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Immobilized Biosystems in Research and Industry

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Introduction

Despite more than 20 years of scientific research into the immobilization of enzymes, industrial applications are limited (Powell, 1984). However, novel developments have been achieved by extending the study of immobilization techniques to include non-enzymic proteins, cells and other molecules, as well as by improving techniques of immobilization for enzymes using new methods of activation for carriers which should increase the range of potential industrial applications. Nowadays, the knowledge obtained from the investigations of enzyme immobilization techniques has led to the immobilization of other bioactive materials such as protein A, antibodies, concanavalin A, cells, nucleic acids, etc., thereby encouraging applications in therapeutic, analytical and industrial processes (Aston and Turner, 1984; Karube, 1984; Neujahr, 1984; Powell, 1984; Akin, 1987; Nilsson, 1988).

The identification of an appropriate source material (e.g. enzyme, cell, antibody or antigen, etc.) is the first consideration before preparing an immobilized biosystem. In theory at least, the selection of a better bioactive agent is the most effective way of meeting requirements. Chemical modification, genetic engineering and immobilization are also ways to improve a biocatalyst's characteristics. Recommendations for desirable minimum requirements for characterization of an immobilized biocatalyst have been listed (Buchholz and Klein, 1987). This paper deals with some of the more recent developments in the immobilization of biosystems and their applications in therapeutic, analytical and industrial processes.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography.

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Immobilization of biosystems

The immobilization of any biosystem will depend on the type of bioactive material that is to be immobilized. It is well known that the attachment of the active agent to a polymer matrix depends on the physical relationship between support and ligand. Immobilization methods may be classified into various groups: adsorption (the catalyst may be physically adsorbed onto the polymer); entrapment (the catalyst may be entrapped inside a semi-permeable matrix or encapsulated in a polymer membrane); crosslinking (the catalyst may be crosslinked with itself or used to strengthen existing polymer–catalyst linkages); and covalent binding (the catalyst is covalently bound to the matrix). The chemistry involved in these methods has been reviewed (Powell, 1984; Kennedy, White and Melo, 1988). However, the factors that influence the choice of the method to be used for the immobilization of biosystems are generally found to be empirical. Few general rules exist, although some more obvious considerations such as requirement for a specific physical form of the immobilized biosystems may rule out certain methods. The choice of methods for the immobilization of biomaterials normally arises from a screening of the different methods available.

For certain applications, for instance processing of high molecular weight polymers or gene manipulation, immobilized biosystems may not be appropriate or even useful, whereas for other applications, such as immobilized enzymes in biosensors, immobilized concanavalin A in affinity chromatography, immobilized antibodies in immunoaffinity processes, immobilization is mandatory rather than simply more effective or cheaper.

Novel techniques for immobilization of biosystems

Although the earliest immobilized enzymes were produced by adsorption, the application of immobilized enzyme technology was limited until methods of covalent coupling with retention of enzyme activity were introduced. Initially there were very few successful methods, and researchers relied chiefly on cyanogen bromide activation of agarose and activation of cellulose derivatives such as diazo- and carboxy-cellulose. Cyanogen bromide is still used extensively (Kohn and Wilchek, 1982, 1983 a, b, 1984) but there is now a large variety of ligand coupling methods available. Recently proposed activating agents (Scouten, 1987) include the following.

SULPHONYL CHLORIDES

Since enzymes are costly and easily degraded it is desirable that the method used for immobilization be efficient and involves the use of mild conditions to enable a high yield of bound active enzyme to be obtained at neutral pH and low temperatures. It is an economic advantage if the enzyme is stabilized by immobilization. It has been found that sulphonate esters are useful reagents not only in organic chemistry but also for immobilization of affinity ligands and enzymes (Nilsson and Mosbach, 1980, 1981, 1984; Nilsson, Norrton and Mosbach, 1981). Leakage of the ligand from the matrix is one of the major

difficulties found with many methods of activating matrices. The formation of stable secondary amide bonds between hydroxylic matrices (e.g. agarose) and amine-containing ligands and proteins has been investigated (Nilsson and Mosbach, 1980, 1981; Bulow and Mosbach, 1982) by using sulphonyl chlorides, which produce sulphonates with a very good leaving group character, for the activation of the matrices. Tresylated carriers allow efficient immobilization at near neutral pH and at 4°C and are now commercially available. Tosylated supports are useful for coupling at pH > 9 and at 25°C.

CHLOROCARBONATES

Supports bearing hydroxyl groups have been activated with chlorocarbonates (*Figure 1*). *N*-Hydroxysuccinimide chlorocarbonate and 4-nitrophenyl chlorocarbonate have been found to be the most useful activating agents (Wilchek and Miron, 1982). Other chlorocarbonates, such as tri- or penta-chlorophenyl chlorocarbonate as well as 4-methylthiophenylchlorocarbonate (Wilchek, Miron and Kohn, 1984), can also be used but with lower efficiency. These reagents react with polyalcohols to give activated carbonates which, on subsequent reaction with amines, yield stable and uncharged carbamates. The extent of activation and coupling can be followed spectrophotometrically (Wilchek and Hurwitz, 1983).

1, 1'-CARBONYLDIIMIDAZOLE

Immobilization of a biospecific ligand onto an inert matrix requires initially the introduction of reactive groups of defined chemical functionality into the inert matrix, and subsequently a coupling reaction with the appropriate ligand, preferably under mildly basic conditions. Until recently, most procedures for immobilizing ligands onto polysaccharides and other soft hydrophilic gels have involved the use of cyanogen bromide activation procedures (Wilchek and Hurwitz, 1983). However, the potential for additional non-specific ion-exchange interactions and leakage of the ligand from the matrix due to the instability of the *N*-substituted isourea linkage constitute the main difficulties inherent in all cyanogen bromide-based systems. It has been observed that imidazole greatly facilitates the phosgene activation of beaded agarose and subsequent immobilization of protein ligands in high yield under mild conditions with excellent stability of the bonds between the ligand and the matrix. Additional studies (Bathell *et al.*, 1979, 1981a, b; Hearn *et al.*, 1983; Hearn 1986) have revealed that carbonyldiimidazole, a product of the reaction between phosgene and imidazole, and related heterocylic carbonylating reagents can convert free hydroxyl groups into imidazolyl-carbamate groups which, on reaction with *N*-nucleophiles, result in the formation of *N*-alkyl carbamates. An important advantage of the 1,1'-carbonyldiimidazole method compared to the standard cyanogen bromide procedure is the absence of any additional charged groups introduced by the functional groups of the activation reagent during either the activation or ligand coupling steps. The activation of gel matrices by 1,1'-carbonyldiimidazole is illustrated in *Figure 2*.

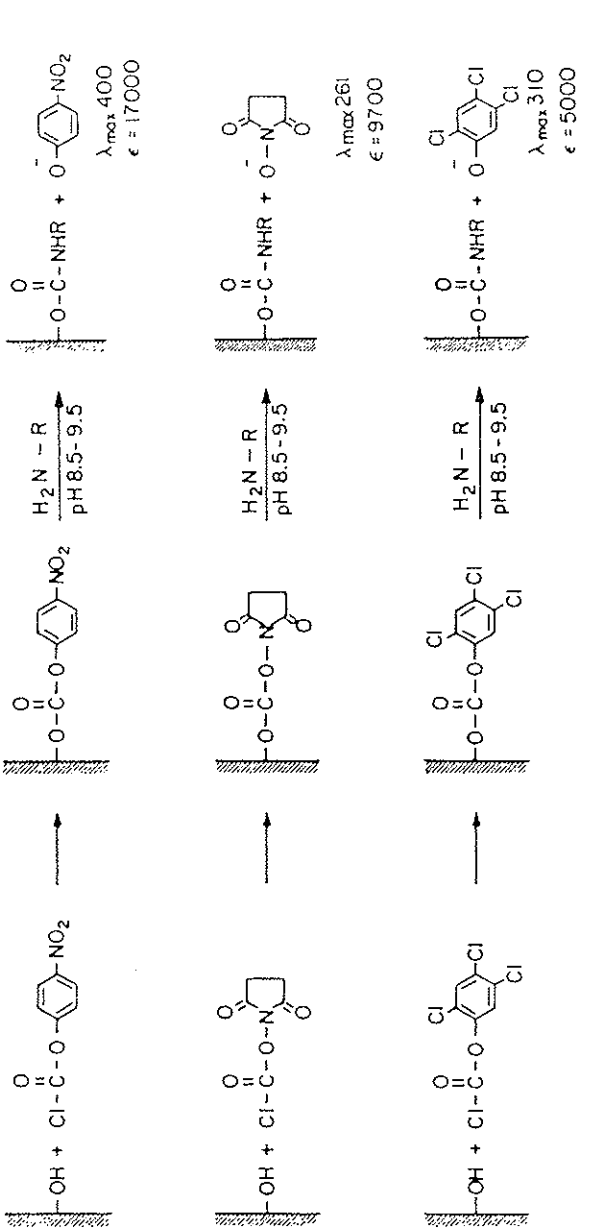

Figure 1. Schematic representation of the preparation, determination and coupling of amino-containing ligands to carriers containing active carbonate (from Miron and Wilcek, 1987).

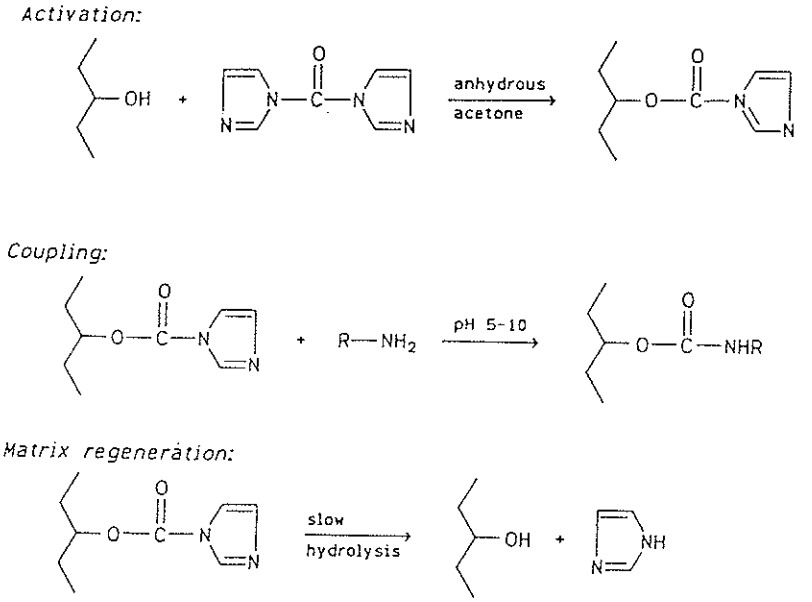

Figure 2. The activation of gel matrices by 1,1'-carbonyldiimidazole and the subsequent coupling of the ligand to the support or regeneration to the parent matrix (from Hearn, 1987).

TRANSITION METAL ACTIVATION

A number of methods of immobilization of biological molecules has been reviewed (Kennedy and Cabral, 1983). However, no one method is perfect for all molecules or purposes. One technique in which matrix derivatization after preparation can be avoided and instantaneous coupling can be achieved under simple conditions is the metal-link chelation process. This procedure is based on the chelation properties of transition metals, namely of titanium and zirconium which seem particularly attractive on account of the non-toxicity of their oxides. Although this method was initially developed using titanium (IV) chloride as metal activator, other transition metal salts can also be used as support activators with subsequent derivatization of the metal-activated support (Cabral *et al.*, 1983).

Immobilization techniques for affinity chromatography

Using 4-nitrophenyl carbonate-Sepharose or *N*-hydroxysuccinimide carbonate-Sepharose, a number of proteins and other ligands have been coupled for their use in affinity chromatography studies, including concanavalin A for glycopeptides and proteins, avidin for isolation of biotin-containing peptides and proteins, different antibodies for isolation of their corresponding antigens, etc. Furthermore, carbohydrate derivatives such as 4-aminophenyl-β-D-glucopyranoside, 4-aminophenyl-β-D-lactopyranoside, etc. have been coupled for the isolation of the corresponding lectins.

In only a few instances have immobilized enzymes been used as reagents for low molecular weight biomolecules. This limited use is probably due to the low capacity and high cost of such systems. With the introduction of high-performance techniques using small volume columns, the analytical potential of such systems may be realized. The reversed affinity high performance liquid chromatography (HPLC), which utilizes immobilized enzyme, is a potential system for the purification of complex isomeric or racemic mixtures. Furthermore, it could provide a method of detection and purification of new chemotherapeutic agents interacting with enzymes. Enzyme-catalysed reactions are slowed down when used in fluid media at subzero temperatures (Douzou, 1977). Under these conditions, enzyme–substrate complexes can be stabilized, permitting their purification by liquid gel filtration (Fink, 1973; Hastings *et al.*, 1973). In order to maintain a fluid medium, antifreeze (usually an organic solvent) must be added to the system under investigation. Two principal applications of subzero temperature separations have been described (Balny and Douzou, 1987): the first concerns the investigation of non-covalent forces involved in protein–protein or protein–ligand interactions, one protein or one ligand being covalently bound to an insoluble carrier, whereas the second covers the improvements in protein fractionation procedures (*Figure 3*).

Immobilization of cells

The idea of using immobilized cells as a research tool was conceived by Updike (Updike, Harris and Shrago, 1969) who used immobilized micro-organisms in a flowing stream configuration for biochemical studies. Immobilized cells have been used to elucidate metabolic reaction pathways (Koshcheenko, Arinbasarova and Skryabin, 1980). *Penicillium urticae* entrapped in polyacrylamide facilitates the isolation of a new intermediate in the biosynthesis of penicillins (Sekigushi, Gaucher and Yamada, 1979), patulin (Deo and Gaucher, 1983),

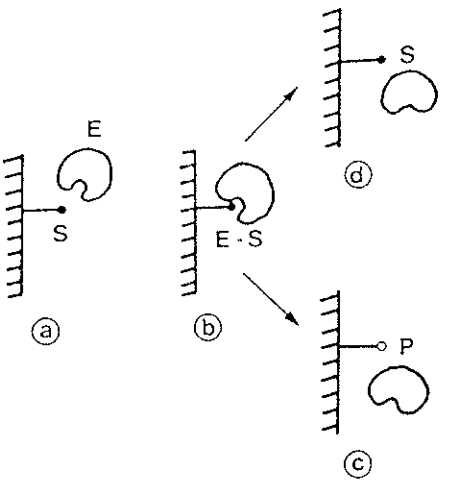

Figure 3. General scheme for affinity chromatography through a 'productive' E–S compound (from Balny and Douzou, 1987).

etc. Combinations of immobilized cells and transducers, such as oxygen electrodes and thermistors, are useful devices for analytical purposes and might also constitute convenient tools in metabolic studies (Mattiasson, Larsson and Mosbach, 1977; Danielsson, Mattiasson and Mosbach, 1981). In addition to microbial and yeast cells, immobilization of plant and animal cells has grown in importance. As already stated, there is no best support, as requirements for particular applications differ. The choice of the proper support will be governed by factors such as cost, mechanical stability, ease of preparation, biocompatibility and resistance against biodegradation. The entrapment technique for organelles and cells is probably a good first choice. However, adsorption as well as other immobilization techniques may be useful in a number of cases.

Microbial enzymes can be excreted from the cell into the broth (extracellular) or retained in the cells during cultivation (intracellular). In order to utilize intracellular enzymes in immobilized form, it is necessary to extract them from the microbial cells. The extracted enzymes are generally unstable and not suitable for use as immobilized enzymes. However, in order to utilize the stabilizing effects of the cell environment, and to avoid the extraction

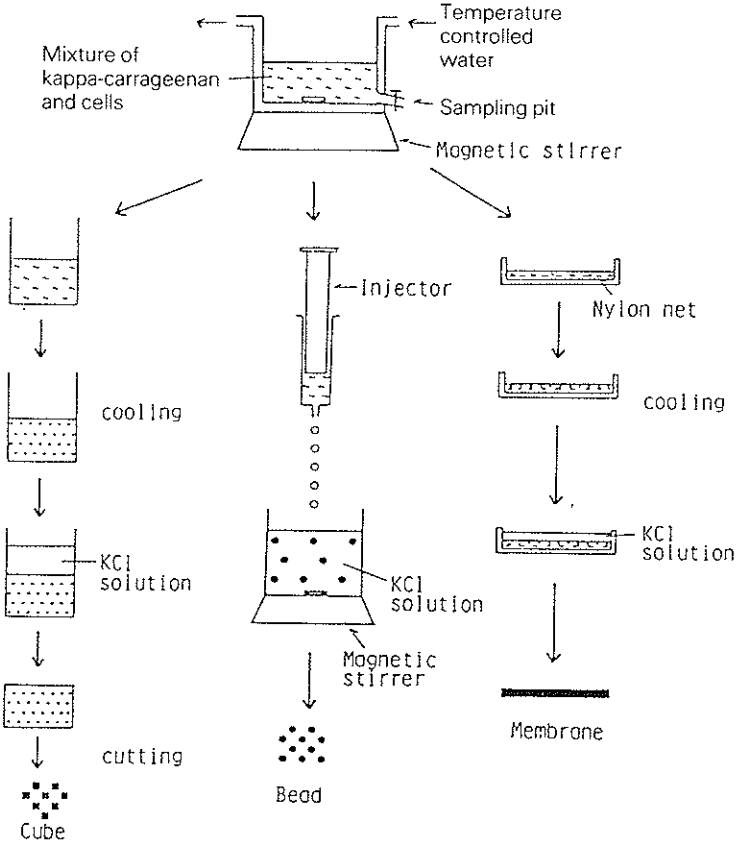

Figure 4. Schematic procedures for immobilization of cells using κ-carrageenan (from Chibata *et al.*, 1987).

of enzymes from the cells and utilize their multi-enzyme systems, direct immobilization of whole cells has been attempted (Bucke, 1987; Chibata *et al.*, 1987; Skryabin and Koshcheenko, 1987; Fukushima *et al.*, 1988). There are advantages and disadvantages in immobilization methods for cells (Akin, 1987; Kennedy, White and Melo, 1988) and, as yet, no universal method has been developed. Therefore, the choice of suitable methods and conditions for immobilization of cells has to be investigated in each case. Advantages of the immobilization method using encapsulation in K-carrageenan are that the immobilization can be performed under mild conditions without the use of reagents that destroy enzyme activity in cells (*Figure 4*).

Applications of immobilized biosystems

The applications of biosystems can be divided into three categories, namely therapeutic uses, analytical uses and industrial uses.

THERAPEUTIC USES

Discussion of the therapeutic uses of immobilized biosystems has been restricted traditionally to the replacement of an enzyme that cannot be synthesized in the body, either as a result of an inborn error of metabolism or organ failure, by a sample of the required enzyme from an alternative source (enzyme replacement); or the use of a non-natural enzyme to remove some unwanted material from the body in the treatment of a disease (enzyme therapy). However, the discussion should be broadened to include the use of immobilized antigens, antibodies, antibiotics, drugs, etc. Biomedical applications of immobilized biosystems are still in their experimental stage, as many necessary prerequisites—absence of toxicity, absence of haemolysis and allergenicity, chemical stability *in vivo* and immunological reactions—must be achieved very precisely before application can be realized.

Enzyme replacement

A particular enzyme, which as a result of genetic or other diseases is not synthesized by the cell, can be replaced in order to reduce the build-up of toxic products in the body. Phenylketonuria is an example of a disease in which the lack of phenylalanine-4-monoxygenase (EC 1.14.16.1) leads to the formation of phenylpyruvic acid (toxic) instead of 4-hydroxyphenylpyruvic acid. In order to control such diseases the use of immobilized enzymes (in encapsulated form) has been attempted as an alternative to injecting the required soluble enzyme, since the immobilized enzyme reduces the immunological responses to the enzyme while controlled release of the enzyme from an unreactive encapsulated form can allow constant low dosages to be available without frequent application of the enzyme. Immobilized biocatalysts can also be used in extracorporeal circulation to control and consequently eliminate the build-up of toxic compounds in the body fluids.

Enzyme therapy

The difference between enzyme replacement and enzyme therapy is that in the latter a non-natural enzyme is used to change the normal environment in the body in order to control the diseased state. In the treatment of leukaemia, for example, asparagine is removed from the blood by the enzyme L-asparaginase (EC 3.5.1.1). The immobilized enzyme is administered to the body in a biodegradable form. This therapeutic application can be extended to control other kinds of diseases using immobilized antibiotics, antibodies, etc. (Cera *et al.*, 1988; Dumitriu *et al.*, 1987).

ANALYTICAL USES

The role of immobilized biosystems, in particular immobilized enzymes in analytical systems, is one of the most interesting and promising areas at present under investigation.

Enzyme electrodes

The determination of enzyme activity is of great importance in clinical studies and in the area of biochemistry. Enzymes are valuable and extremely important aids in the diagnosis of a vast variety of disorders. Some have been used for the determination of the concentration of their substrates or products which exist in serum or plasma. The concept of the soluble enzyme electrode was first introduced in 1962 (Guilbaut, 1984). However, the first working enzyme electrode, using D-glucose oxidase (EC 1.1.3.4) immobilized in a polyacrylamide gel and placed around an oxygen electrode held by a piece of cellulose acetate, was for the determination of the concentration of D-glucose in biological solutions and in tissues, and was reported by Updike and Hicks (1967). The reaction catalysed by the immobilized glucose oxidase is:

$$\beta\text{-D-glucose} + O_2 \rightarrow \text{gluconic acid} + H_2O_2$$

the consumption of oxygen from the solution, which is directly related to the concentration of D-glucose present, being measured by the oxygen electrode. Selection of a suitable gel matrix is of great importance to improve the stability of the enzyme while allowing free passage of substrate to the enzyme (*Figure 5*).

Immunoaffinity

Another application of biological probes is the construction of sensor probes utilizing bound antibodies or antigens. Immobilized creatine kinase M (EC 2.7.3.2) for detection of cardiospecific CK-MB isoenzymes is an example of a system that has been used successfully. The linkage of an antibody or antigen to a matrix followed by enzymatic determination of the immunocomplex formed is called enzyme-linked immunosorbent assay (ELISA) (Louborg, 1984; Johnstone and Thorpe, 1987) (*see Figure 6*). The attachment of

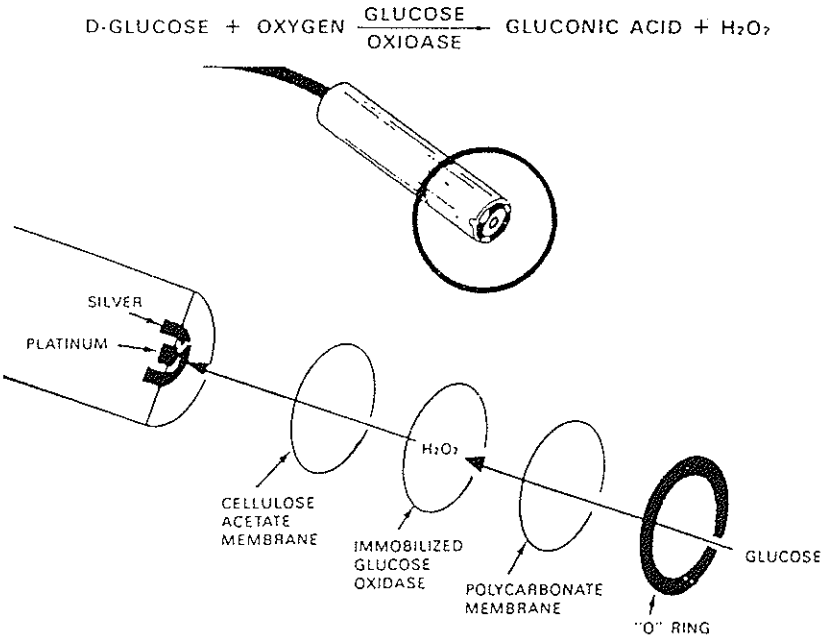

Figure 5. Diagram of a glucose sensor probe.

monoclonal antibodies to insoluble carriers yielding carrier–monoclonal antibody conjugates has been used for the preparation of highly active immobilized enzymes (Solomen *et al.*, 1987). *Figure 7* shows the covalent coupling of a monoclonal antibody onto Eupergit C (oxirane C, Röhm-Pharma) via the oxirane active groups of the polymer (Hannibal-Friedrich, Chun and Sernetz, 1980).

Affinity chromatography

Affinity chromatography has become, in the past 15 years, a particularly powerful tool for the purification of biologically active molecules. With well-designed affinity chromatography systems very specific purification can be achieved in high yields in a single step during the isolation of a specific protein or polynucleotide component. The technique exploits the biological specificity of those substances which can form stable, reversible complexes with matrix-bound biospecific ligands. Until recently, most procedures for immobilizing ligands onto polysaccharide and other soft hydrophilic gels have been based on cyanogen bromide activation procedures (Wilchek, Miron and Kohn, 1984). The problems of unwanted ion-exchange interactions and the leakage of the ligand from the matrix (described above) have led to investigation of a number of alternative methods of activation. This has led to the development of the oxirane approach (Sundberg and Porath, 1974), and the use of *s*-triazines (Lang, Suckling and Wood, 1977), cyanate esters (Kohn and Wilchek, 1983a), and sulphonyl chlorides (Nilsson and Mosbach, 1981,

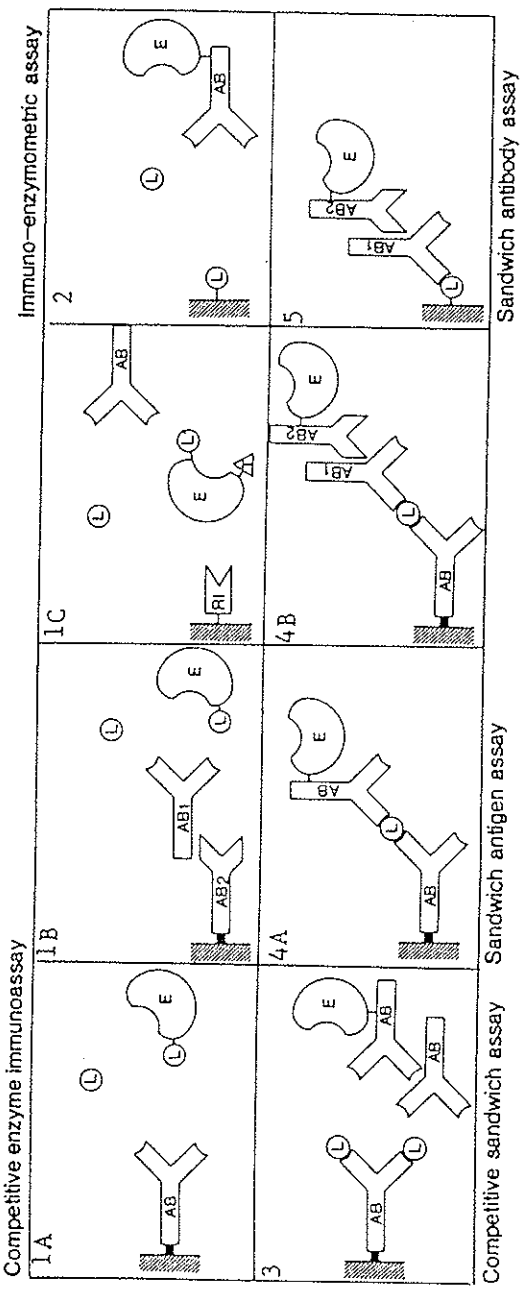

Figure 6. Principals of heterogeneous enzyme immunoassays (ELISA). L, ligand; E–L, enzyme-labelled ligand; E–L–T, tagged enzyme–ligand conjugate; E–AB, E–AB_2, enzyme-labelled antibodies; AB, AB_1, antibodies against ligand under test (1–4) or antibody to be determined (5); AB_2, antibody directed against the immunoglobulin of the species that has been immunized with the antigen to be determined (1–4) or against the antibody to be detected (5); RI, insolubilized receptor.

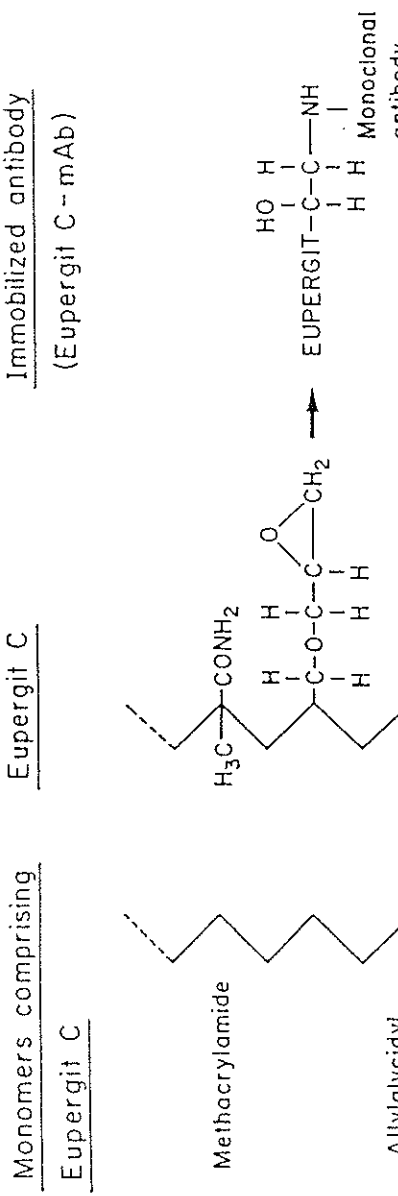

Figure 7. Scheme describing the preparation of the Eupergit C–monoclonal antibody conjugate (from Solomon *et al.*, 1987).

1984) as activating reagents. The use of carbodiimide reagents results in the important advantage (compared to the standard cyanogen bromide procedure) that no additional charged groups are introduced into the product as assessed over the pH range normally used in affinity chromatography (Bethell *et al.*, 1979). A number of applications of affinity chromatography for the purification of biological active molecules have been reviewed (Nishikawa, 1983; Hearn, 1987).

INDUSTRIAL USES

A comparison of the economics of the immobilized and soluble biosystem processes has shown that the use of an immobilized process can halve the cost of production in many cases. It is known that there are only a few successful industrial applications using immobilized enzymes, such as D-glucose isomerase, i.e. xylose isomerase (EC 5.3.1.5), L-aminoacylase (EC 3.5.1.14), etc. Recently, there has been a number of other proposals for the use of immobilized enzymes, most of which have been developed only on the laboratory or occasionally pilot-plant scale. However, several immobilized microbial cell methods have been proposed for the production of ethanol, methane, acetic acid, lactic acid, amino acids, antibodies, and steroid transformation.

The development of a high-fructose corn syrup process is the story of how process engineering bridged the gap between the enzyme manufacturers and the corn syrup industry in the years around 1974. The soluble enzymes α-amylase (EC 3.2.1.1) and amyloglucosidase (glycan-1,4-α-glucosidase, EC 3.2.1.3) are used for the production of glucose syrups and crystalline dextrose from starch. These enzymes are employed as soluble products because of the molecular weight and structure of the substrate—starch. The conversion of dextrose (readily derived from starch) to fructose and high-fructose products was not economically feasible until the necessary enzyme became available in an immobilized and stabilized form bound to an insoluble carrier and capable of re-use (Poulsen, 1984). The past 10 years have shown a tremendous increase in the production of D-fructose from D-glucose, with the enzyme cost of the isomerization step being reduced almost tenfold as a result of immobilization. Hydrolysed lactose is sweeter and more soluble than lactose. Immobilized lactase (EC 3.2.1.108) systems have been designed and operated under industrial conditions to produce lactose hydrolysates from lactic acid wheys (Baret, 1987).

Immobilized cells (e.g. yeasts, bacteria) have been used for continuous ethanol fermentation processes. Various reactor designs, including packed bed, moving bed and suspended bed, have been constructed for ethanol fermentation using immobilized yeast cells.

Xanthine oxidases (EC 1.1.3.22) are able to oxidize a variety of azaheterocyclic compounds (*Figure 8*) other than xanthine itself. Immobilized milk xanthine oxidase has been prepared for its application in organic synthesis (Tramper, 1987).

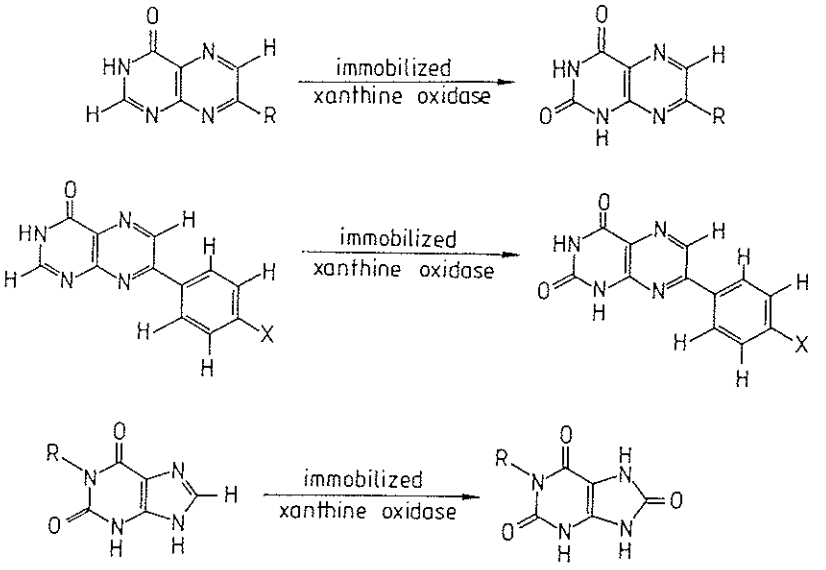

Figure 8. (a) Oxidation of 7-(4-x-phenyl) pteridin-4-ones to 7-(4-x-phenyl) limazines by immobilized milk xanthine oxidase. (b) Oxidation of 7-alkylpteridin-4-ones to 7-alkyllumazines by immobilized milk xanthine oxidase. (c) Oxidation of xanthine (R = H) and 1-methyl xanthine (R = CH_3) to uric acid and 1-methyluric acid by immobilized milk xanthine oxidase (from Tramper, 1987).

Future trends

After this brief description of how immobilization can be brought about, it has to be said that many of the problems associated with immobilized biosystem technologies are being solved in a number of diverse and interesting ways. The indications are that immobilization techniques will be extended rapidly for use in industry, analytical and diagnostic systems, and the main impact of immobilization is still to come.

Acknowledgement

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