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APPLICATION OF VOLTAMMETRY IN BIOMEDICINE – RECENT ACHIEVEMENTS IN ENZYMATIC VOLTAMMETRY

Rubin Gulaboski[1], Valentin Mirceski[2,3]

[1]*Faculty of Medical Sciences, Goce Delčev University, Štip, Republic of Macedonia*
[2]*Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University, Skopje, Republic of Macedonia*
[3]*Department of Inorganic and Analytical Chemistry, University of Lodz, Tamka 12, 91-403 Lodź, Poland*

rubin.gulaboski@ugd.edu.mk; valentin@pmf.ukim.mk

Protein-film voltammetry (PFV) is considered the simplest methodology to study the electrochemistry of lipophilic redox enzymes in an aqueous environment. By anchoring particular redox enzymes on the working electrode surface, it is possible to get an insight into the mechanism of enzyme action. The PFV methodology enables access to the relevant thermodynamic and kinetic parameters of the enzyme-electrode reaction and enzyme-substrate interactions, which is important to better understand many metabolic pathways in living systems and to delineate the physiological role of enzymes. PFV additionally provides important information which is useful for designing specific biosensors, simple medical devices and bio-fuel cells. In the current review, we focus on some recent achievements of PFV, while presenting some novel protocols that contribute to a better communication between redox enzymes and the working electrode. Insights to several new theoretical models that provide a simple strategy for studying electrode reactions of immobilized enzymes and that enable both kinetic and thermodynamic characterization of enzyme-substrate interactions are also provided. In addition, we give a short overview to several novel voltammetric techniques, derived from the perspective of square-wave voltammetry, which seem to be promising tools for application in PFV.

Keywords: protein-film voltammetry; surface electrode mechanisms; enzyme-substrate interactions; modified electrodes; kinetics of electron transfer

АПЛИКАЦИЈА НА ВОЛТАМЕТРИЈАТА ВО БИОМЕДИЦИНАТА – СКОРЕШНИ ПОСТИГНУВАЊА ВО ВОЛТАМЕТРИЈАТА НА ЕНЗИМИ

Волтаметрија со протеински филм (PFV) е наједноставна методологија за студирање на електрохемиските својства на т.н. липофилни редокс-ензими во водна средина. Со апсорпција на даден редокс-ензим на површината од работна електрода е возможно да се студира механизмот и својствата на тој ензим. Покрај тоа, методологијата на PFV овозможува и определување на важни кинетички и термодинамички параметри на интеракциите ензим-електрода и ензим-супстрат, што е особено важно за подобро разбирање на физиолошките функции на ензимите и на голем број метаболички патишта кај живите организми. Со техниката PFV се добиваат важни информации за дизајнирање на биосензори, едноставни медицински инструментални уреди и горивни био-ќелии. Во рамките на овој ревијален труд, е направен преглед на некои скорешни постигнувања на PFV, при што се презентирани нови експериментални протоколи што придонесуваат за подобра комуникација помеѓу редокс ензимите и работната електрода. Притоа посебно внимание е посветено на неколку нови теоретски волтаметриски модели кои помагаат во развој на стратегија за студирање на електродните реакции на апсорбираните ензими и кои овозможуваат разработка на методи за кинетички и термодинамички определувања кај овие системи. Покрај тоа, во трудот е

*We dedicate this work in memory of our great friend and collaborator, Dr. Sci. Sebojka Komorsky-Lovric

даден и краток преглед на неколку нови волтаметриски техники изведени од квадратно-брановата волтаметрија, кои можат да станат значајни алатки во апликацијата на PFV.

Клучни зборови: волтаметрија со протеински филм; површински електродни механизми; ензимско-супстратни интеракции; модифицирани електроди; кинетика на електронски трансфер

1. INTRODUCTION

Electrochemistry is a branch of physical chemistry that mainly considers the process of charge (electrons and/or ions) transfer between two conjoined systems, which are frequently linked to corrosion, metal protection and various types of energy storage systems (batteries). Indeed, these are the most known fields in which electrochemistry plays a major role. However, in the last 30 years we have seen significant progress of electrochemistry in many areas of biomedical sciences [1–3]. Electrochemical techniques most frequently encountered in biomedical investigations are potentiometry [4] and voltammetry [4–5]. Voltammetric techniques are recognized as cheap, simple and powerful tools provided by commercial electrochemical instrumentation. The driving force in voltammetry is the energy of electrons (controlled via the applied electrode potential difference), while the relevant measuring physical parameter is the intensity of the Faradaic current. The Faradaic current occurs due to an electron exchange between the working electrode and electroactive species, either present in the solution or being immobilized on the electrode surface, which undergo redox transformation at the electrode/electrolyte interface.

Voltammetry is used in many laboratories dealing with chemical, biochemical, environmental and physical analyses. It is a versatile technique for the investigation of mechanisms, kinetics and thermodynamics of metal-ligand complexation reactions [6], drug action [7–8] and drug-DNA interactions [9–10]. It is particularly important for the detection and quantification of biochemical and physiological active compounds [3, 8], and is a technique which underlies the operation of many biochemical sensors [11–14]. In addition, important studies related to the application of voltammetry for studying enzyme biochemistry [15] and biophotoelectrochemistry have been already conducted [16].

In the present review, we focus on the application of voltammetry for studying lipophilic redox enzymes and redox active proteins via immobilization on the electrode surface, an approach known as *protein-film voltammetry* (PFV). While addressing relevant experimental studies published in the last several years, we also review several theoretical models which are relevant for the advanced application of pulse voltammetric techniques in PFV. Finally, a few novel voltammetric techniques will be briefly reviewed as promising tools for further advanced applications in PFV.

2. RESULTS AND DISCUSSION

2.1. *Probing electrochemical features of immobilized redox enzymes and proteins*

Since the introduction of PFV, which is considered as the simplest experimental methodology for studying electrochemistry of redox enzymes [17–18], there has been fast progress in the field of enzymatic voltammetry [15–20]. Redox enzymes belong to the class of oxidoreductases and represent more than 30 % of all known proteins which are involved in numerous biological processes comprising electron transfers (e.g., respiration and molecular signalling). Many essential biochemical reactions in living organisms are thermodynamically possible due to their coupling to the hydrolysis of ATP, supported by the action of numerous enzymes. Due to specific catalytic features of the enzymes, the methodology of PFV is acknowledged as a useful approach in designing selective sensors for various substrates [15, 17, 19, 20].

In the context of an electrochemical experiment, it is possible to study electrochemical and chemical features of particular lipophilic enzymes by anchoring the enzyme onto the surface of the working electrode in a form of a monolayer (via self-assembling adsorption or by covalent bonding). The methodology is not limited to the redox enzymes only; in addition, it can be accordingly applied to redox active proteins and various coenzymes (Fig. 1). Commonly, two general experimental approaches can be taken if direct electron transfer takes place between the adsorbed enzyme and the working electrode: (i) voltammetric characterization due to the electrochemical activity of the redox enzyme, conducted in the absence of a substrate (Fig. 2A); and (ii) voltammetry based on the enzymatic catalytic reaction in the presence of the enzyme substrate (the so-called regenerative

surface EC' mechanism) (Fig. 2B). In both scenarios, thermodynamic and kinetic parameters of the enzyme-electrode reaction can be obtained, together with the kinetic characterization of the enzyme-substrate reaction [3–6, 10, 15–21].

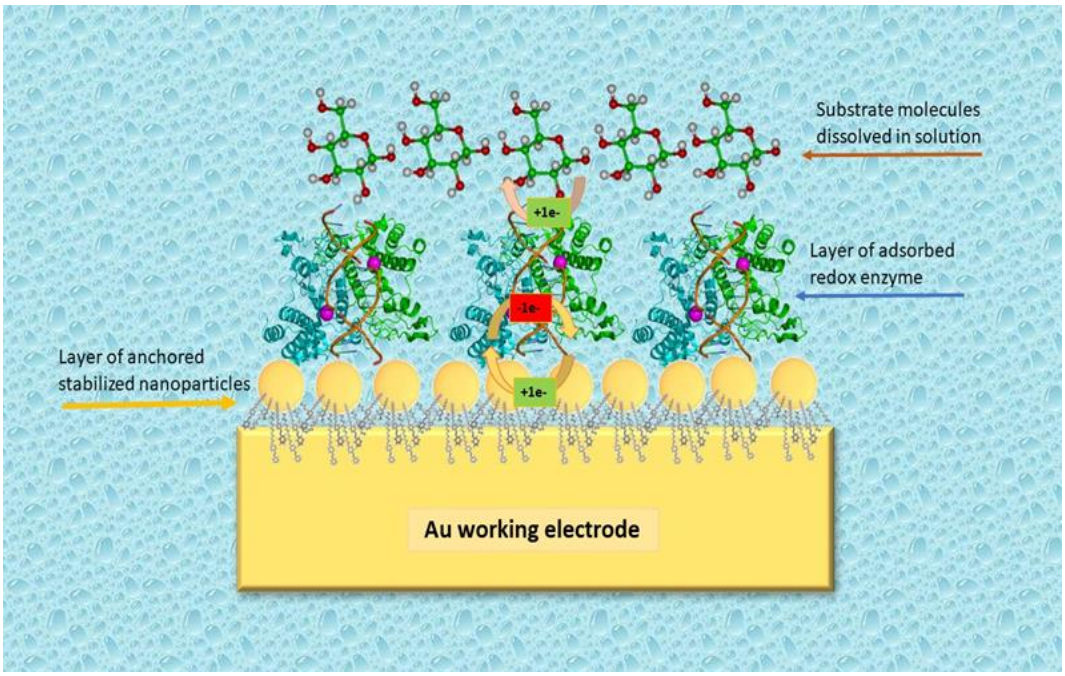

Fig. 1. Schematic representation of protein-film voltammetric set-up, with the Au-working electrode modified with Au-stabilized nanoparticles

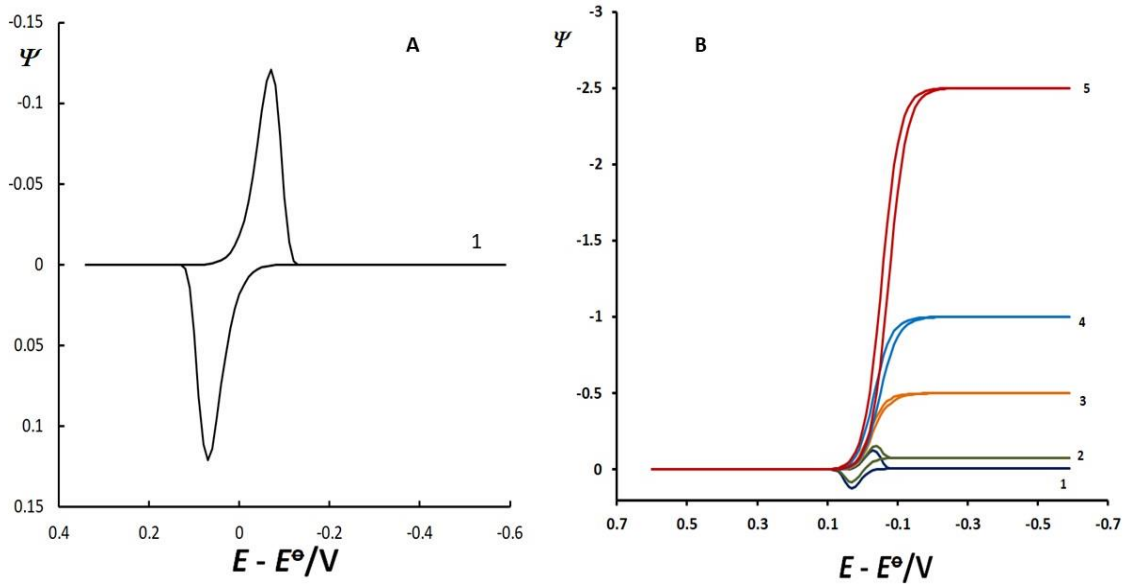

Fig. 2. Theoretical cyclic staircase voltammogram of a lipophilic redox enzyme recorded in the absence of a substrate (A). Cyclovoltammetric patterns in (B) represent the effect of increasing substrate concentration simulated for a regenerative surface EC' (electrochemical-catalytic) electrode mechanism of type A(ads) + ne^- ↔ B(ads) + S → A(ads). The dimensionless kinetic parameter of electron transfer was set to K = 0.025 in all cases. The concentration of the substrate "S" in voltammograms (B) was set to 0 mol/l (1); 0.075 mol/l (2); 0.5 mol/l (3); 1 mol/l (4) and 2.5 mol/l (5). The catalytic rate constant was k_c = 100 mol^{-1} l s^{-1}. Other simulation parameters were: time duration of potential steps t = 0.01 s; potential step dE = 0.01 V, number of electrons n = 1, electron transfer coefficient α = 0.5, and temperature T = 298 K.

Several comprehensive reviews have been dedicated to the experimental achievements of PFV in recent years [8, 15, 19–21]. In addition, valuable theoretical models have been developed under voltammetric conditions [22–40] that provide a means for qualitative characterization of reaction mechanisms. In [6, 15–17, 20–40], important theoretical methodologies to estimate kinetics and thermodynamics of redox enzymes have been presented. However, it must be noted that the main challenge in experimental aspects of PFV is to establish an efficient electronic communication between the electrode and the enzyme, whose bulky structural segments (electrochemically inactive polypeptide backbones) frequently hinder efficient electron exchange [19, 41]. This is because many enzymes contain active sites that are deeply buried in their quaternary structure [15–19], while their polypeptide backbones are commonly attached to the electrode surface and act as electrical insulators. So far, cytochromes, heme-based enzymes, peroxidases, hydrogenases, some quinoproteins and enzymes containing cations of transient metals such as Mo, Co, Cu and W, have been most frequently studied with PFV [19]. Bilirubin oxidase and glucose oxidase are rare examples of enzymes undergoing efficient, direct electron transfer with a few unmodified working electrodes [15–19].

T a b l e 1

An overview of recent studies of redox enzymes conducted at modified electrode surfaces with voltammetric techniques

Enzyme analyzed	**Working electrode**	**Surface modified with**	**Substrate**	**Ref.**
Cellobiose dehydrogenase	Au	Cysteine	Quinones	[60]
Cellobiose dehydrogenase	Graphite electrode	Pt/Pd nanoparticles + nanotubes	Lactose	[61]
Cellobiose dehydrogenase	Au	Positively charged polyethyleneimine gold nanoparticles	Lactose	[62]
Cellobiose dehydrogenase	Glassy carbon	Au-nanoparticles	Glucose	[58]
Cellobiose dehydrogenase	Au	Cysteine + maleimide	/	[63]
Bilirubin oxidase	Au	Maleimide/thiol	Oxygen	[64]
Bilirubin oxidase	Glassy carbon	Cabon nanofibers in presence of NaCl	Oxygen	[65]
Glucose oxidase	Glassy carbon	Phenazine ethosulfate	Glucose	[66]
Lactate oxidase	Au	Multiwalled carbon nanotubes + methylene blue	Lactate	[67]
FAD-glucose dehydrogenase	Au	Multiwalled carbon nanotubes + methylene blue	Glucose	[67]
Glucose dehydrogenase	Glassy carbon	Pyrroloquinoline quinone + poly(4-vinylpyridine) plymer with Os	Glucose	[68]
Glucose dehydrogenase	Glassy carbon	Enzyme fused with cytochrome C	Glucose	[69]
Glucose oxidase	Glassy carbon	3-aminopropyltriethoxysilane	Glucose	[70]
Glucose oxidase	Boron doped diamond	functionalized with 3-aminopropyltriethoxysilane	Glucose	[71]
Pyranose dehydrogenase	Graphite electrode	Osmium redox polymer with poly-(ethylene glycol)(400) diglycidyl ether	Glucose	[72]
Horseradish peroxidase	Carbon paste electrode	Silica sol-gel	2-aminophenol	[73]
Glucose oxidase	Carbon fibers	Graphene oxide	Glucose	[74]
Flavohemoglobin	Graphite	Os-polymer	Oxygen	[75]
Fructose dehydrogenase	Carbon (with cryogel)	Bilirubin	Fructose	[76]
D-Fructose dehydrogenase (Δ1*c*FDH variant with lack of 143 amino acids)	Au	/	Fructose	[77]
Fructose dehydrogenase	Au	Thiol-and diazonium-bound carboxylic acid	Fructose	[78]
Fructose dehydrogenase	Graphite electrode	Ca^{2+} (used as intracomplexating ions)	Fructose	[50]
D-fructose dehydrogenase	Graphite electrode	Thermally reduced graphene oxide	Fructose	[79]
D-fructose dehydrogenase	Carbon electrode	Methoxy-aniline derivatives	D-fructose	[80]
Aldehyde dehydrogenases	Glassy carbon	Pyrroloquinoline quinone	Glucose, aldehydes	[81]

Hence, various protocols have been proposed to overcome poor electron transfer communication between the enzymes and electrode [19], including modification of the working electrode surface with nanoparticles [42–43] and application of redox mediators, which can be either dissolved or attached on the electrode surface [19, 44]. Redox mediators commonly comprise compounds like methylene blue, quinones, hexacyanoferrates, and ferrocene derivatives that are able to shuttle electrons between the working electrode and the active site of the redox enzyme. Most of these protocols are elaborated in detail in [15–21, 45, 46]. All of these studies highlight that "electrostatic compatibility" between the working electrode and the analyzed redox protein is of crucial significance [5, 6, 15–19, 45]. The latter improves electron transfer performances and diminishes thermodynamic and kinetic constraints of the electrode reaction. In some cases, polyvalent cations are added into the electrolyte solution, which facilitate electron transfer between the working electrode and enzymes from the group of dehydrogenases and cytochromes, mainly by affecting the orientation of the enzymes [48–50]. The efficiency of electron exchange can be also affected by activation of the so-called "internal electron tunnelling effects" [51]. "Enzymatic tunnels" are parts of the enzymes tertiary structure that are seen as "conductive pathways" between the enzyme redox active site and the working electrode surface. Such "enzymatic tunnels" are often more active in the presence of "foreign" metal cations trapped in the structure of the immobilized enzyme [52].

Besides modification with nanoparticles [19, 21], "functionalization" of the working electrode surface with conductive polymers has been also reported [47, 53]. An especially promising approach seems to be modification with flexible carbon fibers [54]. Authors in [54] designed a powerful "bioelectrode", where nitrated carbon nanoblisters are anchored on the carbon fibers, which are thereafter used to modify the surface of a graphite working electrode. Such approach enabled the study of important enzymes, e.g., glucose dehydrogenase [55]. A very useful review on modifying carbon fibers for various biomedical applications and voltammetric sensor developments is reported in [56]. Hence, the methodology of PFV has been successfully applied for designing highly selective enzymatic sensors, suitable for the detection of important substrates such as hydrogen peroxide [57], glucose [58], superoxide radicals [57, 59], nitrogen oxide [59] and many other physiologically relevant substrates [19]. Following our last review from 2012 [20], Table 1 provides some of the relevant studies published thereafter.

2.2. *Recent theoretical models relevant to protein-film voltammetry*

Considering the complexity of electrode mechanisms of immobilized enzymes, establishing theoretical models for simulation and elucidation of voltammetric behaviour is of particular importance. In recent years, our group [22–35, 37, 38] and others [6, 15, 36, 39, 40, 82–83] have made efforts to understand and predict voltammetric features by studying various theoretical surface electrode mechanisms. It is worth mentioning that theoretical models under conditions of square-wave voltammetry (SWV) are of special interest, as the technique is considered to be the most advanced member in the family of pulse voltammetric techniques [6, 36].

A variety of electrode mechanisms have been analyzed theoretically, based on adequate models for surface electrode reactions coupled with chemical reactions. The voltammetric features of a redox enzyme undergoing a one-electron electrode reaction (***E*** step), coupled with either a preceding or following chemical step (***C*** step), or by a regenerative follow-up chemical reaction (***C'*** step), are comprehensively studied in the frame of theoretical models for surface ***CE*** [27, 84, 85], ***EC*** [27, 30, 85] and ***EC'*** [27, 33, 84, 85] mechanisms, respectively. These models might be helpful in understanding the voltammetry of cytochromes and other heme-containing proteins, as they undergo redox transformation in a one-electron step [15, 17–19, 21]. In [27, 30, 33, 84, 85], a simple strategy for measuring electrode kinetics is presented, enabling the kinetic characterization of the coupled chemical reactions as well. Especially important insights are obtained in the case of the surface $\boldsymbol{EC_r}$ mechanism, i.e. a mechanism in which the product of the electrode reaction is engaged in a chemically reversible follow-up reaction ($\boldsymbol{C_r}$ step) [25, 27]. This model is closely related to the denaturation of the redox enzymes following the electrode transformation. Although it would be expected that the voltammetric currents would diminish due to the denaturation process, the opposite effect has been predicted under certain conditions in SWV [25, 27]. Specifically, the intensity of the response increases by increasing the rate of the follow-up chemical reaction ($\boldsymbol{C_r}$ step), in particular when the chemical reaction is associated with moderate kinetics. This peculiar phenomenon is a consequence of the interplay between the rate of the follow-up

chemical reaction, the chronoamperometric features of the overall electrode mechanism and the specific current-sampling protocol in SWV [25, 27]. It has been shown that the rate of the follow-up chemical reaction, which proceeds in the time segment of SW potential pulses where the current is not measured, plays a critical role to allow the observation of this feature [25, 30]. Based on such unique voltammetric behaviour, a simple strategy for complete kinetic and thermodynamic characterization of the surface $\boldsymbol{EC_r}$ mechanism has been proposed [25, 27].

Although many enzymes undergo one-electron redox transformation, it is well known that the redox chemistry of many hydrogenases, peroxidases, enzymes with quinone moieties (flavoproteins), and enzymes containing polyvalent cations of Co, Mo or W in their redox active site, proceeds via two-electron redox transformation [15, 17, 18, 20, 21]. Thus, surface electrode mechanisms involving two successive one-electron transfer steps is of particular importance (***EE*** mechanism). Especially complicated situations can exist when one or both of the electrochemical steps are coupled with chemical reactions. If the energies of the two electron transfers differ by at least 200 mV in absolute value, the diagnostic criteria for characterizing each redox step are straightforward [26]. Shown in Figure 3, Figure S1 and Figure S2 are several sets of SW voltammograms of ***ECE***, ***EEC***, and ***EEC'*** mechanisms, respectively. The voltammetric patterns in Figures 3, S1 and S2 represent the influence of the chemical step, i.e. the effect of the substrate concentration in a particular experimental system. In all cases, it is easy to recognize voltammetric features that occur due to the effect of the kinetics of the chemical step, which is in accordance with the expected features reported for the corresponding simpler models [27].

However, when both electron transfers occur at the same potential (or the second step is energetically more favored than the first one), one observes a single SW voltammetric peak which "hides" in its morphology both electron transfer reactions (Fig. 4a). In such a scenario, it is important to find a way to voltammetrically separate the two electron transfer steps, which is possible when the product of the second step is coupled with a follow-up chemical reaction [29]. It has been shown that by altering the kinetics of the follow-up chemical reaction by means of the substrate concentration, the potential of the second electron transfer step can be shifted, resulting in two potentially separated SW voltammetric peaks (Fig. 4d-f).

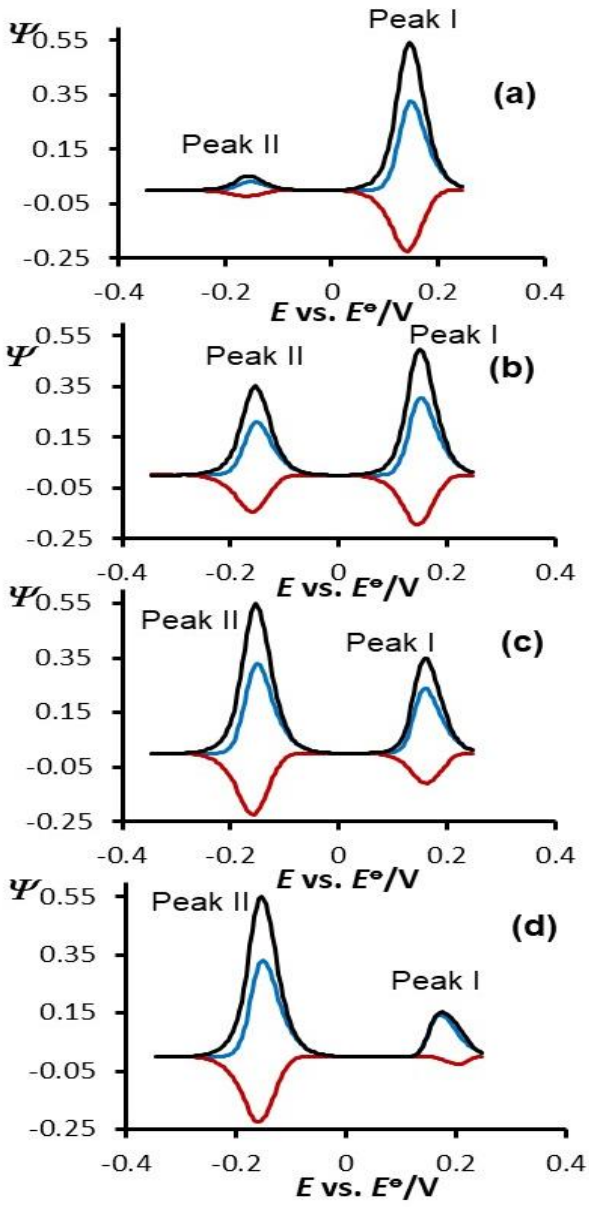

Fig. 3. *Surface ECE mechanism* A(ads) + n_1e- ↔ B(ads) + S → C(ads) + n_2e- ↔ D(ads) in protein-film voltammetry: effect of the substrate concentration c(S) to the features of theoretical SW voltammograms**.** Voltammograms are simulated at a potential separation of |300 mV| between both electrode steps: The values of c(S)/mol·l^{-1} are set to: 0.001 (a); 0.01 (b); 0.05 (c), and 0.2 (d). The chemical rate constant was set to k_{chem} = 10 mol·l^{-1}s^{-1}. Magnitudes of dimensionless kinetic parameters of the first (KI) and second (KII) step were KI = KII = 0.1. Other simulation conditions were: SW frequency f =10 Hz, SW amplitude E_{sw} = 50 mV, potential step dE = 4 mV, temperature T = 298 K. In all simulations, the electron transfer coefficients of the first and second electrode reaction were set to α = 0.5, while the number of electrons exchanged between the working electrode and the redox adsorbates was n_1 = n_2 = 1. The starting potential was set to +0.25 V and all scans were run towards negative potentials. All potentials are referred vs the standard redox potential of the first step.

In addition, detailed studies are reported for two-step enzymatic mechanisms coupled with a follow-up [24, 26, 29] reaction, intermediate re-

generative [26, 33], and preceding [28] chemical steps. While giving diagnostic criteria to recognize a particular mechanism, in [24, 26, 28, 29, 33] simple methodologies are proposed for the complete thermodynamic and kinetic characterizations of both electron transfers and coupled chemical reactions. Additional relevant studies related to the voltammetry of two-step enzymatic mechanisms can be found in the works of Komorsky-Lovric and M. Lovric [86–88]

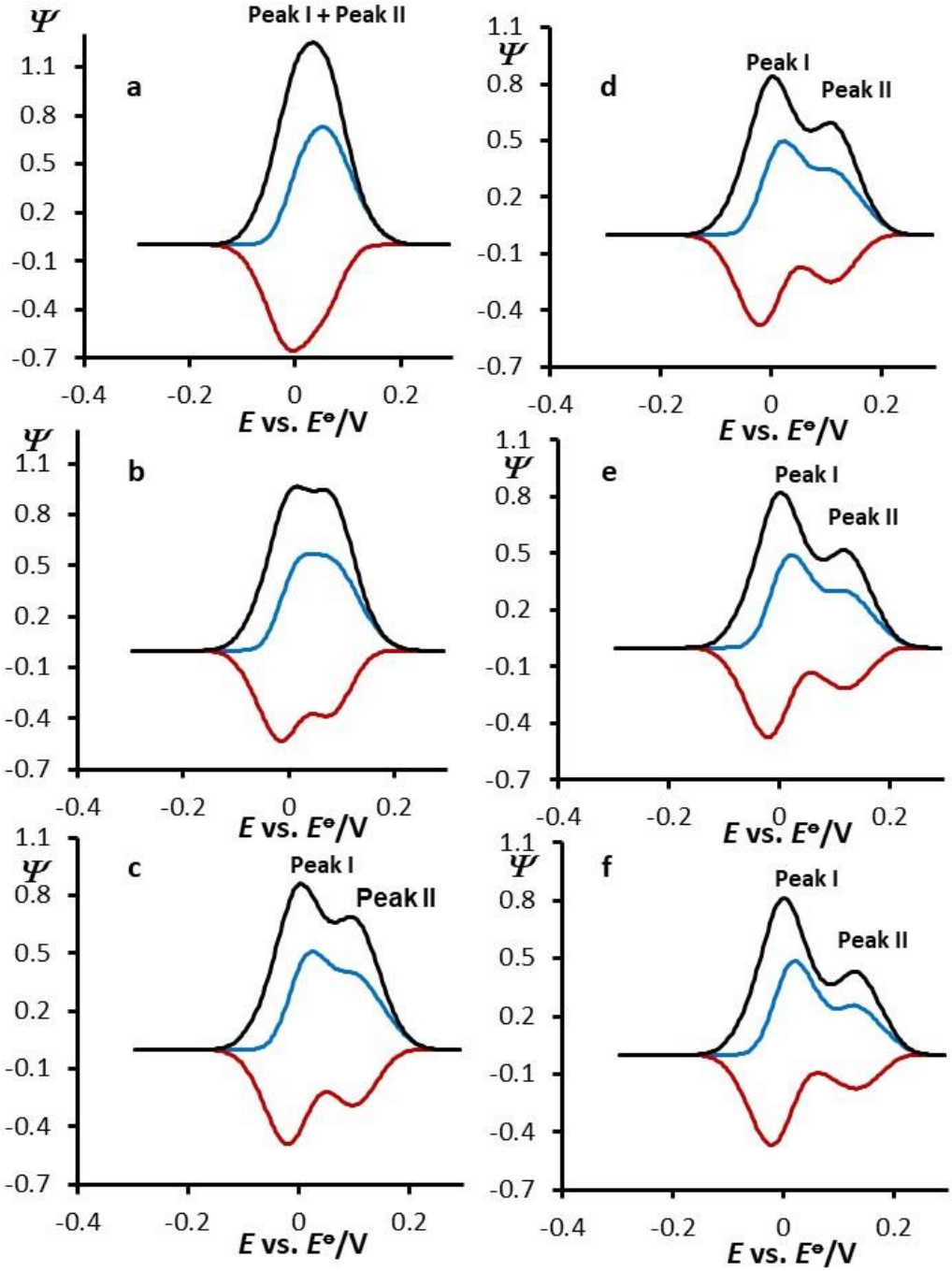

Fig. 4. *Surface EEC mechanism* A(ads) + n_1e- ↔ B(ads) + n_2e- ↔ C(ads) + S ↔ D(ads) in protein-film voltammetry: Effect of the substrate concentration to the features of theoretical SW voltammograms when both electron transfers of an enzyme redox transformation take place at the same potential. The values of c(S)/mol·l^{-1} are set to: 0.0001 (a); 0.02 (b); 0.04 (c); 0.05 (d); 0.06 (e), and 0.075 (f). KI = 1.25; KII = 2.80. The value of the chemical rate constant was set to k_{chem} = 10 mol·l^{-1}s^{-1}. The equilibrium constant of the follow-up chemical reaction was K_{eq} = 0.1. All other simulation parameters were the same as those in Figure 3.

2.3. *Outlooks for the future*

Probing the electrochemical reactions of enzymes offers unprecedented insight into the dynamic properties of these important biocatalysts and their application in various fields related to biosensing and energy conversion systems. Although PFV is probably the simplest experimental set-up designed to study the electrochemical/chemical features of redox proteins, this methodology still suffers from many drawbacks. Major obstacles are seen in the denaturation of the redox proteins and especially in poor electron transfer communication between the working electrode and the active site(s) of analyzed enzymes. To overcome these obstacles, so-called "functionalization" of the working electrode surface must be performed. The modification of electrode surfaces with various conductive polymers and nanoparticles seem to be inevitable scenarios to achieve better compatibility for many electrode-enzyme systems. Indeed, the number of redox enzymes that are suitable for use with PFV is still limited to several hydrogenases, flavoproteins and some metalloenzymes containing ions of Fe, Cu, Co, Mo or W. Even with modification of the working electrode surface, many dynamic features of the enzymatic turnover are still inaccessible by conventional PFV experiments. A promising scenario to overcome some of these drawbacks is seen in the "single molecule" enzymatic studies [89]. This is of exceptional importance, if such systems are studied at a single nanoparticle entity [90–93]. In such an approach, the extraction of kinetic parameters and information on the enzymes mechanism in its natural environment should enable the development of miniaturized voltammetric biosensors. These can be further explored for the detection and quantification of various biomarkers. This can be considered as one of the biggest challenges of PFV methodology applied to biomedicine. For fast and precise kinetic determination of enzymatic systems at molecular levels and to achieve better analytical sensitivity, some of the recent methodological studies might be helpful [31, 35, 94-–98].

In this context, it is worth mentioning several novel chronoamperometric and voltammetric techniques, derived from the perspective of SWV, which bear potential for improved electrochemical study in the context of PFV. One example is "electrochemical faradaic spectroscopy" [97], which utilizes a pulse form of a chronoamperometric experiment that is highly promising in the analytical application of PFV as well as in the development of electrochemical sensors (Fig. 5).

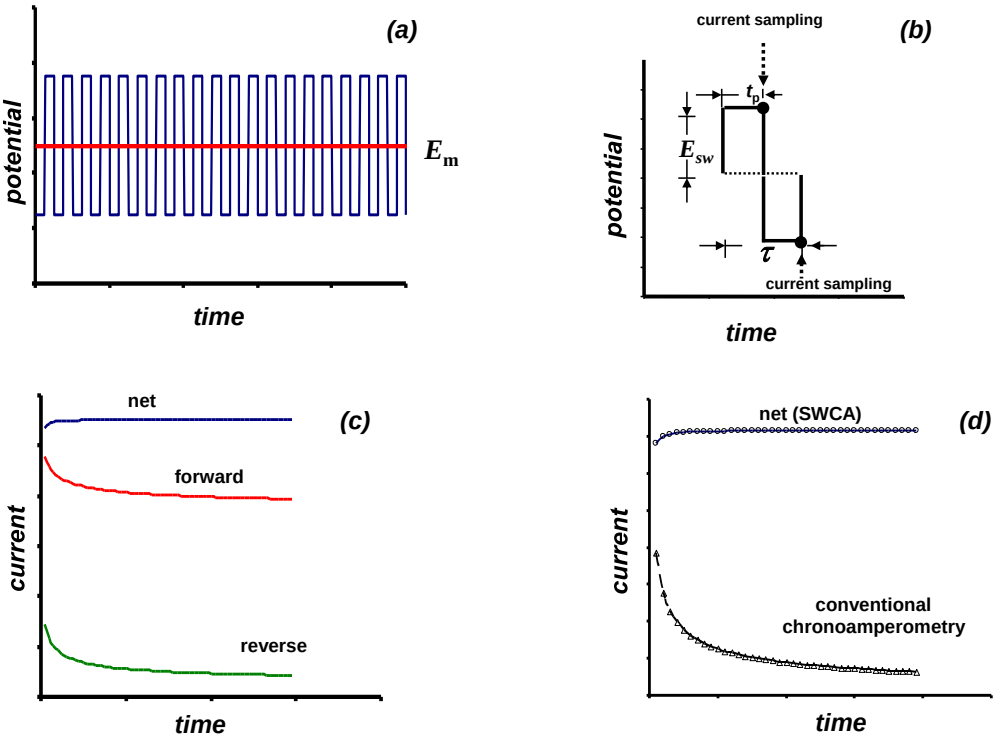

Fig. 5. *SW chronoamperometry (SWCA).* **(a)** Scheme of the excitation signal consisting of a constant mid-potential (E_m) upon which, small, oppositely oriented potential pulses are imposed, analogous to conventional SWV. **(b)** Defining parameters of single square-wave potential cycle, with meaninig identical as in SWV. **(c)** Current responses of this technique have the shape of a square-wave chronoamperogram, showing forward, reverse and net current components as a function of experiment time. **(d)** A comparison of the chronoamperometric response of the technique with the conventional chronoamperometry.

The technique, which can be also termed as "square-wave chronoamperometry", is superior in sensitivity compared to both conventional chronoamperometry and SWV. From the plethora of novel voltammetric techniques, we recall recent techniques such as "differential square-wave voltammetry" [31], "double-sampled differential square-wave voltammetry" [98] and "multisampling square-wave voltammetry" [96]. In differential square-wave voltammetry (Fig. 6), inherent advantages of differential pulse and SWV are unified for the purpose of better discrimination against both charging and background current, while maintaining the ability for the mechanistic and kinetic study of electrode reactions. Differential variants of SWV can also be achieved by introducing a double sampling current protocol, as depicted in Figure 7. This technique is expected to improve mechanistic and kinetic analysis of electrode processes at any degree of electrochemical reversibility [98].

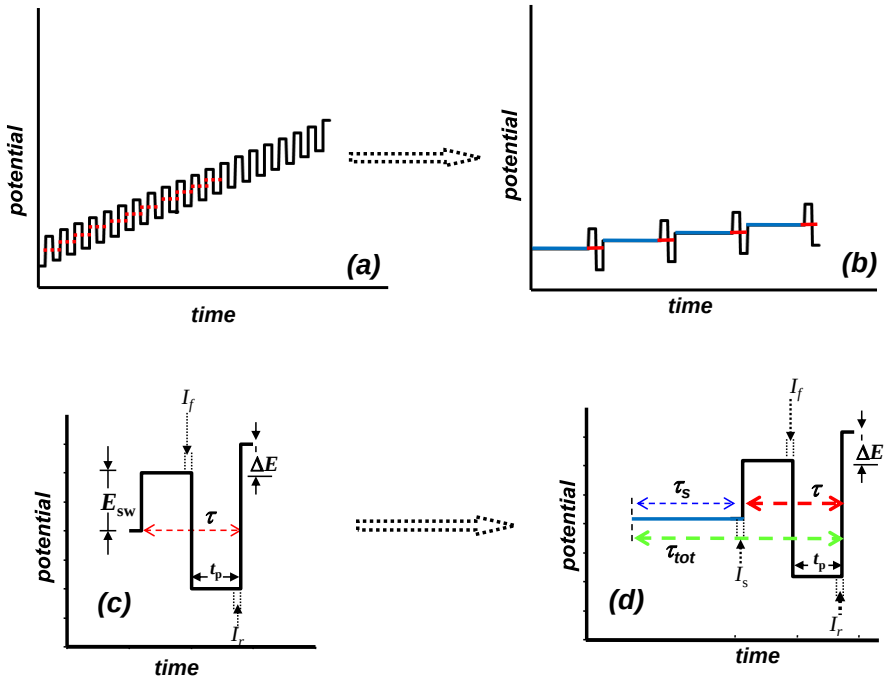

Fig. 6. Potential modulation in (**a**) conventional SWV and (**b**) a new, hybrid technique termed as differential square-wave voltammetry (DSWV). **(c)** Single square-wave potential cycle in SWV, showing the duration of a potential cycle (τ) and the single potential pulse (t_p), the height of the pulse, i.e. SW amplitude (E_{sw}), the step potential (ΔE) and current sampling points (forward (I_f) and reverse current (I_r)). **(d)** Single potential cycle of DSWV including an additional time of the step potential (τ_s) and three current sampling points: I_s, I_f and I_r, referring to the current measured at the end of the potential step, the forward potential pulse and reverse potential pulse, respectively.

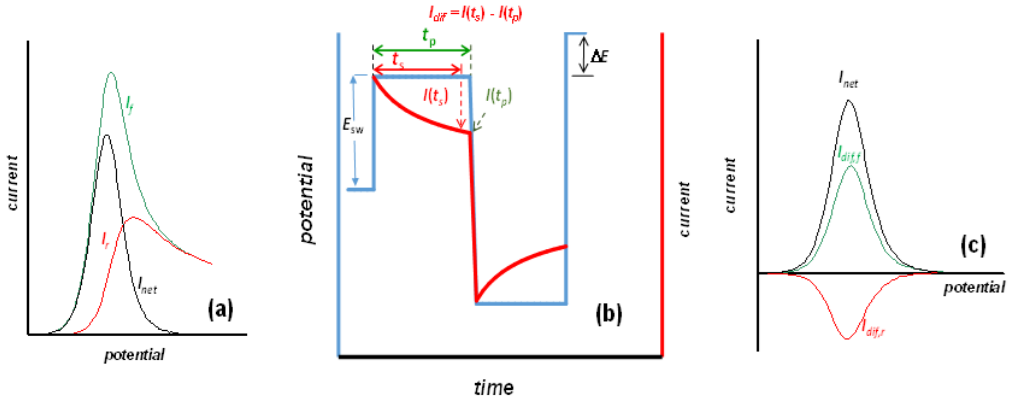

Fig. 7. (a) Current responses of a sluggish redox reaction in conventional SWV. **(b)** Single potential cycle in SWV showing the variation of the potential (blue line, left ordinate) and current (red line, right ordinate) with time. The current is sampled twice in the last quarter of each potential pulse at times t_s and t_p, and the differential current $I_{dif} = I(t_s) - I(t_p)$ for each potential pulse is calculated. **(c)** Expected current responses of a sluggish redox reaction under double-sampling square-wave voltammetry (DSSWV) consisting of differential forward, reverse and net current components.

The idea of the double-sampling current protocol can be further expanded for the purpose of fast characterization of electrode processes [98]. In regards to this goal, SWV is considered as a complex, repetitive double-step chronoamperometric experiment, which provides current-time-potential information in the course of a single experiment (Fig. 8). A typical outcome, presented in a current-time domain (Fig. 8 panel *b*) reveals a wealth of electrochemical data. Applying a multisampling current procedure at different sampling times (t_s), (Fig. 8 panel *c*), several multi-sampled SW voltammograms can be constructed. Hence, out of a single experiment, a series of SW voltammograms can be constructed, the analysis of which can reveal both electrode kinetics and mechanisms. This is highly relevant in the context of enzymatic analysis and sensor applications of voltammetry in biomedicine.

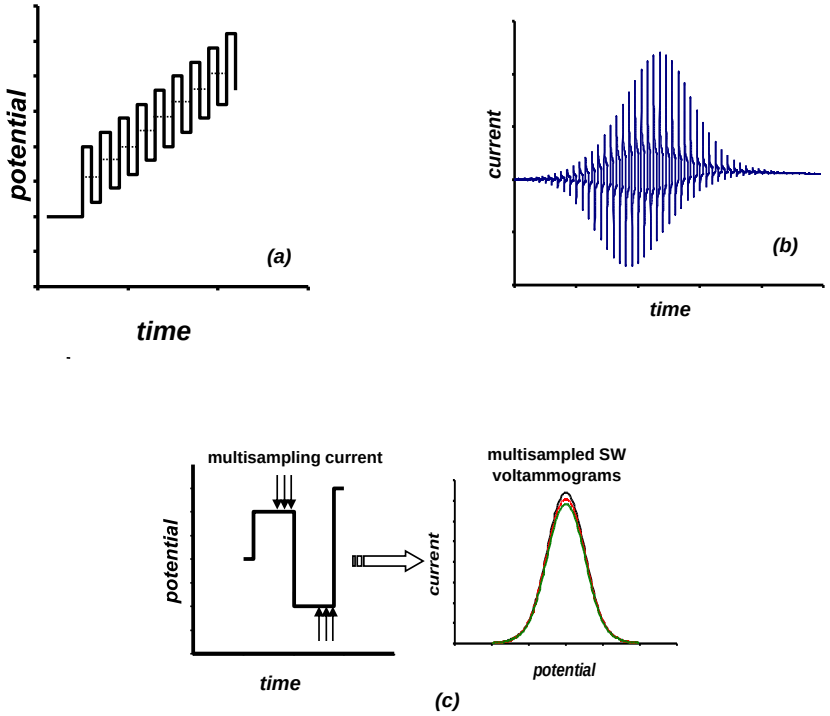

Fig. 8. (a) Potential waveform and **(b)** variation of the current with time, considering SWV as a complex repetitive double-step chronoamperometric experiment. **(c)** Current sampling points at different times of each potential pulse and corresponding multi-sampled net SW voltammograms.

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