



REVIEW PAPER

Immobilized enzyme reactors in HPLC and its application in inhibitor screening: A review

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Abstract This paper sets out to summarize the literatures based on immobilized enzyme bio-chromatography and its application in inhibitors screening in the last decade. In order to screen enzyme inhibitors from a mass of compounds in preliminary screening, multi-pore materials with good biocompatibility are used for the supports of immobilizing enzymes, and then the immobilized enzyme reactor applied as the immobilized enzyme stationary phase in HPLC. Therefore, a technology platform of high throughput screening is gradually established to screen the enzyme inhibitors as new anti-tumor drugs. Here, we briefly summarize the selective methods of supports, immobilization techniques, co-immobilized enzymes system and the screening model.

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1. Introduction

Molecular bio-chromatography is a novel chromatographic technology that integrates interaction mechanism between drug and target with chromatography. It is increasingly used in studying the interactions between drugs and biological macromolecules by many chromatographic parameters. Medicine

interacts with enzymes or receptors to exert biological activities *in vivo*. Enzyme is the biggest target system in drug screening. Many enzymatic models play important roles in drug discovery process, which are important screening methods for potential drugs, and they are important sources of screening potential drugs.

Enzyme is one of the basic symbols for tumor diagnosis. With cancer emergence and metastasis, the activity or expression of enzyme will bring about abnormal phenomena. The abnormal growth of tumor cells and vessels leads to rapid metastasis, and invades surrounding tissues and organs. Therefore, screening drug candidates which are capable of inhibiting tumor from metastasis associated with the key enzymes plays an important role during drug discovery process. At the same time, single-enzymatic model also provides powerful basis to build multiple enzymes bio-chromatograph screening systems.

Immobilized enzymes are used in batch-wise experiments or packed into columns and used in a flow system as immobilized

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enzyme reactors (IMERs). Traditionally, it is difficult to separate products and enzymes after the solvable enzymes interact with substrates. Moreover, the products tend to be low and the enzyme activity variability is large among batches. However, immobilized enzyme maintains high specificity and catalytic efficiency in catalytic reaction, whose abilities against heat, pH, and organic solvents are increased. Therefore, minimizing costs and time of analysis make it economically feasible to operate in a continuous mode [1]. In this way, multiple enzymes that are all relevant to tumor metastasis are co-immobilized onto the chromatographic support and packed into a column for reactions. Reaction time can be shortened and fast screening of the enzyme inhibitor can be performed [2]. In general, IMERs can be coupled with capillary electrophoresis (CE), HPLC, and other modes for on-line analysis [3]. The main purpose of this review is to present the advantages of coupling of an IMER with HPLC method, such as sensitivity, selectivity, rapidity, non-destructivity and reproducibility. In this way, sensitivity of the detection system is considerably enhanced and determination of trace components in complex matrices becomes possible [1].

2. Immobilized enzyme reactors

2.1. Immobilization supports

The main problem in enzyme immobilized process is closely related to the properties of support materials. The ideal material as support should have good biocompatibility, known three-dimensional porosities structure and chemical group. All these properties can modify the mechanical strength, and make it stable in a broad range of temperature, pH, ionic strength and organic solvents. However, the other physical properties, such as form, shape, porosity, pore size distribution, swelling capability, and charges, are also very important because they affect the kinetic process. Indeed, the reaction rates of the immobilized enzyme depend on the enzymatic intrinsic activity, the substrate accessibility to the active sites, the amount of the loaded enzyme, substrate concentration and diffusivity.

Although there is no universal support for all enzymes and their respective applications, a lot of desirable characteristics are common to any material considered for immobilized enzymes. Usually, all the supports that have been studied for immobilized enzymes are classified into inorganic and organic materials, natural and synthetic materials. The morphology and properties of supports are characterized with scanning electron microscopy, transmission electron microscopy, Fourier transform infrared spectroscopy. The component characteristics of supports are determined by X-ray diffraction, elemental analysis and thermo-gravimetric analysis and so on.

2.1.1. Inorganic materials

Inorganic materials have some advantages over polymer materials in good stability, high mechanical strength as HPLC supports, non-toxicity for microorganisms, against microbial decomposition, acid and alkali resistance, low cost and long service, etc. The mainly used supports in enzyme immobilization are porous inorganic solid, such as silica and controlled pore glass (CPG). Silica and its derivatization materials are the typical examples of inorganic materials, and they are widely

used in HPLC as supports due to their large specific surface area and good mechanical properties. Because of the silanol groups on the surface, this kind of material has certain derivative functions as well. However, silanol groups on silica surface generate strong nonspecific adsorption to macromolecules. Recently, the monolithic columns have been proved to be more selective than particle-packed columns for high efficiency separations by HPLC [4]. Due to their small-sized skeletons and wide through-pores, much higher separation efficiency can be achieved than the case with particle-packed columns at a similar pressure drop. In 2011, Miyazaki et al. [5] examined chromatographic properties of a new type of monolithic silica rod columns. Silica rod columns employed for the study were prepared from tetramethoxysilane, modified with octadecylsilyl moieties, and encased in a stainless-steel protective column with two polymer layers between silica and the stainless-steel tubing.

However, research on silica monoliths is tightly restricted by the lack of availability of monoliths having different pore and domain size distributions. Therefore, the faster diffusion of mobile phase on a monoliths silica column than on a particle-packed silica column suggested the possibility of further improvement of the column performance by increasing the structural uniformity of the supports and by decreasing the domain sizes.

The controlled pore glass beads (CPG) played the most important role in enzyme immobilization in the past decade. In fact, their wide surface area, narrow pore size distribution, mechanical strength, rigid structure and chemical inertness satisfy well the requirements for on-line HPLC applications. In addition, the hydroxyl group of CPG leads to easy chemical modification and consequently commercial availability with different bonded reactive groups. Girelli et al. [6] studied that mushroom tyrosinase was covalently bonded with glutaraldehyde, as an activating agent, to aminopropyl-controlled pore glass support by "in situ" immobilization technique. Schiff's base double bond reduction with sodium cyanoborohydride was made as innovation. Catalytic activity and stability of the chromatographic reactor were evaluated using *d*, 1-3, 4-dihydroxyphenylalanine as the substrate.

2.1.2. Organic materials

Organic materials include natural and synthetic supports. Structural proteins, globular protein and its carbohydrates are all natural polymeric materials. Natural polymer materials such as glucan, agarose, chitosan or fibrin polysaccharide matrices have well biocompatibility, soft structures and weak mechanical properties. Natural polysaccharides are suitable for being enzyme carriers, and they are almost non-toxic, having excellent mass transfer performance, and easily available. The synthetic polymer materials include polystyrene, methyl methacrylate divinyl benzene resin, etc. Although synthetic polymer has good chemical and physical properties, polysaccharide has large variability and high maintenance of enzyme activity. Chitosan (CTS) is one of the most popular research carriers, especially being a useful support of immobilized enzymes and cells [7]. However, chitosan swells unsteadily in water and its weakly mechanical strength, researchers usually prepared hybrid chitosan with SiO₂ as solid phase of HPLC.

Due to its variable chemical and physical properties, synthetic organic polymer materials can bear any kind of immobilized enzymes, and they are also resistant to microbial corrosion.

In addition, some researchers try to use some other organic materials, such as poly(glycidyl methacrylate-co-acrylamide-co-ethylene glycol dimethacrylate) monolith [8], Ethylenediamine (EDA) monolithic convective interaction media (CIM) Disks [9–13], Eupergit[®] C or Eupergit[®] C250L [14], and commercial immobilized artificial membrane (IAM) [15] and so on. Each kind of materials has its own strengths. EDA-CIM Disk, available under the trademark CIM (for convective interaction media), is characterized from a well-defined pore-size distribution and excellent separation properties at low back pressure. EDA-CIM Disk is an amine activated monolithic support obtained from the native epoxy groups with a convenient ethylenediamine spacer. EDA monolithic disks can be used for bioconversion after coupling proteins, peptides or other ligands through cross-linking reaction with a suitable bifunctional reagent [10]. The IAM stationary phases produced the covalent binding of phosphatidylcholine to aminopropyl silica using a terminal amide linkage. Such structure limits the access to the unbound amine groups and allows the protein to interact with any combination of polar head groups and hydrophobic chains. It can model flowing membrane lipid on the solid support, and it needs a bio-membrane evaluation system.

2.1.3. Magnetic polymers

Magnetic polymer microsphere is a kind of internal magnetic metal (usually Fe₃O₄) or metal oxides of ultrafine powder, which is the responsiveness of magnetic polymer microsphere, so it can be used as a carrier of immobilized enzymes. The morphology and properties of these magnetic supports are characterized with scanning electron microscopy, transmission electron microscopy, Fourier transform infrared spectroscopy and a vibrating sample magnetometer. Hu et al. [16] demonstrated that a novel-immobilized enzyme strategy created by magnetic nanospheres for monitoring enzyme activity and screening inhibitors followed by HPLC. Through the reaction of the aldehyde groups with amine groups, α -glycosidase was simply and stably immobilized onto magnetic nanospheres by the cross-linking agent glutaraldehyde.

Whereas super paramagnetic nano- or micro-sized particles are commonly used in magnetic bioseparation, a key advantage of the ferromagnetic particles over their superparamagnetic counterparts is their higher magnetic moment that enables easier and more versatile control of the particles by means of a variety of external magnetic fields, not limited to permanent magnets or static DC fields used in most conventional magnetic separation systems. Recently, the applications of immobilized enzymes using magnetic polymer possess more advantages than other porous materials. One of all advantages is that they can be easily removed from the reaction mixture with the assistance of a magnetic field. This facilitates the separation and recycling of immobilized enzyme, as well as the purification of product.

2.1.4. Organic–inorganic hybrid materials

Recently, more and more attention is paid to the preparation of organic–inorganic hybrid carrier materials, because the organic–inorganic hybrid materials contain both advantages of organic materials and inorganic materials. These organic–inorganic hybrid materials have plasticity, easy workability and biological compatibility of the organic materials, and rigidity, magnetism and conductivity performance of the inorganic materials.

The organic–inorganic hybrid materials have high stability and functional composites in great potential applications. After strong interaction (coordination, electrostatic attraction) or weak interaction (hydrogen, etc.) in charge transfer at nanoscale even molecular level, organic and inorganic substances become uniform multiphase even homogeneous materials which are organic–inorganic hybrid materials. At present, many methods are used for the preparation of compound materials, such as layer by layer, sol–gel, layered embedding method and the in-situ polymerization. So far, in HPLC, silica is the most widely used as inorganic carrier material. As an inorganic carrier, surface coating hydrophilic polymer is expanding the application of biological separation of organic–inorganic composites in important ways. Xi and Wu [17] prepared macroporous chitosan layer coated on silica gel to apply to affinity chromatography for trypsin inhibitor purification. In this research, various porous layers could be formed on the bead surface by controlling the content of chitosan and PEG with different molecular mass weight in coating solution. Results proved that this kind of matrix has low nonspecific interaction and could be easily activated for covalently binding of trypsin with high capacity and stability.

Lü et al. prepared a novel chitosan functionalized monolithic silica capillary column using carboxymethyl chitosan as chemical modification reagents. Its performance was investigated through the separation of polar compounds including nucleosides, nucleotides, aromatic acids, and aliphatic acids [18] (Fig. 1).

The objective of this part is to review, in the light of current developments, the methods used for online IMERs from the point of view of the above mentioned aspects, which are also summarized in Table 1.

2.2. Immobilized enzyme solid phase used in HPLC

2.2.1. Immobilizing techniques

According to the properties of the target enzymes, many techniques have been used previously for enzyme immobilization, which can be categorized into entrapment [24,25], cross-linking [16,26,27], adsorption, and a combination of these methods.

Embedding method is divided into net type and micro-encapsulation type. The former is a kind of technique that the enzyme is embedded into the network of polymer gel, and the latter is the method that the enzyme is embedded in a semi-permeable membrane. In embedding methods, the amino acid residue of enzymes usually doesn't react, so the enzyme senior structures are rarely changed. Thus, the enzyme recovery ratio is very high. Because only the small molecules can be diffused into the pore network of polymer gels, the diffusion resistance will cause the changes of kinetic behaviors of the immobilized enzymes and decrease of enzymatic activity. Therefore, the embedding method can be only suitable for small molecular substrates and products.

Physical adsorption immobilizing method is the technique in which insoluble carriers are used to adsorb the enzymes. The affinity forces between enzyme and carrier include Van Der Waals force, ionic bond and hydrogen bond. The commonly used carriers are CPG, activated carbon, bleaching earth, alumina, silica, calcium phosphate, metal oxide and other organic carriers, such as starch, albumin and natural macromolecular carriers.

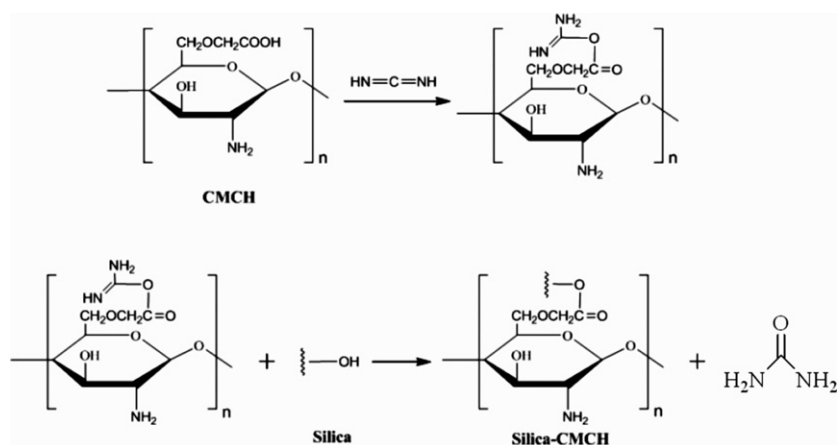


Figure 1 Scheme for the chemical modification of the monolithic silica column using carboxymethyl chitosan. Reproduced from Ref. [16].

Table 1 Supports, immobilizing techniques and enzymes used in IMERs.

| Support | Immobilizing technique | Enzyme | Refs. |
|--|------------------------|--|---------|
| Controlled pore glass(CPG) | Covalent | Tyrosinase | [6] |
| Poly(glycidyl methacrylate-co-acrylamide-co-ethylene glycol dimethacrylate) monolith | Covalent | Trypsin | [8] |
| EDA-CIM | Covalent | Human recombinant butyrylcholinesterase (rBChE) | [9] |
| | | BACE-1 | [10] |
| | | HrBACE-1 | [12] |
| | | Arginase | [13] |
| Eupergit [®] C or Eupergit [®] C250L | Covalent | Sulfite oxidase | [14] |
| Magnetic nanospheres | Cross-linking | α -Glucosidase | [16] |
| Chitosan/SiO ₂ | Covalent | Trypsin | [17] |
| Cellulose | Covalent | Tea polyphenol oxidase | [19] |
| Electrophoresis fused-silica capillary | Covalent | Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | [20] |
| Epoxy monolithic silica | Covalent | MMP-8 | [21] |
| Immobilized artificial membrane(IAM) | Covalent | Purinergic receptor (P2Y1) | [22] |
| Monolithic silica | Entrapment | Lipase | [23] |
| Silica | Entrapment | Butyrylcholinesterase (BuChE) | [24,25] |
| NHS-activated Sepharose beads | Cross-linking | Trypsin | [26] |
| Thermo-responsive hydrogel (synthesized from N-isopropylacrylamide, glycidyl methacrylate, and N, N-dimethyl acrylamide) | Cross-linking | Trypsin | [27] |
| Poly(acrylamide-co-methylenebisacrylamide) (poly(AAm-co-MBA)) monolith | Covalent | Trypsin | [28] |
| Alginate sodium | Entrapment | Cellulose and hemicellulase | [31] |

The biggest strength of physical adsorption is to prohibit centers of enzymatic activity from being destroyed, and rarely change the advanced structure of enzymes, thus decreasing the loss of enzymatic activities. If you can find an appropriate carrier, it will be the most ideal method. This method is very simple, the reaction conditions are usually mild, and the carrier may be used repeatedly. But if the connection is not firm, the enzyme will fall off. For example, Ren and Yu [28] reported a hydrophilic-modified solid support to immobilize enzyme through oriented adsorption. The immobilized enzyme remained 86% of its original activity under the acylation of 1-phenylethanol, much more than those immobilized on other solid supports with lower hydrophilicity (20–35.6%), as this mode of the immobilization could facilitate enzyme to expose its activity site to the substrate.

Double function or multi-function groups of reagents are crosslinked with enzyme molecules in cross-linking immobilization method. Among a variety of cross-linking agents, the most commonly used one is glutaraldehyde. Due to the intensity of the cross-linking reaction conditions, the immobilized enzymes involved in the process of chemical reaction become seriously inactive.

Covalent immobilizing method is an in-depth researched method. Enzymes and polymers are combined with a single carrier by covalent bond. The combination of enzymes and carrier is very strong, but enzymatic activity will decrease because of the violent reaction conditions. The operations of activating covalent carrier and the processes of immobilizing enzymes are relatively complicated. The condition of immobilized enzymes activity must

be strictly controlled. Enzyme immobilization results depend on the type of covalent connection and physical and chemical properties of carrier groups.

At present, the immobilized methods of covalent coupling and cross-linking have been established. These methods include

- (1) Diazotization method: This method is the most commonly used one in covalent methods. It means that the insoluble carriers are treated by dilute hydrochloric acid and sodium nitrite nitrogen, and then transformed into diazo-compound, which can couple with enzyme molecule.
- (2) Peptide bond method: In this method, the carriers which have functional groups will react with the lysines of enzymes.
- (3) Alkylation method and aromatic method: The alkylating or arylating carriers with halogen functional groups will react with the amino of enzymes and proteins in order to immobilize enzyme in these methods.

In general, in order to keep the amount and stability of enzyme, covalent method is usually used to immobilize enzyme in the rigid chromatographic column of HPLC. Before the enzyme is covalently reacted with the support, the support should be firstly functionalized, and then the active functional groups of intermediates are produced. The common active groups of carrier are hydroxyl and amino. Functional process and reagents are shown in Figs. 2 and 3.

2.2.2. Immobilized enzyme evaluation

(1) Determination of immobilized protein.

The amount of enzyme, as a kind of protein, immobilized on the support was calculated from the difference in the amount of immobilized protein contents before and after recycle. The protein concentrations are determined by spectrophotometer measurements at 215 nm and 280 nm

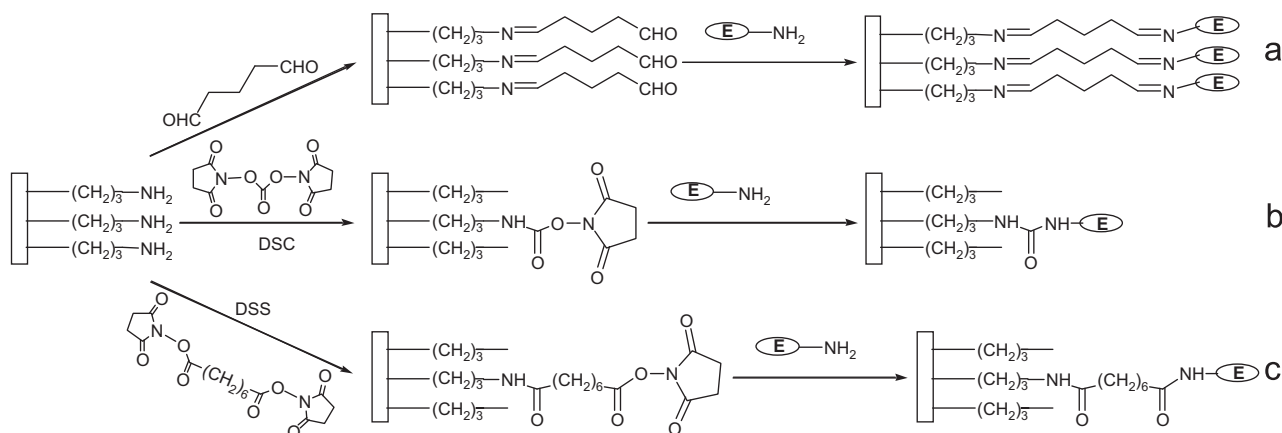


Figure 2 Reaction schemes of covalent immobilization of enzymes with the following activating agents: (a) glutaraldehyde, (b) DSC and (c) DSS.

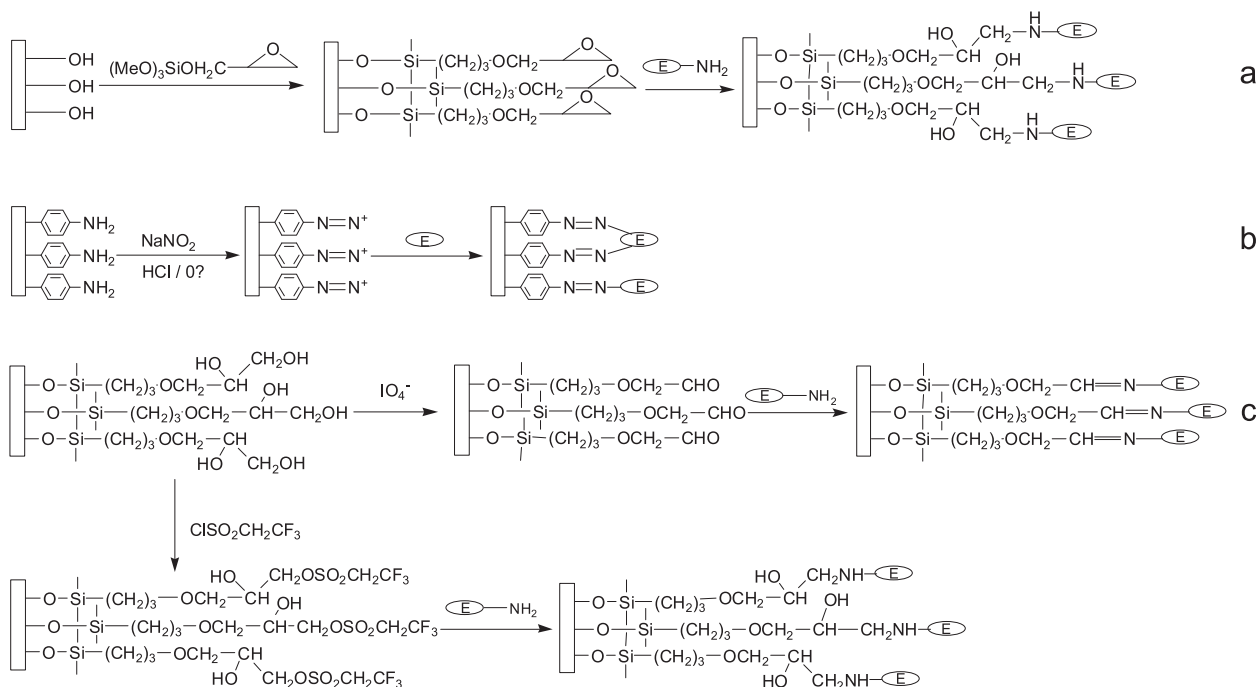


Figure 3 Reaction schemes of some methods for covalent immobilization of enzymes (a) β -Hydroxylamine formation, (b) Schiff's base formation and (c) amine formation via nucleophilic substitution of tresyl group.

and mathematically expressed as

$$Y_{IMER}^{P_{roi}}(\%) = \frac{P_i - P_f}{P_i} \times 100$$

where P_i and P_f are the initial and final UV absorbance values of enzyme solution, respectively [6].

(2) *Removed activity ratio.*

Removed activity ratio, defined by Vilanova et al. [25], represents the activity removed from the enzyme solution as a consequence of the enzyme immobilization as well as its inactivation. The percent removed activity ratio is calculated by

$$R_{rem}^{act}(\%) = \frac{U_i - U_f}{U_i} \times 100$$

where U_i and U_f are the total units of enzyme activity in the solution before and after the immobilization process, respectively [6].

(3) *Determination of immobilized enzyme activity.*

The IMER was placed between the HPLC pump and the detector and then equilibrated with approximate buffer solution. Through enzymatic reaction, we could calculate the activity of immobilized enzyme and make a curve between the concentration of substrate and product that is presented an equation.

(4) *Influence of other factors on immobilized enzyme stability.*

The factors contain chemical and physical parameters on enzyme activity, reaction time, flow rate, appropriate temperature, solvent, the kind of buffer and suitable buffer pH, etc. Immobilized enzymes are usually more stable than free enzymes. However, because of the changes of protein spacial structure, parts of activities of immobilized enzymes are lost in the immobilization chemical process.

3. Co-immobilized enzymes reactor

Co-immobilized enzymes system supplies a practical method to increase the efficiency of screening drugs and it becomes the basis of developing high throughput screening (HTS). In particular, different enzymes on the suitable support have been studied with the aim to develop sensing devices and enzymatic reactors, therefore multi-enzyme systems are particularly used for bio-catalysis and analytical applications. The use of multi-enzyme co-immobilization may be extended to other practical applications that involve in related enzymatic reactions of macromolecular substrates.

For example, Pescador et al. [29] assembled multilayer films of glucose oxidase (GO_x) and horseradish peroxidase (HRP) co-immobilized together with polyelectrolyte layers on the surface of silica microparticles. The influences of different polyelectrolyte combinations on the immobilization and functionality of the enzymes were examined for several multilayer configurations. Dong et al. [11] described a multiple enzyme, one-pot, biocatalytic system for the synthesis of UDP-Glc from low cost raw materials: maltodextrin and uridine triphosphate. Three kinds of enzymes needed for the synthesis of UDP-Glc (maltodextrin phosphorylase, glucose-1-phosphate thymidyltransferase, and pyrophosphatase) were expressed in *Escherichia coli* and then immobilized individually on amino-functionalized magnetic nanoparticles.

4. Screening for the enzyme inhibitors

One of the most useful applications of immobilized enzyme reactor in HPLC is to screen enzyme inhibitors. Studies can be performed for the high throughput screening of candidate drugs, which depend on the enzyme activity changes before and after the reactor by injecting simultaneously both the drugs and substrate.

To verify the feasibility of the model, a kind of definite pharmacological effects enzyme inhibitor can be used as the positive reference. With the determination of concentration-response relationship, and IC_{50} (concentration which reduces by half the product peak obtained at saturating conditions) and/or determining the K_i by the Lineweaver and Burk plot and inhibition ratio of substrate, the feasibility of the model can be evaluated. As reported by Ma and Chen [30], a new immobilized enzyme reactor (IMER) containing human recombinant MMP-9 enzyme was developed and characterized for the on-line screening of MMP-9 inhibitors. The MMP-9 IMER containing active unit of the enzyme ($U=0.08 \mu\text{mol}/\text{min}$) on the disk was inserted into an HPLC system connected to a UV-vis detector for on-line chromatographic screening.

5. Conclusion

As a special kind of HPLC solid phase, IMERs take part in not only separating different components and reacting products, but also in screening inhibitors as candidate drugs. As the result of minimizing cost, shorting analysis time and economically operating in a continuous mode, IMERs are widely used in many fields. In addition, immobilization methods, techniques and evaluations of enzyme immobilization have been presented. In the future, the availability of new support materials will lead to more selective IMERs. Although keeping the amount of immobilized enzyme and delaying the rate of loss enzyme activity are still to be solved, IMERs will become an effective tool to serve for pharmacological, clinical, biological and commodity studies.

Acknowledgments

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