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Review

Advanced strategies for enzyme–electrode interfacing in bioelectrocatalytic systems

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Advances in protein engineering-enabled enzyme immobilization technologies have significantly improved enzyme–electrode wiring in enzymatic electrochemical systems, which harness natural biological machinery to either generate electricity or synthesize biochemicals. In this review, we provide guidelines for designing enzyme–electrodes, focusing on how performance variables change depending on electron transfer (ET) mechanisms. Recent advancements in enzyme immobilization technologies are summarized, highlighting their contributions to extending enzyme–electrode sustainability (up to months), enhancing biosensor sensitivity, improving biofuel cell performance, and setting a new benchmark for turnover frequency in bioelectrocatalysis. We also highlight state-of-the-art protein-engineering approaches that enhance enzyme–electrode interfacing through three key principles: protein–protein, protein–ligand, and protein–inorganic interactions. Finally, we discuss prospective avenues in strategic protein design for real-world applications.

Highlights

Enzyme–electrode systems effectively integrate biological and non-biological components to facilitate 'interfacial electron transfer' (ET) between enzymes and electrodes.

Comprehensive frameworks for enzyme–electrode designs are established according to interfacial ET mechanisms.

Diverse enzyme–electrode interfacing strategies have been developed, taking into account the nature of the enzymatic reactions and the interfacial ET mechanisms involved.

Cutting-edge protein-engineering approaches offer powerful and versatile tools to enhance the efficiency of enzyme–electrode wiring in bioelectronic systems.

Electrochemical communication at enzyme–electrode interfaces

Redox enzymes are critical components in various metabolic pathways of living organisms. Within biological systems, these enzymes are functionally interconnected to facilitate ET, driving essential redox reactions involved in processes such as respiration, photosynthesis, and nitrogen fixation [1]. Integrating isolated redox enzymes with electrodes serving as non-native redox partners can aid harnessing of their electrical properties for powering diverse protein-based bioelectronic systems for bioelectricity generation, biochemical production, medical diagnostics, and environmental monitoring. The 'interfacial ET' between enzymatic redox centers and electrodes is a pivotal feature of so-called enzyme–electrodes, which determines the application scope and performance of protein-based bioelectronics [2]. The directionality, mechanism, and rate of electron flux at the enzyme–electrode interface are crucial parameters to consider from the earliest stages of enzyme–electrode development, regardless of biocatalytic reaction types on electrodes. To facilitate the desired interfacial ET mechanism and drive targeted electrocatalytic reactions, careful selection of design factors, such as enzyme types, electron-shuttling methods, enzyme immobilization strategies, and electrode materials and structures, is essential.

In this review, we provide comprehensive guidelines for designing and constructing enzyme–electrode systems, focusing on ET mechanism types. We highlight recent trends in **protein engineering** (see Glossary)-based enzyme immobilization technologies specifically aimed at enzyme–electrode wiring. We also propose future perspectives for innovative protein-engineering approaches toward the development of enzyme–electrode systems. Ultimately, we provide those interested in, or new to the field of, protein-based bioelectrochemistry with new insights into, and

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a systematic framework of, enzyme–electrode designs, as well as the latest developments in the field, offering a depth of information beyond that currently available elsewhere in protein-based bioelectrochemistry.

Enzyme–electrode design factors tailored for ET mechanisms

Interfacial ET in enzyme–electrode systems occurs via either mediated electron transfer (MET), utilizing external redox mediators, or direct electron transfer (DET), bypassing the need for mediators [3]. The presence or absence of a mediator results in distinct performance-determining factors for each system, highlighting the need for customized enzyme–electrode designs to achieve optimal electrochemical performance. Here, we outline key design considerations based on the selected ET mechanism in enzyme–electrode systems.

Mediated electron transfer

MET uses small redox mediators to facilitate electrons transfer between the redox center of the enzyme and the electrode. Redox mediators, such as viologens and quinones, as well as metal complexes, such as cobaltocene, ferrocene, osmium, and their derivatives, enable electrical connections between the multilayer-immobilized enzymes and the electrode surface, ensuring efficient ET regardless of the distance and orientation of enzymes; this guarantees a higher output in enzymatic electrochemical systems [3] (Figure 1).

Redox potential of enzymes against mediators

When selecting redox mediators, the potential of the catalytically active center of the enzyme should be considered rather than the reaction itself. The difference in the redox potential of this catalytic active center and the mediator is the mediator-induced overpotential (ΔE_{et}), which drives electron flow between the redox enzyme and the mediator [3,4]. Thus, the potential of the redox mediators should be tuned to an optimal distance from the active site of the enzyme. In the **oxidation reaction**, the redox potential of the mediator should be more positive than that of the active center of the enzyme, whereas a **reduction reaction** requires the redox potential of the mediator to be more negative than that of the cofactor of the enzyme. However, Hardt and colleagues [5] demonstrated H_2 uptake and evolution in the same experiment with a [FeFe]-hydrogenase embedded in a low-potential, 2,2′-viologen-modified hydrogel, which supports bidirectional reaction (oxidation or reduction), because its redox potential (–429 ± 8 mV versus standard hydrogen electrode; SHE) matches that of the $2H^+/H_2$ couple (–431 mV versus SHE) at pH 7. Ideally, the redox mediators should be within 50 mV of the catalytic potential of the target enzyme [3,6], because closer values can lead to a low driving force, whereas too distant values may risk higher thermodynamic losses [6].

Reversibility and stability of redox mediators

For enzymes such as hydrogenase and carbon monoxide dehydrogenase with extraordinarily high catalytic rates, and turnover numbers exceeding 1000 s^{-1} [7,8], the rate of redox mediator self-exchange can become rate limiting in enzyme–electrodes. To facilitate quick heterogeneous electron exchange, these redox mediators must match the intrinsic activity of the enzyme and be electrochemically reversible [9,10]. Typically, redox mediators should be chemically and oxygen stable, have appropriate redox potentials, and selectively interact with the target enzymes without undergoing unwanted reactions with the electrolyte or products [11]. Plumeré and colleagues demonstrated a breakthrough in MET-based enzyme–electrodes with the development of a specifically designed viologen-based redox polymer capable of electroenzymatic H_2 uptake by an O_2-sensitive hydrogenase, while simultaneously protecting enzymes from O_2 damage and high potential deactivation [12].

Glossary

Electrosynthesis: in the context of the enzyme–electrode system, refers to the process of using electricity to power enzyme reactions for converting substrates into desired products. It involves electrochemical cells with redox enzymes or other functional proteins immobilized on electrode surfaces for redox reactions. Specifically, the electrode is controlled at a more negative potential; therefore, the enzymatic reactions are driven by high-energy electrons from the electrode.
Enzyme cascade: series of linked enzymatic reactions, in which the product of each reaction serves as a substrate for the subsequent reaction.
Fusion enzymes: also known as chimeric or bifunctional enzymes; genetically engineered proteins that combine the activities of multiple enzymes into one molecule, enhancing reaction efficiency and enabling new functionalities, thereby facilitating complex biochemical processes; for example, combining the solid-binding domain and active site domain of the enzyme.
Oxidation reaction: chemical processes in which a substance loses electrons, leading to an increase in its oxidation state. In the context of this article, electrons flow from the substrate to the redox-active center of the enzyme and subsequently to the electrode directly (DET) or via redox mediators (MET).
Protein engineering: in the context of an enzyme–electrode system, typically aims to modify the native sequence of a protein to tailor new or desirable functions into it to facilitate enzyme immobilization onto the electrode.
Reduction reaction: chemical processes in which a substance gains electrons, leading to a decrease in its oxidation state. In the context of enzyme–electrodes, electrons flow from the electrode to the redox-active center of the enzyme either directly (DET) or through redox mediators (MET).
Solid-binding peptides (SBPs): short amino acid and unstructured sequences that have a high affinity for specific solid surfaces via multiple noncovalent interactions. Solid materials include metals, carbon materials, metal oxides, magnetic materials, and synthetic polymers.
Unnatural amino acids (UAAs): amino acids that are not among the

Biocompatibility of mediators with enzymes

As artificial co-substrates for enzymes, redox mediators should be able to interact with the enzyme, similar to a substrate, to facilitate ET [13]. Milton and colleagues demonstrated that MET with naphthoquinone derivatives was effective for FAD-dependent glucose dehydrogenase (FAD-GDH) but not glucose oxidase (GOx), despite their similar redox cofactor, and potentials suggesting minimal overpotential for MET, indicating its effectiveness [14]. This observation highlights the importance of enzyme compatibility and accessibility to redox mediators. Most redox enzymes have metallocofactors at their active centers, which are prone to inhibition by organometallic complex-based mediators, thereby compromising enzyme activity. Methyl viologen, a common redox mediator, is effective in ET but faces challenges in biological systems due to its propensity to produce reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals, and hydrogen peroxide, which can deactivate enzymes by oxidative damage [15]. Thus, selecting redox mediators that are biocompatible with the target enzymes is crucial for ensuring optimum efficiency of the biocatalyst for maximal output.

common (canonical) 20 amino acids attached to tRNAs in living cells that are used to synthesize proteins. They can be chemically synthesized or biosynthetically incorporated into proteins using modified translation systems.

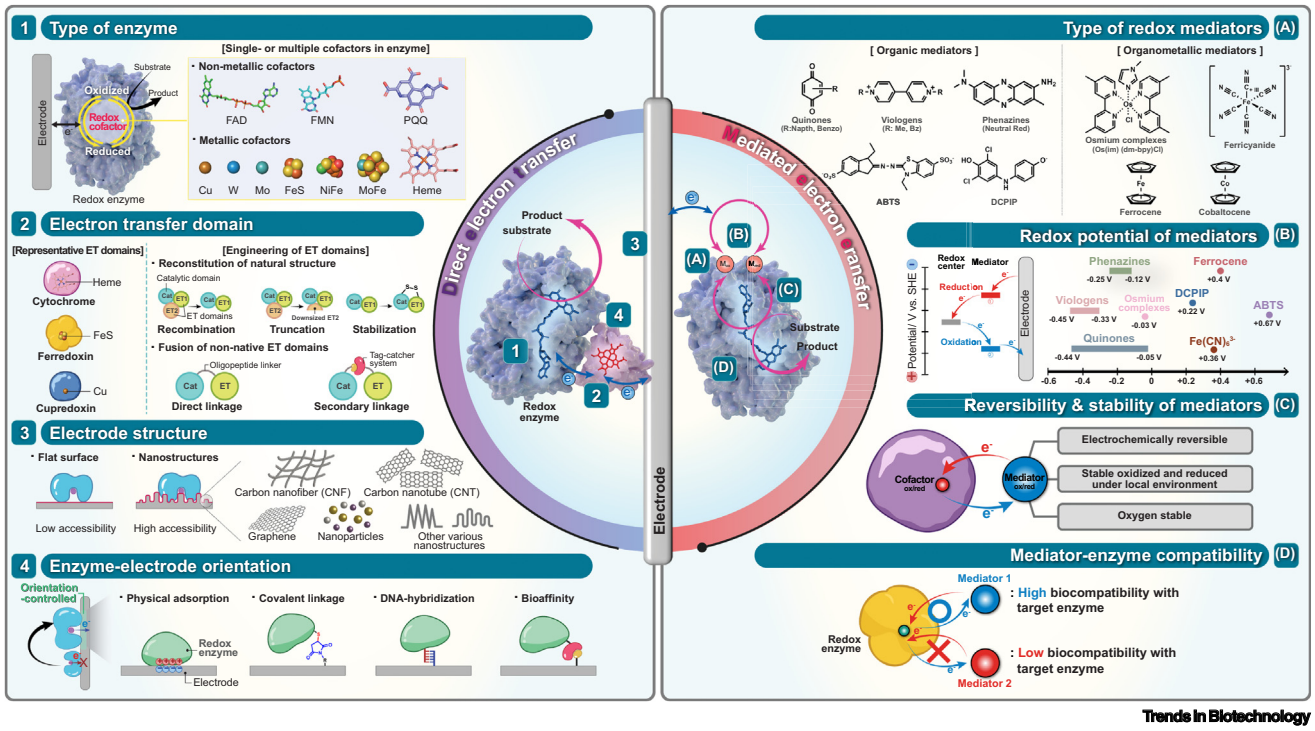

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Figure 1. Design parameters of enzyme–electrodes depending on interfacial electron transfer (ET) mechanisms, direct electron transfer (DET), or mediated electron transfer (MET). (A) Design parameters to be considered for DET-based enzyme–electrodes are the enzyme type, ET domains, electrode structure, and enzyme orientation on the electrode. Given that electron-shuttling molecules are not engaged and electrons should be transferred directly from the enzyme cofactor to the electrode, and vice versa, the enzymatic cofactor–electrode distance should be short. DET-capable enzymes have cofactors that are fixed within the protein structure, and engineering these enzymes through incorporation of native or external ET domains, such as cytochromes, ferredoxins, or cupredoxins, can significantly enhance their electron-transfer capabilities. The electrode material can also be nanostructured to facilitate physical access of electrode surface to the enzymatic cofactor. Most importantly, surface orientation of enzymes on the electrode can be regulated for facile interfacial ET. (B) In the MET system, the properties of redox mediators should be the focus because they are main players in interfacial ET. When selecting a mediator among the various kinds reported thus far, the redox potential of the mediator should be compared with that of the enzyme based on the type of electrocatalytic reaction desired, oxidation or reduction. In addition, the reversibility and stability of the mediator under the given environments (buffer type, pH, or temperature) should be confirmed and the compatibility of the mediator used with the cofactors of coupling enzymes should be considered. Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; DCPIP, 2,6-dichlorophenolindophenol.

Direct electron transfer

DET at the enzyme–electrode interface occurs via electron tunneling without the need for an external redox mediator [13,16]. The electron donor–acceptor distance in enzyme–electrodes should be <14 Å for DET, but this is challenging due to cofactors being deeply buried within the enzyme structure [17–19]. Therefore, researchers have attempted to discover suitable oxidoreductases, reconfigure proteins or electrodes, and control the orientation of enzymes on electrodes (Figure 1).

DET capable enzymes

The primary condition for DET-capable enzymes is that the redox cofactor responsible for electrical communication with the electrodes must be fixed within the enzyme structure [16,20]. Therefore, oxidoreductases with prosthetic groups are promising candidates for constructing DET-capable enzyme–electrodes. Despite most natural enzymes using dissociable NAD(H) or NADP(H) cofactors for metabolism, only ~100 of 1700 oxidoreductases have been applied to direct bioelectrocatalytic systems [21,22]. Such DET-capable oxidoreductases may contain single or multiple cofactors, such as metallocofactors including Cu, W, Mo, FeS, NiFe, MoFe, and heme, or non-metallic cofactors, such as FAD, FMN, and PQQ [23–25].

Electron transfer domain as a built-in electron shuttle

The ET domains, typically containing metallocofactors, may have a crucial role in shuttling electrons since they establish an effective charge-transfer route between the enzyme redox center and electrode. They can either be naturally embedded within monomeric proteins or exist as subunits [26,27]. Natural oxidoreductase structures have been engineered to enhance DET properties through reconstitution or truncation of protein subunits, thereby shortening interfacial ET distances [28,29]. Additionally, the thermal and catalytic stabilities of enzymes have been improved by structural stabilization [30]. For enzymes without intrinsic ET domains, additional wiring routes can be provided using non-native ET proteins. These proteins may harbor prosthetic groups such as heme (cytochromes), iron–sulfur clusters (ferredoxins), or copper (cupredoxins) [26] (Box 1). They can be paired with redox partner proteins for DET on electrodes through methods including co-immobilizations, end-to-end fusion with polypeptide linkers, or bioassembly [31,32]. Rational designs of built-in electron shuttles must carefully consider cofactor exposure, relative redox potential, surface charge, structural similarity, and molecular mobility [33–35].

Electrode materials and structure

Rendering the electrode surface electrically accessible to an enzymatic cofactor is crucial for facilitating interfacial electron exchange. Typically, flat surface electrodes are difficult to wire with enzymes, unless their orientation is carefully controlled, and often fail to load sufficient biomolecules onto a given electrode surface [36]. To improve the electrical connections with the partially conductive sites of enzymes, electrode surfaces are frequently modified with nanostructures. These modifications allow the electrode material to intrude into the nanosized electroactive sites of the enzymes, facilitating improved ET [22,36–39].

Surface orientation of enzymes on electrodes

DET in enzyme–electrodes depends on the distance and orientation of enzymatic cofactors relative to the electrode surface, requiring an electron tunneling distance ≤14 Å [17,18]. Anchoring enzymes on electrodes with the correct orientation is necessary to avoid insulation of enzyme–electrode interfaces due to the randomized surface binding of enzymes [40]. For orientation-controlled enzyme immobilization on the electrode, physical adsorptions, click chemistry-based covalent bonds, DNA-directed hybridization, and bioaffinity have been used [2,23,41–43], as discussed later.

Box 1. Electron carrier proteins for facilitating interfacial DET

Electron carrier proteins are crucial biological components that facilitate ET in various metabolic reactions. They can mediate interfacial DET in enzyme–electrodes as natural alternatives to artificial mediators. Two representative proteins have been extensively studied.

Cytochromes (Cyts), heme-containing proteins, are key facilitators of rapid interfacial ET in various enzyme-based bioelectronics [94]. Classified into types a, b, c, d, f, and o based on the electronic absorption maxima of their heme macrocycle, they exhibit distinct properties in their heme structures and binding conformations [26]. Their redox potentials range from –400 to 400 mV (versus SHE), typically higher than those of iron–sulfur clusters but lower than those of cupredoxins [94]. Facilitated ET by Cyts can be explained by increased polarizability of the protein active site, which lowers the ET activation barrier for ET [95]. The b- and c-type Cyts are commonly used to connect redox enzymes to electrodes. Schachinger and colleagues investigated the charge transfer ability of these Cyts on electrode by co-immobilizing them with FAD-GDH and observed faster electron uptake by c-types due to their higher redox potentials [35]. Viehauser and colleagues produced a Cyt b-glucose dehydrogenase chimeric enzyme to optimize interdomain ET, which ultimately led to enhanced oxidative current output [33].

Ferredoxins (Fds), with diverse FeS clusters ([2Fe-2S], [3Fe-4S], and [4Fe-4S]) are versatile ET domains that facilitate intermolecular charge transfer and serve as redox partners for many enzymes due to their broad redox potential [96,97]. The reduction potentials of FeS clusters in Fds fall below those of hemes in Cyts [E^0 ([2Fe-2S]), –150 to –450 mV; E^0 ([3Fe-4S]), –50 to –450 mV; E^0 ([4Fe-4S]), –250 to –650 mV] with the most negative value of –705 mV reported in the [4Fe-4S] cluster of photosystem I (PSI), rendering them common electron donors in biotransformation processes [96,98,99]. The choice of Fds for coupling with catalytic domains depends on their reduction potential, cofactor exposure, size, and solubility. Li and colleagues emphasized the importance of redox potential of reduced Fds for providing sufficient reducing power [99]. Jin and colleagues demonstrated direct electron uptake from electrodes by [2Fe-2S] Fds adsorbed on polyallylamine-modified electrodes [100]. Subsequently, Yagati and colleagues [101] developed an H_2O_2 biosensor using spinach Fd containing a [2Fe-2S] cofactor, which catalyzes H_2O_2 reduction via direct electron uptake from the electrode. Recent biophotoelectrochemistry studies showed that layering PSI between Fd-paired enzymes and electrodes enhanced electrochemical biotransformation through intermolecular ET [102]. Likewise, the choice of ET domains significantly influences the direction and efficiency of electron flow at the enzyme–electrode interface.

Recent advances in efficient enzyme–electrode wiring approaches

Significant progress has been achieved in enzyme immobilization strategies that enable enzymatic electrocatalysis with enzyme electrodes. Innovative techniques are constantly emerging to improve the catalytic activity of immobilized enzymes, their surface-binding stabilities, and the electrical conductivities between enzymatic cofactors and electrodes. Various principles have been incorporated for enzyme–electrode interfacing: (i) physical adsorption via van der Waals or electrostatic interactions; (ii) enzyme trapping within various architectures [redox polymers, metal–organic framework (MOF), and DNA nanoflowers); (iii) chemical linkage through specific reactions between functionalized electrode surfaces and native/engineered protein residues; (iv) cross-linking of enzymes on electrodes; (v) bioaffinity of enzymes with affinity ligands, such as small molecules, inorganic materials, or other proteins; and (vi) DNA hybridization involving the self-assembly of protein–DNA conjugates with electrode-bound complementary DNA probes [2,39,44–47] (Box 2). The choice of modality for enzyme–electrode interfacing depends on the interfacial ET mechanism (MET or DET), the enzymes of interest, and the working environment. Considering various aspects, single or multiple methodologies should be selected to optimize the electron flux between enzymes and electrodes.

Furthermore, enzyme-based electrocatalytic systems have been developed to provide appealing routes for organic synthesis or higher power generation by introducing multi-enzymatic systems, in which multiple enzymes are coupled for chain reactions. Owing to their potential to address challenges related to the sustainable production of high-value industrial biochemicals, including medicines, fuels, and fertilizers, cascade-type enzymatic electrosynthetic systems are garnering increasing attention [25,48]. When such **enzyme cascades** are co-immobilized on a single-electrode surface, optimizing the intermediate diffusion or channeling becomes crucial because it impacts the overall enzymatic cascade reaction rates on the electrode. This requires precise regulation of the relative orientation and positioning of the coupling enzymes.

Box 2. Enzyme immobilization technologies

Adsorption

Enzyme immobilization via adsorption has been reported on a range of electrode surfaces, including planar, mesoporous, nanoporous, and carbon dots [39,103–109]. Enzyme adsorption on the surface occurs through weak noncovalent forces, such as hydrophobic interactions, van der Waals forces, and hydrogen bonds, or via ionic bonds [110,111]. Therefore, to achieve sufficient and consistent surface coverage, it is essential to consider the polarity and charge of enzyme surfaces.

Entrapment/encapsulation

Enzymes are caged inside fibers, metal organic frameworks, DNA flowers, or redox polymers on the surface [12,45,112–117], which allows for higher enzyme surface coverage, minimizing enzyme leaching, and enhancing mechanical stability [110,111]. Several key considerations in the enzyme entrapment approach include enzyme compatibility with the chemical environment of the polymerization solution, accessibility of substrates and products across the matrices, and enzymes retention within the matrices.

Covalent binding

Covalent bond formation between the surface and the enzymes can occur via amino acid side chains, such as cysteine-thiol groups, lysine-ε-amino groups, histidine-imidazole groups, and aspartic and glutamic acid-carboxyl groups [110,111]. The covalent bond between a specific site in the enzymes and the support surface can be exploited to increase the stereospecificity of the enzymes, control the surface orientation of the enzyme, and position the enzymes within a DET-compatible distance [44,118–124].

Cross-linking

In contrast to covalent linkage and entrapment, cross-linking in enzyme immobilization methods focuses on covalent bonding between enzymes to form enzyme aggregates on a matrix. The method involves using cross-linking agents, such as glutaraldehyde, to enhance enzyme stability and reusability by generating a robust enzyme support structure, although it may cause activity loss due to conformational changes resulting from multiple covalent connections [125].

Bioaffinity

Bioaffinity-based enzyme immobilization leverages the affinity between biomolecules and ligands to anchor enzymes onto a support matrix [126], achieved by precoupling the matrix with affinity ligands followed by introducing enzymes equipped with ligand-binding sites. The host and guest molecules at the enzyme–matrix interfaces are highly specific to each other, which facilitates strong and specific enzyme binding and enables gentle immobilization of enzymes onto the matrix.

DNA scaffold

DNA hybridization is bioaffinity-based enzyme immobilization that utilizes complementary single-stranded DNA (ssDNA) pairs to anchor enzymes onto a matrix [126]. It involves functionalizing the matrix surface with DNA probes, followed by hybridizing with complementary DNA sequences conjugated to enzymes. Highly specific Watson–Crick base pairing minimizes non-specific enzyme binding and precisely directs enzyme attachment positions, which can be predetermined through DNA array designs [127].

Overall, enzyme co-immobilization methodologies should be carefully selected to control both interenzyme and electrode–enzyme interfaces [25,49]. Next, we summarize recent advances in enzyme immobilization for bioelectrocatalysis over the past 10 years, focusing on interfacing principles, highlighting the ET mechanisms involved, and determining whether single or multiple enzymes are (co-)immobilized (Table 1 and Figure 2). The advantages and challenges of these methodologies are detailed in Table 1.

Enzyme–electrode wiring using protein-engineering strategies

Advances in molecular biology-enabled protein engineering have been leveraged as a convenient approach for interfacing proteins with electrode surfaces. Using molecular biology, protein amino acid sequences can be modified through insertion of an immobilization component, which may be in the form of a single amino acid, an affinity tag, or another binding protein.

Table 1. Recent advances in enzyme immobilization techniques for enzyme–electrode systems[a]

Year	Strategy	Principle	Enzyme/electrode	ET mechanisms (MET/DET)	Description	Refs
Universal enzyme–electrode interfacing strategies						
2024	Adsorption	Electrostatic interactions between enzymes and charged cyanamide-modified graphitic carbon nitride	[FeFe]-hydrogenase/charged cyanamide-modified graphitic carbon nitride	DET	Strong electrostatic interaction of [FeFe]-hydrogenase and carbon nitride enabled DET for efficient solar H_2 evolution, achieving turnover frequency of 18 669 h^{-1} (4 h), 4.5 times higher than the previous benchmark (4117 h^{-1}) Stable enzymes linking to carbon nitrides enabled interfacial characterizations of enzyme–photocatalyst biohybrid system	[104]
2022	Adsorption	Electrostatic interactions between enzymes and charged CNT or amorphous CDs	FDH/charged CNT or amorphous CDs	DET	Promoted DET on positively charged CNTs for efficient and reversible bioelectrochemical CO_2 reduction to formate. System exhibited high rates of photocatalytic CO_2 reduction, reaching 3500 h^{-1} on positively charged CDs	[39]
2018	Adsorption	Physical adsorption onto hierarchically. structured ITO electrode	[NiFeSe]-hydrogenase/ inverse opal- ITO (IO-ITO)	DET	IO-ITO provided suitable environment for wiring of [NiFeSe]-hydrogenase in DET regime Combination of [NiFeSe]-hydrogenase-IO-ITO as cathode and photosystem II on dye-sensitized photoanode demonstrated bias-free solar-driven water splitting	[105]
2017	Adsorption	Physical adsorption on mesoporous and planar electrode	W-FDH/Ketjen Black, planar, and mesoporous electrode	DET	Curvature effect of mesoporous structures increased number of enzymes adsorbed with orientations suitable for DET Adsorbed W-FDH on electrode catalyzed DET-type bidirectional interconversion of CO_2/HCOOH and NAD^+/NADH redox couples	[108]
2016	Adsorption	Physical adsorption of enzymes on nanorods electrode	FDH/Cu nanorods deposited GCEs	MET	Cu nanorods with twin crystal structure promoted better enzyme adsorption onto electrode. FDH-Cu nanorods on electrode showed threefold enhancement of formate formation rate, i.e., (6.28 ± 0.02) × 10^{-3} μmol $mg^{-1}min^{-1}$, compared with that reported previously for Cu foil electrodes	[109]
2014	Adsorption	Physical adsorption onto graphite-epoxy electrode	Mo-containing EcFDH-H/graphite-epoxy rotating disk electrode	DET	Adsorbed EcFDH-H retained biocatalytic activity and exhibited reversible formate oxidation and CO_2 reduction EcFDH-H-graphite-epoxy electrode enabled mechanistic investigations of CO_2 reduction	[103]

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Table 1. (continued)

Year	Strategy	Principle	Enzyme/electrode	ET mechanisms (MET/DET)	Description	Refs
2023	Encapsulation	Encapsulation of enzymes in zeolitic imidazolate framework	GDH and NAD^+ cofactor in zeolitic imidazolate/fluorine-doped tin oxide electrode	DET	Encapsulation of both GDH and NAD^+ cofactor in zeolitic imidazolate enhanced biocatalysis of cofactor-dependent enzyme System displayed self-powered biosensor with low detection limits of 2 aM (six copies miRNA-21 in a 5 μl of sample) and exhibited potential for accurate identification of diseases and clinical diagnosis	[117]
2019	Entrapment	Enzymes embedded in cobaltocene-modified poly (allylamine) redox polymer	Diaphorase/cobaltocene-modified poly(allylamine) redox polymer on electrode	MET	Redox polymer contributed to high Faradaic efficiencies (78–99%) and turnover frequencies (2091 h^{-1} and 3680 h^{-1}) of bioelectrocatalytic bioactive 1,4-NADH regeneration by diaphorase System showed enhanced biomethanol and propanol production of 7.1- and 5.2-fold, respectively, compared with negative control when coupled with NADH-dependent alcohol dehydrogenase	[116]
2017	Encapsulation	Encapsulation of enzymes in ZIF-8 with combination of mesoporous and microporous channels	Cytochrome *c*/ZIF-8/screen-printed electrode	DET	Combination of mesoporous and microporous channels in ZIF-8 promoted increment in substrate affinity by 50% and 128% increase in enzymatic activity When immobilized on screen-printed electrode, cytochrome c/ZIF exhibited fast electrochemical detection of residual H_2O_2 in microliter food samples	[113]
2014	Entrapment	Enzymes embedded in viologen-based redox hydrogel	[Ni-Fe]-hydrogenase/ viologen-based redox hydrogel on electrode	MET	Redox film provided self-activated protection of O_2 sensitive [Ni-Fe]-hydrogenase from O_2 damage Redox film served as shield from high potential deactivation	[12]
		Enzyme entrapment in PPy polymer	Urease/PPy polymer on electrode	MET	Presence of anionic cyclodextrin in urease-polymer film enhanced detection limits Urea biosensor exhibited superior sensitivity of 5.79 μC μM^{-1} and good selectivity	[112]
2023	Covalent binding	Amide bond formation between amino groups of enzymes and epoxy group of glycidyl methacrylate	PQQ-dependent aldehyde dehydrogenase/poly glycidyl methacrylate on mesoporous MgO-templated carbon	MET	Low detection limit of 0.02–0.1 ppm achieved by combination of high surface-area electrode, MgO templated carbon, and stable enzyme immobilization MET-based biosensor measured low acetaldehyde gas concentrations from human skin	[120]

Table 1. (continued)

Year	Strategy	Principle	Enzyme/electrode	ET mechanisms (MET/DET)	Description	Refs
		Amide bond formation between acid group of polymer and amide in enzymes	Lac/PAA/CFP electrode	DET	Catalytic activity of Lac/PAA/CFP was double that of enzymes on unmodified CFP Fabricated biosensor for *p*-nonylphenol determination in water samples had low limit of detection (1.74 nM) and broad linear dynamic range (5–250 nM)	[121]
2022	Covalent binding	Amide bond formation between enzymes and carboxylic groups of 4-carboxyphenyl film modified on GNRs on electrode	Lactate oxidase/4-carboxyphenyl film modified GNRs on GCE	MET	Use of modified GNR enhanced performance of electrochemical biosensors for lactate determination, with detection limit of 11 μM Developed biosensor for L-lactate was in good agreement with those obtained with well-established enzymatic spectrophotometric assay kits	[122]
2021	Covalent binding	Amide bond formation between enzymes and 4-MBA-modified graphene-Au nanohybrid on nickel (RGO/Au/Ni electrode)	GOx/4-MBA-modified-RGO/Au/Ni electrode	MET	Modified RGO/Au/Ni promoted higher enzyme loading on electrode Fabricated glucose biosensor had high sensitivity (32.83 μA mM^{-1} cm^{-2}), good anti-interference ability, and repeatability	[123]
		Amide bond formation between enzymes residues and aminophenyl groups on Au surface	FDH/aminophenyl-modified graphite	DET	Covalently immobilized FDH yielded oriented binding on electrode DET-based formate oxidation and CO_2 reduction were considerably enhanced, up to 700 and −200 μA cm^{-2}, respectively	[44]
2020	Covalent binding	Covalent binding between enzymes and carboxylate functional groups on PEDOT interface	LDH/poly-COO^- on PEDOT	DET	Simple covalent chemistry facilitated effective and stable coupling of LDH on PEDOT: poly-COO^- interface PEDOT-based biosensor for lactate detection in human serum sample showed high sensitivity of 8.38 μA mM^{-1} cm^{-2} and good reproducibility	[124]
2024	Cross-linking	Formation of redox cross-linking network in nanostructured porous carbon surface via covalent bonding	FAD-GDH and thionine/MgOC	DET	Current generation performance increased considerably to 0.23 mW cm^{-2} μg^{-1} by diffusion of glucose through deep pores of MgOC Enzyme cross-linking and porous electrode as bioanode improved electron transfer from redox-active center	[46]
2022	Cross-linking	Cross-linking between tannic acid-capped AuNPs and enzymes	GOx/tannic acid-capped AuNPs	MET	Electro-cross-linking approach enabled temporal and spatial control of enzymes Enzyme films demonstrated good stability over 2 weeks following storage at room temperature	[128]

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Table 1. (continued)

Year	Strategy	Principle	Enzyme/electrode	ET mechanisms (MET/DET)	Description	Refs
2020	Cross-linking	Cross-linking between enzymes and LPEIs via GA or EGDGE as cross-linking agents	GOx/mediator modified-LPEI/GA or EGDGE/Au electrode or carbon electrode	MET	combination of enzyme-containing ferrocene-modified LPEI and GA showed higher stability than EGDGE in biosensing context two cross-linking agents showed comparable performance in context of biofuel cells	[129]
2018	Cross-linking	Cross-linking between homobifunctionalized catechol ethylene oxide and enzymes	GOx/homobifunctionalized catechol ethylene oxide and ferrocene methanol on Au electrode	MET	Electro-cross-linking stabilized enzyme film on electrode surface and prevented leakage Electro-cross-linking approach allowed development of miniaturized biosensors through functionalization of single electrode out of a microelectrode array	[130]
2017	Cross-linking	Cross-linking between enzymes and water-soluble amino acids as linkers under carbodiimide coupling conditions forming biohydrogel network	GOx and BSA/carbon cloth electrode	MET	Use of biohydrogels enhanced amount of electroactive enzyme loaded on electrode, reaching 2.2% GOx-BSA biohydrogel enabled efficient enzymatic electron transfer reactions with good stabilization and activity retention over wide range of pH (4–8) for bioelectronics and biofuel cell applications	[131]
		Cross-linking between enzymes and electrode modified with MWCNT using GA as cross-linker	Choline oxidase/GCE-modified MWCNT	DET	Cross-linking method retains choline oxidase bioactivity on electrode Developed inhibition biosensor had high selectivity and low detection limit of 0.04 nM for lead ions for their determination in tap water samples	[132]
2015	Cross-linking	Cross-linking between enzymes and CNTs	GOx/CNT	DET	Cross-linking strategy increased enzyme loading and prevented enzyme denaturation and leaching Optimal GOx/CNT as glucose biosensor achieved both high sensitivity, 16.26×10^{-3} AM^{-1} cm^{-2} and long-term stability over 41 days	[133]
2024	Bioaffinity	ST-SC association between ST-fused-anti-CRP scFvs and SC-fused enzyme	ST-fused-anti-CRP scFvs-immobilized magnetic beads and SC-fused GDH/screen-printed carbon electrode	MET	Bivalent antibody–enzyme complex showed high affinity contributing to high sensitivity with limit of detection of 2.9 nM for CRP detection Assembled system had potential as rapid, convenient, and hand-held detection device without using IgGs	[51]
2023	Bioaffinity	His tagged-enzyme affinity for TIO	[Ni-Fe]-hydrogenase/TIO	DET and MET	His-tag-mediated binding of [Ni-Fe]-hydrogenase increased enzyme surface coverage Homogeneously orientated enzymes enabled DET and promoted [Ni-Fe]-hydrogenase catalytic bidirectionality	[134]

Table 1. (continued)

Year	Strategy	Principle	Enzyme/electrode	ET mechanisms (MET/DET)	Description	Refs
2021	Bioaffinity	CBM-fused enzyme affinity for cellulose electrode	CBM fusion to GOx with natural linker in endo-β-xylanase/cellulose-modified electrode	MET	CBM induced affinity adsorption to cellulose while retaining most of its intrinsic activity Immobilized CBM-fused-glucose oxidase efficiently catalyzed glucose and displayed good sensitivity of 466.7 μA mol^{-1} Lcm^{-2} and stable for over 2 months as biosensor	[135]
2019	Bioaffinity	His-tagged-enzyme affinity for Ni^{2+}-aspartic acid complex of pyrene-KDDD stacked on MWCNT-(COOH)	His-tagged-MoFe nitrogenase/pyrene-KDDD/MWCNT-COOH	MET	Presence of His-tag served dual function for both purification and immobilization onto Ni^{2+}-aspartic acid complex of pyrene-KDDD on MWCNT electrode Strategy promoted high effective concentration of His-tagged nitrogenase at electrode surface allowing kinetics studies and use for bioelectrosynthesis purpose	[136]
2018	Bioaffinity	Biotin–SA interaction	Biotin-modified glucose dehydrogenase/SA-based hydrogel/GCE	MET	SA functioned as protein building block for constructing protein hydrogel that can be used as bridging and immobilization site SA-based hydrogel exhibits ability to serve as scaffold for immobilizing highly active and stable biotin-modified enzymes on electrode	[54]
2016	Bioaffinity	Cysteine-tagged enzyme interaction with vinylphenyl group-modified electrode via thiol-ene click chemistry	Cysteine-tagged D-sorbitol dehydrogenases/vinylphenyl groups-modified GCEs and carbon felt electrodes	DET	Insertion of one or two cysteine moieties at N terminus of enzyme effectively immobilized enzymes in active form on vinylphenyl group-modified electrode Immobilization of cysteine-tagged enzymes via thiol-ene click chemistry on electrode surface did not hinder electrochemical regeneration of NAD^+/NADH cofactor and soluble mediators	[137]
		His-tagged-enzyme affinity for Ni-NTA complex on CNTs	His-tagged-MCO/Ni-NTA complex/CNT	DET	His-tag affinity for Ni-NTA complex on CNT prompted high degree of enzyme surface orientation Immobilized MCO exhibited improved catalytic reaction efficiency with current density of 90 μA cm^{-2} without a mediator	[65]
Multienzyme co-immobilization strategies on electrodes						
2022	Adsorption	Adsorption of multienzymes in random nanopores of ITO electrode	Ferredoxin $NADP^+$ reductase, carboxylic acid reductase, adenylate kinase, and pyruvate kinase/nanoporous ITO electrode	DET	Nanoporous ITO electrode allowed enzyme cascades to be confined, controlled, and monitored in real-time System enabled conversion of cinnamic acid to cinnamaldehyde by optimized enzyme mixture immobilization for simultaneous electrical and chemical energy regeneration	[107]

(continued on next page)

Table 1. (continued)

Year	Strategy	Principle	Enzyme/electrode	ET mechanisms (MET/DET)	Description	Refs
2021	Adsorption	Adsorption of fusion enzymes onto carbon paper	Alcohol dehydrogenase and aldehyde dehydrogenase/carbon paper	MET	Application of substrate channeling in fusion enzyme design resulted in simpler systems with less protein loading needed and greater efficiency Strategy reported improvements in stability demonstrated for 2 days, product selectivity of 90%, and catalyst turnover frequency comparable with that of unbound enzymes	[106]
	Entrapment	Multienzymes embedded in 2,2′-viologen-modified hydrogel	FNR and Ccr/2,2′-viologen-modified hydrogel on GCE	MET	Redox hydrogel wired and accommodated FNR for bioelectrocatalytic NADPH regeneration coupled to Ccr for synthesis of complex molecules from CO_2 Enabled stereoselective formation of (2S)-ethylmalonyl-CoA at rate of 1.6 ± 0.4 μmol cm^{-2} h^{-1} with high Faradaic efficiency of 92 ± 6%	[45]
	Encapsulation	Encapsulation of multienzymes in DFs	HRP/DFs on electrode	DET	Cavity of DF promotes formation of highly ordered and hydrogen-bonded water environment resulting in enhanced cascade catalytic efficiency High density of DNA scaffold ensures encapsulation of GOx/HRP with high efficiency for glucose biosensor and detection of cancerous exosomes and thrombin	[114]
		Encapsulation of enzyme-loaded nanoparticles in porous conductive membranes	GOx and lactate oxidases/PEDOT on HFM electrode	DET	Spatial location of enzymes in PEDOT-HFMs can be modulated through controllable physical entrapment patterns Developed biosensor exhibited excellent sensing with fast response (3 s), wide linear range (glucose, 2–24 mM; lactic acid, 0.1–6 mM), high sensitivity, and low detection limit (glucose, 100 μM; lactic acid, 10 μM) for cancer cell metabolism monitoring	[115]
2017	Covalent binding	Covalent bond formation between multienzymes and carboxylated MWCNTs	DAAO and HRP/MWCNTs and AuNP-modified screen-printed electrode	MET	Combination of MWCNTs/AuNPs provided nanoarchitecture suitable for DAAO/HRP immobilization Co-immobilization of DAAO and HRP formed bi-enzymatic biosensor for selective quantification of D-amino acid in biological samples	[118]
		Covalent binding between multienzymes and SAM	Glutamate dehydrogenase and proline dehydrogenase/SAM/Au electrode	MET	Immobilization strategy retains multienzyme catalytic activity on electrode Multienzyme/SAM/Au electrode showed efficient multienzyme reaction for conversion of L-glutamate to 2-oxoglutarate	[119]

Table 1. (continued)

Year	Strategy	Principle	Enzyme/electrode	ET mechanisms (MET/DET)	Description	Refs
2018	Cross-linking	Cross-linking of multienzymes by TPA as cross-linker on PEI on CNTs	GOx and HRP/TPA/PEI/CNT electrode	DET	TPA as cross-linker promoted stable bonding of catalytic structure Optimal [(TPA/HRP/GOx)]/PEI/CNT exhibited improvements in catalytic activity and power density of 2.0 ± 0.1 mW cm^{-2} as enzyme biofuel cell	[138]
2023	Bioaffinity	GBP-fused enzyme affinity to Au electrode	GBP fused to each invertase and FAD- GDHγα/au electrode	DET	Fusion of GBP co-immobilized invertase and GDHγα on single electrode surface GBP-fused enzyme cascade can be manipulated to induce diverse relative orientations of coupling enzymes while enabling efficient DET at GDHγα–electrode interface	[2]
	DNA directed hybridization	Base-pairing of DNA-modified enzymes with TDNs	DNA-conjugated SOX and HRP/TDN and 16-channel electrochemical chip	DET	Coupling DNA-conjugated multienzymes with TDNs enhanced enzyme activity by 1.44-fold more than that of ssDNA–enzymes Active form of DNA-conjugated SOX and HRP formed cascade reaction on TDNs for sarcosine detection	[139]
2020	DNA-directed hybridization	Base-pairing of DNA-modified enzymes with TDNs	DNA-conjugated sarcosine oxidase, GOx, alcohol oxidase, DNAzyme/thiolated TDN Au electrodes	MET	Bulk enzyme heterojunction strategy enabled by TDN improved overall catalytic cascade efficiency by bringing enzyme pairs within critical coupling length Strategy proved its general applicability with range of enzyme pairs for electrochemically detecting clinically relevant molecular targets	[47]
2019	DNA-directed hybridization	Base-pairing of DNA-modified enzymes with DNA origami	DNA-conjugated GOx and HRP/DNA tile-modified Au electrode	DET	DNA-nanostructured platform can be fine-tuned for controllable interenzyme distances DNA origami-templated enzymatic cascade strategy enabled construction of a programmable and electrochemically driven biomimetic device	[140]
2017	DNA-directed hybridization	Base-pairing of zinc finger DNA-binding protein-fused enzymes and DNA-modified MWCNT	Zinc-finger DNA-binding protein fused- alcohol dehydrogenase and aldehyde dehydrogenase/DNA-modified MWCNT	DET	Zinc finger domains serve as both tags to isolate enzymes from crude cell lysates and anchors to immobilize = enzymes on DNA-modified MWCNT Demonstrated a protein purification-free approach to assemble enzyme cascades as bioanodes	[141]
	DNA hybridization	Enzyme attachment on CL-ODNs	GOx and HRP/CL-ODNs/Au electrode	MET	Bi-enzyme/DNA complex allows enzyme pairing to form complex on microelectrode surface for detection of local glucose distribution relative orientation of bi-enzyme on electrode strongly affected current intensities	[142]

Protein–protein interactions

SpyTag-SpyCatcher system

The SpyTag-SpyCatcher (ST-SC) interaction is based on the formation of spontaneous isopeptide bonds between the Lys residue in the 13 amino acid peptide (SpyTag, ST) and the Asp residue in the complementary 116-residue polypeptide (SpyCatcher, SC) [50]. ST-SC forms spontaneous isopeptide bonds in the temperature range of 4–37°C, pH 5–8, without needing stringent buffering conditions. Despite the remarkable characteristics of the ST-SC bioconjugation, its direct application in enzyme immobilization on electrodes has rarely been reported. However, Miura and colleagues recently demonstrated the immobilization of ST-fused anti-C-reactive protein (anti-CRP) on SC-decorated magnetic beads on an electrode, which subsequently facilitated the binding of a GDH-containing detection module to C-reactive protein [51] (Table 2 and Figure 3).

Sortase-mediated interpeptide conjugation

Sortase A (SrtA)-mediated protein ligation is a bio-orthogonal chemical reaction that covalently attaches two peptides, facilitated by the transpeptidase SrtA from *Staphylococcus aureus*. SrtA recognizes the LPXTG motif on a protein and catalyzes the amide bond formation between its Thr residue and the Gly oligomer (G_n) on complementary proteins or objects [52,53]. This strategy is highly site specific and irreversible under physiological conditions, making it a promising strategy for enzyme immobilization on electrodes with the desired surface orientation. The resulting LPXT$(G)_n$ junction is a short amino acid sequence that allows close enzyme–electrode proximity. Le and colleagues demonstrated SrtA-mediated coupling of LPETG-tagged photosystem I (PSI) with a triglycine-decorated gold (Au) electrode, creating oriented PSI monolayers on the electrode with 94% accuracy [42]. Matsumoto and colleagues [54] also utilized SrtA-assisted interpeptide ligation to bridge GDH-bound streptavidin with a hydrogel on a glassy carbon electrode (Table 2 and Figure 3).

Cohesin-dockerin system

The cohesion (Coh)–dockerin (Doc) system was first constructed by Bayer and colleagues [55], inspired by the cellulosome complex, in which subunits are interconnected by the species-specific interactions between the Coh and Doc domains. The bio-orthogonal Coh-Doc pair facilitates the assembly of multiple enzymes into a single functional complex [56]. However, *in vitro* enzymatic electrochemical systems seldom adopted the principle of Coh-Doc bioconjugation until Meng and colleagues [57] introduced self-assembled synthetic enzyme complexes on electrodes utilizing Coh-Doc affinity and the cellulose-specific binding of the cellulose-binding module (CBM). They constructed a synthetic mini-scaffold to contain the CBM at the N terminus, followed by three different Cohs. The Doc-fused cascade enzymes were then colocalized on the Coh array with controlled spatial organization to optimize the cascade reaction efficiency and bioelectricity generation. Given that it is well established that the

Note to Table 1:
[a]Abbreviations: 4-MBA, 4-mercaptobenzoic acid; AuNP, gold nanoparticles; BSA, bovine serum albumin; CBM, carbohydrate binding-module; Ccr, crotonyl-CoA reductase; CD, carbon dot; CFP, carbon fiber paper; CL-ODN, cross-linked oligodeoxynucleotide; CNT, carbon nanotube; CRP, C-reactive protein; DAAO, D-amino acid oxidase; DF, DNA flower; EcFDH-H, *Escherichia coli* formate dehydrogenase H; EGDGE, ethylene glycol diglycidyl ether; FAD-GDH, flavin adenine dinucleotide-dependent glucose dehydrogenase; FDH, formate dehydrogenase; FNR, ferredoxin $NADP^+$ reductase; GA, glutaraldehyde; GBP, Au-binding peptide; GCE, glassy carbon electrode; GDH, glucose dehydrogenase; GDHγα, FAD-GDH gamma-alpha complex; GNR, graphene nanoribbons; GOx, glucose oxidase; HFM, hollow fiber membranes; HRP, horseradish peroxidase; IgG, Immunoglobulin G; IO, inverse opal; ITO, indium tin oxide; Lac, laccase; LPEIs, poly(ethyleneimine)s; MCO, multicopper oxidase; MgOC, magnesium oxide-templated porous carbon; MWCNTs, multiwalled carbon nanotubes; PAA, polyanthranilic acid; PEDOT, poly(3,4-ethylenedioxythiophene); PEI, polyethyleneimine; poly-COO^-, polycarboxylate; ppm, parts per million; PPy, polypyrrole; PQQ, pyrroloquinoline-quinone; pyrene-KDDD, pyrene moiety-modified polypeptide; RGO/Au/Ni, graphene-Au nanohybrid on nickel; RGO/Au/Ni, nickel nanoparticles loaded on reduced graphene oxide; SA, streptavidin; SAM, self-assembled monolayer; SOX, sarcosine oxidase; ST-fused-anti-CRP scFvs, ST-fused anti-C-reactive protein single-chain variable fragments; ST-SC, SpyTag-SpyCatcher; TDN, tetrahedral DNA nanostructures; TPA, terephthalaldehyde; ZIF-8, zeolitic imidazolate framework-8.

Figure 2. Ten-year timeline of advances in enzyme immobilization technologies for enzymatic electrocatalytic systems (described in Table 1 in the main text). Enzyme immobilization approaches in enzyme–electrodes are categorized by binding principles (physical adsorption, entrapment or encapsulation, covalent linkage, cross-linking, bioaffinity, and DNA-directed hybridization). These strategies have been applied to various enzyme–electrode platforms, differing in ET mechanisms, enzymatic reactions, and applications. The methodology should be carefully selected, considering enzyme properties, reaction environments, and the number of enzymes involved. Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; BSA, bovine serum albumin; CBM, cellulose-binding module; CNT, carbon nanotube; CRP, C-reactive protein; DAAO, D-amino acid oxidase; DET, direct electron transfer; FDH, formate dehydrogenase, GDH, glucose dehydrogenase; GOD, GOx, glucose oxidase; HFM, hollow-fiber membranes; HRP, horseradish peroxidase; INV, invertase; ITO, indium tin oxide; LDH, lactate dehydrogenase; LOx, lactate oxidase; MBA, mercaptobenzoic acid; MET, mediated electron transfer; MWCNT, multiwalled carbon nanotube; PS II, photosystem II; SAM, self-assembled monolayer; SOx, sarcosine oxidase; TDN, tetrahedral DNA nanostructure.

modular assembly of multiple enzymes is feasible using the Coh-Doc system, this approach could be a powerful tool for enhancing the enzymatic chain reaction rate in enzyme cascade-based electrochemical systems (Table 2 and Figure 3).

Table 2. Protein-engineering strategies for enzyme–electrode wiring[a]

Enzyme–electrode interface	Enzyme/inorganic interfacing principle	Protein-engineering method	Inorganic surface modification method	Pros and cons	Refs
Protein–protein interaction					
ST-SC system	Spontaneous isopeptide bonds between lysine residue in ST and aspartate residue in complementary SC	Fusion of SpyTag peptide to anti-CRP	Decoration of magnetic bead with SpyCatcher proteins	Pros: ST-SC bioconjugation is highly specific and irreversible, with isopeptide bond between ST and SC capable of forming across broad range of temperatures and pH levels Cons: gap between enzyme and electrode may increase due to substantial size of proteins	[51]
Sortase-mediated interpeptide interaction	Covalent bonding between LPXTG motif and glycine oligomer by Sortase-guided transpeptidation	Genetic tethering of LPXTG motif to Photosystem I protein	Triglycine-decorated Au electrode	Pros: enzyme–electrode distance can be minimized because resulting LPXT$(G)_n$ junction is short amino acid sequence Cons: involves use of third protein (sortase) during enzyme immobilization on electrode, which can increase procedure complexity	[42]
		Genetic tethering of LPXTG motif to streptavidin that is to bind with biotin-modified GDH	Azido-containing triglycine entrapped within hydrogel modified on GCE		[54]
Coh–Doc system	Complexation of Coh and Doc proteins	Genetic fusion of Doc protein to cascade enzymes	Fusion of Coh with CBM and subsequent binding of fusion protein to cellulose-modified electrode	Pros: Coh-Doc affinity is highly selective and enables co-immobilization of multiple enzymes on electrode with precise positioning control Cons: gap between enzyme and electrode increases due to substantial size of Coh-Doc complex	[57]
Protein–ligand interaction					
Cysteine-maleimide bond	Specific reaction between free thiol group of cysteine and maleimide groups on electrode	Site-directed mutagenesis for cysteine substitution at surface of cellobiose dehydrogenase	Carbon nanotube electrodes modified with maleimide groups	Pros: cysteine–maleimide interaction offers flexibility for selection of enzymes and anchoring position in enzymes. In addition, stable and specific reaction between cysteine and maleimide can be exploited to orient enzymes close to electrode surface for DET Cons: selection of	[58]
		Site-directed mutagenesis for cysteine substitution on surface of flavodehydrogenase domain of flavocytochrome of cellobiose dehydrogenase	Carbon nanotube electrodes modified with maleimide groups		[59]
		Site-directed mutagenesis for cysteine substitution on surface of cellobiose dehydrogenase	Au and GCE modified with maleimide groups		[60]

Table 2. (continued)

Enzyme–electrode interface	Enzyme/inorganic interfacing principle	Protein-engineering method	Inorganic surface modification method	Pros and cons	Refs
		Site-directed mutagenesis for cysteine substitution at surface of bilirubin oxidase	Glassy carbon/multiwalled CNT electrodes modified with maleimide groups	cysteine mutation site requires in-depth understanding of enzyme 3D structure. In addition, there is risk of opportunist bond formation resulting in non-ideal enzyme surface orientation	[61]
Cysteine-maleimide-Au	Specific reaction between thiol group of cysteine and maleimide-modified Au	Site-directed mutagenesis for cysteine substitution near active site of glucose oxidase	Maleimide-modified Au nanoparticle	Pros: cysteine–maleimide–Au interaction enables site-specific immobilization for DET with minimal structure alteration Cons: requires deletion of native surface cysteine residue to avoid nonspecific orientation	[20]
Lysine-pyrene	Specific reaction between lysine and pyrene-modified electrode	Site-directed mutagenesis for surface accessible lysine near active center of laccase	Pyrene-modified CNT electrode/pyrene/β-cyclodextrin-modified Au electrode	Pros: lysine–pyrene interaction enables site-specific immobilization for DET with minimal structure alterations Cons: usually high prevalence of lysine in enzyme polypeptide sequence	[41]
UAA	Specific reaction of azide–alkyne (chemical handle of PrK) cycloaddition	PrK incorporated into copper oxidase at different distances from active sites	Glass carbon electrode modified with copper (I)-catalyzed azide-alkyne cycloaddition (click) reaction to azide-pyrene	Pros: use of UAA guarantees precise and unique site-specific immobilization Cons: UAA incorporation requires complex expression system and may lead to low yield of UAA-containing protein	[62]
		PrK incorporated into FAD-GDH fused to MCD			[63]
His-tag/metal chelate complexes-NTA	Specific interaction between His tagged-enzyme and metal chelate complexes with metalated NTA	His-tag fusion at N or C terminus of each of three subunits of pyrroloquinoline quinone-dependent aldehyde dehydrogenases	Au surface functionalized with Ni-NTA moiety	Pros: presence of His-tag facilitates site-specific enzyme immobilization for DET; His-tag also serves dual function for both purification and immobilization Cons: terminal His-tag fusion limited to one or two possible orientations and requires surface modification with Ni-NTA; fusion site selection complexity increases with number of enzyme subunits	[64]

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Table 2. (continued)

Enzyme–electrode interface	Enzyme/inorganic interfacing principle	Protein-engineering method	Inorganic surface modification method	Pros and cons	Refs
		His-tag fusion at N terminus of multicopper oxidase	CNT functionalized with Ni-NTA moiety	Pros: His-tag fusion enables predetermined enzyme orientation on electrode for DET Cons: limited to one or two possible orientation and requires surface modification with Ni-NTA	[65]
		His-tag fusion at terminus of glucose oxidase	Electropolymerization of pyrrole N-substituted by NTA as chelating center of Cu^{2+}	Pros: His-tag fusion is used for oriented and reversible enzyme immobilization Cons: terminal His-tag fusion is restricted to one or two possible orientations and has strict pH requirements	[66]
		His-tag fusion at N or C terminus of multicopper oxidase	Multiwalled CNTs modified with pyrene/NTA-Ni^{2+} linker	Pros: presence of His-tag orients enzymes in orientation ideal for DET Cons: terminal His-tag fusion is restricted to one or two possible orientations and always requires surface modification; also possibility of steric hindrance of densely packed enzymes	[69]
		His-tag fusion at N or C terminus of multicopper oxidase	Multiwalled CNTs modified with pyrene/NTA-Ni^{2+} linker and 1-PCA as spacer	Pros: presence of His-tag enables oriented immobilization for DET and enzyme loading can be regulated with proper modification of electrode surface Cons: terminal His-tag fusion restricted to one or two possible orientations and always requires intricate surface modifications	[70]
		Incorporation of intrachain His-pair (His-X_3-His) at surface-exposed α-helix of ferredoxin:NADP+ reductase	Au surface modified with self-assembled monolayer of thiols appended with NTA groups complexed with Cu^{2+}	Pros: intrachain His-pair allows for flexible selection of fusion site with minimal enzyme structural alterations Cons: requires in-depth understanding of protein 3D structure for fusion site selection; His-pair has lower binding strength than that of poly-His-tag	[72]

Table 2. (continued)

Enzyme–electrode interface	Enzyme/inorganic interfacing principle	Protein-engineering method	Inorganic surface modification method	Pros and cons	Refs
Streptavidin–biotin complex-based	Specific interaction of streptavidin and biotin	Incorporation of biotinylatable peptide sequences into Φ29 polymerase	Electrode surface functionalized with streptavidin	Pros: strong and specific interaction of streptavidin–biotin facilitates highly stable enzyme immobilization Cons: strong binding interaction could lead to enzyme denaturation upon immobilization	[74]
Protein–inorganic interaction					
Cysteine-gold	Specific reaction between thiol group of cysteine and Au	Site-directed mutagenesis for cysteine substitution at surface of horseradish peroxidase	No modification on Au electrode	Pros: cysteine-Au reaction enables site-specific immobilization for DET and requires no surface modification Cons: requires in-depth understanding of enzyme 3D structure	[75]
		Site-directed mutagenesis for cysteine substitution near active site of bilirubin oxidase	Macroporous Au electrode	Pros: cysteine-Au reaction enables site-specific immobilization for DET and use of macroporous electrode allows control of enzyme loading Cons: requires in-depth understanding of enzyme 3D structure	[76]
Cysteine-silver	Specific reaction between thiol group of cysteine and silver nanocluster	Site-directed mutagenesis for cysteine substitution near to 4Fe-4S cluster and native cysteine deletion in [Ni-Fe] hydrogenase	Silver nanocluster	Pros: cysteine-silver reaction allows site-specific immobilization for DET with minimal enzyme structure alteration Cons: requires in-depth understanding of enzyme 3D structure; native cysteine should be deleted to avoid nonspecific interaction	[77]
GBP-Au	Specific recognition of Au by GBP	GBP fused to lactate dehydrogenase	Au surface (nanoparticles and electrodes)	Pros: GBP fusion enables self-immobilization of enzymes onto range of Au substrates while retaining enzyme bioactivity Cons: GBP-fusion strategy is restricted to one or two possible orientations	[82]

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Table 2. (continued)

Enzyme–electrode interface	Enzyme/inorganic interfacing principle	Protein-engineering method	Inorganic surface modification method	Pros and cons	Refs
		GBP fused to NAD-dependent formate dehydrogenase	Gold layer coated on silicon wafers	Pros: the GBP-fusion enables self-immobilization of enzymes onto range of gold substrates while retain enzyme's bioactivity. Cons: the GBP-fusion strategy is restricted to one or two possible orientations.	[83]
		GBP genetically fused to N or C terminus of FAD-dependent glucose dehydrogenase containing only α- and γ-subunits	Gold film deposition	Pros: the GBP-fusion strategy enables control of enzyme orientation, facilitates a stable immobilization, and promotes formation of enzyme monolayer for a fast interfacial DET. Cons: the GBP-fusion strategy is limited to one or two possible orientations. There is also a possible clash interaction between the solid-binding peptide and the enzyme of interest. This strategy requires in-depth understanding of enzyme structure.	[36,85,86,88,89]
		GBP genetically fused to N- or/and C-terminus of catalytic subunit of carbon monoxide dehydrogenase	No modification on Au electrode		[24]
		GBP genetically fused to N- or/and C-terminus of NAD-dependent formate dehydrogenase			[87]
		GBP genetically fused to N or/and C terminus of FAD-dependent glucose dehydrogenase and invertase	Au film deposition		[2]
CNT-binding peptide-single-walled CNT	Specific recognition of CNT-binding peptide to CNT	CNT-binding peptide genetically fused to terminus of multicopper oxidase	Single-walled CNT	Pros: CNT binding peptide-fusion allows control of enzyme orientation for DET and increased enzyme loading Cons: strategy is limited to one or two possible orientations	[90]
		Carbon nanotube-binding peptide genetically fused to terminus of multicopper oxidase	One-dimensionally oriented single-walled carbon nanotube	Pros: the carbon nanotube binding peptide-fusion allows control of enzyme orientation for DET and increase enzyme loading. Cons: this strategy is limited to one or two possible orientations.	[91]
Silaffin peptide–silica matrix	Specific recognition of silaffin peptide to silica	Silaffin peptide genetically fused to sarcosine oxidase	Silica matrix	Pros: silaffin peptide fusion enables self-immobilization and increased enzyme loading on silica matrix Cons: strategy limited to one or two possible orientations	[92]

[a]Abbreviations: anti-CRP, anti-C-reactive protein; CBM, cellulose-binding module; CNT, carbon nanotube; Coh, cohesion; Doc, dockerin; GBP, Au-binding peptide; MCD, minimal cytochrome c domain; NTA, nitriloacetic acid; PCA, pyrenecarboxylic acid; PrK, propargyl-L-lysine.

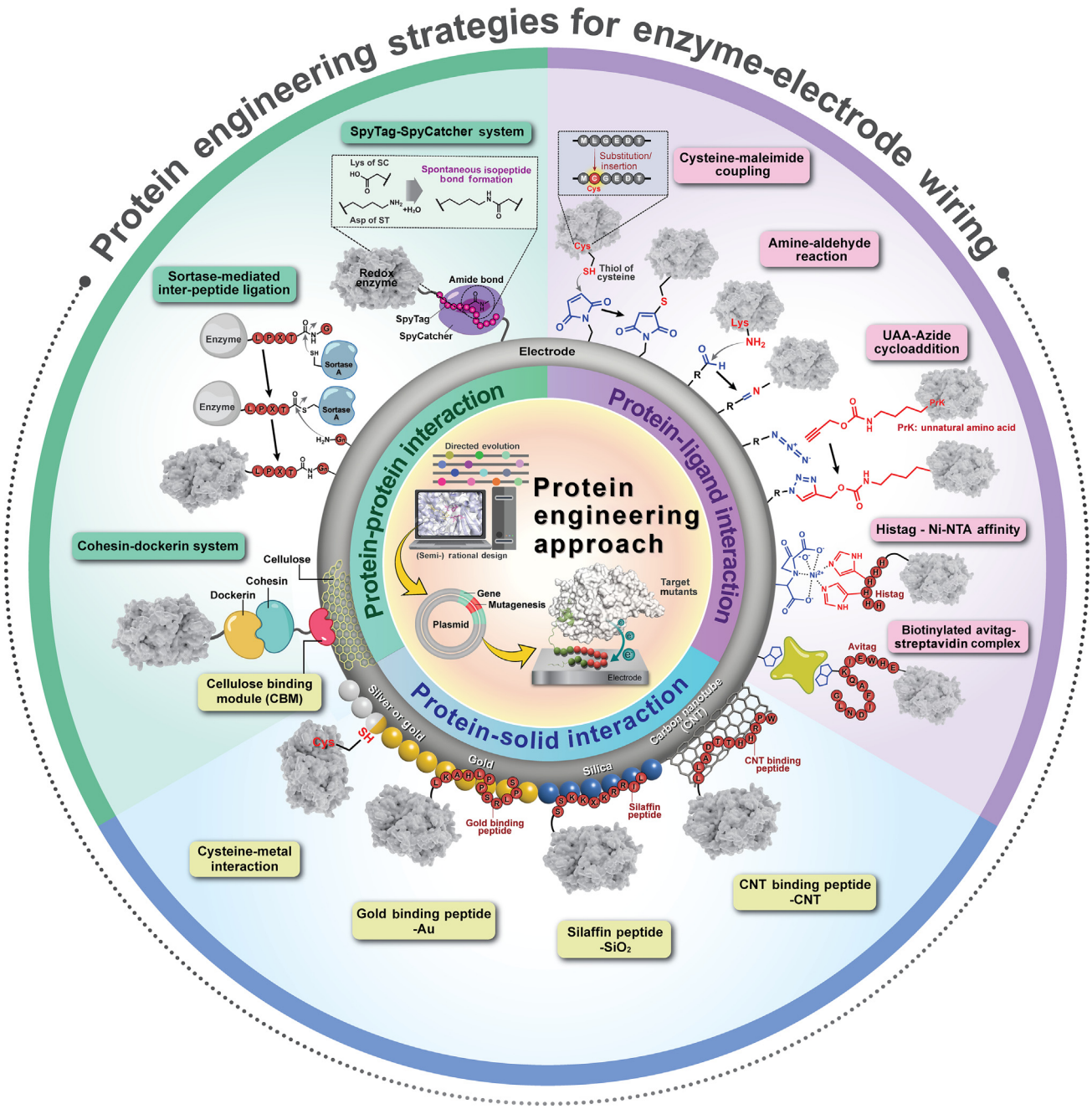

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Figure 3. Cutting-edge protein engineering approaches for enzyme-electrode wiring. Protein engineering-based enzyme immobilization strategies can be categorized based on the principles engaged at the wired enzyme–electrode interface: protein–protein interactions, protein–ligand interactions, and protein–solid interactions. In protein–protein interactions, both host and guest molecules, modified on the enzyme or electrode, are proteins of diverse molecular sizes. They conjugate through affinity interactions, such as the SpyTag-SpyCatcher system and cohesin–dockerin system, or through reactions mediated by a third protein, such as sortase-mediated interpeptide ligation. In protein–ligand interactions, the counterpart molecule is a small molecule, referred to as a ligand. In this regard, enzymes can be conjugated to electrodes via click chemistry, including cysteine–maleimide coupling, amine–aldehyde reactions, and unnatural amino acid (UAA)–azide cyclo-addition. In addition, affinity peptides fused to enzymes can guide enzyme attachment to surface-functionalized ligands, such as His-tag-Ni-NTA affinity and the biotinylated AviTag-streptavidin complex. In protein–solid interactions, without any protein or chemical functionalization of electrode surface, enzymes can directly interact with electrode materials through fused solid-binding affinity peptides. Examples include cysteine–metal interactions, gold (Au)-binding peptide–Au, silaffin peptide–SiO_2, and carbon nanotube (CNT)-binding peptide–CNT. Abbreviations: SC, Spy-Catcher; ST, Spy-Tag.

Protein–ligand interactions

Coupling protein engineering to surface chemistry

Cysteine, a low-prevalence amino acid, is highly appealing because it is the only canonical amino acid with a thiol group (–SH), allowing for unique chemical attributes that can be utilized for cysteine-specific biochemical ligation processes. Individual cysteine residues have been introduced at specific locations of the flavodehydrogenase domain of cellobiose dehydrogenase to immobilize mutants in different orientations via covalent cysteine–maleimide coupling.

DET occurs through the Cyt domain, the mobility and distance of which from the electrode are important for ET [58–60]. The same approach was used to immobilize bilirubin oxidase for electrocatalytic oxygen reduction [61]. Lalaoui and colleagues developed a lysine–pyrene ligation strategy by mutating arginine to lysine at the surface near the T1 copper ion of a fungal laccase to generate the mutant UNIK161 [41]. UNIK161 was further functionalized with a pyrene group that guaranteed the unique single orientation of the laccase on the surface of nanostructured electrodes with extended π-systems. This resulted in robust biocathodes with excellent bioelectrocatalytic dioxygen reduction properties. The use of cysteine or lysine for coupling is promising; however, the addition or deletion of surface-exposed cysteine or lysine residues may adversely impact protein folding, particularly cysteine, which often has a catalytic role in enzymes (Table 2 and Figure 3).

Unnatural amino acids as chemical handles

Unnatural amino acid (UAA) incorporation offers a unique orthogonal 'chemical handle' that allows the conjugation of a linker in single anchoring point in the protein sequence, guaranteeing a precise and highly controlled enzyme orientation on electrode surface. Schlesinger and colleagues incorporated UAA propargyl-L-lysine (PrK) into copper oxidase at various distances from the enzyme active sites to control its surface orientation [62]. An identical strategy was subsequently used to site-specifically wire a FAD-GDH fused to a minimal Cyt c domain to an electrode close to its FAD-binding site and Cyt c domain [63]. This resulted in a 15 times higher ET rate and a tenfold higher sensitivity compared with the nonspecifically wired molecule. Genetic code expansion enables the incorporation of UAA at predefined sites in the protein backbone in response to an amber codon (UAG). However, this involves additional complex translational machinery, which often leads to low yields of UAA-containing proteins (Table 2 and Figure 3).

His-tag-based enzyme immobilization

The orientation of His-tagged enzymes can be precisely controlled by the formation of ternary metal chelate complexes with metalated nitriloacetic acid (NTA), such as Ni- [64,65], Cu- [66,67], or Zn-NTA [68], with functionalities on the electrode. This approach is convenient because the His-tag serves a dual purpose: protein purification and immobilization. Xu and Minteer labeled each of the three subunits of pyrroloquinoline quinone-dependent aldehyde dehydrogenases, producing a complex multisubunit enzyme for site-specific immobilization on a Au surface modified with a Ni-NTA moiety [64]. The orientation of multisubunit enzymes can significantly affect DET by varying the ET distances. Later, Amano and colleagues studied the oriented immobilization of His-tagged multicopper oxidase (MCO) via Ni^{2+} affinity [65] and showed that ET efficiency was dependent on the affinity tag site, orientation, distance between the type 1 copper of MCO and electrode [69], and biomolecular density on electrode [70].

Furthermore, the His-tag-based approach allows for reversible immobilization using imidazole or ethylenediaminetetraacetic acid (EDTA) [66] as chelating or competitive ligands, respectively, or through an electrochemical signal-triggered release [71]. This feature renders the method appealing from a sustainability perspective. Haddour and colleagues [66] generated an electropolymerized NTA film for the reversible and controlled anchoring of Cu^{2+} ions and His-tagged GOx. The amperometric

response of the His-tagged GOx-modified electrode remained stable after five successive cycles of His-tagged GOx release and electrode regeneration via Cu^{2+} removal by EDTA. Although this approach is promising, it is not feasible for applications that require more negative potential input than those of metal complexes.

Alternatively, a shorter, intrachain histidine pair (His-X_3-His) was engineered into the surface-exposed α-helix of ferredoxin:$NADP^+$ reductase (FNR) to control the enzyme orientation on a Au surface modified with a self-assembled monolayer of thiols appended with NTA groups complexed with metal transition ions [72]. The mutation site of the (His-X_3-His) in FNR determines the enzyme-surface orientation and, subsequently, its DET ability on electrode. In contrast to the terminal poly-histidine tag confined at either the N or C terminus, the (His-X_3-His) provides greater flexibility in enzymatic anchoring sites, making it an invaluable toolbox for enzyme–electrode wiring with minimal enzyme modification (Table 2 and Figure 3).

Streptavidin–biotin complex-based approach

The avidin/streptavidin–biotin complex exhibits the strongest known noncovalent interaction, characterized by a femtomolar affinity between a protein and ligand. [73]. Various studies have used this interaction for enzyme–electrode immobilization [54]. Zhang and colleagues [74] engineered a Φ29 polymerase by incorporating biotinylatable peptide sequences that serve as a binding point to an streptavidin-functionalized electrode. Notably, the use of protein engineering to design the streptavidin–biotin complexes for enzyme immobilization on electrodes is not common, because the method by which the enzyme of interest can be chemically biotinylated has been well-established. Another option is Streptag-II (WSHPQFEK) fusion with the enzyme of interest, which binds streptavidin or its engineered form, Strep-Tactin, thus eliminating the need for biotin (Table 2 and Figure 3).

Direct protein–inorganic adsorption

Cysteine–metal interactions

Enzymes engineered with surface cysteine residues close to the active site allow highly specific attachment of the enzyme to Au via the thiol–Au reaction [20,75,76]. Zhang and colleagues demonstrated improved electrocatalytic oxygen reduction and long-term stability by using highly ordered microporous Au electrodes modified with a cysteine-introduced bilirubin oxidase mutant [76]. A similar approach on a [NiFe]-hydrogenase led to enhanced photoevolution of H_2 where a single cysteine residue was introduced close to the surface FeS cluster after deletion of naturally existing cysteine, enabling specific binding to a silver nanocluster [77] (Table 2 and Figure 3).

Solid-binding peptide fusion

The discovery of **solid-binding peptides (SBPs)**, short amino acid sequences (6–21 amino acids) that display binding affinities to solid materials, such as Au, silver, platinum, silica, and carbon, has encouraged many researchers to leverage these special properties, resulting in a multitude of applications. Given that this review covers only use of SBP for enzyme-electrode applications, readers interested in other SBP-based biotechnological applications may refer to reviews by Care and colleagues [78], and Alvisi and de Vries [79].

SBP fusion tags have been used to immobilize proteins on diverse inorganic architectures [80,81]. For instance, an Au-binding peptide (GBP) fused to lactate dehydrogenase (LDH) enabled LDHs to self-immobilize from nanoparticles to electrodes, with good stability, showing promising application toward lactate biosensors and biofuel cells [82]. Later, NAD-dependent formate dehydrogenase (FDH) fused to GBP also self-immobilized on gold electrodes while retaining its catalytic activity, thereby forming a fusion enzyme-integrated circuit-based formate biosensor

[83]. LDH and FDH were integrated into a cascade reaction for efficient L-lactate production with NADH regeneration, utilizing a modular strategy for enzyme orientation and spatial localization on solid supports [84]. This approach opens the possibility for controlled co-immobilization of multiple enzymes on electrodes for higher electricity generation or multistep **electrosynthesis** into value-added products.

Similarly, Lee and colleagues combined enzyme truncation and SBP fusion by using GBP as a molecular linker to FAD-GDH α- and γ-subunits, without the ET subunit, β-subunit [85]. GBP fusion facilitated stable immobilization and uniform monolayer formation of the **fusion enzymes** onto an Au electrode, as well as GBP-fusion site-dependent regulation of enzyme orientation and DET efficiency [86,87]; when combined with electrode patterning, this approach can be used to tune interenzyme spacing [88]. Key factors for optimization of SBP fusion-based enzyme–electrode interfacing include enzymatic fusion site, amino acid composition, and SBP tandem repeats [36,89]. Such a platform further allowed the relative orientations of co-immobilized cascade enzymes to be specifically controlled for efficient intermediate delivery, while enabling efficient DET at the enzyme–electrode interface [2].

Sakamoto and colleagues incorporated a carbon nanotube (CNT)-binding peptide into MCO for oriented enzyme immobilization on single-walled CNTs (SWCNT) [90]. Although the amount of immobilized enzyme was similar for wild-type and fusion enzymes, the latter showed over five times higher current density, indicating enhanced DET due to CNT-binding peptide-guided oriented enzyme attachments. Follow-up work demonstrated that immobilizing fusion-MCO on 1D-oriented SWCNT resulted in a twofold increase in the current density compared with an electrode with randomly stacked CNTs [91]. Chen and Hall [92] constructed a monomeric sarcosine oxidase (mSOx) fusion with a silaffin peptide, R5, which exhibits affinity for silica. The R5-fused-mSOx can be regulated to form a stable thick layer on the silica matrix and to generate a detectable current within the necessary range.

Compared with traditionally used chemical approaches relying on covalent bonds, SBPs bind to inorganic surface through multiple noncovalent interactions, including hydrophobic, electrostatic, polar, and hydrogen bonds, without the need for additional chemical treatment or physical modification [78,93]. However, their main limitation is their obligatory position at the N or C terminus, restricting possible enzyme orientations. Nevertheless, with a vast library of diverse binding peptides and rapid advances in protein structure prediction, shorter SBP tags that can be incorporated into the intrachain will undoubtedly become feasible in the future. This will facilitate engineering enzymes to achieve their intended functionality with greater flexibility for targeted biotechnological applications (Table 2 and Figure 3).

Concluding remarks and future perspectives

Various enzyme immobilization strategies have been used for facilitating efficient wiring of enzyme–electrodes in enzymatic electrocatalytic systems. However, challenges persist in long-term enzymatic catalysis and precise interfacing of enzyme cofactors with electrode surfaces due to a lack of understanding of enzyme features, such as protein structure, surface charge, and cofactor properties. Rapid advances in biotechnology and proteomics have aided structural prediction, computational design, and genetic engineering of enzyme–electrode construction in a more rational manner. In this context, protein engineering has been used to manipulate enzymes to have binding sites for electrode surface-functionalized proteins, ligands, or even bare electrode materials, resulting in site-specific enzyme attachments. This approach not only significantly enhances the binding selectivity and stability of enzymes, but also allows for uniform regulation of the exposure directions of substrate-binding sites or

Outstanding questions

What are the primary challenges in scaling up enzymatic fuel cells and electrosynthesis applications given the significant progress in protein engineering and electrode fabrication in recent years?

Could artificial intelligence serve as a solution for the directed evolution of redox enzymes with superenzymatic properties, or for the creation of novel enzymes that are not found in nature and are not significantly constrained in terms of air, mechanical, and chemical stability?

A substantial body of literature exists regarding enzyme–electrode performance, with numerous research groups focusing on shared interests in CO_2 reduction, nitrogen fixation, H_2 evolution, and uptake. Would standardizing electrochemical cell configurations and characterization techniques enhance knowledge sharing among scientists to promote sustainability?

How can we accurately and simultaneously control the distance and orientation between interenzymes and enzyme–electrodes in enzymatic cascade-based bioelectrochemical cells?

The orientation of enzymes on the electrode is crucial for substrate accessibility and for ensuring intimate electrical contact between the enzymatic cofactor and the electrode. What quantifying technologies are suitable for the accurate visualization of oriented enzymes on the electrode surface?

Reports on protein–protein interaction-guided enzyme–electrode interfacing are limited, despite its exceptional capabilities for site-specific enzyme immobilization. What obstacles exist in leveraging this interfacing principle within an enzymatic electrochemical system, and what strategies can be used to overcome them?

cofactors. Despite the advantages of protein-engineering approaches, several key issues still need to be addressed (see Outstanding questions). Addressing these will help advance these technologies, make them more feasible, and lead to the development of new methodologies.

In terms of the future perspectives of enzyme–electrode platforms, their biosensing applications for biomedical, forensic, and environmental monitoring purposes appear promising for immediate practical usage. Enzyme-based logic gates and circuits comprising multienzymatic chain reactions can be constructed by efficiently interfacing enzyme-based logic systems with electronic transducers, enabling the detection and monitoring of biomarkers or environmentally hazardous substances. Moreover, scaling up enzyme–electrode platforms offers significant opportunities in carbon capture, utilization, and storage (CCUS), which are crucial for fostering green and sustainable environments and societies. Recently discovered plastic-degrading enzymes, such as polyethylene terephthalate hydrolase (PETase) and mono(2-hydroxyethyl) terephthalate hydrolase (MHETase), can be used for carbon upcycling and recycling in this context. While challenges, such as enzyme lifetime and operational stability, remain, optimizing the processes in enzyme-based electrocatalytic reactors will make enzyme–electrode systems more resilient and adaptable for industrial applications.

Author contributions

Conceptualization, H.L. and S.S.R.; investigation, H.L., S.S.R., J.S.S., and M.L.; writing – original draft, H.L., S.S.R., and J.S.S.; writing – review and editing, H.L. and S.S.R.; visualization, H.L.; supervision, I.S.C.; funding acquisition, I.S.C.

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Declaration of interests

The authors declare no conflicts of interest.

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