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Review

Smart chemistry of enzyme immobilization using various support matrices – A review

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ABSTRACT

The surface chemistry, pendent functional entities, and ease in tunability of various materials play a central role in properly coordinating with enzymes for immobilization purposes. Due to the interplay between the new wave of support matrices and enzymes, the development of robust biocatalytic constructs via protein engineering expands the practical scope and tunable catalysis functions. The concept of stabilization via functional entities manipulation, the surface that comprises functional groups, such as thiol, aldehyde, carboxylic, amine, and epoxy have been the important driving force for immobilizing purposes. Enzyme immobilization using multi-functional supports has become a powerful norm and presents noteworthy characteristics, such as selectivity, specificity, stability, resistivity, induce activity, reaction efficacy, multi-usability, high catalytic turnover, optimal yield, ease in recovery, and cost-effectiveness. There is a plethora of literature on traditional immobilization approaches, e.g., intramolecular chemical (covalent) attachment, adsorption, encapsulation, entrapment, and cross-linking. However, the existing literature is lacking state-of-the-art smart chemistry of immobilization. This review is a focused attempt to cover the literature gap of surface functional entities that interplay between support materials at large and enzyme of interest, in particular, to tailor robust biocatalysts to fulfill the growing and contemporary needs of several industrial sectors.

1. Introduction

With extensive industrialization and the looming prospect of anthropogenic climate change, green chemistry has enticed substantial research attention due to its proven benefits. Advances in bioinformatics, genomics, and protein engineering have shaped biocatalysis as an attractive green toolbox compared to chemical catalysis, which generates much hazardous chemical waste. Enzymes are biodegradable, biocompatible, amenable to industrial needs by using protein engineering or directed evolution, obtained from inexpensive and renewable sources, generate relatively less waste, and have exquisite selectivities [1,2]. Compared to metal-based catalysts, enzymes are cost-effective, and less energy is needed to perform enzymatic reactions. However, enzymes in aqueous media are usually on a throw-away basis that is neither economic nor consistent with the circular economy concept, hampering the general practical implementation of the enzymes as robust biocatalysts. Enzyme immobilization might be a practical approach to overcome these limitations. In addition to these factors, the technical need for enzyme immobilization arises from the following characteristics [3]: (1) The hydrophilic protein particles would aggregate when suspended in a hydrophobic environment, leading to enzyme inactivation. (2) The lack of long-term stability, complex downstream processing, low enzyme productivity, and the possibility of product contamination due to free enzyme highlight the need for efficient enzyme immobilization technology.

Immobilized enzymes have shown promising results in terms of thermal, storage, and pH stability [4], easy separation of the product, reusability of the catalyst, catalysis of the unnatural substrates, and

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novel reactions [1] and augmented enantioselectivity [5,6]. Further, immobilized enzymes cannot easily penetrate the skin, so they exhibit low allergenicity. They are easy to be separated from the reaction medium, thus minimizing the protein contamination of the product. High enantio-, chemo- and regioselectivity, and operability under mild experimental conditions have led to a surge in research and industrialization of the enzymes [7–9]. Despite these benefits, immobilized enzymes usually show decreased activity compared to their free counterparts. Generally, a trade-off is needed between enzyme activity and immobilization to harness the benefits of immobilization. It is worth mentioning that the success or failure of the immobilization needs to be proven separately for each enzyme, necessitating in-depth knowledge of the enzyme immobilization chemistry.

Immobilized enzymes have garnered substantial attention as a toolbox for numerous applications. Several approaches have been designed and used for enzyme immobilization, using different support materials for biotechnological and biomedical applications [10–13]. A two-step covalent immobilization approach was employed to immobilize inulinase from *Aspergillus niger* NCIM 945. The immobilized catalyst was applied to the continuous hydrolysis of inulin [14]. Advances in enzyme immobilization approaches have led to increased use of enzymes in biomedical and manufacturing applications [15]. Although challenging, the combination of nanotechnology with enzyme immobilization approaches seems appealing due to the combinatorial catalytic potential of nano-materials and enzymes. Such bio-nanoplatforms are of great interest to produce therapeutic nano-platforms and biosensing devices [15].

Enzyme immobilization entails the interaction between the enzyme and the support material used for immobilization (Fig. 1). For efficient immobilization, it is vital to know the structural and functional entities of enzyme surfaces and support matrices. Further, the composition of the reaction medium for enzyme immobilization and post-immobilization application is also important. This review starts with an overview of the important parameters that need to be considered when planning an immobilization approach for the biocatalyst, followed by a detailed discussion on enzyme immobilization chemistry.

2. Structural and functional entities of support matrices

The important parameters of the support matrix include surface area, particle size, organic or inorganic nature, surface charge, hydrophobicity and hydrophilicity, surface functionalization, chemical, and mechanical stability, and porosity of the support material. The surface properties of both enzyme and support are taken into account for the immobilization process. The support can be modified according to the surface properties of the enzyme to make it chemically compatible with the enzyme [16,17]. The large surface area of the support is crucial that

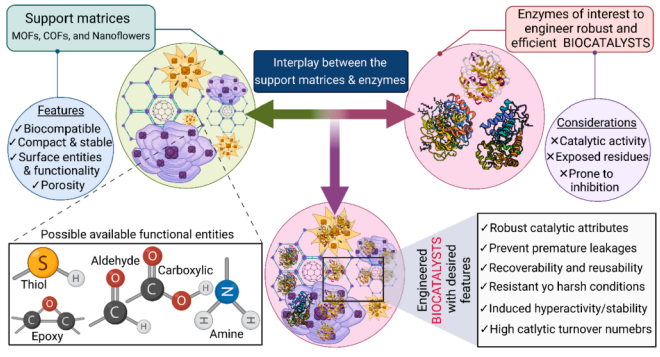

Fig. 1. Illustrates the possible interplay between support matrices (i.e., MOFs, COFs, and nanoflowers) and enzymes of interest to engineer robust and efficient biocatalysts. The scheme also includes possible available functional group entities that interplay along with key considerations and features of support matrices and engineered biocatalysts. Created with BioRender.com.

can be achieved by decreasing the particle size of the support. However, decreasing size may affect the easy separation of the immobilized enzyme. The large pore size of the support facilitates the easy diffusion of substrate and product from the enzyme to the reaction medium and vice versa, improving the catalytic efficiency of the immobilized catalyst. The synthesis of mesoporous materials has garnered substantial research attention over the last few decades [18]. This approach is appealing since the size of the pores and shells are tunable and can be tailored to the desired application [19]. Enzyme immobilization on inorganic supports has furnished biocatalysts with improved catalytic properties [20]. Mesoporous silicates are widely explored support matrices for enzyme immobilization [21]. The size of the mesopores can be altered by optimizing reaction conditions and selecting an appropriate surfactant template to produce ordered pores. The sufficiently large pore size and ordered pore structures help enzyme adsorption and easy entrapment of large-sized enzyme molecules [22].

Solution pH and isoelectric point of the enzyme result in charged enzyme surface that can be either positive or negative depending on the pH of the surrounding medium. The surface charge of the support material has been extensively explored for enzyme immobilization, where ion exchangers efficiently immobilize enzymes based on strong polar interactions. His tag-based enzyme immobilization has been widely reported in the literature [23,24]. The imidazole ring of the histidine residues has been exploited to act as a metal ion binding ligand. Metal ions such as Cu^{2+}, $Co^{2+,}$ or Ni^{2+} have shown promising results for enzyme immobilization.

The mechanical and chemical stability of the support material also governs the success of the immobilization process. Further, the pore size of the immobilization matrix in the case of enzyme encapsulation is of paramount importance for desirable results. For instance, glucose oxidase was entrapped in a semipermeable membrane in a two-tier immobilization process. The surface of the enzyme was first functionalized with vinyl/acryloyl groups by acryloxylation. Then acryloxylated molecules were encapsulated in the stable polymer resulting in a nano encapsulated biocatalyst where the internal core contains enzyme while the external layer forms a thin polymer shell [25]. The immobilized biocatalyst exhibited excellent properties with enhanced enzyme stability and improved activity. Tyrosinase nanocapsules were prepared by enzyme acryloxylation followed by encapsulation in a polymer shell. The immobilized biocatalyst showed enhanced stability and improved electrocatalytic activity [26,27]. The covalent immobilization usually involves functionalizing the surface functional groups of the support matrix where the enzyme is anchored on the modified support.

3. Structural and functional entities of enzyme surfaces

Important factors influencing the efficiency of the enzyme immobilization process include conformational flexibility, isoelectric point, surface functional groups, glycosylation, stability during immobilization, hydrophilic and hydrophobic regions, and presence of additives (Fig. 2). Depending on the pH of the reaction medium and the isoelectric point of the enzyme, the enzyme's surface bears charges. These surface charges have been widely explored for rapid and straightforward enzyme immobilization. This approach significantly retains enzyme activity as the interaction between support and enzyme is relatively weak, and the enzyme's active site is least disturbed upon immobilization [28]. The charges on both the support and enzyme are important for enzyme immobilization. All support materials are not uniquely suitable for the immobilization of each enzyme. Therefore, support materials are modified for immobilization purposes. The supports are modified by using modifiers that can anchor to support from one side and bind to enzymes from the other side [29]. Similarly, the enzyme surface can also be modified by functionalizing the enzyme surface. In addition, protein engineering has enticed substantial attention for tuning enzymes for different applications [30,31]. The support-specific linkers are co-expressed with the enzyme. Upon mixing with the support, the linker

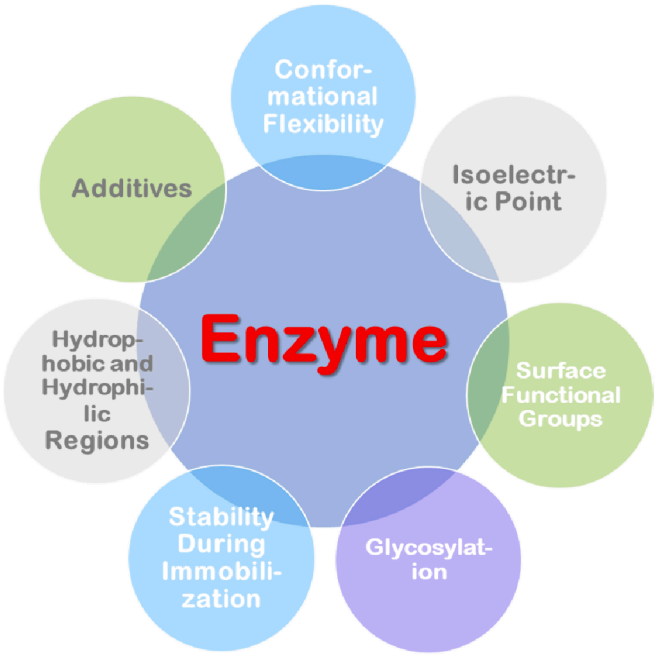

Fig. 2. Essential features of biocatalysts taken into account for designing an immobilization approach.

selectively binds to support, resulting in immobilization of the enzyme [32]. Linkers/spacer arms endow functional flexibility to the enzyme and minimize the steric hindrance, maximizing enzyme activity. Moreover, the surface engineering of the enzyme is another promising approach where the enzyme surface is engineered for oriented enzyme immobilization and optimization of enzyme-surface interactions for enhanced biocatalysis [33].

The presence of inhibitors in the reaction medium hampers enzyme catalysis. Compared to immobilized enzymes, free enzymes are relatively prone to inhibition. Upon immobilization, the orientation of the enzyme inhibition site is changed. As a result, the inhibitor fails to bind the enzyme. The steric exclusion of the inhibitor from the enzyme inhibition site without affecting the enzyme active site minimizes or prevents enzyme inhibition. For instance, it was stated that lactase inhibition might be minimized by inducing partial distortion in its inhibition site or blocking the inhibition site by carrier-based immobilization [34]. The pH effect and presence of detergents in the reaction medium may significantly influence the enzyme activity. Enzyme encapsulation in mesoporous materials may protect the enzyme from inactivation. Since the enzyme remains inside the encapsulating agent, the diffusion of the inhibitors inside the pores may be hindered due to immobilization, resulting in the stability of the enzyme. In addition to encapsulation and isolation of the enzyme from the reaction medium, the support may also have a buffering effect, enabling the enzyme to work in a harsh pH environment [34]. It is worth mentioning that the control of the pore size of the polymer is crucial for the success of the immobilization process. Smaller pore size may lead to diffusion limitation. The enzyme is usually immobilized inside the macropores of the encapsulating polymer. Large pore size may facilitate the diffusion of reagents from the outer environment to the enzyme active site and vice versa.

Heterologous enzyme expression may result in altered post-translational modifications such as glycosylation of the protein. The immobilization behavior of the glycosylated enzymes may significantly differ from the non-glycosylated ones. For instance, the effect of glycosylation on immobilization of feruloyl esterase was studied by Bonzom et al. [35]. The immobilization yield of the glycosylated enzymes was different from the non-glycosylated enzymes that might be due to the difference in surface charge of the protein resulting from glycosylation. Further, significant variation was observed in the enzymes glycosylated in different host organisms. Therefore, glycosylation can also be taken into account while planning an immobilization method. Additives such as bovine serum albumin (BSA), polyols, and sugars are added to the reaction medium during enzyme immobilization. Additives protect the enzyme from the harsh reaction environment. For instance, BSA as a feeder/co-immobilizer has shown stabilizing effect on enzyme stability and also improved immobilization yield in many studies [30,36,37].

4. "Smart" chemistry of immobilization

During reaction or other activities, biomolecules may leach into aqueous media. But the covalent attachment of biomolecule at various surfaces may give immobilized biomolecule. This is formed by the interaction of the enzyme with functional moieties of support. Different functional moieties, including thiol, carboxyl, thiol, amino, etc., can participate in immobilization phenomena. Availability of specific functional groups on biomolecule and substrate determines the type of immobilization reaction. The reaction may occur directly between functional moieties of biomolecules or support, and sometimes activation step is required to activate the functional moieties. Here we have discussed various kinds of chemistries involved in the covalent immobilization of biomolecules onto the support.

4.1. "Click" chemistry involved between the support and catalyst

1,3-Dipolar addition reaction of alkynes and azide catalyzed by a catalytic amount of Cu(I) yielding heterocyclic five-membered triazoles known as click chemistry, and this was introduced by Sharpless in 2001 (Fig. 3) [38]. During the last decades, click chemistry has become an important platform for modifying biomolecules [39]. The azide and alkyne functional moieties can be conveniently incorporated, and they are stable independently and do not react with other functional moieties of the biomolecule. Triazole formation is quantitative and takes place under mild conditions in the presence of a copper catalyst that is indifferent to pH and solvent. This reaction provides various advantages, including high product yield, easy separation of by-products, regioselectivity, etc. Owing to vast potential and scope, this reaction can significantly modify an extensive range of complex substances by transferring its properties to surface-bound reactants, giving access to various emerging functionalized surfaces [40]. In this process, firstly biomolecule can be activated via click functional moiety (either alkyne or azide), which subsequently couple with other surfaces having compatible moiety (either alkyne or azide) to provide stable conjugate. Thereof click chemistry may provide a robust approach for biomolecular surface immobilization through conjugation that involves joining the required substrate with a specific biomolecule. Alkyne and azide functional groups rarely exist in the biological system. Thus, it may add versatility, and alkyne and azide moieties can be incorporated into biomolecules. And this would enable the attachment of biomolecules having either azide or alkyne with different substrates having counter moieties [41]. Recently a study was conducted, and laccase was immobilized on magnetic nanoparticles (Fe_3O_4) via click chemistry, and this synthesized biocatalyst was employed to dimerize phenylacetylene. This heterogeneous enzyme showed great catalytic potential and was easily separated from the reaction mixture [42]. In another study, an alkyne-terminated PEGylated surface was conjugated with azide having biomolecule (protein, biotin, and carbohydrate) through click chemistry in the presence of aqueous media at low temperature [43]. The incorporation of azides and alkyne moieties in biomolecules does not affect their stability. Recently biotin-cellulose conjugate membrane was synthesized immobilizing biotin onto cellulose via click chemistry. Alkyne moieties were substituted in cellulose acetate and coupled with azide containing biotin via click chemistry to yield the biotin-cellulose conjugate membrane [44].

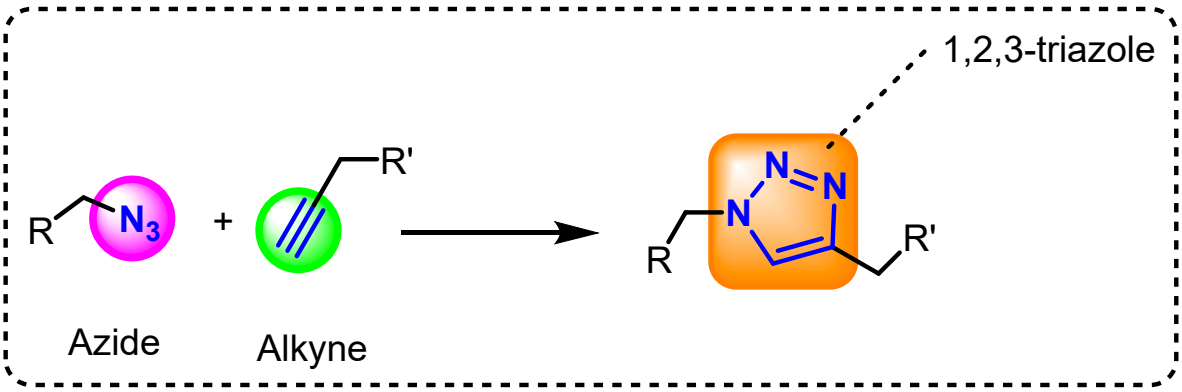

Fig. 3. Representation of triazole formation through the reaction between an azide and alkyne functionalities.

Enzymes can be immobilized on surfaces of materials having excellent surface area and conductivity as multi-walled nano-materials. Using click chemistry, small Laccase enzymes (SLAC) were immobilized on a multi-walled carbon nanotube (MWCNTs) electrode. This was carried out by incorporating an unnatural amino acid (4-azido-*L*-phenylalanine) (AzF) having azide moiety into specific sites of the laccase enzyme. At the same time, MWCNTs electrode was functionalized with alkyne moiety through a linker (cyclooctynyloxyethyl 1-pyrenebutyrate) (PBCO). Then, the copper-free Click reaction directly immobilized the laccase on the MWCNTs electrode. This modified electrode showed high direct electron transfer efficiency and stable current [45].

Site-specific covalent attachment is an interesting approach, and for both basic and applied research regarding proteins, their immobilization is important. Click chemistry was involved in the immobilization of enhanced green proteins (eGFP) [46]. The reaction conditions were robust and better results were obtained. Further triazole linkage was quantified, and it can bear Nn-level force, so the mechanical strength of click bond can be investigated through this. Many click reactions have been reported as excellent immobilization methods [47–49]. These reports focused on azide-alkyne cycloaddition. Copper-catalyzed click reactions have various advantages, including tolerance to a wide range of functional moieties and work over a wide range of temperature and pH (4–12). However, the copper is cytotoxic at threshold concentration, and copper metal residues can cause severe problems if the end use is of biological interest. So other approaches have been reported for azide-alkyne cycloaddition like strain promoted azide-alkyne cycloaddition click reaction (SPAAC) [46], ruthenium (RuAAC) [50] or silver catalyzed azide-alkyne cycloaddition (AgAAC) [51] catalyzed click chemistry leading to variously functionalized triazoles. In SPAAC, the pedant alkyne is replaced with strained alkynes and SPAAC is slower reaction in comparison to CuAAC.

4.2. Amine chemistry involved between the support and catalyst

Replacement of hydrogens of ammonia with alkyl or aryl moieties provides various kinds of amines (primary, secondary, tertiary). Amine functional moieties play an important role in immobilization strategies. For example, two types of Amine moieties are present at surface lysine: (1) N-terminus amine group (pKa < 7.5), which reacts in neutral pH conditions, (2) ε-NH2 groups are present in abundance (pKa $= 10.5$) reacts in alkaline conditions to stimulate protein immobilization on a support having activated electrophilic moieties. The existence of amine moieties on the surface made them valuable for easy and efficient immobilization. Amine chemistry for immobilization does not require pre-activation and catalyst. Thus, unnecessary modification of enzyme surface chemically is impeded, and high efficiency of the enzyme is achieved upon immobilization. The presence of free electron pairs on deprotonated amine moieties endowed them with nucleophilic properties. So they can interact with various electrophiles as epoxy, vinyl or carbonyl functionalities present on the support. Even though lysine has many amine moieties on surface, their reaction with functionalized support materials is difficult, and optimizing immobilization protocols can be achieved. Under alkaline media, the attachment between the ε-NH_2 moiety and electrophile support is obtained [52]. For immobilization of proteins, the reaction of amines with aldehyde functionalities has been practiced widely by employing various supports [53,54]. The reaction between amine and aldehyde creates imine linkage, which further creates secondary amine linkage after reduction, which is more stable than imine [53]. Various selective mild reducing agents, e.g., sodium borohydride, boranes, hydrides, have been used to transform unstable imine linkage to stable secondary amine for strong attachment to support [53].

Stabilization of enzyme through the covalent attachment on a support containing aldehyde functionalities has been reported where picoline borane was used as a reducing agent [53]. Similarly, protein immobilization through NH_2 on the aldehyde functionalized surface has been reported for function determination [55]. Recently *Rhus vernicifera* laccase was immobilized through covalent attachment onto magnetic nanoparticles (Fe_2O_3, Fe_3O_4). Nanoparticles were activated by aminopropyltriethoxysilane followed by glutaraldehyde. The enzyme was immobilized onto Fe_2O_3 particles more efficiently due to its small size and good surface area compared to Fe_3O_4. Immobilized laccase performed an excellent degradation of bisphenol A [56]. Chitosan has been widely used for the immobilization of various enzymes, and for this purpose, chitosan can be activated by the derivatization of the amine group with glutaraldehyde. The exposed aldehyde moiety of glutaraldehyde then can react with an amino group of enzymes for its immobilization. Glutaraldehyde activated chitosan has been used in the immobilization of many lipases. In a study, chitosan was activated with the help of ethylenediamine and glutaraldehyde and employed to immobilize the lipase using a fluidized-bed dryer system [57]. The amine group of proteins (all biomolecules) can readily react with N-hydroxysuccimide (NHS) ester or other activated esters to yield stable, strong amide linkage [58]. The formation of amide linkage has been presented in Fig. 4. This reaction has one disadvantage of using NHS esters because they are not stable in aqueous solutions, so, during the reaction, hydrolysis of the ester may also happen along with immobilization. Alanine was immobilized in kaolinite through amidation, and this reaction was boric acid which leads to the synthesis of biohydride materials [59].

Lysine residues present on the surface of the enzyme also undergo multiple attachments under high pH. This helps to overcome the unstability of the imine linkage between amine and aldehyde. Due to these numerous covalent bond formations, the enzymes quickly immobilized with appropriate functionalized support. This multi-attachment occurs when a greater density of lysine residues is present (at the enzyme's surface). This phenomenon may constrain conformational flexibility [60]. Amine-containing chitosan was used for immobilization of

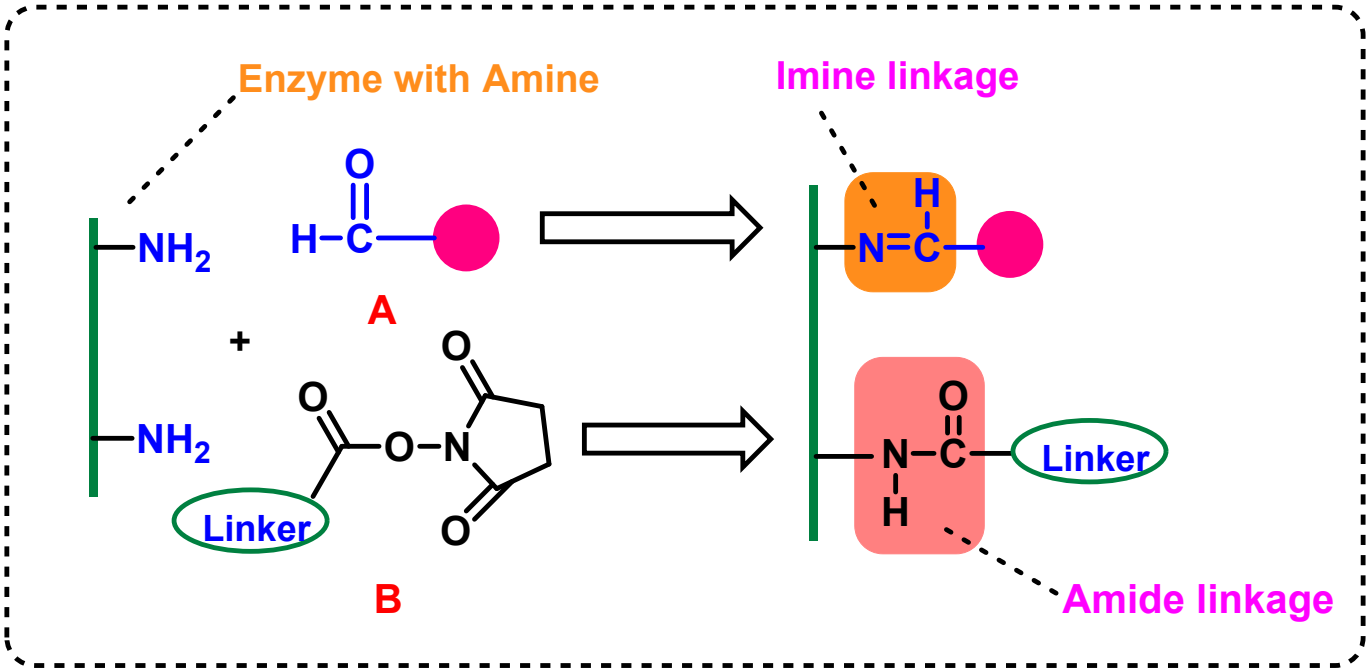

Fig. 4. Representation of amine chemistry for immobilization of enzyme by using surfaces either functionalized with an aldehyde (A) or NHS ester (B).

peroxidase by using gold-based nano-materials [61].

4.3. *Thiol chemistry involved between the support and catalyst*

Coupling of functional thiol moiety with alkenes is a known thiol-ene click reaction. Cysteine is an amino acid present naturally in peptide chains [62] and this amino acid contains thiol side chains, so this chemistry is necessary for the conjugation of biomolecules. Cysteine plays an important role in binding and catalytic practices. The unique features of cysteine impart a key role in synthesizing various molecules, functionalizing surfaces, immobilizing enzymes, and modifying polymers [63]. Thiol moieties of cysteine residues are highly reactive, and are their nucleophilic character made them eligible to react with various kinds of electrophiles. These moieties play an important role in the immobilization of proteins and they undergo a Michael addition reaction with carbon-carbon double bond to form stable thio-ether bonds [64] (Fig. 5). This addition reaction takes place at a wide range of pH values (6.5–7.5), and in this condition, usually, amines are unreactive, but cross reactivity with amines may happen under alkaline conditions. This kind of conjugation has some drawbacks as; 1) artificial modification of biomolecules require if it does not contain cysteine residues 2) if biomolecules contain many cysteine residues, then lack of selectivity would occur 3) if cysteines reside within active sites of biomolecules, then activity may be lost. Cysteine tagged proteins were immobilized on surface of electrodes through thiol-ene click reaction. Firstly, electrode surfaces were modified with vinyl phenyl moieties. Then dehydrogenase-based proteins were immobilized on the surface of the electrode via reaction of the vinyl group with thiol moiety of proteins. These conjugated electrodes were used for bio-electrochemical reactions [65].

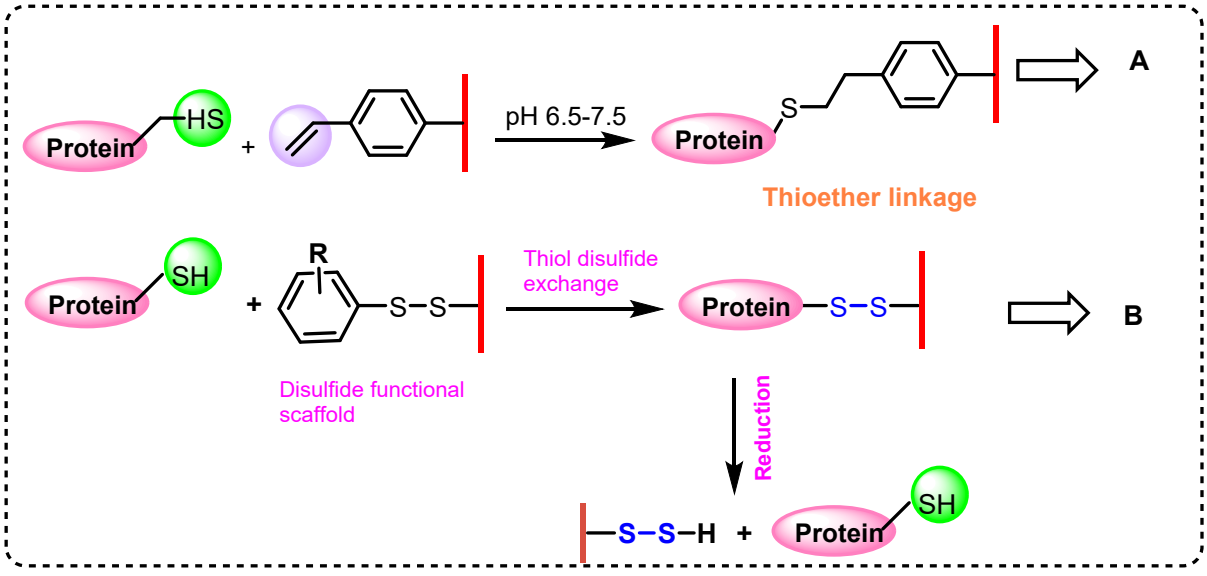

Fig. 5. Thiol chemistry in immobilization of biomolecules. General Representation of A: Immobilization of protein through the reaction of thiol with C═C. B: Reversible Covalent Immobilization of biomolecule via Disulfide Bonds.

Enzymes immobilized in reactors give a fast rate of reaction, easy to use, excellent stability. Immobilized enzyme reactors have many benefits in performing bio-catalytic reactions compared to conventional techniques as they consume less energy, high efficacy, quick exchange of heat, etc. [66]. Thiol ene click reaction played a role in synthesizing immobilized trypsin reactor based on trimethylolpropane trimethacrylate (TRIM) organic monolith. These reactors showed great potential in high-throughput proteomics analysis [67]. Green synthesis of monolithic trypsin immobilized enzyme reactor has also been reported via thiol-ene click reaction used for enzymatic hydrolysis of proteins [68]. Chen and his colleagues reported the fabrication of a monolithic trypsin microreactor through thiol-ene click approach. The -ene functional containing monolithic capillary column was synthesized by sol-gel method, then disulfide bond of trypsin was reduced to thiol moiety. Trypsin thiol moiety was attached with -ene functionality of hybrid monolithic column to form trypsin immobilized microreactor which was used for digestion and separation [69]. Thus thiol-ene click reaction is promising approach for construction of enzyme reactors for selective immobilization of proteins in mild reaction conditions.

Vinyl sulfones addition is an attractive approach for the conjugation of proteins. Thiols react with vinyl sulfones to give stable thioether linkage. Several reports are available in the literature regarding immobilization of biomolecules on support by using vinyl sulfones [70,71]. Various vinyl sulfone with carbohydrates, fluorescent tags, and biotinylation reagents was developed and used to conjugate proteins [72]. Vinyl sulfones have been used to conjugate enzymes to proteins [73]. Polyvinyl sulfones could provide functionalized tags [72]. Thiol moieties can carry out thiol-disulfide exchanges, which assists their reaction with various disulfide moiety-containing carriers, leading to reversible disulfide linkage between protein and carrier [74]. Disulfide linkage can be transformed to irreversible thioether linkage through reduction and this reaction is called as Thio-Mitsunobu reaction [75]. Cysteine is not an appropriate moiety for multi-valent enzymes immobilization; because of this, the biocatalyst may get highly stabilized. Orientation of proteins may be controlled during immobilization by engineering enzyme surfaces with cysteine residues which lead to the synthesis of highly robust and efficient biocatalysts. Chitosan hydrogels containing disulfide linkages towards reversible thiol immobilization were synthesized. Chitosan was modified with polyethylene glycol (PEG) moieties. Disulfide linkage was incorporated into a gel by Michael's addition reaction [74].

4.4. Carboxyl chemistry involved between the support and catalyst

Carboxylic acid functional moieties are present in the structure of a protein. They may be located either at C-terminus or as side chains. Aspartic and Glutamic acid acids contribute as major surface moieties on proteins. Still, because of the low reactivity of carboxylates in aqueous media, it becomes difficult to conjugate proteins at these groups. Covalent immobilization is critical through carboxylic moieties due to bonding within side chains of amino acids to hold the protein in different conformations. Carboxylic acids can be transformed into different reactive esters by using activating groups. During the last decades, carbodiimides have been used as activating groups to modify the acids in protein conformations [60]. Activated carboxyl moiety can react with various nucleophiles (amines etc.) (Fig. 6).

Weakly basic amine should be used because at higher pH the proteins may cross-link. Water in-soluble carbodiimides including N,N′-Dicyclohexylcarbodiimide (DCC), N, N′-Diisopropylcarbodiimide (DIC) are used in the conjugation of proteins [76], but mostly water soluble 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) are exploited for conjugation. EDC stability in aqueous media was described, and optimal pH for conjugation was found in the range of 6 to 7 [77]. Sometimes NHS is incorporated in coupling reaction to enhance efficacy (to produce more stable reaction intermediates). *N*-ethyl-5-phenylisoxazolium-3-sulfonate was used for activation of a carboxylic moiety of trypsin [78]. Fernandez-Lafuente and colleagues employed carbodiimide to activate the carboxylic group of proteins, and they used it to support low pk amine moieties for protein adsorption [79]. These supports have the advantage they allow immobilization at a low concentration of carbodiimide. High concentration of carbodiimide decreases the enzyme/protein efficiency [80]. Peroxidase was modified chemically with different carboxylic anhydrides for the incorporation of carboxylic moieties. Carboxylic anhydrides stabilize the peroxidase enzyme. According to literature introduction of hydrophilic moieties or modification of carboxylic moieties at the surface of proteins gives stabilizing characteristics [81]. Different methods have been employed for modification to enhance the stability of enzymes for good efficiency. Among them, carboxyl chemistry is important to modify protein/enzyme surfaces by incorporating hydrophilic or hydrophobic moieties and cross-linking with amino acid chains of proteins [82]. Modifying the surface of proteins enhances stability by generating additional hydrogen bonds, protecting the hydration shell, and developing electrostatic interactions [83]. Modification changes the positive charge of lysine to negative charge which provides chances of more hydrogen bonding [83].

4.5. Epoxy chemistry involved between the support and catalyst

Since long epoxy chemistry is considered efficient due to various characteristics as easy handling, stability (@neutral pH), wet reaction conditions. Epoxides can react effectively with different nucleophiles present at the protein surface, including thiols, alcohols, and primary amines, leading to thioether formation, ether bonds, and secondary amines, respectively [84]. The covalent attachment between epoxy moiety-containing supports and proteins is slow and takes place at a lower rate even at high Ph. However, according to a report, proteins that were adsorbed previously reacted at a high rate with epoxy moieties of support [85]. Due to the low reactivity of epoxy moieties, direct immobilization of enzymes is difficult even in the presence of all nucleophiles. For instance, for assurance of activation of support functionalized with epoxy moieties, many ligands have to be added. Direct interaction between epoxy supports and proteins can occur physical

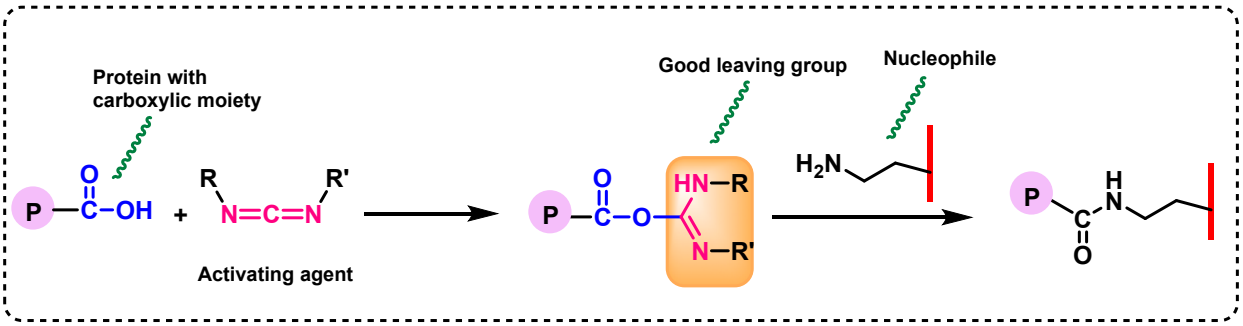

Fig. 6. Carboxyl chemistry of immobilization. General representation of carboxylic acid activation through carbodiimide.

adsorption of proteins on solid support. In this way the protein-based nucleophiles may approach to epoxide moieties and can easily attack. So hypothetically this is two step phenomena, adsorption followed by intramolecular reaction that brings material surface and proteins together [85]. There are several limitations with commercially available epoxy supports. Agarose-based epoxy supports exhibits insignificant immobilization due to the absence of adsorption phenomena (lacking hydrophobic core). Sephabeads functionalized with epoxy show effective immobilization but only at high ionic strength, and such conditions are not suitable for many proteins. The multi-functional carriers having two groups have been designed to improve immobilization efficiency and stability of immobilized proteins. One moiety can induce physical adsorption, and other epoxy moiety can carry covalent attachment (Fig. 7) [85,86]. Epoxy amine supports possessing both amino and epoxy moieties were used to immobilize lipase and amino moiety, which helped in physical adsorption and covalent attachment developed through epoxy moieties. This multi-functional support provides quick immobilization and also enhanced the half-life of immobilized enzymes. Thiol modified epoxy carriers have also been used for protein immobilization through thiol-sulfide exchange followed by covalent attachment through epoxy moiety [86,87]. Various reports are available in literature about increased efficacy of modified epoxy supports and proteins in immobilization. Modified aminated lipase was immobilized on epoxy support and multipoint immobilization enhanced the selectivity, reusability and stability of lipase for fish oil hydrolysis [88]. Similarly, immobilized lipase enzyme on modified (2% Butylamine/gelatin) epoxy supports were carried out, and immobilized lipase showed improved efficiency compared to non-modified support [89]. Yang et al. compared the efficacy of hydroxysteroid dehydrogenase (HSDH) immobilized on epoxy resin supports and aminated modified epoxy resin. Immobilized HSDH on amino epoxy resin support showed 80% enhanced activity compared to un-modified epoxy support [90]. In another report, lipase was immobilized on a novel heterofunctional support (prepared from the reaction of cloistie ad epichlorohydrin). Immobilized lipase efficiently hydrolyzed the olive oil and was also used to synthesize isoamyl acetate and produce biodiesel [91]. This suggests that the efficacy of support-immobilized enzymes depends on the environment of the surface. Enzymes immobilized on epoxy supports show good operational stability and storage as immobilized ketosteroid dehydrogenase on epoxy resins exhibited. This immobilized enzyme played the role of biocatalyst in the dehydrogenation of steroids [92].

5. Engineered catalysts – a drive towards optimum performance

Enzymes are biocatalysts that are used in multiple applications. The efficiency of available enzymes can be enhanced by immobilizing on different substrates. The enzyme engineering may improve the enzyme enantioselectivity, efficacy, catalytic potential, reusability, survival in harsh organic solvents, thermal, storage and pH stability. The enzyme is subjected to an iterative process for engineering with various substrates. This evolution may open a space for the utilization of engineered biocatalysts for enormous applications for well-being.

5.1. Recent advances in metal-organic frameworks (MOFs)

MOFs-enzyme conjugates are developed to keep the enzymes and active and prevent their leaching. The enzyme can be immobilized on engineered supports through three principles: binding, encapsulation, and cross-linking [1]. Different mechanisms are involved for the immobilization of enzymes on MOFs, including physical adsorption, covalent linking, cage encapsulation and in situ MOF formation and enzyme immobilization. These immobilization methodologies have different characteristics and scopes, which we have discussed here.

5.1.1. Physical adsorption

For immobilizing the enzymes on MOFs, adsorption is the facile and economical approach. In this strategy, the enzymes are attached to the surface of MOF via weak forces, including ionic, electrostatic, hydrophobic interactions, hydrogen bonding, and Vander Waals forces, etc., which does not alter the enzyme chemically [93] (Fig. 8A). Through physical adsorption phenomena, the enzymes are bound and cannot leach easily, but the success of confining depends on the surrounding atmosphere around enzyme-MOF conjugate during their application [94]. Furthermore, the balanced structure of MOFs also prevents enzymes from aggregation, passivation, and denaturation in specific environments and shields the active site of enzymes to maintain their catalytic efficacy [95]. Thus, the physical adsorption strength of enzymes on MOFs depends on the surface features of enzymes and MOFs [96].

The broader range of MOFs can act as adsorption supports for immobilization of enzymes and requirement of no extra specific chemicals, minimum operation process made this adsorption process easy, green, and cost-effective for potential immobilization of enzymes [97].

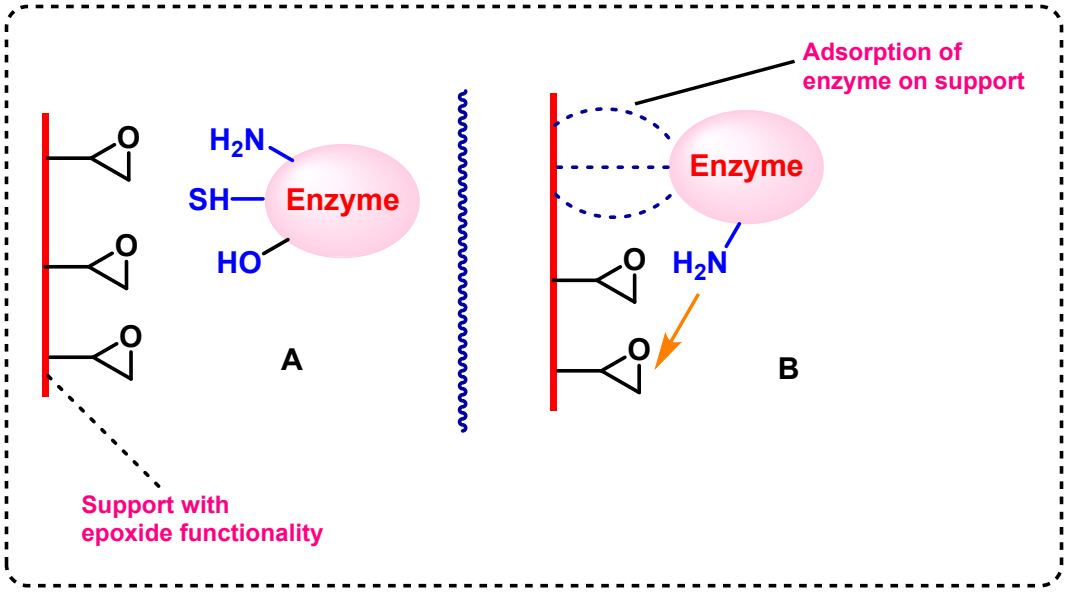

Fig. 7. Epoxide chemistry of immobilization. General representation (A) epoxide moiety can react with various nucleophiles, and direct intermolecular interaction gives slow covalent attachment. (B) Mild physical adsorption followed by intramolecular covalent attachment.

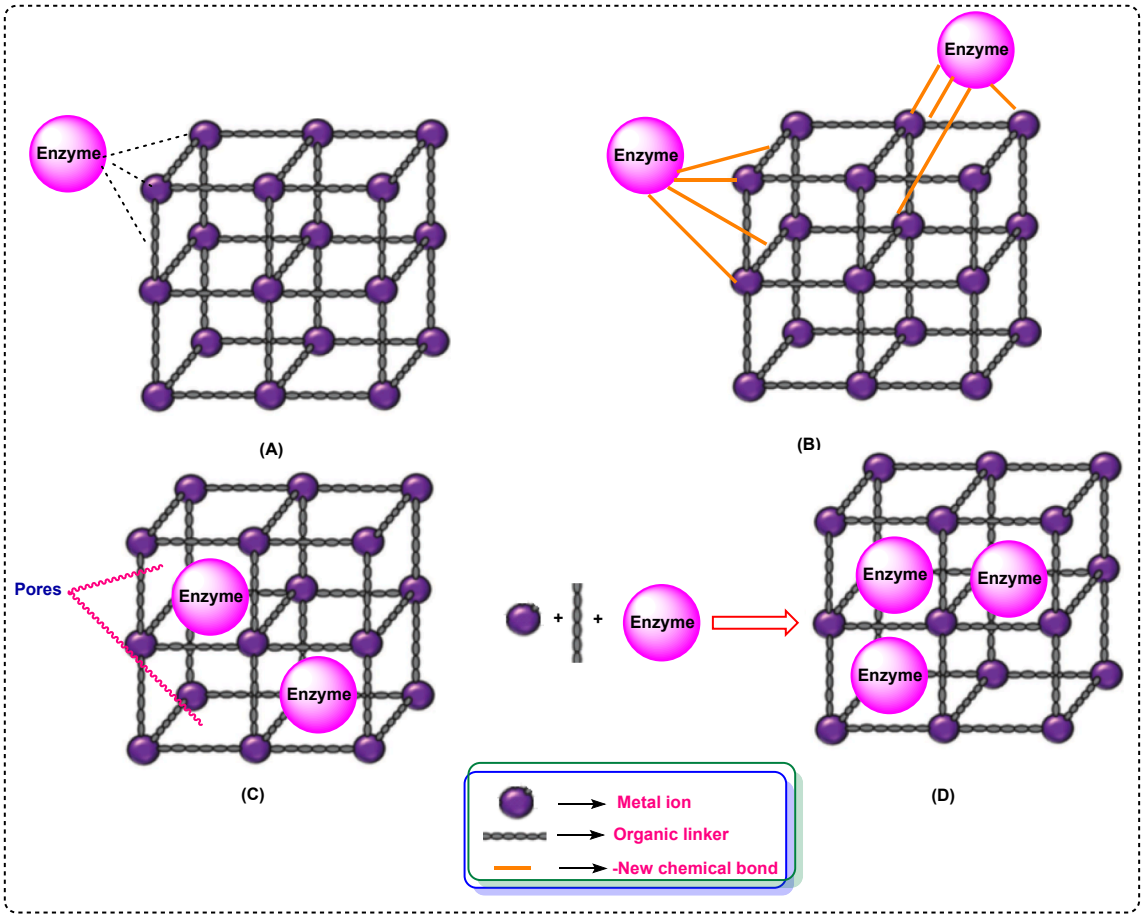

Fig. 8. General representation, (A) physical adsorption of enzyme on the surface of MOF and black dotted lines present electrostatic interactions, van der Waals forces, and hydrogen bonding, etc. (B) Covalent attachment between MOF and enzyme. (C) Enzyme encapsulation in pores of MOF. (D) In situ MOF formation and enzyme encapsulation.

Pisklak's research group made the earliest attempt to immobilize enzymes on MOFs. The enzyme microperoxidase-11 was immobilized on copper (II) dicarboxylate based MOF. The immobilized enzyme was obtained by mixing enzyme and MOF solution through physical mixing at room temperature (rt) [98]. A lot of research was carried out to immobilize enzymes on various MOFs via a physical adsorption strategy. Nanocomposite composed of platinum nanoparticles and MIL-100(Fe) were used as support for immobilizing the glucose oxidase. As platinum nanoparticles possess electroactivity and MOF material with large surface area, good biocompatibility, so their composed bioconjugate with immobilized enzyme (GOx-MIL-100(Fe)-PtNP) showed potential efficacy for sensing of glucose [99]. Jia and her colleagues used meso-MIL-53(Al) for immobilizing laccase. They choose meso-MIL-53(Al) because of its excellent stability and a large number of binding sites. The Lac-MIL-53(Al) was synthesized through physical adsorption, and this conjugate showed potent removal of 99.24% of triclosan in 120 min. The immobilized enzyme showed good stability and re-usability [100].

ZIF-based MOFs act as efficient support for the immobilization of enzymes. Recently ZIF-8 was used as a carrier for immobilization of lipase through physical adsorption. The immobilized enzyme (Lipase@ZIF-8) was used for hydrolysis of propionic ester and the transesterification of phenylethanol. The immobilized enzyme showed the highest activity, re-usability, and stability [101]. Another research group synthesized various ZIF-7, ZIF-8, ZIF-67, ZIF-68, and ZIF-70 to immobilize glucose dehydrogenase. From all synthesized ZIF-based MOFs, the ZIF-70 exhibited great adsorption capacity towards glucose dehydrogenase, further used as glucose biosensors material [102]. In a recent study, Laccase was immobilized on amine-functionalized magnetic MOF through physical adsorption and covalent attachment. The immobilized enzyme efficiently removed the dichlorophenols from wastewater and also exhibited enhanced storage and thermal stability. After reaction completion, the immobilized enzyme can be obtained from media through magnet [103]. So, this magnetic MOF proved as a novel support for the immobilization of enzymes. In a study, UiO-66-COOH was used as support for the immobilization of methioninase through an equilibrium approach. Almost 40% adsorption was obtained after 72 h of equilibrium. The immobilized enzyme was further investigated as an anticancer agent in tumor-bearing mice, getting promising results [104].

5.1.2. *Covalent attachment*

Enzymes can be immobilized on the surface of MOFs through covalent attachments. In physical adsorption, the involved forces are weak enough to immobilize the enzyme tightly on the surface of MOF. At the same time, in covalent attachment, strong bonds are formed between enzyme and MOFs, which prevents the enzyme from leaching, denaturation, and collapsing [93] (Fig. 8B). Multiple functional moieties, including epoxy, amidogens, glyoxal's, and carboxyl's are present at the surface of MOFs, and they can bind with respective active moieties on the enzyme surface. On the enzyme surface, various functional moieties like amines, thiols, and carboxylic in covalent grafting. Most commonly, direct covalent linking occurs between enzyme and MOFs functional moieties. Still, in case if no binding site is available between enzyme and MOF, the enzyme can be first modified or attached to a mediator (dye), which can actively bind with MOF or can reside in cavities of MOFs [105]. Yuan and his colleagues recently synthesized a biocatalyst by

immobilizing PEG-modified lipase on NH_2-MIL-53 MOF through covalent linking between carboxyl moiety of enzyme and amine function group of MOFs. The immobilized lipase was used for the anomeric resolution of fluoromandelic acid. After four consecutive cycles, the immobilized enzyme showed excellent stability with 83% of its initial activity [106].

In another study, lipase was immobilized on Uio-66(Zr) MOF through covalent linkage. Immobilized enzyme was used for hydrolysis and transesterification and excellent results were obtained [107]. Ghasemi et al. reported the immobilization of lipase on MIL-53(Fe) MOF. The MOF was firstly activated with an activating agent (DCC), then lipase was immobilized on activated MOF through covalent attachment [108]. Wand et al. reported the immobilization of D-amino acid transaminase on UiO-66-NH_2 star MOF through covalent linkage. The immobilized enzyme exhibited great catalytic potential, stereoselectivity, and eliminated polluting enzymes' interference [109]. The dye tagging approach is easy and convenient for immobilizing natural enzymes on MOFs. Lin and his colleagues reported a dye tagging approach for immobilization of trypsin. Trypsin was conjugated to a dye, and further it this conjugate was immobilized on MOF through covalent linking [110]. Various approaches are used for immobilization like adsorption, covalent linking, pore diffusion, among all covalent attachment is dominant approach.

5.1.3. Cage encapsulation

MOFs consist of abundant micropores and channels that may be the house of various molecules. This strategy made MOFs eligible candidates for enzyme immobilization [111]. In this approach, enzymes can reside/penetrate the pores of MOFs. MOFs have large pore volumes and wide channel spaces, which allows high enzyme loadings. Encapsulation of enzyme into MOFs made the stable and prevented leaching, aggregation, and unfolding in harsh reaction conditions because of protection around MOFs. With a high degree of freedom, the enzymes diffuse into MOF pores. High dimensional compatibility between MOFs and enzymes is required for infiltration in pores. Therefore MOFs with large pore sizes have been used commonly [112]. MOFs encapsulated enzymes show promising applications in pharmaceuticals, fuel cells, and biocatalysis. Therefore, various reports are available in the literature regarding the encapsulation of enzymes into MOFs. Laccase was encapsulated in zeolitic imidazolate framework-8 (ZIF-8). This conjugated framework gave us an active biocatalyst that was further used to form electrodes and design. The device based on immobilized Laccase/ZIF-8 showed great detection sensitivity of bis-phenol A [107]. Sond research group reported for the first time the encapsulation of multi-enzymes into immisazole based MOF. The enzymes horseradish peroxidase (HRP) and glucose oxidase (GOx) were linked together through DNA scaffold. The multienzyme system has an advantage as it prevents the enzyme from leaching from MOFs. The multienzymes were encapsulated into ZIF-8. The immobilized multienzymes showed excellent stability, reusability in comparison of free enzymes, and also they showed potent activity to detect low concentration of glucose [113]. Therefore, immobilized multienzymes can be used for broad applications in biocatalysis, engineering and biotechnology. In another study amylase-based bioreactor was synthesized by immobilization of amylase into UiO-66. The bioconjugate showed effective catalytic activity for starch hydrolysis and also gave excellent yield of maltose. This conjugate showed efficiency to be re-used even many times (up to 15 times). This was due to the best match of pore size of MOF with amylase [114]. Such kinds of bioconjugates can take part in drug delivery systems. Feng and his coworkers synthesized mesoporous MOFs, and three different enzymes (HRP, Cyt c, and MP-11) were encapsulated in them. Maximum loadings of enzymes were observed. The encapsulated enzymes were re-used many times, and the carrier prevented their leaching. The immobilized HRP showed potent catalytic potential in volatile solvents [115]. The enzyme cutinase was encapsulated in NU-1000 MOF. This study demonstrated that NU-1000 MOF has suitable channels for immobilizing cutinase [33]. Kob and his colleagues carried out the atomic-level study of encapsulation of cutinase into model MOFs. This study was carried out by using quantum mechanics and simulations. Encapsulation of cutinase into IRMOF-74-VI maintained its structural stability at harsh temperatures and protected it from denaturation. This study showed that enzyme encapsulation in porous frameworks retains the enzyme structure and remains reachable in harsh conditions [116].

5.1.4. In situ MOF formation and enzyme encapsulation

Cage encapsulation considers as an efficient approach for immobilization of enzymes into pores of MOFs. Some MOFs have small pore sizes and narrow channels openings so targeted enzymes cannot diffuse into pores. In recent years, the in situ encapsulation approach has been introduced to immobilize large enzymes within the MOF pores. In this approach, MOF fabrication and then immobilization of enzyme occurs in a one-pot reaction, usually comprising co-precipitation and biomineralization methods. In this process, the metal ions, organic linkers, and enzymes combine to lead to MOF formation and enzyme confinement synchronously. The enzyme can be present inside or at the surface of MOFs. Ji et al. presented the In situ encapsulation of lipase and magnetic NPs into ZIF-8 through co-precipitation method, and 88.4% immobilization yield was obtained. The resulting conjugate showed stability in harsh surroundings and was used to hydrolyze para-nitrophenyl acetate. After five times, re-use still retained 82% activity [117]. This strategy is efficient in reducing costs in the industrial sector. In another study, sonicated lipase was immobilized on ZIF-8 through a one-step biomineralization method within 10 min at room temperature. The lipase-MOF retained 54% activity even after seven consecutive cycles of use, and it also showed storage and thermal stability [118]. Lipase was immobilized on ZIF-8 in the presence of proline through the one-pot biomineralization method. And this synthesized conjugate of lipase-proline-ZIF-8 exhibited 135% improved catalytic potential in comparison to free enzyme. And after 6 cycles of re-use, this conjugate retained 72% activity [119]. In another study, lipase was In-situ immobilized onto MIL-53 (Fe) MOF. The support MOF was synthesized in water at room temperature. The immobilized enzyme exhibited stability in harsh conditions and retained 90% catalytic potential after 7 cycles of re-use [108]. Keeping in view various reports, this In-situ encapsulation process has benefits over other immobilization approaches. This involves a one-step/one-pot reaction, simple operation, minimal cost, and potent re-usability. These studies showed that hydrophilic and hydrophobic MOFs implements different effects on immobilization strategies and enzymatic activity.

5.2. Recent advances in covalent organic frameworks (COFs)

Covalent organic frameworks (COFs) are porous materials, and they possess excellent stability, porosity, and tunable features, which made them promising candidates for bio-compatibility with various biomolecules (enzymes, proteins, etc.) for potential applications in multiple fields [120]. The tunable pore size of COFs provides many binding sites for proteins and enzymes, offers a suitable environment for immobilization, and protects them from extreme environmental conditions. Most commonly, enzymes immobilize on COFs through Physical adsorption and covalent linking, and encapsulation. But pore encapsulation approach may lead to poor enzyme loadings and leakage. While encapsulation approach is restricted due to the requirement of harsh synthetic conditions for COFs synthesis, which may destroy the protein and enzymes conformations.

5.2.1. Physical adsorption

Enzymes are immobilized on COFs through the surface physical adsorption approach. Enzymes are adsorbed on the surface of COFs through electrostatic interactions, Vander Waals forces and hydrogen bonding. This approach is suitable for many COFs as this does not need any specific requirements regarding functional moieties and pore size.

The immobilized enzyme obtained after physical adsorption contains high catalytic and retention potential due to the increased surface area of COFs and mild adsorption reaction conditions. While binding force is a bit weak, enzymes can shed quickly and have low stability. In initial efforts, hollow spherical COFs were synthesized, and trypsin was immobilized its pores through adsorption strategy. The immobilized trypsin retained 60% catalytic potential of the free enzyme. This study showed that hollow COFs have internal cavities that provide more active sites for contact with enzymes and improve the catalytic potential of the immobilized molecule [121]. In a recent study, TPMM COFs were synthesized by condensing triformylphloroglucinol (TP) and melamine (MM) in DMSO. The mixture was stirred for 16 h @120 °C. The mixture was cooled and centrifuged to collect respective COF dried in a vacuum chamber @ 80 °C. The COF had several binding sites, structural flexibility and lightweight. Then amylase was immobilized on synthesized COF through physical adsorption. The immobilized enzyme exhibited an excellent affinity for hydrolysis of starch. The immobilized enzyme showed poor leaching and stability. The immobilized enzyme retained 74% activity even after 10 times recycling, so this immobilization approach of enzymes is cost-effective and more utilized in industries [122]. Pyrene-based COF was synthesized by condensation of melamine and 1,3,6,8-tetrakis(4-formylphenyl) pyrene. The structure of COF possessed many functional moieties and DNA aptamer strands were immobilized on it through π-π stacking and electrostatic interaction, which were further used as electrochemical aptasensors in detecting various antibiotics with great accuracy sensing capacity. The COF-based sensors have the potential for sensing other analytes as well [123]. A study was carried out pore environment of COFs to enhance the stability and catalytic potential of lipase enzymes residing inside the pores [124]. Various imine-based COFs were synthesized, and lipase was immobilized in it. The pore volume of synthesized biocomposites was decreased due to partial blocking of pores due to the occupation of enzymes. A unique HRP-pSC_4-AuNPs@COFs nanoprobe was fabricated [125] where COFs have been functionalized with *para*-sulfocalix, arene hydrate (pSC_4)-modified gold nanoparticles (AuNPs) and HRP. This probe was used in detection of colorectal cancer (CRC)-derived exosomes. The performance of detector was found in range from 5×10^2–10^7 particles/μL. An enzyme-based immunosorbent assay was developed for aflatoxins determination. For this purpose, DNAzyme was linked with COF through *π–π stacking*, and then immobilized DNAzyme based electrode was fabricated and employed as a signal amplifier to detect aflatoxins [126]. It is concluded that enzyme preferably resides in larger pores in nano-materials. Further COF based immobilized enzyme shows excellent stability, storage, and reusability even after many cycles. COF-based enzymes showed applications in diversified fields, especially in sensing.

5.2.2. Covalent linkage

The enzyme's surface is bestowed naturally with a variety of functional moieties which can couple with COF functional groups in a covalent fashion. This immobilization strategy provides good stability and reusability. Sometimes support materials have to be functionalized for covalent bonding. Hollow covalent organic framework microsphere (H-COF-OMe) was synthesized by emulsion interfacial polymerization, and this COF showed great surface area spherical hollow microstructure and defects interfaces. Laccase (567 mg/g) was immobilized on H-COF-OMe through covalent interaction [127]. Interesting structural features of synthesized COF stabilized the immobilized laccase from conformation distortion through various binding sites. The immobilized enzyme exhibited thermal storage, high pH stability, and reusability compared to free enzyme. Immobilized showed excellent adsorption potential and degraded the 99% tetracycline within 100 min. This study revealed that a unique synthesized COF as laccase immobilization carrier could be a promising source for the degradation of organic contaminants. Composites of COF@enzyme combine the benefits of both enzymes and COFs and lead to the development of materials with unique features. The first time colorimetric biosensor for determination of glucose concentration was developed by immobilizing glucose oxidase on carboxyl moiety containing COF (COF_{HD}). The composite (COF_{HD}–GOX) based colorimetric sensor showed a wide linear range (0.005 to 2 mM) with a low detection limit (0.54 μM). This sensor was used in various samples as drinks, serum, diabetic-urine and it showed excellent accuracy, selectivity, and storage stability. This sensor possessed the quality of being reused at least five times. This study showed that COF-based enzymes could be a potential source of sensing and diagnostics [128].

5.2.3. Pore diffusion/encapsulation

The pores of COF are tunable, and enzymes can immobilize it through diffusion. For this purpose, the enzyme size should be smaller than the pore to maintain the conformation of the enzyme. The unique environment of pores provides excellent stability and enhances the affinity of enzyme and COF. Lipase was immobilized in two COF-OMe, and COF-V materials with the same structure, and also comparison was carried out with other COFs. Mesoporous COFs with uniform and hydrophobic structures showed great affinity for lipase enzyme and exhibited better catalytic potential for converting phenyl ethanol [129]. The correctly oriented enzyme in pores of COFs enhanced the potential of reaction with substrate and increased the resistance of lipase towards solvents. COF harbored enzyme showed excellent thermal stability and reusability. Thus, COFs are excellent carriers for enzymes. COF with dual pore size was synthesized by the condensation reaction of ammonia and aldehyde [130], and microperoxidase-11 (MP-11) and glucose oxidase (GOD) were immobilized on it for sensing of glucose. The synthesized COFs were crystalline and ordered structures with dual pores (3.06 & 0.87 nm). Both enzymes are embedded in pores of COF through pore encapsulation and hydrogen bonding. The size of both enzymes matched with pores (larger and smaller) of COF. The immobilized enzymes were used for modifying electrodes, and they strongly attached with electrodes due to great flexibility. Then glucose biosensors were prepared, and they showed great stability, reusability, and selectivity. Site-directed spin labeling-electron paramagnetic resonance spectroscopy (SDSL-EPR) exhibited the dynamics and orientation of enzyme or various other biomolecules upon encapsulation in different mesoporous nano-materials (MOFs, COFs), and this technique helped to understand the enzyme behavior and its catalytic potential upon encapsulation [131]. Features of such kinds of immobilized biocatalysts can be improved by the utilization of SDSL-EPR.

5.3. Recent advances in nanoflowers

Hybrid nanoflowers have a large surface area, easy to synthesize, eco-friendly, and straightforward. Due to these features, they have attained attention during the last years as hosts for the immobilization of enzymes [132,133]. Following steps are involved in the formation of hybrid nanoflowers, firstly step protein and metal (II) ions form a primary complex together via coordination between the metal ion and the nitrogen atom of amide moiety present in the biomolecule. Secondly, these complexes offer the site for the nucleation growth of metal (II) phosphate nanoparticles. At the same time, the intramolecular interactions between protein and metal (II) ions promote the development of nano petals. During the final step, a flower-like structure is formed in which proteins act as a glue that binds together the petals (self-assembly) [134,135].

Various kinds of hybrid nanoflowers have been synthesized by utilizing different metal (II) ions as Ca^{+2} [136], Mn^{+2} [137], Cu^{+2} [138], Fe^{+2} [139], and incorporating different biomolecules. These organic-inorganic hybrid nanoflowers showed excellent catalytic potential, reusability, and storage stability. Different techniques like SEM, TEM, EDX, FT-IR, NMR, UV–vis are used to characterize these hybrid nanoflowers. These hybrid nanoarchitectures possess many applications in catalysis, sensing, removal of environmental pollutants, biofuel production, and other devices for biomedical purposes [137,139,140]. Zhong and his research group fabricated magnetic lipase inorganic

hybrid nanoflowers. Surfactant active lipase was used for formation of hybrid nanoflowers by incorporating magnetic nanoparticles into hybrid nanoflowers. Magnetic NPs were embedded into activated lipase hybrid nanoflower in covalent fashion. The synthesized hybrid nanoflower exhibited great catalytic activity, stability and immobilization efficiency in comparison of free enzyme. Hybrid nanoflower possessed excellent reusability potential and after 10 times recycling it retained 84% activity. This active nano-biocatalyst was used for production of biodiesel [140].

The catalyze enzyme was immobilized on Fe_3O_4 NMs and fabricates the enzyme-inorganic hybrid nanoflower where catalase played the role of the organic component and copper ions along with Fe_3O_4 as inorganic counterpart. The synthesized catalase/Fe_3O_4@Cu^{2+} hybrid nanoflower reduced the 4-nitro-benzene and azo dyes at different pH levels. The highest reduction of 4-nitro-benzene (91.52%) to respective amine was obtained at pH 9 in just 30 min [139]. DNA-based hybrid nanoflowers are potential candidates for therapeutic application due to the novel conformation of DNA. Zhao and his group fabricated the DNA-based Mn (II) hybrid nanoflower (DMNF) through biomimetic synthesis. Mn(II) was used as a *co*-factor of DNA polymerase for DNA strands extension. Synthesis was carried out by using the template of a long DNA strand through nucleation and Mn_2PPi growth. The morphological features were controlled through tuning of metal ion concentration and reaction time. The synthesized hybrid nanoflower improved magnetic resonance imaging of tumor sites in acidic media high-resolution imaging of liver and kidneys [137].

6. Concluding remarks and future perspectives

Immobilization is known as a simple way for covalent protein attachment. It has been a topic of incredible interest currently to flourish the biocatalysts-assisted processes in laboratories and industries worldwide. The carrier-immobilized biocatalysts have broached accelerated consideration given a set of unique attributes like biocompatibility, easy separation, non-toxicity, repeatability, high performance, and tolerability to harsh environmental milieus in chemistry, biochemistry industry, and pharmaceuticals. This study spotlighted various chemistries involved in the covalent immobilization of enzymes onto a plethora of support matrices for exploiting as novel biocatalytic candidates in biotechnological applications. The availability of specific functional groups on the surface of biomolecules or enzymes and substrate determines the type of immobilization reaction. The reaction may occur directly between functional moieties of biomolecules or support and an activation step is often required to activate the functional moieties for robust coordination. Functionalized support materials have a notable effect on the inherent mechanical attributes. They are likely to offer better biocompatibility and favorable nano- and microenvironments surrounding the enzyme molecules and consequently contribute to upgrading the biocatalytic performance, reaction efficiencies, and enzyme stability. Furthermore, the development of optimally immobilized biocatalytic conjugate can markedly enhance the robustness and operational durability of the enzymes for multiple uses, leading to reduce the bioprocessing cost. Therefore, fundamental understanding and increased knowledge related to physicochemical features of biocatalysts (structural and mechanistic insights), and nanosupport together with interfacial interactions of enzyme-support conjugate systems are indispensable in developing innovative and sustainable bioprocesses. Despite significant progress, many bottleneck steps need to be resolved for the large-scale application of enzyme-based systems. High cost allied with the fabrication of novel support material, enzyme biosynthesis, and enzyme immobilization is the most important obstacle to bringing this technology to scale-up. Most of the developed and reported biocatalytic cues only display proof-of-concept realization, and very few carriers-insolubilized enzymes have successfully been functioned for up to 10–15 repeated reaction cycles. Thus, mechanical steadiness, biocatalytic performance, stability, and enzyme leaching should be examined for continuous operation. With a collaborative effort of bioprocess engineers, biotechnologists, biochemists, and materials scientists, the immobilized enzyme-based multi-functional systems are remarkable and likely to be industrialized in the near future.

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Declaration of competing interest

The author(s) declare no conflicting interests.

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