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ENZYME ENCAPSULATION IN BIOTECHNOLOGY BY REVERSED MICELLAR SYSTEMS

Definition of reversed micelles

Reversed micelles, also named water–in–oil microemulsions, are water droplets dispersed in organic media obtained by the addition of amphiphile molecules (surfactants). The surfactants molecules are characterised by two–tailed structures. The charged head–groups pointing to the inner side of the water droplets being the main responsible to solubilize water while the hydrocarbon tail facing the organic solvent (Fig. 1).

Surfactants

Surfactants able to form reversed micelles can be anionic, cationic, zwitterionic and non–ionic, which sodium di–2–ethylhexylsulfosuccinate (AOT), hexadecyl–trimethylammonium bromide (CTAB) and poly(oxyethylene(tetramethylbutyl) phenylether) (Triton X–100) are the most common synthetic surfactant used, respectively. The natural biocompatible emulsifiers such as the phospholipid lecithin and derivatives offer an alternative to synthetic surfactants.

Nature of co–surfactants

Many of the available surfactants have a limited solubility in aliphatic organic solvents, and addition of a co–surfactant is necessary to help surfactants to be dissolved in organic solvents and to form suitable reversed micelles to host target proteins and other biomolecules. However, the use of co–surfactants increases the complexity of phase diagrams and difficult to develop a physical picture of the reversed micellar system and their host solutes. It is commonly accepted that co–surfactants usually interpose between the surfactant chains increasing the interface flexibility and the inter droplet interaction.

Type of organic solvent

The type of organic solvent affects the size of the reversed micelles and consequently the water solubilisation capacity. A different micellar structure resulting from the solvent effect can be exploited to

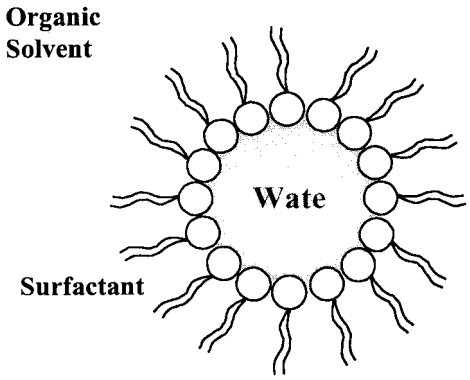

Figure 1. A schematic representation of a reversed micellar iso-octane/water: (b) reversed micelles.

achieve significant changes in the solubilisation of specific protein with different solvents. Several solvents including isooctane, octane, heptane, hexane, cyclohexane, and few others were used to form AOT reversed micelles to encapsulate peroxidases, lipases and other enzymes.

The choice of the solvent is usually determined by its compatibility with surfactant and biocatalyst, and related with the stability of biocatalysts with the logarithm of the partition coefficient of the organic solvent in the system octanol/water (log P). Aliphatic solvents are preferable for use in reversed micellar systems, since they are less likely to denature proteins as compared with aromatic, alcohol, and chloric solvents. However, due to the high hydrophilicity of anionic head groups, such as sulphonic or phosphoric acid, a surfactant with a relatively long hydrophobic tail is required to achieve dissolution in such hydrophobic solvents.

Characteristics of reversed micelles

Reversed micelles are commonly characterised by the parameter Wo, from water–in–oil microemulsion that is calculated by mass balance or measured by Karl Fischer titration, as the ratio between of water to surfactant concentrations. The aqueous core located in the interior of reversed micelles has been characterised by several techniques and the water inside has physico–chemical properties namely pH, viscosity and polarity, different from those of bulk water due namely a structural change in the hydrogen–bonded network.

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Reversed micelles are characterised by nanometer–sized (1–10 nm) water droplets, determined by several techniques, namely, dynamic light scattering, that accomplished with a higher interfacial area (about 100 m^2/ml) than the conventional biphasic systems, minimising mass transfer problems. For this reason, reversed micellar systems form a single optically isotropic and thermodynamically stable liquid solution due to small size and singular solubility capacity of water or aqueous phase containing dissolved compounds which otherwise were insoluble in organic solvent.

The reversed micellar phase is formed in the oil–rich part of the ternary system surfactant/hydrocarbon/water. The AOT ternary system is one of must well defined and characterised (Fig. 2). Among the cationic surfactants, the system CTAB and hexanol as co–surfactant is one of the most extensively studied. The reversed micelles formed by AOT are especially interesting, since they are very stable and are known to solubilize large amounts of water (Wo up to 60) and dissolved compounds, in particular proteins and other biomolecules, over a wide range of concentrations.

Results from different works and techniques, especially dynamic light scattering, are quite coincident for AOT reversed micelles and show a linear increase of the droplet water core size with an increase in the Wo almost independent of droplet concentration and organic solvent.

Water droplet radius (Å) = 1.65*Wo + 3.3

The water molecules are strongly bound to the surfactant polar groups of AOT and to the sodium counter–ions. Some authors consider that "free" water molecules seem to be available only at Wo values larger than about 5–6.

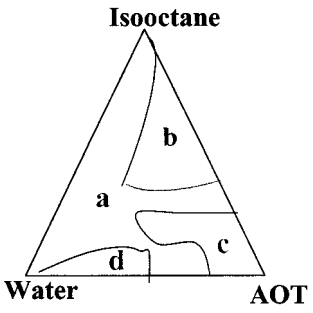


Figure 2. Ternary phase diagrams for AOT/systems (water pool, surfactant, organic solvent).

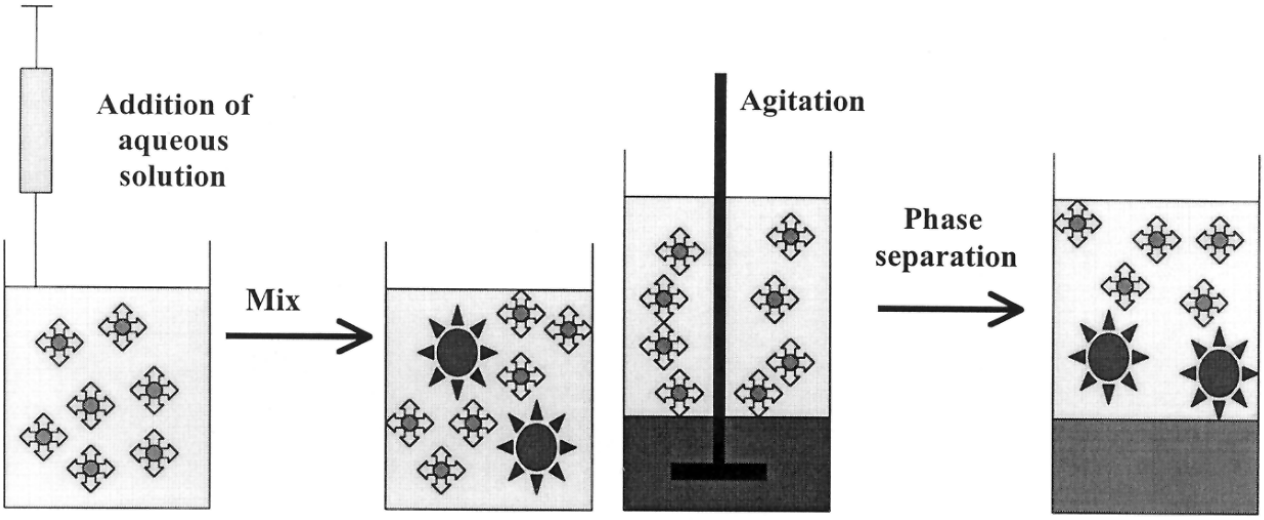
Preparing reversed micelles

There are basically two methods for preparing reversed micelles. In the injection method, a small amount of aqueous solution (containing the dissolved biomolecules) or few mg of dry lyophilized biomolecule, to be encapsulated inside the reversed micelles, are injected into the organic phase loaded with a surfactant (Fig. 3a). Alternatively, the phase transfer method consists in the transfer of water and hydrophilic solutes from the aqueous phase to the organic phase loaded with the surfactant by mixing both phases and phase separation occurs after equilibrium is attained (Fig. 3b).

Localisation of biomolecules in reversed micelles

Reversed micelles can exchange their contents according to a fusion–fusion mechanism based on an encounter process that should be diffusion limited. The proteins can be encapsulated in the water pool or interface of the reversed micelles according to their physico–chemical properties in particular charge and hydrophilic/hydrophobic characteristics. Electrostatic interactions between the biomolecules such as proteins and the charged surfactant are also a major driving force for their solubilization due to the presence of large ionic domains in external surface of the proteins.

The proteins encapsulated in the water pool surrounded by a water–shell (Fig. 4) are the result of



(a) Injection method; (b) Phase transfer method.

Figure 3. Methods for Preparing reversed micelles.

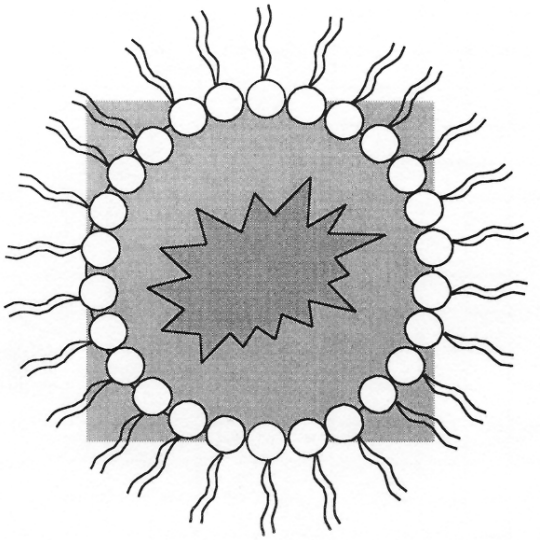


Figure 4. Protein encapsulated in the water pool.

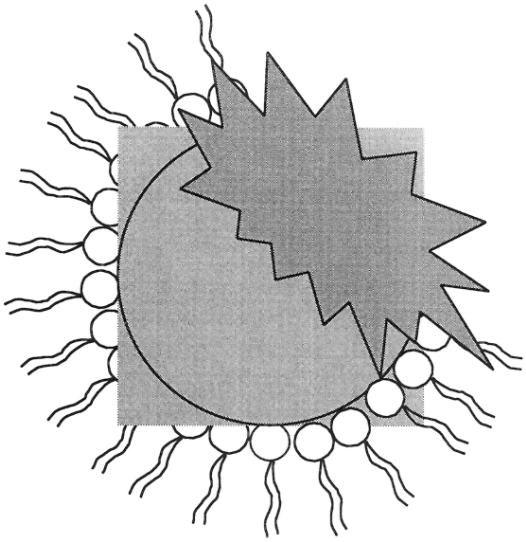


Figure 5. Protein anchored at the AOT interface acting as additional surfactant.

fusion of n empty molecules which means an increase of the dimensions of the resultant micelle but the Wo value per micelle remains the same upon entry of the protein. On the other hand, other authors pointed to no significant effect on the size of the reversed micelle upon solubilization of the proteins except when the inner cavity of the micelle is smaller than the effective protein size (induced fit model).

Other proteins must be localised at the AOT interface as the hydrodynamic radius through dynamic light scattering of the reversed micelles, for instance, with cutinase encapsulated is lower than the radii for empty reversed micelles below Wo 10. This is only explained by this enzyme anchored at the AOT interface acting as additional surfactant (Fig. 5). Electrostatic interactions between the AOT molecule and a positively charged area on the surface of cutinase, or a hydrophobic interaction are possible mechanisms to explain the interfacial localisation. However, the optimisation of experimental protocol and careful selection of surfactants and organic solvents should be previously performed due the high sensibility of proteins to microenvironment changes especially in presence of those chemical compounds.

Characteristics of proteins encapsulated in reversed micelles

It has been, in general, verified that low level of micelle occupancy by proteins occurs in relation to the all reversed micelles available. The dynamic light scattering data for cutinase were obtained for 44 mM protein concentration, which implies that only 3 to 21% of reversed micelles are filled with cutinase, depending on the Wo value. One explanation for less than 100% occupancy is probably due to a process of protein complexing with surfactant molecules to form an insoluble precipitate or another possible explanation is protein aggregation in the aqueous phase which do not solubilize due to steric effects.

Another characteristics observed with some protein encapsulation in reversed micelles is the enhancement of their stability in relation to aqueous solution. However, this stability, in general, critically depends on the Wo value. Stability rate constants for cutinase increase from 0.1 h–1 at Wo 5 to 8 h–1 at Wo 20. For instance, cutinase is highly stable in the reversed micellar system of CTAB/hexanol/isooctane with no unfolding detected for more than 50 days at Wo 20. The high stability of cutinase in these reversed micelles is at least partly due to the presence of hexanol. The effect of polar alcohols on stability is due to the strengthening of intramicellar attractive interactions and also due to an increment of interfacial flexibility that hinders the enzyme–surfactant interactions (especially important when anionic denaturing surfactants, such as, CTAB and AOT are present).

Applications in biotechnology of encapsulated proteins in reversed micelles

Reversed micelles have been successfully used to encapsulate biological molecules such as amino acids and small peptides, nucleic acids and mainly proteins. Reversed micelles have been widely used by our research group to extend the classical liquid–liquid extraction with organic solvents to protein bioseparation towards simultaneously concentration and purification, for instance, cutinase, cytochrome B5, penicillin acylase, and lipases. Additionally, reversed micelles has been also used in large number of enzyme biocatalysis in presence of a bulk organic solvents allowing synthetic and hydrolysis reactions to be performed via a control of water content and the solubilization of hydrophilic substrates.

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[1] E.P. Melo et al. (2001) Reverse micelles and protein biotechnology. Biotechnology Annual Review, M.R. El.Gewely (Ed.), 7, Elsevier Science, 87–129.