ANALYTICAL APPLICATIONS OF IMMOBILIZED ENZYME REACTORS

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Analytical Applications of Immobilized Enzyme Reactors

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Preface

Enzymes are bio-catalysts which effect transformation of substrates to products with high specificity. The usage of enzymes in domestic and industrial applications is well known and has been well documented since the early history of civilization. With the advances in understanding of enzymology, usage of enzymes in industrial and biotechnological processes and molecular medicine has proliferated.

One of the key factors in the widespread application of enzymes in modern technologies is the development of enzyme immobilization techniques, which overcome certain practical, functional and economic constraints. Many natural enzymes can be stabilized by immobilization on solid matrices, with most of the activity retained, for a variety of applications. An important application of immobilized enzymes is in liquid chromatography. In the last decade, post-column enzyme detection has become established as an important discipline in liquid chromatography. The new detection approach offers more sensitive and specific ways for measuring major classes of biomolecules.

Reactors are fabricated by packing the immobilized enzymes into small columns, which can be placed immediately after an HPLC column. When an analyte, which is a natural substrate of the enzyme, passes through the reactor, it will be transformed into a product in the presence of a coenzyme. The enzymatic reaction can be designed in such a way that one of the reaction products will be amenable to optical or electrochemical detection. Hence, compounds that are otherwise difficult to detect by conventional approaches are rendered readily detectable with improved sensitivity. Alternatively, certain reactors can be placed immediately before the injector to transform an analyte into a product that can be chromatographed for subsequent detection. The specificity of the reactor, in general, is dependent on the nature of the immobilized enzymes. Some enzymes have broad specificity, catalysing the transformation of a specific functionality of an entire family of compounds, while other enzymes have absolute specificity, reacting with a single substrate.

This book, the first one on the subject, will describe the theoretical and practical considerations of the design of immobilized enzyme reactors. The integration of the reactors into detection systems in liquid chromatography provides new ways of measuring biomolecules with very high sensitivities. An international team of scientists has drawn on extensive

experience to review the development of a variety of reactors, with emphasis on a wide range of applications, including the analysis of sugars, saccharides, carbohydrates, starch, amino acids, steroids and hormones, bile acids, uremic toxins, glucuronide and sulfate conjugates, and other biomolecules.

The enzyme reaction detection liquid chromatography systems offer new bio-analytical techniques to pharmaceutical scientists and biochemists for effective and sensitive measurement of drugs and conjugated metabolites; starch and carbohydrate ingredients of pharmaceutical formulations; and physiological metabolites. They also offer the broader analytical community a new technique with great potential.

The dedication and commitment of an international team of enthusiastic contributors, and the assistance and patience of the editorial staff of Blackie A & P are gratefully acknowledged.

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Contents

1 Immobilized enzyme reactors: developmental, practical and theoretical considerations

L. GORTON, G. MARKO-VARGA,
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1.1 Introduction

The development and widespread use of immobilized enzymes has been motivated by the need to overcome operative and cost constraints. Nowadays, immobilized enzymes for detection purposes are under intensive research. One of the early preparations and analytical applications of immobilized enzymes, as a reactor (IMER) containing urease in a flow stream for the analysis of urea, has been documented [1]. As early as 1962, Clark and Lyons foresaw the construction of an enzyme electrode [2]; Updike and Hicks [3] in 1967 reported the first such electrode by physically entrapped glucose oxidase in a dialysis membrane close to a Clark type oxygen electrode. They were thus able to follow the oxidation of glucose by the decrease in the tension of molecular oxygen in the solution amperometrically and could correlate the response to the concentration of glucose in the sample. Since then, immobilized enzymes have become an increasingly popular technique for sensitive and selective detection of trace compounds in complex samples in bioanalysis, biotechnology, environmental care, clinical diagnosis and process control.

As a general rule, immobilization of an enzyme is considered a successful procedure to increase its stability, implying a decrease in rate constant of inactivation or an increase in half-time of inactivation under denaturating conditions [4]. However, no serious reasons can be taken from the data in the analytical literature to justify increased stability of enzymes due to immobilization. In this context, the fact that immobilized enzymes are in a state that permits their reuse should not be interpreted as an enhancement of their inherent stability. Moreover, the operational stability of the enzyme-based system, defined as the residual conversion efficiency after operation for different periods [5], depends not only on the enzyme itself but also on the stability of the support, the immobilization procedure and the kind of samples, which may contain different activators and/or inhibitors. However, some considerations can be formulated to predict the operational stability of an immobilized enzyme

[6]. An increased rigidity of the structure of the enzyme may lead to an increased stability preserving the native conformation of the protein and making unfolding less probable. In general, direct adsorption results in the lack of the desirable long-term operational stability. Cross-linking of the enzyme to an inert, generally proteinaceous, material by means of a bifunctional reagent to form intermolecular bonds between the catalyst and the inert protein may result in an extended operativity.

Immobilized enzymes have been used for a long time in industry for biotechnological processes, e.g. in the production of 6-aminopenicillic acid. In the production of penicillin G (phenoxy penicillin) and penicillin V (phenyl penicillin), for example, penicillin G acylase and penicillin V acylase are immobilized on polyacrylamide beads as the support in batch reactors with about 100 kg of enzyme in a 5 m^3 reactor [7]. Other examples include immobilized glucoamylase for finishing in dextrose crystallization and for fructose corn syrup processing; glucose isomerase for isomerization of glucose for fructose production, the largest production process using an immobilized enzyme in existence today.

Analytical development of immobilized enzymes with electrodes, as the physical transducer, have followed two directions: the most common version of the biosensor [8–19] involves the integration of the immobilized enzyme in close proximity to the electrode, and the enzyme reactor electrode version [8] involves the placement of the IMER preceding an electrode in a flow system (see also chapter 3). Each approach has obvious advantages depending on the actual analytical problem; each will find niche applications. Comparatively, enzymes immobilized in reactors prove to be more stable with more activity than enzymes immobilized in close proximity to the electrode surface. This was concluded after evaluation of the operational and/or storage performances of enzyme-based systems. A strict comparison in relation to the different environments and conditions of the enzyme on the electrode and in the reactor cannot be made due to the fact that reactors are generally designed with an excess of reagent (large reactor volume) whereas electrodes provided only limited surface for enzyme immobilization. An empirical comparison between enzyme reactors and enzyme electrodes has been made for L-glutamate oxidase [20]. Experimental conditions were fixed in such a way that the enzyme loading was approximately the same on the enzyme electrode and in the enzyme reactor with identical immobilization procedures (covalent coupling). The stability for both was reported to be the same after repetitive use for 6 months. Further generalization to any other enzyme could be fortuitous.

In a flow system, the enzyme reactor does not have to be in the immediate proximity of the detector as long as the production of the transduction signal is based on either a soluble enzymatic product or co-substrate [8,9,11,12,17,19,21]. The enzymatic reaction occurring in a

reactor can be followed downstream by any appropriate flow-through detector. Some obvious advantages can be obtained by having the enzyme immobilized in a reactor. The amount of enzyme that can be immobilized in a reactor of moderate size (5–500 μl) is very large (up to 1000 units). When a solution containing the substrate passes through the reactor, equilibrium of the enzymatic process can be reached. In many cases, the equilibrium strongly favours product formation (chemically irreversible reaction). Virtually 100% of the substrate can be converted into a detectable product. This is not the case with, e.g. an enzyme electrode, where the amount of immobilized enzyme on the membrane or on the electrode surface is more restricted and will almost always be much too low (1–3 units cm^{-2}) to allow equilibrium of the catalysed reaction to be reached within a reasonable time, particularly when used in a flowing stream. With a reactor containing excess enzyme, a slight variation in the flow rate, pH, ionic strength, temperature and the presence of small concentrations of inhibitors and activators will thus not affect the conversion efficiency of the enzyme.

Immobilization of enzymes by means of covalent coupling to a reactive insoluble support or to the surface of an electrode is the method of choice if a long operational performance is required. The covalent binding should be accomplished through functional groups in the enzyme that are not involved in the catalytic activity. In general, the binding is achieved by a nucleophilic group of the enzyme attacking the activated functional group of the support material. Amino, hydroxyl and thiol groups on the enzyme may participate in the linkage. Cysteine, lysine, tyrosine and histidine residues may be considered the most reactive [22]. This implies a considerable risk for enzyme inactivation during immobilization. A partial loss of enzyme activity is the trade-off for a long operational performance.

1.2 Immobilized enzyme reactors (IMERs)

IMERs are preferably used to derivatize solutes that are inherently difficult to detect or when selective detection is required due to the high complexity of the sample. The enzymatic reaction converts the substrate into a product that is much easier to detect. In some cases, the use of coupled enzyme reactions is necessary to push the unfavourable equilibrium of the detection reaction to the product side or to transfer the analyte into a form active with the actual detection reaction. This can be achieved by the use of several IMERs incorporated into the flow system sequentially or by co-immobilizing multiple enzymes on the same support in the same reactor, a co-immobilized enzyme reactor (CIMER). A special case is where the substrate is a polymer and the actual analysis is

based on detecting the monomeric species. In such cases, an IMER can be used containing a polymer degrading enzyme. The high selectivity that can be obtained by using IMER systems is due to the substrate specificity of the enzyme and the high amounts of active enzyme that can be contained in a reactor of moderate size (10–500 μl). There are several reasons why IMERs in combination with electrochemical detection as a detection unit have gained a lot of attention. The electrochemical step provides both high sensitivity and selectivity by the choice of applied potential.

The growing interest in the analytical use of IMERs is reflected by the number of papers appearing in the literature with IMER-based detection systems used in LC. The field of analytical applications where IMERs have been coupled to LC separations has recently been reviewed [21,23–31]. The first international enzyme meeting, held recently, discussed the use of immobilized enzymes and the latest developments in liquid chromatography (LC) and enzyme chemistry [32]. In most cases, polar compounds are analysed using either pure aqueous, or aqueous phases with the addition of some percentage of organic modifiers, as the mobile phase.

Enzyme catalysed reactions are used for the determination of either enzyme activity, as reported by Schlabach and Regnier in 1978 [33], or substrate concentrations as reported by two independently pioneering research groups, Okuyama *et al.* on the analysis of bile acids [34] and Ögren *et al.* on cholesterol and its auto-oxidation products [35].

The use of IMER(s) has also gained popularity in many other fields of analysis, e.g. the combination of LC with mass spectrometry (MS) was shown to be very powerful for qualitative peptide analysis [36,37]. By utilizing different peptidase IMERs separately or in combination, structural information about the amino acid sequencing of peptides can be obtained using thermospray LC/MS. In one case, the enzymatic cleavage was made directly on the inlet probe tip resulting in enzymatic cleavage by peptidases for the identification of peptides from recombinant proteins [38].

Pioneering work has been carried out by Rassi and Horváth [39] inserting an IMER in a complicated chromatographic system for oligonucleotide synthesis. The oligonucleotides were synthesized by immobilized ribonuclease, fully compatible with preparative scale displacement chromatographic separation by which the product was separated from unreacted reactants. Nearly 100 mg of nucleotides containing the guanosine-3′-phosphate moiety with a purity of 95.7% could be achieved in a 2.4 h run.

Reversed phase chromatography is the most frequently used mechanism for separation in LC using an organic/aqueous mobile phase. When IMERs are to be used in these separations, the stability of the enzymes towards organic modifiers has to be investigated. The separation is generally optimized towards the use of as low an organic additive in the mobile

phase as possible. Bowers and Johnson [40] studied the behaviour of a number of immobilized enzymes in organic solvents, e.g. acetonitrile, ethyl glycol, ethanol and methanol. A buffer with 25% of organic solvent was found to be the upper limit that can support enzymatic reactions. Enzyme activities were found to decrease with increasing hydrophobicity of the solvent.

When using enzymes in organic solvents, an interesting characteristic called 'superactivity' occurs in reversed phase micellar systems [41,42]. In these systems, the enzyme is entrapped in a water cavity, which, surrounded by a shell of surfactant molecules, separates the enzyme from the surrounding organic solvent. The size of the inner cavity becomes larger as the amount of water in the system increases. For a variety of enzymes, an exceptionally high enzyme activity, compared with an aqueous solution, has been observed when the inner cavity of the reversed miscelles almost exactly corresponds to the size of the enzyme. This superactivity was explained by a hypothesis that the relative high rigidity of the surfactant shell may function as a barrier to excessive influx of water that could disturb the catalytic conformation of the enzyme.

Nicotinamide cofactor dependent dehydrogenases and oxidases, the two largest groups of oxidoreductases, are the most frequently used enzymes in LC/IMER systems [21,23,25–31]. Examples where such enzymes have been most frequently reported are for the analysis of acetylcholine and choline using the combination of acetylcholine esterase with choline oxidase, and for bile acids using 3-hydroxysteroid dehydrogenase. The result of the enzymatic reaction is the production of either H_2O_2 or NADH. NADH can be detected by fluorescence, UV, or electrochemistry, and H_2O_2 by chemiluminiscence or electrochemistry respectively (see chapter 2).

1.3 IMER configurations

The conversion efficiency which dictates the sensitivity of the analysis may differ widely depending on the reactor configuration used. The most common are: packed-bed reactors, open-tube wall reactors and single-bead string reactors. In open tubular reactors, the enzyme is bound on the inner wall of the tube. Due to the low surface available for immobilization, these IMERs are necessarily very long to achieve a required surface area [43]. However, these types of reactors were found to operate by flow segmentation, whereby the length of the reactor could be minimized due to increased mass transfer effects. The open tubular heterogeneous enzyme reactors (OTHERs) are not well suited to FI or LC applications. Due to limited enzyme loading, the resulting sensitivity is low while the dispersion is high (large inner diameters and long tubes

are required to achieve a reasonable conversion) which is the reason for the limited applications in analytical work [44]. A compromise between packed-bed and open tubular reactors in terms of dispersion, conversion efficiency and back pressure is offered by single bead string reactors (SBSR) in which a single string of glass beads with the immobilized enzyme are packed in coiled glass or nylon tubing. SBSRs were first reported by Reijn *et al.* [45], who also made kinetic studies and detailed mathematical analysis of dispersion and chemical reaction with these reactors. However, this reactor type also offers a restricted amount of immobilized enzyme per reactor volume.

The packed bed reactor is the most popular amongst those mentioned. This reactor is packed with a solid porous support comprising the immobilized enzyme(s). The efficiency of the packed bed reactor will be dependent on several factors. A higher enzyme activity is attainable by loading on the support with more enzymes. The efficiency of the IMER can be raised by chromatographic purification of crude enzymes for immobilization. The amount (mg)/activity (IU) of enzyme can easily be increased even with the rather purified preparations commercially available [46,47]. This will result in a more efficient reactor loading, thus decreasing the size of the IMER and band-broadening.

To meet the demands of small, highly efficient, and well packed reactors, optimization should be made by considering the choice of solid supports and the binding chemistry. Small particle diameter and small pore sizes will result in high specific surface areas, which are important for high enzyme loading and low additional band broadening [48]. These factors have to be optimized exerimentally since they are difficult to foresee and to determine theoretically. The importance of proper optimization was demonstrated by Gübitz *et al.* [49], who made a comparative study using controlled pore glass (CPG), silica gel and an anion exchange polymer. Variation of the pore sizes and the different immobilization procedures on these supports were made with the aim of developing an optimum activity for a GOD IMER.

1.4 Enzyme kinetics theory

The simplest enzyme catalysed reaction to study in a reactor is one where only one substrate participates in the reaction, obeying the Michaelis–Menten model

$$S + E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P \tag{1.1}$$