Chapter 4
Heterogeneous Enzyme Kinetics

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4.1 Enzyme Immobilization

Because of their excellent functional properties (activity, selectivity, specificity), enzymes have a great potential as industrial catalysts in a number of areas of chemical industry: fine chemistry, food chemistry, analysis and so on (Koeller and Wong 2001). However, the enzymes have been modified during evolution to optimize their behavior in the framework of complex catalytic chains inside the living cells under stress and subjected to regulation. Obviously, enzymes have not been optimized by evolution in order to work as catalysts in industrial reactors so that some of their properties are not well suited for that purpose: they are water soluble, unstable at conditions different from physiological, frequently inhibited by substrates and products of reaction and have rather narrow substrate specificity. In most cases, enzymes have to be greatly improved for their application as industrial catalysts. The engineering of enzymes for such purpose is one of the most exciting, complex and interdisciplinary goals of biotechnology, considering different techniques like: a) the screening, inside the biodiversity, of enzymes with improved properties; b) the improvement of enzyme properties via techniques of molecular biology; c) the improvement of enzyme properties via immobilization and post-immobilization techniques; d) the improvement of enzyme properties via reaction and reactor engineering. These techniques complement each other to succeed in improving enzyme properties for delivering catalysts for a much more sustainable chemical industry, where very complex and useful compounds are synthesized under very mild and cost-effective conditions.

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For technical and economical reasons, most chemical processes catalyzed by enzymes require the re-use or the continuous use of the biocatalyst for a very long time (Chibata et al. 1986; Bickerstaff 1997; Katchalski-Katzir and Kraemer 2000). In this context, immobilization of enzymes may be defined as any technique that allows the re-use or continuous use of the biocatalyst. Immobilization of enzymes is maybe the most relevant approach for stabilization and recovery of enzymes, as already highlighted in Chapter 1. From an industrial perspective, simplicity and cost-effectiveness are key properties of immobilization techniques, but a long term industrial re-use of immobilized enzymes also requires the preparation of very stable derivatives having the right functional properties (activity, selectivity ...) for a given reaction (Gianfreda and Scarfi 1991; Bornscheuer 2003; Cao 2005a). At first glance, the practical development of protocols for immobilization of enzymes is tightly related to simplicity, cost-effectiveness and stabilization. Enzyme immobilization has profound influence on the nature of the catalytic process which is now heterogeneous in nature, since catalysis occurs in the surface or within a solid structure where the enzyme is located while substrates and products of reaction are in the liquid reaction medium where its course is being monitored. Enzyme immobilization will be reviewed in section 4.1 as the necessary framework to treat heterogeneous enzyme catalysis in sections 4.2 to 4.4.

4.1.1 Methods of Immobilization

There is a large number of methods of immobilization that can be broadly divided into those than involve the interaction of the enzyme with a matrix (usually through a chemical bond) and those in which the enzyme is contained within a restricted space, as shown in Fig. 4.1.

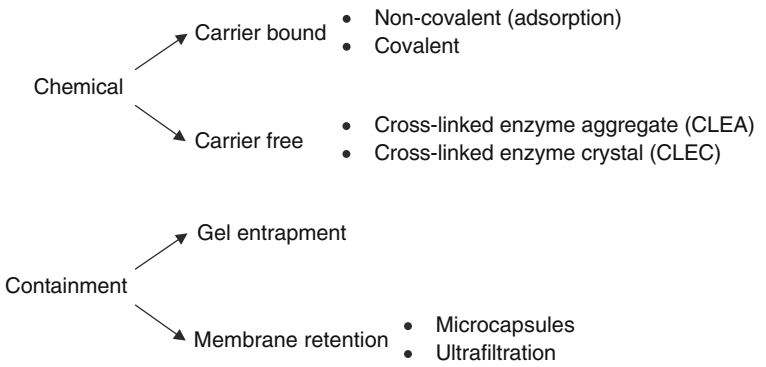

Fig. 4.1 Methods of enzyme immobilization

4.1.1.1 Chemical Bond

Consider those methods that include the chemical binding of the enzyme molecules to an inert carrier (*carrier-bound*), where linkage can be covalent or non-covalent, and those in which the enzyme protein molecules are chemically linked among themselves, usually through a bifunctional reagent, without the participation of an inert carrier (*carrier-free*).

Carrier Bound

Different types of materials have been used as carriers for immobilized enzymes, considering both organic and inorganic compounds. Desirable properties of the carrier are a high surface to volume ratio, high protein binding capacity, compatibility and insolubility in the reaction medium, high mechanical and chemical stability, recoverability after use and conformational flexibility. There is no material that fulfills all these requirements so that in practice all kind of materials have been tested to suit the particular needs of a given process. Many of the materials tested at laboratory scale are, however, not well suited to perform at productive scale, either because of their intrinsic properties or because of their high costs. At the end, availability and cost are key factors in determining the carrier to be used. It must be pointed out that most of the early developments in enzyme immobilization referred to its use in conventional aqueous medium so that a thorough analysis should be made when defining suitable carriers to perform in non-aqueous media (Adlercreutz 2006).

Covalent Immobilization to Solid Supports

A covalent bond is established between the functional groups in the activated carrier and the functional groups in the amino acid residues of the enzyme, like $-OH$, $-SH$, $-NH_2$, and $-COOH$. Covalent immobilization has been thoroughly studied and detailed information on methods and procedures can be found in several publications devoted to it (Zaborsky 1973; Cao 2005b; Guisan 2006). It is a rather complex method where the carrier is hardly recoverable after enzyme exhaustion, immobilization yield is rather low and the kinetic properties of the enzyme can be severely altered. However, operational stability is high and it is quite flexible, so that directed immobilization can be done to suit the particular characteristics of the process.

Among the many systems for covalent enzyme immobilization, multi-point covalent attachment, where the enzyme is linked to the support through several amino acid residues is particularly interesting and has been developed using different solid supports like porous glass, polyacrylamide, cellulose, magnetic particles and so on. Very likely, immobilization on glyoxyl-agarose is the most precise protocol to get very intense enzyme-support multipoint covalent attachments, the enzyme being immobilized through its surface region having the highest density of lysine residues (Pedroche et al. 2007). Multi-point covalent attachment has allowed to significantly

increase the stability of a large number of enzymes like α-chymotrypsin (Guisan et al. 1991), trypsin (Blanco and Guisán 1988; Blanco et al. 1988), carboxypeptidase A (Pedroche et al. 2002), lipases (Otero et al. 1991; Fernández-Lafuente et al. 1998), D-aminoacid oxidases (Fernández-Lafuente et al. 1999), ferredoxin NADP oxidoreductase (Bes et al. 1995), esterases (Fernández-Lafuente et al. 1995), rennin (Penzol et al. 1998) and penicillin G acylase from *Escherichia coli* and *Kluyvera citrophila* (Guisan et al. 1990; Alvaro et al. 1990, 1991). The establishment of a number of attachments between every immobilized enzyme molecule and the support exerts very strong stabilizing effects. When spacer arms (between the enzyme and the support) are very short and the support is very rigid, it can be assumed that all the relative positions among the enzyme residues involved in multipoint immobilization have to remain unmodified during any conformational change induced by any distorting agent (heat, organic cosolvents …). In this way, the intensity of these conformational changes should be strongly diminished. This hypothesis has been raised from the beginnings of enzyme technology to explain the strong stabilizations obtained with conventional immobilization protocols (Martinek et al. 1977; Mozhaev et al. 1990). Despite this, after more than 40 years of research, there are very few immobilization protocols useful to promote very intense enzyme-support multipoint covalent immobilizations. The internal morphology of agarose gels is composed by large fibers with a high geometrical congruence with protein surfaces (Mateo et al. 2006a). At first glance, these gels, when activated with glyoxyl groups (small aliphatic aldehyde groups) are unsuitable for enzyme immobilization. However, at the very end, under tailor-made conditions, they seem to be the most adequate to get dramatic immobilization–stabilization of industrial enzymes. These activated gels are very stable and easy to prepare and are commercially available (Mateo et al. 2005). A scheme is presented in Fig. 4.2. Details on immobilization protocol have been published by Guisan et al. (1997).

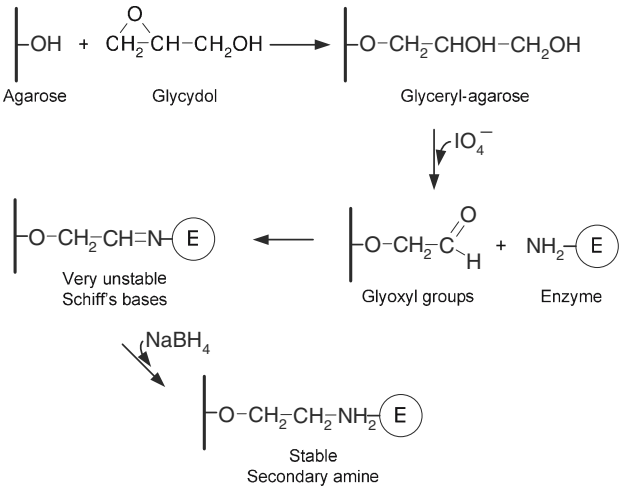

Fig. 4.2 Scheme for enzyme immobilization in glyoxyl-agarose

Table 4.1 Stabilization of Enzymes by Multi-Point Covalent Attachment to Glyoxyl-Agarose

Enzyme	Expressed Activity (%)	Stabilization Factor
Chymotrypsin	70	60,000
Trypsin	75	10,000
Penicillin G acylase (*Escherichia coli*)	70	8000
Penicillin G acylase (*Kluyvera citrophila*)	70	1000
Glutamate racemase	70	1000
Esterase (*Bacillus stearothermophilus*)	70	1000
Lipase (*Candida rugosa*)	70	150
Thermolysin (*Bacillus thermoproteolyticus*)	100	100

The strong stabilization effect promoted by immobilization in glyoxyl agarose can be appreciated in Table 4.1 for different monomeric enzymes with a very small decrease in catalytic activities. Inactivation experiments were made to compare multipoint immobilized derivatives with single point immobilized ones (prepared by using poorly activated supports). In this way, the stabilization factors reported (ratio of half-life of the multi point over the single point immobilization) really represent the 3D stiffening of enzyme structures. Moreover, stiffening of enzymes by a very intense multipoint covalent immobilization should promote stabilization against any distorting agent.

Amino-epoxy supports are other quite interesting matrices for enzyme immobilization by covalent attachment. These derivatives may be easily prepared by reaction of highly activated amino-supports with butanediol diglycidyl ether (BDDGE) and are commercially available from Resindion (Mitsubishi Chem. Co). Ideally, the amino groups on the support should have a pK around 9–10 in order to be easily modified with epoxy reagents and, after modification, to become ionized (as secondary amino groups) at pH 7.0 and 4 °C. In this way a very simple first adsorption of enzymes by anionic exchange may be developed. A three step immobilization protocol on heterofunctional epoxy supports is represented in Fig. 4.3.

Epoxy groups are very poorly reactive towards intermolecular immobilization and hence the first step (at pH 7.0) is always the ionic adsorption of the enzyme through the region having the highest density of negative charges. Then the reaction between the epoxy groups in the support and the amino groups in the enzyme takes place and in the third step (incubation under alkaline conditions) a much more intense multipoint covalent attachment is promoted contributing significantly to enzyme stabilization (Mateo et al. 2007). In addition to commercial supports (i.e. Sepabeads from Resindion), any aminated support may be activated and utilized in a similar way. An additional merit of this immobilization protocol (adsorption plus intramolecular covalent attachment) is the possibility to completely immobilize enzymes that hardly adsorb on the support. The very small percentage of adsorbed molecules becomes also covalently immobilized and hence the equilibrium of adsorption is completely shifted towards the adsorption of enzymes with poor affinity. Epoxy groups in Eupergit C and EP-Sepabeads have been thiolated and the thiol derivative used to immobilize enzymes through their thiol groups via thiol disulfide interchange. For those enzymes lacking cysteine residues, like *E. coli* penicillin

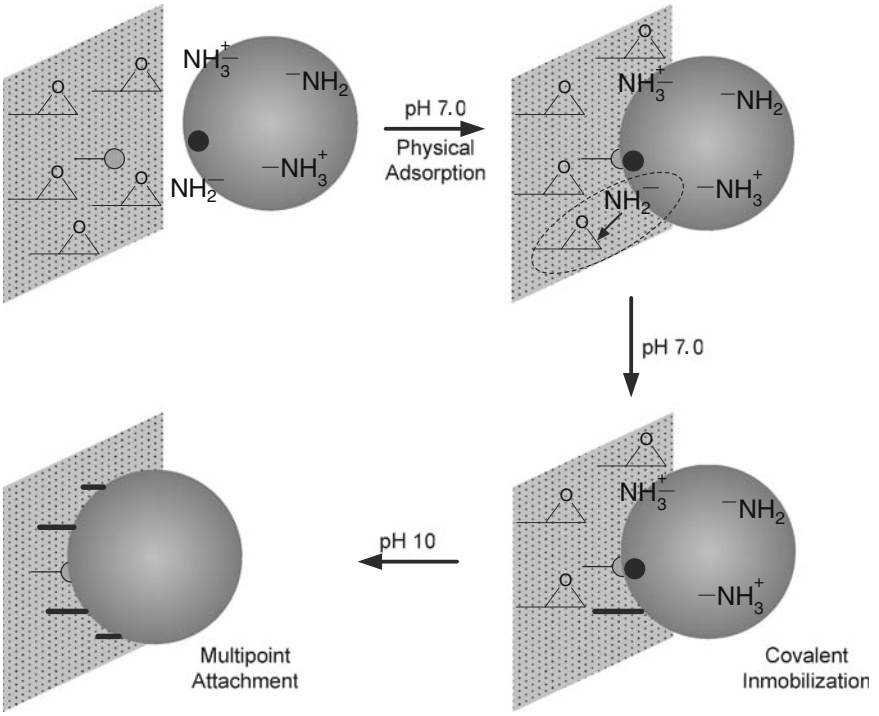

Fig. 4.3 Immobilization of enzymes on heterofunctional epoxy supports

acylase and *Rhizomucor miehei* lipase, they were introduced chemically to promote multipoint covalent attachment to the thiolated support, obtaining dramatic increases in stabilization (Grazú et al. 2005).

Glutaraldehyde is a bifunctional reagent quite useful for developing protocols for covalent immobilization to solid supports (Betancor et al. 2006; Hamerska-Dudra et al. 2006). Enzyme immobilization on amine-activated supports activated with glutaraldehyde is a simple process (Monsan et al. 1975; Alonso et al. 2005; López-Gallego et al. 2005). Amino-supports are chemically very stable and can be stored at 4 °C, for prolonged periods of time and activation is simpler, being glutaraldehyde a non toxic and GRAS reagent frequently used for enzyme immobilization. Nevertheless this method has some drawbacks: multipoint covalent attachment is not very strong nor its chemical stability and the reactivity of the lysine groups cannot be increased further by using more alkaline pH because of the instability of the glutaraldehyde groups.

Non-Covalent Immobilization to Solid Supports

It considers all kind of interactions between the enzyme and the support not involving covalent bonds, including short-range interactions like van der Waals forces, but also stronger ones like hydrophobic interactions and ionic bonds; sometimes they

are referred generically as adsorption. It is a simple method, where the carrier can be easily recovered after enzyme exhaustion by promoting protein desorption, immobilization yields are usually high and no obnoxious reagents are involved. However, its main drawback is that the enzyme can be easily desorbed from its carrier by subtle changes in the reaction medium. This is particularly so in the case of aqueous systems, but it is worthwhile to point out that there is a much lesser tendency to desorption in the case of non-aqueous medium, like organic solvents, where simple immobilization by adsorption can be a good option.

Ionic exchange is a rather simple and effective method for enzyme immobilization since the vast majority of proteins adsorb very fast on anion or cation exchange resins. However, the strength of the interaction between conventional supports (surfaces fairly covered with ionic groups) and proteins is weak: most of the proteins are desorbed at relatively low ionic strengths (200–300 mM) and pH changes may also promote desorption. The use of new solid supports fully covered with ionic polymers like polyethylenimine (PEI) and dextran sulfate (DS), mimicking "ionic flexible and deep beds", has been proposed to increase the strength of the ionic interaction between proteins and supports. These supports have a much higher concentration of ionic groups than conventional ones and their flexible structure allows a better adaptation of the ionic layer to the immobilized protein (Mateo et al. 2000; Torres et al. 2003; Fuentes et al. 2004; Pessela et al. 2005). Many kinds of supports (porous glass, agarose gels, magnetic particles, etc.) covered with such ionic polymers are good candidates to prepare active, stable and selective enzyme biocatalysts that can be used (and re-used) in several types of industrial reactors (see Chapter 5).

Carrier Free

Enzymes can be insolubilized by straight chemical cross-linking of the protein molecules with bifunctional reagents, like glutaraldehyde. Cross-linking can be performed over the soluble enzyme protein (cross-linked enzyme, CLE) (Broun 1976; Tyagi et al. 1999), over a crystallized enzyme protein (cross-linked enzyme crystal: CLEC) (Margolin 1996; Häring and Schreier 1999; Roy and Abraham 2004), or over a protein enzyme aggregate (cross-linked enzyme aggregate: CLEA) (Cao et al. 2000, 2001; López-Serrano et al. 2002; Mateo et al. 2004; Schoevaart et al. 2004; Sheldon et al. 2007). These systems only differ in the protein precursor to be cross-linked and have the obvious advantage that no inert support is involved since the enzyme is auto-immobilized in its own protein mass and, therefore, the specific activity of the biocatalyst is very high, being the enzyme concentration within the biocatalyst close to its theoretical limit of packing (Cao et al. 2003).

CLEs are produced by straight cross-linking of the soluble enzyme. A delicate balance among different factors (amount and type of cross-linking reagent, temperature, pH, ionic strength) is required to control the process. The intermolecular cross-linking of the highly solvated enzyme produces significant losses of activity (immobilization yields are usually below 50%), and results are difficult to reproduce (Cao et al. 2003). Although used for some industrial purposes some decades ago

(Carasik and Carroll 1983), CLEs are no longer used mainly because of their poor mechanical properties.

CLECs are produced by cross-linking of purified enzyme crystals and are endowed with excellent properties: high stability under harsh conditions (high temperatures, extreme pHs, organic solvents), resistance to autolysis (in the case of proteases) and exogenous proteolysis (St Clair and Navia 1992; Vaghjiani et al. 1999) and extremely high volumetric (and specific) activity (Tischer and Kasche 1999; Xu and Klibanov 1996), which is quite relevant for the rather slow reactions of synthesis. They have excellent mechanical properties (Lalonde 1997) and biocatalyst recovery is quite simple (Persichetti et al. 1995). CLECs can be freeze-dried and indefinitely stored at room temperatures (St Clair and Navia 1992; Persichetti et al. 1995). However, a main drawback is the high cost of the biocatalyst that arises from the requirement of a high degree of purity of the enzyme protein to crystallize. So, in practice, despite its excellent properties, very few processes are being conducted with this kind of biocatalysts (Lee et al. 2000). Lowering the costs of production and tailoring existing CLECs to suit particular applications will make them very attractive biocatalysts for organic synthesis (Roy and Abraham 2004).

CLEAs are produced by cross-linking of protein aggregates produced by conventional protein precipitation techniques (see Chapter 2). Stable biocatalysts can be easily prepared in a two-step protocol: firstly, aggregation of soluble enzymes is promoted by the addition of salts (salting-out), organic solvents or polymers under very mild experimental conditions to ensure good retention of enzyme activity; after aggregation strong stirring is promoted to reduce particle size of the enzyme aggregates and, finally, enzyme aggregates are stabilized by cross-linking (usually with glutaraldehyde). A scheme of the preparation of CLEAs is in Fig. 4.4a. This very simple procedure produces insoluble and stable cross-linked enzyme aggregates even after extensive washing to remove the cross-linking reagent. This method was first developed at Delft University by Prof. Sheldon's research group (Cao et al. 2000) and represents a major contribution to biocatalysis because it combines the good properties of non-supported biocatalysts with simplicity and low cost of production since, as opposite to CLECs, CLEAs do not require of a purified enzyme (actually, fractional precipitation is used for enzyme purification of rather crude preparations, as indicated in Chapter 2). This technique has had a great success in producing a number of very active derivatives of industrial enzymes (Cao et al. 2000, 2001; López-Serrano et al. 2002; Mateo et al. 2004, 2006b; Sheldon et al. 2007). Beyond that, several improvements have been introduced. Procedures have been developed for the co-immobilization of different enzymes, producing the so-called combi-CLEAs, either to perform cascade (Sheldon et al. 2007) or non-cascade reactions (Dalal et al. 2006). CLEAs are particularly well suited to develop such multiple-enzyme biocatalysts that can be quite useful for performing complex biotransformations or acting upon complex heterogeneous substrates. Co-immobilization of enzymes and their respective coenzymes is another exciting potential of CLEAs.

Enzymes and highly hydrophilic polymers (i.e. polyethyleneimine and dextran sulfate) can be co-immobilized prior to cross-linking, as shown in Fig. 4.4b creating

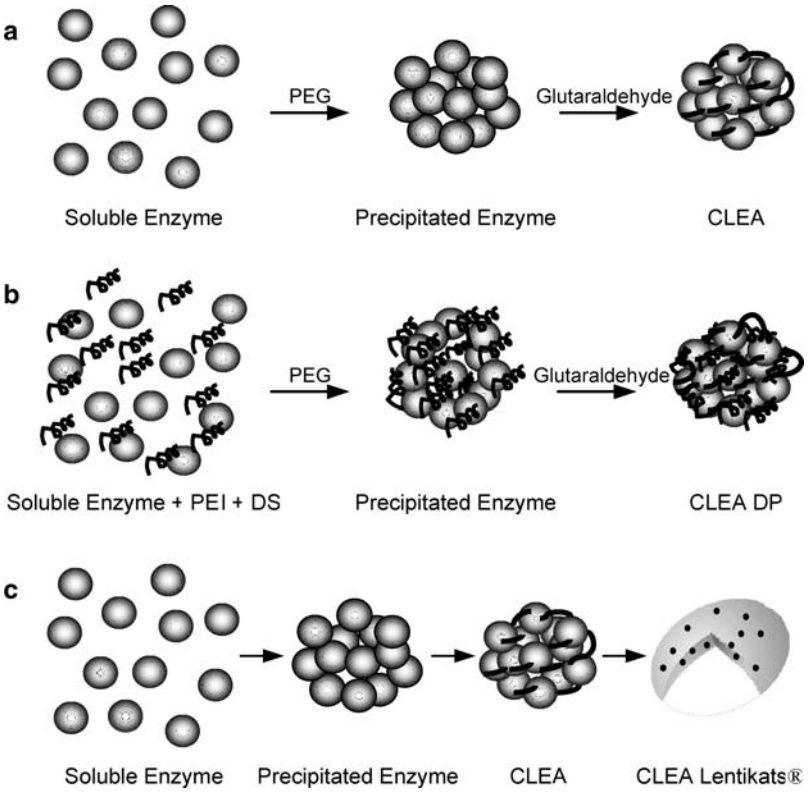

Fig. 4.4 Schematic representation of the preparation of CLEAs: a) using polyethylene glycol (PEG) as precipitating agent and glutaraldehyde as cross-linking agent; b) using highly hydrophilic microenvironment composed by polyethyleneimine (PEI) and dextran sulfate (DS), (PEG) as precipitating agent and glutaraldehyde as cross-linking agent; c) using polyethylene glycol (PEG) as precipitating agent and glutaraldehyde as cross-linking agent and encapsulation into polyvinyl alcohol lens-shaped gel particles (LentiKats)

a highly hydrophilic microenvironment that significantly improves their stability against organic solvents (Abian et al. 2001, 2002). When using high concentrations of organic cosolvents a partition effect of the cosolvent between the bulk solution and the enzyme microenvironment is produced so that the enzyme molecules are now in contact with a much lower concentration of organic cosolvent, diminishing its deleterious effect (Wilson et al. 2004a). These CLEas are particularly well suited to perform in such organic media. Polymers, like polyethyleneimine, that contain several primary amino groups contribute to enhance the intensity of chemical cross-linking in those enzymes that are relatively poor in lysine residues (Wilson et al. 2006).

CLEAs of multimeric enzymes are also very good biocatalysts since the stability is greatly improved. When multimeric enzymes are chemically cross-linked every enzyme subunit becomes also cross-linked and hence no subunit dissociation

is possible (Betancor et al. 2003; Wilson et al. 2004b). CLEAs are then much more stable than their soluble counterparts. In addition to that, inactivation of cross-linked aggregates does not depend on derivative dilution. When using non stabilized derivatives the more diluted suspension inactivates much more rapidly because dissociation of subunits is favored.

Mechanical properties and control of particle size are the main drawbacks of CLEAs. Particles are compressible and shear sensitive and size is usually small so that recovery of the biocatalyst may pose a problem for conventional reactor configurations (see Chapter 5). To solve that problem, basket-type bioreactors can be used or else the biocatalyst can be modified. An interesting approach is the encapsulation of CLEAs within polymer gels, as shown in Fig. 4.4c. Entrapment of CLEAs within polyvinyl alcohol lens-shaped gel particles (LentiKats) produced very robust biocatalysts of a convenient size to be easily recovered (Wilson et al. 2004c).

4.1.1.2 Containment

Consider those methods in which the enzyme is confined to a restricted space and includes molecular entrapment within polymeric gels and also retention within semipermeable membranes that allow free passage of substrates and products of reaction while retaining the enzyme.

Entrapment

The enzyme is confined within the inner cavities of a solid polymeric matrix, compact enough to retain the enzyme molecules within it. Immobilization occurs by polymerization of a monomer solution in which the enzyme is dissolved. Polymerization of the monomer can be induced by physical stimulation (i.e. photopolymerization) or chemically; alternatively, the dissolved polymer can be promoted to sol–gel transition by lowering the temperature. Most popular matrices for gel entrapment are alginate (Cheetham et al. 1979), polyacrylamide (Pizarro et al. 1997), polyurethane (Wang and Ruckenstein 1993); polyvinyl alcohol (Wang et al. 1995) and κ-carrageenan (Tosa et al. 1979). Entrapment in polymeric gels has been a powerful tool for cell immobilization (Klibanov 1983; Cantarella et al. 1997; Muscat and Vorlop 1997), but not as much in the case of enzymes because the tendency to leakage can be counterbalanced only by increasing gel strength which in turns magnifies mass transfer limitations. Polyvinyl alcohol (PVA) is a particularly interesting material because it is innocuous and cheap and also mechanically and chemically robust (Lozinsky and Plieva 1998; Durieux et al. 2000). Even though it has been mainly used for cell immobilization (Wang et al. 1995; Wittlich et al. 1999), it has been successfully applied to the immobilization of enzyme–polymer composites (Czichocki et al. 2001; Gröger et al. 2002) and enzyme aggregates as already mentioned (Wilson et al. 2002, 2004a). Immobilization is performed by mixing a solution of

commercial PVA with the enzyme (or composite or aggregate) and then promoting gelification by dripping the mixture over a cooled surface where regular lens-shaped particles are formed of about 5 mg, 3–4 mm in diameter and 0.2–0.4 mm in height. Biocatalysts of this kind are quite robust and easy to recover during reactor operation.

Sol–gel encapsulation of enzymes is a very attractive system of immobilization that consists in the acid or base catalyzed hydrolysis of tetraalkylosilanes, where the silane precursor undergoes hydrolysis and cross-linking condensation to form a silica matrix in which the enzyme is entrapped (Gill and Ballesteros 1996; Reetz 1997, 2006). It has been successfully applied to a number of enzymes, like esterases (Altstein et al. 1998), proteases (van Unen et al. 2001), organophosphorus hydrolase (Lei et al. 2002), alkaline phosphatase (Braun et al. 2007) and lipases (Schuleit and Luisi 2001; Reetz 2006; Meter et al. 2007). However, applications of sol–gel encapsulation have been more related to chemical analysis than bioprocesses and in that field several enzyme electrodes have been developed for the analysis of organic compounds (Przybyt and Białkowska 2002). Through careful selection of sol–gel precursors and additives, these materials are being designed for specific application, and can produce useful, robust enzyme analytical devices. A comprehensive review on the subject has been published recently (Pierre 2004).

Membrane Retention

The enzyme is retained by a semi-permeable membrane that allows free-passage of the substrates and products of reaction. More than one enzyme can be retained so that cascade reactions can be performed. Retention can be attained by microencapsulation and by containment in ultrafiltration membranes

Microencapsulation

Enzyme microcapsules are produced by promoting a polymerization reaction in the surface of drops of enzyme aqueous solution dispersed in a water-immiscible organic solvent with the aid of a surfactant. Reverse micelles and liposomes are forms of microcapsules. The former are those in which the hydrophilic head of the surfactant is oriented to the inner enzyme aqueous drop while its tail is oriented to the outer organic phase. Liposomes are micelles composed by a double layer of surfactant so that the external solvent is the aqueous enzyme phase. Enzyme reverse micelles have interesting properties since the microenvironment is adequate for the enzyme and mass transfer limitations are negligible, so that several enzymes have been microencapsulated by this technique (Fadnavis and Luisi 1989; Serralheiro et al. 1990; Vicente et al. 1994). The main drawback of enzyme reverse micelles is mechanical weakness and the tendency of the enzymes to denature at the water-organic interface. Lipases are particularly well-endowed to perform at interfaces so their immobilization in reverse micelles has been thoroughly studied (Carvalho and Cabral 2000; Zaman et al. 2005). Liposomes have also been used for enzyme

immobilization (Dufour et al. 1996; Li et al. 2007) though its main potential relies in their biomedical applications (Özden and Hasirci 1990; Esquisabel et al. 2006).

Containment by Ultrafiltration Membranes

In these systems the enzyme is confined within a space delimited by an ultrafiltration membrane that allows the free passage of substrates and products while retaining the enzyme. The enzyme may be free in the inner or outer space of the ultrafiltration device or else attached to either side of the membrane. Nowadays, ultrafiltration membranes are having much better performances being more hydrophilic and inert. One of the drawbacks of enzyme containment in ultrafiltration devices is inactivation by the interaction with interfaces (i.e. air bubbles or any other interface in stirred reactors) and by undesirable aggregation (Caussette et al. 1998; Colombie et al. 2001; Bommarius and Karau 2005). A solution for overcoming those problems is the complete cross-linking of the soluble enzymes with aldehyde-dextran polymers (plus borohydride reduction) to promote the formation of new enzyme molecules fully surrounded by hydrophilic and inert polymers, as illustrated by the dramatic stabilization of glucose oxidase (Betancor et al. 2005). Containment by ultrafiltration membranes is particularly relevant for processes involving coenzyme-requiring enzymes, where both the enzyme and the derivatized coenzyme are retained (Kragl et al. 1996; Liu and Wang 2007).

4.1.2 Evaluation of Immobilization

From a bioprocess perspective the main purpose of enzyme immobilization is to increase its stability and allow its prolonged utilization either in continuous or sequential batch processes (see Chapter 5). Immobilization may also be used for other very relevant purposes, like the modification of enzyme kinetic parameters. The controlled and directed interaction between a solid surface and an enzyme (i.e. immobilization of an enzyme through a specific residue, the stiffening of selected areas of the immobilized enzyme, the interaction of the adsorbed enzymes with polymers) are valuable tools for enhancing the activity and the selectivity when the enzyme is to be used on non-natural substrates or in non-conventional media. The case of lipases (see section 6.3) may serve as a good example to illustrate this. Lipases are mainly active when acting on hydrophobic interfaces of their natural substrates (fats). This catalytic behavior, called *interfacial activation*, involves the opening of a polypeptide chain (*lid*) that covers the active site. The closed (inactive) conformation is favored in aqueous medium, while the open conformation is favored by the presence of interfaces. Lipases interact with any hydrophobic surface much like they do on fat surfaces, so that the enzyme attaches to the surface with the active centre lid wide open (Fernández-Lafuente et al. 1998; González-Navarro et al. 2001). In addition to that, this interaction is quite specific: lipases adsorb on hydrophobic surfaces at very low ionic strength and under these conditions most of the proteins

are unable to do it so that a highly selective adsorption of lipase may occur. When using larger or highly hydrophilic substrates, lower hyperactivation is found. This is presumably due to steric hindrances: the active site of the enzyme is so close to the hydrophobic surface that large and hydrophilic substrate molecules have no access to it (Palomo et al. 2004). Immobilization may also alter enzyme enantioselectivity, which is a very relevant aspect in enzymatic organic synthesis (see section 1.6). Using different immobilization strategies for lipases, regions of the enzyme close to the active site were involved, different degrees of stiffening were promoted and also the interaction of the enzyme with positively or negatively charged polymers, producing several lipase derivatives of quite different enantioselectivity when acting on different chiral ester substrates (Palomo et al. 2002; Turner 2003; Chaubey et al. 2006; Torres et al. 2006).

Enzyme immobilization has evolved to progressively more directed protocols to suit particular process requirements. However, novel methods and materials are still needed to achieve a massive implementation of enzymes as catalysts for complex chemical processes, which explains why, after more than 40 years of work, research in this field keeps on very active. A recently published book covers extensively the most relevant methods of immobilization in the form of critical reviews by the most prominent research groups worldwide (Guisan 2006).

Any immobilization protocol will involve an activation step in which the support (and the enzyme) is activated, and a contacting step in which the activated partners interact with each other to deliver the immobilized enzyme. Optimization of the process of immobilization is a complex task, since many variables are involved in both stages and a sound objective function is required. Some of the most relevant variables to consider are the ratio of activating agent to support in the first stage, the ratio of enzyme contacted to activated support in the second, pH, temperature, time of contact and agitation rate in both stages. These variables are likely to have interactions among them so that a careful statistical design is required for optimization. An even more complex task is to define a proper objective function to optimize. To do so, relevant immobilization parameters have to be taken into account.

4.1.2.1 Parameters of Enzyme Immobilization

During the process of immobilization a fraction of the enzyme protein is immobilized, while the rest remains unbound. The immobilized enzyme protein expresses only a fraction of the expected activity and this can be due to enzyme inactivation, steric hindrance or mass transfer limitations. On the other hand the unbound enzyme protein may be partly inactive, so enzyme immobilization yield (Y_E), as defined by Eq. 4.1, has to be carefully analyzed in its meaning.

$$Y_E = \frac{E_I}{E_C} = \frac{E_I}{E_I + E_R + E_L} \tag{4.1}$$

As defined, Y_E simply represents the fraction of the contacted activity that is expressed in the biocatalyst. Although enzyme is quantified in units of activity (not

mass), Eq. 4.1 is presented as a material balance, so that the term E_L has been included to close that balance. The meaning of E_L is simply the fraction of the contacted enzyme activity which is not expressed either in the biocatalyst or in the medium and may be attributed to inactivation (of the bound, but mostly of the unbound enzyme) or to mass transfer limitations and steric hindrances of the immobilized enzyme. An insight of the reasons underlying the partial expression of the contacted activity may be obtained by defining protein immobilization yield, which is simply the ratio of bound protein to contacted protein, as expressed by Eq. 4.2:

$$Y_P = \frac{P_I}{P_C} = \frac{P_C - P_R}{P_C} \tag{4.2}$$

Comparing P_I with E_I may give an insight of the incidence of mass transfer limitations and steric hindrances. If Y_P is significantly higher than Y_E, then those effects are probably relevant, but immobilization can have degree of selectivity (positive or negative) for the enzyme with respect to the whole protein that cannot be ruled out.

Sometimes enzyme immobilization yield is defined as:

$$Y'_E = \frac{E_I}{E_I + E_L} = \frac{E_I}{E_T - E_R} \tag{4.3}$$

which means that the enzyme remaining active in solution after immobilization is not considered a loss. Although strictly true, for practical purposes it usually represents a loss because it is not economic to recover this small fraction of diluted enzyme. Obviously Y_E is lower than $Y_E{}'$, but the former should be used since it better reflects enzyme recovery.

Other very relevant parameter of enzyme immobilization is biocatalyst mass (or volumetric) specific activity, which is simply the amount of enzyme activity expressed per unit mass (or unit volume) of biocatalyst:

$$A_{sp} = \frac{E_I}{M} \tag{4.4}$$

This parameter may differ significantly from enzyme load, which can be defined as the amount of enzyme loaded to the support. Enzyme load can be calculated by subtracting the total activity in suspension to the activity in solution after immobilization. Such difference simply represents the enzyme protein loaded but gives no insight on the expression of such enzyme protein. Alternatively, protein load can be determined as:

$$P_{load} = \frac{P_C - P_R}{M} \tag{4.5}$$

but protein load may differ from enzyme load since some selectivity of immobilization (whether positive or negative) of the enzyme with respect to protein as a whole may occur. In fact, there are several cases reported where selective immobilization of enzymes has been obtained (Bastida et al. 1998; Fernández-Lafuente et al. 1998).