



Review

Covalent immobilization: A review from an enzyme perspective

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ABSTRACT

Enzymes are indispensable in biotechnology, serving as biological catalysts in applications across various domains, including biosensors, fine chemicals production, pharmaceuticals, and drug development. In this context, enzyme immobilization is crucial for ensuring their retention on devices while preserving their activity over extended periods. Various immobilization methods, both physical (adsorption, affinity bonding, entrapment, encapsulation, and ionic bonding) and chemical (covalent bonding and crosslinking), have been explored, each exerting distinct impacts on enzyme stability and activity. Among these, chemical immobilization typically offers superior stability compared to physical methods due to the formation of stronger bonds between the enzyme and the support material. Covalent immobilization is commonly used due to its efficacy in enhancing enzyme stability. Carbodiimide chemistry and Schiff base reactions are the two most common covalent bond techniques used for immobilization, owing to the functional groups involved in the reaction ($-NH_2$ and $-COOH$), which are commonly found on enzymes surface. This review provides a background on enzymes and the various methods for immobilizing them onto materials, before delving into carbodiimide and Schiff base reaction techniques. The characteristics, advantages, and disadvantages of both these techniques are discussed, including bond formation, reaction condition, and implications for application. Additionally, the review underscores the significance of enzyme orientation, structure, and conformational changes. Achieving optimal orientation and minimizing conformational alterations are critical factors in developing a stable, highly active, selective, scalable, and reproducible enzymatic biosensor.

1. Introduction

Enzymes, often synthesized by biological organisms, are complex proteins that function as biocatalysts [1]. Based on the reactions they catalyse, enzymes are categorised into seven classes, namely oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases [2]. Regardless of their classification, enzymes play crucial roles in various sectors including the pharmaceutical, food, textile industries, and development of biosensors owing to their unique catalytic properties and specificity in facilitating biochemical reactions [3]. Additional advantages of enzymes encompass biodegradability, eco-friendliness, specificity, reduced energy utilization, minimal waste production, and limited hazards to humans, wildlife, and the ecosystem [4]. Despite these favourable attributes, limitations such as low chemical and physical stability and high cost still hinder their optimal

performance [5].

Some of the industrial applications of immobilized enzymes include but are not limited to production of fine chemicals, pharmaceuticals, and medicine, food processing for human and animal nutrition, bio-refinery, wastewater treatment, textiles, bioelectrochemistry, biosensors, environmental remediation, materials and polymers [6]. In these applications, the enzymes need to retain their function for as long as possible while remaining in the catalysis location. Furthermore, parameters such as reusability and the ability to recover the enzyme is crucial to drive cost down and reduce waste production [7,8]. Particularly, when enzymes are utilized as bioreceptors for sensing, it is critical to maintain their stability, activity, specificity, while the manufacturing techniques should also be scalable, reproducible, and cost effective to ensure sensor performance and commercialization potential. In this context, enzymes initiate physicochemical changes upon interaction

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with the targeted analyte, which are subsequently translated into a quantifiable signal by a transducer, facilitating the determination of substrate concentration within the sample via a readable signal [9]. Therefore, the stability of the enzyme is crucial for achieving reproducible and accurate measurements. The primary challenges in developing enzymatic sensors often include their limited shelf-life and narrow working condition range, which restrict their application. To overcome these constraints, several strategies have been proposed to enhance enzyme stability and activity including immobilization, chemical modification, and genetic modification [10]. Among these, enzyme immobilization has gained extensive application in the biological catalysts field [11]. This is primarily due to its direct effect on enzyme stability and activity while being relatively cost-effective [12].

In biosensors, enzymes are typically immobilized on the transducer or carrier support, which varies depending on the sensor type (e.g., electrochemical, optical) or directly on the substrate (i.e., support material). The immobilization technique can be tailored based on the enzyme, application, and substrate. The properties of the support material play an important role for efficient enzyme immobilization, with selection dependant on the enzymes used and the application requirements, such as mechanical properties. Consequently, selecting the appropriate immobilization technique can be complicated, given the numerous variables to take into consideration (Fig. 1) [13]. Covalent immobilization of enzymes, involving the formation of a covalent bond between substrate or support material and enzyme, is one of the most widely used techniques. It is applicable to a variety of enzymes and substrates, offering relatively long shelf lives and stable devices [8]. However, techniques in this category are also characterized by higher costs, increased rate of enzyme denaturation, and a relatively complex optimization compared with other physical immobilization processes [14].

This review offers a comprehensive overview of enzymes structure and the common functional group they exhibit. It subsequently introduces physical and chemical immobilization processes, outlining their respective advantages and drawbacks. The discussion then delves into the two primary types of covalent immobilization, namely carbodiimide and Schiff base. The paper emphasises how the choice of enzyme and immobilization substrate can have an impact on covalent immobilization. The primary constraints are dictated by the selected application and the required sensing performance. Finally, the review

addresses the key parameters to consider for future studies.

2. Factors influencing enzyme activity – Functional groups, pH, temperature, and structure

Enzymes are predominately proteins, biomolecules composed of sequences of amino acids that facilitate or accelerate specific reactions. Some enzymes incorporate non-protein components, such as cofactors or coenzymes, and inorganic molecules such as metal ions (e.g., iron, zinc) [15]. Their catalytic activity is granted by the unique three-dimensional structure formed by these amino acids [16]. Enzymes can comprise over two thousand amino acids linked by peptide bonds in its structure. Most of these amino acids serve a structural function to protect and stabilize the active site, where the chemical reaction is catalyzed. The active site, often a cavity or a cleft in the enzyme, consists of a specific sequence of amino acids that form a characteristic three-dimensional structure and electric charge distribution conferring specificity [17]. The shape and electric state of the active site allow the formation of an enzyme-substrate intermediate through weak bonds (e.g., hydrogen bonds and ionic bonds) only with molecules that “fit” in the active site. The presence of water molecules is also essential for this process, as they facilitate the formation of additional hydrogen bonds. The condition of the active site is crucial for the catalytic properties of enzymes. For instance, some enzymes can catalyze the same reaction with different substrates, due to the malleability of their active site [18]. Conversely, changes in the physical, chemical, or electrical conformation of the active site can lead to loss of activity or specificity [19,20]. Although the exact mechanism is not fully understood, it is known that any chemical or physical variation affecting the mobility or interaction between the amino acids in the peptide structure or the enzyme’s water content can lead to a partial or complete denaturation, and, consequently, a loss of activity. For instance, extreme environmental conditions and the presence of hydrophobic molecules or moieties (e.g., gas bubbles, organic solvents) can cause the enzyme structure to unfold, altering the active site’s electrical conformation [20–22], or result in the loss of water molecules, preventing the formation of a stable enzyme-substrate intermediate. This can occur naturally, as some enzymes have allosteric sites or “switches” to inhibit their activity under certain conditions [17].

It is evident that active sites are highly sensitive to conformational

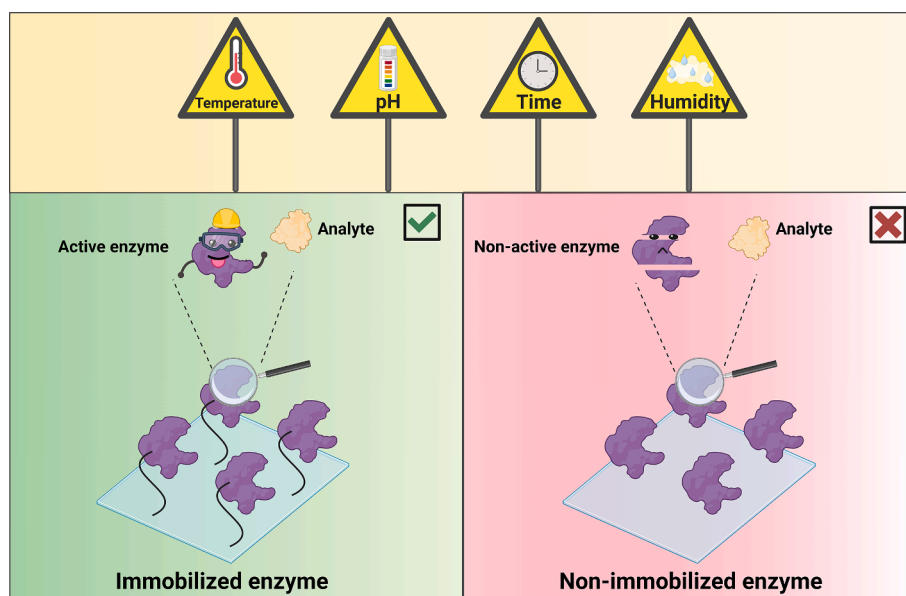


Fig. 1. Schematic summary of the parameters affecting enzyme activity (i.e., temperature, pH, and storage time), and the positive effect that immobilization can have on maintaining the enzyme structure and activity.

changes, regardless of where they occur within the enzyme. This sensitivity is particularly true for native, or raw or free, enzymes, which are not only difficult to recover and reuse but also lack stability due to their high sensitivity to environmental conditions such as temperature, pH, and humidity (or water content) [23,24]. Heat enhances enzyme activity only until the enzyme reaches its optimal temperature, beyond which denaturation occurs due to breakage of weak bonds that maintain the enzyme's structure [25,26]. For example, immobilizing lipase not only elevated the optimal temperature of the enzyme by 9 °C compared to its free counterpart but it also resulted in a two-fold increase in the half-life of the immobilized enzyme, thereby outperforming the free enzyme in maintaining its catalytic activity [27]. α -Amylase, one of the most widely used industrial enzyme, showed an increase in optimum temperature from 55 °C to 70 °C and an increase in half-life from 40.53 min to 115.50 min upon immobilization [28]. Furthermore, immobilized α -amylase retained 43 % of its activity even after six cycles of reuse.

Similarly, variations in pH can affect the weak chemical bonds that maintain the protein structure, leading to denaturation and unfolding, or may hinder the kinetics of the catalyzed redox reaction [29]. For instance, immobilized pectin lyase remained active at pH of 9, whereas free pectin lyase was completely inactive at the same pH. Immobilizing this enzyme not only extends its working pH range but is also highly beneficial in industrial settings as it allows for the reuse of pectin lyase [30].

The presence of water molecules within the enzyme structure, as seen before, enhances the formation of hydrogen bonds, stabilizing the quaternary peptide conformation. Therefore, humidity (or water content), like other parameters, significantly affects enzyme's activity. The activity of certain enzymes, such as urease and dehydrogenases, peaks at specific moisture levels, indicating that both excess and insufficient moisture can impact enzyme performance [31]. For some enzymes, the lower water activity or moisture content can increase the stability and

the activity of the enzyme. For example, immobilized lipase showed low moisture content of 3.84 % w/w and water activity of approximately 0.10, which is necessary in industries when undergoing esterification reactions [32].

Finally, enzymes are susceptible to degradation due to the accumulation of damage from exposure to unusual conditions (as explained above), air, or other chemicals. Processes such as oxidation, racemization, glycation, and other can affect the enzyme at any residue, leading to the destruction of some bonds and the subsequent loss of structure and activity [33]. This degradation typically occurs due to external factors rather than the sole enzyme's catalytic cycle. Therefore, it is crucial to store enzymes in aseptic, dry, and controlled atmosphere containers at low temperatures to preserve their integrity.

As a result, enzymes maintain their activity within specific ranges of pH, temperature, and humidity, which vary depending on their structure [34,35]. For instance, cholesterol oxidase exhibits its highest activity at pH of 7 and temperature of 37 °C in an aqueous medium [36] and retains its activity even at 60 °C [37], while glucose oxidase from *Aspergillus Niger* is chemically stable at room temperature and active within a pH range of 4 – 7 and temperature range of 30 – 60 °C [38–40]. HRP (Horseradish Peroxidase) is stable within a pH range of 5 – 9 and at room temperature [41,42].

To extend their working range, immobilization has been used as a tool to increase the enzymes resilience to environmental changes, improving their stability and shelf-life. For immobilization purposes, the common functional groups of enzymes, such as amino groups ($-\text{NH}_2$), carboxylic groups ($-\text{COOH}$), and hydroxyl groups ($-\text{OH}$), are crucial [43]. Specifically, primary amines and carboxylic acid groups are found on the unbound N-Terminal and C-Terminal of polypeptide chains (Fig. 2). Attempts to add additional functional groups to enzymes, through physical or chemical immobilization may cause the deformation or breakage of the bonds between polypeptide chain residues. Since these bonds confer the enzyme's unique structure and functionality, any

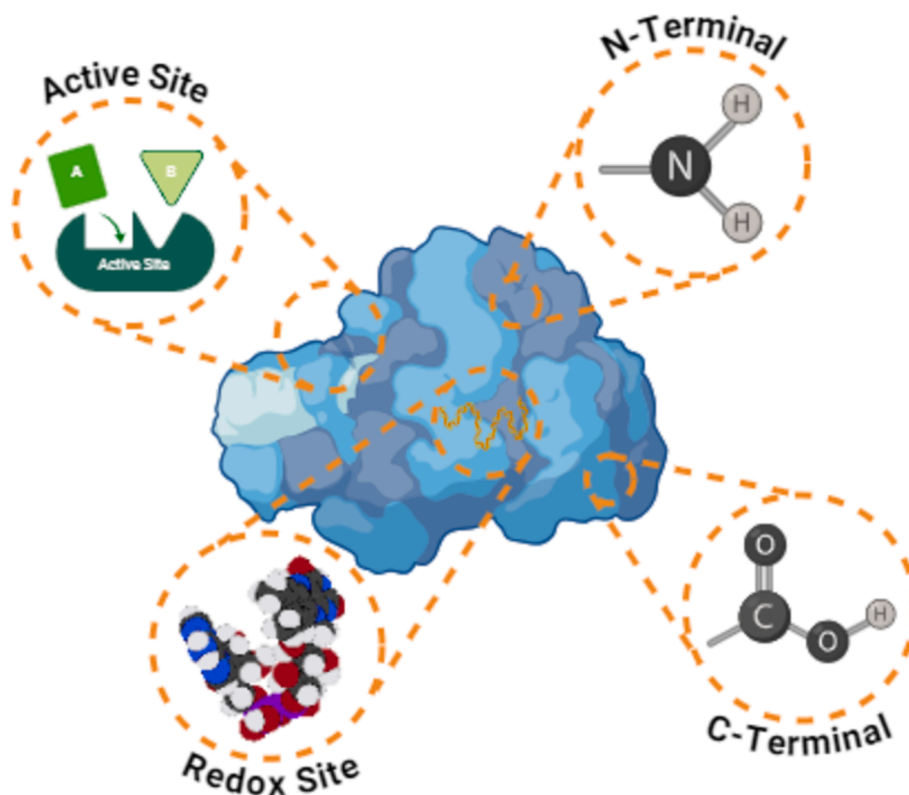


Fig. 2. Fig. 2 Schematic representation of enzyme structure highlighting N- and C-Terminals of the amino acid chain, the active site (lock and key model is shown [17]), and the redox site (in this case FAD is represented).

change may result in loss of activity and/or stability in catalytic properties [44].

The functional groups, active sites, enzyme classes, and applications of some enzymes are listed in Table 1. The most common functional groups present in enzymes are amino and carboxyl groups, while Flavin Adenine Dinucleotide (FAD) is a common cofactor found in the active site of many enzymes.

3. Types of immobilization

As previously stated, the goal of immobilization is to extend the activity of the enzyme over time and over a wider range of working conditions. This can happen in two ways: sheltering and rigidification [69]. Sheltering consists in the protection of the enzyme from possible chemicals or physical conditions that may lead to conformational changes in its structure. Rigidification, instead, is the process of

Table 1

Commonly used enzymes in biosensors, their properties, and their application.

Enzyme	Enzyme class	Cofactors of the Enzyme	Functional Groups Present	Selectivity	Stability	Application	Ref.
Glucose oxidase	Oxidoreductase	FAD	- Amino groups ($-NH_2$) - Carboxyl groups ($-COOH$) - Hydroxyl groups ($-OH$)	Glucose, (reacts partially with other mono- and disaccharides)	5 years at $-20^\circ C$	Used for developing glucose biosensors for diabetic patient.	[45,46,47]
Glucose dehydrogenase	Oxidoreductase	FAD	- Amino groups ($-NH_2$) - Carboxyl groups ($-COOH$) - Hydroxyl groups ($-OH$)	Glucose, maltose, galactose, xylose, and mannose	2 years at $-15^\circ C$	Used for developing glucose biosensors for diabetic patients.	[45,48–50]
Horseradish peroxidase	Oxidoreductase	Heme group	- Amino groups ($-NH_2$) - Carboxyl groups ($-COOH$)	Phenols, aromatic amines, thioanisoles and iodide (hydrogen peroxide – oxidizing agent)	5 years at $2-8^\circ C$	Biosensors, Analysis and Diagnostic Kits and Enzyme Immunoassays	[51–53]
Cholesterol oxidase	Oxidoreductase	FAD	- Amino groups ($-NH_2$) - Carboxyl groups ($-COOH$) - Hydroxyl groups ($-OH$)	Cholesterol	9 months at $-20^\circ C$	Used in measuring the cholesterol levels in Biosensors	[36,54,55]
Choline Oxidase	Oxidoreductase	FAD	- Amino groups ($-NH_2$) - Carboxyl groups ($-COOH$) - Hydroxyl groups ($-OH$)	Choline	1 year at $-20^\circ C$	Utilized in choline biosensors for measuring choline levels	[56,57]
Urease	Hydrolase	(Ni)	- Amine groups ($-NH_2$) - Carboxyl groups ($-COOH$)	Urea and Hydroxyurea	1 year at $4^\circ C$	Employed in urea biosensors for detecting urea levels, Creatinine, Arginine and IgG.	[58–60]
Acetylcholinesterase	Hydrolase	The anionic site and the esteratic subsite	- Amine groups ($-NH_2$) - Carboxyl groups ($-COOH$)	Acetylcholine	2 years at $-20^\circ C$	Applications include treating neuromuscular disorders and Alzheimer's disease	[61,62]
Alkaline Phosphatase	Hydrolase	(Zn) and (Mg)	- Phosphate groups ($-PO_4$) - Amino groups ($-NH_2$) - Carboxyl groups ($-COOH$)	Phosphate esters	N/A	Commonly employed to measure activity in human serum To detect various diseases related to liver and bone.	[63,64]
Carbonic Anhydrase	Lyase	(Zn)	- Amine groups ($-NH_2$) - Carboxyl groups ($-COOH$)	Carbon dioxide	2 years at $-20^\circ C$	Used in biosensors for detecting carbon dioxide levels.	[65,66]
Xanthine Oxidase	Oxidoreductase	(FAD), Molybdenum	- Iron-sulfur clusters - Amine groups ($-NH_2$) - Carboxyl groups ($-COOH$)	Xanthine	$2-8^\circ C$ N/A	Utilized in biosensors for detecting xanthine levels.	[67,68]

Abbreviations: FAD: Flavine Adenine Dinucleotide; etc., Ni: Nickel; Zn: Zinc; Mg: Magnesium; N/A: not available.

anchoring via chemical bonds regions of the enzymes so to make structural changes and unfolding less thermodynamically favourable.

Each immobilization method presents a distinct set of benefits and drawbacks, and it is crucial to exploit them, as they have a pivotal role in ensuring optimal enzyme performance. For instance, biosensors must exhibit high stability, sensitivity, selectivity, and a minimal response time [203]. These attributes are contingent upon the impact of immobilization on the enzyme's functionality and stability. Ideally, the immobilization process should preserve the enzyme's structure, avoiding denaturation or significant conformational changes. However, achieving this is challenging, as various techniques can differently affect the enzyme [70]. Consequently, the suitability of different immobilization methods depends on the intended application, the substrate or support material, and the enzyme.

Immobilization techniques are commonly categorized into two groups: physical and chemical, based on the nature of the bond between the enzyme and the support material [71]. Physical immobilization involves the formation of weak bonds such as Van der Waals forces, hydrophobic interactions, and hydrogen bonds, or the physical confinement and entrapment of the enzyme within the support material, such as porous substrates or micelles [72]. These methods are often preferred due to their cost-effectiveness and minimal impact on the enzyme's structure, thereby reducing the risk of denaturation and activity loss.

Conversely, chemical immobilization relies on the formation of strong bonds, such as covalent and ionic bonds, between the enzyme and the support [73]. While the specific procedures and support materials may vary, chemical immobilization offers a stronger anchoring of the enzyme to the support compared to physical immobilization, often resulting in enhanced stability [7]. The most common strategies for achieving chemical bonding in this context involve the formation of amide or imine linkages, which facilitate chemical linkages between the functional groups of the enzymes and those in the support material. Fig. 3 schematically represent both physical and chemical

immobilization methods, while Table 2 summarizes their advantages and disadvantages.

3.1. Physical immobilization

Physical immobilization methods generally preserve the enzyme's activity by avoiding conformational or structural changes. This preservation is attributed to the weak nature of the bonds formed, which do not strain the enzyme's structure and are crucial for maintaining its function. The support material provides stabilization by protecting and screening the enzyme from inhibitors and factors that may cause denaturation such as hydrophobic molecules, gas bubbles, and extreme environmental conditions. Although conformational changes are limited, minor variations in activity and selectivity can still occur. For instance, Baruch-Shpigler et al. observed that glucose oxidase turned into a general saccharide oxidase, capable of degrading other mono- and di-saccharides, when entrapped in gold with surfactant cetyltrimethylammonium bromide [74]. Physical immobilization can often occur directly between the enzyme and the support material without the need for harmful or hazardous chemicals, making these techniques safer. However, the weak bonds formed can result in enzyme leaching from the immobilization material, leading to a decrease of the catalytic properties [75]. Moreover, these methods often offer limited control over the enzyme's positioning, potentially rendering some enzymes inaccessible to the analyte or obstructing the availability of the enzyme's active site [76]. Consequently, the immobilization substrate must be tailored, for example by adjusting the pores size through various means such as the degree of crosslinking or layer-by-layer constructs [77].

3.1.1. Adsorption

Enzyme immobilization via adsorption represents one of the most versatile, straightforward, and prevalent methodologies. The interactions between the support materials and the enzymes are characterized by relatively weak bonds, such as van der Waals forces and

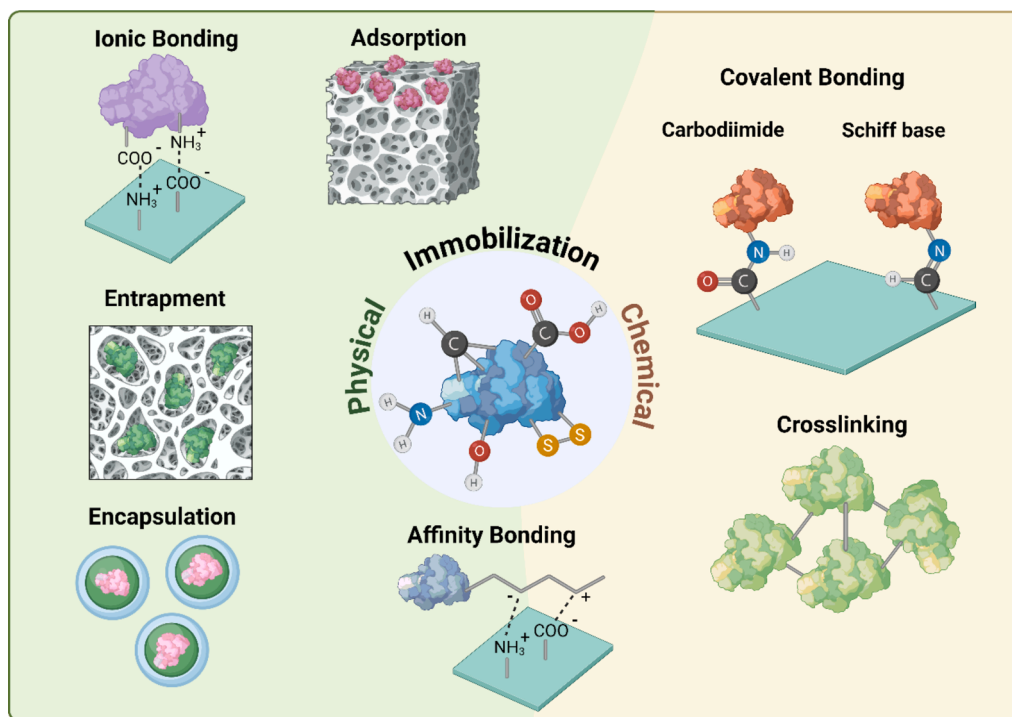


Fig. 3. Schematic of the different types of enzyme immobilization available, including physical (on the left, in green: adsorption, ionic bonding, entrapment, and encapsulation) and chemical (on the right, in yellow: covalent bonding and crosslinking). Affinity bonding sits in between the two areas as the nature of the immobilization depends on the bonds formed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Different immobilization techniques and their advantages and disadvantages.

Immobilisation	Type	Bonds	Reuse/ Regenerated*	Recovered *	Advantages	Disadvantages	Ref
Adsorption	Physical	Van der Waals interactions, Hydrogen bonds	Yes ⁺	Yes	No enzyme conformational changes Cost-effective Simple to use	Possible enzyme leaching	[79,106]
Ionic Interactions	Physical	Ionic bonding	Yes ⁺	Yes	Cost-effective Simple to use	Leaching, can limit enzyme catalytic properties affecting its activity because of excessive charges	[79,106]
Affinity bonding	Physical/ Chemical	Covalent and non-covalent bonds	Yes ^{‡+}	Yes [†]	Less conformation changes, selective and helps in orientation of enzymes.	Affinity tags are limited and expensive	[107]
Covalent binding	Chemical	Covalent bond	Yes [‡]	Yes [†]	No leaching and good high stability.	Conformational changes of enzyme leading to denaturation and low activity.	[79,106]
Entrapment	Physical	Not required	Yes	No	High loading capacity, low cost, good mechanical stability and no conformational changes	Low activity because of limited substrate reaching enzymes and enzyme leakage.	[79]
Encapsulation	Physical	Not required	Yes	No	Stable, No conformational changes of enzyme and low leakage of enzymes	Rapid accumulation of production can cause damage to the membrane	[79]
Cross-linking	Chemical	Covalent bond	Yes [‡]	Yes [‡]	No carriers required, high stability	Expensive and poor mechanical stability and harsh process conditions	[79,106]

* Reused and recovered without any damage to the enzyme and support; ‡ The formation of strong bonds hinder regenerability, yet it does not make it impossible; + Reuse is dependent upon leaching; † It depends on whether the bond is reversible or irreversible; ‡ The enzyme is recovered still crosslinked.

hydrogen bonding, which help preserve both enzyme structure and activity. However, enzymes immobilized through adsorption are highly susceptible to leaching from the support material, and the support material's protective capability is limited [78].

3.1.2. Ionic bonding

Ionic bonding is a reversible immobilization method similar to adsorption. Enzymes are bound to the support substrate through ionic interactions, characterized by the presence of opposite charges between the support and the enzyme. This technique is characterized by its simplicity, cost-effectiveness, and the ability to be easily reversed by changing pH levels. A single enzyme may form multiple ionic bonds with the support, provided the latter has sufficient charges, thereby improving stability. Nevertheless, the presence of charges can influence the structural integrity of the enzyme and its active site, potentially impacting its functional activity [79].

3.1.3. Affinity bonding

Affinity bonding rely on the formation of either covalent or non-covalent bonds between a taggant and the enzyme or support. In this method, a taggant with affinity to the support material is attached to the enzyme, allowing immobilization without compromising the enzyme structure. Conversely, a taggant with affinity to the enzyme can be attached to the support material surface. The nature of the bonding determines whether the interaction is reversible or irreversible. Affinity bonding is characterized by its selectivity, control of orientation, and site-specific immobilization of enzymes. It exerts a lesser impact on the enzyme's structural integrity compared to covalent bonding, thereby better preserving its functional activity. Despite fulfilling most criteria for effective immobilization, affinity bonding is comparatively expensive when juxtaposed with other immobilization methodologies [80,81].

3.1.4. Entrapment

In this method, enzymes are encapsulated within the support matrix, allowing the substrate to permeate through the matrix and interact with the enzyme. Unlike other immobilization techniques that involve bond formation, this approach preserves the enzyme's structural integrity and catalytic activity. However, a significant limitation is associated with

mass transfer dynamics, as the pore size must balance enzyme leaching and substrate molecule permeability and diffusion [82].

3.1.5. Encapsulation

Encapsulation is similar to entrapment, with the enzyme being entrapped within a matrix. However, in encapsulation, enzymes are enclosed within a capsule or spherical structure equipped with porous membranes, facilitating the diffusion of substrates and products. The pore dimensions must be optimized to be smaller than the enzymes. Like entrapment, encapsulation minimally affects the enzyme's structural conformation and functional activity. However, controlling membrane pore size can be challenging, potentially leading to enzyme leaching. This method is unsuitable for enzyme and substrate of similar sizes [79,83].

3.2. Chemical immobilization

Chemical immobilization techniques rely on the formation of strong bonds between the enzyme and the substrate, such as covalent, dynamic covalent, and ionic bonds [84]. This approach offers better control over enzyme's orientation, especially in covalent bonding, compared to physical techniques [85]. These strong bonds result in long-lasting and stable immobilization, reducing enzyme leaching and enhancing stability. Additionally, covalent bonding offers greater control over the immobilization process compared to physical techniques.

The primary mechanism by which chemical immobilization stabilises enzymes is rigidification. The formation of one or multiple strong bonds anchors regions of the enzyme to the support material, thereby making conformational changes and subsequent denaturation more difficult [69]. This often leads to an increased shelf-life and a less pronounced decrease in catalytic activity outside the optimal environmental conditions. Nevertheless, enzyme stability is frequently achieved at the expense of overall activity. The introduction of external bonds with the support material may distort the distribution of electrical charges and induce conformational changes in the enzyme [69,86]. While increased rigidity can enhance stability, it can also reduce activity by hindering the micro-conformational changes necessary for substrate binding and release [87]. Depending on the immobilization conditions, which can also impact the enzyme, the immobilized enzyme activity can

be very close to the free enzyme (*i.e.*, prior immobilization), but it can also decrease, by as much as 80 %, as demonstrated by T. Li et al. in the immobilization of pectinase [88].

3.2.1. Covalent bonding

In this chemical immobilization technique, the interaction between the enzyme and the support material involves covalent bonds formed between their respective functional groups. These covalent bonds can be classified as either reversible or irreversible, depending on their nature. For example, bonds such as the Schiff base bond, disulfide bond, and other types are categorized as dynamic covalent bonds or reversible covalent bonds. Despite their reversible nature, these bonds are stronger compared to those used in physical immobilization methods [89]. Amide bonds synthesized via carbodiimide chemistry are typically considered as irreversible and are among the most robust types of bonding [90]. Due to their stability, high loading efficiency, and reproducibility, covalent immobilization techniques are widely applied across various industries. However, the irreversible and robust nature of covalent bond can alter the enzyme structure, thereby impacting its activity. Consequently, neither the enzyme and nor the support can be reused or recovered without causing damage to either component [14,80].

3.2.2. Crosslinking

Crosslinking is an irreversible immobilization technique that involve forming multiple covalent bonds between various enzymes, without the need for support material. Enzymes immobilized using this technique exhibit high stability, high activity, low leaching rate, and can be relatively easily recovered. However, optimizing the process conditions to achieve a high-quality cross-linked enzyme assembly can be tedious and expensive. In addition, mass transfer limitations can pose a significant disadvantage [91,92].

3.3. Support materials and immobilization

One of the most critical steps in the immobilization process is selecting an appropriate support material, which depends on the chosen immobilization technique and the specific enzymes involved. The selection of support material is typically influenced by various factors such as specificity, biocompatibility, stability, reusability, and economic considerations [93]. A diverse array of support materials is available encompassing organic, inorganic, and hybrid or composite substances. The chosen support material must facilitate the retention of the enzyme's structural integrity and functional capacity. A comprehensive discussion of all materials utilized for support is provided in the work of Zdarta et al. [94].

Key parameters to take into consideration when minimizing the impact of the support material on the enzyme and its catalytic activity include: (i) porosity, (ii) hydrophobicity, (iii) biocompatibility, and (iv) roughness. Porosity enhances the surface area available for binding, thereby augmenting both the stability and catalytic activity of immobilized enzymes [95]. When support materials lack functional groups, pore dimensions must be carefully controlled to minimize leaching without hindering substrate diffusion, thus retaining the maximum reaction rate possible. Commonly used support materials include carboxymethyl-cellulose, starch, collagen, modified sepharose, ion exchange resins, activated charcoal, and silica [75]. Immobilizing the enzyme too close to a hydrophobic material may reduce the enzyme water content, lowering the number of hydrogen bonds within its structure and leading to denaturation. When selecting a hydrophobic material due to other requirements, such as applications or cost limitations, it is then important to use linkers or bridging molecules to distance the enzyme from the surface [11,96]. Furthermore, decorating the surface of the support material has proven beneficial in retaining enzyme activity. Incorporating peptide-modified surfaces, mesoporous silica, chitosan, and others, has shown to enhance both the stability and

activity of enzymes in covalent immobilization. The utilization of alginate–gelatin–calcium hybrid carriers for encapsulation has been documented as an effective strategy, contributing to increased mechanical stability and reducing enzyme leakage [11]. Finally, roughness is another important factor: immobilizing the enzyme within a valley on a highly rough surface may lower activity due to steric hindrance. It is crucial to either avoid “valley immobilization” or reduce the surface roughness [97,98].

Recent advancements in support materials encompass easily separable support materials such as magnetic nanoparticles (MNP), membranes, and capillary columns [95]. For instance, magnetically immobilized enzymes can be retrieved using magnetic fields, facilitating the separation of enzymes from MNPs. The innovation of hybrid supports, particularly those composed of organic–inorganic hybrid materials with magnetic attributes, leveraging organic by-products, presents a promising avenue for enzymatic immobilization, emphasizing sustainable practices in their synthesis. Moreover, membrane-immobilized enzymes are typically affixed within apparatus designed to create membrane bioreactors, allowing for swift separation from the reaction mixture. Additionally, capillary-immobilized enzyme microreactors are commonly integrated into capillary electrophoresis systems for on-line analytical methodologies. Nonetheless, further investigative endeavors are indispensable for the exploration of novel support matrices and the advancement of more efficacious immobilization techniques to enhance the catalytic efficiency, reusability, and stability of immobilized enzymes [99,100].

3.4. Types of immobilization based on the application

Industrial field such as food, chemical, pharmaceutical, cosmetics, and medical device relies on enzymes and their immobilization [101]. The selection of an immobilization technique pertinent to a particular application is contingent upon various factors including process parameters, enzyme lifespan, reusability, and recoverability required, cost considerations, and the characteristics of the support materials employed (*e.g.*, chemical properties, mechanical properties).

In scenarios where the substrate or sample introduces biological contaminants to the enzymes, such as in medical devices, recoverability and reusability are not primary concern. Therefore, immobilization should prioritize stability while maintaining the highest possible activity, potentially favouring physical methods. Conversely, for applications, such as the production of fine chemicals and pharmaceuticals, it is economically advantageous to reuse and recover the enzyme while ensuring its stability [102]. The chemical industry, in particular, shows a clear preference for catalysts that can be reused, hence the use of chemically immobilized biocatalysts. An exemplary biocatalyst in this context is penicillin G amidase, which is employed to hydrolyze penicillin. Through immobilization, the enzyme can be utilized multiple times, thereby providing a cost-effective solution for the industry [103]. In this context, reversible and certain physical immobilization techniques (*e.g.*, ionic bonding, affinity bonding), which allow for easy recovery and reuse of the enzyme without damaging it or the support material, have garnered growing interest in industrial applications [104,105]. Recovery of enzymes immobilized via irreversible immobilization techniques is highly unlikely, as the debonding process would most likely damage both the enzyme and the support. Nevertheless, irreversible immobilizations are still sought after to produce reusable biocatalysts.

4. Types of covalent immobilization

A covalent bond occurs when two atoms with similar electronegativity share one or more pair of electrons [108]. The process of covalent immobilization involves the formation of such bonds, which can be categorized into irreversible covalent linkages or reversible dynamic covalent bonds, between a functional group on the enzyme and a

functional group on the support material.

Two principal approaches are commonly highlighted for enzyme immobilization: carbodiimide chemistry and/or Schiff base reactions. Carbodiimide chemistry results in the formation of an irreversible amide linkage, whereas the Schiff base approach forms imine bonds, which are considered reversible [109,110]. In both methods, the functional groups present on the enzymes interact with those naturally occurring or artificially introduced on the support material.

While the aforementioned methods are extensively employed, alternative approaches for the immobilization of enzymes, such as plasma treatment and thiol bonds/disulfide exchange reactions, have not been explored as much. It is imperative that additional research be conducted in these areas, as they remain in a nascent phase of investigation. Disulfide exchange reactions are also considered reversible, as they can be cleaved by a reduction reaction [111].

4.1. Amide bond/Carbodiimide immobilization method

Enzymes possess both N-terminal and C-terminal regions, which correspond to a free amino group and a carboxyl group, respectively. The establishment of a carbodiimide chemistry is achievable between the carboxyl group of a support material and the amino group of the enzyme, as well as between the carboxyl group of the enzyme and the amino group of the support material [112]. In the latter scenario, care must be taken, as activating the carboxylic acid groups of enzymes might lead to undesirable intermolecular interactions with the enzyme's native primary amine groups of enzymes. This can be detrimental to enzymatic activity and compromise immobilization efficiency. Regardless, this

method is known for the absence of spacer molecules, thereby forming a direct bond between the functional groups of the enzyme and the support in absence of adding any molecular lengtheners or extra atoms [113]. The immobilization process using this method is typically carried out in two steps as follows: (i) a carbodiimide reagent activates the carboxylic acid functionality through the utilization of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), in conjunction with N-hydroxysulfosuccinimide (NHS), resulting in the formation of highly reactive O-acylisourea intermediates under slightly acidic pH (4.5–7.2); (ii) this intermediate is poised to engage with primary amines, serving as nucleophiles, to yield an amide bond along with the byproduct Isoourea (Fig. 4A). The resultant bond exhibits superior strength and stability compared to alternative bonding mechanisms, such as Schiff bases [114].

This method, however, is not free from drawbacks. The reaction involving EDC may result in the formation of undesired byproducts due to the presence of a highly reactive intermediates. These intermediates can interact with other carboxylic groups to produce an anhydride intermediate or, in cases of excess EDC, react with secondary amines to yield an inactivated N-acylisourea derivative. Furthermore, this process may entail certain levels of expenditure and complexity, especially when considering the costs of reagents, efforts for optimization, and factors related to scaling up [114]. A major limitation of this approach is the potential for self-crosslinking caused by the coexistence of carboxylic and amine groups within the enzymes. Moreover, most enzymes undergo denaturation in acidic environments. In fact, the use of a carbodiimide reaction for covalent binding has been found to cause significant denaturation, altering the tertiary structure of glucose oxidase [115].

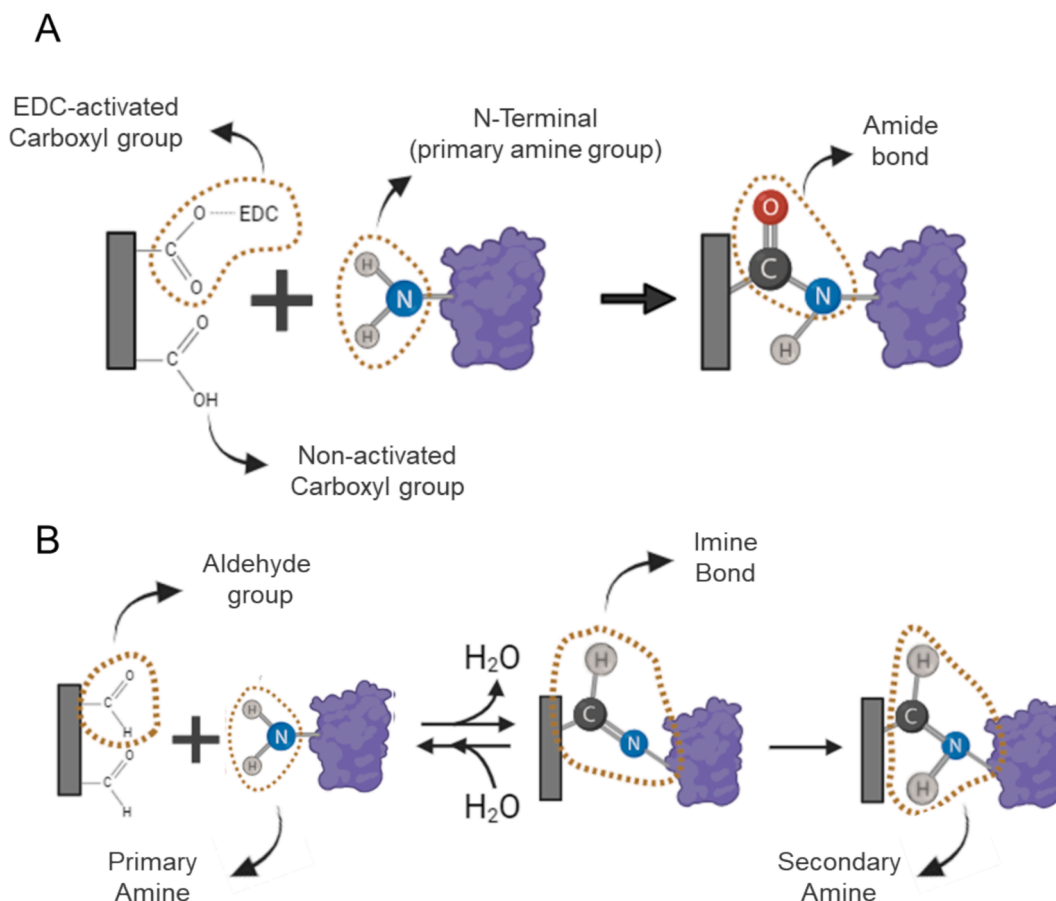


Fig. 4. A) Schematic process involving the formation of an amide bond via EDC activation of a carboxyl group present on the support material and the N-terminal (primary amine) of an enzyme. B) Schematic process of an imine bond formation between support material decorated with aldehyde groups and a primary amine group of an enzyme. Note the reversibility of the imine bond chemistry involving water and also the possible reductive amination of the imine bond (last step of the process) which can occur in the presence of a reducing agent, such as sodium borohydrate.

Therefore, the timing and duration of enzyme addition to the active substrate must be carefully selected. The optimal approach is to attach the amine group of the enzyme to the carboxyl group of the support. Table 3 lists some examples from the literature where carbodiimide chemistry was used to covalently immobilize enzymes. For instance, Feizabadi et al. successfully immobilized horseradish peroxidase on multi-wall carbon nanotubes (MWCNTs) and maintained 90 % of the enzyme activity even after 10 weeks [116,117] (Fig. 5A). HRP exhibited a lower activity when immobilized directly onto MWCNTs compared with the introduction of a spacer (in this case, γ -aminobutyric acid – GABA) when analyzed via cyclic voltammetry. Through computational simulation, it was found that introducing GABA leads to the formation of bond with the lysine amino acids, which both increases the rigidity of the enzyme but also increases the aperture of the active site, leading to a higher activity.

To increase the distance between immobilization support and enzyme and to avoid the formation of a zero-length bond, pre-activated spacers with NHS-activated ester groups have been employed. NHS-esters are highly reactive and do not require harsh conditions, such as acidic pH, to form an amide bond. NHS-esters react with the amine groups of both support material and enzymes, resulting in the formation of an amide bond. NHS activated esters are primarily synthesized using carbodiimide chemistry [118]. These NHS-activated esters are utilised in aqueous solutions at pH 6 to 9 to covalently immobilize antibodies, enzymes, peptides, and other biomaterials [119]. For instance, Cabrita et al. immobilized Laccase on gold substrates using dithio-bis (succinimidyl propionate) as the NHS-activated ester. The amount of laccase immobilized was observed to be higher on pure self-assembled monolayers (SAM) compared to mixed ones, and the current peak displayed for the laccase decreased when mixed SAMs were used [120].

4.2. Imine bond/Schiff-based immobilization method

Aldehydes can undergo a reaction with primary or secondary amines to form a Schiff base or an imine bond (Fig. 4B), which is reversible via hydrolysis in acidic aqueous media (pH < 4) [129]. While this reaction is also possible for ketones, it is less thermodynamically favourable [130]. At the same time, the reaction with primary amines is more favourable compared with secondary and tertiary amines, due to the dissipation of the electric charge on the carbonaceous backbones. However, reaction

with secondary and tertiary amines are still possible via the use of a reducing agent such as cyanoborohydride to expedite the reaction [131]. In enzyme immobilization, the amino groups present in the enzyme structure can directly interact with aldehyde or ketone groups on a support material to establish a Schiff base [132]. Alternatively, the enzyme's amino group can be linked to a bifunctional crosslinking agent like glutaraldehyde, which acts as a bridge between the amino groups of the enzyme and the support [133]. In fact, glutaraldehyde, a homo-bifunctional crosslinker, possesses identical functional groups at both ends. These two reactive groups, typically located at the termini of an organic spacer arm, can be tailored in length to achieve the optimal distance between the conjugated molecules [134]. Due to the absence of additional reagents and the flexibility in the reaction conditions (e.g., the optimal pH for this process depends on the amine and the aldehyde used), the Schiff base reaction offers a cost-efficient alternative to carbodiimide reactions. Table 4 lists some examples from the literature where Schiff base method was used to covalently immobilize enzymes. For example, Manoj et al. successfully immobilized glucose oxidase directly on aldehyde functionalized substrate by maintaining 91 % of activity even after 30 days without using any crosslinker (Fig. 5B) [135]. In their study, the Fourier Transform Infrared spectral analysis showed how the immobilized enzyme showed the same peaks (and in particular the amide I and amide II peaks) of the free enzyme, demonstrating that GOx had retained its structure. Similarly, Šulek et al. used glutaraldehyde as a crosslinker to immobilize cholesterol oxidase, achieving 50 % activity [136]. Despite being a commonly used spacer for Schiff base reaction, glutaraldehyde is cytotoxic [137]. However, other spacers such as aldehyde polysaccharides [109], polyethylene glycol [138], BS3 (Bis(sulfosuccinimidyl)suberate) [139] are available.

One of the primary drawbacks of using the Schiff base reaction for enzyme immobilization is the reversibility of the bonds formed, leading to lower stability compared with carbodiimide bonds [140]. The pH required for a Schiff base reaction depends on the reactants, solvent, and temperature of the reaction. The imine bond can be formed at neutral and basic pH, favoring a base-catalyzed Schiff base reaction [141]. Finally, Schiff base reactions are more susceptible to undesired side reactions than carbodiimide, resulting in the formation of unwanted byproducts. For instance, when utilizing a crosslinking agent, the formation of enzyme-to-enzyme bonds is likely, reducing the efficacy of the immobilization [134].

Table 3

Example of carbodiimide reactions with different supports and enzymes without crosslinkers.

Enzyme	Support	Functional Group of Enzyme	Functional Group of Support	Sensor Type	Shelf Life/ Stability	Ref
Glucose Oxidase	Polypyrrole	–NH ₂	–COOH	Amperometric	Retained 50 % of its initial response after 10 days	[117]
Glucose Oxidase	hybrid sol–gel carbon composite	–COOH	–NH ₂	Amperometric	Retained 85 % of its original response after 1 month	[121]
Lactate oxidase	SWCNT	–NH ₂	–COOH	Amperometric	Covalently immobilized enzyme retained 40 % of its residual activity while adsorbed enzyme retained 20 % after 25 days.	[122]
Alkaline phosphatase	Paper	–NH ₂	–COOH	Colorimetric	–	[123]
HRP	Modified Polymer Single Conical Nanochannels	–NH ₂	–COOH	–	–	[124]
HRP	MWCNTs	–NH ₂	–COOH	Amperometric	90 % of the initial current response after ten weeks when stored at 4 °C	[116]
Cholesterol oxidase	Polyaniline – MWCNT	–COOH	–NH ₂	Amperometric and Spectrophotometric	Retains more than 95 % of activity even after 12 weeks	[125]
Cholesterol oxidase	poly(3-thiopheneacetic acid)	–NH ₂	–COOH	Amperometric	The sensing current still retains 88 % of its original current even after 13 days.	[126]
Urease and glutamate dehydrogenase	N-DNW	–NH ₂	–COOH	Amperometric	80 % of enzyme activity was retained when maintained at 4 to 6 °C for less than 2 weeks.	[127]
Urease	Ethyl cellulose	–NH ₂	–COOH	–	–	[128]

Abbreviations: SWCNT: single-wall carbon nanotube; HRP: Horseradish peroxidase; MWCNT: Multi-wall carbon nanotube; N-DNW: N₂ incorporated diamond nanowire.

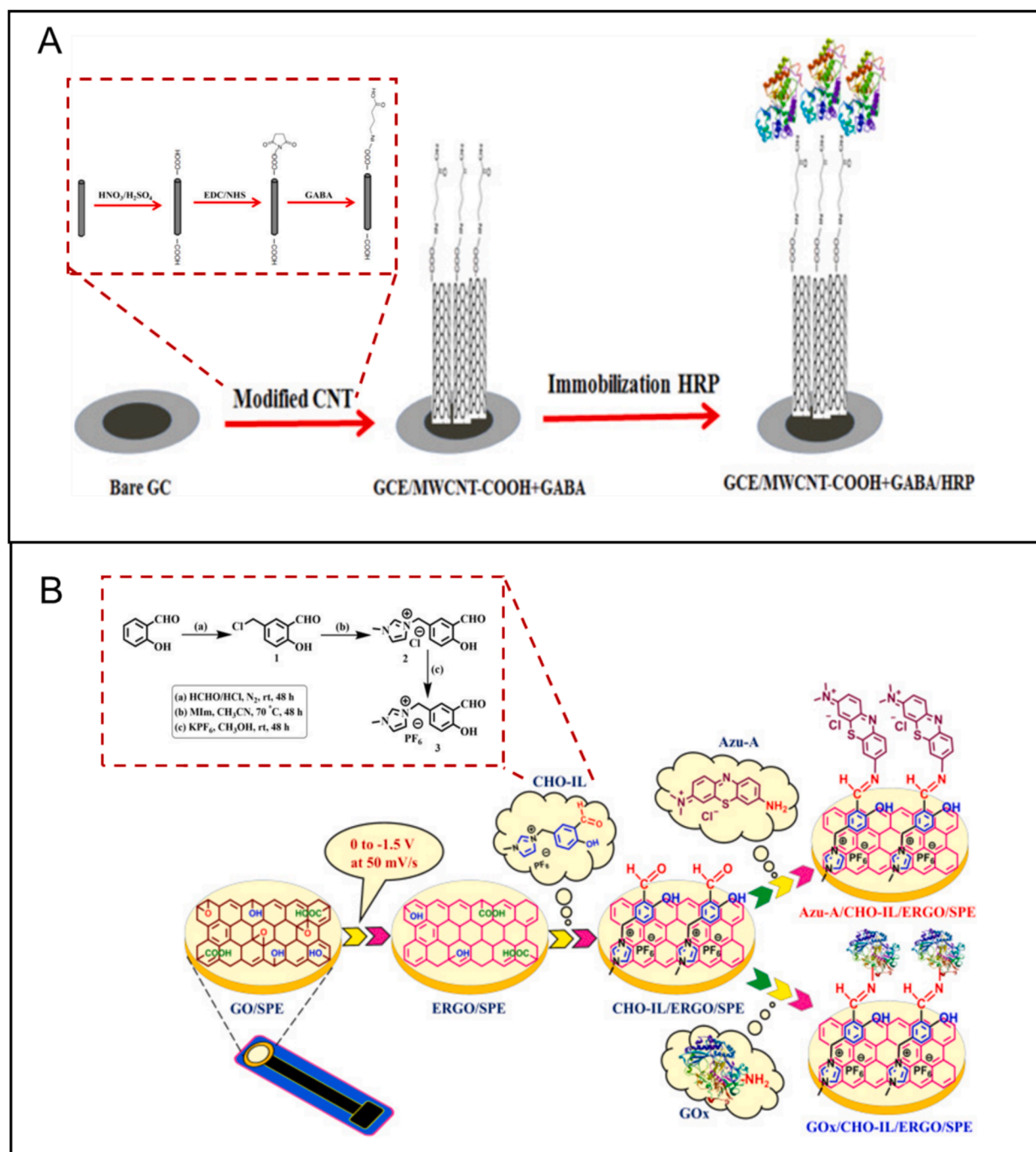


Fig. 5. A) A schematic representation of how Feizabadi et al. managed to modify carbon nanotubes with NHS to immobilize horseradish peroxidase. Adapted from [116]. Copyright 2019, with permission from Elsevier. B) The manufacturing process of the sensor developed by Manoj et al., where an aldehyde functionalized ionic liquid (CHO-IL) has been employed to immobilize glucose oxidase. Adapted from [135]. Copyright 2018, with permission from Elsevier.

4.3. Other covalent immobilization methods

Chemical immobilization techniques can be hazardous and expensive sometimes. Alternatives which are environmentally friendly, clean, dry, chemical-free, and economical like plasma treatment have been increasing in popularity. Plasma treatment creates radicals or adds reactive functional groups like amines and carboxylic acids to the material by modifying the surface properties of the material using ionized gases. Plasma can be used to either immobilize the biomolecules by directly interacting with the support or it can be used to create functional groups which can be later used for immobilization [150,151]. For instance, Yin et al. managed to covalently immobilize HRP using plasma

directly on the surface without using any linkers. Surfaces coated with plasma and immobilized HRP exhibited a significantly greater number of carbon bonds in comparison to plasma coated surfaces lacking HRP [152]. Alternatively, plasma treatment can be employed to decorate the surface of the support material with functional groups such as amines and carboxylic acids, which can subsequently be followed by the covalent immobilization of enzymes utilizing either carbodiimide chemistry or Schiff base reactions. Gancarz et al. effectively achieved the immobilization of glucose isomerase onto plasma-activated surfaces characterized by amine groups by utilizing glutaraldehyde to activate the amine groups present within the enzyme. [153]. Hence, one of the main advantages of plasma is the possibility of introducing functional groups

Table 4

Example of Schiff base reactions with different supports and enzyme.

Enzyme	Support	Functional Group of Enzyme	Functional Group of Support	Crosslinker	Sensor Type	Shelf Life/ Stability	Ref.
Glucose Oxidase	Aldehyde-functionalized ethoxysilane	–NH ₂	–COH	–	–	An increase of enzyme activity over a time of four weeks was observed	[142]
Glucose Oxidase	CHO-IL/ERGO/SPE	–NH ₂	–COH	–	Amperometric	Retained 91 % of its initial response at the end of 30 days	[135]
Lactate dehydrogenase	Optical Fiber	–NH ₂	–NH ₂	Glutaraldehyde	Optical	Good stability in the first 12 h	[143]
Alkaline phosphatase	Screen printed gold electrode	–NH ₂	–NH ₂	Glutaraldehyde	Amperometric	–	[144]
HRP	PANI-MWNT	–NH ₂	–NH ₂	Glutaraldehyde	Amperometric	–	[145]
HRP	CHO-AIL/AuNPs/GCE	–NH ₂	–COH	–	Amperometric	Retained 95 % of the initial response after 30 days.	[146]
Cholesterol oxidase	Polyaniline (PANI)	–NH ₂	–NH ₂	Glutaraldehyde	Amperometric	–	[147]
Cholesterol oxidase	Maghemite (γ-Fe ₂ O ₃) nanoparticles	–NH ₂	–NH ₂	Glutaraldehyde	–	Retained 50 % of its activity compared to free enzyme	[136]
Urease	Dialdehyde porous starch	–NH ₂	–COH	–	–	Retained 75 % of its initial activity	[148]
Urease	Nanocellulose-dialdehyde	–NH ₂	–COH	–	–	Retained 90 % of its activity after 35 days.	[149]

Abbreviations: CHO-IL/ERGO/SPE: Aldehyde functionalized ionic liquid/ electrochemically reduced graphene oxide/ screen printed carbon electrode; PANI-MWNT: polyaniline coated multi-wall nanotube; CHO-AIL/AuNPs/GCE: aldehyde-functionalized redox ionic liquid/ anionic gold nanoparticle/ glassy carbon electrode.

useful to chemical immobilization in materials (or surfaces) which do not naturally possess them. Notwithstanding the numerous benefits, plasma treatment is fraught with challenges, with system dependency being a significant drawback. This indicates that identical flow rates, gas pressures, and power inputs may not yield equivalent concentrations of the desired reactive species [154]. Furthermore, while the groups formed via plasma treatment can be somewhat adjusted via changing the gas used, the variety of groups formed may make this technique difficult to refine and control [155]. In particular, the formation of radical groups may lead to the formation of carbon–carbon bond between support material and enzymes, which may negatively impact enzyme structure and orientation [156].

Another alternative method is thiol-based immobilization of enzymes, which relies on the formation of a sulfur-sulfur bond. Only a few enzymes contain accessible free thiol groups (e.g., glucose oxidase and lactase) [157], while most enzymes contain disulfide bonds (S-S). As a result, one modality to create free thiols on the enzyme is to reduce disulfide bonds to generate thiol functionalities [111,158]. Another approach to introduce thiol functionalities onto the enzyme is to use EDC chemistry in conjugation with Cysteine (an amino acid) to facilitate functionalization. Lastly, Traut's reagent can be used to add thiol functionalities by using the primary amine groups of the enzyme [159]. Regardless, a thiol functionality on the enzyme can allow three types of immobilizations to the substrate: (i) the substrate contain thiols which facilitates formation of disulfide bonds with the enzyme [111,158]; (ii) the substrate contains unsaturated alkenes that can interact with thiols via thiol-ene chemistry (in presence of radical initiator) [157]; or (iii) the substrate contains maleimides which reacts with the thiols through click chemistry [160,161]. Particularly, the latter reaction is efficient at physiological pH (6.5–7.5), where maleimides preferentially react with thiols, while amines remain protonated and unreactive. Although thiols can also react with epoxides and NHS esters, these reactions are slower, and the resulting thioester bonds are less stable [162,163]. The main limitation of using thiol-based immobilization is the jeopardized enzymatic activity caused by artificial modification of the enzyme molecule. The other problem is preventing oxidation of thiol-functionalities of the enzyme, which can easily revert back to disulfide bonds within the enzyme structure.[96]. Overall, the depth of using this modality in enzymatic immobilization is shallow and more thorough research in this area is needed.

5. The effect of carbodiimide and Schiff base immobilization on enzyme activity

Carbodiimide and Schiff base reactions each present distinctive advantages and disadvantages for their application in enzyme covalent immobilization, summarized in Table 5. First, the pH is a crucial determinant, with most enzymes exhibiting peak activity within the pH range of 6 to 8 [164]. Although most enzymes are chemically stable outside this range, Schiff base reaction carried out under acidic conditions should be avoided, as they can significantly affect enzyme's activity, with only mild reaction conditions should be employed [165]. On the other hand, the influence of temperature in both approaches has minimal effect on enzymatic activity, as both reactions occur at room temperature [114]. The Schiff base reaction is more flexible in this regard, as it allows for the incorporation of crosslinkers or spacers such as glutaraldehyde to mitigate the risk of enzyme denaturation and enhance enzyme activity. In particular, the addition of a spacer can reduce the impact of the electrostatic interaction between the support material and the enzyme, thereby reducing the risk of denaturation. Furthermore, the utilization of spacers enhances the accessibility of the enzyme's active site to the substrate, hence increasing activity [166]. Nevertheless, the crosslinking process could inadvertently produce undesired byproducts.

The effect of immobilization methods on the enzymes significantly influences the performance of the biosensors. Given that both methods have distinct advantages and disadvantages, the selection of the immobilization process depends on the specific characteristics of both enzyme and the support material (i.e., the biosensor design). For instance, since Schiff base reactions are typically conducted at lower pH than carbodiimide reactions, it is preferable to use this method with enzymes that can withstand such harsh environmental conditions. For example, HRP has demonstrated to maintain its activity down to a pH as low as 5 [41]. The reversible nature of the imide bonds obtained via Schiff base method may also be suboptimal for biosensors intended for

Table 5

Comparison of carbodiimide and Schiff base reactions.

Parameter	Enzymes	Carbodiimide reaction	Schiff base reaction
pH	6 – 8 [164]	4.5—7.5 [114]	3 – 10 [167,168 141]
Temperature	Room Temperature	Room Temperature [114]	Room Temperature [114]
Spacer	–	Zero Crosslink	Spacer can be used

use in aqueous environments. The introduction of a spacer or linker is another crucial parameter that can significantly impact the performance of a biosensor. The presence of a spacer is particularly important when an enzyme is immobilized on a non-permeable stiff substrate. In soft, porous materials, such as paper or hydrogels, the substrate of the enzymatic reaction (i.e., the analyte) can penetrate and diffuse through the support material, allowing it to reach the enzyme's active site, thereby minimising the impact of its orientation and location [169]. Finally, the cost of the immobilization process can influence the choice of method, depending on the final application of the sensor (e.g., a single use biosensor needs to be as cost-effective as possible).

6. Techniques to assess enzyme activity after immobilization and immobilization efficiency

Although the significance of the immobilization process in maintaining the enzyme activity is well recognised, studies often neglect to quantify such parameters. This include both the immobilization efficiency, which is often well below 100 %, and the resultant enzyme activity. Various techniques have been employed to address this, each with its own advantages and drawbacks. It is also important to note that activity can be indirectly used to estimate immobilization efficiency, as this is assumed to be proportional to the activity of the immobilized enzyme [170]. However, this approach is entirely not accurate given that immobilization can sometimes denature enzymes, and activity is also influenced by other factors, such as enzyme orientation, electron transfer efficiency, and analyte molecules diffusion. Different methods are used for measuring immobilization efficiency such as UV-Vis absorbance, Bradford, and other techniques as mentioned in Nicolás et al. [171].

Sum frequency generation (SFG) spectroscopy relies on the combination of two incident beams, one in the visible range and one in the infrared range, to produce an output beam, characterized by the sum of the frequency of the two incident beams. The intensity of this output beam depends on the structural properties of the molecules or biological entities found at the interface as shown in Fig. 6 [172]. As the output beam is affected by the structural conformation of biological entities, SFG can be used not only to measure the activity but also to detect the structural changes and the orientation of the immobilized enzymes [173–174]. This technique is known to be non-destructive and surface-sensitive, but the traditional SFG is associated with high cost and complexity [175]. Furthermore, it is only suitable for analyzing molecules present at an interface or surface, making it challenging to analyze

systems where enzymes are absorbed or embedded in a matrix. For instance, Niaura et al. employed SFG spectroscopy to study the activity of lipase by monitoring the degradation of an amphiphilic O-palmitoyl-2,3-dicyanohydroquinone monolayer [176].

Spectrophotometry is the traditional method used to indirectly measure activity of immobilized enzymes, using either an assay or a surrogate molecule. This technique measures the intensity of light being absorbed, reflected, scattered, or emitted at a particular wavelength (see Fig. 6). It is applicable not only in the ultraviolet (UV) and visible (vis) ranges but also other wavelengths such as infrared and near infrared. The concentration of a known molecule can be quantified using the Beer-Lambert law [177]. Spectrophotometry does not directly measure the activity of the immobilized enzyme, instead, assays utilize spectrophotometry to measure the enzyme activity. For instance, in Mason et al., assay of p-hydroxybenzoic acid hydrazide in an alkaline solution, which reacts with glucose and fructose to produce yellow anions, was used to quantify invertase enzyme activity [178]. Similarly, in Khoshnevisan et al., the activity and stability of cellulase were measured using UV/Vis absorbance by measuring the amount of glucose released during the hydrolysis of carboxy methyl cellulose solution [179]. While spectroscopic method, including SFG, are generally considered non-destructive, exposing enzymes to various wavelengths, especially at high intensities or for prolonged periods, could potentially have detrimental effects on their activity [180]. The impact on different enzymes, whether free or immobilized, remain unclear and may depend on the enzyme's state and whether the effect is reversible.

Electrochemical methods such as protein film electrochemistry and photo-electrochemistry, impedance spectroscopy, rotating ring disk electrochemistry, microelectrode-based techniques, amperometry and chronoamperometry, and square wave voltammetry can be employed to study the electron transmission from enzymes to the conductor, providing a measure of the catalytic reaction kinetics (see Fig. 6) [181]. As diffusion and electron transfer efficiency may impact such measurement, making comparison between different designs complex, it is also possible to employ models by studying the kinetic and diffusion constants in a dynamic integrated model [182]. Such methods, which can be quite complex and require specific equipment, have variable accuracy (depends on the setup) and may be simpler to apply on an electrochemical sensor rather than other types of applications.

Quartz crystal microbalance (QCM) can also be used to assess the amount of substrate converted into product by the enzymes, quantifying the enzyme activity (see Fig. 6). QCM can weigh substances down to ng per cm², causing a change in the oscillation resonance frequency of the

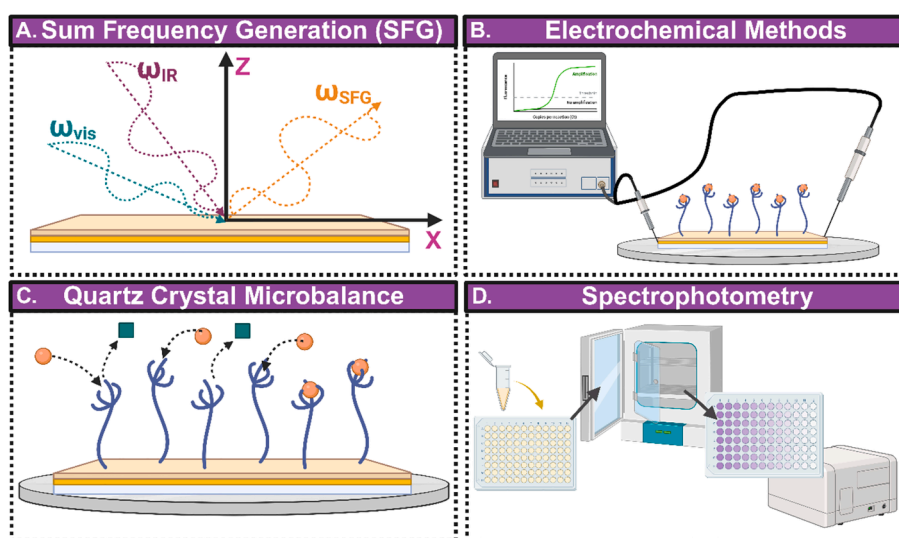


Fig. 6. Characterization techniques used for assessing enzyme activity and enzyme immobilization. Schematic illustration of A) SFG, B) an electrochemical method, C) QCM, and D) spectrophotometry (in this case an assay procedure using a UV/Visible spectrophotometer is shown).

quartz crystal, providing insight on the amount of material present over time [183]. For instance, Santos et al. used QCM to assess the activity of acetylcholinesterase immobilized on a gold electrode placed on the quartz crystal, measuring the amount of product leaving the detection zone [184]. In Su et al. the HRP was immobilized covalently and by non-specific absorption to the poly (ethylene-co-acrylic acid) films. QCM with dissipation monitoring was used to evaluate the activity of the immobilized enzyme, conformational changes and the binding behaviour. It was observed that the covalently bound enzymes showed higher activity compared to the adsorbed enzymes, and moreover, the covalently bound enzymes formed rigid films, whereas enzymes immobilized by adsorption technique formed dissipative films [185]. Both electrochemical and QCM methods require the enzyme to perform its catalyzed reaction, which should not compromise enzyme stability and activity. Nevertheless, other factors, such as the need of an electrolyte in some electrochemical methods, and the limitations imposed by techniques (e.g., necessity to detect electrons movement in electrochemical methods, and the requirement for the products to leave the microbalance in QCM), may limit their application. Table 6 summarizes the properties of the characterization techniques discussed, highlighting their advantages and disadvantages.

7. Future perspectives

Most studies on enzymatic biosensors focus on the effect of immobilization on sensitivity, selectivity, and stability. This focus is understandable, given that changes in enzyme activity directly affect these key performance parameters, which are crucial for evaluating sensor efficacy. In the field of sensing, detecting the analyte within the desired range, while producing the highest possible signal is paramount. However, the multifaceted effect of enzyme immobilization on activity and sensor response are often overlooked. In particular, the orientation of the immobilized enzyme on the support, its structural integrity, and potential conformational changes due to covalent bond formation are underrepresented in the literature (Fig. 7). These factors can directly impact sensitivity, selectivity, and stability.

Firstly, the involvement of specific functional groups of the enzyme in the immobilization process is significant. Covalent linkages can be established either via the N-Terminal or C-Terminal of an enzyme. However, when the N-Terminal is near the enzymatic active site, forming a covalent bond with this functional group may result in steric

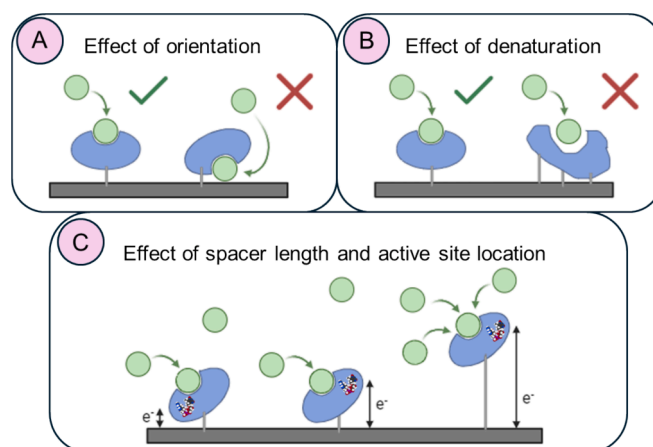


Fig. 7. Schematic representation of A) the potential effect of the immobilization orientation of the enzyme; B) the negative effect that multiple bonds can have on the enzyme conformation, deforming the active site and reducing activity; and C) the effect that the distance between the redox site (in this case indicated by FAD) and the support material or transducer can have on the distance electrons (e^-) have to travel. Note how when the bond is extended (as in the case of the use of a bridging molecule) the distance the electrons have to travel increases, but also the availability of substrate molecules.

hindrance, reducing substrate availability and impacting biosensor performance. This effect is independent of the presence of a spacer. Therefore, it is essential to ascertain the location of the enzyme's active site and functional groups and to allow covalent immobilization only at safe sites. Furthermore, in electrochemical biosensors, the location of the redox centre (often near the active site), is also crucial, as increasing the distance between this centre and the transducer (i.e., the conductor) can hinder direct electrons transfer. For example, immobilizing glucose oxidase through the C-Terminal is advisable as it provides the shortest electron transfer path from the enzyme to the support surface. In contrast, the N-Terminal is closer to the active site, and utilising it for enzyme-substrate binding may result in enzyme structural alteration and inactivation [189]. Additionally, further limitations and requirements may arise when immobilizing multiple enzymes on the same substrate [133]. Site-directed mutagenesis is a powerful technique that

Table 6
Immobilization and immobilization efficiency assessment techniques and their advantages and disadvantages.

Characterisation Test	Testing Mechanism	Effect on Enzyme	Advantages	Limitations	Ref
Sum Frequency Generator	Analyses the output beam resulting from the sum of two incident beam (one visible and one infrared) to provide enzyme structure information	No degradation No loss of enzyme activity	Non-destructive Surface-sensitive Can detect structural and orientation change	Highly complex High cost Cannot detect entities embedded in matrix or adsorbed enzymes	[172 173 175]
Electrochemical Methods	The electron transmission from enzymes to conductor is analysed, providing catalytic reaction kinetics.	Voltage applied can reduce the activity of the enzyme. Electrolyte may affect enzyme	May be hard to compare cross-study results Provides enzyme activity, conformational change and binding behaviour	Requires specific equipment Susceptible to false signals due to cross-reactivity	[186 181]
Quartz Crystal Microbalance	Measures the amount of product mass leaving the scale, quantifying the enzyme activity	Non-destructive as it is a surface sensitive analytical technique, focusing on surface immobilisation of the enzyme	Sensitivity down to ng cm^{-2} Provides enzyme conformational change and binding behaviour Independent from support material	Susceptible to noise from external vibrations QCM crystals are fragile and expensive	[187 183]
Spectrophotometry	Measures the intensity of the absorbed/transmitted/reflected/scattered/emitted light to provide kinetic or structural information about the enzymes (either via assays or spectroscopy)	Intense or prolonged exposure to light can damage the enzyme (especially UV)	Reproducible Sensitive	Indirect activity assessment (assays) Sample pretreatment required	[188 177]

allows for the precise introduction of additional functional groups at specific sites on an enzyme, away from its active site. By strategically placing these functional groups, researchers can achieve directed immobilization, ensuring that the enzyme is attached to a surface in a controlled orientation. For example, De Simone et al. constructed 41 variants of NOV1 dioxygenase and combined the six most active ones to obtain two final variants with two and three mutations each [190]. These variants exhibited an activity ten times higher than the wild enzyme and also a greater operational stability. The addition of functional groups can also enable rigidification through multipoint covalent bonding, which stabilizes the enzyme's structure by forming multiple connections with the support material, although decreasing the activity [87]. In particular, Weltz et al. demonstrated how lipase activity decreases proportionally to the amount of amide bonds formed with the support material [87]. Nevertheless, chemical and physical stability of the enzyme are greatly improved, leading to the enzyme being active up to 80 °C when forming multiple bonds, and only up to 55 °C with a single bond. This approach may be particularly useful for applications in biosensors, biocatalysis, and other areas of biotechnology, where constant catalytic properties are necessary, although further studies are required [191,192].

Secondly, maintaining the enzyme's structure during immobilization is crucial, as it affects the catalytic functions. However, random covalent linkages have the potential to alter the enzyme structure, leading to denaturation [193]. Consequently, it is crucial to investigate how different immobilization methods and support materials influence the enzyme's conformation. Limited research has been conducted on the conformational changes of immobilized enzymes, likely due to the challenges associated with assessing such changes in immobilized proteins. Various analytical techniques such as UV/Visible spectroscopy and FTIR (Fourier transform infrared) spectroscopy can be utilized to study conformational changes in enzymes [194]. While such conformational changes can reduce enzyme activity, it is essential to consider that factors beyond conformational alterations can also affect enzyme activity. The use of linkers or spacers can help alleviate stress between the enzyme and the support material, preventing structural changes and denaturation [195]. Positioning bonds away from the enzyme's active site may lead to fewer conformational changes [196]. Future research should focus on ensuring strong enzyme-substrate interactions with minimal conformational alterations. This may be achievable through computational modelling to verify the effect of a bond on the enzyme structure before conducting experiment, as demonstrated by Feizabadi et al. [116].

In a similar way, immobilization techniques often lack precision in controlling the orientation of the immobilized enzyme, hindering substrate accessibility to the enzyme active site. While covalent bonding allows a better control over orientation compared with other physical immobilization techniques, it is still suboptimal, as bond formation is narrowed down to a family of functional groups rather than a single one. The analysis of the enzyme orientation on the surface is usually carried out via SFG spectroscopy and attenuated total reflection FTIR [195]. Various techniques, including bio/chemical affinity-mediated immobilization, chemically covalent immobilization, and molecular imprinting, are deliberated by Liu et al for the purpose of regulating the orientation of the immobilized enzyme [197]. For instance, slow immobilization processes using lowly activated amine supports have been used to promote specific enzyme orientations under certain conditions [191]. However, these specific orientations do not always align with the desired outcomes and rapid immobilization processes are more effective in retaining enzyme structure and activity, particularly on nano-structured support materials [198]. An alternative approach to control orientation involves incorporating peptide tags at the N- or C-terminal of the protein [191]. These tagged enzymes can subsequently be affixed to the anti-tag antibodies via affinity tags. Although this method results in site-specific immobilization and restores enzyme activity, it is notably complex and limited to enzyme terminals. For instance, glutamate

decarboxylase with SpyTag (a bridging molecule) was immobilized on silica nanoparticles with SpyCatcher mutants, controlling enzyme orientation without affecting its function [199]. The enzyme was oriented away from the support, resulting in better stability and activity compared to the free enzyme, with nearly a 10 % difference in activity after 30 days. As an alternative to tagging, cysteine residues can be integrated into enzymes by conjugating them to thiol-reactive supports [191]. For example, cysteine-modified antibodies have been covalently immobilized to maleimide-group-functionalized support material, increasing sensitivity by up to 15 times [200]. Nevertheless, site-directed immobilization techniques are often costly and intricate. In contrast, electrostatic interactions between the support and enzymes can affect enzyme orientation [197]. Using electric fields to control orientation is an emerging technique that offers a simpler, more economical, and efficient approach. During the immobilization process, electric fields are applied to manage the orientation of biomolecules, which can also be influenced by adjusting the pH or modulating the electric field. For instance, Laccase was immobilized on graphene, and an external electric field was used to orient the enzyme, resulting in higher electron transfer rate compared to physically immobilized enzymes [201,202].

Prospective research endeavors should focus on investigating orientation, structure, and conformational adjustments resulting from diverse immobilization techniques to enhance the efficacy of biocatalysts and biosensors.

CRedit authorship contribution statement

Theja Prabhakar: Writing – review & editing, Writing – original draft, Conceptualization. **Jacopo Giaretta:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Conceptualization. **Riccardo Zulli:** Writing – review & editing, Supervision. **Ronil J. Rath:** Writing – review & editing, Writing – original draft, Visualization. **Syamak Farajikhah:** Writing – review & editing, Supervision. **Sepehr Talebian:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Conceptualization. **Fariba Dehghani:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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