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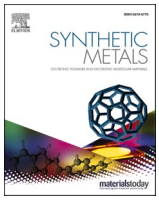

One-step electrochemical approach of enzyme immobilization for bioelectrochemical applications

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ABSTRACT

Enzymatic bioelectrochemistry represents the marriage of electrochemistry and enzymatic biocatalysis, and has led to important applications for biosensors, biofuel cells, and bioelectrocatalysis. Enzyme immobilization is the basis of enzymatic bioelectrochemistry, as immobilization itself determines the enzyme/material interface and thus the electrochemical performance. Amongst the range of methods of enzyme immobilization, one-step electrochemical approaches feature rapid immobilization and good control over the processes, enabling partial or total use of the electrode surface. In this mini-review, we first briefly introduce the operating principles of bioelectrochemical applications based on enzyme modified electrodes. We then overview recent progress in utilizing conductive polymers, redox-active modified polymers, sol–gel silica and electrochemically assistant adsorption for enzyme immobilization via one-step electrochemical approaches. The use of conductive polymers for in situ enzyme immobilization is our major focus. Perspectives for future work are also described.

1. Introduction

Enzymatic bioelectrochemistry studies the electrochemical behavior of oxidoreductases on solid electrode surfaces [1,2]. Electron transfer (ET) pathways between the redox centers of the enzyme and the surface of the electrode are established, via either direct (DET) or mediated ET (MET) [3]. In bioelectrochemistry, the enzyme is turned over in continuous biocatalytic cycles by transferring electrons to or from the electrode surface. The rate of ET is of crucial importance in the performance of bioelectrochemical systems, including biosensors [4], biofuel cells [5], and bioelectrosynthesis [6].

Enzyme immobilization is critical in both bioelectrochemistry and biocatalysis [7]. Effective methods of immobilization require efficient levels of process, with retention of catalytic activity. Immobilization enables reuse of the enzymes (which are often expensive) and can enhance enzyme stability. In bioelectrochemical applications, immobilization should thus lead to a high percentage of “electroactive” enzymes, that enables ET between the enzyme and the electrode surface. In particular for DET, the redox centers of the enzyme should be as close as possible to the electrode surface, as the ET rate decreases approximately exponentially with increasing electron tunneling distance [8–11]. The rate of ET is thus very low for distances longer than about 1.6 nm [12]. Common methods of enzyme immobilization include physical adsorption, polymer entrapment, affinity and covalent binding, and cross-linking [13]. The time required for enzyme immobilization can vary from minutes to hours, or even days. One-step electrochemical approaches are of particular interest, with detailed control of the process, and the ability to address the full area of the electrode surface, irrespective of the geometry.

The concept of a “one-step electrochemical approach” refers to methods that rest on electrochemical techniques (e.g., cyclic voltammetry (CV), linear sweep voltammetry (LSV), chronoamperometry, chronopotentiometry or pulse chronoamperometry) with the electrode in an electrolyte that contains buffer, enzymes and other functional components. The electrochemical steps at the electrode surface can be either oxidation to initiate polymerization, or reduction for the hydrogen evolution reaction to change the local pH and thus trigger solution based processes. *In situ* electropolymerization of a conducting polymer (CP), such as the commonly used polypyrrole (PPy) and poly (3,4-ethylenedioxythiophene) (PEDOT) in the presence of both the monomer and enzyme has been developed into an important and facile one-step method for enzyme immobilization, and is the major subject of

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this review. Several recent reviews related to the application of CPs for enzyme immobilization and development of biosensors and biofuel cells have been published [14,15]. In particular, the biocompatibility of some CPs offers important advantages essential for implantable bioelectronics [15]. Attention is focused on recent progress in the use of derivatives of PPy and PEDOT, leading to new applications e.g. ET mediation and anti-biofouling. Other one-step electrochemical approaches are also discussed, and perspectives for future work described.

2. One-step *in situ* enzyme immobilization in conductive polymer electropolymerization

Electropolymerization of organic monomers to form solid films accompanied by simultaneous enzyme incorporation within polymer matrices is a fast, facile, and attractive approach for enzyme immobilization. In a general electropolymerization process, a standard three-electrode electrochemical cell is used to oxidize the monomers at the electrode surface (Fig. 1). The resulting radical cations undergo polymerization along with deprotonation and incorporation of counter anions to maintain electroneutrality of the newly formed polymer. Enzyme molecules in the electrolyte can be embedded into the polymer network during the growth process. Mediators and co-enzymes can also be immobilized if present in the electrolyte. This one-step electrochemical process is gentle, controllable and reproducible. In particular, the polymer film thickness and the enzyme loading can be controlled by adjusting the electropolymerization parameters. The CPs formed provide a biocompatible micro-environment for enzymes, and the entrapped enzyme molecules retain good activity and stability. We consider first two types of CP, namely PPy and PEDOT. This is followed by a discussion of electrodeposition of Os complex modified redox polymer, sol-gel silica based support materials and finally by a discussion of electrochemically assisted direct adsorption of active proteins and enzymes.

2.1. PPy

PPy is one of the most attractive CPs utilized for enzyme immobilization. This is due to the sufficient solubility and relatively low oxidation potential of the monomer in aqueous solution, comparable with those of enzymes. As shown in Fig. 2A, in the electrochemical polymerization process, the oxidation of pyrrole monomers first takes place at a potential suitable for formation of radical cations at the electrode surface. Two cations then react accompanied by deprotonation, resulting in dimerization. Due to its higher level of conjugation, the dimer has a lower oxidation potential than the monomer. The dimer is thus immediately oxidized to a cation, followed by a subsequent coupling reaction with a monomeric radical cation, hence leading to chain propagation [16,17]. As an insulating polymer chain would terminate its own extension, it is crucial to oxidize the polymer electrochemically to produce a conducting state [18]. As a result, the polymer possesses a positively charged backbone (about one positive charge per four pyrrole units), which is neutralized by incorporation of counter anions from the electrolyte. Enzymes and mediators present in the immediate vicinity of the electrode surface can be physically embedded in the polymer matrix.

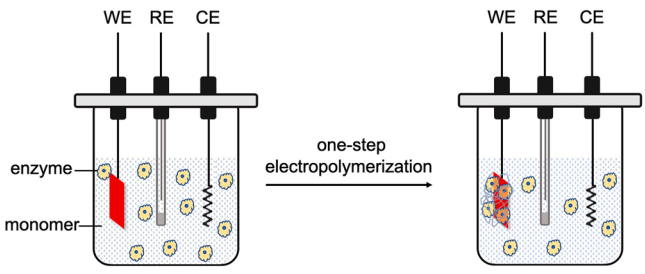

Fig. 1. Schematic drawing of one-step in situ enzyme immobilization via electropolymerization of conductive polymer.

The deposition of pyrrole can be achieved using potentiostatic [19], potentiodynamic [20], or galvanostatic techniques [21]. Potentiostatic polymerization enables synthesizing homogeneous polymer films by controlling the potential. After applying a suitable potential to the working electrode, rapid growth of PPy with increased effective surface area proceeds, accompanied by an increase in current (phase I) [17]. The current then decreases (phase II), until the electrode reaction is blocked due to the increased resistance of the polymer film (Fig. 2B). The thickness of the film can be estimated from the accumulated charge transferred (Q) during the electropolymerization process based on the equation [22]:

$$d = \frac{QM}{nFA\rho} \quad (1)$$

where M is the molar mass of pyrrole (67.09 g mol^{-1}), n the number of electrons associated with the formation of PPy (2.25), F the Faraday constant, A the real electrode surface area, and ρ the polymer density (1.5 g cm^{-3}).

The potentiodynamic process employs a multi-sweep method by cycling the potential that changes between neutral (insulating) and doped (conducting) states of the CP. In the first scan, the current increase (phase I) with increasing potential is a direct measure of the increase in accessible surface and the number of redox sites (Fig. 2C). The current decrease after the first cycle of a multi-sweep cyclic voltammogram at low scan rates is typically seen, underlying phase II as shown in Fig. 2B. The rate of PPy formation is directly visualized, but almost no direct information on the nucleation and growth mechanism can be observed [23]. Galvanostatic methods can also be used, but are less frequently used due to the poor level of control of film growth [17].

The PPy films obtained by electrochemical deposition display good permeability for both ions and neutral species [26,27]. After immobilization of enzymes, the films are therefore widely applied in the fabrication of biosensors for detecting glucose [25], ethanol [28], cholesterol [29,30] and hypoxanthine [31]. Glucose oxidase (GOx) is one of the most stable and commonly used enzymes for glucose sensing. Because the GOx active site, flavin adenine dinucleotide (FAD) is deeply buried in the enzyme, DET between native GOx and the electrode surface is essentially blocked. Dioxygen functions as a natural mediator for GOx, producing H_2O_2, which is a strong oxidant and can chemically oxidize PPy and destroy the polymer conjugation in ring opening steps [17]. On the other hand, the presence of H_2O_2 can initiate the polymerization of pyrrole monomers to form polymeric nanoparticles [32]. To avoid the generation of H_2O_2, suitable artificial mediators such as ferrocene, benzoquinone and their derivatives were introduced [33,34], either in solution or co-immobilized in the polymer. Ramanavicius and associates modified graphite electrodes with a composite layer of PPy, Prussian blue (PB) and GOx (PPy/PB/GOx), displaying a sensitivity of 1.0–1.9 A cm^{-2} mM^{-1} towards glucose in the range 0.1–20 mM (Fig. 2D-F) [25]. Due to the electrocatalytic properties of PB towards the by-product H_2O_2, the optimal potential for the registration of amperometric response towards glucose was at + 0.05 V vs $Ag/AgCl_{KClsat}$. Apart from amperometry, the PPy/GOx electrodes can be used as potentiometric glucose biosensors, by taking advantage of the pH sensitivity of PPy itself. The advantage of potentiometric detection is that the response is independent of the ageing and the size of the biosensor, in contrast to amperometric biosensors. For example, Adeloju and associates fabricated an ultra-thin (~55 nm) PPy/GOx potentiometric biosensor, with a dynamic range from 0.06 to 10 mM glucose. Notably the pyrrole monomer and GOx were here electropolymerized in the absence of supporting electrolyte, the enzyme itself playing the role of counter-ions [35].

Pramanik and associates fabricated a novel biocomposite electrode used for cholesterol sensing prepared by simultaneous

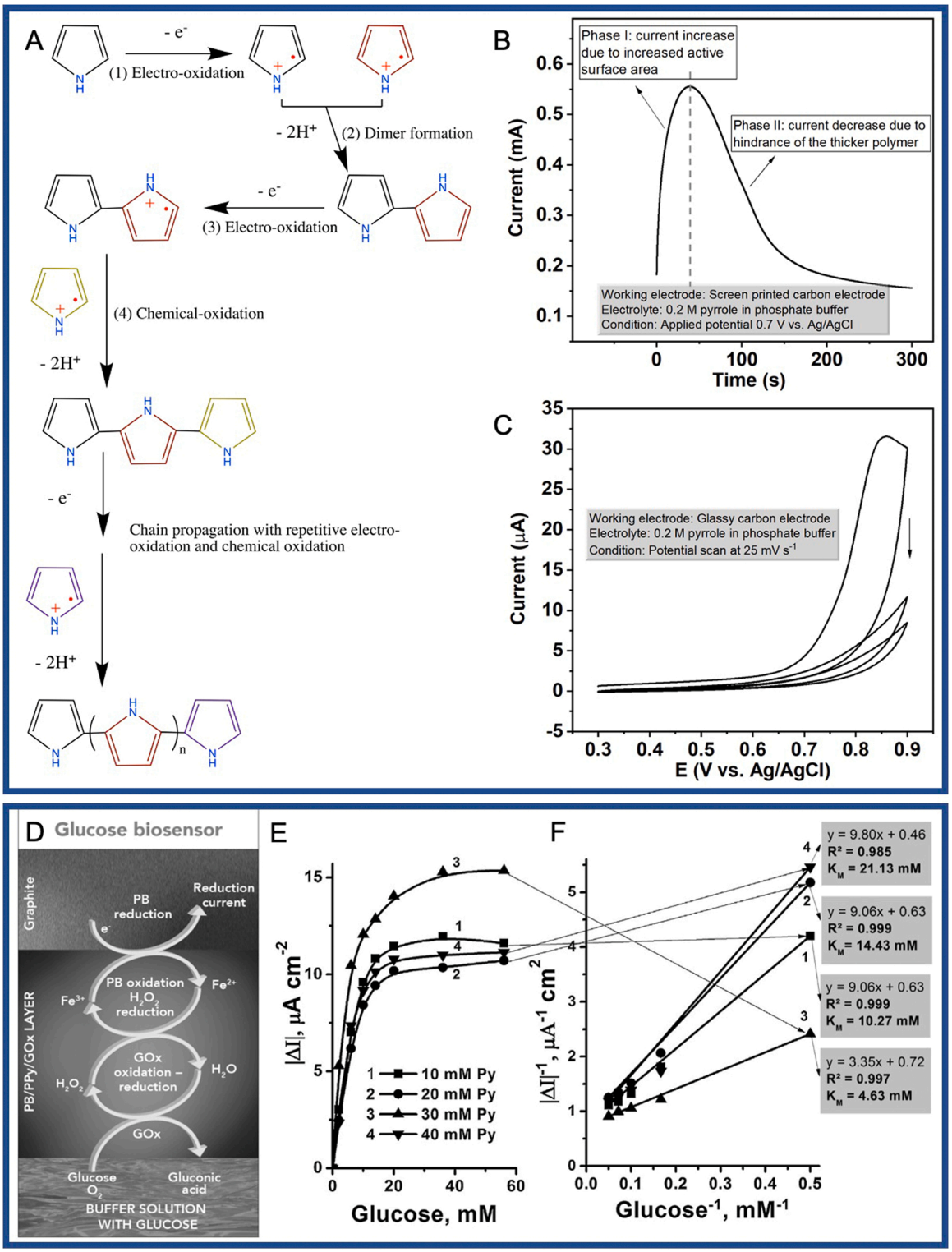

Fig. 2. (A) Schematic drawing of the electrochemical formation of PPy, inspired by Genies et al. [24]; (B) Current-time plot of the potentiostatic polymerization of pyrrole; (C) Multi-sweep potentiodynamic deposition of PPy; (D) Schematic representation of one-step based modification of a graphite electrode by a PPy/PB/GOx composite layer; Registered amperometric responses towards glucose in phosphate buffer registered at 0.05 V vs $Ag/AgCl_{(KClsat.)}$: (E) Calibration curve and the corresponding Lineweaver-Burk plot (F). Adapted with permission from Ref. [25]. Copyright 2017, Elsevier.

electropolymerisation of pyrrole and co-deposition of green reduced graphene oxide (gRGO) and cholesterol oxidase (ChOx). The as-prepared electrode exhibited excellent sensitivity (1095 mA mM^{-1} cm^{-2}), wide linear range (0.01–6 mM), and low detection limit (3.8 mM) of cholesterol [29]. DET between quino-hemoprotein alcohol dehydrogenase (PQQ-ADH) and PPy was also achieved [28]. The cooperative action of the enzyme-integrated prosthetic groups -PQQ and hemes- was assumed to allow this ET pathway between the enzyme's active site and the electrode surface via the conductive polymer backbone. Erol and associates designed a highly selective and stable

amperometric biosensor for the determination of the hypoxanthine (Hx) molecule, prepared by immobilizing the xanthine oxidase and uricase with polypyrrole-paratoluenesulfonate (PPy-pTS) on a platinum surface [31]. The detection limit for Hx of the prepared biosensor was determined to be 5×10^{-3} mM, and the linear working range as 5×10^{-3}–5 mM.

2.2. PEDOT

Another commonly used CP for enzyme entrapment is PEDOT, which can also be simply obtained by one-step electropolymerizing its ethylene-dioxythiophene monomer in aqueous solution. The monomers are oxidized and polymerized to form a p-doped polymer, the oxidation/reduction process of which proceeds reversibly and is accompanied by cation exchange due to the non-extrudable character of the doping anion (Fig. 3A-B) [36]. Compared to PPy, polythiophenes show superior electrochemical stability because of the higher ionization potential (ca. 5 eV versus 4 eV for PPy), protecting PEDOT against oxidative degradation by oxygen and H_2O_2 [37]. Moreover, owing to the outstanding properties such as high optical transparency (90% at d = 100 nm), good flexibility, high conductivity, and high chemical, and thermal/UV stability, PEDOT has attracted significant interest in organic electronics, sensors, and bioelectronics [36]. In spite of its merits, PEDOT, however, still faces challenges for practical application due to the low monomer solubility in water [38], usually requiring surfactants to increase the solubility. Polyethylene glycol (PEG) and poly(styrene sulfonic acid) (PSS) are two co-solvents reported for improving the solubility of EDOT in aqueous media, by increasing the hydrophilicity of the polymer [39,40].

Nanostructured carbon or gold are attractive active electrode materials. Xiao and associates reported a simple, one-step synthesis process of a hybrid film by electropolymerizing EDOT on a nanoporous gold (NPG) film. NPG has a unique nanoporous structure with highly active surfaces, promoting enzyme loading and accelerating ET of mediators. The successful modification of a ~10 nm thick PEDOT/GOx layer on the pore walls of NPG was confirmed by transmission electron microscopy (TEM) (Fig. 3C-F). A wider linear range and a higher sensitivity of glucose detection over a normal polycrystalline flat Au electrode was obtained (Figure G-H) [41]. Maleki et al., constructed a novel biosensor/artificial neural network (ANN) integrated system accomplished by electropolymerizing EDOT along with graphene oxide nano-sheets and laccase as anionic dopants. The biosensor showed good biocatalytic activity toward the oxidation of catechol with a detection limit of 0.032 μM and was successfully applied in analysis of real water samples. The predicted values from ANN modeling agreed well with experimental values [42].

2.3. Derivatives of PPy and PEDOT

Although PPy and PEDOT have been successfully used for enzyme immobilization for more than three decades, challenges remain, especially the poor stability of PPy and limited aqueous solubility of EDOT. Researchers hence focused on functionalizing the pendant groups on the polymer backbones to overcome these inherent limitations. PPy exhibits considerable overoxidation during electrodeposition on the electrode surface due to hydroxyl radicals created during the oxidation of water causing loosely bound polymer [43], while poly(3,4-ethylenedioxypyrrole) (PEDOP) exhibits great stability against overoxidation and higher water solubility over PEDOT [44]. Türkarslan and associates prepared a PEDOP based alcohol biosensor and compared its properties with PPy and PEDOT sensors. Incorporation of an alkenedioxy bridge, as that in PEDOT, into the 3- and 4-positions of the pyrrole ring results in a family of polymers with unique properties, including middle to high bandgaps and low oxidation potentials [45]. The N–H functionality of the PPy derivatives improves the aqueous compatibility in comparison with thiophene polymers. The resulting PEDOP–alcohol oxidase electrode shows superior stability, retaining 80% of initial activity after 28 days storage, in comparison to 50% and 23% activity for the PPy and PEDOT enzyme electrodes, respectively [46].

Besides the addition of surfactants, the aqueous solubility of EDOT can be enhanced by adding pendant groups. Numerous substituted PEDOT derivatives have been investigated by attaching hydrophilic substituents along their backbones [47,48]. Wu and associates selected a zwitterionic sulfobetaine group as the substituent for EDOT [49]. The resulting sulfobetaine-3,4-ethylenedioxythiophene (SBEDOT) monomer

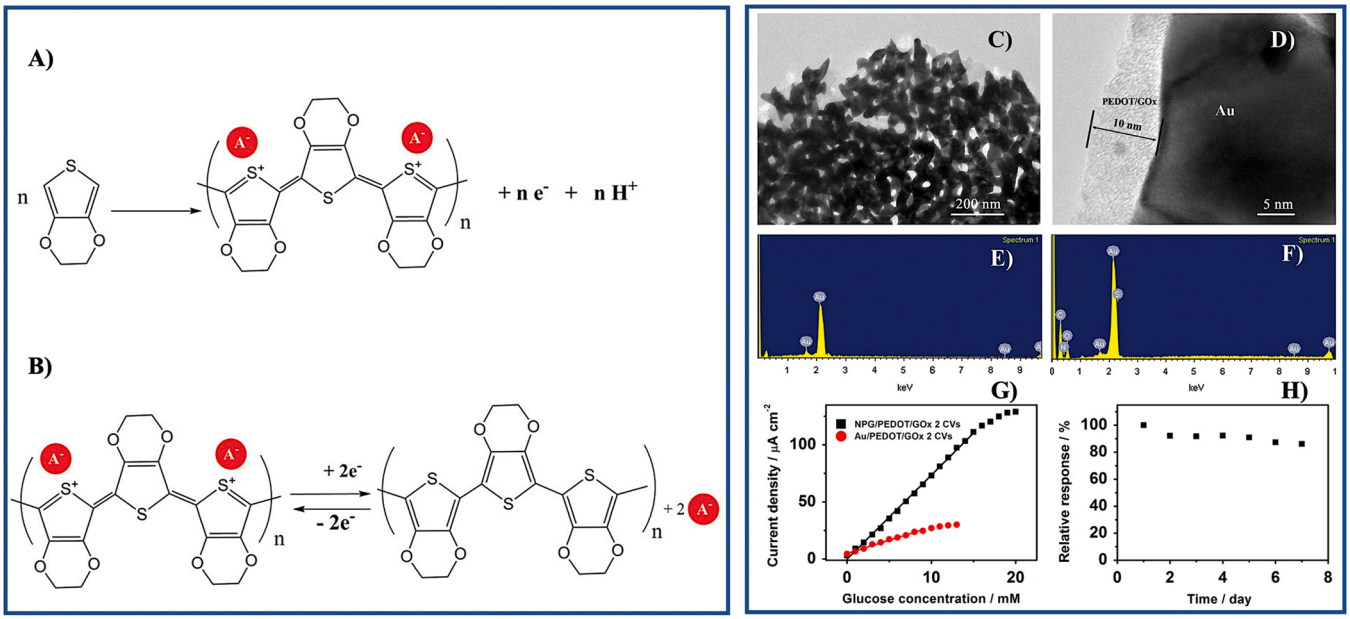

Fig. 3. The oxidation/reduction process of PEDOT. (A) Electropolymerization process of EDOT to produce PEDOT; (B) Polymer reduction reaction for PEDOT. Adapted with permission from Ref. [36]. Copyright 2016, The Royal Society of Chemistry; (C, D) TEM images of NPG/PEDOT/GOx; Energy dispersive X-ray analysis spectra of bare NPG (E) and NPG/PEDOT/GOx (F); (G) Calibration plot of NPG/PEDOT/GOx and Au/PEDOT/GOx; (H) Long term stability of NPG/PEDOT/GOx. Adapted with permission from Ref. [41]. Copyright 2013, Elsevier.

was highly soluble, and the electrostatic attraction among the zwitterionic sulfobetaine sidechains facilitated the deposition of the polymer on the electrode surface. After electropolymerization, PSBEDOT functioned as a three-dimensional CP matrix for the encapsulation of GOx, as well as an electrical conductor for transducing the signal produced via the enzymatic oxidization of glucose. In comparison with the PEDOT–GOx biosensor, the PSBEDOT–GOx electrode demonstrated higher sensitivity and improved stability, benefiting from the compact morphology of the PSBEDOT surface and strong hydrophilicity of the zwitterionic side chains that stabilized the enzyme. Moreover, the employment of zwitterionic materials generated an anti-fouling surface on the electrode, with the zwitterionic group preventing the adhesion of biological molecules, especially, serum albumin proteins [50].

Ferrocene and its derivatives are particularly useful mediators because of the high stability in both oxidized and reduced form in anaerobic environment, good redox reversibility and independence of pH [51]. To overcome the issue of mediator leaching via a simple entrapment by polymers, fusing ferrocene into the monomer before polymerization has been an attractive strategy to immobilize the mediator. Foulds and associates synthesized [(ferrocenyl)amidopropyl] pyrrole (FAPP) by coupling ferrocenecarboxylic acid to *N*-(3-aminopropyl)pyrrole [52]. Entrapment of enzyme in the redox active copolymer (FAPP/pyrrole) was then obtained using potentiodynamic voltammetry in an electrolyte solution containing pyrrole, FAPP and GOx. Ferrocene-pyrrole conjugates were found to be highly efficient for the mediated enzyme turnover. 2,5-di(thienyl)pyrrole could also be covalently bound to ferrocene [51,53].

Os-complexes have been widely used as redox mediators and have also been coupled to PPy chains. Schuhmann and associates developed miniaturized reagentless biosensors by electrochemical copolymerization of $[Os(2,2'\text{-bipyridine})_2(3\text{-}\{\text{pyrrol-1-ylmethyl}\}\text{pyridine})Cl]^{+}$, *N*-methylpyrrole and pyrrole-modified GOx (Fig. 4A) [54]. However, only slow rate of ET were observed, most probably because of the hydrophobicity and rigidity of the CP backbones, as well as the low conformational degree of freedom of the tethered Os complex to the PPy chain. To increase the free-diffusional mobility of Os-complexes for fast ET (ET "gating"), a flexible alkyl spacer chain was introduced to link an Os-bis-*N*,*N*'-(2,2′-bipyridyl) chloride to the *N*-position of a pyrrole unit, forming an Os-complex substituted pyrrole derivative, Os-bis-*N*,*N*'-(2,2′-bipyridyl-*N*-(pyridin-4-yl-methyl-(6-pyrrol-1-yl-hexyl)amine chloro] chloride (Os-Py). Pyrroloquinoline quinone dependent glucose dehydrogenase (PQQ-GDH), as oxygen-insensitive biological recognition element, was entrapped within an Os-complex-modified PPy film by a potentiostatic pulse deposition (Fig. 4B). An efficient ET pathway was achieved by "gated" electron hopping between adjacent Os-complexes [55]. The Os-Py derivative was also utilized as the mediator for GOx, horseradish and tobacco peroxidase (HRP and TBP) to construct biosensors, with fine-tuned properties of enzyme to polymer ratio, counter

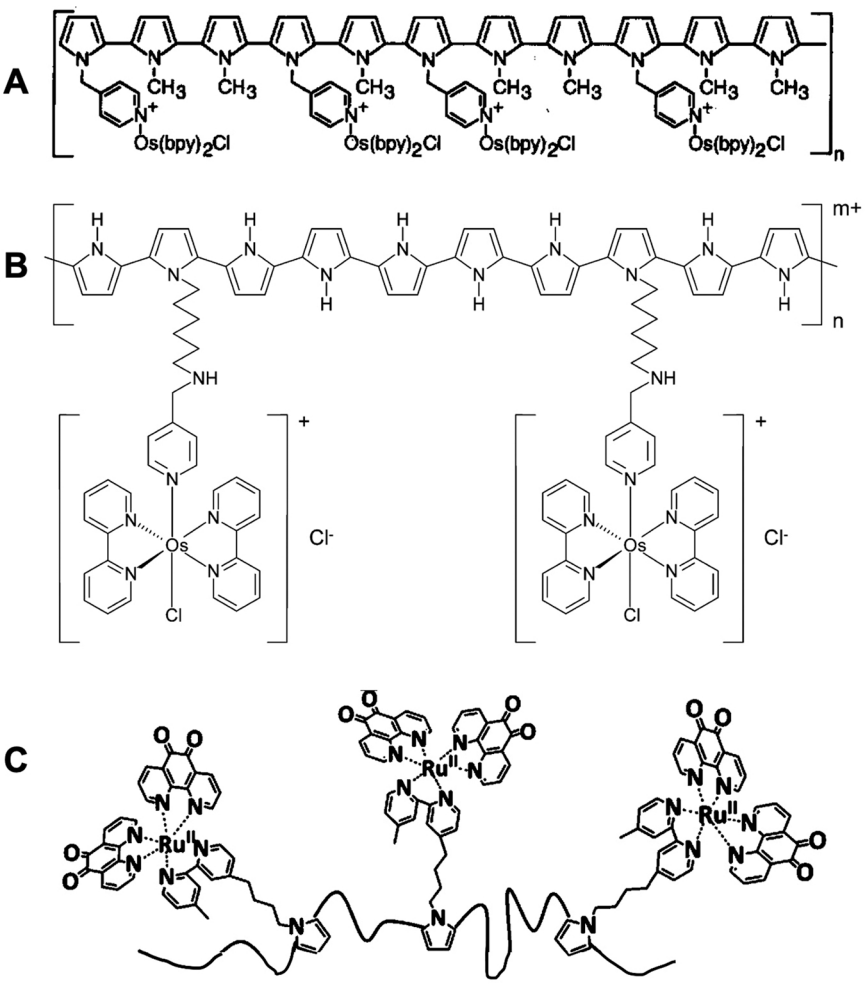

Fig. 4. Structure representations of [Os(2,2′-bipyridine)2 (3-(pyrrol-1-ylmethyl)pyridine)Cl]$^{+}$/*N*-methylpyrrole copolymer (A). Adapted with permission from Ref. [54]. Copyright 1993, Elsevier; Os-bis-*N*,*N*'-(2,2′-bipyridyl-*N*-(pyridin-4-yl-methyl-(6-pyrrol-1-yl-hexyl)amine chloride]/pyrrole copolymer (B). Adapted with permission from Ref. [55]. Copyright 2000, John Wiley & Sons, Inc; and poly-[$Ru^{II}(PhQ)_2$(bpy-pyrrole)] (C). Adapted with permission from Ref. [56]. Copyright 2014, American Chemical Society.

anions of polymer-bound Os-complexes, and intrinsic hydrophilic properties [57–59]. Replacing pyrrole by pyrrole-3-carboxylic acid or co-entrapping polyacrylic acid (PAA) for the formation of co-polymer can improve the hydrophilicity of the backbone, leading to facilitated substrate diffusion within the polymer film [57]. Ru-complexes were also shown to be efficient redox mediators, especially for nicotinamide adenine dinucleotide (NAD)-dependent enzymes. Reuillard and associates reported a novel electropolymerizable Ru(II) complex containing two phenanthrolinequinone (PhQ) ligands, $Ru^{II}(PhQ)_2$(bpy-pyrrole)$(PF_6)_2$, which was used to successfully entrap NAD^+-GDH together with pyrrole monomer [56]. The as-prepared bioelectrode exhibited high current densities for efficient glucose oxidation at low overpotentials.

3. Other one-step electrochemical methods

Other polymeric systems have also been studied to wire enzymes through a one-step electrodeposition strategy. Limited by the electrical communication with the electrode, these polymer layers are generally thinner than those of CPs, and have lower enzyme loading. As compensation, substrates and products diffuse rapidly to and from the enzyme [13]. Redox active complex modified polymers, dopamine polymerization, sol-gel derived silica or direct adsorption immobilization have attracted considerable interest.

3.1. Os complex modified redox polymer

Transition metal ions are able to exchange ligands when electro-reduced and/or electro-oxidized [60,61]. Gao and associates demonstrated that Os-redox polymers containing a labile ligand (Cl^-) in their inner coordination sphere and a robust ligand (such as pyridine or imidazole, exhibiting strong coordinative bonding with Os, but yet uncoordinated) in their backbone could crosslink via ligand exchange onto electrodes along with co-electrodeposited enzyme [62,63]. Os-complexes can be mediators of the immobilized enzymes. Five Os-redox polymers were successfully electrodeposited onto carbon electrodes under mild conditions. These polymers include poly (4-vinylimidazole-co-acrylamide) partially complexed with $[Os(bpy)_2Cl]^{+/2+}$ (I), poly(4-vinylpyridine) (PVP) partially complexed with

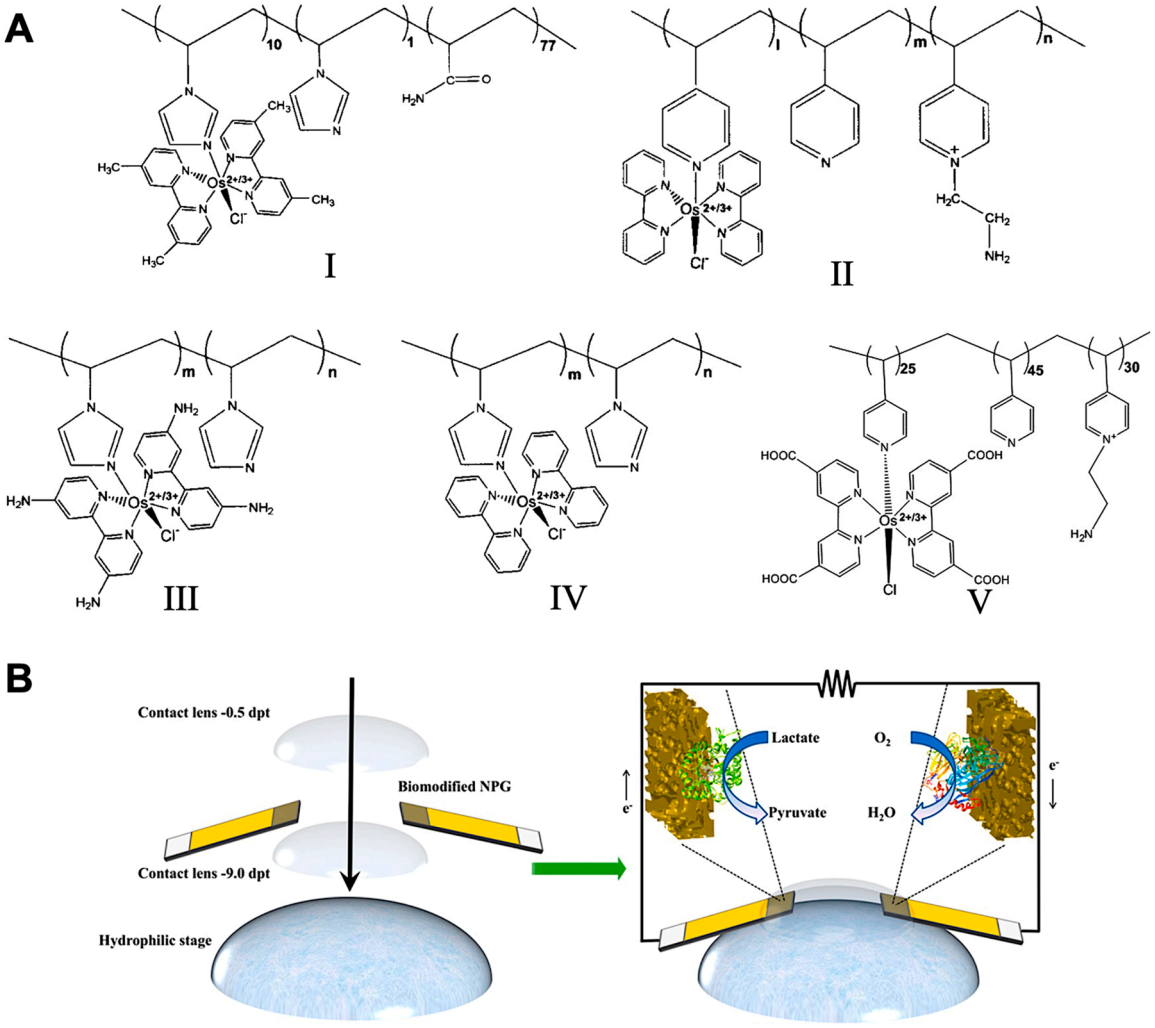

Fig. 5. Examples of Os complex modified redox polymers (A). Adapted with permission from Ref. [62,63]. Copyright 2002, John Wiley & Sons, Inc. Copyright 2013, Elsevier; Scheme of the assembly of a modified contact lens and an EBFC configuration (B). Adapted with permission from Ref. [65] Copyright 2018, American Chemical Society.

$[Os(bpy)_2Cl]^{+/2+}$ and partially quaternized with 2-bromoethylamine (II), poly-(*N*-vinylimidazole) (PVI) partially complexed with $[Os(4,4'$-diamino-$2,2'$-bipyridine$)_2Cl]^{+/2+}$ (III), PVI partially complexed with $[Os(bpy)_2Cl]^{+/2+}$ (IV), and PVP partially quaternized with $[Os(4,4'$-dicarboxylic acid-$2,2'$-bipyridine$)_2Cl]^{+/2+}$ (V) (Fig. 5A). Electronic (gated) conduction takes place in the redox polymers due to the random mobility of their segments, which can exchange electrons upon colliding [64]. After electro-reduction or electro-oxidation, the weakly coordinated Cl^- in the inner sphere could be exchanged by pyridine or imidazole from the neighboring chain, leading to crosslinking and solidification of the redox polymers [62]. Enzymes such as GOx, HRP, soybean peroxidase, or laccase were readily co-electrodeposited with the polymers owing to the histidine, lysine, and arginine coordination to $Os^{2+}/^{3+}$ [62,63].

Xiao et al. electrodeposited $Os(bpy)_2PVI$ with lactate oxidase (LOx) on a NPG electrode to construct a bioanode [65]. Coupled with a *Myrothecium verrucaria* bilirubin oxidase (BOx) based biocathode, a flexible lactate/O_2 enzymatic biofuel cell (EBFC) was fabricated and incorporated in contact lenses (Fig. 5B). Such a device has the potential of operating as an autonomous power supply for wearable electronic devices, for example tear biosensors. An analogous method was used to immobilize diaphorase on NPG, enabling determination of NADH at low overpotentials to avoid interferences [66]. Co-deposition of PEDOT, $Os(bpy)_2PVI$ and enzymes enables the construction of a dual-functional supercapacitor/EBFC hybrid device, taking advantage of the charge storage ability of PEDOT. The hybrid device allowed the energy produced by the EBFC to be stored in the supercapacitor and deliver high-powered pulses [67,68].

3.2. Polydopamine

Dopamine monomers can be polymerized spontaneously or electrochemically onto a wide range of surfaces [69,70]. Generally, dopamine is easily oxidized to dopaminequinone, followed by intermolecular cyclisation to form more readily oxidizable leucodopaminochrome. The leucodopaminechrome obtained then undergoes further oxidation and rearrangement to form 5,6-dihydroxyindole that leads finally to product formation via two pathways, i.e., covalent oxidative polymerization or non-covalent self-assembly [70]. Benefiting from its adjustability, robustness and inertness to harsh environments, polydopamine (PDA) film has been widely utilized in surface coatings, biotechnology and biomedicine, and water purification membranes [71,72].

Lee et al. reported the electrooxidization and polymerization of dopamine monomers to form a compact thin biocatalytic PDA film, in which formate dehydrogenase (FDH) and its co-enzyme NADH were tightly embedded (Fig. 6A) [73]. Hydrogen bonds between the adenosine moiety of NADH with the amino acid residues of FDH stabilized the radical intermediate of NAD after oxidation. Moreover, the quinoidal moiety of dopamine efficiently inhibits the intermediate dimerization to ensure regeneration of NAD^+. The π-stacking structure of PDA offers a robust matrix for enzyme accommodation, with long-lasting enzyme activity. The combination of the FDH/NADH/PDA cathode and a $CoPi/BiVO_4$ photoanode led to a device with efficient self-biased CO_2 conversion to formate driven by light irradiation (Fig. 6B). Almost 100% faradaic efficiency was achieved together with a relatively long period of stability of 25 h (Fig. 6C). Almeida et al. described the immobilization of laccase during potentiostatic deposition of a thin polydopamine film (ePDA) on carbon surfaces. The bioelectrodes were applied as disposable sensors for the detection of phenolic compounds such as caffeic acid, rosmarinic acid and gallic acid [74].

3.3. Silica

Due to their ease of preparation, chemical inertness, negligible swelling and optical transparency, sol–gel prepared silica films have attracted growing interest as a versatile method for the encapsulation of proteins. Electrochemically assisted deposition (EAD) provides a controllable and rapid method to grow silica layers onto a conductive substrate regardless of the roughness of the surface, such as NPG [76], carbon nanotubes (CNTs) [77] or normal GCE.

Walcarius et al. employed sol–gel silica via one-step EAD to immobilize a variety of proteins including diaphorase (DI), GDH, *D*-sorbitol dehydrogenase (DSDH), and cytochrome *c* [75,77–79]. As a result of entrapment in silica gel networks, the immobilized proteins maintained their intrinsic properties without requiring any covalent bond between the support and the biomolecules. For instance, a reagentless electrochemical biosensor was fabricated via one-step deposition of tetraethoxysilane (TEOS) together with GDH, DI, CNT-vitamin K_3 (VK_3),

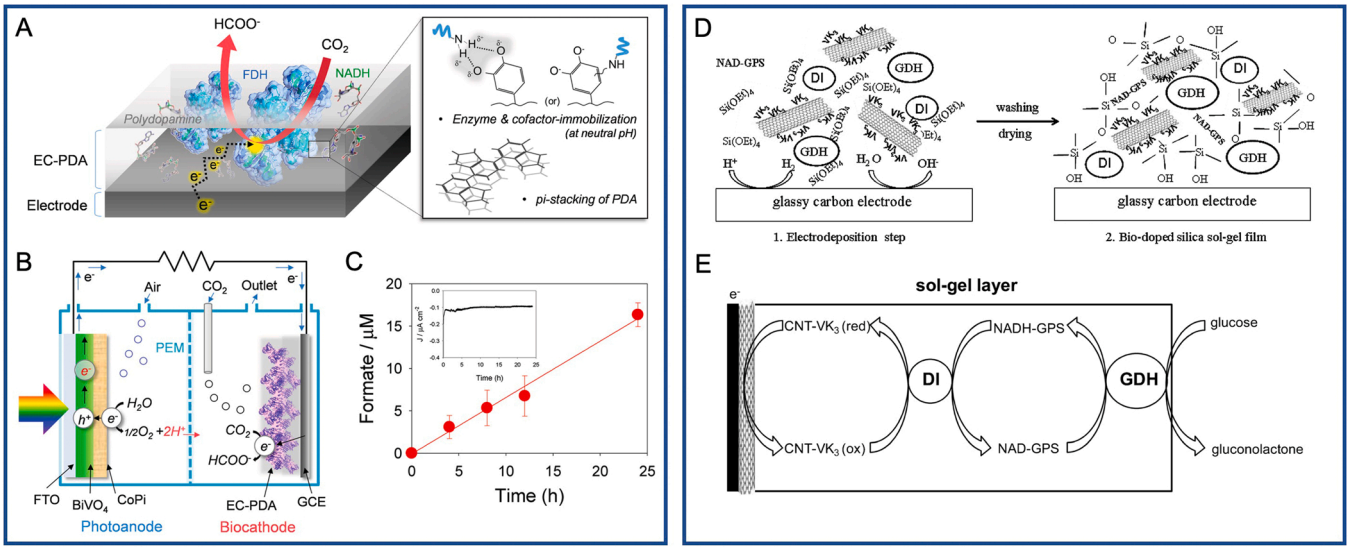

Fig. 6. Schematic drawing of the proposed formation mechanism of a PDA thin film-coated biocathode (A); Illustration of the photochemical system for CO_2 conversion utilizing a $CoPi/BiVO_4$ photoanode and an FDH/NADH/PDA cathode working under visible light irradiation (B); Photocatalytic production of formate as a function of irradiation time over a period of 24 h (C); Adapted with permission from Ref. [73]. Copyright 2016, John Wiley &Sons, Inc; Scheme of the EAD method prepared CNT-VK_3/PEI/NAD-GPS/DI/GDH doped silica sol–gel film (D); Working principle of the bioelectrode (E). Adapted with permission from Ref. [75]. Copyright 2013, John Wiley &Sons, Inc.

polyethylenimine (PEI), NAD-(3-glycidoxypropyl)trimethoxysilane (GPS) (Fig. 6D). GDH served as the catalyst, NAD as the cofactor, VK_3 as the mediator, and DI for the regeneration of cofactor (Fig. 6E) [75]. The EAD process initiated from the hydrolysis of alkoxide ligands at a low pH to yield hydroxylated metal centers, followed by the polycondensation of the hydrolyzed species to form oxypolymers at higher pH. Applying a constant negative potential on the bare electrode induced hydrogen evolution, thus increasing the local pH. As a result, the increase in the concentration of OH^- catalyzed the condensation process, leading to controlled growth of the sol–gel film. The positively charged PEI was used to stabilize the negatively charged GDH, providing a suitable microenvironment for biomolecule entrapment. CNT and GPS were employed to attach VK_3 and NAD respectively, finally tightly entrapped in the sol–gel film.

Electrochemical immobilization of enzymes in solid-gel silica can be used to fabricate a flow reactor for the controlled and stable delivery of the by-product H_2O_2 [80]. In a recent study, GOx was immobilized using different electrochemical methods based on polymers, diazonium coupling, and silica films, which can be further used for *in situ* H_2O_2 production. The immobilization technique used resulted in different rates of formation of H_2O_2 levels of stability of the modified electrodes. GOx encapsulated in silica retained 100% stability with a H_2O_2 production rate of 22 ± 3 nM h^{-1} for 4 h [80].

3.4. Direct adsorption with the assistance of electrochemistry

In addition to immobilizing enzymes along with the electrodeposition of conductive or nonconductive polymers, enzymes can be directly electrochemically adsorbed onto suitable electrode surfaces. Generally, electrostatic forces between biomolecules and the electrode surfaces are assumed to increase the enzyme loading and affinity interactions. By applying a potential between 0.42 and 0.6 V vs. SCE at a highly porous gold (hPG) electrode, Toit et al. successfully immobilized GOx (anionic at pH 7.0) on the electrode [81,82]. The sensor exhibited higher catalytic currents and better long-term stability for the oxidation of glucose than achieved at a drop-cast bioelectrode. Assembling the GOx-hPG anode with a laccase-hPG cathode, two types of miniature EBFCs (parallel channel and single channel) were prototyped using 3D printed fluidic channels (Fig. 7A-D), allowing power generation from continuous flow-through configurations for a period of up to one month. Due to the configuration difference, the parallel channel EBFC registered a higher specific power output by geometric surface areas of anodes, while the single-channel EBFC produced a higher absolute power output (Fig. 7E-F) [82]. Lopez and associates reported a potential pulse-assisted approach to immobilize *Mv*BOx on the NPG surface (Fig. 7G-H). The BOx electrostatic charge is overall negative (at pH 7.0), while its T1 site, which is the primary electron acceptor in DET is surrounded by positively charged amino acids (Fig. 7I). A two-step potential pulse sequence between − 0.8 and + 0.6 V, on either side of the potential of zero charge of gold surfaces, was therefore applied onto NPG in the solution of BOx. Fast switching potential pulses resulted in continued readjustment of enzyme orientation and a concomitant ion stirring effect, which not only allowed a preferential orientation of BOx, but also pushed the biomolecules into the pores of the NPG electrode. An increase in DET current densities and rates of electron transfer of the BOx-NPG electrode compared with a planar electrode, was observed [83].

4. Conclusion and perspectives

We have overviewed one-step electrochemical approaches of enzyme immobilization based on a variety of materials, including CPs and their derivatives, redox complex modified polymers, PDA, and direct adsorption. A comprehensive comparison showing the advantages and limitations of different methods is summarized in Table 1. These one-

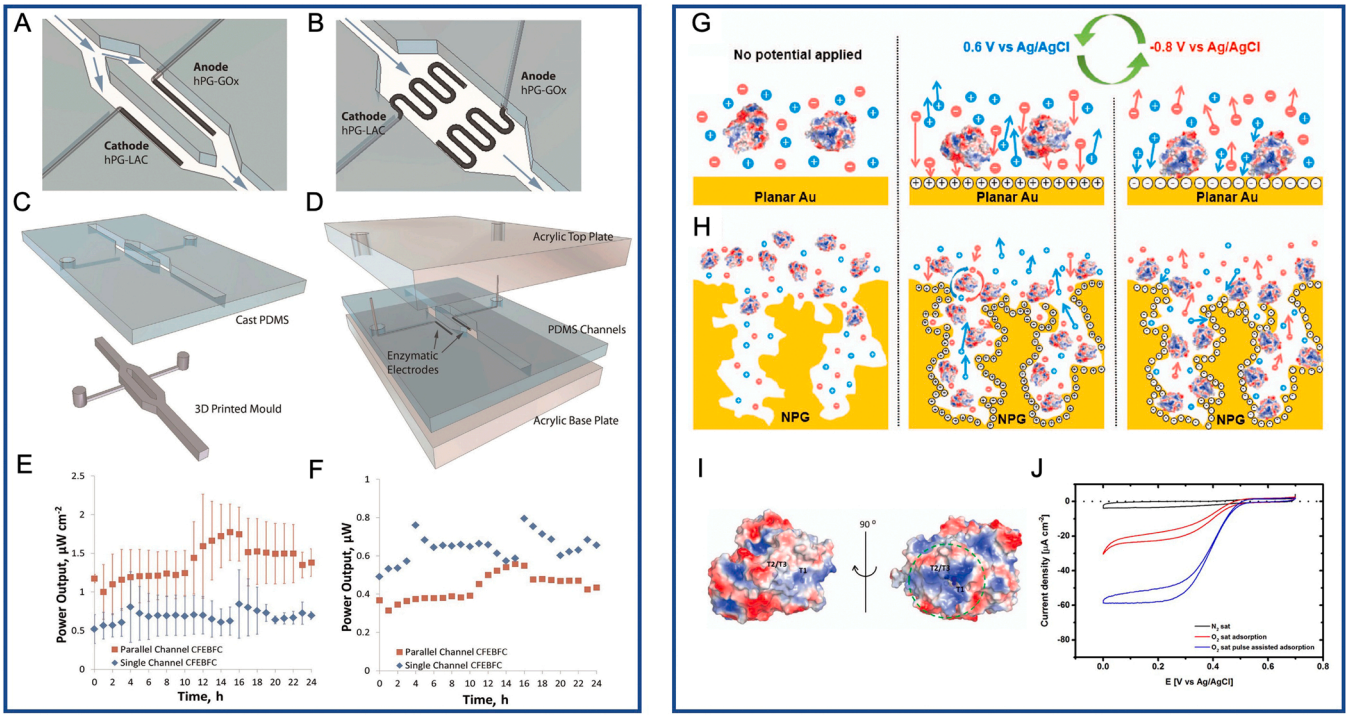

Fig. 7. Scheme and construction of the two types of miniature EBFCs: parallel channel design (A), single channel design with pleated electrodes (B), casting of polydimethylsiloxane (PDMS) channels from 3D printed mould (C), sectional view of the parallel channel EBFC (D); Power output from the two EBFCs over continuous 24 h operation; specific power output by geometric surface areas of anodes (E), absolute power output (F); Adapted with permission from Ref. [82]. Copyright 2015, Elsevier; Potential pulse-assisted immobilization approach at planar gold (G) and NPG electrodes (H); Surface charge distribution pattern of (PBD 2XLL) *Myrothecium verrucaria* BOx (blue color shows positive and red color negative charges) (I); CVs of drop-casting and pulse-assisted adsorbed BOx on NPG electrodes in N_2 or O_2 saturated buffer solution (J). Adapted with permission from Ref. [83]. Copyright 2018, Elsevier.

Table 1
Comparison of the advantages and limitations of different one-step enzyme immobilization methods.

Methods	CP	Redox polymer	Polydopamine	Silica	Adsorption
Electrochemical parameters	Electro-oxidizing to oxidize CP monomers	Electro-reduction to cross-link PVI-Os polymer	Electro-oxidation of dopamine to dopaminequinone to initiate the polymerization	Electro-reduction for hydrogen generation and local pH increase at the electrode	Electrochemical pulses to "stir" the enzyme biomolecules displaying surface charges
Enzyme loading capacity	High	Medium	Medium	Medium	Low
Stability of immobilized enzymes	Medium	Medium	Medium	High	Medium
Advantages	Tunable film thickness and thus enzyme loading; fast process; no limitation on electrode geometry; a wide range of derivates with interesting features.	Tunable film thickness and thus enzyme loading; fast process; no limitation on electrode geometry; electron mediation property; providing better mechanical stability over drop-casting.	Tunable film thickness and thus enzyme loading; fast process; no limitation on electrode geometry; biocompatible.	Tunable film thickness and thus enzyme loading; fast process; no limitation on electrode geometry.	Fast process; no limitation on electrode geometry;
Limitations	Low water solubility of monomers; synthesis efforts required for new CP derivates.	Os complex is expensive; Fc complex is less robust than Os complex.	Ease of oxidation.	Ease of exfoliation; the byproduct ethanol during the process from silica precursor may inhibit enzymes.	Enzyme loading is relatively low.

step protocols all benefit from relatively simple, rapid, and controllable preparation. In particular through the flexible and facile molecular design and synthesis, CPs show extraordinary versatility, such as tunable solubility, conductivity, biocompatibility, and optical and mechanical properties. Recent progress shows the promise of CPs, as one of the most promising electrode materials for implanted biomedical applications, such as biosensors, EBFCs, and other bioelectronics. A key feature of CPs is that they usually do not register well-defined standard redox potentials but vary in a wide potential range [84], which can overlap with those of redox polymers, facilitating ET between the CP and a redox polymer layer. This has led to new innovations such as drug release from CP layers due to the biocatalytically driven Nernstian potential shift of the redox polymer layer [85,86]. Furthermore, a different charge transfer mechanism involving direct "hole" transfer between polymeric semiconductors and enzyme molecules was reported. "Hole transfer" is essentially electron transfer via the highest occupied molecular orbitals (valence band) rather than electron transfer via the lowest unoccupied orbitals (conduction band). This opens new ET opportunities for CPs and biomolecules [87].

The use of one-step electrochemical approaches for enzymatic bioelectronics can be expanded for future development. For example, CPs with unique substituents and dopants (e.g. nanomaterials) can be brought to display new properties, such as anti-biofouling and increased electronic conductivity. The antifouling properties of bioelectrodes both prevent unwanted nonspecific protein adsorption and improve biocompatibility, which could be pivotal in the use of implanted electrodes for long-term metabolic monitoring [88]. New CP microstructures with enzyme incorporated are also of interest. Multifunctional biodevices with CPs based on their versatile nature, such as biodegradability, charge storage capability, mechanical flexibility, electrochromic effects, electrochemically driven actuation and ion release ability can be envisaged. Emerging challenges that include unsatisfactory immobilization ratios and poor enzyme orientation control for DET will be addressed in future studies.

CRediT authorship contribution statement

Fei Shen: Writing – original draft, Writing – review & editing. **Simin Arshi:** Writing – review & editing. **Edmond Magner:** Writing – review & editing. **Jens Ulstrup:** Writing – review & editing. **Xinxin Xiao:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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