healing, anti-inflammation, and prevention of bacterial infections (Takaoka *et al.*, 1972; Efendiev *et al.*, 1991). It has also been reported that lysozyme enhances antibody production in hybridoma reactors (Murakami *et al.*, 1997). Lysozyme also finds application in the treatment of

HIV infections and cancer treatment (Ghosh, 2003). It is also used for lysing *Escherichia coli* and *Streptomyces* species for the extraction of specific antigen and to treat ophthalmic ulcers and infections as well as in ophthalmologic preparation (Ghosh *et al.*, 2000).

Commercially, the most readily available source of lysozyme has been chicken egg white, from which it is industrially extracted. Traditionally, the separation of lysozyme from chicken egg-white has been done by direct crystallization (Alderton & Fevold, 1946) or chromatographic procedures (Li-Chan *et al.*, 1986). However, these methodologies are time-consuming and not well suited for scale-up. Therefore, simple and cost effective separation methodology is needed for mass preparation of lysozyme.

The reverse micellar extraction of lysozyme has been studied using both anionic and cationic surfactants. In an AOT reverse micellar phase, lysozyme was found to denature due to extensive conformational changes although the extraction efficiency was high (Luisi *et al.*, 1988). Noh and Imm (2005) investigated lysozyme extraction using RM formed by the cationic surfactant, cetyl dimethylammonium bromide (CDAB). They reported 96% of total lysozyme activity was recovered after FE. In a trioctylmethylammonium chloride (TOMAC) RMS, no lysozyme was extracted (Wolbert *et al.*, 1989). Continuous extraction of lysozyme from egg white into an AOT reverse micellar phase was obtained using TOMAC as a counter ionic surfactant to back extract the protein from the reverse micellar phase (Jarudilokkul and Stuckey, 2001).

Extraction by non-ionic RMS of lysozyme was also studied in Span 60 RMSs, and lysozyme FE was found to be pH-dependent, with high extraction percentages obtained at neutral pH (Naoe *et al.*, 1998). However, the overall extraction efficiency was not good enough (around 50%) and the alcohols used in the BE process lead to protein denaturation.

1.4.3 Bovine serum albumin (BSA)

BSA has been intensively studied as a model protein in separation techniques and as a vehicle for drug delivery in pharmaceutical research (Sun Q. Y., 2011). It is a globular protein with large molecular size of 3.5-3.7 nm and a MW of 66 kDa (pI 4.8) (Khan, 2012). The single polypeptide chain of BSA is made up of 583 residues folded into three homologous linear domains (I, II, III) with each composed of two subdomains (A and B) (Fig. 1.24) (Carter, 1994). BSA has a high content of charged residues such as Glu and Lys, which results in its strong interactions with anionic and cationic molecules such as metal ions (Wu, 2008). Nine loops are contained in three domains linked by 17 disulphide bonds, each loop formed by six helices; hence, secondary structure is dominated by α-helices (Gelamo, 2002). BSA is also denatured easily under extreme of conditions. A study on thermal denaturation of BSA using vibrational circular dichroism (VCD) spectra indicated that when the temperature was increased to 60°C, the amount of β-sheet structure increased considerably compared to the domination of α-helices in native structure of BSA and the denaturation was found to be irreversible (Shanmugam, 2004).

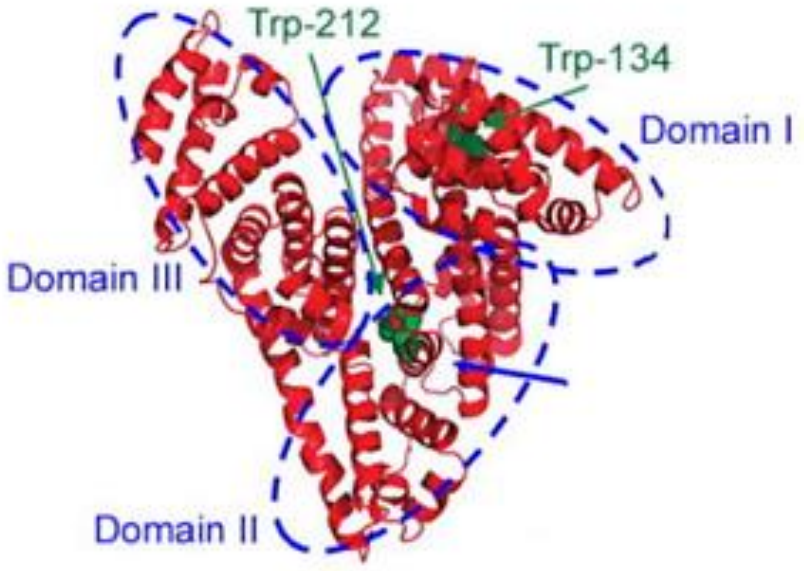

Figure 1.24: The chemical structure and schematic domains of BSA with tryptophan residues (Zhang X. L., 2013)

As a large size protein, BSA (66.5 kDa) was reported to have a low solubility in RM which lead to low extraction efficiency (Hebbar and Raghavarao, 2007; Sun, 2011). Additionally, it has been widely recognized that biological activity of proteins may be damaged during separation processes because of high pH, temperature and strong electrostatic interaction between proteins and surfactants (Liu, 2008). Therefore, attempts to enhance the extraction yield without denaturation of protein have been made by varying parameters affecting the process. Zhang and colleagues studied BSA extraction using CTAB RM in the presence of Cibacron Blue (CB) dye in AQP as an affinity ligand. They reported that the BSA transfer to the RM increased significantly in a wide range of pH by the addition of 1-7 % CB to the AQP based on interaction between anionic dye CB and cationic surfactant CTAB (Zhang, 1999). They also reported the effect of alkyl halides, including 1-chlorobutane (R_4Cl), 1-bromooctane (R_8Br) and 1-iodobutane (R_4I), to BSA extraction using CTAB RM (Zhang, 2002). It was pointed out that the hydrophobic interaction between protein molecules and polar heads of surfactant were reduced due to the addition of alkyl halides leading to increase in BSA recovery yield.

Hebbar investigated BSA extraction using non-ionic surfactant Triton-X-100, ionic surfactant AOT and a mixture of AOT/Triton-X-100 in toluene (Hebbar, 2007). The results showed that although Triton-X-100 could form bigger RM than AOT in toluene, 5.67 nm and 3.36 nm in radius, respectively, weak electrostatic interaction between non-ionic surfactant and protein could not help BSA entering Triton-X-100 RMS.

1.4.4 Haemoglobin

Approximately one third of the mass of a mammalian red blood cell is haemoglobin. Its major function is to carry oxygen from the lungs through the arteries to the tissues and help carry carbon dioxide through the veins back to the lungs. The haemoglobin molecule (64.5 kDa) is made up of four polypeptides chains, which includes 2 α-chains of 141 amino acid residues each

and 2 β-chains of 146 amino acid residues each (Fig. 1. 25). The α-chain and β-chains have different sequences of amino acids, but fold up to form similar three-dimensional structures. The 4 chains are held together by noncovalent interactions. There are 4 binding sites for oxygen on the haemoglobin molecule, because each chain contains one haem group.

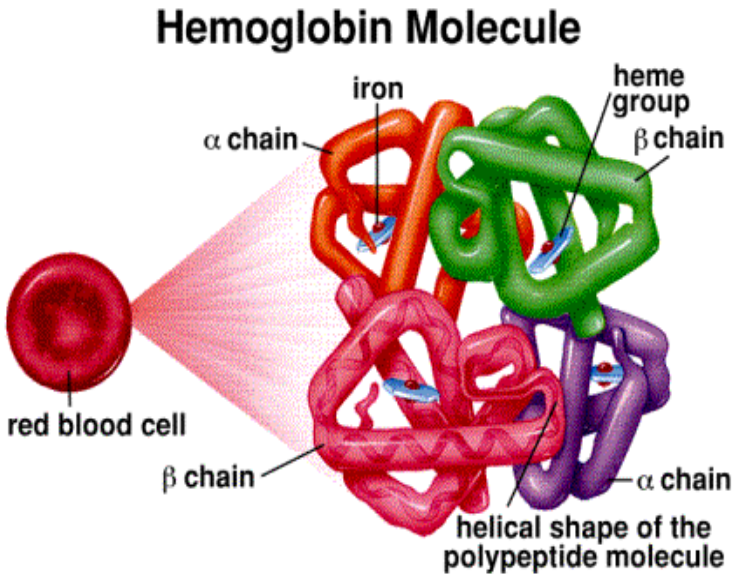

Figure 1.25: The structure of hemoglobin molecule (Itodo *et al.*, 2012)

Surprisingly, only few studies on haemoglobin extraction by reversed micellar solution have been reported. The reason could be the large size of haemoglobin whose diameter is around 5 nm (Erickson, 2009). Although haemoglobin was investigated using reversed micelles formed by a non-ionic surfactant and a co-surfactant, only 50-60% of total haemoglobin was extracted under optimum conditions (Ayala *et al.*, 1992). Kinugase and colleagues have reported that AOT-DEHPA [bis(2-ethylhexyl) phosphate] mixed reversed micelles could extract haemoglobin, and the transfer yield reached around 80% (Kinugasa *et al.*, 1994). Ono *et al.* (1996) studied dioleyl phosphoric acid (DOLPA) RM in haemoglobin extraction. They reported that DOLPA is the first surfactant to extract haemoglobin completely without using any co-surfactants. However, it was also found that haemoglobin, once dissolved into the DOLPA reversed micelles,

was not transferred to the fresh aqueous solution even when the conditions were adjusted to not allow the forward transfer of haemoglobin (Ono *et al.*, 1996).

Summary:

From the current challenges of downstream processing in recombinant protein, the potential of reverse micelles in protein extraction and refolding is promising. RM has studied as refolding agent with a few very first reports. However, these papers mostly used AOT surfactant which is not suitable for pharmaceutical application based on its toxicity. Therefore, a new intoxicated and biocompatible surfactant should be researched as a new agent for protein extraction and refolding using reverse micelles. Pluronics has been investigated for its micelle forming properties and finds application in many therapeutic and pharmaceutical preparations. Some reports have confirmed the ability of pluronics to form reverse micelles, but the study is limited, and the use of pluronics reverse micelles in protein extraction, refolding or precipitation has not been explored in detail. In addition, most of the protein extraction and purification studies use reverse micelles made of ionic surfactants such as AOT, CTAB, CDAB, and TOMAC. However, the drawbacks of strong electrostatic interaction between surfactant and protein resulted in protein denaturation and low protein recovery after backward extraction. Non-ionic surfactants were also investigated for its reverse micelles forming capability and protein extraction with the aim of decreasing electrostatic interaction. However, the hydrogen bonding and hydrophobic interaction were not strong enough for protein extraction and thus lead to low extraction efficiency. The mixed reverse micellar system consisting of ionic surfactants and non-ionic surfactants has been attracting attention of some researchers lately. However, this system was deemed as more complicated, and there has not been much work undertaken so far. Therefore, this project was proposed to study the ability of different surfactant (AOT, CTAB, and pluronics) in forming reverse micelles and mixed reverse micelles in new solvent system, and then apply in protein extraction which aim to establish a new reverse micelles system for overcome the current drawback of pro-

tein extraction in downstream processing. Protein refolding will be considered for next step in selected optimal reverse micelles system.

OBJECTIVES

1. AOT in *p*-xylene RMS and lysozyme extraction in compare with AOT in isooctane RMS.

2. CTAB in mixture of 1-bromooctane, 1-hexanol and petroleum ether reverse micelles system and its application on the extraction of BSA and lysozyme.

3. Triblock copolymer pluronics in *p*-xylene reverse micelles system and its application in lysozyme extraction and refolding.

4. Mixed reverse micelles system of ionic surfactant (AOT, CTAB) and pluronics and lysozyme extraction and refolding.

2.MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

- *p*-xylene (Sigma-Aldrich, ≥ 99%), fluorescien sodium salt (Sigma), rhodamine B (Sigma, ≥95%), sodium phosphate monobasic (Sigma, ≥99%), sodium phosphate dibasic (Sigma, ≥99%), CAPS (Sigma, ≥98%), sodium hydroxide (Sigma-Aldrich, ≥98%), hydrochloric acid (Fluka, 30-35%), 2,2,4-trimethylpentane (isooctane) (Sigma-Aldrich, ≥99%), petroleum ether (Sigma-Aldrich, ≥90%), 1-bromooctane (Aldrich, 99%), 1-hexanol (Sigma-Aldrich, ≥99%), 1-octanol (Sigma-Aldrich, ≥99%), 2-propanol (Sigma-Aldrich, 99.5%), ethanol (Fluka, ≥99.8%), potassium chloride (Sigma, ≥99%), potassium bromide (Sigma, ≥99%), potasium floride (Sigma, ≥99%).

- The model proteins used in this study were lysozyme from chicken egg white (Mw 14.3 kDa), bovine serum albumine (Mw 66.5 kDa), and haemoglobin from bovine blood (Mw 64.5 kDa).

- Surfactants used in this study were docusate sodium salt (AOT, purities ≥99%, Sigma), hexadecyltrimethylammonium bromide (CTAB, purities ≥99%, Sigma), and poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) PEG-PPG-PEG L61 and L81(Aldrich).

2.1.2 Stock solutions:

- Fluorescein sodium salt solution 0.01M in deionized water.
- Rhodamine B solution 0.01 M in deionized water.
- Phosphate buffer stock solution 0.01M in deionized water.
- CAPS buffer solution 0.01M in deionized water.

- Lysozyme stock solution 1 mg/ml in buffer.

- BSA stock solution 1 mg/ml in buffer.

- Organic L61 stock solution 0.1 M in *p*-xylene.

- AOT stock solution 0.1M in *p*-xylene and in isooctane.

- CTAB stock solution 0.1M in petroleum ether, 1-bromooctane, 1-hexanol.

2.2 Equipment:

2.2.1 Karl Fischer titrator coulometers C20/C30

Samples were analysed by injecting 0.1 ml liquid sample into a 100 ml of Hydranal-Coulomat AG (Fluka) was coulometric reagent contained in Karl Fischer cathode chamber. The principle of water content determination of this equipment is based on the reaction

$$H_2O + I_2 + SO_2 + CH_3OH + 3RN \longrightarrow RNH](CH_3OSO_3) + 2(RNH)I.$$

The reaction runs until all the water has been consumed and hence free iodine is detected in the titration solution. The end point is determined using a bivoltametric indicator. The amount of water in the sample is given in ppm or mg/g or percentage unit.

2.2.2 Circular dichroism spectrophotometer

Samples were analysed using a rectangular absorbance macro cell (110-QS, 1 mm pathlength, 350 µl, Hellma). The absorbance of all samples was kept at 0.8 ± 0.05 by diluting. The scan was set up with a wavelength range of 200-280 nm, step size of 1 nm, bandwidth of 1 nm, timer per point of 0.5 second and 10 repeats. The data of CD signal was converted into molar ellipticity using the equation:

$$ME = \frac{CD_S - CD_W}{10 \times C_S \times p} \qquad \textit{(Equation 1)}$$

Where, ME is molar ellipticity data, CD_s is the average data of CD signal of sample, CD_w is data of CD signal of deionized water, C_s is the mole concentration of sample, and p is the pathlength of CD cell in cm.

2.2.3 pH meter

The pH of all solutions in this project was adjusted using Mettler Todedo pH meter made in Switzerland. pH meter was always calibrated using a reference standard buffer of pH 4, 7 and 10 (Sigma) before each measurement. NaOH (0.1 M) and HCl (0.1 M) solution were used for adjusting the pH. Deionized water and 3 M KCl solution were used for washing and storing electrode.

2.2.4 Centrifuge

Centrifugation was done using an Eppendorf centrifuge 5430 with temperature control function. All samples were centrifuged at $\leq$ 4500 rpm, $\leq$ 30 minutes at 20^0C.

2.2.5 Rotator SB3

Mixing process was undertaken using a Stuart rotator. The speed was varied from 10 to 40 rpm. The maximum mixing time was around 90 minutes.

2.2.6 UV-Vis spectrophotometer

All samples were analysed using 1 pair of UV quartz cuvettes (pathlength 10 mm, volume 3.5 ml, Hellma) by Agilent UV-Vis spectrophotometer with temperature control function. The chosen scanning wavelength was from 190-900 nm. Sample was filled to 2/3 in a clean cuvette and placed inside the spectrophotometer with a lid on. All measurements were obtained at stable temperature of 20^0C.

2.2.7 Infrared spectroscopy (IR)

All samples were analysed using liquid transmission cells composed of two CaF_2 windows (1 mm thick) separated by a Teflon spacer. The transmission of OH (deionized water) stretch vibration was recorded in the range of 3000-3700 wavenumber and then converted into absorbance to investigate the presence of water inside the RM. All experiments were run at room temperature (25^0C).

2.3 Methods:

2.3.1 Water solubilisation measurement

Principally, at low surfactant concentration, the ability of the copolymer-oil solution to take in water is very limited and the water solubilisation capacity varies little with the surfactant concentration. The surfactants exist as monomers in this concentration range. Then, an abrupt increase in the water solubilisation capability is noted at an absolute copolymer concentration. This increase is a cooperative process and is indicative of the self-association of the surfactant molecules into reverse micelles that provide a hydrophilic microenvironment suitable for water solubilisation. The "reverse" critical micelle concentration (CMC) can thus be determined from the surfactant concentration at which the water solubilisation capacity starts increasing.

Stock solution of surfactant in nonpolar solvent was prepared in a standard flask and constituted the organic phase. Phosphate buffer at pH 7 was prepared with and without the presence of salt in deionized water and constituted the aqueous phase. The desired concentration of surfactant was obtained by appropriable dilution. At different surfactant concentrations, organic solution was taken and mixed with aqueous solution in a 1:1 ratio using a rotator for 60 minutes at 40 rpm (revolutions per minute). The samples were then centrifuged (if necessary) and 0.1ml of the ORP

of each sample was withdrawn using a syringe and analysed for water content using Karl Fischer Titrator. The experiments were repeated for reproducibility (Fig. 2.1).

2.3.1.1 AOT reverse micelles in isooctane and *p*-xylene

The investigated concentrations of AOT in isooctane and *p*-xylene were 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5, 1.7, and 1.9 (mM).

2.3.1.2 CTAB reverse micelles in petroleum ether, bromooctane and hexanol

The investigated concentrations of CTAB in a mixture of 5 % 1-bromooctane, 20 % 1-hexanol and petroleum ether were 0.02, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 4, and 6 (mM).

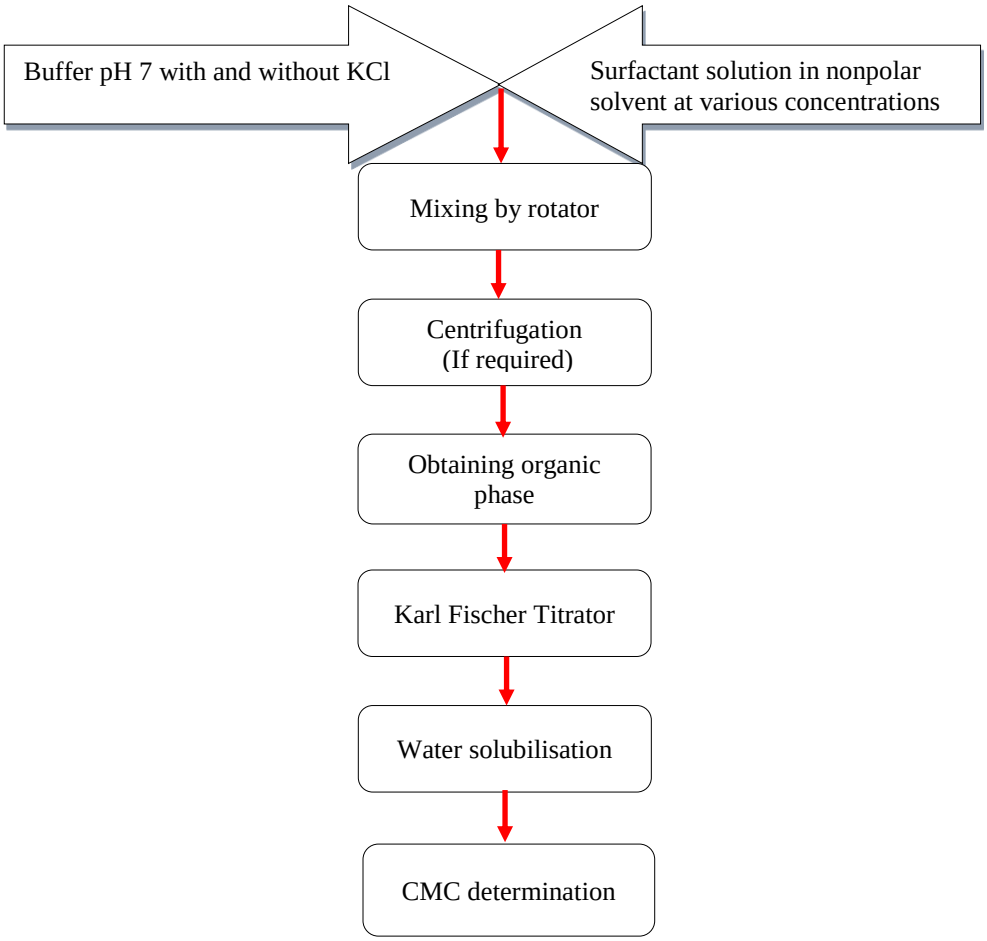

Figure 2.1: Water solubilisation experiment for determining CMC value of RMS

2.3.1.3 L61 reverse micelles in *p*-xylene

The investigated concentrations of L61 in *p*-xylene were 0.0005, 0.0013, 0.005, 0.01, 0.0, 0.1, 0.12, 0.15, 0.18, and 0.2 (mM).

2.3.1.4 Mixed L61 and AOT reverse micelles in *p*-xylene

The concentration of L61 in *p*-xylene was constant at 5 mM. The investigated concentrations of AOT in this mixture were 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.2, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 (mM).

2.3.2 Dye solubilisation measurement

Principally, dye solubilisation and water solubilisation are similar and belong to the group of solubilisation method for CMC determination as presented at Fig. 1.13. At low surfactant concentration, the ability of the copolymer-oil solution to take in water is very limited leads to dye solubilisation inside reverse micelles' cores limited. The surfactants exist as monomers in this concentration range. Then, an abrupt increase in the dye solubilisation capability is noted at an absolute copolymer concentration. This increase is a cooperative process and is indicative of the self-association of the surfactant molecules into reverse micelles that provide a hydrophilic microenvironment suitable for dye solubilisation. The "reverse" critical micelle concentration (CMC) can thus be determined from the surfactant concentration at which the dye solubilisation capacity starts increasing. Dye solubilisation uses hydrophilic dyes to prove the existence of water in the core of the RM.

Stock solutions of surfactant in a nonpolar solvent was prepared in a standard flask and constituted the organic phase. Hydrophilic dye solution was prepared in phosphate buffer at pH 7 with or without the presence of salt and constituted the aqueous phase. Desired concentrations of surfactants were obtained by appropriable diluting. At different concentrations, the organic solution was taken and mixed with the aqueous solution in a 1:1 ratio using a rotator for 60

minutes at 40 rpm (revolutions per minute). The solution was then centrifuged (if necessary), and 1.5 ml of the ORP was withdrawn and analysed on a Shimadzu UV-Vis spectrophotometer. The experiment was repeated for reproducibility (Fig. 3.2).

2.3.2.1 AOT reverse micelles in isooctane and in *p*-xylene with rhodamine B

The investigated concentrations of AOT in isooctane were 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 1and 2 (mM). The investigated concentrations of AOT in *p*-xylene were 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 (mM). 0.1 mM rhodamine B (Fig. 3.3) solution was prepared in phosphate buffer (0.01 M) pH 7 and constituted the aqueous solution. Rhodamine B is a hydrophilic dye that is completely insoluble in isooctane and *p*-xylene. The λ_{max} of Rhodamine B was 554 nm.

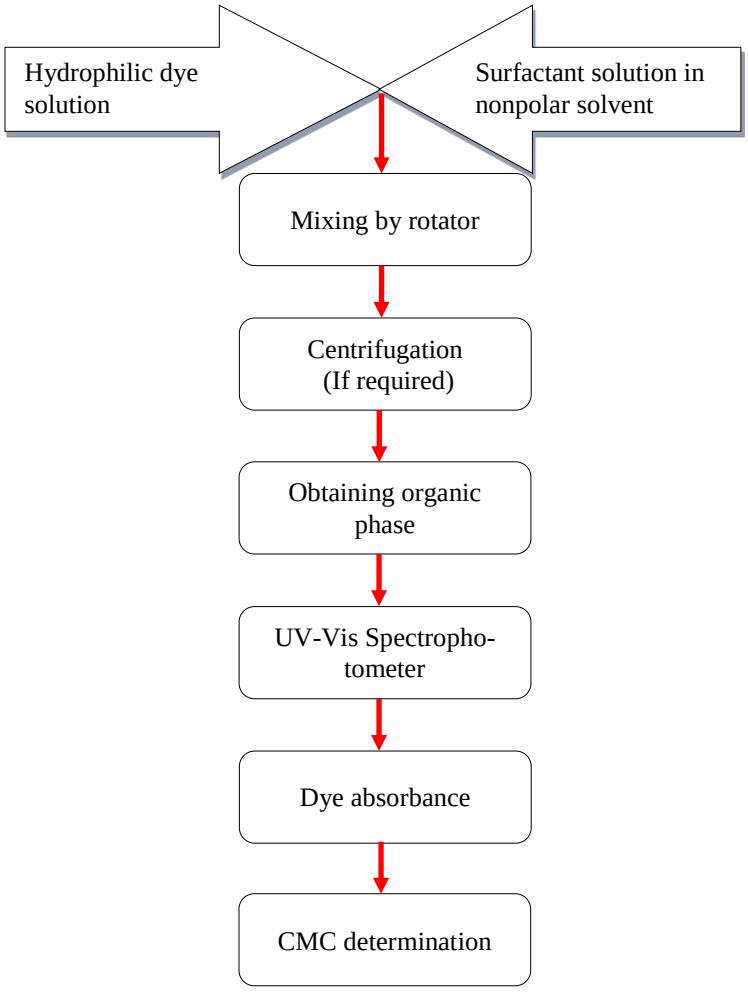

Figure 2.2: Dye solubilisation experiment for determining CMC value of RMS

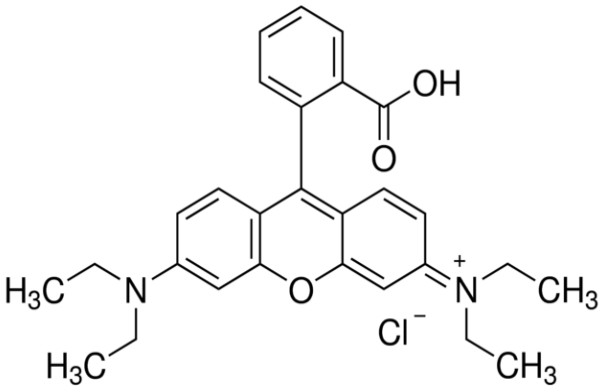

Figure 2.3: Structure of hydrophilic dye Rhodamine B

2.3.2.2 CTAB reverse micelles in petroleum ether, bromooctane and hexanol with rhodamine B

The investigated concentration of CTAB in a mixture of 5 % 1-bromooctane, 20 % 1-hexanol and petroleum ether were 0.1, 0.3, 0.5, 0.7, 0.9, 1.1,1.3, 1.5, 1.7, 1.9, 2, 2.1, 2.3, and 2.5 (mM). Rhodamine B solution was prepared in the same way of section 3.3.2.1.

2.3.2.3 L61 reverse micelles in *p*-xylene and fluorescien sodium

The investigated concentrations of L61 in *p*-xylene were 0.03, 0.05, 0.07, 0.09, 0.11, 0.13, 0.15, and 0.17 (mM). 0.1 M fluorescein sodium (Fig. 3.4) solution (0.1 M) was prepared in phosphate buffer (0.001 M) pH 7. Fluorescein sodium is a hydrophilic dye and is completely insoluble in *p*-xylene. The λ_{max} of fluorescein sodium was 450nm.

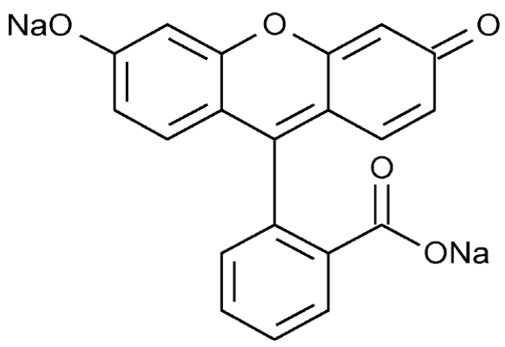

Figure 2.4: The structure of fluorescein sodium

2.3.2.4 Mixed L61 and AOT reverse micelles and fluorescein sodium

The concentration of L61 in *p*-xylene was kept constant as 5 mM. The investigated concentrations of AOT in *p*-xylene were 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.2, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 (mM). Fluorescein sodium solution was prepared in the same way of section 3.3.2.3.

2.3.3 Protein calibration

Desired concentrations (0.05, 0.07, 0.09, 0.1, 0.3, 0.5, 0.7, 0.9, 1.0 mg/ml) of protein solution were prepared from a stock solution in phosphate buffer at pH 7.0. The absorbance of the protein solutions were measured at 280nm (lysozyme and BSA) and 404 nm (haemoglobin) using a UV-Vis spectrophotometer with phosphate buffer as the blank/control solution.

2.3.4 Protein extraction using reverse micelles

Protein (1 mg/ml) was dissolved in phosphate buffer (10 mM constituted the aqueous solution, and surfactant solution solubilised in nonpolar solvent constituted organic solution.

For FE, the aqueous solution was mixed with organic solution in a 1:1 ratio. The two phases were then mixed using a rotator at 40rpm (no heat) for 60 minutes. After a pre-determined time, the samples were centrifuged at 4500 rpm for 5-15 minutes (if necessary) to obtain a clear aqueous phase at the bottom layer. The clear aqueous phase was then carefully withdrawn using a syringe. A double beam UV-Vis spectrophotometer was used to measure the protein concentration in the AQP. The solubilised protein in the reverse micellar phase was determined by calculating the difference between the protein concentration before and after extraction. The protein extraction efficiency was expressed in percentage (Fig. 2.5).

For BE, the ORP obtained from FE experiment was mixed with new fresh aqueous solution with a 1:1 ratio. The two phases were mixed on a rotator at 40 rpm (no heat) for 60 minutes. At the

end of the stipulated time, the contents were centrifuged (if necessary) at 4500 rpm for 5-15 minutes to obtain a clear AQP at the bottom layer. The clear AQP was then carefully removed using a syringe. The protein concentration in the AQP was then measured using a double beam UV-Vis spectrophotometer. The recovered protein from the reverse micellar phase was determined by calculating the difference between the protein concentrations before and after BE. The protein recovery efficiency was expressed in percentage (Fig. 2.5). All the experiments were carried out at room temperature and were repeated for reproducibility.

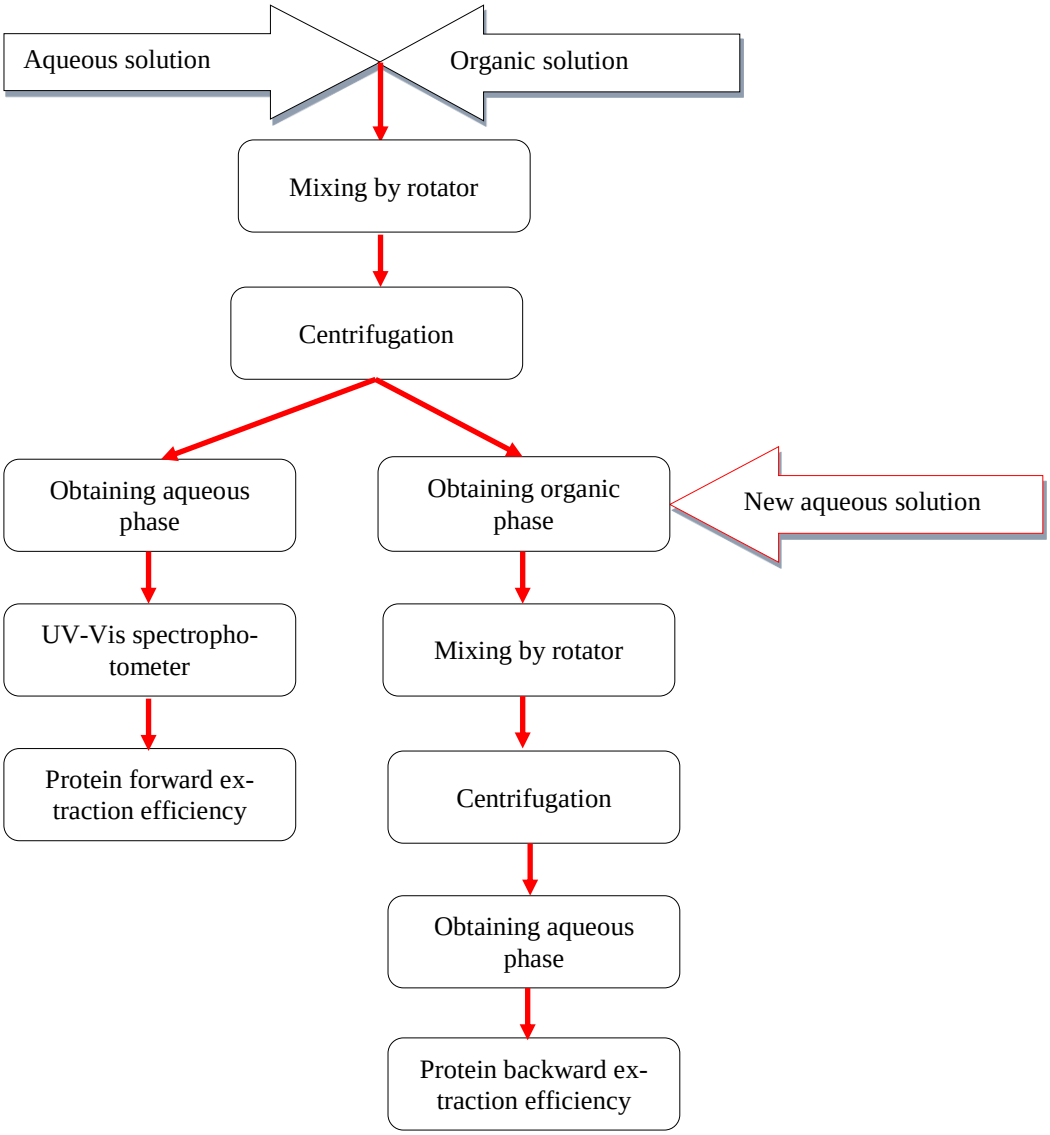

Figure 2.5: The scheme of protein forward and backward extraction using RMS

Protein extraction efficiency was calculated following two equations as shown below. Equation 2 was used to calculate the forward extraction efficiency while equation 3 was used to calculate the backward extraction efficiency.

$$E_f = ((B - A) / B) \times 100\ (\%) \qquad \textit{(Equation 2)}$$

Where, B is the protein concentration in the AQP before extraction and A is the protein concentration in an AQP after extraction.

$$E_b = \frac{C}{(B - A)} \times 100\ (\%) \qquad \textit{(Equation 3)}$$

Where, B is the protein concentration in an AQP before FE, A is the remaining protein concentration in an AQP after FE and C is the protein concentration in AQP after BE.

2.3.4.1 Lysozyme extraction using AOT reverse micelles in isooctane and *p*-xylene

Lysozyme solution (1 mg/ml) was prepared in phosphate buffer pH 8 in the presence of 0.1 M KCl. The optimum pH and salt concentration chosen was based on previous work (Pham, 2010). The investigated AOT concentration for both RMS in isooctane and *p*-xylene were 5, 10, 20, 50, and 100 (mM). For backward extraction, 2 M KCl and pH 11.8 were chosen as optimal conditions based on previous work (Pham, 2010). The secondary structure and activity of lysozyme was studied by CD and *Micrococcus lysodeikticus* cell assay, respectively.

2.3.4.2 Investigating the effect of salt for the backward extraction of BSA using CTAB reverse micellar system

In this study, the effect of different kind of salts such as KCl, KBr and KF on the forward and BE was investigated (Fig. 2.6). The optimised conditions for FE were pH 10, BSA 1 mg/ml and

10 mM CTAB. The optimum pH for BE was 4.3. The secondary structure of BSA was studied using CD. A schematic of the entire process is shown in Fig. 2.6.

2.3.4.3 Investigating lysozyme extraction using CTAB reverse micellar system

For FE, the effect of pH was studied at pH 11, 11.8, 12, and 12.8. The effect of surfactant concentration was investigated at 5, 10, 20, 30, 40, and 50 (mM) of CTAB. The effect of KCl concentration was studied 0.05, 0.1 and 0.2 M.

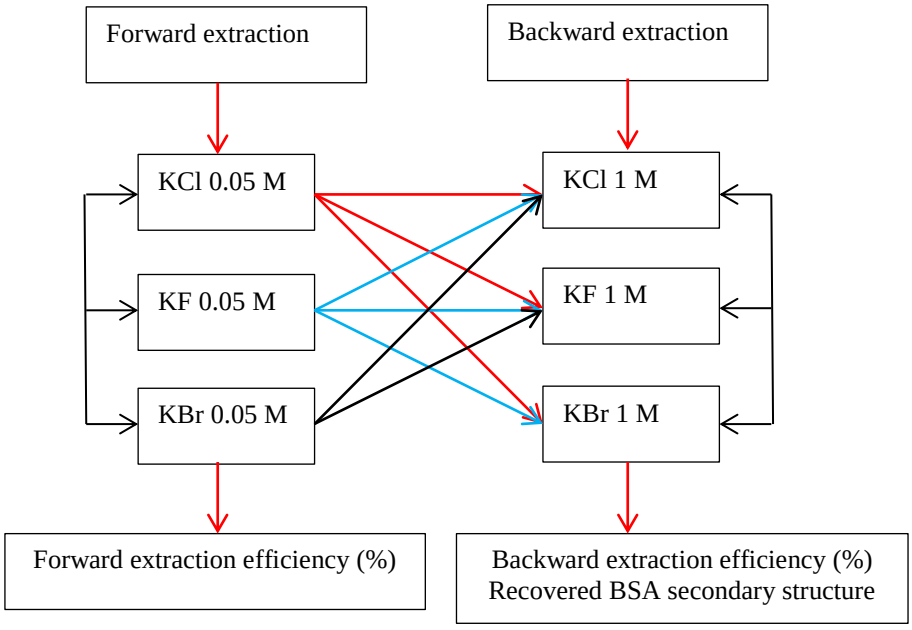

Figure 2.6: Effect of different salt on BSA extraction using CTAB RM in 5 % 1-bromooctane, 20 % 1-hexanol and petroleum ether.

For BE, the recovered lysozyme from CTAB RM was investigated under different conditions. The new aqueous solution was modified by adding varying salts and alcohols to improve lysozyme recovery from CTAB RMS. Different type of salts, include KCl, KBr, KF, NaCl, $CaCl_2$, and $MgSO_4$ were studied at high concentration , 1 M and 2 M. Different types of alcohols, include ethanol, isopropanol, and 1-butanol were studied at 5, 10, 15 and 20 %. Also, oppositely charged surfactants, such as SDS and AOT, were also investigated as co-surfactant.

The secondary structure and activity of lysozyme was studied by CD and *Micrococcus lysodeikticus* cell assay, respectively.

2.3.4.4 Lysozyme extraction using L61 reverse micelles in *p*-xylene

Lysozyme solution (1 mg/ml) was prepared in phosphate buffer (10 mM) and constituted the aqueous solution. The stock L61 solution (20 mM) was prepared in *p*-xylene and constituted the organic solution. For FE, the effect of aqueous pH was studied at pH 2, 3, 4, 5, 7, 9, 11, and 12. The effect of salt was also studied by adding different amount of KCl (0, 0.05, and 0.1 M) to the aqueous phase. The effect of surfactant concentration was investigated at 0.1, 0.12, 0.15, 0.17, 0.19, 2, 5 mM and 10 mM of L61. In addition, the effect of 1-octanol as co-solvent was examined by adding 5, 10, 15 and 20 % 1-octanol to the ORP.

For BE, the effect of pH was studied at pH 7 (using phosphate buffer) and pH 11 and 11.8 (using CAPS buffer). The effect of salt concentration was studied at 0.5, 1, and 2 M of KCl. Also, the effect of two alcohols, ethanol and 2-propanol, was examined by adding 5, 10, 15, and 20 % to the fresh aqueous phase. The mixing time was varied from 60- 120 minutes. The secondary structure and activity of lysozyme was studied by CD and *Micrococcus lysodeikticus* cell assay, respectively.

2.3.4.5 Lysozyme extraction using mixed L61/AOT RM in *p*-xylene

L61 acting as the main surfactant and AOT as a co-surfactant

Lysozyme solution (1 mg/ml) was prepared in phosphate buffer at pH 7 and constituted the aqueous solution. For FE, the effect of salt was studied by adding 0, 0.05, and 0.1 M of KCl to the aqueous phase. L61 was kept constant at 5 mM. The effect of co-surfactant was investigated by adding 0.1, 0.2, 0.4, and 0.5 mM of AOT to L61 solution. Also, the effect of co-solvent was studied by adding 5, 10, 15 and 20 % of 1-octanol to the RMS.

For BE, the effect of varying pH (7, 11, 11.8) were studied by adjusting the fresh aqueous solution using both phosphate buffer and CAPS buffer. The effect of KCl concentration (0.5, 1, 2 M) and two alcohols (5, 10, 15, 20 %), ethanol and 1-propanol, was also investigated. The samples were mixed on the rotator at 40 rpm (no heat) for 60 minutes. At the end of the stipulated time, the contents were centrifuged at 7000 rpm for 15 minutes. The clear bottom layer (AQP) in the tube was then carefully removed using a syringe. The lysozyme concentration in the AQP was then measured using a double beam UV-Vis spectrophotometer. The recovered lysozyme from the reverse micellar phase was determined by calculating the difference between the lysozyme concentrations before and after BE. The lysozyme recovery efficiency was expressed in percentage. The secondary structure and activity of lysozyme was studied by CD and *Micrococcus lysodeikticus* cell assay, respectively.

AOT acting as the main surfactant and L61 as a co-surfactant

Lysozyme solution (1 mg/ml) was prepared in phosphate buffer (10 mM) and constituted the aqueous solution. pH 8 and 0.1 M KCl was chosen as the optimised conditions for FE based on previous experiment (section 3.3.4.1) and previous work (Pham, 2010). Mixtures of L61 and AOT in *p*-xylene constituted the organic solution. AOT concentration was investigated at 0.5, 1, 5 and 10 mM while L61 concentration was investigated at 0.1, 0.5, 1, 5 and 10 mM. L61 concentration was always kept lower than AOT concentration in all samples.

For BE, aqueous phase of pH 11.8 and 2 M KCl were chosen as the optimised condition based on previous experiment (section 2.2.4.1) and previous work (Pham, 2010). The secondary structure and activity of lysozyme was studied by CD and *Micrococcus lysodeikticus* cell assay, respectively.

2.3.5 Precipitate investigation

At the end of FE of protein using L61/*p*-xylene RM and mixed L61/AOT/*p*-xylene RM, some precipitation was observed at the organic-aqueous interface. During FE, the protein must be transferred to the ORP, and it would have been easy to determine the amount of protein directly in the ORP if the solvent absorption did not interfere with that of the protein absorbance. However, *p*-xylene has an absorbance (λmax 278 nm) very close to lysozyme (λmax 281 nm) and therefore the lysozyme in the ORP could not be determined directly. It was, therefore, interesting to investigate the precipitate formed at the interface as it would reveal the amount of lysozyme in the precipitate and thus gives an indirect estimation of the amount of lysozyme trapped inside the pluronics RMS. The precipitation of two proteins, lysozyme and haemoglobin, were investigated using the pluronics mixed RMS.

After FE, the AQP and ORP were removed from the centrifuge tube. The precipitate formed at the interface remained in the tube as a very thin layer. New fresh phosphate buffer (pH 7) or new fresh CAPS buffer (pH 11.8) in the presence of 1 and 2M KCl and ethanol (5, 10, 15, and 20 %) was added to the centrifuge tube that contained the precipitate. The samples were mixed on the rotator at 40 rpm (no heat) for 60 minutes. At the end of the stipulated time, the contents were centrifuged at 7000 rpm for 15 minutes. The clear solution in the tube was then carefully removed using a syringe. The protein concentration was then measured using a double beam UV-Vis spectrophotometer. The secondary structure and activity of lysozyme was investigated using CD and *Micrococcus lysodeikticus* cell assay, respectively. The schematic of the process of analysis of the precipitate is shown in Fig. 2.7.

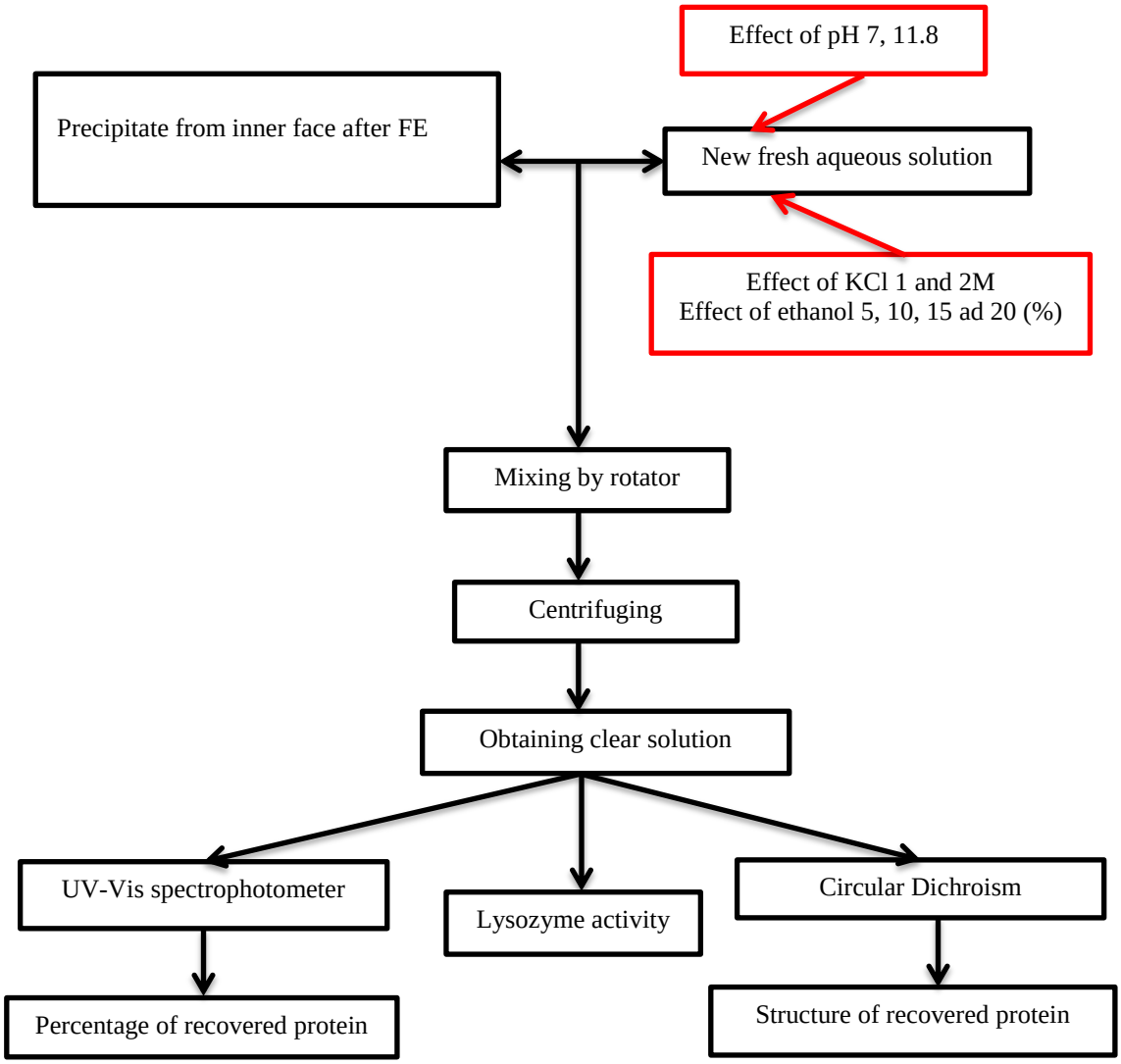

Figure 2.7: Schematic of the lysozyme and haemoglobin precipitate at inner-face between ORP and AQP after FE.

2.3.6 Lysozyme activity using *Micrococcus lysodeikticus* cells

Micrococcus lysodeikticus cell suspension (0.1 mg/ml) and lysozyme solution (1 mg/ml) were prepared in phosphate buffer pH 6.24, and stored at 2-8^{0}C before experiment. UV-Vis spectrophotometer was set at 25^{0}C for 30 minutes to obtain a stable temperature.

Clean UV quartz cuvettes (path-length 10 mm, volume 3.5 ml, Hellma) were filled with 2.5 ml of cell suspension and then equilibrated at 25°C for 10 minutes in the UV-Vis spectrophotometer. These cuvettes were marked as sample and blank for further experiment. The absorbance at 450nm was monitored until the reading was constant. 0.1 ml of phosphate buffer

and 0.1 ml of lysozyme solution were then added to blank cuvette and test cuvette, respectively. The mixture of each cuvette was immediately mixed by inversion and the decrease in absorbance at 450 nm for approximate 5 minutes was recorded. A reading was noted at every 30 seconds for 5 minutes.

From the recorded absorbance, the enzymatic rate of lysozyme was calculated following equation 4 below.

$$\text{Units/ml enzyme} = \frac{\Delta A_{450}.d_f}{(0.001)(0.1)} \qquad \textit{(Equation 4)}$$

Where, d_f was the dilution factor, 0.001 was the change in absorbance at ΔA_{450} as per the Unit and 0.1 was the volume (in millilitres) of enzyme solution.

2.3.7 Investigate of water solubilisation inside the reverse micelles using IR

Stock solution of surfactant in nonpolar solvent was prepared in a standard flask constituted the organic phase. 0.1 M KCl in deionized water was prepared and constituted the aqueous phase. The desired concentration of surfactant was obtained by appropriate dilution. At different surfactant concentrations, an equal volume (1:1 ratio) of organic phase and aqueous phase was mixed on a rotator for 60 minutes at 40 rpm (revolutions per minute). The samples were then centrifuged (if necessary) and 0.5 ml of the ORP of each sample was withdrawn using a syringe and analysed for water solubilisation using IR (Fig. 2.8).

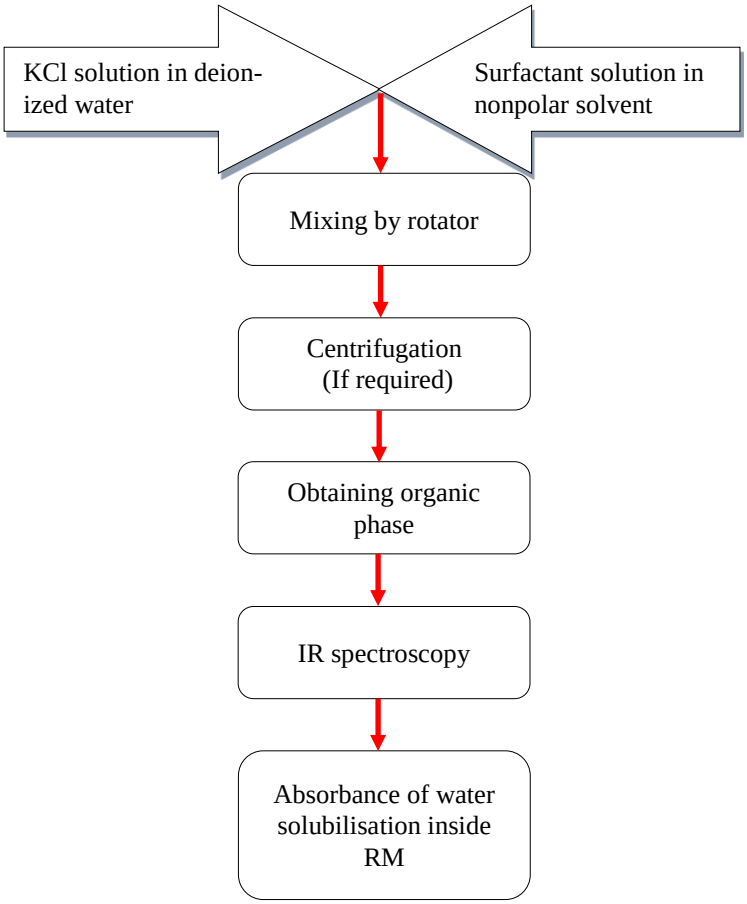

Figure 2.8: Analysis water solubilisation inside RM using IR spectroscopy

3.LYSOZYME EXTRACTION BY ANIONIC AOT REVERSE MICELLAR SYSTEM

This chapter focusses on the results obtained for the extraction of lysozyme in AOT RMS using two solvents: isooctane and *p*-xylene and discussion in detail. This experiment is carried out due to further research of mixed RMS of AOT and pluronics which is presented in chapter 6. There are 3 contents in this chapter, including:

- The size of AOT/isooctane and AOT/*p*-xylene RMS based on water solubilisation and dye solubilisation.
- Water solubilisation of AOT/isooctane and AOT/*p*-xylene RMS using IR spectroscopy.
- Lysozyme forward and backward extraction efficiency by AOT RMS in both solvents.

3.1 The critical micelle concentration of AOT reverse micelles in isooctane

3.1.1 Water solubilisation method

The chosen concentration range of AOT in isooctane was 0.1-1.7 mM. This range was chosen in accordance to the CMC of AOT RM in isooctane from previous reports (0.6-0.9 mM) (Kotlarchyk, 1985; Kelley, 1994; Naoe, 2004).

The water solubilisation was found to be in the range 135-194 ppm from 0.1-0.7 mM AOT which was almost similar (197 ppm) to the amount of water in the sample of pure isooctane (Fig. 3.1). The reason could be because AOT exists in the ORP as monomers or dimers at this concentration range and therefore the water solubilisation is limited because of the absence of RM.

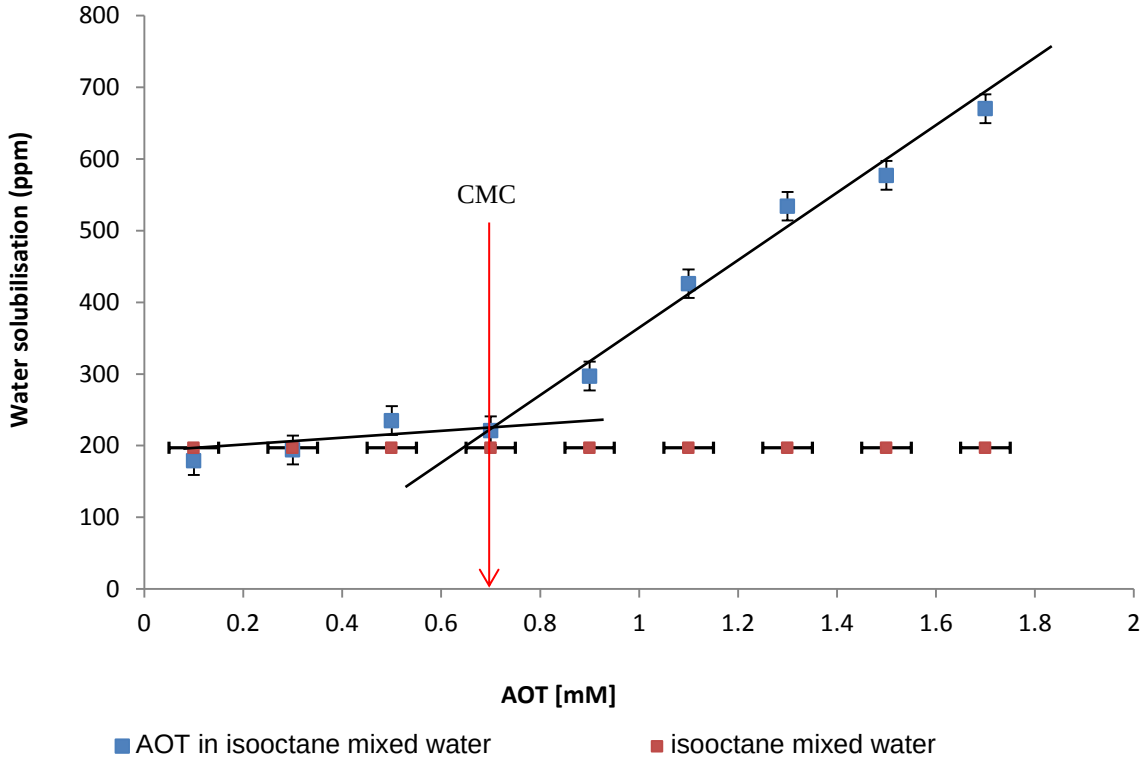

Figure 3.1: CMC of AOT/isooctane RMS determined by water solubilisation method

For AOT concentration from 0.7 to 1.7 mM, there was an abrupt increase (297-670 ppm) in the water solubilisation with the increase in AOT concentration (Fig. 3.1). This increase indicates the self-association of the surfactant molecules into RM, which provides a hydrophilic microenvironment suitable for water solubilisation.

The "reverse" CMC (CMC) can be determined from the surfactant concentration at which the water solubilisation capacity starts increasing (Alexandridis, 1997). In this case, the CMC of AOT (marked by an arrow in Fig. 3.1) was determined as 0.7 mM. This result is slightly low but close to the CMC reported (0.6-0.9 mM) by Jean and colleagues who used the positron annihilation method (Kotlarchyk, 1985).

3.1.2 Rhodamine B solubilisation

In this method, hydrophilic rhodamine B dye was used as a model compound to be solubilised in the reverse micelle. If AOT exist in the ORP as monomers or dimers, then there will be no dye solubilisation. If AOT RM is formed, the dye will be solubilised in the RM, and this can be detected using UV-Vis spectroscopy. Rhodamine B is a hydrophilic dye which is insoluble in isooctane. The concentration range of AOT chosen for the dye solubilisation was 0.05-2 mM, which includes concentration above and below the CMC (0.5 mM) as determined in the water solubilisation method (Fig. 3.2).

Rhodamine B is not soluble in isooctane. However, there is slight solubility of water (197 ppm) in isooctane (Fig. 3.1) which is close to the literature value (0.006% at 20°C) of the solubilisation of water in isooctane (Sigma). As a result of this slight absorbance (0.039) of rhodamine B was observed in the sample of dye and isooctane without AOT (Fig. 3.3). The data also shows an increase in absorbance of the dye with increasing AOT concentration. Thus, it can be reasonably said that the dye is trapped or solubilised inside the reverse micelle, and the solubilisation increases with increasing surfactant concentration. The absorbance of the dye was more or less constant (0.015-0.063) in the AOT concentration range of 0.05-0.4 mM and around the value of blank sample containing isooctane and water only. However, the dye absorbance sharply increased from 0.128 to 0.243 when AOT concentration rose from 0.5 to 3.5 mM. The increase in dye solubilisation is clearly presented in Fig. 3.3. This can be explained based on the hypothesis that RM of AOT in isooctane cannot be formed in the range of 0.05-0.4 mM as the concentration is below the CMC, but once the concentration exceeds the CMC (beyond 0.4 mM) more dye is solubilised inside the RM. This behaviour thus confirms the formation of AOT RM in isooctane and the CMC value using dye solubilisation as 0.45 mM which is close to the CMC value (0.7 mM) using water solubilisation.

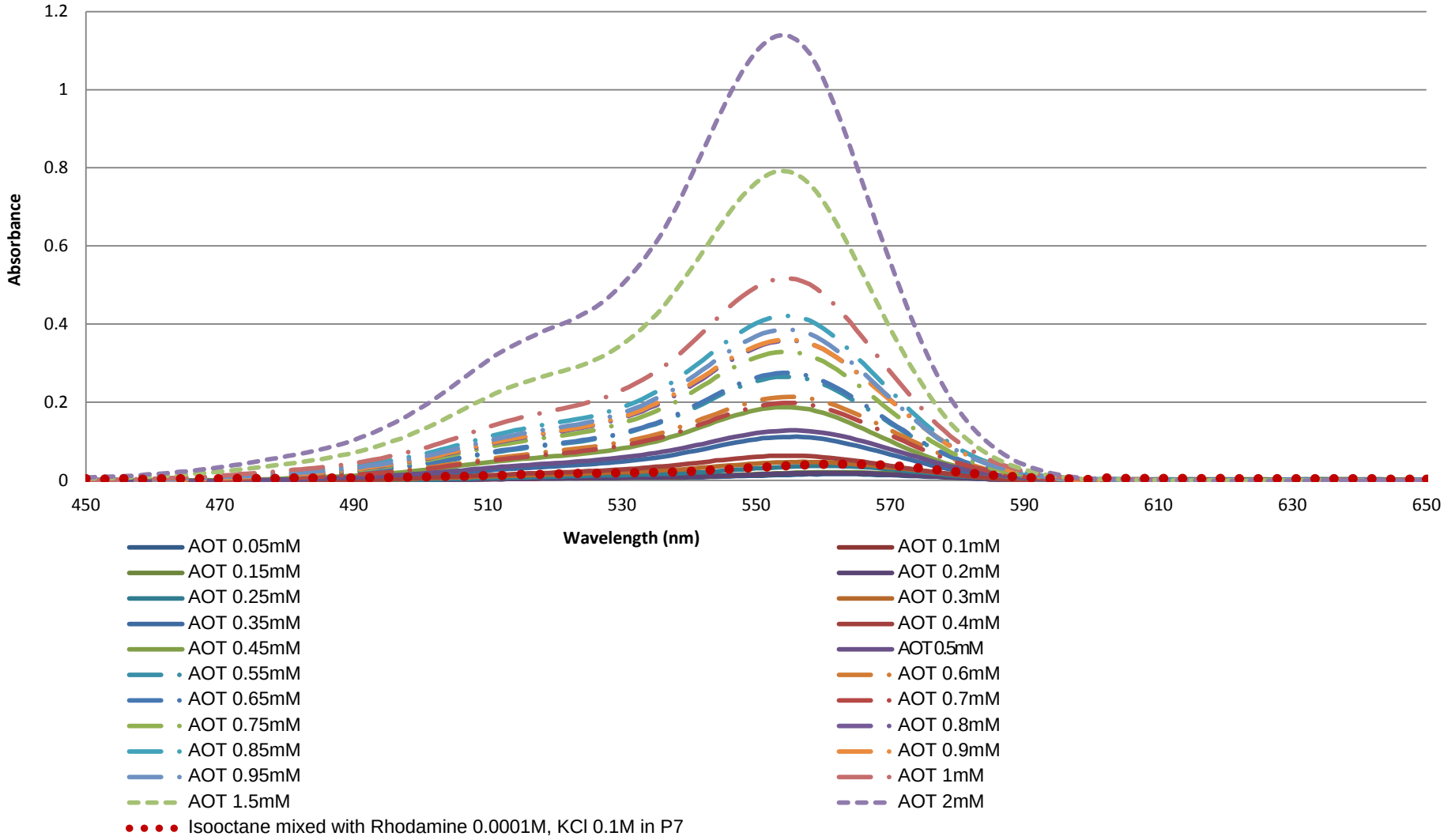

Figure 3.2: Dye solubilisation measurement for AOT RM in isooctane using Rhodamine B solution 0.0001 M, pH 7, KCl 0.1 M.

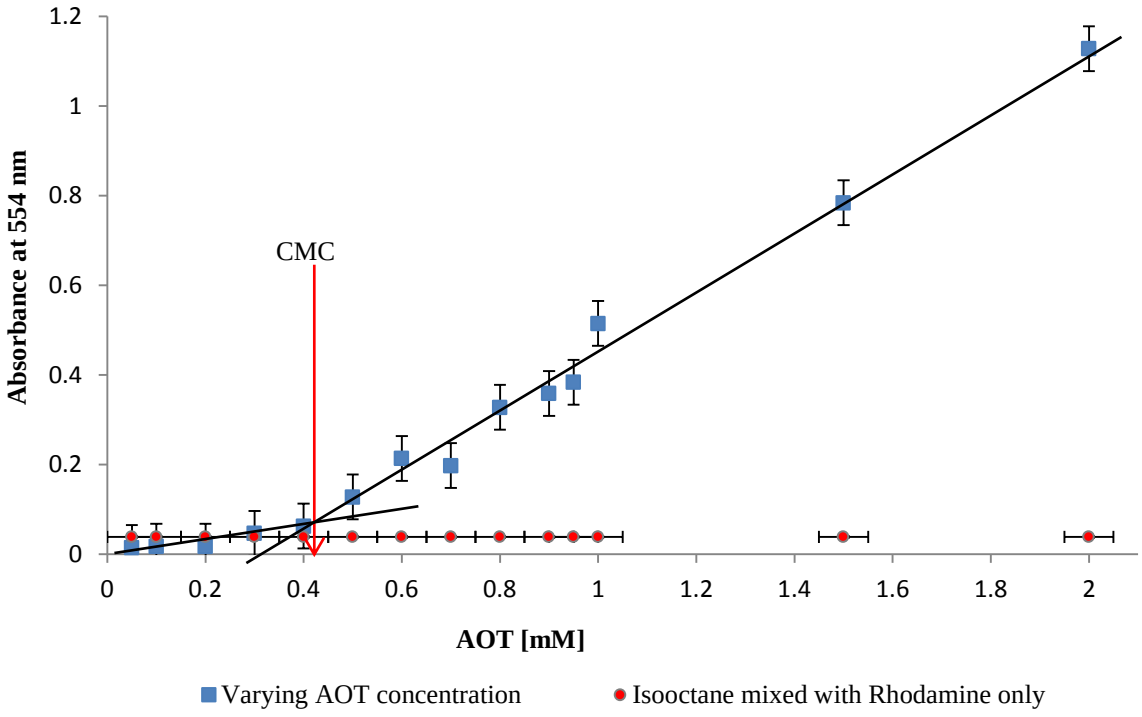

Figure 3.3: CMC of AOT/isooctane RMS determined by the dye solubilisation (Rhodamine B) method.

3.2 The critical micelle concentration of AOT reverse micelles in *p*-xylene

The CMC of AOT RM in isooctane was established to be 0.45-0.7 mM using the water solubilisation and dye solubilisation method. The next step was to investigate if the CMC of AOT would change if a different solvent such as *p*-xylene was used instead of isooctane as *p*-xylene would also be a solvent used for future investigations. Therefore the same method as for AOT in isooctane was repeated but with a different solvent (*p*-xylene) in this study. The reason for choosing *p*-xylene as the solvent was its ability to dissolve triblock copolymer surfactant that would be investigated further for its mixed reverse micelle forming ability with AOT. Tapas and co-workers have studied the behaviour of AOT in non-polar solvent and their report showed that most studies on AOT RM used cyclohexane, benzene, isooctane, 1-octane, *n*-decane, *n*-dodecane, tetrachloromethane, and deuterated chloroform (Tapas, 1995) as the

preferred solvent. There has been no evidence in the literature regarding the CMC of AOT RM in *p*-xylene.

3.2.1 Water solubilisation

The chosen concentration range of AOT in this study was 0.1-1.9 mM based on previous studies related to the AOT RM formation in the literature (Kotlarchyk, 1985; Rodakiewicz-Nowark, 2002).

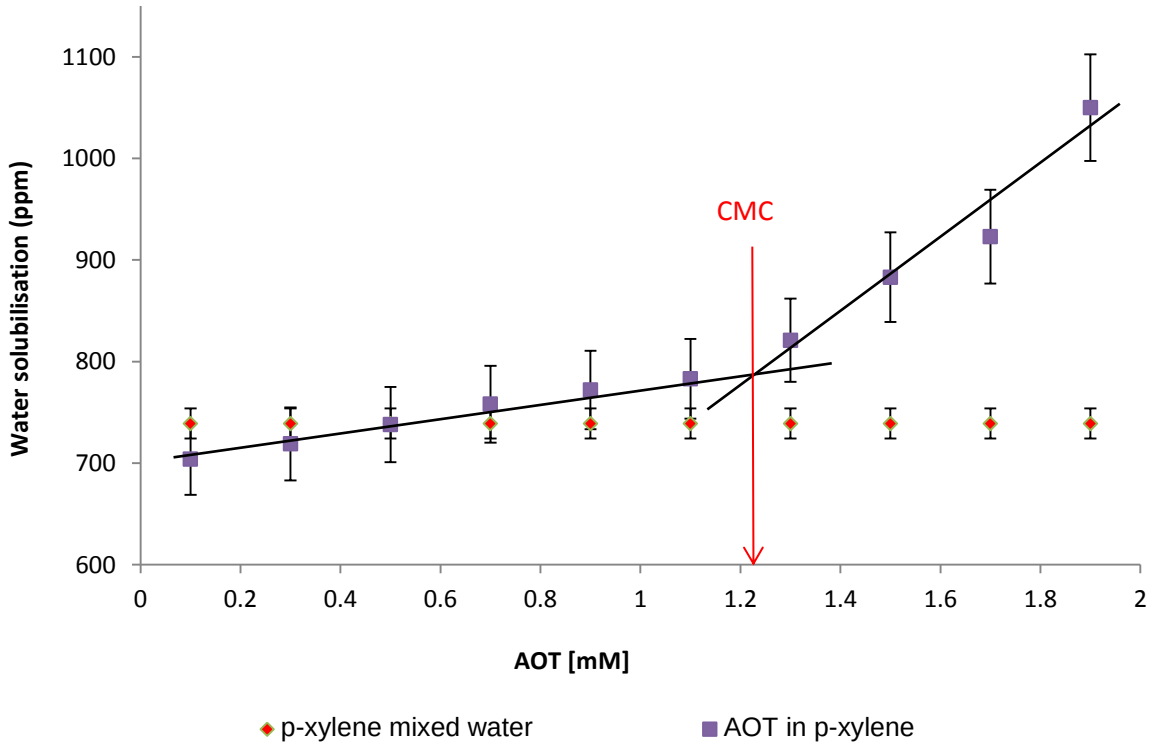

Figure 3.4: CMC of AOT/*p*-xylene RMS determined by the water solubilisation method.

From AOT concentration 0.1 to 0.5 mM, the water solubilisation (704-738 ppm) was low but it gradually increased and was close to the water content value of the sample containing *p*-xylene and water only (739 ppm). The reason could be at the lower concentration range AOT exists in the ORP as monomers or dimers. Since no RM was envisaged at this concentration range there was almost no evidence of any additional water in the system (Fig. 3.4). Higher amount of water solubilisation was recorded here in comparison with isooctane system because the solubilisation

of water in *p*-xylene was 0.0217 mole/litre or 0.023 % at 20^0C while in isooctane it was only 0.006 % (Kirchnerova, 1976; Sadek, 2004).

From 0.5 to 1.1 mM AOT concentration, the amount of water solubilisation increased but gradually from 738 ppm to 783 ppm. However, a sharp increase in water solubilisation (783-1050) was observed from 1.1-1.9 mM of AOT. From this data the CMC of the system was determined to be 1.2 mM which was obtained by the intersection of two linear best fit lines as shown in Fig. 3.4.

The CMC of AOT RM in *p*-xylene was higher than the CMC of AOT RM in isooctane using water solubilisation method, 1.2 mM and 0.7 mM, respectively. The data in Fig. 3.5 shows the comparison of the extent of water solubilisation inside AOT/isooctane/water RM and AOT/*p*-xylene/water RM. After deducing the amount of water solubilisation in isooctane and *p*-xylene, it can be observed that the AOT/isooctane RM can solubilise more water than AOT/*p*-xylene RM. The reason could be because AOT can form bigger RM in isooctane than *p*-xylene. Hsu and colleagues demonstrated that particles can have strong electrostatic interactions in low dielectric constant environments when ions are solubilized by RM (Hsu, 2005). The dielectric constant of *p*-xylene and isooctane are 2.2 and 1.94, respectively which may cause stronger electrostatic interactions of AOT/isooctane RMS than AOT/*p*-xylene RMS. Therefore, larger RM was formed resulting in more water solubilisation in AOT/isooctane system as compared to AOT/p-xylene system.

3.2.2 Rhodamine B solubilisation

The CMC of AOT in *p*-xylene was further investigated using the dye solubilisation method. A hydrophilic dye rhodamine B, insoluble in *p*-xylene, was used as a model compound to be solubilised in AOT/p-xylene reverse micelle system. The range of AOT concentration chosen for the dye solubilisation was from 0.1-5 mM, which includes concentrations above and below the

CMC (1.2 mM) in accordance with the water solubilisation method. The data in Fig. 3.6 shows the increasing solubilisation of rhodamine B in terms of absorbance in the AOT concentration range 0.1-5 mM to confirm the CMC value of AOT/*p*-xylene RMS.

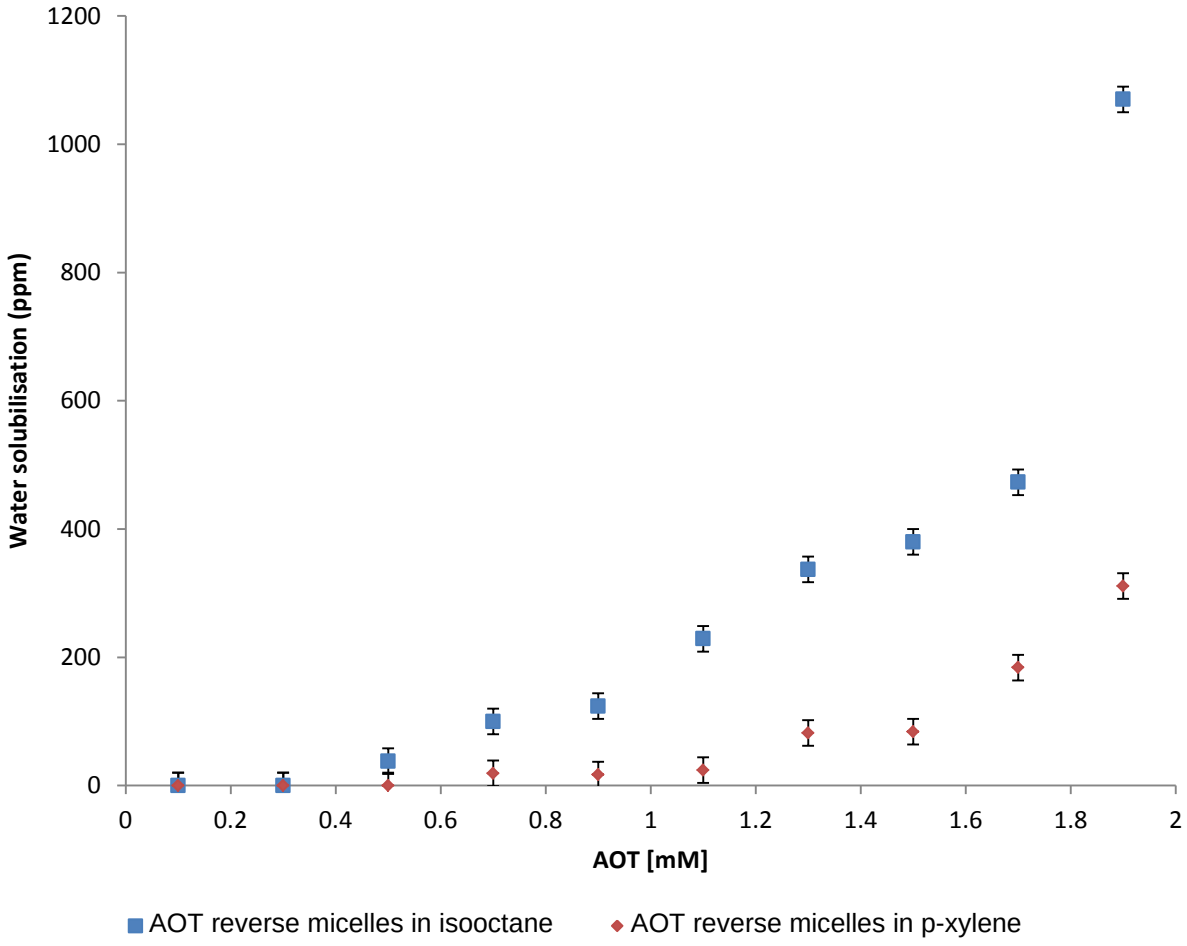

Figure 3.5: Water content of AOT RMS in isooctane and AOT RMS in *p*-xylene after deducing the amount of water solubilisation in isooctane and *p*-xylene.

A small amount of water (739 ppm) was present in *p*-xylene when it was mixed with water under similar solubilising conditions as rhodamine B. Therefore, a small amount of rhodamine B was detected (absorbance of 0.005) in samples containing the dye and *p*-xylene without AOT even though rhodamine B was insoluble in *p*-xylene. The data also shows an increase in absorbance of the dye with increasing AOT concentration. Thus, it can be reasonably said that the dye is

trapped or solubilised inside the reverse micelle, and the solubilisation increases with increasing surfactant concentration.

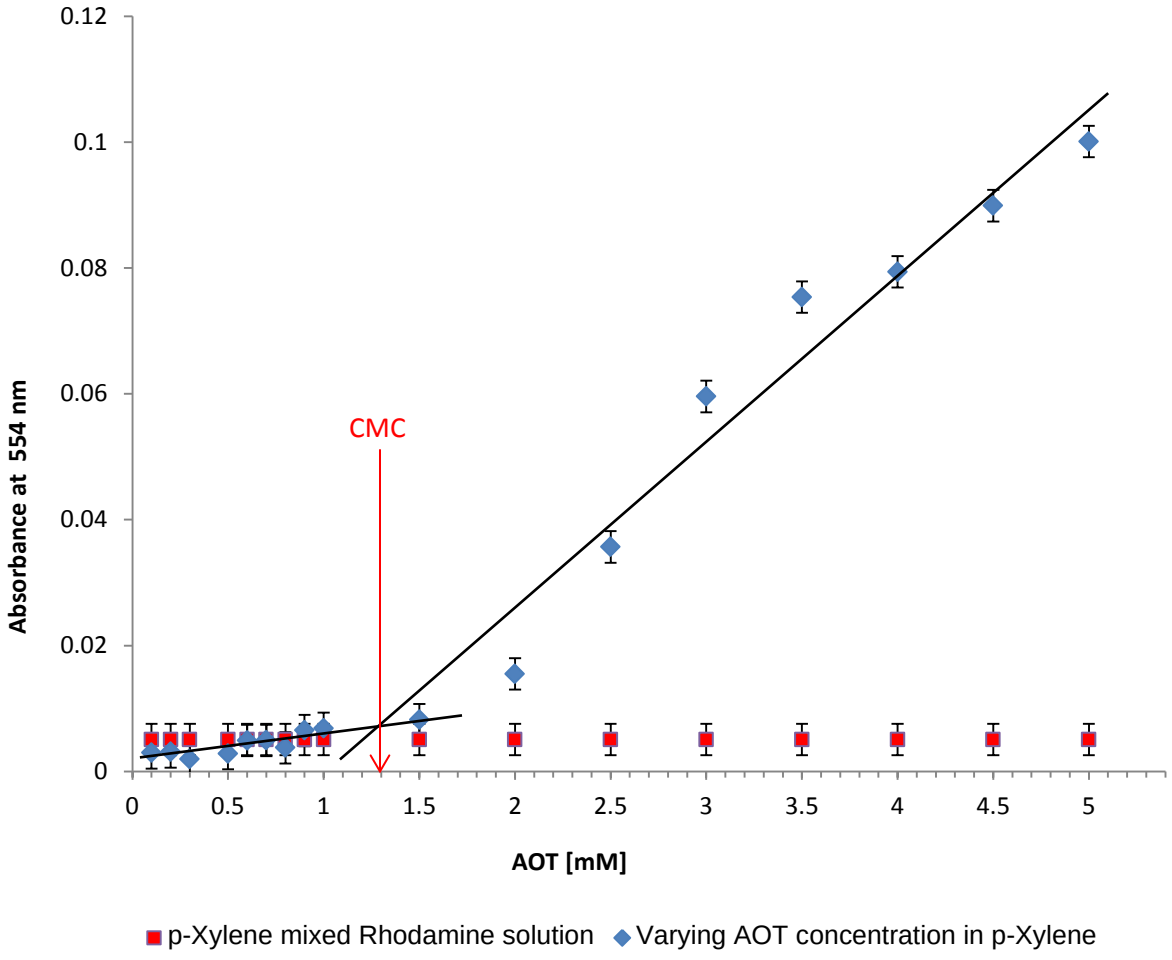

Figure 3.6: Determination CMC of AOT RM in *p*-xylene using the dye (Rhodamine B) solubilisation method.

From 0.1-1 mM AOT concentration the absorbance of rhodamine B was more or less constant (0.0019-0.0068) and around the value of blank sample consisting of *p*-xylene and water only (0.005). However, the dye absorbance sharply increased from 0.008 to 0.1 when the AOT concentration increased from 1.5 to 5 mM. This increase in absorbance can be clearly seen in Fig. 3.6. This phenomena can be explained based on the hypothesis that no RM are formed in the range of 0.1-1 mM, but once the concentration exceeds this point (beyond 1 mM) more dye is solubilised inside the RM. The increase in dye absorbance against increase in AOT concentration was determined and the intersection of two best fit linear lines gave the CMC of this system. Ac-

cording to Fig. 3.6, the CMC value of AOT/*p*-xylene RMS was around 1.3 mM. This result is in agreement with the CMC determinates by water solubilisation method which was also 1.2 mM. Although no CMC value of AOT in *p*-xylene was recorded, the CMC of AOT in benzene using dye (iodine) absorption was reported to be 0.9 mM (Kotlarchyk, 1985). This establishes that the CMC value of AOT in *p*-xylene in this work was close to the value reported in the literature in another AOT aromatic solvent (benzene) system.

3.3 The size of AOT reverse micelles in isooctane and *p*-xylene

The anionic surfactant AOT is known to form spherical RM in a variety of nonpolar solvents (Kinugase, 2002). The size of RM is readily tuneable by adjusting the W_0 value, where W_0 is defined as water content (Scatena, 2001).

$$W_0 = [H_2O]/ [AOT] \qquad \textit{(Equation 5)}$$

Where: $[H_2O]$ = water concentration

[AOT] = AOT concentration

According to the linear relationship between the diameter of water pool and W_0 (Kinugasa T. K., 2003):

$$d_{wp} = 0.29w_0 + 1.1 \text{ (nm)}. \qquad \textit{(Equation 6)}$$

However, this equation is applied for RM in the range W_0 = 2-20 only (Kinugase, 2002).

The data in Fig. 3.7 shows the water content values calculated based on Eq.5. The $[H_2O]$ values were determined from the water solubilisation method for both AOT/isooctane and AOT/*p*-xylene system. Beyond the CMC point, the W_0 value of AOT/isooctane RM increased from 4 to 31 while this value increased from 1 to 9 only for AOT/*p*-xylene RM. The diameter of

the water pool was further calculated based on Eq.6 and the data in Fig. 3.8 clearly shows the difference in size of water pool of AOT RM in isooctane and *p*-xylene. The highest diameter of AOT/*p*-xylene RM was 2.8 nm at an AOT concentration of 1.7 mM as compared to 5.6 mM of AOT/isooctane system at similar AOT concentration

The calculated size of AOT/isooctane in this study was in agreement with previous studies in the literature. David and his colleagues (Moilanen, 2009) reported that AOT/isooctane RM with W_0 = 5, 10 and 16.5, would have water pool diameters of 2.3, 4.0, and 5.8 nm, respectively. In this work, AOT/isooctane RM with W_0 = 4.2, 11.5 and 15.4 have diameters of 2.3, 4.5 and 5.6 nm, respectively (Fig. 3.8). In addition, the size of the RM determined through the water solubilisation method in this study was in general comparable to the size of RM found in the literature which was reported to be in the range of 1.6-20 nm (Moilanen, 2009).

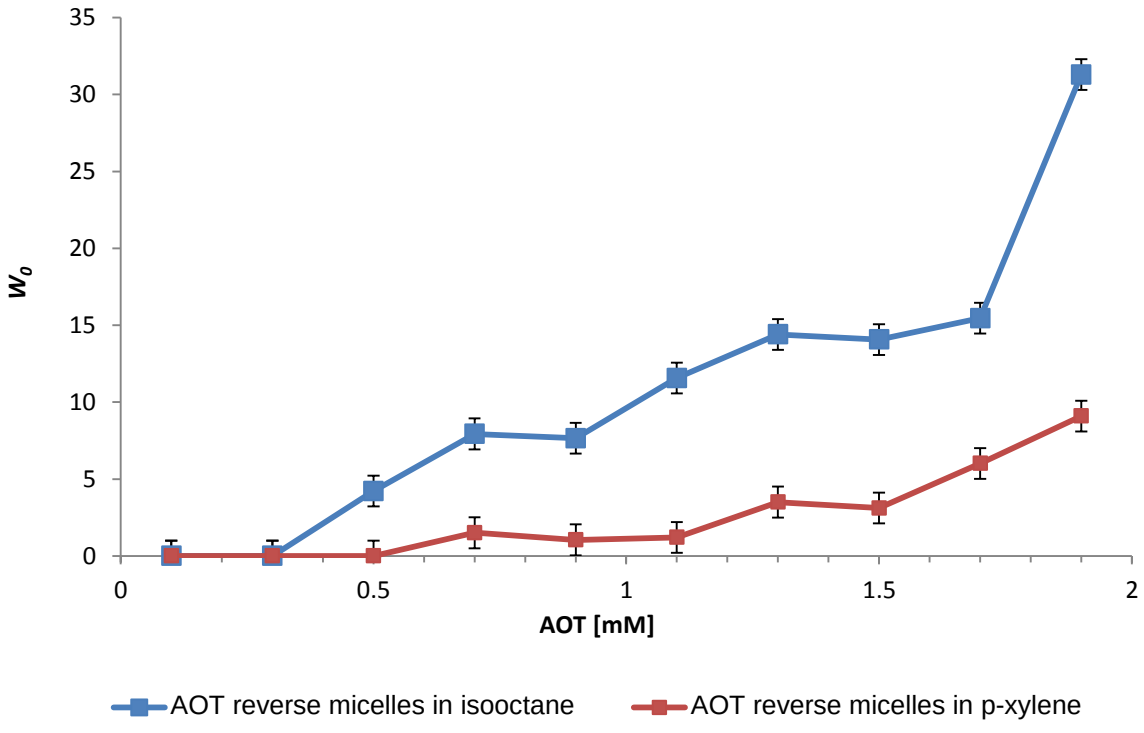

Figure 3.7: Comparing W_0 of AOT RM in isooctane and AOT RM in *p*-xylene according to water solubilisation method.

Therefore, it can be concluded in general that AOT can form bigger revere micelles in isooctane than in *p*-xylene. This result is quite similar to Abuin group's work where they studied the

solubilisation of AOT RM in different solvents, including *n*-heptane, cyclohexane, isooctane, dodecane, benzene, chlorobezene, toluene and tetrachloroethylene (Abuin, 2002). They reported that the characteristics of the micellar aggregates and their capacity to incorporate solutes, is to a large extent dependent on the external solvent. In particular, the thermodynamics of micellization shows that the clustering of benzene molecules around the AOT head-groups appears to be more ordered than is in case of saturated alkanes such as isooctane, cyclohexane and *n*-heptane. It turns out that, at the same value of W_0, the aggregation number of AOT is 5 times greater in *n*-heptane than in benzene. Furthermore, while AOT RM in saturated hydrocarbons is able to solubilize large amount of water, those formed in benzene solubilize only small amounts of water. The limited water solubilisation could lead to smaller W_0 that causes smaller d_{wp} resulting in small RM size (Eq. 5 and Eq. 6).

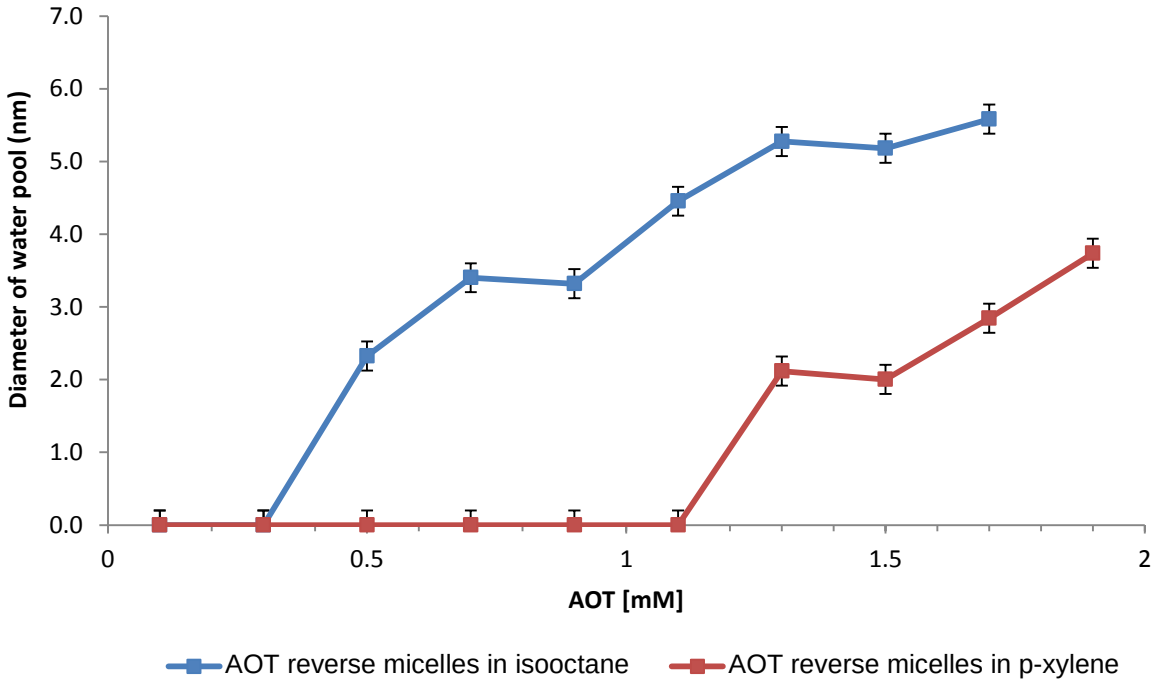

Figure 3.8: The diameter of water pool of AOT reverses micelles system in isooctane and in *p*-xylene.

3.4 Investigation of AOT reverse micellar system using IR

In order to re-confirm the presence of water in the inner core of AOT/isooctane and AOT/*p*-xylene RMS, IR spectroscopy is used as analysed technique. IR is a non-invasive, functional-group selective technique. It has been proved to be sensitive to the chemical environment and has been often employed for the detection and characterization of hydrogen bonding. In this experiment, IR was used to investigate the water inside AOT RMS in the 3000-4000 cm^{-1} range, where absorptions due to O-H stretching modes of H_2O are present (Amico, 1995)

The results from this experiment were presented in Fig. 3.9 and Fig. 3.10. Below the CMC, 0.45-0.7 mM for AOT/isooctane RM and 1.2-1.3 mM for AOT/*p*-xylene RM according to results in sections 3.1 and 3.2, no water was observed. At the CMC point, the absorbance of water was very low which is in agreement with the results of water solubilisation with only 38 ppm and 19 ppm water recorded for AOT/isooctane and AOT/*p*-xylene, respectively (Fig. 3.5). Above the CMC, the absorbance of water increased sharply at 3 mM and 5 mM AOT for both systems. However, the absorbance of water at 5 mM AOT in case of AOT/*p*-xylene RM was 0.0027 which was weaker than the one with 3 mM AOT/isooctane RM (0.0036). Therefore, it is very clear that AOT can form bigger RM in isooctane than *p*-xylene based on the higher amount of water solubilised inside AOT/isooctane RM than AOT/*p*-xylene RM. In addition, this result also proved the concept that increase in surfactant concentration leads to an increase in water solubilisation inside RM which is in agreement with the results in section 3.1and 3.2.

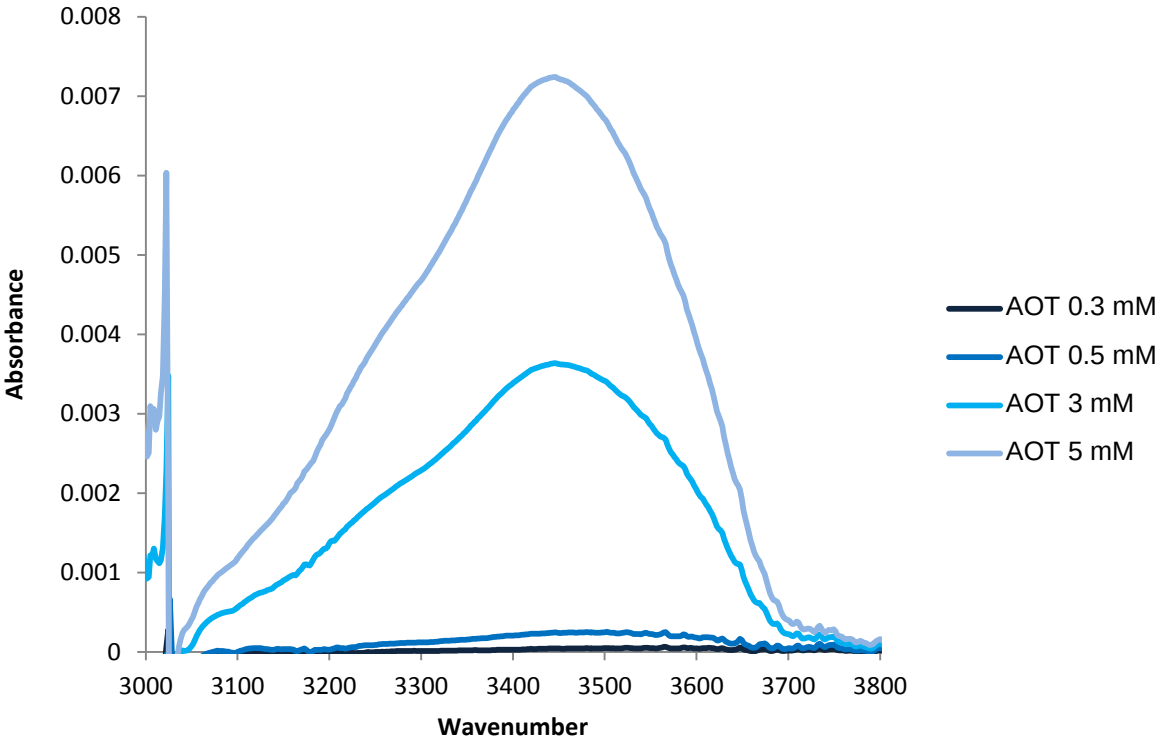

Figure 3.9: The absorbance of water inside AOT/isooctane RMS at different AOT concentration.

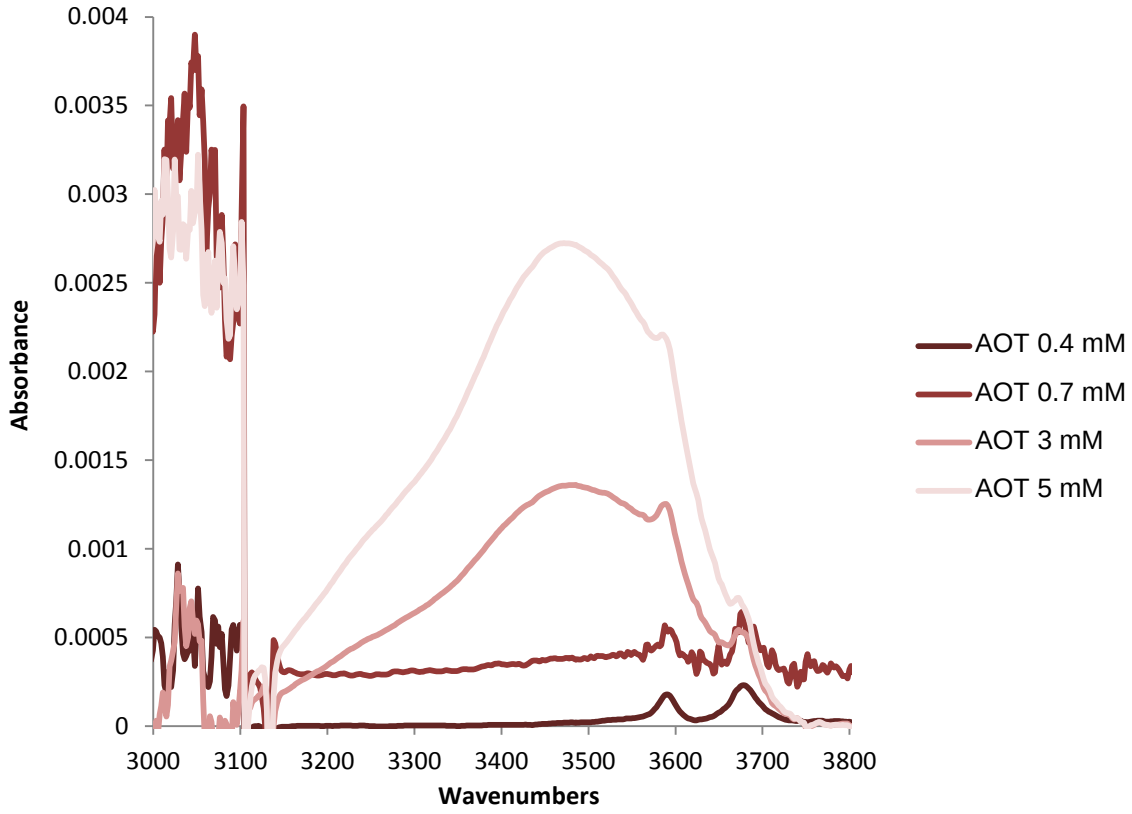

Figure 3.10: The absorbance of water inside AOT/*p*-xylene RMS at different AOT concentration.

3.5 Lysozyme extraction using AOT RM in isooctane and *p*-xylene

Lysozyme extraction using AOT RM in isooctane and *p*-xylene was investigated.

The concentration of lysozyme during the extraction process was determined based on the lysozyme calibration curve in phosphate buffer (Fig. 3.11).

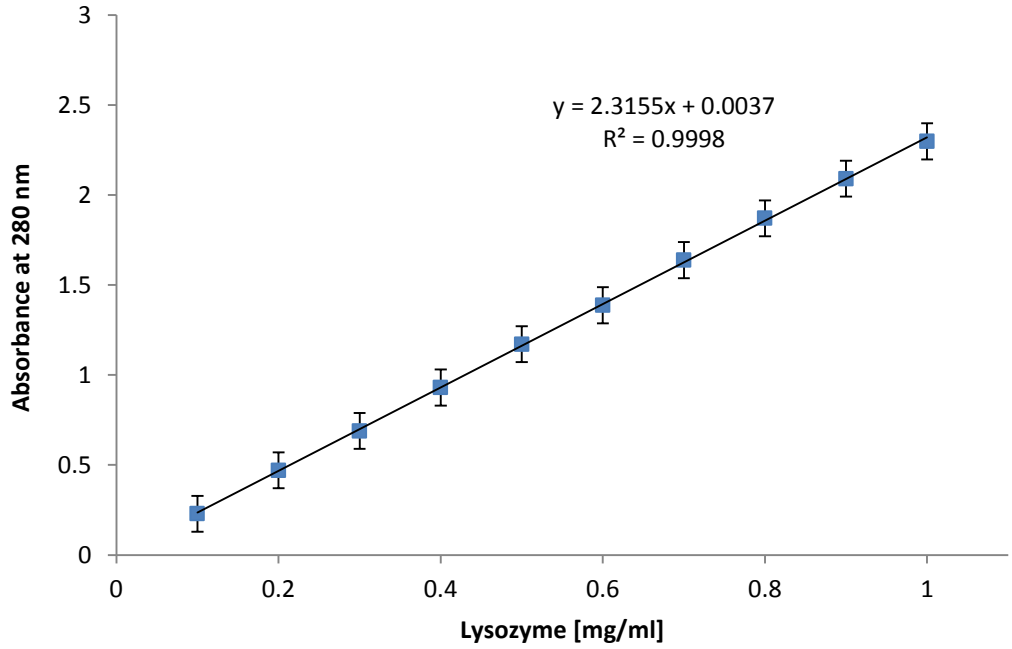

Figure 3.11: Calibration curve of lysozyme (0.1-1 mg/ml) by UV-Vis spectroscopy.

For FE, the studied pH chosen was 4, 5, 6, 7, 8, 9, and 10 as it was below the pI (11.3) of lysozyme (Wetter, 1951). The reason for choosing a pH below pI is that at this pH lysozyme will have a positive charge which is opposite to that on the anionic surfactant AOT (negative charge). This results in electrostatic interaction between the surfactant and the proteins resulting in the entrapment of the protein (lysozyme) in the AOT RM. The result was presented in Fig. 3.12 which showed that pH 8 is the best condition for lysozyme FE with 99 % in extraction efficiency.

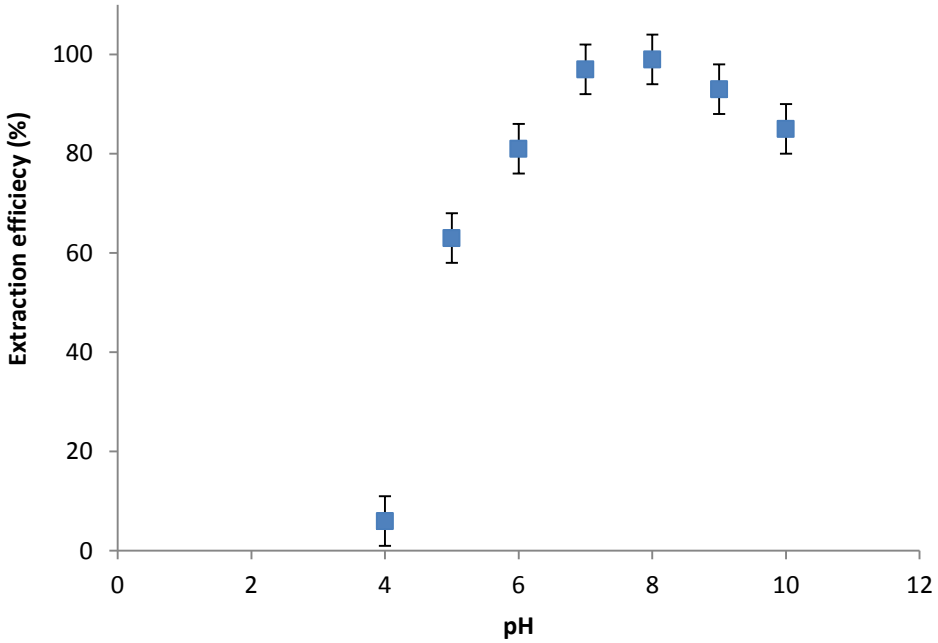

Figure 3.12: Effect of pH to lysozyme FE using AOT/isooctane RMS.

On the contrary, pH 11.8 was best choice for BE because above pI, lysozyme carries a negative charge that is similar to the charge on AOT, resulting in electrostatic repulsion and hence the expulsion of lysozyme out of the AOT RM (Fig. 3.13).

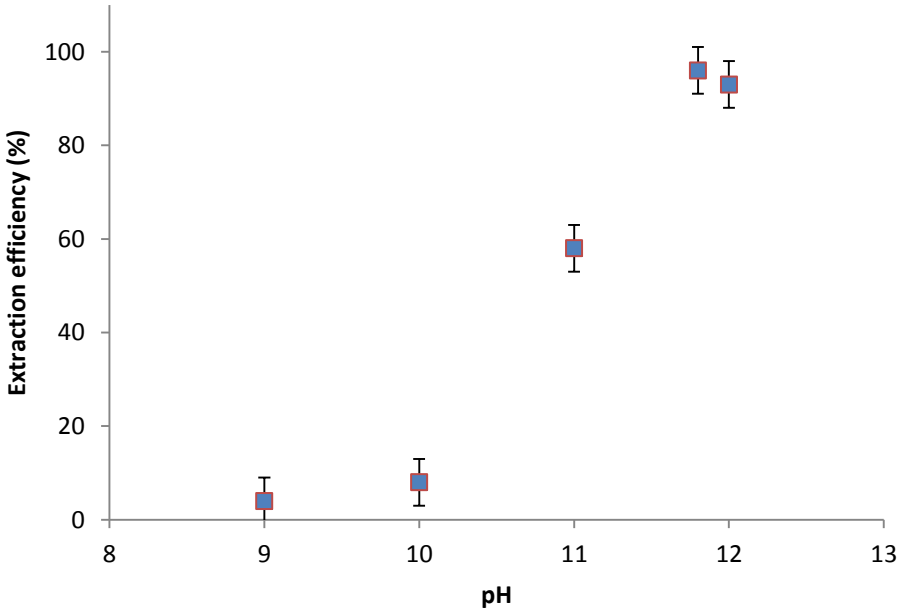

Figure 3.13: Effect of pH to lysozyme backward extraction using AOT/isooctane RMS.

The effect of KCl concentration to lysozyme FE was presented in Fig. 3.14. The result showed that KCl 0.1 M was the best choice for lysozyme FE using AOT/isooctane RMS.

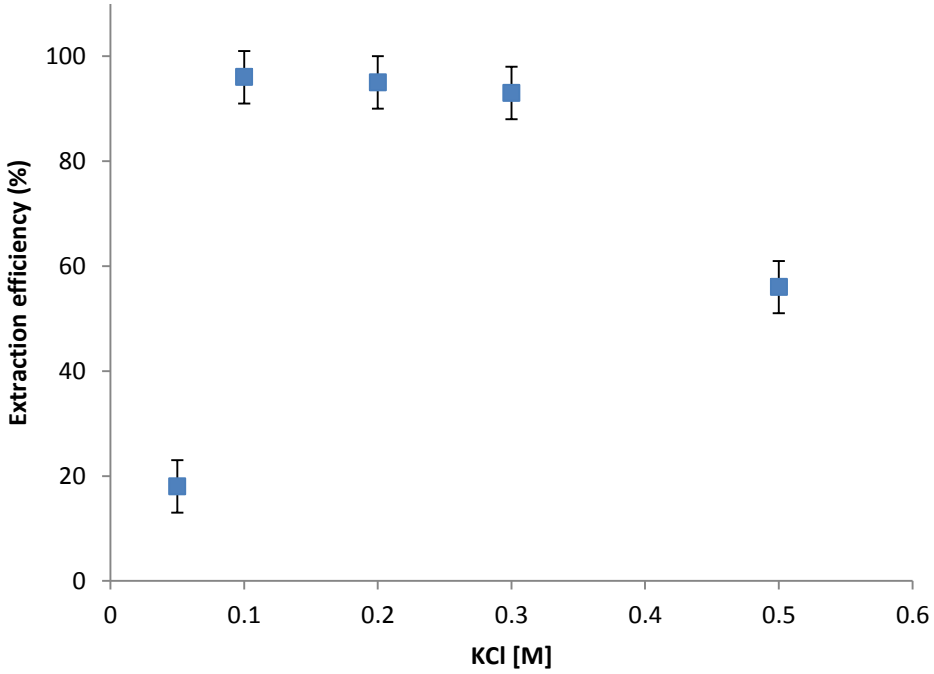

Figure 3.14: Effect of KCl concentration to lysozyme forward extraction using AOT/isooctane RMS.

KCl affects the transfer behavior of protein due to micelle size changes or screening of electrostatic interaction between the protein and the micelle wall (Goklen and Hatton, 1985). In the present investigation, KCl was varied from 0.05 to 0.5 M, and it was observed that the forward extraction efficiency reached the highest value at 0.1 M, (96 %) (Fig. 3.14). Lysozyme extraction increased when KCl concentration ascended from 0.05 to 0.1 M, but decreased beyond KCl concentration of 0.2 M. The probable reason for this behavior could be the decrease of the interaction intensity due to screening effect of KCl, inhibiting the solubilization of the protein and water in reverse micelles (Andrews and Haywood, 1994; Lye *et al.*, 1994; Matzke *et al.*, 1992, Nishiki *et al.*, 1998). At low salt concentration, the surfactant layer is compressed and aggregation occurs at a smaller reverse micelles. At higher salt concentration, the screening dominates and leads to a larger reverse micelles. Upon the formation of micelles-like structures at a high surfactant concentration, the addition of KCl is favorable for larger aggregates to form. As a result,

more lysozyme was extracted by larger reverse micelles, shown by the increase in the extraction of lysozyme from 18 % to approximately 96 % (Fig. 3.14). On the other hand, the extraction of lysozyme decreased with increasing salt concentration (0.2-0.5 M) because of two effects due to a change in thickness of the electric double layer (Kinugase, *et al.*, 1991). One is the size of the reverse micelle, and the other is the electrostatic attractions between the charged groups of the protein and the charged micellar wall. The optimal KCl concentration for lysozyme BE is 2 M which is reported from previous studies (Nishiki, 1998; Pham, 2010).

The effect of varying AOT concentration (5-100 mM) on the extraction of lysozyme was investigated for AOT RMSs in both isooctane and *p*-xylene. KCl and pH conditions for lysozyme FE and BE using AOT/*p*-xylene RMS were used from studied result of AOT/isooctane RMS (Fig. 3.12, Fig. 3.13, Fig. 3.14). The efficiency of FE using AOT/isooctane and AOT/*p*-xylene RM was very high, 96-99 %, but it was easier to recover lysozyme from AOT/isooctane system than AOT/*p*-xylene which is evident from the BE efficiency values in Fig. 3.15 and Fig. 3.16. At 5 mM AOT the recovered lysozyme was 81 % in case of AOT/isooctane which was more than 2 times higher than the AOT/*p*-xylene system (42 %). Following the increase of AOT concentration from 10 to 100 mM, AOT/isooctane system gave a good BE percentage, in the range 81-97 % while AOT/*p*-xylene system gave much lower extraction in the range 56-91 %. The reason could be the size of the AOT/*p*-xylene RM which was found to be smaller than the AOT/isooctane RM (Fig. 3.8). However, the FE efficiency of both AOT systems were similar and therefore there must be some other factor which leads to the decrease in BE efficiency of lysozyme in the AOT/*p*-xylene system. One argument could be that AOT/*p*-xylene RM may interact strongly with lysozyme due to a variety of interactions such as electrostatic, hydrophobic and steric. Moilanen and colleagues reported that with large RMS whose $W_0 \geq 16.5$ (as AOT/isooctane RM in this case), the dynamics of water are separable into a bulk water like core and an interfacial shell, however with small RMS $W_0 \leq 5$ (as AOT/*p*-xylene RM in this case),

nearly all water interacts directly with the interface (Moilanen, 2009) which could result in stronger binding of the protein, leading to difficulty recovery.

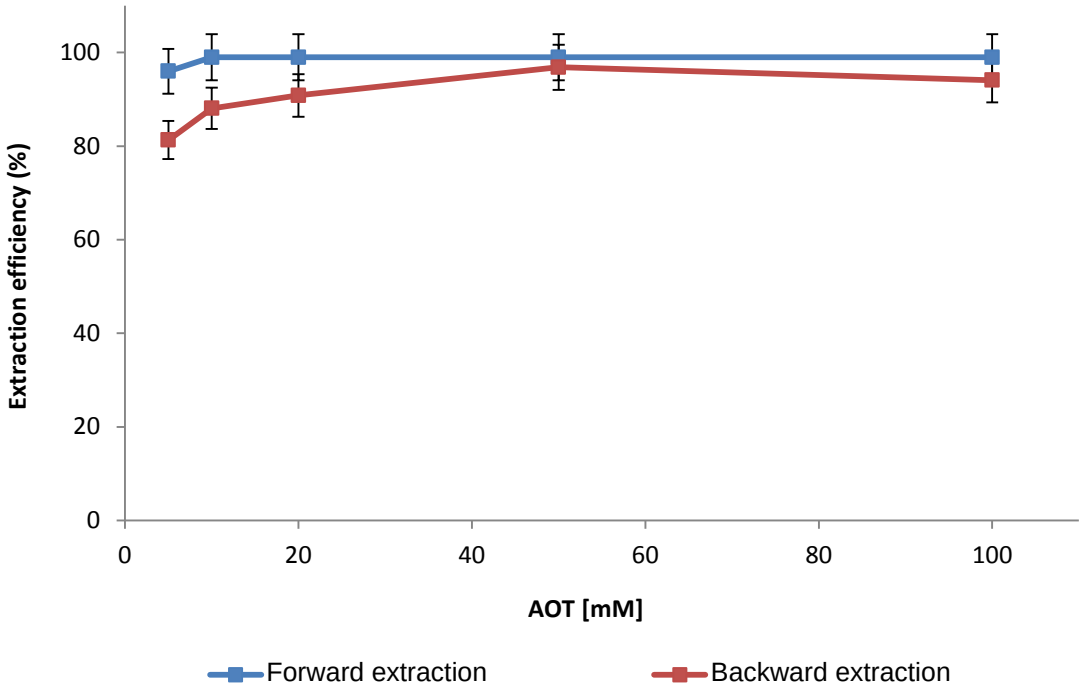

Figure 3.15: Effect of AOT concentration on lysozyme extraction using AOT RM in isooctane. FE: KCl 0.1 M, pH 8. BE: KCl 2 M, pH 11.8

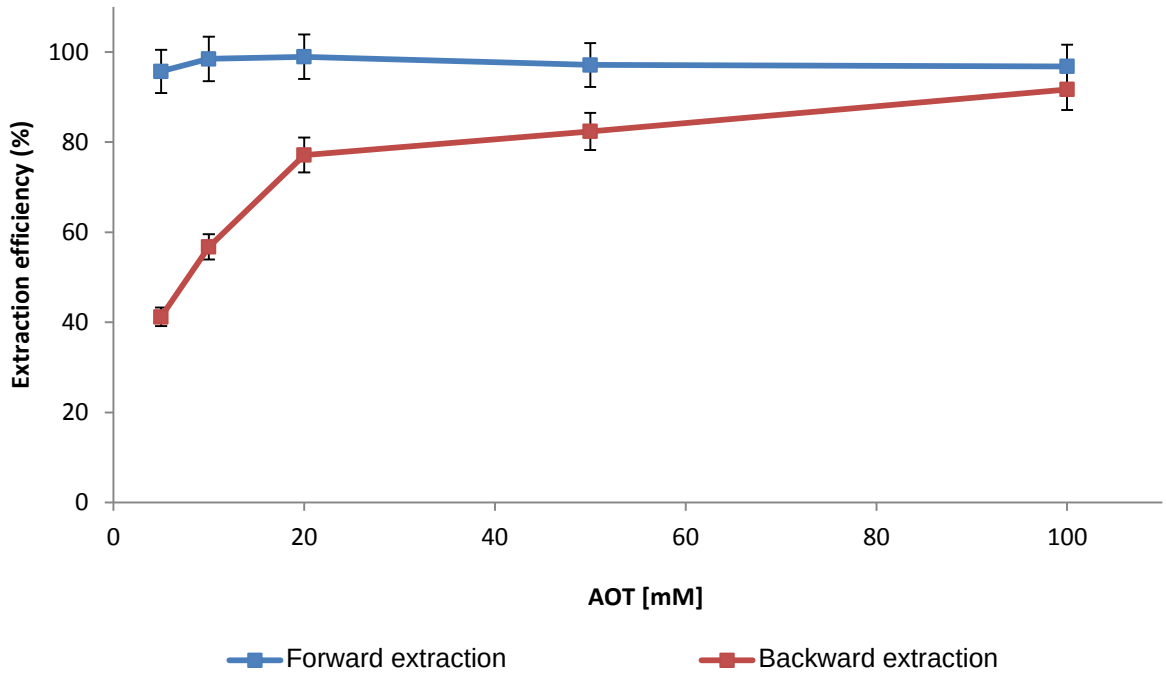

Figure 3.16: Effect of AOT concentration on lysozyme extraction using AOT RM in *p*-xylene. FE: KCl 0.1 M, pH 8. BE: KCl 2 M, pH 11.8

3.6 Structure and activity of recovered lysozyme

3.6.1 Analysis of secondary structure of lysozyme using CD spectroscopy

The secondary structure of recovered lysozymes in case of AOT/isooctane and AOT/*p*-xylene system were analyses by CD and are shown in Fig. 3.17. and Fig. 3.18.

All samples were analysed by CD in the range of 200-280 nm that shows the spectra of α-helical and antiparallel β-sheet conformation (Kelly, 2005).

In case of AOT/isooctane, the spectrum of recovered lysozyme (Fig. 3.17) at different AOT concentrations nearly overlaps each other as well as the spectrum of native lysozyme. This confirms that the secondary structure of recovered lysozyme was maintained which is also in agreement with previous studies in the literature (Kinugasa, 1992; Naoe, 2004).

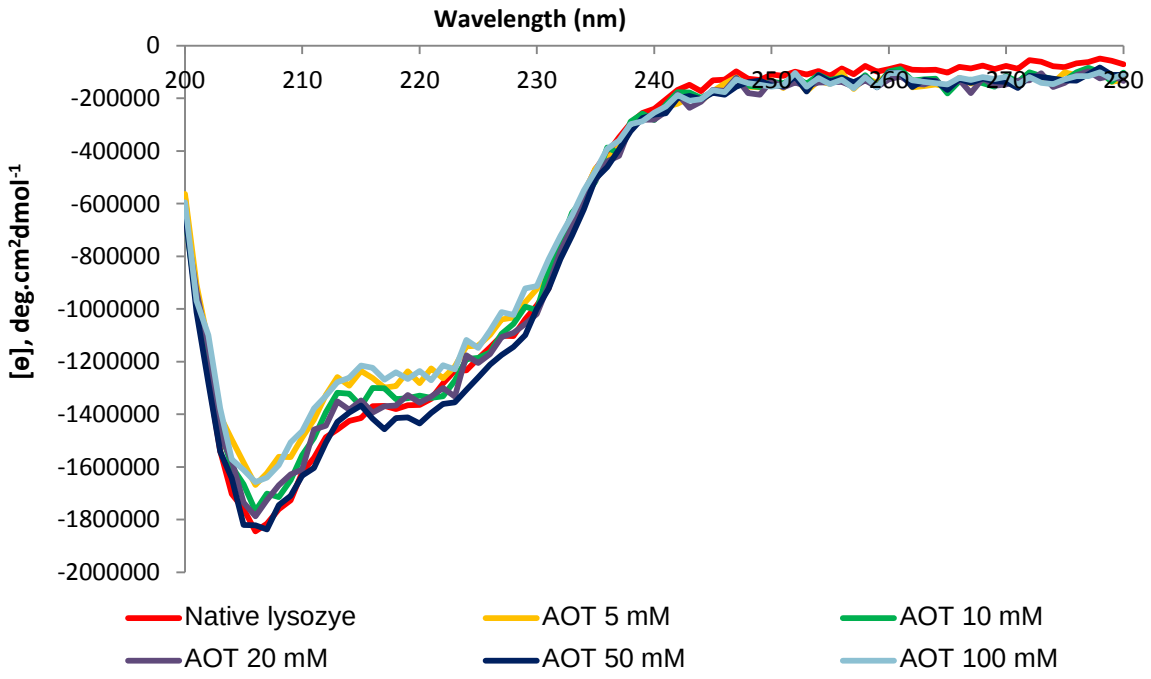

Figure 3.17: CD spectrum of recovered lysozyme using AOT RM in isooctane at varying AOT concentration. FE conditions: KCl 0.1M, pH 8. BE conditions: KCl 1 M, pH 11.8.

These data were also analysed with CDNN programme to determine the percentage of α-helix, antiparallel β-sheet, parallel β-turn and random coil as the major elements of secondary structure of lysozyme (Fig. 3.18). The percentage of antiparallel β-sheet, parallel, and β-turn were relatively similar between all recovered lysozyme samples as compared to native lysozyme. The α-helix quantities of recovered lysozyme samples increased when AOT concentration increased from 5 mM to 50 mM, but decreased at 100 mM AOT. On the other hand, the percent of random coil of recovered lysozyme samples decreased when AOT concentration increased from 5-50 mM, but then increased at 100 mM AOT. When compared to native lysozyme the best recovered lysozyme which kept their secondary structure was the sample from 50mM AOT (Fig. 3.18).

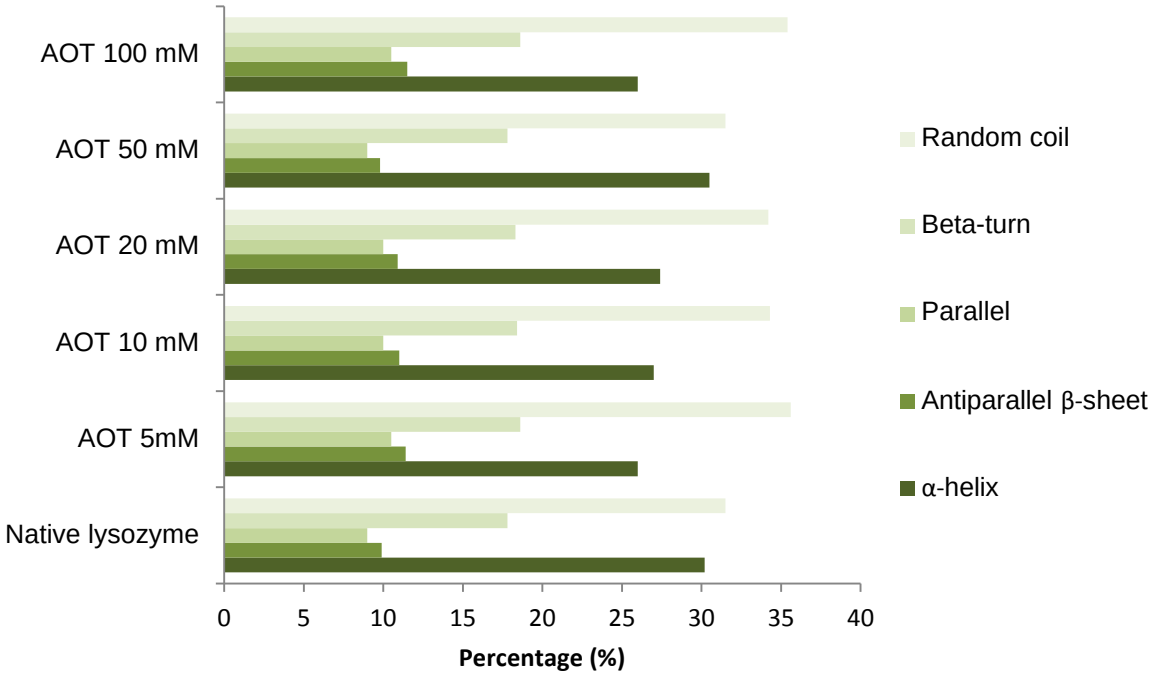

Figure 3.18: CDNN analysis for recovered lysozyme from AOT/isooctane RMS

However, in case of AOT/*p*-xylene system, the molar ellipticity spectrums of extracted lysozyme samples were slightly different in intensities and shape in comparison with native lysozyme spectrum (Fig. 3.19). This could be due to steric interaction or size factor that was explained in the earlier discussion on size of AOT RM in isooctane and *p*-xylene (section 3.3). It can also be

argued that the AOT/isooctane RM was much more suitable to host lysozyme inside the micelles as the lysozyme was easily expelled out because of the absence of very strong interactions thus maintaining the secondary structure. In case of AOT/*p*-xylene RM, the smaller size and stronger interaction between the lysozyme and the AOT/*p*-xylene system led to decreased back extraction and change in the secondary structure of lysozyme mainly due to stronger interaction.

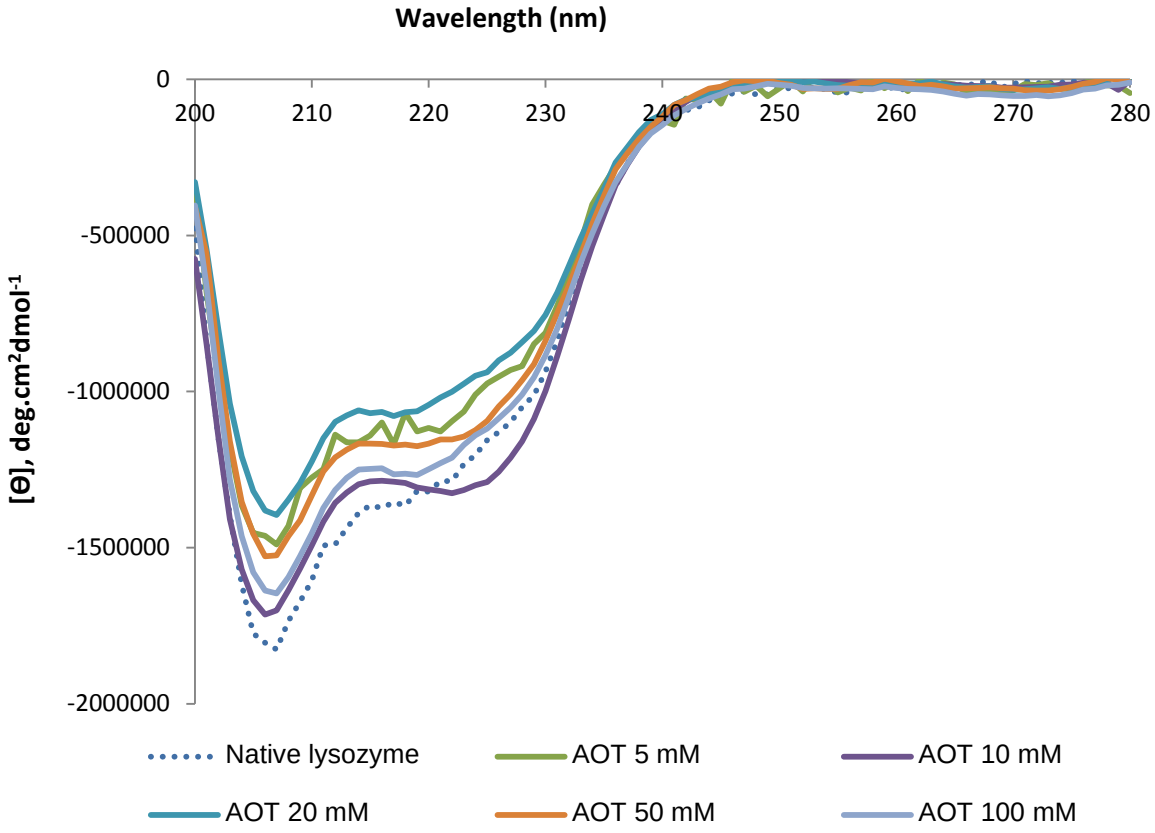

Figure 3.19: CD spectrum of recovered lysozyme using AOT RMS in *p*-xylene at varying AOT concentration. FE conditions: KCl 0.1 M, pH 8. BE conditions: KCl 1 M, pH 11.8.

The CD data from the AOT/*p*-xylene RMS was subjected to CDNN analysis. The percentage of structural elements of recovered lysozyme from this system was slightly different in comparison with native lysozyme. Increase in AOT concentration helped improve the secondary structure of recovered lysozyme by bringing the percent of structural elements closer to the native lysozyme sample (Fig. 3.20). Recovered lysozyme from the sample of 100 mM AOT has almost similar proportion of α-helix, antiparallel β-sheet, parallel β-turn and random coil as compared to native

lysozyme. The reason could be that AOT/*p*-xylene RMS makes the best environment (size) for lysozyme transfer from the aqueous phase to RM phase at 100 mM AOT. This explanation can be supported by the highest efficiency of 91.7 % of recovered lysozyme using AOT/*p*-xylene RMS at AOT 100 mM as shown in Fig. 3.14.

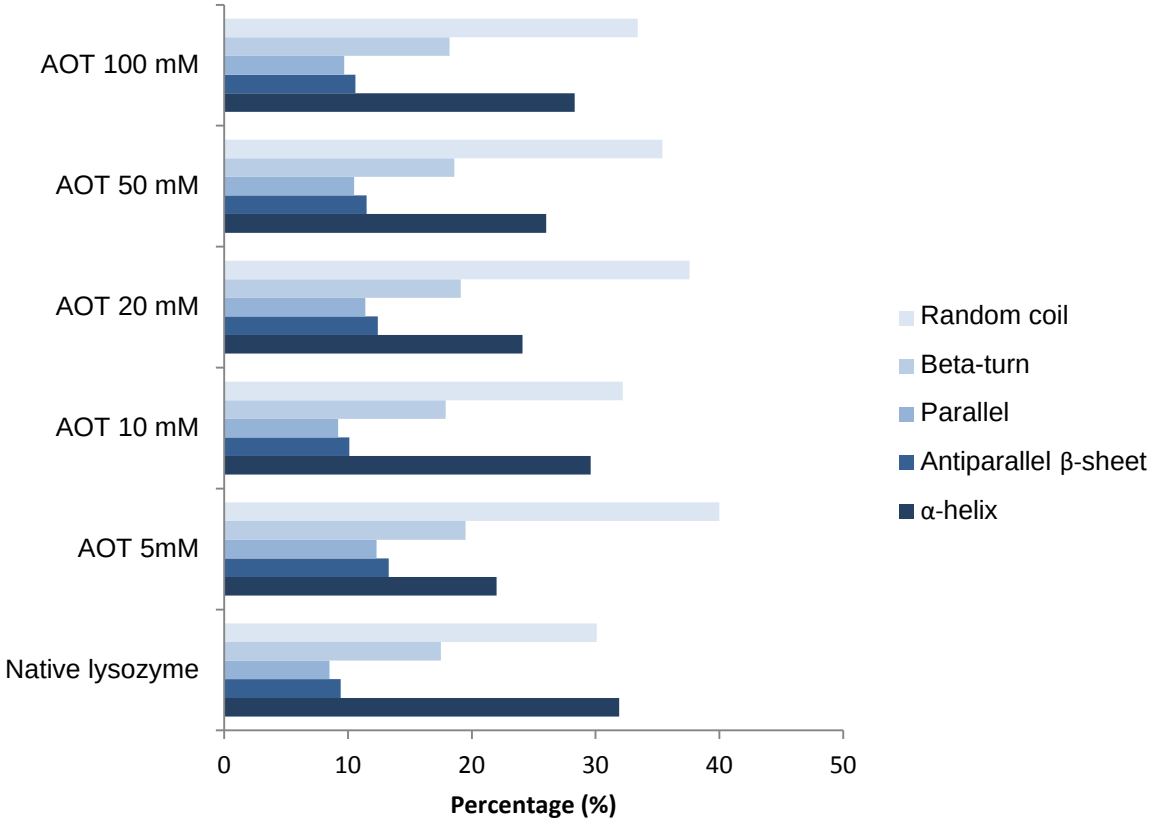

Figure 3.20: CDNN analysis for recovered lysozyme from AOT/*p*-xylene RMS

3.6.2 Recovered lysozyme activity

Lysozyme is a hydrolytic enzyme which has the ability to cleave the cell wall of gram positive and gram negative bacteria. Cleavage of the peptidoglycan wall at the β-1-4 linkage between N-acetyl-glucosamine and N-acetyl galactosamine results in the lysis of the bacterial cell wall (Kato, 1992).

In this study the activity of lysozyme was determined based on its ability to break the bacterial cell wall. If the extracted lysozyme has maintained its biological activity, then the lysozyme will

break the cell walls of *Micrococcus lysodeikticus* bacteria showing a decrease in the absorbance of the solution at 450 nm. If the extracted lysozyme could not maintain its biological activity then there will be no change in the absorbance at 450 nm.

All extracted lysozyme samples were tested with *Micrococcus lysodeikticus* cell assay. The absorbance was read at 450 nm after 60 seconds. The activity of extracted lysozyme using AOT RMS in isooctane and *p*-xylene was calculated using Eq.4.

Table 3.1: Activity of recovered lysozyme from AOT/isooctane and AOT/*p*-xylene RMSs.

Samples	Enzyme activity (units/ml)	Remaining activity based on native lysozyme (%)
Native lysozyme (1mg/ml) in phosphate buffer pH 11.8, KCl 2M	206	100
Recovered lysozyme from AOT isooctane RMS		
5 mM	90	44
10 mM	84	41
20 mM	79	38
50 mM	90	44
100 mM	89	43
Recovered lysozyme from AOT *p*-xylene RMS		
5 mM	100	49
10 mM	110	53
20 mM	117	57
50 mM	159	77
100 mM	150	73

From Tab. 3.1, it can be observed that the activity of recovered lysozyme from AOT/*p*-xylene RMS is slightly higher than AOT/isooctane RMS (49-77 % and 38-44 %, respectively). It was observed that there was a greater change in the secondary structure of recovered lysozyme using AOT/*p*-xylene than the AOT/isooctane RMS based on molar ellipticity spectrum (Fig. 3.15 and Fig. 3.17) and CDNN results (Fig. 3.16 and Fig. 3.18). However, the lysozyme activity assay showed that this change did not make a big effect on the activity of lysozyme. The fact that AOT can form bigger RM in isooctane than *p*-xylene, however, leads to stronger electrostatic interaction between AOT surfactant and lysozyme. This could be one of the reasons for the overall de-

crease in activity of recovered lysozyme as compared to native lysozyme. Similar trend was reported by Hsu and colleagues in their study using AOT RM (Hsu, 2005).

Summary:

It can be concluded that anionic surfactant AOT can form RM in both isooctane and *p*-xylene. Although AOT/*p*-xylene RM forms a smaller size as compared to AOT/isooctane RM causing a comparatively lower lysozyme extraction efficiency at low AOT concentration (<50 mM), the recovered lysozyme from this RMS could maintain its functional integrity much better than the AOT/isooctane RM. This study thus established a new RMS for AOT in aromatic solvent (*p*-xylene) which could maintain the functional integrity of the protein to a large extent as compared to other non-aromatic solvents. This work further leads on to investigate the effect of a cationic RMS and a mixed micelle system (anionic-non-ionic RMS) system on protein extraction.

4.LYSOZYME AND BSA EXTRACTION USING CATIONIC CTAB REVERSE MICELLAR SYS-TEM

This chapter presents the results and discussions related to a cationic (CTAB) RMS in mixture of 1-bromooctane, 1-hexanol and petroleum ether.

- The CMC of this system was determined using the water solubilisation and dye solubilisation method.
- The effect of three salts (KCl, KF, and KBr) on the forward and backward extraction of BSA.
- Lysozyme extraction was investigated by this system.

4.1 Critical micelle concentration of CTAB reverse micellar system

CTAB can only form a stable RM in petroleum ether in the presence of short chain alcohols (Goncalves, *et al*., 2000; Xu, *et al*., 2002), so 20 % 1-hexanol was added to this RMS. However, this system was found to limit the transfer of BSA from the AQP to the ORP resulting in low recovery efficiency (Zhang, 2002). The presence of alkyl halides, 5 % 1-bromooctane, helped to increase the BSA FE efficiency by 12 % and BSA BE by 60 % based on decreasing the electrostatic repulsion between the CTAB molecules and the positive region of proteins as well as the hydrophobic interaction between the hydrophobic regions of protein and surfactant polar heads (Zhang, 2002).

4.1.1 Water solubilisation

In this experiment, CTAB RM were prepared in a solvent mixture consisting of 5% 1-bromooctane, 20% 1-hexanol and petroleum ether. The amount of water in the CTAB RM depends on the concentration of CTAB and follows an increasing trend which was analysed using a Karl Fischer Titrator.

The data in Fig. 4.1 shows the effect of increasing CTAB concentration (0.1-2 mM) on the water content. It was observed that the water solubilisation of the CTAB micelles from 0.1-0.6 mM was in the range of 9257-9533 ppm which was almost similar to the amount of water in blank sample containing the solvent mixture and water only (9331 ppm). The reason could be because in this concentration range, CTAB exists in the ORP as unimers or dimers which limits its water solubilisation capacity due to the absence of RM. In the range of 0.8-1.4 mM of CTAB concentration, the amount of water solubilisation increased but gradually from 10041-10943 ppm. However, a sharp increase in water solubilisation was observed (11529-15173 ppm) beyond 1.4 mM CTAB. This increase indicates the self-association of the surfactant molecules into RM, which provides a hydrophilic microenvironment suitable for water solubilisation. From these data, the CMC of the system was determined to be 1.5 mM which was obtained by the intersection of two linear best fit lines as shown in Fig. 4.1.

From Fig. 4.1, the CMC of CTAB RM in 5% 1-bromooctane, 20% 1-hexanol and petroleum ether was estimated to be around 1.5 mM. Klocova have studied the micellization of CTAB in chloroform in the presence of water using NMR, isothermal titration calorimetry (ITC) and coductometry and they reported the CMC of CTAB in chloroform to be approximately 40 mM (Klicova, 2012). The CMC value of CTAB in chloroform is almost 40 times higher than the CMC of our CTAB RMS. The reason could be the solvent effects on the RMS as was observed in the case of AOT/isooctane and AOT/*p*-xylene RM discussed in section 3.3 (chapter 3). The dielectric constant value of chloroform is 4.8 while that of petroleum ether is 2.0-2.2 (Lide,

2005), CTAB/petroleum ether RM has stronger electrostatic interaction with soluble ions than CTAB/chloroform RM (Hsu, 2005). Therefore CTAB can form RM easily in petroleum ether than in chloroform resulting in a lower CMC value in petroleum ether than in chloroform.

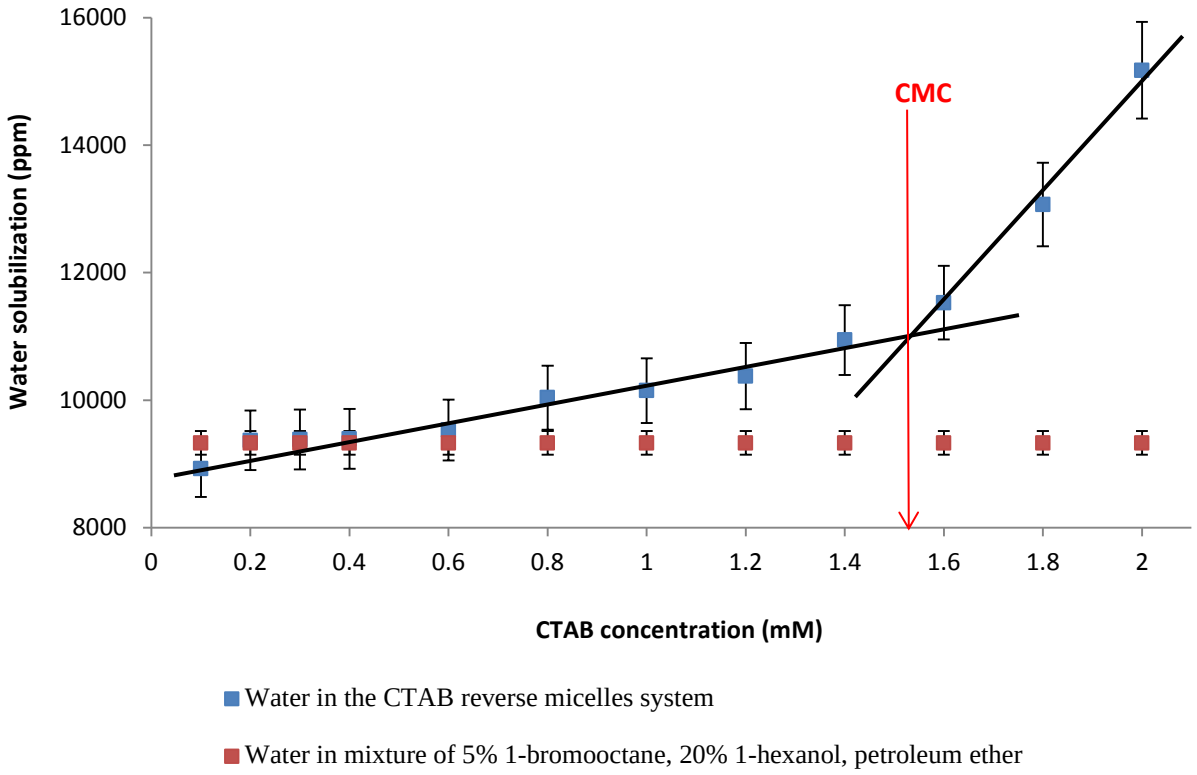

Figure 4.1: CMC of CTAB/5 % 1-bromooctane/20 % 1-hexanol/petroleum ether RMS determined by water solubilisation method.

4.1.2 Rhodamine B solubilisation

Another method to confirm the CMC of RM is the dye solubilisation technique. Hydrophilic dye rhodamine B was used as the model compound to detect the CMC of CTAB in mixture of 1-bromooctane/1-hexanol/petroleum ether reverse micelle system. Rhodamine B was not soluble in the 1-bromooctane, 1-hexanol and petroleum ether mixture. Therefore, the only way rhodamine B could be solubilised was in the water pools of the CTAB reverse micelle system.

From water solubilisation experiment, the mixture of 5% 1-bromooctane, 20% 1-hexanol and petroleum ether can solubilise around 9331 ppm of water. This could be the reason for the initial absorbance (0.0223) of rhodamine B in samples without CTAB (Fig. 4.2).

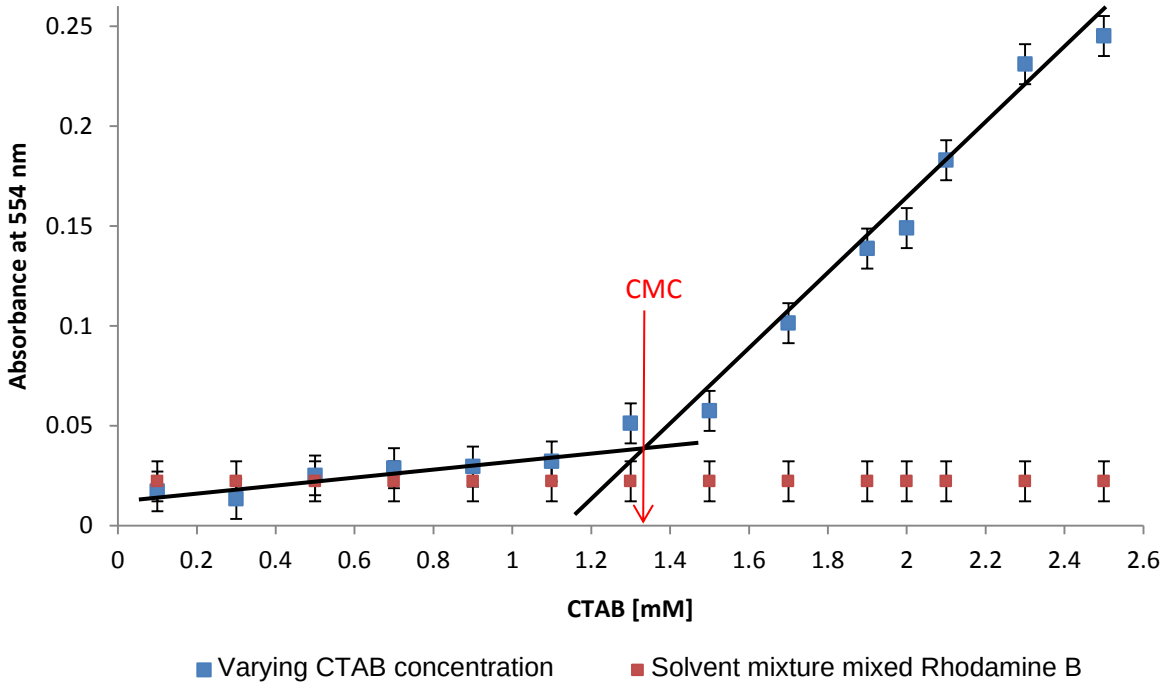

Figure 4.2: CMC of CTAB/5 % 1-bromooctane/20 % 1-hexanol/petroleum ether RMS determined by dye solubilisation (Rhodamine B) method.

From 0.1 to 1.1 mM of CTAB, the absorbance of rhodamine B in CTAB phase was almost constant in the range of 0.017-0.032. However, beyond 1.1 mM, the absorbance of rhodamine B increased sharply from 0.051 to 0.245. The reason could be that below 1.1 mM, CTAB RM were not formed therefore the solubilisation of rhodamine B was limited and just equal to absorbance without CTAB. Above 1.1 mM, CTAB RM was formed resulting in the formation of a hydrophilic area (water-core) inside RM, thus increasing the uptake of rhodamine B into CTAB ORP. From Fig. 4.2, it is clear that the CMC of CTAB RM in 5% 1-bromooctane, 20% 1-hexanol and petroleum ether was 1.3 mM which was close to the CMC determined by water solubilisation experiment in section 4.1.1 as 1.5 mM.

4.2 The size of CTAB reverse micelles

The size of a RM is expressed by the surfactant-to-water ratio W_0 (Hasmann, 2003).

$$W_0 = [H_2O]/[CTAB] \qquad \textit{(Equation 7)}$$

Where, $[H_2O]$ = water concentration

[CTAB] = CTAB concentration

The micellar radius R_m is given by Eq. 8 (Hasmann, 2003)

$$R = (1.64\ W_0)/10\ (nm) \qquad \textit{(Equation 8)}$$

In compare with W_0 value of AOT RMS (31 at 1.9 mM, section 3.3), CTAB RMS had around 5 times higher (162 at 2 mM) (Fig. 4.3). The reported fact that CTAB can held more water in the RM and, also, the size of CTAB RM was bigger than AOT and SDS systems, could be one reason (Ekwal *el al.*, 1971; Fang and Yang, 1999; Li *et al.*, 2006). In addition, incompletely clear ORP even after centrifuging could be another reason caused high amount of water solubilisation analysed by sensitive Karl Fischer Titrator Coulometer.

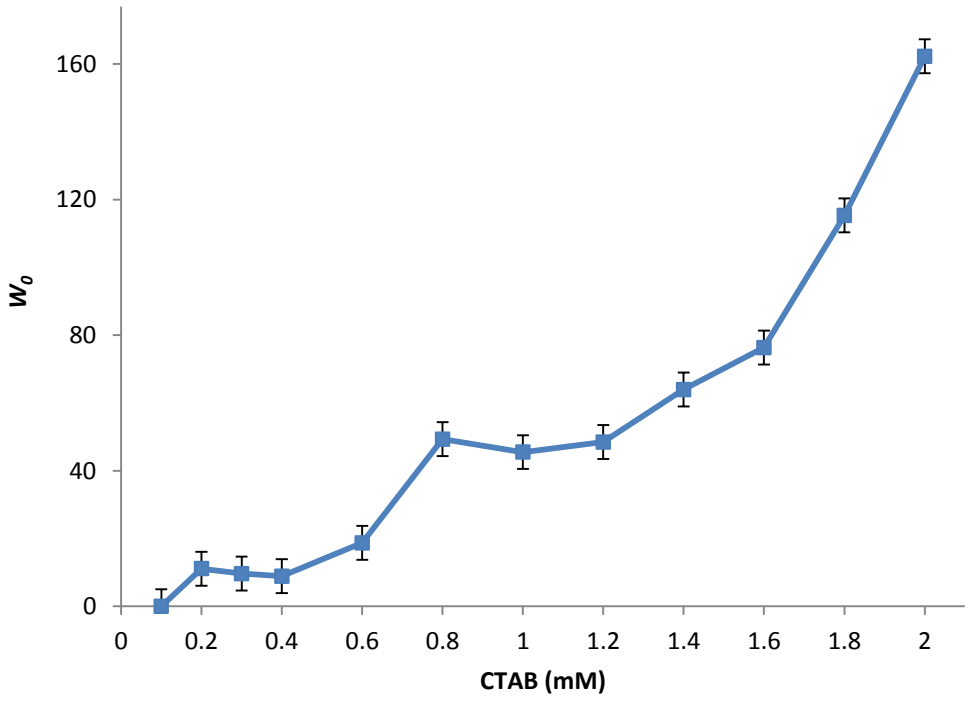

Figure 4.3: Water content W_0 of CTAB/5 % 1-bromooctne/20 % 1-hexanol/petroleum ether RMS

From W_0 value, the reverse micellar radius was calculated following Eq. 8 and presented in Fig. 4.4. The abnormal high value of W_0 have resulted in high this CTAB reverse micellar radius that was 27 nm at 2 mM CTAB. It has been reported that the complete range of RM sizes are 1.6-20 nm in diameter (Moilanne, 2009). In this analyse, the micellar radius of our CTAB RMS was higher than the literature range. However, in the meantime, while CTAB RMS have been widely studied in the literature, droplet sizes of this RMS have been significantly less well characterized than AOT RMS. Especially, no report was recorded for the size of CTAB in mixture of 1-bromooctane, 1-hexanol and petroleum ether RMS. More experiments should be taken using another techniques or methods to explain this result in more detail.

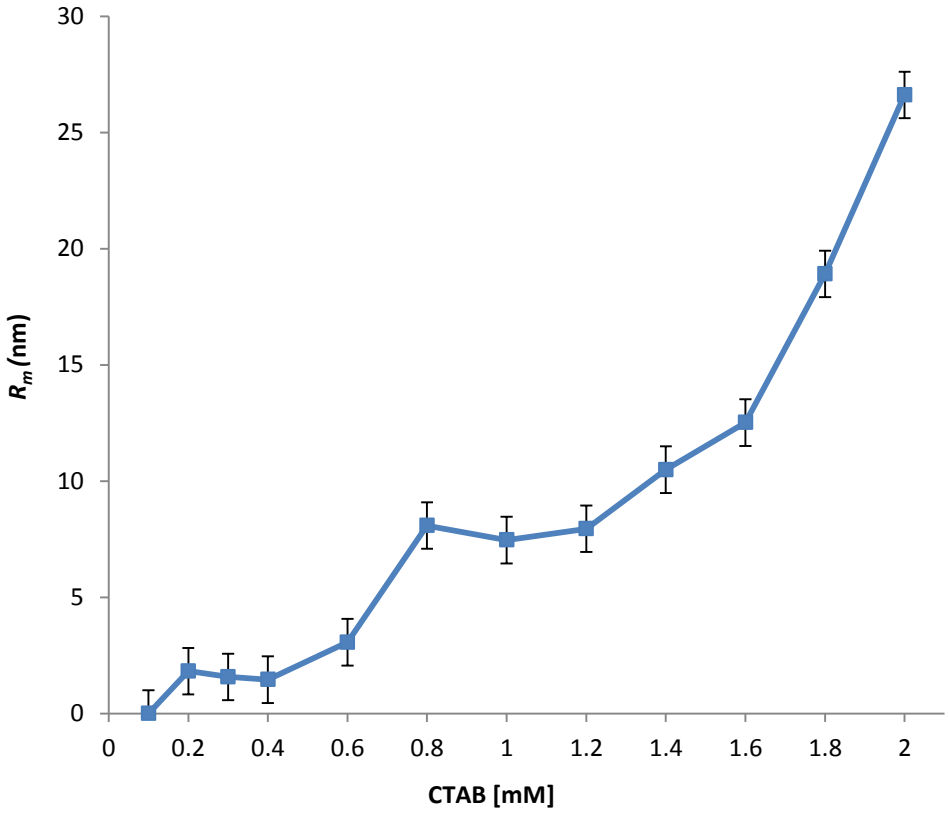

Figure 4.4: The micellar radius of CTAB/1-bromooctane/1-hexanol/petroleum ether RMS

4.3 BSA extraction

BSA extraction using CTAB RMS consisting of 5 % 1-bromooctane, 20 % 1-hexanol and petroleum ether was investigated. 1-bromooctane was used in order to improve the BSA transfer from the AQP to reverse micellar phase, and also improve the BE efficiency of BSA based on decreasing the hydrophobic interaction between RM (Zhang, 2002). The optimal conditions were pH 10 and 10 mM CTAB concentration for FE, and pH 10 for BE based on literature (Zhang, 2002)

The effect of different types of salt (KCl, KF, KBr) with varying concentration on the forward and BE of BSA was investigated. The concentration of BSA during the extraction process was calculated based on the BSA calibration curve (Fig. 4.5).

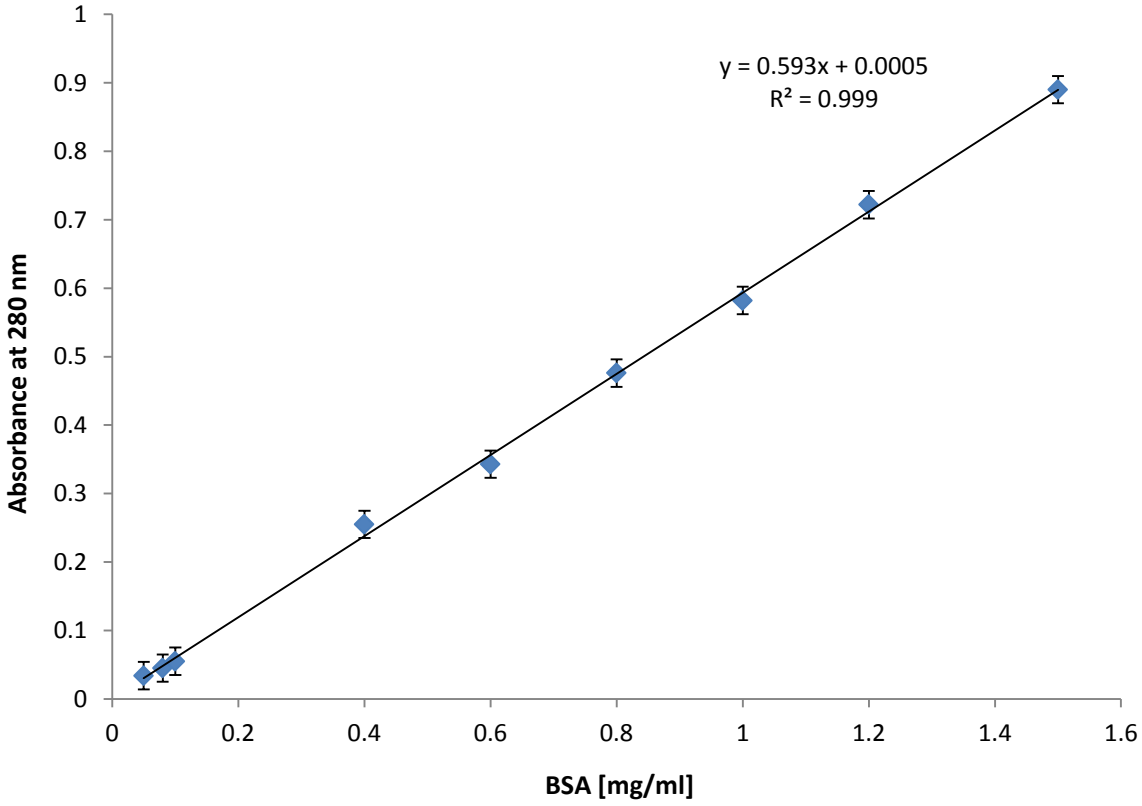

Figure 4.5: Calibration curve of. BSA (0.05-1.5 mg/ml) by UV-Vis spectroscopy.

4.3.1 Extraction efficiency

FE of BSA was conducted at various salt concentrations (0.05, 0.08, 0.1, 0.15, and 0.2M). The data in Fig. 4.6 shows that the maximum extraction of BSA was achieved at a salt concentration of 0.05M, which was 83 %, 33 % and 61 % in case of KCl, KBr and KF, respectively. However, a decreasing trend of extraction yield in all three salt species was observed when the salt concentration was increased (high ionic strength) beyond 0.05 M.

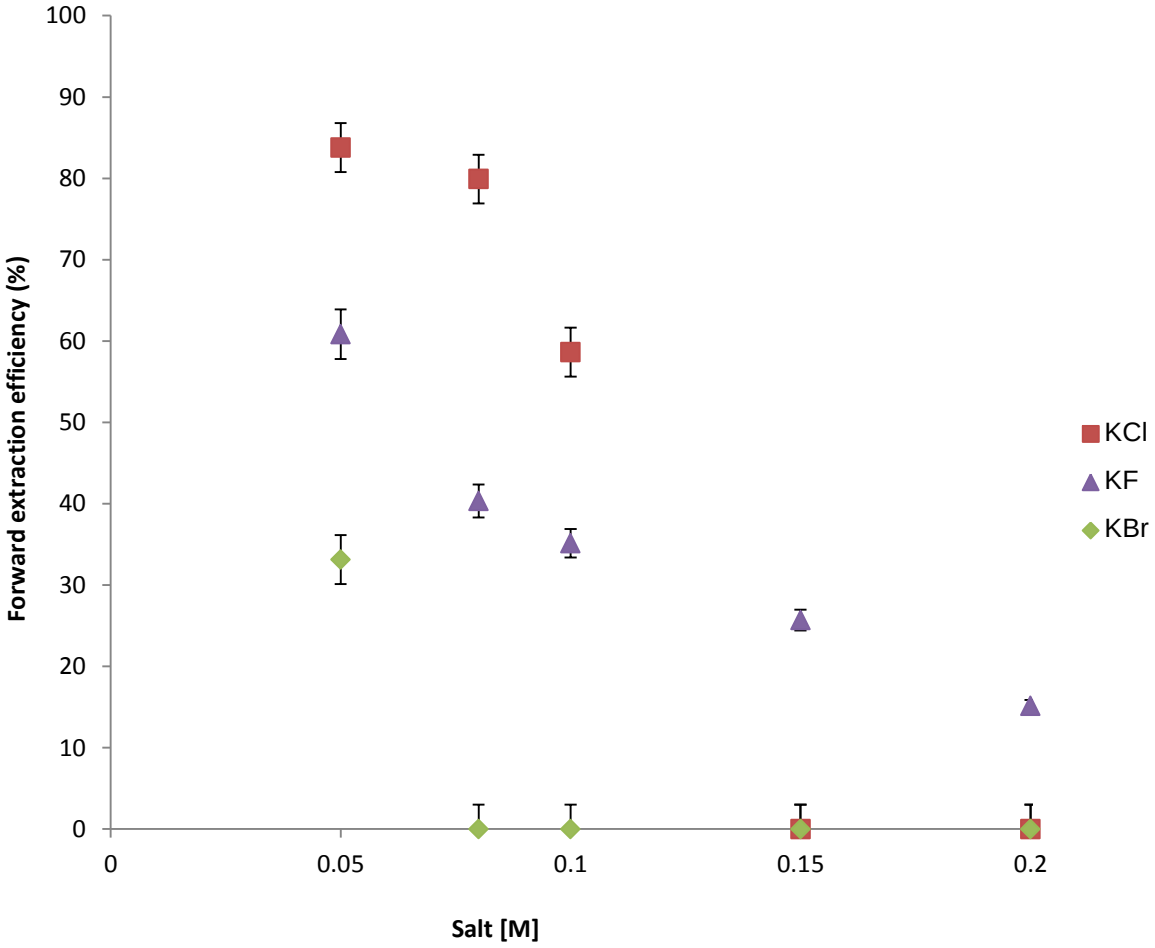

Figure 4.6: Effect of salt concentration on the FE efficiency of BSA using CTAB/5% 1-bromooctane/20% 1-hexanol/petroleum ether.

The effect of ionic strength on the solubilisation of proteins into the reverse micellar phase can be interpreted in two ways. Firstly, the electrostatic attraction between protein and RM polar head groups are weakened due to electrostatic screening effect when ionic strength increases,

which hinders the diffusion of BSA into the reverse micelle aggregates. Another explanation was based on size exclusive effect, which suggests that the increase in ionic strength causes the compression of the electrical double layers of the polar groups on RM, which allows surfactant molecules to move closer to each other leading to reduction in reverse micellar size (Belter, 1988). This phenomenon prohibited incorporation of biomolecules into the water core of the RM droplets, especially in case of BSA which is considered to be a large protein (3.48 nm) (Axelsson, 1978), leading to a decrease in extraction efficiency. However, between the two effects, the electrostatic effect was demonstrated to be more significant due to the small variation of water content over different sizes of RM (Langer, 2006). The reduction of extraction efficiency caused by increasing ionic strength has been reported in several studies using CTAB RMS and AOT RMS (Sun, 2011; Mohd-Setapr, 2009; Noh, 2005). The effect of different types of salts were also investigated by altering anion species, Cl^-, F^- and Br^-. It was shown that the extraction efficiency was greatly influenced by the anionic species. A wider range of KF concentration for FE was investigated. The extraction occurred with KF concentration from 0.05M to 0.2M, while no extraction was observed from 0.15 M KCl and 0.08M KBr (Fig. 4.4). The easiness of extraction for anions, therefore, could be in the order KBr < KCl < KF, which was consistent with the Hofmeister anion series $Br^- < Cl^- < F^-$ which describes the interactions between macromolecules and ions (Zhang, 2002). According to Hofmeister, early members of the series (F^-) increase solvent surface tension and decrease the solubility of nonpolar molecules (salting out), and in effect, they strengthen the hydrophobic interaction; while later salts in the series (Br^-) increase the solubility of nonpolar molecules (salting in) and then they weaken the hydrophobic effect (Hofmeister, 1988). However, in this study, the order of effect of anion species on the extraction yield was $Br^- < F^- < Cl^-$. Therefore, although KCl was not the strongest salt in comparison with KF following Hofmeister series, KCl helped the CTAB RMS to uptake the highest amount of BSA (83 %) while KF extracted 60 % and KBr extracted only 33 % of BSA at 0.05 M con-

centration of CTAB. The reason for this behaviour is not yet clear and needs further investigation.

In BE, same type of salts were studied but with higher concentration (1 M), in order to decrease the electrostatic interaction between CTAB and BSA to release BSA out of CTAB RM. The data in Fig. 4.7 shows the backward extraction yield of BSA using different salt at different concentration. The highest yield of recovered BSA was obtained using the KCl-KCl combination at 64 % followed by KBr-KCl at 47 % and KF-KCl at 30 %. KCl gave the best BE using CTAB RM. Keeping KCl constant as the BE salt it was observed that KBr gave higher recovery yield of BSA in comparison with KF because of the hydrophobic interaction following the Hofmeister series. However, KBr-KBr group gave the lowest BE (<10 %) which could have been the result of very low FE yield (33 %). For KF-KF group, the BE yield was quite low at around 40 % which could also be envisaged as a result of strong hydrophobic interactions.

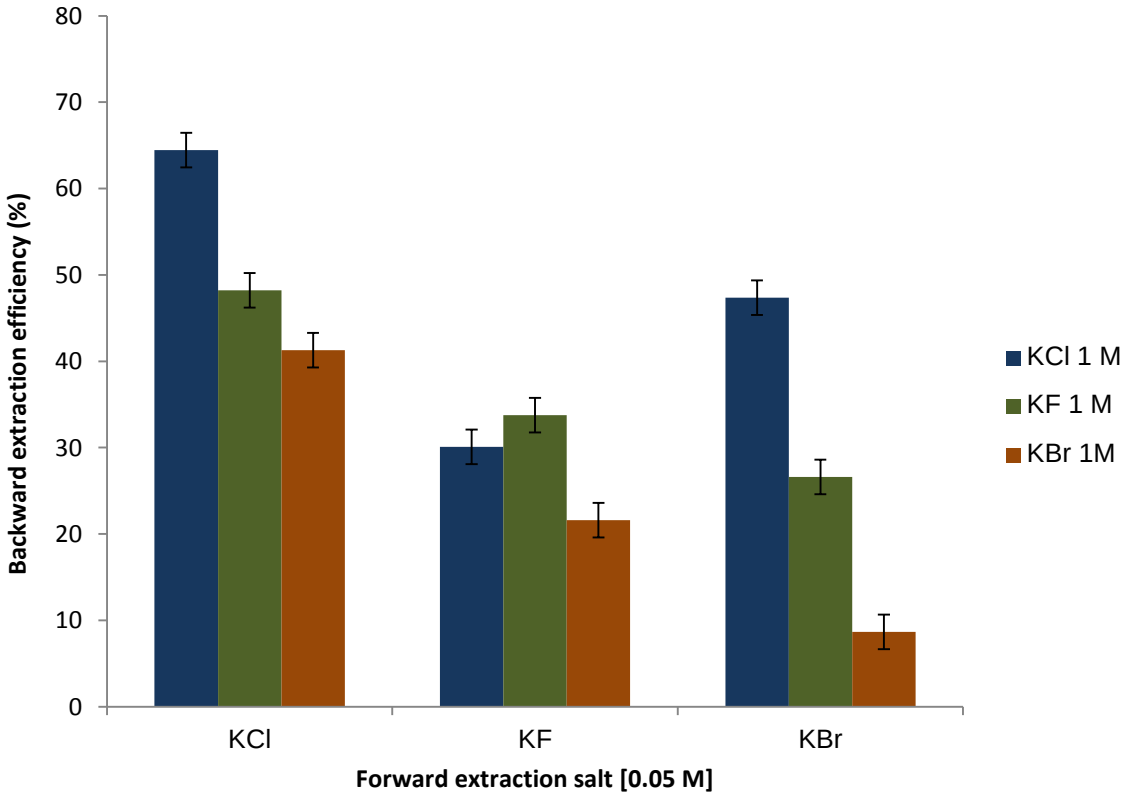

Figure 4.7: BE efficiency of BSA using CTAB/5% 1-bromooctane/20% 1-hexanol/petroleum ether with different salt type.

4.3.2 Extracted BSA structure

All recovered BSA samples were analysed by CD spectroscopy to examine the effect of RM on the secondary structure of BSA. The data in Fig. 4.8, Fig. 4.9 and Fig. 4.10 shows the effect of using different types of salt on the FE and BE of the recovered BSA from the CTAB/1-bromooctane/1-hexanol/petroleum ether reverse micelle system.

If KCl was used for FE, the effect of salt on the secondary structure of recovered BSA was in the order KBr < KCl < KF. This result is in agreement with the literature where Constantinescu and colleagues reported the effectiveness of ionic liquids and Hofmeister series to RNase denaturation (Constantinescu, 2010). The concept of kosmotropic (structure-making) and chaotropic (structure-breaking) ions are traditional explanations for Hofmeister affecting protein hydration (Broering, 2005), enzyme stability (Collins, 1985) and enzymatic functions (Zhao, 2006). However, the interpretation of Hofmeister effects at the molecular level is still complex and unclear.

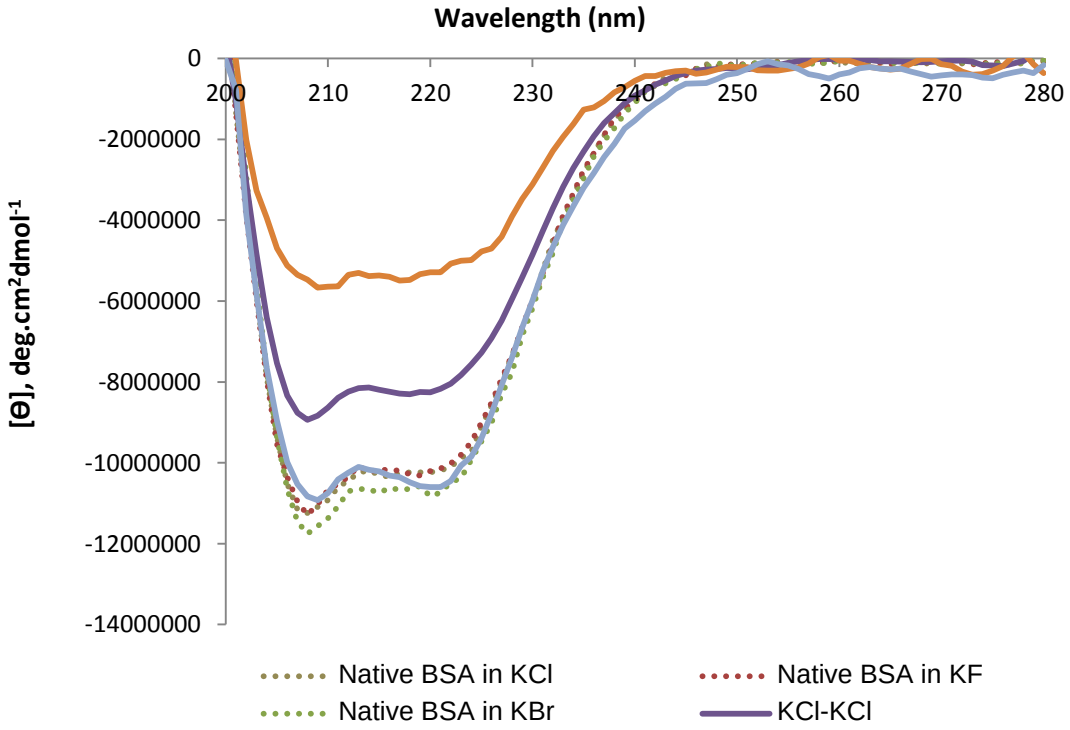

Figure 4.8: CD spectrum of extracted BSA with different salt species.
The FE conditions for all samples were 0.05M KCl. The BE conditions were 1M KCl/KBr/KF

When KF was used as the salt for FE, similar results as KCl were observed. For KF-KF sample, the secondary structure of BSA was almost completely lost (Fig. 4.9) On the contrary, extracted BSA using KF-KCl and KF-KBr showed a CD spectrum similar to the spectrum of native BSA which indicates that the secondary structure of BSA was maintained.

However, when KBr was used in the FE, none of the recovered BSA samples kept their secondary structure according to CD spectrum (Fig. 4.10). This is contrary to the fact that according to Hofmeister series, KBr has the weakest hydrophobic interaction between RM which should be able to expel the protein (BSA) easily out of the CTAB RM. KBr has been reported to be a stronger denaturant of NADPH-cyto-chrome *c*, collagen and ribonuclease in series of $SCN^- = I^- > ClO_4^- > Br^- > NO_3^- > Cl^- > SO_4^{2-} = F^- = CH_3COO^-$ (Nishimura, 1999; Vo, 1964). The secondary structure of BSA may be affected from FE conditions where KBr was used resulting in the change in secondary structure, irrespective of the kind of salt used in BE.

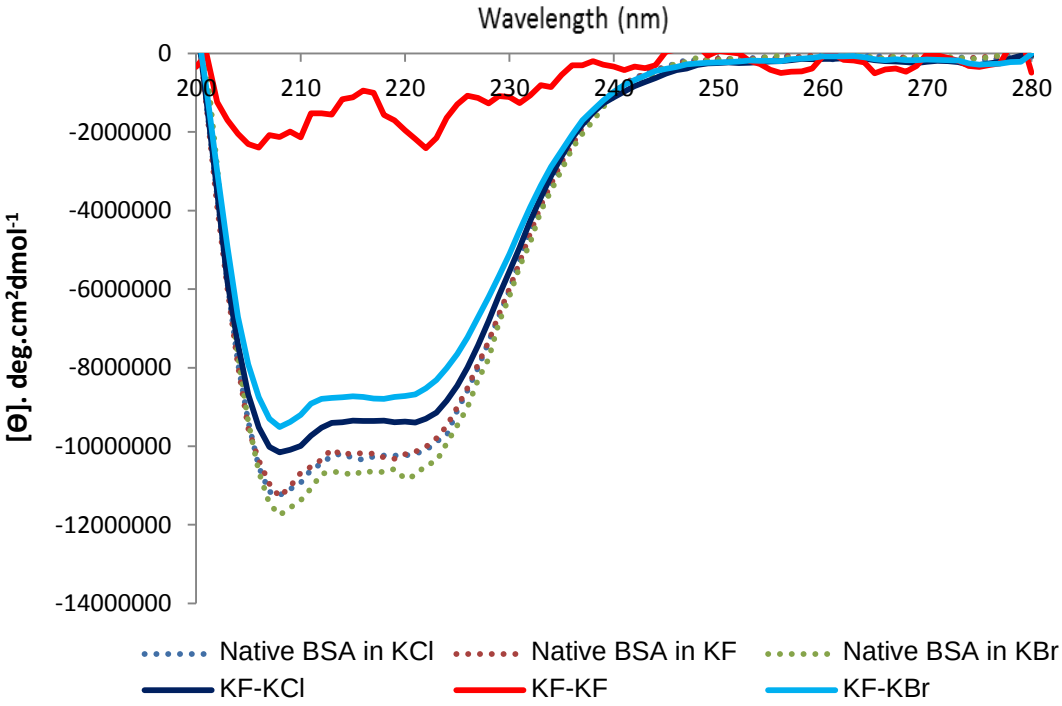

Figure 4.9: Effect of salt concentration on the CD spectrum of extracted BSA. The FE of all sample used 0.05 M KF The BE used 1 M KCl/KBr/KF.

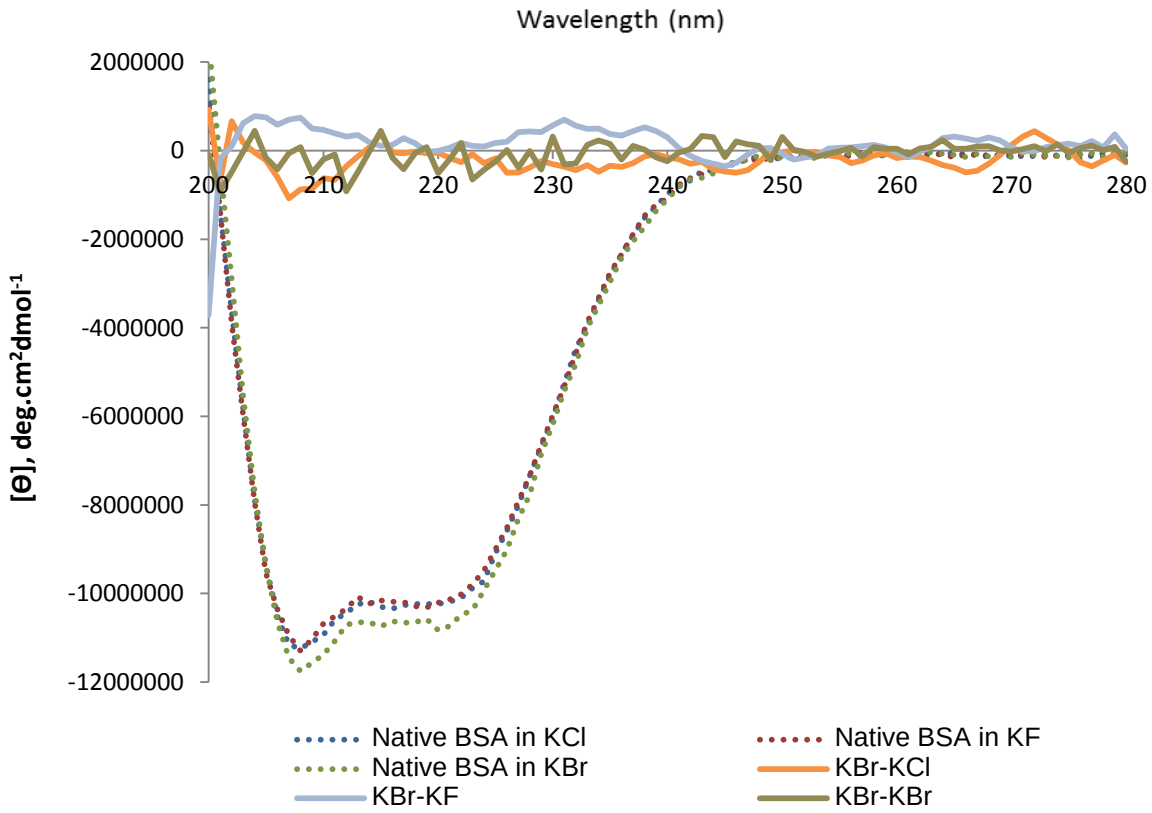

Figure 4.10: Effect of salt concentration on the CD spectrum of extracted BSA. The FE of all sample used 0.05M KBr. The BE used 1M KCl/KF/KBr.

CDNN software was used to determine the percentage of α-helix, β-sheet, parallel, beta-turn and random coil of extracted BSA in comparison with native BSA. The percentage of α-helix, β-sheet, parallel, beta-turn and random coil of extracted BSA using KCl-KCl, KCl-KBr, KF-KCl, KF-KBr are similar with each other and with native BSA in different salt solutions (Fig. 5.11). On the contrary, extracted BSA using KCl-KF, KF-KF, KBr-KCl, KBr-KF and KBr-KBr, showed that the percentage of α-helix reduced while the percentage of random coil increased sharply (Fig. 4.11). This result can be explained based on the effect of surfactant concentration on extraction of proteins. The increase in surfactant concentration can lead to an increase in the number of RM as well as the size of RM. This change can help transfer as well as recover protein easily and also help limit its effect on the protein structure. At very high surfactant concentration, the micellar clustering can take place which decreases the interfacial area available for protein resulting in destroying the protein structure (Nandini, 2009).

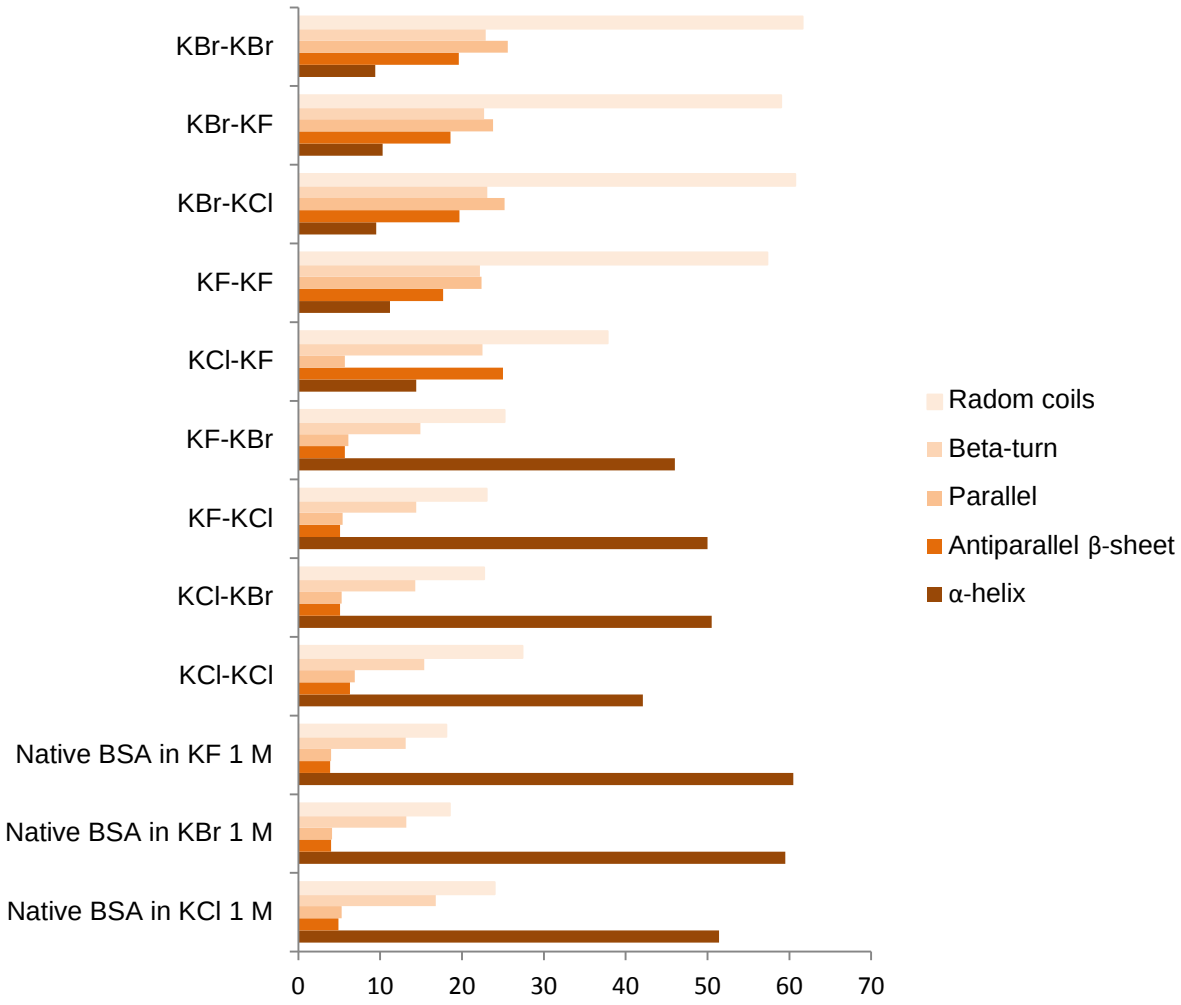

Figure 4.11: CDNN analyse for recovered BSA from CTAB/1-bromooctane/1-hexanol/petroleum ether RMS.

4.4 Lysozyme extraction

The CTAB/1-bromooctane/1-hexanol/petroleum ether RMS was successfully able to back extract BSA and also maintain its biological activity under optimised conditions. Though BSA being a medium sized protein (66 kDa) was successfully extracted it was necessary to investigate if a small sized protein like lysozyme (14 kDa) could be extracted with the same RMS. Therefore, the extraction of lysozyme in CTAB/1-bromooctane/1-hexanol/petroleum ether was investigated. The concentration of lysozyme during extraction process was calculated based on the lysozyme calibration curve (Fig. 3.9) in section 3.4.

4.4.1 Forward extraction

Since electrostatic interaction plays a major role in reverse micellar extraction, changing the solution pH will directly affect the extraction efficiency as pH influences the surface change of molecules in solution. The pI of lysozyme is 11.3 and CTAB is a cationic surfactant, therefore the pH for FE should be higher than 11.3 where lysozyme would be negatively charged as compared to the positively charged CTAB. The data in Fig. 4.12 shows the effect of pH on lysozyme FE.

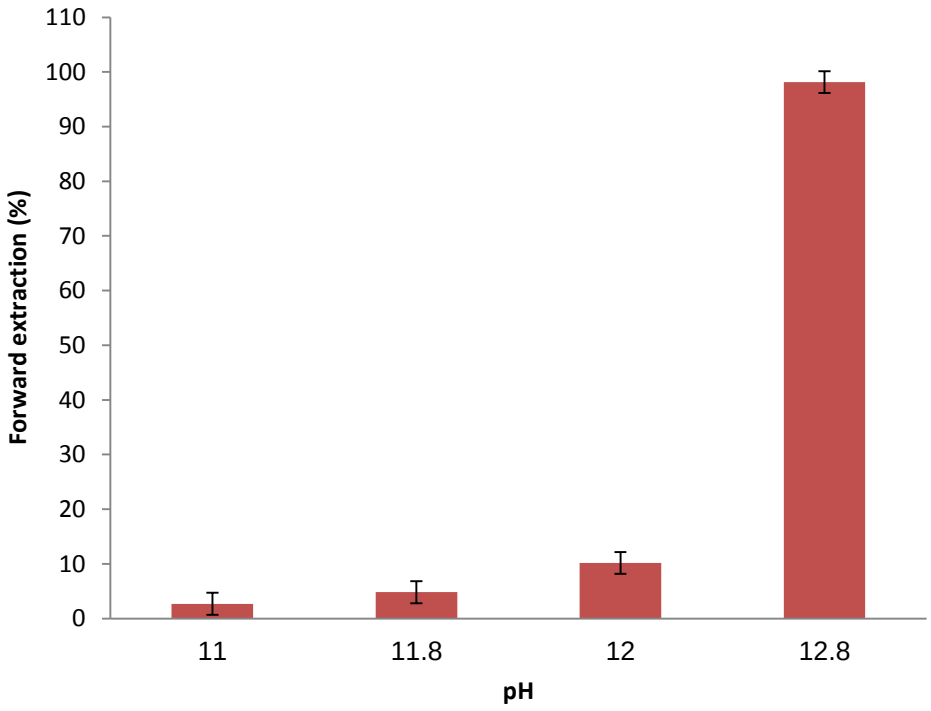

Figure 4.12: Effect of pH on lysozyme FE using CTAB/5 % 1-bromooctane/20 % 1-hexanol/petroleum ether RMS.

At pH 11, which is lower than the pI of lysozyme, only 2 % extraction was obtained. The reason would be similar charges (positive) on both lysozyme and CTAB or lysozyme neutral charge (near pI value) resulting in a very low extraction. From pH 11.8 to 12, the percentage of forward extracted lysozyme increases slowly, 4 - 98 % at pH 12.8 (Fig. 5.12). At pH 11.8, 12 and 12.8, the surface of lysozyme has a positive charge which promotes the uptake of lysozyme into the CTAB RMS. However, pH 11.8 and pH 12 are very close to the pI value of lysozyme (11.3)

which leads to weaker electrostatic attraction between lysozyme and CTAB molecules, thus reducing the FE percentage. It has been previously reported that the difference between the pH and the pI of the protein must oscillate between 1 and 2 points for a higher extraction efficiency and lower loss by denaturation (Pessoa, 1998; Andrews, 1993; Keri, 1995). From experimental results, pH 12.8 was found to be the optimal pH for lysozyme FE using CTAB/5 % 1-bromooctane/20 % 1-hexanol/petroleum ether RMS.

The effect of CTAB concentration on lysozyme FE was also studied. From section 4.1, the CMC of CTAB RM in solvent mixtures of 5 % 1-bromooctane/20 % 1-hexanol/petroleum ether was estimated to be 1.2 mM. Therefore, the range of CTAB concentration for this study was chosen from 5 to 50 mM which was above the CMC. Above 50 mM, CTAB was not completely soluble in the solvent system.

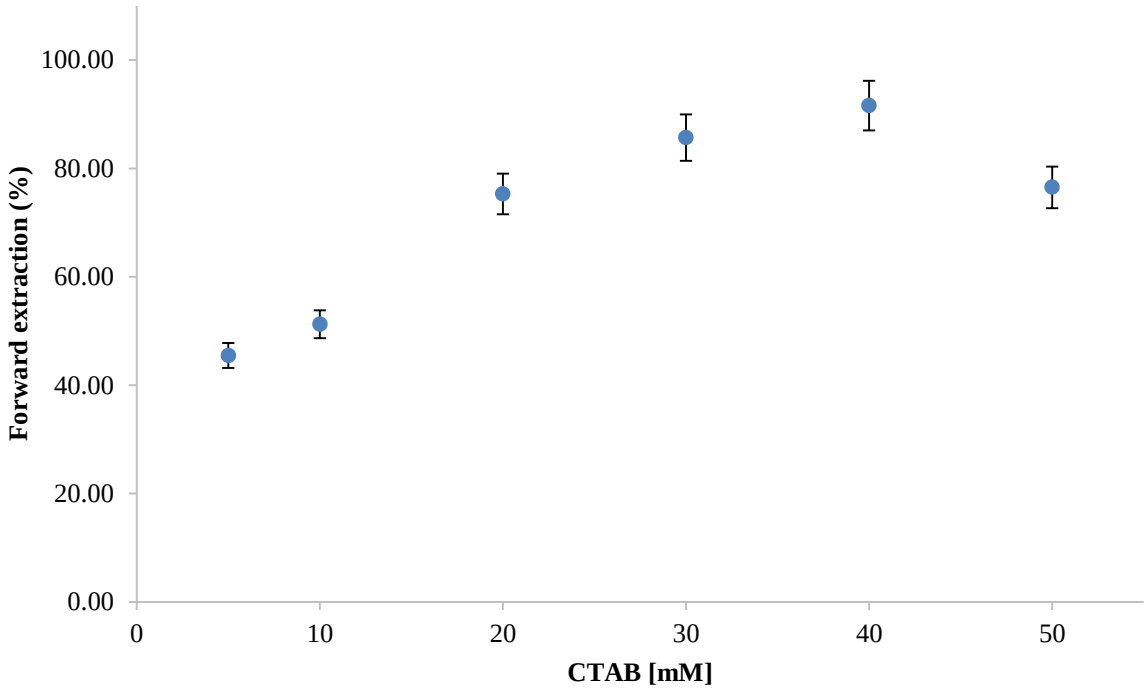

Figure 4.13: Effect of CTAB concentration on lysozyme FE using CTAB/5 % 1-bromooctane/20 % 1-hexanol/petroleum ether RMS.

The data in Fig. 4.13 shows that the FE of lysozyme increased from 45 to 91 % when CTAB concentration increased from 5 to 40 mM. This phenomenon can be explained based on the

hypothesis that increasing surfactant concentration leads to an increase in the number of RM and aggregation number resulting in increased solubilisation of lysozyme in the reverse micellar phase. However, beyond 40 mM, lysozyme FE yield decreased to 76 %. This could be because of the micellar clustering observed at higher concentration, which decreases the interfacial area available for lysozyme resulting in decreased extraction efficiency (Nandini, 2009). In addition, the inter-micellar collisions occur more frequently because of their large population at a higher surfactant concentration (Tonova, 2008), which results in de-assembling/deformation of RM leading to decreased extraction (Hebbar, 2007). Similar results of decrease in extraction efficiency of target biomolecules with increase in the surfactant concentration has been reported for α-amylase (Lazarova, 1999), nattokinase (Liu, 2004) and lysozyme (Noh, 2005). From the experiment results, 30 mM of CTAB was selected as the optimum concentration for further studies using the CTAB/ 5 % 1-bromooctane/ 20 % 1-hexanol/petroleum ether RMS.

The effect of KCl concentration was also investigated. Salt can help improve the electrostatic interaction between RM and proteins in order to transfer proteins from an AQP to the RM phase. In this study, KCl concentration was selected from 0.05 M to 0.2 M. The data in Fig. 4.14 shows the effect of varying KCl concentration on the FE efficiency of lysozyme. At 0.05 M and 0.1 M KCl, the FE of lysozyme was 99 % and 95 % respectively. However, at 0.2 M KCl, the FE decreased sharply to 45 %. Goklen and Hatton reported that protein solubilisation could be controlled by varying the ionic strength of AQP equilibrated with the micellar phase in order to alter the electrostatic interaction between the surface charge of the protein and the charged interior of the RM by Debye screening (Goklen *et al.*, 1985). In this case, higher salt concentration could reduce the electrostatic interaction within CTAB RM, causing weaker lysozyme-CTAB charge interaction and reduction in the size of CTAB RM leading to the decrease in FE efficiency. Therefore 0.05M was chosen as the optimal concentration for lysozyme FE using CTAB/ 5 % 1-bromooctane/ 20 % 1-hexanol/petroleum ether reverse micelle system.

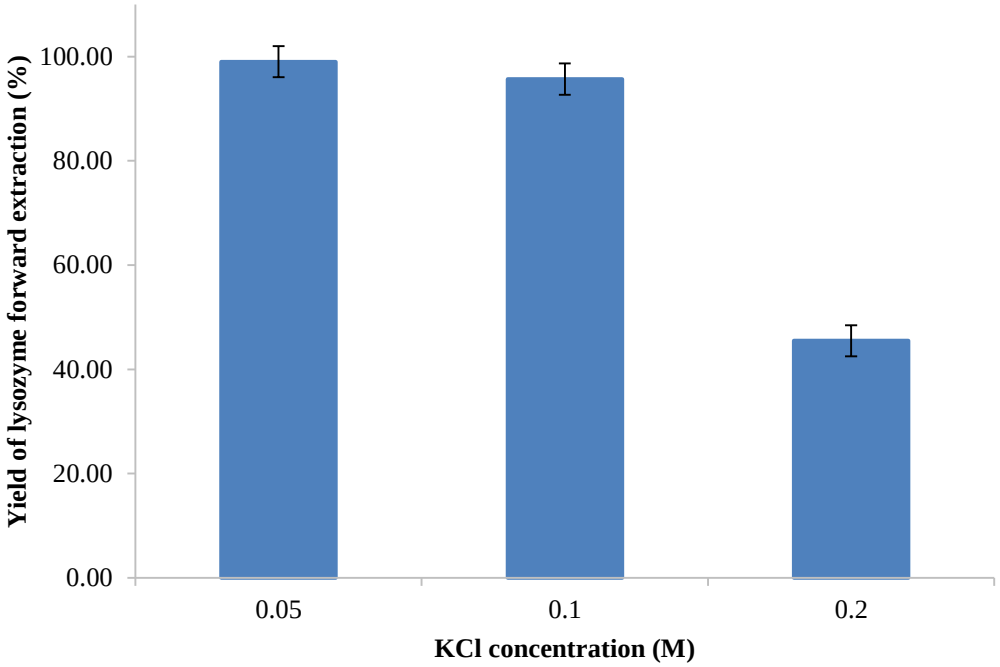

Figure 4.14: Effect of KCl concentration on lysozyme FE using CTAB 30 mM in 5 % 1-bromooctane/20 % 1-hexanol/petroleum ether RMS.

4.4.2 Backward extraction

After the successful transfer of lysozyme from AQP into CTAB RMS, more experiments were studied to recover the lysozyme from CTAB RM.

It was reported that the protein transferred into the reverse micellar phase rapidly undergoes conditions of low ionic strength, and are transferred out of the micelles relatively slowly under conditions of high ionic strength (Goklen, 1985). Therefore, for BE, high salt concentration (1 M and 2 M KCl), was used to maintain high ionic strength environment in order to apply the size exclusion effect to CTAB RM. Also, the pH of the back extraction phase was varied from 4 to 10 so that lysozyme could maintain a positive charge similar to CTAB resulting in electrostatic repulsion leading to the release of lysozyme out of the RMS. However, very low back extraction of lysozyme was obtained under these conditions. The yield of recovered lysozyme was lower than 5 % as compared to a lysozyme forward extracted efficiency of around 98-99 %.

Different kind of salts, including KCl, KBr, KF, NaCl, $CaCl_2$, $MgSO_4$, were also studied at high concentration (2 M) with the aim of optimising a favourable ionic strength which would allow the transfer of lysozyme out of CTAB RM. Different kind of alcohols, including ethanol, isopropanol, 1-butanol, were also used in order to disrupt the interaction between CTAB RM and lysozyme to release the lysozyme. Another technique of adding a counter ionic surfactant was also investigated by adding oppositely charged surfactants such as AOT and SDS to weaken the electrostatic interaction between CTAB RM and lysozyme. However, the recovery yield of lysozyme was still lower than 10 %.

Some precipitation was observed during this extraction, and therefore the precipitate was further analysed to investigate the presence of any lysozyme in it. However, no lysozyme could be recovered from the precipitate. It was therefore concluded that lysozyme was trapped inside the CTAB RMS and could not be recovered as BSA using the same RMS. The reason may be the difference in the size of lysozyme (14.3 kDa) and BSA (65 kDa). It was reported that both protein and polymer solubilisation within RMS depends on the size of the protein relative to that of the RM (Kelly, 1994; Belloc, 1998). Wolbert and co-workers indicated that proteins with MWs larger than approximately 100 kDa should be unable to transfer significantly into RM solutions (Wolbert, 1989). This concept was confirmed by the failure in extracting hexokinase (100 kDa) and lipase A (120 kDa) from an aqueous solution into AOT RMS (Wolbert, 1989; Catarina-Vicente, 1990; Aires-Barros, 1991). On the other hand, Kamihira, Shiomori and Bohidar groups reported that RM is capable of hosting particles with diameter larger than theirs (Shiomori, 1998; Bohidar, 2000; Kamihira, 1994). Gerhardt and Dungan studied the solubilisation of very large molecule IgG (155 kDa) inside AOT-brine-isooctane RM and they envisioned one possible mechanisms for hosting IgG in the ORP is at least two droplet or RM take part in the solubilisation of IgG (Gerhardt, 2002). Their explanation was that the hydrophobic domains in the protein can serve as a “bridge” between two RM, with hydrophilic protein domains residing in the water core. Similar explanation could also be given in the BSA

RMS as being a large sized protein, BSA could be hosted in RM phase by more than one CTAB reverse micelle, so it is easier to recover it by breaking the hydrophobic interaction during BE. On the other hand, lysozyme, being a small protein, could be trapped deeply inside the water core, and the presences of 1-bromooctane, 1-hexanol, in addition, make this CTAB RMS more stable. Therefore, it was difficult to break CTAB RM and get lysozyme out. However, this phenomenon needs to be better understood by further investigation of the interaction between protein and RM at varying protein size which is beyond the scope of this work.

Summary:

It can be concluded that CTAB can form RM in 5 % 1-bromooctane, 20 % 1-hexanol and petroleum ether system. BSA can be extracted using this RMS by maintaining its secondary structure. This system was successful in transferring lysozyme from the AQP to the RMS, but recovery of lysozyme from the reverse micelle core was unsuccessful. Investigation of mixed RMS of cationic CTAB and non-ionic surfactant such as pluronics could be one of the solutions to be considered in the future to reduce the electrostatic effects and expel the proteins out of the RMS.

5.LYSOZYME EXTRACTION USING NON-IONIC TRIBLOCK COPOLYMER PLURONICS RE-VERSE MICELLAR SYSTEM

This chapter aims to investigate the reverse micelle forming ability of triblock copolymers and then, the effectiveness of the reverse micelle will then be tested for the extraction of a small sized protein, lysozyme.

Similar to the AOT and CTAB RMS in chapter 3 and 4, water solubilisation and dye solubilisation methods were used to determine the CMC of these non-ionic pluronics RM. There were several kind of pluronics were investigated in CMC experiment, includes L61, L64, L81, P84, P85, L121 and P123. These pluronics were chosen because the effect of molecular weight (L61, L81 and L121) and effect of PEG percentage (group of L61 and L64; group of L81, P84 and P85; group of L121 and P123) to reverse micelles formation were targeted as the goal of experiment. However, the cloudy organic phase and gel phase formation made it difficult to detect CMC point for L64, P84, P85, L121 and P123. The reason could be very high molecular weight which needs to study further. In the scope of this project, no more experiments were taken for these above pluronics. The target was set up for L61 and L81 those CMC was detected easily using water solubilisation and dye solubilisation methods which are presented in this chapter. L61 and L81 with similar PEG percentage (10 %) but varying surfactant molecule weight (2000 and 2800, respectively) was investigated in order to study the effect of molecular weight of pluronics on their RM forming ability (Alexandridis, 1995). The chosen non-polar solvent for pluronics RMS was *p*-xylene. The reason for choosing this solvent was some previous studies in the literature by Alexandridis and colleagues where they reported that pluronics L64 was completely miscible with *p*-xylene and formed an isotropic solution which solubilized approximately two water molecules per EG segment (Alexandridis, 1997). Also the solubility of these pluronics was

investigated in various solvents such as isooctane, cyclohexane, decane, octane, and alcohols such as decanol, butanol, ethanol, heptanol and isopropanol but no solubilisation was observed. Lysozyme extraction was also studied using L61/*p*-xylene reverse micelles system at the end of this chapter.

5.1 The critical micelle concentration (CMC)

5.1.1 PEG-PPG-PEG L61

5.1.1.1 Water solubilisation

The concentration range of L61 chosen was 0.0001-0.2 mM. The lower limit was chosen based on the CMC of L61 micelles from literature (Kobano *et al.*, 2002). From L61 concentration 0.0001-0.03 mM, there was only a slight change in water content in the range of 688-813 ppm. The reason could be because L61 exists in the ORP as monomers or dimers at this concentration range and therefore limiting the water solubilisation due to the absence of RM. In addition, the water content in this concentration range was nearly similar to the water content of the sample containing *p*-xylene and water only (736 ppm) which supports the argument of the absence of RM.

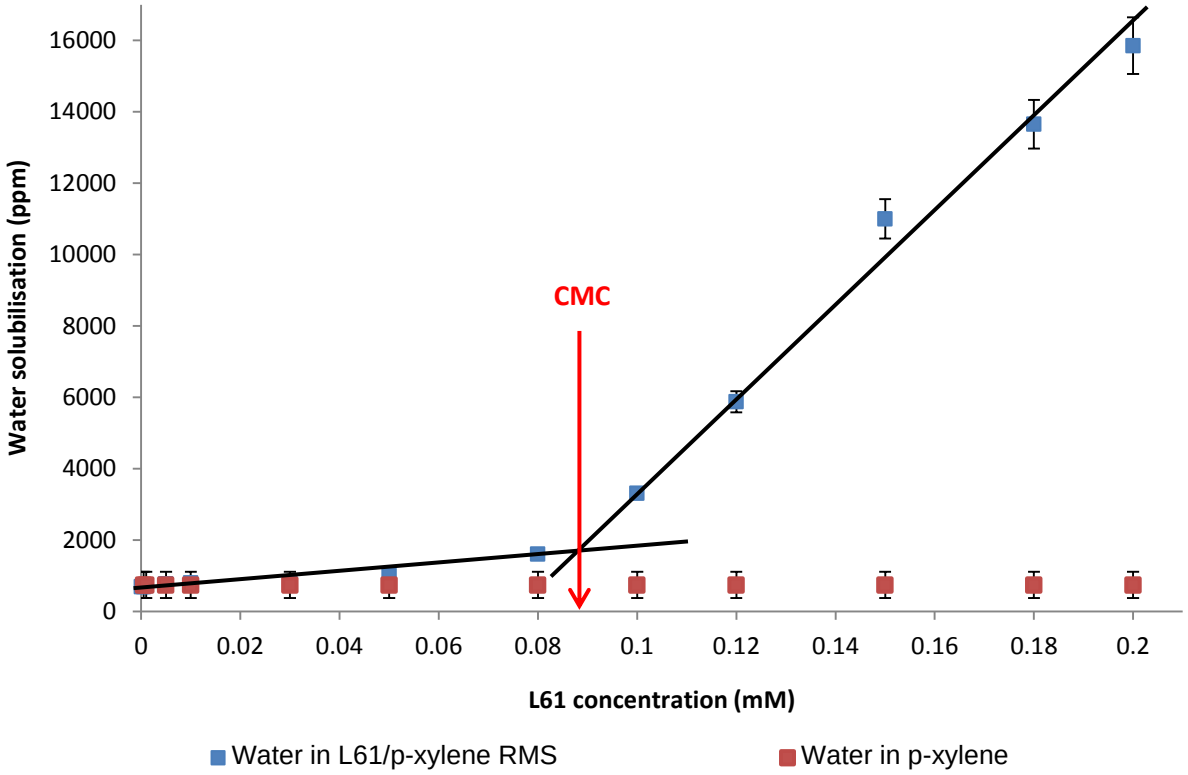

Figure 5.1: CMC of L61/*p*-xylene RMS determined by water solubilisation method.

From 0.05 mM to 0.08 mM L61 concentration, there was a slight increase in water solubilisation from around 1017 to 1608 ppm. This increase indicates the self-association of the copolymer molecules into RM, which provides a hydrophilic microenvironment suitable for water solubilisation. Beyond 0.08 mM L61 concentration, there was an abrupt increase (3311-15852 ppm) in the water solubilisation with the increase in L61 concentration (Fig. 5.1).

The "reverse" CMC (CMC) can be determined from the copolymer concentration at which the water solubilisation capacity starts increasing (Alexandridis, 1997). In this case, the CMC of L61 (marked by an arrow in Fig. 5.1) was determined as 0.09 mM. As per our knowledge there have been no reported studies on the CMC of L61 in organic solvents. However, Kozlov and co-workers (2000) have established a CMC of 0.11mM for the L61 micelles in water. In order to confirm the CMC of L61, dye solubilisation was chosen as the second method. Since there was no reported CMC value of L61 reverse micelle in the literature, dye solubilisation measurement was studied in order to confirm the CMC values obtained using the water solubilisation method.

5.1.1.2 Dye solubilisation

Similar to previous AOT and CTAB reverse micelle system, the dye solubilisation was investigated in a pluronics L61 RMS. A hydrophilic dye rhodamine B was initially investigated as a model compound to be solubilised in the L61 reverse micelle. However, the CMC of L61 RM could not be detected using this dye as there was no increase in absorbance of the dye with increasing L61 concentration. This could be because of the high MW of rhodamine B (479.01 Da) which was unable to enter the RM of pluronics L61 due to the formation of small RM in comparison with AOT and CTAB based on the water content data reported (section 3.1, 3.2, 4.1 and 4.1.1). A smaller hydrophilic dye called fluorescein sodium (376.27 Da) was therefore chosen instead of rhodamine B for further studies. Fig. 5.2 showed the absorbance spectrum of fluorescein sodium with an absorbance maximum at 450 nm.

The data in Fig. 5.2 shows the effect of increasing L61 concentration on the solubilisation of sodium fluorescein in L61 RMS. Fluorescein sodium is a hydrophilic dye which is totally insoluble in *p*-xylene. From the water solubilisation measurement, the CMC of L61 was determined to be 0.09 mM and therefore the range of L61 concentration chosen for the dye solubilisation was narrowed from 0.03-0.17 mM, which includes concentration both above and below the CMC.

In Fig. 5.2, some absorbance of fluorescein sodium was observed in the sample of dye solution mixed with *p*-xylene without L61. This could be because although fluorescein sodium is not soluble in *p*-xylene, there is slight solubility of water in *p*-xylene (736 ppm as shown in Fig. 5.1). Therefore, the dye could be solubilised in this water resulting in a low absorbance of 0.036.

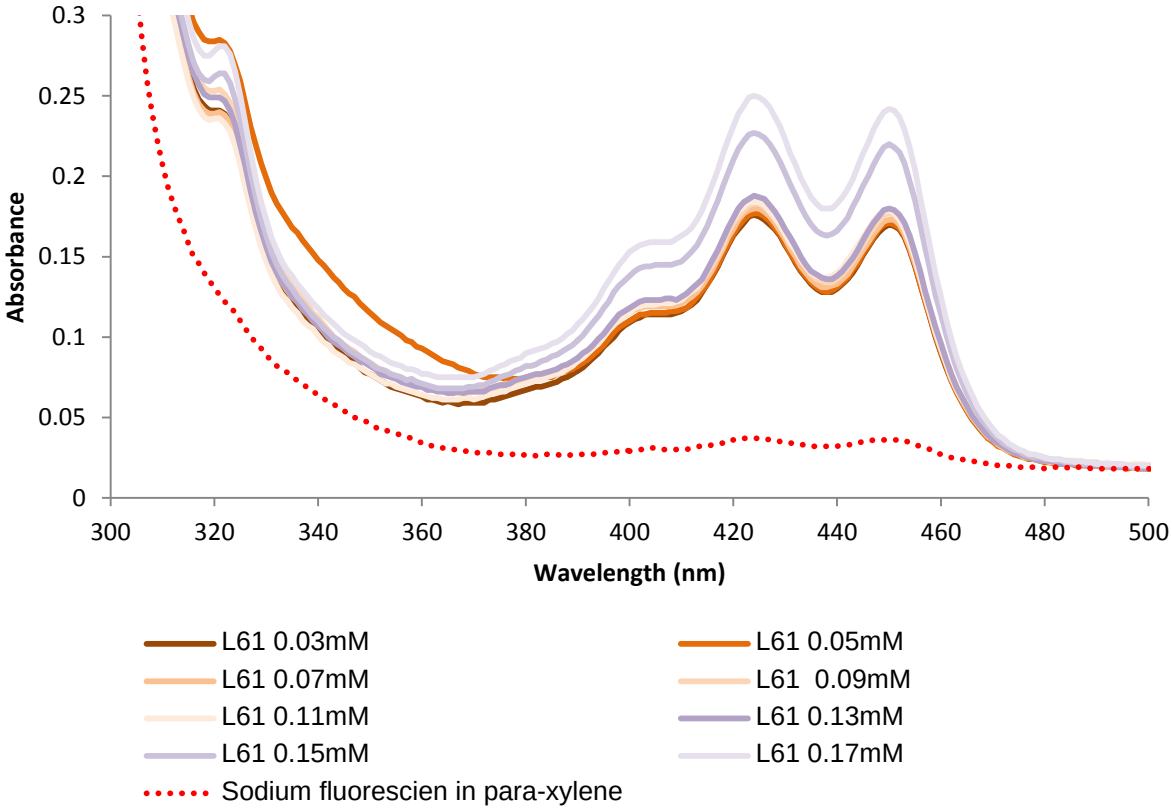

Figure 5.2: The spectrum of fluorescien sodium at different L61 concentration.

The data in Fig. 5.2 also shows an increase in absorbance of the dye with increasing L61 concentration. Thus, it can be reasonably said that the dye is trapped or solubilised inside the reverse micelle, and the solubilisation increases with increasing surfactant concentration. From L61 concentration 0.03-0.11 mM, the absorbance of fluorescein sodium was more or less constant, but there was a considerable increase in the absorbance of the dye from around 0.13 mM and a sharp rise in the absorbance after that (Fig. 5.3). The explanation for this observation is that there is no RM formation in the range of 0.03-0.11 mM as the concentration is below the CMC, but once the concentration exceeds the CMC (beyond 0.11 mM) more dye is solubilised inside the RM. This behaviour thus confirms the formation of L61 RM in *p*-xylene and the detected CMC by dye solubilisation is more or less in agreement with the CMC values of L61/*p*-xylene RMS obtained using the water content method (0.12 and 0.09 mM, respectively).

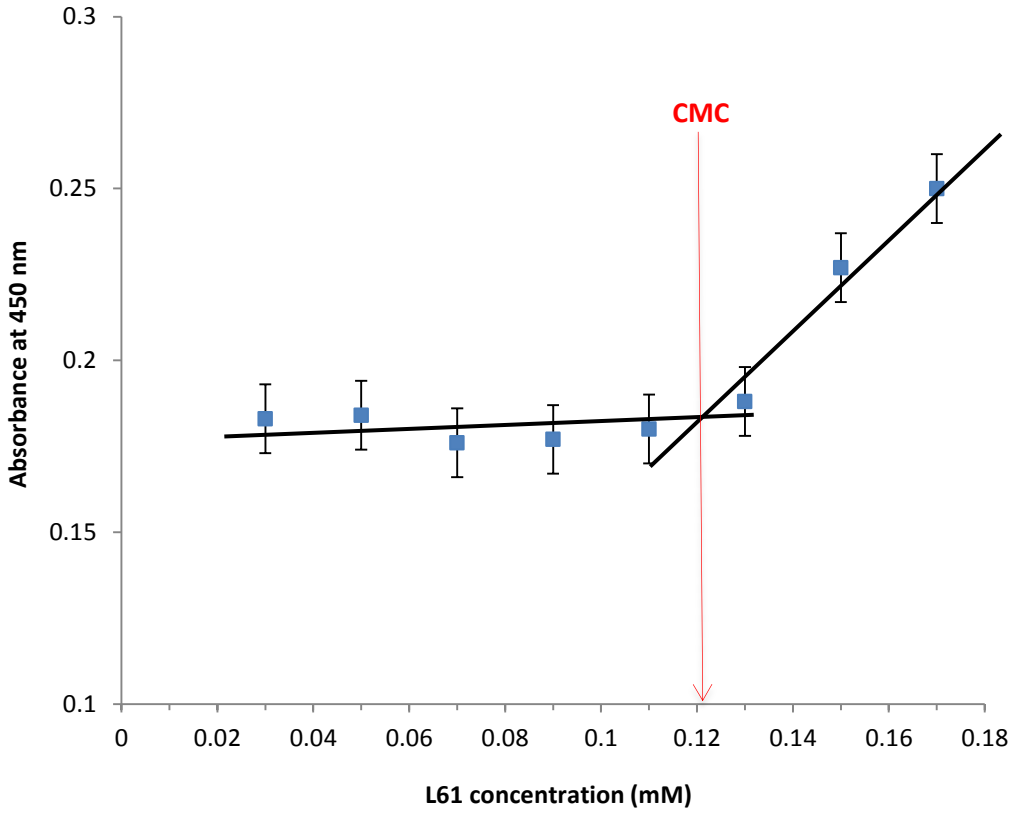

Figure 5.3: CMC of L61/*p*-xylene RMS determined by dye solubilisation (sodium fluorescein) method.

5.1.2 PEG-PPG-PEG L81

The "reverse" critical micelle concentration of L81 in *p*-xylene was also investigated using the water solubilisation method. The CMC of L81 was tested in the concentration range of 0.0001-0.22 mM (Fig. 5.4). From L81 concentration 0.0001 mM to 0.06 mM, the water solubilisation increased only gradually and therefore could not be used for CMC determination. Beyond 0.06 mM L81 concentration there was a sharp increase in the water solubilisation which indicates the formation of RM. From this data, the CMC of L81 in *p*-xylene solution was found to be 0.07 mM.

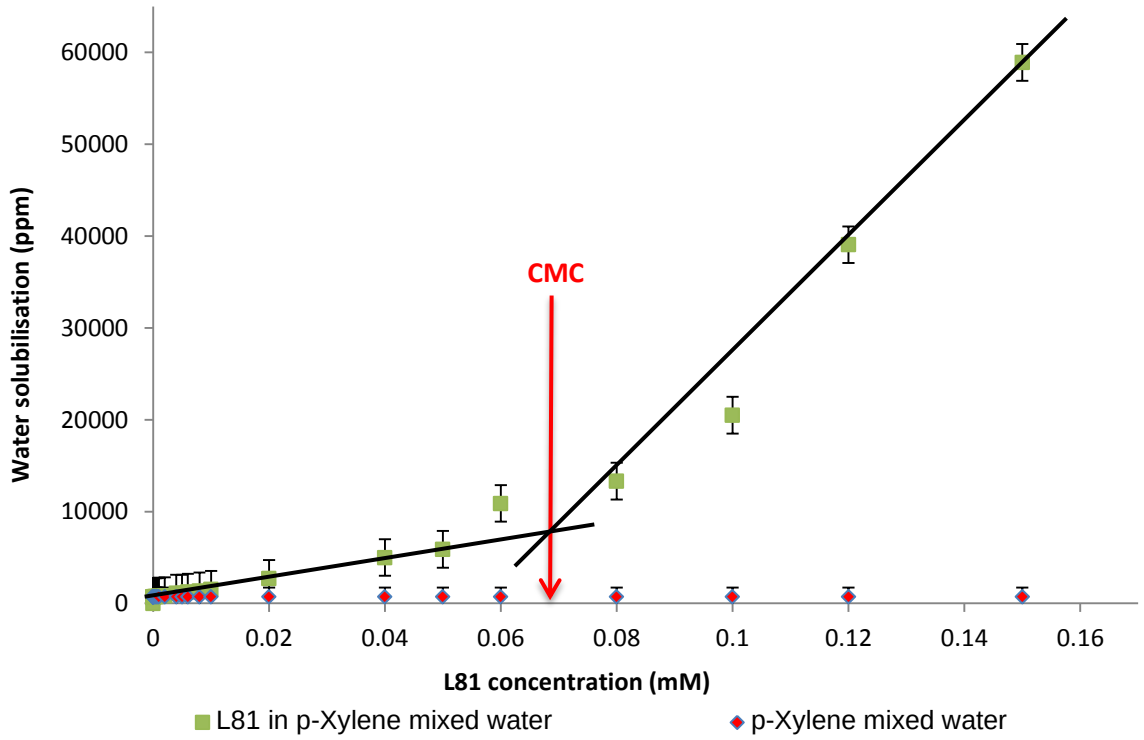

Figure 5.4: CMC of L81/*p*-xylene RMS determined by water solubilisation method.

Also, it can be observed that the CMC of L81 (0.07 mM) was comparatively lower than the CMC of L61 in *p*-xylene (0.08-0.12 mM) which is in agreement with previous studies done by other researchers. Alexanderidis and Anderson (1997) studied the effect of polyoxyalkylene block copolymer molecular mass on RM formation and water solubilisation in xylene. A trend of decreasing CMC with increasing copolymer molecular mass was observed for L62-L92 and L44-L104 groups (Alexandridis and Anderson, 1997). In another research, Zhang and colleagues (2004) investigated the effect of PPG block to pluronics RM formation in L44-L64 and P85-P105.They concluded that smaller molecular mass pluronics were less hydrophilic which does not favour water solubilisation (Zhang *et al.*, 2004). They also explained that short PPG block may make micelle formation difficult because PPG block must be folded to make the two PEG blocks come into the core of the RM. In comparison with L61, L81 has a longer PPG block, thus making it easier to form RM at a lower CMC value. Moreover, L81 is more hydrophilic than L61 because the average number of EO units of L61 and L81 are 4.55 and 6.25 respectively. It was

also reported that the surfactant with high hydrophobicity should have a stronger water-EO interaction, leading to the formation of RM at lower concentrations (Zhang *et al.*, 2004).

Therefore, it can be concluded that L81 can form RM in *p*-xylene at lower concentration than L61. The dye solubilisation with sodium fluorescein was also studied for L81 RM but no dye solubilisation inside L81 RM was recorded. As this study needs further investigation L81 will not be considered for protein extraction studies.

5.1.3 Water content of L61 and L81 reverse micelles in *p*-xylene

Based on the amount of water solubilised the water content W_0 of L61 and L81 RM in *p*-xylene was calculated using the equation: $W_0 = [H_2O]/[\text{Surfactant}]$ (Scatena, 2001). This result was presented in Fig. 5.5.

Again, the ability of L81/*p*-xylene RM in dissolving much more water than L61 at the same concentration (Fig. 5.1 and Fig. 5.4) was confirmed by higher W_0 value (Fig. 5.5). Around the CMC point, L81/*p*-xylene RMS has a W_0 of 7-10 while L61/*p*-xylene RMS had a W0 of 1-2 only. With increase in surfactant concentration, the maximum W_0 value of L61 RMS was 4 as compared to 34 with the L81 RMS system rose up to 34. The higher water content values in L81/*p*-xylene RMS as compared to L61/*p*-xylene RMS could not be explained based on the 5 effect of triblock copolymer molecular weight to their RM formation only. The reason behind this phenomenon is unclear and needs further investigation.

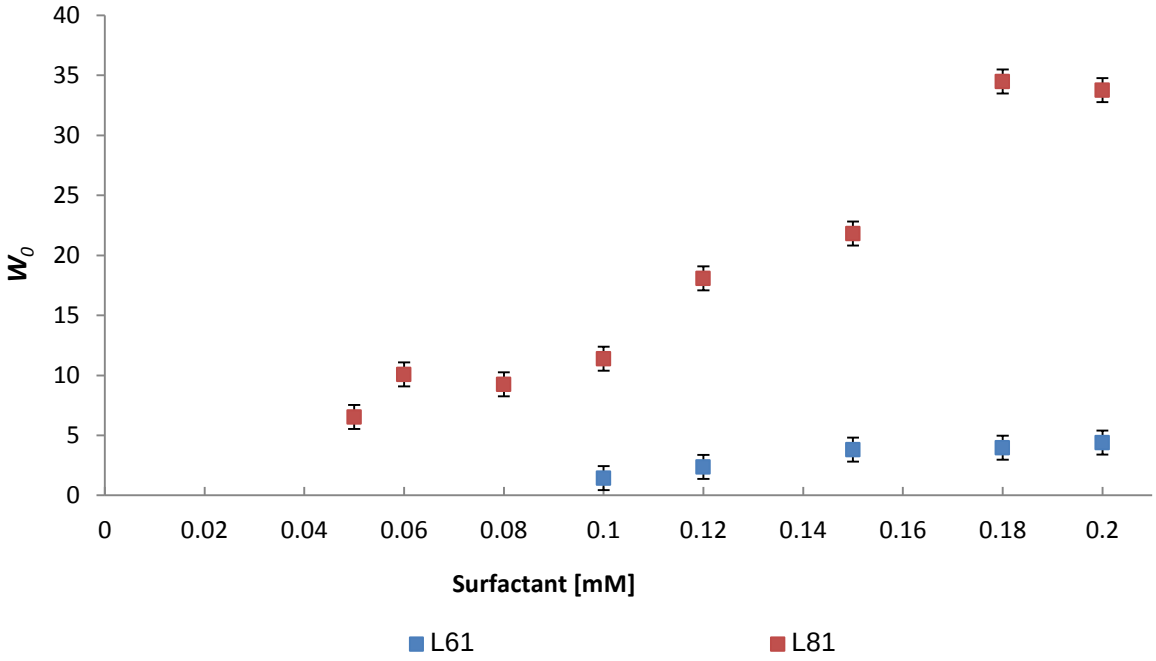

Figure 5.5: Comparing W_0 of L61/*p*-xylene RM and L81/*p*-xylene RMS according to water solubilisation method.

Having proved that pluronics L61 and L81could form RM in *p*-xylene it was interesting to further investigate its application in protein extraction and refolding. The nontoxic and biocompatible nature of pluronics was envisaged as a distinct advantage for many applications in the pharmaceutical and biopharmaceutical sectors if this process was successful. Therefore, lysozyme extraction using pluronics RM was considered as the next step.

5.2 Lysozyme extraction using non-ionic pluronics L61/*p*-xylene reverse micelles

The results of water solubilisation and dye solubilisation show that L61 and L81 can form RM in *p*-xylene. However, it was observed that L81/*p*-xylene formed a cloudy interface when mixed with water at 1:1 ratio as compared to L61/*p*-xylene. This could be because of the larger PPG blocks causing higher micro-viscosity (Singh *et al.*, 2013). Also, L81/*p*-xylene RM could uptake more water than L61 ones at the same concentration (as shown in water solubilisation measurement) which could result in a completely hydrated PEG causing a cloudier phase. In addition, the

failure of L81/*p*-xylene RM in dye solubilisation experiment rise questions about its ability to host chemical or biological molecules. Therefore, although L81 may form much bigger RM in *p*-xylene as compared to L 61, it was not chosen for further investigation in protein extraction.

5.2.1 Effect of L61 concentration

The CMC of L61 was in the range 0.09-0.12 mM, therefore L61 RM was prepared in *p*-xylene over a range of concentration from 0.03-0.2 mM for lysozyme extraction. This concentration range chosen to study the extraction of proteins was both above and below the CMC. However, no extraction of lysozyme was observed in this range (data not shown). The reason could be because the CMC is the point where RM is being formed and the number of micelles would be small resulting in almost no extraction. Therefore, the examined concentration range was too low to extract proteins. It can also be supported with the low water content values obtained in this range thus resulting in smaller RM. The concentration of L61 was, therefore, increased to 5 mM and 10 mM which were much higher than the CMC of L61.

At 5 mM, the FE efficiency of L61/*p*-xylene RM with no salt at pH 7 was around 7-9% (data not shown). It was considered very low and therefore no BE was attempted for these conditions. Further studies involving two parameters: the effect of KCl concentration and the effect of pH were undertaken in order to improve the FE efficiency.

5.2.2 Effect of pH and salt concentration

L61 is a non-ionic surfactant and is envisaged to have little or no effect of pH and salt concentration. In order to confirm this, a range of pH and salt concentration was investigated to study its effect on lysozyme extraction (Fig. 5.6).

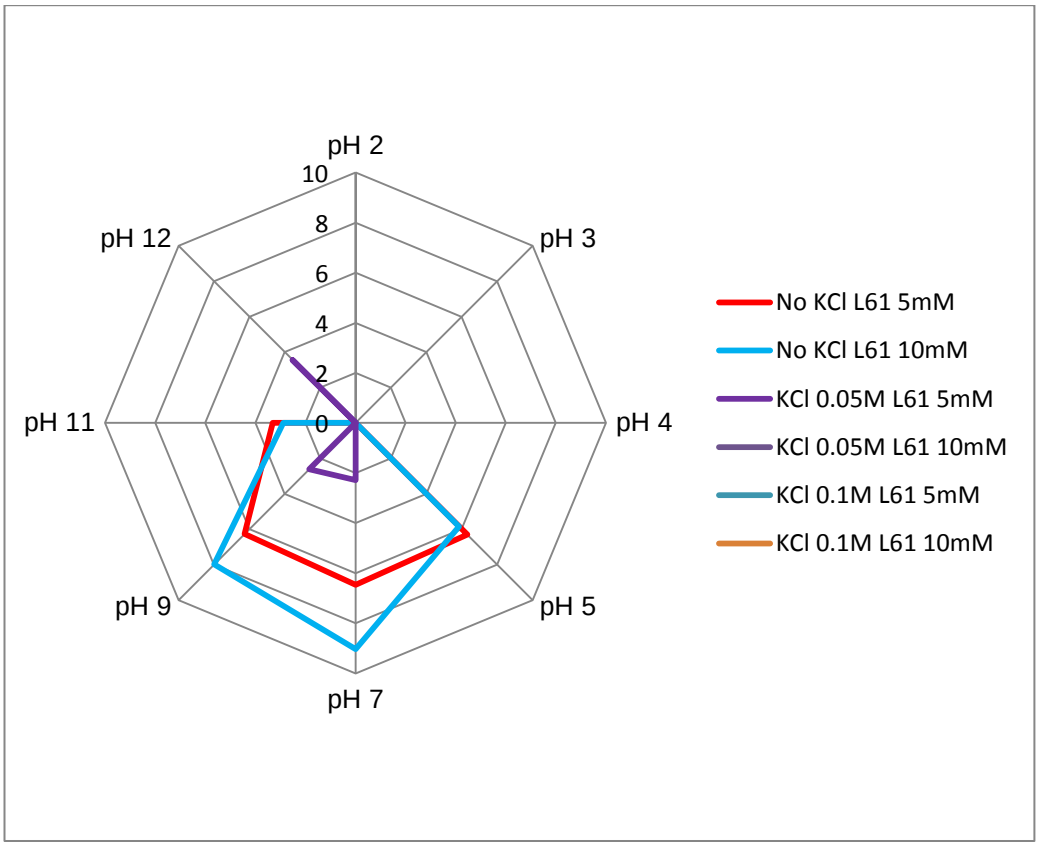

Figure 5.6: Lysozyme FE using L61/*p*-xylene RMS under different pH, KCl and L61 concentration.

From the above experiments, it can be observed that the percentage of FE of lysozyme using L61 RMS was very low, <10 %. In addition, the result also showed that KCl did not play any significant role in the FE process. The contribution of buffer to the ionic strength was eliminated because of the low concentration (0.01 M). This result can be explained on the basis of electrostatic interactions. Ionic surfactants use electrostatic interaction as their main driving force for protein extraction based on the opposite charge of protein and surfactant. However, there is no charge interaction in pluronics RM, and hence, there is no much role for KCl. Non-ionic surfactants depend on hydrophobic interaction that is quite weak to solubilise the proteins. Therefore, less protein or no protein is transferred to the ORP in this case.

In the case of protein extraction using ionic surfactants, the pH and salt concentration are usually adjusted in the aqueous solution which helps govern the behaviour of proteins into the reverse micellar phase. A change in pH can alter electrostatic interaction between the polar head of the surfactant and the protein surface (Goklen *et al.*, 1987). However, in case of non-ionic

surfactants, electrostatic interaction is weak and does not play any role in protein extraction. This explains why the addition of KCl or change in pH did not lead to any protein extraction. The maximum extraction was observed at pH 7 where the charge was neutral.

5.2.3 Effect of 1-octanol

Since L61 RM was able to extract around 7-9 % of lysozyme, it was envisaged that addition of a co-surfactant/co-solvent like 1-octanol could increase the size of the reverse micelle and thus improve the lysozyme extraction. Therefore, the effect of 1-octanol on lysozyme FE using L61/*p*-xylene RM was studied.

As compared to L61 RM alone, the percentage of lysozyme extracted from the AQP was a bit higher (17% as compared to 9 %) using the L61/*p*-xylene/1-octanol RMS. Though addition of 1-octanol increased the extraction of lysozyme, it was not high enough to carry out the BE. In order to carry out an efficient back extraction, there should be more than 25-30% FE (Yu and Chu, 2003; Gomez *et al.*, 2011). In addition, due to the proximity of the absorbance values of lysozyme and *p*-xylene (λ_{max} of lysozyme is 280 nm; λ_{max} of is 273 nm) the concentration of lysozyme in the ORP could not be determined.

In order to investigate the feasibility of L61/*p*-xylene reverse micelle systems a different protein (haemoglobin) which did not absorb at the same wavelength as *p*-xylene was selected for further studies.

5.3 Haemoglobin extraction using L61/*p*-xylene/1-octanol reverse micellar system

The FE of haemoglobin in L61 RM was carried out using the same methodology as lysozyme. The data in Fig. 5.7 shows that around 50 % of haemoglobin was extracted from the AQP after

FE. However, analysis of the ORP did not show the presence of haemoglobin in the reverse micelle core.

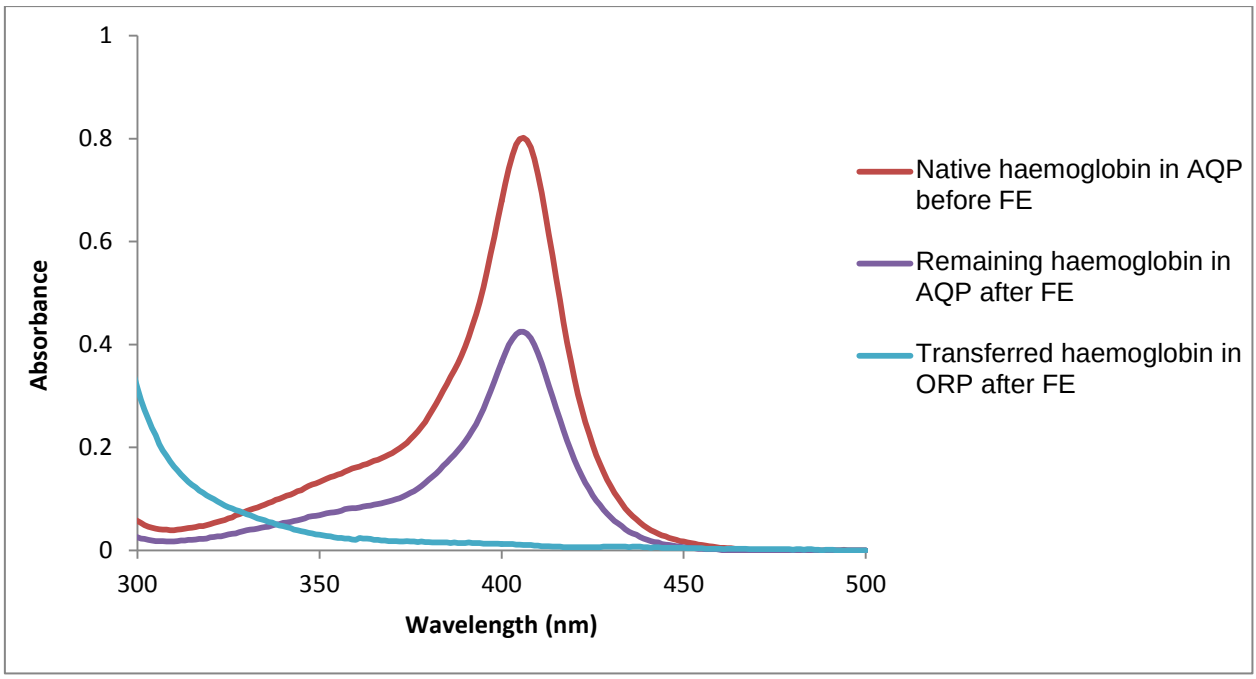

Figure 5.7: Haemoglobin extraction by L61/*p*-xylene/1-octanol RMS.

From the above results, it is clear that no haemoglobin was transferred from the AQP to the ORP after FE. This behaviour could be explained on the basis of weak hydrophobic interaction of the protein and non-ionic L61 RM which is comparable with the lysozyme extraction in the same system. Another explanation for no uptake of haemoglobin in the non-ionic RMS could be its large size (64.5 KDa) which was not compatible with the same reverse micelle formed with non-ionic surfactants.

Summary:

It can be concluded that pluronics reverse micelle systems alone cannot extract proteins efficiently. The next step was therefore to investigate the effect of mixed RM of L61 and an anionic surfactant AOT. The strong electrostatic interaction between anionic AOT surfactants and protein could help L61 extract proteins efficiently. There has been some reports in the literature on the ability of mixed surfactant systems such as a combination of AOT with non-ionic surfactant such

as Tween 85, Brij 56, Brij 58 (Paul, 2006) (Kundu, 2013) in protein extraction. It was therefore, interesting to examine the effect of the addition of an anionic surfactant to a pluronics RMS.

6.LYSOZYME EXTRACTION USING MIXED REVERSE MICELLAR SYSTEM OF ANIONIC AOT AND NON-IONIC L61 SURFACTANT

This chapter deals with the results and discussions related to mixed RMS of AOT and L61 surfactant and investigates three main aspects which includes: the determination of CMC of mixed RMS of L61 and AOT as using the water solubilisation and dye solubilisation method, lysozyme extraction using L61/AOT/*p*-xylene mixed RMS with L61 as the main surfactant and lysozyme extraction using AOT/L61/*p*-xylene mixed RMS with AOT as the main surfactant.

6.1 Critical micelle concentration

The L61/AOT mixed RMS was characterised by determining its CMC using two methods, water solubilisation and dye solubilisation. The concentration of L61 was kept constant at 5 mM. The concentration range of AOT chosen was 0.01-1 mM. The CMC value of L61/AOT mixed reverse micelle system was expected in the range between the CMC of L61/*p*-xylene RMS (0.09-0.12 mM) and AOT/*p*-xylene RMS (1.2-0.13 mM).

6.1.1 Water solubilisation

When different concentration of L61/AOT surfactant in *p*-xylene was mixed with water, the ORP turned milky with the formation of a precipitate at most concentrations. Therefore normal water solubilisation using Karl Fisher titrator could not be investigated. In order to examine the water inside the mixed RM, dye solubilisation experiments were done and the same samples were subjected to water content analysis.

From AOT concentration 0.01-0.1 mM, there was only a slight change in water content in the range of 703-861ppm. The reason could be because L61 and AOT exist in the ORP as unimers or dimers at this concentration range with no RM formation, resulting in limited water solubilisation. In addition, the water content in this concentration range is nearly similar to the water content of the sample containing *p*-xylene and water only (736ppm) which supports the argument of the absence of RM.

The data in Fig. 6.1 shows the effect of varying AOT concentration (L61 was fixed at 5mM) on water solubilisation in the mixed micelle system obtained from the dye solubilisation studies. From 0.1 mM onwards, the water solubilisation increased sharply. This increase indicates the self-association of the surfactant molecules into RM, which provides a hydrophilic microenvironment suitable for water solubilisation. From the data in Fig. 6.1, the CMC of L61/AOT/*p*-xylene mixed reverse micelle system could be assumed as 0.15 mM AOT in the presence of 5 mM L61. There is no record of the CMC of L61/AOT/*p*-xylene mixed reverse micelle system in the literature. Therefore, no comparison could be done. However, dye solubilisation will be examined to confirm this result.

6.1.2 Dye solubilisation

For dye solubilisation method, fluorescien sodium was again selected as the dye and the same methodology used for L61 was adopted (section 5.1.1.2). The concentration of L61 was kept constant at 5 mM while the AOT concentration was varied from 0.01-1 mM.

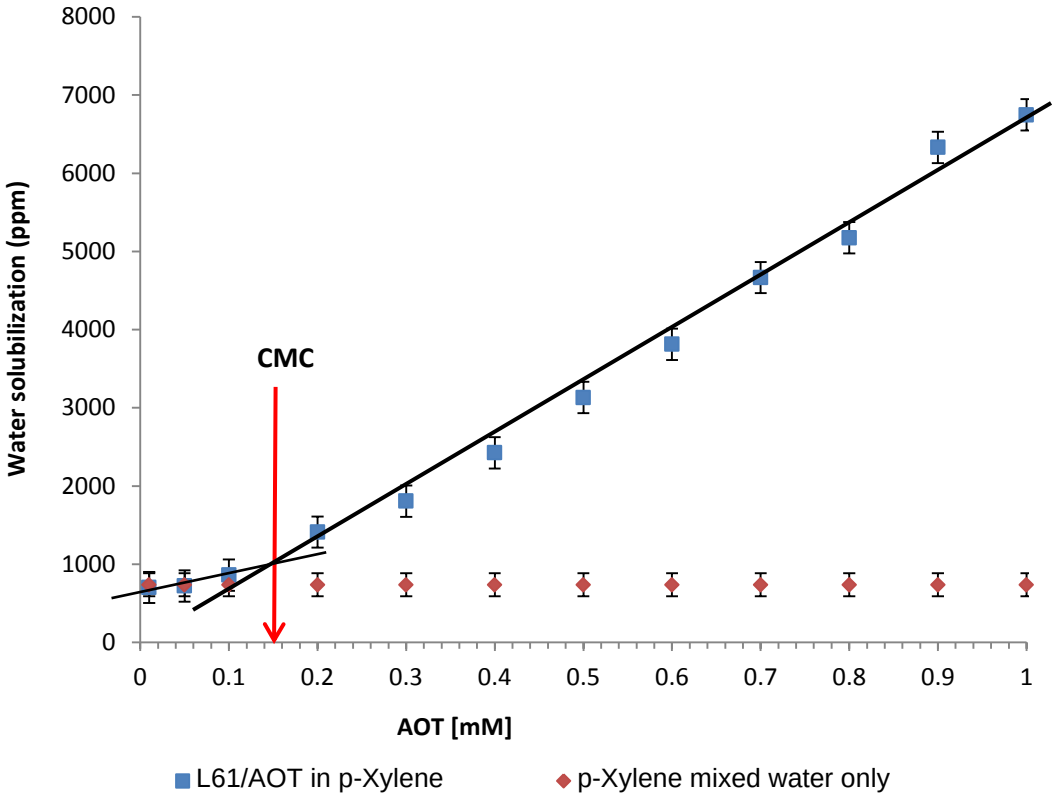

Figure 6.1: CMC of mixed L61/AOT/*p*-xylene RMS determined by water solubilisation method.

The data in Fig. 6.2 shows that the CMC of the mixed RMS was around 0.25 mM of AOT when the concentration of L61 was 5 mM. This CMC value is lower as compared with CMC of AOT in *p*-xylene (1.3 mM) but higher than the CMC of L61 in *p*-xylene (0.09-0.12 mM). Higher CMC of AOT in comparison with L61 can be explained by a strong repulsion between charged AOT headgroups within the RM (Chou *et al.*, 2014). The presence of L61 as a non-ionic surfactant in RM decreases the repulsion between charged AOT headgroups and, as a result, the CMC of the mixture is lower than that of AOT RM alone.

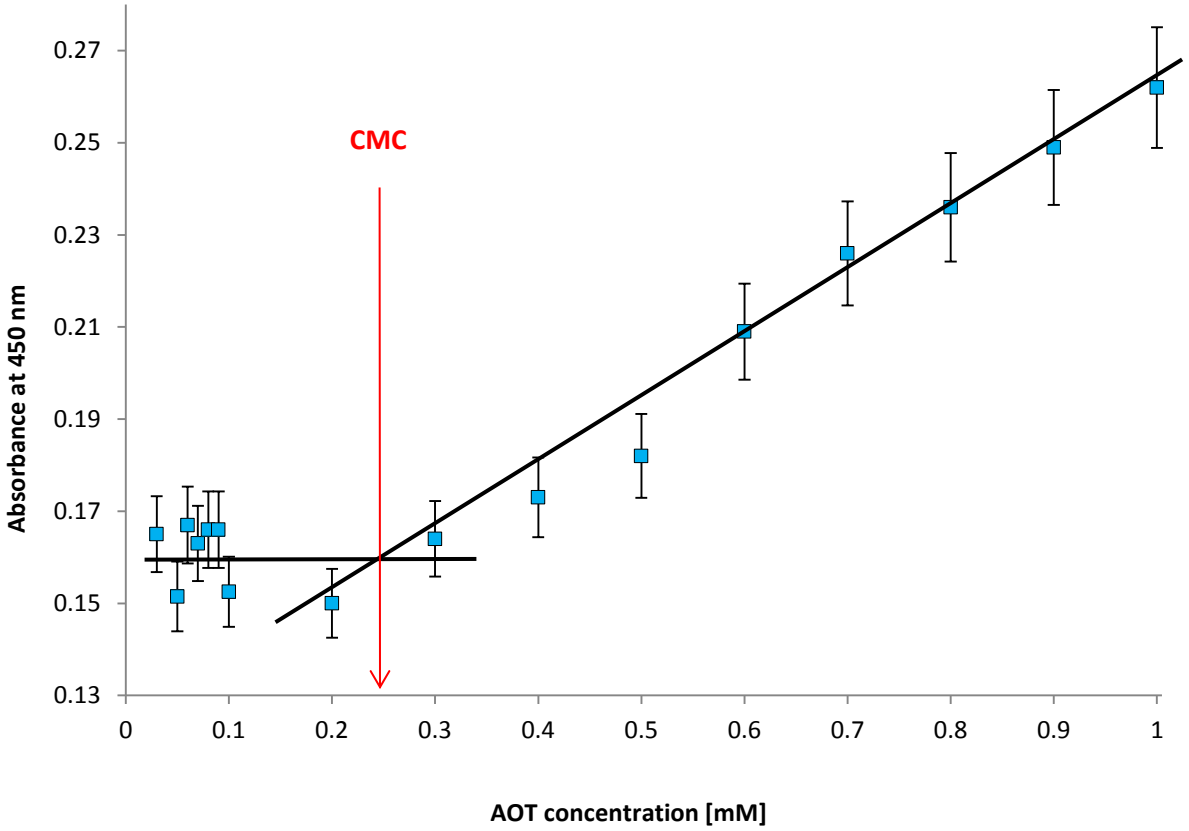

Figure 6.2: CMC of mixed L61/AOT/*p*-xylene RMSdetermined by dye solubilisation (sodium fluorescein) method.

This result was close to the CMC of L61/AOT/*p*-xylene mixed RMS obtained using the water solubilisation method as L61 5 mM and AOT 0.15 mM.

6.2 Lysozyme extraction with L61 as the main surfactant

6.2.1 Forward extraction

6.2.1.1 Effect of AOT concentration

The effect of AOT concentration above and below the CMC of the mixed micelle system (0.1, 0.2, 0.4, 1, 2, 5, 10, 50 and 100 mM) was investigated by keeping the concentration of L61 at 5 mM. The CMC of L61/AOT/*p*-xylene mixed RM was in the range 0.15-0.24 mM with an L61 concentration of 5 mM. Based on the results obtained in the L61 system (section 6.2), pH 7 with

no KCl were chosen as the ideal conditions for this extraction, as it was established that KCl did not play any major role in the extraction of proteins using non-ionic surfactants. Moreover, it is known that lysozyme solubilisation is highest at a pH value slightly less than its pI value (pH 11), so the ideal range was reported be between pH 6-10 (Goklen, 1986). The effect of AOT concentration was assessed based on the yield of lysozyme remaining in the AQP after FE.

At AOT concentration of 0.1 and 0.2 mM, there was no change in the percentage of lysozyme extracted from the AQP (58 and 57 %) (Fig. 6.3). However, the lysozyme extraction efficiency reach 98 % at AOT 0.4 mM (Fig. 6.3). From AOT concentration 0.5 mM to 100mM, no extraction was observed (data not shown). This can be explained based on the role of AOT in the mixed reverse micelle system. AOT was considered as a co-surfactant with L61 (5 mM) in this system. However, beyond 1 mM which is near the CMC value of AOT in *p*-xylene (1.3 mM), AOT may form RM by itself and hence the effect of L61 could have been reduced due to its low concentration as compared to AOT. Though AOT concentration was higher than CMC, there was no extraction of proteins due to the absence of KCl in the system. It has been established in the literature that the FE of proteins can only take place in the presence of small amounts of salt, if an anionic or cationic surfactant is used to form a reverse micelle (Santos *et al.*, 2001).

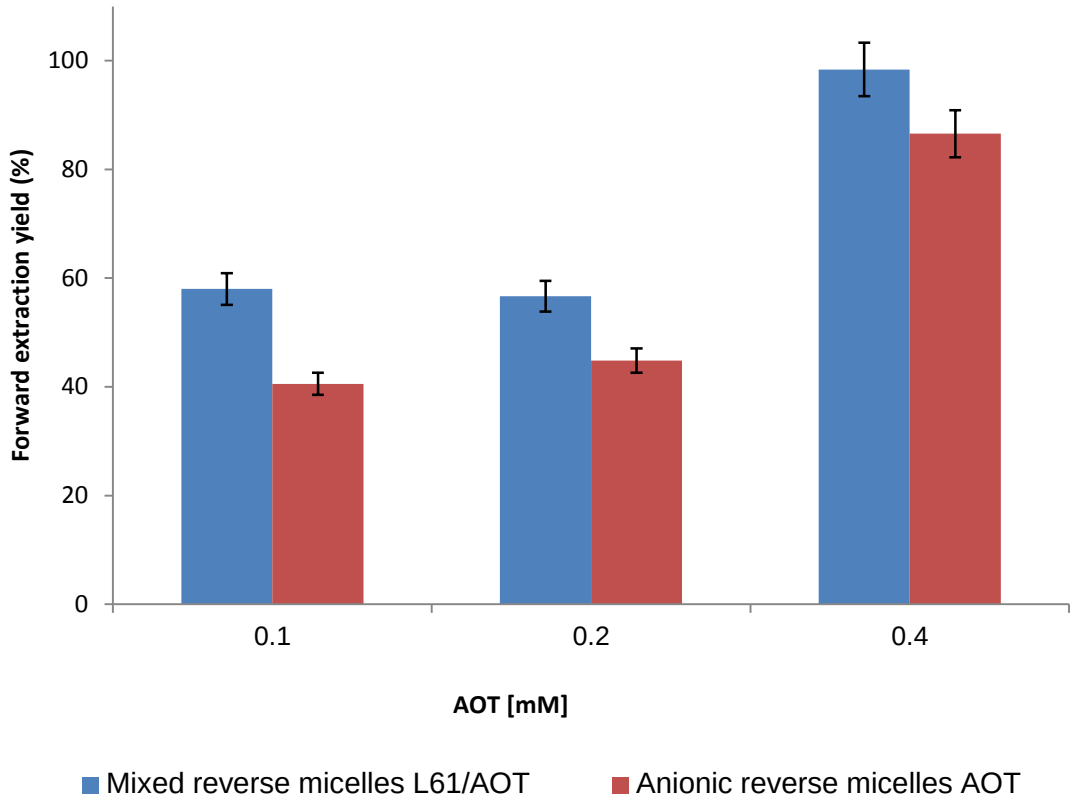

Figure 6.3: Effect of AOT concentration on lysozyme FE using L61/AOT/*p*-xylene mixed RM.

Since the main aim of the investigation was to study the effect of mixed reverse micelle system on the protein extraction and activity, lower AOT concentration was chosen for further experiments. There was no significant difference in the FE yield for 0.1 mM and 0.2 mM AOT, and though 0.4 mM AOT gave higher extraction yield, considerable precipitation was observed. Based on the observations above, 0.1 mM was chosen as the AOT concentration for further experiments.

Another set of experiments using the same conditions as AOT/L61 was performed with AOT reverse micelle only. The results showed that the presence of L61 could help improve the yield of lysozyme FE. In addition, more precipitate was observed after FE in case of AOT RMS than mixed L61/AOT RMS. This phenomenon has been reported in the literature by Sakono and colleagues who studied the refolding of carbonic anhydrase B (CAB) using anionic AOT/isooctane and non-ionic tetra-ethylene glycol dodecyl ether RM (Sakono, 2004). They reported that the

strong interaction of CAB with AOT resulted in the precipitation of CAB at the interface and low yield of BE that was not observed in case of non-ionic surfactant. Therefore, the presence of L61 could help prevent the precipitate formation by weaker electrostatic interaction between AOT and lysozyme.

6.2.1.2 Effect of KCl concentration

The effect of KCl concentration on the extraction of lysozyme in the AOT and mixed reverse micelle system was investigated (Fig. 6.4). No significant difference in extraction yield was observed by varying the concentration of KCl. The reason being that since pluronics is a non-ionic surfactant it does not depend on electrostatic interactions and hence salt does not play a significant role.

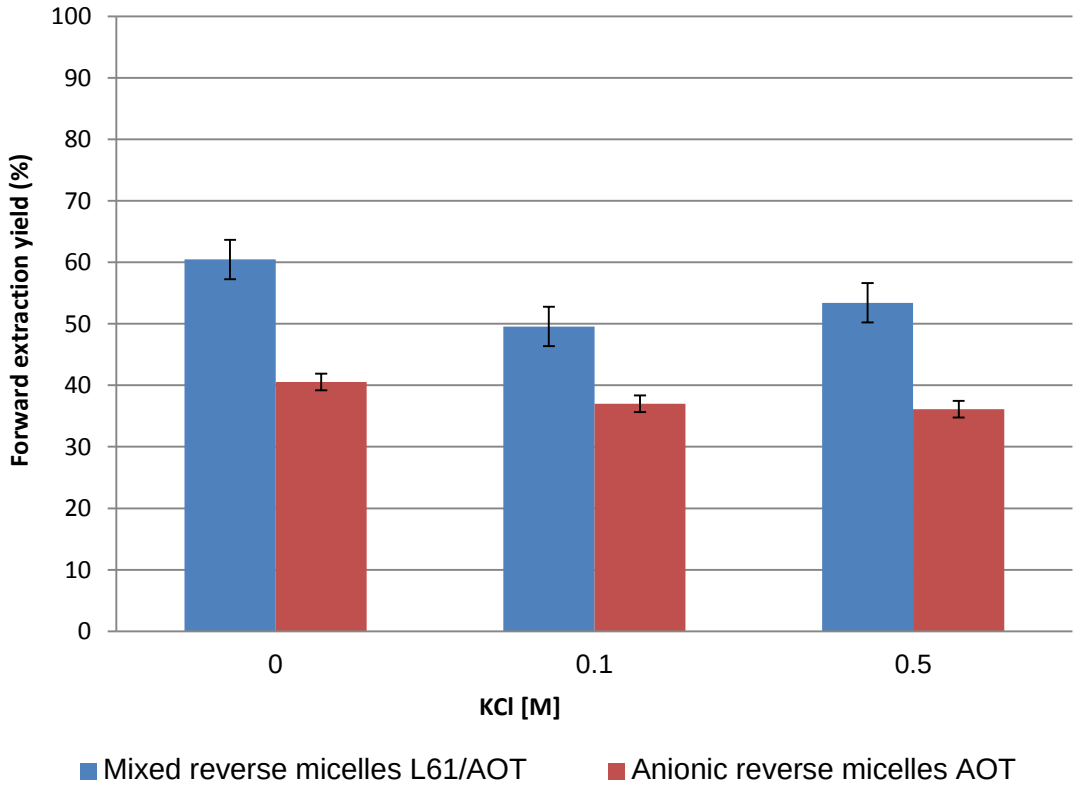

Figure 6.4: Effect of KCl concentration on lysozyme FE using L61 5 mM, AOT 0.1 mM in *p*-xylene.

In the case of AOT reverse micelle system, there was also no significant change in the extraction yield by varying the KCl concentration (Fig. 6.4) The reason being that at 0.1 mM of

AOT (below CMC), no RM were formed and therefore salt does not play any role. However, it was observed that around 40 % of lysozyme was missing from the AQP. This loss of lysozyme could be as a result of protein precipitation, which has also been investigated by other researchers (Decker, 1990; Shin *et al.*, 2003; Cheng and Stuckey, 2012).

6.2.1.3 Effect of 1-octanol as co-solvent

The effect of 1-octanol (5-20 %) in combination with KCl on the extraction of lysozyme in L61/AOT/*p*-xylene mixed RMS was investigated. The concentration of L61 was constant at 5 mM in all experiments.

At 0.1 mM AOT concentration, the presence of 1-octanol resulted in a low extraction yield as compared to the extraction efficiency in the absence of 1-octanol (15 % and 60 %, respectively). This could be because 1-octanol is located in the micellar water pool and interacts only with water at low AOT concentration. However, at high AOT concentration, 1-octanol can form a complex with AOT molecules at the micellar interface that could help forming bigger and more stable RM. This concept has been reported before by Mathew and Juang (Mathew, 2005).

From 0.5-5 mM of AOT concentration, the yield of lysozyme FE increased sharply but was relatively constant, 95-98 % (Fig. 6.5). Moreover, the samples were not milky as in the case of samples without 1-octanol. It can thus be concluded that 1-octanol did play a role in enhancing the solubilisation capacity of proteins in the presence of L61 and AOT which is also in agreement with previous research (Penders and Strey, 1995).

However, at higher AOT concentration, from 10 mM onwards, the lysozyme FE yield decreased with increased precipitate formation at the interface (Fig. 6.5). This could be explained on the basis of a complex formation of 1-octanol with AOT molecules at the micellar interface. According to Perez-Casas and his colleagues (1997), in the presence of long chain alcohol such as 1-octanol, the increase in AOT concentration will lead to two different processes. Firstly,

AOT molecules are withdrawn from the RM to be complied with alcohol. And secondly, the molecules in the bulk of the solution and alcohol molecules penetrate the micelles shell, where they also form a complex with AOT.

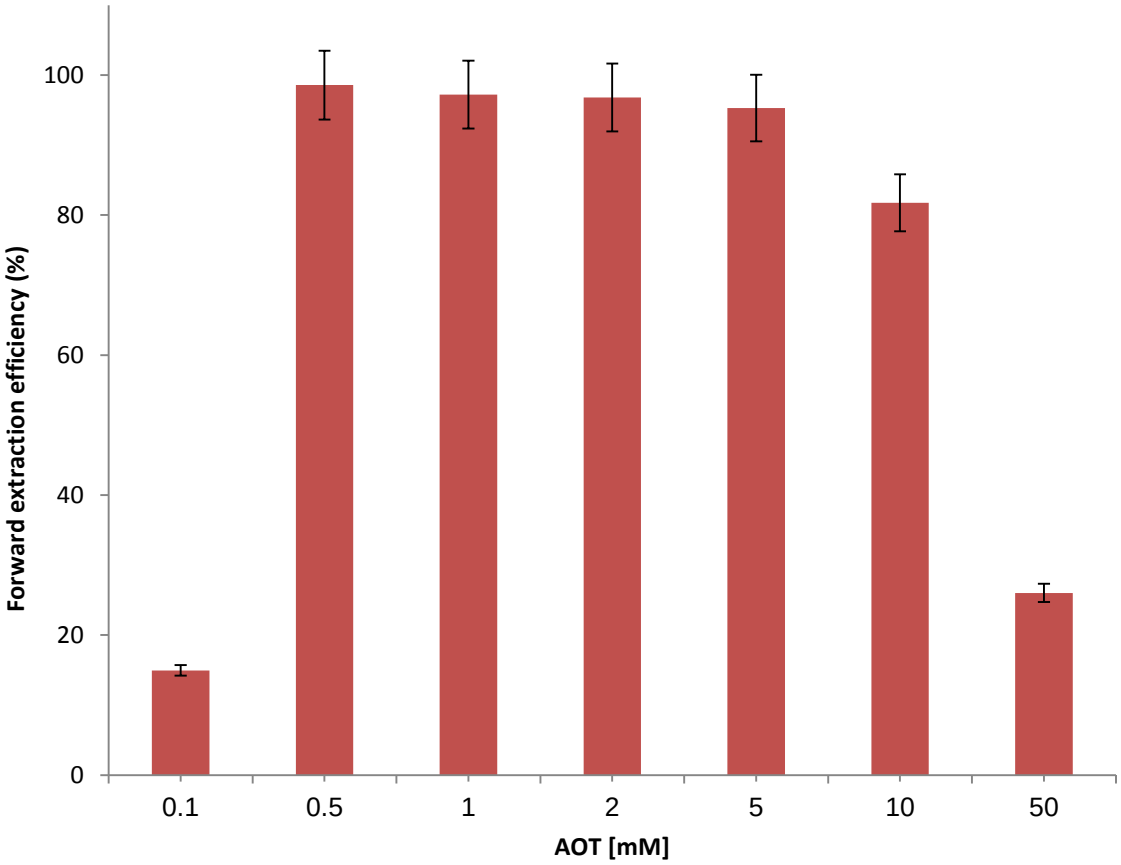

Figure 6.5: Effect of AOT concentration on lysozyme FE using mixed reveres micelles system (L61 5 mM, phosphate buffer pH 7, 1-octanol 20 %).

The effect of salt on lysozyme FE in the presence of 1-octanol was also examined. 0.1M and 0.5 M KCl were added to the aqueous solution with varying 1-octanol and AOT concentration. However, no extraction was observed. According to Bourrel and Schechter (1988), a higher salt concentration shifts the maximum water uptake to lower concentration of alcohol. This behaviour is reported as a common observation in surfactant, alcohol, salt and solvent systems which have also been supported by Wang and co-workers (1994). Mathew and Juang (2006) have reported that a higher salt concentration and a lower alcohol concentration were needed to

begin reverse micelle formation at a fixed surfactant concentration. Therefore, adding KCl to a solution which contains 20 % 1-octanol may not be ideal to enhance protein forward efficiency.

6.2.2 Backward extraction

During reverse micelle extraction, the concentration of protein is determined before and after FE using UV absorbance of the AQP. After FE, if the protein concentration decreases in the AQP, it is assumed that this protein has been solubilized in the organic reverse micelle phase. Once the protein is trapped inside the reverse micelle, it then needs to be back extracted into fresh AQP by optimizing parameters such as pH, KCl concentration and alcohol content.

For the BE experiments, pH 7 and pH 11.8 were investigated. The reason for these pH values was based on the pI of lysozyme which was 11.3 (Wetter, 1951). If the pH of the AQP is higher than the protein pI, the protein charge is negative, but if the pH is lower than the pI, the protein charge is positive. For non-ionic surfactant L61, the driving force for protein extraction is hydrophobic interaction. Therefore, pH is not an important factor. However, the presence of AOT in this mixed reverse micelle system requires the need to investigate the effect of pH for BE. In previous studies, it was found that the best pH for lysozyme BE using AOT RM was in range of 11-12, as at this pH the charge on the protein is negative which is similar to the charge on the anionic surfactant AOT (Hong *et al.*, 2000; Goto *et al.*, 1998; Carlson *et al.*, 1992). This results in electrostatic repulsion, which leads to the release of lysozyme from the organic reverse micellar phase to the AQP. According to Chou and Chiang (1998), pH 11.8 is the optimal pH for lysozyme BE. Moreover, at pH values above 12, insoluble aggregates composed of lysozyme and AOT molecules were formed at the oil-water interface, resulting in a decrease in the recovery ratio (Takumi and Hiroshi, 1992). Therefore, pH 11 and pH 11.8 was selected for the back extraction using CAPS buffer. In addition, the main surfactant in the present study was L61 which is a non-ionic surfactant, and hence pH 7 was also investigated using phosphate buffer.

KCl was also used in the back extraction studies as a higher salt concentration is usually required in the back extraction process using ionic reverse micelle systems. The reason being that high salt concentration causes high ionic strength which not only decreases the repulsive effect of the charged head group of the surfactant, but also leads to a decrease in water solubilisation and a lower W_0 (the molar ratio of water to surfactant) (Andrews and Haywood, 1994; Brandani *et al*, 1994). The KCl used in this experiment was 2 M.

In order to improve extraction, alcohols such as ethanol and 1-propanol were also investigated. In the BE process the presence of alcohol can help break the reverse micelle which aids the release of protein. Hong and colleague (2000) also reported that alcohol suppresses the formation of reverse micelle cluster remarkably and thus increases the rate and fraction of protein BE. The effect of mixing time on BE was also examined at 60, 120 and 180 minutes.

At pH 7 and pH 11, in the presence of ethanol (20 %) and KCl (2 M), no extraction was observed at all. At pH 11.8, in the presence of ethanol at 5, 10 and 15 %, the yield of recovered lysozyme were similar, around 5-7 %; when ethanol was increased to 20 %, the lysozyme recovery rose to 12 % and 23 % in the presence and absence of 2 M KCl, respectively.

However, in case of 1-propanol, no lysozyme was recovered under the same conditions. The reason could be that 1-propanol was much less soluble in the saline solution than in ethanol (The Merck Index) and the BE AQP in this study contained 2 M KCl.

Therefore, the use of different alcohols, high KCl concentration and increased mixing time did not lead to considerable lysozyme recovery from the mixed L61/AOT/*p*-xylene RMS. In addition, there was evidence of considerable precipitate formation at the interface. The lysozyme extracted from the AQP can be analysed either via investigating the precipitate formed or using a fluorescence tag. The precipitate investigation was done first while lysozyme fluorescence tag is beyond the scope of this work.

6.2.3 Lysozyme precipitate investigation

The precipitate obtained from the interface after FE was further investigated. Effect of pH and alcohol on the recovery of lysozyme from the precipitate was analysed.

6.2.3.1 Analysis of the precipitate using UV-Vis spectroscopy

In previous experiments, it was confirmed that there was no effect of pH and salt concentration when the ratio of L61 was high as compared to AOT (L61: AOT is 50:1 or 25:1 or 12.5:1). In the present study, no lysozyme was recovered at pH 7 (Fig. 6.6). This may be because of favourable electrostatic attraction between lysozyme and surfactant, especially AOT. The positively charged lysozyme is bound to the anionic surfactant to form an insoluble protein-surfactant complex. Therefore, lysozyme could not be released from this complex. Ethanol was added to break this complex, and release the lysozyme, but no success was achieved (Fig. 6.6).

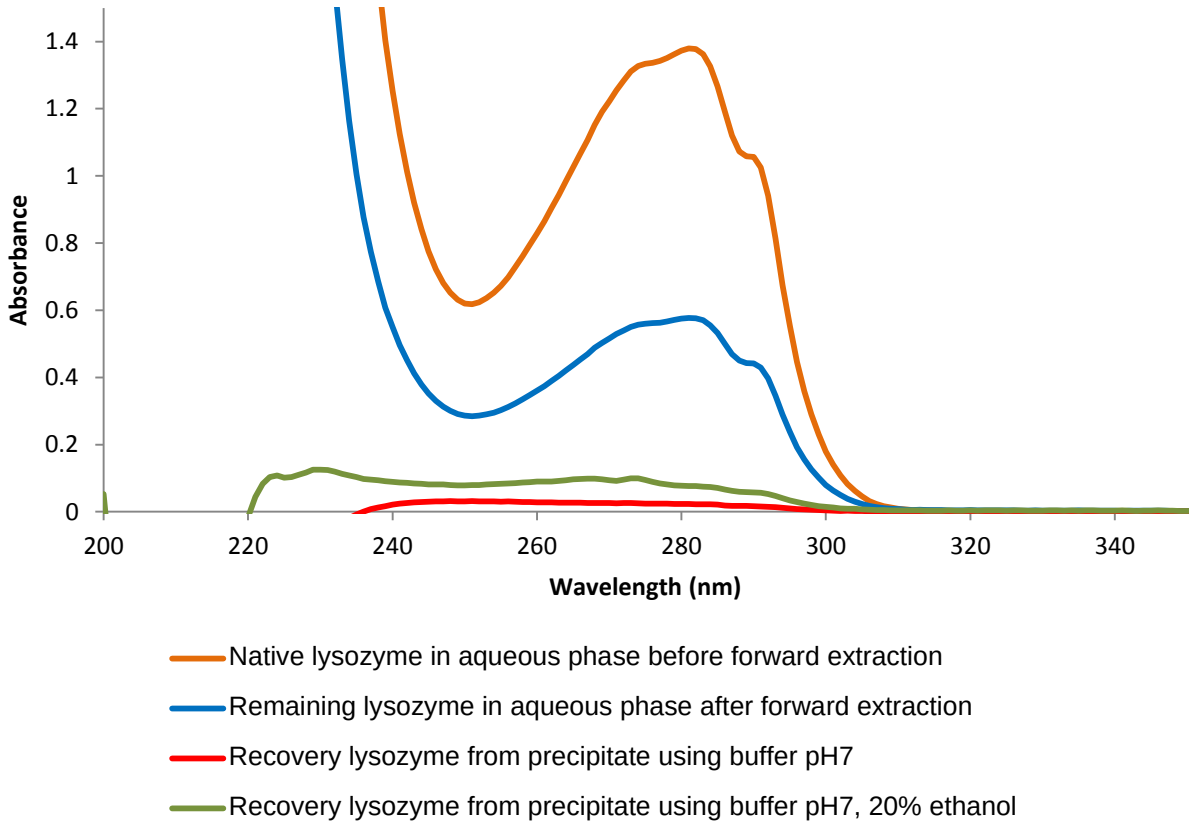

Figure 6.6: Recovered lysozyme from precipitate of L61/AOT/lysozyme mixture at pH 7 and in the presence of ethanol.

At pH 11.8 which is higher than the pI of lysozyme, it would be difficult for lysozyme to bind with the monomers of AOT because of the electrostatic repulsion between the protein and AOT head-groups. Therefore, lysozyme can be released out of protein-surfactant complex. However, no lysozyme was recovered if only CAPS buffer was used to dissolve the precipitate The reason may be that only changing pH conditions was not sufficient to break the protein-surfactant complex. Other factors like the effect of KCl and ethanol concentration could also play a role in weakening the protein-surfactant complex (Fig. 6.7).

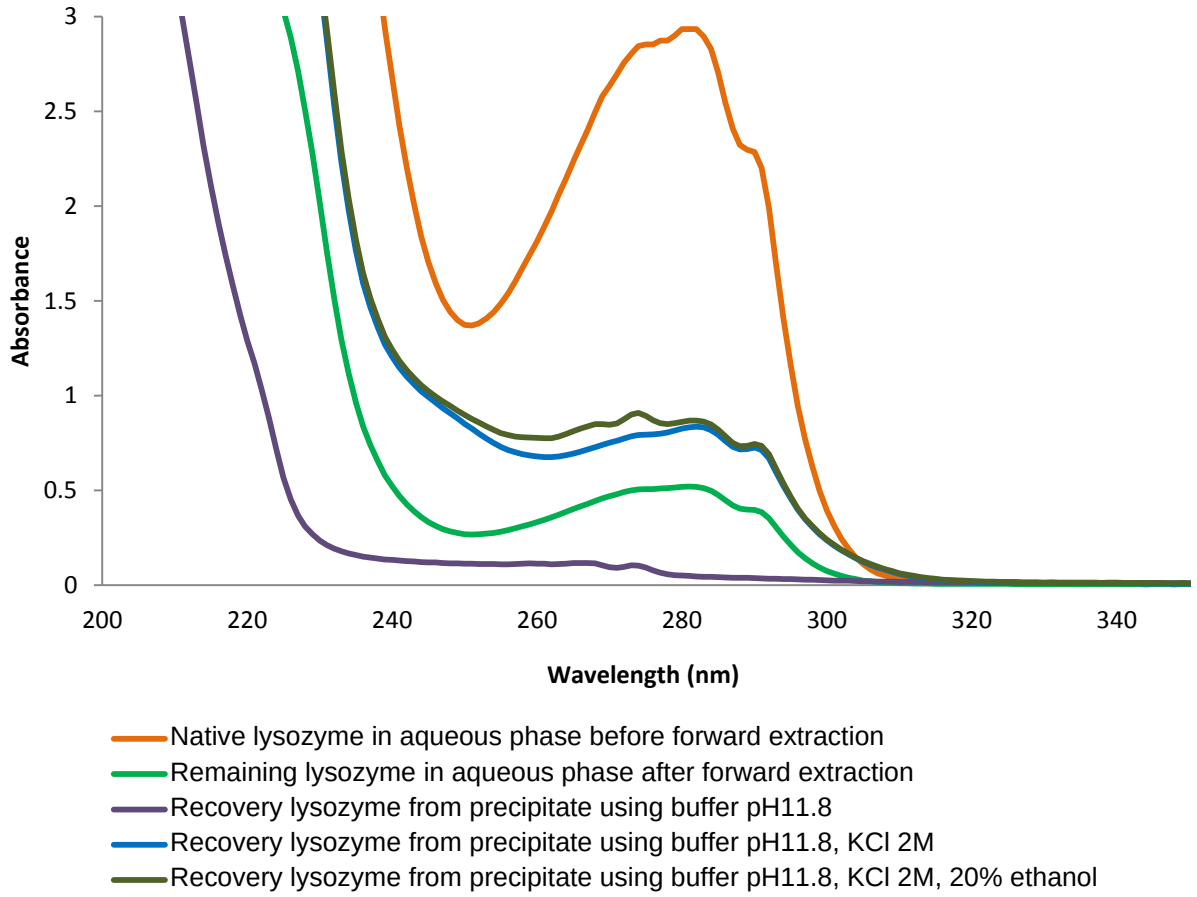

Figure 6.7: Recovered lysozyme from precipitate of L61/AOT/lysozyme mixture at pH 11.8 and in the presence of ethanol.

The data in Fig. 6.8 shows the effect of ethanol and KCl on the recovery of lysozyme from the precipitate at pH 11.8. High KCl concentration assisted the recovery of lysozyme by breaking the L61/AOT/protein complex. 50 % lysozyme was recovered from the precipitate using high

KCl conditions (2M). Also in the presence of 20% ethanol, without KCl, more than 80% lysozyme was recovered from the precipitate.

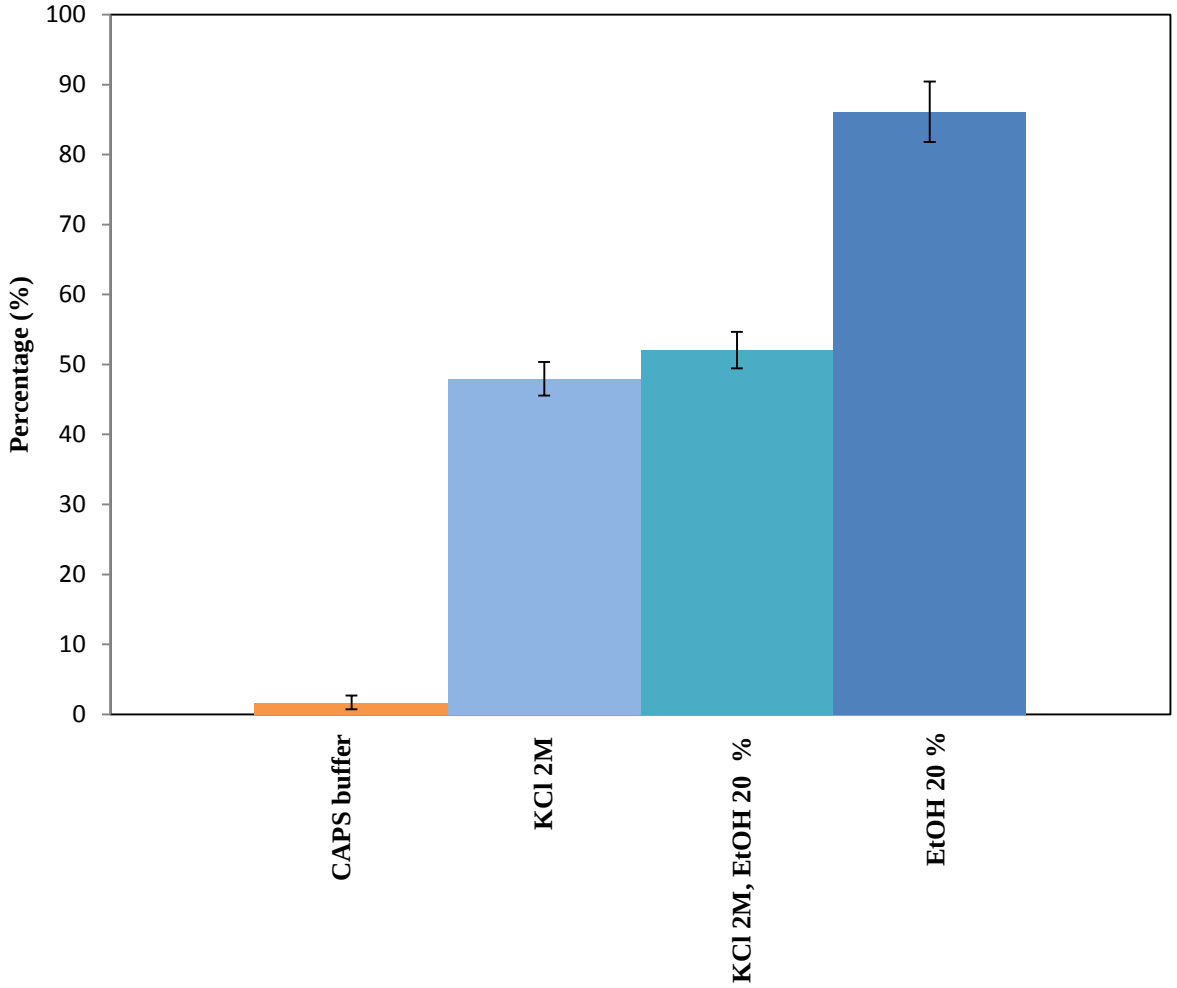

Figure 6.8: Recovered lysozyme from precipitate of L61/AOT/lysozyme at pH 11.8.

In conclusion it can be said that the recovery of lysozyme was successful in the presence of 20% ethanol (86% recovery) and KCl 2 M (50 % recovery). However, it was imperative to analyse the activity of the recovered lysozyme.

6.2.3.2 Activity studies of recovered lysozyme

CD spectroscopy can be used to analyse the secondary structure of proteins. In this case, CD was used to investigate the conformation of the recovered lysozyme from the precipitate. From UV

spectroscopy analysis it was confirmed that around 50% of lysozyme was recovered at pH 11.8 and KCl 2M, both with or without ethanol 20% (Fig. 6.8).

The recovered lysozyme samples were analysed at 200-280 nm. No measurements were done below 200 nm due to noise (Greenfield, 2006). In addition, CD is the method for determining the secondary structure of proteins which is based on negative bands at 222 nm and 208 nm for α-helix and β-sheet and 218 nm for β-pleated sheets. The positive bands at 193 nm and 195 nm are for α-helix and β-sheet structures respectively, however analyses at this wavelength range may lead to an overlap of the spectrum making the analysis difficult (Greenfield, 2006).

The data in Fig. 6.9 shows the CD spectrum of both the native and recovered lysozyme. It can be observed that compared to native lysozyme at pH 7 and pH 11.8, recovered lysozyme in the presence of CAPS buffer at pH 11.8 and KCl 2M maintained its conformation. The recovered lysozyme in the presence of 20 % ethanol completely lost its confirmation, which can be detected by the decrease in the signal (the violet line). The recovered lysozyme from the precipitate using 0.1 M AOT/*p*-xylene solution also lost its secondary structure which is represented as a red dotted line in Fig. 7.9. However the lysozyme recovered from 5 mM AOT/*p*-xylene RM kept its structure and activity (section 4.6). The reason could be that at 0.1 mM (which is below the CMC value), AOT does not form RM and therefore has no aqueous pool for hosting lysozyme inside the ORP. This leads to the direct contact of lysozyme with the non-polar solvent resulting in its denaturation. These results stress the importance of RM in protein extraction as a safe compartment in maintaining the biological activity of proteins. Another conclusion that can be drawn from the results in Fig. 6.9 is that 20% alcohol has the ability of denature lysozyme but the activity of lysozyme can be maintained at high pH and salt concentration.

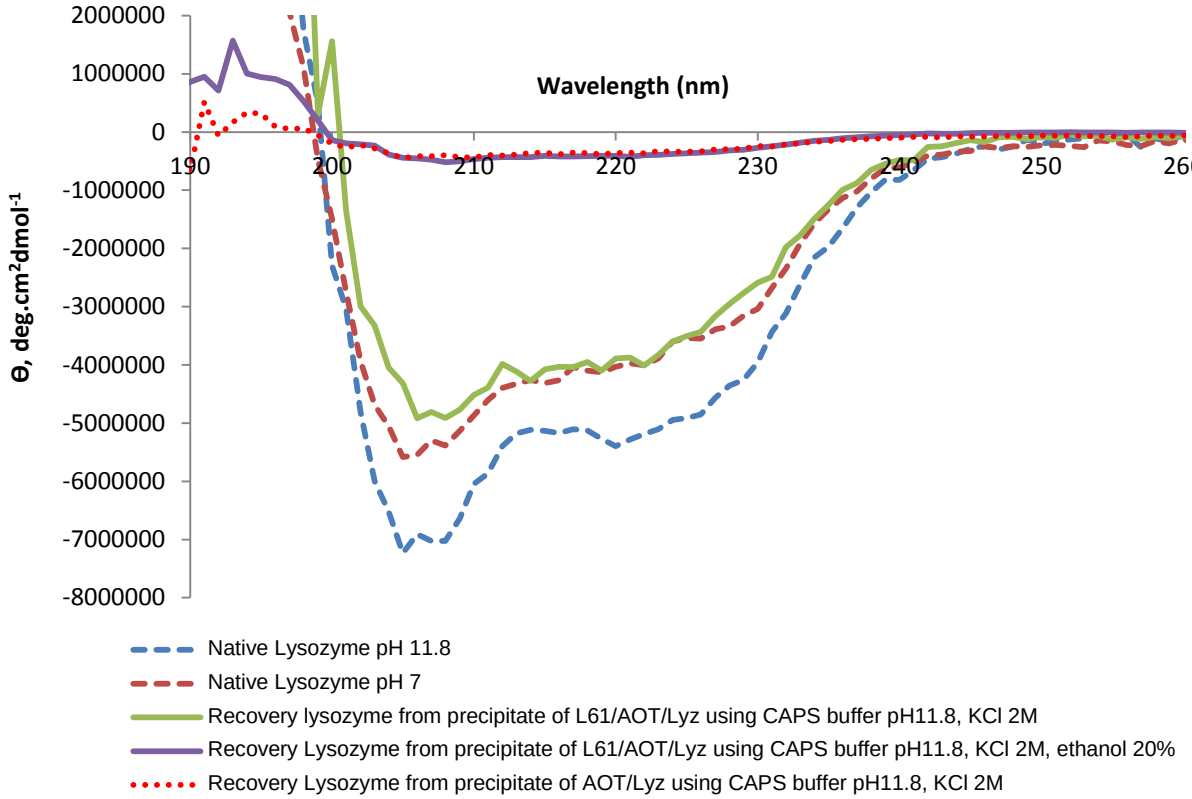

Figure 6.9: CD spectrum of native and recovered lysozyme from precipitate.

The mixed L61/AOT/*p*-xylene RMS with 5 mM L61 and 0.1 mM AOT could not extract proteins into the reverse micellar phase, but resulted in precipitation. It was therefore important to investigate the effect of higher AOT concentration such as 0.2, 0.4 and 0.5 mM by keeping the concentration of L61 constant at 5 mM. Lysozyme was recovered from the precipitate using CAPS buffer at pH 11.8 and 2 M KCl. The percentage of recovered lysozyme was shown in Fig. 7.10. As long as the AOT concentration is kept below and far away from the CMC value of AOT/*p*-xylene RM (1.3 mM), the increase in AOT concentration (0-0.4 mM) leads to increased recovery of lysozyme from the precipitate (6 % to 61 %). However, at 0.5 mM AOT the percentage recovery of lysozyme decreased to 45 %. This decrease could not be explained and needs further investigation in the future.

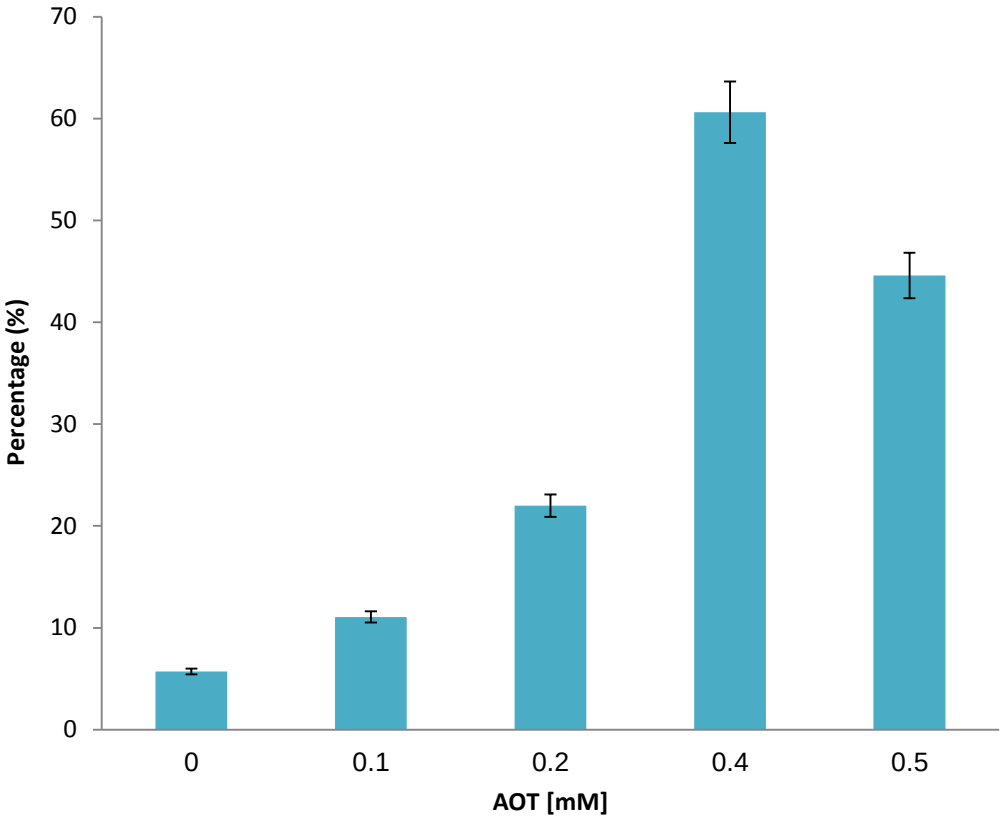

Figure 6.10: Recovered lysozyme from precipitate of L61/AOT/lysozyme using CAPS buffer pH 11.8, 2 M KCl.

Thus it can be concluded that the presence of AOT could not only help pluronics L61 RMS in lysozyme uptake from the AQP into the reverse micellar phase but it also resulted in the precipitation of lysozyme at the interface as a surfactant-protein complex. The advantage of this precipitation was that lysozyme would be recovered from it without losing its biological activity. This can be confirmed from the CD spectrum shown in Fig. 6.11. This precipitation technique could be used in the future as a new method of protein recovery from complex biological systems such as fermentation broth or inclusion bodies. Also the technique uses a nontoxic biocompatible surfactant L61 and the amount of AOT is very low in comparison with other mixed micelle systems.

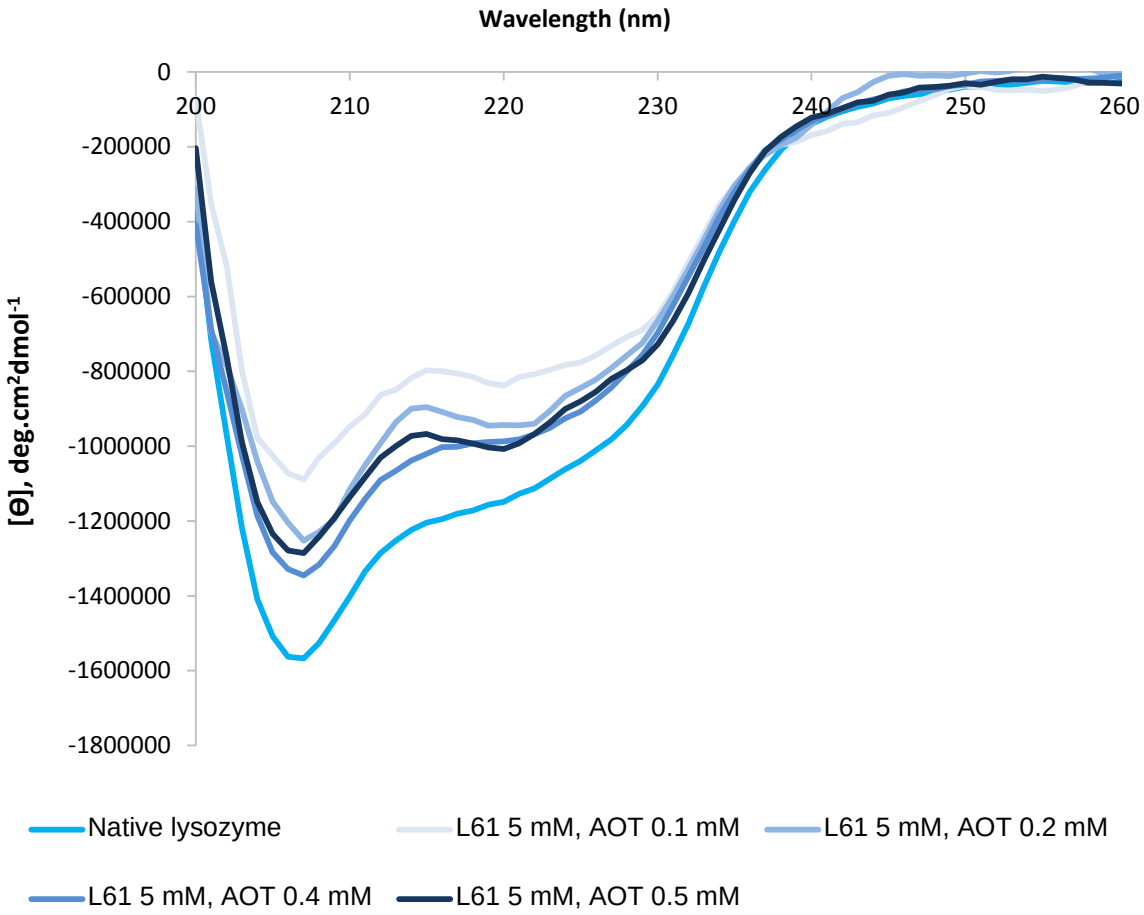

Figure 6.11: CD spectrum of native and recovered lysozyme from precipitate.

The data in Fig. 6.11 shows the CD spectrum of recovered lysozyme from precipitate at different co-surfactant (AOT) concentration as compared with the native CD spectrum. The intensity of the spectrum increased when the AOT concentration increased from 0.1 to 0.5 mM. The shape of the spectrum is quite similar to CD spectrum of native lysozyme. This result can therefore conclude that the secondary structure of recovered lysozyme from the precipitate at different AOT concentrations is maintained. In addition, the activity of recovery lysozyme was tested using Micrococcus *lysodeikticus* cell following the protocol in section 2.3.6.

Table 6.1: Activity recovered of lysozyme from precipitate of L61/AOT/lysozyme at interface after lysozyme FE using L61/AOT/*p*-xylene mixed RMS.

Samples	Enzyme activity (units/ml)	Remaining activity (%)
Native lysozyme (1mg/ml) in phosphate buffer pH 11.8, KCl 2M	206	100
AOT 0.1 mM	136	66
AOT 0.2 mM	131	64
AOT 0.4 mM	104	50
AOT 0.5 mM	119	58

From Tab. 6.1, it can be seen that the recovered lysozyme kept around 50-66 % of its activity in comparison with native lysozyme. This also suggests that the biological activity of proteins cannot be measured by the CD spectrum alone.

6.3 Lysozyme extraction with AOT as the main surfactant

In section 6.2, mixed L61/AOT/*p*-xylene RM was investigated with L61 as the main surfactant. The results concluded that when L61 was the main surfactant, lysozyme could not be forward extracted into the ORP but it precipitated out as a complex of L61/AOT/lysozyme at the interface. Recovered lysozyme from the precipitate kept their secondary structure with around 50-52 % of their activity. It was therefore, necessary to investigate the extraction of lysozyme in the reverse order, which was by keeping AOT as the main surfactant and L61 as the co-surfactant. AOT/*p*-xylene RM could extract around 77 % lysozyme at 20 mM and 82 % at 50 mM on its own. The back extracted lysozyme also kept their secondary structure with 50-72 % activity. So, it would be interesting to see the effect of L61 in the AOT/*p*-xylene system. Almost all extraction conditions were kept similar to the AOT/*p*-xylene RMS. The FE was investigated at pH 8 and 0.1 M KCl whereas, the BE was at pH 11.8 and 2 M KCl.

6.3.1 Extraction efficiency

The AOT concentration selected was 0.5, 1, 5, and 10 mM along with varying concentration of L61 (0, 0.1, 0.5, 1 and 5 mM). L61 concentration was always kept lower than the AOT concentration in all experiments. The reason for this selection was based on previous results where AOT/*p*-xylene RM could only back extract 41-56 % lysozyme (section 3.5) at 5 and 10 mM AOT concentration. From 20 mM AOT concentration, the extraction efficiency was quite high (80-92 %). Therefore, choosing low AOT concentration can explain the effect of L61 as a co-surfactant in a mixed reverse micelle AOT/L61/*p*-xylene system. In addition, the CMC of AOT/*p*-xylene RM was around 1.3 mM (section 3.2), so choosing an AOT concentration of 0.5 mM and 1 mM can help determine the effect of lysozyme extraction near the CMC.

The data in Fig. 6.12 showed the effect of 5 mM and 10 mM AOT concentration with varying L61 concentration on the forward and BE of lysozyme. In case of 10 mM AOT the presence of L61 decreased the FE efficiency of lysozyme between 79-87% as compared to 95% (without L61). However, the lysozyme BE efficiency increased surprisingly from 41.22 % (without L61) to 45.54-88.04 % when L61 concentration increased from 0.1 to 5 mM. The reason could be during FE L61 being a non-ionic surfactant causes the decrease in electrostatic interaction between AOT RM and lysozyme, thus hindering the transfer of lysozyme from the AQP to the ORP as compared to AOT/*p*-xylene RM its own. Contrary to this because of reduced electrostatic interaction, it is easier for the lysozyme to be transferred from the ORP to the AQP during BE. Similar phenomenon has been reported when AOT was mixed with non-ionic surfactants such as Tween 85 and Span 60 (Yamada, 1994; Liu, 1998; Liu, 2008). In the case of 5 mM AOT there was no significant difference in the percentage of forward and BE of lysozyme in the presence of L61. (Fig. 6.12).

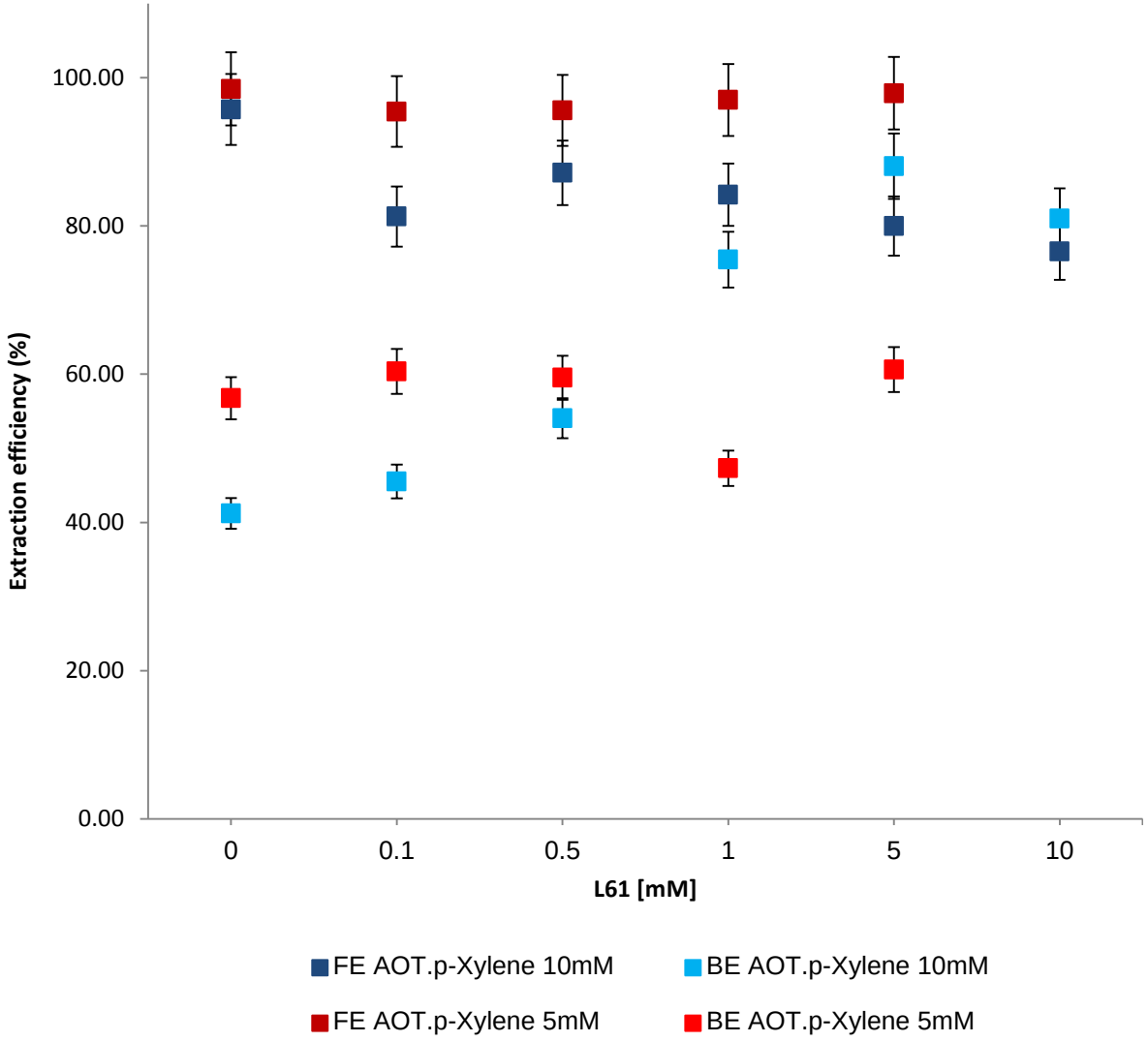

Figure 6.12: Lysozyme extraction using mixed AOT/L61/*p*-xylene RM with varying L61concentration with 5 mM and 10 mM AOT concentration

At lower AOT concentration (0.5 mM and 1 mM), lysozyme FE was very high, 84-89 % and 98 %, respectively, however, no BE was observed. An interesting observation was the formation of considerable precipitate at the interface. This precipitate was further analysed by dissolving into CAPS buffer (pH 12.8, 2 M KCl with and without 20 % ethanol. The data in Fig. 6.13 shows the percentage recovery of lysozyme from the precipitate which was almost similar to the FE efficiency (79-84 %). This phenomenon was also reported by Cheng and Stuckey (Cheng, 2012) where they concluded that below the CMC, surfactants act as a precipitating ligand to form an insoluble protein-surfactant complex.

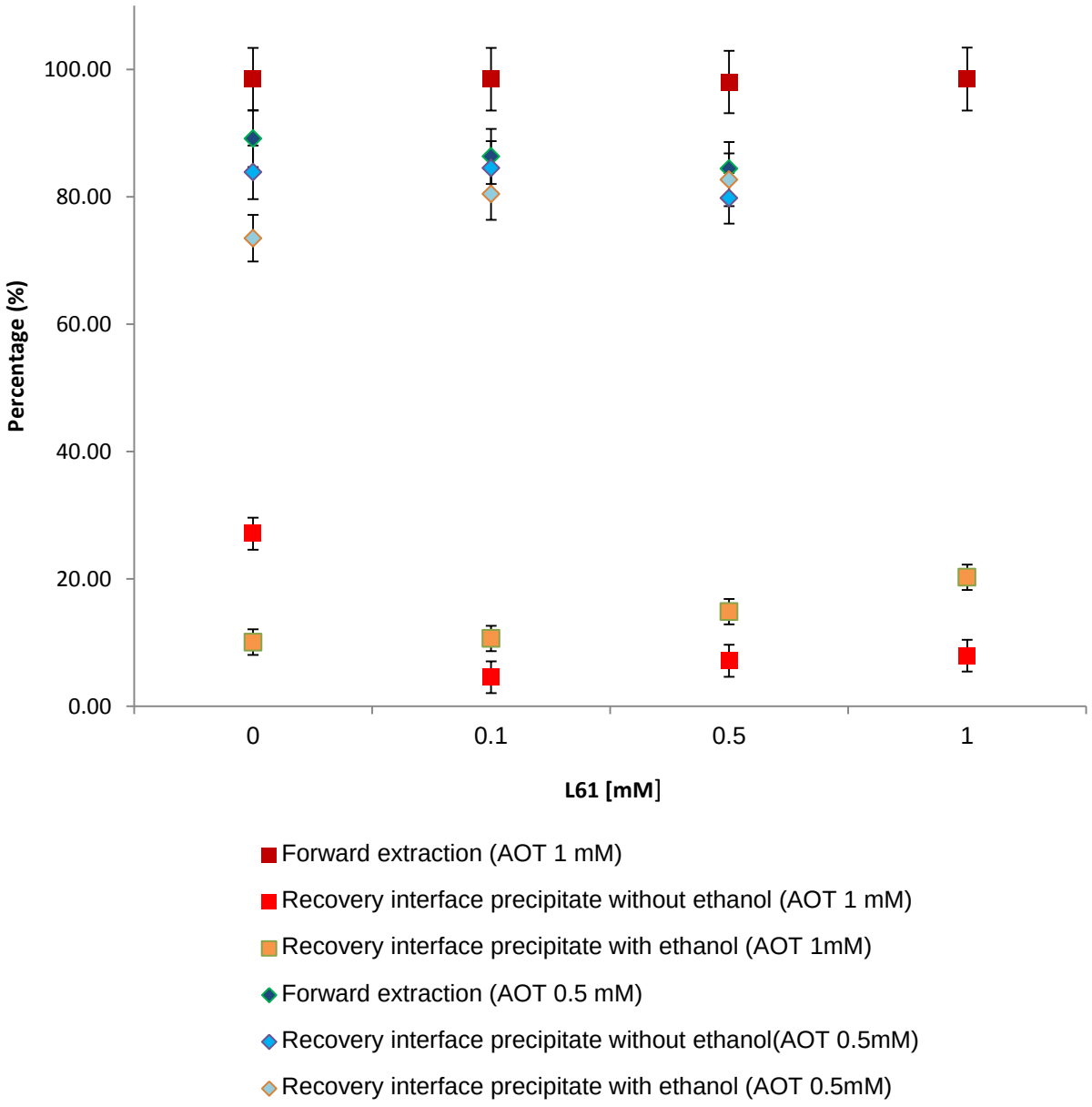

Figure 6.13: Recovered lysozyme from precipitate using mixed AOT/L61/*p*-xylene RM with varying L61concentration and 20 % ethanol.

Unfortunately, this did not happen in case of 1 mM AOT as only 27 % (without L61) and 4-7 % (with L61) lysozyme was recovered from the precipitate. The reason could be that at 1 mM AOT concentration which is close to AOT/*p*-xylene CMC (1.3 mM), the number of RM may be limited as the systems would consist of a mixture of RM and surfactants in monomeric or another form. This results in a complex reverse micelle system leading to both precipitation and extraction of lysozyme. More experiments need to be conducted in order to investigate this phenomenon in greater detail.

In summary, it can be said that the presence of non-ionic surfactant pluronics L61 in AOT/*p*-xylene RMS can improve lysozyme extraction efficiency by decreasing the strong electrostatic interaction between AOT RM and lysozyme. Above the CMC, the optimal ratio for AOT:L61 was 2:1 (AOT 10 mM, L61 5 mM) for mixed anionic-non-ionic AOT/L61 reverse micelle in *p*-xylene. At concentrations below the CMC, all lysozyme was precipitated out and L61 did not play any major role as there was no significant difference in the percentage of lysozyme recovered with and without L61.

6.3.2 Structure and activity of recovered lysozyme

Lysozyme recovered after BE under optimal conditions of 10 mM AOT and varying concentration of L61 (0-5mM) and the recovered lysozyme from the precipitate of 0.05 mM AOT and varying concentration of L61 (0-0.5 mM) were analysed by CD spectroscopy to obtain the secondary structure and the samples were also subjected to the *Micrococcus lysodeikticus* cell assay to determine the activity of lysozyme.

The data in Fig. 6.14 shows the CD spectrum of recovered lysozyme from mixed AOT/L61/*p*-xylene RM has a similar shape to one obtained with native lysozyme spectrum although the intensity was a bit weaker. Therefore, it can be seen that the presence of L61 did not affect the secondary structure of recovered lysozyme but assisted the BE efficiency because of decreasing electrostatic interaction between AOT and lysozyme.

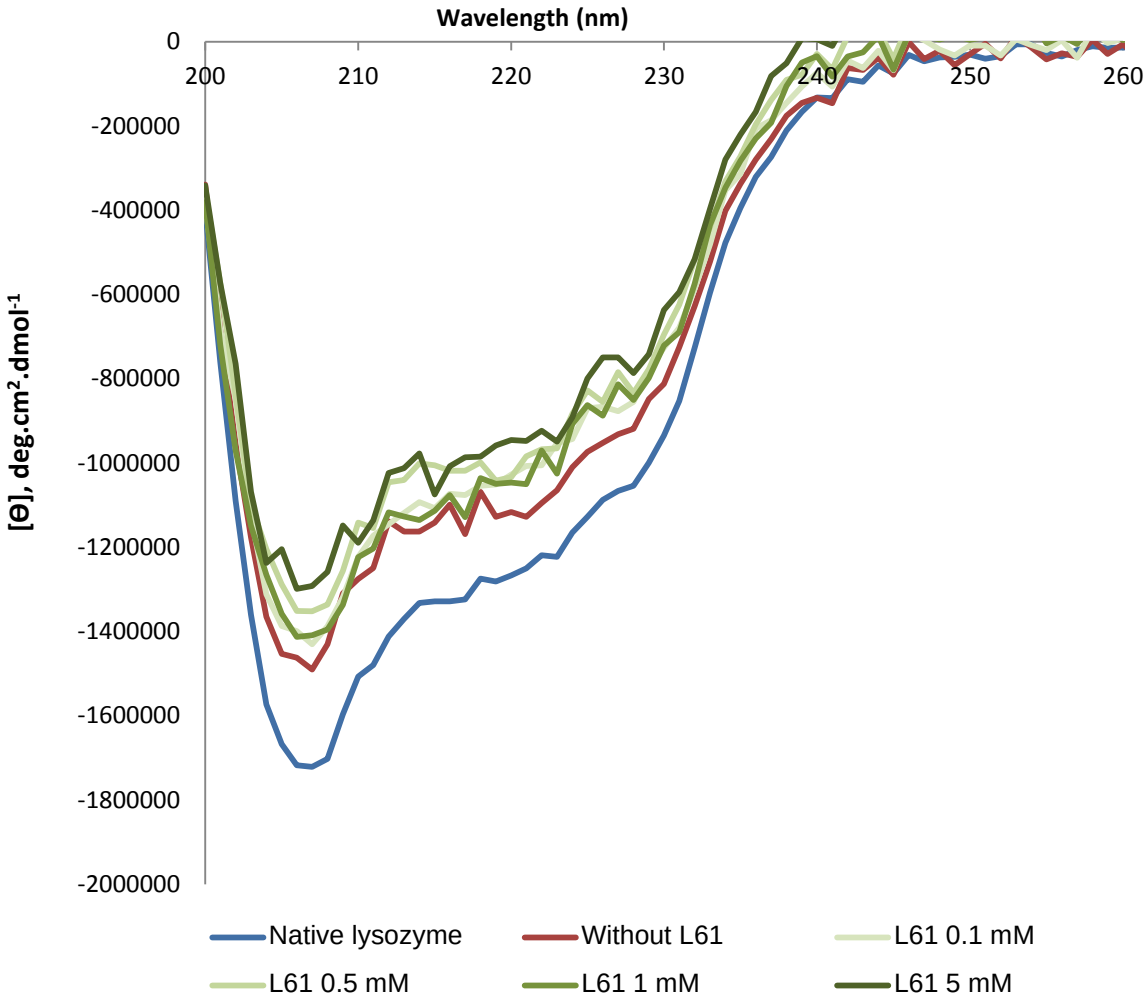

Figure 6.14: CD spectrum of recovered lysozyme from BE using mixed anionic-non-ionic AOT/L61/*p*-xylene RMS. AOT [10 mM] L61 0-5 mM.

The data in Fig. 6.15 shows the CD spectrum of recovered lysozyme from the precipitate as compared to native lysozyme. It was clearly seen from the figure that the presence of L61 assisted in maintaining the secondary structure of lysozyme. The shape of the CD spectrum of recovered lysozyme and native lysozyme was almost similar. However, there was a change in the intensity of the peaks as the intensity increased with the increase in L61 concentration from 0.1-0.5 mM. The intensity was the lowest when L61 was absent. From the above results it can be concluded that L61 plays an important role in maintaining the secondary structure of lysozyme and reduction in electrostatic interaction seems to be the major factor.

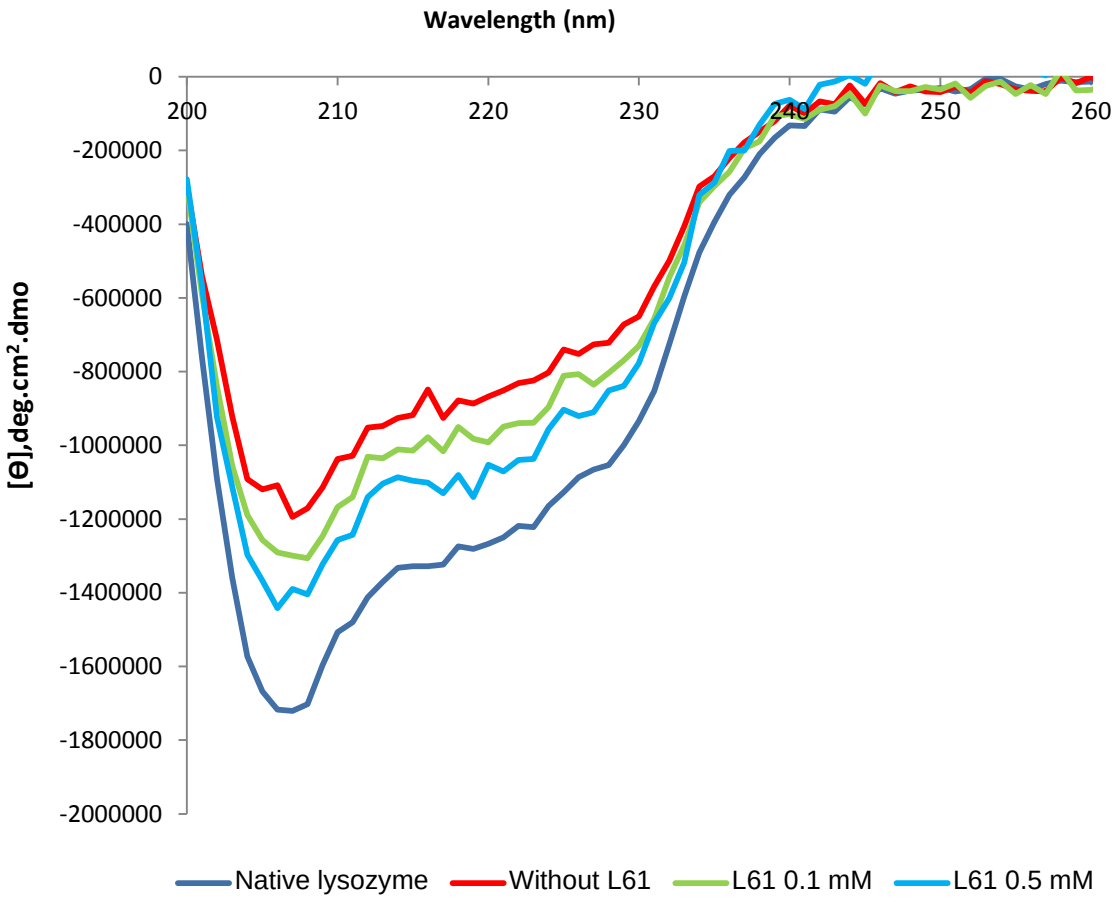

Figure 6.15: CD spectrum of recovered lysozyme from precipitate using mixed AOT/L61/*p*-xylene system. The concentration of AOT kept constant as 0.5 mM. The concentration of L61 is changing from 0 to 0.5 mM.

The CD data obtained was further subjected to analyses using the CDNN software for all recovered lysozyme samples. Similar trend were observed between recovered lysozyme from mixed AOT/L61/*p*-xylene RM with 10 mM AOT and 0-5 mM L61 concentration. The percentage of α-helix changed only slightly at around 3 %. There was no much difference between the percentage of beta-turn, parallel and antiparallel *β*-sheet between the recovered lysozyme sample and native lysozyme. The amount of random coil increased slightly (maximum 3 %) with the increase in L61 concentration (Fig. 6.16). For recovery lysozyme from precipitate of AOT/L61/lysozyme, the presence of L61 helped to keep the percentage of secondary structure elements close to native lysozyme by increasing the quantities of *α*-helix and decreasing the percentage of random coil with the increase in L61 concentration (Fig. 6.17).

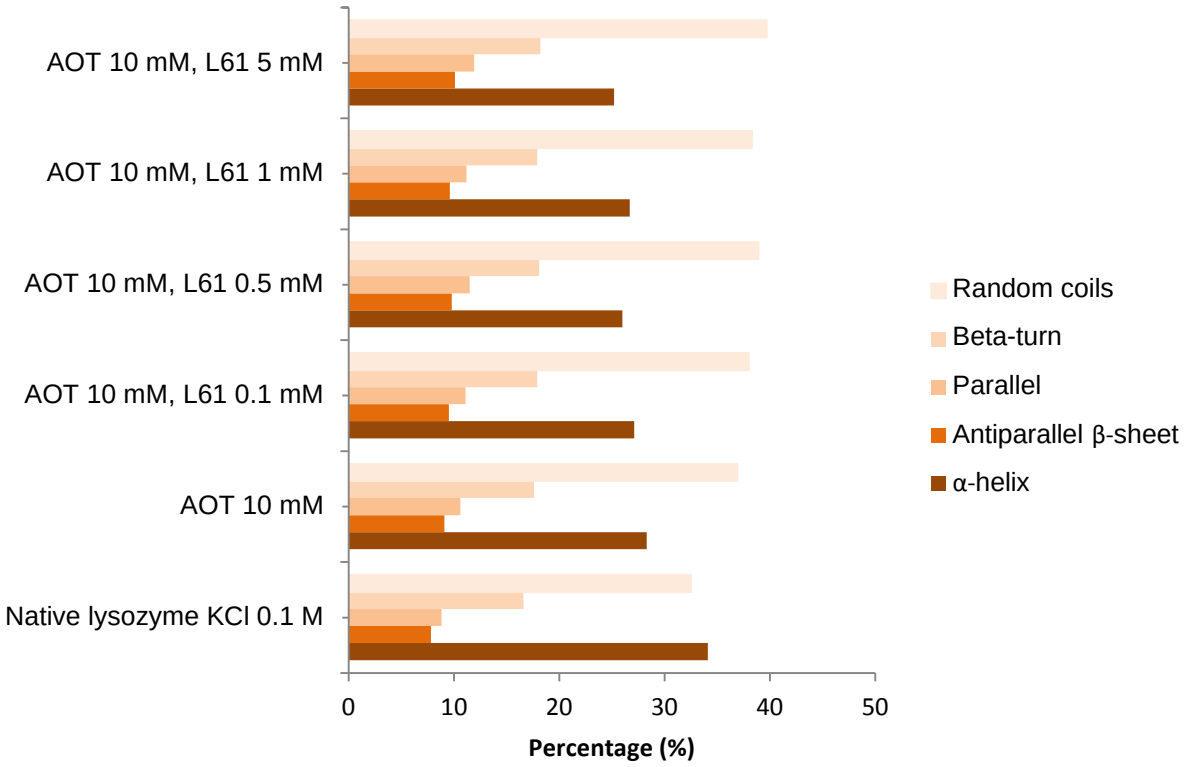

Figure 6.16: CDNN result of recovered lysozyme using mixed AOT/L61/*p*-xylene RMS with 10 mM AOT and varying L61 concentration.

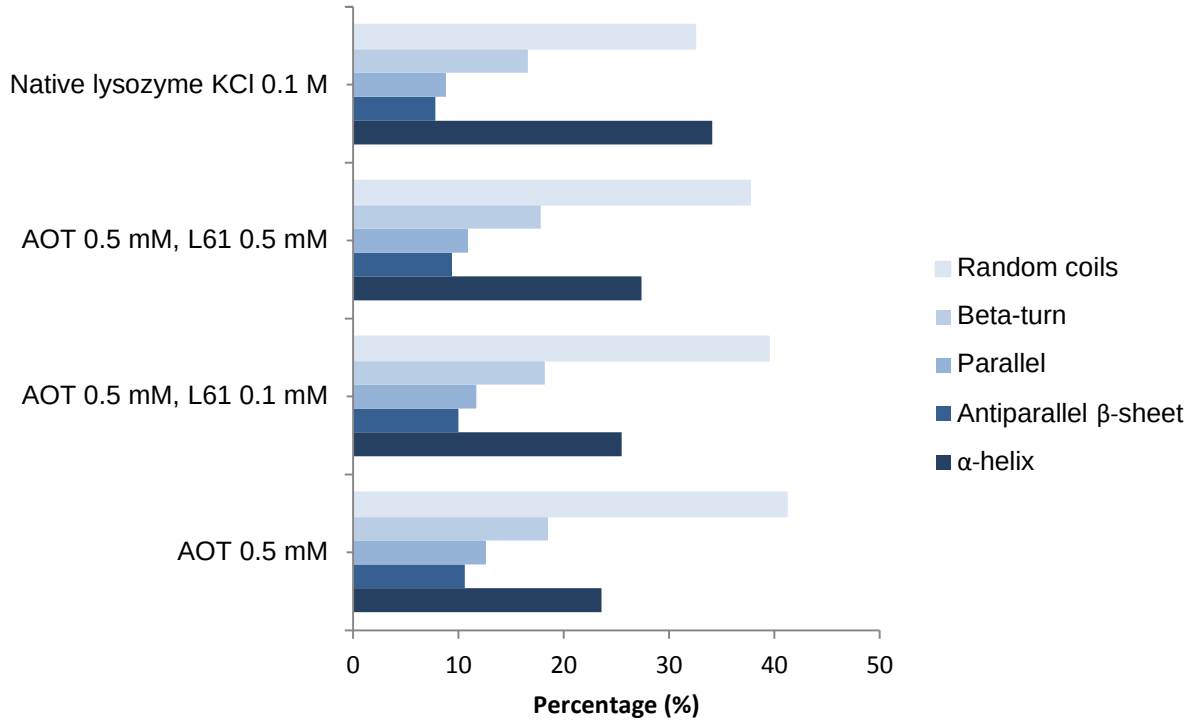

Figure 6.17: CDNN result of recovered lysozyme from precipitate using mixed AOT/L61/*p*-xylene RMS with 0.5 mM AOT and varying L61 concentration.

The activity of recovered lysozyme was tested using the *Micrococcus lysodeikticus* cell assay following the protocol in section 3.3.6. Table 6.2 shows the activity of the recovered lysozyme calculated from Eq. 4.

Table 6.2: Activity of recovered lysozyme from mixed AOT/L61/*p*-xylene RMS

Samples	Enzyme activity (units/ml)	Remaining activity (%)
Native lysozyme (1mg/ml) in phosphate buffer pH 11.8, KCl 2M	206	100
Recovery lysozyme from BE using AOT/L61/*p*-xylene RM (AOT 10 mM)		
Without L61	100	49
L61 0.1 mM	117	57
L61 0.5 mM	110	53
L61 1 mM	80	39
L61 5 mM	80	39
Recovery lysozyme from precipitate using AOT/L61/*p*-xylene surfactants (AOT 0.5 mM)		
Without L61	25	12
L61 0.1 mM	117	57
L61 0.5 mM	105	51

Recovered lysozyme from BE using mixed AOT/L61/*p*-xylene RM kept 39% of its activity when 1mM and 5 mM L61 was present in the mixed micelle system. When the L61 concentrations were 0.5 mM and 0.1 mM, the activity increased to 53 % and 57 %, respectively. For recovered lysozyme from precipitate in the absence of L61, only 12% activity was observed as compared to 51-57 % activity in the presence of L61. This establishes the hypothesis that the presence of L61 not only helps to improve the extraction efficiency but also plays a substantial role in maintaining the protein activity. However, this hypothesis only works at low L61concentration or in other words, the ratio AOT: L61 should be 20:1 or below (AOT 10 mM and L61 0.5 or 0.1 mM). If the ratio changes to 10:1 and further (L61 1 ad 5 mM) the activity of recovery lysozyme will decrease as compared to recovered lysozyme from AOT/*p*-xylene RM.

Summary:

It can be concluded that mixed L61/AOT/*p*-xylene RMS was successful in lysozyme extraction and precipitation. The presence of AOT as co-surfactant could not help L61 extract lysozyme but precipitated it at the interface after FE. Recovered lysozyme from the precipitate maintained their secondary structure and 50-66 % their activity. This result can establish a new method in extraction and purification protein by precipitating them using mixed L61/AOT/*p*-xylene RMS, with the ratio between L61 and AOT as 12.5:1 or below.

Another advantage of the mixed RMS with AOT as the main surfactant and L61 as a co-surfactant was that the presence of L61 not only helps to improve the extraction efficiency by 50 % but also plays a substantial role in maintaining the protein activity (48-56 %) based on reduction in electrostatic interaction when the ratio of AOT: L61 is 20:1 or below. These results established new mixed RMS in protein extraction that has not been reported before. In addition, at low AOT concentration, the presence of L6 helped to maintain the secondary structure and activity of recovered lysozyme from the precipitate at the interface. These studies thus contribute to new methods of protein extraction and precipitation using mixed non-ionic-anionic surfactant in an aromatic solvent.

7. CONCLUSION

Objective 1: AOT in p-xylene RMS and lysozyme extraction in compare with AOT in isooctane RMS.

- From the CMC values determined, it was established that AOT could easily form RM (lower concentration of AOT required) in non-aromatic solvent (isooctane) than in aromatic solvent (*p*-xylene) (0.45-0.7 mM and 1.2-1.3 mM, respectively).
- The AOT/*p*-xylene RM were smaller in size (3.7 nm) as compared to AOT/isooctane RM (5.6 nm) resulting in lower lysozyme extraction efficiency at low AOT concentration (<50 mM).
- Recovered lysozyme from AOT/*p*-xylene RMS could maintain functional integrity to a greater extent in comparison to AOT/isooctane.
- Thus it can be established from this work that the new AOT RMS in aromatic solvent though required a higher AOT concentration for better extraction as compared to AOT in non-aromatic solvents, had the ability to better maintain the functional integrity of the recovered protein which is one of the major requirements in protein extraction.

Objective 2: CTAB in mixture of 1-bromooctane, 1-hexanol and petroleum ether reverse micelles system and its application on the extraction of BSA and lysozyme.

- The cationic CTAB RM was formed in a 1-bromooctane/1-hexanol/petroleum ether system at 1.3-1.5 mM. BSA was successfully extracted by this RMS and the secondary structure of BSA was maintained in the presence of KCl.
- After the successful transfer of lysozyme from the aqueous phase to the RM phase, the protein could not be back extracted to the aqueous phase likely due to strong interaction

of lysozyme and CTAB RM and which was situated deep inside the core of the CTAB RMS.

Objective 3: Triblock copolymer pluronics in p-xylene reverse micelles system and its application in lysozyme extraction and refolding.

- The ability of pluronics L61 and L81 to form RM in *p*-xylene was established and their CMC was determined to be 0.09-0.12 mM and 0.07 mM, respectively. This result confirmed that higher molecular weight surfactants (2800 g/mole) can easily form RM than surfactants with lower molecular weight (2000 g/mole).
- L61 was chosen for further study in lysozyme extraction due to clearer organic phase during reverse micelles forming process. However, no extraction was observed because of weak electrostatic interaction between lysozyme and the non-ionic L61/*p*-xylene RMS. The failure of trapping lysozyme inside L61/*p*-xylene RMS leads to impossible refolding task as the next step.

Objective 4: Mixed reverse micelles system of ionic surfactant (AOT, CTAB) and pluronics and lysozyme extraction and refolding.

- The presence of AOT as a co-surfactant in the L61 RMS did not improve the extraction and led to the precipitation of lysozyme.
- Recovered lysozyme from the precipitate was able to keep its secondary structure and activity intact. This work thus establishes a new method in extraction and purification of proteins by precipitation in a mixed L61/AOT/*p*-xylene RMS, with a 12.5:1or below ratio of L61 and AOT. From this result, refolding lysozyme using L61/AOT/*p*-xylene RMS as precipitate agent could be considered in future work with more studies.
- The presence of L61 as a co-surfactant for AOT/*p*-xylene RMS not only improved the extraction efficiency by 50 %, but also played a substantial role in maintaining protein

activity (48-56 %) based on the reduction in electrostatic interaction when the ratio of AOT:L61 was 20:1 or below. This work therefore led to the invention of new mixed RMS in protein extraction that has not been reported before.

- It was also confirmed that at AOT concentration below CMC, the presence of L61 helped to maintain the secondary structure and activity of the recovered lysozyme from the precipitate formed at the interface.
- The use of pluronics as a non-ionic surfactant in a RMS for protein extraction and precipitation will be a very useful tool in the downstream processing of biomolecules in the future. Based on the limited time, the aim of using mixed AOT/L61/*p*-xylene RMS was noted as future work.
- CTAB could not dissolve inside *p*-xylene, so the aim of forming mixed L61/CTAB/*p*-xylene RMS was fail.

8.FUTURE WORK

8.1 Shape, size and internal structure of AOT, CTAB, L61, L81 and mixed AOT/L61 reverse micelles:

It would be very useful to study the shape, size and structure of the new RMS investigated in this work. This can be achieved by small-angle X-ray scattering (SAXS) or small angle neutron scattering studies (SANS). This information could lead to more insights into the mechanism of the RM formation using mixed surfactant systems. Therefore, the effect of environmental conditions such as solvent, salt, pH, etc. could further be understood in order to set up stable RMS.

8.2 Lysozyme extraction by L81/*p*-xylene RMS

The water content results indicated that L81 could form bigger RM with W_0 value of 34 at 0.2 mM. However, due to the turbidity of the organic phase, more work is needed to be done in order to resolve this issue. If it is success, L81/*p*-xylene RMS also has the potential to be an efficient RMS for protein extraction.

8.3 Lysozyme extraction by mixed cationic CTAB and non-ionic L61 reverse micellar system:

It would be interesting to investigate protein extraction from a mixed RM consisting of a cationic surfactant CTAB and L61. The failure of CTAB RM to recovery lysozyme could be improved with the presence of L61 in the system. New solvent should be studied in using for this mixed RMS instead of *p*-xylene.

8.4 Protein refolding by mixed AOT/L61 reverse micelles:

Lysozyme was successfully extracted in a mixed AOT/L61/*p*-xylene RMS without losing secondary structure and activity. Therefore, studies in protein refolding using this system would be promising as there is a great need for new protein refolding agents especially for recovery of proteins in active form from inclusion bodies.

8.5 Protein refolding by precipitation in L61 RMS in the presence of AOT as a co-surfactant:

The presence of AOT did not improve L61 RMS the extraction of lysozyme but led to the precipitation of proteins at the interface of aqueous phase and organic phase. This is interesting as the recovered lysozyme from the precipitate was able to main the activity and its secondary structure. This system could therefore find application in protein refolding by the precipitation technique.

9.BIBLIOGRAPHY

Abuin, E. L. (2002). Solubilization in AOT-water reverse mielles. Effect of the external solvent. *Langmuir*, 8340-8344.

Aires-Barros, M. a. (1991). Selective separation and purification of two lipases form chromobacterium viscosum using AOT reversed micelles. *Biotechnology and Bioengineering, 38*, 1302-1307.

Alexandridis, P. A. (1997). Effect of solvent quality on reverse micelle formation and water solubilization by poly(ethylene oxide)/Poly(propylene oxide) and poly(ethylene oxide)/poly(butylene oxide) block copolymers in xylene. *Journal of colloid and interface science*, 166-173.

Alexandridis, P. H. (1995). Review: Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer surfactats in aqueous solution and at interfaces: thermodunamics, structure, dynamics and modeling. . *Colloids and surfaces A*, 1-46.

Alexandridis, P. L. (2000). Self assembly and applications. In P. L. Alexandridis, *Amphiphilic block copolymers* (pp. 1-435). Amsterdam: Elservier.

Amico, P. D. (1995). Infrared Absorption and water structure in Aerosol OT reverse micelles. *IL Nuovo Cimento*, 1053-1065.

Andrew R. (1991). *New technologies improve biotech's downstream processing.* The Scientist Magazine.

Andrews, B. P. (1993). Effect of pH and ionic strength on the partitioning of four proteins in reversed micelles systems. *Biotechnol. Bioeng*, 1052-1058.

Attwood, D. F. (2008). Surfactant. In D. F. Attwood, *Physical pharmacy* (pp. 43-62). London: Pharmaceutical Press.

Axelsson, I. (1978). Characterization of protein and other macromolecules by agarose gel chromatography. *Journal of Chromatography A*, 21-32.

Ayala, G. K. (1992). Protein extraction and activity in reverse micelles of a non-ionic detergent. *Biotechnol. Bioeng.*, 806-814.

Battistel, E. (1988). Thermodynamic study of global protein stability in microemulsion. *Jounal of Physical Chemistry*, 50-62.

Bellocq, A. (1998). Phase equilibria of polymer-containing microemilsions. *Langmuir*, 3730-3739.

Belter, P. C. (1988). *Bioseparation.* New York: John Wiley and Sons.

Biotechnology. (2014, 11 18). Retrieved from Koch Modular Process Systems: http://modularprocess.com/liquid-extraction/industrial-applications/biotechnology/

Blake, C. K. (1965). Structure of hen egg-white lysozyme: a three dimesional fourier synthesis at 2 Å resolution. *Nature*, 757-761.

Bluestein, B. H. (1982). *Amphoteric surfactants.* New York: Marcel Dekker.

Bohidar, H. B. (2000). Solubilization of gelatin by water-AOT-isooctane reverse micelles studied by dynamic laser light scattering. *Eur Polym J*, 2463-2470.

Brandani, V. G. (1996). Recovery of alpha-amylase extracted by reverse micelles. *Process Biochemistry*(31), 125-128.

Braun, P. L. (2002). Proteome-scale purification of human proteins from bacteria. *Proc Natl Acad Sci USA*, 2654-2659.

Broering, J. B. (2005). Evaluation of Hofmeister effects on the kinetic stability of protein. *J. Phys. Chem. B*, 20612-9.

Buchacher, A. a. (2006). *Biotechnol. J.*, 148.

Camarinha-Vicente, M. A.-B. (1990). Purification of chromobacterium viscosum lipases using reversed micelles. *Biotechnol Techn*, 137-142.

Capaldi, A. K. (2002). Im7 folding mechanism: misfolding on a path to the native state. *Nat. Struct. Biol., 9*, 209-216.

Carneiro-da-Cuha, M. M.-B. (1996). Liquid-liquid extraction of a recombinant cutinase from fermentation media with AOT reverse micelles. *Bioproc. Eng.*, 151-157.

Carter, D. H. (1994). Structure of serum abumin. *Advances in Protein Chemistry*, 153-203.

Cheng, S. S. (2012). Protein precipitation using an anionic surfactant. *Process Biochemistry*, 712-719.

Choi, J. L. (2004). Secreory and extracellular production of recombinant proteins using Escherichia coli. *Applied microbiology and biotechnology*, 625-635.

Christendat, D. Y. (2000). Structural proteomics of an archaeon. *Bat Struct Biol*, 903-909.

(1998). In Y. Christi, *In Bioseparation and Bioprocessing* (pp. 3-28). London: Wiley-VCH.

Clark, E. (1998). Refolding of recombinant protein. *Curr Opin Biotechnol*, 157-163.

Clarke, K. (2013). Downstream processing. In K. Clarke, *Bioprocess Enginerring. An Introductory Engineering and Life Science Approach* (pp. 209-234). Woodhead Publishing.

Cleland, J. a. (1993). Co-solvent effects on refolding and aggregation. In *Biocatalyst design for stability and specificity* (pp. 151-166). American Chemical Society.

Collins, K. W. (1985). The Hofmeister effect and the behaviour of water at interfaces. *Q. Rev. Biophys*, 323-422.

Constantinescu, D. H. (2010). Protein denaturation by ionic liquids and the Hofmeister series. In N. Plechkove, *In ionic liquids: from knowledge to application* (pp. 107-117). Washington, DC: American Chemical Society.

Crowell, K. (1997). Pharmaceutical application of liquid-liquid extraction. In E. Goldberg, *Handbook of Downstream processing* (pp. 48-69). Chapman & Hall.

Daoud, M. a. (1999). *Soft matter physics.* Springer.

Dobson, C. (2003). Protein folding and disease: a view from the first horizon symposium. *Nature Rev Drug Disc, 2*, 154-160.

Dobson, C. S. (1998). Protein folding: aperspective from theory and experiment. *Angew. Chem. Int. Ed., 37*, 868-893.

Dodsob, C. (2004). Principles of protein folding, misfolding and aggregation. *Seminars in Cell & Developmental Biology*, 3-16.

Dong, R. a. (2010). Complex fluids of poly(oxyethylene) monoalkyl ether non-ionic surfactantant. *Chem. Rev.*, 4978-5022.

Dovyap, Z. B. (2006). Amino acid extraction and mass transfer rate in the reverse micelles system. *Enzyme Microb. Technol.*, 557-562.

Durocher, Y. P. (2002). High-level and high throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cell. *Nucleic Acids Res*, E9.

Ekwall, P. M. (1971). The solution phase with reversed micelles in the cetyl trimethylammonium bromide-hexanol-water system. *J. Colloid Interface Sci*, 266-272.

Ellis, R. (1991). Review: molecular chaperones. *Annu. Rev. Biochem, 60*, 321-347.

Englander, S. M. (2008). Protein folding and misfolding: mechanism and principles. *Quarterly Reviews of Biophysics, 40*(4), 287-326.

Erickson, H. (2009). Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration and electron microscopy. *Biol Proced* , 32-51.

Evansm D.F., W.-s. H. (1994). *The colloidal domain. Where physics, chemistry, biology and technology meet.* New York: Wiley-VCH.

Fang, X. Y. (1999). An experimental study on the relationship between the physical properties of CTAB/hexanol/water reverse micelles and ZrO2-Y2O3 nanoparticles prepared. *J. Colloid Interface Sci*, 242-251.

Frydman, J. (2001). Folding of newly translated proteins in vivo: the role of molecular chaperones. *Annu. Rev. Biochem.*, 603-647.

Gelamo, E. S. (2002). Interaction of bovine (BSA) and human (HSA) serum alumins with ionic surfactats: spectroscopy and modelling. *Biochimica et Biophysica Acta*, 84-99.

Gerhardt, N. D. (2002). Time dependent solubilisation of IgG in AOT-Brine-Isooctane microemulsion: role of Cluster formation. *Biotechnol Bioeng*, 60-72.

Gerhardt, N. D. (2002). Time dependent solubilisation of IgG in AOT-Brine-Isootane microemulsions: role of cluster formation. *Biotechnol Bioeng*, 60-72.

Gerigk, M. B.-N. (2002). Process control for enhanced L-phenylalanine production using different recombinant Escherichia coli. *Biotechnology and Bioengineering*, 746-754.

Gething, M. S. (1992). Protein folding in the cell. *Nature, 355*, 33-45.

Gochman-Hecht, H. a.-P. (2006). Structure modifications of AOT reverse mcielles due to protein incorporation. *J. Colloid Interface Sci*, 276-283.

Goklen, K. a. (1987). Liquid-liquid extraction of low molecular weight proteins by selective solubilization in reversed micelles. *Separation Science and Technology, 22*, 831-841.

Goklen, K. H. (1985). Protein extraction using reverse micelles. *Biotechnology Process*, 69-74.

Guan, Y. L. (1996). Use of aqueous two-phase system in the purification of human interferon-a1 from recombinant Escherichia Coli. *Enzyme Microb. Technol.*, 446-455.

Guan, Y. Z. (1996). Technical aspects of extractive purification of penicillin fermentation broth by aqueous two-phase partitioning. *Sep.Sci.Technol.*, 2589-2597.

Hamley, I. (1998). The physics of block copolymer. In I. Hamley, *The physics of block copolymer* (pp. 131-265). Oxford: Oxford Science Pulication.

Hardesty, B. K. (2001). Folding of a nascent peptide on the ribosome. *Prog Nucleic Acid Res Mol Biol, 66*, 41-66.

Harikrishana, S. S. (2002). Reverse micellar extraction for dowstream processing of protein/enzymes. *Advances in Biochemical Enginering and Biotechnology, 75*, 119-183.

Harikrishna, S. S. (2002). Reverse micellar extraction for downstream processing of proteins/enzymes. *Advances in Biochemical Engineering and Biotechnology, 75*, 119-183.

Harikrishna, S., Srinivas, N., Raghavarao, K., & Karanth, N. (2002). Reverse micellar extraction of downtream processing of proteins/enzymes. *Advances in Biochemical Engineering and Biotechnology, 75*, 119-183.

Harlt, F. H.-H. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*, 1852-1858.

Hart, R. O. (1995). Use of multifactorial analysis to develop aqueous two-phase systems for isolation of nonnative IGF-1. *Bioseparation*, 113-121.

Hartl, F. B.-H. (2011). Molecular chaperones in protein folding and proteostasis. *Nature*, 324-332.

Hasegawa, T. K. (1992). Changes in lipid peroxide levels and activity of reactive oxygen scavenging enzymes in skin, serum and liver follwing UVB irradiation in mice. *Life Science*, 1893-1903.

Hashimoto, Y. O. (1997). Protein refolding by reversed micelles utilizing solid-liquid extraction technique. *Biotechnology and Bioengineering*, 620-623.

Hasmann, F. C. (2003). Optimization of β-xylosidase recovery by reversed micelles using response surface methodology. *Electronic Journal of Biotechnology*, 153-160.

Hebbar, H. R. (2007). Extraction of bovine serum albumin using nanoparticulate reverse micelles. *Process Biochem*, 1602-1608.

Henda, M. G. (2013). Rheological properties and reverse micelles conditions of PEO-PPO-PEO Pluronics F68: effects of temperature and solvent mixtures. *Journal of Polymers*, 1-7.

Hofmeister, F. (1988). *Arch. Exp. Pathol. Pharmakol*, 247-260.

Holmberg, K. J. (2002). *Surfactants and polymers in aqueous solution.* Chichester: Wiley.

Hong, D. L. (2000). Conformation transition and mass transfer in extraction of proteins by AOT-alcohol-isooctane reverse micellar systems. *Journal of Chromatography, 734*, 203-213.

Hong, D. L. (2000). Conformational transition and mass transfer in extraction of proteins by AOT-alcohol-isooctane reverse micellar systems. *Journal of Chromatography*(743), 203-213.

Hsu, M. D. (2005). *Reverse micelles enable strong electrostatic interactions of colloidal particles i nonpolar solvents.* Cambridge, MA 02138, USA: Dept. of Physics and DEAS, Harvard University.

Humphrey, J. (1997). Liquid-liquid extraction. In J. K. Humphrey, *Separation Process Technology* (p. 136). Mc Graw-Hill.

Jarudilokkul, S. P. (1999). Separation and purification of periplasmic cytochrome c553 using reversed mcielles. *Biotechnology Techniques, 13*, 159-163.

Jeon, W. (2010). Retrospective analyses of the bottkeneck in purification of eukaryotic proteins from Escherichia coli as affected by molecular weight, cysteine content and isoelectric point. *BMB rep*, 319-324.

John, R. C. (2001). High pressure refolding of recombinant human growth hormone from insoluble aggregates. Structural transformations, kinetic barriers, and energetics. *J Biol Chem*, 46856-46863.

Kabanov, A. B. (2002). Pluronics block copolymers as novel polymer therapeutics for drug and gene delivery. *Journal of Controlled Release*, 189-212.

Kamihira, M. K. (1992). Purification of recombinant protein-A by aqueous two-phase extractio integrated with affinity precipitation. *Biotenol. Bioeng.*, 1381-1387.

Kamihira, M. Y. (1994). Fractionation of IgG fragments using reversed micellar extraction. *J Ferment Bioeng*, 80-84.

Karplus, M. (1997). The levinthal paradox, yesterday and today. *Fold Des, 2*, 569-576.

Kato, A. T. (1992). Structural and functional properties of hen egg-white lysozyme deamidated by protein engineering. *Biosci Biotechnol Biochem*, 1424-1428.

Kelley, B. R. (1994). Salt and surfactant effects on protei solubilization in AOT-isooctane reversed micelles. In W. inze, *Organized assemblied in chemical analysis: reverse micelles.* (pp. 123-142). Greenwich: JAI Press.

Kelly, S. J. (2005). Review: How to study proteins by circular dichroism. *Biochimica et Biophysica Acta*, 119-139.

Khan, A. K. (2012). Interaction of amphiphilic drugs with human and bovine serum albumins. *Molecular and Biomolecular Spectroscopy*, 119-124.

Kinugasa, T. K. (2003). Effects of ion species in aqueous phase on protein extraction into reverse micellar solution. *Separation and Purification Technology, 31*, 251-259.

Kinugasa, T. W. (1992). Activity and conformation of lysozyme in reverse micellar extraction. *Industrial Engineering and Chemical Research, 31*, 1827-1829.

Kinugasa, T. W. (1992). Activity and conformation of lysozyme in reverse micellar extraction. *Industrial Engineering and Chemical Research, 31*, 1827-1829.

Kinugase, T. K. (2002). Estimation for size of reverse micelles formed by AOT and SDEHP based on viscosity measurement. *Colloids and Surfaces A: Physicochemical and Engieering Aspects*, 193-199.

Klicova, L. S. (2012). CTAB/water/chloroform reverse micelles: a closed or open association model? *Langmuir*, 15185-15192.

Kotlarchyk, M. H. (1985). Structure of AOT reverse micelles determined by small-angle neutron scattering. *Journal of Physical Chemistry*, 4382-4386.

Kotlarchyk, M. H. (1985). Structure of AOT reverse micelles determined by small-angle neutron scattering. *J.Phys.Chem.*, 4382-4386.

Krei, G. M. (1995). Extraction of a-anylase using BDBAC reversed micelles. *Bioseparation*, 175-183.

Krishna, S. S. (2002). Reverse micellar extraction for downstream processing of proteins/enzymes. *Advances in Biochemical Engineering/Biotechnology*, 124-176.

Kundu, K. P. (2013). Physicochemical investigation of mixed surfactant reverse micelles: waer solubilization and conductometric studies. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 154-165.

Langer, E. R. (2006). Capacity bottleneck squeezed by downstream processes. *Bioprocess International*, 14-17.

Langevin, D. (1992). Micelles and microemulsions. *Annual Review of Physical Chemistry*, 341-369.

Lazarova, Z. T. (1999). Integrated reverse micellar extraction and stripping of a-amylase. *Biotechnol Bioeng*, 583-592.

Lee, H. a. (1998). Selective solubilization of alpha-lactalbumin and beta-llactoglobulin into reversed micelles from their mixtures. *Journal of Food Science*, 601-604.

Lesley, S. K. (2002). Structural genomics of the thermotoga maritima proteome implemented in a high-throughput structure determination pipeline. *Proc Natl Acad Sci USA*, 11664-11669.

Levinthal, C. (1998). Mossbauer spectroscopy in biological systems. *Proceesings of a meeting held at allerton house,* (p. 22). Monticello, Illinois: University of Illonois Press,Urbana.

Li, F. L.-Z.-Q.-J. (1997). Studies on cetyltrimethylammonium bromide (CTAB) micellar solution and CTAB reversed microemulsion by ESR and 2H NMR. *Colloids and Surfaces A: Physichemical and Engineering Aspects* , 89-96.

Li, J. Z. (2006). Effect of ionic liquid on the polarity and size of the reverse micelles in supercritical CO2. *Colloid Surface A.*, 208-212.

Lide, D. (2005). *CRC Handbook of Chemistry and Physics.* Boca Raton: CRC Press.

Lindman, B. W. (1980). Topics in current chemistry. *Springer-Verlag, 87*, 6.

Liu, D. M. (1998). Fluorescence probing of mixed reverse micelles formed with AOT and non-ionic surfactants in n-heptane. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 21-26.

Liu, D. M. (1998). Investigation on the conductivity and microstructure of AOT/non-ionic surfactant/watern-heptane mixed reverse micelles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 157-164.

Liu, J. X. (2004). Reverse micelles extraction of nattokinase from fermentation broth. *Biochemistry Engineering Journal*, 273-278.

Liu, Y. C. (2013). Review: Advances in nutritional manipulation rumen functions of surfactants. *African Journal of Microbiology Research*, 1451-1458.

Liu, Y. D. (2008). Reviews: New development of revrese micelles and applications in protein separation and refolding. *Chinese Journal of Chemical Engineering, 16*(6), 949-955.

Manav, B. P. (2008). Application of reverse micelle extraction process for amylase recovery using response surface methodology. *Bioprocess Biosyst*, 379-384.

Manisha, M. M. (2009). Basic and potential application of surfactants - A review. *International Journal of PharmTech Reseach*, 1354-1365.

Martinek, K. L. (1986). Micellar enzymology. *European Journal of Biochemistry*(55), 453-468.

Mathew, D. J.-S. (2005). Improve back extraction of papain from AOT reverse micelles using alcohols and a counter-ioic surfactant. *Biochemical Engineering Journal*, 219-225.

Mathew, D. J.-S. (2007). Review: Role of alcohols in the formation of inverse microemulsions and back extraction of protein/enzymes in a reverse micellar system. *Separation and Purification Technology*, 199-215.

Matzke, A. C. (1992). Mechanisms of protein solubilization in reverse micelles. *Biotechnology and Bioengineering*, 91-102.

Matzke, S. C. (1992). Mechanism of protein solubilization in reverse micelles. *Biotechnology and Bioengineering, 40*, 91-102.

Matzke, S. C. (1992). Mechanisms of protein solubilization in reverse micelles. *Biotechnology and Bioenginerring*(40), 91-102.

Mazzola, P. L.-Y. (2008). Review: Liquid-liquid extraction of biomolecules: an overview and update of the main techniques. *Journal of Chemical Technology and Biotechnology*, 143-157.

Mijts, B. S.-D. (2003). Engineering of secondary metabolite pathway. *Current Opinion in Biotecnology*, 297-207.

Miller, N. (2004). *The misfolding diseases unfold.* Beremans limited.

Milton, T. H. (2007). Chemical, physical, and biochemical concepts in isolation and purification of proteins. In: separation processes in biotechnology. (J. Asenjo, Ed.) *Taylor & Francis*, 17-64.

Mitchell, D. N. (1981). Micelles, vesicles and microemulsions. *J. Chem. Soc. Faraday Trans.*, 601-629.

Mohd-Setapar, S. (2009). Penicilin G solubilisation into AOT reverse micelles. *Chemical Engineering Research and Design*, 833-842.

Moilanen, D. F. (2009). Water dynamics in large and small reverse micelles: From two ensemles to collective behavior. *The Journal of Chemical Physics*, 0147041-9.

Moilanen, D. F. (2009). Water dynamics in large and small reverse micelles: From two esembles to collective behavior. *The joural of chemical physics*, 0147041-9.

Myers, D. (2006). *Surfactant science and technology.* New Jersey: John Wiley & Sons.

Nace, V. (1996). Surfactant sciene series 60. In V. Nace, *Non-ionic surfactant: polyoxyalkylee block copolymers.* (pp. 1-266). New York: Marcel Dekker.

Nandini, K. R. (2009). Reverse micellar extraction for downstram processing of lipase: effect of various parameters on extraction. *Process Biochemistry*, 1172-1178.

Naoe, K. (2004). Higher order structure of protein solubilized in AOT reverse micelles. *Colloids and Surfaces B: Biointerfaces*, 179-185.

Naoe, K. M. (2002). Efficacy of guanidium salts in protein recovery from reverse micellar organic media. *Biochemical Engineering Journal, 10*, 137-142.

Naoe, K. U. (1998). Protein extraction using non-ionic reverse mielles of Span 60. *Biochemical Enginerring Journal*, 113-119.

Ninham, B. C. (1984). Roles of oils and other factors in microemulsion design. *J. Phys. Chem*, 5855-5857.

Nishihama, S. H. (2001). Review of advanced liquid-liquid extraction systems for the separation of metal ions by a combination of conversion of the metal species with chemical reaction. *American Chemical Society*, 3085-3092.

Nishii, Y. K. (2004). Effect of cation species on equilibrium of reversed micellar extraction of lysozyme. *Separation and Purification Technology, 37*, 161-167.

Nishimura, J. N. (1999). The stimulatory effects of Hofmeister ions on the activities of neuronal nitric-oxide synthase. *The Journal of Biological Chemistry*, 5399-5406.

Noh, K. I. (2005). One step separation of lysozyme by reverse micelles formed by cationic surfactant, cetyldimethylammonium bromide. *Food Chemistry*, 95-101.

Odell, J. M. (1988). Non-Newtonian behaviour of hydrolysed polyacrylamide in strong elongatiol flows: a transient network approach. *Polymer*, 1179-1190.

Ohlson, S. H. (1989). High perormance chromatography: A new tool in biotechnology. *Trends in Biotechnology, 7*, 179-184.

Olesik, S. M. (1990). Critical micelles concentration of AOT in supercritical alkanes. *Langmuir*, 183-187.

Paul, B. M. (2006). Conductivity of reverse micellar system of water/AOT + Brij-56 or Brij-58/IPM and their percolation uder varied concentrations of amphiphiles and different additives. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 129-140.

Pena-Pereira, F. L. (2009). Miniaturized preconcentration methods based on liquid-liquid extraction and their application in inorganic ultratrace analysis and speciation: A review. *Spectrochimica Acta Part B*, 1-15.

Pessoa, J. V. (1997). Separation of insulinase from Kluyveromyces marxianus using reversed micellar extraction. *Biotechnol. Techn.*, 421-422.

Pessoa, J. V. (1998). Recovery of inulinase usig BDBAC reversed micelles. *Proc. Biochem.*, 291-297.

Pham, H. (2010). *Lysozyme extraction using reverse micelles.* Medway, Kent: University of Greenwich.

Piirma, I. (1992). Surfactant sciene series 42. In I. Piirma, *Polymeric surfactants.* (pp. 1-285). New York: Marcel Dekker.

Pileni, M. (1989). *Structure and reactivity in reverse micelles.* Amsterdam: Elseviser.

Prasad, N. K. (2010). *Downstream process technology. A new horizon in biotechnology.* New Delhi: PHI Learning .

Prichanont, S. L. (2000). The solubilisation of mycobacterium in a water in oil microemulsion for biotransformation: system selection and characterisation. *Colloid Surface A*, 177-186.

Riess, G. H. (1985). Encyclopedia of polymer science and engineering. In G. H. Riess, *Block copolymers, 2nd ed* (pp. 324-434). New York: Wiley.

Robison, C. S. (1995). Hydrostatic and osmotic pressure as tools to study macromolecular recognition. *Methods Enzymol*, 395-427.

Rodakiewicz-Nowark, J. M. (2002). Enzymatic activity of the A. bisporus tyrosinase in AOT/isooctane water-in-oil microemulsions. *Colloids and Surfaces A*, 347-356.

Rozema, D. (1996). *The development, characterization and application of artificial chaperone protein refolding.* Madison: University of Wisconsin.

Sadana, A. (1998). *Bioseparation of proteins.* New York: Academic Press.

Sakono, M. M. (2004). Refolding of denatured carbonic anhydrase B by reversed micelles formulated with non-ionic surfactant. *Biochem. Eng. J*, 217-220.

Salager, J.-L. (2002). *Surfactants: types and uses.* Venezuela: Universidad de Los Andes.

Scatena, L. B. (2001). Water at hydrophobic surfaces: Weak hydrogen bonding and strong orientation effects. *Science*, 908-912.

Schor, E. (2010). *Ingredients of controversial dispersants used on gulf spill are secrets no more.* The New York Times.

Schramm, L. S. (2003). Surfactant and their applications. *Annu. Rep. Prog. Chem. Sect. C., 99*, 3-48.

Shanmugam, G. P. (2004). Vibrational circular dichroism spectra of protein films: thermal denaturation of bovine serum albumin. *Biophysical Chemistry*, 73-77.

Shetty, K. P. (n.d.). *Food Biotechnology* .

Shiomori, K. E. (1998). Extraction characteristic of bovine serum albumin using sodium bis(2-ethyhexyl) solfosuccinate reverse micelles. *J Ferment Bioeng*, 581-587.

Sigmaaldrich. (2003, Jan 14). *Enzymatic Assay of Lysozyme*. Retrieved Jan 12, 2015, from Sigmaaldrich: http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-lysozyme.html

Simon, L. a. (2005). Aqueous two-phase extraction- a case of study in process analysis and control. *AIChE Annual Meeting*, 279e.

Soto, C. (2001). Minireview: protein misfolding and disease; protein refolding and therapy. *FEBS Letters*, 204-207.

Stefani, M. (2004). Review: protein misfolding and aggregation: new exanokes in medicine and biology of the dark side of the protein world. *Biochimica et Biophysica Acta*, 5-25.

Sun, Q. Y. (2011). Extraction of bovine serum albumin using reverse micelles formed by hexadecyl trimethyl ammonium chloride. *Applied Biochemistry and Biotechnology*, 744-755.

Sun, Q. Y. (2011). Extraction of bovine serum alumin using reverse micelles formed by hexadecyl trimethyl ammonium chloride. *Applied Biochemistry and Biotechnology*, 744-755.

Sundari, C. R. (1999). Hydrophobic surfacesin oligosaccharides: linear dextrins are amphiphilic chains. *Surface B Biointerfaces*, 3-27.

Svensson, B. a. (1999). A SANS investigation of reverse (water-in-oil) micelles of amphiphilic block copolymers. *Macromolecules*, 6725-6733.

Syeinmann, B. (1986). A comparative study of lysozyme conformation in various reverse micellar systems. *Biopolymers*, 1133-1156.

Tapas, K. A. (1995). Solution behaviour of Aerosol OT in non-polar solvents. *Advances in colloid and interface science*, 95-193.

Tonova, K. L. (2008). Reversed micelle solvent as tools of enzyme purification and enzyme-catalyzed conversion. *Biotechnol Advantages*, 516-532.

Vallejo, L. R. (2004). Review: strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins. *Microbial Call Factories*, 3-11.

Vasudevan, M. T. (1995). Surfactant structure effects in protein separation using non-ionic microemulsions. *Biotechnol. Bioeng*, 99-108.

Vasudevan, M. W. (1996). Mechanism of the extraction of proteins into Tween 85 non-ionic microemulsions. *Ind. Eng. Chem. Res.*, 1085-1089.

Verma, S. G. (2013). Activity, stability and kinetic parameters for a-chymotrypsin catalysed reactions in AOT/isooctanne reverse micelles with non-ionic and zwitterionic mixed surfactants. *J. Chem. Sci*, 875-882.

Vo Hippel, P. W. (1964). Neutral salts: the generality of their effects on the stability of macromolecular conformations. *Science*, 577-580.

Watanabes, S. R. (1994). Quanternary ammonium-based surfactants that can recognize choleserol-rich membranes and proton-ionizable analogs that cannot. *Journal of the America Chemical Society*, 5762-5765.

Webbwe, S. M. (1996). Applied sciences. In S. M. Webbwe, *Solvents and self-organization of polymer* (pp. 1-83). Dordrecht: Kluwer Academic Pulisher.

Wetter, L. D. (1951). Immunological studies on egg white proteins: IV. Immunochemical and physical studies of lysozyme. *J.Biol.Chem*, 237-242.

Wolbert, B. H. (1989). Protein transfer from an aqueous into reversed micelles, the effect of protein size and charge distribution. *European Journal of Biochemistry, 184*, 627-633.

Wolf, R. L. (1979). Micellar solubilization of enzyme in hydrocarbon solvents, enzymatic activity and spectroscopic properties of robomuclease in n-octane. *Biochemical and Biophysical Research Communications, 89*, 209-217.

Wong, Y. H. (2002). Structure-activity study of spermicidal and anti-HIV properties of hydroxylated cationic surfactants. *Bioorganic Medicinal Chemistry*, 3599-3608.

Wu, L. M. (2008). Influence of metal ions on folding pathway and conformational stability of bovine serum albumin. *Journal of Molecular Structure*, 44-49.

Yamada, Y. K. (1994). Extraction of enzyme and their activities in AOT reverse micellar systems modified with non-ionic surfactant. *J. Chem. Eng. Jpn.*, 404-409.

Yamaguchi, S. Y. (2013). Review: protein refolding using chemical refolding additives. *Biotechnol*, 17-31.

Yang, H. E. (2003). Effects of bile salts on percolation and size of AOT reversed micelles. *Journal of Colloid and Interface Science, 262*, 531-535.

Yee, A. C.-L. (2002). An NMR approach to structural proteomics. *Proc Natl Acad Sci USA*, 1825-1830.

Yoshii, H. F. (2000). Refolding of denatured/reduced lysozyme at high concentration with infiltration. *Biosci Biotechnol Biochem*, 1159-1165.

Yoshimoto, M. K. (2008). Oxidative refolding of denatured/reduced lysozyme utilizing the chaperone-line function of liposomes and immobilized liposime chromatography. *Biotechnol.Prog, 15*(3), 480-487.

Zhang, W. L. (2002). Forward and backwrd extraction of BSA using mixed reverse micellar system of CTAB and alkyl halides. *Biochemical Engineering Journal*, 1-5.

Zhang, X. L. (2013). Investigation of the interaction of naringin Palmitate with Bovine Serum Albumin: spectroscopic analysis and molecular docking. *PLoS One*, 1-17.

Zhang, Y. C. (2006). Interactions betwee macromolecules and ions: the Hofmeister series. *Current Opinion in Chemical Biology*, 658-663.

Zhao, H. (2006). Are ionic liquids kosmotropic or chaotropic? An evaluation of available thermodynamic parameters for quantifying the ion kosmotropicity of ionic liquids. *Journal of Chemical Technology and Biotechnology*, 877-891.