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Enzyme Immobilization: The Quest for Optimum Performance

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Abstract: Immobilization is often the key to optimizing the operational performance of an enzyme in industrial processes, particularly for use in non-aqueous media. Different methods for the immobilization of enzymes are critically reviewed. The methods are divided into three main categories, *viz.* (i) binding to a prefabricated support (carrier), (ii) entrapment in organic or inorganic polymer matrices, and (iii) cross-linking of enzyme molecules. Emphasis is placed on relatively recent developments, such as the use of novel supports, e.g., mesoporous silicas, hydrogels, and smart polymers, novel entrapment methods and cross-linked enzyme aggregates (CLEAs).

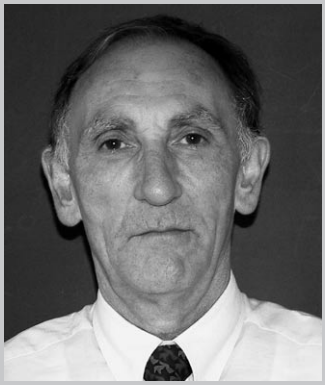
Keywords: biotransformations; cross-linked enzyme aggregates; entrapment; enzymes; immobilization; support binding

1 Introduction

In the drive towards green, sustainable methodologies for chemicals manufacture[1] biocatalysis has much to offer:[2] mild reaction conditions (physiological pH and temperature), a biodegradable catalyst and environmentally acceptable solvent (usually water), as well as high activities and chemo-, regio- and stereoselectivities. Furthermore, the use of enzymes generally obviates the need for functional group protection and/or activation, affording synthetic routes which are shorter, generate less waste and, hence, are both environmentally and economically more attractive than traditional organic syntheses.

Modern developments in biotechnology have paved the way for the widespread application of biocatalysis in industrial organic synthesis.[3–14] Thanks to recombinant DNA techniques[15] it is, in principle, possible to produce most enzymes for a commercially acceptable price. Advances in protein engineering have made it possible, using techniques such as site-directed mutagenesis and *in vitro* evolution *via* gene shuffling,[16–19] to manipulate enzymes such that they exhibit the desired properties with regard to, *inter alia*, substrate specificity, activity, selectivity, stability and pH optimum. Nonetheless, industrial application is often hampered by a lack of long-term operational stability and difficult recovery and re-use of the enzyme. These drawbacks can often be overcome by immobilization of the enzyme.[20–25]

There are several reasons for using an enzyme in an immobilized form. In addition to more convenient handling of the enzyme, it provides for its facile separation from the product, thereby minimizing or eliminating protein contamination of the product. Immobilization also facilitates the efficient recovery and re-use of costly enzymes, in many applications a *conditio sine qua non* for economic viability, and enables their use in continuous, fixed-bed operation. A further benefit is often enhanced stability,[26] under both storage and operational conditions, e.g., towards denaturation by heat or organic solvents or by autolysis. Improved enzyme performance *via* enhanced stability and repeated re-use is reflected in higher catalyst productivities (kg product/kg enzyme) which, in turn, determine the enzyme costs per kg product. As a rule of thumb the enzyme costs should not amount to more than a few percent of the total production costs. In



Roger Sheldon (1942) is Professor of Biocatalysis and Organic Chemistry at Delft University of Technology (Netherlands). He received a PhD in organic chemistry from the University of Leicester (UK) in 1967. This was followed by post-doctoral studies with Prof. Jay Kochi in the USA. From 1969 to 1980 he was with Shell Research in Amsterdam and from 1980 to 1990 he was R&D Director of DSM Andeno. His primary research interests are in the application of catalytic methodologies – homogeneous, heterogeneous and enzymatic – in organic synthesis, particularly in relation to fine chemicals production. He developed the concepts of E factors and atom utilization for assessing the environmental impact of chemical processes.

the production of 6-aminopenicillanic acid (6-APA) by penicillin G amidase (penicillin amidohydrolase, E.C. 3.5.1.11)-catalyzed hydrolysis of penicillin G, for example, 600 kg of 6-APA are produced per kg of immobilized enzyme and the production of the commodity, fructose, by glucose isomerase-catalyzed isomerization of glucose has a productivity of 11,000.[22] Indeed, the development of an effective method for its immobilization was crucial for the application of penicillin amidase in the industrial synthesis of β-lactam antibiotics.[27]

Immobilization is generally necessary for optimum performance in non-aqueous media. In the traditional method of using enzymes as lyophilized (freeze-dried) powders, many of the enzyme molecules are not readily accessible to substrate molecules. Furthermore, lyophilization can cause pronounced structural perturbations often leading to deactivation. In contrast, dispersion of the enzyme molecules by immobilization generally provides for a better accessibility and/or an extra stabilization of the enzymes towards denaturation by the organic medium.

Another benefit of immobilization is that it enables the use of enzymes in multienzyme and chemoenzymatic cascade processes (see later).[28–31] A major problem encountered in the design of catalytic cascade processes is the incompatibility of the different catalysts and a possible solution is compartmentalization (i.e., immobilization) of the different catalysts thus circumventing their mutual interaction which could result in inhibition and or deactivation.

In this review we shall focus on recent developments in novel immobilization techniques, such as the use of novel supports, e.g., smart polymers, novel entrapment methods, and the recent development of cross-linked enzyme aggregates (CLEAs), in the quest for optimum performance in biotransformations. Another approach to facilitating the recovery and re-use of enzymes is to perform the reactions, with the free enzyme, in a membrane bioreactor consisting of an ultrafiltration membrane which retains the enzyme on the basis of its size but allows substrates and products to pass through. This combines ease of recovery and recycling with the high activity of the free enzyme. The methodology is applied commercially, for example, by Degussa in the commercial synthesis of enantiomerically pure amino acids using hydrolases or dehydrogenases.[32] However, since it involves the free enzyme it falls outside the scope of this review.

2 Types of Immobilization

Basically, three traditional methods of enzyme immobilization can be distinguished, binding to a support (carrier), entrapment (encapsulation) and cross-linking.

(i) *Support binding* can be physical (such as hydrophobic and van der Waals interactions), ionic, or covalent in nature. However, physical bonding is generally too weak to keep the enzyme fixed to the carrier under industrial conditions of high reactant and product concentrations and high ionic strength. Ionic binding is generally stronger and covalent binding of the enzyme to the support even more so, which has the advantage that the enzyme cannot be leached from the surface. However, this also has a disadvantage: if the enzyme is irreversibly deactivated both the enzyme and the (often costly) support are rendered unusable. The support can be a synthetic resin, a biopolymer or an inorganic polymer such as (mesoporous) silica or a zeolite.

(ii) *Entrapment via* inclusion of an enzyme in a polymer network (gel lattice) such as an organic polymer or a silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule. The physical restraints generally are too weak, however, to prevent enzyme leakage entirely. Hence, additional covalent attachment is often required. The difference between entrapment and support binding is often not clear. For the purpose of this review we define support binding as the binding of an enzyme to a *prefabricated* support (carrier) irrespective of whether the enzyme is situated on the external or internal surface. Entrapment requires the synthesis of the polymeric network in the presence of the enzyme. For example, when an enzyme is immobilized in a prefabricated mesoporous

silica the enzyme may be situated largely in the mesopores but this would not be entrapment. On the other hand when the enzyme is present during the synthesis of a silica sol-gel the enzyme is entrapped.

(iii) *Cross-linking* of enzyme aggregates or crystals, using a bifunctional reagent, to prepare carrierless macroparticles.

The use of a carrier inevitably leads to 'dilution of activity', owing to the introduction of a large portion of non-catalytic ballast, ranging from 90% to >99%, which results in lower space-time yields and productivities.[22] Moreover, immobilization of an enzyme on a carrier often leads to the loss of more than 50% native activity,[33] especially at high enzyme loadings.[34] The design of carrier-bound immobilized enzymes also relies largely on laborious and time-consuming trial and error experiments, because of the lack of guidelines that link the nature of a selected carrier to the performance expected for a given application.[35] Consequently, there is an increasing interest in carrier-free immobilized enzymes, such as cross-linked enzyme crystals (CLECs),[36] and cross-linked enzyme aggregates (CLEAs).[37] This approach offers clear advantages: highly concentrated enzyme activity in the catalyst, high stability and low production costs owing to the exclusion of an additional (expensive) carrier.

It should be pointed out that, from the literally thousands of papers on enzyme immobilization, it is difficult to make comparisons of the different methodologies as most authors compare the performance of the immobilized enzyme, prepared using a particular technique, with that of the free enzyme but do not compare different methods of immobilization. In addition, details of the immobilization of industrial biocatalysts are often not disclosed.

3 Immobilization on Supports: Carrier-Bound Enzymes

The properties of supported enzyme preparations are governed by the properties of both the enzyme and the carrier material. The interaction between the two provides an immobilized enzyme with specific chemical, biochemical, mechanical and kinetic properties. The support (carrier) can be a synthetic organic polymer, a biopolymer or an inorganic solid.

3.1 Synthetic Organic Polymers

Acrylic resins such as Eupergit® C are widely used as supports. Eupergit® C is a macroporous copolymer of *N,N'*-methylene-bi-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide with average particle size of 170 μm and a pore diameter of 25 nm.[38] It is highly hydrophilic and stable, both chemically and mechanically, over a pH range from 0 to 14, and does not swell or shrink even upon drastic pH changes in this range. It binds proteins *via* reaction of its oxirane moieties, at neutral or alkaline pH, with the free amino groups of the enzyme to form covalent bonds which have long-term stability within a pH range of pH 1 to 12 (see Figure 1). The remaining

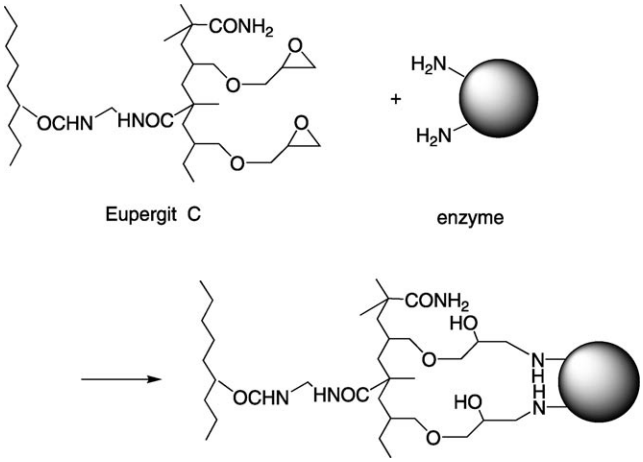


Figure 1. Immobilization of enzymes on Eupergit C.

epoxy groups can be rendered inactive by capping using a variety of reagents (mercaptoethanol, ethanolamine, glycine, etc.) to prevent any undesired support-protein reaction. Due to the high density of oxirane groups on the surface of the beads enzymes are immobilized at various sites of their structure. This "multi-point-attachment" is largely responsible for the high operational stability of enzymes bound to Eupergit® C.

Immobilization by covalent attachment to Eupergit C has been successfully applied to a variety of enzymes for industrial application.[27,38] Penicillin amidase on Eupergit C, for example, maintained 60% of its initial activity over >800 cycles.[38] A major drawback of Eupergit C is diffusion limitations, the effects of which, as would be expected, are more pronounced in kinetically controlled processes. Sepa beads FP-EP (Resindion, Milan, Italy) consist of a polymethacrylate-based resin functionalized with oxirane groups and exhibit characteristics similar to Eupergit C.[39]

Similarly, various porous acrylic resins, such as Amberlite XAD-7, are used to immobilize enzymes *via* simple adsorption without covalent attachment. For example, the widely used enzyme *C. antarctica* lipase B (CaLB),[40] is commercially available in immobilized form as Novozym 435 which consists of the enzyme adsorbed on a macroporous acrylic resin. A disadvantage of immobilization in this way is that, because

it is not covalently bound, the enzyme can be leached from the support in an aqueous medium. In a comparison of the immobilization of lipases of *Humicola lanuginosa, Candida antarctica* and *Rhizomucor miehei* on supports with varying hydrophobicity with Novozym 435 (see below), in the esterification of oleic acid with 1-butanol in isooctane, the highest activity was observed with sepa beads (see above) containing octadecyl chains.[41] This was attributed to the hydrophobic nature of the support facilitating opening of the hydrophobic lid of the lipase.

3.2 Biopolymers

A variety of biopolymers, mainly water-insoluble polysaccharides such as cellulose, starch, agarose and chitosan[42] and proteins such as gelatin and albumin have been widely used as supports for immobilizing enzymes. Indeed, the first industrial application of an immobilized enzyme in a biotransformation is the Tanabe process,[43] first commercialized more than 40 years ago, for the production of L-amino acids by resolution of racemic acylamino acids using an aminoacylase from *Aspergillus oryzae* (Figure 2). The enzyme was immobilized by ionic adsorption on DEAE-Sephadex which consists of cellulose modified with diethylaminoethyl functionalities (Figure 2) and the process was performed in continuous operation in a fixed-bed reactor.

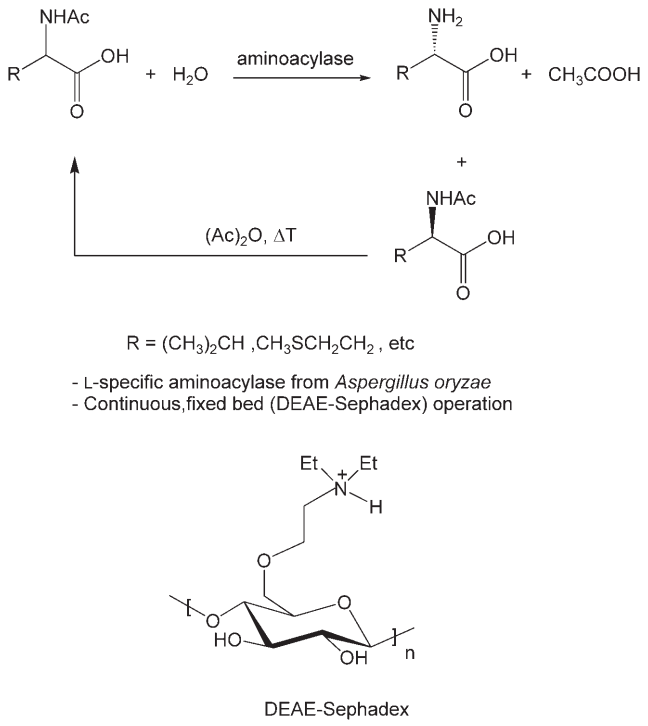


Figure 2. Tanabe aminoacylase process.

This method is still widely used, e.g., in the immobilization of a recombinant epoxide hydrolase from *Aspergillus niger*.[44] An activity retention of 70% was observed in the resolution of *p*-chlorostyrene oxide under biphasic conditions. The immobilized biocatalyst was active at high substrate concentrations (306 g/L) and could be recycled 7 times but it exhibited a slightly lower enantioselectivity compared to the free enzyme ($E=76$ *vs.* $E=90$).

3.3 Hydrogels

In non-aqueous media enzymes can also be immobilized in natural or synthetic hydrogels or cryogels. Polyvinyl alcohol (PVA) cryogels formed by the freeze-thawing method,[45] for example, have been widely used for immobilization of whole cells.[46] A mild and highly efficient method for preparing PVA hydrogels by partial drying at room temperature afforded lens-shaped hydrogels (Lentikats) exhibiting good mechanical stability, easy separation (diameter 3–5 mm and thickness 200–400 μm) and stability towards degradation.[47] Lentikats have been successfully used for the entrapment of whole cell biocatalysts,[48,49] for example, in the immobilization of whole cells of *Rhodococcus equi* A4, which contains nitrile hydratase and amidase activities.[49] However, free enzymes, owing to their smaller size, can diffuse out of the gel matrix and are, consequently, leached in an aqueous medium. In order to entrap free enzymes the size of the enzyme must be increased, e.g., by cross-linking (see later). In contrast, immobilization of free enzymes in PVA hydrogels can be useful in organic media, where the enzyme is not leached from the gel matrix. For example, Ansorge-Schumacher and coworkers reported the co-immobilization of an alcohol dehydrogenase (EC 1.1.1.1) from *Lactobacillus kefir* together with its co-factor, NADP, in PVA beads.[50] The resulting immobilisate was used for the enantioselective reduction of a broad range of hydrophobic prochiral ketones to the corresponding (*R*)-secondary alcohols (Figure 3) in *n*-hexane as solvent. For example, acetophenone afforded (*R*)-1-phenylethanol in >98% *ee*. Co-factor regeneration was achieved within the gel matrix using isopropyl alcohol as the co-substrate (total turnover number of the co-factor 10^2–10^3). In addition to stability towards the organic solvent, the immobilized biocatalyst showed improved thermal stability and long-term stability under the reaction conditions.

The same group reported the enantioselective benzoin condensation of aromatic and heteroaromatic aldehydes, in *n*-hexane as solvent, using a recombinant benzaldehyde lyase (BAL) from *Pseudomonas fluorescens* immobilized in a PVA hydrogel.[51] As noted above, in order to retain the enzyme in a PVA hydro-

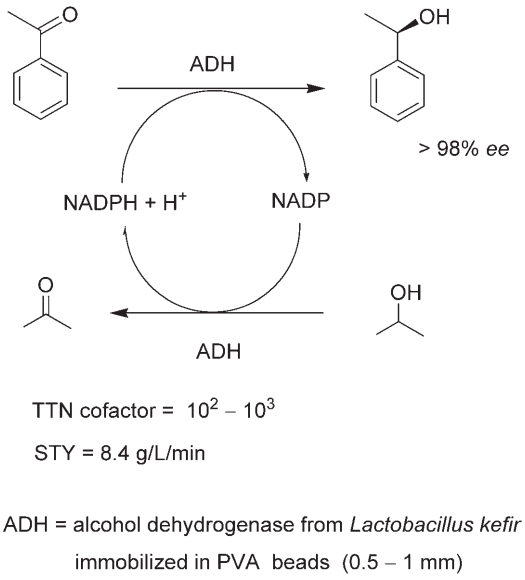


Figure 3. Alcohol dehydrogenase in Lentikat.

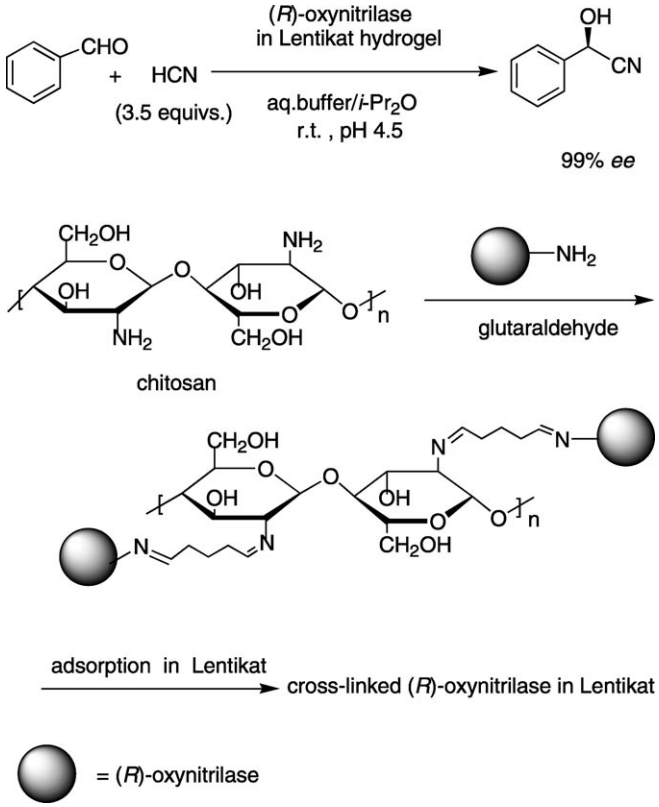


Figure 4. Cross-linked (*R*)-oxynitrilase in Lentikat hydrogel.

gel, in the presence of water, the molecular weight must be increased. Gröger and co-workers[52] achieved this by cross-linking an (*R*)-oxynitrilase using a mixture of glutaraldehyde and chitosan (Figure 4). The cross-linked enzyme was subsequently entrapped in a Lentikat PVA hydrogel. The resulting immobilized biocatalyst had a well-defined particle size of 3–5 mm and showed no leaching in the enantioselective hydrocyanation of benzaldehyde in a biphasic aqueous buffer/organic solvent system. It could be recycled 20 times without loss of yield or enantioselectivity.

An alternative method to increase the size of the enzyme is to form a complex with a polyelectrolyte. Owing to their ampholytic character, proteins exist as polycations or polyanions, depending on the pH of the medium. Hence, they can form complexes with oppositely charged polyelectrolytes. This principle was used by Dautzenberg and co-workers[53] to immobilize amyloglucosidase (EC 3.2.1.3) by coupling to an excess of a complex of sodium polystyrenesulfonate (PSS) or poly-diallyldimethylammonium chloride (PDM). The immobilized biocatalyst retained 45% of its activity and lost no activity over five cycles.

In an interesting recent development, directed evolution of the formate dehydrogenase (EC 1.2.1.2) from *Candida boidinii*, in two rounds of error-prone PCR, was used to create variants that were more suited for immobilization in a polyacrylamide gel.[54] The best mutant had a 4.4-fold higher activity compared to the free enzyme immobilized in the gel. The stabilization resulted from a substitution of lysine, glutamic acid and cysteine residues remote from the active site.

Bruns and Tiller have reported[55] a novel immobilization of enzymes in a prefabricated, nanophase separated, amphiphilic network (see Figure 5), whereby the enzyme is situated in its hydrophilic domains which consist of poly(2-hydroxyethyl acrylate) (PHEA). The substrate that diffuses into the hydrophobic polydimethylsiloxane (PDMS) phase can access the enzyme *via* the large interface, owing to its nanophase separation and peculiar swelling properties. The amphiphilic network was synthesized by UV-initiated radical copolymerization of a silylated 2-hydroxyethyl acrylate monomer and a bifunctional α,ω-methacroyloxypropyl-poly(dimethylsiloxane) (see Figure 5).The hydrophilic domain swells in the presence of water and the polymer is loaded with the enzyme by immersing it in an aqueous solution of the biocatalyst. Upon drying the phase shrinks, trapping the enzyme in an enzyme-friendly environment. When the polymer network is immersed in a non-polar organic solvent the hydrophobic PDMS domain swells allowing access of a dissolved substrate which can approach the enzyme *via* the interface between the two domains. Immobilization of horseradish peroxidase (HRP; EC 1.11.1.7) and chloroperoxidase (CPO; EC 1.11.1.10) in this way afforded immobilisates showing substantially enhanced activities and operational stabilities in heptane, compared to the free enzymes (see Figure 5). In contrast, no enhancement of activity was observed in isopropyl alcohol as solvent, which was

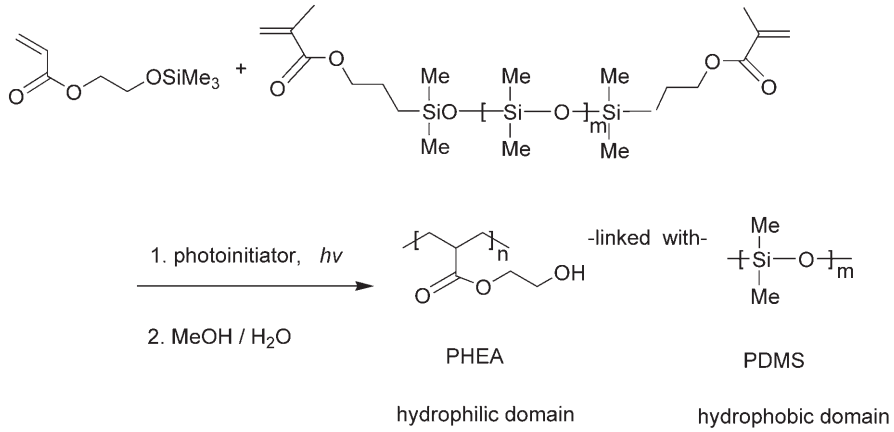


Figure 5. Amphiphilic polymer network for enzyme immobilization.

attributed to swelling of the PHEA phase rather than the PDMS phase in this polar solvent.

3.4 Inorganic Supports

A variety of inorganic solids can be used for the immobilization of enzymes, e.g., alumina, silica,[56] zeolites[57,58] and mesoporous silicas[59–62] such as MCM-41, and SBA-15. One of the simplest and most inexpensive methods to immobilize an enzyme is by silica granulation.[40] It is used, for example, to formulate enzymes for detergent powders which release the enzyme into the washing liquid during washing. Granulation technology was used to immobilize CaLB lipase on silica granules, by first adsorbing the lipase on silica powder followed by agglomeration.[40] Owing to the composition of the granulates, they are intended for use only in organic media. In an aqueous medium the lipase is desorbed and the particle slowly disintegrates. However, the CaLB silica granules can be used in a direct ester synthesis if the water is removed by, e.g., evaporation under vacuum. Applying the granules in packed-bed reactors also minimizes the contact time with high water concentrations. The CaLB silica granules exhibited a similar activity to Novozym 435 in the direct synthesis of the skin emollient, isopropyl myristate.

In order to maintain its integrity in an aqueous environment the enzyme needs to be covalently bonded to the silica support. For example, immobilization of epoxide hydrolase from *Aspergillus niger* by covalent attachment to functionalized silica resulted in 90% activity retention, which was retained over a period of months, in the enantioselective ($E=85$) hydrolysis of *p*-nitrostryrene oxide.[56] Furthermore, an enhanced stability towards 20% DMSO as solvent was observed and no leaching occurred in a filtration test.

Mesoporous silicas, which are nowadays often referred to as nanosilicas, have several advantages as supports: they have uniform pore diameters (2–40 nm), very high surface areas (300–1500 $m^2 g^{-1}$) and volumes (*ca.* 1 $mL g^{-1}$), and are inert and stable at elevated temperatures. Moreover, the surface can be easily functionalized. Because of the large pore sizes of these materials they can accommodate relatively small enzymes in the pores. Whether the enzyme is situated inside the pores or on the outer surface can be determined by comparing immobilization on calcined and non-calcined material (i.e., the latter still contains the template). If these values are roughly the same then most of the enzyme is on the outer surface. On the other hand, when the calcined material adsorbs much more enzyme this indicates that most of the enzyme is situated in the pores.[59]

Covalent binding of α-chymotrypsin (EC 3.4.21.2) to a mesoporous sol-gel glass, which had been modified by reaction of surface hydroxy groups with 3,3,3-trimethoxypropanal, afforded an immobilized catalyst with a half-life one thousand times that of the free enzyme.[61] Immobilization of chloroperoxidase (CPO) from *Caldariomyces fumago* on the same material resulted in increased stability towards organic solvents.[62] The immobilized preparation was used together with free glucose oxidase which generated hydrogen peroxide *in situ,*by aerobic oxidation of glucose.[62] Similarly, immobilization of *Mucor javanicus* lipase (EC 3.1.1.3) on functionalized silica nanoparticles resulted in enhanced thermal stability and a high retention of activity over a wider pH range (Figure 6).[63]

In a variation on this theme, Wang and Caruso[64] immobilized catalase (EC 1.11.1.6) on nanoporous silica spheres, having a surface area of 630 $m^2 g^{-1}$and mesopores with a pore size of up to 40 nm, and subsequently assembled a nano-composite shell coating composed of three layers of poly-dimethyldiallylammonium chloride (PDM) and 21 nm silica nanoparticles. The resulting immobilisate displayed an activity

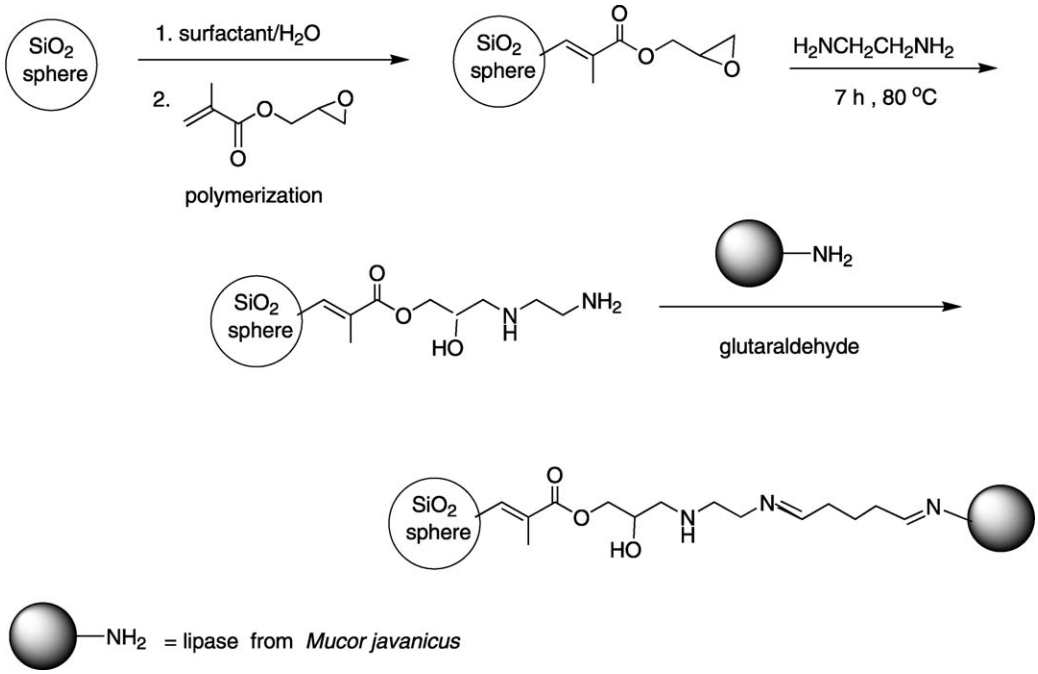


Figure 6. Immobilization of a lipase on silica nanoparticles.

75 times that of catalase immobilized on mesoporous silica spheres.

A novel type of immobilization on inorganic supports comprises the so-called protein-coated microcrystals (PCMCs).[65,66] The stabilization of lyophilized enzyme powders through the addition of lyoprotectants[67] and inorganic salts[68] is well documented. PCMCs are prepared by mixing an aqueous solution of the enzyme with a concentrated solution of a salt such as potassium sulfate (a sugar or an amino acid can also be used). The resulting solution is added dropwise with vigorous mixing to a water-miscible solvent such as isopropyl alcohol, whereupon micron-sized crystals, containing the enzyme on the surface, are formed. A major advantage of the technique is that the enzyme molecules are dehydrated by a mechanism that leaves the majority of the enzymes in an active conformation and minimizes denaturation. The PCMCs can be separated and stored or used as a suspension in an organic solvent. Obviously in an aqueous medium they dissolve to liberate the free enzyme. In a transesterification of *N*-acetyltyrosine ethyl ester with isopropyl alcohol (Figure 7) PCMCs of subtilisin Carlsberg (EC 3.4.21.62) exhibited an activity three

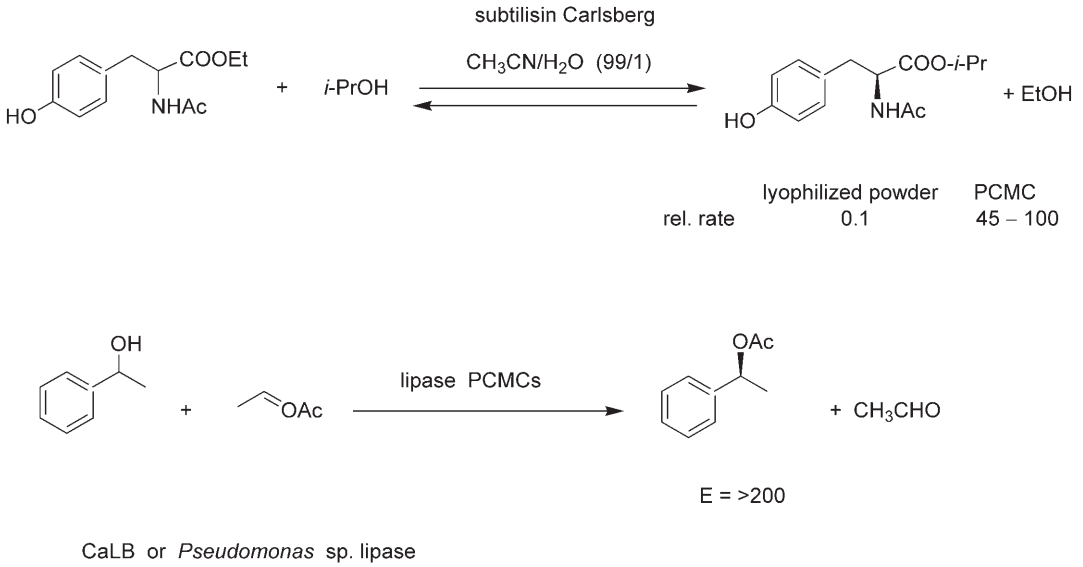


Figure 7. Resolutions with protein coated micro crystals (PCMCs).

orders of magnitude higher than that of the lyophilized powder.[65] Similarly, PCMCs of lipases showed a substantial rate enhancement in most cases, compared to the corresponding lyophilized powders, in the kinetic resolution (Figure 7) of 1-phenylethanol by transesterification.[65] A further elaboration of the technique employs solid state buffers as the support to be coated.[66] The technique was also successfully applied to oxidoreductases – an alcohol dehydrogenase, catalase, soybean peroxidase and horse radish peroxidase – affording PCMCs with enhanced activities in organic media.[66]

3.5 Smart Polymers

A novel approach to immobilization of enzymes is *via* covalent attachment to stimulus-responsive or 'smart polymers' which undergo dramatic conformational changes in response to small changes in their environment, e.g., temperature, pH and ionic strength.[69–71] The most studied example is the thermo-responsive and biocompatible polymer, poly-*N*-isopropylacrylamide (polyNIPAM). Aqueous solutions of polyNIPAM exhibit a critical solution temperature (LCST) around 32 °C, below which the polymer readily dissolves in water while, above the LCST it becomes insoluble owing to expulsion of water molecules from the polymer network. Hence, the biotransformation can be performed under conditions where the enzyme is soluble, thereby minimizing diffusional limitations and loss of activity owing to protein conformational changes on the surface of a support. Subsequently, raising the temperature above the LCST leads to precipitation of the immobilized enzyme, thus facilitating its recovery and reuse. An additional advantage of using such thermo-responsive immobilized enzymes is that runaway conditions are avoided because when the reaction temperature exceeds the LCST the catalyst precipitates and the reaction shuts down.

Two methods are generally used to prepare the enzyme-polyNIPAM conjugates: (i) introduction of polymerizable vinyl groups into the enzyme followed by copolymerization with NIPAM or (ii) reaction of NH_2 groups on the surface of the enzyme with a copolymer of NIPAM containing reactive ester groups or the homopolymer containing an *N*-succinimide ester function as the end group (Figure 8).

For example, penicillin G amidase (PA) was immobilized by condensation with a copolymer of NIPAM containing active ester groups.[72] The resulting enzyme-polymer conjugate exhibited hydrolytic activity close to that of the free enzyme and was roughly as effective as the free PA in the synthesis of cepha-

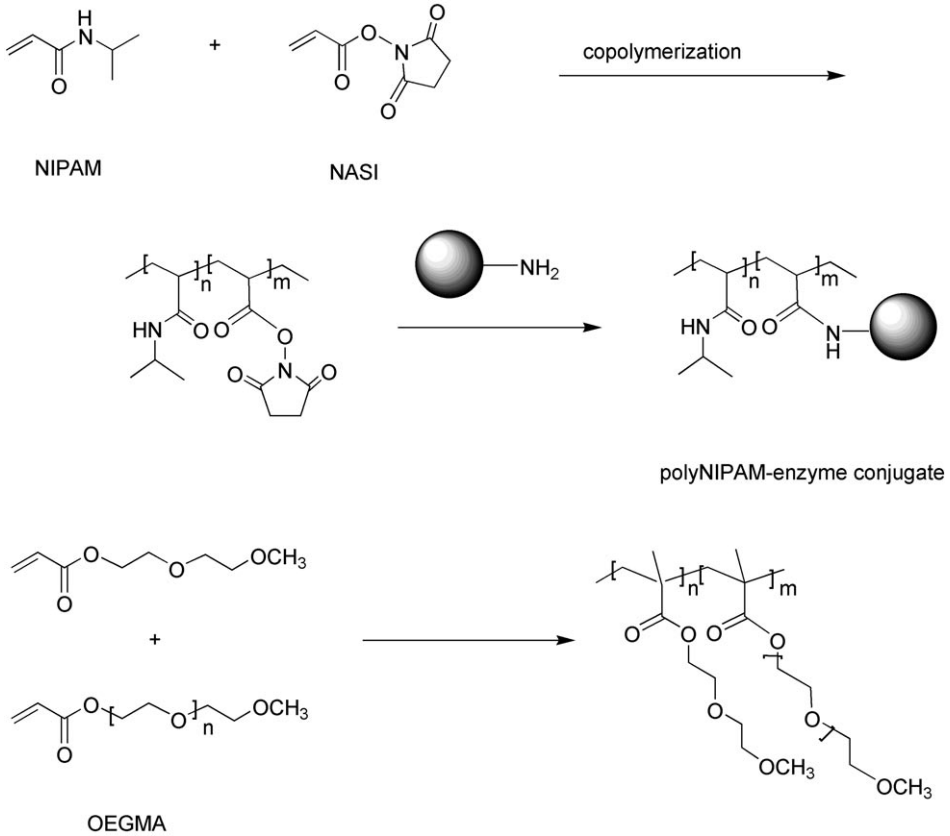


Figure 8. Thermoresponsive polymers for enzyme immobilization.

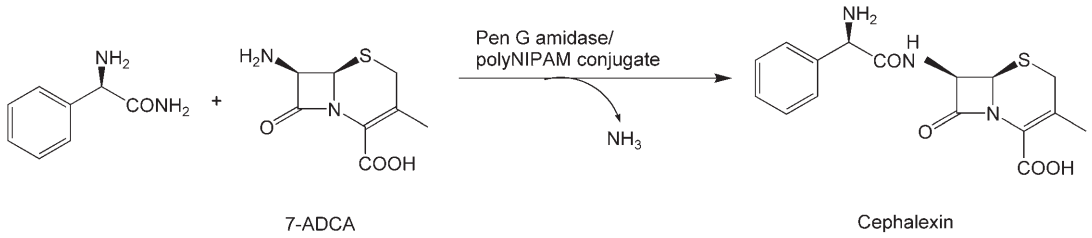


Figure 9. Cephalexin synthesis with pen G amidase/polyNIPAM conjugate.

lexin by reaction of D-phenylglycine amide with 7-ADCA (Figure 9).

More recently, an alternative thermo-responsive polymer has been described.[73] It consists of random copolymers derived from 2-(2-methoxyethoxy)ethyl methacrylate and oligo(ethylene glycol) methacrylate (OEGMA) (see Figure 8) and combines the positive features of poly(ethylene glycol), non-toxicity and anti-immunogenicity, with thermo-responsive properties similar to polyNIPAM. The LCST could be varied between 26 and 90 °C depending on the relative amounts of OEGMA used. These properties make this a potentially interesting support for biocatalysts.

4 Entrapment

Enzymes can be immobilized by entrapment in sol-gel matrices formed by hydrolytic polymerization of metal alkoxides. Immobilization in silica sol gels prepared by hydrolytic polymerization of tetraethoxysilane, in the presence of the enzyme, was pioneered by Avnir and co-workers[74] and has been used for the immobilization of a wide variety of enzymes.[75] It should be pointed out that the morphologies of the silica sol-gels depend on the method of drying.[76] Drying by evaporation affords so-called *xerogels* in which capillary stress causes a shrinkage of the nano cages and pores. When alkylsiloxanes, $RSi(OR)_3$ are used together with $Si(OR)_4$ the surface of the sol-gel is more densely populated by the hydrophobic alkyl groups and the capillary stresses which operate during evaporation are largely attenuated, affording a so-called *ambigel* in which there is no contraction of the nano cages. Alternatively, drying with supercritical carbon dioxide affords a so-called *aerogel* in which the delicate pore structure is maintained. Silica aerogels have a phenomenal porosity; the *Guinness Book of Records* refers to a silica aerogel with a density of 0.001 as the worlds lightest solid.[76]

Reetz and co-workers found that when lipases were entrapped in sol-gels produced from $Si(OEt)_4$ the resulting immobilisates exhibited disappointingly low activities in the esterification of lauric acid by 1-octanol.[77] Reasoning that the microenvironment in the sol-gel may be too hydrophilic they entrapped the lipase in a sol-gel prepared from a mixture of $Si(OMe)_4$ and $RSi(OMe)_3$ containing non-hydrolyzable alkyl moieties, on the premise that the more hydrophobic matrix would facilitate interfacial activation of the lipase. This proved to be the case; they observed rate enhancements of 2–8-fold compared with the traditional lyophilized lipase powder. This method has been widely used for the immobilization of enzymes.[78] An interesting elaboration involves the addition of porous supports such as Celite during the sol-gel process to bind the lipase-containing gels. This "double immobilization" afforded materials with higher thermal stability and activity.[79] More recently, Reetz and co-workers[80] have reported a further improvement of the methodology, involving the use of higher alkyl groups in the $RSi(OMe)_3$ precursor and/or the use of additives such as isopropyl alcohol, crown ethers, surfactants and KCl, with or without the addition of Celite. These second generation sol-gel immobilisates contained high lipase loadings, and were highly active, robust and recyclable.

Pierre and co-workers have reported seminal studies on the entrapment of enzymes in silica aerogels.[76,81,82] For example, entrapment of *Burkholderia cepacia* lipase in a hydrophobic silica sol-gel resulted in an increase of all the kinetic constants, e.g., V_{max} by a factor of 10, in the esterification of lauric acid with 1-octanol.[83] More recently, lipases from *Burkholderia cepacia* and *Candida antarctica* were entrapped in silica aerogels, prepared from mixtures of $Si(OMe)_4$ and $MeSi(OMe)_3$ and reinforced with silica quartz fiber felt to improve their mechanical properties.[84] The resulting biocatalysts showed activities similar to that of Novozym 435 in the synthesis of biodiesel by interesterification of sunflower seed oil with methyl acetate in iso-octane. However, at high substrate concentrations, in the absence of solvent, they were less effective than Novozym 435, presumably owing to diffusion limitations.

Additives such as polyethylene glycol (PEG), polyvinyl alcohol and albumin, can have a stabilizing effect on sol-gel entrapped enzymes. For example, Zanin and co-workers[85] compared three different methods – physical binding, covalent attachment and gel entrapment, in the presence and absence of PEG

1450 – for the immobilization of *Candida rugosa* lipase. Their activities were determined in the hydrolysis of olive oil. Immobilization yields varied from 3 to 32%, the most active biocatalyst resulting from the encapsulation in the presence of PEG.

In an interesting variation on this theme, Naik and co-workers[86] entrapped catalase and horseradish peroxidase (HRP) using a biosilification process. In nature, diatoms are able to synthesize silica nanoparticles by polymerization of silicic acid, catalyzed by enzymes known as silicateins. A peptide subunit of the silicatein from *Cylindrotheca fusiformis* has been shown[87] to catalyze the formation of silica *in vitro*. When this process was performed in the presence of catalase or HRP this resulted in their entrapment.

Enzymes can also be entrapped in silicone elastomers[88] and polydimethylsiloxane membranes.[89] Kobayashi and co-workers[90] have recently described a novel polymer-incarceration methodology for immobilizing enzymes. The immobilization procedure is depicted in Figure 10. It involves dissolving polystyrene containing hydrophilic tetraethylene glycol and glycidol moieties as pendant groups in dichloromethane and then adding a solution of CaLB. This is followed by the addition of 1-hexane which causes coacervation to occur, affording a precipitate containing the lipase in the polymer phase. After decantation of the supernatant the polymeric matrix was cross-linked by reaction of the pendant glycidol groups with a triamine at 60 °C in hexane (see Figure 10) to afford a polymer incarcerated lipase. The immobilisate was used in the kinetic resolution of chiral secondary alcohols and could be reused 5 times without any loss of activity.

5 Carrier-Free Immobilization by Cross-Linking

In the early 1960s, studies of solid phase protein chemistry led to the discovery that cross-linking of dissolved enzymes *via* reaction of surface NH_2 groups with a bifunctional chemical cross-linker, such as glutaraldehyde, afforded insoluble cross-linked enzymes (CLEs) with retention of catalytic activity.[91] However, this method of producing cross-linked enzymes (CLEs) had several drawbacks, such as low activity retention, poor reproducibility, low mechanical stability, and difficulties in handling the gelatinous CLEs. Mechanical stability and ease of handling could be improved by cross-linking the enzyme in a gel matrix or on a carrier but this led to the disadvantageous dilution of activity mentioned above. Consequently, in the late 1960s, emphasis switched to carrier-bound enzymes, which became the most widely used industrial methodology for enzyme immobilization for the next three decades.

5.1 Cross-Linked Enzyme Crystals (CLECs)

The cross-linking of a crystalline enzyme by glutaraldehyde was first described by Quiocho and Richards

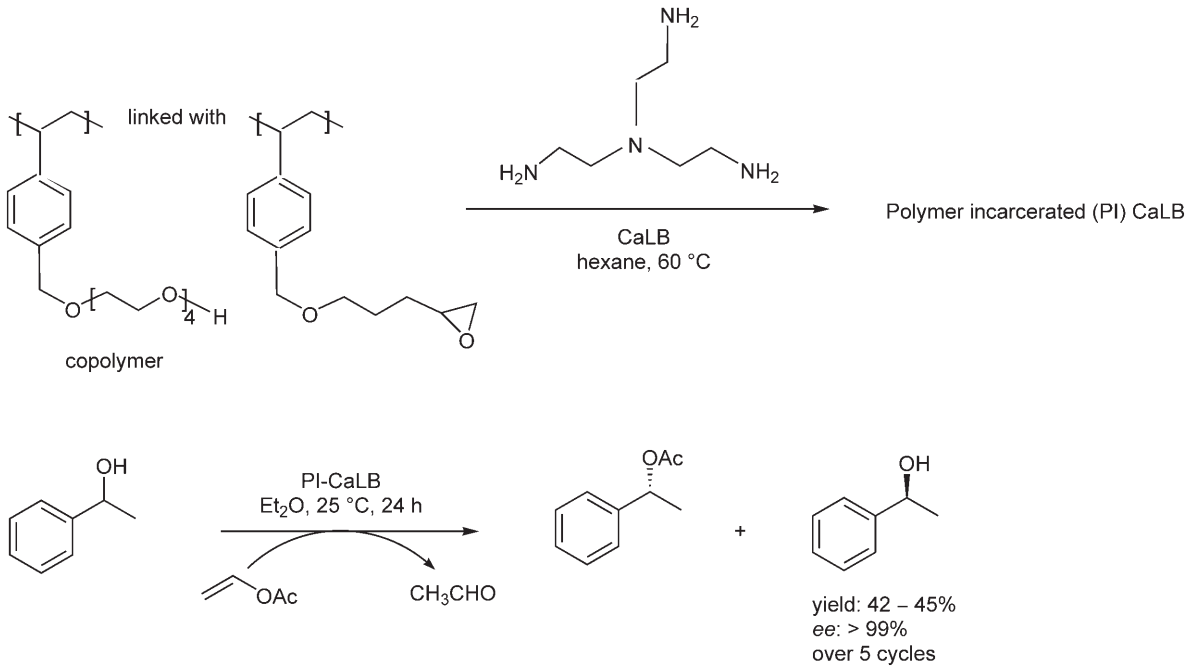


Figure 10. Polymer-incarcerated (PI) *Candida antarctica* lipase.

in 1964.[92] Their main objective was to stabilize enzyme crystals for X-ray diffraction studies but they also showed that catalytic activity was retained. The use of cross-linked enzyme crystals (CLECs) as industrial biocatalysts was pioneered by scientists at Vertex Pharmaceuticals in the early 1990s[93] and subsequently commercialized by Altus Biologics.[36] The initial studies were performed with CLECs of thermolysin (EC 3.4.24.4), of interest in the manufacture of aspartame, but the method was subsequently shown to be applicable to a broad range of enzymes.[36] CLECs proved significantly more stable to denaturation by heat, organic solvents and proteolysis than the corresponding soluble enzyme or lyophilized (freeze-dried) powder. CLECs are robust, highly active immobilized enzymes of controllable particle size, varying from 1 to 100 μm. Their operational stability and ease of recycling, coupled with their high catalyst and volumetric productivities, renders them ideally suited for industrial biotransformations. In a more recent example, a CLEC of chloroperoxidase (CPO) from *Caldariomyces fumago* exhibited a higher thermal stability and tolerance to organic solvents than the free CPO.[94]

5.2 Cross-Linked Enzyme Aggregates(CLEAs®)

An inherent disadvantage of CLECs is the need to crystallize the enzyme, which is often a laborious procedure requiring enzyme of high purity. On the other hand, it is well-known[95] that the addition of salts, or water-miscible organic solvents or non-ionic polymers, to aqueous solutions of proteins leads to their precipitation as physical aggregates of protein molecules, held together by non-covalent bonding without perturbation of their tertiary structure, that is without denaturation. It was reasoned that subsequent cross-linking of these physical aggregates would render them permanently insoluble while maintaining their pre-organized superstructure, and, hence their catalytic activity. This indeed proved to be the case and led to the development of a new family of immobilized enzymes: cross-linked enzyme aggregates (CLEA®) (Figure 11). Since precipitation from an aqueous medium, by addition of ammonium sulfate or polyethylene glycol, is often used to purify enzymes, the CLEA methodology essentially combines purification and immobilization into a single unit operation that does not require a highly pure enzyme. It could be

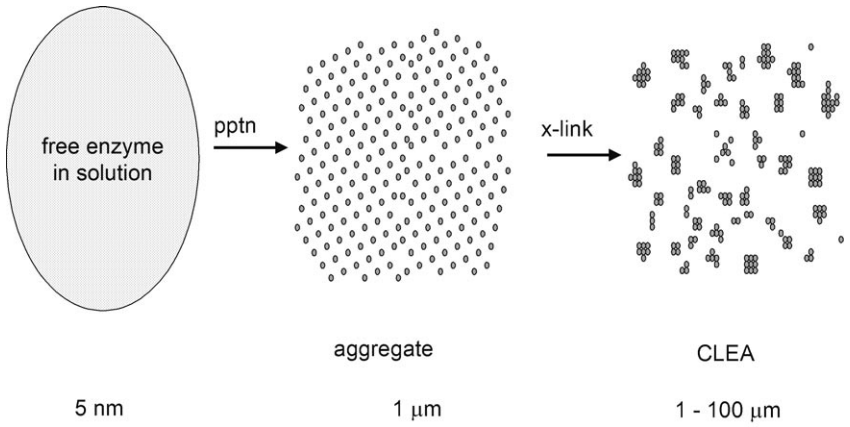
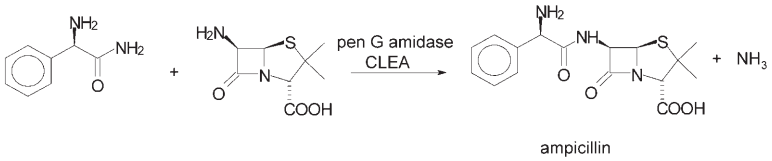


Figure 11. Preparation of a CLEA.



Biocatalyst	Conv. [%]	S/H ratio	Rel. productivity
Free enzyme	88	2.0	100
CLEC	72	0.7	3
T-CLEA	85	1.58	151
PGA-450	86	1.56	0.8

Figure 12. Ampicillin synthesis.

used, for example, for the direct isolation of an enzyme, in a purified and immobilized form suitable for performing biotransformations, from a crude fermentation broth.

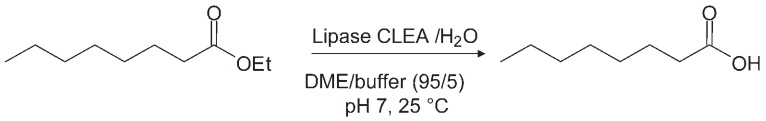
The first examples of CLEAs were derived from penicillin G amidase, an industrially important enzyme used in the synthesis of semi-synthetic penicillin and cephalosporin antibiotics (see earlier). The free enzyme exhibits limited thermal stability and a low tolerance to organic solvents, making it an ideal candidate for stabilization by immobilization. Indeed, penicillin G amidase CLEAs, prepared by precipitation with, for example, ammonium sulfate or *tert*-butyl alcohol, proved to be effective catalysts for the synthesis of ampicillin (Figure 12).[96]

The CLEA exhibited a synthesis/hydrolysis ratio (S/H) comparable with that of the commercial catalyst, PGA-450 (penicillin G amidase immobilized on polyacrylamide), and substantially higher than that of the penicillin G amidase CLEC suggesting that diffusional limitations are more severe in the CLEC. Remarkably, the productivity of the CLEA was higher even than that of the free enzyme that it was made from and substantially higher than that of the CLEC. In stark contrast, the commercial catalyst mainly consists of non-catalytic ballast in the form of the polyacrylamide carrier which was reflected in its much lower productivity. Analogous to the corresponding CLECs, the penicillin G amidase CLEAs also maintained their high activity in organic solvents.[97,98]

CLEAs were subsequently prepared from seven commercially available lipases (EC 3.1.1.3) and the effects of various parameters, such as the precipitant and the addition of additives such as surfactants and crown ethers, on their activities were investigated.[99] The activation of lipases by additives, such as surfactants and crown ethers, is well-documented and is generally attributed to the lipase being induced to adopt a more active conformation.[100] Co-precipitation of such additives with the enzyme followed by cross-linking of the enzyme aggregates, can 'lock' the enzyme in this more favourable conformation. Furthermore, since the additive is not covalently bonded to the enzyme, it can subsequently be washed from the CLEA using, for example, an appropriate organic solvent to leave the immobilized enzyme locked in the favourable confirmation. Using this procedure, a variety of hyperactive lipase CLEAs exhibiting activities up to twelve times that of free enzyme were prepared (Figure 13).[99] The experimental procedure was further simplified by combining precipitation, in the presence or absence of additives, with cross-linking into a single operation.[99]

These results clearly demonstrate the tremendous potential of the CLEAs as immobilized enzymes, with high catalyst and volumetric productivities, prepared in a simple procedure from relatively impure enzyme preparations. Indeed, the simplicity of the operation lends itself to automation, e.g., using 96-well plates.[101]

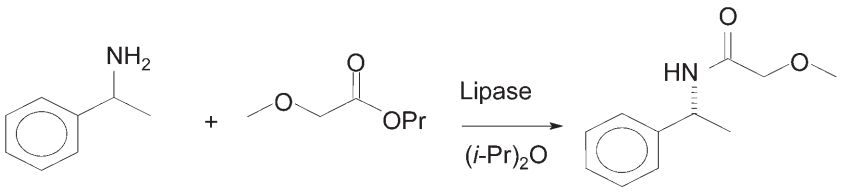
Initial studies of CLEAs derived from the popular *Candida antarctica* lipase B (CaLB) revealed that the excellent performance observed in water, compared to that of the standard immobilized form, Novozym 435 (CaLB immobilized on a macroporous acrylic resin), could not be directly translated to organic media. Consequently the preparation was modified to produce a more lipophilic CLEA which could better accommodate organic solvents. This afforded a dramatic improvement in the activity of CaLB CLEA in the enantioselective acylation of 1-phenethylamine in diisopropyl ether as solvent (Figure 14).[102] Clearly the optimized CaLB CLEAs have activities surpassing those of Nov 435 in both aqueous and organic media.



Lipase	Precipitant/additive	activity CLEA/free enzyme
CaLA	$(NH_4)_2SO_4$/SDS	9
CaLB	DME	0.75
P.alcaligenes	$(NH_4)_2SO_4$/Tr	12
T.lanuginosus	DME	0.8
Rh.miehei	$(NH_4)_2SO_4$/SDS	2.5
A.niger	$(NH_4)_2SO_4$/SDS	2.0
C.rugosa	$(NH_4)_2SO_4$/Tr	4.2

SDS = sodium dodecyl sulfate : Tr = Triton 100 ; DME = 1,2-dimethoxyethane

Figure 13. Hyperactivation of lipase CLEAs.



Enzyme	"Aqueous" activity [U/g]	"Organic" activity [U/g]	Ratio
CALB (lyophilized)	22,000	-	-
Novozym 435	7300	250	29
CLEA-AM[a]	38,000	50	760
CLEA-OM[a]	31,000	1500	21

[a] AM = aqueous medium, OM = organic medium.

Figure 14. Comparison of Novozym 435 with CaLB-CLEAs.

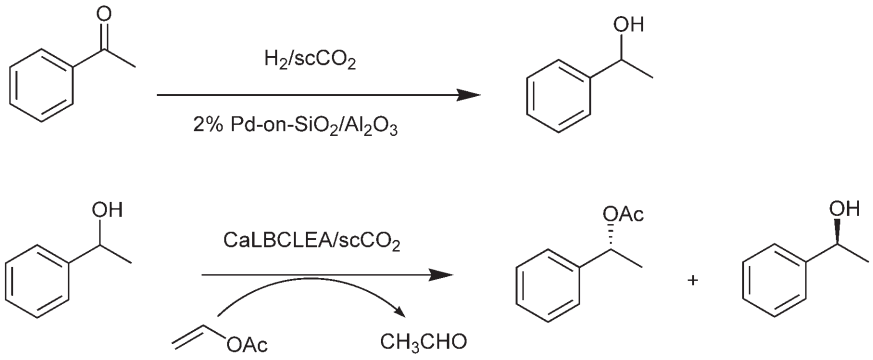


Figure 15. Sequential hydrogenation and resolution in $scCO_2$.

CaLB CLEAs also exhibit excellent activities in supercritical carbon dioxide[103] and ionic liquids.[104] Thus, CaLB CLEA displayed superior activity to that of Novozym 435 in the kinetic resolution of 1-phenylethanol and 1-tetralol, by acylation with vinyl acetate, in $scCO_2$. The enzymatic resolution could also be combined with the production of the 1-phenylethanol substrate, by palladium-catalyzed hydrogenation of acetophenone, using two separate columns in series (one containing the Pd catalyst and the other the CaLB CLEA), without the need for depressurization (Figure 15).[103]

In the kinetic resolution of 1-phenylethanol and 1-phenylethylamine in the ionic liquids, [bmim] $[NO_3]$ and [bmim] $[N(CN)_2]$ the best results were observed with CaLB adsorbed and cross-linked on a polypropylene carrier (Accurel EP100). In contrast, the free CaLB or immobilized as Novozym 435 dissolves in these ionic liquids with complete loss of activity.

An important property of CLEAs from the point of view of large-scale applications is their particle size which obviously has a direct effect on mass transfer limitations and filterability. The enzyme and glutaraldehyde concentrations are, *inter alia*, important factors in determining the particle size of CLEAs as was reported for *Candida rugosa* lipase.[105] Optimum activity was observed with particles of 40–50 nm.

Glutaraldehyde is generally the cross-linking agent of choice as it is inexpensive and readily available in commercial quantities. However, with some enzymes, e.g., nitrilases, low or no retention of activity was sometimes observed using glutaraldehyde as the cross-linker. A possible cause of deactivation is reaction of the cross-linker with amino acid residues which are crucial for the activity of the enzyme. Owing to its high reactivity and small size, which allows it to penetrate the interior of the protein, this will be particularly severe with glutaraldehyde. Hence, this could be avoided by using bulky polyaldehydes, obtained by periodate oxidation of dextrans, as the cross-linkers,[106] followed by reduction of the Schiff's base moieties with sodium borohydride to form irreversible amine linkages. The activity retention of these CLEAs was generally much higher than that observed with CLEAs prepared using glutaraldehyde. Dramatic results were obtained, for example,

Table 1. Effect of cross-linker on recovered activity of CLEA.[106]

Enzyme	Recovered activity [%][a]	
	Glutaraldehyde	Dextran polyaldehyde
Nirilase (*Ps. fluorescens*)	0	50
Nitrilase (Biocatalytics 1004)	0	60
Penicillin G amidase	48	85–90

[a] Relative to the free enzyme.

with a nitrilase (EC 3.5.5.13) from *P. fluorescens* and with a nitrilase from the company Biocatalytics. Cross-linking with glutaraldehyde produced a completely inactive CLEA while with dextran polyaldehyde 50–60% activity retention (not optimized) was observed (see Table 1). Similarly, better activity retentions were observed when penicillin amidase CLEAs were prepared with dextran polyaldehyde compared to glutaraldehyde.[106]

Since cross-linking largely involves reaction of the amino groups of lysine residues on the external surface of the enzyme, every enzyme can be expected to be, and is, different. For electronegative enzymes, that contain a paucity of lysine residues on the surface, cross-linking is expected to be less effective. One way of compensating for this lack of surface amino groups is to co-precipitate the enzyme with a polymer containing numerous free amino groups, e.g., poly-L-lysine[107] or polyethylene imine.[108,109] In a variation on this theme, Gupta and co-workers[110] reported that addition of bovine serum albumin (BSA) as a "proteic feeder" in the preparation of CLEAs from solutions containing low concentrations of enzymes facilitated the formation of the CLEA.

Other examples of hydrolases that have been successfully 'cleated' include pig liver esterase (EC 3.1.1.1), aminoacylase (EC 3.5.1.14), proteases, and glycosidases (EC 3.2.1).[111] For example, the alkaline protease from *Bacillus licheniformis* (alcalase, EC 3.4.21.62, also known as subtilisin Carlsberg), an inexpensive enzyme used in laundry detergents, has been widely used in organic synthesis, e.g., in the resolution of (amino acid) esters[112] and amines[113] and peptide synthesis.[114] An alcalase CLEA showed excellent activities and enantioselectivities in amino acid ester hydrolyses.[115]

Interestingly, a CLEA of aminoacylase prepared from a crude extract from *Aspergillus* sp. fermentation lacked the esterase activity observed with the crude enzyme.[116] This strongly suggests that the esterolytic activity is derived from a protein impurity in the crude aminoacylase preparation and illustrates the potential of the CLEA methodology for performing purification and immobilization in a single operation.

Similarly, CLEAs were successfully prepared from the glycosidase, β-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae* and phytase (EC 3.1.3.26) from *Aspergillus niger*.[101] The former enzyme catalyzes the hydrolysis of lactose in dairy products and is administered as 'tolerase' to people suffering from lactose intolerance and the latter is an acid phosphatase which is added to animal feed in order to hydrolyse phytate (inositol hexaphosphate).

Recyclable CLEAs were also prepared[101] from a variety of oxidoreductases, e.g., glucose oxidase (EC1.1.3.4), galactose oxidase (EC 1.1.3.9) and laccase (EC 1.10.3.2). Laccase, in particular, has many potential applications, e.g., for bleaching in the pulp and paper or textile industries, aqueous effluent treatment and, in combination with the stable radical TEMPO (2,2,6,6-tetramethyl-1-piperidinoxyl), for the catalytic aerobic oxidation of starch to carboxystarch (Figure 16). The latter can be made by TEMPO catalyzed hypochlorite oxidation of starch but greener, aerobic oxidation is more attractive. Immobilization as a CLEA improves the stability of the laccase under the reaction conditions, thereby reducing the enzyme cost contribution.

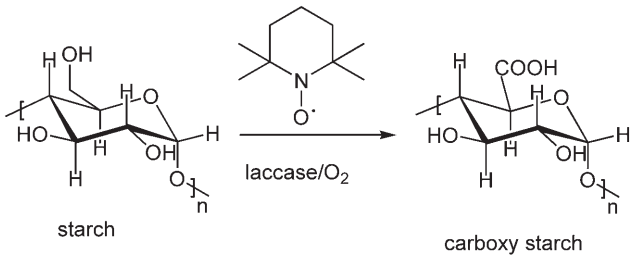


Figure 16. Laccase-catalyzed aerobic oxidation of starch.

Another benefit of the CLEA technology is that it can stabilize the quaternary structures of multimeric enzymes, a structural feature often encountered with redox metalloenzymes. For example, the stability of CLEAs from two tetrameric catalases (EC 1.11.1.6) which, for the soluble enzymes is dependent on concentration, became independent of this parameter in the CLEA, which allowed for the use of low 'concentrations' of catalase.[117] Similarly, CLEAs have been prepared from an alcohol dehydrogenase (EC 1.1.1.1) from *Rhodococcus erythropolis* and a formate dehydrogenase (EC 1.2.1.2) from *Candida boidinii*.[101]

The methodology has also been successfully applied[102] to various *C–C bond forming lyases*, notably the *R*- and *S*-specific oxynitrilases (hydroxynitrile lyases, EC 4.1.2.10) which catalyze the hydrocyanation of a wide range of aldehydes. For example, a CLEA prepared from the (*R*)-specific oxynitrilase from almonds, *Prunus amygdalis* (PaHnL) was highly effective in the hydrocyanation of aldehydes under microaqueous conditions and could be recycled ten times without loss of activity.[118] CLEAs were similarly prepared from the (*S*)-specific oxynitrilases from *Manihot esculenta* and *Hevea brasiliensis*.[119,120] These oxynitrilase CLEAs perform exceptionally well in organic solvents, affording higher enantioselectivities than observed with the free enzymes owing to the essentially complete suppression of competing non-enzymatic hydrocyanation.

A further elaboration of the CLEA methodology involves the immobilization of lipase CLEAs by inclusion in hydrophobic polytetrafluoroethylene membranes for use in membrane bioreactors.[121] This method has advantages compared with physical adsorption or covalent binding. Similarly, inclusion of glucose oxidase CLEAs in magnetic mesocellular carbon foam was used to construct a magnetically switchable bioelectrocatalytic system.[122] The preparation of CLEAs from CO_2 expanded micellar solutions afforded dendritic CLEAs with tunable nanometer dimensions (7–38 nm).[123]

6 Combi-CLEAs and Catalytic Cascade Processes

The ultimate in environmental and economic efficiency is to combine atom-efficient, catalytic, steps into a one-pot, catalytic cascade process without the need for separation of intermediates.[28] Catalytic cascade processes have numerous potential benefits: fewer unit operations, less reactor volume, and higher volumetric and space-time yields, shorter cycle times and less waste generation. Furthermore, by coupling steps together unfavourable equilibria can be driven towards product. In principle, this can be achieved by co-precipitation and cross-linking of two or more enzymes in 'combi CLEAs'. For example, combi CLEAs have been prepared from catalase in combination with glucose oxidase or galactose oxidase. The catalase serves to catalyze the rapid degradation of the hydrogen peroxide formed in the aerobic oxidation of glucose and galactose, respectively, catalyzed by these enzymes.

A combi CLEA containing an *S*-selective oxynitrilase from *Manihot esculenta* and an aselective nitrilase from *Pseudomonas fluorescens*, catalyzed the smooth, one-pot conversion of benzaldehyde to *S*-mandelic acid (Figure 17)[124] in diisopropyl ether/water (9:1) at pH 5.5. The enantioselectivity is provided by the oxynitrilase and *in situ* conversion by the nitrilase serves to drive the equilibrium of the first step towards product. In principle, this could also be achieved by using an *S*-selective nitrilase in combination with non-enzymatic hydrocyanation but, unfortunately, there are no nitrilases that exhibit *S*-selectivity with mandelonitriles. Interestingly, the combi CLEA was more effective than a mixture of the two separate CLEAs. A possible explanation is that the close proximity of the two enzymes inside the combi CLEA is more favourable, compared to the case with two separate CLEAs, for transfer of the product of the first step to the active site of the enzyme for the second step.

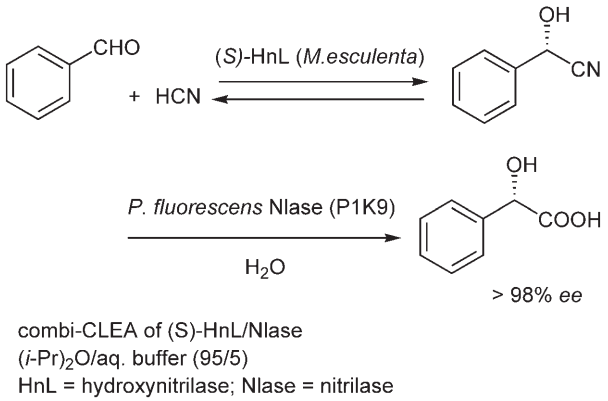


Figure 17. One-pot conversion of benzaldehyde to (*S*)-mandelic acid.

7 Enzyme-Immobilized Microchannel Reactors for Process Intensification

Microreactor technology is an interdisciplinary field that has attracted much attention recently.[125] Process intensification through the use of microchannel reactors (microfluidic devices) has many advantages compared with traditional batch process technologies, such as rapid mass and heat transfer and large surface area to volume ratios. These are attractive features for conducting catalytic reactions in microreactors containing the enzyme immobilized on their inner walls. Maeda and co-workers[107,126,127] developed a microreactor in which enzymes are immobilized as an enzyme-polymer membrane on the inner walls of the microchannels.[127] Thus, a solution of the enzyme (e.g., α-chymotrypsin) in aqueous buffer was mixed with glutaraldehyde and formaldehyde as cross-linkers in commercially available polytetrafluoroethylene (PTFE) tubing (inner diameter 500 μm). This results in the formation of a CLEA membrane on the inner walls of the tubing. This technique has been used to prepare CLEA-based enzyme microreactors (CEMs)

from a wide variety of enzymes.[107] With electronegative enzymes (such as aminoacylase) coprecipitation of the enzyme in the presence of poly-L-lysine (see above) was used to realize fast and efficient CLEA formation.[107] The use of such enzyme-immobilized microchannel reactors clearly has considerable potential for the design of green and sustainable biotransformations.

In the context of incorporating the immobilized biocatalyst in the reactor configuration, the development of a monolithic stirrer reactor by Lathouder and co-workers deserves a special mention.[128] According to this novel concept, the enzyme is immobilized on a honeycomb, ceramic monolith, analogous to those used in catalytic exhaust systems in automobiles. Cordierite monoliths were functionalized, by coating with polyethylene imine or different types of carbon, in order to create adsorption sites for the enzyme (CaLB). These were then employed in a monolithic stirrer reactor in which the monolithic structure is incorporated in the stirrer blades. This concept was tested in the CaLB-catalyzed transesterification of 1-butanol with vinyl acetate.[128]

Carbon nanofiber-coated monoliths performed the best with regard to catalyst productivity and did not exhibit any leaching under the reaction conditions. The activity was lower (by a factor of 2–4) than Novozym 435 or free CaLB but the latter preparations deactivated much faster. In contrast, the monolithic catalysts were operationally stable for several weeks, without significant loss of activity.

8 Conclusions and Prospects

Hopefully, it is clear from this review that the subject of enzyme immobilization continues to attract considerable attention from researchers in both industry and academia. Novel concepts continue to appear, a recent example being single enzyme nanoparticles (SENs) in nanoporous silica.[129] However, many of these innovations involve the use of rather exotic supports which are substantially more expensive than the enzyme to be immobilized. Consequently, they are unlikely to be applied in industrial biotransformations but could be interesting for applications in biosensors or other devices where the enzyme cost contribution is less of an issue. They can also provide important insights into the effects of enzyme immobilization on activity and stability.

For application in industrial biotransformations the cost contribution of the (immobilized) enzyme is an important issue. Clearly, the immobilization methodology, in addition to providing an active and stable biocatalyst, should be a relatively simple operation that does not require a highly pure enzyme preparation or an expensive support that may not be commercially available. Immobilization as silica granulates, for example, meets all these criteria but the methodology is not applicable to aqueous environments (see earlier). Cross-linked enzyme aggregates (CLEAs) would appear to have considerable industrial potential based on their high activity retention and stability coupled with ease of preparation from crude enzyme samples and no requirement for a support. Because they are close to 100% active catalyst they also display high catalyst productivities and space-time yields. However, properties such as mechanical strength and filterability still have to be demonstrated on an industrially relevant scale.

It is also clear that every enzyme is different and, consequently, there is no all-encompassing, 'one size fits all' solution to the problem of enzyme immobilization. Based on the increasing importance of enzymes in a plethora of industrial applications, interest in improving their operational performance will certainly continue unabated. The quest for optimum performance continues.

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