# Two-Phase Systems with Solidified Water Phases – Tools for Technical Use of Sensitive Catalysts

M.B. Ansorge-Schumacher*

*Technical University of Berlin, Institute of Chemistry, Enzyme Technology (TC4), D-10623 Berlin, Germany*

**Abstract**: Aqueous-organic two-phase systems are important tools in organic synthesis to enable reactions that employ compounds with incompatible solubilities. For technical application, different strategies have been developed to prevent coalescence without vast energy input and deactivation of sensitive compounds. A particularly promising one is the solidification of the aqueous phase by forming small distinguished compartments with polymers. The respective systems are assessed with special emphasis on the utility for enzyme catalysed synthesis in organic solvents.

**Keywords:** Biphasic system, enzyme catalysis, solidification, compartmentation, hydrogel, silicone.

Biphasic aqueous-organic reaction systems are important tools in technical chemical processes, particularly where reactants or catalysts with restricted solubility or stability are involved. The individual partition of reactants and products between immiscible phases enables the continuous supply of catalysts located in a favourable reaction environment with educts of low solubility and/or the extraction of products into the non-reactive phase. The clever set-up of partition equilibria can thus ensure high conversion even for reactions with an unfavourable thermodynamic equilibrium [1-3].

A general advantage of using biphasic instead of monophasic reaction systems arises particularly when enzymes are applied to mediate the reaction of hydrophobic compounds. These biocatalysts usually require aqueous solutions as reaction medium, i.e. hydrophobic compounds are in high dilution. This prevents the reaction from gaining maximum velocity, decreases productivity, and complicates downstream processing [4-6]. The overall number of technical processes using such systems is therefore rather limited to date [7]. Biphasic systems help overcome these limitations by minimising the volume of water, keeping educt supply at a maximum, and concentrating products before processing [8-10]. Transfer to a technical scale, however, requires further adaptations.

A major problem in the technical application of standard biphasic reaction systems is the extensive energy input required to keep phases emulsified and thus ensure a proper transfer of educts and products. Enzyme catalysed syntheses additionally suffer from deactivation of biocatalysts [11, 12] due to irreversible unfolding of the three-dimensional active structure upon direct contact to the aqueous-organic interface [13]. Both problems are overcome or reduced when mixing is restricted to the organic phase and movement of catalysts within the aqueous phase is minimised. This can be achieved by stable emulsification of the aqueous phase within the organic solvent. Three general strategies for such a concept can currently be recognised: formation of microemulsions, liquid crystals, and polymer matrices.

**Microemulsions** or **reverse micelles** are thermodynamically stable fine dispersions of water in a hydrophobic solvent. They spontaneously form in the solvent-rich part of a ternary system of water, solvent, and surfactant. The amphiphilic surfactant molecules build monomolecular layers around the water droplets with hydrocarbon tails facing the organic solvent and polar head-groups pointing inwards. Size, shape and solubilising ability of the micelles strongly depend on the nature of the employed surfactants [14]. In biocatalytic systems, sodium(bis-2-ethylhexyl)sulfosuccinate (AOT) has most often been employed achieving good activities with a variety of enzymes, particularly lipases used for esterification and transesterification reactions [14, 15]. However, technical applicability of microemulsions is limited by severe problems with continuous operation, denaturing effects of surfactants, separation and processing of products, and recycling of valuable biocatalysts [15]. Continuous operation can slightly be improved by creating a solid reverse micelle mimetic system termed a microemulsion-based gel (MBG), or organogel [16] via addition of gelatine to a microemulsion of AOT/isooctane (or heptane)/water [17-19] or addition of water to lecithin reverse micelles [20, 21]. The resulting gel materials appear to be almost indefinitely stable if stored in a closed container, and show good catalytic activities in esterification reactions with lipases [22-24]. The mechanical strength of these organogels, however, is very weak and consequently restricted to an application on rather small scale.

**Liquid crystals** are highly viscous to gelatinous thermodynamically stable lyotropic mesophases formed in definite regions of ternary systems consisting of water, solvent and surfactant. Their two- or three-dimensional structure can be hexagonal, cubic, or lamellar, whereas cubic inverse phases are mechanically most stable and thus most interesting for application in biocatalysis [25]. Overlayed with non-miscible organic solvents liquid crystals form stable two-phase systems which compensate problems occurring with reverse micelles, i.e. continuous operation, product and catalyst separation (see preceding paragraph). Several enzymes such as acid phosphatase from wheat germ and alkaline phosphatase from calf intestine, horseradish peroxidase, yeast alcohol dehydrogenase (E.C.1.1.1.1), and mandelate racemase from *Pseudomonas putida* (E.C.5.1.2.2) have been found to display good activities in the hydrolysis of p-nitrophenyl phosphate, the oxidation of pyrogallol, the oxdidation of cinnamic alcohol, and the racemisation of *D*-mandelic acid, respectively, when entrapped in liquid crystals [25-27], sometimes displaying enhanced stability. For use in technical processes, however, additional mechanical stabilisation of the crystals is mandatory. This can be accomplished by attachment to membranes, but usually only at the cost of reaction productivity [26].

**Polymer matrices** are three-dimensional porous networks formed by cross-linking of precursor molecules from various natural and synthetic polymers, synthetic monomers, or inorganic compounds. In many polymer matrices lyophilised or dissolved enzymes can be embedded during network formation [28-35] giving gelatinous to solid catalytically active units that facilitate reuse of the biocatalyst in subsequent reaction cycles or continuous operation in reactors such as batch, plug-flow, or fluidised-bed. A concise overview of types and formation mechanisms commonly used for biocatalyst entrapment is given in Table **1**.

In the presence of hydrophobic solvents polymer matrices with sufficient hydrophilicity form aqueous-organic two-phase systems with a solidified water phase [36-42], while hydrophobic matrices can sometimes stabilise water droplets within their polymer network and

*Address correspondence to this author at the Technical University of Berlin, Institute of Chemistry, Enzyme Technology (TC4), D-10623 Berlin, Germany; Tel: +49-30-314-22127; Fax: +49-30-21126;
E-mail: m.ansorge@chem.tu-berlin.de

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thus form a static emulsion [43]. Both concepts are promising approaches to realise biphasic biocatalysed processes in a technical scale and shall be discussed in more detail.

**Table 1. Mechanisms and Materials for Entrapment of Biocatalysts in a Matrix**

| Mechanisnm of Matrix Formation | Material | |
|---|---|---|
| | **Natural** | **Synthetic** |
| Thermal gelation | Agar, Agarose, κ-Carrageenan, Gelatine, Gellan gum | Polyvinyl alcohol |
| Ionotropic gelation | Alginate, Chitosan, Pectate | - |
| Photo-induced cross-linking | - | Photo-sensitive resin prepolymers |
| Precipitation | Cellulose, Cellulose triacetate | Polystyrol |
| Polymerisation | - | Polyacrylamide, Polymethacrylate, Polyacrylamide hydrazide<br>Photo-cross-linkable resin prepolymers |
| Polycondensation | - | Polyurethane, Epoxy resin |

## AQUEOUS-ORGANIC TWO-PHASE SYSTEMS WITH SOLIDIFIED WATER PHASE

Solidification of aqueous solutions can potentially be achieved by use of hydrogels, i.e. polymer networks with the ability to imbed large quantities of water without subsequent dissolution. Many hydrogels such as polyethylene glycol, polyurethane, alginate, or κ-carrageenan retain their matrix integrity in the presence of non water-miscible organic solvents [14] and thus are suitable material for the formation of biphasic systems. Ionic strength, pH, cofactor concentrations, and other components in the hydrogel can be adapted within wide limits before or after addition of the matrix forming polymer, i.e. individual demands of entrapped catalysts can be accounted for almost regardless of the employed polymer. Also, size and shape of the gels can be designed to match individual requirements of process and biocatalyst stability. Consequently, a rather wide spectrum of different enzymes, ranging from simple lipases [38, 39, 44-46] to complex cofactor-dependent enzymes such as alcohol dehydrogenase from *Lactobacillus kefir* (ADH, E.C.1.1.1.1) [41], carbonyl reductase from *Candida parapsilosis* (CPCR, E.C.1.1.1.1) [42], and benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I (BAL; E.C.4.1.2.38) [47], as well as multi-enzyme systems [41, 42] have successfully been applied to syntheses in gel-stabilised two-phase systems. Use of these systems enabled conversion of hydrophobic compounds with considerably higher productivity than in alternative reaction systems [47], and catalyst stability increased dramatically compared to the stability in pure organic solvents or standard aqueous-organic two-phase systems [41, 42, 48].

The actual synthetic-technical properties of two-phase systems with solidified aqueous phase are influenced by many parameters, particularly the exact composition of the participating phases, and the nature of the gel forming polymer. The ability to solubilise reactants determines the availability of substrates within the gels as well as the elimination of products into the continuous phase. At the same time, the stability of the gel modules and their ability to get suspended or fluidised in a reactor strongly depend on the nature of polymer and solvent. Alginate beads, for example, can only be suspended in a stirred-tank reactor when butanol, hexanol, or octanol are used as solvent [46], while gels from polyvinyl alcohol (PVA) easily agglomerate in hexane [42]. Furthermore, the volume of the hydrogel within the two-phase system can only be kept constant in solvents with a low capacity for water uptake or after previous saturation with water [46].

Chemical and physical robustness of the hydrogel matrices during technical application are mainly determined by the gel forming polymer. However, among the many available materials (see Table **1**), only few combine the required minimum strength with mild gelation conditions and compatibility with the entrapped biocatalysts. While polymers from natural sources such as alginate, κ-carrageenan, or gellan enable entrapment under very gentle conditions and subsequently with a very high residual activity, they lack the mechanical strength required for long-term operation in standard type reactors [49]. Synthetic polymers like acrylate derivatives, on the other hand, reveal excellent resistance against mechanical stress [50, 51], but often cause a considerable deactivation of enzymes before or during polymerisation [50-55]. Nevertheless, a material with promising properties has obviously been found in polyvinyl alcohol (PVA), a synthetic polymer that forms an elastic gel when a concentrated solution of the polymer (10% w/v; polymerisation degree 1,400) and polyethylene glycol 1,000 (10% w/v) is emulsified in chilled silicone oil (-20°C) and slowly thawed to room temperature [41, 42, 47]. The obtained beads have an optimal shape in terms of abrasion and hydromechanical properties in stirred-tank and fluidised-bed reactors [56]. As an additional advantage of this emulsification process no enzymes and cofactors are lost from the matrix during entrapment because of the insolubility of these components in the oily hardening solution.

## STATIC EMULSION

A new type of polymer stabilised two-phase systems for biocatalysis termed static emulsion has been described recently [43]. It consists of a macroscopically large hydrophobic silicon matrix (Ø 0.5 mm to 4 mm) entrapping micrometer-sized droplets of water (Ø 5 μm to 60 μm) with dissolved biocatalysts. Manufacture of these emulsions involves two emulsification steps, first of an aqueous solution in a mixture of silicon prepolymers Sylgard®184 and methylhydrosilan, and second of this slightly pre-solidified emulsion in a solution of polyvinyl alcohol. Up to 25% (w/w) aqueous phase can be included in the silicon matrix without negative impact on the matrix integrity. Thus, considerable amounts of dissolved biocatalyst can be embedded.

Static emulsions have been successfully applied to the entrapment of lipases where an exceptionally high activity was obtained for the esterification of octanol and caprylic acid. Lipase A from *Candida antarctica* (CALA) in static emulsion revealed a 31fold higher activity than the native enzyme under comparable conditions. Lipase from *Thermomyces lanuginosa* (TLL) showed an even 250fold increase in activity. This activation was also better (CALA) or comparable (TLL) to the best activities reported for these enzymes after entrapment in sol-gels, the most successful material for entrapment of lipases to date [56, 57]. However, unlike the brittle sol-gels, static emulsions are highly elastic and were of excellent mechanical robustness during technical application in standard type reactors. Thus, they are most promising material for the scale-up of lipase catalysed reactions in biphasic reaction systems. It will have to be seen, if they can also provide compatible systems for more complex synthetically valuable enzymes, or even for chemical homogeneous catalysts.

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