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Review

Application of immobilized enzyme reactor in on-line high performance liquid chromatography: A review

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Abstract

This review summarizes all the research efforts in the last decade (1994–2003) that have been spent to the various application of immobilized enzyme reactor (IMER) in on-line high performance liquid chromatography (HPLC). All immobilization procedures including supports, kind of assembly into chromatographic system and methods are described. The effect of immobilization on enzymatic properties and stability of biocatalysts is considered. A brief survey of the main applications of IMER both as pre-column, post-column or column in the chemical, pharmaceutical, clinical and commodities fields is also reported.

Keywords: Immobilized enzyme reactor; HPLC; Chromatographic supports

Contents

1. Introduction

Immobilized enzymes are widely used in biocatalysis, bioprocessing and biospecific detection. They are used in batch-wise experiments or packed into columns and used in a flow system as immobilized enzyme reactors (IMERs). This approach does not require highly purified enzymes or a great amount of protein but principally can increase the enzymatic stabilization against heat, organic solvents and pH without too much loss of catalytic activity. In this way a immobilized enzyme becomes a catalyst confined or localized on a suitable support so that it can be used repeatedly for as long as it remains active, thereby minimizing costs, time of analysis and making it economically feasible to operate in a continuous mode.

All these factors improve the accuracy and reproducibility of the analytical method [1–4]. The coimmobilization of

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enzymes participating in the same catalytic sequence allows both the performance of the catalyst in the multisteps reactive processes [5] and the sensitivity of the analytical method [6] to be increased.

In addition ease of automation and control of a continuous process with respect to a batch process has led to the incorporation of the immobilized enzyme column in on-line analytical systems.

The coupling of an IMER with the high performance liquid chromatography (HPLC) method allows the selectivity, rapidity, non-destructivity, reproducibility of this chromatographic system to be combined with the specificity and sensitivity of an enzymatic reaction. In this way sensitivity of the detection system is considerably enhanced and determination of trace components in complex matrices therefore become possible. However, some drawbacks such as band broadening due to non-specific binding to the enzyme [7] and the influence of the flow rate on the substrate conversion efficiency in the case of specific mass transfer regimen can limit the application of IMERs in HPLC. Infact when microparticulate supports are employed, the system operates under diffusion-limiting conditions because the substrate reaches the catalytic sites more slowly than the rate of enzymatic product formation [8]. Now with the introduction of monolithic supports it is possible to achieve a total absence of diffusion limitations so that the enzymatic kinetic parameters were flow-unaffected [9,10].

Several review papers deal with immobilized enzyme reactors in analytical chemistry but they are mainly concerned with practical applications [11–17].

The main purpose of this review is to present the results achieved from 1994 to 2003 in HPLC using on-line immobilized enzymes reactors not only as pre- or post-column but also as chromatographic columns. Some basic paper ante 1994 and some works published in 2004 are also mentioned. A brief overview will be presented on the methods of immobilization which greatly influence the properties of enzymes (stability, lifetime, catalytic activity, deactivation and regeneration) and on analytical applications. Actually choosing the right supports gets more and more complicated because of the increase in the number of natural and synthetic supports available which greatly differ in mechanical and physical properties. Furthermore, it is also necessary, at the same time, to meet many requirements such as low cost, non toxicity, maximum activity, high retention of catalytic activity over a long period, enzymatic stability, ease of protein availability and immobilization. This is why, in this review, particular attention has been focused on supports since for optimising biocatalyzed process it is very important to have a clear state of art of their current development, even if it is impossible to recommend a universal support for all the enzymes taken into consideration. To help the readers to choose the right support we have organized a table taking into account immobilized enzymes, immobilization techniques, activating agents and types of supports.

2. Methods of immobilization

A large number of techniques are now available for the immobilization of enzymes on a variety of natural and synthetic supports. The immobilization of enzymes can be obtained namely "in situ" or "in batch". When employing the "in batch" process the enzyme is firstly immobilized on the support and then is packed into the column using a slurry packing technique, whereas in the "in situ" approach the enzyme is directly immobilized on the pre-packed column. Massolini et al. [18] have compared "in batch" and "in situ" techniques of Penicillin G acylase (PGA) immobilization on various derivatized silica supports concluding that the "in situ" technique is the best way to obtain satisfactory results in terms of bound amount of PGA and enzymatic activity retention since in the "in batch" packing process a loss of catalytic activity can result.

This choice is further confirmed by the greater frequency of "in situ" immobilization revised in this review (Fig. 1) in the decade in question.

The surface of the supports, on which the enzyme is immobilized, has an important role to play in retaining the tertiary structure of the enzyme which highly influences the thermal stability and catalytic activity of the immobilized enzyme. Indeed an immobilized enzyme is known to acquire novel kinetic properties which can modify the Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) and cause a shift of the pH and temperature-activity profile. Likewise the groups involved in the attachment of proteins to the support must be different from the active sites of enzymes. Therefore, the choice of the support as well as the technique depends on the nature of the enzyme, on the nature of the support and on its ultimate application. For this reason it is not possible to recommend any universal immobilization methods. It can

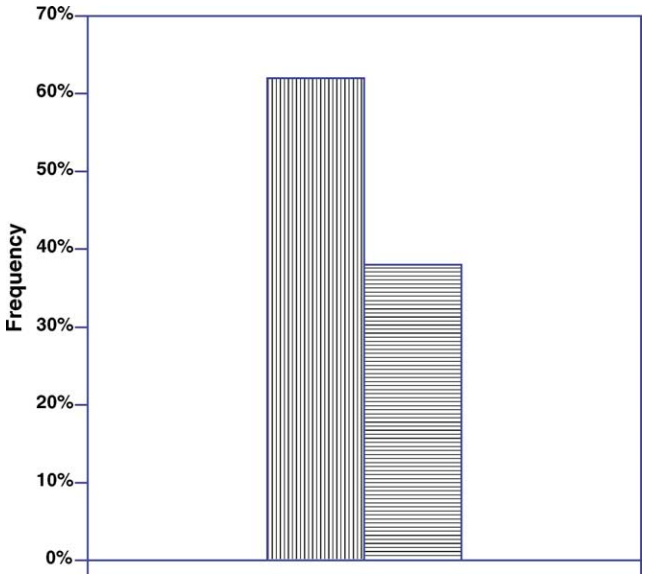

Fig. 1. Frequency in revised literature (1994–2004) of "in situ" (▥) and "in batch" (▤) immobilization method.

only be hoped that this research continues and that attention will be focused on support material selection as well as on the reagents used in the immobilization process. In fact, the toxicity of immobilization reagents should be considered when final applications concern the food processing and pharmaceutical industries. The objective of this part is to review, in the light of current developments, the approaches used for on-line IMERs from the point of view of the above mentioned aspects which are also summarized in Table 1.

2.1. Techniques

The techniques usually employed to immobilize enzymes on solid supports are mainly based on chemical and physical mechanisms. Chemical immobilization methods mainly include enzyme attachment to the matrix by covalent bonds and cross-linking between enzyme and matrix. Physical methods involve: (i) the adsorption of enzyme molecules on a porous support or on a matrix containing ion exchange residues or (ii) the entrapment within an insoluble gel matrix. The simpler method of immobilization, based on a weak binding force, such as the adsorption of protein on the surface of insoluble supports, provides a small perturbation of the native structure of the enzyme but promotes the leak of the adsorbed protein from the support during use. This can occur especially when there is a mild change in temperature, pH, ionic strength or simply because a substrate is present. This situation can be partially overcome by a simple regeneration achieved by the removal of the deactivated enzyme and by reloading with a fresh active catalyst [18,20,70]. The protein leakage is also present when ionic binding mode is implicated [20] even if the binding is stronger than that of physical adsorption.

In the entrapment of enzymes in a gel matrix, the catalyst is mixed with gel formation ingredients and when the gel is formed the enzyme remains “trapped” in the matrix. This mode modifies minimally the properties of the enzyme but requires a skilful operator. To our knowledge, no studies on natural polymers as supports for on-line IMERs in HPLC application, except for only one on a synthetic polymer, polyacrylamide gel [48] have been reported in the last decade. In this case, a toxic gel inducing chemical, is employed which prevents this technique from being used in food and pharmaceutical applications.

A novel kind of embedding is obtained by immobilized artificial membrane (IAM) stationary phases based on the covalent binding of phosphatidylcholine to aminopropyl silica using a terminal amide linkage (Fig. 2). As a result the phospholipid head groups form the surface of the support and the hydrocarbon side chains produce hydrophobic cavities which extend from the charged head groups to the surface of the aminopropyl silica. Such a structure limits the access to the unbounded amine groups and allows the protein to interact with any combinations of polar head groups and hydrophobic chains. This kind of technique has recently gained acceptance among the chiral analysis [43,44] and drug discovery chemists [41,42,45].

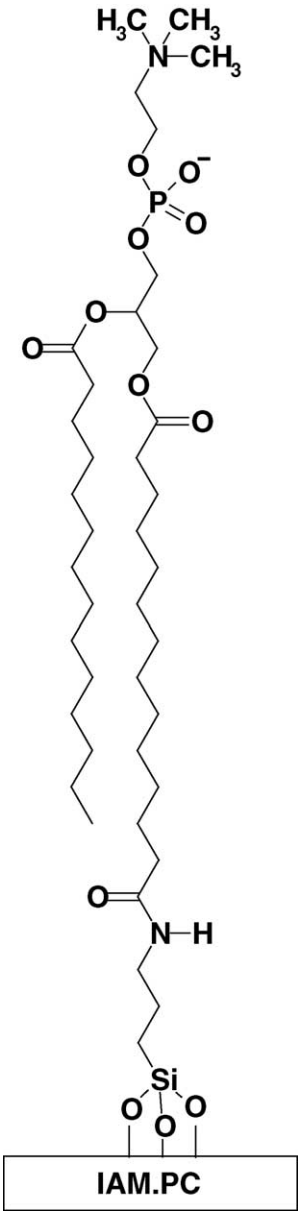

Fig. 2. Schematic IAM structure: the phosphatidylcholine is covalently bound to propylamine groups, which are in turn bound to silica. Taken from http://www.registech.com/iam.

As is evident from Table 1 the most intensely used immobilization technique implicates the formation of a strong binding mode, like the covalent, since it offers the great advantage of preventing the desorption phenomenon also in the presence of substrates and solutions of high ionic strength and of reducing spontaneous enzyme deactivation rates, as in proteases autodigestion. These benefits, involving a longer IMERs lifetime, are counterbalanced by the more easily altered native tertiary structure of the enzyme with the subsequent decrease in catalytic activity.

In addition the use of a covalent binding mode involves a higher enzymatic thermal stability since the strong interaction of enzyme to support causes rigidity of the protein structure

Table 1
List of supports, activating agents, immobilization technique and enzymes used in IMER

Support	Activating agents	Immobilization technique	Enzyme	Comments	Ref.
Agarose	*N*-hydroxy succinimide	Covalent	Allinase	Post-column: detection of L-cysteine sulfoxides in extracts from different allium species	[19]
Aquapore® Ax-300 anion-exchanger		Adsorbtion	Choline oxidase	Post column: detection of choline in plasma; enzyme "in situ" immobilized	[20]
BAS® IMER cartridge		Covalent	Acetylcholinesterase /choline oxidase	Pre- or Post-column	[6–27]
			Lactate oxidase	Post-column: detection of lactate	[28]
Cellulose			Recombinant fused protein cellulose-binding domain-Horseradish peroxidase	Determination of 4-Br-phenol in wastewater	[29]
Cellulose amino derivatized		Covalent	Inulinase	Production of inulo-oligosaccharides	[30]
CPG + γ-aminopropyl-triethoxysilane to form CPG-aminopropyl	Glutaraldehyde	Covalent: Shiff's base formation (Fig. 3a)	Amino acid oxidase type III and horseradish peroxidase	Coupled IMER as post-column: detection of all aminoacids; enzymes "in batch" immobilized	[31]
			Cholesterol oxidase	Post-column; enzyme "in batch" immobilized	[31]
			Glucose dehydrogenase/xylose isomerase/mutarotase	Post-column: pentoses detection; multienzyme "in batch" coimmobilized	[32]
		Covalent: Shiff's base formation (Fig. 3a) followed by reduction to amine bond with cyanoborohydride	Alanine aminotransferase	Column enzyme "in batch" immobilized	[33]
CPG-amino (Trisoperl®)	Glutaraldehyde	Covalent: Shiff's base formation (Fig. 3a)	L-amino acid oxidase or D-amino acid oxidase	Post-column: detection of alanine in beverages; enzyme "in batch" immobilized	[34]
CPG-aminopropyl	Glutaraldehyde	Covalent: Shiff's base formation (Fig. 3a)	Acetylcholine esterase/choline oxidase	Post-column; enzymes "in batch" coimmobilized	[35]
		Covalent: Shiff's base formation (Fig. 3a) followed by reduction to amine bond (sodium borohydride)	Acid phosphatase	Pre-column; enzyme "in batch" immobilized	[36–38]
		Covalent: Shiff's base formation (Fig. 3a) followed by reduction to amine bond (sodium tetrahydroborate)	Acid phosphatase	Pre-column; enzyme "in batch" immobilized	[39]
CPG-aminopropyl + 4-nitrobenzoyl chloride to form nitroaryl-CPG and sodium dithionite to form CPG-aminoaryl	Sodium nitrite	Covalent: diazocopulation (Fig. 4b)	Glucose oxidase	Post-column: detection of glucose and its metabolites in urine samples; enzyme "in batch" immobilized	[35]
Glutaraldehyde-P 40		Covalent: Shiff's base formation (Fig. 3a) followed by reduction to amine bond (sodium cyanoborohydride)	Glyceraldehyde-3-phosphate dehydrogenase	Pre-column; enzyme "in batch" immobilized	[40]
Immobilized artificial membrane (IAM®)		Matrix embedding	Glutamine synthetase	Column; determination of K_m, V_{max}; enzyme "in batch" immobilized; drug discovery	[41]
			Dopamine-beta-hydroxylase	Pre-column; determination of K_m, V_{max}; enzyme "in batch" immobilized; drug discovery	[42]
			Alcohol dehydrogenase	Pre-column; enzyme "in situ" immobilized; chiral separations	[43]
			Lipase	Column; enzyme "in situ" immobilized; chiral separations	[44]
			Monoamine oxidase A and B	Pre-column; determination of K_m V_{max}, efficiency, stability, activity; enzyme "in situ" immobilized; drug discovery	[45]
Metacrilate resin	Avidin	Covalent	Human protein-tyrosine phosphatase	Capillary column	[46]
Microporous glass spheres		Adsorption	α-Amylase	Column; tubular reactor, partial immobilization	[47]
Polyacrylamide gel		–	Horseradish peroxidase	Column, pH influence study	[48]
Poly(glycidyl methacrilate-co-ethylene dimethacrylate) + ethylenediamine to form amine group	Glutaraldehyde	Covalent. Shiff's base formation (Fig. 3a) followed by reduction with sodium borohydride	Glucose oxidase, invertase, trypsin	Column, monolithic disk; enzyme "in situ" immobilized, affinity chromatography	[49]
			Trypsin	Pre-column. monolithic rod; enzyme "in situ" immobilized; affinity chromatography	[50]
Poly (glycidyl methacri late-co-ethylenedimetha crylate)-epoxy (Convective Interaction Media, CIM®) disk		Covalent: β-hydroxylamine formation (Fig. 4a)	Glucose oxidase	Monolithic disk as column	[51,52]
Poly(glycidyl methacri late-co-ethylenedimetha crylate)-ethylenediamine (Convective Interaction Media, CIM®) disk	Glutaraldehyde	Covalent: Shiff's base formation (Fig. 3a) followed by reduction to amine bond (sodium cyanoborohydride)	Human recombinant acetylcholinesterase	Monolithic disk as column for inhibition studies	[53]
Polystyrene	Streptavidin	Covalent	Dopamine-β-hydroxylase and phenylethanolamine *N*-methyltransferase	Two IMER systems coupled as pre-columns; enzymes "in batch" immobilized	[54]
			N-acetylglucosaminyltransferase V	Column; enzyme "in batch" immobilized; affinity chromatography	[55]

Polystyrene-aromatic amino beads	Glutaraldehyde or sodium nitrite/HCl or hexamethylene di-isotio-cyanate	Covalent: Shiff's base formation (Fig. 3a) or diazotization (Fig. 4b) or amide bond formation	β-Lactamase	Column; enzyme "in batch" immobilized; covalent bound studying	[56]
Poly(vinyl alcohol)	Tresyl chloride	Covalent: amine formation (Fig. 4d)	Leucine dehydrogenase	Post-column; enzyme "in situ" immobilized	[57]
			Leucine dehydrogenase/NADH oxidase coimmobilized	Post-column; enzymes "in situ" coimmobilized	[58]
			3-Hydroxybutyrate dehydrogenase/NADH oxidase coimmobilized	Post-column; enzymes "in situ" coimmobilized	[59]
			Phenylalanine dehydrogenase/leucine dehydrogenase	Post-column; enzymes "in situ" coimmobilized	[60]
			3-Hydroxybutyrate dehydrogenase/pyranose oxidase	Post-column; enzymes "in situ" coimmobilized	[59]
Poly(vinyl alcohol) + chloromethyloxirane + aqueous ammonia to form amino groups	Glutaraldehyde	Covalent: Shiff's base formation (Fig. 3a)	Aminoacylase, leucine dehydrogenase	Two IMER systems coupled as post-column; enzyme "in situ" immobilized	[57]
Poly(vinylidene fluoride)		Covalent	Trypsin	μ-Enzymatic reactor coupled with membrane chromatography or with μ-LC	[61,62]
Poroszyme®		Covalent	Trypsin	Column; rapid proteins recognition	[63,64]
			Glutamyl endopeptidase	Pre-column; enzyme "in situ" immobilized	[65]
Resin VA®: epoxide		Covalent: β-hydroxylamine formation (Fig. 4a)	Oxalate oxidase	Post-column; enzyme "in batch" immobilized	[66,67]
			Aldehyde dehydrogenase, NADH oxigenase	Two IMER systems coupled as post-column; enzyme "in batch" immobilized	[5]
Silica (Kromasil® 200 A) + glycidoxypropyltri methoxysilane to form epoxyde-silica gel		β-hydroxylamine formation (Fig. 4a)	Lipase	Column; enzyme "in situ" immobilized; enantioselective synthesis	[68]
Silica (Chromolith Speed Rod®) + 3-glycidoxypropyltrimeth-oxysilane to form epoxide groups		Covalent: β-hydroxylamine formation (Fig. 4a)	Penicillin G acylase	Monolithic column	[69]
Silica (Hypersil-ODS)		Physical adsorption	Lipase	Pre-column; enzyme "in situ" immobilized; high yield immobilization but enzyme desorption	[70]
Silica-amino	Glutaraldehyde	Covalent: Shiff's base formation (Fig. 3a)	Horseradish peroxidase	Post-microbore column; enzyme "in batch" immobilized	[71]
			β-Glucuronidase	Pre-column; enzyme "in batch" immobilized; biological fluids analysis	[72]
Silica-amino (Chromolith)	*N*,*N*′-disuccinimidyl carbonate	Covalent: amide formation (Fig. 3b)	Penicillin G acylase	Pre-column; monolithic type; chiral separations	[73]
			β-Glucuronidase	Pre-column; monolithic type	[74]
Silica-aminopropyl	*N*,*N*′-disuccinimidyl carbonate	Covalent: amide formation (Fig. 3b)	Lipase	Pre-column; enzyme "in situ" immobilized; chiral separations	[70]
			Penicillin G acylase	Column; enzyme "in situ" immobilized	[18]
	Disuccinimidyl suberate	Covalent: amide formation (Fig. 3c)	Penicillin G acylase	Column; enzyme "in situ" immobilized	[18]
			Lipase	Pre-column; enzyme "in situ" immobilized; chiral separations	[70]
Silica-C_{18}	L-Dipalmitoyl phosphatidyl choline		Trypsine	Column; enzyme "in situ" immobilized	[75]
Silica-epoxide		β-Hydroxylamine formation (Fig. 4a)	Acetylcholinesterase	Column; enzyme "in situ" immobilized; determination of K_m, V_{max} and inhibitors' potency	[76]
			β-Glucuronidase	Pre-column; enzyme "in situ" immobilized	[77,78]
Toyopearl®-AF-amino	Glutaraldehyde	Covalent: Shiff's base formation (Fig. 3a)	Acetylpolyamine amidohydrolase, putrescine oxidase or polyamine oxidase	Post-column; enzyme "in batch" immobilized; analysis of metabolites in urine samples	[79]
		Covalent: HRP oxidation with periodate and successive Shiff's base formation	Horseradish peroxidase (HRP)	The IMER was packed in the upper part with immobilized PROD and in the lower part with the immobilized HRP; post-column	[80]
Toyopearl®-TSK gel: epoxide		Covalent: β-hydroxylamine formation (Fig. 4a)	Piranose oxidase (PROD)		
Toyopearl®-AF-tresyl		Covalent: amine formation (Fig. 4d)	Oxalate oxidase	Post-column; enzyme "in batch" immobilized	[81]
Unspecified		Unspecified	Catalase	Post-column; reactor in FIA and HPLC; determination of acethylcholine in animal tissue	[82]
			Acetylcholinesterase/choline oxidase coimmobilized	Pre-column; determination of acethylcholine in animal tissue	[83]
			Acetylpoliamine amidohydrolase/putrescine oxidase coimmobilized	Pre-column; determination polyamines	[84]
			Amino acid acylase	Aqueous two phase system	[85]
Unspecified: aminopropyl		Covalent	Ethylamine oxidase	Column	[86]

and consequently limits the thermal movement of the protein at a high temperature. Therefore, the attached enzyme unfolds with difficulty, inactivation it is not so easily observed and a higher reaction rate and fewer diffusional restrictions can be achieved.

The functional groups that usually take part in this binding are amino, epoxy, carboxyl, diol and phenolic groups which according to the mode of linkage lead to a wide variety of binding reactions such as diazotization, amide bond formation, arylation, Schiff's base formation and amination as shown in Table 1.

The most widely used method is based on the activation of amino supports, independently of their nature: porous [31,32,34–39], siliceous [18,70–73], polymeric [56] or monolithic [53,72], by the use of glutaraldehyde, an omo bifunctional reagent [31,35–39,71,72,79]. The covalent Shiff's base formation between the aldehydic group and the ε-amine group of lysine residue (Fig. 3a) is obtained under mild reaction conditions (T, pH, stirring) in accordance with those required for the optimal catalytic activity and enzymatic stability.

Some researchers have tried to enhance the resistivity against hydrolysis by performing a reduction of the imine bonds and of the residual aldehyde groups using a mild agent such as sodium borohydride [36–38]. Despite previous results reduction with sodium tetrahydroborate, reported by Yamato et al. [37], shows that this approach is not counterbalanced by some gain in catalytic activity.

Other authors [70] have tried to eliminate the presence of imine groups by employing *N*,*N*′-dissucinimidyl carbonate (DSC) as an activating agent (Fig. 3b) or by introducing a longer carbon chain between the amino support and the enzyme to increase the hydrophobicity of IMERs. By employing *N*,*N*′-disuccinimydilsuberate (DSS, Fig. 3c) they obtained a slight increase in the enzymatic rate and almost a complete separation of the product from the unreacted substrate without the analytical column.

Other preferable methods for the covalent immobilization of enzymes on supports are those obtained via reactive epoxy, diol, diazo and tresyl groups following the schemes reported in Fig. 4.

The immobilization mode influences principally the catalytic activity and the bound amount of enzyme but when the IMER is employed as a column it is necessary to combine the previous features with the best chromatographic performance. For example, in a study [87] on chymotrypsin covalently immobilized on three different silica phases with reactive epoxy, aldehyde or tresyl groups the authors noted marked differences on loading capacity, column stability and on chromatographic peak shape and retention values. In the end they chose the aldehydic support for their stereoselectivity study which shows the best enzymatic stability even if it results in a lower loading of enzyme and in a more tailing product peaks. In a study based on PGA immobilized on aminopropyl-silica via amino or carboxylic group of protein and on epoxy-silica, similar effects are shown [18]. In this case the best compromise between protein amount immobilized, enzymatic activity and product separation selectivity is obtained with epoxy-silica. It is evident that it is impossible to establish the best immobilization technique without taking

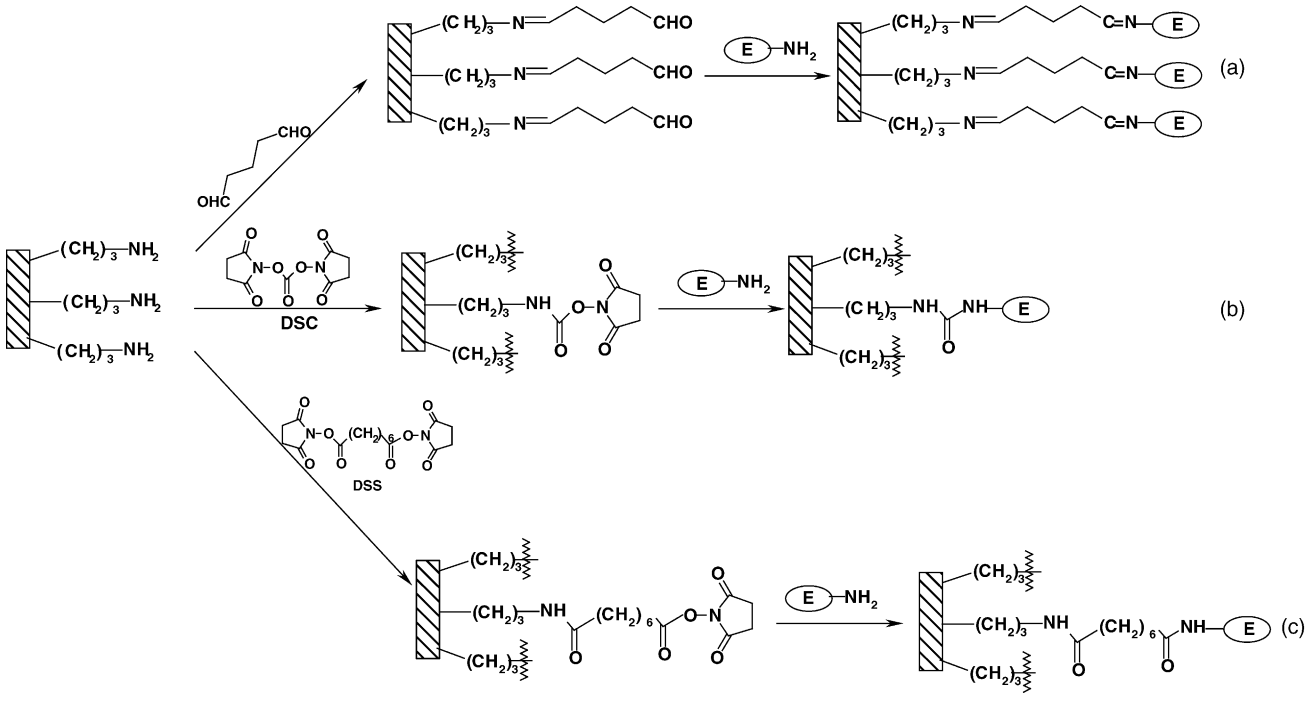

Fig. 3. Reaction schemes of amino-supports for covalent immobilization of enzymes with the following activating agents (a) glutaraldehyde, (b) *N*,*N*′-disuccinimidyl carbonate (DSC), (c) *N*,*N*′-dissuccinimidylsuberate (DSS).

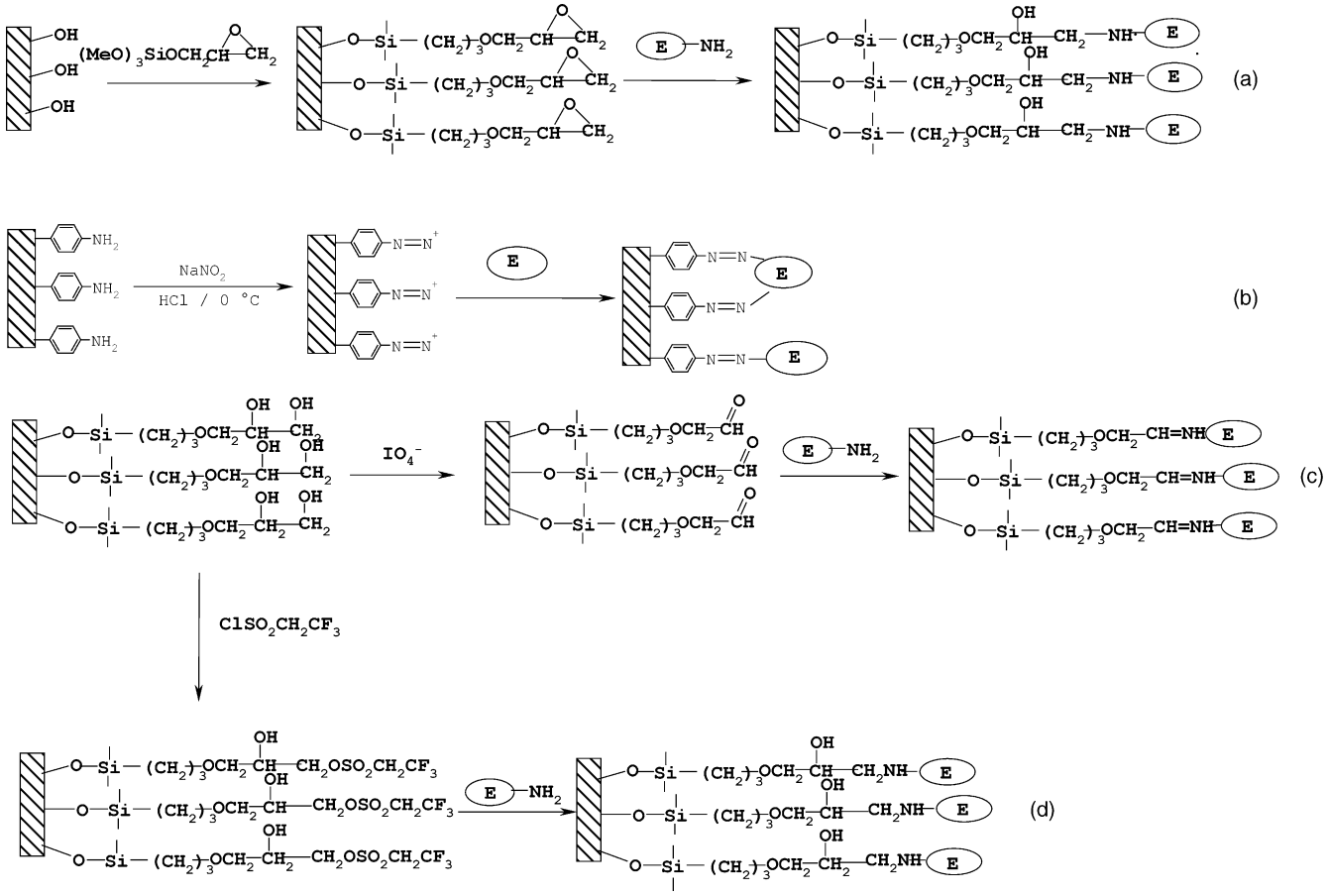

Fig. 4. Reaction schemes of some methods for covalent immobilization of enzymes. (A) β-Hydroxylamine formation, (B) diazonium salt formation, (C) Shiff's base formation and (D) amine formation via nucleophilic substitution of tresyl group.

into consideration the nature of the enzyme which together with the nature of the support regulate the rigidity of the IMER and the protein loading capacity. This latter property, as reported [18,88], strongly influences the chromatographic performance as well as product retentivity, sometimes the selectivity, but never significantly the efficiency.

2.2. Supports

An important factor in the preparation of the bioactive reactor is the structure of the support, since this determines accessibility of active sites to substrates. The ideal support must be inert, stable and resistant to mechanical strength. However, the other physical properties, such as form, shape, porosity, pore size distribution, swelling capability, charges, are also very important because they influence the kinetic process. Indeed, the reaction rates of the immobilized enzyme depend on the enzymatic intrinsic activity, on the substrate accessibility to interact with the active sites, on the amount of the loaded enzyme, and on substrate concentration and diffusivity.

The substrates must be able to diffuse from the bulk phase towards the surface and product away (external diffusion) and within the pores of immobilized enzyme particles (internal diffusion). These effects of diffusion, which are enhanced when the enzyme is entrapped within a matrix, limit the reaction rate because they affect the concentration of the substrate/cofactor in the vicinity of the enzyme. In this way a diffusional layer around the immobilized enzymes is formed and its thickness is correlated to mass transfer effects. A thin diffusion layer, as opposed to a thick layer, results in a low diffusional resistance. Methods to minimize the diffusional effects could be: the decreasing of enzyme loading and the increasing of substrate concentration and diffusivity. This latter feature is strongly influenced by a hydrodynamic parameter such as the flow rate which as it increases, causes a decrease in the diffusional layer.

Care must be taken also in selecting the support materials because their characteristics strongly influence, as previously mentioned, the accessibility of active sites to substrates. For example, the more the pore diameter and size distribution increases the more the surface area decreases. Therefore, it is generally preferable to choose pores with a small diameter if the substrate has similar molecular dimensions. If substrates with high molecular weight are implicated in the enzymatic reaction and their diffusion in the active site is sterically hin-

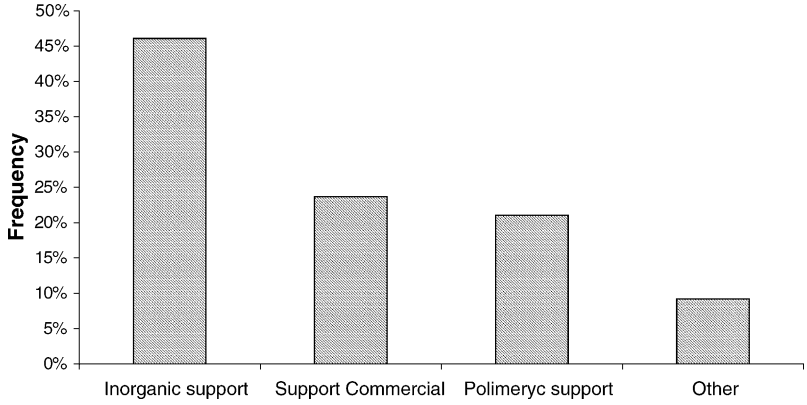

Fig. 5. Supports employed in the reviewed literature (period 1994–2004).

dered, a significant intraparticular mass transfer resistance which in turn significantly decreases the overall reaction rate, must be evaluated.

Another feature influencing the enzymatic activity is the particle size, as is known, the bigger the particle size, the greater the effect of diffusion control and less the activity. To make the correct choice it is also important to consider the relation of particle size versus pressure drop which are correlated in an inverse mode.

Therefore, the evolution of column packing material is to minimize the diffusional limitations by decreasing the size and optimising the geometry of immobilized biocatalyst particles, by decreasing the substrate concentration, by enhancing the flow rate, by increasing the porosity and optimising the biocatalyst distribution in the beads etc. The main supports used in enzyme immobilization are porous inorganic solids (Fig. 5) like the controlled pore glass (CPG) and silica. CPG presents a higher thermal stability and a resistance to acids whereas silica is characterized by a larger specific surface area. Both supports must be derivatized with functional groups which can interact covalently with the enzymes. This feature may be obtained in the laboratory or by using a commercial derivatized support.

When uncoated CPG is employed the researchers use 3-aminopropyltrietoxysilane [31,33] to obtain an aminopropyl-CPG whereas, in the case of uncoated silica glicidoxypropyltrimethoxysilane [68] is used to obtain epoxy-silica. Examples of enzymes immobilized on these kinds of supports are cholesterol oxidase [31], L-aminoacidoxidase [31,34], alanine aminotransferase [33], xylose isomerase, glucose dehydrogenase and mutarotase [32], acid phosphatase [36–39], penicillin G acylase [18,73], β-glucuronidase [72], and lipase [68].

When the enzyme requires an alkaline medium like leucine dehydrogenase [57,58], NADH oxidase [58,59], phenylalanine dehydrogenase [60], pyranose oxidase [59], trypsin [61,62] glucose oxidase [35] and aldehyde dehydrogenase [5] polymeric supports are employed. Among these organic solids poly(vinyl alcohol) activated with tresyl group is most used in “in situ” enzyme immobilization. The performance of these phases is dependent on the kind of enzyme and on the number of injections. In the case of leucine and 3-hydroxybutyrate dehydrogenase coupled to NADH oxidase a satisfactory reproducibility is obtained within 10 days after 300 and 400 injections, respectively, of samples if stored at 4 °C when not in use [59].

A commercial polymeric bioreactor (BAS) containing choline oxidase/acetylcholinesterase or lactate oxidase is generally employed in microbore liquid chromatography coupled to electrochemical detection. This feature ensures low detection limits (fmol for a basal acethylcholine levels in dialysated tissue) and a wide linear range because of the high signal-to-noise ratio and a small flow cell volume. Another commercial IMER, widely employed, is based on poroszyme immobilized peptidases cartridge. This provides an efficient system for peptide mapping of recombinant proteins because of its capacity to minimize enzyme autoproteolysis.

Actually new stationary phases with high quality alternatives to traditional microparticulate sorbents are largely employed, providing important advantages in the chromatographic separation. These phases called “monoliths” or “continuous beds” are disposable as homogenous columns, rods or disks and can be made of macroporous organic polymers [89] or silica material [90]. Contrary to traditional phases consisting of packed particles, the monolithic column is characterized by intricate interconnected cavities which guarantee a porous structure. Additionally, the absence of any interparticle pores creates a rigid structure which enhances the column lifetime but principally modifies the fluid dynamic of the mobile phase inside the monolith with respect to that in conventional porous particles. Indeed, as is well known [91], in conventional chromatography the fluid within the pores is stagnant and the percentage of the adsorptive surface, which is located inside the pores, is only accessible by slow molecular diffusion. In a monolithic stationary phase, the interactive sites are located on the surface of the flow by means of the pores and consequently a very fast mass transfer in and out of the porous material by convection rather than by diffusion is obtained. This characteristic leads generally to fast kinetics, high reactivity and high efficiency

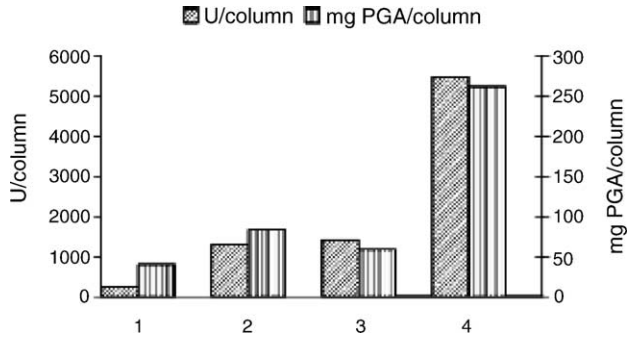

Fig. 6. Amount and activity of PGA immobilized on microparticulate and monolithic silica supports. (1) PGA immobilized on aminopropyl-microparticulate silica, (2) PGA immobilized on epoxide microparticulate silica, (3) PGA-immobilized on aminopropyl-monolithic silica, (4) PGA immobilized on epoxide monolithic silica (taken from ref. [92]).

(200.000–300.000 plates/m in capillary electrochromatography and 30.000–40.000 in HPLC [52]). In the case of an immobilized enzyme such a material, which combines speed and capacity, may be considered an ideal support, as reported in a comparative study [50] where trypsin was immobilized on both macroporous poly(glycidyl methacrylate-co-ethylene dimetacrilate) rods and corresponding epoxide beads. This research revealed not only a greater catalytic activity of the enzyme bound to the monolith, but also a much greater throughput. This is achieved because the monolith can be used even at very high flow rates with modest back pressure, which consequently enables the coupling of the enzymatic column and the analytical column. Same results were obtained by Calleri and co-workers [73,92] in their studies regarding PGA immobilization on epoxide and aminopropyl microparticulate or monolithic silica. Although the author's attention is focused on the optimisation of innovative materials such as monolithic silica columns with a bimodal pore structure (i.e. with throughpores and mesopores), they observed that the amount of immobilized PGA is higher on the epoxy than on aminopropyl-monolithic silica (Fig. 6). In addition, the same authors noted that the epoxy derivatized monolithic silica support presents a higher immobilization yield of PGA compared with the- corresponding PGA-epoxy microparticulate column [69]. Other enzymes, like glucose oxidase [51,52], and acetylcholinesterase [53], have been used for immobilization on monoliths with the geometrical shape of a disk. In these cases the use of ultra short beds (3 mm in thickness) with a diameter of 12–16 mm, as employed in convective interaction media (CIM) disk, shortens time analysis. In addition, the well defined distribution of flow by means of pores allows low back pressure even at high mobile phase flow rates, fast mass transfer (based on convection) and a large surface area which promotes high binding capacities.

3. On-line IMER applications in function of its assembly

When enzymes are immobilized on chromatographic supports, the resulting material can be packed in a small column which, in the liquid chromatographic system, can be placed before and/or after the analytical column. The latter case (post-column), which can be considered really a specific detector, is more commonly employed even if the first tool (pre-column) can be used for clean up purposes, enzymatic pre-treatment of analytes, chiral or achiral compounds synthesis or high throughput screening for enzyme inhibitors.

3.1. Pre-column assembly

Pre-column assembly is easily combined with another chromatographic system by means of a separative column forming two systems which can run independently by using a switching valve (Fig. 7). In this way it is possible to prevent the eventual enzymatic denaturation caused by the use of an organic solvent and pH necessary for the separation on the second chromatographic system [31,72].

In addition this mode is very interesting when it is necessary to obtain by the IMER a very rapid conversion of analytes in substances which, by means of the analytical column, are more easily separated from complex matrices such as urine [72] and biological tissue [6,21] thus resulting in a more accurate and simpler analysis. Indeed, the determination of benzene metabolites in urine, using an immobilized β-glucuronidase reactor, is obtained by injecting directly the sample without resort to additional multisteps such as extraction or steam distillation, which are very time consuming. The successive separation of released phenols from the hydrolytic enzymatic reaction is simply performed on the analytical column without any interferences from the other urinary components [72].

The integration of enzymatic pre-reaction and product separation steps could be very interesting also in stereochemical processes in the case of on-line synthesis, purification of chi-

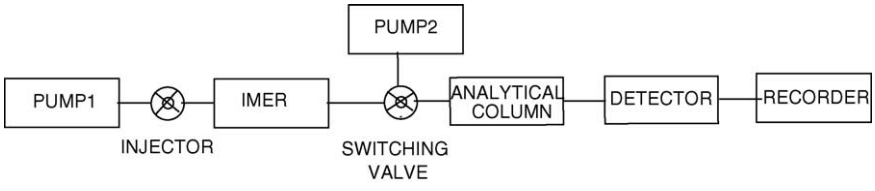

Fig. 7. Schematic representations of the on-line IMER assembly as pre-column: a second pump connected to a switching valve permits to change mobile phase after enzymatic reaction.

ral molecules and in the quick estimation of more suitable conditions for an enantioselective reaction. This approach falls within the enantioselective direct separation methods described by Davankov [93], which are based on either a selective enantiomer transformation at a different rate into new compounds or on labile molecular adduct formation of differing stability with the enantiomers. In the particular case of enzymes, as enantiodiscriminating agents, the direct method is a kinetic enantioselective process leading to the formation of new more easily detectable enantiomeric species.

Actually some enzymes, like lipase [68,70], penicillin G acylase [73] and chimotrypsin [94], have been shown to be useful for this kind of application since they advantageously prove to have a stereoselective behaviour, by their hydrolytic cleavage ability of some particular bonds, producing enantiomerically enriched compounds. Lipase is an esterase which converts lipids to glycerol and free fatty acids or catalyses the reverse reaction (transesterification), penicillin G acylase, acting on penicillin or cephalosporin substrates, catalyses the hydrolysis of benzylpenicillin to release 6-amminopenicillanic acid and phenylacetic acid while chymotrypsin, being a peptidase, cleaves peptides to shorter peptide chains by cutting the bond near the aromatic amino acid. The stereoselective action of all these enzymes, which is strongly dependent on the substrates structure, arises from the presence of a chiral centre near the hydrolysed bond. Generally, it is preferable to connect the IMER to an achiral analytical column when a quick screening of the enzymatic stereoselectivity towards different substrates [70,73] is made while the coupling with separation units, constituted by a chiral stationary phase [44,43,68], is carried out when maximized yield and purity of the products is required in accordance with stereoselective syntheses conditions.

The easy integration of an IMER arranged in pre-column into a multidimensional analytical system makes this approach also very useful when high quality of the products, obtained by proteolysis, is required. Dormady et al. [65] demonstrate that, besides a very rapid action using a proteolytic immobilized enzyme with respect to a free enzyme process and a reduction in contamination derived from autodigestion with the free enzyme, the possibility to connect the IMER and the analytical column to a detector such as MALDI-TOF, makes it possible to eliminate any artefacts and confirm the structure of synthetic proteins.

Another important application of IMER is the possibility to clean up the sample of any interfering species present in the matrix. A typical example is the determination of acethylcholine in a microdialized biological tissue. The removal of the interfering endogenous choline, by the action of the choline oxidase together with catalase [6] or a horseradish peroxidase [21] immobilized reactor converting choline into betaine and H_2O, is realized before of the chromatographic separation. A further coupling of the two chromatographic systems, with a third one, consisting of a combined choline oxidase/acetylcholinesterase post-column IMER, enhances the sensitivity and the selectivity of endogenous acetylcholine determination [6,21] (Fig. 8). In this way the mixed-enzyme post reactor serves to hydrolyse acethylcholine to

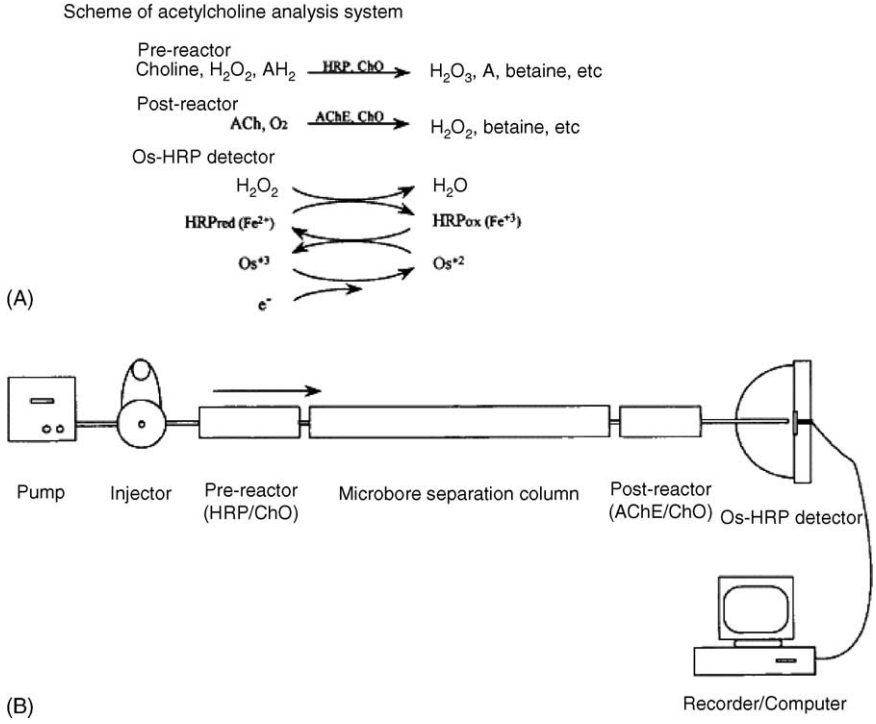

Fig. 8. Scheme of acetylcholine assay method by HPLC-horseradish peroxidase-immobilized glassy carbon electrode with pre- and post-immobilized enzyme reactors (taken from ref. [21]).

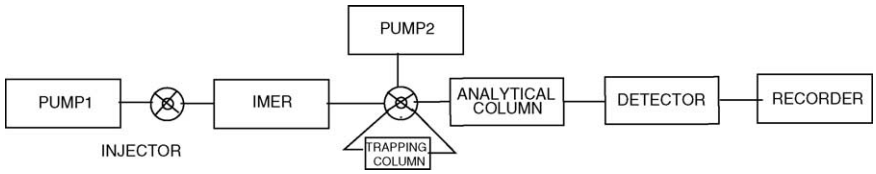

Fig. 9. Schematic representations of the on-line IMER assembly as pre-column: a trap column connected to a switching valve permits pre-concentration of enzymatic reaction product. This can be (eventually) eluted by a different mobile phase pumped by pump 2.

acetate and choline and to convert choline into betaine and an electroactive species, H_2O_2, prior to electrochemical detection. The important result of 5 fmol/μl as a detection limit can be achieved despite the fact that acethylcholine and choline are neither electroactive nor UV absorbing substances.

Another mode of assembly is based on the insertion of a trap column between the IMER and the analytical column with the aim to accumulate and concentrate the enzymatic reaction products taking into account the striking condition of complete conversion of the substrate in product (Fig. 9). As described, for example, by Ono et al. [36] and Yamato and co-workers [37,38] an enzymatic hydrolysis product such as riboflavin, via the switching valve, can be back-eluted from the trap column using an opportune mobile phase containing also organic solvents. In this way, besides preventing the denaturation of the enzyme from the organic phase a concentration at the top of the trap column can be carried out.

In Fig. 10 is depicted the system employed by Pasternyk Di Marco et al. [77,78] for the analysis of chloramphenicol-β-D-glucuronide. In this case the system is composed of a column (ISRP), a β-glucuronidase immobilized enzyme reactor (BG-IMER) column and a C_8 reversed-phase column. The columns are connected by means of three six-port switching valves. Chloramphenicol-β-D-glucuronide is initially separated from the biological matrix by eluting off-line to waste the contaminants, then is deconjugated by IMER to chloramphenicol, and finally it is concentrated on the top of C_8 column and after elution is determined. This way

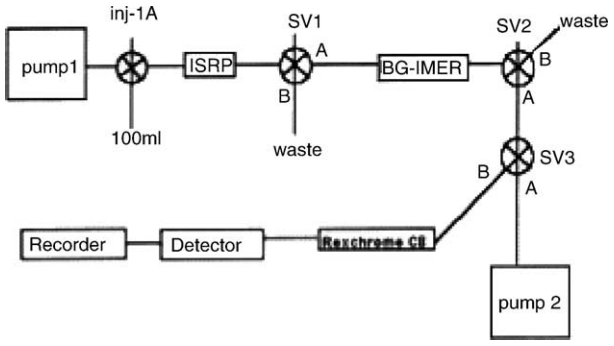

Fig. 10. Schematic diagram of the experimental procedure for off-line/on-line injection. ISRP: intersurface reversed phase column; SV1, SV2, SV3: switching valves; BG-IMER: β-glucuronidase, Rexchrome C_8: analytical column (taken from ref. [77]).

a good and accurate method avoiding any interferences is achieved.

Finally the use of pre-column IMERs connected to an analytical column has proven itself to be a useful a probe for biochemical and pharmacological properties in protein synthesis [46,64] and an on-line highthroughput screening tool for phenylethanolamine *N*-methyltransferase [54], dopamine β-hydroxylase [42] and human recombinant acetylcholinesterase [53] inhibitors. This approach, really could become a feasible alternative, because of its rapidity and efficiency, to the actual conventional control methods used in the production of recombinant proteins and in the discovery of novel therapeutic drugs.

3.2. Post-column assembly

In the case of post-column the analytes are first separated in their original form by a separative column and only through the IMER analytes are advantageously converted, within a few minutes, into products that have an improved absorption, fluorescence or electrochemical detection.

The advantages of post-column IMERs have been well known for long time [95,96]. The main benefits, as reported by Brinkman [95] in a review, are the possibility: (i) to avoid artefact interferences usually observed in a common derivatization reaction on separated analytes and (ii) to obtain high reproducibility also in the case of an incomplete enzymatic conversion or of reaction product instability. The disadvantages are generally due to: (i) band broadening caused by tubes, mixing chambers required for the connection of the IMER to the system, to IMER itself and to dilution effects of the reagent eventually added for product derivatization, (ii) the need, sometimes necessary to enhance the sensitivity of the analytical method, of the enzymatic product derivatization into a more absorbing, fluorescent and electroactive chemical, (iii) the need to modify the experimental conditions for the separative step such as pH, solvent, *T*, etc. to those required for an enzymatic post-column reaction [10,96]. These changes necessitate additional apparatus, namely, a pump and T-junction as reported in Fig. 11.

However, post-column IMER assembly is certainly chosen when the reactor is to be connected to a specific electrochemical detector so that, a successive separative step not being necessary, the enzymatic product may be determined without any possible interference by added reagents for further derivatization. Typical examples are the determination

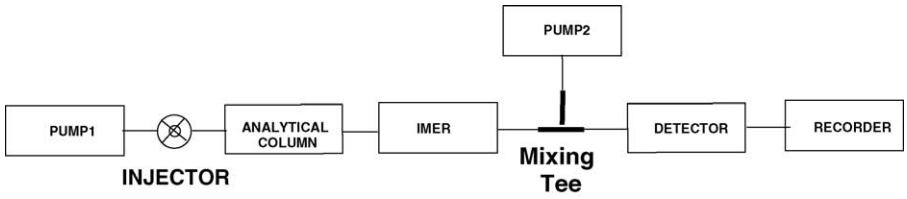

Fig. 11. Schematic representation of the on-line IMER assembly as post-column.

of acetylcholine (Ach), choline (Ch) and oxalate in fluids and tissues. First of all acethylcholine is separated from the matrix interferences on the microbore analytical column and then, by using a post-column reactor containing a acetylcholinesterase/choline oxidase couple is converted to hydrogen peroxide which, being correlated to Ach and Ch concentration, can be easily determined by platinum-black modified electrodes [22,23,26,27,71,97–103]. Oxalate is determined in complex matrices such as plasma [66–67,81] and urine [66,81] by employing oxalate oxidase as an immobilized enzyme after the separation of the metabolite from the other anionic species by ionic exchange. The electrochemical detection of hydrogen peroxide makes this analytical method cheaper than others commonly used because a large number of samples can be analysed using a labour saving process.

Another system with electrochemical detection without having to resort to any pre-treatment step, is based on L-aminoacid oxidase or D-aminoacid oxidase immobilization for the determination of D-alanine. In this case it is necessary to include a mixing chamber to carry out the pH to its optimum value for enzymatic activity. Despite this disadvantage the analytical system renders possible the determination of 1 mg/l amount which can be considered the limit value for bacterial contamination of food [34,104].

Regarding analyses in the field of commodities the determination of some aldehydes, namely, acetaldehyde [105], furfuraldehyde [5] and formaldehyde [106] is of relevant interest in quality controlling of some foodstuff. The system employing aldehyde dehydrogenase coupled to NADH oxidase in the same reactor allows the detection of a wide range of concentrations of aldehydes in various kinds of food, beverages and cosmetics even if it is affected by the short lifetime of the aldehyde dehydrogenase reactor [5,105].

3.3. Column

An interesting technological approach is the use of the integrated enzymatic reaction-separation process with the benefits of maximum time and minimum cost. These findings have led to an increasing interest in the development of immobilized enzyme columns principally in enantioselective analyses [68,85] and in the identification of substrates and inhibitors as potential drugs [41,55,76]. They also offer to researchers a broad selection of powerful synthetic tools for laboratory and large-scale chiral syntheses because of the variety of enzymes that is used, for instance,: lipase [68] horse liver alcohol dehydrogenase [43] and penicillin G acylase [18].

A novel enantioselective mode for the production of chirally pure L-methionine from a racemic mixture of *N*-acetylmethionine, is based on a high-speed countercurrent centrifugal partition chromatography (CPC). This mode consists of an aqueous two-phase system in which, differently from the conventional IMER, the enzyme is dissolved in a liquid stationary phase [85]. This phase is retained in the column because of a combination of special column geometry (channelled disc) and a density difference between the immiscible liquid phases. In this way the reaction occurs in the stationary phase and separation is determined by the partition coefficients of the substances involved in the two phase system.

A particular case of the immobilized enzyme in column assembly is that of a potential drug frontal analysis affinity chromatography as reported in previous reviews [107–110]. This technique can be very useful when required to determine enzyme-substrate kinetic parameters or to characterize the interactions between a candidate drug and the immobilized enzyme without the need to isolate and dissociate drug/enzyme interactions. This rapid and simple reliability approach, is based on the continuous flow of the mobile phase, containing a ligand of the enzyme at known concentration, using the IMER. As the ligand binds to the biocatalyst, the protein becomes saturated and the amount of solute, eluting from the column at retention volume (V), gradually increases until it reaches a plateau (Fig. 12). The degree of difference between the retention parameter (V) and the void volume (V_0), obtained with the passage through the column of a substance without any specific interaction with the enzyme, depends on the concentration of the ligand and can be considered a direct probe for solute-enzyme interaction. Generally this difference ($V - V_0$) decreases as substrate concentration increases. In addition, this approach has a peculiar advantage with respect to the conventional methods. Indeed, it allows to investigate little differences in the binding of solutes in the presence of fast association-dissociation kinetics due to the establishment of a large number of consecutive equilibria between free and bound ligands. A typical application is the development of candidate drug molecules. In this way the bindings of human *N*-acetylglucosaminyltransferase V to a mixture of eight trisaccharide analogues in a single run are achieved [55] since they break at different times according to their affinity to the immobilized enzyme.

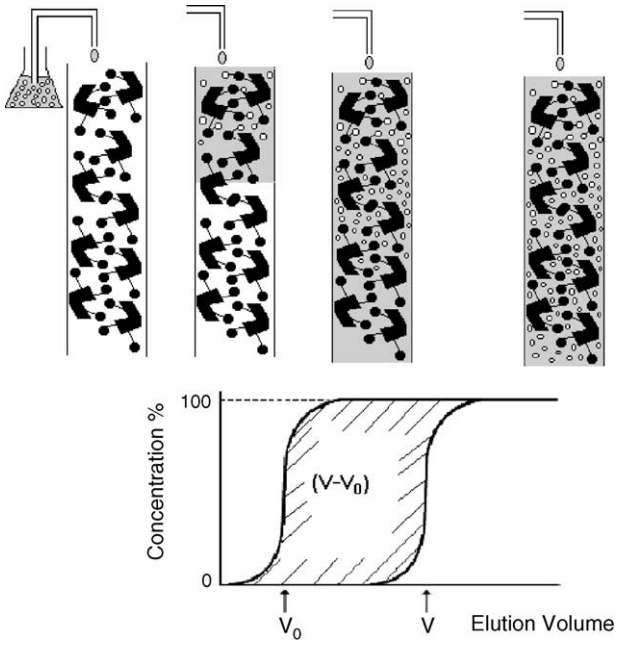

Fig. 12. Elution profiles in frontal affinity chromatography. Curve I, is elution pattern of substance without any specific interaction with enzyme. Curve II is that obtained when specific interactions ligand-immobilized enzyme are present. V_0: void volume; V: elution volume of ligand; ⁘: immobilized enzyme; □: ligand.

4. Conclusion

The papers revised in this review have demonstrated that immobilized enzymes can be used for on-line HPLC pharmacological, clinical and commodity studies. In particular they are very useful as rapid screening tools for the binding investigation of enzymes towards new potential drugs, for the enantiomerical pure compounds syntheses or for its coupling to a detection system and for ultra-micro quantitative analyses. In addition we have presented immobilization methods for HPLC use and the supports mainly employed. Finally, in the light of current developments in this research field, it is to be hoped that the availability of new support materials will lead to more selective IMERs, and a widespread development in bioanalytical science and in applied biotechnology.

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