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Principles, Applications and Design
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Foreword

Enzymes are the biocatalysts of the living cell. Their excellent performance in cellular metabolism is due to their intrinsic catalytic properties. In addition, their activity may be further enhanced by functioning in distinct cellular compartments, e.g., within or attached to cellular membranes, or as multifunctional enzyme complexes such as a cellulose-degrading cellulosome.

Enzyme technologists have reinvented such natural forms of “enzyme immobilization”. They have looked for their own bionic solutions to arrive at immobilized biocatalysts which can be used in analytical devices such as a glucose biosensor or in an industrial plant producing, e.g., chiral amines from racemic precursor material. These initial steps towards a benign “green technology” are presently gaining momentum. In fact, the emerging concept of “white biotechnology” (sustainable chemical processes built on renewable resources and biocatalysts, carried out in “biorefineries”) builds not only on fermentation using metabolically engineered microorganisms, but as much on enzymes improved by protein engineering techniques which are used, attached to carrier material, as heterogeneous catalysts in an enzyme reactor. Quite often, the skill to stabilize and re-use an enzyme catalyst through immobilization has proven one of the key steps to render an enzymatic process economically viable.

With this book on “Carrier-bound immobilized enzymes”, Linqiu Cao provides a comprehensive survey of this important field, covering both the history and the present state of immobilization procedures used in enzyme technology. After a short introduction to 100 years of enzyme immobilization, he discusses in great detail not only the methods by which enzymes can be adsorbed, covalently bound or entrapped, but also the laws governing their behaviour in these artificial environments. In the concluding chapter of his book, he also adds an authoritative survey of most recent developments such as enzyme immobilization using genetically engineered attachment points, artificial tags or enzymes whose properties have been changed through reversible binding to synthetic polymers. He thus provides both the industrial enzymologist and the researcher in an academic environment with a well-structured, easily accessible choice of options and protocols to solve their individual needs.
Foreword

My compliments go to the author for the thorough collection and structuring of a plethora of data, and my wishes to the readers for continuing success in their research on and application of immobilized biocatalysts.

Stuttgart, May 2005

Rolf D. Schmid
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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).

![Applications of Immobilized Enzymes](image)

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
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-
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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Requirement</th>
<th>Benefits</th>
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<tbody>
<tr>
<td>Non-catalytic function</td>
<td>Suitable particle size and shape</td>
<td>Aid separation, easy control of the reaction</td>
</tr>
<tr>
<td></td>
<td>Suitable mechanical properties</td>
<td>Flexibility of reactor design</td>
</tr>
<tr>
<td></td>
<td>Low water regain capability</td>
<td>Easy removal of water</td>
</tr>
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<td></td>
<td>High stability in a variety of organic solvents</td>
<td>No change of pore radius and thus fewer diffusion constraints</td>
</tr>
<tr>
<td>Catalytic function</td>
<td>High volume activity (U g⁻¹)</td>
<td>High productivity and space–time yield</td>
</tr>
<tr>
<td></td>
<td>High selectivity</td>
<td>Fewer side reactions, easier downstream processing and separation of products, and less pollution</td>
</tr>
<tr>
<td></td>
<td>Broad substrate specificity</td>
<td>Tolerance of structural variation of the substrates</td>
</tr>
<tr>
<td></td>
<td>Stability in organic solvents</td>
<td>Shift of reaction equilibrium with the use of organic solvents</td>
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<td>Thermostability</td>
<td>Short reaction time by increasing temperature</td>
</tr>
<tr>
<td></td>
<td>Operational stability</td>
<td>Cost-effective and lower cost-contribution for the product</td>
</tr>
<tr>
<td></td>
<td>Conformational stability</td>
<td>Modulation of enzyme properties</td>
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<tr>
<td>Immobilized enzyme</td>
<td>Recyclability</td>
<td>Low cost-contribution of catalyst</td>
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<td></td>
<td>Broad applicability</td>
<td>Tolerance of process variation</td>
</tr>
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<td></td>
<td>Reproducibility</td>
<td>Guarantee product quality</td>
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<tr>
<td></td>
<td>Easy and quick design</td>
<td>Early insight into process development and avoidance of learning process</td>
</tr>
<tr>
<td>E and E consideration</td>
<td>Lower volume</td>
<td>Lower cost for the solid handling</td>
</tr>
<tr>
<td></td>
<td>Easy disposal</td>
<td>Less environmental concern? Easy biodegradability?</td>
</tr>
<tr>
<td></td>
<td>Rational design</td>
<td>Avoidance of laborious screening</td>
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<td>Safety for use</td>
<td>Meeting safety regulations</td>
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<tr>
<td>IPR</td>
<td>Innovative</td>
<td>Protection of IPR</td>
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<td>Attractive</td>
<td>Licensing</td>
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<tr>
<td></td>
<td>Competitive</td>
<td>Strengthening marketing position</td>
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E and E: Economical and Ecological; IPR: Intellectual Property
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>
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<th>Table 1.2 Survey of enzyme-immobilization techniques in the 1950s</th>
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<td><strong>Carriers</strong></td>
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<tr>
<td>Natural polymers and derivatives</td>
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<tr>
<td>Cellulose</td>
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<tr>
<td>DEAE-cellulose</td>
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<td>Synthetic polymers</td>
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<td>Amberlite</td>
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<td>Diaion</td>
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<tr>
<td>Dowex</td>
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<tr>
<td>Polystyrene</td>
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<tr>
<td>Other polyacrylic polymers and derivatives</td>
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<tr>
<td>Inorganic carriers</td>
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<tr>
<td>Carbon</td>
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<td>Silica</td>
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<tr>
<td>Kaolinite</td>
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<td>Clay</td>
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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.
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-amyrase [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 methacrylic 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 $\alpha$-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>
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<th>Carriers</th>
<th>Activation or coupling methods</th>
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<tr>
<td><strong>Active synthetic carriers</strong></td>
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<tr>
<td>Halogen</td>
<td>Ugi reaction [146, 147]</td>
<td>Reversibly covalent coupling and intra-molecular cross-linking [112]</td>
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<tr>
<td>Epoxy ring</td>
<td>Alkylation with epoxide [107]</td>
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<td>Carbonate</td>
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<td><strong>Functionalized prepolymers (for entrapment)</strong></td>
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<tr>
<td>Carbonate</td>
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<td>Imidoester</td>
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<td>Glutaraldehyde for polyacrylamide [124]</td>
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<td><strong>Inorganic carriers for covalent coupling</strong></td>
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<td>Silica [118]</td>
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<td><strong>Natural polymers and derivatives</strong></td>
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<td>Gelatin</td>
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<td>Collagen</td>
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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

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<tr>
<th>Method</th>
<th>Remarks</th>
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<tr>
<td>Affinity immobilization</td>
<td>Combines mild immobilization conditions and reversibility of binding</td>
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<tr>
<td>Complimentary multi-point attachment</td>
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<tr>
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<tr>
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<td>135</td>
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<tr>
<td>Covalent entrapment</td>
<td>Enzyme entrapment and covalent binding of the enzyme molecules to the matrix occurred concomitantly</td>
<td>139</td>
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</table>
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 Mandl 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].