CHAPTER IV

COMPARATIVE STUDIES ON THE ENZYMATIC ACTIVITY OF HRP ENCAPSULATED IN REVERSE MICELLES OF ‘ANIONIC’, ‘CATIONIC’ AND ‘NON-IONIC SURFACTANT’ SYSTEMS
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4.1 INTRODUCTION

With respect to a variety of practical applications, there is growing interest in microemulsions in order to benefit from their unique physical properties. The applications investigated at present range from classical fields like nutrition science, oil recovery, ground water remediation, soil cleanup and organic chemistry to new subjects like the improvement of fuels or the use as decontamination media. In principle, all these different applications take advantage from the possibility to achieve “thermodynamically stable homogeneous mixtures of non-polar solvents with polar liquids like water, stabilized by a surfactant, which forms an interfacial film separating the two in principle immiscible solvents”. Microemulsions form a multitude of different microstructures. Due to remarkable improvements of experimental techniques (in particular of light-, electron- and neutron scattering, nuclear magnetic resonance and microscopy) there has been great progress during the last few decades in the characterization of the phase behavior of amphiphilic mixtures and of their microstructures. It has been shown that the choice of the microstructure depends on the composition of the samples and on external control parameters such as temperature, added salt, alcohol or dye.

Based on the head group-charge, surfactants are divided into four categories i) cationic, ii) zwitterionic, iii) nonionic and iv) anionic i.e. lipophiles are usually similar from one surfactant to another but hydrophiles show a range of chemical types
and this is the basis for surfactant classification. In anionic surfactants the hydrophile comprises some highly electronegative atoms, making these molecules strongly polar. The counterion is usually a small cation such as sodium but occasionally may be a larger cation such as ammonia or amines. In aqueous solution, the anionic surfactant dissociates giving an anion carrying the amphiphilic properties and an inactive cation (e.g. Na⁺ or K⁺). Cationic surfactants, in contrast, comprise a long chain hydrocarbon as the lipophile with a quaternary amine nitrogen as hydrophile and a halide ion as counterion. In aqueous solutions, cationic surfactants are ionized in a cation carrying the amphiphilic properties and an inactive anion such as Cl⁻ or Br⁻. Non-ionic surfactants are second to anionics in cleaning applications and are frequently used in conjunction with them. An important group of non-ionics includes those where the hydrophile comprises a chain of ethoxy groups and is known as the ethoxylates. Nonionic surfactants as the name implies do not give ions in solution. The hydrophilic part of their molecule contains polar groups such as ether, alcohol, carbonyl or amino groups. Varying the number of ethoxy groups in the chain adjusts the amount of hydrophilic character in the final products.¹⁶

Nonionic surfactants are frequently used in industrial aspect and it has many advantages over the ionic surfactants. For non-ionic systems, the intra and intermicellar head group repulsions are much smaller than for ionic systems and this expected to lead to important differences between two classes of surfactants. One differences lies in the CMC values being almost 100 times smaller for non-ionic than for ionic surfactants for the same alkyl chain length. For ionic surfactant, the CMC is strongly decreased in the presence of electrolyte while the effect is small for non-ionic systems. The solubilization capacity is generally much larger for non-ionic systems than for ionic ones.¹⁷,¹⁸

For the study of interactions between proteins and micellar interface, non-ionic surfactant display, interesting properties by avoiding strong electrostatic interactions which often held responsible for the destabilization of protein structures.¹⁹ Furthermore, the absence of charges on the surfactant head groups allows one to
access the importance of hydrophobic forces in the bilayer insertion mechanism and stability of hydrophobic proteins or peptides.\textsuperscript{20,21,22}

Amphoteric surfactants comprise a long hydrocarbon chain (lipophile) attached to a hydrophilic containing both positive and negative charges, which give it the properties of a zwitterion. The simplest amphotericics can therefore behave as a cation or anion depending on pH.\textsuperscript{23,24}

In aqueous medium, surfactants in pure and mixed states form micelles after a critical concentration, called CMC. Mixed surfactants producing mixed micelles are very often used in industrial preparations and pharmaceutical and medicinal formulations for the purpose of solubilization, suspension, dispersion etc.\textsuperscript{25,26}

Out of the three types of amphiphilicities, it is known that in the mixed states nonionics shows ideal behavior, while other combinations exhibit non-ideality resulting from synergistic (Attractive) or autagonistic (repulsive) interactions between the amphiphilics of different types.\textsuperscript{27,28,29}

The properties of surfactant systems depend mainly upon the molecular structure of surfactant. Hence it is always difficult to optimize the conditions of surfactant systems with reference to requirements. However the surfactant mixtures are useful in adjusting the required properties and a large number of studies were devoted to the mixed surfactant systems in literature.\textsuperscript{30,31} Mixing of similar species has often resulted in some intermediated properties and hence a linear mixing rule is generally accepted.\textsuperscript{32,33}

Reverse micelles and w/o microemulsions represent the L2 domain in the phase diagram of ternary surfactant-oil-water system. The extends of the domain and the nature of the adjoining phases depend on the thermodynamic variables and the identity of surfactant and oil. The introduction of additional components i.e. co-
surfactant, electrolytes etc. usually alter the properties of particles and hence the phase behavior of the system.\textsuperscript{34}

Surfactants have the ability to self-assemble into a variety of microstructures. Surfactant/cosurfactant/water ternary systems, depending on the amount of each component, form different structures such as micelles, reverse micelles, spherical, rod-like, hexagonal, lamellar, and cubic liquid crystals, vesicles and flexible bilayers and at high concentrations a rich variety of lyotropic mesophases\textsuperscript{35,36} and other structures. The surfactant microstructure can have a strong influence on rheological properties. Typically, due to the formation of spherical micelles, dilute solutions of many surfactants exhibit low viscosity. However, under certain conditions or with certain additives, the surfactants can assemble into microstructures which impart viscoelastic and gel-like behaviour to the solution.\textsuperscript{37}

It is well-known that Reverse micelles are nanometer-sized droplets of water or polar solvent, which are surrounded by a layer of surfactant molecules and dispersed in a nonpolar solvent or weakly polar solvent. The hydrophilic head groups of the surfactant molecules are directed toward the core of the micelles and the hydrophobic groups are directed toward the bulk organic solvent. The cosurfactant acts as a “spacer” that minimizes repulsions between the electriferous surfactant heads.\textsuperscript{38}

The size of a reverse micelle in a suspension is characterized by $W_0$, the molar ratio of water or polar solvent to surfactant S, $W_0 = [\text{H}_2\text{O}]/[\text{S}]$; and $W_0$ has been shown to be directly proportional to the micellar radius, so the increase of water content in the system will cause the reverse micelles to enlarge. Confined environments in reverse micelles can be used to carry out a variety of reactions, either by modifying the properties of the encapsulated liquid or by bringing reactants into close contact.\textsuperscript{39} Therefore, the properties of water within the reverse micelles have been extensively studied by many techniques, such as infrared spectroscopy, NMR spectroscopy, Raman and inelastic light scattering, fluorescence upconversion, and molecular dynamics (MD) simulations.\textsuperscript{40,41,42}
When two microemulsions, each containing respective reactants are mixed, the contents of the aqueous droplets are mixed and redistributed very rapidly because of the collisions that involve temporary merging of the droplets into a larger droplet (fusion) and subsequent breakup of this larger droplet (fission). It was found that the mass exchange between the droplets can be extremely fast and consequently, the mass exchange is limited by the collision of the droplets, it may assume that once two droplets are merged through a collision, the contents of the two droplets are mixed rapidly and thoroughly before breakup into two droplets of identical size.\textsuperscript{43, 44}

Several theoretical models have been worked out in order to understand why a random structure of bicontinuous type does not collapse into an ordered phase. An answer has been suggested by De Gennes and Taupin\textsuperscript{45} who first recognized that the stability of the microemulsion phase is controlled by the physics of the amphiphilic film. The interface saturated by surfactant has a nearly vanishing surface tension; in these conditions the elastic constant describing the curvature elasticity of the interface is one of the essential parameters.\textsuperscript{46}

### 4.1.1 Enzyme encapsulation in reverse micelles:

Micellar enzymology is a new physico-chemical approach to biochemical research problems. These model studies of enzymatic catalysis in micro-heterogeneous media are of great importance in understanding of the enzyme functioning in natural lipid systems. In such studies, the free enzymes are not handled in classical aqueous buffers, but in reverse micellar systems of water in organic solvents (microemulsions). In biochemistry, reverse micelles have been used to mimic properties of biological membranes and for catalysis of enzymatic reactions. Enzymes have been found to be active in organic solvents and this finding has prompted a new and expensive field that of bio organic synthesis. Several methods have been developed for carrying out enzymatic catalysis in organic solvents (oils). These includes liquid-liquid two phase systems, solid-liquid two phase systems and various
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homogenous one phase systems such as a mixture of water miscible organic solvents and water and enzymes in microemulsion.\(^{47}\)

Water-in-oil microemulsions or reverse micellar systems, are thermodynamically stable and optically isotropic solutions, with water constituting the dispersed phase, separated from the continuous organic phase by surfactant molecules formed due to the self-organization of surfactants into aggregates in apolar organic solvents with water pools in their polar cores. The enzyme molecules can be encapsulated in the reverse micelles, avoiding direct contact with the organic solvent that may cause denaturation.\(^{48}\) The dispersed water pools act like a microreactor, with a favorable aqueous microenvironment for enzyme activity and an enormous interface, through which lipophilic substrates can be cleaved.\(^{49,50}\).

The polar cores of the reverse micelles are of nanometer size and are therefore able to solubilize significant amounts of water and proteins.\(^{51}\) Previous studies have shown that properties of water molecules at the interface with surfactant polar groups vary significantly from those of bulk water. The water molecules contained within reverse micelles may be bound or free. Approximately five molecules of water are bound tightly to one AOT surfactant head. The bound water has restricted mobility, increasing viscosity and increasing polarity. An increase in the amount of water molecules results in an increase of the size of the water pool and partial recovering of bulk water properties.\(^{52}\)

The study of enzymatic reactions in the aqueous pool of the reverse micellar droplets has become an important field of research owing to the following reasons\(^{53,54}\)

- Enhanced catalytic activity
- Conformational stability
- Various technological applications
- Simulation of the microenvironment of biological enzymes

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• Employment of simple spectroscopic techniques for studying their activity and conformation

In addition, reverse micelles are ideally suited for hosting enzymatic reactions, particularly those involving difficult-to-solubilize substrates, require low water content, or employ multiple biocatalysis.\textsuperscript{55} The hydrated reversed micelles of surfactants in organic solvents offer a unique opportunity for dissolving proteins under such standard conditions so that a protein molecule can choose for itself an optimal microenvironment corresponding to its nature (optimal in terms of thermodynamics).\textsuperscript{56}

Medical science has developed interest in the use of enzymes as drugs. This however poses problems, not yet encountered. After oral administered of enzymes, they are digested in the intestinal tract or excreted. When administered intravenously, they cause immunological reactions. Such effects may be circumvented by masking the antigenic determinants of enzymes, by encapsulation of enzymes, by targeting such capsules to the infected organs.\textsuperscript{57}

A relatively new method of enzyme immobilization in organic media is the entrapment of enzymes in reversed micelles. Microemulsions offer several advantages as reaction media for enzymatic catalysis. In such systems, many biocatalysts regardless of their nature and function, retain catalytic activity e.g. chymotrypsin, trypsin, lysozyme, ribonuclease, pyruvate kinase, some forms of cytochrome, peroxidase, alcohol dehydrogenase and catalase.\textsuperscript{58} Better stability and activity of the biocatalyst as compared to that in water can sometimes be obtained. Product recovery is often easy, enzyme and co-factor can be reused.\textsuperscript{59} The other advantage of this system is the high interfacial area between aqueous and non-aqueous medium. Variation of the surfactant properties might make targeting possible and encapsulation of the drug/enzyme in such a capsule may retard its release, thereby reducing the required frequency of administration.
AOT reverse micelles are commonly used for the solubilization of enzymes and proteins in apolar solvents because they readily dissolve in these solvents and can form reverse micelles without co-surfactants. Other cationic surfactants such as CTAB that form reverse micelles require cosolvents or cosurfactants to form a reverse micellar phase in apolar solvents. The solubilization of proteins and enzymes, such as trypsin, lysozyme, chymotrypsin and amylase, by reverse micellar systems has been well documented in the literature. On several accounts, researchers have been successful in the design of novel reverse micelles by variation of the different microstructural parameters in which both interfacially solubilized enzymes, such as lipase and horseradish peroxidase and water-pool solubilized hydrophilic enzymes, such as trypsin, have displayed superior activity in these nanoreactors. The type of surfactant used to form the reversed micelles can largely influence the enzyme activity.

Rees et al. compared the activity of five microbial lipases in different water in oil (w/o) microemulsions and verified that the lactonisation activity was higher for the systems based on anionic surfactants (AOT) than for those based on cationic surfactants (CTAB). Lipase stability was higher in CTAB, but reached high levels in AOT microemulsions with reduced water content. A comparative study was also carried out by Valis et al. in reversed micelles of anionic, cationic and non-ionic surfactants, using Rhizopus delemar lipase. The assembling conditions of pH, $W_0$ (water to surfactant molar ratio) and temperature, to reach the maximum enzyme activity, significantly differ in each reversed micellar system.

Yamada et al. added non-ionic surfactants (Tween, Triton and Span) to the AOT/isooctane system, forming mixed micelles whereas increasing their number. The hydrolytic activity of Chromobacterium viscosum lipase increased in the presence of the non-ionic surfactants, possibly due to the suppression of electrostatic and hydrophobic interactions between the lipase and the AOT. Cationic surfactants often need a co-surfactant to form reverse micelles, as most of the zwitterionic surfactants. Non-ionic and anionic surfactants must also respect some defined concentrations to
yield a reversed micellar system. The phase diagrams give a picture of the location of phases by relating them with the concentrations of solvent, water and surfactant. Microemulsions can also be used as solvents for multi enzyme catalysed reactions. A chain of three enzymes as used by Laane et.al.\(^69\) to convert a ketosteroid in a CTAB system. The activity of enzymes in microemulsions has been reported to be of the same order of magnitude as that in aqueous solvent.

The main approaches of micellar enzymology has been directed towards studying the optimum conditions like \(W_0\) ([H\(_2\)O]/[Surfactant]), temperature and pH for the maximal activity of the enzyme. Reverse micelles can thus be viewed as microreactors of easily variable dimensions containing aqueous microdroplets (water pools) whose physical properties can be continuously modulated through the amount of water dissolved in the system.\(^70\)

The water pool of the w/o microemulsion has been effectively used in the present study as a nanoreactor for solubilizing and entrapping enzyme in reverse micelles. Water present inside the reverse micelles differs from that of bulk water in its physico-chemical properties. The anomalous properties of water at low \(W_0\) obviously influences the chemical behaviour of solubilized molecules. This water may be thought of as a series of regions in which the water molecules are characterized by different correlation times, which depends on their dipolar interactions with one another and within the polar group of the micellar wall. \(W_0\) i.e. the molar ratio of water to surfactant is the most important parameter in determining the various micellar parameters. This ratio more than the absolute amount of water or surfactant are present in the organic solvents determines most of the structural and physical properties of reverse micelles.\(^71\) For instance at low degrees of hydration the viscosity of water solubilized by AOT in hydrocarbon oil is 200 times higher than that of bulk water, and its polarity corresponds to that of chloroform.\(^72,73\) Another example is that of high chemical activity of solubilized water, namely the nucleophilic capacity of the water molecules solubilized by AOT micelles in octane is at least 103 times higher than that of the bulk water.\(^74\) However reverse micelles usually swell as the amount of
water in the system increases and therefore the difference in the properties of micellar and bulk water becomes less pronounced. Hence reverse micelles can be viewed as microreactors of easily variable dimensions containing aqueous microdroplets whose physical properties can be continuously modulated through the amount of water dissolved in the system.

When a biopolymer is hosted in the water pool, the situation may become complicated. At low levels of water, for example, the protein and the polar micellar wall may compete for water, with the result that at any given $W_0$, the content of free water inside the micelle may be considerably lower in the filled micelles than in the unfilled ones. Furthermore the protein may have an influence on the structure of water. In turn, the structure of water may influence certain dynamic processes and certain physical parameters of the guest proteins. In most of the cases, it has been seen that the space or surface area in the vicinity of the enzyme plays the most crucial role in modulating the catalytic activity of the enzyme. Enzymes localized at the enhanced surface area always exhibited improvement in activity due to the increased substrate concentration and also by attaining a flexible conformation. For some enzymes, an enhancement of the reaction rate in microemulsion over that found in aqueous solutions has been observed. This is often referred to as “superactivity”. No simple explanation is given for this phenomenon, only speculation regarding structural changes of the enzyme substrate concentration or the physical nature of the water in the aqueous microdomains have been put forward.

The regulation of a reaction rate in the microreactor of a reverse micellar droplet would offer several important advantages to study over the reactions in the bulk solvent medium as far as controlled product formation, desirable life time in the mixture or controlled degradation of the reactants are concerned. Reaction kinetics in encapsulated enzymes also offers an interesting area of research.

Catalytic activity of solubilized enzymes are exhaustively discussed in this chapter. One of the most striking effects is the superactivity of the entrapped enzyme.
Concerning the activity of enzymes in reverse micellar systems, the following generalizations are possible:

- Enzymes maintain activity comparable to that found in aqueous solutions there are no significant changes in the kinetic behaviour and a Michaelis-Menten behaviour has been observed in reverse micellar solutions just as in water.
- The maximal activity in reverse micelles is not found at the maximal water content but at rather low $W_0$ values.
- Enzymes in reverse micelles are able to accept not only water soluble substrates, but also water insoluble ones, namely those which are directly solubilized in the hydrocarbons and the stability of enzymes is generally comparable to that in water, being greater at small $W_0$ values and generally rather small at large $W_0$ values.

### 4.2 APPLICATIONS AND PROSPECTS OF MICELLAR ENZYMEOLOGY

Several fields of application have been suggested for protein containing reversed micelles. The conversion of apolar compounds by enzymes entrapped in the aqueous core of reversed micelles have drawn most attention. These aggregates, as already mentioned have a very dynamic character because they can exchange the contents of their water pools by a collision process. Apolar compounds can diffuse from the continuous organic phase into the interphase. Enzyme in the water pool can convert both polar and apolar compounds. This enables conversions that are otherwise difficult to perform.

Protein extraction is another application where several approaches have been described ranging from extraction of proteins to form the solid state, extraction of
proteins from an aqueous solution to extraction of intracellular proteins from intact bacteria.

Reversed micellar system has also been used for analytical purposes. The presence of an organic phase allows detection procedures that are difficult to perform in aqueous media e.g. the assay of apolar compounds. These systems also provide convenient media for studying enzyme mediated processes at sub-zero temperature this in turn considerably slows down the reactions and thus allows the leisurely examination of the kinetics of formation and decomposition of short lived ion intermediates.

Recently application of reversed micelles as drug delivery systems has been suggested.

The major goal of the present work is the rate modulation of an enzyme (HRP) catalytic reaction in reverse micelles of anionic, cationic and non-ionic surfactants and kinetic studies of encapsulated enzymes in reverse micelles.

HRP is a member of the large class of Heme peroxidase which carry out one electron oxidation of variety of substrates at the expanse of $\text{H}_2\text{O}_2$. With this idea in mind, the main objective of present work is-

- To determine $k_{\text{cat}}$ of HRP catalysed oxidation of ODA at the expanse of hydrogen peroxide in reverse micellar systems.
- To investigate the effect of $W_0$ on the catalytic activity of HRP in reverse micelles.
- To investigate the effect of type of surfactant on the HRP activity.
- To investigate the effect of surfactant concentration on HRP activity.

4.3 ENZYME KINETIC STUDIES OF ENCAPSULATED HRP IN MICELLER MEDIA
The study of the enzymatic reaction in the water pool of the reverse micelle droplet has opened up a new approach to the modeling of biochemical processes of the cell. In this chapter, HRP is used for enzyme kinetic studies in all types of reverse micelles. Horseradish peroxidase is a heme-protein containing a heme-iron group. Neutral and amino sugars account for approximately 18% of the enzyme and the active site involves apoprotein as well as heme group. ODA (o-dianisidine) in aqueous solution has been long known as a substrates for peroxidase. The reaction exhibits an optimum pH of 7.2.

4.3.1 Determination of $K_{cat}$:

The main aim of the kinetic studies was to determine $k_{cat}$ for HRP catalysed reaction in reverse micellar medium. ODA in aqueous solution has been known as a substrates of peroxidase.

However, the complexity of mechanism of this reaction, the literature lacks a complete kinetic description.

The data was interpreted according to the following reaction scheme

$$E + S \overset{K_1}{\leftrightarrow} ES \overset{K_{-1}}{\rightarrow} E + P \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldOTS(1)

The Michaelis–Menten kinetics of equation (1) was given as:

$$V = \frac{V_{max} [S]}{K_m + S} \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldOTS(2)$$

The maximum velocity of a reaction, $V_{max}$, occurs at a high substrate concentration when the enzyme is saturated, that is when it is entirely in the form of ES.
\[ V_{\text{max}} = k_{\text{cat}} [E]_0 \] \hspace{1cm} (3)

Where \([E]_0\) is the initial concentration of the enzyme and \(k_{\text{cat}}\) the catalytic constant.

\[ V = \frac{k_{\text{cat}} [E]_0 [S]}{(k_m + [S])} \]

\[ \frac{1}{V} = \frac{k_m}{(k_{\text{cat}} [E]_0 [S])} + \frac{1}{(k_{\text{cat}} [E]_0)} \] \hspace{1cm} (4)

This equation is a linear equation involving \(1/[V]\) and \(1/[S]\). When these quantities are plotted, called the Lineweaver Burk Plot.

\[ \frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}} [S]} \] \hspace{1cm} (5)

One gets a straight line with the intercept equal to \(1/(k_{\text{cat}} [E]_0)\).

Therefore,

\[ k_{\text{cat}} = \frac{1}{(\text{[Intercept]} [E]_0)} \] \hspace{1cm} (6)

Hence, if the total enzyme concentration is known, from the intercept the catalytic constant (\(k_{\text{cat}}\)) can be calculated.

**4.4 ACTIVITY AND ENZYME KINETIC STUDIES OF HORSE-RADISH PEROXIDASE ENTRAPPED IN TERNARY SYSTEMS OF REVERSE MICELLES**

To retain the maximum intrinsic activity of the enzyme in the micellar mediated encapsulated particles, it is always necessary to optimize the conditions of reverse
micellar nanoreactor so that the enzyme in the precursor micellar systems exhibits maximum possible activity.

This study deals with the catalytic behavior of horseradish-peroxidase in three different types of microemulsions systems, by using anionic (AOT), cationic (CTAB) and non-ionic (TX-100) surfactants.

4.4.1 U.V. Spectrophotometeric analysis:

All the reverse micellar systems were sufficiently pure for studying U.V. absorption of guest molecules in reverse micellar system. The ultraviolet absorption spectra of encapsulated HRP catalysed reaction in AOT and TX-100 reverse micellar systems is shown in figures- 1‘a’ and ‘b’ respectively.

For this study, 3ml reverse micellar solution of 0.1M ‘AOT/hexane’ and ‘TX-100/hexanol/cyclohexane’ containing 25 μl of encapsulated HRP (4.165 x 10⁻⁷ M) were taken in a cuvette seperately. In these reverse micellar solutions, 10 μl of 1% ODA and 10 μl of 0.5M H₂O₂ were added. Following the H₂O₂ addition reactions were started. HRP encapsulated in reverse micelles show enzymatic activity on o-dianisidine oxidation by H₂O₂. The time dependent formation of the oxidized dye were measured at 445 nm spectrophotometrically in presence of the enzyme entrapped inside the reverse micelles.

The increase in absorbance at 445nm shows the catalytic activity of HRP in reverse micelles.
Figure-1 U.V. SPECTROPHOTOMETRIC ANALYSIS SHOWING CATALYTIC ACTIVITY OF HRP IN (a) AOT AND (b) TX-100 REVERSE MICELLAR SYSTEM

4.4.2 Effect of $W_0$ on the activity of HRP in reverse micelles:
Water present inside the reverse micelles differs from that of bulk water in its physio-chemical properties. \( W_0 \) i.e. molar ratio of water to surfactant is the most important parameter in determining various properties.

A salient feature displayed by enzymes in reverse micelles is dramatic dependence of their catalytic rate constants on the size of the water pool. By adjusting the parameter \( W_0 \), it is possible to modulate the reversed micelles by influencing the activity/stability of the biocatalyst.

The effect of \( W_0 \) on the activity of enzyme was studied in anionic and non-ionic systems. Nonionic reverse micelles were prepared by the addition of hexanol and varying amounts of buffer (pH 7.2) to a solution of 0.1 M Triton X-100 in cyclohexane. Aqueous buffer solution (pH 7.2) of HRP was added to the reverse micelles. The surfactant/co-surfactant ratio is 1:1. 0.1M Aerosol-OT in hexane was used for the preparation of anionic reverse micelles. In a typical experiment, 10 \( \mu L \) of the substrate stock solution (from 1% stock in water) and 25 \( \mu L \) of the aqueous enzyme stock solution (4.165 \( \times 10^{-7} \) M) were added to the 3ml of W/O microemulsion previously prepared with the desired surfactant concentration and pH, in a cuvette to attain the particular \( W_0 \) and reactant concentrations. The total volume of the reaction mixture was 3.05 ml. Gentle shaking produced clear microemulsion solution within 1 min. The change in absorbance at 445 nm was measured at different interval of time. The enzymatic activity was determined from the slope of Absorbance vs time curve.

In both the above reverse micelles the activity of HRP was spectrophotometrically determined using a spectrophotometer.

Figures 2 ‘a’ and ‘b’ show the variation in activity of HRP as a function of \( W_0 \) at fixed pH of 7.2.
Figure 2: EFFECT OF W₀ ON THE ACTIVITY OF HRP- (a) ANIONIC (AOT/Hexane) AND (b) NON-IONIC (Tx-100/Hexane/Cyclohexane) REVERSE MICELLE
4.4.3 Effect of surfactant concentration on the activity of HRP:

The activity of some enzymes in the reverse micelles, has been reported\(^7^8\), to depend on the surfactant concentration. Surfactant concentration may affect the physicochemical properties of the reversed micellar micro-aggregations, and change the solubilization or activity of the enzymes. In some cases it was attributed to the interaction of the enzymes with the micellar membrane.\(^7^9\) In respect of enzymatic catalysis, surfactant concentration may even influence the distribution of the substrate. Brown et al.\(^8^0\) detected a decrease on *Rhizopus arrhizus* lipase activity with the increase of AOT concentration that reflected on the values of \(k_{\text{cat}}\). The \(W_0\) concept is usually associated only with the hydration level although it is dependent on another variable, the concentration of surfactant. When the surfactant interacts with the enzyme accelerating the denaturing process, which often happens with anionic surfactants e.g., SDS, AOT, one has to consider the water content and surfactant concentration as independent variables, since the same \(W_0\) value can be obtained with different combinations of the two factors.

Figure-3 shows the variation of activity of HRP at different concentrations of AOT in AOT/hexane/water ternary system at a \(W_0\) of 21 and fixed pH 7.2. The substrate ODA was added to the AOT-hexane-buffer system containing the solubilized enzyme HRP. The overall reaction mixture had a volume of 3.055ml and contained 25µl (4.165 x \(10^{-7}\) M) of HRP. Following the addition of 10µl of 0.5M \(H_2O_2\), the mixture was slightly shaken. The reaction was thus initiated. Initial velocities of enzymatic oxidation were measured spectrophotometrically at 25\(^0\)C by recording the increase in absorbance at 445 nm at different interval of time. The activity was calculated from the slope of absorbance vs time curve.
Figure -3 SHOWS THE EFFECT OF AOT CONCENTRATION ON THE ACTIVITY OF ENCAPSULATED HRP IN ‘AOT/HEXANE/WATER’ REVERSE MICELLE

4.4.4 Estimation of $K_{cat}$ and $K_m$ in reverse micelles of anionic and non-ionic systems from activity data:

The kinetics of ODA oxidation was studied as a function of the substrate concentration in aqueous buffer system and in reverse micelles composed of anionic and nonionic surfactants. In both the systems studied, the ODA oxidation followed the classical Michaelis-Menten kinetics. The apparent or observed values of the first-order rate constant $k_{cat}$ and the Michaelis constant $k_m$ were determined by fitting the measured activity at various substrate concentrations to equ.5 and the results are summarized in table-1.

(A) Solubilization of Enzyme HRP in Nonionic Triton X-100 Reverse Micelles-Nonionic reverse micelles were prepared by the addition of hexanol and desired amount of buffer of desired pH to a solution of 0.1 M Triton X-100 in cyclohexane. HRP in buffer at the pH 7.2 was added to the reverse micelles. The total volume of
the reaction mixture was 3.05ml, and it contained 25µl of HRP (4.165 x 10^{-7} M). The ratio of surfactant and co-surfactant used was 1:1.

(B) Solubilization of Enzyme HRP in Anionic AOT Reverse Micelles.

The surfactant AOT was dissolved in hexane, and this solution was used for the enzymatic reactions. The reverse micelles were prepared by adding aqueous buffer of the pH 7.2 to the 0.1M AOT-hexane solution to obtain W_0-21. The ternary system of AOT/water/hexane contains 25µl of 4.165 x 10^{-7} M of HRP.

(C) Activity Measurements.

To both the above reverse micellar systems containing the solubilized enzymes HRP, 10µl (1%) of the substrate ODA was added. The overall reaction mixture had a volume of 3.05 ml. Following the addition of 10µl of H_2O_2 the mixture was slightly shaken. The reaction was thus initiated. Initial velocities of enzymatic ODA oxidation reaction were measured spectrophotometrically at 25°C by continuously recording the increase in absorbance at 445 nm. The activity ‘A’ of the enzyme was determined by the slope of absorbance against time curve.
Figure-4 LINEWEAVER BURK PLOT OF HRP (a) IN AOT/HEXANE REVERSE MICELLES AT W_0=21 AND (b) IN TX-100 REVERSE MICELLE AT W_0=10
Chapter IV

**TABLE-1 COMPARISON OF THE ENZYME KINETICS OF FREE HRP AND HRP ENTRAPPED IN ‘AOT AND TX-100 REVERSE MICELLAR SYSTEM’**

<table>
<thead>
<tr>
<th>Parameters/ Micellar system</th>
<th>Kₘ</th>
<th>Kcat</th>
<th>W₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free HRP</td>
<td>4.55 x 10⁻⁵</td>
<td>6.133 x 10⁷</td>
<td></td>
</tr>
<tr>
<td>HRP entrapped in AOT reverse micelles</td>
<td>2.88 x 10⁻²</td>
<td>9.5 x 10⁷</td>
<td>21</td>
</tr>
<tr>
<td>HRP entrapped in TX-100 reverse micelles</td>
<td>1.3 x 10⁻³</td>
<td>3.4 x 10⁸</td>
<td>10</td>
</tr>
</tbody>
</table>

For kₘₐₜ determination we maintained different W₀ as shown in table-1 because HRP shows higher activity at W₀-21 in case of AOT/hexane reverse micellar system and in TX-100/cyclohexane/n-hexanol reverse micellar system HRP shows maximum activity at W₀-10, which is clear from the figures-2 ‘a’ and ‘b’ respectively.

**4.5 ACTIVITY MEASUREMENT OF HRP IN CATIONIC AND MIXED SURFACTANT REVERSE MICELLAR SYSTEM**

Comparative study of activity of HRP was estimated in CTAB and mixed reverse micelles prepared from surfactants CTAB/AOT and CTAB/TX-100 at W₀ 10, pH 7.2 at 25°C using ODA as the substrate.
(A) Solubilization of Enzyme HRP in Cationic CTAB Reverse micelles:

The reverse micelles were prepared by adding hexanol and buffer to a 0.1M solution of CTAB in iso-octane. The volume of hexanol added was one-tenth the volume of iso-octane in the reaction mixture. The aqueous buffer of the pH 7.2 containing HRP was added to the CTAB/iso-octane/hexanol/buffer reverse micelles.

(B) Preparation of Mixed Reverse Micelles stock solution:

0.1M surfactant mixture of CTAB/AOT and CTAB/TX-100 was dispersed in iso-octane to which n-hexanol was added to attain the corresponding value of 9:1 and shaken vigorously. Then aqueous buffer (phosphate) solution was added to reach the \( W_0 = \frac{\text{[water]}}{\text{[surfactants]}} \) 10, and the whole suspension was vortexed to obtain a macroscopically homogeneous solution.

4.5.1 Measurement of peroxidase activity:

HRP catalysed oxidation of ODA in cationic/nonionic and cationic/anionic mixed reverse micelles were determined with a UV-spectrophotometer. 10µL of the substrate ODA stock solution (1% in water) and 25µL of the aqueous enzyme stock solution (4.165 x 10^-7 M) were added to the 3 mL of W/O microemulsion previously prepared with the 0.1M surfactant concentration and pH 7.2 in a cuvette to attain the \( W_0 = 10 \). Gentle shaking produced clear microemulsion within 1 min. Finally, 10 µl of the hydrogen peroxide stock solution (0.5 M in aqueous phosphate buffer) was added. The absorbance change was monitored instantaneously after the addition of \( \text{H}_2\text{O}_2 \). The progress of the reaction was monitored by the formation of the oxidized product of ODA at wavelength of 445 nm. The initial velocity \( (V) \) of this enzymatic oxidation was determined from the slope of the absorption intensity versus time curve. The enzyme activity was expressed in terms of activity (absorbance/min).
Figure 5 (a) ACTIVITY VS SUBSTRATE CONCENTRATION COMPARATIVE GRAPH OF HRP ENCAPSULATED IN ‘AOT’ & ‘CTAB’ REVERSE MICELLE (b) COMPARATIVE TIME VS ABSORBANCE GRAPH OF HRP ENCAPSULATED IN ‘TX-100/CTAB’, ‘AOT/CTAB’ AND ‘CTAB’ REVERSE MICELLER SYSTEM
The principal goal of this work is to examine comprehensively the factors effecting the activity and kinetics of enzymatic oxidation of ODA in different types of reverse micelles. The following objectives have been achieved: (i) We compare the enzyme kinetics of HRP in reverse micelles composed of the anionic surfactant AOT, the cationic surfactant CTAB, and the nonionic surfactant Triton X-100 with the activity in the aqueous medium. (ii) We investigated the combined effect of both $W_0$ and surfactant concentration and thereby obtain an optimal micellar size. (iii) We also investigated the activity of HRP in mixed surfactant systems. It has been noted that the activity of an enzyme is highly specific to the size of the aqueous core of the micelles and surfactant concentration.75

Towards this goal, the following results are found:

Figure-2 shows, the enzyme activity displays a typical bell-shaped dependence on $W_0$ (H$_2$O/AOT). In AOT reverse micellar system (figure-2a) enzyme exhibits maximum
activity at $W_0$-21, and in case of TX-100 reverse micellar system enzyme exhibits maximum activity at $W_0$-10. Above and below these points the activity is comparatively lower. The maximum activity, which can sometimes much larger than aqueous solution, often coincides with a diameter of the inner cavity roughly of the same dimension as the aqueous core of microemulsion. Enzyme reaction rates in microemulsions are found to vary with the size of the water pool, $W_0$, often an optimum is found where the inner diameter of the micelle equals the diameter of the protein. Figure-3 is the surfactant (AOT) concentration vs activity graph of HRP in AOT/hexane/water reverse micellar system. Graph showing maximum activity of HRP at 0.2M concentration of AOT in hexane. Beyond 0.2M there is considerable decline in activity of enzyme. It should be noted that the effects on substrate distribution are very important.

The catalytic activity of the enzyme is in relation with the substrate which coming into contact with them. The substrate behavior is affected by the surfactant concentration when it distributes between micellar interface and the organic phase. With regards to the kinetics of enzymatic catalysis in reversed micelles, a pseudophase model proposed considers that a part of the overall substrate remains in the micellar sub-phase in close contact with the surfactant due to the electrostatic affinity or hydrophobic adsorption. The increase on surfactant concentration will decrease the substrate accessed by the enzyme, and consequently reduces the activity and also surfactant interacts with the enzyme accelerating the denaturing process.

Table-1 shows the catalytic constants of HRP in microemulsions and in aqueous solutions. The oxidation of ODA by HRP in reversed micelles exhibit a catalytic constant value that is appreciably different from the $K_{cat}$ of the reaction in aqueous buffers. The oxidation of ODA by HRP at the expanse of hydrogen peroxide have been studied in the anionic and non-ionic surfactant system. It was observed that catalytic activity of HRP in miceller system is higher than in aqueous buffer, this is often referred to as ‘superior-activity’. No simple explanation is given for these phenomena. Only the speculation regarding the structural changes of the enzyme
substrates concentration or the physical nature of the water in the aqueous microdomains have been put forward. The $k_{\text{cat}}$ value of HRP in AOT is $9.5 \times 10^7$ and in TX-100 it is $3.4 \times 10^8$. For non-ionic surfactant systems, the intra and intermicellar head group repulsions are much smaller than for ionic systems, which are often held responsible for the high value of $k_{\text{cat}}$. On the other hand cationic surfactants (e.g. CTAB), owing to its positive charge, are basically known to cause attenuation in efficiency of surface-active enzymes through inhibition at the active site of solubilized enzymes. Which is clear from the comparative graph of absorbance vs time shown in figure-5a. But in mixed reverse micelles activity can be improved, compared to that in CTAB probably due to the reduced positive charge density. From the figure-5b and table-2, the highest activity was observed in TX-100-CTAB reverse micellar system. Interestingly, this observed activity is even higher than that obtained in AOT/n-hexane reverse micelles, the most popular W/O microemulsion in micellar enzymology. In every instance, the activities of biocatalysts were improved dramatically, even sometimes better compared to that observed in the AOT-based microemulsion presumably due to the enhanced surface area at the reverse micellar interface or the size of water-pool and thus increasing the local molar concentration of the enzyme and substrate.

### 4.6 CONCLUSION

To retain the maximum activity of the enzyme in the micellar mediated encapsulated particles, it is always necessary to optimize the conditions of reverse micellar nanoreactor so that the enzyme in the precursor micellar systems exhibits maximum possible activity.

It is found that, there are no significant changes in the kinetic behavior and a Michaelis-Menten behaviour has been observed in reverse micellar solutions just as in water. The maximal activity in reverse micelles is not found at the maximal water content (i.e. $W_0$ around 50 to 70) but at rather low $W_0$ values ($W_0$- 21) and bell-shaped curve has been found. The introduction of additional components i.e. co-surfactant,
electrolytes usually alter the properties of particles and hence the phase behavior of the system. Cationic surfactants such as CTAB that form reverse micelles require co-solvents or co-surfactants to form a reverse micellar phase in apolar solvents. In non-ionic and cationic surfactant sometimes n-hexanol act as a competitive inhibitor. In the reverse micelles of sodium bis-(2-ethylhexyl)-sulfosuccinate, in hexane, the most common and best characterized solvent system. To date, the anionic surfactant Aerosol OT (AOT) has been particularly at the center of focus in the field of reverse micellar enzymology in which enzyme HRP show superactivities. AOT reverse micelles are commonly used for the solubilization of enzymes and proteins in apolar solvents because they readily dissolve in these solvents and can form reverse micelles without co-surfactants.
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