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Immobilization of Enzymes

A Literature Survey

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Summary

The term "immobilized enzymes" refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously.". Besides the application in industrial processes, the immobilization techniques are the basis for making a number of biotechnological products with applications in diagnostics, bioaffinity chromatography, and biosensors. Initially, only immobilized single enzymes were used, but the 1970s saw the development of more complex systems—including two-enzyme reactions with co-factor regeneration and living cells. The major components of an immobilized enzyme system are the enzyme, the matrix, and the mode of attachment. The enzymes can be attached to the support by interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds. The covalent reactions commonly employed give rise to binding through amide, ether, thio-ether, or carbamate bonds. As a consequence of enzyme immobilization, some properties such as catalytic activity or thermal stability become altered. These effects have been demonstrated and exploited. The concept of stabilization has been an important driving force for immobilizing enzymes. True stabilization at the molecular level has been demonstrated (e.g., proteins immobilized through multipoint covalent binding).

Key Words: Bioaffinity chromatography; biosensors; enzyme stabilization; immobilization methods; immobilized enzymes.

1. Background

Enzymes are biological catalysts that promote the transformation of chemical species in living systems. These molecules, consisting of thousands of atoms in precise arrangements, are able to catalyze the multitude of different chemical

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reactions occurring in biological cells. Their role in biological processes and in health and disease has been extensively investigated. They have also been a key component in many ancient human activities, especially food processing, well before their nature or function was known ***(1)***.

Enzymes have the ability to catalyze reactions under very mild conditions with a very high degree of substrate specificity, thus decreasing the formation of by-products. Among the reactions catalyzed are a number of very complex chemical transformations between biological macromolecules, which are not accessible to ordinary methods of organic chemistry. This makes them very interesting for biotechnological use. At the beginning of the 20th century, enzymes were shown to be responsible for fermentation processes and their structure and chemical composition started to come under scrutiny ***(2)***. The resulting knowledge led to widespread technological use of biological catalysts in a variety of other fields such as the textile, pharmaceutical, and chemical industries. However, most enzymes are relatively unstable, their costs of isolation are still high, and it is technically very difficult to recover the active enzyme, when used in solution, from the reaction mixture after use.

Enzymes can catalyze reactions in different states: as individual molecules in solution, in aggregates with other entities, and as attached to surfaces. The attached—or "immobilized"—state has been of particular interest to those wishing to exploit enzymes for technical purposes. The term "immobilized enzymes" refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" ***(3)***. The introduction of immobilized catalysts has, in some cases, greatly improved both the technical performance of the industrial processes and their economy (**Table 1**).

The first industrial use of immobilized enzymes was reported in 1967 by Chibata and co-workers, who developed the immobilization of *Aspergillus oryzae* aminoacylase for the resolution of synthetic racemic D-L amino acids ***(4)***. Other major applications of immobilized enzymes are the industrial production of sugars, amino acids, and pharmaceuticals ***(5)*** (**Table 2**). In some industrial processes, whole microbial cells containing the desired enzyme are immobilized and used as catalysts ***(6)***.

Aside from the application in industrial processes, the immobilization techniques are the basis for making a number of biotechnological products with applications in diagnostics, bioaffinity chromatography, and biosensors ***(7,8)***. Therapeutic applications are also foreseen, such as the use of enzymes in extra–corporeal shunts ***(9)***.

In the past three or four decades, immobilization technology has developed rapidly and has increasingly become a matter of rational design; but there is still the need for further development ***(10)***. Extension of the use of immobilized enzymes to other practical processes will require new methodologies and a better understanding of current techniques.

Table 1
Technological Properties of Immobilized Enzyme Systems *(3)*

Advantages	*Disadvantages*
Catalyst reuse	Loss or reduction in activity
Easier reactor operation	Diffusional limitation
Easier product separation	Additional cost
Wider choice of reactor	

Table 2
Major Products Obtained Using Immobilized Enzymes *(3)*

Enzyme	Product
Glucose isomerase	High-fructose corn syrup
Amino acid acylase	Amino acid production
Penicillin acylase	Semi-synthetic penicillins
Nitrile hydratase	Acrylamide
β-Galactosidase	Hydrolyzed lactose (whey)

2. History of Enzyme Immobilization

It is possible to visualize three steps in the development of immobilized biotatalysts (**Table 3**). In the first step, at the beginning of the 19th century, immobilized microorganisms were being employed industrially on an empirical basis. This was the case with both the microbial production of vinegar (by letting alcohol-containing solution trickle over wood shavings overgrown with bacteria) and the development of the trickling filter—or percolating process—for waste water clarification ***(11)***.

The modern history of enzyme immobilization goes back to the late 1940s but much of the early work was largely ignored by biochemists because it was primarily published in journals of other disciplines ***(12)***. The basis of the present technologies was developed in the 1960s and there was an explosive increase in publications. ***(4)***. In the second step, only immobilized single enzymes were used but by the 1970s more complex systems, including two-enzyme reactions with co-factor regeneration and living cells, were developed ***(13)***. As an example of the latter we can mention the production L-aminoacids from α-keto acids by stereoselective reductive amination with L-aminoacid dehydrogenase. The process involves the consume of nicotinamide adenine dinucleotide (NADH) and regeneration of the coenzyme by coupling the amination with the enzymatic oxidation of formic acid to carbon dioxide with concomitant reduction of NAD^+ to NADH, in the reaction catalyzed by the second enzyme, formate dehydrogenase.

Table 3
Steps in the Development of Immobilized Enzymes *(11)*

Step	Date	Use
First	1815	Empirical use in processes such as acetic acid and waste water treatment.
Second	1960s	Single enzyme immobilization: production of L-aminoacids, isomerization of glucose.
Third	1985–1995	Multiple-enzyme immobilization including co-factor regeneration and cell immobilization. Example: production of L-aminoacids from keto-acids in membrane reactors.

The major components of an immobilized enzyme system are the enzyme, the matrix, and the mode of attachment of the enzyme to the matrix. The terms solid-phase support, carrier, and matrix are used synonymously.

3. Choice of Supports

The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. Ideal support properties include physical resistance to compression, hydrophilicity, inertness toward enzymes ease of derivatization, biocompatibility, resistance to microbial attack, and availability at low cost ***(12–14)***.

Supports can be classified as inorganic and organic according to their chemical composition (**Table 4**). The organic supports can be subdivided into natural and synthetic polymers ***(15)***.

The physical characteristics of the matrices (such as mean particle diameter, swelling behavior, mechanical strength, and compression behavior) will be of major importance for the performance of the immobilized systems and will determine the type of reactor used under technical conditions (i.e., stirred tank, fluidized, fixed beds). In particular, pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Nonporous supports show few diffusional limitations but have a low loading capacity. Therefore, porous supports are generally preferred because the high surface area allows for a higher enzyme loading and the immobilized enzyme receieves greater protection from the environment. Porous supports should have a controlled pore distribution in order to optimize capacity and flow properties. In spite of the many advantages of inorganic carriers (e.g., high stability against physical, chemical, and microbial degradation), most of the industrial applications are performed with organic matrices. The hydrophilic character is one of the most important factors determining the level of activity of an immobilized enzyme ***(16)***.

An excellent matrix that has been extensively used is agarose. In addition to its high porosity, which leads to a high capacity for proteins, some other advantages of using agarose as a matrix are hydrophilic character, ease of derivatization, ab-

Table 4
Classification of Supports

Organic
Natural polymers • Polysaccharides: cellulose, dextrans, agar, agarose, chitin, alginate • Proteins: collagen, albumin • Carbon Synthetic polymers • Polystyrene • Other polymers: polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl, and allyl-polymers
Inorganic
Natural minerals: bentonite, silica Processed materials: glass (nonporous and controlled pore), metals, controlled pore metal oxides

sence of charged groups (which prevents nonspecific adsorption of substrate and products), and commercial availability. However, an important limitation in the use of agarose and other porous supports is the high cost. This problem can be circumvented by employing reversible methods that allow matrix regeneration and re-use.

The enzymes can be attached to the support via interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds. One way of classifying the various approaches to immobilizing enzymes is in two broad categories: irreversible and reversible methods ***(17)***. The strength of the binding is usually inversely related to the ease with which it can be reversed. These two conflicting objectives—stability and reversibility—are difficult to fulfill simultaneously. The traditional approach has been to make the bond as strong as possible and sacrifice reversibility.

4. Methods of Irreversible Enzyme Immobilization

The concept of irreversible immobilization means that once the biocatalyst is attached to the support it cannot be detached without destroying either the biological activity of the enzyme or the support. The most common procedures of irreversible enzyme immobilization are covalent coupling, entrapment or micro-encapsulation, and cross-linking (*see* **Fig. 1**).

4.1. Formation of Covalent Bonds

Immobilization of proteins by methods based on the formation of covalent bonds are among the most widely used. An advantage of these methods is that, because of the stable nature of the bonds formed between enzyme and matrix, the enzyme is not released into the solution upon use. However, in order to achieve

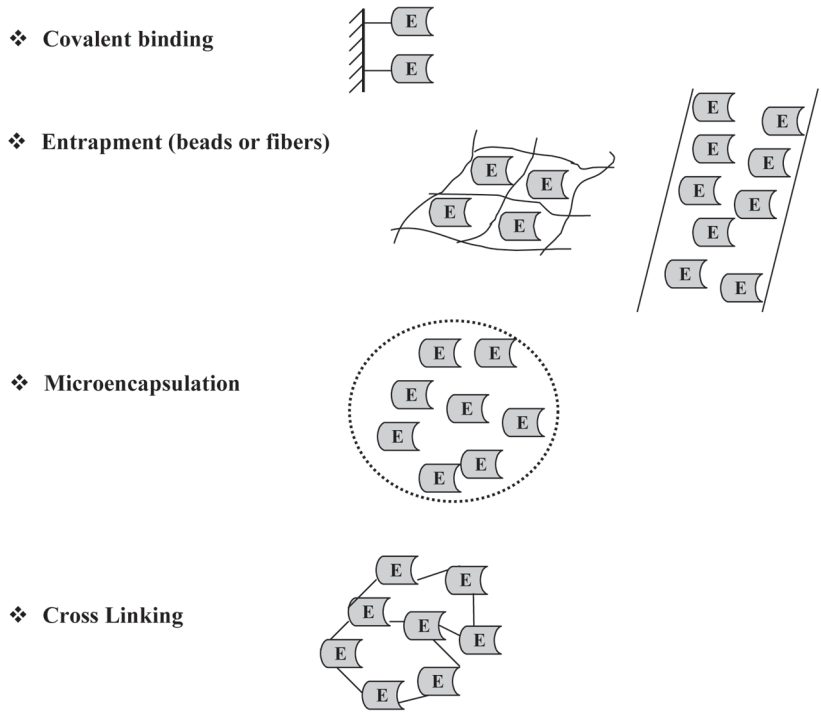

Fig. 1. Approaches to enzyme immobilization, irreversible methods.

high levels of bound activity, the amino acid residues essential for catalytic activity must not be involved in the covalent linkage to the support; this may prove a difficult requirement to fulfill in some cases. A simple procedure that sometimes improves the activity yield is to carry out the coupling reaction in the presence of substrate analogs ***(18)***. Covalent methods for immobilization are employed when there is a strict requirement for the absence of the enzyme in the product.

A wide variety of reactions have been developed depending on the functional groups available on the matrix ***(19)***. Coupling methods in general can be divided in two main classes: (1) activation of the matrix by addition of a reactive function to a polymer and (2) modification of the polymer backbone to produce an activated group (**Table 5**). The activation processes are generally designed to generate electrophilic groups on the support which, in the coupling step, react with the strong nucleophiles on the proteins. The basic principles controlling the course of covalent coupling to the matrices are analogous to those used for the chemical modification of proteins. The most frequently used reactions involve the following side chains of the amino acids: lysine (ε-amino group), cysteine (thiol group), and aspartic and glutamic acids (carboxylic group).

Table 5A
Covalent Coupling Methods of Enzymes: Activation of Matrix Hydroxyl Functions

Activation method	Group that reacts (with activated matrix)	References
Tresyl chloride, sulfonyl chloride	Thiol, amines	***22***
Cyanogen bromide	Amine	***23***
Bisoxiranes (epoxides)	Thiol, amine	***24***
Epichlorohydrin	Thiol, amine	***24***
Glutaraaldehyde	Amine	***24***
Glycidol-Glyoxyl	Amine	***25***
N-Hydroxy-succinimidyl	Amine	***26,27***

There are many commercially available supports for immobilization, the best choice in each case requires the consideration of some relevant properties of the catalyst and the intended use. However, it is usually necessary to try more than one approach and then adapt a method to the specific circumstances ***(20,21)***.

The covalent reactions commonly employed give rise to enzymes linked to the support through either amide, ether, thio-ether, or carbamate bonds. Therefore, the enzyme is strongly bound to the matrix and, in many cases, it is also stabilized, which will be discussed later in **Subheading 6**. However, because of the covalent nature of the bond, the matrix has to be discarded together with the enzyme once the enzymatic activity decays. The benefit of obtaining a leak-proof binding between enzyme and matrix resulting from these reactions is partially offset by the cost, in terms of generally low yield of immobilized activity and by the nonreversible character of this binding. Enzymes attached covalently by disulfide bonds to solid supports represent one way to avoid this problem and will be described in Chapter 17.

4.2. Entrapment

The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through but retains the enzyme ***(35)***. This method differs from the coupling methods described above, in that the enzyme is not bound to the matrix or membrane. There are different approaches to entrapping enzymes such as gel ***(36)*** or fiber entrapping ***(37)*** and micro-encapsulation ***(38)***. The practical use of these methods is limited by mass transfer limitations through membranes or gels.

5. Methods of Reversible Immobilization

Because of the type of the enzyme-support binding, reversibly immobilized enzymes can be detached from the support under gentle conditions (*see* **Fig. 2**). The use of reversible methods for enzyme immobilization is highly attractive, mostly for economic reasons because when the enzymatic activity decays the support can be regenerated and re-loaded with fresh enzyme. Indeed, the cost of the

Table 5B
Modification of the Polymer Backbone to Produce an Activated Group

Polymer	Group that reacts	Reagent	Activated group produced	Group that reacts (with activated matrix)	Refs.
Cellulose agarose	Diol	Periodate	Aldehyde	Amine	***28***
Polyacrylamide	Amide	Hydrazine	Hydrazide	Amine	***29***
Polyacrylamide	Amide	Acid pH	Carboxylic acid	Amine	***29***
Polyester	Ester	Acid pH	Carboxylic acid + alcohol	Amine	***30***
Polyethylene	CH_2	Conc. Nitric acid	Carboxylic acid	Amine	***31***
Polystyrene		Conc. Nitric acid	Nitrated aromatic ring	Histidine, Tyrosine	***32,33***
Nylon	Amide	Hydrazine	Hydrazide	Amine	***34***

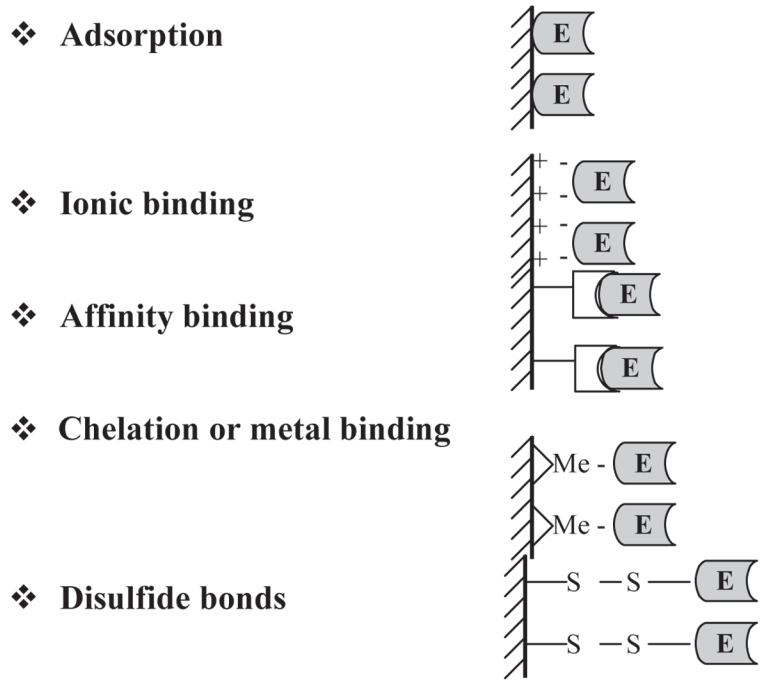

Fig. 2. Approaches to enzyme immobilization, reversible methods.

support is often a primary factor in the overall cost of immobilized catalyst. The reversible immobilization of enzymes is particularly important for immobilizing labile enzymes and for applications in bioanalytical systems ***(17)***.

5.1. Adsorption (Noncovalent Interactions)

5.1.1. Nonspecific Adsorption

The simplest immobilization method is nonspecific adsorption, which is mainly based on physical adsorption or ionic binding ***(39,40)***. In physical adsorption the enzymes are attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions; whereas in ionic bonding the enzymes are bound through salt linkages. The nature of the forces involved in noncovalent immobilization results in a process can be reversed by changing the conditions that influence the strength of the interaction (e.g., pH, ionic strength, temperature, or polarity of the solvent). Immobilization by adsorption is a mild, easy to perform process, and usually preserves the catalytic activity of the enzyme. Such methods are therefore economically attractive, but may suffer from problems such as enzyme leakage from matrix when the interactions are relatively weak.

5.1.2. Ionic Binding

An obvious approach to the reversible immobilization of enzymes is to base the protein–ligand interactions on principles used in chromatography. For example, one of the first applications of chromatographic principles in the reversible immobilization of enzymes was the use of ion-exchangers ***(4,41,42)***. The method is simple and reversible but, in general, it is difficult to find conditions under which the enzyme remains both strongly bound and fully active. More recently, the use of immobilized polymeric-ionic ligands has allowed for modulation of protein–matrix interactions and has thus optimized the properties of the derivative. A number of patents have been filed on the use of polyethyleneimine to bind a rich variety of enzymes and whole cells ***(43)***.

However, problems may arise from the use of a highly charged support when the substrates or products themselves are charged; the kinetics are distorted as a result of partition or diffusion phenomena. Therefore, enzyme properties, such as pH optimum or pH stability, may change ***(44,45)***. Although this could pose a problem it could also be useful to shift the optimal conditions of a certain enzyme towards more alkaline or acidic conditions, depending on the application ***(46)***.

5.1.3. Hydrophobic Adsorption

Another approach is the use of hydrophobic interactions. In this method, it is not the formation of chemical bonds but rather an entropically driven interaction that takes place. Hydrophobic adsorption has been used as a chromatographic principle for more than three decades. It relies on well-known experimental variables such as pH, salt concentration, and temperature ***(47)***. The strength of interaction relies on both the hydrophobicity of the adsorbent and the protein. The hydrophobicity of the adsorbent can be regulated by the degree of substitution of the support and by the size of the hydrophobic ligand molecule. The successful reversible immobilization of β-amylase and amyloglucosidase to hexyl-agarose carriers has been reported ***(48,49)***. Several other examples of strong reversible binding to hydrophobic adsorbents have also been reported ***(50–52)***.

5.1.4. Affinity Binding

The principle of affinity between complementary biomolecules has been applied to enzyme immobilization. The remarkable selectivity of the interaction is a major benefit of the method. However, the procedure often requires the covalent binding of a costly affinity ligand (e.s., antibody,= or lectin) to the matrix ***(53)***.

5.2. Chelation or Metal Binding

Transition metal salts or hydroxides deposited on the surface of organic carriers become bound by coordination with nucleophilic groups on the matrix. Mainly titanium and zirconium salts have been used and the method is known as “metal link immobilization” ***(15,54,55)***. The metal salt or hydroxide is precipitated onto the support (e.g., cellulose, chitin, alginic acid, and silica-based carriers) by heating or neutralization. Because of steric factors, it is impossible for the matrix to occupy all coordination positions of the metal; therefore some of the positions remain free to coordinate with groups from the enzymes. The method is quite simple and the immobilized specific activities obtained with enzymes in this way

have been relatively high (30–80%) However, the operational stabilities achieved are highly variable and the results are not easily reproducible. The reason for this lack of reproducibility is probably related to the existence of nonuniform adsorption sites and to a significant metal ion leakage from the support. In order to improve the control of the formation of the adsorption sites, chelator ligands can be immobilized on the solid supports by means of stable covalent bonds. The metal ions are then bound by coordination and the stable complexes formed can be used for the retention of proteins. Elution of the bound proteins can be easily achieved by competition with soluble ligands or by decreasing pH. The support is subsequently regenerated by washing with a strong chelator such as ethylene diamine tetraacetic acid (EDTA) when desired. These metal chelated supports were named IMA Immobilized Metal-Ion Affinity (IMA) adsorbents and have been used extensively in protein chromatography ***(56,57)***. The approach of using different IMA-gels as supports for enzyme immobilization has been studied using *Eschericia coli* β-galactosidase as a model ***(58)***.

5.3. Formation of Disulfide Bonds

These methods are unique because, even though a stable covalent bond is formed between matrix and enzyme, it can be broken by reaction with a suitable agent such as dithiothreitol (DTT) under mild conditions. Additionally, because the reactivity of the thiol groups can be modulated via pH alteration, the activity yield of the methods involving disulfide bond formation is usually high—provided that an appropriate thiol-reactive adsorbent with high specificity is used ***(59)***. Immobilization methods based on this strategy are discussed in Chapter 17.

6. Properties of Immobilized Enzymes

As a consequence of enzyme immobilization, some properties of the enzyme molecule, such as its catalytic activity or thermal stability, become altered with respect to those of its soluble counterpart ***(11,60)***. This modification of the properties may be caused either by changes in the intrinsic activity of the immobilized enzyme or by the fact that the interaction between the immobilized enzyme and the substrate takes place in a microenvironment that is different from the bulk solution. The observed changes in the catalytic properties upon immobilization may also result from changes in the three-dimensional conformation of the protein provoked by the binding of the enzyme to the matrix. These effects have been demonstrated and, to a lesser extent, exploited for a limited number of enzyme systems.

Quite often when an enzyme is immobilized its operational stability is improved. The concept of stabilization has thus been an important driving force for immobilizing enzymes. In many cases, the observed operational stabilization is usually the result of loading an excess of enzyme, which in turn makes the process diffusion-controlled. However, true stabilization at the molecular level has also been demonstrated, such as the case of proteins immobilized through multipoint covalent binding ***(61)***. Studies carried out by several authors using different methods have demonstrated that there is a correlation between stabilization and the number of covalent bonds to the matrix ***(62–64)***. One of the main problems associated with the use of immobilized enzymes is the loss of catalytic activity, especially when the enzymes are acting on macromolecular substrates. Because of the limited

access of the substrate to the active site of the enzyme, the activity may be reduced to accessible surface groups of the substrate only. This steric restriction may, in turn, change the characteristic pattern of products derived from the macromolecular substrate ***(65)***. There are several strategies to avoid these steric problems such as selection of supports composed by networks of isolated macromolecular chains, careful choice of the enzyme residues involved in the immobilization, and use of hydrophilic and inert spacer arms ***(66)***.

7. The Biology of Enzyme Immobilization

Although the science of enzyme immobilization has developed as a consequence of its technical utility, one should recognize that the advantages of having enzymes attached to surfaces have been exploited by living cells for as long as life has existed. An inquiry into the biological role of enzyme immobilization may provide some lessons for biotechnologists and serve as a second point of departure, in addition to the purely chemical one. In fact, there is experimental evidence that the immobilized state might be the most common state for enzymes in their natural environment. The attachment of enzymes to the appropriate surface ensures that they stay at the site where their activity is required. This immobilization enhances the concentration at the proper location and it may also protect the enzyme from being destroyed. Multimolecular assembly depends normally on weak noncovalent forces and hydrophobic interactions, but sometimes on covalent bonds as well (e.g., disulphide bridges) ***(1,2)***. All these different forces have been exploited in the development of immobilized enzymes.

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