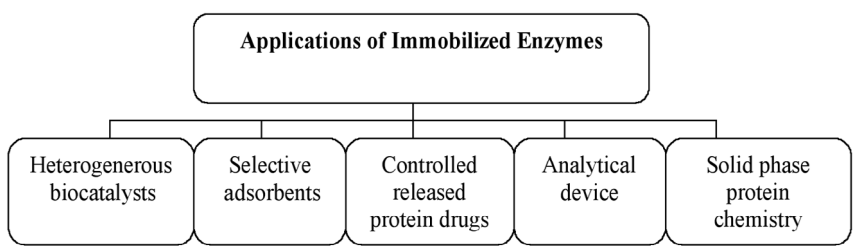
1
Introduction: Immobilized Enzymes: Past, Present and Prospects

1.1
Introduction

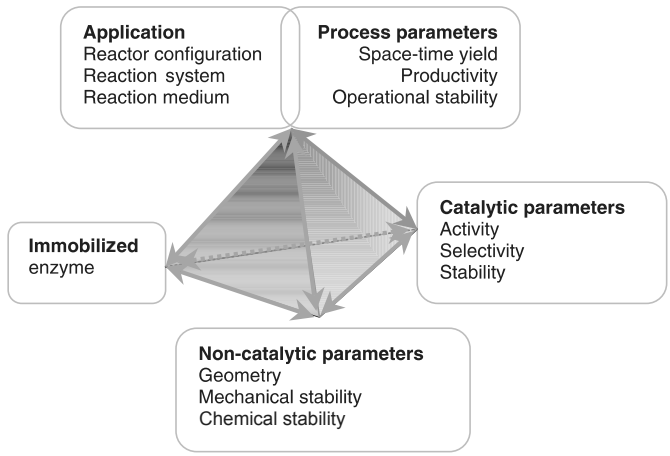
Since the second half of the last century, numerous efforts have been devoted to the development of insoluble immobilized enzymes for a variety of applications [2]; these applications can clearly benefit from use of the immobilized enzymes rather than the soluble counterparts, for instance as reusable heterogeneous biocatalysts, with the aim of reducing production costs by efficient recycling and control of the process [3, 4], as stable and reusable devices for analytical and medical applications [5–11], as selective adsorbents for purification of proteins and enzymes [12], as fundamental tools for solid-phase protein chemistry [13, 14] and as effective microdevices for controlled release of protein drugs [15] (Scheme 1.1).



Scheme 1.1 Range of application of immobilized enzymes.

However, whatever the nature of an immobilized enzyme and no matter how it is prepared, any immobilized enzyme, by definition, must comprise two essential functions, namely the non-catalytic functions (NCF) that are designed to aid separation (e.g. isolation of catalysts from the application environment, reuse of the catalysts and control of the process) and the catalytic functions (CF) that are designed to convert the target compounds (or substrates) within the time and space desired (Scheme 1.2).

NCF are strongly connected with the physical and chemical nature of the non-catalytic part of the immobilized enzymes, especially the geometric properties, e.g. the shape, size, thickness, and length of the selected carrier, whereas the CF are linked to the catalytic properties, for example activity, selectivity, and stability, pH



Scheme 1.2 Relationship between NCF and CF of an immobilized enzyme and its applications.

and temperature profiles. General criteria for selection of these two properties for robust immobilized enzymes as catalysts are proposed in Table 1.1 [16].

In practice, catalytic functions are designed in line with the desired activity, selectivity, substrate specificity, productivity and space–time yield, with the aim of achieving fewer side reactions, high tolerance of structural variation of the substrates, high productivity, high space–time yield, and high durability of the catalyst. On the other hand, the selection criteria for non-catalytic functions, especially geometric properties, are largely dependent on the design of reactor configurations (e.g. batch, stir-tank, column and plug-flow), the types of reaction medium (aqueous, organic solvent, or two-phase system), the reaction systems (slurry, liquid-to-liquid, liquid-to-solid, or solid-to-solid), and the process conditions (pH, temperature, pressure). The objectives when designing the non-catalytic properties are mainly to achieve easy separation of the immobilized enzymes from the reaction mixtures, broad reactor considerations (i.e. flexibility of reactor design), broad applicability in different reaction media and reaction systems, and facilitating process development, down-stream processing and, particularly, control of the process.

It is usually the peculiarities of these two essential elements, i.e. the non-catalytic functions and the catalytic functions that dictate the scope of the final application of the immobilized enzymes obtained. Conversely, the peculiarities of each application also dictate the design and selection of the two essential elements. In general, the NCF and CF of an immobilized enzyme are the two sides of a coin which are the basis of the scope of the final application, as illustrated in Scheme 1.2.

It is, therefore, hardly surprising that the main task of enzyme immobilization is to select a suitable immobilization method (carriers, conditions, and enzymes) to design an immobilized biocatalyst which can meet not only the catalytic needs (expressed as productivity, space–time yield, stability and selectivity) but also the non-catalytic needs (e.g. separation, control, down-streaming process) of a given appli-

Table 1.1 Criteria for robust immobilized enzymes (from Ref. [16])

Parameter	Requirement	Benefits
Non-catalytic function	Suitable particle size and shape	Aid separation, easy control of the reaction
	Suitable mechanical properties	Flexibility of reactor design
	Low water regain capability	Easy removal of water
	High stability in a variety of organic solvents	No change of pore radius and thus fewer diffusion constraints
Catalytic function	High volume activity (U g^{-1})	High productivity and space–time yield
	High selectivity	Fewer side reactions, easier downstream processing and separation of products, and less pollution
	Broad substrate specificity	Tolerance of structural variation of the substrates
	Stability in organic solvents	Shift of reaction equilibrium with the use of organic solvents
	Thermostability	Short reaction time by increasing temperature
	Operational stability	Cost-effective and lower cost-contribution for the product
	Conformational stability	Modulation of enzyme properties
Immobilized enzyme	Recyclability	Low cost-contribution of catalyst
	Broad applicability	Tolerance of process variation
	Reproducibility	Guarantee product quality
	Easy and quick design	Early insight into process development and avoidance of learning process
E and E consideration	Lower volume	Lower cost for the solid handling
	Easy disposal	Less environmental concern? Easy biodegradability?
	Rational design	Avoidance of laborious screening
	Safety for use	Meeting safety regulations
IPR	Innovative	Protection of IPR
	Attractive	Licensing
	Competitive	Strengthening marketing position

E and E: Economical and Ecological; IPR: Intellectual Property

cation. As a result, an immobilized enzyme can be labelled "robust" when its catalytic and the non-catalytic functions both meet the requirements of a specific application. Consequently, it is envisaged there are two possibilities in the development of a biocatalytic process – design of a process around an available immobilized enzyme and the design of an immobilized enzyme around a process.

The first possibility is obviously less desirable, because a ready-made immobilized enzyme (either commercially available or made in-house) is a specific immobilized enzyme only and is thus not necessarily the optimum catalyst for the desired processes, as exemplified by the fact that many types of carrier-bound immobilized penicillin G acylase which are regarded as robust immobilized catalysts for the production of 6-APA are not necessarily good catalysts for the kinetically controlled synthesis of semi-synthetic β-lactam antibiotics [17, 18]. This is largely ascribed to the fact that changing the process conditions often provokes a change of enzyme performance.

By contrast, the diversity of the processes (as reflected by different substrates, reaction types, reactor configurations, down-streaming processes) necessarily requires the design of specific immobilized enzymes which can match process requirements. Thus, it is hardly surprising that design of the immobilized enzyme around a process will dominate the future development of immobilized enzymes.

Although it is becoming increasingly appreciated that the availability of a robust immobilized enzyme in the early stage of process development will definitively enable early insight into process development and save costs not only in process development but also in production, the lack of guidelines to selection of the method of immobilization and the performance to be expected of an immobilized enzyme for a specific application seriously hampers application of a rational approach to the design of such robust immobilized enzymes [19].

In this regard, we attempt to analyse important developments in the history of enzyme immobilization and thus to provide readers with a fundamental basis for understanding and designing robust immobilized enzymes.

1.2 The Past

Although the chronological development of enzyme-immobilization techniques has been discussed intensively for several decades [20–22], it is still worth going back to several historical phases which were important milestones in the history of enzyme immobilization, to appreciate that the roots of enzyme-immobilization techniques are the basis of future development.

For the purpose of discussion, the development enzyme immobilization is classified according to five criteria:

- the number of methods developed,
- the number of materials used for enzyme immobilization,
- the number of binding types established,
- the degree of understanding of the factors influencing the performance of the immobilized enzymes, and
- the number of processes using immobilized enzymes.

Accordingly, the history of bio-immobilization can be divided into several phases:

- the early days (1916–1940s),
- the underdeveloped phase (1950s),
- the developing phase (1960s),
- the developed phase (1970s),
- the post-developed phase (1980s), and
- the rational design phase (1990s–present).

Although there might be some overlap in respect of the time and continuity of development, this classification reflects major developments in enzyme-immobilization techniques. Following this order, we briefly discuss what has been achieved in the last 90 years.

1.2.1 The Early Days (1916–1940s)

Although in 1916, Nelson and Griffin rediscovered that artificial carrier-bound invertase on $Al(OH)_3$ and charcoal was still catalytically active [1], the potential of bio-immobilization as a method of obtaining useful and reusable immobilized biocatalysts was unfortunately not recognized in the succeeding 40 years. This simple fortuitous discovery has, however, been widely recognized as the cornerstone of the various enzyme-immobilization techniques currently available, because in the last half century it actually stimulated much interest and effort in exploration of insolubilized active enzymes for various studies and industrial applications that can be better met with immobilized rather than free enzymes.

In these early days, bio-immobilization techniques were mainly used to prepare adsorbents for isolation of proteins by immunologists, via adsorption on simple inorganic carriers such as glass [23], alumina [24] or hydrophobic compound-coated glass [25].

Along with these prototypes of pseudo-immobilized enzymes (immobilized by reversible non-covalent physical adsorption), few irreversible immobilized enzymes prepared by covalent attachment were also reported in the literature at that time [26].

1.2.2 The Underdeveloped Phase (1950s)

Although in 1950s the method of enzyme immobilization was still dominated by physical methods, i.e. non-specific physical adsorption of enzymes or proteins on solid carriers, for example α-amylase adsorbed on activated carbon, bentonite or clay [27], AMP deaminase on silica [28], and chymotrypsin on kaolinite [29], the method of adsorption was gradually switched from simple physical adsorption to specific ionic adsorption, for instance, chymotrypsin on phosphocellulose [29], catalase on the ionic resin DEAE–cellulose [30, 31], DNase on cellulose [32, 33], lipase and catalase on styrenepolyaminostyrene (Amberlite XE-97) [34], and ribonuclease on the anionic exchanger Dowex-2 and the cationic exchanger Dowex-50 [35].

Along with physical methods of enzyme immobilization, however (e.g. non-specific adsorption, or ionic adsorption), other important methods of enzyme immobilization, for example covalent immobilization, were further investigated. Examples of enzymes were lipases and other enzymes or antibodies covalently bound to polyaminostyrene [34, 36–38], diazotized cellulose [7], poly(acrylic acid) chloride [40, 41], diazotized polyaminostyrene [36, 41, 42], and polyisocyanate [34, 38]. Unfortunately, those early-developed carriers were found to be less suitable for covalent enzyme immobilization, because of poor retention of activity (2–20 % of the native activity), probably attributable to the highly hydrophobic nature of the carriers used at that time [38–45] or the unsuitable active functionality such as diazonium salt, which often affords an immobilized enzyme with lower retention of activity [45].

Apart from the physical adsorption and covalent immobilization used in this period, it was demonstrated for the first time by Dickey that some enzymes such as AMP deaminase entrapped in the sol–gel inorganic matrix formed by silicic acid-derived glasses retained reasonable biological activity [28]. Unfortunately, the importance of this finding was not recognized in the succeeding 40 years [46–48].

In addition to the use of natural polymers, derivatives such as CM-cellulose [30] and DEAE-cellulose [31], and inorganic materials such as carbon [35], glass, kaolinite [39], and clays as carriers for enzyme immobilization, a few synthetic polymers, for example aminopolystyrene and polyisocyanate, prepared directly by poly-

Table 1.2 Survey of enzyme-immobilization techniques in the 1950s

Carriers	Activation or coupling methods	Techniques developed and important observations
Natural polymers and derivatives	Acylazide	Physical adsorption
Cellulose	Diazotium salt	Ionic adsorption [35]
DEAE-cellulose	Polyacrylic acid chloride	Covalent [36]
	Isocyanate [38]	Entrapment in sol-gel glass [28]
		Modification-adsorptive immobilization
Synthetic polymers		pH optimum shifting [35]
Amberlite		
Diaion		
Dowex		
Polystyrene		
Other polyacrylic polymers and derivatives		
Inorganic carriers		
Carbon		
Silica		
Kaolinite		
Clay		

merization of active monomers for covalent enzyme immobilization [37], and synthetic ionic adsorbents such as Amberlite XE-97 [34], Dowex-2, and Dowex 50 [35] for non-covalent enzyme immobilization by ionic adsorption [35, 37, 38, 41] were also added to the family of carriers used for enzyme immobilization (Table 1.2).

1.2.3 The Developing Phase (1960s)

Although different covalent methods of enzyme immobilization were the main focus of bio- immobilization at this time, the long-established non-covalent enzyme immobilization, i.e. adsorption [50] and entrapment [51–53] were further developed, as is reflected in the publications of the time (Ref. [54] and references cited therein). In addition, encapsulation of enzymes in semi-permeable spherical membranes (also called "artificial cells") was first proposed by Chang [55]. Enzyme entrapment techniques were also further extended by the use of synthetic polymeric gels such as PVA (polyvinyl alcohol) [56] or PAAm (polyacrylamide gel) [51] or the use of natural polymer derivatives such as nitrocellulose or starch [56] or silicon elastomers for the sol–gel process [57, 58]. Other techniques of enzyme immobilization, for example adsorptive cross-linking of enzymes on films and membranes [62], or beads for the formation of enzyme envelopes [62], were also developed.

Apart from the development of carrier-bound immobilized enzymes, it was also demonstrated that insoluble carrier-free immobilized enzymes could be prepared by cross-linking of crystalline enzymes [63] or dissolved enzymes [64], by use of a bifunctional cross-linker such as glutaraldehyde. Although the potential of cross-linking of enzyme crystals was not recognized at that time, intensive studies were devoted to preparation of these carrier-free immobilized enzymes, especially CLE (cross-linked dissolved enzymes), as immobilized enzymes. More than twenty enzymes of different classes were either directly cross-linked to form a variety of CLE or first adsorbed on inert supports, such as membranes, and subsequently cross-linked to form supported CLE (Ref. [54] and references cited therein). In the late 1960s, however, research emphasis switched mainly to carrier-bound immobilized enzymes; at this time a wide range of carriers was specifically developed for enzyme immobilization and several important organic reactions for binding enzymes to carriers were established, as is shown in Table 1.3.

From the middle to the end of the 1960s the scope of bio-immobilization was greatly extended owing to the use of more hydrophilic insoluble carriers with defined geometric properties, for example cross-linked dextran, agarose, and cellulose beads (Table 1.3) and particularly as a result of the use of new methods of activation, for example cyanogen bromide [65] and triazine for polysaccharide [66], isothiocyanate for coupling amino groups [67], and Woodward reagents [69] for activation of carboxyl groups. Furthermore, the preparation of synthetic carriers bearing active functionality such as polyanhydride [79] or polyisothiocyanate [67], etc., which could bind enzyme directly (Table 1.3), enabled relatively simple preparation of immobilized enzymes.

Table 1.3 Survey of enzyme-immobilization techniques in the 1960s

Carriers	Activation or coupling methods	Techniques developed and important observations
Synthetic polymers	Cyanogen bromide [65]	Entrapment of whole cells in synthetic gel [53]
Poly(AAc-MAAn)	Triazine method [66]	Encapsulation in artificial cell [57]
PAAm	Glutaraldehyde for crosslinking and coupling [63]	Adsorption-cross-linking [62]
PVA [56]	Woodward reagents [69]	Active site titration [71, 72]
Nylon	Anhydride	First industrial process with immobilized enzymes [52]
Polystyrene [59, 60]		
Natural polymers and derivatives	Isothiocyanate [67]	Modification-covalent immobilization [73, 171]
DEAE-cellulose	Activation of carboxyl groups [68]	Cross-linked enzyme (CLE) [63]
Sephadex	Activation of hydroxyl group with monohalo-acetyl halides [70]	and cross-linked enzyme crystals (CLEC) [63]
Sepharose		Micro-environmental effect [76]
Starch		Immobilization or post-treatment by denaturant [77]
Semi-synthetic carriers		Binding mode was related to the enzyme stability [78]
Collodion		Importance of binding chemistry in terms of activity retention was appreciated [78]
Nitrocellulose		
Epoxy ring-grafted natural polymer		
Inorganic carriers		
Carbon		
Clay		
Silica gel		
Hydroxyapatite		
Kaolinite		

The enzymes studied changed, moreover – from a few classic enzymes such as invertase, trypsin, urease and pepsin to a broad range of enzymes such as galactosidase, amyloglucosidase, urease [78], subtilisin, chymotrypsin [69], lactate dehydrogenase [81], apyrase [83], amino acylase [82], amino acid oxidase [86], catalase, peroxidase [84], hexokinase [85], cholinesterase [91], α-amylase [87], ATPase and adolase, alkaline phosphatase [88], penicillin G acylase [89], β-galactosidase [90], deoxyribonuclease [91], urate oxidase, and cholinesterase, etc., which were expected to have great application potential in chemical, pharmaceutical, and medical industrial sectors.

At the same time it was increasingly appreciated that the physical and chemical nature of the carriers, especially the microenvironment, for example their hydrophilic or hydrophobic nature, the charges on the carriers, and the binding chemistry also strongly dictated the catalytic characteristics of the enzyme, for example activity [76, 79, 92, 93], retention of activity [79, 94] and stability [87].

With increasing awareness that besides functioning as supports, i.e. as scaffolds for the enzyme molecules, the carriers could be used practically as the modifiers of enzyme properties, many carriers of different physical or chemical nature, different hydrophilicity or hydrophobicity, or different shape or size (for example beads, sheet, film, membrane [95] or capsules [55]) were developed to provide carriers with sufficient diversity. This was reflected by the shift of the carriers from a few classics, for example cellulose and its derivatives [44], inorganic carriers [86, 88, 97] and polystyrene and derivatives [37, 59, 60], to a broad variety ranging from naturally occurring materials such as agarose, Sephadex [83], Sepharose [65], glass [97], kaolinite, clay, DEAE-Sephadex, DEAE-cellulose [50], to synthetic carriers such as polyacrylamide [51], ethylene maleic acid copolymer [94], a co-polymer of methylacrylic acid and methylacrylic acid-*m*-fluoroanilide [96], nylon [98, 99], PVA-based carriers for covalent binding or entrapment [56], and a variety of synthetic ion-exchange resins such as Amberlite [100], Diaion and Dowex [101], which have defined chemical and physical properties.

It is also worthy of note that introduction of active-site titration has made it possible to assess the availability of the active site and how this immobilization was affected by incorrect orientation, by deactivation or by diffusion constraints [71, 72]. Meanwhile, the first example of resolution of a racemic compound catalysed by carrier-bound immobilized enzymes was also demonstrated and the first enzyme electrode appeared [6]. Glazer et al. demonstrated that introduction of extra functional groups to the enzyme before immobilization was an efficient means of controlling the mode of binding between the enzyme and the carrier [73, 75]. This technology also has other benefits, for example enzyme inactivation resulting from direct coupling of the enzyme to the resin might be avoided. This concept was later developed as modification–immobilization techniques, with the objective of improving the enzyme, e.g. by enhancement of its stability, activity and selectivity, before immobilization [103].

Remarkably, it was found that not only the soluble enzyme but also the enzyme crystals can be entrapped in a gel matrix with reasonable retention of activity [74].

By the end of 1960s the first industrial application of an immobilized enzyme (ionically bound l-amino acid acylase) for production of l-amino acids from racemic amino acid derivatives had been developed by a Japanese company [50]; this not only exemplified the practical (or industrial) value of immobilized enzymes but also inspired several new research interests; this was subsequently reflected by steadily increasing interest, by an explosive increase in publications on enzyme immobilization, and by the number of new immobilization techniques [174, 175].

1.2.4
The Developed Phase (1970s)

In the 1970s, enzyme immobilization continued to flourish into a maturing phase, although the methods used in this period were still labelled as "less rational". The methods developed in previous phases had been widely extended to several enzymes which were expected to have great industrial potential, for example α-amy-

lase, acylase, penicillin G acylase, and invertase, etc. Achievements in this period have been the subject of several reviews [174, 175, 178].

Although the methods used for enzyme immobilization were not beyond the scope of the four basic methods already previously developed, namely covalent, adsorption, entrapment and encapsulation, many new method subgroups, for example affinity binding and coordination binding [105], and many novel variations of enzyme immobilization were developed (Tables 1.4 and 1.5).

The objective of the sophisticated immobilization techniques developed in 1970s was, primarily, improvement of the performance of the immobilized enzymes which could not be achieved by conventional methods of immobilization. For instance, enzymes can be entrapped in gel-matrix by copolymerization of an enzyme modified with double bonds in the presence of the monomers, leading to the formation of "plastic enzymes" with improved stability [110]. Entrapment of enzyme in the gel matrix can be followed by cross-linking, to reinforce the beads and to

Table 1.4 Survey of enzyme-immobilization techniques in the 1970s

Carriers	Activation or coupling methods	Important techniques developed
Active synthetic carriers	Ugi reaction [146, 147]	Reversibly covalent coupling and
Halogen	Alkylation with epoxide	intra-molecular cross-linking [112]
Epoxy ring	[107]	Affinity immobilization [115]
Aldehyde	Aldehyde activation	Coordination immobilization [116]
Anhydride	Carbonyldiimidazole for	Oriented enzyme immobilization [115]
Acylazide	hydroxyl groups [109]	Introduction of spacer [117]
Carbonate	Oxidization of glucosylat-	Complimentary multipoint
Isocyanate	ed enzymes [120]	attachment [118]
	Benzoquinone [121]	Hydrophilicity–hydrophobicity balance
Functionalized	Carbonate [122]	of the carrier [125]
prepolymers	Imidoester [123]	Enzyme immobilization in organic
(for entrapment)	Divinylsulphone	solvents
PVA-SbQ	Glutaraldehyde for	Enzyme entrapment by reactive
PEG-DMA	polyacrylamide [124]	prepolymers [126, 127]
PEG-CA		Immobilization of enzymes to soluble
ENTP		supports [128]
		Reversibly soluble enzymes [128]
Inorganic carriers		Modification and immobilization [103]
for covalent coupling		Adsorption-covalent binding [104]
Silica [118]		
Natural polymers		
and derivatives		
Gelatin		
Alginate		
Agarose		
Collagen		

Table 1.5 Important technologies developed in the 1970s

Method	Remarks	Ref.
Covalent immobilization via spacer	With the aim of modulating retention of enzyme activity	114
Affinity immobilization	Combines mild immobilization conditions and reversibility of binding	115
Oriented enzyme immobilization	With the aim of enhancing activity retention compared with random immobilization	115
Coordination immobilization	Combines immobilization and regeneration of the carrier	116
Enzyme immobilization in organic solvents	With the aim of exploring other binding chemistry that works exclusively in the absence of water	117
Complimentary multi-point attachment	With the aim of enhancing the enzyme stability	118
Immobilization of enzymes to soluble supports	With the aim of acting on sparingly soluble substrates	128
Modification and immobilization	Combines the techniques of chemical modification with immobilization techniques	129
Reversibly soluble enzymes	Combines the advantages of soluble enzymes and immobilized enzymes	135
Stabilization and immobilization	Combines the techniques of enzyme stabilization with enzyme-immobilization techniques	136
Entrapment by wet spinning technique	High enzyme loading can be obtained	137, 138
Covalent entrapment	Enzyme entrapment and covalent binding of the enzyme molecules to the matrix occurred concomitantly	139

avoid leakage [111]; immobilization of the enzymes (either covalent or by affinity adsorption) via a suitable spacer can improve the enzyme activity [114, 115].

More importantly, inspired by the observation that chemical modification of enzymes often improves their characteristics, for example activity and stability, modified enzymes with improved properties, for example enhanced stability, have been further immobilized by a variety of suitable immobilization methods, for example adsorption on the cationic exchanger by introduction of carboxylic ions to the enzymes by succination [129] or entrapment in a polymeric matrix [130].

Another important discovery in the 1970s was that enzyme immobilization does not necessarily have to be performed in aqueous media – covalent coupling of an enzyme to a solid carrier or entrapment of an enzyme in a gel matrix can be performed in organic solvents [131, 132]; such methods have much attractive potential, for example modulation of enzyme conformation or extending the coupling

chemistry beyond the scope of aqueous media. Unfortunately, this technology was not well developed at the time.

As with the carriers used in 1970s, different polymers with designed characteristics, for example tailored-made hydrophobicity or hydrophilicity, particle size and binding functionality, became available for bioimmobilization. By the end of the 1970s, several new synthetic or natural functionalized polymers with pre-designed chemical and physical nature, particularly natural polymer-based carriers bearing reactive functional groups such as aldehyde, cyclic carbonate, anhydride and acylazide, and synthetic polyacrylic polymers bearing different active functionality such as oxirane ring, aldehyde, anhydrides and carbonate [133], were specifically developed or designed for covalent enzyme immobilization [134].

Among these, synthetic polymers with epoxy groups [140, 141] and derivatives of natural polymers [142], which have defined chemical or physical nature and can be directly used to bind enzymes under mild conditions, attracted much attention [104–143]. An inter-conversion technique which was actually proposed by Manneck at the beginning of 1960s [37] was also widely used to convert the built-in active or inactive functionality into other suitable binding functionality for covalent immobilization [143–145].

More importantly, many new chemical reactions were identified and established for covalent coupling of enzymes to carriers; these included:

- the Ugi reaction [146, 147],
- acylation with an imidoester [149],
- carbohydrate coupling [150],
- use of *N*-hydroxysuccinimide esters for activation of carboxyl groups [151],
- coupling and concomitant purification via thio–disulphide interchange [152],
- oxirane coupling [153],
- the benzoquinone method [154], and
- reversible covalent coupling [112].

Remarkably, increasing attention was also directed toward the preparation of immobilized enzymes with designed geometric properties, for example beads [113], foam [155] or fibres [143], to suit various applications and reactor configurations.

During this period much deep insight was gained into the effect on the performance of the immobilized enzymes of factors such as the microenvironment effect of the carrier [155], the effect of the spacer or arm [158, 160], different modes of binding (chemistry, position and number) [170], enzyme loading [87, 167], changes in the conformation of the enzyme, diffusion constraints [161, 163], orientation of the enzyme [164], and the protective effect of substrate or inhibitor during immobilization, namely prevention of deactivation of enzyme from owing to modification of the active site [166] (Table 1.6).

Consequently, many new strategies were developed to improve the performance of the immobilized enzymes, for example the archetype of site-specific enzyme immobilization on the micelle [164], the stabilization–immobilization strategy [170], intramolecular crosslinking [118] and complimentary multipoint attachment [185]. Some of these achievements have been summarized in books and reviews [174, 159, 259].

Table 1.6 Important factors influencing performance of immobilized enzymes discovered in the 1970s

Factors	Implication or application	Ref.
Hydrophobic partition effect	Enhancement of reaction rate of hydrophobic substrate	125, 157
Microenvironment effect of the carrier	Hydrophilic nature often stabilizes the enzyme, whereas hydrophobic nature often destabilizes the protein	127, 156
Multipoint attachment effect	Enhancement of enzyme thermal stability	–
Spacer or arm various types of immobilized enzyme	With the aim of avoiding deactivation of the enzyme by incompatible interaction with protein-carrier or mitigating the steric hindrance	158–160
Diffusion constraints	Enzyme activity might decrease and stability increases	161, 162
Orientation of the enzyme	Site-specific enzyme immobilization techniques featured	163
Presence of substrates or inhibitor	Higher activity retention	164
Conformational changes or protection	Protection of the enzyme from conformational change during enzyme immobilization process leading to high activity retention	164
Physical post-treatments	Improvement of enzyme performance	165
Enzyme loading	Higher enzyme loading is essential to avoid lower enzyme activity expression	87, 166
Different binding mode	Activity and stability can be affected	167
Enzyme modification	Suitable chemical modification often leads to the improvement of enzyme stability	118
Enzyme modification/ immobilization	Formation of active enzyme, which can be covalently bound to the inert carrier, can control the binding mode such as number of bonds formed between the carrier and enzyme, thus improving the activity retention	168
Physical structure of the carrier such as pore size	Activity retention was often pore-size-dependent	169
Stabilization-immobilization	Enzyme can, moreover, be stabilized before binding of enzyme to carrier	170
Physical nature of the carrier	Carriers with large pore size mitigates diffusion limitation, leading to higher activity retention	171
Hydrophilic-hydrophobic balance of the carrier	A delicate balance of hydrophilic and hydrophobic character of the selected carrier is essential for the activity and stability	172

Because of these in-depth investigations, the potential of enzyme-immobilization techniques in commercial processes has been completely recognized and many other commercial processes with use of enzymes have been under development, for example use of immobilized penicillin G acylase for production of 6-APA – the key intermediate in the synthesis of semi-synthetic β-lactam antibiotics – or the use of immobilized glucose isomerase for production of fructose syrup from glucose [119]. Other proposed applications of immobilized enzymes include controlled-release protein drugs and biomedical application as biosensors or artificial organs [176].

By the end of 1970s enzyme-immobilization techniques had matured to such extent that every enzyme could be immobilized by selecting a suitable method of immobilization (entrapment, encapsulation, covalent attachment, adsorption and combi-methods) or a suitable carrier (organic or inorganic, natural or synthetic, porous or non-porous, film, beads, foam, capsules or disks) and immobilization conditions (aqueous, organic solvents, pH, temperature, etc.). It was also increasingly appreciated that the main problem in enzyme immobilization was not immobilization of the enzymes on the carriers but how to obtain the performance desired for a given application by selecting a suitable immobilization approach from the numerous methods available.

1.2.5 The Post-developed Phase (1980s)

In this period, which spans the beginning to the end of the 1980s, incentives to design robust immobilized enzymes originated from the following potential of the immobilized enzymes:

- Immobilized enzymes might meet the increasing demand by manufacturers of pharmaceuticals and agrochemicals for enantiomerically pure compounds, because of their greater selectivity and specificity.
- Biocatalytic processes might meet increasingly strict environmental regulations, because of their mild reaction conditions and lower energy consumption.
- Biocatalytic processes can provide short-cuts compared with conventional chemical processes, because protecting chemistry can be abandoned, as was demonstrated in the production of 6-APA (6-aminopenicillanic acid, a core intermediate for semi-synthetic penicillins) [174].

It is worth mentioning that another important incentive in the search for robust immobilized enzymes in the 1980s and 1990s was the re-discovery that many enzymes are catalytically active and stable in organic solvents [177–179], thus enabling many reactions which cannot be performed in aqueous media.

Enzyme stability and activity are, however, usually lower in organic solvents, because of distortion of enzyme structure by the organic solvents used [177]. Consequently, much effort was devoted to elucidation of the effects of carriers or immobilization techniques on the catalytic behaviour of the immobilized enzymes obtained and of the effects of organic solvents on the enzyme activity, selectivity and stability under non-aqueous conditions. For example, encaging of enzymes in

Table 1.7 Survey of enzyme-immobilization techniques developed in the 1980s

Carriers	Activation methods or coupling reactions	Important techniques and findings
Synthetic microporous carriers Reactive carriers of pre-designed shape and size and active binding functionalities, e.g. Eupergit C and azalactone, were commercialized *Other types of carrier* More than 100 other types of polymeric carrier have been made commercially available [143]	Azalactone Tosylation (hydroxyl groups) [212] Chloroformate [213] 2-Fluro-1-methylpyridinium toluene-4-sulphonate for hydroxyl group [214] Carbonochloridate for activation of hydroxyl groups [215]	Encagement (double encagement) [180, 181] CLEC might be stable biocatalysts in organic solvents [188] Introduction of aquaphilicity [190] Dynamic immobilization technique [216] Deposition technique [201] Covalent multilayer immobilized enzyme [187] Post-loading entrapment [217] Organosoluble polymer–enzyme complex [191–194] Organosoluble lipid-coated enzyme [195] Introduction of genetically engineered tags [198] Introduction of orientation groups to the carrier Carrier-bound multipoint attachment [182–185] Stabilization and/or immobilization [218] Covalent immobilization of enzyme in organic solvents to design active enzyme in organic solvent [199] Imprinting of enzyme by entrapment [200] Covalent binding of enzyme to carriers might freeze the enzyme conformation induced by the effectors [202] Stabilization of immobilized enzyme by the presence of inhibitors [203]

symplex [180] or sandwich complexes [181] could drastically enhance enzyme stability under non-natural conditions; strengthening multipoint attachment to the carrier [182–185], instead of the complimentary multipoint attachment originally proposed by Martinek et al. [186], could stabilize the overall enzyme scaffold by trapping the hot area that is crucial for stabilization of the enzymes [187]; immobilizing an enzyme by a combination of covalent L–B–L(layer-by-layer) techniques and cross-linking could dramatically enhance enzyme loading and enzyme stability [188]; cross-linking of crystalline enzymes can be used to create stable biocatalysts for biotransformation, especially in organic solvents, because of their high stability in these solvents (see Table 1.7 for details) [189].

Besides efforts to prepare stable immobilized enzymes, several methods and concepts were developed to make the enzymes more active in organic solvents. First, the concept of the water activity of the reaction medium was proposed [190], and reliable comparison of different catalytic processes in low-water media became possible. The introduction of the concept "aquaphilicity" for the carrier enables quantitative measurement and screening of the desired carriers for immobilization of enzymes intended for use in organic solvents with regard to the close relationship between the carrier and the activity and selectivity of the immobilized enzymes [191]. The finding that physical and/or chemical modification of enzymes (for example chemical modification with activated PEG [192–195, 247] or lipid pairs [197], encapsulating the enzyme in micelles [196], and coating the enzyme surface with lipids) renders them soluble in organic solvents enabled the mass transfer limitation associated with the use of lyophilized enzyme powders to be surmounted [197]. Thus, higher activity can be obtained with these techniques.

Along with the cell-free enzymes, whole-cell associated biocatalysts, in the presence or absence of support materials, have also been successfully used in organic solvents [208–211].

Many techniques developed in the 1970s were further implemented to improve enzyme performance [272]. For instance, site-specific enzyme immobilization via a variety of genetically engineered tags attracted much attention [198] because of better retention of activity. Covalent attachment of the enzyme to the carrier in organic media was found to be unique in that the immobilized enzyme obtained is active in organic solvents whereas the immobilized enzyme prepared in aqueous medium is completely inactive, even though their hydrolytic activity was almost identical [199]. This inspired much research interest in preparing active and stable immobilized enzymes in organic solvents in 1990s, as will be discussed below.

1.2.6 Rational Design of Immobilized Enzymes (1990s–date)

Since the 1990s (Table 1.8) there has been an important transition in the development of immobilized enzymes. Approaches used for the design of immobilized enzymes have become increasingly more rational; this is reflected in the use of more integrated and sophisticated immobilization techniques to solve problems that cannot be easily solved by previously developed single immobilization approaches.

In this phase, the major focus of enzyme immobilization was on the development of robust enzymes that are not only active but also stable and selective in organic solvents. Although in the period from the 1970s to the 1980s it was recognized that many enzymes are active and stable in organic solvents under appropriate conditions, the enzymes used are usually less active or stable in organic solvents than in conventional aqueous media [177]. For this reason development of more robust immobilized enzymes which can work under hostile conditions, especially in non-aqueous media came to the forefront of many research interests in this period [219–231].

Table 1.8 Survey of important enzyme-immobilization techniques developed from the 1990s until the present

Strategies	Improvement	Remark	Ref.
Formation of plastic immobilized enzymes in organic solvent	Stability and activity in organic solvents	Stable in organic solvents	240, 241
Introduction of tentacle carriers	Substantial enhancement of enzyme loading	High enzyme loading and less diffusion limitation	242
Chemical post-immobilization techniques	Improvement of enzyme stability and activity	Such as pH-imprinting, consecutive modification, solvent washing and increasing pH, addition of additives	223, 243
Stabilization–immobilization strategy	Enzyme stability in organic solvents, followed by entrapment techniques	The enzyme is stabilized first, followed by another suitable immobilization strategy	245, 246
Engineering the microenvironment	Improvement of enzyme stability and activity	Improvement of enzyme stability in organic solvent	248
Strengthening the multipoint attachment	Improvement of stability	Increasing the number of bonds enhanced the enzyme stability	249
Site-directed enzyme immobilization	Improvement of enzyme activity and stability	Orientation of enzyme on the carrier surface improves activity retention	250
Imprinting-immobilization strategies	Improvement of enzyme selectivity by conformer selectors	Alter the enzyme selectivity by sol–gel techniques and/or cross-linking techniques	251
Improved sol–gel entrapment	Improvement of activity and selectivity	Selection of suitable monomers and conformer selector is essential	47, 48
Cross-linked enzyme aggregates	Improvement of enzyme activity and selectivity	Selectivity relative to CLEC was improved	16
Entrapped CLEA	Improvement of the mechanical stability and tailor-made particle size	CLEA can be prepared in a pre-designed hollow microsphere	252
Non-covalent L-B-L immobilization	Substantial enhancement of enzyme loading	Less diffusion limitation	254
Enzyme deposition techniques	Improvement of enzyme dispersion state in organic solvent	Monolayer principle	201

Among these, many efforts were devoted to the development of cross-linked enzyme crystals (CLEC) suitable for biotransformations in non-aqueous media or in organic–water mixtures, because of the greater stability of the enzymes under hostile conditions [232–234]. Remarkably, it has been noticed that the performance of the CLEC obtained is highly dependent on the predetermined conformation of the enzyme molecules in the crystal lattice. Thus, selection of a highly active enzyme conformation by varying the crystallization conditions becomes crucial for the creation of highly active, stable and selective CLEC.

Because the process of protein crystallization is homogenization of enzyme conformation, and the enzyme conformation in the crystal lattice is predetermined by the crystallization conditions, each type of cross-linked enzyme crystal of the same enzyme might represent only a specific immobilized enzyme whose conformation is homogeneous and fixed by the cross-linking. Although it is possible to crystallize an enzyme in different conformations, and thus to modulate its properties, this technology is obviously laborious and limited compared with carrier-bound methods.

Broad analysis of the performance of CLEC and comparison with conventional carrier-bound immobilized enzymes is still lacking. Cross-linked enzymes, especially CLEC, have occasionally been compared with lyophilized enzyme powder, which was proved to be not only less active but also less selective [235]. A few studies have also shown that the turnover frequency of cross-linked enzymes in organic solvents is generally lower than that of carrier-bound immobilized enzymes based on the same protein mass and the same reaction conditions, suggesting that rigidification or confinement of the enzyme molecules in the compact crystal lattice or diffusion limitation might be major factors responsible for the lower activity [236].

Increasing efforts have also been devoted to developing novel strategies for improving the performance of immobilized enzymes, for example their activity [237], selectivity [207, 251] and stability [239], by combining different immobilization techniques [236]. For example, biocatalytic plastics, which are prepared by polymerizing lipid-coated organic solvent-soluble enzymes bearing attached polymerizable double bonds, were found to be highly stable and active in organic solvents [240, 241] and polymerization of enzyme derivatives bearing unsaturated bonds in the presence of ligands or substrates proved to be an effective means of creation of insoluble enzymes with improved selectivity [207]. The use of dendrimeric (or tentacle) carriers has been found advantageous in that enzyme loading can be dramatically enhanced by at least one order of magnitude, with high retention of enzyme activity and stability [242]. Furthermore, sol–gel techniques were only recognized as an interesting immobilization technique in the 1990s – 40 years after the first use of these techniques for enzyme immobilization [46–48].

Apart from improvement by immobilization strategies, in the 1990s it was also increasingly appreciated that attachment of enzymes to the selected carriers is not the whole story of enzyme immobilization, because the performance of the immobilized enzymes can be substantially improved by use of various post-immobilization techniques; for example, strengthening the multipoint attachment often en-

hances enzyme stability [249] and consecutive treatment of immobilized enzymes by chemical or physical modification, or other activation and stabilization techniques, can dramatically improve enzyme performance [243], as will be discussed elsewhere in this book.

More important, the molecular imprinting techniques originally proposed in the 1970s, has been further developed and extended to several other, related areas [244]. The objective is to improve enzyme performance. For example, pH imprinting of the immobilized enzymes, including enzyme powders, enables maximum activity of the enzyme in anhydrous organic solvents [246], significant activity or selectivity improvement can be achieved by simply lyophilizing the enzymes with the ligands or transition-state analogues or by polymerizing the enzyme–ligand complex under more anhydrous conditions or in aqueous medium.

Remarkably, it has been found that even the stability of the immobilized enzymes can be imprinted. For instance, the temperature optimum of epoxy hydrolase immobilized on DEAE-cellulose was dramatically shifted from 35 to 45 °C if non-ionic detergent Triton X-100 was added during enzyme binding to the carrier [344], suggesting that the stable enzyme conformation induced by the additive was frozen on the carrier. Similarly, it was found that stability of *Candida rugosa* lipase, which was covalently immobilized on silanized controlled-pore silica (CPS) previously activated with glutaraldehyde in the presence of PEG-1500, was increased fivefold compared with the immobilized enzyme without addition of PEG-1500 [209]. Thus, in those cases, the stability or, more precisely, the enzyme conformation induced by the effectors (or additives) was imprinted.

Also worthy of note is that in the last few years of the 1990s it was discovered that not only enzyme crystals but also physical enzyme aggregates could be cross-linked to form catalytically active insoluble immobilized enzymes, nowadays known as CLEA [252, 255, 257]. This discovery might theoretically and/or practically open another possibility for design of robust, highly active, stable and selective immobilized enzymes [16].

As with cross-linked crystalline enzymes, however, the factors which hamper their industrial application lie not in their catalytic properties but in their non-catalytic part – they are usually small and their mechanical stability is usually very poor. This causes difficulties when they are applied to heterogeneous reaction systems, e.g. "solid-to-solid" reaction systems [256], in which large (>100 μm) immobilized enzymes are often chosen to facilitate separation by use of a sieve-plate reactor, as in the kinetically controlled enzymatic synthesis of β-lactam antibiotics [17].

It has recently been found that industrially robust CLEA, with greater activity both in organic solvents and in aqueous media, can be prepared by use of new cross-linking technology [252]. The use of preformed soft hollow microsphere has, moreover, enabled the preparation of CLEA with greater mechanical stability and tailor-made size, and which are thus, in principle, applicable to any reaction system, reactor configuration and reaction medium [253].

By combining the advantages of carrier-bound and carrier-free immobilized enzymes, CLEA with tailor-made properties with regard to both non-catalytic and cat-

alytic function can be designed at will – an attractive proposition for industrial applications.

In general, the techniques currently used for creation of robust immobilized enzymes, which meet both catalytic requirements (desired activity, selectivity, and stability) and non-catalytic requirements (desired geometric properties such as shape, size and length) expected for a given process, are all characterized in that an combined method are used to solve problems that are unsolvable by the straightforward method.

1.3 Immobilized Enzymes: Implications from the Past

Having discussed the historical development of immobilized enzymes in the past 90 years, we are interested in the status of immobilization techniques. In this section, we briefly summarize what has been achieved, what more we can achieve, and what will be achieved in the near future.

1.3.1 Methods of Immobilization

More than 5000 publications, including patents, have been published on enzyme-immobilization techniques [259–263]. Several hundred enzymes have been immobilized in different forms and approximately a dozen immobilized enzymes, for example amino acylase, penicillin G acylase, many lipases, proteases, nitrilase, amylase, invertase, etc., have been increasingly used as indispensable catalysts in several industrial processes.

Although the basic methods of enzyme immobilization can be categorized into a few different methods only, for example adsorption, covalent bonding, entrapment, encapsulation, and cross-linking [264], hundreds of variations, based on combinations of these original methods, have been developed [265]. Correspondingly, many carriers of different physical and chemical nature or different occurrence have been designed for a variety of bio-immobilizations and bio-separations [143, 262, 263]. Rational combination of these enzyme-immobilization techniques with a great number of polymeric supports and feasible coupling chemistries leaves virtually no enzyme without a feasible immobilization route [266].

It has recently been increasingly demonstrated that rational combination of methods can often solve a problem that cannot be solved by an individual method. For instance, the physical entrapment of enzymes in a gel matrix often has drawbacks such as easy leakage, serious diffusion constraints, and lower stability than that for other immobilized enzymes. These drawbacks can, however, be easily solved by rational combination of different methods. For instance, higher stability can be achieved by means of the so-called pre-immobilization stabilization strategy [266] or post-immobilization strategy [267].

In the former case, the enzyme, for instance, can be first crosslinked to form stabilized enzyme preparations e.g. CLEA. Subsequent entrapment endows the CLEA with a suitable particle size and high mechanical stability [266]. Stabilization can be also achieved by chemical modification [267]. For instance, chemical modification of the soluble enzyme with a hydrophilic polymer often stabilizes the enzyme because of the introduction of a favourable hydrophilic microenvironment. Thus, the subsequent entrapment of the stabilized enzyme often leads to the formation of more stable enzyme, compared with the entrapped native enzymes [238, 267, 270].

In the later case, the entrapped enzyme can be further crosslinked, with the aim of enhancing the stability or avoidance of enzyme leakage. For instance, β-amylase from *Bacillus megaterium* immobilized in BSA gel matrix and subsequently covalently crosslinked was fourteen time more thermally stable than the native enzyme [269].

Because of these possibilities, a rational combination of the available methods will definitely facilitate the design of robust immobilized enzymes that can suit various applications. Consequently, use of immobilized enzymes is now becoming commonplace in many fields, for example chemical, medical, pharmaceutical and analytical applications [271–273], with the aim of enabling processes in continuous mode, control of the processes, overcoming cost constraints, and solving problems that were previously approached mainly by chemical means [274] or which could not easily be solved by chemical methods.

There is, nevertheless, still a significant lack of systematic analysis of the methods available. Most enzyme immobilization has been performed without any knowledge of structural information, and the relationship between the performance of the immobilized enzyme and the method selected for immobilization has, so far, rarely been defined or identified. Thus, a central task in the future development of immobilization techniques is probably not to develop new methods of immobilization but to establish guidelines linking the method selected with the performance expected.

1.3.2
Diversity versus Versatility

Despite our increasingly understanding of enzyme-immobilization techniques, and the numerous possible means of obtaining robust immobilized enzymes, development of a robust immobilized biocatalyst which can meet the requirements of modern biocatalytic processes – mild reaction conditions, high activity, high selectivity, high operational stability, high productivity, and low cost [275] – still relies on laborious trial-and-error experimental approaches [276]. Consequently, a crucial question is whether it is possible to develop a generic method or to establish generic guidelines for enzyme immobilization. Obviously, the answer to this question lies both in the reality of different immobilization techniques and the peculiarity of each individual application. The establishment of the guidelines necessitates systematic analysis of the methods available and the experimental information that has been obtained in the past. The poor comparability of many experimental re-

sults (obtained by different groups and people) seriously hampers the establishment of such universally applicable guidelines, however.

On the other hand, the peculiarities of applications, for example the types of reaction (hydrolytic reaction or reverse reaction), reaction medium (aqueous or organic solvents), reaction system (solid-to-solid, liquid-to-solid, liquid-to-liquid), reactor configuration (stir-tank, plug-flow), economic viability (cost contribution of the immobilized enzyme, space-time yield and productivity) and the intrinsic characteristics of the enzymes selected might differ from case to case. Thus differences between the peculiarities of each application also require specific solution of each individual application.

It must, therefore, be expected that choice of the method of immobilization is mainly dictated by the specific conditions and requirements of each application, which should selectively employ the positive attributives of the method selected. In this sense, the diversity of enzyme-immobilization techniques could be a powerful asset in the design of robust immobilized enzymes, because changes in the peculiarities of the applications often require design of new immobilized enzymes which fit the new applications.

It has, for instance, been demonstrated that differently carrier-bound immobilized penicillin G acylase (PGA) is not only suitable for catalysis of the hydrolysis of penicillin G for production of 6-APA – the nucleus of semi-synthetic β-lactam antibiotics (amoxicillin or ampicillin) [277] – it has also recently been increasingly applied to the synthesis of semi-synthetic β-lactam antibiotics (amoxicillin or ampicillin). In the hydrolysis many types of carrier-bound immobilized penicillin G acylase can be used, for example PGA immobilized on Eupergit C (PcA) or PGA immobilized on polyacrylamide, whereas for the synthesis of semi-synthetic antibiotics such as ampicillin, cephalotin, and cephalexin only few carrier-bound immobilized penicillin G acylases, for example gelatin-bead-bound or agarose bead-bound proved advantageous in terms of the high ratio of synthesized antibiotic to hydrolytic product [17, 249, 277].

Another example is the development of immobilized amino acid acylase for use in the production of chiral amino acids [278]. Among a number of preparations obtained by different methods of immobilization, several promising products, for example the enzyme ionically bound to DEAE-Sephadex, covalently bound to iodoacetyl cellulose, or entrapped in PAAm polyacrylamide gel matrix, were screened for further evaluation. Because of the possibility of regenerating the carrier, the stability of the immobilized enzyme, the ease of immobilization, and the cost of the immobilized enzyme, only DEAE-Sephadex was selected for the final process – resolution of racemic amino acid esters. Remarkably, although amino acid acylase immobilized on DEAE-Sephadex was the first enzyme used in commercial processes, the same immobilized enzyme was recently found to be inactive for resolution of racemic amines or alcohols in organic solvents [279].

The importance of diversity in enzyme immobilization techniques has recently been beautifully demonstrated by screening of carriers for immobilization of glycolate oxidase. Twenty-one different carriers were screened, ranging from natural polysaccharide-based carriers such as CNBr-activated agarose sepharose or epoxy

activated agarose or sepharose, ionic exchange CH-sepharose, hydrophobic adsorbents such as phenyl sepharose, to synthetic organic carriers such as epoxy carriers such as Eupergit C, Eupergit C250 L, azalactone carrier such as Emphaze, ionic exchangers Bio-Rex 70, hydrophobic adsorbent such as Amberlite XAD 4, XAD 8, to inorganic carriers such as silanized CPG glass bead derivatives and silanized celite derivatives. The coupling mode covers three types, namely physical adsorption, ionic binding and covalent binding [280].

Remarkably, it was found that among the polysaccharide-based carriers immobilized enzyme with higher activity and retention of activity was obtained with CNBr-activated agarose Sepharose. In contrast, epoxy activated agarose or Sepharose usually afforded lower activity. Remarkably, comparable activity was obtained with synthetic epoxy carrier, i.e. Eupergit C [280]. This example strongly suggests that the performance of a carrier-bound immobilized enzyme is dictated by the physical and chemical nature of the carrier (e.g. chemical composition, binding chemistry, hydrophilicity, pore size and etc.) and that a good carrier or a suitable binding chemistry for an enzyme is not necessary the right one for other enzymes or other applications.

Because of the diversity of carrier nature in terms of the source (synthetic/natural, organic/inorganic), structure (porous/nonporous), the diversity in coupling chemistry, the nature of the interaction (physical, specific adsorption, covalent), designing or screening of a specific immobilized enzyme that suits a specific application becomes possible.

Thus, there is no doubt that changing the type of reaction (hydrolysis or condensation), the reaction medium (aqueous solutions or organic solvents), the reaction system (heterogeneous or homogeneous), the reaction conditions, or even the substrates might lead to a change in the criteria used to assess the robustness of the immobilized enzymes. On the other hand, the diversity of carrier nature (physical and chemical), the binding chemistry, and different immobilization methods provide us an indispensable tool for the design of robust immobilized enzymes.

1.3.3
Complimentary versus Alternative

Enzymes belong to the category of natural catalyst which includes DNA, RNA and catalytic antibodies. A unique function of enzymes is that all the reactions they catalyse, can be performed sequentially, selectively and precisely under mild physiological reaction conditions. This unique feature makes enzymes very attractive for synthetic chemistry, which is usually based on use of hazardous reactants and reaction conditions.

There is, however, no doubt that many are not ideal catalysts for industrial applications, for example in the manufacture of fine chemicals [281, 282] and pharmaceuticals and their intermediates [283], in which the enzymes are usually exposed to non-natural conditions such as high substrate concentrations, high pH, high temperature and the use of deleterious organic solvents. Accordingly, for most industrial applications, they must be modified either by genetic engineering or by

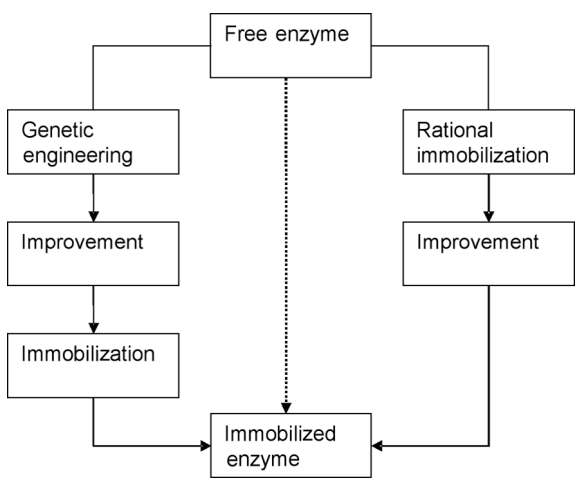
chemical modification, with the objective of improving their selectivity, activity and durability under the process conditions. They must, furthermore, be used in the immobilized forms, to reduce production cost by facilitating downstream processing such as recycling and separation [284].

In the last decade, although it has been increasingly appreciated that genetic engineering is a powerful tool for improvement of enzyme performance, enzyme immobilization is the only technique, which can combine immobilization of an enzyme with improvement of enzyme performance, for example stability, selectivity and activity [285]. Thus, immobilization-improvement strategies might be very attractive for enzymes designed to be used in the immobilized form anyway (Scheme 1.3). In this sense, it is also increasingly recognized that rational immobilization of enzymes by combining immobilization and genetic engineering might be an alternative and complimentary technique for protein engineering.

Many examples have excitingly demonstrated that even for genetically engineered enzymes performance can be further improved by immobilization techniques and many examples have revealed that enzyme-immobilization techniques are indeed an indispensable complimentary tool in enzyme engineering, due to its potential for:

- combination of immobilization and improvement,
- modulation of enzyme performance by selecting appropriate method of immobilization, and
- combination of different immobilization methods.

As shown in Scheme 1.3, improvement by immobilization is obviously straightforward compared with genetic engineering. Furthermore, improvement by immobilization does not normally obviate immobilization of the exact structure.



Scheme 1.3 Comparison of improvement of an enzyme by genetic engineering and by immobilization.

As more information becomes available about the relationship between the performance of the immobilized enzyme and the method selected, design of more robust immobilized enzymes at will, via the use of different immobilization techniques, might be a reality in the near future.

It is currently possible to draw the conclusion that immobilized enzymes might perform better than the native enzymes (improved stability, activity and stability) if the method is correctly selected. This will be discussed in Section 1.3.4 [285–287].

1.3.4 Modification versus Immobilization

As already noted, the problem of enzyme immobilization is not how to immobilize the enzyme but how to achieve the desired performance for a given application by selecting an appropriate means of immobilization. Thus, it is also important to distinguish the two concepts – modification and immobilization – before we enter discussion.

Although enzyme immobilization and improvement of enzyme performance by immobilization share the same principles, the emphasis is different. The former is mainly associated with efforts to find suitable immobilization methods for enzymes that must be immobilized for certain applications. Thus, the immobilization technique developed is mainly intended to retain the major catalytic functions of the native enzymes. In contrast, improvement-by-immobilization is focused mainly on utilization of available immobilization techniques to alter (or improve) enzyme performance, to suit the desired application. Thus, the native enzyme might be not suitable for a desired process, because of its poor performance such as lower activity, or stability or selectivity. Consequently, the technique to be developed should improve the performance of the enzyme besides immobilizing it. Because the success of the latter largely depends on knowledge acquired from experimental information from the former application, we recognize it is essential to provide detailed analysis of the results so far obtained from improvement, by immobilization, of three catalytic characteristics, i.e. activity, stability and selectivity, of the enzymes.

1.3.4.1 Enhanced Stability

Of these three important characteristics of enzymes stabilization by immobilization has been studied since the 1970s, when immobilized enzymes became increasingly used in industrial processes, in which the cost-contribution of the immobilized enzyme is often the indicator of process viability [285]. Since then, many useful strategies have been developed for stabilization of enzymes by immobilization, for example cross-linking, multipoint attachment and engineering of the microenvironment, confining the enzyme molecules, etc.

The stability of a native enzyme (i.e. a non-immobilized or modified enzyme) is principally determined by its intrinsic structure whereas the stability of an immobilized enzyme is highly dependent on many factors, including:

- the properties of its interaction with the carrier,
- the binding position and the number of the bonds,
- the freedom of the conformation change in the matrix,
- the microenvironment in which the enzyme molecule is located,
- the chemical and physical structure of the carrier,
- the properties of the spacer (for example, charged or neutral, hydrophilic or hydrophobic, size, length) linking the enzyme molecules to the carrier, and
- the conditions under which the enzyme molecules were immobilized.

Whatever the reason, the enhanced stability resulting from immobilization can often be ascribed to the intrinsic features of individual immobilization processes, for example:

- molecular confinement (which occurs often in the entrapment process, particularly the sol–gel process) [289];
- favourable microenvironment – achieved by selecting appropriate carriers [256] or engineering the microenvironment by post-immobilization techniques [258];
- chemical modification effect in covalent bonding (such as formation of an extra hydrogen bond as a result of chemical modification in the covalent immobilization process) [290]; and
- rigidification of conformation as a result of multipoint attachment [184].

It might, nevertheless, also be true that many stabilization factors can be integrated into one immobilization process, as in the stabilization and immobilization procedures [291] and three-dimensional immobilization (by cross-linking crystalline enzymes or enzyme aggregates) [16]. Also, it is very difficult to judge which method can give the most stable enzymes, because even the same method (let us say covalent immobilization) might lead to immobilized enzymes of different stability, depending on the carrier selected, the immobilization conditions (e.g. enzyme loading, pH, temperature, ionic strength, additives) [292] or subsequent treatment.

It will, however, never be found that an enzyme cannot be stabilized. Thus, stabilization by immobilization can be always achieved by selecting a suitable immobilization method. One can confidently state that stabilization by immobilization is currently no longer an exception, because of our increasing understanding of the immobilization processes. Remarkably, it has been found that even thermophilic enzymes or extremophilic enzymes [293] can be further stabilized by immobilization [239, 294–296, 230], suggesting that stabilization of enzyme by immobilization can be additive.

1.3.4.2 **Enhanced Activity**

Observation of the enhancement of enzyme activity by immobilization can be dated back to the early 1960s, when Goldstein et al. noted that for trypsin immobilized on a charged carrier the K_m for charged substrates could be reduced by a factor of fourteen [79], because of the so-called microenvironment effect.

It has been found that many types of enzyme immobilized by different immobilization techniques have higher activity than the native enzymes. For instance, ep-

oxy hydrolase adsorbed on DEAE-cellulose by ionic bonding was more than twice as active as the native enzyme [344], lipase–lipid complex entrapped in n-vinyl-2-pyrrolidone gel matrix was 50-fold more active than the native enzyme [298]. Activation by immobilization is, however, often regarded as an extra benefit rather than a rational goal of enzyme immobilization.

Activity retention by carrier-bound immobilized enzymes is usually approximately 50 %. At high enzyme loading, especially, diffusion limitation might occur as a result of the unequal distribution of the enzyme within a porous carrier, leading to a reduction of apparent activity [299]. The conditions for high activity retention are often marginal, thus often requiring laborious screening of immobilization conditions such as enzyme loading, pH, carrier and binding chemistry [292].

Next to the microenvironment effect mentioned above, it has been demonstrated that immobilized enzymes can be more active than the native enzymes, when the inhibiting effect of the substrate was reduced. For example, immobilization of invertase from *Candida utilis* on porous cellulose beads led to reduced substrate inhibition and increased activity [301]. A positive partition effect (enrichment of substrates in the proximity of the enzymes) might also enhance enzyme activity as was observed for kinetically controlled synthesis of ampicillin with penicillin G acylase immobilized on a positively charged carrier [302] or horse liver alcohol dehydrogenase immobilized on poly(methylacrylate-co-acrylamide) matrix [124, 157].

Greater retention of enzyme activity can occasionally be achieved, especially for allosteric enzymes such as some lipases which have lids covering the active centre; conformational change increases the accessibility of the active centre [303–306]. In other instances of improvement of the molecular accessibility of enzymes by immobilization, enhancement of enzyme activity relative to that of the native enzyme powder can be achieved when the enzymes are intended for use in anhydrous organic solvents, for example lipase PS immobilized in sol–gel [236] or protease covalently bonded to silica, because of increasing dispersion of the enzyme molecules and conformation induction.

In general, the activity of the immobilized enzymes can be enhanced by at least ten different effects involved in enzyme immobilization:

- microenvironment effect,
- partition effect,
- diffusion effect (reducing the pH),
- conformational change,
- flexibility of conformational change,
- molecular orientation,
- water partition (especially in organic solvent),
- conformation flexibility,
- conformation induction, and
- binding mode.

For conformation-controlled activity it was found that the enzyme activity (U mg^{-1} protein immobilized) was strongly dependent on the nature of the carriers used. For instance, the activity of lipase PS (*Pseudomonas cepacia*) immobilized on Toyo-

nite, Celite, glass and Amberlite was highly dependent on the nature of the carrier. The highest activity for transesterification in organic solvent was obtained with Toyonite (37.2 μmol min^{-1} mg^{-1}) and the lowest activity was obtained with Amberlite (0.4 μmol min^{-1} mg^{-1}) [307]; the difference in activity is approximately two orders of magnitude!

Enhancement of activity in organic solvents after immobilization by sol–gel processes was clearly demonstrated for lipases, the activity of which in organic solvents, relative to that of the native enzyme powders, can be increased at least fivefold by use of conformer selectors such as surfactants or crown ethers, suggesting that the presence of conformer selectors induced an active conformation, which is, however, frozen by the corresponding immobilization process [47].

Molecular orientation-controlled activity of enzymes was observed early in 1972 [163]. In connection with this observation and enzyme immobilization in organic solvents, it was also demonstrated that the lipase from *Mucor risopus* immobilized in organic solvent was more active in transesterification in organic solvent whereas the lipase immobilized in aqueous medium had almost no activity in organic solvents. The author suggested that the position of binding of the enzymes to the carrier in organic solvents is different from that when immobilization is performed in aqueous medium [199]. Many other types of immobilized enzyme, which can be categorized as immobilized enzymes with orderly oriented enzyme molecules generally have higher activity or stability relative to the counterpart (randomly immobilized enzymes), because of favourable accessibility or avoidance of the modification of the active site [308].

The effect of conformation flexibility is often in contradiction with enzyme activity, i.e. reduction of enzyme conformation flexibility often reduces the enzyme activity. This was confirmed initially by the observation that immobilization of an enzyme on a carrier via a suitable spacer often resulted in better retention of activity than if the enzyme was immobilized without a spacer [309–312].

In contrast, higher activity has been achieved by increasing conformational flexibility. For example, amino acid acylase immobilized ionically on DEAE-cellulose has high activity after post-treatment with a denaturant, which could possibly enhance the enzyme conformational flexibility [352]. For enzymes acting in low-water media, especially, enzyme conformational flexibility is much less than in aqueous media. Thus, if the water content of enzyme preparations is kept to a minimum that enables the enzyme to have the highest conformational flexibility, maximum activity may be achieved in organic solvent because of higher conformational flexibility. For quantitative control of water hydration level, water activity was developed [190].

Nevertheless, water activity is not the whole story of enzyme activity in organic solvents. The fact that dehydration history largely dictates enzyme activity rather than the water activity suggests that some dehydration processes might reversibly deactivate the enzyme [313]. Consequently, it is concluded that the water-activity concept is only valid when the enzyme preparation is not reversibly deactivated by the process for dehydration.

The effect of binding mode on the enzyme activity can be reflected by three factors – the number of bonds formed between the carrier and the enzyme molecules, the position of the bonds and the nature of the bonds. It is easily conceivable that the greater the number of bonds formed between the enzyme and the carrier, the lower the enzyme activity, as demonstrated by immobilization of β-galactosidase *E. coli* and *K. lactis* on thiolsulphinate-agarose and glutaraldehyde-agarose [313]. The greater retention of activity with thiolsulphinate-agarose can be largely ascribed to the fewer bonds formed between the enzyme and the carrier – thiolsulphinate-agarose [314]. Indeed, these two enzymes are much richer in the lysine residues than the cysteine residues. Thus, more bonds can be formed with glutaraldehyde-agarose, resulting in less retention of activity [314].

Interestingly, a recent example showed that α-amylase immobilized on thionyl chloride ($SOCl_2$) activated poly(Me methacrylate-acrylic acid) microspheres has 67.5 % retention of activity whereas 80.4 % was achieved with carbodiimide (CDI)-activated poly(Me methacrylate-acrylic acid) microspheres. Irrespective of whether the enzyme is immobilized on the same carrier with the same binding nature. It was, moreover, found that the former is twice as stable after storage for 1 month. On the other hand the free enzyme lost its activity completely in 20 days. Apparently, this difference can be solely ascribed to the difference in the position of the bonds formed [315].

1.3.4.3 **Improved Selectivity**

The selectivity of enzymes is nowadays becoming a powerful asset of enzyme-mediated asymmetric synthesis, because of the increasing need of the pharmaceutical industry for optically pure intermediates [312].

In general, the selectivity of enzymes includes [317]:

- substrate selectivity – the ability to distinguish and act on a subset of compounds within a larger group of chemically related compounds;
- stereoselectivity – the ability to act on a single enantiomer or diastereomer exclusively;
- regioselectivity – the ability to act exclusively on one location in a molecule;
- functional group selectivity – the ability to act on one functional group selectively in the presence of other equally reactive or more reactive functional groups, for example the selective acylation of amino alcohols [318].

Although a dramatic change of enzyme selectivity by genetic engineering has been beautifully demonstrated [225], there are also numerous attractive examples in which enzyme selectivity has been changed by a variety of immobilization techniques, for example covalent bonding, entrapment, and simple adsorption, as discussed in the following section (for details see Table 1.9). In several extreme instances it has been demonstrated that a non-selective enzyme such as chloroperoxidase was transformed into a stereoselective enzyme after immobilization [319]; the *S*-selective lipase has also been converted to *R*-selective CR lipase by covalent immobilization [320].

Table 1.9 Alteration of selectivity by immobilization

Method of immobilization	Selectivity	Remark	Enzyme	Ref.
Adsorption	Regioselectivity	Non-selective chloroperoxidase was transferred to stereoselective enzyme after immobilization	Chloroperoxidase	318
Adsorption, covalent	Enantioselectivity	Selectivity was improved but dependent on the reaction conditions	Lipases	319 340
Covalent CPG	Product map		Subtilisin	324
Covalent	Product map	Resulted in different product map	Proteases	326
Covalent immobilization on glyoxal agarose	Different selectivity?	Resulted in different selectivity	Urokinase	327
Covalent?	Product map	Immobilized on phenol-formaldehyde resulted in different product composition	α-Amylase	328
Gelatin-entrapped, and surface-bound	Action pattern	The action pattern depended on how the enzymes were immobilized, namely entrapped or linked to the surface	Glucoamylase	329
Adsorption on DEAE-cellulose	Substrate selectivity	Resulted in changed substrate selectivity relative to the native enzyme	Dextransucrease	330
Adsorption	Enantioselectivity	The selectivity of the enzyme is reduced	Lipase CAL-B	331
Covalent on agarose	Enantioselectivity	Selectivity is dependent on the carrier and binding chemistry	Lipase CRL	332 333
Covalent on silica	Enantioselectivity	The enantioselectivity was enhanced 7-fold relative to the free enzyme with trichlorotriazine as activating agent	CRL	332

Table 1.9 Continued

Method of immobilization	Selectivity	Remark	Enzyme	Ref.
Adsorption on celite	Enantio-selectivity	Simple adsorption enhanced the stability of *Candida rugosa* lipase against acetaldehyde, and the selectivity	Lipase CRL	334
Entrapment	Enantio-selectivity	Enhanced selectivity compared with the native enzymes	RML	335
Sol–gel entrapment	Enantio-selectivity	Chiral template influenced enzyme selectivity	Lipase	252
Entrapment in Ca-alginate gel beads	Enantio-selectivity	Increased threefold the enantioselectivity of pegylated PCL in Ca-alginate gel beads	Pegylated PCL	336
Sol–gel entrapment	Enantio-selectivity	Immobilization can trap different enzyme conformation	Fructose-1,6-bisphosphatase	337
Double immobilization technique	Substrate selectivity	(Adsorption on solid carrier, followed by entrapment in alginate beads) resulted in different product spectrum	2-Mannosidase	338
Covalent immobilization	Reaction selectivity	The ratio of condensation to hydrolysis in the kinetically controlled synthesis of β-lactam antibiotics depended on the immobilization methods	Penicillin G acylase	339
Covalent	Enantio-selectivity	The enantioselectivity of lipase MML immobilized on oxirane carrier is dependent on newly introduced functionalities	*Mucor miehei* lipase	340
Ionic binding	Enantio-selectivity	The selectivity is mainly dictated by the nature of pending charged groups	Alkylsulphatase	350

In general, the selectivity that can be influenced by the immobilization techniques can be classified into the following categories, according to the source of the effect:

1. Carrier-controlled selectivity
   a) pore size-controlled selectivity
   b) diffusion-controlled selectivity

2. Conformation-controlled selectivity
   a) microenvironment-controlled selectivity
   b) active centre-controlled selectivity

The effect of steric hindrance on enzyme selectivity, for example the product map, was observed in 1970s [324]. For example, the product pattern of CPG (controlled pore glass)-immobilized subtilsin-catalysed digestion of proteins can be affected by the pore size of the carrier used [325]. Similarly, immobilized ATP deaminase, β-galactosidase [325] and proteases also have different product maps [328, 329]. Urokinase covalently immobilized on glyoxal agarose has different selectivity [326]. α-Amylase immobilized on silica [329] or covalently bound to CNBr-activated carboxymethylcellulose [324] afforded products of composition different from that of the native enzyme. This was largely attributed to the fact that the size of the pores where the enzyme molecules are located determines the accessibility of the substrates, depending on their size.

Diffusion-controlled enantioselectivity was reported recently after a study of the enantioselectivity of lipase CAL-B in the transesterification in organic solvents [332]. For the first time it was reported that diffusion can reduce the enantioselectivity of enzymes. A relevant example worth mentioning is that simple adsorption of lipase CRL on Celite not only enhanced the stability of *Candida rugosa* lipase against acetaldehyde but also enhanced the enantioselectivity up to threefold [331]. It is possible that improvement of enzyme dispersion enhanced the enantioselectivity of the immobilized enzymes relative to the enzyme powders.

The important implication of this discovery is that in diffusion-controlled enantioselectivity reduced enantioselectivity is always accompanied by reduced reaction rate [332]. When screening an enzyme for resolution of racemic compounds it is essential to ensure that the enzyme preparation selected has no diffusion constraints. Otherwise the real potential of the enzyme might be overlooked [332].

Not only can immobilization change the selectivity (product map or enantioselectivity), the presence of diffusion constraints can also affect the selectivity between two reactions that might occur in parallel in the same reaction system. One example is the kinetically controlled synthesis of peptides or β-lactam antibiotics in which one of the reactants, for example an amino acid ester (or generally called active acyl donor), can be integrated into the desired product (*S*) or hydrolysed into the unwanted amino acid (*H*) [17]. Thus the *S*/*H* ratio was regarded as a criterion of the viability of the corresponding process [255].

As with conformation-controlled selectivity, there are often difficulties distinguishing microenvironment effect from conformation change. For instance, entrapment of RML in cellulose acetate–TiO_2 gel fibre improved selectivity in the hy-

drolysis of 1,2-diacetoxypropane, compared with that of native enzymes [336], and the enantioselectivity of pegylated PCL was increased threefold by entrapment in Ca-alginate gel beads [341]. In such cases the lipases might adopt a conformation different from that in the native enzymes owing to interaction between the carrier and the enzyme (change of the enzyme conformation) or to the micro-environmental effect (pH gradient).

The micro-environmental effect has, however, been clearly demonstrated for 1,2-α-mannosidase, for which a double immobilization technique, adsorption on china clay or cellulose DE-52, followed by entrapment in alginate beads, was used; the product spectrum obtained depended on the carrier used for adsorption before entrapment in sodium alginate [339]. Similarly, the substrate selectivity of dextransucrease adsorbed on DEAE-cellulose was different from that of the native enzyme [329].

Most strikingly, it has recently been found that the enantioselectivity of CRL immobilized on silica activated with 2,4,6-trichloro-1,3,5-triazine was approximately seven times higher than that of the soluble enzyme whereas CLR immobilized on agarose activated with tosylate was only four times more selective than the native enzyme [333], implying that chemical modification of the enzyme by active carriers can also affect enzyme selectivity.

Similarly, it was recently found that enzyme activity and selectivity can be also influenced by the nature of the pendant binding functionality. For example, the enantioselectivity of alkylsulphatase immobilized on anionic exchangers such as DEAE-Sephadex, TEAE-cellulose, and Ecetola-cellulose differed substantially, depending on the pendant ionic groups. Immobilization of alkylsulphatase on Ecetola-cellulose enhanced the selectivity severalfold in the hydrolysis of *sec*-alkyl sulphates. Because TEAE-cellulose and Ecetola cellulose differ mainly in the spacer, the selectivity of the immobilized enzyme is mainly dictated by the side chain and the spacer of the binding functionality. Enhancement of the selectivity might be because the charged groups might be able to approach certain negatively charged domains or sites (e.g. the active sites) [351]

Conformation-controlled selectivity was also recently observed for so-called molecular impinging techniques, which are based on the hypothesis that the conformation induced by a ligand can be frozen by physical or chemical means such as lyophilization or cross-linking or molecular confinement. One possible explanation is that the population of some enzyme conformers is enhanced by the conformer selectors used and, consequently, enzyme selectivity toward some substrates can be improved, as is exemplified by the so-called molecular imprinting techniques (MIT) [251].

When improving the selectivity of enzymes by immobilization it is essential to pay attention to medium engineering, because microenvironment–controlled selectivity is not only related to the carrier selected but also to the medium used. Immobilization of the enzyme often results in a change in the optimum pH or temperature. Thus, enzyme characteristics such as activity and selectivity, which are closely related to the pH and temperature, might be correspondingly changed. The optimum pH for selectivity expression might also be different from that of the native enzyme; this was shown by a recent study of catalysis of the resolution of (*R*,*S*)-

mandelic acid methyl ester by immobilized CRL [320]. In this process the extent of selectivity enhancement was strongly related to the pH of the medium used.

In general, improvement of enzyme enantioselectivity by immobilization might be attractive, because of its simplicity and universal applicability and because it usually obviates the need for detailed structural information.

As discussed above, enzyme immobilization can be regarded as a modification process. It is hardly surprising that the performance of the immobilized enzyme depends on the modification (e.g. the immobilization conditions), the nature of the modifier (i.e. the selected carriers) and the nature of the enzymes (source, purity and strain) to be modified.

With regard to the similarity of enzyme immobilization and chemical modification [321], many methods and principles which are widely used for chemical modification of enzymes to enhance enzyme functionality can also be used to improve the performance of the carrier-bound immobilized enzymes. For instance, the stabilization of enzymes by chemical modification can usually be achieved by two major approaches – rigidification of enzyme scaffold with the use of a bifunctional crosslinker and engineering the microenvironment by introduction of new functional groups which favour of hydrophobic interaction (by hydrophobization of the enzyme surface) or hydrophilization of the enzyme surface (because of mitigation of unfavourable hydrophobic interaction) or formation of new salt bridges or hydrogen bonds (because of the introduction of polar groups) [322]. Similarly, these two principles have been also increasingly applied to improve the enzyme performance for instance the stability, selectivity and activity [323].

1.4 Prospective and Future Development

1.4.1 The Room for Further Development

Although the best method of immobilization might differ from enzyme to enzyme, from application to application and from carrier to carrier, depending on the peculiarities of each specific application, criteria for assessing the robustness of the immobilized enzymes remain the same – industrial immobilized enzymes must be highly active (high activity in a unit of volume, U g^{-1} or mL^{-1}), highly selective (to reduce side reactions), highly stable (to reduce cost by effective reuse), cost-effective (low cost contribution thus economically attractive), safe to use (to meet safety regulations) and innovative (for recognition as intellectual property).

As with the volume activity (U enzyme g^{-1} carrier used), most enzymes bound to carriers with particle sizes above 100 μm (minimum size requirements for a carrier-bound immobilized enzyme [284]) have a loading (or payload) ranging from 0.001 to 0.1. The volume ratio of catalyst to reactor is usually in the range 10–20 %. Thus the productivity of most immobilized enzymes is still much lower than in chemical processes, mainly because of the small number of active sites per kg of

biocatalyst (low volume activity) [342]. For currently available porous carriers, moreover, activity retention at maximum enzyme loading is often below 50 %, because of diffusion constraints [299].

Although development of carrier-free enzymes such as CLEA [16] or CLEC [233] can eliminate the use of the extra non-catalytic mass-carrier, the intrinsic drawbacks associated with the carrier-free immobilized enzymes, for example narrow reactor configuration (because of the small sizes), laborious screening of conditions for aggregation, crystallization and cross-linking, can hardly make them the first choice for the bioprocess engineers.

Because the carrier not only functions as a scaffold for the enzyme molecules but also strongly modifies the enzyme characteristics, it is conceivable that abandoning the carrier might simultaneously reject a powerful means of modulating enzyme properties (both non-catalytic and catalytic function) which would easily be obtained by use of appropriate carriers, binding chemistry and immobilization methods.

As a result, it is to be expected that the focus in bio-immobilization should be the development of a new method of enzyme immobilization that combines the advantages of carrier-free and carrier-bound methods. In other words, the new method of enzyme immobilization should be able to provide high enzyme loading (close to that of carrier-free enzymes), high retention of activity, and broad reactor configurations. No currently available method can meet these criteria. Thus, the development of carriers with a predetermined chemical and physical nature, especially suitable geometric properties and binding chemistry, which can bind (or hold) enzyme directly under mild conditions and thus can be used in different reactor configurations, will continue to be the major focus of future developments.

As regards the stability of the immobilized enzymes, it is known that any type of immobilization method (entrapment, encapsulation or covalent entrapment or adsorption [335]) has the potential to stabilize the enzymes relative to the native enzymes or that an immobilized enzyme can be better-stabilized than others immobilized by different methods. For example, lipase from *Candida rugosa* entrapped in alginate gel was found to be more stable than the covalently bound enzyme on Eupergit C or the enzyme encapsulated in a sol–gel matrix [347], and immobilized glucoamylase entrapped in polyacrylamide gels was more stable than that covalently bound to SP-Sephadex C-50 [346]. Another striking example is that pronase and chymotrypsin covalently attached to PDMS film are less stable than the entrapped enzymes [349].

It is, therefore, appreciated that each enzyme-immobilization technique is unique and thus the possibility of improving enzyme performance such as activity, selectivity and stability, and pH optimum, is limited. For example, although multipoint attachment can improve enzyme stability, the extent of this stabilization might be limited because only a part of the protein surface is rigidified. Often, adsorption of enzyme on carriers cannot be used to improve, significantly, enzyme performance such as stability, compared with covalent enzyme immobilization. For instance, covalently immobilized limonoid glycosyltransferase is much more stable than its non-covalent adsorbed counterpart [350]. On the other hand, entrap-

ment of the enzyme in hard sol–gel matrix can often be used to stabilize the overall molecule in a spatially restricted three-dimensional matrix.

Thus, the matrix-entrapped enzymes are, occasionally, even more stable than the covalently immobilized enzymes [349]. In contrast, encapsulation of enzymes in semi-permeable capsules often has less effect on enzyme stability, because neither the microenvironment of the enzyme nor the structure of the enzyme molecules is significantly modified. Thus, it is not surprising that combination of a variety of immobilization techniques will increasingly be used to solve problems which cannot be solved by any single immobilization technique.

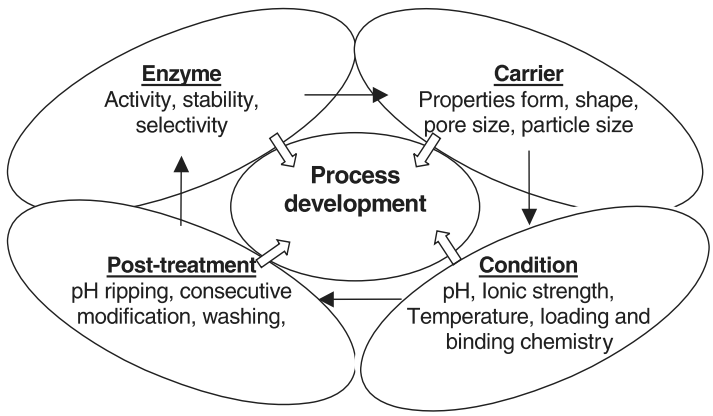
With regard to improvement of enzyme selectivity, although, as noted above, there are many exciting examples of immobilized enzymes for which selectivity, e.g. reaction selectivity, substrate selectivity, stereoselectivity or chemical selectivity, can be affected by the immobilization procedure [339, 343, 344], perhaps combined with reaction medium engineering [341], improvement of enzyme selectivity by immobilization is still, fundamentally, a new endeavour, lacking guidelines that can be used to guide practical experiments. Nevertheless, as with increasing understanding of the relationship between enzyme selectivity and the structural changes resulting from genetic engineering or other chemical modification, increasing interest in improvement of enzyme selectivity by immobilization can be expected in the near future.

1.4.2 An Integration Approach

As noted above, a vast number of methods of immobilization are currently available. Thus, the major problem in enzyme immobilization is not how to immobilize enzymes, but how to design the performance of the immobilized enzyme at will. Unfortunately, the approaches currently used to design robust industrial immobilized enzymes are, without exception, labelled as "irrational", because they often result from screening of several immobilized enzymes and are not designed. As a result, many industrial processes might be operating under suboptimum conditions because of a lack of robust immobilized enzymes.

Another difficulty in rational design is that the comparability of different methods of immobilization is often very poor, mainly owing to inconsistency in the enzymes used (for example source, purity, contamination), the immobilization conditions (time, pH, additives, ionic strength), the assay (substrate, concentration, temperatures), the preconditioning of the carrier and the post-treatment of catalysts. In addition, many data and results reported in the literature are often incomplete, so many conclusions or explanations are not only obscure but also controversial and misleading.

We therefore surmise it might be more realistic to use a Lego approach. In other words, if the enzyme-immobilization method (or approach) can be generally divided into several essential steps (or components), individual optimization of these by use of a rational design might lead to the more rational creation of a robust immobilized enzyme. Analysis of all the methods of immobilization currently avail-



Scheme 1.4 Illustration of general procedures for enzyme immobilization.

able has led to the proposal of a rational general approach to enzyme immobilization based on three stages, selection of enzymes, selection of carriers, and selection of conditions and post-treatments, as shown in Scheme 1.4.

Although many books and reviews dealing with enzyme immobilization appeared in the second half of the last century, the subject still lacks systematic analysis of a general approach to enzyme immobilization, because the books available merely report the feasibility or list the different immobilization techniques [265]. In this context, the author of this book will try to delineate the basic principles governing the individual approaches used to design robust enzymes (Scheme 1.4) and to provide a rational basis for future development of immobilized enzymes.

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