# Analytical Application of Immobilised Enzymes

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Summary: Enzymes bound on solid supports have number of advantages over their soluble counterparts e.g. high stability, no purification, easy handling, economicity and high yield of product. Immobilised enzymes have gained increased signifinance in several areas of analytical chemistry. The use of immobilised enzymes is not only limited for the determination of their substrates but is also gaining attention for the determination of many enzyme inhibitors. This paper gives brief discription about the introduction of immobilised enzymes, methods of immobilisation and their analytical applications particularly in connection with flow injection analysis (F.I.A.)

## Introduction

### Definition

Immobilised enzymes are defined as enzymes that are confined or localised at a defined region of space with retention of their catalytic activity.

### History

The history of immobilised enzymes dates back to 1919 when Nelson and Griffin [1] observed that yeast invertase absorbed on charcoal was able to catalyse the hydrolysis of sucrose. However, their work remained unnoticed until 1953, when Grubhofer and Schleith [2] reported covafent immobilisation of several enzymes on diazotized polyaminostyrene. It was 1969 when Chibita and co workers [3] were successful for the first time in an Industrial application of an immobilised enzyme.

### Advantages of Immobilised Enzymes

Enzymes bound on solid supports have a number of advantages over their soluble counterparts:

1. High yield of product.

2. No purification.

3. Less chance of contamination of product with enzyme.

4. Easy handling, especially while dealing with toxic enzymes.

5. Highly stable toward pH and other changes.

6. Economical.

7. Repeated use

### Methods of Immobilisation

There are four principal methods for immobilisation [4].

#### 1. Carrier Binding Methods [5]

Carrier binding methods are usually further divided into three categories, differing on the mode of binding of the enzyme and carrier:

##### (i) Covalent Binding Method

In this type of immobilisation method a covalent bond is directly formed between the enzyme and the support material. Covalent linkages are strong and, therefore, immobilised preparations are generally stable. There is much less chance of enzyme leakage even i the presence of a high

concentration of substrate or i high inic strength buffer solution. But enzymes immobilised by this fashion generally lose activity upon immobilisation. High enzyme activity cannot be achieved if the immobilisation procedure is not carried out under controlled conditions.

### (ii) Ionic Binding Method

This method involves the ionic binding of enzyme protein to water-insoluble carriers containing ion-exchange resins. Immobilisation by this method can be achieved simply under mild conditions. In this case the immobilised enzyme shows relatively high activity but binding forces between enzyme and carrier are weak. therefore leakage of the enzyme from the carrier may occur by changing the ionic strength or pH of the buffer.

### (ii) Adsorption

This is a simple way of preparing an immobilised enzyme. A wide variety of solids has been used to adsorb enzymes such as activated

carbon, porous glass, alumina and ion exchange resins. Although the method is cheap and easy at the same time it also face the problem of leakage like the ionic binding method due to weak binding forces between the enzyme and the carrier.

### 2. Cross-Linking Method [6]

At present the covalent cross-linking method for immobilisation is the method of choice for many systems which will have long operational use. This method is extensively used in many research investigations. The method involves linking the enzyme with an activated matrix via a bifunctional group. Different types of support materials have been used. like Sephadex, porous glass, polyacrylamide and polystyrene. The most common choice is controlled pore glass, because of its large surface area, using glutaraldehyde as covalent coupling agent [7-9].

The method is based on the following three steps:

1. Silanization of controlled pore glass (CPG) by treatment with 3-aminopropyltriethoxysilane



Carrier
aminoalkyl CPG
(A)
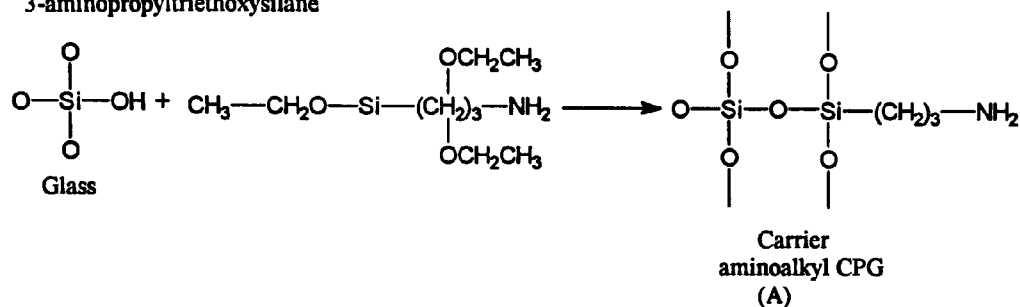
2. Preparation of an aldehyde derivative by treatment of (A) with glutaraldehyde.



Aldehyde Derivative (B)
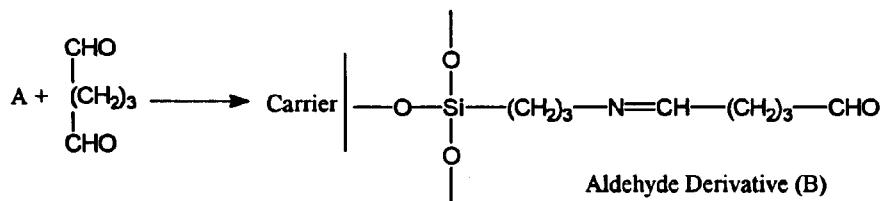
3. Coupling of enzyme by reaction of the CHO group of B and an amino group of enzyme



Immobilized Enzyme
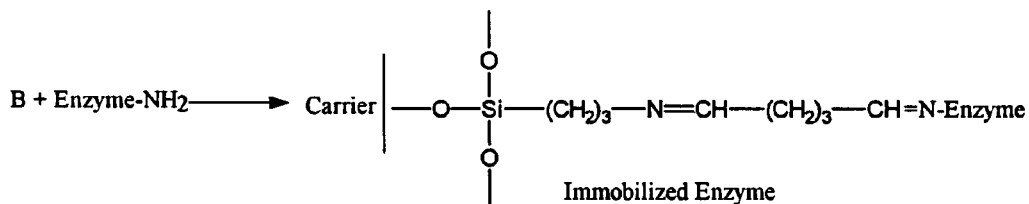
### 3. Entrapment Method [10]

In this method enzymes are entraped in polymers of synthetic or natural origin either by physical entrapment of the enzyme within the carrier i.e. a sort of gel entrapment or by encapsulation of the enzyme in a semipermeable membrane. The most popular gels are polyacrylamide, (polyvinyl alcohol) and silica. A major disadvantage of this method is its limited applicability to small molecules of substrate and product. Entrapped enzyme shows little or no activity for large molecules which cannot penetrate the matrix. Like the ionic binding method there are also chances of enzyme leakage. An outline of these methods for enzyme immobilisation is shown in Fig.(I).

### 4. Membrane Confinement Method [4]

Compared to all methods mentioned above, membrane confinement is the simplest. Membrane confinement depends on the use of a semipermeable membrane; enzyme is placed on one side of the membrane while substrate and product pass freely through the membrane.

### Analytical Applications of Immobilised Enzymes

Immobilised enzymes have been used for a long time in industry for biotechnological purposes, and in clinical, food and environmental analysis. Analytical applications of immobilised enzymes are given in many reviews [10-13] and monographs [4. 10. 14, 15]. In a wide variety of analytical applications, immobilised enzymes have been used in electrodes or in the form of enzyme reactors. A brief description of them is given below:

### Enzyme Electrodes

An enzyme electrode is an electrochemical sensor on whose surface a thin layer of enzyme is attached. The outer surface of the enzyme is exposed to the analyte solution, which is usually stirred continuously to speed up the transfer of analyte to the enzyme to get a quick response.

Enzymes are Immobilised on the solid support by means of different binding chemistries, which are determined by protein nature. Enzymes are often also bound on the electrode surface by pure adsorption, confinement by a membrane or entrapment in carbon paste [16].

The operation of the electrode is simple. The analyte is transported by diffusion to the membrane where it degraded enzymatically to form a product which subsequently can be sensed. Since the conversion of substrate to product reduces the available concentration of substrate, a concentration gradient is formed. A stage is reached where the rate of supply and rate of consumption of substrate become equal. Quatitative measurements can be made either at steady state or during the initial stages of the reaction. Enzyme electrodes have been used for analysis of a single analyte e.g. glucose alone [17] or in a mixture e.g. determination of sucrose in the presence of glucose [18].

### Immobilised Enzyme Reactors

Reactors are columns containing immobilised enzymes. Their conversion efficiency, which indicates sensitivity of analysis, depends on the reactor configuration used. The most common reactors are open tubular, single bead string and packed bed reactors. In open tubular reactors enzymes are immobilised on the inner surface of a tube made of nylon or other material. These reactors have some kinetic problems in that the amount of immobilised enzyme on the surface of a tube is very small. Thus to achieve maximum conversion of substrate to product a long reactor is required, which in turn increases dispersion. On the other hand single bead string reactors, in which a single string of glass beads with immobilised enzyme are packed in coiled glass or nylon tubing, offer back pressure. These reactors also offer limited amount of immobilised enzyme per unit reactor volume.

The packed reactors are the most popular among those mentioned above. These reactors are packed with solid porous supports containing immobilised enzymes. The efficiency of the reactor depends on several factors. A high enzyme activity can be achieved by loading a sufficient amount of enzyme on the solid support and also by chromatographic purification of the crude enzyme immobilised. This will result in more efficient reactor loading thus decreasing the size of the reactor and band broadening and consequently produce better analytical signal [19].

### Enzymes In Flow Injection Analysis

FIA is a technique which has wide applications due to its simplicity and versatility [20].

**1.Covalent binding**

**2.Ionic binding**

**CARRIER BINDING METHOD**

**CROSS-LINKING METHOD**

**1.Lattic type**
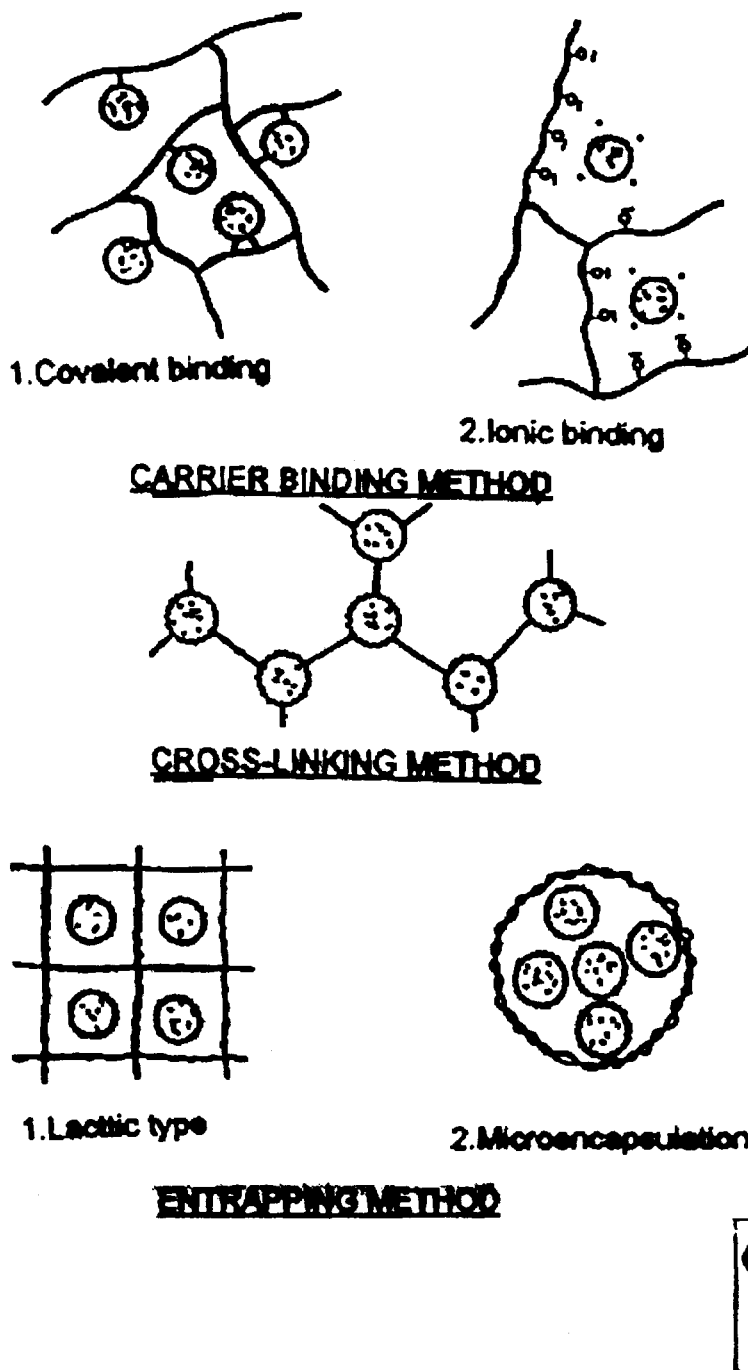
**2.Microencapsulation**

**ENTRAPPING METHOD**

Fig.1:  Schematic diagrams of different modes of enzymes immobilization.

The use of enzymes in FIA has made this technique even simpler and faster. Enzymatic methods in FIA are procedures by which a non detectable species can be converted into a detectable species. Enzymes have been used in FIA in the soluble form [21, 22] or by immobilising them on a solid support.

*Immobilised Enzymes in FIA*

Immobilised enzymes were introduced in a continuous flow (CF) system around 1974 [23]. But their application in flow injection analysis started in 1982 [24]. Since that time a variety of FIA methods

Table 1 Some analytes studied by FIA with Immobilised enzymes

| Enzyme | Analyte | Detection system | Detection limit | Ref |
|---|---|---|---|---|
| 3-Hydroxybutyrate dehydrogenase | Hydroxybutyrate | Chemiluminescence | $1 \times 10^{-5}$M | 35 |
| Luciferase | ATP | Chemiluminescence | $1 \times 10^{-12}$M | 36 |
| Glucoamylase | Starch | Amperometry | 1.0 mg $l^{-1}$ | 37 |
| Aldehyde dehydrogenase | Acetaldehyde | Spectropho-tometry | 20 ng $ml^{-1}$ | 38 |
| Fructose 5-dehydrogenase | Fructose | Amperometry | $2 \times 10^{-4}$M | 39 |
| Carbonic anhydrase | Zn | Spectrophotometry | 0.4 µg $ml^{-1}$ | 40 |
| Invertase, mutarotase, glucose oxidase | Methanol | Fluorimetry | $\geq 1.2 \times 10^{-4}$ M | 41 |

using immobilised enzymes have appeared, which shows their usefulness in many areas of application especially for the determination of enzyme substrates. Table 1 shows a few examples of such analytes.

Until 1988, of publications which appeared for the determination of substrates 30% of them dealt with glucose determination [23]. This may be due to its clinical importance, as it is most frequently determined in clinical laboratories, or it may be due to the availability and cheapness of glucose oxidase, and perhaps also due to the high stability of this enzyme after immobilisation.

There is also increasing interest in food analysis by using immobilised enzymes in FIA. Both areas (clinical and food) take advantage of high enzyme selectivity, so that enzymatic reactions can be applied to complex matrices like whole blood, serum, urine and all types of foods and drinks.

As knowledge of FIA is developing more interesting applications of immobilised enzymes appear. Starting from the determination of a single analyte by using a single immobilised column and progressing toward the determination of five or six analytes [25] in a sample is an example of advances in enzymatic methods in FIA. Analysis of more then one analyte is made possible by placing more than one column in series or parallel like determination of ethanol and aldehyde by immobilised alcohol dehydrogenase and aldehyde dehydrogenase, placed parallel to each other and determination of glucose and sucrose by immobilised invertase and mutarotase placed in seriese [26, 27]. A plug of anatyte mixture is split into subplugs before transfer to enzyme reactors.

In spite of the high selectivity of enzymes for their substrates, sometimes interference appears from real samples e.g. blood and urine, in which ascorbic acid, uric acid and $NH_3$ may reduce system applicability.

However, a system's selectivity can be increased in several ways:

1. By the application of dialyzer units or liquid chromatographic columns [28, 29].

2. By using different reactors which can eliminate interfering species. It may be enzyme reactors (e.g. use of immobilised ascorbate oxidase to eliminate interference of ascorbic acid In the determination of fructose or inorganic species like C11-diethyldimethylthiocarbamate complex immobilised on solid support for the elimination of reducing agents [30, 31].

3. By measuring the difference in response between the signal of the sample in the presence and in the absence of enzyme.

After the exploitation of the advantages of immobilised enzymes in conjunction with FIA for chemical analysis, the technique has also emerged for process analysis. Conventionally, most industrial processes are carried out off line and face problems like limited reliability, time consumption and extra labour to deal with large number of samples for analysis. FIA with immobilised enzyme reactors can give reliable determination of analytes in media originating from food and fermentation. Indeed several applications of FIA for monitoring sugars [32], cholesterol [33] and penicillin [34] by immobilised glucose dehydrogenase, cholesterol oxidase and penicillnase. respectively, have been reported.

The use of immobilised enzymes in FIA is not limited to the determination of enzyme substrates, but is also gaining attention for the determination of many enzyme inhibitors. Applications are found in environmental and agricultural problems. Table 2 gives a list of some enzyme inhibitors studied in this way.

Table 2 Some enzyme inhibitors studied by FIA with Immobilised enzyme

| Enzyme | Inhibitor | Detection method | Detection limit | Ref |
|---|---|---|---|---|
| Alkaline phosphatase | Metal ions and EDTA | Spectrophotometry | $1 \times 10^{-6}$ M | 42 |
| Acetylcholinesterase | Organophosphorus and carbamate compounds | Amperometry | 4.0 and 13.1 nmol $l^{-1}$ | 43 |
| Urease | Hg | Fluorimetry | 2 ng $ml^{-1}$ | 44 |
| Acetylcholinesterase | Paraoxon | Spectrophotometry | $4 \times 10^{-9}$ M | 45 |
| Acetylcholinesterase | Carbamates | Spectrophotometry | $8 \times 10^{-9}$ M | 46 |
| Acetylcholinesterase | Metrifonate | Spectrophotometry | $8 \times 10^{-7}$ M | 47 |
| Acetylcholinesterase | neostigmine/ galanthamine | Spectrophotometry | $1 \times 10^{-7}$ M $5 \times 10^{-7}$ | 48 |

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